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### *The Effect of Antibiotics on Toxin Gene Expression in PVL-positive Staphylococcus aureus Strains*

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**CHAPTER 1**  
**GENERAL INTRODUCTION**

## **1. 1. General Characteristic of the Bacterium**

*Staphylococcus aureus* is a member of the Micrococcaceae, it is gram-positive cocci and can be distinguished from other staphylococcal species on the basis of gold pigmentation of colonies and positive results for coagulase (Lowey et al., 1998). *S. aureus* is identified by the thermo nuclease test, acetoin production, and aerobic fermentation of sucrose, D-mannose, D-cellobiose, D-xylose, L-arabinose, raffinose, D-trehalose, maltose and D-mannitol (da Silva et al., 2005). *S. aureus* has long been recognised as one of the major human pathogens and is by far one of the most common nosocomial organisms, being responsible for most post surgical infections. It is an opportunistic bacterium frequently part of human micro flora, causing disease when the immune system becomes compromised (Aires de Sousa et al., 2004). About 20% of the population are always colonized with *S. aureus*, 60% are intermittent carriers, and 20% never carry the organism (Foster, 2004). The pathogen has been most frequently isolated worldwide from community and hospital acquired infections (Garcia-Lara et al., 2005).

## **1. 2. *S. aureus* Infections and their consequences**

A fundamental biological property of *S. aureus* is its ability to asymptotically colonize healthy individuals. Carriers are at a higher risk of infection and they are presumed to be an important source of the bacterium that spread among individuals (Chambers et al., 2009). *S. aureus* is an extra ordinarily versatile pathogen causing a wide spectrum of infections, ranging from mild to severe and life threatening, in human as well as economically important infections in animals (Bliotta et al., 2006). *S. aureus* has been most frequently isolated worldwide from community and hospital acquired infections of the blood stream, lower respiratory tract, skin and soft tissue (Garcia-Lara et al., 2005). The pathogen can cause a wide variety of infections, which can be divided into three types

- Superficial lesions such as wound infection.
- Toxinoses such as food poisoning, scalded skin syndrome and toxic shock syndrome.
- Systemic and life- threatening conditions such as endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis, and bacteraemia (Aires de Sausa et al., 2004).

In addition to this *S. aureus* has been very adapt at acquiring resistance to antimicrobial drugs, which is an important health, as well as economic, problem. Infections caused by resistant organisms cause increased morbidity, longer hospitalizations, and higher costs when compared with infections caused by susceptible strains; however, the magnitude of effect may vary based on pathogen, resistance to specific antimicrobials, and even mechanisms of resistance (Cosgrove et al., 2009). Isolates that had acquired resistance to methicillin appeared for the first time in 1961 in the UK (Jevons. 1961), and they called methicillin resistant *S. aureus* (MRSA). MRSA is now a problem in hospitals world wide and is increasingly recovered from nursing homes and the community. For most countries badly affected by MRSA there have been many years of debate about its relative virulence compared with methicillin sensitive *S. aureus* (MSSA) and whether it could be controlled. MRSA is endemic in the majority of hospitals around the world and it is clear that it is at least as virulent as MSSA and is an additional burden of healthcare-acquired infection (Gould et al., 2006).

MRSA, besides having established itself as a major hospital pathogen, is now beginning to prevail in the wider community as well. Community acquired (CA)-MRSA strains were appeared in the late 1970s in the USA, primarily among intravenous drug user. In the 1990s, CA-MRSA strains appeared in multiple areas of the world, and spread extensively (Gosbell et al., 2005). The combination of the PVL determinant with the *mecA* gene appears to have created a super adapted *S. aureus* strain that is spreading in the community (Dufour et al., 2002). Gerogianni et al. described a case of necrotizing pneumonia in Greece caused by PVL- producing CA-MRSA carrying *SCCmec* typeV, which was the first report documenting the emergence of PVL-producing CA-MRSA in combination with the *SCCmec* type V in Europe (Gerogianni et al., 2006). Global spread of PVL-positive strains carrying *mecA* is occurring, which exhibit resistance to all  $\beta$ -lactam antibiotics (Monecke et al., 2007).

Typically CA-MRSA strains are less antibiotic resistant than HA-MRSA strains, with many being susceptible to most antimicrobials except for  $\beta$ -lactams. In addition, CA-MRSA strains are reported as being highly transmissible and virulent, associated with the

presence of pore forming leucotoxin PVL (Ellington et al., 2009). PVL is present in most CA-MRSA strains, and they can cause complicated chronic deep-skin infections, or necrotizing pneumonia with an extremely high mortality, even in young and previously health patients (Monecke et al., 2006).

MRSA is responsible for more than 40% of *S. aureus* bacteraemias in the UK and is associated with considerable morbidity and mortality. Between 1990 and 2002, the proportion of *S. aureus* bacteraemias in the UK attributed to MRSA rose from 2% to 43%, and has remained high (Beeston et al., 2009). In the UK, 20,000 cases of *S. aureus* bacteraemia are reported each year, half of which are antibiotic resistant and approximately 4% are fatal, exemplifying a worldwide phenomenon of tremendous economic and human impact (Garcia-Lara et al., 2005). The UK sustains a high burden of hospital acquired (HA)-MRSA infections, with two epidemic MRSA clones predominating; EMRSA-15 and EMRSA-16 (Ellington et al., 2009). The prevalence of PVL among CA-MRSA isolates varies geographically. In the USA, PVL genes are present in 40-95% of CA-MRSA isolates and nearly all USA300 isolates contain PVL (Otter et al., 2009). However, the prevalence of PVL-positive CA-MRSA in the UK is significantly lower than in the USA (Hidron et al., 2009), with fewer than 2 % reported among clinical isolates of *S. aureus* submitted to the national Reference Laboratory (Health Protection Agency 2008). Clones of PVL-producing MRSA are now spreading rapidly throughout the world. Although France, America and Australia have reported cases since 1999, the first recognized British case of PVL-associated pneumonia was in London in 2003 (Klein et al., 2003). Bacterial endocarditis in patients with PVL-producing CA-MRSA furunculosis is an emergency entity, in areas where CA-MRSA skin infections are prevalent, inappropriate initial antibiotic treatments remains a major problem and may result in significant morbidity (Bahrain et al., 2006).

### **1. 3. Genetic Structure of *Staphylococcus aureus***

#### **1. 3. 1. Core Genome**

The core genome makes up about 75 % of any *S. aureus* genome and is highly conserved between isolates. Genes associated with central metabolism and other house keeping

functions, essential for bacteria to absorb nutrients from the environment, synthesize metabolic intermediates, and multiply are the majority of genes comprising the core genome (Lindsay et al., 2004 and Ito et al., 2003). Homology searches for the most closely related orthologues indicates that the genetic background of *S. aureus* has been vertically transmitted from a common ancestor that subsequently diverged to *Bacillus* and *Staphylococcus* species (Ito et al., 2003). Within this part of the genome are also some virulence genes, which are not carried by other staphylococcal species, for example *spa*, encodes protein A, and *hla*, encodes  $\alpha$ -haemolysin (Lindsay et al., 2004).

### **1. 3. 2. Accessory Genome**

Accessory genome seems to have been acquired from other bacterial species via lateral gene transfer and constitutes the genetic information that makes *S. aureus* a notorious hospital pathogen (Hiramatsu et al., 2004). This domain accounts for about 25% of any *S. aureus* genome and mostly consists of mobile (or once mobile) genetic elements that transfer horizontally between strains. These elements include pathogenicity islands, chromosome cassettes, genomic islands, plasmids and transposons (Lindsay et al., 2004). Many of these genetic elements carry genes with virulence and antibiotic resistance functions. The identification and characterization of these elements is providing insight into how the pathogen causes disease and how this is evolving (Lindsay et al 2004). Therefore, the distribution and horizontal spread of these elements can have important clinical implications.

#### **1. 3. 2. 1. Staphylococcal Cassette Chromosome (SCC).**

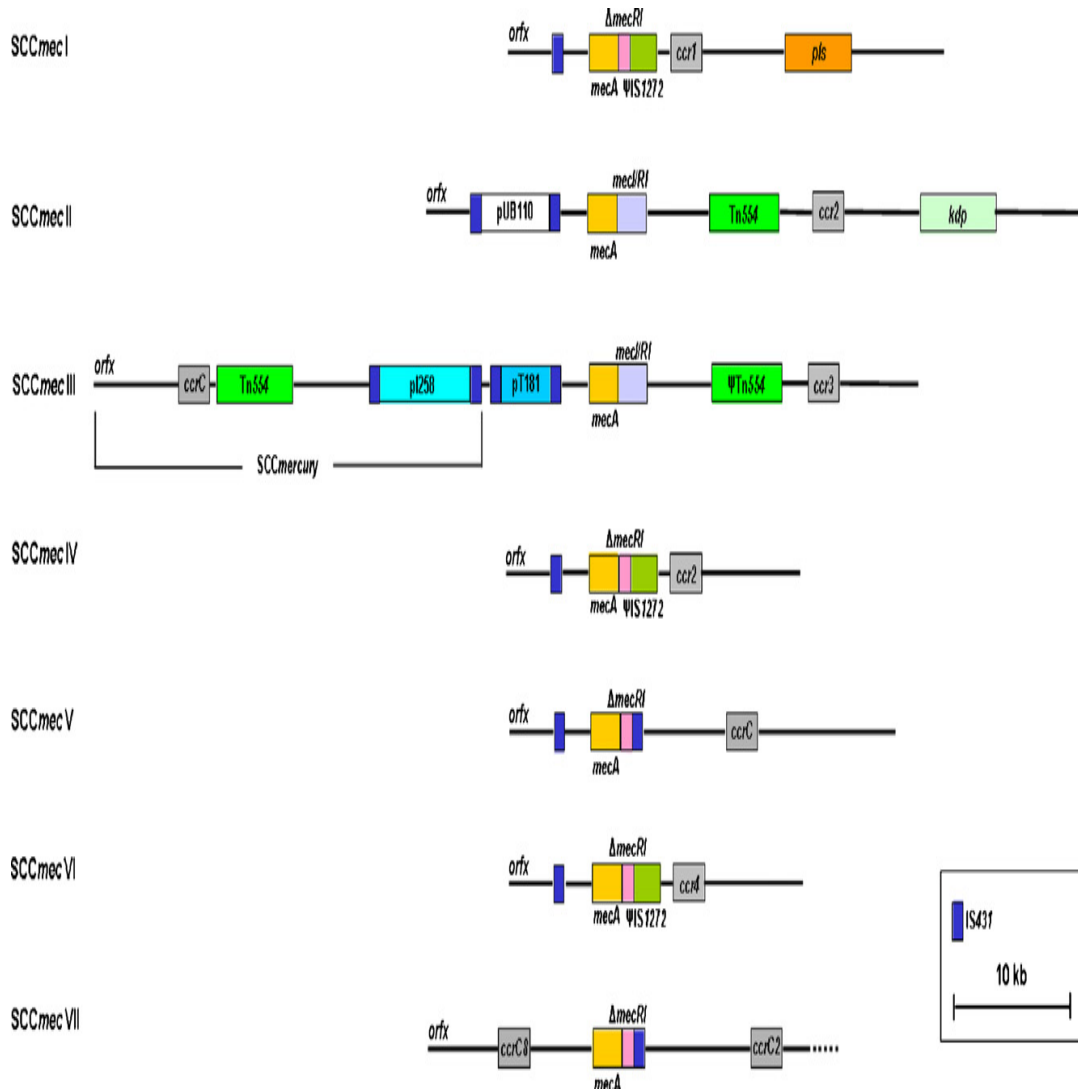
SCC is a basic mobile genetic element that serves as the vehicle for gene exchange among staphylococcal species; *SCCmec* is a member of the SCC family, the members of which specialize as carriers of methicillin resistance. *SCCmec* is a genomic island (G island) that is inserted at the 3' end of *orfX* and that is located near the replication origin of *S. aureus*. *SCCmec* is composed of the *mec* gene complex, which encodes methicillin resistance and the *ccr* gene complex, which encodes the recombinases responsible for its mobility and contains two site specific recombinase genes, *ccrA* and *ccrB* (Ito et al., 2004). There are four different *mec* gene complexes as follows:

- Class A IS431-mecA- mec R1-mec I
- Class B IS431-mecA- $\Delta$ mec R1-IS1272
- Class C IS431-mec A- $\Delta$ mecR1-IS 431
- Class D IS431-mec A - $\Delta$ mecR1.

Different combination of *mec* gene complex classes and *ccr* gene complex types has so far defined eight types of SCC*mec* elements (type I to VIII ) (Chambers et al., 2009), which range in size from 20.9 to 66.9 kb (Deurenberg et al., 2008, Zhang et al., 2009 and Conceicao et al., 2010). SCC*mec* type I (34.3 kb), IV (20.9-24.3 kb), V (28 kb), VI (20.9 kb), and VII (35.9 kb) determine resistance to  $\beta$ -lactam antibiotics only, while types II (53.0 kb) and III (66.9 kb) determine multiresistance, due to additional drug resistance genes as shown in Figure 1. 1. (Deurenberg et al., 2009). SCC*mec* type VIII was identified from Canadian epidemic MRSA strain MRSA9, which is a typical HA-MRSA epidemic strain commonly found in Canada (Zhang et al., 2009).

- Type I, IV and VI  $\rightarrow$  Class B *IS1272- $\Delta$  mecR1 – mecA IS431*
- Type II and III  $\rightarrow$  Class A *mecI-mecR1-mecA-IS431*
- Type V and VII  $\rightarrow$  Class C *IS431-mec $\Delta$  R1-mecA-IS431*
- Type VIII  $\rightarrow$  Class A *IS431-mecA- mec R1-mec I.*

Three types (type I, type II, type III) are carried mostly by hospital acquired (HA-MRSA) strains. On the other hand, types of SCC*mec* elements (type IV, type V and VII) have been widely disseminated among community acquired (CA-MRSA) strains. The type IV, V, VI and VII elements are characterised by their small sizes and lack of resistance genes, other than *mecA* (Hisata et al., 2005). Type III SCC*mec* contains integrated copy of plasmid pT181, transposon Tn554, and pseudo Tn554 that encode resistance to tetracycline, erythromycin, and cadmium respectively (Ito et al 2003). Type II SCC*mec* of N315 and Mu50 contains an integrated copy of plasmid pUB110 and transposon Tn554.



**Figure 1. 1. A schematic drawing of *SCCmec* types I to VII in MRSA.** The major elements of the seven main *SCCmec* types (*ccr* genes, *IS431*, *IS1272*, *mecA*, *mecl/R1*, *orfX*, *pI258*, *pT181*, *pUB101* and *Tn554*) (Deurenberg et al., 2009).

### **1. 3. 2. 2. Pathogenicity Islands of *S. aureus***

Many genes encoding staphylococcal virulence factors, are located on mobile genetic elements, such as bacteriophages, plasmids, and so called "pathogenicity islands" on the bacterial chromosome. *S. aureus* pathogenicity islands (SaPIs) are a family of 15- to 27-kb genetic elements that generally carry one or more superantigen genes, encoding a variety of enterotoxins and/ or toxic shock syndrome toxin 1 (TSST-1). SaPIs are stably integrated at specific chromosomal sites but can be mobilized following infection by certain staphylococcal bacteriophages or by induction of endogenous prophages (Tallent et al., 2007). SaPIs is believed to be formed by integration of extra chromosomal DNAs (plasmids) or by incorporation of bacteriophages carrying toxin genes. Incorporation of SaPIs may transform a non-virulent into virulent strain (Iwatsuki et al., 2006).

### **1. 3. 2. 3. Plasmids in *S. aureus***

Plasmids are another important genetic vehicle that carries antibiotic resistant genes. A number of plasmids found in staphylococci, such as plasmid pUB110, belonging to a group of a small (1-5kb), multicopy (15-60 copies per cell) plasmids, is found integrated in the type II SCC*mec* of some strains of *S. aureus*. This plasmid encodes resistance to aminoglycosides, kanamycin, tobramycin, and bleomycin (Ito et al., 2003). Resistance to penicillins and heavy metals, such as mercury, is encoded by pI258, while tetracycline resistance is encoded by pT181 (Ito et al., 2003).

### **1. 3. 2. 4. Transposons in *S. aureus***

Transposons (Tn) are classical small transferable DNA fragments found in *S. aureus* chromosome, which appear to be involved in inactivation or modification of certain cellular functions by transposing into or besides the genes involved in the function (Hiramatsue et al., 2004). Tn 554 is a site-specific transposon that encodes resistance to spectinomycin and macrolide-lincosamide-streptogramin B antibiotics (MLS) (Kuroda et al., 2001). Tn552 contains the penicillinase gene, *bla*, Tn4001 contains *aacA-aphD*, which encodes for resistance to kanamycin, tobramycin, and gentamycin (Ito et al., 1998) and Tn 58001, which carries a gene (*tet M*) encodes tetracycline and aminocycline resistance (Kuroda et al., 2001).

## **1. 4. Virulence Factors and their Role in Pathogenicity**

Pathogenicity of *S. aureus* is related to a number of virulence factors that allow it to adhere to surfaces, invade or avoid the immune system, and cause harmful toxic effects to the host (Holmes et al., 2005).

### **1. 4. 1. Adherence Factors (Adhesins)**

The attachment of *S. aureus* to surfaces initiating the colonization process is mediated by several adhesins, which are mostly cell wall anchored proteins, and are grouped into a single family called Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) to denote their role (Patti et al., 1994 cited by Belkum et al., 2009). The best characterized MSCRAMMs are the fibronectin-binding proteins (Fnbp), the collagen-binding protein (Can) and the fibrinogen-binding protein clumping factor (ClfA and ClfB) (Table 1. 1) (Garcia- Lara et al., 2005).

### **1. 4. 2. *S. aureus* Exoproteins**

Nearly all strains of *S. aureus* secrete a group of exoproteins such as enzymes and exotoxins which include four haemolysins ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), nucleases, proteases, lipases, hyaluronidases, and collagenase. The main function of these proteins may be to convert local host tissue into nutrients required for bacterial growth (Dinges et al., 2000). This pathogen produces a group of exotoxins called pyrogenic toxin superantigens (SAGs) which include staphylococcal enterotoxins, toxic shock syndrome toxin-1 (TSST-1) and exfoliatins A and B (ETA and ETB) (Poxflvi et al., 2008). The best characterized property of this group is superantigenicity, which refers to the ability of these exotoxins to stimulate proliferation of T lymphocytes without regard for antigen specificity. These toxins cause toxic shock syndrome (TSS) and related illnesses through their capacity to induce massive cytokine release both from macrophages and T cells (Fey et al., 2003). Two former names for TSST-1 were staphylococcal pyrogenic exotoxin C and staphylococcal enterotoxin F (Dinges et al., 2000). Some of the staphylococcal exotoxins belong to the family of bicomponent synergo-hymenotropic toxins such as Panton Valentine Leucocidine (PVL),  $\delta$ -haemolysin and other leukocidins such as LukE-LukD (Gillet et al., 2002).

**Table 1. 1. Surface proteins and their role in *S. aureus* pathogenesis**

<b>Adhesins</b>	<b>Role in Pathogenesis</b>	<b>Reference</b>
Fibronectin- binding protein (FnbpA and FnbpB)	Binding to epithelial and endothelial cells.	Xiong et al., 2004 and Mongodin et al., 2002
Fibrinogen-binding protein (ClfA and ClfB)	Adherence to fibrinogen-containing substances.	Foster and Hook 1998
Collagen-binding protein (Cna)	Adherence to collagenous tissue and substrates.	Foster and Hook 1998
Protein A (Spa)	Binding to Immuno globulin G (IgG)	Clarke et al., 2006
Capsular polysaccharides (CP).	Protection from opsonophagocytosis.	Garcia Lara et al., 2004
Elastin-binding protein (Ebps)	Binding to N-terminal of elastin to promote bacterial colonization.	Downer et al., 2004
Extracellular matrix-binding protein (Ebh)	Adherence to endothelial cells.	Clarke et al., 2006

#### **1. 4. 2. 1. Staphylococcal Haemolysins**

Haemolysins have haemolytic activity against a variety of erythrocytes and also lytic activity against other mammalian cells (Dhople et al., 2005).  $\alpha$ -hemolysin, secreted from *S. aureus* as a water-soluble monomer of 33.2 kDa, assembles on cell membranes to form transmembrane, heptameric channels. This toxin is also a pore forming cytolysin (Chen et al., 1994).  $\beta$ -haemolysin is produced in large quantity by a number of *S. aureus* strains, particularly animal isolates, and is secreted into the culture medium as an exotoxin with a molecular weight 35,000.  $\gamma$ -haemolysin is made by virtually every strain of *S. aureus*. It is a bi-component toxin (Hlg1 of 34kDs and Hlg2 of 32kDs) and is one of the pore forming cytolytic toxins (cytolysin), it is cytolytic toward human and rabbit polymorphonuclear leukocytes and rabbit erythrocytes (Kaneko and Kamio 2004).

#### **1. 4. 2. 2. Toxic Shock Syndrome Toxin (TSST)**

TSST-1 is a potent super antigen and is the most common cause of TSS. The potency of this toxin lies in its ability to efficiently induce T-cell proliferation and activation (10,000-fold more efficiently than other antigens). This is due to bridging the antigen-presenting cells and T-lymphocytes through binding the major histocompatibility complex (MHC) class II on APCs and specific variable regions on the  $\beta$ -chain of both CD4 and CD8 antigen receptors (Deurenberg et al., 2005). It is produced exclusively by *S. aureus* and approximately 20 % of natural human isolates are producers (Novick et al., 2001). TSS is defined as an acute and potentially fatal illness characterized by high fever, a diffuse erythematous rash, desquamation of the skin 1-2 weeks after onset, hypotension and involvement of three or more organ systems (Iwatsuki et al., 2006). TSST-1 is a potent stimulator of interleukin-1 (IL-1) release and stimulates the production of tumor necrosis factor-alpha (TNF alpha). The action of these cytokines are responsible for most of the clinical manifestations of *S. aureus* induced TSS (Reiss et al., 2000).

#### **1. 4. 2. 3. Staphylococcal Enterotoxins (SEs)**

*S. aureus* colonization of food has long been associated with a form of gastroenteritis that is manifested clinically as emesis with or without diarrhoea. This condition is called staphylococcal food poisoning (SFP), and results from ingestion of one or more

performed SEs in food that has been contaminated with *S. aureus*. Table (1. 2) shows a summary of major staphylococcal enterotoxins and their encoded genes. SEs are a family of major serological types of heat stable enterotoxins, they function both as potent gastrointestinal toxins as well as superantigens that stimulate non-specific T-cell proliferation (Balaban et al., 2000). SEA and SED are the serotypes most commonly associated with food poisoning (Villard et al., 2005). SEs are resistant to inactivation by gastrointestinal proteases such as pepsin, as well as by heat, which is the most important properties of these toxins in terms of food safety (Balaban et al., 2000). Food poisoning has a major public health impact. In a study of food poisoning in England, the most prevalent contaminated foods 75% were meat, poultry or their products (Blaban et al., 2000).

#### **1. 4. 2. 4. Exfoliative Toxins (ETs)**

Staphylococcal exfoliatins (ETA and ETB), induce the "acantholytic" infection of *S. aureus* due to the disruption cell-to-cell cohesion, which allows the pathogenic organism to spread within the epithelium (Iwatsuki et al., 2006). The toxin was described for the first time in 1878 by Baron Gottfried Ritter van Rittershain as a severe blistering disease of neonates that is now called staphylococcal scalded skin syndrome (SSSS), or Ritters disease (Amagai et al., 2000). In SSSS, a local infection releases exfoliative toxin A (ETA) into the circulation, which leads to widespread skin blistering, whereas in bullous impitigo the toxin causes blistering locally to the site of infection (Amagai et al., 2000). Exfoliative toxins could also play a role in other disease where superantigens are thought to be involved, such as Kawasaki disease, atopic dermatitis, psoriasis, various autoimmune disorders, and sudden infant death syndrome (Ladhani, 2003). ETB is also a causative agent of staphylococcal scalded-skin syndrome (Jackson et al., 1986), and is plasmid encoded, whereas ETA, is chromosomally encoded (Farrell, 1999).

#### **1. 4. 2. 5. Bicomponent Toxins**

A group of *S. aureus* toxins contain two synergistically acting protein designated S (slow) and F (fast), they also called synergo-hymenotropic toxins. This group includes PVL, gamma haemolysin and other leukocidins such as LukE-LukD (Gillet et al., 2002).

**Table 1. 2. Major Staphylococcal enterotoxins and their encoded genes**

<b>Enterotoxin</b>	<b>Gene</b>	<b>Reference</b>
SEA (27 kDa)	<i>sea</i>	Klotz et al., 2003
SEB (28 kDa)	<i>seb</i>	Bania et al., 2006
SEC (27 kDa)	<i>sec</i>	Klotz et al., 2003, and Bania et al., 2006
SED (26 kDa)	<i>sed</i>	Bayles and Indulo, 1989 and Klotz et al., 2003
SEE (26 kDa)	<i>see</i>	Klotz et al., 2003
SEG (27 kDa)	<i>seg</i>	Munson et al., 1998
SEH (27.3 kDa)	<i>seh</i>	Balaban et al., 2000
SEI (24.9 kDa)	<i>sei</i>	Munson et al., 1998
SEJ (31.2 kDa)	<i>sej</i>	Klotz et al., 2003
SEL (26 kDa)	<i>sel</i>	Orwin et al., 2003

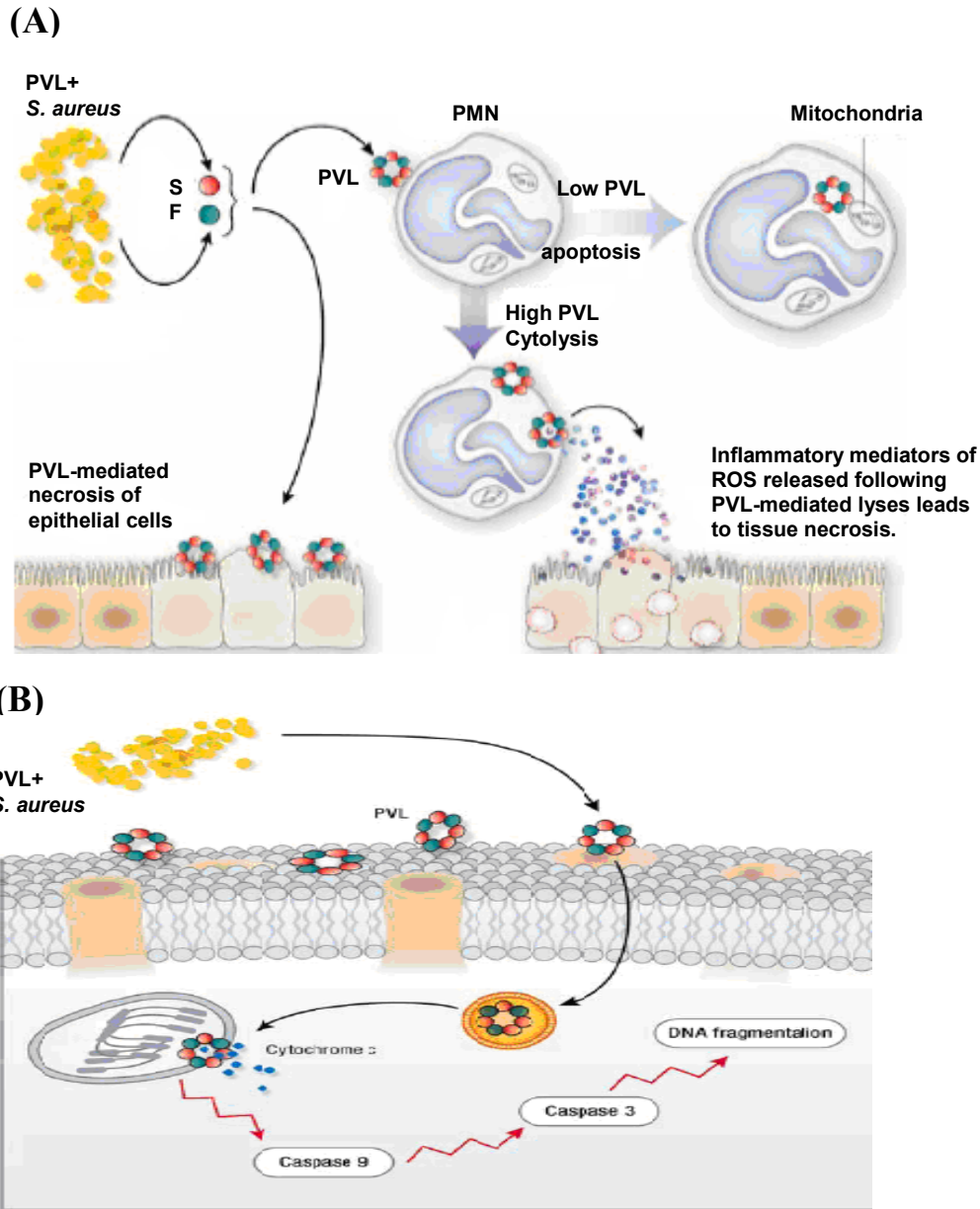
#### **1. 4. 2. 5. 1. Leukotoxin LukE/LukD**

This toxin is a member of the staphylococcal bicomponent leukotoxin family; LukE is 58-68% identical with class S protein and LukD is 71-77% identical with class F protein of the leukotoxin family (von Eiff et al., 2004). LukE/ LukD is as effective as PVL for inducing dermonecrosis when injected in the rabbit skin, but is not haemolytic and is poorly leukotoxic compared to other leukotoxins expressed by *S. aureus* (Gravet et al., 1998 and Kaniko and Kamio, 2003). Various strains of *S. aureus* produce leukocidin LukE/LukD, which binds to leukocyte cell membranes inducing transmembrane pore formation and subsequent cell lysis (Bownik, 2006).

#### **1. 4. 2. 5. 2. Panton Valaentine Leukocidin (PVL)**

PVL is a bicomponent, pore-forming leukotoxin initially designated "substance leukocidin" by Van de Veld in 1894 due to its ability to lyses leukocytes (Boyle-Vavra et al., 2007). Panton and Valantine first associated the leukotoxin with skin and soft tissue infection in 1932, long before penicillin resistant *Staphylococcus aureus* (PRSA) and methicillin resistant *Staphylococcus aureus* (MRSA) were of clinical concern (Boyle-Vavra et al., 2007 and Maltizou et al., 2006). The toxin is produced by fewer than 5% of *S. aureus* strains (Lina et al., 1999). PVL toxin usually targets human and rabbit mononuclear and polymorphonuclear cells (PMNs). Studies have shown that its toxic effect results from synergistic action of two separate exoproteins, namely, LukS-PV and LukF-PV, which are encoded by two contiguous and cotranscribed genes (*lukS-PV* and *lukF-PV*) (Holmes et al., 2005 and Denis et al., 2005). They are separated by a single thymine nucleotide and transcribed as a single mRNA molecules (Johnsson et al., 2004), and they are carried on temperate bacteriophages (Holmes et al., 2005).

The two components of PVL are secreted before they assemble into a pore-forming heptamer on the PMN membrane, leading to PMN lysis (Figure 1. 2). LukS-PV initiates binding to a receptor on PMNs, and subsequently dimerizes with LukF-PV followed by alternate serial binding of both components until the heptamer is assembled (Boyle-Vavra and Daum 2007). It has been reported that the S component of PVL binds to the cell surface ganglioside receptor GM1 on PMNs (Takano et al., 2008).



**Figure1. 2. Model for how PVL might mediate tissue necrosis.**

The two components of PVL, LukS-PV and LukF-PV are secreted from *S. aureus* before they assemble into a pore-forming heptamer on PMN membranes. (A) High PVL concentrations cause PMN lysis and tissue necrosis could result from release of reactive oxygen species (ROS) from lysed PMNs. Alternately, release of granule contents from lysed PMNs could eventually result in tissue necrosis. (B) Low PVL concentrations can induce apoptosis via a novel pathway that is proposed involve PVL-mediated pore formation on the mitochondrial membrane leading to release of cytochrome *c* and induction of caspases 9 and 3 (Boyle-Vavra et al., 2007).

Micek et al., reported that the release of neutrophil chemotactic factors induced by PVL, including interleukin-8 and leukotriene B<sub>4</sub>, in the lung is thought to be the mediator of tissue necrosis accounting for the clinical and radiographic presentation of patients with PVL-positive C-MRSA pneumonia (Micek et al., 2005). Depending on the concentration of PVL, it can cause PMN lysis or apoptosis, the latter via a novel pathway that involves PVL-mediated pore formation in the mitochondrial membrane (Boyle-Vavra et al., 2007) (Figure 1. 2. B). Furthermore LukS-PV has been detected in lung sections of patients with necrotizing pneumonia together with DNA fragmentation, suggesting that PVL induces apoptosis *in vivo* and thereby is directly involved in the pathophysiology of necrotizing pneumonia (Genestier et al., 2005).

Data from a study by Said-Salim et al. demonstrated that PVL genes are differentially distributed among community acquired (CA-MRSA) strains and, when they are present, are always transcribed, albeit with strain to strain variability of transcript level (Said-Salim et al., 2005). To directly test whether PVL is critical for the pathogenesis of CA-MRSA, they evaluated the lysis of human PMNs during pathogenic interaction with PVL-positive and PVL-negative CA-MRSA strains. Unexpectedly, there was no correlation between PVL expression and PMN lyses, suggesting that additional virulence factors underlie leukotoxicity and, thus, the pathogenesis of CA-MRSA (Said-Salim et al., 2005). Conversely, Labendeira-Ray et al. showed that PVL is sufficient to cause pneumonia by infecting mice with PVL producing *S. aureus* and by direct inoculation with native PVL toxin. The mice showed symptoms of severe illness; expression of the leucotoxin induces global changes in the transcription levels of genes encoding secreted and cell-wall anchored staphylococcal proteins, including the lung inflammatory factor staphylococcal protein A (*spa*) (Labendeira-Rey et al., 2007). To date there has not been a complete resolution of this apparently contradictory finding (Schlivert et al., 2010).

The *lukSF-PV* genes in *S. aureus*, despite being exogenously acquired, are regulated and highly integrated into the overall cellular program. Studies using various experimental models have given conflicting results, however, raising the possibility that the role of PVL might depend on the site of infection, as well as the experimental model (Cremieux et al.,

2009). Rodent PMNs, for example, are less susceptible than human PMNs to PVL-induced cytolysis, whereas rabbit PMNs, like those of humans, are highly susceptible to PVL-induced cytolysis. This difference in target cell susceptibility could affect results of experimental models. Diep et al., (2010), developed a rabbit model of necrotizing pneumonia to compare the virulence of a USA300 wild-type strain with that of isogenic PVL-deletion mutant and complemented strains. PVL enhanced the capacity of USA300 to cause severe lung necrosis, pulmonary edema, alveolar hemorrhage, hemoptysis, and death, hallmark clinical features of fatal human necrotizing pneumonia (Diep et al., 2010). This was also supported by Loffler et al., (2010), they demonstrate that the expression of PVL by staphylococcal strains confers strong and rapid cytotoxic activity restrictedly against human neutrophils, as it could not be reproduced in murine or Java monkeys' cells.

### **1. 5. Regulation of Virulence Factors in *S. aureus***

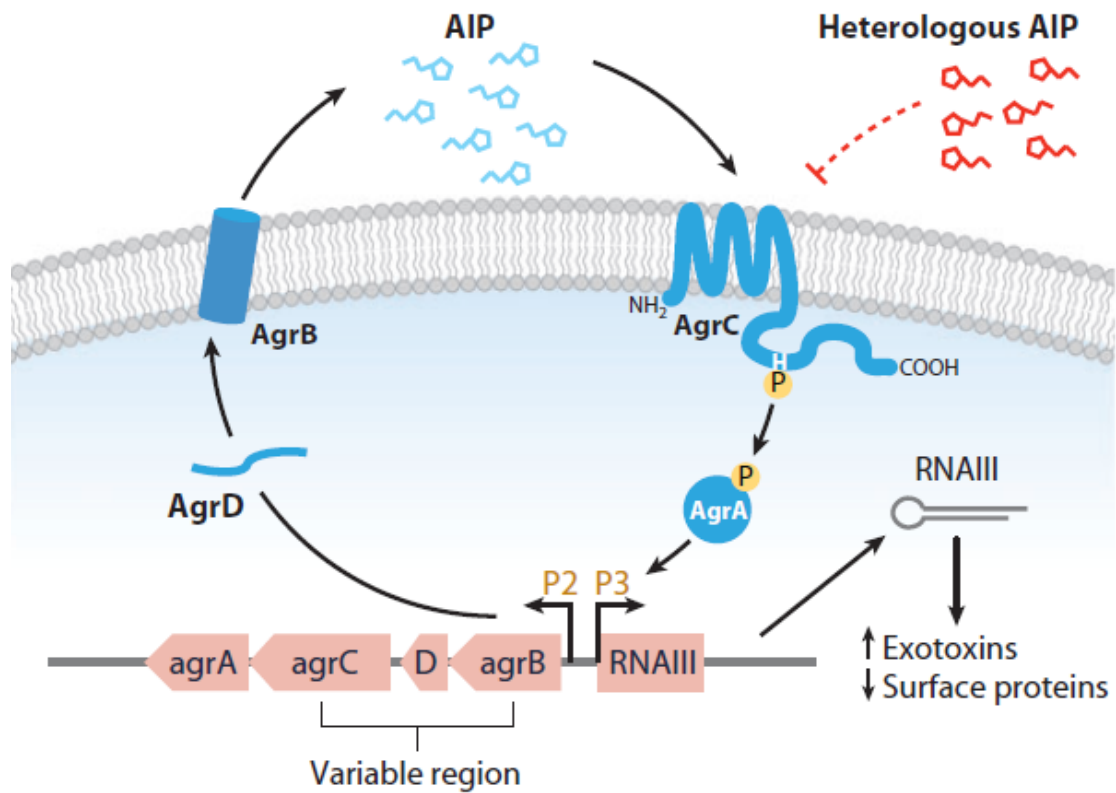
The expression of staphylococcal virulence factor genes is coordinated by global regulators. These regulators help bacteria to adapt to a hostile environment by producing factors enabling the bacteria to survive and subsequently to cause infection at the appropriate time. Several of these global virulence regulators, such as the Agr system, Sar and Sae, have been well characterized. Others such as the Arl, Sar homologues (Rot, MgrA, SarS, SarR, SarT, SarU, SarV, SarX, SarZ and TeaR), the Srr system and TRAP, require further study to determine their role in the virulence regulon (Lindsay 2008).

Most of 50 or more accessory genes involved in pathogenesis encode proteins that are either displayed on the bacterial surface or are released into the surroundings. These enable the organism to evade the host defense, to adhere to cells and the tissue matrix, to spread within the host and to degrade cells and tissues, for both nutrient and protection (Novick, 2003). Changes in nutrient availability, temperature, pH, osmolarity, and oxygen tension are among the environmental signals that have the most potential to influence the expression of virulence factors (Torres et al., 2010). The pathogenicity of *S. aureus* largely depends on the successful adaptation to the human host and the environmentally coordinated expression of virulence factors. Their expression is regulated during the

growth cycle by a network of interacting regulators such as accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sarA*) (Rogasch et al., 2006).

The *sarA* locus encodes a DNA-binding protein that influences the amount of fibronectin and fibrinogen- binding proteins as well as immunodominant antigen A (IsaA), Spa and  $\beta$ -haemolysin (Ziebandt et al., 2001). The *sarA* locus is believed to be necessary for the activation of the *agr* locus (Engelmann et al., 2009). Low *agr* activity at the beginning of an infection allows for the expression of colonization factors, however, high *agr* activity in progressed infection states causes the expression of aggressive virulence factors and degradative exoenzymes, allowing the bacteria to acquire nutrients from host tissue, and eventually to escape, for example from abscess and spread to other infection sites (Otto, 2004). When high cell population densities are reached, they sense a quorum through a cell-cell communication system through the exchange of signalling molecules called auto-inducers in a process referred to as quorum sensing (Bernardo et al., 2004). *agr* encodes a typical autoactivation circuit, as illustrated in (Figure 1. 3).

The locus is expressed from two divergent promoters, P2 and P3, of which the P2 transcript covers a 4-gene operon, containing *agrB*, *D*, *C*, and *A*. AgrA and C constitute a classical two-component signaling module and Agr B and D combine to generate the activating ligand. The autoinducing peptide (AIP) binds to and activates Agr C, which activates AgrA and the activated AgrA upregulates its own promoter P2 as well as that of the adjacent and divergent RNAPIII promoter, P3 (Novick et al., 2008). RNAPIII appears to be the major effector molecule of the *agr* system. It is thought to regulate most target genes at the level of transcription, but has also been shown to affect the translation of some genes (Engelmann et al., 2009). RNAPIII plays a key role in the quorum-sensing-dependent central regulatory circuit and coordinately regulates several virulence associated genes (Huntzinger et al., 2005).



**Figure 1. 3. Schematic representation of the *agr* system.** The AgrD peptide, processed N-terminally by signal peptidase B (SpsB) and C-terminally by AgrB, is secreted in the form of a tailed thiolactone ring, which acts on the transmembrane receptor domain of AgrC, inducing phosphorylation of AgrA, which activates transcription from the two *agr* promoters. The P2 promoter drives the autoinducing circuit, and the P3 promoter drives transcription of RNAIII, which is the regulatory effector of the system (Novick et al., 2008).

SaeRS is a two-component system that has been characterized as a positive regulatory system for the expression of several virulence factors, including coagulase,  $\alpha$ -,  $\beta$ -,  $\gamma$ -heamolysin, nuclease and fibronectin-binding protein in *S. aureus*. Comparison of the transcriptional profile of N315 and KSA (SaeRS null mutant) strains by microarray analysis reveals that the SaeRS system modulates the regulation of coagulase,  $\alpha$ -,  $\beta$ -,  $\gamma$ -heamolysin, nuclease and fibrinogen-binding protein, fibronectin-binding protein and 13 other genes (Kuroda et al., 2007). Two-component signal transduction systems have a key role in mediating the response of bacteria to environmental stimuli. Normally, receptor-mediated detection of a stimulus at the cell surface leads to autophosphorylation of a sensor kinase component, which then phosphorylates the effectors protein component (i.e., the response regulator), enabling the effectors to bind to operator/ promoter sequences of target genes and either increase or repress transcription. (Miller et al., 2004).

It has been suggested that bacterial cell responds to inhibition of peptidoglycan biosynthesis in general, and the genes comprise a cell-wall-stress stimulon. A stimulon refers to the entire set of genes responding together to an environmental stimulus, the environmental stimulus such as cell-wall-active antibiotics at lower concentrations (Utaiida et al., 2003). The pattern of altered gene expression triggered by cell wall active antibiotics forms the cell wall stimulon. A core global *S. aureus* cell wall stress stimulon, consisting of 15 common genes induced by cell wall-active antibiotics in different studies, has been proposed. It is most likely not the single genes but the concerted action of multiple genes that may be relevant for antibiotic resistance. The high number of differentially regulated genes shows that they may form a network, and/or be controlled by common regulators (MCcallum et al., 2006).

SOS response, in its broadest sense, represents all damage-inducible genes, it quickly became synonymous with a dual-component system in which RecA protein is the activator and LexA a negatively acting transcriptional regulator (Little et al., 1980; 1981). DNA polymerase of the SOS system lack intrinsic proof reading activity, which leads to mutations when DNA replication bypasses lesions or errors (Mesak et al., 2008). DNA-damage triggers SOS-response that involves change in the expression of several bacterial

genes. Antibiotics that interfere with DNA metabolism such as quinolones trigger this response. It has been described that some beta-lactams also trigger SOS-response (Miller et al., 2004), although their primary target is not bacterial DNA. The expression of virulence function such as toxins, adhesins and biofilm formation in *S. aureus* is affected by exposure to sub-MIC of antibiotics. Sub-MIC of certain antibiotics, in particular, compounds whose primary mode of action is DNA damage are known to enhance mutation rates in bacteria. This is usually the result of transcriptional changes responsible for DNA repair and preservation of the integrity of the genome, such as the SOS and methyl mismatch repair (MMR) pathways. DNA polymerase of the SOS system lack intrinsic proof reading activity, which leads to mutations when DNA replication bypasses lesions or errors (Mesak et al., 2008).

## **1. 6. Antimicrobial Therapy and Resistance in *S. aureus***

It is possible that empirical prescribing has a significant impact on the management of MRSA infections and ultimately patient outcome (Dancer et al., 2008). Faced with a patient with bacteraemia, clinicians are forced to make an empirical choice of antibiotic for the causative organism, which has not been identified and for which there is no antibiogram. Generic prescribing guidelines and local knowledge help with this choice but increasing resistance has complicated the management of infection (Harbarth et al., 2003). This is well illustrated by the problems surrounding the management of MRSA. There is evidence to show that less than a quarter of patients with MRSA infections receive correct therapy within 48 h of hospital admission, and only about 40% receive appropriate agents after 48 h. Resistant Gram-positive bacteria such as MRSA express a number of virulence determinants, which might explain why patients with MRSA infections are more likely to suffer protracted courses of infection, or even die (Dancer et al., 2008). In a study, A total of 468 patients (52%) had documented bloodstream infection, and 211 patients (23%) received inappropriate initial antimicrobial therapy. The increasing presence of drug-resistant bacterial infections among hospitalised patients has resulted in greater numbers of patients receiving inappropriate antimicrobial treatment. This has led to the development of a novel paradigm guiding the administration of empirical antimicrobial therapy for patients with serious infections in the hospital setting. Antibacterial de-

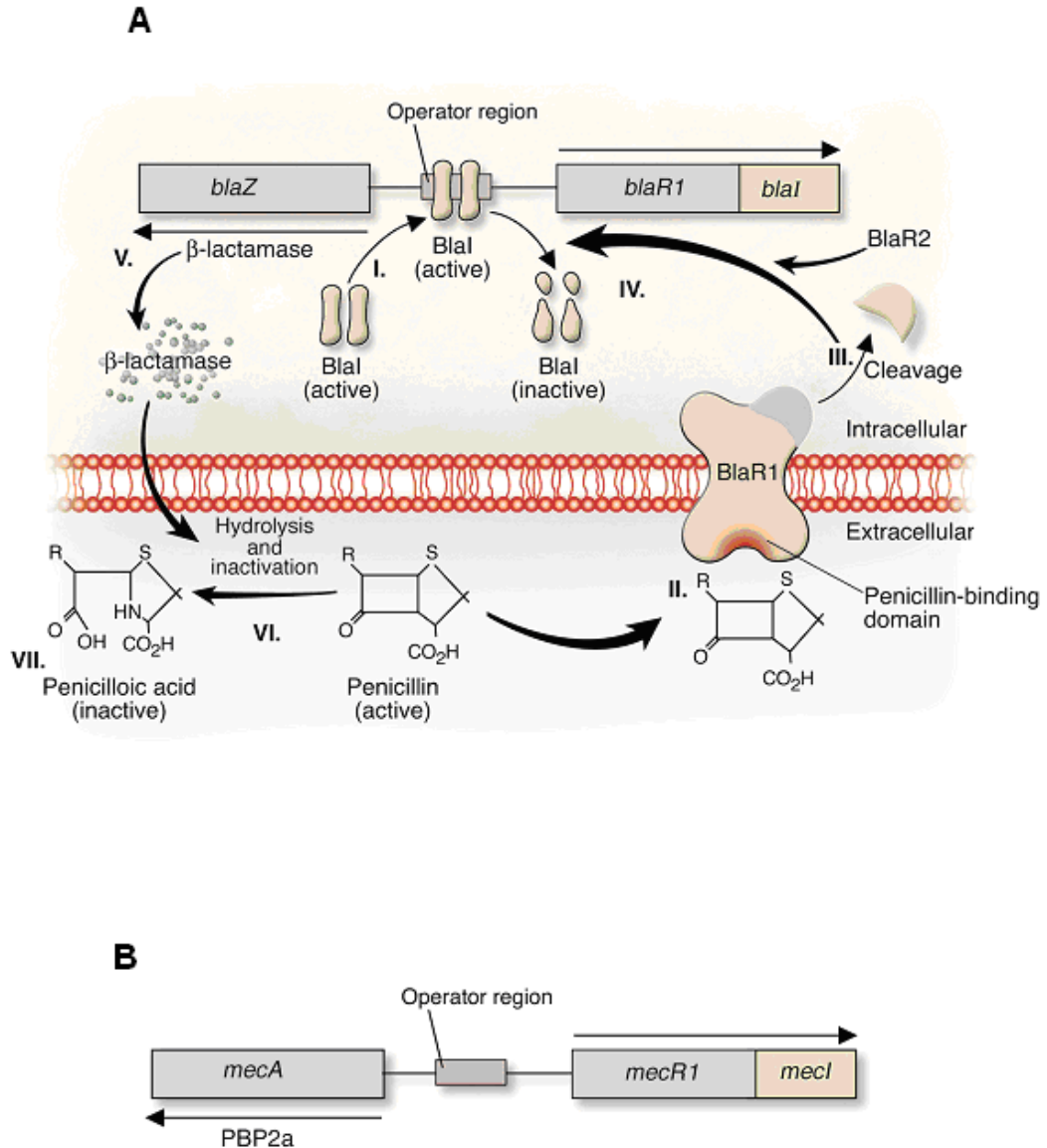
escalation is an approach to antibacterial utilisation that attempts to balance the need to provide appropriate, initial antibacterial treatment while limiting the emergence of antibacterial resistance. The goal of de-escalation is to prescribe an initial antibacterial regimen that will cover the most likely bacterial pathogens associated with infection while minimizing the emergence of antibacterial resistance (Kollef et al., 2003). For example vancomycin use is associated with an increased prevalence of vancomycin-resistant enterococci, and the emergence of *S. aureus* with enhanced resistance to vancomycin is associated with prolonged treatment with vancomycin or glycopeptides (Kim et al., 2006).

Resistance to antimicrobial drugs is an important health, as well as economic, problem. Infections caused by resistant organisms are thought to cause increased morbidity, longer hospitalizations, and higher costs when compared with infections caused by susceptible strains; however, the magnitude of effect may vary based on pathogen, resistance to specific antimicrobials, and even mechanisms of resistance (Cosgrove et al., 2009). Emergence of multi-resistant bacteria correlates with overuse and misuse of antibiotics in clinical practice. The use of antibiotics exerts a selection pressure, leading to the adaptation of bacteria to the presence of antibiotics in the environment. The constant selective antibiotic pressure to which they are submitted elicits stepwise selection of bacteria carrying mutations conferring resistance (Gonzalez-Zorn et al., 2003).

Prudent use of antimicrobial drugs-using the appropriate drug at the appropriate dosage and for the appropriate duration-is an important means of reducing the selective pressure that helps resistant organisms emerge (Tenover et al., 2006). Multidrug resistance is now the norm among many pathogens. *S. aureus* is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections, and its capacity to adapt to different environmental conditions. Virtually all *S. aureus* strains were susceptible to penicillin G when the latter was initially introduced in the early 1940, but by 1942 the first report of penicillin-resistant *S. aureus* had already appeared (Rammelkamp et al., 1942), and today virtually all strains of *S. aureus* are resistant to natural penicillins, aminopenicillins, and antipseudomonal penicillins (Rice et al., 2006). Resistant to these drugs occur because of the acquisition of genes that encode

drug-inactivating enzymes, initially known as penicillinases and now called  $\beta$ -lactamases (Rice, 2007). Staphylococcal resistance to penicillin is mediated by *blaZ*, the gene that encodes  $\beta$ -lactamase, this extracellular enzyme, synthesized when staphylococci are exposed to  $\beta$ -lactam antibiotics (Figure 1. 4), hydrolysis the  $\beta$ -lactam ring, and rendering the  $\beta$ -lactam inactive. *blaZ* is under the control of two adjacent regulatory genes, the antirepressor *blaR1* and the repressor *blaI*, recent studies have demonstrated that the signalling pathway responsible for  $\beta$ -lactamase synthesis requires sequential cleavage of the regulatory protein *blaR1* and *blaI* as shown in (Lowy, 2003).

Methicillin was introduced in 1959 to treat infections caused by penicillin-resistant *S. aureus*. In 1961 there were reports from the UK of *S. aureus* isolates that had acquired resistance to methicillin (Jevons, 1961). In MRSA, a resistance determinant encodes a penicillin binding protein, PBP2a is often inducible by  $\beta$ -lactam antibiotics, allowing the cell to grow in the presence of  $\beta$ -lactam antibiotics at the concentration beyond those achievable pharmacologically (Hiramatsue et al., 2002). Expression of resistance in MRSA strains is similar to regulatory genes for *blaZ* (Figure 1. 4. B).



**Figure 1. 4. Induction of Beta-lactamase synthesis in the presence of the  $\beta$ -lactam antibiotic penicillin.** (A) I. The DNA-binding protein *BlaI* binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin,  $\beta$ -lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer *BlaR1* stimulates *BlaR1* autocatalytic activation. III–IV. Active *BlaR1* either directly or indirectly (via a second protein, *BlaR2*) cleaves *BlaI* into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. V.  $\beta$ -Lactamase, the extracellular enzyme encoded by *blaZ*, hydrolyzes the  $\beta$ -lactam ring of penicillin, thereby rendering it inactive. (B) Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for  $\beta$ -lactamase. Exposure of *MecR1* to a  $\beta$ -lactam antibiotic induces *MecR1* synthesis. *MecR1* inactivates *MecI*, allowing synthesis of PBP2a. *MecI* and *BlaI* have coregulatory effects on the expression of PBP2a and  $\beta$ -lactamase (Lowy et al., 2003).

These genes, *mecI* and *mecRI*, regulate the *mecA* response to  $\beta$ -lactam antibiotics; in a fashion similar to that of the regulation of *blaZ*, by the genes *blaRI* and *blaI* upon exposure to penicillin (Lowy et al., 2003). The incidence of MRSA in the UK has been reduced, HPA reported that, there were a total of 1,898 cases of MRSA reported between April 2009 and March 2010, representing a 35% reduction in cases from the previous year when 2,935 cases were reported (HPA 2010).

Vancomycin, the first glycopeptide antibiotic, was isolated in the mid- 1950s and introduced into clinical practice in 1958, but there is greater interest in vancomycin now, over 50 years after its discovery, than at any time in its history (Levine. 2006). Due to an increasing number of infections caused by multi-resistant (MRSA) worldwide, vancomycin has been the drug of choice for treatment of staphylococcal nosocomial infections for the last 20 years (Aires de Sausa et al., 2004). In 1996, the first clinical vancomycin intermediate resistant *S. aureus* (VISA) with a (MIC) of 8 mg/L was documented. Although VISA strains have now been isolated in many countries around the world, they remain rare (Aires de Sausa et al., 2004). A series of experiments demonstrates that the thickened cell wall of vancomycin intermediate-resistant *S. aureus* (VISA) could protect ongoing peptidoglycan biosynthesis in the cytoplasmic membrane from vancomycin inhibition, allowing the cells to continue producing nascent cell wall peptidoglycan and thus making the cell resistant to vancomycin. They concluded that the cooperative effect of the clogging and cell wall thickening enables VISA to prevent Vancomycin from reaching its true target in the cytoplasmic membrane, exhibiting a new class of antibiotic resistant in Gram-positive pathogens (Cui et al., 2006).

Vancomycin is glycopeptides antibiotic that has as its primary target the D-ala-D-ala subunits of the Gram positive cell wall, which causes cell death by inhibiting cell wall cross-linking vancomycin resistance in *S. aureus* is not mediated by a simple mechanism (e.g. target site modification, production of antibiotic-modifying enzyme, drug efflux or impermeability) (Walsh et al., 2002). The high level of vancomycin resistance occurred because of expression of *van A*, changes the dipeptide terminus from D-alanine –D-

alanine to D-alanine –D-lactate, and the affinity of vancomycin for the new terminus is 1,000 times lower than the native peptidoglycan precursor (Tenover et al., 2006).

To date, seven types of resistance (VanA, -B, -C, -D, -E, -G, and -L) in enterococci have been described; these correspond to specific operons (*vanA*, *-B*, *-C*, *-D*, *-E*, *-G*, and *-L*) responsible for (i) synthesis of a new target (peptidoglycan precursors ending in D-Ala-D-lactate [D-Ala-D-Lac] in VanA, -B, and -D type or D-Ala-D-serine [D-Ala-D-Ser] in VanC, -E, -G, and -L type) having a reduced affinity for glycopeptides and (ii) elimination of the normal D-Ala-D-Ala-terminating precursors. VanA-type resistance, which was the first to be elucidated and which is the most common, is characterized by high levels of resistance to glycopeptides, vancomycin, and teicoplanin and is mediated by transposon Tn1546 or closely related elements that are chromosomally or plasmid located. (Perichon et al., 2009). It has been reported that the *van A* gene complex has transferred from a transposon sitting on a conjugative plasmid in vancomycin-resistant enterococci (VRE) to a typical hospital MRSA (Lindsay et al., 2010). Perichon et al., (2009) reported that acquisition of high-level vancomycin resistance by *S. aureus* strains already multiresistant to antibiotics is a major public health problem. Although it is of serious concern for patients infected with such bacteria, it seems that, due to several biological constraints, dissemination of VRSA has so far been limited.

Clindamycin is one of the protein synthesis inhibitor antibiotics, that inhibit protein synthesis by binding to the bacterial 50S subunit ribosome (Kasten et al., 1999).

Clindamycin is a frequent choice for some staphylococcal infections, particularly skin and soft-tissue infections, and as an alternative in the penicillin-allergic patient (Fiebelkorn et al., 2003). Also, clindamycin has good oral bioavailability making it a good option for outpatient therapy and changeover after intravenous antibiotics. The prescription of clindamycin depends upon site of infection, prevalence of MRSA, antimicrobial susceptibility results, whether the patient is admitted in the hospital or is an outpatient and clinician's own experience (Gupta et al., 2009). It has excellent tissue penetration (except for the central nervous system) and accumulates in abscesses, and no renal dosing adjustments are needed.

Clindamycin belongs to a group of antimicrobial agents called the lincosamides. The lincosamides together with macrolides and streptogramin B are closely related functionally but not structurally. They are often referred to collectively as the MLSB group of antibiotics. The MLSB antibiotics inhibit protein synthesis in susceptible organisms. Two different mechanisms of acquired resistance are commonly involved in MLSB resistance, active efflux encoded by the *msrA* gene and modification of the ribosomal target site encoded by *erm* genes. Resistance as a result of the *erm* genes may be of two types, either constitutive or inducible. Constitutive resistance is permanently expressed and the phenotype demonstrates resistance to all of the MLSB group. However, inducible resistance is only expressed when an inducing agent is present, erythromycin can act as such an agent in vitro (Rich et al., 2005). An *erm* gene, usually *erm(C)* or *erm(A)*, encodes methylation of the 23S rRNA-binding site that is shared by these 3 drug classes. Phenotypically, resistance can be expressed constitutively (the MLSBc phenotype) or only when induced into production (the MLSBi phenotype) (Lewis et al., 2005). Fiebelkorn *et al* (2003) found that out of 114 erythromycin-resistant *S. aureus* isolates, 39 demonstrated constitutive resistances to clindamycin while 33 showed inducible resistance. Various authors have highlighted the relationship of MRSA and MSSA with different phenotypes of clindamycin and erythromycin-resistant isolates.

Linezolid is a synthetic inhibitor of protein synthesis that is active against many Gram-positive bacteria, including such pathogens as methicillin- and vancomycin-resistant staphylococci, vancomycin-resistant enterococci and penicillin-resistant pneumococci. It is often used for treatment of complicated infections when other therapies have failed (Toh et al., 2007). Linezolid, the first oxazolidinone antimicrobial approved for clinical use, inhibits initiation of protein synthesis by preventing the formation of a ternary complex between tRNA, mRNA, and the ribosome by preventing the formation of the formyl methionyl-tRNA:mRNA: 30S subunit ternary complex (Bernardo et al., 2004). It is consistently active against multiresistant gram-positive pathogens including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE).

*S. aureus* isolates exhibiting resistance to linezolid have been selected in vitro but are still a rare phenomenon in the clinical setting (Nannini et al., 2010). The most common mechanism of resistance to linezolid among MRSA isolates is the presence of specific nucleotide substitutions in the central loop of domain V of the 23S rRNA (Tsiodras et al., 2001). Mostly G2576T, T2500A, and G2447T. *S. aureus* isolates possess five or six copies of the 23S rRNA gene and the step-wise accumulation of mutations in the 23S rRNA genes appears to gradually increased linezolid MICs. Indeed, the number of mutated rRNA genes has been associated with the duration of exposure to linezolid (Nannini et al., 2010). Toh et al., (2007) have discovered that linezolid resistance in a methicillin-resistant *S. aureus* hospital strain from Colombia is determined by the presence of the *cfr* gene whose product, Cfr methyltransferase, modifies adenosine at position 2503 in 23S rRNA in the large ribosomal subunit. The molecular model of the linezolid–ribosome complex reveals localization of A2503 within the drug binding site. The natural function of *cfr* likely involves protection against natural antibiotics whose site of action overlaps that of linezolid. In the chromosome of the clinical strain, *cfr* is linked to *ermB*, a gene responsible for dimethylation of A2058 in 23S rRNA. Coexpression of these two genes confers resistance to all the clinically relevant antibiotics that target the large ribosomal subunit (Toh et al., 2007).

### **1. 7. Molecular Typing Methods for *S. aureus***

Molecular typing of *S. aureus* has been used to examine both long-term and global epidemiology and short-term or locale epidemiology. Understanding the genetic structure of the global population over time gives insights into the evolution of bacterial lineages and transmission dynamics (Peacock et al., 2002). Therefore, various techniques for typing have been developed. In general there are ‘band-based’ and ‘sequence-based’ methods to determine the genetic background of the bacterium, but sequence-based methods have advantages because the data are exchangeable (Deurenberg et al., 2009). The most commonly used typing methods are pulsed field gel electrophoresis (PFGE), multi locus sequence typing (MLST), *spa* typing and *SCCmec* typing (Deurenberg et al., 2009).

### **1. 7. 1. PFGE**

This method is based on the digestion of chromosomal DNA with the restriction enzyme *SmaI*, followed by agarose gel electrophoresis in an alternating voltage gradient. The PFGE banding patterns are analyzed with a software package. It is one of the most frequently used staphylococcal typing methods, in which genetic variation between strains can be detected (Belkum et al., 2009). However PFGE is less useful for long-term epidemiology surveillance or for the study of the evolution and phylogenetic relationship among *S. aureus* strains. This is because it does not include a selective amplification step and is less reproducible (Melles et al., 2007).

### **1. 7. 2. MLST**

MLST uses DNA sequencing to uncover allelic variants in several conserved genes, and is one of the most popular genotyping methods for characterizing bacterial strains (Maiden et al., 1998). MLST is a technique suitable for global and local epidemiology of *S. aureus* and other bacterial pathogens (Smith et al., 2005). MLST offers advantages of unambiguous identification and high degrees of typeability and reproducibility based on the nucleotide sequences of 450-500bp internal fragments of seven house keeping genes. In addition, the data obtained by MLST permits investigation of the population structure and the development and testing of evolutionary hypotheses (Smith et al 2005). A major advantage of MLST is the ability to compare sequence data between laboratories via the MLST website on the internet (Berglund et al., 2005).

### **1. 7. 3. *spa* Typing**

The sequence of the polymorphic region X of the *S. aureus* protein A (*spa*) locus has been used by Frenay et al. to developed a single-locus sequence typing technique for the bacterium (Frenay et al., 1996). *spa* typing involves sequence analysis of 24 nucleotide repeats present in the *spa* gene. The number of repeats varies from 1 and 15 and sequence variation in the repeat units allows for the development of binary typing system (Belkum et al., 2009). Diversity of the locus is attributed to deletion and duplication of the repeats, and due to point mutation (Kahl et al., 2005). The main advantage of *spa* typing is its simplicity, since it involves sequencing of only a single locus (Deurenberg et al., 2009).

However, due to recombination events involving *spa* locus, which causes related *spa* repeat patterns within different clonal lineages, *spa* typing sometimes lacks discriminatory power (Deurenberg et al., 2009).

#### **1. 7. 4. *SCCmec* Typing**

A number of methods have been developed to investigate the structure of *SCCmec*. A multiplex PCR assay for *SCCmec* types, in which *mecA* and different loci on *SCCmec* are detected, was developed by (Oliveira et al., 2002).

#### **1. 8. The Aim and Objectives**

The overall aims of this project were to investigate the effect of sub-inhibitory concentrations of cell-wall active and protein-synthesis inhibitor antibiotics on toxin gene expression at mRNA and protein secretion levels. Genes for Panton-Valentine leukocidin and protein A virulence factors, in PVL-positive strains of both MSSA and MRSA were used as target genes in this study. Clindamycin, linezolid (protein synthesis inhibitors) and oxacillin and vancomycin (cell wall active antibiotics) were used to study their effects on *spa* and *lukSF-PV* gene expression in 10 PVL-positive clinical isolates of *S. aureus* obtained from two clinical sources in the UK. For this, strains were characterized, their toxin profile and clonality were investigated.

Research on PVL-positive strains of *S. aureus* has been increased during last decade, fatal cases reported in many places throughout the Europe countries including the UK. USA has also reported several fatal cases. PVL production has been linked to severe infections such as necrotising pneumonia, necrotising fasciitis and osteomyelitis. PVL-associated necrotising pneumonia has a mortality rate of 75%, and complications are more frequent in osteomyelitis caused by PVL-expressing strains. Epidemiological studies showing increase in concern about these strain types. In this regard several studies showed different responses of these strains to sub-MIC of different antibiotics. Therefore, this study will focus on effectiveness of sub-MIC of antibiotics of choices such as linezolid and vancomycin in today's clinical settings against Staphylococcal infections, specifically infections caused by methicillin resistant Staphylococci. Linezolid is also used against

infections cause by vancomycin resistant Staphylococci. The project will also aim to investigate the effect of sub-MIC of these two antibiotics on secretion of other extracellular proteins produced by *S. aureus* strains used in this study. To study if the effect is specific against virulence factors or they generally affecting all staphylococcus extracellular proteins, if this is the case, will that always reduce their amount or it is differential effect.

Furthermore, Linezolid is protein synthesis inhibitor antibiotic at translational level, therefore the project will also study its effect on toxin gene expression at mRNA levels, to see if there any direct or indirect effect of these types of antibiotics at mRNA levels. On the other hand vancomycin is a cell wall active antibiotic, and it has been reported by few studies that it had no effect at subinhibitory concentration on toxin gene expression, by knowing the fact that it is one of the antibiotics of choice it is questionable its effectiveness against protein secretion by some mechanisms or even against transcription. To study this, both antibiotics were used at sub-MIC concentrations against strains used to see the changes happened to virulence factor secretion as well as steady state mRNA levels.

**CHAPTER 2**  
**MATERIALS AND METHODS**

## **2.1. Bacterial Strains**

A total of 10 clinical isolates of *S. aureus* were used in this study. Table (3. 1) shows details of these isolates such as hospital designation number (given by the source of the isolates), site of isolation and presence of PVL and other toxins.

## **2. 2. Bacterial Cultivation and Identification**

### **2. 2. 1. Growth media**

Solid media were as per the liquid media with the addition of 1% (w/v) agar, the incubation was performed at 37 °C, gently shaking (150 rpm) when required.

**LB:** Luria-Bertani medium; 10% (w/v) bacto-tryptone, 10% (w/v) NaCl, 5% (w/v) bacto-yeast extract at pH 7.5. (Oxoid, UK)

**BHI:** brain heart infusion medium (Oxoid, UK).

**ISS:** Iso-Sensitest media (Oxoid, UK).

**Nutrient Agar** (Oxoid, UK)

### **2. 2. 2. Storage of isolates**

Isolates were received on agar slopes; these were sub-cultured onto BHI agar plates and incubated as described in section (2. 2.1). After assessing their purity, for short term storage agar slope was used by inoculation isolated colonies from the agar plate into deep agar slant and incubated overnight at 37°C, and then stored at 4°C. For long term storage, single colonies were grown in BHI broth media and suspended in BHI liquid medium plus 20% glycerol (v/ v) fast frozen in liquid nitrogen and stored as aliquots in sterile plastic vials at -80°C.

### **2. 2. 3. Growth curve**

Growth curves were established to determine the change in viable cell counts and absorbance readings over time. A single colony was inoculated into 5ml of the BHI (Brain Heart Infusion) broth media and incubated overnight at 37°C, an aliquot of 1.0 ml was then transferred to a 100ml of fresh liquid media of the same composition, to give a 1:100 dilution. Growth was monitored for about 15 hours. The number of viable cells (cfu/ ml)

in each sample were determined by placing 10µl of the sample (diluted sample when required) onto the surface of BHI agar plates and incubated at 37 °C overnight. One ml of the liquid culture was aseptically taken to read the absorbance at 600nm. Absorbance readings and cell counts were determined every 20minutes and the data plotted to obtain a growth curve.

#### **2. 2. 4. Minimum Inhibitory Concentration (MIC) Determination**

The macro broth dilution method using Iso-sensitest broth media (Oxoid) was used to determine MIC, the inoculum suspension density was adjusted to be equal that of 0.5 McFarland standards (Andrew JM, 2001). Briefly, liquid media (10ml) was added to 12 universal tubes making the range of antibiotic concentrations of 0.125 to 128.0mg/L. One ml of media containing the antibiotic dilutions was transferred to test tubes with one ml of bacterial suspension. The inoculum suspension was further diluted to give an inoculum size of  $10^5$  cfu/ ml in the same media of antibiotics. Tubes were mixed thoroughly and incubated for 18-20 hrs at 37°C in air and then checked for visible growth. The MIC was determined as the lowest concentration of antibiotic at which there was no visible growth.

### **2. 3. Molecular Biology**

#### **2. 3. 1. Preparation of Genomic DNA**

Total chromosomal DNA was prepared using the method used by (Palomares, Torres et al. 2003) with some modifications. Cells were grown in 5 ml of LB liquid medium overnight and centrifuged. The pellet was resuspended in 1.8 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing lysostaphin (100 µg/µl), mutanolysin (250 µg/ml) , and incubated at 37 °C for one hour. Then 300 µl of 10% sodium dodecyl sulphate (SDS) and proteinase K (250 µg/ml) were added and the suspension incubated at 65 °C for one hour. Afterwards 500 µl of 5 M NaCl and 500 µl of 10% Cetyl Trimethyl Ammonium Bromide (CTAB)/ 0.7 M NaCl were added after being heated in a waterbath at 65°C for 10 min to aid release of DNA due to increased solubility of the suspension. A chloroform-phenol extraction was then performed by adding equal volumes of Phenol/ Chloroform/ Isoamylalcohol 25: 24: 1 (V: V: V), the mixture were then centrifuged at

4000rpm/5minutes. The upper, aqueous phase was recovered and subjected to two further phenol/chloroform/ isoamylalcohol extractions. A final chloroform extraction was carried out to remove residual phenol. The DNA was precipitated with an equal volume of isopropanol and centrifuged at 13, 000 rpm for 10 min, and washed with 70 % ethanol before being air dried and resuspended in 100 µl TE buffer and stored at 4°C overnight and -20°C for longer term storage.

### **2. 3. 2. Polymerase chain reaction (PCR)**

PCR amplifications were performed in a total volume of 50µl and included 100 ng of target DNA, 1x buffer (Bioline), 1.5mM of MgCl<sub>2</sub>, 200 nM of dNTP, 400 nM of each primer and 0.2 µl of Taq DNA polymerase (Bioline). The PCR machine was set for one cycle of 5min at 95°C, 30 cycles of 45 sec at 50°C-60 °C (depends on primers used) and 5min at 72°C, finishing with one cycle of 10 min at 72°C, following the PCR reaction, the products were separated by agarose gel electrophoresis using a  $\phi$ X *Hae* III molecular weight marker. Gels, containing ethidium bromide at a final concentration of 0.5µg/ml, were made at the appropriate agarose concentration to separate the size of the DNA samples. DNA samples were prepared by adding DNA loading buffer (30% sucrose, 100mM EDTA (pH 8.0), 0.05% bromophenol blue) to the sample prior to electrophoresis. DNA bands were viewed by illumination with UV light and images recorded by photography.

### **2. 3. 3. DNA Sequencing**

PCR products for the genes (or part of the genes) required for sequencing were purified using Amersham Biosciences GFX purification kit, and quantified using a NanoDrop1000 spectrophotometer (Labtech). The PCR products were then sequenced (Durham sequencing service) using Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

### **2. 3. 4. RNA Extraction**

RNA extraction was performed using RNeasy mini kit (Qiagen). Briefly cells were grown and centrifuged at 4000g for 15 minutes, pellets then resuspended in TE 1.8 ml of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) containing 200 µg/ ml of lysostaphin, 400

U/ml mutanolysin, and 40 µg/ml proteinase K (final concentrations) and incubated at 37 °C for 1-2 hours with occasional gentle mixing. A volume of 350 µl from lysed cells were used and proceed to elution of RNA, followed manufacture's instructions. DNase I (Qiagen) was used by mixing 10 µl of enzyme with 70 µl of the DNase I supplied buffer and added to each RNA sample tubes according to the manufacture's instructions. RNA samples were eluted 50µl volumes, analyzed using a NanoDrop1000 spectrophotometer (Labtech), and stored at -80°C for longer terms.

### **2. 3. 5. RNA Agarose gel electrophoresis**

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

### **2. 3. 6. cDNA Synthesis**

First strand of cDNA was generated using the Superscript III transcriptase (Invitrogen) system. For this one µg of total RNA was reverse transcribed in a total volume of 20µl containing random hexamer primers (150 ng/µl) (Invitrogen), made up to 11.0µl with sterilized MQ RNase free water, the mixture was heated at 65°C/ 5 minutes, put on ice for 5 minutes and spun briefly. Then 1 x RT buffer (Invitrogen), 10nM DTT (Invitrogen), dNTP (0.5 mM) (Bioline), RNase inhibitor (Promega 2.0u/ µl), and Reverse Transcriptase (RT III) (10u/ µl, Invitrogen) as final concentrations, were added. The suspension of 20 µl was incubated at 42°C for 90 minutes, followed by 72°C for 15 minutes. After confirming that cDNA synthesis was successful by normal PCR, samples were then stored at -20°C.

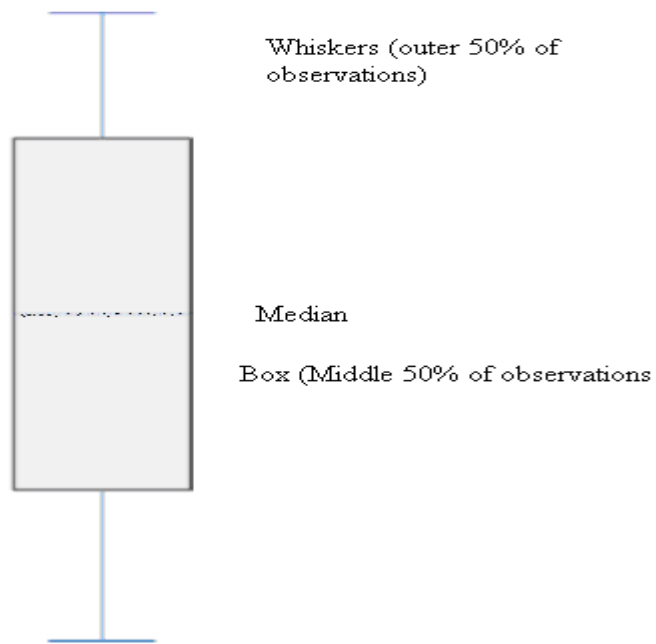
### **2. 3. 7. Real-Time quantitative PCR (RT-qPCR)**

RT-qPCR experiments were performed in a total volume of 20µl, included 10.0 ng of target cDNA, 1x buffer (Bioline), 1.5mM of MgCl<sub>2</sub>, 200 nM of dNTP, 100 nM of each

primer and 0.08 µl of Taq DNA polymerase (Bioline) and fluorescent dye SYTO9 at 5µM. This was added at the last minute as it is a very light sensitive. PCR was carried out in a Rotor-Gene 3000 (Corbett Research) using thermal cycling condition as follows: at 95°C for 10min followed by 95°C for 20sec, 54-56°C (according to the primers melting temperature) for 20sec, and at 72°C for 20sec, with data collection in each cycle at 72°C. Real time qPCR is a powerful tool and the most suitable method for the detection and quantification of mRNA. The method offers high sensitivity, good reproducibility and a wide quantification range. Relative expression is commonly used, where the expression of a target gene is standardised by a non-regulated reference gene (Pffafll et al., 2002). In this study qPCR was used as an assay for the relative quantification of expression of several virulence factor genes. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. Individual reaction are characterized by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known as the threshold cycle (Ct) value. The more target there is in the starting material, the lower the Ct value. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range (Nolan et al., 2006).

### **2. 3. 8. Data Analysis Using (REST-2005)**

Ct values and reaction efficiency were produced in comparative quantification analysis generated by Rotorgen3000 and used for data analysis using REST-2005 software (Corbett Research). The Relative Expression Software Tool (REST-2005) using statistical whisker-box plots (Figure 2. 1), in which the box area encompasses 50% of all observations, the dotted line represents the sample median and the whiskers represent the outer 50 % of observations (Pfaffl et al., 2006). REST 2005 is a software tool used to determine changes in target gene expression standardised by non-regulated reference genes (Corbett Research 2006). The purpose of this software tool in this study was to determine whether there were significant differences in gene expression profile between culture samples exposed to antibiotics and samples without adding antibiotics as controls.



**Figure 2. 1. Whisker box plot in which the box area represents of 50% of observations and dotted line represents the sample median (Pffafle et al., 2006).**

## **2. 4. Proteomics and Protein Analysis**

### **2. 4. 1. Collection and Concentration of Proteins from the Culture**

#### **Supernatant**

Cells were cultured at 37°C under gentle agitating in 100 ml volume of BHI media. Samples were collected in the late exponential phase, following 5 h exposure to antibiotics, by centrifugation for 15 minutes at 4°C, 7000 x g. The supernatant was filtered by passing through 0.22µM pore size filter to ensure that all bacteria were removed from the samples. Extracellular Proteins were concentrated using tri-chloro acetic acid (TCA) precipitation (Engelmann and Hecker 2008), for this freshly prepared ice cold TCA was added to 50 ml of the sample to a final concentration of 10% (v/v). Suspensions were mixed well and left overnight at 4°C to precipitate. The precipitates were then harvested by centrifugation at 4°C and 7000 g for one hour, the supernatant removed very carefully and the pellets washed with 100% ice cold acetone three times and finally with 80% cold acetone. These sequential washing steps aid the removal of any precipitated non-protein compounds, such as salt, which may interfere with isoelectric focusing and electrophoresis. Pellets were collected by centrifugation at 8000g for 10mins each time. The resultant pellet was then air dried for 5 minutes, taking care not to over dry which results in increased resuspension difficulty. Pellets were then resuspended in Urea Buffer and stored at -20°C for short-term or -80°C for long-term storage.

#### **2. 4. 2. Estimation of Protein Concentration**

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

### **2. 4. 3. Modified Bradford Assay**

A modification to the standard Bradford assay was developed by Ramagli *et al.* (Ramagli *et al.* 1985) in order to allow the use of this assay on samples containing carrier ampholytes and thiol-containing compounds. Estimation of protein concentration was performed using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Ltd.). A series of BSA standards ranging from 0-40µg/ µl was prepared from a stock solution of 2mg/ml BSA in Urea buffer (9M urea, 2M thiourea, 4% (w/v) CHAPS) and each made up to a final volume of 25µl in Urea buffer. Aliquots (2µl and 5µl) of samples of unknown concentration were also prepared in a final volume of 25µl Urea buffer. To each of these samples, 10µl 0.1M HCl and 65µl of sterilized MQ water was then added. Finally, 900µl of 20% (v/v) Protein Assay Dye Reagent Concentrate was added to each solution, mixed and incubated at room temperature for 10 minutes. Absorbances were measured at 595nm and the protein concentration of each sample calculated from the plotted BSA standard data.

### **2. 4. 4. Mini-format 1-Dimensional SDS-PAGE**

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

#### **2. 4. 4. 1. Gel Casting**

SDS-PAGE mini gels were performed using 0.75mm thick gels composed of a 12% acrylamide resolving gel and a 5% acrylamide stacking gel. These were cast using the Mini-Protean II Cell (Bio-Rad Laboratories Ltd.) and clean glass casting plates. Resolving gel solutions were prepared containing 10% or 12% (w/v) acrylamide (acrylamide:bis-acrylamide 37: 5: 1) (Bio-Rad Laboratories Ltd.), 375mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate (Bio-Rad Laboratories Ltd.), and 0.2% (v/v) TEMED (Bio-Rad Laboratories Ltd.). Stacking gels were similarly prepared containing 5% (w/v) acrylamide and 125mM Tris-HCl pH 6.8 as the buffer. The resolving gel was cast first and overlaid with water-saturated butan-1-ol. Once this gel had set, the butan-1-ol was rinsed out and excess water removed by blotting. The stacking gel was then

overlaid onto the level surface of the resolving gel and individual sample loading wells were created by insertion of a Teflon comb. Wells were then rinsed free of any unpolymerised acrylamide.

#### **2. 4. 4. 2. Sample Preparation & Gel Loading**

Protein samples in urea buffer were solubilised in 5x SDS sample loading buffer (12% (w/v) SDS, 5% (w/v) DTT (Melford Laboratories Ltd., Ipswich, UK), 0.05% (w/v) bromophenol blue, 0.312 M Tris-HCl pH 6.8, 50% (v/v) glycerol) to give a 1x concentration in a volume of up to 20 $\mu$ l. All samples were then denatured at 100°C for few minutes and centrifuged for one minute to pellet cell debris and insoluble material. SDS-PAGE gels were placed in Tris-glycine running buffer (25mM Tris-HCl, 190mM Glycine, 0.1% SDS) and prepared protein samples were loaded (up to 20 $\mu$ l per well). Protein ladder 7 (66, 45, 36, 29, 24, 20.1, 14.2 kDa, Sigma) protein standards were loaded beside the protein samples to allow estimation of protein size.

#### **2. 4. 4. 3. Electrophoresis**

Electrophoresis through the stacking gel was conducted at 100V, after which the voltage was increased to 200V until the dye front reached the bottom of the resolving gel. Gels were then carefully detached from the apparatus and proteins visualized by either Coomassie Brilliant Blue R-250, silver stain or SYPRO Ruby staining.

#### **2. 4. 5. Mini-format 2-Dimensional SDS-PAGE**

Mini-format two-dimensional electrophoresis (2-DE) was performed using a MultiPhor II Electrophoresis Unit with an accompanying Immobiline DryStrip kit (Amersham Biosciences) for first dimension isoelectric focusing (IEF) and a Mini Protean II vertical gel apparatus (Bio-Rad Laboratories Ltd.) for second dimension SDS-PAGE. IEF was conducted using 7cm Immobiline Dry strips (Amersham Biosciences), also known as immobilised pI gradient (IPG) strips, with linear pI gradients of 3-10 ranges.

#### **2. 4. 5. 1. Protein Loading by In-gel Rehydration**

Protein samples were loaded onto 7cm IPG strips (Amersham Biosciences) by in-gel rehydration. Strips were rehydrated in an Immobiline DryStrip Reswelling Tray (Amersham Biosciences) using rehydration solution (9M urea, 2M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT (Melford Laboratories Ltd.), 2% (v/v) IPG Buffer (Pharmalyte), 0.002% (w/v) bromophenol blue) containing the desired quantity of urea buffer solubilised protein sample in a final volume of 125 $\mu$ l. DTT, Pharmalytes and protein sample were added to the rehydration solution immediately prior to strip rehydration. Samples were placed into separate grooves of the reswelling tray and the strip carefully laid gel-side down on top of the sample and covered with 2 ml of paraffin oil, to prevent evaporation and urea crystallization and left overnight at room temperature.

#### **2. 4. 5. 2. First Dimension Isoelectric Focusing**

The MultiPhor II Electrophoresis Unit and accompanying DryStrip kit were assembled as per manufacturer's instructions. The ceramic cooling plate was maintained at a constant temperature of 20°C using a circulating water bath (Grant Instruments Ltd., Cambridgeshire, UK), preventing overheating of IPG strips during IEF. Hydrated IPG strips were removed from the reswelling tray, rinsed with MQ water, and placed into the grooves of the DryStrip aligner tray gel side up with the acidic ends towards the anode and basic ends towards the cathode and level with other strips. Electrode wicks were cut to the required size, moistened with MQ water and placed perpendicularly across each end of the IPG strips and in direct contact with the gel surface. Anodic and cathodic electrodes were then placed on top of the appropriate wicks and the strips fully submerged with paraffin oil to prevent urea crystallisation. IEF was performed using a 3-step program detailed in Table 2. 1.

**Table 2. 1. Multiphor IEF program for 7cm IPG strips.**

Step	Volts (V)	Current ( $\mu$ A/strip)	Power (W)	Time (hrs:mins)	Volt hours (Vh)
1	200	50	5	0:01	-
2	3,500	50	5	1:30	2,800
3	3,500	50	5	1:05	3,700
Total				2:36	6,500

### **2. 4. 5. 3. IPG Strip Equilibration**

On completion of IEF, strips were removed from the focusing unit and rinsed with ddH<sub>2</sub>O and incubated in 2ml equilibration buffer (6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50mM Tris-HCl pH 8.8, 0.002% (w/v) bromophenol blue) containing 1% (w/v) DTT for 15 minutes at room temperature in the reswelling tray with gentle agitation on an orbital shaker. A second incubation was then performed under the same conditions using equilibration buffer containing 4.8% (w/v) iodoacetamide. These incubations serve to reintroduce SDS to the protein and ensure permanent reduction and alkylation which is a prerequisite for subsequent second dimension resolution.

### **2. 4. 5. 4. Second Dimension SDS-PAGE**

Second dimension SDS-PAGE was performed using 1mm thick 12% acrylamide resolving gels. Equipment and buffers used were the same as for 1-D SDS-PAGE without stacking gel. The void volume in the cassette above the resolving gel was filled with ddH<sub>2</sub>O and then rinsed, equilibrated IPG strip was laid onto the resolving gel. Water was then removed by blotting, taking care not to introduce bubbles. IPG strips were then overlaid with agarose sealing solution (1% (w/v) low melting point agarose, 0.002% (w/v) bromophenol blue in Tris-glycine SDS electrophoresis buffer) and subjected to

electrophoresis at 60V for 30 minutes followed by 120V until the dye-front reached the bottom of the resolving gel. Gels were then removed and spots visualized by staining.

## **2. 4. 6. Large-format 2-Dimensional SDS-PAGE**

### **2. 4. 6. 1. Preparation of Backed Gels**

Glass plates were soaked for several hours in 1% (v/v) Decon, rinsed with ddH<sub>2</sub>O, soaked in 1% HCl (v/v) for 1 hour and then thoroughly rinsed with ddH<sub>2</sub>O. Backed gels, in which the gel is immobilised on a glass plate (260 x 200 x 1.5mm and low fluorescence glass cassettes 260 x 200 x 1mm) using a silane based reagent. 2-4ml of Bind-silane solution (80% (v/v) ethanol, 0.1% (v/v) PlusOne Bind-Silane (Amersham Biosciences), 2% (v/v) glacial acetic acid) was applied to the surface of the glass plate (without spacers) and gently wiped with a lint free cloth and then covered with lint free cloth and allowed to dry for 1.5 hours.

### **2. 4. 6. 2. Large-format Gel Casting**

Glass cassettes were prepared and 12 large format homogeneous gels were cast using a 2DE Optimizer (Nextgenesciences). The tank is pre-calibrated for 12 gels and followed on screen step-by-step instructions. One ml of butanol was then pipette over each gel to ensure a straight finished gel; the tank was then left without being moved for the gels to set overnight. Resolving gel solutions were prepared as outlined in Table 2. 2. Butan-1-ol was rinsed out with MQ water prior to use.

**Table 2. 2. Composition of large format 12% acrylamide SDS-PAGE gels.**

<b>Constituent</b>	<b>Volume (ml)</b>	<b>Final conc.</b>
10 % (v/v) SDS	10 0.	1 %
1.5 M Tris-HCl	250	375 mM
40 % (v/v) acrylamide	300	12 %
ddH <sub>2</sub> O	434	-
10 % (v/v) APS	5	0.05 %
TEMED	1	0.1 %

### 2. 4. 6. 3. Protein Sample Preparation

Protein samples were prepared as described for mini-format 2DE (2. 4. 1).

### 2. 4. 6. 4. Protein Loading by In-gel Rehydration

In-gel rehydration of 18cm IPG strips was performed as described for mini-format 2DE with protein samples in a final volume of 500  $\mu$ l rehydration buffer. Rehydrated strips were then transferred to the Ettan IPGphor (Amersham Biosciences) on the IEF unit, gel side up with the acidic end at the anode. Individual electrode wicks (5 x 12mm), soaked with ddH<sub>2</sub>O, were blotted with filter paper and placed at both ends of each strip.

Electrodes were firmly clipped in place, ensuring good contact with each electrode wick.

### 2. 4. 6. 5. First Dimension IEF using the Ettan IPGphor IEF system

IPGphor Strip Holders containing the rehydrated IPG strips and samples were correctly positioned on the Ettan IPGphor unit. IEF was conducted at 50 $\mu$ A per strip for 70kVh at 20°C using a 5–step program (Table 2. 3).

**Table 2. 3. Ettan IPGphor IEF program for 18cm IPG strips<sup>1</sup>.**

Step	Step type	Volts (V)	Time (hrs: mins)	Volt hours (Vh)
1	Gradient	500	00:10	41
2	Gradient	1,000	01:20	1,000
3	Gradient	4,000	01:40	41,66
4	Step n' Hold	6,500	10:00	65,000
5	Step n' Hold	1,000	60:00	-

<sup>1</sup> Ettan IPGphor IEF program was performed over night for 70 kVh, ~15 hours.

### 2. 4. 6. 6. IPG Strip Equilibration

On completion of IEF, strips were removed from the focusing unit and rinsed with ddH<sub>2</sub>O to remove any cover fluid. Strips were then incubated in 5ml equilibration buffer (6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50mM Tris-HCl pH 8.8, 0.002% (w/v) bromophenol blue) containing 1% (w/v) DTT for 15 minutes at room temperature. A

second incubation was then performed under the same conditions using equilibration buffer containing 4.8% (w/v) iodoacetamide. Equilibration was performed in equilibration tubes (Amersham Biosciences) at 100rpm on an orbital shaker.

#### **2. 4. 6. 7. Second Dimension SDS-PAGE**

Focused sample IPG strips were laid onto the resolving gel as described in section (2. 4. 5. 4), and subjected to electrophoresis using the Ettan DALT*twelve* (Amersham Biosciences) large format vertical electrophoresis systems. Electrophoresis using the Ettan system required 7.5 litres of Tris-glycine electrophoresis buffer in the lower reservoir, 2.5 litres of 2 x Tris-glycine electrophoresis buffer in the upper reservoir of the tank and was performed 5W per gel for 30 minutes followed by 17W per gel for 4 hours or until the bromophenol blue dye front reached the bottom of the gel. Temperature was maintained at a constant 25°C.

#### **2. 4. 6. 8. 2DE Gel Staining Using MS-Compatible Silver**

This method was performed essentially as described for the Plus One Silver Stain (Amersham Biosciences) and is similar to Shevchenko's modified silver stain (Shevchenko *et al.* 1996). All solutions were prepared immediately prior to use and incubations performed with gentle agitation. Gels were fixed twice in fixing solution (40 % (v/v) methanol, 10 % (v/v) glacial acetic acid) for at least 15 minutes each (1 hour for large-format gels), transferred to sensitising solution (30% (v/v) methanol, 6.8 % (w/v) sodium acetate, 0.2 % (w/v) sodium thiosulphate) and incubated for 30 minutes. Gels were washed three times in MQ water for 10 minutes each, followed by incubation in 0.25 % (w/v) silver nitrate for 20 minutes. Gels were then washed twice with MQ water for 1 minute each, followed by incubation in developing solution (2.5 % (w/v) sodium carbonate, 100 µl formaldehyde) for up to 5 minutes until protein spots were suitably visible with negligible background staining. At this point gels were transferred to stop solution (1.46 % (w/v) EDTA) and incubated for at least 10 minutes. Gels were then extensively washed in MQ water prior to scanning.

#### **2. 4. 6. 9. SYPRO Ruby Red**

Staining solution was filtered prior to use to remove precipitated material. Gels were fixed twice in fixing solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid) for 2 hours each, followed by over-night incubation in SYPRO Ruby Protein Stain (Genomic Solutions Ltd.) in the absence of light. Staining solution was removed and the gels rinsed twice with MQ water for 1 minute each, before incubating twice in destain (10% (v/v) methanol, 6% (v/v) acetic acid) for 1-2 hours.

#### **2. 4. 6. 10. Gel Imaging with the Typhoon 9400 Variable Mode Imager**

The Typhoon Variable Mode Imager (Amersham Biosciences) was used in fluorescence acquisition mode at normal sensitivity, using the 610nm band pass 30 emission filters for the scanning of SYPRO Ruby stained gels. Loose gels were scanned directly on the glass platen while backed gels were supported by the +3mm Gel Alignment Guides and scanned at the +3mm focal plane. Appropriate scanning areas were defined using the scanner control software. Pre-scans were performed at 500µm pixel resolution until a suitable photon multiplier tube (PMT) voltage had been identified at which final imaging could be performed. This ensured maximum use of the dynamic range of the stain while avoiding pixel saturation. Final images were acquired at a resolution of 100µm and saved as GEL files, a modified TIF file format.

#### **2. 4. 7. Identification of Proteins**

Protein Identification was performed by Joanne Robson, Proteomic services, School of Biological and Biomedical Sciences, Durham University. This included picking selected spots, MALDI-TOF mass spectrometry, and MASCOT search software. A list of identified proteins with their MASCOT score are produced to use in this study.

### **2. 5. 2DE DIGE Technique Experiments**

#### **2. 5. 1. Sample Preparation and Labeling process**

Protein samples were prepared as in previous section with the exception that labeling buffer was used instead of urea buffer. Labeling buffer was prepared by adding 25µl of a

1M Tris-HCl pH 8.5 stock solution to 1.0 ml of Urea buffer(9 M urea, 2 M thiurea, 4% (w/ v) CHAPS) so that samples were ready for CyDye DIGE Fluor minimal dye labeling. This precipitates the protein removing any compounds that may affect the CyDye labeling process and was performed according to the manufactures recommendations. Protein pellets were dried in air for 2-5 minutes, taking care not to over dry, and solubilised by vortexing for two hours in labeling buffer (9 M urea, 2 M thiurea, 4% (w/ v) CHAPS, 25 mM Tris-HCl pH 8.5) to a protein concentration between 1-5mg/ ml. The pH of theses protein samples was measured by pipetting a small volume, typically 1 $\mu$ l, onto pH indicator strips (pH 7-14) and adjusted, if necessary, using NaOH or HCl to between pH 8.0-9.0 (preferred pH 8.5) which is essential for optimal CyDye labeling.

### **2. 5. 2. Reconstitution of stock CyDye DIGE Fluor minimal dyes in dimethylformamide (DMF)**

CyDye DIGE Fluor minimal dyes (Amersham Biosciences) are supplied as solids and are reconstituted in DMF for use. High quality DMF (>99.8% pure) was used and care was taken to ensure it was not contaminated with water. CyDye vials (Cy3 and Cy5) were removed from the freezer and allowed to equilibrate to room temperature for at least 5 mins. Vials were spun briefly to bring the powder contents to the bottom before opening. Vial contents were resuspended in 20 $\mu$ L of DMF by vortexing for 30 sec to get the primary stock. This gives a stock of 0.5nmol/ $\mu$ L (500pmol/  $\mu$ l), which is stable for maximum of 2 months at  $-20^{\circ}\text{C}$ . After closing, vials were sealed with parafilm and placed in a heat sealed bag containing desiccant. Working CyDyes solution was then prepared by removing stock solution from the freezer, allowed to warm up to room temperature for at least 5 minutes and spun briefly to bring the contents to the bottom. From each of these Dyes (Cy3 and Cy5) solutions 0.8  $\mu$ L was mixed with 1.2 $\mu$ L of DMF (fresh aliquot) to give a final volume of 2  $\mu$ L. The concentration of this working dilution was 200 pmol/ $\mu$ L (0.02 mM). CyDye solutions were then vortexed and spun to make them ready for labeling. These working solutions were used on the same day they prepared.

### **2. 5. 3. Protein Labeling with CyDyes DIGE Fluor Minimal Dyes**

The minimal Dye labeling experiment was designed in which all 27 samples (controls and treated with antibiotics) were labeled with Cy5 and a pooled internal standard was created containing equal amounts of all samples and labeled with Cy3. All labelling reactions were performed at a dye to protein ratio of 200 pmol dye: 25 µg protein. Minimal labelling of each protein sample was performed using 25 µg of protein in a final volume of 32 µl Tris-urea labelling buffer. Sufficient internal standard was prepared to allow it to be included in each experiment at a final concentration matching that of the test samples. Volumes of each protein sample equivalent to 25 µg were dispensed into fresh microfuge tubes, made up to 32 µl with labeling buffer and numbered 1-27, mixed by vortexing and placed on ice. To prepare the pooled standard, volumes of each protein sample equivalent to 25 µg were dispensed into one tube and the volume made up to 864 µl with labeling buffer. This was then divided into 5 tubes with equal volumes of 172.8 µl and numbered with 28a, 28b, 28c, 28d and 28e; the tubes are mixed by vortexing and placed on ice. To each of the 27 samples, 1 µl (200 pmol) of Cy5, and to the pooled standard tubes 5.4 µl (200 pmol) of Cy3 working Dye solutions were added. Samples were mixed thoroughly by vortexing and incubated on ice for precisely 30 minutes in the dark. After this time labeling reactions were stopped by adding of 1 µl of 10 mM L-Lysine, therefore the final volume of each of the 27 samples was 34 µl in 0.73 µg/ µl concentration. Samples were mixed by vortexing and left in the dark for a further 10 minutes, after which they were stored at -20°C.

### **2. 5. 4. Labeling efficiency Quality Control**

To check the labeling efficiency, 2 µg of each of the protein samples was resolved via SDS-PAGE. Resolved protein bands were visualized using a Typhoon 9400 variable mode imager directly on the glass platen in fluorescence acquisition mode using the Cy-Dye detection parameters and settings for each Cy-Dye.

### **2. 5. 5. Large format 2DE of CyDye Labelled Protein Samples**

Equal quantities (12.5 µg) of each of the 27 samples (Cy 5 labelled) were mixed together with the same quantity of pooled internal standard (Cy3 labelled). Consequently each tube

contained a total of 25 µg in 34 µl; this was made up to 100 µl with ddH<sub>2</sub>O and 400 µl of ice cold acetone was added and mixed by vortexing. The suspension was left at room temperature for 60 minutes before centrifugation at 13,000 rpm for 10 minutes to collect the proteins. Pellets were air dried for 2-5 minutes then resuspended in 100 µl of urea buffer by shaking at room temperature for 1-2 hrs. Samples were loaded using IGR; IEF and Electrophoresis were all performed as described in sections (2. 4. 6. 4), (2. 4. 6. 5), (2. 4. 6. 6) and (2. 4. 6. 7).

### **2. 5. 6. DIGE Gel Imaging**

The Typhoon Variable Mode Imager (Amersham Biosciences) was used in fluorescence acquisition mode at normal sensitivity, using the appropriate laser and filter combinations for each CyDye as detailed in Table 2. 4.

**Table 2. 4. Imaging parameters for visualisation of fluorescently labelled proteins.**

<b>Dye</b>	<b>Emission filter (nm)</b>	<b>Laser</b>
Cy3	580	Green (532)
Cy5	670	Red (633)

DIGE gels were imaged in the glass cassettes immediately after completion of second dimension electrophoresis after being rinsed with ddH<sub>2</sub>O and dried with a lint-free tissue. Cassettes were supported by the +3mm Gel Alignment Guides and scanned at the +3mm focal plane. Cassettes were pressed during scanning to prevent movement and distortion of the acquired images. Appropriate scanning areas were defined using the scanner control software. Pre and full scans were then performed as described in section 2. 4. 6. 10. Each gel resulted in two separate images that were overlaid and saved as a multi-channel dataset file.

### **2. 5. 7. 2DE Data Analysis using Progenesis Software**

Progenesis (Nonlinear Dynamics) same spot analyses software was used to analyze 2DE gel images. 2DE scanned gel images were imported to Progenesis software, the software

was used for alignment of the images and statistical data generated by the software. Briefly a reference 2DE gel image produced for a pooled sample made from all ten protein samples, was chosen to which all other images were matched by software transformation. Several identical anchor points across all the gels to serve as reference land marks were used. The parameters used to determine the choice of reference image were overall quality of the image and number of spots. Then the software automatically matched all spots across all gels (SYPRO-Ruby stained or CyDye labeled), which were manually checked and altered if necessary. The software was subsequently used to determine the average changes in protein abundance across all gels for each protein spot between different samples. The software then quantified volume, normalised volume and background (i. e. the abundance) of each spot.

## **2. 6. Western Blotting**

### **2. 6. 1. Electrophoretic Transfer**

Proteins were separated by 1D SDS-PAGE before being transferred to nitrocellulose membrane (Amersham Biosciences) using a Mini-Protean II Western Transfer cell (Bio-Rad Laboratories Ltd.). Transfer was performed by electrophoresis at 70V for 1 hour at 4°C in transfer buffer (25mM Tris-HCl, pH 8.3; 0.15M glycine, 10% methanol). Ponceau S stain was then added to the membrane for few minutes to confirm that proteins are transferred.

### **2. 6. 2. Immunoblotting**

Membranes were blocked to prevent non-specific binding of antibody using 5% non-fat dried milk (Marvel) in Tris-buffered saline (TBS: 20mM Tris-HCl, 500mM NaCl; pH 7.5) for at least 1 hour at room temperature or overnight at 4°C and then washed twice for 10 minutes in Tween-TBS (TBS-T: 0.05 % tween-20 in TBS). Membranes were then incubated in primary antibody (diluted as required in 1% Marvel in TBS) solution for 1 hour at room temperature with agitation and then washed twice with TBS-T for 5 minutes to remove excess antibody. A further 1 hour incubation in the appropriate secondary antibody solution (Cy3-conjugate; diluted as required in 5 % Marvel in TBS-T) was then

performed after which any unbound secondary antibodies were removed by two 10 minute washes in TBS-T, followed by a single wash in TBS for 10 minutes.

### **2. 6. 3. Blotted Membrane Densitometry**

Densitometry was performed on blotted membrane using the Typhoon Machine 9400. Digitised greyscale images were generated by scanning in specific emission filters for fluorescence of Cy3 dye and were analyzed using ImageQuant (Molecular Dynamics) software packages. Data were taken using volume values in which background subtraction was performed based on local background.

## **CHAPTER 3**

### ***S. aureus* Strain Characteristics**

### 3. 1. Introduction

The presence of the PVL toxin is more common among CA-MRSA than HA-MRSA isolates, however, the prevalence of PVL among CA-MRSA isolates varies geographically; in the USA, PVL genes are present in 40-95% of CA-MRSA isolates and nearly all USA300 isolates contain PVL (Otter et al., 2009). However, the prevalence of PVL-positive CA-MRSA in the UK is significantly lower than in the USA (Hidron et al., 2009). As the prevalence of PVL-positive strains amongst *S. aureus* isolates is only around 2 % in the UK (Otter et al., 2009). Clones of PVL-producing MRSA are now spreading rapidly throughout the world; although France, America and Australia have reported cases since 1999, the first recognized British case of PVL-associated pneumonia was in London in 2003 (Klein et al., 2003).

*S. aureus* evolves primarily by acquiring single nucleotide polymorphisms in its core genome and by acquiring mobile genetic elements through horizontal gene transfer (Shukla et al., 2009). DNA sequencing is an efficient way to identify these genetic changes in order to differentiate and phylogenetically classify bacterial strains (Smith et al., 2005). Many epidemiological studies are concerned with the relationship between isolates that are recovered from clusters of infections of a small community within a short period or longer term of a broader geographic range. MLST can be used to identify if the causative agent is a single strain or whether infections are due to a number of different strains (Spratt ., 1999). It has proven to be an excellent method to study the molecular evolution of *S. aureus* (Deurenberg et al., 2008). A major advantage of MLST is the ability to compare sequence data between laboratories via the MLST website on the internet (Berglund et al., 2005). In addition, the data obtained by MLST permits investigation of the population structure and the development and testing of evolutionary hypotheses (Smith et al., 2005). MRSA and VRSA *S. aureus* clones being the most important examples, the MLST data reveal that these clones have evolved from genotypes which were already common in the population (Enright et al., 2002 cited by Feil et al., 2003). MLST is a technique suitable for global and local epidemiology of *S. aureus* and other bacterial pathogens (Smith et al., 2005). In which isolates that have identical sequences at all seven loci are considered to be a clone and are assigned a unique

sequence type (ST). STs that differ by single nucleotide polymorphisms (SNPs) at fewer than three loci are closely related and are grouped into clonal complexes (CCs). This grouping is accomplished by using MLST data to group closely related strains into a CC (Chambers et al., 2009).

*S. aureus* populations consist of about ten dominant human lineages and many minor lineages. The dominant human lineages of *S. aureus* are often referred to by their clonal complex (CC) numbers, although they can be detected by MLST, *spa* typing or microarray analysis. They are CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51 (Feil et al., 2003). Isolates from the same lineage have remarkably conserved genomes (with the exception of their mobile genetic elements) despite wide geographic, temporal and presumably selective diversity. In contrast, isolates from different lineages have hundreds of genes that vary between them. The major differences were identified and associated with lineages using microarray technology and referred to as “core variable” (CV) genes. Some of the variations are small inserts or variant regions within a gene, while others might include the presence or absence of a gene or series of genes, including small ‘islands’ of DNA and up to substantial variation in the genomic islands alpha and beta (Lindsay et al., 2010). Most human nasal carriage isolates belong to about 10 dominant lineages. In general, human lineages are thought to have been relatively stable for the last 40 years. Most of hospital *S. aureus* strains also belong to a few of these same lineages CC5, 8, 22, 30 and 45. Surprisingly, in each geographical location only one or two of these MRSA lineages predominate in hospitals (Feil et al., 2003 and Gomes et al., 2006).

The UK sustains a high burden of hospital acquired (HA)-MRSA infections, with two epidemic MRSA clones predominating; EMRSA-15 (ST-22/ CC22) and EMRSA-16 (ST-36/ CC30) (Ellington et al., 2009). In a study on a collection of 199 isolates obtained from non-hospitalized patients presenting skin and soft tissue infections 96% of these isolates were genetically related to EMRSA-15 or -16, this suggests nosocomial MRSA spreads within the community settings as well (Rollason et al., 2010). Despite the fact that EMRSA-16 seems to be reduced during the past decade, Ellington et al., found that about

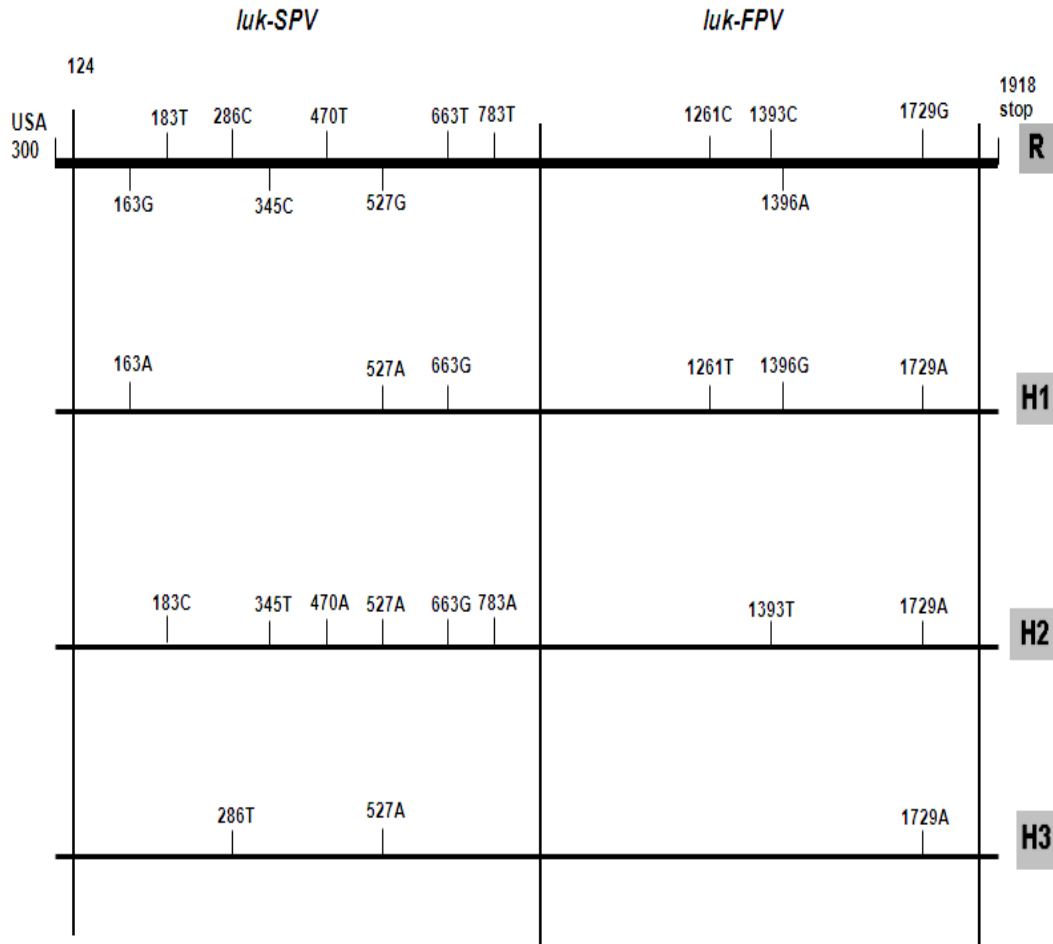
95 % of 295 isolates belong to EMRSA-15 and EMRSA-16. However, the proportion of EMRSA-16 declined from 21.4 % in 2001 to 9 % in 2007, whilst the proportion of EMRSA-15 rose dramatically from 75.7 % in 2001 to 85.4 % in 2007 (Ellington et al., 2010). In a study by Rasigade et al., analysis of 211 PVL-positive *S. aureus* isolates that were collected in 19 countries throughout the world, between 1981 and 2007, found that the predominant lineages of PVL-positive isolates were CC-30, CC-121, CC-1, CC-5 and CC-80 (Rasigade et al., 2010).

It was believed that the dissemination of PVL-positive CA-MRSA clones was related to continents, for example USA 400 (ST-1) and USA300 (ST-8), which are mostly found in the USA and Canada, whereas clones with ST-80 are mostly in Europe, and ST-30 clones in Australia (Vandensech et al., 2004 and Gilbert et al., 2006). However recent studies showing that five major PVL-positive CA-MRSA clones are disseminating worldwide. ST-1 is observed in Asia, Europe, and USA, the ST-8 clone in Europe and the USA, the ST-30 clone in the USA, Japan, Australia, Europe and South America, the ST-59 clone in Asia, the USA and the ST-80 clone in Asia, Europe, and the Middle East (Diep et al., 2004, Takano et al., 2008 and Deurenberg et al., 2008).

Although variation in the genetic background of strains of *S. aureus* has been investigated, only a few studies to date have focused on evaluating the genetic variation within the *lukSF-PV* genes. The gene nucleotide sequence is highly conserved in the *S. aureus* genome, but there were 12 sites that varied (John et al., 2008). Much of the sequence divergence in the genomic DNA is due to single nucleotide polymorphisms (SNPs), which occur when a single nucleotide differs between members of a given species, and it is the most common type of genetic variation. The functional effect of SNPs depends on its nature and position (Schork et al., 2000). Many SNPs within coding regions will be phenotypically silent, as the nucleotide change introduced will not result in a change in the amino acid encoded (synonymous substitution) and are more likely to have a functional effect if they occur in the first or second base pair of a codon (Lindsay et al., 2006). Nucleotide polymorphism analysis of PVL genes in *S. aureus* shows 12 variable positions scattered along the nucleotide sequence as shown in (Figure 3. 1) (O'Hara et al., 2008).

PVL toxin consists of S and F protein subunits, and it is considered that S binds to PMNs first, followed by subsequent binding of the F protein (Boyle Vavra et al., 2007). Interestingly amino acid changes were only reported in the S protein region, indicating the possibility that PVL variants (with amino acid changes) show different host specificity (Takano et al., 2008). Variation at position 527 of the PVL gene resulted in a change of a histidine (H) to arginine (R) substitution (H 176 R) at amino acid 176 with potential functional implications. O'Hara et al. reported that the substitution could enable S subunit to form pores with subunits other than F subunit of PVL toxin. This will amplify the rate of pore formation. It could also alter interaction with PMN surface receptors required for competent pore formation (O'Hara et al., 2008). On the bases of this synonymous change of amino acid at 176 position, PVL-positive strains could be classified into R and H variants, and the H variants can be further classified into three groups H1, H2 and H3 (o'Hara et al., 2008). H variants have a broader geographic distribution compared to R variants and the H variant has spread among several clonal complexes and contains more genetic variation (Dumitresco et al., 2008). They reported that R variants are mostly among isolates recovered from USA, and belong to CC1 and CC8. However, H variants (all three types) were mainly among isolates recovered from Europe, but were also recovered from USA, Asia, and North Africa (Dumitresco et al., 2008).

The aims of this part of the project were to determine toxin gene profiles of several clinical isolates of PVL-positive methicillin-resistant and methicillin-susceptible *S. aureus* and characterize these isolates by molecular methods, genotypic variation among isolates of *S. aureus* obtained from two sources in the UK, to investigate whether *S. aureus* genotypes of these isolates similar to those genotypes prevalent in the UK, or to the global *S. aureus* population structure. In addition to that it was also to find if the variation occurring within the *lukSF-PV* genes.



**Figure 3. 1. *lukSF-PV* gene sequence variants from PVL-positive MRSA and MSSA strains.** Black horizontal line at the top represents the sequence from USA300 genome, shown nucleotide variation at 12 sites indicated by short vertical lines. At right, the four type variants are shown according to how they are grouped into R, H1, H2 and H3 types (O'Hara et al., 2008).

## **3. 2. Results and Discussion**

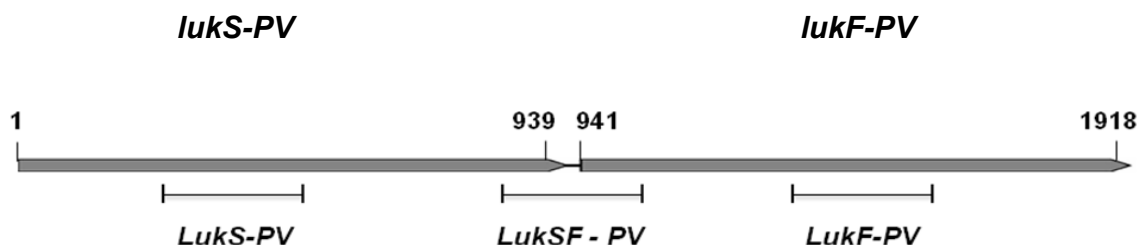
### **3. 2. 1. Clinical Isolates Used In This Study**

For this study 10 clinical isolates of *S. aureus* were obtained from two sources, eight of which were PVL-positive. Five of the clinical isolates were obtained from South Tyneside District General Hospital (STDGH), and the other five were provided by the UK Staphylococcal national reference laboratory in London. Identification and characteristics of these strains are summarised in Table (3. 2). Four of them are MRSA, six are MSSA and they were isolated from different site of infections. Strain 3, one of the MSSA strains was used to initiate the research. This strain was isolated from a fatal case of necrotizing pneumonia in a fourteen year old boy, by Dr R Ellis, Consultant Microbiologist at South Tyneside District General Hospital (STDGH). The patient presented initially with a sore throat and pyrexia, but deteriorated rapidly, developing hypotension, multiple organ failure and purpura fulminans. *S. aureus* was isolated from the tracheal aspirate; the strain was unusual in that it expressed PVL, TSST-1, and SEC; PVL-positive strains of *S. aureus* expressing more than two virulence factors are extremely rare. It has been postulated that the combination of virulence factors in this particular organism was responsible for the fulminating clinical course of the case (Mushtaq et al., 2008). In addition to strain 3, strains 1, 6, 7, 8 and 9 are also MSSA. Strain 6 was another strain isolated from a pneumonia patient. Most other strains used in this study were isolated from skin and soft tissue infections such as boils, nasal swabs and wound swabs as reported by the source of the strains (Table 3. 1). The remaining 4 strains 2, 4, 5 and 10 are MRSA, from that two are are positive for PVL toxin.

### **3. 2. 2. PCR Analysis to Determine the Presence of Toxin Genes**

PCR was carried out to check for the presence of *lukSF-PV* and *mecA* genes, in order to confirm PVL-positive strains and the presence of *mecA* gene in MRSA strains. In addition to that, toxin profile of each isolate was determined using several primers to check for these toxin genes (Table 3. 1). Three of these were used to confirm the presence of *lukSF-PV* genes. They were designed to detect both *lukS-PV* and *lukF-PV* subunits, which are 939 and 978 nucleotides in size, respectively, which are separated by a single thymine nucleotide (Johnson et al., 2004). Agarose gel electrophoresis of PCR experiments are

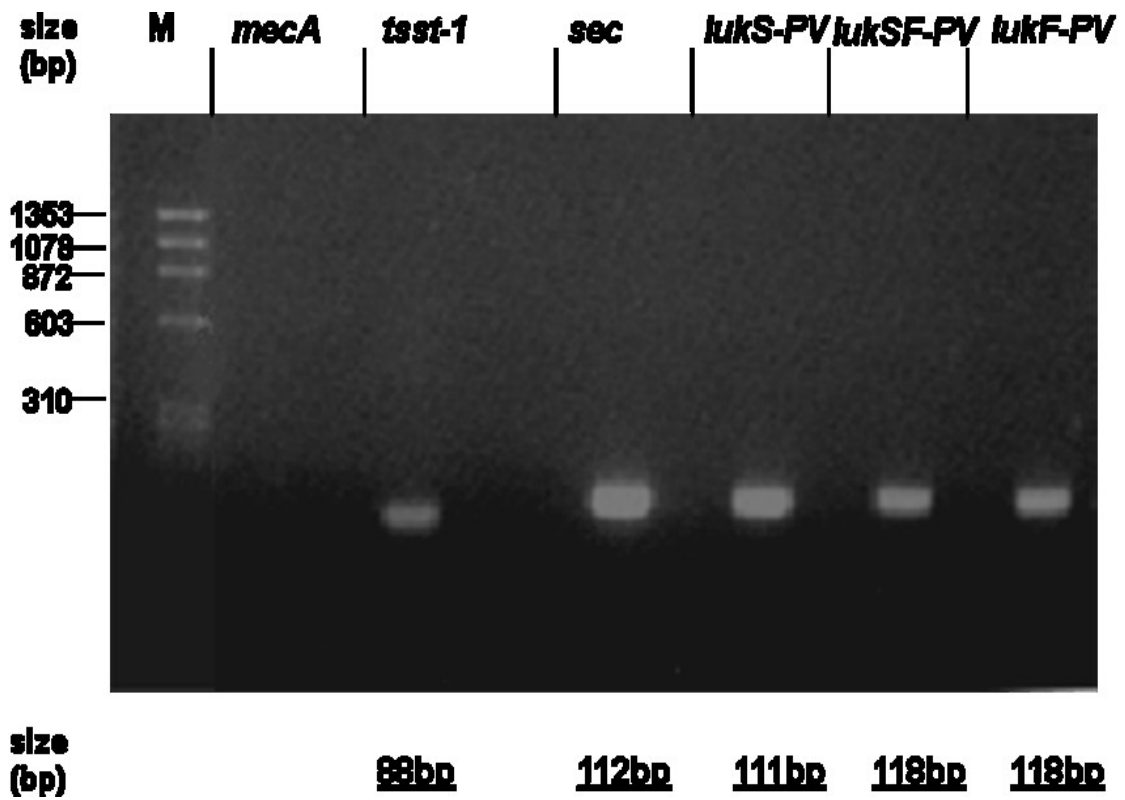
exemplified by those for strain 3, which was confirmed as positive for the *tsst-1*, *sec* and *lukS-PV* and *lukF-PV* genes and negative for *mecA* (Figure 3. 3). Similar PCR analyses were carried out for each strain to confirm their toxin profiles with respect to *lukSF-PV*, and other toxin genes and the presence of the *mecA* gene. The results confirmed that with exception of strain 1 and 5, all strains were positive for PVL genes. Detail of toxin profile of isolates used in this study using PCR is summarized in Table 3. 2. The most noticeable point here was about strain 3, in which in addition to the presence of three toxin genes (*lukSF-PV*, *tsst-1* and *sec* as provided by the source of this isolate Table 3. 2), this strain was also found to be positive for another enterotoxin *sel*. In addition to this strain, *tsst-1* was also found to be present in one of the MRSA strains, strain 5. Two of the isolates strain 4 and 10, were found to be positive for *seq* which they both are MRSA.



**Figure 3. 2. The structure of *lukSF-PV* genes, which consists of two regions *lukS-PV* and *lukF-PV*. Three primer pairs (Nakagwa et al., 2005) to amplify fragments of each subunit and one of both subunits as indicated.**

**Table 3. 1. Primers used to detect the presence of toxin genes and *mecA*.**

<b>Gene name</b>	<b>Primer sequence(5'-3')</b>	<b>Amplicon size</b>	<b>Reference</b>
<i>lukSF-PV</i>	F: ttacacagttaaatatgaagtgaactgga R: agcaaaagcaatgcaattgatg	118bp	Nakagwa et al., 2005.
<i>lukS-PV</i>	F: gcaaggtttatcaattcaagactactt R: gggtcatttgtttgagaccaatat	111bp	Nakagwa et al., 2005.
<i>lukF-PV</i>	F: ttacacagttaaatatgaagtgaactgga R: agcaaaagcaatgcaattgatg	119bp	Nakagwa et al., 2005.
<i>seb</i>	F: atgtaattttgatattcgcagtg R: tgcaggcatcatatcatacca	643bp	Monday et al., 1999
<i>tsst-1</i>	F: tcacagtaactcaaatacatggatt R: tgtgatccgctcattcattgtt	88bp	Deurenberg et al., 2005.
<i>sec</i>	F: ctgcaccaggcgataagttt R: ccattctttggttaaggtggac	112bp	Deurenberg et al., 2005.
<i>sel</i>	F: caccagaatcacaccgctta R: tccccttatcaaaaccgctat	410bp	Holtfreter et al., 2006
<i>seq</i>	F: gaacctgaaaagcttcaagga R: attcgccaacgtaattccac	209bp	Holtfreter et al., 2006
<i>mecA</i>	F: tggatgtggaagtagattgggat R: gccaatcacaggaacacatatgagattag	115bp	Nakagwa et al., 2005.



**Figure 3. 3. Amplification of virulence factors from strain 3.** Agarose gel electrophoresis of PCR amplicons for *tsst-1*, *sec*, *lukS-PV*, *lukF-PV* and *lukSF-PV*. DNA molecular weight marker (M)  $\phi$ X *Hae* III indicated on the left. The size of each product in bp is indicated underneath.

**Table 3. 2. Characteristics of clinical isolates used in this study.**

<b>Strain/ Ho. Des. No.</b>	<b>Isolation site</b>	<b>Present Toxin genes</b>	<b>ST/ Clonal complex</b>	<b>PVL-Type variant</b>
<b>1 (MSSA)/ 684126</b>	Tracheal aspirate	<i>seg</i> and <i>sel</i>	ST-5/ CC5	
<b>2 (MRSA)/ 758503</b>	Umbilical swab	<i>lukSF-PV</i>	ST-88/ CC88	H1
<b>3 (MSSA)/ 681111</b>	Pneumonia	<i>lukSF-PV</i> , <i>tsst-1</i> , <i>sel</i> and <i>sec</i>	ST-776/ CC30	H1
<b>4 (MRSA)/ 830817</b>	Pus swabs	<i>lukSF-PV</i> and <i>seg</i>	ST-8/ CC8	R
<b>5 (MRSA)/ 834924</b>	Wound swabs	<i>tsst-1</i>	ST-217/ CC22	
<b>6 (MSSA)/ 70200329</b>	pneumonia	<i>lukSF-PV</i> and <i>seg</i>	ST-30/ CC30	H2
<b>7 (MSSA)/ 70280140</b>	Nasal swab	<i>lukSF-PV</i> and <i>seg</i>	ST-217/ CC22	H1
<b>8 (MSSA)/ 70320124</b>	Nasal swabs	<i>lukSF-PV</i> and <i>seh</i>	ST-1/ CC1	H2
<b>9 (MSSA)/ 70340454</b>	Boils	<i>lukSF-PV</i> and <i>seb</i>	ST-121/ CC121	R
<b>10 (MRSA)/ 45260142</b>	Boils	<i>lukSF-PV</i> and <i>seg</i>	ST-8/ CC8	R

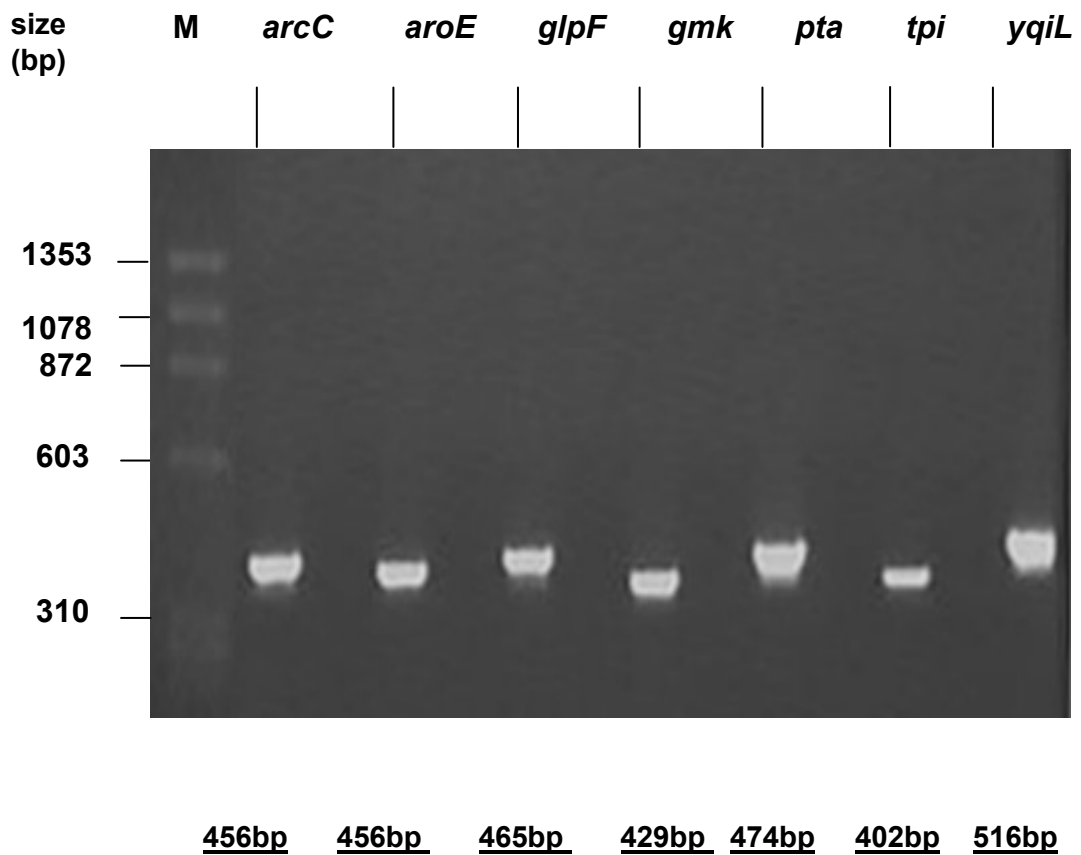
Ho.Des.No. is Hospital designation number, STDGH indicates South Tyneside District General Hospital. UKSRL indicates UK Staphylococcal National Reference Laboratory.

### **3. 2. 3. Determination of *S. aureus* sequence types (ST).**

The ten clinical isolates of *S. aureus* were subjected to MLST using the method of (Enright et al., 2000). Fragments of seven house keeping genes were amplified by PCR using genomic DNA as templates and the primers summarised in Table 3. 3. The PCR products which were between 402-516 bp (Figure 3. 4), were then sequenced with the same primers used for amplification using Big Dye Terminator v3.1 Cycle Sequencing kit (applied biosystems). In MLST analysis a distinct allele is assigned to each of the different sequences of each house keeping gene and the alleles of the seven genes defines the *S. aureus* lineage, resulting in an allelic profile (Deurenberg et al., 2008). Comparison with all *S. aureus* isolates in the database ([www.mlst.net](http://www.mlst.net)) revealed the presence of eight STs (ST-1, ST-5, ST-8, ST-30, ST-88, ST- 121, ST-217 and ST-776). Most of these STs were represented by a single isolate, while each of ST-8 and ST-217 were represented by two isolates. The isolates were then assigned into seven CCs (CC5, CC22, CC88, CC30, CC8, CC1 and CC121) (Table 3. 4).

**Table 3. 3. Sequences of primers used in MLST analysis (Enright et al., 2000).**

<b>Gene</b>	<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Amplicon size</b>
<b>Carbamate kinase (<i>arcC</i>)</b>	<i>arcC</i> -F	ttgattcaccagcgcgtattgtc	456 bp
	<i>arcC</i> -R	aggtatctgcttcaatcagcg	
<b>Shikimate dehydrogenase (<i>aroE</i>)</b>	<i>aroE</i> -F	atcggaatcctatttcacattc	456 bp
	<i>aroE</i> -R	gggtgttgattaataacgatatc	
<b>Glycerol kinase (<i>glpF</i>)</b>	<i>glpF</i> -F	ctaggaactgcaatcttaatcc	465 bp
	<i>glpF</i> -R	tggtaaaatcgcatgtccaattc	
<b>Guanylate kinase (<i>gmk</i>)</b>	<i>gmk</i> -F	atcgttttatcgggaccatc	429 bp
	<i>gmk</i> -R	tcattaactacaacgtaatcgta	
<b>Phosphate acetyltransferase (<i>pta</i>)</b>	<i>pta</i> -F	gttaaaatcgattacctgaagg	474 bp
	<i>pta</i> -R	gaccctttgtgaaaagcttaa	
<b>Triosephosphate isomerase (<i>tpi</i>)</b>	<i>tpi</i> -F	tcgttcattctgaacgtcgtgaa	402 bp
	<i>tpi</i> -R	tttgcaccttctaacaattgtac	
<b>Acetyl coenzyme A acetyltransferase (<i>yqiL</i>)</b>	<i>yqiL</i> -F	cagcatacaggacacctattggc	516 bp
	<i>yqiL</i> -R	cgttgaggaatcgatactggaac	



**Figure 3. 4. Amplification of seven house keeping genes from strain 3.** Agarose gel electrophoresis of PCR products for fragments of seven genes indicated above. DNA molecular weight marker (M)  $\phi$ X *Hae* III with bp sizes indicated on the left. The size of each product in bp is indicated underneath.

**Table 3. 4. MLST Sequence typing and clonal complex of *S. aureus* isolates used in this study.**

Strain	House keeping genes							ST/ Clonal complex
	<i>arcC</i>	<i>aroE</i>	<i>glpF</i>	<i>gmk</i>	<i>pta</i>	<i>tpi</i>	<i>yqiL</i>	
<b>1</b>	1	4	1	4	12	1	10	ST-5/ CC5
<b>2</b>	22	1	14	23	12	4	31	ST-88/ CC88
<b>3</b>	92	2	2	2	2	2	2	ST-776/ CC30
<b>4</b>	3	3	1	1	4	4	3	ST-8/ CC8
<b>5</b>	7	6	1	5	8	5	6	ST-217/ CC22
<b>6</b>	2	2	2	2	6	3	2	ST-30/ CC30
<b>7</b>	7	6	1	5	8	5	6	ST-217/ CC22
<b>8</b>	1	1	1	1	1	1	1	ST-1/ CC1
<b>9</b>	6	5	6	2	7	14	5	ST-121/ CC121
<b>10</b>	3	3	1	1	4	4	3	ST-8/ CC8

This genotyping method is suitable for tracking global pathogen evolution over decades because it focuses on stable housekeeping genes and provides information that places isolates into genetic categories that provide perspective on a global scale (Hall et al., 2009). For example, clonal complex (CC) 5, the most abundantly populated CC in the MLST database, comprises 124 sequence types and CC 8, the next-most-abundant CC, comprises 109 sequence types (Hall et al., 2009).

In this study among 10 isolates, one was found to belong to CC5, and two isolates were belonging to CC8. The remaining 7 isolates were found to belong to 5 CCs, CC88, CC30, CC22, CC1 and CC121. These data are in a good accordance with what Chambers et al., reported for a collection of *S. aureus* isolates between 1961 and 2004 show that 88% of the collected strains can be assigned to one of 11 CCs (CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45 and CC51/ 121) (Chambers et al., 2009). Although most of the CCs resulted in this study, were among the most 11 CCs found worldwide, but none of these isolates have the same ST as EMRSA-15 and EMRSA-16, ST-22 (CC22) and ST-36 (CC30), respectively (Aires-de-Sousa et al., 2008) the most prevalence STs in the UK. However four of the isolates were found to belong to the same CCs of EMRSA-15 and EMRSA-16, which are strain 5 and 7 (CC 22) and strains 3 and 6 (CC30). These two clones emerged in the UK in 1991, spread widely, and account for 93 to 95% of MRSA isolates seen in the UK (Johnson et al., 2005 and Ellington et al., 2010).

This diversity in PVL-positive strains might be due to the fact that the PVL genes are located on several different phages (Rijenders et al., 2009). Furthermore, this might also support the idea that the PVL genes might be transferred from one strain to another with different clonality via phage transduction, as the gene is carried on a prophage (Narita et al., 2001). Data presented here suggested that two of the clinical isolates used belong to ST-8/ CC8, same or identical to sequence type of USA300 isolates (Chambers et al., 2009). Similar isolates have been recovered from a variety of community populations, including children particularly in USA, which is a PVL-positive CA-MRSA. The other STs found here were also found elsewhere, for example ST-5 was previously found in Germany and ST-88 was found in Hong Kong, Taiwan, China, Belgium and Sweden, suggested that such isolates belong to globally disseminated clones (Ellington et al., 2009).

MLST also provides a logical nomenclature for MRSA clones as the ST precisely defines a strain as having a unique and unambiguous allelic profile and identifies those MRSA isolates that have descended from the same recent common ancestor (Enright et al., 2002). For example in this study strain 5 (MRSA) and 7 (MSSA) were both found to have the same ST-217. This may explain that MLST data may address basic questions about the evolutionary and population biology of bacterial species. This is because the expected frequency occurrence of any allelic profile by chance is too low as the product of the observed frequencies of each of the seven alleles in the population is hardly expected. Therefore it is unlikely that unrelated MSSA and MRSA isolates would be assigned to the same ST by chance.

### **3. 2. 4. *lukSF-PV* Gene Polymorphism.**

Although genotypic variation among PVL-positive *S. aureus* strains has been focused during last few years, however only a few studies to date have focused on genetic variation within the *lukSF-PV* genes using different clinical isolates. In a recent study by O'Hara et al., using a collection of 174 PVL-positive *S. aureus* isolates, nucleotide variations were detected at 12 positions of the *lukSF-PV* genes. They found that most of the isolates obtained from Europe harbour what they called H variant, whereas most of the US isolates harbour R variants of the genes (O'Hara et al., 2008). They reported that an arginine at site 176 can extend further than histidine and thus can interact with the 3' end of the LukF-PV protein, stabilizing the LukSF-PV interaction. A more stable interaction between these two subunits of PVL toxin could allow faster and more efficient pore formation, which could be critical to toxicity; this may explain why the R variant has a more severe phenotype than H variant.

To find the type variants of PVL-positive isolates, DNA fragments of *lukSF-PV* genes were amplified by PCR of genomic DNA and sequenced. Six primer pairs, which were designed to cover the areas where nucleotide polymorphism had previously been reported (Figure 3. 5) were used (O'Hara et al., 2008). Their amplicon sizes and sequences are summarized in Table 3. 5. PCR products were then purified using Amersham Biosciences GFX purification kit, and directly sequenced in both the forward and reverse directions, with the set of synthetic oligonucleotide primers in

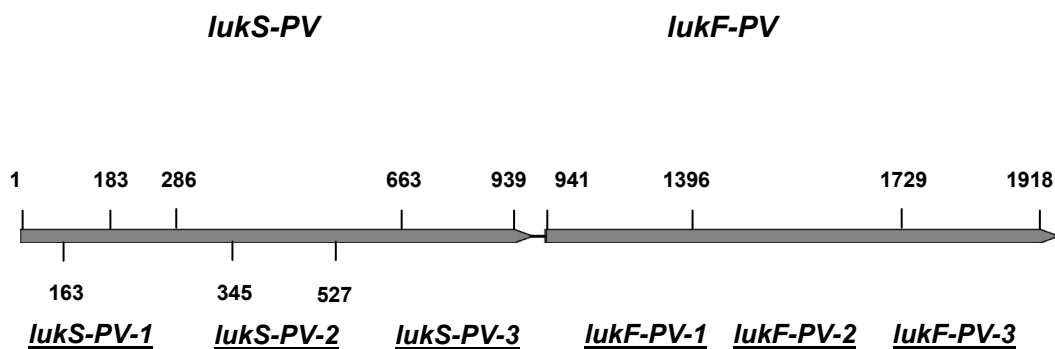
Table 3. 5 using Big Dye Terminator v3.1 Cycle Sequencing kit (applied biosystems).

Three parts of each subunit were sequenced to cover all the areas where polymorphism occurs. PVL genes from all eight PVL positive strains were sequenced; the data was then analyzed using multiple sequence alignment software (<http://bioinfo.genotoul.fr/multalin/multalin.html>). This is an extension of pair wise alignment, to incorporate more than two sequences at a time. The data identifies that these eight clinical isolates show polymorphisms at only five positions (345, 527, 663, 1396 and 1729) they did not show any polymorphism at other seven sites (Figure 3. 6). This might be due to low number of isolates used here. Therefore the diagram (Figure 3. 6) presented here is only a part of sequenced DNA, where the polymorphisms occurred. Data showed that three isolates were identified as R type variants and the remaining five were identified as H variants. Among the isolates of H variants three were found to belong to H1 variants, two belong to H2 variants and none of them were found to belong to H3 variants (Table 3. 6).

With one exception, all of the polymorphisms were synonymous (phenotypically silent). The exception was SNPs at position 527 where the polymorphism was non-synonymous as it resulted in change of encoded amino acid histidine (H) to arginine (R) at amino acid 176. By using the type variant classification tool of the main R and H variants produced by O'Hara et al., (2008), it was possible to identify the PVL-positive isolates by checking nucleotide changes at variable positions as shown in Figure 3. 1. On the basis of nucleotide variation at position 527, G results in an arginine (R) and A resulting in a histidine (H) at amino acid 176. Table (3. 6) shows the polymorphic sites in each strain with their PVL type variants.

**Table 3. 5. Primers used for sequencing *lukSF-PV* gene subunits.**

<b>Primer name</b>	<b>Primer sequence(5'-3')</b>	<b>Amplicon size in bp (amplified region)</b>
<i>lukS-PV-1</i>	F: tggcaaaaaagactattagctgc. R: ttgtattggaaaggccacctcat.	319 (2-321)
<i>lukS-PV-2</i>	F: aaagcaatgaggtggcctttcc. R: tggatcatgtccagacattttacc.	318 (291-609)
<i>lukS-PV-3</i>	F: tagtggccatcaacaggaggta. R: ttgaaaccactgtgtactaatggg.	267(437-704)
<i>lukF-PV-1</i>	F: tgctggaaacatttattctggc. R: tatgtgcttcaacatccaacc	344 (1176-1520)
<i>lukF-PV-2</i>	F: tcaggtggaggaatgggtc. R: ttctggattgaagtacctctgg.	287 (1397-1684)
<i>lukF-PV-3</i>	F: agacaaagcaactaaatgctggac. R: ctgtatgatttccaatcaactc.	271(1600-1871)



**Figure 3. 5. DNA polymorphic sites of the *lukSF-PV* gene.** The gene contains 2 subunits *lukS-PV* (nucleotide 1- 939) and *lukF-PV* (941- 1918). Numbers above and below the gene structure indicate the positions of nucleotide polymorphisms within PVL-positive strain variants. Six primers to include all areas of the DNA where polymorphism occurs are indicated below the diagram.

Data showed that three out of eight were identified as R type variants (Table 3. 6), in which two isolates 4 and 10 were similar to that of CA-MRSA USA300. Both of these were isolated from superficial skin infections as they were isolated from pus swabs and boils respectively. Strain 9 also had a PVL sequence very similar to that of USA 300 a part of at one position 1729, in which strain nine had A instead of G in strain 4 and 10 as shown in (Table 3. 6). This strain is MSSA, which is not in a good accordance with what O'Hara group reported; as they found that 91.3 % of MRSA were R variants and the same ratio of MSSA have the H variant.

Similar to our results R variant was seen among MSSA isolates elsewhere by Enany et al., (2010) they also found that the identification of PVL sequence of the H2 variant were mostly found in CC30. The remaining five isolates in this study were identified to harbour the H variant. Strain 6 in CC30, and strain 8 in CC1 were found to harbour the H2 variants, and strains 2, 3 and 7, were all identified to harbour the H1 variants. These data showed that five of the H variant isolates belong to four different CCs (CC88, CC30, CC22 and CC1), while the three R variant isolates belong to only 2 CCs (CC8 and CC121). These data are consistent with data presented by Dumitresco et al., showing that H variants have spread among more different CCs, and contains more genetic variation (Dumetresco et al., 2008).

345

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PVS-2 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
PVS-3 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
PVS-6 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
PVS-7 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
PVS-8 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
PVS-9 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
PVS-10 ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA
Consensus ACAGATCATATAAAGCAATGAGGTGGCC TTCCCAATACAAAACCAATGACCCCAATGGTAGATTTAATAAATTATCTA

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527

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PVS-2 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
PVS-3 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
PVS-6 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
PVS-7 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
PVS-8 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
PVS-9 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
PVS-10 AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC
Consensus AACAGGAGGTAATGGTTCATTTAATTATTCAAAACCAATTAGTTATAATCAACAAACTATATCAGTGAAGTAGAACATC

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663

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PVS-2 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
PVS-3 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
PVS-6 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
PVS-7 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
PVS-8 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
PVS-9 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
PVS-10 ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC
Consensus ATATAAACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAAATGAATTACCCCATTAGTACAC

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1396

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PVF-2 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-3 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-7 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-8 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-6 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-9 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-4 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
PVF-10 ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG
Consensus ATCTCTAACGGCTTGCAGGTGGAGGTAATGGTTCAAAATCTTTTTCAGAGACAAATTAACATATAAACAGGAAGCTATAG

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1729

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PVF-2 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-3 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-7 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-6 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-8 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-9 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-4 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
PVF-10 GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT
Consensus GAAACCAAAACGCTGCAAAAAATCAAAATTAAGTACTTATCAAGAGAAATGGATAGATATACAACCTTTTGGAAAT

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Figure 3. 6. Alignment of *lukSF-PV* gene sequences. Multalin analysis of *lukSF-PV* gene sequence (<http://bioinfo.genotoul.fr/multalin/multalin.html>), PVL gene subunit names and lab numbers are indicated on the left and nucleotide numbers 345, 527, 663, 1396, and 1729 above the nucleotides are indicated to positions where polymorphism occurs in this study.

**Table 3. 6. PVL type variants according to nucleotide variations at several positions in the *lukSF-PV* genes.**

Strain	Polymorphic sites to determine PVL type variants									Type variant
	163	183	286	345	470	527	663	1396	1729	
<b>2</b>	G	T	C	C	T	A	G	G	A	<b>H1</b>
<b>3</b>	G	T	C	C	T	A	G	G	A	<b>H1</b>
<b>4</b>	G	T	C	C	T	G	T	A	G	<b>R</b>
<b>6</b>	G	T	C	C	T	A	G	A	A	<b>H2</b>
<b>7</b>	G	T	C	C	T	A	G	G	A	<b>H1</b>
<b>8</b>	G	T	C	T	T	A	G	A	A	<b>H2</b>
<b>9</b>	G	T	C	C	T	G	T	A	A	<b>R</b>
<b>10</b>	G	T	C	C	T	G	T	A	G	<b>R</b>

**CHAPTER 4**  
**The Effect of Antibiotics on Steady-state mRNA**  
**levels**

## 4. 1. Introduction

The clinical efficacy of antibiotics is not only determined by their respective bactericidal or bacteriostatic activity, but also by their action on bacterial virulence factor release, especially when present at suboptimal concentrations. In principle antibiotics can both up and down modulate the synthesis and release of virulence factors (Bernardo et al., 2004).

One crucial feature of almost all bacterial infections is the need for the invading pathogen to reach a critical cell population density sufficient to overcome host defenses and establish the infection (Williams et al., 2000). Since microbial adherence is the initial step of many infectious processes, the ability of antibiotics to affect this property may be an important criterion in selecting an antibiotic for therapy (Shibl, 1985). Thus, antibiotic induced modulation of virulence factors might lead to either aggravation or attenuation of the disease (Gemmell, 1991; Lowy, 1998). Subinhibitory concentrations of certain antibiotics, especially those that inhibit bacterial protein biosynthesis, may have an impact on host-pathogen interactions, such as the adherence of bacteria to epithelial cells (Shibl, 1987).

The effect of antibiotics on global bacterial transcription is dose dependent. At low concentration, the number of transcripts modulated by the antibiotic is low, increasing until around 5% of total bacterial transcripts at concentrations close to those that inhibit growth. The number of transcripts modulated decreases at higher concentrations. This curve reflects the different effects of antibiotics on bacterial transcriptome: at low concentration antibiotics might act as signaling molecules modulating expression of specific genes. Upon increase in concentration, stress responses sum up to the panel of regulated genes and at high, inhibitory concentrations, the observed changes mainly deal with the mechanism of action of the antibiotic and cell growth inhibition (death) (Fajardo et al., 2008). Furthermore each antibiotic triggers a specific response, and these responses may have adaptive values. The fact that a number of antimicrobials not used for therapy (like lantibiotics) are involved in quorum sensing and that classical quorum sensing autoinducers have antimicrobial activity further support this dual role of antibiotics (Fajardo et al., 2008).

The expression of virulence function such as toxins, adhesins and biofilm formation in *S. aureus* is affected by exposure to sub-MIC of antibiotics. Sub-MIC of certain antibiotics, in particular, compounds whose primary mode of action is DNA damage, are known to enhance mutation rates in bacteria. This is usually the result of transcriptional changes responsible for DNA repair and preservation of the integrity of the genome, such as the SOS and methyl mismatch repair (MMR) pathways. DNA polymerase of the SOS system lack intrinsic proof reading activity, which leads to mutations when DNA replication bypasses lesions or errors (Mesak et al., 2008). DNA-damage triggers SOS-response that involves change in the expression of several bacterial genes. Antibiotics that interfere with DNA metabolism such as quinolones trigger this response. It has been described that some beta-lactams also trigger SOS-response (Miller et al., 2004), although their primary target is not bacterial DNA. Besides SOS-response, other types of stress responses are modulate by antibiotics. This modulation does not necessarily imply induction of certain genes, as could be predicted. For instance heat shock genes are induced by gentamycin and repressed by chloamphenicol in *Bacillus subtilis* (Fajardo et al., 2008).

Ohlsen et al. studied the effect of 31 antibiotics at sub-MIC levels (one-fourth MIC) on the expression of *hla* (encoding  $\alpha$ -hemolysin), the most significant observation was almost complete inhibition of *hla* gene expression by clindamycin, strong induction of the gene by sub-inhibitory concentration of  $\beta$ -lactam antibiotics and glycopeptide antibiotics had no effect. The authors suggested that the effect of certain antibiotics on virulence properties may be relevant for the clinical management of *S. aureus* infection (Ohlesen et al., 1998). In a study, using Western blotting and proteomic approaches, it has been found that for the management of toxic *S. aureus* infections,  $\beta$ -lactam antibiotics are unfavorable, because even sub-inhibitory concentration of these antibiotics led to an induce toxin gene expression through stimulation of exoprotein synthesis (Koszczol et al., 2006). In another study, using Western blotting for protein assay and Northern blotting for RNA assay, Stevenson et al., (2007), also established that sub-MIC of  $\beta$ -lactam antibiotics may fail in infections with toxin-producing organisms due to their effect on the cell wall, in contrast to protein synthesis inhibitors, they fail to suppress toxin production (Stevenson et al., 2007). Vancomycin, the first glycopeptide antibiotic, was isolated in the mid- 1950s and introduced into clinical practice in 1958. Due to an increasing number of infections caused by multi-

resistant (MRSA) world wide, vancomycin has been the drug of choice for treatment of staphylococcal nosocomial infections for the last 20 years (Aires de Sausa et al., 2004).

Protein synthesis inhibitor antibiotics such as clindamycin and linezolid have been recommended for the treatment of some *S. aureus* infections, however, neither linezolid nor clindamycin is expected to be effective for the treatment of *S. aureus* infections in neutropenic patients, which call for bactericidal rather than bacteriostatic antibiotics. In contrast to linezolid, quinopristin/ dalforpristin exerts bactericidal activity against *S. aureus*, on the other hand, comprehensive investigation of toxin production using proteomic approaches showed that while sub-inhibitory concentration of quinopristin dalforpristin efficiently impedes the secretion of most exotoxins by *S. aureus*, the release of a few exoproteins increased (Koszczol et al., 2006). It has been reported that exposure to linezolid at sub-MIC levels led to significant decrease in *spa* gene expression, which caused an increase in susceptibility of *S. aureus* phagocytosis by human neutrophils (Gemmel et al., 2002). Similarly Bernardo et al., (2002) studied the effect of sub-MIC levels of linezolid on several virulence factors; results showed that the expression of virulence factors in *S. aureus* is especially sensitive to the inhibition of protein synthesis by linezolid.

The effect of these antibiotics on other bacteria has been studied too, for example macrolides and clindamycin inhibit biofilm formation in *Pseudomonas aeruginosa*. Sub-inhibitory concentration of mupirocin show important biological effect against *P. aeruginosa* and *P. mirabilis* by suppressing flagellin expression and flagellin formation (Horii et al., 2003). Miller et al. reported a mechanistically novel type of defense mechanism in *E. coli* that uses a bacterial two-component signal transduction system to induce the SOS response and temporally inhibit cell division during exposure to  $\beta$ -lactam antibiotics, consequently limiting the bactericidal effects of these drugs (Miller et al., 2004). Goh et al., reported that sub-MIC of different antibiotics activate or repress a wide variety of promoters in *Salmonella typhimurium*, similar effects have been obtained with *E. coli* and *Pseudomonas aeruginosa*. For example erythromycin and rifampicin, inhibitors of translation and transcription respectively, modulate (activate or repress) transcription of approximately 5%

of genes in *S. typhimurium* and *E. coli*, confirming that sub-MIC of antibiotics with different chemical structures and mode of action exerts effects on bacterial transcription (Goh et al., 2002).

The consequences of sub-MIC concentrations of antibiotics on a range of global responses in the cell have been identified, thus providing important information on metabolic interaction associated with the target node. Perhaps more importantly, many small molecule inhibitors identified thus far have been shown to exhibit contrasting properties when tested at low concentrations; this is the phenomenon of hormesis (Davies et al., 2006). Table (4. 1) provides different physiological effects of sub-MIC of several antibiotics in different bacteria.

It has been suggested that bacterial cell responds to inhibition of peptidoglycan biosynthesis in general, and the genes comprise a cell-wall-stress stimulon. A stimulon refers to the entire set of genes responding together to an environmental stimulus, the environmental stimulus being a cell-wall-active antibiotic in this case (Utaiida et al., 2003). The pattern of altered gene expression triggered by cell wall active antibiotics forms the cell wall stimulon. A core global *S. aureus* cell wall stress stimulon, consisting of 15 common genes induced by cell wall-active antibiotics in different studies, has been proposed. Whether these differentially regulated genes have a beneficial, a deleterious or no effect on survival under such adverse conditions, is unknown in most cases. It is most likely not the single genes but the concerted action of multiple genes that may be relevant for antibiotic resistance. The high number of differentially regulated genes shows that they may form a network, and/or be controlled by common regulators (MCcallum et al., 2006).

**Table 4. 1. Effect of sub-MIC of Several Antibiotics on *S. aureus* and other Bacteria (Davies et al., 2006).**

<b>Antibiotics</b>	<b>Organism</b>	<b>Effect</b>
<b>Tetracyclin</b>	<i>Bacteroides sp.</i> <i>S. epidermidid</i> <i>Streptococcus sp</i>	Enhanced gene transfer (conjugation of antibiotic resistance genes). Stimulation of bacterial adhesion. Changes in exoprotein secretion
<b>β-lactams</b>	<i>S. sp.</i>	Decreased biofilm formation.
<b>Ceruline</b>	<i>S. aureus</i>	Inhibition of protein secretion.
<b>Aminoglycoside</b>	<i>P. aeruginosa</i> <i>S. pneumoniae</i>	Increased biofilm formation. Increased mutation frequency.
<b>Fluroquinolones</b>	<i>E. coli</i>  <i>S. aureus</i> <i>S. pneumoniae</i> <i>Mycobacterium fortuitum</i>	Reduced hemolytic activity. Induction of colicin synthesis. Increased adhesion. Increased mutation frequency. Increased mutation frequency.
<b>Macrolides</b>	<i>Mycobacterium avium</i> <i>P. aeruginosa</i>	Decreased biofilm formation. Inhibition of quorum sensing (virulence suppression).
<b>Lincosamide</b>	<i>Bacillus fragilis</i>  <i>S. aureus</i>	Altered cell morphology and increased DNA fragmentation. Changes in exoprotein expression.
<b>Oxazolidinone</b>	<i>S. aureus</i>	Decreased secretion of virulence factors.
<b>Mupirocin</b>	<i>P. aeruginosa</i>	Reduced biofilm formation. Reduced flagellin expression.
<b>Rifampicin</b>	<i>E. coli</i>	Reduced toxin secretion.

In this study RT-qPCR was used as a method of choice to study the effect of several antibiotics on steady state levels of mRNA of selected genes. Several time periods (4 hrs, 8 hrs, 10 hrs and 24 hrs) post antibiotic addition were used for the similar studies by other researchers (Ohelsen et al., 1998, Bernardo et al., 2004 and Stevenson et al., 2007). Here cells were exposed to antibiotics for one and five hrs, so that the expression analysis in early and late exponential phase would be studied. Low *agr* activity at the early exponential phase allows for the expression of colonization factors, however, high *agr* activity in late stages of growth causes the expression of virulence factors (Otto, 2004).

The aims of this chapter were to determine the MIC of several antibiotics in 10 clinical isolates of PVL-positive *S. aureus* strains. It was also to establish a growth curve of the bacterial cells with and without adding sub-MIC amounts of antibiotics, to study their effect on growth at different exponential phases. In addition to these the main objective of this part of the study was to investigate the effect of sub-MIC of clindamycin, oxacillin, linezolid and vancomycin on *lukSF-PV* and *spa* virulence factor steady state mRNA levels in clinical isolates used.

## 4. 2. Results and Discussion

Experiments were designed to study the effect of sub-MIC of four antibiotics, clindamycin, oxacillin, linezolid and vancomycin, on the transcription of selected virulence factor genes. *LukSF-PV* and *spa* genes were studied in all ten clinical isolates and *tsst-1* and *sec* toxin genes were also studied in strain 3. It was known that strain 3 is positive for these two toxin genes in addition to *LukSF-PV* and *spa* genes. The experiments determined the steady-state levels of mRNA for each gene in the presence and absence of antibiotics. As pre-requisites for these experiments, it was necessary to determine the MIC for each antibiotic for all used strains and the growth curve characteristics of each strain in the presence and absence of antibiotics. In order to illustrate these processes, detailed experimental data is presented for strain 3 only; the data for all strains is then presented in summary tables.

### 4. 2. 1. MIC Determination

“Minimum inhibitory concentration (MIC) is defined as the lowest concentration (maximum dilution) of antimicrobial that will inhibit the visible growth of microorganisms after overnight incubation” (Andrew et al., 2001). MIC determination was carried out for these antibiotics using the macro broth dilution method following the British Society for Antimicrobial Chemotherapy (BSAC) standard methods (Andrew et al., 2001). Iso Sensitest Broth (ISB) media was used which was prepared following manufactures instructions. Overall MIC determination showed different oxacillin MICs (range 0.25-16.0mg/L) were observed amongst the strains tested in this study. Within MRSA isolates the highest MIC (16mg/L) was seen in strain 2, the other MRSA strains (strain 4, 5 and 10) had lower MICs of 8.0mg/L. These are within the defined range of MICs for MRSA strains (Andrew et al., 2001). MSSA isolates were found to have a very narrow MIC range of 0.25-0.5mg/L (Table 4. 2). The clindamycin MICs showed that all tested isolates were in a very narrow range (0.25-0.5mg/L) and all are susceptible to clindamycin. Linezolid and vancomycin MICs were also in the susceptible ranges of 1.0-4.0mg/L (Andrew et al., 2001). No vancomycin or linezolid resistance was found among the isolates used in this study according to BSAC brake points.

**Table 4. 2. MIC of Clindamycin, Oxacillin, Linezolid and Vancomycin for each strain.**

Strains	MIC in mg/L			
	clindamycin	oxacillin	linezolid	Vancomycin
<b>1.</b>	0.5	0.5	2.0	4.0
<b>2.</b>	0.5	16.0	4.0	4.0
<b>3.</b>	0.25	0.25	2.0	2.0
<b>4.</b>	0.5	8.0	2.0	2.0
<b>5.</b>	0.5	8.0	4.0	2.0
<b>6.</b>	0.25	0.25	2.0	1.0
<b>7.</b>	0.5	0.5	2.0	1.0
<b>8.</b>	0.25	0.5	4.0	2.0
<b>9.</b>	0.25	0.5	4.0	2.0
<b>10.</b>	0.5	8.0	2.0	2.0

#### 4. 2. 2. Effects of sub-MIC of Antibiotics on Growth

Growth curves were determined for all 10 clinical isolates used in this study using absorbance readings at 600nm. Figure (4. 1) shows a representative standard growth curve that was established for strain 3 using viable cell count and absorbance readings. BHI broth media was inoculated with an overnight culture to an A600 of 0.03. The cultures were incubated at 37°C with gently shaking (150 rpm); growth was monitored for each of three replicates for about 15 hrs. Viable cells (cfu/ ml) were estimated using the Miles and Misra technique, in which 10µl of the sample (diluted sample when required) was placed onto the surface of BHI agar plates and incubated at 37°C overnight.

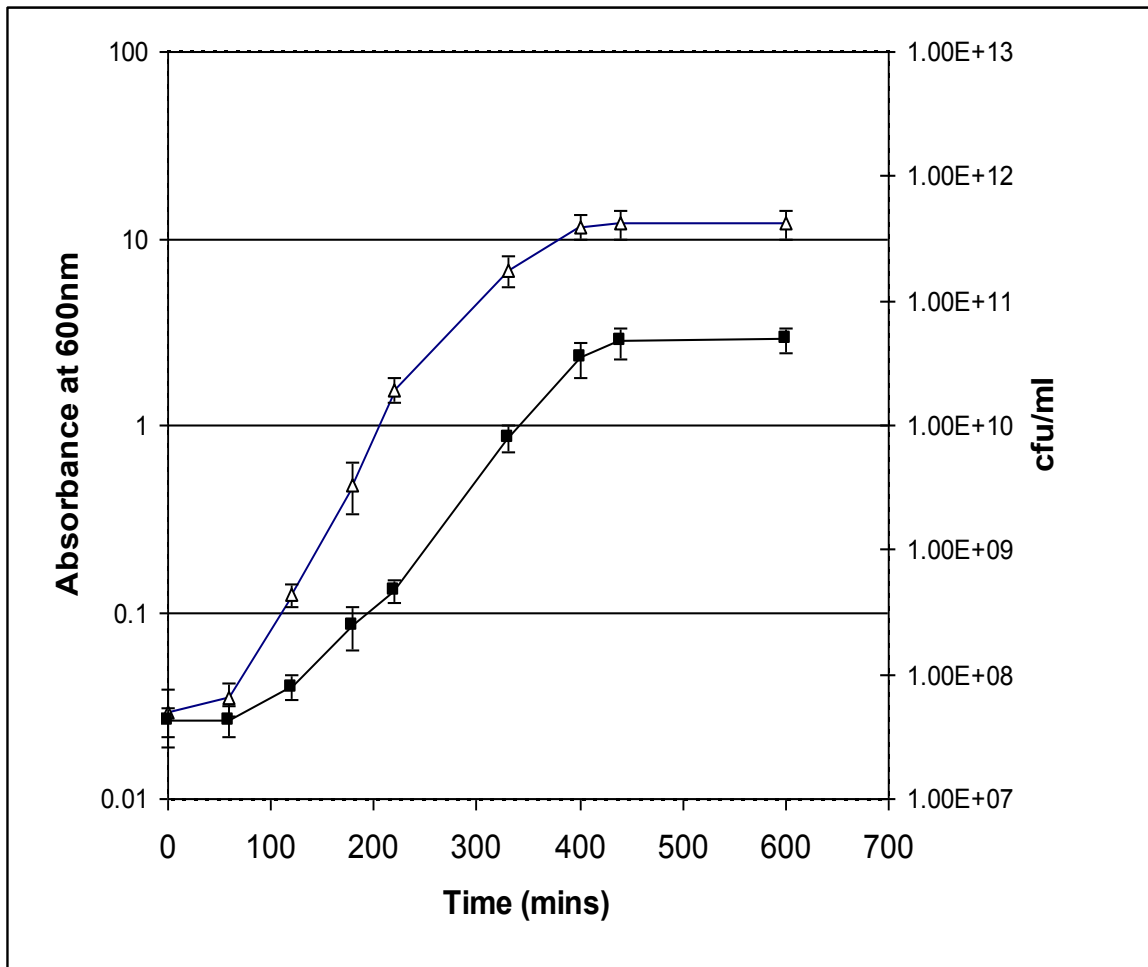
Isolates were grown under the standard conditions, established above, for 180mins when they reached an absorbance at 600nm of 0.5-0.6 (Figure 4. 2); at this point the cultures were divided into two flasks (50ml each), and antibiotic was added to one flask. The cells were allowed to grow under the same conditions. Samples were taken at several time intervals to monitor growth and at one hour and five hours post antibiotic additions for toxin gene expression analysis. Antibiotics were added at a final concentration of one fourth of MIC (sub-MIC), this amount was multiplied by five for clindamycin, linezolid and vancomycin (Table 4.3). This amount of antibiotic was used to compensate the fact that the inoculum size at the time of adding antibiotics ( $2.5 \times 10^8$ ) was greater than the inoculum size of cells when growth started ( $5 \times 10^7$ ). Oxacillin was added without multiplying the value, as previously used by (Stevens et al., 2007), so that the antibiotic would not inhibit growth.

Antibiotics were used at sub-MIC levels, allowing the cells to grow and PVL toxin is associated with intense necrosis *in vivo*, possibly leading to poor antibiotic diffusion and sub optimal concentrations at sites of infections (Cars et al., 1990 cited by Dumerisco et al., 2007). Antibiotics were added at early exponential phase of the growth, so that the bacterial cells are active and at the same physiological state. Growth curves following the addition of these concentrations of antibiotics were established for each strain and the data for strain 3 is shown in Figure (4. 2). During the first hour some slowing of the growth was noticed in samples exposed to antibiotics. Samples were taken at the one hour time point will therefore represent the

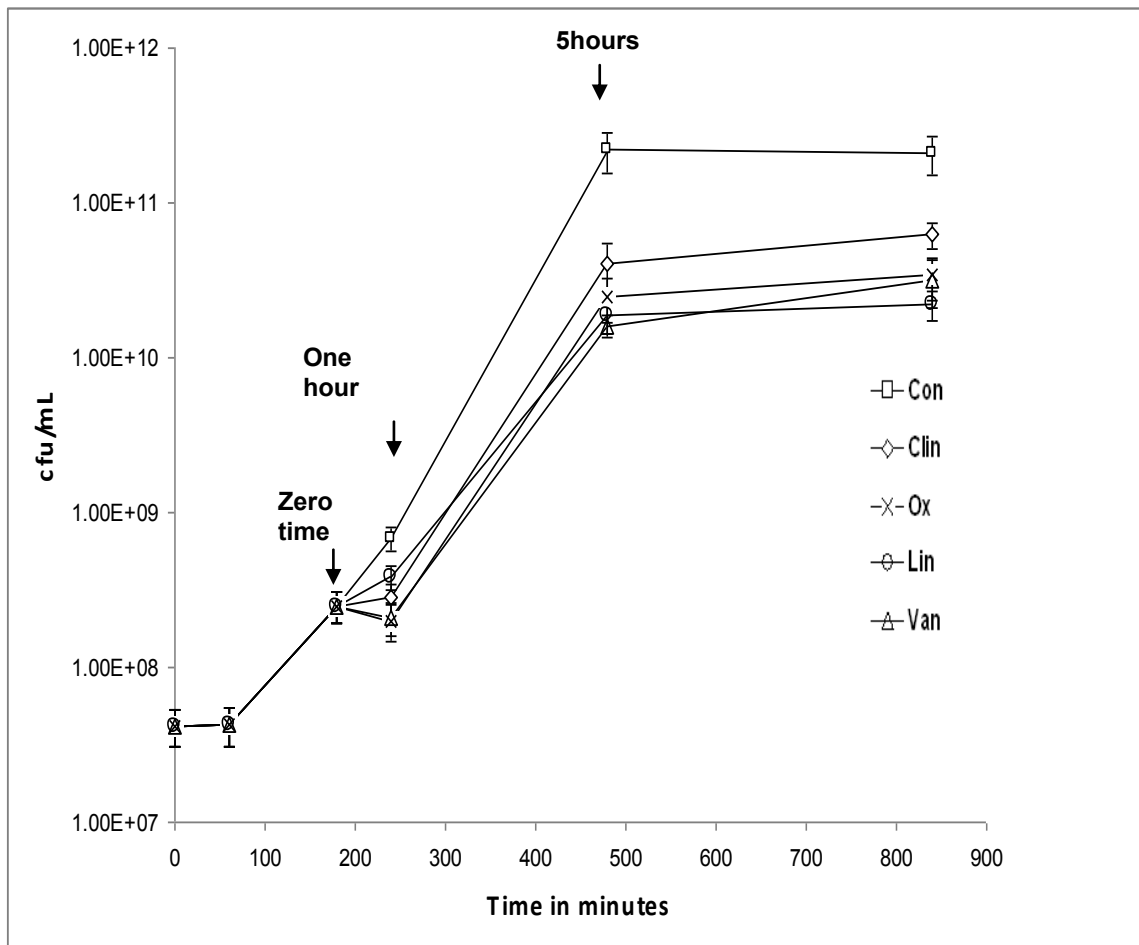
effect of growth inhibition by antibiotic treatment. The cells in each case then entered an exponential phase or even post exponential phase, from which samples were taken at five hours post antibiotic additions, for relative expression purposes using qPCR, as well as for proteomic studies. This is because the production of staphylococcal exoproteins is regulated in a coordinated, growth phase dependent manner, occurring preferentially during the post-exponential phase of growth (Bernardo et al., 2004).

**Table 4.3. Antibiotic concentrations added to each strain of *S. aureus* used in this study.**

<b>Strains</b>	<b>Antibiotic concentration in mg/L</b>			
	<b>clindamycin</b>	<b>oxacillin</b>	<b>linezolid</b>	<b>Vancomycin</b>
<b>1.</b>	0.625	0.125	2.5	5.0
<b>2.</b>	0.625	4.0	5.0	5.0
<b>3.</b>	0.312	0.0625	2.5	2.5
<b>4.</b>	0.625	2.0	2.5	2.5
<b>5.</b>	0.625	2.0	5.0	2.5
<b>6.</b>	0.312	0.0625	2.5	1.25
<b>7.</b>	0.625	0.125	2.5	1.25
<b>8.</b>	0.312	0.125	5.0	2.5
<b>9.</b>	0.312	0.125	5.0	2.5
<b>10.</b>	0.625	2.0	2.5	2.5



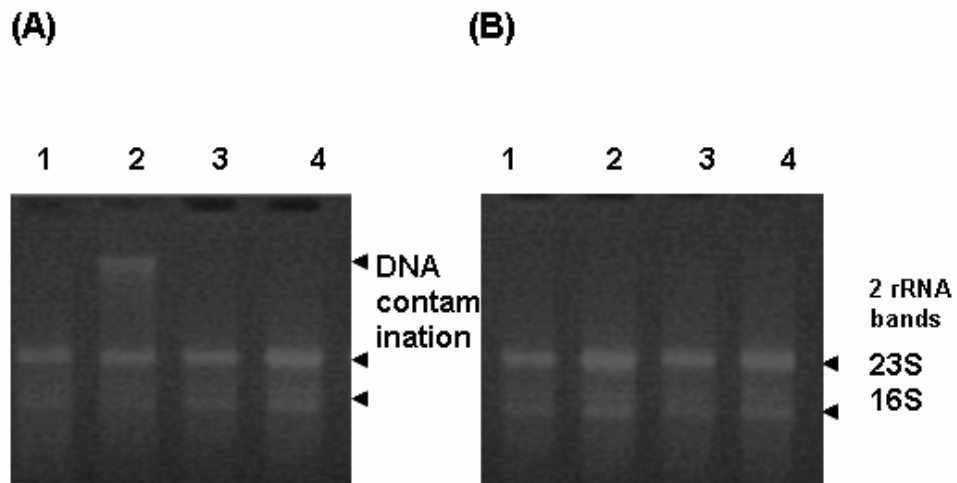
**Figure 4. 1. A standard growth curve established for strain 3. Absorbance (A 600nm Δ) and viable cell count (cfu/ml ■).** Cultures were started with inoculum size of  $5 \times 10^7$  cfu/ ml with A600 was 0.025-0.035, cells were allowed to grow for about 15 hrs. Samples were taken at several time points; absorbance and cfu/ml were taken each time. At 180 minutes when the absorbance was 0.48 and number of viable cells was  $2.5 \times 10^8$  cfu/ml. Values are means of three replicates, and indicated by showing stander deviation error bars.



**Figure 4. 2. Growth curves for strain 3 in the presence and absence of antibiotics.** The number of viable cells was determined in cultures incubated in the presence and absence of antibiotics. No antibiotic (control □), clindamycin (◇), oxacillin (X), with linezolid (○) and with vancomycin (Δ). Values are means of three experiments, indicated by stander deviation error bars. Cultures were started with inoculum size of  $5 \times 10^7$  cfu/ ml. Antibiotics were added at early exponential phase (zero time), and samples were taken at one hour and 5hours periods as indicated.

### 4. 2. 3. RNA Isolation and Reverse Transcription to cDNA

RNA extraction was carried out using RNeasy mini kit (Qiagen) with RNA protect bacteria reagent (Qiagen) and also DNase treatment. RNA samples were analyzed for concentration, purity and integrity using a NanoDrop1000 spectrophotometer (Labtech) based on absorbance readings at 260nm. RNA samples were analyzed for integrity and to check for DNA contamination by separating on RNA agarose mini gels (1.2 % agarose). Some samples showed DNA contamination (e.g. lane 2 of Figure 4. 3A). These samples were subjected to further DNase treatment, as any amount of DNA would affect the PCR results. First strand cDNA was synthesized from 1 $\mu$ g of total RNA by reverse transcription using Superscript III transcriptase (Invitrogen) in a total volume of 20 $\mu$ l. Standard PCR was carried out using *spa* primers, which confirmed that cDNA synthesis was successful and could be used in qPCR experiments.



**Figure 4. 3. RNA agarose gel analysis.** Show two rRNA bands with DNA contamination in lane 2 (A), and without any DNA contamination following DNase treatment (B).

#### **4. 2. 4. Primer design and testing**

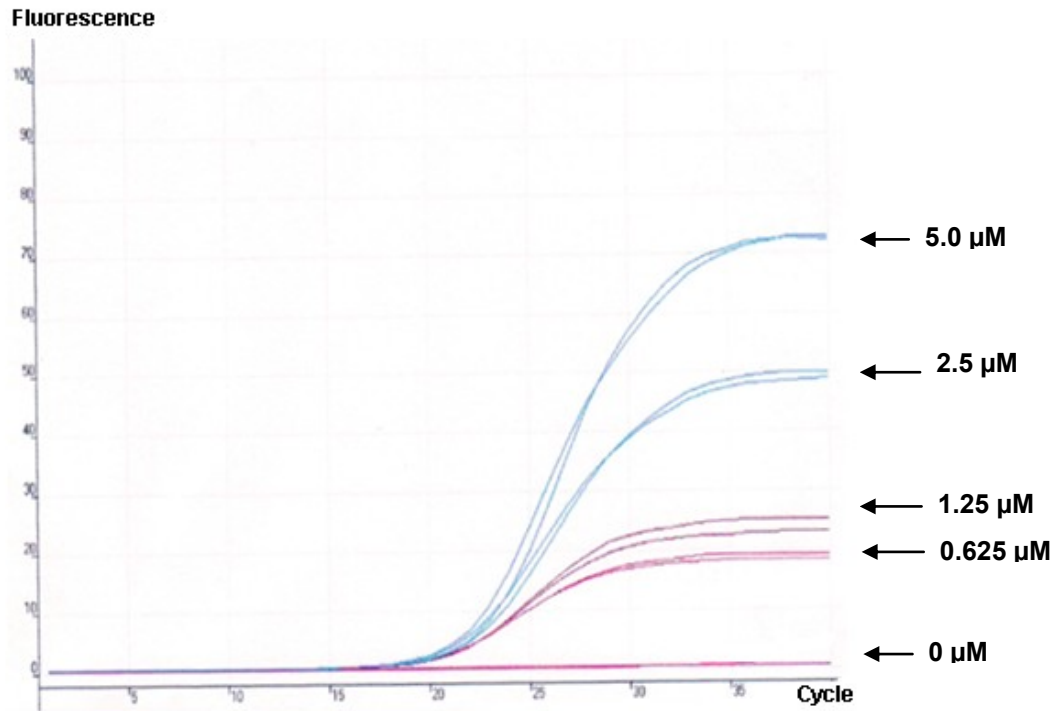
A number of primer pairs were used in qPCR experiments. Several considerations were taken into account to design primers: most were between 15-25 base pairs long, with a G/C content of around 50%, and avoiding sequence complementary at 3' ends to prevent primer dimers as shown in Table (4. 4). Primers were chosen with one or more G or C residues at the 3' end to increase binding efficiency due to stronger hydrogen bonds of G/ C residues (Nolan et al., 2006). The specificity of each primer was analysed by standard PCR and agarose gel electrophoresis, to establish that they gave PCR products of the expected sizes. Three of these primers *gyrA*, *nuc* and *rho* (DNA gyrase subunit A, Nuclease and Transcription termination factor respectively) were used to represent fragments of three internal control genes that had previously been used for a similar purpose (Theis et al., 2007). qPCR experiments were used to test these internal gene primers and their stability in cultures with and without adding antibiotics. *gyrA* was found to be the most reliable primer as it showed stable in cultures with and without antibiotics, therefore this gene was chosen as a control reference gene to use in qPCR experiments.

#### **4. 2. 5. Fluorescent dye SYTO9**

It is possible to use a variety of fluorescent dyes in qPCR experiments; the common characteristic of these dyes is that they do not fluoresce in the presence of single strand DNA. But as the PCR reaction progresses, double stranded products are generated the fluorescent dye intercalates into the double stranded DNA and begins to fluoresce (Bustin et al., 2000). An optimization experiment was carried out for SYTO9 dye to monitor DNA amplification in qPCR using *spa* as primer pairs and the same amount of cDNA. Increasing dye concentration produced higher fluorescent levels without inhibition of the PCR reaction (Figure 4. 4).

**Table 4. 4. Primers used in qPCR experiments**

<b>Gene</b>	<b>Accession number</b>	<b>Primer sequence forward (F) and reverse (R).</b>	<b>Amplicon size</b>	<b>Reference.</b>
<i>nuc</i>	EF529608	F: tgtagttcaagtctaagtagctcagcaa R: tgcactatatactgttgatcttcagaa	96bp	Alarcon et al., 2005.
<i>rho</i>	AF333962	F: cgacgttcagacaatttacg R: gaaatattgaagcgggtgg	145bp	This study.
<i>gyrA</i>	AP009351	F: gttgatggccaagtaactttgg R: gatactgatgcaccattgg	210bp	This study.
<i>lukSF-PV</i>	AB331245	F: ttacacagttaaatgaagtgaactgga R: agcaaaagcaatgcaattgatg	118bp	Nakagwa et al., 2005.
<i>spa</i>	EF562612	F: cagcaaaccatgcagatgcta R: cgctaatgataatccaccaaataca	101bp	Nakagwa et al., 2005.
<i>tsst-1</i>	JO2615	F: tcacagctaactcaaatacatggatt R: tgtggatccgctcattcattgtt	88bp	Deurenberg et al., 2005.
<i>sec</i>	X51661	F: ctgcaccaggcgataagttt R: ccattctttgttgaagggtggac	112bp	Deurenberg et al., 2005.



**Figure 4. 4. Optimization of SYTO9 in qPCR experiments.** Several concentrations, 0, 0.625, 1.25, 2.5 and 5.0 $\mu$ M were used, 5.0 $\mu$ M was chosen due to producing the highest fluorescent level without inhibiting the reaction. As they all gave the same Ct values 19.2.

#### **4. 2. 6. Real Time Quantitative qPCR**

For RT-qPCR analysis Rotor-Gene 3000 (Corbett Research) was used, from which Ct value and reaction efficiency was produced. Ct value is defined as the point at which the fluorescence rises appreciably above the background (Pfaffle et al., 2002), and is the most important parameter for qPCR as it is used to quantify the amount of template in samples. Reaction efficiency is the probability of replication of a DNA molecule at a replication cycle (Lalam et al., 2006). The data were then subjected to analysis using the Relative Expression Software Tool (REST) program. REST 2005 is a software tool used to determine changes in target gene expression standardised by non-regulated reference genes (Corbett Research 2006). Graphical output data using whisker box-plots provided a visual presentation of variation of each gene that highlights potential issues such as distribution skew. The purpose of this software tool in this study was to determine whether there were significant differences in gene expression profiles between culture samples exposed to antibiotics and samples without added antibiotics.

#### **4. 2. 7. Relative Expression of toxin and pathogenicity genes**

Data were subjected to relative expression analysis of the target genes compared to reference genes. The data for Strain 3 is presented below, as an example of the data along with summary tables for all strains. Strain 3 was selected for this purpose because all four target genes *tsst-1*, *sec*, *lukSF-PV* and *spa* are present in this strain.

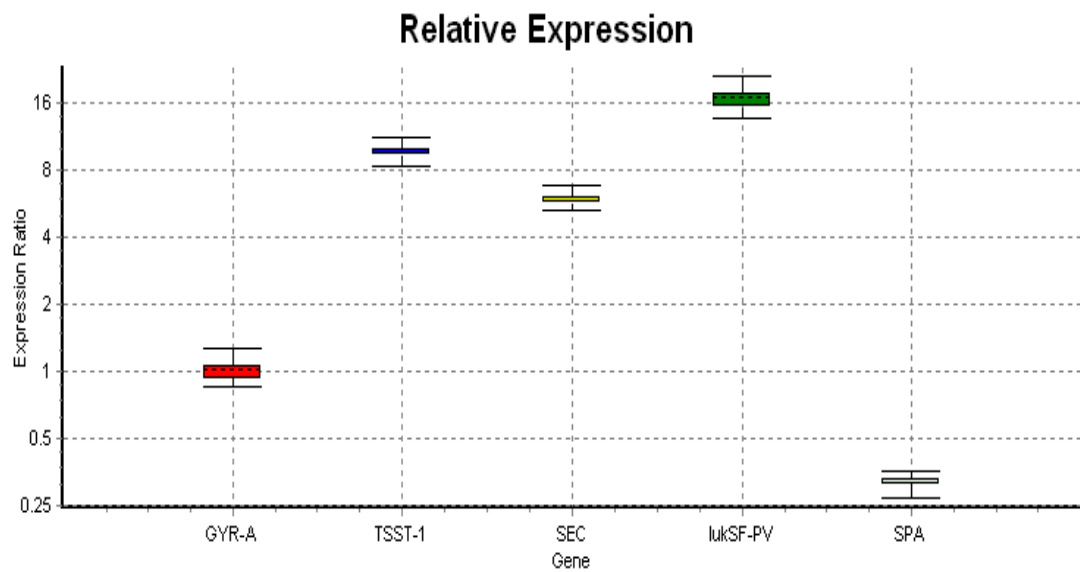
##### **4. 2. 7. 1. The Effect of Protein Synthesis Inhibitors**

Cells of *S. aureus* strain 3 were grown with and without clindamycin and linezolid for one hour and the relative steady-state mRNA levels were quantified using qPCR and REST2005. This included analyses of the relative expression of target genes versus reference genes to determine if the relative changes were statistically significant, using minimum three replicates. Clindamycin treatment had different effects on toxin mRNA steady-state levels and that of virulence factor protein A at the one hour time point. This antibiotics caused an increase in mRNA levels of the three toxin genes (*tsst-1*, *sec* and *lukSF-PV*), but a decrease of *spa* mRNA levels (Table 4. 5; Figure 4. 5). Similar results were found in samples exposed to linezolid with some differences in fold changes (Table 4. 6; Figure 4. 6).

**Table 4. 5. Relative Expression Results of the effect of sub-MIC of clindamycin on strain 3.**

Gene	Type	Expression	P value	Result
<i>gyrA</i>	REF	1.0		
<i>tsst-1</i>	TRG	9.56	0.004	UP
<i>sec</i>	TRG	5.80	0.001	UP
<i>lukSF-PV</i>	TRG	16.80	0.016	UP
<i>spa</i>	TRG	0.316	0.008	DOWN

REF indicates reference gene, TRG target gene, UP to significantly increased, and DOWN to significantly decreased if P value is <0.05.

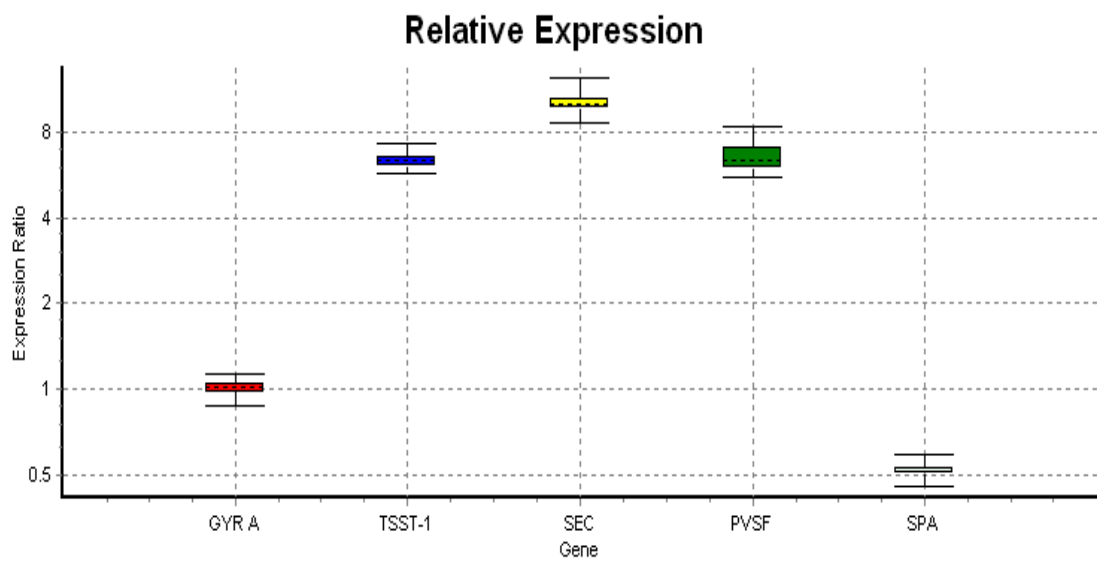


**Figure 4. 5. Relative Expression Ratio of four target genes in strain 3 exposed to the sub-MIC of clindamycin.**

**Table 4. 6. Relative Expression Results of the effect of sub-MIC of linezolid on strain 3.**

Gene	Type	Expression	P value	Result
<i>gyrA</i>	REF	1.0		
<i>tsst-1</i>	TRG	6.324	0.032	UP
<i>sec</i>	TRG	10.022	0.008	UP
<i>lukSF-PV</i>	TRG	6.485	0.008	UP
<i>spa</i>	TRG	0.524	0.003	DOWN

REF indicates reference gene, TRG target gene, UP to significantly increased, and DOWN to significantly decreased if P value is <0.05.



**Figure 4. 6. Relative Expression Ratio of four target genes in strain 3 exposed to sub-MIC of linezolid.**

For example *lukSF-PV* mRNA showed an increase of 16.8 fold in cultures exposed to clindamycin, while in cultures exposed to linezolid, it increased 6.4 fold, though the change were significant in both cases. The mRNA levels of *tsst-1* and *sec* were both significantly increased with similar fold changes when cells were exposed to these two antibiotics. Sub-MIC levels of clindamycin and linezolid both significantly reduced *spa* mRNA levels approximately 3 and 2 fold, respectively.

#### **4. 2. 7. 2. The Effect of Cell Wall Inhibitors.**

Oxacillin and vancomycin were both used to study their effect on steady-state mRNA levels of the same toxin and virulence factor genes as above. Samples incubated with these antibiotics were tested and data showed that the steady-state mRNA levels of all target genes were down-regulated by 2-4 fold changes with the greatest reduction being in *spa* mRNA levels (Table 4. 7, Figure 4. 7). In vancomycin treated samples *spa* mRNA level was also the most affected and down-regulated by 0.187 fold change (Table 4. 8, Figure 4. 8). The other three toxin genes were also significantly down-regulated in cultures exposed to sub-MIC of vancomycin.

#### **4. 2. 8. *lukSF-PV* and *spa* Gene Expression in Cultures Exposed to Antibiotics for one hour**

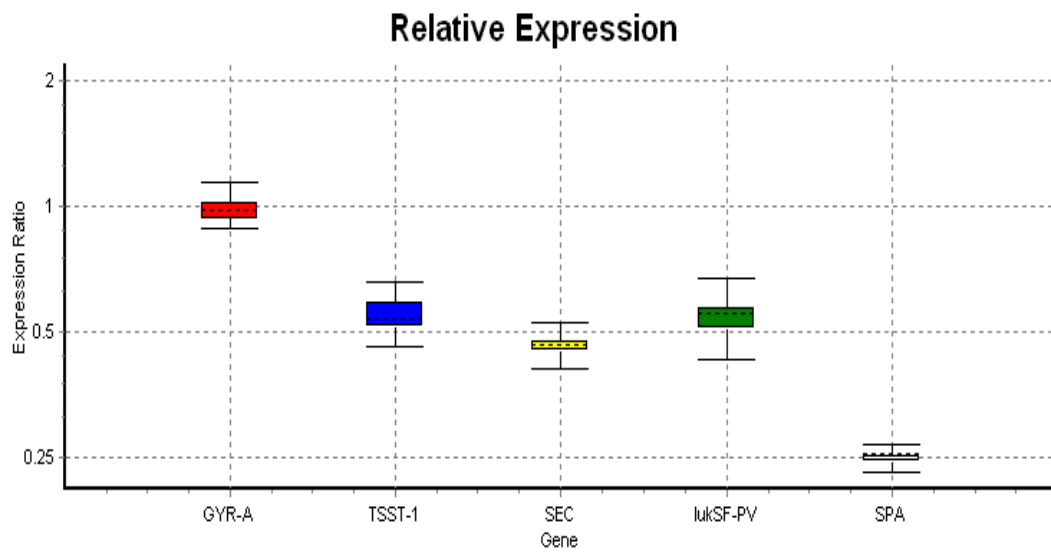
Experiments were designed to test the effect of sub-MIC levels of used antibiotics on *lukSF-PV* and *spa* mRNA steady state levels. Analysis of the relative expression of *lukSF-PV* and *spa* mRNA reveals a general pattern that is consistent across all strains. Data showed that both cell wall inhibitors reduced *lukSF-PV* gene transcription at different fold changes significantly (Table 4. 9). In contrast, both protein synthesis inhibitors significantly increased *lukSF-PV* mRNA levels in the majority of the strains, and decreased *spa* mRNA levels. The effect of these antibiotics on *spa* gene was also studied. *spa* mRNA levels decreased in cultures grown with all antibiotics used in this study (Table 4. 10). The reason for that could be the effect of these antibiotics on growth of these bacterial cells, as the growth was lowered for the first hour of growth. It is clear that cell-wall-active antibiotics inhibit bacterial growth by inhibiting peptidoglycan biosynthesis. However, following inhibition of peptidoglycan biosynthesis, various secondary cellular responses are possible: cells may lyse, cells may die and lyse, cells may die without lysis or growth may be inhibited but the cells

survive (Utaiida et al., 2003). In this study both oxacillin and vancomycin caused a decrease in number of cells immediately after addition to the cultures, after which, the cultures returned to log phase again (Figure 4. 3). One hour post antibiotic additions of sub-MIC levels of both cell wall inhibitors reduced *lukSF-PV* and *spa* mRNA levels. The fold change was higher in *spa* mRNA levels than that of *likSF-PV* mRNA levels. The reason might be due to differential localization of these two virulence factors, as protein A is cell wall located.

**Table 4. 7. Relative Expression Results of the effect of sub-MIC of oxacillin on strain 3.**

Gene	Type	Expression	P value	Result
<i>gyrA</i>	REF	1.0		
<i>tsst-1</i>	TRG	0.543	0.004	DOWN
<i>sec</i>	TRG	0.463	0.004	DOWN
<i>lukSF-PV</i>	TRG	0.546	0.005	DOWN
<i>spa</i>	TRG	0.250	0.017	DOWN

REF indicates reference gene, TRG target gene, UP to significantly increased, and DOWN to significantly decreased if P value is <0.05.

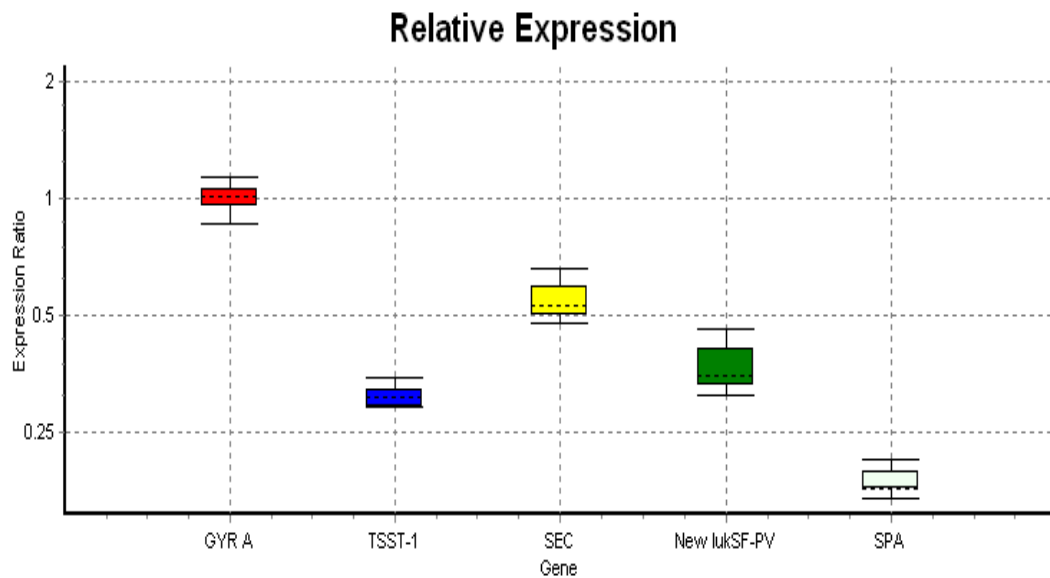


**Figure 4. 7. Relative Expression Ratio of four target genes in strain 3 exposed to sub-MIC of oxacillin.**

**Table 4. 8. Relative Expression Results of the effect of sub-MIC of vancomycin on strain 3.**

Gene	Type	Expression	P value	Result
<i>gyrA</i>	REF	1.0		
<i>tsst-1</i>	TRG	0.311	0.032	DOWN
<i>sec</i>	TRG	0.552	0.031	DOWN
<i>lukSF-PV</i>	TRG	0.372	0.047	DOWN
<i>spa</i>	TRG	0.187	0.017	DOWN

REF indicates reference gene, TRG target gene, UP to significantly increased, and DOWN to significantly decreased if P value is <0.05.



**Figure 4. 8. Relative Expression Ratio of four target genes in strain 3 exposed to sub-MIC of vancomycin.**

**Table 4. 9. *lukSF-PV* mRNA levels in cultures incubated with antibiotics for one hour.**

Strain	Fold change in level of <i>lukSF-PV</i> gene in the presence of antibiotics			
	Clindamycin	Linezolid	Oxacillin	Vancomycin
<b>2</b>	8.197 ↑	5.867 ↑	0.559 (p=0.083)	0.306 ↓
<b>3</b>	19.722 ↑	5.900 ↑	0.546 ↓	0.497 ↓
<b>4</b>	1.789 (p=0.134)	3.427 ↑	0.634 (p=0.695)	0.604 ↓
<b>6</b>	4.914 ↑	7.169 ↑	0.246 ↓	0.167 ↓
<b>7</b>	2.571 ↑	4.564 ↑	0.414 ↓	0.228 ↓
<b>8</b>	2.691 ↑	2.440 ↑	0.362 ↓	0.377 ↓
<b>9</b>	2.684 ↑	2.184 ↑	0.242 ↓	0.213 ↓
<b>10</b>	4.555 ↑	3.629 ↑	0.797 (p=0.969)	0.183 ↓

Green downward arrows indicate significant reduction and red upward arrows indicate significant induction (p<0.05 unless indicated).

**Table 4. 10. *spa* mRNA levels in cultures incubated with antibiotics for one hour.**

Strain	Fold change in level of <i>spa</i> gene in the presence of antibiotics			
	Clindamycin	Linezolid	Oxacillin	Vancomycin
2	0.196 ↓	0.272 ↓	0.164 ↓	0.126 ↓
3	0.632 ↓	0.524 ↓	0.242 ↓	0.187 ↓
4	0.284 ↓	0.861 (p=0.678)	0.390 ↓	0.596 ↓
6	0.393 ↓	0.373 ↓	0.101 ↓	0.306 ↓
7	0.269 ↓	0.174 ↓	0.413 ↓	0.214 ↓
8	0.180 ↓	0.299 ↓	0.216 ↓	0.225 ↓
9	0.214 ↓	0.112 ↓	0.124 ↓	0.134 ↓
10	0.183 ↓	0.388 ↓	0.172 ↓	0.821 (p=0.984)

Green downward arrows indicate significant reduction ( $p < 0.05$  unless indicated).

#### **4. 2. 9. *lukSF-PV* and *spa* gene Expression in Cultures Exposed to Antibiotics for 5hrs.**

Three strains (3, 9 and 10) were chosen to study the effect of these antibiotics on the mRNA level of *lukSF-PV* and protein A using qPCR following growth in the presence of antibiotics for 5 hrs. These strains all contain the *lukSF-PV* gene and are representatives of both MSSA and MRSA; strains 3 and 9 are MSSA and strain 10 is MRSA. mRNA levels of the target genes were modulated differentially in cultures exposed to antibiotics.

The three strains produced significantly lower *lukSF-PV* mRNA in cultures exposed to linezolid and vancomycin with similar fold change (Table 4. 9). Five hours post antibiotic addition clindamycin remained to increase *lukSF-PV* mRNA levels in these cultures and with different fold changes ranging from 2.3 fold to 6.2 fold in those three strains (Table 4. 9). Similarly oxacillin increased *lukSF-PV* mRNA levels in all three strains with the highest fold change was obtained in strain 3 exposed to oxacillin, where the *lukSF-PV* mRNA increased 13.5 fold (Table 4. 11). *spa* mRNA level was decreased in cultures in the presence of clindamycin, linezolid and vancomycin. Oxacillin was the only antibiotic which caused increase of *spa* mRNA level with 5.4 fold in strain 3, and 1.8 and 1.6 fold in strain 9 and 10 respectively (Table 4. 12). Similarly, other studies were also found that sub-MIC of clindamycin stimulates synthesis of coagulase and fibronectin binding protein at transcription levels (Herbrt et al., 2001).

The striking point here was oxacillin in contrast to vancomycin (two cell wall inhibitors) caused an increase in steady-state mRNA levels of these two virulence factors following 5hrs growth in all three isolates. This is particularly important in PVL-positive strains of *S. aureus*, as producing PVL toxin in response to these antibiotics even at low concentrations could lead to worsening outcomes. This is due to the fact that, the PVL toxin at low concentrations can induce apoptosis via a novel pathway that has been proposed to involve PVL-mediated pore formation of the mitochondrial membrane (Boyle-Vavra et al., 2007).

**Table 4. 11. *lukSF-PV* mRNA level in cultures incubated with antibiotics for 5hrs.**

Strain number	Fold change in level of <i>lukSF-PV</i> gene in the presence of antibiotics			
	Clindamycin	Linezolid	Oxacillin	Vancomycin
3	6.208 ↑	0.684 ↓	13.506 ↑	0.435 ↓
9	2.339 ↑	0.227 ↓	3.238 ↑	0.636 ↓
10	2.831 ↑	0.460 ↓	2.901 ↑	0.291 ↓

Green downward arrows indicate significant reduction and red upward arrows indicate significant increase ( $p < 0.05$  unless indicated).

**Table 4. 12. *spa* mRNA level in cultures incubated with antibiotics for 5hrs.**

Strain	Fold change in level of <i>spa</i> gene in the presence of antibiotics			
	Clindamycin	Linezolid	Oxacillin	Vancomycin
3	0.45 ↓	0.464 ↓	5.443 ↑	0.127 ↓
9	0.523 ( $p=0.104$ )	0.403 ↓	1.897 ↑	0.543 ↓
10	0.352 ↓	0.591 ( $p=0.194$ )	1.687 ↑	0.816 ( $p=0.462$ )

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

The induction of virulence factors by  $\beta$ -lactam antibiotics has been seen in other studies.  $\beta$ -lactam antibiotics of different classes induced  $\alpha$ -haemolysin, PVL and TSST-1 expression at both the mRNA and protein levels (Ohlsen et al., 1998, Stevens et al., 2007 and Dumitresco et al., 2007). These findings led to the speculation that  $\beta$ -lactam therapy might enhance the virulence of some *S. aureus* strains, in turn worsening the symptoms of serious staphylococcal infection. The reason for this reduction in one hour cultures and induction in 5 hrs cultures with effect of this antibiotic, possibly due to the fact that  $\beta$ -lactam antibiotics are effective only when the number of cells are low (Stevens et al., 2007). They reported that the reason is ‘Eagle effect’, which demonstrates that  $\beta$ -lactam antibiotics are ineffective during the stationary phase of bacterial growth-or, that they are only effective in rapidly growing organisms (Eagle, 1952 cited by Stevens et al., 2007).

Both protein synthesis inhibitors clindamycin and linezolid caused an increase of *lukSF-PV* mRNA levels in all isolates when cultures were grown with these antibiotics for one hour. These antibiotics had opposing effects on mRNA levels when cells were incubated in their presence for 5 hours. Clindamycin incubated cells increased *lukSF-PV* mRNA levels, however linezolid caused *lukSF-PV* mRNA levels to drop in all three isolates. In addition, *spa* mRNA levels decreased in all isolates used, and in both time periods when cells were grown with these two protein synthesis inhibitors. Similarly in a study of toxin genes, clindamycin markedly suppressed translation but not transcription of several virulence factor genes, which supports the contention that protein-synthesis inhibition is an important consideration in the selection of antimicrobial agents to treat serious infection caused by toxin-producing *S. aureus* (Stevens et al., 2007). The reduction of *spa* mRNA expression in strains of *S. aureus* exposed to these protein-synthesis inhibitors would lead to increased bacterial susceptibility to phagocytosis and suggesting the additional therapeutic efficiency (Koszczoł et al., 2009). Bernardo et al. showed that the expression of virulence factors in *S. aureus* is especially sensitive to the inhibition of protein synthesis by linezolid, which should be an advantage in the treatment of infections with toxin producing *S. aureus* (Bernardo et al., 2004).

**CHAPTER 5**  
**Proteomic Analysis of *S. aureus* Extracellular  
Proteins**

## 5.1. Introduction

Complete genome sequences of several strains of *S. aureus* have opened a new avenue for the study of virulence factors and pathogenicity in this bacterium. When taken together, genome-wide profiling via transcriptomics and proteomics would allow the comprehensive measurement of the *S. aureus* biological system (Scherl et al., 2005). The evolution towards rapid protein identification has made large scale proteome analysis possible. This is mainly due to achievements in protein and peptide separation, mass spectrometry techniques, and the combination of these technologies with powerful bioinformatic tools (Scherl et al., 2005). Nearly all strains of *S. aureus* secrete a group of core exoproteins to facilitate host tissue degradation into nutrients required for bacterial growth (Pocsfalvi et al., 2008). Examples of these exoproteins are enzymes (nucleases, proteases, lipases, hyaluronidases and collagenases) and exotoxins (Dinges et al., 2000). Virulence factors are of special interest since they might be in direct contact with the host tissue as they enable the organism to evade the host defence, to adhere to cells and the tissue matrix, to spread within the host and to degrade cells and tissues, for both nutrient and protection (Novick, 2003). Regarding the secretome of *S. aureus* it is expected that between 5 and 15% of the whole staphylococcal proteome is exported during a bacterial life time in a growth phase dependent manner (Pocsfalvi et al., 2008). According to their function, staphylococcal virulence factors can be divided into at least four groups:

1. Proteins involved in adhesion and invasion of host cells.
2. Proteins mediating degradation of host cells for both nutrition and spreading.
3. Proteins that enable the bacteria to evade the immune response.
4. Proteins for utilization of nutrients that predominantly exist in the host (Hecker et al., 2010).

The secreted virulence factors, typically produced during the post exponential phase and stationary phases including a large group of exoenzymes such as proteases and nucleases that make nutrients available to the microorganisms (Schlivert et al., 2010). The secreted virulence factors also include a large group of exotoxins such as highly inflammatory cytolytins and PVL, super antigens such as staphylococcal enterotoxins, toxic shock syndrome toxin and exfoliative toxins (A and B) (Schlivert et al., 2010).

The function of at least 70% of extracellular proteins is not yet clear and their possible role in virulence has to be elucidated (Hecker et al., 2010). The secretome of *S. aureus* consists of all proteins that are directly secreted via different secretion pathways.

All proteins that have to be transported from the cytoplasm across the membrane to the extra-cytoplasmic compartments of the cell, or the extracellular milieu, need to contain a specific sorting signal for their distinction from resident proteins of the cytoplasm.

Like all living organisms *S. aureus* employs several specific and non-specific pathways to transport proteins to an extra-cytoplasmic location, of which the general secretory (Sec) pathway is the most well known and best described (Sibbald et al., 2006). In addition to Sec pathway, examples of others are the twin-arginine translocation or ‘‘Tat pathway’’, the ATP-binding cassette (ABC) transport system, and the type IV prepilin-like export operate as ‘‘special-purpose’’ pathways (Buist et al., 2006). Most well known virulence factors that are secreted by Gram-positive pathogens have a typical N-terminal signal peptide that would lead them to the Sec-pathway. In contrast to the Sec pathway which transports proteins in an unfolded manner, the Tat pathway serves to actively translocate folded proteins across a lipid membrane bilayer (Mori et al., 2001).

Proteins without signal peptides could also be secreted by using non specific and /or unknown mechanisms (Pocsflavi et al., 2008). Recently, it was found for the first time that Gram-positive bacteria naturally produce membrane vesicles (MVs) during their growth, which share many common features with outer membrane vesicles of Gram-negative bacteria (Lee et al., 2009). Using proteomic approach, they identified a total of 90 protein components of *S. aureus*-derived MVs.

In addition to the information about the presence or absence of virulence factors proteomic techniques can provide information about the expression of individual virulence factors and their possible post translational modifications (PTM) (Engelman et al., 2009). Identification of extracellular proteins showed that many proteins secreted via the Sec pathway appear as multiple spots on 2D gels. Such multiple spots may be due to charge alteration (e.g. enterotoxins) or to fragmentations e.g. lukF and lukM (Engelman and Hecker 2009). One of the most common types of PTMs is direct protein phosphorylation, which reversibly modulates protein activity (Eymann et al., 2007). Phosphorylation of extracellular proteins could play an important role in

pathogenesis of *S. aureus*. Lams – Lopez and co-workers provided evidence that the phosphorylation of enolase, which is mediated by a serine/ threonine kinase utilizing ATP as a phosphoryl donor, might play a role in allowing bacteria to adhere to the laminin-containing extracellular matrix (Lomas-Lopez et al., 2007). Protein damage and oxidation can also be investigated by proteomics, e.g. the irreversible oxidation of glyceraldehyde 3-phosphate dehydrogenase by hydrogen peroxide treatment (Weber et al., 2004).

Integral membrane proteins (IMPs) lie at the critical junctions between intracellular compartments and cells and their environment. As such, IMPs are in a unique position to mediate a host of cellular processes, including intercellular communication, vesicle trafficking, ion transport, protein translocation/ integration, and propagation of signaling cascades. IMPs are predicted to make up 20-25% of all ORFs in most genomes (Wolff et al., 2008). There are several special features of membrane proteins that render their analysis so difficult. Their hydrophobic character conveys poor solubility in aqueous buffer and makes a separation via 2D PAGE complicated. Furthermore membrane proteins are disguised by the high abundance of many cytosolic proteins requiring an efficient isolation procedure (Engelmann et al., 2009 and Wolff et al., 2008). They reported that, the implementation of three different technologies, one-dimensional gel-Liquid chromatography (LC), two-dimensional LC, and a membrane shaving approach combined with MS/MS analyses, enabled an identification of most membrane proteins. Especially the MS-based techniques have undergone dramatic developments during the last 15 years (Han et al., 2008). Nevertheless, gel-based proteomics is still a powerful tool to address many physiological issues because (i) most of the metabolic pathways and the most obvious stress/ starvation responses can be visualized on 2D gels, (ii) very rapid stress/ starvation responses can be directly followed because gel-based proteomics can discriminate between protein synthesis and protein level accumulated in the cells, and (iii) protein modifications such as protein processing, protein phosphorylation or protein damage, and even proteolysis at a proteome-wide scale can be visualized. MS-based procedures, on the other hand, are absolutely required to cover the entire proteome including membrane proteins or low-abundance proteins (Hecker et al., 2010). The theoretical extracellular proteome map of *S. aureus*, which considers the proteins that are actively secreted via different pathways, indicates that most of the proteins that

belong to the core and the variable exo-proteome can be allocated to the PI of 3-10 and molecular weights (MW) of 10-140kD. Based on this calculation, 2D gel electrophoresis combined with mass spectrometry represents a very efficient tool to identify all of the proteins which are present in the extracellular milieu and to analyze the secreted proteins pattern under different growth conditions and strains (Engelman et al., 2009). From an infection-related point of view, secreted proteins still bound to the cell surface or secreted into the extracellular space form the most crucial class of *S. aureus* proteins, because most of the virulence factors can be found in these two sub-proteomic fractions (Becher et al., 2009). Accordingly, when secreted in detectable amounts, about 90% of the predicted extracellular proteins should thus be present on 2D gels in PI 3-10. 2DE represents one of the most powerful and widely used techniques for the simultaneous resolution of thousands of proteins from a single complex biological source (Klos et al., 1999). In the first dimension proteins are separated by PI in a process called isoelectric focusing (IEF). The proteins will move along the gel and will accumulate at their isoelectric point, the point at which the overall charge on protein is zero. Following IEF, the proteins are then separated on the basis of their MW using SDS-PAGE. The resulting protein profile can then be visualized in a number of ways, including staining and pre-labeling with fluorescent dyes or radiolabels.

The development of 2D Difference in-gel electrophoresis (DIGE) circumvents many of the technological limitations of traditional 2D PAGE. 2-D DIGE provides increased confidence of protein differences between samples, due to the capacity to include internal standards in gels, and because multiple samples can be analyzed in a single gel, reducing the number of gels that must be run (Tonge et al., 2001). Two protein samples are pre labeled with different cyanine dyes, thus enabling two different samples to be run on the same gel in both dimensions. Hence, the two samples are subjected to the same procedure and environment throughout the experiment. Protein spots can be detected by fluorescence imaging immediately after electrophoresis with a high sensitivity (Unlu et al., 1997). In this study for 2D-DIGE, the experimental design utilized an internal standard created by pooling aliquots of all the samples in the experiment. Therefore, in this experiment, an aliquot of each of the control and treated samples were pooled and labeled with Cy3 as internal standard. This was mixed with a

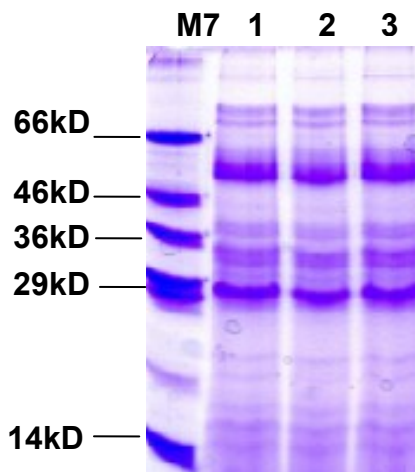
control or treated sample labelled with Cy5 prior to electrophoresis. This experimental design reduces gel-to-gel variation over conventional 2-D PAGE (Yan et al., 2002).

The aims of this part of the project were to produce secretome maps for quantitative analyses for 10 clinical isolates of *S. aureus* and identification of extracellular virulence factors and other extracellular proteins by MALDI-TOF Mass spectrometry. The study will also analyze the phenotypic diversity of the strains used, based on their extracellular proteins. The main objective was to determine changes in the quantity of virulence factors and other extracellular proteins in the presence of cell wall and protein synthesis inhibitor antibiotics.

## 5. 2. Results and Discussion

### 5. 2. 1. Protein Sample Preparation and SDS-PAGE Analyses

In this work, a comparative secretomic analysis of 10 clinical isolates of *S. aureus* was performed, using 2D gel SDS-PAGE combined with MALDI-TOF mass spectrometry. For protein sample preparation cells were cultured at 37°C under gentle agitating in 100 ml volume of BHI media, samples were collected at 5 hrs time after antibiotic addition, so that the cells were grown with antibiotics for 5 hrs (section 4. 2. 2). Extracellular Proteins were concentrated using tri-chloro acetic acid (TCA) precipitation. The concentration was determined using Bradford assay and samples stored at -20°C. Protein samples were subjected to SDS-PAGE analysis to establish the uniformity of protein profiles in terms of band patterns between samples obtained from cultures of all ten strains. For this 10µg of each sample were solubilised in 1x SDS sample loading buffer (10% SDS, 5%DTT), in a total volume of 25µl. Samples were boiled for a few minutes, spun at 10000rpm for two minutes and resolved via SDS-PAGE using 12 % polyacrylamide gel. Gels were stained using Coomassie blue (Figure 5. 1). Protein fractions have distinct profiles, with the most abundant protein bands in 14-60 kD regions.



**Figure 5. 1. Evaluation of reproducibility and solubility of protein samples on SDS PAGE gel analyses.** Samples were taken from three separate cultures of strain 10 (1, 2, and 3 lanes). Protein samples were quantified using the Bradford assay, and 10µg were resolved via SDS-PAGE using 12 % PAGE and proteins were then stained with Coomassie blue.

### **5. 2. 2. 2DE Gel Analysis**

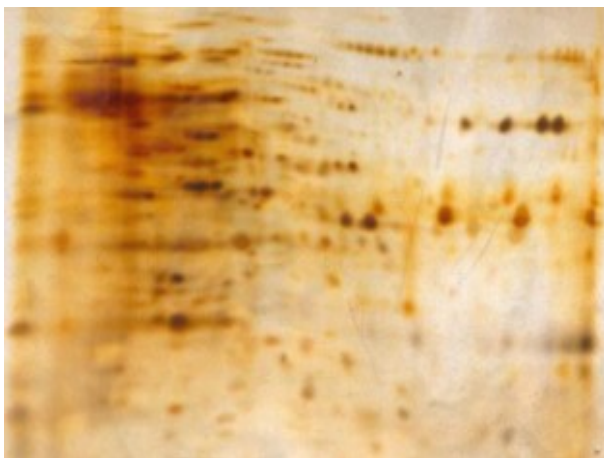
2-DE separates a mixture of proteins by two physical factors, pI and molecular weight. In this study 7cm and 18cm IPG strips were used in combination with silver, SYPRO-RUBY and some times Coomassie blue staining methods. Small 7cm IPG strips (pI 3-10) with 12% polyacrylamide gels were routinely used as an analytical tool for testing sample solubility and 2D gel reproducibility (Figure 5. 2). Identical gels were stained with silver and SYPRO-Ruby protein gel stains, which are both very sensitive staining methods. It has been reported that they both interact with lysine amino acid of proteins, and SYPRO-Ruby in addition to lysine with arginine and histidine residues too (Chou et al., 1990). However when silver stained gels remained for longer in the developing solution, highly abundant protein spots become saturated and most low abundant spots become clearly visible (Figure 5. 2). This occurred particularly in the low pI area of the gels, as it seems that the number of spots in pI 4-7 on silver stained gels is higher than that stained with SYPRO-Ruby stain.

The advantage of SYPRO-Ruby protein gel stain is that it has a broader linear dynamic, from approximately 1-2 ng to 1-2 µg compared to silver stain, which displays a linear dynamic range only from 8-60ng of protein (Lopez et al., 2000). In addition, SYPRO-Ruby gel stain has a significant advantage since it allows peptide recovery and therefore identification of lower abundant protein spots than silver stain (Macri et al., 2000). Therefore SYPRO-Ruby protein gel stain was chosen for staining purposes in the rest of the proteomic experiments.

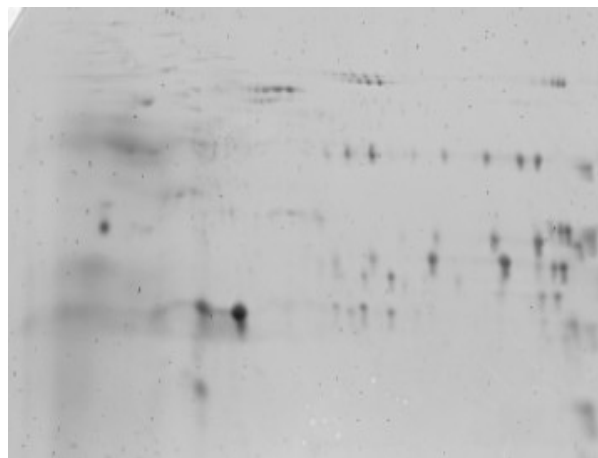
### **5. 2. 3. Secretome Analysis for *S. aureus* Extracellular proteins**

To perform a comparative secretome analysis, the secreted proteins of all 10 strains were separated using 2DE. Samples were loaded using IGR into 18 cm IPG strips and reswelled overnight at room temperature. IEF was carried out using six phases of stepped or gradient voltage from 300 to 10,000volts, with a total focusing of 70,000 volt hours. Samples were then resolved using 12% polyacrylamide in large format gels. Proteins were stained using SYPRO-Ruby protein gel stain and images were taken using a Typhoon machine (Amersham Biosciences).

**(A)**



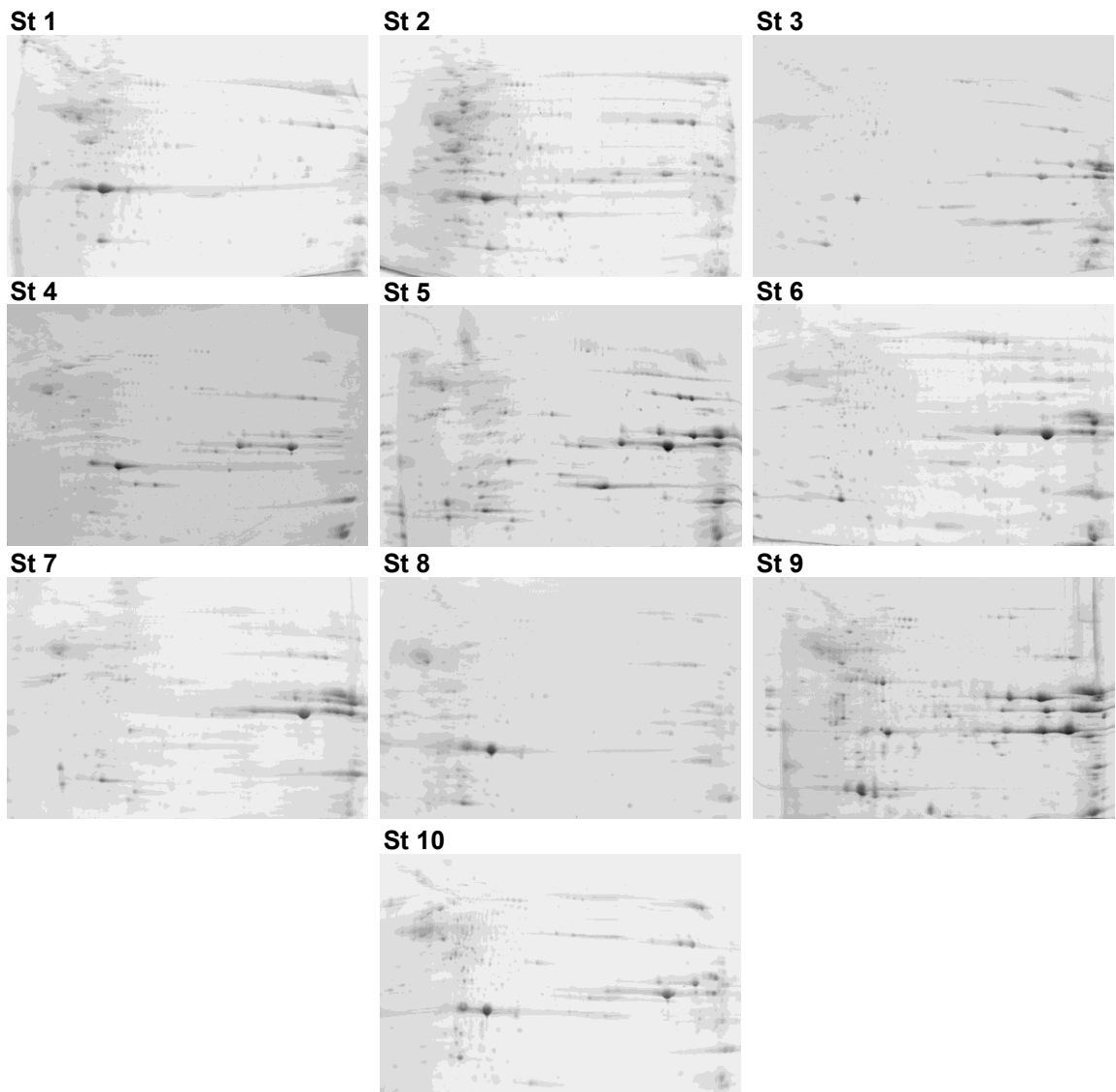
**(B)**



**Figure 5. 2. 2DE analysis of protein samples from strain 10, stained with silver (A), and SYPRO Ruby (B).** The protein amounts of 20 $\mu$ g for silver, and 50 $\mu$ g for SYPRO Ruby were loaded using IGR onto 7cm mini IPG pI 3-10 strips and resolved using 12% polyacrylamide gels. SYPRO-Ruby stained gel was scanned using Typhoon 9400 (Amersham Biosciences).

Progenesis SameSpot analyses software (Nonlinear Dynamics) was used to analyse the data, in which 2DE image from the internal pooled standard from all ten strains was used as a reference for comparative analyses. Visual appearance of 2DE protein profiles revealed a high degree of exoproteome heterogeneity among these different clinical isolates (Figure 5. 3). Results showed that in general most of the strains showed very different 2DE maps. For example strain 5 and 7 belong to the same CC22, but have quite different 2DE profiles (Figure 5. 3). On the other hand strain 4 and 10 have very similar 2DE profile; both of these strains are very similar to USA300; a CA-MRSA strain, and has the same MLST type. Different 2DE profiles may reflect the different ecological niches that each strain can inhabit. As strain 5 and 7 were isolated from wound swab and nasal swabs, respectively, whereas strain 4 and 10 were both isolated from pus swabs. These results agree with data published by Hecker et al., in which they showed that three clinical isolates of *S. aureus* within the same MLST type can have extremely variable exoproteome profiles (Hecker et al., 2010).

Having obtained all 2DE images from 10 isolates, in order to perform a comparative 2DE mapping exercise, they were all imported into the Progenesis SameSpots software (Nonlinear Dynamics). 2DE images were cropped first, selecting only the area of the gel within which proteins had resolved and boundary regions without proteins were removed. The pooled internal standard image, produced from a pooled sample made from all 10 isolates, was then selected as a reference map to which all other gels were matched by making a number of vectors manually before matching all 2DE images automatically by the software. 2DE images were aligned so that cognate spots are at corresponding locations across different gels. The protein spots volume (abundance) was subsequently normalised and quantified by the software. The software was able to detect all resolved protein spots in each gel, and given p value to each spot using ANOVA test for statistical analysis. The total number of proteins was 667 and these were ranked by ANOVA (p value). This number (667) represents the combined number of spots, as it was detected from the internal reference map, which contains equal amounts of proteins from all 10 isolates. Since the Progenesis software is designed for differential analysis only, it does not tell if the spot is present or not. To determine the spots present in each strain cut-off value of the protein volume abundance was required. This can be obtained from several known and identified proteins in each strain.



**Figure 5. 3. 2DE gel protein patterns of 10 clinical isolates of *S. aureus*.** In total 350 $\mu$ g of the protein extract of each isolate was separated on 2D gels, using IPG strips (pI 3-10) for the first dimension. Protein spots were stained with SYPRO-Ruby stain and scanned using Typhoon 9400 (Amersham Biosciences).

#### 5. 2. 4. Identified Proteins using MALDI-TOF Mass Spectrometry

Most of the virulence factors that are in the focus of infection-related proteomic studies of *S. aureus* are either cell surface-exposed or secreted into the extracellular space. By knowing the MW and PI of target virulence factors such as both subunits of PVL, enterotoxins, TSST-1, and protein A, a number of spots were chosen from the secretome map for identification using MALDI-TOF mass spectrometry. Spots were taken from a pooled sample separated on a large format gel stained with SYPRO-Ruby protein stain, either by using a ProPic spot picking robot (Genomic Solutions Ltd.) or manually. A total of 60 proteins were identified representing 51 different proteins (Figure 5. 4). For each protein a mascot search result from MALDI-TOF was generated with several protein scores. A protein score greater than 82 means significant  $p$  value  $< 0.05$ , where  $p$  value is the probability that the observed match is a random event. It also gave the identification number (ID) of the protein identified and matched peptides to the protein identified. PSORT analysis (J:\ PSORT prediction. mht) was used to determine localization of each protein identified. Identified proteins were found to be differently localized, as they showed to be extracellular, cytoplasmic and membrane proteins. Details of identified virulence factors are summarized in (Table 5. 1).

Most of these virulence factors are known to have predicted cleavable Sec-type signal peptides, which means that they use the most commonly used pathway for bacterial protein transports Sec pathway. This was expected since the fact that extracellular proteome represents a reservoir of virulence factors and are usually actively secreted to the supernatant of the bacterial culture. The other proteins identified in this study were a mixture of cytoplasmic, membrane and extracellular proteins (Table 5. 2). From this several proteins involved in metabolic pathways such as glycolysis, amino acid synthesis and proteins with other different functions. Among cytoplasmic proteins, candidates of glycolysis (glyceraldehyde 3 phosphate dehydrogenase, Enolase, Fructose-biphosphate aldolase, and Pyruvate dehydrogenase E1) and proteins involved in protein synthesis, folding and degradation were also among those identified, such as elongation factors G, Ts, Tu, Trigger factor, Dnak protein, and 50S protein subunits. These proteins might secrete to the supernatant via MVs, as a result of cell damage or through an unidentified transport pathway.



**Figure 5. 4. 2DE protein reference map of *S. aureus* secretome.**

The secretome of a pooled sample of *S. aureus* was separated by 2DE, using IPG strip of (pI 3-10). Protein sample of 300 $\mu$ g of a pooled sample from 10 different isolates of the bacterium was resolved via IEF and large format SDS-PAGE at 12% polyacrylamide concentration. Proteins were stained with SYPRO-Ruby protein gel stain and scanned by Typhoon machine. A total of 51 proteins were identified using MALDI-TOF mass spectrometry after tryptic digestion.

**Table 5. 1. Extracellular virulence factors identified by MALDI-TOF mass spectrometry, their identification number and combined protein score.**

<b>Spot No.</b>	<b>Protein name</b>	<b>Identification No.</b>	<b>Combined protein score &gt;82</b>
1	LukF-PV subunit of PVL	157833587	739
2	LukS-PV subunit of PVL	52695626	571
3	LukS-PV subunit of PVL	52695626	686
4	Alpha-hemolysin S subunit.	2914569	247
5	leukocidin subunit precursor	57650693	607
6	leukocidin subunit precursor	57650693	664
7	SEB (Enterotoxin B).	108515206	743
8	SEB	108515206	718
9	SEB	108515206	680
10	SEC (Enterotoxin C)	93278709	265
11	SEC	93278709	599
12	TSST-1 (Toxic shock syndrome toxin).	2780937	690
13	SEQ (Enterotoxin Q)	87161054	517
38	SEL (Enterotoxin L)	21282489	510
30	Leukocidin subunit precursor	57650693	160
14, 15 and 43	Immunodominant antigen A	gi 15925559	191
50	Thermonuclease precursor	gi 253731423	398
53	Secretory antigen precursor SsaA-like protein	gi 253729965	261
61	Thermonuclease precursor	gi 145581406	379

A score greater than 82 indicates a significant value ( $p < 0.05$ ).

They might be released to the supernatant due to cell lyses, or transported through as yet unknown mechanisms. Membrane vesicles might play a role in translocation of some of these proteins, which were found for the first time in 2009 in gram positive bacteria by. These MVs contained many proteins from all locations (extracellular, membrane and cytoplasm), of which about 60 % were cytoplasmic proteins. They reported that these MVs share many common features with outer membrane vesicles of Gram-negative bacteria (Lee et al., 2009). For example N-acetylmuramoyl-L-alanine amidase, which act as peptidoglycan hydrolase, might also, be involved in vesicle generation in *S. aureus* similar to the action of murein hydrolase in Gram-negative bacteria (Oshida et al., 1995).

### **5. 2. 5. Secretome Maps of the Isolates are Highly Variable**

In order to perform a comparison of secretome map patterns among isolates used and to investigate phenotypic diversity, data were subjected to different analysis. Cutt-off value was determined first, for which the mean volume (protein abundance produced by Progenesis software) for several identified proteins were taken, by knowing that some of these proteins are present in some strains and absent in others, it was possible to determine a threshold. For example PCR data showed that strain 3 and strain 5 among these isolates were positive for *tsst-1*, and this was also confirmed using MALDI-TOF identification. By taking the average volume of three replicates of TSST-1 in strain 3 and 5, the minimum mean in strains 3 and 5 was 1.83, and the maximum mean in other strains was 1.15. SEB is present only in strain 9, the minimum value was 2.9 in that strain, and the maximum volume in other strains was 1.45. On the base of this analysis for 5 proteins in all 10 isolates (Table 5. 4), the value 1.5 was determined as a threshold. This means any values equal or greater than 1.5 indicate to that the protein is present in certain strain. There may be some proteins present and having the mean less than 1.5, however to avoid including any false positive and to be in a conservative side 1.5 was determined. The mean volumes of all 667 protein spots were then subjected to Multidimensional scaling analysis, to show the proximity and distance among strains. This analysis was performed by Allan Seheult, Department of Mathmatical Sciences, Durham University.

**Table 5. 2. Proteins with different functions identified by MALDI-TOF mass spectrometry, their identification number and combined protein score (>82 indicates a significant value).**

Spot No.	Protein name	Identification No.	Combined protein score >82 is significant.	Protein localisation
<i>Central carbon metabolism: glycolysis, TCA cycle and pentose phosphate.</i>				
44	Glyceraldehyde-3-phosphate dehydrogenase	gi <a href="#">15923762</a>	574	Cytoplasm
49	Enolase	gi <a href="#">15923766</a>	327	Cytoplasm
46	Fructose-bisphosphate aldolase	gi <a href="#">15925115</a>	209	Cytoplasm
55	Pyruvate dehydrogenase E1	gi <a href="#">15924084</a>	403	Cytoplasm
56	D-fructose-6-phosphate amidotransferase	gi <a href="#">21283809</a>	421	Cytoplasm
40	Phosphoglycerate kinase	gi <a href="#">15923763</a>	325	Cytoplasm
48	Malate:quinone oxidoreductase	gi <a href="#">15925597</a>	128	Cytoplasm
36	Transketolase	gi <a href="#">15924332</a>	421	Cytoplasm
37	Transketolase	gi <a href="#">15924332</a>	510	Cytoplasm
<i>Amino acid, Protein synthesis and protein fate.</i>				
20	Serine protease	gi <a href="#">82751394</a>	227	Membrane
27	Cysteine proteinase	gi <a href="#">22218028</a>	317	Extracellular
31	Glutamyl endopeptidase	gi <a href="#">9968803</a>	281	Extracellular

19	Elongation factor G	gi 15923537	393	Cytoplasm
22	Glutamyl-tRNA synthetase	gi 15923518	244	Cytoplasm
32	Elongation factor Ts	gi 21282869	586	Cytoplasm
33	Trigger factor	gi 15924665	510	Cytoplasm
34	DnaK protein	gi 15924570	271	Membrane
52	50S ribosomal protein	gi 49482728	86	Cytoplasm
59	Elongation factor Tu	gi 15923538	336	Membrane
45	DNA-directed RNA polymerase	gi 148267002	130	Cytoplasm
<i>Oxidative stress</i>				
57	Superoxide dismutase	gi 15924543	170	Cytoplasm
58	alkyl hydroperoxide reductase subunit C	gi 15923371	268	Cytoplasm
<i>Cell wall biosynthesis</i>				
17	N-acetylmuramoyl-L-alanine amidase	gi 15925634	571	Extracellular
18	N-acetylmuramoyl-L-alanine amidase	gi 15925634	365	Extracellular
21	Dihydrolipoamide dehydrogenase	gi 87161349	670	Cytoplasm
23	Putative cell wall surface anchor family protein	gi 87162069	780	Cytoplasm

24	Glycerol phosphate lipoteichoic acid synthetase	gi 239636668	307	membrane
25	Chain A, of Lipoteichoic Acid	gi 222446944	439	Cytoplasm
26	Chain A, of Lipoteichoic Acid	gi 222446944	494	Cytoplasm
<i>Nucleotide biosynthesis</i>				
54	guanine nucleotide regulatory protein	gi 439548	87	Cytoplasm
61	Inositol-monophosphate dehydrogenase	gi 227558072	243	Cytoplasm
<i>Others</i>				
28	Phosphatidylinositol-specific phospholipase C	gi 425478	530	Cytoplasm
29	Phosphatidylinositol-specific phospholipase C	gi 425478	501	Cytoplasm
35	Hypothetical protein SACOL0479	gi 21282124	270	Extracellular
51	Hypothetical protein SAV0613	gi 15923603	570	Extracellular
60	Alpha S1 Casien	gi 159793197	366	Cytoplasm

Our data showed that any two strains shared a number of proteins; for example strain 1 has 300 proteins and strain 4 has 232 proteins, 132 of these proteins were found to be shared between these two strains (Table 5. 5). Based on the shared proteins between any two isolates, it is possible to know how close these two strains are to each other. The most similarity was seen between strains 4 and 10, and 2 and 8, as they shared 194 and 218 proteins respectively. To see how these strains are different to each other, this means the distance between any two strains, a multidimensional scaling plot was generated based on the differences between these strains (Figure 5. 5), which showed that isolates have different distances from one other. Data presented here, revealed that the secretome profiles of the clinical isolates was highly variable. Results showed that most of the strains showed very different 2DE maps. For example strain 5 and 7 belong to the same clonal complex (CC22), but have quite different 2DE profiles. MDS analysis showed that 139 out of 667 proteins were shared between these two strains. Data presented here showed that only 20 out of 667 proteins were found to be produced by all 10 isolates, some of these shared proteins were among identified proteins such as Isa A, Elongation factor G, thermonuclease, Enolase and fructose-biphosphate aldolase. Similarly Ziabedt et al., (2010) found IsaA and Nuc were shared between 25 clonally different isolates of *S. aureus*. The different 2DE profiles could be due to different sites of infections, and therefore may reflect the different ecological niches that each strain can inhabit. As strain 5 and 7 were isolated from wound swabs and nasal swabs respectively, whereas strain 4 and 10 were both isolated from pus swabs. These results agree with data published by Hecker et al., in which they showed that three clinical isolates of *S. aureus* within the same CC can have extremely variable exoproteome profiles (Hecker et al., 2010). They were taken from three different sites of infections. Research has focused on identifying genotypic diversity to explain phenotypic diversity, as there is extensive phenotypic variation among clinical isolates of pathogenic bacteria; however, phenotypic diversity is not always reflected in genotypic diversity. The phenotypic differences between different strains have, in part, been attributed to difference at the level of expression (Encheva et al., 2007). Most of these strain phenotypic differences may be caused by changes in the global regulatory networks that control gene expression (Jelsbak et al., 2010).

**Table 5. 3. Determination the lowest abundance of the protein in Positive and the highest abundance of the protein in Negative Isolates for TSST-1.**

Strain	TSST-1	Av Vol	Av BG	Row Vol	RV/ BG	Min +Ve	Max -Ve
1	Negative	0.058	0.44	0.498	1.131818	1.834081	1.150242
2	Negative	0.078	0.858	0.936	1.090909		
3	Positive	0.558	0.669	1.227	1.834081		
4	Negative	0.052	0.96	1.012	1.054167		
5	Positive	0.837	0.725	1.562	2.154483		
6	Negative	0.08	0.79	0.87	1.101266		
7	Negative	0.056	0.739	0.795	1.075778		
8	Negative	0.05	0.51	0.56	1.098039		
9	Negative	0.093	0.619	0.712	1.150242		
10	Negative	0.05	0.4	0.45	1.125		

Av Vol indicates to average volume, Av BG, to average background, Row Vol, to row volume before subtracting the background value, Min +Ve, to minimum value among positive strains for that protein, and Max-Ve indicates to maximum value among positive strains for that protein. Each value is the mean of three biological replicates.

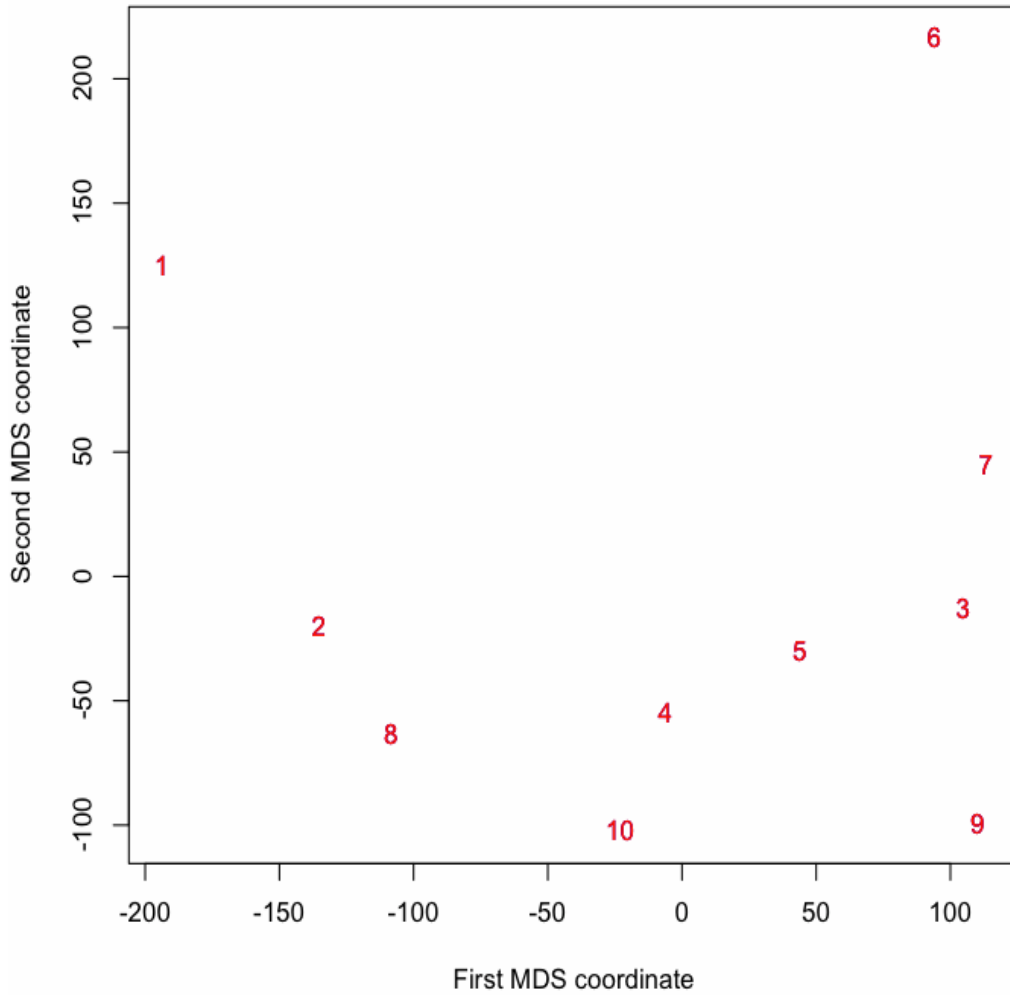
**Table 5. 4. Determine threshold for present and absent proteins in isolates used.**

Protein	Minimum mean volume positive	Maximum mean volume Negative
SEC	4.79	1.26
SEB	2.9	1.45
TSST-1	1.83	1.15
PVF	2.35	1.22
PVS	2.92	1.25

**Table 5. 5. Strain Similarities Based on Number of Shared Spots between iaolates**

<b>Strains</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>
<b>1</b>	<b>300</b>	208	104	132	142	98	90	179	86	151
<b>2</b>		<b>341</b>	147	180	188	111	120	218	140	223
<b>3</b>			<b>227</b>	135	160	92	109	134	124	151
<b>4</b>				<b>232</b>	157	92	110	173	126	194
<b>5</b>					<b>278</b>	104	139	178	144	183
<b>6</b>						<b>197</b>	87	90	74	104
<b>7</b>							<b>181</b>	112	100	131
<b>8</b>								<b>290</b>	133	211
<b>9</b>									<b>224</b>	164
<b>10</b>										<b>300</b>

Numbers in bold represents the number of proteins present in each strain, the other numbers represent the number of proteins shared between any two strains.

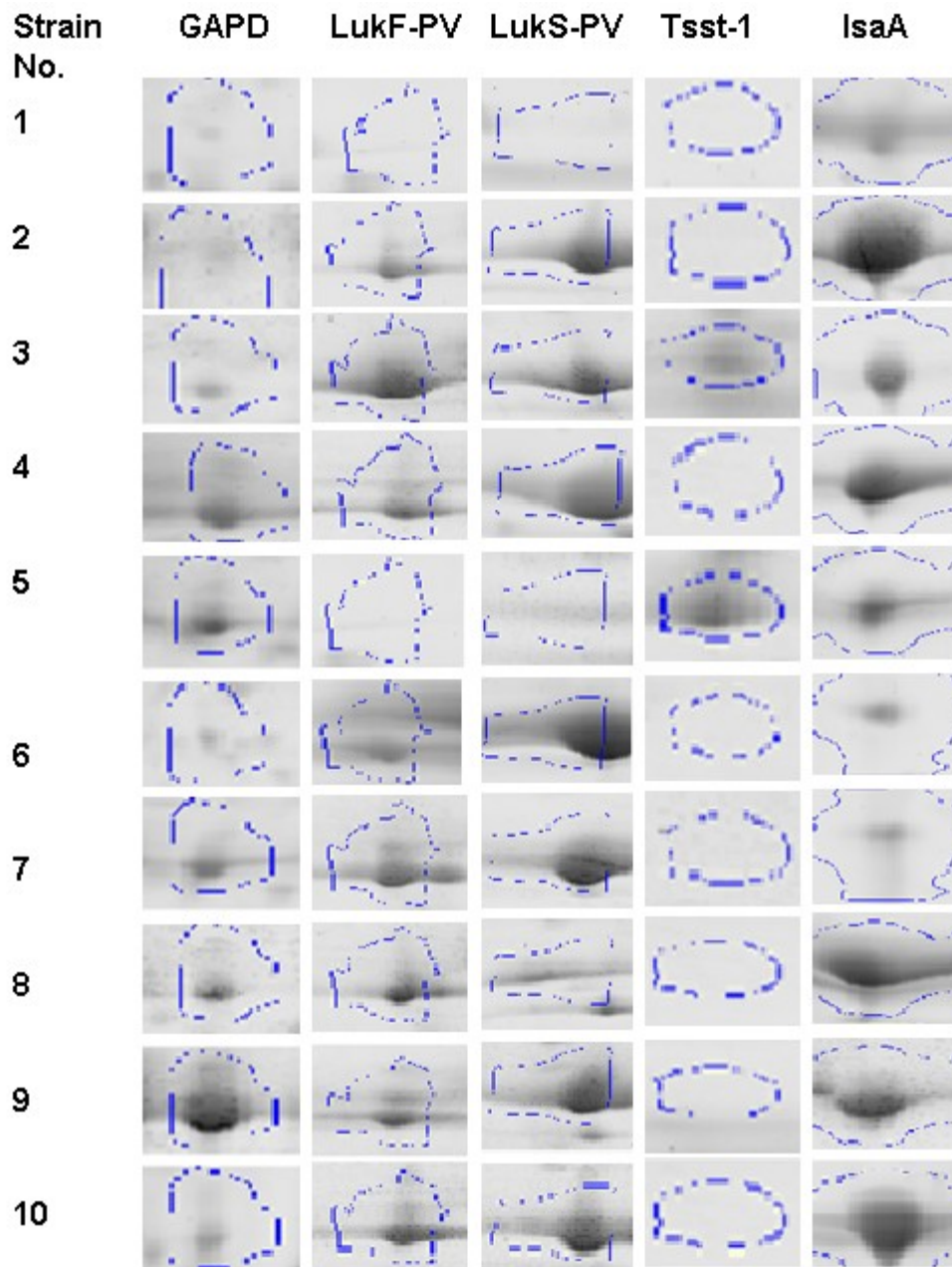


**Figure 5. 5. Multidimensional scaling (MDS) plot of strain similarities.** The distance between any two strains show how their phynotypic characteristics related to each other.

Ziebandt et al., (2010) used 25 clinical isolates belonging to 17 clonal complexes with a high degree of secretome heterogeneity. They suggested that variation in the expression levels of virulence genes may relate to differential activities of specific *S. aureus* gene regulators.

### **5. 2. 5. Individual Protein Expression Variability among Isolates**

In addition to the variability of whole 2DE protein patterns, results also showed differences in the quantities of individual proteins among different clinical isolates (Figure 5. 6). Both subunits of PVL toxin appeared in different abundance among these clinical isolates, the difference were also noticed between both subunits in strain 2 the amount of LukS-PV was several times higher than amount of LukF-PV in the same strain. Proteins involved in central carbon metabolism, for example Glycerinaldehyde 3-phosphate dehydrogenase (GPDH), also showed variability among these isolates for example in strain 9, it was highly expressed; however in strain 1, it was hardly detected. Variation in the expression levels of proteins may relate to differential activities of staphylococcal gene regulators, specifically in the case of virulence factors as the activity of staphylococcal *agr* (Novick et al., 2003). A study by Ziebandt et al., (2004) showed that loss of RNAIII, which is the main regulator of *agr*, dramatically affected the extracellular protein patterns of *S. aureus*. By comparing the extracellular protein pattern of the wild-type with that of an isogenic *agr* mutant, they showed that the levels of most protein spots were decreased in the mutant (Ziebandt et al., 2004). Several proteins on 2DE gels have shown to resolve in more than one spot and sometimes in trains, as a series of spots either as a result of PI variation such as SEB and SEC, and some times due to MW variation such as for IsaA. The most extensively modified *S. aureus* proteins such as SEB in strain 9, in which three spots were identified ( 7, 8, and 9 Figure 5. 5), and Isa A (3 spots 14, 15 and 43 Figure 5. 5) These variations might be attributed either to post-translational modification (PTM) or to modification occurring during the preparation of protein samples. Deviations in the MW of these proteins may indicate specific processing of these proteins. Similarly it has been reported that several proteins secreted via the Sec pathway appeared as multiple spots on 2D gels, due to charge alteration and fragmentation (Hecker et al., 2010).



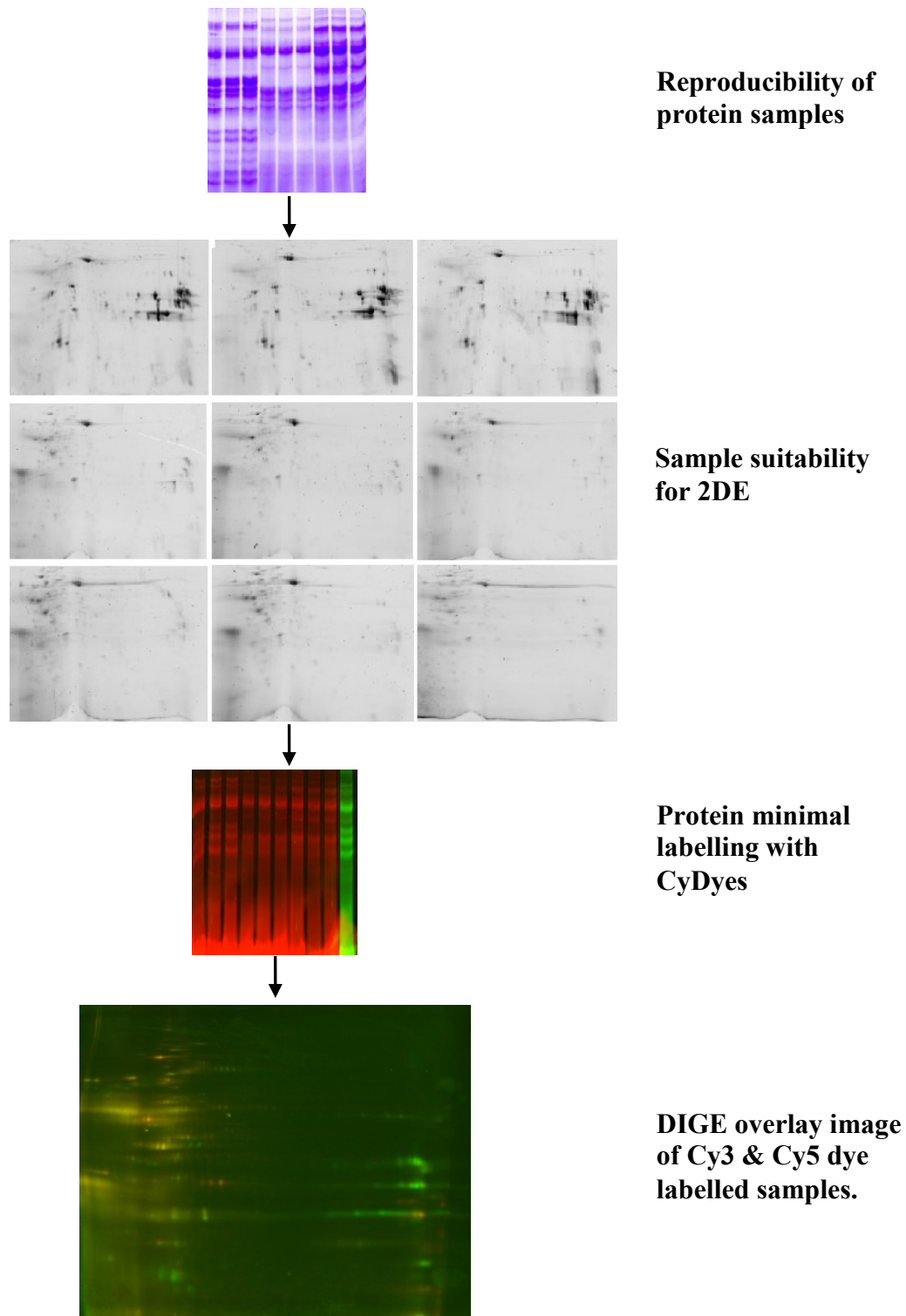
**Figure 5. 6. Variability in the abundance of several proteins in 10 clinical isolates of *S. aureus*.** The respective sector on the 2D gel of each isolate is shown for the protein indicated.

### **5. 3. The Effect of Linezolid and Vancomycin on Secreted Proteins**

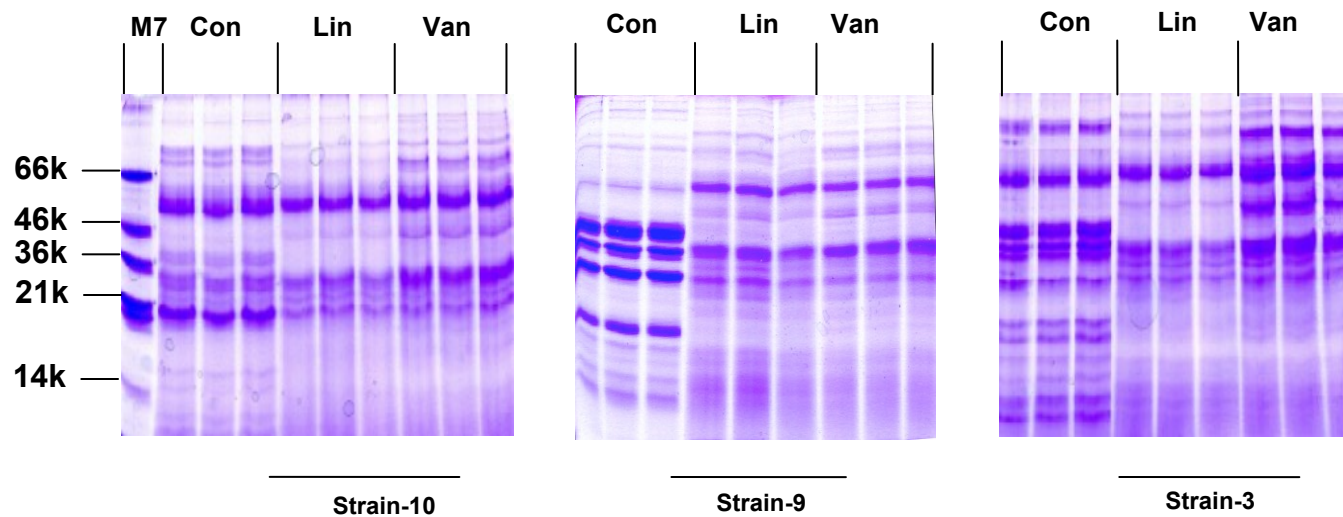
Experiments were designed to examine the effect of linezolid (a protein synthesis inhibitor) and vancomycin (a glycopeptide cell wall inhibitor) on secreted proteins of three strains of *S. aureus*. The clinical isolates represented both MRSA (strain 10), and MSSA (strain 3 and 9). The reason for reducing the number of the strains and antibiotics used, in this part of the study, was due to the economic and time costs involved with carrying our DIGE experiments. To examine the influence of these antibiotics on extracellular virulence factors, protein samples from culture supernatants that had been exposed to antibiotics were compared to control culture supernatants by 2D DIGE; samples from the culture supernatants were collected after 5hrs exposure to antibiotics. Figure 5. 7 shows the strategy followed to carry out the DIGE experiment.

#### **5. 3. 1. Preparation of Protein Samples for CyDye Labelling**

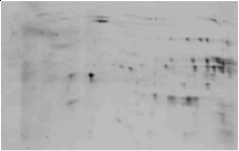
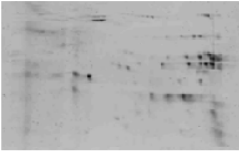
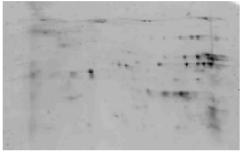
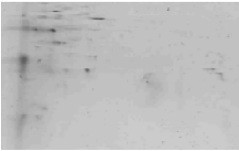
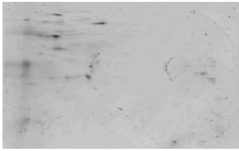
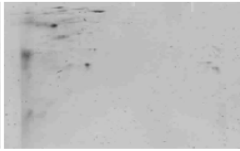


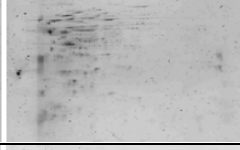

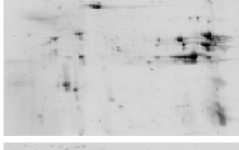


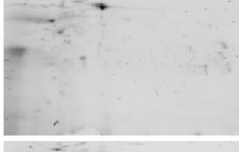





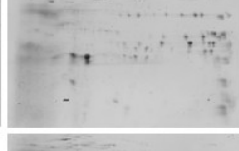
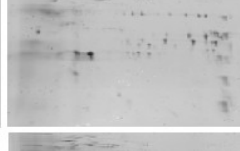






Reproducibility of protein samples was assessed by 1D SDS PAGE in which 10µg of protein samples from three biological replicates of each strain, either in the absence of antibiotics or in the presence of linezolid and vancomycin were analysed (Figure 5. 8). A volume of each urea-solubilised protein sample corresponding to approximately 50µg protein was then subjected to analysis by 2DE on a small gel format. Using this strategy, the validity and suitability of each sample for 2DE electrophoresis was confirmed before starting CyDye labeling (Figure 5. 9). These analyses showed that replicates were equivalent and suitable for labeling purposes. In addition to that, data also showed obvious differences in 1D and 2DE profiles between samples grown in the absence and presence of linezolid and vancomycin. This was an essential requirement for subsequent CyDye minimal labeling of protein for 2D-DIGE analyses. A volume of each urea-solubilised sample corresponding to approximately 200µg protein was then subjected to acetone precipitation and the resulting pellets were resolubilized again in 100µl of labeling buffer (Tris-urea buffer, pH 8.5). Protein samples were then manually adjusted to approximately pH 8.5 if required and the concentration assessed using a modified Bradford assay.



**Figure 5. 7. Diagrammatic strategy for 2DE-DIGE analysis of culture supernatant proteins.**



**Figure 5. 8. Reproducibility of samples by 1D SDS-PAGE prior to proteomic 2D DIGE analysis.** Image shown is representative of 27 samples, from three strains, each grown in the absence (con) and presence of linezolid (lin) and vancomycin (van). Three replicates for each treatment were performed. Quantity of 10 $\mu$ g of each protein sample was solubilised in 1X SDS sample buffer and resolved via SDS PAGE.

Strain treatments	Biological Replicates		
	1	2	3
ST-3 Con			
ST-3 Lin			
ST-3 Van			
ST-9 Con			
ST-9 Lin			
ST-9 Van			
ST-10 Con			
ST-10 Lin			
ST-10 Van			

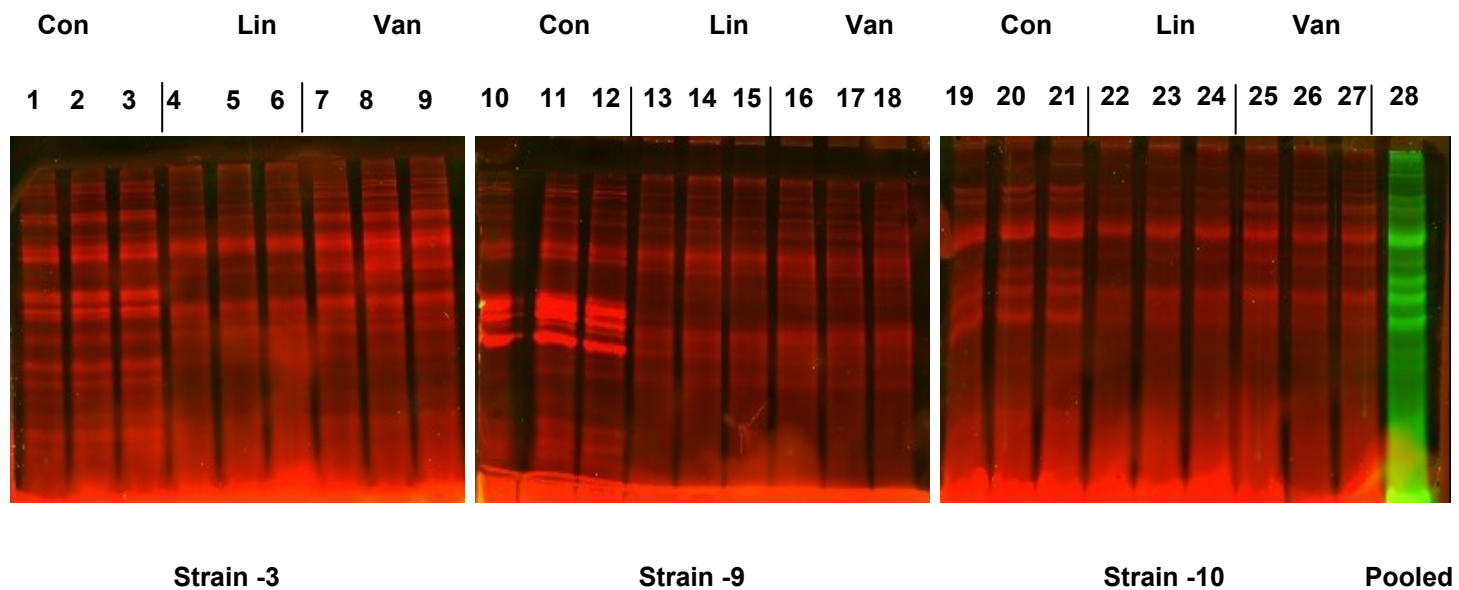
**Figure 5. 9. Reproducibility and Validity of Protein samples for CyDye labelling and DIGE analyses.** Protein samples (50 µg) were loaded via in gel rehydration (IGR) into 7cm pI 3-10 IPG strips and resolved via mini SDS PAGE using a 12 % acrylamide resolving gel and stained with SYPRO-Ruby protein stain. Samples, control and treated with linezolid and vancomycin from three strains 3, 9 and 10; and in three biological replicates.

### **5. 3. 2. Labelling Conditions**

Having established sufficient repeat material for DIGE analyses and established that protein samples were reproducible; 25µg of each protein sample was labelled with CyDye using the minimal CyDye labelling method (Manufacture instructions). This method limits the extent of labelling to only 1% of all lysine residues. All 27 samples, (control and those treated with antibiotics) were labelled with Cy5 and a pooled standard sample, which contains same quantity of all 27 samples, numbered 28, and was labelled with Cy3. The success and efficiency of the CyDye minimal labelling of protein samples was assessed by 1D SDS-PAGE analysis. This was performed by running 2 µg of each protein sample on 1D SDS-PAGE gel (Figure 5. 10), which also demonstrated that samples were equally loaded. Gels were visualised by fluorescent imaging using a Typhoon 9400 (Amersham Biosciences). This demonstrated that total protein could be efficiently labelled using the minimal labelling DIGE chemistry.

### **5. 3. 3. 2DE DIGE Analyses**

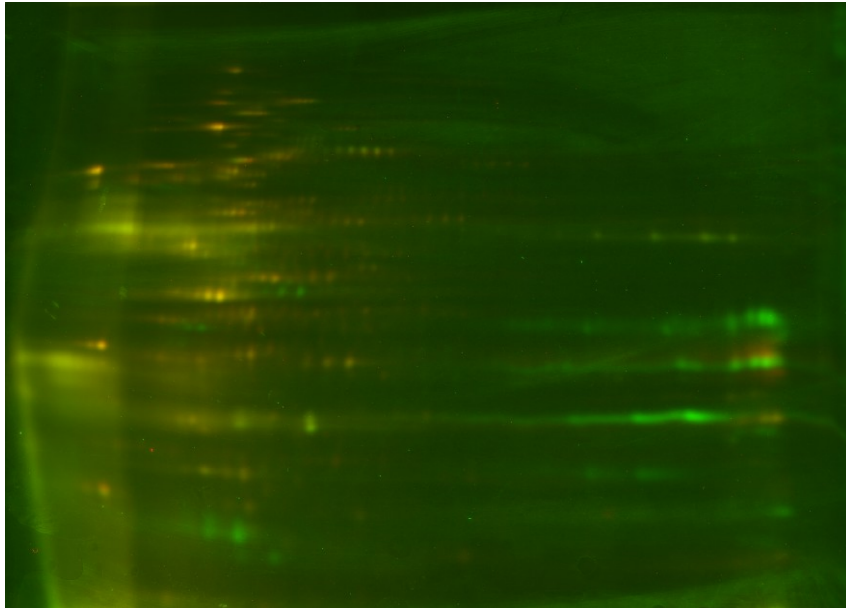
Once successful CyDye labelling was established, large format pH 3-10, 12% polyacrylamide 2DE was performed. Here equal, 12.5µg, quantities of Cy5 labelled sample and Cy3 labelled pooled standard were mixed and loaded into 18cm pH 3-10 IPG strips using the same methodology as for the secretomic analysis (section 5. 2. 1. 2). Gels were then immediately visualised via fluorescence imaging using a Typhoon 9400 variable mode imager (Amersham Biosciences) detecting both Cy images simultaneously, and obtaining a maximum pixel intensity above 60,000 but below 100,000 (saturation) for each image by selecting appropriate photon multiplier tube (PMT) values. Fluorescent images from each DIGE gel can be viewed either as individual Cy images or as a double overlay image (Figure 5. 11). An overlay image of both Cy images gave some indication of the spots that changed in abundance following antibiotic treatment. Green spots are those showing a decrease and red spots are those showing an increase in abundance in that sample compared with the internal standard sample. However, this kind of information is not quantitative as spot abundance have not been normalised at this stage.



**Figure 5. 10. Labelling Efficiency of protein samples using Minimal CyDye labeling.** Protein samples belongs to strain 3 (1-9), strain 9 (10-18) and strain 10 (19-27) were labelled with Cy5, the pooled standard sample (lane 28) was labelled with Cy3. Quantity of 2  $\mu$ g of each protein sample was resolved via SDS PAGE using 12 % acrylamide resolving gel.

pI 3

10



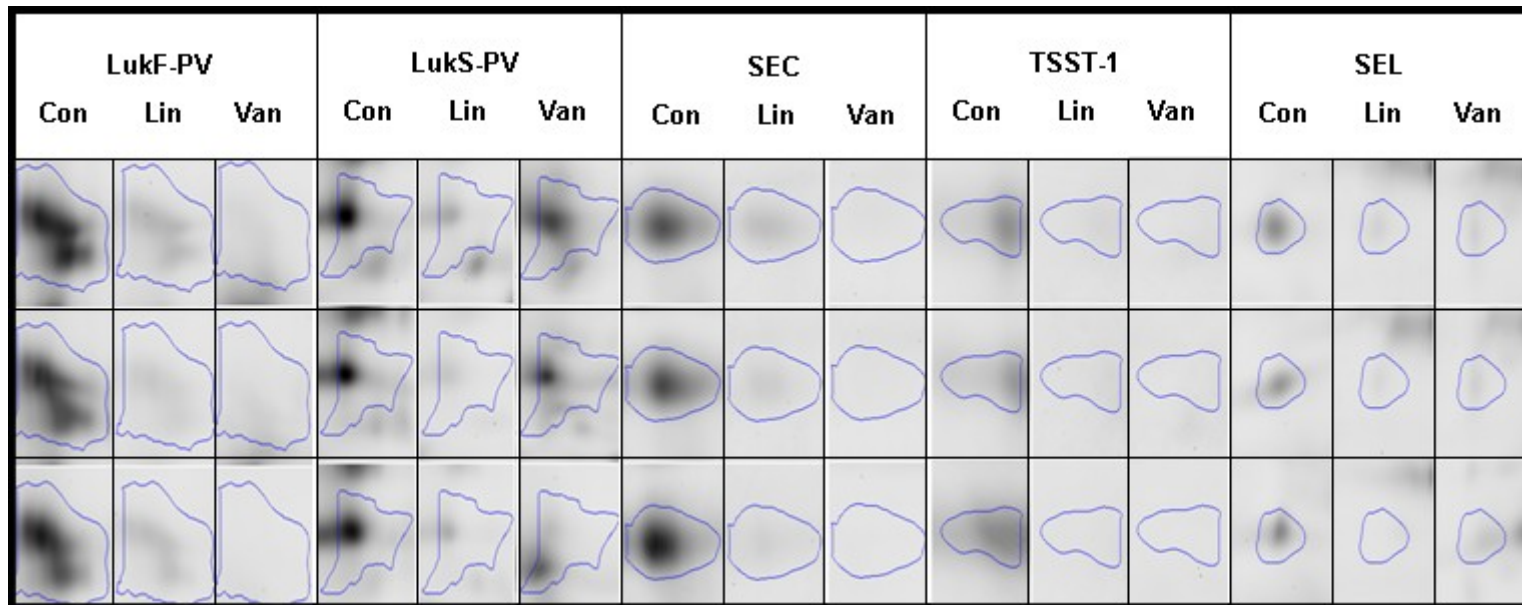
**Figure 5. 11. A typical example of 2DE-DIGE overlay image**

The gel generated for the labelled protein sample, from strain 10 exposed to vancomycin labelled with Cy5 mixed with pooled standard labelled with Cy3. Green spots indicate spots that have a reduced abundance in the presence of vancomycin; red spots have an increased abundance and yellow spots do not change in abundance when treated with this antibiotic.

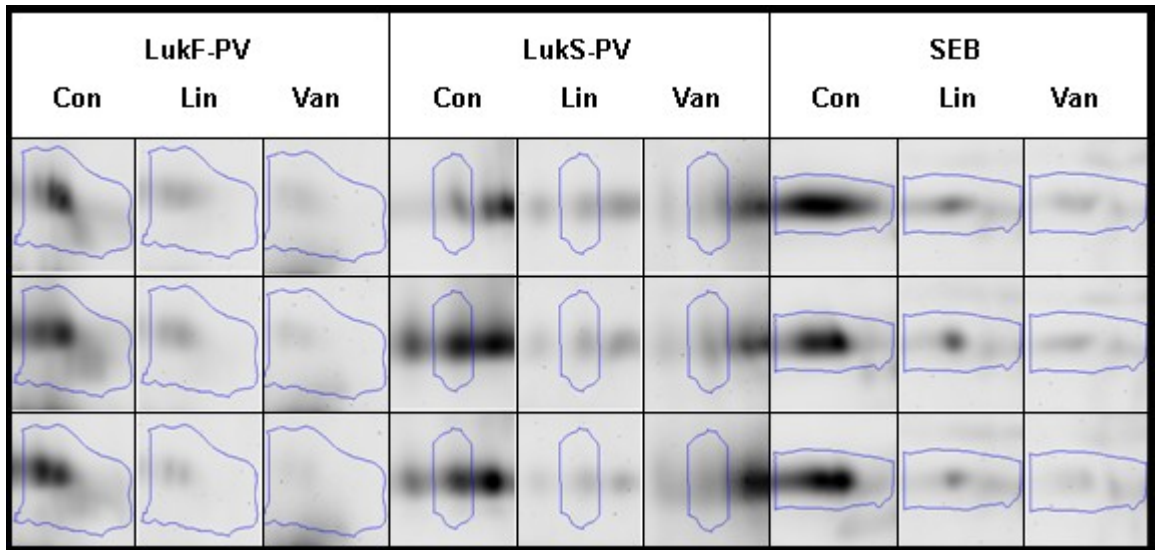
Having obtained all 2DE DIGE images for all 27 gels, they were imported into the Progenesis SameSpots software (Nonlinear Dynamics) and subjected to data analyses as explained in section 5. 2. 2. 2DE images were first cropped, selecting only the area of the gel within which proteins had resolved and boundary regions without proteins, were removed. The pooled internal standard image was then selected as a reference map to which all other gels were matched by making a number of vectors manually before matching all 2DE DIGE images automatically by the software. 2DE DIGE images were aligned so that cognate spots are at corresponding locations across different gels. The protein spots volume (abundance) was subsequently normalised and quantified by the software. To show the significance of the expression differences of proteins between two groups, grown in the absence and in the presence of certain antibiotic, ANOVA test (equivalent t-test for two groups) was used. The tests return a p-value that takes into account the mean difference and the variance and also the sample size of each spot.

#### **5. 3. 4. The Effect of sub-MIC of Linezolid and Vancomycin on Toxin Abundance in Culture Supernatants.**

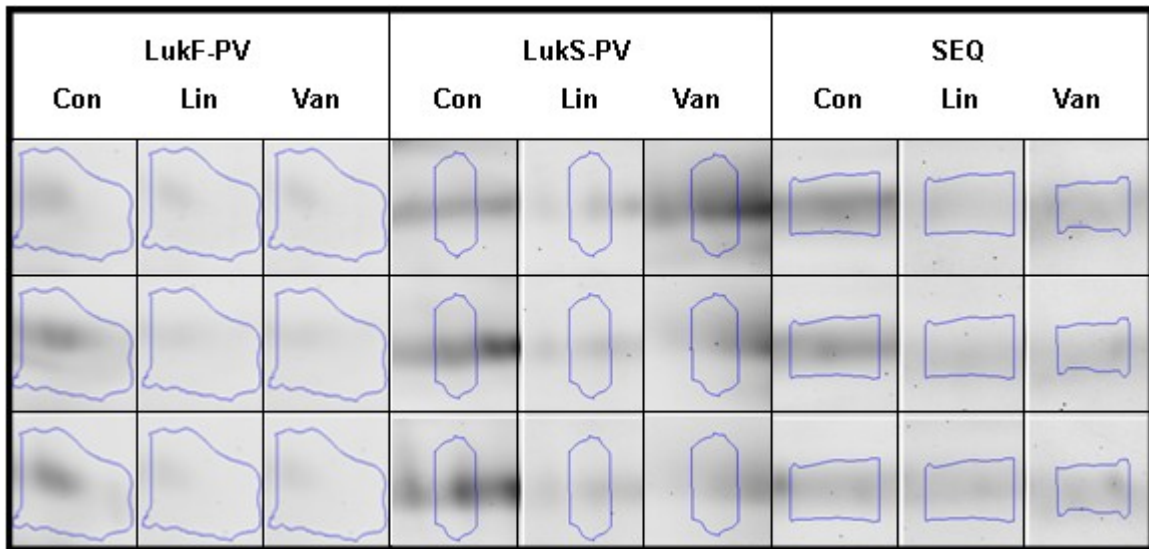
The Progenesis SameSpot software was used to determine the average changes in protein abundance across all 27 gels for each protein spot between controls and antibiotic treated samples. The three strains used in this analysis contained both subunits of the PVL toxin. In addition, strain 3 contained SEC, SEL and TSST-1; strain 9 contained SEB and strain 10 contained SEQ. All strains produced significantly lower levels of the PVL subunits in cultures exposed to either linezolid or vancomycin, ranging from 3.8 to 29-fold reductions (Figures 5. 12, 5. 13, 5. 14 and Table 5. 3). Reductions in the levels of the two PVL subunits were usually of a similar magnitude for a particular strain and antibiotic treatment. The exceptions to this were strains 3 and 9, which showed a wide variation in PVL subunit levels when treated with vancomycin; no clear pattern was present in these data with a greater reduction in the PVF subunit observed in strain 3 and a greater reduction in the PVS subunit is strain 9. The abundance of enterotoxins and TSST-1 were also dramatically reduced following either linezolid or vancomycin treatment but, again, there was no clear pattern indicating that one of these antibiotics was superior in terms of reducing virulence factor abundance in culture supernatants.



**Figure 5. 12. Modulation of the abundance of PVL toxin F and S subunits, staphylococcal enterotoxis SEC, SEL and TSST-1 in the absence (con) and the presence of linezolid (lin) and vancomycin (van) treatment of strain 3.**



**Figure 5. 13. Modulation of the abundance of PVL toxin F and S subunits and staphylococcal enterotoxin SEB in the absence (con) and the presence of linezolid (lin) and vancomycin (van) treatment of strain 9.**



**Figure 5. 14. Modulation of the abundance of PVL toxin F and S subunits and staphylococcal enterotoxin Q (SEQ) in the absence (con) and the presence of linezolid (lin) and vancomycin (van) treatment of strain 10.**

**Table 5. 6. Changes in toxin levels in culture supernatants of three *S. aureus* strains five hours after antibiotic treatment.**

Strain	Antibiotic	Fold change of each virulence factor, in each strain exposed to two antibiotics.						
		PVS	PVF	SEC	TSST-1	SEL	SEB	SEQ
3	Linezolid	0.10 ** ↓	0.076 ** ↓	0.076 ** ↓	0.129 ** ↓	0.090 ** ↓		
	Vancomycin	0.22 ** ↓	0.034 ** ↓	0.185 ** ↓	0.066 ** ↓	0.217 * ↓		
9	Linezolid	0.22 ** ↓	0.26 ** ↓				0.25 ** ↓	
	Vancomycin	0.05 ** ↓	0.15 ** ↓				0.083 ** ↓	
10	Linezolid	0.113 ** ↓	0.16 ** ↓					0.25 ** ↓
	Vancomycin	0.153	0.116 * ↓					0.20

Green downward arrows indicate significant decrease of the virulence factor abundance. \*  $p < 0.05$  and \*\*  $p < 0.001$ , were produced by the Progenesis software using one way ANOVA test. Seven virulence factors were studied here, which are PVL subunits PVS and PVF, Toxic shock syndrome toxin TSST-1, Enterotoxins C, L, B and Q.

For example in strain 3, linezolid was more effective at reducing the levels of SEC and SEL but vancomycin was more effective at reducing the levels of TSST-1. Nevertheless, both vancomycin and linezolid proved to be very effective at reducing the levels of toxin and virulence factors in culture supernatants.

DIGE data also showed that in addition to virulence factors many other proteins were significantly down-modulated by both linezolid and vancomycin. However there were also a number of proteins that were up-modulated in the presence of each antibiotic. Table 5. 7. shows the number of proteins up and down-modulated with effect of each antibiotic used. Virulence factors were among those down-modulated, with effect of both antibiotics. However, most proteins identified to be cytoplasmic proteins were among those proteins up-modulated in the presence of these two antibiotics. Similarly Bernardo et al., (2004) found that sub-MIC of linezolid induced the secretion of a number of cytoplasmic proteins into bacterial supernatant, but reduced the release of virulence factors (Bernardo et al., 2004). How linezolid increase cytoplasmic protein levels remains to be investigated, it could be speculated that linezolid may stimulate MVs by unknown mechanism.

**Table 5. 7. General profile of *S. aureus* whole proteins changing with effect of linezolid and vancomycin using DIGE technique.**

Strain	Number of proteins changed in cultures grown in the presence of linezolid		Number of proteins changed in cultures grown in the presence of vancomycin	
	Up-modulated	Down-modulated	Up-modulated	Down-modulated
<b>3</b>	185	127	228	125
<b>9</b>	119	132	140	130
<b>10</b>	9	94	29	90

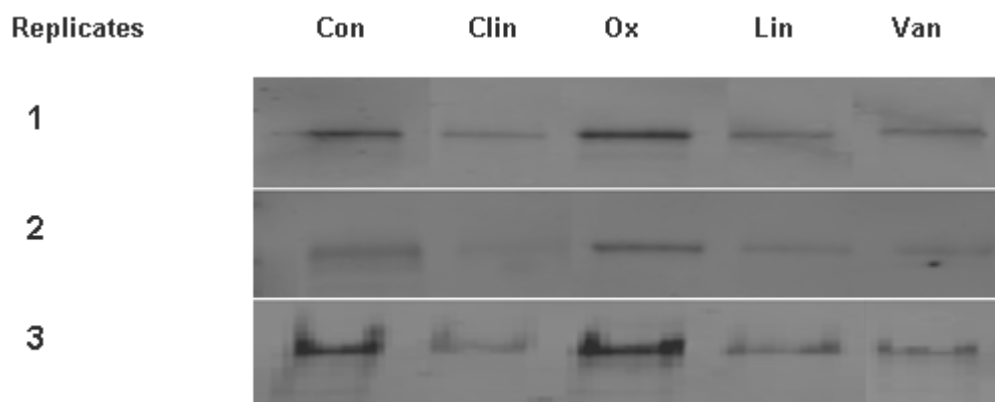
At the protein level, linezolid, caused both PVL protein subunits PVS and PVF to be dramatically reduced in all three isolates. The other virulence factors were also significantly reduced at the protein level by linezolid (e.g. TSST-1, SEC and SEL in strain 3, SEB in strain 9, and SEQ in strain 10). Similarly in a study of toxin genes, clindamycin and linezolid markedly suppressed translation but not transcription, which supports the contention that protein-synthesis inhibition is an important consideration in the selection of antimicrobial agents to treat serious infection caused by toxin-producing *S. aureus* (Stevens et al., 2007). Data showed that the protein synthesis inhibitors might be a better choice for treatment of infections caused by these bacteria particularly those producing high amount of toxins. However there are some limitations of these types of antibiotics, for example clindamycin can not be used for many MRSA infections because MRSA strains, particularly HA-MRSA strains, harboring *mecA* type II and III, are multi-drug resistant, which includes resistance to clindamycin (Koszczol et al., 2006).

Dumitrescu et al. studied the effect of several antibiotics on PVL producing *S. aureus*, their results shows that subinhibitory concentration of clindamycin and linezolid significantly reduced PVL release; they suggested that the possible mechanism is through their impact on bacterial protein synthesis (Dumitrescu et al., 2007). Recent studies are confirming that linezolid appears to be the most promising antimicrobial agent especially against CA-MRSA strains as it has been used successfully in three patients with necrotizing pneumonia to date (Morgan, 2007). Linezolid has been found to be more effective against PVL producing *S. aureus* infections *in vivo* than vancomycin. This may be due to the fact that linezolid may inhibit PVL production, probably resulting in reduced pathogenicity of PVL positive *S. aureus*, as linezolid markedly suppresses translation of the PVL gene, and a subinhibitory concentration of linezolid inhibits the PVL level in a concentration-dependent manner (Yangihara et al., 2009). The efficiency of linezolid may be due, in part, to the high concentrations achieved in skin, as well as the more subtle action of inhibition of toxin and enzyme production by *S. aureus* (Gemmell et al., 2002). Rayner et al, reported that antibiotics such as linezolid and quinopristin/dalfopristin have a good anti-staphylococcal activity but are very expensive and should be reserved for patients

who fail on, or are intolerant of conventional therapy, or who have highly resistant strains such as hetero-VISA (Rayner et al., 2005).

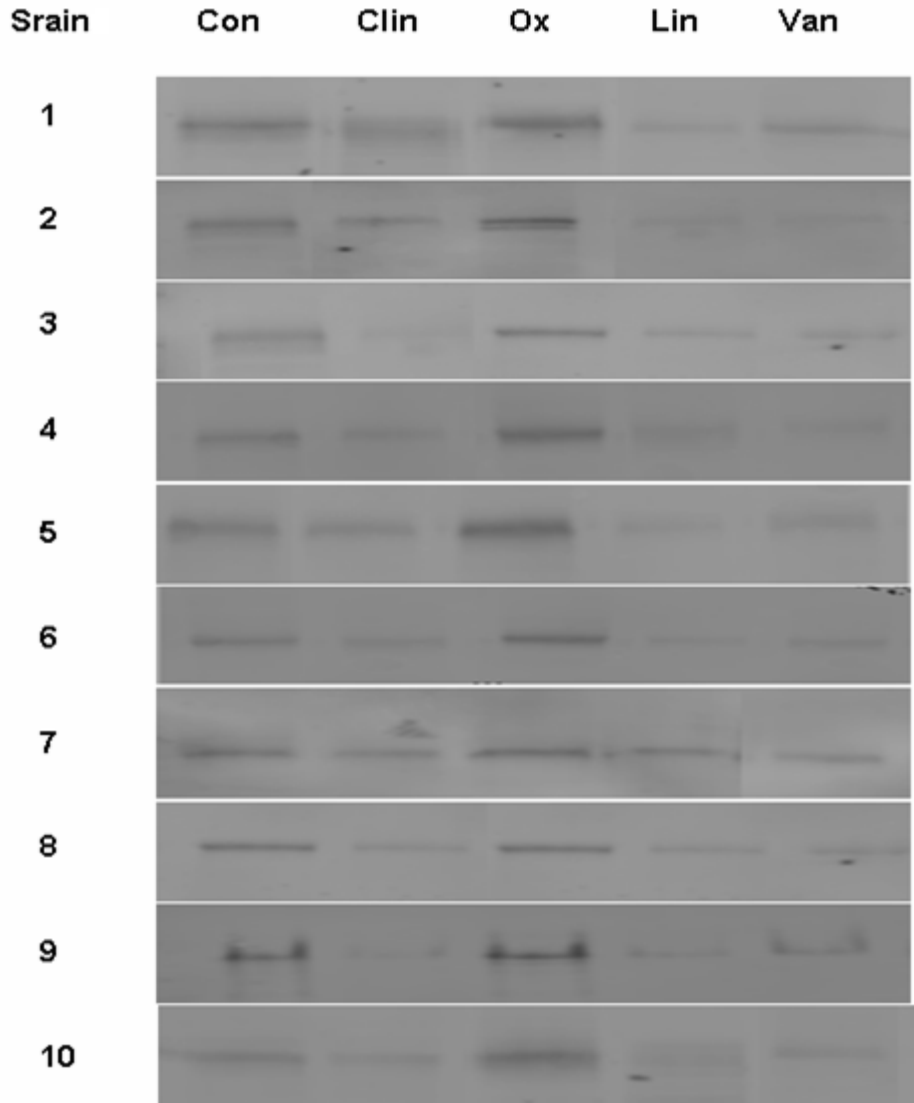
#### **5. 4. Effect of Antibiotics on Spa levels.**

A number of attempts were made to identify Spa during the secretome analysis, by picking spots from the area of the 2D gel corresponding to the PI and molecular weight of Protein A and subjecting them to MALDI-TOF, but these were not successful. As an antibody against Protein A is commercially available, western blotting was used to determine the effect of antibiotic treatment on Protein A levels. Proteins from culture supernatants were separated by 1D PAGE then transferred to nitrocellulose membrane (Amersham Biosciences). The cross-reacting proteins with primary antibody were detected using Cy3-conjugated secondary antibody (Sigma) and quantified following scanning on a Typhoon 9400. Three technical replicate western blots were used for each antibiotic treatment and the average volume of these used to determine what effect the antibiotic treatment had on protein A levels (Figure 5. 15). In strain 3, for example, oxacillin was the only antibiotic that caused an increase in the amount of Spa protein. Here the protein two times greater in cultures incubated with oxacillin than without antibiotic. The other three antibiotics (clindamycin, linezolid and vancomycin) significantly reduced Spa protein levels, with the greatest reduction seen in cultures grown with clindamycin, as the amount of Spa protein was 7 times less compared to control sample (Figure 5. 15). With the exception of oxacillin the other three antibiotics decreased protein A abundance after five hours incubation of cells with those antibiotics (Figure 5. 16. and Table 5. 8). Spa increased in all strains in the presence of oxacillin with the highest change in strain 8, in which the protein was 3.26 times greater than in cultures grown without adding antibiotics. Figure (5. 16) summarizes the up and down-modulation of Spa. In which linezolid, clindamycin and vancomycin down-modulated the virulence factor with different fold changes, however oxacillin caused up-modulation of the protein in all strains.



**Figure 5. 15. A typical example of western immunoblotting technique for strain 3, showing the effect of antibiotics on protein A (Spa) levels.** Cells were grown in the absence (con) and presence of clindamycin (clin), oxacillin (ox), linezolid (lin) and vancomycin (van). Data are represents three technical replicates numbered 1, 2 and 3. The intensity volume mean and standard deviation of control (30 and 0.75), clin. (4.34 and 1.83), ox. (91.31 and 3.62), lin. (6.23 and 1.40) and van. (8.64 and 1.61) respectively. Replicate means and standard deviations of all strains summarized in Table (5. 9).

In a study by Veringa et al., (1986) The inhibition of protein A synthesis by sub-MIC of clindamycin in *S. aureus* caused enhanced uptake of clindamycin-treated *S. aureus* by PMNs. Because clindamycin is considered to be a general inhibitor of protein synthesis, it is reasonable to assume that clindamycin inhibits the synthesis of protein A (Veringa et al., 1986). This suggesting additional therapeutic efficiency of clindamycin for treatment of these infections (Koszczol et al., 2006). Staphylococcal *agr* mutant are known to over express protein A, thus antibiotics affecting the synthesis of regulator protein, may cause to increase in Spa expression (Gemmel et al., 1995). It has been reported that the influence of sub-MIC of clindamycin must interfere with translation of one or more regulatory gene products that in turn, affect transcription of the protein (Herbert et al., 2001)



**Figure 5. 16. The effect of four antibiotics on SPA abundance in culture supernatants.** Western immuno blots using anti-Protein A primary antibody and Cy3 conjugated secondary antibody either in the absence (Con) or presence of antibiotics. Clin, Clindamycin; Ox, Oxacillin; Lin, Linezolid; Van, Vancomycin. Three replicates were used for each treatment as indicated by standard deviation and P value (Table 5. 9).

**Table 5. 8. Fold change in Protein A abundance in culture supernatants five hours after incubation in the absence (con) and presence of clindamycin (clin), oxacillin (ox), linezolid (lin) and vancomycin (van).**

Strain	Con	Clin	Ox	Lin	Van
<b>1</b>	1	0.318 ↓	1.77 ↑	0.312 ↓	0.67
<b>2</b>	1	0.512 ↓	2.032 ↑	0.11 ↓	0.098 ↓
<b>3</b>	1	0.144 ↓	3.042 ↑	0.207 ↓	0.288 ↓
<b>4</b>	1	0.479 ↓	1.713 ↑	0.106 ↓	0.179 ↓
<b>5</b>	1	0.465 ↓	1.63 ↑	0.209 ↓	0.325 ↓
<b>6</b>	1	0.103 ↓	2.026 ↑	0.160 ↓	0.147 ↓
<b>7</b>	1	0.445 ↓	2.77 ↑	0.103 ↓	0.129 ↓
<b>8</b>	1	0.285 ↓	3.215 ↑	0.132 ↓	0.30 ↓
<b>9</b>	1	0.359 ↓	3.26 ↑	0.313 ↓	0.118 ↓
<b>10</b>	1	0.121 ↓	2.186 ↑	0.90	0.806

Green downward arrows indicate significant decrease and red upward arrows indicate significant increase (p<0.05).

**Table 5. 9. Western Blotting data of the effect of clindamycin, oxacillin, linezolid and vancomycin on the secretion level of Spa virulence factor in 10 nclinical isolates of *S. aureus*.** Three replicates were used, as indicated by standard deviation (STDEV) and standard error (STERR). Statistical T-TEST was performed as indicated by P-values.

Con.	Replicates			Mean	STDEV	STERR		
Strain	1	2	3				Effect Clin.	P value
1	29.13	28.71	30.45	29.43	0.907965	0.524229		
2	47.17	49.19	49.17	48.51	1.160517	0.670045		
3	30.72	29.21	30.07	30	0.75743	0.437315		
4	39.23	40.21	39.54	39.66	0.500899	0.289203		
5	31.22	29.71	30.27	30.4	0.763348	0.440732		
6	35.27	36.33	35.86	35.82	0.531131	0.306658		
7	20.33	18.16	19.23	19.24	1.085035	0.626463		
8	19.92	20.87	21.19	20.66	0.66053	0.381368		
9	20.17	19.47	20.57	20.07	0.556776	0.321464		
10	23.82	22.42	22.67	22.97	0.746659	0.431097		
<b>strain</b>								
	1	2	3	Mean	STDEV	STERR	Effect Clin.	P value
1	8.22	10.41	9.51	9.38	1.100772	0.63555	0.318722	1.69E-05
2	22.32	27.12	25.08	24.84	2.408983	1.390868	0.512059	1.06E-04
3	5.81	4.92	2.29	4.34	1.830273	1.05674	0.144667	2.34E-05
4	18.67	20.71	17.68	19.02	1.545024	0.892046	0.479576	2.52E-05
5	15.23	13.19	13.94	14.12	1.031843	0.595752	0.464474	2.54E-05
6	4.32	2.93	3.82	3.69	0.70406	0.406501	0.103015	3.78E-07
7	7.12	10.11	8.45	8.56	1.498032	0.864915	0.444906	5.62E-04
8	4.29	7.11	6.27	5.89	1.447895	0.835967	0.285092	8.76E-05
9	5.95	8.83	6.88	7.22	1.469796	0.848612	0.359741	1.44E-04
10	2.11	3.57	2.69	2.79	0.735119	0.424434	0.121463	4.82E-06
<b>strain</b>	<b>Ox</b>							
	1	2	3	Mean	STDEV	STERR	Effect Ox.	
1	55.71	50.32	50.24	52.09	3.135267	1.8102	1.769963	2.74E-04
2	101.22	99.33	95.28	98.61	3.034749	1.752164	2.032777	1.17E-05
3	87.24	94.21	92.48	91.31	3.629311	2.095445	3.043667	8.84E-06
4	64.21	68.71	70.96	67.96	3.436932	1.984372	1.713565	1.46E-04
5	52.13	50.72	46.43	49.76	2.968788	1.714081	1.636842	3.97E-04

<b>6</b>	68.72	75.12	73.96	72.6	3.409868	1.968746	2.026801	5.07E-05
<b>7</b>	49.75	54.32	56.1	53.39	3.275561	1.891202	2.774948	6.79E-05
<b>8</b>	67.32	63.72	68.31	66.45	2.415512	1.394638	3.21636	5.92E-06
<b>9</b>	63.22	66.81	66.71	65.58	2.044431	1.180388	3.267564	3.12E-06
<b>10</b>	47.45	53.82	49.45	50.24	3.257653	1.880862	2.187201	1.46E-04
<b>strain</b>	<b>Lin</b>							
	1	2	3	<b>Mean</b>	<b>STDEV</b>	<b>STERR</b>	<b>Effect Lin.</b>	
<b>1</b>	8.24	10.29	9.01	9.18	1.035519	0.597875	0.311927	1.41E-05
<b>2</b>	4.12	6.45	5.45	5.34	1.168888	0.674878	0.11008	1.41E-06
<b>3</b>	7.81	5.12	5.76	6.23	1.40524	0.81134	0.207667	1.34E-05
<b>4</b>	3.02	6.24	3.43	4.23	1.752741	1.011975	0.106657	4.64E-06
<b>5</b>	4.65	7.24	7.22	6.37	1.489597	0.860045	0.209539	1.55E-05
<b>6</b>	4.12	6.92	6.24	5.76	1.460411	0.843193	0.160804	4.73E-06
<b>7</b>	1.73	3.04	1.2	1.99	0.947154	0.546855	0.10343	3.19E-05
<b>8</b>	1.94	4.12	2.16	2.74	1.200167	0.692937	0.132623	2.25E-05
<b>9</b>	5.11	8.13	5.78	6.34	1.58597	0.915687	0.315894	1.45E-04
<b>10</b>	18.94	22.35	20.9	20.73	1.711345	0.988074	0.902481	1.06E-01
<b>strain</b>	<b>Van</b>							
	1	2	3	<b>Mean</b>	<b>STDEV</b>	<b>STERR</b>	<b>Effect Van.</b>	
<b>1</b>	18.21	22.01	19.75	19.99	1.911335	1.103542	0.679239	1.51E-03
<b>2</b>	3.94	5.21	5.13	4.76	0.711266	0.410662	0.098124	6.23E-07
<b>3</b>	6.91	8.89	10.12	8.64	1.619537	0.935068	0.288	3.22E-05
<b>4</b>	6.21	9.04	6.14	7.13	1.654479	0.955242	0.179778	5.28E-06
<b>5</b>	11.24	9.03	9.4	9.89	1.183681	0.683418	0.325329	1.47E-05
<b>6</b>	4.02	7.01	4.75	5.26	1.558878	0.900045	0.146845	5.59E-06
<b>7</b>	2.11	3.42	1.94	2.49	0.809877	0.467596	0.129418	2.81E-05
<b>8</b>	5.34	7.25	6.1	6.23	0.961613	0.555204	0.301549	2.81E-05
<b>9</b>	2.02	3.48	1.61	2.37	0.982904	0.567496	0.118087	1.10E-05
<b>10</b>	17.23	21.33	16.97	18.51	2.445649	1.412038	0.805834	3.91E-01

**CHAPTER 6.**  
**GENERAL DISCUSSION**

## 6.1. Clonal Lineages of *S. aureus* Strains used in This Study

The population structure of *S. aureus* has been studied previously by a variety of techniques, including multilocus enzyme electrophoresis, PFGE, and MLST. These studies have revealed a highly clonal population structure in *S. aureus* organism (Feil et al., 2003). This clonal structure of the population in *S. aureus* is an indication that recombination has had negligible impact on the diversification of the core genome of this species. Such a view is consistent with an examination of intra-clonal diversity, which suggests that the vast majority of clonal variants arise by point mutation, rather than recombination (Feil et al., 2003). A major advantage of MLST is the ability to compare sequence data between laboratories via the MLST website on the internet (Berglund et al., 2005). In addition, the data obtained by MLST permits investigation of the population structure and the development and testing of evolutionary hypotheses (Smith et al., 2005). The major disadvantage of MLST is that a single base pair sequencing error can result in assignment to a wrong ST.

Data generated in this study using 10 isolates confirmed a high degree of clonality, with seven CCs identified (CC5, CC8, CC88, CC30, CC22, CC1 and CC121). These CCs belong to the same *S. aureus* population structure and most prevalent strains worldwide (Chambers et al., 2009. Feil et al., 2003, Larsen et al., 2008 and Rasigade et al., 2010). CC22, the genetic background for the EMRSA-15 clone, which is the most prevalent CC type in the UK and Ireland, was also found in this study within two isolates. However the two CC22 isolates belonged to ST-217, not ST-22, which is the ST of EMRSA-15., ST-217 is one of the rare ST types in the UK, but it has been found in the other countries previously. For example, it has previously been reported in the Netherlands, where together with ST-22 representing 24 isolates out of 398 isolates of *S. aureus* during several years in 14 medical centres (Rijnders et al., 2009). ST-217 was also identified in a study of Swiss strains; ST217 was the predominant clone (18 isolates from total 178 isolates) and generally exhibited high levels of oxacillin and ciprofloxacin resistance (Qi et al., 2005). One of the isolates, originally isolated from a superficial infection, belonged to CC121; other isolates from this CC have previously been reported from superficial infections (Wiese-Posselt et al., 2007). ST-121, the historical PVL-positive MSSA genetic

background in France has been gradually replaced over the past decade by MSSA lineages related to CA-MRSA (mostly ST-80 PVL-positive MSSA) (Resigade et al., 2010).

From the strains studied here two isolates were found to have the same sequence type of CA-MRSA USA300 strain. This is a common strain and spread widely among different community and health care settings in the USA. The, USA300 lineage, is not prevalent in the UK, or elsewhere in Europe, with only isolated cases having been identified (Monecke et al., 2007). It was possibly imported into the UK by carriers of USA 300 from the USA, indicating that the world is now such a small village; through increased human movements around the world, the spread of bacterial strains is easier than ever before.

## **6. 2. The Effect of Antibiotics on Toxin Gene Expression in *S. aureus***

The increasing incidence of bacterial infections due to *S. aureus*, and the rise in MRSA and VRSA therein, is of great concern. As the resistance to most antibiotics rises, modulation of virulence factor expression by antibiotic treatment may be of increasing importance (Gemmel et al., 2002). The effect of sub-MIC of antibiotics on toxin production is an important consideration in the selection of an antimicrobial agent to combat staphylococcal infections (Stevens et al., 2007). The possible up and down regulation of exoprotein release is especially important for *S. aureus* infections because this bacterium produces a large number of toxins and virulence factors. This determines, at least in part, the pathogenesis of the infections caused by different strains of *S. aureus*. This antibiotic-induced modulation could lead to either worsening or attenuation of the disease (Gemmel et al., 1991).

The exposure of bacteria to sub-inhibitory concentrations of antibiotics under clinical conditions is highly probable. For example, drug–drug interaction, drug–food interaction or certain clinical or health conditions of the patient could result in decreased bioavailability of antibiotics in systemic circulation, and therefore, sub-inhibitory levels of antibiotics may be established. Moreover, systemic absorption of topically applied antibiotics will result in the establishment of sub-therapeutic levels of these antibiotics for asymptomatic infections or chronic bacterial colonizers in other parts of the body. In

addition, in certain situations, sub-inhibitory concentrations are probable in the internal milieu of biofilms even though the surrounding environment may have lethal concentrations, a condition that is attributed to inactivating enzymes or physical and chemical barriers to antibiotic penetration (Hogan and Kolter 2002; Fux et al. 2005). Therefore, the effects of sub-MIC concentrations of antibiotics on microorganisms is of continuing interest to microbiologists (Haddadin et al., 2010). Sub-inhibitory concentrations of certain antibiotics, especially those that inhibit bacterial protein biosynthesis, may have an impact on host-pathogen interactions, such as the adherence of bacteria to epithelial cells. Since microbial adherence is the initial step of many infectious processes, the ability of antibiotics to affect this property may be an important criterion in selecting an antibiotic for therapy. Thus, antibiotic-induced modulation of virulence factors might lead to either aggravation or attenuation of the disease (Bleckwede et al., 2005).

SpA as an important virulence factor has been chosen as one of the target proteins to see the effect of studied antibiotics on its levels in the supernatant. Spa, which exists in both secreted and membrane-associated forms, possesses two distinct Ig-binding activities: each domain can bind Fc region of IgG, involved in effector functions and Fab, the Ig fragment responsible for antigen recognition (Graille et al., 2000). The virulence factor Spa, is among staphylococcal proteins with potential Sec-type signal peptides and potential LPXTG cell wall sorting signal for covalent cell wall binding (Sibbald et al., 2006). Spa is primarily known for its ability to bind to the Fc region of IgGs and inhibit opsono-phagocytosis. This is due to its interaction with IgG Fc and becomes coated with IgG in an inappropriate conformation not recognized by the Fc receptor on neutrophils (Gomez et al., 2006). As to the role that Spa is playing, this molecule can interfere with innate host defense in several ways other than its well-known anti-opsonic activity due to binding the Fc portion of immunoglobulin. It activates TNFR1, the receptor for TNF- $\alpha$ ; it interacts with the epidermal growth factor receptor; it activates platelets by binding von Willebrand factor; and it is a superantigen for B cells (Chambers et al., 2009). A novel function of Spa was identified by Merino et al (2009) in the promotion of cell-to-cell interactions and biofilm formation by investigating the protein-dependent biofilm

production of an *agr arlRS* double mutant. The covalent attachment of Spa to the bacterial surface is not required for its ability to promote cell-to-cell interactions. Thus, secreted Spa from *S. aureus* strains or a Spa variant lacking the carboxy-terminal LPXTG domain is sufficient to induce biofilm development (Merino et al., 2009).

In this study sub-MIC of clindamycin and linezolid (protein synthesis inhibitor antibiotics) both caused a reduction in protein A amounts at both the protein and mRNA levels. Clindamycin induced PVL mRNA levels, while linezolid caused a significant reduction of both virulence factors (Spa and PVL) at both levels in all three strains used (Table 6. 1). The reduction of protein secretion by these protein synthesis inhibitor antibiotics could explain their mode of action, as they both inhibit protein synthesis at ribosome levels. How linezolid reduced *spa* mRNA levels in this study remains to be investigated. It has been suggested that these antibiotics must interfere with translation of one or more regulatory gene products that, in turn, affect transcription of these genes (Herbert et al., 2001). Because clindamycin and linezolid are considered to be a general inhibitor of protein synthesis, it is reasonable to assume that they inhibits the synthesis of protein A and other structural proteins and enzymes in *S. aureus* (Veringa et al., 1986). Similarly in a study of toxin genes, clindamycin and linezolid markedly suppressed translation of several virulence factors, which supports the contention that protein-synthesis inhibition is an important consideration in the selection of antimicrobial agents to treat serious infection caused by toxin-producing *S. aureus*, they suggested that the possible mechanism is through their impact on bacterial protein synthesis (Dumitrescu et al., 2008, Stevens et al., 2007).

**Table 6.1. Effect of antibiotics on the levels of PVL toxin and protein A, at the mRNA and protein levels, following 5 h incubation.**

Strains	Virulence factor	<u>Linezolid</u>		<u>Vancomycin</u>		<u>Clindamycin</u>		<u>Oxacillin</u>	
		mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein
3	<u><i>lukS-PV</i></u>		0.10		0.22				
	<u><i>lukF-PV</i></u>	0.684 ↓	0.076 ↓	0.435 ↓	0.034 ↓	6.208 ↑		13,50 ↑	
	<i>spa</i>	0.464 ↓	0.207 ↓	0.127 ↓	0.288 ↓	0.45 ↓	0.144 ↓	5.44 ↑	3.042 ↑
9	<u><i>lukS-PV</i></u>		0.22		0.05				
	<u><i>lukF-PV</i></u>	0.227 ↓	0.26 ↓	0.636 ↓	0.15 ↓	2.339 ↑		3.238 ↑	
	<i>spa</i>	0.403 ↓	0.313 ↓	0.543 ↓	0.118 ↓	0.523 ↓	0.359 ↓	1.89 ↑	3.26 ↑
10	<u><i>lukS-PV</i></u>		0.11		0.15				
	<u><i>lukF-PV</i></u>	0.460 ↓	0.16 ↓	0.291 ↓	0.11 ↓	2.831 ↑		2.901 ↑	
	<i>spa</i>	0.591	0.90	0.816	0.806	0.352 ↓	0.121 ↓	1.68 ↑	2.186 ↑

Green downward arrows indicate significant decrease and red upward arrows indicate significant increase ( $p < 0.05$ ). qPCR was used to quantify the mRNA levels and 2D-DIGE technique for protein levels of both subunits of PVL toxin and Western blotting for SpA protein level.

The induction in *lukSF-PV* mRNA by sub-MIC of clindamycin might be through an indirect effect on regulatory proteins. These data are in good accordance with the observations of Herbert et al. (2001) who demonstrated increased amounts of *coa*- and *fnbB*-specific transcripts in *S. aureus* strains NCTC8325 and WCUH29 when they were treated with sub-MIC concentration of clindamycin. Clindamycin may have a direct or an indirect effect on the regulator proteins that are important in the production of exoproteins. In principle, protein synthesis inhibitors interact directly with the ribosome and stop peptide translation from mRNA. Therefore, it is puzzling that these antibiotics have at the transcriptional levels. With regard to *S. aureus*, it was suggested (Herbert et al., 2001) that clindamycin blocks the translation of several regulation factors, and as a result, the levels of transcription of exoproteins increase or decrease. Increases in exoprotein levels may have been observed because the synthesis of negative regulators is inhibited, and as a result, this may remove the suppressive effects and allow the effect of decreased translation to be overcome by the direct effects of antibiotic treatment. The other possibility is that the proteins whose levels are increased are necessary for the bacteria to survive in stressful surroundings, such as in the presence of antibiotics, and that the mechanism for increases in protein levels occurs at the transcriptional level (Deneve et al., 2008 and Tanaka et al., 2005), these points require further analysis. It can be speculated that antibiotics can induce stress conditions which interact on regulatory genes, in particular genes involved in two-component regulatory systems, which have been well described in *S. aureus*. It has also been suggested that antibiotics at sub-MIC bind to their known target sites on the ribosome (albeit transiently), causing minor perturbations in ribosome function. These effects must be responsible for a mechanism coupling translation to transcription, resulting in promoter-selective modulations of the latter. The transmission of signals from ribosome to RNA polymerase due to sub-MIC of these antibiotics could involve the release of small amounts of incomplete polypeptides, interference with ribosome assembly, induction of translation errors, or possibly interactions of small molecules with RNA. The sequelae of all these events may be low-level stress responses that act through one of the many bacterial sigma factors to activate or repress specific sets of transcripts. These changes might also result in compensating effects on the transcription of nodes of linked metabolic networks (Tsui et al., 2004).

It is tempting to speculate that linezolid-induced inhibition of global regulators might result in the decreased virulence factor secretion observed in our study. However, the pattern of individual exoproteins reduced in the presence of linezolid is not consistent with the pattern to be expected after inactivation of a specific global regulator. For example, protein A is a prototypical surface protein anchored into the cell wall, as shown in this study and a study by Gemmell and Ford linezolid reduces protein A expression. If the primary effect of linezolid was to decrease expression of the gene regulator, then protein A expression would be expected to increase rather than decrease. Gemmel and Ford also found that lipase was increased in the supernatants of linezolid-treated *S. aureus* and suggested that shorter half-life of virulence factors would explain why virulence factors were more sensitive to linezolid than other proteins (Gemmell and Ford, 2002). However, little is known about possible relationships between gene function and protein or mRNA half-life or abundance, and the apparent sensitivity of virulence factors for the action of linezolid requires further investigations (Bernardo et al., 2004).

In addition to that, in this study linezolid caused an increase in the secretion of a number of cytoplasmic proteins involved in different metabolic activities (Table 5. 7). How linezolid increased the secretion of cytoplasmic protein remains to be investigated, it could be speculated that linezolid may stimulate MVs by an unknown mechanism. These MVs were described for the first time in 2009 in Gram positive bacteria by Lee et al., they were found to contain many proteins from all locations (extracellular, membrane and cytoplasm) and share many common features with outer membrane vesicles of Gram-negative bacteria (Lee et al., 2009). Similarly Bernardo et al., found that sub-MIC of linezolid induced the secretion of a number of cytoplasmic proteins into bacterial culture supernatant, but reduced the release of virulence factors (Bernardo et al., 2004). They suggested that, linezolid prevents the formation of the formyl methionyl-tRNA: mRNA: 30S subunit ternary complex. Thus, the linezolid-induced failure of ribosomal assembly might promote the leakage of small-sized individual ribosomal proteins. It is known that bacterial metabolism is a complex network of interacting pathways, and negative effects on one pathway often lead to compensatory adjustment in other pathways (a form of

homeostasis). This may occur through coordinate changes in transcription rates, which would be reflected as an apparent activation or repression of promoter activity. In prokaryotes, regulation of transcription in response to external signals is rapid and efficient (Goh et al., 2002). In this study up- and down-modulation of a large number of cytoplasmic proteins may be explained by the global transcription changes occurred in the presence of low antibiotic concentration as signal molecules.

Regarding cell wall inhibitor antibiotics (oxacillin and vancomycin) used in this study, oxacillin caused a significant increase of protein A at protein and mRNA levels, as well as *lukSF-PV* mRNA levels. This is in a good agreement with several studies (Koszczol et al., 2006, Stevenson et al., 2007 and Dumetrisco et al., 2008), they reported that  $\beta$ -lactam antibiotics are unfavorable, because even sub-inhibitory concentration of these antibiotics led to an induce toxin gene expression through stimulation of exoprotein synthesis or other mechanisms. Ohlsen et al., (1998) showed that sub-MIC of  $\beta$ -lactam antibiotics strongly induced *hla* expression, they suggested that this induction depends on a specific interaction of the agents with penicillin binding proteins. As a consequence, such an interaction may induce signal transduction mechanisms, resulting in activation of the *hla* promoter.

Vancomycin, on the other hand reduced both virulence factors at both the level of mRNA and protein secretion. Although  $\beta$ -lactam and glycopeptides inhibit the synthesis of peptidoglycan by very different mechanisms, there is a common set of genes that is up-regulated in response to both classes of antibiotics. Studies on genome-wide transcriptional profiling of the response of different strains of *S. aureus* to the presence the various cell wall active antibiotics have led to the identification of a ‘‘cell wall stimulon’’, which comprises the entire set of genes that respond together to cell wall damage (Lindsay 2008).

Besides indicating a specific response to cell wall damage,  $\beta$ -lactam antibiotics also induce a *bona fide* SOS response in *S. aureus*, characterized by the activation of the RecA and LexA proteins, the two master regulators of the SOS response (Maiques et al., 2006,

Miller et al., 2004). It has been hypothesized that oxacillin may be involved in SOS pathway stimulation to enhance these virulence factor transcription (Dumitresco et al., 2008). Staphylococcal *agr* mutants are known to over express protein A, thus antibiotics affecting the synthesis of this regulatory protein, may cause an increase in Spa expression (Gemmel et al., 1995). It has been reported that for the management of toxic *S. aureus* infections,  $\beta$ -lactam antibiotics are unfavorable, because even sub-inhibitory concentration of these antibiotics led to an induce toxin gene expression through stimulation of exoprotein synthesis (Koszczol et al., 2006). Stevenson et al., (2007), also established that sub-MIC of  $\beta$ -lactam antibiotics may fail in infections with toxin-producing organisms due to their effect on the cell wall, in contrast to protein synthesis inhibitors, as they fail to suppress toxin production (Stevenson et al., 2007). Similarly in our study oxacillin caused a significant increase of *spa* and *lukSF-PV* expression as well as Spa protein secretion.

Several studies previously reported that vancomycin at sub-MIC levels had no effect on toxin gene expression in *S. aureus* strains (Ohleson et al., 1998, Stevenson et al., 2007). However, our data showed a dramatic reduction of steady state mRNA and secretion of virulence factors studied here. The main effect of these types of antibiotics could be through their effect on the SarA regulator system, which in turn indirectly affect the transcriptional levels of the SaeRS system. The main product of the *sar* locus is a DNA-binding protein, SarA, which is transcribed from three different promoters. SarA acts on the expression of virulence factors indirectly by up-regulating RNAPIII transcription and also stimulating or repressing the transcription of several virulence genes such as *hla*, *fnbA*, *cna*, *spa*, and *ssp* (Girauda et al., 2003). The SaeRS system appears to be activated at the post exponential growth phase, and is also affected by the Agr, SarA and other environmental conditions (Kuroda et al., 2007). The observations of a study on *sae* as well as work on *sar* and *agr* regulators reveal that the production of exotoxins and cell wall-associated proteins in *S. aureus* is controlled through a complex interactive regulatory network (Girauda et al., 2003).

Vancomycin increased the secretion of several cytoplasmic proteins in this study, which could be through the SaeRS regulatory system. It is probable that vancomycin treatment

generates signals in diverse physiological pathways, which are recognized by multiple signal sensors that in turn activate multiple response regulators. From the point of view of virulence regulation, vancomycin might activate certain virulence factors through the activation of SaeRS system. SaeRS is known as the sensor-regulator system for haemolysins and coagulase (Kuroda et al., 2003). Similarly, it was reported by several laboratories, that exposure of staphylococci to cell wall inhibitors caused the rapid and extensive up-regulation of a unique set of genes which had a wide range of functions, such as determinants of cell wall synthesis (*mgtB*, *murZ*, and *pbpB*); genes of intermediary metabolism (for instance, *thrB* and *thrC*, *dapA*, and *serA*); and genes for chaperones, heat shock proteins, and osmoprotectant transporters (*prsA*, ORF SA1549, *opuD*, and *proP*) (Gardete et al., 2006).

Proteolytic degradation of proteins is one of the critical problems in 2DE gel techniques (Han et al., 2005). Proteolysis may account for differences in global 2-DE patterns, and may result from the cleavage of a single peptide bond, yielding two lower MW fragments, from partial degradation generating multiple forms that are dispersed on the 2-DE map, or even from extensive degradation, with the complete disappearance of the protein and the fragments thereof (Castellanos-Serra et al., 2002). Several proteases are secreted by *S. aureus*, serine protease (SspA), Cysteine protease (SspB) or staphopain B and Cysteine proteinase inhibitor (SspC) also known as staphostatin B. Staphopain A (ScpA) is a cysteine protease encoded by a gene of *scpAB* operon. These proteins have been implicated principally in the degradation or activation of external staphylococcal proteins (Lindsay 2008). In terms of enzyme specificity, the cleavage of peptide bonds by proteases may be specific, e.g. limited proteolysis, which depends on the amino acid sequence surrounding the hydrolysed site, or unspecific, which results in complete degradation of proteins to oligo-peptides and/or amino acids (Wladyka et al., 2008). Since SspB is a secreted protein and SspC is an intracellular protein, it was hypothesized that SspC functions as a cytoplasmic inhibitor that is required to protect cytosolic proteins from degradation by prematurely folded or activated SspB and as such the enzyme itself and its endogenous inhibitor could be attractive targets for the development of anti-staphylococcal therapies (Shaw et al., 2005). Normally during the sample preparation, proteolysis problems are

tackled by the addition of proteinase inhibitors, by the use of a basic pH (Tris base) during protein extraction, by boiling the sample in SDS for a few minutes, or by the denaturing effect of the lysis solutions used for protein extraction. In this study in addition to those mentioned above, all samples all samples were precipitated in very low pH by using tri chloro acetic acid and containing 2 M thiourea, which both are actively proteolysis inhibitors.

The emergence of drug resistant bacteria poses a serious threat to human health, and the preservation of the ‘antibiotic era’ will likely require the discovery of novel antibiotics that act via novel mechanisms. One potential novel mechanism is the inhibition of bacterial type I signal peptidase (SPase). SPase is conserved across both Gram-positive and Gram-negative bacteria and is required to process cell surface bound preproteins during export from the cytoplasm (Harris et al., 2009). Guidance for the treatment of PVL-*S aureus*-related infections in England by Health Protection Agency (HPA) recommends agents such as linezolid, clindamycin and rifampicin for the treatment of serious disease and mupirocin for decolonization purposes. However resistant to clindamycin and mupirocin has been detected in multiple lineages of PVL-MRSA (Ellington et al., 2010). Linezolid and vancomycin are still drugs of choice for treatment of patients with CA-MRSA pneumonia (Hidron et al., 2009).

Further studies are needed to answer the question of whether bacteriostatic antibiotics could prove beneficial for patients with an infection for which bactericidal antibiotic have been traditionally used. The data showed in this study confirmed that growing *S. aureus* in the presence of oxacillin induce toxin expression and might enhance the virulence of this bacterium, therefore using these antibiotics to treat *S. aureus* infections may contribute to worse outcomes. These data also confirmed that linezolid and vancomycin, are both important selections of antimicrobial agents to treat serious infections caused by the bacterium.

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# APPENDIX

## Hous keeping gene sequences and their correspondent alleles in all 10 isolates.

### *arcC*

#### >ST-1 *arcC* ALLELE NUMBER IS 1

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTCAATAACCCAACCAACCAATTGGTCCTTTTTATACGAA  
AGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAAATACCTATGAAGGTGTTGAAGCG

#### >ST-2 *arcC* ALLELE NUMBER IS 22

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTTGATAACCTAACTAAACCAATTGGTCCTTTTTATACGAAA  
GAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAAATACCTATGAAGGTGTTGAAGCG

#### >ST-3 *arcC* ALLELE NUMBER IS 92

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAAG  
GTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAAT  
CGTAACACGTGTGGAAGTAGATAAAGATGATCCACGATTTGATAACCTAACTAAACCAATTGGTCCTTTTTATACGAAA  
GAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAAATACCTATGAAGGTGTTGAAGCG

#### >ST-4 *arcC* ALLELE NUMBER IS 3

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTTGATAACCCAACCTAAACCAATTGGTCCTTTTTATACGAAA  
GAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAAATACCTATGAAGGTGTTGAAGCG

#### >ST-5 *arcC* ALLELE NUMBER IS 7

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTCAATAACCCAACCAACCAATTGGTCCTTTTTATACGAA  
AGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTT  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAAATACCTATGAAGGTGTTGAAGCG

#### >ST-6 *arcC* ALLELE NUMBER IS 2

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAAG  
GTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAAT  
CGTAACACGTGTGGAAGTAGATAAAGATGATCCACGATTTGATAACCCAACCTAAACCAATTGGTCCTTTTTATACGAAA  
GAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGGCTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA

GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG

**>ST-7 arcC ALLELE NUMBER IS 7**

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTCAATAACCCAACCAAAACCAATTGGTCCTTTTTATACGAA  
AGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTT  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG

**>ST-8 arcC ALLELE NUMBER IS 1**

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTCAATAACCCAACCAAAACCAATTGGTCCTTTTTATACGAA  
AGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG

**>ST-9 arcC ALLELE NUMBER IS 6**

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAAATGATCCACGATTTGATAACCCAACCTAAACCAATTGGTCCTTTTTATACGAAA  
GAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG

**>ST-10 arcC ALLELE NUMBER IS 3**

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGAATGTCACAG  
GGTATGATAGGCTATTGGTTGGAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAA  
TCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTTGATAACCCAACCTAAACCAATTGGTCCTTTTTATACGAAA  
GAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATAGAAAAGTA  
GTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAATATTGTCATTGC  
ATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG

***aroE***

**>ST-1 aroE ALLELE NUMBER IS 4**

AATTTTAATTCCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAAATTCCTCATAAAGAACGTATCATACCGTATTTAGATTATGTTGATG  
AACAAGCGATTAATGCAGGTGCAGTTAACACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAGGCCAAATTAACCTGTTGCGAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAACCAAATTTTCATTGGCAGATGCTGAAAAGTATTTA

**>ST-2 aroE ALLELE NUMBER IS 1**

AATTTTAATTCCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATG  
AACAAGCGATTAATGCAGGTGCAGTTAACACTGTTTTGATAAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAGGCCAAATTAACCTGTTGCGAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAACCAAATTTTCATTAGCAGATGCTGAAAAGTATTTA

**>ST-3 aroE ALLELE NUMBER IS 2**

AATTTTAATTCCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCAAAAAAGAATTAGATGGCTTTAATATCACAAATTCCTCATAAAGAGCGTATCATACCGTATTTAGATCATGTTGATG  
AACAAGCGATTAATGCAGGTGCAGTTAACACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGAGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAGGCCAAATTAACCTGTTGCGAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAACCAAATTTTCATTGGCAGATGCTGAAAAGTATTTA

**>ST-4 aroE ALLELE NUMBER IS 3**

AATTTTAATTCCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGAAGGCTTTAATATCACAAATTCCTCATAAAGAACGTATCATACCGTATTTAGATTATGTTGATG

AACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAAACCAAATTTCAATAGCAGATGCTGAAAAGTATTTA

**>ST-5 aroE ALLELE NUMBER IS 6**

AATTTTAATTCCTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAATTCCTCATAAAGAGCGTATCATACCGTATTTAGATCATGTTGAT  
GAACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTA  
TTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTG  
AAGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCGTTTTG  
AATCTTGGAATTTAAATATAAACCAAATTTCAATAGCAGATGCTGAAAAGTATTTA

**>ST-6 aroE ALLELE NUMBER IS 2**

AATTTTAATTCCTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAATTCCTCATAAAGAGCGTATCATACCGTATTTAGATCATGTTGATG  
AACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGAGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAAACCAAATTTCAATAGCAGATGCTGAAAAGTATTTA

**>ST-7 aroE ALLELE NUMBER IS 6**

AATTTTAATTCCTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAATTCCTCATAAAGAGCGTATCATACCGTATTTAGATCATGTTGAT  
GAACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTA  
TTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTG  
AAGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCGTTTTG  
AATCTTGGAATTTAAATATAAACCAAATTTCAATAGCAGATGCTGAAAAGTATTTA

**>ST-8 aroE ALLELE NUMBER IS 1**

AATTTTAATTCCTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAATTCCTCATAAAGAACGATCATACCGTATTTAGATCATGTTGATG  
AACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAAACCAAATTTCAATAGCAGATGCTGAAAAGTATTTA

**>ST-9 aroE ALLELE NUMBER IS 5**

AATTTTAATTCCTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAGAAATTA  
TTTCGAAAAAAGAATTAGATGGCTTTAATATCACAATTCCTCATAAAGAGCGTATCATACCGTATTTAGATCATGTTGATG  
AACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGATGGTAT  
TGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTGGTGCA  
AGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCGTTTTGA  
ATCTTGGAATTTAAATATAAACCAAATTTCAATGGAAGATGCTGAAAAGTATTTA

**>ST-10 aroE ALLELE NUMBER IS 3**

AATTTTAATTCCTTAGGATTAGATGATACTTGAAGCCTCCTAAATTCCTCAATTGAAGAATCCTTCATTTAATTAAGA  
AATTATTTTCGAAAAAAGAATTAGAAGGCTTTAATATCACAATTCCTCATAAAGAACGATCATACCGTATTTAGATTATGT  
TGATGAACAAGCGATTAATGCAGGTGCAGTTAACTGTTTTGATAAAAAGATGGCAAGTGGATAGGGTATAATACAGAT  
GGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAAATTTTGGGCGCAGGTG  
GTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAAATTTGTAAGGCCCAAATTAAGTGTTCGCAATAGAACGATGGCTCG  
TTTTGAATCCTTGGAATTTAAATATAAACCAAATTTCAATGGAAGATGCTGAAAAGTATTTA

***glpF***

**>ST-1 glpF ALLELE NUMBER IS 1**

GGTGTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGTCTGTCGGTCAATTCTCA  
GGTGACATTTAAACCCAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCTGGTTATA  
TTGTTGCTCAAAATGTTAGGTGCAATGTCGGAGCAACAATGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGA  
AGAAGCTGGCGCAAAATAGGTGTTTTCTACAGCACCGGCTATTAAGAATTACTTTGCCAACTTTTAAAGTGAGATT  
ATCGGAACAATGGCATTAACTTTAGGTATTTATTTATCGGTGTAACAAAATGCCGATGGTTTAAATCCTTTAATTGTC  
GGAGCATTAAATGTTGCAATCGGATTAAGTTTAGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT

**>ST-2 glpF ALLELE NUMBER IS 14**



TTGTTGCTCAATGTTAGGTGCAATTGTCGGAGCAACAATTGTATGGTTAATGTACTTGCCACATTGAAAAGCGACAGA  
AGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAGAATTACTTTGCCAACTTTTTAAGTGAGATT  
ATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAACAATAAATGCCGATGGTTTAAATCCTTTAATTGTC  
GGAGCATTAAATTGTTGCAATCGGATTAAGTTTAGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT

**pta**

**>ST-1 pta ALLELE NUMBER IS 12**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTGATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGAGACACTGTGCGTCCAGCTTTACAA  
ATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTGG  
TGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-2 pta ALLELE NUMBER IS 12**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTGATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGAGACACTGTGCGTCCAGCTTTACAA  
ATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTGG  
TGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-3 pta ALLELE NUMBER IS 2**

GCAACACAATTACAAGCAACCGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTAATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGCGACACTGTGCGTCCAGCTTTACA  
AATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAGTACATCTTTG  
GTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-4 pta ALLELE NUMBER IS 4**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTGATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGCGACACTGTGCGTCCAGCTTTACA  
AATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTG  
GTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**ST-5 pta ALLELE NUMBER IS 8**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTAATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCGCAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGAGACACTGTGCGTCCAGCTTTACAA  
ATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTGG  
TGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-6 pta ALLELE NUMBER IS 6**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTAATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGCGACACTGTGCGTCCAGCTTTACA  
AATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAGTACATCTTTG  
GTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-7 pta ALLELE NUMBER IS 8**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAA  
AACTTAATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAAGCTGAATTAGTTCAATCATTTG  
TTGAACGACGTAAGGTAAAGCGACTGAAGAACAAGCGCAAGAATTATTAACAATGTGAACTACTTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTTAGTGGTGCAGCACATTCAACAGGAGACACTGTGCGTCCAGCTTTACAA  
ATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTGG  
TGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-8 pta ALLELE NUMBER IS 1**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAA  
AACTTGATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTG  
TTGAACGACGTAAGGTAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACACTCTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTAGTTAGTGGTGCAGCACATTCAACAGGAGACACTGTGCGTCCAGCTTTACAA  
ATCATCAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGTACAATACATCTTTGG  
TGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGACAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-9 pta ALLELE NUMBER IS 7**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAA  
AACTTAATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTG  
TTGAACGACGTAAGGTAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACACTCTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTCTAGTTAGTGGTGCAGCACATTCAACAGGCGACACTGTGCGTCCAGCTTTACA  
AATCATCAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTG  
GTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGACAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

**>ST-10 pta ALLELE NUMBER IS 4**

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAA  
AACTTGATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTG  
TTGAACGACGTAAGGTAAGCGACTGAAGAACAAGCACAAGAATTATTAACAATGTGAACACTCTCGGTACAATGCT  
TGTTTTATGCTGGTAAAGCAGATGGTTAGTTAGTGGTGCAGCACATTCAACAGGCGACACTGTGCGTCCAGCTTTACA  
AATCATCAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAGGTGATGAACAATACATCTTTG  
GTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGACAGAAATTGCAGTAGAAAAGTGCAAAATCAGCATT

***tpi***

**>ST-1 tpi ALLELE NUMBER IS 1**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGA  
AGATGCAATGAAATGTGTGCATTTGTACGTCAAACATTTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGT  
ATTCAATATGGTGGTAGTGTTAAACCTAACACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAG  
GTGGCGCA

**>ST-2 tpi ALLELE NUMBER IS 4**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATATGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAA  
GATGCAATGAAATGTGTGCATTTGTACGTCAAACATTTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTA  
TTCAATATGGTGGTAGTGTTAAACCTAACACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
TGGCGCA

**>ST-3 tpi ALLELE NUMBER IS 2**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAA  
GATACGAATGAAATGTGTGCATTTGTACGTCAAACATTTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTA  
TTCAATATGGTGGTAGTGTTAAACCTAACACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
TGGCGCA

**>ST-4 tpi ALLELE NUMBER IS 4**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATATGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAA  
GATGCAATGAAATGTGTGCATTTGTACGTCAAACATTTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTA  
TTCAATATGGTGGTAGTGTTAAACCTAACACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
TGGCGCA

**>ST-5 tpi ALLELE NUMBER IS 5**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATATGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTAT  
CTGAAATCAACTAAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAA  
GATGCAATGAAATGTGTGCATTTGTACGTCAAACATTTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTA

TTCAATATGGTGGTAGTGTTAAACCTAACAAACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
TGGCGCA

**>ST-6 tpi ALLELE NUMBER IS 3**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAA  
GATGCGAATGAAATGTGTGCATTTGTACGTCAAACCTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTA  
TTCAATATGGTGGTAGTGTTAAACCTAACAAACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
TGGCGCA

**>ST-7 tpi ALLELE NUMBER IS 5**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGCGT  
GAAACAGAACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTNGTAGGTGAGCAAGTTAAGAAAAGCTGTTGCAGGTTT  
ATCTGAAAATCAACTAAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTG  
AAGATGCGAATGAAATGTGTGCATTTGTACGTCAAACCTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCG  
TATTCAATATGGTGGTAGTGTTAAACCTAACAAACATTAAGAATACATGGCACAAACTGATATTGATGGGGCA

**>ST-8 tpi ALLELE NUMBER IS 1**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGA  
AGATGCAAAATGAAATGTGTGCATTTGTACGTCAAACCTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGT  
ATTCAATATGGTGGTAGTGTTAAACCTAACAAACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAG  
GTGGCGCA

**>ST-9 tpi ALLELE NUMBER IS 14**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCATGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGA  
AACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAAGCTGTTGCAGGTTTATC  
TGAAGATCAACTTAAATCAGTTATAATTGCTTATGAACCAATTTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAG  
ATGCGAATGAAATGTGTGCATTTGTACGTCAAACCTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTAT  
TCAATATGGTGGTAGTGTTAAACCTAACAAACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
GGCGCA

**>ST-10 tpi ALLELE NUMBER IS 4**

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATATGTGTTGGTG  
AAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAAGCTGTTGCAGGTTTAT  
CTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAACCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGA  
GATGCAAAATGAAATGTGTGCATTTGTACGTCAAACCTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTA  
TTCAATATGGTGGTAGTGTTAAACCTAACAAACATTAAGAATACATGGCACAAACTGATATTGATGGGGCATTAGTAGG  
TGGCGCA

***yqiL***

**>ST-1 yqiL ALLELE NUMBER IS 10**

GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAGAGACGGGTTTGAATCCAA  
GTGAGATTGATGAAGTTATCATCGGTAACGTAACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGA  
AAGGTGGCTTGCCAGAAAACAGTACCTGCATTACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGC  
ATATCAATCTATTGTGACTGGTGAAAATGACATCGTGTAGCTGGCGGTATGGAGAATATGTCTCAGTCACCAATGCTT  
GTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATGATGGTTTAAACAGATG  
TATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTAGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA  
TTTGCTGTAAACTCACAACATAAAGCAGTACGTGCACAGCAA

**>ST-2 yqiL ALLELE NUMBER IS 31**

GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAGAGACGGGTTTGAATCCAA  
GTGAGATTGATGAAGTTATCATCGGTAACGTAACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGA  
AAGGTGGCTTGCCAGAAAACAGTACCTGCATTACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGC  
ATATCAATCTATTGTGACTGGTGAAAATGACATCGTGTAGCTGGCGGTATGGAGAATATGTCTCAGTCACCAATGCTT  
GTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATGATGGTTTAAACAGATG  
TATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTAGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA  
TTTGCTGTAAACTCACAACAAAAGCAGTACGTGCACAGCAA

**>ST-3 yqiL ALLELE NUMBER IS 2**



GTCAACAACAGTCGCTTTGGTTTTAAAATGGGACATCAATCAATGTTGATAGCATGGTATATGATGGTTAACAGATG  
TATTTAATCAATATCATATGGGTATTACTGCTGAAAAATTTAGTAGAGCAATATGGTATTTCAAGAGAAGAACAGATACA  
TTTGCTGTAAACTCACAAACAAAAGCAGTACGTGCACAGCAA

## **gmk**

### **>ST-1 gmk ALLELE NUMBER IS 4**

CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATTAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCGTTATTTATTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCCAATGAGAAAATACAAAGTCGTATTAACGAAGCGCGTAAAGA  
AGTTGAAATGATGAATTTATACGATTACGTT

### **>ST-2 gmk ALLELE NUMBER IS 23**

CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATTAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCGTTATTTATTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCCGATGAGAAAATACAAAGTCGTATTAACGAAGCGCGTAAAGA  
AGTTGAAATGATGAATTTATACGATTACGTT

### **>ST-3 gmk ALLELE NUMBER IS 2**

CGAATATTTGAAGATCCAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATTAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCGTTATTTATTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCACGTAAGA  
AGTCGAAATGATGAATTTATACGATTACGTT

### **>ST-4 gmk ALLELE NUMBER IS 1**

CGAATATTTGAAGATCCAGTACATCATATAAGTATTCTATTTCAATGACAACACGCTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCGCTATTTATTTCTTAGCACCTCCAAGTTTAGAAC  
ACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCGCGTAAAG  
AAGTTGAAATGATGAATTTATACGATTACGTT

### **>ST-5 gmk ALLELE NUMBER IS 5**

CGAATATTTGAAGATCCAGTACATCATATAAGTATTCTATTTCAATGACAACACGCTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCATTATTTATTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCCGATGAGAAAATACAAAGTCGTATTAACGAAGCACGTAAGA  
AGTCGAAATGATGAATTTATACGATTACGTT

### **>ST-6 gmk ALLELE NUMBER IS 2**

CGAATATTTGAAGATCCAGTACATCATATAAGTATTCTATTTCAATGACAACACGCTCTAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATTAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCGTTATTTATTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCACGTAAGA  
AGTCGAAATGATGAATTTATACGATTACGTT

### **>ST-7 gmk ALLELE NUMBER IS 5**

CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTTTTAGAAATT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAAGAAATTTCCAGATGCATTATTTATTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCCGATGAGAAAATACAAAGTCGTATTAACGAAGCACGTAAGA  
AGTCGAAATGATGAATTTATACGATTACGTT

### **>ST-8 gmk ALLELE NUMBER IS 1**

CGAATATTTGAAGAACCAAGTACATCATATAAGTCTTCTATTTCAATGACAACACGTCAAATGCGTGAAGAGTGAAGTC  
GATGGCGTAGATTACTCTTTTAAAACCTAGGGACGCGTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAA  
TATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGATTTTTAGAAAT  
TGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCGCTATTTATTTTCTTAGCACCTCCAAGTTTAGAA  
CACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCGCGTAAA  
GAAGTTGAAATGATGAATTTATACGATTACGTG

**>ST-9 gmk ALLELE NUMBER IS 2**

CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGA  
TGGCGTAGATTACTTTTTAAAACCTAGGGATGCGTTTGAAGCTTTAATTAAGATGACCAATTTATAGAATATGCTGAAT  
ATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGATTTTTAGAAAT  
GAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCGTTATTTATTTTCTTAGCACCTCCAAGTTTAGATCA  
CTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCACGTAAAGA  
AGTCGAAATGATGAATTTATACGATTACGTG

**>ST-10 gmk ALLELE NUMBER IS1**

CGAATTTGGAAGANCCAGTCCATCCATATNAGTCTNCTNTTNTCAATNGACAACACGTCAAATNGCGTGAAGGGTGA  
AGTNGCANGGCGTNGATTACTCNTTTAAAACCTAGGGACGCGTTNGAAGCTTTAATCAAAGATGNCCAATTTATAGAATA  
TGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGATTTT  
TAGAAATTTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCGCTATTTATTTTCTTAGCACCTCCAAGT  
TTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCG  
CGTAAAGAAGTTGAAATGATGAATTTATACGATT

## **Protein Identification**

### **Excision of Proteins for PMF-MS**

Large format 2D gels were transferred on to a clean low fluorescence glass plate (290 x 250 mm) using a nylon mesh support matrix. Spots were carefully excised using One Touch Plus Spot Picking Pipettes (The Gel Company) into separate wells of a 96-well microtitre plate (Genomic Solutions). All spot picking was performed in a lamina flow sterile air cabinet while wearing gloves, face-mask and hair net to prevent keratin contamination. Robotic excision was achieved using a ProPic spot picking robot (Genomic Solutions Ltd.) in combination with Phoretix Evolution image analysis software (Nonlinear Dynamics). Spots were excised into separate wells of a 96-well microtitre plate (Genomic Solutions Ltd.). Gels were periodically rehydrated with ddH<sub>2</sub>O to maintain gel integrity.

### **Peptide Mass Fingerprinting Mass Spectrometry (PMF-MS)**

Microtitre plates containing gel plugs were transferred to a ProGest Workstation (Genomic Solutions Ltd.) for automated tryptic digestion and peptide extraction according to the ProGest long trypsin digestion protocol. Gel plugs were rehydrated with 50mM ammonium bicarbonate containing 6.6% (w/v) trypsin (Promega) and digested overnight.

Peptides were extracted using 50% (v/v) acetonitrile, 0.1% (v/v) TFA into a final volume of 50 $\mu$ l (2 x 25 $\mu$ l extractions) and lyophilised in a vacuum concentrator. Peptides were re-suspended in 10 $\mu$ l of 0.1% formic acid and sonicated in an ultra-sonic water bath for 1 minute. Samples were then analysed directly by Matrix-assisted Laser Desorption/Ionisation Time of Flight PMF MALDI-ToF.

### **MALDI-TOF PMF**

MALDI-ToF PMF was performed by (Proteomic services, School of Biological and Biomedical Sciences, Durham University) using a Voyager-DE™ STR BioSpectrometry™ Workstation (Applied Biosystems, Warrington, UK). Briefly, approximately 0.3 $\mu$ l of thin film solution (2 parts 1% nitrocellulose in 50% (v/v) acetone, 50% (v/v) isopropanol to 3 parts saturated  $\alpha$ -cyano-4-hydroxy-cinnamic acid in acetone) was applied to each grid position. Subsequently, 0.5 $\mu$ l of concentrated peptide solution was applied onto each position and allowed to dry for 1 hour. Individual positions were then washed with 5 $\mu$ l ice-cold 0.1% (v/v) TFA to remove salts and other impurities. Once dry, spotted target plates were inserted into the MALDI-TOF mass spectrometer and analysed. Data acquisition and analysis was performed in fully automated mode. Spectra were acquired from 480 laser shots using system parameters optimized for the mass range 800-3500 amu. Online data analysis was performed using Applied Biosystems Data Explorer software. This carried out peak detection, noise reduction and peak de-isotoping. De-isotoped spectra were internally calibrated against the trypsin autolysis peaks 842.5100 and 2211.1046 m/z present in the spectra and calibrated peak lists of peptide masses for each sample were generated.

### **Mascot search software for protein identification**

Protein identification from MALDI-PMF peptide mass data was achieved using the MASCOT ([www.matrixscience.com](http://www.matrixscience.com)) mass spectrometry database search software. This software queried all available entries in the NCBI nr database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Returned matches are ranked based on the MOWSE score. This score is calculated from the number of matched peptides, the number of matched fragment ions, the accuracy of the match and a weighting for large peptide fragment matches. Higher scores indicate

more probable matches. The highest ranked match was usually considered as the successful identification of the protein.

**Mascot Search Result Table produced by MALDI-TOF mass spectrometry, for spot number 3, which showed the 686 protein score for S subunit of PVL toxin of *S. aureus*. As indicated above the score above 82 is significant ( $p < 0.05$ ).**

<b>{MATRIX}</b> <b>{SCIENCE}</b> Mascot Search Results			
User : Email : Search title : SampleSetID: 1022, AnalysisID: 1540, MaldiWellID: 12013, SpectrumID: 60286, Path=\Dups\Dups1_207\Combo Database : NCBIInr_090909 090909 (9655479 sequences; 3300246437 residues) Timestamp : 17 Sep 2009 at 08:13:10 GMT Warning : A Peptide summary report will usually give a much clearer picture of MS/MS search results. Top Score : 686 for <b>gi 52695626</b> , Chain A, Structure Of The Panton-Valentine Leucocidin S Component From Staphylococcus Aureus			
<b>Probability Based Mowse Score</b>  Ions score is $-10 * \log(P)$ , where P is the probability that the observed match is a random event. Protein scores greater than 82 are significant ( $p < 0.05$ ). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.			
Matched peptides shown in <b>Bold Red</b>  1 <b>DNNIENIGDG AEVVKR</b> TEDT SSDKWGVTQN IQFDFVKDKK <b>YNKDALILKM</b> 51 <b>QGFINSKTTY YNYKNTDHIK</b> AMRWPFQYNI <b>GLKTNDPNVD LINYLPKNKI</b> 101 DSVNVSQTLG YNIGGNFNSG PSTGGNGSFN YSK <b>TISYNQQ NYISEVEHQN</b> 151 <b>SKSVQWGIKA NSFITSLGKM SGHDPNLFVG YKPYSQNPRD</b> YFVPDNELPP 201 LVHSGFNPSF IATVSHEKGS <b>GDTSEFEITY GRNMDVTHAT RRTHYGNSY</b> 251 <b>LEGSRIHNAF VNRNYTVKYE VNWK</b> THEIKV KGHN			
	Accession	Score	Description
1.	gi 52695626	686	Chain A, Structure Of The Panton-Valentine Leucocidin S Component From Staphylococcus aureus
2.	gi 156153053	561	PVL toxin [Staphylococcus aureus]

3.	gi 156152999	560	PVL toxin [Staphylococcus aureus]
4.	gi 156152731	559	PVL toxin [Staphylococcus aureus]
5.	gi 156152737	559	PVL toxin [Staphylococcus aureus]
6.	gi 156152757	559	PVL toxin [Staphylococcus aureus]
7.	gi 156152879	559	PVL toxin [Staphylococcus aureus]
8.	gi 156152739	559	PVL toxin [Staphylococcus aureus]
9.	gi 9635191	550	LukS-PV [Staphylococcus phage PVL]
10.	gi 166197608	550	Panton-Valentine Leukocidin S [Pseudomonas putida]
11.	gi 21283108	549	Panton-Valentine leukocidin chain S precursor [Staphylococcus aureus subsp. aureus MW2]
12.	gi 119443709	549	Panton-Valentine leukocidin chain S precursor [Staphylococcus phage phiPVL108]
13.	gi 116735192	549	Panton-Valentine leukocidin chain S precursor [Staphylococcus aureus]
14.	gi 251814417	549	Panton-Valentine leukocidin subunit S [Staphylococcus aureus subsp. aureus TCH70]
15.	gi 156152897	519	PVL toxin [Staphylococcus aureus]
16.	gi 551670	480	lukS [Staphylococcus aureus]
17.	gi 38683286	386	Panton-Valentine leukocidin [Staphylococcus aureus]
18.	gi 40795871	385	Panton-Valentine leukocidin S [Staphylococcus aureus]
19.	gi 72536463	385	Panton-Valentine leukocidin [Staphylococcus aureus]
20.	gi 253730096	272	Panton-Valentine leukocidin subunit S [Staphylococcus aureus subsp. aureus USA300_TCH959]