The Effects of Antibiotic Stress on the Expression of Virulence Factors by Strains of *Staphylococcus aureus* Displaying Vancomycin-Intermediate Resistance

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ABBREVIATIONS

ABBREVIATION	FULL TITLE
ABTS	2,2'-azino-di(3ethybenthiazoline sulphanate)
BHI	brain heart infusion broth
BSA	bovine serum albumin
CA-MRSA	community acquired-methicillin resistant *Staphylococcus aureus*
CBA	columbia blood agar
CEF	cefpirome
CNS	coagulase-negative staphylococci
CP	capsular polysaccharide
DAP	daptomycin
ELISA	enzyme-linked immunoabsorbent assay
EF	elongation factor
EMRSA	epidemic methicillin resistant *Staphylococcus aureus*
FCS	foetal calf serum
GTP	guanosine triphosphate
GRI	Glasgow Royal Infirmary
H_2O_2	hydrogen peroxide
HBSS	hanks balanced salt solution
HRP	horse radish peroxidase
Ig	immunoglobulin
Il	interleukin
LPS	lipopolysaccharide
Luminol	5-amino 2,3-dihydrophthalazinedione2
LZD	linezolid
MAb	monoclonal antibody
MAC	minimum antibiotic concentration

mRNA	messenger RNA
MHB	mueller hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
MSSA	methicillin sensitive *Staphylococcus aureus*
NADPH	nicotinamide adenine dinucleotide phosphate
NPS	normal pooled serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMNL	polymorphonuclear leucocytes
PMA	phorbol myristate acetate
PNP	p-nitrophenyl phosphate
RF	RNase free
RNA	ribonucleic acid
RT	reverse transcription
SAAP	streptavidin alkaline-phosphatase
SAg	superantigen
s.d	standard deviation
SYN	Synercid
Synercid	dalfopristin/quinupristin
TEM	transmission electron microscopy
THB	todd hewitt broth
tRNA	transfer RNA
TSB	tryptone soya broth

SUMMARY

Strains of *Staphylococcus aureus* have caused a wide variety of ailments and diseases in humans and animals for centuries. The original antimicrobial agents of choice for treatment of pathogenic strains were the penicillins and cephalosporins, which disrupt the bacterial cell wall. The emergence of strains producing β-lactamase enzymes that hydrolysed the penicillins and related antimicrobials led to the development of new cell wall agents lacking the β-lactam ring for example vancomycin, or have been modified to confer β-lactamase resistance, for example methicillin and oxacillin. The prevalence of such resistance is now thought to be as high as 90% of all *S.aureus* strains.

One of the cell wall inhibitors developed in response to β-lactamase activity was methicillin. Methicillin was introduced in to the pharmaceutical market in 1960 but by 1961 a strain of *S.aureus* displaying reduced methicillin susceptibility (MIC ≥25μg/ml) had already been isolated. Today, resistance to methicillin in staphylococci is wide spread and has been reported in strains of *S.aureus* and coagulase-negative staphylococci. Over the years the number of methicillin-resistant *S.aureus* (MRSA) isolates has increased significantly and are particularly problematic within medical institutes although the incidence of community-acquired MRSA (CA-MRSA) is increasing. Infection of an individual with a strain of MRSA is aided by a deficiency in an individuals immune defence system whether it is through injury or surgery. The ability of the strain to persist in a host can be attributed to the difficult eradication of the infection by antimicrobial agents as numerous resistance mechanisms are carried by strains of MRSA.

In many instances the drug of choice to treat acute cases of MRSA infection is the glycopeptide vancomycin, which until relatively recently has been very successful. The occurrence of vancomycin resistance among enterococci has been known for many years but its resistance mechanisms have only been demonstrated to be transferable to *S.aureus in vitro*. The prospect of vancomycin resistance spreading to multi-resistant strains of staphylococci is one not many clinicians like to consider as this would further limit the spectrum of agents available to treat infected individuals. In 1997, the first strain of

MRSA displaying reduced susceptibility to vancomycin was isolated from a patient in Japan and it has rapidly been succeeded by strains in the United Kingdom, United States and across Europe. The mechanism of resistance employed by these strains has not been elucidated as the *van* genes that confer high-level vancomycin resistance in strains of enterococci are not carried by these isolates of *S.aureus*. However, the United States have recently isolated the *vanA* gene from two clinical isolates of *S.aureus* confirming the potential for acquisition of genes conferring high-level resistance to vancomycin by *S.aureus*.

With the high prevalence of MRSA in many countries the need for new and improved antimicrobial agents with novel modes of action has been highlighted. Among this group of new antimicrobials are the oxazolidinones, for example linezolid, the streptogramin quinupristin/dalfopristin (Synercid), and the lipopeptide daptomycin each with a unique mechanism of action. Studies using the aforementioned antimicrobials have been carried out, and have given encouraging results in animal models of endocarditis and bacteremia, and in compassionate use programs leading to the launch of linezolid in 1999 and phase III clinical trials of daptomycin that are due for completion in the near future.

In this study, we examined the investigational protein synthesis inhibitors linezolid and quinupristin/dalfopristin, and daptomycin, a disrupter of cell membrane potential, along with cefpirome, a cell wall inhibitor, against strains of *S.aureus* displaying vancomycin-intermediate resistance (VISA). Six VISA strains were chosen from three countries, Japan (Mu3 and Mu50), United Kingdom (3700w and 3759v) and the United States (5827 and 5836) along with an epidemic strain of MRSA, EMRSA-16, and a standard methicillin sensitive *S.aureus* (MSSA) Cowan. Sub-inhibitory concentrations of cefpirome, daptomycin, linezolid and synercid were used to investigate their effects on cell growth and morphology, virulence factor expression, and strain susceptibility to opsonophagocytosis. Characteristics of individual strains in the absence of antimicrobial stress were compared to standard strains EMRSA16 and MSSA in addition to antimicrobial treatment being compared to the antibiotic free control for individual strains.

It was found that each strain of VISA has distinctive characteristics and that the vancomycin resistant genotype does not confer the same phenotypic characteristics on every strain. A feature observed by other researchers and confirmed here, was the existence of much thickened cell walls in selected VISA strains. The lack of surface protein A was also apparent by its presence on VISA 5827 only in addition to the MSSA

and EMRSA-16 strains. Extracellular protein A was also not uniformly detected but was expressed by four of the VISA strains. Exposure to sub-inhibitory concentrations of all four of the antimicrobial agents was generally found to reduce the expression of the surface proteins, clumping factor and protein A, in the majority of strains but produced variable responses in their effects on alpha-haemolysin and toxic shock syndrome toxin 1 (TSST-1) expression. Analysis of mRNA transcription of the protein A gene, *spa*, revealed increased levels of mRNA in response to antimicrobial stress however, the levels of mature protein were reduced confirming that linezolid and quinupristin/dalfopristin are inhibitors of protein synthesis.

With regard to opsonophagocytosis, through microscopic visualisation, each strain appeared to be ingested to similar degrees by polymorphonuclear cells (PMNL) with the exception of Mu50 which was ingested to significantly higher degrees following exposure to cefpirome, linezolid and synercid. Using chemiluminescence as an indicator of phagocytic ingestion strain 3759v induced significantly less chemiluminescence in comparison to all of the other strains, including the standards, whereas, Mu3 and Mu50 induced greatly enhanced results. Exposure of the cell cultures to any of the antimicrobial agents did not have a uniform effect on opsonophagocytosis of the strains but chemiluminescence was found to be reduced in strains where antimicrobial agents exerted an effect.

Protein A, amongst others, is regarded as an antiphagocytic protein. The absence of protein A on the surface of the majority of VISA strains was not found to increase the susceptibility of strains to opsonophagocytosis. Several strains possessed thickened cell walls that may be compensatory for the lack of protein A and affect phagocytosis as expression of thickened cell walls in response to antimicrobial stress reduced the level of chemiluminescence observed in several of the strains. Thus, the effects of different antimicrobial agents on a bacterial cell are not necessarily those exerted upon all bacterial strains. The characteristics assayed for here only represent a small fraction of a wider picture of the interaction between bacteria and antimicrobial agent.

CHAPTER ONE

INTRODUCTION

1.1 Staphylococci

Staphylococci are relatively large (0.8μm in diameter) Gram-positive, clump forming, facultative aerobic cocci. The genus comprises of both animal and human specific strains some of which are potentially serious pathogens. At present, there are greater than twenty strains of staphylococci with strains of the same species having 80-100% sequence identity. In humans there are two major species; *Staphylococcus epidermidis* (*S.epidermidis*) found as a commensal organism of the skin and mucous membranes or as a contaminant of artificial devices, and *Staphylococcus aureus* (*S.aureus*).

Staphylococcus aureus has been associated with a wide variety of ailments and diseases including abscesses, food poisoning, pneumonia, bacteraemia, endocarditis, osteomyelitis, meningitis, toxic shock syndrome (TSS), and scalded skin syndrome (SSS), thus causing a high degree of morbidity and mortality. Not all strains of *S.aureus* are pathogenic. Many people carry the bacterium asymptomatically in their nasal cavity, throat and skin. The development of a diseased state in a host can arise as a result of the acquisition of additional genetic material by the bacterium or by the expression of a phenotype encoded by the genetic material, for example the expression of extracellular enzymes and toxins ("virulence factors"). In other instances a compromised host immune system is all that is required for an opportunistic pathogen to cause an infection.

Genetic analysis of the *S.aureus* genome shows the presence of 1-60kbp plasmids that can be grouped into 4 general classes (Table 1).[258] Additional genetic material such as resistance plasmids and virulence genes can be acquired by conjugation and transduction influencing the pathogenic status of the organism. Other species that carry toxin genes on mobile genetic elements include *Escherichia coli* (*E.coli*), *Vibrio cholerae* and *Corynebacterium diphtheriae*, however genetic elements acquired by Gram-positive bacteria tend to be more stably integrated in to the bacterial genome.

Plasmid Group	Characteristics
I	1-5kb; high copy number; single antibiotic resistance
II	20-40kb and copy number; β-lactamase and inorganic ion resistance; comprised mainly of transposons
III	40-60kb; multiple antibiotic resistance markers; comprised mainly of transposons
IV	Intermediate size; heavy metal and antibiotic resistance

Table 1. Staphylococcal plasmid groups and their characteristics

The acquisition of DNA encoding methicillin resistance (*mecA* operon) by *S.aureus* (MRSA) is currently giving rise to potentially life-threatening illnesses and therefore causing major concerns in clinical institutions. The emergence of MRSA as a nosocomial pathogen was initially reported in Europe during the 1960s soon after the introduction of methicillin. The first reported isolate in the United Kingdom was by Jevons[176] and Barber[24] in 1961. An early case study into the prevalence of MRSA by Parker and Hewitt classified strains resistant to methicillin with an MIC≥25ug/ml by broth micro-dilution.[276] They discovered the ability of MRSA to express large quantities of penicillinase and display resistance to several non-penicillin antibiotics. It has also become apparent that strains are not confined to exposed populations as community acquired-MRSA (CA-MRSA) has been reported. Isolates have also been reported from countries where methicillin and penicillinase-resistant penicillins were not available such as Poland, Turkey and India, and in populations with no apparent risk factors to the acquisition of methicillin resistance.[21,45,111,267,330]

A decline in the isolation frequency of multi-resistant strains occurred in the 1970s possibly due to a reduction in the use of tetracycline during this time.[21] However, from 1978 'new' MRSA strains emerged causing intra- and inter-hospital outbreaks in Australia, the Republic of Ireland and the United States of America, and in 1981 epidemic MRSA strain-1 (EMRSA-1) emerged in London hospitals spreading across the country. Since then, and particularly from 1991, different strains of EMRSA have continued to appear and are continually being isolated from patients. At present there are seventeen strains of EMRSA in the UK many of which have heterogenous resistance.[18] All strains of MRSA express an additional 78KDa cell wall protein PBP2a (or 2') active at concentrations of antibiotics that inactivate other PBPs retaining its transpeptidase activity.[236]

The treatment of these highly resistant strains is sometimes dependent upon the antibiotic vancomycin however, strains of enterococci already display high-level vancomycin resistance and strains of *S.aureus* and coagulase-negative staphylococci (CNS), with intermediate to high-level resistance have been isolated.[316,332] The first reported strain of vancomycin intermediate resistant *S.aureus* (VISA) with an MIC of 2μg/ml was in Japan in 1997 by Hiramatsu (hVISA Mu3) followed by its clonal variant VISA Mu50 (MIC=8μg/ml) and two cases in the United States of America from patients receiving extended vancomycin treatment, strains 5827 and 5836 (MIC=8μg/ml).[48,49,161] To date, more strains of VISA have been isolated in the United States and across Europe.[50,52,282,296,332] In the UK, the first VISA strain did not appear until 1998 when a strain of EMRSA-15 (vancomycin MIC=4μg/ml) was recovered from a patient receiving vancomycin therapy in Bristol.[166] This was followed in 1999 by the isolation of two strains, 3700w and 3759v (MIC=6μg/ml), within days of each other from different patients at Glasgow Royal Infirmary.[164]

Analysis of glycopeptide resistant strains, in particular Mu50, has revealed major alterations in its cell wall composition. Increased levels of glutamine non-aminated murapeptides, PBP2 and PBP2' expression, reduced levels of peptidoglycan cross linking and dimer:monomer muropeptides and an overall thickened cell wall have all been described, but not all together and not in all strains.[40,84,149,150,224,242,314,317] Additional features such as longer mean generation times, small colony size on solid agar, reduced methicillin resistance, and reduced 'cellular amounts' of PBP4 with increasing vancomycin MIC's have also been described.[84,296,317,329]

1.2 Staphylococcal Proteins and Toxins

The initiation of a diseased state is dependent upon the penetration of the host's physical barriers and the ability to survive the inhibitory effects of antimicrobial secretions and cells of the immune system (section 1.7). Over time bacteria and other microorganisms have developed ways of evading or overwhelming host defences. Many species of bacteria display proteins on their cell surface that aid attachment of the bacterial cell to host cells as well as protect it from phagocytosis. Selected extracellular proteins or toxins function to destroy target host cells facilitating the infection process. The expression of virulence factors in *S.aureus* is multifactorial and subject to environmental and internal regulation

(section 1.3). The systems that eventually succumb to staphylococcal infection depend upon the genetic makeup of the infecting strain and its environment. Table 2 summarises the range of proteins and toxins expressed by *S.aureus* and their role in human disease.

Toxin	Target	Mode of Action	Disease
α-, β-, δ-haemolysin	Red and white cells	Haemolysis	Abscesses
γ-haemolysin	Leukocytes	Leukolysis	Abscesses
Panton –Valentine leukocidin (PVL)	Leukocytes	Leukolysis	Abscesses, Necrotising pneumonia
Toxic shock syndrome toxin (TSST-1)	Tcells	Release of cytokines, Systemic shock	Toxic shock syndrome (TSS)
Enterotoxins A to O	Intestinal cells	Induce vomiting, diarrhoea, shock	Food poisoning
Exfoliative toxin A and B	Epidermis	Desquamation of skin, shock	Scalded skin syndrome (SSS)
Staphylococcal enterotoxin- like protein (SET-1)	?	?	?
Coagulase	Fibrinogen	Induces fibrin clotting	Resistance to phagocytosis
Protein A	Fc portion of IgG antibodies	Reduced activation of complement pathways and opsonisation	Resistance to phagocytosis

Table 2. Proteins and toxins produced by *S.aureus*.

1.2.1 Cell Surface Proteins

Cell surface components are often implicated in bacterial adherence to host cells, a number of which have been designated microbial surface components recognising adhesive matrix molecules or MSCRAMM. Fibronectin-binding protein A (FnBPA), collagen-binding protein and fibrinogen clumping factor A are only a few of such proteins that mediate adherence to plasma clots and collagenous tissues, and are important for colonisation and infection.[109] Other non-MSCRAMM surface proteins are involved in the

evasion of cell recognition and phagocytosis by the host immune system, for example coagulase and protein A, as is the expression of polysaccharide capsules.

1.2.1.1 Clumping Factor and Coagulase

Currently, staphylococci are classified as either coagulase-negative or coagulase-positive depending upon their ability to coagulate fibrin at their surface. The majority of pathogenic staphylococci readily produce coagulase, a18kDa heat-labile protein, although coagulase-negative staphylococci (CNS) have been isolated from infections particularly in infants and immunocompromised individuals.[107]

There are two types of coagulase: surface bound (clumping factor) and free coagulase. Free coagulase is a fibrin-clotting enzyme that binds to prothrombin (68kda) in plasma to form staphylothrombin (89kda). Staphylothrombin mimics thrombin, stimulating plasma clotting of fibrinogen to insoluble fibrin, a form more resistant to the action of fibrinolysin than a normal fibrin clot. Fibrin is deposited around the surface of bacterial cells thus protecting them from phagocytic attack. In contrast, staphylococcal clumping factor does not form true plasma clots, but acts directly upon fibrinogen to induce cell clumping. Cells in the centre of the clump are provided with a degree of protection from phagocytosis. The role of free coagulase or clumping factor in virulence has not been concluded due to contradicting data. Strains of coagulase-negative staphylococci (CNS) are known to be less virulent than coagulase-positive strains however mutations within the coagulase gene do not alter virulence. Both forms of coagulase are not always expressed; some strains are known to produce one or the other.[174]

1.2.1.2 Protein A

Greater than 90% of coagulase positive *S.aureus* strains express protein A as a cell wall component, comprising up to approximately 7% of the total cell wall, although expression is variable.[107] In strains of MRSA as many as 50% do not express cell bound protein A, but secrete it extracellularly, as do some strains of CNS.[107,367]

The gene encoding protein A, *spa*, generates a protein with a molecular weight of 42kDa although size heterogeneity exists among serologically distinct strains.[35,106] The gene comprises three distinct regions: the Fc portion, X region containing varying numbers of

highly polymorphic 24bp repeats differing in number and sequence between strains, and a C-terminus.[342] The development of typing techniques using the polymorphic X-region for MRSA typing in outbreak situations has provided promising results,[268,333] although its use in discriminating between epidemic and sporadic strains of MRSA has provided variable results.[113,163] Structural determinants of the Fc portion promote binding to host immunoglobulins (Ig) to form protein A-Ig complexes through binding to discontinuous epitopes within the Fc region of human IgG antibodies, reactions with heavy chain variable regions of V_HIII antibodies, or through protein A-specific antibody.[90,106]

Negatively regulated by the regulatory locus *agr* (section 1.3), optimal expression occurs during late logarithmic phase and decreases during the stationary phase. The absence of protein A on dividing cell septum during cell division indicates late incorporation into the outer cell wall.[253] Characteristically, cell bound and extracellular protein A are very similar and it has been suggested that they are synthesised from the same mRNA template. The incorporation of protein A into the cell wall occurs in one of two ways: linked to cytoplasmic or membrane bound peptidoglycan monomers which are then transported through the cell membrane and inserted into the pre-existing peptidoglycan network; or are transported through the membrane as a single molecule then linked into growing peptidoglycan chains or an already completed chain via covalent bonds. Free protein A is the proportion of protein not incorporated into the cell wall.[245]

The expression of protein A in either form confers antiphagocytic properties on bacterial cells through the formation of protein A-IgG complexes in competition with host phagocytic cells. Bacterial cell surface complement activation in the absence of IgG and C3b fixation is a crucial requirement for rapid phagocytic ingestion (section 1.7).[279] Protein A-IgG complex formation reduces IgG-mediated opsonisation of cells via the activation of both the alternative and classical complement pathways.[279,327] Enhanced phagocytosis of protein A deficient strains, such as *S.aureus* Wood 46 compared to rich strains in normal serum gives evidence to this, as is the relationship between the degree of protein expression and resistance to phagocytosis.[279,327] Dossett et al (1969)[90] and Spika et al (1981)[327] have suggested that the expression of protein A may function to mask alternative complement-activating sites or prevent the attachment of specific opsonins through non-specific reactions with IgG, as isolated protein A will inhibit phagocytosis of *S.aureus* and *E.coli*.

The precise role of protein A as a virulence factor is highly debated as experimental results vary depending on the animal model used. No toxin or enzymatic activity has been associated with protein A,[145] however *spa* mutants have been found to be less virulent in mouse models of peritonitis and skin abscesses, mortality rate is reduced and skin lesions from subcutaneous injection are significantly smaller. Contrary to this it does not appear to be a major virulence factor in the staphylococcal keratitis rabbit model.[46] A report by Jung *et al.* (2001) suggested the ability of protein A to bind cytoskeletal β-actin in epithelial cells.[182] These authors proposed that protein A might facilitate the adherence and invasion of *S.aureus* in oral epithelial cells through the polymerisation of actin filaments, however the presence of the appropriate receptors on epithelial cells and *S.aureus* has not been proven. It has also been considered as the cause of hypersensitivity reactions, inflammation and tissue damage through complexes with immunoglobulins.[106,144,145]

1.2.1.3 Polysaccharide Capsule

It has been predicted that approximately 90% of *S.aureus* strains produce a polysaccharide capsule (CP) of some kind attached to the exterior of the cell wall.[326] Several capsule polysaccharides (CP) have been identified belonging to strains of *S.aureus* where type CP5 or CP8 occurs in >80% of clinical isolates.[326]

Reports from Dassy *et al* (1993)[83] and Luong *et al* (2002)[221] show that CP5 and CP8 production is positively regulated by *agr* and reduced in *agr* mutant strains, as well as being influenced by environmental conditions such as iron limitation, alkaline conditions, CO_2, and anaerobiosis.[82,209] Promoter regions upstream of CP5 and CP8 share almost identical sequence homology resulting in similar regulation mechanisms. *Agr* is the predominant regulator here, although *SarA* (section 1.3) does exert minor control at the transcription level, but *SarA* also plays a role at the post-translation level affecting CP8 integrity.

Expression of CP8 during the stationary phase along with the exotoxins, suggests a role in infection, however contradictory reports exist.[221] Mutation studies with $CP5^-$ strains have indicated no loss of virulence compared to the parental strain while $CP5^+$ or $CP8^+$ strains did not confer resistance to opsonophagocytic killing by human PMNL.[8,370] Several

authors, however, have reported that CP5 and CP8 are indeed antiphagocytic by interfering with opsonisation via the classical and alternative complement pathways and enhanced virulence.[185,254,280,337] Nilsson *et al.* (1997)[254] and Thakker *et al.* (1998)[337] also reported that $CP5^{+}$ strains caused a higher incidence of arthritis and bacteraemia than non-producers. Bacterial strains grown on solid media have been reported to produce >100 fold more CP5 and CP8 than broth grown cells.[209,337] This may account for the conflicting data as Albus and Baddour used broth cultures whereas Nilsson and Thakker both used agar cultures.

Extracellular polysaccharide material loosely associated with the bacterial cell, slime, plays an important role in the adhesion of cells to biologically inert surfaces e.g. plastics and animal cells that are also largely polysaccharide, and bone.[229,230] Coagulase negative staphylococci, a common cause of catheter related infections, once adhered to a surface may use the slime layers to establish firmer adhesion initiating the formation of microcolonies and eventually biofilms, thus providing increased access to organic nutrients and a protective barrier from antibacterial agents.

1.2.2 Extracellular Toxins

Extracellular toxins are a heterogeneous group of proteins of which there are two principal groups: enzymes such as proteases and nucleases; and toxins including exfoliative toxins, enterotoxin, PV-leucocidin (PVL), and toxic shock syndrome toxin (TSST-1). The necessity to produce these products is probably driven by the need to extract nutrients from available resources for bacterial growth and survival, an effect of which is the establishment of a diseased state within a host environment. Only the toxins produced by *S.aureus* are discussed here.

1.2.2.1 Haemolysins

Four staphylococcal haemolysins have been described to date, alpha (α), beta (β), delta (δ), and gamma (γ)-haemolysin. All four haemolysins are positively regulated by the *agr* locus therefore expression is growth phase dependent, with maximal expression during late logarithmic phase to early stationary phase.[197] Alpha-haemolysin transcription is

somewhat more complex and is believed to be influenced by at least two additional regulatory systems as well as osmotic regulation and catabolite repression (section 1.3).[54,58,265,348]

Alpha-haemolysin: Of the haemolysins α-haemolysin (34kDa), encoded by the chromosomal *hla* gene, is the most dominant being produced by 95% of *S.aureus* strains forming 33% of the total bacterial protein expressed.[1,141] Primarily coagulase-positive strains tend to be α-haemolysin positive, whereas coagulase-negative strains are negative for the α-haemolysin gene.[32,44] First sequenced in 1984 by Gray and Kehoe,[141] α-haemolysin exerts cytolytic, haemolytic, necrotic and lethal effects on numerous cells particularly those with membranes rich in phosphatidylcholine and sphingomyelin, including epithelial cells and cells of the immune system.[30,32,87] Lateral aggregation of toxin monomers into heptomeric structures within target membranes results in pore formation (1-2nm diameter) allowing the rapid efflux of potassium and influx of sodium and calcium. Ion imbalance consequently causes swelling and rupture of cells as well as indirectly stimulating cytokine release and apoptosis.[31,179,246] The specificity of the toxin for membranes comprised of phosphatidylcholine and sphingomyelin can be linked to a number of clinical features observed in a host including vasoconstriction, increased vascular permeability, and pulmonary oedema.[87]

In vitro, rabbit erythrocytes are 100 fold more sensitive to α-haemolysin than those from sheep and humans,[30,97] whereas β- and δ-haemolysins preferentially lyse sheep and human erythrocytes respectively.[97,246] This demonstrates the special attention required in choosing an appropriate animal model for studying α-haemolysin. The relevance of α-haemolysin in human disease has been questioned due to its poor activity against human erythrocytes. However, human platelets and monocytes are more susceptible than erythrocytes to α-toxin activity and may provide the primary target *in vivo*.[31,32,315] For example, high toxin levels of 10-50 haemolytic units (HU) consume components C3 of the classical complement pathway reducing their opsonic activity whilst low doses potentiate phagocytic and bactericidal activities of human PMNL.[121,125,126] Animal models of *S.aureus* keratitis in rabbits, murine sepsis and murine septic arthritis have all shown toxin related cell damage, while mutagenesis studies with *hla*⁻ mutant strains display reduced virulence compared to the parental strains following intraperitoneal or intravenous inoculation.[43,46,255,334] Evidence that the regulation of *hla* expression is not a simple one

has been demonstrated by Goerke *et al* (2001) in a guinea pig model of device-related infection.[136] Here, *hla* transcription was induced independently of *agr* and *sarA* but was dependent upon *sae* regulation (section 1.3). *In vitro*, *hla* expression is dependent upon the activity of all three regulatory systems. Despite the evidence of alpha haemolysins biological activity in a variety of animal models, its common occurrence within the staphylococcal genome and complex regulation, the perceived role of α-haemolysin as a major virulence factor in disease pathogenesis remains ambiguous.

Beta-haemolysin (β) is an Mg^{2++}-dependent sphingomyelinase C ("hot-cold" haemolysin), expressed by 11% of staphylococcal strains, predominantly bovine strains, although it has been isolated in humans.[97,246] Activity is through the degradation of sphingomyelin in the outer phospholipid layer of erythrocyte membranes, particularly those of sheep due to their high sphingomyelin content.[360] Haemolytic action of β-haemolysin does not actually involve cell lysis, but sensitises cells to the action of other agents.[338] Selected strains of glycopeptide intermediate *S.aureus* (GISA) and *agr*$^-$ strains have been shown only to express β-haemolysin.[300]

Delta-haemolysin (δ) is a 26 amino acid peptide secreted as part of the RNAIII transcript during *agr* activation forming cationic-selective channels in phospholipid bilayers, in particular those of human erythrocytes.[169] The toxin has also been identified as a putative contributor to TSS pathogenesis. Lyon *et al.* (2000) have shown that strains of GISA do not express δ-haemolysin due to a loss of *agr* function or truncated AgrA (section 1.3).[300]

Gamma haemolysin (γ) differs from the other three haemolysins in that it is secreted as two individual proteins termed 'S' and 'F' for slow and fast eluting proteins. The *luk* locus encodes these components on two transcription units with the first encoding a single open reading frame (ORF) and the second encoding two ORFs. Two forms of the 'S' component exist, HlgC and HlgA, whilst there is only one form of the 'F' component, HlgB, giving the potential formation of two toxin combinations.[76] Preferential binding to membranes containing phosphatidylinositol results in activity against mammalian erythrocytes, neutrophils and macrophages. Detection of γ-toxin is possible in 99% of *S.aureus* strains and is predominant in the culture supernatants of TSS isolates.[73,283]

Toxin	Most susceptible erythrocyte species	Target Cells	Mode of Action
α-haemolysin	Rabbit	Phosphatidylcholine and sphingomyelin membranes	Pore formation
β-haemolysin	Sheep	Sphingomyelin membranes	Degrades sphingomyelin
δ-haemolysin	Human, Horse	Phospholipid bilayers	Cationic channel formation
γ-haemolysin	Rabbit	Phosphatidylinositol membranes	Granule secretion and the release of inflammatory mediators by leukocytes

Table 3. Haemolysin activity

Panton-Valentine Leukocidin (PVL)

PV-leukocidin is a phage (ϕSLT) encoded toxin similar to γ-haemolysin but is only expressed in approximately 2% of *S.aureus* strains.[95,283] As with γ-haemolysin PVL is a two-component toxin. PVL components lukS-PV and lukF-PV, when expressed together with γ-haemolysin, are capable of forming up to six different combinations of toxin with HlgA, HlgB and HlgC.[283] Individually, these components are relatively inactive and, depending upon the combination of 'S' and 'F' components, elicit varying levels of activity against variable targets; LukS-PV + LukF-PV, LukS-PV + HlgB > HlgC +LukF-PV, HlgC + HlgB > HlgA + LukF-PV, HlgA + HlgB exert activity towards PMNL, monocytes and macrophages of rabbits and human; hlgA + hlgB also display activity towards human Tcells and erythrocytes; HlgA + LukF-PV has no erythrocyte activity.[103,199,284]

Pathogenesis of PVL and γ-haemolysin involves the sequential, irreversible, binding of the 'S' component followed by the 'F' component to target membranes resulting in the formation of hexameric pores.[74,235] The activation of calcium channels and increased permeability to monovalent cations leads to leukocyte destruction by granule secretion, the release of inflammatory mediators, and tissue necrosis as evidenced in cases of furunculosis, impetigo, cellulitis and cutaneous absesses.[74,140,215,283] In *in vivo* studies,

intradermal administration of PVL elicits a severe inflammatory lesion, PMN infiltration and skin necrosis in rabbits.[199,321]

In France, a study of community acquired MRSA (CA-MRSA) revealed a prevalence of PVL in 100% of community-acquired MRSA with a total of 2% of all clinical isolates being PVL-producing strains.[95] Susceptibility to PVL^+ strains appears not to be in immunocompromised individuals but in young healthy individuals who present no obvious risk factors for the acquisition of MRSA. Infections appear to be secondary to an influenza-like illness that rapidly develops into severe pneumonia and leucopenia.[133] PVL has also been isolated from strains associated with antibiotic-associated diarrhoea (AAD).[139]

1.2.2.2 Superantigens

Around 40% of *S.aureus* isolates and 6% of CNS isolates from healthy carriers and clinical infection are capable of expressing one or more superantigen, while most healthy adults will have antibody titres to common superantigens, probably following exposure to concentrations that are not associated with clinical effects.[6,112,343]

Of the staphylococcal toxins, enterotoxins A through E, and H, exfoliative toxins A and B, and toxic shock syndrome toxin (TSST-1) are all classed as superantigens (SAg) along with the streptococcal pyrogenic exotoxins (SPE) A, B, and C.[16] The SAgs are a class of small non-glycosylated polypeptides that share 22-80% amino acid sequence identity, with TSST-1 being the most divergent.[87] The biological properties that set them aside from other staphylococcal toxins are outlined in Table 4. Genetically, the toxin genes are plasmid, bacteriophage or pathogenicity island borne and are regulated by the *agr* and staphylococcal accessory regulatory (*sar*) systems (section 1.3).

Shared Biological Properties of Superantigens
Pathogenicity
Enhancement of susceptibility to endotoxin shock (up to 100,000 fold)
Non-specific T-lymphocyte mitogenicity
B-lymphocyte suppression
Erythrophagocytosis
Tumour Necrosis Factor (TNF) and Interlukin-1 (IL-1) release from macrophages

Table 4. Biological properties of superantigens

The presence of foreign bodies within the host are recognised by cells of the immune system, ingested by antigen presenting cells (APC) and presented to T-lymphocytes as major histocompatibility complex (MHC) class II peptide products (section 1.6). Specific binding between Tcell receptors (TCR) and the processed antigen triggers Tcell proliferation and release of cytokines initiating an immune response. Superantigens differ in their mode of Tcell activation in that they do not require processing by APCs.

A superantigenic toxin will non-specifically bind directly with MHC class II molecules bridging the gap between it and particular vβ TCRs blocking antigen-specific Tcell recognition (Figure 1).[23] This high frequency binding stimulates the proliferation of $CD4^{+}CD8^{-}$ T_{helper}/delayed type hypersensitivity T cells, and $CD8^{+}CD4^{-}$ $T_{cytotoxic}$/suppressor T cells and release excess quantities of cytokines such as, interleukin-1 (Il-1), Interferon gamma (IFN-γ) and tumour necrosis factor (TNF), the causative agents of hypotension and shock through an overwhelming inflammatory response.[16,102] Fever has been attributed directly to the stimulation of the hypothalamus fever response control centre, or indirectly by the release of Il-1 and TNF from monocytes and macrophages. An interesting response to superantigen exposure is the failure of the majority of patients to develop neutralising antibodies upon recovery. Other characterised effects of superantigen release include the inhibition of immunoglobulin (Ig) secretion through the induction of a non-specific T-suppressor factor, and the enhancement of host susceptibility to endotoxin such as the Gram-negative lipopolysaccharide (LPS).[16]

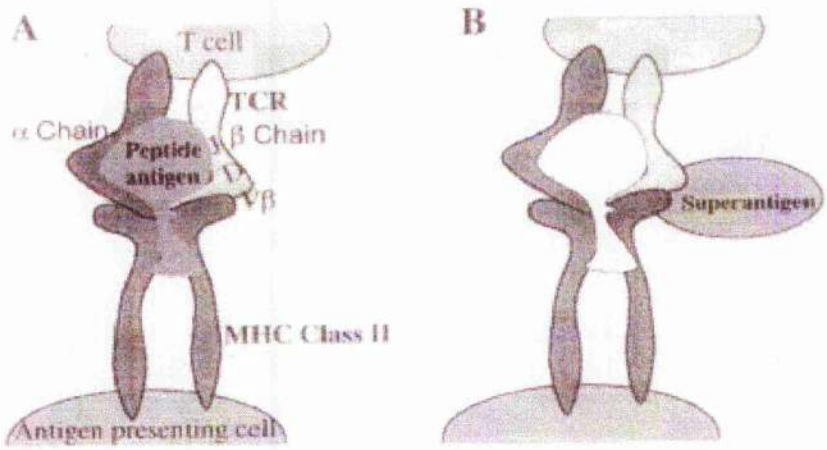


Figure 1. Model for the MHC class II–Tcell receptor interactions with (A) conventional antigen, (B) superantigen. (A) A processed antigen presented by MHC class II attracting specific Tcell with antigen specific Tcell receptor (TCR) variable (vβ) domain. (B) A superantigen binding directly to the outside of the MHC molecule cross-linking it to the vβ-chain. Initiating non-specific cell activation.[23]

1.2.2.2.1 Toxic Shock Syndrome Toxin 1 (TSST-1)

TSST-1 is symptomatically related to the staphylococcal enterotoxin group, but shares little sequence homology with staphylococcal enterotoxins or streptococcal pyrogenic enterotoxins.[16,36] The protein is also the only SAg capable of passing through mucosal membranes in an intact form.[148]

The synthesis and secretion of the low molecular weight polypeptide occurs primarily during late logarithmic phase via positive regulation by the *agr* locus, not as a result of cell lysis.[197,302] The chromosomally encoded TSST-1 gene (*tst*) was cloned by Kreiswirth *et al.* (1983)[201] and has been located to pathogenicity island-1 (SaPI1) by Lindsay *et al.* (1998).[219] As yet, *tst* has never been detected in strains of CNS, but has been detected in a bovine strain of *S.aureus* on pathogenicity island SaPIbov.[105]

Bergdoll *et al.* (1981)[28] and Schlievert *et al.* (1981)[301] first identified TSST-1,[29] initially named staphylococcal enterotoxin F (SEF) and pyrogenic exotoxin C respectively, as being associated with cases of menstrual toxic shock syndrome (TSS). Today, TSST-1 is associated with 100% of menstrual cases and 50% of non-menstrual cases such as post-surgical wound infection, and staphylococcal scalded skin syndrome (SSSS)-like illness especially in AIDS patients.[87,301] Reports have also indicated a putative role in sudden

infant death syndrome (SIDS) cases along with SEA, B, and C, and Kawasaki syndrome, an acute febrile disease of children under 5 years of age where symptoms resemble TSS, but the presence of TSST-1$^+$ strains has not been conclusive.[210,251,372,378]

In addition to *agr* regulation, TSST-1 transcription is subject to temperature regulation with toxin levels detectable at 37°C to 40°C, but not at 30°C. The possible role of a temperature regulated transport mechanism such as an activating factor for example, a protease required for toxin modification that is activated at 37°C but not at 30°C, or a repressor has been suggested.[302] Low glucose concentrations, magnesium ions, and elevated concentrations of oxygen and carbon dioxide are also required for optimal expression.[188,339,373] All these conditions are established in the vagina, and are associated with tampon usage. TSST-1 is also a negative regulator of exoprotein gene transcription, repressing most other exoproteins whilst positively regulating its own transcription.[356] Enterotoxin B has similar properties.[356]

Numerous authors have attempted to recreate the pathogenesis of TSS in animal models, but the contribution of other factors released as a consequence of TSST-1 superantigenicity hinders the recreation of a true TSS model. Strains of TSST-1$^+$ MSSA and MRSA do not exert lethal effects in a murine sepsis model and TSST-1 has been reported to be a poor immunogen in rabbits.[303,334] However, the toxin causes reactivation of cell wall induced arthritis, induces systemic symptoms of food poisoning in rabbits, and produces in mice many clinical features of TSS except for the rash and desquamation.[307]

1.2.2.2.2 Enterotoxins

There are currently eleven staphylococcal enterotoxins (SEA through SEK) that have been associated with human food poisoning however, enterotoxins SEL through SEO have recently been identified but further investigations into their biological activity are required. To date, all enterotoxins possess emetic properties and are the only SAg with this activity.[87] Enterotoxin genes have been detected on the bacterial chromosome, on plasmids and on bacteriophage. Transcription is generally independent and not regulated by a polycistronic transcript.

Within the enterotoxins, SEA, SED, SEE, SEG, SHE and SEJ share 53-83% sequence homology, and SEB, SEC_1 and streptococcal pyrogenic exotoxin A (SPEA) share 42-67%

homology.[16,23] Despite the relatedness between each enterotoxin, antibodies to one enterotoxin do not necessarily confer immunity to food poisoning or to the other enterotoxins.[25,87] The more recently described enterotoxins I, K and L differ structurally from SEA-E by their lack of a cysteine loop, a region thought to be important for emetic activity.[247,270] SHE and SEI are the most divergent of the enterotoxins. Homology to the SEA-E group and the SEB-C group have both been described with significant regions of homology across the groups thus making it difficult to assign SHE and SEI to a particular group.[23] Here, they have been linked to the SEA-E subclass (Figure 2).

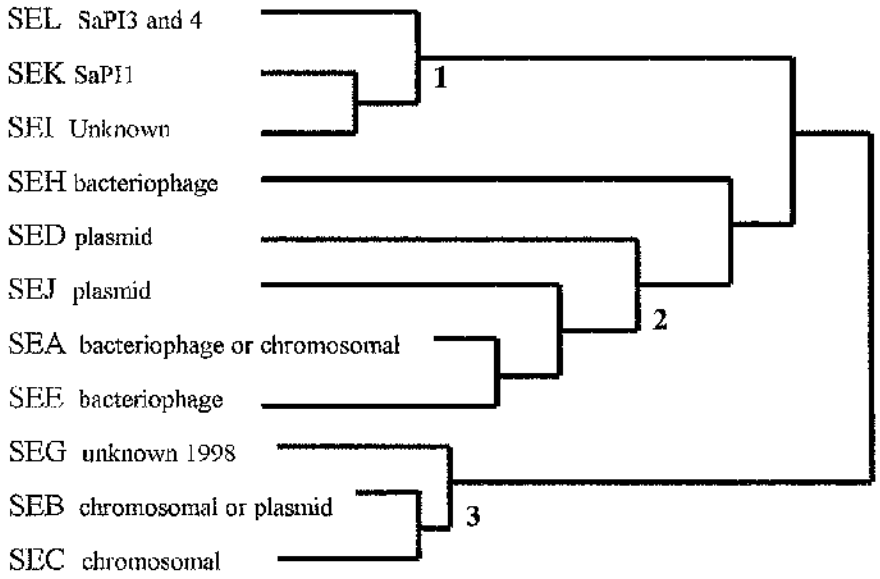


Figure 2. Phylogenic tree showing the relatedness of staphylococcal enterotoxins. Three groups are recognisable. Adapted from Orwin *et al.* (2001),[270] Alouf *et al.* (1991),[16] and Novick *et al.* (2001)[261]

To date, SEA and SED, and to a lesser extent SEB and SEC, are responsible for the majority of food poisoning cases.[23,87] Over the years there has been a gradual shift in the carriage of enterotoxins in strains of MRSA. During the 1960's, the majority of strains produced SEB whereas the primary enterotoxin produced by MRSA is highly dependent upon country and strain. In Scotland, 70% of MRSA carry SEC and are classified as EMRSA-15 whereas strains of EMRSA-16 are primarily carriers of SEA (Dr D. Morrison, MRSA Reference Laboratory 2002; personal communication). The production of SEA, SEB or both in strains of septicaemic MRSA infections also indicates a putative role for enterotoxins in pathogenesis, as does the isolation of SEB, SEC, SEG and SEI in cases of menstrual and non-menstrual TSS and SSS.[171,182] Recombinant SEK has been shown to demonstrate lethality in the rabbit model of TSS.[270]

In 2000 a novel family of at least five enterotoxin-like genes (*set1-set5*) was identified encoding staphylococcal enterotoxin-like proteins or SET proteins.[366] Four of the *set* genes encode proteins of 227-234 amino acids with 38-53% homology to other known enterotoxins and TSST-1. So far SET-1 has been identified in numerous strains of *S.aureus*, but not from *S.epidermidis*. Activities of recombinant SET1 (rSET1) on PMNL cells results in cell stimulation and a 10-fold greater production of Il-6 and Il-1β compared to that produced by SEB at higher concentrations thus suggesting an important role of SET-1 in pathogenicity.[366] Further studies into the extent of *set* gene expression amongst *S.aureus* and their putative role as virulence factors are required.

1.2.2.2.3 Exfoliative Toxins

Exfoliative toxins ETA and ETB are chromosomally and plasmid encoded proteins respectively, with superantigenic properties.[203,240,266] Production of these toxins by bacterial strains present on the skin surface results in the onset of diseases such as staphylococcal scalded skin syndrome (SSSS) or Ritter's disease, primarily in infants and children.[203] Toxin expression by *S.aureus* on the skin causes the formation of skin lesions that spread through the destruction of the skin's intercellular connections separating the layers within the epidermis, thus forming a rash and bullous impetigo.[140,203,214] Exfoliative toxin A was isolated by Kapral and Miller[184] and, Arbuthnott[17] *et al.* in 1971 followed by the characterisation of ETB by Kondo *et al.* in 1974.[196] Both toxins display 40%-50% sequence homology to each other[208,264] and 25% identity to staphylococcal V8 protease indicating serine protease activity, although conclusive evidence is lacking.[81,349] There is also evidence to support activity against melanocyte stimulating hormones (MSH) present in the epidermis, a possible target in cases of SSS.[289] Murine studies indicate that toxin susceptibility ceases in mice >7 days old due to efficient clearing of the toxin in serum. Insufficient removal of toxins occurs in immunocompromised individuals who are more at risk of succumbing to SSSS suggesting a threshold level of toxin is required for skin exfoliation.[281]

1.3 Regulation of Virulence

The three phases of bacterial growth, lag phase, exponential phase, and post-exponential or stationary phase, are very different with respect to their metabolic requirements. During the exponential phase cell metabolism is at its most efficient with its regulation systems coordinated to provide constant growth and to maintain cell composition. Upon entering the post exponential phase energy is directed more towards long-term survival. Due to this enzymes used for the exploitation of environmental nutrients are no longer required. They become an unnecessary burden and are no longer synthesized in response to restructuring of the cell's regulatory systems. It is as a result of this restructuring that pathogenic strains of bacteria produce phase dependent virulence factors. In *S.aureus* surface proteins such as protein A, coagulase and fibronectin binding proteins (Fnbp), products of the exponential phase, are repressed during the post exponential phase making way for the expression of extracellular proteins such as toxins and haemolysins.[207,212] Similar regulatory system reorganization is observed in other bacterial species; *Bacillus subtilis* induces exoprotein synthesis, competence and sporulation in response to nutrient deprivation[155] and the *ArcA* and *FNR* systems of *E.coli* respond to changes in osmolarity.[328]

Characteristically, regulation systems are two component signal-transducing systems with a common mechanism of action and shared essential protein sequences. In many cases transmembrane histidine kinase signal receptor proteins detect environmental signals although some are cytoplasmic and respond to intracellular signals. Activated signal receptors undergo allosteric modification activating latent histidine phosphokinase (HPK) activity. This triggers an autophosphorylation cascade that ultimately results in the phosphorylation of a cytoplasmic response regulator activating it to trigger repression or stimulation of target gene transcription.

The ability of *S.aureus* to produce a multitude of virulence factors is attributed to the coordinated activation and regulation of a set of genes contained within the chromosomal polycistronic accessory gene regulator (*agr*) locus.[243,290] Species of coagulase negative staphylococci including *S.epidermidis, S.warneri* and *S.simulans* have also been shown to carry similar regions of the *agr* operon of *S.aureus*.[94,335,347] The *agr* locus, cloned and sequenced by Peng *et al.* (1988), is composed of 2 divergent transcripts RNAII (P2) and

RNAIII (P3) transcribed in opposite directions.[259, 278] A weaker third promoter, P1, lies 5' to P2 and is transcribed constitutively throughout the growth cycle, but as yet, has an unknown role.[278] In 1986, Recsei *et al.* demonstrated that the majority of a*gr* regulated exoproteins were weakly synthesized or absent in *agr* negative mutants, but expression of surface protein was increased, indicating a strong correlation between *agr* regulation and the post-exponential phase.[290] It has also been recognized that *agr* mutants are less virulent in animal models compared to *agr* wild type strains.[2,197]

Figure 3 illustrates a map of the *agr* locus and its transcripts. Promoter two and P3 initiate transcripts in opposite directions giving rise to a 3.5kb transcript (RNAII) and 0.5kb transcript (RNAIII) respectively. RNAII is comprised of 4 unlinked open reading frames (ORFs) *agrA*, agr*B*, agr*C* and agr*D* whilst RNAIII encodes delta (δ)-haemolysin.[169,260]

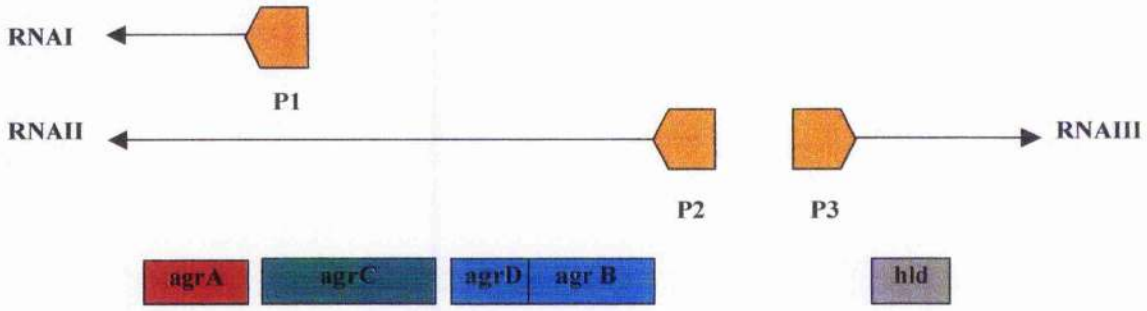


Figure 3. *S.aureus agr* regulatory locus. Adapted from Novick *et al* (1995)[260]

Each of the ORFs encoded on the RNAII transcript are essential for optimal activation of the RNAIII promoter.[260] Early data reviewed by Kornblum *et al.* (1990) identified *agrB* as encoding a histidine phosphokinase sensor protein, however this has since been revised.[197] It is now accepted that products of *agrA* and *agrC* which display homology to components of a classical quorum sensing 2-component signalling pathway encode a response regulator and a histidine phosphokinase sensor protein respectively.[260] *AgrB* and a*grD* function to generate a relatively hydrophobic 1kDa autoinducing octapeptide (AIP) with quorum sensing properties.[177] The gene *agrD* encodes a putative precursor peptide, AgrD, thought to be processed through an unusual intramolecular thioester modification linking the thiol group of a central cysteine residue to the C-terminus carboxyl group, and secreted by AgrB, an integral membrane protein.[232,271]

All stains of *S.aureus* do not produce the same AIP. Several structures of AIP have been identified enabling strains to be grouped by the peptide they produce. As a specific group they will activate the *agr* locus of strains within the same group, but inhibit that of strains from other groups and staphylococcal species, for example *S.epidermidis.*[178,232] Despite this *S.aureus* is more sensitive to inhibition from those produced by *S.epidermidis* than *vice versa.*[272,273] The ability to produce variable AIPs is a result of sequence variability in *agrB, agrD* and agr*C* thus determining the specificity of the processing reaction, the propeptide, and the receptor-ligand reaction.[178] The only conserved region of the AIP is a central cysteine residue five residues from the C-terminus linked by an essential thiolester bond.[271] Activation of *agr* is sensitive to the amino acid sequence and stereochemical nature of a particular AIP, and it has been proposed that inactivation occurs when a different class of peptide binds to AgrC in an alternate orientation blocking binding sites.[231]

Polymorphism within the hypervariable region of *agrC-agrD-agrB* alters the genetic sequence of AIP and the response regulator AgrC allowing the classification of strains into one of four *agr* groups.[172,178] Within each group the genetic sequence of the hypervariable region is highly conserved permitting the activation of the *agr* locus in strains belonging to the same *agr* group but inhibiting the activation of strains from the other groups without affecting growth.[172] Interstrain activation or inhibition takes place at the AgrC level.[222] Two closely related *agr* groups are group I and group IV, differing only in the sequence of P3 as observed by a weak activation of group I strains by group IV culture supernatants.[172]

Jarraud *et al.* (2002) suggested that *agr* groups could be associated with specific genetic backgrounds and that particular *agr* types were associated with certain diseases.[173] The system is not 100% accurate but it is generally agreed that CA-MRSA *mecA*$^{+}$ PVL^{+} strains and *tst*$^{+}$ strains belong to *agr* group III, ET–producing and CA-MRSA *mecA*$^{-}$ PVL^{+} strains are group IV (some strains also belong to groups I and II), strains resulting in enterotoxin mediated diseases belong to groups I or II, and that *agr* group II strains are more frequently associated with disease in hospitals, including strains of glycopeptide resistant *S.aureus* (GISA).[95,172,173,241,300] Contradicting reports which support and rebut the latter statement were made by Sakoulas *et al.* (2002)[300] who classified all GISA strains as being *agr* group II, and van Leeuwen *et al.* (2000)[346] who reported that only 6% of MSSA and 5% of MRSA clinical isolates belonged to *agr* groups II and III respectively. No MSSA have been isolated that can be classified as *agr* group IV.[241]

Low-level quantities of RNAII transcript are detectable during the exponential phase ensuring the presence of low levels of AgrA and AgrC that would be available to respond to an external signal along with low levels of AIP. During exponential growth AIP levels are increased, ultimately reaching a threshold concentration that triggers RNAII and RNAIII transcription. Once activated both promoters become stimulated thus initiating autocatalytic activation of RNAII that in turn rapidly increases the level of RNAIII transcription and hence increases exoprotein synthesis whilst depressing surface protein expression.[177,260] RNAIII, an antisense RNA effector molecule, is responsible for regulated transcription of target exoprotein genes and in some cases translation.[244,259] Deletion within RNAIII results in an agr⁻ phenotype and the loss of delayed translation of δ-haemolysin.[259] RNAIII has also been found to independently regulate translation of α-haemolysin through direct mRNA binding.[244] It has been elucidated that in the absence of a fully functional RNAIII α-haemolysin translation is impaired adopting an untranslatable configuration.[244,259] In addition, Janzon and Arvidson (1990) have suggested that the δ-haemolysin transcript, when transcribed as part of the RNAIII transcript, plays a role in positive and negative regulation of exoprotein synthesis, but its mode of action is still under investigation (Figure 4).[170] Loss of *agr* function can arise as a result of a number of factors including; point mutation, loss of gene function required for *agr* expression, *agr* suppression via mutation within promoter regions or a disruption of quorum sensing pathways.[300] The serial passage of strains of *S.aureus* has been shown to induce mutations within the *agrC* gene or *agrC-agrA* region.[325] Some strains of GISA have been identified with mutations within *agrB* but demonstrate no obvious loss of *agr* and only reduced *hld* function whereas VISA 5836 from the United States has a nonsense mutation in *agrA* and is devoid of *agr* function.[300]

An early study by Wesson *et al.* (1988) hypothesised that in an environment where low levels of AIP are present, cell surface proteins are optimally expressed promoting adhesion to host endothelial cells and ingestion.[365] Enclosed within an endosomal membrane AIP concentrations may increase due to cell density triggering expression of *agr*-regulated exoproteins that in turn may aid release from the endosome by inducing host cell apoptosis.[365] Quzi *et al.* (2001) confirmed this hypothesis using green fluorescent protein (GFP) and luciferase (*lux*) reporter cassettes.[287] These authors reported that the *agr* P3 promoter was induced within 100mins of internalisation releasing replication competent cells into the host cell cytoplasm. Similar observations were made by Bayles *et al.* (1998)

with bovine epithelial cells therefore it would appear that the *agr* locus contributes to endosomal lysis by ingested bacteria.[27] Similarly, the high levels of RNAII and RNAIII expression have been reported within vegetations in an experimental endocarditis model implicating a role for *agr* in *in vivo* invasiveness.[369] Differences in the levels of expression were variable depending upon the target tissue, thus suggesting that host factors may play a role in gene expression.[369]

In summary, the pattern of gene regulation observed by the *agr* system is thought to mimic that displayed during pathogenic invasion of host cells. Initial infection is characterised by adhesion and colonization through cell surface proteins. As a result of cell division, cell density increases activating toxin production, some of which degrade surrounding tissues allowing for release and spread of infection.

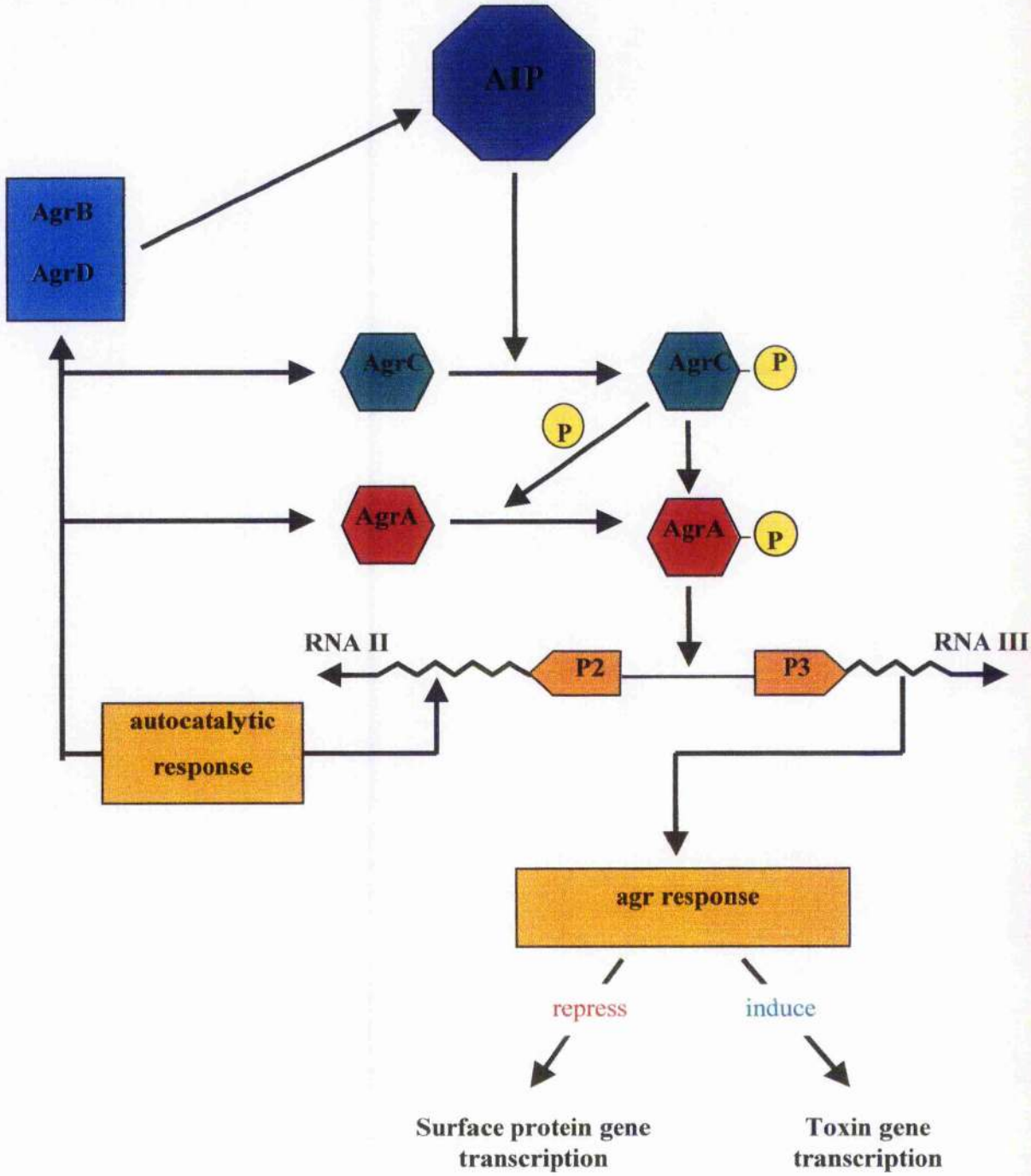


Figure 4. *Agr* locus regulation. Adapted from Kornblum *et al* (1990)[197] and Cheung *et al* (1997)[63]

In addition to *agr* there is a second major regulatory system that, in conjunction with *agr*, forms a complex growth phase-dependent regulatory system. In 1992, Cheung *et al.* identified a new putative global regulatory locus through transposon insertion studies.[58] This new staphylococcal accessory regulator (*sar*), now termed the *sarA* locus,[57] when

interrupted by mutation was found to increase α-haemolysin, lipase and serine protease production whilst decreasing β- and δ-haemolysin levels and cell wall proteins with the exception of cell bound protein A.[58,61,96,186] *SarA* is required for optimal expression of *agr* RNAIII levels and gene activation.[57,59,68,96]

The 1.2kb *sarA* locus is comprised of three overlapping transcripts, *sarA* (0.56kb), *sarB* (0.8kb) and sar*C* (1.2kb) each with its own promoter, P1, P2, and P3 respectively, but all transcripts include the SarA ORF (Figure 5).[26]

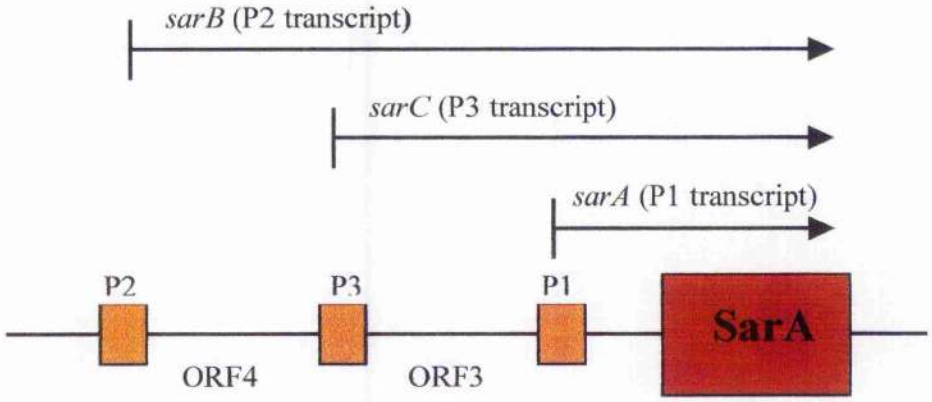


Figure 5. *sarA locus* promoters and transcripts. Adapted from Bayer *et al* (1996)[26]

Each transcript is not uniformly expressed throughout the growth cycle; *sarA* and *sarB* are optimally produced during the exponential phase whereas *sarC* is optimal during the post-exponential phase.[26] Complete transcription of the *sarB* transcript; a putative 39-amino acid (ORF3) and a putative 18-amino acid (ORF4) is essential for the optimum expression of the DNA binding protein SarA, a protein specific for *agr* P2 and P3 thereby optimising expression of RNAII and RNAIII.[62,67,156] Of the three promoters P1 is the strongest promoter and along with P2 is sigma A (σ^A)-dependent. Promoter P3 is σ^B-dependent but contradicting evidence exists to the role of σ^B in the activation of the *sarA* locus.[26,33,227] However, up-regulation of SarA expression, α-toxin, coagulase and clumping factor transcription, but not the *agr* locus is associated with *sigB* inactivation, suggesting additional *agr*–independent activity on target gene activation.[55,59,64] *Agr*-dependent and -independent regulation has been proposed by Chien *et al.* (1999) through the discovery of a conserved SarA-binding site within the promoter sequences of *agr*, *fnb*, *hla,* and *spa* genes to which binding exerts the required response whether it is activation or repression.[69]

It is now widely accepted that protein A (*spa*) is co-regulated by *agr* and *sarA* loci at the transcription level, and that repression via *sarA* can be *agr*-dependent or *agr*-independent which may account for variations in protein production.[63,212] Strains deficient in either or both *agr* and *sarA* regulatory systems are less virulent in murine bacteraemia indicating that *sarA* activity is essential for the development of bacteramia.[158] *SarA* has also been reported to play a role in the initial adherence of *S.aureus* in rabbit endocarditis and in apoptosis of mammalian cells.[59,60,365]

As part of the regulatory system SarA is under the control of its own by-products. A 345bp transcript designated *sarR*, which shares 51% sequence homology with SarA, has been identified by Manna *et al.* (2001).[226] These authors report that rSarR binds to *sar* promoters repressing SarA expression, possibly through down-regulation of *sarA* P1 transcription.[226] The joint repression of protein A by *sarA* and *agr* led to the assumption by Cheung *et al* (2001) that there must be a protein that activated protein A expression.[65] As a result they discovered another ORF with sequence homology to SarA, this time upstream of *spa* and with the ability to bind to *spa* promoter. The ORF was termed *sarS* for gene supplemental to SarA (also identified as SarH1 by Tegmark *et al*).[336] SarS is repressed by *agr* and *sarA*, yet increased yields of expression in *sarA*$^-$ and *agr*$^-$ mutants differs between the two regulatory loci.

Yet another homolog to *sarA* is *sarT*, a repressor of α-haemolysin (*hla*) expression and repressed by *agr* and *sarA* itself.[305] Studies have shown that *sarA*, but not *agr*, represses *sarT* promoting α-haemolysin synthesis and that *sarT* mutants give rise to increased levels of RNAIII suggesting a negative effect on RNAIII transcription probably through a *sarA* independent mechanism. Transcription of *SarT* is induced between late exponential and post-exponential phases. Prior to this sarA represses it. As a repressor of *hla*, it was found that *sarA*, and not *agr*, is able to activate transcription through *sarT* repression, the details of which are very complex (Figure 6).[305] Additional extracellular proteins shown to be under the influence of SarA include: V8 serine protease, staphylococcal enterotoxin B (*seb*), and toxic shock syndrome toxin-1 (*tst*) genes.[55]

From these studies it has been elucidated that the *sarA* locus and indeed SarA itself exerts partial control of the regulation of RNAIII at the transcription level, *agr*-dependent and independent control of positive and negative gene regulation.

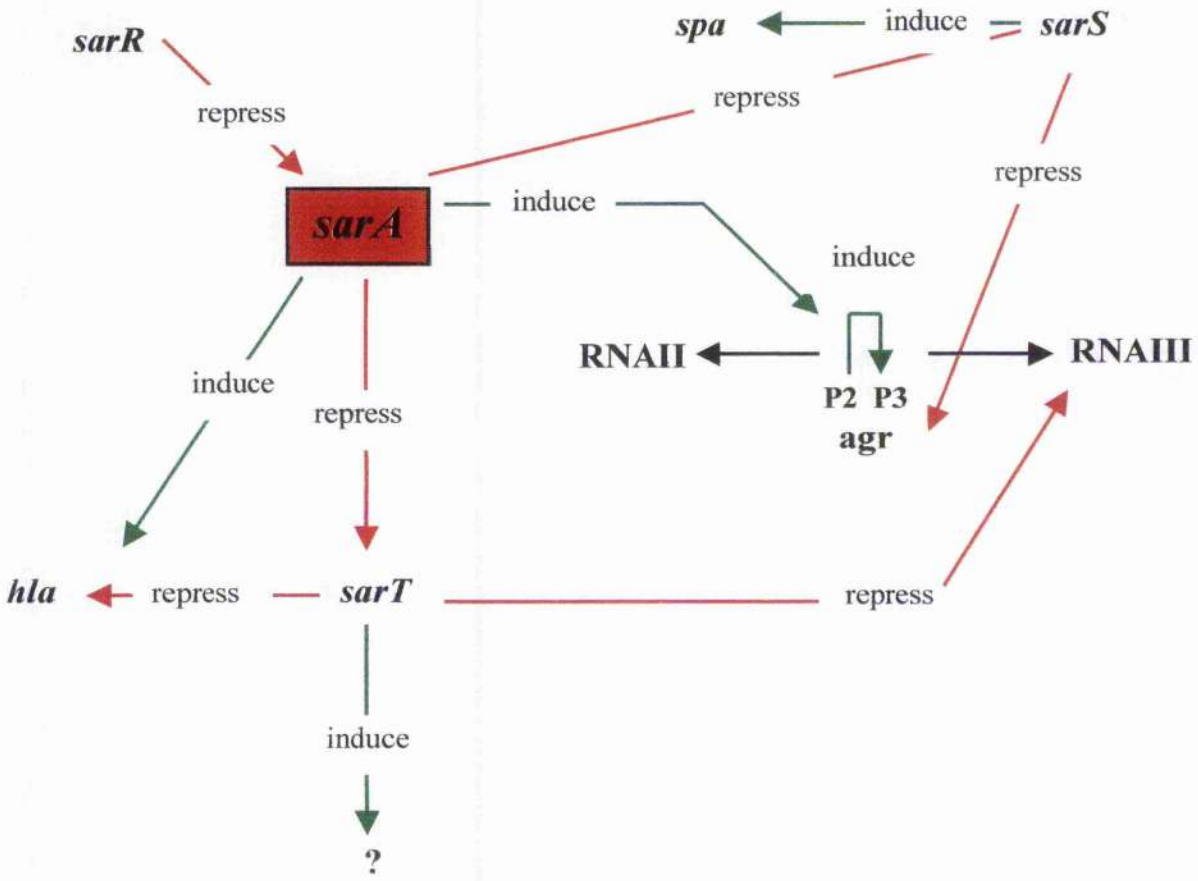


Figure 6. Proposed model of *sarA* regulation. Adapted from Schmidt *et al* (2001)[305]

Further pleiotropic regulatory elements involved in the control of virulence factor expression are still being described. One such element is the most recently described *sae* locus, another two-component system (*saeRS*).[135] Within *sae*, *saeR* encodes an activator gene protein and *saeS* a sensor histidine kinase protein. Mutations within *sae* result in disrupted transcription of numerous genes including *hla, hlb, coa* and DNase genes, but appear to have no effect on *agr* or *sar*.[134,135] The exact mechanism of action is still being questioned.[134] McNamara *et al.* identified the *rot* gene, putative toxin repressor that is assumed to be inactivated by RNAIII during post-exponential phase although the exact mode of action is not clear.[233] The ArlS-ArlR two-component system described by Fournier *et al.* (2001) is not an auto-regulated system but under the control of the *agr* and *sarA* systems.[110] Mutations in either component increase α- and β-haemolysin, coagulase, protein A, serine proteases and lipase suggesting a role in transcription repression.[110] Additional regulatory systems that have been identified include the staphylococcal

respiratory response (*ssr*) and the extracellular protein regulatory (*xpr*) systems but their role in the regulation of virulence is unclear.[154,322,374]

1.4 Antimicrobial Agents

There have been considerable developments in antibiotics since their discovery. Many drugs are no longer toxic and many are naturally occurring products of bacteria themselves. To date there are over thirteen families of antimicrobial agents including the aminoglycosides, β-lactams, cephalosporins, fluoroquinolones, glycopeptides, lincosamides, lipopeptides, macrolides, oxazolidinones, polymixins, quinolones, streptogramins and tetracyclines. Each family has its own mode of action that can be classified into one of five groups of inhibitors as follows:

1 Protein synthesis
2 Cell wall synthesis
3 Cell membrane function
4 Cell metabolism
5 Nucleic acid synthesis

The majority of antibiotics on the market today target protein synthesis. This thesis will study selected inhibitors of protein synthesis, cell membrane function and cell wall synthesis.

1.4.1 Protein Synthesis

The process of protein synthesis is a complicated one involving a number of sequential stages with each stage dependent upon the completion of the previous stage. Every component and protein required by a bacterial cell to function is encoded on individual genes, the template for mRNA assembly. The transfer of data from DNA to RNA, the process of transcription, requires the presence of RNA polymerase enzymes that bind to specific start codon sequences encoded within the DNA of individual genes. Once bound these enzymes uncoil the DNA double helix allowing the attachment of free nucleotides in a 5' to 3' direction until the transcription of a stop codon. As the mRNA elongates it detaches itself from the DNA template allowing recoiling. Translation of mRNA occurs

with the aid of ribosomes present in the cell cytoplasm along with a pool of amino acids and transfer RNA. Ribosomes are composed of two individual subunits, 30S and 50S subunits, that remain separated until required for protein synthesis. In the cytoplasm mRNA is bound by an fMet initiation signal. This in turn binds to the 30S ribosomal subunit, facilitated by IF2 and IF3 accessory initiation factors, to a 50S ribosome to complete the 70S initiation complex and stabilized by guanosine triphosphate (GTP). Upon formation of the 70S initiation complex the elongation phase commences with the aid of GTP and three elongation factors: EF-Tu, EF-Ts, and EF-G. Elongation is a three-stage cycle (codon recognition, peptide bond formation, and translocation) with each cycle resulting in the formation of a peptide bond and the addition of one amino acid residue and eventually the release of a complete protein. Codon recognition involves the formation of hydrogen bonds with the anticodon region of aminoacyl-tRNA coupled to its appropriate amino acid. Peptidyl transferase enzymes catalyse the formation of peptide bonds between the amino acids to form a growing polypeptide chain as the mRNA moves through the ribosome during the translocation phase. To complete the process a protein release factor bound to the termination codon causes the addition of a water molecule instead of an amino acid to the polypeptide, resulting in the release of the polypeptide and separation of the ribosome into its 2 subunits ready to start the process again. Figure 7 summarises the stages involved in protein synthesis.

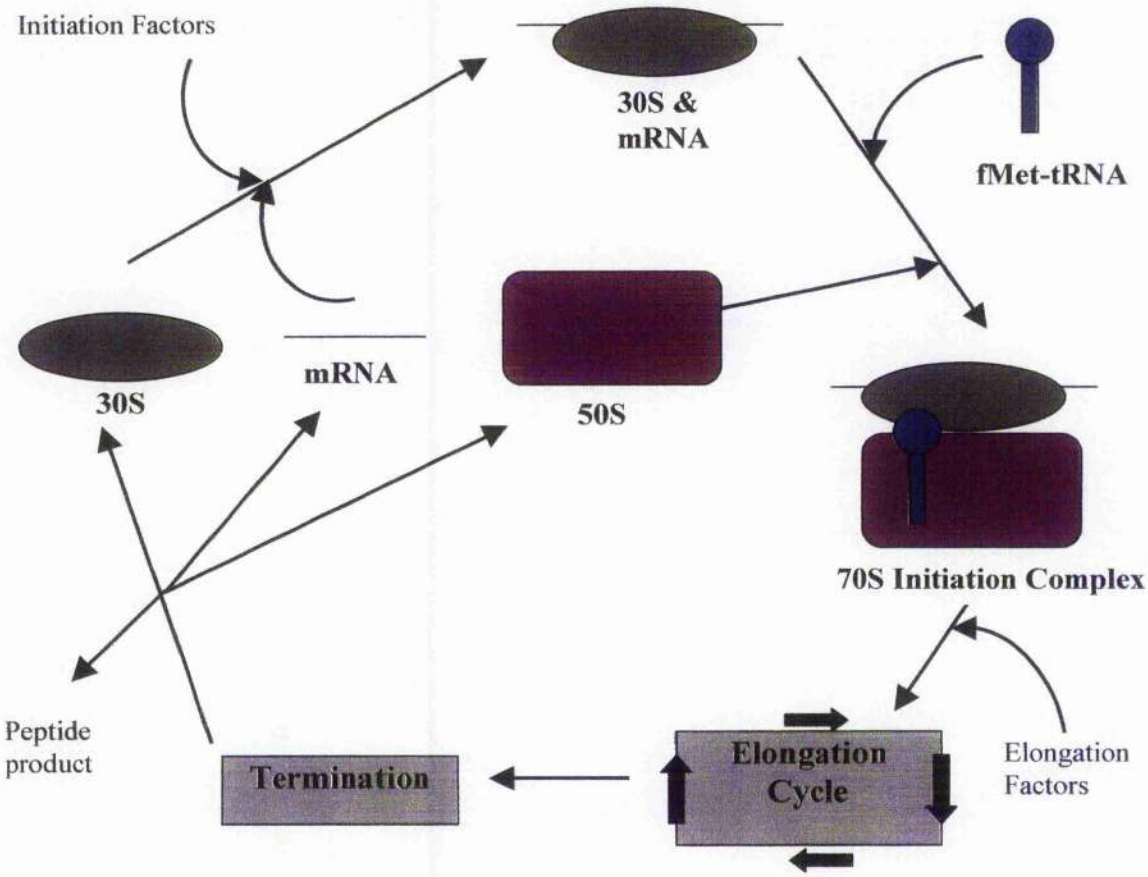


Figure 7. Summary of the stages involved in protein synthesis

1.4.1.1 Protein Synthesis Inhibitors

Most protein synthesis inhibitors are bacteristatic by nature with the exception of the aminoglycosides that are bactericidal.[198] It is unclear why but it is possibly due to their multiple effects on the cell membrane, RNA metabolism and misreading of codons through activity at the 30S ribosomal level (Table 5).

Antimicrobial Family	Examples	Bacterial Target	Mode of Action
Aminoglycosides	streptomycin, gentamicin, kanamycin, neomycin	Gram +ve Gram -ve	Codon misreading; translocation impairment; RNA metabolism
Chloramphenicol	chloramphenicol	Gram +ve Gram –ve	Inhibits peptide bond formation on 50S ribosome; disrupts aminoacyl-tRNA binding and peptidyltransferase activity
Lincosamides	clindamycin lincomycin	Gram +ve Gram -ve	Binds to 50S ribosomal subunit, interfering with peptidyltransferase reactions.
Macrolides	erythromycin clarithromycin	Gram +ve	Premature release of incomplete polypeptides from 50S ribosomes; inhibits assembly of new ribosomal subunits
Oxazolidinones	linezolid	Gram +ve Gram -ve	Inhibits initiation complex formation
Streptogramins	dalfopristin/quinupristin (Synercid)	Gram +ve	Inhibits substrate attachment and peptidyltransferase elongation
Tetracyclines	tetracycline minocycline	Gram +ve Gram -ve	Inhibits cell free RNA translation on 30S ribosome; loss of membrane transportation; oxidative phosphorylation

Table 5. Properties of antimicrobial agents affecting bacterial protein synthesis

1.4.1.1.1 Oxazolidinones

The discovery of a new class of synthetic protein synthesis inhibitors, the oxazolidinones, was made in 1987 by E.I du Pont de Nemours and Co, Inc. Following this the first antimicrobial agent, linezolid, was launched into the UK pharmaceutical market in February 2001 for use against skin and skin structure infections, bacteraemia and pneumonia associated with Gram-positive bacteria.

Linezolid is bacteristatic by nature with a broad spectrum of activity spanning Gram-positive bacteria (MIC=1-4μg/ml) including multi-resistant species,[181,230,298,306,368,377] *Clostridium* species (MIC=0.25-8μg/ml) and Gram-negative anaerobes for example, *Bacteroides* species (MIC=2-8μg/ml).[371] However *E.coli* (MIC>128μg/ml) is unaffected. Bactericidal effects have been observed in pre-clinical studies against *Streptococcus pneumoniae* (MIC=1-4μg/ml).[368]

The mode of action of this drug is different from that of any other protein synthesis inhibitor. Linezolid acts not by affecting peptidyl transferase or translation termination, but by preventing the formation of the N-formylmethionyl-tRNA-mRNA-70S ($tRNA^{fMet}$-mRNA-70s) on the 30S subunit ternary complex via binding to sites on the 16S or 23S ribosomal RNA (rRNA) (Figure 8).[313,331] Recent publications have supported the hypothesis that linezolid has dual functions. Strong binding of linezolid to the 50S ribosomal subunit has suggested that the drug only binds to the 50S subunit inhibiting the initiation complex formation and through the binding of 23S within 50S subunits preventing the assembly process.[53,213,228]

Comparative studies with other drugs have shown that linezolid is as effective as vancomycin and β-lactams in clinical cure rates and microbiological success rates *in vitro* and *in vivo* against antibiotic sensitive and resistant staphylococci and enterococci.[66,104,180,257,277,297,309] The maintenance of drug levels at concentrations greater than the MIC appears to be critical for optimal activity in rabbit endocarditis models.[80,269]

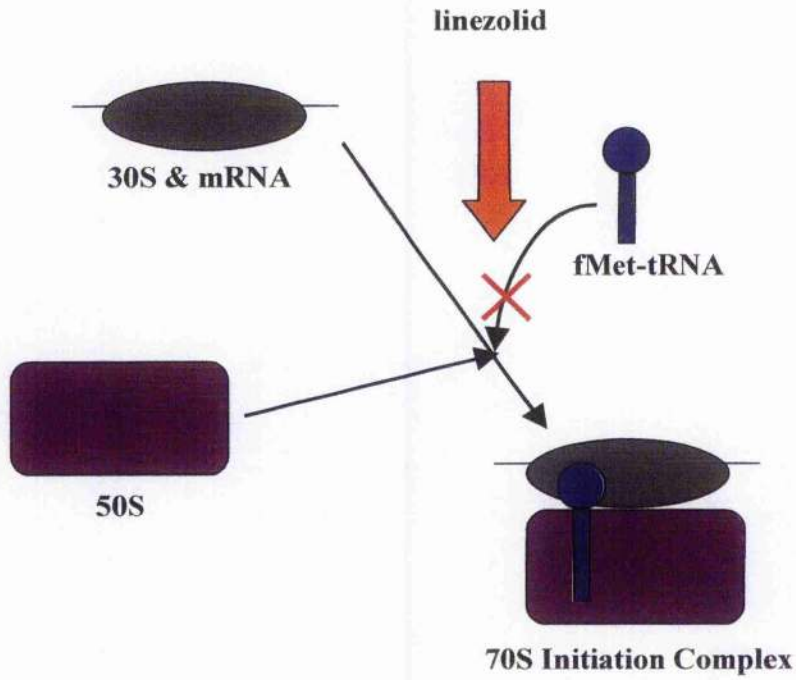


Figure 8. Mechanism of action of linezolid

1.4.1.1.2 Streptogramins

Synercid is the first injectable streptogramin combining the two streptogramin derivatives of pristinomycin, dalfopristin (Type IIA) and quinupristin (Type IA) in a 70:30 ratio. Streptogramins individually inhibit different stages of the protein synthesis process in a bacteriostatic manner. Type A (IIA) is thought to bind only to the free ends of peptidyl transferase at the termination-initiation phase, whereas Type B (IA) blocks peptide bond synthesis *in vitro* inducing the release of incomplete protein chains. The combination of these individually bacteriostatic compounds gives synercid its bactericidal capabilities.[217]

Inhibition by synercid occurs through an induced conformational change in the 50S ribosomal subunit by dalfopristin. Reversible binding to the peptidyltransferase domain of the ribosome and the resulting conformational change improves the binding capabilities of the quinupristin subunit at a different site, rendering the drug bound ribosome unable to carry out substrate attachment and peptidyl transferase elongation stages (Figure 9).[217]

Although most staphylococcal and streptococcal species are susceptible to the lethal effects of synercid (MIC=0.12-2 μg/ml), it is only bacteriostatic or weakly bactericidal to *Enterococcus faecium* (MIC≤2μg/ml) but not *E.faecalis.*[75,99,114,358,377] *In vitro* studies in to the effectiveness of synercid against strains of VISA, VRE, MSSE, MRSE, and ciprofloxacin sensitive and resistant strains of *S.aureus* compared to other leading antimicrobial agents, such as vancomycin, has yielded promising results.[180,218,299,357] The use of synercid in patients with MRSA and VRE infections failing alternate therapy has also provided promising results.[93,218,239,252] Despite this, treatment with synercid has also been reported to fail in an endocarditis rat model with erythromycin resistant MRSA due to the short half-life of dalfopristin in serum.[101]

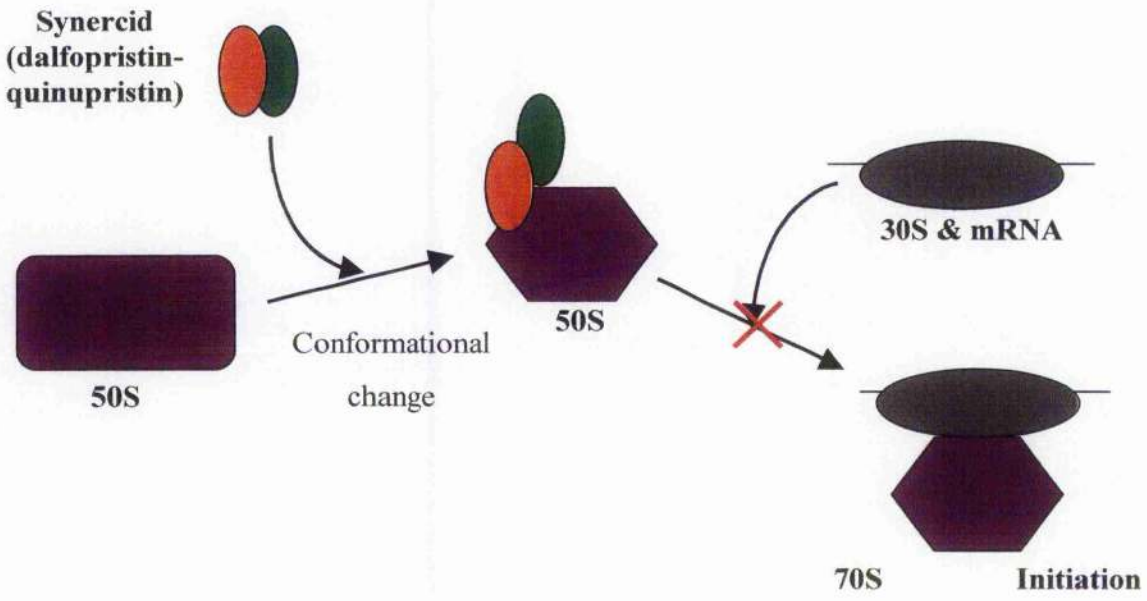


Figure 9. Mechanism of action of Synercid

1.4.2 Gram Positive Cell Walls and their Biosynthesis

The composition of the bacterial cell wall is not uniform for all species, but can be grouped into two main categories: Gram-positive and Gram-negative bacteria, depending upon their reaction to the Gram stain. As Gram-positive bacteria are the subject of this study only the Gram positive cell wall structure will be discussed.

The structure and composition of a bacterial cell wall is influenced by an organism's external environment in order to provide cell integrity. The cell wall of Gram-positive bacteria is composed of two layers, a cytoplasmic membrane and a thick (15-80nm) lattice like layer of peptidoglycan and teichoic acids. Peptidoglycan is composed of parallel chains of glycerophosphate or ribitol phosphate residues linked by β1-4 glycoside bonds and cross-linked by pentapeptides. It is the presence of components such as N-acetylmuramic acid, D-glutamic acid and D-alanine that give selective toxicity for antibiotics like the β-lactams.

Peptidoglycan biosynthesis only occurs during cell growth and division. It involves controlled splicing of existing bonds by autolysins and the simultaneous insertion of new pieces of peptidoglycan in a three-stage cycle. Stage one takes place in the cytoplasm where cell wall precursors are formed using soluble enzymes. At the cytoplasmic membrane the precursors become bound to the recyclable lipid carrier, bactoprenol, which gives molecules enough hydrophobicity to pass across the membrane, prior to insertion into the cell wall. The third and final stage involves cross linkage of new molecules into the pre-existing peptidoglycan network via the transpeptidation reaction. Here, transpeptidase enzymes remove a D-alanine group from the new molecule and use the exposed peptide bond to cross link with a pentaglycine bridge.

1.4.2.1 Inhibitors of Cell Wall Synthesis

This group of antibiotics includes the penicillins and cephalosporins, which contain a β-lactam ring hence, are designated β-lactam antibiotics. Both target the enzyme transpeptidase involved in the transpeptidase reaction of peptidoglycan synthesis. The first β-lactam, penicillin G, was primarily a Gram-positive antibiotic. Derivatives of penicillin G developed by chemical synthesis possess altered properties that give broader spectrum activity that includes some Gram-negative bacteria. Beta-lactam antibiotics bind with transpeptidase enzymes, penicillin-binding proteins (PBPs), on the surface of bacterial cells through their β-lactam ring rendering them inactive and unable to carry out transpeptidase reactions. As a result, cross-linking of the bacterial cell wall is inhibited producing a weakened structure that is susceptible to osmotic pressure. Antibiotic-PBP complexes have also been found to stimulate the release of the enzyme autolysin accelerating cell wall digestion. Production of the enzyme β-lactamase by bacterial strains

inactivates the majority of β-lactam antibiotics, however production of semi-synthetic penicillins such as oxacillin and methicillin, with altered side chains, renders these drugs resistant to β-lactamase activity. Methicillin is a semi-synthetic penicillin modified by penicillin amidase activity to remove penicillin side chains that are replaced with an acyl side chain thus providing a broader spectrum of activity and reduced susceptibility to β-lactamase enzymes.

Cephalosporins differ from β-lactams by the presence of a six-membered dihydrothiazine ring in place of a five-member thiazolidine ring. In this study we used cefpirome, an example of a 4th generation cephalosporin with improved anti-staphylococcal activity. Its structure is similar to that of the penicillins, but has been modified though the 'generations' to give a broader activity spectrum and resistance to β-lactamase degradation. The addition of a methyl-cyclopentapyridinium hydroxide at position 3 of the cephem ring has increased the drugs bactericidal activity to include the staphylococci, Enterobacteriaceae and *Pseudomonas aeruginosa*, including Type 1 β-lactamase-hyperproducer strains.[275] The mode of action of these antibiotics however remains the same as the penicillins binding irreversibly to PBPs preventing peptidoglycan cross-linking.

Glycopeptides were introduced into clinical practice in 1956, but were not widely used due to their poor tolerability and toxic side effects. Vancomycin, although a cell wall inhibitor targets a different stage of peptidoglycan synthesis from the β-lactams. Binding of vancomycin to the terminal D-alanine-D-alanine amino acids on the undecaprenyl peptidoglycan precursors occurs at the bacterial cell membrane surface blocking transglycosylase and transpeptidase reactions. Teicoplanin is chemically similar to vancomycin, but displays lower toxicity levels and less frequent side effects.

1.4.3 Drugs That Act on Cell Membrane Function

Bacterial membranes are composed of 50-70% protein, 20-30% phospholipid, with the rest made up from glycolipids. Polymyxins and polyenes are the two major groups active against bacterial cell membranes. Their structure is similar to that of detergents with a polypeptide head and a long hydrophobic tail promoting binding to the cell membrane causing leakage of the cell cytoplasm. Unfortunately polymyxin activity is non-specific

and can cause mammalian cell damage particularly of the liver, kidney, and nervous system.

Daptomycin is a semi-synthetic, water-soluble, cyclic lipopeptide or peptolide. Structural similarities can be seen with the polymyxins but their modes of action are very different. The exact mechanism by which daptomycin exerts its activity is not completely understood but the biosynthesis and assembly of peptidoglycan, teichoic acid and lipoteichoic acid (LTA) appears to be affected. Other authors have detailed the disruption of cell membrane potential through the loss of potassium ions.[7,9,10,37]

The enzymes involved in LTA synthesis are localised at the cytoplasmic membrane. It is these membrane proteins that are believed to be the site of daptomycin activity, not LTA itself.[37] At concentrations of daptomycin equivalent to the MIC of *S.aureus* and *E.faecalis* and *E.faecium* irreversible binding of daptomycin to the cytoplasmic membrane through a calcium bridge has been proposed.[47] Different phenotypes displayed by enterococci and staphylococci in response to daptomycin were also described. These authors report the inhibition of growth and peptidoglycan synthesis in *S.aureus* but not in enterococci, however *E.faecium* was unable to undergo cell separation forming rod-shaped cells whereas *S.aureus* cells remained unchanged. The inhibition of growth was suggested to result from the inhibition of LTA synthesis and resulting loss of LTA function as a carrier of teichoic acid or as a regulator of autolysins.[47] It has been suggested that as LTA are a component of adhesins daptomycin may prevent cell proliferation in an adhered state.[47]

The bactericidal activity of daptomycin against Gram-positive organisms is concentration dependent but is heavily influenced by calcium concentrations with evidence of reduced activity in the presence of serum or albumin, unlike other antimicrobial agents such as vancomycin, linezolid and synercid.[115,151,206,323] Complicated skin and soft tissue infections, and bacteraemia have been the focus of clinical trials, especially those caused by multi-resistant organisms. Animal models of endocarditis, osteomyelitis and bacteraemia caused by strains of VISA, MSSA, MRSA, MRSE, MSSE, VS*E.faecium* and VR*E.faecium* (MIC≤2μg/ml) and *in vitro* studies have all shown positive results with daptomycin therapy.[5,98,116,159,192,193,299,323]

1.5 Antimicrobial Resistance

Bacterial susceptibility to antibiotics varies dramatically within and between strains. Antibiotic resistance can be acquired through the acquisition of transposable resistance genes carried on plasmids, transposons and insertion sequences encoding for example β-lactamase enzymes and efflux systems, or through genetic mutations. Some organisms are intrinsically resistant to some antibiotics including strains of streptomycetes. Streptomycetes naturally produce antimicrobial agents as by-products so resistance mechanisms exist within strains towards the antimicrobial that they produce. Additional natural resistance traits of a particular organism may be due to: lack of target structure, for example Mycobacteria lack a typical cell wall and are resistant to penicillins; agent impermeability e.g. Gram-negative bacteria are impermeable to penicillin G.

Examples of resistance in *S.aureus* include drug inactivation of penicillins but not methicillin (β-lactamase), chloramphenicol (efflux system), and aminoglycosides (phosphorylation, acetylation or adenylation of drug).[70] Multiple resistance characteristics can be presented by a single strain. Continual exposure to a particular agent can also create a selective pressure for higher levels of resistance among strains particularly within a hospital environment. Cross-resistance between groups of antibiotics is also prevalent as seen with the MLS_B phenotype.

1.5.1 MLS_B Resistance

The MLS_B phenotype confers multiple resistances to macrolides, azalides, lincosamides, and Type B streptogramins. Macrolide resistance arose shortly after the introduction of erythromycin into clinical use and is inducible by low concentrations of the drug. There are several mechanisms of macrolide resistance, the main type is through the increased expression of the *ermC* gene encoding a methyltransferase enzyme that methylates 23S ribosomal RNA at a site of key importance to the binding of MLS_B antibiotics, resulting in multiple cross resistance.[354]

1.5.1.1 Streptogramin Resistance

Resistance to dalfopristin/quinupristin (synercid) is at present relatively low, although resistance towards its individual components exists in strains of staphylococci, *E.faecalis* and *E.faecium*. Resistance to group B streptogramins, including quinupristin, can take one of two forms: constitutive or inducible (Table 6). The most common form is constitutive resistance conveying cross-resistance to macrolides and lincosamides (MLS_B phenotype).[216] For substantial loss of antibacterial activity to be achieved resistance to group A streptogramins or both group A and B streptogramins is required, as synercid remains active due to the synergy of its components.[41,364]

Since synercid was launched into the UK market in 1999 it is interesting that already all strains of *E.faecalis* are resistant to synercid and that clinical isolates of *E.faecium* (MIC ≥4μg/ml) and isolates of CNS, MRSA and GISA (MIC=2-8μg/ml) have been recovered displaying low-level synercid resistance.[71,91,364] High level resistance (MIC>32μg/ml) in *E.faecium* is associated with the *vatD* and *vatE* genes.[324] The mechanism of resistance in *E.faecalis* remains unknown.

Strain	Gene	Mode of Action	Resistance	Ref
enterococci	*vat* (D, E)	dalfopristin acetyltransferase	Group A streptogramin, synercid	153,175, 291,363,
staphylococci	*vga* (A, B)	dalfopristin ATP-binding efflux pump	Group A streptogramin	14,364
	vat (A, B, C)	dalfopristin acetyltransferase	Group A streptogramin; synercid	15,91,216, 364
	erm (A, C, Y)	Methylation of 23S rRNA;	MLS_B phenotype; quinupristin; synercid	216
	msrA	quinupristin ATP-binding efflux pump	Inducible Macrolide and group B streptogramin	295
enterococci staphylococci	*vgb* (A,B)	quinupristin hydrolase	Group B streptogramin	13,41,175, 364,
	erm(B)	Methylation of 23S rRNA	MLS_B phenotype; quinupristin; synercid	41

Table 6. Streptogramin resistance mechanisms using the revised nomenclature proposed by Roberts *et al* [292]

1.5.2 Oxazolidinone Resistance

As with synercid, linezolid was only recently introduced into the pharmaceutical market and already reports of resistance are emerging in strains of *E.faecium* and *S.aureus*. Several strains of linezolid-resistant *E.faecium* (MIC=8-64μg/ml) have been reported in individuals taking part in a compassionate use program and in patients receiving long-term courses of the drug.[19,138] Strains of *E.faecalis* (MIC=64μg/ml) and *S.aureus* (MIC>32ug/ml) have also been isolated from patients receiving linezolid.[19,340] Analysis of these strains has revealed mutations within the 23S rRNA encoding for a central loop and within the peptidyltransferase.[19,286,340] The mechanisms providing resistance to linezolid have yet to be fully established but no evidence exists of known resistance mechanisms conveying resistance to the oxazolidinones.[180,368]

1.5.3 Lipopeptide Resistance

Resistance to daptomycin has been achieved *in vitro* through serial passage of strains of *S.aureus, S.epidermidis, S.pneumoniae, E.faecium* and *E.faecalis* at sub-inhibitory levels. Spontaneous resistance at x8 MIC concentrations was not documented.[319] Accompanying increased MIC values of 8- to 32–fold, variable changes in strain characteristics were observed which could be divided in to three categories (Table 7).[319] The exact mechanism of resistance remains to be defined.

Class	Characteristics
1	Normal growth rate; cross resistance to nisin antibiotic
2	Reduced growth rates on Muller Hinton agar (MHA), poor / no growth on chemically defined medium (CDM); no antibiotic cross resistance
3	Severe growth defects in Mueller Hinton broth (MHB) and CDM; poor or no pigmentation on MHA; no antibiotic cross resistance

Table 7. Characteristics of *S.aureus* following serial passage in sub-MIC daptomycin

1.5.4 Methicillin Resistance

Kreiswirth *et al.* (1993)[200] proposed the clonal theory of MRSA evolution, which has been disproved, and it is now widely believed that the evolution of MRSA is polyclonal.[100] In staphylococci, methicillin resistance is chromosomally encoded on a staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* is characterised by inverted and direct repeats and site specific recombinase genes (*ccrA* and *ccrB*) in addition to the *mecA* complex, the gene responsible for methicillin resistance.[160,189] Expression of an additional 78KDa cell wall protein PBP2a (or 2') encoded by the *mecA* gene is responsible for methicillin resistance. The low affinity of PBP2' for β-lactam antibiotics allows transpeptidase activity to continue in the presence of β-lactam concentrations that inactivate other PBPs.[160,236]

To date there have been four groups of SCC*mec* DNA described with each displaying differences in structure and nucleotide sequence but are conserved in the *mecA* gene: Type I - 39kb, Type II - 52kb, Type III - 67KB, Type IVa - 20kb and Type IVb - 24kb.[160,168,223] All strains of community acquired MRSA (CA-MRSA) have been found to carry the Type IVa SCC*mec* a much smaller area of DNA that is not associated with additional resistance genes seen with the other SCC*mec* types, therefore remaining susceptible to numerous antibiotics.[22,45,162,223,267]

A mecR1-mecI regulator controls the regulation of mecA expression. Strains of staphylococci displaying low level methicillin resistance possess a functional *mecR1-mecI* regulator that inhibits the complete expression of the resistance phenotype.[167,190] Deletion of the *mecI* gene through the insertion of IS1272 and/or a mutated or deleted *mecI* gene along with partial deletion of the *mecR* gene (IS1272-Δ*mecR1-mecA*-IS431R) de-represses *mecA* transcription.[190,194,362] The latter is commonly found amongst strains of MRSA.[194,362]

A family of five chromosomally encoded *fem* genes (*femA* through *femE*), considered to be involved in cell wall metabolism, and essential for the expression of methicillin resistance have been described.[146] Inactivation or loss of these genes resulting in reduced levels of resistance has been demonstrated in *agr*-null strains and in strains of VISA where methicillin resistance is reduced.[146] Inactivation of the *femC* gene appears to decrease the transcription of the *glnA* gene involved in glutamine production and the level of

methicillin resistance expressed.[146] The full extent of the involvement of the *fem* genes in the expression of methicillin resistance is unclear.

In addition to the presence of the *mecA* gene, low-level resistance to methicillin can also be achieved through β-lactamase over expression, over production of PBP4, which has lower affinity for β-lactams, and mutations of PBP2.[236]

1.5.5 Vancomycin Resistance

Effective treatment of MRSA is often limited to vancomycin and teicoplanin and until recently vancomycin resistance in staphylococci did not exist. Vancomycin resistance has, however, existed in strains of enterococci since 1987 and has rapidly increased since the 1990s. The transfer of high-level vancomycin resistance from *E.faecalis* to *S.aureus* has been achieved *in vitro*, but had not been proven to occur *in vivo*.[256] During 1995 the first isolate of *S.aureus* with reduced vancomycin susceptibility (vancomycin-intermediate resistance *S.aureus*; VISA) was isolated in a French hospital but was not reported until 1998.[282] The first reported strains of VISA were isolated from a Japanese hospital in 1997 (strains Mu3 and Mu50).[161] Following this report several isolates of *S.aureus* displaying vancomycin-intermediate resistance (VISA) have been reported across Europe and in the United States of America.[48,49,50,52,143,164,296,341]

Vancomycin resistance in strains of enterococci is associated with a set of *van* genes taking the form of either the vanA phenotype (plasmid encoded vancomycin and teicoplanin resistance) or vanB phenotype (chromosomally encoded vancomycin resistance) although *vanD* through *vanH* genes have also been described.[137] The substitution of D-alanyl-D-alanine with D-alanyl-D-lactate reduces the binding affinity of vancomycin up to 1000 fold. The vancomycin resistance mechanisms identified in strains of staphylococci, including CNS, appear not to be the same as that found in enterococci.[308,316,332] In July 2002, a strain of VRSA displaying a vancomycin MIC>128μg/ml and a teicoplanin MIC of 32μg/ml was isolated in the United States.[52] The strain was recovered from an infection containing VRE and was found to be carrying the *vanA* gene. This is the first staphylococcal isolate reported to carry the enterococcal *vanA* gene suggesting horizontal transfer had occurred between the species during infection and that the transfer of genes conferring high level vancomycin resistance is

feasible *in vivo*.[52] A second, unrelated, isolate of VRSA (MIC=32μg/ml) carrying the *vanA* gene was isolated in the United States in September of 2002.[51] It has not been made clear whether this isolate was recovered from a mixed culture containing VRE but it is suggested than the gene was acquired through conjugation events with enterococci.[51]

All of the VISA strains to date, except the UK strains 3700w and 3759v, have been isolated from patients who have received vancomycin therapy. Strains of VISA share similar characteristics including longer generation times and increased cell walls as discussed earlier (section 1.1). Cell wall structure modifications such as increased glutamate muropeptides that actively bind vancomycin, and the overall thickened cell wall are thought to increase the consumption of vancomycin reducing the amount able to reach D-Ala-D-Ala pentapeptide precursor terminus target in the cell membrane.[149,318] Boyle-Vavra *et al* (2001) could not find consistency in the peptidoglycan composition between the Japanese and United States strains and suggested that cell wall composition was not solely responsible for resistance.[40]

In studies of Mu3, Mu50 and Mu50ω (vancomycin susceptible revertant of Mu50) seven novel ORFs identified as vancomycin resistance associated genes (*vra*), an up-regulation in fructose operon genes, and a down-regulation of protein A in Mu3 and Mu50 were recorded.[202] The genes *vraS* and *vraR* encode a histidine kinase and response regulator respectively, forming the components of a two component regulatory system. Additional components of the regulatory system are encoded by the genes *vraA* (CoA ligase), *vraB* (acetyl-CoA-acetyltransferase), *vraC* (unknown), *vraD-vraE* and *vraF-vraG* (ABC transporters with homology to *B.subtilis* two-component regulatory systems).[202] The cloning of the *vraS* and *vraR* genes in to a strain of vancomycin susceptible *S.aureus* (VSSA) was found to reduce the level of vancomycin susceptibility by that strain.[202] Homology of the ABC transporters with the glutamine transporter (*glnQ*) in *S.aureus* is thought to contribute to vancomycin resistance through the increased transportation of cell wall amino acids consumed in peptidoglycan synthesis. Increased consumption of acetyl CoA during the synthesis of N-acetyl-glucosamine and N-acetyl-muramic acid during peptidoglycan synthesis may also be compensated for by the *vraA* encoded CoA ligase.[891] Increased activity of the fructose genes (*fruAB*) has been shown to correlate with increased glucosamine synthase (GlmS) activity for enhanced peptidoglycan synthesis (section 2.1).

Kuroda *et al.* (2000) postulated that increased activity could be through the inactivation of a repressor but more detailed studies are required.[202]

The level of methicillin resistance reported for some strains of VISA is reduced in comparison to those of MRSA.[317] Full methicillin resistance is thought to require a functional *agr* regulatory system and *fem* genes, both of which are absent from *agr-null* strains. Several VISA have been described that have a defective *agr* locus and as a result display reduced methicillin resistance.[300]

1.6 The Effects of Sub-Inhibitory Concentrations of Antibiotics on Gram-Positive Bacteria

The concentrations at which antimicrobial agents are tested against strains of bacteria can be classified into four categories: the minimum antibiotic concentration (MAC), minimum bactericidal concentration (MBC), minimum inhibitory concentration (MIC), and the post-antibiotic effect (PAE). The effects of antibiotics at concentrations lower that that which inhibits growth is termed the sub-minimal inhibitory concentration (sub-MIC).

The effect that antimicrobial agents have *in vivo* is dependent upon the concentration they reach within the host. The effects observed at concentrations greater than the MIC are vastly different from those seen at sub-inhibitory concentrations. At the MAC and sub-MIC, changes in bacterial cell morphology, growth, adherence and protein expression are often encountered and the study of these effects is important due to the presence of such concentrations during antimicrobial therapy.[131,311,312] The range of sub-MIC's at which an antibiotic is active is highly variable. For example, penicillin will induce morphological changes at $^1/_{16}$ MIC whereas cephaloridine is only active up to $^1/_3$ of the MIC.[131] In addition, the effects induced by a particular antibiotic may not be the same as those induced by all antibiotics of the same group of the same bacterial species, and antibiotic effects may be different between bacterial species due to variations in the bacterial target.[131]

Numerous authors have demonstrated the activity of antibiotics at sub-MIC levels on both Gram-negative and Gram-positive bacteria *in vitro*. Examination of the morphological

structure of bacteria by electron microscopy has revealed changes in the bacterial cell wall structure induced by both inhibitors of the cell wall and of protein synthesis. The stages of peptidoglycan synthesis take place at numerous sites on the cell wall, some of which are linked to the extension of the cell wall and are susceptible to protein synthesis inhibitors. Other sites that are related to cell wall thickening are independent of protein synthesis, thus protein synthesis antibiotics are capable of inhibiting cell wall extension but cell wall thickening continues resulting in expression of thickened cell walls.[131]

Alterations of the bacterial cell surface can interfere with adherence to and colonisation of host cells. An increase in the release of preformed adhesin, suppression of adhesin formation and expression, and the induced formation of defective adhesins can all result from exposure to sub-MIC antibiotics.[131,312]

Numerous authors have demonstrated the ability of sub-inhibitory concentrations of antibiotics to affect the expression of virulence factors, and susceptibility to opsonophagocytosis. The expression of surface bound and extracellular proteins can be inhibited or enhanced by protein synthesis inhibitors depending upon the protein in question and the antibiotic. As reported with cellular morphology, antibiotic groups other than protein synthesis inhibitors can alter protein expression. The interaction of antibiotics with phagocytic cells of the immune system is also variable and can occur directly or indirectly through their effects on phagocytic cell function or the effects exerted upon the infecting bacterium respectively. An example of the latter is shown by the enhancement of opsonophagocytic uptake and oxidative metabolism in *S.aureus* and *S.pyogenes* by clindamycin.[72,120,128,238,351,352] The increased levels of phagocytosis have been linked to the reduced expression of surface proteins that provide antiphagocytic protection such as, protein A and M protein, or those that promote adhesion to surfaces.[120,128,352] Some contradictory reports have been published where no significant alteration in phagocytosis has been observed.[344,359]

Table 8 summarises the effects of sub-MIC antibiotic concentrations on cellular morphology, adherence and virulence factor expression by staphylococci.

Antimicrobial Agent	Sub-MIC Effects			Reference
	Cell Morphology	Adherence	Virulence Factors	
β-lactams	Inhibition of septum lysis; formation of multiple cross walls; thickened septa; increased cell size	Increased fibronectin binding; Increased adhesion	Increase α-haemolysin, TSST-1 expression	131,220,266, 285,312
	Increased cell size and thickened cell walls; thickened, dual cross wall formation	Reduced adherence and colonisation; decreased binding to fibronectin	Inhibition of haemolysin, coagulase, cell bound protein A, enterotoxin, TSST-1 and ETA+B expression; increases extracellular protein A, penicillinase	117,123,127, 230,266,304, 311,312,352
chloramphenicol		Reduced fibronectin binding;	Inhibition of haemolysin, coagulase, protein A	89,311,312
daptomycin	No effect			47
Glycopeptides	Aggregation, inhibition of autolysis	Inhibits adherence Increased fibronectin binding	Enhanced activity and no effect on alpha-haemolysin; no effect on enterotoxin and protein A expression	92,129,195, 266,285,312, 316
linezolid		Reduced adhesion	Inhibition of coagulase and haemolysin activity; reduced SEA, SEB, protein A, α- and β-haemolysin, and lipase expression	92,132,274
methicillin			Enhances alpha-haemolysin activity but no effect on protein A	195,245,266, 311
synercid	Thickened, layered cell wall			131
Tetracyclines			Inhibition of alpha-haemolysin, lipase, coagulase	89,311

Table 8. The effects of sub-inhibitory concentrations of antibiotics on staphylococci. Abbreviations: Toxic shock syndrome toxin (TSST-1), Exfoliative toxins A and B (ETA + B)

1.7 The Immune System

The defence mechanisms employed by a healthy individual normally provide a harmless relationship between host and bacteria. The natural protective properties of the skin and antimicrobial substances secreted in sweat and internal secretions such as mucus and tears provide a highly resistant barrier to invading organisms. The development of an infection and progression to cause disease is usually related to a breach in the skin and/or the impairment of the host's defence mechanisms allowing opportunistic bacteria to become pathogens.

The human immune system can be divided into two parts: innate and acquired immunity. The innate immune system is present in individuals from birth and provides non-specific defence to an array of foreign bodies. The acquired immune system on the other hand relies on the exposure to foreign bodies to stimulate the proliferation of lymphocyte cells expressing specific receptors to the antigen(s) that stimulated their synthesis.

1.7.1 Cells of the immune system

Both innate and acquired immune systems exploit differentiated stem cells albeit for different purposes (Figure 10).[361]

The two main groups of phagocytic cells are the polymorphonuclear leucocytes (PMNL) and mononuclear phagocytes of the innate immune system. The neutrophils are the most abundant and efficient phagocytic cell circulating in the bloodstream, and entering tissues in response to infection or injury. Mononuclear cells (monocytes) can further differentiate into macrophages within tissues and adhere to surfaces. The cells of the acquired immune system are derived from different precursors to form antigen-specific B- and T-lymphocytes.

Recruitment of phagocytic cells to a site of infection is mediated by the release of chemotactic factors such as the complement component C5a, histamine and cytokines from other phagocytic cells.

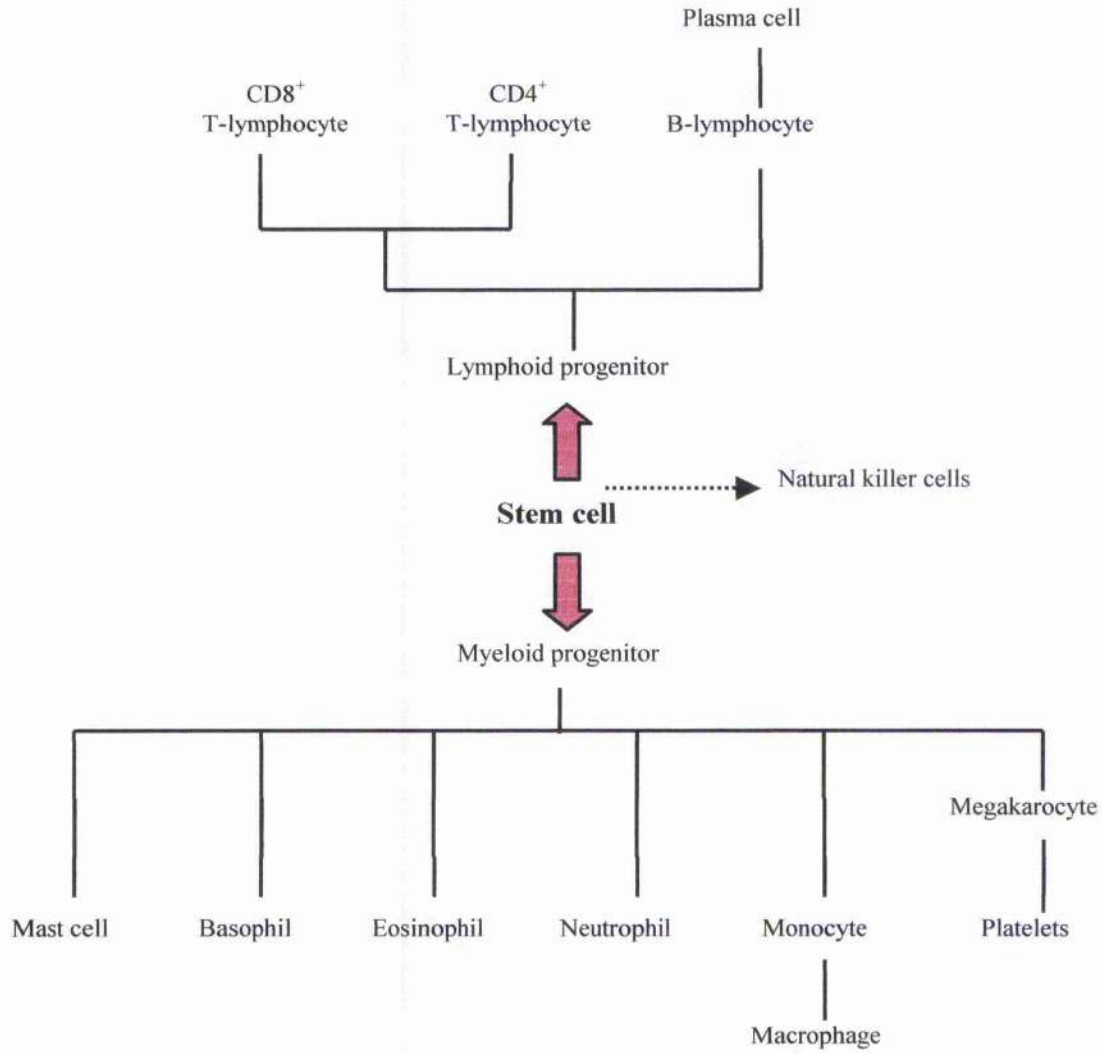


Figure 10. Cells of the immune system. From Weir and Stewart.[361]

1.7.2 Phagocytosis

In the presence of a foreign body within the host, for example a bacterium, stimulates circulating phagocytes to release cytokines that stimulate the release of other proteins including fibrinogen and C-reactive protein that bind to the microbial cell walls thus activating the classical complement pathway (Figure 11). Major features of the innate immune system are the serum components of the alternative and classical complement pathways which are initiated by different stimuli. Antibody-microbe immune complexes activate the classical pathway whereas the alternative pathway continually generates the component C3b that is inactivated unless it is attached to a foreign body. Component C3b

deposited on the surface of a bacterial cell opsonises the cell as well as triggering a cascade of events leading to the formation of a membrane attack complex (MAC) causing cell membrane damage and lysis of some bacteria.

Phagocytic cells carry receptors for component C3b facilitating the attachment of complement-coated microbes.[3] Complement opsonisation of bacteria through the classical complement pathway is required for optimal attachment and phagocytosis, opsonisation by the alternate pathway occurring at a slower rate.[108,350]

Opsonisation of bacteria increases bacterial surface hydrophobicity whilst decreasing surface charge, reducing the repulsion forces between bacterium and phagocyte. Attachment of a bacterial cell to a phagocyte stimulates the polymerisation of actin and microfilaments within the phagocyte to form a membrane bound phagosome around the bacterium.[3] Within the phagocyte, the phagolysosomes fuse with intracellular lysosomes containing microbicidal enzymes and molecules produced by respiratory burst that are capable of killing the ingested organism.

The process of phagocytosis increases the oxygen consumption of the phagocytic cells increasing the cell's respiratory burst. Enhanced enzymatic activity of NADPH / NADH oxidase, and the enzymes superoxide dismutase and myeloperoxidase convert oxygen to bactericidal oxygen species such as superoxide anion (O_2^-), H_2O_2, singlet oxygen and hydroxyl radicals. Damage to the microbial cell wall and cell death occurs as a result of exposure to these oxygen derivatives.[130,147,191]

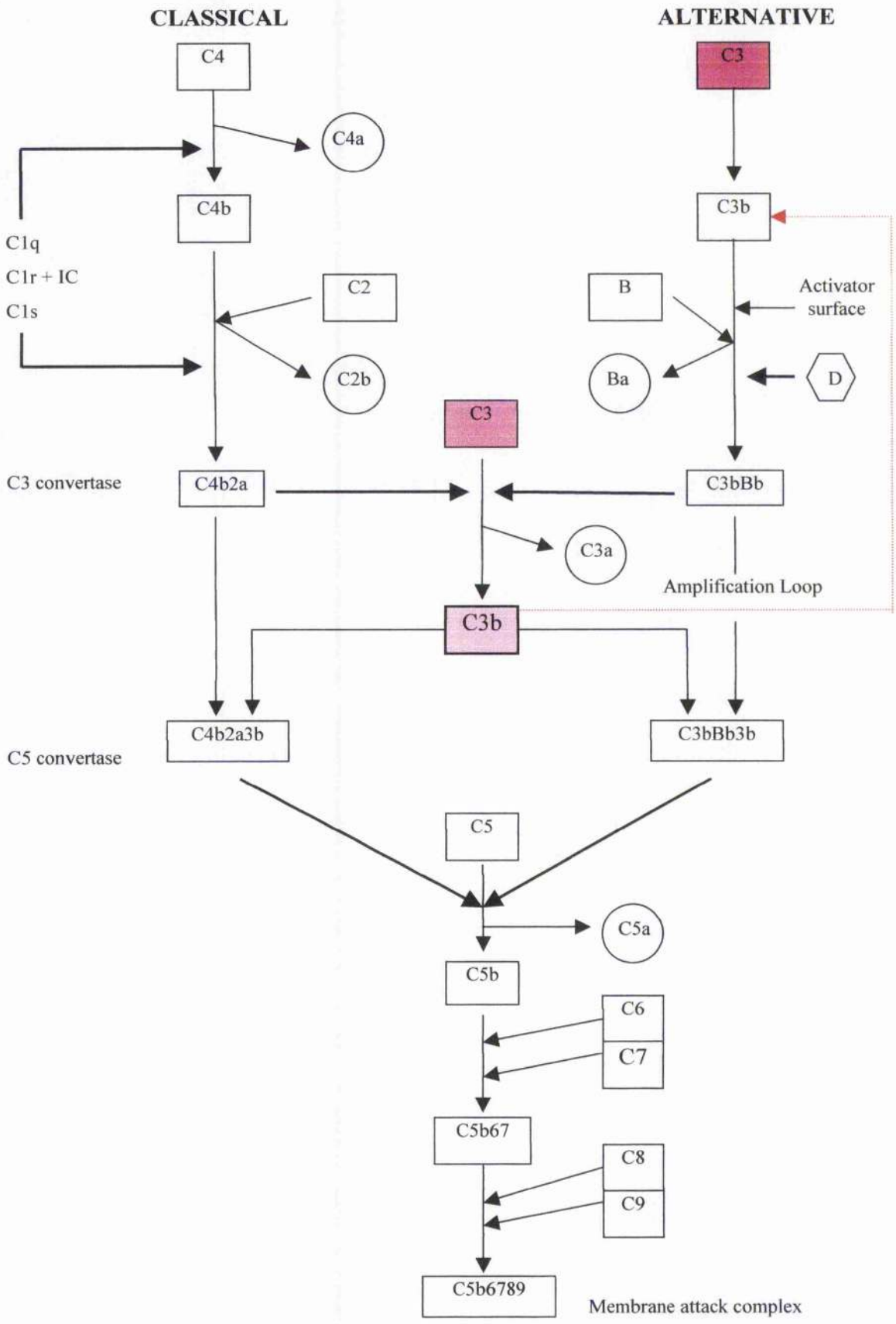


Figure 11. Complement activation.

1.7.3 Resistance to phagocytosis

Many bacterial species have developed cell surface or secreted proteins that can protect the cell from phagocytosis. The most common mechanism of resistance is the expression of a polysaccharide capsule. Polysaccharide capsules can prevent the binding of complement components to bacterial cell walls or inhibit the binding of phagocytes to bound opsonins for example, the highly capsulated *S.pneumoniae.*

S.aureus produces a surface protein, protein A. As mentioned previously in section 1.2.1.2, protein A binds to the Fc portion of IgG. The release of cell free protein A prevents the attachment of IgG to surface bound antigens and subsequently to phagocyte receptors. The clotting factor coagulase converts fibrinogen to fibrin to form a fibrin net around the bacterium inhibiting phagocytic ingestion.

In some instances species of bacteria do not inhibit ingestion, but once ingested release agents that render them resistant to the bactericidal actions of the lysosome, for example Salmonella Typhimurium Vi antigen, Mycobacteria and *Legionella pneumophila* cytotoxin, and catalase from *S.aureus* and *Listeria monocytogenes.* Intracellular pathogens such as *E.coli* and *Salmonella Typhimurium* can survive within phagocytes retaining structural and functional integrity, but lose the ability to multiply.[3,191]

The action of antimicrobial agents can also influence a bacterial strain's resistance to phagocytosis. Drug-damaged bacteria display altered characteristics that change the way in which phagocytes perceive them through altered antigen expression reducing the activation of phagocytic cells and antibody response, altered yields of exotoxins that act upon PMN function, abnormal cell morphology, and loss or exposure of surface antigens involved in opsonisation and attachment.[130]

CHAPTER TWO

Comparison of the Morphology and Physiology of Strains of *Staphylococcus aureus* with Increased Resistance to Vancomycin

2.1 INTRODUCTION

The structure and composition of the bacterial cell wall is influenced by an organism's external environment in order to provide sufficient cell integrity. The composition of the bacterial cell wall is not uniform for all species, but can be grouped into two main categories: Gram-positive and Gram-negative species, depending upon their reaction to the Gram's stain. As Gram-positive bacteria are the focus of this study, only the Gram-positive cell wall structure will be discussed.

The cell wall of Gram-positive bacteria is composed of two layers, a cytoplasmic membrane and a thick (15-80nm) lattice like layer of peptidoglycan and teichoic acids. The main constituent, peptidoglycan is unique to prokaryotes and highly conserved between bacterial species. The ability to withstand extreme environmental pressures can be attributed to cross-linkage of peptidoglycan layers via pentaglycine bridges. The structure of the peptidoglycan layer comprises two alternating sugar derivatives N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) linked by very strong glycosidic β-1,4 bonds, and the amino acids L-alanine, D-alanine, D-glutamic acid, and lysine forming glycan pentapeptides.

Biosynthesis of the peptidoglycan layer is a growth phase dependent process involving two metabolic pathways: The Embden-Meyerhof (EMP) pathway and one that involves the uptake of GlcNAc from the environment (Figure 12). In general, the process involves three precise stages: Stage one: MurNac-pentapaptide is formed in the cytoplasm by soluble enzymes. At the cytoplasmic membrane the nucleotide becomes bound to recyclable lipid carriers, uridine diphosphate and undecaprenolphosphate (bactoprenol), that provide molecules with sufficient hydrophobicity for passage across the membrane prior to insertion into the cell wall. Here GlcNAc and a pentaglycine bridge are bound to MurNAc (stage two). The final stage involves cross linkage of new molecules into the pre-existing peptidoglycan network via the transpeptidation reaction. Ultimately the enzyme autolysin precisely cuts β1,4 bonds and new peptidoglycan segments simultaneously insert into the existing glycan backbone. Cross links are formed by removal of the terminal D-alanine group from the new molecule enabling cross linkage with pentaglycine bridges of adjacent glycan chains.

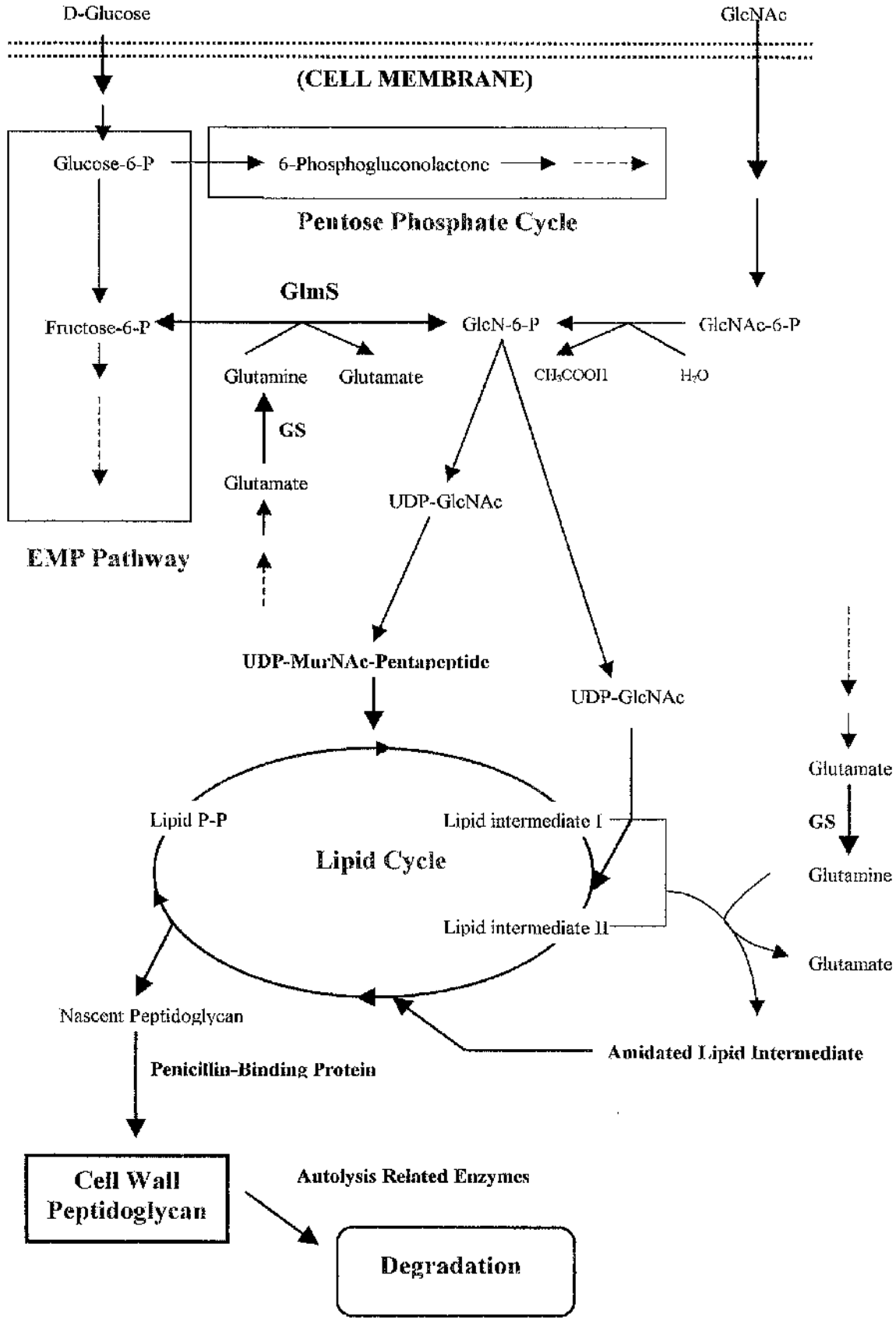


Figure 12. Peptidoglycan synthesis. Abbreviations: glucosamine synthase (GlmS), glutamine synthetase (GS). Adapted from Cui *et al.* (2000)[78]

Other important components of the cell wall include teichoic acids, lipoteichoic acids, and protein A. External to the cell wall some bacterial species synthesise layers of glycoproteins and polysaccharides termed the glycocalyx. Together with protein A, the glycocalyx is believed to play a role in attachment, phagocytic evasion and resistance to dessication whereas the teichoic acids are partially responsible for a cell's negative charge and putatively facilitate the passage of ions across the membrane (Figure 13).

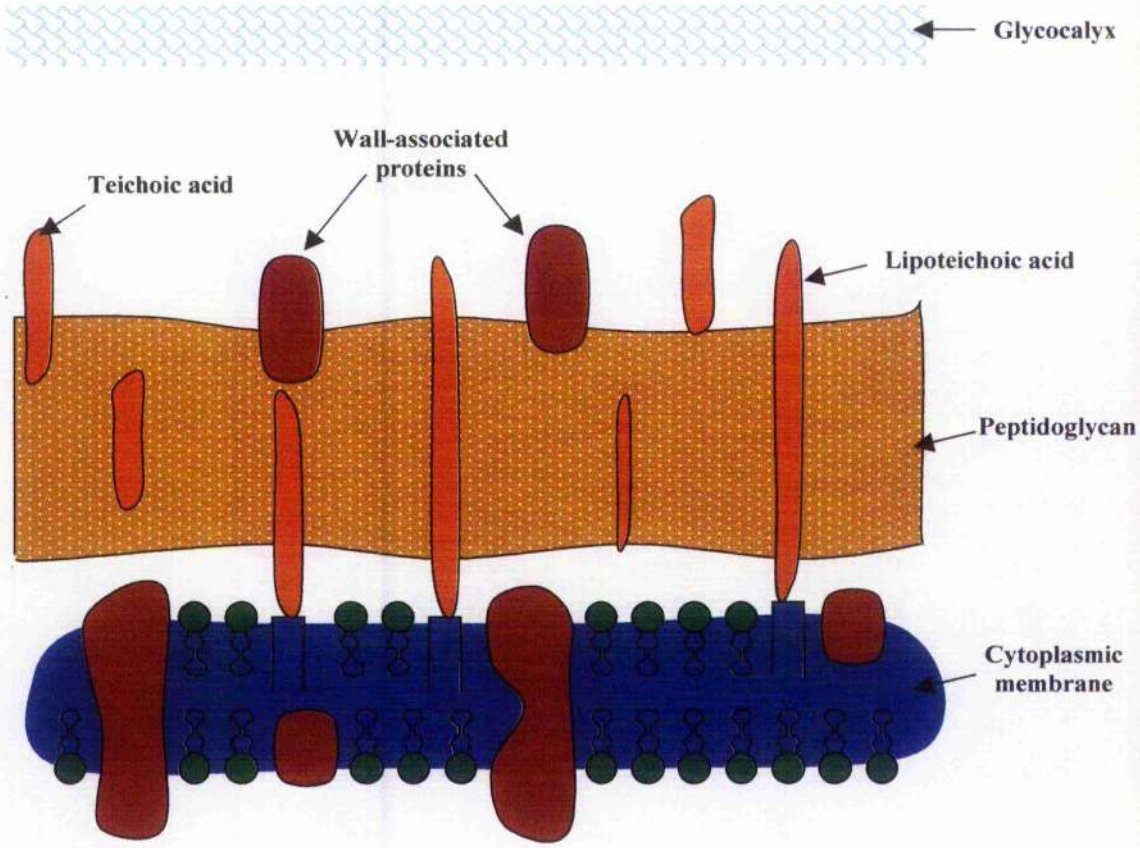


Figure 13. Gram-positive cell wall structure. Adapted from *Brock*[898].

The cell wall composition of several strains displaying increased glycopeptide resistance including the VISA strains Mu3 and Mu50, and to a lesser extent the 5836 and 5827 has been intensively studied.[149,150,242,314] The following characteristics of increased cell wall thickness, increased glutamine-non-aminated muropeptides and incorporation of GlcNac and UDP-N-acetyl-muramylpentapeptides within the cell wall, raised PBP2 and PBP2' expression, decreased cross linkage of peptidoglycan and, elevated or reduced autolytic activity have all been reported from strains of VISA. None of these characteristics are uniformly present in all VISA strains indicating two or more mechanisms of resistance

(Hiramatsu 2002 personal communication). In Mu50 and Mu3 incorporation of GlcNAc from the environment is enhanced. However, Mu50 and to a much lesser extent Mu3 also utilise glucose and glutamine more efficiently through enhanced *glmS* activity thus presenting a plausible explanation for the differences observed in cell wall thickness between the two clonal strains.[20,78,149] The increased *glmS* activity exerts an increased requirement for glutamine that may not be met. Glutamine deficiency has been shown in *femC* mutants to cause increased nonaminated muropeptides within the cell walls as seen in Mu50.[78,146]

For this study we employed transmission electron microscopy (TEM) to examine morphological changes in several strains of *S.aureus* exposed to sub-inhibitory concentrations of antibiotics

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Strains

Staphylococcus aureus strain Cowan (NCTC 8520) was kindly provided by Prof C. G. Gemmell (Glasgow Royal Infirmary, Glasgow) and strains 3700w, 3759v, 5827, 5836, Mu3 and Mu50 by Dr D. Morrison (MRSA Reference Laboratory, Stobhill Hospital, Glasgow). Each strain was stored on protect beads at –70°C and cultured on Columbia blood agar (CBA, Appendix 1) at 37°C prior to use (Oxoid). Strains 3700w and 3759v are clinical isolates from Glasgow Royal Infirmary (1999) with intermediate resistance to vancomycin (VISA).[164] Strains 5827 and 5836 are clinical VISA isolates from Michigan and New Jersey respectively.[48,49] Mu50, an intermediate vancomycin resistant strain and its hetero-vancomycin resistant precursor strain Mu3, were isolated in Japan by Hiramatsu in 1997.[161] Strains were periodically characterised by PFGE and phage typing at the MRSA Reference Laboratory, Stobhill Hospital (Appendix 2).

2.2.2 Preparation of Bacteria

Bacteria taken from protect beads were transferred on to CBA plates and sub-cultured for single colonies on three consecutive days before use and every week thereafter for a maximum of three weeks.

2.2.3 Preparation of Antimicrobial Agents

Antimicrobial agents used were obtained in their white powder form from their manufacturers: cefpirome (CEF, Roussel Uclaf, Paris, France), daptomycin (DAP, Cubist, Cambridge, Massachusetts, USA), linezolid (LZD, Pharmacia, Kalamazoo, Michigan, USA) and Synercid (SYN, Rhône-Poulenc Rorer, Paris, France). Agents were dissolved in sterile distilled water to the required concentrations using estimated potencies of 1000µg/ml. Stocks were stored at –70°C until use and discarded after three months.

2.2.4 Minimum Inhibitory Concentration – Based on NCCLS Methodology

MHB was dispensed in 100µl volumes in a 96 well microtitre plate. One hundred microliters of the antimicrobial agents, at x2 the required concentration, was added to column 1 and diluted 1:2 along each row except column 12 (growth control). The bacteria were adjusted to McFarland turbidity standard 0.5 through direct broth suspension of isolated colonies from a CBA plate. This was further diluted 1:10 in MHB and 5µl added to each well of the microtitre plate (final inoculum $5x10^5$cfu/ml). Plates were incubated overnight at 37°C. Broth used for daptomycin MIC determination and all subsequent experiments was supplemented with 10mg/ml Ca^{++} (final concentration) as per the manufacturer's instructions. For vancomycin MIC determination, additional Etest strips were used: 100µl of the bacterial suspension prepared as above was plated on to a fresh CBA plate onto which an Etest strip was aseptically placed. Plates were incubated as above. MIC's were carried out in triplicate.

2.2.5 Growth curve

Each strain (five single colonies from a 24hour old CBA plate) was inoculated in to 10ml of fresh MHB containing no drug, ½ MIC and ¼ MIC of the desired antibiotic. Cultures were immediately vortexed and an OD_{620}nm reading of each suspension taken using a CE272 linear readout ultraviolet spectrophotometer (Cecil Instruments, Cambridge, England). Each suspension was incubated for up to 24hrs at 37°C in a shaking incubator (150rpm) with OD_{620}nm readings being taken every hour. Samples were vortexed prior to each reading. Each experiment was carried out in duplicate.

2.2.6 Electron Microscopy

Overnight cultures (16hrs) of selected strains were prepared in MHB containing no drug and ½ MIC of each antibiotic then centrifuged in a IEC Centra-4x centrifuge (International Equipment Company, Dunstable, England) at 3000rpm for 15mins. Cell culture supernatants were discarded and the cell pellet fixed in 500µl 2.5% PBS-glutaraldehyde overnight at 4°C. Excess glutaraldehyde was discarded and the cells washed thrice in PBS. Pellets were saturated with 1% osmium tetroxide (OsO_4) for 1-3hrs and washed a further three times with PBS. Increasing concentrations of ethanol (30%, 50%, 70%, 90% and several changes of 100%) were used to dehydrate the samples before being left overnight in Epoxy resin (Epon 812). Samples were transferred into fresh Epoxy resin and left for a further 1-2 days at room temperature. Polymerisation was carried out at 68°C over a period of 2-3 days after which thin sections, 60-70 nm thick, were cut. Each section was stained and counterstained with uranyl actetate (UrAc) and lead citrate respectively. Examination of the sections was under a JEOL JEN 1200ExII Electron Microscope with LaB_6 filament. Sample preparation post fixation in PBS-glutaraldehyde was carried out by Jim Aitken, Department of Virology, University of Glasgow, Glasgow, UK.

2.3 RESULTS

2.3.1 Determination of Minimum Inhibitory Concentration

To achieve an accurate estimate of the minimum inhibitory concentration a range of concentrations were used, the same range being used for each antibiotic. The MIC was taken as the lowest concentration that inhibited growth (Table 9).

Antibiotic (μg/ml)	linezolid	cefpirome	synercid	daptomycin	vancomycin	
Strain	broth	broth	broth	broth	broth	Etest
Cowan (NCTC 8520)	1.56	0.39	0.19	0.125	0.78	2
3700w	0.78	8	0.125	0.78	3	6
3759v	0.78	25	0.5	0.39	3	6
5827	0.78	6.25	0.125	0.78	3	12
5836	0.39	4	0.125	0.39	3	8
Mu3	1.56	6.25	0.39	0.39	1	2
Mu50	1.56	8	0.39	0.39	1.56	8
EMRSA 16	1.56	32	0.5	0.195	0.39	1.25

Table 9. Minimum inhibitory concentrations (μg/ml) of bacterial strains to various antibiotics

The differences in vancomycin MIC values between the broth micro-dilution assay and the Etest were not expected but can may be explained by the reduced surface area available to vancomycin on the CBA plate and easier detection of hetero-resistant colonies.

2.3.2 Growth curves in the presence of sub-inhibitory concentrations of antibiotics

Growth in the presence of antibiotics was determined by optical density readings over a 24hour period. Figures 14-21 show the growth of each strain in broth only and in the presence of ½ and ¼ MIC linezolid, cefpirome, synercid and daptomycin. Each strain used was affected to different degrees by the addition of sub-inhibitory concentrations of antibiotics.

Sub-MIC cefpirome was shown to cause a reduction in optical density of *S.aureus* Cowan during the first hour of exposure after which exponential growth ensued but remained less than optimal. Severe disruption to the growth pattern of 3700w and 3759v was apparent. One half MIC exerted bacteristatic properties for 8 and 4 hrs respectively with growth not exceeding a 1.5fold increase in optical density. Exponential growth then proceeded with 24hr readings similar to that of the no drug control. One quarter MIC was also bacteristatic towards both of these strains but to a lesser extent. Strains 5827 and Mu50 displayed marginally restricted growth at both sub-MIC's of cefpirome whereas slightly increased

growth was observed with strain 5836. Mu3 appeared unaffected by exposure to cefpirome.

Linezolid exposure produced relatively consistent growth characteristics between each of the strains tested. Reduced growth rates were noted in strains of Cowan, 3759v, Mu3, Mu50 and E16 at ½ and ¼ MIC, and against 3700w at ½ MIC only. Indeed linezolid was the most effective growth inhibitor of Cowan, Mu3 and Mu50. The growth pattern of 5827 was not shown to be affected whereas a marginal increase in the growth of 5836 compared to the no drug control was evident.

Synercid (½ MIC) exerted bacteristatic properties on *S.aureus* Cowan to the same degree as ¼ MIC linezolid whereas ¼ MIC synercid exposed cultures were only slightly inhibited. Both concentrations were bacteristatic towards cultures of 3700w resulting in a reduced growth rate with cell density increasing by only half a log by 5hrs. Recovery of these cells by 24hrs did not take place. VISA 3579v was also inhibited by both ½ and ¼ MIC synercid with a 3fold reduction in optical density of ½ MIC treated cells after 8hrs. Synercid was the only antibiotic tested to inhibit growth of strain 5836. Exponential growth proceeded during the initial 3hrs of growth after which the generation time of the cells was rapidly depressed only increasing 2fold by 8hrs. Bacteristatic growth conditions were observed with E16 at both ½ and ¼ MIC synercid and was the most effective antibiotic against this strain also. Marginal inhibition of strain 5827 and no effect on Mu3 and Mu50 was detected.

The only strain to be seriously inhibited by exposure to daptomycin was VISA 3759v which displayed bacteristatic growth for 5-6hrs after which time exponential growth occurred resulting in cell densities comparable to the no drug control after 24hrs. *S.aureus* Cowan was also slightly inhibited by ½ MIC daptomycin but not at ¼ MIC. With 3700w, ½ MIC a slight enhancement of growth of noticeable whereas ¼ MIC daptomycin slightly reduced growth, these differences were negligible by 6hrs. The remaining strains E16, 5827, 5836, Mu3 and Mu50 showed very little response to daptomycin exposure although Mu50, 5836 displayed a slight enhancement of growth.

Although measurement of growth by optical density is highly valuable in the determination of a bacterial strain's ability to divide in the presence of sub-inhibitory concentrations of

antibiotic, it does not provide us with details regarding the state of the cells, for example, cell size or clumping.

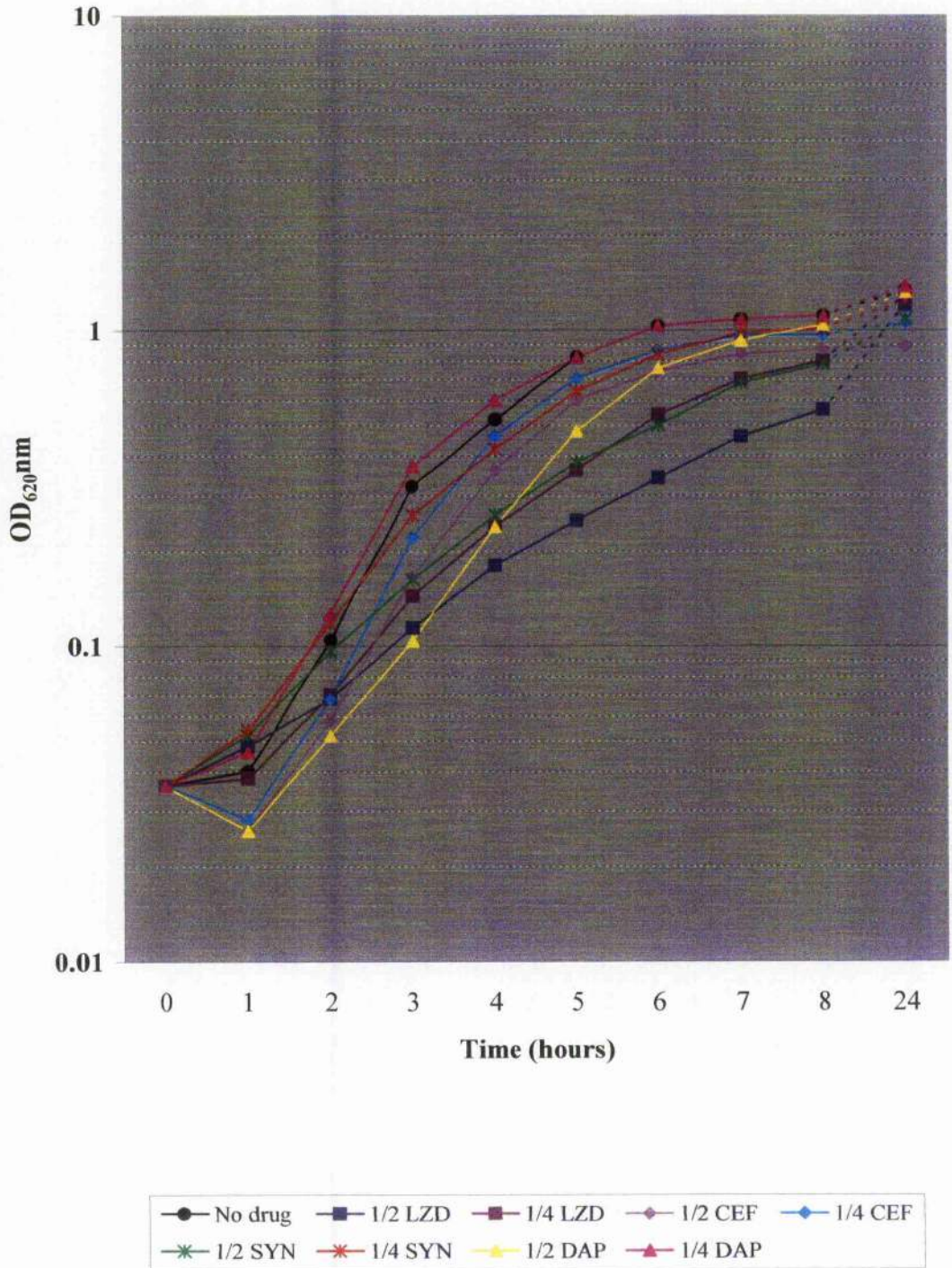


Figure 14. Growth of *S.aureus* Cowan in the absence and presence of sub-inhibitory concentrations of antibiotics

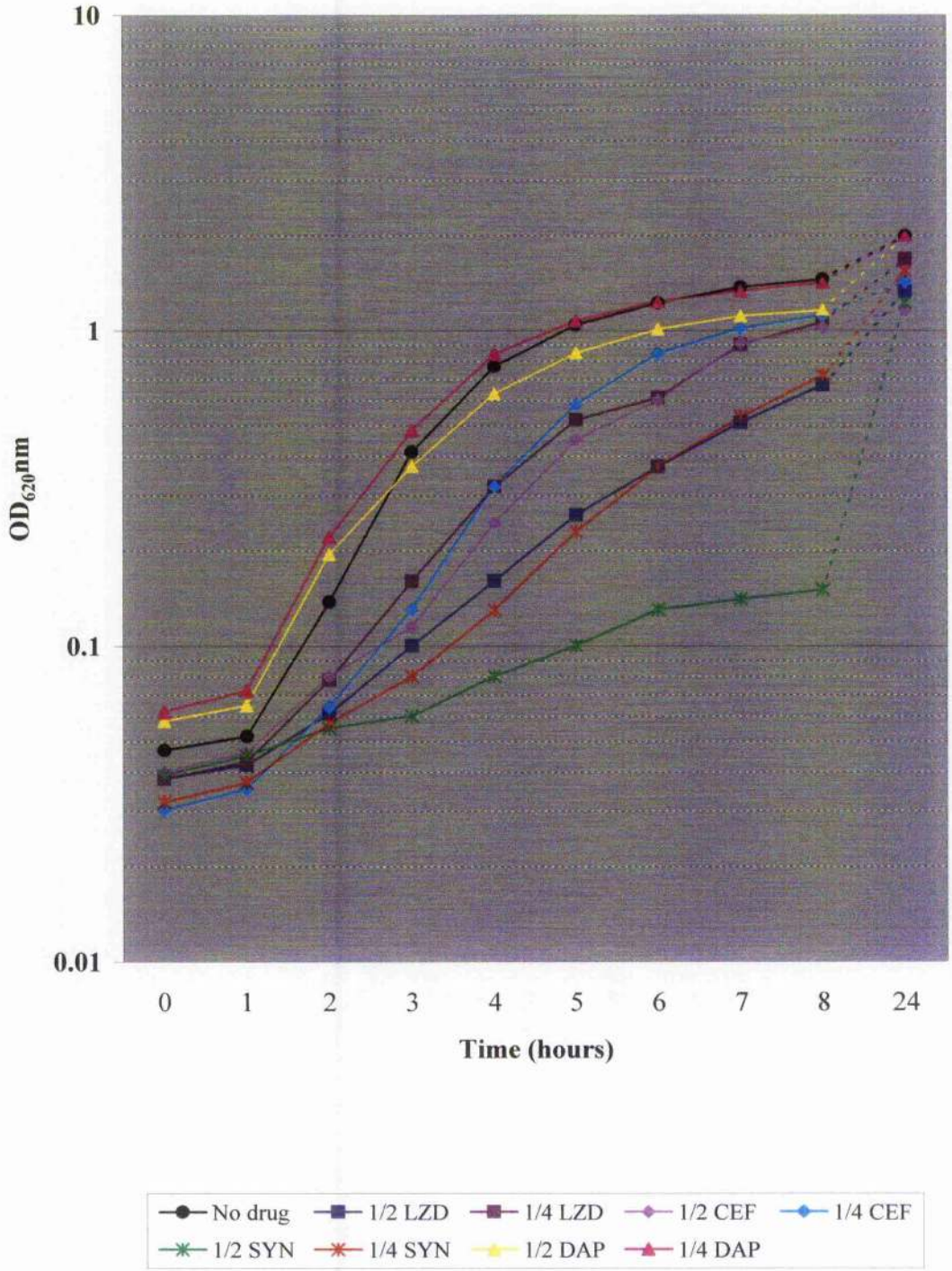


Figure 15. Growth of EMRSA-16 in the absence and presence of sub-inhibitory concentrations of antibiotics

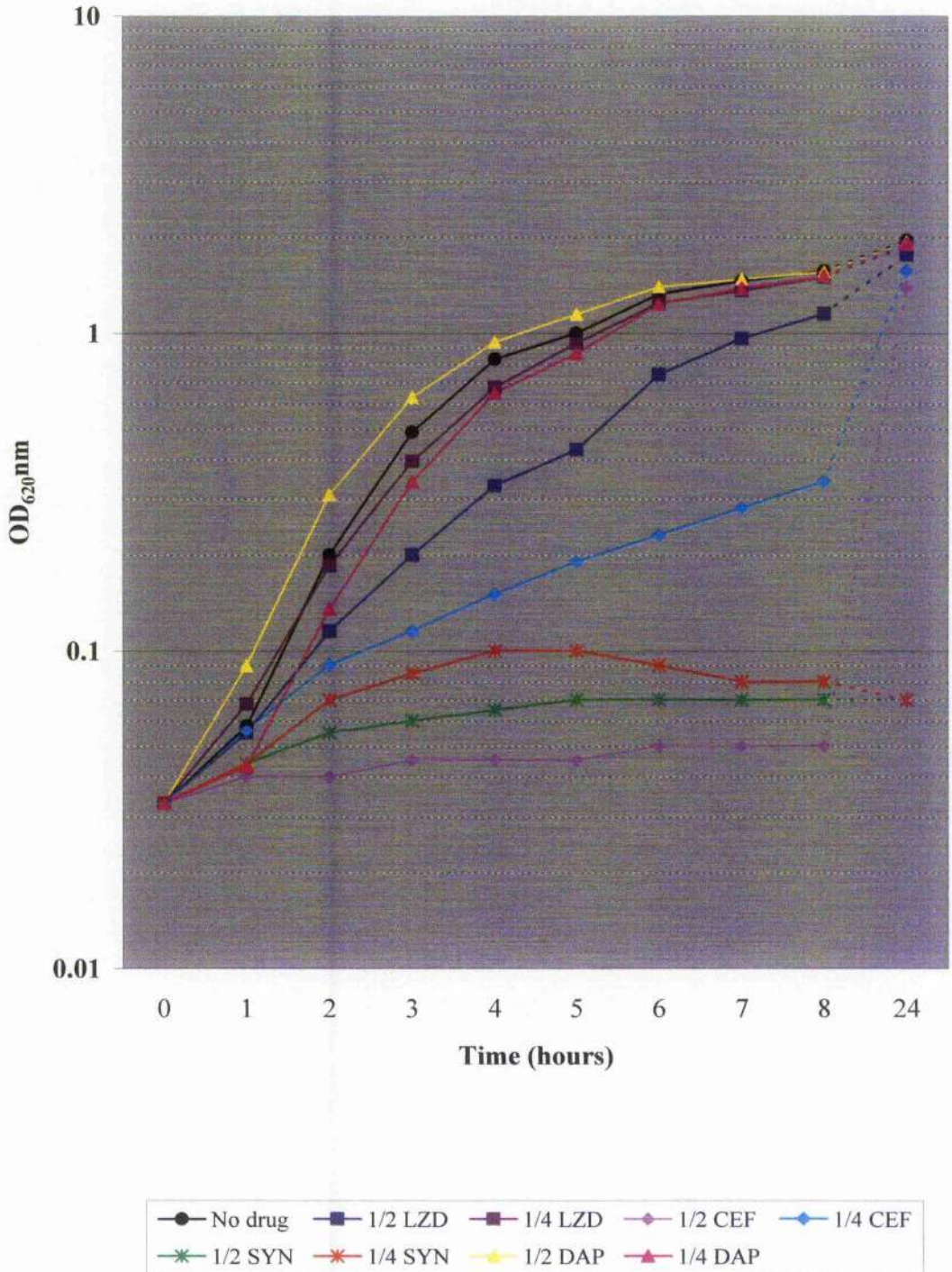


Figure 16. Growth of VISA 3700w in the absence and presence of sub-inhibitory concentrations of antibiotics

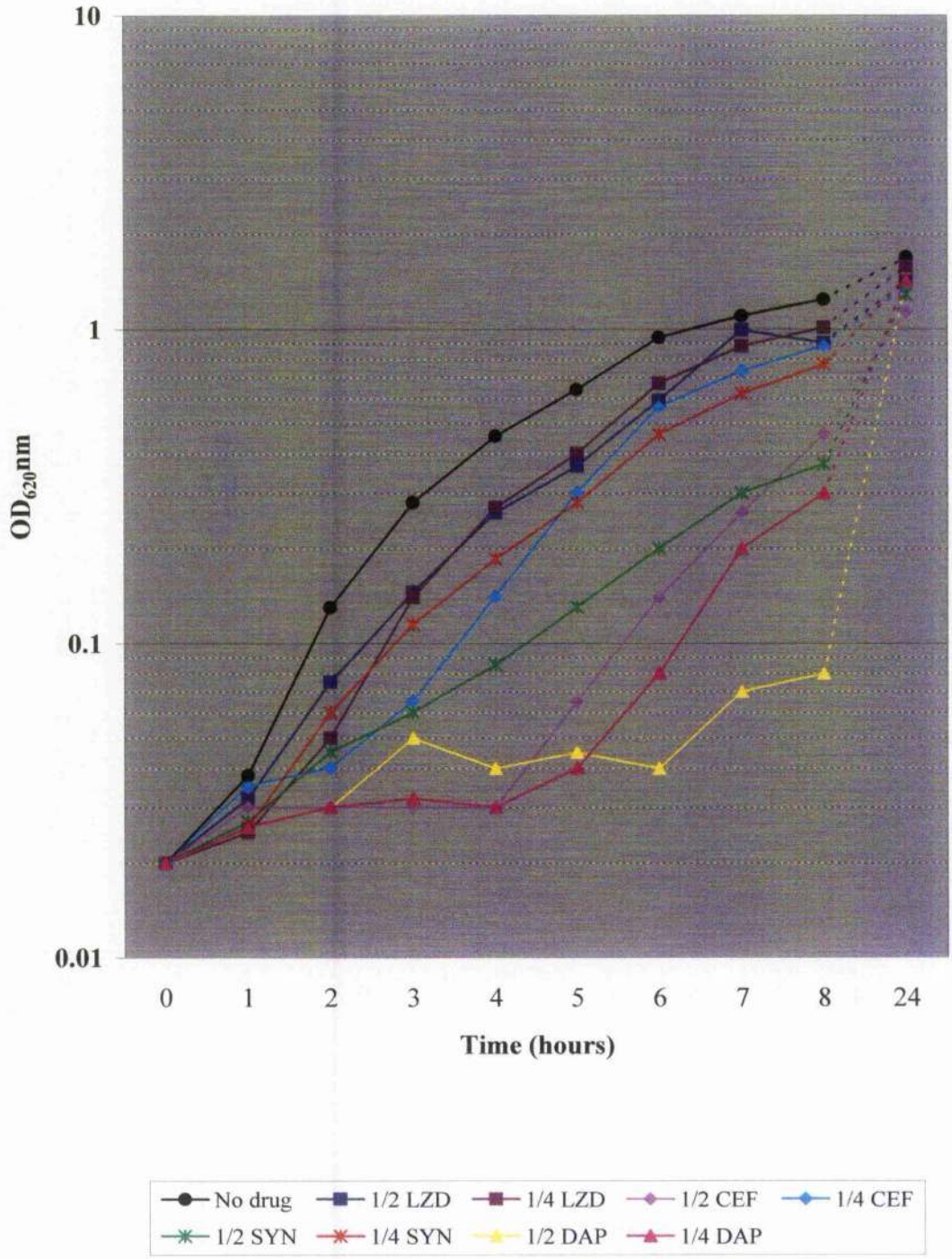


Figure 17. Growth of VISA 3759v in the absence and presence of sub-inhibitory concentrations of antibiotics

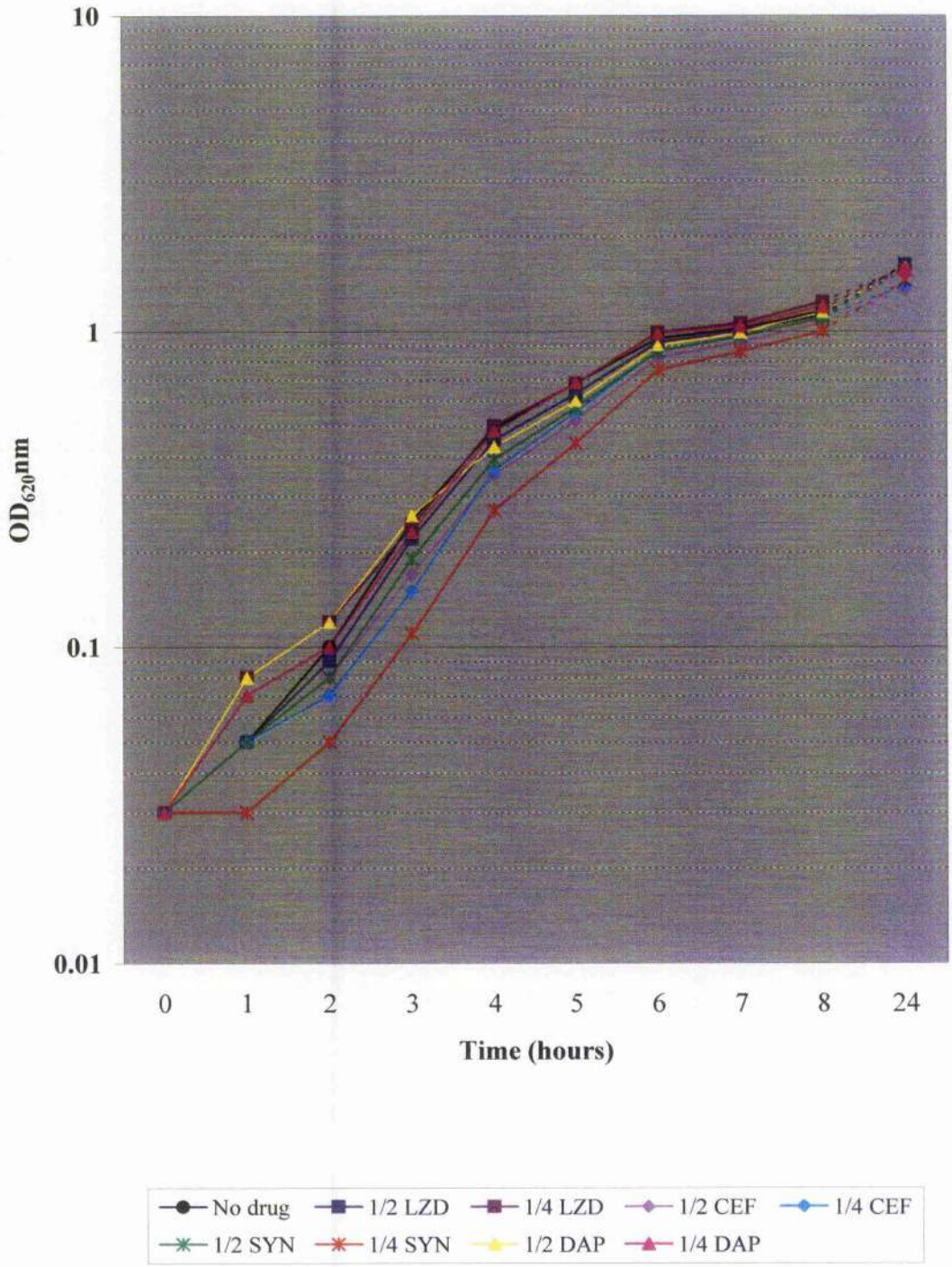


Figure 18. Growth of VISA 5827 in the absence and presence of sub-inhibitory concentrations of antibiotics

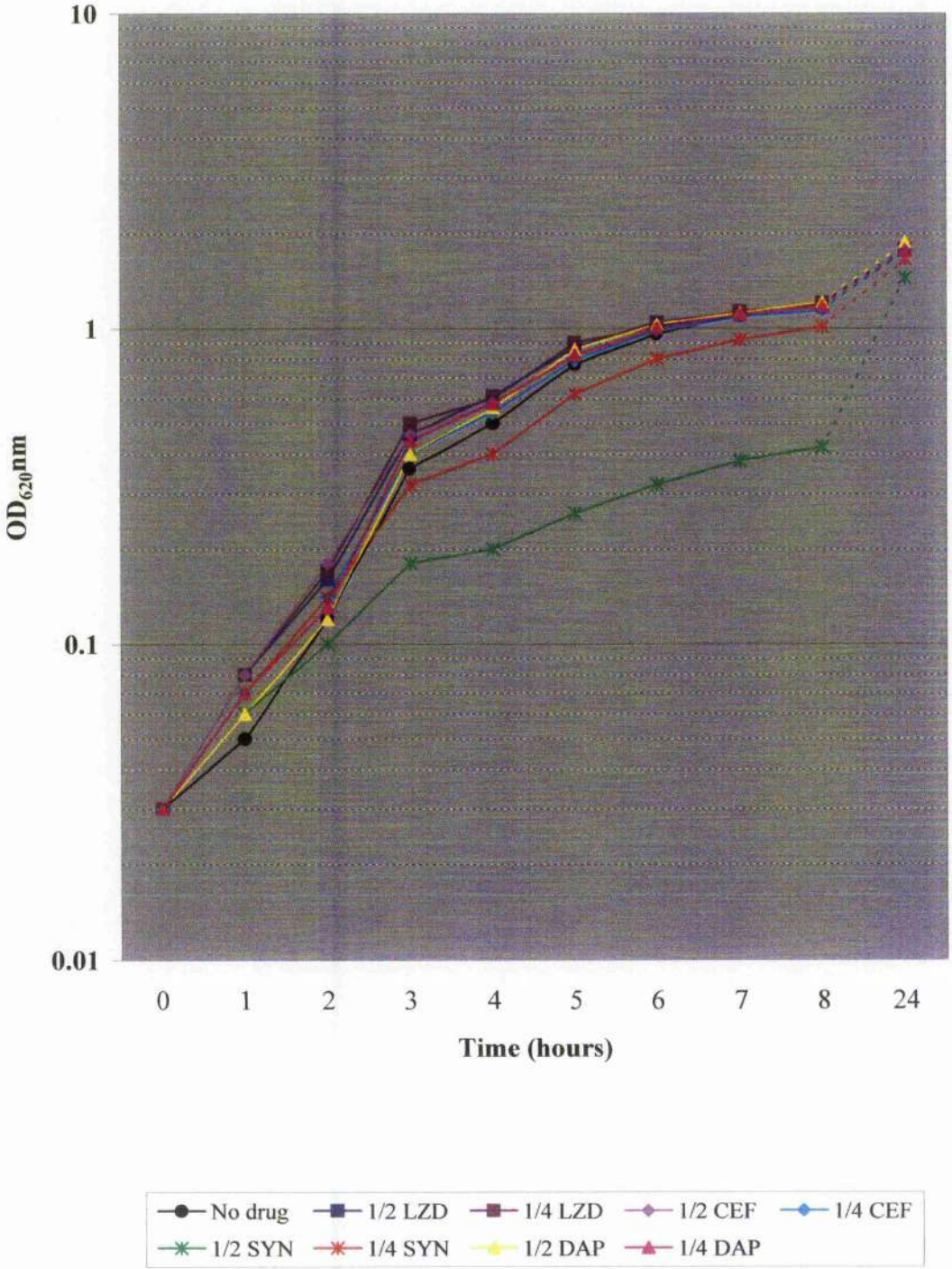


Figure 19. Growth of VISA 5836 in the absence and presence of sub-inhibitory concentrations of antibiotics

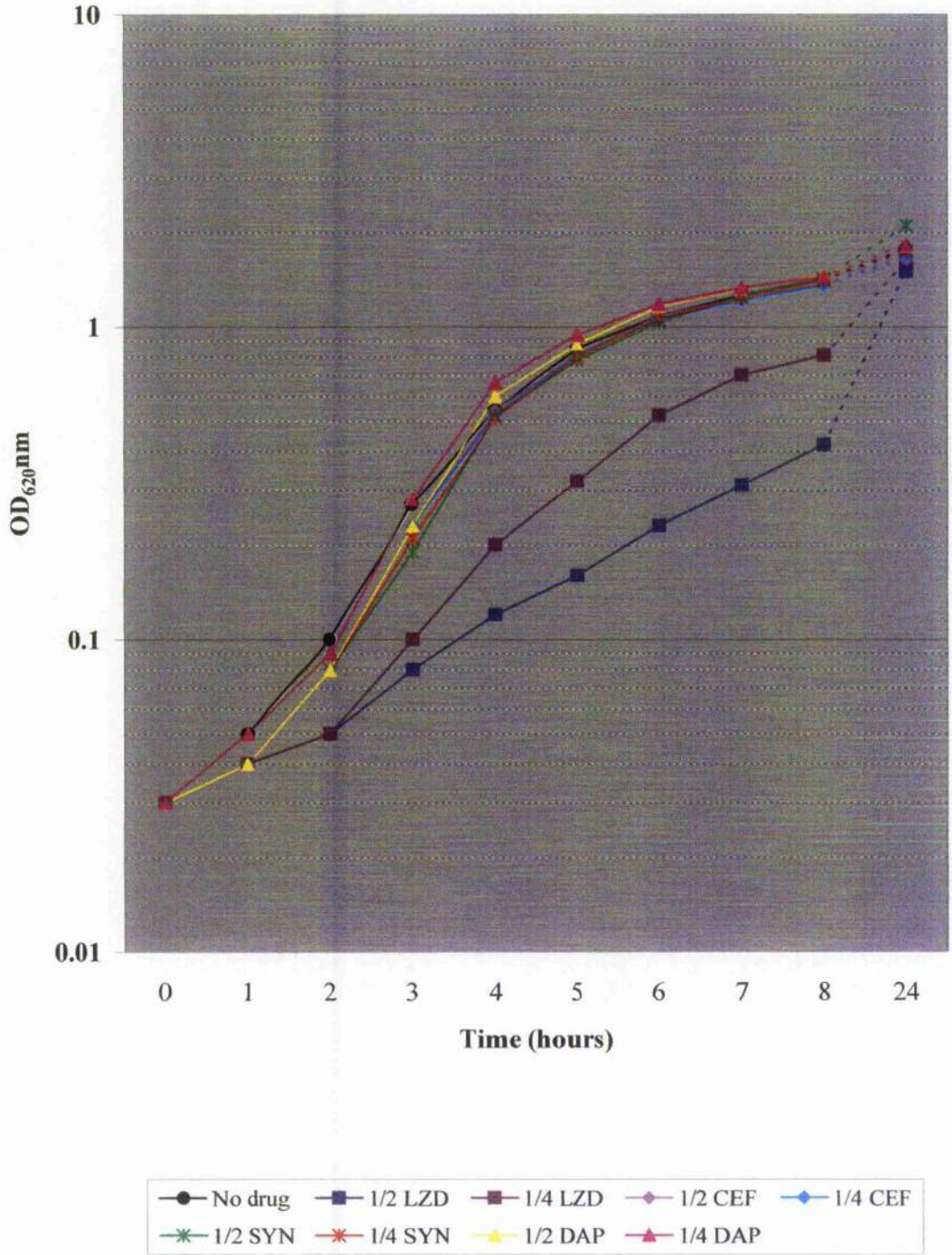


Figure 20. Growth of VISA Mu3 in the absence and presence of sub-inhibitory concentrations of antibiotics

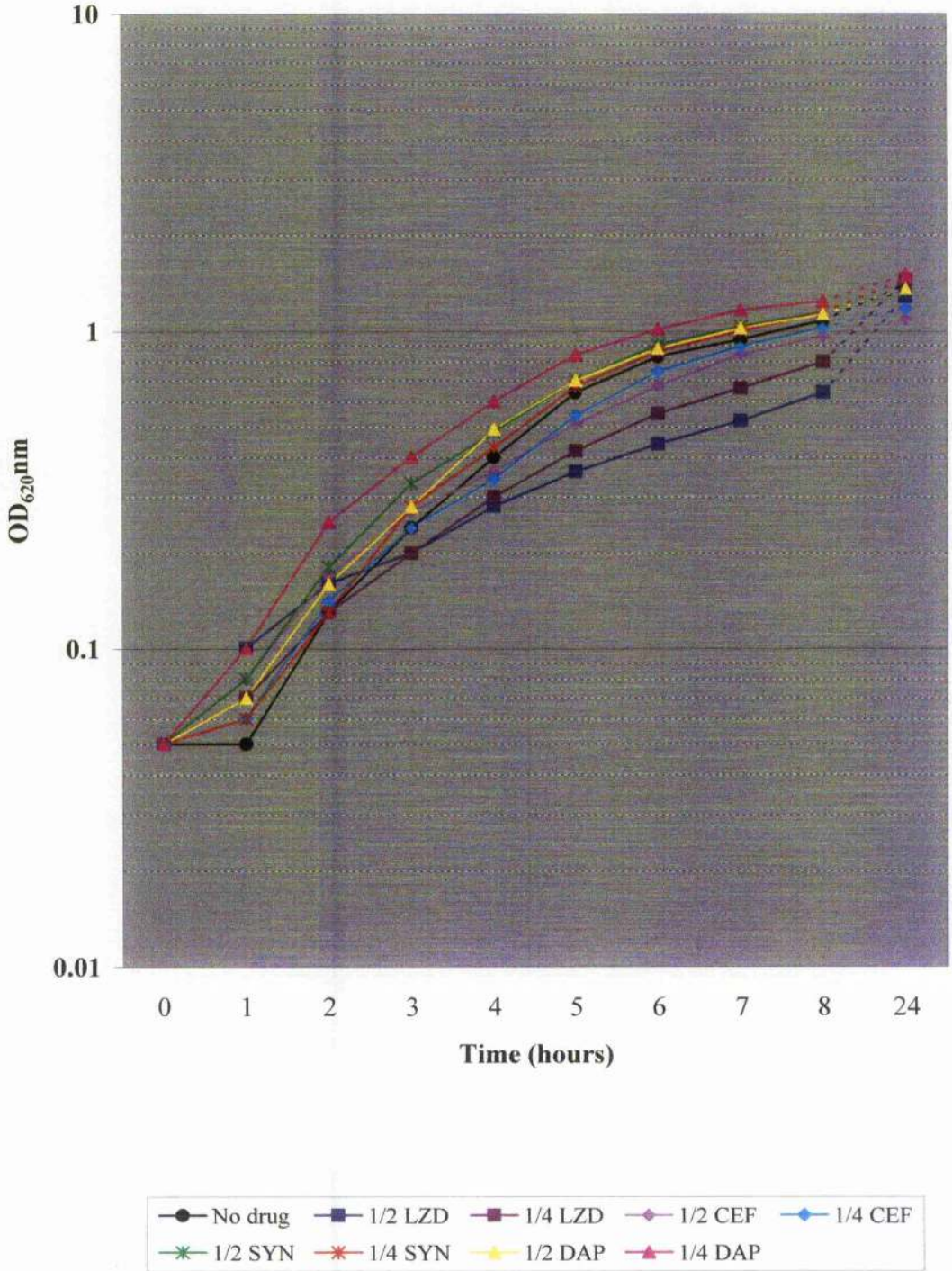


Figure 21. Growth of VISA Mu50 in the absence and presence of sub-inhibitory concentrations of antibiotics

2.3.3 Electron Microscopy Studies of Microbial Cell Walls and Cell Surface Material

Sections of cell preparations were observed through an electron microscope at a magnification of x40000. Random fields of view were chosen and digital images taken of typical and atypical cell formations at a magnification of x100000. The key feature of this exercise was to examine cell wall structure, alterations in cell morphology and the presence of any surface material. We found that cell size and the extent of cell division among cultures varied between strains (Table 10) and between drug treated cells of the same strain (Table 11). Bacterial cultures grown in unsupplemented MHB all showed typical growth characteristics with many cells undergoing cell division. Cell wall associated material, which is assumed capsular material or slime, was not observed in all strains and was not reproducible. Loss of loosely associated slime layers may have occurred during handling of cell cultures prior to fixation in PBS-glutaraldehyde.

Cowan	E16	3759v	5836	Mu3	Mu50
24.5 ± 4.3	28.1 ± 2.3	27.5 ± 3.4	51.2 ± 7.4*	30.9 ± 8.3	27.8 ± 3.8

Table 10. Comparison of cell wall thickness between strains of *S.aureus*. Statistical analysis compares strains with MSSA Cowan. Readings represent mean +/- standard deviation, * = pvalue ≤0.05.

Table 11 shows the variation in cell wall thickness associated with antibiotic treatment and the presence of cell wall material. Cell wall thickness was calculated using a conversion grid where 1mm ≠ 6.6128 nm. Mean values and standard deviations were calculated by a two sample T-test using a Minitab statistical package. Figures 22 and 23 illustrate the effects antibiotics have on the morphology of individual cells. The electron microscope images presented here are representative of the majority of cells present in a section. Photographs at a final magnification of x60000 showing a wider spectrum of cells are available upon request.

	Antibiotic Exposure / Cell Wall Thickness (nm ± s.d)				
	No Drug	½ MIC LZD	½ MIC CEF	½ MIC SYN	½ MIC DAP
Cowan	24.5 ± 4.3	31.4 ± 5.3	26.4 ± 6.6	32.2 ± 5.7	25.4 ± 9.6
E16	28.1 ± 2.3	44.4 ± 5.4*	33.9 ± 4.2	59.5 ± 6.6*	33.9 ± 4.2
3759v	27.5 ± 3.4	31.4 ± 4	34.2 ± 5	39.5 ± 6.7*	18.2 ± 1.9*
5836	51.2 ± 7.4	36 ± 4*	51.2 ± 2.3	53 ± 2.1	27.8 ± 3.8*
Mu3	30.9 ± 8.3	70.5 ± 8.3*	56.2 ± 15.2*	39.7 ± 0.01*	34.2 ± 5
Mu50	27.8 ± 3.8	86 ± 9.3*	58 ± 25.7	65 ± 9.5*	26.4 ± 6.6

Table 11. Alteration of cell wall thickness of strains of *S.aureus* exposed to ½ MIC linezolid (LZD), cefpirome (CEF), synercid (SYN) and daptomycin (DAP). Readings represent mean +/- standard deviation, * = pvalue ≤0.05.

Exposure to ½ MIC linezolid significantly increased the cell wall thickness of strains E16, Mu3 and Mu50 (pvalue ≤0.05). The largest increases were noticed in strains Mu3 (128%) and Mu50 (209%). The cell walls of Mu3 also gave an uneven 'moth eaten' appearance. In contrast, strain 5836 developed significantly thinner cell walls in response to linezolid. *S.aureus* Cowan and 3759v were not affected. In addition to thickened cell walls, Mu50 displayed abnormal cell growth characteristics in response to ½ MIC cefpirome. Many of the cells were devoid of cytoplasmic material and appeared to have burst; a similar phenomenon was observed with the same strain grown in the presence of ½ MIC synercid. The increase in cell wall thickness observed in Mu50 was very similar to that of Mu3 exposed to ½ MIC cefpirome but the differences were found not to be significant and significant respectively. Only two images of Mu50 were suitable to take cell wall readings from (76.05 and 39.68) resulting in a high standard deviation. Nevertheless, both of these measurements are substantially higher than that of the control strain in the absence of antibiotic. Strain Mu3 also appeared to be smaller in comparison to the untreated culture. The other strains grown in ½ MIC cefpirome displayed normal cell walls and cell size although septum formation in strain 5836 was slightly deranged.

Synercid produced variable effects on different strains; the strain most affected at ½ MIC levels was 5836. This strain did not show an altered thickness but had 'moth eaten' cell walls and the majority of cells were seen to be dividing in multiple planes and 'sticking together'. Multiple septum formation was also observed in strain 3759v in addition to a thickened cell wall, whilst E16 displayed thickened 'moth eaten' cell walls but normal cell

division. Mu3 again grew with a thicker cell wall whilst *S.aureus* Cowan remained unaffected. Only two strains, 3759v and 5836, displayed altered cell morphology in response to ½ MIC daptomycin. Both strains possessed significantly thinner cell walls (pvalue ≤0.05) but, cell size and division were not altered.

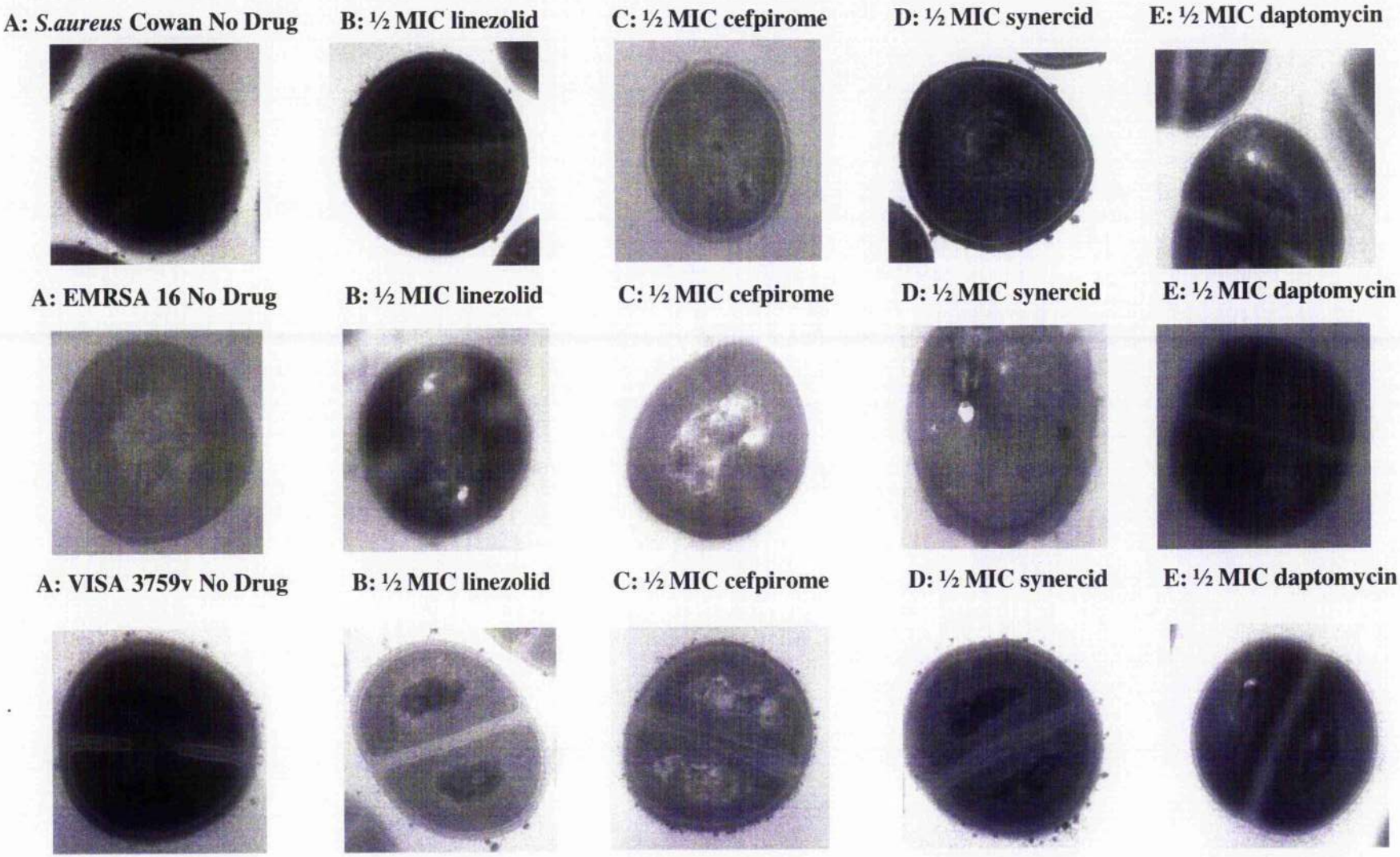


Figure 22. The effects of ½ MIC linezolid, cefpirome, synercid and daptomycin on the morphology of strains of *S.aureus*

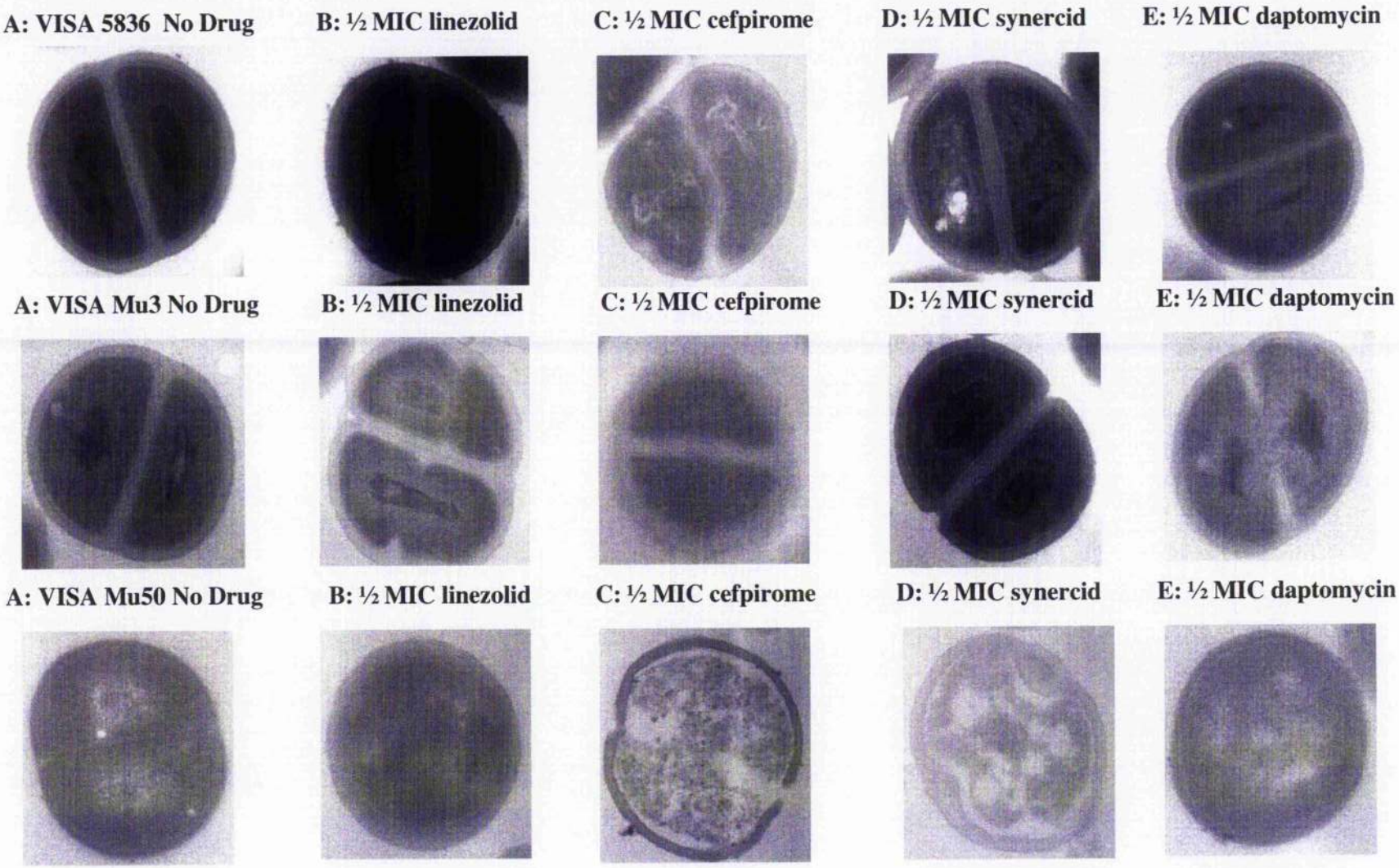


Figure 23. The effects of ½ MIC linezolid, cefpirome, synercid and daptomycin on the morphology of strains of *S.aureus*

2.4 DISCUSSION

The activity of antibiotics on bacterial growth and cell morphology has been studied intensively in recent years, mainly with the β-lactams. At sub-inhibitory concentrations, antibiotics can cause a range of effects that are not unique to any individual agent. Such effects include: prolonged lag phase, reduction in rate of exponential growth, reduced population size reaching stationary phase, the prevention of cross-wall formation and/or cell wall division without any effect on viability or rate of cell growth, abnormal cell size and multiple thickened cell walls.[4,77,129,293,294] The Minimum antibiotic concentration (MAC) according to Gemmell and Lorian is the 'minimum quantity of antimicrobial agent necessary to induce alteration in bacterial structure, as seen by electron microscopy, and/or growth producing one-log decrease in cell numbers compared to the control'.[131]

Recently, interest has developed in the cell wall morphology of VISA strains as a possible mechanism for increased vancomycin resistance. Strain Mu50 (MIC=8g/ml) has been reported to possess a cell wall twice as thick as that of the average staphylococcal cell wall, including its related strain Mu3, which was similar to sensitive strains.[78,150] The results presented in Figure 23 show that the cell wall of Mu3 is marginally thicker than that of Mu50 and that although both have thicker walls than MSSA Cowan it is not significant. MHB was used as our growth medium however Hiramatsu (2002 personal communication) recommends the use of BHI for the optimum expression of cell wall thickness to be achieved through full use of the EMP-*glmS* pathway in peptidoglycan biosynthesis as detailed in this chapters introduction. This is supported by Cui *et al.* (2000) who found that media containing cell wall component amino acids, and glucose was better for supporting vancomycin resistance in VISA although they did not find the cell walls of Mu3 and Mu50 to be vastly different either.[78] Measurements of 26.17 ± 2.17nm and 31.88 ± 1.56nm respectively were reported which are similar to our measurements of 30.9 ± 8.3nm and 27.8 ± 3.8nm (Mu3 and Mu50) in MHB. Studies of VISA cell walls have found a degree of variation between research groups.[78,79]

In our determination of the minimum inhibitory concentration (MIC) we used broth micro-dilution assays with MHB for all antimicrobial agents. It was noticed that by this method the vancomycin MIC values were less than those expected for all VISA strains. The use of E-test strips however increased the level of resistance range to between 2μg/ml and

12μg/ml. The use of MHB in the micro-dilution assays and in the above electron microscopy experiments may not be providing the strains with adequate nutrients for full resistance to be expressed but as vancomycin is not being used here as an investigational drug this discrepancy is not of major importance.

Upon examination of sections under TEM we discovered no consistent effects of an antimicrobial agent on all of the strains tested. In general, exposure to ½ MIC linezolid, cefpirome and synercid resulted in increased cell wall thickness although some strains displayed no such increase. The finding that 1μg/ml linezolid caused no morphological alterations of GISA were reported by Grif *et al.* (2001) however in this instance whole cells were being examined not sections.[142] In contrast to this, cell walls were reduced upon exposure to ½ MIC daptomycin, an expected outcome due to the drug's ability to affect biosynthesis and assembly of peptidoglycan. An interesting observation regarding Mu50 treated with ½ MIC synercid was that the majority of cells were devoid of cytoplasmic material and that ½ MIC cefpirome caused granulation of the cytoplasm. This phenomenon was also reported in *S.aureus* associated with bone infections, and has been attributed to phenotypic modification in response to a changing environment.[77]

In relation to growth, again there was no uniform outcome by a particular drug. However there is a strong correlation between reduced growth rate and increased cell walls irrespective of the antibiotic involved. Strains 3759v and E16 exposed to ½ MIC synercid, and strains E16, Mu3 and Mu50 exposed to ½ MIC linezolid are good examples of this. Increased energy consumption involved in increased peptidoglycan synthesis may be a key factor to these findings (Hiramatsu 2002; personal communication). The reverse of this may also be true in the finding of reduced cell wall thickness and accelerated cell growth with strain 5836 exposed to ½ MIC linezolid. Out of all the strains studied, strains 3700w and 3759v were the only strains where growth was inhibited by ½ MIC cefpirome. However, re-growth occurred by 24hrs and after 4 hrs respectively suggesting low-level hetero-resistance within the populations as evidenced by the normal appearance of the cell wall of 3759v at 16hrs. In contrast, strain 3700w exposed to ½ and ¼ MIC synercid did not recover by 24hrs suggesting complete inhibition of growth by synercid at these concentrations, the production of an inhibitory protein or possible depletion of nutrients.

CHAPTER THREE

The Effects of Antimicrobial Agents on the Expression of Virulence Factors and mRNA Transcription

3.1 INTRODUCTION

The production of virulence factors within a host serves to aid survival of the fittest. The production of such factors in an infection process contributes to microbial pathogenicity through their interactions with host-defence mechanisms, ability to facilitate establishment of an ecological niche and their direct, potentially lethal effects on host tissues.

Here we consider proteins that are considered virulence factors of *S.aureus*, some of which are secreted, attached to the bacterial cell wall, or both. Irrespective of their final destination transcription of all of these factors is believed to be regulated by the global regulatory system *agr*. As discussed in section 1.3, cell wall associated proteins are transcribed during the exponential growth phase when the *agr* locus is being repressed. In response to an autoinducing peptide the *agr* system is activated stimulating the transcription of extracellular protein genes. Two cell-associated proteins have been chosen: clumping factor and protein A, and two extracellular proteins or toxins: Toxic shock syndrome toxin (TSST-1) and alpha–haemolysin (α-toxin). In addition, the expression of free coagulase and protein A was also investigated.

For each virulence factor a different culture medium is used provide to optimal conditions for toxin production although the growth conditions remain constant. For the detection of toxins we employed enzyme linked immunsorbent assays (ELISA) and biological activity assays both of which provide highly sensitive, reproducible results. We also used a fluorescence-activated cell sorter (FACS) and fluorescein isothiocyanate (FITC) conjugate probes to detect CD69 surface receptors on human Tcells, a receptor that is over-expressed as a result of Tcell stimulation, and therefore an indicator of Tcell stimulation by culture supernatants. The levels of protein A and TSST-1 mRNA at a particular time point are also investigated using a LightCycler instrument.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains

The bacterial strains used are those referred to in section 2.2.1. Additional strains are used as standards due to their ability to produce high levels of particular toxins as detailed in

Table 12. Minimum inhibitory concentrations for the strains mentioned were carried out as described in section 2.2.4.

Strain	Toxin produced	Source
DU5723	Protein A negative mutant	Prof. T Foster, Dublin University
Cowan (NCTC 8520)	Protein A positive TSST-1 negative	Prof. C. Gemmell, University of Glasgow
Wood 46 (NCTC 7121)	α-Haemolysin	Prof. C. Gemmell, University of Glasgow
NCTC 6571	Coagulase	Glasgow Royal Infirmary Diagnostic Laboratory
NCTC 11963	TSST-1	Prof. C. Gemmell, University of Glasgow
119993 (clinical strain)	Coagulase negative staphylococcus	Glasgow Royal Infirmary Diagnostic Laboratory

Table 12. Standard strains of staphylococci. Abbreviations: Toxic shock syndrome toxin (TSST-1)

3.2.2 Preparation of Antimicrobial Agents

The antibiotics used were cefpirome, daptomycin, linezolid and synercid as previously described in section 2.2.3. Each antibiotic was freshly prepared to the required concentration in 10ml volumes except for cultures in Todd Hewitt broth (THB) for α-toxin detection where 50ml flasks were used. Broth supplemented with 10 mg/ml Ca^{++} was used with daptomycin. The minimum inhibitory concentrations of all of the strains in variable culture medium (section 3.2.3) were tested and were found to be the same as those achieved in MHB.

3.2.3 Preparation of Bacteria

Cultures of the appropriate bacterial strains were grown for 16hrs in the presence and absence of ½ and ¼ MIC of each antibiotic. The following culture media were used: Brain Heart Infusion broth (BHI) – clumping factor and coagulase; MHB – Protein A; THB - α-haemolysin; Tryptone Soya broth (TSB) – TSST-1.

3.2.4 Preparation of Peripheral blood mononuclear cells (PBMC)

The isolation of peripheral blood mononuclear cells (PBMC) was carried out using Histopaque 1077 (Sigma, Poole, UK) according to the manufacturer's instructions. Fresh whole blood was obtained by venipuncture from healthy adult volunteers and carefully layered in 6ml volumes on top of 6ml of Histopaque 1077 in sterile conical test tubes (Life Technologies, Paisley, UK). Centrifugation was carried out in an IEC Centra-4x centrifuge (International Equipment Company, Cambridge, UK) at 1900rpm for 30mins. Mononuclear cells were present in an opaque layer below the plasma layer. This layer was removed using a sterile pasteur pipette and washed with RPMI/10% Foetal Calf Serum (FCS) for 15mins at 1800rpm. The washed cells were pooled, counted in a haemocytometer and adjusted to a concentration of $2x10^6$ PMBC/ml with RPMI/10%FCS.

3.2.5 Maintenance of Jurkat Tissue Culture Cell Line

Jurkat tissue culture cell lines were donated by the University of Glasgow Department of Medicine, Glasgow Royal Infirmary, Glasgow. Maintenance of the Jurkat cell line required supplementation with fresh RPMI tissue culture medium twice weekly. Cells were centrifuged at 13000rpm for 10mins then handled under strict sterile conditions in a laminar flow cabinet. Spent culture medium was discarded and the cell pellet resuspended in 2ml of fresh culture medium. The cell suspension was transferred into a vented flask and made up to 40ml with fresh supplemented RPMI medium (Appendix 1). Jurkat cells were incubated at 37°C in the presence of 5%CO_2 for 3 days before being fed again.

3.2.6 Polymerase Chain Reaction (PCR)

Bacterial DNA from sample and PCR control strains (Table 13) was extracted by incubating colonies with 100µl NET buffer (Appendix 1) and 10µl achromopeptidase (10units/µl) for 15mins at 50°C. The PCR reaction was carried out using ready-to-go PCR beads (Amersham) to which 19µl PCR water (Sigma), 2µl of forward and reverse test primers mix (MWG, Appendix 2), 2µl of DNA positive control, nuclease or 16S rRNA, primer mix (MWG, Appendix 2), and 2µl of lysed cell suspension were added. One reaction bead was used per primer per strain. Amplification conditions for each primer were programmed into a Techne Genius thermal cycler (Techne Ltd, Cambridge, UK,

Appendix 3). Samples were run on a 1.5% agarose gel in x10 TBE buffer (BioWhittaker) for 115mins at 140 volts. Gels were stained with ethidium bromide (Sigma) and photographed under ultra violet using a UV transluminator (UVP Ltd, Cambridge, UK) connected to an Elonex computer system and analysed by 'Grab-IT' version 2.53 software.

Strain	Toxin Gene	Toxin produced	Source
S.aureus Cowan (NCTC 8520)	*spa*$^+$	protein A	Prof. C. Gemmell, University of Glasgow
NCTC 10657	*sea*$^+$/*seb*$^+$	SEA / SEB	MRSA Reference Laboratory, Stobhill
NCTC 11963	*tst*$^+$/*sec*$^+$	TSST-1 / SEC	MRSA Reference Laboratory, Stobhill
NCTC 10656	*sed*$^+$	SED	MRSA Reference Laboratory, Stobhill
FRI 326	*see*$^+$	SEE	Public Health Laboratory, London
ETA	*eta*$^+$	Exfoliative toxin A	Public Health Laboratory, London
ETB	*etb*$^+$	Exfoliative toxin B	Public Health Laboratory, London
Wood 46 (NCTC 7121)	*hly*$^+$	α-toxin	Prof. C. Gemmell, University of Glasgow

Table 13. PCR standard control strains. Abbreviations: Alpha-toxin (α-toxin); staphylococcal enterotoxin A-E (SEA-E); toxic shock syndrome toxin (TSST-1)

3.2.7 Detection of Protein A by ELISA

Sixteen hour bacterial cultures in MHB were centrifuged at 3000rpm for 15mins decanting the culture supernatants into sterile universals. Cell pellets were adjusted to an optical density, OD_{620}nm = 0.025 ($1x10^7$cells/ml) in sterile saline. Immulon 4-HB plates (Dynex) were coated with 100µl rabbit monoclonal anti-protein A antibody (MAb anti-SpA) (Sigma, 1:10000) overnight at 4°C. Plates were washed thrice in PBS/0.05% Tween-20 (PBS/T) then blocked for 2hr at 37°C with PBS/0.05%Tween-20/0.01% Bovine serum albumin (PBS/T/BSA, 200µl). Following washing a further three times in PBS/T plates were incubated with 100µl of purified protein A (Sigma) and samples diluted in PBS/T/1% normal rabbit serum (NRS), in triplicate, for 2hrs at 37°C. The washing steps were repeated as before prior to incubation with 100µl Biotinylated MAb anti-SpA (Sigma, 1:40000) for 1hr at 37°C followed by 100µl Streptavidin Alkaline-phosphatase (Sigma, 1:1500) for 1hr at 37°C. A final six washes were carried out before incubation with 100µl

2Mm p-nitrophenyl phosphate (PNP, Sigma) for 30mins at room temperature. Each plate was read using a Titertek® Multiscan Plus plate reader MK II (ICN) at 405nm. Experiments were carried out five times.

3.2.8 Immunogold-labelling of Protein A

Sixteen hour cultures of selected strains were prepared in MHB only and centrifuged at 3000rpm for 15mins. The cell culture supernatant was discarded and the cell pellets were fixed in 4% PBS-paraformaldehyde for 1hr at 4°C. Excess PBS-paraformaldehyde was discarded and the samples incubated with 500µl rabbit anti-protein A (1:500, Sigma) for 1hr at room temperature. Rabbit anti-protein A was removed and 250µl anti-rabbit gold conjugate (10nm, Sigma) added and incubated for a further 1hr at room temperature. Samples were fixed in 2.5% PBS-glutaraldehyde overnight at 4°C and treated as described in section 2.2.6 for viewing by electron microscopy.

3.2.9 Detection of TSST-1 by ELISA

The detection of toxic shock syndrome toxin (TSST-1) was carried out based on a protocol from Toxin Technology (Sarasota, USA). Sixteen hour bacterial cultures in TSB were centrifuged at 3000rpm for 15mins decanting the culture supernatants into sterile universals, discarding the cell pellets. Immulon 4-HB plates (Dynex) were coated with 10µg/ml anti-TSST-1 (Toxin Technology) for 12-18hrs in a humid chamber at 37°C, washed thrice with PBS/0.05% Tween-20 (PBS/T) then blocked with 200µl PBS/T/bovine serum albumin (BSA) for 25mins. Plates were washed with PBS/T then incubated for 2hrs at 37°C with 100µl of purified TSST-1 (Toxin Technology) and samples diluted in PBS/T/normal rabbit serum (NRS), in triplicate. One hundred microlitres horse radish peroxidase (HRP) conjugated anti-TSST-1 IgG 1:100 (Toxin Technology) was added post washing and incubated for 1hr in a humid chamber at 37°C. A final 5 washes in PBS/T was followed by the addition of 100µl 2,2'-azino-di(3ethybenthiazoline sulphanate) solution (ABTS, Sigma) for 30mins at room temperature. Each plate was read on a Titertek® Multiscan Plus plate reader MK II (ICN) at 405nm. Experiments were carried out five times.

3.2.10 Activation of CD69 Receptor on Jurkat cells by TSST-1

The number of cells present in fresh culture were counted in a haemocytometer and adjusted to a concentration of $1x10^6$ cells/ml with fresh RPMI medium, 1ml of which was added to wells of a 24-well microtitre plate. Stock concentrations of 100 µg/ml, 10µg/ml, 1µg/ml, 0.1µg/ml and 0.01µg/ml purified TSST-1 made in RPMI and neat culture supernatants were added in 10µl volumes to the microtitre plate, one well is always used as a blank. Cells were incubated for 24hr at 37°C, in the presence of 5%CO_2. Five hundred millilitres from each reaction was transferred into two FACS tubes labelled 'IgG' and 'FITC' then centrifuged at 1500rpm for 5mins. Supernatants were discarded and the pellet resuspended in residual medium. To the 'IgG' tube 5µl of Mouse IgG-2a fluorescein isothiocyanate (FITC) conjugate was added and to the 'FITC' tube 5µl of Human CD69 FITC conjugate. The tubes were incubated for 30mins at 4°C. Cells were washed with 500µl PBS/1% foetal calf serum (FCS) at 1500rpm for 10mins, resuspended in 500µl PBS/1% FCS, then run through a fluorescence activated cell sorter (FACS) (FACScan, Becton Dickinson) and analysed by CellQuest software.

3.2.11 Stimulation of T-cell proliferation by TSST-1

Freshly isolated PBMCs from a healthy donor adjusted to $2x10^6$cell/ml were added to wells of a 96-well tissue culture plate (Greiner bio-one) in 100µl volumes. Culture supernatants were the same as those used in section 3.2.8. Stock concentrations of 100µg/ml, 10µg/ml, 1µg/ml, and 0.1µg/ml purified TSST-1, 1:10 and 1:50 dilutions of culture supernatants were in RPMI/10% FCS. Standards and samples were added in 100µl volumes to the 96-well plate in triplicate alongside an RPMI/10%FCS control. RPMI/10%FCS was used to buffer the plates to prevent them drying out. Cells were incubated for 48hrs at 37°C, in the presence of 5%CO_2. Methyl ^{3H}thymidine (Amersham) was diluted 1:20 prior to 10µl being added to each test well and re-incubated for a further 18hrs at 37°C, in the presence of 5%CO_2. Tcell proliferation was recorded as counts per minute on a radioactive counter (Packard Matrix 96 Direct Beta Counter).

3.3.12 Haemolytic Activity of alpha-haemolysin

Bacterial cultures (16hrs) in THB were centrifuged at 3000rpm for 15mins discarding the cell pellet. Fresh rabbit blood (in Alsever's solution 50% v/v stock, SAPU, Diagnostic Scotland, Edinburgh, UK) was prepared to a 2% suspension in sterile saline following three washes in saline. Sterile saline (50µl) was inoculated into wells of a 96 well V-bottomed microtitre plate (Dynex) in which 50µl of sample was mixed and doubling dilutions made across the plate. Sterile saline and 1% Tween-20 was used as negative and positive controls respectively. To each well 50µl rabbit blood was added and plates incubated for 1hr at 37°C. Plates were centrifuged at 1000xg for 10mins before being read visually. The haemolytic titre of a sample was determined as the reciprocal of the dilution causing 50% haemolysis compared with the negative control.

3.2.13 Clumping Factor and Cell Free Coagulase Assays

Overnight cultures (16hrs) prepared in BHI were centrifuged at 3000rpm for 15mins. Culture supernatants were decanted in to sterile universals and cell pellets adjusted to an optical density, $OD_{620}nm$ = 0.025 ($1x10^7$cells/ml) in sterile saline. Culture supernatants (cell free coagulase, 100µl) or cell pellets (clumping factor, 100µl) were added to 96-well microtitre plates pre-inoculated with 100µl BHI/rabbit plasma (E&O Laboratories)/sterile saline (Appendix 1) and doubling dilutions made across the plate. The plates were incubated in a shaking incubator at 37°C at 150rpm for 8hrs. The coagulase titre was determined as the reciprocal of the dilution causing coagulation after 8hrs.

3.2.14 RNA Isolation adapted from TRIzol® Reagent Manufacturer's Instructions

Four and 12hr cultures of E16 (50ml) were set up in RNase-free MHB (RF-MHB) only and in the presence of ½ MIC linezolid and synercid. Cultures were centrifuged in pre-chilled (-70°C) universals at 3000rpm for 15mins at 4°C. The culture supernatants were discarded and the cell pellets kept on ice for the remainder of the isolation process. Cell pellets were adjusted to $1x10^7$cells/ml then centrifuged at 3000rpm for 15mins at 4°C. The cell pellets were lysed in 1ml TRIzol reagent (Sigma) per $1x10^7$ cells by vortexing for 5mins. To the lysed cells 0.2ml chloroform was added, the cells vortexed for a further 15sec and incubated for 5min at room temperature. Released RNA was present within the aqueous phase of the preparation and was transferred to a fresh 1.5ml eppendorf. The RNA

preparation was precipitated with 0.5ml isopropyl alcohol overnight at 4°C. The precipitate was centrifuged for 10mins at 10000xg and the supernatant discarded. Washing of the RNA was carried out with 1ml 75% ethanol, vortexed then centrifuged for 5mins at 7000xg. The RNA pellet was air dried for 5-10min. Once dry the RNA preparation was dissolved in RNase-free water an incubated for 10min at 55-60°C. RNase-free-DNase (Roche, 10units/µl) in 500µl dH_2O plus 1xDNase buffer (Appendix 1) was added to the RNA and incubated for 1hr at 37°C. RNA was precipitated overnight in 0.5ml isopropyl alcohol at 4°C. Samples were stored at –20°C until required.

3.2.15 RNA Isolation using a Qiagen RNeasy (Mini) Isolation kit

RNA isolation was carried out using a Qiagen RNA isolation kit (Qiagen) according to the manufacturer's instructions. In brief, 4hr and 12hr 50ml cultures of E16 were set up in RNase-free MHB (RF-MHB) only and in the presence of ½ MIC linezolid and synercid. Cultures were centrifuged in pre-chilled (-70°C) universals at 3000rpm for 15mins at 4°C. The supernatants were discarded and the cell pellets kept on ice for the remainder of the isolation process. Cultures were adjusted to OD_{620}nm = 0.115 in 50ml RF-MHB ($1x10^8$cells/ml) then centrifuged at 3000rpm for 15mins at 4°C. The cell pellet was resuspended in 100µl lysozyme/TE buffer, vortexed and incubated for 10mins at room temperature after which time 350µl RLT buffer was added. Cells were vigorously vortexed and centrifuged at 11000rpm for 2mins, then the cell pellets were discarded. To the supernatant (approx 450µl), 250µl 100% ethanol was added and mixed thoroughly before the sample was transferred (approx 700µl) to an RNeasy mini column in a 2ml collection tube. Samples were spun at 11000rpm for 15sec and the flow through discarded. RW1 buffer (700µl) was added to the column, spun for a further 15sec at 11000rpm, and the flow through and collection tube discarded. The column was transferred to a new 2ml collection tube and 500µl RPE buffer was twice added to the column, spun for 15sec at 11000rpm and the flow through discarded each time. The column was transferred to a 1.5ml eppendorf tube where 50µl RNase-free water was added directly on to the membrane and spun for 1min at 11000rpm. To remove any DNA present in the samples 500µl of RF-H_2O, 50µl DNase buffer, and 3µl RNase-free-Dnase (10000Units stock, Appendix 1) were added and incubated for 1hr at 37°C. Isopropylalcohol (500µl) was added to the samples and left to precipitate overnight at –20°C. Samples were spun for

10min at >10000rpm and the pellet resuspended in 50µl of RF-H_2O. RNA samples were quantified as detailed below then stored at -20°C until required.

3.3.16 RNA Quantification

Sample RNA was quantified by spectrophotometry. Dilutions (1:20) of the samples were made in RF-H_2O and transferred in to a Hellma quartz cuvette (Type 105.202-QS, Germany). Readings were taken at wavelengths of 260nm and 280nm and the ratio between the readings used to estimate the purity of nucleic acid in the sample ($^{260nm}/_{280nm}$). The following calculation was used to quantity the RNA in 50µl of sample where 40µg/ml corresponds to the quantity of single stranded DNA and RNA at an OD of 1:

40µg/ml x 260nm reading x dilution x sample volume

3.3.17 LightCycler Primer Design

New primers (MWG) for use in the LightCycler were designed using a LightCycler probe design software version 1.0 (Roche). The gene sequence for each gene was inserted into the program from Genbank: protein A accession number J01786, TSST-1 accession number J02615, 16s RNA accession number X80725, and the program set to generate primers 16 bases in length (Appendix 2).

3.3.18 LightCycler DNA Standards

DNA standards were prepared using the method described in 3.2.5. Several PCR reactions of E16 with LightCycler primers for 16s RNA, TSST-1 (*tst*) and protein A (*spa*) were performed and pooled together.

3.3.19 Real Time PCR

Amplification of RNA was carried out using a LightCycler (Roche) and an RNA amplification kit SYBR green1 (Roche) according to the manufacturer's instructions. All reagents were kept on ice whilst the reactions were being set up. To each reaction capillary tube the following reagents were added; PCR grade H_2O (10.2µl), LightCycler-

RT-PCR reaction mix SYBR Green 1 (4µl), Resolution solution (3µl), $MgCl_2$ (6mM stock solution, 2.4µl), LightCycler-RT-PCR enzyme mix (0.4µl) plus 1µl each of the appropriate forward and reverse primer and 3µl of the appropriate RNA sample or DNA standard (Table 14). The capillary tubes were capped, centrifuged for 5sec at 3000rpm then placed into the LightCycler carrousel. The cycling conditions are comprised of five programs that were followed exactly as stated in the LightCycler-RNA Amplification Kit SYBR Green 1 instruction manual:

1 Reverse Transcription (RT) of template RNA
2 Denaturation of cDNA/RNA hybrid
3 Amplification of cDNA
4 Melting curve analysis for product identification
5 Cooling the rotor and thermal chamber

In brief, RT was carried out for 10mins at 55°C followed by the following amplification conditions: denaturation for one cycle at 95°C for 30sec, 45 cycles at 95°C for 0sec, (temperature transition 20°C/sec), 55°C for 10sec (temperature transition 20°C/sec), 72°C for 13sec (temperature transition 2°C/sec) and fluorescence acquisition at 72°C in single mode. Melting curve analysis was performed during one cycle of 95°C for 0sec, (temperature transition 20°C/sec), 65°C for 10sec (temperature transition 20°C/sec), 95°C for 0sec (temperature transition 0.1°C/sec) with step-wise fluorescence acquisition. Sequence-specific standard curves were generated by using 10-fold dilutions of specific RNA standards (1:10, 1:00, 1:1000). The quantity of mRNA transcripts from each sample were determined using the LightCycler software version 3.2 (Roche).

Reaction	Sample plus primer
1	E16 MHB 4hr culture + *spa* primers
2	E16 ½ linezolid 4hr culture + *spa* primers
3	E16 ½ synercid 4hr culture + *spa* primers
4	Blank (no RNA) + *spa* primers
5	E16 MHB 4hr culture + 16s RNA primers
6	E16 ½ linezolid 4hr culture + 16s RNA primers
7	E16 ½ synercid 4hr culture + 16s RNA primers
8	Blank (no RNA) + 16s RNA primers
9	E16 MHB 12hr culture + *tst* primers
10	E16 ½ linezolid 12hr culture + *tst* primers
11	E16 ½ synercid 12hr culture + *tst* primers
12	Blank (no RNA) + *tst* primers
13	E16 MHB 12hr culture + 16s RNA primers
14	E16 ½ linezolid 12hr culture + 16s RNA primers
15	E16 ½ synercid 12hr culture + 16s RNA primers
16	*spa* DNA 1:10 + *spa* primers
17	*spa* DNA 1:100 + *spa* primers
18	*spa* DNA 1:1000 + *spa* primers
19	*tst* DNA 1:10 + *tst* primers
20	*tst* DNA 1:100 + *tst* primers
21	*tst* DNA 1:1000 + *tst* primers
22	16s RNA DNA 1:10 + 16s RNA primers
23	16s RNA DNA 1:100 + 16s RNA primers
24	16s RNA DNA 1:1000 + 16s RNA primers

Table 14. LightCycler reactions

3.3 RESULTS

3.3.1 Determination of the MIC of standard strains

The MIC (μg/ml) for the control strains used in this section are shown in Table 15.

Strain	cefpirome	daptomycin	linezolid	Synercid
Wood 46	0.39	0.20	0.78	0.08
NCTC 11963	0.39	0.13	1.56	0.19
NCTC 6571	0.39	0.20	1.56	0.06
119993	1	0.20	0.78	0.10

Table 15. Minimum inhibitory concentration (μg/ml) of standard strains to various antibiotics

3.3.2 Detection of Virulence Genes

Each strain was subjected to PCR analysis to determine their genetic complement. In each case all the test strains plus a positive control were tested for a single toxin gene at a time. The distribution of virulence genes in our strains is displayed in Table 16 where N = no gene detected and P = positive gene detection. This distribution was used to determine which strains would be used in future experiments.

As expected, the genetic complement varied between strains but all carried the genes for protein A and α-haemolysin. The clonal strains Mu3 and Mu50 identically carried genes comprising 5 enterotoxins (including enterotoxin-like protein-1 (SET-1)) and TSST-1. Strains 3700w and 3759 are also identical but only carried enterotoxin A and SET-1, differing from EMRSA 16 by one gene, EMRSA 16 has TSST-1 in place of SET-1. VISA strains 5827 and 5836 only carry the genes for enterotoxins SEG, SEI and SET-1.

From these findings we chose to investigate the expression of TSST-1, α-haemolysin, protein A, and coagulase. The presence of the coagulase gene was not measured as all strains demonstrated a positive slide test.

Toxin \ Strain		Cowan	E 16	3700w	3759v	5827	5836	Mu3	Mu50
sea	120bp	N	P	P	P	N	N	P	P
seb	163bp	N	N	N	N	N	N	N	N
sec	271bp	N	N	N	N	N	N	P	P
sed	319bp	N	N	N	N	N	P	N	N
see	178bp	N	N	N	N	N	N	N	N
seg	327bp	P	P	N	N	P	P	P	P
sei	465bp	N	P	N	N	P	P	P	P
sej	142bp	N	N	N	N	N	P	N	N
set1	677bp	P	N	P	P	P	P	P	P
tst	445bp	N	P	N	N	N	N	P	P
eta	741bp	N	N	N	N	N	N	N	N
eta	629bp	N	N	N	N	N	N	N	N
spa	variable	P	P	P	P	P	P	P	P
hly	535bp	P	P	P	P	P	P	P	P
nuc	280bp	P	P	P	P	P	P	P	P
16S rRNA	479bp	P	P	P	P	P	P	P	P

Table 16. Distribution of virulence genes

3.3.3 Expression of Protein A

An ELISA-based standard curve of different purified protein A concentrations against OD_{405}nm readings was constructed (Figure 24) and used to determine the concentration of cell bound protein A ($1x10^7$cells/ml) and that which is present free in the culture supernatant per ml.

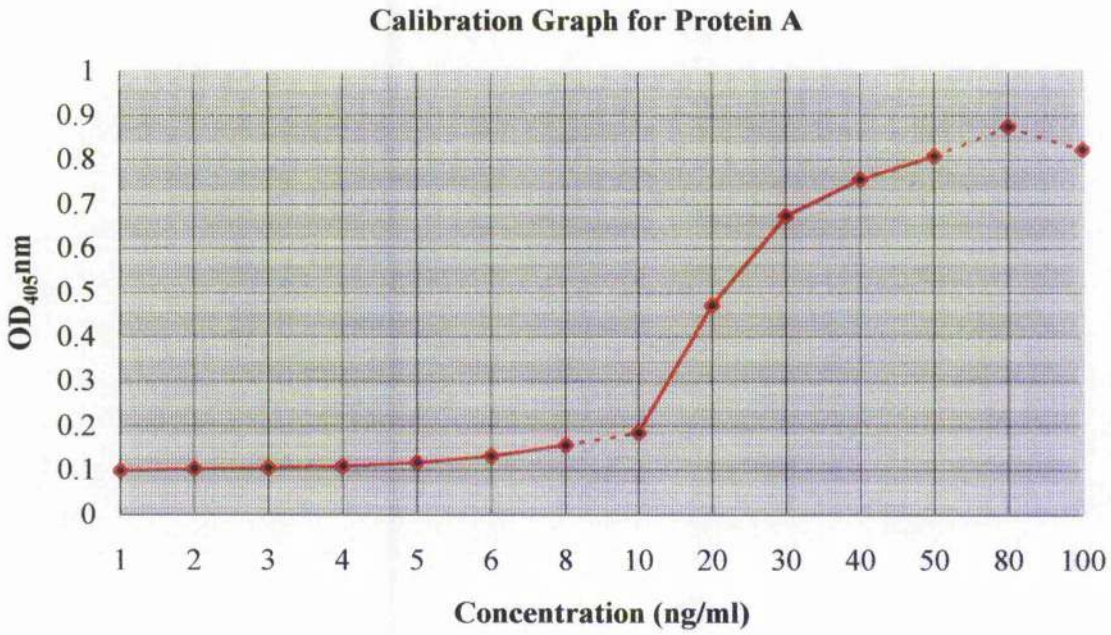


Figure 24. Purified protein A concentration and optical density (OD_{405}nm) calibration graph

For each strain five separate cultures were prepared and used in five separate assays. Within each assay a culture was tested in triplicate wells to obtain a mean reading. The concentration of protein A detected in each of these assays was then calculated from the mean OD_{405}nm readings using the calibration graph above. Statistical analysis was in the form of a Student's t-test. Mean values and standard deviation are given where n = 5.

Strains of VISA vary greatly in their ability to produce protein A either in a surface bound or free form even though all carry the *spa* gene. The protein A negative mutant DU5723 was included for assay validity and was negative for both surface bound and free protein A. Figure 25 shows the production of surface bound protein A per $1x10^7$cells/ml for strains *S.aureus* Cowan, E16 and VISA 5827. It should be noted that the results for strains 3700w, 3759v, 5836, Mu3 and Mu50 are not shown as levels were below the detection limit (10ng/ml) of the assay but do not necessarily indicate no expression.

Surface bound protein A: i) ELISA

As expected *S.aureus* Cowan, a high protein A producer, expressed the greatest quantity of surface bound protein A at 20.5ng/1x10^7cells/ml (Figure 25). In addition, only EMRSA-16 (E16) and 5827 produced detectable surface bound protein A but at lower levels than that of Cowan, strain 5827 was significantly less (pvalue ≤0.05). None of the antibiotics tested had any significant affect on strain 5827 expression of this protein. *S.aureus* Cowan and E16 were affected in different ways by some of the antibiotics. Cefpirome was found to significantly increase expression of surface protein A in Cowan but not in E16 whereas synercid reduced levels in both strains to undetectable levels, as did linezolid in E16 alone. Daptomycin had no significant effect on any strain. It is interesting to note that six out of seven VISA strains produced no surface bound protein A and have been omitted from Figure 25. The lack of protein A expression has been described previously for strains of MRSA by other research groups. To confirm that protein A is not expressed at the cell surface and not just undetectable at the concentration of cells tested here the strains were incubated with an anti-protein A gold label.

ii) Immunogold-labelling

Several of the strains tested above, including Wood 46 a known low protein A producer, were examined for their ability to display surface bound protein A by immunogold-labelling. Figure 26, as expected, shows *S.aureus* Cowan displaying a uniform distribution of protein A around its surface whereas Wood 46 produced only a single positive reaction in comparison. Strains 3759v, 5836, and Mu50 all gave negative results that correlate with those achieved by ELISA. Strain E16 gave a strong positive reaction by immunogold-labelling similar to that of *S.aureus* Cowan but was quantified by ELISA to be slightly less than that of Cowan. Mu3 was negative by both ELISA and immunogold-labelling therefore is a true non-producer of surface bound protein A.

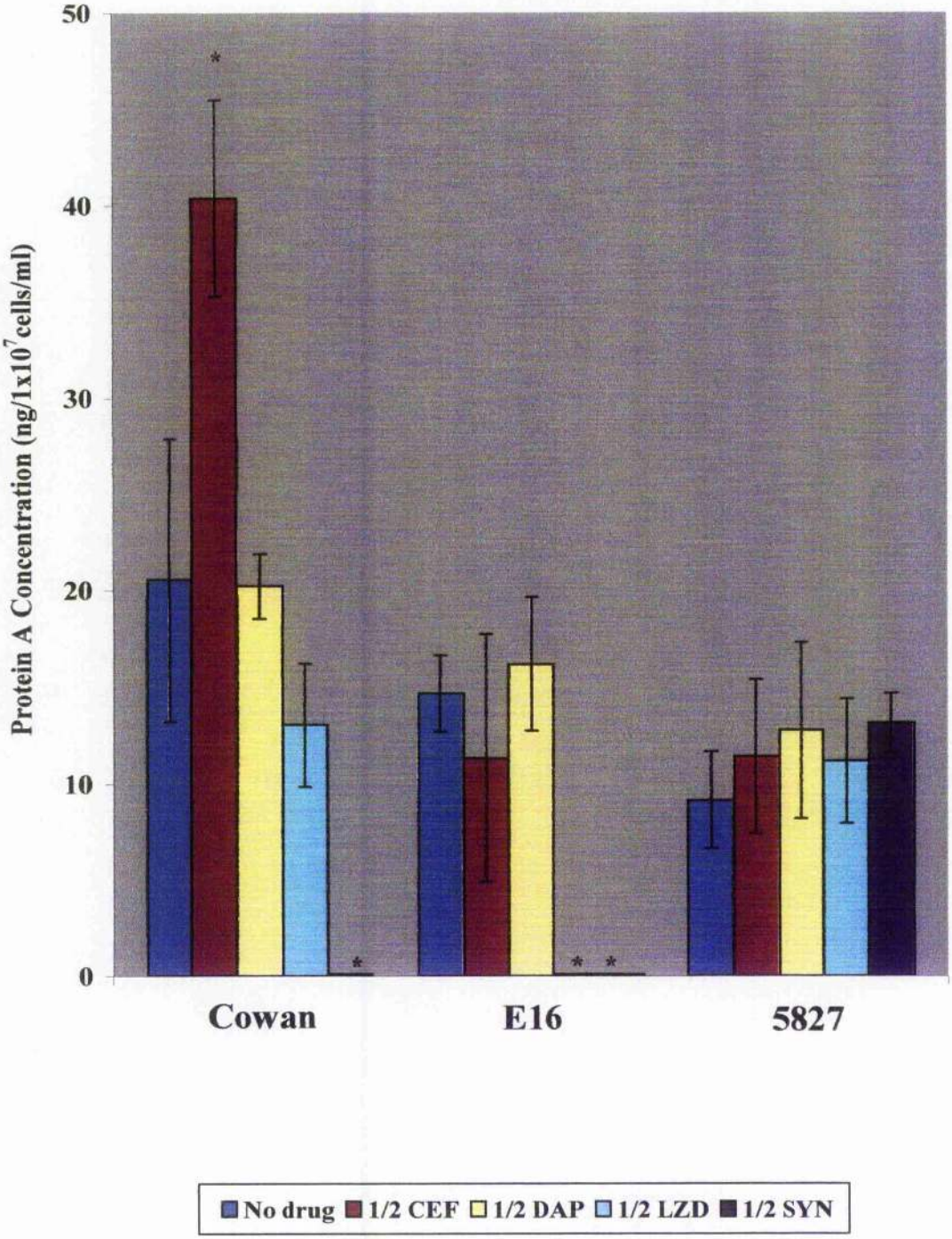


Figure 25. Expression of surface bound protein A by strains of *S.aureus* in the absence and presence of ½ MIC antibiotics. Readings represent mean +/- standard deviation error bars. * = pvalue<0.05.

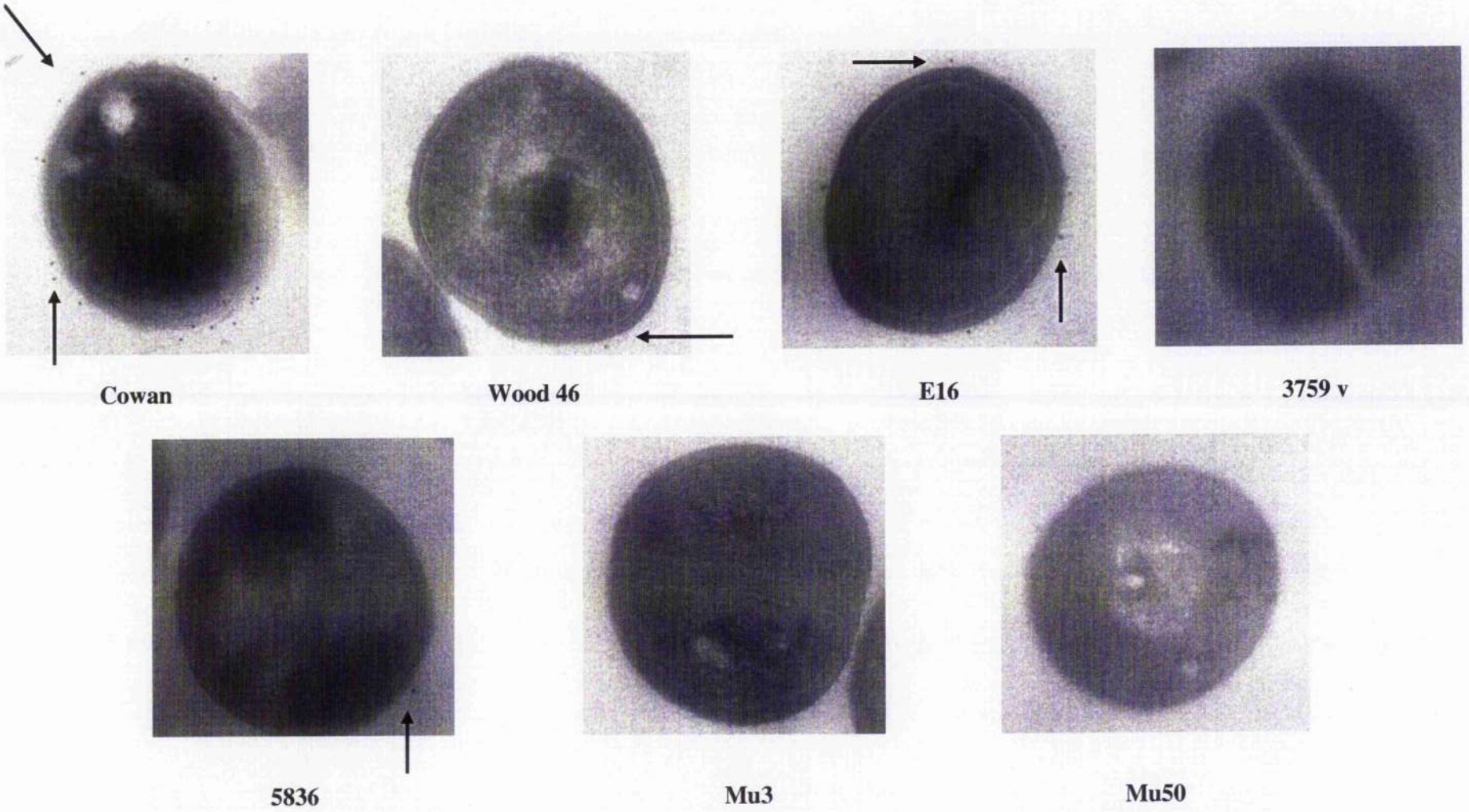


Figure 26. Immunogold-labelling of protein A on the surface of strains of *S.aureus*. Particles of protein A can be visualised by black dots denoted by arrows (↑) on the surface of the cell.

Free protein A – Preliminary Experiment

To establish the limits at which various drugs had no effect on extracellular protein A, the protein was quantified by ELISA in culture supernatants of *S.aureus* Cowan grown in MHB in the presence and absence of ½, ¼, $^1/_8$, $^1/_{16}$ and $^1/_{32}$ MIC linezolid and synercid (Figure 27 and 28). Each graph represents five individual experiments (1-5) with any significant difference indicated (* – pvalue ≤0.05). It was shown that linezolid is effective at ½ and ¼ MIC in 4 out of 5 experiments and that synercid is effective at ½ MIC in 5 out of 5 experiments. In some instances over expression of protein A was recorded for $^1/_{16}$ and $^1/_{32}$ MIC linezolid and synercid. This is possibly due to loose association of surface bound protein A with the cell wall and its release in to culture supernatants, and is variable depending upon factors affecting the culture conditions. From these findings the decision was made to examine each drug at ½ MIC only for the effect on protein A expression.

Free protein A

Expression of free protein A in MHB is far greater than that produced by any of the strains in a cell bound form. The presence of small amounts of extracellular protein A in strains that did not produce detectable levels of surface bound protein A was observed. All of the strains tested can be divided into three levels of protein expression. *S.aureus* Cowan, E16 and VISA 5827 expressed similar levels of >2000ng/ml protein A in culture media alone (Figure 29); VISA strains 3759v, Mu3 and Mu50 produced between 30 and 60ng/ml protein (Figure 30) whereas 3700w and 5836 failed to produce detectable levels of protein A (<10ng/ml) (not shown).

The protein synthesis inhibitors linezolid and synercid both reduced free protein A expression. Linezolid dramatically reduced the quantity of free protein in strains Cowan, E16, 5827 and Mu3 (Pvalue ≤0.05). Strains 3759v and Mu50 also displayed reduced expression but to non-significant degrees. Synercid gave similar results to linezolid with the exception that strains 3759v and 5827 showed significant and insignificant reductions in protein A expression respectively. In contrast, daptomycin was found to increase protein A expression albeit only significantly in strain 5827. The cell wall inhibitor cefpirome both increased and decreased the quantity of protein A detected. The most significant increase was observed with *S.aureus* Cowan. This may possibly be due to the partial disruption of the cell wall releasing surface bound protein A. Other authors have

discovered that some strains of MRSA express free but not surface bound protein A as was clearly illustrated with strains 3759v, Mu3 and Mu50.[107,367] As these strains displayed <10ng/ml of surface bound an increase in expression would not have been expected and indeed levels of free protein A were actually reduced in strains 3759v and Mu3 exposed to ½ MIC cefpirome (Pvalue ≤0.05).

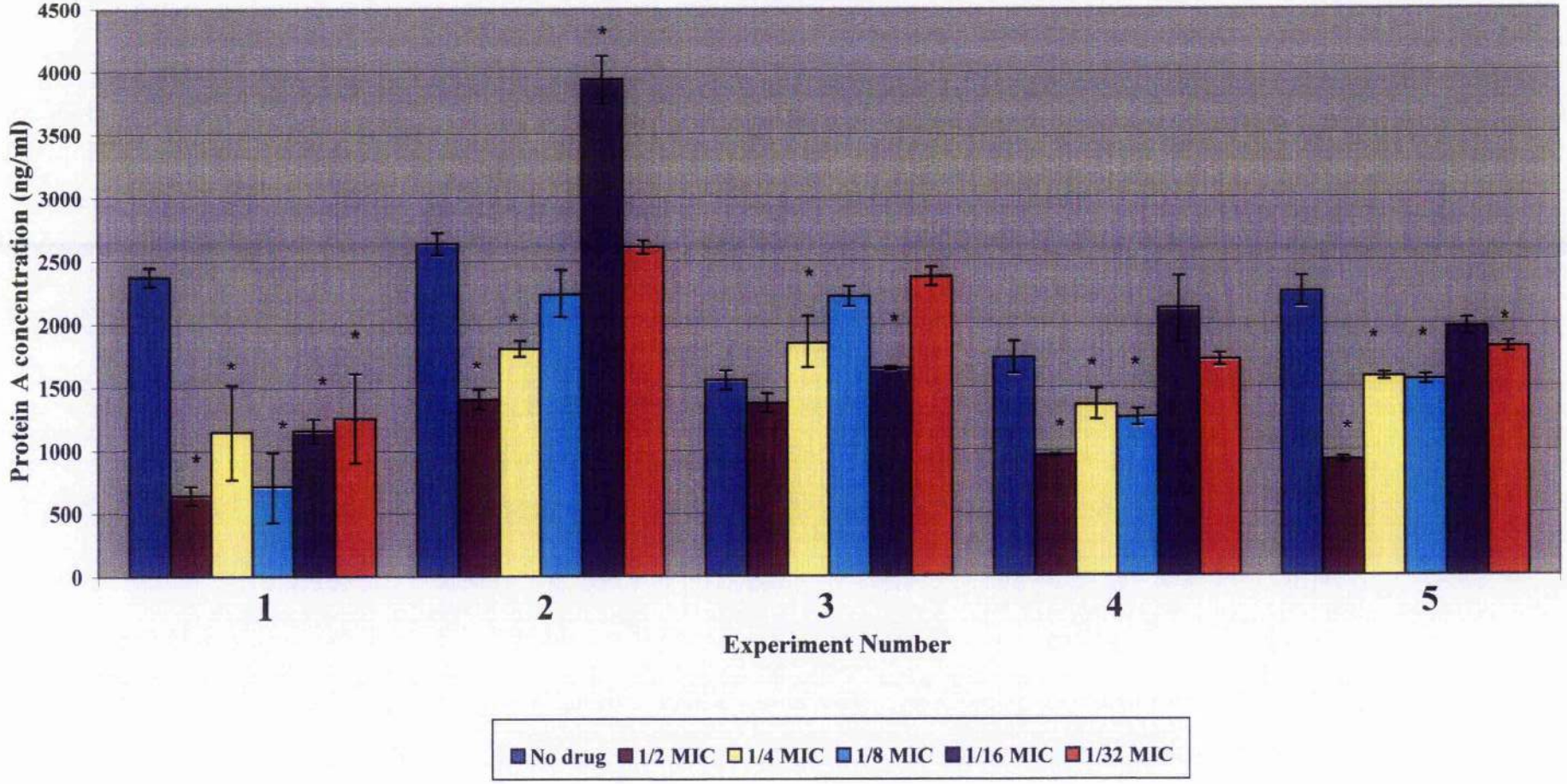


Figure 27. Effective sub-inhibitory concentrations of linezolid against cell-free protein A expression by S.aureus Cowan

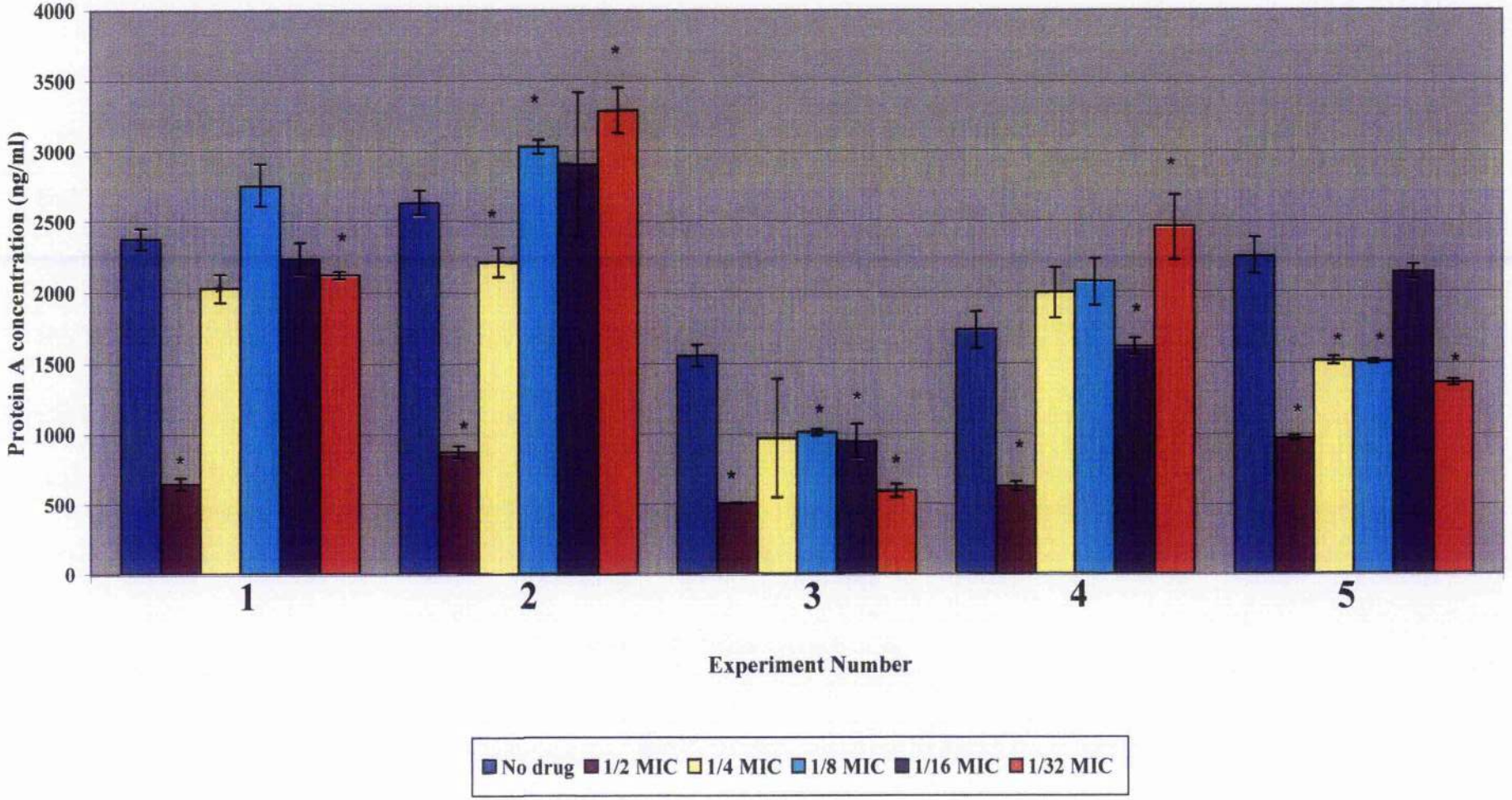


Figure 28. Effective sub-inhibitory concentrations of synercid against cell-free protein A expression by S.aureus Cowan

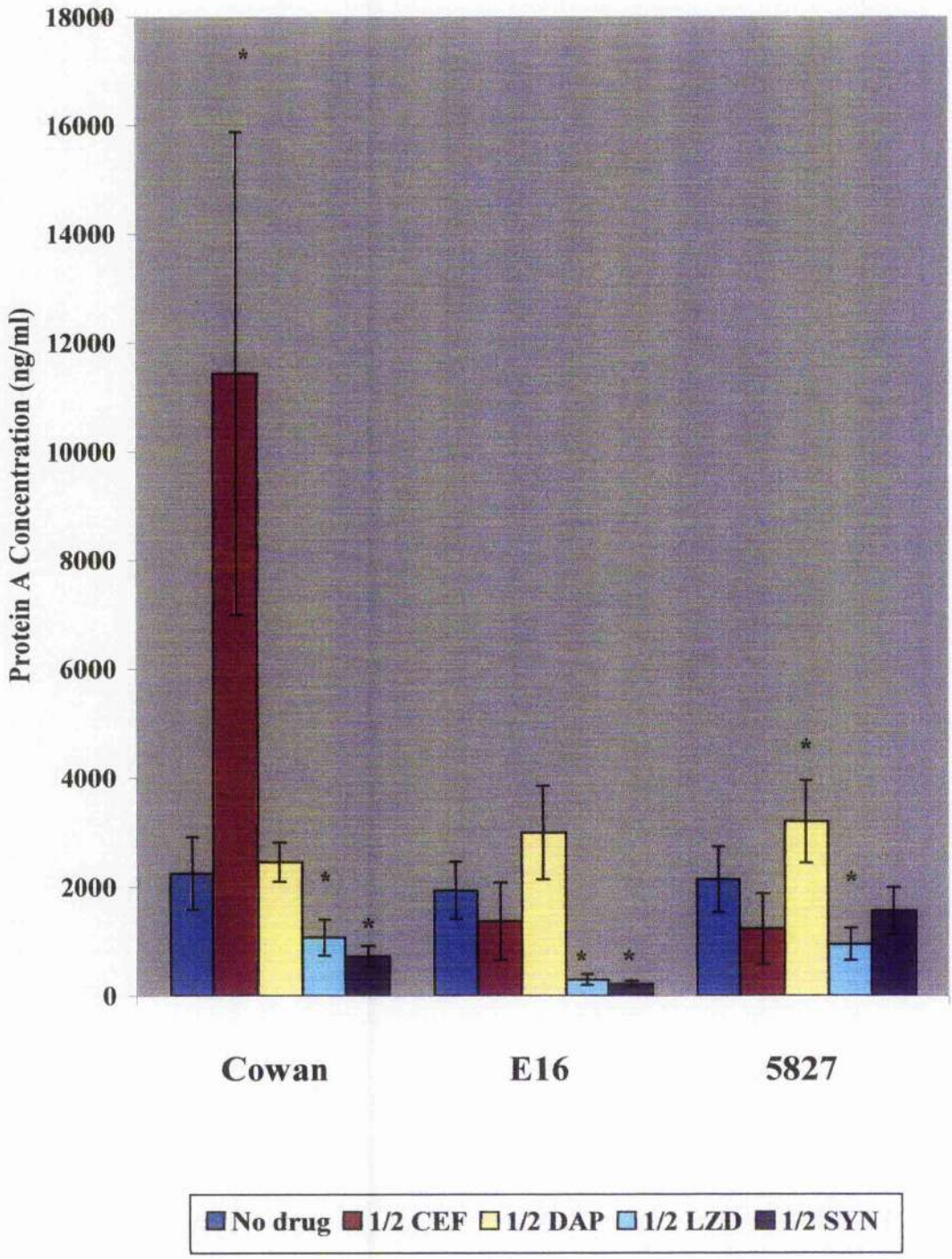


Figure 29. Expression of cell-free protein A by strains of *S.aureus* in the absence and presence of ½ MIC antibiotics. Readings represent mean +/- standard deviation error bars, * = pvalue<0.05

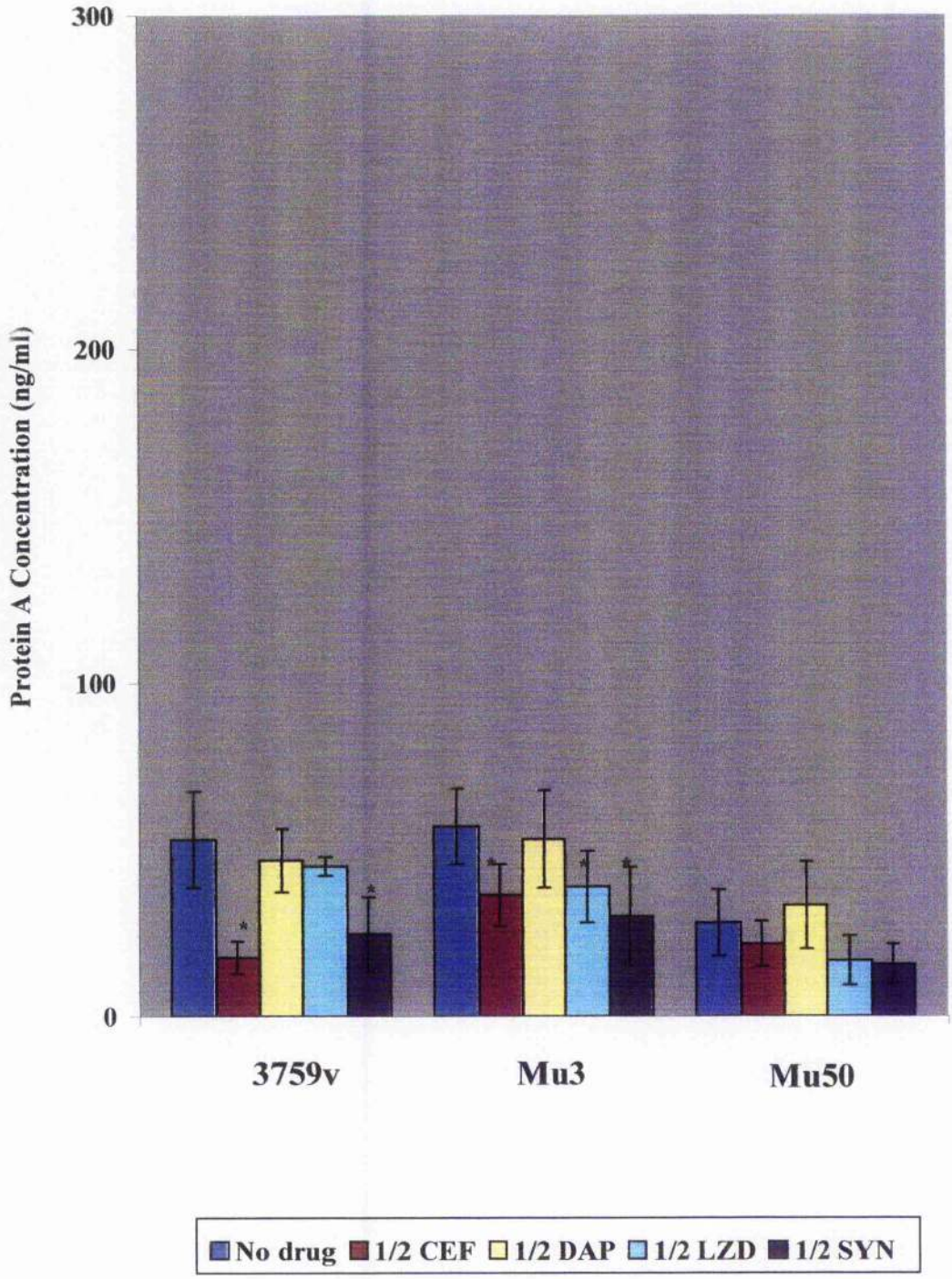


Figure 30. Expression of extracellular protein A by strains of *S.aureus* in the absence and presence of ½ MIC antibiotics. Readings represent mean +/- standard deviation error bars, * = pvalue<0.05

3.3.4 Production of TSST-1

An ELISA-based standard curve of purified TSST-1 concentration against OD_{405}nm readings was constructed (Figure 31) and used to determine the concentration of TSST-1 in culture supernatants. Statistical analysis was in the form of a Student's t-test. Mean values and standard deviation are given where n = 5.

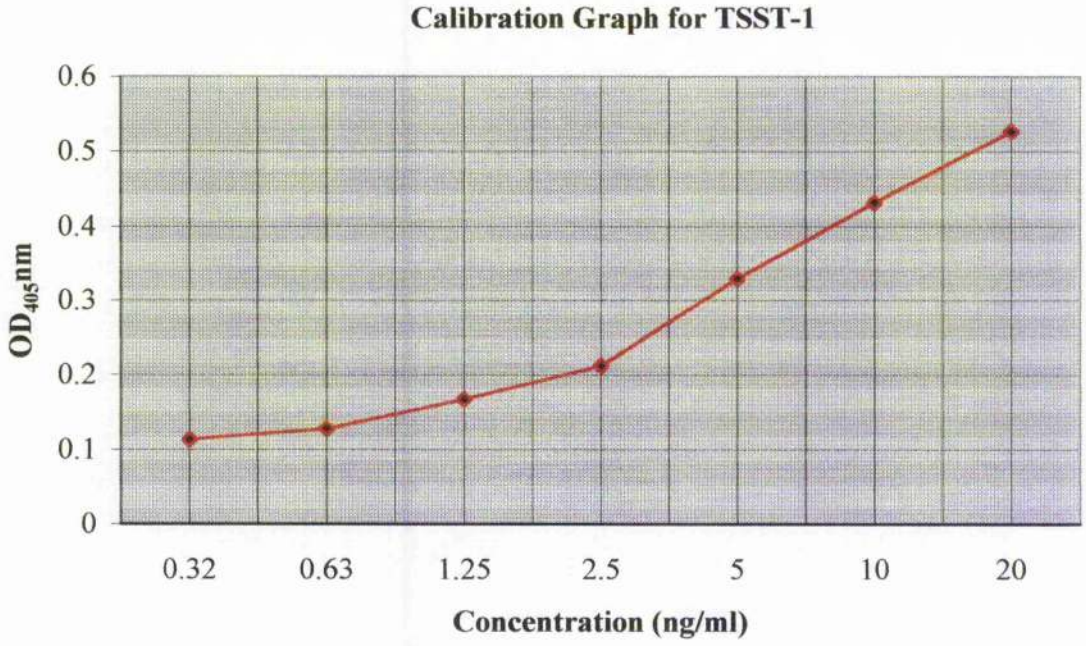


Figure 31. Purified TSST-1 concentration and optical density (OD_{405}nm) calibration graph

From Table 16 the three strains carrying the *tst* gene were chosen, as was *S.aureus* Cowan, a TSST-1 negative strain and NCTC 11963, a TSST-1 positive strain. *S.aureus* Cowan was included for assay validity and was negative in all assays. As with the protein A ELISA there is a detection limit, in this case of 0.32ng/ml. Again levels below 0.32 are depicted as zero but does not necessarily mean no expression.

Figure 32 illustrates the varying extent of TSST-1 expression. By far, strains Mu3, Mu50 and NCTC 11963 (positive control) produced greater quantities of toxin compared to strain E16. As Mu3 and Mu50 are clonal similar degrees of expression and its response to antimicrobial agents might be expected. This was true for the levels of toxin detected in TSB culture medium only and in the presence of ½ MIC linezolid and synercid which significantly reduced toxin expression (pvalue ≤ 0.05). Synercid also reduced expression

of TSST-1 in NCTC 11963 strain but had no effect on E16 whereas linezolid had the opposite effect of increasing expression in E16 but had no effect on NCTC 11963. Cefpirome both induced and repressed TSST-1 expression in Mu3, E16 and Mu50, NCTC 11963 respectively. Daptomycin had no significant effect on any of the strains tested.

In order to verify that the toxin measured by ELISA was truly TSST-1 as measured in standard biological assays Tcell stimulation and Tcell proliferation assays were carried out with both crude and purified toxin preparations.

3.3.5 Activation of Tcell Receptor CD69

Jurkat cells are a Tcell tissue culture cell line with similar characteristics to those of Tcells. Numerous attempts to activate CD69 receptor failed with both our own culture supernatants containing TSST-1 and purified TSST-1 even though stimulation was observed with the phytohaemaggultanin / phorbol myristate acetate (PHA/PMA) positive control (Figure 33). The same assay using freshly isolated PBMCs from healthy donors was also tried, but this also proved to be an inadequate assay. The cells were being killed by components within the culture supernatants. As the TSST-1 has not been purified all of the proteins and enzymes expressed by our strains under antibiotic stress are still present and clearly have a detrimental effect on human Tcells (Figure 34).

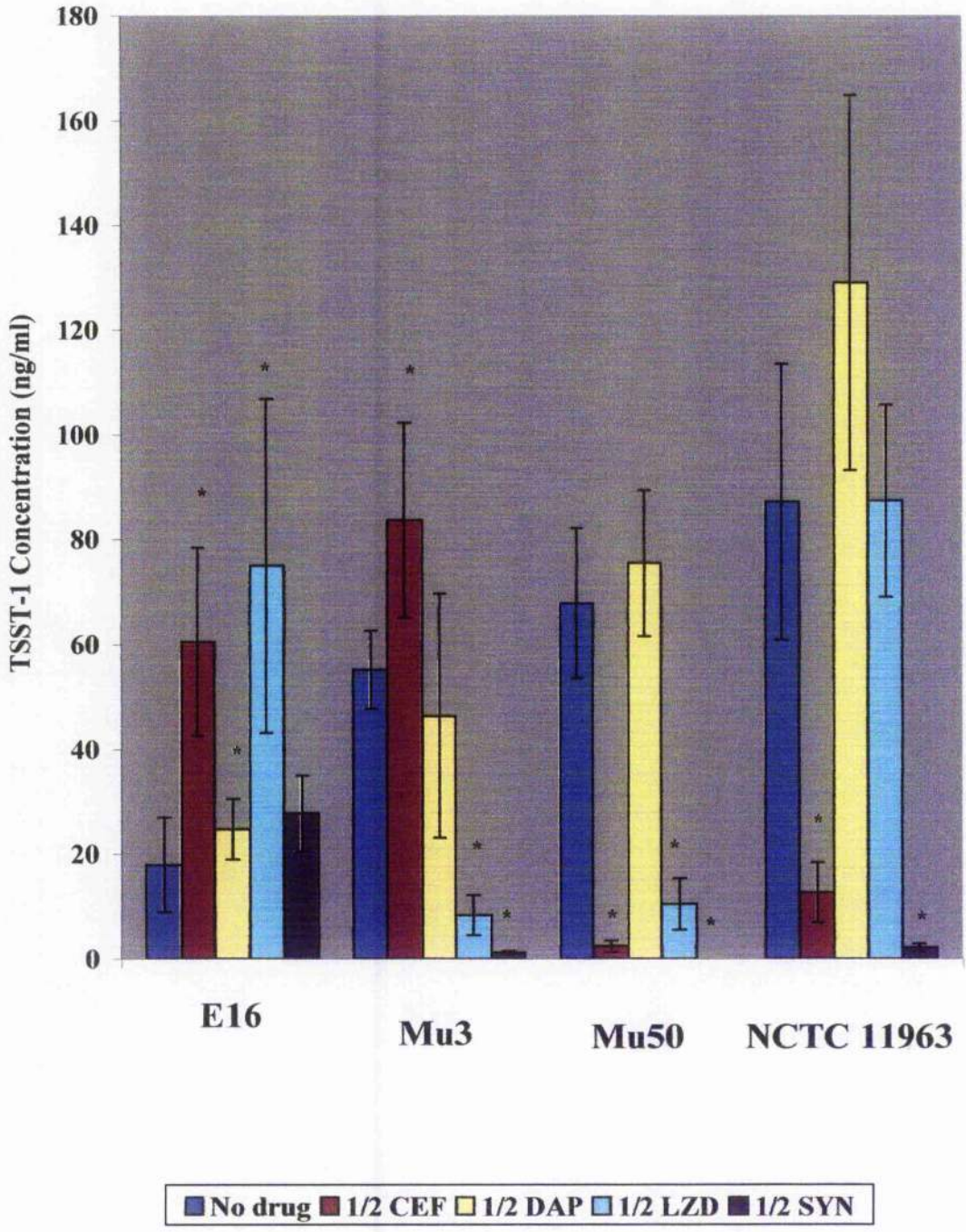


Figure 32. Expression of TSST-1 by strains of *S.aureus* in the absence and presence of ½ MIC antibiotics. Readings represent mean +/- standard deviation error bars, * = pvalue<0.05

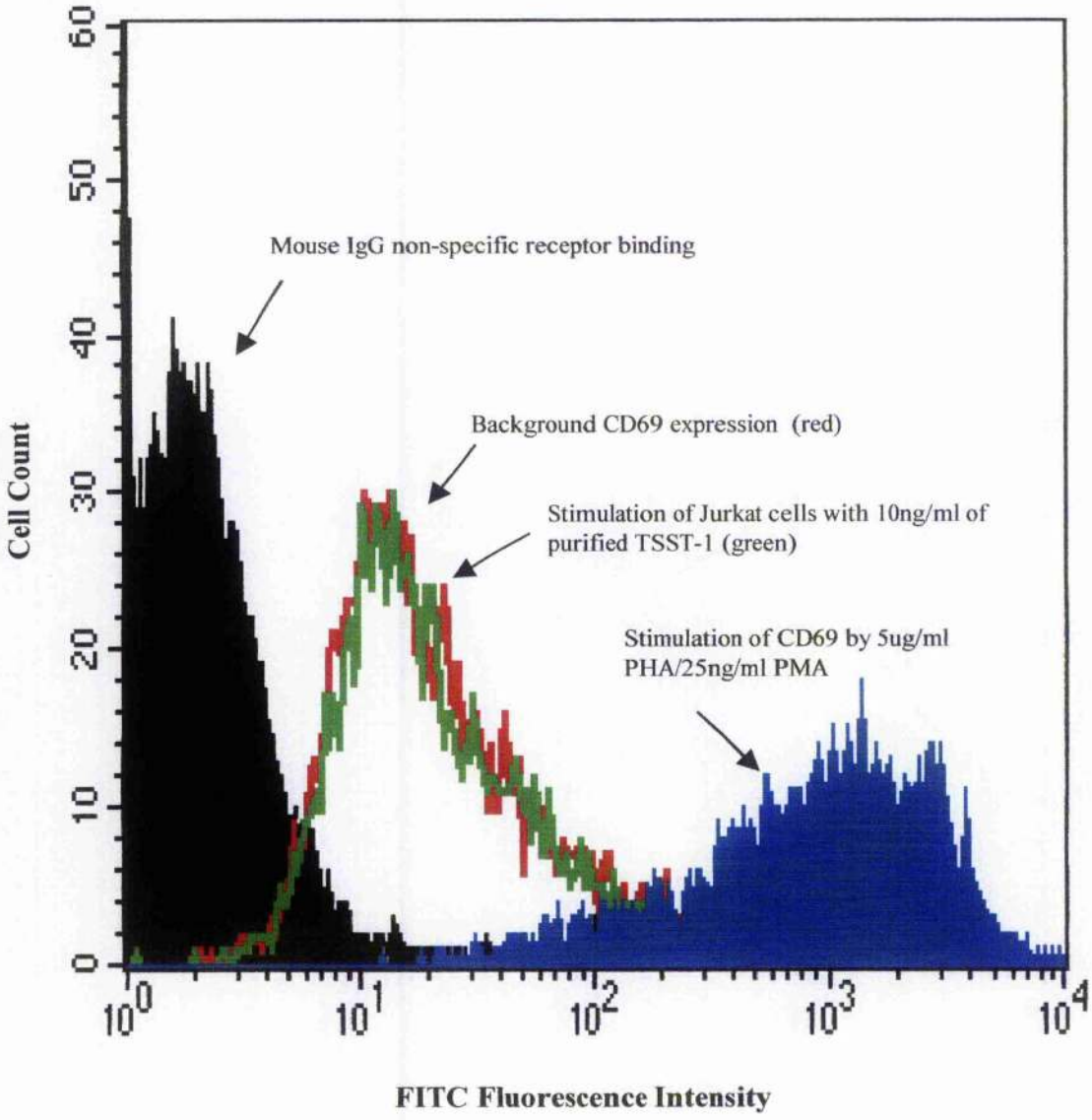


Figure 33. Expression of CD69 receptor by Jurkat cells following stimulation by purified TSST-1 and PHA/PMA.

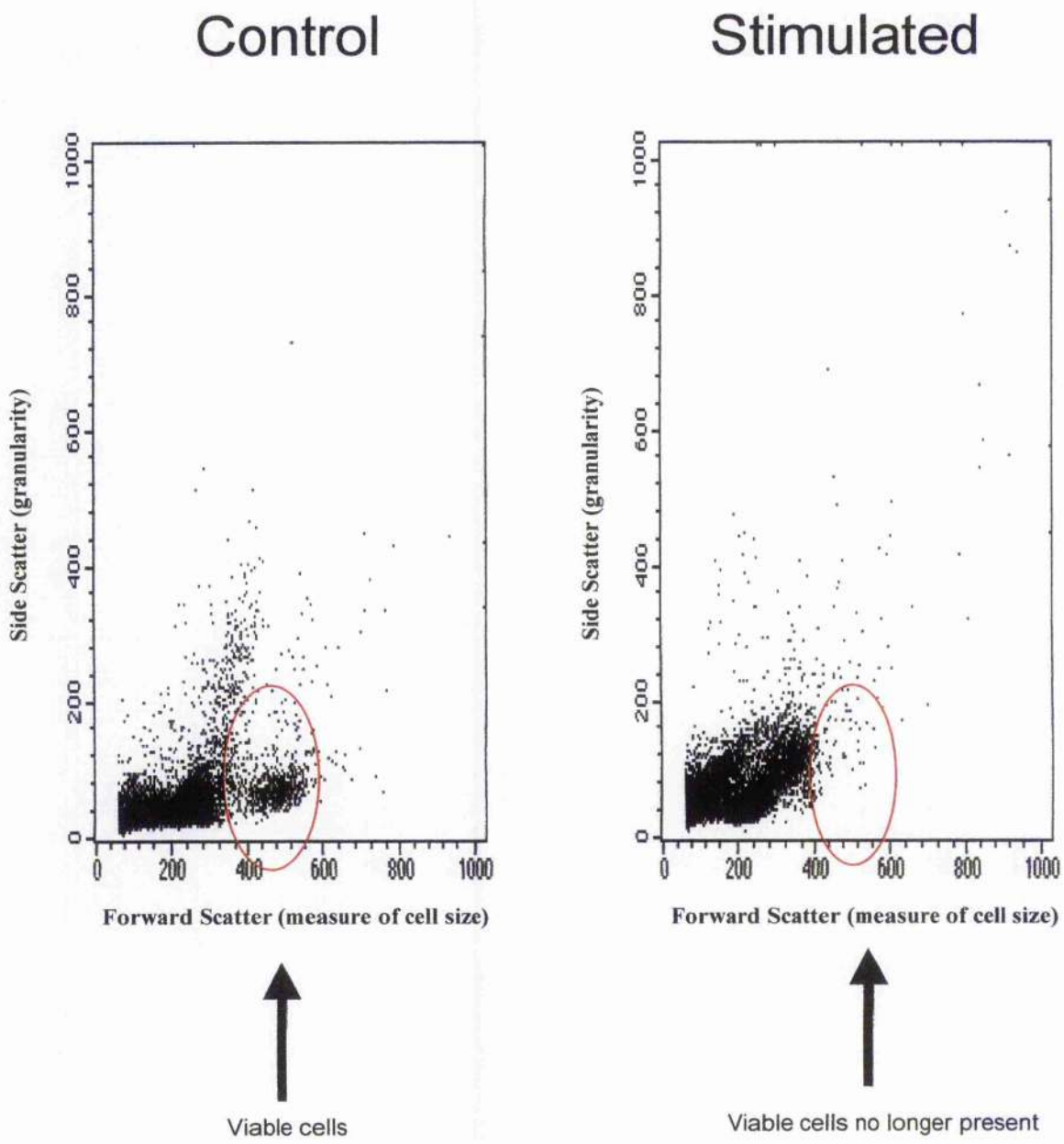


Figure 34. Expression of CD69 on PBMC in RPMI culture medium (control) and after exposure to the culture supernatants of strain NCTC 11963 exposed to ½ MIC daptomycin (stimulated). Viable cells from the population are not detected following stimulation with culture supernatants.

3.3.6 Tcell Proliferation Induced by Culture Supernatants

With the above results in mind the proliferation of human Tcells was examined using the same culture supernatants tested in the ELISA. Each culture was tested in triplicate and the mean count of methyl ^{3H}thymidine incorporation calculated and compared to the Tcells plus medium control (Figure 35, Table 17).

One in ten dilutions of E16, Mu3 and Mu50 culture supernatants all stimulated Tcell proliferation in excess of typical Tcell proliferation. The degree of proliferation does not appear to be concentration dependent as indicated by the TSST-1 standards. The reduced level of proliferation seen using 100μg/ml of purified TSST-1 may be due to killing of the PMBCs through apoptosis. With regard to the reduced levels of TSST-1 produced by strains upon exposure to ½ MIC synercid as detected by ELISA, there is no apparent relationship between the level of toxin produced and the degree of Tcell proliferation observed. As we are only looking at the production of one individual toxin the reduction in TSST-1 production by ½ MIC synercid may not be true of all superantigens some of which may be being stimulated. The high level of proliferation observed with the bacterial supernatants is greater than that caused by 0.01μg/ml to 100μg/ml of purified TSST-1 indicating that there are other factors present within the culture supernatants that may exert specific or non-specific Tcell stimulation, such as the enterotoxins. This would be a fitting assumption especially as Mu3 and Mu50 have the capability to express an additional four enterotoxins. Strain E16 only carries SEA in addition to TSST-1 which would agree with the reduced levels of Tcell proliferation in comparison with Mu3 and Mu50. Thus it can be established that the levels of TSST-1 detected with antibiotic treatment is expressed in a biologically active form capable of exerting superantigenic properties upon Tcells.

Standard	methyl ^{3H}thymidine incorporation (cpm)
TSB Broth	7141
PBMC	7324
0.1μg/ml TSST-1	27540
1μg/ml TSST-1	29511
10μg/ml TSST-1	33898
100μg/ml TSST-1	16512

Table 17. Standards included in an assay of PBMC proliferation

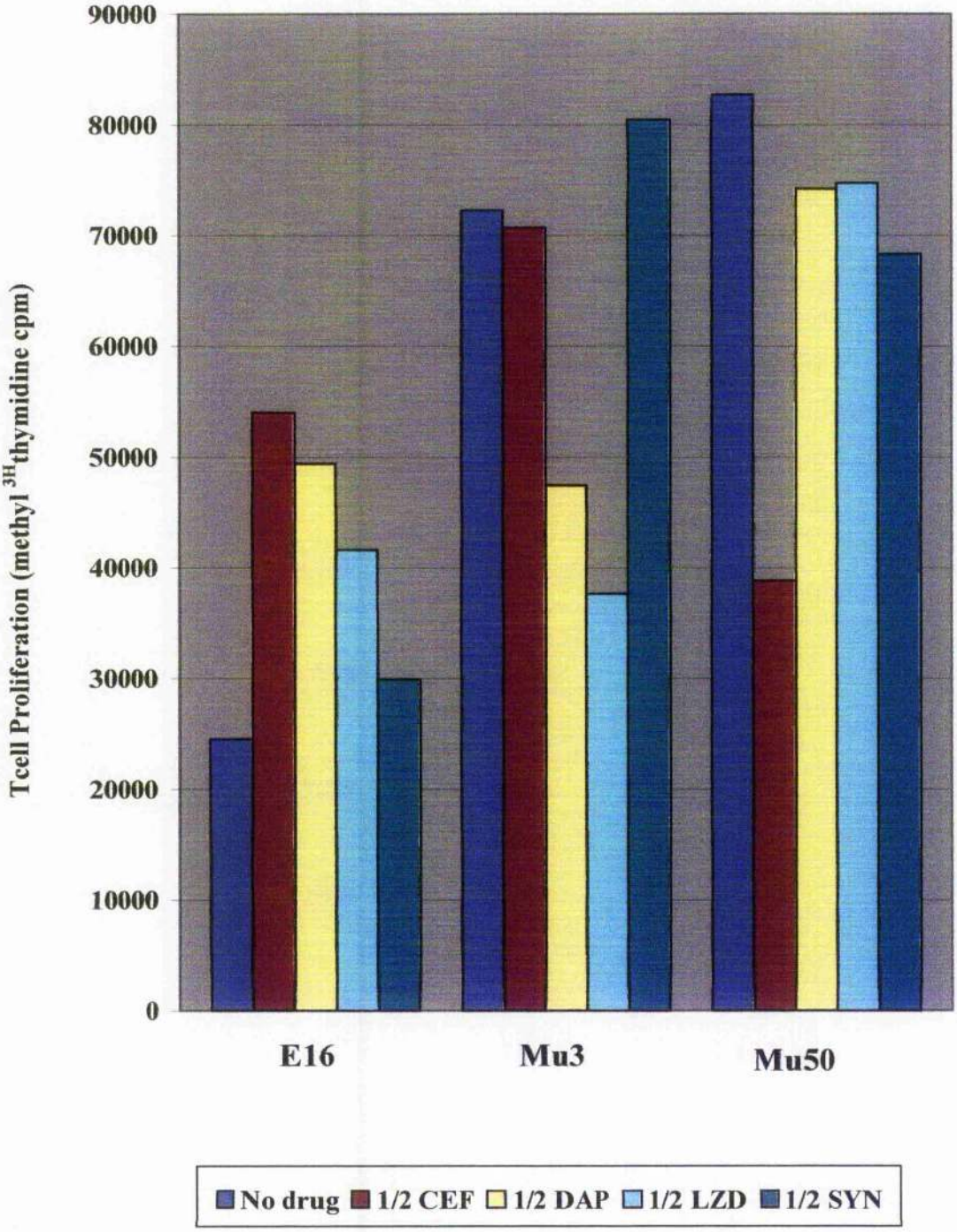


Figure 35. Human peripheral blood mononuclear cell (PBMC) proliferation induced by 1:10 dilutions of culture supernatants from strains of *S. aureus* grown in the absence and presence of ½ MIC of each antibiotic.

3.3.7 Haemolytic Activity of Alpha-Haemolysin (α-toxin)

Haemolytic activity in E16 and the VISA strains, with the exception of Mu50, is very low in comparison to *S.aureus* Cowan and Wood 46 the positive control strain (Table 18). The haemolytic activity of the VISA strains ranges from zero to 32 haemolytic units (HU). Strains Mu50, Cowan and Wood 46 display titres of 64HU, 64HU and 1024HU respectively. VISA 5836 did not have any apparent haemolytic activity in culture medium alone and was only detectable with neat culture supernatants in cultures grown in the presence of ½ and ¼ MIC of all drugs.

One half linezolid was the only drug to have a uniform effect against all of the strains tested reducing activity to between zero and 4HU, and reducing Wood 46 activity from 1024HU to 512HU. Synercid reduced the level of α-toxin expression at ½ MIC in 16hr cultures of *S.aureus* Cowan, E16, 3759v and Mu50, had no effect on Mu3, and increased expression in strains 3700w and 5827 but only by one dilution. Daptomycin decreased activity in Cowan, 3759v and Mu50 and increased it in 5827 and Mu3 but again only by one dilution. No effect was observed in cultures of E16 and 3700w. At ½ MIC, cefpirome was most effective at reducing alpha haemolytic activity of Mu50 from 64HU to 8HU. Other strains with reduced activity were 3700w, 3759v, 5827 and Mu3 by one dilution and strain 5836 was increased by one dilution. Haemolytic activity of Cowan and E16 was unaltered. One-quarter MIC's were also effective against some strains.

STRAIN / DRUG	Wood 46	Cowan	E16	3700w	3759v	5827	5836	Mu3	Mu50
No drug	1024	64	8	2	32	2	0	16	64
½ MIC CEF	1024	64	8	0	16	0	Neat	32	8
¼ MIC CEF	2048	128	16	Neat	32	4	Neat	64	64
½ MIC DAP	1024	32	8	2	16	4	Neat	32	32
¼ MIC DAP	1024	32	8	4	16	4	Neat	32	128
½ MIC LZD	512	2	2	0	4	Neat	Neat	4	0
¼ MIC LZD	512	32	16	4	16	4	Neat	16	8
½ MIC SYN	1024	32	2	4	4	4	Neat	16	8
¼ MIC SYN	1024	64	2	4	8	4	Neat	16	64

Table 18. Haemolytic activity of alpha-haemolysin against rabbit erythrocytes. Results represent the mean of 4 experiments

3.3.8 Coagulase

3.3.8.1 Clumping Factor

Table 19 shows the 8hr coagulase titres for clumping factor present on the surface of $1x10^7$cells/ml following growth in the absence and presence of ½ and ¼ MIC linezolid, cefpirome, synercid and daptomycin. The activity of clumping factor was determined after 8hrs incubation as this time proved optimal; later readings (24hrs) were not taken as the microtitre plate contents had dried out.

Linezolid had the greatest effect on clumping factor expression by *S.aureus* Cowan increasing titres from 1024 units to 8192 units with ¼ MIC. Strains 3700w and Mu3 were the only other strains to produce increased levels of expression but only by a single dilution. The remaining strains all displayed reduced expression. Strains E16, 3759v, 5827 and Mu50 all displayed reduced clumping factor activity when grown in the presence of ½ MIC cefpirome and surprisingly clumping factor titres of Cowan, 5836 and Mu3 were reduced by one or two dilutions when grown in the presence of ¼ MIC but not ½ MIC cefpirome. Clumping factor production by strain 3700w remained unaffected by cefpirome. Synercid was also a potent reducer of coagulase activity in all strains with the exception of Cowan and 3700w grown in the presence of ¼ MIC. Daptomycin generally had little impact on the production of clumping factor; activity of strains 3759v and 5827 was reduced by ½ MIC and both ½ MIC and ¼ MIC respectively. The remaining strains showed no real variation in their titres of clumping factor.

STRAIN / DRUG	Cowan	E16	3700w	3759v	5827	5836	Mu3	Mu50	NCTC 6571
No drug	1024	4096	128	128	256	32	128	256	256
½ MIC CEF	1024	1024	128	64	64	32	128	64	256
¼ MIC CEF	512	4096	128	128	64	8	64	256	512
½ MIC DAP	1024	4096	128	64	128	32	256	256	512
¼ MIC DAP	4096	2048	128	128	128	32	64	256	256
½ MIC LZD	2048	1024	256	128	32	16	128	64	128
¼ MIC LZD	8192	512	256	64	64	16	512	128	256
½ MIC SYN	512	256	64	32	64	16	32	128	128
¼ MIC SYN	4096	512	256	64	64	16	64	128	256

Table 19. Clotting activity of staphylococcal clumping factor. Results represent the mean of 4 experiments

3.3.8.2 Cell Free Coagulase

Figures 36-39 illustrate the titres of cell free coagulase in 16hr culture supernatants from bacteria grown in the absence and presence of ½ and ¼ MIC linezolid, cefpirome, synercid and daptomycin. Each graph represents the mean (of four assays) titre of coagulase.

Titres of free coagulase were generally found to be lower than those of clumping factor. In response to the presence of antibiotics in the growth medium the majority of strains exhibited reduced coagulase titres. Exceptions were observed in cultures of *S.aureus* Cowan where greater activity in the presence of ½ and ¼ MIC linezolid was recorded. Coagulase expression by strains 3759v and NCTC 6571 was increased in the presence of all of the antibiotics. Strain Mu50 was unaffected by the presence of daptomycin in the culture medium.

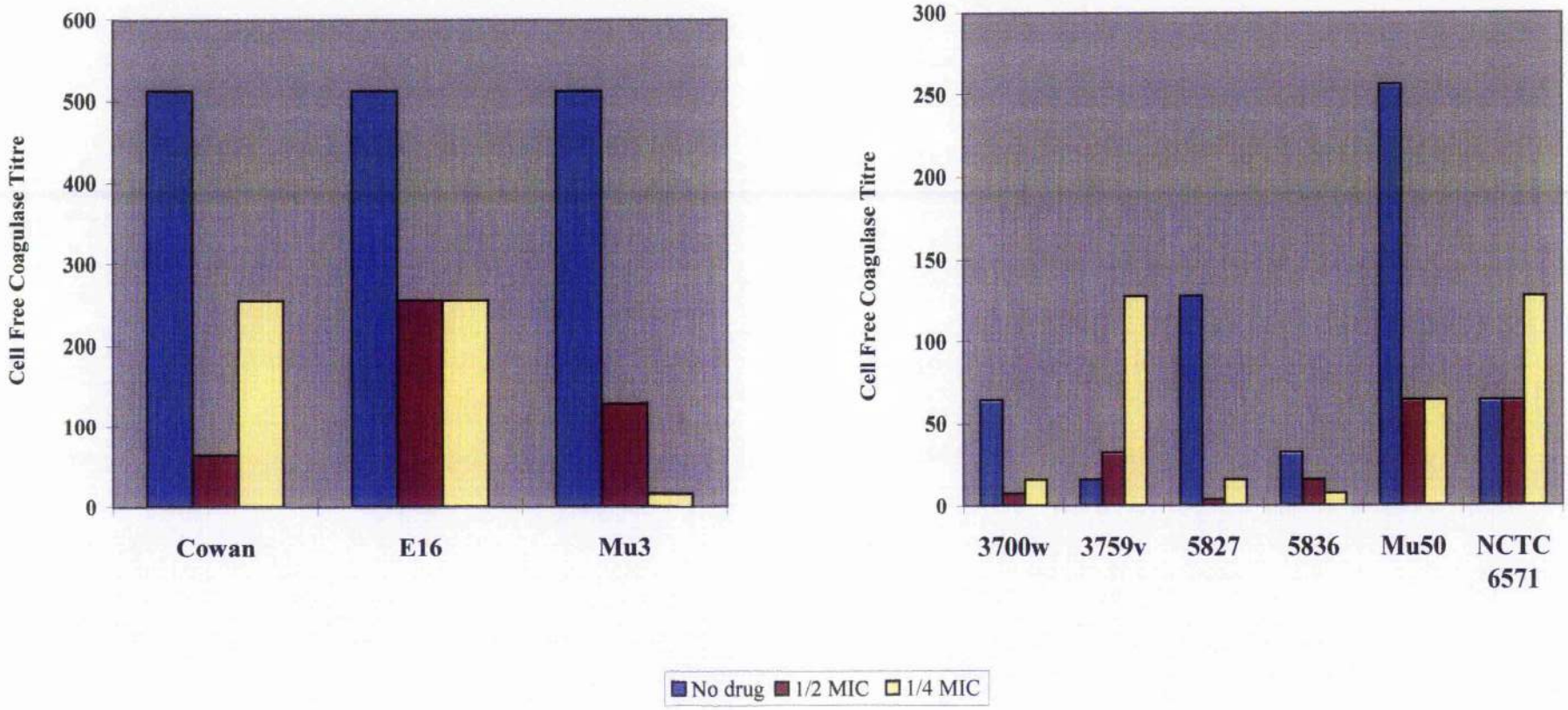


Figure 36. Expression of cell free coagulase by strains of S.aureus in the absence and presence of 1/2 and 1/4 MIC cefpirome.

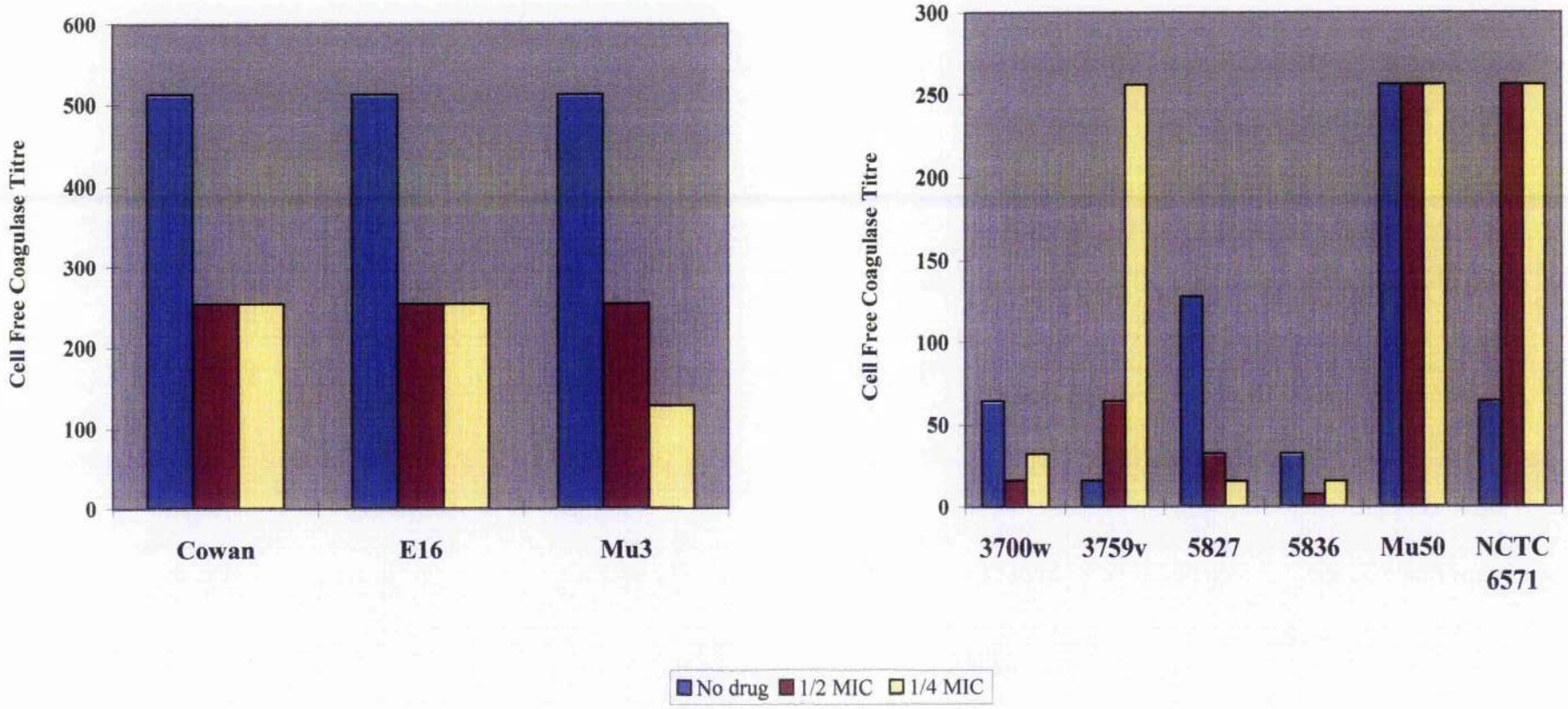


Figure 37. Expression of cell free coagulase by strains of S.aureus in the absence and presence of 1/2 and 1/4 MIC daptomycin.

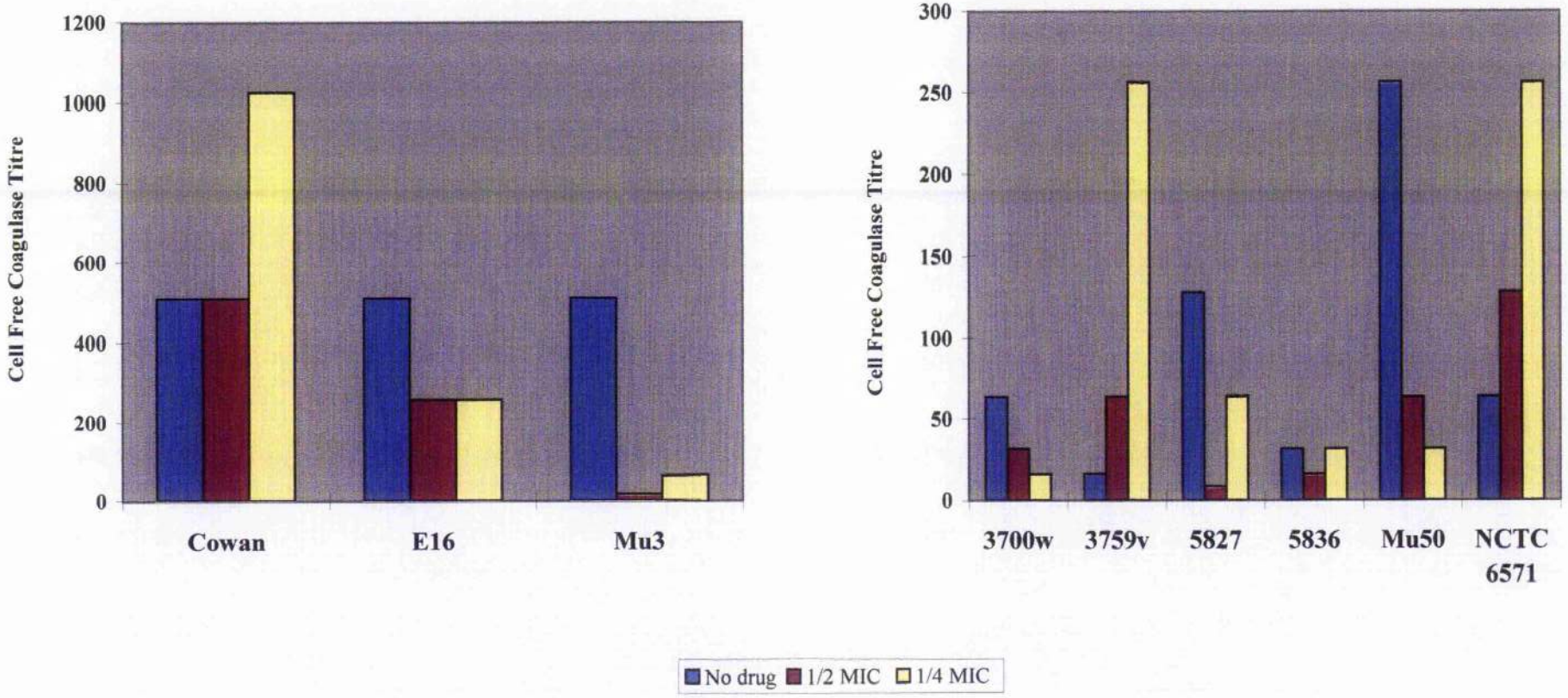


Figure 38. Expression of cell free coagulase by strains of S.aureus in the absence and presence of 1/2 and 1/4 MIC linezolid.

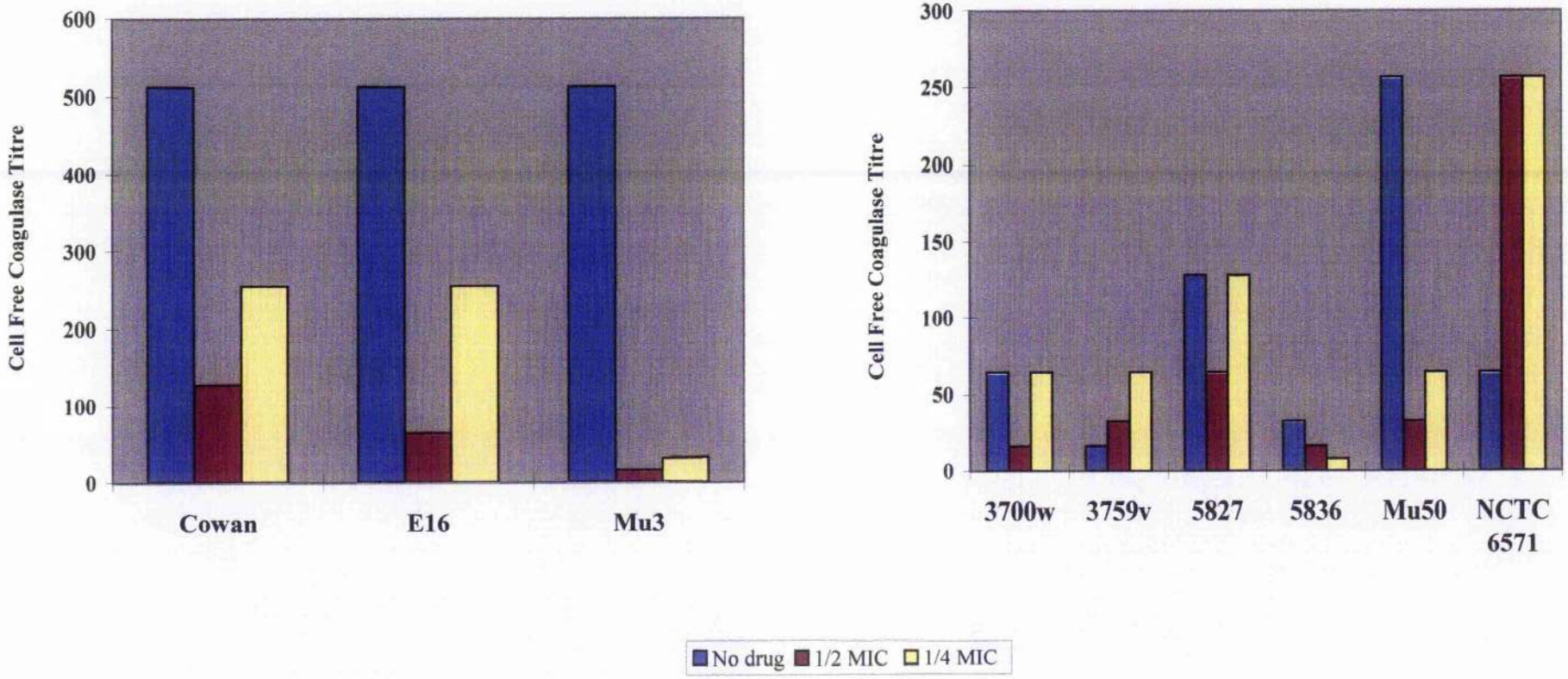


Figure 39. Expression of cell free coagulase by strains of S.aureus in the absence and presence of 1/2 and 1/4 MIC synercid.

3.3.9 RNA Quantification

RNA isolation from cell cultures proved to be difficult. Several attempts were made to isolate RNA using a method adapted from the manufacturer's instructions for TRIzol (Sigma). However, this method was time consuming and laborious and was substituted by the Qiagen RNeasy Mini RNA isolation kit due to its rapid isolation procedure.

Several preparations of RNA were made although RNA was undetectable by spectrophotometry and on agarose gels. Those preparations that were quantifiable are detailed in Table 20.

Sample	$OD_{260}nm$	$OD_{280}nm$	$^{260}/_{280}$	Yield ng/ml
E16 4hr No drug	0.44	0.24	1.8	11
E16 4hr ½ MIC LZD	0.40	0.27	1.45	10
E16 4hr ½ MIC SYN	-	-	-	-
E16 12hr No drug	0.5	0.4	1.25	12.5
E16 12hr ½ MIC LZD	0.33	0.25	1.32	8.25
E16 12hr ½ MIC SYN	0.17	0.095	1.79	4.25

Table 20. Quantification of RNA isolated from cultures of EMRSA-16.

For each time point, 4hrs and 12hrs, a culture of E16 in RF-MHB only and in the presence of ½ MIC linezolid and ½ MIC synercid were prepared and the RNA extracted. Using the following calculation: 40µg/ml x 260nm reading x dilution x sample volume (0.05ml), the purity of nucleic acid and the yield of RNA from each culture was determined. RNA from 4hr cultures of E16 in the presence of ½ MIC synercid could not be quantified by spectrophotometry but RNA was visible on agarose gels (Figure 40).

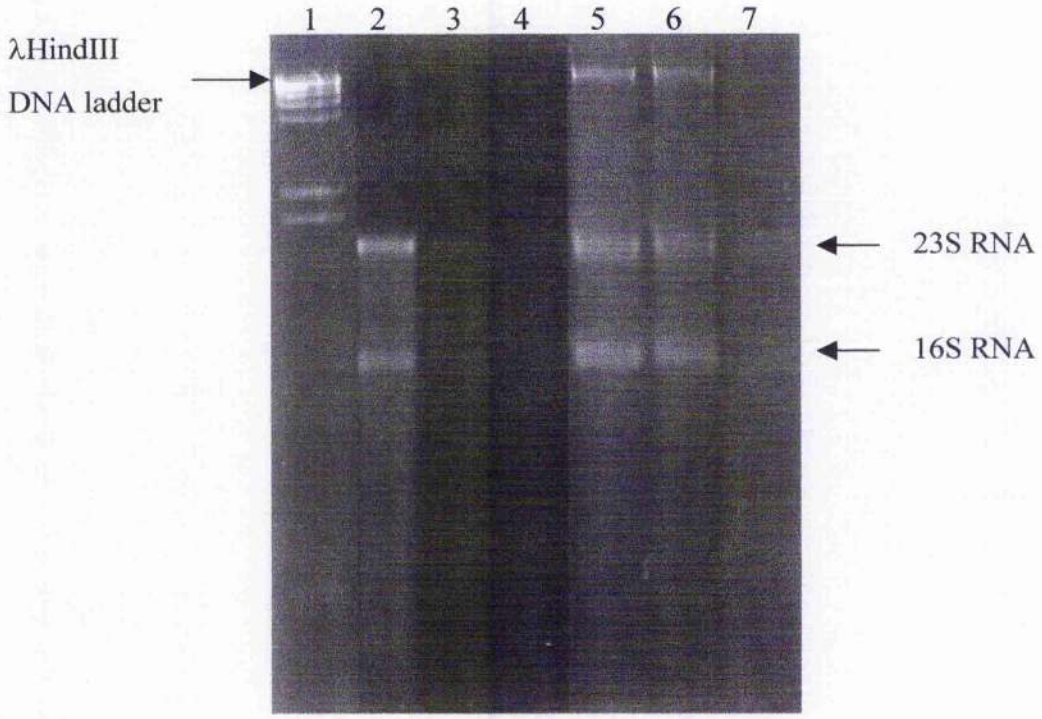


Figure 40. RNA extracted from 4hr and 12hr cultures of E16 in the absence (lanes 2 and5) and presence of ½ MIC linezolid (lanes 3 and 6) and synercid (lanes 4 and 7) respectively

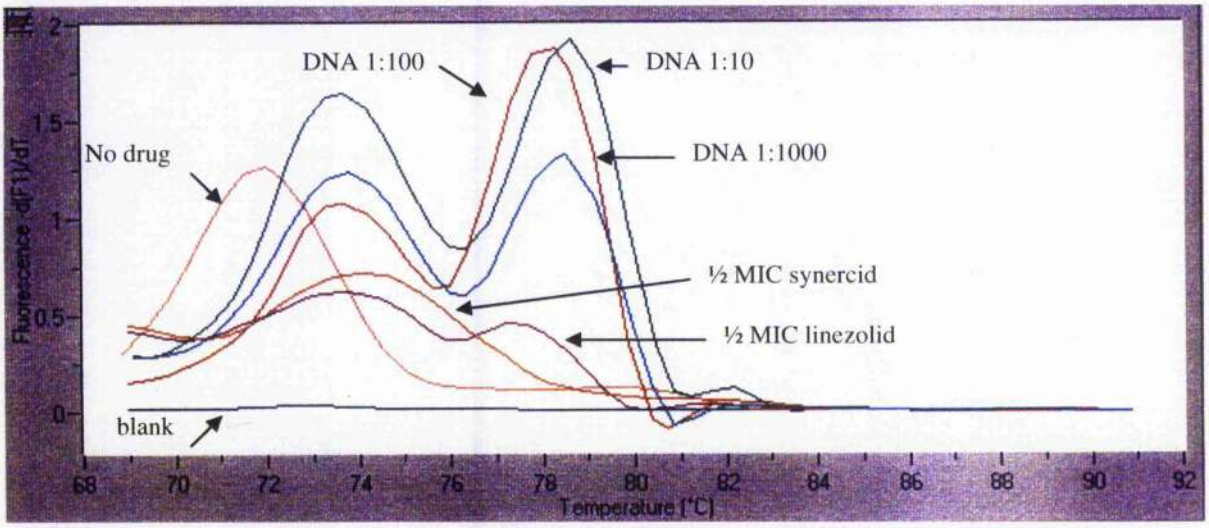
3.3.10 Transcription of *spa* and *tst* mRNA

Cultures of EMRSA-16 were set up at two time points, 4hrs and 12hrs in drug free medium and in the presence of ½ MIC linezolid and synercid. From this the levels of *spa* (protein A) and *tst* (TSST-1) mRNA transcripts were quantified respectively. Difficulties with non-specific annealing of the *tst* primers were encountered using a range of annealing temperatures (50–55°C) resulting in primer-dimer and non-specific PCR products. Figures 41 and 42 show the melting curves and DNA products produced using the RNA isolated from 12hr cultures of E16 and *tst* primers with annealing temperatures of 54°C and 50°C respectively. As illustrated in Figure 41 there are two main products in the TSST-1 DNA standards with a different set of peaks being observed in the three RNA samples. The products were run on an agarose gel (Figure 41) and were found not to be of 467bp, the expected size of any TSST-1 product. As some of the products are probably as result of mispriming and primer-dimer formation the annealing temperature was lowered to 50°C and the experiment repeated using the same samples of RNA (Figure 42).

Reducing the annealing temperature to 50°C did not improve the specificity of the *tst* gene primers. Here, the products amplified in the DNA standards were all the same but differed from those found in the sample of RNA. From the melting curve it would appear that there is very little product in the three RNA samples so the products were run on an agarose gel (Figure 42). No products could be detected in the RNA samples. Why the *tst* primers should amplify multiple products is unclear. One reason may be the lack of specificity of the primers but this should not be the case as the primers were designed using the LightCycler probe design software.

The above problems were not encountered with the 4hr RNA samples. Although some primer-dimer products were observed, the main product amplified from the protein A DNA standards and the three RNA samples were identical. Using 16S mRNA as the reference transcript it was shown, that after 4hrs incubation, growth in ½ MIC linezolid gave rise to twice as much *spa* mRNA as that present in drug free cultures, and that cultures with ½ MIC synercid produced double the quantity of *spa* mRNA (Figure 43). In Chapter Two partial inhibition of bacterial growth due to exposure to ½ MIC linezolid and synercid was found such that at 4hrs, growth was reduced by more than ½ a log by ½ MIC linezolid and 1log by ½ MIC synercid. This has not, however, appeared to have had an effect on mRNA synthesis. It is surprising therefore that, according to the findings by ELISA, both surface bound and extracellular protein A are reduced upon exposure to these antimicrobial agents, thus suggesting that the mRNA is transcribed, but is not translated into mature protein.

A



B

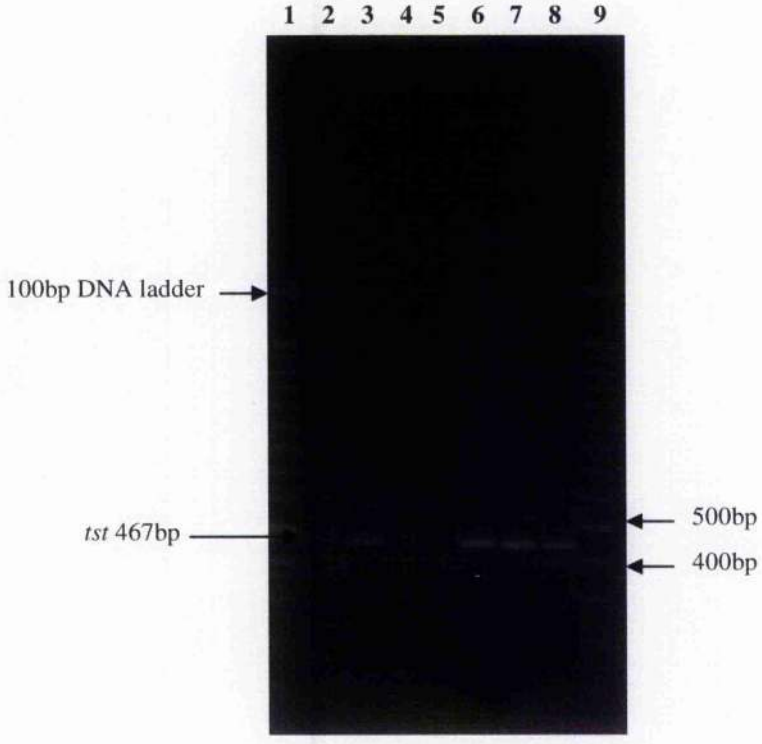
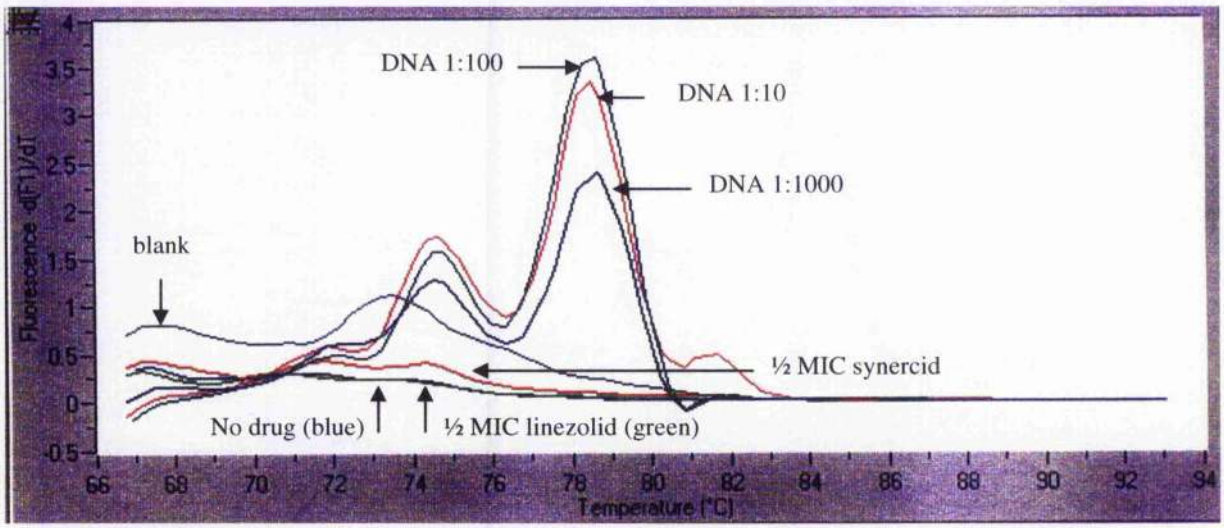


Figure 41. **A**: Melting curve analysis of mRNA isolated from 12hr cultures of E16 in the absence and presence of ½ MIC linezolid and synercid. Annealing temperature 54°C. **B**: LightCycler products producing the melting curve in A run on an agarose gel. Key: lanes 1+9 = DNA ladder, lane 2 = no drug, lane 3 = ½ linezolid , lane 4 = ½ synercid, lane 5 = blank, lane 6 = 1:10 DNA standard, lane 7 = 1:100 DNA standard, lane 8 = 1:1000 DNA standard

A



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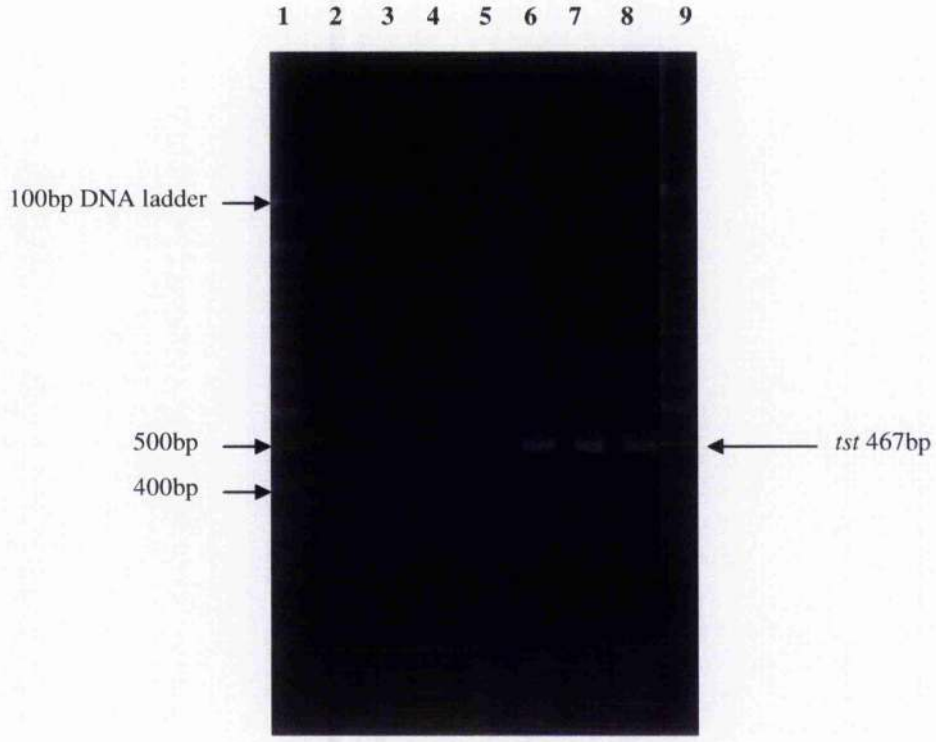

Figure 42. **A**: Melting curve analysis of mRNA isolated from 12hr cultures of E16 in the absence and presence of ½ MIC linezolid and synercid. Annealing temperature 50°C. **B**: LightCycler products producing the melting curve in A run on an agarose gel. Key: lanes 1+9 = DNA ladder, lane 2 = no drug, lane 3 = ½ linezolid , lane 4 = ½ synercid, lane 5 = blank, lane 6 = 1:10 DNA standard, lane 7 = 1:100 DNA standard, lane 8 = 1:1000 DNA standard

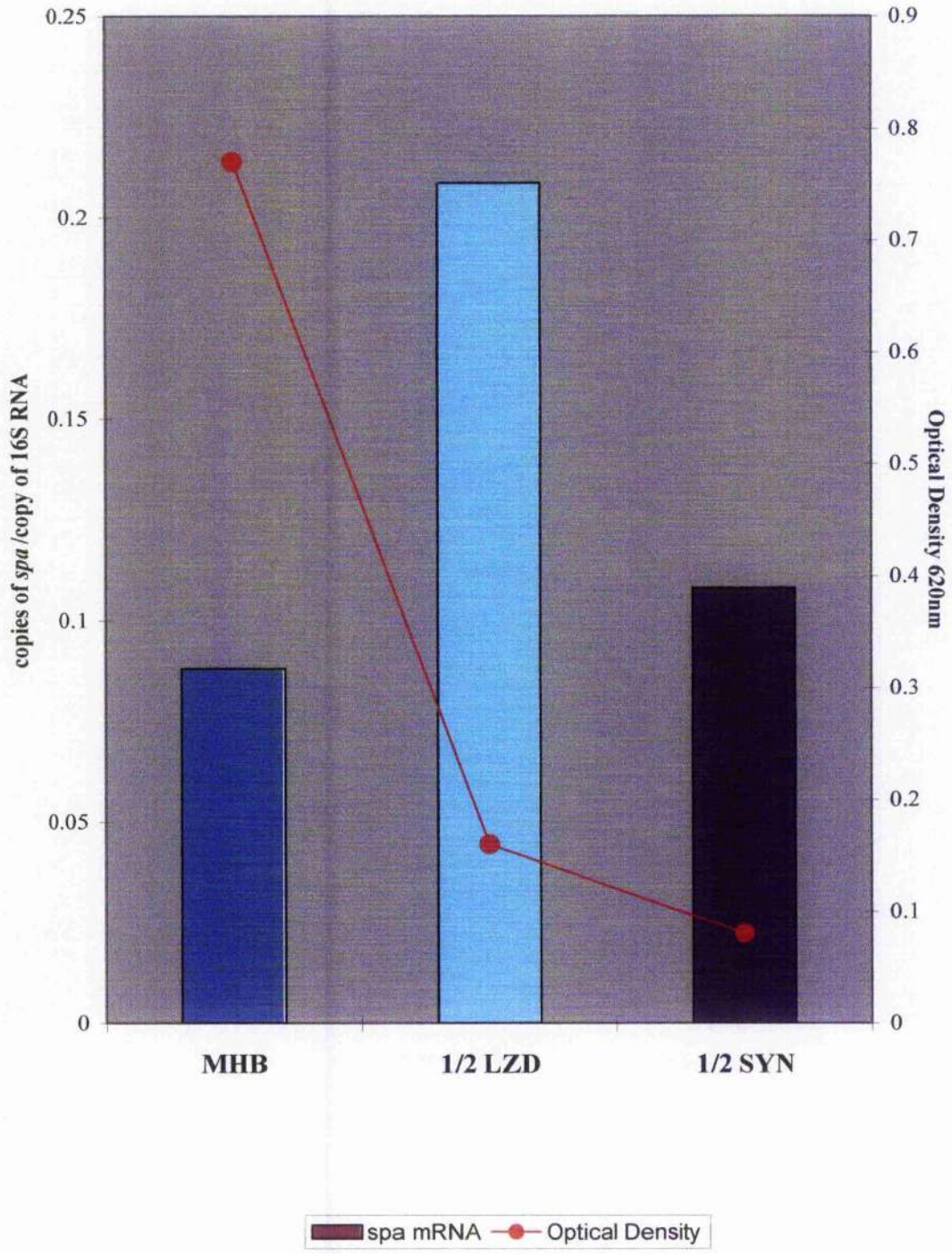

Figure 43. Expression of protein A (*spa*) mRNA transcripts by EMRSA-16 in comparison with optical density of 4hr cultures in the absence and presence of ½ MIC linezolid (LZD) and synercid (SYN).

3.3 DISCUSSION

Although the precise mechanism of action is known for the antibiotics linezolid, cefpirome and synercid, the bacterial target of daptomycin remains to be clarified. Studies in to the efficacy of these agents in animal infection models and in compassionate use programs against Gram-positive bacteria have provided promising results. Their effects on protein and toxin biosynthesis remain to be clarified.

The strains of vancomycin-intermediate *S.aureus* (VISA) used in this study differ from strains of MRSA and MSSA in their phenotypic characteristics. In Chapter Two I showed that several of the VISA strains exhibited thickened cell walls in comparison to a strain of MRSA, EMRSA-16, and MSSA *S.aureus* Cowan. In this chapter it was noticed that only a single strain of VISA, strain 5827, was capable of expressing surface bound protein A. Several strains, including strain 5827, expressed extracellular protein A. Strains 3700w and 5836 were negative for both forms of protein A. It has been described previously that some strains of MRSA do not express cell bound but extracellular protein A. The reasons for this are unclear but is a phenomenon mirrored here in strains of VISA.[107,367]

The action of other antimicrobial agents on the expression of surface proteins and exotoxins from numerous bacterial species has been extensively reported. The activity of antibiotics has been demonstrated to vary between bacterial species and strains often with contradictory results. For example, sub-inhibitory concentrations of clindamycin inhibit the production of coagulase, protein A, TSST-1 and α-haemolysin by *S.aureus* but can also stimulate the expression of enterotoxins in other Gram-positive and Gram-negative bacteria.[117,123,124,127,130,152,211,266,304,310]

The results presented here indicate that the activity of the antimicrobial agents cefpirome, daptomycin, linezolid and synercid varies towards different strains of *S.aureus*. In each of the experiments employed here the quantification and/or activity of surface proteins and extracellular toxins was measured following the incubation of strains in sub-MIC antibiotics for 16hrs. Sub-MIC linezolid and synercid were found to have no effect or reduce the expression of surface proteins protein A, and coagulase/clumping factor in the majority of strains but differed in their effect on alpha-haemolysin and TSST-1 activity.

Synercid increased alpha-haemolysin activity in three out of eight strains and reduced it in three out of eight strains, whereas linezolid reduced alpha-haemolysin activity in seven out of the eight strains. Both reduced TSST-1 expression in two out of three strains, Mu3 and Mu50. A recent publication by Gemmell and Ford (2002) showed that linezolid reduced alpha-haemolysin and coagulase expression at concentrations of ½ MIC to $^{1}/_{8}$ MIC in two strains of MSSA.[132] As yet, no reports of any activity of synercid on virulence factor expression have been published. However its ability to inhibit protein biosynthesis albeit at a different site from linezolid, would suggest similar results. This was not the case. Sub-MIC synercid increased alpha-haemolysin expression in strains 3700w, 5827 and 5836, but it should be emphasised that in the absence of any drug, haemolysin expression was very low and that increases were by a single dilution only.

Quantification of protein A messenger RNA (*spa* mRNA) transcripts from 4hr cultures of strain E16 was carried out to establish whether sub-MIC linezolid and synercid affected the levels of mRNA expression in addition to the levels of protein. At 4hrs, the presence of ½ MIC linezolid and synercid exerted bacteristatic properties on the growth of E16; however, the number of copies of *spa* mRNA was increased in comparison to the no drug control. This would suggest that these protein synthesis inhibitors inhibit the translation of mRNA in to mature protein resulting in decreased levels of protein A expression. These findings support the mechanisms of action suggested for linezolid and synercid

A hypothesis to the variable expression of virulence factors in response to clindamycin was proposed by Gemmell (1982).[124] It was suggested that the ribosomes involved in the transcription of mRNA are dispersed through out the bacterial cell, some of which are associated at the cell membrane. It is the ribosomes present at the cell membrane that would interact with and be affected by antibiotics penetrating the bacterial cell allowing a degree of protein expression to be carried out by ribosomes present in the cell cytoplasm (Figure 44). It is also possible that protein synthesis inhibitors may influence toxin expression, in part, through the impairment of growth.

Bacterial Cell

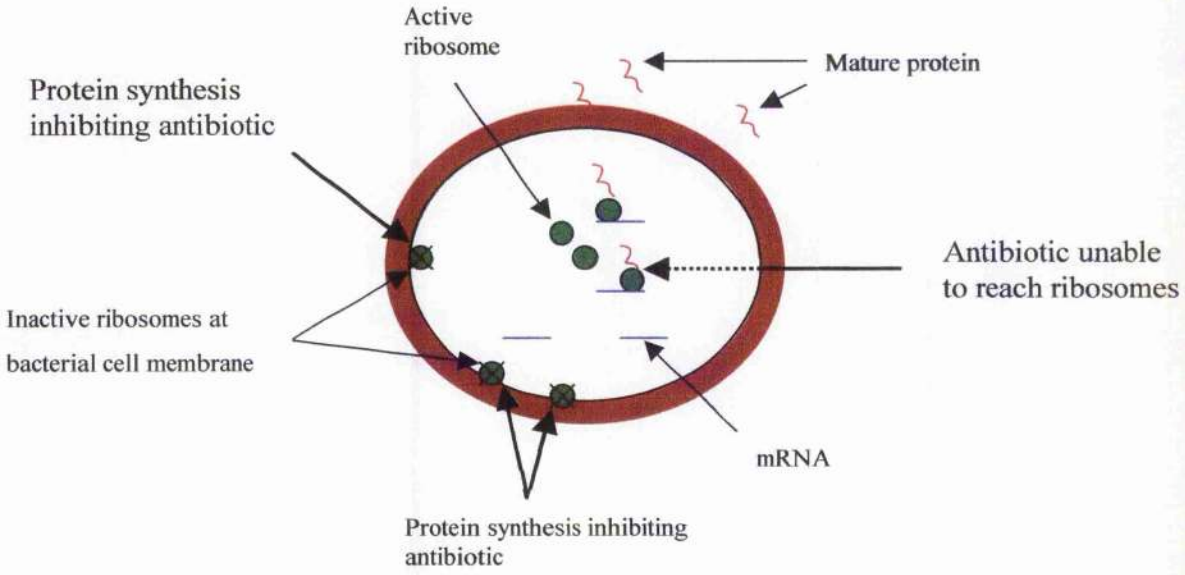

Figure 44. Proposed model to explain how protein synthesis inhibiting antibiotics might affect expression of proteins and toxins in *S.aureus*

Daptomycin, an antibiotic with cell membrane activity, had no effect on the expression of protein A in either form from any strain or on clumping factor in five out of the eight strains. A possible reason for our findings may be that at ½ MIC neither the expression of protein A and clumping factor is sufficiently affected nor is peptidoglycan biosynthesis sufficiently affected at this concentration for any effects on the cell wall and the incorporation of surface proteins to be detected. Extracellular coagulase activity, however, was reduced in the majority of strains suggesting that daptomycin inhibited the release of extracellular coagulase through interference of the bacterial cell membrane potential. Nevertheless why the export of extracellular protein A should not be affected is unclear; it is possible that the protein had become detached from the cell surface. Daptomycin exposure did not alter the expression of TSST-1 in any strain and only altered the expression of alpha-haemolysin by a single dilution.

The bacterial target for daptomycin is believed to be lipoteichoic acid (LTA) synthesis at the cell membrane although instances of cell membrane disruption and inhibition of peptidoglycan synthesis have also been proposed as sites for daptomycin activity. It has been demonstrated that daptomycin cannot enter the cytoplasm so its effects can only be

exerted on those proteins present at the cell membrane. In the presence of daptomycin at 1 x MIC *S.aureus* has been shown to display inhibited growth but strains of enterococci grew equally as well as cells in no drug cultures suggesting that daptomycin is bacteristatic.[47] In this study only strain 3759v was inhibited at ½ MIC daptomycin but as the aforementioned study was carried out at 1 x MIC, sub-inhibitory concentrations may not be sufficient to inhibit growth in *S.aureus*.

Should sub-MIC daptomycin be sufficient to disrupt bacterial cell membrane potential, leakage of the cell cytoplasm would occur releasing any intracellular proteins and toxins. As this was not the case it would suggest then, at ½ MIC and ¼ MIC, daptomycin does not influence the expression of virulence factors through the disruption of the bacterial cell membrane. It is possible that as certain aspects of protein synthesis do take place at the cytoplasmic membrane, daptomycin may exert an effect on protein synthesis. Strain 3759v appears to react differently from the other strains in response to the stress exerted upon the cell by daptomycin. Growth inhibition and the increased expression of cell free coagulase may result indirectly from LTA inhibition as proposed by Canepari *et al* (1990)[47] or directly through an action on ribosomes at the cell membrane (Figure 45).

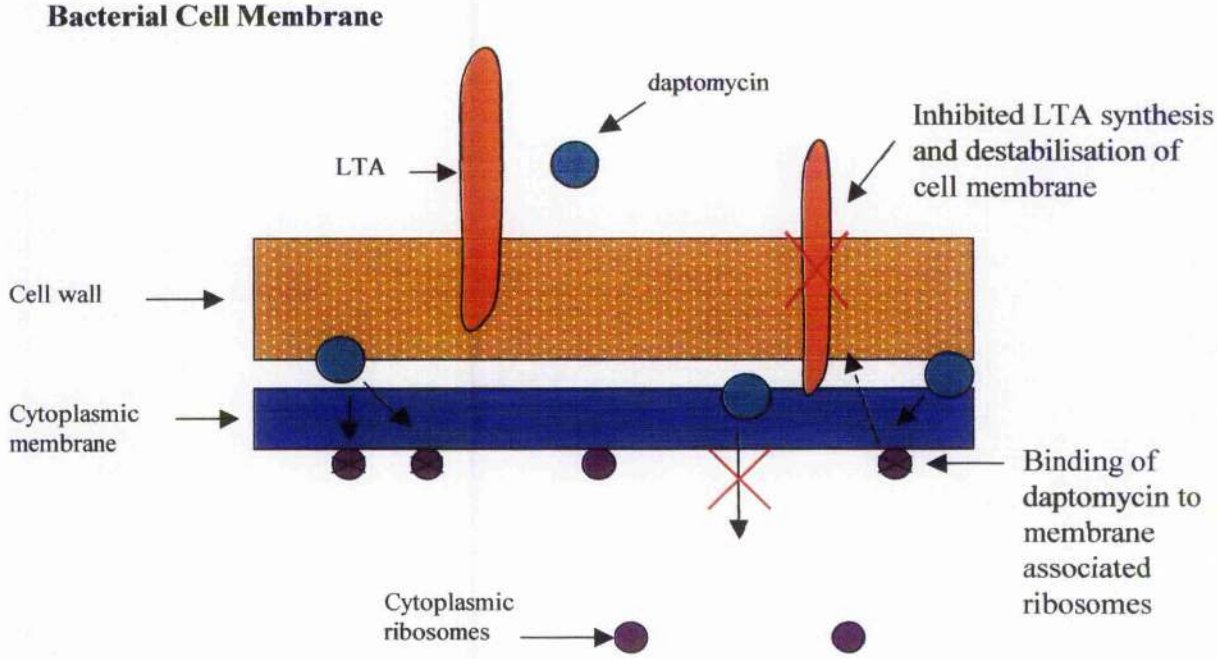

Figure 45. Proposed model for daptomycin inhibition of lipoteichoic acid (LTA) synthesis and low-level expression of proteins and toxins in Gram-positive bacteria

The cell wall inhibitor cefpirome was found to increase the expression of both surface bound and extracellular protein A on strain *S.aureus* Cowan, probably as a result of disruption of the cell integrity releasing fragments in to culture supernatants. All of the other strains found to express extracellular protein A and the expression of clumping factor by all strains were either unaffected or expressed at reduced levels. Disruption of the cell wall may be responsible for reducing the incorporation of surface proteins in to the bacterial cell wall. Toxin and cell free coagulase expression was found to be reduced in the majority of strains. Alpha haemolysin expression was only altered by a single dilution in all strains except for strain Mu50 where expression was reduced 3fold.

In a report by Ohlsen *et al* (1998) on the expression of alpha-haemolysin by *S.aureus* strain Wood 46 in the presence of ¼ MIC of β-lactam, cephalosporin and aminoglycoside antibiotics, alpha–haemolysin production was found to be increased due to increased *hla* mRNA expression.[266] In the same study, glycopeptides were found to have no effect on alpha-haemolysin expression whilst protein synthesis inhibitors had variable effects.

Using ¼ MIC methicillin and strains of MSSA and MRSA, it was possible to demonstrate that increases in *hla* mRNA were strain specific and that strain specific regulatory mechanisms determined the extent of alpha-haemolysin expression in the presence of methicillin. Non-stimulation of alpha-haemolysin expression by a β-lactam with no anti-staphylococcal activity suggests induction of *hla* expression may depend upon interaction of β-lactams with PBPs inducing a signal transduction mechanism that activates the toxin promoter (Figure 46).[266] It was also established that the enhanced expression observed in the presence of β-lactam antibiotics is specific, independent of RNAIII regulation, and not due to the destabilization of the bacterial cell wall or accumulation of cell wall precursors.[266]

In contrast to the above findings, we found that toxin expression was inhibited by the presence of ½ MIC cefpirome. It is possible that as the strains studied here display vancomycin-intermediate resistance in addition to being strains of MRSA that the signal transduction mechanisms have an altered function in strains of VISA in response to additional factors that differentiated VISA from strains of *S.aureus* and MRSA.

Bacterial Cell

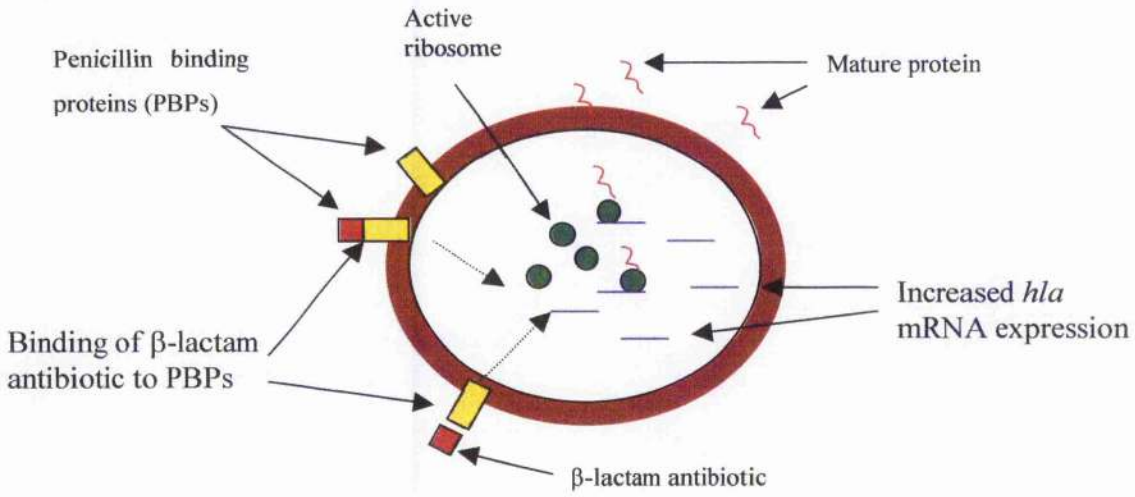

Figure 46. Proposed model for the activation of alpha-haemolysin expression in the presence of β-lactam antibiotics in Gram-positive bacteria

CHAPTER FOUR

Do Antimicrobial Agents Influence Bacterial Susceptibility to Phagocytosis?

4.1 INTRODUCTION

As discussed in section 1.6, neutrophils will form phagosomes around opsonised bacteria attached to their surface. Once contained intracellularly, the phagosome is fused with lysosomes forming a phagolysosome. During the process of bacterial ingestion, extracellular oxygen is taken in leading to an increased respiratory burst. It is this process of respiratory burst, the breakdown of oxygen into O_2^- + H_2O_2, that can be measured as an indicator of bacterial ingestion and presumably killing. Resistance to phagocytosis can also be measured.

In 1972, Allen *et al.* first described the phenomenon of PMNL chemiluminescence during bacterial phagocytosis and it has been used in many applications since, one of which is as an indicator of leukocyte metabolic activity and has been used to screen for oxidative metabolism defects in neutrophils such as those occurring in patients with chronic granulomatous disease (CGD).[85] Chemiluminescence provides simple, wide ranging yet highly sensitive measurements. The reaction is dependent upon the availability of oxygen, O_2^- and H_2O_2, and the activity of oxidase and myeloperoxidase (MPO).[151]

Chemiluminescence originates from the relaxation of singlet oxygen and electronically excited oxidative radicals such as H_2O_2, O_2^- and hydroxyl radicals produced by metabolic burst.[56] Relaxation of these excited molecules back to the ground state dissipates energy as light emission. Light emissions can be amplified by the addition of luminol, increasing sensitivity. In the presence of substrates such as uric acid and glucose, luminol is oxidised to aminophthalic acid generating hydrogen peroxide (H_2O_2). Relaxation of aminophthalate anions results in light emission.[11] Luminol-enhanced chemiluminescence is primarily from myeloperoxidase (MPO) dependent production of H_2O_2 although hypochlorous acid (HOCl) formation from H_2O_2 and Cl^- by MPO may diffuse from the PMN aiding the conversion of luminol to aminophthalate anion and light.[86]

To assess the susceptibility of our strains to phagocytic ingestion we pre-incubated each strain with pooled human serum so that complement components, mainly C3b, bind to the bacterial surface as in an *in vivo* infection, promoting phagocytic ingestion. Isolation of fresh human PMNL cells from blood donations provided phagocytically active neutrophils.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial Strains

The bacterial strains used are those referred to in section 2.2.1. Strains *S.aureus* Cowan, EMRSA-16, VISA 3700w, 3759v, 5827, 5936, Mu3 and Mu50 were maintained on fresh CBA plates and grown for 16hrs in MHB only, and in the presence of ½ or ¼ MIC concentrations of each of the antimicrobial agents, in a shaking incubator at 37°C 150rpm prior to each experiment. Cells were washed thrice in sterile saline and adjusted to $OD_{620}nm = 1x10^7$cells/ml.

4.2.2 Preparation of Antimicrobial Agents

The antibiotics used were cefpirome, daptomycin, linezolid and synercid as previously described in section 2.2.3. Each antibiotic was freshly prepared to the required concentration in 10ml volumes of MHB. Broth supplemented with 10mg/ml Ca^{++} was used with daptomycin.

4.2.3 Serum Isolation

Fresh blood from healthy adult donors was obtained by venipuncture and allowed to clot. The serum of five donors was pooled and aliquoted after centrifugation at 1000rpm for 15min. Aliquots were stored at –70°C until use.

4.2.4 Opsonization of Bacteria

Prior to use bacteria were adjusted to the required optical density then opsonised in an equal volume of 10% normal pooled serum (NPS) (Appendix 1) for 15mins in a shaking incubator at 37°C 150rpm. Cells were centrifuged at 3000rpm for 15min and re-suspended in their original volume of gel-Hank's (Appendix 1) for immediate use.

4.2.5 Preparation of Polymorphonuclear Leukocytes

The isolation of polymorphonuclear leukocytes (PMNL) was carried out according to the method described by Bøyüm *et al.*[42] Fresh whole blood was obtained by venipuncture from healthy adult volunteers and carefully layered in 5ml volumes on top of 5ml of

polymorphprep (Nycomed Pharma AS, Oslo, Norway) in 10ml sterile conical test tubes (Life Technologies, Paisley, UK). Centrifugation was carried out in an IEC Centra-4x centrifuge at 1800rpm for 30min. Two layers of white blood cells are visible, the lower of which contains the PMNL. This layer was removed using a sterile Pasteur pipette and washed with gel-Hanks at 18000rpm for 15min. The washed cells were pooled, counted in a haemocytometer and adjusted to a concentration of $1x10^7$ PMNL/ml with gel-Hank's (Appendix 1).

4.2.6 Phagocytic Ingestion

Phagocytic ingestion of bacteria was examined using cytospin preparations. Equal volumes of opsonised bacteria ($1x10^7$) and PMNL ($1x10^7$) were incubated in a Stuart orbital shaking incubator SI50 (Bibby Sterilin Ltd, Stone, Staffordshire, England) at 37°C, at 150rpm for 30min. Ice-cold PBS was used to stop the reaction and to wash the cells three times to remove any unphagocytosed bacteria. Cells were re-suspended in their original volume of gel-Hanks from which 100µl was removed into 1ml PBS to dilute samples. Five hundred microlitres of this solution was transferred to a cytospin chamber assembled with silane coated slides (Appendix 1) according to the manufacturer's instructions. Slides were spun at 500rpm for 5min in a Cyto-tek centrifuge (Miles Inc, Elkhart, Indiana, USA), air dried, then stained with Leishman's stain (Appendix 1). PMNL with ingested bacteria were examined at x1000 magnification.

4.2.7 Measurement of Chemiluminesence

One hundred microlitres of opsonised bacteria ($5x10^8$) were incubated with 50µl freshly isolated PMNLs ($1x10^7$cells/ml) and 50µl luminol (10^{-5} M) (Merck, Darmstadt, Germany) to give a ratio of 100:1. The luminescence output was recorded continuously for 75min in a Bio-Orbit Luminometer 1251 (Bio-Orbit, Turku, Finland) connected to an IBM PS/1 computer terminal with 'MultiUse' software package installed. The reaction was carried out at 37°C and the peak activity expressed in mV.

4.3 RESULTS

4.3.1 Phagocytic Ingestion of Bacteria

Phagocytic ingestion was measured by counting the number of bacteria within 100 randomly selected PMNL and the average number of bacteria per cell calculated. The percentage of PMNL ingesting bacteria was also calculated using the same 100 randomly selected cells (Figures 48-49, Tables 21 and 22). Readings represent the mean +/- standard deviation for 3 experiments as determined by a Student's T-test using a Minitab statistical package.

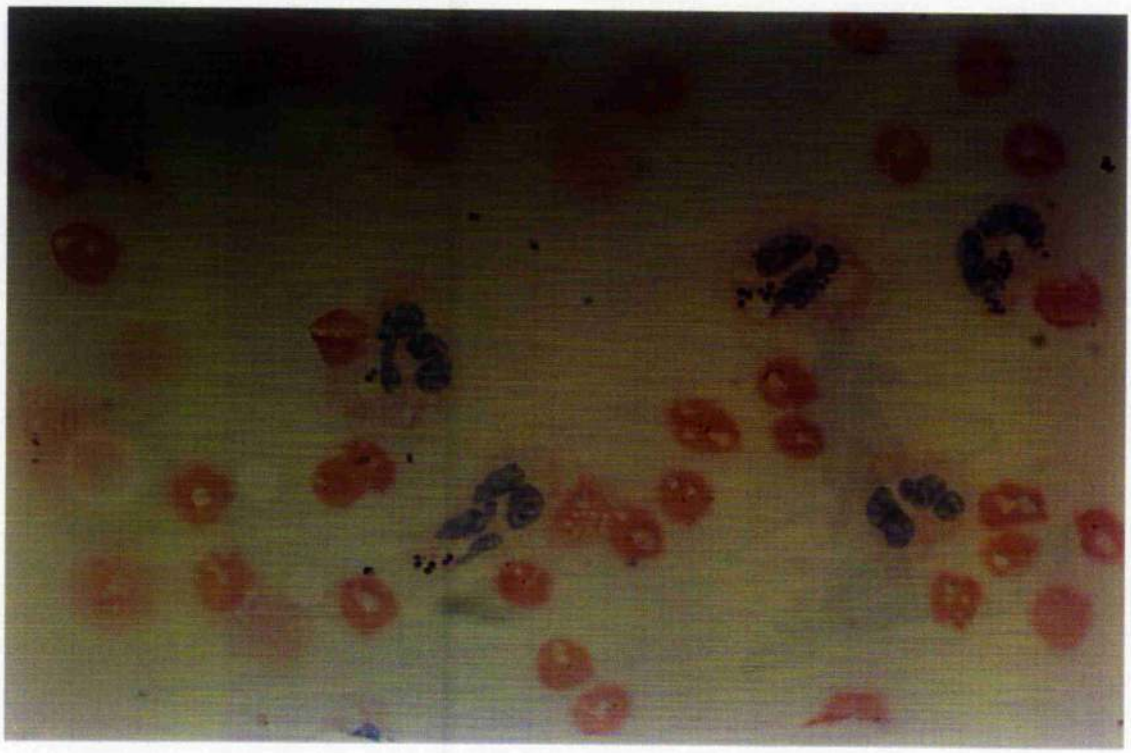

Figure 47. Bacterial ingestion by PMNL x7000 magnification

The mean number of bacteria per PMNL was between 7 and 10 for all strains grown in culture medium alone occurring within 35 to 61% of PMNL. A higher proportion of PMNL (>50%) ingested strains E16, Mu3 and Mu50 compared to the other strains even though the numbers of bacteria per cell were similar. Strain 5827 was the only strain to have its uptake affected by antimicrobial action. Cefpirome, linezolid and synercid each increased the number of bacterial cells per PNML (pvalue ≤ 0.05) however, the apparent

increased susceptibility to opsonophagocytosis was not correlated with an increased percentage of PNML actively phagocytosing strain 5827. Significant differences in the percentage of phagocytosing PMNL were noted with PMNL exposed to ½ MIC cefpirome and linezolid treated Mu3 and ½ MIC cefpirome, linezolid and synercid treated Mu50. It is possible that the 1:1 ratio of bacteria:PMNL was insufficient and that the majority of available bacteria have been ingested as seen by the consistent counts, and that the variable PMNL activity is representative of the different ingestion capabilities observed with PMNL preparations.

	Cowan	E16	3700w	3759v	5827	5836	Mu3	Mu50
Bacteria Per PMNL	8.2 ± 1.2	7.6 ± 1.3	9.7 ± 1.3	9.1 ± 1.8	7.3 ± 0.7	9.3 ± 1.6	7.8 ± 1.4	6.8 ± 0.5
% ingestion by PMNL	37.7 ± 7	57 ± 11	38.7 ± 14	49.7 ± 8.5	36.3 ± 19	41 ± 6	61 ± 8.9*	55.3 ±7.2

Table 21. Mean number of ingested bacteria per PMNL and the percentage (%) of PMNL ingesting bacteria in comparison with MSSA Cowan. Readings represent mean +/- standard deviation. *= pvalue ≤0.05.

	No drug	cefpirome	daptomycin	linezolid	synercid
Cowan	8.2 ± 1.2	8.7 ± 1.2	8.9 ± 1.5	7.3 ± 1.4	6.9 ± 2.5
E16	7.6 ± 1.3	6.9 ± 1.3	8.3 ± 1.7	7.3 ± 0.8	6.1 ± 0.7
3700w	9.7 ± 1.3	13.8 ± 2.8	10.7 ± 1.5	11.7 ± 2.7	10.9 ± 0.8
3759v	9.1 ± 1.8	6.8 ± 1.2	8.6 ± 0.6	7.8 ± 0.7	7.2 ± 1.5
5827	7.3 ± 0.7	14.9 ± 2.8 *	9.7 ± 1.3	13.1 ± 0.3 *	12.5 ± 1.7 *
5836	9.3 ± 1.6	11.4 ± 1.6	10.3 ± 1.1	10.9 ± 3	10.4 ± 1.9
Mu3	7.8 ± 1.4	6.2 ± 0.9	10 ± 0.04	6 ± 1.3	6.1 ± 0.7
Mu50	6.8 ± 0.5	6.3 ± 1.3	8.8 ± 2	5.7 ± 1.3	5.3 ± 1.5

Table 22. Mean number of ingested bacteria per PMNL. Readings represent mean +/- standard deviation, *= pvalue ≤0.05. It should be noted that a variety of PMNL preparations were used in the above experiments and comparisons should be made within an experiment.

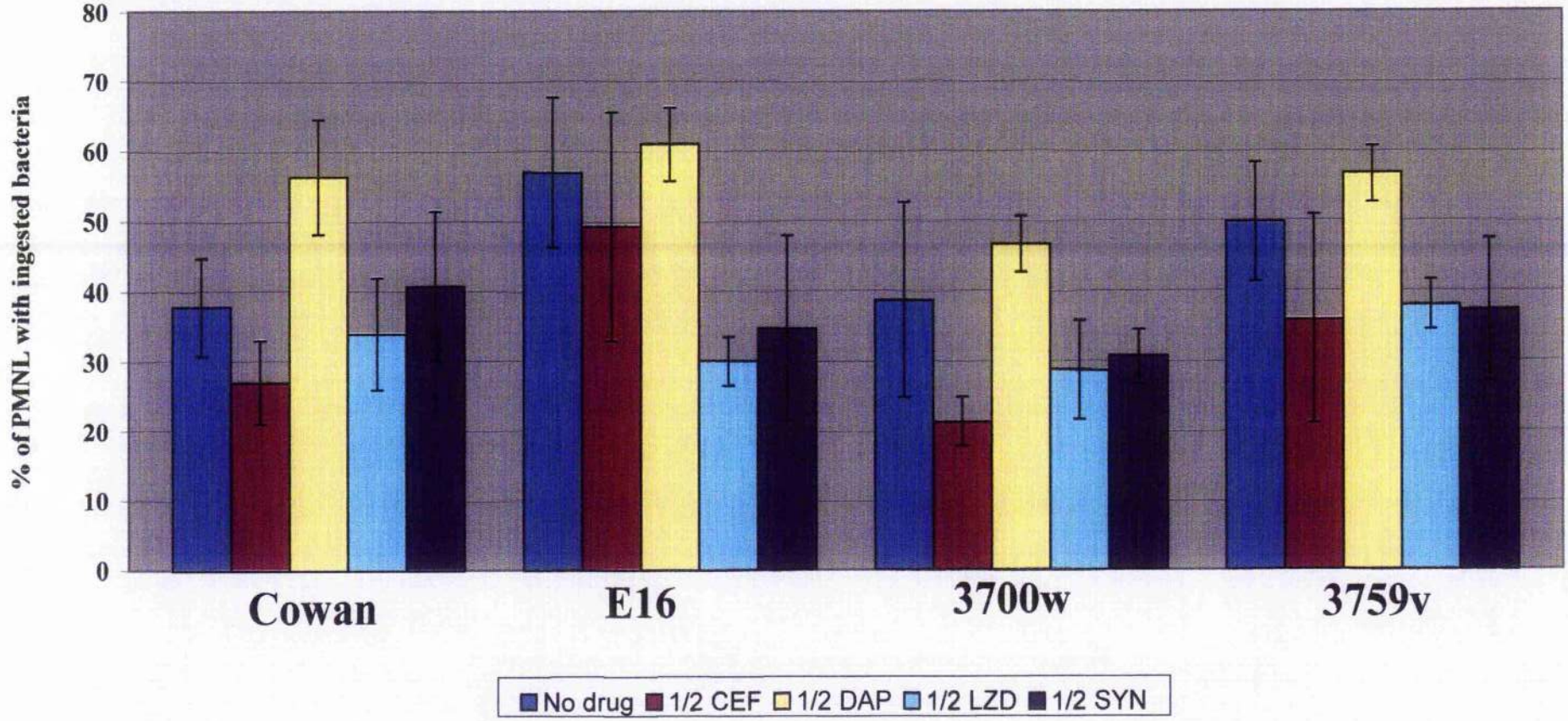

Figure 48. Percentage of PMNL containing ingested strains of opsonised S.aureus following growth in the absence and presence of 1/2 MIC of all antibiotics. Readings represent mean +/- standard deviation error bars. *= pvalue <0.05.

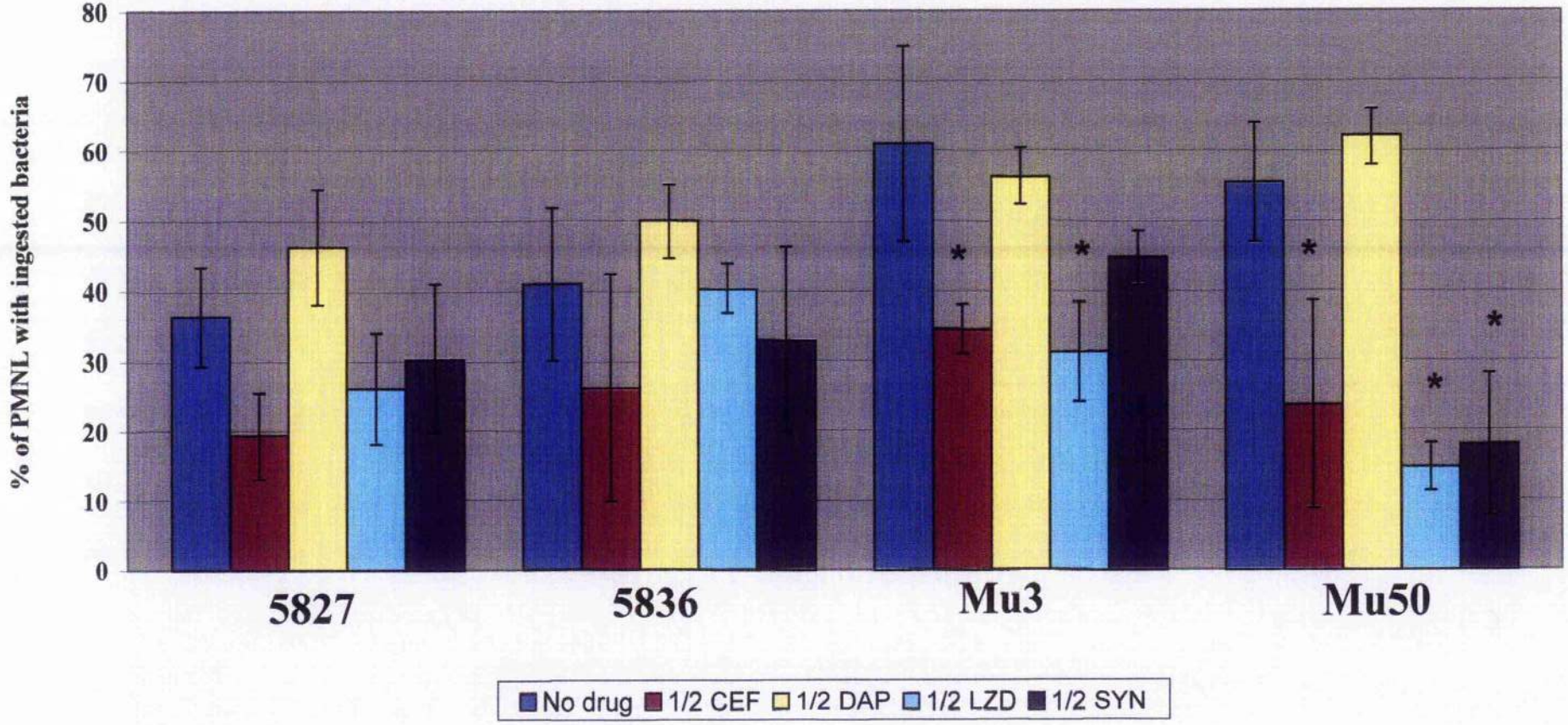

Figure 49. Percentage of PMNL containing ingested strains of opsonised S.aureus following growth in the absence and presence of 1/2 MIC of all antibiotics. Readings represent mean +/- standard deviation error bars. *= pvalue <0.05.

4.3.2 Phagocytic ingestion of bacteria as determined by the release of a chemiluminescence response

Table 23 and Figures 50 and 51 illustrate the chemiluminescence peak response (mV) elicited by E16 and the VISA strains in comparison to Cowan, and the effect of drug treatment on this response within strains respectively. The peak response represents the maximum light emission, in millivolts (mV), achieved by each strain that can be interpreted as maximal respiratory burst and therefore phagocytic uptake. As more oxygen is taken up from the environment during phagocytosis there is more available for conversion to H_2O_2 and detected by chemiluminescence. Statistical analysis was in the form of a Student's t-test. Mean values and standard deviation are given where n = 10.

	Cowan	3700w	3759v	5827	5836	Mu3	Mu50	E 16
Peak Response (mV)	27.1 (s.d 14.3)	29.4 (s.d 7.2)	9.05 (s.d 5.7)	24.9 (s.d 11.9)	29.3 (s.d 8.5)	34.7 (s.d 13.4)	40.7 (s.d 13.1)	42.6 (s.d 8.7)
P-value	*****	0.653	0.003	0.717	0.687	0.237	0.041	0.011

Table 23. Chemiluminescence response (mV) by strains of *S.aureus* compared to that of MSSA Cowan standard strain

In comparison to *S.aureus* Cowan VISA 3759v produced a much lower chemiluminescence response whilst Mu3 and Mu50 produced elevated responses (pvalue ≤ 0.05). Observations not recorded by the microscopic assay of stained cells.

Growth of cultures in the presence of ½ MIC and ¼ MIC cefpirome, daptomycin, linezolid and synercid did not alter the opsonophagocytic susceptibility of strains *S.aureus* Cowan, 3700w, 5827 or 5836. Daptomycin in particular made no significant impact on any of the strains tested. Cefpirome both increased and decreased chemiluminescence responses with 3759v at ½ MIC, and with E16 and Mu50 at ½ and ¼ MIC respectively. Linezolid and synercid effectively reduced chemiluminescence at ½ MIC and ¼ MIC concentrations. Linezolid expressed activity against E16 and Mu50 at both sub-MIC's and Mu3 exposed to ½ MIC only. One half MIC and ¼ MIC synercid exposed E16 also produced reduced chemiluminescence responses.

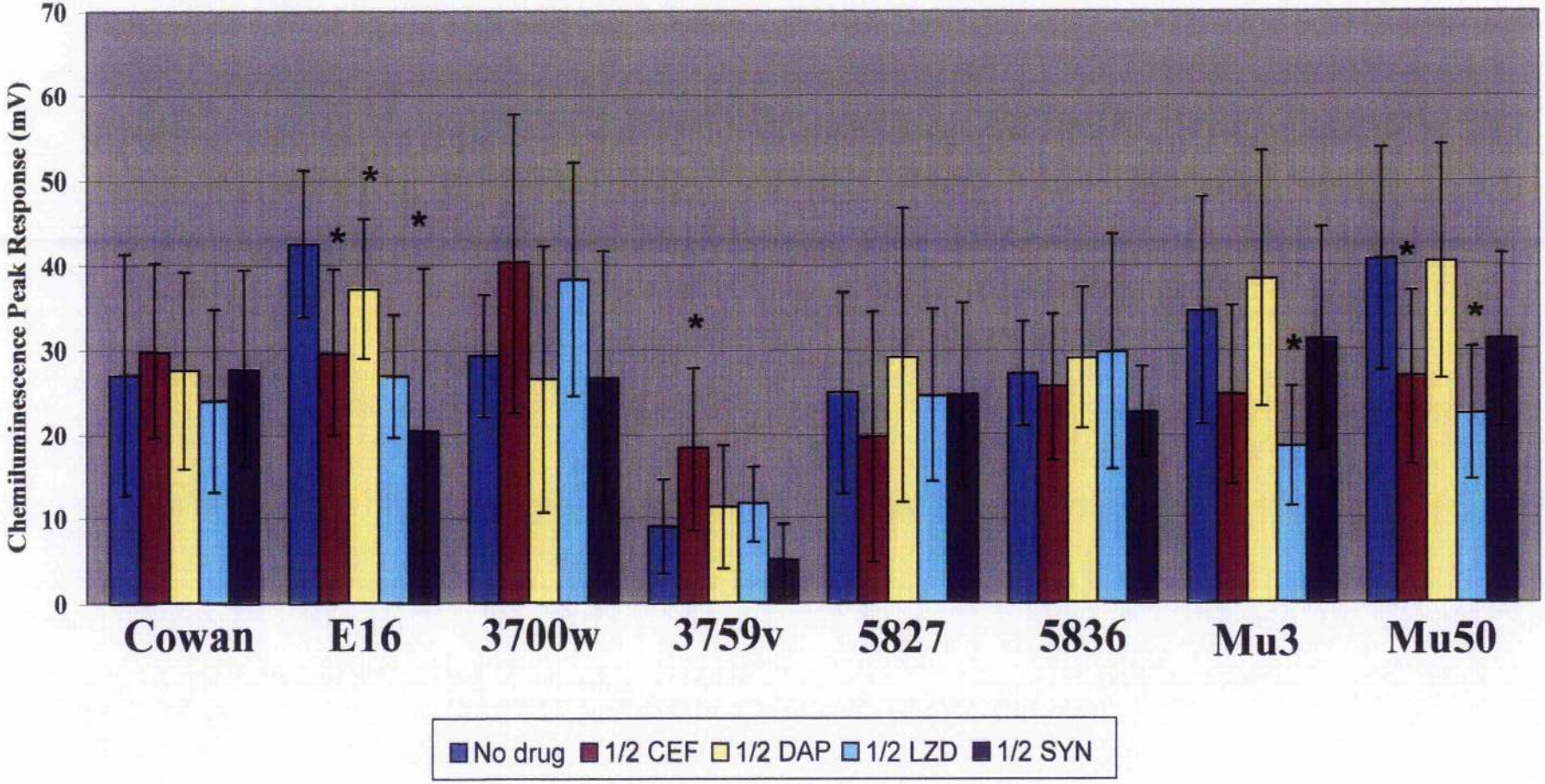

Figure 50. Chemiluminescence response (mV) of PMNL initiated by 1/2 MIC drug-treated strains of S.aureus. Readings represent mean +/- standard deviation error bars. *= pvalue < 0.05.

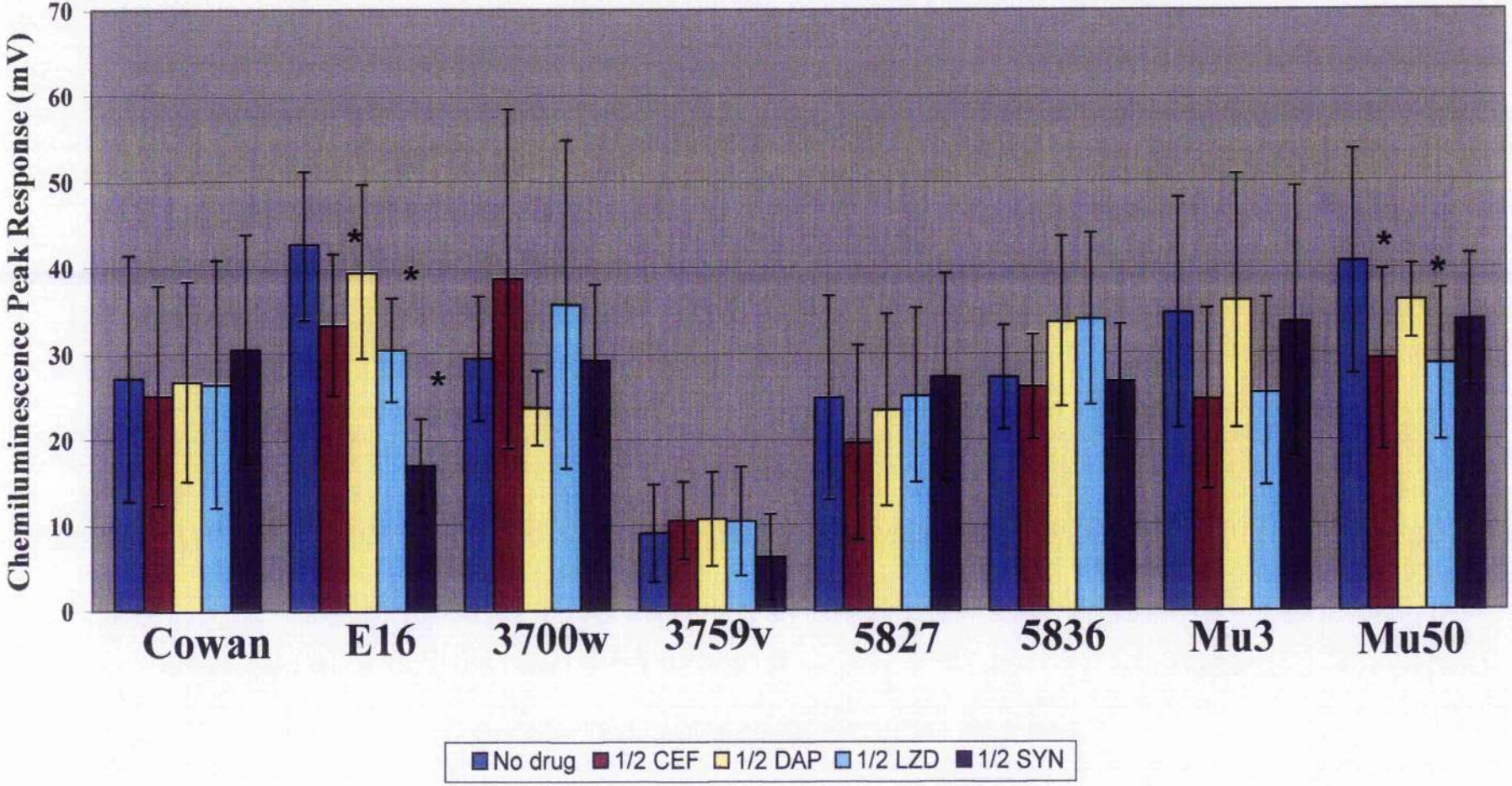

Figure 51. Chemiluminescence response (mV) of PMNL initiated by 1/4 MIC drug-trested strains of S.aureus. Readings represent mean +/- standard deviation error bars. *=pvalue <0.05.

4.4 DISCUSSION

Both of the investigations conducted in this section sought to examine the susceptibility of VISA to opsonophagocytosis by human PMNL cells. Discrepancies were seen between manual counts of ingested bacteria and the automated quantification of respiratory burst as an indicator of phagocytic uptake. This could be due to a number of reasons including the different ratios of bacteria:PMNL used, test variability and human error.

Some correlation was found in the reduced percentage of PMNL ingesting bacteria and the reduced chemiluminescence response produced by linezolid and cefpirome treated Mu50 and cefpirome treated Mu3. Antibiotic treatment of EMRSA16 produces a marked reduction in chemiluminescence response that is not observed statistically by bacterial counts however, if it were not due to high experimental variance a reduction in ingestion of linezolid and synercid can clearly be seen. Similar correlations between phagocytosis and chemiluminescence have been described by Gemmell and O'Dowd (1983).[127] Investigations by Bramley *et al.* (1989) reported the ingestion of many bacteria by some PMNL and none by others in a mouse mammary gland model.[43] They suggested that this might reflect the arrival of PMNL into the gland at different times or different phagocytic potential of neutrophil subpopulations. The latter could be an explanation of the variable numbers of PMNL phagocytosing bacteria in our study.

Research by other authors has investigated the effects of various protein synthesis inhibitors and cell wall antibiotics on bacterial ingestion, the most extensively studied drug being the protein synthesis inhibitor clindamycin (clin). The discovery that sub-MIC's of lincosamides and the macrolide erythromycin (ery) potentiates bacterial uptake of several species including staphylococci and streptococci has been made, and is generally correlated to the loss of an antiphagocytic surface protein.[119,122,127,129,237,352] Cell wall inhibitors have produced more varied results with reports of either increased uptake or no effect.[237,352]

The process of respiratory burst and chemiluminescence is one that progresses rapidly during the initial 10mins of contact with bacteria and can be influenced by the age of PMNL preparation.[12,320] Neutrophils from individuals with bacterial infections can also affect results as their activity can vary and as a result variability in study populations and methods can occur. For the purposes of this study our PMNL cells were isolated from

healthy blood donors and used within half an hour of their isolation. In our assay we have utilised the peak response as our indicator of maximal respiratory burst; however Allen (1977) has suggested that the efficacy of phagocytosis measured as a rate of ingestion (mV/cell/min) calculated over the initial 10 minutes is a more accurate estimate of phagocytic activity.[12] The use of PMNL preparations from blood donors results in PMNL with variable activity. In each of the experiments here, a single PMNL preparation was used to examine one strain under all experimental conditions therefore comparisons should be made within an experiment.

Protein A is considered to confer antiphagocytic properties upon a bacterial cell. In Chapter Three it was found that all of the VISA strains, with the exception of strain 5827, did not express surface bound protein A. A feature observed with some strains of MRSA.[107,367] Extracellular protein A was more readily detected in the VISA strains with strains 3759v, 5827, Mu3 and Mu50 recorded as positive producers by ELISA. EMRSA-16 expressed less but not significant, levels of protein A to *S.aureus* Cowan, a high protein A producer. The chemiluminescence response elicited by these strains was significantly different (pvalue ≤ 0.05) with E16 inducing a higher level of respiratory burst suggesting that the strain was more readily phagocytosed than the Cowan strain. In contrast strain 5827 produced significantly reduced levels of surface bound protein A but produced a chemiluminescence response similar to *S.aureus* Cowan. This suggests that protein A alone is not sufficient to alter the phagocytic susceptibility of these strains to ingestion by PMNL. Exposure to sub-MIC cefpirome, linezolid, and synercid were found to reduce the chemiluminescence response of PMNL incubated with strains E16, Mu3 and Mu50 but not to the same extent. One half MIC cefpirome was shown to significantly increase ingestion of strain 3759v. The increased susceptibility to phagocytosis of strains exposed to antibiotics does correlate with reduced levels of cell free protein A observed for cefpirome, linezolid and synercid exposed strain of E16, 3759v, Mu3 and Mu50. This suggests that these antibiotics are altering the bacterial cells, in addition to reducing protein A expression, affecting phagocytic susceptibility.

CHAPTER FIVE

GENERAL DISCUSSION

Ailments and diseases resulting from staphylococcal infection are numerous and variable in severity from folliculitis and boils, to food poisoning, toxic shock syndrome (TSS), scalded skin syndrome (SSS) and bacteraemia amongst others. The onset and progression of a diseased state is multifactorial involving the production of factors that aid in the primary attachment of a bacterium to host cells, invasion and spread of the bacterium within host tissues.

Strains of *S.aureus* are commonly associated with invasive diseases through the production of cell-bound and/or extracellular virulence factors. Expression of particular proteins and toxins can sometimes be associated with specific symptoms and disease, for example, the enterotoxins are food poisoning agents, TSST-1 causes TSS and the exfoliative toxins are the causative agents of SSS. The carriage of genetic elements encoding virulence factors as well as those encoding antibiotic resistance is highly variable amongst staphylococci and does not necessarily complement the observed phenotype. Whether it is the expression of proteins and toxins or resistance, the genes that encode them are under tight regulation by a number of mechanisms. The well-documented *agr* and *sarA* regulatory loci of *S.aureus* are the key regulators of virulence factor expression although homologous components of the *agr* locus have also been identified in strains of CNS. It is believed that the *agr* locus functions *in vivo* to activate extracellular toxin transcription in response to increasing levels of auto-inducing peptide to aid the destruction of host tissues and spread of the bacterium.

Antibiotic resistance is regulated by the expression of antibiotic specific mechanisms that can inactivate, prevent binding of, or export the antibiotic out of the bacterial cell. The occurrence of antibiotic resistance is widespread among bacteria and is increasingly problematic in the effective treatment of severe infections. The development of resistance to methicillin in *S.aureus* (MRSA) in the 1960s reduced the number of antibiotics available for use against *S.aureus*, leaving vancomycin as the drug of choice in many clinical situations. Resistance to vancomycin is already prevalent in strains of enterococci due to the carriage of *van* genes. The prospect of vancomycin resistance spreading to staphylococci is one that has caused concern among clinicians. In 1992, Noble *et al.* demonstrated that vancomycin resistance could be transferred from *Enterococcus faecalis* to *S.aureus* but not between strains of *S.aureus in vitro*.[256] The first *in vivo* isolate of *S.aureus* displaying reduced susceptibility to vancomycin (VISA) was reported in Japan in 1995 and has been followed by several strains throughout the Western world, MIC=2μg/ml to 16μg/ml. None of these isolates carried the enterococcal *van* genes conferring high-

level resistance but were phenotypically different from strains of MRSA. Two isolates of *S.aureus* from the United States have recently been reported to carry the enterococcal *vanA* gene displaying vancomycin MIC's of 30μg/ml to >128μg/ml, thus having the designation VRSA. One of the isolates was isolated alongside *E.faecalis* and it is thought that the genes were transferred to *S.aureus in vivo.*[51,52]

Common characteristics shared by the VISA strains is the production of cell walls that vary in composition and in many cases are thicker than those of normal strains of methicillin sensitive *S.aureus* (MSSA) and MRSA. The consistency of some of the characteristics between VISA isolates has led researchers to believe that the cell walls may form a barrier to vancomycin preventing it from reaching its true target. Other features in addition to thickened cell walls have been reported suggesting that there may be one or more mechanism of resistance. The VISA strains used in this study were isolated in Japan (Mu3 and Mu50), the United States (5827 and 5836) and the United Kingdom (3700w and 3759v). All displayed vancomycin MIC's of 2-16μg/ml.

With the appearance of VISA and VRSA strains, the development of new antimicrobial agents with novel modes of action towards Gram-positive bacteria is increasingly important. An advance in new drug development has been made within the oxazolidinone, streptogramin, and lipopeptide antibiotic groups. The protein synthesis inhibitors linezolid (oxazolidinone) and synercid (streptogramin), and daptomycin (lipopeptide), a cell membrane inhibitor, have each shown promising results in the treatment of Gram-positive infections. Here, the effects of these antibiotics plus the cephalosporin cefpirome, at sub-inhibitory concentrations, were examined for activity against the aforementioned VISA strains, MSSA *S.aureus* Cowan and a local isolate of EMRSA-16 (E16). The phenomenon of antibiotic stress, as examined here, can be related to how an organism reacts to an unfavourable situation. The addition of antibiotics at sub-inhibitory concentrations to the culture medium will have variable effects on different bacterial strains depending upon their ability to cope with this form of stress. Bacteria that have been exposed to antibiotics in the past, for example strains of MRSA and VISA, may be expected to react differently than strains that have had no prior contact to antibiotics, for example *S.aureus* Cowan due to the expression of compensatory mechanisms.

5.1 Bacterial Growth and Cell Morphology

Since the isolation of the Japanese strains Mu3 and Mu50 by Hiramatsu *et al.* interest in the phenotype of these strains, in particular the thickened cell wall has developed. Abnormal levels of glutamine-non-aminated muropeptides, N-acetylglucosamine, and UDP-N-acetyl-muramylpeptides, reduced peptidoglycan cross linkage and enhanced expression of the penicillin binding proteins PBP2 and PBP2' have been described. It is interesting to note that not all of the characteristics mentioned occur in every VISA isolate.

The mechanisms conveying vancomycin resistance to *S.aureus* have not been fully characterised. It is thought however, that the thickened cell wall plays a major role. Investigations into the Japanese strains Mu3 and Mu50 established that activity of the *glmS* pathway involved in the conversion of fructose-6-phosphate and glutamine to glucose-6-phosphate was enhanced and more efficient, suggesting a role in thickened cell wall expression.[78] Glutamine deficiency resulting from increased demand may account for the levels of nonaminated muropeptides within the cell wall.[146] The type of medium used to examine cell wall production has been reported to produce variable results with medium containing high levels of amino acids and glucose for example, Brain Heart Infusion (BHI) broth is regarded to be the best medium for the expression of cell wall features.[78]

In this study the growth kinetics of the VISA strains, MSSA *S.aureus* Cowan and E16 were studied over a twenty-four hour period in the absence and presence of sub-inhibitory concentrations (½ and ¼ MIC) of the antibiotics linezolid, cefpirome, synercid and daptomycin. Representative strains were also examined under electron microscopy to identify the characteristic thickened cell walls of the VISA strains and to study the effects that ½ MIC of the antibiotics had on the bacterial cell wall. It was shown that none of the antibiotics exerted a uniform effect on all of the strains either during growth or on the bacterial cell wall.

As previously mentioned the use of BHI is recommended for investigations of the bacterial cell wall structure. In this study Mueller Hinton Broth (MHB) was used, as this is the medium of choice for NCCLS antibiotic susceptibility testing. The use of MHB as the culture medium of choice was made prior to the recommendations by Cui *et al.*[78] The culture medium was not changed in light of these findings due to the quantity of work that had already been carried out and the financial constraints surrounding the electron microscopy work. As the aim of this section was to examine the effects of ½ MIC

antibiotics on the bacterial cell wall in comparison to no drug controls and strains of MSSA and E16, the culture medium used it not critical. However, it would be interesting to re-examine the VISA isolates following culture in BHI to ascertain whether any differences in cell wall morphology occur in comparison to our findings in MHB.

Under electron microscopy, the cell wall thickness of strains *S.aureus* Cowan, E16, 3759V, Mu3 and Mu50 was very similar. The cell wall of strain 5836 was found to be significantly thicker than all of the other strains. In response to ½ MIC of antibiotics each strain reacted differently. Only the clonal strains Mu3 and Mu50 were affected in the same manner by the same antibiotics. Sub-MIC linezolid, cefpirome and synercid each significantly increased cell wall thickness. Other strains to be affected by ½ MIC linezolid were strains E16 and 5836 whose cell walls were found to have increased and been reduced respectively. Cell walls of strains E16 and 3759v were also increased by ½ MIC synercid. Daptomycin produced interesting results in that exposure to ½ MIC reduced the cell wall size in strains 3759v and 5836. It is likely that the ability of daptomycin to inhibit the incorporation of peptidoglycan into the cell wall by its action on cell membrane function is a contributing factor to these findings.

When comparing the growth of the strains in ½ and ¼ MIC antibiotics a strong relationship was found between cell wall thickness and growth rate observed over the initial eight hours of the growth cycle. For strains Mu3 and Mu50, the most dramatic increase in cell wall thickness was observed following exposure to ½ MIC linezolid. When examining the growth patterns of these strains it was found that both of these strains displayed impaired growth in the presence of ½ and ¼ MIC linezolid compared to the no drug controls. Strain E16 also produced similar patterns of growth inhibition with the greatest degree of inhibition correlating to the degree of cell wall thickening in response to ½ MIC synercid followed by linezolid and cefpirome. Gemmell and Lorian have also reported thickened cell walls in response to sub-inhibitory levels of synercid.[131] Although, this was not always the case as seen with other cell wall changes, it would appear that a thickened cell wall exerts a burden upon growing cells.

The minimum antibiotic concentration (MAC) has been defined by Lorian as the minimum concentration of antibiotic necessary to induce alterations in bacterial structure as seen by electron microscopy, and/or growth producing one log decrease in numbers compared to the no drug control.[131] It would appear that the MAC for linezolid, cefpirome, synercid

and daptomycin for the majority of VISA strains used in this study is observed at concentrations of ½ MIC.

5.2 Virulence factor expression

Humans, as a host, provide a niche for numerous commensal organisms that help to maintain the health of an individual. Infection by an organism does not necessarily lead to disease, but depends on the pathogenic nature of the organism and the host in question. The ability of a bacterium to express proteins, enzymes and toxins inadvertently makes them potential pathogens. Infection by *S.aureus* is multifactorial due to the vast spectrum of virulence factors that can be carried on its genome and subsequently expressed. The ability of an organism to colonise a host relies upon evasion of the host's defence mechanisms. The initial barrier to bacterial colonisation is the skin and antimicrobial secretions. Following attachment to the epithelial cell layer or breaching of the skin through injury, a bacterium is able to invade cells through the production of enzymes and toxins that destroy host cells releasing nutrients for growth and spread through host tissues.

The effects of antibiotics on the expression of virulence factors has been explored extensively in an attempt to evaluate how antibiotics contribute to the cure of an infection, and the advantages and disadvantages of using a particular antibiotic in particular situations. Evidence for the latter scenario is given by the preferred use of clindamycin, an inhibitor of TSST-1 production, in place of β-lactam antibiotics due to its strong inhibitory effects.[266] Protein synthesis inhibitors are generally regarded to have inhibitory effects on most virulence factors, although increased extracellular protein A expression has been reported with sub-MIC clindamycin, as has the enhanced expression of toxins by other bacterial species.[152]

Using the polymerase chain reaction (PCR) for the detection of virulence genes, each strain was examined for carriage of the staphylococcal enterotoxins (SE) A through J, exfoliative toxins (ET) A and B, staphylococcal enterotoxin-like proteins (SET-1), alpha-haemolysin, TSST-1 and protein A genes. Coagulase expression was determined by a positive reaction on a slide agglutination test. The genetic complement of each of the VISA strains was shown to be different in comparison to each other and to either *S.aureus* Cowan or E16. The only strains to carry identical genes were the clonal strains Mu3 and Mu50. From the genes detected, protein A (surface bound and extracellular) clumping factor, cell free

coagulase, alpha-haemolysin and TSST-1 were chosen to be examined further. A time point of sixteen hours was chosen to quantify the proteins and toxins to allow optimal expression and for convenience.

Enzyme-linked immunosorbent assays (ELISA) employ simple and reliable methodology to detect immune complexes formed with the desired antigen and detected by a coloured substrate. Here, we used sandwich ELISA's to quantify the levels of TSST-1, surface bound and extracellular protein A. Biological assays with rabbit plasma and rabbit erythrocytes were used to determine the activity of clumping factor and cell free coagulase, and alpha-haemolysin respectively.

5.2.1 Protein A

The expression of surface bound protein A was found to be absent from all of the VISA strains with the exception of strain 5827. The levels of extracellular protein A were detectable in some of the VISA strains but were dramatically lower than those expressed by *S.aureus* Cowan and E16. Strains 3700w and 5836 did not express either form of protein A. The lack of surface bound protein A expression, but expression of extracellular protein A by strains of MRSA and coagulase negative staphylococci (CNS) has previously been described.[107,367] The presence of thickened cell walls in strains 3759v, 5836, Mu3 and Mu50 in comparison to the high protein A producers *S.aureus* Cowan may be responsible for the lack of cell bound protein A. However, strains 3759v, Mu3 and Mu50 were of similar thickness to the protein A producer E16.

The exposure of *S.aureus* Cowan to ½ MIC synercid and E16 to ½ MIC linezolid and synercid resulted in significant increases in their cell wall thickness but reduced the amount of detectable surface bound and extracellular protein A. Similar results were observed in the levels of extracellular protein A from 3759v and Mu3 following antibiotic exposure suggesting that protein A expression may be being inhibited directly and that a thickened cell wall may reduce the quantity of protein A able to be expressed. It would have been interesting to examine the cell wall of strain 5827, the only VISA to produce both forms of protein A, under the electron microscope to determine any altered structure that may account for its difference from the other VISA strains in protein A expression. Unfortunately it was not chosen for inclusion in the limited electron microscopy study.

5.2.2 Clumping factor and Cell Free Coagulase

The expression of clumping factor by all of the VISA strains was relatively low (32 units to 256 units) in comparison to *S.aureus* Cowan and E16 (1024 units and 4096 units). None of the antibiotics tested affected clumping factor expression by any of the strains by more than one or two dilutions, with the exception of strain E16. The exposure of strain E16 to sub-MIC cefpirome, linezolid and synercid reduced titres of clumping factor from 4096 units to 1024, 1024 and 256 units respectively. Why the VISA isolates should have reduced levels of clumping factor expression is unclear, however, reduced coagulase activity in strains of VISA has been previously reported and it has been indicated that misidentification of VISA isolates could occur if the coagulase test is not incubated for greater than four hours.[242] The levels of cell free coagulase expressed by the VISA strains were also significantly lower than those produced by *S.aureus* Cowan and E16. Strain Mu3 was an exception with a titre of 512 units, the same as the Cowan and E16 strains. Of the low producers, titres ranged from 16 units in strain 3759v to 256 units in strain Mu50. The exposure of strains to ½ and ¼ MIC cefpirome reduced titres considerably, with the exception of strain 3759v where coagulase expression was increased by ½ and ¼ MIC. Daptomycin also reduced the expression of coagulase in several strains but not to the same extent as cefpirome. Neither ½ nor ¼ MIC daptomycin made any impact on the expression of coagulase by Mu50. The protein synthesis inhibitors linezolid and synercid were also found to exert inhibitory effects upon the strains but to varying degrees. It was interesting to note that strain 3759v and the positive standard strain NCTC 6571 expressed more coagulase with decreasing antibiotic concentration of all the test antibiotics. The most notable effects were observed in ¼ MIC linezolid and ¼ MIC daptomycin treated cultures of strain 3759v (increased titre from 16 units to 256 units) and ½ and ¼ MIC linezolid, daptomycin and synercid treated cultures of NCTC 6571 (increased titre from 64 units to 256 units).

Why is strain 3759v different from the other VISA strains? Strain 3759v expressed the lowest level of cell free coagulase in the absence of antibiotics yet antibiotics with three different modes of action stimulated coagulase expression. The cell walls of strain 3759v are increased in thickness by the presence of ½ MIC linezolid, cefpirome and synercid, but reduced by daptomycin. The thickest cell wall is observed under the influence of ½ MIC synercid that also reduces the clumping factor titre, and gives the lowest rise in cell free coagulase expression. It is possible that the cell wall density of strain 3759v may differ from that of the other VISA strains in its ability to allow the secretion of proteins. The

degree of peptidoglycan cross-linkage can determine the resulting density of the cell wall. If this is altered by antibiotic stress the expression of cell components in culture supernatants may be different from the levels expressed in the cell cytoplasm with the effects observed governed by the ability of the bacterium to transport them from the cell. For example, should the degree of peptidoglycan cross-linkage be reduced, it would be conceivable that larger and/or more proteins can be transported out of the cell. This would only apply to cell free proteins as cell wall bound proteins would require binding to cell wall components for expression. In this instance it could be suggested that peptidoglycan cross linkage is being reduced therefore reducing the quantity of surface proteins that can be attached but allows the export of proteins out of the cell. The reduction in cell free protein A expression is probably due to the effects of the antibiotics on protein synthesis.

5.2.3 Alpha-Haemolysin

Alpha-haemolysin is one of four haemolytic toxins produced by *S.aureus*. The haemolysin is produced by 95% of *S.aureus* strains, and is generally linked to the coagulase-positive phenotype.[1,32] *In vitro*, rabbit erythrocytes are the most sensitive cell type to the lytic action of alpha-haemolysin.[30,97] Fresh suspensions of rabbit erythrocytes were incubated with serial dilutions of culture supernatants to determine the haemolytic titre of the strains. *S.aureus* Cowan and the VISA Mu50 were found to produce the highest haemolytic titres (64 HU) followed by the VISA strains 3759v (32HU) and Mu3 (16HU). The other strains of VISA and E16 were very low producers of alpha-haemolysin with titres ranging from zero to 8HU. The absence of haemolysin activity by strains of glycopeptide resistant *S.aureus* (GISA) has been attributed to a loss of function in haemolysin production and/or an inability to secrete the haemolysin through the thickened cell wall.[300] The classification of GISA as *agr*-null strains provided evidence for the low expression of proteins and toxins.[300] (Richard Novick 2002; personal communication)

The presence of sub-MIC cefpirome in the culture medium reduced haemolytic titres by a single dilution or had no effect with the exception of reduced expression (64HU to 8HU) by Mu50. Increased haemolysin expression has been described previously in strains of staphylococci and streptococci during exposure to glycopeptide and β-lactam antibiotics respectively.[89,245,266,312] Linezolid and synercid inhibit expression by the majority of strains. It is likely that the activity of linezolid and synercid is a result of their activity on protein synthesis, inhibiting alpha-haemolysin (*hla*) mRNA translation albeit through different mechanisms. Gemmell and Ford have also reported reduced expression of

haemolysin by another strain of *S.aureus* (Wood 46) by sub-MIC linezolid. Daptomycin reduced titres by one dilution or had no effect on strains.[132] The ability of daptomycin to disrupt bacterial cell membrane potential is obviously insufficient to increase the levels of haemolysin detected and has no apparent effect on the translation and expression of alpha-haemolysin.

5.2.4 Toxic shock syndrome toxin-1 (TSST-1)

TSST-1 is responsible for the symptoms of toxic shock syndrome (TSS) although involvement of the enterotoxins SEA, SEB and SEC has been reported.[251,301,378] The classification of TSST-1 as a superantigen as a result of its biological properties makes it an interesting virulence factor to study. Only three of our strains, E16, Mu3 and Mu50, carried the *tst* gene. Detection and quantification of TSST-1 in culture supernatants in these strains was performed by ELISA.

The initial levels of toxin detected in drug-free medium were found to be significantly higher in the VISA strains than in E16 (Mu50>Mu3>E16). The fact that strains Mu3 and Mu50 are both high producers of TSST-1 and alpha-haemolysin with respect to the other VISA strains contradicts many reports detailing the lack of dual expression of these toxins. It is widely reported that TSS isolates carry the gene for alpha-haemolysin but fail to express detectable levels of the haemolysin due to a single, reversible, gene mutation.[73,262] Non-clinical strains however express both toxins.[73] Mu3 and Mu50 are clinical isolates but were not associated with TSS so phenotypically they fall into the latter category. Strains Mu3 and Mu50 behaved similarly in response to ½ MIC linezolid and synercid where toxin levels were significantly reduced. E16 on the other hand expressed significantly increased levels of toxin when grown in the presence of ½ MIC linezolid. One half MIC cefpirome was also found to increase toxin levels in strains E16 and Mu3 but reduced them in strain Mu50.

The ability of the protein synthesis inhibitors to reduce TSST-1 expression in the two VISA strains indicates an effect on toxin translation as has been demonstrated with the protein synthesis inhibitor clindamycin.[89,117,123,127,304,345,352] Why ½ MIC linezolid should increase toxin expression and synercid should have no effect in E16 is unclear considering that both of these antibiotics inhibited normal cell growth. Both of these drugs also induced thickened cell walls thus the release of TSST-1 could be affected by altered cell wall density as previously mentioned. Alternatively, the ribosomal organisation of E16

may differ from Mu3 and Mu50 and the levels of expression result from antibiotic activity on TSST-1 translation at or near to the cell membrane.

Despite the effects of the antibiotics on TSST-1 expression, the ability of culture supernatants to stimulate Tcell proliferation, a property of superantigens, was not affected to the same extent. There is no apparent relationship between the level of toxin expression and mitogenic activity. From the standards included in the assay (range 0.1μg/ml to 100μg/ml) it is clear that Tcell proliferation induced by TSST-1 is not concentration dependent. It is likely that, as the culture supernatants were used in their crude form, other superantigens may be present in since the genes for enterotoxin A (SEA), SEG and SEI are carried by the three strains and additionally SEC by strains Mu3 and Mu50. The potential expression of the enterotoxins may contribute to the levels of Tcell proliferation observed.

5.2.5 Protein A (*spa*) mRNA transcription

Determination of the levels of protein A (*spa*) mRNA from E16 during growth for 4hrs in the absence and presence of ½ MIC linezolid and synercid showed that the levels of *spa* mRNA transcript were actually increased even though the growth rate of E16 was severely inhibited in comparison to the no drug control. The significantly reduced levels of surface bound and extracellular protein A, as measured by ELISA, in response to linezolid and synercid suggests that mRNA transcripts are being produced but not being transcribed into mature protein. The increased levels of transcript observed may be due to a backlog of untranscribed mRNA but this is usually degraded suggesting that the antibiotics may be up regulating transcription or that degradation enzymes are being inhibited. Although the levels of mRNA for the other proteins and toxins studied in this thesis were not determined it seems logical to assume that, as all four of the antibiotics were found to reduce the expression of both extracellular toxins and surface bound proteins in the majority of strains, inhibition of mRNA translation is taking place.

5.3 Susceptibility to Phagocytosis

Phagocytosis is a key process in the eradication of infecting bacteria from a host. Many bacterial species express surface components that mask phagocyte binding sites or form a protective barrier around the cell preventing the adherence of opsonic complement factors. Numerous authors have investigated the process of phagocytosis and the effects that antibiotics have on the process. Of interest is the ability of antibiotics to modify a

bacterium such that its susceptibility to phagocytic uptake by polymorphonuclear cells (PMNL) is increased. There are several studies of the effects of clindamycin on strains of staphylococci and streptococci and their subsequent susceptibility to phagocytosis. The loss of protein A and M protein expression by these species by sub-MIC clindamycin increased their susceptibility to phagocytosis.[118,120,352]

In staphylococci the antiphagocytic protein of interest is protein A. The ability of protein A to bind the Fc portion of immunoglobulin (IgG) reduces activation of complement and thus IgG-mediated opsonisation of the bacterial cells, a crucial requirement for rapid phagocytic ingestion.[279,327]

The process of chemiluminescence was used to measure the quantity of light being emitted in response to PMNL respiratory burst, a process closely related to bacterial ingestion. It was noticed that strain 3759v produced a significantly reduced chemiluminescence response in comparison to *S.aureus* Cowan and all of the other strains whereas Mu3 and Mu50 produced significantly enhanced responses suggesting reduced and enhanced susceptibility to phagocytosis respectively. This would indicate that these strain 3759v expresses additional characteristic(s) that are different from the other strains enabling it to inhibit opsonophagocytosis more effectively. Strains Mu3 and Mu50 on the other hand do not express such effective antiphagocytic properties. Incubation of the strains in ½ and ¼ MIC linezolid, cefpirome and synercid produced variable but reduced chemiluminescence responses of strains E16, Mu3 and Mu50 only. Cultures of strain 3759v in ½ MIC cefpirome demonstrated increased susceptibility to PMNL ingestion.

The presence of protein A is considered to confer antiphagocytic properties upon a cell but reduced protein A expression by *S.aureus* Cowan in response to ½ MIC synercid does not alter the PMNL chemiluminescence response to the treated organism. This strain does however possess a slightly thicker cell wall in the presence of synercid. The balance between protein A expression and cell wall thickness and/or composition appears to be important in the determination of phagocytic susceptibility. Thickened cell walls are present in strain Mu3 exposed to ½ MIC linezolid, and strain Mu50 exposed to ½ MIC linezolid and synercid. The levels of protein A expressed by these strains were also significantly reduced by these antibiotics as is the chemiluminescence response, thus suggesting that modification of the cell walls may compensate for the lack of protein A in terms of susceptibility to phagocytosis. The presence of a thickened cell wall alone is not sufficient to induce antiphagocytic properties upon a cell as demonstrated by the reduction

in cell wall thickness and lack of protein A expression by strain 5836 in response to ½ MIC linezolid and daptomycin, but no alteration in the PMNL ingestion of this strain. This would suggest that other surface and cell free proteins not included in this study are being expressed and/or that the actual composition and density of the cell wall plays a role in the susceptibility of VISA strains to phagocytosis by PMNL. This conclusion was supported by the irregular observation, by electron microscopy, of electron dense material loosely associated with the cell wall. These observations were in addition to, and apparently unrelated to any expression of protein A.

There are no doubt other characteristics of the VISA strains that contribute to their resistance to phagocytic ingestion as the absence of protein A expression by these strains does not appear to render them more susceptible to opsonophagocytosis. This is evidenced by the characteristics of strain 3759v. In normal cell culture strain 3759v possesses a cell wall that is slightly thicker than that of *S.aureus* Cowan. It is a non-producer of surface bound protein A but produces low levels of cell free protein A, clumping factor and coagulase, and produces moderate levels (32HU) of alpha-haemolysin but is the most resistant strain to opsonophagocytosis.

5.3 Relationship of Present Study to Previous Investigations

The effects of sub-MIC antibiotics have been studied on both Gram-positive and Gram-negative species of bacteria. The activity of the lincosamine, clindamycin, is perhaps the best studied of the protein synthesis inhibitors. Sub-MIC clindamycin, in addition to chloramphenicol and erythromycin, have been reported to reduce the expression of fibronectin binding proteins (Fnbp) in *S.aureus* thereby reducing the strains susceptibility to phagocytosis.[88,285] In contrast, clindamycin inhibits the expression of protein A in *S.aureus* and M protein in *S.pyogenes* increasing opsonophagocytosis.[118,120,352] Extracellular proteins and toxins including coagulase, alpha-haemolysin, TSST-1 and nucleases of *S.aureus*, *E.coli* haemolysin and shiga-like toxin, and the glycocalyx of *S.aureus*, *E.coli* and *Bacillus species* are also subject to inhibition by clindamycin.[39,123,248,304,345] The latter plays a role in the inhibition of bacterial adherence to bone surfaces and potentiation of phagocytosis.[122,165,204,205,230,351,353]

Exposure to some antibiotics stimulates protein and toxin expression. For example, clindamycin enhances expression of *Clostridium difficile* enterotoxin,[158] synercid increases

the expression of the *ica* operon (involved in adhesion) in *S.epidermidis*[288] and lincomycin has been shown to increase the expression of heat labile toxin and choleragen by *E.coli* and *Vibrio cholerae* respectively.[211] Erythromycin and chloramphenicol have no effect on toxin expression and phagocytosis of group A streptococci.[118]

It is interesting to note that Milatovic *et al.* (1982,1983) reported thickened cell walls in isolates of *S.aureus* exposed to sub-MIC clindamycin.[237,238] Increased binding of the complement component C3 leads to enhanced phagocytosis of the bacterium. It was hypothesised that the thickened cell wall exposed more C3 binding sites thus promoting phagocytosis.

The cell wall active antibiotics have also been shown to affect protein biosynthesis. Increased levels of Fnbp in *S.aureus* have been reported during exposure to the β-lactams, cephalosporins, vancomycin, and ciprofloxacin, probably due to the inhibition of peptidoglycan cross-linkage thus exposing more Fnbp.[34,205] Enhanced levels of alpha-haemolysin occur in the presence of methicillin[10] and increased phagocytosis of *Pseudomonas aeruginosa* has been reported to coincide with altered morphology through β-lactam action.[375]

5.4 Summary

Throughout this thesis I have endeavoured to characterise the variable morphology and the extent of surface protein and extracellular toxin expression by strains of *S.aureus* displaying intermediate resistance to vancomycin in comparison to a strain of methicillin susceptible *S.aureus* and a local isolate of methicillin resistant *S.aureus*, and to determine the effects that antibiotic stress has on the above factors.

The results presented here suggest that strain- and species-dependent effects take place in response to antibiotics at sub-inhibitory concentrations. The ability of strains of VISA to express thickened cell walls is believed to play a role in the strain's resistance to vancomycin though the binding of vancomycin molecules at the peripheral cell wall may prevent bactericidal activity. A thickened cell wall may also hinder the passage of antibiotics into and proteins and toxins out of the bacterial cell. The composition of the cell wall is also of major importance. A lower degree of peptidoglycan cross-linkage in the cell wall, a common characteristic of VISA strains, affects the density of the cell wall and

it is plausible that the passage of molecules and proteins through the cell would also be affected. In instances of reduced cross-linkage the passage of molecules through the cell wall would be expected to be more efficient and vice versa (Figure 52). A similar efflux of proteins out of the cell may be expected to occur in response to peptidoglycan cross linkage i.e. cell walls with low-level cross linkage should allow the passage of a greater number and indeed larger molecules out of the cell.

The increased levels of toxin expression by selected strains in this study may be due to the above hypothesis or the inhibition of the enzymes that degrade toxins in the culture supernatant as proposed by Levner *et al.* (1977).[211] However, most strains of *S.aureus* produce one or more proteolytic enzymes[187] and without actually measuring the levels of protease produced by different VISA strains this idea is difficult to prove or disprove. As the majority of strains reduce surface protein and extracellular toxin expression in response to antibiotic stress it is likely that this is a result of antibiotic effects on protein synthesis as demonstrated by the effects of linezolid and synercid on *spa* mRNA and the reduced levels of protein A detectable by ELISA.

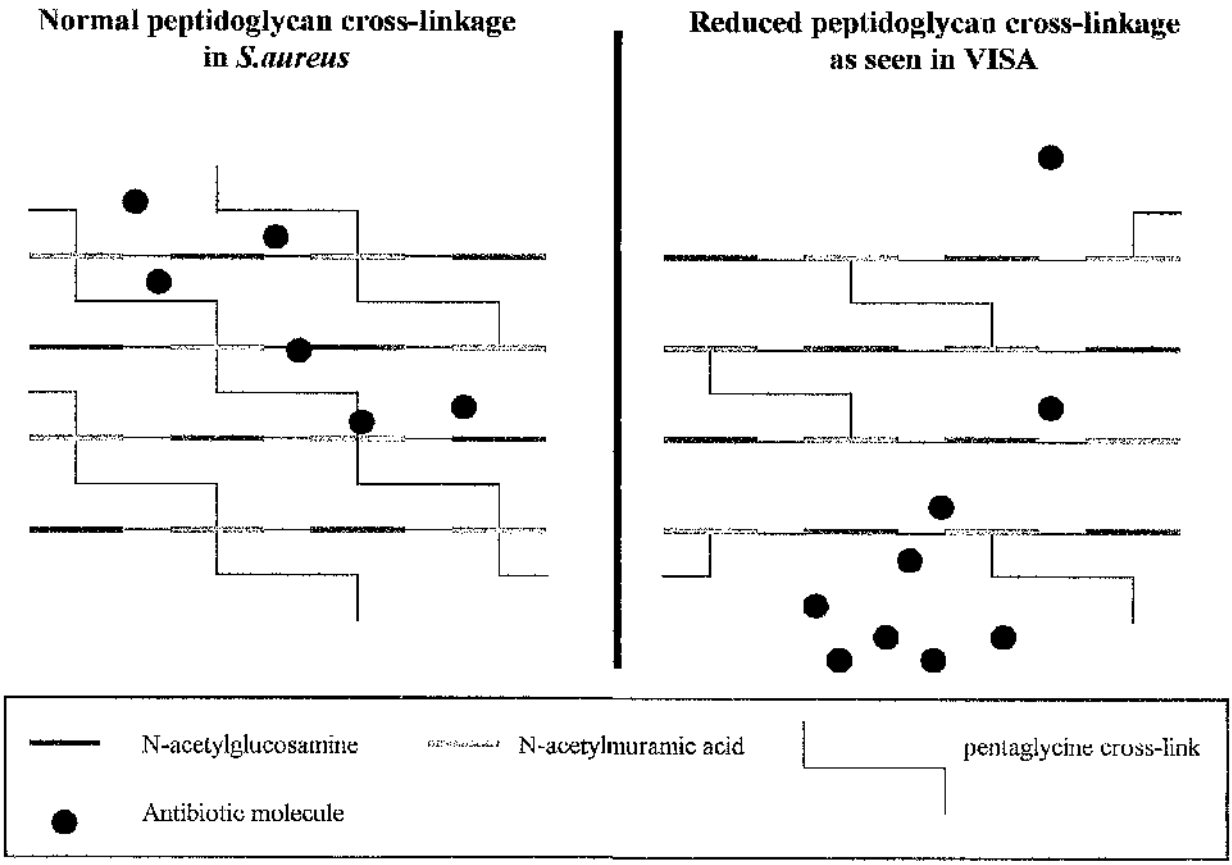

Figure 52. Proposed model of the passage of antibiotic molecules through cell walls that differ in their intrinsic density

FUTURE WORK

To develop this research further the following avenues may yield interesting results:

- To examine the cell wall morphology and extent of cross-linkage of VISA isolates to attempt to explain protein secretion and antibiotic resistance in relation to the transportation of molecules across the cell wall

- To establish whether there are pools of proteins in the cytoplasm of VISA strains in the presence and absence of antibiotic induced stress in order to support the hypothesis of inhibited protein transportation through the cell wall.

- To measure mRNA synthesis in strains of VISA in relation to the proteins and toxins examined in this study during linezolid, synercid and daptomycin stress

- To establish if there any similarities between strains of VISA and VRSA in their ability to express surface bound and cell free proteins and toxins and their consequent susceptibility to phagocytosis

- To establish what effects these agents have on linezolid resistant MRSA?

- Carry out further investigations into protein A positive VISA 5827 as this strain was the only VISA is this study to express protein A.

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APPENDIX ONE

REAGENTS

Achromopeptidase solution

1ml	TE buffer (x1)
1 vial	achromopeptidase powder (100,000 units/ml)

Vortex, leave in 50°C waterbath 15min make sure powder is dissolved → 100 units/µl

Dilute to 10 units/µl:- add 90µl TE buffer or PCR water to 10µl achromopeptidase (100 units/µl). Store -20°C

Anti-rabbit IgG gold conjugate Buffer

0.1%	BSA
0.05%	Tween 20
5%	Foetal calf serum

Dilute in 0.5M NaCl

Coagulase Tubes

120ml	BHI
20ml	Rabbit plasma
60ml	sterile saline

x1 DNase buffer

50µl	x10 DNase
500µl	RF-H_2O

Gel-Hank's solution

10ml	10x Hank's balanced salt solution (HBSS) (ICN)
10ml	1% Gelatin
80ml	dH_2O

Adjust pH with 0.1M NaOH

Leishman's Stain

Soak slides in 100% Leishman's stain for 5 minutes

Soak slides in 50% Leishman's stain in Methanol for 5 minutes

Rinse off with dH_2O

Air dry

Luminol (10^{-2}M)

1.77mg	Luminol (5-amino-2,3-dihydro-1,4 phthalazinedione)
1ml	Dimethyl sulphoxide

Dilute to 10^{-5}M with sterile PBS before use.

NET Buffer

1ml	Tissue Culture Water
1ml	TE Buffer (x1)
200µl	5M NaCl

Store at room temperature

Phosphate Buffered Saline (x10 PBS)

3.54g	KH_2PO_4
14.8g	$NaHPO_4$ anhydros
85g	NaCl

Dissolve in 1 Litre ddH_2O and filter sterilise.

PBS/0.05% Tween-20

100ml	x10 PBS
0.5ml	Tween-20
900ml	ddH_2O

PBS/Tween 20/Bovine Serum Albumin (PBS/T/BSA)

1L	PBS/0.05% Tween-20
10.8g	Bovine Serum Albumin

PNP (Stock 0.1M PNP)

0.026318g	p-nitrophenyl substrate
10ml	1M Tris

Dilute 3ml 0.1M PNP in 7ml 1M Tris → 2mM PNP

RPMI medium (Supplemented)

500ml	RMPI-1640 (Sigma)
5ml	penicillin (5000U/ml)/streptomycin (5000U/ml)
5ml	L-glutamine (200mM)
2.5ml	fungazome
2%	heat inactivated foetal calf serum

Silane Coated Slides

Soak slides in 2% Decon overnight

Rinse and leave for 1 hour in dH_2O

Soak in acetone for 5 minutes

Soak in 2% Silane in Acetone for 5 minutes

Rinse in dH_2O for 30 minutes

Dry in an oven before use

0.1M Sodium Hydrogen Carbonate ($NaHCO_3$ pH 8.2)

8.4g	$NaHCO_3$
1L	ddH_2O

0.01M $NaHCO_3$

0.84g	$NaHCO_3$
1L	ddH_2O

1M Tris

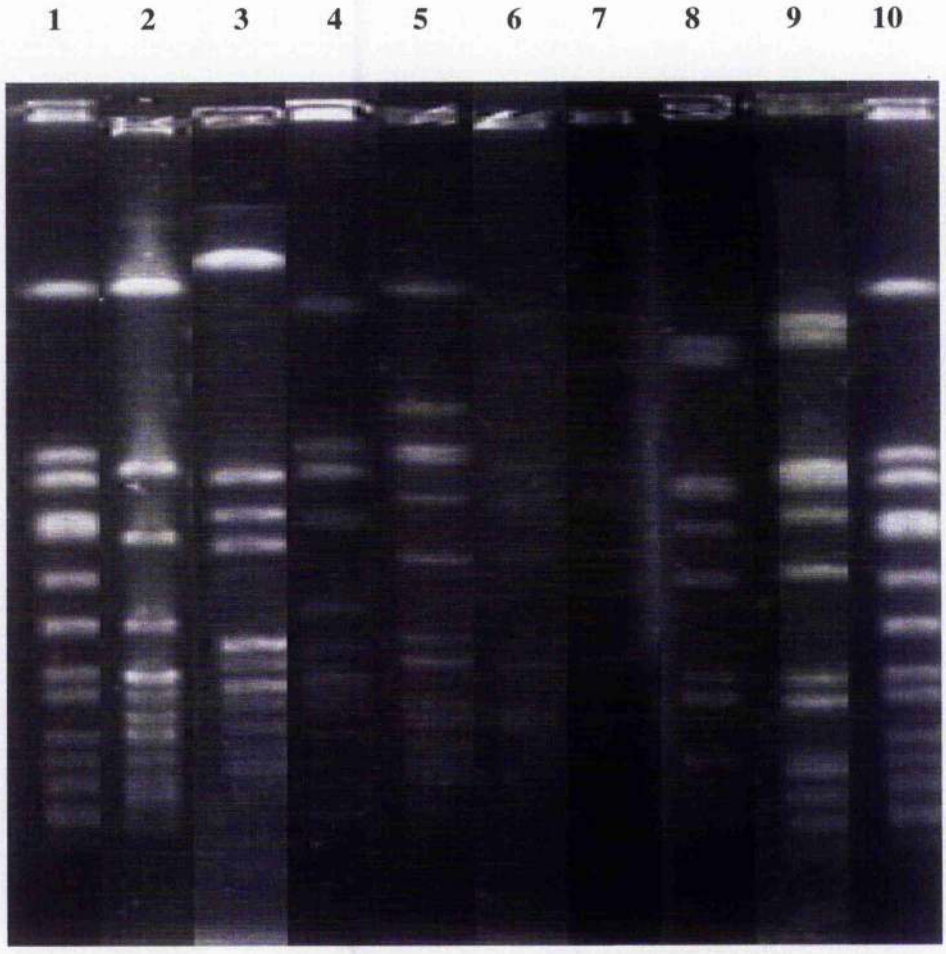
30.2758g	Trizma Base
220ml	ddH_2O

Dissolve and make up to 250ml

APPENDIX TWO

Phenotypic and Genotypic Characterisation of Bacterial Strains

A. Restriction enzyme digests (smaI) of *S.aureus* Cowan, E16 and strains of VISA by Pulse Field Gel Electrophoresis (PFGE).



Key: lanes 1 + 10 = NCTC 8325 control strain, lane 2= *S.aureus* Cowan, lane 3 = E16, lane 4 = VISA 3700w, lane 5 = VISA 3759v, lane 6 = VISA 5827, lane 7 = VISA 5836, lane 8 = VISA Mu3, lane 9 = VISA Mu50

B. Phage Type and antibiogram of bacterial strains

Strain Origin	Strain	ANTIBIOGRAM	METH (MIC)	PHAGE TYPE
Glasgow VISA	3700W	PnMtCxErGnCpTrSuTeKmStTbAk	R	Not Typable
Japan VISA	MU3	PnMtCxErClGnCpTeKmTbAk	R	6ih/85ih
LHI Type	E16	PnMtCxErClCpKmTb	R	83Cw/29ih/75w/77/83A
USA VISA	5827	PnMtCxErClGnCpTrKmTb(SuAk)	R	932ih/6ih/54w/85ih
Glasgow VISA	3759V	PnMtCxErClGnCpSuTeRfKmStTbAk	R	83C/42E/47/53/54/75/77/83A/84/85/88A/81 +
USA VISA	5836	PnMtErClCpRfKmTb	R (32)	Not Typable
Japan VISA	MU50	PnMtCxErClGnCpTeRfKmTb(Ak)	R	6ih/85ih
Cowan Strain	8530	Fully sensitive.	S (0.094)	29w/52/52A/80

APPENDIX THREE

PCR and LIGHTCYCLER PRIMERS

A. Preparation of Primer Stocks

Each primer was adjusted to a concentration of 100pmol/µl in tissue culture water (Sigma) according to the Oligo synthesis report provided (MWG primers).

Alpha-Haemolysin (*hla*) – 535bp [265]

Forward 5' GGT/ TTA/ GCC/ TGG/ CCT/ TC **3'**
Reverse 5' CAT/ CAC/ GAA/ CTC/ GTT/ CG **3'**

Enterotoxin A (*sea*) - 120bp [345]

Forward 5' TGG/ GAA/ ACG/ GTT/ AAA/ ACG/ AA **3'**
Reverse 5' GAA/ CCT/ TCC/ CAT/ CAA/ AAA/ CA **3'**

Enterotoxin B (*seb*) - 163bp [MRSA Ref Lab]

Forward 5' GTA/ TGG/ TGG/ TGT/ AAC/ TGA/ GC **3'**
Reverse 5' CCA/ AAT/ AGT/ GAC/ GAG/ TTA/ GG **3'**

Enterotoxin C (*sec*) - 271bp [MRSA Ref Lab]

Forward 5' CTC/ AAG/ AAC/ TAG/ ACA/ TAA/ AAG/ CTA/ GG **3'**
Reverse 5' TCA/ AAA/ TCG/ GAT/ TAA/ CAT/ TAT/ CC **3'**

Enterotoxin D (*sed*) - 319bp [MRSA Ref Lab]

Forward 5' CTA/ GTT/ TGG/ TAA/ TAT/ CTC/ CTT/ TAA/ ACG **3'**
Reverse 5' TTA/ ATG/ CTA/ TAT/ CTT/ ATA/ GGG/ TAA/ ACA/ TC **3'**

Enterotoxin E (*see*) - 178bp [345]

Forward 5' CAG/ TAC/ CTA/ TAG/ ATA/ AAG/ TTA/ AAA/ CAA/ GC **3'**
Reverse 5' TAA/ CTT/ ACC/ GTG/ GAC/ CCT/ TC **3'**

Enterotoxin G (*seg*) - 327bp [4]

Forward 5' CGT/ CTC/ CAC/ CTG/ TTG/ AAG/ G **3'**
Reverse 5' CCA/ AGT/ GAT/ TGT/ CTA/ TTG/ TCG **3'**

Enterotoxin H (*seh*) - 360bp [4]

Forward 5' CAA/ CTG/ CTG/ ATT/ TAG/ CTC/ AG **3'**
Reverse 5' GTC/ GAA/ TGA/ GTA/ ATC/ TCT/ AGG **3'**

Enterotoxin I (*sei*) - 465bp [4]

Forward 5' CAA/ CTC/ GAA/ TTT/ TCA/ ACA/ GTT/ AC **3'**
Reverse 5' CAG/ GCA/ GTC/ CAT/ CTC/ CTG **3'**

Enterotoxin J (*sej*) - 142bp [4]

Forward 5' CAT/ CAG/ AAC/ TGT/ TGT/ TCC/ GCT/ AG **3'**
Reverse 5' CTG/ AAT/ TTT/ ACC/ ATC/ AAA/ GGT/ AC **3'**

ExfoliativeToxin A (*eta*) - 741bp [MRSA Ref Lab]

Forward 5' CTA/ TTT/ ACT/ GTA/ GGA/ GCT/ AG **3'**
Reverse 5' ATT/ TAT/ TTG/ ATG/ CTC/ TCT/ AT **3'**

Exfoliative Toxin B (*etb*) - 629bp [MRSA Ref Lab]

Forward 5' ATA/ CAC/ ACA/ TTA/ CGG/ ATA/ AT **3'**
Reverse 5' CAA/ AGT/ GTC/ TCC/ AAA/ AGT/ AT **3'**

Nuclease (*nuc*) - 280bp [MRSA Ref Lab]

Forward 5' GCG/ ATT/ GAT/ GGT/ GAT/ ACG/ GTT **3'**
Reverse 5' AGC/ CAA/ GCC/ TTG/ ACG/ AAC/ TAA/ AGC **3'**

Panton-Valentine Leukocidin (*pvl*) – 433bp [215]

Forward 5' ATC/ ATT/ AGG/ TAA/ AAT/ GTC/ TGG/ ACA/ TGA/ TCC/ A **3'**
Reverse 5' GCA/ TCA/ AST/ TGT/ ATT/ GGA/ TAG/ CAA/ AAG/ C **3'**

Protein A Variable X-region (*spa*) - variable [113]

Forward 5' TGT/ AAA/ ACG/ ACG/ GCC/ AGT/ GCT/ AAA/ AAG/ CTA/ AAC/ GAT/ GC **3'**
Reverse 5' CAG/ GAA/ ACA/ GCT/ ATG/ ACC/ CCA/ CCA/ AAT/ ACA/ GTT/ GTA/ CC **3'**

Toxic Shock Syndrome Toxin (*tst*) - 445bp [MRSA Ref Lab]

Forward 5' AAG/ CCC/ TTT/ GTT/ GCT/ TGC/ G **3'**
Reverse 5' ATC/ GAA/ CTT/ TGG/ CCC/ ATA/ CTT/ T **3'**

Staphylococcal Enterotoxin like-protein (*set-1*) - 677bp [366]

Forward 5' GAA/ TTC/ AGA/ TTG/ GGA/ GAA/ TAA/ TAC/ TAT **3'**
Reverse 5' AGA/ TCT/ CAA/ CGT/ TTC/ ATC/ GTT/ AAG/ CTG/ C **3'**

16S rRNA - 479bp [MRSA Ref Lab]

Forward 5' GGA/ ATT/ CAA/ A(T:G 1:1)G/ AAT/ TGA/ CGG/ GGG/ C **3'**
Reverse 5' CGG/ GAT/ CCC/ AGG/ CCC/ GGG/ AAC/ GTA/ TTC/ AC **3'**

B. LightCycler Primers

Each primer was adjusted to a concentration of 100pmol/µl in tissue culture water (Sigma) according to the Oligo synthesis report provided (MWG primers).

16sRNA – 580bp

Forward 5' TAT/ TGC/ ACA/ ATG/ GGC/ G **3'**
Reverse 5' CGA/ ATT/ AAA/ CCA/ CAT/ GCT **3'**

Protein A (*spa*) – 556bp

Forward 5' GAC/ GAT/ CCT/ TCG/ GTG/ A **3'**
Reverse 5' TCG/ CCA/ GTT/ TCT/ GGT/ A **3'**

Toxic shock syndrome toxin (*tst*) – 467bp

Forward 5' CGA/ CAA/ CTG/ CTA/ CAG/ AT **3'**
Reverse 5' TGA/ GTT/ AGC/ TGA/ TGA/ CG **3'**

C. Thermal Cycler Programs for PCR Detection of Genes

Toxins, nuclease and 16s RNA [MRSA Ref Lab]

1 cycle 95^0C 5min
30 cycles 95^0C 30sec, 55^0C 30sec, 72^0C 1min
1 cycle 72^0C 5min
10^0C HOLD

Staphylococcal exotoxin-like protein 1 (SET-1) [366]

1 cycle 94^0C 5min
30 cycles 94^0C 1min, 45^0C 1min, 72^0C 1min
1 cycle 72^0C 10min
10^0C HOLD

Protein A [163]

1 cycle 95^0C 5min
25 cycles 95^0C 1min, 60^0C 1min, 72^0C 1min
1 cycle 72^0C 5min
10^0C HOLD

Enterotoxins G-J [4]

1 cycle 95^0C 10min
15 cycles 95^0C 1min, 68^0C 45sec, 72^0C 1min
16 cycles 95^0C 1min, 64^0C 45sec, 72^0C 1min
1 cycle 72^0C 10min
10^0C HOLD

VIRULENCE CHARACTERISTICS OF VISA ISOLATED FROM DIFFERENT PARTS OF THE WORLD

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Abstract

Vancomycin-intermediate *Staphylococcus aureus* (VISA) strains may differ phenotypically from strains of methicillin-resistant *S.aureus*. We compared six strains from America, Japan and the United Kingdom, a methicillin sensitive *S.aureus* (MSSA) and EMRSA16.

Cell wall thickness was examined using electron microscopy and selected strains were assayed for the production of protein A and toxic shock syndrome toxin (TSST-1) by ELISA, and alpha-haemolysin through haemolysis of rabbit erythrocytes. All strains were tested for susceptibility to phagocytosis by human polymorphonuclear (PMN) cells and their ability to induce respiratory burst.

All VISA cell walls were thicker than EMRSA16 and MSSA. Cell bound protein A was detected on only one VISA strain. Three VISA produced 640-1280 haemolytic units (HU)/ml of culture and three produced 80-160HU/ml. The VISA strains produced three-fold more TSST-1 than EMRSA16.

The presence of a thickened cell wall made no significant difference to the susceptibility of four VISA strains to phagocytic uptake. The absence of surface protein may have been compensatory. Induction of respiratory burst by four VISA strains was significantly lower than EMRSA16. We speculate that increased cell wall thickness whilst not affecting the susceptibility of VISA to phagocytic ingestion, it does affect their ability to generate respiratory burst. Higher protein A expression in MSSA may be responsible for reduced respiratory burst compared to EMRSA16.

Introduction

At present isolates of VISA strains occur sporadically albeit in several countries. It has been shown that these strains have altered cell wall composition and thickness[1,3,4]. Their ability to produce extracellular toxins or susceptibility to phagocytosis has not yet been studied.

Methods

• **Strains.** Methicillin sensitive *S.aureus* (MSSA) strain Cowan and EMRSA16 were obtained from our own collection. Vancomycin-intermediate *S.aureus* strains 3700w, 3759v (UK), 5827, 5836 (USA), Mu3 and Mu50 (Japan) were kindly donated by Dr. Donald Morrison, MRSA Reference Laboratory, Stobhill Hospital, Glasgow.

• **Electron Microscopy.** Overnight cultures pellets were fixed with 2.5% PBS-glutaraldehyde then saturated with 1% osmium tetroxide. Samples were dehydrated using ethidium hydroxide, polymerised in epoxy resin from which 60-80nm sections were cut. Sections stained/counter stained with uranyl acetate and lead citrate respectively then examined under transmission electron microscopy.

• **Phagocytic Uptake and Respiratory burst.** Fresh human PMN cells were isolated using the method of Boyum *et al* (1968)[2]. Phagocytic uptake was recorded by visual detection of ingested bacteria following a 1:1 incubation of PMN:serum opsonised bacteria for 30minutes at 37°C. Respiratory burst was measured by the chemiluminescence produced through incubation of a 1:50 ratio of PMN:bacteria in the presence of luminol at 37°C.

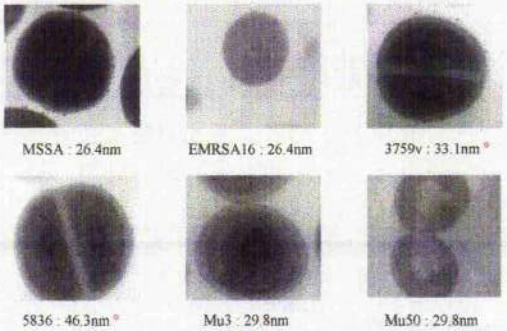
• **ELISA.** (Adapted from Yarwood and Schlievert, 2000)[5]. Protein A and TSST-1 were both detected by sandwich ELISA. Immulon4HB plates were coated with anti-protein A-TSST-1 overnight and blocked with PBS / 0.05% Tween20 / 0.01% Bovine serum albumin (P/T/B) for 2hours. Cell pellets ($1x10^7$cells/ml) for cell-bound protein A or culture supernatant for extracellular protein A and TSST-1 were incubated for 2hours after which biotin-anti-protein A or anti-TSST-1-HRP was added. Measurement of proteins was made using a plate reader 405nm following incubation with PNP (protein A) or ABTS (TSST-1) for 30minutes. Plates were washed several times with P/T after each step.

• **Alpha Haemolysin.** Doubling dilutions of culture supernatants in saline were incubated with a 2% suspension of rabbit erythrocytes for 1hour at 37°C. Titres of haemolysin were taken as the reciprocal of the dilution giving 50% haemolysis.

Results

Our studies suggest that a thicker cell wall is the primary feature that distinguishes strains with reduced vancomycin susceptibility. The ability to express proteins and toxins is not dissimilar from that of MSSA and MRSA.

Cell Wall Thickness



MSSA : 26.4nm | EMRSA16 : 26.4nm | 3759v : 33.1nm *

5836 : 46.3nm * | Mu3 : 29.8nm | Mu50 : 29.8nm

Magnification x100,000; * $p<0.05$.

Stains Mu3 and Mu50 exhibited only marginally thicker cell walls to those of MSSA and EMRSA16 whereas 3759v and 5836 displayed a significantly enlarged cell wall.

Phagocytic Uptake/Respiratory Burst

Strain	Ingested Bacteria per PMN	Respiratory Burst (mV)
MSSA	8.2 +/- 1.2	27.1 +/- 14.3 *
EMRSA16	7.6 +/- 1.3	42.6 +/- 8.7
3700w	9.7 +/- 1.3	29.4 +/- 7.2 *
3759v	9.1 +/- 1.8	9 +/- 5.7 *
5827	7.4 +/- 0.7	24.9 +/- 11.9 *
5836	9.3 +/- 1.6	29.3 +/- 8.5 *
Mu3	7.8 +/- 1.4	34.7 +/- 13.4
Mu50	6.8 +/- 0.5	35 +/- 5.9 *

Despite being ingested to the same extent there is a significant reduction in respiratory burst between EMRSA 16 and all strains (* $p<0.05$) excluding Mu3.

Virulence Factor Expression

Strain	Protein A* ($ng/1x10^7$cells/ml)	TSST-1 (ng/ml)	Alpha-Haemolysin (HU/ml)
MSSA	20.54	N/D	1280
EMRSA16	14.7	17.92	160
3700w	<10	N/D	80
3759v	<10	N/D	640
5827	<10	N/D	40
5836	<10	N/D	0
Mu3	<10	55.2	320
Mu50	<10	67.8	1280

Notes: N/D indicates not done due to absence of TSST-1 gene

* $<10ng/1x10^7$cells/ml indicates levels were below the detection limits of the ELISA

Cell-bound protein A was not observed on the VISA cell walls whereas it was readily detectable on EMRSA16 and MSSA. Only two VISA strains, Mu3 and Mu50, produced TSST-1 and did so at levels that exceed that of EMRSA 16. Alpha-haemolysin was produced by all strains but the level of expression varied. Mu50 and MSSA expressed titres of 1280HU/ml whereas 5836 failed to produce any haemolysin.

Discussion

It has been established that VISA strains:

- differ in their complement of genome encoded proteins and toxins
- vary in their ability to express proteins and toxins
- have thickened cell walls
- lack cell wall associated protein A

The absence or presence of protein A does not appear to affect the ability of human PMN to successfully ingest the bacteria however, all of the VISA and MSSA induce a lower level of respiratory burst, an indicator of bactericidal killing. Whether the increased cell wall plays a role in intracellular survival is unclear at this stage. Together with the expression of alpha haemolysin it would appear that strains of VISA are not necessarily more pathogenic with respect to their toxin complement.

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