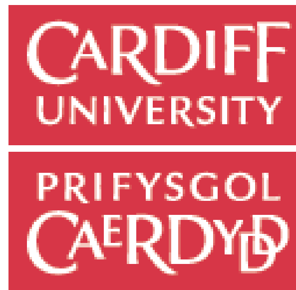
Small colony variants in *Staphylococcus aureus* and other species: antibiotic selection, antimicrobial susceptibility, and biofilm formation



Thesis presented for the Degree of Philosophiae Doctor

by

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September 2011

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SCIENTIFIC CONFERENCES, PRESENTATIONS AND PUBLICATIONS

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Norville, P., Plummer S., and Day, M. Stress responses of *Staphylococcus aureus* small colony variants. 13th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI) – Cairns, Australia, September 2008.

Norville, P., Plummer S., and Day, M. Selection and characterisation of *Staphylococcus aureus* small colony variants after aminoglycosides exposure. South Wales and South West Microbiology Forum (SWSWF) – Cardiff, Wales, September 2010.

Norville, P., Aminoglycoside selection and biofilm formation in *Staphylococcus aureus* small colony variants. Oral presentation at Cultech/Obsidian Research Open Day. Port Talbot, Wales, October 2010.

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SUMMARY

Staphylococcus aureus is one of the leading causes of hospital acquired infections. The ability of *S. aureus* to acquire resistance to a diverse range of antimicrobial compounds, results in limited treatment options, particularly in methicillin-resistant *S. aureus*. A mechanism by which *S. aureus* develops reduced susceptibility to antimicrobials is through the formation of small colony variants (SCVs). Reduced antimicrobial susceptibility in *S. aureus* SCVs is not related to 'classical' mechanisms of resistance, but occurs as a direct result of the development of the SCV phenotype. *S. aureus* SCVs are frequently associated with defects in the bacterial electron transport chain and these defects are responsible for the characteristics associated with the SCV phenotype.

This study aimed to investigate and characterise the selection of *S. aureus* SCVs in the presence of various antibiotics and also to examine their biofilm forming capabilities. Four members of the aminoglycoside family of antibiotics were shown to select for *S. aureus* SCVs. In addition, a broad range (X 0.25 MIC – X 4 MIC) of aminoglycoside concentrations were shown to select for *S. aureus* SCVs. Characterisation of these isolates revealed that differences in auxotrophy, biochemical profiles, carotenoid production, haemolysis, levels of intracellular ATP, mutation frequency and reversion rate were present. Members of the tetracycline family of antibiotics were also shown to select for *S. aureus* SCVs. Tetracycline selected *S. aureus* SCVs show attenuated catalase, coagulase and heamolysis activity and reduced production of extracellular DNase and lipase and reduced susceptibility to various antimicrobial agents. As SCVs have been linked to persistent and recurrent infections their ability to form biofilms was also investigated. A range of *S. aureus* SCVs isolated from various backgrounds were shown to form greater biofilms in comparison to parent strains, which was attributed to increased production of polysaccharide intracellular adhesin. In addition *S. aureus* SCV biofilms displayed a more pronounced reduction in antimicrobial susceptibility, which was attributed to a reduction in antimicrobial penetration through SCV biofilms.

Limited discovery of novel antibiotics in recent years and the observation that *S. aureus* SCVs can be selected for by various antimicrobial compounds highlights the need for novel antimicrobial compounds. Accordingly, an investigation into the susceptibility of *S. aureus* to various plant compounds was undertaken. Both *S. aureus* SCVs and parent strains showed susceptibility to five plant antimicrobials tested, of which SCVs were more susceptible to cinnamon bark, green tea and oregano. Resistance to these plant antimicrobials could not be induced and synergistic relationships between certain plant antimicrobials and antibiotics were demonstrated. Finally, formation of SCVs in bacterial species other than *S. aureus* was examined. Gentamicin induced SCV selection in *Escherichia coli*, *Pseudomonas aeruginosa* and *S. epidermidis* as well as chloroamphenicol and ciprofloxacin in *E. coli* and tetracycline in *S. epidermidis*. SCVs from these bacterial species shared common characteristics associated with the SCV phenotype including altered growth and biochemical profiles, auxotrophy for compounds involved in electron transport, reduction in expression of virulence factors and reduced antimicrobial susceptibility. Additionally all SCVs showed an increased capacity to form biofilms.

The ability of certain antibiotics to select for SCVs and their increased capacity to form biofilms suggest that SCV are an important adaptation to aid survival and persistence in times of stress. Reduced susceptibility to commonly used antibiotics in SCVs signifies that the development of new antimicrobial compounds is required. Harnessing naturally occurring plant antimicrobials and their synergistic relationship with antibiotics may offer a novel approach to treating antibiotic resistant infections whilst overcoming antibiotic selection for SCVs.

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TABLE OF ABBREVIATIONS

AAC	aminoglycoside acetyltransferase
agr	accessory gene regulator
AIDS	acquired immunodeficiency syndrome
AIP	autoinducing peptide
AMEs	aminoglycoside modifying enzymes
AMIR	antibody-mediated immune response
APH	aminoglycoside phosphotransferase
ANT	aminoglycoside nucleotidyltransferase
ATP	adenosine triphosphate
BAP	biofilm associated protein
BSAC	British Society for Antimicrobial Chemotherapy
CAMHB	cation adjusted Muller Hinton broth
CA-MRSA	community acquired methicillin resistant *Staphylococcus aureus*
CCs	clonal complexes
CFS	chronic fatigue syndrome
CFU	colony forming unit
CIP	ciprofloxacin
CLSI	Clinical Laboratory and Standards Institute
Clf	clumping factor
CMIR	cell mediated immune response
CF	cystic fibrosis
CFS	chronic fatigue syndrome
CoNS	coagulase negative staphylococci
c-di-GMP	cyclic-diguanylate GMP
DECO	decomplexation solution
DMMB	dimethyl methylene blue
DMSO	dimethylsulfoxide
DNase	deoxyribonucleic acid
DOX	doxycycline
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EAP	extracellular adherence protein
EC	-epicatechin
ECG	-epicatechin gallate
ECM	extracellular matrix
EGC	-epigallocatechin
EGCG	-epigallocatechin gallate
EHEC	enterohaemorrhagic *Escherichia coli*
EMRSA	epidemic methicillin-resistant *Staphylococcus aureus*
EO	essential oil
EPEC	enteropathogenic Escherichia coli
EPS	extracellular polymeric substances
ETC	electron transport chain
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAB	formic acid buffer
FADH2	flavin adenine dinucelotide
FDA	Food and Drug Administration
FIC	fractional inhibitory concentration
FICi	fractional inhibitory concentration indices
FnBPs	fibronectin-binding proteins
GA	glycerol alanine
GI	genomic island

HAC	hydroxyapatite cement
HGT	horizontal gene transfer
HQNO	4-hydroxy-2-heptylquinoline-N-oxide
H%	cell-surface hydrophobicity percentage
ica	intracellular adhesin
IVET	*in vivo* expression technology
IS	insertion sequence
J	junkyard
kb	kilobase
LB	Luria broth
LPS	lipopolysaccharide
MBC	minimum bactericidal concentration
MFS	major facilitator superfamily
MGEs	mobile genetic elements
mg/L	milligrams per litre
MIC	minimum inhibitory concentration
MH	Mueller Hinton
MLS	macrolide–lincosomide–streptogramin B
MLST	multilocus sequence typing
MRSA	methicillin-resistant *Staphylococcus aureus*
MMR	methyl-directed mismatch repair
MSCRAMMs	microbial surface components recognising adhesive matrix molecules
MSSA	methicillin-sensitive *Staphylococcus aureus*
MSCVPC	minimum small colony variant prevention concentration
NADH	nicotinamide adenine dinucelotide
nm	nanometer
OD	optical density
OM	outer membrane
OXA	oxacillin
OXY	oxytetracycline
PBP 2a	penicillin binding protein 2a
PBS	phosphate buffered saline
PDF	peptide deformylase
PFGE	pulse field gel electrophoresis
PIA	polysaccharide intracellular adhesin
PKS	polyketide synthases
PM	phenotypic microarray
PNAG	polymeric N-acetyl glucosamine
PVL	Panton-Valentine leukocidin
qPCR	quantitative PCR
QRDR	quinolone resistance-determining region
RAPD	random amplified polymorphic DNA
RLUs	relative light units
RRPs	ribosomal protection proteins
sar	staphylococcal accessory regulator
SCC*mec*	staphylococcal cassette chromosome mec
SCVs	small colony variants
SCV^{GEN}	SCV selected for in the presence of gentamicin
SCV^{TET}	SCV selected for in the presence of tetracycline
SD	standard deviation
SE	standard error
STM	signature tagged mutagenesis
ST	sequence type
SNPs	single nucleotide polymorphisms
SXT	trimethoprim-sulphamethoxazole

TA	toxin-antitoxin
TCA cycle	tricarboxylic acid cycle
TD-SCVs	thymidine-dependent SCVs
TEM	transmission electron microscopy
UPEC	uropathogenic *E. coli*
WHO	World Health Organisation
$\Delta\Psi$	membrane potential
σ^B	sigma B

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CHAPTER 1: GENERAL INTRODUCTION

1.1 *Staphylococcus aureus*

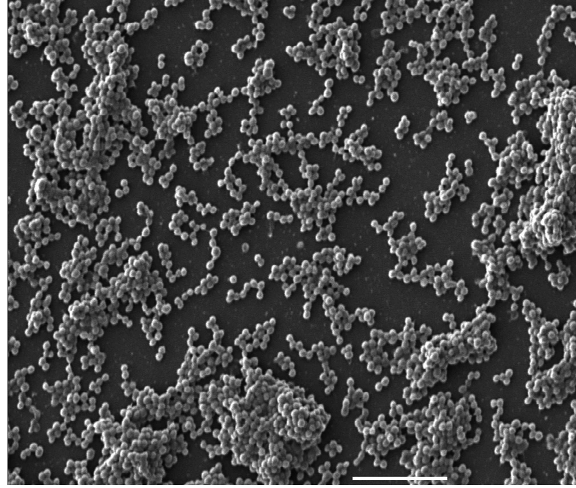
The term 'staphylococcus' (from the Greek 'staphyle' meaning bunch of grapes) was originally coined by Sir Alexander Ogston to describe the grape like clusters of bacteria found in the pus of acute abbesses (Newsom, 2008; Ogston, 1882). To date there are 35 recognised species of *Staphylococcus* all of which are Gram-positive, non-motile, non-spore forming and grow optimally under aerobic conditions (Peacock, 2005). The majority of species grow at a temperature range of 18-40°C and have a high salt tolerance, defined as the ability to grown in the presence of 10 % w/v sodium chloride.

Shortly after the discovery of the *Staphylococcus* genus, Anton J Rosenbach isolated a strain of staphylococci and named it *Staphylococcus aureus* due to the pigmented appearance of its colonies. *S. aureus* is a coccoid shaped bacterium that occurs singly, in pairs, short chains or grape like clusters (Figure 1.1). Approximately 20-30 % of the general human population are carriers of the bacterium, which is commonly found on the skin and mucous membranes (particularly the nose; Kluytmans *et al.*, 1997). The golden pigment of some *S. aureus* on rich medium is due to the presence of carotenoids which are thought to protect cells from oxidants, produced by the immune system (Liu *et al.*, 2005). The production of catalase and coagulase are two important characteristics that allow differentiation from other bacterial genera and species. Catalase production differentiates *S. aureus* from *Streptococcus* species and coagulase production allows differentiation from coagulase negative staphylococci (CoNS) such as *S. epidermidis*.

1.1.1 Features of the *S. aureus* genome

S. aureus strains Mu50 and N315 were the first to be sequenced and published in 2001 (Kuroda *et al.*, 2001). As of Summer 2011, the genomes of 25 different *S. aureus* strains have been sequenced (National Centre for Biotechnological Information, 2011). All the sequenced genomes are between 2.7 and 3 million base pairs (bp) in size and have a low G+C content (between 32.7 and 32.9 %). Their genomes contain a single circular chromosome which

A)



B)

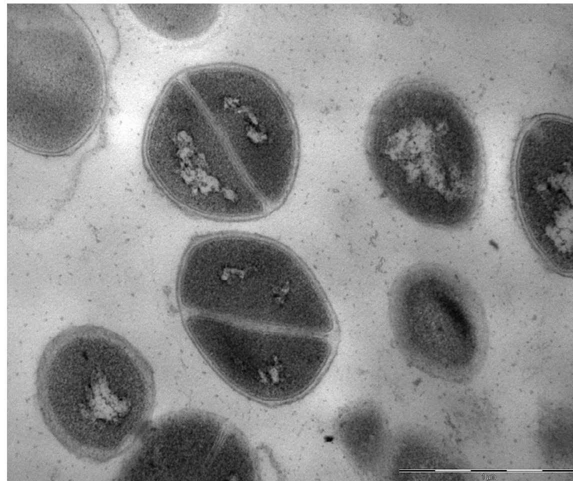


Figure 1.1 Arrangement and morphology of *S. aureus* cells A) - Scanning electron micrograph of *S. aureus* exhibiting typical cocci morphology with the presence of irregular grape-like clusters. B) - Transmission electron micrograph showing *S. aureus* daughter cells which have divide and are yet to split apart. A) Scale bar represents 10 µm, magnification X 4000. B) Scale bar represents 1 µm, magnification X 31,000.

encodes approximately 2700 coding sequences as well as structural and regulatory RNAs (Holden & Lindsay, 2008). Bacterial genomes are composed of a core genome, comprising of genes essential for growth and functionality coupled with an auxiliary set of genes (Frost *et al.*, 2005). The *S. aureus* genome comprises approximately 75% genes involved in processes such as central metabolism and other housekeeping functions (Lindsay & Holden, 2004). This gene set is highly conserved between strains and makes up the core genome. The auxiliary gene set is composed of genes associated with virulence and drug resistance, which are frequently carried on mobile genetic elements (MGEs; Holden & Lindsay, 2008). MGEs consist of bacteriophages, genomic islands, plasmids and transposons all of which can move between the genomes of bacteria via the process of horizontal gene transfer (HGT). Analysis of closely related orthologues indicates that the genetic background of *S. aureus* has been vertically transmitted from a common ancestor that subsequently diverged to *Bacillus* and *Staphylococcus* species (Ito *et al.*, 2003).

1.1.2 *S. aureus* infections

S. aureus commonly colonises humans as a commensal; however under certain conditions *S. aureus* behaves as an opportunistic pathogen and is the frequently the causative agent of various human diseases. As such it is of the most intensively studied bacterial species (Plata *et al.*, 2009). *S. aureus* infections have been linked to a diverse range of medical conditions including skin, soft-tissue, respiratory, bone, joint, and endovascular disorders (Lowy, 1998) and are associated with prolonged hospital stay, increased morbidity and mortality, as well as increased healthcare costs (Que & Moreillon, 2010). Pathogenesis requires the combination of various virulence factors including; secreted proteins, cell surface-bound proteins and cell surface components (Tenover & Gorwitz, 2000). These factors are regulated by a network of interacting genetic and environmental factors.

1.2 Antibiotic resistance

1.2.1 Origins of antibiotic resistance

Antibiotics have revolutionised medicine in many respects; their introduction as a means to combat bacterial infection is regarded as one of the most important events in medical history (Davies & Davies, 2010; Overbye & Barrett, 2005). The discovery of penicillin by Sir Alexander Fleming in 1928 marked the birth of the antibiotic era.

Following the clinical success of penicillin, the development of chloramphenicol, tetracycline, erythromycin, rifampicin and vancomycin was achieved between 1940 and 1960 (Yoneyama & Katsumata, 2006). However, resistance to two of the first commonly used antibiotics (penicillin and streptomycin) was reported in clinical isolates a few years after their introduction (Barber & Rozwadowskadowzenko, 1948; Waksman *et al.*, 1945). Before the introduction of penicillin in the 1940s as a therapeutic agent, a bacterial penicillinase was identified (Abraham & Chain, 1940) revealing that bacterial resistance mechanisms were components of natural microbial populations rather than purely a consequence of human medical intervention (Bentley *et al.*, 2002; D'Costa *et al.*, 2006). Resistance elements are predicted to have been circulating in bacterial populations for millennia rather than emerging since the advent of the antibiotic era (Wright, 2007). This is strengthened by the fact that the majority of antibiotics originate from soil dwelling actinomycetes and resistance elements for self-protection are often clustered in antibiotic biosynthetic operons (Bentley *et al.*, 2002; Cundliffe *et al.*, 2001). Accordingly the mechanisms of resistance to many antibiotics in clinical isolates have their origins in the environmental resistome (Alonso *et al.*, 2001).

Bacteria have the ability to adapt and adjust to changes in their environment and have subsequently developed protective mechanisms to reduce their susceptibility to antibiotics (Hogberg *et al.*, 2010). Although this reduced susceptibility can be achieved via spontaneous mutation and alteration of the target gene, the majority of antibiotic resistance genes are acquired through HGT. Antibiotic resistance genes are commonly carried on mobile genetic elements such as bacteriophages, plasmids and transposons, allowing them to be transferred among bacteria. HGT has facilitated the spread of antibiotic resistance genes via gene exchange processes such as conjugation, transduction and transformation. The liberal widespread use of antibiotics (between 100,000 and 200,000 tonnes per annum worldwide) has provided the requisite conditions to mobilise resistance genes that circulate in the environmental, into pathogenic bacteria (Wise, 2002; Wright, 2007). Consequently resistance to every antibiotic ever used in clinical practice has now been reported (Payne *et al.*, 2007).

1.2.2 The cost of antibiotic resistance

A recent conservative estimate on the cost of antibiotic resistance to member states of the European Union (EU) was €1.5 billion with approximately 25,000 patients in the EU dying each year from infections caused by multi drug resistant pathogens (So *et al.*, 2010). In both Europe and the USA the most problematic pathogens associated with antibiotic resistance and nosocomial infections are; *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species which have been termed the ESKAPE pathogens (Rice, 2008). Rice (2008) reported that this collection of pathogens are of exceptional importance as they represent paradigms of pathogenesis, transmission, and resistance. Currently rational and prudent use of antibiotics coupled with strict and meticulous hygiene policies are employed to combat the threat posed by antibiotic resistance (World Health Organisation, 2001). However these policies alone cannot solve the problem of antibiotic resistance and development of novel antibiotics is crucial for the future of healthcare systems across the world (Hogberg *et al.*, 2010)

1.2.3 Antibiotic resistance in *S. aureus* and the spread of methicillin-resistant *S. aureus*

Before the antibiotic era, prognosis for patients with staphylococcal infections was poor. For example, a study in a Boston hospital in 1941 reported that the mortality rate of patients with *S. aureus* bacteremia was 82% (Skinner & Keefer, 1941). The introduction of penicillin in the 1940s greatly improved the prognosis and decreased mortality rates for patients that had succumb to staphylococcal infections. Soon after the introduction of penicillin, approximately 60% of *S. aureus* hospital isolates in the UK were reported as penicillin resistant (Barber & Rozwadowskadowzenko, 1948). Penicillin resistant strains carried a gene (*blaZ*) encoding a ß-lactamase, which inactivates penicillin through hydrolysis of the ß-lactam ring (Projan & Ruzin, 2006). The 1950s saw the spread of virulent penicillinase-producing strains that disseminated through hospitals (Shanson, 1981). This was followed by the development of semisynthetic penicillins such as methicillin, which resisted the action of ß-lactamase enzymes due to the presence of an acyl side chain, preventing hydrolysis (Gilmore *et al.*, 2008; Kirby & Bulger, 1964; Rolinson *et al.*, 1960). Similarly to penicillin, the introduction of methicillin was closely followed by the development of resistance. The first case of methicillin-resistance in *S. aureus* was reported in Britain in 1961,

(Barber, 1961) but resistance remained at low frequencies through the 1960s (approximately 1% of hospital isolates in the UK) and therefore methicillin was viewed as clinically effective (Parker & Hewitt, 1970). Outbreaks of methicillin-resistant *S. aureus* (MRSA) began to be observed in hospitals in the 1960s (Barrett *et al.*, 1968; Benner & Kayser, 1968; Colley *et al.*, 1965) and the late 1960s and 1970s saw the spread of MRSA across international borders (Lowy, 2003). This was followed by a decline in the prevalence of MRSA in the late 1970s and 1980s due to the implementation of infection control policies and the introduction of gentamicin (Ayliffe, 1997).

In the following decades epidemic strains of MRSA disseminated worldwide and various individual MRSA strains became prevalent in geographically distinct areas of the globe (Chambers & Deleo, 2009). Outbreaks of MRSA are of great concern as the prognosis for infected patients is worse than those infected with methicillin-sensitive *S. aureus* (MSSA). Meta analysis of *S. aureus* cases over a 20 year period, showed a significant increase in mortality was associated with MRSA bacteremia (Cosgrove *et al.*, 2003). Differences in mortality have been attributed to the lower availability of bactericidal drugs to treat MRSA and the underlying healthcare problems in the old and sick rather than enhanced virulence of MRSA strains (Lowy, 2003). Furthermore, the cost of treating MRSA patients is twice that of treating patients with MSSA infections (Capitano *et al.*, 2003), which is attributed to the increasing costs of vancomycin as well as implementing patient isolation practices within hospitals (Gilmore *et al.*, 2008). MRSA is still prevalent and a major concern in hospitals today and is responsible for 19,000 deaths a year in the US (Klevens *et al.*, 2007). This accounts for more deaths than the combined number of deaths from patients suffering from acquired immunodeficiency syndrome (AIDS) and tuberculosis (Boucher & Corey, 2008).

The glycopeptide, vancomycin is the antimicrobial of choice for treatment of MRSA patients. Those who cannot tolerate vancomycin can be treated with fluroquinolones, trimethoprim sulfamexazole or clindamycin (Lowy, 1998). However *S. aureus* can also become resistant to these antimicrobial agents through various mechanisms (Table 1.1). In recent years other novel antibiotics such as linezolid and daptomycin have suffered a similar fate with resistance being reported soon after their introduction (Figure 1.2).

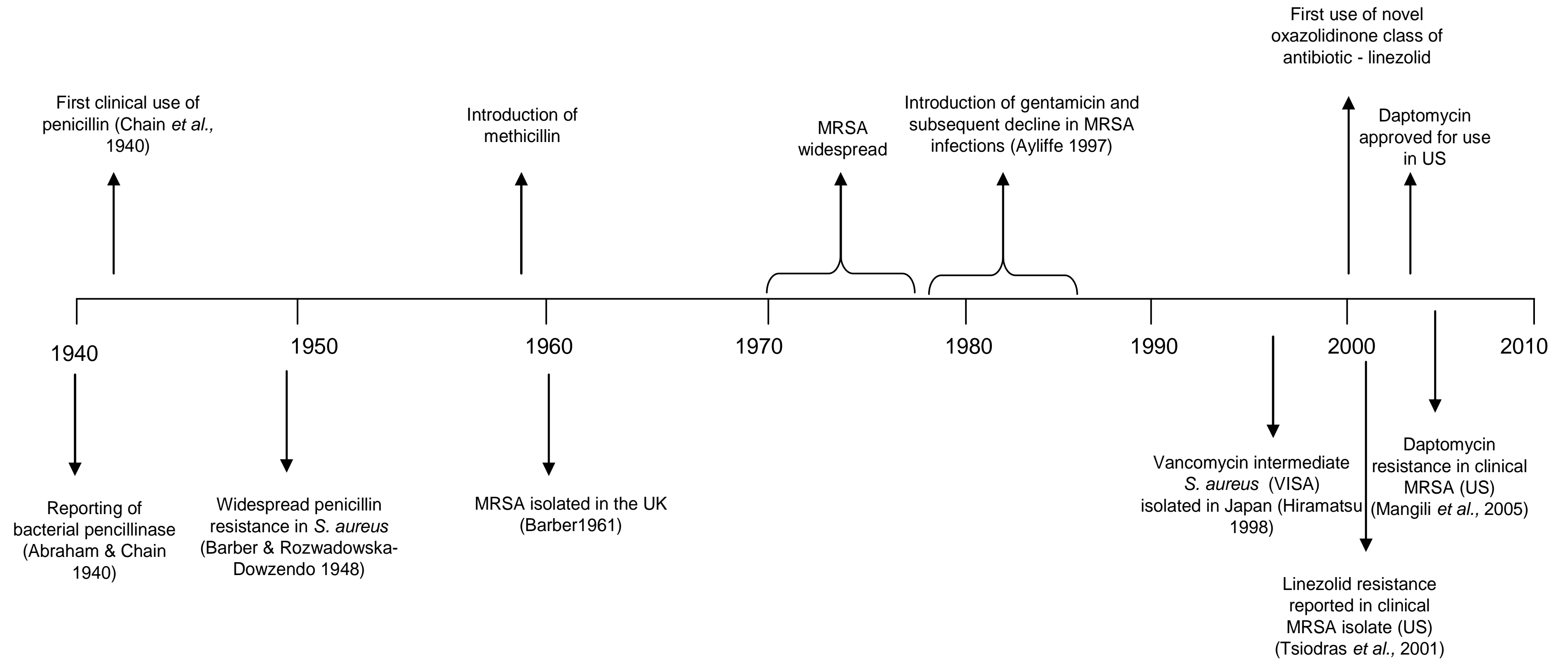


Figure 1.2 The development of antibiotics and progression of antibiotic resistance in *S. aureus*

Table 1.1 Mode of action of antibiotics and mechanism of resistance in *S. aureus*. Adapted from al - Masaudi *et al.*, (1991), Jensen & Lyon (2009) and Woodford (2005)

Antibiotic	Mechanism of action	Resistance mechanism(s)	Gene(s)	Genomic Location
Aminoglycosides	Protein synthesis – inhibit translocation	AMEs – inactivation of antibiotic	*aac*, *aph*, *ant*	Chromosome, plasmids and transposons
ß-lactams (Penicillin)	Peptidoglycan synthesis – inhibits cell wall synthesis enzymes	Enzymatic hydrolysis of ß-lactam ring	*blaZ*	Plasmid and transposons
Semisynthetic ß-lactams (Methicillin)	Peptidoglycan synthesis – inhibits cell wall synthesis enzymes	Modified PBP2a with reduced affinity for ß-lactams	*mecA*	Chromosome
Chloramphenicol	Protein synthesis – inhibit transpeptidation	Chloramphenicol aceltyransferase – inactivation of antibiotic	*cat*	Plasmid
Daptomycin	Cell membrane depolarisation via leakage of potassium and other cellular components	Not completely understood – mutations in RNA polymerase ß-subunit implicated	*rpoB*	Chromosome
Quinolones	DNA synthesis – inhibit DNA gyrase	Alteration in QRDR, reducing affinity of enzyme-DNA complex for fluroquinolones	*grlA*/*B*,	Chromosome
Fusidic acid	Protein synthesis – forms a stable complex with elongation	Decreased affinity of the G factor for the antibiotic. Impermability	*fusA*, *fusB*	Chromosome and plasmid

	factor G and ribosome inhibiting translocation	and efflux also implicated		
Glycopeptides	Cell wall synthesis – prevent incorporation of late peptidoglycan precursors into the peptidoglycan matrix	VISA – thickening of cell wall	-	
		VRSA - alteration in composition of precursors resulting in reduced affinity for vancomycin	*vanA*	Plasmid
Macrolides, Lincosamides and Streptogramin B	Protein synthesis – stimulates dissociation of peptidly-tRNA during elongation	Methylation of adenine on 23S component – reduced affinity for antibiotics	*ermA*, *ermB*, *ermC*	Plasmid and transposons
Mupirocin	Protein synthesis – inhibits isoleucyl-tRNA synthestase preventing incorporation of isoleucine into nascent peptides	Alteration of target site	*ileS*	Chromosome
		Acquisition of novel isoleucyl-tRNA synthestase	*mupA*	Plasmid
Oxazolidinones	Protein synthesis – prevent formation of the 70S ribosomal initiation complex	Alteration of domain V component of the 23S rRNA	23S rRNA	Chromosome
Rifampicin	RNA polymerase – binds to ß-subunit of DNA dependant RNA polymerase	Alteration of target site	*rpoB*	Chromosome
Tetracycline	Protein synthesis – inhibit binding of aminoacyl-tRNAs	Tetracycline efflux proteins – energy dependent efflux of	*tet*(*K*), *tet*(*L*)	Chromosome and plasmid

		tetracycline		
		Ribosomal protection protein – promotes release of bound tetracycline	*tetA*(*M*)	Transposons
Trimethoprim	Tetrahydrofolic acid synthesis – competes with DHFR inhibiting reduction of dihydrofolate acid to tetrahydrofolic acid	Chromosomal mutations, reduced affinity for trimethoprim	*dfrB*	Chromosome
		Acquisition of unique DHFR with reduced affinity for trimethoprim	*dfrA*,	Plasmid

AMEs - aminoglycoside modifying enzymes; DHFR - dihydrofolate reductase; PBP - penicillin binding protein; QRDR - quinolone resistance determining region; VISA - vancomycin intermediate *S. aureus*; VRSA - vancomycin resistant *S. aureus*

1.2.4 Mechanism of methicillin-resistance in *S. aureus*

Methicillin was the first synthetic penicillin to be developed and is resistant to hydrolysis by ß-lactamase enzymes (Kirby & Bulger, 1964; Rolinson *et al.*, 1960). Methicillin binds to penicillin binding proteins (PBP) in sensitive *S. aureus* strains and inhibits cross linking of peptidoglycan, resulting in cell lysis (Wise & Park, 1965). *S. aureus* possess several PBPs that are responsible for catalysing cross-linking reactions between peptidoglycan polymers, one of the final steps in bacterial cell wall assembly (Chambers, 1988). MRSA strains posses an additional PBP called PBP2a (also known as PBP') which confers resistance to methicillin and all other ß-lactam antibiotics (Brown & Reynolds, 1980; Georgopapadakou *et al.*, 1982). PBP2a has a low affinity for ß-lactam antibiotics (Hartman & Tomasz, 1984) and thus can continue to catalyse the formation of cross-bridges in bacterial cell wall peptidoglycan in the presence of ß-lactams (Berger-Bachi & Rohrer, 2002; Hartman & Tomasz, 1984). PBP2a is thought to have initially evolved by recombination of two genes: an inducible type I penicillinase gene and a PBP gene of *E. coli*, resulting in a β-lactam-inducible MRSA PBP (Song *et al.*, 1987). The genetic basis of this resistance is the *mecA* gene which encodes PBP2a. *mec* DNA is known to be present in *S. aureus* (Sjostrom *et al.*, 1975), however additional DNA is present in MRSA strains (Beck *et al.*, 1986; Hiramatsu *et al.*, 2001). *mecA* is believed to have originated in the animal related staphylococcal species *S. fleurettii*, the sequence of which is nearly identical to the *mecA* region found in MRSA strains (Tsubakishita *et al.*, 2010). *mecA* is found on a mobile genetic island (GI) termed the staphylococcal cassette chromosome, *mec* (SCC*mec*) and is a well-developed vehicle for transmission of genes among staphylococcal species (Ito *et al.*, 1999; Katayama *et al.*, 2003).

1.2.5 Staphylococcal chromosome cassette *mec*

SCC*mec* is a 21 – 67 kb fragment of DNA that integrates into the *S. aureus* chromosome at a unique site (*attBscc*), near the origin of replication (Chambers & Deleo, 2009; Hiramatsu *et al.*, 2001). *attBscc* is highly conserved among clinical strains of *S. aureus* and is found within an opening reading frame of unknown function (*orfX;* Ito *et al.*, 1999). The integration off SCC*mec* near the origin of replication may provide an advantage allowing the instant utilisation of imported antibiotic resistance genes (Ito *et al.*, 2003). SCC*mec* is a variable genetic element,

but contains conserved elements such as the *mec* operon (composed of *mecA* and its regulatory genes) and the cassette chromosome recombinase complex *ccr* (Holden *et al.*, 2004; Ito *et al.*, 2001). The *ccr* genes encode recombinases crucial for excision of the cassette from the chromosome and subsequent integration in the correct orientation (Katayama *et al.*, 2000). Different types of SCC*mec* have been classified according to the class of the *mec* gene complex and the type of *ccr* complex that they contain (Table 1.2). Currently three distinct *ccr* genes have been identified; *ccrA*, *ccrB* and *ccrC* which are regarded as phylogenetically diverse as they share less than 50% DNA sequence similarity (Ito *et al.*, 2009).

The regions of SCC*mec* which border the *ccr* and *mec* complexes are know as junkyard (J) regions and are divided into three regions. J1 ranges from the chromosome right junction to the *ccr* genes, and J2 ranges from the *ccr* genes to *mec* complex (Ito *et al.*, 2003). Finally, the J3 region is located between the *mec* complex and the left extremity of SCC*mec*. Various insertion sequences (IS), transposons, and plasmids have been found in SCC*mec*, including Tn*554*, IS*1272*, IS*431*, pUB110, pT181, and p1258; many of which carry resistance to other classes of antimicrobial agents (Table 1.2).

1.2.6 Community-acquired MRSA

Although MRSA is widely regarded as a nosocomial problem there are increasing reports of outbreaks and transmission in the community. Community-acquired MRSA (CA-MRSA) infections refer to those caused outside the hospital setting and are prevalent in previously healthy individuals. CA-MRSA is commonly associated with skin and soft tissue infections (such as furunculosis) but has also been associated with severe necrotising pneumonias (Zetola *et al.*, 2005). Such infections have been associated with certain groups of society including injected drug users (Huang *et al.*, 2008), men who have sex with men (Diep *et al.*, 2008) and people who engage in contact sport (Lindenmayer *et al.*, 1998). CA-MRSA strains differ from hospital acquired strains as they show susceptibility to many antibiotics. This is due to the fact that they carry the type IV SCC*mec* element which carries no additional resistance genes (Said-Salim *et al.*, 2003). The presence of additional virulence genes is thought to contribute to the pathogenicity of CA-MRSA. Genome sequencing of CA-MRSA

Table 1.2 Classification of SCC*mec* types in MRSA. (Chambers & Deleo, 2009; Deurenberg & Stobberingh, 2008; Ito *et al.*, 2001; Ito *et al.*, 2003; Ito *et al.*, 2009)

SCC*mec* Type	Size (kb)	ccr genes	mec complex	Additional resistance elements
SCC*mec* I	34	*ccrA1*, *ccrB1*	Class B	
SCC*mec* II	53	*ccrA2*, *ccrB2*	Class A	puB110, Tn*554*
SCC*mec* III	67	*ccrA3*, *ccrB3*	Class A	pI258, pT181, Tn*554*, ΨTn*554*
SCC*mec* IV	21-24	*ccrA2*, *ccrB2*	Class B	
SCC*mec* V	28	*ccrC*	Class C	
SCC*mec* VI	24	*ccrA4*, *ccrB4*	Class B	
SCC*mec* VII	41-49	*ccrC*	Class C	
SCC*mec* VIII	32	*ccrA4*, *ccrB4*	Class A	Tn*554*

Five (A–E) different classes of *mec* elements have been defined, of which three (A–C) are common in SCC*mec*. pUB110 carriers the *ant* (4') gene providing resistance to several aminoglycosides. Tn*554* encodes *ermA* providing constitutive and inducible macrolide–lincosomide–streptogramin B (MLS) resistance. Additional resistances to cadmium, mercury and tetracycline are observed in SCC*mec* III via ΨTn*554*, pI258 and pT181 respectively.

strain MW2 revealed the presence of additional virulence genes, including the presence of additional toxins such as the Panton-Valentine leukocidin (PVL) toxin (Baba *et al.*, 2002). The increasing prevalence of community-acquired MRSA coupled with the substantial morbidity and mortality associated with these infections suggests that CA-MRSA has the potential to cause serious issues in public health (Yamamoto *et al.*, 2010).

1.2.7 Searching for novel antibiotics targets

The dramatic increase in the emergence of antibiotic resistance coupled with the current difficulties facing the pharmaceutical industry means there is a pressing need for the development of new approaches to antibiotic discovery. One such approach encompasses a new generation of screening methods. Screening novel antibiotics against an *Escherichia coli* strain that possesses resistance to commonly used antibiotics preselects any hits to be of a potential novel class of antimicrobial agent (Gullo *et al.*, 2006). Secondly, the utilisation of techniques such as *in vivo* expression technology (IVET) and signature tagged mutagenesis (STM) may prove useful in novel candidates through identifying genes that are essential for the infection process (Alksne & Projan, 2000; Chiang *et al.*, 1999). Yoneyama & Katsumata (2006) point out that the expression profiles of pathogens during infection *in vivo* vary greatly in comparison to those recreated in rich media *in vitro*. Therefore the use of techniques that can potentially identify these targets may provide novel targets for antibiotic development.

Utilising the wealth of knowledge generated from microbial genome sequencing projects offers great potential in generating leads for novel antibiotics. Analysis of pathogen genome sequences allows for the identification of conserved enzymes that are essential for bacterial growth and replication (Fischbach & Walsh, 2009). A study by Rosamond & Allsop (2000) analysed the genomes of several respiratory pathogens for essential conserved genes, not present in humans. Through detailed bioinformatic analysis three possible candidates were selected as potential targets for the development of novel respiratory tract antibiotics. Finally identification of novel ecological niches inhabited by antibiotic producing bacteria may yield previously unidentified bioactive compounds (Wright & Sutherland, 2007). Abyssomicin C (which inhibits bacterial *p*-aminobenzoic acid biosynthesis) has been isolated from a

recently identified marine actinomycete bacterium (Bister *et al.*, 2004). Other sources of novel antibiotic producing bacteria include bacterial symbionts of insects (Kaltenpoth *et al.*, 2005) and arthropods (Gebhardt *et al.*, 2002). Re-examining the soil dwelling actinomycetes may hold potential in the discovery of novel antibiotics as it has been predicted that ~ 2% of the antibiotics produced by *Streptomyces* have been identified (Baltz, 2005). Potential new targets for novel antibiotics include peptide deformylase (PDF) (Yuan *et al.*, 2001), bacterial fatty acid synthesis (Payne *et al.*, 2001), the non-mevalonate pathway (Yoneyama & Katsumata, 2006) and signalling networks (two component systems and quorum sensing pathways) (Barrett & Hoch, 1998).

1.3 Molecular typing of MRSA

Molecular typing of MRSA strains is an important tool in tracing outbreaks and cases of transmission. Various molecular epidemiological techniques have been employed to type and track isolates including; macrorestriction of chromosomal DNA (generated via restriction enzyme digestion) and profiling via pulse field gel electrophoresis (PFGE), nucleotide polymorphisms in the *mecA* gene and Tn*554* insertion patterns (Oliveira *et al.*, 2002). The need for more rapid methods has seen the development of several polymerase chain reaction (PCR) based methods including coagulase gene typing, random amplified polymorphic DNA (RAPD) and repetitive element sequence-based PCR (rep-PCR; Weller 2000).

1.3.1 Pulse field gel electrophoresis

Restriction enzymes (such as *Sma*I) are used in PFGE to digest bacterial genomes; strain-specific banding patterns are then generated using a specialised form of electrophoresis (Tenover *et al.*, 1997). PFGE is considered to be a highly discriminatory typing method for studying outbreaks and hospital-to-hospital transmission of MRSA isolates (Deurenberg *et al.*, 2007). This technique has been used to investigate the spread of clones through European countries (Deplano *et al.*, 2000) as well as to trace the source, transmission, and spread of nosocomial infections (Ichiyama *et al.*, 1991). PFGE is reproducible and highly discriminatory typing method, but criticisms of the technique include its speed and the lack of a common nomenclature (Deurenberg *et al.*, 2007; van Belkum *et al.*, 1998).

1.3.2 Random amplified polymorphic DNA

RAPD uses low stringency PCR with short arbitrary primers to amplify portions of genomic DNA followed by the separation of resulting fragments by electrophoresis (Williams *et al.*, 1990). RAPD has been used to investigate a nosocomial outbreak of a non-phage typeable MRSA strain (Tambic *et al.*, 1997) and the use of multiple primers has been demonstrated to improve discriminatory power (Cheeseman *et al.*, 2007). Although RAPD is considered simpler and less time consuming, comparison with PFGE reveals that RAPD is less discriminatory (Saulnier *et al.*, 1993).

1.3.3 Multilocus sequencing

MLST was first applied to *Neisseria meningitidis* in order to overcome the problems associated with traditional and molecular typing methods (Maiden *et al.*, 1998). MLST differs from PFGE as it is a sequence-based technique and exploits the differences in the nucleotide sequence of several house keeping genes. Fragments (~ 450 bp) of these house keeping genes are sequenced and a single polymorphism results in the assignment of a new allele number. The combination of the seven alleles generates an allelic profile, which translates into a single sequence type (ST; Enright & Spratt, 1999). The discriminatory power of MLST was validated through comparison with PFGE profiles and the power of MLST as an epidemiological monitoring tool has been facilitated by an easily accessible online database which is expanded as novel STs are identified (Urwin & Maiden, 2003). MLST has been successfully applied to *S. aureus* using the seven housekeeping genes; carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) (Enright *et al.*, 2000). MLST has proved instrumental in analysing and tracing the origins and evolutionary history of MRSA (Enright *et al.*, 2002; Oliveira *et al.*, 2002).

1.4 Small colony variants

The first incidence of small colony variants (SCVs) was reported in 1910 when an irregular form of *Eberthella typhosa* (now known as *Salmonella enterica*) was observed (Jacobsen, 1910; Proctor, 2001). SCVs have been observed in several bacteria species and have been extensively studies in *S. aureus*.

1.4.1 *S. aureus* small colony variants

S. aureus SCVs are slow growing, morphological variants that exist as a staphylococcal subpopulation and often arise after exposure to antimicrobial chemotherapeutics (Proctor *et al.*, 2006). The phenotypic trait of slow growth leads to the development of microcolonies which are approximately 10 times smaller than wildtype *S. aureus* colonies (Proctor *et al.*, 1995). *S. aureus* SCVs also demonstrate a number of other characteristics that are atypical for *S. aureus* including reduced α-toxin production, delayed coagulase activity, the production of non pigmented colonies and deficiencies in certain biochemical reactions (Balwit *et al.*, 1994; Proctor *et al.*, 1995; von Eiff *et al.*, 2000). *S. aureus* SCVs have been associated with persistent and recurrent infections and SCVs with defects in the components of the bacterial electron transport chain (ETC) and the biosynthesis of thymidine have been consistently recovered.

1.4.1.1 Electron transport deficient SCVs

Electron transport chain deficient SCV isolates are commonly auxotrophic for haemin and menadione (McNamara & Proctor, 2000; Proctor *et al.*, 1995; Proctor & Peters, 1998). Both play crucial roles in bacterial electron transport (Figure 1.3). Haemin is required for the biosynthesis of cytochromes which accepts electrons from menaquinone and completes the ETC. Menadione is isoprenylated to form menaquinone and is the acceptor of electrons from nicotinamide adenine dinucelotide (NADH) and flavin adenine dinucelotide ($FADH_{2;}$ von Eiff *et al.*, 2001b). Haemin and menaquinone are both used in aerobic electron transport and mutations in their biosynthetic genes results in a disrupted ETC and blockage of oxidative respiration. Haemin and menaquinone are synthesised by the *heme* and *men* operons respectively (Tien & White, 1968), and mutations in genes of these operons result in the SCV phenotype (Bates *et al.*, 2003; von Eiff *et al.*, 1997b).

1.4.1.2 Haemin and menadione auxotrophy

S. aureus hemB mutants display typical characteristics associated with the SCV phenotype including micro colonies, reduced coagulase activity and reduced susceptibility to aminoglycosides. However, these characteristics can be reversed by growing *hemB* mutants in the presence of haemin, or by complementing the mutant with intact *hemB* (von Eiff *et al.*, 1997b). Mutation of the *menD* gene (encoding 2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase) also results in

mutants displaying characteristics associated with the SCV phenotype (Bates *et al.*, 2003; Kohler *et al.*, 2008). Without oxidative respiration SCVs can only produce ATP through fermentation, which is significantly less efficient, resulting in ATP shortage in SCVs. ATP is required for various essential cellular functions including cell wall biosynthesis, the generation the electrochemical gradient and carotenoid biosynthesis (Figure 1.3). As a result of reduced ATP production SCVs display slower growth (hence smaller colonies), reduced uptake of aminoglycosides and cationic peptide transport and decreased pigment formation.

Analysis of SCVs recovered from the clinical environment has identified further mutations in the *heme* and *men* operons. Haemin auxotrophy has been linked to mutations in *hemH* which is involved in the final step in haemin biosynthesis (Schaaff *et al.*, 2003). The genetic basis for clinically isolated menadione auxotrophy in clinically isolated SCVs has also been demonstrated. DNA sequencing of the nine genes involved in menadione biosynthesis from osteomyelitis isolates revealed mutations in the gene encoding naphthoate synthase *menB* (Lannergard *et al.*, 2008). In both studies, supplementation with haemin or menadione resulted in reversion to the wildtype phenotype.

1.4.1.3 Thymidine dependent SCVs (TD-SCVs)

TD-SCVs are frequently recovered from cystic fibrosis (CF) patients that have received long term treatment with trimethoprim sulphamethoxazole (SXT; Gilligan *et al.*, 1987; Kahl *et al.*, 1998). TD-SCVs share characteristics with electron transport deficient SCVs, such as reduced α-toxin production and lack of pigmentation (Kahl *et al.*, 1998). TD-SCVs frequently display reduced susceptibility to SXT and show increased persistence in CF patients in comparison to wildtype strains (Kahl *et al.*, 2003b). In terms of their morphology TD-SCVS exhibit two distinct phenotypes when grown on Columbia sheep blood agar; either a ‘fried egg’ morphology (translucent edges surrounding a smaller, elevated pigmented centre) or pinpoint colonies (approximately 10 times smaller than wildtype *S. aureus*; Kahl *et al.*, 2005). Transmission electron microscopy (TEM) revealed abnormal cell size and morphology (Figure 1.4). TD-SCVs are up to 8 times larger than wildtype cells due to ‘swollen cells’ with uncompleted cross walls (Kahl *et al.*, 2003a). Thymidine auxotrophy in TD-SCVs is due to mutations in *thyA* (which encodes thymidylate

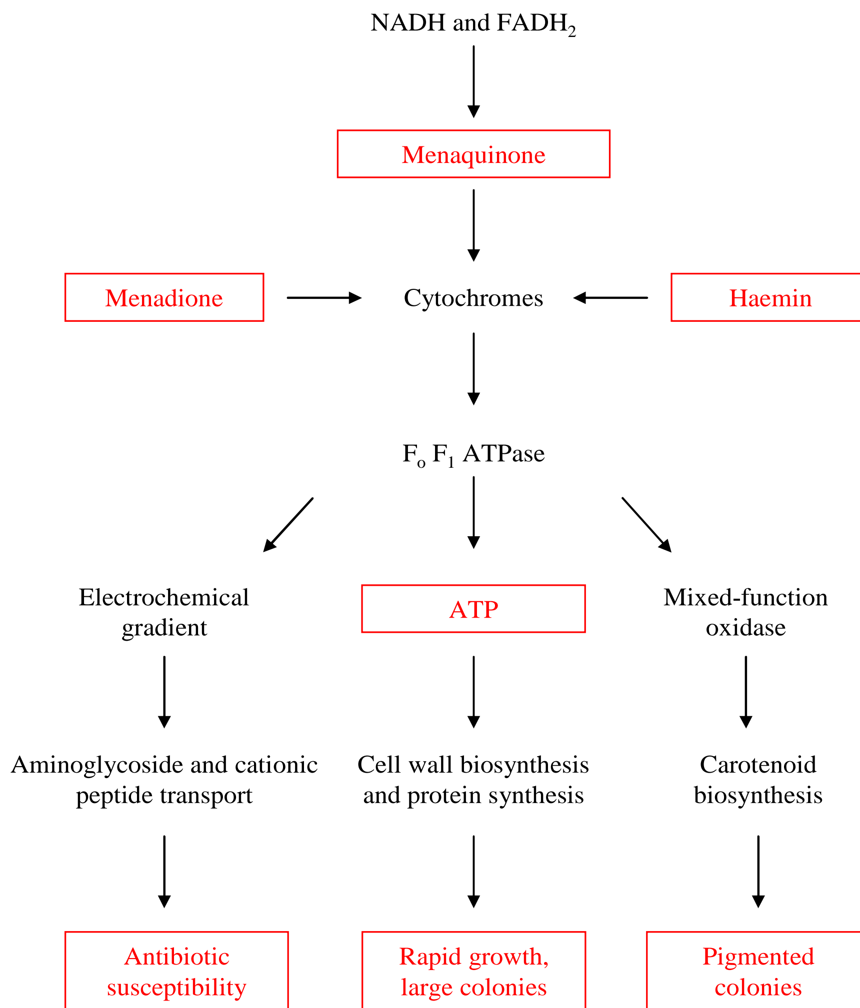


Figure 1.3 Relationship between electron transport, ATP production and characteristics associated with the SCV phenotype in *S. aureus* (adapted from Proctor *et al*., 2006) Reduced ATP production results in reduced cell wall and protein bioysythesis resulting in reduced growth rate and production of microcolonies. ATP is also required for carotenoid biosynthesis, hence SCVs produce non-pigmented colonies. Reduced antibiotic susceptibility to aminoglycosides and cationic peptides is observed due to a reduction in electrochemical gradient, required for the uptake of these compounds.

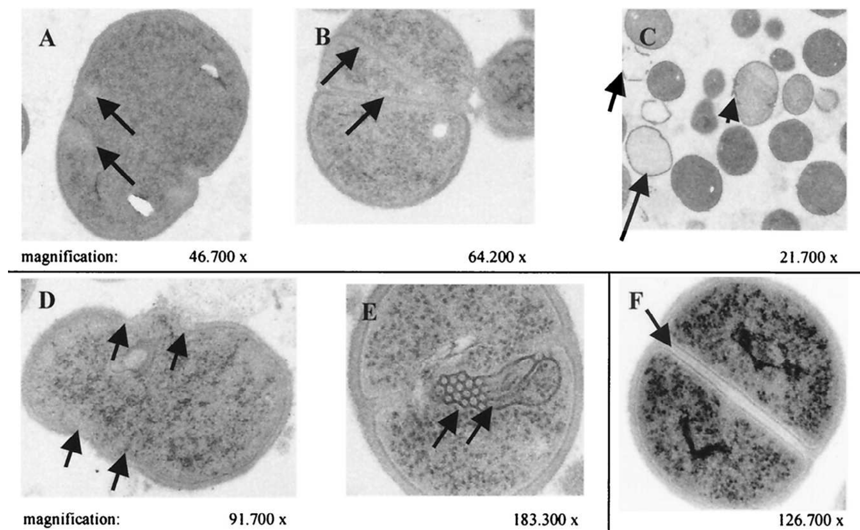


Figure 1.4 TEM images of SCVS exhibiting 'fried egg' morphology (A, B and C), pinpoint SCVs (D and E) and wildtype *S. aureus* (F) Incomplete cell walls, irregular cell size and empty cells are represented by the arrows in A-E. Regular cell wall separation can be seen in wildtype *S. aureus* (F). Images reproduced from Kahl *et al.* (2003a).

synthase in *S. aureus*) and this mutation is also responsible for reduced SXT susceptibility (Besier *et al.*, 2007). SXT interferes with tetrahydrofolic acid which acts as a co-factor for thymidylate synthase (Besier *et al.*, 2007). Thymidylate synthase plays a role in DNA synthesis, catalysing the synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). Analysis of clinical TD-SCVs and a *thyA* knock-out mutant revealed that a series of different mutations in *thyA* are responsible for the TD-SCV phenotype. Mutations in *thyA* permits TD-SCVs to bypass the SXT inhibited pathway. Thymidine is still required by TD-SCVs as it is essential for DNA synthesis. In order to compensate for the lack of thymidine, TD-SCVs increase expression of *nupC*, which is responsible for the transport of nucleotides into the cell (Chatterjee *et al.*, 2008; Saxild *et al.*, 1996). If large amounts of thymidine are present TD-SCVs can revert to the wildtype form (Kahl *et al.*, 1998) accounting for the phenotypic switching observed in TD-SCVs.

Mutations in *thyA* have also been linked to hypermutability in TD-SCVs (Besier *et al.*, 2008a). Clinical TD-SCVs isolates were analysed for mutations in the methyl-directed mismatch repair (MMR) system. The MMR system is composed of a series of genes (*mutS, mutL, mutH*, and *uvrD*,) which are responsible for DNA repair in *S. aureus* (Miller, 1996). Hypermutability is associated with a truncation in *mutL* in TD-SCVs. This leads to replication errors (due to a defective DNA mismatch repair system), that combined with the selective pressure of SXT favour the emergence of mutations in the *thyA* gene and the formation of the TD-SCV phenotype (Besier *et al.*, 2008a). Another characteristic associated with TD-SCVs is enhanced post-stationary phase survival (Chatterjee *et al.*, 2007). Thymidine auxotrophy in TD-SCVs results in a delay in tricarboxylic acid (TCA) cycle function, preventing entry into the death phase. Chatterjee *et al.*, (2007) further established that TD-SCVs utilise contrasting metabolic pathways during stationary phase in comparison to *hemB* mutants.

Although TD-SCVs are frequently isolated from CF patients, patients suffering from other medical conditions are known to harbour TD-SCVs (Besier *et al.*, 2008b). In all cases, patients were suffering from various chronic infections and had been administered long term SXT treatment. Similarly to TD-SCVs isolated from CF patients, sequence analysis showed mutations in *thyA* were responsible for the SCV phenotype. Besier *et al.* (2008) also showed that dTMP (a metabolite of thymidine)

was found in the majority of specimens analysed. The level of dTMP is crucial to the phenotypic appearance of *S. aureus* SCVs as high concentrations result in reversion to the wildtype phenotype (Zander *et al.*, 2008).

1.4.1.4 CO_2 Auxotrophy

Auxotrophy for CO_2 has been reported in *S. aureus* SCVs (Hale, 1951; Thomas, 1955). Growth in the presence of CO_2 restores pigment and α-toxin activity (Thomas, 1955), similar to the supplementation with auxotrophic compounds observed in ETC deficient SCVs. Recently a Spanish hospital reported the isolation of CO_2-dependent SCVs of *S. aureus* in which auxotrophy for CO_2 was confirmed by growth in 5% CO_2 for 18 hours (Gomez-Gonzalez *et al.*, 2010). SCVs were isolated from a range of patients suffering from infections such as catheter-related bacteremia, and wound and respiratory infections. The authors concluded that they were unaware of any alterations in bacterial metabolism that may cause this type of variant or the genetic mechanism for reversion to a rapidly growing form. Auxotrophy for CO_2 is rarely reported in *S. aureus* SCVs, however the isolation of CO_2 auxotrophs suggests that specific atmospheric requirements may be needed to isolate and characterise these variants (Pinto & Merlino, 2011).

1.4.2 SCV formation

The mechanisms behind the formation/generation of SCVs are yet to be fully understood. Regulatory as well as genetic events might be involved, especially since many SCVs are unstable and form revertants growing as large colonies. Schaaff *et al.*, (2003) have examined whether an increased mutation rate favours the formation of SCVs by comparing *E.coli* wildtype with *mutS* $^{-}$ mutants (part of the damage-directed MMR crucial for proofreading during DNA replication; Horst *et al.*, 1999). Results showed that the emergence of spontaneous SCVs was 556-fold higher in the *mutS* $^{-}$ mutants than in the wildtype strain, concluding that a high mutation rates favours the emergence of SCVs. These finding are similar to those observed in TD-SCVs (see section 1.4.1.3), suggesting that mutations may play a role in the formation of SCVs. The widely reported ability of antimicrobials such as aminoglycosides to select for SCVs (Balwit *et al.*, 1994; Musher *et al.*, 1977) suggests that certain antimicrobials may induce SCV formation. Recently, aminoglycoside-induced SCV formation has

been linked to the activity of an alternative sigma factor in *S. aureus* (see section 1.4.8.2).

1.4.3 Infections associated with *S. aureus* SCVs

A range of human infections have been associated with *S. aureus* SCVs (Table 1.3), many of which have been linked to persistent and recurrent outbreaks. In humans cystic fibrosis and osteomyelitis infections have been frequently attributed to the presence of *S. aureus* SCVs, as well as bovine mastitis in dairy cattle (von Eiff *et al.,* 1997; Kahl *et al.,* 1998; Atalla *et al.,* 2008).

1.4.3.1 Cystic fibrosis

TD-SCVs are frequently recovered from CF patients and has been linked to treatment with SXT (see section 1.4.1.3). SCVs have the ability to replace wildtype strains in the CF environment and demonstrated increased levels of persistence (Kahl *et al.*, 2003b). Transcriptional analysis of TD-SCVs showed low levels of expression of the accessory gene regulatory (*agr*; which acts as a genetic control for various virulence factors) and increased expression of genes regulated by the alternative sigma factor, σ^B (Moisan *et al.*, 2006). Altered transcriptional profiles may serve as an optimised adaptation to the CF lung, facilitating increased persistence in comparison to wildtype strains (Proctor *et al.*, 2006).

1.4.3.2 Osteomyelitis

S. aureus SCVs have also been isolated from patients being treated for osteomyelitis, which is often treated with beads that provide a slow release of an antimicrobial (von Eiff *et al.*, 2001b). SCVs isolated from osteomyelitis patients frequently display, auxotrophy for haemin and/or menadione and up to a 32 fold greater gentamicin minimum inhibitory concentration (MIC; von Eiff *et al.,* 1997a). Analysis of patient case history revealed that patients infected with *S. aureus* SCVs were more likely to undergo relapses of osteomyelitis, strengthening the link between SCVs and recurrent infection. As gentamicin treatment for *S. aureus* SCV infection is no longer effective in osteomyelitis patients, treatment with hydroxyapatite cement (HAC) loaded with vancomycin has been trialled in animals (Joosten *et al.*, 2005). HAC/vancomycin-

Table 1.3 Isolation of *S. aureus* small colony variants from human infections

Source information	Auxotrophy	SCV associated observations	Reference
Patients treated with penicillin	CO_2	-	Sherris, (1952)
Sepsis and osteomyelitis patients	Menadione and thymidine	Reduced aminoglycoside susceptibility	Acar *et al.*, (1978)
Septic arthritis patient	Menadione	Reduced aminoglycoside susceptibility	Spearman *et al.*, (1996)
Osteomyelitis patients treated with gentamicin beds	Haemin and/or menadione	Clonal PFGE profiles, SCVs associated with recurrent infections	von Eiff *et al.*, (1997a)
CF patients	Haemin, thymidine, and/or menadione	Clonal PFGE profiles, resistance to antifolate agents	Kahl *et al.*, (1998)
Sepsis in an AIDS patient	Thymidine and menadione	Reduced antibiotic susceptibility, delay in identification resulting in patient mortality	Seifert *et al.*, (1999)
Persistent wound infection treated with clindamycin	Haemin and menadione	Misidentified as CoNS	Abele-Horn *et al.*, (2000)
Patient with Darier's disease	Haemin	Reduced antibiotic susceptibility, increased intracellular persistence	von Eiff *et al.*, (2001a)
Recurrent ventriculoperitoneal shunt-related meningitis	Haemin	Clonal PFGE profiles, misidentified as CoNS by automated identification systems	Spanu *et al.*, (2005)
Prosthetic joint infection	-	Clonal PFGE profiles, SCV infection resulted in removal of prosthetic device	Sendi *et al.*, (2006)

AIDS – acquired immunodeficiency syndrome; CoNS – coagulase negative staphylococci; CF – cystic fibrosis; PFGE – pulse field gel electrophores

treated animals showed eradication of infection and furthermore HAC/vancomycin treatment did not induce SCV formation.

1.4.3.3 Bovine mastitis

Bovine mastitis is commonly associated with dairy cattle and is defined as 'inflammation of the mammary gland' (Bradley, 2002). Although limited studies are available, the presence of *S. aureus* displaying the SCV phenotype has been detected in *S. aureus* positive milk samples produced by cows suffering from chronic mastitis (Atalla *et al.*, 2008). Persistent infection in bovine mastitis may be due to the ability of SCVs to persist for longer periods in bovine mammary epithelial cells, creating a reservoir of bacteria for persistent or relapsing infections (Atalla *et al.*, 2010a). Antibody-mediated (AMIR) and cell-mediated immune responses (CMIR) display marked differences in cows infected with SCVs in comparison to those infected with wildtype *S. aureus* (Atalla *et al.*, 2010b). A lack of immune activation in the host is thought to aid SCV persistence in cows with chronic mastitis.

1.4.4 Identification and susceptibility testing of SCVs

As *S. aureus* SCVs display many characteristics that are abnormal for wildtype strains they pose difficulties in identification and isolation in the laboratory (Figure 1.5). The production of non-pigmented, non-hemolytic colonies on solid agar may lead to *S. aureus* not being detected. Additionally, SCVs can be missed as they are easily overgrown by wildtype *S. aureus* due to a much slower dividing rate of SCVs (180 minutes for SCVs and 20 minutes for wildtype; von Eiff *et al.*, 2001b). Lack of coagulase production and reduced haemolytic activity mean staphylococcal SCVs and are often misidentified as CoNS (McNamara & Proctor, 2000; Seaman *et al.*, 2007). A study evaluating commonly used media for recovery of *S. aureus* showed that SCVs often failed to produce the phenotypic characteristics required in order to obtain a positive *S. aureus* culture result (Kipp *et al.*, 2005). In order to avoid misidentification the extension of conventional culture techniques is desirable. It was concluded that laboratories should be specifically looking for SCVs in samples from patients who have received long-term therapy or when an infectious disease has been unusually persistent. Suspected SCVs that provide abnormal standardised testing results should be confirmed as *S. aureus* by testing the species-specific genes (von Eiff, 2008). A non-conventional diagnostic approach using a 16S rRNA-directed *in situ*

hybridisation technique has been shown to be successful in correctly identifying SCVs as *S. aureus* (Kipp *et al.*, 2003).

1.4.5 Reduced antimicrobial susceptibility in SCVs

Reduced antimicrobial susceptibility in SCVs does not result from the classical mechanisms of antimicrobial resistance such as production of β-lactamases (to inactivate β-lactam based antibiotics) or efflux pumps (to pump antimicrobials out from the cell; al Masaudi *et al.* 1999). The mechanism of reduced susceptibility relates to characteristics associated with the SCV phenotype. For example, reduced aminoglycoside susceptibility is related to the lack of a functional electron transport chain (Figure 1.3). The uptake of positively charged aminoglycoside molecules requires the presence of a differential charge to be present across the bacterial membrane (Kohanski *et al.*, 2010). In aerobically growing bacteria this is present due to the presence of an electrochemical gradient (due to an active ETC). In SCVs however, interruption of the ETC reduces the electrochemical gradient across the bacterial membrane, decreasing the uptake of aminoglycosides (Proctor, 2006). Interruption of the ETC is also responsible for reduced susceptibility to cell wall specific antibiotics as limits on ATP production reduce cell wall biosynthesis which is associated with a four fold increase in the MIC of cell wall specific antibiotics (McNamara & Proctor, 2000). Finally, the survival of *S. aureus* SCVs within host cells reduces the effectiveness of antibiotics that have a limited ability to penetrate eukaryotic cells, such as ß-lactams and vancomycin (Darouiche & Hamill, 1994).

Various antimicrobial agents can select for SCVs which result in reduced susceptibility to these selecting compounds through unique mechanisms. Pan *et al.,* (2002) discovered that exposure of *S. aureus* to sparfloxacin and a ciprofloxacin derivative gave rise to SCVs which exhibited reduced susceptibility to fluroquinolones. In order to identify a genetic basis for the mutations that gave rise to reduced fluroquinolone susceptibility the quinolone resistance-determining region (QRDR) of the *gyrA*, *gyrB*, *grlA*, or *grlB* genes were sequenced. No alterations in the sequence of the QRDRs were detected. The reduced fluroquinolone susceptibility may be related to depletion of intracellular ATP levels which is known to protect against quinolone killing via reducing gyrase-mediated DNA cleavage (Li & Liu, 1998).

Triclosan, a synthetic bisphenol antimicrobial agent, has also been shown to select for *S. aureus* SCVs (Seaman *et al.*, 2007). The target of triclosan is enoyl reductase (encoded by *fab*I) responsible for bacterial fatty acid bioysythesis (Sivaraman *et al.*, 2004). Mutations in *fabI* have been shown to result in reduced triclosan susceptibility in *S. aureus* (Fan *et al.*, 2002). Sequencing of *fab*I in *S. aureus* SCVs however revealed 100% sequence similarity with wildtype susceptible *fab*I sequences, suggesting a novel mechanism for reduced triclosan susceptibility. Despite the aforementioned examples, there are examples where reduced antimicrobial susceptibility is in *S. aureus* SCVs is due to mutations in the target gene. Norstrom *et al.*, (2007) identified novel mutations in *fusA* (translation elongation factor) and *rplF* (ribosomal protein L6) in SCVs selected for in the presence of fusidic acid. These mutations gave rise to reduced fusidic acid susceptibility through altering the structural conformations of translation elongation factor EF-G on the ribosome.

1.4.6 Construction of mutants representing the SCV phenotype

Several mutants have been artificially generated in order to provide insights into the SCV phenotype. von Eiff *et al.* (1997b) constructed a stable *S. aureus* SCV by interrupting the *hemB* gene with an erythromycin cassette. The resulting mutants displayed a classic SCV phenotype with reduced coagulase and pigment production, reduced susceptibility to aminoglycosides and increased intracellular persistence. The same methodology has been used to interrupt the *S. aureus menD* gene, resulting in a stable menadione auxotrophic SCV (Bates *et al.*, 2003). Both mutants have been used to further the understanding of the SCV phenotype and to demonstrate the link between deficiencies in electron transport and SCV characteristics.

1.4.6.1 Microarray and protein profiling analysis of *hemB*$^-$ and *menD*$^-$ mutants

Microarrays allow the production of a ‘gene expression profile’ or ‘signature’ for a particular organism under certain environmental conditions and provide high levels of data output to investigate bacterial metabolism (Ehrenreich, 2006; Lucchini *et al.*, 2001). Seggewiss *et al.*, (2006) performed a comparative, genome-wide transcriptome analysis of the *S. aureus hemB*$^-$ mutant. A difference in the expression of 170 genes was recorded with 122 genes significantly up-regulated in the *hemB*$^-$ mutant in comparison to the parent strain. Glycolytic and fermentation pathways were upregulated in the *hemB*$^-$ mutant due to deficiencies in the ETC. Similar expression

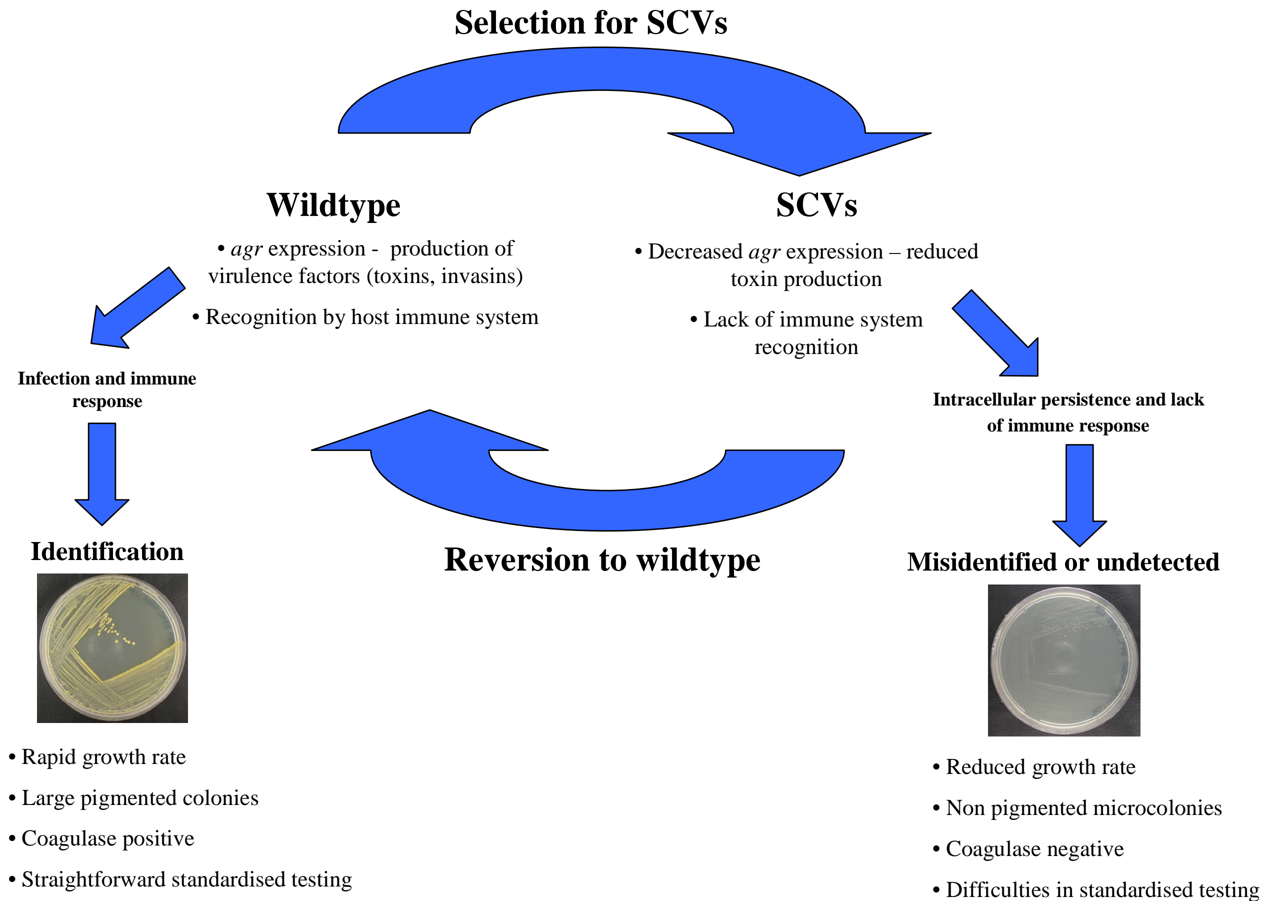


Figure 1.5 Cycling of the SCV phenotype and identification issues Antibiotic treatment of *S. aureus* infections can select for SCVs. Reduced expression of virulence factors may lead clinicians to believe antibiotic therapy has eradicated infection and antibiotic therapy ceases. Removal of the antibiotic selective pressure that maintains SCV phenotype, permits reversion to the virulent wildtype form

profiles have been generated from microarray analysis of SCV *menD*$^-$ mutants, although genes involved in nitrate respiration and fermentation pathways were further upregulated in the *menD*$^-$ mutant (Kohler *et al.*, 2008).

Phenotype microarray (PM) technology consists of preconfigured well arrays in which each well tests a different cellular phenotype (Bochner *et al.*, 2001). Individual wells are continuously monitored and results are combined with bioinformatic software to allow high throughput screening of cellular phenotypes. PM technology has been applied to *hemB*$^-$ and *menD*$^-$ SCV mutants to screen over 1,500 phenotypes (von Eiff *et al.*, 2006). Analysis showed that both mutants were defective in utilizing a variety of carbon sources. The *menD*$^-$ mutant was found to be more metabolically restricted than the *hemB*$^-$ mutant as more metabolic pathways in *S. aureus* utilise menaquinone than haemin. An absolute protein quantification approach by Kriegeskorte *et al.*, (2011) showed an agreement with previous microarray studies; 1019 cytoplasmic proteins in *S. aureus* were identified of which 154 were differentially regulated in SCVs. Proteins involved in the TCA cycle were down regulated as well as certain virulence markers. Notably, differences in the expression of proteins involved in glycolysis were observed between SCV isolates, suggesting that variation exists in SCVs isolated from different environments.

1.4.6.2 Virulence and persistence of *hemB*$^-$ and *menD*$^-$ mutants in animal models

Incorporating SCVs into several established animal models has provided insights into *S. aureus* SCV host/pathogen interactions. Both *hemB*$^-$ and *menD*$^-$ mutants have been investigated in a rabbit model of endocarditis to study virulence and rates of infection (Bates *et al.*, 2003). The capacity to induce experimental endocarditis was similar in the two mutants when compared to the wildtype parent strain. This was surprising as SCVs produced reduced amounts of α-toxin and exhibit reduced rates of growth. The *menD*$^-$ mutant accumulated at lower densities than the *hemB*$^-$ mutant and parent strains, which may be related to its slower growth rate of the *menD*$^-$ mutant. The nematode *Caenorhabditis elegans* has also been used to study host/pathogen interactions of both the *hemB*$^-$ and *menD*$^-$ mutants. In contrast to the rabbit model of endocarditis, both mutants (as well as clinically derived SCVs) were shown to be less virulent in nematodes than parental strains (Sifri *et al.*, 2006). Reduced exoprotein

production due to the loss of a functional electron transport chain was suggested to be responsible for the reduced virulence of SCVs. Finally, the *hemB*⁻ mutant also been examined in a murine model of septic arthritis (Jonsson *et al.*, 2003). In contrast to other animal model results that concluded that the *hemB*⁻ mutant was more virulent in comparison to parent strains. Mice inoculated with the *hemB*⁻ mutant developed more severe clinical arthritis which was linked to higher protease production and decreased adhesive properties of the *hemB*⁻ mutant, enhancing invasiveness. Although the animal models discussed show varying degrees of virulence it is clear that SCVs are associated with altered virulence profiles. Alterations in the expression of virulence factors are considered key to increased intracellular persistence of *S. aureus* SCVs, allowing the ability to cause persistence and recurrent infections.

1.4.7 Intracellular persistence

SCVs have the potential to persist in the presence of antimicrobials via methods that extend beyond classical mechanisms (see section 1.4.5). One such method is encompassing the intracellular environment known as the 'intracellular milieu'. Bacteria have to meet several criteria in order to be able to survive intracellulary, including; not killing the host cell by inducing apoptosis, resisting intracellular host defences, not activating the host immune system and the ability to replicate in the host cytoplasm (Sendi & Proctor, 2009). Assays have demonstrated that SCVs fulfil these criteria by persisting intracellulary in endothelial cells (Balwit *et al.*, 1994; von Eiff *et al.*, 1997b), and persisting in keratinocytes in a patient suffering from Darier's disease (a inherited rare cutaneous disease; von Eiff *et al.*, 2001a; Copper & Burge 2003). SCVs isolated from the Darier's disease patient were shown to persist >100 fold more in comparison to the normal phenotype.

The intracellular milieu itself can trigger the emergence of SCVs in *S. aureus* (Vesga *et al.*, 1996). Intracellular bacteria were shown to develop the SCV phenotype at a much greater rate than bacteria not exposed to an intracellular environment in bovine endothelial cells. Tuchscherr *et al.*, (2010) showed that during infection in endothelial cells SCVs exhibit increased expression of fibronectin-binding proteins (FnBPs; aiding host cell invasion) and avoid activation of the host cell immune system. The latter is linked to the down regulation of virulence factors such as haemolysins, which when produced by *S. aureus* induce inflammation and tissue destruction. The lack of

expression of virulence factors, and reduction of damage to host cells in SCVs can be correlated to low levels of RNAIII and *agr* which act as genetic controls to various virulence factors (Proctor *et al.*, 2006). The study by Tuchscherr *et al.*, further illustrated that SCVs were able to persist in endothelial cells for prolonged periods of time (in comparison to wildtype *S. aureus*), which was attributed to differences in gene expression.

Another recent study by Tuchscherr *et al.*, (2011a) highlighted the importance of the SCV phenotype when *S. aureus* is exposed to intracellular conditions. Over the course of infection in various *in vivo* and *in vitro* models, the *S. aureus* phenotype gradually changed to favour the SCV phenotype. However, SCVs recovered were shown to be extremely unstable, reverting to the wildtype form following subculture. These studies suggest that *S. aureus* has the ability to adopt different phenotypes, adapting to hide or attack host cells in certain conditions. The switch to the SCV phenotype appears to serve as an adaptation for persistence in *S. aureus* and the intracellular milieu may act as reservoir for recurrent infections as patients infected with SCVs can experience disease free periods, lasting several years only for the infection to re-emerge years later (Proctor & Peters, 1998; Tuchscherr *et al.*, 2011a).

1.4.8 Global regulators of *S. aureus* with relevance to the SCV phenotype

1.4.8.1 Accessory gene regulator (*agr*)

The *agr* system consists of two divergently transcribed loci which are controlled by two promoters (Peng *et al.*, 1988). These neighbouring promoters (P2 and P3) regulate the transcription of two transcripts RNAII and RNAIII, respectively (Novick *et al.*, 1993). The P2 transcript contains four genes *agrB, D, C*, and *A*, of which *agrB* and *agrD* together produce an autoinducing peptide (AIP; Novick *et al.*, 1995). AIP increases transcription from the *agr* promoters P2 and P3 corresponding to an increase in transcription of the effector molecule RNA III. *agr* is responsible for the regulation of 138 genes in *S. aureus* of which approximately 20 are putative virulence determinants (Dunman *et al.*, 2001). These include cell wall associated factors (that facilitate attachment to the host) and secreted exotoxins that aid in the invasion process. Due to this role in the regulation of virulence factors, *agr* plays an important role in the infection process. During the initial stages of infection *agr* activity is low

and adhesion factors are expressed, but as infection progresses *agr* activity increases inducing the production of exotoxins (Otto, 2001).

Due to the absence of certain virulence factors in SCVs, the role of *agr* in SCVs has been investigated. Analysis of thymidine auxotrophic SCVs isolated from CF patients and SCV isolates from bovine mastitis has revealed that levels of *agr* expression are reduced or almost absent in SCVs (Atalla *et al.*, 2008; Kahl *et al.*, 2005). *agr* controls the expression of α-hemolysin, which correlates to the lack of α-hemolysin frequently observed in SCVs. The expression of the exoprotein coagulase is also regulated by the *agr* locus (Atalla *et al.*, 2011) and reduced expression of *agr* correlates with the lack of or complete absence of coagulase activity observed in SCVs.

1.4.8.2 Sigma B

Alternative sigma factors in bacteria are involved in regulating gene expression in response to environmental signals such as changes in temperature or pH shifts (Hecker *et al.*, 2007; Kullik *et al.*, 1998). Three alternative sigma factors have been identified in *S. aureus*; sigma A (Deora & Misra, 1996) sigma B (Wu *et al.*, 1996) and sigma H (Morikawa *et al.*, 2003), all of which are closely related to their respective forms in *Bacillus subtilis*. Regulation of σ^B is modulated by *rsbU*, *rsbV*, *rsbW* gene products which sit in a chromosomal cluster along with *sigB* (Bronner *et al.*, 2004). Under non stress conditions RsbW acts as an anti-sigma factor and holds σ^B in an inactive complex (Pane-Farre *et al.*, 2006). Under stress conditions RsbV is dephosphorylated by RsbU and forms a RsbV–RsbW complex. This permits the release of free σ^B, which binds to RNA polymerase to form an active σ^B-holoenzyme (Bronner *et al.*, 2004). Microarray based analysis has shown that σ^B influences the expression of 251 genes, 198 of which are positively influenced (Bischoff *et al.*, 2004). Cell envelope biosynthesis, signalling pathways and various virulence factors were among some of the diverse cellular processes influenced by σ^B. Numerous studies have examined σ^B activity in *S. aureus* SCVs. A collection of TD-SCVs isolated from CF patients exhibited decreased σ^B activity in comparison to isogenic wildtype strains (Kahl *et al.*, 2005). σ^B activity in these strains however was restored upon supplementation with thymidine suggesting that regulatory mechanisms are responsible for the various alterations observed. In contrast, another study showed

SCVs isolated from CF patients exhibited increased σ^B activity in compassion to parent strains (Moisan *et al.*, 2006). Increased σ^B activity was correlated to persistence in eukaryotic host cells which was impaired in a σ^B deficient mutant. Mitchell *et al.* (2008) demonstrated that this may be related to increased expression of FnBPs, (which are positively influenced by σ^B) which permits increased adhesion of *S. aureus* to host tissues.

Recently σ^B has been implicated in the emergence of SCVs following exposure to aminoglycosides (Mitchell *et al.*, 2010a). Sub-inhibitory concentrations of gentamicin and tobramycin significantly increased the frequency SCV formation in *S. aureus* strains with σ^{B+} background, where-as fewer SCVs were recovered from a σ^{B-} constructed mutant (Mitchell *et al.*, 2010a). Quantitative PCR (qPCR) also demonstrated that sub-inhibitory concentration of gentamicin and tobramycin induced σ^B activity. Exposure to 4-hydroxy-2-heptylquinoline-N-oxide (HQNO; an antistaphylococcal exoproduct produced by *P. aeruginosa*; Machan *et al.*, 1992) has also been shown to select for *S. aureus* SCVs via a σ^B dependent mechanism. Exposure to HQNO resulted in a concomitant activation σ^B of and repression of *agr* (Mitchell *et al.*, 2010b). Additionally, elevated σ^B levels were responsible for increased FnBP expression and increased biofilm formation.

1.5 SCVs in bacterial species other than *S. aureus*

Although *S. aureus* SCVs are the most extensively studied, SCVs have been isolated from a broad range of Gram-negative and Gram-positive bacteria including *Pseudomonas aeruginosa* (Haussler *et al.*, 1999b; Haussler *et al.*, 2003b), *E. coli* (Lewis *et al.*, 1991), CoNS such as *S. epidermidis*, and *S. capitis* (von Eiff *et al.*, 1999) *E. faecalis* (Wellinghausen *et al.*, 2009) and *Burkholderia pseudomallei* (Haussler *et al.*, 1999a). Notably, the majority of these species are of significant medical importance in conditions such as cystic fibrosis (CF), nosocomial infections and device related infections. Research bias in favour of clinically-important species many account for the reporting of SCVs in the aforementioned groups and little is know about SCVs in the environment. Despite this bias, there is paucity of publications on SCVs in species other than *S. aureus*. SCVs of these species share some of the characteristics associated with *S. aureus* SCVs, including auxotrophy

(Funada *et al.*, 1978; Wellinghausen *et al.*, 2009), reduced susceptibility to aminoglycosides (Gerber & Craig, 1982; Haussler *et al.*, 1999a), and altered biochemical profiles (Langford *et al.*, 1989; Voureka, 1951).

1.6 Project aims

- Investigate the ability of various aminoglycosides to select for *S. aureus* SCVs across a range of concentrations.

- Characterise aminoglycoside selected SCV isolates to determine if variation is present among SCV isolates and if any variation is influenced by the concentration of selecting aminoglycosides.

- Investigate if the tetracycline group of antibiotics can select for *S. aureus* SCVs and to characterise any tetracycline selected SCVs.

- Examine biofilm formation in *S. aureus* SCVs and compare biofilm formation with parent strains.

- Examine biofilm susceptibility to antimicrobials and the effects of antimicrobial agents on biofilm mass and cells within biofilms.

- Identify if a difference in antimicrobial susceptibility of biofilms formed by *S. aureus* SCV and parent strains is present. If a relationship is present, elucidate a mechanism for difference in susceptibility.

- Investigate the anti-staphylococcal effects of various plant compounds produced by the CASE sponsor, Cultech Ltd, and compare SCV and parent susceptibilities.

- Investigate if resistance to these compounds can be induced and if they have a synergistic relationship with clinically used antibiotics.

- Identify if antibiotics can select for bacterial species other than *S. aureus* and characterise any antibiotic selected SCVs using species specific assays.

- Examine biofilm formation in SCVs of other bacterial species.

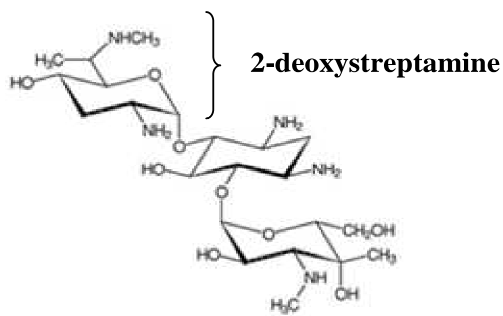
2 CHAPTER 2: AMINOGLYCOSIDE SELECTION FOR *STAPHYLOCOCCUS AUREUS* SMALL COLONY VARIANTS

2.1 Introduction

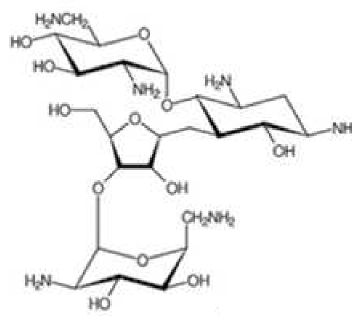
2.1.1 Aminoglycoside classification and mode of action

Gentamicin, neomycin, streptomycin and tobramycin are all examples of amino sugars that are members of the aminoglycoside family. Aminoglycosides have a backbone structure which is made up of an aminocyclitol ring saturated with amine and hydroxyl substitutions (Shakil *et al.*, 2008). Aminoglycosides can be distinguished on the basis this aminocyclitol ring. For example, streptomycin contains streptidine as its aminocyclitol unit (Figure 2.1), where as gentamicin, kanamycin and neomycin are 2-deoxystreptamine containing aminoglycosides (Magnet & Blanchard, 2004).

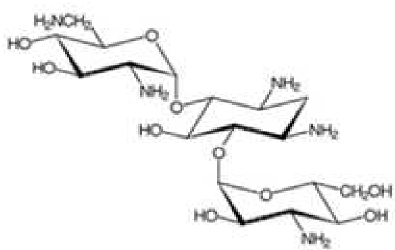
Aminoglycosides exhibit activity against a range of clinically important pathogens including *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Pseudomonas* spp., *Staphylococcus aureus* and some streptococci (Vakulenko & Mobashery, 2003). Aminoglycosides display good synergetic activity with other antibiotic classes (such as β-lactams) and are therefore used in the treatment of a range of bacterial infections including meningitis, pneumonia, tuberculosis and even plague (Nakamura *et al.*, 2000; Shakil *et al.*, 2008). Aminoglycosides bind to the 16S rRNA, at the tRNA acceptor A site and interfere with protein synthesis (Magnet & Blanchard, 2004). The exact mechanism of action of aminoglycosides is uncertain and multiple mechanisms may be involved. It has been suggested that the binding of aminoglycosides induces the misreading of messenger RNA producing defective proteins, but aminoglycosides may also interfere with initiation complexes (Wirmer & Westhof, 2006). Aminoglycoside uptake in Gram-positive bacteria takes place in two distinct phases. Firstly, energy-independent binding of aminoglycosides to phospholipids and teichoic acids occurs. This is followed by an energy-dependent transport across the cytoplasmic membrane, provided a sufficient membrane potential ($\Delta\Psi$) is present (Taber *et al.*, 1987).



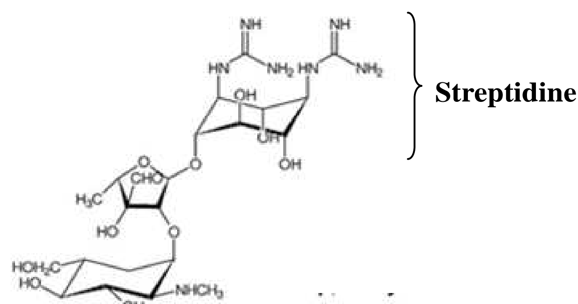
Gentamicin



Neomycin



Kanamycin



Streptomycin

Figure 2.1 Chemical structure of aminoglycosides used in this study. Adapted from Klostermeier *et al.*, (2004).

2.1.2 Aminoglycoside resistance

Streptomycin was the first aminoglycoside to be discovered following its isolation from the actinomycete *Streptomyces griseus* in 1944 and was shown to be active against *Mycobacterium tuberculosis* (Schatz & Waksman, 1944{Schatz, 1944 #773)}. The antibiotic was later shown to be clinically effective in the treatment of tuberculosis (Hinshaw *et al.*, 1946). Resistance however was reported just two years later (Youmans *et al.*, 1946). Currently resistance to aminoglycosides can be acquired by four different mechanisms: i) alteration of the target; ii) interference with transport of the antibiotic; iii) enzymatic inhibition of the antibiotic; iv) substitution of the target (Veyssier & Bryskier, 2005).

2.1.2.1 Aminoglycoside resistance in *S. aureus*

Aminoglycoside resistance is common in staphylococci and has been surveyed by the SENTRY Antimicrobial Surveillance Programme (Schmitz *et al.*, 1999). Nineteen European hospitals were surveyed and found that 23% of *S. aureus* isolates were resistant to gentamicin, 29% to tobramycin, 31% to kanamycin and 21% to streptomycin. High level aminoglycoside resistance in *S. aureus* can be conferred by mutations in the 30S domain of the bacterial ribosome, leading to altered aminoglycoside binding (Schito, 2006). However, the most common mode of resistance is through aminoglycoside modifying enzymes (AMEs) which inactivate many aminoglycosides of therapeutic importance (Jensen & Lyon, 2009). AMEs can be grouped into three categories according to their mode of action on the aminoglycoside substrate; acetyltransferase (AAC), phosphotransferase (APH) and nucleotidyltransferase (ANT; Woodford, 2005). Aminoglycosides are chemically modified by AMEs (through covalent modification of specific amino or hydroxyl functions), which results in poor binding to the ribosome (Mingeot-Leclercq *et al.*, 1999). AMEs are often located on plasmids, transposons and integrons permitting lateral transfer of aminoglycoside resistance among certain bacterial populations (Shakil *et al.*, 2008).

2.1.2.2 Reduced aminoglycoside susceptibility in *S. aureus* small colony variants

Small colony variants (SCVs) however do not follow these ‘classical mechanisms’ of resistance. Reduced susceptibility to aminoglycosides in SCVs is due to a failure to accumulate aminoglycosides intracellulary (Miller *et al.*, 1980). As SCVs are

defective in electron transport, SCVs display a reduced $\Delta\Psi$. Reduced $\Delta\Psi$ across the bacterial membrane results in a lowered uptake of positively charge compounds such as aminoglycosides (Proctor & Peters, 1998). Monitoring $\Delta\Psi$ in *S. aureus* parent and SCV strains Baumert *et al.* (2002) showed that following the accumulation of glucose, $\Delta\Psi$ in SCVs dropped rapidly. The reduction in $\Delta\Psi$ was directly linked to a 10-30 fold reduction in aminoglycoside susceptibility. Low level or complete absence of $\Delta\Psi$ in anaerobic bacteria confers intrinsic resistance to aminoglycosides (Bryan *et al.*, 1979) highlighting the importance of $\Delta\Psi$ for aminoglycoside susceptibility.

2.1.3 Aminoglycoside selection for *S. aureus* SCVs

Various classes of antimicrobial agents have been demonstrated to select for *S. aureus* SCVs most notably aminoglycosides and trimethoprim-sulfamethoxazole (SXT) (Gilligan *et al.*, 1987; Kahl *et al.*, 1998). Other antibiotics such as fusidic acid (Norstrom *et al.*, 2007), members of the fluroquinolones (pazufloxacin and sparfloxacin; Mitsuyama *et al.*, 1997; Pan *et al.*, 2002) and biocides such as triclosan (Seaman *et al.*, 2007) have all been shown to induce SCV formation. Extracellular products produced by other bacteria have also been implicated in the formation of *S. aureus* SCVs. 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) and pyocyanin produced by *P. aeruginosa* both interfere with the *S. aureus* electron transport chain (ETC) and have been demonstrated to select for SCVs *in vitro* (Biswas *et al.*, 2009; Hoffman *et al.*, 2006).

Aminoglycosides are the most commonly reported antimicrobial to be associated with the selection of *S. aureus* SCVs, with cases of both *in vivo* and *in vitro* selection for SCVs being documented. In the clinical setting SCVs are often recovered from patients who are being treated for osteomyelitis (von Eiff *et al.*, 1997), which is often treated by surgical placement of gentamicin beads in bones where infection is present. The use of gentamicin beads provides a steady release of antimicrobial to the site of infection over the course of weeks or months if required (Evans & Nelson, 1993). It is hypothesised that the slow release of low levels of gentamicin into the infected area is an efficient way to select for SCVs (von Eiff *et al.*, 1998). Several studies have demonstrated aminoglycosides can readily select for SCVs *in vitro* (Balwit *et al.*, 1994; Kaplan & Dye, 1976; Musher *et al.*, 1977). SCVs selected for by aminoglycosides *in vitro* share characteristics with those isolated from patients

receiving aminoglycoside treatment. Aminoglycoside selected SCVs are frequently auxotrophic for haemin and menadione (Balwit *et al.*, 1994; von Eiff *et al.*, 1997), which relates to defects in the ETC and ATP generation (see section 1.4.1.1).

2.1.3.1 Molecular mechanisms for aminoglycoside induced SCV selection

The formation of SCVs in *S. aureus* has been postulated to involve underlying regulatory and genetic mechanisms. Schaaff *et al.* (2003) demonstrated that mutations are involved in the emergence of aminoglycoside selected SCVs. Sequencing of the haemin operon of a gentamicin selected, stable haemin auxotroph revealed the presence of a deletion in the *hemH* gene. Mutation in *hemH* leads to the inactivation of last step of haemin biosynthesis and is responsible for the auxotrophy for haemin displayed. Global regulators have also been demonstrated to play a role in SCV formation. Recently the alternative transcription sigma factor, sigma B (σ^B) has been implicated in the formation of SCVs (Mitchell *et al.*, 2010a). *S. aureus* strains with a σ^{B+} and σ^{B-} background were exposed to subinhibitory concentrations of gentamicin and gene expression was monitored. Emergence of SCVs was promoted in the presence of aminoglycosides in the σ^{B+} strain; however SCVs were not detected from the σ^{B-} strain. Additionally the presence of aminoglycosides in the exponential phase of growth significantly increased the expression of σ^B and correlated with the emergence of SCVs.

2.1.4 Aims

Although aminoglycosides such as gentamicin are known to select for *S. aureus* SCVs, little is known about the effect of different aminoglycosides and different concentrations on SCV selection. Differences in auxotrophy have been reported in aminoglycoside selected SCVs, no study has conducted analysis on variations that may exist in SCVs selected from different aminoglycoside concentrations. Therefore the aims of this study were to:

- Examine the ability of gentamicin, kanamycin, neomycin and streptomycin to select for *S. aureus* SCVs
- Examine the effect of aminoglycoside concentration on growth rate
- Determine SCV selection frequencies in the presence of various aminoglycoside concentrations
- Confirm SCV isolates are *S. aureus* via species specific multiplex PCR
- Characterise SCV isolates on the basis of auxotrophy, ATP concentration, heamolysis, carotenoid biosynthesis, and reversion rate
- Examine and compare mutation frequency in SCV isolates

2.2 Materials and methods

2.2.1 Bacterial strains

S. aureus American Type Culture Collection (ATCC) strain 25923 and epidemic methicillin-resistant *S. aureus* (EMRSA) strain 15 were included in this study in order to represent well characterised MSSA and MRSA strains respectively. Both strains are susceptible to a range of aminoglycosides according to published CLSI guidelines. Strains were maintained at -80°C in Mueller Hinton (MH) broth supplemented with 8% dimethyl sulfoxide (DMSO) and re-isolated on MH agar plates when required.

2.2.2 Preparation of aminoglycoside stock solutions

Gentamicin, kanamycin, neomycin and streptomycin were all obtained from Sigma Aldrich (UK). Manufactures potencies were used to determine amounts of each aminoglycoside powder required to produce a 10,000 mg/L solution. All powders were dissolved in sterile deionised water, dissolved thoroughly (through vortex mixing) and filter sterilised with a 0.2 µm filter (Minisart, UK). Reduced strength stock solutions were made where required in deionised water and all stock solutions were stored at 4°C for a maximum of 14 days.

2.2.3 Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined according to Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2006). Cation adjusted Mueller Hinton (CAMHB) was used for MIC determination and stock solutions of $CaCl_2$ and $MgCl_2$ were prepared and added to MH broth to ensure each batch contained the correct concentrations of $CaCl_2$ (20 mg/L) and $MgCl_2$ (10 mg/L). Individual *S. aureus* colonies (3-4) were inoculated into CAMHB and incubated at 37°C with shaking at 150 rpm. Cultures were grown to the end of logarithmic phase and cell densities were adjusted to match the turbidity of a 0.5 McFarland standard at 625 nm. The range of antimicrobial concentrations to be tested was decided and concentrations were made in CAMHB at double the required concentration to allow for dilution by the inoculum. Microtitre well plates (Fisher, UK) were inoculated with 100 µL of required antimicrobial concentrations and 100µL of inoculum to provide a test inoculum of 5 X 10^5 CFU/mL. Microtitre plates were incubated at 37°C without shaking and the MIC was recorded as the lowest concentration that inhibited visible growth after 18 hours.

2.2.4 Determination of minimum bactericidal concentrations

Minimum bactericidal concentrations (MBC) were determined according to CLSI guidelines (CLSI, 1999) with recommendations from Peterson & Shanholtzer (1992). All non-turbid wells from MIC experiments were further examined to determine MBCs. Wells were stirred gently with a pipette tip and 100 µL aspirated onto the surface of an antimicrobial free MH agar plate. In order to avoid carryover of antimicrobial agents, samples were dispensed onto the centre of a MH agar plate and streaked down the centre to allow the broth to be absorbed into the agar. The inoculum was then spread over the plate with a sterile glass rod. Plates were then incubated at 37°C and examined for growth after 24 and 48 hours. Following incubation, plates were analysed for growth and the number of colony forming units (CFU) recorded. The MBC was recorded as the lowest concentration that provided a 99.9% reduction from the initial inoculum.

2.2.5 SCV selection assays

Aminoglycosides were prepared in CAMHB at concentrations of X 0.25 MIC, X 0.5 MIC, MIC, X 2 MIC and X 4 MIC and dispensed into wells of a microtitre well plate. *S. aureus* strains ATCC 25923 and EMRSA 15 were grown in CAMHB as described previously (section 2.2.3), and dispensed into wells contain aminoglycosides to achieve a starting inoculum of 5 X 10^5 CFU/mL. Aminoglycoside concentrations were prepared to double the final concentration to allow for dilution by the inoculum and control wells containing aminoglycoside free CAMHB were also included. Microtitre plates were incubated for 24 hours at 37°C without shaking. Following incubation, wells were analysed for the presence of SCVs by aspirating 100 µL of individual well contents onto MH agar containing the defined MIC for the aminoglycosides tested. Agar plates were inverted and incubated for 48 hours at 37°C. Additionally serial dilutions of wells were prepared in phosphate buffered saline and plated onto aminoglycoside free MH agar to calculate the number of wild type CFU. Suspect SCVs were subjected to coagulase analysis using Staphylase test kit (Oxoid Ltd, UK). Several colonies of suspected SCVs were smeared onto a test circle on a reaction card. A drop of test reagent was added and colonies mixed into reagent using a sterile wire loop. The presence of agglutination indicated the colonies being tested were coagulase positive, whereas the absence of agglutination was recorded as coagulase negative. Microcolonies that were coagulase negative were recorded as

SCVs and the SCV formation frequency was calculated as the number of SCVs per CFU counts on aminoglycoside free MH agar.

2.2.6 *S. aureus* growth characteristics in the presence of aminoglycosides

Growth dynamics in a range of aminoglycoside concentrations were examined using a Bioscreen C analyser (ThermoFisher UK). Aminoglycoside concentrations and *S. aureus* cells were prepared as described previously (section 2.2.3) and inoculated into honeycomb bioscreen plates (Growth Curves Ltd, Finland). Plates were incubated for 48 hours at 37°C with shaking for 5 seconds before every optical density measurement at intermediate intensity. Optical density was read using the wideband filter (450 – 580 nm) every 10 minutes. Aminoglycoside free CAMHB was used to obtain growth curves in the absence of aminoglycosides which served as controls.

2.2.7 DNA extraction

S. aureus DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, UK). Overnight *S. aureus* parent and SCV cultures were pelleted by centrifuging at 10,000 rpm in microfuge tubes. 200µL of lysis buffer (consisting of 200 U/mL lysostaphin and 2 x 10^6 U/mL lysozyme) was used to resuspend pellets, which were subsequently incubated for 30 minutes at 37°C. Proteinase K solution (20 µL) was then added followed by addition of 200 µL of manufacturers specific lysis solution. Samples were vortexed thoroughly in order to create a homogenous mixture and incubated at 55°C for 10 minutes. DNA binding columns were optimised for binding using ethanol, followed by the addition of the previously prepared lysate. Samples were centrifuged at 8,000 rpm for 1 minute, followed by 2 additional washing steps before eluting bound DNA by the addition of 200 µL manufactures elution solution. Finally sample were centrifuged for 1 minute at 10,000 rpm and the remaining elute (regarded as pure genomic DNA) stored at 4°C until required.

2.2.8 Species confirmation

As *S. aureus* SCVs are frequently difficult to identify a modified version of the quadriplex PCR protocol developed by Zhang *et al.* (2004) was employed to confirm that SCV isolates were in fact *S. aureus*. PCR targeted 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), and *mecA* (a determinant of methicillin resistance) using the primers in Table 2.1.

All PCR reagents were supplied by Qiagen (UK). PCR was carried out in 25 µL reactions with 2 µL template DNA (approximately 50 ng/µL) being added to a 23 µL PCR mixture consisting of; sterile polished deionised water, 1 X Coralload buffer, 1 X Q solution, 1.5 mM $MgCl^2$, 0.12 µM each 16S rRNA and *mecA* primers, 0.04 µM each *nuc* primer, 200 µM dNTPs, and 1 unit *Taq* DNA polymerase. PCR was conducted in a Flexigene Thermal Cycler (Techne Ltd., UK) with the following cycle; 5 min at 94°C, followed by 10 cycles of 94°C for 40 seconds, 68°C for 40 seconds, and 72°C for 1 min and 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, and a final hold at 72°C for 10 min. PCR products (12 µL) were run on 2% w/v agarose (Sigma Aldrich, UK) gels and were visualised with ethidium bromide (0.5 µg/mL final concentration in TAE buffer; 40 mM Tris-acetate, 1 mM EDTA) for 30 minutes. Molecular standards were run on gels using Hyperladder I (Invitrogen, UK).

2.2.9 Analysis of SCV isolates

For the purpose of following experiments, where required SCV and parent strains were grown in MH broth at 37°C with shaking at 150 rpm. Parents were incubated for 24 hours whereas SCVs were incubated for 48 hours to allow sufficient growth.

2.2.9.1 Biochemical analysis

API STAPH strips (Biomerieux, France) were used to analysis the ability of SCV isolates to ferment certain carbohydrates and to examine enzyme production. A direct colony suspension of SCV and parent cells was prepared in API STAPH suspension medium (Biomerieux, France) and 100 µL added to all tubes. Anaerobic conditions were created by the overlaying of mineral oil to ADH and URE tests. Parent test strips were incubated at 37°C for 24 hours where as the incubation period was extended to 48 hours for SCV isolates. Positive/negative results were read based on colour changes outlined by the API STAPH guide. The catalogue of the enzymatic reactions tested and resulting colour changes are listed in Appendix 1.

2.2.9.2 Haemolytic assay of growth medium

Haemolytic activity of parent and SCV strains was analysed using the method of Brouillette *et al.*, (2004). Optical density of cultures was measured at 650 nm and recorded. Cultures were then centrifuged at 10,000 rpm for 5 minutes and the supernatant collected. 1 mL of supernatant was incubated with 5 mL of sheep red

Table 2.1 Primers used in multiplex PCR for species confirmation of SCVs

Primer	Sequence (3'- 5')
Staph756F	AACTCTGTTATTAGGGAAGAACA
Staph750R	CCACCTTCCTCCGGTTTGTCACC
MecA1	GTAGAAATGACTGAACGTCCGATAA
MecA2	CCAATTCCACATTGTTTCGGTCTA
Nuc1	GCGATTGATGGTGATACGGTT
Nuc2	AGCCAAGCCTTGACGAAC TAAAGC

Lyophilised primers were obtained from MWG Eurofins (Germany). 100 pmol stocks were obtained by the addition of nuclease free H_2O.

blood cells (Oxoid, UK) for 1 h at 37 °C to allow lysis. Red blood cell debris was pelleted by centrifugation and released haemoglobin was measured at 540 nm. Haemolytic activity was estimated based on the ratio of OD_{540}/OD_{650}.

2.2.9.3 Reversion rates

Rates of reversion were calculated using the method of Seaman *et al.*, (2007). Ten SCV colonies were suspended in 3 mL of 0.9% NaCl and used to inoculate an antibiotic free MH agar plate. After 48 hours growth at 37°C the number of wildtype colonies was counted and the frequency of reversion was determined as the number of wildtype CFU per SCV.

2.2.9.4 Carotenoid production

Carotenoid production was quantified using a methanol extraction protocol (Morikawa *et al.*, 2001). Cells were pelleted by centrifuged at 10,000 rpm for 5 minutes and washed once with PBS. Cells were resuspended in 200 µl methanol and heated at 55°C for 3 minutes. The supernatant was removed from the cell debris after spinning for 1 minute at 13,000 rpm and methanol added to yield a final volume of 1 mL. Absorption spectra of the methanol extracts were measured in a quartz cuvette and the absorbance at 465 nm was recorded.

2.2.9.5 Quantification of intracellular ATP

In order to compare levels of ATP between SCVs and parents an enzyme based luciferase assay was employed. The BacTiter-Glo™ Microbial Cell Viability Assay (Promega, UK) quantifies ATP using mono-oxygenation of luciferin to produce a light signal which can be detected using a luminometer. Cultures were adjusted to 1 X 10^7 CFU/mL and 100 µL added to individual wells of 96 microtitre well plates. BacTiter-Glo reagent (100 µL) was added to each well and gently shaken for 5 minutes at room temperature. Control wells contained no bacteria and were used to determine background luminescence. Luminescence was detected using relative light units (RLU) with a LUMIstar OPTIMA plate reader (BMG, UK). RLU/ATP was determined from a standard curve using dilutions of ATP standard (Promega, UK) and the concentration of ATP per mL was calculated.

2.2.9.6 Auxotrophy profiles

SCVs are commonly auxotrophic for haemin, menadione and thymidine (individually or a combination), therefore the auxotrophy profiles of various SCV isolates were examined. Stock solutions (1,000 mg/L) of haemin, menadione and thymidine (Sigma Aldrich, UK) were prepared by dissolving powders in DMSO (haemin) or sterile deionised H_2O (menadione and thymidine). Stock solutions were stored at 4°C for a maximum of 14 days. Lawns of SCVs were prepared by spreading 100µL of overnight culture onto the surface of a MH agar plate. Sterile filter paper discs plated in the centre of the plate and 10 µL of haemin, menadione or thymidine stock solutions added to filter discs and plates were incubated for 48 hours. SCVs were confirmed as auxotrophic if a zone of wildtype like growth (i.e. large colonies, restoration of pigment) was present surrounding the filter disc.

2.2.9.7 Mutation frequency to rifampicin resistance

For the measurement of mutation frequency to rifampicin resistance of SCV and parent strains the method of Besier *et al.*, (2008a) was applied. Cultures were pelleted by centrifugation at 10,000 rpm for 5 min and resuspended in 1 mL MH broth. A 100 µL sample of this suspension was plated onto MH agar plates as well as MH agar containing rifampin at a concentration of 100 mg/L. After 48 hours incubation at 37°C, CFU were counted and mutation frequencies determined by dividing the number of CFU on rifampicin-supplemented agar by the number of CFU on rifampicin-free agar. Colonies growing on rifampicin containing plates were streaked onto another rifampicin containing in order to prove the stability of the mutants.

2.2.9.8 Statistical analysis

Minitab statistical software 16 was used to investigate significant difference between parent and SCV isolates. Provided the data met the requirement of being normally distributed and showed equal variance, data was subject to 2 sampled t tests with 95% confidence intervals. If the assumptions of the t test were violated the Mann-Whitney test (non-parametric(with 95% confidence intervals was applied.

2.3 Results

2.3.1 MIC and MBCs

S. aureus strains ATCC 25923 and EMRSA 15 were tested for their susceptibilities to the aminoglycosides gentamicin, kanamycin, neomycin and streptomycin. Both strains were found to be sensitive to all aminoglycosides tested (Table 2.2). MIC values for the four aminoglycosides tested were 2-4 times greater for EMRSA 15 in comparison to ATCC 25923. ATCC 25923 MBCs for gentamicin, kanamycin and neomycin were 2 times greater than MIC values where as the MBC for streptomycin was 4 times greater than the MIC. During MBC testing however SCVs were frequently detected and values shown in Table 2.2. These represent 99.9% elimination of wildtype and do not account for the selection of SCVs.

2.3.2 Minimum small colony variant prevention concentration (MSCVPC)

As SCVS were frequently detected at concentrations deemed bactericidal for wildtype *S. aureus* the term minimum concentration that prevents formation of SCVs during routine MIC and MBC testing (MSCVPC) was implemented. During MBC testing, plates regularly contained a mixture of wildtype and SCV colonies (Figure 2.2). As the antibiotic concentration was increased wildtype colonies were completely eradicated leaving only SCV colonies. CLSI guidelines stipulate that a 99.9% reduction from the 'original' inoculum is required to achieve MBC criteria. Consequently the MBC was recorded as the lowest concentrations at which 99.9% of wildtype were eliminated and MSCVPC was introduced to cover the concentration at which SCVs were not detected. The MSCVPC varied for each aminoglycoside but was always within the range of being equal to 2 times greater than the MBC (Table 2.2).

2.3.3 SCV selection frequencies

All four aminoglycosides were successful in isolating SCVs although differences in frequencies after exposure to different concentrations and between strains were observed (Table 2.3). Notably the presence of subinhibitory concentrations (X 0.25 and X 0.5 MIC) of all aminoglycosides significantly increased the recovery of SCVs in comparison to untreated control conditions ($P = < 0.01$). SCVs were recovered from control conditions that were plated on kanamycin and streptomycin in both

Table 2.2 Aminoglycoside MICs, MBCs and MSCVPC (mg/L) for *S. aureus* ATCC 25923 and EMRSA 15 determined by broth microdilution

	ATCC 25923			EMRSA 15		
	MIC	MBC	MSCVPC	MIC	MBC	MSCVPC
Gentamicin	0.25	0.5	1	1	2	4
Neomycin	0.5	1	2	2	5	10
Kanamycin	2	4	8	4	12	20
Streptomycin	2	8	8	4	20	30

Modal MIC, MBC and MSCVPC values are presented. Modal values were obtained from three independent replicates and three independent biological replicates.

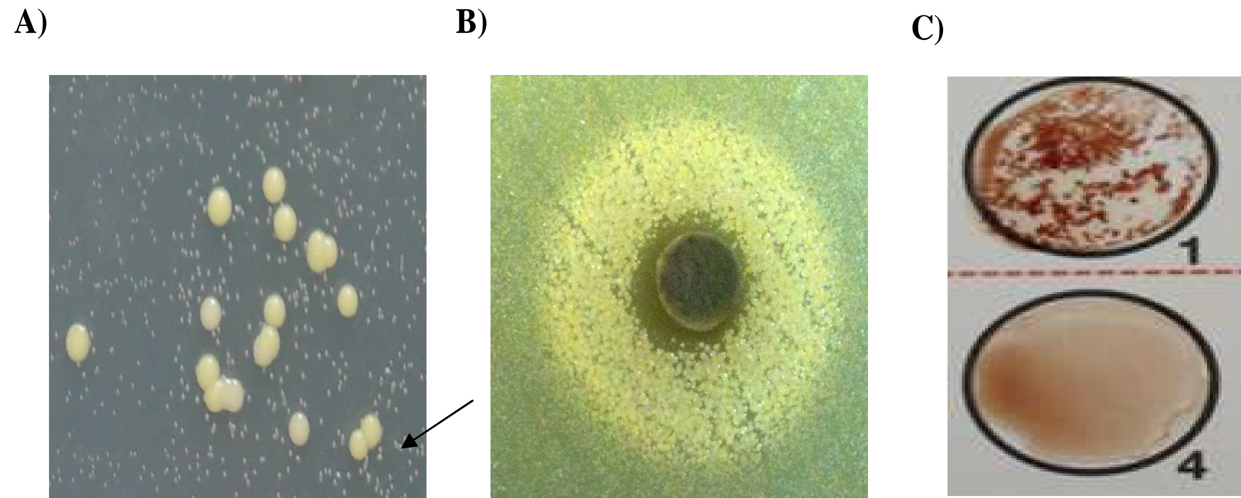


Figure 2.2 Characteristics associated with *S. aureus* SCVs recovered following aminoglycoside exposure A) - Presence of parent and SCV colonies (arrow) recovered following MBC testing; B) - Restoration of wildtype growth and pigmentation around a filter disc impregnated with haemin indicating haemin auxotrophy; C) - Agglutination positive *S. aureus* (top) and agglutination negative *S. aureus* SCVs (bottom). The differences in colony size, pigmentation and coagulase activity demonstrate the difficulties in identifying *S. aureus* SCVs using traditional methods.

strains and from only EMRSA 15 in the case of neomycin. Interestingly concentrations of aminoglycosides that are inhibitory (exceeded MIC) to ATCC 25923 and EMRSA 15 were also found to select for SCVs. Exposure of EMRSA 15 to all tested combinations across the range of aminoglycoside gave rise to SCVs, expect for X 4 MIC gentamicin (Table 2.3). In contrast SCVs were not detected from ATCC 25923 after to exposure to any aminoglycosides at X 4 MIC concentration. Exposure to X 4 MIC for EMRSA 15 proved to be the most efficient concentration in selecting for SCVs for neomycin, kanamycin and streptomycin.

2.3.4 Identification of SCVs as *S. aureus* using multiplex PCR

Amplification of 16S rRNA and *nuc* genes was successfully employed to ensure that all SCVs were *S. aureus* and not contaminants (Figure 2.3). SCV isolates recovered following exposure to various aminoglycoside concentrations show the presence of 16S rRNA (756 bp) and *nuc* (279 bp), which can also be observed in the parent strain (Figure 2.3). Additionally the presence of *mecA* (310bp) can be observed in SCV isolates recovered from EMRSA 15, permitting discrimination between MRSA and MSSA strains.

2.3.5 Reversion rates

The rate at which SCVs reverted to the parent form varied according to the aminoglycoside concentration applied. SCV isolated at X 2 and X 4 MIC of the four aminoglycosides tested were deemed as stable isolates as no revertants were detected (Table 2.4). SCVs isolated from X 0.25 MIC of all aminoglycosides showed high rates of reversion, and reversion rates were significantly higher than reversion rates at X 0.5 MIC ($P = < 0.05$). As aminoglycoside concentrations increased, the frequency at which revertants from both strains were detected decreased. For example the reversion frequency of EMRSA 15 SCV isolated from X 0.5 MIC neomycin exposure (2.2×10^{-4}) was significantly higher than the reversion frequency of EMRSA 15 SCV isolated from MIC neomycin exposure (8.4×10^{-5}; $P = < 0.05$). Comparison of SCVs selected from different aminoglycosides revealed no significant difference in reversion rates.

Table 2.3 SCV selection frequencies (expressed as SCVs per CFU) after 24 hours exposure to increasing concentrations of aminoglycosides

	Control		**X 0.25 MIC**		**X 0.5 MIC**		**MIC**		**X 2 MIC**		**X 4 MIC**	
	25923	E15	25923	E15	25923	E15	25923	E15	25923	E15	25923	E15
Gentamicin	ND	ND	$2.1 X 10^{-4}$	$8.7 X 10^{-4}$	$3.5 X 10^{-2}$	$2.5 X 10^{-3}$	$6.2 X 10^{-2}$	$2.1 X 10^{-2}$	$2.1 x 10^{-1}$	$2.5 X 10^{-1}$	ND	ND
Neomycin	ND	$1.1 X 10^{-6}$	$8.3 X 10^{-4}$	$3.2 X 10^{-5}$	$2.6 X 10^{-3}$	$3.9 X10^{-3}$	$1.7 X 10^{-2}$	$4.2 X 10^{-2}$	$2.6 X 10^{-1}$	$8.5 X 10^{-1}$	ND	$2.5 X 10^{2}$
Kanamycin	$2.7 X 10^{-6}$	$6.2 X 10^{-6}$	$5.1 X10^{-5}$	$2.1 X 10^{-5}$	$2.1 X 10^{-2}$	$7.6 X 10^{-3}$	$8.4 X 10^{-2}$	$1.6 X 10^{-2}$	$5.4 X 10^{-1}$	$5.9 X 10^{-1}$	ND	$3.2 X 10^{2}$
Streptomycin	$1.5 X 10^{-6}$	$7.3 X 10^{-5}$	$4.2 X 10^{-4}$	$8.9 X 10^{-5}$	$5.9 X 10^{-3}$	$5.7 X 10^{-3}$	$5.3 X 10^{-3}$	$7.5 X 10^{-3}$	$2.3 X 10^{-1}$	$4.2 X 10^{-2}$	ND	$4.8 X 10^{2}$

25923 – ATCC 2923; E15 – EMRSA 15; ND – SCVs not detected

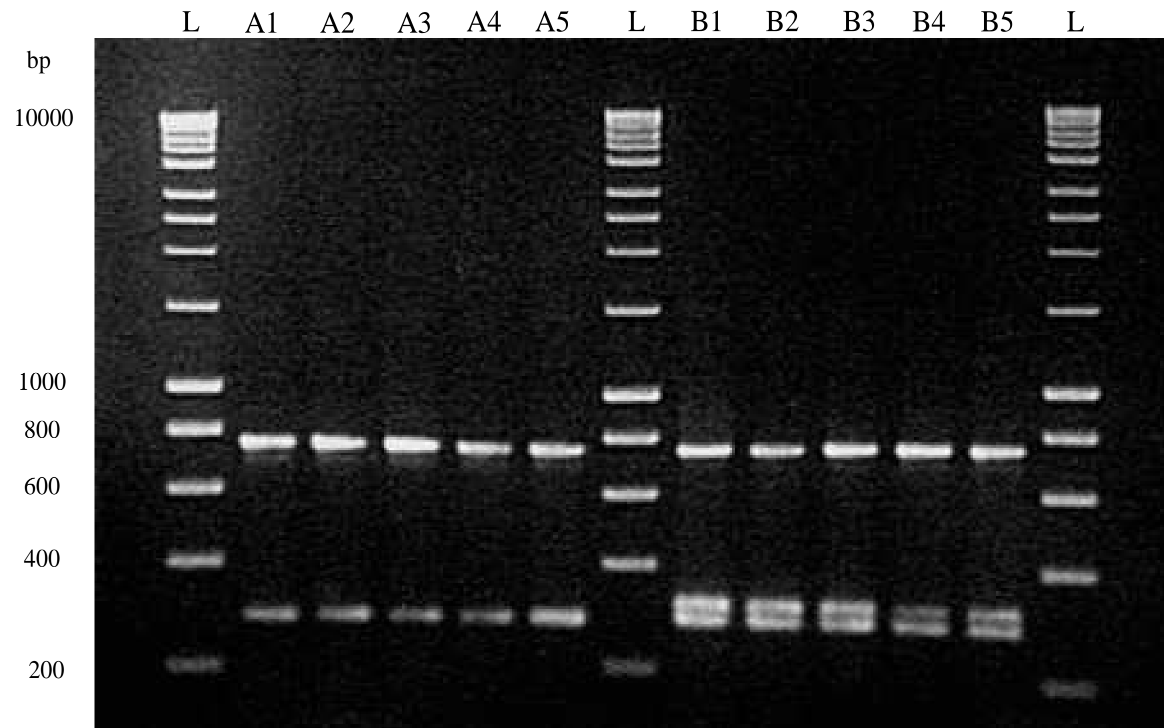


Figure 2.3 Multiplex PCR of SCV isolates recovered from various concentrations of gentamicin L - Hyperladder 1; A1 – ATCC 253923 WT; A2 - ATCC 25923 SCV X 0.25 G; A3 - ATCC 25923 SCV X 0.5 G; A4 - ATCC 25923 SCV MIC G; A5 - ATCC 25923 SCV X 2 MIC G; B1 – EMRSA 15 WT; B2 – EMRSA 15 SCV X 0.25 G; B3 – EMRSA 15 SCV X 0.5 G; B4 – EMRSA 15 SCV MIC G; B5 – EMRSA 15 SCV X 2 MIC G. Multiplex PCR confirmed SCV isolates as *S. aureus*. The presence of the additional band in EMRSA 15 SCV isolates is attributed to the presence of *mecA* (310bp).

Table 2.4 Reversion frequencies (expressed as CFU per SCV) of SCV isolated from increasing aminoglycoside concentrations

	X 0.25 MIC		X 0.5 MIC		MIC		X 2 MIC		X 4 MIC	
	25923	E15	25923	E15	25923	E15	25923	E15	25923	E15
Gentamicin	5.8 X 10^{-2}	3.5 X 10^{-2}	2.2 X 10^{-3}	2.7 X 10^{-4}	1.2 X 10^{-6}	4.1X 10^{-5}	S	S	NT	NT
Neomycin	3.1 X 10^{-1}	7.1 X 10^{-1}	6.1 X 10^{-4}	2.2 X10^{-4}	5.7 X 10^{-5}	8.4 X 10^{-5}	S	S	NT	S
Kanamycin	4.2 X10^{-2}	5.3 X 10^{-2}	5.1 X 10^{-3}	4.1 X 10^{-4}	4.2 X 10^{-6}	5.4 X 10^{-6}	S	S	NT	S
Streptomycin	6.3 X 10^{-2}	5.6 X 10^{-2}	4.6 X 10^{-3}	5.2 X 10^{-4}	3.6 X 10^{-5}	2.6 X 10^{-5}	S	S	NT	S

25923 – ATCC 2923; E15 – EMRSA 15; S – stable isolates (no revertants detected); NT – not tested. Large rapidly growing pigmented colonies were recorded as revertants.

2.3.6 Growth rates in the presence of aminoglycosides

Parent strains ATCC 25923 and EMRSA 15 displayed typical *S. aureus* growth rates when grown in unmodified MH broth (Figure 2.4). The presence of X 0.25 MIC gentamicin and kanamycin significantly increased the lag phase in strain ATCC 25923 ($P = < 0.001$). The same conditions in strain EMRSA 15 however resulted in growth profiles similar to unmodified conditions. As *S. aureus* strains were exposed to increasing concentrations of aminoglycosides lag phase duration increased in a concentration dependant manner (Figure 2.4). For example, exposure to X 0.5 MIC gentamicin concentration resulted in an average 9 hour lag phase duration where as exposure to X 2 MIC gentamicin concentration resulted in an average lag phase duration of 16.5 hours. Exposure of ATCC 25923 to X 2 MIC gentamicin and kanamycin resulted growth profiles with a significantly lower growth rate in comparison to other concentrations ($P = < 0.05$), similar to the growth rate of SCVs. Sampling of wells following the completion of growth rates analysis confirmed the presence of a *S. aureus* SCVs population in gentamicin and kanamycin concentrations of X 2 MIC for ATCC 253923. Although concentrations of X 4 MIC gentamicin and kanamycin resulted in no change in optical density, sampling of EMRSA 15 exposed to X 4 MIC kanamycin revealed the presence of a SCV population. SCVs were not detected for ATCC 25923 exposed to X 4 MIC for gentamicin and kanamycin and EMRSA 15 at X 4 MIC gentamicin. This is in agreement with MSCVPC obtained previously, as X 4 MIC values are equal to MSCVPC values.

2.3.7 Biochemical profiles

Contrasting biochemical profiles between parent and SCV isolates were observed for both strains, and biochemical profiles of EMRSA 15 and corresponding SCV isolates are shown in Table 2.5. Parent strains displayed the ability to ferment a range of carbohydrates as well as production of alkaline phosphatase, arginine dihydrolase and urease enzymes. All SCV isolates were unable to ferment mannitol where as SCVs selected from higher aminoglycosides concentrations were unable to ferment lactose, maltose and sucrose (Table 2.5). Furthermore, loss of enzymatic activity was also observed in several SCV isolates. Alkaline phosphatase activity was not detected in SCVs isolated from X 2 MIC gentamicin and kanamycin and X 4 MIC kanamycin.

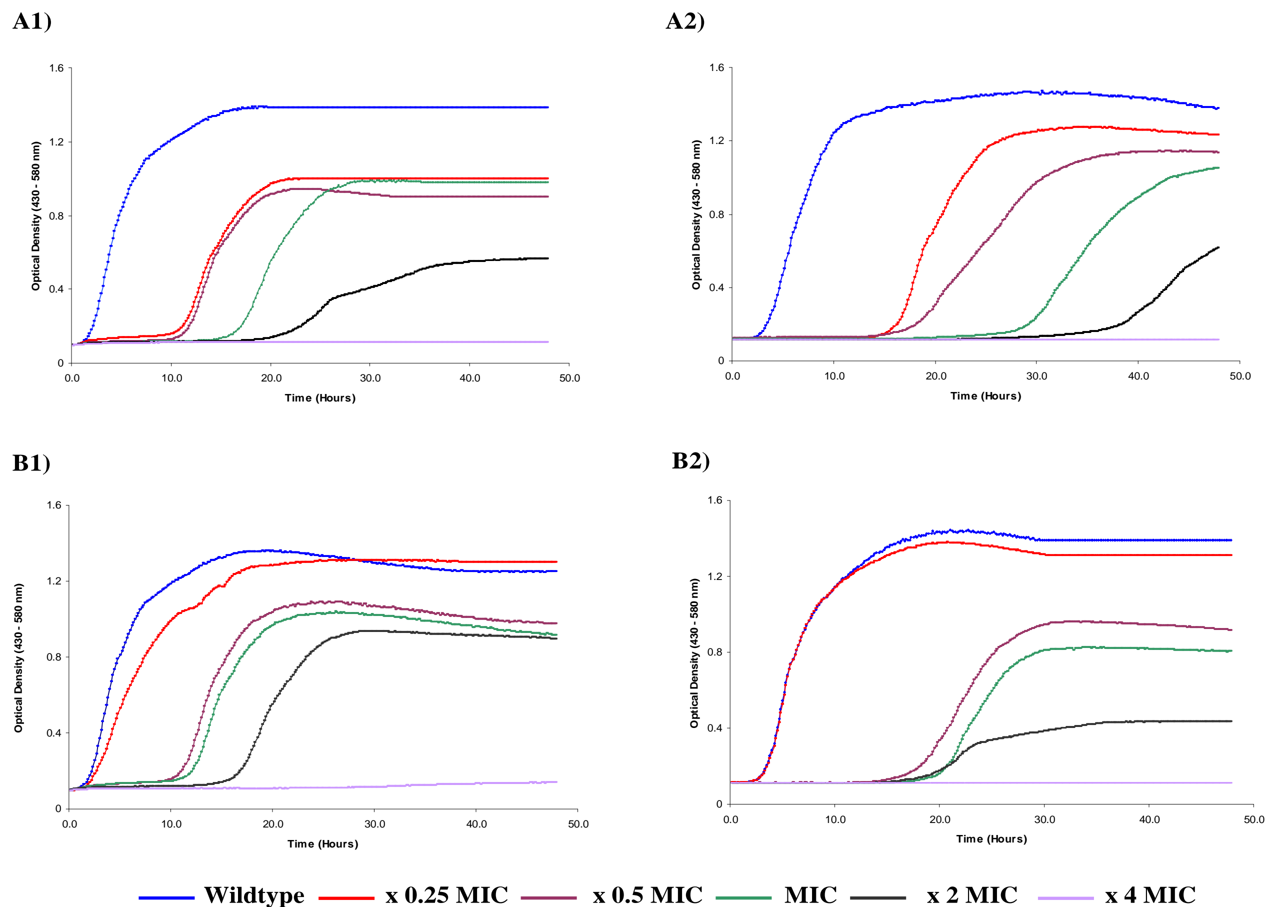


Figure 2.4 Growth rates of ATCC 25923 and EMRSA 15 in varying concentrations of gentamicin and kanamycin A1) - ATCC 25923 gentamicin; A2) - ATCC 25923 kanamycin; B1) - EMRSA 15 gentamicin; B2) - EMRSA 15 kanamycin. Generally increasing aminoglycoside concentration results in an increase in lag phase duration and a reduction in final optical density. Data is the mean of three independent replicates.

Table 2.5 Biochemical profiles of EMRSA 15 and corresponding SCVs selected for in the presence of different concentrations of gentamicin and kanamycin

	Parent	SCV X 0.25 MIC		SCV X 0.5 MIC		SCV X MIC		SCV X 2 MIC		SCV X 4	
		G	K	G	K	G	K	G	K	G	K
GLU	+	+	+	+	+	+	+	+	+	NT	+
FRU	+	+	+	+	+	+	+	+	+	NT	+
MNE	+	+	+	+	+	+	+	+	+	NT	+
MAL	+	+	+	+	+	-	+	-	-	NT	-
LAC	+	+	+	+	+	+	+	-	-	NT	-
TRE	+	+	+	+	+	+	+	+	+	NT	+
MAN	+	-	-	-	-	-	-	-	-	NT	-
XLT	-	-	-	-	-	-	-	-	-	NT	-
MEL	-	-	-	-	-	-	-	-	-	NT	-
NIT	+	+	+	+	+	+	+	+	+	NT	+
PAL	+	+	+	+	+	+	+	-	-	NT	-
VP	+	+	+	+	+	+	+	+	+	NT	+
RAF	-	-	-	-	-	-	-	-	-	NT	-
XYL	-	-	-	-	-	-	-	-	-	NT	-
SAC	+	+	+	-	+	-	+	-	-	NT	-
MDG	-	-	-	-	-	-	-	-	-	NT	-
NAG	+	+	+	+	+	+	+	+	+	NT	+
ADH	+	+	+	+	+	+	+	+	+	NT	+
URE	+	+	+	+	+	+	+	+	+	NT	-

Differences between SCV and parents are shaded in grey. G - Gentamicin; K – Kanamycin; + - Positive (enzyme activity, fermentation of carbohydrate); - = Negative (lack of enzyme activity, inability to ferment carbohydrates); NT – not detected. Test abbreviations are shown in Appendix 1.

A lack of urease activity was also observed in SCV isolated from X 4 MIC kanamycin.

2.3.8 Carotenoid production

Methanol extraction of carotenoids revealed similar absorbance values corresponding to similar levels of carotenoids in parent strains (ATCC 25923 = 0.20 ± 0.01, EMRSA 15 = 0.22 ± 0.007). All SCV isolates produced lower amounts of carotenoid in comparison and differences in the amount of carotenoid produced by SCV isolates was observed according to the concentration of the selecting aminoglycoside (Figure 2.5). SCVs isolates from both strains following exposure to MIC, X 2 MIC and X 4 MICs of gentamicin and kanamycin were significantly reduced in comparison to parent strains ($P = < 0.001$). Complete absence of carotenoid was observed in EMRSA 15 SCVs recovered from exposure to X 2 MIC gentamicin and X 2 and X 4 MIC kanamycin (Figure 2.5).

2.3.9 Intracellular ATP concentrations

Similar intracellular ATP concentrations were recorded for parent strains (Figure 2.6). Overall SCV isolates contained lower intracellular ATP levels in comparison to parent strains (Figure 2.6). ATP concentrations decreased as the concentration of the selected aminoglycoside increased. Average ATP concentrations of SCV isolates from both strains following exposure to MIC, X 2 MIC and X 4 MIC gentamicin and kanamycin concentrations were significantly reduced in comparison to parent strains ($P = < 0.001$).

2.3.10 Auxotrophy profiles

Auxotrophy was confirmed if SCVs appeared as fast growing pigmented colonies around filter discs impregnated with haemin, menadione or thymidine. Auxotrophy was detected in 26/40 (65 %) isolates examined in this study (Figure 2.7). Haemin auxotrophy was the most frequently auxotrophy detected with 12/40 (30%) of isolates. Auxotrophy for a combination of haemin and menadione was the second most frequent auxotrophy detected with 10/40 isolates (25%) displaying this auxotrophy profile. The remaining isolates in which auxotrophy was identified were menadione auxotrophs (5/40; 10%). Auxotrophy for thymidine was not detected in any of the SCV isolates tested.

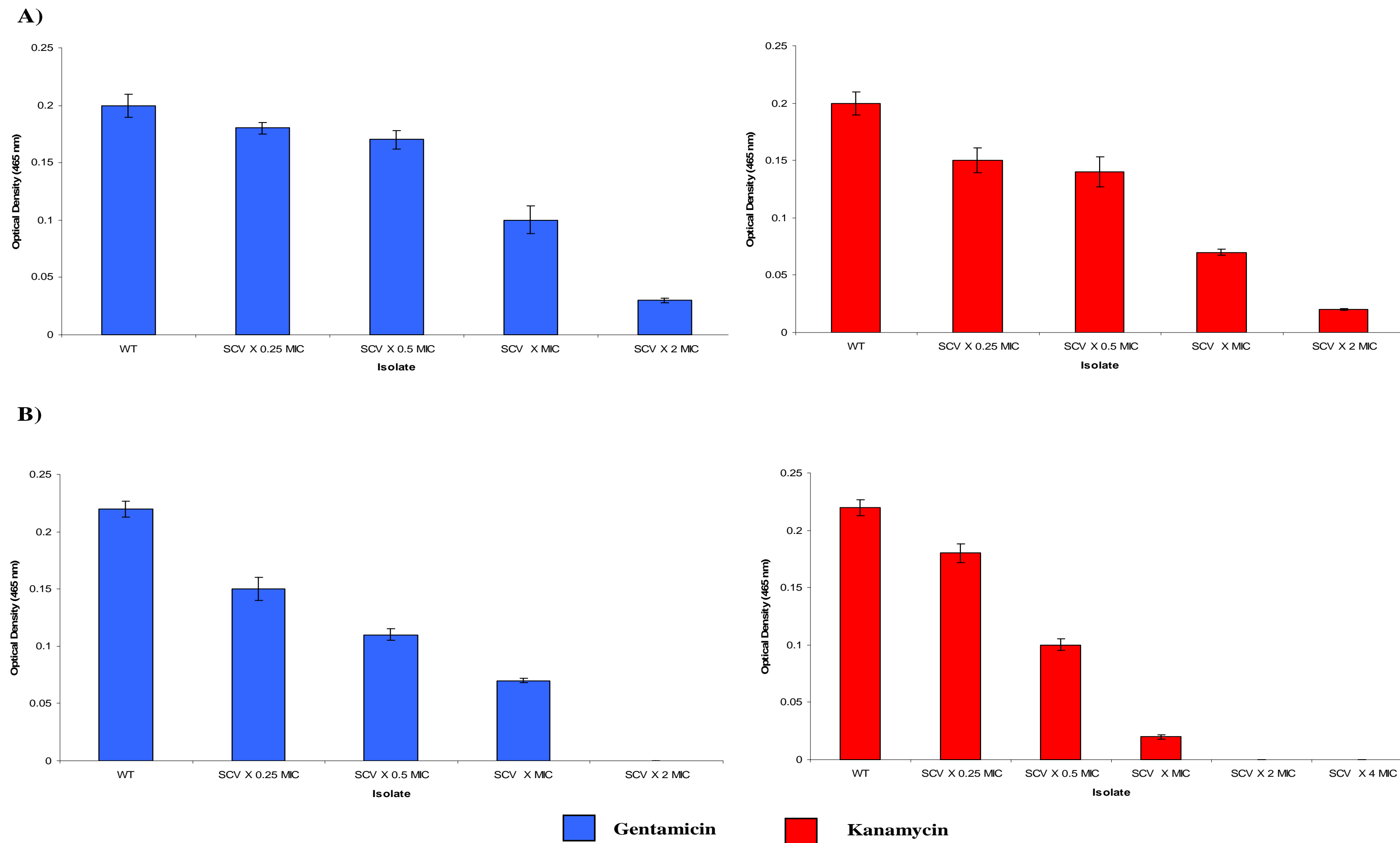


Figure 2.5 Carotenoid production in SCVs isolated following exposure to different aminoglycoside concentrations A) - ATCC 25923; B) - EMRSA 15. Carotenoid production was completely absent in SCV isolated from higher aminoglycoside concentrations. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

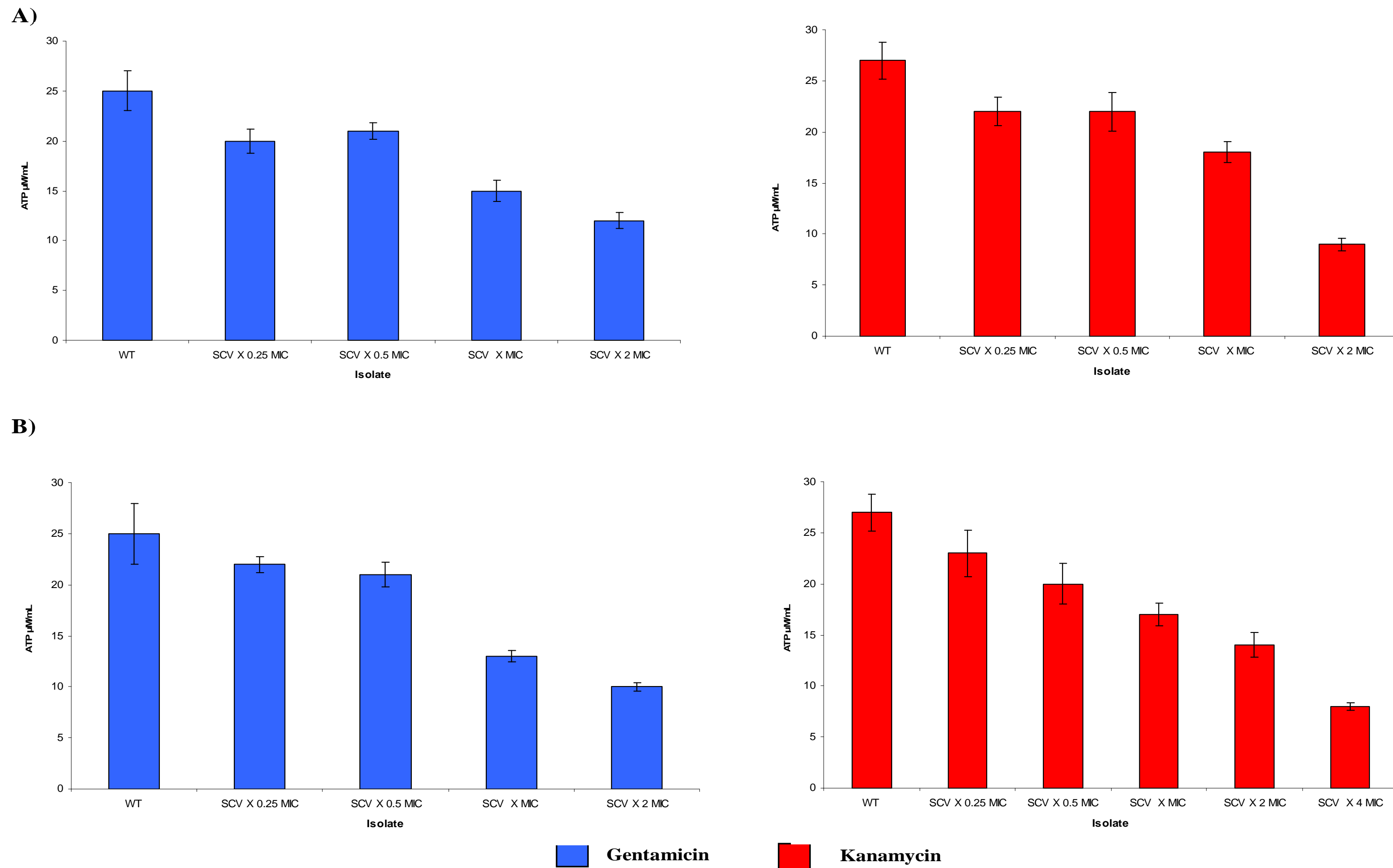


Figure 2.6 Intracellular ATP concentrations of SCVs isolated following exposure to different aminoglycoside concentrations A) - ATCC 25923; B) - EMRSA 15. SCVs isolated following exposure to MIC, X 2 and X 4 MIC aminoglycoside concentrations contained significantly lower levels of intracellular ATP in comparison to parent strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error

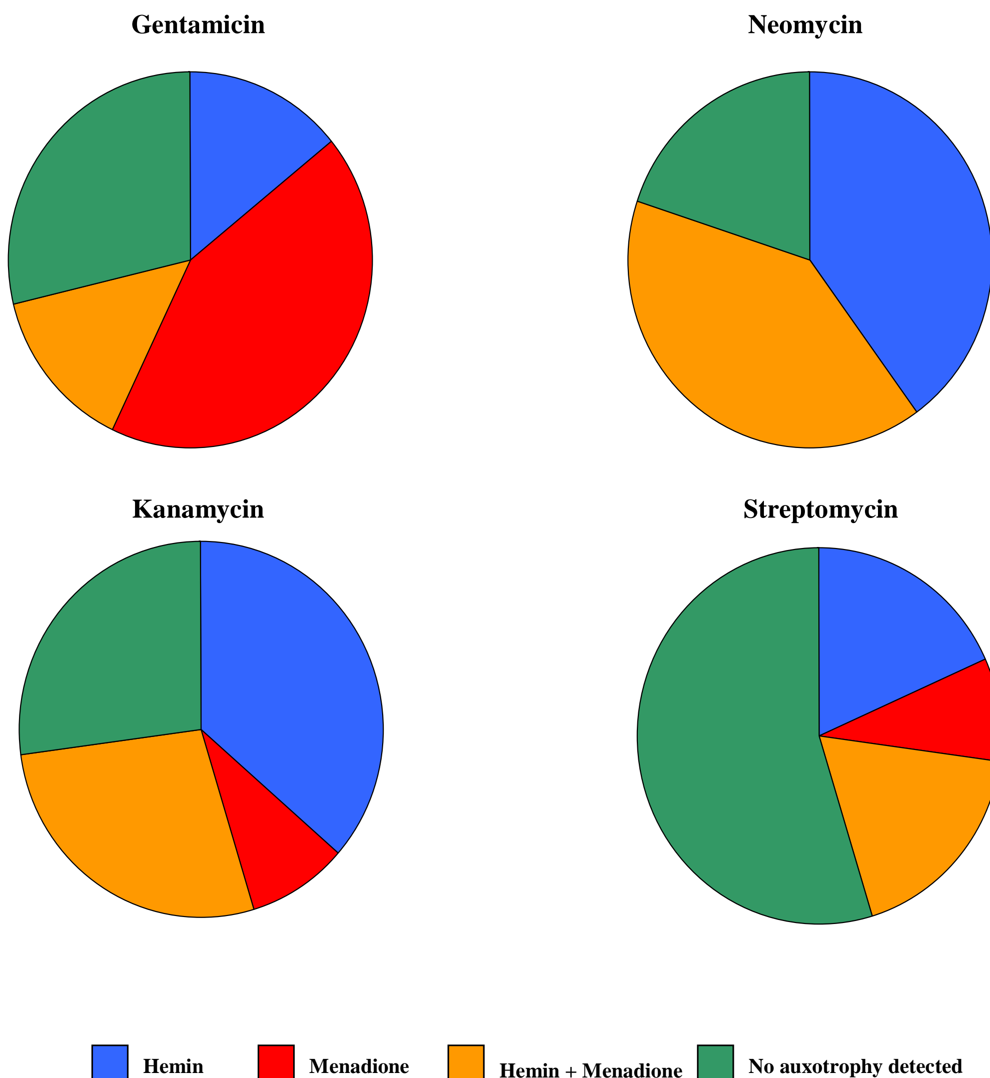


Figure 2.7 Auxotrophy profiles of SCV isolates following exposure to different concentrations of four aminoglycosides A complete absence of thymidine auxotrophy was observed. Number of isolates examined; gentamicin n = 8, kanamycin n = 11, neomycin n = 10 and streptomycin n = 11.

2.3.11 Mutation frequency to rifampicin resistance

Differences in mutation frequency were observed between SCV isolates and parent strains. SCV isolate isolated from exposure to X 0.25, X 0.5 and X MIC gentamicin and kanamycin concentrations displayed mutation frequencies significantly greater than parent strains ($P = < 0.05$; Table 2.6). ATCC 25923 SCV isolates recovered from exposure to X 2 MIC gentamicin and EMRSA 15 SCV isolates recovered from exposure to X 2 MIC gentamicin and X 2 and X 4 MIC kanamycin concentrations displayed significantly higher mutation rates than SCV isolates recovered following exposure to lower aminoglycoside concentrations and corresponding parent strains ($P = < 0.05$). In SCV isolates where the mutation frequency to rifampicin resistance was $> 10^{-7}$ isolates were termed as hypermutators due to their high mutation frequencies (Table 2.6).

Table 2.6 Mutation frequency to rifampicin resistance of SCV isolates and corresponding parent strains

Gentamicin isolates

Isolate	ATCC 25923	EMRSA 15
Parent	2.1×10^{-10}	1.5×10^{-10}
SCV X 0.25	1.9×10^{-9}	1.8×10^{9}
SCV X 0.5	8.9×10^{-9}	7.2×10^{-9}
SCV X MIC	2.1×10^{-9}	6.7×10^{-8}
SCV X 2 MIC	**8.5×10^{-6}**	**8.1×10^{-6}**

Kanamycin isolates

Isolate	ATCC 25923	EMRSA 15
Parent	2.1×10^{-10}	1.5×10^{-10}
SCV X 0.25	7.8×10^{-9}	1.2×10^{-9}
SCV X 0.5	5.9×10^{-9}	7.8×10^{-9}
SCV X MIC	5.4×10^{-9}	2.1×10^{-8}
SCV X 2 MIC	**2.2×10^{-6}**	**6.1×10^{-6}**
SCV X 4 MIC	-	**2.6×10^{-6}**

Mutation frequencies are the mean of three independent replicates. Isolates were regarded as strong hypermutators if mutation frequency was greater that 10^{-7} and are shown in bold.

2.4 Discussion

Aminoglycosides (particularly gentamicin) have been demonstrated to select for SCVs of *S. aureus*, as well as other bacterial species, including *E. coli* (Lewis *et al.*, 1991) and *Pseudomonas aeruginosa* (Langford *et al.*, 1989). This study highlights the ability of gentamicin, neomycin, kanamycin and streptomycin to select for *S. aureus* SCVs at various concentrations. To our knowledge this is the first study that has compared SCV selection and corresponding isolates following exposure to various aminoglycosides across a broad range of concentrations.

One of the important implications of this work relates to aminoglycoside susceptibility testing of *S. aureus*. CLSI guidelines stipulate that MICs are defined as 'the lowest concentration of antimicrobial agent that inhibits growth of the organism in microdilution wells as detected by the untrained eye' (CLSI, 2006). In well plate susceptibility testing this is based on the absence of turbidity following incubation. The results of this study clearly highlight certain shortcomings of this form of susceptibility testing as concentrations exceeding aminoglycoside MICs, resulted in the growth of *S. aureus* through the formation of SCVs. CLSI guidelines state that an incubation time of 16-20 hours should be adhered to before reading MICs. However, in this study increases in absorbance were frequently detected following 15 or more hours incubation which may not be sufficient to be detected by laboratory personal when recording MICs by eye. Aminoglycoside susceptibility testing for *S. aureus* and other bacterial species should therefore take into consideration the formation/selection of SCVs. Increasing the incubation time to 48 hours will allow changes in optical density (caused by the growth of SCVs) to be recorded. Furthermore, SCVs were often recovered from aminoglycoside concentrations that exceeded concentrations that were bactericidal for parent *S. aureus*. We therefore implemented the term 'minimum SCV prevention concentration'. The application of the MSCVPC may have applications in susceptibility testing as it ensures the elimination of the whole population regardless of whether the test compound has led to the selection of SCVs.

The phenotypic appearance of aminoglycoside selected SCVs on solid agar also presents identification difficulties. Contrasting colony size and the lack of pigmentation observed in SCVs may result in the conclusion that *S. aureus* is not

present. Mannitol salt agar is a selective, differential medium that has been used for the isolation of *S. aureus* (Sharp & Searcy, 2006). The observation that SCV isolates in this study failed to ferment mannitol and observations of other mannitol fermentation deficient SCVs (Atalla *et al.*, 2008) suggests that SCVs will not be able to grow on mannitol salt agar and may go undetected. Furthermore rapid identification tests such as the presence of coagulase (to differentiate between *S. aureus* and CoNS) are subject to limitations when testing SCVs due to their lack of coagulase activity. In this study molecular identification was used to successfully confirmed SCV isolates as *S. aureus*. Molecular identification overcomes the failings of phenotypic testing concerning the identification of SCVs. The use of multiplex PCR also allows the identification of the presence of resistance determinants (such as *mecA*) which may be an important consideration when considering treatment of *S. aureus* infections. If an SCV infection is suspected applying molecular identification can provide important evidence regarding the causative agent.

In total 40 different isolates were recovered following the application of various aminoglycoside selection conditions. Various differences were observed between these isolates. Contrast in levels of intracellular ATP between SCV isolates and parents was observed. No correlation between the concentration of the selecting aminoglycoside and the resulting auxotrophy was observed. Reduced ATP production in SCVs is related to defects in electron transport (Proctor *et al.*, 2006). In this study we were able to identify auxotrophy for haemin, menadione or a combination of both in 65% of isolates. Haemin and menadione have crucial roles in electron transport as discussed previously (see section 1.4.1.2). The reduced levels of ATP in SCVs can be linked to auxotrophy for these compounds. In the remaining SCV isolates in which it was not possible to determine auxotrophy profiles defects in other complexes or components of the electron transport chain may be responsible for the SCV phenotype. Possible candidates include defects in unsaturated fatty acid biosynthesis or in the F_0F_1-ATPase. Unsaturated fatty acid biosynthesis is required to form the isoprenoid tail that is added to form menaquinone (Collins & Jones, 1981; Kaplan & Dye, 1976), so a defect in the production of this lipid would disrupt formation of the electron transport chain. Mutations or inhibition of the F_0F_1-ATPase (which is required for ATP generation) have also been suggested to produce the SCV phenotype (McNamara & Proctor, 2000). Mutations in the genes involved in the biosynthesis of

the F_0F_1-ATPase can generate SCVs in *E. coli* (Jensen & Michelsen, 1992), therefore the same may be apparent in *S. aureus*.

Differences in intracellular ATP concentrations were apparent between isolates and a correlation between the selecting aminoglycoside concentration and levels of ATP observed. SCVs selected at higher aminoglycosides concentrations had lower levels of intracellular ATP in comparison to those isolated at lower concentrations. As the electron transport chain is SCVs is not fully operational, an upregulation of glycolytic and fermentative pathways is required in order to generate ATP (Kohler *et al.*, 2003). However in order to utilise other carbohydrates, a fully functional tricarboxylic acid cycle (TCA cycle) is required. Differences in carbohydrate utilisation were observed in the SCVs isolated in this study, which has been reported previously using phenotypic microarray profiling (von Eiff *et al.*, 2006). The failure to ferment carbohydrates can be related to accumulation of NADH, which occurs as a result of defects in electron transport (Proctor, 2006). The differences in biochemical profiles between the isolates in this study may therefore by related to more pronounced defects in TCA cycle function. For example isolates that have lost the ability to ferment multiple carbon sources may show a further, down regulation of enzymes involved in TCA cycle blocking the fermentation of certain carbohydrates (Proctor, 2006). Another plausible explanation is that reduction in cellular ATP indirectly blocks the uptake of complex carbohydrates and also indirectly blocks the steps of the phosphotransferase systems inhibiting the utilisation of lactose, mannitol, maltose and sucrose (Reizer *et al.*, 1988). Further pronounced deficiencies in ATP availability may explain why SCVs isolated following exposure to higher aminoglycoside concentrations may show further deficiencies in carbohydrate utilisation.

The deficiencies in ATP production appear to correlate with deficiencies in carotenoid production. SCVs isolated at higher aminoglycoside concentrations showed undetectable carotenoid production, whereas as isolate recovered from a lower aminoglycoside concentration showed carotenoid production similar to parent strains. In order to drive carotenoid biosynthesis and produce pigment, *S. aureus*, must expend energy and process ATP as well as a functional ETC to function (Proctor, 2000). The lower ATP levels observed in *S. aureus* SCVs results in the variation in carotenoid production observed in this study. Although the presence of pigmentation

in *S. aureus* protects cells from oxidative stress (Clauditz *et al.*, 2006), SCVs may expend the limited available energy for essential cellular process such as cell wall biosynthesis at the cost of pigment production.

Several isolates showed high levels of mutational resistance towards rifampicin and were deemed hypermutators. These isolates were recovered from the higher aminoglycoside concentrations tested. Hypermutability is considered to be a key force in driving bacterial evolution and bacteria displaying the associated increases in mutation frequency are recovered from cystic fibrosis (CF) patients (Oliver *et al.*, 2000). CF patients infected with hypermutable isolates can suffer from increased infection duration and isolates often display raised levels of antibiotic resistance (Macia *et al.*, 2005; Mena *et al.*, 2007). The hypermutator phenotype has been linked to defects in the methyl-directed mismatch repair (MMR) system (Oliver *et al.*, 2002), allowing for adaptation (through increasing mutation frequency) whilst reducing the risk of accumulating deleterious mutations (Jolivet-Gougeon *et al.*, 2011). The hypermutator phenotype in SCV isolates observed in this study may aid persistence and long term survival. SCV isolates recovered from higher aminoglycosides concentrations in this study also show reduced energy levels. Coupled with the observation that the hypermutator phenotype permits a fitness disadvantage (Jolivet-Gougeon *et al.*, 2011), SCV isolates are severely impaired. The hypermutator phenotype may provide a survival mechanism through the SCV phenotype allowing long term persistence and the development of antibiotic resistance.

The alternative sigma factor, σ^B is an important regulator in *S. aureus* influencing the expression of many genes including various virulence factors (Bischoff *et al.*, 2004), many of which show altered levels of expression in SCVs (Kohler *et al.*, 2003). σ^B has been implicated in the formation of SCVs following aminoglycoside exposure (Mitchell *et al.*, 2010a) and sustained σ^B activity in *S. aureus* SCV isolates has been associated with ‘locking’ SCVs into a constant state of colonisation (Mitchell *et al.*, 2008). It is feasible that expression of σ^B varies between the aminoglycoside select SCV isolates examined in this study. Several isolates from higher aminoglycoside concentrations showed a lack of reversion to the wildtype form. However SCVs isolate from lower aminoglycoside concentrations were associated with higher reversion frequencies. σ^B expression therefore may directly correlate to the

concentration of the selecting aminoglycoside and isolates selected for in the presence of higher aminoglycoside concentrations may display a sustained rate of increased σ^B expression maintaining the SCV phenotype.

This study highlights the ability of a range of aminoglycosides to select for SCVs and that formation of the SCV phenotype provides a survival mechanism to bypass the inhibitory effects of aminoglycosides. Through analysis of various isolates it is proposed that aminoglycoside selection directly impacts on energy availability and σ^B expression. Differences in energy levels results in differences in carotenoid biosynthesis and carbohydrate utilisation. σ^B expression may correlate with aminoglycoside in a concentration dependant manner and serve as a governing global regulator influencing SCV characteristics. The observation of hypermutability suggests that the SCV phenotype in various isolates may be further geared towards survival and persistent in order to over overcome environmental stress. On the outset the variants are observed as slow growing variants with attenuated virulence where as in real terms they have the characteristics for persistence and antibiotic resistance. With this in mind the analogy ‘a wolf in sheep’s clothing’ is useful in characterising the SCV phenotype.

2.5 Conclusions

- Increasing aminoglycoside concentrations increase the duration of lag time in *S. aureus* and prolonged incubation can result in the growth of *S. aureus* through the formation of SCVs
- Examining SCV selection in the presence of aminoglycosides revealed that aminoglycoside susceptibility testing of *S. aureus* may be hindered through the formation of *S. aureus* SCVs, resulting in the implementation of a 'minimum SCV prevention concentration'
- Multiplex PCR targeting *S. aureus* specific genes is a useful tool in overcoming the uncertainty surrounding SCV identification and can be used to confirm SCVs as *S. aureus*
- *S. aureus* SCVs can be selected for following exposure to kanamycin, gentamicin, neomycin and streptomycin at a range of concentrations including those that exceeding MBC
- Auxotrophy for haemin and menadione is responsible for the disruption of the electron transport chain and the SCV phenotype in the majority of aminoglycoside selected SCVs
- Variation in carbohydrate utilisation, carotenoid production and levels of intracellular ATP are apparent in SCVs selected in the presence of aminoglycosides
- SCV isolates selected at higher aminoglycoside concentrations can be classified as hypermutators and these isolates also show a high level of stability

3 CHAPTER 3: TETRACYCLINE SELECTION FOR *STAPHYLOCOCCUS AUREUS* SMALL COLONY VARIANTS

3.1 Introduction

3.1.1 History of tetracycline

The tetracycline group of antibiotics, discovered in the 1940s exhibit antimicrobial activity against a broad range of Gram-negative and positive microorganisms (Chopra & Roberts, 2001). Chlortetracycline was the first member of the family to be isolated (from *Streptomyces aureofaciens*) and is classed as a first generation tetracycline along side oxytetracycline and tetracycline (Roberts, 1996). These compounds were discovered by systematic sampling of fermentation products of spore forming soil bacteria and were rapidly introduced into clinical practice (Thaker *et al.*, 2010). This was followed by the development of minocycline and doxycycline constituting the second generation tetracyclines, followed by the development of the glycylcyclines e.g. tigecycline (third generation tetracyclines; Chopra & Roberts, 2001). Second and third generation tetracyclines are the products of semi-synthesis, i.e. synthetic organic manipulation of natural product antibiotic scaffolds (Thaker *et al.*, 2010).

Several aspects of the tetracyclines make them excellent therapeutic agents; they exhibit good oral absorption and low toxicity, are relatively inexpensive and most importantly they are active against common pathogens (Schnappinger & Hillen, 1996). Tetracyclines have been used for the clinical treatment of a wide range of infections including respiratory tract infections and sexually transmitted diseases, as well in the management of acne (Zhanel *et al.*, 2004).

3.1.2 Mode of action

The antimicrobial activity of tetracyclines is achieved via inhibition of bacterial protein synthesis. Several tetracycline binding sites on the ribosome are present, but the key binding appears to be in the region of the tRNA acceptor site (Anokhina *et al.*, 2004). 'Typical' tetracyclines (such as tetracycline and doxycycline) bind to the bacterial 30S ribosomal subunit and exert bacteriostatic activity via preventing the attachment of aminoacyl t-RNA to the ribosomal receptor site (Roberts, 1996). 'Atypical' tetracyclines exhibit bactericidal activity (Chopra, 1994) and studies in *E. coli* demonstrated that they are poor inhibitors of protein synthesis and are thought to

target the cytoplasmic membrane interfering with membrane permeability (Oliva *et al.*, 1992).

3.1.3 Mechanisms of resistance

The use of tetracyclines has declined in recent decades due to the emergence of resistant strains of bacteria (Griffin *et al.*, 2010). The first incidence of tetracycline resistance was reported in *Shigella dysenteriae* in 1953 (Roberts, 1996). Four different mechanisms of bacterial resistance to the tetracyclines have been identified (Figure 3.1). These include: 1) protection of the antibiotic target i.e. the ribosome; 2) reduction of tetracycline intracellular concentration via efflux; 3) inactivation of the tetracycline molecule by modifying enzymes; and 4) target modification (Schnappinger & Hillen, 1996). Tetracycline resistance is normally due to the acquisition of new genes often associated with either a mobile plasmid or transposon (Roberts, 1996). Tetracycline resistance genes are diverse in sequence and recent analysis reported that 1,189 tetracycline genes have been reported in more than 84 genera and 354 species of Gram-positive and Gram-negative bacteria (Liu & Pop, 2009).

3.1.3.1 Ribosomal protection proteins

Ribosomal protection proteins (RPPs) were first identified in streptococci, in which the TetM protein was shown to provide resistance to tetracycline (Burdett, 1986). RPPs are soluble cytoplasmic proteins that display sequence similarity with the elongation factors involved in protein synthesis (Zakeri & Wright, 2008). RPPs are thought to interact with the protein at the base of the ribosome disrupting the primary tetracycline binding site, causing tetracycline molecules to be released from the ribosome (Burdett, 1996; Roberts, 2005). There are various different RPPs spanning Gram positive and Gram negative bacterial genera, where Tet(O) and Tet(M) are the most prevalent and the best studied classes (Zakeri & Wright, 2008). RPPs are approximately 72.5 kDa in size and the genes encoding them are divided into three groups based on the amino acid sequence of encoded proteins (Connell *et al.*, 2003; Taylor & Chau, 1996). Group-1 includes *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32), and *tet*(36), whereas Group-2 includes *tetB*(P). Finally Group-3 is contains a smaller number and includes *tet*(Q) and *tet*(T) (Thaker *et al.*, 2010).

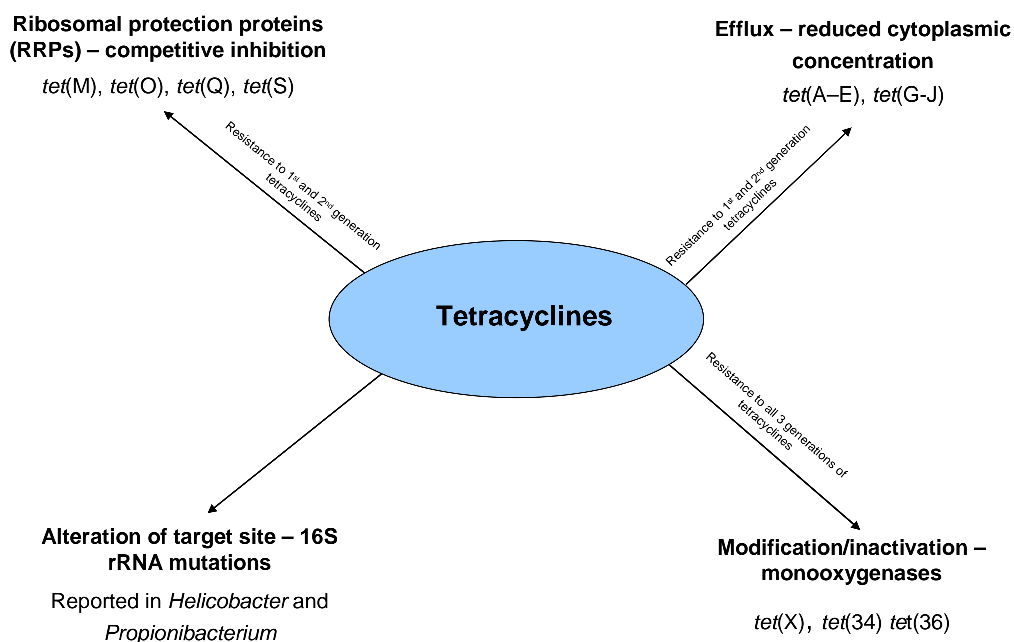


Figure 3.1 Bacterial mechanisms employed for resistance to the tetracycline class of antibiotics. Efflux, ribosomal protection proteins are widespread in bacteria, mediated through horizontal gene transfer whereas target mutation and inactivation have been sparsely reported. Adapted from Zakeri & Wright (2008) and Thacker *et al.*, (2010)

3.1.3.2 Efflux mediated resistance

Efflux mediated resistance reduces the concentration of tetracycline in the cytoplasm via the *tet* efflux genes coding for 46 kDa membrane associated proteins that export tetracycline out of the cell (Chopra & Roberts, 2001). Efflux predominantly occurs via proteins that are members of the major facilitator superfamily (MFS) group of membrane transporters (Paulsen *et al.*, 1996). Energy-dependent removal of tetracyclines involves the exchange of a proton for a tetracycline-cation complex via these membrane associated transporters (Butaye *et al.*, 2003). Since the original reports of efflux mediate tetracycline resistance in *E. coli* (McMurry *et al.*, 1980), several other classes of *tet* efflux genes have been described. The first group includes *tet*(A)–(E), (G), (H), (J), and (Z) as well as *tet*(30) (Roberts, 2005). Classes A, B, D, and H are associated with non-conjugative transposons or transposon-like elements where as classes C, E, and G are often found on plasmids (Roberts, 1996). The other group of the *tet* efflux genes (including *tet*(K) and *tet*(M)) are distributed among Gram-positive bacteria including *Bacillus* and *Staphylococcu*s species. The substrate spectrum of the Tet efflux proteins present in bacteria commonly includes tetracycline, oxytetracycline, chlortetracycline and doxycycline, but not minocycline and glycylcyclines (Schnappinger & Hillen, 1996).

3.1.3.3 Enzymatic inactivation

The first tetracycline inactivating enzyme to be discovered was *tet*(X) from a strain of *Bacteroides fragilis*, although it did not convey resistance in its host (Speer *et al.*, 1991). The *tet*(X) gene product is a 44-kDa cytoplasmic protein that chemically modifies tetracycline (Chopra & Roberts, 2001). Tet(X) is monooxygenase enzyme that acts on first and second generation tetracyclines, but it is also active against the recently approved third generation tetracycline; tigecycline (Moore *et al.*, 2005; Yang *et al.*, 2004). To date *tet*(X) has not be found amongst clinically-derived bacterial strains (Moore *et al.*, 2005), however the fact that it was originally found on a mobile genetic element (Speer *et al.*, 1991) suggests that it can be passed between bacteria via horizontal gene transfer. Two other genes *tet*(34) and *tet*(36) have been proposed as tetracycline inactivators, but their exact mechanism of tetracycline inactivation is not fully understood (Thaker *et al.*, 2010).

3.1.3.4 Target modification

Target modification is the least prevalent of all tetracycline resistance mechanisms. Studies using tetracycline resistant propionibacteria (*Propionibacterium acnes*) revealed that single base mutations in the 16S rRNA sequence were responsible for tetracycline resistance (Ross *et al.*, 1998). Several studies have also shown that substitutions in the primary binding site of the 16S rRNA of *Helicobacter pylori* can mediate tetracycline resistance (Gerrits *et al.*, 2002; Trieber & Taylor, 2002).

3.1.3.5 Tetracycline resistance in *S. aureus*

Two mechanisms of tetracycline resistance have been identified in *S. aureus*. Active efflux via *tet(*K*)* and *tet*(L) and ribosomal protection mediated via *tet*(M) (Fluit *et al.*, 2005). Both *tet*(K) and *tet*(L) confer resistance to tetracycline and chlortetracycline, but in the majority of cases tetracycline efflux in *S. aureus* is mediated by *tet*(K), which is commonly carried on the 4.4 kb plasmid pT181 (Guay *et al.*, 1993; Werckenthin *et al.*, 1996). The conjugative transposon Tn*5801* carries *tet*(M) which mediates tetracycline and minocycline resistance through ribosomal protection (Kuroda *et al.*, 2001). A survey regarding the distribution of *S. aureus* tetracycline resistance mechanisms in various European hospitals concluded that *tet*(K) and *tet*(M) were the most prevalent resistance mechanisms (Fluit *et al.*, 2005).

3.1.4 Tigecycline

Tigecycline belongs to a recently developed derivative of the tetracycline class of antibiotics know as the glycylclines (Stein & Craig, 2006). Tigecycline is a structural derivative of minocycline, differing from it by the long side chain at the 9 position of carbon atom of the D ring of the tetracyclic nucleus (Seputiene *et al.*, 2010). The molecular basis of tigecycline action is similar to the tetracyclines, binding to the ribosome 30S subunit and preventing attachment of aminoacylated tRNAs to the ribosomal A site (Bauer *et al.*, 2004). Tigecycline however posseses an advantage over the first and second generation tetracyclines as it can overcome the previously described mechanisms of tetracycline resistance. Ribosomal protection resistance is overcome as tigecycline remains bound to ribosomes that have been modified by *tet*(M) (Petersen *et al.*, 1999). Secondly the long side chain of tigecycline makes the molecule a poor substrate for tetracycline efflux pumps (Someya *et al.*, 1995).

A broad spectrum of human pathogens including penicillin resistant *S. pneumonia*, vancomycin-resistant enterococci, methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* are all widely susceptible (Noskin, 2005). Tigecycline is primarily bacteriostatic with time dependent activity against MRSA and other Gram-positive pathogens (Stryjewski & Corey, 2009). Although tigecycline resistance has been reported in medically important pathogens such as *Enterococcus faecalis* and (Werner *et al.*, 2008) and *Acinetobacter baumannii* (Navon-Venezia *et al.*, 2007) tigecycline resistance is yet to be reported in clinical isolates of *S. aureus*. However *in vitro* studies have shown increased expression of a member of the multidrug and toxin extrusion (MATE) family of efflux pumps (*mepA*) can result in reduced tigecycline susceptibility (McAleese *et al.*, 2005).

3.1.5 Aims

Various antimicrobial agents are known to select for *S. aureus* SCVs; however no study has examined the ability of the tetracycline class of antibiotics to do so. Therefore the aims of this study were to:

- Determine if members of the tetracycline class of antibiotics can select for *S. aureus* SCVs and compare selection frequencies between various strains
- Characterise any SCVs on the basis of auxotrophy, growth rate, MIC profiles and *S. aureus* specific enzymes
- Compare morphology differences of SCVs and parent stains using transmission electron microscopy
- Determine whether SCVs display clonality with parent strains through random amplified polymorphic DNA analysis
- Sequence the *S. aureus* 16S rRNA of any SCVs to identify mutations that may be responsible for reduced tetracycline susceptibility

3.2 Materials and methods

3.2.1 Bacterial strains

In this study four *S. aureus* strains that demonstrated susceptibility to a range of tetracyclines were included. *S. aureus* American Type Culture Collection (ATCC) strain 25923 and National Collection of Type Cultures (NCTC) strain 6571 were chosen as they represent well characterised methicillin-sensitive reference stains. Epidemic methicillin-resistant *S. aureus* (EMRSA) strain 15 and N315 were chosen to represent well studied clinical MRSA strains. Strains were maintained at -80°C in Mueller Hinton (MH) broth supplemented with 8 % dimethyl sulfoxide (DMSO) and re-isolated on MH agar plates when required.

3.2.2 Preparation of tetracycline stock solutions

Doxycycline, tetracycline and oxytetracycline were obtained from Sigma Aldrich (UK). Antibiotic stocks (10,000 mg/L) were prepared by adding 100 mg of the required antibiotic to 10 mL of sterile deionised water. Solutions were dissolved thoroughly by vortex mixing and where required 1 M NaOH was added dropwise to aid dissolving. Solutions were filter sterilised by passing them through 0.2 µm filters (Minisart, UK), and diluted to reduced strength stock solutions in sterile deionised water. Stocks solutions were maintained at 4°C for a maximum of 14 days.

3.2.3 Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined as described previously (section 2.2.3).

3.2.4 Tetracycline SCV selection assays

Overnight *S. aureus* cultures grown in cation adjusted Mueller Hinton broth (CAMHB) were diluted to achieve a starting density of 5 X 10^5 CFU/mL and used to inoculate individual universal tubes containing fresh CAMHB containing a range of doxycycline, tetracycline and oxytetracycline concentrations based on previously defined MIC values (section 3.2.3). Tetracycline exposed cultures were incubated at 37°C with shaking at 150 rpm. After 6 hours incubation, dilutions were prepared in sterile phosphate buffered saline (PBS) and 100 µL dispensed onto MH agar plates containing 2 mg/L doxycycline, 2 mg/L tetracycline or 4 mg/L oxytetracycline. Solutions were spread over the surface of agar plates using a sterile cotton swab. Additionally dilutions of wells were plated onto tetracycline free MH agar to calculate

the number of wild type CFU. Agar plates were inverted and incubated for 48 hours at 37°C. Following incubation, plates were examined and SCVs were recorded as presence of non-pigmented microcolonies. The frequency of SCV formation was determined as the number of SCVs per total CFU counts on tetracycline free MH agar.

3.2.5 Characterisation of SCV isolates

S. aureus parent and SCV strains were grown in CAMHB, with SCV cultures being supplemented with 1 mg/L tetracycline to prevent reversion to the parent phenotype. Overnight parent cultures were prepared by inoculating individual colonies in CAMHB followed by incubation at 37°C with shaking at 150 rpm for 18-24 hours. SCVs were incubated for additional time (36 – 48 hours) to allow similar cell densities to be achieved.

3.2.5.1 Auxotrophy

Auxotrophy for haemin, menadione and thymidine in SCV isolates was determined as described previously (section 2.2.9.6).

3.2.5.2 Catalase production

Addition of 1% w/v hydrogen peroxide (H_2O_2) (Sigma-Aldrich, Poole, UK) to overnight *S. aureus* parent and SCV cultures was used to detect catalase production. Catalase activity was recorded using a 3 point scale. Immediate and rapid bubbling following addition of H_2O_2 was recorded as strong catalase production, bubbling observed after longer than 15 seconds was recorded as weak and lack of bubbling was recorded as absence of catalase production.

3.2.5.3 Coagulase production

Parent and SCV isolates were examined for coagulase activity using the Staphylase test kit (Oxoid Ltd, UK) as described previously (section 2.2.5). An absence of agglutination was recorded as coagulase negative. Strong agglutination was recorded as strong coagulase production and weak agglutination as weak coagulase activity.

3.2.5.4 DNase production

DNase agar was prepared by adding 39 g of dehydrate DNase culture media (Oxoid, UK) to 1 L of deionised water. Overnight cultures of *S. aureus* parent and SCV strains were adjusted to a density of 1 x 10^7 CFU/mL and of this suspension 10 µL drops

were spotted onto the surface of a DNase agar plate. Following incubation for 24-48 hours at 37°C, plates were flooded with 1 M hydrochloric acid (HCl) and left for 5 minutes at room temperature. The addition of HCl causes the hydrolysis of DNA resulting in the agar turning opaque. In the presence of DNase enzymes, DNA is digested and no DNA is available to be hydrolysed. Therefore clear zones indicated a presence of DNase enzymes. Excess hydrochloric acid was removed from the plate and zones of clearing were measured digitally using IMAGE J (NIH).

3.2.5.5 Haemolysis and lipase activity

MH agar was supplemented with 5% defibrinated sheep blood (Oxoid, UK) to examine haemolysis activity. Plates were inoculated with densities stated previously and incubated under the same conditions (section 3.2.5.4). Haemolysis activity was detected by the production of zones of clearing surrounding bacterial growth. Heamolysis activity was quantified on the degree of clearing observed, ranging from absent to weak to strong. To examine production of extracellular lipases MH agar was supplemented with 10% w/v egg yolk (Sigma Aldrich, UK). Following incubation, the diameter of zones of clearing (indicating hydrolysis of lipids) were measured digitally using IMAGE J.

3.2.5.6 Growth rate analysis

Parent and SCV cultures were adjusted to achieve a starting density of 5 X 10^5 CFU/mL in 250 mL conical flasks containing 50 mL MH broth. Flasks were incubated at 37°C with shaking at 150 rpm. At set time points 1 mL samples were taken and OD_{630} measured. Following completion of growth rate experiments, flasks were aseptically sampled and serial dilutions in PBS made. Final viable counts were performed by drop counting on MH agar.

3.2.5.7 Transmission electron microscopy

Cells in logarithmic growth phase were fixed by the addition of 1.5% v/v glutaraldehyde (TAAB, UK), which was incubated for 12 hours at 37°C. Following fixation cells were washed twice in double distilled water (5 minutes each wash) and subsequently dehydrated with graded concentrations of ethanol (50%, 70%, 90% and 100%) with each dehydration step lasting 15 minutes. Following dehydration cells were embedded in LR White embedding resin (London Resin Company, UK) and cut into sections. Ultrathin sections were stained firstly with 4% w/v aqueous uranyl

acetate (5 minutes), water washed and air dried. Cells were then counterstained with lead acetate (30 seconds), water washed and air dried. Sections were viewed with a CM12 transmission electron microscope (FEI, USA) with images recorded using a SIS MegaView III digital camera.

3.2.5.8 DNA extraction

DNA extraction from parent and SCV isolates was performed as described previously (section 2.2.7)

3.2.5.9 Random amplified polymorphic DNA PCR (RAPD-PCR)

All PCR reagents were supplied by Qiagen (UK). RAPD-PCR was carried out as described previously by (Mahenthiralingam *et al.*, 1996). Primers 208 (5′-ACGGCCGACC-3′) 268 (5′-AGGCCGCTTA-3′) 272 (5′-AGCGGGCCAA-3′) (MWG Biotech, UK) were used as they have be found to produce good discriminatory patterns in *S. aureus* (Cheeseman *et al.*, 2007). RAPD-PCR was carried out in 25 µL reactions consisting of 2 µL template DNA (approximately 50 ng/µL) being added to a 23 µL PCR mixture consisting of; sterile polished deionised water, 1 X Coralload buffer, 1 X Q solution, 3 mM $MgCl^2$, 1.6 µM each RAPD primer, 200 µM dNTPs, and 1 unit *Taq* DNA polymerase. PCR was conducted in a Flexigene Thermal Cycler (Techne Ltd., UK) with the following cycle; 5 min at 94°C, followed by 4 cycles of 36°C for 5 min, 72°C for 5 min and 94°C for 5 min and a further 30 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 5 min and a final hold at 72°C for 5 minutes. PCR products (12 µL) were run on 1.5 % w/v agarose (Sigma Aldrich, UK) gels and were visualised with ethidium bromide (0.5 µg/mL final concentration in TAE buffer; 40 mM Tris-acetate, 1 mM EDTA) for 45 minutes. Molecular standards were run on all gels using molecular weight maker Hyperladder I (Invitrogen, UK).

3.2.5.10 16S rRNA amplification

Primers rRNS-1 (5’-AGAGTTTGATCCTGGCTCAG-3’) and rRNS-2 (5’-AAGGAGGTGATCCA(A/G)CCGCA-3’; MWG Biotech, UK) were used to amplify the *S. aureus* 16S rRNA gene of parent and SCV isolates. PCR was carried out in 25 µL reactions consisting of: 1 X PCR buffer, 1 X Q Solution, 3 mM $MgCl_2$, 100 µM dNTPs mixture, 0.4 µM primer, 1 U of *Taq* DNA polymerase and 2 µL of template DNA. PCR was conducted with the following cycle; 5 min at 94°C, followed

by 35 cycles of 94 ºC for 30 seconds, 30 seconds at 50ºC, 60 seconds at 72ºC for and a final hold at 72ºC for 5 min.

3.2.5.11 Sequencing

Prior to sequencing, amplified products were treated and cleaned with Montage PCR Centrifugal Filter Devices (Millipore, UK), using various centrifugation steps to purify PCR products. PCR products were sequenced with six additional primers listed in Table 3.1. Sequencing reactions were performed using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit and run on an ABI 3130×1 Genetic Analyzer. Sequences were viewed using Chromas lite v2.01 (Technelysium Pty Ltd). Following sequence determination 16S rRNA sequences of SCV and parent strains were compared using BioEdit Sequence Alignment Editor (Hall, 1999).

3.2.6 Statistical analysis

Analysis of significant differences between characteristics of SCV isolates and parent strains were performed as described previously (section 2.2.9.8).

Table 3.1 ***S. aureus*** **16S rRNA sequence primers (Werner** ***et al.*****, 2008)**

Primer	Sequence (3'- 5')
rRNS-357F	CTCCTACGGGAGGCAGCAG
rRNS-704F	GTAGCGGTGAAATGCGTAGA
rRNS-1114F	GCAACGAGCGCAACCC
rRNS-neu1R	CCTACTGCTGCCTCCCGTAG
rRNS-685R	TCTACGCATTTCACCGCTAC
rRNS-1100R	GGGTTGCGCTCGTTG

3.3 Results

3.3.1 Tetracycline selection for SCVs

Doxycycline, tetracycline and oxytetracycline all selected for *S. aureus* SCVs (Figure 3.2). SCVs were recorded as non-pigmented, microcolonies that were approximately 5-10 times smaller in comparison to parent colonies. SCVs were termed according to the antibiotic that selected for them. For example N315 DOX was a SCV derived from N315 following exposure to doxycycline. SCVs were detected in control experiments (where no antibiotic was added to broth before agar plating) but frequencies were significantly lower in comparison to conditions where antibiotics were present ($P = < 0.01$). SCV selection frequencies were highest following exposure of cultures to X 0.5 MIC for all 4 strains tested across the panel of tetracyclines examined.

3.3.2 Characterisation of tetracycline selected SCVs

3.3.2.1 Auxotrophy and SCV characteristics

SCVs auxotrophic for haemin and menadione were frequently observed in tetracycline selected SCVs (Table 3.2). Auxotrophy was for haemin, menadione or thymidine was not detected in 25% of the SCVs analysed. All SCVs showed reduced catalase, coagulase and haemolysis activity in comparison to their parent counterparts with certain SCVs showing a complete absence of catalase, coagulase and/or heamolysis activity. To confirm auxotrophy requirements SCV cultures were supplemented with either haemin or menadione and examined for restoration of parent characteristics. In all cases SCVs reverted to the parent phenotype and produced catalase, coagulase, and haemolysis activity comparable with that activity recorded in parent strains.

3.3.2.2 Cellular morphology

Differences in cellular morphology were observed between parent and SCV strains (Figure 3.3). Transmission electron micrographs showed SCV cell walls to be significantly thicker in comparison to parent cell walls ($P = < 0.01$). Individual parent cells showed regular patterns of division with a clear regularly shaped septum. SCVs frequently displayed an irregular shaped septum, with a curved appearance in contrast to the straight septum shown in parent cells.

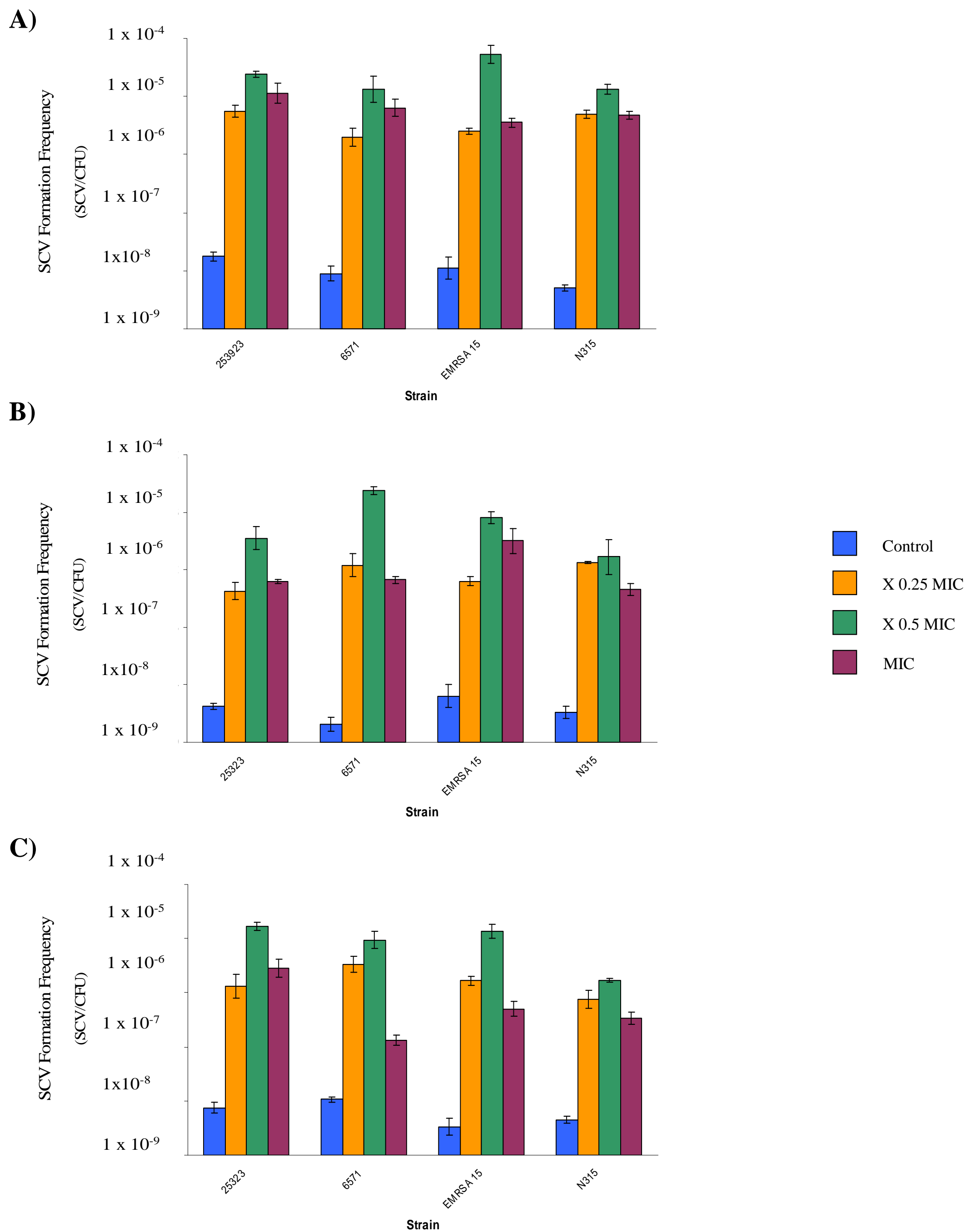


Figure 3.2 SCV formation frequencies after exposure to various concentrations of tetracycline antibiotics A) - Doxycycline; B) - Tetracycline; C) - Oxytetracycline. Control conditions had no prior exposure to tetracycline. SCV formation frequencies were determined as the number of SCVs per total CFU counts on antibiotic free MHA. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

Table 3.2 Characteristics of SCVs isolated after exposure to various tetracyclines

Strain	Auxotrophy	Catalase	Coagulase	Haemolysis
ATCC 25923	NT	+ +	+ +	+
ATCC 25923 SCV DOX	Haemin	-	-	-
ATCC 25923 SCV TET	ND	+	+	-
ATCC 25923 SCV OXY	Haemin	+	+	-
NCTC 6571	NT	+ +	+ +	++
NCTC 6571 SCV DOX	Haemin	+	+	+
NCTC 6571 SCV TET	Menadione	-	-	-
NCTC 6571 SCV OXY	Menadione	-	-	-
N315	NT	+ +	+ +	++
N315 SCV DOX	ND	+	+	+
N315 SCV TET	Haemin	-	-	+
N315 SCV OXY	ND	-	+	-
EMRSA 15	NT	+ +	+ +	++
EMRSA 15 SCV DOX	Menadione	+	-	-
EMRSA 15 SCV TET	Haemin	-	-	+
EMRSA 15 SCV OXY	Haemin	+	+	-

++ - Strong catalase/coagulase/haemolysis activity; + - Weak catalase/coagulase/ haemolysis activity; - = Absence of catalase/coagulase/ haemolysis activity; DOX - doxycycline; TET - tetracycline; OXY - oxytetracycline; ND - not detected; NT =- not tested

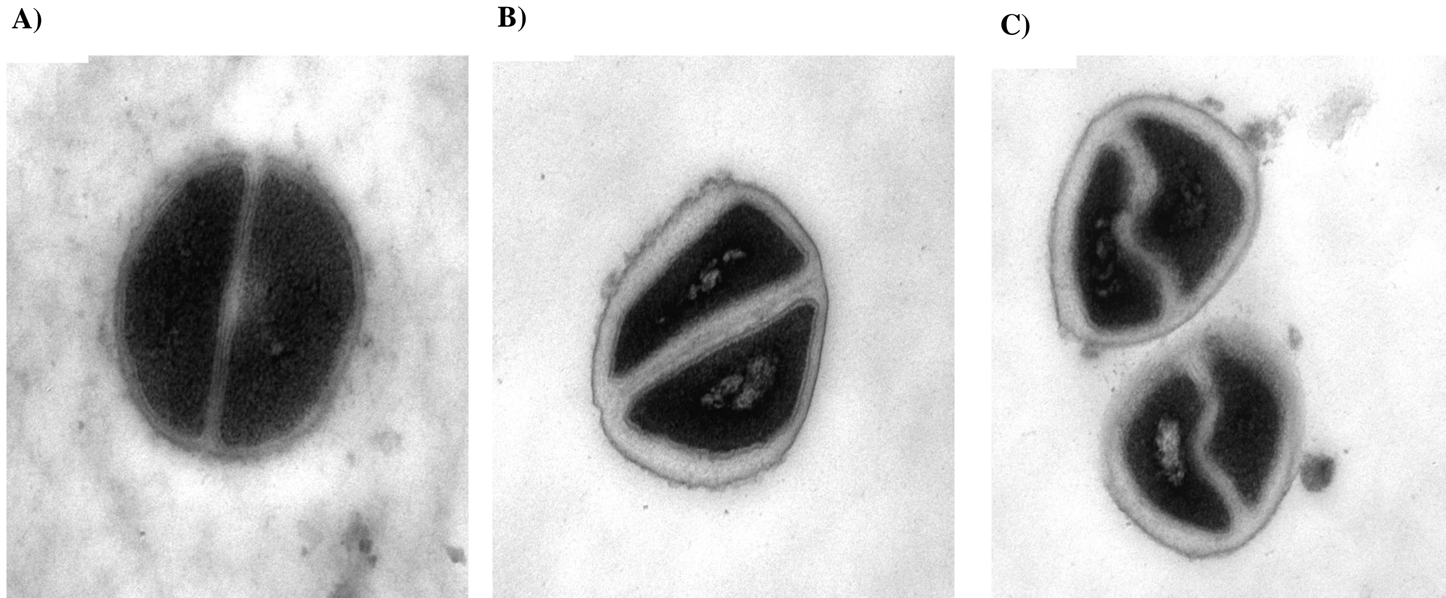


Figure 3.3 Transmission electron micrographs of SCV selected in the presence of tetracycline and parent cells A) - Parent individual cocci showing regular cell wall and septum, B) - Individual SCV showing increased cell wall thickness, C) - SCVs showing irregular septum formation. All images magnification X 31,000.

3.3.2.3 DNase and lipase activity

A measurement of extracellular DNase activity was obtained by measuring zones of DNA hydrolysis (Figure 3.4). All SCV isolates from all three tetracycline antibiotic produced significantly less DNase in comparison to parent strains ($P = < 0.01$). The average zone of DNA hydrolysis in parent strains was 18.75 mm where as the SCV average was over 4 times smaller at 4.24 mm (Figure 3.4). SCVs also produced significantly less extracellular lipase activity than parent strains ($P = < 0.01$). Zones of clearing on MH agar containing egg yolk were an average of 11.7 mm in diameter for parent strains were as SCV isolates produced average diameter zones of lipase activity of 1.04 mm (Figure 3.4). A complete absence of lipase activity was observed in 3 out of the 12 SCV isolates examined.

3.3.2.4 Growth rate analysis

Growth rate analysis revealed changes in parent and tetracycline selected SCV growth profiles. As shown in Figure 3.5 the SCVs of ATCC 25923 and EMRSA 15 in the presence of doxycycline, tetracycline and oxytetracycline all exhibited increases in the duration of the lag phase of growth. The duration of lag phase in parent strains was approximately 1.5 hours in comparison to an average of 14 hours for the 6 representative SCVs shown in Figure 3.5. Furthermore SCVs also reached lower maximum cell densities reflected by the lower optical density values (Figure 3.5). Viable counts revealed that on average SCVs reached a maximum cell density of 2.7×10^{8} CFU/mL. In comparison both parent strains reached higher average cell densities of 5×10^{9} CFU/mL.

3.3.2.5 Susceptibility profiles

MICs of parent and SCV isolates were obtained for all tetracycline antibiotics examined as well as for a fluroquinolone (ciprofloxacin), an aminoglycoside (gentamicin) and a ß lactam (oxacillin) according to CLSI guidelines (Table 3.3). SCV isolates exhibited an increase in MIC for all 3 members of the tetracycline class of antibiotics regardless of the tetracycline antibiotic used for selection. For example ATCC 25323 DOX (selected for in the presence of doxycycline) exhibited an 8 fold increase in doxycycline susceptibility, as well as a 4 fold increase in tetracycline and oxytetracycline susceptibility (Table 3.3). SCVs also showed reduced susceptibility to gentamicin and oxacillin. Gentamicin MICs for SCV isolates were 2-8 fold higher

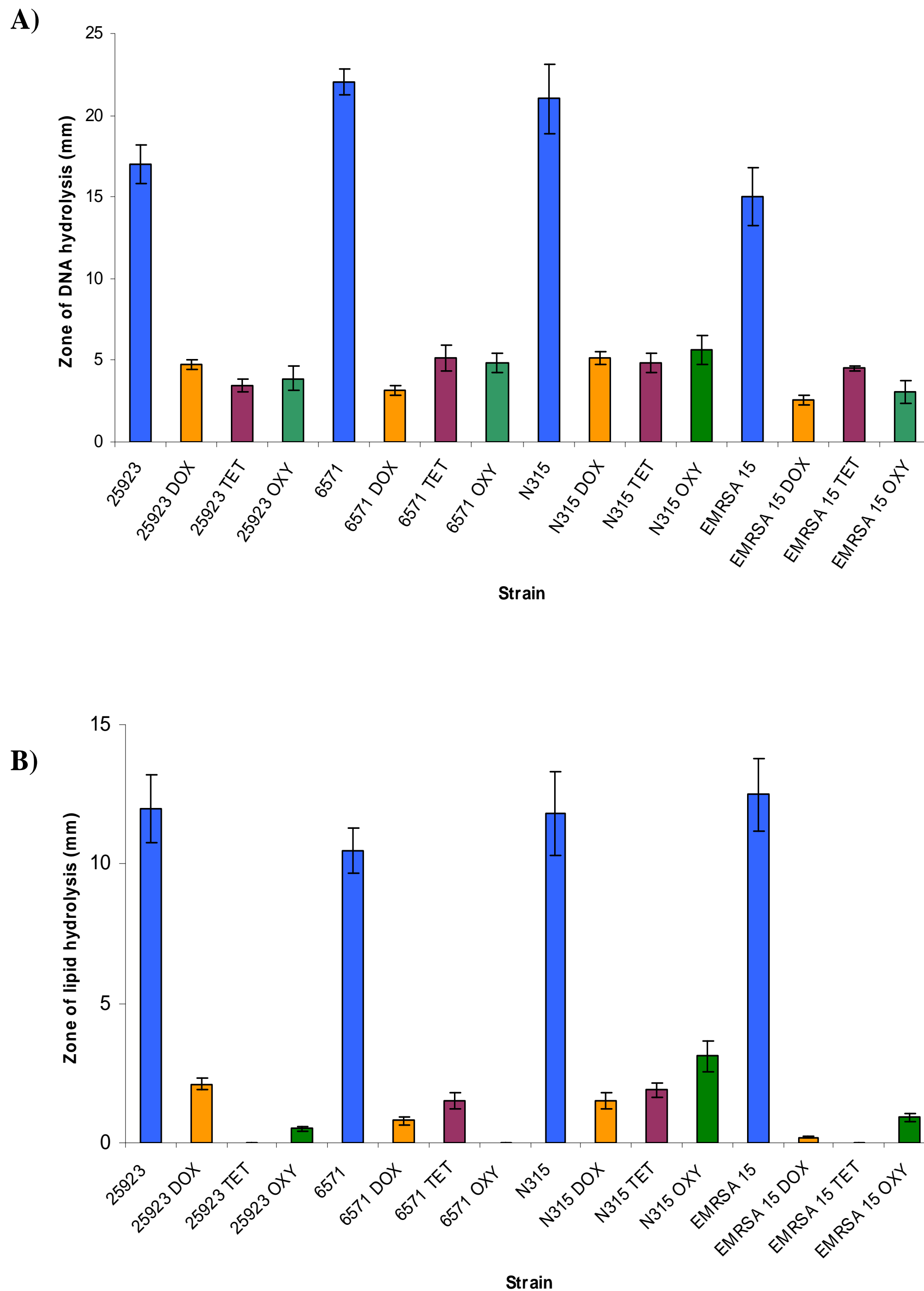


Figure 3.4 DNase and lipase activity in SCVs and corresponding parent strains A) - DNase zones represent hydrolysis of DNA and extracellular DNase activity; B) - Lipase activity zones representing breakdown of lipids. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

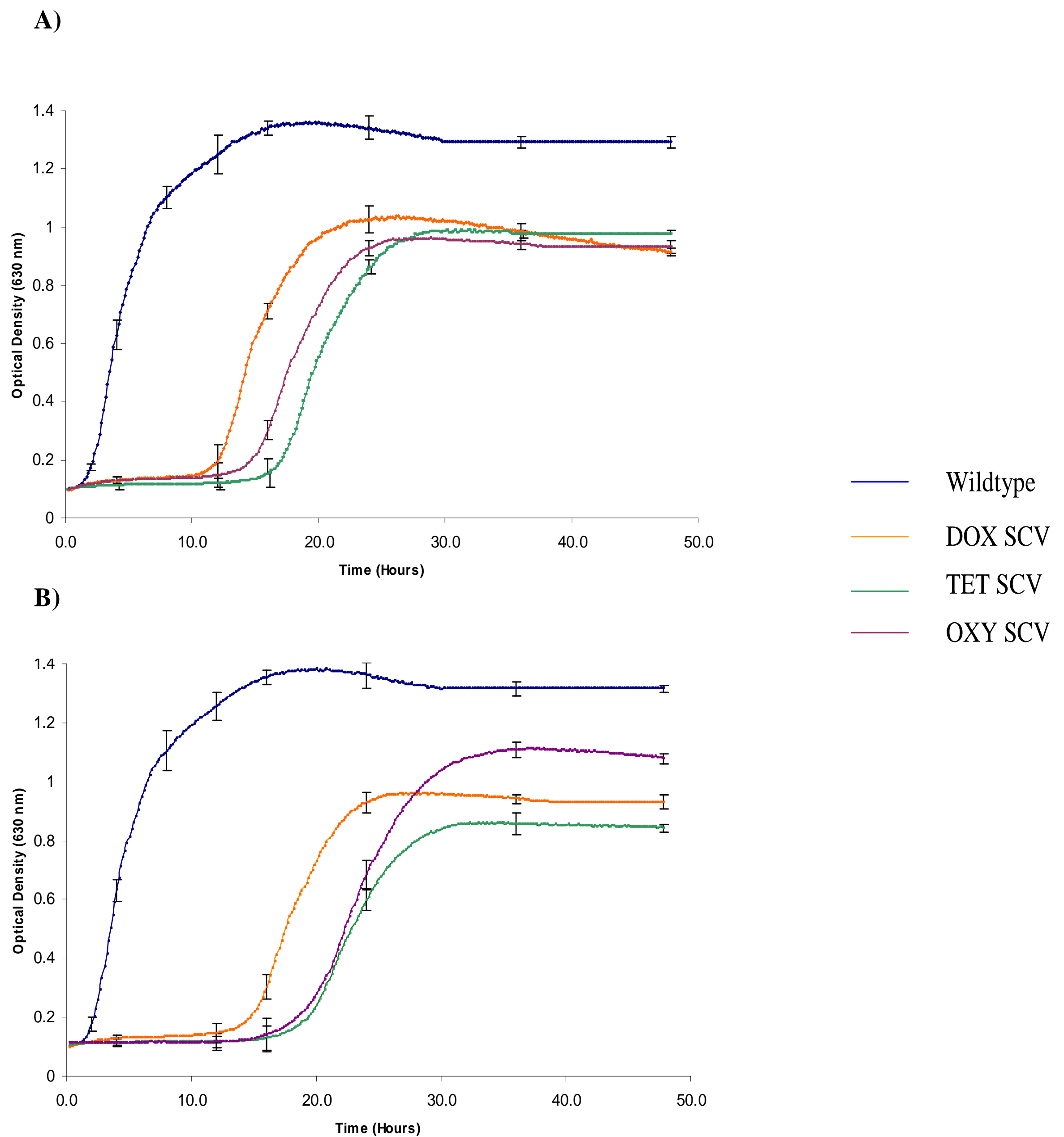


Figure 3.5 Tetracycline selected SCVs and parent growth dynamics A) - ATCC 25923 and SCV derivatives B) - EMRSA 15 and SCV derivatives. SCVs exhibited extended lag phases and reached lower maximum cell densities in comparison to parent strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

Table 3.3 MIC (mg/L) for SCV isolated in the presence of various tetracycline antibiotics and their corresponding parent strains

Strain	DOX	TET	OXA	CIP	GEN	OXA
ATCC 25923	0.25	0.5	1	0.75	0.25	0.5
ATCC 25923 SCV DOX	2	2	4	0.5	1	2
ATCC 25923 SCV TET	1.5	2	3	0.75	1.5	1
ATCC 25923 SCV OXY	2	2	4	0.75	1	1
NCTC 6571	0.5	0.5	2	0.5	0.125	0.5
NCTC 6571 SCV DOX	2	1.5	5	0.5	1	1.5
NCTC 6571 SCV TET	2	2	4	0.75	0.75	1
NCTC 6571 SCV OXY	2	2	4	0.5	1	1
N315	0.5	1	2	0.75	0.5	NT
N315 SCV DOX	2	2	5	0.75	1.5	NT
N315 SCV TET	2	2.5	5	0.75	2	NT
N315 SCV OXY	2	2	4	0.5	2	NT
EMRSA 15	0.25	0.5	1	0.75	1	NT
EMRSA 15 SCV DOX	2	2	3	0.5	2	NT
EMRSA 15 SCV TET	1.5	1.5	4	0.75	2	NT
EMRSA 15 SCV OXY	2	2	4	0.75	3	NT

CIP - Ciprofloxacin; DOX - Doxycycline; GEN - Gentamicin; OXA - Oxacillin; OXY - Oxytetracycline; TET - Tetracycline; NT - not tested. Modal MIC values are presented. Modal values were obtained from three independent replicates and three independent biological replicates.

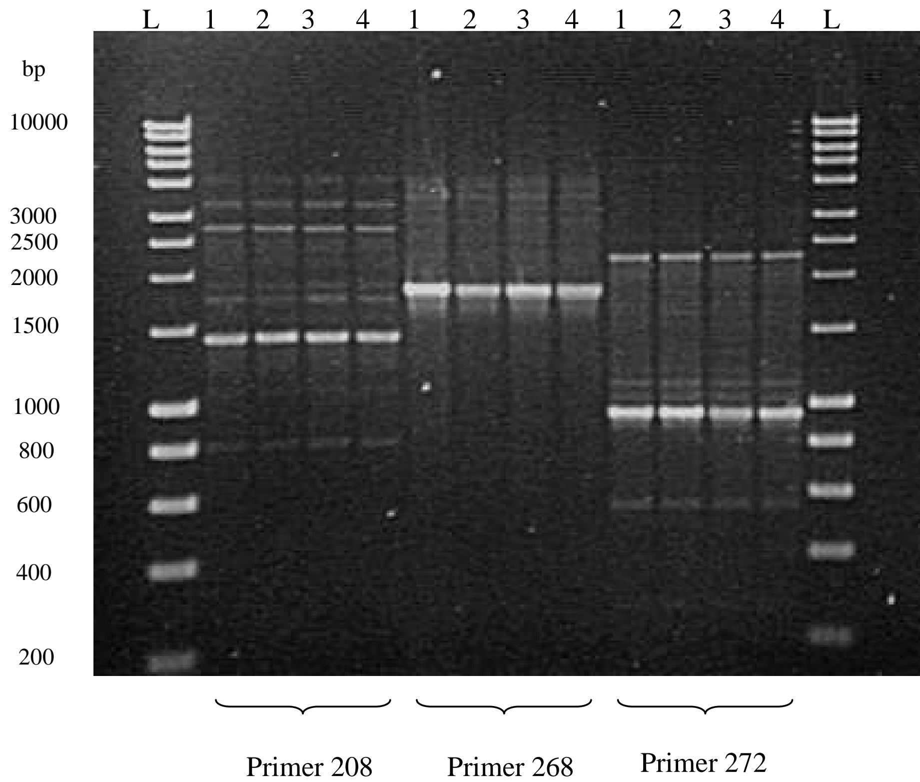


Figure 3.6 Typical SCV and parent RAPD profiles L - Hyperladder 1; 1 – ATCC 25923; 2 – ATCC 25923 SCV DOX; 3 – ATCC 25923 SCV OXY; 4 – ATCC 25923 SCV TET. RAPD analysis of SCVs selected in the presence of various tetracyclines showed SCVs displayed identical profiles in comparison to parent strains with three different primer sets.

than parent MICs and 2-4 fold increase in SCV susceptibility to oxacillin susceptibility was also recorded. MICs for the DNA replication inhibitor ciprofloxacin showed no consistent variation in comparison of parent and SCV isolates. Although though increases in MIC for all SCV isolates were detected in 5 out of the 6 antibiotics tested none of the increases were large enough to classify them as resistant according to CLSI guidelines.

3.3.2.6 RAPD profiles

RAPD fingerprints for SCVs isolated from different tetracycline antibiotics were identical to parent fingerprints generated from all three primer sets (Figure 3.6). The same banding patterns were also observed between SCV isolates (from the same parent strain) recovered from different selection backgrounds.

3.3.2.7 16S rRNA sequence analysis

Sequence analysis of the 16S rRNA showed a 99.9 % sequence similarity between *S. aureus* parent strains. Sequence compassion of *S. aureus* parents and SCVs selected in the presence of tetracycline however revealed no difference in 16S rRNA sequence.

3.4 Discussion

Exposure of wildtype cultures to doxycycline, tetracycline and oxytetracycline resulted in the selection of SCVs in both MSSA and MRSA strains. SCVs isolated displayed morphological characteristics that correlate with other studies that report SCV as non-pigmented microcolonies that take 24-48 hours to appear on solid agar (Pan *et al.*, 2002; Seaman *et al.*, 2007). The appearance of these atypical phenotypes (in comparison to wildtype strains) was attributed to defects in the *S. aureus* electron transport chain (ETC).

Auxotrophy for haemin and menadione was regularly detected (75 % of total isolates) in the tetracycline selected SCVs isolated this study. Auxotrophy for haemin and menadione has been widely reported in *S. aureus* SCVs as well as SCVs isolated from several other bacterial species (Colwell, 1946; Sasarman *et al.*, 1970). Haemin and menadione are both crucial components of the bacterial ETC. Haemin is required for the synthesis of cytochromes and menadione is isoprenylated to form menaquinone and is the acceptor of electrons from nicotinamide adenine dinucelotide (NADH) and flavin adenine dinucelotide ($FADH_2$) (von Eiff *et al.,* 2001b; Bates *et al.,* 2003). The inability to produce menaquinone and haemin in the SCVs selected for by tetracyclines results in a disrupted ETC resulting in reduced ATP levels. The reduction in cellular ATP levels results in the characteristic non-pigmented microcolonies observed. The production of pigment in *S. aureus* requires energy to drive carotenoid biosynthesis, whilst ATP is essential to driver cellular process such as protein and cell wall synthesis (Proctor *et al.*, 2006). The attenuated production of catalase, coagulase, DNase and lipase production agrees with previous research which shows that SCVs produce lower amounts of extracellular accessory proteins (Proctor *et al.*, 2006; Seaman *et al.*, 2007). Many of these proteins are under the control of the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sarA*) which has been shown to be downregulated in SCVs (Kahl *et al.*, 2005). The reduction in various extracellular accessory proteins suggests the expression of global regulators is also altered in tetracycline selected SCVs. The selection of SCVs following exposure to tetracyclines may act as a survival mechanism in which the switching to the SCV phenotype renders the loss of certain functions but most importantly permits survival and persistence.

All SCVs were also examined for the presence of thymidine auxotrophy. Thymidine auxotrophy has been reported in *S. aureus* SCVs and has been associated with SCVs recovered from cystic fibrosis patients (CF) (Kahl *et al.*, 1998). Thymidine auxotrophic SCVs share characteristics with haemin and menadione auxotrophs but a link between thymidine auxotrophy and exposure to trimethoprim sulphamethoxazole (SXT) is apparent (Gilligan *et al.*, 1987). SXT is commonly used to treat CF and thymidine auxotrophic SCVs are commonly recovered from CF patients. Exposure to SXT results in mutations in *thyA* which encodes thymidylate synthase in *S. aureus* (Besier *et al.*, 2007). The failure to detect thymidine auxotrophs in this and previous studies (Chapter 2) suggests that thymidine auxotrophy is not accountable for the SCV phenotype following exposure to aminoglycosides and tetracycline. However analysis of isolates from different sources (such as clinical derived specimens) may yield thymidine auxotrophs. In several of the SCVs isolated, no auxotrophy profile was detected. Various studies have reported the isolation of SCVs where no auxotrophy profile can be detected including SCVs selected in the presence of linezolid, sparfloxacin and triclosan (Gao *et al.*, 2010; Pan *et al.*, 2002; Seaman *et al.*, 2007). Possible candidates for the site of auxotrophy are unsaturated fatty acid biosynthesis or in the F_0F_1-ATPase as discussed previously (section 2.4).

SCVs commonly display reduced susceptibility to a variety of antimicrobial compounds as a direct result of the SCV phenotype. The SCVs isolated in this study showed reduced susceptibility to tetracyclines as well as gentamicin and oxacillin in comparison to parent strains. It is well documented that SCVs are less susceptible to aminoglycosides antibiotics such as gentamicin (Proctor *et al.*, 1998). The presence of an electrochemical gradient across the bacterial membrane is essential for the uptake of positively charged molecules such as aminoglycosides (Balwit *et al.*, 1994). As SCVs have a reduced electrochemical gradient, less uptake of these positively charged molecules occurs, resulting in reduced susceptibility. The reduced susceptibility to ß-lactam antibiotics observed is has been related to the slow growth rate of SCVs reducing the effectiveness of these cell wall active antibiotics (Schnitzer *et al.*, 1943; Youmans *et al.*, 1945). Although tetracycline selected SCVs produced atypical growth profiles, following the extension of lag phase only subtle differences were observed in growth rate, which suggests others mechanisms are responsible for

reduced oxacillin susceptibility. The thickening of the SCV cell wall observed in electron micrographs may be responsible for reduced susceptibility to oxacillin. Cell wall thickening has been observed in *S. aureus* strains displayed reduced susceptibility to vancomycin (Cui *et al.*, 2003) and has also been shown to be responsible for reducing susceptibility to ß-lactam antibiotics (Morikawa *et al.*, 2001). Thickening of the cell wall may impact on the penetration of cell wall specific antibiotics (such as oxacillin), which must cross the cell wall in order to reach their cellular target (Lambert, 2002). No difference in susceptibility between SCV and parent strains to the DNA gyrase/topoisomerase inhibitor ciprofloxacin was observed. Similarly to the reduced susceptibility to ß-lactam antibiotics, it would be hypothesised that a slower growth rate would result in reduced susceptibility to ciprofloxacin due to lower rates of DNA synthesis. The similarities in growth rate and ciprofloxacin susceptibility suggest that DNA synthesis in the exponential phase is proceeding at similar levels in parent and SCV strains.

RAPD has been applied to study and track the epidemiology of *S. aureus* in both clinical and environmental settings (Lee, 2003; VandenBergh *et al.*, 1999). RAPD was successfully applied to SCV isolates which had been selected for in the presence of various tetracyclines. SCV isolates displayed fingerprint patterns that were identical to their parent counter parts and identical fingerprints of SCV isolated from different tetracyclines were also observed confirming the clonality of the tetracycline selected SCV isolates. Although RAPD is not as powerful as other molecular techniques for studying epidemiology (such as pulse field gel electrophoresis; PFGE) the homology observed between SCV isolates and parent strains confirms clonality is associated with tetracycline selected SCVs.

In order to identify the genetic basis for the mutations that give rise to the SCV phenotype investigators have sequenced genes that may be potential targets for mutations. Norstrom *et al.,* (2007) have shown that fusidic acid can select for SCVs via mutations in *fusA* and *rplF* (ribosomal protection protein). Fusidic acid targets protein synthesis through preventing the release of translation elongation factor EF-G (Bodley *et al.*, 1969). Mutations in *fusA* reduce the affinity of fusidic acid for its target where as mutations in *rplF* results in alteration (directly or indirectly) of the structural conformations of EF-G on the ribosome (Norstrom *et al.*, 2007). These mutations

represent novel mechanism for fusidic acid that give rise to the SCV phenotype. In this study we sequenced the 16S rRNA sequence of parent and SCV *S. aureus* isolates in order to identify mutations that may be responsible for the tetracycline selected SCV phenotype. However no alterations in the DNA sequences were detected. Studies have reported an absence of mutation(s) in the gene(s) targeted by the selecting antibiotic that results in SCVs. An outcome of this is no known genetic basis for the SCV phenotype. For example Mitsuyama *et al.,* (1997) demonstrated that pazufloxacin (a broad spectrum fluoroquinolone) selected for *S. aureus* SCVs but no mutations were detecting in the quinolone resistance determining regions of either DNA gyrase of topoisomerase IV. Therefore the authors suggest that the fluoroquinolone resistance may follow a similar mechanism of resistance to aminoglycosides in SCVs i.e. reduced uptake due to reduced electrochemical gradient across the bacterial membrane. The mechanisms of reduced tetracycline susceptibility in SCVs may follow the similar mechanism. Uptake of tetracycline in susceptibile *E. coli* involves both energy dependent and energy independent systems (McMurry & Levy, 1978). Furthermore the uptake of tetracycline in *E. coli* has been partially attributed to the presence of a proton motive force (Smith & Chopra, 1984). As SCVs are deficient in the generation of a proton motive force (due to electron transport chain interruption), this may be responsible for a reduction in tetracycline uptake in tetracycline selected SCVs, reducing their susceptibility in comparison to parent strains.

The findings in this chapter illustrate the ability of several members of the tetracycline class of antibiotics to select for SCVs *in vivo*. Although several other antibacterial compounds have been shown to select for SCVs this is the first case to our knowledge where tetracyclines have been demonstrated to select for the SCV phenotype. The SCVs isolated shared similar characteristics with those that have been reported in various other studies. The ability of tetracycline to select for SCVs raises concerns regarding the clinical use(s) of tetracycline. Although large reductions in antimicrobial susceptibility were not observed, the selection of SCVs is unfavourable due to their ability to hide inside host cells, which can serve a reservoir for chronic and therapy-refractive infections (Tuchscherr *et al.*, 2011b).

3.5 Conclusions

- Doxycycline, tetracycline and oxytetracycline can select for *S. aureus* SCVs at a range of concentrations.
- Exposure to X 0.5 MIC results in the highest SCV formation frequencies in the three tetracyclines analysed.
- Tetracycline selected SCVs display increased cell wall thickness and irregular septum formation.
- Auxotrophy for haemin and menadione was widely observed in SCV isolates correlating to defects in electron transport.
- Tetracycline selected SCVs show attenuated catalase, coagulase and heamolysis activity and reduced production of extracellular enzymes.
- Tetracycline selected SCVs showed reduced susceptibility to a range of tetracycline, gentamicin and oxacillin.
- Reduced tetracycline susceptibility in SCVs is not related to mutations in 16S rRNA.
- Tetracycline selected SCVs and parents produced identical RAPD profiles indicating clonality.

4 CHAPTER 4: BIOFILM FORMATION IN *STAPHYLOCOCCUS AUREUS* SMALL COLONY VARIANTS

4.1 Introduction

4.1.1 Biofilms

Bacteria have the ability to produce a protective hydrated matrix of polysaccharide and protein, forming a slimy layer known as a biofilm (Stewart *et al.,* 2001). A biofilm can be further defined as an 'assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material' (Donlan, 2002). This polysaccharide is composed of extracellular polymeric substances (EPS) and consists of a variety of different biopolymers which immobilise biofilm cells, keeping them within close proximity of one another (Flemming & Wingender, 2010). Various bacterial infections in the modern world are thought to involve biofilm formation (Costerton *et al.*, 1999), underlying the importance of this area in the clinical setting. Biofilms constitute a protected mode of growth that allows survival in a hostile environment and therefore treatment of biofilm related infections depends on long term, high-dose antibiotic therapies and may require the removal of indwelling devices (Fux *et al.*, 2005).

4.1.2 Staphylococcal biofilms

Staphylococcus aureus and *S. epidermidis* biofilms are among the most commonly encountered organisms in the clinical setting and are responsible for a large proportion of biofilm-mediated device-related infections (O'Gara & Humphreys, 2001). Staphylococci can form biofilms on frequently used medical devices such as catheters (Marrie & Costerton, 1984b), pacemakers (Marrie & Costerton, 1984a) and prosthetic knee and hip joints (Rohde *et al.*, 2007). Staphylococcal biofilms represent a focus of infection which may allow clusters of cells to detach from the biofilm, resulting in bloodstream infection and metastatic spread (Fitzpatrick *et al.*, 2005).

4.1.2.1 Staphylococcal biofilm formation

Staphylococcal biofilm formation is a two step process, attachment of cells to a surface, followed by accumulation of cells to form a multilayered cell cluster (Gotz, 2002). Figure 4.1 shows that adhesion to a surface is the crucial transition stage from free-floating planktonic cells to a biofilm and involves the interplay of several

adhesion molecules enabling the physico-chemical interactions between the cells and the surface (Kong *et al.*, 2006). The transition from planktonic to multicellular lifestyle requires the co-ordinated expression of a variety of specialised extracellular and cellular components (Valle *et al.*, 2003).

4.1.2.2 Molecules contribution to adhesion

Adhesion to a surface in staphylococci is mulifactoral. Interactions between the bacterial surface, substrate surface and the surrounding environment all play a role (Wang *et al.*, 1995). Nutrient flow, pH and hydrodynamic flow are all examples of environmental factors that contribute to the adhesion process, although the physiochemical properties of the bacterial and substrate surface determine the non specific interactions that determine attraction or repulsion (Higashi & Sullam, 2006).

Although various environmental factors are play a role, bacterial surface proteins are crucial for initial adhesion. Many surface proteins are produced in staphylococci and a large proportion belong to the microbial surface components recognising adhesive matrix molecules (MSCRAMM) family (Higashi & Sullam, 2006), the majority of which are anchored to cell wall peptidoglycan (Patti *et al.*, 1994). Fibronectin-binding proteins (FnBPs), a collagen-binding protein, Cna, and clumping factor (Clf) are all adhesins belonging to MSCRAMM family (Foster & Hook, 1998). These components bind to the extracellular matrix of host tissues and mediate initial attachment. *S. aureus* also produces secreted proteins to facilitate adhesion. One such example is extracellular adherence protein (EAP; Palma *et al.*, 1999) which is required for the adherence of *S. aureus* to eukaryotic cells and also facilitates internalisation (Haggar *et al.*, 2003). Although a variety of different surface proteins exist they all share the same principal role; bacterial adhesion. This adhesion is crucial for the first step in biofilm biogenesis to allow the subsequent steps of biofilm formation to proceed.

4.1.2.3 Molecules responsible for exopolysaccharide production

Following adhesion the next stage in staphylococcal biofilm formation is the synthesis of exopolysaccharide, which aids aggregation and cellular adhesion. The principle exopolysaccharide produced by staphylococci is polysaccharide intracellular adhesin (PIA) (Mack *et al.*, 1996) which is considered essential in mediating cellular accumulation (Figure 4.1) and biofilm development (Gotz, 2002). PIA, also termed

polymeric N-acetyl glucosamine (PNAG), is composed of 2 polysaccharide fractions, polysaccharide I (~ 80%) and polysaccharide II (~20%; Mack *et al.*, 1996). Polysaccharide I is composed of linear ß 1, 6-linked N-acetylglucosamine residues containing up to 15% de-N-acetylated amino groups and substitution with succinate and phosphate residues introducing simultaneously positive and negative charges into the polysaccharide (Cramton *et al.*, 2001a; Cucarella *et al.*, 2001; Mack *et al.*, 1996). Polysaccharide II is structurally related to polysaccharide I but has a lower content of non N-acetylated D-glucosminyl residues (Gotz, 2002).

4.1.2.4 PIA production and the intracellular adhesin locus

PIA production is under the control of the intracellular adhesin (*ica*) operon (Figure 4.2), which was first identified in *S. epidermidis* (Heilmann *et al.*, 1996). Using transposon mutagenesis Heilmann *et al.,* (1996) isolated a biofilm-negative mutant with an insertional inactivation of the *ica*ABC gene cluster which had lost the ability to form biofilms on a polystyrene surface. Complementation with an *ica*ABC-carrying plasmid restored the mutant's biofilm forming capacity and the expression of PIA. The *ica* operon was later identified in *S. aureus* and sequence comparison with the *S. epidermidis ica* genes revealed 59 to 78% amino acid identity (Cramton *et al.*, 1999). The *ica* operon consists of PIA biosynthesis genes (*ica*ADBC) and a regulator (*ica*R) (Higashi & Sullam, 2006). *ica*R is located upstream of *ica*ADBC (Figure 4.2) and is a member of the *tet*R family of transcriptional regulators that is divergently transcribed and functions as a negative regulatory protein (Conlon *et al.*, 2002). IcaR binds the *ica* operon promoter region close to the IcaA start codon and exerts its repressor activity by obscuring the binding site of the *ica* promoter (Jefferson *et al.*, 2003; Jefferson *et al.*, 2004). *IcaADBC* encode the biosynthetic genes that are responsible for PIA biosynthesis; *ica*A encodes a transmembrane protein that synthesises *N*-acetyl-glucosamine oligomers (Gerke *et al.*, 1998). The surface-attached protein *ica*B is then responsible for deacetylation of the N-acetylglucosamine polymer (Vuong *et al.*, 2004b). *IcaC* is involved in translocation and externalisation of the growing polysaccharide to the cell surface (O'Gara, 2007) and *icaD* is thought to function as a chaperone, directing the correct folding and membrane of insertion of IcaA (Gotz, 2002).

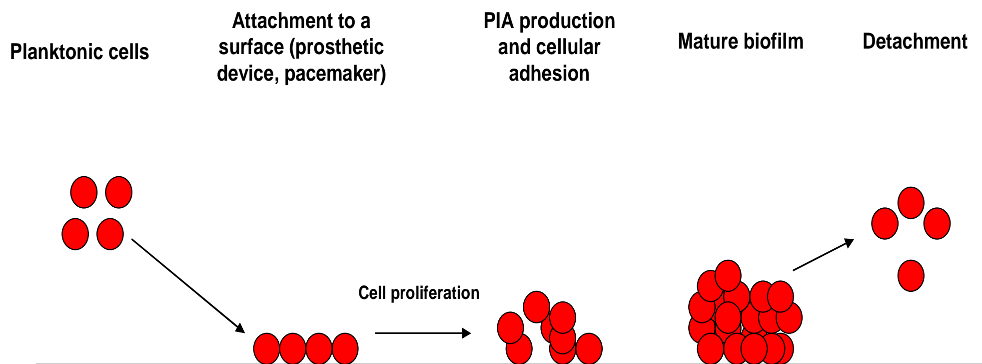


Figure 4.1 Formation of *S. aureus* biofilms Planktonic cells firstly attach to a surface substrate, followed by cell proliferation. As cell numbers increase, PIA is produced in increasing amounts encapsulating the bacteria in slime like matrix. With time a large community of cells forms a mature biofilm from which individual cells may detach to initiate formation of other biofilms. Adapted from Vuong & Otto (2002).

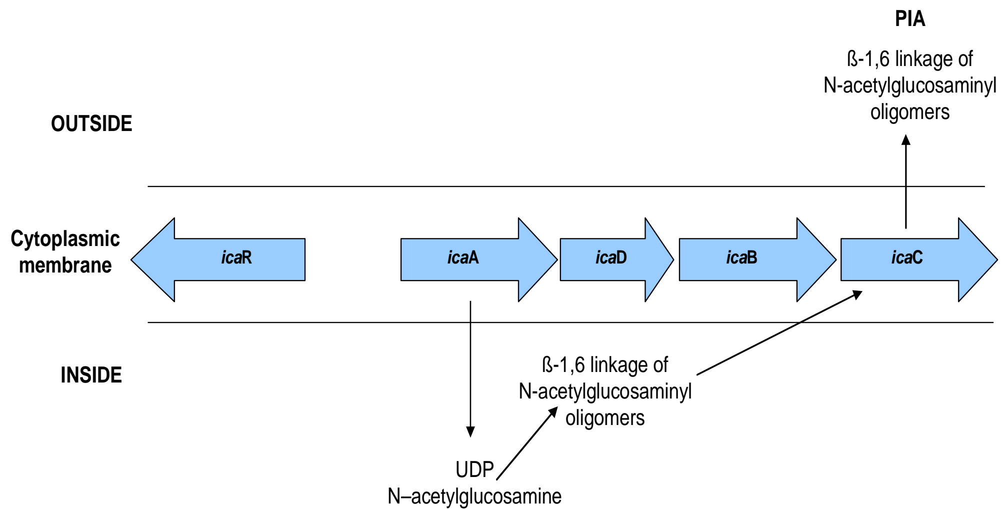


Figure 4.2 Organisation of *ica* locus in *S. aureus* Oligomers derived from UDP N-acetylglucosamine are synthesised by *icaA*, which are then modified with longer oligomers produced by *icaD*. Deacetylation of the poly-N-acetylglucosamine molecule is carried out by *icaB* and *icaC* is finally responsible for translocation of the completed PIA to the cell surface. Adapted from Gerke *et al.,*(1998) and Gotz (2002).

4.1.2.5 Other molecules involved in biofilm formation

As well as PIA, other *ica*-independent molecules are known to impact on staphylococcal biofilm formation. The biofilm associated protein (Bap) was first identified by Cucarella *et al.,* (2001) using transposon mutagenesis. The mutant strain displayed a significant decrease in attachment to inert surfaces, intercellular adhesion, and biofilm formation. Bap is a multidomain surface-associated protein that promotes both primary attachment to surfaces and intracellular adhesion (Lasa & Penades, 2006). Bap negative strains produce lower levels of PIA; however a PIA producing *S. aureus* strain complemented with Bap produced PIA in significantly greater quantities (Cucarella *et al.*, 2001). Staphylococcal isolates that contain Bap are strong biofilm producers (despite not containing the *ica* operon) indicating Bap mediates a alternative mechanism of biofilm development, to the regular PIA-dependent mechanism (Tormo *et al.*, 2005a).

4.1.2.6 Influence of environmental stimuli and global regulators on staphylococcal biofilm formation

The role of environmental stimuli and their impact on biofilm formation has been the subject of much research. Certain environmental stimuli can induce changes in global gene expression which can promote biofilm formation. The diverse environmental conditions encountered by staphylococci therefore have a direct impact on the extent to which strains can form biofilms (Table 4.1). The accessory gene regulator (*agr*) effects the regulation of various virulence genes in *S. aureus* and plays a role in quorum sensing. *Agr* is an example of a two component system consisting of a sensor protein that subsequently activates a response regulator protein (Higashi & Sullam, 2006). *Agr* mutants display increased capacity to form biofilms, which appears to be independent of PIA production (Vuong *et al.*, 2000). Increased biofilm formation is attributed to the inability of cells to detach from the mature biofilm, as *agr* plays a role in the detachment process (Kong *et al.*, 2006; Vuong *et al.*, 2004a). Other global regulators also affect biofilm formation in staphylococci, most notably the staphylococcal accessory regulator (*sarA*; Tormo *et al.*, 2005b; Valle *et al.*, 2003) and the alternative sigma factor, σ^B (Knobloch *et al.*, 2004). *sarA* mutants showed down-regulation of the *ica* operon transcription and subsequent decrease in PIA production (Valle *et al.*, 2003). Valle *et al.* (2003) suggesting that *sarA* enhances *ica* operon transcription and suppresses transcription of a protein involved in the turnover

of PIA. *S. epidermidis* mutants with mutations in σ^B show an upregulation of *icaR* (the repressor of the *ica* locus), resulting in reduced PIA synthesis (Knobloch *et al.*, 2004).

4.1.2.7 Antimicrobial resistance in staphylococcal biofilms

Formation of biofilms leads to a reduction in antimicrobial susceptibility in staphylococci (Amorena *et al.*, 1999). Even antimicrobial-sensitive bacteria that do not have a known genetic basis for resistance demonstrate reduced susceptibility when they form a biofilm (Stewart & Costerton, 2001), a trait that which appears to be multi-factorial. One mechanism is the failure of an agent to penetrate the full depth of the biofilm. Components of the biofilm (such as the exopolysaccharide matrix) can limit the transport of antimicrobial agents to the cells within the biofilm (Mah & O'Toole, 2001). Farber *et al.,* (1990) for example has demonstrated that addition of *S. epidermidis* PIA to microdilution susceptibility plates increased the MIC of glycopeptide antimicrobials, as these large molecules are poorly absorbed. The presence of an exopolysaccharide matrix can create a permeability barrier, meaning the antimicrobial cannot penetrate the biofilm (Stewart, 1996).

Secondly, the growth rate of cells within a biofilm is substantially reduced in comparison with planktonic cells as cells growing in biofilms are commonly nutrient-depleted (Mah & O'Toole, 2001). Reduced growth rates lead to reduced susceptibility to antimicrobials designed to target fast growing bacteria (Tuomanen *et al.*, 1986). Eng *et al.,* (1991) demonstrated that bacteria exposed to nutrient limitation showed reduced antimicrobial susceptibility to a range of antimicrobial classes. The reduced metabolic activity of cells embedded in the biofilms mimic this nutrient depleted state correlating with reduced antimicrobial susceptibility in biofilms (Dunne, 2002) Finally, it has been hypothesised that cells present in a biofilm may induce a specific ‘biofilm phenotype’. This ‘biofilm phenotype’ has been likened to a spore-like state entered into by some of the bacteria resulting in reduced susceptibility to antibiotics and disinfectants (Stewart & Costerton, 2001). Reduced antimicrobial susceptibility to ß-lactams, quinolones and glycopeptides has been observed in biofilms formed by *S. aureus* (Chuard *et al.*, 1997). Specifically a recent study by Singh *et al.* (2010a) has highlighted the poor penetration of certain antimicrobial agents through staphylococcal biofilms. ß-lactam antibiotics (oxacillin and cefotaxime) and a

Table 4.1 Effect of environmental stimuli on biofilm formation in staphylococci

Factor	Effect	Reference
Anaerobic conditions	Stimulate *ica* transcription, increasing PIA production	(Cramton *et al.*, 2001b)
Ethanol	Reduced transcription of the *icaR* repressor	(Conlon *et al.*, 2002)
Supplementation with carbohydrates	Increased adherence to surfaces	(Mack *et al.*, 1992)
Iron limitation	Increased PIA production	(Deighton & Borland, 1993)
Sodium chloride	Increased biofilm formation, *ica* independent mechanism	(Lim *et al.*, 2004)
Subinhibitory antibiotic concentrations	Increased *ica* expression and PIA production	(Rachid *et al.*, 2000)
High temperature	Increased *ica* expression and PIA production	(Rachid *et al.*, 2000)

glycopeptide (vancomycin) show a significantly reduced penetration through *S. aureus* and *S. epidermidis* biofilms. Biofilms produced by *S. aureus* may also protect against clearance by host immune systems. Presence of PIA and biofilm formation has been shown to protect cells from phagocytosis and killing by polymorphonuclear leukocytes (Vuong *et al.*, 2004c). Other studies have shown that although leukocytes can penetrate the biofilm matrix they are unable to penetrate bacterial cells and promote phagocytosis due to unfavourable conditions (Leid *et al.*, 2002).

4.1.3 Biofilm formation in small colony variants

As *S. aureus* SCVs are frequently recovered from patients with infections typically associated with biofilm formation (endocarditis, soft tissue infections and osteomyelitis) there is a suggestion that SCVs and biofilms may have a similar underlying physiology (Higashi & Sullam, 2006). Several studies have shown that SCVs from different bacterial species are capable of forming biofilms (Al Laham *et al.*, 2007; Haussler *et al.*, 2003b; Sendi *et al.*, 2006; Singh *et al.*, 2010b). For example *P. aeruginosa* SCVs isolated from cystic fibrosis (CF) patients have been shown to display a marked increase in biofilm forming capacity (Haussler *et al.*, 2003b). SCVs also displayed increased binding to an eukaryotic cell line and increased pilli mediated twitching motility which may play a role in adaptation the CF environment. In staphylococci SCVs biofilm formation has been attributed to various mechanisms.

Vaudaux *et al.* (2002) demonstrated that *S. aureus* SCVs increase surface display of the MSCRAMMs, in particular, fibronectin binding proteins FnBPs. Increased transcript levels of clumping factor A (*clfA*) and fibronectin protein (*fbn*) were responsible for the increased production of these adhesins, and increased adhesion to fibronectin coated surfaces. Mitchell *et al.,* (2008) also demonstrated increased expression of FnBPs in SCVs and an increased capacity to bind fibronectin. Furthermore the authors showed the importance of the alternative sigma factor σ^B, on the expression of FnBPs, suggesting that sustained σ^B activity in *S. aureus* SCVs locks SCVs into a constant state of colonisation.

Increased capacity to form biofilms has also been attributed to PIA dependent mechanisms in *S. epidermidis* SCVs (Al Laham *et al.*, 2007). The study demonstrated that increased production of PIA (and augmented expression of the *ica* operon) is

responsible for increased adhesion to surfaces and increased biofilm forming capacity in SCVs. Reduced antimicrobial susceptibility has also been investigated in SCVs. *S. aureus* SCVs exhibit more pronounced reductions in antimicrobial susceptibility than parent strains when adhering to a fibronectin surface (Chuard *et al.*, 1997). SCVs were highly resistant to the bactericidal action of vancomycin and oxacillin whereas the parent strain showed a reduction in viable cell numbers.

4.1.4 Aims

Several studies have examined the ability of SCVs to form biofilms however a limited number of studies are available regarding biofilm formation in *S. aureus* SCVs as well as the susceptibility of SCV biofilms to antimicrobial agents. The aims of this study were to:

- Assess the ability of *S. aureus* parent and SCV isolates to produce polysaccharide using a simple agar screen.
- Quantify biofilm formation to provide a comparison of biofilm formation in parent and SCV isolates.
- Examine antimicrobial susceptibility of parent and SCV biofilms to a range of antimicrobial agents.
- Examine the mechanism for any difference in antibiotic susceptibility between parent and SCV biofilms.

4.2 Materials and methods

4.2.1 Bacterial strains

Methicillin-sensitive *S. aureus* (MSSA) strains ATCC 25923 and NCTC 6571 and methicillin-resistant *S. aureus* (MRSA) strains COL, EMRSA 15, EMRSA 16 and N315 and their SCVs were all examined for their ability to form biofilms. Additionally *S. aureus* strains Sa 6538 (biofilm positive) and Sa 5374 (biofilm negative; Tote *et al.,* 2008) were used as positive and negative controls respectively. SCVs previously selected for in the presence of gentamicin (SCV^{GEN}) and tetracycline (SCV^{TET}) were subject to multiplex PCR (as described previously) to confirm SCVs were *S. aureus*. Strains were maintained at -80°C in Mueller Hinton (MH) broth supplemented with 8% dimethyl sulfoxide (DMSO) and re-isolated on MH agar plates when required.

4.2.2 Congo red agar screen

Before biofilm quantification was assessed a simple morphological screen was performed using Congo red agar to detect polysaccharide production in *S. aureus* (Freeman *et al.*, 1989). Congo red agar was prepared by adding 50 g sucrose and 10 g of purified agar to 1 L of brain heart infusion broth (Oxoid, UK). After autoclaving the agar was cooled to 55°C and a filter sterilised solution of Congo red (Sigma, UK; 0.8 g in 10 mL) added. Several individual *S. aureus* colonies were used to inoculate Congo red agar plates which were incubated at 37°C. After 48 hours incubation plates were examined for the presence of black crystalline colonies indicative of polysaccharide production. Absence of polysaccharide production was recorded following the appearance of pink colonies with occasional darkening at the centre of the colony.

4.2.3 Quantification of biofilms

Biofilms formed by *S. aureus* strains were quantified using the method of Tote *et al.*, (2008). This assays uses the cationic dye dimethyl methylene blue (DMMB) to quantify biofilms as it binds specifically to *S. aureus* PIA.

4.2.3.1 Preparation of dimethyl methylene blue working solution

DMMB (32mg) powder (Sigma, UK) was dissolved in 25 mL of ethanol and filter sterilised. Two formic acid buffer (FAB) were prepare; FAB1 was prepared by adding 4.77 g guanidine hydrochloride (Sigma, UK) and 0.5 g sodium formate (Sigma, UK)

to 237 mL of ultrapure water, followed by the addition of 0.5 mL formic acid (Sigma, UK) and 12.5 mL ethanol to create a final working volume of 250 mL. FAB 2 was prepared by adding 4.77 g guanidine hydrochloride and 0.5 g sodium formate to 49.5 mL of ultrapure water followed by the addition of 0.5 mL formic acid. The final DMMB working solution was prepared by adding FAB 1 and FAB 2 to 12.5 mL of the filtered DMMB solution which was stored at room temperature and protected from light.

4.2.3.2 Preparation of decomplexation solution

Decomplexation solution (DECO) was prepared by adding 50 mL of 1- propanol (Sigma, UK) to 500 mL of 50 mM sodium acetate buffer. This was used to dissolve 380 g of guanidine hydrochloride to achieve a final concentration of 4 M.

4.2.3.3 Biofilm growth

Several individual *S. aureus* colonies were inoculated into MH broth supplemented with 0.5% glucose and incubated at 37°C with shaking at 150 rpm, until reaching a start inoculum of 1×10^6 CFU/mL. Bacterial suspensions (100 µL) were added to individual wells of 96 well flat bottom microtitre plates (Fisher, UK) with replicates for each strain. Plates were incubated on a horizontal shaking platform for 72 hours at 37°C. Growth medium was discarded every 24 hours and fresh medium added to avoid the build up of toxic metabolites.

4.2.3.4 Quantification of biofilms

Following 72 hours incubation, growth medium was discarded and adhering biofilms were washed twice with phosphate buffered saline (PBS). DMMB working solution (200 µL) was added to each individual well and incubated at room temperature for 30 minutes whilst protected from light. DMMB was then discarded by centrifuging well plates for 20 minutes at 6000 rpm. Wells were then washed with 200 µL of ultrapure water to remove any unbound DMMB, before adding 250 µL of DECO solution. Plates were incubated at room temperature for 30 minutes and optical density was measured at OD_{630} using a DYNEX Technologies MRX® Microplate Absorbance Reader with Revelation™ application programme.

4.2.4 Adhesion to silicone

Adhesion of *S. aureus* to silicone was assessed using the method of Williams *et al.*, (1997). Silicone catheter discs were prepared by cutting silicone sheets to equal dimensions (0.5 cm^2). Discs were sterilised by overnight submersion in 90% ethanol, followed by 2 hour incubation at room temperature to allow the evaporation of excess ethanol. Overnight *S. aureus* cultures grown in MH broth were adjusted to 1 X 10^5 CFU/mL and catheter discs were placed in the suspension. Discs were incubated statically at 37°C for 7 days hours to allow attachment of the bacteria. The discs were aseptically transferred (ensuring adhering cells were not disrupted) to fresh MH broth every 48 hours to prevent build-up of toxic metabolites. After 7 days incubation discs were washed gently in PBS and transferred to fresh MH broth. In order to enumerate bacteria, discs were sonicated for 5 minutes and vortexed for a further 1 minute to remove adherent bacteria. Serial dilutions were performed in PBS and viable counts performed on the resulting suspensions, using drop counts.

4.2.5 Cell-surface hydrophobicity

Cell-surface hydrophobicity has been implicating in enhancing the ability of the SCVs of *P. aeruginosa* to form biofilms and to influence cell clumping. This was investigated in *S. aureus* using a microbial adhesion to hydrocarbons assay (Perez *et al.*, 1998). Overnight cultures of *S. aureus* strains grown in MH broth, were pelleted by centrifugation at 10,000 rpm for 5 min and subsequently washed twice with PBS. Cells were adjusted to an optical density of 0.5 at OD_{600} and 2 mL mixed with 400 µl xylene and vortexed for 2 min. After 30 min incubation at room temperature, the aqueous phase was collected carefully and its OD_{600} was determined. The OD_{600} of the aqueous phase relative to the initial suspension was taken as a measure of cell-surface hydrophobicity (H%), which was calculated with the formula:

$$\mathbf{H\% = [(OD_0 - OD)/OD_0] \times 100}$$

Where OD_0 and OD are the optical density before and after extraction with xylene.

4.2.6 Scanning electron microscopy

Biofilms were prepared on Thermanox glass coverslips (Fisher Scientific, UK). Coverslips were aseptically transferred to *S. aureus* strains growing in MH broth. Coverslips were incubated statically at 37°C for 7 days, with growth medium being

changed every 48 hours. Biofilms formed on coverslips were fixed by the addition of 1.5% v/v glutaraldehyde (TAAB, UK), which was incubated for 12 hours at 37°C. Following fixation biofilms were washed twice in double distilled water (5 minutes each wash) and subsequently dehydrated with graded concentrations of ethanol (50%, 70%, 90% and 100%) with each dehydration step lasting 5 minutes. Samples were then treated with three 5 mins applications of hexamethyldisilazane and sputter coated with gold. Samples were then viewed with JEOL 840A scanning electron microscope (JEOL Ltd, UK) with images recorded on SIS Imaging Software.

4.2.7 Activity of antimicrobial agents against *S. aureus* biofilms

S. aureus strains ATCC 25923 and EMRSA 15 and corresponding SCVs were chosen to investigate the effect of antimicrobial agents on biofilms. A modification of the DMMB assay was applied to determine the effect if antimicrobials on biofilms (Tote *et al.*, 2009). Biofilms were grown as described previously (section 4.2.3.3) and culture medium carefully removed with a pipette, ensuring biofilms were not disrupted. Ciprofloxacin, chloramphenicol, gentamicin, tetracycline, rifampicin (Sigma, UK) and triclosan (Ciba, Germany) were prepared at the following concentrations – X 16 MBC; X 8 MBC; X 4 MBC; X 2 MBC; MBC, and MIC. 50 µL of the antimicrobial and 50 µL of MH broth were added to individual wells with established biofilms. Due to the dilution with MH broth double the required antimicrobial concentrations were prepared initially. Ultrapure water was used to replace antimicrobials for the formation of untreated control biofilms. Plates were subsequently incubated on a horizontal shaking platform for 48 hours at 37°C and biofilms quantified as described previously (section 4.2.3.4). Differences in absorbance were used to quantify the % reduction in antimicrobial treated biofilms in comparison with untreated controls.

4.2.8 Preparation of colony biofilms and susceptibility to antimicrobial agents

The method of Anderl *et al.,* (2000) was used to form colony biofilms which were assessed for susceptibility to various antimicrobial agents. *S. aureus* strains were grown in MH broth and adjusted to ~ 1 x 10^7 CFU/mL and 10 µl of this suspension was used to seed black polycarbonate membrane filters (Fisher, UK; 13 mm diameter, pore size 0.4 µm). Filters were inverted and placed onto MH agar and incubated at 37 °C for 4 days. Membrane-supported biofilms were transferred to fresh culture

medium every 48 hours. Following incubation, biofilms were washed with PBS to remove non-adherent cells. Adherent bacteria were enumerated by suspending membranes in 1 mL PBS and vortexing at high speed for 2 min. PBS was used to perform serial dilutions on the resulting suspension and dilutions were plated onto MH agar and incubated for 48 hours at 37°C. To examine the effect of the antimicrobial agents on biofilms formed. Colony biofilms were prepared as stated previously and following incubation transferred to MH agar containing ciprofloxacin, chloramphenicol, gentamicin, tetracycline, triclosan and rifampicin at concentrations ranging from 0 – 256 mg/L. Plates were incubated at 37 °C for 48 hours and antibiotic-treated biofilms were enumerated as stated above for control biofilms.

4.2.9 Antimicrobial penetration through biofilms

The method of Singh *et al.*, (2010a) was used to measure the penetration of the six antimicrobial agents examined previously through *S. aureus* ATCC 25923 and EMRSA 15 and corresponding SCVs biofilms. This method uses zones of inhibition to measure the penetration of antimicrobial agents through biofilms (Figure 4.3). Colony biofilms were prepared as described previously (section 4.2.8) and transferred to MH agar plates inoculated with *S. aureus* ATCC 25923 in order to provide confluent lawn growth. A 6 mm diameter nitrocellulose membrane (Fisher, UK; pore size 0.4 µm) was placed on the surface of each biofilm along with an antibiotic disc (ciprofloxacin 5 µg, chloramphenicol 30 µg, gentamicin 10 µg, rifampicin 5 µg, tetracycline 30 µg, (Oxoid, UK) and triclosan 10 µg). Each disc was moistened with 24 µL of sterile polished water to prevent antibiotic movement through biofilms via capillary action. Control conditions were prepared replicating previous conditions with sterile membrane filters being excluded so no biofilms were present. Plates were incubated at 37 °C for 48 hours and the zone of inhibition measured digitally using IMAGE J (NIH). Inhibition zones produced by control conditions were taken to represent 100% penetration through *S. aureus* biofilms.

4.2.10 Statistical analysis

Analysis of significant differences between biofilm formation and antimicrobial susceptibility of SCV and parent biofilms was performed using the statistical analysis described previously (section 2.2.9.8).

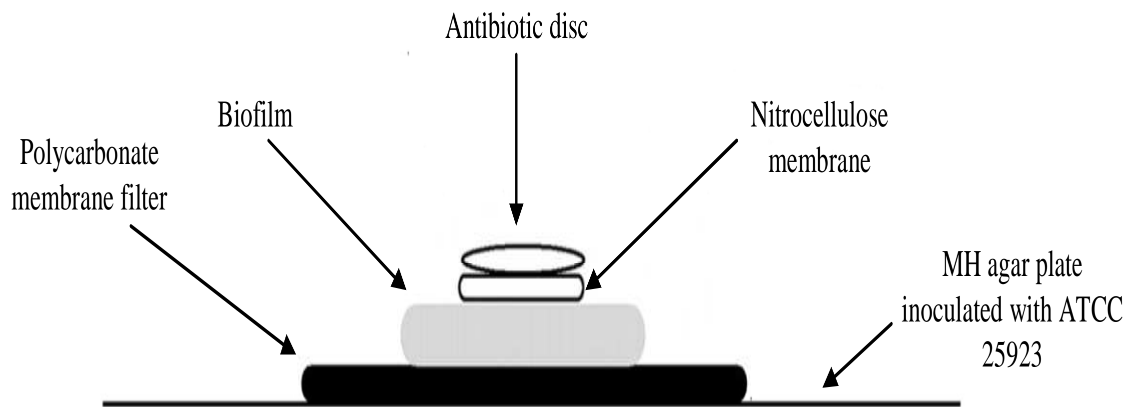


Figure 4.3 Schematic displaying experimental setup to examine antibiotic penetration through *S. aureus* biofilms Adapted from Singh et al., (2010a).

4.3 Results

4.3.1 Congo red agar screen

Eight *S. aureus* strains and their SCV derivatives (selected for in the presence of gentamicin or tetracycline) were investigated for their ability to form biofilms using an agar screen. All parent strains grown on Congo red agar produced a black crystalline morphology, except the biofilm negative Sa 5374 strain which produced pink colonies. Black crystalline colonies indicated the ability to produce polysaccharide and were recorded as biofilm positive. All SCV strains (including SCVs isolated from Sa 5374) produced a black crystalline morphology. The positivity of Sa 5374 SCVs was surprising, although subsequent repeats confirmed Sa5374 SCVs produce polysaccharide. SCV colonies were distinctly smaller than parental colonies but produced the black crystalline morphology associated with polysaccharide production.

4.3.2 DMMB biofilm quantification and adhesion to silicone

A DMMB microtitre well plate assay was applied to the same panel of strains using optical density measurements to correspond to biofilm production. Biofilm formation was observed in all parent strains (except the negative control; Sa 5374) and all SCVs including Sa 5374 (Figure 4.4). Excluding the negative control no significant difference was observed in biofilm formation between MRSA and MSSA strains ($P = > 0.05$). Across all strains tested biofilm formation was significantly increased in SCVs compared to parents ($P < 0.01$; Figure 4.4). As the DMMB assay used directly quantifies PIA production these results confirms the presences of increased PIA in SCV biofilms. Similar results were also observed using silicon catheter disc biofilm quantification assay (Figure 4.4). Significantly greater numbers of SCV viable cells were recovered from silicone discs in comparison to parent strains ($P = < 0.01$). Consistently lower numbers of Sa 5374 parent viable cells (~ 1×10^2 CFU/mL) were recovered from silicone discs compared to corresponding SCVs (~10^6 CFU/mL).

4.3.3 Cell-surface hydrophobicity

In order to confirm that the difference in biofilm formation between SCV and parent strains was related to differences in PIA production rather than cell surface hydrophobicity, their ability to adhere to hydrocarbons was observed. No significant difference in cell-surface hydrophobicity values were observed for SCV and parent

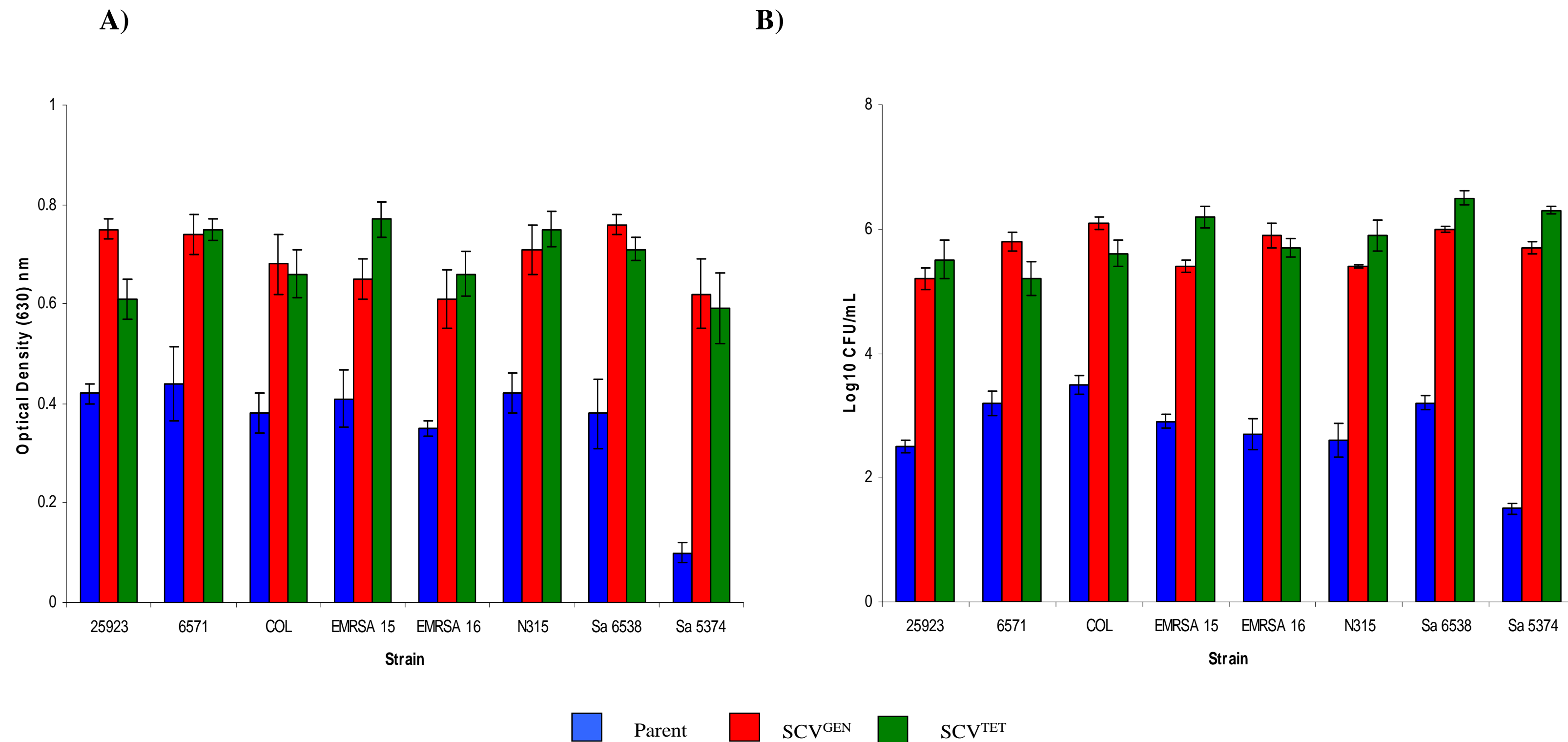


Figure 4.4 Biofilm formation in *S. aureus* SCV and parent strains A) - Biofilm formation assessed by DMMB assay; B) – Bacterial counts recovered from silicone catheter discs assessed by viable count. SCVs formed greater biofilms and consistently higher numbers of SCVs were recovered from silicone catheter discs in comparison to parent strains. Results are means values of three independent replicates and three independent biological replicates. Error bars represent standard error.

strains (P = > 0.05); mean H% = 65.2 (±3.2) in SCV strains and 67.7 (±4.8) in parent strains.

4.3.4 Scanning electron micrograph analysis of biofilms

Dense multilayered biofilms were observed in SCVs adhered to Thermanox coverslips (Figure 4.5 C). Although clustering and aggregation of cells was observed in parent biofilms, an absence of multilayer clusters of cells is apparent (Figure 4.5 A). SEM images at increased magnification (Figure 4.5 B and D) demonstrate the presence of extracellular polysaccharide in SCV biofilms which appears absent in biofilms formed by parent strains. The SCV cellular cluster (Figure 4.5 D) is clearly covered in a 'slimy' extracellular polysaccharide substance whereas parent biofilms lack this substance (Figure 4.5 B).

4.3.5 Susceptibility of biofilms to antimicrobial agents

The effect of antimicrobial treatment on established biofilms was evaluated using the DMMB method. Six different antimicrobial agents were applied at various concentrations to *S. aureus* parent ATCC 25923 and EMRSA 15 biofilms, and biofilms formed by their corresponding SCVs. Both parent and SCV biofilms showed reduced susceptibility to concentrations of antimicrobial agents that normally inhibited planktonic cells (Tables 4.2 and 4.3). Application of MIC and MBC concentrations to both parent and SCV biofilms resulted in no reduction of established biofilms in both strains. Furthermore treatment with X 2 MBC concentrations of all six antimicrobials tested resulted in no reduction of EMRSA 15 parent and SCV biofilms.

Comparison of the effects of antimicrobial agents on SCV and parent biofilms SCV biofilms revealed SCV biofilms were significantly less susceptible to all six antimicrobial compounds tested (P = < 0.01). Application of X 2 MBC of ciprofloxacin, triclosan and rifampicin to ATCC 25923 parent biofilms resulted in reduction in biofilms in comparison to control; however the same concentrations produced no reduction in ATCC 25923 SCV biofilms (Table 4.2). Similar results were observed in EMRSA 15 biofilms. Application of X 4 MBC ciprofloxacin, tetracycline and rifampicin resulted in a reduction of EMRSA 15 parent biofilms, but the same concentrations produced no effect on SCV biofilms (Table 4.3).

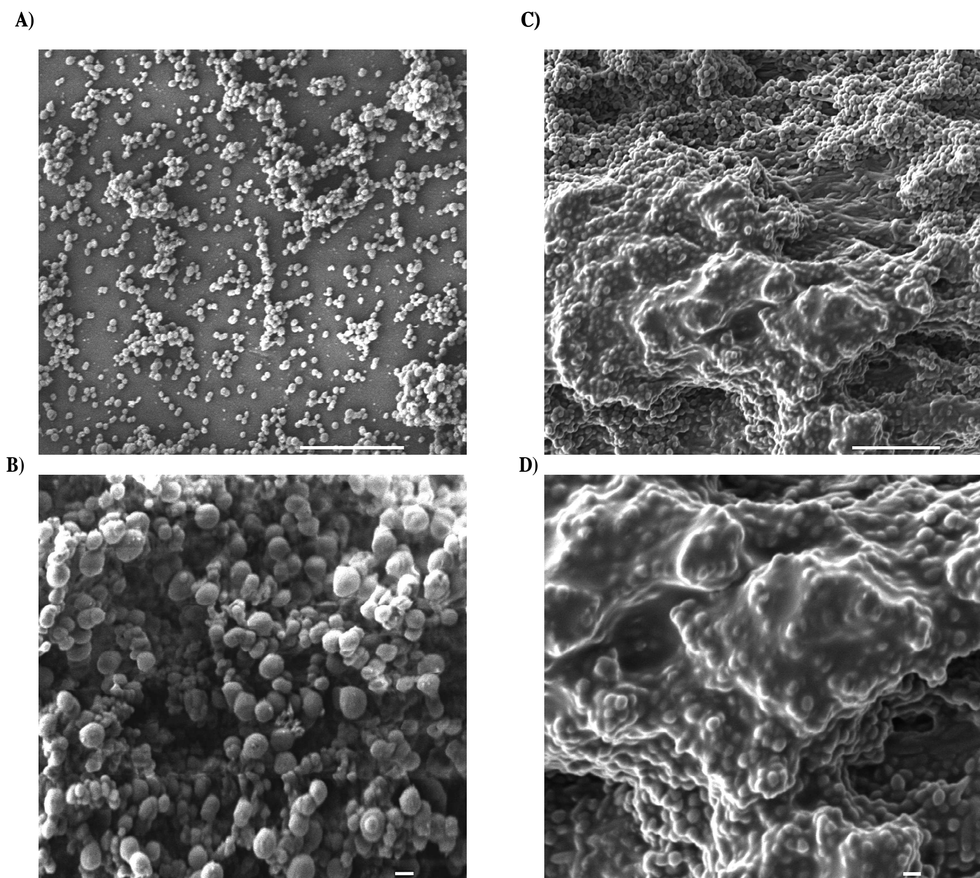


Figure 4.5 Scanning electron micrographs of *S. aureus* SCV and parent biofilms formed on Thermanox coverslips Images A and B show wildtype biofilms, and SCV biofilms are displayed in images C and D. Images A and C x 2000 magnification, scale bar represents 10 microns. Images B and D x 4000 magnification, scale bar represents 1 micron.

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Table 4.2 Effect of antimicrobial agents on biofilm formation in *S. aureus* ATCC 25923 SCVs and corresponding parent strain

	X 2 MBC			X 4 MBC			X 8 MBC			X 16 MBC		
	P	SCV^{GEN}	SCV^{TET}	P	SCV^{GEN}	SCV^{TET}	P	SCV^{GEN}	SCV^{TET}	P	SCV^{GEN}	SCV^{TET}
Ciprofloxacin	11 ± 1	0	0	11 ± 1	0	8 ± 1	27 ± 2	18 ± 2	24 ± 3	79 ± 5	47 ± 4	55 ± 3
Chloramphenicol	0	0	0	0	0	0	18 ± 3	0	0	29 ± 2	0	0
Gentamicin	0	0	0	4 ± 1	0	0	14 ± 3	0	0	32 ± 4	0	0
Tetracycline	0	0	0	17 ± 3	7 ± 1	11 ± 2	38 ± 5	0	0	68 ± 2	39 ± 4	41 ± 3
Triclosan	15 ± 2	0	0	24 ± 2	0	0	49 ± 4	6 ± 1	8 ± 1	79 ± 6	21 ± 2	19 ± 2
Rifampicin	17 ± 3	0	0	28 ± 3	14 ± 4	18 ± 2	41 ± 2	25 ± 4	32 ± 2	87 ± 4	69 ± 3	74 ± 2

MIC and MBC concentrations showed no activity against *S. aureus* SCV 25923 and parent biofilms. Data shown is % reduction from control conditions (untreated biofilms) and is the mean of eight replicates and two independent biological replicates.

Table 4.3 Effect of antimicrobial agents on biofilm formation in *S. aureus* EMRSA 15 SCVs and corresponding parent strain

	X 4 MBC			X 8 MBC			X 16 MBC		
	P	**SCV^{GEN}**	**SCV^{TET}**	**P**	**SCV^{GEN}**	**SCV^{TET}**	**P**	**SCV^{GEN}**	**SCV^{TET}**
Ciprofloxacin	9 ± 2	0	0	33 ± 4	15 ± 2	9 ± 1	67 ± 3	41 ± 2	37 ± 4
Chloramphenicol	0	0	0	19 ± 2	0	0	39 ± 4	0	0
Gentamicin	0	0	0	17 ± 1	0	0	31 ± 2	0	0
Tetracycline	11 ± 1	0	0	35 ± 2	0	0	59 ± 4	38 ± 3	27 ± 2
Triclosan	0	0	0	29 ± 2	14 ± 1	0	66 ± 2	31 ± 4	21 ± 1
Rifampicin	15 ± 2	0	0	41 ± 5	31 ± 3	24 ± 3	85 ± 3	67 ± 6	57 ± 2

MIC, MBC and X 2 MBC concentrations showed no activity against *S. aureus* EMRSA 15 SCV and parent biofilms. Data shown is % reduction from control conditions (untreated biofilms) and is the mean of eight replicates and two independent biological replicates.

Concentrations of X 16 MBC ciprofloxacin, tetracycline, triclosan and rifampicin and triclosan produced the greatest reduction (73 ± 3.625 %) in biofilm formed by parent strains. Application of the same antimicrobial concentrations to SCV biofilms resulted in a reduction, however the reduction (43 ± 3 %) was significantly lower in comparison to parent biofilms ($P = < 0.01$). The highest concentrations of chloramphenicol and gentamicin examined produced a 33 ± 3 % reduction in parent biofilms. The same concentrations however resulted in no reduction of SCV biofilms. No single antimicrobial agent completely eradicated parent and SCV biofilms at all concentrations examined. Rifampicin was the most active antimicrobial in reduction of both SCV (67 ± 3.25 %) and parent (86 ± 3.5 %) biofilms in both strains examined (Tables 4.2 and 4.3).

4.3.6 Effect of antimicrobial agents on colony biofilms

A colony biofilm assay was implemented to determine the total viable bacterial burden within a biofilm and to allow the detection of any SCV disseminating from parent biofilms. The six antimicrobial agents tested previously were tested against parent and SCV isolates. Initial observations showed that significantly higher numbers of SCVs (~ 1 x 10^7 CFU/mL) were recovered from colony biofilms formed by both strains in comparison to parent colony biofilms (~ 1 x 10^5 CFU/mL; $P = < 0.01$). Ciprofloxacin, rifampicin, tetracycline and triclosan all reduced viable cell counts in ATCC 25923 and EMRSA 15 parent biofilms, although no antimicrobial concentration tested completely eliminated viable cells (Figure 4.6 and Figure 4.7). Viable cell counts showed that the highest concentrations of the four antimicrobials tested achieved an average 3.8 $\log_{10}$ reduction in CFU/mL in comparison to controls.

Chloramphenicol and gentamicin were less active against parent biofilm with the highest concentrations examined achieving a mean 1.35 $\log_{10}$ reduction in viable cell counts. Similarly to parent strains, chloramphenicol and gentamicin showed the lowest reduction in SCV viable cell counts (1.175 $\log_{10}$ reduction). Ciprofloxacin and rifampicin showed the greatest reduction in viable counts of SCVs in both strains. The highest concentration of ciprofloxacin produced an average 2.75 $\log_{10}$ reduction, where as treatment with the highest rifampicin concentration resulted in average 4.15 $\log_{10}$ reduction in SCV viable cell counts (Figures 4.6 and 4.7). Although higher numbers of SCVs were recovered from

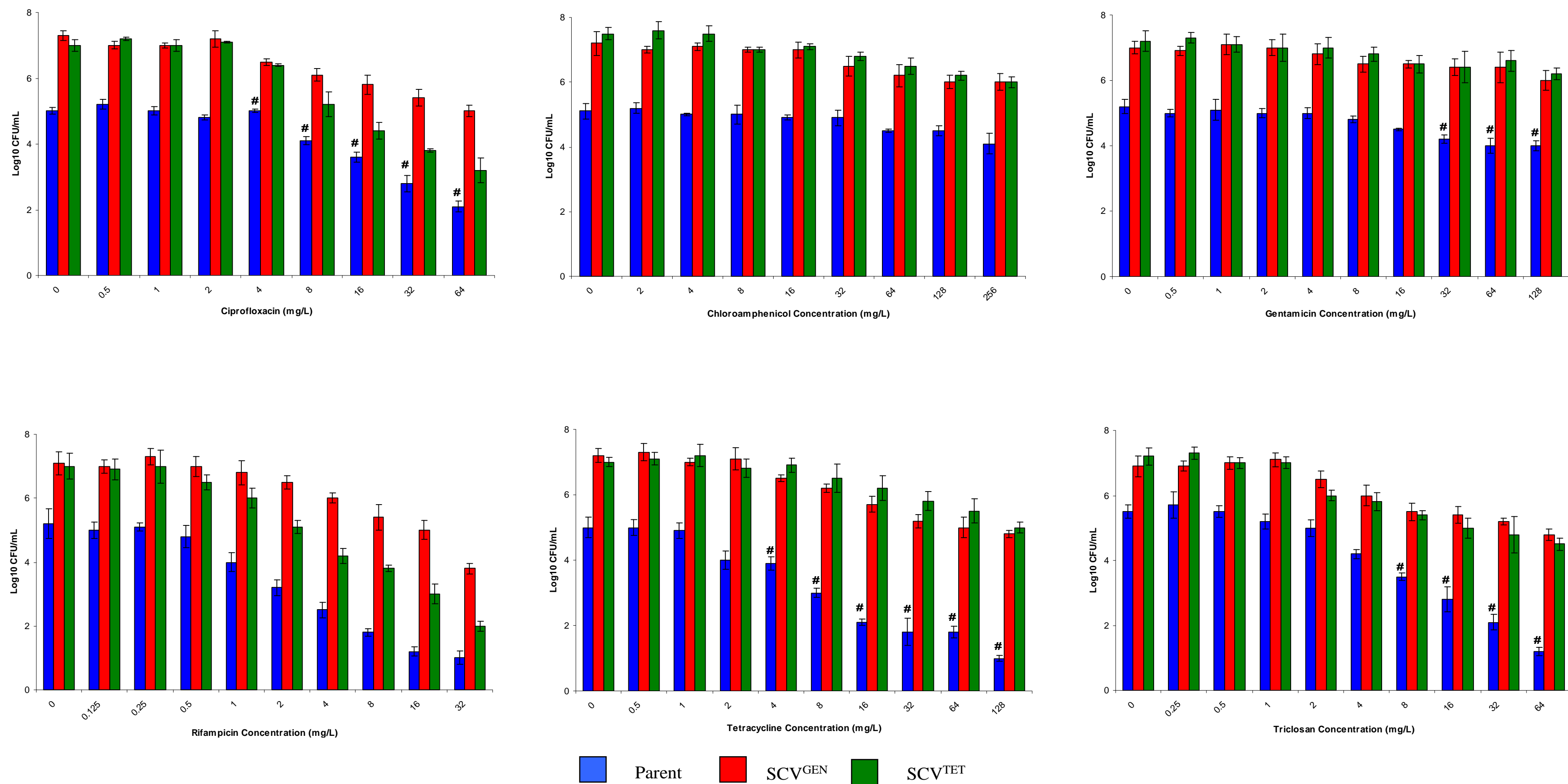


Figure 4.6 Effect of exposure to six antimicrobials on colony biofilms formed by *S. aureus* ATCC 25923 SCVs and corresponding parent strain Exposure to various concentrations of ciprofloxacin, gentamicin, tetracycline and triclosan yielded SCVs in 25923 colony biofilms represented by #. Results are means values of eight independent replicates and two independent biological replicates. Error bars represent standard error.

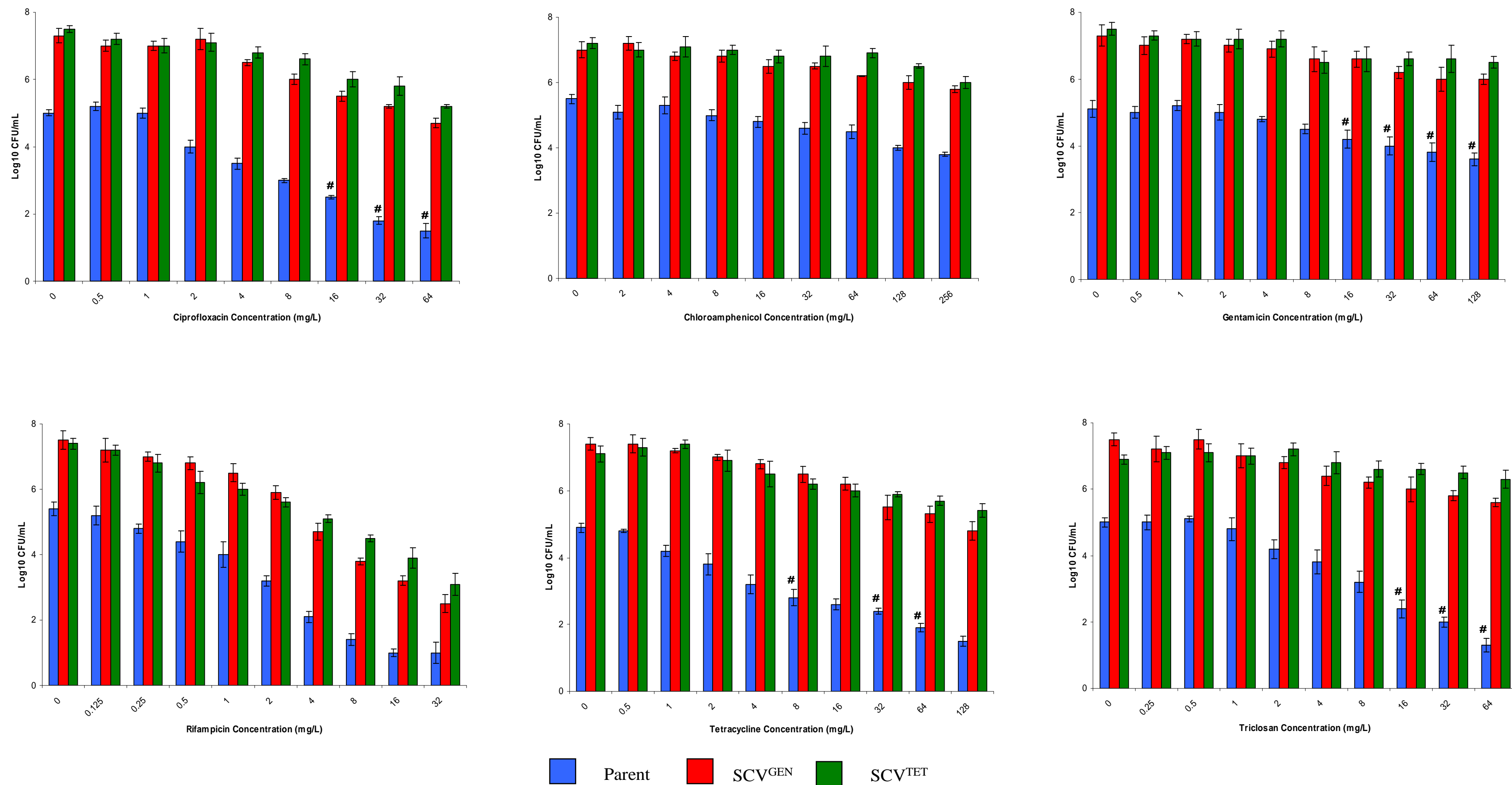


Figure 4.7 Effect of exposure to six antimicrobials on colony biofilms formed by *S. aureus* EMRSA 15 SCVs and corresponding parent strain Exposure to various concentrations of ciprofloxacin, gentamicin, tetracycline and triclosan yielded SCVs in EMRSA 15 colony biofilms represented by #. Results are means values of eight independent replicates and two independent biological replicates. Error bars represent standard error.

Table 4.4 Penetration of antimicrobial agents through *S. aureus* ATCC 25923 and EMRSA 15 SCV and parent biofilms

Antimicrobial	Concentration (µg)	Control inhibition zone (mm)	25923 parent biofilm zone diameter	25923 SCV^{GEN} biofilm zone diameter	253923 SCV^{TET} biofilm zone diameter
Ciprofloxacin	5	27 ± 1.05	25 ± 0.45	24 ± 0.64	24 ± 1.46
Chloramphenicol	30	24 ± 0.55	<13	<13	<13
Gentamicin	10	22 ± 0.71	<13	<13	<13
Rifampicin	5	32 ± 1.22	29 ± 1.02	28 ± 1.27	27 ± 1.15
Tetracycline	30	28 ± 0.64	25 ± 0.74	12 ± 0.76	15 ± 0.52
Triclosan	10	32 ± 1.88	28 ± 1.09	19 ± 1.51	22 ± 1.01

Antimicrobial	Concentration (µg)	Control inhibition zone (mm)	EMRSA 15 Parent	EMRA 15 SCV^{GEN}	EMRSA 15 SCV^{TET}
Ciprofloxacin	5	27 ± 1.05	24 ± 1.63	22 ± 1.38	23 ± 0.67
Chloramphenicol	30	24 ± 0.55	<13	<13	<13
Gentamicin	10	22 ± 0.71	<13	<13	<13
Rifampicin	5	32 ± 1.22	27 ± 1.25	28 ± 0.87	26 ± 0.74
Tetracycline	30	28 ± 0.64	25 ± 1.17	19 ± 0.87	17 ± 0.83
Triclosan	10	32 ± 1.88	26	21 ± 1.59	18 ± 1.12

Limit of detection was 13 mm due to the presence of the membrane filter that biofilms were grown on. Results are means values of three independent replicates.

biofilms, the reductions achieved by ciprofloxacin, rifampicin, tetracycline and triclosan were significantly lower in SCV biofilms in comparison to parents ($P = < 0.05$). SCVs were not detected in any of the control biofilms and were only detected in the presence of higher antimicrobial concentrations of ciprofloxacin, gentamicin, tetracycline and triclosan (Figures 4.6 and 4.7). For example in parent biofilms treated with gentamicin no SCVs were observed upon treatment with concentrations of 0-16 mg/L, but SCVs were subsequently detected at 32, 64 and 128 mg/L concentrations in ATCC 25923 biofilms. No SCVs were detected in biofilms treated with chloramphenicol and rifampicin.

4.3.7 Antimicrobial penetration of biofilms

As differences in the efficiency of antimicrobials at reducing biofilms and viable cell counts were observed the penetration of antibiotics through biofilms was examined using the method of Singh *et al.*, (2010a). The penetration of chloramphenicol and gentamicin through SCV and parent biofilms of both strains was significantly reduced in comparison to control conditions (Table 4.4; $P < 0.05$). No significant difference was observed in the penetration of ciprofloxacin, rifampicin, tetracycline and triclosan through parent biofilms formed by ATCC 25923 and EMRSA 15 ($P = > 0.05$). Ciprofloxacin and rifampicin also showed no significant reduction in the penetration of SCV biofilms formed by both strains ($P = > 0.05$; Table 4.4). In contrast to parents the penetration of tetracycline and triclosan through SCV biofilms was significantly reduced ($P = < 0.01$).

4.4 Discussion

S. aureus is well known for its ability to form biofilms on a range of materials including indwelling catheters (Marrie & Costerton, 1984b) and prosthetic devices (Litzler *et al.*, 2007; Rohde *et al.*, 2007). This study has identified the ability of several *S. aureus* parent and SCV strains to form biofilms using a simple agar screen. In addition quantification of biofilms was achieved using DMMB biofilm assay developed by Tote *et al.*, (2008). Although biofilm formation in staphylococci involves various steps and stages the accumulation of cellular aggregates and the ability to form biofilms is dependent on the production of exopolysaccharide (Heilmann *et al.*, 1996). In staphylococci this is dependent on the production of PIA which is regulated by the *ica*ADBC genes (making up the *ica* operon). This study demonstrates that SCVs produced significantly greater biofilms in comparison to parent strains. As the DMMB assay directly quantifies PIA and SCVs showed no difference in cell surface hydrophobicity these results suggest that PIA production in increased is SCVs and hence they exhibit an increased capacity to form biofilms.

Anaerobic conditions are known to increase the expression of PIA in *S. aureus*, which has been attributed to increased *ica* gene transcription (Cramton *et al.*, 2001b). As the SCV phenotype draws parallels with *S. aureus* when it is grown anaerobically (Balwit *et al.*, 1994), increased *ica* transcription may be responsible for the increased PIA production in SCVs observed in this study. Inhibition of the TCA cycle has been shown to impact on PIA production in *S. epidermidis* (Vuong *et al.*, 2005). Subsequently this has been suggested as the mechanism for increased PIA production in a *S. epidermidis* SCV constructed mutant (Al Laham *et al.*, 2007) and menadione auxotrophic *S aureus* SCV (Singh *et al.*, 2010a). As part of this study a lack of utilisation of carbohydrates was observed (Chapter 2). This suggests inhibition of the TCA cycle may also play a role in increased PIA production in the SCVs studied for biofilm formation.

SCVs display important differences in their expression profile in comparison to parent strains (Seggewiss *et al.*, 2006) and therefore it is feasible that variation in gene expression may relate to differences in biofilm production. Perhaps the most attractive candidate to explain the difference between biofilm formation in parent and SCV

strains is the staphylococcal accessory regulator (*sarA*). Studies have shown that *sarA* can influence the expression of the *ica* operon (Beenken *et al.*, 2004; Valle *et al.*, 2003). In *S. aureus sarA* mutants *ica* transcription is decreased, decreasing PIA production. *S. aureus* SCVs (recovered following aminoglycoside exposure) however, show increased expression levels of *sarA* (Mitchell *et al.,* 2010a). Alterations in the expression of *sarA* may be therefore responsible for increased *ica* transcript and subsequent increased PIA production.

Antimicrobial susceptibility of *S. aureus* biofilms is notably reduced in comparison with planktonic cells (Amorena *et al.*, 1999). This has been attributed to several factors including the presence of exopolysaccharide matrix and a reduced growth rate (Mah & O'Toole, 2001). Coupled with resistance mechanisms such as biofilm exopolysaccharide and slow growth rate of cells growing within biofilms, *S. aureus* biofilms provide a unique mechanism for colonisation and reduced antimicrobial susceptibility. In this study, SCV and parent biofilms displayed reduced susceptibility to ciprofloxacin, chloramphenicol, gentamicin, tetracycline, triclosan and rifampicin at concentrations normally bactericidal to planktonic cells. Antimicrobial susceptibility testing showed antimicrobial agents to have significantly smaller inhibitory effect on SCV biofilms in comparison to biofilms formed by parent strains. These results agree with previous studies that have shown SCV biofilms to exhibit reduced susceptibility to antimicrobials in comparison with parents (Chuard *et al.*, 1997; Williams *et al.*, 1997).

Antibiotic penetration was shown to be an important factor in the reduction of biofilm mass and cell quantity by antimicrobial agents. Chloramphenicol and gentamicin showed a significant reduction in penetration of parent and SCV biofilms correlating with the lowest overall reduction in biofilm mass and viable cell count. A difference in the penetration of antimicrobial agents was observed between parent and SCV strains; no significant reduction in penetration of tetracycline and triclosan penetration was observed in parent biofilms however the opposite was apparent in SCV biofilms. The differences in responses between SCV and parents may be explained by the difference in PIA production observed, which is known to reduce the activity of various antimicrobials (Souli & Giamarellou, 1998). Increased PIA production in SCVs may limit the diffusion and/or inactivate the antibiotics examined, accounting

for the differences observed between SCV and parent strains. Furthermore SEMs in this study show a difference in biofilm structure formed by SCV and parent strains. The multilayered complexity of SCV biofilms may contribute to reduced antibiotic penetration and the observed reduction in antimicrobial susceptibility observed. Rifampicin proved to be the best agent in reducing biofilm mass and also reducing viable counts in both SCV and parent biofilms. Previous studies have shown that rifampicin can penetrate biofilms formed by *S. epidermidis* (Zheng & Stewart, 2002) and that PIA does not inhibit rifampicin activity (Souli & Giamarellou, 1998). Biofilms formed by both parent and SCV strains showed no significant effect on the penetration of rifampicin which may account for the activity of rifampicin against parent and SCV biofilms observed in this study.

Although differences in the susceptibility of SCV and parent biofilms to antimicrobial agents was observed, no single antimicrobial agent eradicated biofilms or completely eliminated viable cells. The remaining cells encountered may represent the formation of biofilm-specific, drug-resistant or drug-tolerant physiologies, including the presence of persister cells (Stewart & Costerton, 2001). Persisters represent a subpopulation of bacteria that exhibit the ability to survive at lethal concentrations of antimicrobials without any clear resistance mechanism (Lewis, 2005). A recent study has shown large numbers of persisters to be present in *S. aureus* biofilms that confer resistance to various antimicrobials (Singh *et al.*, 2009). Persisters may explain the ability of parent biofilms to withstand elevated concentrations of antimicrobials tested in this study.

Although this study did not actively assay for persisters, SCVs were isolated from parent biofilms. The presence of SCVs in biofilms has been observed in *P. aeruginosa* (Haussler *et al.*, 2003b) and *S. pneumonia* (Allegrucci & Sauer, 2007) which as well as persisters may contribute to the reduced antimicrobial susceptibility of biofilms. Singh *et al.,* (2009) have suggested that SCVs contribute significantly to reduced susceptibility in *S. aureus* biofilms. Although we did not observe the presence of SCVs in biofilms that were not treated with antimicrobial agents their presence would clearly result in further reduced antimicrobial susceptibility. *S. aureus* SCVs display reduced susceptibility to cell wall specific antibiotics and aminoglycosides as a direct result of interruption of the electron transport chain and slow growth characteristics

(McNamara & Proctor, 2000). As SCVs already exhibit reduced susceptibility in their planktonic state this would suggest that in a biofilm, this reduced susceptibility would be further amplified. A biofilm formed completely of SCVs would exhibit further reduced susceptibility in comparison to a biofilm composed solely of parent/wildtype cells. This may also contribute to the reduced susceptibility of SCV biofilms to antimicrobial agents observed in this study.

The enhanced biofilm forming capacity of SCVs may correlate to their ability to cause persistent and recurrent infections. The ability of SCVs to form biofilms on materials such as silicone (frequently used in catheter; Jones *et al.,* 2006) may provide an increased opportunity to cause disease and persist in the hospital setting. SCVs have been isolated from CF pulmonary infections, osteomyelitis, and prosthetic device related infections (Proctor *et al.*, 2006) all of which have been linked to the presence of biofilms (Costerton *et al.*, 1999). This suggests that SCVs may play a significant role in biofilm related infections. Currently no antimicrobial drug has been found that completely eradicates adherent microbial populations, meaning biofilm infections are rarely resolved and usually persist until the removal of the effected medical device (Cos *et al.*, 2010). Novel approaches to combat biofilm associated infections including antibiotic lock therapy, inhibition of quorum sensing, and degradation of biofilms by genetically engineered phage are prospective treatment options (Agarwal *et al.*, 2010). The enhanced resistance of SCVs may in turn contribute to the adverse therapeutic outcome in these infections (Singh *et al.,* 2009).

4.5 Conclusions

- The Congo red agar screen is a useful method to screen polysaccharide production in *S. aureus* SCVs.
- *S. aureus* SCVs have an increased capacity to form biofilms in comparison to parent strains
- No difference in cell surface hydrophobicity between SCV and parent strains suggest increased PIA production is the mechanism for increased biofilm formation in SCVs.
- *S. aureus* biofilms are less susceptible to ciprofloxacin, chloramphenicol, gentamicin, tetracycline, triclosan and rifampicin than planktonic forms.
- SCVs are present in *S. aureus* biofilms following treatment with antimicrobial agents.
- SCV biofilms display further reductions in antimicrobial susceptibility which can be attributed to reduced antibiotic penetration through biofilms.

5 Chapter 5: Susceptibility, Resistance Induction and Synergistic Effects of Various Plant Antimicrobials Against *Staphylococcus aureus* Small Colony Variants

5.1 Introduction

5.1.1 Issues surrounding antibiotic discovery

Following the development of the sulphonamides in the 1930s and penicillin in the 1940s many new classes of antibiotics have been developed, however in the past 30 years only two new classes of antibiotics (the oxazolidinones and lipopeptides) have been developed (Norrby *et al.*, 2005; Silver, 2011). Various challenges to antibacterial drug discovery have kept the output of new classes of antibacterial agents extremely low. These difficulties have further exacerbated the current crisis of increasing antibiotic resistance in clinically-relevant bacteria. One of the key problems regarding the discovery of novel antibacterial agents is that the majority of targets which allow selective toxicity have already been exploited (Moellering, 2011). Furthermore, the difficulty and the time taken to develop novel agents mean a huge financial investment by the pharmaceutical industry. Payne *et al.,* (2007) estimate that for each individual agent, from the initial target identification to the file to launch procedure takes an average of 14 years. In addition the expenses associated with the pharmaceutical research and development of each individual agent is \$400 - \$800 million (DiMasi *et al.*, 2003). This has resulted in several large global pharmaceutical companies (GlaxoSmithKline, Eli Lilly and Proctor and Gamble,) reducing their investment in or completely deserting antibiotic discovery (Overbye & Barrett, 2005).

Further issues have arisen regarding the regulations which govern antibiotic development, and the bodies that control the approval of new antibiotics agents have received criticism. The Food and Drugs Administration (FDA) in the US has been accused of 'shifting the goalposts' in antibiotic approval after changing the approval criteria for antibiotics (Lancet, 2006). In addition, the lack of clinical trial guidelines, difficulties in recruiting sufficient subjects for clinical trials and the ambiguity surrounding the acceptability of model based evidence provide further hindrances (Spellberg *et al.*, 2008). Consequently it is not surprising that the number of novel agents in the antibiotic pipeline are far and few between.

5.1.2 Plant products as antimicrobials

The rapid decline in the development of novel antimicrobial has resulted in alternative approaches to antimicrobial development being sought. Natural products offer potential for the development of new antibacterial drugs and a new avenue to overcome the productivity crisis facing those engaged in drug discovery and development (Newman *et al.*, 2003). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, many of which display antimicrobial properties *in vivo* (Cowan, 1999). Approximately 420,000 plant species are present on earth (Vuorela *et al.*, 2004), with only 5% of known plants being systematically investigated for the presence of bioactive compounds (Verpoorte *et al.*, 2000). Therefore plants may represent a huge reservoir of potential new compounds, which remains untapped.

5.1.3 Essential oils

Essential oils (EOs) are aromatic oily liquids obtained from a variety of different plant materials including bark, flowers, leaves and roots (Burt, 2004). The main constituents of EOs are mono- and sesquiterpenes (including carbohydrates, alcohols and ethers) which are responsible for the fragrant and biological properties of aromatic plants (Kalemba & Kunicka, 2003). They are frequently liquid, volatile, rarely coloured, and soluble in organic solvents (Bakkali *et al.*, 2008). EOs are used in the food industry as flavour additives and for the preservation of foodstuffs (Bouhdid *et al.*, 2010) and EOs and their components have been deemed safe for use in food and beverages by the FDA (USFDA, 2009). Previous years have seen a revival in the use of EOs in protecting livestock and food from disease, due to their spectrum of antibacterial activity (Dorman & Deans, 2000). It is well documented that EOs posses antibacterial, (Deans & Ritchie, 1987; Holley & Patel, 2005), antimycotic (Azzouz & Bullerman, 1982) and antiparasitic activity (Pandey *et al.*, 2000).

Although the exact antimicrobial effects of EOs have not been determined, it is accepted that the action is dependent on the lipophilic character of their hydrocarbon skeleton and the hydrophobic character of their functional groups (Kalemba & Kunicka, 2003). As EOs are composed of several different chemical compounds, their antibacterial activity is not attributed to one specific mechanism and there are several known targets in the bacterial cell. EOs are known to cause degradation of the

bacterial cell wall (Helander *et al.*, 1998), damage the cytoplasmic membrane (Sikkema *et al.*, 1994) and damage membrane proteins (Ultee *et al.*, 1999).

Gram positive and Gram negative bacteria display differences in susceptibility to EOs due to differences in there cellular composition. The outer membrane (OM) of Gram negative bacteria contains lipopolysaccharide (LPS) molecules, providing the bacteria with a hydrophilic surface (Nikaido, 1994), which in turn serves as a penetration barrier to hydrophobic EOs. *Pseudomonas aeruginosa* for example displays intrinsic resistance to a variety of EOs due to the hydrophilic nature of it's OM.

5.1.3.1 Oregano – *Origanum vulgare*

Oregano EO is composed of two main antibacterial components; thymol and carvacrol, the precursors of which are the monoterpene hydrocarbon molecules, γ-terpinene and *p*-cymene (Nostro *et al.*, 2004). Thymol and carvacrol are phenolic compounds known to posses bacteriostatic or bactericidal activity depending on the concentration (Dorman & Deans, 2000). Thymol is structurally very similar to carvacrol, the major difference being a different location of the hydroxyl group on the phenolic ring (Burt, 2004). Carvacrol is the major component of oregano EO fraction (60 – 74%) (Ultee *et al.*, 1999) with thymol concentrations ranging from 0 - 33% (Faleiro *et al.*, 2005). The compositions can vary greatly depending upon the geographical region, variety and age of the plant, the method of drying and the method of extraction of the oil (Jerković *et al.*, 2001).

Several studies have shown a wide range of human pathogens to be susceptible to oregano oil, including *Candida albicans*, *Escherichia coli Staphylococcus aureus*, and *Salmonella typhimurium* (Dorman & Deans, 2000; Friedman *et al.*, 2002; Hammer *et al.*, 1999). Oregano EO, thymol and carvacrol have all been shown to disrupt the bacterial cell membrane, causing increased permeability to the nuclear stain ethidium bromide (Lambert *et al.*, 2001). Studies in *Bacillus cereus* have also shown carvacrol to interact with the cell membrane, dissolving the phospholipid bilayer, increasing membrane fluidity (Ultee *et al.*, 2000; Ultee *et al.*, 2002). Interaction with the lipid bilayer of the cytoplasmic membrane results in leakage of cellular material such as ions, ATP and nucleic acid (Helander *et al.*, 1998; Trombetta *et al.*, 2005).

5.1.3.2 Cinnamon – *Cinnamomum zeylanicum* (Synonym: *Cinnamomum verum*)

Many species of cinnamon yield volatile oils on distillation (Chericoni *et al.*, 2005). Variability in the composition of EOs from the same *Cinnamomum* species is common and is related to the geographical source of plant (Cheng *et al.*, 2004). *Cinnamomum zeylanicum* is one of the worlds most commonly exported species of cinnamon and its primary constituents are cinnamaldehyde from the bark oil, eugenol from the leaf oil, and camphor from the root-bark oil (Wijesekera & Chichester, 1978). Cinnamaldehyde, an aromatic aldehyde is the main component of bark extract (Ali *et al.*, 2005) and has been demonstrated to display strong antibacterial activity against a range of bacterial pathogens including *Helicobacter pylori* (Ali *et al.*, 2005), *P. aeruginosa* and *S. aureus* (Bouhdid *et al.*, 2010). Eugenol is also present in bark oil (but at lower concentrations) and has a similar range of activity to cinnamaldehyde, with activity against human pathogens such as *E. coli, Listeria monocytogenes* (Friedman *et al.,* 2002), *S. aureus* and *Klebsiella pneumonia* (Prabuseenivasan *et al.*, 2006).

Cinnamaldehyde induces changes in the membrane but does not result in the disintegration of OM (Helander *et al.*, 1998). Treatment of exponentially growing *B. cereus* cells with cinnamaldehyde results in filamentation and strong inhibition of cell separation (Kwon *et al.*, 2003). Further studies have shown that cinnamaldehyde inhibits FtsZ which is responsible for the regulation of bacterial cell division (Domadia *et al.*, 2007).

5.1.3.3 Ginger (*Zingiber officinale*)

Ginger is used in pharmaceutical, cosmetic, and food and beverage industries and it's EO has applications as an analgesic, anti-inflammatory, and antirheumatic (de Melo *et al.*, 2011). Ginger oil contains considerable concentrations of phenolic compounds including eugenol, gingerol and zingerone (Singh *et al.*, 2008). The antibacterial activity of ginger EO is related to these phenolic compounds and although an exact mechanism of action has not been established it is likely to involve the synergistic action of all constituents.

5.1.4 Green tea (*Camellia sinensis*)

Camellia sinensis belongs to the 'non-fermented' class of tea in which the leaves are dried and steamed in the manufacturing process (Cabrera *et al.*, 2006). The chemical

composition of green tea is complex and contains a diverse array of amino acids, carbohydrates, vitamins, minerals and pigments (Friedman, 2007). The polyphenol fractions have been widely investigated for their antimicrobial properties, of which the simplest compounds are catechins. Approximately 10% of green tea is made up of catechins, of which 4 main compounds are present; epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) (Hamilton Miller, 1995). Green tea catechins exert antimicrobial activity against human pathogens such as *S. aureus*, *S. epidermidis*, *Vibrio cholerae* (Toda *et al.*, 1989), *Clostridium* spp. (Ahn *et al.*, 1991) and *P. aeruginosa* (Yi *et al.*, 2010). Similarly to EOs, several cellular targets have been proposed for green tea. Targets include the FabI and FabG reductase steps in the *E. coli* fatty acid elongation cycle (Zhang & Rock, 2004) and DNA gyrase (Gradisar *et al.*, 2007). Certain green tea catechins (EGC and EGCC) can also inhibit bacterial efflux pumps Tet (K) (Roccaro *et al.*, 2004) and NorA (Gibbons *et al.*, 2004), which reduced susceptibility to tetracycline and norofloxacin respectively. Green tea has also been shown to reverse methicillin resistance in methicillin-resistance *S. aureus* (MRSA), inhibiting the synthesis of penicillin binding protein 2' (PBP2'; Yam *et al.*, 1998).

5.1.5 Candicidin

Candicidin is a unique antimicrobial produced and formulated by Cultech Ltd., composed of a variety of EOs including; oregano, clove leaf oil, ginger oil and wormwood oil. The main antibacterial component of clove leaf oil derived from *Eugenia carophyllus is* eugenol (Farag *et al.*, 1989) which shows activity against various pathogenic bacteria (Sanla-Ead *et al.*, 2011). Clove oil possesses antioxidant properties and shows potential as a natural preservative or as a source of natural antioxidants for use in pharmaceutical applications (Chaieb *et al.*, 2007). Wormwood oil is derived from *Artemisia absinthium*, and has antibacterial activity against pathogens such as *K. pneumonia* (Viljoen *et al.*, 2006). The monoterpene ketone thujone is considered as the main 'active ingredient' (Lachenmeier, 2010) however limited information regarding its antibacterial application are available.

5.1.6 Synergy between plant antimicrobials and antibiotics

Formulations of different antibacterial compounds that complement each others' action offers a strategy to overcome the problem of antibiotic resistance (Hemaiswarya *et al.*, 2008). For example, the combination of a ß-lactam antibiotic together with ß-lactamase inhibitor will render the ß-lactamase enzyme redundant allowing the antibiotic to remain active. Synergy is the term used to described when the combined effect of two compounds is greater than the sum of the effects of each compound alone (Berenbaum, 1978; Rand *et al.*, 1993). Several examples in the literature have demonstrated successful combinations of plant antimicrobials with many different classes of antibiotics. Shiota *et al.* (2000) demonstrated that extract from the petals of *Rosa canina* (rose red) significantly increased the ß-lactam susceptibility of MRSA strains. Erybraedin and eryzerin isolated from the roots of *Erythrina zeyheri* (member of the *Fabaceae* plant family, found in South Africa) decreased the susceptibility of vancomycin resistant enterococci (VRE) to vancomycin (Sato *et al.*, 2004). Furthermore, certain plant compounds have shown synergy with multiple antibiotics such as *Punica granatum* (pomegranate) which displayed a synergistic relationship with chloramphenicol, gentamicin, tetracycline, and oxacillin against MRSA (Braga *et al.*, 2005).

5.1.7 Aims

Cultech Ltd. produces various naturally derived compounds which are available in the form of EOs and emulsified powders. This study is the first to examine the action of naturally-derived plant EOs and plant compounds against *S. aureus* SCVs. As *S. aureus* SCVs are difficult to treat in the clinical setting and can be selected for in the presence of various antibiotics (Chapters 2 and 3), naturally derived compounds may offer an alternative method for treatment of SCV related infections.

- Examine the susceptibility of a range of *S. aureus* parent and SCV strains to various EOs and green tea using a disc diffusion method.
- Apply the CLSI broth dilution method(s) to obtain MIC and MBC values for plant antimicrobial powders against *S. aureus* parent and SCV strains.
- Investigative time kill dynamics to validate plant antimicrobial MBC values.
- Examine the ability of *S. aureus* parent and SCV strains to develop resistance to plant antimicrobial compounds.
- Investigate synergistic relationships that may exist between plant antimicrobials and various classes of conventional antibiotics.

5.2 Material and methods

5.2.1 Essential oils and powders

Cinnamon bark, oregano and ginger oils and green tea (Cultech Ltd.) were examined for there antibacterial activity against *S. aureus* SCV and parent strains using a disc diffusion method. Emulsified powders of cinnamon bark and oregano oil as well as candicidin and green tea (Cultech Ltd.) were used for microdilution susceptibility testing. The major known antibacterial constituents of EOs and the emulsified powders used in this study are shown in Table 5.1. Stock solutions (100,000 mg/L) of the powders candicidin, cinnamon, green tea and oregano were prepared by dissolving 1 gram of each powder in 10 mL of sterile deionised water. Suspensions were vortexed thoroughly for 5 min followed by centrifuged for 10 min at 10, 000 rpm. Dilutions of stocks were prepared in sterile deionised water and stock stored at 4°C for a maximum of 7 days.

5.2.2 Bacterial strains

Several reference methicillin sensitive *S. aureus* (MSSA) strains and well characterised MRSA strains were used to investigate the anti-staphylococcal effects of plant antimicrobials (Table 5.2). SCVs derived from these strains following antibiotic exposure, as well as laboratory constructed SCVs and a SCV human isolates were also examined (Table 5.3). Strains were maintained at -80°C in Mueller Hinton (MH) broth supplemented with 8 % dimethyl sulfoxide (DMSO) and re-isolated on MH agar plates.

5.2.3 Disc diffusion

Disc diffusion method was carried out following guidelines from the British Society of Antimicrobial Chemotherapy (BSAC) standardised disc susceptibility testing method (Andrews & Susceptibility, 2009). Individual *S. aureus* colonies (3-4) were inoculated into cation adjusted Mueller Hinton broth (CAMHB) and incubated at 37°C with shaking at 150 rpm. Cultures were grown to the end of logarithmic phase and cell densities were adjusted to match the turbidity of a 0.5 McFarland standard at 625 nm. Occasionally SCV strains failed to reach the densities required by the McFarland standard, in which case densities of parent strains were adjusted accordingly to ensure similar inoculum concentrations. A sterile cotton swab was dipped into the suspension and spread evenly over the surface of MH agar plate. This

procedure was shown to consistently produce semi confluent lawns recommend by the BSAC for susceptibility testing. Sterile filter discs (5mm diameter) were applied to the centre of the agar onto which 5 µL of plant antimicrobial stock solutions (100,000 mg/L) were dispensed. Plates were allowed to dry for 10 minutes before being incubated (24 hours for parent and 48 hours for SCVs) at 37°C. Following incubation inhibition zones were measured digitally using IMAGE J (NIH).

5.2.4 Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined according to Clinical Laboratory Standard Institute (CLSI) guidelines as described previously (section 2.2.3). As no information regarding the susceptibility of *S. aureus* SCVs to plant antimicrobials was available, a wide range of plant antimicrobial concentrations were tested. Stocks of plant antimicrobials were used to prepare concentrations double the required concentration to allow for dilution by the inoculum.

5.2.5 Determination of minimum bactericidal concentrations

Non turbid wells from MIC experiments were used to determine MBCs as described previously (section 2.2.4).

5.2.6 Time kill assays

Time kill assays were performed for two reasons. Firstly, to confirm that concentrations obtain from well plate based MBC assays reached the required reduction in inoculum and secondly to assess the rate at which bactericidal activity was achieved. Overnight cultures of *S. aureus* parent and SCVs strains were prepared in CAMH broth and then inoculated into 50 mL of CAMHB (in 250 mL narrow neck conical flasks) to achieve a starting density of 5 x 10^5 CFU/mL. Required volumes of plant antimicrobial agents were then added so flasks contained bactericidal concentrations of plant antimicrobials and incubated at 37°C with shaking at 150 rpm. Samples were taken every 2 hours for 10 hours. Serial dilutions were performed in phosphate buffered saline (PBS) and dilutions were plated onto MH agar. Plates were incubated at 37°C and colonies were enumerated after 48 hours.

Table 5.1 Composition of the major antibacterial components of plant antimicrobials used in this investigation

Essential oil/powder	Antibacterial composition
Candicidin powder	7.6 % (v/v) oregano oil 3.6 % (v/v) clove leaf oil 3.6 % (v/v) ginger oil 3.6 % (v/v) wormwood oil
Oregano oil	74.3 % (v/v) carvacrol 0.35 % (v/v) thymol
Oregano powder	25 % (w/v) oregano oil emulsified with tapioca starch
Cinnamon bark oil	70 % (v/v) cinnamaldehyde 4.5 % (v/v) eugenol
Cinnamon bark powder	33 % (w/v) cinnamon bark oil emulsified with tapioca starch
Green tea powder	45 % EGCG
Ginger oil	84 % (v/v) gingerol

Table 5.2 MSSA and MRSA strains used in this study

Strain	Description	Resistance(s)	Source/Reference
ATCC 25923	*S. aureus* reference strain	-	ATCC
NCTC 6571	*S. aureus* reference strain	-	NCTC
MRSA COL	Genome sequence early MRSA strain originally isolated in 1960s	TET, OX	Dyke *et al.*, 1966 (Dyke *et al.*, 1966); Gill *et al.*, 2005 (Gill *et al.*, 2005)
EMRSA 15	Epidemic MRSA type 15 – isolated in 1991, prevalent in UK hospitals	OX	Richardson & Reith, 1993
OMB 299	Revertant of SCV isolated from wound infection	ERY	University Hospital Münster, Germany
N315	Genome sequenced MRSA strain isolated in 1982	ERY, NEO, OX	Kuroda *et al.*, 2001(Kuroda *et al.*, 2001)

ATCC – American Type Culture Collection; NCTC – National Collection of Type Cultures; ERY- Erythromycin; NEO – Neomycin; OX – oxacillin; TET - Tetracycline

Table 5.3 *S. aureus* SCV strains used in this study

Strain	Description	Auxotrophy	Source/Reference
ATCC 25923 SCV^{GEN}	SCV derived from ATCC 25923 following gentamicin exposure	Hemin	This study
ATCC 25923 SCV^{KAN}	SCV derived from ATCC 25923 following kanamycin exposure	N/D	This study
NCTC 6571 SCV^{GEN}	SCV derived from NCTC 6571 following gentamicin exposure	Menadione	This study
NCTC 6571 SCV^{TET}	SCV derived from NCTC 6571 following tetracycline exposure	Menadione	This study
MRSA COL *hemB*	Laboratory generated SCV mutant of MRSA COL	Hemin	vonEiff *et al.*, 1997
MRSA COL *menD*	Laboratory generated SCV mutant of MRSA COL	Menadione	Bates *et al.*, 2003
EMRSA 15 SCV^{GEN}	SCV derived from EMRSA15 following gentamicin exposure	Menadione	This study
EMRSA 15 SCV^{NEO}	SCV derived from EMRSA15 following neomycin exposure	N/D	This study
OMB 299 SCV	SCV isolated from wound infection	Hemin	University Hospital Münster, Germany
N315 SCV^{GEN}	SCV derived from N315 following gentamicin exposure	N/D	This study
N315 SCV^{TET}	SCV derived from N315 following tetracycline exposure	Haemin	This study

N/D – No auxotrophy detected. SCVs recovered following antibiotic exposure were previously confirmed as *S. aureus* via multiplex PCR.

5.2.7 Short-term resistance training by exposure to a single sub-lethal concentration of plant antimicrobials (Cooper *et al.*, 2010)

S. aureus strains ATCC 25923 and MRSA COL and their respective SCVs ATCC 25923 SCV^{GEN} and MRSA COL *hemB* were examined to test if resistance to the plant antimicrobials could be induced. 250 mL conical flask containing X 0.5 MIC (in 50 mL CAMHB) candicidin, cinnamon bark, green tea and oregano powders were prepared. Starting densities of 5 x 10^5 CFU/mL were prepared by appropriate dilutions of overnight parent and SCV cultures. Flasks were incubated at 37°C with shaking at 150 rpm. On 10 successive days, similarly prepared flasks were inoculated with 40 µL from each preceding day's culture. The MIC for each strain was determined every day preceding subculture to assess any change in susceptibility after continuous exposure.

5.2.8 Effect of plant antimicrobials on antibiotic susceptibility of *S. aureus*

The chequerboard method was used to determine the interactive effects between plant antimicrobials and antibiotics (White *et al.*, 1996). The range of plant antimicrobial and antibiotic concentrations to be examined was determined in accordance with previously defined MIC values.

Suspensions of cinnamon, green tea and oregano were prepared at four times the required final concentration in CAMHB, in order to allow for dilution by inoculum and antibiotic. Increasing concentrations of the plant antimicrobial suspensions (50 µL volumes of each) were dispensed into each column of a microtitre plate, increasing from right to left (Figure 5.1). Increasing antibiotic solutions (50 µL volumes of each) were dispensed into each row, increasing in concentration from top to bottom. Optically adjusted *S. aureus* parent and SCV inoclua (1 x 10^6 CFU/mL) were prepared as described previously (section 2.2.3) and 100 µL inoculated into each individual well. This resulted in final total volume of 200 µL containing 5 x10^5 CFU/mL. Control wells were prepared by replacing the plant antimicrobial and antibiotic with 100 µL of CAMHB. Microtitre plates were incubated statically at 37°C for a total of 24 hours for parent strains. The incubation period was extended to 48 hours for SCV strains. Following incubation fractional inhibitory concentrations (FICs) and FIC indices (FICi) were calculated using the following formula, in order to determine the interactions between plant antimicrobial and antibiotics.

$$\textbf{FICi} = \textbf{FIC (Plant antimicrobial)} + \textbf{FIC (Antibiotic)}$$

Where:

$$\textbf{FIC (Plant antimicrobial)} = \frac{\text{MIC of plant antimicrobial in combination}}{\text{MIC of plant antimicrobial alone}}$$

$$\textbf{FIC (Antibiotic)} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

Synergism was defined by a FICi ≤ 0.5. Indifference was defined as an FIC index of > 0.5 but of < 4. Antagonism was defined as an FIC index of >4 (White *et al.*, 1996).

S. aureus N315 and its corresponding SCV N315 SCV^{GEN} are resistant to erythromycin, neomycin and oxacillin so these strains were chosen to investigate synergy with the plant antimicrobials in this study. *S. aureus* MRSA COL and its corresponding SCV MRSA COL *hemB* are resistant to oxacillin and tetracycline and therefore these strains were also selected to investigate synergy.

5.2.9 Statistical analysis

Analysis of significant differences between susceptibilities of SCV isolates and parent strains was performed using the statistical analysis described previously (section 2.2.9.8).

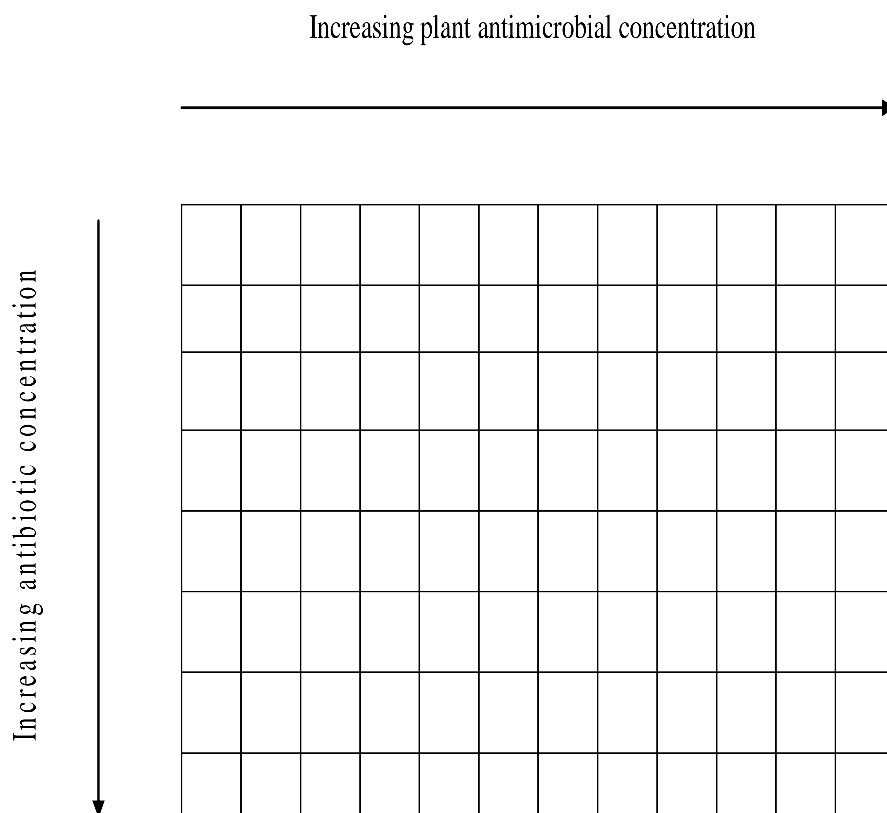


Figure 5.1 Layout of plant antimicrobial and antibiotic solutions in chequerboard plates

5.3 Results

5.3.1 Anti-staphylococcal effects of EOs and green tea

5.3.1.4 Disc diffusion

The antimicrobial action of candicidin, cinnamon bark, ginger grass, green tea and oregano oil against *S. aureus* SCVs and their parent strains was assessed using a simple disc diffusion method. Cinnamon bark oil, ginger grass, green tea and oregano oil antimicrobials produced inhibition zones in all SCVs and their respective parent strains examined (Figure 5.2). Candicidin showed no activity against all isolates examined. Oregano oil produced the largest inhibition zones in SCV and parent strains (mean 36 mm), followed by cinnamon bark oil (30 mm), green tea (17 mm) and ginger grass oil (11 mm). No significant difference was observed in diameter of inhibition zones produced by all plant antimicrobials in MRSA and MSSA strains ($P = > 0.05$). Mean inhibition zones produced by cinnamon bark oil (Figure 5.2 A), green tea (Figure 5.2 C) and oregano oil (Figure 5.2 D) were significantly greater in SCVs in comparison to inhibition zones in parent strains ($P = < 0.05$). No significant differences in the diameter of inhibition zones produced by ginger grass oil were detected between SCV and parent strains ($P = > 0.05$).

5.3.1.5 Microdilution susceptibility

Emulsified forms of candicidin, cinnamon bark oil, green tea and oregano oil were employed for microdilution testing against the same panel *S. aureus* SCV and parent strains. Although no guidelines to determine sensitivity and resistance for plant antimicrobials are available, all SCV and parent strains showed susceptibility to the concentrations examined (Table 5.4). Across the four compounds tested, per milligram green tea produced the lowest MICs (50 – 250 mg/L) in all *S. aureus* SCV and parent strains. Candicidin produced the highest MICs across all stains (2250 – 3000 mg/L; Table 5.4). Comparison of MICs between SCV and parent strains demonstrated that SCVs were more sensitive than their parents to cinnamon bark, green tea and oregano which agreed with previous disc diffusion results. A trend in susceptibility of SCVs compared to parent strains to candicidin was not observed. MBCs correlated with previously determined MICs, with green tea producing the lowest mean MBCs followed by oregano and cinnamon bark respectively (Table 5.4). MBCs of cinnamon bark, green tea and oregano were lower in SCVs in comparison to

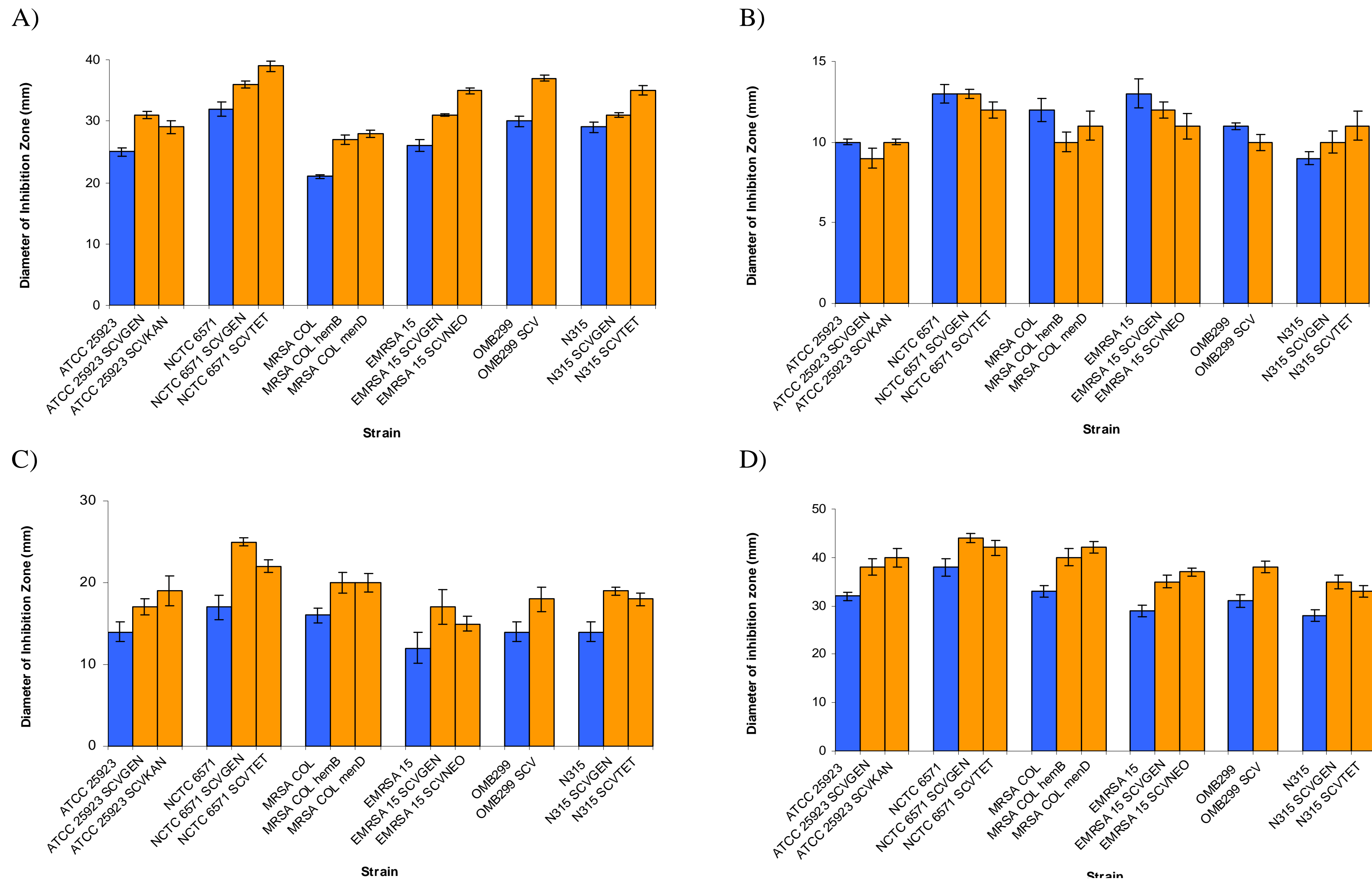


Figure 5.2 Antibacterial effects of various plant antimicrobials on *S. aureus* SCVs and their parent strains A) - Cinnamon bark oil B) - Ginger grass oil C) - Green tea D) - Oregano oil. Inhibition zones are the results of three independent replicates and three independent biological replicates. Error bars represent the standard error of the mean

Table 5.4 Plant antimicrobial MICs and MBCs (mg/L) of *S. aureus* SCVs and their parent strains

Strain	Candicidin		Cinnamon Bark		Green Tea		Oregano	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ATCC 25923	3000	5500	2000	3250	175	1250	1250	2000
ATCC 25923 SCV^{GEN}	3250	6000	1250	2000	75	750	750	1500
ATCC 25923 SCV^{KAN}	3000	6250	1000	2500	75	750	750	1250
NCTC 6571	2750	5000	2250	2750	150	1500	1500	2500
NCTC 6571 SCV^{GEN}	3000	5500	1750	2250	75	1000	1000	1500
NCTC 6571 SCV^{TET}	3250	6000	1750	2500	50	750	750	1250
MRSA COL	2500	5750	2000	3250	250	1750	1750	2750
MRSA COL *hemB*	2500	5750	1250	1750	125	1000	1000	1500
MRSA COL *menD*	2500	6000	1000	1500	150	1000	1000	1750
EMRSA 15	2250	5750	3000	3750	200	1500	1500	2250
EMRSA 15 SCV^{GEN}	2500	6000	2250	3000	125	1000	1000	1500
EMRSA 15 SCV^{NEO}	2500	5250	2500	3250	150	750	750	1250
OMB 299	3250	6250	2500	3000	175	1750	1750	2500
OMB 299 SCV	3000	6000	1750	2250	125	1500	1500	1750
N315	3500	6000	3000	4000	150	1500	1500	2250
N315 SCV^{GEN}	3250	6250	2000	2750	125	1250	1250	1500
N315 SCV^{TET}	3500	6250	2250	2750	125	1000	1000	1250

Modal MIC and MBC values are presented. Modal values were obtained from three independent replicates and three independent biological replicates.

parent strains. Although green tea showed the lowest MIC and MBC values across the panel of strains examined, average MBCs were eight fold greater than MICs. Average MBCs for cinnamon bark, oregano and candicidin were 1.3, 1.5 and 2 times greater than MIC values.

5.3.1.6 Time kill assays

Four plant antimicrobials (as above) were tested against *S. aureus* ATCC 25923 and MRSA COL and their corresponding parent SCVs to determine the rate at which plant antimicrobials achieved bactericidal activity using time kill assays (Figure 5.3). All previously determined MBCs (derived from the microtitre method above) achieved the required 99.9 % reduction (from the starting inoculum) required by the CLSI to be classed as bactericidal. Differences in the time required for bactericidal activity to be achieved varied between plant antimicrobials and difference between SCV and parent strains were also observed for certain compounds (Figure 5.3). Although all compounds examined achieved bactericidal activity within 10 hours, oregano (Figure 5.3 D) showed a substantially greater kill rate, reaching the 99.9 % reduction level in 4 hours for both SCV and parent strains. Green tea achieved the second most rapid time kill of between six and eight hours for SCV and parent strains respectively (Figure 5.3 C). Candicidin took 10 hours to achieve elimination of both SCV and parent strains. Differences in the time taken for green tea to exert the required reduction from the initial inoculum were apparent between SCV and parent strains. ATCC 25923 SCV^{GEN} and MRSA COL *hemB* reached the bactericidal threshold within 6 hours where as both respective parent strains took a further 2 hours to reach the same reduction. Similar contrasting time kill kinetics were apparent when exposing SCV and parent cultures to cinnamon bark (eight hours for SCV and ten hours for parents). Uniform time kill kinetics were observed for candicidin between SCV and parent strains, which both reached the required reduction in 10 hours. Strains were also analysed for their responses to exposure to plant antimicrobials at previously defined MICs. All four plant antimicrobials over the 10 hour period showed no increase in viable cell count from the starting inoculum density. No increase in viable count is comparable with the original MIC determination.

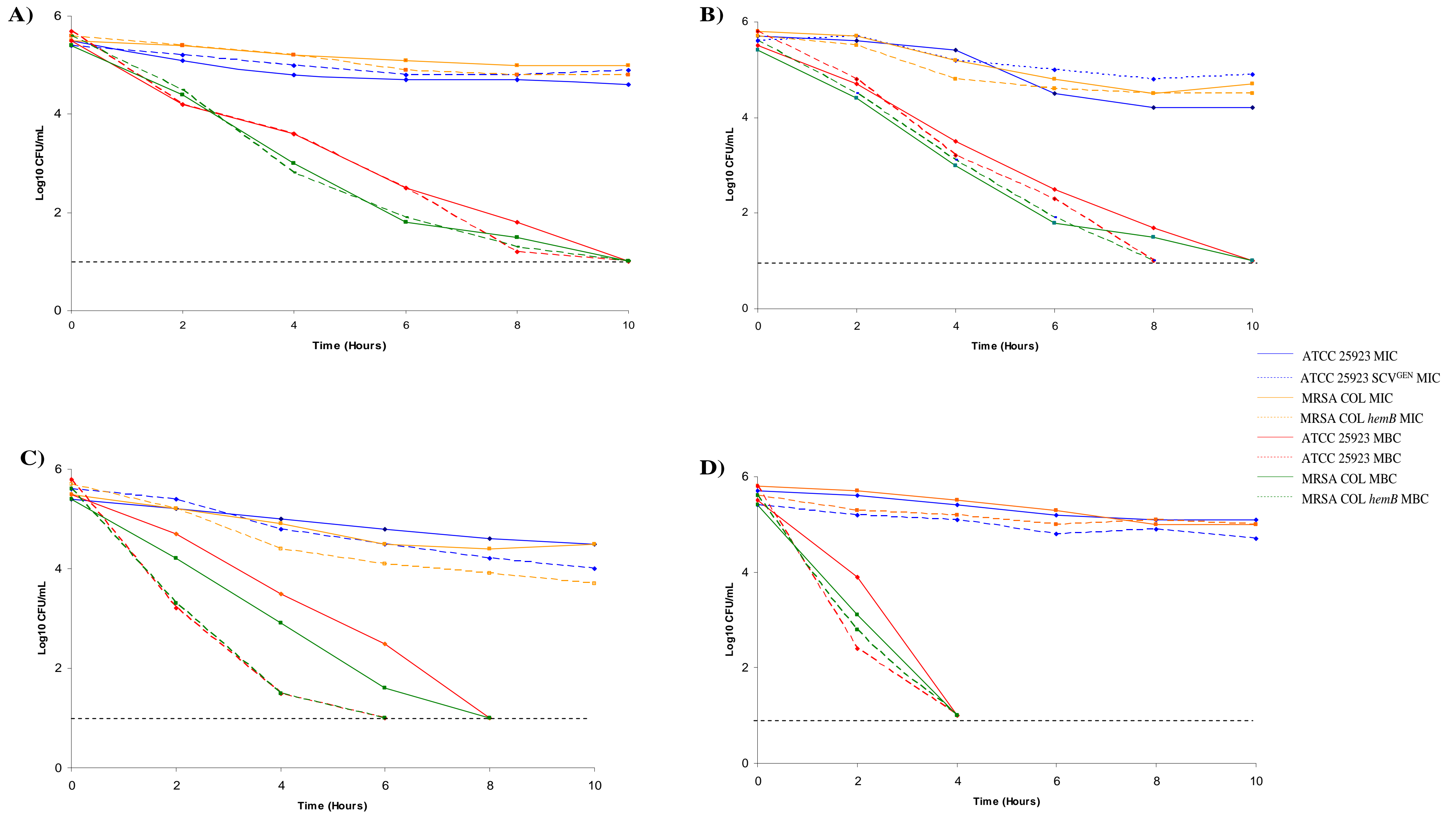


Figure 5.3 Time kill curves of plant antimicrobials against *S. aureus* SCVs ATCC 25923 SCV^{GEN} and MRSA COL *hemB* and their corresponding parent strains A) – Candicidin; B) - Cinnamon Bark; C) - Green tea; D) – Oregano. Dotted line represents limit of detection.

5.3.1.7 Synergy between plant antimicrobials and antibiotics

Synergistic relationships between three plant extracts (cinnamon, green tea and oregano) and four conventional antibiotics (erythromycin, neomycin, tetracycline and oxacillin) were examined in two *S. aureus* and SCV strains (Tables 5.5 and 5.6). Various synergistic relationships were observed with green tea displaying the largest number of synergistic relationship (three antibiotics). In the combinations in which synergy was reported the same trends were observed in SCV and parent strains. Cinnamon and oxacillin consistently showed a synergistic relationship achieving a FICi < 0.05 in both N315 and MRSA COL parent and SCV strains. All plant antimicrobial-antibiotic indifferences observed in SCVs were also reported in corresponding parent strains. Green tea showed a synergistic relationship with both neomycin and oxacillin in N315 SCV its parent strain and with tetracycline and oxacillin in MRSA COL *hem B* and its parent strain. No antagonistic combinations (FICi > 4) were observed in all of the plant antimicrobial-antibiotic combinations tested (Tables 5.5 and 5.6).

5.3.2 Resistance induction

Two strains of *S. aureus* (one SCV and one parent of each) were exposed to sub lethal (X 0.5 MIC) concentrations of candicidin, cinnamon bark, green tea and oregano for 10 days (Figure 5.4). An alteration of MIC of both SCV and their respective parent strains to all four plant antimicrobials was observed. MICs of all four strains tested displayed increasing MICs after exposure to candicidin, cinnamon bark and green tea. This was with the exception of MRSA COL exposed to green tea and oregano the MIC of which remained the same after 10 days continuous exposure (Figure 5.4). Exposure of SCVs and parent strains to green tea and candicidin produced small changes in MIC (0.26 and 0.15 fold increase respectively) after 10 days. Exposure of ATCC 25923 parent and SCV strains to cinnamon saw a two fold increase in MIC. The two fold increase was apparent in the MRSA COL *hemB* mutant; however the parent strain showed a lower 1.375 fold increase. Inversely exposure to oregano resulted in an increase in susceptibility in ATCC 25923 SCV^{GEN} and parent strains as well as the MRSA COL *hemB* mutant.

Table 5.5 The effects of plant antimicrobials on erythromycin, neomycin and oxacillin susceptibility of *S. aureus* N315 SCV^{GEN} and its corresponding parent strain expressed as FICi

N315 SCV^{GEN}

Combination tested	MICs (mg/L) Plant Anti.	Antibiotic	FICi	Relationship
Cinnamon and erythromycin	2000	64	1.39	Indifference [a]
Cinnamon and neomycin	2000	128	1.85	Indifference [a]
Cinnamon and oxacillin	2000	64	0.20	Synergy [b]
Green tea and erythromycin	125	64	2.15	Indifference [a]
Green tea and neomycin	125	128	0.15	Synergy [b]
Green tea and oxacillin	125	64	0.36	Synergy [b]
Oregano and erythromycin	1250	64	2.8	Indifference[a]
Oregano and neomycin	1250	128	1.9	Indifference[a]
Oregano and oxacillin	1250	64	0.21	Synergy [b]

N315 Parent

Combination tested	MICs (mg/L) Plant Anti.	Antibiotic	FICi	Relationship
Cinnamon and erythromycin	3000	64	1.25	Indifference [a]
Cinnamon and neomycin	3000	128	1.57	Indifference [a]
Cinnamon and oxacillin	3000	64	0.41	Synergy [b]
Green tea and erythromycin	150	64	2.5	Indifference [a]
Green tea and neomycin	150	128	0.37	Synergy [b]
Green tea and oxacillin	150	64	0.25	Synergy [b]
Oregano and erythromycin	1500	64	1.72	Indifference [a]
Oregano and neomycin	1500	128	2.1	Indifference [a]
Oregano and oxacillin	1500	64	0.33	Synergy [b]

Indifference ([a]) was defined as an FIC index of > 0.5 but of < 4. Synergism ([b]) was defined by a FICi ≤ 0.5. Antagonism was defined as an FIC index of >4 (White *et al.*, 1996).

Table 5.6 The effects of plant antimicrobials on oxacillin and tetracycline susceptibility of *S. aureus* COL *hemB* and its corresponding parent strain expressed as FICi

MRSA COL *hem B*

	MIC's (mg/L)			
Combination tested	**Plant Anti.**	**Antibiotic**	**FICi**	**Relationship**
Cinnamon and oxacillin	1250	128	0.32	Synergy [b]
Cinnamon and tetracycline	1250	64	1.57	Indifference [a]
Green tea and tetracycline	125	64	0.32	Synergy [b]
Green tea and oxacillin	125	128	0.22	Synergy [b]
Oregano and tetracycline	1000	64	1.96	Indifference [a]
Oregano and oxacillin	1000	128	0.17	Synergy [b]

MRSA COL Parent

	MIC's (mg/L)			
Combination tested	**Plant Anti.**	**Antibiotic**	**FICi**	**Relationship**
Cinnamon and oxacillin	2000	128	0.27	Synergy [b]
Cinnamon and tetracycline	2000	64	1.25	Indifference [a]
Green tea and tetracycline	250	64	0.25	Synergy [b]
Green tea and oxacillin	250	128	0.27	Synergy [b]
Oregano and tetracycline	1750	64	2.15	Indifference [a]
Oregano and oxacillin	1750	128	0.24	Synergy [b]

Indifference ([a]) was defined as an FIC index of > 0.5 but of < 4. Synergism ([b]) was defined by a FICi ≤ 0.5. Antagonism was defined as an FIC index of >4 (White *et al.*, 1996).

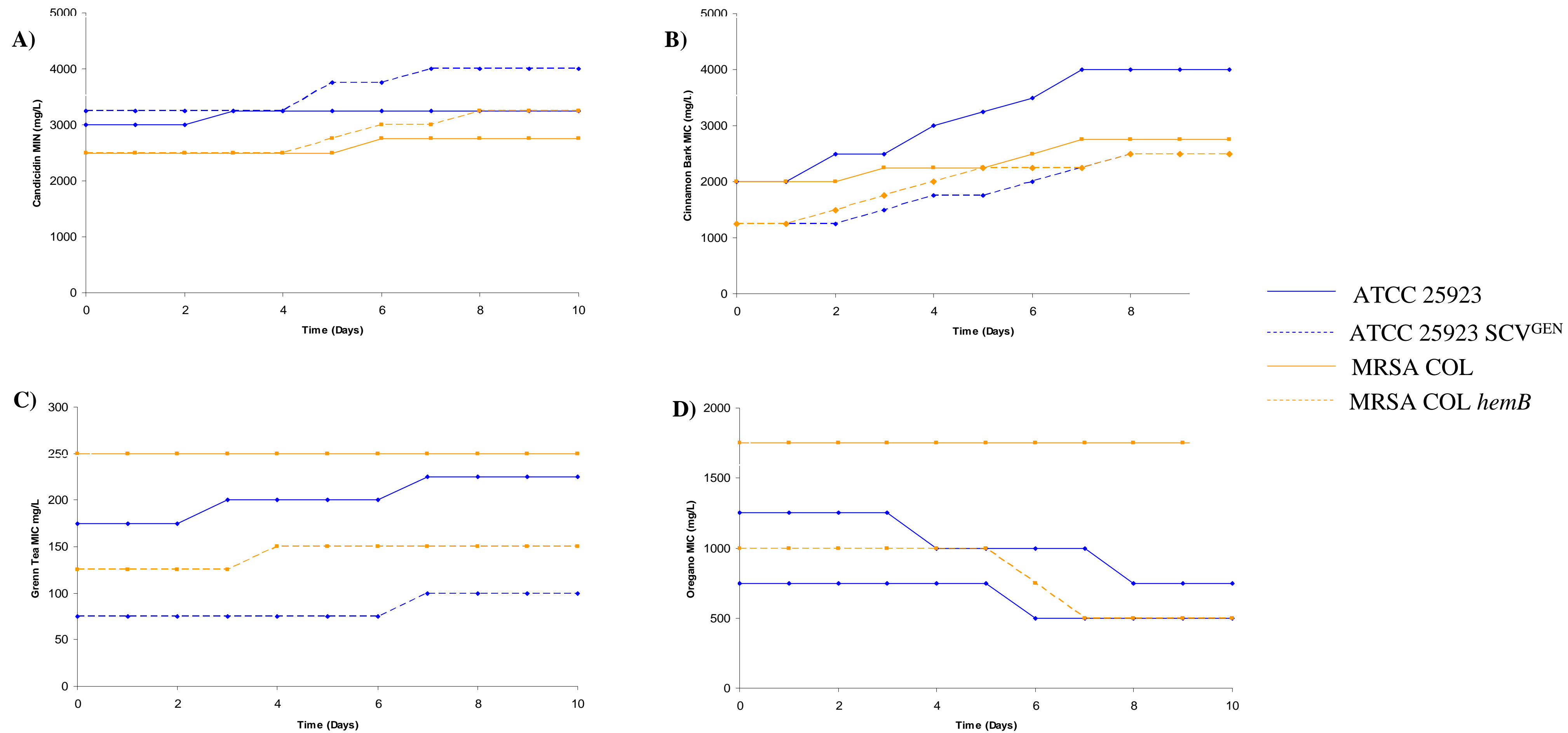


Figure 5.4 Susceptibility of *S. aureus* SCVs ATCC 25923^{GEN} and MRSA COL *hemB* and their corresponding parent strains following continuous exposure to plant antimicrobials A) - Candicidin B) - Cinnamon Bark C) - Green tea D) - Oregano

5.4 Discussion

Disc diffusion has been implemented to assess the antimicrobial susceptibility of several different bacterial species to various EOs (Burt & Reinders, 2003; Kalemba & Kunicka, 2003). In this study disc diffusion was applied to a collection of *S. aureus* SCV and parent strains, and inhibitions zones measurements were successfully obtained for all plant antimicrobials except candicidin. EOs contain a mixture of several different components and their antibacterial effect is poorly understood (Holley & Patel, 2005). Consequently efforts have focused on identifying the individual components of EOs to develop an understanding of antibacterial activity.

Oregano oil produced the largest mean inhibition zones in comparisons to the other EOs examined. The main component of the oregano oil used in this study was carvacrol, with the active compound making up 74.3% of the total composition. Carvacrol is responsible for the antibacterial activity of various EOs (thyme, savory as well as oregano) and has been shown to disrupt bacterial cell membranes (Lambert *et al.*, 2001). The disruption of the cell membrane causes the leakage of cellular constituents resulting in bacterial death. SCVs were shown to be more susceptible to oregano compared to parent strains in both the disc diffusion and broth dilution method. This may be related to the differences in membrane potential ($\Delta\Psi$) and ATP availability. Studies in *B. cereus* have shown that exposure to carvacrol results in depletion of the intracellular ATP pool, however this was not attributed to leakage of ATP through the membrane (Ultee *et al.*, 1999). Ultee *et al.* (1999) also showed that carvacrol induces membrane damage but concluded that ATP is not lost through this damage but is affected due the effect of carvacrol on $\Delta\Psi$. SCVs have a reduced $\Delta\Psi$ as a direct consequence of their phenotype (Balwit *et al.*, 1994), thus it can be postulated that carvacrol increases lethality in SCVs, as levels of ATP diminish at quicker rate in comparison to parent strains.

The main component of the cinnamon bark used in study was cinnamaldehyde (70 % of total composition). All SCV and parent strains examined were shown to be susceptibile to preparations of cinnamon bark, with SCVs producing larger inhibition zones and lower MIC and MBC values. Although cinnamaldehyde acts on the bacterial membrane, unlike carvacrol treated cells, cells treated with cinnamaldehyde

do not show depletion of intracellular ATP or disintegration of the membrane (Helander *et al.*, 1998). Cinnamaldehyde has been shown to inhibits FtsZ which is responsible for the regulation of bacterial cell division (Domadia *et al.*, 2007) therefore it is surprising that SCVs show increased susceptibility to cinnamon. SCVs exhibited reduced rates of cell wall synthesis and an increase in cell wall thickness in comparison to parent strains, which results in reduced susceptibly to antibiotics that target cell wall synthesis. Slower rates of cell division would suggest that SCVs would exhibit reduced susceptibility to antimicrobials targeting the cell division process. Other cellular components may therefore be the target of cinnamaldehyde.

Green tea exerts strong antibacterial activity against a range of bacterial pathogens, which is related to its composition of polyphenolic catechins, one of which is EGCG (Friedman, 2007). Several different targets for the antibacterial activity of green tea catechins have been published. Examples include the FabG and FabI reductase steps in the *E. coli* fatty acid elongation cycle (Zhang & Rock, 2004), cell division machinery (West *et al.*, 2001) and DNA gyrase (Gradisar *et al.*, 2007). Similar to the effects of cinnamon and oregano SCVs were also more susceptible to green tea. As the exact mechanism of activity of green tea is yet to be eluded, the difference in susceptibility between SCV and parent strains in difficult to postulate. If green tea targets cell division or DNA gyrase, increased susceptibility in SCVs is striking as SCVs would be expected to be more susceptibile, due to the slower growth rate observed in many SCVs.

Combined use of antibiotics with plant antimicrobial compounds may offer a strategy to overcome the problem of increasing antibiotic resistance through synergism; where the combined effect of two compounds is greater than the sum of the effects of each compound alone (Berenbaum, 1978; Rand *et al.*, 1993). Many studies have proven the synergistic action of EO fractions from different plants with synthetic drugs as well as antifungal agents (Hemaiswarya *et al.*, 2008). This study has identified synergistic relationships between several antibiotics and plant antimicrobials. Green tea displayed the highest number of synergistic relationships in both SCV and parent strains; include synergy with neomycin, oxacillin and tetracycline. The synergistic relationship between green tea and oxacillin has been explained by Zhao *et al.* (2001). EGCG of green tea directly binds to peptidoglycan on the cell wall interfering with

the integrity of the cell wall. The action of green tea directly facilitates the action of cell wall specific antibiotics, such as oxacillin.

Tetracycline resistance in *S. aureus* is often related to active efflux of tetracycline thus reducing the accumulation of tetracycline molecules within the cell. One example of this efflux pump is the Tet(K) efflux pump, which is widespread in staphylococcal species. Synergy between the EGCG and tetracycline has been observed via the inhibition of the Tet(K) efflux pump, drastically decreasing the MIC of tetracycline (Roccaro *et al.*, 2004). The strain tested for green tea and tetracycline synergy in this study (MRSA COL) also possess the tetracycline efflux pump Tet(K) , confirming the inhibition of tetracycline efflux by green tea (Liu & Pop, 2009). Synergy between green tea and neomycin was also observed in *S. aureus* N315 SCV and the parent strain. Resistance to various aminoglycosides in *S. aureus* N315 is due to the presences of nucleotidyltransferases, enzymes that modifies the antibiotics rendering them inactive (Kuroda *et al.*, 2001). The mode of synergy may involve green tea catechins, such as EGCG, inhibiting the activity or production of these modifying enzymes. EGCG can inhibit antibiotic modifying enzymes (Zhao *et al.*, 2003) and in this case may inhibit activity of aminoglycoside modifying enzymes (AMEs), allowing an active aminoglycosides to exert antibacterial activity. The observation that green tea has a synergistic relationship with three separate antibiotic classes makes it an attractive candidate for further investigation. This is supported by evidence that the components of green tea have antibacterial activity against various human pathogens (Anand *et al.*, 2006; Gordon & Wareham, 2010; Osterburg *et al.*, 2009) and are also active against *S. aureus* SCVs. SCVs display reduced susceptibility to cell wall specific antibiotics (such as ß-lactams) and protein synthesis inhibitors (aminoglycosides) as a direct result of their phenotype. Although the mechanisms surrounding the synergistic relationships may not be directly applicable to combating the reduced susceptibility mechanisms in SCVs, the susceptibility of SCVs to green tea may have potential in treatment of SCV infections.

Among the other plant antimicrobials examined cinnamon and oregano both showed synergy with oxacillin. The literature provides several examples of other plant antimicrobials that present synergy with ß-lactam antibiotics. For example, baicalin (a flavonoid used in traditional Chinese medicine) has been shown to inhibit ß-lactamase

activity (Liu *et al.*, 2000). Oxacillin is an example of a ß-lactam antibiotic that is resistant to the action of ß-lactamase enzymes, where the ß-lactam ring is protected from hydrolysis. Therefore if cinnamon and oregano are not involved in enzymatic inactivation, they may disrupt the integrity of the cell wall facilitating the action of ß-lactam antibiotics in a similar manner to green tea.

Bacterial resistance to antibiotics and biocides is well documented and patterns of resistance are closely observed by clinicians and healthcare workers. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) state that a clinically resistant organism is one in which the level of antimicrobial susceptibility has a high likelihood of clinical failure (EUCAST, 2009). Organisations such as EUCAST and the CLSI publish breakpoints that can be used in order to interpret whether an organism is susceptible or resistant to a certain antibiotic. Such guidelines are unavailable for plant antimicrobial compounds, mainly due to their lack of use in the clinical setting. The breakpoints stipulated by the CLSI usually specify an four to eight fold difference in MIC between sensitive and resistance organisms. For example *S. aureus* strains that are classified as susceptible to tetracycline have an MIC < 4 mg/L where as strains classified as resistance have a MIC > 16 mg/L. Increases in MIC for candicidin, cinnamon, and green tea were detected although the largest increases across the panel of four strains was a doubling in the MIC against cinnamon of *S. aureus* ATCC 25923. Therefore none of the *S. aureus* strains examined in this study would meet the criteria for a 'resistant' classification.

Classical mechanisms of resistance to antibiotics include enzymatic inactivation, efflux or modification of the target site (Almasaudi *et al.*, 1991). Although these mechanisms may be applied to combat the antibacterial effects of plant antimicrobials, studies have shown a lack of resistance to plant antimicrobials such as garlic and honey (Cooper *et al.*, 2010; Wood, 2009). It is likely that the bacterial resistance encountered today is due, in part, to the overuse of antibiotics in clinical and agricultural setting. The lack of resistance to plant antimicrobials may be partly due to the lack of implementation in treating bacterial infections and consequently the opportunity for bacteria to develop and evolve resistance mechanisms has not arisen. Another explanation may be the combination effects exerted by the different components of plant antimicrobials. EOs contain several groups of chemical

compounds resulting in multi-factorial antibacterial activity and therefore have several targets in the bacterial cell (Burt, 2004). For instance, the main component of the cinnamon used in this study is cinnamaldehyde; however eugenol is also present at lower concentrations. The multi-target nature of plant antimicrobial compounds suggests resistance is less likely to occur in comparison to exposure to an individual antimicrobial agent.

The problem of bacterial resistance to clinically important antibiotics has resulted in national and local surveillance networks being established to monitor antibiotic resistance trends (Nwosu, 2001). At present, plant antimicrobials are not used as systemic antibiotics due to their low level of activity, especially against Gram-negative bacteria. The reported MIC is often orders of magnitude higher than those of common broad-spectrum antibiotics from bacteria or fungi (Tegos *et al.*, 2002). The MICs of the plant antimicrobials assessed in this study are substantially higher than MICs for conventional antibiotics, although this study demonstrates that inhibition of *aureus* SCV and parent strains can be achieved. Additionally throughout the course of susceptibility testing it was observed that none of the plant antimicrobials examined selected for SCVs in parent strains. This is another advantageous characteristic of the plant antimicrobials examined as SCV selection can lead to persistent and recurrent infections. Whether or not these concentrations are achievable in the treatment of infections requires further investigation. An attractive option is the use of plant compounds as a topical agent for the treatment wounds or burns. Manuka honey is a plant derived antimicrobial that offer potentials in this particular application (Cooper & Molan, 1999; Cooper *et al.*, 1999; Dunford *et al.*, 2000). Plant antimicrobials may also be implemented in the decolonisation of patients who are known carriers of MRSA to prevent dissemination and spread of nosocomial infection (Caelli *et al.*, 2000; Dryden *et al.*, 2004). Coupled with the lack of resistance and the synergistic combinations with several commonly used antibiotics, plant antimicrobials offer an attractive avenue for antimicrobial chemotherapy. More research is required to understand the complete molecular mechanism of the drug action in the presence and absence of the natural compounds, as well as the stability, selectivity and bioavailability of these natural products (Hemaiswarya *et al.*, 2008).

5.5 Conclusions

- *S. aureus* SCV and parent strains are susceptible to candicidin, cinnamon bark, ginger, green tea and oregano as confirmed by a combination of disc diffusion and microdilution methods.
- *S. aureus* SCVs exhibit larger zones of inhibition and/or lower MIC and MBCs of candicidin, cinnamon bark, green tea and oregano.
- Resistance to candicidin, cinnamon bark, green tea and oregano could not be induced in SCV or parent strains following short term exposure to MIC.
- Cinnamon and oregano combined with oxacillin produces a synergistic relationship against *S. aureus* SCV and parent strains.
- Green tea combined with neomycin, oxacillin and tetracycline produces a synergistic relationship *S. aureus* SCV and parent strains.

6 CHAPTER 6: SMALL COLONY VARIANTS IN SPECIES OTHER THAN *STAPHYLOCOCCUS AUREUS*

6.1 Introduction

6.1.1 Small colony variants of bacterial species other than *S. aureus*

SCVs have been extensively studied in *Staphylococcus aureus*, however SCVs have been isolated from a variety of other bacterial species have been isolated (Table 6.1). These species include important Gram-negative pathogens such as *Pseudomonas aeruginosa* and Gram-positive pathogens such as *Enterococcus faecalis* and *Streptococcus pneumonia*.

6.1.1.1 *Escherichia coli*

E. coli is rod shaped facultatively anaerobic bacterium that is a normal component of the intestinal flora of humans. Although *E. coli* is generally viewed as a commensal, certain strains have acquired virulence factors that allow colonisation of novel niches and the ability to cause disease in otherwise healthy hosts (Kaper *et al.*, 2004). Different pathovars of *E. coli* have been described including enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), and uropathogenic *E. coli* (UPEC) (Croxen & Finlay, 2010). EPEC and EHEC cause disease in the small bowel and large bowel, respectively, where as UPEC colonises the bladder causing cystitis which can lead to kidney failure if left untreated. Comparison of a benign laboratory strain (K12) and an enterohaemorrhagic strain (O157:H7) identified the presence of 4.1 Mb sequence highly conserved between strains, regarded as the backbone of the *E. coli* chromosome (Hayashi *et al.*, 2001). Genome analysis concluded that O157:H7 diversified from a common lineage via the acquisition of foreign DNA, much of which encodes virulence related functions accounting for the difference in pathogenicity between strains.

SCVs of *E. coli* have been isolated from patients suffering from prosthetic joint infections and chronic urinary tracts infections (Roggenkamp *et al.*, 1998; Tappe *et al.*, 2006). Analysis of isolates revealed they show *E. coli* SCV share characteristics associated with the conventional SCV phenotype observed in *S. aureus* including the

Table 6.1 Small colony variants isolated from various bacterial species

Species	Source of isolation	Observations associated with SCV phenotype	Reference
Brucella abortus	Mouse model of infection	Slow growth rate, increased persistence in mouse model	Jacob *et al*., (2006)
Burkholderia cepacia	Lung transplant in CF patient	Increased serum resistance	Haussler *et al*., (2003a)
Burkholderia pseudomallei	*In vitro* exposure to ceftazidime, ciprofloxacin and gentamicin	Identical biochemical, PFGE and electron microscopy profiles; reduced susceptibility to various antimicrobials	Haussler *et al*., (1999a)
Brucella melitensis	Bacterial endocarditis (blood culture)	Reduced streptomycin susceptibility through development of SCV phenotype	Hall & Spink (1947)
Cryptococcus neoformans	Chronic meningitis	Negative result for cryptococcal antigen; implications in identification	To *et al*., (2006)
Enterococcus faecalis	Amyloid arthropathy in chickens	Identical PFGE and MLST profiles to WT; increased virulence and persistence	Petersen *et al*., (2008)
Enterococcus faecalis	Chronic aortic valve endocarditis	Identical PFGE profile to WT; auxotrophy for haemin; abnormal cell wall and cell size	(2009) Wellinghausen *et al*.,
Neisseria gonorrhoea	Gonorrhoea patient	Reversion to WT following subsequent culture	Morton & Shoemaker (1945)
Salmonella enterica serovar Typhimurium	Prolonged intracellular fibroblast infection	Increased intracellular persistence; reduced virulence in mice model; reduced susceptibility to aminoglycosides	Cano *et al*., (2003)
Streptococcus pneumoniae	Isolated from *S. pneumonia* biofilms during initial attachment phase	Increased adherence, aggregation and biofilm formation	Allegrucci & Sauer (2007)

CF - cystic fibrosis; PGFE – pulse field gel electrophoresis; MLST - multilocus sequence typing; SCV - small colony variant; WT - wildtype

production of microcolonies, slow growth, reduced aminoglycoside susceptibility and auxotrophy. In cases of infection with *E. coli* SCVs, infection duration and persistence are increased (Roggenkamp *et al.*, 1998; Sendi *et al.*, 2010) which has been documented in patients with *S. aureus* SCV infection (Kahl *et al.*, 1998; von Eiff *et al.*, 1997a). Laboratory studies have shown that antimicrobial agents such as gentamicin can select for *E. coli* SCVs (Lewis *et al.*, 1991). These variants showed similar characteristics associated with the SCV phenotype and auxotrophy for haemin was attributed to mutations in *hemB*.

6.1.1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a rod shaped motile bacterium. On solid agar colonies often appear as green-blue in colour, characterised by the production of the pigments pyocyanin and pyoverdin (Pier & Ramphal, 2010). Genome sequencing of several *P. aeruginosa* strains has revealed the presence of a large genome (6.2 – 6.6 Mb) that permits nutritional versatility and environmental adaptability (Stover *et al.*, 2000; Winstanley *et al.*, 2009). A large proportion of genes in *P. aeruginosa* encode environmental sensors and transcriptional regulators that aid adaptational response to environmental fluctuations (Stover *et al.*, 2000). *P. aeruginosa* is responsible for various community acquired infections (ear infections and keratitis), and is also the causative agent of nosocomial infections of the respiratory tract, urinary tract and burns (Rossolini & Mantengoli, 2005). Intrinsic resistance to a range of antimicrobial agents (quinolones, chloramphenicol, tetracycline, trimethoprim and sulphonamides) due the presence of multidrug efflux systems (Poole, 2001) results in limited treatment options for *P. aeruginosa* infections.

SCVs of *P. aeruginosa* have been isolated from *in vitro* exposure to aminoglycosides as well as experimental models of infection (Gerber & Craig, 1982; Gerber *et al.*, 1982). Common characteristics of SCVs included reduced uptake of aminoglycosides, reversion to the wildtype phenotype and changes in biochemical profiles. Chronic pulmonary infections in CF patients are also a source of *P. aeruginosa* SCVs. The link between the prevalence of SCVs in CF patients has been linked to the use of ventilator administered antimicrobials, which may induce the SCV phenotype (Haussler *et al.*, 1999b). These isolates showed reduced susceptibility to antipseudomonal agents and are associated with reduced lung function in comparison

with patients infected with wildtype *P. aeruginosa* strains. *P. aeruginosa* SCVs also display many characteristics corresponding to niche adaptation with the lungs of CF patients. These characteristics include increased biofilm formation and increased association with a eukaryotic cell line (Haussler *et al.*, 2003b). Auxotrophy for compounds such as haemin, menadione and thymidine has not been previously reported in *P. aeruginosa* SCVs.

6.1.1.3 *Staphylococcus epidermidis*

S. epidermidis is a member of the coagulase negative staphylococci (CoNS), which are differentiated from *S. aureus* by an inability to coagulate rabbit plasma (i.e. coagulase negative). *S. epidermidis* shares a core set of 1,681 open reading frames with *S. aureus* but lacks many of the genomic islands that encode virulence factors such as toxins (Gill *et al.*, 2005). Recently the importance of *S. epidermidis* as an opportunistic pathogen has been recognised. Its natural niche on the human skin permits access through implanted devices resulting in *S. epidermis* being the most common source of infections associated with indwelling medical devices (Rogers *et al.*, 2009). This opportunistic pathogen is one of most frequently recovered microorganisms in the hospital environment, showing high levels of incidence in nosocomial bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat (Vuong & Otto, 2002). The affinity of *S. epidermidis* for foreign materials commonly used in modern medicine has further contributed to the increased incidence in nosocomial infections (Huebner & Goldmann, 1999). Antibiotic resistance is also prevalent in *S. epidermidis* and other coagulase negative CoNS. Recent surveillance studies in North America and the UK reported a high rate of ciprofloxacin, erythromycin penicillin, and oxacillin resistance (Hope *et al.*, 2008; Streit *et al.*, 2004).

SCVs of *S. epidermidis* have been isolated from pacemaker-related infections (von Eiff *et al.*, 1999), and SCVs of other CoNS species including *S. capitis* and *S. lugdunensis* have been isolated from prosthesis related infections (Seifert *et al.*, 2005; von Eiff *et al.*, 1999). Prosthetic device related infections caused by *S. epidermidis* SCVs respond poorly to antimicrobial chemotherapy, often resulting in the removal of the prosthesis. Similarly to *S. aureus* SCVs, identification of CoNS SCVs is a challenge due to their abnormal colony phenotypes, slow growth rate and altered

biochemical characteristics; amplification and sequence analysis of 16S rRNA is vital for accurate species identification (von Eiff *et al.*, 1999). Recently a link has been postulated between CoNS SCVs and patients suffering from myalgic encephalomyelitis (known as chronic fatigue syndrome; CFS). CFS is a condition characterised by long periods of fatigue, short term memory loss and musculoskeletal pain (Fukuda *et al.*, 1994). Increased carriage of CoNS on skin and the presence of atypical macrococci in blood cultures from CFS patients (unresponsive to antibiotic treatment) allude to the involvement of a SCV phenotype (Onyango *et al.*, 2008; Tarello, 2001). The presence of a SCV phenotype may explain the persistence and duration of the disease as SCVs frequently persist within host cells and abate the immune response.

The construction of a stable *S. epidermidis hemB* laboratory mutant (via allelic replacement) has aided the study of SCVs of this species. Stable *S. epidermidis* SCVs have permitted developments regarding the understanding of pharmcodynamic responses to antistaphylococcal agents (Wu *et al.*, 2009). This approach has demonstrated that SCVs of *S. epidermidis* show reduced susceptibility to vancomycin in comparison to wildtype strains due to enhanced adhesion properties. Furthermore, increased capacity to form biofilms in *S. epidermidis hemB* mutants has been reported (Al Laham *et al.*, 2007). Increased expression of *icaA* resulted in increased production of polysaccharide intracellular adhesin and the formation of large clusters of cells.

6.1.2 Aims

SCVs of *E. coli*, *P. aeruginosa* and *S. epidermidis* have been isolated and characterised, however the majority of cases have been isolated from the clinical environment. The following work aimed to investigate whether various antimicrobial agents could select for SCVs *in vitro* and characterise these isolates to determine if an overlap was present between lab and clinically derived isolates through the following aims:

- Examine the ability of several antimicrobial agents to select for SCVs in *E. coli*, *P. aeruginosa* and *S. epidermidis*.
- Characterise any SCVs using species specific assays.
- Determine antimicrobial susceptibility profiles of SCV isolates.
- Analyse biofilm formation in SCV isolates.
- Generate RAPD profiles for SCV isolates and compare parent RAPD profiles.

6.2 Materials and methods

6.2.1 Bacterial strains and growth medium

E. coli ATCC 25922 and NCTC 10418, *P. aeruginosa* ATCC 27853 and PAO1 and *S. epidermidis* LTN (obtained from Heath Hospital, Cardiff) were all maintained on Mueller Hinton (MH) agar and grown in MH broth at 37°C with shaking (150 rpm) unless otherwise stated. All growth medium was obtained from Oxoid (UK), with the exception of purified agar which was obtained from Difco (UK). Additional components required for certain agar assays were obtained from Sigma (UK).

6.2.2 Preparation of antimicrobial agents

Ampicillin, ciprofloxacin, chloramphenicol, gentamicin, tetracycline were obtained from Sigma Aldrich (UK) and triclosan from Ciba Speciality Chemicals (Germany). Ciprofloxacin, chloramphenicol, gentamicin and tetracycline powders were dissolved in sterile deionised water. Saturated $NaHCO_3$ solution was used to dissolve ampicillin and triclosan was dissolved in dimethyl sulfoxide. Reduced strength stocks were made where required in deionised water and all stock solutions were stored at 4°C for a maximum of 4 days.

6.2.3 Determination of minimum inhibitory concentrations

MICs were determined using Clinical Laboratory and Standards Institute (CLSI) guidelines as described previously (section 2.2.3).

6.2.4 SCV selection assays

Following the determination of MICs, strains that showed susceptibility were exposed to antimicrobial agents to examine their ability to select for SCVs *in vitro*. *E. coli*, *P. aeruginosa* and *S. epidermidis* cultures were prepared by inoculating 3-4 individual colonies into cation adjusted Mueller Hinton broth CAMHB and incubating at 37°C with shaking at 150 rpm. Cultures were grown to mid exponential phase and antimicrobial agents added at concentrations determined as the MIC in previous susceptibility testing (section 6.2.3). Following incubation for a further 6 hours, 100 µL of culture was spread over the surface of a MH agar plate (containing X 2 MIC the selecting antimicrobial agent), using a sterile glass rod. Plates were incubated for 48 hours at 37°C. The presence of colonies with altered phenotypic appearances were suspected as SCVs and chosen for further analysis.

6.2.5 Characterisation of SCVs

6.2.5.1 Growth rate analysis

Overnight cultures of *E. coli*, *P. aeruginosa* and *S. epidermidis* parent and SCV strains were used to inoculate 250 mL conical flasks containing 50 mL of MH broth at a starting density 1 X 10^5 CFU/mL. Flasks were incubated at 37°C with shaking (150 rpm) for 48 hours. Optical density was measured at 580 nm and used to calculate lag time and growth rate. Lag time was recorded as the time taken for to the culture to reach the beginning of exponential growth from initial inoculation. Growth rate was calculated using the formula *ln*NT – *ln*NO/ TIME. Viable counts were obtained following the end of growth rate experiments by performing relevant serial dilutions in phosphate buffered saline (PBS) and enumerating cells on MH agar.

6.2.5.2 Auxotrophy

Auxotrophy profiles of *E. coli*, *P. aeruginosa* and *S. epidermidis* SCVs were determined as described previously (section 2.2.9.6).

6.2.5.3 Antimicrobial susceptibility testing of SCVs

Initially susceptibility testing was planned to be investigated via microdilution methods using CLSI guidelines. However the production of cellular aggregates and biofilms were frequently observed during microtitre susceptibility testing lead to inconsistencies and errors in observations. Therefore the British Society for Antimicrobial Chemotherapy (BSAC) standardised disc susceptibility testing method (section 5.2.3) was applied which improved result interpretation and consistency. Disc impregnated with antimicrobial agents were all obtained from Oxoid (UK). The antimicrobial discs used were 10 µg ampicillin, 30 µg chloramphenicol, 5 µg ciprofloxacin, 10 µg gentamicin, 30 µg tetracycline.

6.2.5.4 *S. epidermidis* DNA extraction

DNA extraction from *S. epidermidis* isolates was performed as described previously for *S. aureus* (section 2.2.7).

6.2.5.5 *E. coli* and *P. aeruginosa* DNA extraction

DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, UK), according to manufactures instructions. Overnight cultures were centrifuged at 10,000 rpm in microfuge tubes to pellet cells. 180 µL of lysis buffer was used to

resuspend pellets and create a homogenous mixture. This was followed by the addition of 20 µL of Proteinase K, which was subsequently incubated for 30 minutes at 55°C. Manufactures specific lysis C solution (200 µL) was then added and incubated for a further 10 minutes at 55°C. DNA binding columns were optimised for binding using ethanol followed by the addition of the previously prepared lysate. Samples were centrifuged at 8,000 rpm for 1 minute, followed by 2 additional washing steps before eluting bound DNA by the addition of 200 µL manufactures elution solution. Finally, samples were centrifuged for 1 minute at 10,000 rpm and the remaining eluate (regarded as pure genomic DNA) was stored at 4°C until required.

6.2.5.6 Biofilm formation assay

SCVs isolated from *E. coli*, *P. aeruginosa* and *S. epidermidis* were analysed for the ability to form biofilms using a standardised multiwell microtitre plate assay (O'Toole *et al.*, 1999). Overnight cultures of SCV and parent strains were adjusted to 1 x 10^6 CFU/mL in fresh medium (Luria Broth for *E. coli* and *P. aeruginosa* and tryptone soy broth for *S. epidermidis*) supplemented with 0.5 % glucose and 200 µL was dispensed into wells of 96 microtitre well plates (Fisher, UK). Microtitre plates were incubated at 37°C for 48 hours and subsequently stained with 0.1 % safranin (dissolved in H_2O and filter sterilised). Biofilms were stained for 15 minutes at room temperature before being washed twice with PBS to remove unattached cells and residual dye. To elute safranin from biofilms 200 µL of solvent was added to individual wells (95% ethanol for *P. aeruginosa* and *S. epidermidis* and 80% ethanol, 20% acetone for *E. coli*) and incubated at room temperature for 30 minutes. Subsequently optical density was measured at OD_{490} using a DYNEX Technologies MRX® Microplate Absorbance Reader with Revelation™ application programme.

6.2.5.7 Cell-surface hydrophobicity

The microbial adhesion to hydrocarbons assay described previously (section 4.2.5) was used for determine cell-surface hydrophobicity.

6.2.5.8 Random amplified polymorphic DNA analysis

RAPD was carried out as described previously (section 3.2.5.9) with the inclusion of the additional primers 228 (5'-GCTGGGCCGA-3') and 270 (5'-TGCGCGCGGG-3').

6.2.6 Characterisation of *P. aeruginosa* SCVs

P. aeruginosa is a motile organism therefore various motility assays were employed to compare flagella and pili activity in SCV and parent strains.

6.2.6.1 Swarming assay (Rashid & Kornberg, 2000)

Swarming plates (0.5% purified agar, 8 g/L nutrient broth, 5 g/L glucose) were prepared and allowed to dry overnight before being used. SCV and parent strains were adjusted to 1 X 10^6 CFU/mL in PBS and 5 uL inoculated onto the centre of swarming agar plates. Plates were incubated at 30°C for 48 hours and the furthest branching point from the initial point of inoculation measured.

6.2.6.2 Swimming (Rashid & Kornberg, 2000)

SCV and parent strains were adjusted to 1 X 10^6 CFU/mL in PBS and used to inoculate tryptone swim plates (10 g/L tryptone, 5 g/L NaCl, 0.3 % purified agar). Tryptone swim plates were inoculated with the use of a sterile toothpick and incubated at 30°C for 48 hours. Swimming motility was assessed by examining the circular turbid zone formed by the bacterial cells migrating away from the initial point of inoculum.

6.2.6.3 Twitching (Darzins, 1993; Deziel *et al.*, 2001)

SCV and parent strains were adjusted to 1 X 10^6 CFU/mL in PBS and used to inoculate thin (3 mm) LB agar plates. Using a sterile toothpick cells were stabbed into the agar so the toothpick reached the Petri dish surface. Bacterial growth at the interface between the plastic surface and the agar was measured by removing agar and staining with 1 % crystal violet. Twitching motility was determined by measuring the diameter of the crystal violet stained area.

6.2.6.4 Pyocyanin assay (Schaber *et al.*, 2004)

SCV and parent strains were grown in glycerol alanine minimal (GA) medium (1 % glycerol, 6 g/L L-alanine, 2 g/L $MgSO_4$, 0.1 g/L K_2HPO_4, and 0.018 g/L $FeSO_4$) to investigate pyocyanin production. Overnight SCV and parent cultures were centrifuged at 10,000 rpm for 5 minutes and the supernatant collected. Individual strain supernatants (5 mL) were mixed with 5 mL chloroform and left at room temperature for 5 minutes to allow separation. To the lower organic layer 1.5 ml 0.2 M hydrochloric acid was added and the pyocyanin-rich organic layer was

separated. The absorbance of the extracted layer was measured at OD_{520} to quantify pyocyanin production.

6.2.6.5 Elastin Congo red assay

To quantify elastolytic activity the method of Schad *et al.,* (1987) was applied. Supernatants of SCV and parent strains grown in LB were collected as described previously (section 6.2.6.4) and 100 µL added to 2 ml of 10 mM $NaHPO_4$ containing 30 mg elastin Congo red (Sigma Aldrich, UK). The mixture was incubated at 37 °C for 14 hours, centrifuged at 10,000 rpm for 5 minutes and released Congo red measured at 495 nm.

6.2.7 Characterisation of *E. coli* SCVs

6.2.7.1 Biochemical analysis

API 20E strips (Biomerieux, France) were used to compare biochemical profiles between *E. coli* SCV and parent isolates. A direct colony suspension of SCV and parent cells was prepared in API suspension medium (Biomerieux, France), and 200 µL used to inoculate CIT, GEL and VP reactions (in order to fill both tube and cupule) with 100 µl being added to all remaining tubes. Anaerobic conditions were created by the overlaying of mineral oil to ADH, LDC, ODC, H_2S and URE tests. Wildtype test strips were incubated at 37°C for 24 hours where as the incubation period was extended to 48 hours for SCV isolates. Following incubation various tests required the addition of reagents which can be found in the Appendix. The catalogue of the enzymatic reactions tested and resulting colour changes are seen in Appendix 1.

6.2.7.2 Swimming motility

Swimming agar used for *P. aeruginosa* (section 6.2.6.2) was used to assay swimming motility in *E. coli* SCVs and parents.

6.2.8 Characterisation of *S. epidermidis* SCVs

6.2.8.1 Catalase production

Catalase production was determined by the addition of 10 uL hydrogen peroxide (Sigma, UK) to glass slides containing a homogenous mixture of several *S. epidermidis* colonies. The production of bubbles was indicative of catalase activity.

6.2.8.2 Biochemical analysis

API *Staph* strips (Biomerieux, France) were used as described previously (section 2.2.9.1).

6.2.9 Statistical analysis

Analysis of significant differences between characteristics of SCV isolates and parent strains were performed as described previously (section 2.2.9.8).

6.3 Results

6.3.1 SCV selection

E. coli ATCC 25922 and NCTC 10418 strains showed susceptibility to ampicillin, ciprofloxacin, chloramphenicol, gentamicin, tetracycline and triclosan and thus all were analysed for their ability to select for SCVs (Table 6.2). *P. aeruginosa* ATCC 27853 and PAO1 however displayed resistance (MIC > 32 mg/L) to ampicillin, chloramphenicol, tetracycline and triclosan. Both strains were sensitive to gentamicin and ciprofloxacin which were used in SCV selection assays (Table 6.2). Finally, *S. epidermidis* LTN showed susceptibility to chloramphenicol, gentamicin and tetracycline, which were employed in SCV selection assays.

SCVs were successfully isolated from *E. coli*, *P. aeruginosa* and *S. epidermidis*. Gentamicin was the only antimicrobial agent that selected for SCVs in all three species. Furthermore, SCVs were recovered after exposure of *E. coli* to ciprofloxacin and chloramphenicol and *S. epidermidis* with tetracycline. Exposure to ampicillin, and triclosan did not result in the detection of SCVs from any of the species examined. Although the species examined exhibit morphological differences on solid agar, when observing SCVs, colonies with a smaller diameter (in comparison to parent strains) were consistently observed in all three species (Figure 6.1). *E. coli* and *P. aeruginosa* SCV colonies also appeared less mucoid. SCV were named to reflect the selecting antimicrobial. For example *P. aeruginosa* PAO1 SCV^{GEN} was selected for after exposure of strain PAO1 in the presence of gentamicin.

6.3.2 Auxotrophy profiles

Auxotrophy for haemin, menadione and thymidine was tested for in SCVs isolated from *E. coli*, *P. aeruginosa* and *S. epidermidis* (Table 6.3). Autotrophy for haemin was detected in all *E. coli* SCVs recovered from both ATCC 25922 and NCTC 10418. Menadione auxotrophy was observed in *S. epidermidis* LTN SCVs. Auxotrophy was not detected in any of the gentamicin selected SCVs isolated from *P. aeruginosa* ATCC 25922 or PAO1. Furthermore, no auxotrophy was detected in SCVs isolated following exposure to ciprofloxacin and no SCVs isolated showed auxotrophy for thymidine.

Table 6.2 Minimum inhibitory concentrations of *E. coli*, *P. aeruginosa* and *S. epidermidis* parent strains

	Ampicillin	Chloramphenicol	Ciprofloxacin	Gentamicin	Tetracycline	Triclosan
***E. coli* ATCC 25922**	1	2	0.125	0.25	0.5	0.125
***E. coli* NCTC 10418**	2	4	0.25	0.25	1	0.125
***P. aeruginosa* ATCC 27853**	> 32	> 32	0.5	1	> 32	> 32
***P. aeruginosa* PAO1**	> 32	> 32	1	2	> 32	> 32
***S. epidermidis* LTN**	> 32	4	> 32	0.5	1	4

Modal MIC values are presented. Modal values were obtained from three independent replicates and three independent biological replicates.

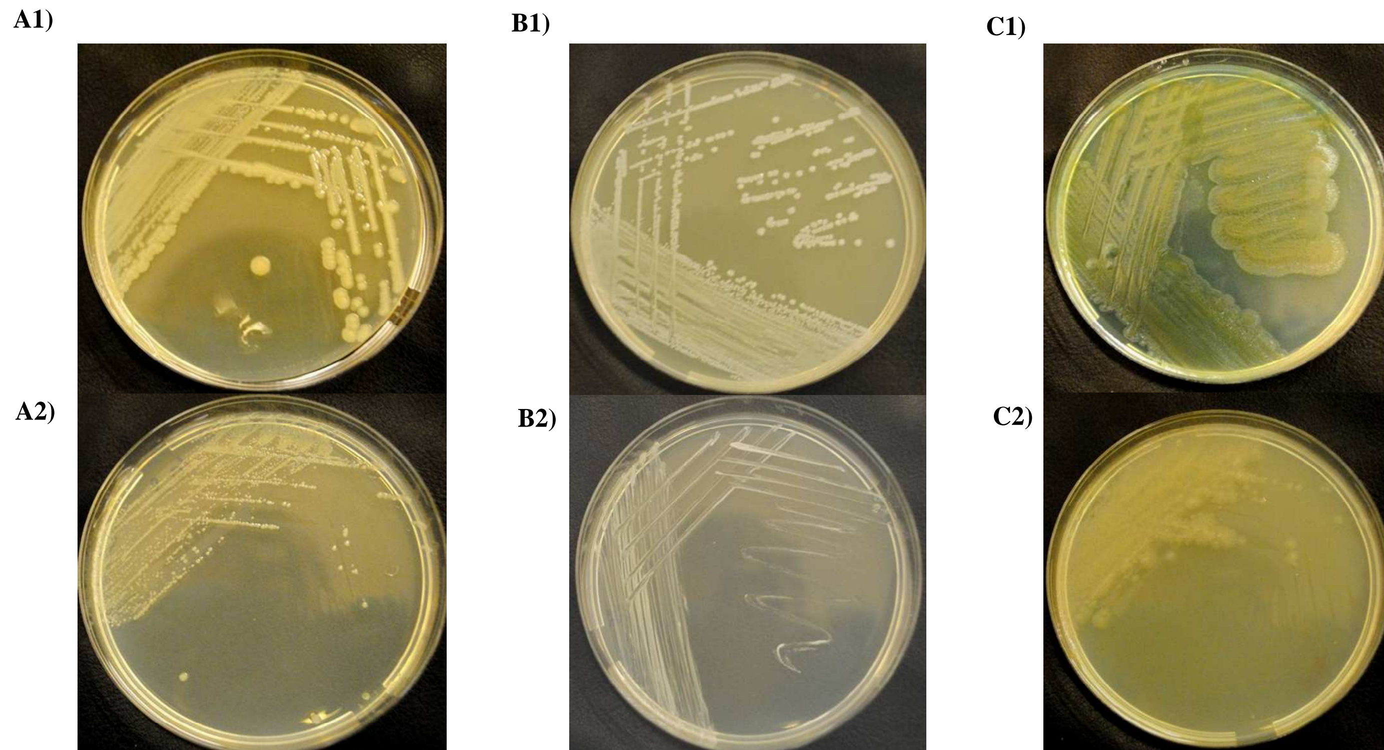


Figure 6.1 Morphological differences between wildtype and SCV strains A1) - *E. coli* ATCC 25922; A2) - *E. coli* ATCC 25922 SCV^{GEN}; B1) - *S. epidermidis* LTN; B2) - *S. epidermidis* LTN SCV^{GEN}; C1) - *P. aeruginosa* PAO1; C2) - *P. aeruginosa* PAO1 SCV^{GEN}. Colony diameters are reduced in all SCVs. Additionally a loss of a mucoid appearance in *E. coli* ATCC 25922 SCV^{GEN} and loss of pyocyanin production in *P. aeruginosa* PAO1 SCV^{GEN} is also apparent.

6.3.3 Growth profiles

All SCVs isolated produced dissimilar growth profiles to parent strains (Table 6.3). A significant decrease in growth rate and an increase in the duration of lag time was observed in all SCV in comparison with parent strains ($P = < 0.01$) For example, lag time duration for *E. coli* was 1.64 hours, in comparison to an average 4.43 hour lag time observed in SCV isolates (Table 6.3). Similar extension of lag times was observed in *P. aeruginosa* and *S. epidermidis* SCVs. The longest increased in lag time was observed in *E. coli* SCVs selected for in the presence of ciprofloxacin. Reduced maximum cell density was consistently observed amongst SCVs from all species. Wildtype strains consistently reached > 10^9 CFU/mL where as all SCV strains produced significantly lower final cell density in comparison ($P = < 0.01$).

6.3.4 Antibiotic susceptibilities

The BSAC disc diffusion method was used to examine the susceptibility of *E. coli*, *P. aeruginosa* and *S. epidermidis* SCV and parent strains. *E. coli* SCVs (selected for in the presence of chloroamphenicol, ciprofloxacin and gentamicin) produced significantly smaller zones of inhibition for all 5 antibiotics tested (Figure 6.2), including those targeting cell wall synthesis (ampicillin), DNA replication (ciprofloxacin) and protein synthesis (chloramphenicol, gentamicin and tetracycline) ($P = < 0.05$). *E. coli* SCVs selected in the presence of ciprofloxacin (*E. coli* SCV^{CIP}) displayed the most pronounced degree of reduced susceptibility, producing the smallest inhibition zones for every antimicrobial agent examined.

Due to the intrinsic resistance of *P. aeruginosa* to various antimicrobial agents, susceptibility profiles were only determined for gentamicin and ciprofloxacin. *P. aeruginosa* SCV^{GEN} produced significantly smaller zone of inhibition for gentamicin ($P < 0.01$). Mean zones of inhibition for ciprofloxacin were smaller but not statistically significantly (Figure 6.2). *S. epidermidis* SCVs showed a significant reduction in the diameter of inhibition zones for gentamicin ($P = < 0.01$). *S. epidermidis* LTN SCV^{TET} produced significantly smaller zones of inhibition when exposed to the antibiotic that had selected for them (tetracycline).

Table 6.3 Auxotrophy profiles and growth characteristics of *E. coli*, *P. aeruginosa*, *S. epidermidis* SCV and parent strains

Strain/isolate	Auxotrophy	Lag time duration (hours)	Growth rate	Maximum cell density (CFU/mL)
E. coli ATCC 25922	N/T	1.70	0.88	5.2×10^9
E. coli ATCC 25922 SCV^{CHL}	Haemin	4.08	0.27	5.8×10^8
E. coli ATCC 25922 SCV^{CIP}	N/D	4.80	0.20	7.2×10^7
E. coli ATCC 25922 SCV^{GEN}	Haemin	4.13	0.24	5.0×10^8
E. coli NCTC 10418	N/T	1.58	0.85	3.2×10^9
E. coli NCTC 10418 SCV^{CHL}	Haemin	4.23	0.31	1.5×10^8
E. coli NCTC 10418 SCV^{CIP}	N/D	4.87	0.22	9.5×10^7
E. coli NCTC 10418 SCV^{GEN}	Haemin	4.53	0.27	2.6×10^8
P. aeruginosa ATCC 27853	N/T	2.15	0.81	5.1×10^9
P. aeruginosa ATCC 27853 SCV^{GEN}	N/D	5.91	0.36	3.6×10^7
P. aeruginosa PAO1 WT	N/T	2.06	0.77	2.8×10^9
P. aeruginosa PAO1 SCV^{GEN}	N/D	5.68	0.41	6.3×10^7
S. epidermidis LTN	N/T	2.20	0.81	7.5×10^9
S. epidermidis LTN SCV^{GEN}	Menadione	5.21	0.34	2.2×10^8
S. epidermidis LTN SCV^{TET}	Menadione	5.46	0.30	4.3×10^8

N/D – not detected; N/T – not tested

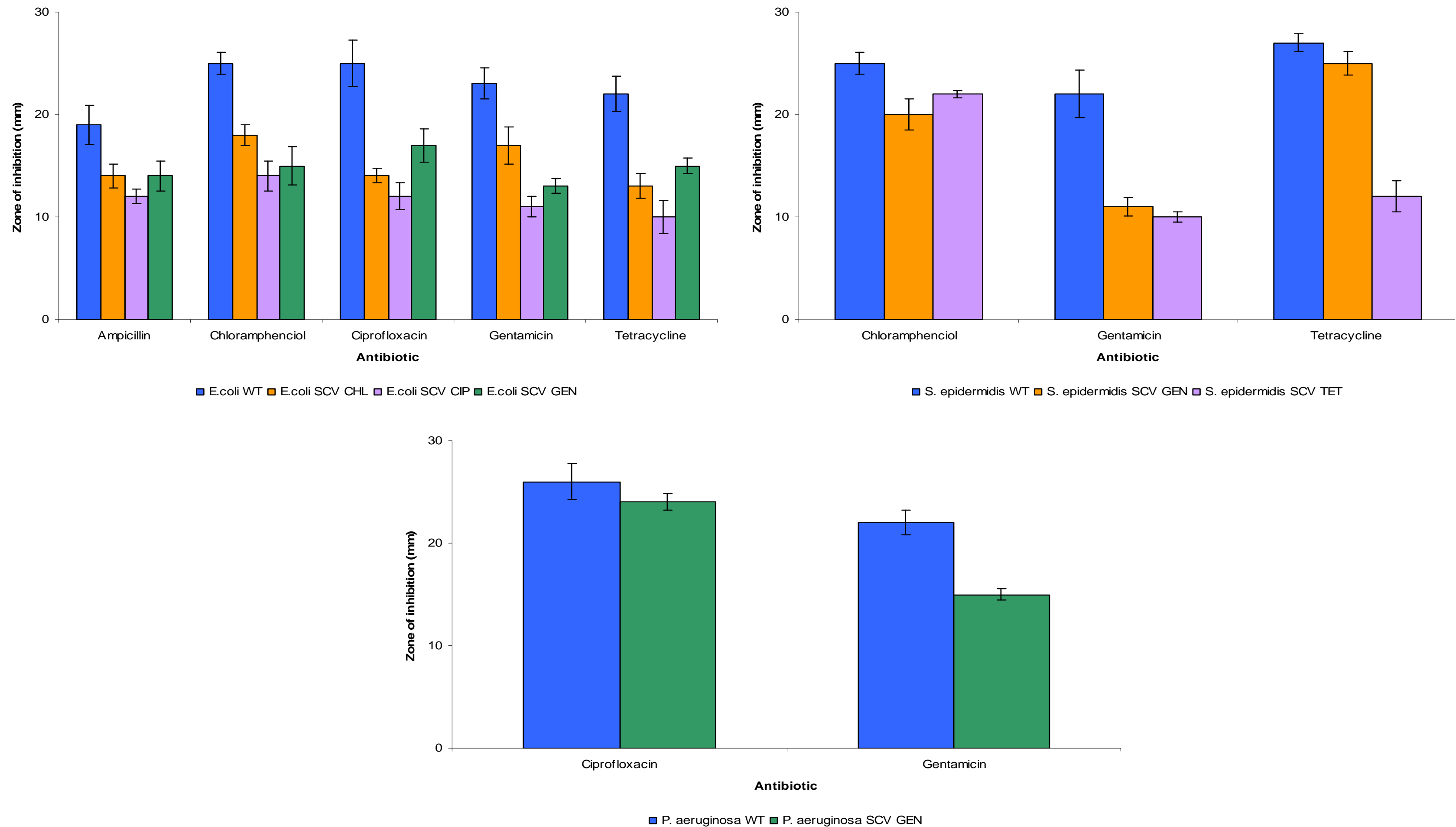


Figure 6.2 Inhibition zones of *E. coli*, *P. aeruginosa*, *S. epidermidis* SCV and parent strains measured by disc diffusion Results represent the mean of three independent replicates and three independent biological replicate. Error bars represent standard error

6.3.5 Biofilm formation and cell-surface hydrophobicity

E. coli, *P. aeruginosa* and *S. epidermidis* all formed biofilms using the multiwell biofilm assay. *S. epidermidis* produced the greatest biofilm mass, where as *E. coli* and *P. aeruginosa* produced biofilm at similar levels (Figure 6.3). SCVs of *E. coli*, *P. aeruginosa* and *S. epidermidis* showed increased biofilm formation in comparison to parent strains regardless of the selecting antibiotic ($P = < 0.01$; Figure 6.3). No significant difference in cell-surface hydrophobicity was observed between *S. epidermidis* parent and SCV isolates ($P = > 0.05$; Figure 6.3). SCV isolates of *E. coli* and *P. aeruginosa* however displayed a significant increase in cell surface hydrophobicity in comparison with parent strains ($P = < 0.01$).

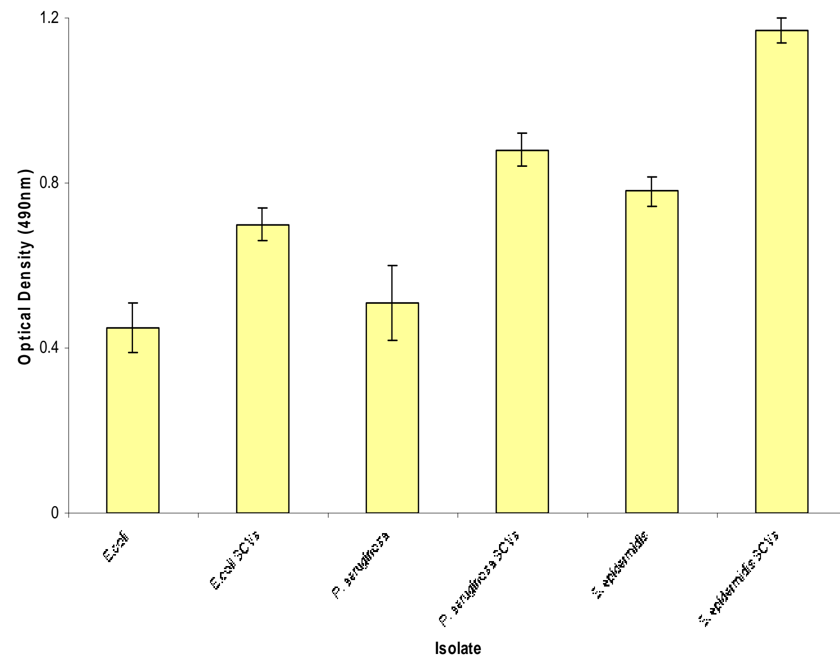
6.3.6 RAPD profiles

RAPD primers 228 and 272 produced reproducible complex banding patterns for both *E. coli* and *S. epidermidis*. SCV isolates for both species showed identical RAPD profile regardless of the selecting agents, indicating clonality (Figures 6.4 and 6.5). *P. aeruginosa* RAPD profiles lacked the banding complexity seen in *E. coli* and *S. epidermidis* however parent and SCV isolates produced identical RAPD profiles with primers 268 and 270 (Figure 6.5).

6.3.7 Characterisation of *P. aeruginosa* SCV isolates

Various agar based motility assays were applied to *P. aeruginosa* SCV^{GEN} isolates to examine flagella and type IV pili activity. Twitching motility was measured by crystal violet staining growth at the interface between the plastic surface of a Petri dish and the agar. *P. aeruginosa* SCVs isolated from PAO1 and ATCC 25923 strains showed a significant increase in the diameter of this zone correlating to an increase in twitching motility ($P = < 0.01$; Figure 6.6). Using the swarming motility assay parent strains demonstrated far reaching irregular branching patterns. In contrast SCV strains failed to produce the complexity of branching patterns observed and a significant reduction in branching diameter was observed ($P = < 0.01$; Figure 6.6). Similarly SCV isolates were also deficient in swimming motility producing smaller diameter concentric rings ($P = < 0.01$; Figure 6.6) in comparison to parent strains. Elastase activity was measured via the release of Congo red from Congo red bound elastin. SCVs produced significantly lower elastolytic activity in comparison to parent strains ($P = < 0.05$; Figure 6.6). On agar plates *P. aeruginosa* SCV isolates appeared deficient in

A)



B)

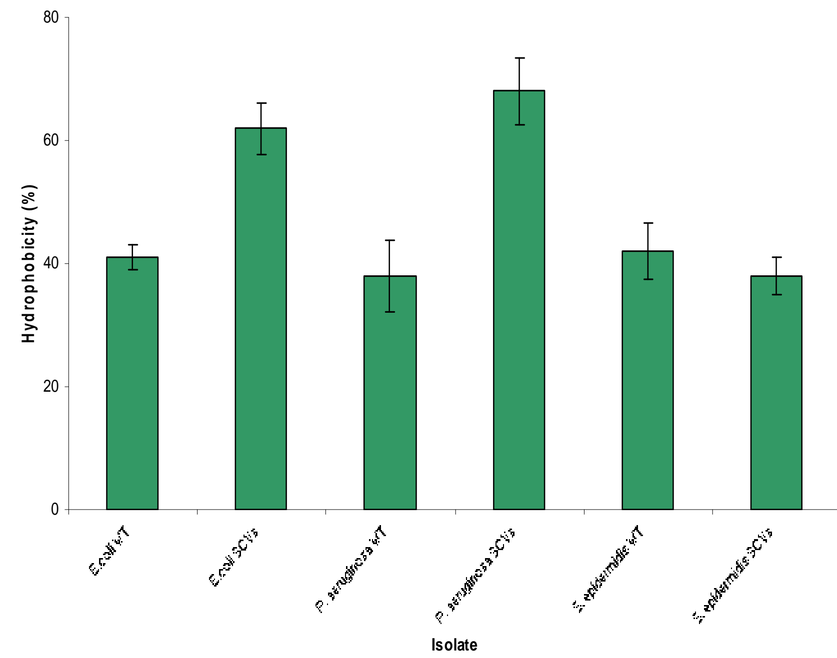


Figure 6.3 Biofilm formation (A) and cell-surface hydrophobicity (B) in *E. coli*, *P. aeruginosa*, *S. epidermidis* SCV and parent strains

Results represent the mean of three independent replicates and three independent biological replicate. Error bars represent standard error

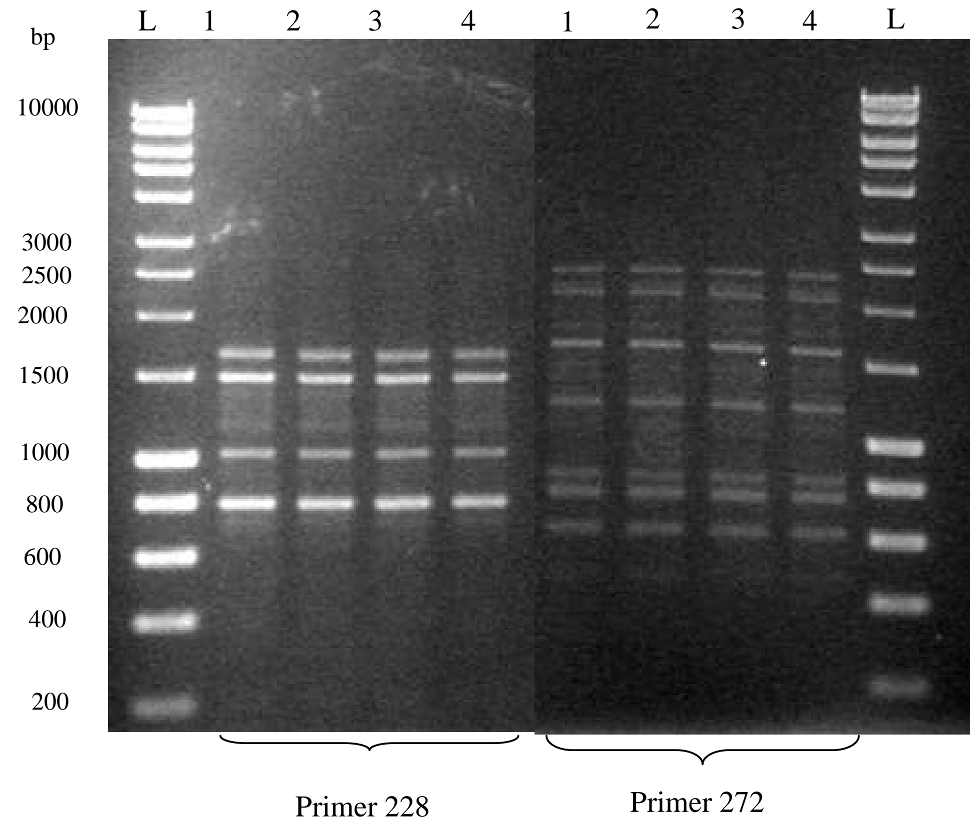


Figure 6.4 *E. coli* SCV and parent RAPD profiles L - Hyperladder 1; 1 - *E. coli* ATCC 25922; 2 - *E. coli* ATCC 25922 SCV^{CIP}; 3 - *E. coli* ATCC 25922 SCV^{CHL}; 4 - *E. coli* ATCC 25922 SCV^{GEN}. RAPD analysis of *E. coli* SCVs selected in the presence of various antimicrobials displayed identical profiles in comparison to parent strain.

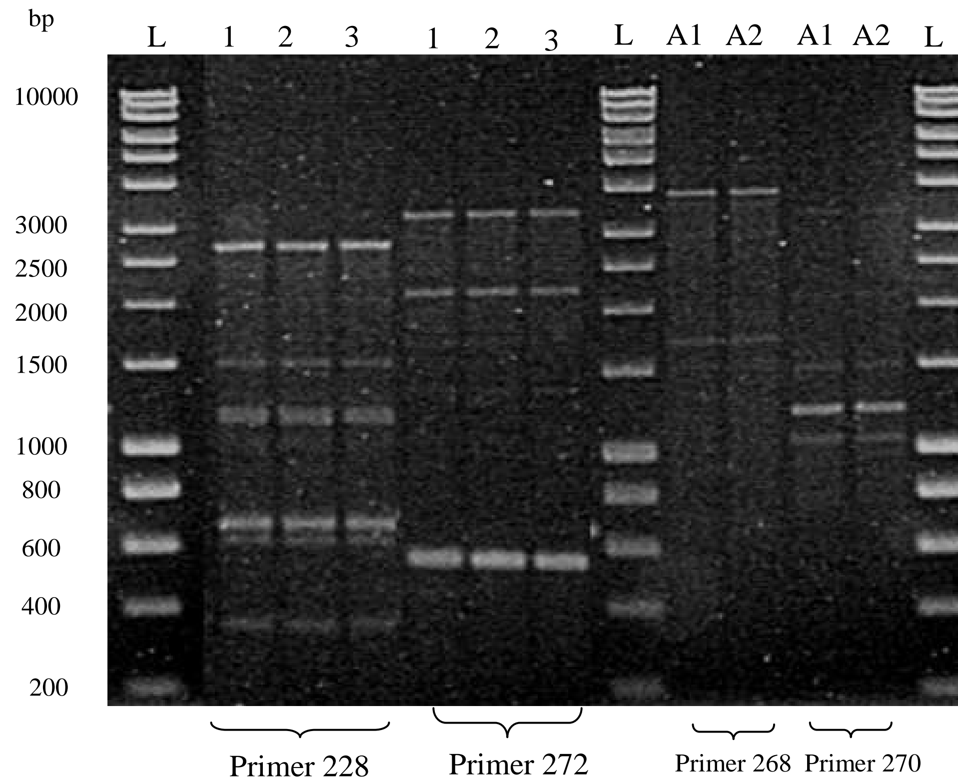


Figure 6.5 *S. epidermidis* and *P. aeruginosa* SCV and parent RAPD profiles L - Hyperladder 1; 1 - *S. epidermidis* LTN; 2 - *S. epidermidis* LTN SCV^{GEN}; 3 - *S. epidermidis* LTN^{TET}; A1 - *P. aeruginosa* PAO1; A2 - *P. aeruginosa* PAO1 SCV^{GEN}. RAPD analysis of *S. epidermidis* SCVs selected in the presence of gentamicin and tetracycline and *P. aeruginosa* SCVs selected in the presence of gentamicin displayed identical profiles in comparison to parent strain.

pyocyanin production (Figure 6.1). Although the presence of pyocyanin was visualised with increasing incubation, quantitative analysis confirmed SCVs were significantly deficient in pyocyanin production ($P = < 0.01$). Parent strains produced an absorbance of 0.31 (±0.08) in comparison to an average of 0.11 (± 0.05) in SCV strains.

6.3.8 Characterisation of *E. coli* SCV isolates

Biochemical analysis of wildtype *E. coli* demonstrated the production of β-galactosidase through the hydrolysis of *ortho*-nitrophenyl-galactopyranoside (Table 6.4). The production of β-galactosidase facilitates the hydrolysis of lactose which is a common characteristic of *E. coli*. All *E. coli* SCV isolates showed an absence of β-galactosidase activity even after prolonged incubation. SCVs were negative for mannitol fermentation, for which the wildtype was positive. Other differences between SCVs and wildtype were seen in an inability to produce indole (SCV^{CIP} and SCV^{GEN}), and inability to ferment sorbitol (SCV^{CIP} and SCV^{GEN}) and melibioise (SCV^{CIP}). Motility assays revealed that all *E. coli* SCV regardless of the selecting antimicrobial were deficient in swimming motility (Figure 6.7). A significant reduction in the mean average diameter was observed in SCV isolates in comparison to parent strains ($P < 0.01$)

6.3.9 Characterisation of *S. epidermidis* SCV isolates

S. epidermidis SCVs (SCV^{GEN} and SCV^{TET}), produced different biochemical profiles in comparison to parent strains (Table 6.5). Fermentation of maltose and lactose was not observed in both SCV isolates as well as a lack of production alkaline phosphatase production. Addition of hydrogen peroxide to wildtype strains resulted in rapid bubbling indicative of the action of catalase. However all SCV isolates showed a weak catalase response. As the assay did not allow a quantitative parameter all SCVs were regarded as weakly catalase positive.

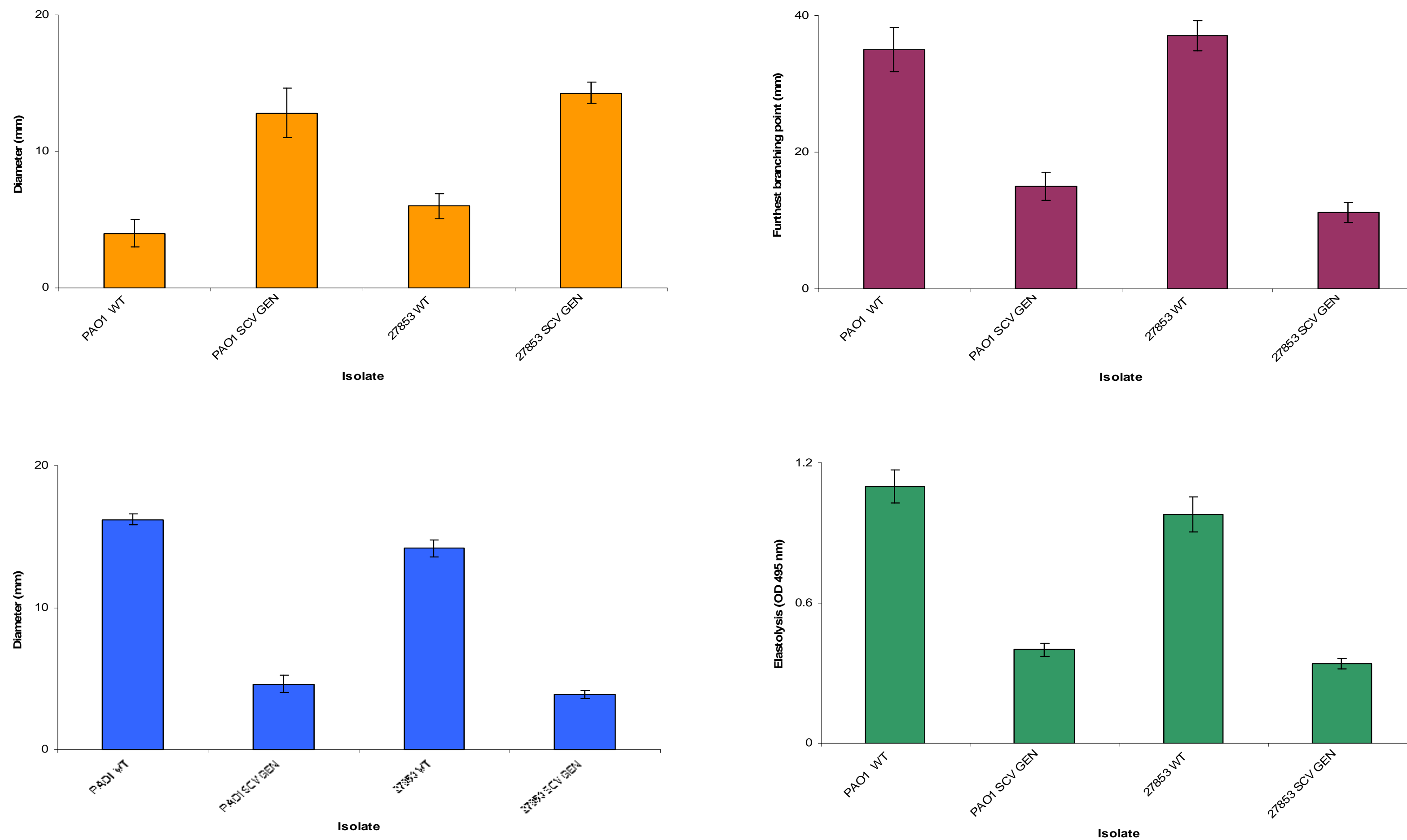


Figure 6.6 Characteristics of *P. aeruginosa* SCVs A) - Twitching motility; B) - Swarming motility; C) - Swimming motility; D) - Elastolysis activity. Results represent the mean of three independent replicates and three independent biological replicate. Error bars represent standard error

Table 6.4 Biochemical analysis of *E. coli* ATCC 25922 SCVs

	Parent	*E. coli* SCV^{CIP}	*E. coli* SCV^{CHL}	*E. coli* SCV^{GEN}
ONPG	+	-	-	-
ADH	-	-	-	-
LDC	+	+	+	+
ODC	+	+	+	+
CIT	-	-	-	-
H_2S	-	-	-	-
URE	-	-	-	-
TDA	-	-	-	-
IND	+	-	+	-
VP	-	-	-	-
GEL	-	-	-	-
GLU	+	+	+	+
MAN	+	-	-	-
INO	-	-	-	-
SOR	+	-	+	-
RHA	+	+	+	+
SAC	-	-	-	-
MEL	+	-	+	+
AMY	-	-	-	-
ARA	+	+	+	+

+ - Positive (enzyme activity, fermentation of carbohydrate); - = Negative (lack of enzyme activity, inability to ferment carbohydrates). Differences between SCV and parents are highlighted in grey.

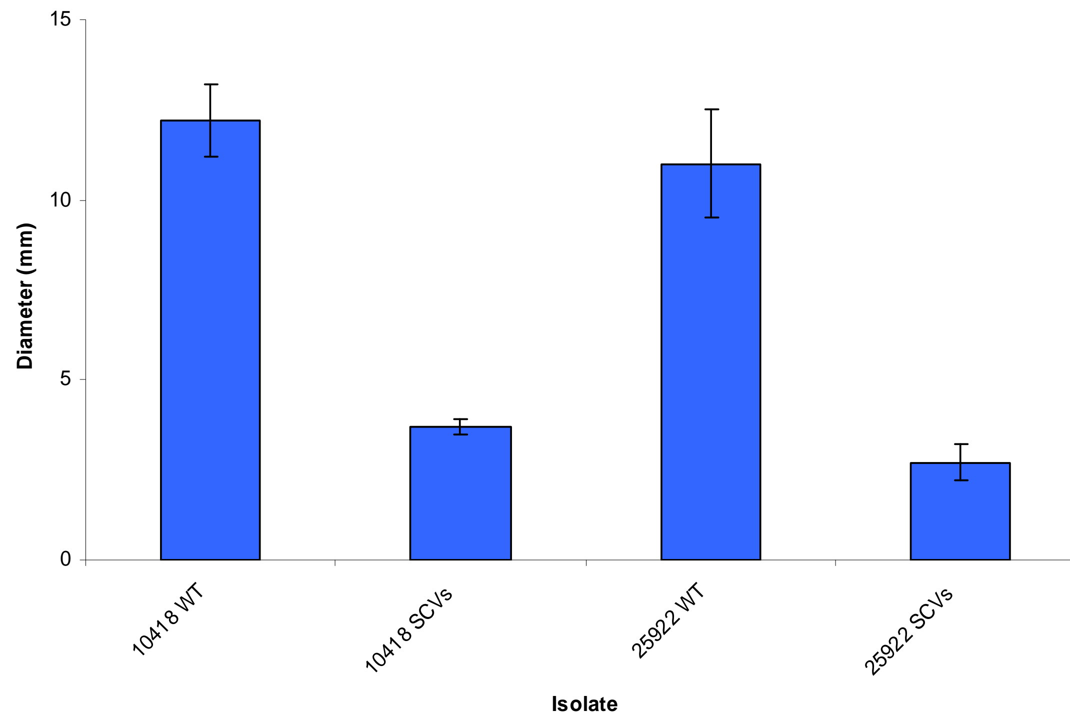


Figure 6.7 Swimming motility of *E. coli* SCV and parent strains. *E. coli* SCVs produced reduced zones of swimming motility in both strains examined. Results represent the mean of three independent replicates and three independent biological replicate. Error bars represent standard error

Table 6.5 Biochemical profiles of *S. epidermidis* LTN SCVs

	Parent	*S. epidermidis* SCV^{GEN}	*S. epidermidis* SCV^{TET}
GLU	+	+	+
FRU	+	+	+
MNE	+	+	+
MAL	+	-	-
LAC	+	-	-
TRE	-	-	-
MAN	-	-	-
XLT	-	-	-
MEL	-	-	-
NIT	+	+	+
PAL	+	-	-
VP	+	+	+
RAF	-	-	-
XYL	-	-	-
SAC	+	+	+
MDG	-	-	-
NAG	-	-	-
ADH	+	+	+
URE	+	+	+

+ - Positive (enzyme activity, fermentation of carbohydrate); - = Negative (lack of enzyme activity, inability to ferment carbohydrates). Differences between SCV and parents are highlighted in red

6.4 Discussion

This study on *E. coli*, *P. aeruginosa* and *S. epidermidis* illustrates that SCV formation can be readily promoted by commonly used antibiotics. Although not all antibiotics examined result in the selection of SCVs, SCV selection with antibiotics (ciprofloxacin, chloramphenicol and tetracycline) that have previously not been known to select for SCVs, was observed.

Regardless of the bacterial species all SCVs isolated produced atypical phenotypic characteristics in comparison with parent strains and therefore phenotypic identification is prone to misidentification. Phenotypic variation has been well studied in *P. aeruginosa* and is known to produce a diverse array of morphological characteristics (Hogardt & Heesemann, 2010). *P. aeruginosa* is easily recognisable when grown on solid agar due to the production of pyocyanin, which was shown to be attenuated in SCVs. The atypical morphology and slow growth rate of SCVs may lead to them being misidentified or the determination of a culture negative in the laboratory testing. Similar problematic issues may arise during phenotypic identification of *E. coli* SCVs. Due to abnormalities in carbohydrate utilisation and growth profiles, SCVs can be misidentified when grown on selective differential medium (Kipp *et al.*, 2005). MacConkey agar is commonly used for the identification of lactose fermenting Gram-negative bacteria. The utilisation of lactose in the medium results in an acidification of pH and a subsequent colour change. However all *E. coli* SCV isolates were unable to utilise lactose which may lead to misidentification.

These results underline the importance of molecular identification as gold standard. In this study RAPD analysis was applied to determine whether SCVs showed clonality with parent strains. Consistent and easily interpretable banding patterns were produced confirming clonality between SCVs and parents. These results are similar to the findings found in RAPD profiles generated from *S. aureus* SCVs recovered following exposure to tetracycline (Chapter 3). RAPD analysis has been applied to *P. aeruginosa* isolates recovered from patients suffering from CF (Mahenthiralingam *et al.*, 1996). Many of these isolates displayed alteration in colonial morphology and motility but continued to produce a consistent RAPD fingerprints. The RAPD profiles generated in this study are in agreement with the work of Mahenthiralingam *et al.*,

(1996), as the various differences alterations observed in SCVs do not correspond to a change in RAPD profiles. Although RAPD strain typing is highly discriminatory, the technique is subject to variability between laboratories. There are alternative PCR identification methods which could also be employed to aid SCV identification. Several *P. aeruginosa* specific gene targets are available but *ecfX* (which encodes an extracytoplasmic function sigma factor) has been demonstrated to be both discriminatory and reliable (Lavenir *et al.*, 2007). Multiplex PCR protocols are available for the identification of *E. coli* (Toma *et al.*, 2003) which allows differentiation of different *E. coli* pathovars. Finally multiplex PCR allowing identification of *S. epidermidis* as well as differentiation from *S. aureus* (Zhang *et al.*, 2004) would permit accurate determination of *S. epidermidis* SCV isolates.

P. aeruginosa is intrinsically resistant to many antimicrobial agents (most β-lactams the older quinolones, chloramphenicol, tetracycline, macrolides, and rifampin) and thus very few antipseudomonas agents are available (Rossolini & Mantengoli, 2005). The observation that gentamicin can select for SCVs is thus important as it remains one of the few agents available to treat *P. aeruginosa* infection. SCVs have been implicated in persistent and recurrent infections (Roggenkamp *et al.*, 1998; von Eiff *et al.*, 1998) and thus treatment with gentamicin (and possibly other aminoglycosides) may present risks through the selection of SCVs. Several antibiotics were also able to select for SCVs of *E. coli* and *S. epidermidis*. Although these species do not show intrinsic resistance to the same degree as *P. aeruginosa*, antibiotic resistance in isolates has become apparent (Arciola *et al.*, 2005; Mathai *et al.*, 2001). The observation that various antibiotics can select for the SCV phenotype therefore has implications in treatment and management of infections.

The bacterial signal molecule cyclic-diguanylate GMP (c-di-GMP) has been linked to SCV phenotype in *P. aeruginosa* (Meissner *et al.*, 2007). c-di-GMP controls several cellular functions in many bacteria, but is principally related to the regulation and transition of the motile to the sessile form (Jenal & Malone, 2006). Further investigation has shown that the *yfiBNR* genes in *P. aeruginosa* produce a signalling molecule that regulates c-di-GMP levels in *P. aeruginosa* (Malone *et al.*, 2010). Disruption of the YfiR (which regulates YfiN and subsequently c-di-GMP) leads to the production of the SCV phenotype through increased c-di-GMP production.

Antibiotics such as aminoglycosides influence the expression of c-di-GMP (Hoffman *et al.*, 2005), which may explain the ability of gentamicin to select for SCVs. Parallels can be drawn between this mechanism and the alternative sigma factor, sigma B (σ^B) in *S. aureus* which influences formation of the SCV phenotype (Mitchell *et al.*, 2010a). It is feasible that σ^B activity governs SCV formation in *S. epidermidis* as comparison of σ^B between the two species show a similar organisation although there are variations in their role and function (Kazmierczak *et al.*, 2005).

The identification of auxotrophy in *E. coli* and *S. epidermidis* SCVs confirmed disruption of the bacterial electron transport chain (ETC). Auxotrophy is a common occurrence in SCV isolates recovered from antibiotic exposure (Balwit *et al.*, 1994; Lewis *et al.*, 1991). The resulting defects in electron transport result in major energy deficiencies resulting in atypical colony morphologies and growth profiles. Although auxotrophy was not detected in *P. aeruginosa*, SCV isolates shared characteristics with SCVs from other species, suggesting defects in ETC are responsible for the SCV phenotype. Haemin and menadione both play roles in *P. aeruginosa* electron transport (Matsushita *et al.*, 1980), therefore defects in other components of the ETC may be responsible for the SCV phenotype. *E. coli* and *S. epidermidis* SCVs also displayed atypical biochemical characteristics in comparison to parent strains. This is an agreement with previous analysis in *E. coli* (Lewis *et al.*, 1991) and *S. epidermidis* SCV (Al Laham *et al.*, 2007) This may be attributed to interruption of the ETC and inability to utilise the tricarboxylic acid cycle (TCA cycle; Proctor, 2006).

P. aeruginosa is a motile organism that can move via the process of swarming and swimming; both functions are dependant on the action of flagella (Henrichsen, 1972). The observations in this study suggest that *P. aeruginosa* SCVs are flagella deficient in comparison to parent strains. *P. aeruginosa* flagella have been shown to be an important virulence factor, and are required for the establishment of respiratory tract infections (Feldman *et al.*, 1998). The loss of flagella activity may relate to an adaption after infection has been established, which has been observed in *P. aeruginosa* recovered from CF patients (Luzar *et al.*, 1985; Starkey *et al.*, 2009). The findings presented in this study suggest that exposure of *P. aeruginosa* to gentamicin, results in the production of the SCV phenotype similar to that observed in CF, which serves as an environmental adaptation.

The various physiological changes that have been reported in *P. aeruginosa* isolates from CF patients are often the result of adaptation to the CF lung (Smith *et al.*, 2006; Sriramulu *et al.*, 2005). *P. aeruginosa* SCVs recovered from CF patients have been shown to exhibit similar characteristics suggesting the SCV phenotype may play a key role in the pathogenesis of *P. aeruginosa* lung infection (Haussler *et al.*, 2003b). Although the *P. aeruginosa* SCVs isolates in this study share characteristics with CF SCV isolates, this study additionally reports reduction in pyocyanin production and elastolytic activity. Pyocyanin mediates tissue damage and necrosis during lung infection (Lau *et al.*, 2004). This stimulates the immune system which increases the number of macrophages, CD4 (+) T cells and neutrophils at the site of infection (Caldwell *et al.*, 2009). Elastase is a zinc metalloprotease secreted by *P. aeruginosa* that causes tissue destruction during infection due to its proteolytic activity (Galloway, 1991). The reduced activity of these virulence factors shares common features with *S. aureus* SCVs which show reduced production of certain virulence factors (Tuchscherr *et al.*, 2010). Reduction in virulence factors in the CF lung serves as an adaptation to a less aggressive lifestyle geared towards persistence (Hogardt & Heesemann, 2010) which *P. aeruginosa* SCVs are suited to. The SCV phenotype may serve as a survival mechanism to respond to a change in environment (exposure to antibiotics), similar to adaptations that occur in the CF lung.

Disc diffusion assays were used to provide a measure of antimicrobial susceptibility. All SCVs (regardless of bacterial species or selecting agent) showed a reduction in susceptibility to gentamicin. Auxotrophy was defined in the majority of SCV isolates, confirming that disruption of the ETC is present in these isolates. Disruption of the ETC results in a decreases in the uptake of gentamicin. The isolates in which auxotrophy were not detected, reduced gentamicin susceptibility may be attributed to disruption of the ETC, since they share many characteristics with the auxotrophic SCV isolates. *E. coli* and *S. epidermidis* SCVs also showed reduced susceptibility to a variety of different classes of antimicrobial agents in comparison to parent strains. An explanation for this reduced susceptibility is the reduced growth rate observed in all SCV isolates. Antibiotics target various bacterial cellular processes, including protein, DNA and cell wall synthesis and thus altered rates of bacterial growth rate coincides with the response to antimicrobial agents (Tuomanen *et al.*, 1986). As SCV isolates displayed reduced growth rate these processes work at a slower rate thus reducing

susceptibility. This is supported by the observation that *E. coli* SCV^{CIP} showed the slowest growth rate in comparison to other *E. coli* SCV isolates and the greatest reduction in susceptibility to all antibiotics examined.

Hydrophobicity is an important factor in bacterial adhesion to surfaces and to each other (van Loosdrecht *et al.*, 1987). Increased hydrophobicity has been attributed to the abundance of pili in both *E. coli (*Drumm *et al.,* 1989*)* and *P. aeruginosa (*Speert *et al.,* 1986*).* As *E. coli* and *P. aeruginosa* SCVs isolated in this study displayed increased hydrophobicity, it is postulated that these SCVs show an increased abundance of pili in comparison to parent strains. This is further supported by the increase in twitching motility observed in *P. aeruginosa* SCVs as twitching motility in *P. aeruginosa* is dependent on the action of pilli (Bradley, 1980) and mutants defective in pili production are deficient in twitching motility (Shan *et al.*, 2004). The increase in the abundance of pili may increase the capacity of SCVs to form biofilms. *P. aeruginosa* mutants that are deficient in the synthesis of type IV pili are also deficient in the formation of biofilms on abioitic surfaces (O'Toole & Kolter, 1998). Similarly in *E. coli*, mutants deficient in pili production are also deficient in biofilm formation (Pratt & Kolter, 1998). Although both of these studies show the crucial importance of pili in biofilm formation it is important to note that the deficiencies in different type pili systems impacts on different stages of biofilm development. Nevertheless the data shown in this study suggest that the presence of pili may be linked to biofilm formation in *P. aeruginosa* SCVs. An increased abundance of pili has been reported in *P. aeruginosa* SCVs isolated from CF patients and linked to biofilm formation (Haussler *et al.*, 2003b) adding evidence to the links proposed.

In contrast, *S. epidermidis* SCVs displayed no difference in cell surface hydrophobicity in comparison with parent strains but still showed an increased capacity to form biofilms. The σ^B mediated selection mechanism proposed previously correlates with the enhanced biofilm capacity in *S. epidermidis* SCVs. Previous studies have demonstrated that *S. epidermidis* mutants constitutively expressing σ^B displayed increased production of polysaccharide intracellular adhesin (PIA) therefore increasing biofilm formation (Jager *et al.*, 2009). Increased expression of σ^B can be proposed as the genetic mechanism for increased biofilms formation in *S. epidermidis* SCVs. Biofilm formation in *S. epidermis* is considered as one of the organism's key

virulence factors in mediating device associated infections (McCann *et al.*, 2008). A switch to the SCV phenotype may facilitate persistence through the increased capacity to form biofilms.

The production of the SCV has been linked to persistence and survival in *S. aureus* (Proctor *et al.*, 2006). SCVs isolated from diverse bacterial species that inhabit many different environments share similar characteristics that have come to be associated with the SCV phenotype. The SCV phenotype appears to be a common mechanism utilised by bacteria to permit survival in the presence of antimicrobial compounds which subsequently permits persistence via reduced antimicrobial susceptibility and biofilm formation. The switch from a fast growing virulent phenotype to a slow growing SCV seems a small price to pay for survival.

6.5 Conclusions

- Exposure of *E. coli* to ciprofloxacin and chloroamphenicol and *S. epidermidis* to tetracycline results in selection for SCVs.
- Exposure of *E. coli*, *P. aeruginosa* and *S. epidermidis* to gentamicin results in selection for SCVs.
- *E. coli*, *P. aeruginosa* and *S. epidermidis* SCVs share known characteristics with SCVs including atypical colony morphology and growth profiles.
- *E. coli* and *S. epidermidis* SCVs are auxotrophic for compounds which have roles in electron transport.
- Susceptibility to various antimicrobial agents is reduced in SCVs.
- *E. coli* and *P. aeruginosa* SCVs have an increased capacity to form biofilms which appears to be related to increased abundance of pili.
- RAPD analysis confirmed SCVs show clonality with parent strains.
- *E. coli* and *S. epidermidis* SCVs display atypical biochemical profiles.
- *P. aeruginosa* SCVs are deficient in swarming and swimming motility, as well as deficient in the production of virulence factors; elastase and pyocyanin.

7 CHAPTER 7: GENERAL CONCLUSIONS AND DISCUSSION

7.1 Conclusions

The work presented in this study aimed to investigate the ability of various antimicrobials to select for *S. aureus* SCVs and investigate their capacity to form biofilms as well as examine their susceptibility to a range of plant antimicrobial compounds. Antibiotic selection for SCVs in other bacterial species was also examined and these isolates were characterised and their capacity to form biofilms investigated. In this chapter the general conclusions of each main area of work will be reiterated with respects to the aims mentioned in Chapter 1.

- Various aminoglycosides can select for *S. aureus* SCVs at a range of concentrations.
- The formation of SCVs is a hindrance to accurate aminoglycoside susceptibility testing, which led to the development of a 'minimum SCV prevention concentration', which ensures eradication rather than SCV selection at higher concentrations.
- Variations in carbohydrate utilisation, carotenoid production, levels of intracellular ATP, mutation frequency and rates of reversion are apparent between SCVs selected at different aminoglycoside concentrations.
- Members of the tetracycline family of antibiotics can select for *S. aureus* SCVs
- Tetracycline selected SCVs show attenuated catalase, coagulase and heamolysis activity and reduced production of extracellular DNase and lipase and reduced susceptibility to various antimicrobial agents.
- *S. aureus* SCVs show increased biofilm formation in comparison to parent strains which appears to be linked to increased production of polysaccharide intracellular adhesin.
- *S. aureus* SCV and parent biofilms show reduced susceptibility to various antimicrobial agents in comparison to planktonic cells.
- The reduction in antimicrobial susceptibility is further pronounced in *S. aureus* SCV biofilms which is linked to reduced antimicrobial penetration through *S. aureus* SCV biofilms.

- *S. aureus* SCV and parents are susceptible to various plant antimicrobial compounds of which SCVs are more susceptible to cinnamon bark, green tea and oregano.
- Resistance to plant antimicrobials was not detected following continuous exposure of *S. aureus* SCVs to sub lethal concentrations.
- Various plant antimicrobials display a synergistic relationship against *S. aureus* with various antibiotics including oxacillin, neomycin and tetracycline.
- Gentamicin can select for SCVs in *Escherichia coli*, *Pseudomonas aeruginosa* and *S. epidermidis*. Additionally exposure of *E. coli* to ciprofloxacin and chloramphenicol and *S. epidermidis* to tetracycline selected for SCVs.
- SCVs from these bacterial species share characteristics with *S. aureus* SCVs including altered growth and biochemical profiles, auxotrophy for compounds involved in electron transport, reduction in virulence factors and reduced antimicrobial susceptibility.
- All SCVs showed an increased capacity to form biofilms compared to their parent strains.

7.2 General discussion

7.2.1 Identification and treatment of SCV infections

Despite the observation that *S. aureus* SCVs are often associated with infections commonly caused by biofilms, planktonic cells are still of the utmost importance in species and strain identification and determining susceptibility profiles in clinical laboratories. However the identification of SCVs is riddled with difficulty. It is well documented that identification of *S. aureus* SCVs is difficult and automated systems can misidentify SCVs (Seifert *et al.*, 1999; Spanu *et al.*, 2005). Atypical growth rates can lead to their presence being missed on agar plates and their atypical phenotypic and enzymatic characteristics can also lead to misidentification. In this study multiplex PCR was successfully employed for the identification of *S. aureus* SCVs. Molecular identification of SCVs has been applied previously and proved successful (Sendi *et al.*, 2006; von Eiff *et al.*, 1999). RAPD fingerprinting also showed that SCV isolates showed clonality with parent strains. Although molecular diagnostics may increase expense compare with conventional diagnostic microbiology it overcomes the uncertainty that may surround the identification of SCVs.

In order for susceptibility testing to be performed cultivation is required. When investigating SCVs in species other than *S. aureus* conventional CLSI microdilution susceptibility proved difficult due to the formation of biofilms in microtitre plates. However the use of disc diffusion method of susceptibility overcame these associated problems. Again these issues highlight difficulties during susceptibility testing of SCVs. Molecular determination of resistance determinants such as *mecA* may prove useful for determining SCV susceptibility. Furthermore, the instability of the SCV phenotype represents further challenges. SCVs may be the causative agent of infection, but the subsequent cultivation may result in reversion to the parent phenotype which may have implications for the chosen treatment regime. Taking into account these issues, an awareness of the SCV phenotype (particularly in the diseases they have been associated with such as CF and osteomyelitis) may aid accurate diagnostics and change treatment course.

Research presented here documents for the first time for the tetracycline class of antibiotics to select for *S. aureus* SCVs. The SCV phenotype represents a novel

mechanism for reduced tetracycline susceptibility in *S. aureus*. As the uptake of tetracycline is partially energy dependent (McMurry & Levy, 1978) and attributed in part to the presence of a proton motive force (Smith & Chopra, 1984), the reduced susceptibility to tetracycline in SCVs may be related to reduced uptake. Tetracycline and other tetracycline antibiotics have been successfully applied for the treatment of MRSA infections (Ruhe *et al.*, 2005). A follow up of patients in the latter study would have provided an interesting observation to determine if any relapse of infection were reported. This would perhaps correlate with the presence of SCVs. Although the selection of SCVs does not result in a large reduction in antimicrobial susceptibility, the finding that tetracycline can select for *S. aureus* (and also *S. epidermidis*) SCVs may have implications in its clinical use. Investigating the ability of the recently developed tigecycline to select for SCVs would be an interesting further investigation.

Exposure to several aminoglycosides at a wide range of concentrations also resulted in the selection of *S. aureus* SCVs. Although gentamicin has a long history of SCV selection this study was the first to address concentration dependent selection. Interestingly the concentration of selecting aminoglycoside impacts on the characteristic of the SCV selected for and a clear correlation between aminoglycosides concentration and SCV characteristics are apparent. The observation that a broad range of concentrations selects for *S. aureus* SCVs is also an important finding. The concentrations examined range from bacteriostatic to bactericidal; however the distinction between bacteriostatic and bactericidal activity can often be arbitrary in the clinical sense. Achieving bactericidal activity is crucial for the effective treatment of various bacterial infections (Pankey & Sabath, 2004). As aminoglycosides are considered as bactericidal agents, they may be employed a high concentrations to treat infection. However the use of bactericidal concentrations can select for a SCV population, hence the development of the term 'minimum SCV prevention concentration'. This ensures eradication of wildtype and thwarts the selection of SCVs.

7.2.2 SCV and persisters

Some overlap exists between the SCV phenotype and a state of bacteria termed persisters which are often recovered following antibiotic exposure. Persisters have been described as a 'subpopulation of dormant cells that have been implicated in a

range of chronic and recurrent infections through their ability to survive antibiotic treatments' (Jermy, 2011). Although persisters are antibiotic tolerant, they differ from antibiotic resistance mutants as their antibiotic tolerance is not hereditary and can be reversed when grown in the absence of antibiotics (Jayaraman, 2008). Persisters have also been implicated in chronic infectious diseases, including cystic fibrosis (CF) patients infected with *Pseudomonas aeruginosa* and *Candida albicans* in oral thrush patients (Lewis, 2010). The observation that SCVs have also been implicated in persistent infections suggests that some similarities exist between the two phenotypes. Some overlap in gene expression profiles in SCVs and persisters is apparent; for instance, expression of stress response proteins (heat shock, SOS response) and operons involved in oxidative phosphorylation (NADH dehydrogenase, ATP synthase, and cytochrome *O*-ubiquinole oxidase) are altered in persisters (Keren *et al.*, 2004). Similarly in *S. aureus* SCVs alteration in the expression of genes with functions in electron transport and global regulators involved in stress response and virulence are altered (Moisan *et al.*, 2006; Seggewiss *et al.*, 2006). An important contrasting characteristic between SCVs and persisters is growth rate. SCVs are metabolically active although they exhibit atypical growth profiles. On the other hand persisters are regarded as non-growing, dormant cells that exist as a distinct physiological state (Balaban *et al.*, 2004; Shah *et al.*, 2006).

SCVs and persisters have been associated with antimicrobial resistance. Although SCVs and persisters do not follow classical resistance mechanisms, there are differences in the mechanisms that contribute to reduced antibiotic susceptibility/tolerance. In persisters of *Escherichia coli* toxin-antitoxin (TA) modules and other genes block translation in protein synthesis (Keren *et al.*, 2004). This results in the shutting down of cellular processes and targets which antimicrobial agents required to be active in order to effective. The non-dividing state results in the tolerance observed in persisters. In SCVs however, reduced susceptibility to antimicrobial agents rather than tolerance is observed. A perturbed electron transport chain results in a reduced membrane potential and uptake of positively charged antimicrobials such as aminoglycosides is reduced. Defects in electron transport result in a slower growing phenotype however cellular processes are still active in contrast to persisters. As such, it is important to document that although persisters and SCVs

can both be linked to recurrent and refractory infections and some overlap exists between the states, they must be viewed as separate entities.

7.2.3 Biofilm formation in SCVs

In this study SCVs from several bacterial species displayed an increased capacity for biofilm formation in comparison to parent strains. Biofilms have been suggested to be the root of many chronic and persistent infections and are associated with increasing healthcare cost and morbidity. Furthermore, approximately 60% of bacterial infections are thought to involve biofilms (Costerton *et al.*, 1999) yet the role of SCVs in these biofilm-related infections remains largely unknown. The ability of SCVs to form biofilms has clear implications in biofilm associated infections. It is well documented that bacteria present in biofilms showed a reduced growth rate which contributes to reduced antimicrobial susceptibility (Mah & O'Toole, 2001). Nutrient limitation and oxygen limitation is thought to be responsible for slow growth rate in biofilms (Brown *et al.*, 1988). The SCV phenotype and the biofilm phenotype appear to share similarities. SCVs were not detected from parent biofilms, however it is important to note that SCVs are frequently unstable and reversion during plate counting may have meant SCVs went undetected. SCVs were shown to disseminate from *S. aureus* parent biofilms that had been exposed to antimicrobials and their growth characteristics and biofilm forming capacity suggest the SCV phenotype permits an optimised phenotypic state for biofilm growth and proliferation of biofilms. Couple with their reduced growth rate and reduced antimicrobial susceptibility the formation of a SCV phenotype serves as an adaptive plasticity.

7.2.4 Novel biofilm treatment strategies

This study also demonstrates the reduced antimicrobial susceptibility of biofilms. Biofilms displayed reduced susceptibility to various antimicrobials which we observed in several wildtype *S. aureus* strains. However, biofilms formed by SCV isolates showed further reduced susceptibility in comparison. One of the novel approaches to treating biofilm associated infections is the use of bacteriophage. Bacteriophages are viruses that infect bacteria and can follow a virulent lytic or lysogenic lifestyle. The use of bacteriophage to control device associated infections offers several advantages over antimicrobial agents. Firstly, whilst older biofilms are more difficult to eradicate using conventional antimicrobials, bacteriophage treatment

is not affected by biofilm age (Amorena *et al.*, 1999; Hanlon *et al.*, 2001). Hanlon *et al.,* (2001) also documented that biofilm thickness and the presence of polysaccharide did not limit the diffusion of phage through biofilms, which in the study antibiotic penetration has a direct impact on antimicrobial susceptibility. Finally, bacteriophage can be engineered to express biofilm degrading enzymes. The treatment of biofilms with such degrading enzymes, such as dispersin B (which causes hydrolysis of glycosidic linkages in biofilm polysaccharide) can eradicate bacterial biofilms (Itoh *et al.*, 2005) and use of bacteriophage expressing these enzymes enabled high levels of anti-biofilm activity (Lu & Collins, 2007). Bacteriophage K (a member of the *Myoviridae* phage family) has been shown to inhibit various clinically isolated *S. aureus* strains and other staphylococcal species (O'Flaherty *et al.*, 2005), although it has yet to be tested on biofilms formed by *S. aureus*. Bacteriophage K has been demonstrated to reduce biofilm mass in biofilms formed by *S. epidermidis* (Cerca *et al.*, 2007). The engineering of bacteriophage K to produce biofilm degrading enzymes such as dispersin B may offer an attractive option to combat SCV biofilms. Other novel approaches for the eradication of staphylococcal biofilms include the inhibition of quorum sensing (Balaban *et al.*, 2007) and the impregnation of biomaterials with novel antibacterial compounds (such as usnic acid, a secondary lichen metabolite) to inhibit biofilm formation (Francolini *et al.*, 2004).

7.2.5 The SCV phenotype as a survival strategy

An overview of the research presented here and a review of the literature implicates the role of the SCV phenotype as survival mechanism in *S. aureus* and other bacterial species. The ability to switch to an altered phenotype in the presence of antimicrobial agents is clearly favourable if it permits survival. SCVs 'trade in' many characteristics that are associated with rapid growth in order to survive in unfavourable conditions. Various reports of phenotypic switching have been reported in the literature including the formation of persisters, in the presence of antibiotics. Other examples include variation of membrane surface lipoprotein antigens in *Mycoplasma bovis* (Lysnyansky *et al.*, 1996) and altered expression of surface determinants in *Burkholderia pseudomallei* (Chantratita *et al.*, 2007). Formation of the SCV phenotype in *S. aureus* can also be viewed as a phenotypic switching system which appears to be strongly influenced by the alternative sigma factor, σ^B (Mitchell *et al.*, 2010a). The observation that SCVs characteristics correlates to the concentration of

the selecting aminoglycoside suggests that σ^B is influenced in a concentration dependent manner. Rates of reversion in SCV isolates showed differentiation with SCVs selected a higher concentrations remaining stable. In these variants σ^B may constantly upregulated thus locking them into the SCV phenotype. The influence of σ^B and the locking in of the SCV phenotype has been observed in *S. aureus* SCV isolated from CF patients (Mitchell *et al.*, 2008). Clearly σ^B plays an important role in the SCV phenotype in *S. aureus* SCVs and their selection in the presence of aminoglycosides. Various other antimicrobial agents are known to select for *S. aureus* SCVs and it would be interesting to monitor the expression of σ^B (via real time PCR) to determine whether these antibiotics influence the expression of the global regulator and are involved in SCV formation.

7.2.6 Novel antimicrobials for *S. aureus* and SCVs

The ability of *S. aureus* to develop resistance to variety of antimicrobial agents and the ability to switch to the SCV phenotype results in diminishing therapeutic options for treatment of these infections caused by these organisms. As discussed previously the difficulties surrounding antibiotic resistance and the lack of novel antibiotics in development have worrying implications in the healthcare setting. Cinnamon bark, ginger grass, green tea and oregano all have activity against *S. aureus* as demonstrated via disc diffusion and broth dilution methods. Broth dilution testing revealed *S. aureus* SCVs to be more susceptible to cinnamon bark, green tea and oregano which is encouraging as SCV show reduced susceptibility to various antimicrobial agents. Another significant observation was that none of the plant antimicrobials examined showed selection for *S. aureus* SCVs, which taking into consideration of previous finding may be a consideration in the treatment of *S. aureus* infection. Although the plant antimicrobials concentrations that were inhibitory are a lot higher than the concentrations of commonly used antibiotics, this research also highlights their possible use in combination with antibiotics. Various synergistic relationships were observed thus there is that these plant antimicrobials offer a novel avenue to combat antibiotic resistance.

The susceptibility of *S. aureus* SCVs to plant antimicrobials may have applications in the treatment of biofilm associated infections. For example carvacrol and eugenol are effective against biofilms formed by *E. coli* O157:H7 and *Listeria monocytogenes*

(Perez-Conesa *et al.*, 2011). Specifically carvacrol and thymol can inhibit the first step in biofilm formation (initial adherence) in *S. aureus* (Nostro *et al.*, 2007). The application of these compounds to biomaterials such as catheters and prosthetics may provide a useful strategy to block biofilm formation.

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APPENDIX 1

Enzymatic Reactions of API STAPH Test Strip

Tests	Substrates	Reactions/Enzymes	Result	
			Negative	Positive
0	No Substrate	negative Control	red/orange	-
GLU FRU MNE MAL LAC TRE MAN XLT MEL	D-glucose D-fructose D-mannose maltose lactose D-trehalose D-mannitol xylitol D-melibiose	acidification due to carbohydrate utilization	red	yellow
NIT	potassium nitrate	reduction of nitrate to nitrite	colourless-Light pink	red/purple*
PAL	B-naphthyl-acid phosphate	alkaline phosphatase	yellow	violet#
VP	sodium pyruvate	acyl-methyl-carbinol production	colorless/light pink	pink/violet^
RAF XYL SAC MDG NAG	raffinose xylose sucrose α-methyl-D glucoside N-acetyl-glucosamine	acidification due to carbohydrate utilization	red	yellow
ADH	arginine	arginine dihydrolase	yellow	orange-red
URE	urease	urease	yellow	red-Violet

*1 drop of the reagents NIT-1 and NIT-2 was added, and left for 10min before result being read.

1 drop of the reagents ZYM A and ZYM B were added, and left for 10min before result being read.

^1 drop of the reagents VP-1 and VP-2 was added, and left for 10 min before result being read.

ADH and URE- Anaerobic conditions needed for these reactions. This was achieved by filling up to the cupule meniscus with mineral oil.

API Staph medium (composition per litre) 0.5 g yeast, 10 g bactopeptone, 5 g NaCl, 10 mL trace elements

Enzymatic Reactions of API E Test Strip

Tests	Substrate	Reactions/Enzymes	Result	
			Negative	Positive
ONPG	2-nitrophenol-ßD-galactopranoside	ß-galactosidase	colorless	Yellow
ADH	L-arginine	arginine dihydrolase	yellow	red/orange
LDC	L-lysine	lysine decarboxylase	yellow	red/orange
ODC	L-omithine	omithine decarboxylase	yellow	red/orange
CIT	trisodium citrate	citrate utilisation	yellow	blue/green
H_2S	sodium thiosulfate	H_2S production	colorless	black deposit
URE	urea	urease	yellow	red/orange
TDA	L-tryptophane	tryptophane deaminase	yellow	red*
IND	L-tryptophane	indole production	yellow	red ring#
VP	sodium pyruvate	acetoin production	colorless	pink/red^
GEL	gelatin	gelatinase	no diffusion	diffusion of pigment
GLU	D-glucose	fermentation/oxidation	blue/green	yellow
MAN	D-mannitol	fermentation/oxidation	blue/green	yellow
INO	inositol	fermentation/oxidation	blue/green	yellow
SOR	D-sorbitol	fermentation/oxidation	blue/green	yellow
RHA	L-rhamnose	fermentation/oxidation	blue/green	yellow
SAC	D-sucrose	fermentation/oxidation	blue/green	yellow
MEL	D-melibose	fermentation/oxidation	blue/green	yellow
AMY	amygdalin	fermentation/oxidation	blue/green	yellow
ARA	L-arabinose	fermentation/oxidation	blue/green	yellow

*1 drop of the TDA reagent added and result read immediately

1 drop of the JAMES reagent added and result read immediately

^1 drop of the reagents VP-1 and VP-2 was added, and left for 10 min before result being read.

ADH, LDC, ODC, H2S and URE - Anaerobic conditions needed for these reactions. This was achieved by filling up to the cupule meniscus with mineral oil.