UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Biological Sciences

Influence of MRSA Biofilm Phenotype on Resistance to Antimicrobial Agents

by

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Thesis for the degree of Doctor of Philosophy

September 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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INFLUENCE OF MRSA BIOFILM PHENOTYPE ON RESISTANCE TO ANTIMICROBIAL AGENTS

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Staphylococcus aureus is an important pathogen in foreign body-related infections. In the past five years, patient deaths due to methicillin resistant *S. aureus* (MRSA) nosocomial infections have more than doubled from 5,000 in 2001, to an estimated 11,000 in 2005, with increasing mortality. This may be related to its growing genotypic resistance to many commonly used antibiotics, including vancomycin, and biofilm-related phenotypic resistance to antibiotics and disinfectants, as an estimated 60% of all nosocomial infections are biofilm-related. Importantly, many promising antimicrobial agents may fail at clinical trial due to initial *in vitro* screening against standard planktonic cultures, which do not express the resistant biofilm phenotype present *in vivo*.

Accordingly, the aim of this study was to investigate the influence of the MRSA biofilm phenotype on resistance to antimicrobial agents, including a range of novel antimicrobial chemistries. This involved optimisation of a rapid biofilm screening protocol, using a 96-well microtitre plate format, and mutants and reagents that have been implicated in quorum sensing and biofilm physiology. A chamber slide system was used for image analysis of biofilm structure and metabolic heterogeneity of respiratory activity and EPS production. Cell viability and biomass production of immature and mature biofilms was assessed by *in situ* cell vitality, using tetrazolium salt (XTT) reduction and Safranin staining, respectively, and culturability on agar media.

Treatment with hypochlorite, hypochlorite based hospital disinfectants and peroxygen, and phenolic-based laboratory disinfectants prevented MRSA biofilm formation. However, immature and mature biofilms were partially and completely resistant, respectively, to these disinfectants. This may be one rationale for difficulty in controlling hospital MRSA outbreaks and may also be one factor in the increase in nosocomial MRSA infections.

Mature biofilms of MRSA responded to high concentrations of vancomycin (64 and 256 mg l^{-1}) by increasing the biomass and were found to be resistant to vancomycin concentrations not previously reported (2048 mg l^{-1}), suggesting that vancomycin may not be suitable for the treatment of biofilm-associated infections. Therefore, a range of antibiotics with different modes of action were also assessed for their ability to prevent biofilm formation and their activity against preformed immature and mature *S. aureus* biofilms. The ionophore antibiotics, which act against the plasma membrane, were the only group active against mature MRSA biofilms at the low concentrations of the planktonic MIC and ten times the MIC. In addition, synergistic effects were observed when ionophores were used in combination with antibiotics, including vancomycin.

Several promising novel chemistries were also identified with activity against mature *S. aureus* biofilm phenotype, even at low concentrations of ten times the planktonic MIC or less.

The QS signals PIA and AI-2 were investigated for their effects on biofilm formation and the biofilm phenotypic resistance. *agr*$^{-}$ mutants had increased biofilm formation compared to wild-type *agr*$^{+}$ strains; however, no differences in antibiotic resistance of the two biofilms were seen. On the other hand, chemically synthesised AI-2, at high concentrations, caused a reduction in biofilm formation and had a synergistic effect on biofilm phenotypic resistance.

Nitric oxide (NO), an important eukaryotic signalling molecule, was recently found to cause the dispersal of *P. aeruginosa* biofilms. It also caused a reduction in biofilm formation by MRSA, and its addition in the mid concentration range caused increased susceptibility of the mature biofilm phenotype. It is possible that NO induces cells within the biofilm to revert to the susceptible planktonic phenotype, although the mechanism for this is currently unrecognised.

It is therefore concluded that the biofilm phenotype is an important contributor to the resistance of MRSA strains, and that it is possible to affect this resistance with signalling molecules. This work highlights the importance of screening new antimicrobial agents for activity against the biofilm phenotype.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor Bill Keevil for supervising this project, and for making it possible to attend the British Biofilm Club meeting, the ASM biofilm meeting in Canada 2003 and the Clean Hospitals Summit in 2005.

I would also like to thank Sandra Wilks for helping me find my feet and for support and encouragement throughout my PhD; Ian Lipscomb for assistance with microscopy procedures and office entertainment and Jonathan Noyce for technical help with MRSA culture and information. Many thanks must also go to all of the postdoctoral staff, technicians and other PhD students that have been a part of the Environmental Healthcare Unit at the University of Southampton, for their friendship and support during my PhD.

In addition I would also like to thank Dr Richard Novick, Dr. F. Götz and Dr Bonnie Bassler for donating their genetically modified *S. aureus* strains, and to Professor Shoolingin-Jordan for donating his cyclic AI-2 analogue, for use in this project.

This work has been carried out with financial support from a BBSRC CASE studentship (02/A2/P/08380), with further financial support from Syngenta. From Syngenta, I would like to thank Fergus Early and Chris Longshaw for providing thought-provoking discussions, their patience and support throughout the later part of my PhD.

Finally, thanks must go to my friends and family, particularly to Iain and my grandparents without whose support and editing skills this PhD would never have been finished.

LIST OF ABBREVIATIONS

AB	Autoinducer Bioassay Broth
ABC	ATP Binding Cassette
ACP	Acyl-Carrier-Protein
ADI	Arginine Deiminase Pathways
agr	Accessory Gene Regulator
AI-1	Auto-Inducer 1
AI-2	Auto-Inducer 2
AIP	Auto Inducing Peptide
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
ATCC	American Type Culture Collection
AMC	Activated Methyl Cycle
BB	Bonnie Bassler (strains)
BHI	Brain Heart Infusion
$BHI_{25\%}$	Quarter Strength Brain Heart Infusion
CA-MRSA	Community Acquired Methicillin Resistant *S. aureus*
CAT	Chloramphenicol Acetyl Transferase
CCR	Chromosomal Cassette Recombinase
CFU	Colony Forming Units
CRA	Congo Red Agar
CRC	Catabolite Repression Control
c-di-GMP	Cyclic-di(3'→5')-Guanylic Acid
dH_2O	Distilled Water
DHF-A	Dihydrofolic Acid
DIC	Differential Interference Contrast Microscopy
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DPD	4;5-Dihydroxy-2;3-Pentonedione
EDIC	Episcopic Differential Interference Contrast Microscopy
EF-G	Elongation Factor G
EMRSA	Endemic Methicillin Resistant *S. aureus*
EPS	Extracellular Polysaccharide
FDA	Food and Drug Administration
FUR	Ferric Uptake Regulator
GDP	Guanosine Diphosphate
GISA	Glycopeptide Intermediate Resistant *S. aureus*

GSK	GlaxoSmithKline
GTP	Guanosine Triphosphate
HPA	Health Protection Agency
HSL	Homoserine Lactone
ICA	Intercellular Adhesion Cluster
INT	Iodonitrotetrazolium Violet 2-[4-Iodophenyl]-3-[4-Dinitrophenyl]-5-Phenyltetrazolium Chloride
LB	Luria-Bertani Broth
MAA	Marketing Authorization Application
MDR	Multidrug Resistance
MH	Muller-Hinton Broth
MHC	Major Histome Compatibility
MIC	Minimum Inhibitory Concentration
MLS	Macrolide, Lincosamide and Streptogramin$_B$ Resistance
MP-SCLM	Multi-Photon Scanning Con-Focal Laser Microscopy
MRSA	Methicillin Resistant *S. aureus*
MSCRAMM	Microbial Surface Component Recognising Adhesive Matrix Molecules
MSSA	Methicillin Susceptible *S. aureus*
MTT	Thiazolyl Blue 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyl-Tetrazolium Bromide
NAD^+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NAG	N-Acetylglucosamine
NAMA	N-Acetyl Muramic Acid
NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
NDA	New Drug Application
NHS	National Health Serivce
NM	Nano Meters
N-MRSA	Nosocomial Methicillin Resistant *S. aureus*
NO	Nitric Oxide
OD	Optical Density
PAA	Peracetic Acid
PABA	Paraminobenzoic Acid
PBP	Penicillin Binding Proteins
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pfs	5'Methylthioadenosine/S-Adeonsylhomocysteine Nucleosidase
PIA	Polysaccharide Intercellular Adhesion
PSMs	Phenol-Soluble Modulins
PMS	Phenazine Methosulphate

PNAG	Poly-N-Acetyl Glucosamine
PS	Capsular Polysaccharide
PVL	Panton-Valentine Leucocidin
QS	Quorum Sensing
RN	Richard Novick (strains)
RNA	Ribonuleic Acid
RNI	Reactive Nitrogen Intermediates
RPM	Revolutions per Minute
Sae	*S. aureus* Exprotein Expression
SAH	S-Adenosylhomocystein
SAM	S-Adenosylmethionine
sar	Staphylococcal Accessory Regulator
SCC	Staphylococcal Cassette Chromosome
SCLM	Scanning Confocal Laser Microscopy
SCV	Small Colony Variant
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SNP	Sodium Nitroprusside
SRH	S-Ribosylhomocystein
THF-A	Tetrahydrofolic Acid
TSB	Triptic Soy Broth
$TSB_{25\%}$	Quarter Strength Triptic Soy Broth
TSS	Toxic Shock Syndrome
TTC	Triphenyltertrazolium Chloride-2,3,5-Triphenyl- 2H- Tetrazolium Chloride
VNC	Viable Non-Culturable
VRE	Vancomycin Resistant Enterococci
VISA	Vancomycin Intermediate Resistant *S. aureus*
VRSA	Vancomycin Resistant *S. aureus*
v/v	Volume per Volume
w/v	Weight per Volume
XTT	Sodium 3,3-{-[(Phenylamino) Carbonyl]-3-4-Tetrazolium}-Bis(4-Methoxy-6-Nitro)

0. THE PUBLIC PERCEPTION OF MRSA

(Highfield 2002)

1. GENERAL INTRODUCTION

Since the first discovery of methicillin-resistant *S. aureus* (MRSA) in 1961 in England (Jevon *et al.* 1961), MRSA has become one of the most prevalent pathogens that cause nosocomial infections. In the past five years, patient deaths due to MRSA nosocomial infections have more than doubled from 5,000 in 2001 to an estimated 11,000 in 2005. These bacteria are gaining genetic resistance to antimicrobial agents, and there is the added complication of biofilm phenotypic resistance as an estimated 60% of nosocomial infections are biofilm related (Costerton *et al.* 1999).

1.1 Staphylococci

Staphylococcus is a bacterial genus that contains both commensal and versatile pathogens in the human body. Staphylococci are Gram-positive cocci; they are non-motile, non-spore forming, sometimes encapsulated, catalase positive, and extremely hardy facultative anaerobes. They grow by aerobic respiration or by fermentation.

Staphylococci cells are about 1 μm in diameter and are perfectly spherical; they divide in two planes allowing them to grow individually, in pairs or in irregular grape-like structures; indeed *staphyle* is a Greek term meaning a bunch of grapes. Staphylococci produce colonies that are 1 to 2 mm in diameter, smooth and translucent.

1.1.1 Taxonomy and classification

Alexander Ogston (1882) described a group of micrococci isolated from wound infections or associated with inflammation and named them staphylococci. The taxonomy and nomenclature of the genus *Staphylococcus* has been controversial and has undergone revision several times since the 1880's. Rosenbach (1884) classified staphylococci based on colony colour; *Staphylococcus aureus* produces orange/yellow colonies on blood agar, due to the production of carotenoids, and *Staphylococcus albus* produces white colonies. Ogston (1882), Verneuil (1880) and Elek (1959) supported this classification by claiming that there was a connection between the yellow appearance of pus and the growth of the infecting staphylococci.

At the end of the nineteenth century, all cocci were included in the one family Coccaceae. Taxonomic classification of the staphyloccoci was further confused when Migula (1900) subdivided the family into the genera *Streptococcus, Micrococcus, Sarcina, Planococcus* and

Planosarcina. In this classification staphyloccoci were grouped with the micrococci in the genus *Micrococcus*, despite studies showing the two genera to be different. Many further revisions were made in the early twentieth century, but it was not until Evans (1955) that staphylococci and micrococci were again separated, based on their relationship to oxygen. Species that were facultative anaerobes were placed in the genus *Staphylococcus*; species that were obligate aerobes were considered as *Micrococcus*. Schleifer (1972) also reported differences in cell wall composition, while Silvestri (1965) reported a clear distinction, based on DNA base composition, with staphylococci having a G+C molar ratio of 30-39%, while micrococci have a ratio of 63-73% (Table 1).

Table 1 *Summary of differences between staphylococci and micrococci*

Character	Staphylococci Sp	Micrococci Sp	Reference
DNA G+C Ratio	30-39%	63-73%	Silvestri *et al.* 1965
Teichoic acids	+	-	Schleifer *et al.* 1972; Kloos 1975
Aliphatic hydrocarbons in neutral lipids	-	+	Kloos *et al.* 1974
Respiration	Facultative anaerobe	Aerobic	Evans *et al.* 1955
Glucose fermentation	+	-	Evans *et al.* 1955

This resulted in the removal of *Staphylococcus* from the family *Micrococcus*, which has been dissected into new genera (Stackebrandt *et al.* 1995). *Staphylococcus* is now in the class Bacilli with other low G+C ratio bacteria.

Phylum: Firmicutes
Class: Bacilli (Low G+C ratio)
Order: Bacillales
Family: Staphylococcaceae
Genus: *Staphylococcus*

(Bergey's Manual of Systematic Bacteriology 2nd edition).

1.1.2 *Staphylococcus* species

Based on the analysis of DNA-DNA hybridisation, there are currently 38 species and 24 subspecies of *Staphylococcus* recognised. However, only a small number of these are able to colonise humans (Table 2), and two species are thought to be particularly important in human disease, *S. epidermidis* and *S. aureus*.

Table 2 *Species of Staphylococcus*

Species	Sub-Species	Natural Host	Reference
S. aureus	*Anaerobius*	Primates (inc. humans) Poultry, + Cattle	De La Fuente *et al.* 1985
	Aureus	Primates (inc. humans) Poultry, + Cattle	Rosenbach 1884
S. auricularis		Primates (inc. humans)	Kloos *et al.* 1983
S. capitis	*Capitis*	Goats + Primates (inc.	Kloos *et al.* 1975b
	Urealyticus	Goats + Primates (inc.	Bannerman *et al.* 1991
S. caprae		Goats + Primates (inc.	Devriese *et al.* 1983
S. epidermidis		Primates (inc. humans) + Eisenia fetida (Red Worm)	Winslow *et al.* 1908
S. haemolyticus		Primates (inc. humans)	Scheifer *et al.* 1975
S. hominis	*Hominis*	Primates (inc. humans)	Schlefier *et al.* 1975
	Novobiosepticus	Primates (inc. humans)	Kloos *et al.* 1998
S. intermedius		Felines, Canines, + Humans	Hajek 1976
S. lugdunensis		Primates (inc. humans)	Freney *et al.* 1988
S. pulvereri		Mammals (inc. humans)	Zakrzewska-Czerwinska *et al.* 1995
S.		Primates (inc. humans)	Kilpper-Balz *et al.* 1981
S. simulans		Felines, Canines + Humans	Kloos *et al.* 1975
S. warneri		Poultry, Primates (inc.	Kloos *et al.* 1975b
S. xylosus		Primates (inc. humans), Rodentia, Gypsy moth larva	Kloos *et al.* 1975b

Adapted from the National Centre for Biotechnology Information (NCBI)
(http://www.bacterio.cict.fr/s/staphylococcus.html)

1.1.3 Staphylococcus aureus

S. aureus is found in the normal flora of around 30% of adults. It can colonise areas such as the axillae (under arm), the inguinal (groin), the perineal (perineum) and the anterior nares (nostrils) (von Eiff 2001). According to Wenzel *et al.* (1995), there are three patterns of carriage for *S. aureus*; those people who always carry a strain (11-32%), people who carry the organism intermittently with various strains, and a small minority of people who never carry *S. aureus.*

S. aureus possesses many properties that contribute to its virulence, which can be both surface attached or extracellular. Clumping factor is an example; it is a cell-fixed protein that attaches specifically to fibrinogen and fibrinin in the plasma, thus aiding colonisation of wound surfaces. Plastic devices become coated with fibrinogen shortly after insertion, thus making them a target for colonisation. Another virulence factor that aids colonisation of wounds is Protein A. Protein A, another component of the cell surface, binds to IgG by the Fc portion of the immunoglobulin molecule. Not only does it compete with the Fc receptors of phagocytes for antibody molecules, but it also coats the bacteria with host proteins and therefore hides them from phagocytes and the cells responsible for immunity. Table 3 summarises the properties of *S. aureus* that contribute to its virulence. The bacterium also produces a variety of extracellular enzymes that probably do not contribute directly to its virulence, but supply it with nutrients by degrading blood cells and components of damaged tissue.

Table 3 *Properties of S. aureus implicated in its virulence*

Virulence Factor	Action Site	Action
Clumping factor	Bacterial surface	Attaches bacterium to fibrin, fibrinogen and plastic devices
Fibronetin-binding protein	Bacterial surface	Attaches bacterium to acellular tissue - endothelium, epithelium, clot and indwelling plastic devices
Protein A	Bacterial surface, Extracellular	Competes with Fc receptors of phagocytes; coats bacterium with host's immunoglobulin
α-Toxin	Extracellular	Makes holes in host cell membranes
Leukocidin	Extracellular	Kills neutrophills or causes the release of their enzymes
Enterotoxins	Extracellular	Superantigen, if systemic, cause toxic shock; and food poisoning if ingested
Toxic shock syndrome toxin-1	Extracellular	Superantigen, if systemic, causes toxic shock

(Taken from Nester *et al.* 2004)

S. aureus causes many clinically important infections, being responsible for 50% of skin infections. Morbidity in normal and immunocompromised patients can be affected by three main syndromes: (i) superficial lesions, abscesses (such as furuncles, carbuncles and folliculitis), and wound infections (staplylococcal impetigo); (ii) deep-seated and systemic infection e.g. osteomyelitis, endocarditis, pneumonia and bacteraemia; and (iii) toxaemic syndromes, e.g. Toxic shock

syndrome (TSS) caused by TSS toxin-1 (TSST-1), staphylococcal scarlet fever, scalded skin syndrome (SSS) caused by exofoliatins, and staphylococcal food poisoning (Arbuthnott 1990; Lina 1997 and Novick 2000). Mortality can occur in the young, old and immuno-compromised patients.

1.1.4 Superantigens

Around 75% of all of TSS cases are due to the production of TSST-1; a particular kind of exotoxin produced by about 5% of *S. aureus* strains. The remaining 25% are caused by exotoxins called enterotoxins. Both *S. aureus* exotoxins are superantigens.

Superantigens, unlike conventional antigens, bind simultaneously to the outer portion of the MHC-II molecules and the T-cell receptor. They effectively short-circuit the normal control mechanisms inherent in antigen processing and presentation. Whereas most antigens stimulate about 1 in 10,000 T cells, superantigens can stimulate as many as 1 in 5 T cells. The activation of very large numbers of T4-lymphocytes results in the secretion of both lymphocyte-derived cytokines (interleukin (IL-2), tumour necrosis factor ß, g-interferon) and monocyte-derived cytokines (IL-1, IL-6, tumour necrosis factor A). Circulation of high levels of IL-2 in the blood can lead to symptoms such as fever, nausea, vomiting, diarrhoea, and malaise.

S. aureus enterotoxins are unusual in that they are heat-stable and are retained in cooked food. When ingested, *S. aureus* enterotoxins causes nausea and vomiting, and may lead to TSS. *S. aureus* can grow well on high salt foods, such as ham, which is not always adequately refrigerated, making it a particularly dangerous pathogen.

1.2 Antibiotic resistant mechanisms

Antibiotic resistance now occurs on a regular basis for the majority of microbial species that will have been exposed to antibiotic treatment when infecting patients or applied in animal husbandry and veterinary practice. The principle resistance mechanisms involve a phenotypic adaptation to the local environment, involving biofilm formation and quorum sensing, and changes in the genotype through mutation and / or the acquisition of resistance genes. These phenomena will now be described.

1.2.1 Biofilm phenotype

A biofilm is a highly ordered sessile community of multilayered micro-colonies, interspersed with water channels and embedded in extracellular polymers (polysaccharides, glycoproteins and

proteins). Anton Van Leeuwenhoek discovered the biofilm phenotype when he examined dental plaque in 1683. However, it was not until the 1970's that biofilm was recognised as the dominant structure for bacterial colonisation. An estimated 99% of bacteria exist in biofilms, while only 1% exists in a free-floating (planktonic) state (Fergie *et al.* 2004). Biofilms are thought to be the stable point in the biological cycle for many bacterial species.

Pathogenic and commensal bacteria are especially adaptable, having to cope with the transition between life in the environment and life within the human body, especially the dramatic changes in nutrient availability and the host's primary and secondary immune defences (Jefferson 2004a). The ability to grow in a biofilm is a clinically relevant phenotypic adaptation, helping biofilm-forming bacteria to cope with the stress of its ever-changing environment and providing resistance to the bodies' defence mechanisms. Indeed, biofilm formation has recently been recognised as an important aspect of many bacterial diseases. An estimated 65% of bacterial diseases involve biofilm formation (Costerton *et al.* 1999), including common bacterial infections such as osteomyelitis, dental caries, middle ear infections and biomedical implant infections (Jefferson 2004a). In fact, the biofilm-forming ability of *S. epidermidis* and *P. aeruginosa* has long been shown to support infections of these opportunistic pathogens (Baldassarri *et al.* 2001) (Table 4). Established biofilms can tolerate antimicrobial agents at concentrations many times that needed to kill genetically identical planktonic cells (Lewis 2001).

Table 4 *A list of the most common diseases / infections involving biofilms*

Disease or infection	Bacterial species
Dental caries	*Streptococcus* sp.
Otitis media	*Haemophilus influenzae*
Cystic fibrosis pneumonia	*Pseudomonas aeruginosa*
Endocarditis	Streptococci / staphylococci
Necrotizing fascitis	Group A streptococci (*S. pyogenes*)
Bacterial prostatits	*Escherichia coli*
Infected sutures	Staphylococci
Mechanical heart valves	Staphylococci
Vascular grafts	Gram-positive cocci
Arteriovenous shunts	Staphylococci
Urinary Catheter infections	*E. coli* / staphylococci
Orthopedic prosthesis	Staphylococci

(Adapted from Costerton *et al.* 1999)

Biofilm formation

Although biofilms formed in human infections and on medical implants can be single species, infection-associated and environmental biofilms are mostly heterogeneous, containing both anaerobic and aerobic species (O'Toole *et al.* 2000). Biofilm formation is initiated in response to environmental cues including local nutrient availability, osmotic stress, temperature, and toxic molecules.

Staphylococcal biofilm formation is a well-characterised process consisting of two main stages. In the first stage, primary attachment, the bacterium encounters a substratum; this stage is reversible and the cells may easily detach. Within the host's body, initial adherence is likely to be mediated in part by cell wall–associated adhesins, including the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).

S. aureus produces an abundance of MSCRAMMs, which bind to host extracellular matrix proteins including clumping factors A and B, fibronectin-binding factors A and B (FnbpA and FnbpB), fibrinogen-binding protein clumping factors A and B (ClfA and ClfB) and a collagen- binding protein (Cna) (Froman *et al.* 1987; McDevitt *et al.* 1997; Ni Eidhin *et al.* 1998 and Patti *et al.* 1994). *S. aureus* protein A has not only been found to bind to the Fc domain of IgG, but also to mediate attachment of *S. aureus* to van Willebrand factor, a protein required for normal platelet adhesion (Hartleib *et al.* 2000). In many species, especially the oral staphylococci, once a biofilm is established, the expression of MSCRAMMs is suppressed, suggesting that the main role of MSCRAMMs is in the initial attachment and, when the biofilm is fully formed, these proteins are no longer needed (Jefferson 2004a).

In the second stage of biofilm development the cells become irreversibly attached, multiply and form a mature, multi-layered, structured community. New cells attach to the substratum or to existing biofilm cells (Cramton *et al.* 1999). This stage is associated with the production of extracellular factors, including the production of extracellular polysaccharide (EPS). EPS or 'slime' is a component of the cell surface and biofilm layer that mediates cell adherence to biomaterials. EPS is a complex hydrated polyanionic polysaccharide matrix produced by polymerases in a phase variable manner correlating with biofilm formation. EPS can act as an ionic exchange matrix capable of trapping nutrients, nucleic acids, proteins and metal ions (Dunne 2002). It also binds water to help resist desiccation and gives mechanical stability to the biofilm, helping to prevent sloughing. The exact chemical constituents of the *S. aureus* EPS have yet to be determined (Baldassarri *et al.* 1996 and Mack *et al.* 1996), although, a major component of EPS is poly-N-acetyl glucoasamine (PNAG) polymer, which is closely related to the polysaccharide intercellular

adhesin (PIA) found in *S. epidermidis*. PIA / PNAG is synthesized by four enzymes, IcaA, D, B and C, which are encoded by the intercellular adhesion cluster (*ica*); they mediate the aggregation of bacterial cells (Cramton *et al.* 1999).

IcaA, a glucosyltransferase, and its chaperone, IcaD, together synthesise sugar oligomers using UDP-N-acetylglucosamine as a substrate (Cramton *et al.* 2001). IcaB, a de-acetylase, and the IcaC protein are thought to be involved in secretion of the polymer (Jefferson 2004a). *S. aureus ica* deletion mutants are no longer able to form biofilms, demonstrating that the *ica* genes and, therefore, PIA / PNAG production are required for biofilm formation (Cramton *et al.* 1999; Heilmann *et al.* 1996). Not only is PIA / PNAG critical for biofilm formation, but it also plays an important role in virulence. Presence of the *ica* locus is important for certain types of human infections and animal infection models (Jefferson *et al.* 2006).

In recent years, several factors have been identified that influence PIA / PNAG production or biofilm production, which have been summarized in Table 5 below.

Table 5 *Summary of factors that influence PIA and biofilm formation*

Factor	Reference
Factors that influence PIA / PNAG expression	
Glucose	Mack *et al.* 1992
Glucosamine, N-acetylglucosamine	Gerke *et al.* 1998
Oleic acid	Campbell *et al.* 1983
Urea (*S. saprophyticus*)	Hjelm *et al.* 1991
Anerobiosis	Cramton *et al.* 2001
Fe limitation	Deighton *et al.* 1993; Elci *et al.* 1995 and Evans *et al.* 1994
High osmolarity, high temperature	Rachid *et al.* 2000
Ethanol (*S. epidermidis*)	Knobloch *et al.* 2001
Global regulators	
SigB (*S. aureus*)	Rachid *et al.* 2001
RsbU (*S. epidermidis*)	Knobloch *et al.* 2001
agr (*S. aureus*)	Vuong *et al.* 2000
Sar (*S. aureus*)	Pratten *et al.* 2001

The *ica* locus also codes for capsular polysaccharide adhesin (PS/A). PS/A is a component of the cell surface and biofilm layer that also mediates cell adherence to biomaterials. The exact chemical components of PS/A have yet to be determined (McKenney *et al.* 1998). It is related but distinct

from PIA, with both sharing the same β-1-6-linked-polyglucoasmine backbone but differing in the primary constituent on the amino groups.

Biofilm biomass is determined by a balance between attachment, growth, and detachment processes. Some of the factors that have been suggested to be important in biofilm detachment include nutrient levels (Hunt *et al.* 2003), fluid shear stress, abrasion / erosion (Picioreanu *et al.* 2001), quorum-sensing signals, and matrix-degrading enzymes (Allison *et al.* 1998). Detachment of viable cells from established biofilms may then allow staphylococci to spread and colonize new sites.

Recently there have been several papers published on the subject of why bacteria form biofilms; there are four main points of view: (i) colonisation; the nutrient rich human body is an ideal environment for bacteria, and most commensal bacteria exist as biofilms, especially in the gut; (ii) community; living in a community can bring advantages, such as the division of labour and cooperative/altruistic behaviour, and biofilm formation is the ideal environment for horizontal gene transfer (Cvikovvitch *et al.* 2003); (iii) that biofilm is actually the default mode of growth; (iv) the most interesting point is one of defence, as biofilms are generally more resistant to antimicrobial treatment than planktonic cultures (Jefferson 2004a).

Biofilm resistance

Biofilms have increased resistance to antibiotics and there are several theories to explain this, e.g. the physical barrier theory (slime); it was generally considered that antimicrobials could not penetrate the full depth of the biofilm. However, Jefferson *et al.* (2004b), found that vancomycin can penetrate the very deepest layers of an *S. aureus* biofilm in around 60 minutes; other compounds and antibiotics such as tetracycline have also been shown to readily penetrate biofilms (Stone *et al.* 2002).

A second theory, the enzymatic obstacle theory, states that a biofilm exhibits an altered microbial metabolism or a different genotypic response to adherence (Gorman *et al.* 2001). Adherent bacteria may express different genes from their planktonic counterparts, thereby modifying their phenotype. Some cells in a biofilm may experience nutrient limitation and therefore exist in a slow-growing or starved state; slow-growing or non-growing cells display reduced susceptibilities to many antimicrobial agents (Becker *et al.* 2001). Indeed, many studies have shown that planktonic cells in the stationary phase show striking similarities to cells within a biofilm; both are affected by nutrient limitation and high cell densities and express similar degrees of antibiotic tolerance (Fux *et al.* 2004). However, although bacterial starvation explains antimicrobial tolerance in the depths of a

biofilm, surface layers should remain susceptible, allowing biofilms to be cleared layer by layer, but this is not the case.

Beenken *et al.* (2004) identified 580 differently expressed genes in biofilm by comparison to either or both exponential- or stationary-phase planktonic cultures. Several of the operons that were induced in biofilms have been found to be important in acid tolerance in other bacterial species. Including genes from the urease and arginine deiminase (ADI) pathways, which are involved in the generation of ammonia, which bacteria use to neutralise acidic environments. Also, a large number of genes that are part of the *sarA* operon were also differentially expressed in biofilms. The *sarA* operon in *S. aureus* is also implicated in acid tolerance response. It has been speculated that the difference in gene expression is also affected by oxygen limitation in the deeper biofilm layers, which has been shown for *E. coli* reporter strains in sample biofilms (Robinson *et al.* 1996).

Biofilms provide the perfect environment for gene transfer

Biofilms also provide the ideal niche for the accelerated exchange of extra-chromosomal DNA responsible for antibiotic resistance, virulence factors and environmental survival capabilities, thus making it the perfect environment for the emergence of multi-drug resistant pathogens (Donlan 2002; Hausner *et al.* 1999). Resistance genes are often introduced from other bacterium via the transfer of a plasmid. These resistance-conferring plasmids are present in virtually all bacteria. Plasmids and chromosomes are both capable of individual existence and replication in the cell. The most probable reason for increased conjugation within biofilms is that the biofilm environment provides minimal shear and cells are in close contact with one another (Prakash *et al.* 2003).

1.2.2 Quorum sensing influencing cell phenotype

Clearly an important feature of biofilm formation is the cohabitation of microbial communities with high species diversity in dense populations. It is noteworthy that many species of microorganisms have evolved signaling systems that allow gene expression to be coordinated in a multicellular fashion, particularly at high cell concentrations, as in biofilms. Production and accumulation of signal molecules, also called 'autoinducers', enables individual cells to sense when the minimal number of bacteria has been achieved for a coordinated response, where gene expression is controlled in a cell density-dependent manner; this phenomenon is termed quorum sensing (QS) (Fuqua *et al.* 1994). The signal is received by a surface protein, which activates a transduction signal pathway, causing the up-regulation of the signal molecule. This auto-induction caused by cell density explains why some disease-causing virulence factors are not expressed during early stages of infection.

QS-controlled multicellular behavior includes a variety of physiological and morphological processes, e.g. bioluminescence, antibiotic biosynthesis, biofilm differentiation, plasmid conjugal transfer, competence for DNA uptake, and the production of virulence determinants (Bassler *et al.* 1999 and Winzer *et al.* 2002).

The *lux* system

The cell communication QS system was first discovered in the Gram-negative luminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* over 25 years ago. Both are most commonly known for their symbiotic relationships with many marine eukaryotes, where they exist at high population densities with other species of bacteria, and luminesce (Nealson *et al.* 1979). However, both can also be found free-living in the sea (Bassler *et al.* 1997).

In *V. harveyi*, two QS systems (signalling system 1 and 2) function in parallel, to control the density-dependent expression of bioluminescence (the luciferase structural operon *luxCDABE*) (Surette *et al.* 1999). Sensory information from both systems is integrated via a shared regulatory protein (Bassler *et al.* 1994; Freeman *et al.* 1999 and 1999B). Genetic analysis of *V. harveyi* has shown that activation of system 1 or 2 is sufficient for the density-dependent expression of luminescence (Surette *et al.* 1999). Signalling system 1 is a high specificity, species-specific system, whereas signalling system 2 is a species non-specific system (Surette *et al.* 1998). Each system is composed of a sensor (LuxN and LuxQ), which are two-component proteins of the hybrid-Kinase class, each containing a sensor periplasmic domain, a histodine-kinase domain and a response regulator domain (Bassler *et al.* 1995). They also contain a cognate autoinducer (AI-1 and AI-2 respectively) (Miller *et al.* 2001), belonging to a family of bacterial two-component adaptive regulatory proteins.

AI-1 is N-(3-hydroxybutanoyl)-homoserine lactone (HSL). It is, however, different to the acyl-HSL of *V. fischeri* and many other Gram-negative bacteria, in that its synthesis is not dependent on a LuxI-like protein (autoinducer synthases). However, the production of AI-1 does require the LuxL and LuxM genes (Cao *et al.* 1989), which share no homology with the LuxI family, although the biosynthetic pathway is thought to be similar (Miller *et al.* 2001).

AI-2 is a novel furanosyl borate diester consisting of two-fused five membered rings, bearing little or no resemblance to other characterised autoinducers (Chen *et al.* 2002). AI-2 is thought to be the ultimate 'Universal' signal for inter-species communication. Indeed, Surette *et al.* (1998) identified LuxS (a synthase responsible for production of AI-2 in *V. harveyi*, *Escherichia coli* and *Salmonella typhimurium*) and AI-2 in over 40 species of Gram-negative and Gram-positive bacteria. AI-2 is produced from S-adenosylmethionine (SAM) in three enzymatic steps (Figure 1). SAM is an

essential co-factor for processes such as DNA, RNA and protein synthesis. The use of SAM as a methyl donor (catalysed by methyltransferases) in these and other metabolic processes produces the toxic intermediate S-adenosylhomocysteine (SAH), which is hydrolysed to S-ribosylhomocysteine (SRH) and adenine by the nucleosidase Pfs (5'methylthioadenosine/S-adeonsylhomocysteine nucleosidase). LuxS catalyses the conversion of SRH to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (Chen *et al.* 2002). The DPD then forms a cyclic molecule and spontaneously forms a furanone (pro-AI-2) and an active furanosyl borate diester is formed in the presence of borate.

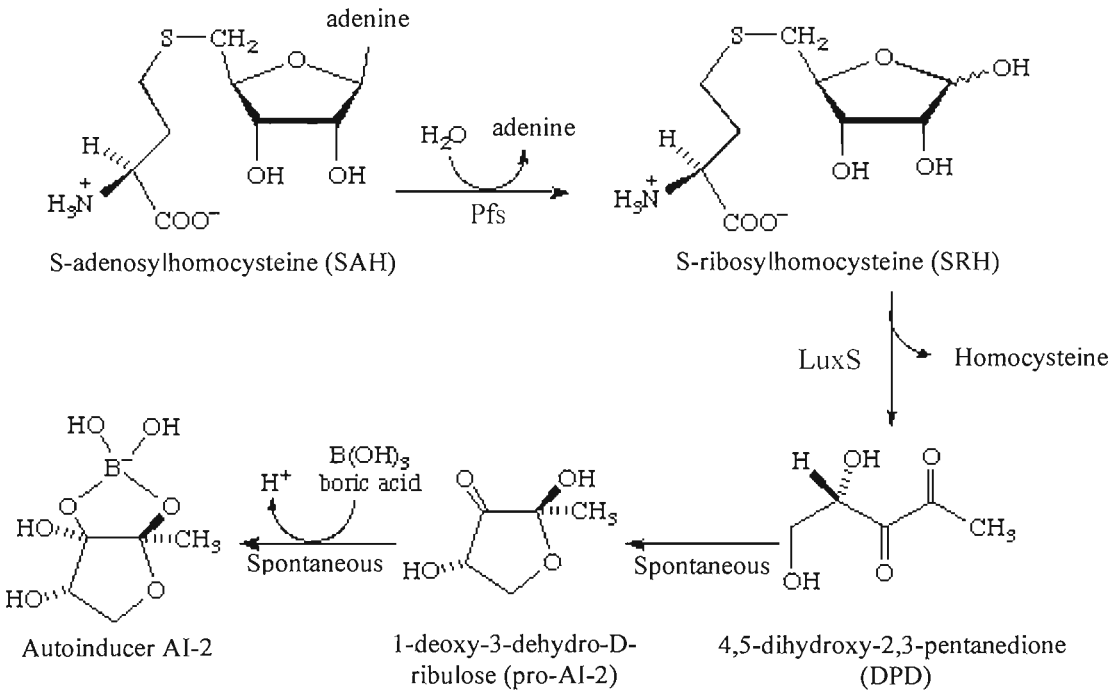


Figure 1 *A simplified scheme of Autoinducer AI-2 biosynthesis, showing the enzymes involved (Green). Adapted from http://www.chem.qmul.ac.uk.*

The detection of the AI-2 signal molecule occurs via a complex phospho-relay system (Winzer *et al.* 2002). Two proteins are required, LuxP and LuxQ (Miller *et al.* 2001). LuxP, a soluble periplasmic protein resembling the ribose and galactose binding proteins of *E. coli* and *S. typhumurium*, is thought to be the primary AI-2 receptor as it is free in the cytoplasm and binds tightly to AI-2 (Chen *et al.* 2002). LuxQ is a two-component hybrid sensor kinase, bound within the bacterial inner membrane. Interaction of the sensors LuxN and LuxPQ with their cognate autoinducing ligands AI-1 and AI-2 is proposed to transduce a signal to a shared response regulator protein called LuxO, a two-component response regulator (Bassler *et al.* 1994b). LuxO mutants express luminescence continuously (Bassler *et al.* 1994b) indicating that LuxO is a negative regulator of luminescence in *V. harveyi*. Freeman 1999, proposed that un-phosphorylated LuxO is

inactive and phosphorylated LuxO (P-LuxO) is the repressor. Phosphorylation of LuxO is carried out by LuxU, a phosphotransferase protein, which accepts phosphate from LuxN and LuxQ, and transfers the signal to LuxO. At low cell densities LuxN and LuxQ autophosphorylate and convey phosphate through LuxU to LuxO. Phospho-LuxO indirectly represses *lux*CDABE expression. Therefore, no light is produced under these conditions. At high cell densities, when LuxN and LuxQ interact with their autoinducer ligands, they change from kinases to phosphatases that drain phosphate away from LuxO via LuxU. Unphosphorylated LuxO is inactive. LuxR binds the *lux*CDABE promoter and activates transcription. Therefore, under these conditions, the bacteria produce light (Figure 2) (Freeman *et al.* 1999 and Miller *et al.* 2001). Recent evidence suggests that the alternative sigma factor, sigma-54 (σ^{54}), is required for LuxO function and that LuxO-σ^{54} repression of *luxCDABE* is indirect. Therefore, LuxO-σ^{54} are dependent activators of an as yet unidentified repressor of *luxCDABE* expression (Lilley *et al.* 2000).

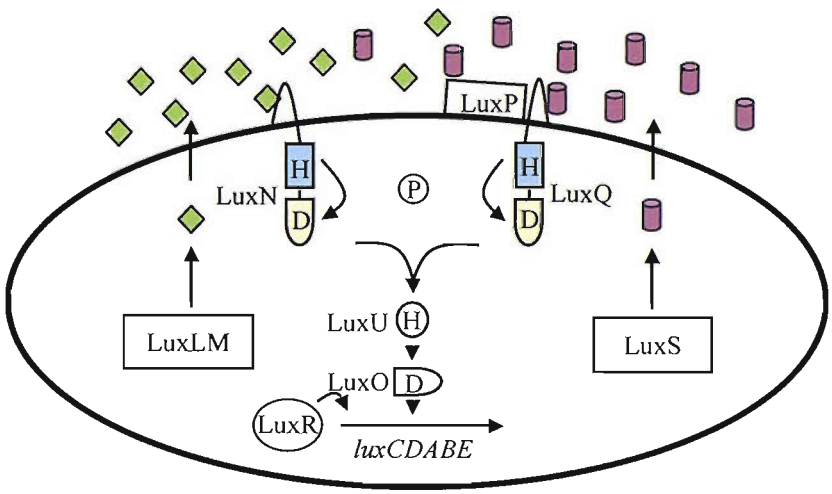


Figure 2 *The hybrid HSL-two-component quorum sensing circuit of V. harveyi (Miller et al. 2001).*

AI-1 are represented as Green Diamonds and AI-2 as Purple Cylinders. The sensor kinase domains of the LuxN and LuxQ histidine sensor kinases (Blue), are autophosphorylated on a conserved histidine residue (H) and subsequently the phosphoryl group is transferred to a cognate response regulator protein. The response regulator (Yellow) is phosphorylated on a conserved aspartate residue (D). P represents the phosphorylation cascade.

Quorum sensing in *S. aureus*

The temporal expression of many *S. aureus* virulence factors is coordinately regulated by several well-characterized genetic loci: the accessory gene regulator (*agr* operon), the staphylococcal accessory regulator (*sarA* gene) and *S. aureus* exoprotein expression (*sae)* (Cheung *et al.* 1992).

Accessory gene regulator (*agr*)

An RNA molecule called RNAIII regulates density-dependent pathogenicity in *S. aureus*. RNAIII has the unique property of acting as a messenger while it also encodes δ-hemolysin gene *hld*, which is not involved in regulation (Janzon *et al.* 1989; Morfeldt *et al.* 1995). RNAIII acts primarily at the level of target gene transcription and independently regulates the transcription of at least two exoproteins, and also has multiple regulatory functions. Expression of RNAIII is temporal, with maximal expression occurring in the transition from the post-exponential to the stationary phase. It has been postulated that this temporal expression is responsible for the repression of the expression of diverse surface proteins such as the cell surface proteins, protein A, coagulase and fibronecting-binding protein, during the exponential phase; and the enhanced expression of extracellular toxins and exoproteins such as proteases, haemolysins, TSSS-1 and enterotoxin B, during the post-exponential phase (Janzon 1990; Kornblum *et al.* 1990; Novick *et al.* 1993 and Novick 1999).

The *agr* locus encodes a self-inducing, pheromone-sensing, two-component signal transduction system (Figure 3). It consists of two divergent transcription units, RNAII and RNAIII, controlled by the promoters P2 and P3. The P3 transcript, RNA III, is the intracellular effector of the *agr* response (Novick *et al.* 1993), and it is the environmental concentration of the autoinducing peptide (AIP), encoded by the RNAII transcript, that is partially responsible for regulating the level of RNAIII (Ji *et al.* 1995 and Novick *et al.* 1995).

The RNA II transcript encodes four genes, *agrA*, *agrB*, *agrC* and *agrD*, which are co-transcribed (Rechtin *et al.* 1999) and required for the positive regulation of the P2 and P3 promoters (Figure 3). *agrD* encodes a precursor of the AIP and has a highly conserved motif, including the absolutely conserved Glu-Asp pair, essential for processing of the C-terminal end of the AIP (Dufore *et al.* 2002). *agrB* encodes a transmembrane protein, thought to be responsible for the processing, including proteolytic digestion, intramolecular thioester bond formation and secretion of the AIP (Zhang *et al.* 2002). Together, *agrC,* which encodes a membrane sensor protein, and *agrA*, a response regulator protein, form a structure homologous to a classical two-component signal transduction system (Lyon *et al.* 2002).

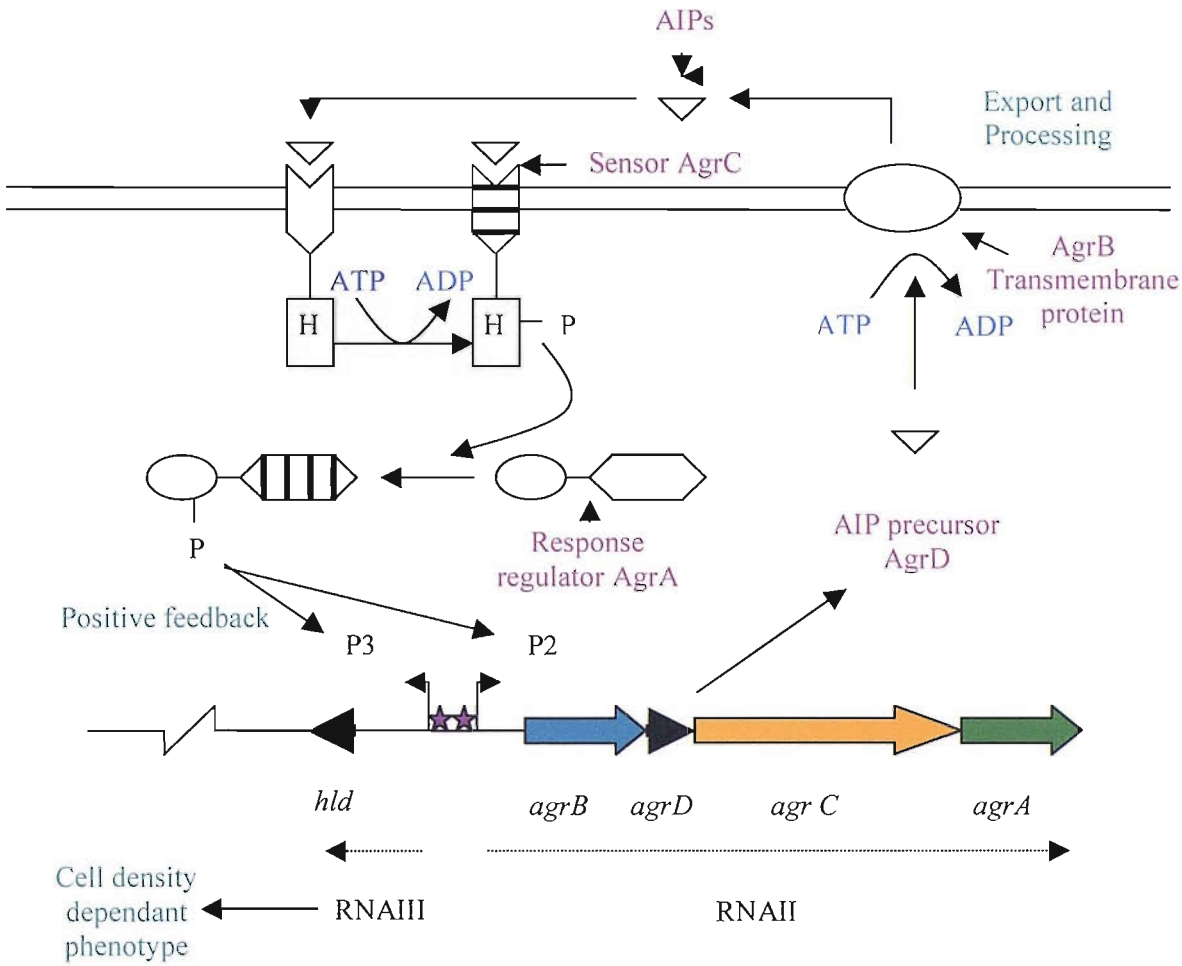


Figure 3 *A diagrammatic representation of the accessory gene regulator (agr) locus, showing the genes agr A, B, C and D in S. aureus, and a model of the function of the agr QS system (Kleerebezem et al. 1997).*

The amino acid sequence of the AIP is highly polymorphic between *S. aureus* strains, segregating them into four distinct alleric groups. AIP's have between 7 and 9 amino acids; they all share a common central cysteine, located 5 amino acids from the C-terminal end. A catalytic thioester bond is formed between the conserved cysteine and the C–terminal carboxyl (Lyon *et al.* 2002B). The thiolactone structure is essential for the AIP's full biological activity (Otto 1999). Within a group, a specific AIP can activate the *agr* response via interaction with its specific AgrC. AIP's produced by other groups are generally mutually inhibitory (Jarraud *et al.* 2002; Lyon *et al.* 2002b).

A limited number of studies that address the quorum response in staphylococcal biofilms appear at first glance to be somewhat conflicting in their results and interpretation. Pratten *et al.* (2001) found little difference between wild-type *S. aureus* and an *agr* mutant in adherence to either uncoated or fibronectin-coated glass under flow conditions, even though *hld* was expressed. In another study, RNAIII expression decreased *S. aureus* adherence to fibrinogen under static

conditions, but increased adherence to fibronectin and human endothelial cells in both static and flow conditions (Shenkman *et al.* 2002). Vuong *et al.* (2000) found that those *S. aureus* strains with a nonfunctional *agr* were much more likely to form biofilms under static conditions and were hyper-biofilm-forming strains. However, α-toxin, positively regulated by the *agr* system, was recently shown to be required for biofilm formation under both static and flow conditions (Caiazza *et al.* 2003).

Staphylococcal accessory regulator (*sar*)

Another regulatory locus, designated *sar*, was identified by Cheung *et al.* (1992). The *sar* locus encodes three overlapping but distinct promoters that produce three distinct transcripts (*sarA*, *sarB* and *sarC*), which are expressed at different times during growth, with *sarA* and *sarB* transcripts preferentially expressed during the exponential phase, and maximal expression of the *sarC* transcript occurring during the late stationary phase (Bayer *et al.* 1996; Manna *et al.* 1998 and Blevins *et al.* 2002). However, all of the *sar* transcripts encode SarA, a DNA-binding protein.

Phenotypic analysis has revealed that the *sar* locus is necessary for δ-hemolysin production. SarA interacts with the *agr* locus during the mid-to-late exponential phase of growth by binding to RNAIII, regulating its transcription (Cheung *et al.* 1994; Cheung *et al.* 1997). Mutation of *sar* results in reduced expression of both RNAIII and the *agr* transcript RNAII (Heinrichs *et al.* 1996; Cheung *et al.* 1997). In the case of the RNAIII P3 promoter, there is evidence to suggest that the binding of SarA compensates for inappropriate spacing of the −10 and −35 boxes and thereby enhances the binding of RNA polymerase (Morfeldt *et al.* 1996). Furthermore, results from animal model studies suggest that *agr* and *sar* also interact *in vivo* to control genes that affect pathogenesis of *S. aureus* infection. Unlike *agr*, the *sar* locus activates the synthesis of both extracellular (e.g. hemolysins) and cell wall-associated proteins (e.g. Fibronecting-binding protein) (Cheung *et al.* 1994).

Beenken *et al.* (2003) and Valle *et al.* (2003) both demonstrated that mutation of *sarA* in *S. aureus* resulted in a reduced capacity for biofilm formation. In contrast, Pratten *et al.* (2001) found that a *sarA* mutant was as capable of forming a biofilm as the corresponding wild-type strain.

***S. aureus* exprotein expression (*sae*)**

sae is an important regulator of virulence gene expression. It was initially identified in a mutant that was defective in the production of several exoproteins (Giraudo *et al.* 1994, 1999). The *sae* locus is composed of four genes, two of which encode a complex two-component system (Novick *et al.* 2003 and Steinhuber *et al.* 2003). Mutations in the *sae* have no effect on *agr* or *sarA* expression

(Giraudo *et al.* 1997). However, *sae* is activated by *agr*, at least in some strains, by an unknown mechanism (Giraudo *et al.* 2003; Novick *et al.* 2003; Goerke *et al.* 2005). The expression of *sae* is essential, as demonstrated in several animal models, for virulence-gene expression *in vivo* (Rampone *et al.* 1996; Benton *et al.* 2004; Goerke *et al.* 2005).

1.2.3 The nitric oxide chemical signal

A very recent development has been the recognition that nitric oxide (NO) is an important signal for modulating formation and detachment of *P. aeruginosa* biofilms (Barraud *et al.* 2006). NO has been recognised as an important signalling molecule in mammalian systems for many years, influencing such events as cell apoptosis, differentiation and cell proliferation (Moncada *et al.* 1998). The realisation that NO is also an important signalling molecule for some microbial species will have a profound impact on our knowledge of biofilm physiology. Barraud *et al.* (2006) found that low sub-lethal concentrations of the NO donor, sodium nitro-prusside (SNP), greatly enhanced the efficacy of antibacterial compounds in the removal of established *P. aeruginosa* biofilms from glass surfaces. To date it is not known if NO will modulate not only MRSA biofilm formation and detachment but also whether this will convert the biofilm phenotype back to the planktonic phenotype and render the cells more susceptible to antibiotic treatment.

1.2.4 Genotype modification: methicillin resistant *S. aureus*

As mentioned above, mutation or acquisition of resistance genes will alter the genotype and confer resistance to certain antibiotics. It is pertinent at this point to consider the acquisition of resistance genes by *S. aureus* and the emergence of the superbug MRSA.

Penicillins, monobactams, carbapenems, and cephalosporins are known as β-lactam antibiotics because, as part of their chemical structure, they contain a β-lactam ring (Figure 4). The illustration overleaf shows the basic structure of penicillin and cephalosporin. "R" represents sites where different chemical side chains attach, depending on the particular antibiotic.

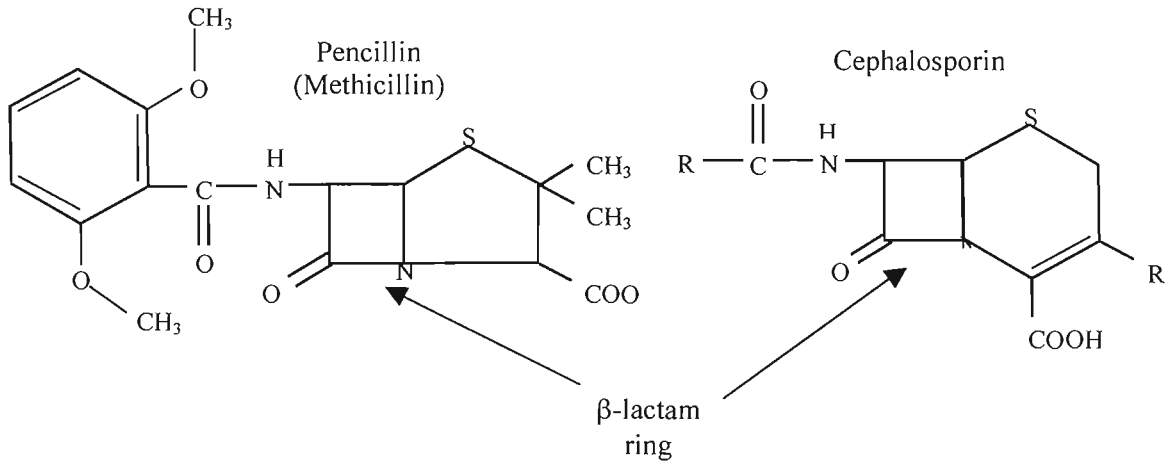


Figure 4 *The β-lactam rings of penicillins and cephaloporins (Image taken from www.cat.cc.md.us/courses/bio141)*

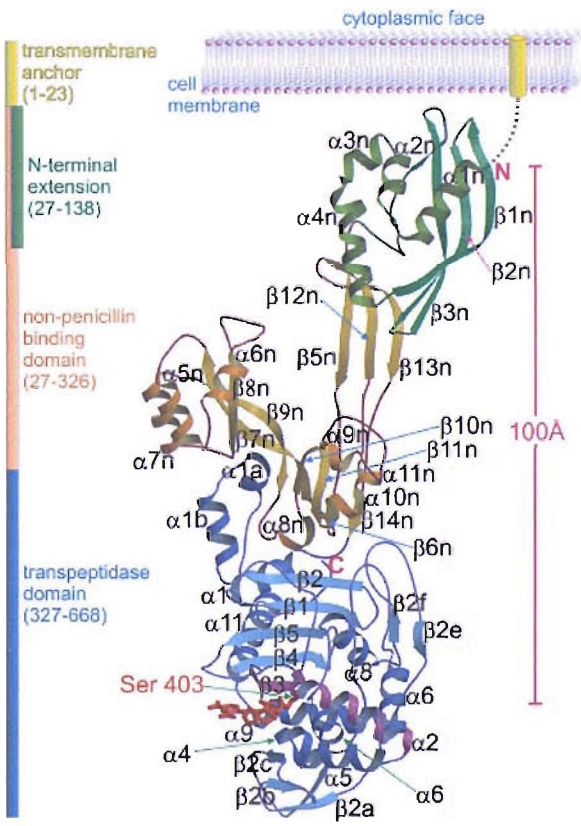
β-Lactam antibiotics exert their action by inhibiting a class of enzymes, commonly referred to as the penicillin-binding proteins (PBPs), that catalyse the synthesis of the peptidoglycan (Ghuysen 1991). Over the last 50 years, many strains of *S. aureus* have acquired resistance to penicillin and other β-lactam antibiotics through the acquisition of a gene encoding the enzyme β-lactamase, which breaks down the critical β-lactam ring, thus destroying the antibiotics activity. Until recently, infections with β-lactamase producing *S. aureus* could be treated with methicillin or other β-lactamase-resistant (2nd generation) β-lactams. However, new strains have emerged that, not only produce β-lactamase, but also have modified PBPs with low affinity for all β-lactam drugs. These strains are called methicillin-resistance *S. aureus*. Resistance to methicillin began to appear just one year after its introduction, but it was not until the 1990s that MRSA strains were prevalent in hospitals worldwide. In the UK the proportion of blood isolates of *S. aureus* that are resistant to methicillin has risen from just a few percent in the late 1980s to over 30%, with rates of over 40% seen in tertiary referral hospitals (Harbarth *et al.* 2003).

MRSAs produce a novel PBP, distinct from the PBPs normally found in *S. aureus,* called PBP 2a. PBP2a and the normal PBPs produced by *S. aureus* share 21% or less sequence identity. The PBP2a possesses a reduced affinity for binding to β-lactam antibiotics (Utsui *et al.* 1985).

In general, most low molecular mass PBPs possess only carboxypeptidase / transpeptidase activity; the high-molecular mass PBPs can possess both transpeptidase and transglycosylase activities; all mediate the formation of the peptide bridges between adjacent strands of peptidoglycan. PBP transpeptidase activity is inhibited by β-lactam antibiotics because the β-lactam ring bears a structural similarity to the normal substrate of the PBPs (muropeptides), and thus β-lactams form

a serine-bound acyl-enzyme intermediate with the protein, leading to competitive inhibition (Ghuysen 1991).

MRSA strains continue to grow in the presence of high concentrations of β-lactam antibiotics owing to the expression of PBP 2a, which is encoded by the *mecA* gene. The crystal structure of PBP2a was determined by Lim *et al.* (2002) (Figure 5); they found that the active site of the PBP2a is distorted relative to those of non-resistant PBPs. Kinetic studies have shown that, while the initial binding affinities of PBP2a are comparable to those of non-resistant PBPs, the subsequent acylation step, during which the PBP and the β-lactam bind with an RCO- group, is 1000-fold slower in the PBP2a (Lim *et al.* 2002). The reduction in acylation rate confers resistance to β-lactam antibiotics, but also means the peptidoglycan produced by these strains is of abnormal muropeptide composition, particularly with respect to the low degree of cross-linking (Wyke *et al.* 1982).



The bilobed N-terminal (nPB) domain is coloured orange with the N-terminal lobe (N-terminal extension) coloured green. The transpeptidase domain is coloured blue with the position of the active site indicated by the red nitrocefin adduct (shown in stick rendering). The secondary structure elements of the transpeptidase domain were labelled in accordance with the labelling scheme used for R6 PBP2x. The N- and C-termini are labelled N and C, respectively. Shown to the left of the ribbon representation is a linear representation of the domain structure of PBP2a with the residue numbers shown in parentheses.

Figure 5 *Structure of penicillin-binding protein of S. aureus (Lim et al. 2002)*

Methicillin resistance in MRSA strains is due to the acquisition of the *mec*A gene *via* horizontal transfer from an unidentified species. The *mecA* gene complex is carried on a specific integrative genetic element known as the staphylococcal cassette chromosome (SCC), which corresponds to about 1-2% of the entire *S. aureus* chromosome (Hiramatsu 2004). The molecular anatomy of SCC*mec* is distinctive and consists of a mec complex, composed of the *mec*A gene and it's variably present regulatory elements *mec*I and *mec*RI (Figure 6). The *mecA* gene is highly conserved

among clinical MRSA isolates (>90% sequence identity between strains). Also present are a pair of novel chromosomal cassette recombinase (ccr) genes, *ccr*A and *ccr*B, that mediate insertion and excision of SCC*mec* from the bacterial genome (Mongkolrattanothai *et al.* 2003). Other distinctive elements include the characteristic direct and inverted repeats at either end of the SCCmec element and the site-specific integration of the entire element at the 3' end of a gene of unknown function called *orf* (Ito *et al.* 2001) (Figure 6).

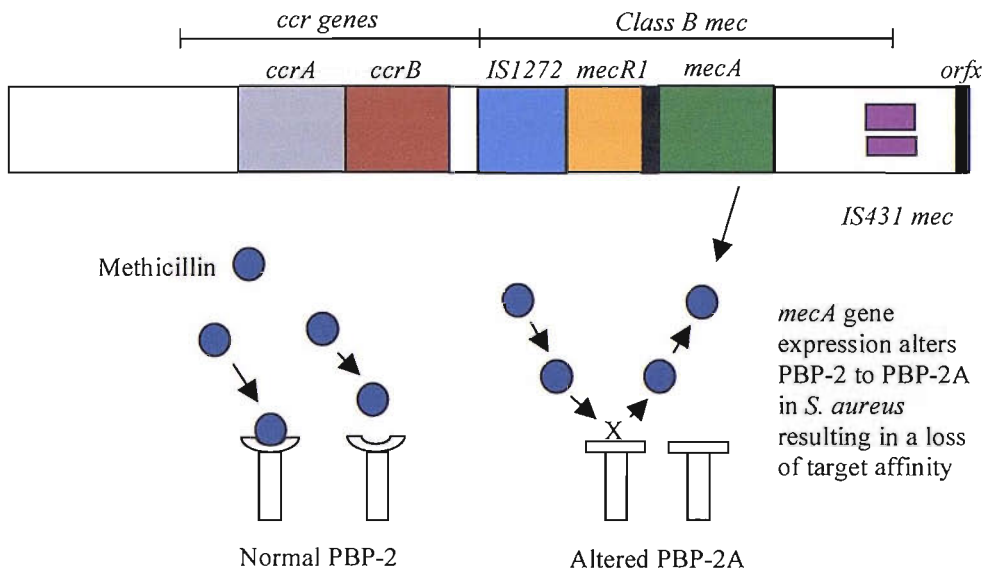


Figure 6 *Structure of the Staphylococcal cassette chromosome mec, with the recombinase genes complex upstream of the mec complex (Rybak et al. 2005).*

Five SCC*mec* types have been identified for *S. aureus* (Table 6). The gene elements differ in size, composition and associated antimicrobial resistance expression (Daum *et al.* 2002; Ito *et al.* 2004 and Ma *et al.* 2002).

Table 6 *The five types of mec gene complex*

SCCmec Types	Size (kb)	Features
I	34.3	Lacks other resistance genes
II	53.0	Associated with multiple drug resistance
III	66.9	Associated with multiple drug resistance
IV	20.9 – 24.3	Resistant to β-lactam antibiotics
V	28.0	Lacks antibiotic resistance genes other than mecA

(Rybak et al. 2005)

The SCC*mec* types I, II and III are predominantly found in nosocomial (hospital acquired) MRSA (N-MRSA) isolates. These isolates carry a number of inserted plasmids and transposable genetic elements downstream of the *mec*A complex. Types II and III are responsible for the multiple non-β-lactam antimicrobial resistance expressed by N-MRSA strains. SCC*mec* type IV is smaller, lacking other multi-drug-resistance genes, being typically found in community acquired MRSA (CA-MRSA) strains. The SCC*mec* type V is similar to type IV in size but does not contain antimicrobial resistance genes other than *mec*A. Type V SCC*mec* is also predominantly found in CA-MRSA strains (Ito *et al.* 2004; Rybak *et al.* 2005).

Infection with MRSA has become endemic in hospitals around the world. MRSA is widely thought to be transmitted between patients on the hands of hospital staff, and hand washing is widely recognised as the single most important factor for prevention of colonisation and infection (Teare 1999). However, despite the implementation of standard infection control practices, MRSA infection rates remain high. Other authors have argued that cross infection from the hospital environment is therefore as important an infection route as hand contamination. French *et al* (2004), found that, before cleaning, up to 90% of environmental swabs taken from areas such as floor corners, floor areas beside the bed, over-bed tables, bed frames, bedside chairs, lockers, door handles, light switches, sink taps, televisions and remote controls were positive for MRSA colonisation. More surprising is that, after terminal cleaning of the same environmental areas, which complied with current NHS guidelines, 66% still yielded MRSA (French *et al.* 2004).

Nosocomial MRSA

Nearly all MRSA infections are acquired in hospital and many of them are potentially preventable (National Audit Office 2000). Many measures have been implemented to reduce the spread of nosocomial MRSA infection such as hand washing, gloves, masking, gowning, appropriate device handling, and the appropriate handling of laundry. Despite all these precautions, nosocomial MRSA infection is still on the increase.

Community acquired MRSA

In recent years, there have been several reports of community-acquired MRSA (CA-MRSA) infections throughout the world (Abudu *et al.* 2001). Most of these outbreaks have been associated with a single-clone strain. Transmission has occurred by close physical contact in situations involving children in day-care centres, children and adults on Indian reservations, athletes, military personnel and inmates in correctional facilities (CDCP 2003; CDCP 2003b).

Investigators have revealed several characteristics that differentiate CA-MRSA from health-care-associated nosocomial MRSA (N-MRSA) strains. Community acquired isolates tend to be

susceptible to a variety of non-β-lactam antibiotics whereas N-MRSAs are typically resistant to multiple antibiotics. In addition, the genotypes of community isolates are not the same as those of nosocomial derived isolates. Community strains harbour a novel methicillin resistance cassette gene element; type V, not identified in N-MRSA isolates. Community acquired isolates also often occur in patients lacking the typical risk factors for MRSA (Karchmer 2000). Finally, community acquired isolates are more likely than nosocomial isolates to encode putative virulence factors, such as Panton-Valentine leucocidin (PVL), a cytotoxin virulence factor. Strains that produce PVL have slightly altered pathogenesis and epidemiology. They have been strongly associated with necrotising pyogenic cutaneous infections, especially furuncles (boils) (Gladstone *et al.* 1957).

Vancomycin resistant *S. aureus*

Vancomycin is a 1.5 kD glycopeptide antibiotic. It is the antibiotic of choice for treating MRSA infections. But now, forty years after vancomycin was first introduced, the medical community is facing a crisis as more and more bacteria are gaining vancomycin resistance. The development of vancomycin-resistant (MIC >32 mg l^{-1}) enterococci in 1988 led the way for the emergence of vanocmycin-resistant *S. aureus* strains (VRSA). VRSA were first recognised in 2002 in the USA (CDC).

Vancomycin binds to the D-alanyl-D-alanine residue of the peptidoglycan monomers, thus preventing peptidoglycan polymerization. There are two possible factors leading to vancomycin resistance; thickening of the peptidoglycan layer, and / or modification of the peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-lactate. Enterococci gain resistance to vancomycin by the accumulation of the *vanHAX* genes. The VanH enzyme is involved in the creation of a new enzymatic pathway that produces D-lactate from pyruvate. VanA enzyme catalyses the addition of D-lactate to the D-alanine at the end of the peptide cross-bridge, rather than D-alanine. Finally, metallodipeptidase VanX hydrolyzes the normal D-Ala-D-Ala moiety, reducing the cellular pool of the D-Ala-D-Ala dipeptide. These changes do not affect functioning of the peptidoglycan layer or its ability to cross-link the glycan strands, but they do have a lower (1000-fold) binding affinity for vancomycin, which is therefore rendered ineffective (Walsh 2001) (See Chapter 7).

1.3 Antimicrobial strategies

There are several well-recognised approaches to prevent infection. These involve the principles of prevention and cure. In terms of prevention good hygiene is paramount, involving for example the efficient cleaning of hospitals to prevent cross-contamination of patients. This can be supplemented

with the prophylactic administration of appropriate antibiotics. Alternatively, where patients are already infected, the cure principle involves the therapeutic administration of appropriate antibiotics. These principles will now be discussed.

1.3.1 Antibiotic treatment

The discovery of antibacterial drugs has been one of the most important medical achievements of the last 60 years. The 'golden' period of discovery (1940-1960) produced virtually all clinically important antibiotics in use today. However, antibiotic success has been undermined by the discovery of resistant bacteria. This ability of bacteria to become resistant to antimicrobials in a relatively short time frame creates a continuous requirement for new and improved antibiotics.

In recent years, there has been a relentless increase in the occurrence of antibiotic resistance in many common bacterial pathogens, such as *S. aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*. Most multi-drug resistant bacteria are isolated in hospital wards or outpatient centres, as it is in these environments that natural selection breeds antibiotic resistant microbes.

Several strategies are employed for the discovery of new antibacterial drugs, including the chemical manipulation of existing drugs to improve their range of activity, and to circumvent existing bacterial resistance mechanisms (Chopra *et al.* 2000). However, often the improvements achieved are small and relatively short-lived, with the evolution of modified mechanisms of resistance encompassing the newer analogues. The pharmaceutical industries have found it increasingly difficult to meet the needs of the medical community, thus there is an important demand for the discovery and development of new classes of antibiotics. Genomic-based approaches have been used to search for novel drug targets; these targets need to be sufficiently different in their construction or organisation from those of any mammalian homologues, decreasing the likelihood of cross-resistance through existing mechanisms of bacterial resistance (Chopra *et al.* 2000).

Antibiotics can be classified by their chemical structure, their microbial origin or their mode of action. The main modes of action are depicted below (Figure 7).

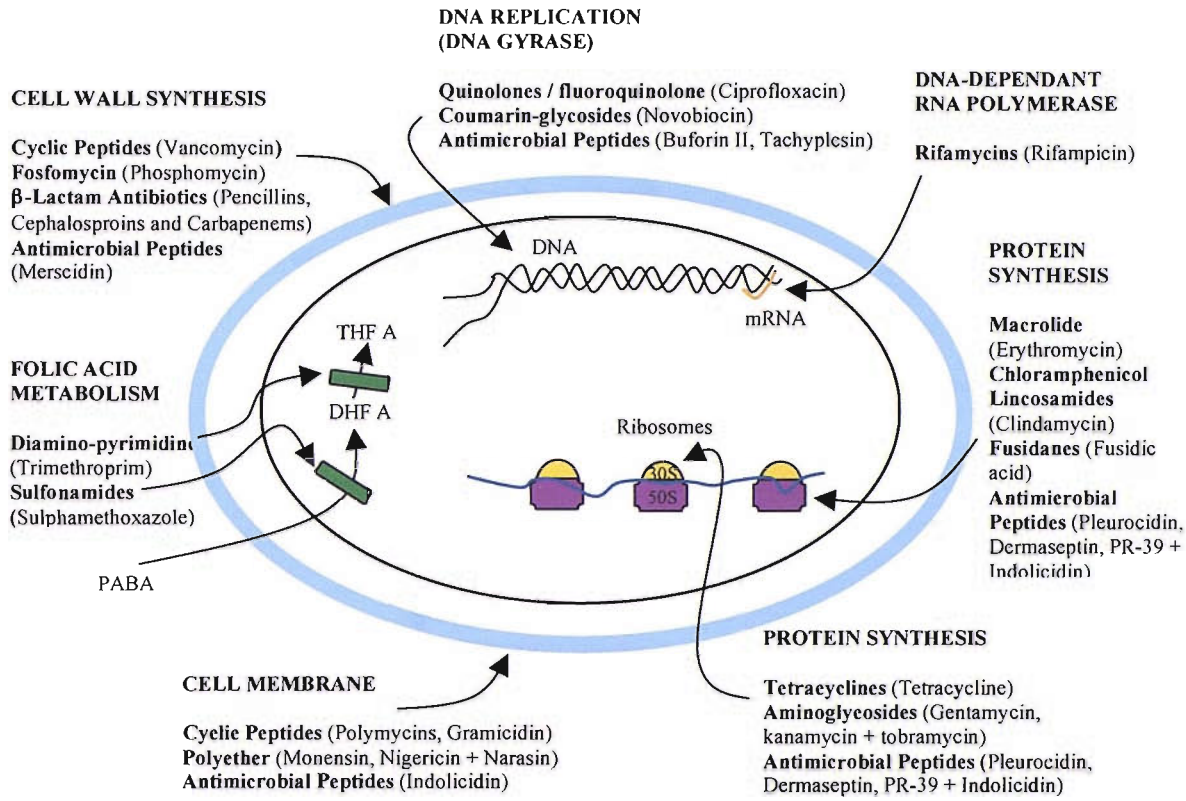


Figure 7 *Sites of action of different antimicrobial agents (Baron 1997). PABA, paraminobenzoic acid; DHF A, dihydrofolic acid; THF A, tetrahydrofolic acid.*

Antimicrobial agents that target the cell wall

Peptidoglycan is specific to bacteria; therefore antibiotics that interfere solely with the cell wall synthesis do not affect eukaryotic cells, and usually have a high therapeutic index. The cell wall is absolutely crucial to the survival of the bacterium for it provides the entire cell with mechanical support. The cell faces a huge intracellular osmotic pressure, and the rigidity of the peptidoglycan helps to maintain the cell's shape and prevent it from lysing (Gale 1972). Inhibition of the formation of the peptidoglycan layer in bacteria therefore causes the cell to lyse. Penicillins, cephalosporins and other β-lactam agents, as well as phosphomycin, cycloserine, bacitracin and the glycopeptides vancomycin and teicoplanin, selectively inhibit different stages in the construction of the peptidoglycan (Figure 8 and 9).

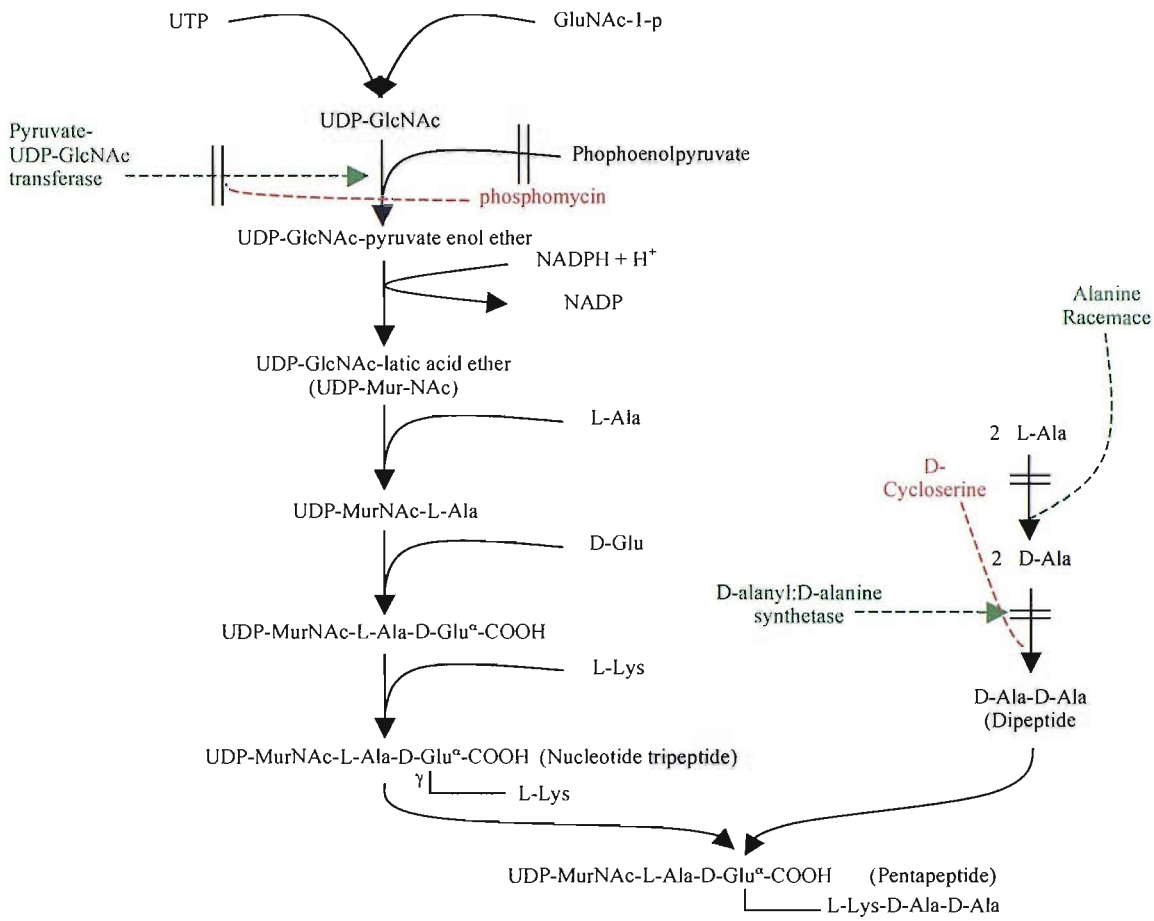


Figure 8 *A simplified scheme of bacterial peptidoglycan synthesis, showing the enzymes involved (Green) and the sites of action antibiotics (Red). Modified from Gale et al. (1972)*

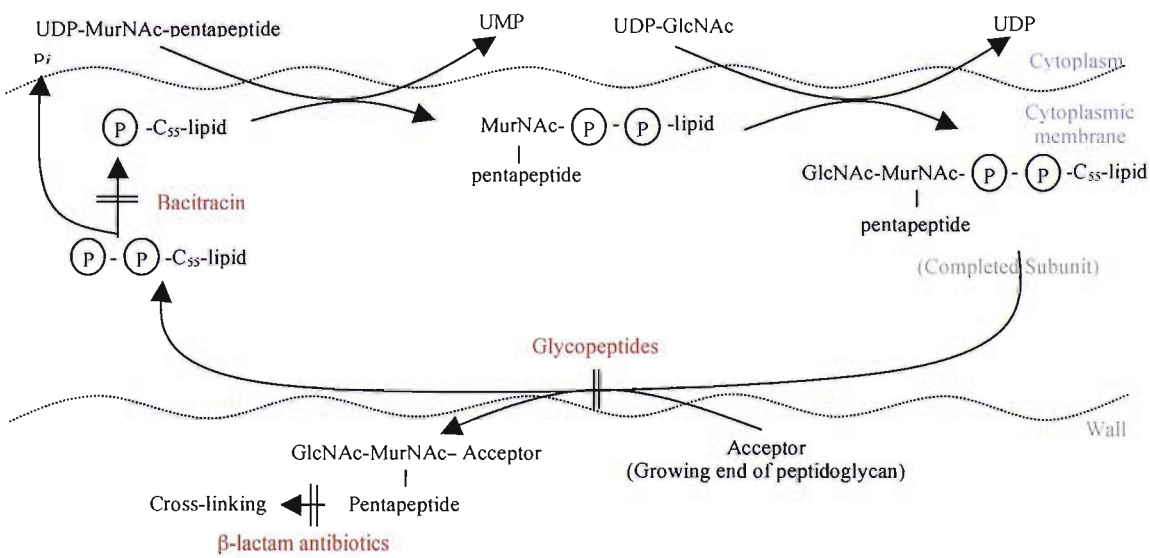


Figure 9 *A simplified scheme of the incorporation of NAG and NAM into membrane bound lipid intermediates and the subsequent transfer of the completed subunit to the growing end of the peptidoglycan. Sites of action of antibiotics shown in red (Gale et al. 1972).*

The β-lactam ring of the penicillins and other β-lactam antibiotics bears structural similarity to the normal substrate of the penicillin-binding proteins (PBPs), enzymes that mediate the formation of the peptide bridges between adjacent strands of peptidoglycan (Figure 9), and thus competitively inhibit their enzymatic activity. This causes a disruption in the cell wall biosynthesis, eventually leading to cell lysis. As the cell wall is only synthesised in actively multiplying cells, β-lactam antibiotics are only effective against growing bacteria. The bactericidal effect exhibited by β-lactam antibiotics in Gram-positive bacteria is due to the release of lipoteichonic acid; an event that appears to trigger autolysis of the peptidoglycan.

Resistance to β-lactam antibiotics often occurs when the bacteria produce β-lactamases. However, the penicillin analogues, methicillin and oxacillin, have bulky "shields" in the acyl side-chain that prevent attack by β-lactamases. Resistance to these antibiotics occurs through altered bacterial genes coding for structurally modified PBP, PBP2a, which does not bind any β-lactam antibiotics (Baron 1997).

The glycopeptides vancomycin and teicoplanin inhibit mucopeptide synthesis by complexing with the C-terminal residues of the peptidoglycan precursor, acyl-D-alanyl-D-alanine peptide side chain of NAM molecules (Figure 9). Complex formation at the outer surface of the cytoplasmic membrane prevents the transfer of these precursors from a lipid carrier to the growing peptidoglycan wall by transglycosidases. Biochemical reactions in the cell wall catalyzed by transpeptidases and D,D-carboxypeptidases are also inhibited by vancomycin and other glycopeptide antimicrobials (Baron 1997). In addition, vancomycin affects the synthesis and functioning of the protoplast membrane, although these effects occur after the immediate cell wall inhibition and are secondary.

Resistance of planktonically grown cells to vancomycin occurs in two ways; a thickening of the peptidoglycan layer or the possession of transposable genetic elements that encode special cell wall-synthesizing enzymes, the *van* genes, that change the structure of the normal acyl-D-alanyl-D-alanine side chain in the peptidoglycan assembly pathway. There are five *van* genes, *van* S, R, H, A and X, which are carried on a bacterial plasmid (Arther *et al.* 1993).

- VanH Generates D-lactate by reducing pyruvate
- VanA Generates an ester acyl-D-alanyl-D-lactate
- VanX Hydrolyses acyl-D-alanyl-D-alanine amide so only acyl-D-alanyl-D-lactate is built into the cell-wall

In the absence of vancomycin the VanH, A, and X genes are transcriptionally inactive. The presence of vancomycin is somehow detected by VanS (a sensor kinase) and the transcription of VanH, A, and X initiated by VanR. The result is peptidoglycan intermediates with acyl-D-

alanyl-D-lactate termini instead of the usual acyl-D-alanyl-D-alanine. The altered side chain (acyl-D-alanyl-D-lacate) significantly reduces the binding of vancomycin and allows normal peptidoglycan polymerization to occur in the presence of the drug (Baron 1997).

Phosphomycin is another antibiotic that affects the synthesis of the cell wall; it inhibits the enzyme, pyruvate-UDP-GlcNAc transferase (Figure 8), blocking the addition of a lactic acid constituent derived from phosphoenolpyruvate to the N-acetylmuramic acid component of peptidoglycan.

Antimicrobial agents that target the plasma membrane

A few antibiotics damage bacterial membrane. Unfortunately, these cell membrane-acting antibiotics do not discriminate between prokaryotic and eukaryotic cells, which they bind to a lesser extent, limiting their use to topical applications. Polymyxin B and the closely related compound colistin (polymyxin E) are the only membrane-active antimicrobial agents to be administered systemically in human medicine. They act like cationic detergents; they disrupt the cytoplasmic membrane of the cell, probably by attacking the exposed phosphate groups of the membrane phospholipid.

Several antibiotics, known collectively as ionophores, disrupt transmembrane ion concentration gradients, required for the proper functioning and survival of microorganisms, by transporting ions across the lipid bilayer of the cell membrane. They are lipid-soluble molecules, usually synthesized by certain microorganisms to act as a defense against competing microbes. There are two broad classifications of ionophores: i) small molecules (mobile ion carriers) that bind to a particular ion, shielding its charge from the surrounding environment, and thus facilitating its crossing of the hydrophobic interior of the lipid membrane; and ii) channel formers that introduce a hydrophilic pore into the membrane, allowing ions to pass through while avoiding contact with the membrane's hydrophobic interior. Ionophores include the topical antibiotic gramicidin A, and some agents used in veterinary medicine, such as monensin. *Bacillus brevis* synthesizes a mixture of linear gramicidins, which belong to the peptide class of antibiotics. Commercial samples contain a mixture of the three main gramicidins A, B and C. The channel through the membrane formed by gramicidin has low ion specificity ($Rb^+ > Cs^+ > K^+ > Na^+$) (Blasko *et al.* 1989). Monensin, nigericin and narasin are all polyether antibiotics. Monensin and narasin, produced by *Streptomyces cinnamonensis* and *Streptomyces aureofaciens* respectively, both form complexes with monovalent alkali cations, with selectivity of $Na^+ > K^+$ (monensin) and $Na^+ > K^+ = Rb^+ > Cs^+ > Li^+$ (narasin) (Caughey *et al.* 1986). The ionophore / ion complexes are able to cross the cellular membrane and therefore collapse the transmembrane Na^+ potential. Nigericin, on the other hand, preferentially forms complexes with K^+ and permits electroneutral (1:1) K^+ / H^+ exchange across a membrane, thus collapsing ΔpH (Ramos *et al.* 1976).

Antimicrobial agents that interfere with transcription and DNA synthesis

Compounds that bind directly to the DNA double helix are generally highly toxic to eukaryotic cells and only a few, which interfere with DNA-associated enzymatic processes, exhibit sufficient selectivity for systemic use as antimicrobial agents; these include the quinolones (and fluoroquinolones), novobiocin and rifampicin. However, diaminopyrimidines, sulfonamides and nitrofuans also affect DNA synthesis.

Quinolones bind to the cleavage complex composed of DNA and DNA gyrase. DNA gyrase, a topoisomerase, acts to maintain an optimum supercoiling state of DNA in the cell. DNA gyrase is essential for relieving torsional strain during replication of circular chromosomes in bacteria (Baron 1997). The interaction of quinolones and the α-subunit of DNA-gyrase act to stabilize the cleavage intermediate, which has a detrimental effect on the normal DNA replication process. Another enzyme topoisomeras IV is also affected. Indeed, in Gram-positive bacteria, topoisomerase IV seems to be the main target (Ng *et al.* 1996). Bacterial resistance to quinolones can occur in several ways: mutations in a highly conserved region in the DNA gyrase subunits α (gyr A) or β; mutations which reduce outer membrane permeability in Gram-negative bacteria or active efflux transporters. Novobiocin also acts on DNA gyrase; it competes with ATP binding at the β-subunit of DNA gyrase, inhibiting initiation of DNA synthesis.

Tetrahydrofolate is an important enzyme that is essential in the biosynthesis of amino acids, purines and pyrimidines (Figure 10). Bacteria lack a transport system for the uptake of preformed folic acid from their environment, whereas mammalian cells only utilise preformed folates from their diet, so selectivity occurs (Mann *et al.* 1996). The production of tetrahydrofolate is inhibited by sulfonamides and trimethroprim. Sulfonamides block the conversion of pteridine and para-aminobenzoic acid (PABA) to dihydrofolic acid by acting as a competitive inhibitor of the enzyme dihydropteroate synthase (Figure 10). Bacteria resistant to sulfonamides produce a modified form of dihydropteric acid synthase. The modified enzyme has a poor affinity for sulfonamides and therefore preferentially binds PABA. Trimethroprim prevents the reduction of dihydrofolate to tetrahydrofolate; it has an affinity for the bacterial dihdrofolate reductase 10,000 to 100,000 times greater than that of the mammalian enzyme (Baron 1997). The modification of the enzyme is also the mechanism of resistance to trimethroprim. In *E. coli*, the gene for the enzyme dihydrofolate reductase leads to the production of both normal and a modified enzyme. The modified enzymes have an affinity for trimethoprim 20,000 times lower than the native enzyme. The modified enzyme can thus catalyse the production of the folic acid required for growth. However, because the modified enzyme is still able to bind trimethroprim, albeit at a much-reduced affinity, resistance can be overcome by increased trimethroprim concentrations.

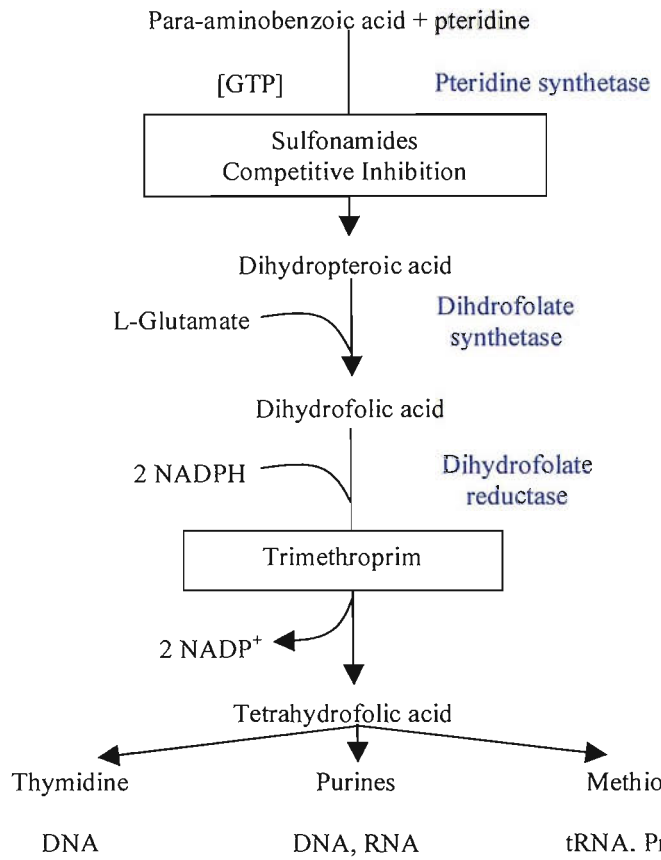


Figure 10 *Folic acid metabolism; showing sites of inhibition by sulfonamides and trimethroprim (Baron 1997).*

Rifampicin is an extremely efficient inhibitor of DNA-dependent bacterial RNA polymerase, preventing the transcription of RNA species from the DNA template; however the eukaryotic RNA polymerase is not affected. RNA polymerases consists of a core enzyme made up of four polypeptide subunits, two α subunits, one β and one β', and an additional component, the sigma subunit, which is required to associate with the core enzyme for selective and high affinity binding to the DNA. Rifampicin specifically binds to the β-subunit.

Antimicrobial agents that interfere with translation

While all cells synthesise proteins, the structure of the prokaryotic ribosomes (70S) is different enough from eukaryotic ribosomes (80S) to make it a suitable target for selective toxicity. Many antibiotics interfere with protein synthesis, including naturally accruing antibiotics such as chloramphenicol, tetracyclines, aminoglycosides, fusidic acid, macrolids, lincosamides and stretogramins. In the first stage of bacterial protein synthesis, messenger RNA (mRNA) binds to the 30S ribosomal subunit; this binding attracts *N*-formylmethionyl transfer RNA (tMet-tRNA) to the initiator codon AUG. The 50S subunit is then added to form a complete initiation complex. fMet-tRNA occupies the P (peptidyl donor) site; adjacent to it is the A (aminoacyl acceptor) site, which is aligned with the next trinucleotide codon of the mRNA. Transfer RNA (tRNA) bearing

the appropriate anticodon, and its specific amino acid, enters the A site, and a peptidyl transferase joins the *N*-formylmethionine to the new amino acid with loss of the tRNA in the P site, forming the first peptide bond of the new protein. A translocation event then moves the remaining tRNA with its dipeptide to the P site, aligning the next triplet codon of the mRNA with the vacant A site.

The aminoglycosides act by binding to specific ribosomal subunits via free NH_4 and OH groups, which are essential to their activity. Gentamycin, tobramycin and kanamycin are 2-deoxystreptidine derivatives. They bind to bacterial ribosomes at different sites, inhibiting bacterial protein synthesis. Kanamycin and tobramycin bind to the 30S ribosome subunits, while gentamycin also binds to the L6 protein of the 50S ribosomal subunit. They cause mis-reading of the tRNA code and initiator tRNA (Tanaka *et al.* 1967). Cross-resistance of tobramycin with gentamycin is almost complete. Drug-modifying enzymes mediate the most common form of aminoglycoside resistance. Aminoglycosides are modified outside the cell; a number of enzymes can acetylate free amino groups and phosphylate or adenylate the hydroxyl groups that are essential to their activity. Resistance is therefore partly due to poor uptake of the altered compound. A less common form of resistance involves the alteration of the binding site on 30S ribosomes (Figure 11) (Baron 1997).

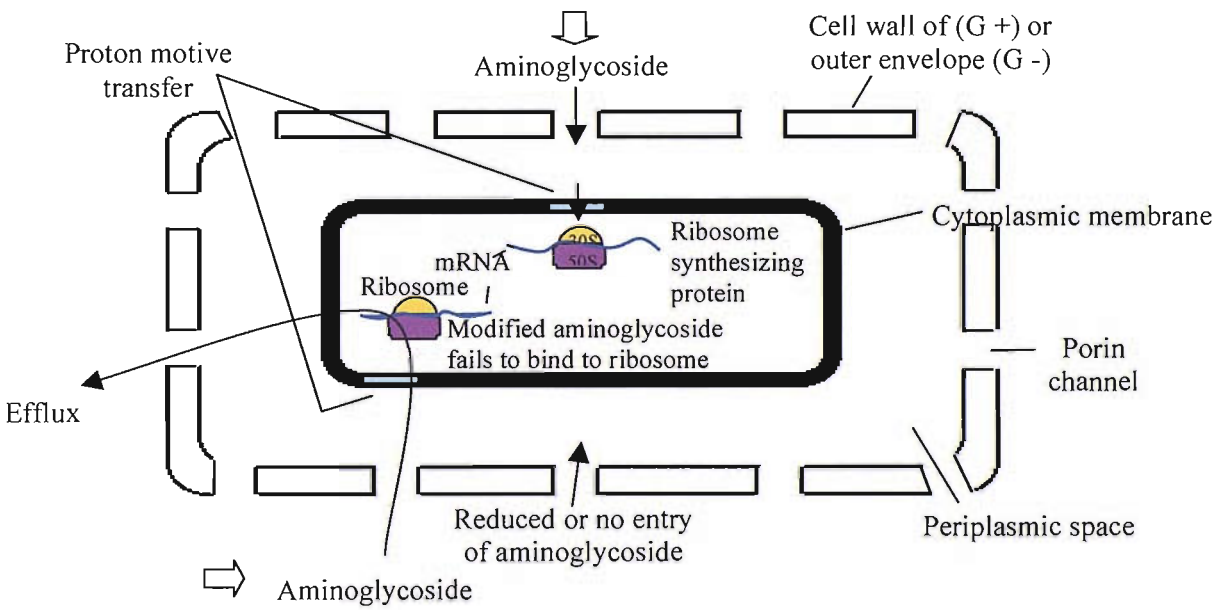


Figure 11 *Diagrammatic representation of transfer and transfer reduction of aminoglycosides across the bacterial cell wall (Baron 1997).*

Tetracyclines interfere with the transfer of amino acids from the aminoacyl tRNA to the protein. They compete with the binding of aminoacyl tRNA to the A site on the 30S ribosomal subunits. All antibiotics in the tetracycline group are based on a hydronaphthacene nucleus containing four fused rings (Franklin 1963). Tetracycline resistance genes in *S. aureus* are carried on small multi-copy plasmids. Normal resistance is due to a decrease in the levels of drug accumulation; decreased

uptake and increased efflux both probably participate. Resistant bacteria bind less tetracycline and an energy-dependent process removes the tetracycline that they do accumulate.

There are three important classes of antimicrobial drugs that bind to the 50S ribosomal subunit, chloramphenicol, erythromycin and the marcrolides. Chloramphenicol, a widely used broad-spectrum antibiotic, binds to a peptidyl transferase enzyme. The enzyme links amino acids in the growing peptide chain; therefore, the binding of chloramphenicol freezes the process of chain elongation, bringing bacterial growth to an abrupt halt. The process is completely reversible so chloramphenicol is a bacteristatic and not a bactericidal agent. Many Gram-positive and Gram-negative bacteria are resistant to chloramphenicol because they possess the enzyme chloramphenicol acetyl transferase (CAT). Chloramphenicol acetyl transferase acetylates hydroxyl groups on the chloramphenicol structure using acetyl co-enzyme A as the acetyl donor; this modification leads to reduced binding to the 50S ribosome. Other acetyl transferase enzymes are responsible for the acetylation of many aminoglycoside antibiotics e.g. streptomycin, spectinomycin and the gentamycins.

Erythromycin belongs to the macrolide antibiotics which all contain a macrocyclic lactone ring linked glycosidically to unique sugars. Erythromycin inhibits protein synthesis in growing cultures (Otaka *et al.* 1970) by binding to the 50S subunit of ribosomes. It appears to impair a pepidyltransferase reaction or translocation, or both (Baron 1997). Clindamycin belongs to a small group of antibiotics that have a novel structure unlike that of any other antibiotic, called the lincosamides. They appear to interfere indirectly with the peptidyl transferase reaction, possibly by blocking the P site.

Macrolides, lincosamides and streptogramins are structurally different classes of antibiotics, but they bind to closely related sites on the 50S ribosomal subunit. One consequence of this is that staphylococci exhibiting inducible resistance to erythromycin also become resistant to other macrolides, lincosamides and streptogramin$_B$, this is termed MLS resistance. Genes that specify MLS resistance include the genes *erm*C and *erm*A that encode normally repressed methyltransferases, which causes the methylation of two adenine nucleotides in the 23S component of 50S RNA. The methylated RNA binds macrolide and lincomycin-type antibiotics less well than unmethylated RNA. The gene *mrs*A, another determinant of MLS, it encodes a protein responsible for the active excretion of macrolides and streptogramins but not of lincosamides. Another gene - *lin*A, which is also less frequently encountered, it encodes a protein product, which modifies (adenylylation) and thus inactivates lincosamide antibiotics.

Fusidic acid forms stable complexes with an elongation factor (EF-G), which is involved in translocation and with guanosine triphosphate (GTP), which provides energy for the translocation process. One round of translocation can occur resulting in hydrolysis of GTP (GDP), but the fusidic acid-EF-G-GDP-ribosome complex blocks further chain elongation, leaving peptidyl-tRNA in the P site.

1.3.2 Disinfection strategies

Antiseptics and disinfectants are used extensively in hospital and other health care setting for a variety of topical and hard-surface application. In particular, they are an essential part of infection control practices and aid in the prevention of nosocomial infections. A wide variety of active chemical agents are available, many of which have been used for hundreds of years for antisepsis, disinfection and prevention. Despite this, less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. Most chemical biocides react irreversibly with proteins, and the cytoplasmic membrane, although their precise mechanisms of action are often not understood. Numerous different chemicals are marketed for medical and industrial use under a variety of trade names. Frequently they contain more than one antimicrobial chemical as well as other chemicals such as buffers that can influence their antimicrobial activity. Selecting the appropriate biocide is a complex decision. Some of the points for consideration include: toxicity, activity in the presence of organic matter, compatibility with the material being treated, residue, cost and availability, storage and stability, and the environmental risk. There are several classes of biocide chemicals; each type has characteristics that make it more or less appropriate for specific uses (Table 7).

Chemical	Characteristics	Uses
Alcohols (ethanol and isopropanol)	Easy to obtain and inexpensive. Rapid evaporation limits their contact time.	Aqueous solutions of alcohol are used as antiseptics to disinfect skin in preparation for procedures that break intact skin, and as disinfectants for treating instruments
Aldehydes (glutaraldehydes and formaldehyde)	Capable of destroying all forms of life. Irritating to the respiratory tract, skin and eyes	Glutaraldehyde is widely used to sterilise medical instruments. Formalin is used in vaccine preparation
Biguanides (chlorhexidine)	Relatively low toxicity, destroys a wide range of microbes, adheres to and persists on skin mucous membranes.	Chlorhexidine is widely used as an antiseptic in soaps and lotions, and more recently, impregnated into catheters and surgical mesh.
Ethylene Oxide Gas	Easily penetrates hard-to-reach places and fabrics and does not damage moisture sensitive material. It is toxic, explosive and potentially carcinogenic.	Commonly used to sterilise medical devises.
Halogens (Chlorine and Iodine)	Chlorine solutions are inexpensive and readily available; however organic compounds and other impurities neutralise the activity. Some forms of chlorine may react with organic compounds to form toxic chlorinated products. Iodine is more expensive than chlorine and does not reliably kill endospores.	Solutions of chlorine are widely used to disinfect inanimate objects, surfaces, drinking water and wastewater. Tincture of iodine and ionophores can be used as disinfectants or antiseptics.

Ozone	This unstable molecular form of oxygen readily breaks down.	Used to disinfect drinking water and wastewater.
Heavy metal derivatives (Silver and Mercury compounds)	Most metal compounds are too toxic to be used medically.	Silver sulfadiazine is used in topical dressings to prevent infection of burns. Silver nitrate drops can be used to prevent eye infections caused by *Neisseria gonorrhoeae* in newborns. Some metal compounds are used to prevent microbial growth in industrial processes.
Peroxygens (Hydrogen peroxide and peracetic acid)	Readily biodegradable and less toxic than traditional alternatives. The effectiveness of hydrogen peroxide as an antiseptic is limited because then enzyme catalase breaks it down. Peracetic acid is a more potent germicide than hydrogen peroxide.	Hydrogen peroxide is used to sterilise containers for aseptically packaged juices and milk. Peracetic acid is widely used to disinfect and sterilise medical devices.
Phenolic Compounds (Triclosan and hexachlorophene)	Wide range of activity, reasonable cost, remains effective in the presence of detergents and organic contaminants, leaves an active antimicrobial residue.	Triclosan is used in a variety of personal care products, including toothpastes, lotions and deodorant soaps. Hexachlorophene is highly effective against *S. aureus*, but its use is limited because it can cause neurological damage.
Quaternary Ammonium Compounds (benzalkonium chloride + cetylpyridinium chloride)	Non-toxic enough to be used on food preparation surfaces. Inactivated by anionic soaps and detergents.	Widely used to disinfect inanimate objects and to preserve non-food substances

1.4 Methods to study biofilm formation and resistance

An assessment of viability and growth is important when working with microorganisms; for example, when investigating the antimicrobial effects of a compound. In the relatively new field of biofilm studies, research has increased rapidly, resulting in a plethora of different and often ingenious antimicrobial testing strategies to measure biofilm characteristics. These assays have been used for a number of applications, including determination of adherence, quantification of inhibition of biofilm formation, antimicrobial screening of natural products and the determination of biofilm susceptibility patterns to antimicrobials (Cassey *et al.* 2004).

The simplest biofilm test involves growing bacteria on congo red agar (CRA) (Freeman 1989), strains that are biofilm-positive (slime producing), grow as black crystalline colonies, while biofilm-negative (non-slime producing) form smooth colonies that are red/pink in colour. This assay only infers slime production and, therefore, gives no quantitative information regarding the amount of biofilm produced, strength of attachment, cell viability or the effect of antimicrobial treatment. The assay is therefore seldom used in isolation.

Various model systems have been proposed to study biofilm formation and control under defined or semi-defined conditions. These include devices such as the chemostat (Keevil 2001), the annular reactor (Characklis *et al.* 1982) and the Propella (Wilks *et al.*, 2005), which can operate under strictly defined nutrient and environmental conditions, as well as controlling the shear force on the abiotic surfaces investigated; and the Robbins device flow cell, which may be less successful at controlling the growth environment under low nutrient conditions where gradients may occur along the length of the device at low flow rates (McCoy *et al.* 1982).

Although not so good for investigating the influence of shear on biofilm formation, significant advances have been made using microtitre plate biofilm-generating systems. These offer the ability of high throughput for mass screening, e.g. selection of specific mutants that may be involved in biofilm formation, maturation and sloughing processes, or screening of a range of antimicrobial agents (Gabrielson *et al.* 2002). In addition, quantitative measurements such as optical density (OD) can be made of the dried, stained material. In terms of objectivity and accuracy, the microtitre test is superior because it eliminates subjectivity in reading of results and it gives a quantitative value necessary in detailed comparisons.

Many investigators have subsequently modified the microtitre plate biofilm assay and, although deceptively simple, these methods require careful attention to detail in order to obtain meaningful and reproducible results. The Calgary biofilm device is one such modified microtitre plate assay,

which consists of a microtitre plate and a lid sporting 96 pegs on which the biofilm is grown; it is often used in clinical laboratories for antimicrobial susceptibility testing (Ceri *et al.* 1999).

Biofilms can be quantified by a variety of techniques, such as direct microscopic enumeration, dry weight, total viable plate counts, turbidity measurements, metabolically active dyes, radiochemistry and luminometry. For example, sample discs are inserted into a circulating bacterial suspension, the discs are aseptically removed at specific times and biofilm formation is assessed by several methods, i.e. scraping or sonicated removal of bacteria from the surface for direct plating or scanning electron microscopy, epifluorescence via light microscopy or episcopic differential interference contrast (EDIC) microscopy (Keevil 2003).

Knobloch *et al.* (2001) underlines the need for an improved microtitre plate assay that can better discriminate between weak biofilm producers and biofilm-negative strains. Development of a standardised assay needs to consider many factors that differ depending on the assays intended end use. These factors include the strains used, biofilm age and growth conditions (stress, excessive or physiologically relevant). The most important qualities that need to be incorporated into a standardised method are ease of use, accuracy and reproducibility. The method should also be rapid and inexpensive.

1.5 Aims

1.5.1 Assay development and optimisation

It is clear that many promising antimicrobial agents fail at clinical trial due to initial screening against standard planktonic cultures, which do not express the resistant biofilm phenotype (Nicholls 1993). Accordingly, this study has sought to develop and validate a rapid screening technology.

Many assays already exist for the formation of biofilm. The majority of these assays determine biofilm biomass, but give no information about cell viability within the biofilm, and are not suitable for assaying the effects of antimicrobial agents against preformed biofilms. Also, growth conditions and methods of detection used in the different biofilm assays vary greatly. Accordingly, the first aim of this work was to optimise growth conditions for *S. aureus* biofilm formation; then to optimise existing assays, or develop a new quantitative biofilm assay capable of detecting not only changes in biofilm biomass but also biofilm viability, but also suitable for determining the efficacy of antimicrobial agents in preventing initial attachment and their effectiveness against preformed MRSA biofilm.

1.5.2 Determination of *S. aureus* biofilm resistance to disinfection

The majority of MRSA infections are contracted in the hospital environment. It is therefore important that the disinfectant cleaning chemistries used by the hospitals are capable of eliminating MRSA in the environment.

Several hypochlorite-based hospital cleaning chemistries (not previously evaluated for their effects on biofilm) were donated by Southampton General Hospital, with the aim of determining their efficacy in preventing initial cell attachment and their effectiveness in disrupting and killing preformed MRSA biofilms. Similarly, peroxygen and phenolic-based disinfectants used in microbiological laboratory disinfection, which had not previously been investigated against MRSA biofilms, were tested to determine their effects on biofilm development and preformed biofilm formations.

1.5.3 The efficacy of vancomycin against MRSA biofilms

Previously Jones *et al.* (2001) had shown that MRSA biofilm formed on the surface of catheters were resistant to vancomycin. However, only surface coverage was determined, and no indication

of cell viability was given. Therefore, an aim of this work was to add to this knowledge, and determine the viability of the MRSA biofilms after treatment with vancomycin.

1.5.4 The determination of the effects of antibiotics with differing modes of action on MRSA biofilms

The biofilms of many species of bacteria have been shown to be resistant to several different antibiotics. However, only a small number of antibiotics have been tested against the MRSA biofilm mode of growth, making it difficult to draw general conclusions about biofilm resistance mechanisms. Therefore, the aim was to conduct a comprehensive study looking at a large number of antibiotics with differing modes of action to try to determine if one mode of action was more successful at disrupting and killing preformed MRSA biofilms.

1.5.5 Screen a library of novel antimicrobial agents for activity against MRSA biofilms

Novel antimicrobial agents provided by Syngenta will be screened against *S. aureus* for activity specifically against the biofilm phenotype.

1.5.6 Influence of quorum sensing on biofilm structure and antimicrobial resistance

The *agr* system has been reputed to have varying effects on biofilm formation. An *agr* mutant and its wild-type counterpart were tested for their ability to form biofilm, and resistance of that biofilm to antimicrobial agents was tested using the combined biofilm assay. Also, AI-2, a QS signal, produced by mainly Gram-negative bacteria and thought to be a universal signal, was tested for its production by the Gram-positive *S. aureus* strains and for the effect of exogenous application of biofilm growth, structure and its effect on biofilm resistance to antibiotics.

1.5.7 Assessment of the chemical signal NO on biofilm structure and antibiotic resistance

To date it is not known if NO will modulate MRSA biofilm formation and detachment but also whether this will not only convert the biofilm phenotype back to the planktonic phenotype, but render the cells susceptible to antibiotic treatment.

2. GENERAL METHODOLOGY

2.1 Glassware

All glassware was cleaned at high temperatures using Guard professional concentrated detergent (containing 0-5% non-ionic surfactants and 5-20% anionic surfactants) at a concentration of 1 / 400 parts H_2O. Glassware was repeatedly rinsed with distilled H_2O, then left to air-dry.

2.2 Sterilization

Glassware, heat resistant plastics, culture media and non-heat-labile solutions were sterilized by autoclaving at 121°C for an appropriate amount of time. Heat-labile solutions were sterilized by membrane filtration (0.2 μm; Sartorius Minisart).

2.3 Culture media

Both solid and liquid media were used for bacterial subculture as detailed in Table 8. Media components were dissolved in one litre of distilled H_2O and sterilized by autoclaving at 121°C for 15 minutes. Heat-labile supplements were added aseptically to cooled media (55°C).

Table 8 *Culture Media*

Name	Abbreviation	Supplier
Brain Heart Infusion broth	BHI	Oxoid UK
Mueller Hinton broth	MHB	Oxoid UK
Nutrient broth	NB	Oxoid UK
Tryptone Soy broth	TSB	Oxoid UK
Luria-Bertani broth	LB	Sigma UK
Autoinducer Bioassay broth	AB	Produced in-house
Mueller Hinton II agar	MHA	Oxoid UK
Nutrient agar	NA	Oxoid UK
Congo Red agar	CRA	Produced in-house

In addition to using the manufacturers stated concentration, BHI and TSB, were also prepared at a quarter their normal strength. Quarter strength BHI and TSB, where used, were represented as $BHI_{25\%}$ and $TSB_{25\%}$ respectively throughout this work.

AB medium was prepared as previously described by Greenberg *et al.* 1979, containing 0.3 M NaCl, 0.05 M $MgSO_4$ and 0.2% (w/v) vitamin-free casamino acids (pH 7.5). After autoclaving (121°C for 15 minutes), 1 ml of sterile 1 M potassium hydrogen phosphate (pH 7.5), 1 ml of 0.1 M L-arginine (free base) and 500 µl of 50% (w/v) glycerol were added per 100 ml.

Congo red agar (Freeman *et al.* 1989) was prepared using BHI or TSB as base medium; supplemented with 1% (w/v) agar (Oxoid UK) and 0.08% (w/v) Congo red (Sigma-Aldrich UK). Congo red stock solution was prepared as a concentrated aqueous solution, autoclaved (121^0C for 15 minutes) separately from other medium constituents, and added upon cooling (55°C).

2.4 Storage of antimicrobial agents

Antimicrobial powders were obtained directly from the manufacturer or from commercial sources. Syngenta test chemicals were supplied direct from Syngenta, UK. Each agent was supplied with a stated potency (mg or International Units per g powder, or as percentage potency) and an expiry date. Powders were sealed and stored in the dark at 4°C with a desiccant unless otherwise recommended by the manufacturer. The containers were allowed to warm to room temperature before opening to avoid condensation of water on the powder.

2.5 Preparation of stock solutions

Antimicrobial stock solutions were prepared to a concentration of 10,000 mg l^{-1} using the manufacturers stated diluent (Table 9, overleaf). Stock solutions were stored at -20°C for up to one month, and diluted in sterile dH_2O upon use.

Table 9 *Diluents for making stock solutions of antimicrobial agents.*

*Antimicrobial Agent	Diluent	*Antimicrobial Agent	Diluent
Actinomycin D	DMSO	Narasin	Ethanol
Ampicillin	DMSO	Nigericin	Ethanol
Bacitracin	DMSO	Nitrofurantoin Crystalline	H_2O
Chloramphenicol Hydrochloride	Ethanol	Novobiocin Sodium Salt	H_2O
Ciprofloxacin	H_2O	Oxacillin	H_2O
Clindamycin Hydrochloride	H_2O	Phosphomycin	DMSO
Erythromycin	Ethanol	Rifampicin	DMSO
Fusidic Acid Sodium Salt	DMSO	Sulphamethoxazole	DMSO
Gentamycin	H_2O	Tetracycline Hydrate	Ethanol
Gramicidin	DMSO	Tobramycin	H_2O
Irgasan	Ethanol	Trimethroprim	DMSO
Kanamycin	H_2O	Vancomycin Hydrochloride	H_2O
Monensin	Ethanol	Syngenta Test Chemicals	DMSO

*All antibiotic standards were obtained from Sigma-Aldrich, UK.

2.6 Preparation of working solutions of antibiotics and test chemicals

Working concentrations of antibiotics and test chemicals were obtained by diluting the stock solutions (10,000 mg l^{-1}) in 5 ml of sterile dH_2O to eleven times the desired final concentration. An aliquot of 20 µl was dispensed into the wells containing 200 µl of culture (freshly inoculated culture in the case of the 0 hour-old microtitre plates, and biofilms and fresh media in the case of the 8 and 24 hour-old microtitre plates), such that the final desired concentration was obtained.

To obtain the working concentrations for the determination of MICs, serial dilutions were set up across microtitre plates (200 µl per well) at twice the desired antimicrobial concentration, using sterile media as the diluent. A positive control was included containing sterile broth only. The wells were inoculated with a ten-fold diluted starter culture (200 µl per well) resulting in a final inoculum of 5×10^5 CFU ml^{-1}.

2.7 Bacterial strains

Several strains of *Staphylococcus aureus, Vibrio harveyi* and *Escherichia coli* were used in this study (Table 10):

Table 10 *Bacterial Strains*

Name	Characteristics	Source
NCTC 12973	Methicillin susceptible *S. aureus* (MSSA)	ATCC, USA
NCTC 10442	MRSA	TCS Biosciences, UK
NCTC 11939	Endemic MRSA (EMRSA-1)	HPA, UK
NCTC 13143	Endemic MRSA (EMRSA-16)	HPA, UK
RN6390B	*agr* positive MSSA	Dr. R. Novick (Novick *et al.* 1993)
RN9611	*agr* negative MSSA	Dr. R. Novick (Novick *et al.* 1993)
ATCC 35556	Wild-type MSSA	Dr. F. Götz (Cramton *et al.* 1999)
ATCC 35556Δ*ica*::tet	*ica* deletion mutant, MSSA	Dr. F. Götz (Cramton *et al.* 1999)
BB120	Wild-type *V. harveyi*	Dr. B. Bassler, (Bassler *et al.* 1993)
BB170	Sensor 1^-, Sensor 2^+; *V. harveyi*	Dr. B. Bassler (Bassler *et al.* 1993)
NCTC 12900	*E. coli* O157:H7, Non-toxogenic	HPA, UK
DH5α	AI-2 negative, *E. coli*	HPA, UK

2.8 Storage of viable organisms

In the short term (<1 week), bacteria were stored on agar. For intermediate (<6 months) and long-term (>6 months) storage of bacteria, the 'Protect' micro-bead system (Fisher Life Sciences, UK) was used. Briefly, a bacterial colony was used to inoculate a micro-centrifuge tube containing the 'Protect' beads in a glycerol suspension. Once inoculated, the tube was inverted six times to ensure thorough mixing. The excess glycerol was then aspirated off, and the beads were stored at -20 or -80°C until needed. To minimise phenotypic change during storage and retrieval treatments, cultures were examined to ensure that the characteristics of the parent culture were retained.

2.9 Starter cultures and the standard inoculum

To obtain starter cultures of *S. aureus* and *E. coli*, one 'Protect' bead was used to inoculate 5 ml of sterile BHI. The culture was grown for 12 hours at 37°C with no agitation. Starter cultures of *V. harveyi* were grown from one 'Protect' bead in 50 ml of AB medium, incubated at 30°C for 48 hours on a rotary shaker (175 rpm). The culture was diluted 1:100 in AB media and incubated for a further 12 hours at 30°C (175 rpm) to ensure cells were in the late log phase.

To produce a standardised inoculum, 1 ml of starter culture was adjusted with sterile broth to a turbidity of 0.08-0.1 (620 nm); a correctly adjusted suspension contained approximately 1.5×10^8

CFU ml^{-1}. The inoculum was diluted 1 in 1000, giving a final standard inoculum of 1.5×10^5 CFU ml^{-1}.

2.10 Standard planktonic growth conditions

Starter cultures were used to inoculate standard volumes (200 µl per well or 10 ml in culture tubes) of sterile media. Unless otherwise stated, broth cultures and agar plates of *S. aureus* and *E. coli* strains were incubated at 37°C without agitation. Broth cultures of *V. harveyi* strains were incubated at 30°C with agitation on a rotary shaker (175 rpm).

2.11 Growth and preparation of biofilms

Sterile media ($BHI_{25\%}$+0.09% (w/v) glucose) was inoculated from the appropriate starter culture. Aliquots of 200 µl were dispensed into the wells of NuncSurface microtitre plates and incubated at 37°C for the required time. After incubation, the planktonic culture phase was gently aspirated and the biofilm washed with 200µl of distilled H_2O to remove unbound cells.

2.12 Estimation of bacterial growth

2.12.1 Determination of turbidity

Growth of bacterial cultures was routinely determined by measurement of turbidity, measured as optical density (OD) at 620 nm, using a Tecan SunRise microtitre plate reader.

2.12.2 Determination of viable count

Viable counts were determined for coloured / stained cultures, for which turbidity cannot easily be estimated (Miles 1938). Starter cultures were 10-fold serially diluted in phosphate buffered saline (PBS) from neat to 10^{-8}. A 20 µl volume of each dilution was spread on to an MHA-plate. Agar plates were incubated at 37°C for 24 to 48 hours. Colonies were counted and colony number was expressed as colony forming units per millilitre (CFU ml^{-1}).

To obtain a viable count from a biofilm, the planktonic culture phase was carefully aspirated, leaving the biofilm intact. Attached biofilm cells were gently washed with 200 µl of distilled H_2O, and PBS (100 µl) was added to each biofilm. Biofilms were removed by vigorously scraping the surface of the well with a pipette tip. The well contents were aspirated into a micro-centrifuge tube, and the cell clumps were further dissociated by being drawn though a syringe fitted with a 23-

gauge needle (Heilmann *et al.* 1996). The well contents were serially diluted, and a viable cell count obtained as above.

2.12.3 Determination of total cell numbers

In situations where both the number of dead and live cells were important, the total cell number was determined. Starter cultures were 10-fold serially diluted in PBS from neat to 10^{-8}. The total cell number was determined by the use of a haemocytometer counting chamber, and the cells were counted using a light microscope at 1000 x magnification; the total cell number was expressed as total cell number ml^{-1}.

2.13 Viability staining for microtitre plate reader quantification

Both resazurin and the tetrazolium salts were investigated for their potential use as viability indicators in the biofilm assay (Table 11, overleaf).

2.13.1 Resazurin (alamar blue) staining

Resazurin is both a chromogenic and fluorescent dye. During reduction, resazurin displays three colours (blue-pink-colourless). Resazurin (blue and non-fluorescent) is reduced to resorufin (pink and highly fluorescent) by cellular enzymes. The enzymes that may be involved in resazurin reduction include mitochondrial enzymes such as flavin mononucleotide dehydrogenase, flavin adenine dinucleotide, nicotinamide adenine dinucleotide dehydrogenase, nicotinamide adenine phosphate dehydrogenase and cytochromes (O'Brien *et al.* 2000). The reduction product, resazurin, rapidly leaks from the cell.

For preparation of the resazurin stock solution, one tablet of Alamar Blue (Sigma-Aldrich, UK) was dissolved in 20 ml of sterile distilled H_2O in the dark for 10 minutes before being diluted to a working concentration of 0.005% (w/v). An aliquot (100 µl) of diluted resazurin was dispensed into wells containing biofilms and incubated in the dark for five minutes.

A standard curve for resazurin reduction was obtained as previously described using MRSA NCTC 10442. The standard curve was plotted with CFU ml^{-1} on the X-axis against optical density 540 nm on the Y-axis. The assay was found to be non-linear due to the multiple colour changes exhibited by reduction of resazurin.

Table 11 *Viability Stains*

Name	Molecular Weight	λ. Max.	Reference
Alamar Blue (resazurin) 7-hydroxy-3H-phenoxazin-3-one 10-oxide	229	600 nm (Blue) 570 nm (Pink)	Wang *et al.* 1998
INT (Iodonitrotetrazolium violet 2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride)	505	490 nm	http://chemfinder.cambridgesoft.com
MTT (Thiazolyl blue 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)	414	540 nm	Koning *et al.* 1987
TTC (Triphenyltertrazolium chloride- 2,3,5-triphenyl- 2H- tetrazolium chloride)	334.8	510 nm	
XTT (Sodium 3,3-{-[(phenylamino) carbonyl]-3-4-tetrazolium}-bis(4-methoxy-6-nitro))	674	490 nm	

2.13.2 Tetrazolium salt staining

Tetrazolium salts precipitate and change colour when electron transfer in viable cells reduces them. Therefore, they are used for vitality assays. Four low molecular weight tetrazolium salts were investigated in this study.

Tetrazolium salt stock solutions were prepared in dH_2O. Tetrazolium stock solutions (100 µl) were added to microtitre plate wells, at a final working concentration of 0.05% (w/v). The microtitre plates were cultured at 37°C, in the dark for 24 hours. Absorbance was measured at 492 or 540 nm.

To increase the speed of XTT reduction, an electron carrier, phenazine methosulphate (PMS; Sigma-Aldrich, UK), was added. Solutions were prepared to a working concentration of 0.05% (w/v) XTT and 0.00039% (w/v) PMS.

To stain intact biofilms, an aliquot of 100 µl of XTT+PMS was added to each biofilm containing well; the microtitre plates were then incubated at 37°C for 20 minutes in the dark. Absorbance was measured at 492 nm and compared to a standard curve of XTT+PMS (OD_{492}) against CFU ml^{-1}.

2.14 Total cell staining for microtitre plate reader quantification

2.14.1 Safranin staining of cell pellets and intact biofilms

The total cells numbers (biomass) of the biofilm were determined using Safranin (VWR, UK) staining (O'Toole *et al.* 1999), which absorbs maximally at 420 nm. Safranin stains cells irrespective of whether the bacteria are dead or alive, therefore giving an indication of total cell count.

Fresh quarter strength BHI ($BHI_{25\%}$) + 0.09% (w/v) glucose was inoculated with a starter culture of MRSA (NCTC 10442) and incubated at 37°C for 18 hours. Microcentrifuge tubes (1.5 ml) containing 1 ml of the culture were centrifuged at 2500 x g for 20 minutes. The cell pellets were re-suspended in 250 µl of Safranin solution (Ready to use solutions for Gram staining kit; Sigma-Aldrich, UK), for 15 min before being re-centrifuged and the remaining unbound stain aspirated. All samples were then washed three times in 1 ml of distilled H_2O, vortexed for 2 minutes, and centrifuged (2,500 x g, 20 min). This process was repeated two further times or until the supernatant was clear, ensuring all unbound Safranin was removed. Lastly, bound stain was removed from the cell pellet by re-suspension in 250 ml of 70% (v/v) ethanol and mixed for 2 hours. Absorbance of a 200 µl aliquot of each treatment was measured at 540 nm using a Tecan SunRise microtitre plate reader.

For intact biofilms, the supernatant was carefully aspirated, and biofilms were dried for 5 minutes before the addition of Safranin (40 µl) for 20 minutes. Unbound Safranin was removed by gently washing twice with 200 µl of dH_2O. Bound Safranin was extracted with 50 µl of ethanol, for a minimum of 5 hours. The absorbance (540 nm) of the Safranin stained biofilm was measured using a Tecan SunRise plate reader. Optical density was compared to the standard curve of Safranin (OD_{540}) against total cell number ml^{-1}.

2.14.2 Crystal violet staining of cell pellets and intact biofilms

Crystal Violet staining of cell pellets and intact biofilms was carried out as previously described for Safranin staining (2.14.1), using the Sigma 'Ready to use solution for Gram staining' kit.

2.14.3 Alcian blue staining of cell pellets and intact biofilms

Alcian Blue staining of control cell pellets and cell pellets that had undergone polysaccharide removal was carried out as described for Safranin staining (2.14.1), with the exception that samples were stained with 250 µl of 0.05% (w/v) Alcian Blue 8 GX (Sigma-Aldrich, UK) in 2% (v/v) acetic

acid for 10 minutes. In addition, instead of washing in dH_2O, samples were washed twice with 1 ml of 2% (v/v) acetic acid. Bound stain was solubilised in 1 ml of 1% (w/v) sodium dodecyl sulphate (SDS) before the absorbance was read at 540 nm (Telang 2000).

Alcian Blue was also used to stain biofilms grown in chamber slides. After the planktonic phase of the culture was removed, biofilms were stained with 100 µl of Alcian Blue 8 GX (0.05% w/v in 2% acetic acid; Sigma-Aldrich, UK) for 10 minutes. Biofilms were then washed with 2 ml of acetic acid. Stained biofilms were left to dry for 5 minutes before investigation under the microscope (Telang *et al.* 2000).

2.14.4 Cell staining following removal of capsular polysaccharide

Microcentrifuge tubes (1.5 ml) containing 1 ml of 12 hour-old culture were centrifuged at 2,500 x g for 20 minutes and the supernatant aspirated. To remove capsular polysaccharide, the cell pellets were suspended in 1 ml of PBS (pH 7.0) containing 0.5 M sodium chloride (Sutra *et al.* 1990). Controls were suspended in 1 ml of PBS (pH 7.0). All samples were stirred vigorously for 10 min on an orbital shaker at 100 rpm. The micro centrifuge tubes were re-centrifuged and the supernatant removed. Cell pellets were stained using Safranin (Section 2.14.1), Crystal Violet (Section 2.14.2) or Alcian Blue (Section 2.14.3).

2.15 Visualisation of the biofilm

2.15.1 Microscopic validation

MRSA biofilm formation was examined and compared using episcopic differential interference contrast (EDIC) microscopy. For many years, biofilms were observed using scanning electron microscopy (SEM) because of the high magnification and image contrast that could be achieved. However, due to the need to dehydrate samples several incorrect theories were created; for example, that biofilms were flat confluent films (Keevil 2003). However, with the use of EDIC microscopy, which is a more rapid and cheaper alternative to scanning confocal laser microscopy (SCLM) and multi-photon SCLM (MP-SCLM), there is no need to dehydrate the samples so the specimen remains in a more natural state.

Conventional differential interference contrast (DIC) microscopy implements the destructive / constructive nature of light waves. The source light is split into two polarised parallel beams before it reaches the specimen, having traversed the object; the wave paths alter in accordance with thickness, slopes and refractive index. Variations in the sample cause interference between the two

beams, and the details are visualised in a pseudo 3-dimentional appearance. EDIC microscopy is based on conventional Nomarski DIC microscopy. However, DIC is commonly used with transmitted light and is therefore unsuitable for viewing biofilms on opaque materials such as rocks or plumbing tube. However, in episcopic DIC (EDIC) microscopy, the prism is placed above the stage, and it is refracted light that is visualised. EDIC is therefore particularly useful for studying biological contamination and biofilm formation on opaque surfaces, such as metals, plastics, tissue surfaces and indwelling medical devices (Rogers *et al.* 1992; Keevil *et al.* 1995 and Keevil 2001). Biofilm biomass was investigated by the analysis of percentage biofilm surface coverage.

2.15.2 Congo red staining of PIA / PNAG

Biofilms of different ages, grown on glass chamber slides were washed in 200 µl of dH_2O, and stained with 100 µl of an aquius solution of Congo red 0.08% (w/v) for 10 minutes. For biofilms less than three hours old, the Congo red stain was aspairated and the biofilms examined by EDIC microscopy. Congo red stained biofilms older than three hours, were first washed with 200 µl of dH2O, then visualised by EDIC microscopy.

2.15.3 SYTO-9 / propidium iodide viability staining

Conformation of viability data was also obtained by the use of *BacLight* viability kit (Molecular Probes, UK). *BacLight* is a combination dye system consisting of SYTO-9, which labels all cells green and propidium iodide (PI), which can only cross damaged cellular membranes; hence, viable cells are stained green and non-viable cells are stained red. The two BacLight stains were dissolved in DMSO, mixed together (300 µl / 300 µl) and diluted 1:10 in dH_2O, proving 6 ml of *BacLight* stock solution. The stock solution was kept at -20°C and protected from light. When needed, 200µl of BacLight was added to MRSA biofilms grown in glass chamber slides, from which the planktonic culture had been aspirated, the slides were incubated in the dark at room temperature for 20 minutes, then excess *BacLight* was gently aspirated off. The stained biofilms were visualised by epifluorescent EDIC microscopy.

2.15.4 TTC staining

MRSA biofilms grown in chamber slides were also stained with the tetrazolium salt TTC. Stock solutions of TTC were diluted using dH_2O to a working concentration of 0.05% (w/v). An aliquot of 200 µl was added on inoculation of the chamber slide. MRSA biofilms were grown in the presence of TTC at 37°C for 24 hours, the planktonic culture phase was aspirated and the biofilms

washed gently in 200 µl of dH_2O. The TTC stained biofilms were visualised using EDIC microscopy.

2.16 Autoinducer-2 bioassay

2.16.1 Preparation of cell-free supernatants containing AI-2 from planktonic and biofilm cultures

The isolation of the AI-2 pheromone was carried out as described previously (Surette *et al.* 1999). To obtain cell free supernatants, starter cultures of *V. harveyi* (BB 170 and BB120) were diluted 1:1000 in AB medium and grown at 30°C for 8 hours on a rotary shaker (175 rpm). For *S. aureus* (NCTC 10442) and *E. coli* (NCTC 12900 and DH5α), starter cultures (Section 2.9) were diluted 1:1000, into 100 ml of sterile $BHI_{25\%}$ + 0.09% glucose with and without 0.5 or 1 mM of boric acid (Sigma, UK), and incubated at 37°C for 12 hours. At various time points (5, 6, 7 and 8 hours for *V. harveyi* and 1-24 hours for *S. aureus* and *E. coli*) 10 ml samples were filtered through a 0.2 µm filter (Minisart, Sartorius).

Cell free supernatants were also obtained from biofilms; *S. aureus* and *E. coli* biofilms were grown in NuncSurface microtiter plates at 37°C for up to 24 hours. The planktonic culture phase was removed and the biofilm washed with 200 µl of dH_2O (to remove any residual planktonic cells) and air-dried. The biofilms were re-suspended in 50 µl PBS; cells were vigorously dispersed using a pipette tip. The liquid containing the re-suspended biofilm cells was aspirated and filtered using a 0.2 µm filter. All cell free supernatants were stored at -20°C for up to one month (Greenberg, 1979).

2.16.2 Autoinducer AI-2 assay

Cell free supernatants of *S. aureus* and *E. coli* strains and a chemically synthesised cyclic analogue of the AI-2 molecule (Kindly donated by Professor Shoolingin-Jordan) were tested for the ability to induce luminescence in the *V. harveyi* (BB170) reporter strain. Surrette and Bassler (1998) previously described an assay for the production of AI-2.

V. harveyi BB170 (sensor 1^-, sensor 2^+) starter cultures were diluted 1:5000 to below the quorum sensing threshold concentration and were dispensed into the microtiter plate wells (90 µl). The positive control wells contained 90 µl of the diluted *V. harveyi* culture and 10 µl of *V. harveyi* (BB120 or BB170) supernatant, while negative control wells contained 10 µl of sterile AB

medium. Test wells contained 90 µl of diluted *V. harveyi* BB170 culture and 10 µl of cell-free supernatant. To isolate wells and eliminate interference, India ink was placed in all wells adjacent to the test wells and also between the wells. The microtiter plates were incubated at 30°C for 1 to 8 hours on a rotary shaker (175 rpm). Luminescence was measured hourly using a Fluostar plate reader (BMG Labtechnologies) in the luminescence mode. Also, using the same machine the absorbance at 620 nm was measured and the luminescence normalised.

2.17 Assessment of antimicrobial agents and chemical signals

Stock solutions of disinfectants and antibiotics were first diluted in dH_2O, and then in to sterile $BHI_{25\%}$ + 0.09% (w/v) glucose to obtain their correct working concentrations. Inoculated aliquots (200 µl) of the supplemented media were dispensed into microtitre plate wells for 0 hour assays, or un-inoculated aliquots were dispensed into wells containing washed, preformed, immature (8 hour-old) or mature (24 hour-old) biofilms. All microtitre plates were incubated at 37°C for a further 18 hours. The treated biofilms were then assessed for total cell number, viability and structure using the methods described above. In some experiments stock solutions of the chemical signals, chemically synthesised AI-2 or the NO donor SNP (Sigma-Aldrich, UK), diluted in dH_2O, were also added to the growth media at 0, 8 or 24 hours.

3. EVALUATION OF EXISTING AND DEVELOPMENT OF A BIOFILM ASSAY SYSTEM TO ASSESS RESISTANCE

3.1 Introduction

Nosocomial infections, especially those associated with implantable medical devices, are on the increase. An estimated 60% of nosocomial infections are biofilm related, underlining the need for a standardised disinfectant and antimicrobial susceptibility testing protocol for biofilm formation.

For this investigation the microtitre plate format was chosen as it is high throughput, quantitative and can easily be modified. However, a wide range of microtitre plate assays have been described in the literature. Most microtitre plate assays are based around a similar format; cultures are grown in wells, the planktonic cells are removed, biofilm washed and the attached material stained and analysed. Generally, assays measure the total cellular material, which is useful if the treatment affects biofilm formation. However, if the treatment is tested against preformed biofilm, total biomass (total cell number) may be unaffected, while the biofilm viability is reduced. This would not be identified by the standard microtitre plate assays; therefore, measuring cell viability is essential when assessing antimicrobials against preformed biofilms.

The aims of this investigation were firstly to evaluate the best of the present microtitre assays and then adapt them to the needs of this study, giving quantitative data about biofilm formation by clinical and laboratory strains. The chemicals need to be evaluated for activity against biofilm formation (as a prophylactic treatment) and against preformed biofilm (therapeutic treatment) at different stages of development, such as initial cell attachment, and immature and mature biofilms. Therefore, it was important that information about viability (cytotoxic effects), as well as biomass, was obtained in order to determine whether biofilm formation is merely halted or removed.

3.2 Methods

Methods are as described in chapter 2.

3.3 Results

3.3.1 Development of the microtitre plate system to ensure reproducible MRSA growth and biofilm formation

The production of biofilm by some *S. aureus* strains has been well documented. However, the formation of a biofilm by the MRSA strains used in this study has not previously been defined. Not all *S. aureus* strains are capable of biofilm formation. Indeed, Knobloch et al. (2002) evaluated 128 *S. aureus* strains, of which only 73 were deemed biofilm positive using Christensen's tube test. Therefore, it was important that the strains used in this study were investigated for their ability to form biofilm.

Biofilm formation by *S. epidermidis* and *S. aureus* requires the synthesis of an intercellular polysaccharide adhesin (PIA) and the PIA-related polysaccharide PNSG (poly-N-Succinyl-β-1,6,glucosamine), respectively, which mediate cell-to-cell adhesion (Mack *et al.* 1996). PIA and PNSG are synthesised by the gene products of the *ica*ADBC locus (Cramton *et al.* 2001 and Gerke *et al.* 1998). Recently, Articola (2001) found that all *ica*ADBC-positive *S. aureus* strains displayed a biofilm / EPS (slime) positive phenotype on congo red agar (CRA), whereas all *ica*ADBC-negative strains were biofilm-negative on CRA. Screening on congo red agar was first established by Freeman (1989), but the method has since been modified by different investigators (Deighton *et al.* 2001; Heilmann *et al.* 1998) Congo red can form complexes with PIA and PNSG resulting in a visible colour change. Strains that are biofilm / slime positive form black colonies that have a dry crystalline morphology, and biofilm / slime negative strains form colonies that are red and have a smooth surface (Figure 12).

Therefore, the *S. aureus* strains were evaluated for their ability to form biofilm. Diluted (10^{-5}) starter cultures of the four *S. aureus* strains, including two EMRSAs (NCTC 11939 and NCTC 13143), one MRSAs (NCTC 10442) and one MSSA (ATCC 29213), were used to inoculate CRA plates, which were incubated at 37°C for 24 hours (Figure 12).

The colonies of all four *S. aureus* strains displayed the dry crystalline morphology indicative of a slime producing (biofilm forming) isolate. The smooth red colony morphology was not observed. Therefore, all four *S. aureus* strains were deemed biofilm positive. These results were also confirmed using Christensen's microtitre plate assay (Figure 13).

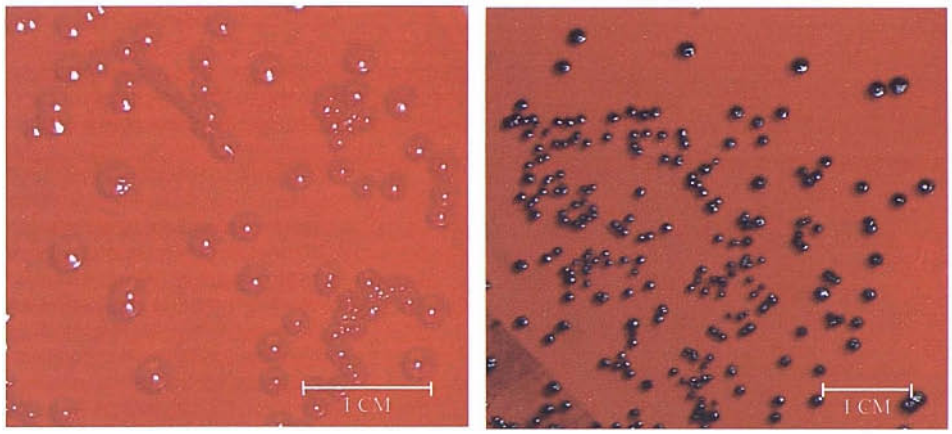


Figure 12 *Examples of colony morphologies produced by S. aureus on CRA. Images taken with a FinePix A210, Fujifilm 3.2 mega-pixels digital camera.*

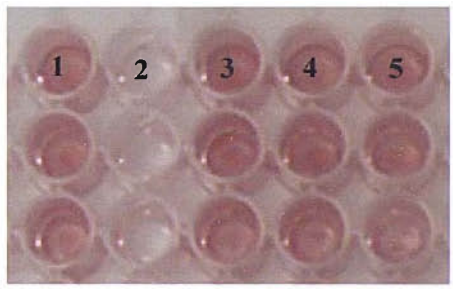


Figure 13 *Safranin staining of biofilms produced by S. aureus strains. Lanes 1 to 5 contain MRSA (NCTC 10442), control lane (medium only), MRSA (NCTC 11939), MRSA (NCTC 13143) and MSSA (NCTC 12973). Images taken with a FinePix A210, Fujifilm 3.2 mega-pixels digital camera.*

3.3.2 Identification of the immature and mature biofilm phase of growth

Biofilm formation has been described as occurring in three distinct stages. The first stage comprises of initial attachment of single cells to a surface, followed by the second stage of cell-to-cell attachment, which is achieved by the production of PIA / PNAG. Lastly, the attached cells become embedded in a "slime" or ESP matrix. It is difficult to define these stages in terms of the biofilm growth curve. However, Congo red complexes with β-glucans such as PIA / PNAG and PS/A, which are all found in abundance in the biofilm slime / matrix, a visible colour change (from red to black) occurs (Wood 1980).

Firstly, it was confirmed that Congo red would complex with PIA / PNAG, and that this would induce a colour change in the Congo red stain. Congo red agar has been used to show that colonies on the agar surface produce PIA / PNAG, but to this aurthers knowledge this has not been shown to occur in biofilm culutres. Therefore, using an *ica* delection mutant (ATCC 35556 *ica::tet*) and its wild-type strain (ATCC 35556), it was determined that Congo red would indeed form a coloured complex with the PIA / PNAG / EPS matrix of biofilm and planktonic cultures (Figure 14).

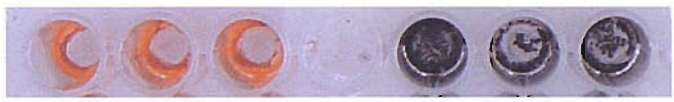


Figure 14 *Congo red stained cultures of the ica delection mutant (ATCC 35556Δica::tet), and its wild-type strain (ATCC 35556).*

Therefore, Congo red was used to define the stages of biofilm formation with respect to PIA / PNAG expression. MRSA (NCTC 10442) starter cultures were used to inoculate sterile BHI (2 ml) in glass chamber slides, culture incubated at 37°C for 1- 24 hours. After incubation, the planktonic phase was aspirated and the remaining attached cells were gently washed with distilled H_2O and stained with congo red (0.08%, w/v) solution for five minutes, after which residual stain was gently removed by washing with distilled H_2O. Biofilm formation and slime production was monitored over a 24-hour period using EDIC (Section 2.15.1) microscopy (Nikon Eclipse 600) (Figure 15, overleaf).

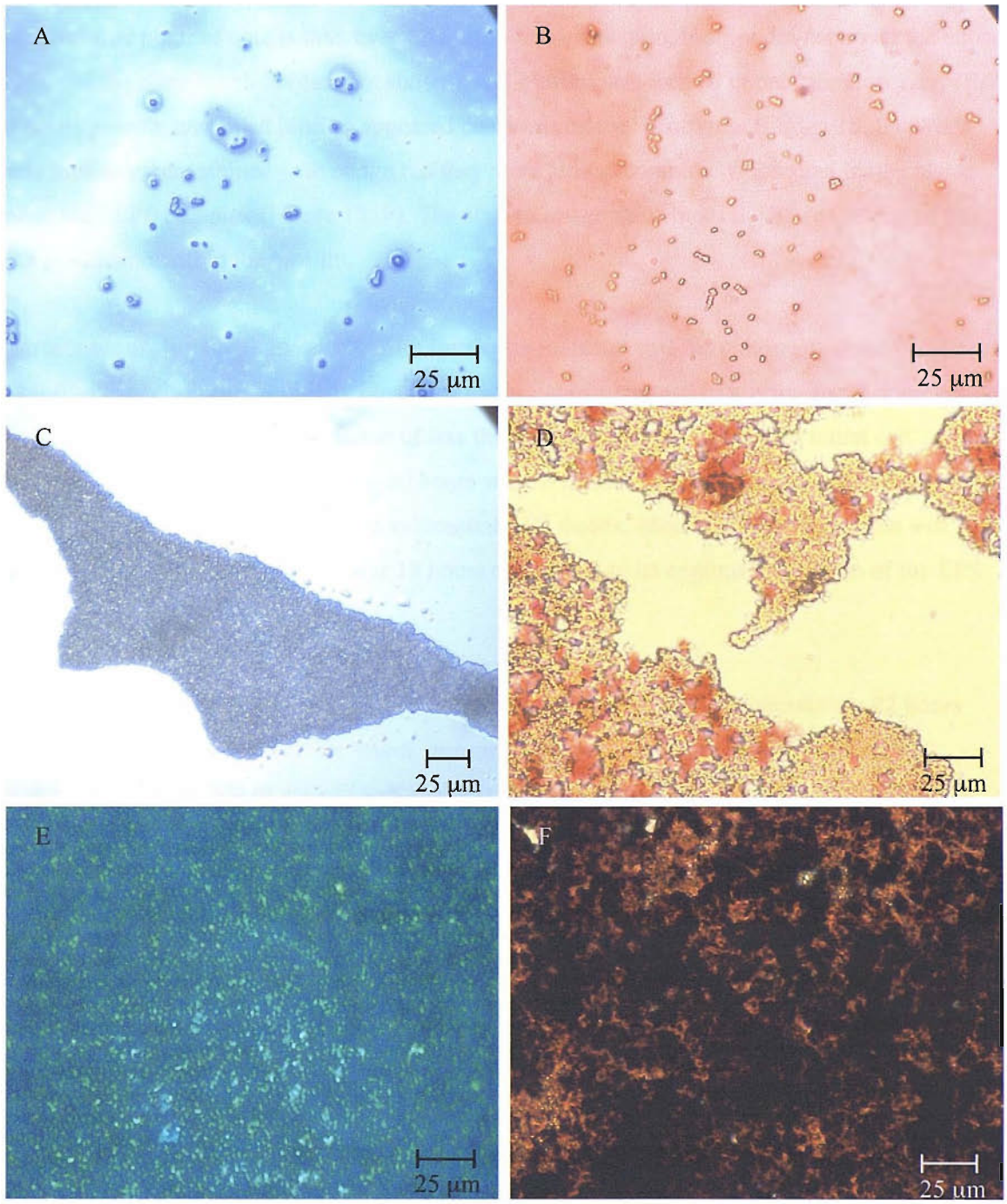


Figure 15 *An EDIC microscopy image (x1000) of MRSA (NCTC 10442) biofilm formation on the surface of a glass chamber slide. Unstained and congo red stained biofilm, after 2 (A and B), 8 (C and D) and 24 hours (E and F) incubation.*

After only two hours incubation, the community of attached MRSA cells were discrete and the cells were not close enough for cell-to-cell attachment to occur; also, the lack of colour change exhibited after congo red staining implied that no ESP matrix was being produced (Figure 15, A and B). After 5-9 hours incubation, more cells attached to the surface and aggregated over the surface. For biofilms of this age, stained with congo red (Figure 15, D), little or no colour change

was again observed, indicating that there was little to no EPS present for congo red to complex with. Another point of note is that, even after 10 hours incubation, biofilm did not cover the entire surface of the chamber slide (data not shown), and biofilm was formed in patches. However, after 24 hours growth, unstained biofilm appeared confluent on the substratum (Figure 15, E). When these biofilms were stained with congo red they were almost completely obscured by the black congo red / EPS complex (Figure 15, F). The stained image also shows the heterogeneity of the EPS production within the biofilm.

Microscopic analysis of *S. aureus* biofilm formation indicates that the previously described stages of biofilm formation can be clearly identified and roughly attributed to biofilm age. For the purpose of this investigation, biofilm formation of less than 3 hours will be regarded as initial cell attachment. Biofilm formation of 6 to 10 hours will be considered as immature biofilm, relating to its semi-confluent nature but lack of a substantial EPS matrix. Mature biofilm formation will therefore be regarded as anything over 18 hours old, owing to its copious production of the EPS matrix.

Christensen's microtitre plate assay was also used to monitor biofilm formation over 22 hours (Figure 16). Starter cultures of the four *S. aureus* strains were diluted 1:1000 into fresh BHI medium; aliquots of 200 µl were placed in the wells of a microtitre plate (NuncSurface), which were incubated at 37°C for 1-22 hours. After incubation, the planktonic phase of the culture was aspirated and the biofilms washed gently with 200 µl of distilled H_2O. The attached cells were stained for 20 minutes with a ready-to-use Safranin solution (40 µl per well); Unbound Safranin was removed with gentle washing in distilled H_2O.

Biofilm formation by the EMRSA strains, NCTC 11939 and 13143, was significantly (one way ANOVA, confidence level of 95%) reduced compared to biofilm formation by the laboratory MRSA and MSSA strains, NCTC 10442 and 12973, respectively (Figure 16). However, there was no significant difference in biofilm formation between the two EMRSA strains or between the MRSA and MSSA strains.

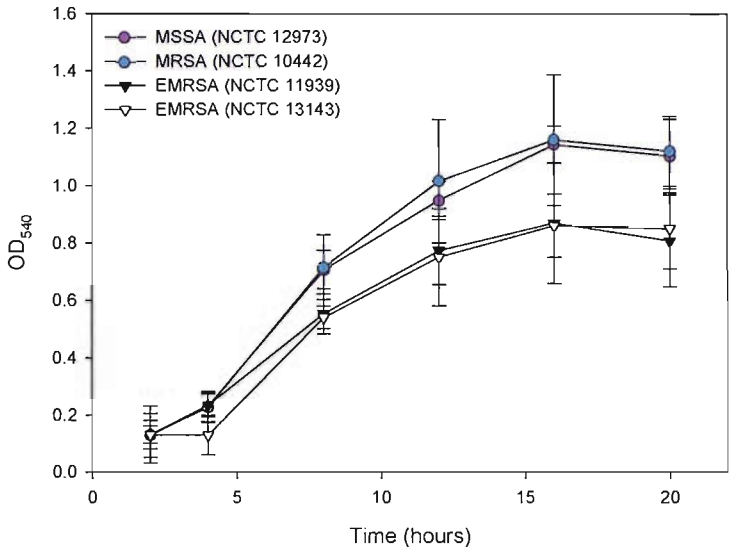


Figure 16 *A comparison of biofilm formation by the MSSA strain NCTC 12973, the MRSA strain NCTC 10442, and the EMRSA strains, NCTC 11939 and NCTC 13143.*

Although differences in biofilm formation were observed between the *S. aureus* strains, all four were deemed biofilm positive. It was, therefore, important to identify the optimum conditions for biofilm formation.

3.3.3 Comparison of culture media to provide optimum MRSA Biofilm formation

Many different media have been used in previous biofilm models, the most popular being Tryptone Soy Broth (TSB), although Mueller Hinton Broth (MHB), Brain Heart Infusion (BHI), Nutrient Broth (NB) and Luria-Bertani Broth (LB) have also been employed. Preliminary experiments compared the accumulation of biofilm biomass of several *S. aureus* strains (MSSA NCTC 12973, MRSA NCTC 10442 and the EMRSAs NCTC 11939 and NCTC 13143) in different commercial media over time. Biofilm formation was significantly increased for all strains when they were grown in BHI at one-quarter strength ($BHI_{25\%}$) (data not shown).

Bai *et al.* (2002) previously investigated biofilm formation by five *S. aureus* (MRSA) strains on orthopaedic screws made of stainless steel or titanium alloys. Biofilm formation in undiluted and four-fold diluted BHI ($BHI_{25\%}$), TSB and minimal media (MM) were compared. In accordance with the results of this investigation, Bai *et al.* (2002) showed that $BHI_{25\%}$ favoured biofilm formation and was superior to $BHI_{100\%}$, TSB and MM. However, this was not true for planktonic growth, but the author neglects to identify which media were best (Bai *et al.* 2002).

Many existing microtitre plate biofilm assays use media supplemented with a sugar, such as glucose and sucrose, at concentrations ranging from 0.25% to 5% (w/v) (Wu *et al.* 2003). Preliminary experiments compared biofilm formation in $BHI_{25\%}$ media supplemented with 0.09 (the highest physiological concentration that can be present in the blood of a healthy human (Nester *et al.* 2004)), 1 and 3% (w/v) glucose. Regardless of the glucose concentration, biofilm biomass was increased between 31 and 40% for all strains (data not shown). In the light of the above results, all subsequent biofilm experiments were carried out using $BHI_{25\%}$ + 0.09% glucose (w/v) as the growth medium.

Also, regardless of the media / glucose concentration used, biofilm formation of the EMRSA strains, NCTC 11939 and 13143, was retarded compared to the MRSA and MSSA strains, NCTC 10442 and 12973.

3.3.4 Influence of substratum physico-chemistry on biofilm formation

Many factors affect the initial attachment and the strength of attachment of a bacterium to a surface including: electrostatic and hydrophobic interactions, steric hindrance, Van der Waals forces, temperature and hydrodynamic forces (Dunne 2002). These factors can apply to the surface of the bacteria, the substratum or the medium in which they are suspended.

Initial experiments compared the influence of different physico-chemical substrata on biofilm formation. A surface neutral microtitre plate (NuncSurface) was compared to several commercial microtitre plates with varying surface properties. The data showed no significant difference in biofilm formation over a 14-hour period on different polystyrene surfaces. However, biofilms formed on NuncSurface microtitre plates were the most resistant to vigorous washing (data not shown), indicating that the strength of attachment was increased in biofilms formed on NuncSurface microtitre plates. Therefore, these plates were used throughout the remainder of this investigation.

3.3.5 The investigation of different viability dyes for use in the biofilm assay

The majority of existing biofilm assays measure only biofilm biomass and therefore give no indication of biofilm viability. These existing biofilm assays can only be used to assess the ability of antimicrobial agents to retard biofilm formation; they cannot effectively be used to assess antimicrobial activity against existing biofilms. Therefore, several viability dyes were investigated for their potential use in this biofilm assay including resazurin and the tetrazolium salts TTC, INT,

MTT and XTT. The two main criteria for the viability dyes was that they were sufficiently sensitive to react to low cell numbers, and they react in a reasonable amount of time for the assay to be performed.

Resazurin was considered for its potential use in the biofilm assay. Resazurin is both a chromogenic and a fluorescent dye. When reduced, resazurin goes though several colour changes, from navy blue to dark purple, then to bright pink and finally to very faint pink / clear.

As reduction involves several colour changes, the relationship between absorbance (single wavelength OD_{540}) and CFU ml^{-1} is not linear, making it very difficult to express the relationship in an equation. Additionally, as preliminary experiments demonstrated (data not shown) 15-hour-old biofilms contained between 2.5 and $3.0x10^6$ CFU ml^{-1}. At this viable cell number, the reduction of resazurin through one colour change, navy blue to dark purple, (therefore requiring the use of only one wavelength) took only one and a half minutes. This short incubation time was not practical; indeed, the addition of resazurin to a 96-well microtitre plate took approximately one minute, during which time the first wells had already completed their reduction; this would require wells to be measured separately, reducing the number of chemicals able to be processed.

Therefore, due to the complications of multiple colour changes and short incubation requirements, resazurin was not used in the biofilm assay. Instead, tetrazolium salts were investigated for their potential use.

3.3.6 Comparison of different Tetrazolium salts for use as a viability dye in the biofilm assay

Four tetrazolium salts were looked at as possible candidates (INT, XTT, MTT and TTC). Initial results showed that MRSA biofilms were capable of reducing each of the tetrazolium salts. However, the reduction of XTT, MTT and TTC to their coloured formazans (orange, blue and red) produced the strongest colour change (data not shown). When INT, MTT and TTC are reduced, however, they form a water insoluble formazan, so, when the tetrazolium salt is reduced within the cell, the insoluble formazan stays in place, in effect staining the cells. This caused a problem, because depth of colour within the biofilm was sufficient that the microtitre plate reader was unable to reliably read through the biofilm. One way to overcome this problem was to solubilize the formazan and biofilm in a suitable organic solvent; however, it was important that the biofilm remained intact. The formazan of XTT is soluble in water, and, once reduced, it is released into the liquid above the biofilm and not trapped within the biofilm, making it a more suitable choice.

However, a second problem was encountered; often the thickness of the biofilm formation was sufficient to obscure the absorbance readings of the above XTT containing supernatant (data not shown). This problem was overcome by the removal of a specific volume of reduced XTT containing supernatant and measuring its absorbance in a separate microtitre plate.

Also, in existing biofilm assays where biofilm viability is measured, often both the viability dye and chemistries to be tested are added at inoculation and their effect on biofilm formation is assessed. However, within this assay the effect of antimicrobial chemicals on biofilms of different ages needed to be measured. If the viability stain were to be added at the beginning of the assay, this would give false positive results, as reduction could occur before treatment began. This assay required a viability dye that was sensitive but could still be fully reduced within a few hours, so it could be added after treatment. Therefore, it was important to measure XTT reduction verses time.

3.3.7 The relationship between XTT reduction and incubation time

Initial experiments investigated XTT (0.005% (w/v)) reduction by 24 hour-old washed MRSA (NCTC 10442) biofilms over time. The biofilms were incubated in darkness in the presence of XTT, for 0-300 minutes. The absorbance of the stained biofilms was measured at 492 nm, using a Tecan SunRise microtitre plate reader, at 20-minute intervals for five hours (data not shown). The reduction of XTT over time proved to be a linear relationship, until an absorbance of 0.65 was reached (Data not shown). However, it took five hours to obtain an absorbance of just 0.4. The length of time required for the full reduction of XTT made it impossible to produce a standard curve for absorbance of XTT stained biofilm against CFU ml^{-1}, as, over the course of the experiment, the viable cell number would have changed dramatically.

Bioreduction of XTT by murine cells was also found not particularly efficient by Roehm *et al.* (1991). Roehm *et al.* (1991) found that they could improve XTT reduction by addition of electron coupling agents such as phenazine methosulphate (PMS) or menadione (MEN). Addition of one of these mediators, phenazine methosulfate (PMS), was therefore investigated for the reduction of XTT by *S. aureus* biofilms. MRSA (NCTC 10442) biofilms were grown in $BHI_{25\%}$ + 0.09% glucose, as described above; 24 hour-old biofilms were washed and incubated in darkness in the presence of 0.005% (w/v) XTT and 0.00039% (w/v) PMS, for 0 to 40 minutes. The absorbance of 50 µl of the reduced XTT / PMS containing supernatants was measured at 492 nm using a Tecan SunRise microtitre plate reader.

The reduction of XTT to an absorbance of 0.4 had taken a little over 4 hours, but, when XTT was reduced in the presence of PMS, it took around 20 minutes to reach this same level of absorbance (data not shown). This meant that, by the addition of PMS, the reduction reaction of XTT occurred 12 times faster than XTT alone; therefore, the reaction could be accomplished in a reasonable amount of time. It was then necessary to determine the relationship between XTT / PMS reaction and viable cell number.

3.3.8 The relationship between XTT / PMS reduction and viable cell number

In order to relate the absorbance of XTT / PMS dyed biofilms to viable cell number (CFU ml^{-1}), a standard curve was developed. Fresh culture medium ($BHI_{25\%}$ + 0.09% (w/v) glucose) was inoculated from a starter culture of MRSA (NCTC 10442); aliquots of 200 µl were placed in the wells of microtitre (NuncSurface) plates. The microtitre plates were incubated at 37°C for 24 hours. The culture was serially diluted and incubated for 20 minutes with XTT / PMS (0.005% / 0.00039% (w/v)). The absorbance of 50 µl of the reduced XTT / PMS containing supernatants was measured at 492 nm using a Tecan SunRise microtitre plate reader for each dilution, along with a determination of the viable cell number (Figure 17).

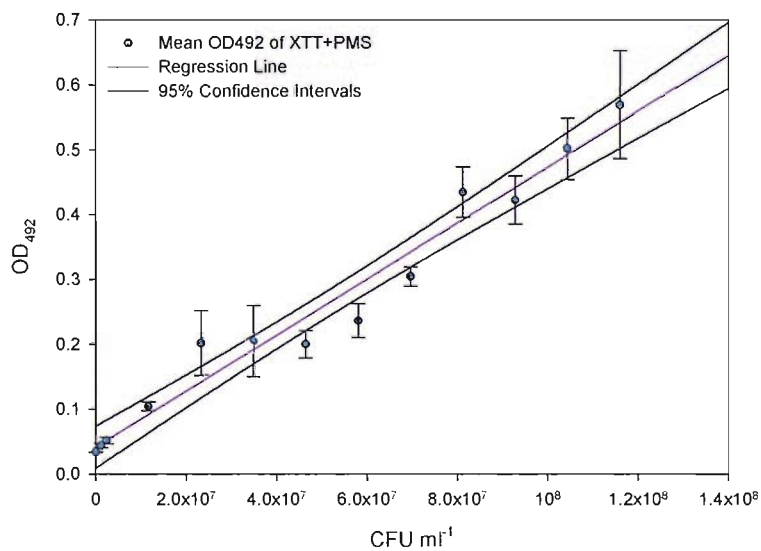


Figure 17 *The relationship between XTT / PMS dyed MRSA cells and viable cell number (CFU ml^{-1}). A regression line (Y=(X*4.31e-8)+0.042) was plotted showing the 95% confidence intervals.. Data are the mean of five experiments.*

The maximum absorbance achieved by XTT / PMS reduction was 1.05, which was obtained after 70 minutes incubation. This equates to a CFU ml^{-1} of 3.6 x 10^7, which is well above the maximum

viable cell number in a biofilm (grown in $BHI_{25\%}$ + 0.09% (w/v) glucose), which was 2.5-3.0 x10^6 CFU ml^{-1}. Therefore, the standard curve was valid up to and above the maximum viable cell number present in the biofilm.

It was noticed that repeats of this experiment, undertaken with different stock solutions of XTT / PMS, produced slightly different results. In order to eliminate these variances, which were due to slight inconsistencies in the preparation of XTT / PMS stock solutions, a new standard curve of XTT / PMS reduction versus viable cell number was undertaken for each new batch of stock solutions.

3.3.9 Assay of Total Cells

As well as a viable cell count, it was important to know the total number of attached cells, dead and alive. The majority of existing biofilm assays routinely use Safranin or Crystal Violet for this purpose. It was important therefore, only to determine which best correlated to total cell number and to ensure that neither of the stains were affected by the amount of EPS produced by the biofilm; otherwise, test chemicals that induced EPS production would artificially appear to have increased the total cell number relative to the viable cell number. Therefore, the intensity of Safranin and Crystal Violet-stained biofilm recovered in the presence of salt (facilitates removal of EPS; Sutra *et al.* 1990) was compared to the absorbance of untreated recovered biofilms. Alcian Blue staining was used to confirm that the EPS had indeed been removed by the salt treatment, because Alcian Blue selectively stains EPS and not bacterial cells. The absorbencies of Safranin and Crystal Violet staining for both treated and untreated recovered biofilms were not significantly different, indicating that both Safranin and Crystal Violet stain the bacterial cells only and not the EPS (data not shown).

The relationship between total cell number and Safranin staining (absorbance at 540 nm) was also investigated, so that absorbance measurements could be reliably related to total cell number. Starter cultures (Section 2.9) of MRSA (NCTC 10442) were used to inoculate fresh $BHI_{25\%}$ + 0.09% glucose media (100 ml), which was incubated at 37°C for 18 hours. The cultures were two-fold serially diluted with fresh media, and microcentrifuge tubes containing 1 ml of each dilution were centrifuged at 2,500 x g for 20 minutes, and the supernatant aspirated. The cell pellets were re-suspended in 200 µl of a commercial Safranin solution; cells were stained for 5 minutes. Unbound Safranin was removed by repeated washing, dH_2O was added to the samples and mixed for 1 minute; the samples were then centrifuged and the supernatant aspirated. This washing step was repeated until the supernatant was clear, then bound Safranin was removed by re-suspending the cell pellet in 200 µl of 70% (v/v) ethanol. Both the total cell number (via the use of a

haemocytometer) and the absorbance (540 nm; using a Tecan SunRise microtitre plate reader) for each dilution were determined (Figure 18).

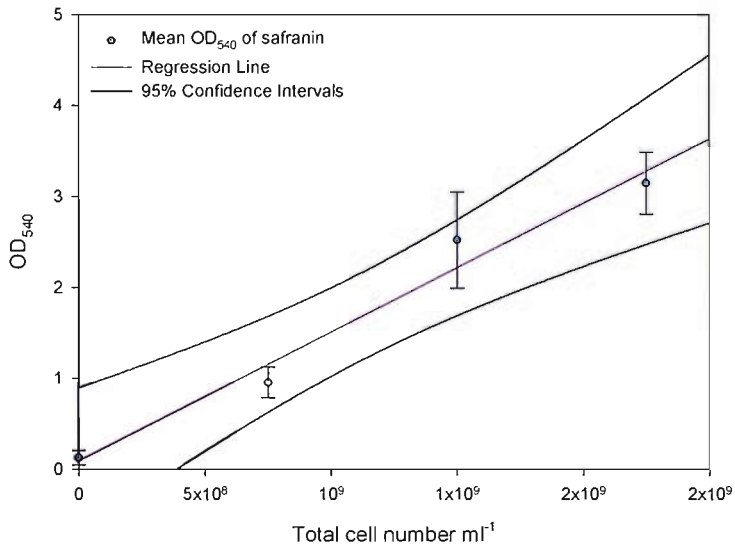


Figure 18 *Scatter graph showing the relationship between Safranin stained biofilm and total cell number ml^{-1}. A regression line (Y=(X*1.41e-8)+0.13) has been plotted showing the 95% confidence intervals Data are the mean of five experiments.*

The relationship between total cell number (ml^{-1}) and absorbance (540 nm) of Safranin-stained cells was linear. The equation of the regression line was used to relate the absorbance of Safranin-stained biofilms to total cell number. In the light of these results, it was decided that Safranin would be used to detect total attached cell numbers.

3.3.10 Validation of the combined biofilm assay

The cell viability procedure using XTT / PMS would have to be conducted first, as Safranin staining requires the biofilm to be solubilized with ethanol (killing any viable cells) and produces a much deeper colour, which would interfere with any subsequent staining. To determine if the cell viability assay procedure interfered with the assay of total cells, Safranin staining of control biofilms and biofilms that had previously undergone the cell viability assay were compared. No significant difference was found between the biofilm samples (data not shown), indicating that no interference had occurred between the assay procedures.

Biofilm Variability

Biofilms formation is a dynamic process of attachment, growth and detachment. Due to the heterogeneity of the EPS production, it was decided that a measure of the variability of the biofilm biomass and viability should be conducted (Figure 19). *S. aureus* starter cultures were used to

inoculate fresh $BHI_{25\%}$ + 0.09% (w/v) glucose. Aliquots of 200 µl were decanted into the wells of six replicate NuncSurface microtitre plates and incubated at 37°C for 24 hours. After incubation, the planktonic phase of the culture was gently aspirated off; unbound cells were removed washing the biofilms in 200 µl of dH_2O. An XTT / PMS stock solution (100 µl) was added to each well and incubated at 37°C in the dark for 20 minutes; 50 µl of the reduced viability stain (supernatant) was then gently aspirating and placed in a second microtitre plate. The absorbance of each well in this second microtitre plate was measured at 492 nm using a Tecan SunRise microtitre plate reader.

The biofilms in the original microtitre plates were then stained with a commercial Safranin solution (40 µl) at room temperature for 20 minutes. Unbound Safranin was removed by twice washing the biofilm in 200 µl of dH_2O, and the absorbance was measured at 540 nm (Figure 19). The total number of cells within the first replicate biofilm (blue bars, Figure 19) was normalised to 100%. The number of viable cells (black bars, Figure 19) within the first replicate biofilm was therefore represented as a percentage of its total cell number. For replicates 2 to 6, the total cell number and the percentage of viable cells, were expressed as a percentage of the total cell number in replicate 1.

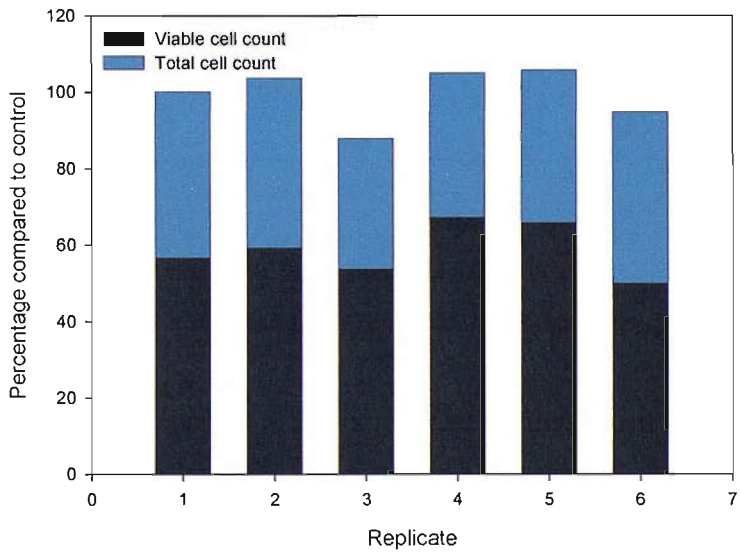


Figure 19 *The total biomass and the proportion of viable cells within replicate (1-6) biofilms, expressed as a percentage of replicate 1. Biofilms were grown for 24 hours at 37°C.*

The total cell number (biomass) in the six replicate biofilms varied by 18.1%, which equates to 4.2 x 10^5 cells (total cell number ml^{-1}). The percentage of viable cells within the biofilms varied between 50 and 67% of the total cell number, a difference of 17%; this equates to a difference in the viable cell number of 4.0 x 10^5 CFU ml^{-1}. However, using one-way ANOVA, the differences in total cell number and the percentage of viable cell were shown to be not significant, to a confidence level of 95%.

To determine whether the combined assay accurately depicted the viable and total cell numbers within mature biofilms, the above experiments were repeated. However, after incubation biofilms were removed from the microtitre plate surface by mechanical agitation (pipette tip), cell clumps were dissociated by drawing the resulting cell suspension repeatedly through a 23-gauge needle. Total cell numbers were estimated by direct cell counting of diluted samples of the cell suspension in a haemocytometer. A determination of viable cell numbers was carried out by plating out 20 µl aliquots of the serially diluted cell suspension on to nutrient agar plates, after incubation at 37°C for 24 hours the CFU ml^{-1} was determined. Using these methods viable and total cell numbers within mature 24-hour-old *S. aureus* biofilms varied by 19 and 22% respectively (data not shown), implying that the combined biofilm assay was as accurate as direct counting and culturing methods.

To assess the variability in viability, TTC stained biofilms were examined by EDIC microscopy (Figure 20). Earlier experiments had shown that the production of EPS was heterogeneous, and images of control biofilms showed that they varied in the amount of biofilm surface coverage (data not shown). MRSA biofilms were grown in chamber slides at 37°C for 24 hours; the biofilms were washed with 200 µl of dH_2O to remove non-adhered planktonic bacteria. The biofilms were then stained with TTC (0.05% (w/v)), at 37°C in the dark for 5 hours. The excess TTC solution was aspirated off and the biofilms were visualised using EDIC microscopy.

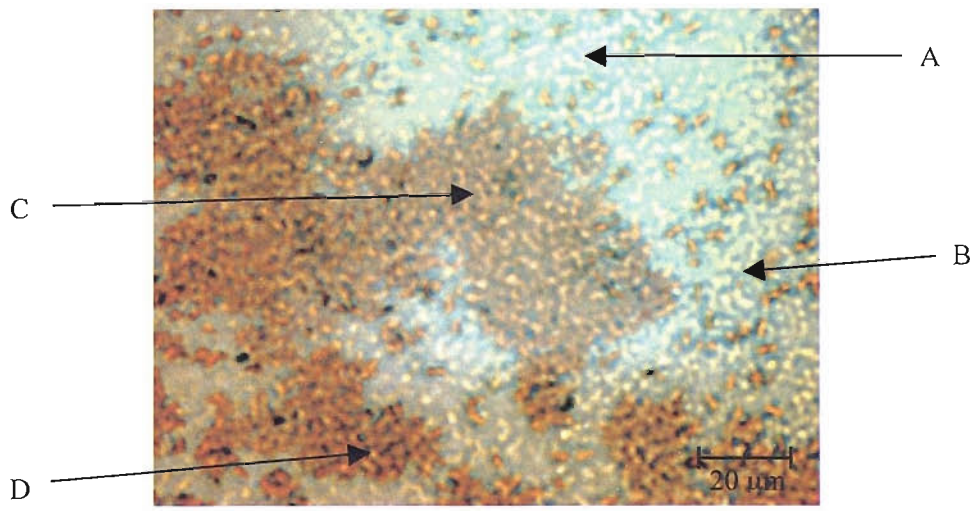


Figure 20 *An EDIC microscopy image showing the heterogeneity of respiratory activity (TTC stain) of 24-hour-old MRSA biofilm, formed on the glass surface of a chamber slide (x1000).*

This image shows that TTC reduction by biofilm cells was also heterogeneous, as reduction appears to occur in distinct patches. There appear to be four clearly defined states of respiratory

activity: A) no respiratory activity (dead cells), B) low respiratory activity, C) intermediate respiratory activity, D) higher respiratory activity.

Although the viability, biomass and EPS production had all been shown to be heterogeneous in MRSA biofilms, these differences did not significantly affect the results obtained with the combined (XTT / PMS and Safranin) biofilm assay. Therefore, the combined assay was used to define the effects of the addition antimicrobial agents on MRSA biofilms.

3.4 Discussion

The aim of the work presented was to assess and optimise existing microtitre plate assays and thereby to identify optimum growth conditions for good *S. aureus* biofilm formation. In addition, the heterogeneity of *S. aureus* biofilms was recognised in terms of PIA / PNAG - EPS production, and cell respiration.

3.4.1 Determination of biofilm formation

Knobloch *et al.* (2002) found that not all *S. aureus* strains were capable of biofilm formation. This was thought to be mainly due to the absence of the *ica* operon responsible for PIA / PNAG expression. Indeed both McKenney *et al.* (1998) and Articola *et al.* (2001) found a strong correlation between the possession of the *ica* genes and biofilm formation. However, all four *S. aureus* strains used in the present study were shown to produce PIA / PNAG by the formation of black colonies on CRA, and were also shown to produce biofilm in the Christensen *et al.* (1985) Safranin absorbance assay.

Biofilm formation followed the same sigmoid growth curve expected for planktonic growth, consisting of a clearly defined lag phase, exponential growth phase and static growth phase. However, it is noteworthy that biofilm formation by the *S. aureus* (EMRSA) strains NCTC 11939 and 13143 was slower, and the total biofilm yield or 'biomass' was retarded, as this was not a consequence of the planktonic growth rate, which was not significantly different to the other *S. aureus* strains (data not shown). One possibility for the difference in biofilms formation is the fact that both of the EMRSA strains are agr^{+} strains (Saunders *et al.* 2004). However, the MRSA and MSSA strains are possibly agr^{-} strains (preliminary experiments, data not shown), which tend to be hyper-biofilm forming strains.

Staphylococcal biofilm formation is a well-characterised process, comprising primary attachment of the bacteria to a substratum; then the attached cells multiply and form a mature, multi-layered, structured community, which finally becomes enclosed in an EPS matrix (Cramton *et al.* 1999). EPS production is important as it can be used as a marker of biofilm maturity. The EDIC microscopy images of Congo red stained biofilms indicate that in *S. aureus* the classic stages of biofilm formation can be clearly identified and visualised and also separated by time. Initial attachment of distinct singular cells was observed between 0-3 hours growth, where no EPS was detected. A second discrete stage of biofilm formation occurred between 3 and 9 hours growth, where biofilm formed in patches across the chamber slide surface and remained void of any significant amounts of EPS; these biofilms were therefore termed 'immature'. Lastly, the third

discrete stage in MRSA biofilm formation was defined by confluent biofilm surface coverage and the copious production of EPS. This stage of biofilm formation was designated 'mature' biofilm. The production of PIA in this time dependent manner is supported by the findings of Resch *et al.* 2005, who showed that in *S. aureus* biofilms the *ica* genes were expressed in an exponential manner between 3 and 10 hours growth, and expression gradually decreased between 10 and 16 hours, at which time expression was also most non-existent; implying that formation of the mature EPS matrix was complete. These stages have been described by the use of staphylococcal mutants deficient in one of the steps: *dlt*A mutants, which express teichoic acids that lack the D-alanine and are defective in initial cell attachment (Gross *et al.* 2001); and *ica* mutants, which are deficient in PIA / PNAG production, and are therefore defective in EPS matrix formation (Heilmann *et al.* 1996). However, to this author's knowledge, biofilm formation has not been visualised by Congo red staining in this way and has not been so successfully separated in time.

EPS production was heterogeneous across the biofilm surface. However, biofilms themselves are heterogeneous; biofilms contain channels through which nutrients circulate, and therefore cells in different zones of the biofilm cells experience differences in nutrient limitation, CO_2, O_2 and pH to name just a few (Brown *et al.* 1993). Cells also express different genes, depending on their position within the biofilm (Prakash *et al.* 2003); it is not surprising therefore that these differences lead to even greater variations in the physical makeup of the biofilm (e.g. EPS expression).

3.4.2 Biofilm growth conditions

Existing biofilm assays use an array of different media, some supplemented with various sugars. However, few investigators have identified reasons for their choices. Therefore, biofilm formation of all four *S. aureus* strains was monitored in several different media +/- glucose. $BHI_{25\%}$ proved superior to $BHI_{100\%}$ and $TSB_{100\%}$, and, when $BHI_{25\%}$ medium was supplemented with 0.09, 1 or 3% (w/v) glucose, regardless of the concentration, biofilm biomass of all four *S. aureus* strains was increased between 31 and 40% relative to the controls (data not shown).

Bai *et al.* (2002) recently investigated biofilm formation by five *S. aureus* (MRSA) strains on orthopaedic screws made of stainless steel or titanium alloys. Biofilm formation in undiluted and four-fold diluted BHI ($BHI_{25\%}$), TSB and minimal media (MM) was compared. In accordance with the results of this investigation, Bai *et al.* (2002) showed that $BHI_{25\%}$ favoured biofilm formation and was superior to $BHI_{100\%}$, TSB and MM. (Bai *et al.* 2002).

Biofilm is often formed as a defence against unfavourable conditions such as osmotic stress, heat shock, presence of toxic molecules (heavy metals and biocides), limited nutrient or O_2 availability

and a rise in pH. Indeed, under *in vivo* conditions, where iron and certain trace elements are scarce, bacteria produce proteins that bind or capture such elements (Hoyle *et al.* 1990). In most species, the EPS is predominantly anionic and creates an efficient scavenging system for trapping and concentrating essential minerals and nutrients from the surrounding environment (Costerton *et al.* 1987). Indeed, Cramton *et al.* (1999) demonstrated that anaerobic growth conditions led to increased expression of the *ica* operon and PIA / PNAG (major component of EPS) production in both *S. epidermidis* and *S. aureus*. Rachid *et al.* (2000) subsequently demonstrated that expression of *ica* is at least partially controlled by the stress response transcription factor σ^B. Nutrient limited stress conditions, which may be encountered earlier in the diluted $BHI_{25\%}$, medium, may be sufficient to kick start the induction of genes involved in acquiring scarce nutrients, and therefore induce the expression of PIA / PNAG e.g. EPS and the biofilm mode of growth.

However, contradictory to the above hypothesis that biofilms form in response to unfavourable environments, it has also been well-documented that biofilm formation in medium supplemented with glucose is significantly increased. The addition of glucose has been shown by several investigators to dramatically increase the number of slime-producing strains on CRA (Knobloch *et al.* 2002), suggesting that the genes for slime production / biofilm formation is inducible by a suitable metabolic source. In agreement with numerous other studies, Knobloch *et al.* (2002) and Stepanovic *et al.* (2000) found significant increases in adherence / biofilm formation of *S. aureus* isolates, when the growth media was supplemented with glucose or sucrose (Christensen *et al.* 1985; Deighton *et al.* 1990; Mulder *et al.* 1998). As in the present study, Stepanovic *et al.* (2000) also found enhanced biofilm formation in the presence of glucose was independent of concentration.

Addition of glucose to the growth media increases general bacterial growth (planktonically) by acting as a substrate for the glycolysis pathway, glucose is oxidised to pyruvate, producing a net gain of 2 ATP molecules. The increase in biofilm formation could simply be a by-product of the general increase in bacterial growth. However, glucose is known to induce the transcription of the *ica* genes that control PIA / PNAG production in *S. epidermidis* and *S. aureus,* respectively (Mckenney *et al.* 1998). The production of PIA / PNAG is currently the best-understood mechanism of staphylococcal biofilm induction, when the *ica* genes are up-regulated biofilm formation is induced. Jefferson (2004a) suggests that glucose induced EPS production may be multifunctional. Glucose may simply serve as a substrate in EPS synthesis, or EPS production may function as a mechanism for glucose storage. Alternatively, she suggests that bacteria may have evolved to interpret elevated glucose levels as a cue that they are in the blood stream; therefore, the formation of a biofilm removes it from circulation and protects it from the immune system.

3.4.3 Microtitre plates

Biofilm formation by *S. aureus* (NCTC 10442) was not affected by microtitre plate type, whether hydrophobic (PolySorp™), hydrophilic (MaxiSorp™) or neutral (NuncSurface). However, the strength of biofilm attachment was affected, with stronger attachment occurring on neutral microtitre plates, and the weakest attachments occurring on hydrophobic microtitre plates. Quirymen *et al.* (1989) found that the formation of dental plaque in the oral cavity occurred far less on hydrophobic than on hydrophilic surfaces. Also Everaert *et al.* (1997) found that, after six weeks in the human body, hydrophobic silicone rubber voice prostheses in laryngectomised patients harvested less biofilm than a hydrophilic surface prosthesis. Both these findings support the conclusions reached in this investigation; as biofilm produced in both situations would encounter shear forces in which weakly attached biofilm would be removed from the hydrophobic surfaces.

Many factors affect attachment of a bacterium to a surface and strength of attachment including: electrostatic and hydrophobic interactions, steric hindrance, Van der Waals forces, temperature and hydrodynamic forces (Dunne 2002). These factors can apply to the surface of the bacteria, or the substratum and the medium in which they are suspended.

Initial attachment occurs between the bacteria and a "conditioned" surface (a layer of adsorbed organic molecules from the media, present on a surface generally called a 'conditioning film'). As demonstrated in the oral cavity, the formation of a conditioning film can change the physico-chemical properties of a substratum (Bruinsma *et al.* 2001). The composition and hydrophobicity of absorbed conditioning films is dictated by the material's surface hydrophobicity, so each conditioning film is unique to its specific substratum (Vroman *et al.* 1975). Mittelman (1996) noted that a number of host-produced conditioning films, such as blood, tears, urine, saliva, intervascular fluid, and respiratory secretions, influence the attachment of bacteria to biomaterials. Therefore, $BHI_{25\%}$ medium may produce a unique conditioning film on the neutral microtitre plate that favours biofilm formation in *S. aureus*.

The physico-chemistry of microbial cell surfaces (phenotypic component) is an important determinant in the adherence of bacteria to living and non-living surfaces (Rosenberg 1984). Several groups have reported that *S. aureus* is hydrophobic, whereas *S. epidermidis* was said to be hydrophilic (Rosenberg *et al.* 1980). However, the growth environment can affect the cell surface hydrophobicity. Indeed, Hogt *et al.* (1983) showed that *S. epidermidis* cells were more hydrophobic following growth in TSB when glucose was present. However, these forces probably only affect initial attachment of single cells, when the cell is within 1 nm or less of the surface. Also, a ubiquitously valid description of the role of hydrophobicity on adhesion in different habitats is still

not available (Bruinsma *et al.* 2001), making it difficult to draw any definite conclusions as to why bacterial cells attach more readily to certain surfaces.

The chemical composition and structural arrangement of the cell surface is extremely complex due to the presence of a large variety of chemical components, most of which are involved in biofilm attachment. Heilmann *et al.* (1997) showed that attachment of *S. epidermidis* cells to polymer surfaces may be aided by an autolysin and therefore by its homologue *atl* in *S. aureus*. Teichoic acid structure is also extremely important in the development and attachment of biofilm. Specifically, the addition of D-alanine esters to teichoic acids via DltA may be an important factor in acquiring the correct charge balance on the Gram-positive cell surface to enable initial attachment and subsequent biofilm formation (Shirtliff *et al.* 2002). In addition, a two-component regulatory system, encoded by *arlRS*, a member of the OmpR-PhoB family of response regulators, is able to mediate adhesion and affect biofilm formation in *S. aureus* (Fournier *et al.* 2000).

In general, staphylococci do not posses surface appendages, such as flagella or pili (fimbriae) that many Gram-negative bacteria use to attach to surfaces. However, Rupp *et al.* (2005) found that rolling *S. aureus* biofilm microcolonies were attached to the underlying biofilm layer or the glass surface by "tethers," which were detected on the individual cocci associated with them. These tethers, which measured up to a few micrometers in length, initially formed at the leading edge of the microcolony and were gradually stretched to lengths of between 70 and 180 μm over periods of 1 to 3.5 hours before finally breaking, allowing a jerky forward motion. It is possible that these tethers also have a role in the attachment of static biofilm cells to polystyrene surfaces, in which case they may have a greater affinity for attachment to the neutral surface of the NuncSurface microtitre plate than other microtitre plates investigated.

In conclusion, regardless of the explanation, attachment was increased when biofilms were grown on NuncSurface microtitre plates. Therefore, these plates were used throughout this investigation, as the biofilm assay requires several washing steps, and biofilms grown on these microtitre plates are more resistant to washing.

3.4.4 Viability dyes

Measuring absorbance is one way in which biofilms can be evaluated, and this is often undertaken using Safranin or Crystal Violet staining. Absorbance measurements are easy to perform, but are often only valid within a limited concentration interval. Furthermore, they give no information about cell viability. The most common method for obtaining a viable cell count from a biofilm involves the physical removal (via mechanical agitation, vortexing or sonication) of attached

biofilm cells and a determination of the CFU ml^{-1} for the resulting cell suspension. This method is easy to undertake, relatively quick to repeat and inexpensive. However, a major disadvantage is that, due to the inconsistent release of biofilm cells from a surface, reproducibility with this method is considered low. Incomplete release of biofilm is most likely due to the heterogenic nature of many man-made materials, including plastics and glass, which often have rough surfaces that support denser biofilm formation and protection from agitation (Deighton *et al.* 2001).

In this investigation biofilm was removed from the polystyrene surface by repeatedly scraping the surface with a sterile pipette tip. Removing the biofilm was difficult and time consuming; when the experiment was first performed, results were spurious, due to the difficulty in dissociating biofilm clumps into single cells. When repeating the experiment, biofilm clumps were drawn several times though a 23-gauge needle to aid dissociation and the results were more reliable (Pettit *et al.* 2005). Another problem with this method concerns the physiological state of the cells themselves. Biofilm bacteria have been shown to have a decreased rate of respiration due to the limitation of nutrients and gaseous exchange. If detached biofilm cells are directly inoculated onto high nutrient agar, they may experience shock, possibly leading to cell death. Therefore, complicated and time-consuming resuscitation steps are often required (Cellini *et al.* 1998).

Other methods of estimating cell number include: counting attached cells using microscopy, suitable only for initial cell attachment; estimating growth using the incorporation of radioactively labelled substrates (Veenstra *et al.* 1996). However, as shown by Christensen *et al.* (1995) after the first few hours of incubation the ratio of colony forming units to counts per minute can become progressively unstable. Other methods include; assaying for secreted bacterial products (Deighton *et al.* 2001), such as the bioluminescence protocols; for urease (Dunne *et al.* 1991) and ATP (Schumacher-Perdreau *et al.* 1994). However, while both of these bioluminescent assays overcome problems associated with the inconsistent release of adherent bacteria from biomedical surfaces (Deighton *et al.* 2001) they require genetic modifications to include bioluminescent or fluorescent reporter genes in the *S. aureus* isolates. Therefore, not only are these assays inappropriate for testing clinical strains but it is also unclear whether these genetic modifications affect other aspects of the cell's behaviour (Amorena *et al.* 1999; Gracia *et al.* 1999; Tenhami *et al.* 2001).

A non-invasive and non-toxic way of measuring cell viability is the use of metabolically active dyes in a colorimetric assay. There are three main classes of metabolically active dyes measuring viability and cytotoxicity: i) fluorogenic esterase substrates, which measure enzymatic activity and cell membrane integrity; ii) nucleic acid stains that simultaneously detect both live and dead-cell populations by uptake or exclusion; iii) vitality dyes, stains that measure the oxidation or reduction state of the cell, providing a measure of cell viability and overall cell health; these are particularly

useful when cells have a tendency to adhere together or where other additives affect the colour of the growth medium (Eloff 1998).

Resazurin (Alamar Blue) has been extensively used as an oxidation-reduction indicator to detect bacteria in broth cultures and milk. However, the correlation between resazurin reduction and bioluminescent assays for ATP has been reported to be poor (Squatrito *et al.* 1995). Resazurin is both a chromogenic and fluorescent dye. When reduced, resazurin yields the product resorufin, which rapidly leaks from the cell. The major advantage of resazurin is that it is capable of detecting very low cell numbers, but this ability also makes it extremely sensitive. There were drawbacks to using resazurin; during its reduction, resazurin displays three colours (blue-pink-colourless) making it difficult to read at a single wavelength. In addition, as other authors have noted, the variations (or nuances) in the purple colouration, although clearly visible by eye, are not detected as obviously by a spectrophotometer (Gabrielson *et al.* 2002). For that reason, and because of the speed of reduction through multiple colour changes, resazurin was not used in this assay.

Therefore Tetrazolium salts were investigated, they are widely used for detecting the redox potential of cells for viability, cytotoxcity and proliferation assays (Hussain *et al.* 1993 and Roehm *et al.* 1991). They are colourless or yellowish dye precursors and, following reduction, these water-soluble compounds form uncharged, highly coloured but non-fluorescent salts known as formazans (Gabrielson *et al.* 2002). Tetrazolium salts detect oxidative enzyme systems by acting as electron acceptors (Liu 1981). Cleavage of the tetrazolium salt to a formazan occurs via the succinate tetrazolium reductase and the NADH-tetrazolium reductase systems of metabolically active cells. NAD(P)H formed during enzymatic reduction (dehydrogenase activity) transfers hydrogen and electrons to most tetrazolium salts (http://www.serva.de). The relationship between the amount of precipitated formazan / colour change and the bacterial concentration is well-established (Gabrielson *et al.* 2002; Mattila-Sandholm *et al.* 1991 and Tengerdy *et al.* 1967).

Initially, four, tetrazolium salts were considered (INT, XTT, MTT and TTC) for use, and, although TTC proved to be the most reactive of all the tetrazolium salts, TTC, INT and MTT were immediately ruled out for use in this biofilm assay as they form insoluble formazans. When these tetrazolium salts are reduced, they form coloured pellets within the biofilm cells. Due to the heterogeneous nature of the biofilm, these tetrazolium salts produced patchy coloured deposits, resulting in highly variable absorbance readings. Also, a second problem encountered was that, for particularly thick biofilm formations, absorbance readings were made artificially low due to the reader being unable to measure the absorbance of the coloured formazans through the biofilm itself; this particular problem has not been identified by any other authors. A possible solution to both of these problems is to introduce solubilization step, thereby releasing the formazans from the

biofilm cells. However, this is undesirable as it destroys the biofilm and does not allow for further investigation.

However, unlike most tetrazolium salts, the orange-coloured formazan of XTT is extremely water-soluble. This means that the highly coloured reduction product can leave the cells and re-enter the liquid, giving a more even colour distribution. XTT therefore does not require solubilization prior to quantification, thereby solving the problem of biofilm heterogeneity, and after reduction the liquid containing the XTT formazan can be removed for separate quantification, thereby solving the problem caused by distorted reading through think biofilms.

Moreover, the correlation of cell number versus reduction of XTT is reported to be similar or to be better than that of MTT (Meshulam *et al.* 1995). However, the reduction of XTT by *S. aureus* cells was not particularly efficient; this was also noted by Roehm *et al.* (1991) for murine cells. Yet, by the addition of phenazine methosulfate (PMS) (an electron mediator which effectively shuttles electrons across the cytoplasmic membrane), the time taken for reduction XTT could be increased, while still offering equivalent sensitivity and maintaining a good correlation between absorbance and CFU ml^{-1}.

At the time of undertaking this work, to the author's knowledge no one had used XTT to measure the viability of *S. aureus* or MRSA biofilms. However, El-Azizi *et al.* (2005) has since published a paper where viable cells within MRSA biofilms were monitored using XTT with menadione (MEN) as the electron carrier.

Viability data alone does not give sufficient information to make a valid conclusion about the prevention of biofilm formation or the disruption of preformed biofilms. Therefore, the XTT / PMS assay was run in conjunction with the standard Safranin assay developed by Christensen *et al.* (1985).

3.4.5 Assay of total Cells

The use of both Safranin and Crystal Violet is well-established in biofilm biomass assays. Neither one has any obvious advantages over the other. One modification that was made to the Christensen *et al.* (1985) assay was the re-solubilization of the washed Safranin biofilms. Safranin was re-solubilized in 50 µl of 70% (v/v) ethanol; this was undertaken so that the variability in the biofilm formation did not affect the readings of the microtitre plate reader.

It was important to confirm that both Safranin and Crystal Violet do not stain the EPS of biofilm cells; otherwise, test chemicals that induce EPS production would appear to have artificially increased total cell numbers / biomass. Therefore, Safranin and Crystal Violet staining of control and salt-washed (has been shown to remove ESP) (Sutra *et al.* 1990) samples was compared. Alcian Blue, a cationic dye widely used for the demonstration of glycosaminoglycans as it selectively stains acid mucopolysaccharides, was used to demonstrate the presence or absence of EPS in the treated samples. The salt wash proved successful in removing EPS from recovered *S. aureus* biofilms, and both Safranin and Crystal Violet were shown to uniformly stain bacterial cells regardless of the presence or absence of EPS. Therefore, the absorbance of Safranin and Crystal Violet was considered indicative of the concentration of bacteria. The work of Christensen *et al.* (1985) supports these findings; these authors removed polysaccharide from *S. epidermidis* and found no difference in Crystal Violet staining with untreated cells. Using direct cell counting Safranin absorbance was successfully related to total cell number within the biofilm.

XTT / PMS staining was used in conjunction with the Safranin staining method in order to create a picture of biofilm health. The interference between the two assays was measured (data not shown), Safranin staining of *S. aureus* biofilms was constant between biofilms previously stained with XTT / PMS (which was aspirated off before the Safranin staining commenced) and control biofilms.

3.4.6 Validation of biofilm assay

Replicate control biofilms were studied using the combined XTT / PMS / Safranin biofilm assay. The percentage of viable cells had an average value to 55%. One possible reason for this low percentage of viability in untreated control biofilms is that cells at the surface or edges of the biofilm are dead; a covering of dead cells would provide a mechanism of resistance for the biofilms as antimicrobial agents that try to penetrate the biofilm would first encounter dead cells and be 'mopped up'. However, these results were confirmed by the determination of total cell number by direct cell counting and the total viable cell number by undertaking of CFU ml^{-1} experiments. Therefore, this low percentage viability is not a factor of the combination of assay, but is a true representation of mature 24-hour-old *S. aureus* biofilms.

The percentage viability and the total cell number within the mature biofilms varied by 17 and 18%, respectively. However, statistical analysis (one way ANOVA) proved that these differences were not significant. The respiratory activity of cells within mature biofilms was shown to be heterogeneous, by TTC reduction; also EDIC microscopy images of replicate biofilms showed that biofilm percentage coverage of control 24-hour-old biofilms varied between 96 and 100% (data not shown).

Differences in surface coverage and biofilm thickness go some way to accounting for the variances in the total cell number of the replicate biofilms. However, variances in the staining procedure also probably account for variability.

However, biofilm formation is a dynamic process of attachment, growth and detachment, and, when a biofilm is at steady state, cells are shed from it at a constant rate (Potera 1999). Therefore, it has been widely recognized that biofilms are structurally and physiologically heterogenic; they contain water channels through which nutrients, O_2, CO_2, waste products and signalling factors circulate, creating concentration gradients. As a consequence of these gradients, differences in growth rate also occur. This situation creates bacterial populations with metabolic differences, leading to further differences in gene expression (Prakash *et al.* 2003). Heterogeneity within the biofilm has been shown for protein synthesis (Huang *et al.* 1998), respiratory activity (Xu *et al.* 2000), and by the present study, EPS production.

In conclusion, the interest in antibiotic susceptibility tests for biofilm bacteria has increased in the last few years (Dunne *et al.* 1993); and while the widely used systems, of the modified Robbins device or chemostat system, allow a continuous growth medium flow, rather than a discontinuous one, they require expert handling (Domingue *et al.* 1994). Also, neither of these methods are appropriate for the initial screening of numerous chemicals as they are not high through put, labour intensive and experiments need to be observed over several days or weeks. The 96-well microtitre plate assay used in this work follows the present tendency towards time-efficient bacterial viability tests (Stepanovic *et al.* 2000) and, despite the limitations of these assays, such as discontinuous nutrient supply and limited shear forces, it is possible using this methodology to predict the *in vivo* outcomes of device-related infections (Widmer *et al.* 1990). Microtitre plate assays are simple, reliable and cost effective.

The combined biofilm assay used here facilitates the automation of bacterial viability and total cell determination through absorbency of colored products, minimizing sample handling, and allowing the study of different factors within a single test sample. The main advantage that this combined assay is that it gives valuable information regarding the relative proportions of viability and biomass single samples of disinfectant or antibiotic treated biofilms, that easily and effectively be related reliably back to cell numbers within the actual biofilm, this is not determined by any other currently available microtitre plate assay.

4. DETERMINATION OF THE EFFICACY OF DISINFECTANTS USED IN HOSPITAL AND LABORATORY ENVIRONMENTS IN PREVENTING AND ERADICATING MRSA BIOFILM.

4.1 Introduction

As already discussed, MRSA is an important nosocomial infection; infection can occur from contact with contaminated surfaces, equipment and through contamination of health care worker's hands and gloves. MSSA and MRSA strains can survive on dry surfaces for prolonged periods, (Hirai 1991; Talon 1999) and many investigations have shown that these organisms can contaminate surfaces and equipment touched by hospital staff (Rutala *et al.* 1983; Blythe *et al.* 1998; Talon 1999). Standards of hospital cleaning have declined (Rampling *et al.* 2001), and floors, furniture and medical equipment may now be sources of MRSA transmission (Talon 1999; Rampling 2001).

It is, therefore, of the utmost importance that the hospital environment be cleaned and disinfected regularly and sufficiently to control the build-up of MRSA colonies and biofilm on contact surfaces and equipment in order to prevent cross-contamination to non-infected patients; especially as non-MRSA patients often reside in rooms / wards previously occupied by MRSA-infected patients. MRSA-infected patients are normally barrier nursed in private single rooms or specifically designated MRSA wards. Terminal cleaning is carried out after the MRSA patient has vacated the room / ward (French *et al.* 2004). However, French *et al.* (2004) found that the hospital environment could become extensively contaminated with MRSA which was not eliminated by standard cleaning methods. This perhaps suggests that the *S. aureus* found in the hospital environment were resistant to the disinfectants used, or that these bacteria were growing in a resistant biofilm.

Biofilm bacteria can be up to 1000-fold more resistant to antimicrobial agents than the same organisms grown planktonically (Costerton *et al.* 1999). The resistance is demonstrated, not only towards antibiotics and antiseptics, but also to highly reactive chemicals, including isothiazolones, quaternary ammonium compounds (Costerton *et al.* 1984) and halogens (Favero *et al.* 1983).

Several categories of germicidal chemicals are regularly utilised for cleaning and disinfecting healthcare environments, including: alcohols, aldehydes, halogens, phenolic compounds and quaternary ammonium compounds. However, hypochlorite compounds are one of the principle cleaning chemistries used in hospital disinfection, because they are readily available and

inexpensive. Hypochlorites are commonly available in different forms, such as solutions of sodium hypochlorite and powdered or tabulated sodium dichloroisocyanurate (NaDCC).

It was therefore important to determine the ability of the cleaning chemistries actually employed in the hospital and laboratory environment to kill / remove MRSA biofilm at the concentrations at which they are used. For that reason, along with hypochlorite itself, two cleaning chemistries containing hypochlorite compounds (sodium dichloroisocyanurate) (donated by the Southampton General Hospital), Sanichlor and Chemisan Plus, and two other cleaning chemistries, Hycolin, a phenolic-based compound, and Virkon, a multi-component peroxygen based oxidizing agent (disinfects aimed specifically at microbiology laboratories), were investigated for their competence in preventing initial cell attachment and their ability to disrupt preformed immature and mature MRSA biofilms. The efficacy of the germicidal agents in controlling MRSA biofilms was assessed by the following methods: direct staining procedures in the biofilm assay, advanced microscopy techniques and culture recovery growth on agar to determine viable cell counts.

To this author's knowledge, there is no published data describing the effects of the two hospital cleaning chemistries or Hycolin and Virkon against biofilms of MRSA or any other bacterial species. Plus, there is no information on how the viability of biofilm remaining after hypochlorite treatment is affected.

4.2 Methods

The methods used in this work were as described in chapter 2.

4.3 Results

4.3.1 The effect of hypochlorite on MRSA initial attachment and preformed immature and mature biofilms

The efficacy of hypochlorite on planktonic cultures was first determined. MRSA (NCTC 10442) starter cultures were used to inoculate 10 ml of sterile $BHI_{25\%}$ + 0.09% (w/v) glucose, which were then incubated at 37°C for 18 hours to a CFU ml^{-1} of approximately $3.4x10^8$. Hypochlorite stock solution was added to the cultures to give working concentrations of 100, 500 and 1000 mg l^{-1}. After a further 0.5 or 2.5 hours incubation, 100 µl aliquots, serially diluted in PBS, were used to inoculate nutrient agar plates, which were subsequently incubated at 37°C for 24 hours and the CFU ml^{-1} determined.

Culture viability was reduced to zero within 0.5 hours for the higher hypochlorite concentrations of 1000 mg l^{-1}, and 2.5 hours for the lower hypochlorite concentrations of 100 and 500 mg l^{-1} (Figure 21).

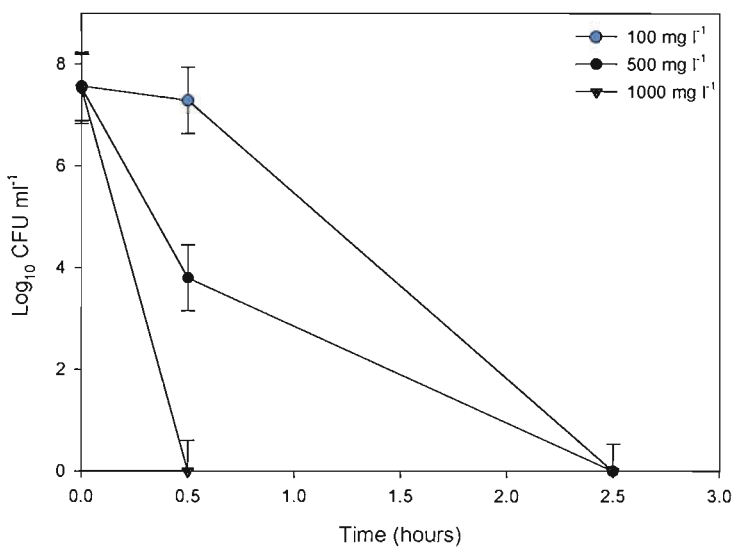


Figure 21 *The number of viable cells within static planktonic MRSA cultures after treatment with hypochlorite at concentrations of 100, 500 or 1000 mg l^{-1}. Data are the mean of two experiments.*

Although hypochlorite concentrations as low as 100 mg l^{-1} were capable of killing static planktonic MRSA cultures, biofilms have previously proved more resistant to disinfection (Luppens *et al.* 2001). Hypochlorite was therefore evaluated for its ability to affect biofilm formation and the disruption of preformed biofilms.

To investigate the ability of hypochlorite in preventing MRSA biofilm formation, biofilms were grown in $BHI_{25\%}$ + 0.09% glucose (Section 2.9) in the presence of varying concentrations (100, 500 and 1000 mg l^{-1}) of hypochlorite at 37°C for 18 hours. Control biofilms were grown without hypochlorite under the same environmental conditions.

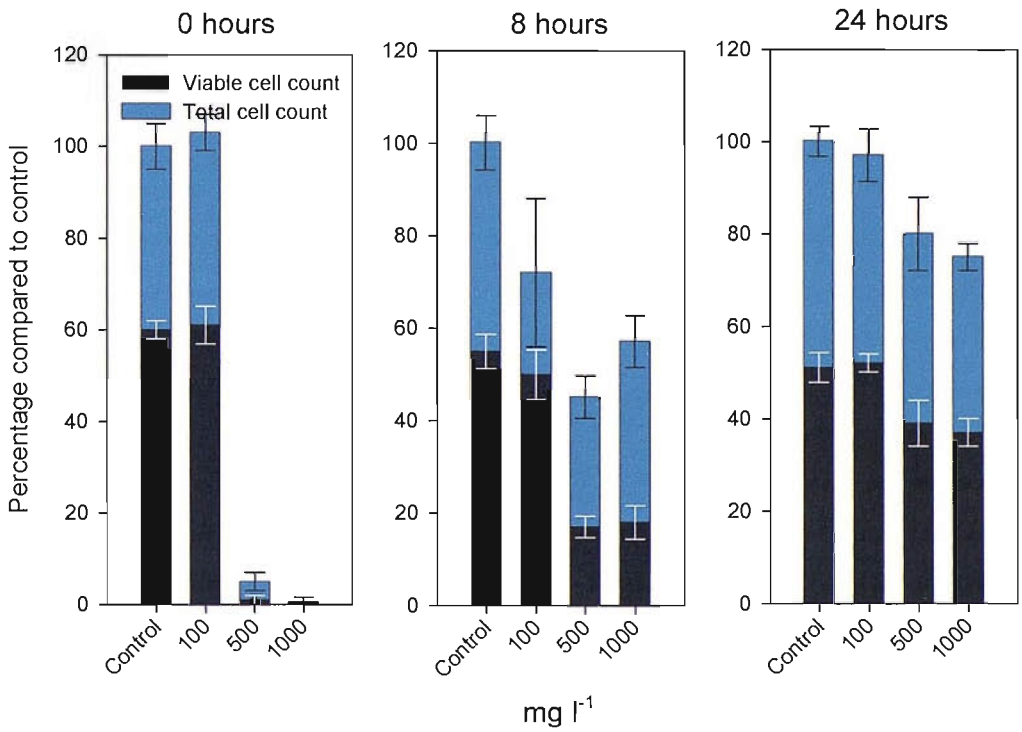


Figure 22 *The effect of hypochlorite treatment (100, 500 or 1000 mg l^{-1}) on the formation of MRSA biofilms and against preformed immature (8 hour-old) and mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data are the mean of four experiments.*

Control biofilms were represented as the total number of cells within the biofilm or biomass (blue bars; Figure 22), normalised to 100%, and the number of cells deemed viable (using the XTT / PMS assay procedure, Section 2.11.2) within the biofilm was represented as a percentage of the total biomass (black bars, Figure 22). The biofilm biomass and the relevant percentage viability of hypochlorite treated biofilms were therefore expressed as a percentage of the normalised control biofilm biomass.

The formation of MRSA biofilm was unaffected by the addition of 100 mg l^{-1} hypochlorite (Figure 22). The biofilms exhibited a comparable biomass (total cell number) and percentage viability to control biofilms. However, biofilm formation in the presence of 500 and 1000 mg l^{-1} hypochlorite was significantly retarded compared to control biofilms. The biomass of these biofilms was

reduced by 95%, and the viability reduced to less than 10% of the total. Therefore, hypochlorite at high concentrations was effective in preventing biofilm formation.

The effect of hypochlorite was also investigated against preformed MRSA biofilms. Biofilms were grown at 37°C for 8 (immature) or 24 (mature) hours, the planktonic phase of bacterial culture was aspirated and the biofilms washed gently with distilled H_2O to remove unbound cells. Aliquots of sterile media or media containing varying concentrations of hypochlorite were placed in the microtitre plate wells and the biofilms were incubated at 37°C for a further 18 hours.

Hypochlorite, at a concentration of 100 mg l^{-1} hypochlorite, caused a significant (30%) reduction in the biomass of immature biofilms. However, the percentage of viable cells was increased (Figure 22), suggesting either that there had been a slight under estimation of the biomass (the SEM was twice that of other treatments) or that there were fewer attached cells but more were viable. Hypochlorite at this concentration had no significant effect on mature biofilms.

Immature and mature biofilms treated with higher hypochlorite concentrations (500 and 1000 mg l^{-1}) showed a marked decrease in biomass of between 40-50% for immature biofilms and 20-25% for mature biofilms. The percentage of viable cells was reduced from 50% in the control biofilms to 40-30% for the immature biofilms (number of viable cells remaining equates to $2.29\text{-}2.53 \times 10^7$ CFU ml^{-1}). However, the percentage viability in treated mature biofilms was unaffected.

To confirm the results of hypochlorite treatment, EDIC microscopy images (x 1000) (Figure 23) were captured of each treatment for each biofilm age. An analysis of biofilm surface area coverage was undertaken, the results are displayed in Table 12.

Table 12 *The percentage surface area coverage of hypochlorite treated and untreated biofilms, grown on glass chamber slides*

Treatment	Biofilm Age (hours)		
	0	8	24
Control (0 mg l^{-1})	99%	100%	98%
Hypochlorite 100 mg l^{-1}	82%	97%	100%
Hypochlorite 500 mg l^{-1}	0%	49%	78%
Hypochlorite 1000 mg l^{-1}	0%	62%	67%

The surface area coverage of control biofilms was typically 98-100%. However, this was reduced with hypochlorite treatment. Figure 23 shows that biofilm formation was reduced in the presence of

100 mg l^{-1} hypochlorite and almost completely prevented in the presence of 500 and 1000 mg l^{-1} hypochlorite. The data in Table 12 confirms these results, showing 0% surface coverage (no biofilm) for biofilm formed in the presence of 500 or 1000 mg l^{-1} hypochlorite. For preformed biofilms, treated with hypochlorite, the percentage surface coverage was also reduced to as much as 49% for immature (8 hour old) biofilms treated with 500 mg l^{-1} hypochlorite and 67% for mature 24-hour-old biofilms treated with 1000 mg l^{-1} hypochlorite.

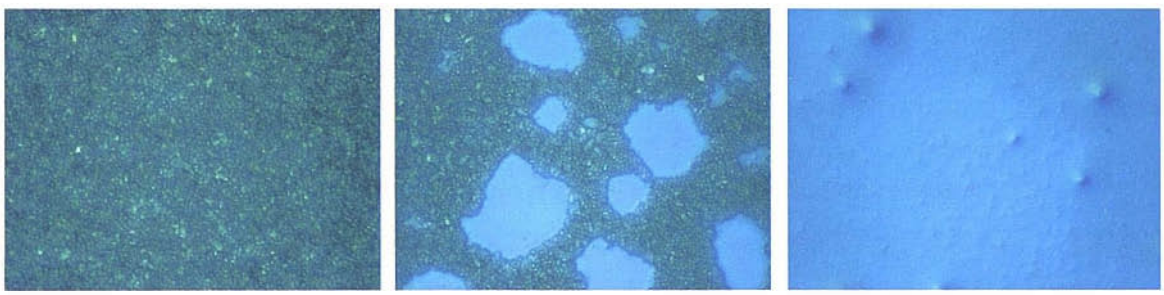


Figure 23 *Examples of EDIC microscopy images (x 1000) showing the different percentage surface coverage of biofilm formation by MRSA (NCTC 10442) with and without hypochlorite treatment.*

4.3.2 The effect of two commonly used hospital disinfectants on initial attachment of MRSA and preformed biofilms

Chemisan Plus

Chemisan Plus (Chemblend ltd, UK) claims that it has excellent cleaning properties and provides complete bactericidal properties and is effective against HIV and Hepatitis A, B and C. It is routinely used to decontaminate body fluid spillages and specifically for MRSA decontamination. For cleaning of body fluid spillages and MRSA decontamination, Chemisan Plus is used at a concentration of 50 g l^{-1}, equating to 10000 mg l^{-1} chlorine; for general cleaning of hard surfaces, a solution of 10 g l^{-1} is used (equating to 2000 mg l^{-1} chlorine).

Both concentrations were tested for their potential to kill static planktonic MRSA cultures (Figure 24), prevent MRSA biofilm formation and disrupt preformed biofilms (Figure 25).

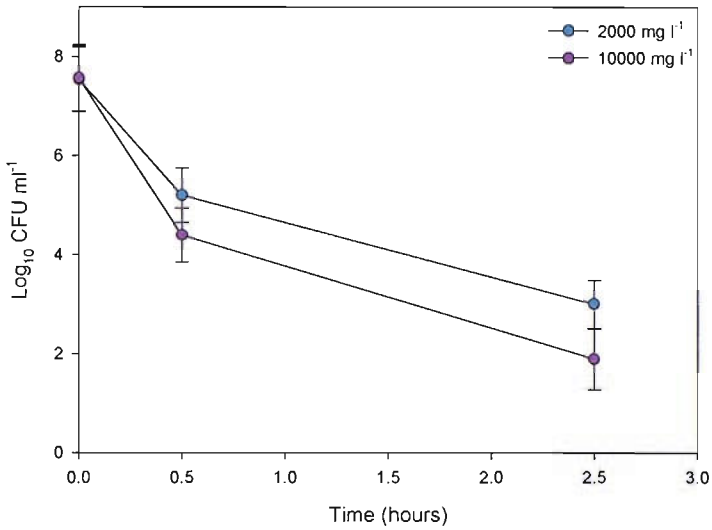


Figure 24 *The number of viable cells within static planktonic MRSA cultures after 0.5 and 2.5 hours treatment with Chemisan Plus at concentrations of 2000 and 10000 mg l^{-1}. Data are the mean of two experiments.*

A short 0.5 hour incubation in the presence of Chemisan Plus at both concentrations, produced a rapid two logarithmic reduction in the number of viable planktonic MRSA cells (Figure 24). However, after 2.5 hours incubation in the presence of Chemisan Plus, a significant number (between 8.7 x 105 and 9.5 x105) of MRSA cells were still viable in the static planktonic culture.

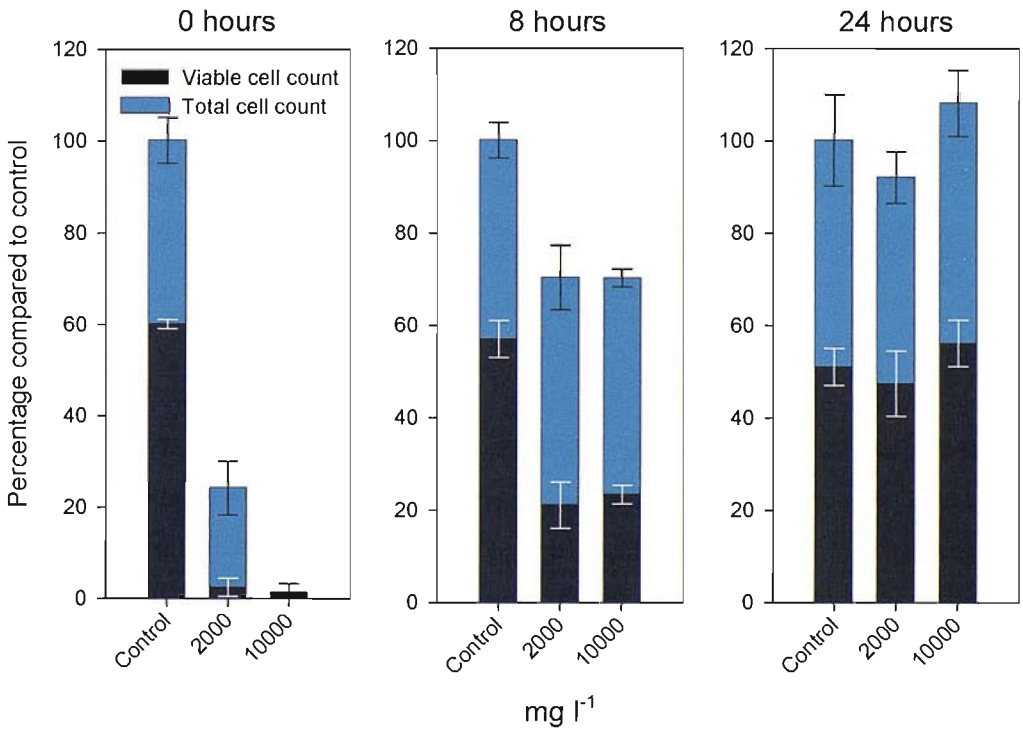


Figure 25 *The effect of increasing Chemisan Plus concentrations (2000 and 10000 mg l^{-1}) on initial MRSA biofilm formation and against preformed immature (8 hour-old) and mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data are the mean of five experiments.*

Biofilms formed in the presence of Chemisan Plus at 2000 mg l^{-1} and 10000 mg l^{-1} (Figure 25) showed a 75% and a 99% reduction in biofilm biomass respectively, compared to control biofilms. The percentage of viable cells in the biofilm treated with 2000 mg l^{-1} was reduced from 60% to 20% of the total biomass. However, the percentage of viable cells in the 10000 mg l^{-1} treated biofilm remained at 60%, but this may be a consequence of the XTT / PMS assay. Chemisan Plus was subsequently tested against immature (8 hour old) and mature (24 hour old) preformed biofilms (Figure 25).

When immature biofilms were treated with Chemisan Plus (Figure 25), the total biomass was reduced by 70% regardless of the concentration of Chemisan Plus used. The percentage of viable cells within the biofilms was also reduced from 50% of the total in the control biofilms to 35% of the total in the treated biofilms.

Mature biofilms treated with 2000 and 10000 mg l^{-1} Chemisan Plus displayed only a 6 or 9% difference in biomass (when compared to control biofilms) and 0 or 3% difference respectively in the percentage viability (Figure 25). However, the natural variability in biofilm biomass can be as much as 17% (Figure 19), consequently this result was not significant. It was therefore concluded that mature MRSA biofilms were unaffected by treatment with Chemisan Plus, indicating that mature biofilms are highly resistant.

Sanichlor

The second cleaning chemistry to be investigated for its efficacy in killing static planktonic MRSA cells, preventing biofilm formation and its disruption of preformed MRSA biofilms, was Sanichlor (Henkel-ecolab ltd, UK), (Figures 26 and 27). Sanichlor powder is marketed as a broad-spectrum disinfectant, used for the destruction of pathogenic organisms including HIV and hepatitis B viruses. The active ingredient is sodium dichloroisocyanurate, and its recommended concentration for use is 1000 mg l^{-1}.

Sanichlor, at a concentration of 1000 mg l^{-1}, was sufficient to reduce the CFU ml^{-1} of the planktonic MRSA culture to zero within 0.5 hours incubation (Figure 26).

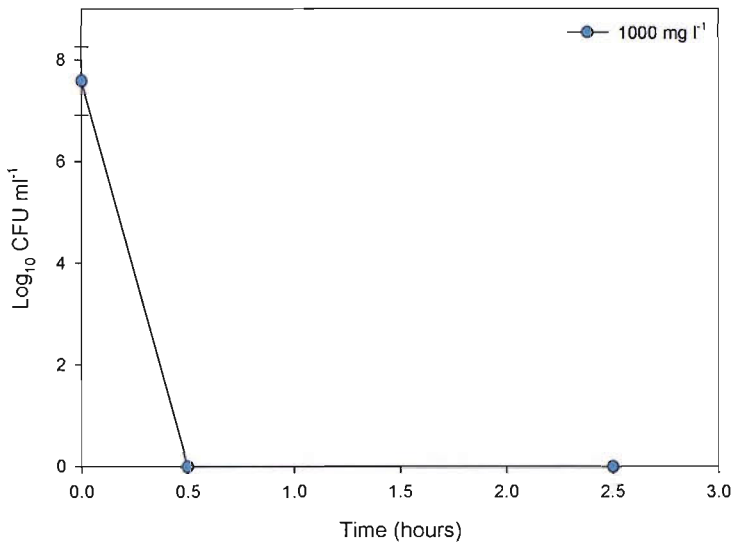


Figure 26 *The number of viable cells within static planktonic MRSA cultures after 0.5 and 2.5 hours treatment with Sanichlor at a concentration of 1000 mg l^{-1}. Data are the mean of two experiments.*

The biomass of biofilms grown in the presence of Sanichlor (1000 mg l^{-1}) was less than 20% of the control biofilms (Figure 27). The percentage viability was also reduced from 50% in the control biofilm to less than 10% of the total cell number in the treated biofilm.

When Sanichlor (1000 mg l^{-1}) was used to treat preformed immature MRSA biofilms (Figure 27), the biomass was reduced by 30% compared to control biofilms. Additionally, the percentage of viable cells was reduced from 55% in the control biofilms to 10% of the total biofilm biomass.

As seen in previous experiments, the biomass of mature 24 hour-old biofilms treated with Sanichlor (1000 mg l^{-1}) was slightly but not significantly increased. However, the percentage of viable cells was significantly decreased from 50% to 34% (Figure 27). Therefore, unlike Chemisan Plus, Sanichlor did demonstrate a small amount of disinfectant activity against mature preformed biofilms.

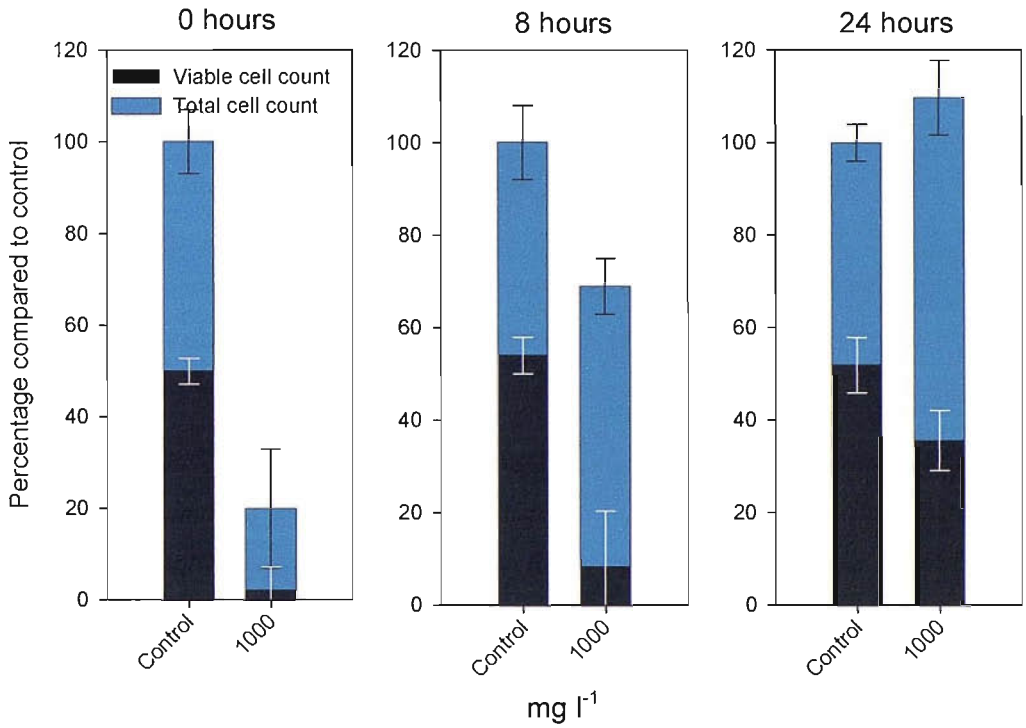


Figure 27 *The effect of Sanichlor (1000 mg l^{-1}) on initial biofilm formation and against preformed immature (8 hour-old) and mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data are the mean of five experiments.*

4.3.3 The efficacy of two well-known laboratory disinfectants in preventing MRSA biofilm formation and their effect on preformed MRSA biofilms.

The recommended working concentration for Hycolin is 2%; it is used for cleaning hard surfaces and discard pots. It is effective against bacteria, fungi and viruses. Virkon is used as a 1% solution; it is suitable for use on hard surfaces and for disinfection of laboratory equipment.

The efficacy of Hycolin and Virkon in killing static planktonic cultures was determined as for hypochlorite (Section 4.3.1) (Figure 30 and 32). Stock solutions of Hycolin or Virkon were added to the cultures to the desired working concentrations; the static planktonic cultures were further incubated for 2.5 hours. After incubation, serial dilutions of each treatment were used to inoculate sterile Nutrient agar plates, which were incubated at 37°C for 24 hours before being counted and the CFU ml^{-1} determined.

The ability of Hycolin and Virkon in preventing biofilm formation by MRSA was assessed by the addition of these chemistries at the point of media inoculation. The viability of biofilms formed in the presence of these cleaning chemistries was determined by the reduction of XTT / PMS (Section 3.13.2), and total biofilm biomass was determined by the Safranin assay (Section 3.14.1) The ability of the cleaning chemistries to disrupt and kill preformed biofilms was also determined. MRSA biofilms were grown in microtitre plates at 37°C for 8 or 24 hours. Biofilms were washed with distilled H_20 to remove non-adhered planktonic cells. Sterile media ($BHI_{25\%}$ + 0.09% glucose) was supplemented with Hycolin or Vircon at varying concentrations; these supplemented media were added to the biofilms, which were incubated at 37°C for a further 18 hours.

Hycolin

Hycolin is kept in aqueous solution by a detergent. It is effective against vegetative bacteria, including *Mycobacterium spp*, fungi, and has some activity against a limited range of viruses. The recommended working concentration for Hycolin is 2%; it is used for cleaning hard surfaces and discard pots. It is effective against bacteria, fungi and viruses.

All concentrations of Hycolin tested were capable of complete killing of the static planktonic MRSA cells within 0.5 hours of treatment (Figure 28). In addition, the data shown in Figure 29 demonstrates that Hycolin was successful in preventing MRSA biofilm formation at high (2 and 4% v/v) concentrations. Lower concentrations (1% v/v), reduced biofilm biomass to 20% of the control, and the percentage viability from 54 to 35%.

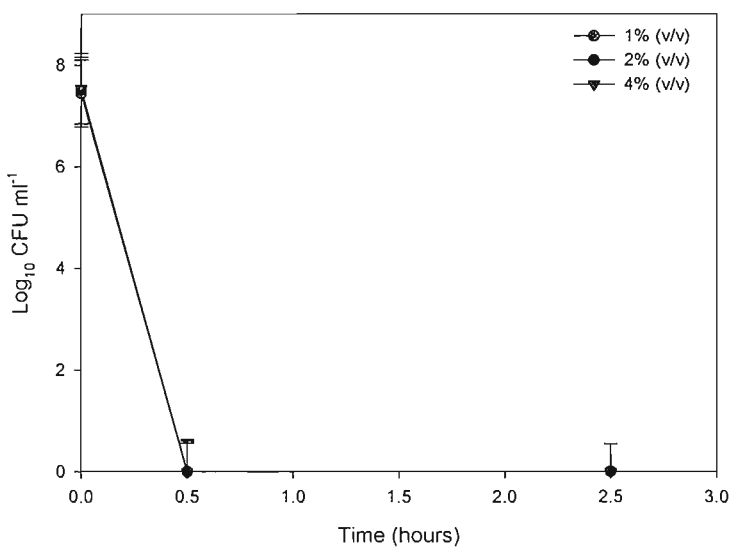


Figure 28 *The number of viable cells within static planktonic MRSA cultures after 0.5 and 2.5 hours treatment with Hycolin at concentrations of 1, 2 and 4% (v/v). Data are the mean of two experiments.*

Hycolin also possessed some activity against preformed biofilms. The total biomass of immature and mature biofilms was unaffected by treatment with Hycolin at concentrations of 1 and 2% (v/v) (Figure 29). However, the biomass of biofilms treated with 4% (v/v) Hycolin was reduced by 30% and 15% for immature and mature biofilms respectively. Using one-way ANOVA the differences in biomass between treated and untreated biofilms were found to be significant for the immature biofilms and not significant for the mature biofilms.

Treatment of these biofilms with Hycolin also resulted in a reduction in percentage viability of approximately 15% for 1 and 2% (v/v) for both immature and mature biofilms. A reduction in percentage viability from 50% to 18% or 50% to 37% for immature and mature biofilm respectively was also seen for biofilms treated with 4% (v/v) Hycolin (Figure 29).

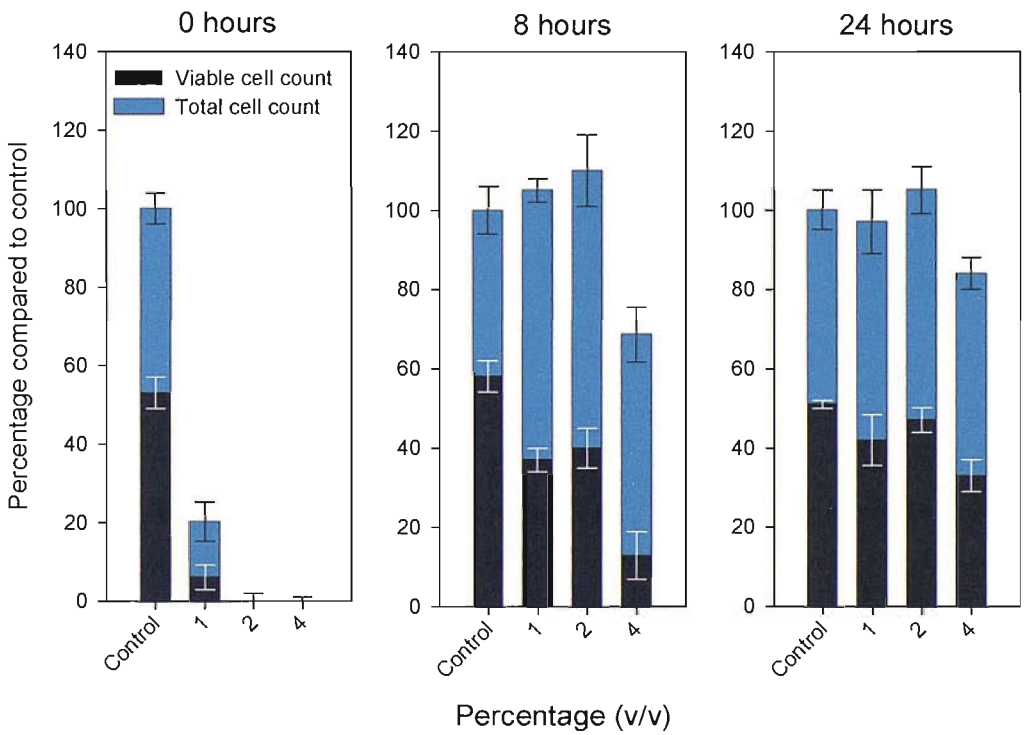


Figure 29 *The effect of Hycolin (1, 2 or 4% (v/v)) on initial biofilm formation and against immature (8 hour-old) and mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data are the mean of six experiments.*

Virkon

Virkon is suitable for use on hard surfaces such as floors, workbenches, trolleys and sinks, and for the disinfection of laboratory equipment including pipettes and centrifuges, as a 1% solution. Virkon is also used in the health care setting for the cleaning of bed frames and stainless steel instruments, as it is less corrosive than hypochlorite solutions.

Static planktonic cultures of MRSA (NCTC 10442) were killed within 0.5 hours of treatment with Virkon at concentrations of 0.5, 1 and 2% (w/v) (Figure 30).

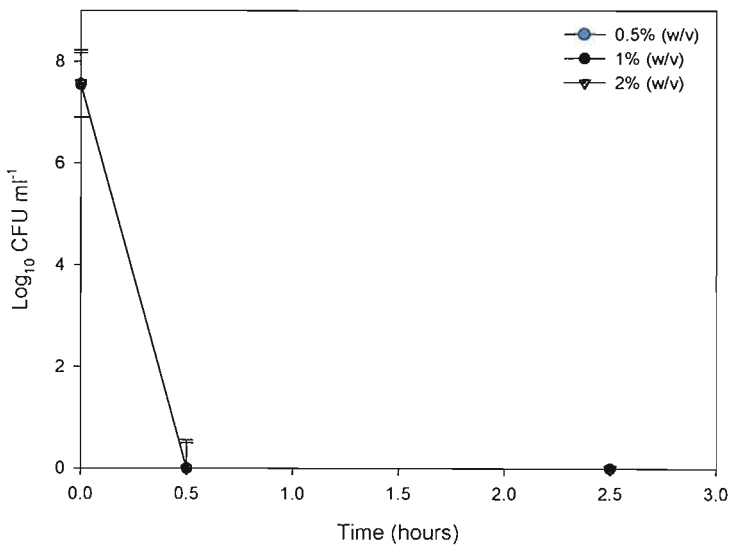


Figure 30 *The number of viable cells within static planktonic MRSA cultures after 0.5 and 2.5 hours treatment with Virkon at concentrations of 0.5, 1 and 2% (w/v). Data are the mean of two experiments.*

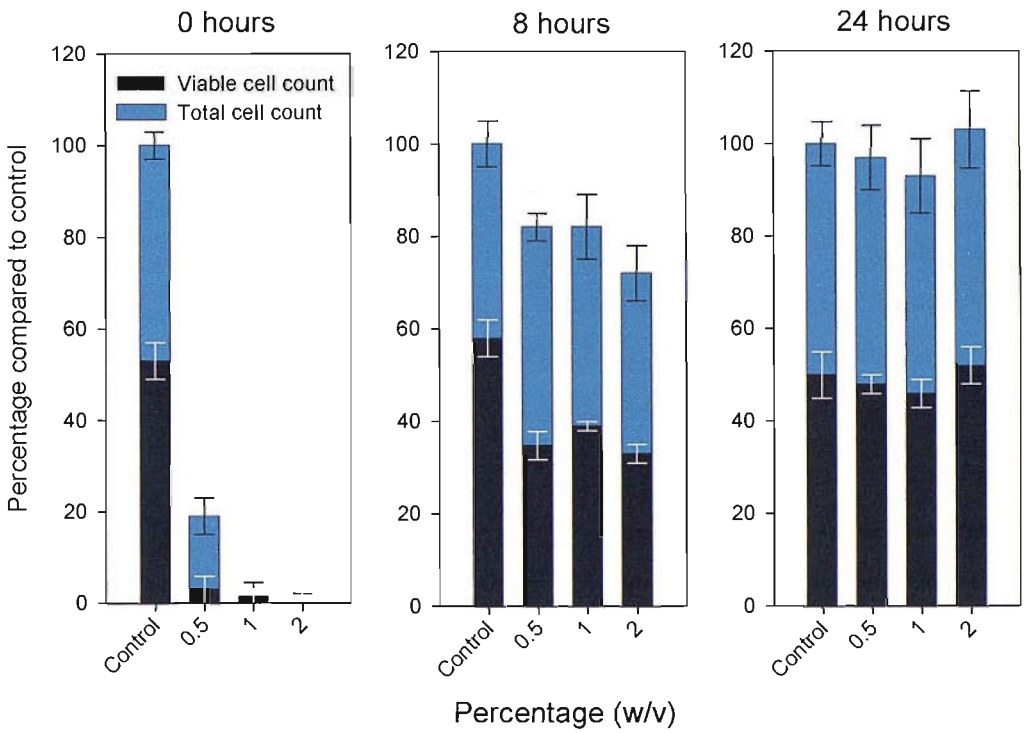


Figure 31 *The effect of Vircon (0.5, 1 or 2% (w/v)) on initial biofilm formation and against immature (8 hour-old) and mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data are the mean of six experiments.*

The biomass of biofilms formed in the presence of the lowest concentration of Virkon (0.5% (w/v) was reduced to 80% compared to the control biofilms (Figure 31); and the percentage viability was reduced from 55% in control biofilms to 20% in these treated biofilms. Biofilm formation was completely prevented when concentrations of 1% and 2% (w/v) Virkon were used. The biomass of preformed immature biofilms treated with Virkon at 0.5% and 1% or 2% (w/v) was reduced by 20% or 30% respectively (Figure 31). Also, biofilm viability was reduced from 57% of the total biomass in control biofilms to between 43% and 46% of the total biomass in Virkon treated biofilms. However, mature MRSA biofilms were resistant to Virkon at concentrations of 0.5%, 1% and 2% (w/v).

4.4 Discussion

The disinfectants investigated here are used at Southampton General Hospital and most other NHS hospitals throughout Hampshire. Overall, the results of this investigation show that, although hypochlorite itself and the hypochlorite-based disinfectants were capable of killing planktonic MRSA cultures in the static growth phase, both immature and mature MRSA biofilms were at least partially resistant, and in some cases were totally resistant, even after 18 hours treatment. Moreover, mature biofilms treated with Chemisan and Sanichlor actually showed increased biomass relative to the control biofilms. One simple explanation for this increase is the fact that hypochlorite is an oxidant; therefore, it may help to break down complex organic components within the medium, making them more assessable for use by the bacterial cells.

The non-hypochlorite based disinfectants, Hycolin, a phenolic-based, and Vircon, a peroxygen-based disinfectant, which have not previously been tested against biofilms, were investigated for their activity against static planktonic cultures and immature and mature biofilms. It was hypothesized that these non-hypochlorite-based disinfectants might have a more pronounced effect on mature biofilm as they would not be deactivated ('mopped up') in the EPS matrix. However, while both were capable of killing plankontic MRSA cultures in the static growth phase, and had significant effects on both biomass and relative percentage viability of immature biofilms, mature biofilms were totally resistant.

These results are significant, as contamination of the hospital environment is thought to be an important factor in the increase in MRSA nosocomial infections. French *et al.* (2004) found extensive MRSA contamination of door handles and sink taps in hospital wards previously occupied by colonised or infected patients. Boyce *et al.* (1997) also found similar MRSA environmental contamination, and also demonstrated that nurses contaminated their gloves with MRSA while performing activities that involved touching objects in the rooms of MRSA patients. Contamination of hospital wards with MRSA is not surprising, although it is usually supposed that the patient contaminates the room rather than the other way around. However, French *et al.* (2004) found that wards were often contaminated with multiple MRSA strains, suggesting survival of MRSA strains from previous occupants. Indeed, MRSA biofilms are able to survive for long periods in the environment. Both Beard-Pegler *et al.* (1998) and Wagenvoort *et al.* (2000) demonstrated that epidemic MRSA (EMRSA) strains (isolated from patients) survived 1 to 3 months longer on cotton and on hard surfaces than either strains that caused sporadic MRSA infection or hospital strains of MSSA, which were able to survive for 3 to 6 months. Surface dirt would possibly aid MRSA survival by giving protection from desiccation and possibly acting as a

nutrient source. Indeed, Rampling *et al.* (2001) found that a prolonged outbreak of EMRSA-16 could not be controlled until the organism was eliminated from the ward environment.

In the media, a reduction in and poor standards of hospital cleaning have often been blamed for the rise in MRSA nosocomial infections. Upon patient discharge hospitals employ terminal cleaning with disinfectants such as hypochlorite, which is widely thought to reduce viable adherent bacteria. However, French *et al.* (2004) compared MRSA contamination, prior to and immediately following terminal cleaning, and found only a small reduction in MRSA contamination. MRSA was common in sites that might transfer organisms to the hands of staff, and was isolated from areas and bed frames used by non-MRSA patients. They concluded that the hospital environment could become extensively contaminated with MRSA that was not eliminated by standard cleaning methods, perhaps suggesting that the attached cells were resistant to the disinfectants used. However, the results presented here suggest that the persistence of MRSA in the hospital environment may not only be due to ineffective cleaning, but due also to the failure of hypochlorite-based disinfectants in adequately removing and killing MRSA biofilms.

The failure of the disinfectants to successfully kill and remove mature MRSA biofilms can only exacerbate the ineffectiveness of, and supposed reductions in, hospital cleaning. Many studies have shown that planktonic cells in the stationary phase show striking similarities to cells within a biofilm; both are affected by nutrient limitation and high cell densities and express similar degrees of antibiotic tolerance (Fux *et al.* 2004). However, significant differences were seen in the relative susceptibilities of *S. aureus* cultures in the stationary phase and biofilms, implying that resistance of the biofilms is a function of the biofilm phenotype, not displayed by stationary phase cultures. By examining the modes of action of these disinfectants, it is possible to hypothesise mechanisms that lead to their failure in biofilm disinfection.

Hypochlorites are very strong oxidizing agents, the exact mechanism of killing bacteria has not been determined, but they are thought to cause the oxidation of sulfhydryl groups on essential enzymes, leading to cell death. Hypochlorite in water exists in three forms (as a dissolved gas (Cl_2), hypochlorous acid (HOCl), and / or hypochlorite ions (OCl^-)), which exist together in equilibrium. The three forms of hypochlorite have varying bactericidal strengths; hypochlorous acid is 100 times more powerful an oxidant and disinfectant than the hypochlorite ions. Consequently, hypochlorite solutions are most effective at pH 5 to 7 whereas hypochlorous acid is the predominant form (www.edstrom.com). The pH within and the surrounding MRSA biofilms was measured at 5.5 (data not shown), implying that hypochlorite should be at its most active.

The cell wall of staphylococci is mainly composed of peptidoglycan and teichoic acid, neither of which appears to act as an effective barrier to the entry disinfectants. Since high-molecular-weight substances can readily traverse the cell wall of staphylococci, this may explain the sensitivity of the cells in the planktonic mode of growth to these disinfectants. However, the plasticity of the bacterial cell wall is a well-known phenomenon (Poxton 1993). Reduced growth rate can affect the physiological state of the cells, and, under such circumstances, the thickness and degree of cross-linking of peptidoglycan are likely to be modified. However, while this may effect some cell wall active antibiotics, there is no evidence that it effects the activity of these disinfectants.

Hypochlorite reacts strongly with organic material, which consumes free hypochlorite, reducing its effectiveness; indeed, in situations when excessive organic material is present, concentrations of up to 1000 mg l^{-1} may be required, where a 500 mg l^{-1} would normally suffice. It may be argued that the hypochlorite disinfectants were deactivated by the media constituents. However, even in copious volumes of medium, there was sufficient free hypochlorite remaining to kill MRSA static planktonic cultures, containing approximately $3.5x10^7$ CFU ml^{-1} viable bacteria; significantly more than the total number of cells within the mature MRSA biofilms ($3.5x10^6$ CFU ml^{-1}, data not shown). Nevertheless, mature biofilms were resistant even with a contact time of 18 hours.

If the media is not sufficient in mopping up the hypochlorite, then, arguably the most important resistance mechanism of biofilms when considering hypochlorite is the formation of the ESP matrix. Indeed, increased resistance to hypochlorite has been reported for a *Vibrio cholerae* mutant, which expressed an amorphous exopolysaccharide, causing cell aggregation ("rugose" morphology) (Morris *et al.* 1996). In addition, using a chlorine detecting microelectrode, De Beer *et al.* (1994) were able to show that EPS prevented hypochlorite from reaching the majority (<20%) of cells in both *Klebsiella pneumoniae* and *P. aeruginosa* biofilms. The penetration profile was suggestive of a substrate being consumed within the matrix. This implies that the hypochlorite, in the presence of mature MRSA biofilms, was reacting with the EPS components and therefore being mopped up, reducing the levels of available hypochlorite; consequently, only cells in the upper biofilm layer were affected. However, it is doubtful whether the partial resistance of the immature biofilms can be entirely explained by the deactivation of hypochlorite in the limited amount of PIA / PNAG (a major constituent of the EPS matrix) that they produce.

Hycolin is a composition of phenolics blended with anionic surfactant. Phenolic disinfectants have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound (Nester *et al.* 2004). Gram-positive bacteria are generally more sensitive than Gram-negative to Phenol and its derivatives, which exhibit several types of bactericidal action. Active

phenol species penetrate and disrupt the cell wall and bind to the cytoplasmic membrane, causing inhibition of membrane bound enzymes and a change in membrane permeability. They also precipitate cellular proteins (Lambert *et al.* 1973). Whilst the most likely reason for the failure of the hypochlorite disinfectants is their deactivation in the EPS matrix, Kolawole (1984) found there was no difference in the killing of mucoid and non-mucoid strains of *S. aureus* by phenols, suggesting that the production of EPS matrix is not an important factor in the resistance of mature biofilms to phenols. Conversely, Converti *et al.* (1997) showed that the diffusion rate of phenols through biofilms of *Pseudomonas putida* was only 0.6% of that calculated for phenol diffusion in water. However, more importantly, Srivastava *et al.* (1966) proposed that phenol acts only at the point of separation of pairs of daughter cells, with young bacterial cells being more sensitive than older cells to phenol. Bacterial cells within biofilms exhibit slow growth (or even non-growth). Indeed, as a consequence of the biofilm structure, there are zones of cells within the biofilm, which experience differences in nutrient limitation, CO_2, O_2 and pH (Brown *et al.* 1993), leading to further differences in gene expression and growth. However, temping as it may be to ascribe mature biofilm resistance to a consequence of the reduced growth rate, this does not explain the susceptibility of static growth phase planktonic cultures, as they to have reduced growth rates. Therefore, resistance of mature biofilms to Hycolin is probably due to a combination of retardation in the EPS and the reduced growth rate.

Virkon is billed as the first new approach to disinfection in forty years, possessing an unrivalled spectrum of activity including staphylococci and, in particular, MRSA. Unlike hypochlorite-based disinfectants, Virkon is resistant to inactivation by organic material, and therefore cannot be mopped up by the EPS matrix. Virkon is a balanced, stabilised blend of peroxygen compounds, surfactants, organic acids and an inorganic buffering system. Peroxygens include hydrogen peroxide, peracetic acid and ozone. Hydrogen peroxide (H_2O_2) is a widely used biocide for disinfection, sterilization, and antisepsis. H_2O_2 acts as an oxidant by producing hydroxyl-free radicals (OH), which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted (Block 1991). In general, greater activity is seen against Gram-positive than Gram-negative bacteria. However, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations. Peracetic acid (PAA) (CH_3COOOH) is considered a more potent biocide than hydrogen peroxide, being sporicidal and bactericidal at low concentrations (<0.3%) (Block 1991). PAA has the added advantage of being free from decomposition by peroxidases, unlike H_2O_2, and remaining active in the presence of organic loads (McDonnell 1999). Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (McDonnell 1999). Similar to H_2O_2, PAA probably denatures proteins and enzymes and increases cell wall

permeability by disrupting sulfhydryl (-SH) and sulfur (S-S) bonds (Block 1991). Potassium peroxymonosulphate (Ozone R) is used as an efficient, chemo-selective and stereo-selective oxidizing agent for a wide variety of phosphorous, phosphothio- and phosphoseleno-compounds. Therefore, Virkon works by oxidising proteins and other components of the protoplasm, resulting in inhibition of enzyme systems and the loss of cell wall integrity (www.dustless.com/chemicals/virkon).

The diffusion coefficient of H_2O_2 within biofilms was shown to be about half their value in pure water (Stewart 1998). Therefore, there does not appear to be a significant diffusion barrier. Failure to penetrate the biofilm is more likely to depend on a neutralizing reaction between the hydrogen peroxide and some constituent of the biofilm (Stewart *et al.* 2000).

In general, stress response regulators are involved in biofilm formation. Indeed Knobloch (2001) found that, if the gene coding for the positive regulator of σ^B (rsbU) in *S. epidermidis* was mutated, a biofilm-negative phenotype was observed. This supports the fact that biofilm is formed for defence reasons, and perhaps sheds some light on why they are more resistant than planktonic cultures. The exact role of the stress response regulators is still unclear (Jefferson 2004a). However, the alternative sigma factor σ^B plays a prominent role in the stress response of the Gram-positive bacteria, including *Staphylococcus aureus.* In all Gram-positive bacteria the activation of the σ^B, leads to up-regulation of a diverse array of genes that confer protection to the cell against adverse conditions; including Staphylococcal coagulase, activation of SarA, osmolye, amino acid, and, cation and anion transporters, and the production of neutralizing enzymes to prevent cellular damage (including peroxidases, catalases, glutathione reductase) and to repair DNA lesions (e.g., exonuclease III) (Demple 1991). Catalases are enzymes that neutralize peroxygens such as hydrogen peroxide (Nester *et al.* 2004); Elkins *et al.* (1999) and Stewart *et al.* (2000) found that biofilms of catalase (*KatA*) positive *P. aeruginosa* strains were more resistant to hydrogen peroxide killing than the biofilms of *katA* knock out mutants. Catalyses have been shown to be up-regulated in *P. aeruginosa* biofilm and secreted in to the EPS matrix (Stewart *et al.* 2000), and, indeed, Beenken *et al.* (2006) showed that in *S. aureus* biofilms catalase (*KatA*) was also slightly up-regulated compared to stationary phase planktonic cultures. Also, Resch *et al.* (2005) found that genes encoding superoxide dismutase and glutathione peroxidase were also up-regulated in *S. aureus* biofilm cells. Therefore, biofilm resistance to Virkon is at least partly conferred by the increase in these neutralizing enzymes and helps to explain the differences in resistance between static-growth-phase planktonic cultures and biofilm.

In general, while some potential resistance mechanisms have been discussed, the results presented in this chapter do not explain why biofilms are resistant to the disinfectants tested,

especially as differences in immature and mature biofilm resistance could not only be due to variations in EPS production, but also to alterations in gene expression, as a result of differences in stress conditions, such as nutrient limitation and pH. While the author acknowledges that these biofilms, which are formed in complex growth media with glucose, are in no way indicative of MRSA biofilms formed in the hospital environment, the hospital disinfectants, Chemisan and Sanichlor, have a recommended contact time of only 5 minutes. However, the biofilms in these investigations were exposed for 18 hours, which in some ways overcomes the problem of enhanced biofilms growth. This work does suggest that the rise in nosocomial MRSA infections rather than being totally the result of poor cleaning standards, and genetically acquired resistance mechanisms, is also due to MRSA biofilm formation in the hospital environments and the use of ineffective hypochlorite based cleaning solutions. This resistance raises serious doubts about the Government's proposed control and reduction in nosocomial MRSA infections through increased hospital cleaning, as this not only requires good cleaning practices but the use of efficient and effective disinfectants, used at the appropriate concentrations. If a disinfectant is used at a concentration that is too low, it risks infection and the acquisition of resistance to the disinfectant: however, a concentration that is too high increases the cost and environmental burden. The hospital disinfectants used here were ineffective in removing and killing both immature and mature MRSA biofilms at their recommended concentrations.

If hospitals are unable to curb MRSA transmission in a health care environment and therefore prevent nosocomial infection from occurring, what treatment is available for patients that do become infected, and, more importantly, are these prophylactic and therapeutic antibiotics capable of killing MRSA in the biofilm mode of growth? Currently, the last line of defence for MRSA infections is treatment with vancomycin. Given that MRSA biofilms were resistant to cleaning chemistries containing hypochlorite, it was important to confirm that MRSA biofilms were still susceptible to vancomycin.

5. DETERMINATION OF THE EFFICACY OF VANCOMYCIN TREATMENT IN PREVENTING MRSA BIOFILM FORMATION AND ERADICATION OF ESTABLISHED MRSA BIOFILMS

5.1 Introduction

Vancomycin was isolated in 1956 from cultures of *Streptomyces orientalis* by McCormick *et al.* (1956). Vancomycin is active against Gram-positive cocci, notably staphylococci, including β-lactamase-producing and methicillin-resistant strains, *streptococci,* and, to a lesser extent, *clostridia* (Mann *et al.* 1996). It was introduced into hospitals more than forty years ago in response to new strains of staphylococci that were becoming resistant to penicillin. As the former wonder drug penicillin became increasingly ineffective in hospitals around the world, physicians began to see vancomycin as the new antibiotic that would keep humans ahead of Gram-positive pathogen infection. The introduction of methicillin decreased the use and importance of vancomycin for a few years; however, when methicillin-resistant *S. aureus* strains appeared in the past two decades, the glycopeptide antibiotic was reinstated as an essential therapeutic agent (Mayhall 2005). Vancomycin is now seen as the last-resort drug, because it is often the last opportunity that a physician may have to eliminate a Gram-positive bacterial infection, since bacteria have become resistant to so many other drugs.

Vancomycin's action is bactericidal, but its toxicity precludes its use except for the most serious of infections. Vancomycin is not absorbed from the gastrointestinal tract and is, therefore, for systemic action and has to be administered intravenously as intramuscular injection causes pain and necrosis. This presents an additional risk, as patients are required to stay in hospital for the length of their antibiotic treatment. Lengthy hospitalisations have shown to be a risk factor for MRSA infection.

The hope that vancomycin would be a cure-all antibiotic came to an end in 1987 when vancomycin-resistant enterococci (VRE) appeared in hospitals (Mann *et al.* 1996); resistance was due to a mutation in the first steps of peptidoglycan synthesis. Since then, VRE strains have spread extremely rapidly; indeed, between 1989 and 1993, the number of VRE strains have increased from 0.3% to 7.9%, raising serious concerns within the scientific and health care communities. In 1997, the first clinical isolate of vancomycin-intermediate *S. aureus* (VISA) was first identified in Japan (Hiramatsu 1998). Subsequently, VISA strains have been isolated in hospitals in England, France, South Africa, the USA, Asia and Brazil, confirming that emergence of vancomycin resistance in *S. aureus* is a global issue. It is also termed GISA (glycopeptide-intermediate *S. aureus*) as they are resistant to all glycopeptide antibiotics. The GISA phenotype is somewhat unique, compared with

the phenotypes associated with resistance to other antibacterial agents, because it is the result of a thickening of the cell wall, which is believed to deplete the vancomycin available to kill the bacteria. However, recently the emergence of *vanA* mediated vancomycin resistant *S. aureus* has occurred (Sievert *et al.* 2002).

Although the majority of MRSA strains are deemed susceptible to vancomycin inhibition, the majority of studies have involved planktonically grown cells. There have been two previous studies, suggesting that the MRSA biofilm mode of growth confers phenotypic resistance to a range of vancomycin concentrations (Jones *et al.* 2001; El-Azizi *et al.* 2005). This biofilm resistance phenomenon has been further examined to determine its prevalence in a range of epidemic MRSA strains and the effect of high concentrations of vancomycin on immature and mature biofilm viability and biomass.

5.2 Methods

The methods used in this work were as described in chapter 2.

5.3 Results

5.3.1 Determination of the planktonic minimum inhibitory concentration of vancomycin for the *S. aureus* strains

The minimum inhibitory concentration (MIC) of vancomycin was determined for the planktonic *S. aureus* cultures (NCTC 11939, 13143, 10442 and 12973), using the standard broth micro-dilution susceptibility method of the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Briefly, a two-fold dilution series of the antimicrobial agents was set up in four replicate microtitre plates (NuncSurface) with 200 µl of $BHI_{25\%}$ + 0.09% glucose per well. The microtitre plates were inoculated with starter cultures (turbidity of 0.08-0.1; 620 nm) of the four *S. aureus* strains to give a final inoculum of 5×10^5 CFU ml^{-1}. The plates were incubated at 37°C for 24 hours and growth inhibition was determined by a visual assessment of culture turbidity (Table 13).

Table 13 *The MICs (mg l^{-1}) of vancomycin for the four S. aureus strains, including the EUCAST target strain, in planktonic culture.*

Chemical / Agent	NCTC 11939	NCTC 13143	NCTC 10442	NCTC 12973	*EUCAST NCTC 12973
Vancomycin hydrochloride	2	1	1	1	1

*Target MIC reported by EUCAST (2000)

The target vancomycin MIC for the standard *S. aureus* EUCAST (NCTC 12973) strain was included to ensure reliability of the MIC procedure. The vancomycin MIC ranged between 1 and 2 mg l^{-1}.

5.3.2 The effect of vancomycin at various concentrations on initial cell attachment and efficacy against preformed immature and mature MRSA biofilms

The effects of non-inhibitory and inhibitory planktonic concentrations 0.5 (1/4 MIC), 2 (MIC) and 20 (10*MIC) were investigated for their ability to prevent initial cell attachment and their effects on immature (8-hours-old) and mature (24-hours-old) preformed MRSA biofilms. MRSA (NCTC 11939, 13143, 10442 and 12973) starter cultures were used to inoculate, 200 µl, aliquots of $BHI_{25\%}$ + 0.09% (w/v) glucose, in the wells of microtitre plates (NuncSurface). Media contained

vancomycin at 0.5, 2 or 20 mg l^{-1} for initial attachment experiments; but no vancomycin for preformed biofilm experiments. The microtitre plates were incubated at 37°C for 18 hours for initial cell attachment experiments or for 8 or 24 hours for immature and mature biofilms respectively. For the preformed biofilm experiments, after incubation (for 8 or 24 hours), the planktonic culture phase was aspirated off, and the remaining attached biofilm cells were gently washed in dH_2O (200 µl). Sterile growth media (200 µl), with or without vancomycin at 0.5, 2 or 20 mg l^{-1}, was returned. The microtitre plates were then incubated at 37°C for a further 18 hours.

The results for the efficacy of vancomycin treatment of MRSA biofilms are given below only for the MRSA strain NCTC 10442, as all strains followed the same pattern of susceptibility and resistance. The percentage of viable cells (determined using the XTT / PMS assay procedure, Section 2.13.2) (black bars, Figure 32) and the biomass (Safranin staining, Section 2.14.1) (blue bars, Figure 32) of vancomycin-treated biofilms were expressed as a percentage of the total biofilm biomass of the mature (24-hour-old; vancomycin minus) control biofilms, which was normalised to 100%.

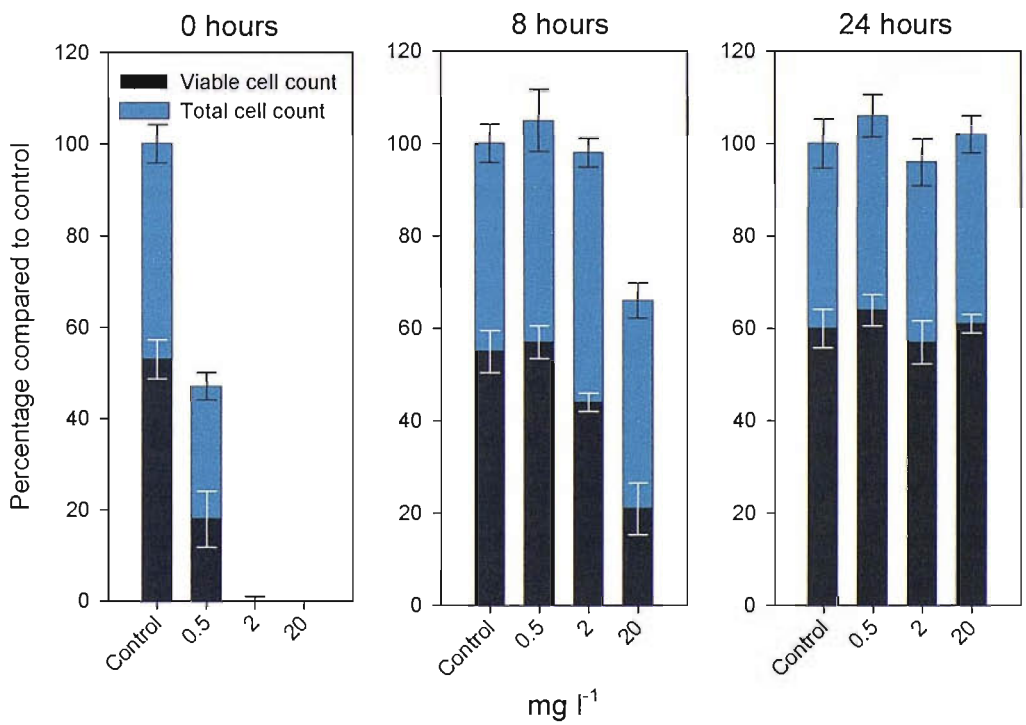


Figure 32 *The effect of vancomycin treatment (0.5, 2 or 20 mg l^{-1}) on initial biofilm formation and against immature (8 hour-old) and mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars).*

As expected, vancomycin, at the sub-planktonic MIC of 0.5 mg l^{-1}, did not prevent initial cell attachment. However, the biomass of the subsequent biofilm formation was significantly reduced to

48% of the control biofilm, while the percentage viability of the treated biofilm remained at the same level as the control biofilms, at approximately 45-50%. Initial cell attachment and, therefore, biofilm formation in the presence of vancomycin at the higher concentrations of 2 and 20 mg l^{-1} was completely prevented.

The effect of vancomycin was also investigated against preformed biofilms. No effect on the biomass of immature biofilms (total cell number) or the percentage of viable cells was observed for biofilms treated with 0.5 or 2 mg l^{-1} vancomycin (Figure 32). However, immature biofilms that were treated with vancomycin at a concentration of 20 mg l^{-1} showed a significant reduction in biomass of 40% compared to control biofilms, and also the percentage viability was slightly but significantly reduced from approximately 50% in the control to 40% in these biofilms.

Vancomycin was also investigated for its efficacy against mature (24-hour-old) preformed biofilms. Vancomycin treatment, at concentrations of 0.5, 2 and 20 mg l^{-1}, had no significant effect on biofilm biomass or percentage viability when compared to control (untreated) biofilms (Figure 32), implying that mature 24-hour-old biofilms were completely resistant to vancomycin concentrations of at least ten times the planktonic MIC.

To validate the above results, EDIC microscopy images (x 1000) were captured of each treatment for each biofilm age. An analysis of biofilm surface area coverage was determined using ImagePro software (Table 14). Figure 38 shows examples of the EDIC microscopy images.

Table 14 *The percentage surface area coverage of vancomycin treated and untreated biofilms, grown on glass chamber slides*

Treatment	Biofilm age (hours)		
	0	8	24
Control (0 mg l^{-1})	99%	100%	98%
Vancomycin 0.5 mg l^{-1}	47%	89%	99%
Vancomycin 2 mg l^{-1}	0%	95%	96%
Vancomycin 20 mg l^{-1}	0%	51%	94%

The percentage surface area coverage of control biofilms was typically between 98 and 100%; however, this was reduced with vancomycin treatment. The data in Table 14 shows that the percentage surface area coverage of biofilm formed in the presence of vancomycin 0.5 mg l^{-1} was reduced to below 50% of the control biofilms and completely retarded (0%) in the presence of 2 and 20 mg l^{-1} vancomycin. For preformed immature (8 hour old) biofilms treated with vancomycin (20 mg l^{-1}), the percentage surface area coverage was reduced to 51%, similar to the Safranin

stained biofilm biomass data in figure 37. Also, surface coverage of immature biofilms treated with 0.5 mg l^{-1} vancomycin was slightly but significantly reduced; however, when treated with the higher concentration of 2 mg l^{-1}, no significant difference was observed. Mature (24-hour-old) biofilms treated with all vancomycin concentrations were resistant and, therefore, showed little or no reduction in the percentage biofilm surface area coverage (Figure 33).

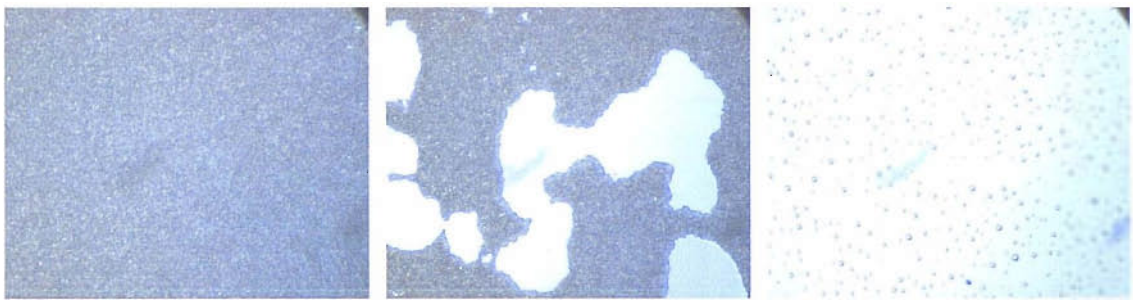


Figure 33 *EDIC microscopy images (x 1000) showing the surface coverage of MRSA (NCTC 10442) biofilm formed in the presence of 0.5, 2 and 20 mg l^{-1} vancomycin.*

5.3.3 Determination of the extent of mature biofilm resistance to high vancomycin concentrations

The extent of mature biofilm resistance to high vancomycin concentrations was further investigated. MRSA (NCTC 10442) biofilms were grown in 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose in microtitre plate wells at 37°C for 24 hours. After incubation, the planktonic culture phase was removed and the remaining attached cells were washed gently in 200 µl of dH_2O. Aliquots (200 µl) of sterile media containing 64, 256, 1024, or 2048 mg l^{-1} of vancomycin were replaced and the biofilms were incubated at 37°C for a further 18 hours. As above, the percentage of viable cells was determined by the XTT / PMS assay, and the total biofilm biomass by the Safranin assay. The biomass of control biofilms was normalised to 100%, and the percentage viability and biomass of treated biofilms was given as a percentage of the control biofilm biomass (Figure 34).

Vancomycin, at both 64 and 256 mg l^{-1} caused a small but significant increase in biofilm biomass of around 18%. However, no increase was observed in biofilms treated with the high vancomycin concentration of 1024 mg l^{-1} (Figure 34). Vancomycin, at a concentration of 64 mg l^{-1}, had no significant effect on mature biofilm percentage viability, while a significant reduction of 20 and 30% was seen for biofilms treated with 256 and 1048 mg l^{-1} respectively. However, vancomycin concentrations as high as 2048 mg l^{-1} were not sufficient to completely remove and / or kill mature

MRSA biofilms. For biofilms treated with this vancomycin concentration, biomass was reduced by 20%, and the percentage viability from 60%, in the control mature biofilms, to 8%. However, this percentage of viability still equates to approximately 3.3 x 10^5 CFU ml^{-1} viable cells, which would be sufficient to cause illness. The highest vancomycin concentration of 4096 mg l^{-1} reduced the biomass further to 20% of the control biofilm biomass and was the only treatment that succeeded in completely killing these mature biofilms.

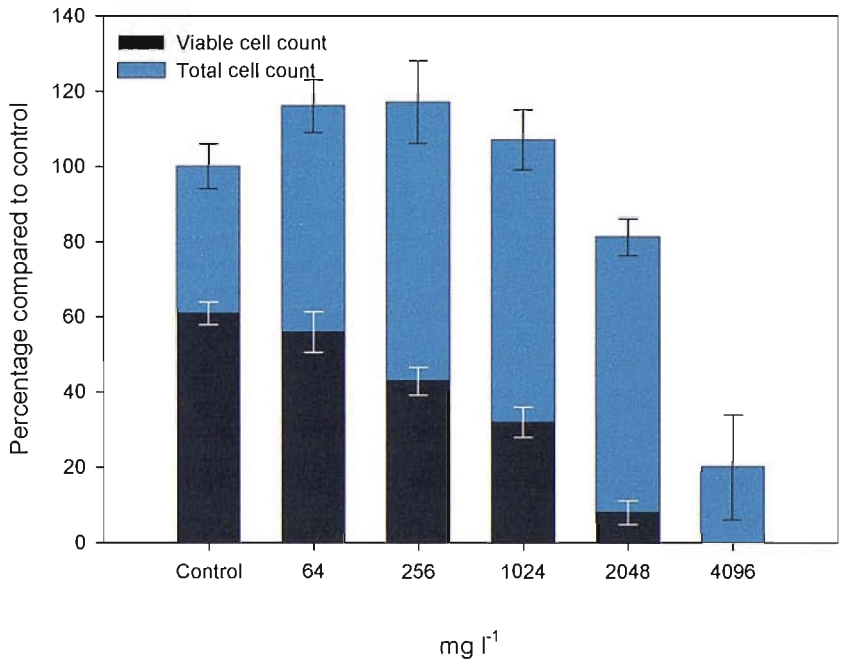


Figure 34 *The effect of vancomycin treatment (64, 256, 1024 2048 or 4096 mg l^{-1}) on mature (24 hour-old) MRSA biofilms. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars).*

These results were supported by biofilm recovery experiments, where biofilm cells were removed from the surface of the microtitre plate wells, serially diluted in PBS, and plated on agar plates that were incubated at 37°C for 48 hours. The number of viable cells recovered, after vancomycin treatment, at concentrations of 2048 and 4095 mg l^{-1}, was less than 5 and 0%, respectively, of the number of cells recovered from control biofilms (data not shown). These experiments also indicate that the cells are viable and the remaining viability seen in these biofilms is not a by-product of an abiotic XTT / PMS conversion process.

5.4 Discussion

Due to the emergence of MRSA strains, vancomycin has become the antibiotic of choice, 'the last line of defence'. In the last five years there has been a large increase in the incidence of MRSA infection and the number of deaths caused by MRSA infections has doubled from 5000 to 11000 in the UK. However, many patients, infected with vancomycin susceptible MRSA isolates, die regardless of vancomycin treatment.

Some vancomycin resistant *S. aureus* isolates have been found to mediate resistance through a non-VanA-related mechanism. Hiramatsu (2001) found that the cell walls of 16 VRSA strains from seven different countries were significantly thicker (mean 31.3 nm), compared to vancomycin susceptible *S. aureus* (VSSA) (mean 23.4 nm). Transmission electron microscopy of these VRSA strains suggests that they contain 30-40 layers of peptidoglycan; substantially more than in VSSA, which possesses less than 20 (Hiramatsu 2001). The increase in peptidoglycan layers substantially increases the number of vancomycin-binding sites. Vancomycin becomes trapped in the outer peptidoglycan and so cannot penetrate to the inner cell-wall layers (Cui *et al.* 2000). Hiramatsu (2001) observed thickened cell walls in planktonic bacteria, and, consequently, the planktonic MIC of vancomycin was increased. The planktonic MICs of the *S. aureus* strains tested here were low (1 mg l^{-1}); therefore, unless biofilm formation triggers an increase in peptidoglycan production, of which no evidence has been published in the literature, this resistance mechanism cannot account for the biofilm resistance to vancomycin.

A second possible reason for this non-VanA resistance to vancomycin is the formation of biofilm itself. Biofilms are now recognised as an important virulence factor in bacterial infection; biofilms are capable of forming on implantable medical devices such as prosthetic heart valves (Blouse *et al.* 1978), cerebrospinal fluid shunts (Schoenbaum *et al.* 1975), orthapeadic appliances (Patterson *et al.* 1972) and intravascular catheters (Bender *et al.* 1980). Physicians have reported the failure of some antibiotics in clearing biofilm infections, which represent a source of persisting and relapsing infections, especially those associated with in-dwelling medical devices (Costerton *et al.* 1999).

Biofilm formation may also have been an important factor in VRSA strains acquiring the *vanA* gene, as the resistance to vancomycin by the biofilms may have given them sufficient time and protection in which to acquire the genes from bacteria, such as entericocci. As discussed in the following chapter, biofilm formation provides the perfect environment for gene transfer, and the *vanA* gene is widespread throughout enterococci species including: *faecium and gallinarum* (Panesso 2005), *Bacillus circulans* (Ligozzi *et al.* 1998), *Oerskovia turbata*, *Arcanobacterium*

(Corynebacterium) haemolyticum (Power *et al.* 1995), *Paenibacillus popilliae* (Guardabassi 2005), and can also be found in *S. aureus* (Hiramatsu 1998).

Prakash *et al.* (2003) argued that eradication of a bacterial biofilm infection is possible if the antibiotic therapy is implemented early enough and if sufficiently high concentrations of the antibiotics are used. However, this may not be the case for vancomycin treatment if the growth rates *in vivo* are similar to those *in vitro*. Here, immature biofilm, just 8-hours-old at the time of treatment, was able to resist vancomycin up to concentrations of 20 mg l^{-1}, with a reduction of only 40% in the biofilm biomass, and mature biofilms, only 24-hours old, were completely resistant to vancomycin concentrations up to 64 mg l^{-1}. El-Azizi *et al.* (2005) determined that the viability of mature *S. aureus* biofilms was reduced by up to 40% when treated with vancomycin at concentrations of 500 and 1000 mg l^{-1}. Concurrently, in this investigation mature biofilms were still viable, even after treatment with vancomycin concentrations as high as 2048 mg l^{-1}, which, to this author's knowledge, has not before been reported.

Although it is often quoted that bacteria within biofilms are resistant to antibiotic levels 10 to 1000-fold higher than genetically identical planktonic bacteria, the work presented here shows that biofilm bacteria of the MRSA strain NCTC 10442 can be more than 2000 times more resistant to vancomycin than genetically identical planktonic bacteria.

Furthermore, although it may be possible to administer greatly increased concentrations of some antibiotics, vancomycin has several dangerous side-effects, including anaphylactoid reactions, pseudomembranous colitis (infection of the colon), renal failure, interstitial nephritis (inflammation of the kidneys), hearing loss and reversible neutropenia (reduction in white blood cells) (Rocha *et al.* 2002). Therefore, the maximum dose of 30 and 50 mg l^{-1} cannot be exceeded, suggesting that delayed vancomycin therapy would be ineffective, leading to increased patient morbidity and mortality.

The properties of biofilms that result in antibiotic resistance may include slow growth, phenotypic heterogeneity, the presence of persister cells, inactivation of antibiotics within the biofilm EPS matrix, and limitations on antibiotic penetration imparted by the biofilm matrix (Stewart *et al.* 2002). However, the relative contributions of these properties to resistance are not well understood. In particular, a role for the staphylococcal EPS matrix (composed primarily of PNAG) as a diffusion barrier has not been conclusively established (Jefferson 2004a). A study with *S. epidermidis* suggested that PNAG restricts glycopeptide penetration (Konig *et al.* 2001), and another found that "slime," containing an unknown quantity of PNAG extracted from staphylococcal isolates caused a 2 to 16-fold increase in the MICs of vancomycin and teicoplanin

(Mathur *et al.* 2005). The work presented here shows that the *S. aureus* biofilm is heterogeneous in the production of PIA / PNAG (slime), implying that certain areas should be more susceptible to vancomycin treatment if this was the case. In contrast, others report free diffusion of certain antibiotics through staphylococcal biofilms (Dunne *et al.* 1993) and, although Wilcox *et al.* (2001) found that vancomycin concentrations in central-venous-catheter (CVC)-associated biofilms formed *in vivo,* varied markedly ranging from 1 to 22 mg g^{-1}, the median vancomycin concentration within the biofilm grossly exceeded the planktonic MIC for their *S. aureus* isolate. It has, in fact, been argued that the diffusion coefficient of small molecules, such as antibiotics, through the biofilm EPS matrix, is roughly equivalent to the diffusion coefficient of water (Stewart 1998).

The rate of antibiotic penetration is important because, if the rate is decreased, then the bacteria may be exposed to a gradually increasing dose of the antibiotic and may have time to mount a defensive response to the compound. In support of this idea, bacteria have been shown to increase transcription of stress-associated genes, such as heat shock protein homologues and cell wall synthesis genes, within an hour of exposure to low doses of cell wall-active antibiotics (Utaida *et al.* 2003).

Most published studies of diffusion in biofilms fail to address the rate with which antibiotics are transported. However, Jefferson *et al.* (2004b) used a fluorescently labeled vancomycin derivative, BodipyFL-vancomycin, to show that binding of vancomycin to the bacterial cells within the deepest layers of a *S. aureus* biofilm did not occur for nearly 60 min, whereas maximal binding of the antibiotic to planktonic cells occurred within about 5 min. It is possible that biofilm resistance is an artifact of this reduced antibiotic dose. Indeed, El-Azizi *et al.* (2005) showed that, although disrupted MRSA biofilms were still 40% viable after treatment with 1000 mg l^{-1} vancomycin, they were significantly more susceptible to vancomycin than intact MRSA biofilms.

However, Jefferson *et al.* (2004b) argue that, as bacteria have been shown to mount a transcriptional stress response to low doses of cell wall-active antibiotics within an hour (Utaida *et al.* 2003), it is conceivable that the resistance of bacteria in the deeper layers of the biofilm may actually increase after administration of the antibiotic. The cells may be able to mount a response at the level of transcription or protein secretion in time to defend themselves against the lethal effects of the antibiotic. Indeed, the work presented here shows that mature biofilm biomass actually increased at sub-lethal vancomycin concentration. For example, the alternative sigma factor σ^B, which controls a large regulon of genes that encode efflux pumps, multidrug resistance determinants, heat shock proteins, and other stress-related proteins, could be induced so that the cells become more resistant to the antibiotic challenge (Bischoff *et al.* 2004). In contrast to the popular belief that characteristics specific to bacterial biofilms make them "constitutively" resistant

to antibiotics, this idea suggests that resistance may be at least partially inducible (Jefferson *et al.* 2004b).

The phenotypic resistance of biofilm cells to vancomycin is probably more to do with the fact that biofilm cells are slow-growing and are, therefore, not producing new cell wall components at a fast rate. Indeed, the biomass of 24-hour-old biofilms does not significantly increase if the biofilm is grown for a further 24 hours (data not shown). However, there is evidence linking the induction of the stringent stress response and slow-growth; indeed in *E. coli,* the expression of ppGpp in the stringent response inhibits peptidoglycan synthesis (Ishiguro *et al.* 1976). If ppGpp is expressed in *S. aureus*, biofilms, and also causes a reduction in peptidoglycan synthesis, it could help explain the resistance of both immature (partial resistance) and mature biofilms to vancomycin.

One major omission of the results obtained by El-Azizi *et al.* (2005), and those of this investigation, are that they are obtained from one prolonged dose. In medicine, several therapeutic regimes are routinely utilised for vancomycin administration. They include 0.5g every 6 hours, 1g every 12 hours or 2g every 24 hours, which equate to maximal concentrations in the sera of 10, 25 and 50 mg l^{-1} respectively (Garrod *et al.* 1973). Therefore, further work should be undertaken first to determine the concentration of vancomycin in the biofilms and whether the concentration decreases over time; and, secondly, to determine if multiple doses of vancomycin have any significant effect on mature biofilms.

The main conclusions of this work are that immature and mature MRSA biofilms are variably resistant to vancomycin and that mature MRSA biofilms are resistant at much higher concentrations (2000 times the planktonic MIC) than previously reported. Also, the MIC of mature MRSA biofilms was nearly 4000 times the planktonic MIC.

Given that MRSA biofilms are resistant to the last line of defence in MRSA treatment and vancomycin resistance cannot be overcome simply by increasing the dose, it is important that antibiotics specifically aimed at the biofilm mode of growth be identified. In the following chapter (6), antibiotics with a range of modes of action were investigated for their activity against *S. aureus* biofilms, in the hope of identifying any that would be useful in the clinical setting as an alternative to vancomycin when the infection was deemed to be biofilm-related.

6. *S. AUREUS* BIOFILM SUSCEPTIBILITY TO ANTIBIOTICS WITH DIFFERENT MODES OF ACTION

6.1 Introduction

The previous chapter has shown that both immature and mature MRSA biofilms are highly resistant to vancomycin (the last line of defence). It is therefore imperative that antibiotics, active specifically against the biofilm phenotype, are identified, so that they can be used instead of vancomycin when the infection is thought to be biofilm mediated.

Antibiotics can be classed by their mode of action. A number of bacterial processes utilise enzymes or structures that are either different, absent, or not commonly found in eukaryotic cells. Several microbial processes, including the synthesis of bacterial cell walls, proteins and nucleic acids, metabolic pathways, and the integrity of the cytoplasmic membrane, are targets of most antimicrobial drugs (Nester *et al.* 2004). As resistance mechanisms generally tend to be specific, it is sensible to assume that some modes of action may be more active against the biofilm phenotype than others.

This chapter therefore looked at a range of antimicrobial agents with different modes of antibiotics to determine if any were more active against the MRSA biofilm mode of growth. All of these antibiotics were tested for their ability to prevent biofilm formation and their activity against preformed MRSA biofilms at low, planktonic MIC concentrations, with a view to determining whether certain modes of action are more effective against the biofilm mode of growth.

6.2 Methods

The methods used in this chapter are as described in chapter 2.

6.3 Results

Antibiotics, representing all common modes of action, were chosen for the study according to their common use in research, human medicine and veterinary practice. Twenty-two antibiotics were used including: the cell wall active antibiotics, phosphomycin and the penicillin, ampicillin; the ionophore antibiotics, gramicidin and the polyethers, monensin, nigericin and narasin, which interfere with the permeability of the cell membrane. Of the antibiotics that interfere with transcription or DNA synthesis, the peptide, actinomycin D, the fluoroquinolone, ciprofloxacin, the coumarin-glycoside, novobiocin, the macrolide, rifampicin and the sulfonamides, sulphamethoxazole and trimethroprim were investigated; and lastly of the antibiotics which interfere with protein synthesis, the phenicol, chloramphenicol hydrochloride, the macrolide, erythromycin and the related lincosamide, clindamycin hydrochloride; the steroid, fusidic acid, the aminoglycosides, gentamycin, kanamycin and tobramycin, as well as tetracycline were all considered.

Also, the cell wall active antibiotics, ceftriaxone a cephalosporin, the carbopenams, imipenem and ieropenem, the glycopeptide teichoplanin, the lipoglycopeptide dalbavancin and the cyclic peptide bacitracin, along with the cell membrane active, lipopeptide, daptomycin, were requested from their suppliers / manufacturers, but were not obtained.

6.3.1 Planktonic susceptibility of MSSA and MRSA strains to a range of antibiotics

The aim of this chapter was to try to determine the extent to which the *S. aureus* biofilm mode of growth was more resistant to antibiotics than the planktonic mode of growth. Two epidemic strains, EMRSA-1 (NCTC 11939) and 16 (NCTC 13143), one MRSA strain (NCTC 10442) and an MSSA (NCTC 12973) strain, were evaluated for the susceptibility of planktonically grown cells to 22 antibiotics of several different modes of action. The MICs for each antibiotic was determined for planktonic *S. aureus* cultures in MH broth, using the standard broth micro-dilution susceptibility method of the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Briefly, a two-fold dilution series of the antimicrobial agents was set up in four replicate microtitre plates (NuncSurface) with 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose per well. The microtitre plates were inoculated with starter cultures (turbidity of 0.08-0.1; 620 nm) of the four *S. aureus* strains to give a final inoculum of 5×10^5 CFU ml^{-1}. The plates were incubated at 37°C for 24 hours and growth inhibition was determined by a visual assessment of culture turbidity.

To ensure consistency, the MIC results obtained for the *S. aureus* strain NCTC 12973 were compared to the target MICs published by EUCAST (2000). If the MIC was more than one dilution

factor out, the results were discarded and the experiment repeated (MIC results - Appendix A).

MRSA strains have gained multiple genetic mechanisms of resistance to several classes of antimicrobials, including macrolides, aminoglycosides, fluoroquinolones, tetracyclines and lincosamide antibiotics such as clindamycin. Of the 22 antibiotics tested, the two EMRSA strains, NCTC 11939 and 13143, were planktonically and therefore genetically resistant to 9 and 10 respectively. The standard laboratory MRSA strain, NCTC 10442, was also resistant to 6 of the 22 antibiotics. However, the MSSA strain, NCTC 12973, was planktonically resistant to just one, sulphamethoxazole.

The difference in the planktonic resistance phenotypes of the *S. aureus* strains is due to the specific SCC*mec* type that they carry (Table 6). The *S. aureus* strain NCTC 10442 has an SCC*mec* type I (Suzuki *et al.* 1993) which lacks other associated resistance genes. The *S. aureus* strain NCTC 11939 (EMRSA-1) has an SCC*mec* type III (Holmes *et al.* 2005); while NCTC 13143 (EMRSA-16) has an SCC*mec* type II (Murchan *et al.* 2004), both of these SCC*mec* types are associated with multiple resistance genes (carried on the same plasmid). Therefore, it is expected that the EMRSA strains would have resistance to a broader spectrum of antibiotics, than the non-epidemic MRSA strain.

6.3.2 Efficacy of antibiotic with differing modes of action in preventing initial MSSA and MRSA cell attachment

To investigate the ability of the antibiotic to prevent initial cell attachment, starter cultures were used to inoculate 200 µl of $BHI_{25\%}$ + 0.09% glucose, with or without the antibiotics at the planktonic MIC or ten times the MIC, in the wells of microtitre plates. The microtitre plates were incubated at 37°C for 18 hours. After incubation, the planktonic culture phase was aspirated; the remaining attached cells were washed in 200 µl of dH_2O. The percentage of viable cells was determined using the XTT / PMS assay, and total biofilm biomass was determined using the Safranin assay.

As expected, all of the antibiotics, to which the *S. aureus* strains were planktonically resistant, were successful in completely preventing initial cell attachment at both the planktonic MIC and ten times the MIC (data not shown).

6.3.3 The effect of the addition of antibiotic with differing modes of action to preformed immature and mature MSSA and MRSA biofilms

To determine the efficacy of the antibiotics against preformed *S. aureus* biofilms, immature and mature biofilms were grown from *S. aureus* starter cultures in microtitre plate (NuncSurface) wells, containing 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose. The microtitre plates were incubated at 37°C for 8 or 24 hours for immature and mature biofilms respectively. After incubation, the planktonic culture phase was removed and the remaining attached cells were gently washed in dH_2O. Growth media (200µl), with the antibiotics at their planktonic MIC or ten times the MIC, were replaced, and the microtitre plates were incubated at 37°C for a further 18 hours. Control biofilms were grown in media without antibiotics under the same environmental conditions. The percentage of viable cells (% V) within the biofilm was determined using the XTT / PMS assay (Section 3.13.2), and biofilm biomass (B) by the Safranin assay (Section 3.14.1). The biomass and percentage viability of the treated immature and mature biofilms were expressed as a percentage of the total biomass of the control mature biofilms, which was normalised to 100%. The results for those biofilms not significantly different in either biomass or percentage viability from the control biofilms are not given, and instead are represented by a dash (-). Those antibiotics that had no effect on either the immature or mature biofilms of any of the four *S. aureus* strains, were omitted from the results tables (Tables 15 - 20).

The efficacy of antimicrobial agents that interfere with cell wall synthesis against preformed immature *S. aureus* biofilms

Planktonic cultures of the MRSA strains NCTC 11939, 13143 and 10442 were resistant to ampicillin. Therefore, the efficacy of ampicillin was only investigated for the *S. aureus* strain NCTC 12973 at concentrations of 1 and 10 mg l^{-1}. Immature, MSSA NCTC 12973, biofilms treated with ampicillin displayed a small but significant decrease in percentage of viable cells from 64% in the control biofilms to 39% and 42% for planktonic MIC and ten times the MIC treated biofilms respectively (Table 15).

Immature biofilms of the *S. aureus* strains NCTC 11939, 10442 and 12973, treated with phosphomycin at the planktonic MIC, were not significantly different in biomass or percentage viability to the control biofilms. Immature biofilms of NCTC 13143, however, had both significantly decreased biomass and percentage viability when treated with phosphomycin at the planktonic MIC (Table 15). Immature biofilms of all the *S. aureus* strains treated with the higher phosphomycin concentration exhibited significantly reduced biomass from 100% in control biofilms to 55% or less. The percentage of viable cells within the biofilms was also significantly

reduced for all but the NCTC 11939 biofilm, which showed an increased percentage viability compared to control biofilms.

Table 15 *The efficacy of cell wall synthesis interfering antibiotics against immature (8-hour-old), S. aureus biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
Ampicillin	X	X	X	X	X	X	39	102
1 mg l^{-1}							(±3.9)	(±8.2)
Ampicillin	X	X	X	X	X	X	42	103
10 mg l^{-1}							(±3.7)	(±4.0)
Phosphomycin	-	-	19	43	-	-	-	-
8 mg l^{-1}	(±3.4)	(±3.7)	(± 4.1)	(±7.6)	(±5.5)	(±3.3)	(±7.5)	(±5.4)
Phosphomycin	79	33	18	36	42	55	45	39
80 mg l^{-1}	(±5.8)	(±5.1)	(± 3.4)	(±7.1)	(±3.2)	(±9.6)	(±4.5)	(±7.1)

The efficacy of antimicrobial agents that interfere with cell wall synthesis against preformed mature (24-hour-old) *S. aureus* biofilms

Mature biofilms of all *S. aureus* strains were resistant to both concentrations (the planktonic MIC and ten times the MIC) of ampicillin and phosphomycin.

Assessment of antimicrobial agents that interfere with cell membrane against preformed immature *S. aureus* biofilms

The lower MIC of gramicidin (4 mg l^{-1}) had no significant effect on the percentage viability of immature biofilm of the four *S. aureus* strains (data not included), although the biomass of these biofilms was reduced to around 60% of the control biofilm biomass. However, while the addition of the other three ionophore antibiotics, at their MIC, caused a significant reductions in biomass, percentage viability, or both, for the immature biofilms of NCTC 12973 and NCTC 10442, only monensin and narasin had significant effects on biofilms formed by NCTC 13143 (Table 16).

The addition of the ionophore antibiotics at the higher concentrations of ten times the MIC led to a significant reduction in both biofilm biomass (between 60% and 80% reduction) and percentage viability (from 60% to between 30% and 8%) for the *S. aureus* strains NCTC 11939, 13143 and 12973, and just percentage viability for the *S. aureus* strain NCTC 10442 (Table 16).

Table 16 *The efficacy of the ionophore antibiotics, which interfere with cell membrane permeability against immature, 8-hour-old, S. aureus biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
Gramicidin	52	54	62	60	43	59	31	42
4 mg l^{-1}	(±1.1)	(±5.2)	(±3.5)	(±5.0)	(±7.9)	(±8.2)	(±5.9)	(±5.9)
Gramicidin	31	29	25	43	22	41	30	40
40 mg l^{-1}	(±5.9)	(±4.4)	(±3.8)	(±5.5)	(±3.6)	(±6.8)	(±5.9)	(±8.4)
Monensin	-	-	29	57	13	48	18	46
1 mg l^{-1}	(±6.9)	(±7.5)	(±5.3)	(±4.4)	(±2.8)	(±10.1)	(±2.5)	(±4.9)
Monensin	36	19	19	44	10	77	25	24
10 mg l^{-1}	(±3.8)	(±3.0)	(±2.1)	(±5.7)	(±1.9)	(±6.0)	(±3.7)	(±4.3)
Nigericin	-	-	-	-	22	60	23	91
0.06 mg l^{-1}	(±8.1)	(±8.7)	(±9.4)	(±9.6)	(±8.3)	(±7.1)	(±3.4)	(±8.5)
Nigericin	15	24	26	30	7	75	17	31
0.6 mg l^{-1}	(±3.1)	(±2.0)	(±2.8)	(±3.3)	(±2.1)	(±9.1)	(±3.5)	(±6.6)
Narasin	-	-	44	55	46	71	13	78
0.25 mg l^{-1}	(±8.8)	(±8.6)	(±6.4)	(±8.8)	(±8.9)	(±9.7)	(±3.6)	(±9.1)
Narasin	29	14	29	34	8	85	16	39
2.5 mg l^{-1}	(±5.6)	(±2.9)	(±4.1)	(±6.6)	(±3.2)	(±9.9)	(±2.4)	(±4.8)

Assessment of antimicrobial agents that interfere with cell membrane against preformed mature (24-hour-old) *S. aureus* biofilms

The biomass and percentage viability of mature biofilms were variably affected by the addition of the ionophore antibiotics at their planktonic MIC (Table 17). While monensin caused a small, but sometimes significant, reduction in biofilms for all four strains, the other ionophore antibiotics were effective only against certain strains. The percentage viability of susceptible ionophore treated mature biofilms varied depending on the *S. aureus* strain, with the NCTC 13143 and 12973 strains exhibiting a greater decrease, to 35% and 20% respectively.

The higher ionophore concentrations of ten times the planktonic MIC produced significant differences, compared with control biofilms in both biofilm biomass and percentage viability for all four *S. aureus* strains (Table 17). While all the ionophore antibiotics caused a significant reduction in the biomass of NCTC 11939 biofilms, only a small non-significant reduction in the percentage viability of these biofilms was observed. However, the biomass and the percentage of viable cells

were significantly reduced, compared with control biofilms, for the other three *S. aureus* strains, when treated with all four ionophore antibiotics.

Table 17 *The efficacy of the ionophore antibiotics, which interfere with cell membrane permeability against mature, 24-hour-old, S. aureus biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
Gramicidin	68	54	17	79	50	60	62	49
40 mg l^{-1}	(±7.2)	(±6.7)	(±3.4)	(±5.1)	(±6.5)	(±3.3)	(±4.8)	(±6.6)
Monensin	60	62	26	80	35	93	18	82
1 mg l^{-1}	(±10.0)	(±7.8)	(±3.3)	(±9.0)	(±7.1)	(±6.9)	(±4.5)	(±8.3)
Monensin	50	26	37	52	16	28	18	66
10 mg l^{-1}	(±9.1)	(±4.6)	(±3.9)	(±8.4)	(±3.0)	(±2.1)	(±2.9)	(±4.6)
Nigericin	-	-	35	89	39	85	20	86
0.06 mg l^{-1}	(±8.2)	(±9.9)	(±6.0)	(±9.2)	(±6.6)	(±7.6)	(±2.9)	(±9.4)
Nigericin	53	56	40	47	16	64	22	55
0.6 mg l^{-1}	(±6.7)	(±8.0)	(±4.9)	(±7.2)	(±3.6)	(±9.8)	(±3.9)	(±7.1)
Narasin	-	-	-	-	-	-	21	85
0.25 mg l^{-1}	(±7.5)	(±5.9)	(±6.1)	(±7.6)	(±6.6)	(±8.1)	(±2.9)	(±9.2)
Narasin	40	58	37	56	24	71	27	53
2.5 mg l^{-1}	(±5.9)	(±6.0)	(±5.4)	(±8.2)	(±3.1)	(±7.5)	(±3.4)	(±8.2)

The efficacy of antimicrobial agents that interfere with transcription or DNA synthesis against preformed immature (8-hour-old) *S. aureus* biofilms

Immature biofilms of all planktonically susceptible *S. aureus* strains were resistant to treatment with trimethroprim at the planktonic MIC (Table 18). The biomass and percentage viability of biofilms treated with ciprofloxacin, rifampicin and novobiocin at their MIC, were variable, reduced, depending on the *S. aureus* strain used.

Immature biofilms treated with the higher concentrations of rifampicin, novobiocin and trimethroprim, all exhibited significantly reduced biofilm biomass and percentage viability, apart from the biofilms formed by NCTC 10442, which were planktonically resistant to rifampicin (Table 18).

Table 18 *The efficacy of the antibiotics that interfere with transcription or DNA synthesis against immature, 8-hour-old, S. aureus biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
Ciprofloxacin	31	67	X	X	22	104	-	-
0.125 mg l^{-1}	(±4.2)	(±6.4)			(±3.6)	(±9.1)	(±9.9)	(±8.2)
Ciprofloxacin	20	22	X	X	20	62	36	56
1.5 mg l^{-1}	(±4.2)	(±3.9)			(±6.0)	(±10.3)	(±5.7)	(±6.6)
Rifampicin	-	-	12	48	X	X	11	48
0.5 mg l^{-1}	(±0.8)	(±0.3)	(±2.0)	(±8.1)			(±2.4)	(±5.8)
Rifampicin	22	25	10	55	X	X	8	44
5 mg l^{-1}	(±3.5)	(±4.7)	(±2.3)	(±5.4)			(±1.9)	(±6.8)
Novobiocin	-	-	16	48	13	103	34	94
1 mg l^{-1}	(±0.4)	(±0.8)	(±3.7)	(±6.1)	(±2.0)	(±9.9)	(±6.4)	(±8.3)
Novobiocin	37	14	9	56	0	54	0	44
10 mg l^{-1}	(±5.4)	(±3.9)	(±3.1)	(±7.5)	(±0.8)	(±9.6)	(±0.2)	(±8.0)
Trimethroprim	6	64	0	60	12	85	37	102
80 mg l^{-1}	(±1.2)	(±7.3)	(±0.5)	(±9.1)	(±1.1)	(±8.2)	(±6.3)	(±9.5)

The efficacy of antimicrobial agents that interfere with transcription or DNA synthesis against preformed mature (24-hour-old) *S. aureus* biofilms

Mature biofilms of the four *S. aureus* strains demonstrated resistance to all of the transcription and DNA synthesis-interfering antibiotics, apart from novobiocin, at both the planktonic MIC and ten times the MIC (data not shown). Mature biofilms of the *S. aureus* strains, NCTC 11939 and NCTC 10442, demonstrated a significant reduction in the percentage of viable cells, from 64% in control biofilms to 38% or 37% respectively, when exposed to novobiocin at the higher concentration of ten times the planktonic MIC (Table 19).

Table 19 *The efficacy of the antibiotics that interfere with transcription or DNA synthesis against mature, 24-hour-old, S. aureus biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
Novobiocin	38	102	-	-	37	99	-	-
10 mg l^{-1}	(±7.3)	(±9.4)	(±4.7)	(±9.2)	(±2.3)	(±8.2)	(±4.6)	(±7.8)

The efficacy of antimicrobial agents that interfere with translation against preformed immature (8-hour-old) *S. aureus* biofilms

The *S. aureus* immature biofilms exposed to tetracycline, erythromycin, the aminoglycosides, gentamycin, kanamycin and tobramycin and clindamycin were resistant at the planktonic MIC. The immature biofilms of the two EMRSAs, NCTC 11939 and 13143, were also resistant to chloramphenicol and fusidic acid at their planktonic MICs. However, immature biofilms of the *S. aureus* strains, NCTC 10442 and NCTC 12973, exhibited significantly reduced viability when treated with tetracyline. Also, significantly reduced viability was reported for the strain, NCTC 10442, treated with the planktonic MIC of fusidic acid. However, both the biomass and the percentage of viable cells were both significantly reduced in the case of the *S. aureus* strain, NCTC 12973, treated with chloramphenicol at the planktonic MIC (Table 20).

Table 20 *Biofilm assay for the activity of antibiotics with a mode of action that interferes with translation against immature, 8 hour old, MRSA biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
Tetracycline 5 mg l^{-1}	X	X	X	X	X	X	38 (±4.3)	47 (±6.2)
Erythromycin 0.5 mg l^{-1}	X	X	X	X	26 (±3.8)	102 (±10.2)	46 (±6.3)	104 (±9.9)
Erythromycin 5 mg l^{-1}	X	X	X	X	16 (±4.5)	105 (±9.8)	24 (±2.3)	65 (±5.4)
Gentamycin 40 mg l^{-1}	X	X	X	X	31 (±4.2)	82 (±8.1)	21 (±3.6)	53 (±6.7)
Tobramycin 40 mg l^{-1}	X	X	X	X	33 (±5.1)	95 (±9.8)	30 (±7.6)	101 (±8.6)
Clindamycin 1.5 mg l^{-1}	X	X	X	X	X	X	47 (±5.9)	57 (±6.6)
Chloramphenico 18 mg l^{-1}	- (±9.6)	- (±9.4)	- (±7.5)	- (±8.8)	- (±6.5)	- (±7.3)	24 (±3.2)	67 (±7.4)
Chloramphenico 180 mg l^{-1}	30 (±5.0)	34 (±5.8)	27 (±3.2)	60 (±7.1)	25 (±2.6)	55 (±9.1)	25 (±4.2)	11 (±3.2)
Fusidic Acid 1 mg l^{-1}	-(±8.7)	- (±9.2)	- (±9.5)	- (±8.1)	26 (±4.1)	102 (±9.7)	- (±9.4)	- (±8.4)
Fusidic Acid 10 mg l^{-1}	30 (±6.5)	37 (±5.1)	31 (±4.9)	67 (±8.6)	38 (±3.2)	68 (±8.6)	30 (±6.0)	79 (±7.3)

The higher concentrations of ten times the planktonic MICs of all the antibiotics, apart from kanamycin and tobramycin, caused significant reductions in the biomass of immature biofilms of all the *S. aureus* strain. The percentage of viable cells within all of the treated biofilms was significantly reduced from 62% in the control to below 30% (Table 20).

The efficacy of antimicrobial agents that interfere with translation against preformed mature (24-hour-old) *S. aureus* biofilms

Mature biofilms of the four *S. aureus* strains were resistant to all of the above antibiotics at both the planktonic MIC and ten times the MIC (data not shown).

6.3.4 The synergistic effect of the ionophore antibiotics in combination with antibiotics with differing modes of action, against preformed immature and mature *S. aureus* biofilms

It was noted that the ionophore antibiotics had significant effects on mature biofilms, but were not successful in completely removing / killing these biofilms when used at concentrations of up to ten times the planktonic MIC. Therefore, a comprehensive assessment was made of the possible antimicrobial synergy when different classes of antibiotics were used in combination with ionophores against preformed immature and mature *S. aureus* biofilms.

Immature and mature biofilms were grown in microtitre plate wells containing 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose inoculated from *S. aureus* (NCTC 10442) starter cultures. The microtitre plates were incubated at 37°C for 8 or 24 hours, for immature and mature biofilms respectively. After incubation, the planktonic culture phase was aspirated off and the biofilms washed in 200 µl of dH_2O to remove non-adherent cells. Aliquots of sterile media, containing the ionophores in combination with different antibiotics, were added to the washed biofilms, and the microtitre plates were incubated at 37°C for a further 18 hours. The percentage viability and biomass were determined using the XTT / PMS and Safranin assays respectively.

For immature biofilms the only significant effect on biofilm biomass was observed when fusidic acid was used in combination with the ionophores, gramicidin and monensin, resulting in a 20% reduction from 60% in control fusidic acid-treated biofilms to 40% in combination treated biofilms (Table 21). However, treatment with monensin and nigericin caused a 10% reduction in the

percentage viability of immature biofilms when used in combination with all but novobiocin (Table 21). Novobiocin-treated control biofilms already possess very low percentage viabilities of less than
10% of the total biomass. The addition of gramicidin also caused a significant reduction in the percentage viability of immature biofilms when used in combination with fusidic acid.

The biomass of mature biofilms was unaffected by the use of ionophores in combination with antibiotics of differing modes of action (Table 22). However, the percentage viability of mature biofilms was significantly reduced by 10% to 20%, further than individual antibiotic treatment, for biofilms treated with gramicidin and narasin for all combinations tested. Monensin used in combination with phosphomycin, ciprofloxacin, chloramphenicol and fusidic acid also had synergistic effects and caused further reductions of 10% to 20% in the percentage of viable cells within the biofilms. However, combinations with nigericin produced no significant synergistic reduction in the percentage viability.

Table 21 *The synergistic effects of the ionophore antibiotics used in combination with antibiotic of differing modes of action against immature (8-hour-old) biofilm.*

	Control		Vancomycin (20 mg l^{-1})		Phosphomycin (80 mg l^{-1})		Ciprofloxacin (1.5 mg l^{-1})		Novobiocin (10 mg l^{-1})		Chloramphenicol (80 mg l^{-1})		Fusidic acid (8 mg l^{-1})	
	% V	B	% V	B	% V	B	% V	B	% V	B	% V	B	%V	B
Control	69 (±3.5)	100 (±3.7)	22 (±11.7)	66 (±5.8)	43 (±9.6)	54 (±5.4)	22 (±4.0)	55 (±3.5)	8 (±3.7)	50 (±11.7)	28 (±4.7)	50 (±11.0)	39 (±5.6)	61 (±7.5)
Gramicidin (4 mg l^{-1})	42 (±12.4)	52 (±10.9)	19 (±2.5)	36 (±4.1)	40 (±5.0)	52 (±10.6)	18 (±3.2)	49 (±12.4)	8 (±1.9)	41 (±12.5)	27 (±4.8)	38 (±6.1)	18 (±1.4)	44 (±3.7)
Monensin (1 mg l^{-1})	18 (±4.3)	45 (±6.7)	9 (±1.6)	44 (±12.4)	8 (±1.6)	50 (±6.8)	9 (±4.7)	42 (±4.3)	9 (±4.7)	48 (±11.6)	8 (±2.4)	46 (±10.4)	10 (±2.1)	43 (±12.3)
Nigericin (0.06 mg l^{-1})	17 (±10.2)	54 (±8.8)	8 (±2.6)	51 (±7.8)	7 (±3.4)	51 (±13.9)	6 (±2.8)	55 (±10.2)	8 (±5.8)	48 (±8.6)	7 (±2.0)	68 (±8.9)	10 (±1.9)	63 (±9.4)
Narasin (0.25 mg l^{-1})	59 (±4.2)	60 (±6.0)	22 (±2.7)	56 (±9.3)	49 (±11.1)	56 (±5.6)	19 (±4.4)	48 (±4.2)	6 (±1.0)	49 (±12.7)	24 (±9.3)	68 (±11.3)	56 (±2.9)	67 (±8.4)

Table 22 *The synergistic effects of the ionophore antibiotics used in combination with antibiotic of differing modes of action against mature (24-hour-old) biofilm.*

	Control		Vancomycin (20 mg l^{-1})		Phosphomycin (80 mg l^{-1})		Ciprofloxacin (1.5 mg l^{-1})		Novobiocin (10 mg l^{-1})		Chloramphenicol (80 mg l^{-1})		Fusidic acid (10 mg l^{-1})	
	% V	B	% V	B	% V	B	% V	B	% V	B	% V	B	%V	B
Control	63 (±4.3)	100 (±6.7)	54 (±12.5)	98 (±8.9)	52 (±5.9)	104 (±10.8)	51 (±4.2)	98 (±6.0)	30 (±12.7)	98 (±9.3)	55 (±5.0)	100 (±4.2)	52 (±8.0)	93 (±11.9)
Gramicidin (4 mg l^{-1})	46 (±10.2)	96 (±8.8)	29 (±8.6)	93 (±2.0)	21 (±11.8)	90 (±5.1)	26 (±10.4)	91 (±3.5)	24 (±3.2)	90 (±11.7)	22 (±10.5)	92 (±11.3)	23 (±6.9)	89 (±2.2)
Monensin (1 mg l^{-1})	31 (±7.0)	109 (±6.0)	28 (±12.7)	108 (±7.3)	25 (±9.4)	104 (±3.9)	25 (±3.0)	100 (±12.4)	27 (±10.9)	103 (±12.5)	23 (±2.4)	108 (±12.2)	26 (±10.3)	99 (±7.7)
Nigericin (0.06 mg l^{-1})	35 (±9.3)	97 (±3.5)	28 (±3.7)	97 (±9.3)	27 (±7.1)	97 (±2.4)	28 (±7.8)	94 (±4.3)	31 (±6.7)	98 (±8.6)	33 (±13.7)	95 (±6.9)	31 (±5.3)	88 (±5.4)
Narasin (0.25 mg l^{-1})	57 (±5.8)	104 (±12.4)	30 (±10.9)	100 (±11.7)	25 (±12.1)	98 (±13.8)	31 (±4.0)	97 (±10.2)	24 (±8.8)	101 (±11.6)	26 (±12.6)	98 (±7.2)	40 (±8.3)	85 (±7.6)

6.4 Discussion

The antibiotics were placed into groups that shared similar modes of action in order to identify if one mode of action was more effective against the MRSA biofilm phenotype. Nearly all of the 22 antibiotics, regardless of their mode of action, had some effect on the immature (8-hour-old) biofilm phenotype. However, only novobiocin, which caused a significant reduction in the percentage viability for the MRSA strains NCTC 11939 and 10442, when used at a concentration of ten times the planktonic MIC, and the ionophore antibiotics, which function against the cell membrane, had any significant effect on the mature (24-hour-old) biofilm phenotype, at both the planktonic MIC and ten times the MIC.

There have been no previous reports, investigating the resistance of bacterial biofilms to ionophore antibiotics. Amorena *et al.* (1999) found that novobiocin at 16 mg l^{-1} had no effect on the biofilms of four slime-producing *S. aureus* isolates grown in tryptone soy broth (TSB), but, at 100 mg l^{-1}, novobiocin caused a significant reduction in viability of both 6 and 48-hour-old *S. aureus* biofilms.

Planktonic bacteria utilize several ingenious mechanisms to develop resistance. These include degradation of the drug, inactivation of the drug by enzymatic modification, and alteration of the drug target (Davies *et al.* 1994). However, these mechanisms are all quite specific for a single drug or a single class of drug, and, since resistant biofilms are formed by planktonically susceptible strains, these planktonic resistance mechanisms are, at least not the only cause of biofilm resistance. It is well known that bacterial cells within a biofilm are able to withstand host immune responses, and they are less susceptible to antimicrobials than their non-attached individual planktonic counterparts. As already discussed in the previous chapter, there are several proposed ideas as to the mechanisms of biofilm specific resistance. However, for some of the antibiotics used in this chapter, there is both supporting evidence, and evidence to the contrary, for these resistance mechanisms in the biofilms of other species of bacteria. It is therefore likely that biofilms evade antimicrobial challenges by multiple mechanisms (Costerton *et al.* 1999). Each of these biofilm resistant mechanisms will now be discussed.

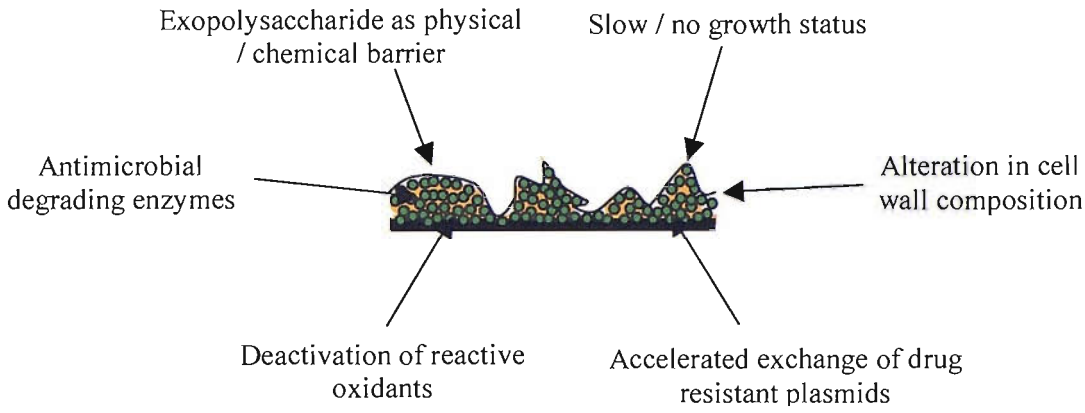


Figure 35 *Illustration of mechanisms of antimicrobial resistance by biofilm (adapted from Prakash et al. 2003).*

6.4.1 Biofilm phenotype

The first mechanism of reduced biofilm susceptibility, is more speculative than the proceeding hypotheses. It hypothesises that at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype. This phenotype is not a response to nutrient limitation; it is a biologically programmed response to growth on a surface (Costerton *et al.* 1999). This concept is of particular interest, because the control of key biofilm genes would offer excellent options to overcome tolerance.

A multitude of strategies has been applied to compare gene and protein expression patterns in biofilms with those in planktonic cultures. Staphylococcal biofilm formation has been attributed to the staphylococcal accessory regulator (*sar*A) (Beeken *et al.* 2004) and the *ica* ADBC gene cluster (Cramton *et al.* 1999). However, Knobloch (2002) reported that virtually all *S. aureus* strains contain the *ica* gene cluster, but do not necessarily produce biofilms. This observation underlines the importance of additional control mechanisms, such as sub-inhibitory antibiotic concentrations (Rachid *et al.* 2000), phase variation (Ziebuhr *et al.* 1999), quorum sensing (Valle *et al.* 2003) or *ica*R (Conlon *et al.* 2002), a transcriptional repressor of *ica* expression under environmental control.

Beenken *et al.* (2004) identified a total of 580 genes that were differentially expressed in biofilms and either or both exponential and stationary phase planktonic cultures. Several of the operons that were induced in biofilms have been found to be important in acid tolerance in other bacterial species. One way in which bacteria combat acidic environments is to produce alkaline compounds, such as ammonia, that can neutralise acids; it is generated through the urease and arginin deiminase

(ADI) pathways (Cunin *et al.* 1986), multiple genes from which were induced in *S. aureus* biofilms by comparison to planktonic conditions. Under anaerobic conditions, some bacteria are also able to generate ATP as an energy source through catabolism of arginine via the ADI pathway (Cunin *et al.* 1986); this process involves five enzymes, all of which are also significantly induced in *S. aureus* biofilms (Beenken *et al.* 2004). Three genes, of a second operon involved in pH homeostasis, *kdp* operon (*kdp*DE), were induced in *S. aureus* biofilms.

Also included among the genes induced in biofilms were seven genes that comprise the urease operon (*ure*ABCEFGD) (Beenken *et al.* 2004). Urease is an enzyme that catalyses the hydrolysis of urea to yield two molecules of ammonia and one molecule of CO_2. Recently, Saïd-Salim *et al.* (2003) found that genes of the urease operon in *S. aureus* are negatively regulated by the SarA homolog Rot (repressor of toxin). Urease synthesis by *Klebsiella aerogenes* is stimulated under conditions of nitrogen limitation (Friedrich *et al.* 1977), and in *Helicobacter pylori*, the expression of the urease operon is up-regulated by a pH-dependent, post-transcriptional regulatory mechanism, and, in acidic conditions up-regulation of the operon occurs (Akada *et al.* 2000). Therefore it is possible one or both of these environmental conditions are the cause of the up-regulation of these genes in *S. aureus* biofilms.

A theory concerning the formation of persister cells in the biofilm is often cited as the possible explanation or precursor to the biofilm 'phenotype'.

6.4.2 Persister cells

Although most resistance of *S. aureus* to aminoglycosides is due to aminoglycoside-modifying enzymes, small-colony variants of staphylococci also show resistance, which may be due to a defect in adenylate cyclase or in cyclic adenosine 5'-monophosphate (cAMP)-binding proteins such that cells with a reduced growth rate do not transport aminoglycosides into the cytoplasm. Small colony variants exist within biofilms to a greater extent than in planktonic cultures (data not shown); therefore, resistance of the biofilm may be increased though the increase production of this group of cells.

Bacterial populations produce persister cells that neither grow nor die in the presence of antimicrobial agents (Keren *et al.* 2004). It is possible that persisters are cells in a state of deep dormancy (Nystrom 2003). Scherrer (1988) suggested that persisters represent a particular stage in the cell's life cycle, but, since early exponential cells undergo a cell cycle but do not produce persisters, this theory can be ruled out (Scherrer *et al.* 1988). Persisters have often been over-

looked, partly due to the definition of minimum bactericidal concentration (MBC), which is the death of $\geqslant 3$ log of cells, as, in most cases, persisters fail beyond this threshold and are therefore not reported (Moore *et al.* 1997).

The reason for persister tolerance remains unknown, and the suggestion that persisters are simply non-growing (Bigger 1944) does not explain their phenotype. Indeed, fluoroquinolones kill non-growing cells, but leave persisters intact (Keren *et al.* 2004). Keren *et al.* (2004) proposed that the persisters were largely responsible for the high levels of tolerance of biofilms to fluoroquinolones, even though they represent a small faction of the population, typically 10^{-6} to 10^{-4} cells. Biofilms are not unique in producing large persister cell populations; stationary phase cultures of *Pseudomonas aeruginosa* produce persisters at high levels (10^{-3}-10^{-2}) and exhibit tolerance comparable to that found in biofilms (Brooun *et al.* 2000; Spoering *et al.* 2001). Similarly, stationary-state cultures of *S. aureus* (ATCC 55585) and *E. coli* (CGSC4401) produce large populations of persisters (Keren *et al.* 2004). Keren *et al.* (2004) found that the rate of persister production in *S. aureus* cultures over time was higher than the overall growth rate of the culture (14 minutes doubling time compared to 30 minutes).

Karen *et al.* (2004) suggested that biofilm survival was based on the presence of persisters and not on expression of possible biofilm-specific resistance mechanisms. However, this author does not agree, for, although mature *S. aureus* biofilms were found to be resistant to the fluoroquinolone, ciprofloxacin, and the percentage of SCVs was increased in *S. aureus* biofilms, they did not however, account for all of the viable bacteria within the ciprofloxacin treated biofilms (data not shown).

However, persister cells are generally considered to be cells in the viable but non-culturable state (VNC) and are, therefore, not the cause of biofilm resistance, as the percentage of cells deemed viable by the XTT / PMS assay were recoverable and culturable without the need for a resuscitation step (data not shown).

6.4.3 Penetration of antimicrobials though the biofilm matrix

One proposed mechanism for biofilm specific resistance to antimicrobial agents is the failure of an agent to penetrate the full depth of the biofilm. Indeed, many investigators believe that the EPS matrix acts as a physical / chemical barrier. The diffusion of antimicrobial agents across biofilm has been assessed by elegant concentration measurements and the visualization of bactericidal effects on the opposite side of *in vitro* biofilms (Anderl 2003). Although most studies have

documented unimpaired antimicrobial penetration (Anderl 2003), there are some exceptions (Ishida *et al.* 1998).

The EPS is negatively charged and functions as an ion-exchange resin. It is thought to be capable of binding a large number of positively charged molecules, retarding their penetration through to the embedded cells (Prakash *et al.* 2003). The aminoglycoside antibiotics are positively charged (gentamycin and tobramycin, +5 and kanamycin, +4). Nichols *et al.* (1988) were able to show a concentration dependent binding of tobramycin to the alginic acid EPS, produced by mucoid strains of *P. aeruginosa*, suggesting that these molecules would be retained within the EPS matrix. The aminoglycosides (gentamycin, kanamycin and tobramycin) were evaluated in the combined biofilm assay; none had any effect on mature biofilms, which could accordingly be explained by the retention of these antibiotics in the copious amount of PIA / EPS shown to be present in mature *S. aureus* biofilms (Section 3.3.2). Conversely, only tobramycin (and then only at the highest concentration) was active against immature biofilms. Immature biofilms, as previously shown, have limited EPS production and therefore, it is unlikely that their resistance is due to the formation of a diffusion barrier, and more likely due to other biofilm specific resistance mechanisms.

Furthermore, Farber *et al.* (1990) found that extracts of *S. epidermidis* EPS interfered with the antibacterial activity of glycopeptide antibiotics. The addition of 0.5% (v/v) EPS extract increased the planktonic MIC of both vancomycin and teichoplanin approximately five-fold versus both 'slime'-positive and negative strains. The authors suggested that the EPS either physically complexes with and inactivates glycopeptides or coats the cell wall to create a permeability barrier. However, Dunne *et al.* (1993) showed that 'slime'-positive *S. epidermidis* biofilm did not prevent the perfusion of vancomycin or rifampin. Therefore, it is likely that the inactivation of glycopeptide antibiotics by *S. epidermidis* EPS extracts shown by Faber *et al.* (1990) was due to complex formation between the two, and not the formation of a permeability barrier.

Indeed, another biofilm resistance mechanism linked to the EPS matrix is the production of and immobilization of antimicrobial-degrading agents such as β-lactamases within the EPS, which lead to the inactivation of the incoming antimicrobial molecules. It is interesting to note that *P. aeruginosa* biofilm cells have been shown to produce 32-fold more β-lactamase than cells of the same strain grown planktonically (Potera 1999 and Tuomanen *et al.* 1986). It is plausible that production of β-lactamase and other antimicrobial-degrading molecules, such as aminoglycoside-modifying enzymes, are also increased in *S. aureus* biofilms, although no literature on the subject has been published. However, once more, this does not explain the resistance of immature, EPS / PIA-reduced biofilms; and indeed, further studies by Anderl *et al.* (2000) showed that biofilms of

K. pneumoniae β-lactamase mutant were also resistant to ampicillin, suggesting again that there is more than one resistance mechanism at work.

Lastly, although not strictly related to EPS, another diffusion barrier presented by the cell wall may also play a part in biofilm resistance. Not only has peptidoglycan production increased in vancomycin resistant planktonic *S. aureus* strains (Hiramatsu 2001), but *S. aureus* has also been shown to O acetylate its peptidoglycan (Johannsen *et al.* 1983). To date, a total of 28 species of both Gram-negative and Gram-positive bacteria, including both *E. faecalis* and *S. aureus,* are known to modify their peptidoglycan by O acetylation. The O acetylation of peptidoglycan occurs on the C-6 hydroxyl moiety of component *N*-acetylmuramoyl residues, producing the corresponding *N,O*-diacetylmuramoyl derivatives in the bacterial cell wall heteropolymer (Pfeffer *et al.* 2006). In *E. faecalis,* production of O aceytlated peptidoglycan was found to vary depending on the growth phase of the culture (Pfeffer *et al.* 2006). When entering the stationary growth phase, there was an increase in peptidoglycan production of between 10% and 40%, however, cells in the VNC state had the highest levels of peptidoglycan. It is reasonable to believe that this may also occur in *S. aureus*; however, if this were the case and O acetylation of peptidoglycan was increased in biofilm cells, it is uncertain how this would affect antibiotic resistance.

There is lots of evidence supporting the theory of reduced penetration of antibiotics either by the EPS acting as a permeability barrier, or the presence of antibiotic deactivating molecules. Although this may occur for specific antibiotics, it cannot be used as a blanket theory, as the ionophore antibiotics were effective against mature biofilm cells, and therefore they were not retarded or deactivated in the abundant EPS of these mature biofilms.

6.4.4 Slow growing or starved state leaded to slower metabolism

A second hypothesis to explain reduced biofilm susceptibility to antibiotics posits that at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state (Brown *et al.* 1988). Spatial heterogeneity in the physiological state of the bacteria within model biofilms has been demonstrated by a variety of micro-slicing and microscopic techniques (Kinniment *et al.* 1992; Wentland *et al.* 1996 and Xu *et al.* 1998). Such heterogeneity of biofilms constitutes an important survival strategy, because at least some of the cells, which represent a wide variety of different metabolic states, are almost certain to survive any metabolically directed attack (Costerton *et al.* 1999).

Eng *et al.* (1991) provided evidence in support of the theory that altered rates of bacterial growth dictate the response to antibiotics. The authors were able to demonstrate that, by controlling the

growth rate of bacteria through nutrient limitation, only the fluoroquinolone antibiotics produced bactericidal effects against stationary-phase Gram-negative organisms, and that no class of antibiotic was bactericidal versus growth-limited *S. aureus*. Also, Anwar *et al.* (1992) were able to show age-related differences in the response of *S. aureus* biofilms to antimicrobial therapy. Exposure of 4-day-old chemostat grown biofilms to tobramycin produced a rapid reduction in viable cell counts, whereas biofilms, developed over a 13-day period, demonstrated marked resistance.

The efficacy of antimicrobial agents that interfere with translation, and those that inhibit enzymatic pathways directly (such as trimethroprim, which inhibits tetrahydrofolate) may be affected by a decrease in metabolism and reduced protein synthesis. Indeed, the mature biofilms of *S. aureus* were resistant to all antibiotics with this mode of action, apart from novobiocin. It is possible that the reduced growth-rate goes someway to explaining mature biofilm resistance, as it would also help explain why the ionophore antibiotics, whose activity does not depend on inhibition of transcription, translation or enzymatic pathways, were so successful in disrupting mature *S. aureus* biofilms. However, the mature biofilms of planktonically susceptible *S. aureus* strains were resistant to ciprofloxacin, a floroquinolone antibiotic, which is active against slow / non-growing cells. Also, the mature biofilms of EMRSA-1 (NCTC 11939) and one MRSA strain (NCTC 10442) were susceptible to novobiocin, which inhibit the enzyme DNA gyrase, suggesting that this is not the only resistance mechanism active in mature biofilms.

The penetration of aminoglycosides through the cell membrane of the bacterium depends partly on oxygen-dependent active transport by a polyamine carrier system. If this activity of these transporters was reduced in slow-growing biofilms cells, it would lead to reduced uptake and therefore reduced effectively of the aminoglycoside antibiotics. Indeed, anaerobic organisms such as *Bacteroides* species, are resistant to aminoglycosides, because they lack an oxygen-dependent transport system to move the antibiotics across the cytoplasmic membrane (Mann *et al.* 1996).

Recently, it has been suggested that the slow growth rate of some cells within the biofilm is not due to nutrient limitation *per se*, but to a general stress response initiated by growth within a biofilm. This idea is an attractive possibility, because the stress response results in physiological changes that act to protect the cell from various environmental stresses. Thus, the cells are protected from the detrimental effects of heat shock, cold shock, changes in pH and many chemical agents (Foley *et al.* 1999). The starvation and stress responses in *S. aureus* have been wel-characterized; the alternative sigma factor σ^B, which is required for biofilm formation (Rachid *et al.* 2000), responds to environmental stress by modulating the expression of a wide range of stress and stationary-phase proteins. The alternative sigma factor σ^B is regulated by the *sar* locus, which has been shown to

control stress responses and is up-regulated in biofilms. Also, when slow growth is induced by an insufficient supply of amino acids and / or carbon source, bacteria adapt to this lack of nutrients with a series of regulatory events know as the stringent response. In *E. coli,* the stringent response activates the *relA*-dependent synthesis of ppGpp, which inhibits anabolic processes in bacterial cells (Cashel *et al.* 1996). The occurrence of ppGpp is also widespread among staphylococci (Cassels *et al.* 1995). Interestingly, ppGpp suppressed the activity of a major *E. coli* autolysin, SLT (Betzner *et al.* 1990). Homologous genes in *S. aureus* may also be suppressed in biofilm formation and may help explain the tolerance of antimicrobial agents in these slow growing cells.

As already discussed in Chapter 5 the expression of ppGpp in the stringent response in *E. coli* also inhibits peptidoglycan synthesis (Ishiguro *et al.* 1976). If this is also true for *S. aureus* biofilms, it gives an explanation for the resistance of both immature (partial resistance) and mature biofilms to other the cell wall active antibiotics, e.g. ampicillin and phosphomycin.

6.4.5 Does the microenvironment affect antimicrobial activity?

For some antimicrobials, biofilm resistance may be simply the case of unfavourable conditions, which reduce the uptake of the antimicrobial agent into the bacterial cell. It is likely that the same factors that adversely influence antimicrobial activity *in vitro*, including pH, CO_2, O_2, divalent cation concentration, hydration level and pyrimidine concentration, will also produce undesirable effects in the deepest layers of a bacterial biofilm where acidic and anaerobic conditions persist (Jorgensen *et al.* 1999).

Although not much data is available for how the microenvironment of a biofilm affects antibiotic activity, based on the results of disk diffusion and broth microdilution susceptibility testing it has been predicted that the activity of the aminoglycosides (gentamycin, kanamycin and tobramycin), macrolides (erythromycin), and tetracyclines would likely be compromised in an acidic milieu with increased CO_2 (Dunne 2002).

An example of how the biofilm microenvironment can affect activity of antibiotics is given by rifampicin. Williams *et al.* (1998) investigated the mechanisms of rifampicin transport into *S. aureus* cells. For wild-type *S. aureus,* accumulation of rifampicin was unaffected by antibiotic efflux inhibitors; therefore, they concluded that rifampicin accumulation was dependent on the membrane potential and not efflux inhibitors. They concluded that rifampicin accumulation was temperature and pH dependent; the lower the temperature and pH (<7) the lower the concentration of rifampicin accumulated. The author's argue that the most likely

explanation for the lower concentrations of rifampicin accumulated by *S. aureus* at low pH, is that rifampicin undergoes a structural change at acidic pH. Rifampicin contains a weak basic methylpiperazine group ($pK_a = 7.9$), which may become protonated in an acidic environment. Membranes are less permeable to ions and polar molecules (Nikaido *et al.* 1993) and, therefore, *S. aureus* would be expected to accumulate less rifampicin at lower pH values. It is possible, therefore, that the pH of the culture fluid surrounding the *S. aureus* biofilms in this investigation, which was measured at pH 5.5 for mature biofilms (biofilms grown in the presence of glucose, data not shown) was sufficient to cause a structural change in rifampicin and inhibit its accumulation of the biofilm cells, therefore providing resistance.

In addition, Mates *et al.* (1983) provided evidence, which showed that under anaerobic growth conditions (increased pH and decreased O_2), aminoglycosides (gentamycin) remained unbound within *S. aureus* cells, suggesting therefore, that effective aminoglycoside binding requires not only sensitive ribosomes but also critical intracellular conditions (e.g., pH, redox potential, etc.) not present in anaerobic growth.

This illustrates the importance of testing new antibiotics specifically against the biofilm microenvironment to ensure activity against the biofilm, and not just the planktonic mode of growth.

6.4.6 Composition of the cell membrane

One low specificity mechanism of biofilm resistance is the formation of low permeability plasma cell membranes, in order to decrease the influx of the drug into the cells. In Gram-negative bacteria, the outer membrane, with its unusual, high saturation, extensively cross-linked, lipopolysaccharide (LPS), is much less fluid than the normal glycerophospholipid-containing plasma membrane. This reduction in fluidity leads a reduced diffusion rate of 50 to 100 times for antibiotics (Vaara *et al.* 1990). However, the vast majority of clinically important antibiotics and chemotherapeutic agents show some hydrophobicity, which allows them to diffuse across the lipid bilayers of the cytoplasmic membrane (prominent exceptions include fospomycin and aminoglycosides) (Nikaido 1994), in the Gram-positive bacteria. Under normal planktonic conditions, it is not possible to make the cytoplasmic membrane of Gram-positive much less permeable, because this would require decreasing the membrane fluidity and interfering with the proper functioning of membrane proteins. Although, within the biofilm, it may be possible to change the permeability of the cytoplasmic membrane as often membrane proteins are not needed

or down-regulated anyway. Also, under aerobic and anaerobic planktonic growth conditions, the magnitude of the electrical potential ($\Delta\Psi$) and therefore passive diffusion rates, vary. Although, changes in the cytoplasmic membrane may lead to increased resistance to a number of antibiotics, any changes that were made within the cytoplasmic membrane, however, did not affect the activity of the ionophores, as they were the only group of antibiotics to have a significant effect on both the percentage viability and biomass of mature *S. aureus* biofilms.

However, Mates *et al.* (1983) demonstrated that manipulations that change the magnitude of $\Delta\Psi$ in planktonic *S. aureus* cultures (ionophores, external pH changes) did affect effective aminoglycoside (gentamycin) uptake and killing (Mates *et al.* 1982). They showed that at acidic external pH, as in the *S. aureus* biofilm, gentamycin accumulation within the cell was significantly reduced, suggested that deficient uptake under anaerobic conditions in these facultative organisms may be due to a low magnitude of $\Delta\Psi$ when the electrochemical proton gradient is generated via ATP hydrolysis (Miller *et al.* 1980). It has also been suggested, however, that electron transport *per se* is necessary for aminoglycoside uptake (Campbell *et al.* 1980) and that aminoglycoside resistance, under anaerobic conditions, is due to a lack of electron transport (Bryan *et al.* 1979). The magnitude of $\Delta\Psi$ may also affect the entry of other antibiotics into anaerobic *S. aureus* biofilm cells.

Antibiotics can be pumped out of the cell in an energy-dependent fashion (Nikaido 1994). Multidrug efflux is an increasingly reported phenomenon and has been described for many organisms. Bacteria possess a wide array of drug efflux proteins and a number of clinically relevant species, including *S. aureus*, utilize these transporters as part of their planktonic resistance strategy. Examples of efflux-related resistance mechanisms that have been described for *S. aureus* include those conferred by QacA (broad-range of chemicals) and NorA (quinolones, chloramphenicol), which are multidrug resistance (MDR) transporters, and the more specific MsrA (macrolides and type B streptogramins) and TetK (tetracycline) transport proteins (Littlejohn *et al.* 1992 and Guay *et al.* 1993). These export proteins were originally described to efflux quaternary ammonium salts (antiseptics), fluoroquinolones, macrolides and tetracyclines, respectively, although these efflux proteins, especially QacA and NorA, actively export a broad array of structurally dissimilar drugs from the bacterial cell. Beeken *et al.* 2006 found that the EmrB/QacA, a subfamily of multidrug resistance proteins, were slightly up regulated in biofilms compared to stationary growth. Therefore, resistance may occur because membranes of these biofilm cells are better equipped to pump out antibiotics before they can cause damage, or even antibiotic targets disappear (Prakash *et al.* 2003).

The ionophore antibiotics were the only group of antibiotics that were active against the mature biofilm phenotype. They act by disrupting transmembrane ion concentration gradients that are required for the proper functioning and survival of microorganisms. Antibiotics representing both classifications of ionophores, the mobile ion carriers (monensin, nigericin and narasin) and the channel formers (gramicidin), had equal effectiveness against the mature biofilm phenotype. If the cytoplasmic membrane is damaged, it is the disruption of the proton motive force that leads to cell death. Proton extrusion during respiration or ATP hydrolysis leads to the generation of a transmembrane electrochemical gradient of hydrogen ions (protons) ($\Delta\mu_{H+}$) which is the immediate driving force for a wide variety of biological processes (Nester *et al.* 2004). This thermodynamic entity is composed of electrical and chemical parameters according to the following relationship: $\Delta\mu_{H+} = \Delta\Psi - Z\,\Delta pH$, where $\Delta\Psi$ represents the electrical potential across the membrane, and ΔpH is the chemical difference in H^+ (proton) concentration across the membrane (Z is equal to 61.7 mV at 38°C, or 58.8 mV at room temp) (Anand *et al.* 1960). With regard to active transport, the chemiosmotic hypothesis predicts that transport is driven by $\Delta\Psi$ (interior negative) for cationic substrates, by ΔpH (interior alkaline) for anionic substrates, and by $\Delta\mu_{H+}$ for neutral substrates.

The reason why the ionophore antibiotics were so successful (at low concentrations) against MRSA mature biofilms is unclear. However, one possible reason for their activity may relate to the fact that they do not have to enter the cells in order to have an effect and so are not affected by efflux proteins. Also, as they do not inhibit translation, transcription or any specific enzymatic pathways, they are not affected by the reduction in growth rate. Indeed, obviously the EPS matrix does not act as a diffusion barrier to the ionophores, and there are no ionophore deactivating molecules. Lastly, none of the genes involved in stress responses that are induced in biofilm cells interfere with ionophore activity. Therefore, it is likely the ionophore antibiotics evade multiple biofilm resistance mechanisms.

6.4.7 Synergistic effects of the ionophore and antibiotics with differing modes of action

Interestingly, when the ionophores were used in combination with antibiotics with differing modes of action, they had synergistic effects (regardless of the mode of action) against both immature and mature MRSA biofilms. There are several possible explanations for this synergism; for example, the addition of the ionophore antibiotics would cause disruption of the multidrug efflux pumps, thereby reducing the amount of antibiotic molecules pumped out of the cell and so increasing the overall concentration of those antibiotics inside the cell, leading to more effective / quicker killing.

Indeed disruption of the NorA MDR transporter could partly explain the observed increased resistance to ciprofloxacin and chloramphenicol. Also, in the presence of the ionophore antibiotics, slow growth and the reduction in translation and transcription normally seen in biofilm cells may be reversed in an effort to right the imbalances in $\Delta\Psi$. In addition, the synergy observed between these antibiotics could simply be the cumulative effects of the killing of separate distinct populations of cells within the biofilm, by the different antibiotics.

However, while Mates *et al.* (1983) were investigating the relationship between the magnitude of $\Delta\Psi$ in planktonic *S. aureus* cultures and anaerobic gentamycin up-take, they found that when sub-lethal levels of nigericin were added, the $\Delta\Psi$ in aerobic planktonic cells was not measurably changed, but in anaerobic planktonic cells the $\Delta\Psi$ was increased to the aerobic level, therefore under anaerobic conditions, there was minimal uptake of gentamycin and no killing, when nigericin was added. However, gentamycin uptake under anaerobic conditions was stimulated along with an increase in killing. This change in the magnitude of $\Delta\Psi$, induced by the addition of the ionophores, could also be an important resistance mechanism in biofilm cells, which could also explain the increased resistance of other antibioses used in combination with the ionophores. This suggests that under anaerobic conditions the cytoplasmic membrane in biofilm cells is a barrier to antibiotic influx, and that this is destroyed by ionophore activity.

However, this does not explain why the addition of the ionophores should increase the activity of vancomycin as it is active at the cell wall and does not have to pass through the cell membrane. If the mechanism of biofilm resistance is that vancomycin is mopped up by the EPS matrix, it is unclear what effect the addition of the ionophore antibiotic would have on the efficacy of this resistance mechanism; although, as discussed, there is the possibility that the synergism seen is due to an increase in peptidoglycan production, induced by the addition of the ionophores, or that it is simply the cumulative effect of the vancomycin and the ionophores on different cells within the biofilm, and that no actual synergism occurs.

Unfortunately, these cell membrane-acting antibiotics do not discriminate between prokaryotic and eukaryotic cells, which they bind to a lesser extent, limiting their use to topical applications. Polymyxin B and the closely-related compound colistin (polymyxin E), which act like cationic detergents, are the only membrane-active antimicrobial agents to be administered systemically in human medicine. However, they have little to no effect on gram-positive bacteria, since the cell wall is too thick to permit access to membrane. Gramicidin is used primarily as a topical antibiotic, used in the treatment of infected surface wounds, and in eye, nose, and throat infections. However, monensin, nigericin and narasin, the mobile ion carriers, are only used in veterinary medicine as an additive to cattle feed.

In conclusion, biofilms of *S. aureus*, MRSA and MSSA strains, are phenotypically resistant to a wide-variety of antibiotics. Although work has been previously published looking at the activity of a few antibiotics against *S. aureus* biofilms, it has been difficult to draw general conclusions about global resistance, as these investigations were carried out using different methods and *S. aureus* strains. The work presented here shows that *S. aureus* biofilms are resistant to antibiotics with differing modes of action at the same time. However, more importantly, the ionophore antibiotics are active against mature *S. aureus* biofilms.

As discussed, the results for *S. aureus* biofilm resistance strongly suggests that multiple mechanisms are required for overall antibiotic resistance, including: the induction of a biofilm phenotype, a barrier to diffusion, presented by the EPS matrix, the reduced growth rate of biofilm cells, and extreme micro-environmental conditions of the biofilm. However, the work here also suggests that the cytoplasmic membrane acts as an effective diffusion barrier to antibiotics, at least in biofilm cells, and that this barrier can be disrupted by addition of the ionophore antibiotics, which has not previously been identified.

As the ionophore antibiotics are not suitable for human use, this work underlines the need for novel antimicrobial agents, capable of circumventing all of the biofilm resistance mechanisms, or the discovery of co-drugs, which can disarm the biofilm resistance mechanisms, making the biofilms more susceptible to existing antibiotics.

7. SCREENING OF NOVEL ANTIMICROBIAL AGENTS FOR ACTIVITY AGAINST MSSA AND MRSA BIOFILMS

7.1 Introduction

The investigation into the efficacy of a range of antibiotics has demonstrated that the mature MRSA biofilm was phenotypically resistant to many antibiotics with different modes of action, save the ionophores, which affect the cellular membrane. However, treatment with ionophores at the planktonic minimum inhibitory concentration (MIC) or ten times the MIC was not sufficient in completely removing or killing mature *S. aureus* biofilms. In addition, although synergistic effects were observed when the ionophore antibiotics were used in combination with antibiotics with differing modes of action, these combination treatments also failed to completely eliminate mature *S. aureus* biofilms. Furthermore, the ionophore antibiotics are toxic to mammalian cells and, therefore, they are limited either to veterinary medicine or topical applications.

A problem commonly faced by medical professionals today is the resistance of bacterial infections to antibiotics that planktonic studies have deemed as inhibitory. It is possible that the resistance is related to the biofilm phenotype. Consequently, it is important that new novel antimicrobial agents and antimicrobial classes are specifically tested against the biofilm phenotype at an early stage of drug evaluation.

Therefore, a complete assessment was made of a library of 79 novel potential antimicrobial agents. The library included: three agents related to diaryl-ureas that were possible inhibitors of the electron transport chain (DC03, DC41 and DC42); three putative inhibitors of FabI (the acyl carrier protein enoyl reductase), which have the same mode of action as triclosan (DC28, DC29 and DC30); two derivatives of tiamulin, a protein synthesis inhibitor used in animals (DC15 and DC16); one oxazalidinone derivative, a possible protein synthesis inhibitor (DC05); and two rifampicin analogues (DC10 and DC11).

7.2 Methods

The methods used in this chapter are as described in chapter 2.

7.3 Results

7.3.1 Planktonic susceptibility of MSSA and MRSA strains

The MICs of 79 novel antimicrobial agents were determined for planktonic *S. aureus* cultures (NCTC 11939, 13143, 10442 and 12973) in MH broth, using the standard broth micro-dilution susceptibility method of the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Briefly, a two-fold dilution series of the antimicrobial agents was set up in four replicate microtitre plates (NuncSurface) with 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose per well. The microtitre plates were inoculated with starter cultures (turbidity of 0.08-0.1; 620 nm) of the four *S. aureus* strains to give a final inoculum of 5 x 10^5 CFU ml^{-1}. The plates were incubated at 37°C for 24 hours and growth inhibition was determined by a visual assessment of culture turbidity.

Only the 42 chemicals (DC 1-42) that produced planktonic MICs of 8 mg l^{-1} or below, for three or more of the *S. aureus* strains, were assessed in the biofilm assay (for MICs see Appendix B). The EMRSA strain NCTC 11939 was the only strain planktonically resistant to some (12 out of 42) of the remaining novel antimicrobial agents, determined as an MIC of 32 mg l^{-1} or more, confirming its broad-spectrum antimicrobial genotype. Also, the MICs of the antimicrobial agents for this strain were at least twice that of the other *S. aureus* strains. Apart from the antimicrobial agents DC03 and DC13 showing lower activity for the *S. aureus* strains NCTC 10442 and 11939 respectively (MICs of 16 mgl^{-1}), MICs of 8 mg l^{-1} or less were achieved for the other 40 antimicrobial agents against all four strains.

7.3.2 Efficacy of novel antimicrobial agents in preventing initial MSSA and MRSA cell attachment

To investigate the ability of the novel antimicrobial agents to prevent initial cell attachment, starter cultures were used to inoculate 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose, with or without the novel antimicrobial agents at the planktonic MIC or ten times the MIC, in the wells of microtitre plates. The microtitre plates were incubated at 37°C for 18 hours. After incubation, the planktonic culture phase was aspirated; the remaining attached cells were washed in 200 µl of dH_2O. The percentage of viable cells was determined using the XTT / PMS assay and total biofilm biomass was determined using the Safranin assay.

As expected, all 42 novel antimicrobial agents were successful in significantly reducing or preventing initial cell attachment of planktonically susceptible *S. aureus* strains at both

concentrations (data not shown). At the planktonic MIC, the total biomass was reduced by a minimum of 90%, and the percentage viability was reduced to between 0 and 10% from 60% in the control biofilms. For cultures treated with ten times the MIC, both biomass and percentage viability were reduced in all cases to zero.

7.3.3 The effect of the addition of novel antimicrobial agents to preformed biofilms

As all antimicrobial agents were successful in preventing initial cell attachment, it was desirable to determine their efficacy against preformed *S. aureus* biofilms. Immature and mature biofilms were grown from *S. aureus* starter cultures in microtitre plate wells containing 200 µl of $BHI_{25\%}$ + 0.09% (w/v) glucose, which had been incubated at 37°C for 8 or 24 hours, for immature and mature biofilms respectively. After incubation, the planktonic culture phase was aspirated off and the remaining biofilms were gently washed in dH_2O. Growth media (200 µl), with or without the novel antimicrobial agents at the planktonic MIC or ten times the MIC, were replaced, and the microtitre plates were incubated at 37°C for a further 18 hours. The percentage of viable cells (% V) within the biofilm was determined using the XTT / PMS assay, and biofilm biomass (B) by the Safranin assay (Tables 23-26).

For immature biofilms grown in the presence of five of the novel antimicrobial agents (DC04, DC28, DC31, DC30 and DC40) at both concentrations, the total biomass (100%) and percentage of viable cells (63%) was not significantly different to the control immature biofilms. Also, of the 9 antimicrobial agents effective against the *S. aureus* immature biofilms, the *S. aureus* strain NCTC 11939 was planktonically resistant to 1 agent and its immature biofilm was resistant to 4 of the antimicrobial agents (Table 23).

However, the novel antimicrobial agents DC01, DC02, DC05, DC11, DC14, DC17 and DC21 all had an effect at the planktonic MIC on immature biofilms of each of the four *S. aureus* strains (Table 23). The antimicrobial agents DC05, DC14 and DC21 significantly reduced the percentage of viable cells from 63% to between 38% and 5%. The biomass of the biofilms treated with these agents was, in the majority of cases, also significantly reduced by up to 50%. Furthermore, for immature biofilms that were susceptible to the antimicrobial agents DC01, DC02 and DC11 at the planktonic MIC, a smaller but still significant reduction in both biomass and percentage viability was seen. Immature biofilms of *S. aureus* strains, that were not resistant to DC26 and DC27, showed no significant difference in biomass, but a significant reduction of half or more was seen in the percentage of the viable cells (Table 23).

A greater number of novel antimicrobial agents had a significant effect on immature biofilms when used at a concentration of ten times the planktonic MIC (Table 24). Of the 31 antimicrobial agents effective against immature biofilms at ten times the planktonic MIC, all but 10 caused very significant reductions (to below 20%) in the percentage of viable cells. The biomass of these biofilms was variably affected, being generally more reduced for the biofilms of the strains NCTC 11939 and 13143 than the *S. aureus* strains NCTC 10442 and 12973. The other 10 antimicrobial agents also caused significant but smaller reductions in the percentage viability from 60% to 40% of the total biofilm biomass. The biomass of these treated biofilms was again variably affected.

Table 23 *The efficacy of novel antimicrobial agents at the planktonic MIC on immature (8-hour-old) S. aureus biofilms.*

	NCTC 1939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
DC01	- (±2.9)	- (±5.4)	34 (± 1.7)	50 (±6.9)	- (±8.5)	- (±13.5)	- (±7.7)	- (±4.4)
DC02	- (±11.0)	- (±7.7)	34 (±1.2)	65 (±11.2)	- (±4.3)	- (±4.5)	30 (±3.6)	84 (±12.8)
DC05	28 (±2.0)	55 (±3.6)	10 (±4.1)	5 (±12.3)	15 (±1.9)	98 (±6.7)	24 (±4.3)	81 (±5.9)
DC11	X	X	- (± 3.5)	- (±7.6)	53 (±9.0)	54 (±10.2)	16 (±1.1)	70 (±2.2)
DC14	76 (±5.9)	38 (±11.8)	20 (±7.5)	27 (±3.3)	22 (±3.1)	63 (±3.1)	15 (±1.1)	43 (±10.8)
DC17	62 (±11.4)	55 (±3.2)	36 (±5.4)	17 (±10.8)	13 (±2.7)	109 (±2.8)	18 (±3.7)	79 (±1.8)
DC21	37 (±12.6)	93 (±3.6)	13 (±3.2)	48 (±12.4)	23 (±8.1)	94 (±2.9)	38 (±7.7)	60 (±4.6)
DC26	- (±13.0)	- (±6.7)	21 (±1.9)	108 (±3.9)	- (±12.0)	- (±5.9)	20 (±1.5)	105 (±7.4)
DC27	- (±6.3)	- (±5.2)	17 (±9.2)	72 (±6.5)	28 (±6.4)	102 (±7.4)	33 (±2.2)	87 (±4.0)

(- Not significantly different to control; X planktonically resistant)

Table 24 *The efficacy of high concentrations (at ten times planktonic MIC) of the novel antimicrobial agents on immature (8-hour-old) Biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
DC02	54 (±7.6)	69 (±3.7)	34 (± 1.7)	26 (±4.4)	39 (±6.4)	102 (±2.6)	32 (±2.4)	81 (±2.0)
DC05	21 (±2.3)	60 (±7.5)	4 (± 1.2)	47 (±13.1)	8 (±4.7)	104 (±3.7)	11 (±5.5)	86 (±3.3)
DC06	75 (±4.8)	52 (±12.1)	66 (± 4.1)	27 (±6.4)	9 (±1.2)	102 (±4.2)	13 (±4.0)	68 (±8.7)
DC07	12 (±9.4)	16 (±2.0)	0 (± 3.5)	12 (±4.3)	5 (±3.5)	76 (±3.3)	0 (±5.5)	11 (±11.6)
DC09	X	X	54 (± 7.5)	67 (±9.0)	28 (±1.6)	83 (±10.0)	26 (±3.3)	78 (±7.2)
DC10	- (±7.8)	- (±10.2)	22 (±5.1)	86 (±5.0)	36 (±6.8)	51 (±7.6)	54 (±5.1)	62 (±11.8)
DC11	X	X	43 (±2.3)	58 (±13.3)	16 (±2.1)	64 (±10.1)	10 (±7.2)	58 (±3.5)
DC12	4 (±1.0)	79 (±8.7)	0 (±6.8)	15 (±11.9)	9 (±5.3)	100 (±7.0)	8 (±4.4)	86 (±8.8)
DC13	17 (±4.3)	86 (±2.9)	10 (±2.7)	30 (±13.3)	23 (±9.3)	114 (±3.4)	16 (±3.0)	85 (±9.1)
DC14	0 (±2.1)	5 (±11.8)	0 (±6.2)	16 (±5.0)	14 (±5.7)	72 (±11.4)	9 (±1.5)	27 (±8.2)
DC16	X	X	- (±5.8)	- (±8.5)	16 (±1.0)	95 (±3.2)	19 (±8.0)	92 (±5.0)
DC17	45 (±5.2)	40 (±8.0)	6 (±3.7)	15 (±9.0)	11 (±1.7)	100 (±9.2)	14 (±1.6)	73 (±9.4)
DC18	X	X	19 (±5.2)	38 (±8.2)	22 (±4.2)	114 (±6.6)	9 (±4.2)	97 (±2.3)
DC19	X	X	16 (±0.4)	30 (±7.3)	8 (±9.1)	102 (±9.3)	15 (±2.3)	90 (±6.1)
DC20	39 (±2.2)	69 (±2.4)	7 (±2.4)	28 (±3.4)	28 (±1.1)	47 (±5.8)	7 (±7.8)	72 (±3.0)
DC21	14 (±4.8)	63 (±13.1)	9 (±5.3)	11 (±4.4)	6 (±4.3)	116 (±3.7)	12 (±1.9)	76 (±2.5)
DC22	X	X	24 (±6.5)	68 (±13.0)	32 (±6.1)	107 (±4.5)	18 (±1.3)	59 (±5.5)

Table 24 continued.

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
DC24	32 (±2.7)	93 (±12.2)	7 (±2.4)	55 (±5.8)	- (±3.7)	- (±7.8)	12 (±2.1)	96 (±3.1)
DC25	36 (1.6)	77 (±6.2)	2 (±1.0)	33 (±6.0)	20 (±4.8)	102 ±3.8)	12 (±1.5)	59 (±9.7)
DC26	6 (±3.8)	70 (±12.4)	0 (± 1.1)	12 (±4.5)	13 (±2.1)	108 (±4.3)	3 (±0.9)	61 (±10.1)
DC27	47 (±6.6)	29 (±5.8)	4 (± 0.2)	18 (±11.1)	10 (±1.6)	94 (±11.5)	11 (±1.8)	73 (±2.2)
DC29	X	X	15 (± 2.4)	67 (±7.5)	10 (±2.5)	97 (±4.9)	15 (±4.3)	73 (±6.8)
DC32	X	X	- (±5.6)	- (±8.9)	26 (±3.1)	86 (±2.5)	38 (±9.1)	90 (±13.4)
DC33	X	X	- (±7.1)	- (±9.6)	16 (±3.7)	106 (±9.8)	27 (±2.9)	79 (±12.0)
DC34	13 (±3.0)	54 (±10.4)	15 (±5.7)	20 (±10.0)	5 (±2.0)	108 (±5.1)	8 (±1.7)	91 (±7.5)
DC35	- (±6.7)	- (±11.3)	26 (±1.5)	85 (±3.4)	- (±13.3)	- (±11.3)	32 (±8.8)	55 (±10.5)
DC36	41 (±0.4)	74 (±12.5)	8 (±2.0)	35 (±3.1)	23 (±1.2)	104 (±8.6)	6 (±1.6)	62 (±12.6)
DC37	- (±6.5)	- (±11.4)	16 (±2.1)	49 (±7.8)	- (±5.6)	- (±9.1)	10 (±4.4)	63 (±11.9)
DC38	34 (±7.5)	41 (±7.5)	2 (±0.7)	22 (±11.5)	7 (±3.0)	47 (±2.4)	10 (±2.7)	64 (±5.4)
DC39	56 (±3.7)	39 (±3.6)	47 (±6.8)	36 (±4.1)	13 (±5.4)	72 (±7.9)	12 (±1.1)	84 (±3.5)
DC42	- (±7.3)	- (±6.7)	15 (±2.4)	64 (±12.6)	8 (±1.0)	54 (±3.6)	24 (±3.7)	87 (±8.4)

Mature (24-hour-old) *S. aureus* biofilms were more resistant to the novel antimicrobial agents than the immature (8-hour-old biofilms). Indeed, 15 of the 42 agents tested had no effect on the mature *S. aureus* biofilms (Table 25).

Only five of the novel antimicrobial agents had a significant effect on mature *S. aureus* biofilms when used at the planktonic MIC (Table 25). The addition of these five agents to mature biofilms resulted in a significant decrease in the percentage of viable cells, from 60% in the control biofilms to between 20% and 40%. In general the biomass of the *S. aureus* strains NCTC 13143, 10442 and 12973 was not significantly affected by antimicrobial addition. However, the *S. aureus*

strain NCTC 11939 showed a significant, 30%, reduction in biomass when treated with three of the five antimicrobial agents.

When the higher concentrations (ten times the planktonic MIC) were used, 22 antimicrobial agents had a significant effect on the percentage of viable cells and the total biomass of mature *S. aureus* biofilms (Table 26). All caused a significant reduction in the percentage viability from 60% to less than 35%, with six being reduced to less than 21% viability for all strains. The biomass of mature biofilms produced by the *S. aureus* strains NCTC 13143, 10442 and 12973 was not significantly different from the control biofilm biomass for any of the antimicrobial treatments, apart from those biofilms treated with the antimicrobial, DC17, which caused a significant reduction in biomass of 30% for NCTC 13143 and 12973, and 60% for the *S. aureus* strain NCTC 11939.

Table 25 *The efficacy of novel antimicrobial agents active at the planktonic MIC on mature (24-hour-old) S. aureus biofilms.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
DC05	18 (±3.8)	81 (±5.3)	36 (±6.4)	124 (±8.1)	34 (±9.2)	117 (±12.3)	40 (±3.0)	127 (±8.0)
DC14	36 (±11.0)	68 (±12.6)	25 (±4.1)	77 (±3.9)	24 (±5.4)	114 (±13.5)	29 (±6.8)	91 (±6.3)
DC17	37 (±13.6)	67 (±2.9)	13 (±2.8)	102 (±12.1)	13 (±6.9)	100 (±13.2)	41 (±2.3)	69 (±9.9)
DC21	41 (±6.4)	68 (±5.4)	9 (±1.8)	105 (±7.4)	34 (±1.1)	76 (±6.7)	27 (±4.4)	104 (±12.9)
DC27	27 (±4.5)	109 (±12.1)	37 (± 7.5)	94 (±11.4)	- (±4.7)	- (±9.3)	- (±5.9)	- (±10.0)

Table 26 *The efficacy of the Syngenta chemicals in affecting the total cell number and the percentage of viable cells of mature 24-hour-old biofilms at ten times the MIC.*

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
DC02	- (±4.9)	- (±11.3)	30 (±3.0)	77 (±6.8)	15 (±2.3)	99 (±13.5)	27 (±1.9)	99 (±3.9)
DC05	18 (±5.0)	87 (±4.3)	17 (±6.6)	112 (±11.7)	19 (±1.8)	104 (±7.6)	26 (±6.7)	108 (±2.3)
DC06	- (±7.6)	- (±8.2)	28 (±9.2)	104 (±6.8)	40 (±8.4)	106 (±9.5)	30 (±2.7)	104 (±9.5)
DC07	21 (±6.2)	58 (±8.9)	4 (±2.2)	115 (±4.0)	18 (±5.5)	102 (±2.7)	16 (±5.2)	106 (±5.0)
DC11	X	X	63 (±7.1)	100 (±11.4)	11 (±0.2)	13 (±11.5)	39 (±6.5)	87 (±4.1)
DC12	17 (±2.0)	81 (±3.0)	8 (±6.4)	109 (±10.9)	17 (±6.5)	115 (±8.7)	21 (±2.5)	97 (±8.4)
DC13	38 (±5.2)	75 (±5.9)	64 (±8.4)	100 (±8.7)	32 (±3.6)	110 (±7.4)	20 (±7.7)	82 (±2.0)
DC14	10 (±3.9)	73 (±5.3)	10 (±4.1)	81 (±9.6)	18 (±9.8)	108 (±4.1)	16 (±6.0)	83 (±2.9)
DC17	21 (±1.0)	37 (±3.4)	14 (±1.4)	67 (±3.9)	11 (±3.5)	105 (±6.2)	22 (±3.3)	68 (±3.5)
DC18	X	X	37 (±1.3)	119 (±4.7)	39 (±2.2)	118 (±11.9)	26 (±5.0)	90 (±4.8)
DC19	X	X	14 (±1.8)	120 (±4.4)	25 (±6.1)	101 (±5.6)	30 (±1.8)	106 (±3.3)
DC20	43 (±11.0)	70 (±5.2)	13 (±1.1)	120 (±3.2)	18 (±3.5)	104 (±11.9)	19 (±1.3)	86 (±4.1)
DC21	35 (±0.3)	37 (±9.3)	8 (±3.1)	102 (±13.5)	26 (±2.0)	86 (±5.4)	23 (±7.5)	105 (±10.5)

Table 26 continued:

	NCTC 11939		NCTC 13143		NCTC 10442		NCTC 12973	
	% V	B	% V	B	% V	B	% V	B
DC22	X	X	15 (±1.6)	101 (±3.0)	36 (±5.3)	108 (±3.5)	31 (±7.2)	102 (±8.0)
DC24	26 (±2.8)	64 (±3.8)	22 (±2.2)	92 (±8.6)	22 (±7.9)	119 (±12.1)	13 (±1.3)	121 (±3.1)
DC26	9 (±3.9)	98 (±3.1)	15 (±1.5)	71 (±12.0)	8 (±2.9)	108 (±7.8)	12 (±4.2)	106 (±7.0)
DC27	30 (±2.0)	100 (±8.9)	25 (±5.6)	79 (±11.5)	- (±4.3)	100 (±10.7)	- (±3.6)	- (±9.8)
DC29	X	X	21 (±9.3)	106 (±3.8)	25 (±1.2)	104 (±5.0)	20 (±1.4)	106 (±3.7)
DC34	- (±5.4)	- (±7.6)	13 (±3.9)	87 (±11.6)	21 (±5.4)	104 (±10.3)	20 (±5.6)	121 (±12.3)
DC36	34 (±1.7)	82 (±10.6)	7 (±3.6)	108 (±5.8)	32 (±9.4)	103 (±13.6)	29 (±4.5)	97 (±4.4)
DC38	21 (±1.4)	78 (±2.9)	8 (±1.0)	109 (±7.5)	15 (±5.4)	102 (±5.5)	34 (±2.1)	97 (±7.0)
DC39	28 (±6.8)	117 (±7.7)	38 (±8.5)	113 (±12.2)	26 (±3.1)	101 (±10.6)	16 (±3.3)	87 (±8.9)

7.4 Discussion

For the majority of the novel antimicrobial agents very little can be said about their activity, as their mode of action is unknown and they were completely novel structures unrelated to any existing antimicrobial agents (Syngenta – personal communication). However, for five of these chemicals (DC05, DC14, DC17, DC21 and DC27) it is important that further work is undertaken as they had significant effects at the planktonic MIC in reducing the biomass or percentage viability of mature biofilms. Apart from the ionophores tested in this investigation, to this author's knowledge no other antibiotics have been shown to have such a large effect on mature *S. aureus* biofilms at this low concentration.

The antimicrobial agents DC14 and DC17 (Figure 43 – Appendix C) are related compounds; they have some structural similarity to PIRATE insecticide, which has an uncoupler mode of action. However, DC17 has been tested by Syngenta in an uncoupler assay on cockroach mitochondria and was found to be inactive (Syngenta – personal communication), indicating that also, although structurally similar to the insecticide Chlorfenapyr (a.k.a PIRATE), they do not share its mode of action. Also, both had moderate to high toxicity against the Sf21 cell line (an insect cell line derived from the lepidopteran moth species, *Spodoptera frugiperda*, which indicates basic cellular toxicity, such as respiration or metabolism).

The antimicrobial agents DC21 and DC27 (Figure 44 – Appendix C), also had moderate to high toxicity against the Sf21 cell line; however, no information is available about their modes of action.

However, for a small number of the active antimicrobial agents tested, limited information is available; consequently, these agents can now be discussed.

DC03, DC41 and DC42 derivatives of diarylurea

Three of the novel antimicrobial chemicals tested (DC03 (Figure 45 – Appendix C), DC41 and DC42) were related to the diarylureas (DAU) (Figure 45 – Appendix C). The DAUs were discovered in a screening process designed to find compounds that would inhibit the *S. aureus* alpha-toxin and the electron transport chain. Many electron transport inhibitors are too toxic to consider using in humans. However, Proctor *et al.* (2002) studied two DAU compounds in which the effective dose for the inhibition of bacterial respiration was 50 to 3500 times lower than the concentration required to cause similar inhibition of mammalian mitochondrial respiration. Proctor *et al.* (2002) argued that, as human and animal exposure studies have previously shown a lack of acute toxic responses to this group of compounds, exemplified by the related herbicide diuron,

which even at massive oral doses (38 mg kg^{-1}), have proven to be non-fatal (Geldmacher-von *et al.* 1971), the development of drugs based on the DAU compounds would therefore be rational.

The DAU compounds tested by Proctor *et al.* (2002) reduced toxin production in *S. aureus* and reduced the damage caused by *S. aureus* when co-cultured with endothelial cells. This raises the possibility that compounds of the DAU type that inhibit bacterial respiration might prove valuable for the prevention of toxin production in *S. aureus*. Nevertheless, no studies have so far been reported on the possible inhibitory effects of DAU on biofilm formation, which is arguably a more valuable attribute. The MICs determined for the two DAUs studied by Proctor *et al.* (2002) for their *S. aureus* isolate 6850 were between 1 and 2 mg l^{-1}. The DAU derivatives investigated here had higher MICs of between 2 and 16 mg l^{-1}. However, it is difficult to compare these results as they were performed using different *S. aureus* strains and culture conditions.

Proctor *et al.* (2002) did not investigate the effect of the DAUs on initial cell attachment or their effect on preformed biofilm. The work presented here shows that one of the three DAU derivatives did indeed show efficacy against immature but not mature *S. aureus* biofilms. This might suggest that new analogues or new derivatives should be synthesized and evaluated for activity against immature and mature *S. aureus* biofilms.

As the DAU compounds are a novel group, there should be less likelihood that the bacteria would show cross-resistance to drugs that are currently available. However, the planktonic MIC of the *S. aureus* strain NCTC 10442 was already twice or three times as high as the other *S. aureus* strains, suggesting that this strain is already partially resistant.

Proctor *et al.* (2002) found that growth in the presence of the two DAUs caused the *S. aureus* strain 6850 to produce small colony variants (SCV) at a much higher rate than cultures without DAUs. This increased production of SCVs might be problematic since they are more resistant to antibiotics (Chuard *et al.* 1997). If the *S. aureus* strain, NCTC 11939, more readily produces SCVs than the other *S. aureus* strains tested here in the presence of the DAU derivatives, it may also explain why that strain had higher planktonic MICs. Further work would be needed to determine if that was the case.

The development of drugs that are able to reduce the virulence of bacteria, even if they fail to kill pathogens, may allow for the discovery of agents that are unrelated to currently used antibiotics. Also, the compounds decreased the virulence of bacteria at concentrations much lower than that required to kill the bacteria; hence, this allows for lower doses and less toxicity.

Caiazza *et al.* (2003) found that alpha-toxin (encoded by the *hla* gene) is required for biofilm formation by *S. aureus*; *hla* deletion mutants' defective in alpha-toxin production failed to form biofilms under both static and flow conditions. The strains lacking alpha-toxin had an apparent defect in cell-to-cell interactions. This may partially explain the reduction of biomass seen for immature biofilms treated with the DAU derivative (DC42), although cells within the existing biofilm may be unaffected, i.e. new biofilm cells may be unable to form due to the inhibition of alpha-toxin production.

The DAU compounds investigated by Proctor *et al.* (2002) caused a reduction in the electron transport chain activity. Indeed, it is the respiratory activity that the XTT/ PMS assay measures. Therefore, it could be argued that the reduction in viability seen with the addition of DC42 to immature biofilm was not due to its killing effect but due to a reduction in the electron transport chain activity. On the one hand, the biofilm recovery experiments described here, where biofilms were removed from the surface, homogenized and subsequently grown on agar, showed lower viability, supporting the proposal that DC42 kills cells. On the other hand, it could also be argued that the cells with reduced electron transport chain were viable but non-culturable. Consequently, further work using animal infection models would have to be performed to determine if this was the case.

DC28, DC29 and DC30 putative inhibitors of FabI

Three of the novel antimicrobial agents (DC28, DC29 and DC30) have a similar mode of action to triclosan, the diazaborines (a class of heterocyclic antimicrobial agents) and isoniazid. All three inhibit the FabI enzyme (McMurry *et al.* 1998) or, in the case of isoniazid, a mycobacterial FabI homolog (termed InhA) (Banerjee *et al.* 1994; Rozwarski *et al.* 1998). Although the three novel antimicrobial agents share their mode of action with the above antibiotics, they are not structurally related to any of them (Syngenta – Personal communication, active project - structures not shown).

FabI, encoded by the enoyl-acyl-carrier-protein (ACP) reductase gene (*fabI*), is one of the key enzymes in the bacterial fatty acid biosynthetic cycle (Bergler *et al.* 1996; Heath *et al.* 1995). Fatty acids are necessary for reproducing and building cell membranes. Humans do not have an enoyl-ACP reductase enzyme, so are not affected.

Triclosan, the diazaborines and isoniazid all bind to the enoyl-ACP reductase enzyme, increasing the enzyme's affinity for NAD^+, which results in the formation of a stable ternary complex of FabI-NAD^+-antibiotic, which is unable to participate in fatty acid synthesis (Baldock *et al.* 1998; Banerjee *et al.* 1994; Heath *et al.* 1999; Levy *et al.* 1999; Stewart *et al.* 1999).

Bacterial fatty acid biosynthesis is carried out by a universal series of reactions catalyzed by a collection of enzymes each encoded by a separate gene (Cronan *et al.* 1996; Rock *et al.* 1996). Fatty acids are assembled two carbon units at a time in a cyclical sequence of reactions. There are four basic reactions that constitute a single round of elongation. The first step is the condensation of malonyl-ACP with either acetyl-CoA to initiate fatty acid synthesis (FabH) or with the growing acyl chain to continue cycles of elongation (FabB or FabF). Then the β-ketoacyl-ACP is reduced by an NADPH-dependent β-ketoacyl-ACP reductase (FabG). Only a single enzyme is responsible for this step (Zang *et al.* 1998). There are two β-hydroxyacyl-ACP dehydrases (FabA and FabZ) capable of forming *trans*-2-enoyl-ACP. The product of the *fabA* gene is specifically involved in the introduction of a *cis* double bond into the growing acyl chain at the β-hydroxydecanoyl-ACP step and most efficiently catalyzes dehydration of short-chain β-hydroxyacyl-ACPs, whereas the FabZ dehydratase has a broader substrate specificity (Heath *et al.* 1996)). FabI catalyzes the last reaction in each elongation cycle; it therefore plays a regulatory role in determining the rate of fatty acid synthesis (Heath *et al.* 1995; 1996).

Triclosan has a broad spectrum of antibacterial activity and is effective against both Gram-negative and Gram-positive organisms. Since its introduction in the 1960s, it has been used variously as an antiseptic additive in a wide range of consumer goods, including cutting boards, mattress pads, facial cleansers, and hand soaps (Bhargava *et al.* 1996). However, although these antimicrobial agents had good activity against staphylococci, they were not active against streptococci or enterococci (Syngenta – personal communication). A possible reason for this resistance is due to the presence of a fabI homologue, either fabK or fabL, which are insensitive.

For Gram-positive species, resistance to triclosan is mediated through the over-expression of wild-type FabI, increasing the resistance (MIC) 30-fold over wild-type strains. McMurry *et al.* (1998) observed that in *E. coli*, in which wild-type *fabI* was expressed on a multicopy plasmid, resistance to triclosan increased 16 to 32-fold. In some Gram-negative organisms such as *P. aeruginosa* and *E. coli*, resistance to triclosan can also be attributed to efflux by the MexAB-OprM or AcrAB systems rather than to over-expression of FabI (McMurry *et al.* 1998 1; Hoang *et al.* 1999).

In 1998, triclosan was recommended for the control of MRSA, after being successfully used to control MRSA outbreaks in a neonatal nursery (Zafar *et al.* 1995) and a cardiothoracic surgical unit (Brady *et al.* 1990), and to provide an alternative to expensive vancomycin administration (Webster *et al.* 1994).

Limited information is available for the effect of triclosan on *S. aureus* biofilms. Jones *et al.* (2006) showed that triclosan was capable of preventing biofilm formation by *S. aureus* on the surface of catheters, but did not test triclosan against mature preformed biofilms. However, the results from some recent studies conclusively demonstrated that long-term use of oral care formulations containing triclosan reduced gingival plaque, an oral biofilm, and gingivitis gum disease (Sreenivasan *et al.* 2002); although, it was unclear whether this was through the prevention of new biofilm formation or an effect of triclosan on preformed plaque biofilms. However, McBain *et al.* (2003) concluded that the long-term exposure of domestic-drain biofilms to triclosan did not affect bacterial vitality i.e. the agent is bacteriostatic.

Therefore, the present study has sought to determine whether novel antimicrobial agents with a similar mode of action have even greater anti-biofilm effects. The data indicate that only one of the three derivatives was active against both immature and mature biofilms but only at ten times the planktonic MIC.

The MIC of triclosan was determined by Suller *et al.* (2000) for 33 *S. aureus* (with some MRSA) strains, and ranged between 0.025 and 1 mg l^{-1}. However, some *S. aureus* strains have emerged that exhibit low-level resistance to triclosan (2-4 mg l^{-1}) (Cookson *et al.* 1991). The *S. aureus* strain NCTC 11939 was resistant (MIC > 32 mg l^{-1}) to all three novel antimicrobial agents (DC28, DC29 and DC30). The MIC of DC28 for the other *S. aureus* strains was between 0.25 and 0.5 mg l^{-1}, which is comparable to the planktonic MIC of triclosan. The other two novel chemicals, DC29 and C30, had MICs of between 1 and 8 mg l^{-1}. However, it was DC29 that was active against the mature biofilm phenotype, albeit at ten times the planktonic MIC.

The recent discovery of platensimycin, a previously unknown class of antibiotics produced by *Streptomyces platensis* by Wang *et al.* (2006), has renewed interest in the fatty acid pathway as a target. Platensimycin demonstrates strong, broad-spectrum activity and was particularly active against MRSA, VISA and VRE, and indeed treatment with platensimycin eradicated *S. aureus* infection in mice. Platensimycin is the most potent inhibitor reported for β-ketoacyl-(acyl-carrier-protein (ACP)) synthase I/II (FabF/B), also found in the synthetic pathway of fatty acids.

The ubiquitous occurrence of the fatty acid synthase systems in bacteria and the essential nature of the FabI reaction make this enzyme an attractive target for antibacterial drugs. The design and development of second-generation FabI inhibitors, based on their ability to form ternary FabI-NAD^+-drug complexes, will supplement the arsenal against a broad spectrum of bacteria (Heath *et al.* 1999).

DC15 and DC16 derivatives of tiamulin

Two of the novel antimicrobial agents (DC15 and DC16; Figure 46, Appendix C) were derivatives of tiamulin. Tiamulin is a semi-synthetic derivative of the natural antibiotic pleuromutilin (Egger *et al.* 1976 and Kavangh *et al.* 1951), a natural product of *Clitopilus scyphoides*, an edible mushroom. It consists of a tri-cyclic nucleus composed of a cyclo-pentanone, cyclo-hexyl and cyclo-octane, and a (((2-(diethylamino)ethyl)thio)-acetic acid) side-chain on C14 of the octane ring. This side-chain differs for all antibiotics of the pleuromutilin group.

Tiamulin is a prokaryotic ribosome inhibitor; it binds tightly to the 50S ribosomal subunit, exclusively binding to domain V of the 23S RNA at the peptidyl transferase site. By occupying the peptidyl transferase site, it sterically hinders the correct positioning of the tRNAs for peptide transfer, directly inhibiting peptide bond formation. Both analogues showed good activity in an *in vitro* transcription / translation assay (Syngenta – Personal communication). The *in vitro* transcription translation assay works by incubating a plasmid, encoding the gene for a reporter (β-galactisidase) along with the S30 cell extract from *E. coli,* which contains all the necessary proteins for protein biosynthesis (RNA polymerase, tRNAs and ribosomes etc). This supports the idea that the mode of action of the two tiamulin derivatives is against protein biosynthesis and, therefore, likely to be the same as tiamulin.

The activity of tiamulin is bacteriostatic and largely confined to Gram-positive bacteria, such as streptococci and staphylococci, and also highly active against *mycoplasmas*. The range of MIC against streptococci and staphylococci is between 0.125 and 0.15 mg l^{-1}. However, the average planktonic MIC for the *S. aureus* strains tested with the tiamulin derivatives was 1 mg l^{-1}, 8 to 10 times higher than tiamulin itself. Also, the *S. aureus* strain NCTC 11939 was planktonically resistant to both agents.

So far, pleuromutilin derivatives are not used in human medicine, but tiamulin is used for the treatment, control and prophylaxis of swine dysentery, swine pneumoniae and mycoplasma diseases in swine and poultry. However, the increasing number of pathogens resistant to common antibiotics has raised a new interest in pleuromutilin derivatives, which may be suitable for human therapy (Schlünzen *et al.* 2004). Indeed, GlaxoSmithKline (GSK) have 3 compounds from the pleuromutilin class in their current development pipeline; one topical pleuromutilin (retapamulin) has been submitted as a New Drug Application (NDA) to the FDA in 2005 and the European equivalent MAA (Marketing authorization application) in 2006. They also have two oral pleuromutilins, 565154 and 742510, currently in phase I clinical trials, where an evaluation of their clinical pharmacology will be tested in volunteers before moving on to phase II, where dose

determination and efficacy trials will be carried out (http://www.gsk.com). For this reason the two derivatives of tiamulin are of great interest.

Jones *et al.* (2001B) found tiamulin to be active against 250 *S. aureus* strains growing planktonically with an MIC of 1 mg l^{-1}. The *S. aureus* strain NCTC 11939 was resistant to both the tiamulin derivatives tested here. However, the three other *S. aureus* strains had planktonic MICs of 0.5 to 2 mg l^{-1}, comparable to tiamulin itself.

The novel antimicrobial agent DC16 had some activity against the immature non-EMRSA biofilms, but at the higher concentrations. To this author's knowledge, tiamulin has not been tested against biofilms of any bacterial species. Therefore, it is important that efficacy of tiamulin itself is determined against the *S. aureus* biofilm phenotype, as it may be more effective than its derivatives.

DC05, an oxazolidinone derivative

The oxazolidinones (linezolid and eperezolid) represent a new class of antimicrobial agents, which are active against multidrug-resistant staphylococci, streptococci, and enterococci (Brickner *et al.* 1996). After first being identified as prospective antimicrobial agents in 1987 (Slee *et al.* 1987), oxazolidinones were abandoned for some time due to their high toxicity. Later on, new derivatives with superior pharmacological properties were found (Brickner *et al.* 1996; Ford *et al.* 1996), and recently one of the oxazolidinone antibiotics, linezolid, has been approved for clinical use. One of the novel antimicrobial agents was a derivative of the oxazolidinones (DC05; Figure 47, Appendix C).

Oxazolidinones inhibit protein synthesis by binding the ribosomal 50S subunit, and they have no affinity to the 30S subunit. Oxazolidinones compete with chloramphenicol and lincomycin for the binding of the 50S subunit, which indicates that they have close binding site, even though oxazolidinones do not inhibit peptidyl transferase like chloramphenicol and lincomycin (Lin *et al.* 1997). It has also been reported that oxazolidinones do not inhibit formation of fMet-tRNA or elongation or termination step (Shinabarger *et al.* 1997). Although the binding site of the oxazolidinones is on the 50S subunit, Swaney *et al.* (1998) recently demonstrated that oxazolidinones inhibit formation of the initiation complex, which is composed of 30S subunit, fMet-tRNA, mRNA, GTP, and initiation factors 1-3. Oxazolidinones inhibit fMet-tRNA binding to the P site (Aoki *et al.* 1997; Bobkova *et al.* 2003). The effect of oxazolidinones binding to the 50S subunit is inhibition of 70S formation. If the 70S is already formed, binding of oxazolidinones inhibits translocation of the peptide chain to the P site from the A site during formation of the peptide bond (Bozdogan *et al.* 2004). DC05 had potent

activity in an *in vitro* transcription / translation assay (Syngenta – personal communication). It is therefore likely that this antimicrobial agent's mode of action is against protein biosynthesis, and acts in a similar, if not identical, way to the oxazolidinones.

Resistance to other protein synthesis inhibitors does not affect oxazolidinone activity. However, rare development of oxazolidinone resistance cases, associated with 23S rRNA alterations during treatment, have been reported. Most mutations defined in various species associated with linezoid resistance occur by G to U substitution in the peptidyl transferase centre of 23S rRNA at position 2576 (Auckland *et al.* 2002; Prystowsky *et al.* 2001). This base is in the proximity of the P site, which confirms the mechanism of action.

The planktonic MIC of the antimicrobial chemical, DC05, for the *S. aureus* strains used in this investigation ranged between 0.125 and 2 mg l^{-1}. This is consistent with the findings of Bozdogan *et al.* (2004) and El-Azizi *et al.* (2005), who both found the planktonic MIC of linezolid to be approximately 2-4 mg l^{-1} for both methicillin resistant and susceptible *S. aureus* strains. El-Azizi *et al.* (2005), who also measured biofilm viability using an XTT assay, also found that MSSA and MRSA biofilms treated with linezolid, even up to concentrations as high as 1000 mg l^{-1}, were resistant and maintained equal viability to control biofilms. However, Curtin *et al.* (2003), who looked at *S. epidermidis* biofilms in a modified Robbins device, found that the addition of linezolid at much higher concentrations of 2 mg ml^{-1} reduced biofilm viability to zero after 24 hours treatment. Similarly, *S. epidermidis* biofilms treated with high concentrations (4 mg ml^{-1}) of eperezolid yielded no viable cells after 168 hours.

However, the oxazolidinone derivative tested in this work (DC05) was highly effective at disrupting both immature and mature biofilms of all four *S. aureus* strains used, at both the planktonic MIC and ten times the MIC, much lower concentrations than linezoid.

The oxazolidinones are a relatively new class of antimicrobials and it is therefore hoped that many of their derivatives will be active against the *S. aureus* biofilm phenotype and will go on to clinical trial.

DC10 and DC11 analogues of rifampicin

Within the library, there were two antimicrobial agents that were analogues of rifampicin (DC10 and DC11, Figure 48 – Appendix C). Rifampicin or rifampin is a bacteriocidal antibiotic of the ansamycin class of antibiotics, characterised by a long aliphetic bridge connecting two non-adjacent positions of an aromatic nucleus. It is a semi-synthetic compound derived from

Streptomyces mediterranei. It is typically used to treat *Mycobacterium* infections, including tuberculosis and leprosy, and also has a role in the treatment of MRSA (Heinz *et al.* 2005).

Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding its β subunit, thus preventing transcription of messenger RNA (nRNA) and subsequent translation to proteins. Eukaryotic enzymes are 10^2 to 10^4 times less sensitive to inhibition by rifampicin than prokaryotic RNA polymerases (Hartmann *et al.* 1968).

By far the predominant mechanism of resistance to rifamycins is modification of the drug target by mutation, *rpoB*, which codes for the β subunit. Resistance by modification of the antibiotic (inactivation) has also been described, but does not seem to be of high clinical significance (Heinz *et al.* 2005). Pathogens develop resistance to rifampicin at a high rate. This is the reason the antibiotic is used almost exclusively in drug combinations, and why its use is restricted to the treatment of tuberculosis or clinical emergencies (Campbell *et al.* 2001).

Indeed, the *S. aureus* strain NCTC 10442 was planktonically resistant to rifampicin; the other three *S. aureus* strains had MICs of between 0.01 and 0.5 mg l^{-1}. However, all the *S. aureus* strains were susceptible to the novel rifampicin analogues apart from NCTC 11939, which was planktonically resistant to DC11. The MIC of DC10 for the *S. aureus* strains was low (0.03 mg l^{-1}), and a little higher (2-4 mg l^{-1}) for the novel agent DC11.

The efficacy of rifampicin in treating bacteria adhered to biomaterials has been broadly demonstrated *in vitro* (Saginur *et al.* 2006 50) and in clinical trials (Marciante *et al.* 2003 31). Rifampicin was previously tested in the present study against immature and mature *S. aureus* biofilms (Chapter 6). Treatment with rifampicin on immature biofilms caused a significant reduction in both biomass and the percentage of viable cells at both the planktonic MIC and ten times the MIC. However, it did not have any effect on mature *S. aureus* biofilms. Furthermore, both of the novel antimicrobial agents were active against the *S. aureus* immature biofilm phenotype; DC11 was active at both concentrations and DC10 only at the higher concentration of ten times the planktonic MIC. However, DC11 was also active against the mature biofilm phenotype (at ten times the planktonic MIC), meaning that it has greater activity against the mature biofilm phenotype than rifampicin itself. This suggests that there may be other rifampicin analogues that have even more activity against mature biofilms that need to be tested.

Significantly, Gualtieri *et al.* (2006) recently demonstrated that a new relatively hydrophobic rifampicin derivative (SB13) with high antistaphylococcal (planktonic) activity, in the micromolar range, was also highly active against *S. epiermidis* biofilms. Its effects were fast and kinetically

related to those of rifampicin, but unlike rifampicin, which triggers the appearance of resistance due to point mutation at the surface of the polymerase, SB13 did not select for resistant bacteria. It would be interesting to determine if this was also the case with the rifampicin analogues used here.

However, rifampicin itself has many other adverse effects, which are chiefly related to the drug's hepatotoxicity. Patients receiving rifampicin often undergo liver function tests, including aspartate aminotransferase (AST). Rifampicin is an inducer of hepatic cytochorme P450 enzymes (such as CYP2D6 and CYP3A4) and will increase the metabolism of a many drugs that are cleared by the liver through this enzyme system. This results in numerous drug interactions. Therefore, analogues of rifampicin may also have similar or more severe adverse effects, which will have to be determined before further research into their antimicrobial activity is continued.

In view of the increasing resistance of *S. aureus* to multiple antibiotics, it is absolutely essential to find drugs aimed at novel susceptible targets. The majority of the work has focused on bypassing the existing resistance genotypic markers, such as MecA. Nevertheless, the present study has shown the critical importance of the biofilm phenotype in resisting antimicrobial agent efficacy. However, assessment of the novel antimicrobial library indicated that several of the novel compounds were as effective against the biofilm phenotype as the planktonic phenotype. This is significant and encouraging, and should stimulate further development of such agents to combat not only emerging 'superbugs' but also more common infections that rely on biofilm formation (Table 4).

8. INFLUENCE OF THE *AGR* OPERON AND AI-2 SIGNALLING ON MRSA BIOFILM STRUCTURE AND ANTIMICROBIAL RESISTANCE

8.1 Introduction

The previous work has demonstrated that *S. aureus* biofilms are highly resistant to disinfectants and antibiotics. Although the ionophore antibiotics, both alone or in combination with antibiotics of differing modes of action, had significant effects on mature *S. aureus* biofilms at low concentrations, none were able to completely remove or kill mature *S. aureus* biofilms; and, while chapter 7 has shown some promising novel antimicrobial agents, active against mature *S. aureus* biofilms, it will conceivably be years before these compounds are available for therapeutic use.

Other workers have suggested that the biofilm resistance may be primarily due to the biofilm phenotype rather than the EPS acting as a diffusion barrier or reduced cell growth. For the former case, the evidence suggested that QS plays an important role in biofilm formation and *in vivo* pathogenicity (Otto 2004). In staphylococci, there are two possible QS systems. The accessory gene regulator (*agr*) is genus specific and uses a post-translationally modified low molecular weight peptide as an autoinducing signal, (autoinducing peptide - AIP). In the pathogens *S. aureus* and *S. epidermidis*, agr controls the expression of a series of toxins and virulence factors and the interaction with the innate immune system. However, the role of *agr* during infection is controversial.

A possible second QS system of staphylococci, *luxS*, is found in a variety of Gram-positive and Gram-negative bacteria. AI-2, a novel furanosyl borate diester consisting of two-fused five membered rings, bearing little or no resemblance to other characterised autoinducers (Chen *et al.* 2002), is the signalling molecule encoded by *luxS* system. AI-2 has been proposed to be the ultimate 'Universal' signal for inter-species communication (Surette *et al.* 1998). As well as bioluminescence in *Vibrio harveyi*, AI-2 has also been shown to regulate several behaviours in other bacterial species (Federle *et al.* 2003). However, LuxS also plays a distinct role within central metabolism as part of the activated methyl cycle (AMC) to recycle *S*-adenosylhomocysteine, which would otherwise have toxic effects on the cell (Winzer *et al.* 2002; 2002B) and may not be involved in QS at all.

Intriguingly, *S. aureus* contains the *luxS* gene, but there have been no reports on whether the pathogen produces AI-2, nor, indeed, whether this molecule would exhibit modulatory activity on *S. aureus* biofilm structure and physiology.

Therefore, the aim of this investigation was to determine whether AIP exhibited any modulatory activity on biofilm structure and antimicrobial resistance using an *S. aureus agr* deletion mutant; furthermore, to ascertain whether MRSA cultures produce AI-2, and to investigate the effects of exogenous chemically synthesised AI-2 on biofilm formation and resistance.

8.2 Methods

The methods used in this work were as described in chapter 2.

8.3 Results

8.3.1 Biofilms formation by *agr* positive and negative *S. aureus* strains

Previous studies investigating the role of *agr* in biofilm formation have lead to conflicting results, with evidence for both increased and decreased biofilm formation in agr^- mutants. The effect of agr knockouts on biofilm formation appears to be assay specific; therefore, it was important to determine the effect of *agr* deletion on biofilm formation in this biofilm assay. Starter cultures of RN6390B (agr^+) and RN6911 (agr^-) were used to inoculate 200 µl aliquots of $BHI_{25\%}$ + 0.09% (w/v) glucose in microtitre plates, which were incubated at 37°C for 24 hours. The percentage of viable cells within the biofilms was determined, using the XTT / PMS assay procedure (Section 2.13.2), and the total cell number (biomass) by Safranin staining (Section 2.14.1).

The biomass of the RN6390B (agr^+) biofilms was normalised to 100% (blue bars, Figure 36). The number of cells deemed viable within the biofilm was represented as a percentage of that total biomass (black bars, Figure 36). The biofilm biomass and the relevant percentage viability of RN9611 (agr^-) biofilm were therefore expressed as a percentage of the normalised RN6390 (agr^+) biofilm biomass (Figure 36). The biomass of biofilm formed by agr^- *S. aureus* strain, RN9611, was increased by 70% compared to its corresponding agr^+ strain, RN6390B. However, the percentage of viable cells within the biofilm was unaffected, remaining at approximately 60% of the total biomass.

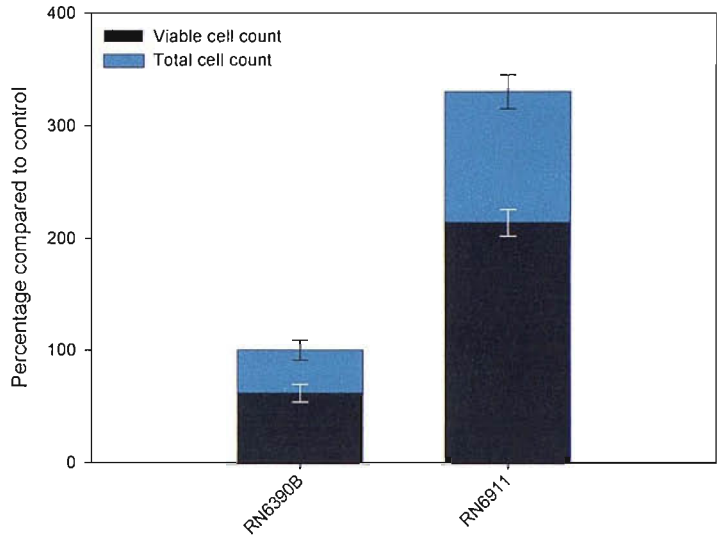


Figure 36 *Biofilm formation by the S. aureus strains RN6390B (agr^+) and RN9611 (agr^-).*

8.3.2 The influence of the *agr* operon expression on the resistance of immature and mature MRSA biofilms to several antibiotics

The influence of the *agr* operon was investigated on biofilm resistance against several commonly used antibiotics. *S. aureus* starter cultures (Section 2.9) of RN6390B (agr^+) and RN6911 (agr^-) were used to inoculate 200 µl aliquots of $BHI_{25\%}$ + 0.09% (w/v) glucose in microtitre plate (NuncSurface) wells. The microtitre plates were incubated at 37°C for 8 or 24 hours, for immature and mature biofilms, respectively. After incubation, the planktonic culture phase was aspirated and the biofilms gently washed in 200 µl of dH_2O. Sterile $BHI_{25\%}$ + 0.09% (w/v) glucose (200 µl), containing the antibiotics at their planktonic MIC or ten times the planktonic MIC were replaced, and the microtitre plates were incubated at 37°C for a further 18 hours.

The percentage of viable cells within the biofilms was determined using the XTT / PMS assay procedure (Section 2.13.2), and the total cell number (biomass) by Safranin staining (Section 2.14.1), which was normalised to 100% for untreated control biofilms. The biomass of treated biofilms was given as a percentage of the normalised control biofilm biomass, and the percentage of viable cells for each treated biofilm was given as a percentage of the total biomass of that biofilm. The results are only shown for those treatments that caused a significant difference in biomass or percentage viability (Table 27 and 28); data were the mean of three independent experiments.

Table 27 *The effect of the deletion of the agr operon on the resistance of immature biofilms to antibiotics with differing modes of action*

	RN9611 (*agr*$^-$)		RN6390B (*agr*$^+$)	
Antibiotic treatment	% V	B	% V	B
Control	62 (±4.7)	100 (±9.2)	59 (±2.9)	100 (±10.9)
Ampicillin (1 mg l^{-1})	30 (±8.6)	107 (±10.7)	36 (±8.8)	99 (±5.0)
Ampicillin (10 mg l^{-1})	33 (±5.9)	98 (±5.2)	30 (±8.6)	92 (±3.6)
Vancomycin (20 mg l^{-1})	33 (±4.2)	87 (±9.2)	38 (±4.3)	95 (±6.2)
Gramicidin (40 mg l^{-1})	20 (±6.7)	80 (±5.6)	19 (±2.0)	88 (±3.3)
Monensin (1 mg l^{-1})	18 (±6.5	74 (±10.7)	16 (±7.8)	80 (±5.6)
Monensin (10 mg l^{-1})	10 (±6.7)	44 (±4.6)	14 (±3.1)	40 (±6.7)
Nigericin (0.1 mg l^{-1})	8 (±2.1)	44 (±8.9)	10 (±2.1)	41 (±8.1)
Narasin (0.125 mg l^{-1})	34 (±4.5)	44 (±10.1)	29 (±8.7)	39 (±6.6)
Narasin (1.25 mg l^{-1})	11 (±2.4)	43 (±9.0)	11 (±3.1)	39 (±5.9)
Ciprofloxacin (0.25 mg l^{-1})	24 (±3.0)	105 (±10.2)	23 (±4.1)	99 (±10.2)
Ciprofloxacin (2.5 mg l^{-1})	30 (±6.6)	81 (±8.1)	35 (±4.5)	93(±9.6)
Rifampicin (0.01 mg l^{-1})	22 (±6.1)	88 (±7.9)	26 (±5.2)	97 (±10.0)
Rifampicin (0.1 mg l^{-1})	24 (±3.6)	82 (±7.4)	22 (±4.7)	89 (±8.9)
Novobiocin (0.6 mg l^{-1})	32 (±4.7)	102 (±9.1)	28 (±5.0)	97 (±9.3)
Trimethroprim (80 mg l^{-1})	43 (±6.6)	102 (±10.9)	38 (±4.1)	89 (±7.6)
Erythromycin (0.05 mg l^{-1})	28 (±3.3)	108 (±10.8)	29 (±6.2)	97 (±5.5)
Erythromycin (0.5 mg l^{-1})	15 (±3.9)	54 (±5.1)	14 (±4.4)	60 (±7.5)
Gentamycin (0.5 mg l^{-1})	49 (±6.6)	59 (±6.8)	53 (±4.9)	57 (±5.1)
Tobromycin (5 mg l^{-1})	28 (±4.1)	103 (±6.9)	27 (±5.3)	107 (±8.7)
Clindamycin (1.25 mg l^{-1})	31 (±4.2)	77 (±7.8)	31 (±6.5)	81 (±8.2)
Fusidic acid (1.25 mg l^{-1})	36 (±3.9)	71 (±6.6)	45 (±5.2)	65 (±6.1)

As expected from the previous work presented in this study, many of the antibiotics tested had significant effects, causing reductions in both the percentage viability and biomass of the immature *agr* positive and negative biofilms. However, there were no significant differences in effect of these antibiotics between the *agr*$^+$ and *agr*$^-$ *S. aureus* strains.

Table 28 *The effect of the deletion of the agr operon on the resistance of mature biofilms to antibiotics with differing modes of action*

	RN9611 (*agr*$^-$)		RN6390B (*agr*$^+$)	
Antibiotic treatment	% V	B	% V	B
Control	57 (±8.7)	100 (±8.1)	64 (±6.1)	100 (±7.6)
Ampicillin (10 mg l^{-1})	37 (±4.2)	74 (±8.4)	40 (±5.9)	98 (±7.6)
Gramicidin (40 mg l^{-1})	16 (±2.2)	97 (±9.5)	20 (±3.6)	99 (±9.2)
Monensin (1 mg l^{-1})	14 (±2.6)	101 (±8.7)	11 (±2.5)	89 (±6.4)
Monensin (10 mg l^{-1})	11 (±1.9)	93 (±5.6)	11 (±4.0)	96 (±8.5)
Nigericin (0.01 mg l^{-1})	19 (±3.7)	98 (±8.2)	22 (±5.4)	103 (±10.3)
Nigericin (0.1 mg l^{-1})	8 (±2.2)	60 (±6.4)	9 (±2.3)	64 (±7.1)
Narasin (0.125 mg l^{-1})	25 (±4.0)	95 (±8.6)	31 (±3.9)	105 (±9.9)
Narasin (1.25 mg l^{-1})	12 (±2.9)	77 (±6.3)	17 (±3.3)	72 (±9.4)
Ciprofloxacin (0.25 mg l^{-1})	27 (±4.1)	102 (±10.2)	30 (±4.3)	97 (±10.1)
Ciprofloxacin (2.5 mg l^{-1})	25 (±6.0)	101 (±8.9)	18 (±3.6)	95 (±8.7)
Novobiocin (0.6 mg l^{-1})	32 (±5.8)	107 (±10.8)	36 (±4.4)	100 (±7.7)
Erythromycin (0.5 mg l^{-1})	39 (±6.2)	84 (±7.1)	39 (±3.6)	99 (±7.5)

As previously shown with MRSA (NCTC 10442), EMRSA (NCTC 11939 and 13143) and MSSA, the biofilm percentage viability and biomass of mature RN6911 and RN6390B biofilms, were reduced in the presence of the ionophore antibiotics and high (ten times the planktonic MIC) concentrations of novobiocin. However, RN6911 and RN6390B biofilms were also affected by the addition of ciprofloxacin, at the planktonic MIC, and ampicillin ciprofloxacin and erythromycin at ten times the planktonic MIC (Table 27).

Again there was no significant difference in the patterns of resistance between the *agr* positive and negative strains, regardless of the antibiotic used. This suggests that the *agr* operon is not involved in immature or mature biofilm-specific resistance. It was therefore, important to investigate the effects of AI-2, the proposed universal cell signal, on biofilm formation and resistance.

8.3.3 AI-2 production by MRSA biofilms

luxS is present in the genomes of a wide variety of Gram-negative and Gram-positive bacteria, including *S. aureus*, and every bacterium, the genome of which contains a functional *luxS* gene, has been shown to be capable of producing an activity in planktonic cultures that can be detected by an AI-2–specific *V. harveyi* reporter strain (Federle *et al.* 2003). Therefore, the extent of AI-2

production in MRSA biofilm cultures was determined as previously described (Surette *et. al.* 1999).

The cell free supernatants were obtained for biofilm cultures of the MRSA strains, NCTC 10442, 11939 and 13413; the *E. coli* strain O157, which has previously been shown to produce AI-2; the *E. coli* strain DH5α, an AI-2 deficient mutant; and from planktonic cultures of the *V. harveyi* strains BB120 and BB170, grown in the presence of 5 mM boric acid, (which had no significant effects on planktonic or biofilm growth of the *S. aureus* strains – data not shown). The addition of boric acid to the media was important, since boron is needed as a cofactor for the generation of active AI-2 (Chen et al. 2002).

The cell-free supernatants were tested for the presence of AI-2, by their ability to induce luminescence in the *V. harveyi* BB170 (sensor 1^-, sensor 2^+) reporter strain (Surrette and Bassler 1998). *V. harveyi* was grown for 12 hours at 30°C in a rotary shaker, and then diluted 1:5000 to below the QS threshold concentration. A 90 µl aliquot of this diluted culture was then mixed to a 10 µl volume of each of the cell-free supernatants. Luminescence and absorbance (620 nm) were measured hourly using a Fluostar plate reader (BMG Labtechnologies); luminescence was normalised, using the absorbance measurements, and fold induction of luminescence was calculated. The positive control was 10 µl of *V. harveyi* BB170 supernatant (data not shown); the negative control was mixed with 10 µl of sterile AB medium (Figure 37).

Two different concentrations of the chemically synthesised AI-2 (donated by Professor Shoolingin-Jordan) (2.5 mM – Black bar; 5 mM - Dark grey bar), which has previously been show capable of inducing luminescence in *V. harveyi*, and of having AI-2 activity against *E. coli* O157 (H. Geier - un-published work) were included to show that it could indeed induce luminescence in *V. harveyi*, and therefore has AI-2 activity; also it gives an indication of the amount of AI-2 present in the cell supernatants.

Cell free supernatants of *E. coli* O157 contained a substance, which will be referred to as AI-2, capable of significantly inducing luminescence production by the *V. harveyi* BB170 cultures. For *E. coli* O157, AI-2 production increased exponentially between 2 and 6 hours, after which, the production or the activity of the AI-2 decreased slowly, as shown in the 12 and 24 hours cell free supernatants. Excluding the 12-hour supernatant of MRSA NCTC 10442, all other supernatants of *E. coli* DH5α, MRSA NCTC 10442, NCTC 11939 and NCTC 13143 did not contain any substance capable of inducing luminescence in the *V. harveyi* cultures greater than the negative media control. However, the 12-hour supernatant of MRSA NCTC 10442 was significantly different to the negative media control and appeared to produce a modest concentration of AI-2.

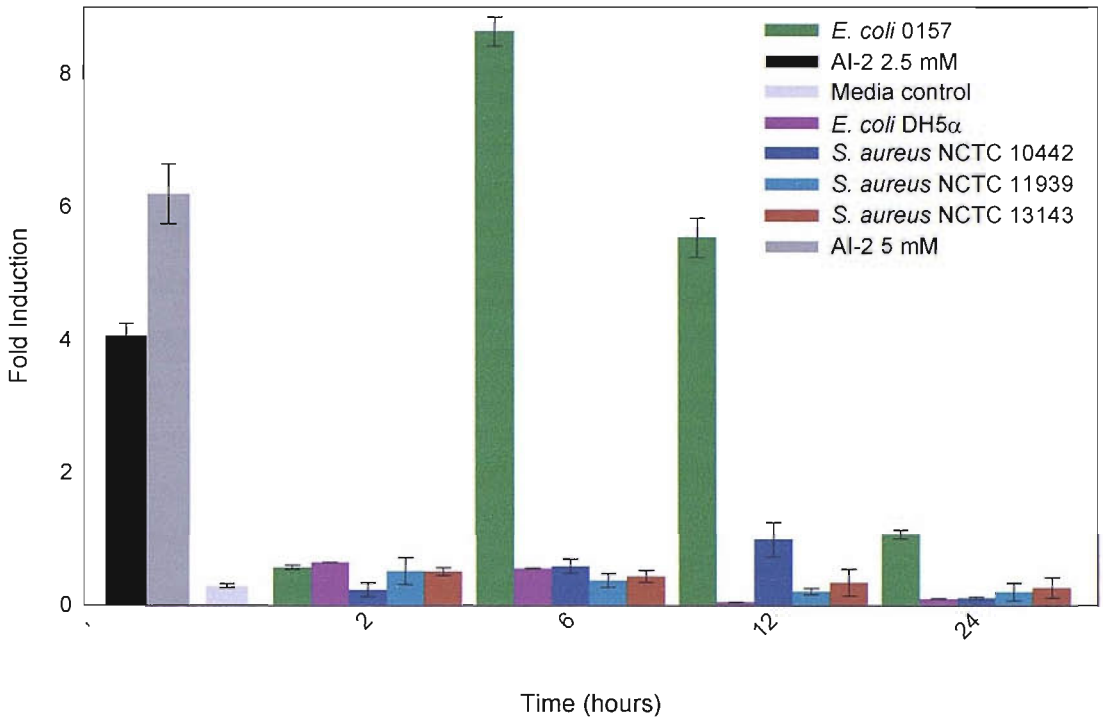


Figure 37 *Autoinducer bioassay examining the AI-2 production in various MRSA and E.coli biofilm cultures. The black and dark grey bars are a positive control, showing that the chemically synthesised AI-2 was capable of activating luminescent production in the V. harveyi culture.*

Although it appeared that MRSA cultures under the tested conditions did not produce noteworthy amounts of AI-2 in biofilm cultures, it has been shown that *S. aureus* strains possess the relevant genes for doing so. Therefore, it was necessary to next determine whether MRSA planktonic or biofilms cultures could respond to the addition of exogenous chemically synthesised AI-2.

8.3.4 The effects on planktonic growth of MRSA cultures grown in the presence of exogenous chemically synthesised AI-2

The effect of exogenous chemically synthesised AI-2 was determined for the planktonic growth of MRSA. Aliquots of sterile $BHI_{25\%}$ + 0.09% (w/v) glucose (10 ml) containing the chemically synthesised AI-2, at concentrations ranging from 0.1mM to 10 mM, were added to MRSA (NCTC 10442) starter cultures (Section 2.9) and incubated with shaking (175 rpm) at 37°C for up to 24 hours. Culture turbidity (620 nm) was measured hourly.

No significant difference in the planktonic growth rate for MRSA (NCTC 10442) cultures grown in the presence of varying concentrations (0.1 – 10 mM) of the chemically synthesised AI-2 was observed (data not shown).

8.3.5 Influence of exogenous AI-2 analogue on biofilm formation

To investigate the effects of the addition of exogenous chemically synthesised AI-2 on the formation of biofilm by MRSA (NCTC 10442), starter cultures (Section 2.9) of MRSA NCTC 10442 were used to inoculate 200 µl aliquots of $BHI_{25\%}$ + 0.09% (w/v) glucose containing varying concentrations (0.1, 2 and 10 mM) of the chemically synthesised AI-2. Biofilms were formed in NuncSurface microtitre plates at 37°C for 6, 12 or 24 hours. Control biofilms were grown without exogenous chemically synthesised AI-2 under the same environmental conditions. The percentage of viable cells within the biofilms was determined, using the XTT / PMS assay procedure (Section 2.13.2); and the total cell number (biomass) was determined by Safranin staining (Section 2.14.1).

The biomass of the mature control biofilms (24 hours-old) was normalised to 100% (blue bars, Figure 38). The number of cells deemed viable within the mature control biofilm was represented as a percentage of that total biomass (black bars, Figure 38). The biofilm biomass and the relevant percentage viability of the immature control biofilms and the AI-2 treated biofilms were therefore expressed as a percentage of the normalised mature control biofilm biomass.

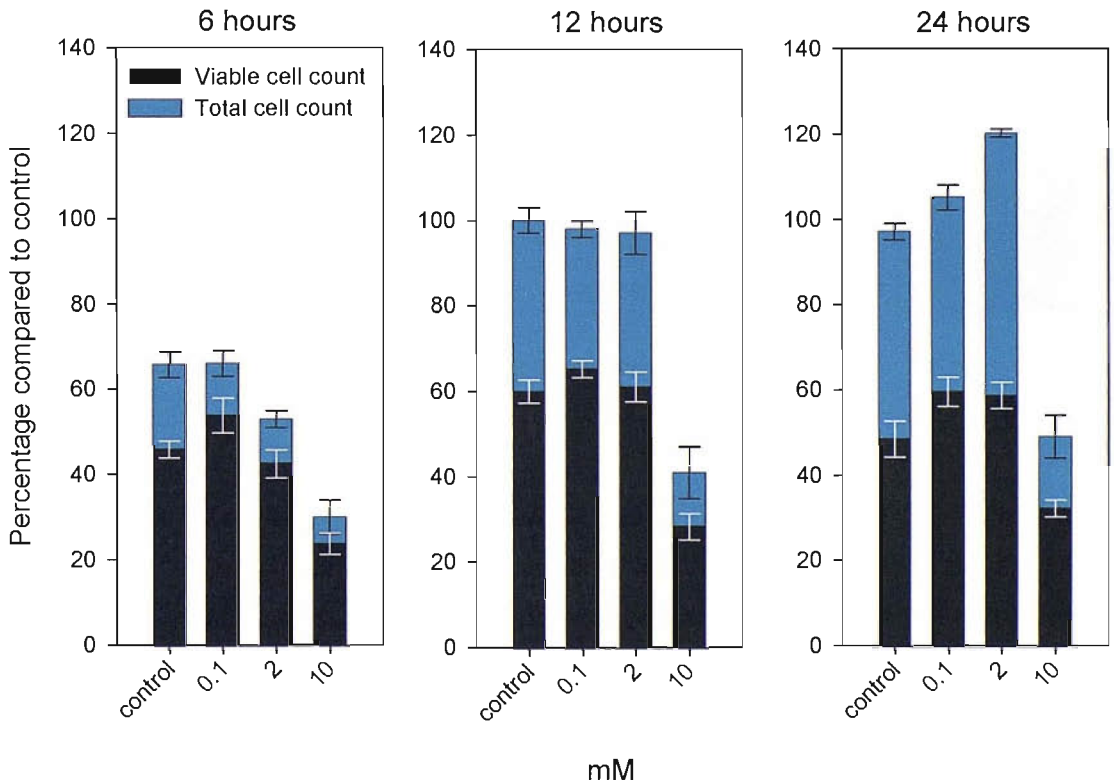


Figure 38 *The effect of the addition of chemically synthesised AI-2 (0.1, 2 or 10 mM) on the formation of MRSA biofilm over 6, 12 and 24 hours. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data were the mean of four experiments.*

The above experiments were repeated with mature (24-hour-old) MRSA (NCTC 10442) biofilms grown on glass chamber slides. EDIC microscopy images were captured of biofilm formed in the presence of 0.1, 2 and 10 mM of chemically synthesised AI-2 (Figure 39).

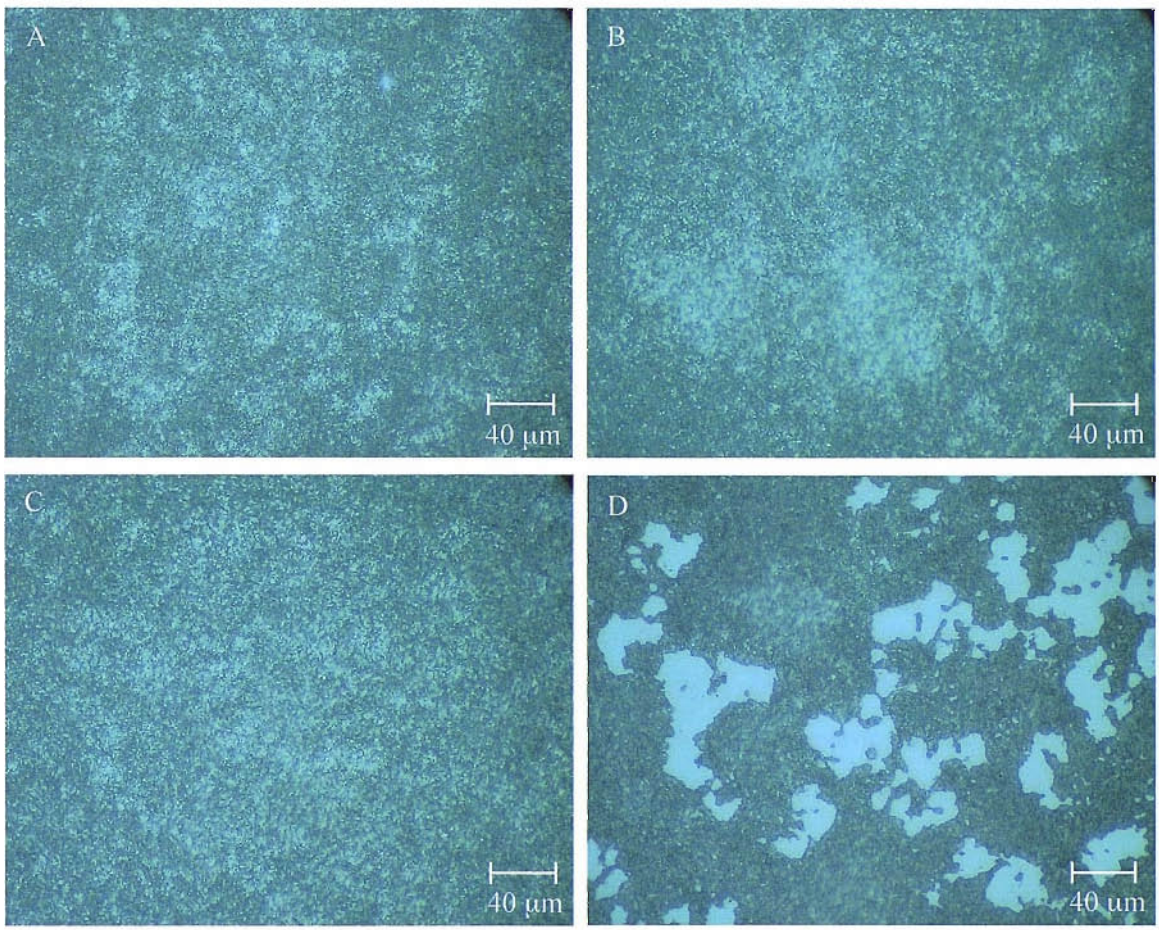


Figure 39 *EDIC microscopy images (x1000) of mature (24-hour-old) MRSA (NCTC 10442) control biofilms (A) and biofilms formed in the presence of chemically synthesised AI-2, at concentrations of 0.1 mM (B), 2mM (C), and 10 mM (D).*

Biofilm biomass increases with age, but the percentage of viable cells within the biofilms decreases with age, from approximately 70% in 6-hour-old biofilms to 55% in 24-hour-old biofilms. This reduction is probably due to oxygen / nutrient limitations, which led to cell death or reduced respiratory activity in the mature biofilms. The addition of the chemically synthesised AI-2 at the lowest concentration of 0.1 mM had no significant effect on biofilm formation over the 24-hour growth period (Figure 40 and 41). The addition of 2 mM AI-2 caused a significant reduction in biomass (approximately 20%) for biofilm formed over six hours, although, after 12-hours biofilm growth, there was no significant difference between these biofilms and the 12-hour control biofilms, either in biomass or percentage viability. Conversely, after 24-hours biofilm growth, biofilms grown in the presence of 2 mM AI-2 analogue showed a significant increase of 20% in

biomass and 15% in the relevant percentage of viable cells, when compared to control biofilms of the same age (Figure 40).

Also, MRSA biofilm formation was significantly affected by the addition of AI-2 at a concentration of 10 mM, resulting in a considerable reduction of biomass (54, 59 and 49%, respectively) in all three ages of biofilm (Figure 38 and 39), while the percentage of viable cells within those biofilms was increased, but not significantly, for all three.

8.3.6 The effect of the addition of varying concentrations of AI-2 analogue on the resistance of mature MRSA biofilms to several antibiotics

The effects of the addition of exogenous AI-2 analogue on biofilm resistance were investigated for several common antibiotics. MRSA (NCTC 10442) starter cultures (Section 2.9) were used to inoculate 200 µl aliquots of $BHI_{25\%}$ + 0.09% (w/v) glucose containing varying concentrations (0.1, 2 and 10 mM) of the AI-2 analogue. Biofilms were formed in NuncSurface microtitre plates at 37°C for 24 hours. After incubation, the planktonic culture phase was aspirated and the biofilms gently washed in 200 µl of dH_2O. Fresh $BHI_{25\%}$ + 0.09% (w/v) glucose (200 µl), containing the various concentrations of the AI-2 analogue, were added back to the appropriate wells, along with high concentrations of antibiotics. These biofilms were incubated at 37°C for a further 18 hours.

Control biofilms were grown in the presence of varying concentrations of exogenous AI-2 analogue without the additional antibiotics. The percentage of viable cells within the biofilms was determined using the XTT / PMS assay procedure (Section 2.13.2) and the total cell number (biomass) by Safranin staining (Section 2.14.1). These results are presented in Table 31; data shows the biomass of biofilms for each treatment as a percentage of the total biomass of the control biofilm, which were normalised to 100%. Also, the percentage of viable cells for each treated biofilm is given as a percentage of the total biomass for that biofilm.

As previously, for control biofilms (Table 29) grown in varying concentrations of exogenous AI-2, biomass was increased for concentrations of 0.1 and 2 mM and decreased for the higher concentration of 10 mM. The percentage of viable cells within biofilms grown in the presence of 0.1 and 2 mM AI-2 remained at around 55%, comparable to the control biofilm, and was increased to approximately 70% in biofilms grown in the presence of 10 mM, AI-2. For mature biofilms grown in control conditions, the addition of various antimicrobials caused reductions in biomass and viability as expected for each different antimicrobial treatment (hypochlorite – Chapter 4; antibiotics – Chapter 6).

For biofilms grown in the presence of 0.1 mM AI-2, there was no significant synergistic effect on percentage viability or biomass when these biofilms were additionally treated with the antimicrobial agents, apart from those biofilms treated with gramicidin. For biofilms treated with gramicidin, there was a small but significant further reduction in percentage viability of 12%. By increasing the concentration of the AI-2 to 2 mM, the increase in biomass seen in the control biofilms was significantly reduced in biofilms treated with all antimicrobial agents, apart from chloramphenicol. Also, there was a significant synergistic reduction in percentage viability for those biofilms treated with hypochlorite (10%) and chloramphenicol (20%).

When mature biofilms formed in the presence of 10 mM AI-2, and were additionally treated with high concentrations of antimicrobial agents, there were significant synergistic reductions in biofilm biomass of between 30-60%. However, there were no synergistic reductions in percentage viability.

Table 29 *The effect of the addition of the AI-2 analogue (0.1, 0.5, 2, 5 or 10 mM) on the resistance of mature biofilms to several antibiotics*

	Control		Chlorine (1000 mg l^{-1})		Gramicidin (32 mg l^{-1})		Vancomycin (16 mg l^{-1})		Chloramphenicol (64 mg l^{-1})	
	% Viability	Biomass	% Viability	Biomass	% Viability	Biomass	% Viability	Biomass	% Viability	Biomass
Control	56 (± 1.6)	100 (± 4.6)	23 (± 1.7)	91 (± 2.1)	64 (± 8.3)	82 (± 6.1)	65 (± 5.9)	78 (± 4.9)	52 (± 8.1)	84 (± 7.6)
0.1	59 (± 6.1)	94 (± 8.4)	19 (± 1.2)	103 (± 7.2)	52 (± 3.2)	78 (± 9.7)	64 (± 4.0)	74 (± 10.1)	54 (± 6.9)	75 (± 5.7)
2	46 (± 3.3)	121 (± 7.4)	12 (± 3.5)	105 (± 7.6)	69 (± 8.8)	87 (± 5.6)	66 (± 3.8)	71 (± 3.2)	33 (± 3.4)	112 (± 9.9)
10	72 (± 2.2)	62 (± 8.9)	20 (± 2.7)	29 (± 9.1)	71 (± 4.4)	19 (± 10.2)	78 (± 10.3)	12 (± 8.3)	69 (± 4.4)	49 (± 6.9)

(Data were the mean of three independent experiments)

8.4 Discussion

The effect of *agr* on biofilm formation and resistance

The best-known QS system in staphylococci is the *agr* locus. The *agr* system of *S. aureus* consists of four genes (*agr*A, *agr*C, *agr*D and *agr*B) that are co-transcribed (RNAII) with the gene for the effector molecule for the *agr* system, RNAIII, which also encodes the gene for δ-toxin (*hld*) (Peng *et al.* 1988). The *agr* system is activated during the transition from the exponential growth phase to the stationary growth phase (Novick *et al.* 1999B). The *agr* system up-regulates production of secreted virulence factors, including the alpha, beta and delta hemolysins, and down-regulates production of cell-associated virulence factors (Novick *et al.* 1993; Recsei *et al.* 1986).

QS has been shown to be involved in biofilm development of several Gram-positive and Gram-negative bacteria, including *Streptococcus mutants* (Li *et al.* 2002) and *Pseudomonas aeruginosa* (Davies *et al.*1998). In general, the formation of a biofilm involves several distinct stages, including initial attachment, cell-to-cell adhesion and proliferation, maturation, and, finally, detachment (Otto 2004). Interestingly, in *Staphylococcus*, QS appears to influence biofilm formation at many of these stages, Table 30:

Table 30 *Biofilm-associated factors regulated by QS systems in S. aureus*

Biofilm Phase	Factor	QS system	References
Attachment	Phenol-soluble modulins (PSMs)	*agr*	Vuong *et al.* 2000
Cell-to-cell adhesion	PIA	*luxS*	Xu *et al.* 2006
Detachment	PSMs	*agr*	Vuong *et al.* 2004

However, Several questions regarding the role of *agr* expression in biofilm behavior and function, particularly in the second stage of biofilm formation, remain to be addressed; e.g. Can *agr* QS affect the structure and development of *S. aureus* biofilms? Does QS affect the antibiotic resistance of *S. aureus* biofilms? (Yarwood *et al.* 2004). These questions have been investigated here, using the combined biofilm assay and EDIC microscopy visualisation of biofilm structure; the influence of *agr* on biofilm-specific resistance was investigated for antibiotics with differing modes of action.

Previous studies investigating *agr* QS in staphylococcal biofilms appear conflicting in their results and interpretation. Although Vuong *et al.* (2000) found a strong correlation between the *agr* negative genotype and enhanced biofilm formation, only 6% of clinical isolates with a functional *agr* system formed biofilm on polystyrene, compared to 78% of *agr*-defective isolates under static growth static conditions. Pratten *et al.* (2001) found little difference in adherence to either uncoated or fibronectin-coated glass, under flow conditions, between wild-type *S. aureus* and an *agr*$^-$,

hld-expressing mutant; and Shenkman *et al.* (2002) found that RNAIII expression in *S. aureus* decreased adherence to fibrinogen under static conditions, but increased adherence to fibronectin under both static and flow conditions.

Taken together, these studies imply that the precise role of *agr* expression in *S. aureus* biofilm development is dependent upon the conditions in which the biofilm is grown, highlighting the importance of examining biofilm formation by agr^+ and agr^- strains in the combined biofilm assay used here. However, as in many other microtitre plate assays, biofilm formation by the agr^- mutant was increased (70%) compared to the wild-type agr^+ strain.

Vuong *et al.* (2000) proposed that the reduced ability of *S. aureus* wild-type agr^+ strains to adhere to surfaces and produce biofilm was due in part to the surfactant properties of δ-hemolysin. However, they have recently shown that the increase in biofilm thickness of *S. epidermidis* agr^- mutants is not attributed to cell growth or death, but to the inability of cells to detach from the mature biofilm (Vuong *et al.* 2004). They used time-lapse confocal microscopy to show that detachment of cell from the biofilm coincided with *agr* expression (Yarwood *et al.* 2004). Vuong *et al.* (2004) proposed that the failure of cells to dislodge from the mature biofilm was likely due to the abolished production of a group of small peptides in the *S. epidermidis agr* mutants, known as the phenol-soluble modulins (PSMs). These amphipathic peptides, that include the RNAIII-encoded δ-toxin, are produced in an *agr*-dependent manner. Kong *et al.* (2006) suggest that, due to their amphipathic nature, PSMs facilitate the detachment of bacterial cell patches from the biofilm. Three strains of *S. aureus* (COL, Mu50 and N315) have recently been shown to contain the genes for *bete* and *hld* PSMs (Gill *et al.* 2005).

The observation of a large number of *S. aureus* virulence factors that are regulated by the *agr* operon has led to the assumption that it plays a crucial role in staphylococcal pathogenesis. Indeed, many reports have shown that the loss of the *agr* system correlates with reduced virulence in various *S. aureus* infection models. A study by Gillaspy *et al.* (1995) implied that mutation of the *agr* system reduces the incidence and severity of disease in a rabbit model of osteomyelitis. Also, partial attenuation of *S. aureus* virulence has been demonstrated for agr^- mutants in a rabbit endophthalmitis model (Booth *et al.* 1997). Finally, *agr* mutation significantly impaired the ability of *S. aureus* to cause an invasive pulmonary infection (Heyer *et al.* 2002).

However, there is also some doubt about the importance of the *agr* system in staphylococcal pathogenesis. Yarwood *et al.* (2002) showed that the *agr* operon appears to be unnecessary in certain infections for the expression of secreted virulence factors, *agr* expression in a rabbit abscess model was decreased at the same time that the animals developed TSS through exotoxin

production. Also, the *agr* mutant was as effective at causing TSS as the isogenic wild-type organism. In addition, Vuong *et al.* (2004B) used an indwelling medical device model to show that while the *agr* wild-type *S. epidermidis* strains infiltrated the tissue surrounding the devices more successfully than the *agr*-negative counterpart; *agr* mutant strain showed an enhanced ability to colonize the devices. This indicates that whilst *agr* is crucial for invasiveness of *S. epidermidis, agr* deletion enhances the capacity to cause biofilm-associated infection on medical implant devices. They proposed that this was due to the loss of the expression of the PSM peptides in the *agr* mutant strains, as PSM have strong pro-inflammatory properties.

Indeed, evidence of selection for an agr^- phenotype in chronic infections is emerging. Schwan *et al.* (2003) studied chronic wound infections using a murine abscess model. When a mixture of normal, hyper-hemolytic (presumably agr^+) and non-hemolytic (presumably agr^-) strains were inoculated into the mouse, the population of hyper-hemolytic isolates decreased over time (44-9%), while the percentage of non-hemolytic isolates increased over time. Conversely, Yarwood *et al.* (2003) found that infections established with single strains resulted in decreased cell numbers recovered from agr^- infections, compared with agr^+ infections; suggesting that the agr^+ strain somehow assists the agr^- strains in establishing infection.

Evidence for the natural selection of agr^- phenotype is shown by the expression of virulence factors in epidemic methicillin-resistant strains, which is shifted away from extracellular toxins, regulated by *agr,* and, toward expression of surface proteins and colonization factors, not regulated by *agr* (Papakyriacou *et al.* 2000). Furthermore, *agr* mutants can frequently be found in isolates from clinical settings (Vuong *et al.* 2000 and Vuong *et al.* 2003) and arise spontaneously *in vitro* (Somerville *et al.* 2002). These studies support the idea that, whereas secreted virulence factors may be important during the acute phase of infection, loss of *agr* function may enhance the long-term survival of staphylococci in the host and contribute to persistent (often biofilm-associated) infections. The enhanced survival of *agr* mutants might be due in part to the decreased production of immunostimulatory factors, such as superantigens, and increased expression of immune-evading factors, such as protein A (Yarwood *et al.* 2003).

One area of investigation, which has been largely over-looked, is the effect of *agr* on biofilm resistance; indeed, only one article has been published on the subject. Yarwood *et al.* (2004) studied the contribution of *agr* to *S. aureus* biofilm development and resistance to both rifampicin and oxacillin. They used different biofilm generating systems and found, that in a microtitre plate assay the agr^- mutant produced increased biofilms compared to controls; in the spinning disk reactor the agr^- mutant had decreased biofilm formation compared to agr^+ controls; and flow cells

showed no difference. They showed that biofilms of an *agr*D mutant, formed in a spinning disk reactor, exhibited enhanced biofilm sensitivity to rifampicin, but not to oxacillin, compared with the wild-type strain. However, under environmental conditions where the *agr*$^-$ strain showed enhanced biofilm formation (microtitre plate assay), this difference in resistance was not seen.

In this investigation, the resistance of immature and mature biofilms *agr*$^-$ strain and its corresponding *agr*$^+$ wild-type strain was determined against antibiotics with differing modes of action. Although the percentage viability and biomass of immature biofilm and mature biofilms were affected by antibiotic treatment, especially treatment with the ionophore antibiotics, there was no difference in resistance between the strains, implying that the biofilm resistant phenotype and any associated genes, at least under these conditions, are not regulated by the *agr* QS system. This is surprising as the *agr* system is involved in other aspects of biofilm formation and up-regulates the production of secreted virulence factors, including α, β, and δ-toxins, and down-regulates the production of cell associated virulence factors (Novick *et al.* 1993 and Recsei *et al.* 1986). However, the up-regulation of secreted factors makes sense if the cells are to remain in one location for any length of time, as in a biofilm, if the cells are in the blood system of the gut, these secreted factors would be diluted or removed, therefore, making it more likely that cells on the move would express cell associated virulence factors.

Alternatively, if *agr*$^-$ strains are naturally selected for, in biofilm related infections, as previously shown by Vuong *et al.* (2000), it is common sense that any inducible biofilm specific resistance mechanisms, for example a specific biofilm phenotype, would not be under the control of the *agr* system, as these inducible mechanisms would be lost, in the very instance where there expression is warranted. Also, if biofilm resistance were due to the formation of a diffusion barrier, the loss of *agr* would have no effect, as PIA expression has been shown to be regulated by *lux* and not the *agr* system.

Therefore, contrary to other pathogenic bacteria such as *Pseudomonas aeruginosa*, the inactivation or down-regulation of QS appears to be a crucial prerequisite for biofilm-associated infections in staphylococci to develop.

The effect of exogenous chemically synthesised AI-2 on biofilm formation and resistance

Unlike for HSL and oligopeptide autoinducers, the biosynthetic pathway and chemical intermediates in AI-2 production, and possibly the AI-2 molecule itself, are identical in all AI-2 producing bacteria studied to date. This, and the fact that AI-2 levels have been shown to be affected by environmental parameters like osmolarity and pH (Surrette *et al.* 1999), implying that

AI-2 production and degradation are tightly linked with the physiological and metabolic state of the cells, led to the proposal that AI-2 is the ultimate 'Universal' signal for interspecies cell-to-cell communication that can be produced and detected by a diverse range of both Gram-negative and Gram-positive bacterial species (Bassler 1999 and Miller *et al.* 2001).

It has been clear for several years that AI-2 production is widespread in bacterial species. However, demonstrations that it acts as a signal in bacteria other than in *V. harveyi* have not been as readily established and remain a matter of debate (Bassler 1999; Winzer *et al.* 2002B and Xavier *et al.* 2003). This is mainly due to the dual function of LuxS, which is involved in both metabolic and signaling processes. Indeed, presently, outside of the genus *Vibrio*, the only definitive genes shown to be regulated by the AI-2 signal, and not the loss of LuxS, are those involved in its own uptake, phosphorylation, and (probably) degradation in *Salmonella enterica* serovar Typhimurium (Taga *et al.* 2001) and *E. coli* (Xavier *et al.* 2005), i.e., the *lsr* system, comprising an ABC transporter, an AI-2 kinase, and putative enzymes for the subsequent conversion of phosphorylated AI-2.

AI-2 is produced from S-adenosylmethionine (SAM) in three enzymatic steps (See Chapter 1). During SAM-dependent methyltranserase reactions, SAM is converted to SAH, a potent feedback inhibitor of the SAM-dependent methyltransferases (Gordon *et al.* 1987). In some bacteria, e.g. *P. aeruginosa*, the hydrolysis of SAH to adenosine and homocysteine, is catalyzed by SAH hydrolase. In other bacteria, e.g. *E. coli* and *S. aureus*, the nucleosidase Pfs and the SRH cleavage enzyme (LuxS) catalyse the conversion of SRH to DPD and homocystine (Chen *et al.* 2002). AI-2 is produced from DPD and is secreted during exponential growth and possibly degraded at a later stage. MHF, a secondary product of the SRH cleavage has DNA-damaging properties. This could explain why AI-2 (and MHF) are excluded from the cell during exponential growth, later to be taken up and degraded in a controlled fashion, preventing the build-up of toxic intracellular levels (Winzer *et al.* 2002). One interesting question that is often asked is why do some bacteria employ two enzymatic steps to achieve what others can accomplish in a single step. It has been suggested that the Pfs enzyme is sufficient for detoxification, and that one reason for employing the Pfs-LuxS variant of the AMC, is because it allows them to generate the AI-2 signal (Schauder *et al.* 2001). It is, however, as pointed out by Winzer *et al.* (2002), remarkable that the synthesis of AI-2, which is clearly used as a signal molecule by *V. harveyi*, relies on the degradation of a metabolite, whereas other signal molecules, for instance the *N*-acyl-HSLs, are synthesized by dedicated synthases.

In most bacteria examined, extracellular AI-2 activity peaks in mid-late exponential phase and declines suddenly in stationary phase. Indeed, Doherty *et al.* (2006) found that AI-2 formation by *S. aureus* planktonic cultures was strictly *luxS* dependent and that AI-2 expression peaked during the transition to stationary phase under a range of different conditions, including anaerobiosis, and

after entry into stationary phase, AI-2 activity was significantly reduced in the absence of glucose, suggesting either uptake or degradation. Although AI-2 expression in the positive control organism, *E. coli* O157, followed this pattern of production and degraded, AI-2 was only minimally produced in MRSA biofilms. The reason for this limited production is unclear, especially as Doherty *et al.* (2006) found that *luxS* gene in *S. aureus* is under the control of a σ^{70}-dependent promoter and was continuously expressed under all conditions tested, implying that LuxS is required throughout growth. However, the magnitude of light stimulation in *S. epidermidis* was lower than that of AI-2 in *V. harveyi* BB170 (Xu *et al.* 2006). They hypothesised that this might be due to unique modifications to the basic AI-2 structure or to a lower production level of AI-2 in *S. epidermidis* under the growth conditions that were used. In fact, interspecies signals secreted by *Salmonella* serovar Typhimurium have recently been demonstrated to be a distinct form of the AI-2 signal, although they are both derived from the same precursor (Miller *et al.* 2004). The same can be hypothesised for the limited AI-2 production in the MRSA biofilm; it could be due to modifications in the structure of the AI-2 produced by *S. aureus*; or due to the lack of glucose in the media. Further work is required to determine AI-2 production in MRSA biofilms under different environmental and growth conditions.

It is clear that, in both Gram-negative and Gram-positive bacteria, *luxS* inactivation affects the expression of an assortment of apparently 'niche-specific' genes, Table 31.

Table 31 *LuxS/AI-2–regulated behaviors*

Species	Function	Reference
Actinobacillus actiomycetemcomitans	Virulence factors; leukotoxin, iron acquisition	Fong *et al.* 2003
Borrelia burgdorferi	Expression of ErpA-1 and –N proteins	Stevenson *et al.* 2002
Campylobacter jejuni	Motility	Elvers *et al.* 2002
Clostridium perfringens	Expression of alpha, kappa and theta toxin	Ohtani *et al.* 2002
Escherichia coli W3110	Cell division, DNA processing, morphology	DeLisa *et al.* 2001
E. coli, EHEC (O157:H7)	Type-III secretion, shiga toxin, flagella	Sperandio *et al.* 1999
E. coli, EPEC (O127:H6)	Motility (Flagellin expression)	Sperandio *et al.* 1999
Neisseria meningitidis	Bacteremic infection	Winzer *et al.* 2002C
Photorhabdus luminescens	Antibiotic production (carbapenem)	Derzelle *et al.* 2002
Porphyromonas gingivalis	Protease, hemagglutinin, heme acquisition	McNab *et al.* 2003
Salmonella enterica serovar Typhimurium	Biofilm formation	De Keersmaecker *et al.* 2005
Salmonella typhi	Biofilm formation	Prouty *et al.* 2002
Salmonella typhimurium	AI-2 ABC transporter expression	Taga *et al.* 2001
Shigella flexneri	Expression of *virB*	Day *et al.* 2001
Streptococcus mutans	Biofilm formation (granular appearance)	Merritt *et al.* 2003
Streptococcus pneumoniae	SpeB cysteine protease secretion	Stroeher *et al.* 2003
Streptococcus pyogenes	SpeB cysteine protease / hemolysin SagA secretion	Lyon *et al.* 2001
Vibrio cholerae	Cholera toxin, toxin-co-regulated pilus	Miller *et al.* 2002
Vibrio harveyi	Luminescence, type III secretion, protease and siderophore production	Bassler *et al.* 1994 Lilley *et al.* 2000
Vibrio vulnificus	Virulence factor expression	Kim *et al.* 2003

Table taken from Federle *et al.* (2003).

The effect of *luxS* expression on the biofilm formation of several species has been previously investigated. Interestingly, *luxS* mutants of certain bacteria have been described as being impaired in the ability to form monospecies or mixed-species biofilms. These include *P. gingivalis*, *S. gordonii*, *S. mutans*, *S. enterica* subspecies, and *K. pneumoniae* (Balestrino *et al.* 2005; Blehert *et al.* 2003; Fong *et al.* 2003; McNab *et al.* 2003; Merritt *et al.* 2003 and Prouy *et al.* 2002). However, both Gonzalez-Barrios *et al.* (2006) and Sela *et al.* (2006) found that the biofilm formation of *E. coli* of *L. monocytogenes* respectively was stimulated *luxS*-deficient mutants. Sela *et al.* (2006) hypothesised that, in *L. monocytogenes,* the *luxS* gene is associated with repression of components required for attachment and biofilm formation. Recent investigations also indicate

that *luxS*–mediated interspecies cell-cell communication is necessary to coordinate biofilm production. Indeed, McNab *et al.* 2003 showed that *luxS* was required for the formation of mixed-species biofilms of two oral bacteria, *S. gordonii*, a commensal species, and *P. gingivalis*, an oral pathogen associated with the progression of periodontitis. While *luxS* was dispensable for monospecies biofilm formation in *P. gingivalis* and *S. gordonii*, its expression was required in one of the two species for the mixed biofilm to form. The effect of *luxS* deletion on biofilm formation appears to be species-specific and is probably dependent on the other niche-specific behaviours influenced by *luxS*.

However, an intriguing question raised in the current *luxS* / AI-2 debate is whether the phenotypic differences seen with the *luxS* mutants are due to a loss of the AI-2 signal, or due to the disruption of the AMC metabolic pathway. Although these phenotypes have generally been attributed to the loss of AI-2-dependent signalling, this conclusion is based on indirect evidence, i.e., the phenotype of *luxS* mutants, addition of AI-2-containing spent culture supernatants, or co-culture of the wild type and mutant. Furthermore, only a few of the above-mentioned studies attempted to use a defined preparation of AI-2 to restore the biofilm characteristics of the wild-type. However, De Keersmaecker *et al.* (2005) demonstrated that AI-2 derived from synthetic DPD could not restore biofilm formation by an *S. enterica* serovar Typhimurium *luxS* mutant, whereas the introduction of *luxS* under the control of its own promotor restored the wild-type phenotype. Similarly, the addition of synthetic AI-2 failed to restore type III secretion and motility defects in enterohemorrhagic *E. coli* (O157:H7) (Sperandio *et al.* 2003), phenotypes previously attributed to *luxS* / AI-2-based QS by Sperandio *et al.* (Sperandio *et al.* 1999 and 2001). Due to these difficulties in determining the exact cause of *luxS* deletion phenotypes, it was decided not to investigate *S. aureus luxS* mutants, but instead to determine the effects of exogenously added, chemically synthesised, AI-2 on MRSA biofilm formation and antibiotic resistance, as no data on the subject has as yet been published.

In this study, high concentrations of a chemically synthesised AI-2, did not significantly affect the growth of planktonic cultures. However, these same high concentrations caused significant reductions in biofilm biomass. Nonetheless, it can not be argued that this was due to the toxic effects of the AI-2 molecule, as the percentage of viable cells within the treated biofilms was actually increased compared to control biofilms. Therefore, it is more likely that the reductions in biomass were due to decreased attachment or increased detachment from the biofilm.

Auger *et al.* (2006) also showed that chemically synthesised AI-2 inhibited biofilm formation of the Gram-positive *Bacillus cereus* in a concentration-dependent manner. They hypothesised that AI-2 down-regulated components associated with biofilm attachment. Indeed, in a very recent study, Xu *et al.* (2006) found that in the closely related *S. epidermidis*, *luxS* mutation led to biofilms with

significantly increased biomass (1.7 times) compared to that of wild-type biofilms, and further showed that the increase in biomass could be repressed by the exogenous addition of AI-2 containing supernatants. They used immuno-dot blot analysis to determine the amount of PIA / PNAG in AI-2 treated and untreated biofilms, and found that PIA production was up-regulated in the *luxS* mutant. Thus, these data indicate that the *luxS* gene of *S. epidermidis* is involved in repressing biofilm formation through a cell-to-cell signalling mechanism based on AI-2 secretion and that *luxS* / AI-2-dependent QS regulates the expression of PIA. Therefore, this author suggests that it is possible that PIA / PNAG production is down-regulated in MRSA biofilms grown in the presence of AI-2. To ascertain whether PIA / PNAG is also reduced in *S. aureus* AI-2 treated biofilms, further work to compare the relative quantities of PIA / PNAG in treated and untreated biofilms, using Congo red staining, needs to be undertaken. However, the reduction in PIA production itself can not directly account for the reduction in biomass observed in this study, as it was shown previously that the Safranin assay is not affected by the presence / absence of EPS / PIA. However, the loss of PIA / PNAG would weaken cell attachment to the microtitre plate surface, and the author suspects that the 'plaques' observed in biofilms grown in the presence of high concentrations of synthetic AI-2, maybe caused by clumps of biofilm becoming detached from the surface of the plate during the washing procedures used in the combined biofilm assay.

If exogenous AI-2 does lead to a reduction in PIA expression in MRSA biofilms, it would also partly explain the synergistic effects seen for AI-2 / antibiotic treatments of mature biofilms. The removal of PIA / PNAG, a main constituent of the *S. aureus* EPS matrix, would weaken biofilm attachment and remove a possible barrier to antibiotic penetration. However, as already discussed, the diffusion barrier presented by the EPS matrix cannot completely account for biofilm resistance, as it was previously shown that immature biofilms, which lack a copious PIA / PNAG containing EPS matrix, are still partially resistant to numerous antibiotics. Indeed, although synergistic effects were seen in AI-2 treated biofilms, and the biofilms, even in the presence of high concentrations of antimicrobial agents, were still partially resistant. Unfortunately this leads to the conclusion that other unidentified biofilm resistance mechanisms are still in place and unaffected by AI-2 addition.

In order to inhibit biofilm formation, the bacterial cells must be able to sense AI-2. However, in *S. aureus,* none of the genomes published to date contain potential homologues for either the *lsr* AI-2 uptake system present in a diverse range of bacteria (including several Gram-positive *Bacillus* spp.) or the LuxP/LuxQ AI-2 signal transduction system (also not present in *Bacillus* spp.) found in *Vibrio* species. Therefore, if AI-2 is used as a signalling molecule, responsible for a reduction in PIA production by *S. aureus*, it must be sensed via a different and as yet unknown mechanism (Doherty *et al.* 2006).

The main conclusion of this work is that AI-2 addition does modulate both MRSA biofilm formation and resistance. It is hypothesised that this may be due to an AI-2 induced reduction in PIA / PNAG, as seen in *S. epidermidis*. Therefore, it is promising that AI-2 or AI-2-derivatives could in theory be used as co-drugs for the treatment of MRSA biofilm-related infections, although much further investigation needs to occur.

9. THE INFLUENCE OF EXOGENOUS NITRIC OXIDE ADDITION ON MRSA BIOFILM FORMATION AND BIOFILM RESISTANCE

9.1 Introduction

Vancomycin has been recognised as the last line of defence to treat patients with MRSA infections. However, as the present work (Chapter 5) and that of Jones *et al.* (2001) have shown, the mature MRSA biofilm phenotype is resistant to very high concentrations of the antibiotic. Also, the ionophore antibiotics that had some effect on MRSA biofilms (Chapter 6), both alone and in combination with antibiotics of differing modes of action, were not able to completely remove or kill the mature biofilms. In addition, the effect was not significantly modulated in *agr* QS mutants. However, the exogenous addition of the controversial QS molecule AI-2 did have modulatory effects, but even in combination with antibiotics, mature MRSA biofilm was not eradicated. Given that resistance is biofilm-associated, the aim is to combine strategies that modulate biofilm development and dispersal with antibiotic treatments. One emerging strategy for the control of biofilm development and dispersal in *P. aeruginosa* biofilms is use of the chemical signal, nitric oxide (NO). It was therefore decided to determine whether NO could modulate MRSA mature biofilm formation and antibiotic resistance.

The generation of oxidative or nitrosative stress inside biofilm microcolonies was recently linked to processes of cell lysis and dispersal in *P. aeruginosa* biofilms (Webb *et al.* 2003). Subsequently it was established that production of NO, and its downstream reaction product peroynitrite (ONOO-), were key to these processes. Nitrosative stress involves production and ensuing damage from reactive nitrogen intermediates (RNI) that include nitric oxide (NO), peroxynitrite ($ONOO^-$), nitrous acid (HNO_2), nitrogen trioxide (N_2O_3) and others. RNI are small and potentially highly reactive molecules that can be continuously produced in the organisms as by-products of anaerobic respiration. Excessive production of RNI can cause damage to DNA, lipids and proteins (Barraud *et al.* 2006). However, in addition to their damaging properties, RNI, are also involved in many signalling and regulatory pathways in both eukaryotic and prokaryotic organisms (Nathan 2003; Thannickal 2003). In eukaryotic biology, NO and other RNI derived from arginine; have been shown to be critical in many physiological signalling roles, including the regulation of blood pressure and flow (Kilbourn *et al.* 1993; Palmer *et al.* 1987), neurotransmission (Garthwaite 1991), and immune responses, including some of the effector functions of macrophages, such as cytotoxicity toward tumour cells and microorganisms (Liew *et al.* 1991; Nathan 1992; Nathan *et al.* 1991), and cell apoptosis, differentiation and cell proliferation (Moncada *et al.* 1998). Also,

numerous studies in *E. coli* have shown that RNI activate global regulatory networks such as the SOS response (Lobysheva *et al.* 1999).

NO was also recently shown to effectively kill the mucoid form of *P. aeruginosa* in both planktonic and biofilm cultures, when added back in sterile supernatants from CF transplant patients and in a mouse model of chronic CF airway infection (Yoon *et al.* 2006). While the roles of RNI have been studied extensively in planktonic bacterial physiology in the context of protective mechanisms, there is a paucity of information as to their role in multicellular biofilm development and differentiation processes. Barraud *et al.* (2006) found that NO, the main precursor of $ONOO^-$ *in vivo* (Beckman *et al.* 1990), was able to induce dispersal at concentrations that were non-toxic to wild-type *P. aeruginosa* (in the nanomolar range). Furthermore, bacteria that were exposed to low levels of NO were more effectively removed from surfaces using combined antimicrobial treatments than were control antibiotic treated biofilms.

The only work published, looking at the effect of NO on *S. aureus* biofilms, was done by Nablo *et al.* (2005), who looked at the *in vivo* antibacterial activity of NO-releasing xerogel coatings against an aggressive subcutaneous *S. aureus* infection in a rat model. An 82% reduction in the number of infected implants was achieved with the NO-releasing coating. These results suggest that NO-releasing coatings may dramatically reduce the incidence of biomaterial-associated infection. The objective of this study was therefore, to determine if exogenous NO addition also modulates MRSA biofilm development and antibiotic susceptibility. This may lead to novel applications for NO as a co-drug for the control of persistent MRSA biofilms.

9.2 Methods

The methods used in this work were as described in chapter 2.

9.3 Results

9.3.1 Planktonic growth of *S. aureus* cultures grown in the presence of the NO donor SNP

The growth of planktonic cultures of the *S. aureus* strain NCTC 10442 was monitored in the presence and absence of varying concentrations of the NO donor SNP. SNP was used as it has the advantage of established steady state levels of NO, which mimic endogenous NO production during bacterial denitrification (Kwiatkowski *et al.* 1996).

Culture tubes containing sterile $BHI_{25\%}$ + 0.09% glucose (10 ml) with varying concentrations of SNP (10 nM, 500 nM, 10 μM or 10 mM) were inoculated using MRSA (NCTC 10442) starter cultures (Section 2.7) and incubated with shaking (175 rpm) at 37°C for 0 to 12 hours. The turbidity (620 nm) of the cultures was measured hourly (Data were the mean of three experiments).

Planktonic *S. aureus* cultures grown in varying concentrations of SNP had comparable viability and growth rate when compared to planktonic cultures grown in control conditions without SNP (Data not shown). Therefore, it was assumed that the concentrations of SNP tested were sub-lethal for planktonic *S. aureus* cultures.

9.3.2 Biofilm formation in the presence of SNP at varying concentrations

A range of concentrations of the NO donor, SNP, were tested for their effects on the formation of biofilm by the MRSA strain, NCTC 10442. Starter cultures of NCTC 10442 were used to inoculate microtitre plate (NuncSurface) wells containing 200 μl of $BHI_{25\%}$ + 0.09% glucose with or without varying concentrations (10 nM – 10 mM) of SNP. The microtitre plates were incubated at 37°C for up to 24 hours. Biofilm physiology and growth were investigated by the determination of the percentage of viable cells, using XTT / PMS assay procedure (Section 2.13.2) and the total cell number (biomass) by Safranin staining (Section 2.14.1).

The biomass of the mature (24 hours-old) control biofilms was normalised to 100% (blue bars, Figure 40), and the number of viable cells within the biofilm was given as a percentage of that total biomass (black bars, Figure 40). The biofilm biomass and relevant percentage viability of 6 and 12-hour control, and SNP treated biofilms, were therefore expressed as a percentage of the mature control biofilm (Figure 40).

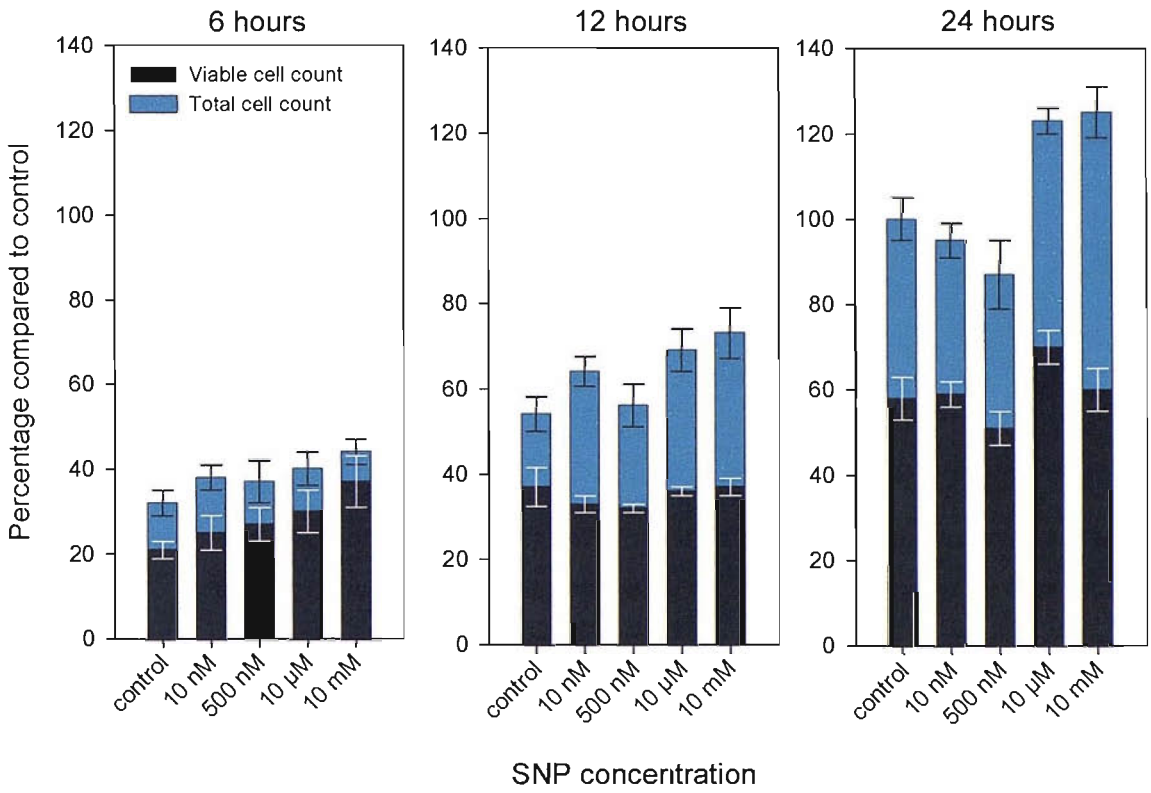


Figure 40 *The effects of the addition of SNP (10 nM - 10 mM) on the formation of MRSA biofilms over 6, 12 and 24 hours. Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data were the mean of three experiments.*

The lowest concentration of SNP (10 nM) had a small but not significant effect on biofilm formation, increasing biomass compared to control biofilms at 6 and 12 hours but reducing biomass compared to control biofilms after 24 hours growth. At a concentration of 500 nM, a marked decrease in biofilm biomass was observed, which became more significant as the biofilm formed, so that, after 24 hours of biofilm formation, the difference between the biomass of control biofilms and those treated with 500 mM SNP was 20%. However, further increases in SNP concentration (10 µM and 10 mM) caused significant increases in biofilm biomass of approximately 20%, for biofilm at all ages. Alternatively, the addition of SNP, at all concentrations, had no significant effect on the percentage of biofilm viability when compared to control biofilms of the same age.

EDIC microscopy images of mature MRSA biofilms, grown in the presence of varying concentrations of SNP, show no change in the structure or percentage surface coverage of the biofilm (Figure 41).

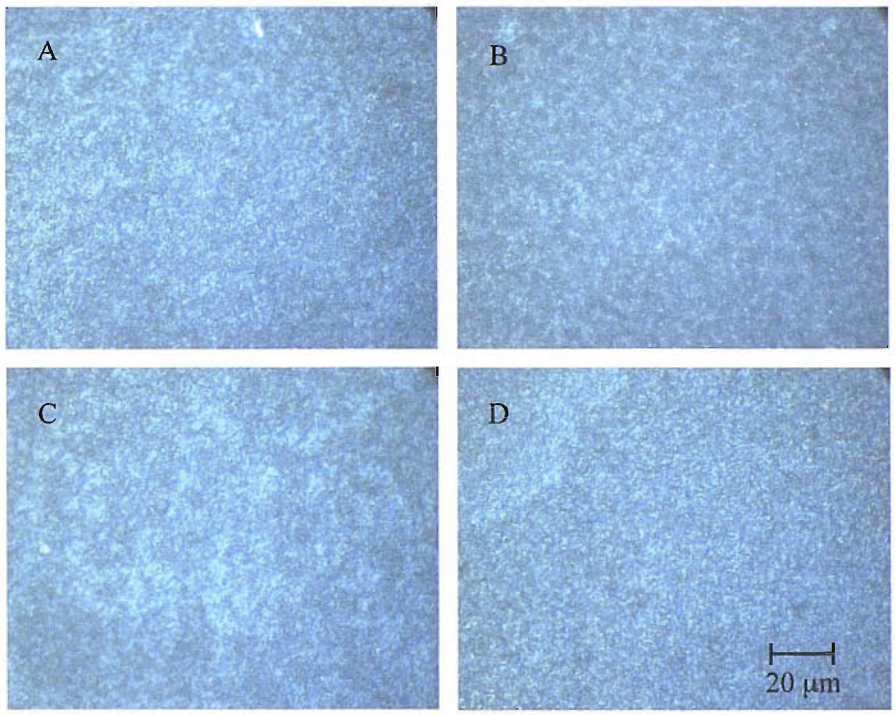


Figure 41 *Addition of SNP to MRSA biofilms in tissue culture plates does not effect biofilm structure or percentage surface cover. Examples of EDIC microscopy images (x 1000) showing the structure and surface coverage of mature MRSA (NCTC 10442) biofilms grown in the presence of 10 nM (A), 500 nM (B), 10 µM (C) and 10 mM (D) of the NO donor SNP.*

9.3.3 The effect of SNP at various concentrations on the resistance of immature and mature MRSA biofilms to vancomycin

The effect of combined NO and vancomycin treatment was investigated for immature and mature MRSA biofilms. MRSA (NCTC 10442) starter cultures were used to inoculate the wells of microtitre plates containing 200 µl of $BHI_{25\%}$ + 0.09% glucose, with or without SNP, at varying concentrations (10 nM – 10mM); the microtitre plates were incubated at 37°C for 8 or 24 hours for immature and mature biofilms respectively. After incubation, the planktonic culture phase was aspirated, the biofilms were gently washed in dH_2O (200 µl) and sterile growth media (200 µl), with or without SNP, and vancomycin at ten times the planktonic MIC (20 mg l^{-1},) was returned. The microtitre plates were then incubated at 37°C for a further 18 hours.

Control biofilms were grown in the presence and absence of SNP (10 nM – 10 mM), or in the presence / absence of vancomycin (20 mg l^{-1}). The percentage of viable cells (determined using the XTT / PMS assay procedure; Section 2.13.2) and the biomass (Safranin staining; Section

2.14.1) were expressed as a percentage of the total biofilm biomass of the SNP- / vancomycin+ control (Figure 42).

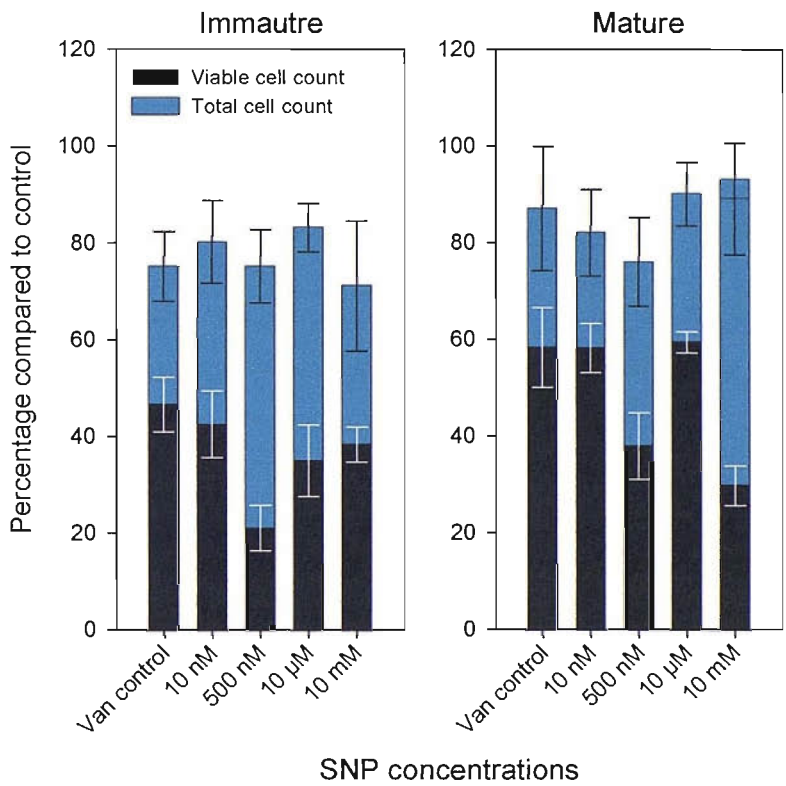


Figure 42 *The effects of the addition of SNP (10 nM - 10 mM) on the resistance of immature and mature MRSA biofilms to vancomycin (16 mg l^{-1}). Data shows the total biofilm biomass (blue bars) and the percentage of viable cells (black bars). Data were the mean of three experiments.*

Vancomycin treated immature and mature biofilms grown in the presence of 10 nM SNP, were not significantly different either in biomass or percentage viability to control vancomycin-treated biofilms. However, although the biomass of immature biofilms treated with both SNP at 500 nM and vancomycin was unaffected, the percentage of viable cells within the biofilm was significantly reduced from 64% to 30%. For mature biofilms treated with SNP at 500 nM and vancomycin, both the biomass and percentage viability were significantly reduced by 20% and 25% respectively, compared to control vancomycin-treated biofilms. The higher SNP concentration of 10 µM caused further significant reductions in viability for immature but not mature biofilms. This concentration of SNP caused a significant increase in biomass for both immature and mature biofilms, even in the presence of vancomycin. However, the highest concentration of SNP, 10 mM, caused significant reductions in both biomass and percentage viability for vancomycin-treated immature biofilms and only in viability for vancomycin-treated mature biofilms.

9.3.4 The effect of varying concentrations of SNP on the resistance of immature and mature MRSA biofilms to other antibiotics

The above work shows that the NO donor SNP has significant modulatory activity on the biofilm phenotypic resistance to vancomycin of both immature and mature MRSA biofilms. It was therefore important to determine whether this modulation extended to other classes of antibiotics (Table 32 and 33).

The addition of SNP, at 10 nM, to immature biofilms had significant effects on biofilm biomass. Biofilms that were also treated with gramicidin showed a small but significant drop in biomass of 16% (Table 32), and immature biofilms treated with phosphomycin showed a significant rise in biomass of 21%. For biofilms grown in the presence of SNP (10 nM), the percentage of viable cells was reproducibly, but not significantly, reduced for all but the gramicidin and fusidic acid-treated biofilms.

Immature biofilms grown in the presence of 500 nM SNP showed a significant decrease in biomass of 27% and 34% when treated with phosphomycin and gramicidin respectively. Also, the viability of biofilms grown in 500 nM SNP, and treated with all but phosphomycin and gramicidin, showed significant reductions of up to half.

For immature biofilms treated with 10 μM SNP and antimicrobial agents, there were no significant differences in either the percentage viability or biomass, compared to antimicrobial-treated control biofilms. When biofilms were grown in the presence of the highest concentration of SNP (10 mM), again the biofilm viability was reduced for all but the phosphomycin and gramicidin treated biofilms.

Table 32 *Immature (8-hour-old) MRSA biofilm resistance to several antibiotics in the presence of varying concentrations of the NO donor SNP.*

	Control		Phosphomycin (64 mg l^{-1})		Gramicidin (32 mg l^{-1})		Novobiocin (8 mg l^{-1})		Chloramphenicol (64 mg l^{-1})		Fusidic acid	
	% V	B	% V	B	% V	B	% V	B	% V	B	% V	B
Control	62 (±8.7)	100 (±9.8)	38 (±9.0)	117 (±8.0)	27 (±6.8)	98 (±9.7)	46 (±8.2)	106 (±9.2)	41 (±8.9)	110 (±9.4)	43 (±8.0)	95 (±2.8)
10 nM (8 h)	44 (±1.0)	87 (±3.5)	35 (±6.6)	114 (±3.3)	30 (±9.0)	89 (±3.1)	41 (±5.7)	107 (±9.7)	35 (±4.2)	107 (±8.8)	48 (±4.8)	77 (±9.6)
500 µM (8 h)	68 (±4.2)	123 (±3.9)	52 (±9.6)	90 (±3.6)	28 (±4.7)	64 (±1.7)	31 (±7.6)	98 (±7.9)	24 (±6.6)	119 (±2.3)	29 (±2.7)	104 (±3.2)
10 µM (8 h)	49 (±4.1)	113 (±8.8)	35 (±9.6)	138 (±9.7)	26 (±5.3)	93 (±3.2)	48 (±6.8)	97 (±7.0)	36 (±9.4)	113 (±2.8)	41 (±12.8	109 (±8.3)
10 mM (8 h)	40 (±1.0)	109 (±3.1)	36 (±5.7)	99 (±8.5)	26 (±2.2)	78 (±3.8)	33 (±4.0)	116 (±8.3)	30 (±2.9)	101 (±3.1)	25 (±7.5)	105 (±2.0)

For mature biofilms grown in the presence of 10 nM SNP, there was no significant difference in the percentage viability or biomass from the antibiotic control biofilms (Table 33). There were significant reductions in both the percentage viability and biomass for biofilms treated with 500 nM SNP and the antibiotics. However, as seen with the immature biofilms, these reductions were not seen for biofilms grown in the higher SNP concentration of 10 µM. Indeed, although the percentage viability remained comparable to the antibiotic control biofilms, the biomass was

significantly increased (20-43%) for all antibiotic treatments. However, contrary to the 10 mM SNP control biofilms, which showed an even further increase in biomass than the 10 μM SNP control biofilms, no further significant increase in biomass was seen when these biofilms were also treated with antibiotics; However, these biofilms showed significant reductions in percentage viability.

Table 33 *The effect of the addition of the NO donor SNP at varying concentrations on the resistance of mature MRSA biofilms to several antibiotics.*

Control			Phosphomycin (64 mg l^{-1})		Gramicidin (32 mg l^{-1})		Novobiocin (8 mg l^{-1})		Chloramphenicol (64 mg l^{-1})		Fusidic acid	
	% V	B	% V	B	% V	B	% V	B	% V	B	% V	B
Control	63 (±9.4)	100 (±8.8)	53 (±10.7)	95 (±3.4)	38 (±11.6)	105 (±8.6)	58 (±12.4)	100 (±8.9)	48 (±5.6)	116 (±4.4)	52 (±5.4)	100 (±3.9)
10 nM (24 h)	59 (±10.1)	98 (±5.9)	60 (±4.9)	89 (±12.5)	30 (±5.7)	135 (±12.1)	43 (±7.2)	138 (±5.4)	49 (±4.2)	118 (±2.6)	42 (±4.7)	130 (±9.0)
500 μM (24 h)	51 (±2.9)	95 (±13.1)	40 (±12.6)	83 (±6.7)	27 (±7.2)	110 (±5.3)	40 (±6.5)	92 (±9.7)	44 (±10.0)	81 (±5.6)	35 (±4.5)	91 (±9.6)
10 μM (24 h)	70 (±4.6)	123 (±8.7)	47 (±11.2)	111 (±9.1)	30 (±3.8)	118 (±11.9)	43 (±3.8)	143 (±11.2)	47 (±8.4)	128 (±10.7)	50 (±3.7)	115 (±6.6)
10 mM (24 h)	50 (±7.7)	125 (±6.8)	24 (±6.9)	91 (±4.5)	20 (±6.4)	102 (±2.6)	30 (±5.6)	105 (±6.5)	30 (±6.7)	108 (±4.2)	32 (±6.2)	97 (±11.0)

9.4 Discussion

NO is produced as a by-product of the anaerobic reduction of nitrite (NO_2) and is involved in the regulation of anaerobic processes. NO exposure was found to activate genes for anaerobic metabolism in *P. aeruginosa*, a process that involves the anaerobic regulator ANR (Firoved *et al.* 2004).

Planktonic MRSA cultures were able to grow in the presence of high concentrations of NO with no change in growth rates. One explanation for the ability of *S. aureus* to divide, despite the presence of relatively high levels of NO, might be the efficiency of staphylococcal NO scavenging. Indeed, Richardson *et al.* (2006) found that *S. aureus* cultures in the presence of high concentrations of NO exhibited a marked increase in NO consumption

NO mediated dispersal and effects on biofilm formation

Bacterial biofilms periodically undergo regulated and co-ordinated dispersal events where sessile biofilm cells convert to planktonic bacteria (Sauer *et al.* 2004). Barraud *et al.* (2006) showed that endogenous RNI production was intimately correlated with differentiation and dispersal events *in situ* in *P. aeruginosa* biofilms. Also, exogenous NO and/or its downstream derivatives, at sub-lethal concentrations, were able to induce a transition from biofilm to the planktonic phenotype, facilitating the dispersal of *P. aeruginosa* biofilm bacteria. In evidence of this, they also demonstrated that a *P. aeruginosa* mutant, lacking the only enzyme in *P. aeruginosa* capable of generating metabolic NO through anaerobic respiration (nitrite reductase, Δ*nirS*), did not disperse; whereas a *P. aeruginosa* NO reductase mutant (Δ*norCB*) exhibited greatly enhanced dispersal when treated with NO at these low-concentrations.

Barraud *et al.* (2006) showed that low levels of NO caused a reduction in biofilm biomass and an increased number of planktonic organisms; they proposed that low levels of NO induce *P. aeruginosa* cells in the biofilm mode of growth to detach and revert to the planktonic phenotype. This is supported by the fact that *P. aeruginosa* microarray studies have revealed that, upon exposure to NO, genes involved in adherence are down-regulated (Firoved *et al.* 2004), suggesting a mechanism by which the bacteria can detach from the biofilm.

In *S. aureus,* 66 genes are up-regulated in the presence of NO, and 14 are repressed (Richardson *et al.* 2006). Of the up-regulated genes, 45% are involved in iron homeostasis, many of which are regulated by the ferric uptake regulator (FUR). However, there is no information available as to whether genes involved in adherence are also down-regulated in *S. aureus* in the presence of NO. It is interesting to note that in *S. aureus* biofilm cells, Nitrite reductase (*nirB*), is up-regulated three times in comparison with to stationary phase cells (Beenken *et al.* 2004).

MRSA biofilms treated with the same sub-lethal nanomolar NO concentrations, as used by Barraud *et al.* (2006), also displayed reduced biomass, implying that the effect we are seeing is a genuine one. It also suggested that exogenous NO might also induce MRSA biofilm cells to disperse, explaining the reduction in biomass observed for MRSA biofilms grown in the presence of low sub-lethal concentrations of NO. However, further work, looking at the ratio of biofilm to planktonic cells in MRSA cultures treated with NO at varying concentrations, will have to be undertaken to ensure that this is the case.

At higher exogenous NO concentrations, the biomass of MRSA biofilms was significantly increased; Barraud *et al.* (2006) found similar effects in *P. aeruginosa* biofilms at high concentrations of NO, although they reported no increase in the biofilm / planktonic ratio, as previously seen with the lower exogenous concentrations of NO. Possible explanations for this increase in biofilm biomass include: an increase in anaerobic gene transcription upon exposure to NO as previously described for MRSA and *P. aerginosa* (Firoved *et al.* 2004; Richardson *et al.* 2006 and Yoon *et al.* 2002), which may be favourable for biofilm growth. Also, NO is rapidly converted back to NO_2^- and NO_3^- at slightly acidic pH (Yoon *et al.* 2006), which would lead to enhanced anaerobic metabolism and biomass production. Indeed, the pH of mature MRSA biofilms in this study was approximately 5.5, since these biofilms were grown in the presence of glucose (data not shown).

Lastly, NO is also one component of slightly acidified nitrite that was shown recently to effectively kill the mucoid form of *P. aeruginosa* in planktonic and biofilm culture (Yoon *et al.* 2006). Indeed, when the production of RNIs, (which are small and potentially highly reactive molecules, continuously produced in organisms as by-products of anaerobic respiratory metabolisms), overwhelms the capacity of the cell to remove such molecules, RNI can mediate nitration of protein tryosine residues, peroxidation of lipids and deamination of DNA bases (Radi *et al.* 1991; Wink *et al.* 1991 and Schopfer *et al.* 2003). Therefore, a logical explanation would be that the organisms would be safer within the confines of the biofilm, given the high millimolar NO levels, rather than risk single-cell suicide on their own once dispersed from the biofilm.

NO meditated biofilm susceptibility to antimicrobial agents

Barraud *et al.* (2006) also looked at the efficacy of NO addition to increase *P. aeruginosa* biofilm susceptibility to antimicrobial agents (tobramycin, hydrogen peroxide and sodium dodecyl sulphate). For biofilms treatment with combined NO (500 nM) and tobramycin, a two- fold logarithmic reduction in CFU ml^{-1} was observed, and biomass / surface coverage was reduced by around 80%, compared to biofilms treated with tobramycin alone.

If the observation by Barraud *et al.* (2006) that the addition of NO at low concentration induced *P. aeruginosa* biofilms cells to revert to the planktonic phenotype was also true of MRSA biofilm cells, then it could be argued that MRSA biofilms treated with low levels of NO may also have increased susceptibility to antimicrobial agents. Indeed, mature MRSA biofilms treated with NO, in combination with vancomycin, showed a 20% reduction in biomass and 25% reduction in the percentage viability of the biofilm compared to vancomycin-only treated biofilms. Also, NO was tested in combination with a number of other antimicrobial agents with differing modes of action against both immature and mature MRSA biofilms. The results were variable, but, compared to antimicrobial treatment alone, a reduction in both the percentage viability and biofilm biomass was seen for nearly all of the antimicrobial / NO combinations, when NO was used at a concentration of 500 nM.

In theory, if NO exposure could induce the transition of MRSA biofilm cells to a planktonic phenotype, it could explain why the biofilms displayed altered antimicrobial resistance in response to NO exposure. However, the exact mechanism by which exposure to NO increases sensitivity to antimicrobials is not yet understood. Nevertheless, these effects were observed using several different classes of antimicrobial compounds, suggesting that a generalized mechanism of tolerance to antimicrobial stress is affected.

Several factors have been suggested to contribute to the increased resistance of biofilm cells, including reduced metabolic activity and growth rates of the sessile cells (Spoering *et al.* 2001), limited antibiotic penetration due to the protective structure of the biofilm (Costerton *et al.* 1999 and Davies 2003), and phenotypic diversification of biofilm cells (Drenkard *et al.* 2002 and Lewis 2005). Biofilm cells display a decreased metabolic activity and resemble the physiology of stationary phase cells (Hentzer *et al.* 2005 and Sternberg *et al.* 1999). Many antimicrobial treatments are known to be more efficient against metabolically active cells; for example, the aminoglycoside tobramycin and β-lactam antibiotics are effective only at killing actively dividing cells (Evans *et al.* 1994). Thus, one possibility is that low concentrations of NO induce a transition to a planktonic physiology that is more characteristic of actively growing cells in both *P. aeruginosa* and MRSA biofilms. Thus, upon NO exposure, both cells remaining attached to the

surface and planktonic cells are more sensitive to antimicrobials. Further research needs to be undertaken to determine a more detailed understanding of this process.

Also, as low concentrations of NO alone induced dispersal in *P. aeruginosa* biofilms and caused significant reductions in the biomass of both *P. aeruginosa* and MRSA biofilms, the antimicrobials may by more able to penetrate the remaining thinner biofilm. However, for some antibiotics (including tobramycin), limited penetration of the biofilm does not appear to be the cause of biofilm tolerance (Walters *et al.* 2003). Also, the biofilm structure appears to be unchanged (no plaque formation; Figure 41) by the addition of NO, suggesting, therefore, that any changes in the resistance of these biofilms are physiological rather than changes in the physical barrier.

Combined exposure to both NO and antimicrobial agents may therefore offer a novel strategy to control pre-established persistent *P. aeruginosa* and MRSA biofilms and biofilm-related infections.

Evolutionary perspectives

Recent analyses of microbial genomes have suggested that homologous NO-sensing receptor domains are common to both prokaryotic and eukaryotic regulatory proteins (Aravind *et al.* 2003; Iyer *et al.* 2003). In eukaryotes, NO signalling is known to play an important role in cGMP turnover and the regulation of diverse processes, including apoptosis, cell proliferation and differentiation. Intriguing similarities exist between the signalling role of NO in eukaryotes and its control of biofilm cell differentiation, death and dispersal, as demonstrated in this study and by Barraud *et al.* (2006). Biofilms are thought to exhibit development analogies with multicellular eukaryotes (Branda *et al.* 2004; Webb *et al.* 2003); therefore, the examination of bacterial biofilm populations may provide information about the origins of key regulatory processes in these more complex organisms. The NO-mediated control of biofilm development in both MRSA and *P. aeruginosa* may point to a conserved role for NO signalling in the regulation of differentiation and development events across prokaryotic and eukaryotic physiology.

NO is an important effector of host innate immunity, attributed both to its antimicrobial activity and immunomodulatory role. Inflammatory NO has been shown to be indispensable for normal clearance of diverse intracellular and extracellular pathogens including viral, fungal, bacterial and parasitic microorganisms (De Groote 1999). NO is generated through the action of an inducible nitric oxide synthase (iNOS) (Beckerman *et al.* 1993; Boockvar *et al.* 1994; Chan *et al.* 1995; Lewis *et al.* 1995 and Nalwaya *et al.* 2005). Evolution may have led mammalian systems to also use the iNOS system to generate NO at the low concentrations that modulate MRSA biofilm infection. Concentration of NO have been measured *in vivo* between 25 nM – 15 nM for spleen cells, and activated leucocytes can generate local concentrations of NO in the micromolar range

(Firoved *et al.* 2004). NO at these concentrations could induce dispersal of biofilm cells and induce a return to the less resistant planktonic phenotype. However, these concentrations, are also low enough that they would not compromise the essential homeostatic mechanisms of the host such as blood pressure. This supports the proposal of Sasaki *et al.* (1998) that NO produced by the iNOS system has a protective role in *S. aureus* infection in mice, resulting in lower mortality levels.

Biofilm Control

Strategies to induce biofilm dispersal are of interest due to their potential to prevent biofilms and biofilm-related infections. Here, with low non-toxic doses of NO, induced reductions in MRSA biofilm biomass, and also by further treatment with antimicrobial agents, both the percentage viability and biomass were further significantly reduced.

Although previous studies have demonstrated that NO can reduce initial bacteria attachment to surfaces, the mechanism for reduced attachment was assumed to be that of NO toxicity and of nitrosative stress (Firoved *et al.* 2004 and Nablo *et al.* 2003). The present investigation, and that of Barraud *et al.* (2006), are the first to demonstrate a role for NO in dispersal events from mature biofilms and the first to suggest that NO may be involved in regulated processes of differentiation within multicellular biofilms.

Further research needs to be undertaken to determine how to deliver the relevant concentrations of NO to bacterial biofilms in environmental and medical settings.

10. GENERAL CONCLUSIONS AND FURTHER WORK

In the industrialised world, acute bacterial infections, caused by rapidly proliferating planktonic cells, have been gradually replaced by chronic infections due to environmental organisms growing as biofilms (Fux *et al.* 2005). Now more than 60% of the bacterial infections currently treated by physicians in the developed world are considered to involve biofilm formation (Costerton *et al.* 1999). Bacterial biofilms are difficult to detect in routine diagnostics and the failure of conventional culture techniques to predict antibiotic susceptibilities of biofilm communities explains part of our failure to eradicate biofilm-related infection. Also many promising antimicrobial agents fail at clinical trial due to initial screening against standard planktonic cultures, which do not express the resistant biofilm phenotype (Nicholls 1993).

A variety of model systems have been designed to measure biofilm formation and specific biofilm characteristics, of these the microtitre plate format was chosen because of its simplicity of use, low cost and high throughput. These assays have been successfully used previously to assay biofilm formation by *S. epidermidis* but not for *S. aureus* strains until now. Numerous microtitre biofilms assays were evaluated, but they gave little or no information about cell viability within the biofilms, and were not suitable for assaying the effects of antimicrobial agents against preformed biofilms. However, more importantly these assays were not suitable for *S. aureus* biofilms, as they are not as robust as the *S. epidermidis* biofilms and detach more easily.

Using the Christensen *et al.* (1995) Safranin assay and Congo red staining it was shown that *S. aureus* biofilms form in a similar manner to *S. epidermidis* biofilms and that there was no difference between methicillin resistant and methicillin susceptible strains. This is important because biofilm formation has not long been recognised as an important virulence factor for *S. aureus* infections.

The existing Christensen *et al.* (1985) microtitre plate Safranin assay was improved by optimising the growth conditions for *S. aureus* biofilm formation and was modified to give quantitative data for the determination of total cell numbers in the biofilms. There was no adequate pre-existing assay for determining the viable cells number; therefore, the chromogenic viability dye XTT was investigated. XTT in conjunction with PMS give a reliable determination of number of viable cells within the biofilms. The variability within the two assays, although around 18%, was found not to be significant. Control biofilms (24-hours-old) contained around 3.5×10^6 total cells; between 50% and 67% of these cells were deemed viable by the XTT / PMS assay. The variation in viability was

also observed by the reduction of TTC by MRSA 24-hour-old biofilms. The reason that only 50-67% of the cells were viable in untreated biofilms is not known; however, it could be a factor of using the two assays in conjunction. Further work is needed to determine whether this is the case and how the percentage viability and total biomass of untreated *S. aureus* biofilms varies depending on environmental factors such as temperature, pH and growth media.

The production of EPS within MRSA biofilms was found to be heterogeneous; this was not surprising, given the nature of the biofilm (nutrient and oxygen gradients) and its complex structure. MRSA biofilm formation was increased when cultures were grown in quarter strength BHI medium, supplemented with 0.09% (w/v) glucose. Glucose is a known inducer of EPS production, and it was thought that limitation of other nutrients in the diluted medium induced stress responses that control biofilm formation; for example glucose metabolism caused a decreased pH that may have affected biofilm formation. In future work it will be important to determine whether the observation of increased biofilm formation in $BHI_{25\%}$ + 0.09% glucose is due to overall increased planktonic growth rate (maintaining the same planktonic cell to biofilm ratio), or whether it is due to a larger percentage of cells in the culture becoming biofilm cells. This could be achieved using a chemically defined medium, in which the components could be systematically changed in order to identify essential components for *S. aureus* biofilm formation.

Microscopy observations of differently aged biofilms eluded to the fact that immature (8-hour-old) biofilms and mature (24-hour-old) *S. aureus* biofilm formations could be distinguished purely by the amount of EPS present, which could be observed / quantified by the amount of Congo red staining. However, several *S. aureus* mutants that are deficient in one of more of the stages of biofilm development (i.e. adherence to plastic, intercellular adhesion and the production of a slime matrix) have been previously described in the literature; and using Congo red stained images of the these biofilm mutants it should be possible to more clearly define the stages of biofilm development and link them more fully to the times points used in the biofilm assay. This therefore will provide support for evidence presented in this work, that there is a measurable physiological difference between the three time points tested. To achieve this it would be necessary to obtain *S. aureus* biofilm developmental mutants (e.g. the *dltA* mutant and the *ica* mutant). The *dlt*A mutant described by (Peschel *et al.* 1999) expresses teichoic acids that lack the D-alanine. These cells have an increased negative charge and it has been postulated that this increase leads to an increased scavenging of positively charged antimicrobial peptides, thus leading to hypersensitivity. The mutant is biofilm negative and is severely affected in adherence, the first step of biofilm formation, to polystyrene or glass surfaces (Gross *et al.* 2001). Biofilm formation of the *dltA* mutant can be completely restored by the addition of Mg^{2+} ions. The *ica* operon controls the production and expression of polysaccharide intercellular adhesin. An *ica* mutant leads to a pleiotrophic

phenotype; the cells are biofilm and heamagglutination negative, less virulent and less adhesive on hydrophilic surfaces (Heilmann *et al.* 1996b). If Congo red can be used to determine the stage of biofilm development, it would be useful to determine if it could be used to obtain the same information about clinical biofilms *in vivo*. If this was the case then in the future modified Congo red assays could be used to determine the stage and / or rate of biofilm development formed on accessible surfaces, such as the skin, which could be biopsied and stained. This information could be used to learn more about how *in vivo* biofilm formation compares to *in vitro* biofilm formation, and could be useful in deciding relevant treatment strategies.

Work in this thesis has provided an explanation as to why it is difficult to treat patients with antibiotis such as vancomycin once the infection has become well established. I believe this is due to the biofilm that is formed. Consequently it is very important to prevent MRSA survival in the hospital environment to reduce patient infection. The number of nosocomial MRSA infections is on the rise; MSSA and MRSA can survive on dry surfaces for prolonged periods, therefore it is important that surfaces are regularly cleaned and disinfected to prevent cross-contamination to non-infected patients. Using the combined biofilm assay the efficacy of a range of disinfectants used to control contamination by the Southampton General Hospital were tested for their ability to prevent biofilm formation and their efficacy against pre-formed immature and mature biofilms. All of the hypochlorite-based hospital cleaning chemistries failed to kill mature MRSA biofilms, highlighting the fact that the recent increases in nosocomial MRSA infections could be a by-product of the failure of the cleaning procedures and / or the products used to remove MRSA cells and biofilm from the environment. A peroxygen-based disinfectant Virkon was also ineffective in killing mature MRSA biofilms. The phenolic-based disinfectant, Hycolin, did have a small effect on mature MRSA biofilms, however, the reduction in viability was only 15%, and the biofilms are therefore still 35% viable, which equates to $1.0x10^6$ CFU ml^{-1}. The fact that the MRSA mode of growth is resistant to hospital cleaning chemistries is of great concern, this data supports the work done by French *et al.* (2004) and Dancer *et al.* (2006) who previously determined that the hospital environment plays a large role in MRSA nosocomial infection and that MRSA can survive for long periods of time. An important implication arising from the data presented in this thesis is that hospital surfaces, or any other surface for that matter, should be cleaned thoroughly and regularly to prevent MRSA adapting its physiology on the surface to become resistant to the biocides.

The hospital cleaning chemistries were tested only at their stated working concentrations, further work should be undertaken to determine their MIC against mature MRSA biofilms. One failure of the work is that the combined biofilm assay in no way represents an environmental MRSA biofilm; by changing the temperature, type and volume of medium, the combined biofilm assay could be modified to better represent an environmental biofilm e.g. more dehydrated. Using this modified

assay the disinfectant work needs to be repeated, so that the efficacy of cleaning chemistries can be evaluated in a model more representative of the environment where they are used. Following on from this it would be of great hygienic value if the efficacy of these cleaning chemistries could be evaluated against MRSA biofilms actually in the hospital environment. Many ward trials have already been successfully undertaken; therefore it should be possible to set up a trial to evaluate the efficacy of these cleaning chemistries in a working ward. Of great scientific value will be the elucidation of the mechanism(s) of biofilm resistance to these aggressive disinfectants. Is resistance due to EPS production, cell membrane modification, or protection or internal macromolecules such as nucleic acids and proteins? The use of appropriate mutations, for example in *ica* and *dltA*, and sigma factors, DNA repair mechanisms etc. should be investigated in future studies.

The biocide resistance data should not be considered solely in terms of hospital hygiene. Indeed the resistance of MRSA biofilms to disinfectants suggests that more care should also be taken in the microbiology research laboratory to protect research workers, and that we should not rely on the disinfectant chemistries investigated so far for complete disinfection of the laboratory where biofilm formation may have occurred as a result of accidental spills.

Since the discovery of penicillin in 1938, antibiotics have proven tremendously successful in controlling acute bacterial infections. Microbiologists have learned to predict antibiotic effects *in vivo* by evaluating the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) *in vitro*. MIC and MBC assess the effect of antibiotics against planktonic organisms in the exponential phase of growth and therefore correctly predict antibiotic efficacy against rapidly dividing bacteria in acute infections, such as septicemia. However, in a growing number of chronic and device-related infections in which antibiograms of the causative organism show sensitivity to standard antibiotics in readily attainable concentrations, the infection fails to be cleared. Microscopic evaluations of these infections have revealed the bacteria to the growing as surface-adherent biofilms. Bacteria in the biofilm mode of growth are inherently tolerant to host defenses and antibiotic therapies and are therefore of major clinical importance. Successful treatment in these cases often depends on long-term, high-dose antibiotic therapies and the removal of any foreign-body material. Biofilm growth therefore represents a major risk factor for the spread of antibiotic resistance by promoting horizontal gene transfer (Fux *et al.* 2005).

MRSA cultures were tested against vancomycin treatment; planktonic cultures had MICs of between 1 and 2 mg l^{-1} but, again, mature biofilms were found to be more resistant than previously thought. Indeed, viable cells were still found within the biofilm even after treatment with vancomycin at 2048 mg l^{-1} for 18 hours. The reason for this considerable resistance is not clear; however, it possibly involves decreases in peptidoglycan production, reduced transport through,

and deactivation in the EPS matrix. Given this phenotypic resistance of biofilm to vancomycin, it is suspected that formation of MRSA biofilms might account for the increasing persistence of MRSA infections even though treatment appropriate for treating the planktonic organism is being given.

The resistance of mature MRSA biofilms to vancomycin is important as vancomycin is often quoted as being the last line of defence against MRSA infection (Costerton *et al.* 1999). One way of overcoming the resistance of medical biofilms is to increase the dosage of the antibiotic; however, the dosing of vancomycin is tightly controlled and increased concentration leads to serious side-effects, such as hearing loss and kidney failure. Once more further work needs to be undertaken, vancomycin is rarely used alone in the treatment of clinical infections and is often used in conjunction with rifampicin and other antibiotics, it is important that these antibiotic combinations are tested against MRSA biofilms to see if they have any significant effects on preformed MRSA biofilms. Also vancomycin needs to administered intravenously (IV) for systemic therapy since it does not cross through the intestinal lining. Due to difficulties with its use, including side effects and the increase in vancomycin resistance, other alternatives need to be found for MRSA biofilm treatment.

A range of antibiotics with differing modes of action was tested against MRSA, EMRSA and MSSA biofilms. Only one group of antibiotics, the ionophores, was capable of reducing the biomass and percentage viability of mature biofilms. The antimicrobials ceftriaxone, dalbavancin, daptomycin, imipenem and meropenem, were requested from their manufacturers but were not received during the course of this investigation. It is important that in the future these antibiotics are tested against the MRSA biofilm phenotype as some of them may be adequate replacements for vancomcyin in treating MRSA infections; therefore it is important to know what effect, if any, they have on preformed MRSA biofilms.

Several mechanisms for biofilm resistance were identified including: the adoption of a biofilm phenotype, the formation of persister cells, retarded penetration of antimicrobial agents through the biofilm matrix, a reduction in growth or a starved state leading to slower metabolism, the effect of the microenvironment of the antimicrobial agents, and changes in the composition of the cell membrane. However, biofilm resistance is probably a combination of several mechanisms, which vary, depending on the antibiotic in question. At present, there is no plausible explanation as to why the ionophores are able to act against mature biofilm when other antibiotics are ineffective, however, the results suggest that their activity may relate to the fact that they do not have to enter the cells to have an effect and so are not affected by efflux proteins or a reduction in growth rate / metabolism. Also the results show that the presence of the EPS matrix had little or no effect on the ionophore activity, suggesting that the EPS matrix does present a diffusion barrier to these

molecules and also that the ionophores are not degraded / deactivated in any way by proteins within the matrix.

Two patterns of damage have been observed by direct microscopy. First, a top-to-bottom gradient of decreasing antibiotic susceptibility. This gradient originates in the surface layers of biofilms where there is almost complete consumption of oxygen and glucose, leading to anaerobic nutrition-depleted niches with restricted metabolic activity in their depths (Anderl *et al.* 2003). Metabolic inactivity might reach an extent where biofilm bacteria are still viable, but have lost culturability. Second, there is a patchy distribution of bacterial survival within presumably identical microenvironments (Huang *et al.* 1995). Microscopy images of ionophore-treated MRSA biofilms (not shown) suggest that the second pattern of killing occurs more dramatically than the first, evidenced by clear plaque formation (holes) within the biofilm. Further biochemical and molecular investigation of cells in areas where plaque formation is just occuring and surrounding the biofilm where no plaque formation is obvious may help us to understand mechanisms of antibiotic suseptibility and tolerance within these biofilms.

Synergistic effects were seen when the ionophores were used in combination with antibiotics of differing modes of action. Mates *et al.* (1983) demonstrated that changes in the magnitude of $\Delta\Psi$, that occur when cells are grown in anaerobic conditions, led to a reduction in aminoglycoside diffusion, and that this reduction in diffusion was reversed in the presence of the ionophore nigericin, leading to a possible explanation for the observed synergistic effects. Indeed, the work here suggests that the cytoplasmic membrane may play an important role in the biofilm-resistant phenotype, which has not so far been recognised.

Only four ionophore antibiotics were investigated in this work, further work needs to be undertaken to see if other ionophore antibiotics have the same efficacy against mature MRSA biofilm, and also whether the ionophores are active against other biofilm forming bacteria. Also, further investigation to determine the exact mechanism of action of the ionophores against biofilm bacteria needs to be undertaken, starting with labelling of the ionophores to see if they are indeed binding to the cell membrane and how well they are able to diffuse though the biofilm. Conversely, the ionophore antibiotics are not used in human medicine, as they do not discriminate between prokaryotic and eukaryotic membranes, and their use is therefore limited to topical applications and as a bacterial inhibitor in animal feeds. However, although the ionophore antibiotics are relativly expensive, additional work should be undertaken to investigate the possibility of using the ionophore antibiotics as disinfectant agents for the cleaning of MRSA wards in hospitals, as they may prove to be very effective.

As the ionophore antibiotics are not suitable for human use, this work underlines the need for novel antimicrobial agents, capable of circumventing all the biofilm resistance mechanisms, or the discovery of co-drugs, which can disarm the biofilm resistance mechanisms, making the biofilms more susceptible to existing antibiotics. To this end, a library of novel antimicrobial agents was screened. Several chemicals were found to be active at low concentrations against the mature MRSA phenotype, including: three related to the diarylureas, three putative inhibitors of FabI (not structurally related to triclosan), one oxazolidinone derivative and two analoges of rifampicin. However, at present the full mechanisms of action for each of these chemicals are unknown. Therefore, further work needs to be undertaken to find out their precise mechanism of action, their MIC against immature and mature biofilms, their range of antimicrobial activity, and their toxicity before they can be considered for animal trials.

The observation of antimicrobial tolerance in biofilms that are too thin to represent a relevant diffusion barrier for metabolic substrates suggests that starvation-induced dormancy is not the only reason for antimicrobial tolerance. It was this observation that led to the hypothesis of a genetically controlled biofilm-specific phenotype. This concept is of particular interest because the control of key biofilm genes would offer excellent options to overcome tolerance. A multitude of strategies have been applied to compare gene and protein expression patterns in biofilms with those in planktonic cultures. When assessed by DNA microarrays, gene expression in biofilms differed from that in planktonic cultures by 580 genes, many of which were involved in acid tolerance or were part of the *SarA* operon (Beenken *et al.* 2004). Staphylococcal biofilm formation has been attributed to the staphylococcal accessory regulator (*SarA*) (Beenken *et al.* 2004) and the *icaADBC* gene cluster (Cramton *et al.* 1999). However, Knobloch *et al.* (2002) reported that virtually all *S. aureus* strains contain the *ica* gene cluster, but do not necessarily produce biofilms. This observation underlines the importance of additional control mechanisms, such as subinhibitory antibiotic concentrations, phase variation, quorum sensing or icaR, a transcriptional repressor of ica expression under environmental control (Valle *et al.* 2003).

Antibiotic resistance of the mature MRSA biofilm phenotype was investigated in the presence of two *S. aureus* QS systems, *agr* and the 'universal' signal AI-2. In general, the accessory gene regulator (*agr*) system in *S. aureus* represses several surface adhesins that mediate contact with the host matrix (Yarwood *et al.* 2003). These include fibrinogen and fibronectin-binding protein. Under certain conditions, *agr* mutants adhere more efficiently than wide-type strains (Vuong *et al.* 2000). However, while a mutant of the *agr* system showed greatly enhanced biofilm formation / cell adherence, there were no differences in the biofilm-specific resistance of the *agr* mutants and its wild-type strain. The increase in biofilm formation is thought to be due to the loss of δ-toxin,

which has been implicated in decreased detachment of cells from the biofilm. Further work needs to be conducted to determine whether AIP is expressed in MRSA biofilms and if so at what stage of biofilm development AIP is expressed, and the agr system activated. Also it would be useful to determine how the secretion of AIP is affected by changes in the environmental conditions.

However, when antibiotic resistance was compared between the *agr* positive and negative strains there was no significant difference for any of the antibiotics tested. This strongly suggestes that under the environmental conditions tested here *agr* plays very little or no role in biofilm phenotypic antibiotic resistance. It has been suggested by Yarwood *et al.* (2004) that under different environmental conditions the role of *agr* in biofilm formation changes. Further work is needed to see if *agr* plays a greater role in biofilm phenotypic resistance under different environmental conditions not investigated in the current work.

The QS signal AI-2 is considered to be the universal signal for interspecies cell-to-cell communication. Reports have shown that *S. aureus* posesses the relevant genes for AI-2 production, however, it is interesting to note that *S. aureus* has also been shown not to contain any potential homologues for either the *lsr* AI-2 uptake system or the LuxP / LuxQ AI-2 signal transduction system (Doherty *et al.* 2006). Using the Bassler *et al.* (2003) AI-2 assay it was shown that under the conditions used in the combined biofilm assay AI-2 was not significantly produced in MRSA planktonic cultures or in biofilms. However, further work is needed to determine whether AI-2 is produced by *S. aureus* cultures under differening environmental conditions.

The effect of the addition of chemically synthesised exogenous AI-2 on biofilm formation showed that high concentrations caused a significant reduction in biofilm biomass while having no effect on the percentage of biofilm viability. Therefore, it was concluded that the reduction in biomass in the presence of exogenous AI-2 could not be related to toxicity. In addition, biofilms grown in the presence of AI-2 were less resistant to several antibiotics. In *S. epidermidis* it has been suggested that AI-2 down-regulates the production of PIA / PNAG; if this is also the case for *S. aureus,* it could provide a plausable explanation for the increased susceptibility seen in the presence of exogenous AI-2. However, further work is needed to determine whether PIA / PNAG is indeed reduced in the presence of exogenous AI-2. Congo red staining of treated and untreated biofilms should give a quick observation of the amount of PIA / PNAG present. It is also extremely important to determine the mechanism by which AI-2 regulates biofilm formation and PIA / PNAG production since none of the currently known AI-2 signal transduction systems are found in *S. aureus*. If a new system is discovered it may lead to a better understanding of QS systems in general.

The mammalian signilling molecule nitric oxide (NO) was recently shown to control the development and dispersal of *P. aeruginosa* biofilms; it was therefore decided to see if NO could modulate MRSA biofilm formation and antibiotic resistance. The addition of very low concentrations of the chemical signal, NO, caused reductions in MRSA biofilm formation and significantly increased biofilm susceptibility to a range of antimicrobials. From the previous work done with *P. aeruginosa* it is thought that NO causes biofilm dispersal and also possibly causes a reversion to the planktonic (more susceptible) phenotype; indeed NO may already be employed for a similar purpose in the innate immune system. Although the addition of NO had similar effects on the MRSA biofilms to the *P. aeruginosa* biofilms, further work needs to be undertaken to determine if the reduction in biofilm biomass is due to either increased dispersal of live cells from the biofilm or increase in cell death and subsequent detachment from the biofilm. It was proposed by Barraud *et al.* (2006) that detachment was occuring in the *P. aeruginosa* biofilm due to the cells reverting back to a planktonic phenotype; it would therefore be important to determine whether cells depersing from the MRSA biofilms in the presence of NO were reverting back to the planktonic phenotype in terms of their antibiotic resistance. This work is very promising as there is the potential of NO as a co-drug in order to promote the susceptible planktonic phenotype, and allow existing antibiotics to be used with renewed susses.

The studies with NO herald the potential for a new era in biofilm formation and control. This is especialy pertinent to MRSA survival in the hospital environment and transmission to patients. It is proposed that future studies with disinfectants and antibiotics should be undertaken with a view to understanding the potential syergistic advantages of NO co-addition.

APPENDIX A

A.1 Minimum inhibitory concentrations determined for antibiotics with differeing modes of action

Table 34 *MICs (mg l^{-1}) determined for antibiotics that interfere with cell wall synthesis.*

Chemical / Agent	NCTC 11939	NCTC 13143	NCTC 10442	RN 9611	NCTC 12973	*EUCAST NCTC 12973
Ampicillin	>32	>32	>32	1	0.5	0.5
Vancomycin Hydrochloride	2	1	1	2	1	1
Phosphomycin	16	16	8	4	4	
Bacitracin	>32	>32	8	>32	>32	

*Target MICs EUCAST (2000)

Table 35 *MICs (mg l^{-1}) determined for antibiotics that interfere with cell membrane permeability.*

Chemical / Agent	NCTC 11939	NCTC 13143	NCTC 10442	RN 9611	NCTC 12973	*EUCAST NCTC 12973
Gramicidin	2	4	4	4-8	4-8	
Monensin	1	1	1	1	1	
Nigericin	0.06	0.06	0.03	0.01	0.01	
Narasin	0.25	0.25	0.25	0.125	0.06	

*Target MICs EUCAST 2000

Table 36 *MICs (mg l^{-1}) determined for antibiotics that interfere with transcription and DNA synthesis.*

Chemical / Agent	NCTC 11939	NCTC 13143	NCTC 10442	RN 9611	NCTC 12973	*EUCAST NCTC 12973
Ciprofloxacin	0.25	>32	0.125	0.25	0.25	0.25
Rifampicin	0.5	<0.01	32	<0.01	0.01	0.01
Nitrofurantoin Crystalline	>32	16	16	16	16	16
Actinomycin D	>32	16	>32	32	8	
Novobiocin Sodium Salt	1	1	1	0.06	0.03	
Sulphamethoxazole	>32	>32	>32	32	>32	
Trimethroprim	16	8	8	8	2	2

*Target MICs EUCAST 2000

Table 37 *MICs (mg l^{-1}) determined for antimicrobial agents that interfere with translation.*

Chemical / Agent	NCTC 11939	NCTC 13143	NCTC 10442	RN 9611	NCTC 12973	*EUCAST NCTC 12973
Tetracycline Hydrate	>32	>32	>32	16	0.5	0.5
Erythromycin	32	>32	0.5	0.5	0.5	0.5
Gentamycin	>32	>32	4	0.5	0.25	0.25
Kanamycin	>32	>32	0.5	0.25	0.25	0.25
Tobramycin	>32	>32	4	0.5	1	0.5
Clindamycin Hydrochloride	>32	>32	>32	0.125	0.125	
Chloramphenicol Hydrochloride	16	8	4	1	1	0.5
Fusidic Acid Sodium Salt	1	1	1	0.125	0.125	0.06

*Target MICs EUCAST 2000

APPENDIX B

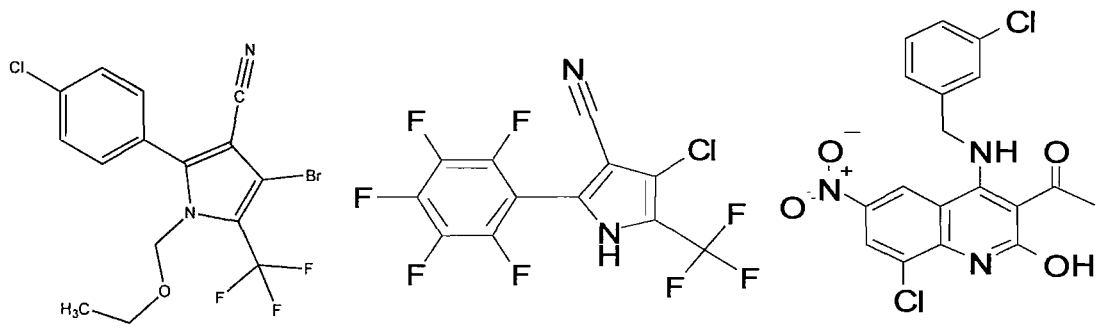
B.1 Minimum inhibitory concentrations of the novel antimicrobial agents

Table 38 *The MICs (mg l^{-1}) of the novel antimicrobial agents determined for four S. aureus strains.*

Chemical Number	NCTC 11939	NCTC 13143	NCTC 10442	NCTC 12973
C01	8	1	4	2
C02	2	0.5	1	1
C03	4	2	16	8
C04	4	2	2	2
C05	0.125	0.5 – 1	0.5 – 1	2
C06	8	8	4	4
C07	8	1	2	2
C08	2	1	2	2
C09	> 32	0.5	0.5 – 1	1
C10	0.5	0.03	0.015	0.015
C11	>32	2	4	2
C12	8	4	8	8
C13	4	2	4	4
C14	16	2	2	2
C15	> 32	1	0.5	2
C16	> 32	1	0.5	1
C17	2	1	1	2
C18	32	4	2	2
C19	> 32	8	2	2
C20	8	2	4	4
C21	8	8	16	8
C22	>32	2	2	1
C23	4	4	4	4
C24	8	8	4	4
C25	4	1	4	4
C26	2	0.5	4	2

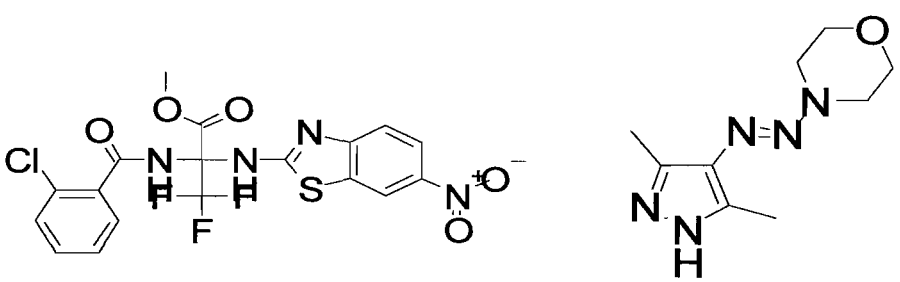
C27	8	4	8	8
C28	32	0.5	0.25	0.25
C29	> 32	8	4	2
C30	> 32	2	1	1
C31	> 32	2	6	0.5
C32	> 32	8	4	4
C33	16	1	0.5	0.5
C34	8	4	4	4
C35	8	1	4	2
C36	8	0.25	8	4
C37	4	2	2	2
C38	8	1	1	2
C39	8	0.5	1	1
C40	2	1	2	2
C41	8	4	8	8
C42	8	4	8	8

APPENDIX C



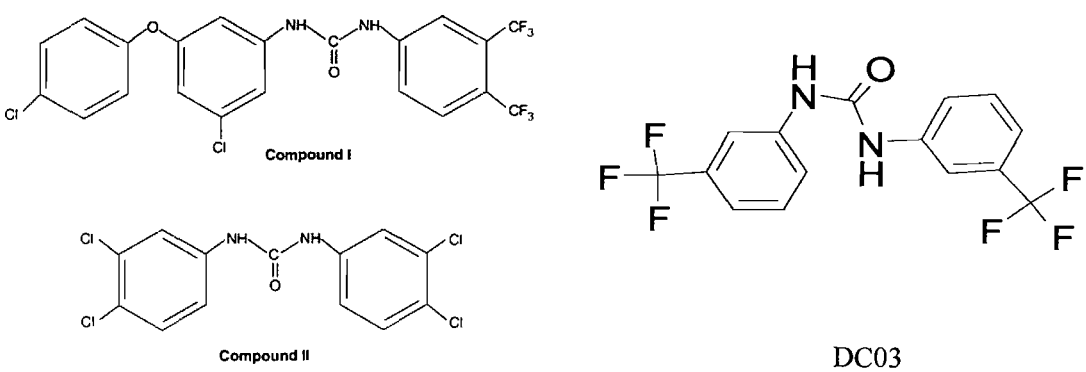
Chlorfenapyr DC14 DC17

Figure 43 *DC14 and 17; structurally related to the insecticide Chlorfenapyr (a.k.a. Pirate) (www.wuzhouchem.com/cataloged/agro/insecticide/chlorfenapyr.htm).*



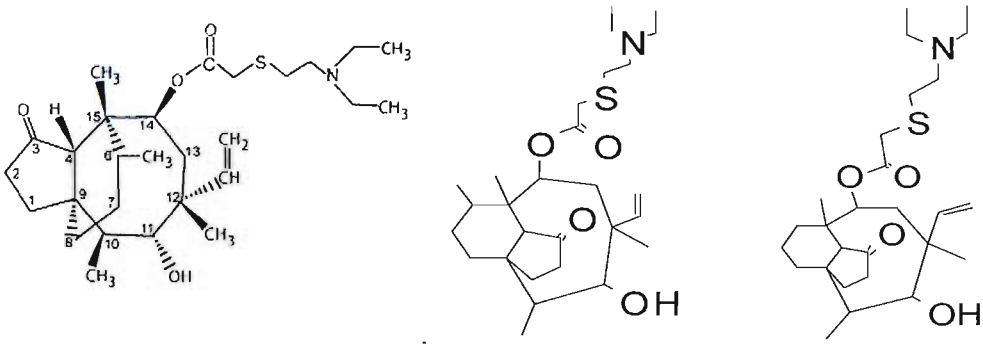
DC21 DC27

Figure 44 *The structure of DC21 and 27; both have high toxicity against the Sf21 cell line*



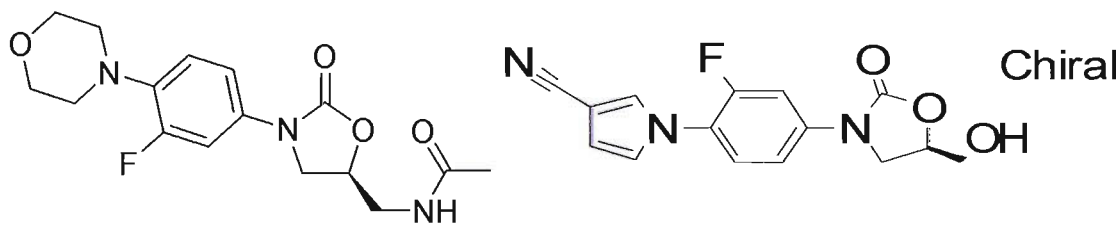
DC03

Figure 45 *The structure of the two DAU compounds used by Proctor et al. (2002) and the related DAU, DC03.*



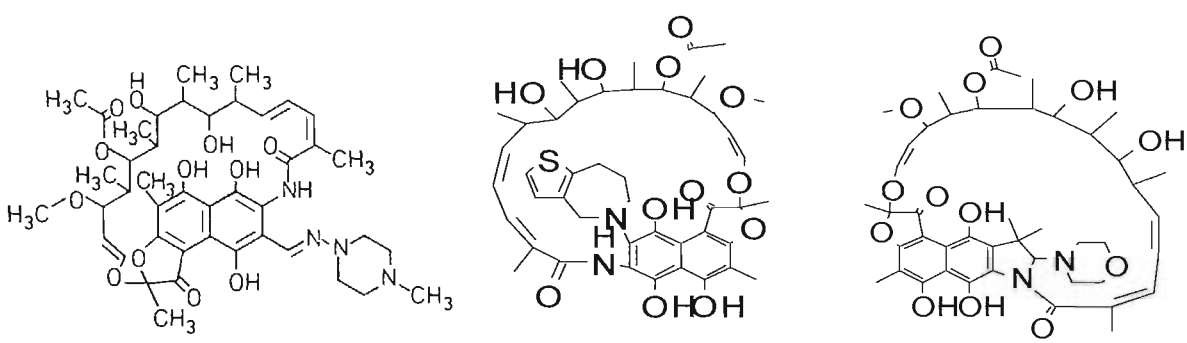
Tiamulin DC15 DC16

Figure 46 *The structure of tiamulin (Schlünzen et al. 2004) and its two derivatives, DC15 and DC16*



Linezolid DC05

Figure 47 The *structure of linezolid (www.wikipedia.org/linezolid) and the oxazolidinone derivative DC05.*



Rifampicin DC10 DC11

Figure 48 *The structures of DC10 and DC11, analogues of rifampicin (www.infektionsnetz.at/AntibiotikaRifampicin.phtml).*

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