Staphylococcus aureus: salt stress, cell wall deficiency, and Biofílm formation

Beckingsale, Thomas

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Thomas Beckingsale

School of Biological and Biomedical Science
Durham University

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Abstract

Staphylococcus aureus (*S. aureus*) is a major cause of chronic infection and is of particular concern in the infection of implanted prostheses. Biofilm formation offers bacteria innate protection from antimicrobial agents and prevents the eradication of prosthetic infection *in vivo*.

Current work shows a positive correlation between salt (NaCl) stress and cell wall deficiency in *S. aureus*. Cell wall deficient cells, produced in response to salt stress, demonstrate an increase in penicillin G resistance which increases with further salt stress, but returns to baseline levels after removal of the salt stress, indicating a phenotypic change in response to salt stress. Lysostaphin susceptibility decreases after salt stress, indicating changes to the structure of the cell wall in response to salt stress. Biofilm assays also show a positive correlation between biofilm formation and increased NaCl concentration. Further investigation using other Group I metals showed a positive correlation between increasing levels of KCl concentration and biofilm formation. This appears to be an ion specific effect related to potassium and sodium, as other Group I metals (Lithium, Rubidium and Caesium) did not have a similar effect.

RT-PCR showed that both KCl and NaCl stress lead to a decrease in transcription of several genes, including some involved in cell wall synthesis, global gene regulation and polysaccharide intercellular adhesin production, over the baseline level. However, RT-PCR shows no significant difference between cells stressed with either KCl or NaCl.

The work in this thesis shows a positive link between cell wall deficiency, antibiotic resistance and biofilm formation in *S. aureus* cells stressed with NaCl or KCl.

Declaration

I declare that the work within this thesis is my own work. Any Material generated through joint work has been acknowledged and the appropriate publications cited. No part of the work contained within this thesis has been previously submitted for any other degree or qualification at Durham University or any other university or institute of learning.

Signed:

Date:

Statement of Copyright

Staphylococcus aureus: Salt Stress, Cell Wall Deficiency, and Biofilm Formation.

For Alex

Research has been likened to gathering flowers in a large meadow. If that is so then the field of microbiology is a huge and bountiful plain. In preparing this thesis I have searched and botanised in just some of those vast acres and I have gratefully benefited from the bouquets of learning plucked by many other researchers. What I have gathered from their labours and cultivated myself, will hopefully be of use to all those who have been picking flowers in this particular field. Any weeds of error are of my own harvesting (Beckingsale, 1967).

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1.1.0 Acknowledgements

Ask a Doctor to describe a typical Orthopaedic Surgeon and various stereotypes will spring forth. "Carpenter," is one common description. "Bull in a china shop," is another. We are also reputed to have, "The strength of an ox but only half the intelligence." Hence I imagine it was with some trepidation that the scientists of Lab 5 welcomed me into their fold, but welcome me they did. I would like to thank them all for their kindness, support and tireless patience. In particular I would like to thank Colleen for keeping the entire lab supplied and running smoothly, Val and Jo for their calm explanations no matter how facile my questions, John R for his computing expertise, and Wen and Johan for their tuneful musings.

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1.2.0 Introduction

1.2.1 Natural Order

Until Zacharias Jannsen invented the microscope, understanding of the natural world consisted only of what could be seen with the human eye and a single lens. Aristotle, the father of modern science and the earliest, and perhaps greatest, of all naturalists, was the first to attempt to rationalise and bring some order to nature when he proposed a two kingdom system of classification in which all living things were grouped as either Plants or Animals. The discovery of microscopic life and in particular that of bacteria, named from the Greek word *bakterion,* meaning little staff or rod, which could be classified as neither plants nor animals brought this long held order into question.

As knowledge grew, distinctions between eukaryotic and prokaryotic cells were discovered. The fundamental differences between them include not only the method of genomic storage, as described above, but also the mechanism of control and expression of the genome, the structure of the translation apparatus, and the function of numerous cellular processes. Woese's made a huge scientific advance by sub-classifying prokaryotic cells under the broad headings of eubacteria, meaning literally true little rods from *eu* and *bakterion*, and archaebacteria, from *archae* meaning early (Makins, 1993). These two classes are now known simply as bacteria

and archaea respectively, and together with eukaryota, they make up the most modern classification, a *three-domain* system (Woese, 1987). Woese sequenced the 16S subunit of ribosomal ribonucleic acid (rRNA) and hence produced a universal phylogenetic tree, showing the evolutionary distances between the three kingdoms of bacteria, archaea and eukaryotes (Woese, 1987). Further sequence comparisons of 16S rRNA lead to broad sub-classifications within bacteria.

Not everyone agrees with the *three*-domain system, but whatever the disagreements and discussions that may occur, one thing is clear (Mayr, 1998). Technology is providing ever changing means of investigating and classifying the evolution and relationships of the natural world (Scamardella, 1999). Historical classification systems, like those of Aristotle, have tended to group living organisms based on their physical similarities. However, this type of classification, which tends to overemphasise the importance of morphology, is ineffective and inefficient when it comes to the sub-classification and investigation of bacteria. Bacteria are characterised by marked metabolic diversity, and are more accurately classified experimentally, for instance by sequencing their 16S rRNA. Hence classification can best be made according to their metabolic and biochemical evolutionary lines (Woese, 1987).

Appreciating the phylogenetic origins of bacteria, and their place in the natural world, is important for understanding the evolutionary roots of cells. The micro-organisms present today, although seemingly basic forms of life, are the successful lineages of evolutionary events. They are genetically, biochemically and metabolically diverse whilst also being adaptable and ubiquitous.

1.2.2 Bacterial Classification

The structural unit of bacteria is the cell. Bacteria are prokaryotes, meaning that they do not have a nucleus, which distinguishes them from unicellular eukaryotic life such as yeasts. Although most bacteria which are pathogenic to humans have a cellular diameter of 1 to 2 micrometers (μm), bacterial cells can not be readily classified according to size (Barton, 2005).

Bacteria are often described according to their morphology, for instance bacilli are rod shaped, cocci round and vibrios s-shaped. Further distinctions can be made according to group morphology of the bacteria. For instance, Streptococci, *streptos* meaning twisted, form long twisted chains of cells (Makins, 1993).

One broad and common bacterial classification is to experimentally ascertain and hence divide bacteria into two groups known as Gram-positive and Gram-negative. This eponymous classification, named after Christian Gram, categorises bacteria according to the staining characteristics of their cell wall.

1.2.2.1 The Gram Classification

The *Gram stain* involves staining a smear of bacteria with *crystal violet* and *Lugol's iodine*. The bacterial cells are then washed with a solvent, such as the ketone, acetone. Cells with one class of cell wall retain the violet dye after such treatment and are termed Gram-positive. Cells with the other class of cell wall are decolourised by solvent treatment, do not retain the violet dye, and are termed Gram-negative. Colourless, Gram-negative bacteria are counterstained with *safranin* or *carbolfuchsin* to aid visualisation by microscopy.

1.2.2.1.1 Differences between Gram-positive and Gram-negative Cell Walls

Gram-positive bacteria, which retain the crystal violet of the Gram stain, have cytoplasmic membranes outside of which lie thick cell walls, multifaceted matrices composed of cross-linked peptidoglycan, polyanionic teichoic acids and surface proteins (Neuhaus & Baddiley, 2003). More recent work has demonstrated a periplasmic space between the cytoplasmic membrane and the cell wall. This periplasmic space, or inner wall zone, is bereft of the cross-linked polymeric network, characteristic of the cell wall. Outside the inner wall zone, lies the outer wall zone, which comprises the peptidoglycan-teichoic acid matrix and its associated proteins (Matias & Beveridge, 2006).

Gram-negative bacteria also have a peptidoglycan cell wall, usually thinner than that of the Gram-positive bacteria, but this is only a part of a multilayered and complex structure. The cytoplasmic membranes of Gram-negative bacteria are surrounded by a periplasm, a layer which contains many enzymes crucial to cellular function and in which the peptidoglycan cell wall resides. Outside the peptidoglycan cell wall lies a further layer, the outer membrane. The outer membrane of Gram-negative bacteria is essentially a second lipid bilayer, but it also contains variable amounts of polysaccharide and protein. These polysaccharides are linked primarily to the outer surface of the lipid bilayer forming a lipopolysaccharide outer layer or LPS. These lipopolysaccharides can be divided into three sections; the inner Lipid A section, the middle core polysaccharide section and the outer O-specific polysaccharide. The LPS layer, and in particular Lipid A, is toxic to humans and is termed endotoxin. The outer membrane of Gram-negative bacteria also contains porins, proteins which function as channels for low-molecular-weight hydrophilic substances.

1.2.2.1.2 Differences between Gram-positive and Gram-negative Peptidoglycan

It is the rigid peptidoglycan layer of both Gram-positive and Gram-negative cell walls which gives them their strength. The peptidoglycan, also called murein, has a very similar chemical composition in both Gram-positive and Gram-negative cell walls and is composed of two sugar derivatives, *N-acetylglucosamine* and *N-acetylmuramic acid*, and the amino acids *L-alanine, D-alanine, D-glutamic acid* plus either *L-lysine* or *diaminopimelic acid* (DAP). These basic components are joined in a repeating structure. *N-acetylglucosamine* and *N-acetylmuramic acid* join alternately to form a glycan backbone. To each *N-acetylmuramic acid* residue is attached a tetrapeptide made up of *L-alanine, D-alanine, D-glutamic acid* and DAP (Madigan, Martinko & Parker, 2000). DAP is present in the tetrapeptides of all Gram-negative cell walls but many Gram-positive cell walls contain *L-lysine* instead, as is the case in *S. aureus.* The overall repeating unit produced is known as a *glycan tetrapeptide.* Of note, the presence of the *D* enantiomers of *alanine* and *glutamic acid* in peptidoglycan is of interest. In nature proteins are made exclusively of *L* stereoisomers and so the utilization of dextrorotatory amino acids in the cell wall is unusual. It has been suggested that bacteria utilise the *D* enantiomers as a mechanism of avoiding digestion by the proteolytic enzymes of higher organisms (Friedman, 1999).

The strength of the cell wall peptidoglycan comes from further cross-linking between the long chains of glycan tetrapeptides, and it is these cross-links which differ between Gram-positive and Gram-negative bacteria.

In Gram-negative bacteria, direct peptide linkage occurs between the tetrapeptide chains, usually joining the amino group of DAP in one peptide chain, to the carboxyl group of the terminal D-alanine in another.

In Gram-positive bacteria however, the cross-linkage is usually via a peptide bridge. The types and numbers of amino acids in these bridges varies between different Gram-positive bacteria. In *S. aureus*, for example, the bridges linking the chains are composed of five molecules of the amino acid glycine, forming so called pentaglycine cross bridges (Madigan et al., 2000). In *S. aureus*, penicillin binding protein 4 (pbp4) plays a vital role in peptidoglycan cross-linkage. Mutants with no pbp4 show hypo-cross-linkage and a slight increase in β-lactam antibiotic sensitivity (Sieradzki, Pinho & Tomasz, 1999).

1.2.2.1.3 Peptidoglycan Cell Wall Biosynthesis

The biosynthetic pathway of peptidoglycan is performed and controlled by the Mur enzymes in the cytoplasm. These are a series of enzymes which catalyse the formation of *N*-acetylglucosamine-*N*-acetylmuramyl pentapeptide, the basic monomer of peptidoglycan. They are the subject of research into novel antibiotic agents because of their importance in bacterial cell wall production (El Zoeiby, Sanschagrin & Levesque, 2003).

Another enzyme which plays an important role in the biosynthesis and maintenance of the peptidoglycan cell wall in *S. aureus* is Atl, a peptidoglycan hydrolase called autolysin. It is encoded by the gene *atl* and it is essential for cell division and separation, cell wall turnover and antibiotic-induced cell lysis (Fitzpatrick, Humphreys & O'Gara, 2005b).

AtlE, the autolysin of *S. epidermidis*, has been shown to adhere directly to hydrophobic surfaces and also has the ability to bind vitronectin suggesting a role in the early adhesion phase of biofilm formation. Hence, due to its marked similarity to

AtlE, it is hypothesised that Atl is also capable of playing a similar role in biofilm formation.

1.2.2.1.4 Teichoic Acids in Gram-positive Bacterial Cell Walls.

Teichoic acids are an essential part of the multifaceted matrix called the cell wall, *teichos* being the Greek word for wall.

Teichoic acids are broadly subdivided into 2 main groups, the wall teichoic acids and the lipoteichoic acids. The wall teichoic acids are covalently linked to peptidoglycan via a linkage unit, while the lipoteichoic acids are anchored to the membrane by a glycolipid anchor, with their tails extending into the cell wall across the periplasmic space (Matias & Beveridge, 2006; Neuhaus & Baddiley, 2003).

Teichoic acids are acidic polysaccharides, and contain either glycerophosphate, or ribitol phosphate residues. These acidic polysaccharides are negatively charged, and hence teichoic acids are partially responsible for the negative charge of the cell surface (Neuhaus & Baddiley, 2003). Interestingly, biofilm formation on polystyrene and glass surfaces is decreased in mutant *S. aureus* cells with increased teichoic acid expression, and hence an increased negative charge at the cell surface, suggesting an electrostatic repulsion (Neuhaus & Baddiley, 2003).

The polyanionic cell wall, a matrix of cross-linked peptidoglycan, wall teichoic acids, lipoteichoic acids and proteins, fulfils many functions. It affords tensile strength whilst still providing elasticity, and it imparts a physical barrier, encapsulating and withholding the cellular contents, whilst still exhibiting permeability (Schäffer & Messner, 2005). The teichoic acids along with cell wall proteins act to effect the passage of ions, proteins, nutrients and antibiotics through

the cell wall, and Teichoic acids in particular play an essential role in metal cation homeostasis (Neuhaus & Baddiley, 2003; Schäffer & Messner, 2005).

The teichoic acids are also an important factor in determining cell surface hydrophobicity and they are inextricably linked to the presentation of autolysins. As such, they help regulate the action of the autolysins, peptidoglycan hydrolases which are themselves essential to the process of cell wall biosynthesis and have been implicated in biofilm formation (see section 1.2.2.1.3). Atl, an autolysin of *S. aureus*, is itself an example of a protein which docks with lipoteichoic acid (Neuhaus & Baddiley, 2003). Some cell surface adhesins too, are bound ionically to teichoic acids indicating their significance in cell-cell and cell-surface adhesion (Neuhaus & Baddiley, 2003; Schäffer & Messner, 2005).

A further notable action of teichoic acids, is their ability to interact directly with host receptors. This, combined with both their importance to adhesin presentation and their direct role in surface adhesion, makes teichoic acids significant contributors to bacterial pathogenicity. Furthermore, studies using teichoic acid deficient *S. aureus* have shown significantly lower rates of sepsis and septic arthritis in mouse infection models than with wild-type *S. aureus*, confirming a crucial role in virulence (Neuhaus & Baddiley, 2003).

1.2.2.1.5 Relationship of the Cell Wall Structure to the Gram Stain

When the Gram stain is performed, the *crystal violet* and *Lugol's iodine* form an insoluble complex inside the bacterial cells being tested. This complex is then washed out of Gram-negative bacteria by a separate solvent. This process does not occur in Gram-positive bacteria because their thicker peptidoglycan cell wall becomes dehydrated by the solvent. This process of dehydration has the secondary effect of

closing the pores in the cell wall and thus preventing the escape of the crystal violet-iodine complex. In Gram-negative bacteria, the solvent readily penetrates the lipid-rich outer membrane and the much thinner peptidoglycan cell wall does not prevent the transit of the crystal violet-iodine complex (Madigan et al., 2000).

1.2.2.1.6 Lysostaphin and its Effect on *S. aureus* Peptidoglycan.

Lysostaphin is a zinc-containing endopeptidase enzyme which causes lysis of *S. aureus* (Trayer & Buckley III, 1970). It was originally described by Schindler and Schuhardt, who isolated it from cell-free isolates of *Staphylococcus staphylolyticus* (Schindler & Schuhardt, 1964).

Bacterial cell walls are made of long chains of glycan tetrapeptide, known as peptidoglycan. Cross-linking of these chains is vital to the strength of the cell wall. In *S. aureus* the bridges linking the chains are composed of five molecules of the amino acid glycine, forming so called pentaglycine cross bridges. Lysostaphin specifically cleaves these pentaglycine cross-bridges in the cell walls thus weakening them (Wu et al., 2003). Bacteria maintain a high internal osmolarity which exerts a high turgor pressure on the cell wall, and hence, weakening of this primary physical barrier leads to cell lysis.

The action of lysostaphin has been shown to be independent of resistance to other antibiotics, phage type or capsulation (Trayer & Buckley III, 1970). However, Fuller et al have shown increased resistance to lysostaphin in cell wall deficient bacteria (Fuller et al., 2005).

1.3.0 *Staphylococcus aureus*

The Staphylococci, from the Greek *staphule*, meaning bunch of grapes, and *kokkos* meaning berry, are a genus of round, Gram positive bacteria, which tend to grow in clumps, hence their descriptive name (Makins, 1993). Staphylococci are non-motile, non-sporing and usually non-capsulate. They grow over a wide range of temperatures (10-42°C) with an optimum growing temperature of 37 °C. They are normally aerobic but are also facultatively anaerobic.

S. aureus is a common species of this genus and is so named due to its characteristic golden colonies, the Latin word for gold being *aurum* which, literally translated means 'glowing dawn' after Aurora, the Goddess of dawn in Roman mythology (Makins, 1993).

Staphylococci were first identified by Louis Pasteur whose work followed on from Robert Koch and his famous postulates. Pasteur also realised the significance of micro-organisms in infection and championed the germ theory of disease (Porter, 2001). He isolated *S. aureus* from cases of puerperal septicaemia and rather colourfully described the bacteria as *microbes en amas de grains,* translated as, "microbes in a cluster of grains" (Castiglioni, 1975).

However, in 1881 Sir Alexander Ogston showed categorically that a number of pyogenic diseases in humans were associated with a cluster-forming micro-organism. His detailed diagrams and florid descriptions of *S. aureus* demonstrated the bacteria's importance in surgical infection and abscess formation (Ogston, 1881).

S. aureus is distinguished from other Staphylococci by the production of coagulase (Elliot, Hastings & Desselberger, 1997; Greenwood, Slack & Peutherer, 2003). Coagulase is an extra-cellular enzyme which converts plasma fibrinogen to fibrin. It is aided by an activator present in plasma. The deposition of fibrin results in

coagulation of blood and also interferes with phagocytosis, thus increasing the ability of the bacteria to invade host tissues unhindered (Elliot et al., 1997). Staphylococci other than *S. aureus* do not produce this enzyme and are thus often collectively referred to as coagulase-negative staphylococci.

Most *S. aureus* strains, and some other staphylococci, also produce a thermostable deoxyribonuclease, an enzyme which breaks down DNA. The presence or absence of this enzyme can be used to help distinguish the particular strain of a staphylococcus.

Individual strains of *S. aureus* can be identified by bacteriophage typing. Bacteriophages are viruses that infect bacteria by attachment to the bacterial surface. This attachment is specific and phages have a very narrow host range and are even unique to strains within bacterial species (Elliot et al., 1997). This fact has been utilised to develop a set of specific anti-staphylococcal bacteriophages. These can then be tested against cultures of *S. aureus* to distinguish one strain from another (Elliot et al., 1997; Nester, Roberts & Nester, 1995).

1.3.1 *Staphylococcus aureus* and Medicine.

S. aureus is a widespread human pathogen. Outside the body it causes no ill effects and is carried, without detriment, on the skin and in the upper respiratory tract by 25 percent of the population (Kumar & Clark, 1998). [Figures for carriage vary, and range from 15 percent (Russel, Williams & Bulstrode, 2004) to 33 percent (Elliot et al., 1997)]. If, however, it enters the body through abrasions, ulcers, burns or surgical or traumatic wounds, it can cause a multitude of clinical conditions (Russel et al., 2004). Patient susceptibility to staphylococcal infection is increased by metabolic

abnormalities, dysfunction of the humoral or cellular immune system, malnutrition and senescence (Kumar & Clark, 1998).

Staphylococcal infection can affect many sites and organs of the human body. Invasion of the skin can cause impetigo, cellulitis, furuncles and carbuncles. In the lungs abscesses and pneumonia are the result. Infection of the heart leads to endocarditis and of the pericardium, pericarditis. Meningitis and abscess formation can be the result of infection to the central nervous system and osteomyelitis and septic arthritis if bones and joints are affected. Strains that produce toxins can lead to scarlet fever, toxic shock syndrome and gastroenteritis (Kumar & Clark, 1998). It can form a synergistic relationship with certain coliforms and species of *bacteroides* leading to necrotising fasciitis, a condition known in the tabloid media as the "flesh eating bug" (Russel et al., 2004). It colonises and infects foreign bodies such as catheters, cannulae, ventilatory tubing and cardiac and orthopaedic prostheses. The majority of these conditions lead to local abscess formation but the infection can spread via the blood stream leading to a bacteraemia and metastatic infection (Kumar & Clark, 1998).

1.3.2 Treatment of Staphylococcal Infections

Throughout recorded civilization, physicians have understood that the drainage of pus is associated with healing of an abscess. Galen, the Greek philosopher and physician, incorrectly surmised that it was the pus itself that heralded healing, when he referred to it as *pus bonum et laudabile,* good and laudable pus (Gorbach, 1995; Russel et al., 2004). However, the old adage, "Never let the sun set on undrained pus" still applies and an essential part of abscess treatment is surgical incision and drainage (Gorbach, 1995).

For systemic infection treatment with antibiotics is mandatory. Until the advent of penicillin, medical treatment was restricted to antisepsis and surgical prophylaxis was not possible. The discovery of penicillin is attributed to Sir Alexander Flemming, who observed that bacteria were killed on an agar plate that had been contaminated with *penicillium* spores (Porter, 2001). He was unable to isolate the penicillin, a feat later achieved by Ernst Chain and Howard Florey in Oxford, but it was his observations which must stand as one of the most important discoveries of modern medicine (Porter, 2001; Russel et al., 2004). Since the advent of penicillin, a host of antibiotics have been developed. They have revolutionised the ability of the medical professional to treat infections. However, indiscriminate use of antibiotics has led to the development of resistance.

1.3.3 Methicillin Resistant *Staphylococcus aureus*

Penicillin, as discovered by Sir Alexander Flemming, is the archetypal antibiotic of a class of drugs known as the penicillins, of which methicillin is another member. Penicillins, in turn, are part of a larger family of drugs known as the β-lactams. The β-lactams have in common a β-lactam ring. A lactam is a cyclical amide, the term amide signifying a carbonyl group (C=O) joined to a nitrogen atom. A Greek character is used to prefix lactams and signifies the size of the ring, in this case β, indicating that it is a 4 membered ring comprising 3 carbon atoms and a nitrogen atom (Neal, 1997). β-lactam antibiotics mimic the structure of *D*-alanyl-*D*-alanine and thus target the transepeptidation reaction of peptidoglycan synthesis. The β-lactam ring occupies the active site serine residue of penicillin binding proteins (PBPs), which catalyse the glycan polymerisation reactions, thus inhibiting their action (Navarre & Schneewind, 1999).

Methicillin resistant *Staphylococcus aureus* (MRSA) was first noted in 1961, shortly after the advent of methicillin (Kumar & Clark, 1998). Referred to in lay parlance as the "superbug" its name suggests only resistance to methicillin. MRSA is however, usually resistant to all β-lactam antibiotics and many others, including the aminoglycosides. Hence a more appropriate meaning for MRSA is perhaps, multiply resistant *Staphylococcus aureus.* MRSA can produce β-lactamase, an enzyme which breaks down the β-lactam ring of penicillin based antibiotics, hence its resistance to them.

MRSA has also developed alternative metabolic pathways, such as for the production of folic acid which has lead to resistance to sulphonamide antibiotics (Neal, 1997). However, perhaps the most important factor in methicillin resistance is the acquisition of the *mecA* gene, which encodes an altered penicillin binding protein PBP2a (de Lencastre, Oliveira & Tomasz, 2007). Worryingly, horizontal transfer of this gene has been demonstrated between staphylococcal species, which will inevitably lead to a more widespread prevalence of β-lactam resistance (de Lencastre et al., 2007). Thankfully, for the moment, MRSA is usually still treatable with vancomycin and teicoplanin.

The rates of recorded MRSA infection initially increased after reporting was made mandatory, with a 5 per cent rise in MRSA bacteraemia between 2002/3 and 2003/4 being documented and published (White, 2004). However, between 2005/6 and 2006/7 there was a 10 per cent drop in the rate of MRSA bacteraemia, perhaps due to greater awareness and hence more timely and appropriate treatment (HPA, 2008).

The financial costs associated with nosocomial infections, of which MRSA is the most important, are huge, and are thought to exceed 1 billion pounds per year in

England (Bourn, 2000). The cost is also enormous in human terms, with an estimated 5000 excess deaths resulting from hospital acquired infections (Mayor, 2000).

1.3.4 Endoprostheses

Many surgical procedures require the placement of foreign material within the body of the patient. In many cases this amounts to no more than suture material, but in an increasing number of interventional procedures, implantation of foreign material is required. In neurosurgery metallic clips, ventricular drains and cerebrospinal fluid shunts are used. Pacemakers and prosthetic valves are used to treat cardiac problems. Prosthetic vascular grafts are used in vascular surgery. Stents are an essential part of treatment for many urological disorders and synthetic lenses are used for cataract surgery. This list is far from exhaustive, but gives a flavour of the scale of foreign material used in surgery. Perhaps the specialty which uses the most foreign implants is orthopaedic surgery. Plates and screws are often used to hold fractures. Rods of metal are placed within bones to treat many long bone fractures. Large metal prostheses are inserted in joint replacement surgery and entire bones can be replaced with metal equivalents in the treatment of orthopaedic tumours.

The most feared complication, common to all surgical specialties, is infection. Surgical cleanliness has advanced dramatically since the introduction of the principles of antisepsis by Baron Lord Joseph Lister in the nineteenth century, which he published in *The Lancet* in 1870 (Firkin & Whitworth, 2002; Porter, 2001). His use of carbolic acid to kill bacteria at the surgical site has been replaced with filtered air theatres and the use of sterile equipment. Despite the improvements in equipment and the theatre environment it is not possible to sterilise the patient; antisepsis is the best that is attainable.

Bacteria are ubiquitous on the skin, the first barrier that must be crossed during an operative procedure, and can hence be taken into a wound. Bacteria can also be introduced to the bloodstream by procedures like cannulation and catheterisation and hence infection can spread to the operative site haematogenously. Physiological stress, either from a condition requiring an operation, or from the operative procedure itself, tends, perversely, to diminish the effectiveness of the hosts' immune system and further predispose to infection.

Prophylactic antibiotics are frequently used perioperatively to reduce the risk of infection both locally and by haematogenous spread. In fact, it has been suggested that judicious use of prophylactic antibiotics could reduce surgical site infections by 40 to 60 percent (Odom-Forren, 2006). Antibiotic resistance is of huge concern in the use of prophylactic antibiotics. Most commonly used antibiotics are ineffectual against MRSA. There is also genuine and appropriate fear that improvident and pervasive use of antibiotics will worsen the problem of resistance.

Surgical site infection is all the more sinister if it involves an implanted foreign body. Infective bacteria can evade both the host immune system and antibiotic administration by forming biofilm on prosthetic surfaces. Once a biofilm has been established, eradication is only possible by removing the prosthesis (Miller, 2004). In orthopaedic surgery biofilm may persist at the prosthetic-bone interface as there is very poor blood supply at the cortical bone surface. Only once eradication of the infection has been confirmed, can the insertion of a new prosthesis be entertained (Miller, 2004).

1.4.0 Biofilm

A biofilm is a conglomeration of microbial cells in a sessile multicellular community (Bryers, 2008; Cucarella et al., 2001). Within the biofilm, bacteria are embedded in a self-produced, hydrated matrix of polysaccharide, teichoic acid and protein (Stewart & Costerton, 2001). Scientific understanding of biofilms is increasing and with it an appreciation of its clinical relevance. The role of biofilms in persistent and chronic bacterial infections and infections of implants and prostheses is of particular concern (Costerton, Stewart & Greening, 1999). Biofilm effects chronic infection by providing innate protection to micro-organisms from opsonophagocytosis and antibiotic agents (Cramton et al., 2001; Stewart & Costerton, 2001). Antibiotic treatment of patients harbouring a biofilm will lead to temporary suppression of infective symptoms, by killing free-floating bacteria shed from the biofilm population (Stewart & Costerton, 2001). However, it does not kill the sessile bacteria within the biofilm which are afforded between 1000 and 1500 times the antibiotic resistance of their planktonic counterparts and so, when the antimicrobial chemotherapy is stopped, the biofilm acts as a nidus for recurrence of the infection (Stewart & Costerton, 2001; Wu et al., 2003). Although some methods of biofilm removal from abiotic surfaces have been described, there remains no safe method of removing the biofilm *in vivo* and hence, biofilm formation on prosthetic implants necessitates their removal to eradicate the infection (Wu et al., 2003).

1.4.1 The Formation of Biofilm:

There are 2 major steps in the formation of biofilm (Cramton et al., 1999). Firstly there is a requirement for attachment of the bacterial cells to a surface, so called early adhesion. The second action is growth-dependent accumulation of

bacteria in multilayered cell clusters which requires intercellular adhesion (Cucarella et al., 2001; Heilmann et al., 1996).

1.4.1.1 Requirement 1 for Biofilm Formation: Early Adhesion.

The phase of early adhesion is mediated by bacterial surface proteins, particularly the microbial surface components that recognise adhesive matrix molecules (MSCRAMMs) (Cucarella et al., 2001). The MSCRAMMs not only mediate adhesion of bacteria to host cells, but also to abiotic surfaces once they are covered with plasma constituents. Fibrinogen (Fg) is the major blood protein deposited on implanted biomaterial. In the case of the gram-positive bacterium *S. aureus*, there are at least 4 separate MSCRAMMs that recognise and bind to Fg (Davis et al., 2001; Walsh et al., 2004; Wann, Gurusiddappa & Hook, 2000). Two of these MSCRAMMs, fibronectin binding proteins A and B (FnbpA and FnbpB), were first discovered and thus named due to their ability to bind fibronectin (Fn), but more recent research has demonstrated a second binding site for Fg (Wann et al., 2000). The other 2 Fg binding proteins are clumping factors A and B (ClfA and ClfB) (Walsh et al., 2004). Over 20 MSCRAMMs have been discovered on the surface of *S. aureus* so far (Roche et al., 2003). These bind a variety of host proteins and molecules and there is much interaction between them.

Also, a novel surface protein has been isolated which is directly associated with biofilm formation on abiotic surfaces *in vitro* in the absence of host plasma constituents called biofilm associated protein (Bap) (Cucarella et al., 2001). This novel protein has marked similarities to surface proteins previously discovered on gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi) and gram-positive (*Enterococcus faecalis*) micro-organisms. All Bap-positive *S.aureus*

strains tested in one study were highly adherent and strong biofilm producers (Cucarella et al., 2001).

Due to this abundance of surface proteins *S. aureus* has many mechanisms in its arsenal to initiate and mediate this early adhesion and many of these have been linked to increased virulence and pathogenicity.

1.4.1.1.1 *Staphylococcus aureus* Surface Proteins:

Much work has been done investigating the interactions between MSCRAMMs of *S. aureus* and host extracellular matrix proteins to elicit the mechanisms of adhesion between the two.

Fibronectin Binding Proteins A and B.

As their name suggests, these two MSCRAMMs bind fibronectin (Fn). Fibronectin binding protein A (FnbpA) and fibronectin binding protein B (FnbpB) are expressed during the early exponential phase of growth (Wann et al., 2000), Fn binding activity being lost as the cells enter the stationary phase, possibly as the result of degradation of both surface proteins by V8 serine proteases (Roche, 2004). They are encoded by two closely linked genes fnbA and fnbB, the transcripts of which are under the control of *agr* and *sar*A (Roche, 2004). *agr* and *sar*A act diametrically in the production of these two surface proteins where *agr* is a repressor and *sar*A is an activator (Heinrichs, Bayer & Cheung, 1996).

FnbpA and FnbpB are also able to bind to elastin and FnbpA has the ability to bind fibrinogen (Fg) too (Walsh et al., 2004; Wann et al., 2000).

Clumping Factors A and B.

ClfA and ClfB primarily bind fibrinogen (Fg), and are the primary cell adhesins for Fg (Walsh et al., 2004; Wann et al., 2000). However, in *S. aureus* mutants that do not express ClfA and ClfB, cell interaction with Fg continues and is then mediated by fibronectin binding protein A (FnbpA) (Wann et al., 2000).

Fg is a glycopeptide found in blood. It is important in coagulation where it mediates platelet adherence and is cleaved by thrombin to fibrin, which is the major component of crosslinked clots. Fg is also the major blood protein deposited on implanted bio-materials. Fg is composed of 6 polypeptide chains, two Aα-, two Bβ- and 2γ-chains. ClfA binds to the γ-chain of Fg. This is the same site on Fg that FnbpA and platelet integrin (αIIbβ3) bind to (Wann et al., 2000).

ClfA has a Ca^{2+} binding site. This induces a conformational change, incompatible with binding to the C-terminus of the γ-chain of Fg. Ca^{2+}-dependent regulation of ClfA may prevent occupation of all the receptors of intravascular *S. aureus* from being occupied by soluble Fg, thus allowing the bacteria, under the right conditions, to adhere to solid-phase Fg as laid down on implanted prostheses or in fibrin clots (O'Connell et al., 1998).

ClfB has the ability to bind to the tail region of type I cytokeratin 10 (K10) (Walsh et al., 2004). K10 is expressed on the surface of squamous epithelia and keratinocytes and hence ClfB may play an important role in skin colonisation with *S. aureus*.

Elastin Binding Protein.

Elastin binding protein (Ebps) promotes binding of soluble elastin peptides and tropoelastin to *S. aureus*. This is an important distinction because Ebps-negative

cells still bind to immobilised elastin via fibronectin binding protein A (FnbpA) and fibronectin binding protein B (FnbpB). However, FnbpA and FnbpB defective cells with normal Ebps do not bind to immobilised elastin. Interestingly, cells treated with fibronectin prior to experimentation are still able to bind to immobilised elastin which suggests that FnbpA and FnbpB have a secondary binding site, specific to elastin (Roche, 2004). Hence, FnbpA and FnbpB probably play the more significant role in attachment to immobilised human lung and aortic elastin and thus in the pathogenesis of endocarditis and pneumonia.

Collagen Adhesin Gene.

Collagen adhesin gene (*cna*) encodes the only the *S. aureus* MSCRAMM that mediates a high level of collagen binding. Like fibronectin binding protein A (FnbpA) and fibronectin binding protein B (FnbpB), *cna* is temporally regulated. The regulation of *cna* is at least partially dependent on the *sar* locus. However, in the case of *cna*, this regulation is independent of the interaction between *sar* and *agr* (Gillaspy et al., 1998).

There is variation in the collagen binding capacity (CBC) among *cna*-positive strains and this is due, in part, to differences in transcription levels but also it is due to variations in capsule production. This is demonstrated by the observation that in *cna*-positive cells with high encapsulation there was little or no collagen binding, but if capsule deficient strains were induced, they had markedly increased CBC over their encapsulated parents (Gillaspy et al., 1998).

Cna is relatively rare, being present in only 10 out of 25 strains in one series (Gillaspy et al., 1998). In one study all *S. aureus* isolates from bone and joint infections were *cna*-positive. This suggested that *cna* was very important in the

pathogenesis of such infections. However, a further study found only the same level of *cna*-positive cells from infected bone and joint isolates as in control samples (Foster & Hook, 1998). Hence, the importance of *cna* in musculoskeletal infections is not yet fully described or understood and merits further investigation.

Staphylococcal Protein A.

Staphylococcal protein A (SpA) is a further well described surface protein which has numerous adhesive capabilities. It can bind the crystalline fragment (Fc) region of immunoglobulin G (IgG) thus diminishing IgG-mediated opsonisation and hence phagocytosis by macrophages. This is of huge benefit in avoiding host immunity. It also has the capability to bind the antigen binding fragment (Fab) region at an alternative site. This allows *S. aureus* to act as a super-antigen by crosslinking membrane bound immunoglobulin M (IgM) on B lymphocyte cells, leading to their activation. SpA also has the ability to activate complement through the classical pathway and this ability too, contributes to the virulence of *S. aureus*. SpA also binds von Willebrand Factor (vWF) and it has been speculated that this ability is important in the pathogenesis of endovascular infections and endocarditis (Hartleib et al., 2000).

vWF is a large glycoprotein which is extremely important in haemostasis. vWF binds to and stabilises factor VIII which is essential for the survival of this factor within the circulation. Free vWF also undergoes protease cleavage in the plasma. The products of this cleavage bind to sub-endothelial components like collagens, proteoglycans and glycosaminoglycans at sites of endovascular injury. vWF then acts as a bridge between a specific glycoprotein on the surface of platelets (GPIb/IX) and collagen fibrils (Kumar & Clark, 1998). This is pivotal for platelet adhesion at sites of vascular injury and hence crucial for coagulation and haemostasis.

The importance of vWF to platelet adhesion and the fact that *S. aureus* binds it so readily has led to the suggestion that platelets play a primary role in the early steps of *S. aureus* adhesion to damaged endothelium and spur the growth of infective vegetation (Hartleib et al., 2000).

SpA is also very important in the virulence of *S. aureus*. In a murine septic arthritis model, in the cases that were inoculated with SpA deficient *S. aureus* there was decreased severity of arthritis, decreased erosive damage to the cartilage and bone and a decreased mortality rate. However, there was no decrease in the bacterial load *in vivo* and when recombinant SpA was injected with SpA deficient *S. aureus*, there was no increase in virulence compared to controls. SpA itself lacked direct inflammatogenic properties when injected intra-articularly (Palmqvist et al., 2002). Hence, SpA seems to play an important role in virulence but this must be multifactorial in nature and suggests interactions between it and other surface proteins.

SpA has not classically been described as an MSCRAMM although current opinion is that it probably should. In consistency with other MSCRAMMs it undergoes sortase mediated cell wall linkage via an LPXTG motif, it is an adhesin molecule and it is co-regulated by 2 genes, *sar* and *agr*, which in this case, both act as repressors, leading to decreased expression in the stationary growth phase (Hartleib et al., 2000).

LPXTG: The Cell Wall Sorting Signal.

There are six well-defined mechanisms for protein anchoring to the cell wall, of which the sortase-mediated linkage is the most common and most well known.

In sortase-mediated cell wall anchoring, the protein for cell wall expression carries a cell wall sorting signal on its C-terminal end. This includes a peptide with an LPXTG motif, a hydrophobic domain and a positively charged tail. The N-terminal signal peptide promotes translocation across the bacterial membrane and it has been suggested that the hydrophobic and positively charged tail, hold the translocated protein within the plasma membrane until the substrate is recognised by sortase (Ton-That, Marraffini & Schneewind, 2004). In *srt*A defective cells, there is no LPXTG mediated protein expression on the surface.

Sortase, encoded by *srt*A, is a membrane bound transpeptidase that cleaves the polypeptides between the threonine and glycine residues of the LPXTG motif. It then covalently links the carboxyl group of the threonine to the adjacent pentaclycine cross-bridge in the cell wall peptidoglycan (Foster & Hook, 1998).

11 named surface proteins with the LPXTG motif were previously described, but Roche et al. have updated this, with the discovery of a further 10 sortase mediated surface proteins (Roche et al., 2003). The 11 previously described were SpA, ClfA and ClfB, *cna*, FnbpA and FnbpB, Serine Aspartate Repeat Proteins SdrC, SdrD and SdrE and 2 Plasmin Sensitive Proteins, Pls and FmtB. The 10 newly discovered surface proteins were named *S. aureus* Surface Proteins A-J (Sas A-J). Interestingly 8 of the 10 new Sas proteins carried the characteristic LPXTG peptide but 2 (SasD and SasF) carried an LPXAG motif. Antibodies to SasA, G, H, I and J were subsequently discovered in convalescent patients of culture positive *S. aureus* infected patients. This raises the question as to whether these surface proteins are particularly expressed during in vivo infection or whether they are virulence associated (Roche et al., 2003).

In *srt*A defective cells, there is no LPXTG mediated protein expression on the surface. In L-form bacteria where the cell wall is defective, proteins are still

expressed on the surface. However, their binding is very unstable as there is no cell wall to covalently bind them to. In these conditions the proteins are located on the cell membrane. In the case of SpA there was less than one quarter of the amount expressed on the surface of the L-form than there was on the cell walled parent which may have been due to its easy detachment and subsequent loss (Ivanova et al., 1989).

***srt*B**

*srt*B plays no role in LPXTG sorting but cleaves an NPXTG sorting signal instead. This motif is found in the C-terminus of IsdC. IsdC is a haem-binding surface protein which is only expressed in conditions of iron starvation. It is possibly the only sorting substrate of srtB (Ton-That et al., 2004).

1.4.1.2 Requirement 2 for Biofilm Formation: Intercellular Adhesion.

Intercellular adhesion is the second prerequisite for biofilm formation. Fundamental to this process is the formation of a self-produced extra-cellular polysaccharide matrix. Essential polysaccharides in this matrix are polysaccharide intercellular adhesin (PIA) a linear β-1,6-linked *N*-acetylglucosaminoglycan (PNAG) in *S. epidermidis*, and poly-*N*-succinyl-β-1,6-glucosamine (PNSG) in *S. aureus* (Cramton et al., 2001; Mack et al., 1996). These polysaccharides are both synthesised by products of the intercellular adhesion (*ica*) gene locus, which is present in both the respective bacteria (Cramton et al., 1999; Cramton et al., 2001). Importantly, antibodies raised against either PNAG or PNSG, recognise both antigens (Cramton et al., 2001). This has lead to the suggestion that purified PNAG could have potential as a broadly protective vaccine against clinically important strains of staphylococci, a suggestion backed up by animal studies (Maira-Litran et al., 2004).

1.4.1.2.1 The *ica* Gene Locus.

The *ica* operon (*icaADBC*) consists of 4 open reading frames, with an assumed regulatory gene (*icaR*) upstream and in the opposite orientation (Cramton et al., 1999; Cramton et al., 2001). IcaA and IcaD work synchronously to produce sugar oligomers from UDP-*N*-acetylglucosamine in *in vitro* assays (Cramton et al., 2001). This *N*-acetylglucosaminyltransferase activity combined with the activity of IcaC forms a product that is recognised by an antibody raised against *S. epidermidis* polysaccharide intercellular adhesin (PIA) (Gerke et al., 1998). If the *ica* locus is mutated or deleted in wild-type biofilm-forming strains to produce isogenic mutants then the ability to form biofilm *in vitro* is lost. This demonstrates that the *ica* genes, and therefore PIA/PNSG production, is important to biofilm formation (Cramton et al., 1999; Cramton et al., 2001). However, while *icaADBC* gene locus is currently the best understood mechanism of biofilm formation, more recent studies have shown that biofilm formation is possible independent of the *ica* genes (Fitzpatrick, Humphreys & O'Gara, 2005a; Toledo-Arana et al., 2005).

The environmental triggers for PIA/PNSG expression are not well understood but a wide variety of exogenic environmental factors certainly result in synthesis of PIA and this seems to be, at least in part, controlled by the alternative sigma factor, σ^B. For instance, work by S.E.Crampton et al in 2001 has suggested that anaerobic conditions increase PIA synthesis and work by S.Dobinsky et al in 2003 has shown a link between PIA expression in *S. epidermidis* and glucose concentration in the growth medium (Cramton et al., 2001; Dobinsky et al., 2003). This latter work threw up some surprising results. Maximum transcription of *ica* was detectable in the stationary phase of growth in the absence of glucose despite a PIA- and biofilm-

negative phenotype. The resultant abundance of *ica* mRNA was shown to be functionally active, as induction of stationary phase cells with glucose led to immediate PIA synthesis. However, induction of biofilm formation could be completely inhibited by the addition of chloramphenicol, used to inhibit protein synthesis from the abundant mRNA upon the addition of glucose. When this was given at a later stage of biofilm accumulation, it also inhibited further development of preformed biofilm. This suggests that continuous translation of a further, *ica*ADBC-independent factor is required for the expression of a biofilm positive phenotype (Dobinsky et al., 2003).

1.4.1.2.2 Other Important Substances in Matrix Formation.

Carbohydrate-rich polymers are essential in the production of matrix and hence biofilm formation, and polysaccharide intercellular adhesin (PIA)-like substances have been isolated from other biofilm-positive bacteria, both gram-positive and gram-negative. For instance a PIA-like polymer was recently shown to play a role in biofilm formation by *E. coli* strain MG1655. The genes that encode this polymer in *E. coli* are designated *pga*, and they share surprising similarity with the *ica* gene locus in *S. aureus* (Wang, Preston III & Romeo, 2004).

Cellulose has long been recognised as an extra-cellular polysaccharide produced by many bacteria, most notably *Gluconacetobacter xylinus*, whose bacterial cellulose synthesis (*bcs*) gene is found in many of the cellulose producing micro-organisms. It has also been shown to be an essential part of the extracellular matrix formed by *Salmonella typhimurium* and *E. coli* (Zogaj et al., 2001).

Extracellular DNA has been shown to play an important role in biofilm formation (Witchurch et al., 2002). Also, dead cells have been observed in some

biofilms, suggesting that cell detritus can be considered part of the extracellular matrix (Yarwood et al., 2004). Bacterial cellular death within a biofilm has also been postulated to be advantageous to the remaining biofilm cells as lysed cells provide a new nutrient reservoir (Resch et al., 2005).

1.4.2 Quorum Sensing in Biofilm Formation:

Intercellular signalling, often referred to as quorum sensing, has been shown to be involved in biofilm formation in several different bacteria. For instance, *Pseudomonas aeruginosa* uses an acyl-homoserine lactone system for quorum sensing. *S. aureus* however, has a different system for quorum sensing which is encoded by the accessory gene regulator (*agr*) locus. The *agr* system contributes to virulence in model biofilm-associated infections including endocarditis and osteomyelitis. The precise role of the *agr* system does, however, vary with the type of infection model used (Yarwood et al., 2004).

1.4.2.1 What is Quorum Sensing?

Bacteria have, in the past, often been considered as individual units whose primary function is to find nutrients and multiply. However, the discovery of cell-to-cell communication, so-called quorum sensing, has shown that bacteria are, in fact, capable of coordinated activity. This has obvious advantages to the bacteria, which are thus able to collectively migrate towards better nutrient supplies or adopt new modes of growth, such as biofilm formation, which affords collective protection from adverse environments and leads to innate protection from antimicrobial agents (De Kievit & Inglewski, 2000; Stewart & Costerton, 2001; Wu et al., 2003).

Intercellular communication is mediated by small, self-produced signal molecules called auto-inducers (AIs). The dictionary definition of quorum is, "A minimum number of members of an assembly, etc., required to be present before any business can be transacted" (Makins, 1993). This definition helps to explain the phenomenon of quorum sensing, in that AI release by a single bacterium will lead to a concentration too low to be detected in the environment, and hence no business transactions will take place. However, when sufficient bacteria are present in the "assembly", the concentration of AIs reaches a threshold level, which allows the bacteria to sense a critical cell mass, and start business transactions, in bacterial terms, by activation or repression of target genes.

Gram-positive quorum-sensing bacteria usually make use of small post-translationally processed peptide signal molecules. These peptide signals interact with the sensor element of a histidine kinase, two component signal transduction system (De Kievit & Inglewski, 2000). Quorum sensing is used to regulate virulence in *S. aureus*. The virulence of *S. aureus* is dependent on the temporal expression of a diverse array of virulence factors, including both cell associated products, such as protein A, collagen- and fibronectin-binding proteins and the fibrinogen binding clumping factors, and secreted products including lipases, proteases, alpha-toxin, toxin-1, beta-haemolysin and enterotoxins B, C and D (De Kievit & Inglewski, 2000). Expression of the *agr* locus down-regulates expression of the exponential phase proteins such as protein A whilst up-regulating expression of post-exponential proteins such as the enterotoxins (Lina et al., 1998).

During the early stages of *S. aureus* infection, surface proteins involved in attachment (e.g. collagen- and fibronectin-binding proteins and the clumping factors) and defence (e.g. protein A) predominate. Timely, correct expression of the virulence

factors is essential for the establishment of an infection and is a highly regulated process (Kullik, Giachino & Fuchs, 1998). However, once a high cell density at the site of infection is achieved, expression of the *S. aureus* surface proteins is decreased and secreted proteins are preferentially expressed. The bacteria also have to recognise and respond to certain signal provided by the host. Such signals could be temperature (increases upon infection), peroxide (released by macrophages), pH shifts, or the presence or absence of specific carbon or energy sources (Kullik et al., 1998). The genetic basis for this temporal gene expression depends on two individual genes, which control several distinct phenotypic effects, so-called pleiotropic regulatory loci namely the accessory gene regulator (*agr*) and staphylococcal accessory gene regulator (*sar*) (De Kievit & Inglewski, 2000).

1.4.2.2 The Global Regulatory Genes, *sar* and *agr*, in *Staphylococcus aureus*.

The pathogenesis of *S. aureus* is dependent on the expression of cell wall-associated adhesins and the secretion of extracellular toxins. Control of this expression and excretion is a complex process but the global regulatory genes, the accessory gene regulator (*agr*) and the staphylococcal accessory gene regulator (*sar*), are known to play an important role in the regulation of expression of multiple virulence factors, including cell wall adhesins, in *S. aureus* (Shenkman et al., 2001). In orthopaedics in particular the *agr* system has been implicated in biofilm-associated osteomyelitis (Yarwood et al., 2004).

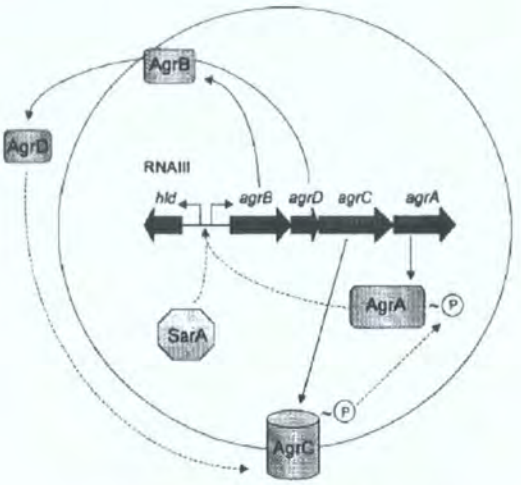
1.4.2.2.1 The *agr* Locus:

The agr locus consists of two divergently transcribed operons, RNAII and RNAIII. The RNAII contains the *agrBDCA* genes that encode the signal transducer

(AgrC) and response regulator (AgrA), and AgrB and AgrD which are involved in generating the quorum-sensing signal molecule (De Kievit & Inglewski, 2000). The RNAIII operon encodes a δ-haemolysin and is itself a regulatory RNA that plays a key role in the *agr* response (De Kievit & Inglewski, 2000). During *S. aureus* quorum sensing, the AgrC signal transducer is autophosphorylated in response to the octapeptide signal molecule, which in turn leads to phosphorylation of the AgrA response regulator (Ji, Beavis & Novick, 1995). Phosphorylated AgrA stimulates transcription of RNAIII. RNAIII, in turn, up-regulates expression of numerous *S. aureus* exoproteins as well as the *agrBDCA* locus (De Kievit & Inglewski, 2000). The latter leads to a rapid increase in the synthesis and export of the octapeptide signal molecule (Ji et al., 1995). At the second regulatory locus, the *sar* gene product (SarA) functions as a regulatory DNA-binding protein to induce expression of both the RNAII and RNAIII operons of the *agr* locus (De Kievit & Inglewski, 2000). Figure 1.1 shows the role of the *agr* locus and SarA in quorum sensing.

However, there is a further product of the *sar* locus, SarX, expressed maximally during the post-exponential phase of growth, which has a negative effect on the transcription of *agr* RNAII and RNAIII (Manna & Cheung, 2006).

Quorum Sensing in *Staphylococcus aureus*



(De Kievit & Inglewski, 2000)

Figure 1.1: The diagram above depicts quorum sensing in Staphylococcus aureus. At the *agr* locus, *agrBDCA* encodes proteins required to produce and detect AgrD, the peptide signal molecule. Another, divergent operon, *hld,* encodes δ-haemolysin and RNAIII. AgrD is an octapeptide produced via cleavage of the *agrD* gene product. It is passed extra-cellularly by AgrB, which is a membrane-associated protein. Once a threshold concentration of AgrD is reached, autophosphorylation of a transmembrane protein, AgrC occurs. AgrC in turn phosphorylates the response regulator, AgrA, which, once phorphorylated, stimulates expression of RNAIII. RNAIII is regulatory, and a rise in its concentration results in an increased production of numerous factors, reduced expression of specific surface proteins, and induced expression of the *agrBDCA* operon. A DNA-binding protein, SarA, encoded by the *sar* locus, adds to the picture by upregulating expression of both *agrBDCA* and *hld* operons (De Kievit & Inglewski, 2000).

1.4.2.2.2 The *sar* locus:

The *sar* locus is composed of 3 overlapping transcripts, designated *sarA*, *sarC* and *sarB* (Manna, Bayer & Cheung, 1998). These originate from 3 distinct promoters, P1, P3 and P2 respectively. The expression of the 3 transcripts varies during the growth cycle. The expression of *sarA* and *sarB* is maximal during the exponential-phase of growth, but *sarC* is maximally expressed during the post-exponential phase (Manna et al., 1998). Molecular analysis has shown that the *sarB* transcript is essential for the full expression of RNAII and RNAIII, the 2 operons of the agr locus, in *S. aureus*. Hence *agr* activation is at least partially mediated by the binding of the *sar* gene products to the *agr* promoter (Manna et al., 1998). One can also conclude from this that the mechanism by which *sar* is activated from its own promoter will have a bearing on *agr* expression. P3 is the central promoter of *sar*, and studies have shown that this is σ^B dependent (Manna et al., 1998).

More recently however, SarA has been shown to be involved directly in biofilm formation by enhancing transcription of the *ica* operon, the gene controlling production of polysaccharide intercellular adhesin (PIA). Furthermore, this function has been shown to be σ^B independent (Valle et al., 2003). SarA also plays an important role in the expression of Bap and so it is perhaps not surprising, that *sarA* mutants have a decreased ability to form biofilm (Beenken, Blevins & Smeltzer, 2003; Trotonda et al., 2005).

Another recent discovery, SarX, maximally expressed during the post-exponential phase of growth, has a negative effect on the transcription of *agr* RNAII and RNAIII. The transcription of SarX though, is uniquely controlled by MgrA, a SarA-like protein. Hence *sar* has both a positive and a negative effect on *agr* transcription and is, itself, subject to multiple controls (Manna & Cheung, 2006).

1.4.2.2.3 Alternative Sigma Factor: Sigma B.

In bacteria, alternative sigma factors are known to play a role in regulating gene expression upon major changes in the environment. An alternative sigma factor, σ^B, has been identified in *S. aureus* and its induction has been shown to occur during the stationary phase of growth and on heat shock (Kullik et al., 1998). In work on other bacteria, such as *Bacillus subtilis*, the corresponding sigma factor has been shown to be itself, a target of a complex regulatory network, which controls gene expression in response to stress and certain stationary-phase-specific signals (Hecker, Schumann & Volker, 1996). In *S. aureus*, σ^B modulates several other functions, including peroxide resistance, possibly alkali stress response, cell aggregation and production of lipase and thermonuclease. The transcription of the global regulator *sar* is also at least partially controlled by σ^B (Kullik et al., 1998). The Sar protein is required for expression of the regulator *agr*, which is itself a global regulator involved in the expression of virulence genes. Thus it seems that σ^B is a stress- and stationary-phase specific global regulator which is directly and indirectly associated in the expression of virulence genes (Kullik et al., 1998).

Sigma-B is itself subject to higher control mechanisms by the *rsbU* gene (Kennedy & O'Gara, 2004). The functional protein produced by this *rsbU*, RsbU, is an activator of σ^B (Knobloch et al., 2001). RsbU has thus been shown to have a positive effect on σ^B regulated biofilm formation in *S. epidermidis* (Knobloch et al., 2004).

1.4.3 Stress Resistance

1.4.3.1 The *clpC* Locus

clpC encodes ATPase ClpC, a class III group heat shock protein. The Clp protein complexes play a crucial role in intracellular homeostasis under stress conditions. They are involved in energy-dependent proteolysis, their name, Clp, being an abbreviation of caseinolytic protease. The Clp complex is sigma-B independent in its effect on stress resistance. Furthermore, the Clp complex is vital for reactivation and refolding of proteins damaged by conditions of stress (Chatterjee et al., 2005).

1.5.0 L-Forms of Bacteria

The peptidoglycan cell wall of bacteria serves as a mechanical framework and is a major determinant of cell shape. The peptidoglycan cell wall can expand or contract greatly in response to changes in environmental osmolarity or pH, but ultimately provides a strong barricade to withhold the high turgor pressures present inside bacteria (Doyle & Marquis, 1994). Cell walls also protect bacteria from the environment and help regulate the flow of nutrients and toxins into, and out of, the cells (Fung, 1971).

However, under certain environmental conditions, for example in the presence of cell wall-active antibiotics, phenotypic variants of bacteria grow, which are cell wall deficient (CWD). Cell wall deficient bacteria (CWDB), often referred to as L-forms, can continue to grow, metabolise and even divide, and can be propagated indefinitely on suitable media (Fuller et al., 2005; Fung, 1971). L-forms are defined as cell-wall-deficient organisms that are induced from a bacterium following suppression of the synthesis of its rigid cell wall. Spheroplasts and protoplasts are not the same thing as L-forms. Instead, they are spherical structures, produced from bacteria, by

complete (protoplasts) or partial (spheroplasts) enzymatic removal of the cell wall in hyperosmolar environments (Balows et al., 1992). Fung defines 2 types of L-forms, stable and unstable. Stable L-forms are described as those, "Which are capable of dividing, but unable to revert to the original bacteria". Unstable L-forms are those which are able to revert to the original bacteria (Fung, 1971).

L-forms were first isolated and described by Klieneberger in 1935. The L-forms she grew from *Streptobacillus moniliformis*, had an altered morphology and Klieneberger termed them L1, perhaps in acknowledgement of the Lister Institute of Preventative Medicine at which she worked at the time (Balows et al., 1992; Klieneberger, 1935). At first she incorrectly surmised that the abnormal growth was that of a symbiotic organism. Dienes, however, demonstrated that the L-forms were derived from the bacilli, and reverted into bacilli on further transfer to broth media. He observed that the L-forms were also serologically similar to the bacilli. When he made similar observations in other bacteria, he became convinced that L1, and the more stereotypical bacilli of *Streptococcus moniliformis* were in fact one and the same, and not symbionts (Dienes & Weinberger, 1951).

Bacterial cells normally maintain an internal osmolarity markedly higher than that of their outside environment. The high concentration of intracellular solutes creates a considerable turgor pressure which can exceed 200 kPa (Madigan et al., 2000). This pressure is normally withheld by the strong peptidoglycan cell wall, and hence CWDB, which lack this sturdy barrier, require media of high osmolarity to survive (Fung, 1971).

S. aureus is however, classed as halotolerant, meaning that it can tolerate some increase environmental osmolarity, but that it generally grows best in the absence of the added solute, a fact exploited in the pathology laboratory to aid its isolation and

identification. High osmolarity has though, in itself, also been implicated in the formation of L-forms in *S. aureus*, and Yabu described L-form formation on solid media containing 4.8 percent sodium chloride. (Yabu, 1995)

There is increasing evidence that CWDB are important clinically, particularly as persisters in chronic infection. This topic however, is shrouded in controversy. CWDB grow only in specific conditions and are notoriously difficult to isolate from clinical specimens. Standard laboratory media may in fact act to suppress atypical forms and encourage growth of normally walled bacteria. Furthermore, their ability, in many cases, to revert to wild-type bacteria in vitro, may lead to a significant underestimation of their prevalence and their importance in disease processes. Although understanding and awareness is improving, CWDB are still often dismissed in the laboratory as quirks or coincidences and treated as nothing more than a curiosity (Domingue & Woody, 1997).

CWDB may form in response to antimicrobial therapy *in vivo*, or may form as a means of evading the host immune system. They can live and replicate in host cells and have a much greater capacity to avoid and survive phagocytosis. It is suggested that these abilities of CWDB play a crucial role in chronic and persistent infection, where CWDB may effectively lie dormant (Domingue & Woody, 1997; Michailova et al., 2007).

Much of the evidence surrounding CWDB and disease persistence, however, revolves around rat and guinea pig models. Work here has been fruitful and may highlight the role of CWDB in chronic and persistent infections of humans. One example from the literature shows a positive correlation between L-forms of *S. epidermidis* and secretory otitis media in guinea pigs, while another demonstrates a link between L-forms of *S. aureus* and chronic and latent lung infection in rats (Göksu

et al., 1996; Michailova et al., 2007). CWDB have also been isolated from urinary tract infections, glomerulonephritis, rheumatic fever, infective endocarditis, Crohn's disease, osteomyelitis, septic arthritis, ocular infections and many more (Domingue & Woody, 1997; Onwuamaegbu, Belcher & Soare, 2005).

Although some, like Onwuamaegbu et al., remain sceptical about the clinical significance of CWDB, there is a growing interest and discussion in this field (Onwuamaegbu et al., 2005). Evidence linking inter-related phenomena, like small colony variants (SCVs), to persistent infections is also growing, adding further weight to this argument (von Eiff, Peters & Becker, 2006).

1.6.0 Osmotic Regulation in Bacteria

1.6.1 Mechanosensitive Channels

During the evolution of living organisms, mechanosensation is believed to be one of the first signal transduction processes to have appeared (Martinac, 2001). In bacteria, mechanosensation is important in osmoregulation, but the evolution of mechanosensation has resulted in pain sensation, hearing, blood pressure regulation, proprioception and many other functions in animals (Martinac, 2001).

Mechanosensitive (MS) channels were first discovered in *E. coli,* but homologues have since been discovered in most bacterial groups and even in Archaea, suggesting their ubiquitous presence in microbes (Anishkin & Kung, 2005). The primary MS channels of bacteria respond to changes in water concentration rather than changes in specific solutes. Bacterial MS channels combine sensor and effector in one molecule (Anishkin & Kung, 2005).

E. coli has two MS channels, the mechanosensitive channel of large conductance (MscL), and the mechanosensitive channel of low conductance (MscS).

Experiments using double mutants of *Escherichia coli,* which contained neither the MscL gene (*mscL*), nor the MscS gene (*mscS*), showed that even mild osmotic down-shift of the environment led to cell lysis. This demonstrated that MscL and MscS are emergency valves which open and discharge solute in response to high intracellular turgor pressures (Anishkin & Kung, 2005). Searching GenBank® revealed orthologues of MS channels in *S. aureus* (http://www.ncbi.nlm.nih.gov/Genbank/).

1.6.2 Bacterial Potassium Channels

The most abundant intracellular cation is potassium, which acts as one of the major osmolytes within the cell. Together with several counter molecules like organic phosphates, and neutral molecules like glycine, potassium draws water across the lipid bilayer and into the cytoplasm sustaining a high turgor pressure. Potassium is the major intracellular cation because it interacts little with macromolecules in the cytoplasm, even at high concentration. This is in contrast to sodium which does interact due to its higher surface charge density (Kuo et al., 2005).

A putative bacterial potassium channel, called Kch, homologous to eukaryotic potassium channel protein structure, has been discovered in *E. coli* (Milkman, 1994). Milkman found the channel in all reference (ECOR) *E. coli*, and reported only very conservative variation suggesting the channels importance to the species. He suggested that the channel plays an important role in cellular response to osmotic shock (Milkman, 1994). More recently Roderick MacKinnon has overseen and managed much research into the exact structure of this potassium channel using a homologue in *Streptomyces lividans*, work which earned him a Nobel Prize for Chemistry in 2003. His team has investigated how the potassium channel achieves such impressive fidelity in distinguishing between sodium and potassium and yet is

able to maintain a throughput rate approaching the diffusion limit (Doyle et al., 1998). However, a recognisable code for this potassium channel has not been found in *Staphylococci* (Kuo et al., 2005). Nevertheless, other mechanisms of potassium transport do exist in bacteria, notably the potassium efflux system, KefC, of *E. coli* (Munro et al., 1991). KefC is also present in many other Gram-negative bacteria although not generally in Gram-positive bacteria. In spite of this it is found in *S. aureus* (Douglas et al., 1991). The presence of a putative *kefC* gene in the *Staphylococcus aureus* genome has been confirmed in this work with BLAST searches.

1.7.0 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was invented in 1983 by Kary Banks Mullis, an achievement which won him a Nobel Prize for chemistry a decade later. (Brock, 1997) It is a method of greatly amplifying DNA such that significant amounts can be produced from, in theory, just a single initial target DNA molecule. It requires the design of two short DNA oligonucleotide primers which are complimentary and can base pair to the target gene. The primers bind to opposite ends and opposite strands of the target DNA molecule. The reaction then makes use of a heat-stable DNA polymerase enzyme (*Taq*) which extends the primer until a copy of the target DNA is produced. The two copies are then heated to dissociate the double-stranded DNA molecules, the primers bind, and the process begins again. A chain reaction then ensues whereby copies are made from copies.

Taq polymerase, first isolated from the thermophile, *Thermus aquaticus,* is essential to the PCR process as its structure and function are not affected by the

heating process necessary to split the double-stranded DNA molecules and allow primer binding. (Brock, 1997)

Reverse-transcriptase or Real-time PCR uses the principles of PCR to quantify the levels of transcription occurring within cells for specific genes. It requires, firstly, that cells are harvested during a period of maximum RNA transcription as occurs in the mid-exponential growth phase. The RNA must then be recovered from the cells before undergoing reverse transcription to convert it back to DNA. The principles of PCR can then be used to quantify the level of transcription that was occurring in the original cells.

1.8.0 The Impetus for Current Research

1.8.1 Background

Recent work at Durham University has researched the induction of Cell Wall Deficient (CWD) *S. aureus* by sub-lethal doses of antibiotic, and investigated the resistance of these CWD bacteria to penicillin and lysostaphin (Fuller et al., 2005). CWD bacteria have generally been associated with antibiotic resistance since the early days of their discovery (Dienes & Weinberger, 1951).

Salt (NaCl) stress has previously been implicated as an alternative mechanism of inducing CWD *S. aureus* (Yabu, 1995). In addition, increased NaCl concentration has also been implicated in increased biofilm formation in *S. aureus* (Lim et al., 2004). Toledo-Arana et al. have noted that biofilm formation can be induced by the addition of 0.5 M NaCl to Typticase soy broth medium (Toledo-Arana et al., 2005). They suggest that this indicates that NaCl either induces or depresses genes important to biofilm formation and go on to postulate that this hints at a mechanism in *S. aureus* that senses and responds to environmental signals (Toledo-Arana et al., 2005).

1.8.2 Aims of Current Work

The first aim current work was to investigate CWD bacteria induced by NaCl stress, and elucidate whether this lead to penicillin and lysostaphin resistance. It was hypothesised that CWD bacteria induced by NaCl stress would have a higher minimum inhibitory concentration (MIC) of penicillin, and be less susceptible to lysostaphin than wild-type bacteria.

The second aim of this work was to investigate the formation of biofilm in response to NaCl stress and confirm the positive correlation noted in previous work (Lim et al., 2004; Toledo-Arana et al., 2005). A further aim of this arm of the project was to investigate whether the increase in biofilm, noted in response to increased sodium concentration, was unique to sodium, or whether it was common to other group I metals (lithium, potassium, rubidium and caesium). The hypothesis was that increased biofilm formation was caused by environmental sodium and not by an osmotic effect or other Group I metals.

The final aim of this work was to investigate the transcriptional response to increased concentrations of either sodium or potassium ions, the hypothesis being that there would be an up-regulation of biofilm genes, and a down-regulation of genes involved in production of the cell wall.

Section 2: Methods

2.1.0 Laboratory Equipment

2.1.1 Consumables

Brain Heart Infusion broth powder. CM0225. Oxoid Ltd, England, UK.

Tryptic-Soy Broth. 22092. Sigma-Aldrich Inc, USA.

Agar Bacteriological. (Agar No.1) LP0011. Oxoid Ltd, England, UK.

Yeast Extract. LP0021. Oxoid Ltd, England, UK.

Bovine Serum Albumin. A4503. Sigma-Aldrich Inc, USA.

BBL™ Tryticase™ Peptone. 211921. Becton Dickinson and Company, USA

Accustain® Safranin O Solution. HT90432. Sigma-Aldrich Inc, USA.

Accustain® Crystal Violet Solution. HT90132. Sigma-Aldrich Inc, USA.

Lugol's Iodine Solution. 351901G. VWR International Ltd, England, UK.

Gram colour, decolourising solution. 351964V. VWR International Ltd, England, UK.

Lithium Chloride. L4408. Sigma-Aldrich Inc, USA.

Sodium Chloride. 301237S. VWR International Ltd, England, UK.

Potassium Chloride. 295945C. VWR International Ltd, England, UK.

Rubidium Chloride. R2252. Sigma-Aldrich Inc, USA.

Caesium Chloride. C3032. Sigma-Aldrich Inc, USA.

Penicillin G E-test strips, 0.002-32. ABBiodisk, Sweden.

Lysostaphin. Sigma-Aldrich Inc, USA.

Proteinase K. Sigma-Aldrich Inc, USA.

Mutanolysin. Sigma-Aldrich Inc, USA.

RNeasy Protect Bacteria Mini Kit. 74524. Qiagen Ltd, England, UK.

SuperScript™ III Reverse Transcriptase. Invitrogen, England, UK.

Biotaq™ DNA polymerase PCR kit. Bioline, England, UK.

2.1.2 Hardware

PCR PxE 0.5 Thermal Cycler. Thermo Electron Corporation, USA.

Ultrospec 1100 pro. Spectrophotometer. Amersham Biosciences, England, UK.

Centrifuge 5415D. Eppendorf, USA.

RT-PCR Rotor-Gene. RG-3000. Corbett Research, England, UK.

Nanodrop® ND-1000 Spectrophotometer. Nanodrop Technologies, England, UK.

2.1.3 Bacterium Used for Experimentation.

S. aureus ATCC 9144 was used for all experiments.

It is also known as the "Oxford" strain, is β-lactamase deficient and is sensitive to penicillin.

2.1.4 Stock Cultures

A stock culture of cells was stored at -80°C in 15% glycerol. Cultures for experimentation were grown on trypticase-soy broth (TSB) agar and stored at 4°C. Cells were transferred to fresh TSB plates at least weekly, and new cultures made from the -80°C stock at least monthly.

2.2.0 Basic Techniques

2.2.1 Growth Media

All amounts given are to be made up in 1 litre of distilled, deionised water.

Trypticase-Soy Broth.

30g Trypticase-soy broth powder.

Brain Heart Infusion Broth. (BHI)

37g Brain Heart Infusion broth powder.

Yabu Medium (Yabu, 1995).

10g Peptone.

5g Yeast Extract.

48g Sodium Chloride.

50ml 10% Bovine serum albumin.

N.B. The bovine serum albumin (BSA) must be filter sterilised and added to the broth mixture after autoclave sterilisation. Addition of the BSA prior to sterilisation will result in denaturation and precipitation of the protein during the autoclave process.

Durham Brain Heart Infusion Broth (Page, unpublished).

10g Peptone.

5g Yeast Extract.

37g Brain Heart Infusion broth powder.

The above recipe is the basic broth into which was added Group I metal chloride salts for biofilm and RT-PCR experiments.

Where necessary, bacteriological agar (Oxoid Ltd, England) was added at 1% to solidify the media.

2.2.2 Photograph of an Agar Plate

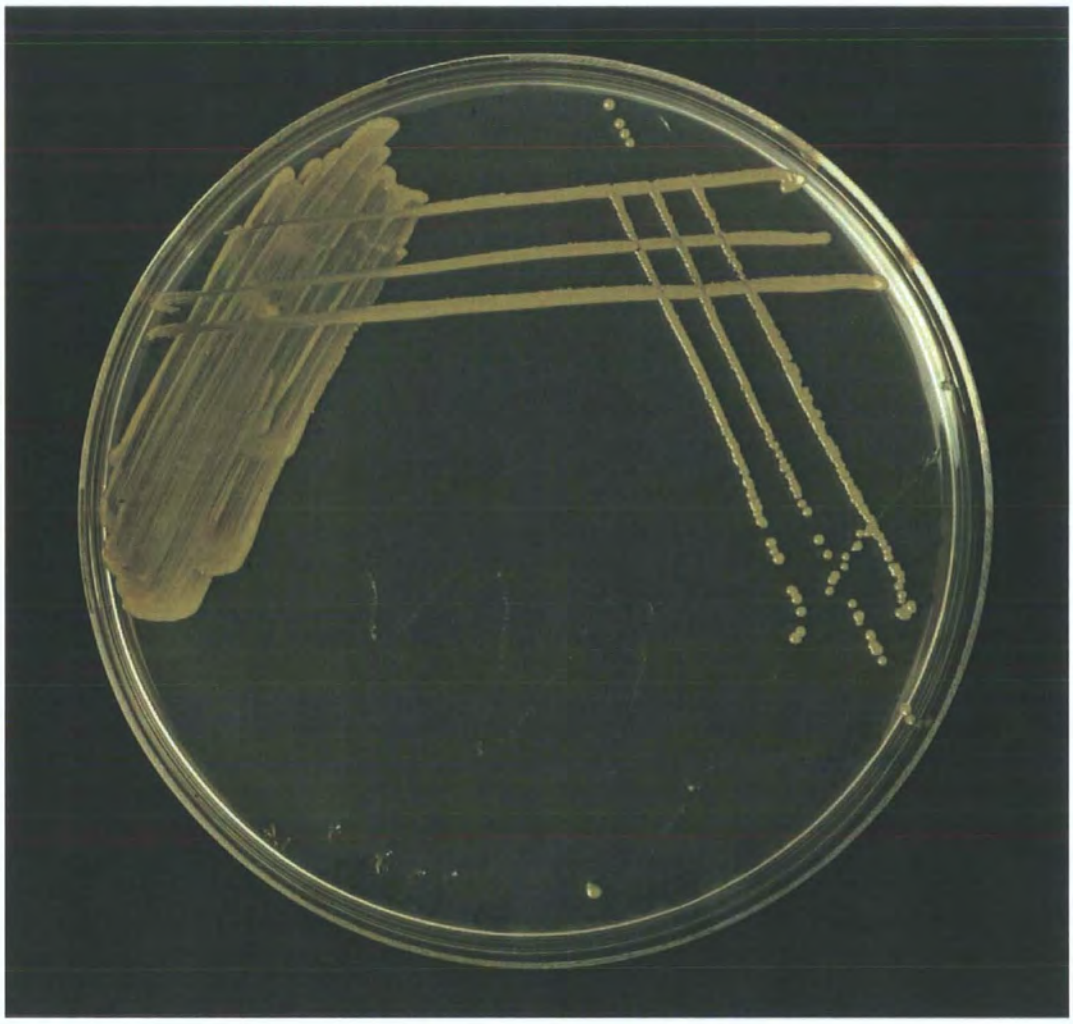


Figure 2.1: Photograph of an agar culture, showing the smearing and streaking pattern used.

2.3.0 Experiments

2.3.1 Gram Staining

2.3.1.1 Preparing a Slide from a Culture on Agar

For each slide, a flamed, cooled loop was used to place a drop of sterile water on a cleaned, labelled slide. The loop was re-flamed, cooled and used to extract half a colony from an agar plate culture which was then smeared in the drop of water, leaving a thin film. Slides were then air dried.

2.3.1.2 Preparing a Slide from Broth Culture

For each slide, a flamed, cooled loop was used to extract a loopful of medium from a broth culture, which was smeared directly onto a cleaned, labelled slide to leave a thin film. Slides were then air dried.

2.3.1.3 The Staining Technique

Slides were not heat fixed. Experimental trial showed that cell wall deficient (CWD) bacteria were destroyed by heat fixation and hence this process was not used.

Prepared slides were placed on a rack and bathed with oxalate crystal violet for 60 seconds. Slides were then washed with water, bathed in Lugol's iodine solution for 60 seconds, then drained and blotted gently. Decolourisation was performed for 30 seconds with 95% ethanol and the slides were then washed for 2s with water. Counterstain was performed with Safranin-O for 5 seconds. The slides were then washed with water, blotted gently and allowed to air dry. Coverslips were applied prior to visualisation with a microscope.

2.3.2 Lysostaphin Susceptibility Assay

For each experimental scenario, cultures were prepared overnight on agar plates. Cells were then transferred to liquid medium of the same type as the agar from which they were taken. Cells were grown to mid-exponential phase (OD_{620} 0.7-1.0), collected into a pellet by centrifugation then washed three times by re-suspension in 20mM potassium phosphate buffer pH 7.5. Finally, the washed cell pellet was re-suspended in the same buffer to an OD_{620} of approximately 0.25. The change in optical density at 620nm was then recorded against time following the addition of 1 unit of lysostaphin to 1ml of the suspension (Kusuma & Kokai-Kun, 2005).

2.3.3 E-Testing on Agar Plates

For each experiment half a colony was sampled from overnight agar culture and suspended in sterile 0.85% saline or appropriate broth to a McFarland standard of 0.5. A sterile cotton bud was used to smear the suspension evenly over an agar plate which was then dried prior to application of the E-test strip. The plate was incubated overnight and the MIC value was then read directly from the graduated E-test strip.

Trypticase soy broth (TSB) was used as the medium of choice. If exact MICs are required, such as in hospitals where antibiotic regimens are decided on the results, then iso-sensitive media should be used. In these experiments MIC trend was more important than exact MIC readings and hence TSB was an acceptable substitute.

2.3.3.1 Diagram of E-test Smearing Technique

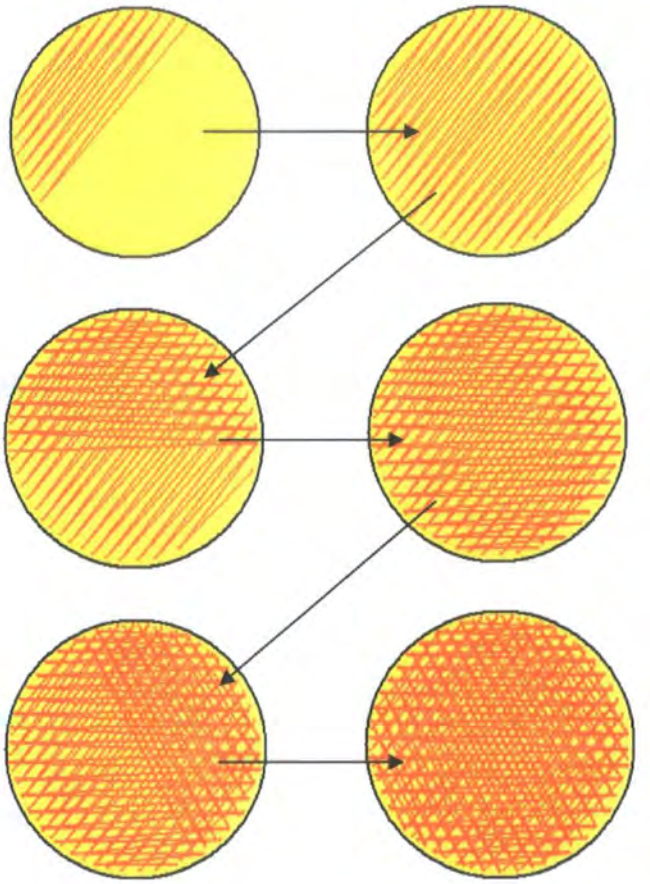


Figure 2.2: Diagram showing the smearing technique of an agar plate with a suspension of cells using a cotton bud to ensure an even spread, prior to placement of an E-test strip for antibiotic MIC assay.

2.4.0 Basic Method of Biofilm Formation and Assay on 96-Well Microtiter Plates

Method adopted from Christensen et al. (Christensen et al., 1985).

A 500µl sample of an overnight stationary-phase broth culture was diluted 1:100 in fresh, sterile broth media which was then grown to mid-exponential phase (OD_{620} 0.7-1.0) at 37°C. 200µl of this culture was pipetted into each well of a 96 well microtiter plate which was then incubated for four hours at 37°C. After incubation the contents of the wells was gently aspirated. Each well was washed three times with 200µl of sterile phosphate buffered saline. 200 µl of safranin-O dye was then pipetted into each well to stain any resultant biofilm and was then rinsed out with tap water. The plate was then dried in an incubator. Next, 200 µl of 70% Ethanol was pipetted into each well and the plate was placed on a shaker at 100 revolutions per minute for 15 minutes. The resultant solution in the microtiter plate wells was then read using a plate reader the results were recorded.

2.4.1 Group I Metal Chloride DBHI Broth Preparation

Durham Brain Heart Infusion (DBHI) broth was prepared (1g peptone, 0.5g yeast extract, 3.7g Brain Heart Infusion broth powder per 100ml). Appropriate amounts of LiCl, NaCl and KCl were added to 100ml aliquots to produce 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 molar concentrations respectively, and appropriate amounts of RbCl and CsCl were added to 50ml aliquots to produce the same concentrations. All of these were then subdivided into 5ml aliquots and sterilised in an autoclave prior to experimentation.

1.4M concentrations were chosen as the maximum concentrations used because of previous documentation in the literature that generally only *S. aureus* can

grow on media supplemented with 7.5 percent NaCl (approximately 1.4mol/l) (Chapman, 1945).

2.4.2 Table of Group I Metal Chloride Amounts Used.

Concentration	LiCl	NaCl (*)	KCl	RbCl (#)	CsCl (#)
0.1mol/l	0.42g	0.08g	0.75g	0.61g	0.84g
0.2mol/l	0.85g	0.67g	1.49g	1.21g	1.69g
0.4mol/l	1.7g	1.84g	2.98g	2.42g	3.37g
0.6mol/l	2.54g	3.01g	4.47g	3.63g	5.05g
0.8mol/l	3.39g	4.18g	5.95g	4.84g	6.74g
1.0mol/l	4.24g	5.34g	7.46g	6.05g	8.42g
1.2mol/l	5.09g	6.51g	8.95g	7.26g	10.10g
1.4mol/l	5.93g	7.68g	10.44g	8.47g	11.79g

Figure 2.3: The table above shows the masses of each Group I metal added to 100ml of DBHI to achieve the concentration listed in the left hand column.

*The above amounts of NaCl are each 0.5g lower than the calculated amounts required to produce the respective molar concentrations. This takes into account the fact that the Brain Heart Infusion broth powder used as one of the ingredients to make the DBHI results in a solution that already contains 5g NaCl per litre or 0.5g per 100ml. Hence it also needs to be acknowledged that all of the other Group I metal chloride solutions will contain 0.5% NaCl as well as the respective concentration Group I metal chloride in that solution.

The rubidium chloride and caesium chloride amounts were mixed with 50ml of DBHI and not 100ml, and are correspondingly lower.

2.4.3 Group I Metal Chloride Biofilm Assay.

A stationary-phase culture was prepared overnight in Trypticase-soy broth. 50µl of the overnight culture was added to a test tube containing 5ml of sterile DBHI to form a 1:100 dilution. The same operation was performed to inoculate 5ml test tubes of DBHI broth containing 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 M concentrations of LiCl respectively. These tubes were incubated to mid-exponential phase in aerobic conditions, at 200rpm, at 37°C.

96-well microtiter plates were then filled with the respective cultures as demonstrated in the diagram below. Wells A1-A8 contained sterile DBHI as a no bacteria control. The other wells contained cultures as shown.

2.4.3.1 Diagram of a 96-Well Microtiter Plate Demonstrating the Filling Procedure.

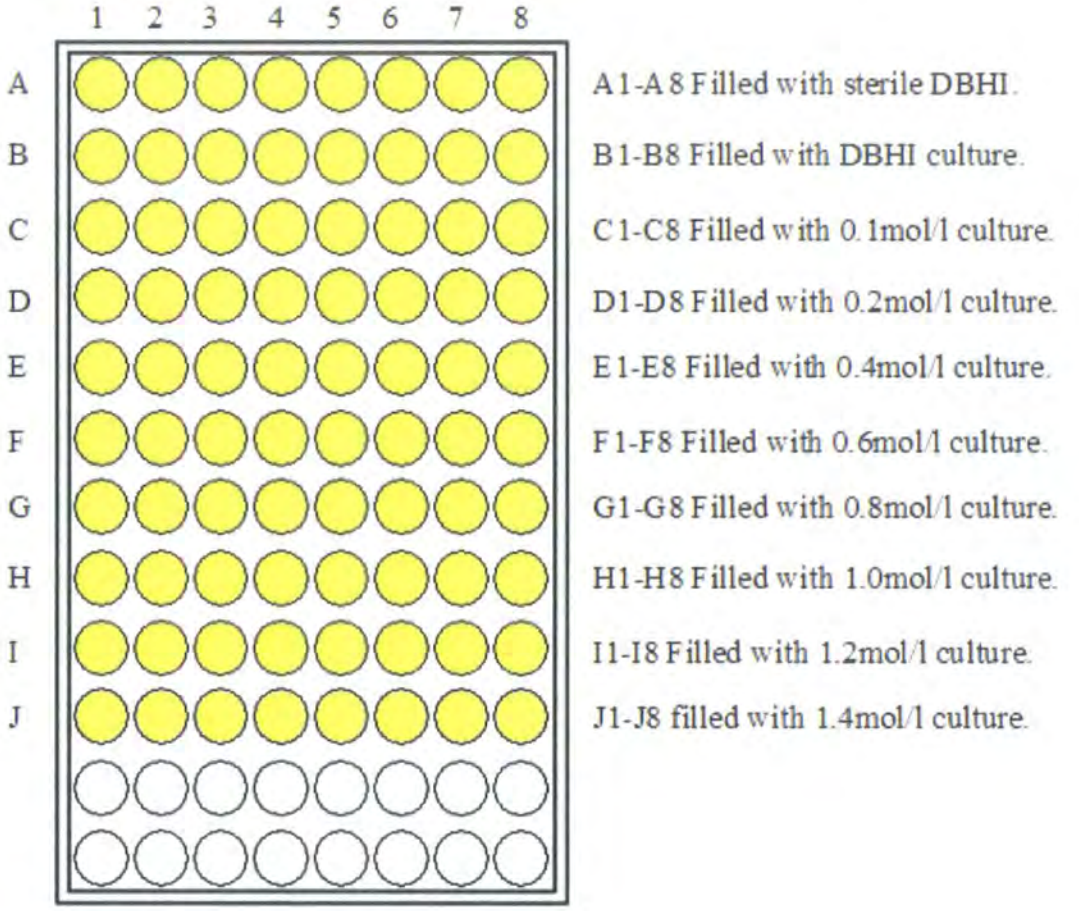


Figure 2.4: The figure above shows a representative diagram of a 96-well microtiter plate. The numbers along the top op the plate and the letters down the left hand side of the plate indicate the nomenclature of the wells. The text to the right of the diagram describes which culture was placed in each well. By filling eight wells with each respective culture, each experiment performed has eight technical replicates.

Once the plates were filled as above, they were incubated for 4 hours at 37°C without agitation in aerobic conditions. The rest of the method of plate preparation follows the basic method of biofilm assay as described above and the plates were then read in a microtiter plate reader

The above process was repeated in exactly the same way substituting the test tubes containing LiCl with appropriate test tubes of NaCl, KCl, RbCl and CsCl respectively. To ensure uniformity and comparability of results, all experiments were performed in parallel, and the same overnight culture was used to inoculate all the test tubes.

2.5.0 Polymerase Chain Reaction.

2.5.1 Crude Isolation of DNA from Bacterial Cells.

A 1ml sample of stationary-phase culture was centrifuged at 5000xg for 10 minutes. The supernatant was decanted and the pellet re-suspended in 1ml of buffer containing 10mM Tris, 1mM EDTA at pH 8.0 ($T_{10}E_1$). The sample was centrifuged again for further 5 minutes at 5000xg and the supernatant decanted prior to further re-suspension in 1ml of $T_{10}E_1$ buffer. The process of centrifugation and decantation was repeated once more but the pellet was then re-suspended in 200µl $T_{10}E_1$ buffer. An Eppendorf tube containing the resultant suspension was then placed in boiling water for 10minutes.

After boiling, the suspension was again centrifuged for 5 minutes at 5000xg and the supernatant collected and placed in a separate sterile Eppendorf tube. The supernatant collected contains the cellular DNA. The concentration of the nucleic acid can be checked with a nanodrop spectrophotometer (nanodrop technologies, England).

Note: This is a crude method of DNA harvesting. It is suitable for production of DNA used to check primers and optimise PCR. Some staphylococci, and in particular *S. aureus,* often produce a thermostable deoxyribonuclease (DNAase) (Greenwood et al., 2003). This crude production of DNA does not take account of DNAase enzymes and should therefore be used promptly and not stored for long periods of time.

2.5.2 Design of Primers.

Target genes of experimental interest were identified from the literature. The genetic sequences of the chosen genes were ascertained using GenBank® (http://www.ncbi.nlm.nih.gov/Genbank/).

These genetic sequences were used to design primers. For each gene, two primers were designed, one forward and one reverse. The following general principles of primer design were used:

1. Complimentary sequences in the primers were avoided to prevent the formation of primer dimers.
2. The primers were designed to have similar melting temperatures (Tm).

   N.B. The melting temperature can be roughly calculated by the following equation: $Tm = 4(G+C) + 2(A+T)$
3. The primers were designed to produce amplicons of approximately the same size, in this case 100-200 base pairs. A small size of amplicons was preffered to facilitate RT-PCR in later experiments.

The OligoPerfect™ Designer on the Invitrogen website was used to aid primer design (http://www.invitrogen.com/content.cfm?pageid=9716).

Primers were synthesised by Sigma-Aldrich.

2.5.3 PCR Reaction Mixture

A standard single reaction mixture of 50µl in volume was used for each experiment.

The 50µl reaction mix was made up of:

5µl	10x NH_4 buffer.	1X
1.5µl	50mM $MgCl_2$	1.5mM
1.0µl	10mM dNTPs (deoxy-nucleotide building blocks).	0.2mM
1.0µl	20µM Primer 1. (Forward primer).	0.4µM
1.0µl	20µM Primer 2. (Reverse primer).	0.4µM
0.2µl	*TAQ* polymerase.	
3ng	Target Nucleic Acid.	
	Sterile, deionised H_2O to total of 50µl.	

N.B. The figures at the right show the final concentrations when made up to 50µl.

In order to appropriately control each experiment, 4 reactions for each target gene were performed. The main reaction contained both primers and the target DNA for amplification. The control reaction contained both primers but no DNA. One reaction contained only the forward primer with DNA to verify that no product was formed from only that primer. The final reaction contained only the reverse primer with DNA to verify that no product was formed from only that primer.

Master-mixes were made for ease of experimentation. These were large mixes that contained all the common elements for a series of PCR reactions. The unique components were left out and pipetted separately into each reaction as required. The *TAQ* was only added to the master-mix when the reaction was ready to begin.

2.5.4 PCR Amplification Cycles

Once reaction mixtures have been prepared they need to go through a process of heating, to denature the strands of DNA, followed by a series of cycles of heating, annealing and extension. They then require a final extension to ensure that all amplicons are double-stranded and full length.

The typical reaction cycle used was:

1 cycle of:	95°C for 5 minutes.
30 cycles of:	95°C for 30 seconds.
	55°C-65°C for 30 seconds.
	72°C for 1 minute (per kilobase)
1 cycle of:	72°C for 5 minutes.

The annealing temperature is variable and needs to be optimised for each reaction.

2.5.5 Agarose Gel Electrophoresis.

The basic gel recipe used for running the products of the PCR reactions was:

1.5% Agarose.

1x TRIS-Acetate-EDTA buffer (1x TAE)

7.5µl Ethidium Bromide per 100ml

The concentration of agarose can be adjusted according to the amplicon size. A higher concentration will favour the passage of smaller molecules whereas a lower concentration will favour the passage of larger molecules. In these experiments ethidium bromide was used as the stain to visualise the passage of the amplicons through the agarose gel.

The agarose was dissolved in the TAE solution in a microwave and was not allowed to boil or splash. The solution was then allowed to cool, but NOT solidify, and the ethidium bromide was added ensuring that it was mixed thoroughly. Finally the gel was poured into mould of appropriate size and air bubbles eliminated. Proper formation of the wells was also ensured.

10μl of each completed reaction solution was placed in sterile Eppendorf tubes and 1μl of 10x Orange G solution added to each. This was mixed thoroughly by gently flicking the tube before briefly centrifuging.

Agarose gels were removed from the moulds and immersed in TAE buffer in an electrophoresis tank. 5μl of each reaction mix was pipetted into separate wells of the prepared agarose gel. In one well, a molecular weight marker was placed to compare against the bands produced by the reaction mixes.

Correct alignment was assured (DNA is negatively charged and will migrate towards the positively charged anode) and 100 volts was applied across the gel. N.B. At higher voltage the DNA will move faster through the gel and at lower voltage it will move slower.

The orange G dye used can be seen with the naked eye as it moves through the gel. When it had moved 2/3 of the way across the gel, the gel was removed from the tank and placed under an ultraviolet light to allow visualisation of the bands. Photographs were taken to aid analysis.

2.6.0 Reverse-Transcriptase PCR

The following processes need to be performed in order to proceed with RT-PCR in bacteria.

1. Bacterial cells need to be harvested in mid-exponential growth when they are maximally transcribing RNA in response to their environment to optimise their growth and survival.
2. The RNA needs to be collected from the cell with minimal loss.
3. The RNA then needs to undergo reverse transcription to produce copy DNA (cDNA) which can then be used for the RT-PCR reaction.
4. Appropriate genes need to be identified and thence primers designed for use in the RT-PCR experiment.

In RT-PCR, if the gene of interest has been up-regulated by the cells in the experiment, then there will be more cDNA of that gene and hence it will be detected earlier when its rapid amplification begins in the polymerase chain reaction. If the gene of interest is down-regulated, then there will be less cDNA of that gene and hence its presence will be detected later when the polymerase chain reaction is started.

2.6.1 RNA Preparation

For bacterial RNA extraction and protection the RNeasy® Mini Kit (Qiagen, UK) was used. All buffers and solutions were prepared, and experiments performed as described in the RNeasy® Mini Handbook protocols.

Bacterial broth cultures were prepared and cells grown to mid-exponential phase. A 0.5ml sample of the culture was taken and placed in an RNA-free Eppendorf tube to which was added 1ml of RNAprotect reagent (Quiagen, UK). Bacterial pellets

were harvested by centrifuging at 5000g for 5 mins at 4°C. The supernatant was then decanted and any remaining media was carefully removed by aspiration.

Cell lysis was performed by re-suspending the pellet in 100µl TE buffer containing 100unit/ml lysostaphin, 0.2mg/ml Proteinase K and 500unit/ml mutanolysin and incubating at room temperature for 1 hour with occasional vortexing.*

After incubating 350µl of Buffer RTL was added to the sample and mixed thoroughly by vortexing vigorously. If insoluble material was visible, the sample was centrifuged for 2 mins in a microcentrifuge at maximum speed and only the supernatant was used in subsequent steps.

Next 250µl of ethanol (96-100%) was added to the lysate and mixed thoroughly by pipetting and NOT centrifugation. The sample (usually 700µl), including any precipitate that may have formed, was then added to an RNeasy mini column placed in a 2ml collection tube. The tube was then centrifuged for 15s at 8000g and the flow-through discarded.

Next, 700µl Buffer RW1 was added to the RNeasy column and the tube centrifuged for 15s at 8000g to wash the column. The flow-through and collection tube were then discarded before transferring the RNeasy column into a new 2ml collection tube.

500µl of Buffer RPE were then pipetted onto the RNeasy column and the tube centrifuged again for 15s at 8000g to wash the column and the flow-through was discarded.

Another 500µl of Buffer RPE was added to the RNeasy column as per the manufacturer's instructions and the tube centrifuged for 2 mins at 8000g to dry the RNeasy silica-gel membrane. To eliminate any chance of possible Buffer RPE

carryover, the extra step of centrifuging in a microcentrifuge for 1 min at full speed was taken.

The elution step was performed by transferring the RNeasy column to a new 1.5ml collection tube, pipetting 30-50µl RNase-free water directly onto the RNeasy silica-gel membrane, and centrifuging for 1 min at 8000g. The concentration of the eluted RNA was measured with a nanodot machine. Serial elutions were performed until the RNA yield declined.

* The above protocol is as described in the Qiagen RNeasy Mini handbook, Third Edition (06/2001) apart from the process of cell lysis. The process of cell lysis described here was developed by Dr Tony Fawcett, Durham University, specifically for use with *S. aureus*. It results in a more specific and efficient cell lysis and hence a greater and more accurate RNA yield.

2.6.2 Reverse Transcriptase Reaction

The concentration of RNA in the sample to be used was measured using a nanodrop machine and a volume containing a total of 1μg of RNA was then placed in an RNA-free Eppendorf tube using a sterile, filter-tipped pipette. 1μl of hexamers (Invitrogen, UK) was added and a volume of sterile, RNA-free water, such that the total volume was 11μl. This mixture of RNA, hexamers and water was then heated to 65°C for 5 minutes before being cooled on ice for 5 minutes.

The other reactants were prepared in a master-mix which contained the following amounts per reaction:

4μl 5x ST Buffer.

2μl 0.1M DTT.

1μl 10mM dNTPs

1μl RNA sin

1μl SuperScript™ Reverse Transcriptase III

This gives a total of 9μl per reaction.

9μl of the master-mix was then added to each 11μl RNA mix to give total reaction volumes of 20μl. The mixtures were then incubated at room temperature for 10 minutes, 50°C for 50 minutes and 85°C for 5 minutes. The resultant DNA was assed with a nanodrop machine and stored at -20°C.

2.6.3 RT-PCR (Chatterjee et al., 2005)

The standard single reaction mixture used for RT-PCR was 20 microlitres in volume. This was made up of:

2µl 1x NH_4 buffer.

1.2µl 3mM $MgCl_2$

0.4µl 0.2mM dNTPs

4µl 400nM primers

0.08µl *TAQ*

0.2µl *SYBR* Green

25ng cDNA (Recombinant DNA)

Sterile, deionised water to a total of 20µl.

Master-mixes were made for ease of experimentation that contained all the common elements for a series of RT-PCR reactions. The unique components were left out and pipetted separately into each reaction as required (Primers). The *TAQ* and *SYBR* Green were added to the master-mix only when the reactions were ready to be started. All master-mixes and tubes were kept on ice.

Once the reaction tubes were fully prepared they were secured in the RT-PCR machine and the program set-up. The typical program used ran a heating step for 5 mins at 95°C, followed by 40 cycles of: 95°C for 20s, 56°C for 20s, 72°C for 30s. Three reactions were performed for each specific gene using the appropriate primers. This was done to give an average reading and ensure validity of results. The Relative Expression Software Tool V1.9.12 (REST 2005) was used to evaluate the results (Pfaffl, Horgan & Dempfle, 2002).

N.B. *SYBR* Green is light-unstable and must be kept in the dark. It is necessary to mix up new batches regularly to ensure effective performance. Reactions need to be optimised for each new batch of *SYER* Green to ensure efficiency. Whenever possible, repeat or multiple experiments should be performed in succession using the same batch of *SYBR* Green to ensure comparability of both experimentation and results.

2.6.4 Comparative RT-PCR on Cells Grown in DBHI Under NaCl Stress and Under KCl Stress.

S. aureus ATCC 9144 was cultured in Trypticase-Soy Broth to stationary-phase. Three biological replicates were then grown from this stationary phase culture.

1. A 1:50 culture was prepared in DBHI.
2. A 1:50 culture was prepared in DBHI supplemented with 1.4M NaCl.
3. A 1:50 culture was prepared in DBHI supplemented with 1.4M KCl.

The three cultures were then grown to mid-exponential phase, checked by spectrophotometric absorbance. 1ml was taken from each culture and mRNA was protected and removed from the cells using the RNEasy© Mini Kit (Quiagen, UK). (See Section 2.6.1) The collected mRNA was then converted to copy DNA (cDNA) by reverse transcriptase reaction (See Section 2.6.2). Comparative RT-PCR was then performed individually on the three cDNA samples. The results were then analysed with the Relative Expression Software Tool V1.9.12 (REST 2005) to assess any differences in the transcription of the genes under experimentation.

Section 3: Results

3.1.0 The Effect of Salt (NaCl) Stress on *Staphylococcus aureus.*

NaCl stress has previously been implicated in the growth of colonies of L-forms of *S. aureus* (Yabu, 1995). Kunihiko Yabu reported the visualisation of classic "fried-egg" colonies when *S. aureus* strain 209P was grown on his medium. The medium comprised of 1.0g peptone, 0.5g yeast extract, 1.2g agar and 4.8g NaCl per 100ml. This was supplemented with 5ml of 10% bovine serum albumin. He reported that when exponentially growing cells were plated on the agar medium, more than 99% of the cells inoculated developed L-form colonies. These experiments were repeated in the current study using *S. aureus* Oxford, strain ATCC 9144.

3.1.1 Cell Wall Deficient Bacterial Growth on Yabu Agar

Growth of *S. aureus,* strain ATCC 9144, on the Yabu medium led to a mixed appearance of colonies. The plate appeared, at first glance, to be contaminated, with two distinct colony types visible (See Figure 3.1). There was growth of small colonies, which stained Gram-negative, and large colonies, which stained predominantly Gram-positive. This result is contrary to Yabu's results with a different strain of *S. aureus.*

The mixed growth of the *S. aureus,* strain ATCC 9144, on the Yabu agar initially looked like contaminated growth, but repeated experiments consistently showed that the two colony types both originated from the same bacterium, in a similar manner to the classical L-form description. The fact that they originated from the same bacterium was confirmed by simultaneously inoculating culture onto trypticase-soy agar and Yabu agar. The small, Gram-negative colonies only formed

on the Yabu agar. Subsequent serial growth of Gram-negative cells from the Yabu agar onto trypticase-soy agar, resulted in reversion to classical golden colonies of Gram-positive clusters of cells (See Section 3.1.3).

3.1.1.1 Photograph of *Staphylococcus aureus* Growing on Yabu Agar

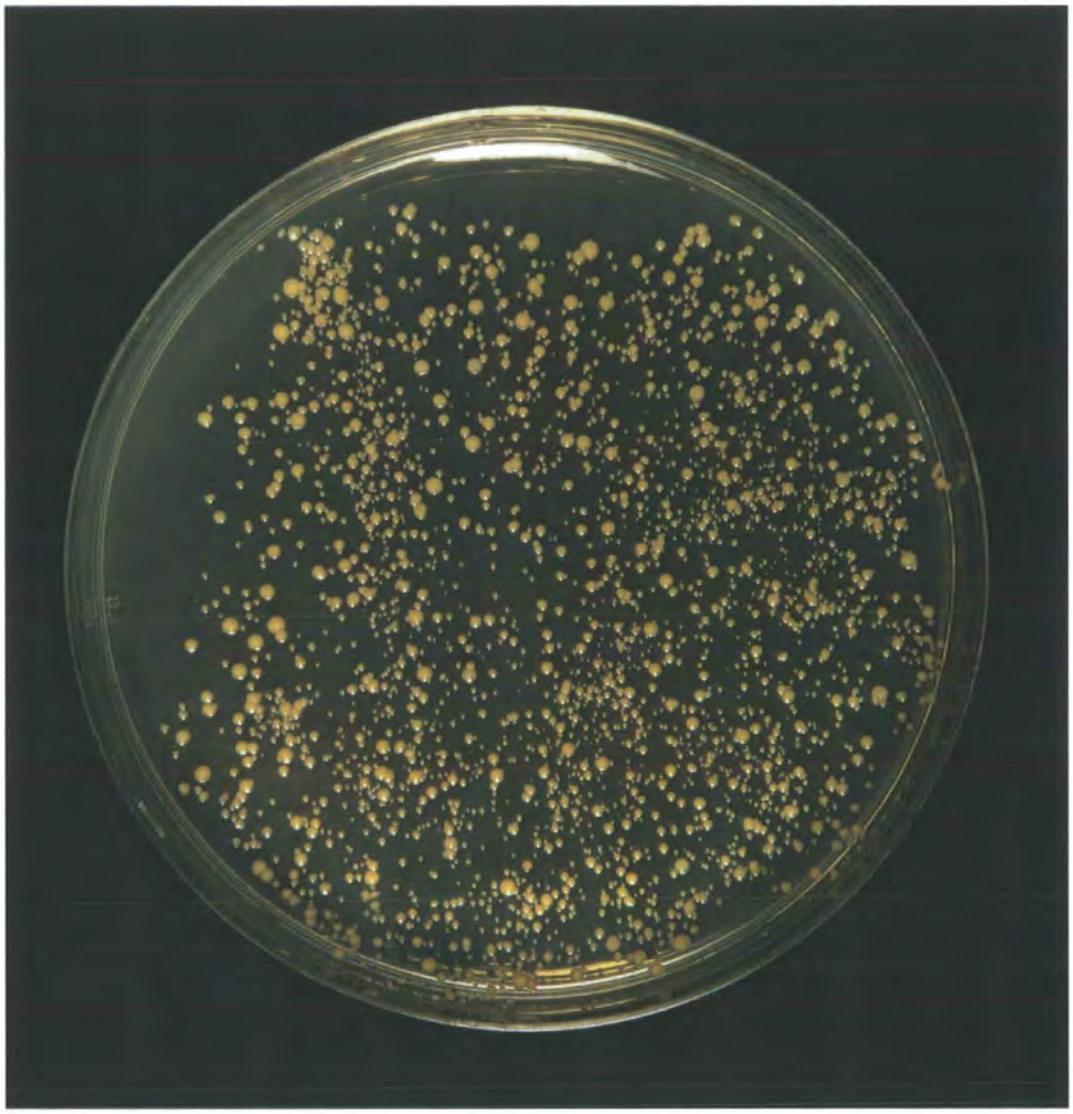


Figure 3.1: Photograph of *S. aureus* ATCC 9144 growth on Yabu agar showing a mixed growth pattern of small colonies (Gram-negative, cell wall deficient bacteria) and large colonies (Gram-positive, cell wall intact bacteria).

3.1.2 Differential Cell Counts on Yabu Agar, a Salt Stress Medium, Verses Trypticase-Soy Agar, a Standard Growth Medium.

Cell counts were performed on *S. aureus* ATCC 9144 cultured in trypticase-soy broth. The cell counts were performed on plates of trypticase-soy broth agar (TSB), a standard growth medium, and the Yabu agar, a salt (NaCl) rich medium. An exponential phase culture was used with an absorbance of 0.742 (OD_{600}).

The same cultures and dilutions were used to inoculate plates of the 2 different agars, but the cell suspensions used gave an apparent 15-fold difference in the cell count. The experiments were done in triplicate.

On TSB agar an average of 42 cells grew on the plates inoculated with 100µl of 10^{-6} dilution. This equates to $4.2x10^{7}$ cells per 100µl or $4.2x10^{8}$ cells per ml.

On the Yabu agar, an average of 277 cells grew on the plates inoculated with 100µl of 10^{-4} dilution. This equates to $2.77x10^{6}$ cells per 100µl or $2.77x10^{7}$ cells per ml.

The differences between the cell counts performed on the 2 different agars suggests that the high salt (NaCl) content of the Yabu agar has, at least, an inhibitory effect on the cell growth. This is consistent with classification of *S. aureus* as halotolerant. Of the cells that do grow, some persist with a Gram-positive phenotype and produce normal colonies, but others adopt a Gram-negative phenotype and grow into smaller colonies.

3.1.3 Salt (NaCl) Stress Leads to an Increased Minimum Inhibitory Concentration of Penicillin G.

Kleineberger was the first to describe what were termed L-forms and are now often referred to as cell wall deficient (CWD) bacteria, when she described the L1

form of *Streptobacillus moniliformis* in 1935 (Klieneberger, 1935). In 1942, Cynthia Pierce noted that these L1 forms were resistant to penicillin (Pierce, 1942). Early work linked the formation of L-forms to antibiotic usage (Fung, 1971). More recently, research has demonstrated more clearly a role of cell wall deficient bacteria in penicillin resistance (Fuller et al., 2005). It was, however, the antibiotic agent itself which was the key ingredient in both the initiation of the CWD bacteria and also in the generation of the antibiotic resistance in that research (Fuller et al., 2005). The current work looks at NaCl induced L-forms, and investigates whether they have any increased resistance to penicillin.

S. aureus, ATCC 9144, was grown alternatively on Trypticase-soy broth agar (TSB) and Yabu, high salt, agar. Cultures were labelled with the addition of a T after every passage on TSB and with the addition of a Y added after every passage on Yabu agar. Hence, the initial culture grown on TSB was labelled T, and the subsequent transfer and subculture onto Yabu, TY. Figure 3.2 shows the order of sub-cultures performed in table form and figure 3.3 shows the same in diagrammatic form.

Colonies were harvested, suspended in 0.85% saline, as per the manufacturers instructions, and inoculated onto agar plates with penicillin G E-test strips to assess the minimum inhibitory concentration (MIC). Colonies were also Gram stained to evaluate their cell wall status. Other colonies were re-suspended in 20mM potassium phosphate buffer and lysostaphin susceptibility assays performed. The results of the E-test and Gram stains are summarised in the following table (See Figure 3.2).

3.1.3.1 Table of Results of E-Test After Salt (NaCl) Stress

	Penicillin G E-test MIC in mg/l	Result of Gram Stain
Culture on TSB agar (T)	MIC 0.008 mg/l	Gram-positive cocci
Subculture onto Yabu agar (TY)	MIC 0.016 mg/l	90% Gram-negative cocci
Subculture onto TSB agar (TYT)	MIC 0.023 mg/l	Mixed Gram-negative and Gram-positive cocci
Subculture onto Yabu agar (TYTY)	Not Determined	Not Determined
Subculture onto TSB agar (TYTYT)	Not Determined	Not Determined
Subculture onto Yabu agar (TYTYTY)	MIC 0.047 mg/l	Gram-negative cocci
Subculture onto TSB agar (TYTYTYT)	MIC 0.023 mg/l	Mixed Gram-positive and Gram-negative cocci
Subculture onto TSB agar (TYTYTYTT)	MIC 0.012 mg/l	90% Gram-positive cocci
Subculture onto TSB agar (TYTYTYTTT)	MIC 0.012 mg/l	Gram positive cocci
Subculture onto TSB agar (TYTYTYTTTT)	MIC 0.008 mg/l	Gram positive cocci

Figure 3.2: The table above summarises the results of the E-test and Gram stains performed. It shows that the MIC results increase with increasing episodes of salt stress, but that this increase is unstable and returns to baseline levels after removal of the salt stress. It also shows that salt stress leads to a Gram-negative phenotype but that this change too, is unstable. The cells return to a Gram-positive phenotype after removal of the salt stress.

3.1.3.2 Illustration of Results of E-Test After Salt (NaCl) Stress

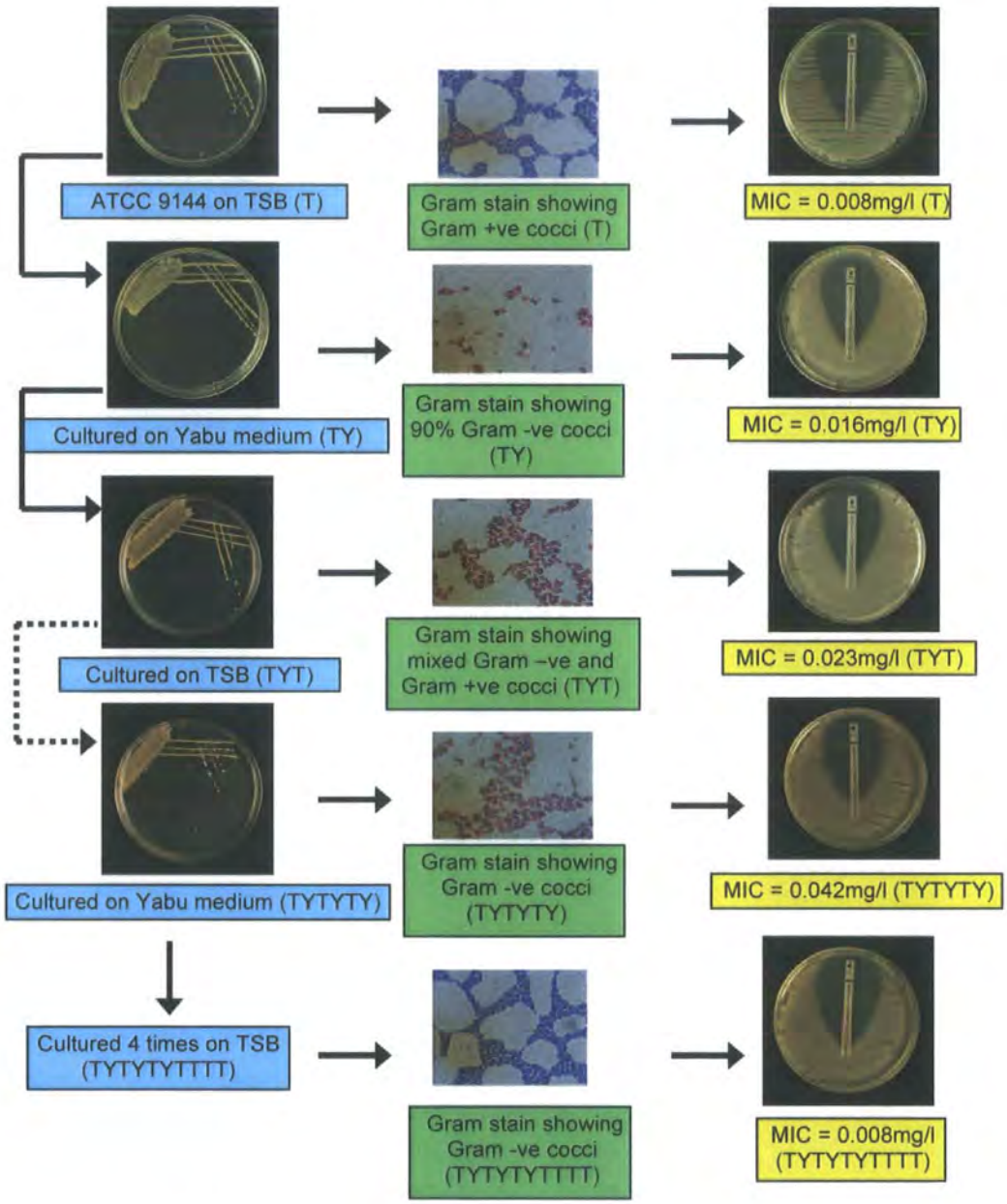


Figure 3.3: The above illustration shows the key results of Gram-stains and E-tests under NaCl stress. It shows the change in Gram stain from Gram positive to Gram negative when NaCl stress is applied. It also shows increasing penicillin G resistance with increasing exposure to NaCl stress. It shows that the increase is unstable and reverts to baseline levels after four passages without salt stress on TSB.

The above lines of experiments were performed on two occasions from separate stock cultures. The results were the same in each experimental lineage.

The baseline MIC was 0.008mg/l. This rose to 0.047mg/l after three passages of salt stress, a six-fold rise. This increase in penicillin resistance was, however, unstable and reverted to the baseline level of 0.008mg/l after four passages on TSB. Salt stress also lead to a Gram-negative phenotype, and this too was unstable, regressing to a Gram-positive phenotype upon removal of the salt stress.

The rise in penicillin resistance demonstrated here in cells stressed only with salt, is modest compared to the levels demonstrated in the CWD bacteria produced by penicillin stress which were in excess of 256mg/l (Fuller et al., 2005). However, the six-fold increase in penicillin resistance above the baseline demonstrated here is achieved in cells that are entirely antibiotic naïve, and hence seems to be in response to the salt stress. The modest raise of penicillin resistance is transient and returns to baseline levels once the salt stress is removed. The CWD phenotype assumed under the salt stress is also transient and the cell-line reverts to a Gram-positive phenotype upon removal of the salt stress. This is contrary to the CWD bacteria produced by penicillin stress, which retained their antibiotic resistance in a stable manner even after the causative stress was removed (Fuller et al., 2005). Although both cells stressed with salt, and cells stressed with penicillin assume a Gram-negative phenotype, the levels of antibiotic resistance and the stability of that resistance is considerably different between the two, suggesting very different causative mechanisms.

3.1.4 Salt (NaCl) Stress Leads to a Decrease Susceptibility to Lysostaphin.

The lysostaphin susceptibility assay was used here to assess whether CWD bacteria produced on the high salt (NaCl) concentration Yabu agar developed lysostaphin resistance in response to this salt stress.

Lysostaphin susceptibility assays were performed on the following cell cultures:

1. Three separate cultures of T (Stock "Oxford", ATCC 9144, *S. aureus* grown overnight on Trypticase-Soy broth (TSB)).
2. Three separate cultures of TY (Cells from the above culture, T, transferred to Yabu media and grown overnight).
3. Two separate cultures of TYT (Cells from culture TY transferred to TSB and grown overnight).
4. Two separate cultures of TYTYTY (Cells from TYT transferred to Yabu media and grown overnight (TYTY), then transferred to TSB and grown overnight (TYTYT), then transferred to Yabu media again).

The following figure shows, in graphical form, the results of the above experiments.

The cells stressed with salt show a Gram-negative phenotype and were less susceptible to lysostaphin. Cells stressed once with salt (lineage TY) were less susceptible to lysis by lysostaphin than the baseline cells grown on TSB (lineage T). The increase in resistance to lysostaphin noted after one pass on salt stress decreases slightly after removal of the salt stress by a single passage on TSB (lineage TYT). Three passages of salt stress (lineage TYTYTY) lead to a greater resistance to

lysostaphin than is noted after a single passage of salt stress. All these results mirror and complement the results noted in the E-test experiments (Section 3.1.3), where salt stress lead to progressive increased levels of penicillin resistance.

The increased resistance to lysostaphin in salt stressed cells suggests that the cell-wall deficient (CWD) bacteria, which stain Gram-negative, contain less pentaglycine cross-bridges than they do in their Gram-positive state. It also seems apparent that the CWD cells rely on mechanisms other than the strength of their cell wall to maintain cellular integrity.

3.1.4.1 Graph of Lysostaphin Susceptibility Assays

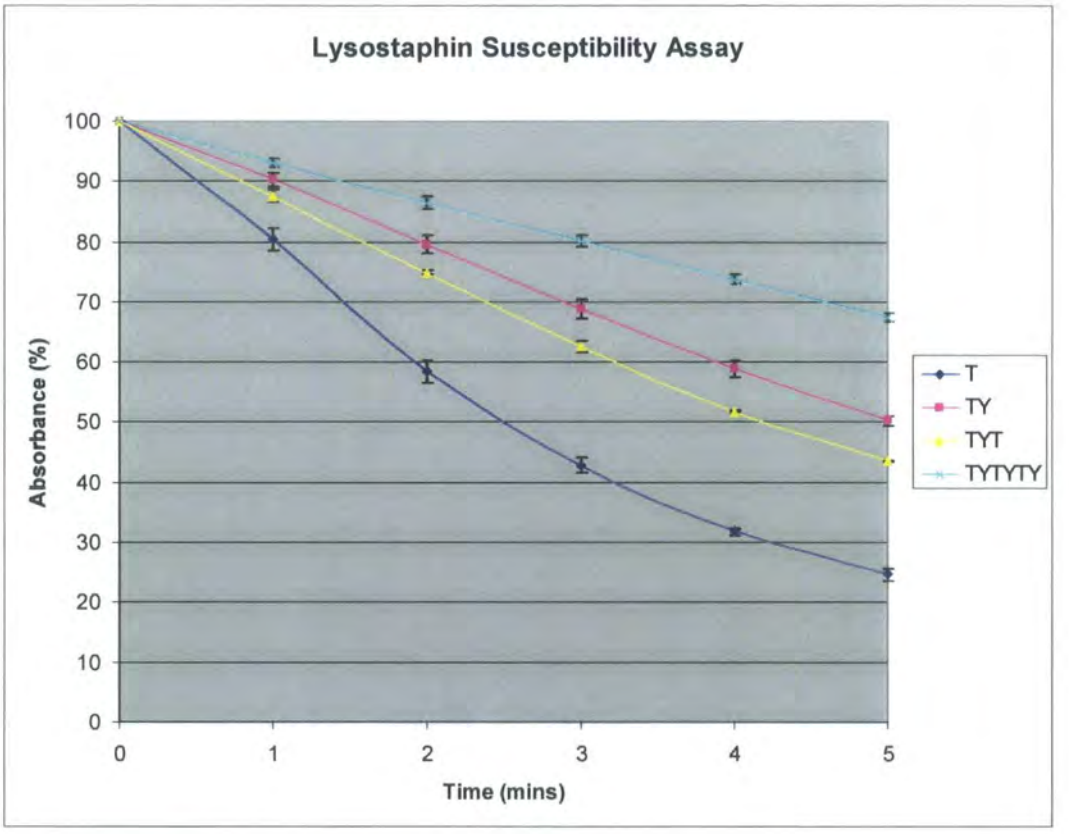


Figure 3.4: A graph of absorbance against time for the lysostaphin susceptibility assays on cultures of T, TY, TYT and TYTYTY. Absorbance decreases as cells are lysed and is equivalent to cell intactness. The graph demonstrates that resistance to lysostaphin is increased in cells that have been stressed with NaCl on Yabu agar. (Compare T to TY). The increased resistance to lysostaphin remains once the NaCl stress is removed by a single transfer (compare TY to TYT), and increases further with subsequent exposures to salt stress. (compare TY and TYT to TYTYTY). Error bars of the standard deviation are included.

3.2.0 Biofilm Formation in 96-Well Microtiter Plates

High concentrations of Sodium Chloride and Glucose have previously been implicated in increased biofilm formation in *S. aureus* (Lim et al., 2004). The aim of the following experiments was to ascertain whether this increase in biofilm formation was the result purely of high osmolarity, whether it was specific to sodium or whether it occurred with other Group I metals. Chlorides of Lithium, Sodium, Potassium, Rubidium and Caesium were used.

The method of Christensen et al. was used (Christensen et al., 1985). However, in early experimentation with sodium chloride it was noted that much biofilm formation occurred on the walls of the wells, which hence could not be recorded by the plate reader. The book on biofilms of the Society for General Microbiology entitled, "Community Structure and Co-operation in Biofilms" states that biofilms normally occur at a phase boundary (Allison et al., 2000). Hence it is perhaps to be expected that much of the biofilm formation will be on the walls of the wells.

To overcome this problem of biofilm on the well walls, a further technique was developed. The wells were stained with Safranin O solution, washed, dried and then refilled with 70% ethanol. The ethanol acted as a solvent to the Safranin which had been taken up by the biofilm on the walls of the wells (See Figure 3.5). By taking plate readings with the ethanol still in the wells, and setting the wavelength of the plate reader to 490nm (within the absorbance range of safranin) the absorbance of the safranin was taken into account and hence the biofilm on the walls of the wells was recordable. In each experiment, one row of wells was inoculated with sterile growth media. These wells were prepared in the same way as all experimental wells and were stained with Safranin, washed and dried. Seventy per cent ethanol was also added to

these wells prior to plate reading and was thus used as the zero point to control all other experimental wells.

The following pages contain the results of the biofilm experiments for the different Group I metal chlorides.

Photograph Showing Biofilm Formation on the Walls of a 96-Well Microtiter Plate Under NaCl Stress.

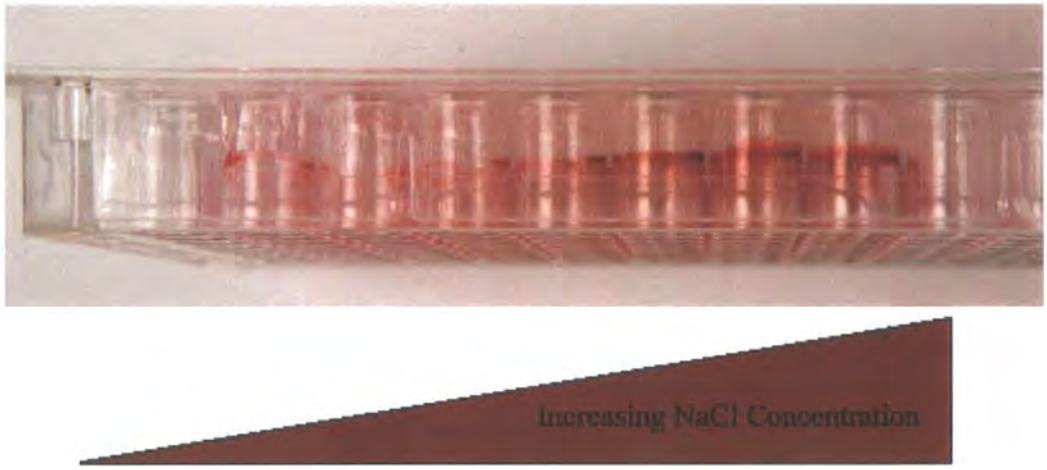


Figure 3.5: Photograph of an early experiment showing a stained and dried 96-well microtiter plate from the side. The wells contained increasing concentrations of NaCl from left to right. This picture shows increased biofilm formation with increased NaCl concentration. Much of the biofilm however, is present on the walls of the wells, making recording of it with a plate reader inaccurate and unreliable, and prompting the development of the new technique.

3.2.1 Lithium Chloride Biofilm Assay Results

The lithium chloride (LiCl) biofilm assay did not show any standard effect on biofilm formation, as measured by spectrophotometry (OD_{490}). At low concentrations of LiCl (0.4 to 0.6 mol/l) biofilm formation decreased. At higher concentrations of LiCl (1 to 1.2 mol/l) biofilm formation increased. At a concentration of 1.4 mol/l biofilm formation was equivalent to the baseline level. Linear regression appears to show a slight but steady increase in biofilm formation in response to increasing concentrations of LiCl. However, there is no significant difference between the level of biofilm formation at 0 mol/l and 1.4 mol/l (Student T test: $p = 0.485$).

3.2.1.1 Graph of the LiCl Biofilm Assay Results

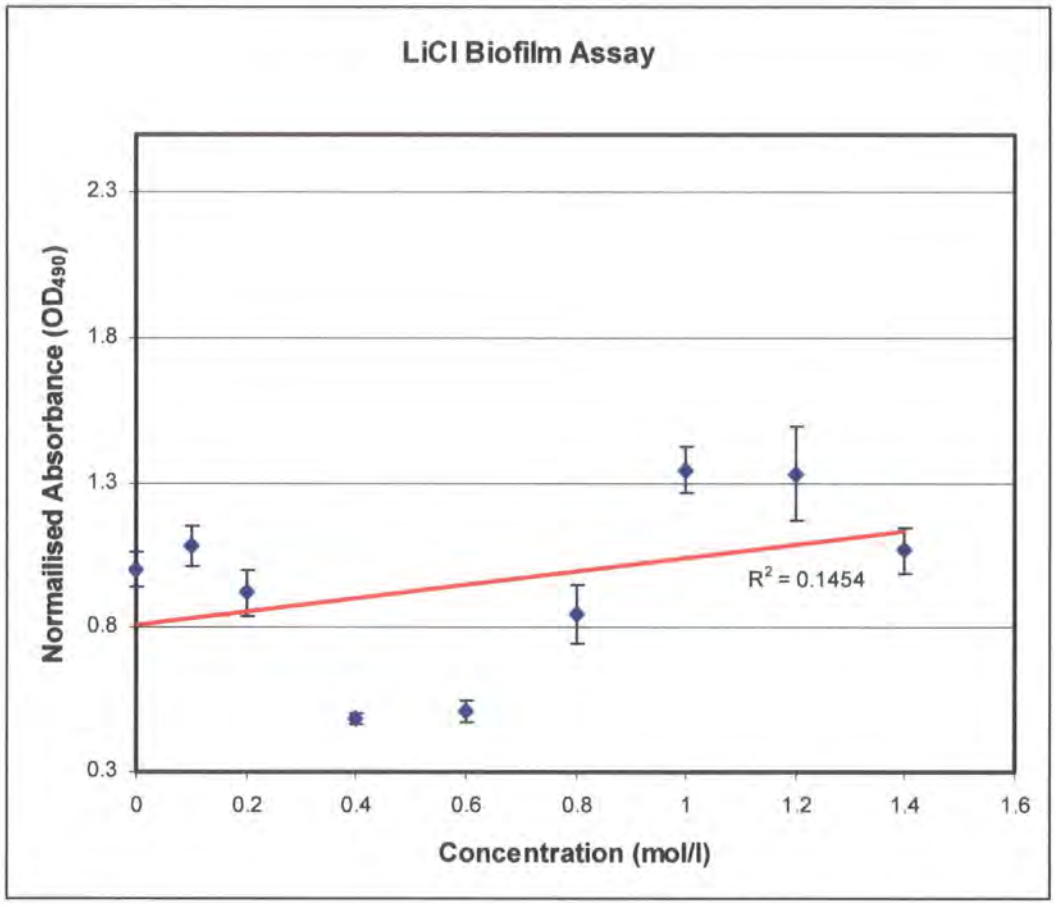


Figure 3.6: Graph of changing effect of LiCl concentration verses *S. aureus* biofilm formation as measured by spectrophotometry of 96 well microtiter plates. The results have been normalised such that the reading at 0mol/l is 1. Results represent the averages of three independent experiments each with eight technical replicates. Error bars indicate the standard error of the mean. Mild increases in LiCl concentration (0.4M – 0.6M) led to decreased biofilm formation, but higher levels (1.0M – 1.4M) led to a slight increase in biofilm formation. The black line shows a linear regression of the results ($R^2 = 0.1454$).

Statistics:

Comparing 0mol/l to 1.4mol/l No difference Student T test: p = 0.485

3.2.2 Sodium Chloride Biofilm Assay Results

Increased sodium chloride (NaCl) concentration is known to have a positive effect on biofilm formation in *S. aureus* (Lim et al., 2004). Current work confirms this relationship, and demonstrates a fifty per cent increase in biofilm formation, as measured by spectrophotometry (OD_{490}), at an NaCl concentration of 1.4 mol/l compared to 0.6 mol/l. This rise is statistically significant (Student T test: $p = 7.01 \times 10^{-5}$).

The pattern of biofilm formation appears to be bi-phasic. Biofilm formation appears constant between NaCl concentrations of 0mol/l and 0.6mol/l (shown by the red linear regression on the graph). However, biofilm formation increases above NaCl concentrations of 0.6mol/l (shown by the blue linear regression on the graph).

3.2.2.1 Graph of NaCl Biofilm Assay Results

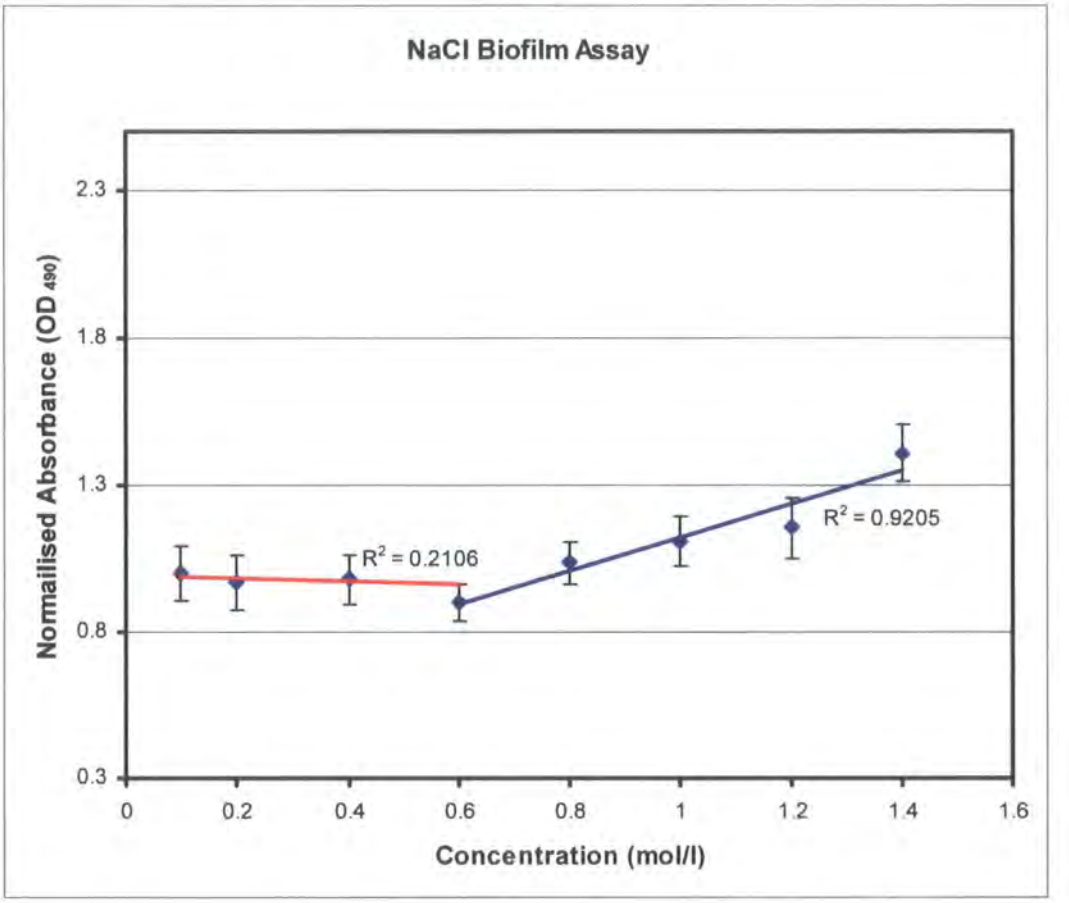


Figure 3.7: Graph of changing effect of NaCl concentration verses *S. aureus* biofilm formation as measured by spectrophotometry of 96 well microtiter plates. The results have been normalised such that the reading at 0.1mol/l is 1. Results represent the averages of three independent experiments each with eight technical replicates. Error bars indicate the standard error of the mean.

A bi-phasic growth pattern is shown. Biofilm formation remained constant between NaCl concentrations of 0mol/l and 0.6mol/l as shown by the red linear regression (R^2 = 0.2106). Increases in NaCl concentration above 0.6mol/l led to an increase in biofilm formation as shown by the blue linear regression (R^2 = 0.9205).

Statistics:

Comparing 0.1mol/l to 0.6mol/l	Not Different	Student T test: p = 0.378
Comparing 0.6mol/l to 1.4mol/l	Different	Student T test: $p = 7.01 \times 10^{-5}$

3.2.3 Potassium Chloride Biofilm Assay Results

Increased concentrations of potassium chloride (KCl) lead, in a similar way to increased NaCl concentrations, to a bi-phasic response in biofilm formation, as measured by spectrophotometry (OD_{490}). The amount of biofilm formation remained static between KCl concentrations of 0mol/l and 0.4mol/l (shown by the red linear regression on the graph). However, above KCl concentrations of 0.4mol/l, biofilm formation increased with increasing KCl concentration (shown by the blue linear regression on the graph). The rise in biofilm formation between a KCl concentration of 0.4mol/l and 1.4mol/l was statistically significant (Student T test: $p = 1.53x10^{-13}$).

The statistical significance of the increase in biofilm in response to increase potassium concentration is greater than the statistical significance of the increase in biofilm formation in response to increase sodium concentration ($p = 7.01x10^{-5}$ vs. $p = 1.53x10^{-13}$). The reason for this is two-fold. Firstly, the proportional rise in increased biofilm formation was greater in response to increased potassium ion concentration than to increased sodium concentration. Secondly, the potassium biofilm assays were repeated four times rather than the three times the sodium biofilm assays were performed. This increase in repetition, lead to a greater number of readings and a lower standard error, which thus lead to a tightening of the significance values.

3.2.3.1 Graph of KCl Biofilm Assay Results

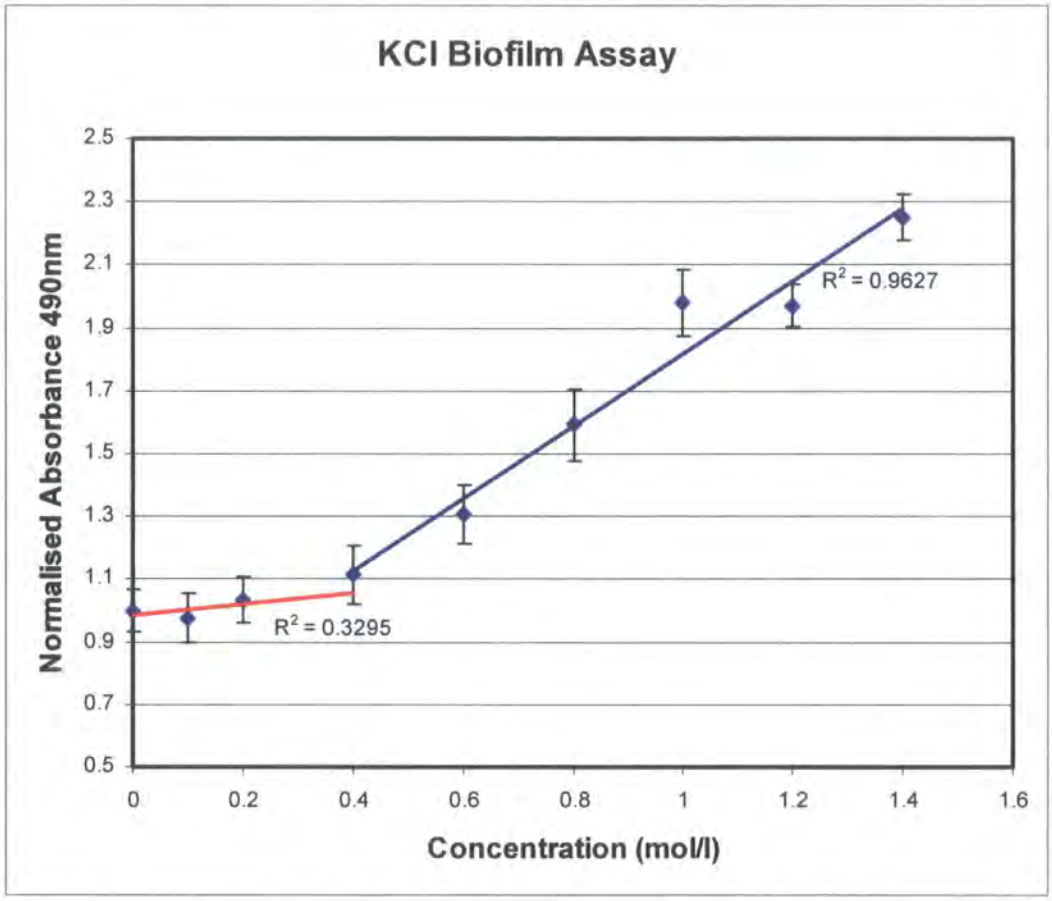


Figure 3.8: Graph of changing effect of KCl concentration verses *S. aureus* biofilm formation as measured by spectrophotometry of 96 well microtiter plates. The results have been normalised such that the reading at 0mol/l is 1. Results represent the averages of four independent experiments each with eight technical replicates. Error bars indicate the standard error of the mean.

A bi-phasic growth pattern is shown. Biofilm formation remained constant between KCl concentrations of 0mol/l and 0.4mol/l as shown by the red linear regression (R^2 = 0.3295). Increases in KCl concentration above 0.4mol/l led to an increase in biofilm formation as shown by the blue linear regression (R^2 = 0.9627).

Statistics:

Comparing 0.4mol/l to 1.4mol/l	Different	Student T test: $p = 1.53 \times 10^{-13}$
Comparing 0mol/l to 0.4mol/l	Not Different	Student T test: $p = 0.355$

3.2.4 Rubidium Chloride Biofilm Assay Results

Increased concentrations of rubidium chloride (RbCl) lead to increased levels of biofilm formation, as measured by spectrophotometry (OD_{490}). This increase however was not linear, and seemed to represent more of an on-off switch.

Between concentrations of 0 mol/l and 0.4 mol/l the amount of biofilm formation appeared stable (shown by the red linear regression on the graph). Statistically, the readings taken at 0 mol/l and 0.4 mol/l were not different (Student T test: $p = 0.635$). However, at RbCl concentrations of 0.6 mol/l and above, there was a rise in biofilm formation above the baseline level. Higher concentrations of RbCl (0.8 mol/l to 1.4 mol/l) did not however lead to increases in biofilm formation above the level noted at 0.6 mol/l (shown by the blue linear regression on the graph).

Statistical analysis showed a significant difference between the amount of biofilm formation recorded at a RbCl concentration of 0.4 mol/l and the amount of biofilm formation recorded at 0.6 mol/l (Student T test: $p = 0.002$). Analysis also showed no difference between the recordings of biofilm formation at 0.6 mol/l of RbCl and at 1.4 mol/l RbCl (Student T test: $p = 0.792$)

3.2.4.1 Graph of RbCl Biofilm Assay Results.

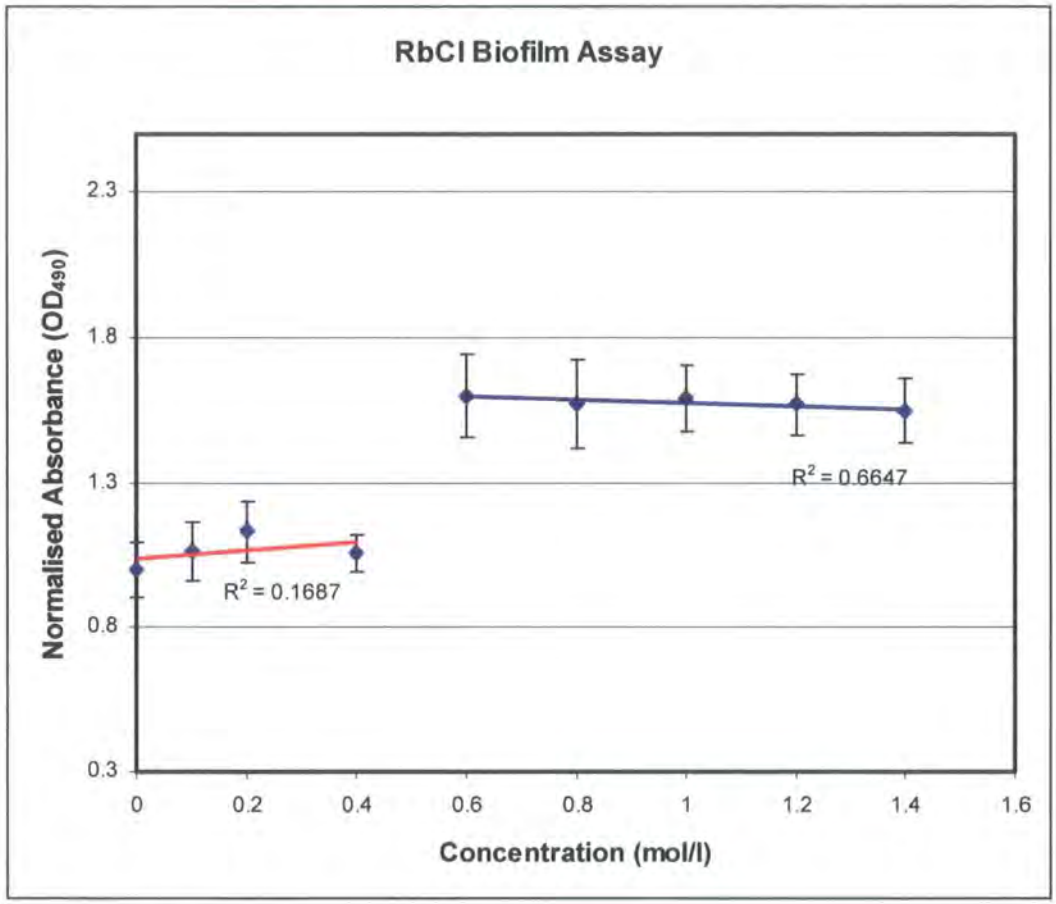


Figure 3.9: Graph of changing effect of RbCl concentration verses *S. aureus* biofilm formation as measured by spectrophotometry of 96 well microtiter plates. The results have been normalised such that the reading at 0mol/l is 1. Results represent the averages of three independent experiments each with eight technical replicates. Error bars indicate the standard error of the mean.

A possible on-off phenomenon is shown by the graph. RbCl concentrations between 0mol/l to 0.4mol/l lead to a stable level of biofilm formation, as shown by the red linear regression on the graph ($R^2 = 0.1687$). However, RbCl concentrations above 0.4mol/l led to an increase in biofilm formation but this remained at a stable level, as shown by the blue linear regression on the graph ($R^2 = 0.6647$).

Statistics:

Comparing 0 mol/l to 0.4 mol/l	No difference	Student T test: $p = 0.635$
Comparing 0.4 mol/l to 0.6 mol/l	Different	Student T test: $p = 0.002$
Comparing 0.6 mol/l to 1.4 mol/l	No Difference	Student T test: $p = 0.792$

3.2.5 Caesium Chloride Biofilm Assay Results

Increased concentrations of caesium chloride (CsCl) lead to a variable effect on biofilm formation, as measured by spectrophotometry (OD_{490}).

Small increases in CsCl concentration (0.1 mol/l to 0.2 mol/l) lead to a significant increase in biofilm formation. The difference between the amount of biofilm formation at a CsCl concentration of 0 mol/l and 0.2 mol/l was statistically significant (Student T test: $p = 5.04 \times 10^{-15}$) Higher increases in CsCl concentration (0.4 mol/l to 1.4 mol/l) lead to only slight changes in biofilm formation compared to the baseline level. There was no statistical difference between the amount of biofilm formed at 1.2 mol/l of CsCl and 0 mol/l (Student T test: $p = 0.354$). The amount of biofilm formation at 1.4 mol/l was however significantly lower than the baseline level at 0 mol/l (Student T test: $p = 0.004$).

Biofilm formation varies considerably with changes to CsCl concentration. Small increases in concentration (0.1 mol/l to 0.2 mol/l) lead to increased biofilm formation, higher levels (0.4 mol/l to 1.2 mol/l) have little effect on biofilm formation and even higher levels (1.4 mol/l) lead to a decrease in biofilm formation.

3.2.5.1 Graph of CsCl Biofilm Assay Results.

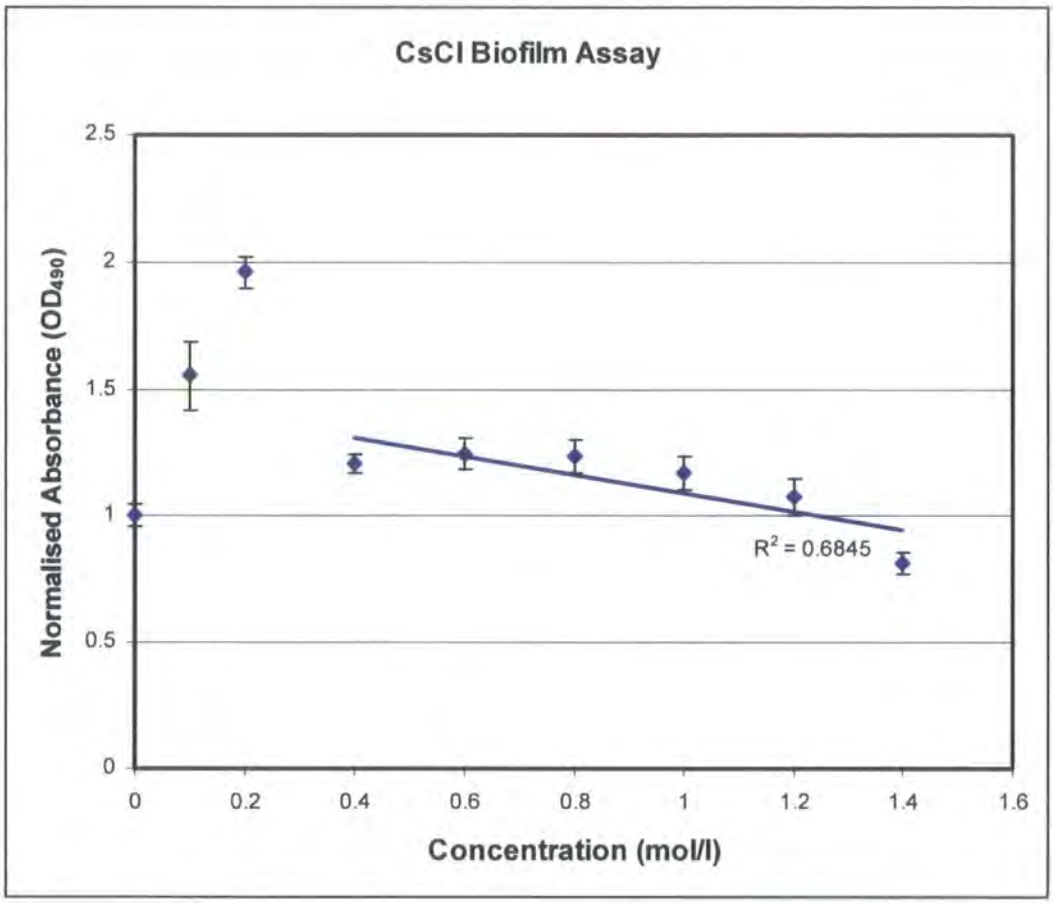


Figure 3.10: Graph of changing effect of CsCl concentration verses *S. aureus* biofilm formation as measured by spectrophotometry of 96 well microtiter plates. The results have been normalised such that the reading at 0mol/l is 1. Results represent the averages of three independent experiments each with eight technical replicates. Error bars indicate the standard error of the mean. Small increases in CsCl concentration (0.1mol/l to 0.2mol/l) led to an increase in biofilm formation. Higher increases in CsCl concentration (0.4mol/l to 1.2mol/l) did not have a significant effect on biofilm formation compared to the baseline level. However, a CsCl concentration of 1.4mol/l appeared to cause a decrease in biofilm formation. The blue line shows a linear regression of the results suggesting that higher concentrations of CsCl, of 0.4mol/l and above, lead to a gradual decline in biofilm formation.

Statistics:

Comparing 0 mol/l to 0.2 mol/l	Different	Student T test: $p = 5.04 \times 10^{-15}$
Comparing 0 mol/l to 1.2 mol/l	No Difference	Student T test: $p = 0.354$
Comparing 0 mol/l to 1.4 mol/l	Different	Student T test: $p = 0.004$

3.2.6 Biofilm Assays: Summary of Results

1. **LiCl:** Changes in LiCl concentration lead to a variable and unpredictable effect on biofilm formation. A slight increase in LiCl concentration (0.4mol/l) leads to an apparent small reduction in biofilm formation but a higher concentration (1.0mol/l) leads to a slight increase in biofilm formation. Yet higher concentrations (1.4mol/l) seem not to affect biofilm formation.
2. **NaCl:** Increasing NaCl concentration leads to a bi-phasic effect on biofilm formation. Concentrations of NaCl above 0.6mol/l lead to a progressive increase in biofilm formation. Concentrations below 0.6mol/l do not affect biofilm formation above the baseline level.
3. **KCl :** Increasing KCl concentration also leads to bi-phasic effect on biofilm formation. Concentrations of KCl above 0.4mol/l lead to a progressive increase in biofilm formation. Concentrations below 0.4mol/l do not affect biofilm formation above the baseline level.
4. **RbCl:** Low concentrations of RbCl (0mol/l to 0.4mol/l) do not alter biofilm formation, but at concentrations of 0.6mol/l and above, biofilm formation is increased above the baseline level. This increase in biofilm formation does not increase any more with further increases in RbCl concentration.
5. **CsCl:** Low concentrations of CsCl (0.2mol/l) increases biofilm formation, but higher concentrations of CsCl (1.4mol/l) lead to a progressive decrease in biofilm.

3.2.7 Comments on Biofilm Assays

The biofilm assays of Lithium, Potassium, Rubidium and Caesium were all performed against a background of 0.5% NaCl. This is because the BHI used to

produce the basic growth media, to which the various concentrations of LiCl, KCl, RbCl and CsCl were added for the above assays, contained this concentration of NaCl. This equates to an NaCl concentration of less than 0.1mol/l. An increase in biofilm in response to increased NaCl concentration was not seen below a concentration of 0.6mol/l. Hence, it seems unlikely that a background concentration of just 0.5% NaCl would have a significant effect on the assays of Lithium, Potassium, Rubidium and Caesium. However, this is a potential source of error and efforts should be made to circumvent this in future work, perhaps by formulating a replacement BHI powder from constituent parts, excluding the NaCl.

3.3.0 Polymerase Chain Reaction

The following sections portray the process and results of primer design, primer checking with PCR, primer checking with RT-PCR, and finally the results of RT-PCR on the experimental cultures.

3.3.1 Primer Design

Genes of interest were identified in the literature and putative sequences were found using GenBank® (http://www.ncbi.nlm.nih.gov/Genbank/). The primers were designed from these sequences for use in RT-PCR experimentation but were first used in PCR experiments. PCR was performed firstly to confirm the presence of the target genes in *S. aureus* ATCC 9144, and secondly to confirm the activity of the designed primers.

3.3.2 Genes of Interest

Experiments, both historical and those described in this thesis, have shown that NaCl stress leads to the formation of cell wall deficient bacteria and also an increase in biofilm formation in *S. aureus* (Lim et al., 2004; Yabu, 1995). Furthermore, current work also shows an increase in biofilm formation in *S. aureus* in response to KCl stress. Genes were chosen, and primers designed to identify the cellular processes occurring at a transcription level in response to either NaCl or KCl stress. The genes chosen were a representative selection of proteins/genes affecting biofilm formation, cell wall production, gene regulation, stress response and normal cell physiology.

3.3.2.1 Table of Gene Products Chosen for Experimentation.

Gene Product	Effect
Staphylococcal Protein A	Binds Fc and Fab region of antibodies. Binds vWF.
Collagen Adhesin	Binds Collagen.
Elastin Binding Protein	Binds Elastin peptides.
Clumping Factor B	Binds Fibrinogen and Type I Cytokeratin.
PIA	Intercellular adhesion.
MurG	Catalyses peptidoglycan production.
MurB	Catalyses peptidoglycan production.
Autolysin	Cell division and cell wall turnover.
Pbp4	Peptidoglycan cross-linkage.
MscL	Emergency solute valve.
KefC	Potassium efflux.
SarX	Negative regulation of the *agr* locus.
SarA	Positive regulation of the *agr* locus.
RsbU	Positive regulator of σ^B.
ClpC	Refolding damaged proteins.
Thermostable Nuclease	Used for *S. aureus* identification.
16S	rRNA.

Figure 3.11a: Table of gene products chosen for experimentation. *nuc* and *16s*, which encode a thermostable nuclease and 16S respectively, are universal *S. aureus* genes and hence are often used for *S. aureus* detection in PCR experiments (Brakstad, Aasbakk & Maeland, 1992; Donlan, 2005). Furthermore, 16S can be used to sub-classify bacteria (Woese, 1987).

3.3.3 Table of Primers for the Corresponding Proteins in Figure 3.11a.

SpA Sense Primer:	5'-CAGCAAACCATGCAGATGCTA-3'	Tm: 66°C
SpA Reverse Primer:	5'-CGCTAATGATAATCCACCAAATACA-3'	Tm: 64°C
Cna Sense Primer:	5'-TGCTGTCCACCTTGAATCTG-3'	Tm: 63.9°C
Cna Reverse Primer:	5'-GGGAGATATGCTGCCAGAAG-3'	Tm: 63.6°C
EbpS Sense Primer:	5'-CAACATTTTCCGGTGAACCT-3'	Tm: 63.6°C
EbpS Reverse Primer:	5'-CAACGCTAATCAAGCGAACA-3'	Tm: 63.8°C
*ClfB Sense Primer:	5'-TAATACGATGCCAATTGCAGAC-3'	Tm: 63.5°C
*ClfB Reverse Primer:	5'-GTTCCTGATTTAGGTGCCTTTG-3'	Tm: 63.4°C
**ica* Sense Primer:	5'-ACCCAACGCTAAAATCATCG-3'	Tm: 63.6°C
**ica* Reverse Primer:	5'-TTTTCAATGTTTAAAGCAACACG-3'	Tm: 62.5°C
*MurG Sense Primer:	5'-TGGAGCAACAATTCGAGAAG-3'	Tm: 62.9°C
*MurG Reverse Primer:	5'-AAGCTTCCACCCATAACGAC-3'	Tm: 62.9°C
Mur B Sense Primer:	5'-CACTGCACCACCAATTGAAC-3'	Tm: 64.1°C
MurB Reverse Primer:	5'-AGGTGGTATTCGCGGTATTG-3'	Tm: 63.5°C
Atl Sense Primer:	5'-GGATGTGCAGGATTCCATCT-3'	Tm: 63.9°C
Atl Reverse Primer:	5'-AAACAAGCTGGTTGGGACAC-3'	Tm: 63.9°C
*Pbp4 Sense Primer:	5'-CACATTTCGTCAATCCAACG-3'	Tm: 63.9°C
*Pbp4 Reverse Primer:	5'-TAACTGCATGCGTTGTTGGT-3'	Tm: 64.1°C
MscL Sense Primer:	5'-TGAATAAAGCAAACGCGATG-3'	Tm: 63.5°C
MscL Reverse Primer:	5'-GGTGCAGCTTTCAACAAGATT-3'	Tm: 63.0°C
Kef Sense Primer:	5'-CAATGGTCAAGGCGACAGTA-3'	Tm: 63.7°C
Kef Reverse Primer:	5'-ATACCGATTTGCGTCGTACC-3'	Tm: 63.5°C
SarX Sense Primer:	5'-TATTTGGGGGTGCAACATTT-3'	Tm: 63.7°C
SarX Reverse Primer:	5'-ATCTTTGCAATGCTTCATCGT-3'	Tm: 63.4°C
SarA Sense Primer:	5'-TCAATTTCGTTGTTTGCTTCA-3'	Tm: 63.0°C
SarA Reverse Primer:	5'-CAACCACAAGTTGTTAAAGCAGTT-3'	Tm: 63.0°C
RsbU Sense Primer:	5'-GGCTCATGACCAGCTGAACT-3'	Tm: 64.4°C
RsbU Reverse Primer:	5'-TTGGCATGGATTCTTATGGAC-3'	Tm: 63.5°C
ClpC Sense Primer:	5'-CACAGCGCGTATTAGCACAT-3'	Tm: 63.7°C
ClpC Reverse Primer:	5'-GCAGCAATTCCTTCAGGTTC-3'	Tm: 63.6°C
**nuc* Sense Primer:	5'-TTTATTTTCAAGTCTAAGTAGCTCAGCAA-3'	Tm: 65°C
**nuc* Reverse Primer:	5'-TGCACTATATACTGTTGGATCTTCAGAA-3'	Tm: 64°C
*16s Sense Primer:	5'-CGGTCCAGACTCCTACGGGAGGCAGCA-3'	Tm: 80°C
*16s Reverse Primer:	5'-GCGTGGACTACCAGGGTATCTAATCC-3'	Tm: 69°C

Figure 3.11b: Table of primers designed for PCR and RT-PCR experimentation. Primers marked (*) were already available in the laboratory, all others were designed de novo.

3.3.4 PCR to Check the Primers

PCR was performed on a crude preparation of DNA from *S. aureus* ATCC 9144 to confirm the presence of the genes chosen for experimentation, and to check the function of the primers designed for this purpose.

All the primers gave a positive result, confirming the presence of the genes.

3.3.4.1 Photograph of a Typical PCR Gel

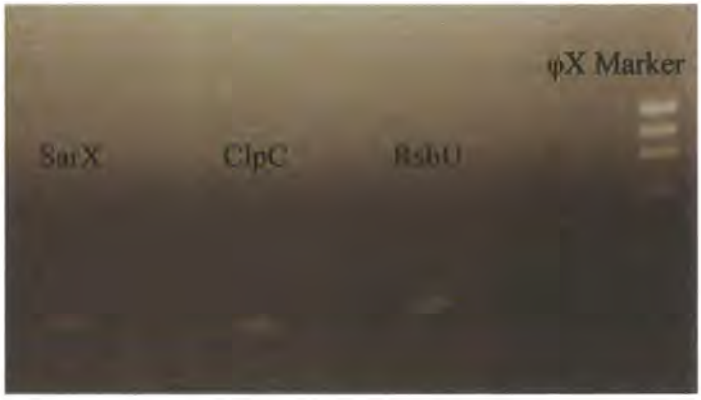


Figure 3.12: Photograph showing a typical PCR agarose gel. The fluorescing bands show a positive result. The genes under experimentation are labelled.

3.3.5 RT-PCR to Check Primers

RT-PCR was performed on a crude preparation of DNA from *S. aureus* ATCC 9144 to optimise the experiment. It was also performed to further check the presence of the genes chosen for experimentation, and the function and efficiency of the primers designed for RT-PCR. The take off curves showed good grouping, indicating that the primers functioned appropriately and that the chosen genes were present in the ATCC 9144 strain of *S. aureus* used. Three No-DNA control experiments were performed. These controls each had very late and reduced take-off confirming the efficacy of the assay. Each experiment was performed in triplicate which meant that individual outliers could be ignored (See Figure 3.13).

A table, Figure 3.14, showing the absolute values of take-off and amplication demonstrates close similarity and good grouping of the take-offs around the 17 cycle mark, confirming the conclusions made about figure 3.13 that the genes chosen for experimentation are present in the genome of *S. aureus* ATCC 9144, and that the primers function correctly.

The positive results gained in this RT-PCR experiment confirm the positive results gained from the PCR experiments performed on the bacterial DNA, and corroborate the presence of the experimental genes in *S. aureus* ATCC 9144.

3.3.5.1 Graph of RT-PCR Take Off and Amplification for DNA

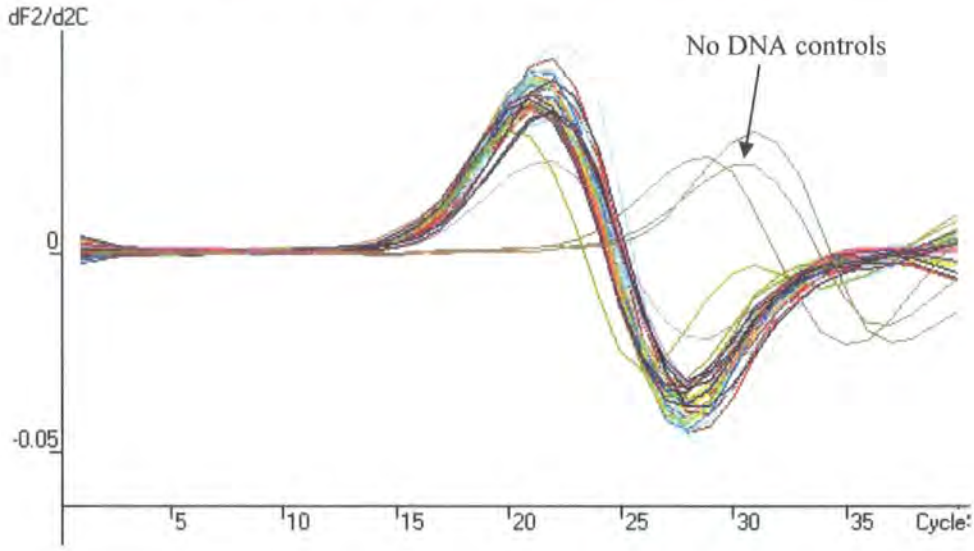


Figure 3.13: Graph of take off and amplification against number of cycles of PCR. In this experiment bacterial DNA was used. There is no significant difference in the take off of any of the genes. There a couple of outlying results close to the main grouping, which can be disregarded, as each experiment was performed in triplicate. The three outlying results at the right-hand end of the graph are the no DNA controls which confirm efficacy of the assay. The close grouping of the take-off curves shows that all the primers functioned appropriately and hence, that all the chosen genes were present in *S. aureus* ATCC 9144.

3.3.5.2 Table of Results for RT-PCR of DNA

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
1		Ebps	17.6	1.74	1.02E+00	17.6	[17.5,17.8]
2		Ebps	17.6	1.77	1.02E+00		
3		Ebps	17.7	1.70	9.65E-01		
4		MurB	17.1	1.69	1.33E+00	16.9	[16.4,17.5]
5		MurB	17.0	1.66	1.40E+00		
6		MurB	16.7	1.70	1.64E+00		
7		Kef	17.2	1.70	1.26E+00	17.3	[17.1,17.5]
8		Kef	17.3	1.69	1.19E+00		
9		Kef	17.4	1.78	1.13E+00		
10		SarA	17.0	1.70	1.40E+00	17.0	[16.8,17.2]
11		SarA	16.9	1.81	1.47E+00		
12		SarA	17.1	1.65	1.33E+00		
13		SarX	16.8	1.70	1.56E+00	17.0	[16.5,17.6]
14		SarX	17.2	1.77	1.26E+00		
15		SarX	17.1	1.79	1.33E+00		
16		MscL	18.1	1.66	7.81E-01	17.7	[16.7,18.7]
17		MscL	17.3	1.74	1.19E+00		
18		MscL	17.6	1.73	1.02E+00		
19		Atl	17.8	1.80	9.15E-01	17.6	[17.3,18.0]
20		Atl	17.6	1.49	1.02E+00		
21		Atl	17.5	1.65	1.07E+00		
22		RsbU	17.0	1.66	1.40E+00	16.8	[16.1,17.4]
23		RsbU	16.5	1.92	1.82E+00		
24		RsbU	16.8	1.58	1.56E+00		
25		ClpC	16.9	1.73	1.47E+00	16.7	[16.2,17.3]
26		ClpC	16.8	1.55	1.56E+00		
27		ClpC	16.5	1.69	1.82E+00		
28		nuc	17.9	1.74	8.68E-01	17.8	[17.7,18.0]
29		nuc	17.8	1.65	9.15E-01		
30		nuc	17.8	1.73	9.15E-01		
31		Ebps Control	26.9	1.66	7.37E-03	25.8	[22.5,29.1]
32		Ebps Control	24.3	1.59	2.92E-02		
33		Ebps Control	26.1	1.68	1.13E-02		

This report generated by Rotor-Gene Real-Time Analysis Software 6.0 (Build 19)

Figure 3.14: The table above shows the take off and amplification of the genes under experimentation when RT-PCR was performed on a preparation of bacterial DNA.

3.4.0 Comparative RT-PCR to Detect Changes in Genetic Transcription in Response to NaCl and KCl Stress.

Current work has shown increased biofilm formation in *S. aureus* in response to increased potassium concentration, and has confirmed an increase in biofilm formation in response to increased sodium concentration. Changes in biofilm formation in response to changes in lithium, rubidium and caesium concentration show more unpredictable patterns. Sodium and potassium are also the two most abundant and important cations within cells. Hence, RT-PCR was performed using the primers identified in section 3.3.3 on *S. aureus* ATCC 9144 cultured in DBHI, DBHI supplemented with 1.4mol/l NaCl, and DBHI supplemented with 1.4mol/l KCl, to investigate what changes occur at a transcription level in response to sodium and potassium stress. These three cultures were prepared from the same overnight stationary phase culture to ensure comparability. All three cultures were grown to an absorbance of 0.75 (OD_{620}), i.e. mid-exponential growth. An identical volume of cells (1ml) was harvested at the same absorbance for each culture, again, to ensure reliability and comparability of results. mRNA was then harvested from each sample, reverse transcriptase reaction was performed, and the resulting cDNA samples were then subjected to comparative RT-PCR.

The table below shows the RNA yield from the three cultures measured by spectrophotometry (OD_{260}). The 260/230 and 260/280 ratios show the purity of the samples harvested, all of which are satisfactory.

3.4.0.1 Table of RNA Purity and Yields

	260/230	260/280	ng/µl
DHBI culture	2.27	2.13	503
DBHI + NaCl culture	2.05	2.15	483
DBHI + KCl culture	1.77	2.12	161

Figure 3.15. A table of the 260/230 ratio, 260/280 ratio, and RNA yield, all of which are satisfactory.

3.4.1 DBHI cDNA RT-PCR Results: Comparative Quantitation Report

The following results are for RT-PCR performed on the cDNA obtained from a culture of *S. aureus* ATCC 9144 grown in DBHI medium. This experiment is the control arm. The cells have not been stressed with sodium or potassium. The results obtained in this line of experimentation were used to compare and contrast with the results obtained in response to sodium or potassium stress.

The following pages contain a take-off graph and a table of values for the take-off and amplification. A melt curve analysis is also included to show that for each individual primer pair, all the PCR products have the same melting temperature. This is an important feature as it rules out primer dimers, contamination or mispriming.

When comparing the graph for this experiment (Figure 3.16) to the graph of RT-PCR performed on pure bacterial DNA (Figure 3.13), there is much more variability and spread in the take off. This is as expected with cDNA, as some genes will be transcribed more than others, leading to larger amounts of mRNA, thus larger amounts of cDNA and thus earlier take offs. Genes that take off after more PCR cycles (further to the right on the graph) have been transcribed less than genes which take off after fewer PCR cycles (further to the left on the graph). The table showing the absolute values for take-off and amplication in this experiment confirms the variability that one would expect with cDNA which indicates variability in the transcription of the genes in question.

3.4.1.1 Experiment Information

Run Name	Run DBHI cDNA
Run On Software Version	Rotor-Gene 6.0.19
Run Signature	The Run Signature is valid.
Gain FAM/Sybr	5.

3.4.1.2 Take Off Graph for DBHI cDNA RT-PCR

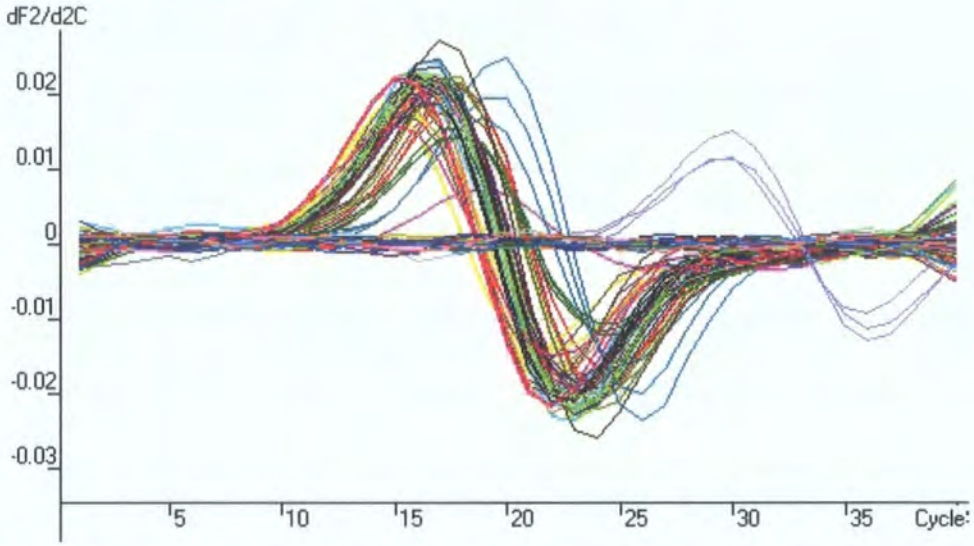


Figure 3.16: The graph above shows the take off and amplification against number of cycles of PCR for cDNA from DBHI culture.

3.4.1.3 Table of Results for DBHI cDNA RT-PCR

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
A1		nuc	14.0	1.64	8.15E-01	13.6	[12.6,14.5]
A2		nuc	13.4	1.64	1.08E+00		
A3		nuc	13.3	1.78	1.13E+00		
A4		16s	11.8	1.81	2.30E+00	11.5	[10.4,12.7]
A5		16s	11.0	1.56	3.36E+00		
A6		16s	11.8	1.65	2.30E+00		
A7		Spa	12.3	1.33	1.82E+00	12.5	[11.4,13.6]
A8		Spa	13.0	1.70	1.31E+00		
B1		Spa	12.2	1.71	1.91E+00		
B2		pbp4	12.1	1.68	2.00E+00	12.2	[11.4,12.9]
B3		pbp4	11.9	1.58	2.20E+00		
B4		pbp4	12.5	1.75	1.65E+00		
B5		ClfB	15.3	1.60	4.41E-01	15.5	[14.7,16.2]
B6		ClfB	15.8	1.75	3.48E-01		
B7		ClfB	15.3	1.59	4.41E-01		
B8		MurG	13.0	1.82	1.31E+00	13.8	[10.0,17.5]
C1		MurG	12.8	1.56	1.44E+00		
C2		MurG	15.5	0.93	4.01E-01		
C3		icaD	11.2	1.65	3.06E+00	11.0	[10.7,11.4]
C4		icaD	10.9	1.53	3.52E+00		
C5		icaD	11.0	1.31	3.36E+00		
C6		Atl	11.4	1.32	2.78E+00	11.6	[11.2,11.9]
C7		Atl	11.6	1.61	2.53E+00		
C8		Atl	11.7	1.59	2.41E+00		
D1		ClpC	12.5	1.60	1.65E+00	12.6	[12.0,13.1]
D2		ClpC	12.4	1.74	1.73E+00		
D3		ClpC	12.8	1.67	1.44E+00		
D4		SarX	13.8	1.64	8.96E-01	13.1	[11.6,14.7]
D5		SarX	12.6	1.23	1.58E+00		
D6		SarX	13.0	1.46	1.31E+00		
D7		EbpS	13.4	1.59	1.08E+00	13.3	[12.4,14.2]
D8		EbpS	12.9	1.77	1.37E+00		
E1		EbpS	13.6	1.73	9.84E-01		
E2		RsbU	12.0	1.61	2.10E+00	11.9	[11.6,12.2]
E3		RsbU	11.8	1.51	2.30E+00		
E4		RsbU	11.8	1.61	2.30E+00		
E5		Kef	12.4	1.53	1.73E+00	12.6	[12.2,12.9]
E6		Kef	12.7	1.60	1.51E+00		
E7		Kef	12.6	1.74	1.58E+00		
E8		SarA	12.3	1.54	1.82E+00	12.7	[11.7,13.7]
F1		SarA	12.8	1.65	1.44E+00		
F2		SarA	13.1	1.61	1.25E+00		

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
F3		MurB	11.9	1.60	2.20E+00	12.1	[11.2,13.0]
F4		MurB	12.5	1.64	1.65E+00		
F5		MurB	11.9	1.63	2.20E+00		
F6		MscL	11.1	1.61	3.21E+00	11.1	[10.4,11.8]
F7		MscL	11.4	1.74	2.78E+00		
F8		MscL	10.8	1.57	3.69E+00		
G1		nuc control	25.8	1.09	3.10E-03	25.5	[24.8,26.2]
G2		nuc control	25.4	1.39	3.74E-03		
G3		nuc control	25.3	1.40	3.92E-03		

This report generated by Rotor-Gene Real-Time Analysis Software 6.0 (Build 19)

Figure 3.17: The table above shows the number of cycles at which take off for each gene occurred and the amplification of each gene when RT-PCR was performed on a culture of *S. aureus* ATCC 9144 in DBHI. It shows variability between genes as expected with recombinant cDNA.

3.4.1.4 Melt Curve Analysis for DBHI cDNA RT-PCR

Melt curves are an important part of RT-PCR analysis. They are used to assess for primer dimers, contamination or mispriming. The traces produced for each gene under investigation in an experiment can be inspected for double peaks. A double peak would be indicative of primer dimers.

The melt curve (Figure 3.18) shows single peaks with good amplification for each gene. There are no secondary peaks indicative of primer dimers, contamination or mispriming in the main experimental genes. Each set of three lines for each gene shows good clustering and similar peak heights, apart from the occasional single outlier which can be disregarded as each experimental arm was performed in triplicate. The no DNA control lines (mauve) show a small peak indicative of primer dimers but this only just breaks the threshold line. The peaks for the different genes occur at different temperatures because the products are of variable length.

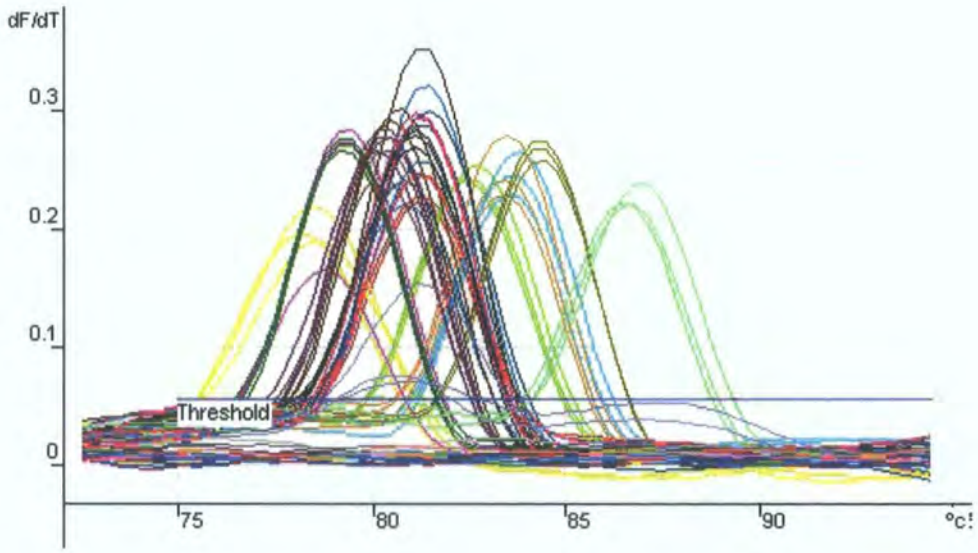


Figure 3.18: The melt curve shows single peaks with good amplification for each gene.

3.4.2 NaCl cDNA RT-PCR Results: Comparative Quantitation Report

The following results are for RT-PCR performed on the recombinant DNA obtained from a culture of *S. aureus* ATCC 9144 grown in DBHI medium supplemented with 1.4mol/l of NaCl.

The following pages contain a take-off graph and a table of values for the take-off and amplification. A melt curve analysis (not included) was performed which again showed single peaks for each individual primer pair and demonstrated no contamination, primer dimers or mispriming.

When the graph for this experiment (Figure 3.19) is compared to the graph of RT-PCR performed on cDNA from DBHI culture (Figure 3.16), it shows a different pattern, suggesting different genetic transcription of the experimental genes in response to NaCl stress. Analysis with the Relative Expression Software Tool V1.9.12 (REST 2005) was used to highlight these differences. (See Section: 3.3.8)

The table of take-off and amplication (Figure 3.20), shows, in absolute terms, the differences when compared to the cDNA from the DBHI experiment (see Figure 3.17).

3.4.2.1 Experiment Information

Run Name	Run NaCl cDNA
Run On Software Version	Rotor-Gene 6.0.19
Run Signature	The Run Signature is valid.
Gain FAM/Sybr	5.

3.4.2.2 Take off Graph for NaCl cDNA RT-PCR

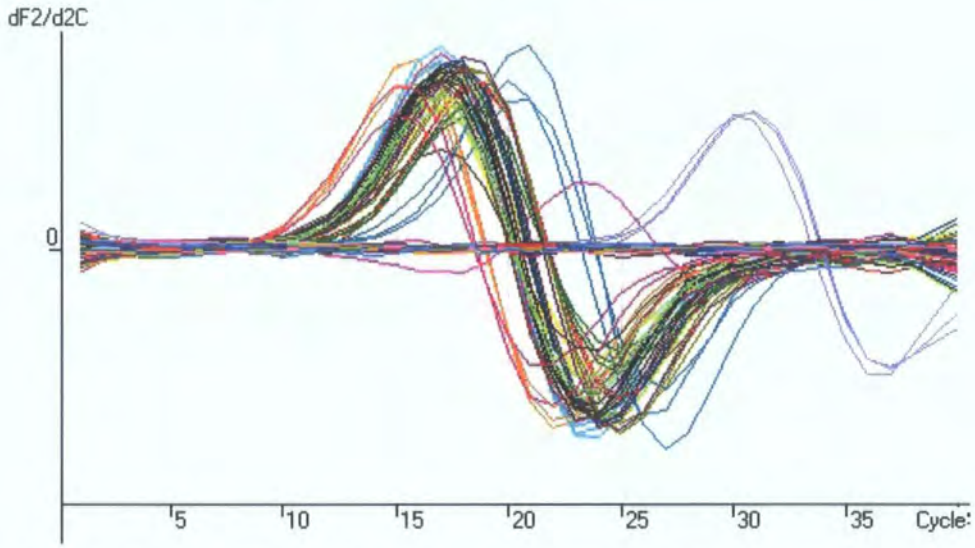


Figure 3.19: The graph above shows the take off and amplification against number of cycles of PCR for a culture of *S. aureus* ATCC 9144 in DBHI supplemented with 1.4mol/l NaCl.

3.4.2.3 Table of Results for NaCl cDNA RT-PCR

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
A1		nuc	14.4	1.72	1.00E+00	14.4	[14.4,14.4]
A2		nuc	14.4	1.78	1.00E+00		
A3		nuc	14.4	1.69	1.00E+00		
A4		16s	13.5	1.79	1.63E+00	13.2	[12.7,13.8]
A5		16s	13.1	1.93	2.02E+00		
A6		16s	13.1	1.79	2.02E+00		
A7		Spa	13.7	1.74	1.46E+00	13.6	[13.2,14.0]
A8		Spa	13.7	1.78	1.46E+00		
B1		Spa	13.4	1.74	1.72E+00		
B2		pbp4	12.8	1.70	2.38E+00	12.9	[12.5,13.3]
B3		pbp4	12.8	1.70	2.38E+00		
B4		pbp4	13.1	1.80	2.02E+00		
B5		ClfB	16.7	1.72	2.87E-01	16.4	[15.1,17.6]
B6		ClfB	15.8	1.60	4.68E-01		
B7		ClfB	16.6	1.61	3.03E-01		
B8		MurG	20.4	1.15	3.86E-02	15.4	[4.6,26.2]
C1		MurG	12.8	1.72	2.38E+00		
C2		MurG	13.0	1.79	2.14E+00		
C3		icaD	12.7	1.67	2.51E+00	12.9	[12.3,13.4]
C4		icaD	12.8	1.66	2.38E+00		
C5		icaD	13.1	1.82	2.02E+00		
C6		Atl	11.5	1.86	4.82E+00	11.6	[11.0,12.1]
C7		Atl	11.4	1.70	5.09E+00		
C8		Atl	11.8	1.73	4.10E+00		
D1		ClpC	13.6	1.63	1.54E+00	13.5	[13.4,13.7]
D2		ClpC	13.5	1.84	1.63E+00		
D3		ClpC	13.5	1.88	1.63E+00		
D4		SarX	14.6	1.69	8.97E-01	14.2	[12.9,15.6]
D5		SarX	14.5	1.96	9.47E-01		
D6		SarX	13.6	1.66	1.54E+00		
D7		EbpS	13.9	1.58	1.31E+00	13.8	[12.9,14.7]
D8		EbpS	14.1	1.63	1.18E+00		
E1		EbpS	13.4	1.62	1.72E+00		
E2		RsbU	12.9	1.70	2.26E+00	13.0	[12.7,13.3]
E3		RsbU	13.1	1.65	2.02E+00		
E4		RsbU	13.1	1.79	2.02E+00		
E5		Kef	13.8	1.67	1.38E+00	13.9	[13.3,14.4]
E6		Kef	13.7	1.72	1.46E+00		
E7		Kef	14.1	1.76	1.18E+00		
E8		SarA	13.2	1.64	1.92E+00	13.2	[12.3,14.0]
F1		SarA	13.5	1.75	1.63E+00		
F2		SarA	12.8	1.77	2.38E+00		

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
F3		MurB	13.0	1.67	2.14E+00	13.0	[12.8,13.1]
F4		MurB	12.9	1.73	2.26E+00		
F5		MurB	13.0	1.60	2.14E+00		
F6		MscL	10.9	1.67	6.68E+00	12.2	[7.7,16.7]
F7		MscL	11.5	1.65	4.82E+00		
F8		MscL	14.3	0.41	1.06E+00		
G1		nuc control	26.7	1.59	1.27E-03	26.6	[25.9,27.4]
G2		nuc control	26.3	1.66	1.57E-03		
G3		nuc control	26.9	1.73	1.13E-03		

This report generated by Rotor-Gene Real-Time Analysis Software 6.0 (Build 19)

Figure 3.20: This table show the number of cycles at which take off for each gene occurred, and the amplification of each gene.

3.4.3 KCl cDNA RT-PCR Results: Comparative Quantitation Report

The following results are for RT-PCR performed on the recombinant DNA obtained from a culture of *S. aureus* ATCC 9144 grown in DBHI medium supplemented with 1.4mol/l of KCl.

The following pages contain a take-off graph and a table of values for the take-off and amplification. A melt curve analysis (not included) was performed which again showed single peaks for each individual primer pair and demonstrated no contamination, primer dimers or mispriming.

When the graph for this experiment (Figure 3.21) is compared to the graph of RT-PCR performed on cDNA from DBHI culture (Figure 3.16), it shows a different pattern, suggesting different genetic transcription of the experimental genes in response to KCl stress. Analysis with the Relative Expression Software Tool V1.9.12 (REST 2005) was performed to highlight these differences (See Section: 3.3.9).

The table of take-off and amplication (Figure 3.22), shows, in absolute terms, the differences when compared to the cDNA from the DBHI experiment (see Figure 3.17).

3.4.3.1 Experiment Information

Run Name	Run KCl cDNA
Run On Software Version	Rotor-Gene 6.0.19
Run Signature	The Run Signature is valid.
Gain FAM/Sybr	5.

3.4.3.2 Take off Graph for KCl cDNA RT-PCR

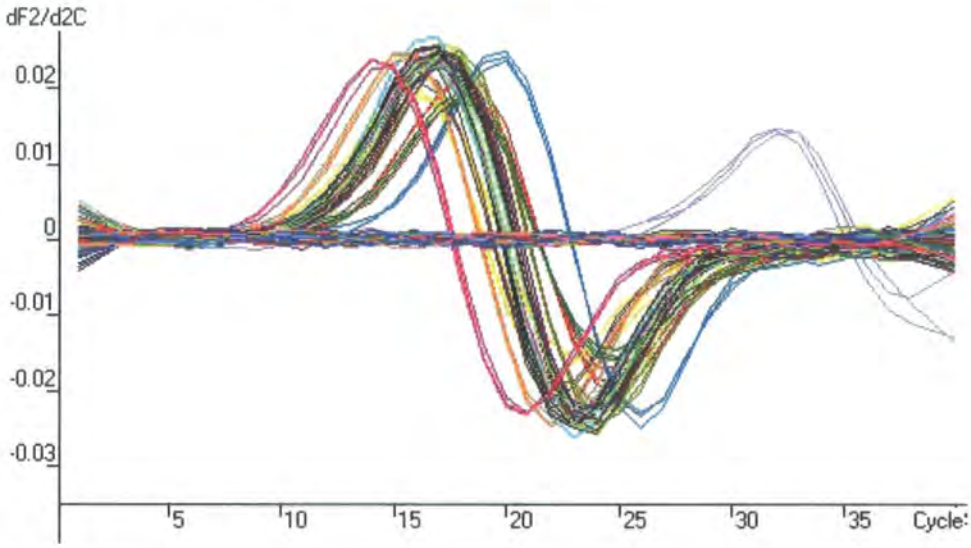


Figure 3.21: This graph shows the take off and amplification against number of cycles of PCR for cDNA from 1.4M KCl culture.

3.4.3.3 Table of Results for KCl cDNA RT-PCR

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
A1		nuc	14.1	1.07	9.39E-01	14.0	[13.4,14.5]
A2		nuc	13.7	1.40	1.13E+00		
A3		nuc	14.1	1.62	9.39E-01		
A4		16s	11.7	1.66	2.91E+00	12.1	[11.2,13.0]
A5		16s	12.2	1.82	2.30E+00		
A6		16s	12.4	1.37	2.09E+00		
A7		Spa	13.3	1.69	1.37E+00	13.3	[13.0,13.7]
A8		Spa	13.2	1.67	1.44E+00		
B1		Spa	13.5	1.49	1.25E+00		
B2		pbp4	12.3	1.35	2.19E+00	12.3	[12.1,12.5]
B3		pbp4	12.4	1.47	2.09E+00		
B4		pbp4	12.2	1.39	2.30E+00		
B5		ClfB	15.8	1.81	4.21E-01	15.8	[15.8,15.8]
B6		ClfB	15.8	1.77	4.21E-01		
B7		ClfB	15.8	1.53	4.21E-01		
B8		MurG	13.0	1.66	1.58E+00	12.7	[12.1,13.4]
C1		MurG	12.7	1.74	1.82E+00		
C2		MurG	12.5	1.89	2.00E+00		
C3		icaD	12.3	1.73	2.19E+00	12.3	[12.0,12.7]
C4		icaD	12.5	1.47	2.00E+00		
C5		icaD	12.2	1.79	2.30E+00		
C6		Atl	11.4	1.80	3.35E+00	11.5	[11.2,11.8]
C7		Atl	11.4	1.67	3.35E+00		
C8		Atl	11.6	1.65	3.05E+00		
D1		ClpC	13.1	1.23	1.50E+00	13.0	[12.6,13.4]
D2		ClpC	13.1	1.22	1.50E+00		
D3		ClpC	12.8	1.65	1.73E+00		
D4		SarX	13.7	1.65	1.13E+00	14.1	[13.2,15.1]
D5		SarX	14.4	1.74	8.15E-01		
D6		SarX	14.3	1.64	8.55E-01		
D7		EbpS	13.5	1.66	1.25E+00	13.4	[12.8,13.9]
D8		EbpS	13.1	1.64	1.50E+00		
E1		EbpS	13.5	1.40	1.25E+00		
E2		RsbU	12.8	1.71	1.73E+00	12.5	[11.4,13.5]
E3		RsbU	12.6	1.90	1.90E+00		
E4		RsbU	12.0	1.60	2.53E+00		
E5		Kef	13.1	1.74	1.50E+00	13.3	[12.8,13.8]
E6		Kef	13.3	1.34	1.37E+00		
E7		Kef	13.5	1.69	1.25E+00		
E8		SarA	12.8	1.70	1.73E+00	12.9	[12.6,13.2]
F1		SarA	12.8	1.55	1.73E+00		
F2		SarA	13.0	1.78	1.58E+00		

No.	Colour	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)
F3		MurB	12.6	1.42	1.90E+00	12.6	[11.9,13.3]
F4		MurB	12.3	1.36	2.19E+00		
F5		MurB	12.9	1.66	1.65E+00		
F6		MscL	10.3	1.59	5.63E+00	10.3	[10.0,10.7]
F7		MscL	10.2	1.67	5.90E+00		
F8		MscL	10.5	1.66	5.12E+00		
G1		nuc control	27.3	1.50	1.87E-03	27.9	[26.4,29.4]
G2		nuc control	28.5	1.35	1.06E-03		
G3		nuc control	27.8	0.96	1.48E-03		

Figure 3.22: This table show the number of cycles at which take off for each gene occurred and the amplification of each gene. It shows differences when compared to the cDNA from DBHI experiment (See Figure 3.17).

3.4.4 Statistical Analysis Using REST 2005 V1.9.12

The Relative Expression Software Tool (REST) developed by Michael Pfaffl in conjunction with Corbett Research was used to analyse the raw data from the experiments. The program compliments the Rotor-Gene machine used, also manufactured by Corbett Research. REST has been well validated and the latest incarnation, REST 2005 V1.9.12, which increased the number of iterations performed from 2000 to 50,000 to improve the consistency of statistical tests, was used in these experiments (Pfaffl et al., 2002).

The REST program can be used to produce boxplots to show the relative expression of the genes in a visual form. Boxplots are useful because they show a large amount of information. The boxes represent the interquartile range, or middle 50%. The dotted line within each box represents the median gene expression. Whiskers represent the minimum and maximum observations. Boxes below a relative expression ratio of 1 show genes that have been down-regulated, and boxes above a relative expression ratio of 1 show genes that have been up-regulated in the sample group relative to the control.

3.4.5 Statistical Comparison of DBHI vs. NaCl: Relative Expression Report

The results from the DBHI culture were set as the control group, and the results from the DBHI culture supplemented with 1.4mol/l NaCl were set as the sample group. MurG and Atl were set as the reference genes as they were expressed in a 1:1 ratio.

The results of this statistical comparison, shown in table form in Figure 3.23, shows that several genes are down regulated under NaCl stress and that several of these changes are statistically significant. 16s, Spa, pbp4, icaD, ClpC, RsbU, Kef and MurB are down-regulated with statistical significance. Nuc and ClfB are also down-regulated with very nearly statistical significance.

A boxplot of the results (Figure 3.24) shows that most genes have been down-regulated in the NaCl group relative to the DBHI control group. MurG and Atl both have a value of 1 as they are the reference genes are were expressed in a 1:1 ratio. No genes have been up-regulated. Not all the apparent down-regulations seen in this boxplot achieve statistical significance, as shown in the table in Figure 3.23.

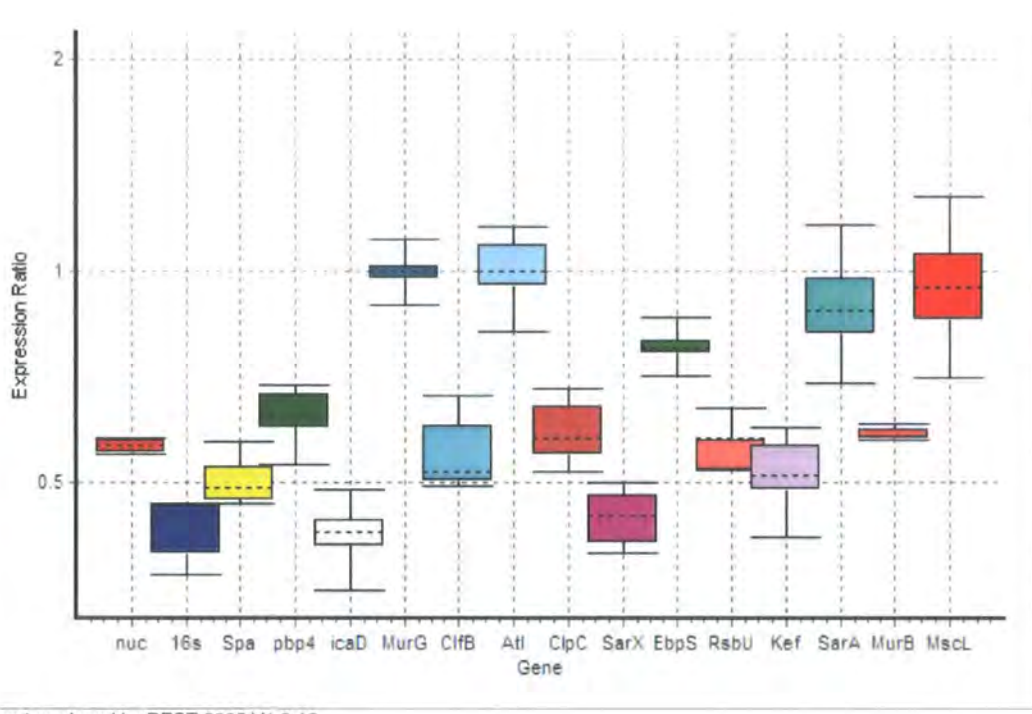
3.4.5.1 Table of Statistics for DBHI vs. NaCl

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
nuc	TRG	0.722	0.565	0.550 - 0.581	0.550 - 0.581	0.067	
16s	TRG	0.794	0.433	0.370 - 0.468	0.370 - 0.468	0.000	DOWN
Spa	TRG	0.66	0.504	0.468 - 0.550	0.468 - 0.569	0.000	DOWN
pbp4	TRG	0.692	0.623	0.579 - 0.692	0.539 - 0.692	0.034	DOWN
icaD	TRG	0.607	0.419	0.379 - 0.462	0.356 - 0.486	0.000	DOWN
MurG	REF	0.723	1.000	0.946 - 1.060	0.905 - 1.106	1.000	
ClfB	TRG	0.654	0.551	0.494 - 0.643	0.494 - 0.665	0.066	
Atl	REF	0.635	1.000	0.919 - 1.103	0.838 - 1.148	1.000	
ClpC	TRG	0.727	0.590	0.548 - 0.672	0.525 - 0.682	0.032	DOWN
SarX	TRG	0.585	0.447	0.407 - 0.490	0.399 - 0.499	0.143	
EbpS	TRG	0.633	0.783	0.744 - 0.825	0.715 - 0.857	0.141	
RsbU	TRG	0.645	0.560	0.524 - 0.578	0.524 - 0.627	0.033	DOWN
Kef	TRG	0.67	0.513	0.470 - 0.569	0.427 - 0.593	0.032	DOWN
SarA	TRG	0.684	0.893	0.788 - 1.035	0.709 - 1.148	0.536	
MurB	TRG	0.646	0.588	0.578 - 0.608	0.578 - 0.608	0.033	DOWN
MscL	TRG	0.648	0.951	0.796 - 1.142	0.719 - 1.261	0.737	

Legend: P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance. Sample = DBHI + 1.4M NaCl, Control = DBHI. TRG – Target. REF – Reference. 50,000 iterations.

Figure 3.23: Table of Relative Expression Results for DBHI vs. NaCl. Expression values of less than 1 indicate that the gene has been down-regulated in the sample group relative to the control. Expression values above 1 indicate that the gene has been up-regulated in the sample relative to the control. The standard error, 95% confidence intervals and P values are shown. The P value indicates whether a change in gene expression, whether up or down-regulated, is statistically significant or could have happened by chance.

3.4.5.2 Boxplot of DBHI vs. NaCl: Relative Expression Report



Report produced by REST 2005 V1.9.12
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Figure 3.24: The boxplot above shows boxes representing the interquartile range, or the middle 50% of observations. The dotted line within each box represents the median gene expression. Whiskers or error bars represent the minimum and maximum observations. Boxes below a relative expression ratio of 1 show genes that have been down regulated. Boxes above a relative expression ratio of 1 show genes that have been up-regulated. N.B. The colour scheme of this boxplot was produced by the REST 2005 program, and is not changeable. The colours do not link with the quantitation report and should not be used for gene identification. The genes are marked on the x-axis.

3.4.5.3 Interpretation of Results for DBHI vs. NaCl

16s, Spa, pbp4, icaD, ClpC, RsbU, Kef and MurB are all down-regulated under salt stress with a significance value of p=0.05 or better. nuc and ClfB are also down regulated although only if the significance values are stretched to p=0.1. MurG and Atl, used as the reference genes are expressed in a 1:1 ratio. The other genes, SarX, Ebps, SarA and MscL are also down regulated under salt stress although these changes are not statistically significant.

3.4.6 Statistical Comparison of DBHI vs. KCl: Relative Expression Report

The results from the DBHI culture were set as the control group, and the results from the DBHI culture supplemented with 1.4mol/l KCl were set as the sample group. MurG and Atl were set as the reference genes as, again, they were expressed in very nearly a 1:1 ratio.

The results of this statistical comparison, as shown in table form in Figure 3.25, shows that several genes are down regulated under KCl stress and that several of these changes are statistically significant. nuc, 16s, Spa, pbp4, icaD, RsbU and Kef are all down-regulated with statistical significance. ClfB, ClpC, SarX and MurB are also down-regulated and very nearly achieve statistical significance.

A boxplot of the results shows that most genes have been down-regulated in the KCl group relative to the DBHI control group. Not all the apparent down-regulations seen in this boxplot achieve statistical significance, as shown in the table in Figure 3.25. One gene, MscL appears to have been up-regulated but, when this is compared to the table in Figure 3.25, it is not statistically significant.

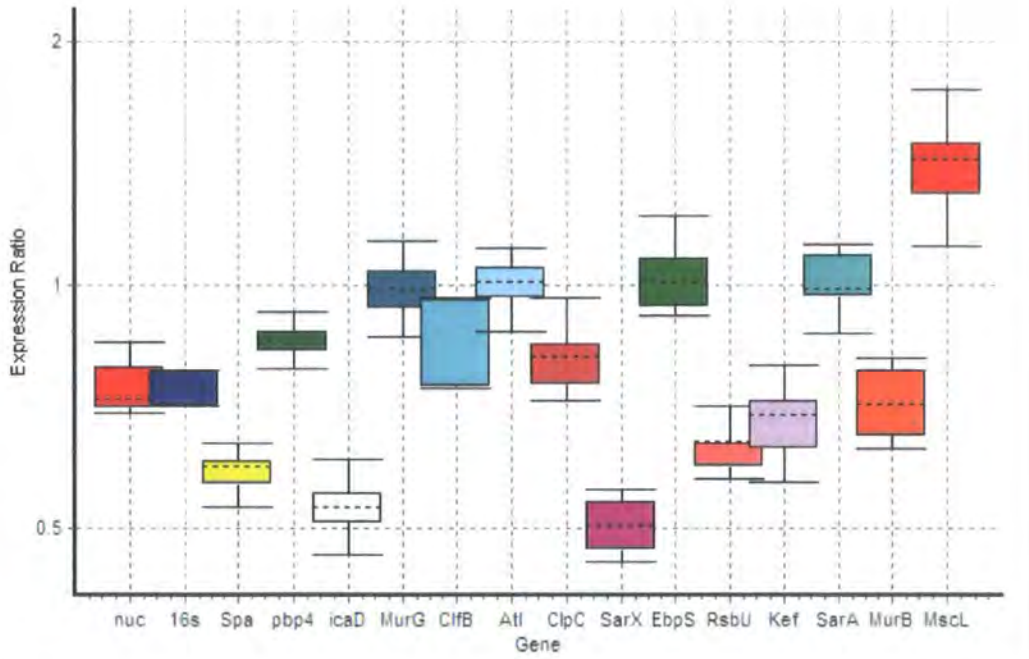
3.4.6.1 Table of Statistics for DBHI vs. KCl

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
nuc	TRG	0.502	0.749	0.696 - 0.826	0.696 - 0.848	0.033	DOWN
16s	TRG	0.663	0.747	0.710 - 0.786	0.710 - 0.786	0.000	DOWN
Spa	TRG	0.578	0.588	0.552 - 0.616	0.535 - 0.635	0.000	DOWN
pbp4	TRG	0.494	0.854	0.814 - 0.896	0.792 - 0.921	0.034	DOWN
icaD	TRG	0.58	0.531	0.491 - 0.575	0.468 - 0.604	0.000	DOWN
MurG	REF	0.736	0.990	0.911 - 1.080	0.870 - 1.128	1.000	
ClfB	TRG	0.675	0.811	0.744 - 0.963	0.744 - 0.963	0.064	
Atl	REF	0.607	1.010	0.963 - 1.096	0.893 - 1.110	0.937	
ClpC	TRG	0.518	0.804	0.727 - 0.850	0.719 - 0.941	0.081	
SarX	TRG	0.518	0.504	0.464 - 0.549	0.456 - 0.558	0.072	
EbpS	TRG	0.604	1.026	0.919 - 1.132	0.919 - 1.206	0.834	
RsbU	TRG	0.668	0.629	0.577 - 0.654	0.577 - 0.700	0.035	DOWN
Kef	TRG	0.607	0.680	0.628 - 0.750	0.583 - 0.789	0.017	DOWN
SarA	TRG	0.658	1.005	0.944 - 1.121	0.882 - 1.121	1.000	
MurB	TRG	0.534	0.714	0.628 - 0.812	0.628 - 0.812	0.068	
MscL	TRG	0.64	1.407	1.250 - 1.617	1.141 - 1.727	0.129	

Legend: P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance. Sample = KCl, Control = DBHI.
TRG – Target. REF – Reference. 50,000 iterations.

Figure 3.25: Table of Relative Expression Results for DBHI vs. KCl. Expression values of less than 1 indicate that the gene has been down-regulated in the sample group relative to the control. Expression values above 1 indicate that the gene has been up-regulated in the sample relative to the control. The standard error, 95% confidence intervals and P values are shown. The P value indicates whether a change in gene expression, whether up or down-regulated, is statistically significant or could have happened by chance.

3.4.6.2 Boxplot of DBHI vs. KCl: Relative Expression Report



Report produced by REST 2005 V1.9.12
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Figure 3.26: This boxplot shows boxes representing the interquartile range, or the middle 50% of observations. The dotted line within each box represents the median gene expression. Whiskers represent the minimum and maximum observations. Boxes below a relative expression ratio of 1 show genes that have been down regulated. Genes above a relative expression ratio of 1 show genes that have been up-regulated.

N.B. The colour scheme of this boxplot was produced by the REST 2005 program, and is not changeable. The colours do not link with the quantitation report and should not be used for gene identification. The genes are marked on the x-axis.

3.4.6.3 Interpretation of Results for DBHI vs. KCl.

nuc, 16s, Spa, pbp4, icaD, RsbU and Kef are all down regulated under KCl stress with a significance value of p=0.05 or better. Several other genes, MurB, ClfB, ClpC and SarX also appear to be down regulated although this is only significant if the significance values are stretched to p=0.1. MscL appears to be up regulated although this is not statistically significant. MurG and Atl, used as the reference genes, were expressed with a ratio close to 1:1, as was SarA and EbpS.

3.4.7 Statistical Comparison of NaCl vs. KCl: Relative Expression Report

The results from the DBHI culture supplemented with 1.4mol/l NaCl were set as the control group, and the results from the DBHI culture supplemented with 1.4mol/l KCl were set as the sample group. MurG and Atl were set as the reference genes as they were again expressed in close to a 1:1 ratio.

The results of this statistical comparison, as shown in table form in Figure 3.27, show that several genes are slightly up-regulated under KCl stress relative to NaCl stress. However, there is no statistically significant difference between the two groups. The results column is empty because there has not been any statistical up or down regulation of any gene.

A boxplot of the results (Figure 3.28) shows that most genes have been up-regulated in the KCl group relative to the NaCl control group. However, none of the up-regulations seen in this boxplot achieve statistical significance, as shown in the table in Figure 3.27.

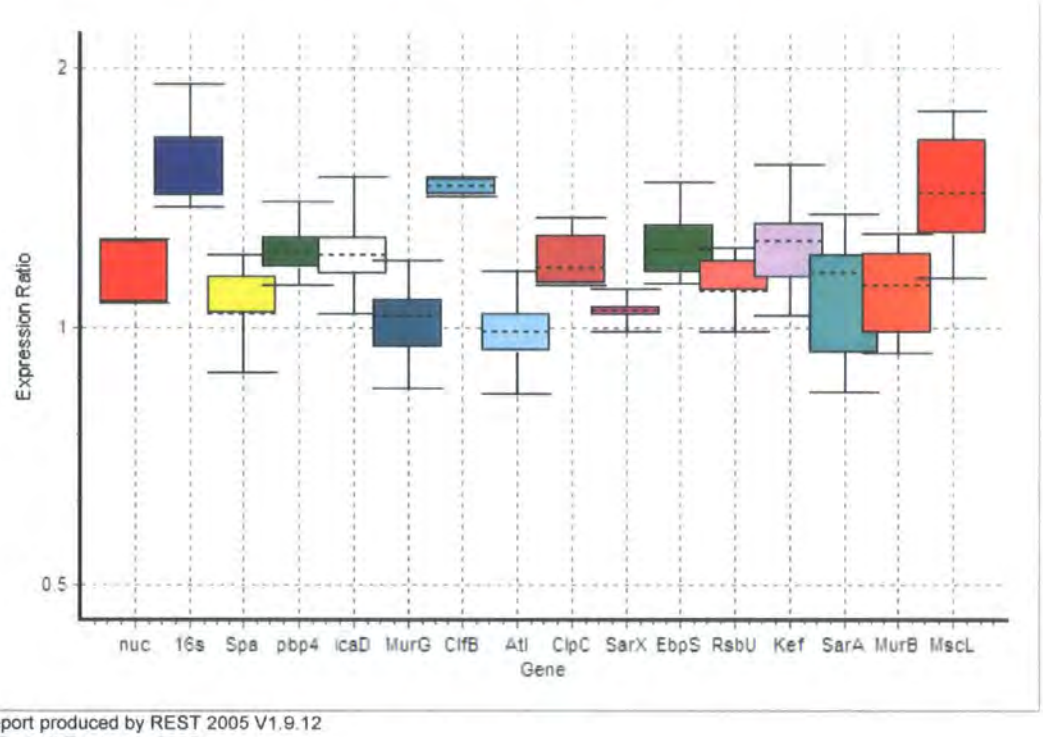
3.4.7.1 Table of Statistics for NaCl vs. KCl.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
nuc	TRG	0.547	1.128	1.064 - 1.267	1.064 - 1.267	0.357	
16s	TRG	0.74	1.566	1.376 - 1.759	1.376 - 1.894	0.168	
Spa	TRG	0.685	1.073	0.998 - 1.195	0.906 - 1.212	0.581	
pbp4	TRG	0.568	1.223	1.132 - 1.322	1.118 - 1.388	0.230	
icaD	TRG	0.69	1.235	1.109 - 1.382	1.048 - 1.482	0.226	
MurG	REF	0.633	1.013	0.916 - 1.105	0.858 - 1.179	0.901	
ClfB	TRG	0.688	1.457	1.420 - 1.496	1.420 - 1.496	0.099	
Atl	REF	0.735	0.987	0.897 - 1.131	0.846 - 1.164	0.854	
ClpC	TRG	0.575	1.190	1.120 - 1.283	1.120 - 1.331	0.257	
SarX	TRG	0.758	1.045	1.015 - 1.077	0.992 - 1.102	0.713	
EbpS	TRG	0.582	1.249	1.122 - 1.374	1.122 - 1.461	0.267	
RsbU	TRG	0.752	1.126	1.081 - 1.236	1.002 - 1.236	0.367	
Kef	TRG	0.653	1.242	1.101 - 1.377	1.043 - 1.514	0.246	
SarA	TRG	0.698	1.095	0.934 - 1.316	0.859 - 1.353	0.502	
MurB	TRG	0.573	1.103	0.977 - 1.267	0.943 - 1.282	0.484	
MscL	TRG	0.648	1.440	1.236 - 1.719	1.155 - 1.777	0.195	

Legend: P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance. Sample = KCl, Control = NaCl.
TRG – Target. REF – Reference. 50,000 iterations.

Figure 3.27: Table of Relative Expression Results for KCl vs. NaCl. Expression values of less than 1 indicate that the gene has been down-regulated in the sample group relative to the control. Expression values above 1 indicate that the gene has been up-regulated in the sample relative to the control. The standard error, 95% confidence intervals and P values are shown. The P value indicates whether a change in gene expression, whether up or down-regulated, is statistically significant or could have happened by chance.

3.4.7.2 Boxplot of NaCl vs. KCl: Relative Expression Report



Report produced by REST 2005 V1.9.12
© Corbett Research, 2005

Figure 3.28: This boxplot shows boxes representing the interquartile range, or the middle 50% of observations. The dotted line within each box represents the median gene expression. Whiskers represent the minimum and maximum observations. Boxes below a relative expression ratio of 1 show genes that have been down regulated. Genes above a relative expression ratio of 1 show genes that have been up-regulated. N.B. The colour scheme of this boxplot was produced by the REST 2005 program, and is not changeable. The colours do not link with the quantitation report and should not be used for gene identification. The genes are marked on the x-axis.

3.4.7.3 Interpretation of Results for NaCl vs. KCl.

Comparing NaCl stress to KCl stress, there was no significant difference in the results. Under KCl stress there seemed to be general up regulation of the genes compared to NaCl stress, but without statistical significance. MurG and Atl, once again used as the reference genes, were expressed with close to a 1:1 ratio.

Section 4: Discussion

4.1.0 NaCl Stress: Summary of Results

1. Salt stress on Yabu agar leads to the formation of Cell Wall Deficient bacteria.
2. Salt stress leads to inhibition of growth and decreased cell counts.
3. Salt stress leads to an increase in penicillin resistance from an MIC of 0.008mg/l to 0.047mg/l after three passages on Yabu agar. This resistance is un-stable and returns to the base-line MIC of 0.008mg/l after 4 passages on trypticase-soy broth agar.
4. Salt stress leads to a decrease in susceptibility to lysostaphin.

4.1.1 Discussion of NaCl Stress Results

Since the original discovery of L-forms at the Lister Institute by Klieneberger in 1935, many different mechanisms of inducing them have been described (Klieneberger, 1935). L-forms, or cell wall deficient bacteria, are often induced by sub-lethal doses of cell wall-active antibiotics. Yabu has previously described the formation of L-forms in *S. aureus* on a high concentration NaCl media (Yabu, 1995). Current work, described here, has repeated his work but with slightly differing results.

The Yabu paper quoted a 99% conversion of inoculated cells into L-form colonies (Yabu, 1995). However, in current experiments, the Yabu agar led to a mixed growth of large and small colonies, the small being cell wall deficient, the large being normal Gram-positive cocci. It is important to note though, that a different strain of *S. aureus* was used (ATCC 9144 instead of 209P). Although the results differ slightly, NaCl stress did induce L-form formation.

S. aureus is a halotolerant bacterium, meaning that it can grow under conditions of high salt concentration, but that it does not necessarily need a high salt concentration to grow. The cell count experiments performed on Yabu agar show that high NaCl concentrations are inhibitory, leading to a significantly decreased cell count when compared to Trypticase-soy broth agar (15-fold less). This is an important consideration because inhibition of growth by high concentrations of NaCl may adversely affect other experiments which make comparisons of the actions of *S. aureus* when grown in low and high concentrations of NaCl.

Previous work has shown that high concentrations of NaCl can be used to isolate *S. aureus* from mixed cultures (Chapman, 1945). Chapman quotes Koch, who noted that usually only staphylococci are able to grow on agar media containing 7.5 percent NaCl (approximately 1.4mol/l). Chapman concludes that, even amongst *Staphylococci*, there is a difference in growth patterns on media supplemented with 7.5 percent NaCl. He states that pathogenic staphylococci grow luxuriantly whereas non-pathogenic staphylococci grow poorly (Chapman, 1945). The findings of Chapman support the observations made in this work, that *S. aureus* ATCC 9144 grows in the presence of high NaCl concentration, but less well.

Cell wall deficient bacteria have been implicated in antibiotic resistance since the early days of their discovery (Dienes & Weinberger, 1951). Recent work at Durham University has shown that sub-lethal levels of penicillin not only lead to the formation of antibiotic resistant L-forms in *S. aureus*, but that the resistance is stable even after removal of the inducing stress (Fuller et al., 2005). The cell wall deficient bacteria induced by a high concentration of NaCl in the experiments in this thesis did gain slight resistance to penicillin. The level of this resistance increased with

subsequent NaCl stresses, such that it rose from a baseline MIC of 0.008mg/l to an MIC of 0.047mg/l after three passes of NaCl stress. In contrast to the work of Fuller et al., the increase in penicillin resistance was modest. It was also unstable, and the MIC returned to the baseline level of 0.008mg/l after removal of the NaCl stress.

The cell wall deficient bacteria induced by NaCl stress were also subjected to lysostaphin susceptibility assays. The results showed that increasing NaCl stress lead to a decrease in lysostaphin susceptibility.

Intuitively, the results of these experiments can be explained. Stressful environmental conditions have long been known to induce cell wall deficiency in bacteria (Dienes & Weinberger, 1951). The cell walls of bacteria normally provide a rigid and strong barrier to withhold the turgor pressure on the inside of the cell (Doyle & Marquis, 1994). However, in the osmotically stressful environment of high NaCl concentration, the cells do not require this strong barrier, and so can survive in a cell wall deficient state. Furthermore, in a cell wall deficient state it also follows that some degree of resistance to cell wall active antibiotics should develop, as the bacteria are not producing their cell walls in the normal way. Also, if the cell walls are deficient, it seems likely that there will be less pentaglycine cross-brides, which normally bind the peptidoglycan in the cell wall of *S. aureus*. Hence, enzymes which act on cell wall, such as lysostaphin, which breaks down the pentaglycine cross-bridges, should also be less efficacious. This is borne out by the results in this thesis.

Other work has also shown resistance of *S. aureus* to antibiotics after salt stress. McMahon et al. showed that NaCl stress lead to resistance to gentamicin, oxacillin and erythromycin in *S. aureus* (McMahon et al., 2007). In their work, NaCl concentrations of greater than 4.5 percent were used to stress the cells. Gentamicin resistance increased between 1.5 and greater than 4 times the baseline level in

different strains. Erythromycin resistance increased between 0 and 4 times the baseline level in different strains. Oxacillin resistance was, however, quite variable. A 1.5 to 2-fold decrease in resistance compared to the baseline was noted in two strains whereas a 2.1 and 4-fold rise was noted in others. Furthermore, McMahon et al. stated that the increases in antibiotic resistance noted in response to NaCl stress were stable.

This is in contrast to the results in this thesis, where an increase resistance to penicillin was noted in response to NaCl stress. A single episode of NaCl stress lead to a 2-fold increase in penicillin resistance, but three episodes of NaCl stress lead to an 8-fold increase in resistance over the baseline level. This was unstable, returning to normal after four passes on trypticase-soy broth agar without added salt stress.

It is interesting to note that resistance to gentamicin and erythromycin has been noted after NaCl stress. Gentamicin effects its antibiotic function by interfering with the reading of mRNA at the level of the ribosome. Erythromycin also affects protein synthesis but at a slightly different site, by inhibiting translocation. (Neal, 1997) Salt stress, as stated before, leads to cell wall deficiency in *S. aureus* and this would offer a putative mechanism of resistance to cell wall active antibiotics. However, it is intuitively less obvious how salt stress affects the resistance of cells to gentamicin and erythromycin, which both act by inhibiting protein synthesis.

4.2.0 Biofilm Assays: Summary of Results

1. **LiCl:** Changes in LiCl concentration lead to a variable and unpredictable effect on biofilm formation. A slight increase in LiCl concentration (0.4mol/l) leads to an apparent small reduction in biofilm formation but a higher concentration (1.0mol/l) leads to a slight increase in biofilm formation. Yet higher concentrations (1.4mol/l) seem not to affect biofilm formation.
2. **NaCl:** Increasing NaCl concentration leads to a bi-phasic effect on biofilm formation. Concentrations of NaCl above 0.6mol/l lead to a progressive increase in biofilm formation. Concentrations below 0.6mol/l do not affect biofilm formation above the baseline level.
3. **KCl :** Increasing KCl concentration also leads to bi-phasic effect on biofilm formation. Concentrations of KCl above 0.4mol/l lead to a progressive increase in biofilm formation. Concentrations below 0.4mol/l do not affect biofilm formation above the baseline level.
4. **RbCl:** Low concentrations of RbCl (0mol/l to 0.4mol/l) do not alter biofilm formation, but at concentrations of 0.6mol/l and above, biofilm formation is increased above the baseline level. This increase in biofilm formation does not increase any more with further increases in RbCl concentration.
5. **CsCl:** Low concentrations of CsCl (0.2mol/l) increases biofilm formation, but higher concentrations of CsCl (1.4mol/l) lead to a progressive decrease in biofilm.

4.2.1 Discussion of Biofilm Assay Results

Biofilm formation is an extensive research topic and has aroused much interest in the scientific community (Branda et al., 2005). The role of biofilm in chronic and

persistent infections, particularly those associated with endoprostheses, is well documented (Costerton et al., 1999; Stewart & Costerton, 2001).

Previous work has highlighted an increase in biofilm formation in response to increased NaCl concentration (Lim et al., 2004). The findings by Lim et al. have been reiterated by this work, although the increase in biofilm formation seen, while statistically significant, was less than in their study. The role of other Group I metals in biofilm formation has not been published previously.

Lithium chloride gave an unpredictable result in terms of its effect on biofilm formation. Comparing a 0mol/l concentration with a 1.4mol/l concentration there was no difference in biofilm formation. There were however, slight changes at lower concentrations which are not readily explicable. Growth of *S. aureus* was not inhibited by higher concentrations of LiCl. High concentrations of NaCl have been shown to inhibit most contaminating bacteria other than *S. aureus* (Chapman, 1945). Lithium too can be used to help isolate *S. aureus*. HiChrome™ Aureus Agar Base, manufactured by Fluka, Sigma-Aldrich, USA, contains lithium chloride and potassium tellurite, a combination which inhibits most bacterial contaminants except *S. aureus*. This agar is very similar in its constituents to the one described by Zebovitz et al., which was shown to inhibit most contaminating microflora except *S. aureus* (Zebovitz, Evans & Niven, 1955).

Increasing sodium concentration, as mentioned above, does have a positive effect on biofilm formation. NaCl stress also leads to the development of a Gram-negative phenotype in *S. aureus*. One possible explanation that links both these observations could be answered by some work previously performed on *Lactococcus lactis* (Mercier et al., 2002). Biofilm formation is often attributed to MSCRAMMs. However, Mercier et al. showed a positive correlation between peptidoglycan breaks,

rather than particular molecules, and increased biofilm formation. They demonstrated decreased biofilm formation in *Lactococcus lactis* that was defective in peptidoglycan hydrolase. However, when lysosyme, a peptidoglycan hydrolase was added, the decrease in biofilm formation was reverted, showing that it was the peptidoglycan breaks and not the intrinsic bacterial enzyme that was essential to biofilm formation (Mercier et al., 2002). NaCl stress leads to Cell Wall Deficient bacteria with a Gram-negative phenotype. If an increase in the number of peptidoglycan breaks is a feature of cell wall deficiency, then this would explain the increase in biofilm noted in response to an increase in NaCl concentration. One other possible explanation regards teichoic acids. These are covalently bound to peptidoglycan and hence a deficient cell wall inevitably leads to an alteration in surface expression of teichoic acid. Teichoic acids are negatively charged, and hence are partially responsible for the negative charge of the cell surface (Neuhaus & Baddiley, 2003). Biofilm formation on polystyrene and glass surfaces is decreased in mutant *S. aureus* cells with increased teichoic acid expression, and hence an increased negative charge at the cell surface, suggesting an electrostatic repulsion (Neuhaus & Baddiley, 2003). It thus seems plausible that CWDB, with compromised teichoic acid structure, would cause less electrostatic repulsion and may be more readily adherent to glass and polystyrene surfaces. Future work could look at the nature of the deficient cell wall produced in response to NaCl stress, and ascertain the structure and cross-linkage of the peptidoglycan as well as the attachment and amount of teichoic acid.

Potassium has not previously been implicated in biofilm formation. However, in this work it has been shown to exert an effect on biofilm formation. An increase in KCl concentration, lead to an increase in biofilm formation, which was highly statistically significant. Potassium is the most abundant cation in intracellular fluid

and is required by all organisms. Many enzymatic processes including several involved in protein synthesis specifically require potassium (Madigan et al., 2000). Knowledge of potassium channels is increasing, but a specific channel has yet to be found in *Staphylococcus aureus* (Kuo et al., 2005). (See Section 1.6.2) A potassium efflux channel, present in *E. coli*, has however, been identified in *S. aureus*, although not in other Gram-positive bacteria (Douglas et al., 1991).

The increase in biofilm formation in response to increase KCl concentration is potentially an important surgical consideration. Cellular injury or insult can lead to cell necrosis and lysis. The intracellular concentration of potassium in human cells is 30 times higher than that of the surrounding extracellular fluid. Hence, cell necrosis leads to markedly increased local concentrations of potassium. This raises the concern that biofilm formation, and hence intractable infection of implanted prostheses may be more likely in areas of major tissue trauma such as in large soft tissue and tumour resections, crush injuries and major trauma and prosthetic revision surgery.

The effects on biofilm formation noted in response to changes in RbCl and CsCl concentrations are not readily explicable. Neither Rubidium, nor Caesium is considered to be a major trace element, vital to cellular function (Madigan et al., 2000). Work published previously has not looked at the effect of rubidium or caesium on biofilm formation and so the findings presented here merit further investigation.

Osmolarity has not been completely excluded as a cause of increased biofilm formation. However, if the changes seen in response to increased concentrations of NaCl and KCl were purely in response to changes in osmolarity of the media, then one would expect to see the same effect from equimolar concentrations of LiCl, RbCl and CsCl, which was manifestly not the case. Future work could include the use of sorbitol, a non-metabolisable osmotic agent, as an osmolarity matched control to

investigate this further. Other ideas for research would be to perform similar experiments using the chlorides of divalent Group II metals such as magnesium and calcium, both of which are extremely important to cellular function. Calcium has in fact been shown previously to have a negative effect on biofilm-associated protein (Bap) dependent biofilm formation, although this seems to be a Bap specific phenomenon and this work has not been extended to *S. aureus* biofilm in general (Arrizubieta et al., 2004).

4.3.0 Discussion of RT-PCR

RT-PCR is one method of investigating the expression profile of experimental cells. It was used in this work to investigate cellular changes, at a transcription level, in response to either NaCl or KCl stress.

RT-PCR has several drawbacks. Firstly it can only be used to investigate specific genes of interest, which need to be identified before the experiment and have specific primers designed for them. Secondly it is a multi-step process with possibilities for error at each step. It requires perfect collection of the mRNA, faithful reverse transcription and well-optimised RT-PCR. Finally, it is prone to contamination, and hence extreme care must be taken at every step of the process.

RT-PCR, as mentioned above, can only be used to investigate specific, pre-identified genes. In this work, various genes, representative of many processes involved in biofilm formation, cell wall production, osmoregulation, stress response and gene regulation were identified in the literature. Primers were designed and assessed using both PCR and RT-PCR. The genes chosen for investigation were only

a "best guess" about which cellular processes may have been affected by NaCl or KCl stress.

An alternative method of investigating cellular changes in response to NaCl or KCl stress would have been to perform microarrays. Having identified some changes amongst the few genes chosen in this work, microarray may be a more suitable method for future research.

Regarding the RT-PCR experiments described here, it is worth commenting on some potential improvements that could be made to the technique in the future. In all 3 experimental situations (DBHI only, DBHI supplemented with 1.4mol/l NaCl and DBHI supplemented with 1.4mol/l KCl), *murG* and *atl* were used as the reference genes for statistical comparison. This is because they were expressed in a 1:1 ratio in all 3 experiments and thus provided a baseline against which to compare the transcription of all the other genes under experimentation. However, future work may be supplemented by using other constitutive or "housekeeping" genes as the reference genes. A multilocus sequence typing scheme, which has been developed for *S. aureus*, uses 7 such housekeeping genes, and it may be appropriate to use some or all of these in future work. These housekeeping genes are, carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) (Enright et al., 2000). The use of the gene for *S. aureus* specific thermostable nuclease (*nuc*), which was used in the experiments in this thesis, has been described in the literature. However, *nuc* is often used more for *S. aureus* identification and quantification than for comparison between rates of transcription of different genes (Alarcon, Vicedo & Aznar, 2005; Brakstad et al., 1992).

4.4.0 Summary and Discussion of DBHI vs. NaCl RT-PCR Results.

Comparison of RT-PCR performed on *S. aureus* culture from DBHI and from DBHI supplemented with 1.4M NaCl showed that several genes were down-regulated with statistical significance. These were *16s*, *Spa*, *pbp4*, *icaD*, *ClpC*, *RsbU*, *Kef*, and *MurB*. No genes were up-regulated.

The down-regulation of *16s* suggests a pleiotropic effect and a probable global reduction in transcription. Micro-array could be used to investigate this hyothesis further.

It is interesting to note that pbp4 has been down regulated. Penicillin binding protein plays an important role in peptidoglycan cross-linking (Sieradzki et al., 1999). Results above, show that NaCl stress leads to cell wall deficiency, increased resistance to lysostaphin and penicillin, and an increase in biofilm formation in *S. aureus*. (See Sections: 3.1.1, 3.1.3, 3.1.4.1 and 3.2.3.1) A reduction in pbp4 production seems to offer one possible mechanism for some of these changes. Cell Wall Deficient cells have an altered cell wall structure by definition, and hence it fits with this picture that there should be a down-regulation of a major protein involved in crosslinking the peptidoglycan structure. The down-regulation of pbp4 also offers a putative mechanism for penicillin resistance in that the cells are not producing their cell walls in the normal way. The down-regulation of pbp4 may also explain the increase in resistance to lysostaphin. Lysostaphin lyses *S. aureus* cells by cleaving the pentaglycine cross-bridges present in the peptidoglycan. It seems likely that in their cell wall deficient state, *S. aureus* cells do not require extensive cross-linking of their peptidoglycan to maintain cellular integrity and hence have a lesser requirement for pbp4 and a greater resistance to lysostaphin.

The down-regulation of MurB might also be expected from the results in this work. MurB is an important enzyme in the production of the basic building blocks of peptidoglycan (El Zoeiby et al., 2003). Under NaCl stress, the *S. aureus* cells adopt a cell wall deficient phenotype. A deficient cell wall implicitly requires less peptidoglycan in its formation and hence in is not surprising that MurB has been down regulated under NaCl stress. MurG, however, was unaffected by NaCl stress.

Kef is a putative potassium efflux channel. In low-osmolar environments it is postulated that it is used to dump potassium, the most abundant intracellular cation, thus redressing the difference between the internal and external osmolarities and so protecting the cell from osmotic lysis (Douglas et al., 1991). In the high osmolar conditions of NaCl stress, there is little requirement for this channel and hence it is perhaps to be expected that its production should be down-regulated, as indeed is the case.

The down-regulation of *icaD* is perhaps the most unexpected result. The intercellular adhesin locus, of which *icaD* is a vital part, controls the production of polysaccharide intercellular adhesin (PIA). This intercellular adhesin is an extremely important part of biofilm formation and many papers have investigated both its production and the control mechanisms thereof (Cramton et al., 1999; Dobinsky et al., 2003). NaCl stress leads to an increase in biofilm formation, but a decrease in *icaD* transcription, which suggests that the biofilm is forming via another mechanism. One possible alternative mechanism of biofilm formation, previously suggested in this thesis (See Section 4.2.1), is that of peptidoglycan breaks. This mechanism has in its favour the fact that NaCl stress leads to cell wall deficiency and therefore a probable large increase in the number of peptidoglycan breaks.

The down-regulation of RsbU lends further weight to the suggestion that biofilm formed in response to NaCl stress is PIA independent. RsbU increases PIA production by a rather convoluted mechanism. RsbU enhances the function of the alternative sigma factor, σ^B, which in turn represses the negative regulator of the ica operon, *icaR*, leading to an increase in PIA synthesis (Knobloch et al., 2004). The down-regulation of both *icaD* and RsbU in response to NaCl stress, whilst demonstrating an increase in biofilm formation under the same environmental circumstances, suggests that an alternative mechanism is at work. This would certainly be worthy of further investigation and research. Micro-array, as mentioned earlier, may be a more appropriate means of investigating which genes are up or down regulated under salt stress.

4.5.0 Summary and Discussion of DBHI vs. KCl RT-PCR Results.

Comparison of RT-PCR performed on *S. aureus* culture from DBHI and from DBHI supplemented with 1.4M KCl showed that several genes were down-regulated with statistical significance. These were *nuc*, *16s*, *SpA*, *pbp4*, *icaD*, *rsbU*, and *Kef*. MscL appeared to be slightly up-regulated although not with statistical significance.

The pattern of down-regulation under KCl stress is very similar that seen under NaCl stress. Once again, there is down-regulation of *16s* which may suggest a global down-regulation of transcription. Also, the two genes that directly, *icaD*, and indirectly, *rsbU*, affect the production of polysaccharide intercellular adhesin, are down-regulated in a similar manner to that seen under NaCl stress. However, in response to KCl stress, biofilm formation actually increases significantly. Hence, some other mechanism of biofilm formation must be functioning.

Kef is down-regulated in a similar manner under KCl stress as under NaCl stress. Again it seems likely that this occurs due to the lesser demands placed on this potassium efflux channel in the high osmolar conditions of KCl stress.

The fact that pbp4 is down-regulated under KCl stress as well as NaCl stress, suggests that there may be similar phenotypic changes noted. Future work should include repetition of the experiments looking at NaCl stress using the Yabu media, but instead substitute KCl for NaCl. Questions to be answered are whether KCl stress leads to cell wall deficiency, increased penicillin resistance and increased lysostaphin resistance in the same way that NaCl stress does. One would predict that it would.

4.6.0 Summary and Discussion of NaCl vs. KCl RT-PCR Results.

Comparison of RT-PCR performed on *S. aureus* culture from DBHI supplemented with 1.4M NaCl and from DBHI supplemented with 1.4M KCl showed no significant differences. Overall, many of the genes appeared to be slightly up regulated in the KCl stressed cells as compared to the NaCl stressed cells. However, none of these changes were statistically significant.

NaCl stress and KCl stress both lead to an increase in biofilm formation. The fact that both stresses lead to an alteration in gene transcription compared to the unstressed state, and that there is no significant difference between the genetic transcriptions of the NaCl and KCl stressed states, suggests that the cells react to these two stresses in a very similar manner.

4.7.0 Small Colony Variants of *Staphylococcus aureus*

Small colony variants or SCVs are a phenotype most commonly associated with *S. aureus,* which is gaining increasing attention in the medical literature. They

are slow-growing, morphological variants, which are particularly associated with persistent and antibiotic resistant infections (Besier et al., 2007). In orthopaedic surgery, SCVs have been linked to persistent infection of implanted prostheses as well as cases of osteomyelitis and septic arthritis (Sendi et al., 2006; von Eiff et al., 2006). SCVs are characterised by their slow growth as tiny, hypo-pigmented, non-haemolytic colonies (Besier et al., 2007). SCVs have the ability to persist intracellularly in *in vitro* systems and can also readily revert to a normal phenotype (von Eiff et al., 2006). These two factors in particular explain the difficulties encountered in culturing and identifying SCVs from clinical samples (Vaudaux, Kelley & Lew, 2006). SCVs also display an increased level of resistance to cell-wall-active antibiotics and aminoglycosides, and the possible link to intracellular persistence further complicates their eradication (Sendi et al., 2006; Vaudaux et al., 2006).

Results presented in this thesis suggest that the phenomena noted in response to NaCl stress, may in fact be inter-related to those described in the SCV literature. NaCl stress lead to small, hypo-pigmented colonies of Gram-negative cells interspersed among normal colonies of Gram-positive cells. (See Figure 4.1) Cells stressed with NaCl also developed increased resistance to penicillin, and, as has previously been demonstrated, showed an increased propensity to develop biofilm (Lim et al., 2004). The salt stressed cells were also less susceptible to enzymatic cell wall attack by lysostaphin.

Von Eiff et al. have described SCVs as displaying decreased pigment formation and being approximately 10-fold smaller than their normal colonies. This matches the colonies seen in *S. aureus* as a result of NaCl stress (See Figure 4.1) (von Eiff et al., 2006). SCVs are associated with antibiotic resistant infections (von Eiff et al., 2006). NaCl stress also lead to a degree of antibiotic resistance.

Biofilm formation has been implicated as playing a major role in persistent prosthetic infections (Trampuz & Zimmerli, 2005). Prosthetic infections are difficult to treat and can present as low-grade infections and sometimes with no clinical evidence of infection at all. SCVs are also associated with orthopaedic prosthetic infections and lead to many of the same clinical problems of identification and treatment as biofilm. Results presented in this thesis link possible SCVs, produced by salt stress, with increased biofilm formation, also in response to increased NaCl stress. It is interesting that a recent review in the literature about SCVs did not mention biofilm formation (von Eiff et al., 2006). Equally, a recent review of biofilm in orthopaedics did not mention SCVs (Trampuz & Zimmerli, 2005).

Infection of orthopaedic prostheses can be a catastrophic complication. Biofilm formation has long been implicated as a cause of persistent and intractable orthopaedic infection, however, a recent article by Sendi, Rohrbach et al, has highlighted SCVs as the cause of the same scenario (Sendi et al., 2006). They stress the difficulty of isolating SCVs from clinical specimens and highlight the fact that their presence may be grossly under-reported in the orthopaedic literature.

It seems possible, from the results presented in this thesis, that scientists investigating Cell Wall Deficient bacteria, scientists investigating biofilm formation and scientists investigating SCVs may all in fact be investigating the inter-related phenomena.

SCVs are frequently resistant to aminoglycosides like gentamicin, but can also be induced from normal cultures of *S. aureus* by exposure to aminoglycosides (von Eiff et al., 2006). NaCl stress, which seems to induce SCVs, as described in this thesis, has also been implicated in the primary induction of gentamicin resistance (McMahon et al., 2007). Once again, it seems possible that these observations are one

and the same thing and that the authors are climbing the same mountain but from different sides.

4.7.1 Photograph of Mixed Colonies on Yabu Agar Showing Possible SCVs

Figure 4.1: An enlarged photograph showing the colonies formed on high NaCl concentration Yabu agar. It shows the normally sized yellow colonies of *S. aureus,* which stained Gram-positive, interspersed with small hypo-pigmented colonies, which stained Gram-negative. It seems likely that these small colonies are Small Colony Variants.

4.8.0 Salt Levels *in vivo.*

The absolute salt levels described here that have lead to L-form formation, increased antibiotic resistance and increased biofilm formation are unlikely to be achieved *in vivo*. The urinary system produces the highest concentration of sodium during normal physiology in the human body. Even in concentrated urine, the maximum physiological concentration of sodium is only around 0.3mol/l, half the level required to cause increased biofilm formation (Coulthard & Haycock, 2003; Plough & Baker, 1959). Urinary catheterisation is an extremely common procedure

both in hospital and in the community, with over 30,000,000 being used in the United States every year. Urinary catheterisation also has a very high rate of infection of between 10 and 30 per cent, and biofilm is a major problem (Darouiche, 2001). Although *S. aureus* is not the most common urinary pathogen, it is an important one, particularly regarding MRSA and its added implication for therapeutic eradication (Jones et al., 2001). Hence, sodium concentration on it own, does not explain the common nature of biofilm in the urinary tract, or elsewhere in the body. The development of L-forms, the nature of antibiotic resistance and the causative mechanisms of biofilm formation are hugely complex and interconnected phenomena and although salt stress is only a small part of the picture, it does add to the intricate model that is building.

Section 5: Bibliography

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