SCV'S: FORMATION AND CHARACTERISATION IN STAPHYLOCOCCUS SP

Thesis presented for the Degree of Philosophiae Doctor

by

Naiyf Sultan Alharbi

Cardiff School of Biosciences

Cardiff University

February 2013

TO MY PARENTS FOR THEIR CONSTANT SUPPORT AND DEVOTION

TO MY WIFE AND CHILDREN FOR THEIR LOVE,
ENCOURAGMENT AND UNDERSTANDING

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed …… (candidate) Date ……

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD

Signed …… (candidate) Date ……

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.
Other sources are acknowledged by explicit references.

Signed …… (candidate) Date ……

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed …… (candidate) Date ……

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Graduate Development Committee.**

Signed …… (candidate) Date ……

ACKNOWLEDGEMENTS

I am very grateful to my supervisor Dr. Martin Day for his continuous advice, encouragement and friendship throughout this study. I am also indebted to Dr. Andrew Morby for support, guidance and assistance in the genome sequencing component of my research.

During my PhD I have received support from many different people:
I would like to thank Dr. Phillip Norville and Dr. Gordon Webster for their help and advice throughout my research.

I would like to thank my uncle Battal, brothers, sisters and cousins for their continual support. I also extend my thanks to James Evans for his help in teaching me the process of statistical analysis I used on my data, and to Fahad Almuzini for help in the design some of the drawings.

Acknowledgements are also due to my fellow PhD researchers, Nicolas Passiotis, Angharad Williams, Laura Thomas, Matt Bull, Othman Boshi, Laura Morris and Kwestan Hama Chawash. I also give a big ‘thank you’ to Joan for all her kindness during my PhD studies. Thanks you all for your friendship and helpful discussions.

I gratefully acknowledge the award of a grant from king Saud University, Saudi Arabia.

SUMMARY

Staphylococcus aureus is the most common cause of hospital-acquired infection and contributes significantly to patient morbidity and mortality. The ability of *S. aureus* to switch to an alternative phenotype in the presence of antimicrobial agents is clearly favourable. One of these alternatives are small colony variants (SCVs). The novel phenotypes include changes to colony morphology, antibiotic susceptibility, haemolytic activity and many other physiological activities. It is now recognised that SCVs have a deficiency in electron transport, owing to mutations affecting its efficacy.

This study investigated SCVs in various ways. In the evolution component changes (mutations) occurring sequentially in successive cycling (15 cycles), were identified. In this experiment selection was made for sequentially SCV mutants and wild type revertants. Two sequenced clinical MRSA strains COL and N315 were chosen so changes in sequence in SCVs and wild type revertants could be compared. Selection for SCVs was made independently for triclosan and gentamicin for both strains. The final SCV and WT strains isolated were compared physiologically and genetically and showed differences in frequency, biochemical profiles, pigment production, haemolysis, catalase, coagulase, levels of intracellular ATP and phage yield. The genomic sequence of the final 4 cycle isolates (SCV15) showed numerous and diverse mutations occurred COL and N315 SCVs. Over 70 mutations were found and 33 were determined as *historic* mutations and the rest were termed *novel* mutations. The novel mutations occurred during the cycling process. The *historic* mutations occurred prior to the experiment and these mutations were acquired during growth in laboratory culture. Only one mutation was found to be common between COL and N315 and this was in the *fab*I gene. These data indicate mutations occurring in ~1.3% of the genome (~ 40 Kb) can generate mutants with the SCV phenotype.

Susceptibility to phage 80α and transduction of *S. aureus* wild type and their SCVs 1-3 was studied. Wild type strain of *S. aureus* and SCV3 both yielded a high number of lysogens (~68%) the remaining being resistant mutants. SCV 1 and SCV 2 provided a much lower proportion of lysogens (4-10%). There was no obvious relationship between cellular ATP levels and lysogen formation. Consequently the frequency of lysogen formation (or that of resistance mutants) cannot be related to energy status.

Transduction of ciprofloxacin resistance (*grlA*) was observed into COL wild type at a 5-10-fold higher frequency than into SCV1. Transduction of rifampicin resistance (*rpoB*) into SCVs was reduced almost 10-fold. As transduction was significantly decreased into SCVs it is hypothesised this process was influenced by ATP levels. The data thus suggests that SCV strains will be less efficient in gene exchange by transduction *in vivo*.

Three SCVs previously isolated from *S. aureus* COL on the basis of different growth rate were further studied. Results clearly support the hypothesis that there is a physiological diversity in SCV populations.

Sensitivity of *S. aureus* wild type and SCVs strains to various antimicrobial was determined. The SCV strains were more sensitive to some antibiotics and heavy metals than the wild type strain.

LIST OF FIGURES

LIST OF TABLES

Page

TABLE OF ABBREVIATIONS

A	adenine
AAC	aminoglycoside acetyltransferase
ADP	adenosine diphosphate
Ag	argent
agr	accessory gene regulator
Al	aluminium
AMEs	aminoglycoside modifying enzymes
Amp	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BHI	Brain Heart Infusion
bp	base pairs
BSAC	British Society for Antimicrobial Chemotherapy
C	cytosine
CAMHB	cation adjusted Muller Hinton broth
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
Cd	cadmium
CF	cystic fibrosis
CFS	chronic fatigue syndrome
CFU	colony forming unit
Cip-r	ciprofloxacin resistance
CLSI	Clinical Laboratory and Standards Institute
CoNS	coagulase negative staphylococci
CoPS	coagulase-positive staphylococci
Cr	chromium
CRE	catabolite responsive element
Cu	copper
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EES	ethyl ethane sulphonate
EF-G	elongation factor
EMRSA	epidemic methicillin-resistant *Staphylococcus aureus*
Ery	erythromycin
ETC	electron transport chain
FADH2	flavin adenine dinucelotide

G	guanine
GEN	gentamicin
GFP	green fluorescent protein
GMP	guanine monphosphate synthetase
Hg	mercury
HGT	horizontal gene transfer
HQNO	4-hydroxy-2-heptylquinoline-N-oxide
HX	hypoxanthine
Kan	kanamycin
kb	kilo base
LSD	least significant difference
M	molar
MeC	methylcytosine
MGEs	mobile genetic elements
mg/L	milligrams per litre
MIC	minimum inhibitory concentration
MH	Mueller Hinton
µL	microliter
MLS	macrolide–lincosomide–streptogramin B
MLST	multilocus sequence typing
MMR	methyl-directed mismatch repair
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *Staphylococcus aureus*
Mup	mupirocin
NADH	nicotinamide adenine dinucelotide
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
Neo	neomycin
nm	nanometer
Nov	novobiocin
OD	optical density
ORFs	open reading frames
Oxa	oxacillin
Pb	lead
PBP 2a	penicillin binding protein 2a
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis

PFU	Plaque forming unit
PMF	proton Motive Force
qPCR	quantitative PCR
QRDR	quinolone resistance-determining region
RAPD	random amplified polymorphic DNA
Rif-r	rifampicin resistance
RLU	relative light units
rpm	rotation per minute
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-TRI	SCV selected for in the presence of triclosan
SD	standard deviation
SE	standard error
SEM	Scanning electron microscopy
SEPHCHC	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate
Sn	tin
ST	sequence type
SXT	trimethoprim-sulphamethoxazole
T	thiamine
TAE	tris-acetate EDTA
TCA cycle	tricarboxylic acid cycle
Tec	teicoplanin
Tet	tetracycline
TF	transduction frequency
TRI	triclosan
TSST	toxic shock syndrome toxin
U	uracil
UV	ultraviolet
Van	vancomycin
WT	wild type
XMP	xanthosine monophosphate
$\Delta\Psi$	membrane potential
σ^B	sigma B factor

CONTENTS

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Phenotypic characteristics of *Staphylococcus aureus* parental strains

Staphylococcus aureus is Gram-positive coccus which forms large golden yellow colonies on agar, and it is often haemolytic on blood agar. The bacteria are catalase-positive and oxidase-negative. Nearly all strains of *S. aureus* produce the enzyme coagulase (Todar, 2005). The cell wall contains peptidoglycan and teichoic acid. *S. aureus* is resistant to temperatures as high as 50°C, to high salt concentrations, and to drying. It is perfectly spherical cells about 1 micrometer in diameter and grows in clusters.

Paster and Koch (1878) were the first to observe and culture staphylococci, but the earliest detailed studies on staphylococci were performed by Ogston in 1881 and Rosenbach in 1884 (Ogston, 1882; Cookson *et al.,* 2003). Ogston coined the name *Staphylococcus* to describe grape-like clusters of bacteria (Figure 1.1). He observed in pus from human abscesses and by 1884 a scientist named Rosenbach was able to isolate and grow these microorganisms in pure culture and described two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (golden) and *Staphylococcus albus* (white) (Kloos & Schleifer, 1986) the latter species is now known as *Staphylococcus epidermidis.* The data there are 35 recognised species of staphylococci all of which are Gram-positive non-motile, non-spore and grow optimally under aerobic conditions (Peacock, 2005). The production of coagulase is an important characteristic that allow differentiation from other bacterial genera and species. Staphylococci are generally divided in two main groups, coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) such as *S. epidermidis* based on the production of coagulase, an enzyme-like factor that causes fibrin to coagulate and form a clot, a trait which is generally associated with pathogenicity (Bannerman & Peacock, 2007). Catalase production differentiates *S. aureus* from streptococcal species.

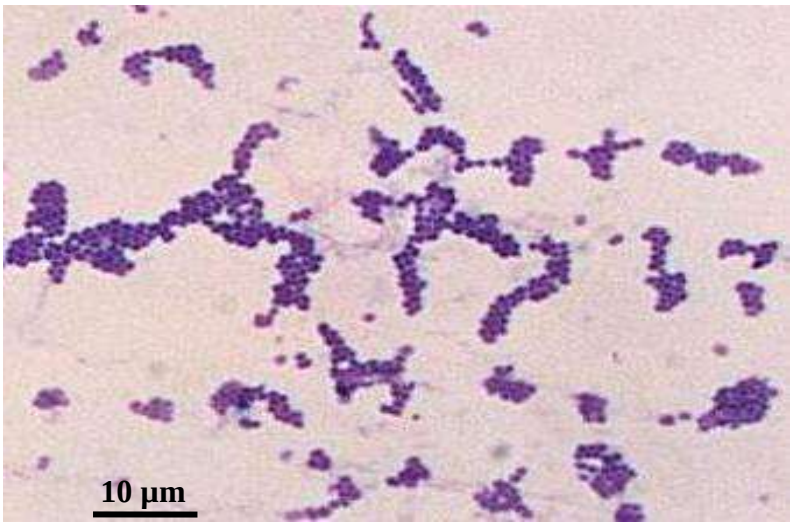
Those *Staphylococcus aureus* strains resistant to antimicrobial agents are clinically significant since they commonly cause problems, both in developing and industrial countries (Wenzel *et al.,* 1981). *S. aureus* is the most common staphylococcal pathogen while *S. epidermidis* is not pathogenic but may cause infections in some circumstances. Since both are inhabitants of the skin, most lesions are superficial and develop as boils, pustules, pimples, furuncles, carbuncles, or impetigo contagiosa (Sun, 1996).

1.2 Pathogenicity of *Staphylococcus aureus*

S. aureus is an opportunistic pathogen and the major causative agent of numerous hospital- and community-acquired infections. 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). *S. aureus* infection generally occurs in those who are immunodeficient and have provided a means of entry into the body, i.e. a cut or wound (Lowy, 1998). It is for this reason that *S. aureus* is prevalent within post-operative infections. *S. aureus* possesses some of the various virulence factors as shown in Table 1.1. Pathogenesis requires the combination of various virulence factors including; secreted proteins, cell surface-bound proteins and cell surface components (Tenover & Gorwitz, 2000). Some global regulatory genes that coordinate the expression of various groups of *S. aureus* virulence genes have been identified (Peng *et al.,* 1988; Dufour *et al.,* 2002; Lindsay *et al.,* 2006). Of the most extensively studied is *agr*, which induces the expression of extracellular protein whilst suppressing the expression of surface protein. *agr* and other regulators appear to coordinate the pathogenesis of staphylococcal infection in a sequential nature; surface protein are predominantly synthesized during the exponential growth phase and secreted proteins during the stationary phase. Thus different stages of infection require differential expression of virulence determinants (Lowy, 1998).

In addition staphylococci have proven very successful at developing and acquiring antimicrobial resistances through mutation and gene exchange (World Health Organisation, 2011). Section 1.8 discusses the potential for gene exchange to contribute to evolution in staphylococci.

(A)



(B)

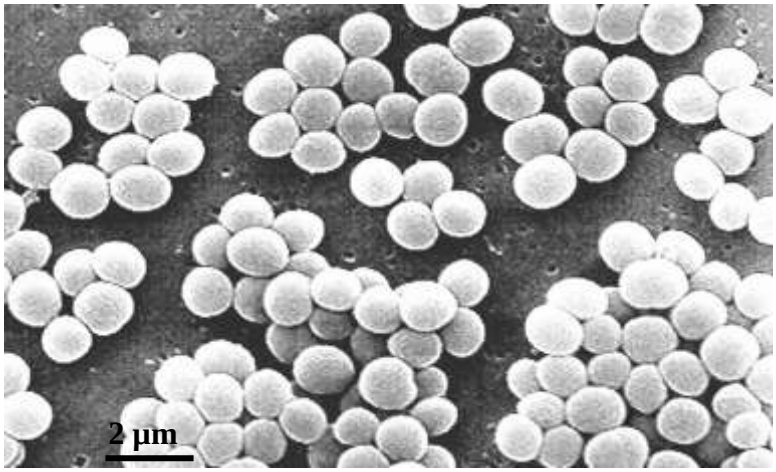


Figure 1.1 Shape and arrangement of *S. aureus* (A) - Light microscopy shows Gram-positive *S. aureus*, arranged in grape-like clusters and magnified about 100x. (B) - Scanning electron microscopy (SEM) shows a higher resolution image of *S. aureus.* Magnified about 10,000x (Seaman, 2007).

Table 1.1 Virulence and related factors contributing to the pathogenicity of *S. aureus*

Virulence factor	Role	Example	References
Adhoidanesins Adhesins	Surface proteins that promote attachment to host proteins such as laminin and fibronectin.	Coagulase and fibronectin-binding protein.	Moreillon *et al.*, 1995 & Menzies, 2003.
Invasins	Enable invasion by damage of host membranes.	α-toxin, β-toxin, δ-toxin, γ-toxin and leukocidin.	Novick, 2003a & Gillet *et al.*, 2002
	Promote bacterial spread by dissolution of fibrin clots.	Staphylokinase.	Collen, 1998
	Enhance survival through nutrient provision.	Proteases, lipases, haemolysins and deoxyribonucleases.	Urban *et al.*, 2006
Avoidance of host defences	Prevention of opsonization and phagocytosis by immunological disguise. Enhance survival in phagocytes.	Capsule polysaccharide, protein A and leukocidin. Carotenoids, catalase production	O'Riordan & Lee, 2004; Peterson *et al.*, 1977
Exotoxins	Disrupt host membranes or otherwise promote symptoms of disease.	Toxic shock syndrome toxin (TSST-1), enterotoxins A-M and other superantigen toxins.	(Novick *et al.*, 2001
	Cleave desmoglein 1, a cadherin that is found in desmosomes in the epidermis.	Scalded skin syndrome .Exfoliative toxin (A & B)	Raygada *et al.*, 2009
Intrinsic and acquired antimicrobial resistance	Prevent infection by chemotherapeutics.	β-lactamases, penicillin binding protein 2a (PBP2a)	Matsuhashi *et al.*, 1986 & Kaatz *et al.*, 2005; Ojo *et al.*, 2006

The genes encoding for *S. aureus* pathogenicity functions are located in a variety of genomic locations, with mobile genetic elements being significant repositories.

1.3 Multi-antibiotic resistant *Staphylococcus aureus*

Resistance to penicillin was reported in *S. aureus* within 5 years of its introduction in 1943. Currently it is estimated that more than 90% of *S. aureus* isolates worldwide are resistant to this antibiotic (Lyon & Skurray, 1987; Swartz, 1997). The incidence of penicillin-resistant *S. aureus* in clinical isolates was less than 1% in 1940, increased to 14% in 1946 and approximately 60% by 1948 in UK hospitals (Barber & Rozwadowska-Dowzenko, 1948). Levels of penicillin-resistance *S. aureus* continued to rise (Table 1.2), and owing to the high incidence of penicillin-resistance it was necessary to introduce other antibiotics to control the infections. Antibiotics such as streptomycin, tetracycline, chloramphenicol and erythromycin were introduced but these similarly followed by the emergence of resistant strains (Shanson, 1981). Resistance to several different antibiotics, such as tetracycline, chloramphenicol, streptomycin, neomycin, kanamycin and the MLS group that were introduced in the late 1940s and early 1950s, (Novick, 1989). Multi-resistant *S. aureus* was first reported in the UK in 1961 (Parker & Hewett 1970). It was also responsible for outbreaks of nosocomial infection in several other countries (Rountree & Beard 1968; King *et al.*, 1982; Naidoo *et al.*, 1984).

Resistance to the ß-lactamase-resistant penicillins, such as methicillin, arose in the mid 1960s and is associated with the *mecA* gene (Koch, 2003). Early in the 1980s methicillin-resistant *S. aureus* (MRSA) strains re-emerged and many showed additional resistances to several antimicrobial agents including gentamicin and unrelated agents such as antiseptics. Several strains of *S. aureus* have shown resistance to as many as 20 antimicrobial agents, including antiseptics and heavy-metal ions (Lyon & Skurray 1987).

Resistance to gentamicin first arose in the mid 1970s and is mediated by the *aac*A-*aph*D gene, which also confers resistance to other aminoglycosides kanamycin and tobramycin (Casman & Bennett, 1963). Emerged after some 10 years use and was associated with its extensive use as a topical antibiotic (Noble & Naidoo 1978).

Tetracycline, erythromycin and chloramphenicol-resistant strains of *S. aureus* were detected between 1951-1960 in Australian hospitals (Horinouchi & Weinblum 1982) Resistance to amikacin, chloramphenicol, erythromycin, tetracycline,arsenate, cadmium and mercury was reported from Australia between 1940-1974 (Lacey, 1975).

Trimethoprim resistance was first reported in 1980, more than 15 years after it is clinical introduction (Young *et al.*, 1987; Amyes & Towner, 1990). Although the use of trimethoprim for more than 15 years, the incidence of trimethoprim-resistant

staphylococci has since remained almost stable (Young *et al.,* 1987; Amyes *et al.,* 1989; Amyes & Towner, 1990).

Mupirocin was first prescribed in 1986 and resistance strains were reported immediately following its clinical use (Rahman *et al.,* 1990). Reduces susceptibility can be achieved via spontaneous mutation and alteration of the target gene, the majority of antibiotic resistance genes are acquired through horizontal gene transfer (HGT). Antibiotic resistance genes are commonly carried by mobile genetic elements such as bacteriophage, plasmids and transposons, allowing them to transferred among bacteria. HGT has facilitated the spread of antibiotic resistance genes via gene exchange processes such as conjugation, transduction and transformation (Wise, 2002; Wright, 2007).

1.3.1. Appearance of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Methicillin-Resistant *Staphylococcus aureus* MRSA was first described in 1961, shortly after the introduction of penicillinase-resistant β-lactams antibiotics into clinical practice. Since then, hospitals worldwide have reported varying proportions of MRSA among *S. aureus* isolates (de Lencastre, 1998). The incidence of methicillin resistance of *S. aureus* after clinical treatment of aminoglycoside antibiotics decreased in the UK. After the clinical introduction of new penicillin and cephalosporin derivatives, the MRSA strains re-emerged in UK in the early 1980s (Marples and Reith 1992). Gentamicin resistance began to emerge in *S. aureus*, in the late 1970s and early 1980s gentamicin-resistant MRSA became a problem by the mid-1980s and a strain designated epidemic MRSA 1 was widely distributed in the UK, the Irish Republic and Australia (Ayliffe, 1997).

1.3.2. Mechanisms of methicillin-resistance in *S. aureus*

There are two principle mechanisms by which MRSA exhibit resistance to ß lactams, namely altered PBPs and penicillinases. The first is achieved through a mutation in penicillin-binding protein (PBP; *mecA*). The gene *mecA* and regulatory sequences that encode for production of a low-affinity penicillin-binding protein (PBP-2a) are not present in methicillin sensitive strains (Greenwood 1995). This enzyme can continue to catalyse the formation of cross-bridges in bacterial cell wall peptidoglycan in the presence of β-lactams (Berger-Bachi & Rohrer, 2002; Hartman & Tomasz, 1984). PBPs are the target for β lactam antibiotics, so in the case of MRSA by altering PBP, it is not allowing the drug to target it, therefore it is insensitive to ß lactams. For a drug to be effective the affinity between the PBP and the drug must be high (Mulligan *et al*, 1993). Thus in sensitive strains methicillin binds to PBP and inhibits cross linking of peptidoglycan, resulting in cell lysis (Wise & Park, 1965). *S. aureus* has several PBPs that are responsible for catalysing cross-linking reactions between peptidoglycan polymers, one of final steps in bacterial cell wall assembly (Chambers, 1988).

The gene encoding PBP is *mecA*, and mutations in *mecA* modify the PBP. The widespread presence of MRSA is thus partially due to the lateral gene transfer of *mecA* (Ghuysen, 1991). The *mec* gene is believed to have originated in the animal related staphylococcal species *S. fleurettii*, as the sequence is nearly identical to the *mecA* region found in MRSA strains (Tsubakishita *et al.,* 2010).

The second mechanism is the enzymatic degradation of the penicillin by ß-lactamase. The enzyme was termed it a penicillinase (Wise, 1982). The importance to MRSA strains was recognised by de Lencastre (1998).

Table 1.2 Relationship between the clinical introduction of an antibiotic and appearance of resistance

Antibiotic	Date of introduction	Delay prior to Appearance of resistance	Date of		References
			Clinical problem	% Strains Resistance (date)	
Chloramphenicol	1950	8	1958	2-34 (1953-1958)	Kock, 1960; Lowbury & Ayliff, 1974
Erythromycin	1952	1	1953	43 (1952)	Ministry Health 1959
Fusidic acid	1962	8	1970	1-2	Godtfredson *et al.*, 1962; Ayliff *et al.*, 1979
Gentamicin	1950	25	1975	27 (1976-1980)	Rountree & Beard 1965; Richardson & Marple 1982
Kanamycin	1950	25	1975	4 (1976-1980)	Rountree & Beard 1965; Richardson & Marple 1982
Methicillin	1960	1	1961	37 (1976-1980)	Jevons 1961; Richardson & Marple 1982
Mupirocin	1986	1	1987	0.01-3	Rahman *et al.*, 1987; Cookson 1990
Neomycin	1950	10	1960	40	Waisbren, 1958; Rountree & Beard 1965; Parker & Hewitt, 1970
Penicillin	1941	9	1948-1950	60	Barber 1947; North & Christie 1946; Barber & Rozwadoska, 1948
Tetracycline	1940	9	1949	60	Finland *et al.*, 1950; Ministry Health 1959
Trimethoprim	1980	1	1985	26	Lyon *et al.*, 1983; Archer *et al.*, 1986
Vancomycin	1958	24	1982	20	Kirby *et al.*, 1959; Cafferkey *et al.*, 1982; Watanakunakorn, 1984

Table 1.3 Mechanism of resistance to several antibiotics and their genetic location in *S. aureus*
(Adapted from al Masaudi *et al.*, 1991, Jensen & Lyon, 2009 and Woodford, 2005)

Antibiotics	Mechanism of action	Main resistance mechanism	Gene*(s)*	Genomic location
β-lactam (penicillins & cephalosporines)	Peptidoglycan synthesis-inhibits cell wall synthesis enzymes	Enzymatic hydrolysis of β-lactam ring.	*blaZ*	Plasmids & transposons
Semisynthetic β-lactams (Methicillin)	Peptidoglycan synthesis-inhibits cell wall synthesis enzymes	Modified PBP2a with reduced affinity to β -lactams antibiotics	*mecA*	Chromosome
Aminoglycosides	Protein synthesis-inhibit translocation	AMEs-inactviation of antibiotic	*aac*, *aph*, *ant*	Chromosome, plasmids & transposons
MLS antibiotics	Protein synthesis-stimulatesdissociation of peptidly-tRNA during elongation	Enzymatic methylation of adenine on 23S rRNA rduced affinity for antibiotics	*ermA, ermB, ermC*	Plasmids & transposons
Quinolones	DNA synthesis-inhibit DNA gyrase	Alteration in QRDR, reducing affinity of enzyme-DNA complex for fluroquinolones	*grlA/B*	Chromosome
Oxazolidinones	Protein synthesis-prevent formation of the 70S ribosomal initiation complex	Alteration of domain V component of the 23S rRNA	23S rRNA	Chromosome
Chloramphenicol	Protein synthesis-inhibit transpeptidation	Chloramphenicol acetyl transferase-inactivation of antibiotic	*cat*	Plasmid
Fusidic acid	Protein synthesis-forms a stable complex with elongation factor G and ribosome inhibiting translocation	Decreased affinity of the G factor for the antibiotic. Impermability and efflux also implicated	*fusA, fusB*	Chromosome & plasmid

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 synthetase	*mupA*	Plasmid
Rifampicin	RNA polymerase-binds to β-subunit of DNA dependant RNA polymerase	Alteration of target site	*ropB*	Chromosome
Tetracycline	Protein synthesis-inhibit binding of aminoacyl-tRNAs	Tetracycline efflux proteins-energy dependent efflux of tetracycline	*tet(K), tet(L)*	Chromosome & plasmid
		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

PBP, penicillin-binding protein; AMEs, aminoglycoside modifying enzymes; QRDR, quinolone resistance determining region; DHFR, dihydrofolate reductase

1.4. General features of Small Colony Variants

Small colony variants (SCVs) constitute a slow-growing subpopulation of bacteria with distinctive phenotypic and pathogenic traits. Since their first description in 1910 (Proctor *et al.,* 2001), SCVs have been described in a wide range of bacterial species, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (Proctor *et al.,* 2006). SCVs all display a small colony size, a slow growth rate, and atypical colony morphology. Since their discovery *S. aureus* SCVs have been associated with persistent, recurrent and antibiotic resistant infections (Proctor *et al.,* 2006).

1.4.1. *S. aureus* small colony variants

SCVs of *S. aureus* arise as a drug-resistant sub-population during exposure to antimicrobial chemotherapeutics (Proctor *et al.,* 2006). In contrast to the normal *S. aureus* phenotype typical SCV colonies are non-pigmented colonies and also about 10-fold smaller (von Eiff *et al*., 2001). *S. aureus* SCVs exhibit many other atypical characteristics including reduced susceptibility to a range of antimicrobial compounds, lack of coagulase and haemolytic activity. Table 1.4 summarises the general characteristics reported for many SCVs.

Although their microscopic morphology and Gram staining of SCVs are normal, clinical microbiologists may fail to detect them because of their atypical biochemical characteristics and very slow growth (Proctor *et al.,* 2001). This makes them a challenge for clinical microbiologists to identify. Further characteristics include altered expression of virulence genes; intracellular persistence *in vitro* systems; auxotrophy for growth factors, such as thymidine, hemin, and/or menadione; and the ability to revert to the normal phenotype (Kahl *et al.,* 2005 & Proctor *et al.,* 2006).

Table 1.4. General differences in phenotypic characteristics between wildtype *S. aureu* and small colony variants (SCVs)

Differences	Wild type	SCVs	References
Colony size (approx.)	2 mm	0.2 mm	Bergoge-Berezin, 2000; Roggenkamp *et al.,* 1998
lag phase (mean)	2.8 hours	4.5 hours	Kahl *et al.,* 2005
Cell Wall	Thin	Thick	Peschel, 2002
Coagulase production	Positive	Negative	Seifert *et al.,* 2003
Haemolytic	Positive	Negative	Balwit *et al.,* 1994
Pigmentation	Golden yellow	Non pigmented	Balwit *et al.,* 1994; Proctor *et al.,* 1998
Auxotrophy for hemin and/or menadione	Negative	Positive	Kahl *et al.,* 2003; Proctor & Peters 1998
Intracellular survival	Poor	Increased	Kahl *et al.,* 1998; Vaudaux *et al.,* 2002; von Eiff *et al.,* 2001
Amino glycoside antibiotics	Sensitive	Reduced susceptible	Rusthoven *et al.,* 1979; Proctor & Peters 1998

1.4.2 Antibiotic selection for *S. aureus* SCVs

Aminoglycosides such as gentamicin have been shown to select for *S. aureus* SCVs in patients receiving treatment for osteomyelitis (Musher *et al.,* 1977). Other antibiotics such as chloramphenicol, streptomycin and a number of different quinolones have also displayed ability to select for *S. aureus* SCVs (Seligman, 2006; Mitsuyama *et al.,* 1997). Recently, triclosan (commercially know as Irgasan; 2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a synthetic bisphenol antimicrobial agent, has been shown to select for *S. aureus* SCVs (Seaman *et al.,* 2007). Interestingly in 1998 it was recommended for the control of methicillin-resistant *S. aureus* (MRSA) (Duckworth, 1998). Triclosan has been used in some skin care products for over 30 years and has been employed as surgical scrubs, hand washes and body washes to control MRSA (Russell, 2004). Thus the topical use of triclosan against staphylococci is widespread, but the fact it selects for SCVs (Seaman *et al.,* 2007) appears not to be considered a health risk.

1.4.3 Infections associated with *S. aureus* SCVs

S. aureus SCVs have been associated with relapsing or persistent infections, in particular in osteomyelitis, some foreign body-associated infections (Sendi *et al.,* 2006) and attributed to the pathogenesis of relapsing or persistent infections in cystic fibrosis patients (Kahl *et al.,* 1998; Abele *et al.,* 2000 & Gilligan *et al.,* 1987). Further investigation has revealed that patients infected with *S. aureus* SCVs were more likely to undergo relapses of osteomyelitis and the MICs for gentamicin were up to 32-fold greater for the SCVs when compared to the wild type strain MIC (von Eiff *et al.,* 1997). The ability of SCVs to enter into and persist within host cells that are not naturally phagocytic, such as epithelial cells and endothelial cells provides a source for these persistent infections (Schroder *et al.,* 2006). A study in CF patients isolated *S. aureus* SCVs and showed SCVs to persist for extended periods in comparison to the wildtype (Kahl *et al.,* 2003). Of further concern is that *S. aureus* SCVs were shown to persist in CF patients after antibiotic therapy was halted (Kahl *et al.,* 2003).

One study has demonstrated the virulence of a site-directed hemin-auxotrophic *S. aureus* SCV mutant in a murine model of septic arthritis (Jonsson *et al.,* 2003). The study concluded that the small colony variants of S. *aureus* were more virulent on a per organism basis than its isogenic parental strain in the model of septic arthritis. This can at

least in part be explained by the ability of SCV to produce high amounts of destructive proteases (Jonsson *et al.,* 2003).

The study included normal and SCV strains from CF patients as well as control strains were tested for the susceptibility to defences, killing activity of professional phagocytes and adhesion to A549 cell line **(**Sadowska *et al.,* 2002).

Thus the SCV state is obviously of clinical significance, but establishing a clear link between recurrent infection and SCVs is clearly hampered by diagnostic failures on occasion.

1.4.4 The survival advantage of the SCV condition to *S. aureus*

There are many advantages to be gained by growing in the cytoplasm of host cells, inside non-professional phagocytes, such as endothelial cells and osteoblasts (Hudson *et al.,* 1995 & von Eiff *et al.,* 2000). These rewards include protection from antibodies, complement and antibiotics that penetrate poorly into mammalian cells and a highly nutritious environment. *S. aureus* SCVs have a reduced production of cytotoxins and this may down-regulate the induction of cell lysis or apoptosis (Proctor *et al.,* 2006). Consequently and unlike the wild type, they do not kill the cells because they produce very little α-toxin (von Eiff *et al.,* 2000). They are thus in a safe haven and can multiply without obvious limitation. *S. aureus* SCVs can revert to the highly virulent rapidly growing wild type form and lyse the host cell. If the host immune response has dropped and antibiotic therapy is complete (von Eiff *et al.,* 2000) then an infection results. The continued persistence of the SCV strain and its phenotype indicates a survival advantage compared to strains with their normal phenotype (von Eiff 2008). It has also been demonstrated that the intracellular location 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, suggests that SCVs are induced by the intracellular *milieu* (Vesga *et al.,* 1996).

1.4.5 Reduced antibiotic susceptibility in *S. aureus* SCVs

The ability to form a variant sub-population affords *S. aureus* a number of survival advantages. SCV do persist intracellularly, within non-professional phagocytes, and this shields them from host defences and decreases exposure to antimicrobial agents (Balwit *et al.,* 1994). *S. aureus* SCVs also show reduced antimicrobial susceptibility. Seaman *et al.*

(2007) showed *S. aureus* SCVs have raised minimum inhibitory concentrations (MICs) to several antimicrobials including gentamicin, erythromycin and linezolid. However, resistance in SCVs does not result from the classical mechanisms of resistance such as production of β-lactamases (to break β-lactam based antibiotics) or efflux pumps (to pump antimicrobials out from the cell) (al Masaudi *et al.,* 1991). Resistance to antimicrobials in SCVs is a direct consequence of the defects in the ETC and the SCV phenotype itself (Proctor 1998). Interruption of the ETC reduces the electrochemical gradient across the bacterial membrane, which in turn decreases the uptake of antimicrobial agents such as aminoglycosides, which require a charge differential to be present (von Eiff *et al.* 2006). Limits on ATP production reduce the growth rate of *S. aureus* SCVs, and this reduction is associated with a four-fold increase in the MICs of cell wall specific antibiotics. The decrease in energy bestows a characteristic pleiotropic phenotype including slow growth (hence small colonies), lack of pigmentation, non-production of virulence factors and reduced spectrum of carbohydrate utilization (McNamara & Proctor 2000). The survival of *S. aureus* SCVs within host cells reduces the effectiveness of antibiotics that have a limited ability to cross eukaryotic membranes (Darouiche & Hamill, 1994). This reduced susceptibility of *S. aureus* SCVs to antimicrobial agents is the main factor why SCVs have the ability to produce persistent and reoccurring infections. Antimicrobial agents that penetrate host cells and exert optimal intracellular bactericidal activity, especially against slow growing bacteria, have been suggested for use against *S. aureus* SCVs (Vaudaux *et al.* 2006). Rifampin is the single antistaphylococcal agent considered to be effective for treating intracellular infections (Sendi *et al.,* 2006).

1.5 Isolating and identifying SCVs

It is recognised that the recovery and identification of SCVs by clinical microbiologists can be difficult (Proctor, 1994 & von Eiff *et al.,* 1997). SCVs and parents *S. aureus* have the same appearance when Gram stained and SCVs are morphologically identical and often in a mixed population with parents *S. aureus*. Additionally and even when present as a small proportion of the total number of bacteria, normally growing organisms rapidly overgrow SCVs in liquid medium in an overnight culture. Thus rendering SCV isolation and susceptibility testing difficult (Kipp *et al.,* 2005). Also the slow growth rate of SCVs and revertability makes standardization of testing difficult, because the slow growth rate

alters diffusion tests and times for measuring susceptibility. Lastly irrespective of their auxotrophism, errors can occur when these variants are resistant to oxacillin and are tested by disc-diffusion tests, Etests (AB Biodisk), microdilution tests, automated susceptibility testing systems, as well as anti-penicillin-binding-protein-2a (PBP2a) latex agglutination tests (MRSA-Screen, Denka Seiken) (Kipp *et al.,* 2004).

Since their colonial morphology is atypical, their biochemical reactions are unusual, such as reduced coagulase activity (often SCVs are only coagulase positive in the tube test after incubation for 18 hours) their identification is difficult (Kahl *et al,*. 1998).

The failure to recover SCVs results in a major susceptibility reporting error, in that the more resistant of the two populations of organisms exhibited by a strain will be missed. Similarly, in instances where *S. aureus* SCV is misidentified, physicians may not be alerted to the possibility of recurrent infections or treatment failures (Proctor & Peters, 1998).

Thus SCVs are characterized by a strong reduction in growth rate, an atypical colony morphology, and unusual biochemical characteristics. All characteristics which have clinical health care issues (Proctor *et al* 2006)

1.6. Biochemical basis of SCVs

SCVs have a deficiency in electron transport, owing to mutations in the bacterial electron transport chain (ETC), or in thymidine biosynthesis (Proctor *et al.,* 2006).

1.6.1 Menadione and haemin SCVs

Clinical isolates of SCVs are commonly auxotrophic for menadione and haemin, two compounds that are each crucial to the functioning of the electron transport chain (ETC; von Eiff *et al.,* 1997, Proctor & Peters 1998). Both haemin and menadione have roles in bacterial electron transport. Menadione is isoprenylated to form menaquinone and is the acceptor of electrons from nicotinamide adenine dinucelotide (NADH) and flavin adenine dinucelotide ($FADH_2$) (Bates *et al.* 2003). Haemin is required for the biosynthesis of cytochromes, which accepts electrons from menaquinone and completes the ETC. Haemin and menaquinone are used both by aerobic electron transport. Mutations in the genes encoding these components of the ETC chain therefore interrupted ATP generation (Proctor *et al.* 2006).

Thus mutations in genes that result in the SCV phenotype include *men*D (Bates *et al.* 2003) and *hem*B (von Eiff *et al.* 1997). Other mutations occur in *cat*A block the

biosynthesis of haemin, and therefore *catA* mutants also display the SCV phenotype (Clements *et al.* 1999).

von Eiff *et al.* (1997) showed that laboratory constructed *hem*B mutants display characteristics typical of SCVs (micro colonies, reduced coagulase activity and increased resistance to aminoglycosides), indicating that mutations in *hemB* result in the SCV phenotype. Interestingly all characteristics of the SCV phenotype in this mutant were reversed by growing in the presence of haemin or by complementing the mutant with intact *hemB* (von Eiff *et al.* 1997). Without oxidative respiration SCVs can only produce ATP through fermentation, which is significantly less efficient resulting in severe ATP reduction, by 92%. ATP production is directly linked to cell wall biosynthesis, the generation the electrochemical gradient and also carotenoid biosynthesis. The reduction of ATP production in SCVs results in slower growth (hence smaller colonies), reduced uptake of aminoglycosides and cationic peptide transport and also decreased pigment formation in SCVs.

1.6.2 Thymidine biosynthesis deficient SCVs

The other group of SCVs that are constantly recovered from clinical specimens are those deficient in thymidine biosynthesis (Proctor *et al.* 2006). SCVs that are auxotrophic for thymidine have been commonly isolated from cystic fibrosis (CF) patients after long-term treatment with trimethoprim sulphamethoxazole (SXT) (Kahl *et al.* 1998). A further study by Kahl *et al.* (2005) showed *S. aureus* SCVs auxotrophic for thymidine to display two distinct colony morphologies: pin point colonies (one tenth the size of wildtype *S. aureus*) and 'fried egg' type colonies, with translucent edges that surround an elevated pigmented centre. Thymine auxotrophic SCVs have similar characteristics to SCVs deficient in electron transport; however the two morphological variants tested by Kahl *et al.* 2005 showed the pinpoint colonies to exhibit an even longer doubling time and did not reach as high cell densities as the 'fried egg' colonies. Procter *et al.* (2006) suggest that thymidine auxotrophic SCVs may have a mutation in the *nupC* gene, which as Figure 1.2 shows is involved in the thymidine uptake from the environment. This is supported by the notion that *B. subtilis nupC* mutants, were shown to have a thymidine uptake 5 times slower than the wildtype (Saxild *et al.* 1996). Recently Beiser *et al.* (2007a) have shown mutations in the thymidylate synthestase gene, may be responsible for the formation of thymidine auxotrophic SCVs in *S. aureus*. Defects in thymidylate synthestase causes metabolic

impairments in the cell (Beiser *et al.* 2007a), which correlates with features of SCVs, such as reduced ATP production.

Reduced thymidine uptake, ensures that the SCV phenotype is maintained, however supplementation with thymidine results in phenotypic reversion to the wildtype in *S. aureus* SCVs isolated from CF patients (Kahl *et al.* 1998). In thymidine auxotrophic SCVs there is also down regulation of the virulence regulators *agr* and *sarA*, and dependent virulence genes such as *hla* and *spa* (Kahl *et al*. 2005). Also showed that thymidine auxotrophic SCVs displayed decreased expression of *agr* and *sarA*, although supplementation of thymidine increased the transcription of the *agr* locus and the *agr*-dependent virulence genes *spa* and *hla* to full or partial transcription. Similarly to the *hemB* mutant supplementation of the compound the SCVs are deficient in this case thymidine, results in reversion to the wildtype and restores transcription of virulence associated genes (Kahl *et al*. 2005).

1.6.4 Prototrophic SCVs

In all of the discussions of SCVs, phenotypes and genotypes there is an absence of analysis of where the mutations in non-auxotrophic mutants are. Clearly if these are not shown to become wild type by supplementation then their mutations are in other genes. There is no conjecture in the literature. Logically therefore these mutations must be in genes not related to the biosynthesis of precursor chemicals but in the structural composition of an active ETC. Since there are many components, which comprise the ETC one could estimate crudely auxotrophs are a small subset of potential mutations accusing the SCV phenotype.

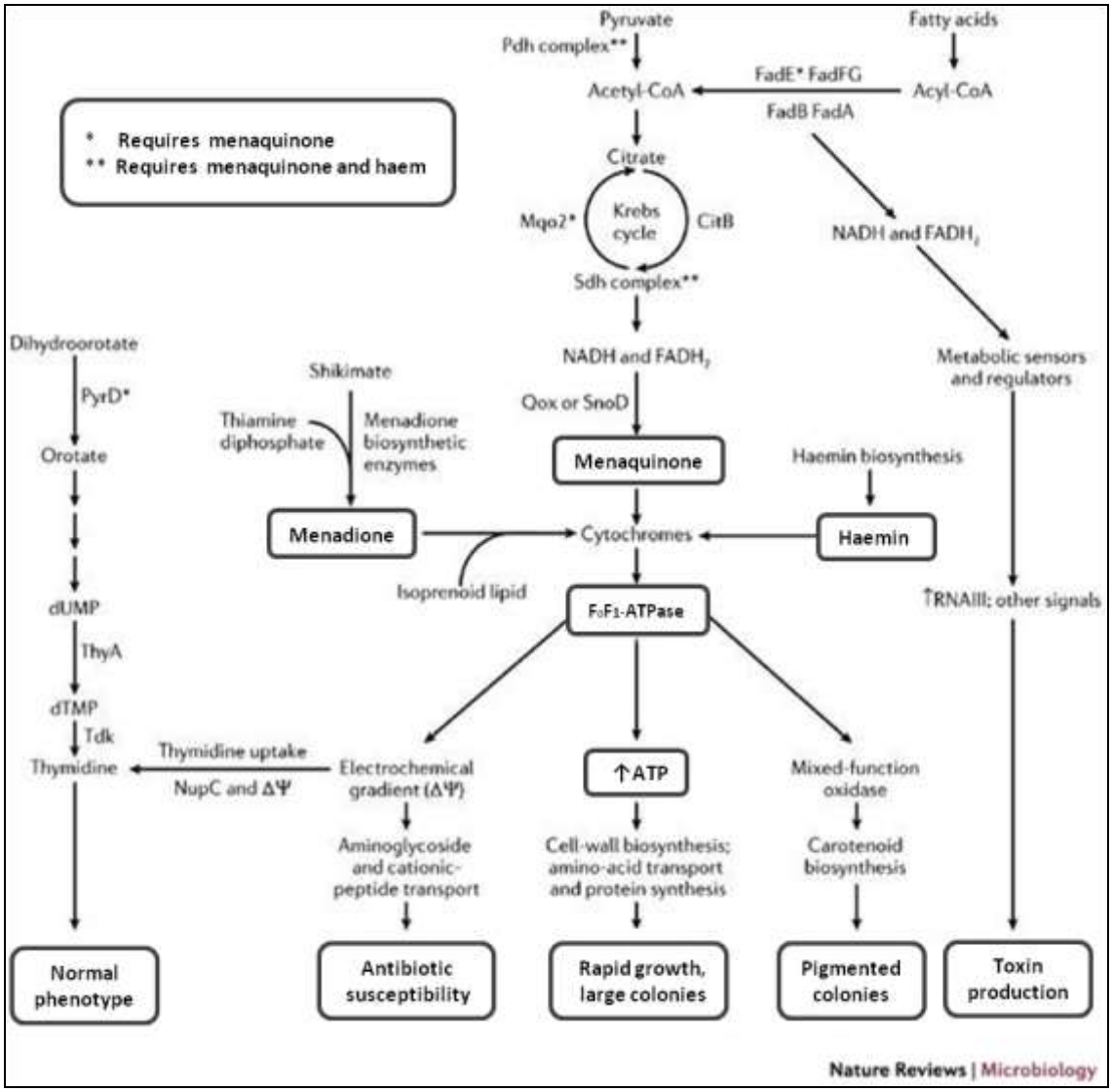


Figure 1.2 Relationship between electron transport and the small colony variants phenotype in *S. aureus* (Proctor *et al.* 2006). Defects in electron transport reduce the yield of ATP (92 %) effecting all metabolism including that available for cell-wall biosynthesis, which leads to a slower growth rate and production small colonies. Since ATP is required for carotenoid biosynthesis and maintaining membrane potential. Thus SCVs produce non-pigmented colonies and decreased uptake of cationic compounds.

1.6.4 SCV formation

The molecular mechanisms behind the emergence of SCVs are not well understood and regulatory as well as genetic mechanisms seemed conceivable to Schaaff *et al* (2003). This was due to the fact many SCVs are unstable and form wild type revertants (Becker *et al.* 2007), although this seems a little naive as many mutations revert at high frequencies. Schaaff *et al.* (2003) have examined whether an increased mutation rate favours the formation of SCVs by comparing an *E. coli* strain with *mutS* gene knocked out with wildtype strains. *mutS* along with *mutH* and *mutL* make up the dam-directed mismatch repair system in *E. coli* which is crucial in proofreading during DNA replication (Horst *et al.* 1999). The results of Schaaff *et al.* (2003) showed that the emergence of spontaneous SCVs was 556-fold higher in the *mutS* mutant than in the wildtype strain, demonstrating that a higher mutation rate favours the emergence of SCVs, and suggest that point mutations play a substantial role in the formation SCV populations. The widely reported ability of antimicrobials such as aminoglycosides and triclosan to select for SCVs (Musher *et al.* 1977, Balwit *et al.* 1994 Seaman *et al.* 2007) suggests that the presence of antimicrobials may induce SCV formation. Recently, aminoglycoside-induced SCV formation has been linked to the activity of an alternative sigma factor in *S. aureus*. Formation of the SCV phenotype in *S. aureus* can be viewed as a phenotypic switching system which appears to be strongly influenced by the alternative sigma factor, σ^B (Mitchell *et al.*, 2010a). 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.

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 Quantitative PCR (qPCR) also demonstrated that sub-inhibitory concentration of gentamicin and tobramycin induced σ^B activity (Mitchell *et al.*, 2010a).

1.7 Molecular identification techniques for *S. aureus* SCVs

Identification of clinical strains *S. aureus* SCVs is difficult because of their slow growth rate and unusual colony morphology (Proctor *et al.* 1994). Thus SCVs cause numerous problems for diagnosis and treatment, and are also rapidly overgrown (Proctor, 2000). Population sizes as low as 0.01% of wildtype *S. aureus* will overgrow *S. aureus* SCVs in an overnight culture without selection (von Eiff *et al.,* 2001a). The standard biochemical tests for identification of *S. aureus* are made obsolete by changes in carbon source utilisation and virulence factors, and their lack of coagulase production and reduced haemolytic activity meaning staphylococcal SCVs are difficult to recognise and are often misidentified as coagulase negative staphylococci (CoNS) (Seaman *et al.* 2007, McNamara and Proctor 2000). Standardisation testing for *S. aureus* SCVs is difficult; as their slow growth rate lengthens times for measuring antimicrobial susceptibility and errors can also occur when SCVs are tested via automated susceptibility testing systems (Kipp *et al.* 2004). Rapid identification of *S. aureus* SCVs had been demonstrated by the use of multiplex polymerase chain reaction (PCR), targeting the 16S rRNA (*Staphylococcus* genus specific), and *nuc* (*S. aureus* species specific) genes (Seaman *et al.* 2007and Zhang *et al.* 2004). The identification of microorganisms based on their 16S rRNAs has many advantages; each bacterial cell contains multiple copies of the 16S rRNA in its ribosomes. Hence, the technique is sensitive enough to detect single bacterial cells and 16S rRNA genes are highly conserved throughout bacterial evolution **(**Krimmer *et al.*, 1999).

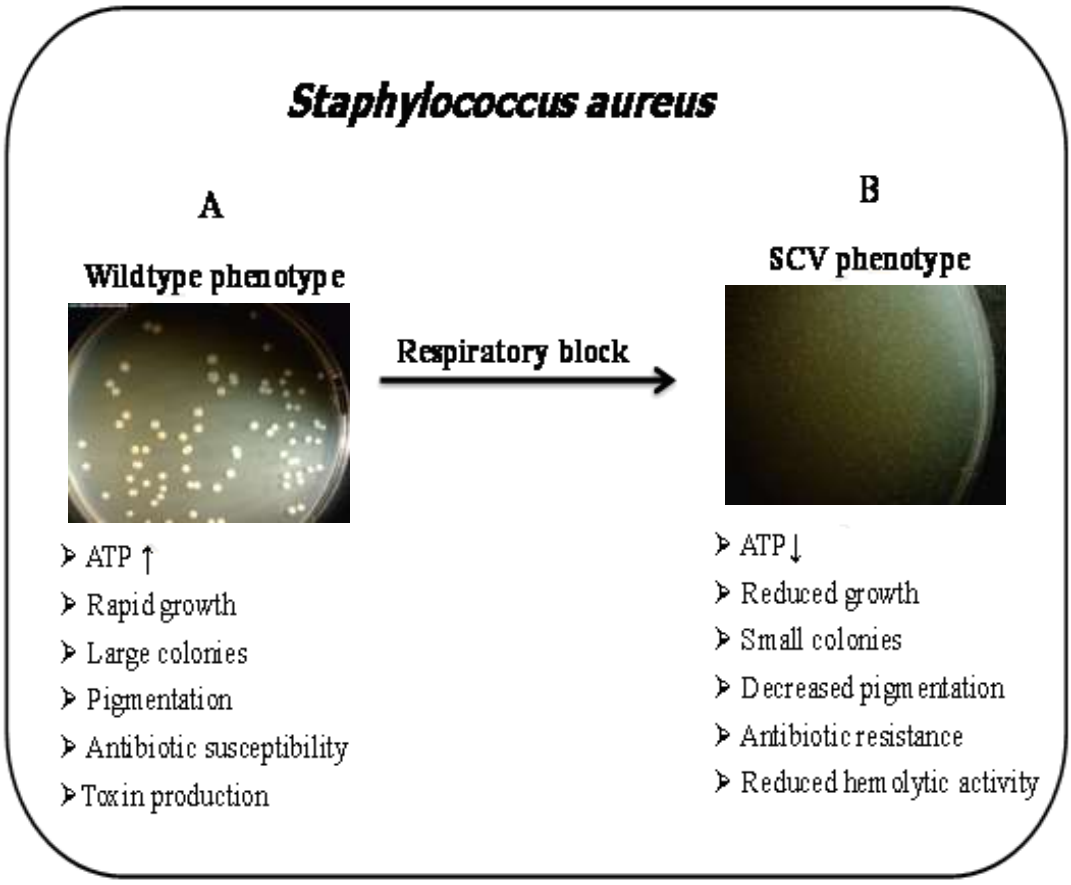


Figure 1.3 Summary of characteristics of (A) wild type *S. aureus* (B) *S. aureus* SCV.

1.8 Gene transfer in *Staphylococcus aureus*

Genetic transfer is the mechanism by which DNA is transferred from a donor to a recipient. There are four transfer mechanisms for transfer of resistance genes between *staphylococcus aureus.* These are transformation, phage-mediated conjugation, conjugation and transduction.

1.8.1 Transformation

Transformation was first demonstrated in 1928 by British bacteriologist Frederick Griffith and the identification of the "transforming principle" as DNA (Avery *et al.,* 1944). Transformation is a process by which naked DNA is taken up and integrated into the genome of a recipient cell and occurs with chromosomal or plasmid DNA under certain conditions (Lindberg *et al.* 1972). The transformation of *S. aureus* requires a high concentration of calcium ions or bacteriophage in either the prophage or vegetative state. In the absence of these conditions and the presence of nuclease, transformation is reduced (Lyon & Skurray, 1987).

Transformation of staphylococci *in vitro* has been described but most cultures of *S. aureus* contain high levels of nucleases which probably prevent transformation *in vivo* (Sjostrom, *et al.,* 1979). The ability to absorb free DNA from the environment is relatively rare in species of Gram-positive bacteria. However *S. aureus*, under specific conditions, is able to take up (naked) DNA from the environment. The natural competence system of *S. aureus* in induced only in very early exponential growth (Novick, 1989). Chromosomal or plasmid DNA can be transformed into *S. aureus*, although competence requires high calcium concentration (Lacey, 1984).

1.8.2 Phage-mediated conjugation

Phage-mediated conjugation is the transfer mechanism most likely to occur between staphylococci in nature. MRSA have evolved from a single clone and are now heterogeneous in properties (Lacey, 1986). It is a mechanism which needs viable cell-to-cell contact as well as the presence of lysogenic phage in either the donor or recipient cell (Lacey, 1980). This mechanism occurs in *vivo* between staphylococci at the high frequencies ($\geq 10^{-1}$) when the recipient is a lysogen (Lacey, 1980; Lacey & Kruczenyk, 1986). In this transfer high cell densities and calcium ions are both required for high rates of gene transfer (Witte, 1981). Gentamicin resistance is transferred at frequencies between 10^{-3} and 10^{-6} by phage-mediated conjugation in *vivo* and *vitro* (Lacey & Lord, 1980;

Naidoo, 1984). High frequency of transfer suggests that transfer of small plasmids and chromosomal genes between staphylococci is likely to occur under natural conditions by this mechanism (Lacey, 1984).

1.8.3 Conjugation

The transfer mechanism is not affected by DNAase, chelating agents and human serum (McDonnell *et al.* 1983). Conjugative plasmid is able to mobilize non-conjugative plasmid between clinical isolates of S. *aureus* (Udo & Grubb, 1990).

Bacterial conjugation is a highly specific process whereby DNA is transferred from donor to recipient bacteria by a specialized multi-protein complex, termed the conjugation apparatus (Figure 1.4; Lederberg and Tatum 1946). An important prerequisite for conjugative transfer is an intimate association between the cell surfaces of the interacting donor and recipient cells. In Gram-negative bacteria, this physical contact is established by complex extracellular filaments, designated sex pili. For the majority of Gram-positive bacteria, the means to achieve this intimate cell-to-cell contact have not yet been identified (Grohmann *et al.,* 2003).

Staphylococcal conjugation was first described in 1982 with the description of the transfer of plasmids carrying gentamicin resistant (Jaffe *et al.,* 1982). These plasmids occur very commonly in *S. epidermidis* and may responsible for the spread of gentamicin resistance to *S. aureus* (Forbes & Schaberg1983). Conjugative staphylococcal plasmids isolated carried resistance to gentamicin and other aminoglycosides, ethidium bromide and quaternary ammonium compounds (Grohmann *et al.,* 2003). These findings show that conjugation systems are probably of significant in the dissemination of antimicrobial resistance genes among the *staphylococcus* genus (Novick, 1989).

1.8.4 Transduction

Transduction is a mechanism by which chromosomal or plasmid DNA from the donor is carried by a bacteriophage to the recipient (Jawetz *et al.,* 1987). Two types of transduction mechanisms, general and restricted, have been described (Jawetz *et al.,* 1987). In generalized transduction the phage either carriers a phage genome or a segment of chromosome or plasmid from the donor, certain temperate phage strains can transfer only few genes of bacterial chromosome this is termed restricted transduction (Jawetz *et al.,* 1987). Transduction in *S. aureus* was first carried out in *vitro* in 1958 (Ritz and Baldwin, 1958) and frequencies of $\leq 10^{-7}$ per recipient were reported. Phage mediated transduction

has been postulated to be a mechanism by which β-lactamase genes have spread among staphylococci (Novick, 1989). The principles of transduction are shown in Figure 1.4.

1.9 The susceptibility of *S. aureus* strains to bacteriophage 80α

There are over 250 staphylococcal bacteriophages reported in the literature (Pantucek *et al*., 2004). Recognition of the importance of viral-mediated virulence gene transfer is growing (Rice, 2006). While most strains of *S. aureus* contain at least one prophage, rare strains contain ≥ 15 phage types, suggesting a potential for a protective effect from lysis by phage integration (Lindsay & Holden, 2004). *S. aureus* bacteriophage 80α is a temperate phage that is capable of generalized transduction and also serves as a helper phage for the mobilization of several different SaPIs, including SaPI1, SaPI2, SaPIbov1, and SaPIbov2 (Novick *et al.*, 2010 & Christie *et al.*, 2010).

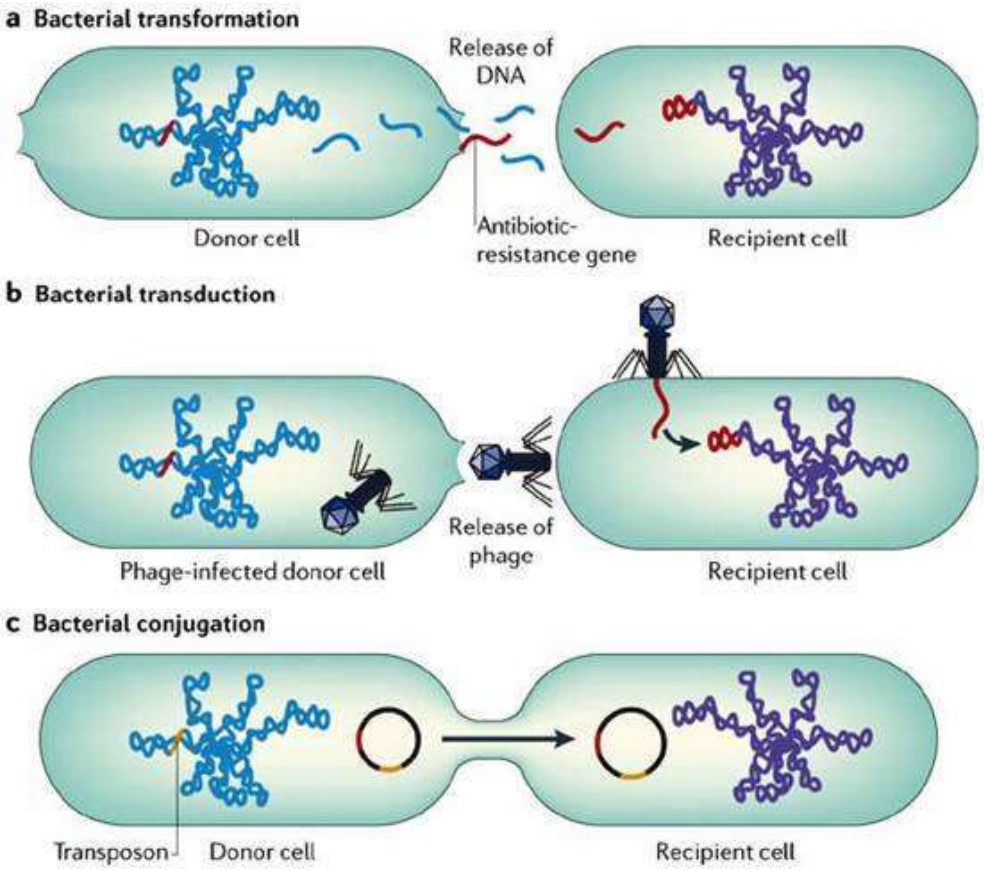


Figure 1.4 Transfer of DNA between bacteria cells. (a) Transformation Involves acquisition of DNA from the extracellular environment, for example when DNA is released on lysis of an organism and subsequently picked up by another organism. The acquired gene can be integrated into the chromosome or into a plasmid of the recipient cell. **(b) Transduction** Involves acquisition of foreign DNA by means of bacteriophages. **(c) Conjugation** Occurs by direct contact between two bacteria and the formation of a mating bridge across the bacteria in which the DNA is exchanged (Furuya & Lowy, 2006).

1.10 Aims

The aims of this investigation were to:

- Examine the ability of gentamicin and triclosan to select for *S. aureus* SCVs.
- Compare the sensitivity and resistance to an antibiotic (gentamicin) and a biocide (triclosan) of MRSA strains.
- Examine the effect of forward and reverse mutation cycles on *S. aureus* wildtype strains.
- Make a physiological and genetical comparison of SCVs and wildtype strains.
- Make a comparative analysis of staphylococcal genomes.
- Examine the potential of SCVs to acquire and disseminate antimicrobial resistance by transduction.
- Compare aerobic and anaerobic growth of *S. aureus* COL and SCV strains.
- Compare the effects of inhibitors on the growth of *S. aureus* COL and SCV strains.

CHAPTER 2 :

MUTATION IN *STAPHYLOCOCCUS AUREUS*

2.1 Introduction

2.1.1 History of mutation

A mutation is a change in the sequence of genomic DNA. Mutations come from errors made during the replication of DNA or from exposure to mutagens as radiation, viruses, transposons and mutagenic chemicals (Burrus, & Waldor, 2004; Denamur & Matic, 2006). Mutations include deletion (loss of DNA bases), duplication or insertion (gain of DNA bases), transposition and missense or nonsense (substitution of a DNA base). Such mistakes are rare (Alderson & Rowland, 1995), but occur consistently during the life of cells. Many mutations are recognized because the phenotype or resistant of the organism has changed (LeClerc *et al.,* 1996). Bacteria undergo mutation at a rate of between about 10^{-6} to 10^{-9} per cell generation. This mutation rate can be increased by subjecting bacteria to certain chemicals or to irradiation. Many of these mutations physiologically impair the bacterium in some way and so for example gaining resistance to an inhibitor will often be at a cost (Saunders, 1984). Novel genes are produced by several processes, commonly through the duplication and mutation of an ancestral gene, or by recombining parts of different genes to form new combinations with new functions (Long *et al.,* 2003).

Most if not all bacteria transfer and receive genes and this process maybe mutagenic if recombination or transposition occurs. There are three transfer processes called transformation, transduction, and conjugation (Davison, 1999). Gene transfer has significance in antibiotic resistance as it allows the rapid transfer of ecologically relevant genes between different pathogens (Hastings *et al,* 2004).

2.1.2 Causes of mutation

2.1.2.1 Spontaneous mutation

Spontaneous mutations arise from a variety of sources, including natural errors in DNA replication and spontaneous lesions. An error in DNA replication can occur when base is changed by the repositioning of a hydrogen atom, altering the hydrogen bonding pattern of that base resulting in incorrect base pairing during replication this

is called tautomerism (Griffiths *et al.,* 2000). In addition to replication errors, spontaneous lesions**,** naturally occurring damage to the DNA, can generate mutations. Two of the most frequent spontaneous lesions result from depurination (loss of a purine base (A or G) to form an apurinic site) and deamination which is hydrolysis of a base to an atypical base containing a keto group in place of the original amine group such as C→U and A→HX (hypoxanthine), which can be corrected by DNA repair mechanisms; and 5MeC (5-methylcytosine) → T, which is less likely to be detected as a mutation because thymine is a normal DNA base (Montelone, 1998). The rates of such mutations have been determined for many species. *E. coli* has a spontaneous mutation rate of 10^{-8} (one error in every 10^{8} nucleotides replicated) (Khanna, 2008).

2.1.2.2 Induced mutation

Induced mutations are mutations that caused by agents in the environment. Induced mutations increase the mutation rate over the spontaneous rate (Burrus, & Waldor, 2004). Mutations can be caused by interactions of the genome with chemical agents, radiation and transposon insertion (Khanna, 2009).

2.1.2.2.1 Chemical mutagens

The first report of mutagenic action of a chemical was in 1942 by Charlotte Auerbach, who showed that nitrogen mustard (component of poisonous mustard gas used in World Wars I and II) could cause mutations in cells. Since that time, many other mutagenic chemicals have been identified (Montelone, 1998). Chemical mutagens may induce point mutations that occur when a single base pair of a gene is changed. These changes are classified as transitions or transversions. Transitions occur when a purine is converted to a purine (A↔G) or a pyrimidine is converted to a pyrimidine (T↔C) (Khanna, 2009). A transversion results when a purine is converted to a pyrimidine or a pyrimidine is converted to a purine. A transversion example is adenine (A) being converted to a cytosine (C). Two main classes of chemical mutagens are routinely used. These are alkylating agents and base analogs. Each has a specific effect on DNA. Alkylating agents (such as ethyl methane sulphonate (EMS), ethyl ethane sulphonate (EES) and mustard gas) can mutate both replicating and non-replicating DNA (Tripathi, 2010). In contrast, a base analog (5-bromouracil and 2-aminopurine) only mutate DNA when the analog is incorporated into replicating DNA

(Ogino *et al.*, 2002). Each class of chemical mutagen has specific effects that can lead to transitions, transversions or deletions.

2.1.2.2.2 Radiation

Radiation is also capable of inducing mutations such as Ionizing radiation (X-ray, γ-ray), even non-Ionizing radiation (UV). Ionizing radiation has various effects on DNA depending on the type and energy of the radiation, can create free radicals that result in problems ranging from point mutations (changes in a single nucleotide) or deletions (loss of a chromosomal segment) (Larsen *et al.*, 2006). Some types of ionizing radiation act directly on DNA, others act indirectly by stimulating the formation of reactive molecules such as peroxides in the cell (Brown, 1999). In addition to targeted effects of damage induced directly in cells by irradiation, a variety of untargeted effects may also make important short-term and long-term contributions to determining overall outcome after radiation exposures (Coates *et al.*, 2004). Non-ionizing radiation (UV radiation) induces dimerization of adjacent pyrimidine bases, especially if these are both thymines (Brash *et al.*, 1991). UV light can induce adjacent pyrimidine bases in a DNA strand to become covalently joined as a pyrimidine dimer. UV radiation, particularly longer-wave UVA, can also cause oxidative damage to DNA (Kozmin *et al.*, 2005).

2.1.2.2.3 Transposon insertion

Transposable elements are mobile pieces of DNA that can move from one location in a genome to another (Nagy & Chandler, 2004) and the presence of a transposable element in a wild type gene disrupts the normal function of that gene. The mechanism of transposition can be either (copy and paste) or (cut and paste).

Transposons carry many genes, so for example some encode for antibiotic resistance and others for catabolic phenotypes (Reznikoff, 2003). Although transposon insertion in a gene usually causes complete loss of function, in a few rare exceptions, transposon insertions mutations are null alleles (Kleckner, & Botstein, 1977).

2.1.3 Effects of mutation

Mutations can affect individual cells in a variety of ways. Mutations can affect the organism's phenotype, which effects how successfully the organism interacts with the environment. Thus some of these mutations are beneficial to the organism. However

generally mutations have a negative impact and so cause biochemical variation. So if a mutation occurs in a gene that encodes an enzyme involved in a metabolic pathway causing its' inactivation, such as an enzyme involved in the biosynthesis of an amino acid (Snyder& Champress, 2007), the consequence is the cell dies. Such mutations are termed lethal as they lead to the death of the organism. Mutations change the genome sequence and can be diverse, ranging from single base pairs changes, inversions, transpositions and deletions. All but the latter are revertable and these can be either true revertants, where the original sequence is recovered, or pseudorevertants where a second mutation suppresses the first (Ellis *et al.,* 2001).

Mutations can effect the cell's phenotype, but in many cases there is no visible evidence of genomic change. In this instance these are termed silent.

2.1.4 Antibiotic and biocide selection for *S. aureus* SCVs

2.1.4.1 Selection of *S. aureus* SCVs by gentamicin

Gentamicin is member of the aminoglycoside family. Aminoglycosides are distinguished by a backbone of an aminocyclitol ring saturated with amine and hydroxyl substitutions (Shakil *et al.,* 2008; Figure 2.1). Gentamicin is 2-deoxystreptamine containing aminoglycosides (Magnet & Blanchard, 2004). Gentamicin 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 have a variety of effects within the bacterial cell but principally they inhibit protein synthesis by binding to the 30S ribosomal subunit to prevent the formation of an initiation complex with messenger RNA. They also cause misreading of the messenger RNA message, leading to the production of nonsense peptides. Another important function of the aminoglycosides is that they increase membrane leakage intracellular contents (White *et al.,* 2005). Various classes of antimicrobial agents have been demonstrated to select *Staphylococcus aureus* SCVs most especially aminoglycosides and trimethoprim-sulfamethoxazole (SXT) (Gilligan *et al.,* 1987). A number of different quinolones have also shown to select for *S. aureus* SCVs (Mitsuyama *et al.,* 1997), and other antibiotic such as fusidic acid (Norstrom *et al.,* 2007) and biocides such as triclosan (Seaman *et al.,* 2007) have all been shown to

select for the SCV phenotype. 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 by *P. aeruginosa* both interfere with *S. aureus* electron transport chain (ETC) and have been demonstrated to select for SCVs *in vitro* (Biswas *et al.*, 2009). 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.*, 1997a). Gentamicin selected SCVs maybe auxotrophic for haemin and menadione that confers defects in the ETC and consequently in ATP generation (Balwit *et al.*, 1994; von Eiff *et al.*, 1997a).

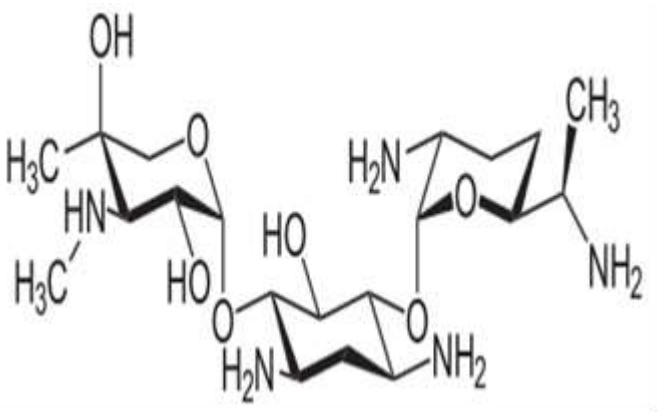


Figure 2.1 The chemical structure of gentamicin. (Klostermeier *et al.*, 2004)

2.1.4.2 Selection *S. aureus* SCVs of by Triclosan

Triclosan (2, 4, 4'-trichloro-2'-hydroxydiphenyl ether), (Irgasan DP300), (Irgacare MP) and (Irgacid LP10) (Figure 2.2) is a broad spectrum biocide that has been in use for more 35 years. It was developed in the 1960s by J.R. Geigy AG (Basel Switzerland) and is now manufactured predominantly by Ciba Specialty Chemicals GmbH (Grezach-Wyhlen, Germany) (Seaman, 2007). Usage was increasing, but has now stabilized, with about 350 tonnes of triclosan being used within countries of the European every year. Of this, the UK is estimated to use 60-90 tonnes (Fan, 2002). Recently, triclosan, a synthetic bisphenol antimicrobial agent that has a broad range of activity against Gram-positive and Gram-negative bacteria, has been shown to select for *S. aureus* SCVs (Seaman *et al.* 2007).

Triclosan has been used in some skin care products for over 30 years and has been employed as surgical scrubs, hand washes and body washes to control MRSA (Russell, 2004). The wide use of triclosan in consumer products and in control of MRSA, coupled with its ability to select for SCVs of *S. aureus* has many implications in the healthcare setting (Bhargava & Leonard, 1996). In 1987 triclosan was successfully used to control an outbreak of MRSA and more recently it was used successfully to control MRSA outbreaks in a neonatal nursery (Tuffnell *et al.,* 1987 & Zafar *et al.,* 1995). Subsequently, in 1998 it was recommended for the control of nosocomial MRSA and 2% triclosan baths are now one of the recommended rationales for skin decolonisation of MRSA carriers (Ayliffe *et al.,* 1998 & Coia *et al.,* 2006). Triclosan has been reported to achieve growth inhibitory activities by blocking fatty acid synthesis and more specifically by explicitly inhibiting an NADH-dependent enoyl-acyl carrier protein (ACP) reductase, encoded by the *fabI* gene. (Payne *et al.,* 2001 & Heath *et al.,* 2000). Seaman *et al*. (2007) have found *in vitro* that triclosan can select for *S. aureus* colonies showing the characteristic SCV phenotype with low-level triclosan resistance and which coincidently have reduced susceptibility to penicillin and gentamicin. Additionally, triclosan-isolated SCVs were shown to have an increased tolerance to the lethal effects of triclosan.

Despite all the research into SCV formation a description of the classes of SCVs remain obscure. A description of prototroph and auxotroph is hardly sufficient as specific auxotrophic classes are found. Why are these classes unique? Additionally auxotrophs are found in clinically isolated SCVs, but to our knowledge have not been

found in laboratory isolations. Thus the cycling experiment was designed to try and understand and determine what events took place in the formation and reversion of SCV mutants. Critical to this investigation and understanding was the determination to sequence the final mutants and determine the locations of the mutations conferring the SCV phenotype. Simply this will inform us to whether the mutations are site specific (forward - point mutations occurring and reverting at just one site) or of a more general nature. Logic says the latter is the case.

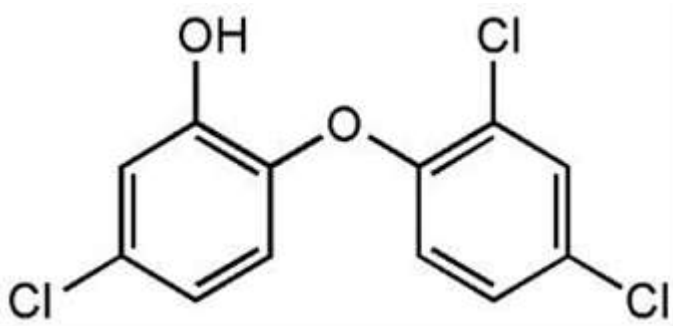


Figure 2.2 The chemical structure of triclosan (Heath *et al.,* 1989)

2.1.5 Identification characteristics *mecA* gene and *nuc* gene

mecA gene is embedded in a large heterologous chromosomal cassette, the SCC*mec* genetic element and gene encodes the penicillin binding protein (PBP 2A) with reduced affinity for β-lactams (Song *et al.,* 1987; Ito *et al.,* 1999). The characteristic methicillin resistance phenotype in *S. aureus* is due to the presence of *mecA* (Hartman & Tomasz, 1981; Ito & Hiramatsu, 1998). The *mecA* gene confers resistance to antibiotics such as methicillin because it prevents penicillin-like antibiotics inhibiting enzymes forming the cell wall of the bacterium (Abd El-Moez *et al.,* 2011)

The staphylococcal nuclease (*nuc*) gene from *Staphylococcus aureus* has been cloned and sequenced (Kovacevic *et al.,* 1985). *S. aureus* strains produce an extracellular

thermonuclease (Madison & Baselski, 1983). This maybe important because the enzyme produces thymidine from DNA possibly making it available to the pathogen during infection. Thus, success or failure with trimethoprim-sulfamethoxazole may well depend on the amount of tissue damage and organism burden, rather than acquisition of a resistance gene (Proctor, 2008).

2.1.6 The experimental strategy and design of the cycling experiment

The aim was to examine the evolutionally changes (mutations) occurring sequentially in successive cycling selections of SCV mutants and wildtype revertants. Two sequenced clinical MRSA strains MRSA COL (Dyke *et al.,* 1966) and N315 (Kuroda *et al.,* 2001) were chosen so changes in sequence in SCVs and revertants could be compared. Selection for SCVs was made independently for triclosan and gentamicin resistance for both strains. Then WT revertants were obtained from these SCVs by growth on nutrient medium and selected by large wild type colony form. Successive cycles of forward and reverse selection were made. From each selection cycle a representative SCV isolate and its WT revertant was stored.

The experiment was to be terminated if the SCV or wild type was unable to revert or when the mutation rate between the two isolates became consistent. Due to time constraints we terminated the experiment after 15 cycles. At this point DNA was obtained from the final SCV and wild type strains and sent for gnomic sequencing. This was examined to determine where mutational change had occurred. The evolutionary history of the two strains selected on two antimicrobial stored as isolates from each stage in the 15 cycles. Thus potentially all 60 strains could be examined to see when and what events have occurred. An analysis of the results should provide an idea of the mutational capacity and be suggestive of the evolutionary potential of the organism. A comparison of the results between two antimicrobials and two genetic backgrounds will provide a sound basis for the analysis and interpretation. So for example it may show what is a general phenomenon or a strain specific one.

2.1.7 Aims

- To determine the forward (to SCV) and reversion (to WT) mutation rates of S. *aureus* isolates.
- To compare the phenotypes exhibited by strains isolated in the successive cycles of selection
- To analyse these strains which represent successive mutational steps in the selection cycle by a comparison of their genetic differences.
- To determine if independent MRSA strains respond in a similar evolutionary manner to selection by antimicrobials.

2.2 Materials and Methods

2.2.1 Bacterial strains and bacteriophage

In this study two *S. aureus* strains that demonstrated susceptibility to a mutation of gentamicin and triclosan were included. Methicillin-resistant *S. aureus* (MRSA) COL (Dyke *et al.*, 1966) and N315 (Kuroda *et al.*, 2001) were the parental strains.

Strain terminology was as follows. The terms SCV01 and WT01 refer to the parental type (SCV or WT) and generation cycle (01, 02, 03, etc.). The terms COL-GEN, COL-TRI, N315-GEN and N315-TRI refer to strain (COL or N315) and the selection medium containing gentamicin (GEN) or triclosan (TRI). The original WT were thus termed COL WT00 and N315 WT00. Strains were maintained at -80°C in Mueller Hinton Broth (MH; CM0405: Oxoid Ltd, UK) supplemented with 8% dimethylsulphoxide (DMSO; 154938: Sigma Aldrich, UK) and re-grown on Mueller Hinton Agar (MH; CM0337: Oxoid Ltd, UK) plates when required. Bacteriophage Φ 80α used to detect phage sensitivity (Novick, 1963).

2.2.2 Preparation of gentamicin and triclosan stock solutions

Gentamicin was obtained from Sigma Aldrich (UK G-3632) and triclosan (Irgasan DB300) was from Ciba Spezialitätenchemie Grenzach GmbH (Grenzach-Whlen, Germany) Manufactures potencies were used to determine amounts of each of gentamicin and triclosan powder required to produce a 10,000 mg/L solution. Gentamicin powder was dissolved in sterile deionised water, as for the triclosan powder was dissolved in sterile DMSO , 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 sterile deionised water. Stock solutions were maintained at 4°C.

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 Storage of wild type and SCV cultures

The wild type and SCV strains were inoculated into 10 ml MH broth (plus 0.031 mg/L triclosan or 0.25 mg/L gentamicin for the SCV strains). Wild type strains were incubated at 37°C in an orbital shaker at 150 rpm for 24 hours and SCV strains for 48 hours. The optical density at 700 nm (OD_{700}) of each suspension was recorded and 1 ml of each culture in 8% DMSO was prepared and stored at -80°C.

2.2.5 Isolation of SCV mutants

Appropriate serial dilutions from the wild type broth cultures of COL and N315 strains (and labelled as appropriate e.g. COL WT00; COL WT01, etc) were made in sterile phosphate buffered saline (PBS; BR0014G: Oxoid, UK), and then plated out onto MH agar plus gentamicin or triclosan (concentrations in Table 2.1). These plates were incubated at 37°C for 48 hours. Colony counts were made at 48 hours yielding SCVs from strain-antimicrobial (e.g. COL SCV 01-TRI, N315 SCV01-GEN).

One representative SCV colony was chosen per cycle (e.g. COL SCV01-GEN, etc.) from each of the four assays. The strain was inoculated into MH broth plus selective agent (gentamicin or triclosan; Table 2.1). These were incubated at 37°C for 48 h in an orbital shaker, their optical density recorded and stored as described previously (section 2.2.3).

2.2.6 Isolation of wild type revertants

Appropriate serial dilutions were made in PBS of an SCV (e.g. COL SCV01-GEN) culture was made and 100 µl spread-plated out onto MH agar. Plates were incubated at 37°C and colony counts of wild type and SCV were determined at 24/48 hours. Wild type revertants were represented by large colonies. A representative individual

colony was chosen and termed WT01 (e.g. COL-GEN, COL-TRI, N315-GEN and N315-TRI). It was inoculated onto MH broth and incubated overnight. The OD_{700} was recorded and a sample was stored at -80^{o}C, as described above (section 2.2.4) and labelled appropriately, e.g. COL WT01-GEN.

Table 2.1 The concentrations of gentamicin and triclosan used to isolate SCVs

Strains	Antimicrobial	MIC (mg/L)	Agar medium (mg/L)	Broth medium (mg/L)
COL	gentamicin	0.25	1.00	0.25
	triclosan	0.125	0.5	0.03125
N315	gentamicin	0.25	2.00	0.30
	triclosan	0.125	0.50	0.03125

Modal values were obtained from three independent replicates and three independent biological replicates.

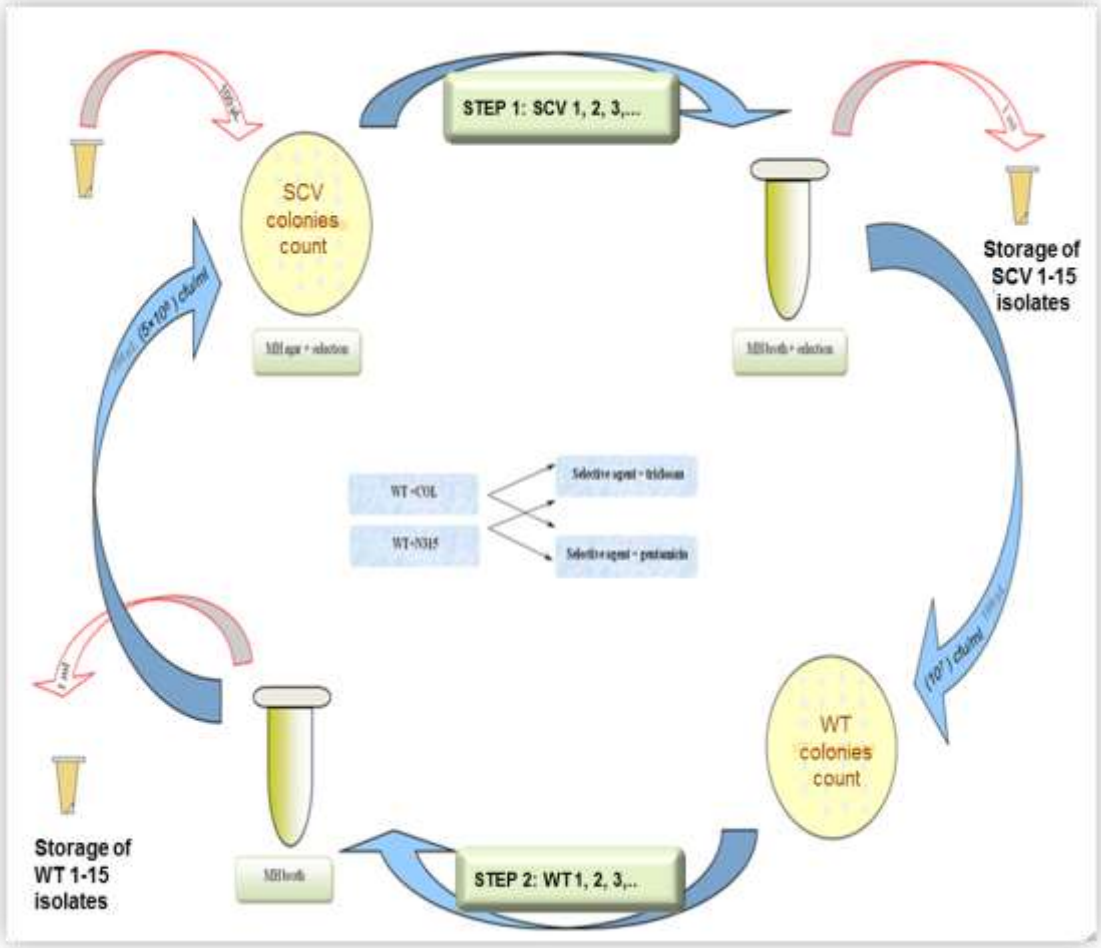


Figure 2.3 A schematic of the steps for isolating SCV mutants and wild type revertants. There were 15 successive cycles of selection.

2.2.7 Characterisation of SCVs isolates

S. aureus SCV strains were grown in MH broth, supplemented with 1 mg/L of either gentamicin or triclosan to prevent overgrowth by revertants with the parental phenotype. Cultures were prepared by inoculating individual colonies in supplemented MH broth followed by incubation at 37°C with shaking at 150 rpm for 36-48 hours.

2.2.7.1 Determination of auxotrophy

SCVs may be auxotrophic for haemin, menadione or thymidine, hence the auxotrophic profiles of SCVs were examined. Stock solutions (1,000 mg/L) of haemin, menadione and thymidine (H9039; M5625; T1895; Sigma Aldrich, UK) were prepared by dissolving powders in DMSO (haemin) or sterile deionised water (menadione and thymidine). Stock solutions were stored at 4°C for maximum 14 days. Lawns of SCVs were prepared by spreading 100 µL of a 48 h culture onto the surface of a MH agar plate. Sterile filter paper discs (151202; Whatman 42) plated in the centre of the plate and 10 µL of haemin, menadione or thymidine stock solutions added to filter discs and plate 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.

2.2.7.2 Coagulase activity

Coagulase production was determined by using Staphylase test kit (DR0595A; Oxoid Ltd, UK). Individual colonies were smeared into a test circle and a control circle on a reaction card. 1 drop of the Test Reagent was then added to the test circle and 1 drop of Control Reagent was added to the control circle. A colony was then mixed separately into the test reagent and into the control reagent. The presence of agglutination was recorded as coagulase positive and the absence of agglutination as coagulase negative.

2.2.7.3 Haemolysis activity

MH agar was supplemented with 5% defibrinated sheep blood (SB054; TCS Biosciences Ltd, UK) to examine haemolysis activity. 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 blood agar plate. Following incubation for 24-48 hours at 37°C. Haemolysis activity was detected by the

production of zones of clearing surrounding bacterial growth. Heamolysis activity was quantified by measuring the zone of clearing observed and related to the wild type control (100%).

2.2.7.4 Catalase activity

Addition of 1% w/v hydrogen peroxide (H_2O_2; H1009: Sigma-Aldrich, Poole, UK) to *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.

2.2.7.5 DNase activity

DNase agar was prepared by adding 39 g of dehydrate DNase culture media (CM0321: Oxoid, UK) to 1.0 L of deionised water. Overnight cultures of *S. aureus* parent and SCV strains were adjusted to a density of $1x10^7$ 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 0.5 M hydrochloric acid (HCl; 84415: Sigma Aldrich, UK) 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 using callipers. DNAse activity was quantified by measuring the zone of clearing observed. The relative activity (R) was calculated relative to the wild type control (100%), so R= SCV diameter/WT diameter.

2.2.7.6 Pigment production

Pigment 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 (34860: Sigma Aldrich, UK) 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. Pigment production was quantified and absorbance reported relative to the wild type control (100%).

2.2.7.7 Quantification of intracellular ATP

Intracellular levels of ATP were determined by an enzyme based luciferase assay. The BacTiter-Glo™ Microbial Cell Viability Assay (G819A: Promega, UK) quantifies ATP using mono-oxygenation of luciferin to produce a light signal that 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.

2.2.7.8 Phage sensitivity and phage yield by Φ 80α

Aliquots (~10^7 CFU/ mL) of host culture were inoculated in 10 ml of Brain Heart Infusion Broth (BHI; CM1137: Oxoid Ltd, UK). Supplemented with 10 mM $CaCl_2$ and $MgSO_4$ (containing 1 mg/L gentamicin for the SCVs cultures) with (~10^5 PFU/ mL) of Φ80α. Incubated at 37°C for 24 (WT) or 48 (SCV) hours. Supernatants of samples were passed through a sterile nitrocellulose filter membrane (Millipore; pore size 0.2 µm). Then 500 µl (~5 x 10^8 CFU/ mL) cells of a freshly grown overnight culture of host were mixed into an overlay of 3 ml BHI semi agar (BHI; CM1136: Oxoid Ltd, UK) supplemented with $CaCl_2$ (10 mM) and $MgSO_4$ (10 mM). This was poured over the agar surface and let to dry for 10 min. Aliquots (10 µl) of serial dilutions of phage Φ80α lysate were applied and incubated at 37°C for 24 hours. The presence of plaques indicated phage sensitivity. The phage titre/ml was determined by counting the plaques and multiplying by the dilution.

2.2.7.9 Growth rate

Growth dynamics were examined using a Bioscreen C analyser (ThermoFisher, UK) Overnight culture of *S. aureus* parent and SCVs strains were diluted to a density of 5 x 10^5 CFU/mL were inoculated with 100 µL of MH broth 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. Optical density was read using the wideband filter (450 – 580 nm) every 10 minutes.

2.2.7.10 DNA extraction

DNA was extracted using the GenElute Bacterial genomic DNA Kit (NA2100: Sigma Aldrich, UK). Overnight cultures of *S. aureus* parent and SCV culture were pelleted by centrifuging at 10,000 rpm in microfuge tubes. 200 µL of lysis buffer (consisting of 200 U/mL lysostphin 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 200 µL of manufactures 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 of manufacturer's elution solution. Finally samples were centrifuged for 1 minute at 10,000 rpm and the remaining elute (regarded as pure genomic DNA) stored at 4°C until required.

2.2.7.11 Species confirmation

As *S. aureus* SCVs are frequently difficult to identify and so a modified version of the quadriplex PCR protocol developed by Zhang *et al.* (2004) was employed to confirm that SCV isolates were *S. aureus*. PCR target 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), and *mecA* (a determinant of methicillin resistance) primers were employed (Table 2.2). All PCR reagents were obtained from 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 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 5 minutes at 94°C, followed by 10 cycles of 94°C for 40 seconds, 68°C for 40 seconds, and 72°C for 1 minute, and 25 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes, and a final hold at 72°C for 10 minutes. PCR products (12 µL) were run on 2% w/v agarose (A4718: Sigma Aldrich, UK) gels and were visualised with ethidium bromide (E8751: Sigma Aldrich, UK) (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 (33025: Bioline, UK).

Table 2.2 Primers used in multiplex PCR for species confirmation of SCVs

Primer	Sequence (3'- 5')
Staph756F	AACTCTGTTATTAGGGAAGAACA
Staph750R	CCACCTTCCTCCGGTTTGTCACC
*mecA*1	GTAGAAATGACTGAACGTCCGATAA
*mecA*2	CCAATTCCACATTGTTTGGGTCTA
*nuc*1	GCGATTGATGGTGATACGGTT
*nuc*2	AGCCAAGCCTTGACGAACTAAAGC

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

2.2.8 Statistical analysis

An ANOVA analysis (IBM SPSS software 20) followed by LSD (Least Significant Difference) was used to investigate significant difference between parent and SCV isolates.

2.3 Results

2.3.1 Wild type revertants

Figure 2.4 shows the isolation frequency of wild type revertants from their respective SCV parental strains over 15 cycles.

Statistical analysis shows that the selection of COL wild type revertants (gentamicin cycles; $2.08x10^9$ CFU/mL and triclosan cycles; $1.60x10^9$ CFU/mL) shows significantly reduced average frequency compared to N315 wildtype revertants (gentamicin cycles; $3.25x10^9$ CFU/mL and triclosan cycles,; $2.96x10^9$ CFU/mL; P = < 0.05). Thus the average N315 reversion rate to wild type was 1.75-fold higher than COL (Table 2.3).

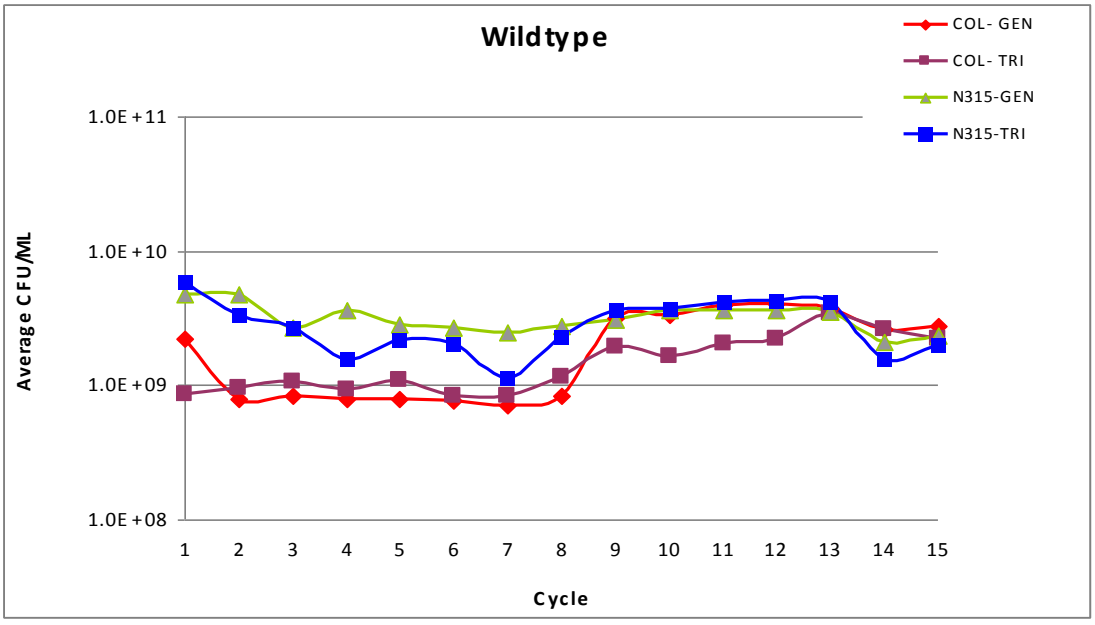


Figure 2.4 The number of wild type revertants, recorded at each cycle, from the SCV isolates of *S. aureus* COL and *S. aureus* N315.

Figure 2.5 compares the frequency of WT formation (ie reversion) of COL and N315 from their respective SCVs and locates their cycle identifier. The presence of the majority of the dots above the line signifies that the COL strain reverts at a lower frequency than N315 overall.

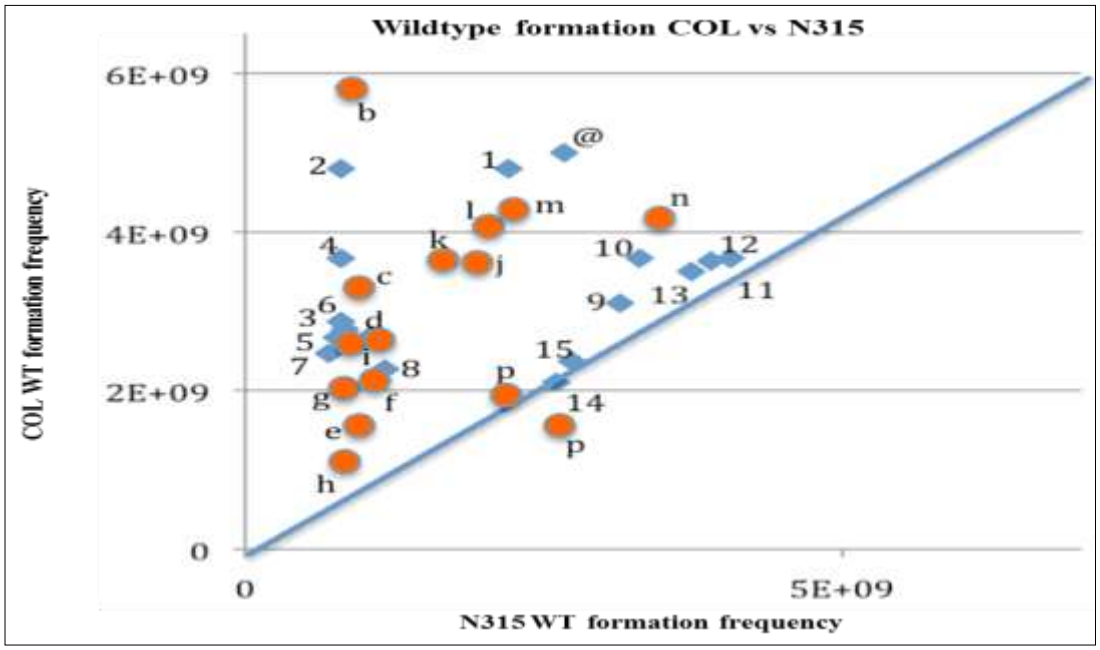


Figure 2.5 Shows the distribution of reversion isolates with respect to triclosan isolated SCVs (a-p, red symbols) and gentamicin SCVs (1-15). The initial count is represented by @

2.3.2 SCV formation

Figure 2.6 compares the frequencies of formation of SCVs from COL and N315 after triclosan and gentamicin selection. There is a downward trend (implying a reduction in mutation rate) to the data over the first 8 cycles, after which it appears to remain reasonably constant. Thus both strains are behaving (evolving?) in a similar manner.

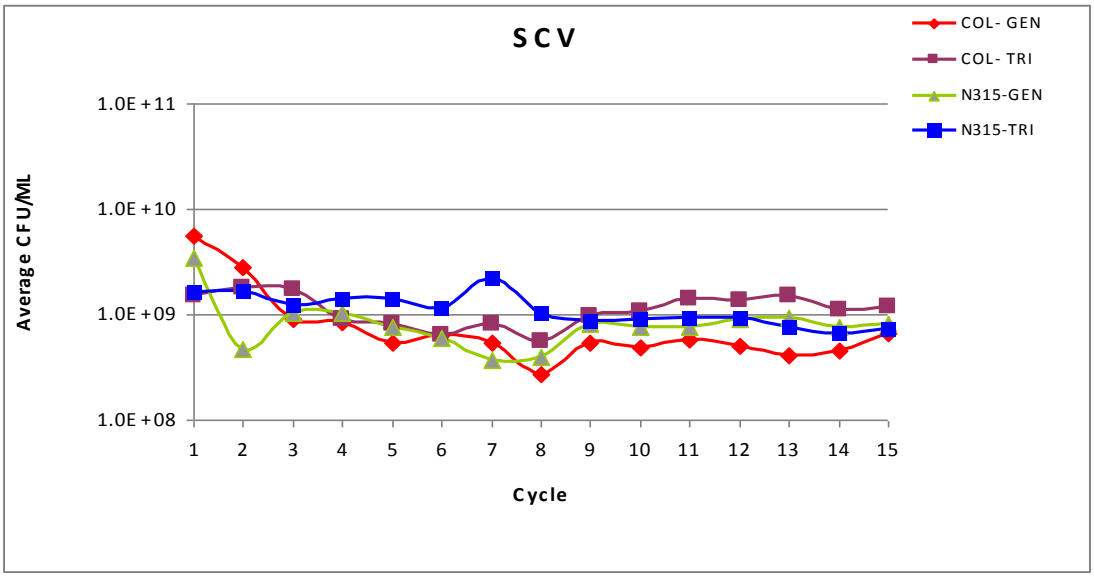


Figure 2.6 The number of SCV mutants, recorded at each cycle, from the wild type strains *S. aureus* COL and *S. aureus* N315.

Figure 2.7 shows the frequencies of SCV formation and locates the cycle number. Interestingly there is a clear distinction between triclosan and gentamicin shown by both strains. Triclosan shows a lower frequency of occurrence (1-2x10^9) as the data clusters towards the axis. The implication is that SCV formation by gentamicin is a more common occurrence (4x10^9). This could imply that there are more genes that could become mutated to provide the SCV phenotype. There is a wide distribution of frequencies of SCV formation when selection is made by gentamicin. There is no correlation in the distribution with cycle number so the changes appear random.

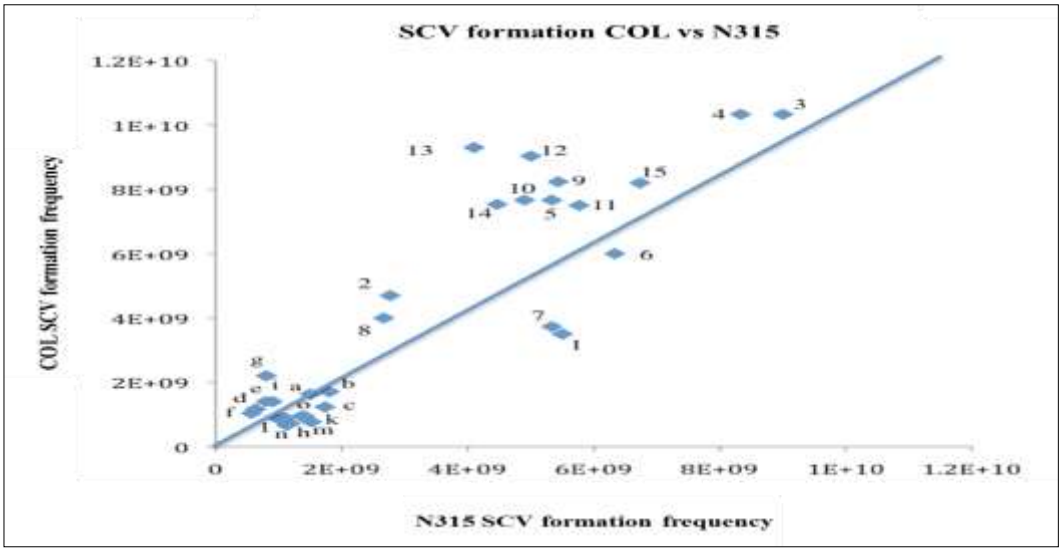


Figure 2.7 A comparison of the frequency of SCV formation by COL and N315 at each cycle after selection by triclosan (a-m) and gentamicin (1-15).

2.3.3 Mutation frequency

The SCV isolates occur at a frequency that averages at $1.17x10^9$ CFU/mL, for triclosan selection and $6.31x10^9$ CFU/mL for gentamicin selection (Table 2.3). This indicates that COL SCVs have adopted a lower mutation frequency than that of the wild type strain.

Table 2.3 A summary of the average of forward and reverse mutation frequencies

Strain	*GEN	*TRI
SCVs (COL+N315)	$6.31x10^9$	$1.17x10^9$
WT reversion (COL+N315)	$2.66x10^9$	$2.28x10^9$

*: CFU/mL

2.3.4 The rates of reversion between wild type and SCV

Figure 2.8 A-D compares the frequencies for the formation of triclosan and gentamicin SCVs plotted against their respective wild type reversion frequencies. It is clear that frequencies for SCV formation (COL: $5.44x10^9$ CFU/mL; N315: $7.18x10^9$ CFU/mL) are higher than wildtype reversion for gentamicin treatments (COL: $2.08x10^9$ CFU/mL; N315: $3.25x10^9$ CFU/mL). In contrast the SCV formation with triclosan selection (COL: $1.16x10^9$ CFU/mL; N315: $1.17x10^9$ CFU/mL) are lower than wildtype reversion (COL: $1.6x10^9$ CFU/mL; N315: $3.0x10^9$ CFU/mL).

The degree of scatter on the graph is suggestive of a variation in spontaneous mutation rate, which is related to the type of drug/antimicrobial the organism is exposed to. Is this an example of a cell's mutation rate being modified by the environment?

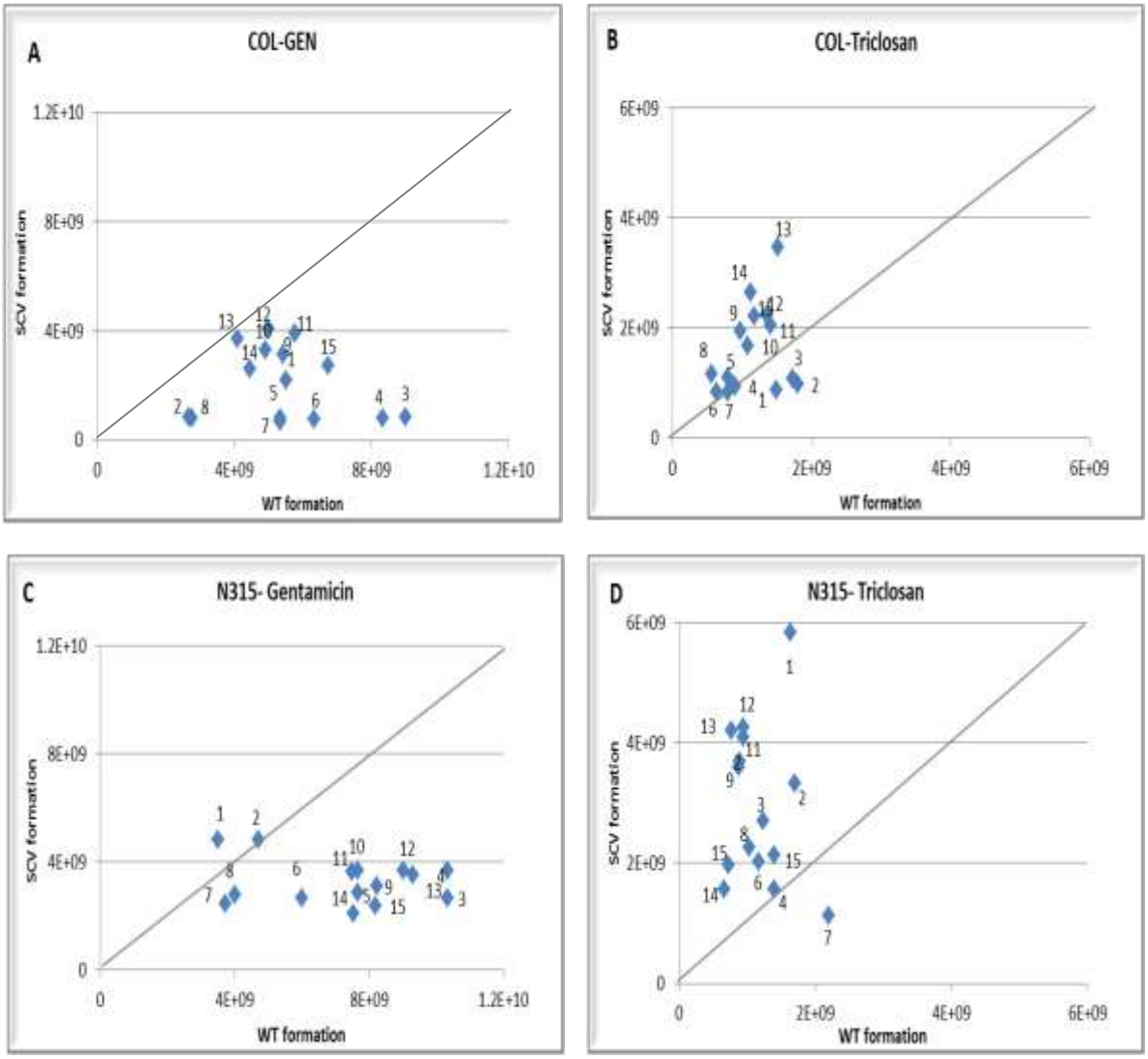


Figure 2.8 The frequency of reversion to wild type plotted against the mutation frequency to SCV. A) - *S. aureus* COL-Gentamicin; B) - *S. aureus* COL-Triclosan; C) - *S. aureus* N315-Gentamicin and D) - *S. aureus* N315-Triclosan.

Figure 2.9 A-D shows the relationship within the same strain to the two selective agents. The reversion graphs (2.9A and C) forward

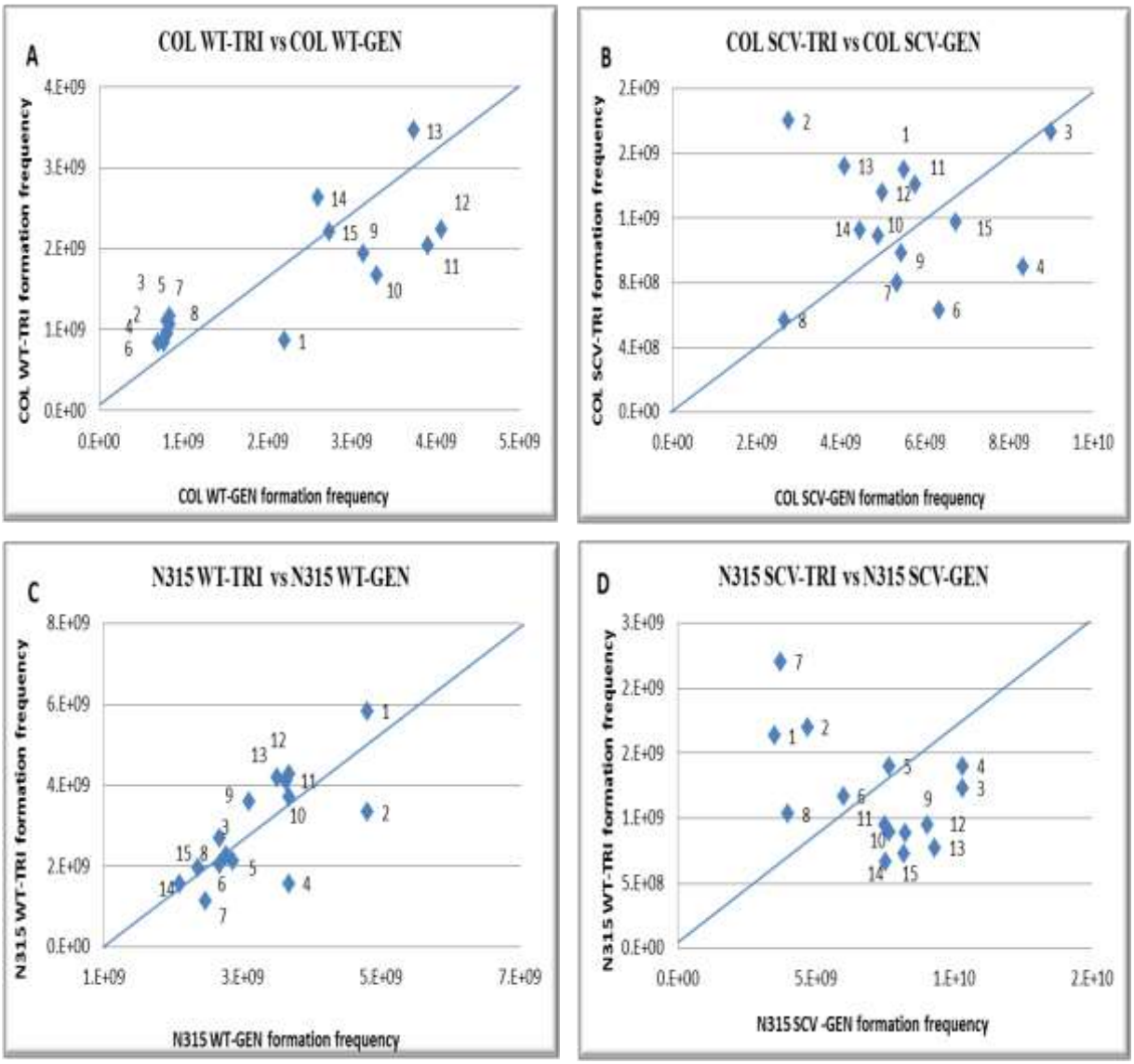


Figure 2.9 The frequency of strains from triclosan agent plotted against the mutation frequency to strains from gentamicin. A) - *S. aureus* COL-wild type; B) - *S. aureus* COL-SCV; C) - *S. aureus* N315-wild type and D) - *S. aureus* N315-SCV. There is no significant difference between gentamicin and triclosan treatments.

2.3.5 Characterisation of gentamicin and triclosan selected SCV15 isolates

All SCV15 isolates showed reduced or absented catalase, coagulase and haemolysis activity in comparison to their parental strains (Table 2.6). All SCV15s showed a complete absence of heamolysis activity, and all had a reduced catalase, coagulase, DNase activity. Fig 2.17 illustrates the tests used. There were differences in DNase activity by isolates obtained by triclosan selection that showed less DNase activity in comparison to those isolated by gentamicin (Table 2.6). Auxotrophy was confirmed if SCVs appeared as large pigmented colonies around filter discs impregnated with haemin, menadione or thymidine. All SCV15 isolates were sensitive to phage 80α. This also confirmed their identity as only *S. aureus* shows sensitivity to this phage (2.17 D).

2.3.5.1 DNase activity

DNase activity was determined by measurement of zones of extracellular DNA hydrolysis (Figure 2.10, Table 2.4). All SCV15 isolates showed significantly less DNase activity (reduced between 17-40 %) in comparison to parent strains (P = < 0.05). This was also true of the revertant wild type isolates (WT15) and so illustrates the fact that some characteristics are attenuated in the isolates as they increase in cycle number. Both COL SCV isolates (from gentamicin and triclosan) showed significantly fewer DNase activity reduced between 7-18 % in comparison to both COL WT15 isolates (from gentamicin and triclosan; P = < 0.05). No significant different DNase activity between both N315 SCV isolates (from gentamicin and triclosan) and both N315 WT15 isolates (from gentamicin and triclosan; P = < 0.05).

2.3.5.2 Pigment production

All SCV15 isolates produced significantly lower amounts of pigment (52-79%) in comparison to wild type strains (P = < 0.05). Wild type revertants also produced significantly less (13-22% down) than the original strains (P = < 0.05). So pigment production is also attenuated in these mutants (Figure 2.11, Table 2.4).

2.3.5.3 Intracellular ATP concentrations

(Figure 2.12, Table 2.4 shows that all SCV15 isolates contained significantly lower intracellular ATP levels (39-47%) in comparison to parent strains (P = < 0.05). ATP concentrations also significant different reduced in the wild type revertant strains (67-73%; P = < 0.05).

2.3.5.4 Phage sensitivity and phage yield by Φ 80α

All isolates were sensitive to phage 80α. The selection of N315 (WT00, WT15-GEN, WT15-TRI and SCV15-GEN; mean $3.7x10^8$ PFU/mL) gave significantly higher phage yields than COL (mean $1.84x10^8$ PFU/mL) generally. However SCV 15-TRI and N315 ($2.23x10^8$ PFU/mL) and COL ($1.6x10^8$ PFU/mL) were not significant different ($P = < 0.05$). Also there was no significant difference between wild type original (COL and N315) and wild type revertants isolate from gentamicin (COL WT15-GEN and N315 WT15-GEN; $P = < 0.05$; Figure 2.13 and Table 2.4).

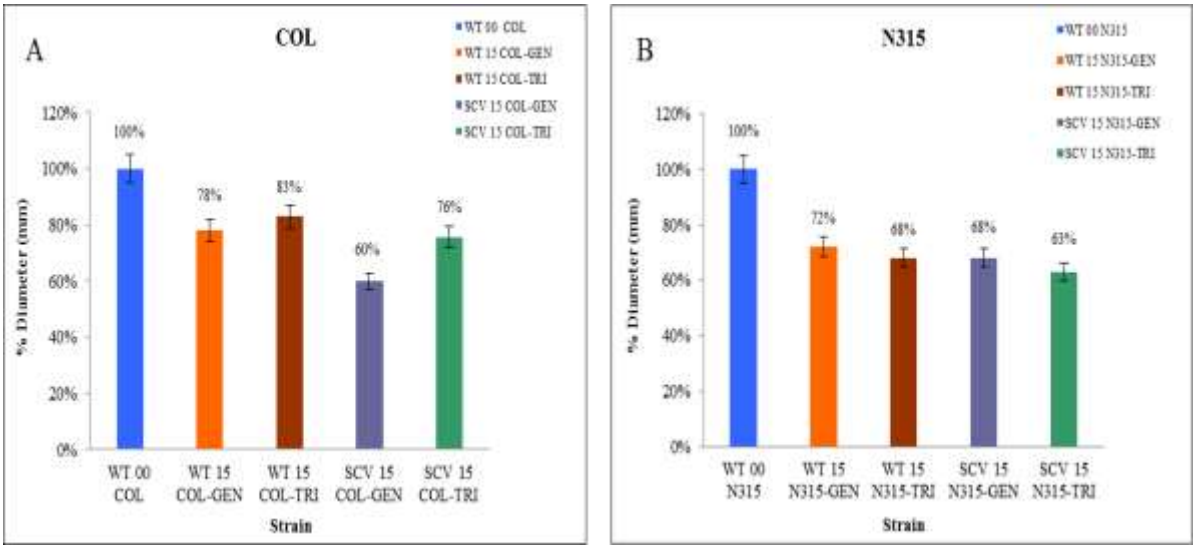


Figure 2.10 Extracellular DNase activity. A) by parental COL WT00, SCV15 and wildtype 15 strains; B) by parental N315 WT00, SCV15 and wildtype 15 strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

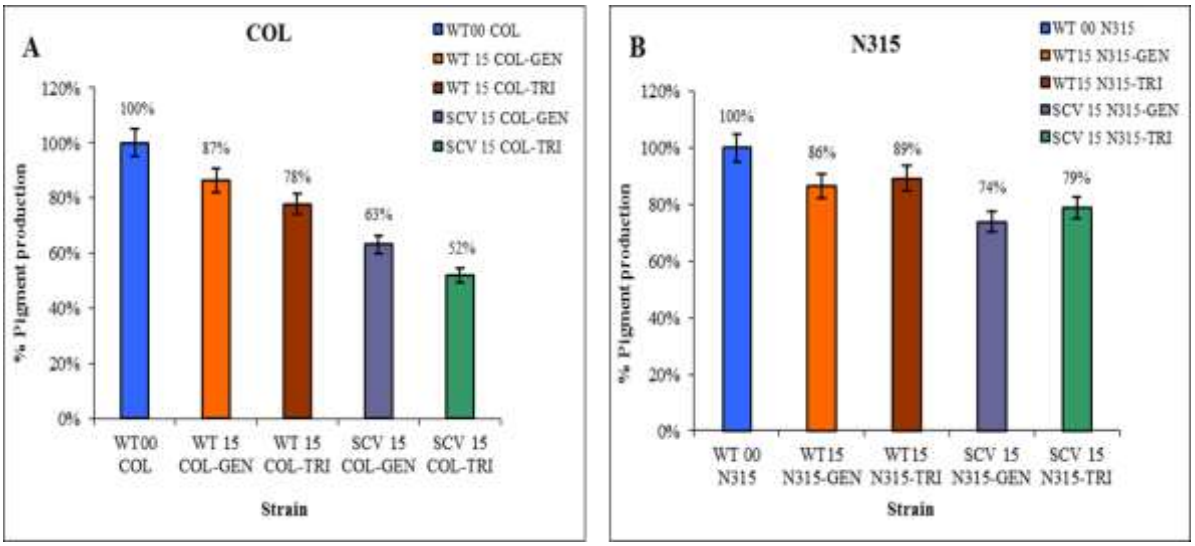


Figure 2.11 Pigment production. A) parental COL WT00, SCV15 and wildtype 15 strains; B) parental N315 WT00, SCV15 and wildtype 15 strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

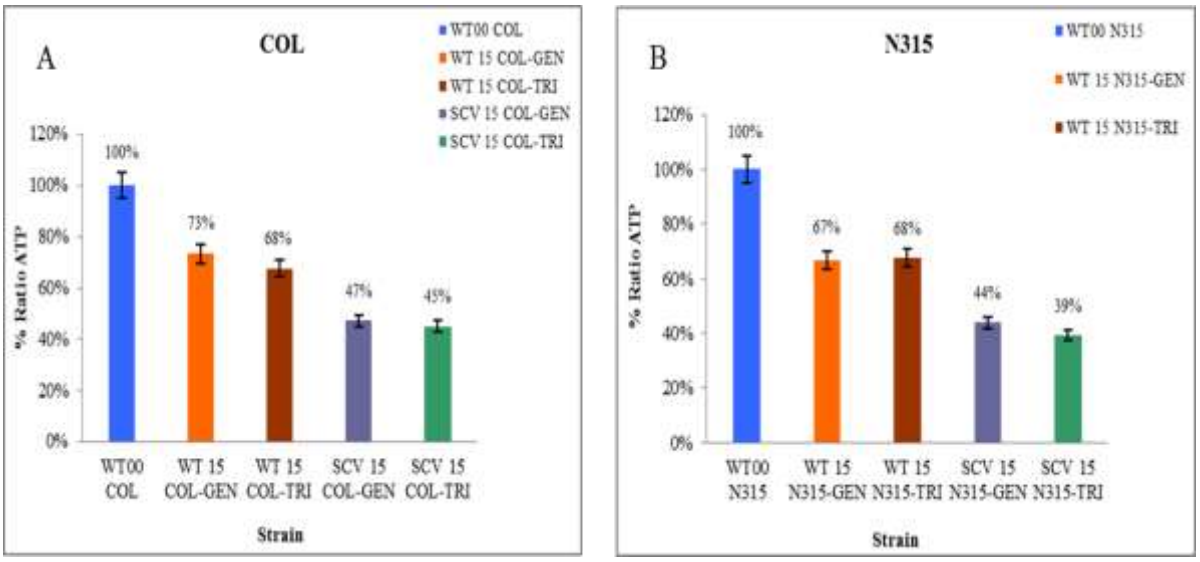


Figure 2.12 Intracellular ATP concentration. A) parental COL WT00, SCV15 and wildtype 15 strains; B) parental N315 WT00, SCV15 and wildtype 15 strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error

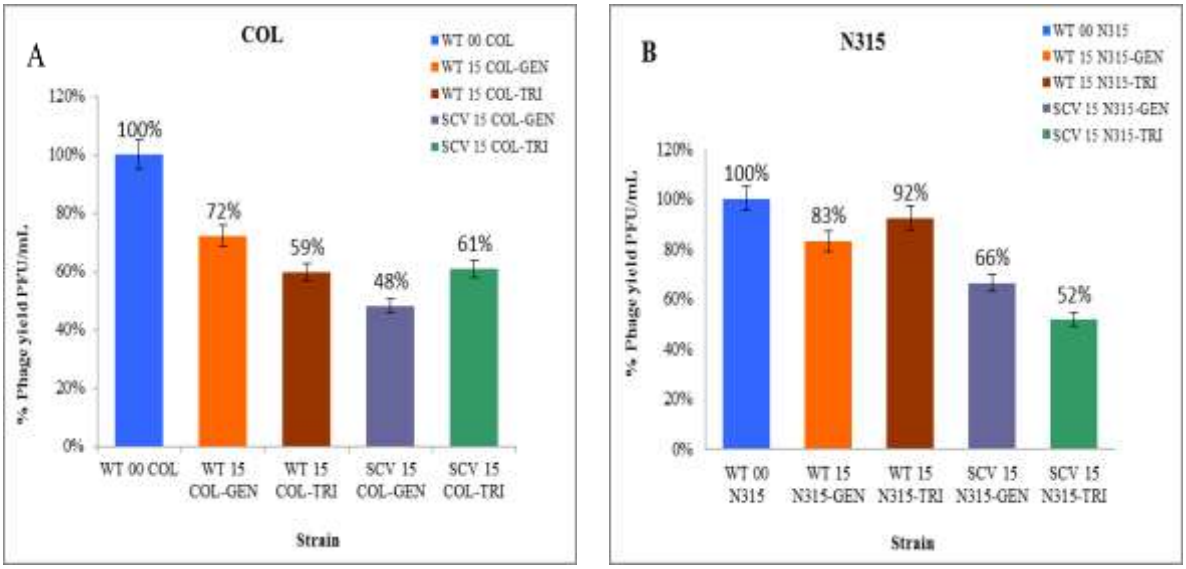


Figure 2.13 The ratio phage yield from *S. aureus* strains. A) phage yield by parental COL WT00, SCV15 and wildtype 15 strains; B) by parental N315 WT00, SCV15 and wildtype 15 strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

2.3.5.5 Growth rate and maximum cell density

Parental strains COL (WT00) and N315 (WT00) displayed typical *S. aureus* growth rates when grown in unmodified MH broth (Figure 2.14 A & B) and reached a final cell density at 24 hours of $1.35x10^9$ CFU/mL. The wild type revertant isolates obtained after 15 cycles (COL WT15 and N315 WT15) gave similar growth profiles to their WT00 original parental strains and there was no significant different between them (P = < 0.05). However at 24 hours COL WT15 was slightly lower at $1.0 x10^9$ CFU/mL compared to WT00 of $1.3x10^9$ CFU/mL.

All SCVs had extended lag phases of between 4-6 hours in comparison to the wildtype (Figure 2.14). All the SCV isolates gave significantly reduced optical densities (0.77- 0.82 OD) at 24 hours (P = < 0.05); reduced to 58-63% of their respective parental strains (1.21-1.40 OD).

Wild type revertants (COL 83% and N315 95%) were no significant to compared the original strains (P = < 0.05; Figure 2.14; Table 2.4).

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

Amplification of 16S rRNA, *mecA* and *nuc* genes confirmed all SCVs were *S. aureus*. Figure 2.7 shows presence of 16S rRNA (756 bp), *nuc* (279 bp) and *mecA* (310 bp), which can also be observed in the parent strain (Figure 2.18) in wild type and SCV isolates.

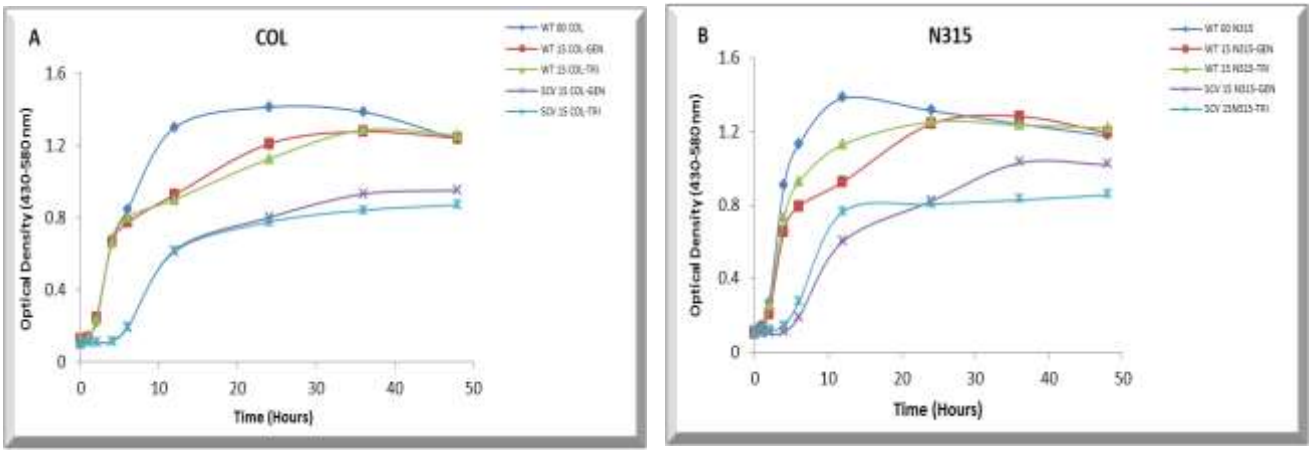


Figure 2.14 Growth curves of COL and N315. A) COL strains; B) N315 strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

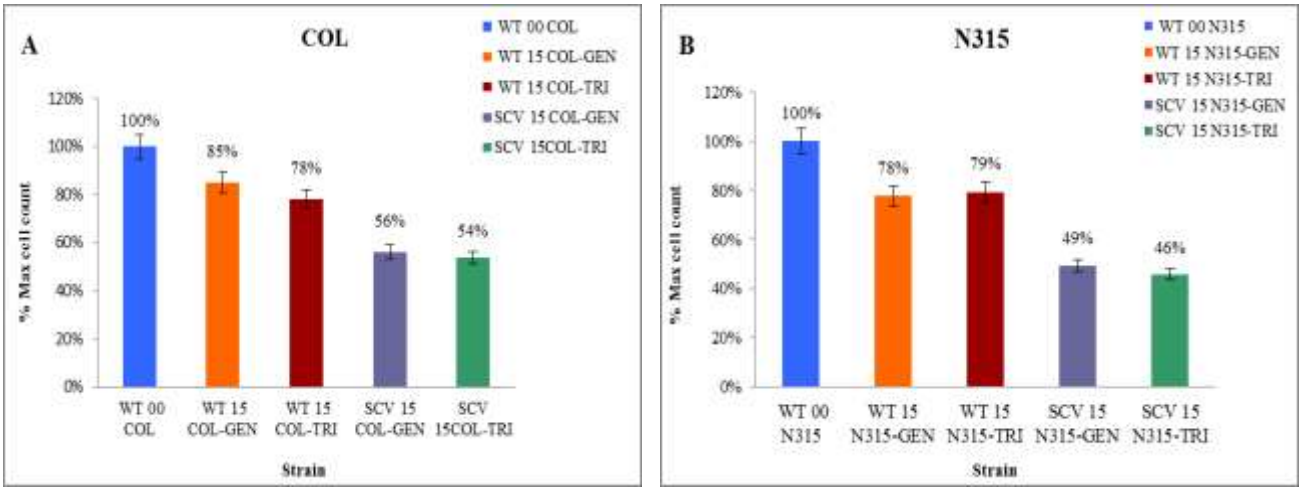


Figure 2.15 Maximum cell count of COL and N315. A) COL strains; B) N315 strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

Table 2.4 A comparison of physiological tests between SCVs and wild type strains

Strain	Catalase	Coagulase	Haemolysis	Auxotrophy	DNAse activity (% WT00)	Phage Φ 80 α yield [a] (%WT00)	Pigment production (% WT00)	Intracellular ATP (concentration; RLU) (%WT00)	growth ($OD_{430-580\ nm}$ at 24 h) (%WT00)	Max cell yield (% WT00)
COL WT 00	***	***	***	NT	100	100	100	100	100	100
COL WT 15 -GEN	***	***	**	NT	78	72	87	73	86	83
COL WT 15 -TRI	***	***	**	NT	83	59	78	68	80	78
COL SCV 15 -GEN	**	**	-	+	60	48	63	47	57	56
COL SCV 15 -TRI	**	**	-	+	76	61	52	45	55	54
N315 WT 00	***	***	***	NT	100	100	100	100	100	100
N315 WT 15 -GEN	***	***	**	NT	72	83	86	67	95	78
N315 WT 15 -TRI	***	***	**	NT	68	92	89	68	95	79
N315 SCV 15 -GEN	**	**	-	+	68	66	74	44	63	49
N315 SCV 15 -TRI	**	**	-	+	63	52	79	39	61	46

***: Strong catalase/coagulase/haemolysis activity; **: Medium catalase/coagulase/ haemolysis activity; -: Absence of catalase/coagulase/ haemolysis activity; +: Auxotrophic; NT – not tested; RLU: Relative light units by using luminescence; GEN: gentamicin and TRI: triclosan.

[a] Phage Φ 80 α titres on N315 were 6.5×10^9 and COL 3.2×10^9 pfu ml^{-1}.

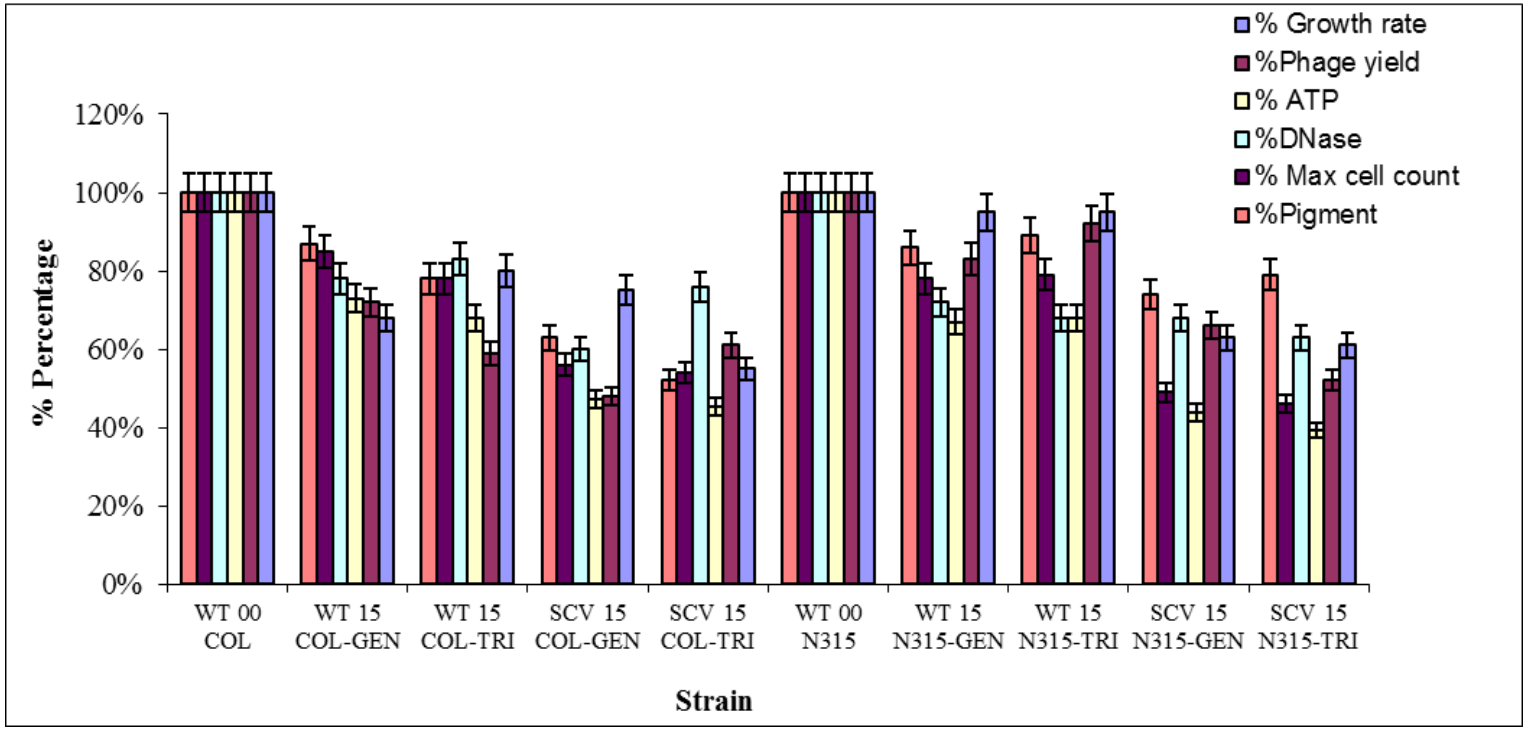


Figure 2.16 Summarises *S. aureus* COL and N315 wildtype test results with those of wildtype 15 and SCV 15. Error bars represent standard error.

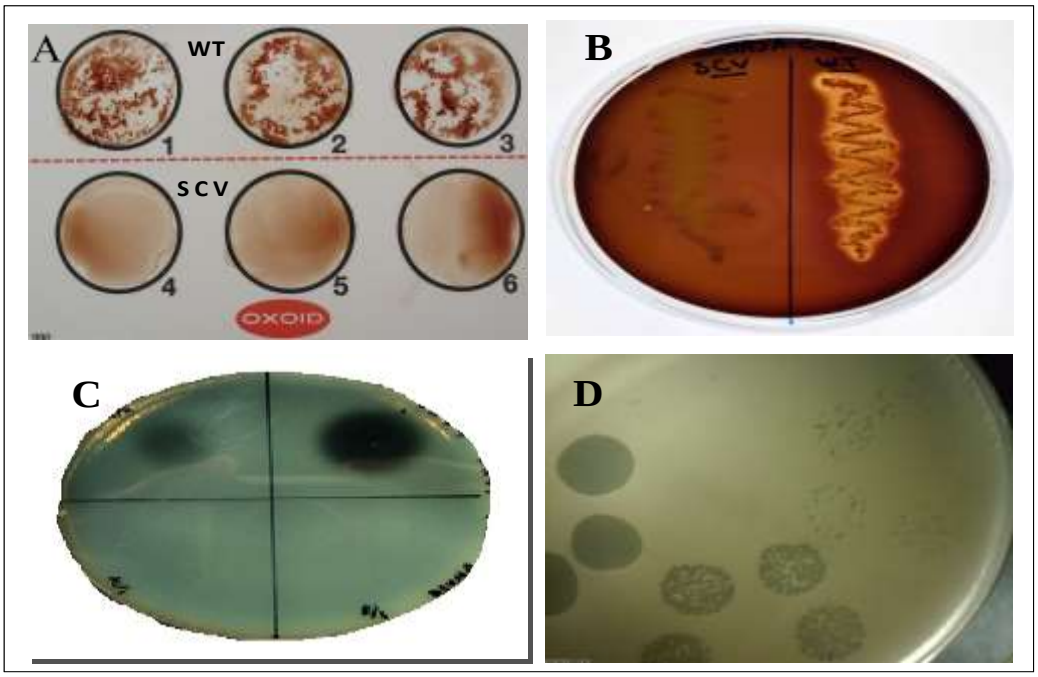


Figure 2.17 Characteristics associated with *S. aureus* SCVs A) Agglutination positive *S. aureus* (WT) and agglutination negative *S. aureus* SCVs (SCV); B) - Comparison of haemolytic activity of wildtype (right hand side of plate) and COL SCV; C) - Comparison of wildtype and SCVs of *S. aureus* on DNAse agar; D) - Plaques of bacteriophage 80α in a dilution series plated onto a soft agar overlay of *S. aureus* WT.

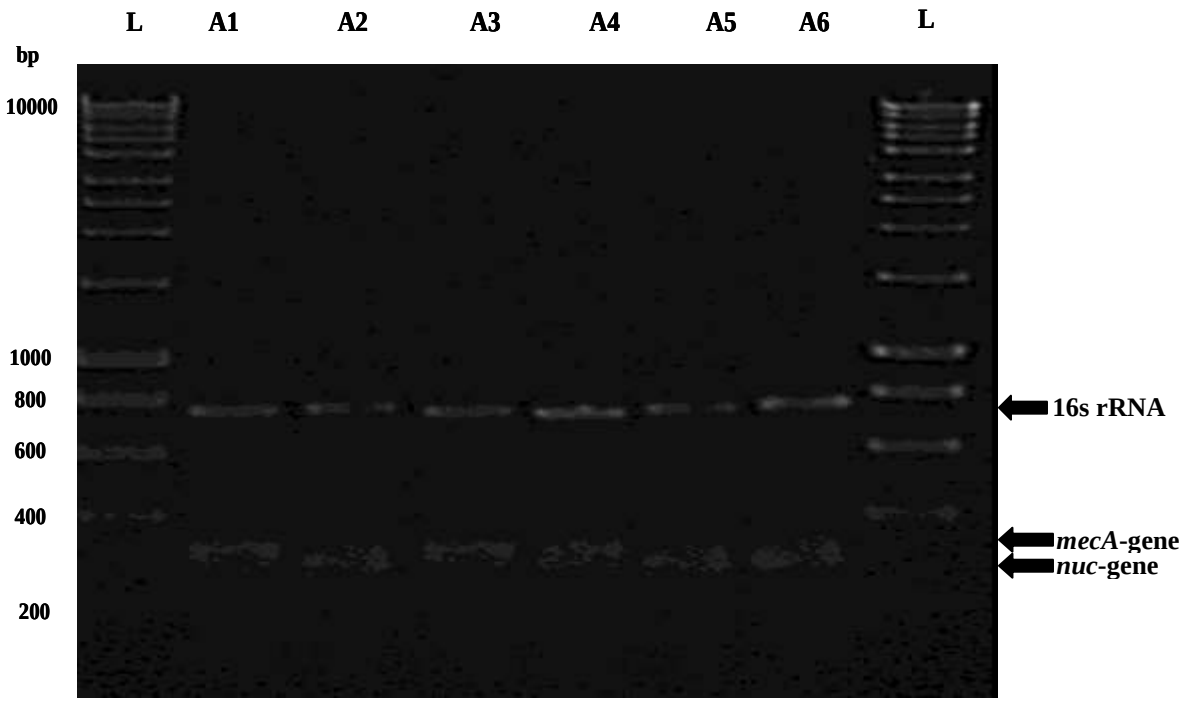


Figure 2.18 Multiplex PCR of SCV isolates from gentamicin and triclosan. L - Hyperladder 1; A1 – WT COL; A2 – WT N315; A3 – SCV15 COL-GEN; A4 - SCV15 COL-TRI; A5 - SCV15 N315-GEN; A6 – SCV15 N315-TRI. Amplification of the staphylococcal 16s rRNA (765 bp), *nuc*-genes (279 bp) and *mecA*-genes (310 bp) are shown.

2.3 Discussion

The fact that the cycling of the cultures was successful shows that no deletion SCV mutants occurred. So all the mutations to SCV were revertable point mutations. Figure 2.3 illustrated the steps in the cycle of repeated selection of SCV and WT strains. The cycling experiment resulted in the isolation of fifteen independent wild type and SCV strains, 60 in all. Multiplex PCR and sequencing of the staphylococcal 16S rRNA, *nuc*-gene and *mec*-gene provides 100% clarity that isolates SCVs are indeed *S. aureus.* Phage sensitivity also confirms that the isolates are *S. aureus.*

Figures 2.4 and 2.6 indicate that the rate of formation of wild type and SCV strains remains within an order of magnitude. Thus a mutational change to modify the spontaneous mutation rate has not been subject to selection.

Figure 2.5 and 2.7 compare the mutational changes occurring between COL and N315 strains and the data displayed indicates the cycle selection number. If the strains were identical and all other parameters were equal then the points should all fall on the 45 slope. The fact they do not illustrates a basic difference in responses by and between the strains and possibly to the selective agents. Some antibiotics like ciprofloxacin induce the SOS system and thus promote spontaneous mutation rate (Drlica & Zhao, 1997). Thus the potential is there for some antimicrobials to promote spontaneous mutation and thus raise the forward mutation rate to SCVs.

Physiological tests (Table 2.4) were done on WT00 and cycling isolates WT15 and SCV15. The data is normalised to the WT strain values and shown in Figure 2.16. This figure shows that all the WT15 strains had reduced physiological activity compared the parental strain (WT00). Thus although they acquired visually a normal morphology and physiological reactions, when measured most were clearly impaired. It is only the growth rates and phage yield by N315 WT15- TRI, that appear not to be statistically different for any WT15 isolates.

All the assays for strain COL WT15 are significantly different from the SCV15 results, except for phage yield and DNA's by SCV15-TRI.

Within these COL SCVs there is a lot of variation, but this is spread across the two strains. Both strains perform better in some assays and so neither strain is

physiologically equivalent. Thus the regulation of phenotypes by energy shortage caused by the mutations to SCV is not having the same effect on physiology. This indicating that SCV formation is not a unique physiological state.

The SCV strains all show reduced activities too. In all case (except DNase activity) SCV15-TRI shows lower activity than SCV15-GEN. The one exception between these SCV15 and the WT15 strains is DNase activity. Statistically these are not different.

Auxotrophy for haemin and menadione was regularly detected in SCVs isolated in this study (Table 2.4). Auxotrophy for haemin and menadione has been reported in *S. aureus* SCVs as well as SCVs isolated from several other bacterial species (Colwell, 1946; Sasarman *et al.,* 1970). The failure to produce menaquinone and haemin in the SCVs selected by gentamicin and triclosan results in a disrupted ETC resulting in reduced ATP levels. Thus this provides evidence for a reduction in energy generation capacity seen as intracellular ATP changes (Table 2.4). The physiological consequences are clear. Pigment concentrations were reduced (Table 2.4) and *S. aureus* requires energy to drive carotenoid biosynthesis, as well as other essential cellular process, such as protein and cell wall synthesis (Proctor *et al.*, 2006).

These effects on SCVs have been noted previously (Proctor *et al.*, 2006; Seaman *et al.*, 2007), but no one has examined revertant physiology or activities before. The fact that N315 and COL wild type revertants reacquire some wild type characteristics and yet others do not requires further investigation.

2.4 Conclusions

- There were no issues in the experimental design
- Multiplex PCR and sequencing confirmed all isolates were *S. aureus*.
- All mutations were revertable, therefore no deletion mutants occurred
- The rate of formation of SCVs was about the same as the rate of reversion.
- SCVs isolates had a reduction in a range of physiological parameters (intracellular ATP levels, pigment, catalase, coagulase, haemolytic, phage yield and DNase activity) that appear related to ATP generation capacity.
- Wild type revertants were not true revertants. They showed a reduction in most physiological activities.

CHAPTER 3:

BACTERIOPHAGE 80α STUDIES

3.1 Introduction

3.1.1 Gene transfer in staphylococci

Staphylococcus aureus is an effective opportunistic pathogen. This organism has acquired antimicrobial resistances through mutation and gene exchange. These antibiotic resistance determinants occur in both the chromosome and plasmid (Kayser *et al.,* 1972; Townsend *et al.,* 1985). It was recognised quickly that the organism acquires resistance soon after the introduction of new antibiotics (Lyon & Skurray, 1987). Antibiotic resistance phenotypes may be transferred by transformation, conjugation or transduction (Stiffler *et al.,* 1974; Archer & Armstrong, 1983). The transfer and consequent spread of antibiotic resistance among strains of *S. aureus* presents a major concern in the treatment of staphylococcal infections. The outbreaks of nosocomial infections by gentamicin and other antibiotic resistant strains of *S. aureus* has increased in some countries (Gillespie & Skurray, 1986).

3.1.2 Features of the *rpoB*, *grlA* and *sarA* genes

The *rpo* gene:

Rifampicin interacts specifically with the RNA polymerase beta-subunit encoded by the gene *rpoB* (Wichelhaus *et al.,* 1995). Rifampicin resistance in *S. aureus*, as in other bacteria, is associated with mutations in particular regions (cluster I and II) of the gene *rpoB* (Aubry-Damon *et al.,* 1998 & Aboshkiwa *et al.,* 1999).

The *grl* gene:

Quinolones are a group of compounds which target DNA topoisomerase IV (*grl;* Ferrero *et al.,* 1995). They have wide-ranging and potent antibacterial activity against Gram-negative and Gram-positive organisms, and are known under the names ciprofloxacin, ofloxacin, and norfloxacin (Shalit *et al.,* 1989 & Kojima *et al.,* 1990**)**. Quinolone resistance genes of *S. aureus* reported thus far include *norA* (Yoshida *et al.,* 1990**)**, *gyrA* (Fasching *et al.,* 1991 & Goswitz *et al.,* 1992), *gyrB* (Ito *et al.,* 1994), *flqA* (Trucksis *et al.,* 1991), and *grlA* (Ferrero *et al.,* 1994). The *norA* gene is normally present on the bacterial chromosome and encodes a membrane protein acting as a quinolone efflux pump (Kaatz *et al.,* 1993). The mutated *grlA* genes responsible for quinolone resistance were dominant over the wild-type allele (Yamagishi *et al.,* 1996).

The *grlA* and *grlB* genes encode the structural proteins of DNA topoisomerase IV, and mutations of the former gene are found in quinolone-resistant *S. aureus* strains, with or without *gyrA* mutations (Ferrero *et al.*, 1995).

The *sar* gene:

SarA is a 14.5-kDa DNA binding protein that modulates the transcription of over 120 genes both directly and indirectly (Dunman *et al.*, 2001) and its expression is carefully controlled during bacterial growth (Manna & Cheung, 2001). Recognizing that *SarA* can control over 120 genes both directly and indirectly (Dunman *et al.*, 2001). The *sarA* promoter region of *S. aureus* is complex, with three promoters (P2, P3, and P1) in an extensive region (~800 bp). The P1 promoter is most active and the *sigB*-dependent P3 promoter also contributes to *sarA* expression (Cheung *et al.*, 1999 & Manna *et al.*, 1998). The distal P2 promoter is weak *in vitro* and *in vivo* and appears to play a relatively minor role in driving *SarA* expression (Cheung & Manna, 2005).

3.1.3 Transduction in *S. aureus*

There is a long history of antibiotic resistance transfer amongst staphylococci or from staphylococci to other bacteria by transduction (Ritz & Baldwind, 1958; Dowd *et al.*, 1983). Transduction frequency ($0.2\text{-}8.2 \times 10^{-6}$) of methicillin resistance in *S. aureus* by bacteriophage 80 was stimulated by a penicillinase plasmid (Stiffler *et al.*, 1974), and other factors effecting gene exchange recognized include cell density temperature and media composition (Jarolmen *et al.*, 1965; Tartera *et al.*, 1988). All bacteriophages from *S. aureus* have either a relative or an absolute requirement for calcium ions (Rountree, 1951). Figure 3.1 shows a schematic of generalised transduction.

3.1.4 Temperate bacteriophages of *S. aureus*

Temperate (lysogenic) bacteriophages do not automatically enter the virulent cycle. Depending on a number of conditions, these bacteriophages may integrate their genome into that of the host cell where it remains until induced to become autonomous again and start replication and cell lysis (Brock & Madigan, 1988). The bacteriophage genome may also replicate autonomously in the cytoplasm of host cells without the production of new complete phage particles and lysis of the host cell (Hayes, 1968). There are over 250 staphylococcal bacteriophages reported in the literature (Pantucek *et al.*, 2004). A large number of temperate double-stranded DNA bacteriophages that infects *S. aureus* have been characterized, while most strains of *S. aureus* strains contain at least one prophage and rare

strains contain >15 bacteriophage types, suggesting a widespread potential for a protective effect from lysis due to resident phage integration (Lindsay & Holden, 2004). Comparative analysis of genome sequences revealed that *S. epidermidis* vB_SepiS_phiIPLA5, vB_SepiS_phiIPLA7, phiPH15 and phiCNPH82 are closely related, while phi909 showed a high similarity to *S. aureus* phages, as similar size and overall genome organization

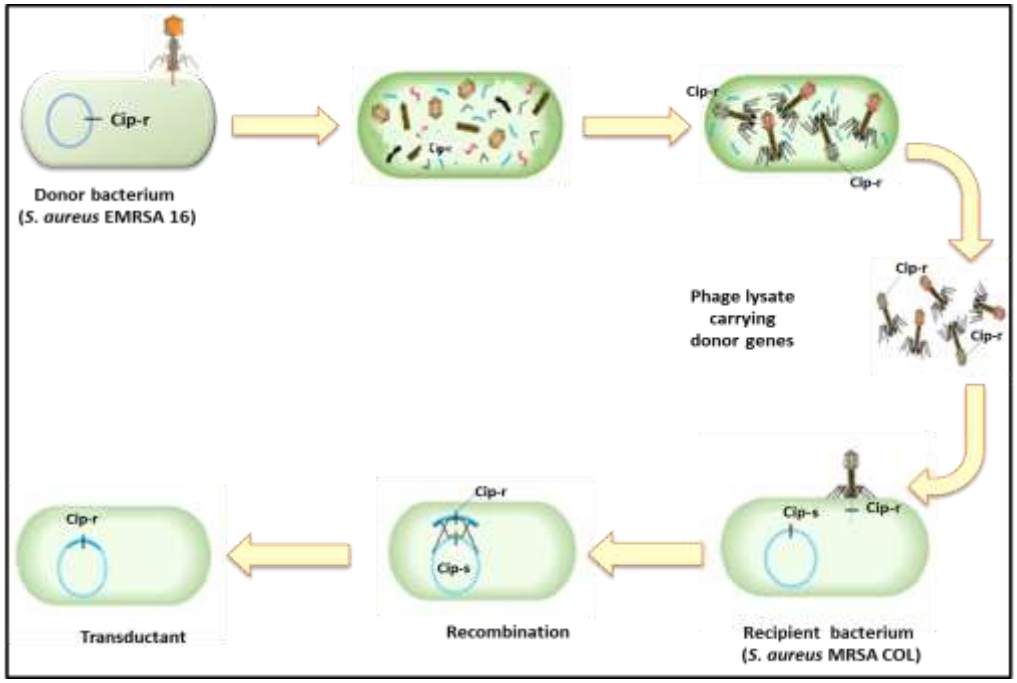


Figure 3.1 A schematic of generalized transduction. DNA genome of a temperate bacteriophage (i.e. Φ80α) inserts into the chromosome as a prophage; it later replicates, lyses the cell, and infects a simple recipient cell in which the novel DNA recombines into the recipient host cell chromosome. Adapted (Griffiths *et al*, 2000).

(Gutierrez *et al.*, 2012; Rosenstein *et al.*, 2009), also detected close relationships between *S. aureus* and CoNS phages (Deghorain *et al*., 2012). Thus it is clear a bacteriophage population could play a significant role in the evolution of their host cells. *S. aureus* is a bacterial pathogen known to cause infections in epidemic waves and maybe transduction contributes in this. One such epidemic was caused by a clone known as phage-type 80/81, a penicillin-resistant strain that rose to world prominence in the late 1950s (DeLeo *et al.*, 2011).

Phage 80α is a generalized transducing phage (Novick, 1963). Bacteriophage 80α belongs to a class of related staphylococcal *Siphoviridae* that are highly mosaic but retain a conserved organization of genes in functional modules (Kwan *et al.*, 2005). Some staphylococcal *Siphoviridae* encode virulence factors, such as staphylokinase, exfoliative toxin A, enterotoxin A, Panton-Valentine leukocidin (Winkler *et al.*, 1965; Yamaguchi *et al.*, 2000; Betley & Mekalanos, 1985; van Wamel *et al.*, 2006). The genome length of bacteriophage 80α is 43,864 bp, encoding approximately 73 ORFs and is deposited in Genbank under accession number DQ517338 (Christie *et al.*, 2010).

3.1.5 Bacteriophage-host interaction

The role of bacteriophages in genetic interactions with their hosts has been discussed by Fry & Day (1992), as is the use of phages in molecular biology (Freifelder, 1987). Transduction in natural environments may be a rare event, as generalised transduction has only been demonstrated once in freshwater ecosystems (Saye, 1987).

Bacteriophage adsorption is the first step towards infection. This occurs at defined receptors thus giving phage:host relations a specificity. This particular step is sensitive to environmental conditions such as ionic composition, temperature and pH (Shao & Wang, 2008). After adsorption the bacteriophage genome is introduced into the host cell. Once in the cytoplasm the most frequent outcome is expression on the phage genome. This leads to synthesis of phage proteins and replication of the phage genome. The phage genome is then packaged into capsids to produce infectious virions. The host cell then bursts releasing progeny phage (Freifelder, 1983).

Certain bacteriophages delay the reproductive stage and these are termed temperate and remain as a prophage in association with the host. The host is termed a lysogen. In this response the lytic functions are repressed by a phage encoded DNA binding protein (Ptashne, 1967). There are benefits to the host as the prophage confers immunity to further infection and possibly encodes other phenotypic traits (see 3.1.4). This is termed "lysogenic

conversion" (Rudin *et al.,* 1974; Low & Porter, 1978). Lysogenic conversion in *S. aureus* strains can result in either loss or acquisition of phenotypic characters. The conversion of lipase-positive strains to lipase-negative strains has been reported by different authors (Rosendal & Bulow, 1965). After adsorption the bacteriophage genome is introduced into the host cell, lysogeny is favored when this occurs; similar host monitoring abilities have been reported in bacteriophage D3 of *P. aeruginosa* (Kokjohn & Miller, 1988) and P22 of *S. typhimurium* (Levine, 1957). Thus, phages clearly impact virulence by positive lysogenic conversion, since they provide novel functions and activities to the host, and virulent bacteriophages can only enter the lytic pathway, but a lysogenic-like stage has been recorded (Canchaya *et al.,* 2003; Brussow *et al.,* 2004; Malachowa & DeLeo, 2010) This has been termed pseudolysogeny and bacteriophages were observed to exist in an arrested state of the lytic cycle due to host inactivity (Figure 3.2). As soon as host cell vegetative growth was restored, the bacteriophage lytic pathway ensued (Romig & Brodetsky, 1961).

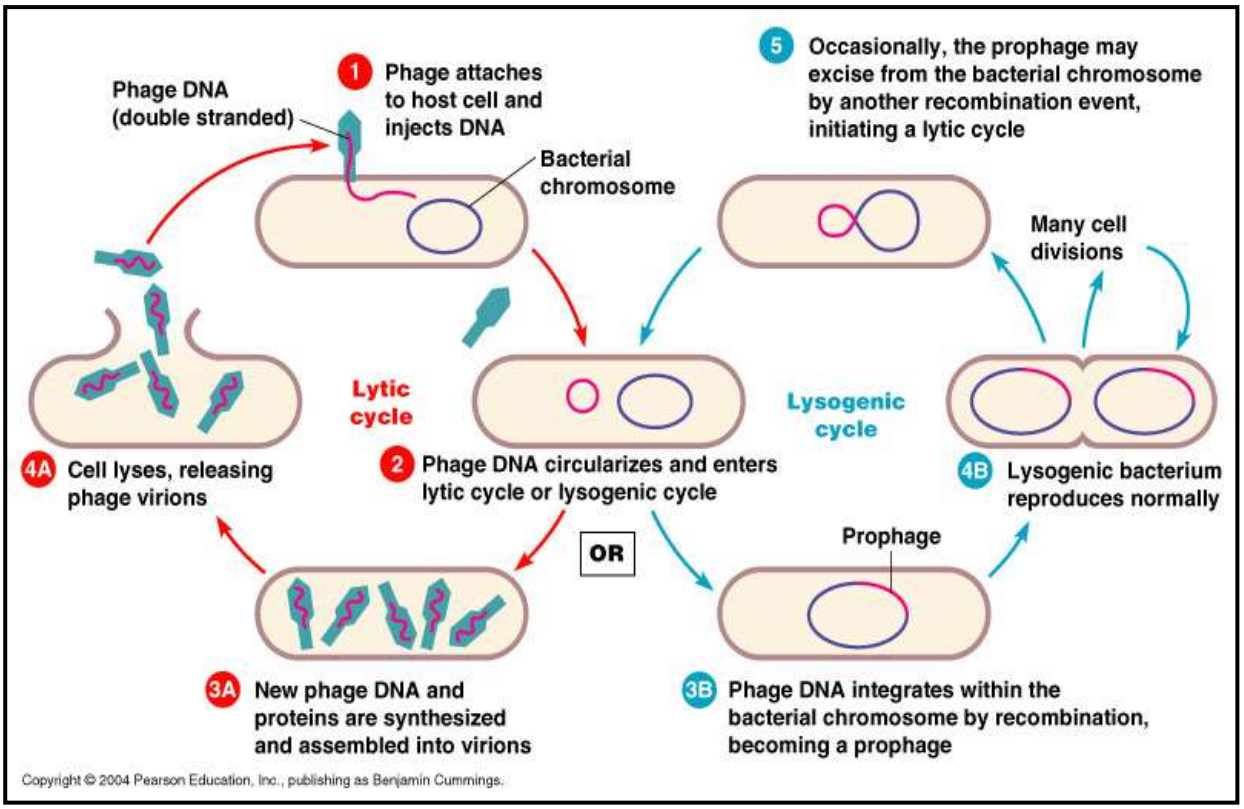


Figure 3.2 A schematic of generalized bacteriophage replication. There are two cycles of replication temperate bacteriophage. **Lytic cycle:** bacteriophage enter cell, multiply and lyse cell. **Lysogenic cycle:** bacteriophage may integrate their genome into that of the infected host cell (Bauman, 2004).

3.1.6 Aims

- To examine the ability to transduce *rpoB*, *grlA* and *sarA* genes between the wild type and SCV strains.

- To determine whether transfer frequency was influenced by the recipient or donor lysate.

- To determine relative susceptibilities of *S. aureus* wildtype and SCVs to bacteriophage 80α.

- Determine the relative frequency of phage 80αresistance and lysogeny occurring between the strains.

- Compare lysate yields from *S. aureus* wildtype, SCVs and their lysogens.

- Analyse the effect of wild type and SCV their physiological status on phage interactions.

3.2 Materials and Methods

3.2.1 Bacterial strains

The bacterial strains and bacteriophage used in this study are listed in Table 3.1. These strains were selected to represent a broad range of methicillin-susceptible and methicillin-resistant phenotypes. *S. aureus* MRSA COL; MRSA COL-SCV 1, 2 and 3 are sensitive to rifampicin and ciprofloxacin, Epidemic methicillin-resistant *S. aureus* (EMRSA) strain 16 is strain containing *rpoB* gene which encodes rifampicin resistance, MRSA N315 is strain containing *grlA* gene which encodes ciprofloxacin resistance, and *gfpuvr* with *sarA* P2 promoter. 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 Transduction related protocols

3.2.2.1 Preparation rifampicin and ciprofloxacin stock solutions

Rifampicin (Rif: R3501) and ciprofloxacin (Cip: C17850) 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 DMSO for rifampicin and 10 mL of deionised water. Solutions were dissolved thoroughly by vortex mixing and 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.

3.2.2.2 Minimum inhibitory concentrations

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

3.2.2.3 Bacteriophage propagation and titration

Bacteriophage lysates were made with bacteriophage 80α (Novick, 1963). The phage was propagated on EMRSA 16, MRSA N315 and ALC 1473 (~ 10^7 CFU/mL) and bacteriophage 80α (~ 10^5 CFU/mL) were mixed with 10 mM $CaCl_2$ and $MgSO_4$ and poured onto the surface of MH agar and incubated at 37°C for 24 hours. The surface growth was harvested in 4 mL PBS (supplemented with 10 mM $CaCl_2$ and $MgSO_4$) and cleared by centrifugation for 10 minutes at 4000 rpm to remove cell debris. The supernatant was sterilised by passing through 0.2 µm filters (Minisart, UK) and titred on BHI. Then an aliquot of cells (~5 x 10^8 CFU/ mL), from a freshly grown overnight culture of the host, were mixed into an overlay of

3 mL BHI semi agar (supplemented with 10 mM $CaCl_2$ and $MgSO_4$). This was poured over the agar surface and let to dry for 10 minutes. Aliquots (10 µl) of serial dilutions of bacteriophage 80α lysate were applied and incubated at 37°C for 24 hours. The bacteriophage titre/mL was determined by counting the plaques and multiplying by the dilution.

3.2.2.4 Viable bacterial cell count

10 µL aliquots of a 10-fold serially diluted culture were deposited onto corresponding sectors of MH agar, incubated at 37°C for 48 hours, and then colonies were enumerated.

3.2.2.5 Transduction procedure

An overnight culture was grown in MH broth, harvested by centrifugation (5000 rpm for 5 min), and the pellets resuspended in 1 ml fresh MH broth to ~10^9 CFU/mL. All media for bacteriophage work were supplemented with $CaCl_2$ (10 mM) and $MgSO_4$ (10 mM). Then 100 µL (~10^9 CFU/mL) of the host strain was mixed with 100 µL of bacteriophage 80α, prepared on EMRSA 16, N315 or ALC1437. The culture was incubated at 37°C for 30 minutes. Aliquots (100 µL) were plated out onto selective media MH agar containing either 50 mg/L rifampicin or 2 mg/L ciprofloxacin. Plates were incubated at 37°C for 48 hours, and transductants enumerated. Phage and cultures without bacteriophage were plated separately on selective media as controls.

3.2.2.6 Preparation of lysogens

Ninety six individual overnight (microtiter plate) cultures of COL-wildtype and COL-SCV1, 2 and 3 were prepared by inoculating 10 ml of brain heart infusion (BHI; CMO135: Oxoid Ltd, UK) supplemented with 10 mM $CaCl_2$ and $MgSO_4$ (and 1 mg/L gentamicin for the SCVs cultures). Aliquots of host culture (~10^9 CFU/ mL) and Φ80α-9789 (~10^8 CFU/ mL) were inoculated in 200 µL BHI supplemented with 10 mM $CaCl_2$ and $MgSO_4$ (containing 1 mg/L triclosan for the SCV cultures) and incubated at 37°C for 24 (WT) or 48 (SCV) hours. A sample of each well was streaked to single colonies on MH agar (for the SCVs this contained 1 mg/mL gentamicin) and these were incubated at 37°C for 48 hours. A single isolated colony from each plate was stabbed into an overlay (5 ml MH semi agar; 6 gm/L; plus 10 mM $CaCl_2$ and 100 µL of an overnight grown culture of strain COL). The plates were incubated at 37°C for 24 hours. A zone of lysis indicated phage production and thus the presence of a lysogen.

Table 3.1 Bacterial strains and bacteriophage used in this study of transfer of antimicrobial resistance amongst staphylococci.

Strain	Description	Resistance	Source/Reference
COL	An MRSA strain originally isolated in 1961 Genome sequenced	Tet, Oxa	(Dyke *et al.*, 1966 & Gill *et al.*, 2005)
COL$^{\text{Rif-R}}$	derived from COL following rifampicin selection	Rif	This study
COL SCV 1	derived from COL following triclosan selection	Tet	(Day *et al.*, 2012)
COL SCV 2	derived from COL following triclosan selection	Tet	(Day *et al.*, 2012)
COL SCV 3	derived from COL following triclosan selection	Tet	(Day *et al.*, 2012)
EMRSA 16	Epidemic methicillin-resistant *S. aureus* strain containing *rpoB-gene* which encodes rifampicin resistance(isolated 1992)	Cip, Ery, Neo, Kan	(Murchan *et al.*, 2004)
MRSA N315	MRSA strain (isolated Japan 1982). *grlA*-gene (ciprofloxacin resistance). Genome sequenced	Cip, Ery, Neo, Oxa	(Kuroda *et al.*, 2001)
ALC1437	RN6390 with pALC1421 (*gfpuvr* with *sarA* P2 promoter)	–	(Cheung *et al.*, 1998)
Mu50	MRSA strain (isolated Japan 1997). Genome sequenced	Van	(Hiramatsu, *et al.*, 1997)
EMRSA 15	Epidemic MRSA type 15 (isolated UK 1991)	Oxa	(Richardson & Reith, 1993)
ATCC 25923	Methicillin-sensitive isolated USA, Seattle 1945	–	American Type Culture Collection
ATCC 29213	Methicillin-sensitive isolated USA, Wichita 1955	–	American Type Culture Collection

NCTC 9789	Methicillin susceptible *S. aureus* isolated UK	–	The National Collection of Type Cultures
NCTC 6571	Oxford strain, isolated in 1940, methicillin-sensitive	–	The National Collection of Type Cultures
IA48	MRSACOL *hemB*::*ermB*, SCV	–	Vaudaux *et al.*, 2002
DB24	8325-4 *menD*::*ermC* (8325-4: Prophage-cured derivative of wild-type strain NCTC 8325, *rsbU* mutant)	–	Bates *et al.*, 2003
OM1B	Phenotype SCV indistinguishable from OM1a	–	Lannergard *et al.*, 2008
Eagles	Carries plasmid-borne, high-level mupirocin resistance	Mup, Ery, Tec, Amp,	Thomas *et al.*, 1999
F89	Carries plasmid-borne, high-level mupirocin-resistant strain	Mup	Thomas *et al.*, 1999
RN450	Plasmid-free recipient strain cured of Φ11, Φ12 & Φ13	Nov, Rif	Novick, 1967
Bacteriophage 80α	Mosaic genome related to Φ11 (transducing phage)	–	Novick, 1963

Tet – Tetracycline; Oxa – Oxacillin; Cip – Ciprofloxacin; Ery – Erythromycin; Kan – Kanamycin; Neo – Neomycin; Van – Vancomycin; Mup – Mupirocin; Tec – Teicoplanin; Amp – Ampicillin; Nov – Novobiocin; Rif – Rifampicin; – No information

3.2.2.7 Preparation of lysates from lysogens strains

Overnight cultures of the lysogen (L-COL, L-SCV 1, L-SCV 2 and L-SCV 3**)** were prepared by inoculating 10 ml of BHI supplemented with 10 mM $CaCl_2$ and $MgSO_4$ (and 1 mg/L triclosan for the SCVs cultures). An aliquot (~10^7 CFU/ mL) of host culture were inoculated in 10 ml BHI supplemented with 10 mM $CaCl_2$ and $MgSO_4$ (containing 1 mg/L triclosan for the SCV cultures) and ~10^5 CFU/ mL of Φ80α-9789 was added. The culture was incubated at 37°C for 24 (WT) or 48 (SCV) hours. Supernatants of samples were passed through a sterile nitrocellulose filter membrane (Millipore; pore size 0.2 µm). Then ~5 x 10^8 CFU/ mL of a freshly grown overnight culture of host were mixed into an overlay of 3 ml BHI semi agar supplemented with $CaCl_2$ (10 mM) and $MgSO_4$ (10 mM). This was poured over the agar surface and let to dry for 10 min. Aliquots (10 µl) of serial dilutions of bacteriophage Φ80α lysate were applied and incubated at 37°C for 24 hours. The bacteriophage titre/ml was determined by counting the plaques and multiplying by the dilution.

3.2.2.8 Preparation of lysates from parental strains

Overnight cultures of the parental strains (COL-wildtype, SCV 1, SCV 2 and SCV 3**)** were prepared by inoculating 10 ml of BHI supplemented with 10 mM $CaCl_2$ and $MgSO_4$ (and 1 mg/L triclosan for the SCVs cultures). Aliquots (~10^7 CFU/ mL) of host culture were inoculated in 10 ml BHI supplemented with 10 mM of $CaCl_2$ and $MgSO_4$ (containing 1 mg/L triclosan for the SCVs cultures) and ~10^5 CFU/ mL of Φ80α-9789. Incubated at 37°C for 24 (WT) or 48 (SCV) hours. The phage titre/ml was determined as described previously (section 3.2.3.7).

3.2.2.9 Calculation of transduction frequency

Transfer frequencies were calculated by dividing the total number of transductants/mL by total viable cell count per mL.

3.2.3 Statistical analysis

The mean of at least three independent experiments was used in all calculation. IBM SPSS software 20 was used for analysis of data. The ANOVA method was used to calculate the least significant difference (LSD), as described previously (section 2.2.8).

3.3 Results

3.3.1 Transduction experiments

3.3.1.1 Transduction of ciprofloxacin resistance (cip-r; *grlA* gene)

The transduction frequency of ciprofloxacin resistance into wildtype COL and SCVs by bacteriophage 80α-EMRSA 16 was determined. Table 3.2 shows that the transduction frequency of cip-r was significantly higher (P = < 0.01) at $1.8x\ 10^{-7}$ into the wild type COL host than into SCV 1. No transfer into SCV 2 and SCV 3 was observed. The limit of detection was $\leq 1.0x10^{-9}$ cfu

Table 3.2 Transduction of ciprofloxacin resistance into *S. aureus* COL and SCV strains by Φ 80α-EMRSA 16

Donor EMRSA 16 phage $80\alpha^{Cip-r}$	Recipient cell count	Number of phage	Number of transductants	Transductants /recipient	Transduction frequency/phage	% WT Transduction frequency
COL-wildtype	$2.3x10^{9}$	$1.0x10^{9}$	$1.8\ x10^{2}$	$7.8x\ 10^{-8}$	$1.8x\ 10^{-7\ A}$	100
COL-SCV 1	$1.2x10^{9}$	$1.0x10^{9}$	20	$1.7x\ 10^{-8}$	$2.0x\ 10^{-8}$	11
COL-SCV 2	$1.3x10^{9}$	$1.0x10^{9}$	0	$\leq 7.7x10^{-9}$	$\leq 1.0x10^{-9}$	≤ 1
COL-SCV 3	$1.1x10^{9}$	$1.0x10^{9}$	0	$\leq 9.1x10^{-9}$	$\leq 1.0x10^{-9}$	≤ 1
Mean	$1.5x10^{9}$	$1.0x10^{9}$	$1.0x10^{2}$	$4.7x10^{-8}$	$1.\ 0x10^{-7}$	56

[A] The transduction frequency was significantly higher than SCV1 (P = < 0.01).
Transduction frequency values were obtained from three independent replicates and three independent biological replicates. The limit of detection was $\leq 1.0x10^{-9}$ cfu/mL

3.3.1.2 Transduction of rifampicin resistance (*rpoB* gene)

A rifampicin resistant mutant of strain COL was made by selection on MH plates containing 50 mg/L rifampicin and this was used in the following experiment. Lysates were prepared on $COL^{Rif\text{-}r}$ (Table 3.3).

Table 3.3 and Figure 3.3 shows the transduction frequency was significantly higher into the wildtype COL ($4.8x10^{-7}$) than SCV 3, SCV2 and SCV 1 which was the lowest ($3.6x10^{-7}$).

There was no significant difference in transduction frequency between SCV 1, SCV 2 and 3 (P = < 0.05).

Table 3.3 Transduction of rifampicin into *S. aureus* COL and SCVs by Φ 80α $COL^{Rif\text{-}r}$

Donor COL phage 80α $^{Rif\text{-}r}$	Recipient cell count	Number of phage	Number of transductants	Transductants /recipient	Transduction frequency/phage	% WT Transduction frequency
COL-wildtype	$2.5x10^{10}$	$2.5x10^{10}$	$1.2x10^{4}$	$4.8x10^{-7}$	$4.8x10^{-7}$	100
COL-SCV 1	$4.9x10^{9}$	$2.5x10^{10}$	$9.0x10^{3}$	$1.8x10^{-6}$	$3.6x10^{-7}$	75
COL-SCV 2	$2.9x10^{9}$	$2.5x10^{10}$	$9.5x10^{3}$	$3.3x10^{-6}$	$3.8x10^{-7}$	79
COL-SCV 3	$1.3x10^{9}$	$2.5x10^{10}$	$1.0x10^{4}$	$7.7x10^{-6}$	$4.0x10^{-7}$	84
Mean	$8.5x10^{9}$	$2.5x10^{10}$	$1.0x10^{4}$	$3.3x10^{-6}$	$4.5x10^{-7}$	84.4

Transduction frequency values were obtained from three independent replicates and three independent biological replicates.

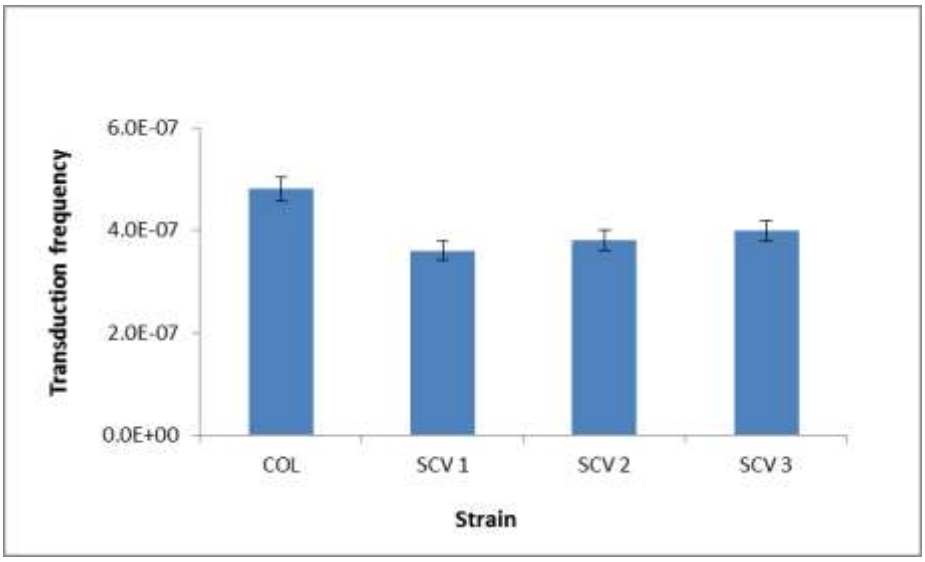


Figure 3.3 A comparison of the transduction frequencies of rifampicin resistance into *S. aureus* COL wildtype and SCV 1, 2 and 3 by Φ 80*α*-COL^{Rif-r}. *The transduction frequency into strain COL was significantly higher than into the SCVs. Error bars represent the standard error of the mean.

3.3.1.3 Transduction of *sarA* P2 by Φ80*α*-ALC1437

Table 3.4 shows an inability to transduce GFP into any strains. The limit of detection was about $6x10^{-9}$ and for two strains it was $3x10^{-11}$. Thus it remains a possibility that if the recipient numbers were raised by another order of magnitude it may have resulted in transfer.

Table 3.4 *S. aureus* strains used in an attempt to transfer *gfpuvr* by Φ 80α-ALC1437

Donor ALC1437 phage 80α	Recipient cell count	Number of phage	Number of transductants	Transduction frequency/phage
COL-wildtype	$2.1x10^{9}$ $3.2x10^{11*}$	$6.4\ x10^{9}$ $3.4x10^{11*}$	0 0	$\leq 6.4x10^{-9}$ $\leq 3.4x10^{11*}$
COL-SCV 1	$2.0x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
COL-SCV 2	$2.1x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
COL-SCV 3	$1.7x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
EMRSA15	$1.2x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
EMRSA 16	$1.6x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
MRSA N315	$4.7x10^{9}$ $3.2x10^{11*}$	$6.4x10^{9}$ $3.4x10^{11*}$	0	$\leq 6.4x10^{-9}$ $\leq 3.4x10^{11*}$
Mu50	$1.7x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
ATCC 25923	$2.3x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
ATCC 29213	$3.5x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
NCTC 9789	$3.5x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
NCTC 6571	$4.1x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
IA48	$4.4x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
DB24	$1.7x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
OM1B	$1.6x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
Eagles	$2.1x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
F89	$2.0x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$
RN450	$2.1x10^{9}$	$6.4x10^{9}$	0	$\leq 6.4x10^{-9}$

*Repeated with high number of cells and phage

3.3.2 Lysogeny of *S. aureus* COL

3.3.2.1 Rates of resistance to and lysogen formation by bacteriophage 80α

Figure 3.4 shows a *clear* zone of lysis of the indicator lawn due to phage reproduction in some spots. The growth of lysogens causes the *cloudy* appearance which can be seen in some zones. 192 samples from (768 samples in all) each of four stains were tested for lysogeny or resistance (Figure 3.5). COL and SCV 3 were similar both gave a similar and greater proportion of lysogens (68.2 and 68.8% respectively) than either SCV 1 or SCV 2 (9.9 and 4.2 % respectively). These two groups were significantly different (P = < 0.01).

Figure 3.6 shows the relative proportions of resistant and lysogens formed by each strain. Wildtype COL and SCV 3 were significantly different from SCV1 and 2 (P = < 0.01). There is no obvious reason for the different response between these strains. The error bars represent the standard error of the mean.

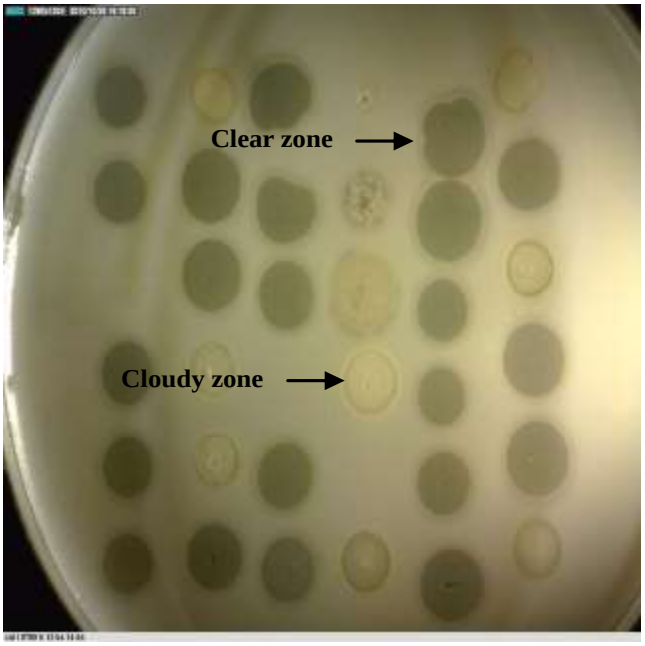


Figure 3.4 An illustration of bacteriophage 80α release by COL lysogens and lysis of the indicator lawn. Zones of clearing with **cloudy centres** were made by the release of bacteriophage from 80α COL lysogens.

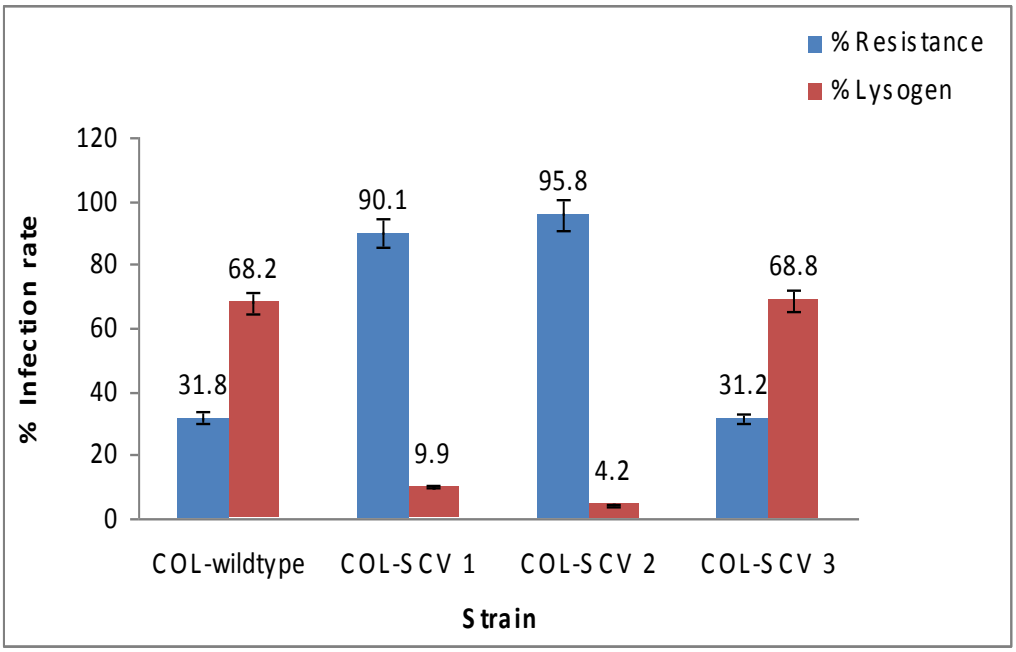


Figure 3.5 A comparison of the frequency of formation of phage 80 α lysogens and resistant mutants of *S. aureus* COL and SCV strains 1, 2 and 3. Error bars represent the standard error of the mean**.**

3.3.2.2 Viable cell counts of lysogens and parental strains

The data in Table 3.5 (illustrated in Figure 3.6) compares the final viable cell counts after 48 hours incubation of parental strains and their respective lysogens. As expected the wild type has about a 10-fold higher cell yield than the SCVs. It is clear the final cell yields of lysogens are reduced compared to their parental strains, except for SCV3 (% CFU lysogen/parental strain column). The effect is greatest in the wild type strain (53%) and least for SCV3 (98%). The cell yields of lysogens of COL, SCV 1, 2 and 3 were reduced compared to the wildtype, by 47, 26, 38 and 2% respectively. Thus phage carriage clearly has a significant effect ($P = < 0.01$), but a decreasing one in the slower growing SCVs.

The relative amount of phage produced by lysogens of SCV 1, 2 and 3 (compared to COL) was raised by 39, 16 and 84% respectively (Table 3.5). Thus although lysogeny reduces cell yield significantly it impacts phage production by about 10-fold at worst.

3.3.2.3 Phage 80α yield from lysogens

Figure 3.7 compares the phage yields of lysogen and lysates. Phage yields from lysogens are 5-10-fold higher than from lysates (Table 3.5) and were significantly greater in comparison to phage yield by lysates ($P = < 0.05$).

Table 3.5 A comparison of phage 80α yields from lysogens and parental strains of COL and SCVs 1, 2 and 3

Strain	CFU/mL (lysogen)[1]	CFU/mL (parental strain)[1]	PFU/mL lysogen	PFU/mL lysate	% PFU:CFU (phage yield: lysogen CFU)	% PFU:CFU (phage yield :parental strain CFU)	% CFU lysogen / CFU parental strain	% WT phage yield	% Ratio of phage yield lysate/lysogen lysate
COL-wildtype	$2.24x10^9$	$4.20x10^9$	$2.70x10^8$	$5.90x10^7$	12.05	1.40	53	100	12
COL- SCV 1	$3.08x10^8$	$4.16x10^8$	$2.50x10^7$	$6.50x10^6$	8.12	1.56	74	39	19
COL- SCV 2	$2.14x10^8$	$3.46x10^8$	$1.20x10^7$	$1.60x10^6$	5.61	0.46	62	16	8
COL- SCV 3	$2.16x10^8$	$2.20x10^8$	$3.40x10^6$	$7.00x10^5$	1.57	0.32	98	8	20

[1] Final viable cell counts; values were obtained from at least three independent replicates and three independent biological replicates.

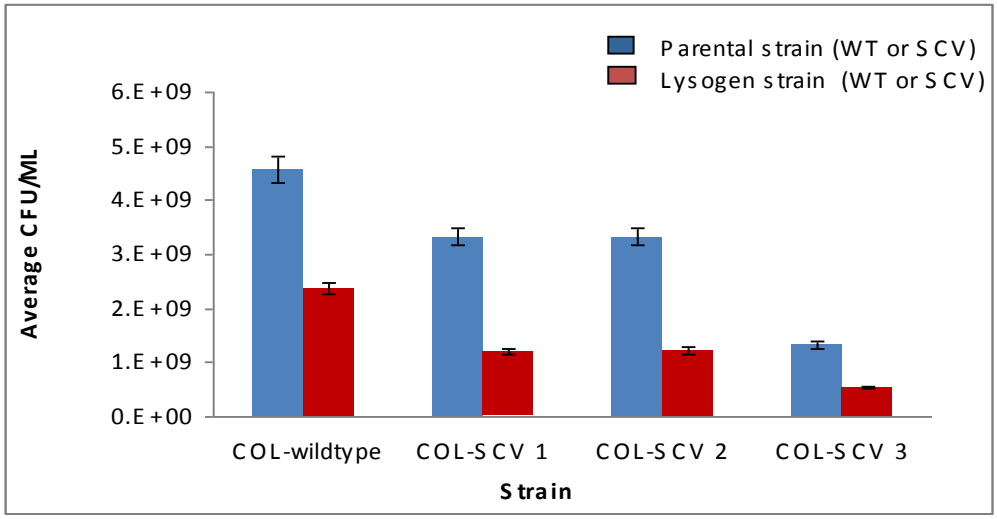


Figure 3.6 Comparison of the average viable counts of *S. aureus* COL and SCV strains and their lysogens. Lysogeny reduces cell yield when this is compared to that of their parental strains. Error bars represent the standard error of the mean**.**

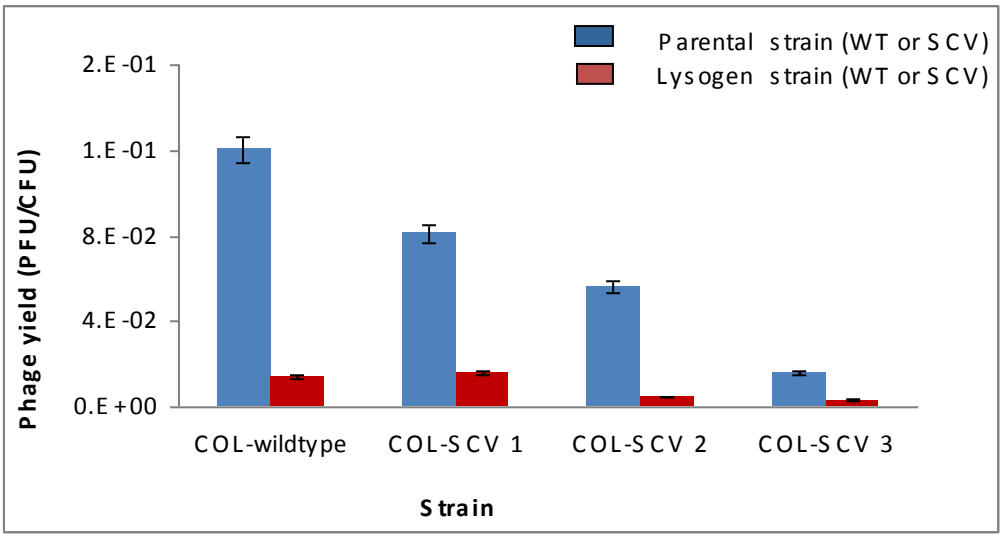


Figure 3.7 A comparison of the phage yield from a lysate and lysogen strains of COL and SCV 1-3. The ratio between the phage yield of lysogens and lysates was highest for the wildtype. Error bars represent the standard error of the mean.

3.3.2.4 Lysate reproducibility

Figure 3.8 A and B show the titres obtained for 14 individual lysates prepared from COL strains and their lysogens over a 3 month period. Table 3.6 shows the average lysate titre for the lysogens of COL wildtype was ($3x10^8$ PFU mL^{-1}) and SCV 1-3 ($30\text{-}3x10^6$ PFU mL^{-1}).

It is clear that the COL wildtype lysogen gave the highest average lysate titre. The SCV lysogens were 10-100 fold lower (Table 3.6).

Table 3.6 The average phage titre from several lysates.

Strain	Lysogen[1]	Lysate[1]
COL- wildtype	$3x10^{8\,\$}$	$6x10^{7\,\&}$
COL-SCV1	$3x10^{7\,*}$	$7x10^{6\,\&}$
COL-SCV2	$1x10^{7\,*}$	$2x10^{6\,\&}$
COL-SCV3	$3x10^{6\,*}$	$7x10^{5\,\&}$

[1]n= 14; $ significantly different from all SCV lysogens; * no significant difference; & no significant difference

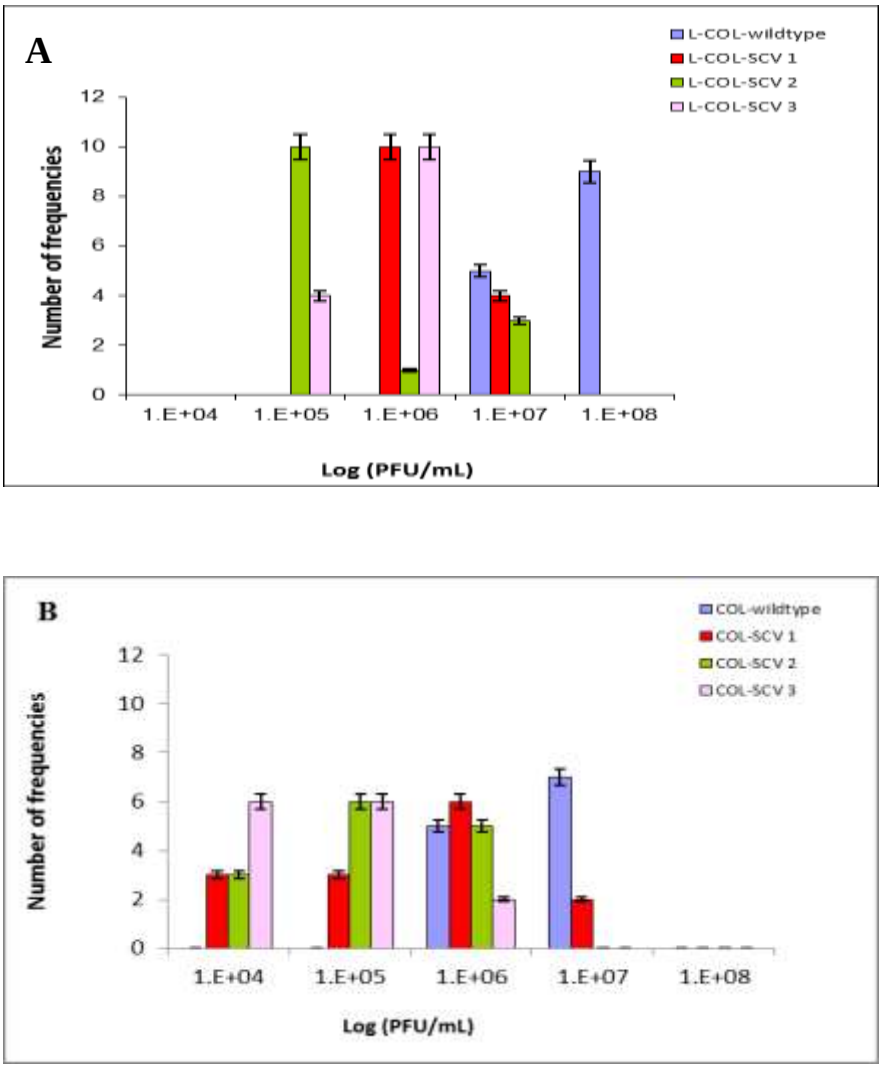


Figure 3.8 Distribution of phage yields obtained from temporally separate A) lysogen and B) lysate cultures. Error bars represent the standard error of the mean.

3.4 Discussion

Transduction:

Transduction of ciprofloxacin resistance (*grlA* from EMRSA 16) was observed into COL wildtype (mean $4.7x10^{-8}$, Table 3.2) and at a 5-10-fold higher frequency than into SCV1. Thus transduction of ciprofloxacin resistance was significantly decreased into SCV1 and not observed ($\leq 1.10^{-9}$) into either SCV2 or 3. This experiment also examined transfer between genetically distinct MRSA strains (donor lysate prepared on EMRSA 16 and recipient was strain COL) and indicates a potential for interstrain antibiotic gene transfer. This data also suggests that SCV strains are less efficient in gene exchange by transduction than wild type strains. Intra strain transductions (e.g. between SCVs and COL) experiments (Table 3.2) confirm this reduction into SCVs is real. However transfer is only reduced by around 20%. There is no explanation for the absence of ciprofloxacin resistance transfer into SCV2 and 3.

Transduction of rifampicin resistance (Table 3.3) was observed into COL wildtype ($4.8x10^{-7}$) and all SCVs at almost 10-fold reduced frequency (average $3.8x10^{-6}$).

Transduction frequencies may be higher in wild type cells because that cell walls of SCVs are thicker than those in the wildtype, Thus phages could less effective in lysing the cell and hence yield a lower lysate phage population. Others have suggested this may impact on transduction as it would not take place as efficiently (Bulger & Bulger, 1967; Sendi & Proctor, 2009).

Table 3.7 summarises the transfer data. Transfer of ciprofloxacin resistance was significantly lower than rifampicin resistance. Transduction of ciprofloxacin resistance and rifampicin resistance were similar (Table 3.8), but about 10-fold lower at worst, to those reported by Stiffler *et al.*, (1974).

Table 3.7 A summary of the transduction frequencies for ciprofloxacin and rifampicin resistance.

Strain	% WT rifampicin Transduction frequency	% WT ciprofloxacin resistance Transduction frequency
COL-wildtype	100 ($8.0x10^{-7}$)*	100 ($1.8x10^{-7}$)*
COL-SCV1	75	11
COL-SCV 2	79	≤1.0
COL-SCV3	83	≤1.0

*The transduction frequency into COL wildtype is in parenthesis

Transduction of *gfpuvr* with *sarA* P2 promoter was not seen in any *S. aureus* strains examined (Table 3.4). However the P2 promoter is weak and so expression may not have occurred (Cheung & Manna 2005).

Table 3.8 Comparison antibiotic resistance transduction frequencies between an earlier study and this investigation.

Recipient strain	Antibiotic resistance	Transduction frequency	Sources
***S. aureus* RN450**	Chloramphenicol Tetracycline	$2\text{-}30x10^{-7}$ $26\text{-}82x10^{-7}$	Stiffler *et al.*, 1974
***S. aureus* COL**	Ciprofloxacin Rifampicin	$1.8x10^{-7}$ $4.8x10^{-7}$	This study

Lysogen formation:

The initial experiments examined the rate of formation of lysogens. The COL wildtype strain of *S. aureus* and SCV3 both yielded a high number of lysogens (~68%; Figure 3.4), the remaining being resistant mutants. SCV 1 and SCV 2 provided a much lower proportion of lysogens (4-10%) as most were resistant to bacteriophage 80α. In the summary Table 3.9 below these strains were shown to have different intracellular ATP levels. There is clearly not a relationship between cellular ATP levels and % lysogen formation. Therefore the frequency of lysogen formation (or that of resistance mutants) cannot be directly related to energy status. It does not even relate to a predicted ATP yield ratio between wildtype and SCVs 1-3 (32:20:10:2; Day *et al.*, 2012).

Table 3.9 A comparison of ATP levels and lysogen formation.

Strain	COL-wildtype	COL-SCV 1	COL-SCV 2	COL-SCV 3
ATP (RLU)	38663	12575	10968	10930
% ATP	100	32	28.4	28.3
predicted ATP yield	32	20	10	2
Lysogen*	131	19	8	132
% Lysogen formation	68.2	9.9	4.2	68.8

* Represents number lysogens found from within 192 samples for each strain; RLU-Relative Light Units.

Lysate reproducibility:

Figure 3.8 shows that all strains produced phage with a distribution of between 2-3 orders of magnitude. Thus it is not a consistent process, but one modified by parameters unknown at present. So these lysate yield differences presumably reflect environmental impact, even under relatively controlled laboratory conditions. It does appear that the amount of variation in yield is less in the lysates from lysogens than from growth through overlays.

3.5 Conclusions

- Transduction is not consistent between the strains. Ciprofloxacin resistance only transferred between *S. aureus* strain COL and SCV1, and was not detected into SCV 2 or 3. GFP was not detected into any strain tested.
- Rifampicin resistance was transferred into all strains.
- The frequency of transduction was $1.8x10^{-7}$ to ciprofloxacin and $4.8x10^{-7}$ to rifampicin. This is about equivalent to published data.
- Phage 80α resistance was significantly different in some SCVs strains.
- Phage carriage in the lysogen strains had a physiological cost and reduced cell yields by 53-98%.
- Phage yields from lysates or lysogens were significantly reduced in SCV hosts compared to wildtype by 1-2 orders of magnitude.
- Lysate reproducibility is lower by SCVs than the wild type. Environmental factors impact significantly resulting in 2-3 orders of magnitude in yields.

CHAPTER 4:

CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* COL SCV 1, 2 AND 3

4.1 Introduction

4.1.1 Small colony variants of bacterial species

Small colony variants (SCVs) were first described in 1910, in an aberrant form of *Salmonella enterica* serovar Typhi (Proctor *et al.* 2001), and have been since described in a number of different bacterial genera. SCVs have been isolated from a variety of other bacterial species have been isolated. These species include important Gram-negative pathogens such as *Pseudomonas aeruginosa* (Bryan & Kwan 1981), Gram-positive pathogens such as *Enterococcus faecalis* (Petersen *et al*., 2008) and *Streptococcus pneumonia* (Alleqrucci & Sauer 2007). SCVs have been isolated from methicillin resistant *Staphylococcus aureus* (MRSA) (Bulger 1967). Although SCVs have been isolated from a number of different bacterial genera, SCVs of *S. aureus* have been most widely studied (Proctor *et al.* 2006). Since their discovery *S. aureus* SCVs have been associated with persistent, recurrent and antibiotic resistant infections (Seaman 2007).

SCVs constitute a slow-growing subpopulation of bacteria with distinctive phenotypic and pathogenic traits. Since their discovery *S. aureus* SCVs have been associated with persistent, recurrent and antibiotic resistant infections (Seaman 2007). SCVs have been described in a wide range of bacterial species, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (Proctor *et al.,* 2006)**.** Generally, SCVs display a small colony size, a slow growth rate, and atypical colony morphology. Since their discovery *S. aureus* SCVs have been associated with persistent, recurrent and antibiotic resistant infections (Proctor *et al.*, 2006). *S. aureus* is a member of the coagulase positive staphylococci (CoPS), which are differentiated from *S. epidermidis* by an ability to coagulate rabbit plasma (i.e. coagulase positive).

S. aureus shares a core set of 1,681 open reading frames with *S. epidermidis* (Gill *et al.*, 2005). *S. aureus* SCVs, identification of CoPS SCVs is a challenge due to their abnormal colony phenotypes, slow growth rate and altered biochemical

characteristics. Techniques such as amplification and sequence analysis of 16S rRNA are vital for accurate species identification (von Eiff *et al.*, 1999).

4.1.2 Molecular typing of MRSA

The typing techniques are used in tracking sources, pathways of spreading infections and studying the population genetics. MRSA typing is essential for the establishment of national and international surveillance networks. In MRSA typing both phenotypic and genotypic methods are used (Weller, 2000).Various molecular epidemiological techniques have been employed to type and track isolates including; macrorestriction of chromosomal DNA 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 and random amplified polymorphic DNA (RAPD) (Weller, 2000).

4.1.2.1 Random amplified polymorphic DNA

RAPD uses low stringency PCR of genomic DNA; it is performed with single short primers with arbitrary sequences to amplify portions 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-type able MRSA strain (Tambic *et al.*, 1997) and the use of multiple primers has been demonstrated to improve discriminatory power (Cheeseman *et al.*, 2007). The advantage of RAPD over others is the relative speed and simplicity of the technique (Tambic *et al.*, 1997). Although RAPD is considered simpler and less time consuming, comparison with PFGE reveals that RAPD is less discriminatory (Saulnier *et al.*, 1993).

4.1.2.2 DNA sequence analysis-based typing methods

DNA sequence analysis is an objective genotyping method as the genetic code is highly portable, easily stored and can be analysed in a relational database. Two different strategies have been used to type the isolates by analysing their DNA sequences: Multilocus sequence typing (MLST) and Single-locus sequence typing (SLST) (Mehndiratta & Bhalla, 2012). 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 proved instrumental in analysing and tracing the origins and evolutionary history of MRSA (Enright *et al.*, 2002; Oliveira *et al.*, 2002).

4.1.3 Aims

- Compare physiological and genetic differences between SCVs 1, 2 & 3 and *S. aureus* COL wildtype.

- Study the relationship between the effects on phenotype, growth curve and other factors.

4.2 Materials and methods

4.2.1 Bacterial strains and growth medium

S. aureus COL, COL-SCV 1, COL-SCV 2 and COL-SCV 3 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 media were obtained from Oxoid (UK), with the exception of components required for certain agar assays, which were obtained from Sigma (UK).

4.2.2 Preparation of antimicrobial agents

Gentamicin was obtained from Sigma Aldrich (UK) and triclosan from Ciba Speciality Chemicals (Germany). Gentamicin powders were dissolved in sterile deionised water, 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.

4.2.3 Determination of minimum inhibitory concentrations

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

4.2.4 Characterisation of *S. aureus* COL SCV 1, 2 & 3

4.2.4.1 Determination of auxotrophy

Auxotrophy was determined using haemin, menadione or thymidine, as described previously (section 2.2.7.1).

4.2.4.2 Coagulase production

Coagulase production was determined by using Staphylase test kit (DR0595; Oxoid Ltd, UK) as described previously (section 2.2.7.2).

4.2.4.3 Haemolysis activity

Haemolysis activity was determined by MH agar was supplemented with 5% defibrinated sheep blood (SB053; TCS Biosciences) as described previously (section 2.2.7.3).

4.2.4.4 Catalase production

Catalase production was determined by 1% w/v hydrogen peroxide (H_2O_2) (H-1009; Sigma-Aldrich, Poole, UK) to *S. aureus* parent and SCVs as described previously (section 2.2.7.4).

4.2.4.5 DNase production

DNAse activity was quantified by measuring the zone of clearing observed and related to the wild type control (100%), by DNase culture media (CM0321; Oxoid, UK) as described previously (section 2.2.7.5).

4.2.4.6 Pigment production

Pigment production was quantified using a methanol extraction protocol (Morikawa *et al.*, 2001) as described previously (section 2.2.7.6).

4.2.4.7 Quantification of intracellular ATP

Intracellular levels of ATP were determined by an enzyme based luciferase assay. The BacTiter-Glo™ Microbial Cell Viability Assay (G819A; Promega, UK), as described previously (section 2.2.7.7).

4.2.4.8 Phage sensitivity

Phage sensitivity by Φ 80α was studied by overlays of 3 ml 50% w/v agar Brain Heart Infusion agar (BHI; CM1136, Oxoid) (6 gm/L), plus 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 500 µL of culture, as described previously (section 2.2.7.8).

4.2.4.9 Growth curve

Growth rate were examined using a Bioscreen C analyser (ThermoFisher, UK) as described previously (section 2.2.7.9).

4.2.4.10 DNA extraction

DNA was extracted using the GenEute Bacterial genomic DNA Kit (NA2100, Sigma Aldrich, UK) as described previously (section 2.2.7.10).

4.2.4.11 Aerobic and anaerobic growth

S. aureus COL and their SCVs strains were inoculated into 10 ml MH broth (plus 1 mg/L of gentamicin for the SCV strains). Aerobic cultures were incubated at 37°C in an orbital shaker at 150 rpm for 24 hours and SCV strains for 48 hours. For the

anaerobic cultures 2 ml paraffin oil (76235; Fluka Analytical) was added to 10 ml MH broth and this was sterilised by autoclaving for 15 minutes at 121°C. These were inoculated and incubated at 37°C without shaking. Their optical density recorded after 24 or 48 hours.

4.2.4.12 Species confirmation

4.2.4.12.1 Random amplified polymorphic DNA PCR (RAPD-PCR)

RAPD-PCR was carried out as described previously by (Mahenthiralingam *et al.*, 1996). All PCR reagents were supplied by Qiagen (UK). Primers 208 (5′-ACGGCCGACC-3′) 268 (5′-AGGCCGCTTA-3′) 272 (5′-AGCGGGCCAA-3′) (MWG Biotech, UK). 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, 1mM EDTA) for 45 minutes. Molecular standards were run on all gels using molecular weight maker Hyperladder I (Invitrogen, UK).

4.2.4.12.2 Multiplex PCR

As *S. aureus* SCVs are frequently to identify a modified version of the quadriplex PCR protocol developed by Zhang *et al.* (2004) was employed to confirm that SCV isolates were *S. aureus*. PCR target 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), and *mecA* (a determinant of methicillin resistance) as described previously (section 2.2.7.11).

4.3 Results

4.3.1 Minimum Inhibitory Concentrations

The susceptibility profiles to gentamicin and triclosan of *S. aureus* COL-SCVs were significantly raised (P = < 0.05). The MIC of COL-SCVs for gentamicin was increased by 0.5 fold and MICs for triclosan increased between 0.5-0.8 fold (Table 4.1).

4.3.2 Characterisation of COL and SCV 1, 2 & 3

4.3.2.1 Catalase, haemolysis and auxotrophy

SCV1, 2 and 3 isolates showed reduced or absented catalase, coagulase and haemolysis activity in comparison to their wild type strain (Table 4.2). Certain SCVs showed a complete absence of heamolysis activity, and all had a reduced catalase and coagulase activities. Auxotrophy was confirmed for all three SCVs. Growth of fast growing pigmented colonies around filter discs impregnated with haemin, menadione or thymidine. All isolates were sensitive to phage 80α.

4.3.2.2 DNase activity

DNase activity was obtained by measurement of zones of DNA hydrolysis. DNase activity in COL-SCV 1 was about 65% of the wildtype strain and this was significantly reduced (P = < 0.05). The SCV 2 and 3 displayed an absence of DNAase activity (Figure 4.1; Table 4.2).

4.3.2.3 Pigment production

S. aureus COL and SCV strains 1, 2 and 3 produced lower amounts of pigment (61; 43; 21%) respectively in comparison to wild type strains (Figure 4.2). Figure 4.10 and 4.11 show images of the colony morphologies at two magnifications to illustrate the lack of visual pigment. Despite this the SCVs strains retained 40-80 % of the pigment produced by the COL-wildtype (Table 4.2).

4.3.2.4 Intracellular ATP concentrations

All SCVs strains had reduced intracellular ATP levels (around 30%) compared to COL-wildtype (Figure 4.3; Table 4.2).

Table 4.1 MIC (mg/L) for *S. aureus* COL wildtype and SCVs

Strain	GEN	TRI
COL-wildtype	0.25	0.125
COL-SCV 1	1	2
COL-SCV 2	2	4
COL-SCV 3	4	5

GEN - Gentamicin; TRI - Triclosan. Modal values were obtained from three independent replicates and three independent biological replicates. These are significantly different.

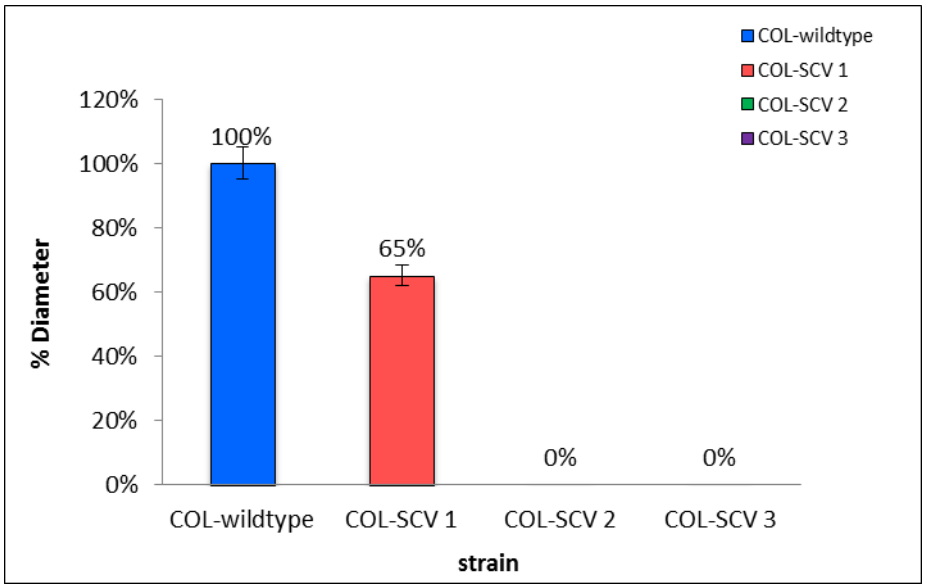


Figure 4.1 Extracellular DNAase activity of COL wildtype and SCV strains; Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

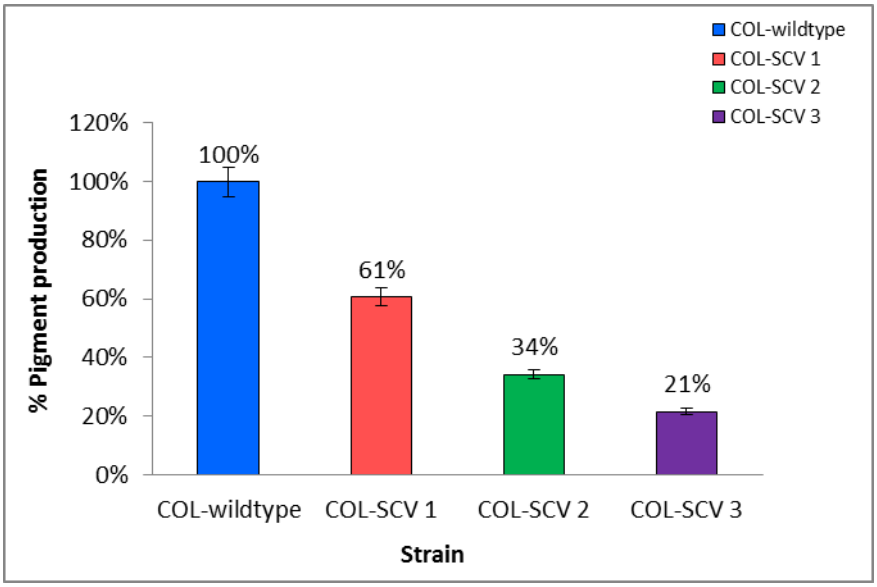


Figure 4.2 Pigment production by COL wildtype and SCV strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error, showing all are significantly different.

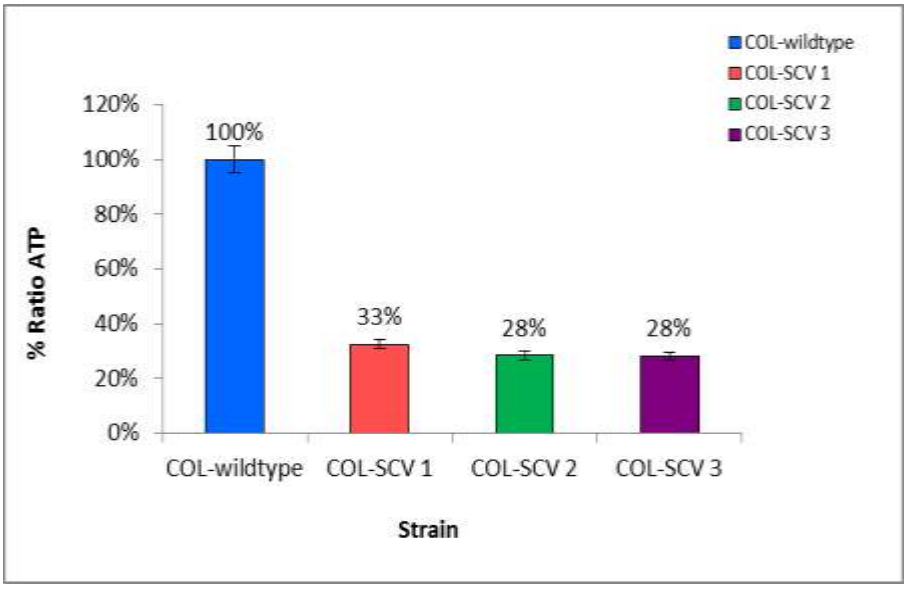


Figure 4.3 Intracellular ATP concentrations of COL wildtype and SCV strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

4.3.2.5 Growth rate and maximum cell count

The growth profile of *S. aureus* COL-wildtype is typical (Figure 4.4). It shows a short lag phase and then increases in cell density during exponential phase, with the culture reaching high cell densities upon entering the stationery phase.

The SCVs strains are atypical; their lag phase clearly extended. The duration of lag phase in wildtype strain was approximately 1.5 hours in comparison to an average of 8-12 hours for the SCVs (Figure 4.4). Furthermore SCVs also reached lower maximum cell densities reflected by the lower optical density values (Figure 4.5). Viable counts revealed that SCV1, 2 and 3 reached a maximum cell density of $3x10^8$, $1.3x10^8$ and $7x10^7$ CFU/mL respectively. In comparison the wildtype strain reached a higher average cell density of $2.7x10^9$ CFU/mL (Figure 4.4).

4.3.3 Aerobic and anaerobic growth

Figure 4.6 shows the effect of aerobic growth on stationary phase cell densities. Cell densities of COL-SCV 1, 2 and 3 were decreased between 0.35-0.84-fold compared to COL-wildtype.

Surprisingly perhaps the stationary phase densities after anaerobic growth were similar between 0.36-0.49 (Figure 4.6).

4.3.4 Species confirmation - RAPD

RAPD fingerprints for SCVs isolated were identical to wildtype fingerprints generated from three primer sets (Figure 4.7). Thus confirming the identity of the strains.

4.3.5 Species confirmation - multiplex PCR

Amplification of 16S rRNA, *mecA* and *nuc* genes confirmed all SCVs were *S. aureus*. Figure 4.8 shows presence of 16S rRNA (756 bp), *nuc* (279 bp) and *mecA* (310 bp), which can also be observed in the parent strain (Figure 4.8). Again confirming the identity of the strains.

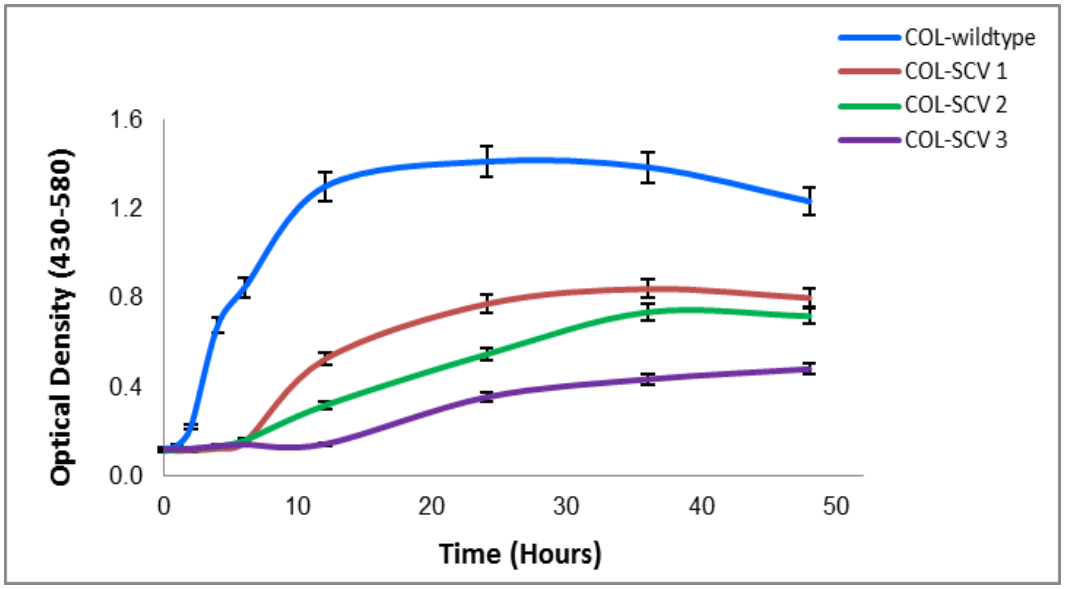


Figure 4.4 Growth curves of COL wildtype and SCV strains.
SCVs exhibited significantly extended lag in comparison to wildtype strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

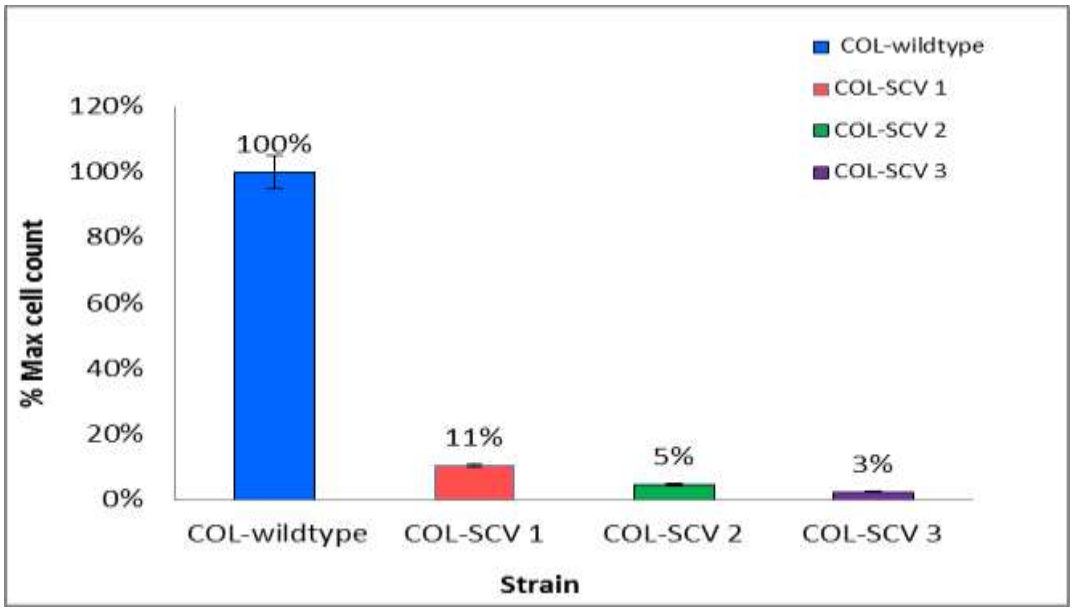


Figure 4.5 Maximum cell count of COL wildtype and SCV strains.
The wildtype was significantly different from the SCV strains. Results are the means of three independent replicates and three biological replicates. Error bars represent standard error.

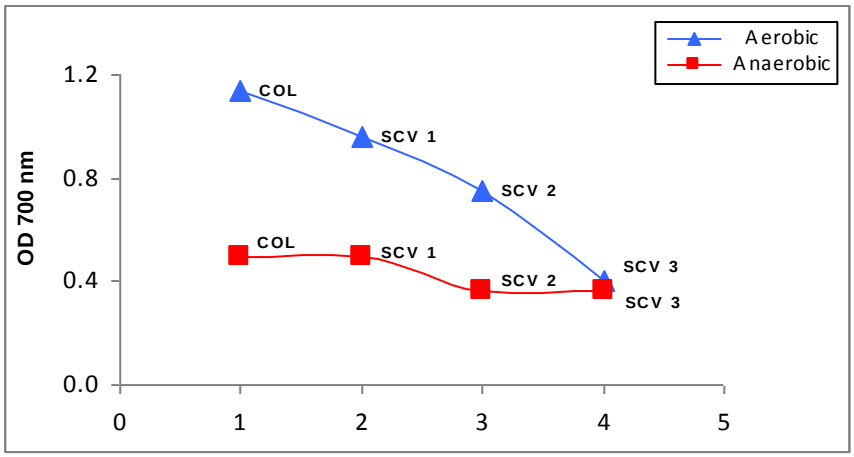


Figure 4.6 Comparison of aerobic (Δ) and anaerobic (□) growth yield of COL wild type and SCV strains. Results are the means of three independent replicates and three biological replicates.

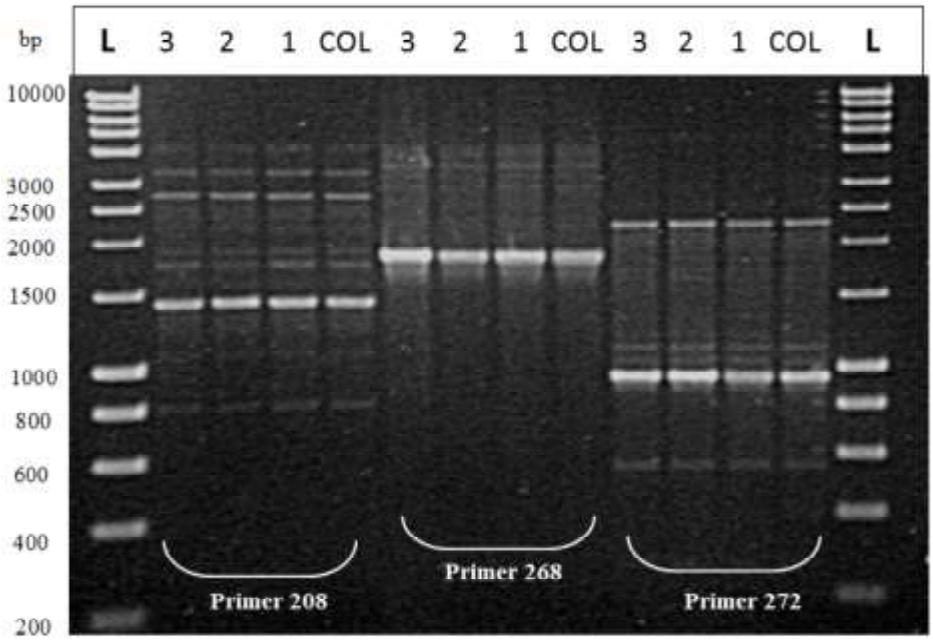


Figure 4.7 RAPD profiles of COL wild type and SCV strains.

L – Hyperladder; lane; COL wildtype; lane 1 –SCV 1; lane2 –SCV 2; lane 3 –SCV 3. All strains displayed identical profiles with three different primer sets.

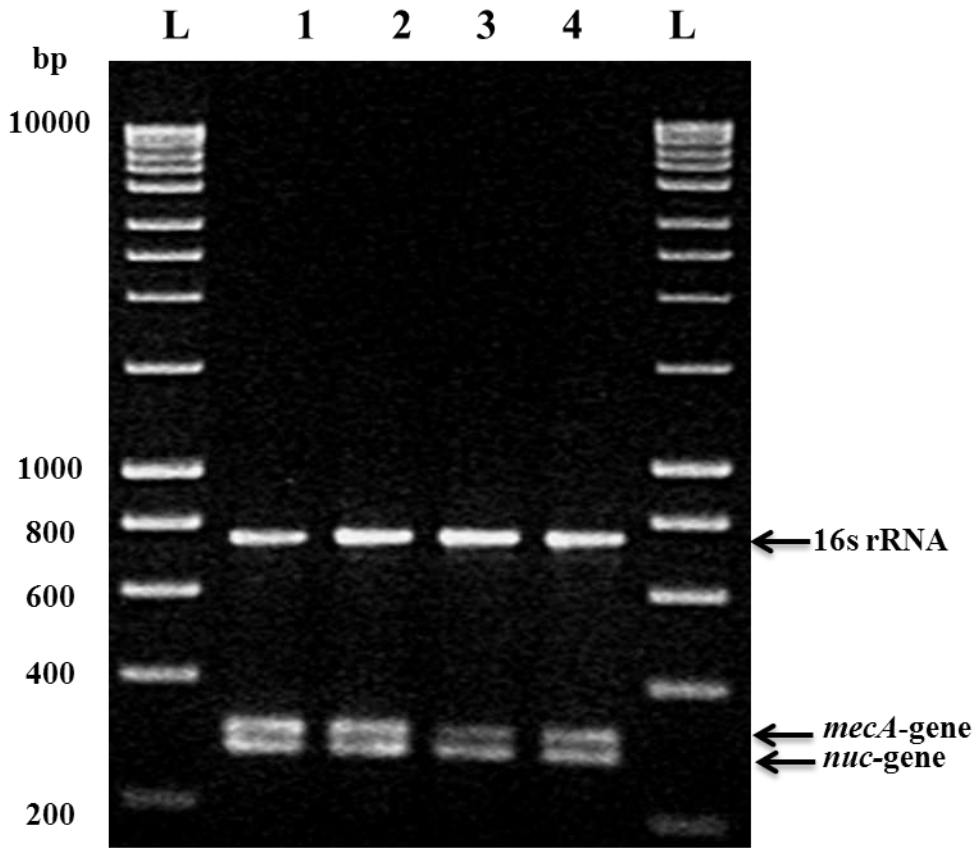


Figure 4.8 Multiplex PCR of *S. aureus*.

L – Hyperladder, lane 1; –COL-wildtype; lane 2 –-SCV 1; lane 3 –SCV 2; lane 4 - SCV 3. Amplification of the staphylococcal 16s rRNA (765 bp), *nuc*-genes (279 bp) and *mecA*-genes (310 bp) are shown.

Table 4.2 Summary of the characteristics of *S. aureus* COL wildtype and SCV strains

Strain	Catalase	Coagulase	Haemolysis	Phage 80α sensitivity	Auxotrophy	Triclosan MIC (mg/L)	Gentamicin MIC (mg/L)	DNAse (% wild type)	Pigment production (% wild type)	Intracellular ATP concentration (%wild type)	Growth rate (OD_{530}; %wild type)	Max cell count (CFU/mL; %wild type)	Lag phase (hours)
wildtype	***	***	***	+	NT	0.125	0.25	100	100	100	100	100	100 (1-2)
SCV 1	**	*	*	+	+	2	1	65	61	32	40.5	11	433 (6-7)
SCV 2	**	-	-	+	+	4	2	-	34	28.4	24.5	5	633 (9-10)
SCV 3	*	-	-	+	+	5	4	-	21	28.3	11.2	3	733 (10-12)

***: Strong catalase/coagulase/haemolysis activity; **: Medium catalase/coagulase/ haemolysis activity *: Weak catalase/coagulase/ haemolysis activity; -: Absence of catalase/coagulase/ haemolysis activity; +: Positive; NT: not tested.

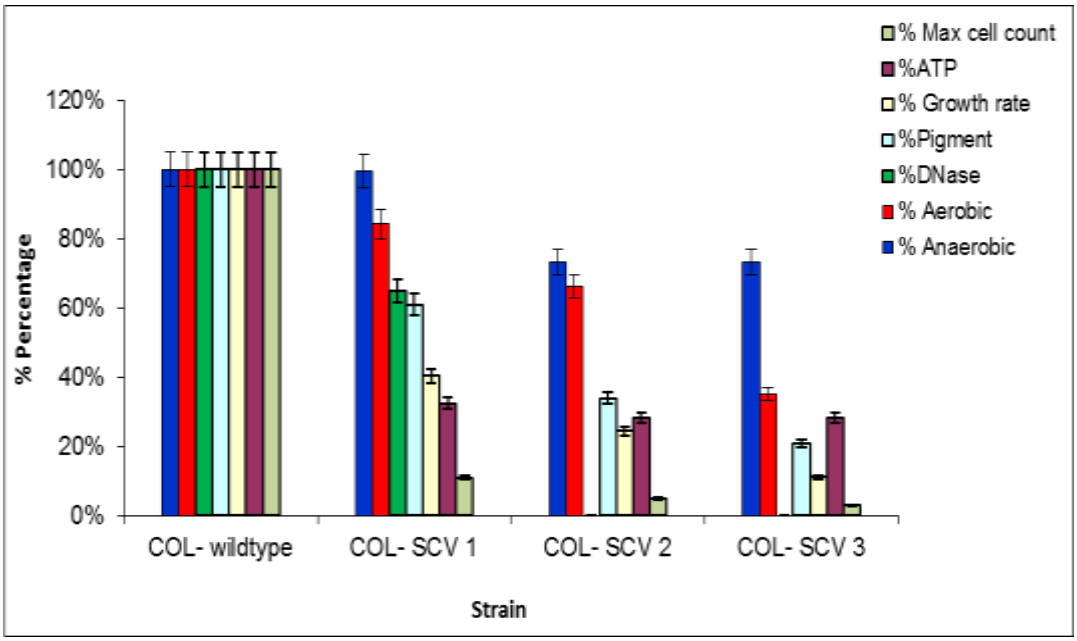


Figure 4.9 A comparison of *S. aureus* COL wildtype test results with SCV strains. Error bars represent standard error.

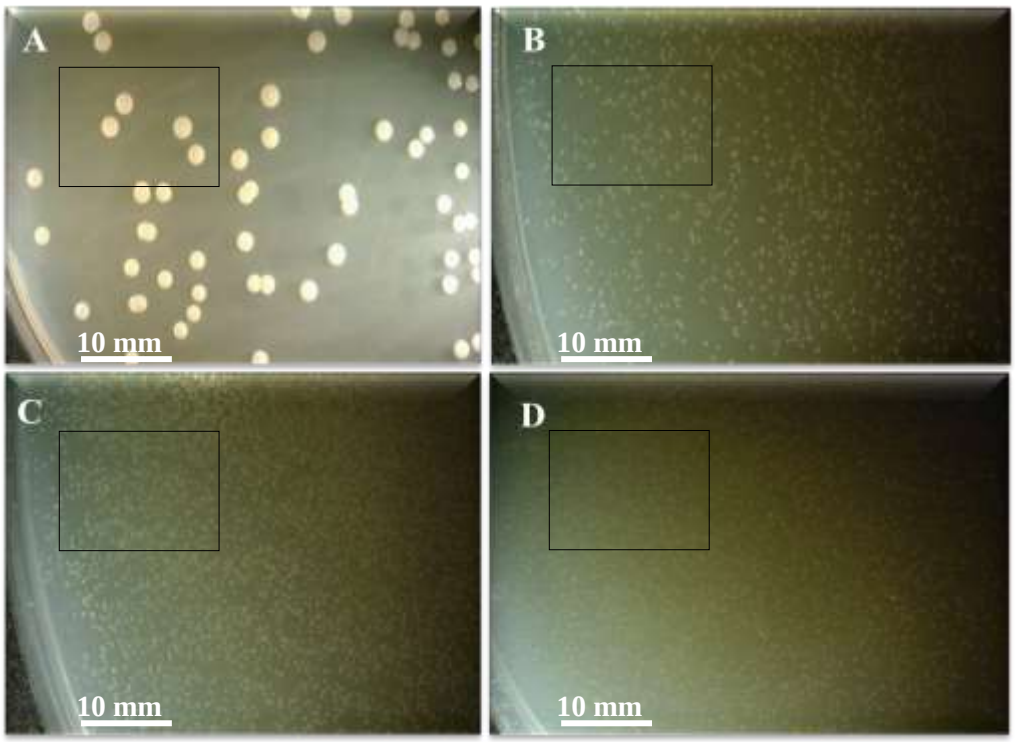


Figure 4.10 Morphological differences between *S. aureus* wildtype and SCVs strains A) **COL-wildtype; B) SCV 1; C) SCV 2; D) SCV 3.**

SCVs colonies were approximately one tenth the size of wild type colonies.

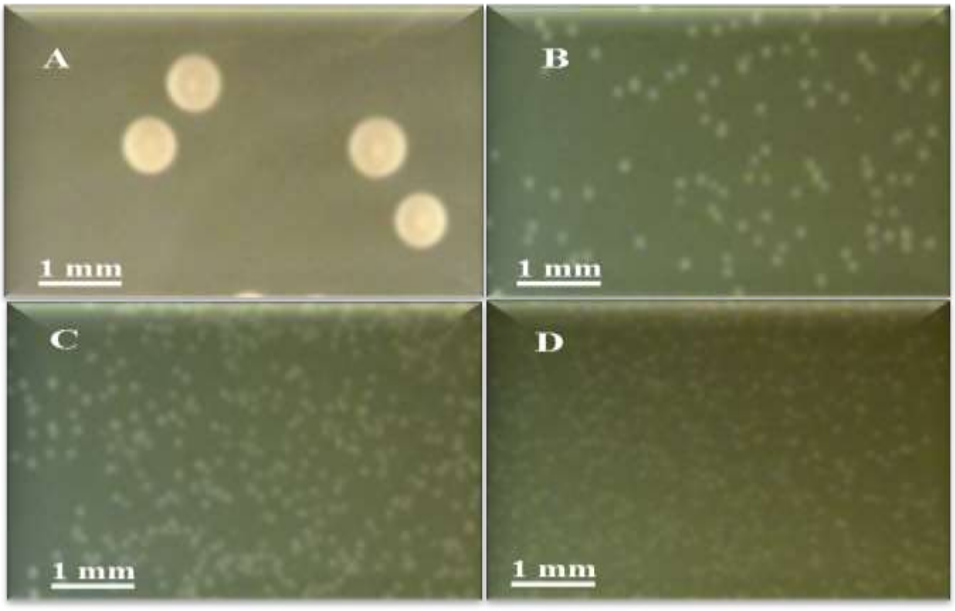


Figure 4.11 Magnified morphological differences between *S. aureus* wildtype and SCVs colonies A) **COL-wildtype; B) SCV 1; C) SCV 2; D) SCV 3.**

Note of the lack of pigmentation of SCVs.

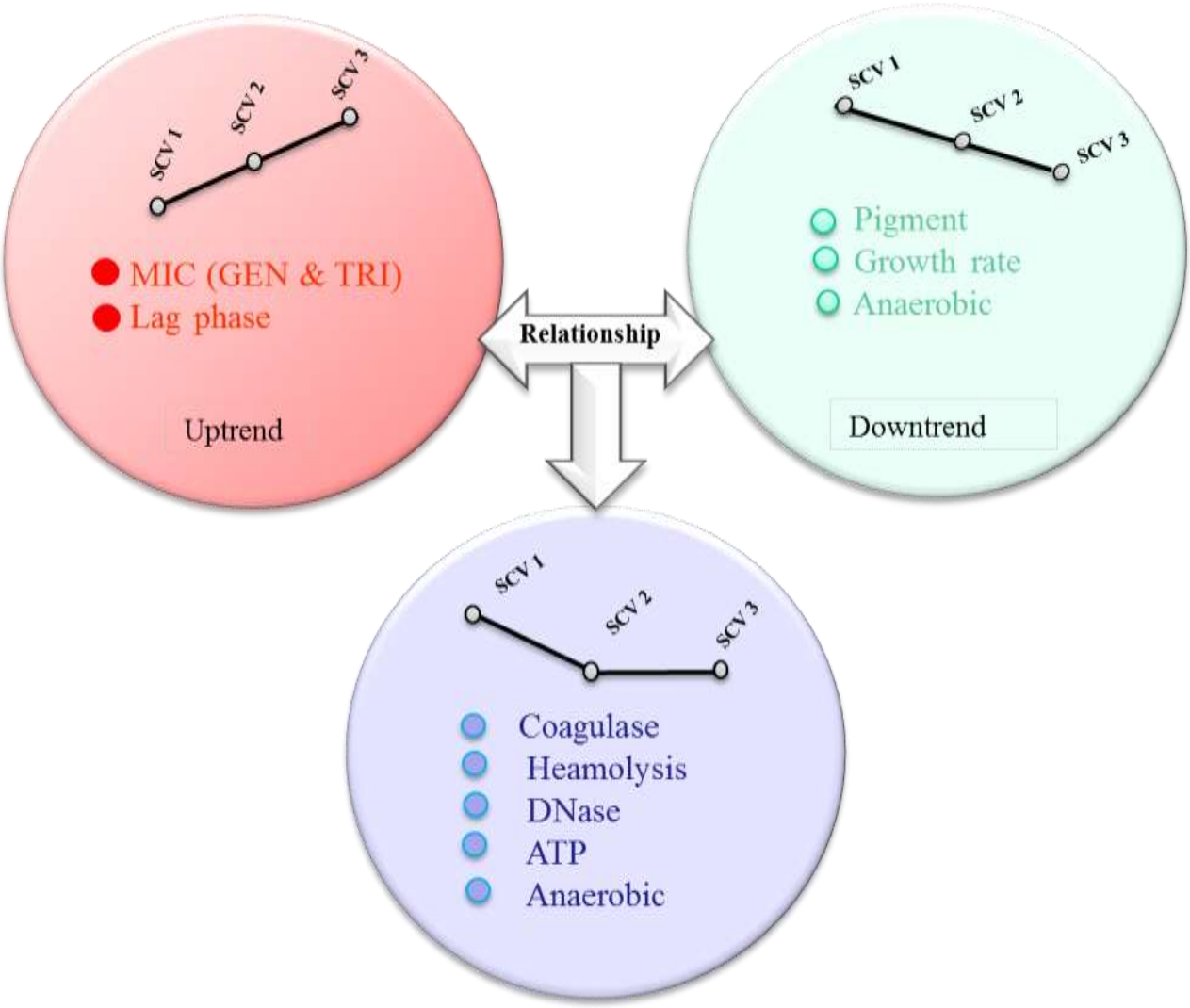


Figure 4.12 A schematic of differentially expressed phenotypes common to *S. aureus* and SCVs strains.

4.4 Discussion

Phenotype and expression:

The small colony phenotype and the lack of pigmentation of COL SCVs selected on triclosan medium are typical. This contrasts with the wild type colony size. These SCVs displayed morphological characteristics (Figure 4.10 and 4.11) that correlate well with other studies (e.g. Seaman *et al.*, 2007, Proctor *et al* 2006). The presence of pigment in *S. aureus* protects them from oxidative stress (Clauditz *et al.*, 2006), so the absence in SCVs is interesting. Thus presumably the limit in energy generation means it is utilised first for essential cellular process such as cell wall biosynthesis at the cost of pigment production. Although the colonies look to have lost pigment, analysis shows that it is still present. This indicates that the pigment could still be essential for survival.

Table 4.1 is important to an understanding the SCV phenotype. Firstly the SCVs were isolated as triclosan resistant. They are clearly gentamicin resistant to SCVs. Also there is a crossover in resistance in SCVs with other antimicrobials. Also Table 4.1 shows a progressive lack of sensitivity by the SCVs compared to the wild type strain. When compared to the growth rates of the strains (Table 4.2) it shows the slowest growing strain, SCV3, has the highest MIC.

Table 4.2 showed each SCV shows a reduction in intracellular ATP levels, catalase, coagulase, DNase activity and maximum cell yield, compared to the wild type strain. The absence of haemolytic activity in SCVs has been noted previously (Proctor *et al.*, 2006; Seaman *et al.*, 2007). Presumably the loss of these functions (catalase, haemolysis, coagulase and DNase) are related to the pathogenicity success of these organisms.

Aminoglycoside (gentamicin) insensitivities increased and growth rate, together with gentamicin resistance, were found to be inversely and linearly related (Day *et al.*, 2012; Figure 4.12).

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 gentamicin (Table 4.1) even though they were selected on triclosan. It is well documented that SCVs are less susceptible to aminoglycosides antibiotics such as gentamicin (Proctor *et al.*, 1998). This is because 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,

fewer uptakes of these positively charged molecules occurs, resulting in reduced susceptibility. Interestingly the reduced susceptibility to ß-lactam antibiotics 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).

Growth and lag:

These SCVs were selected on triclosan and produced growth profiles substantially different from the wildtype strain. The extension of the lag phase in comparison to the wildtype was between 6-12 hours (Table 4.2). In addition their growth rates (Figure 4.5), with a gradual increase in cell density during exponential phase and with the culture reaching maximum cell densities upon entering the stationary phase of growth. COL wildtype was 1.412 and SCVs were 0.84 - 0.435 (Figure 4.4).

Compared with *S. aureus* COL wildtype the SCV isolates were less efficient in aerobic respiration thus had reduced growth. Anaerobic cell yield was almost equal (Figure 4.6). Presumably SCVs obtain less energy per glucose molecule aerobically than the wild type. However when these are grown anaerobically when both SCVs and wild type utilise the same pathway to generate ATP. In common with other facultative aerobes, *S. aureus* can grow in the absence of oxygen either by fermentation or by using an alternative terminal electron acceptor, such as nitrate (Clements & Foster, 1999). These terminal electron acceptors have smaller reduction potentials than O_2, meaning that less energy is released per oxidized molecule (Yarwood & Schlievert, 2000). Presumably these are the pathways being utilised by SCVs to generate ATP.

Identity of SCV strains:

The identity of the culture in these experiments is critical. This is why the strains were tested critically to ensure their identity. RAPD-PCR has been applied to study and track the epidemiology of *S. aureus* in both clinical and environmental settings (Lee, 2003; VandenBergh *et al.*, 1999) and so was an appropriate test. The SCV isolates were confirmed as *S. aureus* by both RAPD-PCR (Figure 4.7), and Multiplex PCR (Figure 4.8). We also used phage sensitivity to confirm these isolates were *S. aureus.*

4.5 Conclusions

- Multiplex PCR and sequencing successfully confirmed all isolates were *S. aureus*.
- SCVs and COL produced identical RAPD profiles indicating clonality.
- There is a physiological diversity between SCV strains 1-3.
- *S. aureus* SCVs show reduced activity of several enzymes.
- The lag phase is extended in SCVs
- The growth rate of the SCVs is by 60-89% reduced
- Final cell densities (stationary phase) are reduced in SCVs compared to the wild type
- Growth rate is related to final cell densities
- Anaerobic growth of the *S. aureus* wildtype and SCVs was identical, suggesting they were using the same energy generating pathway.

CHAPTER 5:

SUSCEPTIBILITY AND RESISTANCE OF *STAPHYLOCOCCUS AUREUS* COL AND THEIR SMALL COLONY VARIANTS TO INHIBITORY AGENTS

5.1 Introduction

5.1.1 Brief history of resistance in S*taphylococcus aureus*

Staphylococcus aureus strains comprise one of the significant human pathogens. They are major cause of hospital-acquired infection and frequently cause severe infection and the persistent increase in the number of methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) (Wadworth *et al.,* 1992; Office for National Statistics, 2006). The rise and spread of microorganisms with reduced susceptibility to antimicrobials occurs around the world (Lu *et al.,* 2005). Staphylococci are no different with MRSA strains acquiring resistance to newly introduced antibiotics. They thus pose a clinical problem as they spread rapidly and are difficult to treat even with newer antibiotics (Pechere *et al.,* 1992). Multi-resistance *S. aureus* strains are potential hazard in the hospital environment (Russell, 1991; Udo & Grubb, 1993). MRSA can encode resistance to >20 antibiotics, and to antiseptics such as quaternary ammonium compounds (Lyon & Skurry, 1987). Plasmid encoded resistance occurs also to various other agents (e.g. ethidium bromide and acriflavine; Emslie *et al.,* 1985). Many metals (e.g. $AgNO_3$, $CuSO_4$, $HgCl_2$, and $ZnSO_4$) have antimicrobial properties and are used in disinfectant and antiseptic formulations. For example $AgNO_3$ was used to prevent gonococcal eye infections, mercurochrome and merthiolate (mercury salts) are applied to skin after minor wounds, and zinc is used in antifungal antiseptics, while $CuSO_4$ is used as an algicide (Ronald, 1995).

The relationship between the use of antibiotic and development of antibiotic resistance has been discussed at length (Livermore, 2003). More recently the increased use of biocides as disinfectant, antiseptics and preservatives has also created discussion about their role in the development of resistance (Russell, 1997). To compound the problem of insensitivity there are 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 (Norville, 2011). One of the key issues to the discovery of novel antibacterial agents is that the majority of targets that allow selective toxicity have already been exploited (Moellering, 2011).

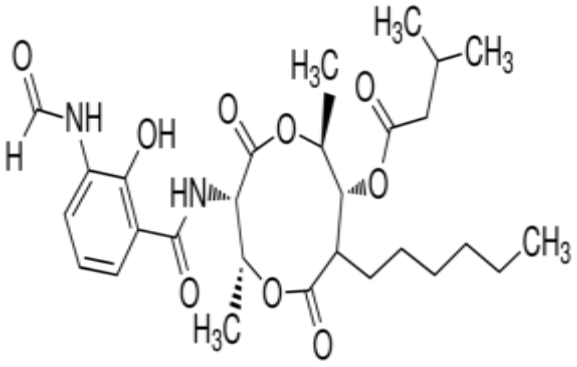
5.1.1.1 The susceptibility of *S. aureus* strains to antimycin, oligomycin and carbonyl cyanide m-chlorophenylhydrazone (CCCP)

The absence of oxidative phosphorylation in cells is the consequence of the absence of components of both the electron transport chain and the ATP synthase complex (Epstein *et al.,* 2001). This occurs normally when cells are grown in anaerobic conditions. Aerobic growth eukaryotic cells when exposed to different inhibitors of oxidative phosphorylation antimycin, CCCP, and oligomycin have their mitochondrial functions affected by inhibition of oxidative phosphorylation (Epstein *et al.,* 2001).

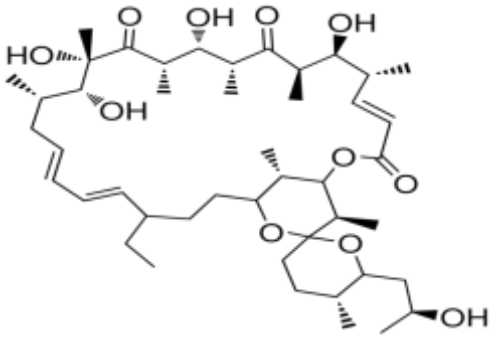
Antimycin, was first isolated from *Streptomyces* sp. in 1949 (Neft & Farley, 1972), and targets the Qi site of cytochrome c reductase, thereby inhibiting the oxidation of ubiquinol (Figure 5.1). The inhibition of this reaction disrupts the formation of the proton gradient across the inner membrane. The production of ATP is subsequently inhibited, as protons are unable to flow through the ATP synthase complex in the absence of a proton gradient (Dairaku *et al.,* 2004). Antimycin demonstrated significant activity as a growth inhibitor of *B. subtilis* and *S. aureus* (Arsianti *et al.,* 2010).

CCCP uncouples the proton gradient established from the normal activity of electron carriers in the electron transport chain (Park *et al.,* 1997). Tynecka *et al* (1999) showed *S. aureus* 17810R was inhibited by CCCP as it prevented ATP synthesis via oxidative phosphorylation.

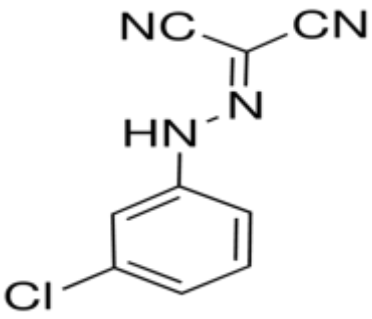
Oligomycin is isolated from *Streptomyces diastatochromogenes* and inhibits mitochondrial H^+-ATP synthase by blocking its proton channel (F_0 subunit) (Kagawa & Racker, 1966), responsible for oxidative phosphorylation of ADP to ATP. A characteristic feature of the structure in oligomycin probably plays a role in the interaction with F_1 and F_0 moieties of the of *E. coli* ATPase complex (Ovchinnikov *et al.,* 1984). Figure 5.1 shows chemical structure to antimycin, oligomycin and CCCP. Figure 5.2 shows a summary of the various metabolic pathways predicted or confirmed to be affected in respiratory-deficient staphylococci by Proctor *et al.* 2006.



Antimycin A



Oligomycin A



Carbonyl cyanide m-chlorophenylhydrazone (CCCP,)

Figure 5.1 Chemical structure of antimycin A, oligomycin A and CCCP (Ulanovskaya, *et al.*, 2008).

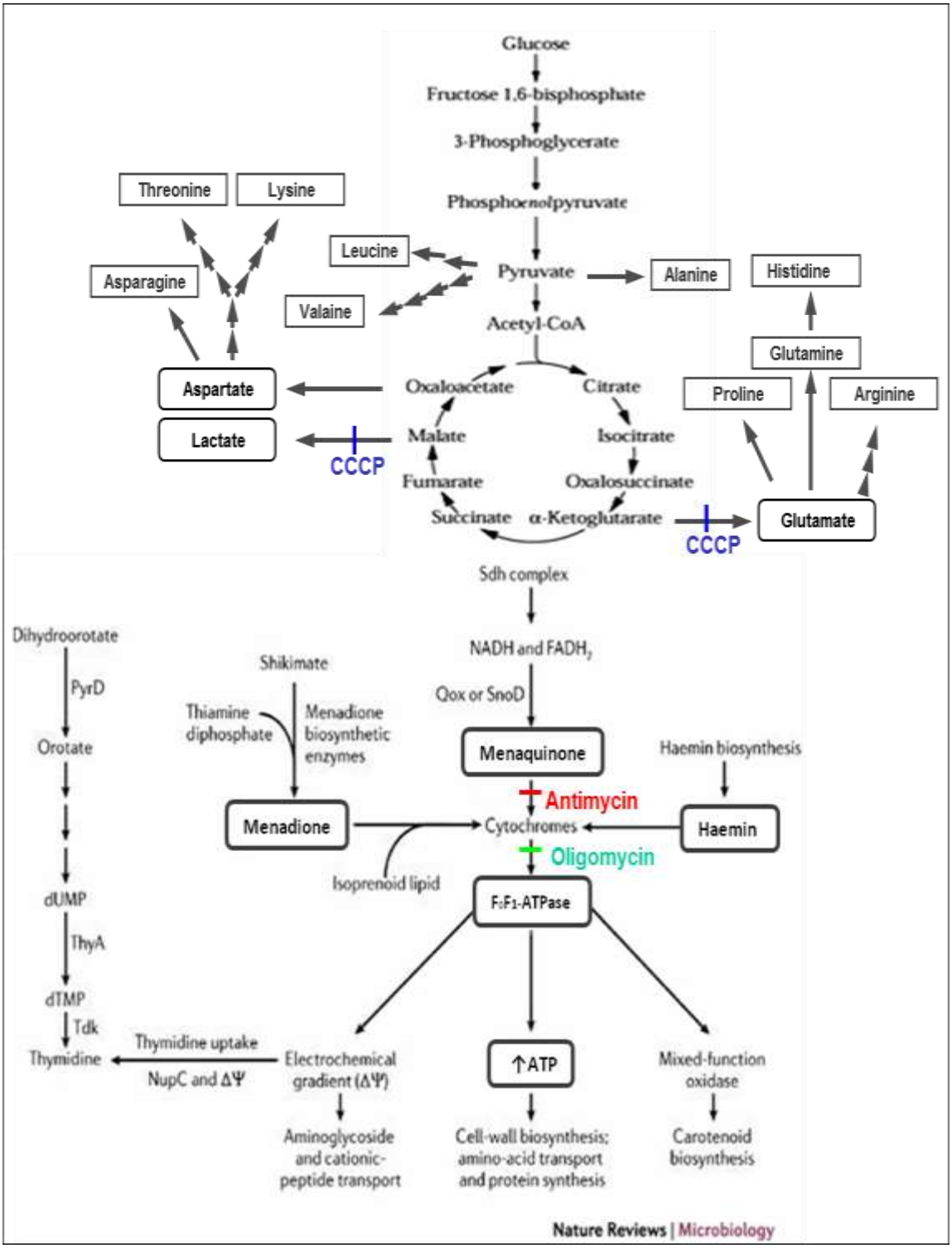


Figure 5.2 Metabolic pathways affected in respiratory-deficient cells as inferred from transcript profiling. Solid coloured bars indicate sites of inhibition by antimycin, oligomycin and CCCP, have their mitochondrial functions affected by inhibition of oxidative phosphorylation (adapted Proctor *et al.* 2006).

5.1.1.2 Susceptibility to heavy metals

Metal ions are directly and indirectly important to microbial metabolism. Although ions (e.g. Al, Ag, Cd, Sn, Cu, Cr, Hg and Pb) have essential biological roles, many are frequently inhibitory at higher concentrations (Gadd, 1988). Staphylococci are often resistant to heavy metal ions, especially cadmium and mercury, and arsenic (Dyke *et al.,* 1970) and this is thought to be related to their exposure in clinical environments.

In some instances resistance is plasmid encoded, so for example the penicillinase plasmids in *Staphylococcus aureus* also carry genes determining resistance to several heavy metal ions (Novick & Roth, 1968). Resistance to penicillin G in *S. aureus* depends mainly upon the production of a penicillinase enzyme (Rosdahl & Rosendal, 1983). Richmond *et al*. in 1964 investigated 181 selected strains and found that the active production of penicillinase closely correlated with multiple antibiotic resistance and resistance to mercury salts. Rosdahl and Rosendal (l980) found that changes in staphylococcal flora in Denmark were associated with changes in resistance to heavy metals. Resistance to mercury was most often found in multi-antibiotic resistant strains but rarely in strains resistant only to penicillin.

Thus metals may play a role in maintaining retention of antibiotic resistance once resistance is achieved. Many antibiotic resistance genes are plasmid-borne and in clinical isolates tend to be present on the same plasmids as resistance factors for metals such as mercury (Christon *et al.,* 1997).

Resistance to cadmium (Cd^{2+}) is widespread in *Staphylococcus aureus*. There are two separate Cd^{2+}resistance determinants, *cadA* and *cadB*, located on plasmids (Witte *et al.,* 1986; Novick *et al.,* 1979). The resistance function coded by the *cadA* determinant results from decreased intracellular accumulation of Cd^{2+} (Weiss *et al.,* 1978). The *cadB* gene product may protect the cell by binding Cd^{2+} (Perry & Silver, 1982). Resistance to mercury has been found in a number of *Staphylococcus aureus* strains, especially in those connected with outbreaks of infections in hospitals (Hall, 1970; Witte *et al.,* 1980), mercury resistance has been plasmid encoded on plasmid p1258 the *merA* gene codes for the mercuric reductase enzyme, and the *merB* gene codes for an organomercurial lyase enzyme (Weiss *et al.,* 1977). Free copper ions can be toxic to cellular systems, as these can create harmful oxygen species by reacting with hydrogen peroxide (Gaetke & Chow, 2003). Copper ions may also disrupt proteins/enzymes by binding to free protein thiol groups or by competing with other metals for cofactor binding sites (Teitzel *et al*., 2006).

5.1.2 Aims

- Study the relative sensitivity of *S. aureus* parent and SCVs strains to various antibiotics and chloride salts of heavy metals using a disc diffusion method

- Determine if any agents selected for SCVs

5.2 Materials and methods

5.2.1 Bacterial strains

Methicillin-resistant *S. aureus* (MRSA) strain COL and the small colony variants 1, 2 and 3. Strains were stored at -80°C in Mueller Hinton (MH) broth supplemented with 8% dimethyl sulfoxide (DMSO) and re-isolated on MH agar plates when required.

5.2.2 Preparation of antibiotics stock solutions

Antimycin A (A8674), oligomycin A (O4876) and CCCP (C2759) were obtained from Sigma Aldrich (UK). Antibiotic stocks (10,000 mg/L) were prepared. 100 mg of the antimycin and CCCP were dissolved in 10 mL of ethanol. 100 mg oligomycin was dissolved in 10 mL of DMSO. Solutions were mixed thoroughly by vortex mixing and then filter sterilised by passing them through 0.2 µm filters (Minisart, UK). Stocks solutions were maintained at 4°C.

5.2.3 Preparation of chloride salts of heavy metals stock solutions

Cadmium chloride (10064: BDH chemicals; fw:183.32), chromium chloride (022887: Aldrich Chemical Company; fw: 158.136), copper chloride (10088: BDH chemicals; fw:170.48) and mercury chloride (3450; Fisons Scientific; fw:271.50). Stock solutions (5 M) were prepared by adding formula weight of the required chloride salts to 10 mL of sterile deionised water and filter sterilised by passing them through 0.2 µm filters (Minisart, UK). Reduced strength stock solutions were prepared in sterile deionised water and held at 4°C.

5.2.4 Disc diffusion

The standardised disc susceptibility testing method (Andrews, 2009) was carried out following guidelines from the British Society of Antimicrobial Chemotherapy (BSAC). Briefly 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. SCV strains failed to reach the densities required by the McFarland standard, in which case densities of parent strains were adjusted to ensure similar inoculum concentrations. A sterile cotton swab was dipped into the suspension and spread evenly over the surface of MH agar plate. Sterile filter discs (150 mm; Whatman) were applied to the centre of the agar onto which 5 µL of antibiotics stock solutions (1,000 mg/L) and 5 µL

chlorides salts heavy metals stock solutions (5 M) were dispensed, as shows Table 5.1 and 5.2. Plates were allowed to dry for 10 minutes before being incubated for either 24 hours (wildtype) or 48 hours (SCVs) at 37°C and then zones of inhibition were measured.

Table 5.1 The concentrations of antibiotics used

Antibiotics	Drop (µL)	Stock (mg/L)	Final concentration (mg)
Antimycin	5	1000	5
Oligomycin	5	1000	5
CCCP	5	1000	5

Table 5.2 The concentrations of chloride salts of heavy metals used

Biocides	Drop (µL)	Stock (M)	Final concentrations (mM)
Cadmium chloride	5	5	25
Chromium chloride	5	5	25
Copper chloride	5	5	25
Mercury chloride	5	5	25

5.3 Results

5.3.1 Disc diffusion

5.3.1.1 The activity of antimycin, oligomycin and carbonyl cyanide m-chlorophenylhydrazone (CCCP)

The antimicrobial action of antimycin, oligomycin and CCCP against *S. aureus* SCVs and their parent strains was assessed using a simple disc diffusion method. All antimicrobials produced inhibition zones in all SCVs (1, 2 and 3) and their respective parent strains (Figure 5.3).

Table 5.3 show the inhibition zone size of COL wild type was 1.8-2 folds less susceptible to CCCP than SCV 1-3 and mean 1.3-2.2 folds less were each of antimycin and oligomycin. All were almost equally sensitive to antimycin and oligomycin. CCCP produced the largest inhibition zones in SCV and parent strains (mean 21 mm), followed by antimycin (4 mm) and oligomycin (3 mm).

Statistical analysis shows that the SCV strains from the three antimicrobial produced significantly more sensitive (the diameter of inhibition zones were larger) in comparison to wildtype strain ($P = < 0.05$). There was no significant difference in sensitivity to oligomycinin by SCV 1-3 ($P = > 0.05$). Wildtype and SCV1 show the same sensitivity to antimycin and oligomycin and both are significantly more resistant than SCV 2 and 3.

Three small colony variants arose in the CCCP zone of inhibition of the wildtype strain COL. One of these (SCV-CCCP) was stored and later sequenced.

Table 5.3 Relative sensitivities of *S. aureus* wildtype strain and their SCVs to CCCP, antimycin and oligomycin.

Antimicrobial	Concentration (mg)	COL-wildtype[a]	COL-SCV 1[a]	COL-SCV 2[a]	COL-SCV 3[a]
Antimycin	5	4	5	7	9
Oligomycin	5	3	4	5	6.5
CCCP	5	21	38	41	43.5

[a] Inhibition zone diameter (mm)

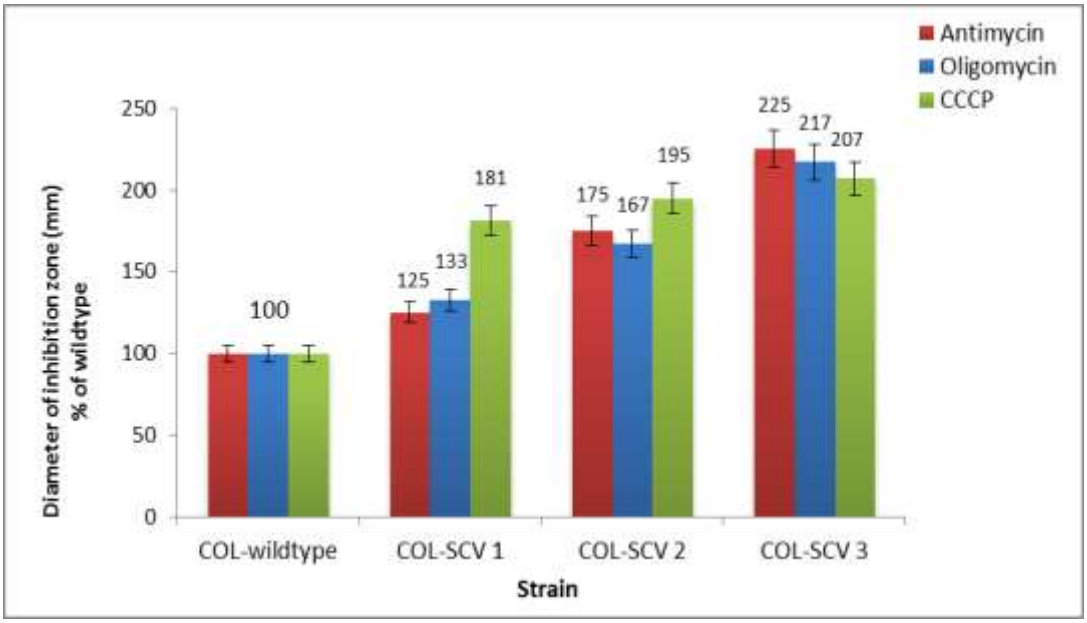


Figure 5.3 The relative % differences in sensitivity to antimycin, oligomycin and CCCP of *S. aureus* strain COL and SCV 1-3. Inhibition zones are the results of three independent replicates and three independent biological replicates. Error bars represent standard error. 100% is the wild type arbitrary resistance level.

5.3.1.2 The activity of chloride salts of cadmium, chromium, copper and mercury

The wildtype COL strain was significantly less sensitive to the chloride salts of four heavy metals than the SCVs strains (P = < 0.05). The average diameter of inhibition zone in wildtype strains was 7.4 mm where as the SCV average was 1.6 times greater at 12.3 mm (Figure 5.4).

With copper, cadmium and mercury salts the relative sensitivity increased, such that SCV3 was the most sensitive and the least was SCV strain 1.

Sensitivity to the SCVs to the chromium salt was significantly higher compared to the wild type, but there was no difference between them.

There is little difference between the SCV strains in sensitivity to cadmium chloride (Table 5.4).

SCV 2 is significantly more susceptible to copper chloride than the other two SCV strains (P = < 0.05).

SCV 3 is significantly more susceptible to both cadmium and mercury chloride than the other two SCV strains

SCVs were significantly more sensitive to cadmium chloride than the wildtype strain (P = < 0.05).

Small colony variants were not obtained from any of the chloride salts.

Table 5.4 Relative sensitivities of *S. aureus* wildtype strain and their SCVs to heavy metal chloride salts

Heavy metal chloride salts	Concentration (mM)	COL-wildtype[a]	COL-SCV 1[a]	COL-SCV 2[a]	COL-SCV 3[a]
Chromium chloride	25	9	13	13.5	17.5
Copper chloride	25	3.5	7	8.5	8
Cadmium chloride	25	6.5	10	11	12.5
Mercury chloride	25	10.5	12.5	14	19.5

[a] Inhibition zone diameter (mm)

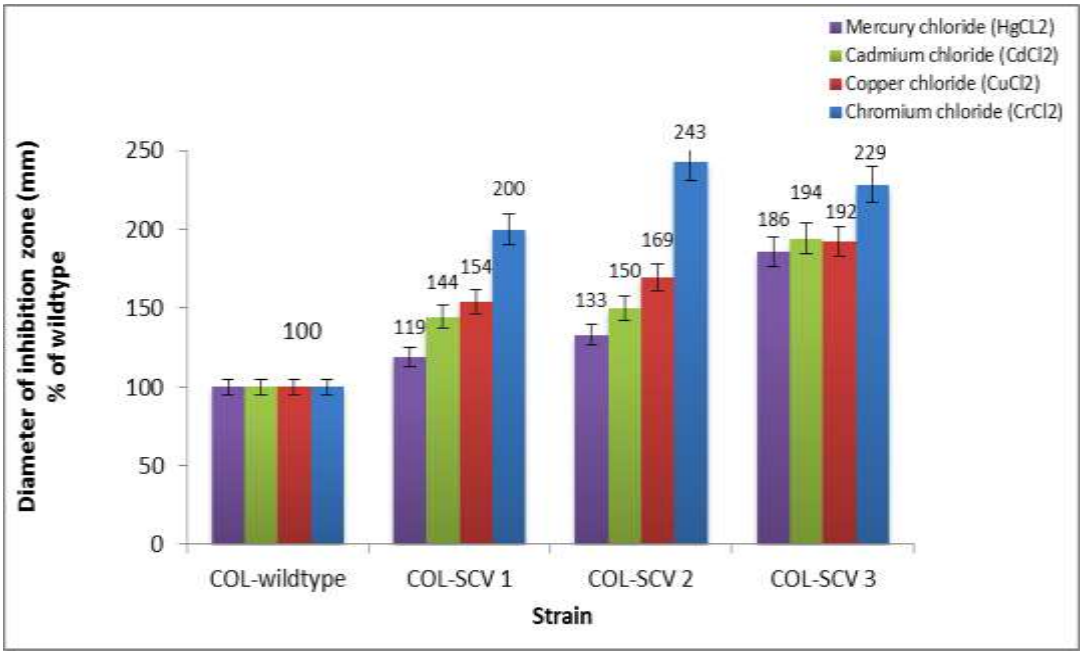


Figure 5.4 The relative % differences in sensitivity to cadmium, chromium and copper salts of *S. aureus* parent strain and their SCVs. Wildtype produced significantly smaller inhibition zone compared to the SCV strains (P = < 0.05). The results of three independent replicates and three independent biological replicates. Error bars represent standard error.

5.4 Discussion

Disc diffusion was applied to assess the antimicrobial susceptibility to various antibiotics and salts heavy metals.

All strains of *S. aureus* tested were sensitive to antimycin, oligomycin and CCCP (Figure 5.3). These different inhibitors of oxidative phosphorylation, namely antimycin, CCCP, and oligomycin, each inhibit oxidative phosphorylation (Epstein *et al.,* 2001).

The data (Table 5.3 and Figure 5.3) shows that the SCV strains were significantly more sensitive to antimycin (the diameter of inhibition zones were larger) in comparison to the wild type. Also SCVs 1 and 2 and 3 showed significantly increased sensitivities between themselves ($P = < 0.05$). This is probably because antimycin affects the Qi site of cytochrome and this then disrupts the formation of the proton gradient across the inner membrane (Dairaku *et al.,* 2004; Arsianti *et al.,* 2010).

There was no significant difference in sensitivity to oligomycinin between SCVs 1, 2 or 3 but they were significantly more sensitive than the wild type ($P = < 0.05$). Oligomycin targets the interaction with F_1 and F_0 moieties of the of ATPase complex (Ovchinnikov *et al.,* 1984). Why the SCVs were more sensitive remains undetermined, but maybe it was due to interference of the proton gradient.

SCVs 1, 2 and 3 were significantly more sensitive to CCCP than the wild type. Also they were significantly different themselves. CCCP is a protonophores and, disrupts Proton Motive Force (PMF) (Eva *et al*., 1994) and so inhibits efflux (molecular traffic) across the cell membrane. Thus CCCP inhibits growth by affecting the cell's PMF. The NADH dehydrogenase complex, functioning as the first energy coupling site in the respiratory chain, the activity of this hypothetical coupling device in *S. aureus,* which seems to be required for tight coupling between respiration and phosphorylation, was observed only in whole cells but not in broken ones. Thus it appears that all three SCVs have a common functional pathway up to this point in oxidative ATP generation. Hence that in the L-lactate oxidizing system operating at the second energy coupling site in the respiratory chain of *S. aureus,* such a controlling device may be absent (Tynecka *et al.,* 1999). Thus the effect on SCVs cannot be explained.

Small colony variants arose in the CCCP zone of inhibition of the wildtype strain COL. These were not studied further. None was seen for the other inhibitors.

The SCV strains were more sensitive to three heavy metals than the wildtype strain (Figure 5.3). SCVs strains exposed to cadmium, copper chromium and mercury showed significantly higher sensitivity (the diameter of inhibition zones were larger) in comparison with wild type.

The clinical use of and hence exposure to heavy metals results in the selection of bacterial strain also able to resist antibiotics. This occurs for genes encoding heavy metals are located together with antibiotic resistance genes. So for example the penicillin binding protein A2 (PBP A2) in *Staphylococcus aureus* also carries genes determining resistance to several heavy metal ions (Novick & Roth 1968) as well as those to erythromycin and other antibiotics (Mitsuhashi 1963). Many staphylococci are resistant to heavy metal ions, especially cadmium and mercury, and arsenic (Dyke *et al.*, 1970).

No small colony variants were observed with any of the heavy metal chloride salts. This is strange as Hirsch (1954) reported SCVs from *E. coli* strain B after exposure to copper sulphate. However another important fact not commented upon is that he did some isolations anerobically and found no SCV were isolated. This correlates with the phenotypes of the SCVs we have isolated. To our knowledge this is the only paper to report an attempt to isolate SCVs anaerobically.

Perron and colleagues (2004) studied resistance to carbepenem and heavy metals in *Pseudomonas aeruginosa*. Part of the reason for their high levels of tolerance/resistance is the presence of multiple efflux pumps. They found a RND proton driven pump conferring resistance to cadmium/cobalt/zinc that also conferred resistance to carbapenem. This cross resistance as the result of a single mutation, thus there is the possibility that in SCVs an analogous mutation may lead to either raised or lowered sensitivities to antimicrobials.

To have raised or lowered sensitivities to these inhibitors suggests that the mutations conferring the SCV phenotype co-incidentally impacts on their tolerance or sensitivity to these inhibitors. Therefore to explain this change in sensitivity it is important to consider the potential physiological impact of reduced ATP generation capacity.

The ability of *S. aureus* to switch to an alternative phenotype in the presence of antimicrobial agents is clearly favourable if it permits survival. SCVs have many characteristics that are associated with survival in unfavourable conditions. Various reports of phenotypic switching have been published (Mitchell *et al.*, 2010a). Formation of the SCV phenotype in *S. aureus* was hypothesised as a potential phenotypic switching system that could be influenced by the alternative sigma factor, σB (Mitchell *et al.*, 2010a). This was a hypothesis discussed early in the work, however the sequencing data on the cycling mutants discussed later (chapter 6) suggests strongly that this is not involved.

It had been hoped that there may have been some differences in sensitivities between the SCVs and then this could have been used to try and understand and explain their physiological changes.

5.5 Conclusions

- CCCP can select SCV at higher concentration. Antimycin and oligomycin were not observed to select for SCV from *S. aureus* COL.

- No SCV were select heavy metal chloride salts.

- SCV1, 2 and 3, wild type strains show significant differences in sensitivity for reasons that remain unknown.

CHAPTER 6:

SEQUENCING OF THE *S. AUREUS* GENOME

6.1 Introduction

6.1.1 Structure of the *S. aureus* genome

Whole genome sequences of several different *S. aureus* strains have been determined. The first two *S. aureus* strains to be sequenced were N315 and Mu50 (Kuroda *et al.*, 2001), which were followed by nine, including strain COL, additional strains (Baba *et al.*, 2002; Diep *et al.*, 2006 & Gill *et al.*, 2005). The sequenced genomes are between 2.7-3 million base pairs (bp) in size and have a low G+C content (~32%). Their genomes contain a single circular chromosomes which encodes approximately 2700 coding sequences as well as structural and regulatory RNAs (Holden & Lindsay, 2008).

Bacterial genomes are commonly viewed as having a core genome, comprising of genes essential for growth and functionality, coupled with an auxiliary set of genes (Frost *et al.*, 2005). It has been proposed that the auxiliary genes confer traits which may be beneficial under certain circumstances (Dobrindt *et al.*, 2004). In the *S. aureus* genome ~75% of genes are highly conserved between isolates and make up the core genome (Lindsay & Holden, 2004). 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). *S. aureus* contains many types of MGEs, including plasmids, transposons, bacteriophages, genomic islands, plasmids and staphylococcal cassette chromosomes all of which can move around bacterial genomes via the process of horizontal gene transfer (Malachowa & DeLeo, 2010). Analysis of closely related orthologues (genes in different species that originated by vertical descent from a single gene of the last common ancestor) indicates that the genetic background of *S. aureus* has been vertically transmitted from a common ancestor that subsequently diverged into *Bacillus* and *Staphylococcus* species (Ito *et al.*, 2003).

There are several possible mutations that could occur in these genomic sequences. These could be point mutation (substitutions) which are termed a transition when a purine replaces a purine (adenine, guanine or a pyrimidine replaces a pyrimidine

(thymine, cytosine and uridine); or a transversion when a purine replaces a pyrimidine or *vice versa*. Other sequence changes are defined as duplications, deletions and transpositions. These potentially involve the change or movement of several to hundreds of bases.

6.1.2 Relevant biochemical features in staphylococci.

6.1.2.1 *dnaC* gene

dnaC gene encoded DNA helicases which cut the hydrogen bonds between bases in double stranded DNA, which serve as a single strand DNA template for DNA polymerases (Arai *et al.,* 1981). A protein encoded by the *S. aureus dnaC* gene has 44% homology with *E. coli dnaB* and 58% homology with *B. subtilis dnaC* replicative DNA helicases (Kaito *et al.,* 2002). Kaito and colleagues suggested that the *dnaC* is involved in DNA repair and that *S. aureus dnaC* helicase is a promising target for antibiotics providing bactericidal effects.

6.1.2.2 *mecA* gene (penicillin binding protein A2)

The *mecA* gene allows staphylococci to be resistant to antibiotics such as methicillin and other penicillin-like antibiotics. *mecA* gene is spread on the SCC*mec* genetic element (Ito & Hiramatsu, 1998). The gene encodes the penicillin binding protein (PBP), called PBP 2A, has a low affinity for β-lactam antibiotics. This enables transpeptidase activity in the presence of β-lactams, preventing them from inhibiting cell wall synthesis (Hartman & Tomasz, 1984; Ubukata *et al.,* 1989). In addition to *mecA*, more than 30 kb of flanking sequences is also acquired, and the entire region is called *mec* locus (Beck *et al.,* 1986). Both *mecA* and *mec* locus DNAs hybridize among all species of staphylococci that display the methicillin-resistant phenotype, suggesting the horizontal transfer of *mec* DNA (Archer & Pennell**,** 1990). Kipp and colleagues study evaluate the influence of SCV phenotype on the detection of methicillin resistance (*mecA*) and detect *mecA* gene in SCVs in comparison with (wild type) normal-phenotype (Kipp *et al.,* 2004).

6.1.2.3 *fusA* gene (Elongation factor-G "EF-G")

Fusidic acid inhibits bacterial protein synthesis by preventing release of EF-G from the ribosome (Bodley *et al.,* 1969). Resistance to fusidic acid in *Staphylococcus aureus* may result from transfer of the *fusB* determinant or from spontaneous mutations in the *fusA* gene that encodes the drug target, the elongation factor G (EF-G; O'Neill *et al.,* 2007). Mutations in the EF-G gene (*fusA*) which prevent binding by fusidic acid protect the staphylococcal translation apparatus from inhibition (O'Neill *et al.,* 2004). *S. aureus* as SCV phenotype on the basis of resistance to aminoglycosides belong to either the *fusA*-SCV or *fusE* resistance class (Norstrom *et al.,* 2007).

6.1.2.4 *ccpA* (catabolite control protein A)

ccpA is global transcriptional regulator of carbon catabolite repression and carbon catabolite activation, which ensures optimal energy usage under diverse conditions (Gill *et al.,* 2005). The catabolite control protein A is a member of the LacI/GalR family of transcription factor that binds to specific *cis*-acting DNA sequences designated the catabolite responsive element (CRE; Henkin, 1996; Stulke & Hillen, 1999). In *S. aureus*, *ccpA* appears to function as a global regulator, affecting not only carbon metabolism but also the production of virulence determinants, antibiotic resistance, biofilm formation, and nitrogen metabolism (Seidl *et al.*, 2008; Seidl *et al.*, 2006). Recent transcriptome and proteome analysis confirmed the broad effects of *ccpA* on gene expression in *S. aureus* (Seidl *et al.,* 2009).

6.1.2.5 *fabI* (enoyl acyl carrier protein reductase)

Fatty acid biosynthesis in staphylococci is typically catalyzed by the type II fatty acid synthase system, utilises several discrete enzymes effecting fatty acid chain elongation and subsequent membrane synthesis (Heath *et al.,* 2000). The enoyl-acyl carrier protein (ACP) reductase, which catalyzes the last step of each cycle of fatty acid elongation, is a promising target because it plays a key role in the regulation of the pathway (Heath & Rock, 1995; Heath *et al.,* 2001). The *fabI* enzymes are receiving increased attention not only because of their regulatory significance, but also because of the recent discovery that inhibitors of this step in the pathway are effective antibacterials. The diazoborines inactivate *fabI* through the formation of a covalent bond with NADH in the active site of the enzyme (Baldock *et al,* 1996; Baldock *et al,* 1998). Triclosan, widely used as a disinfectant agent with broad-

spectrum antibacterial activity, was found to inhibit *fabI*, the enoyl-ACP reductase of *S. aureus* (Heath *et al.,* 2000). *fabI* gene reduced susceptibility within the SCVs phenotype. This implies that *fabI* is not the sole target for triclosan (Seaman *et al.,* 2007).

6.1.2.6 *mnh gene* (Na^+/H^+ antiporter)

The Na^+/H^+ antiporter has several roles in bacterial cells. Firstly it is to establish an electrochemical potential across the cytoplasmic membrane (Chen *et al.,* 1958; Jackowski & Alix, 1900). Secondly to extrude Na^+ and Li^+, ions which are toxic if accumulated at high concentrations (Inaba *et al.,* 1994; Nozaki *et al.,* 1996). Thirdly it is to regulate intracellular pH under alkaline conditions and cell volume (Krulwich, 1983; Padan & Schuldiner, 1994).

Antiporters are composed of multiple subunits, were shown in *Staphylococcus aureus* to be part of an operon encoding seven genes *mnhA-G*. (Hiramatsu *et al.*, 1998). The *mnh* cluster in *S. aureus* is required to confer significant resistance to sodium ions and alkaline pH (Hiramatsu *et al.,* 1998).

6.1.2.7 *menD* gene (Menaquinone biosynthesis)

Menaquinone is one of the bacterial forms of vitamin K and is an important component of the electron transport system (Lin & Kuritzkes, 2003; Taber**,** 1980). Menaquinoneis is derived from the shikimate pathway via isochorismate (Weische & Leistner, 1985). The *menD* gene of *S. aureus* codes for the first enzyme of menaquinone biosynthesis, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC) synthase (Gill *et al.,* 2005). The *menD* mutation provides the *S. aureus* SCVs with a survival advantage during antimicrobial therapy, compared with its parent strain (Bates *et al.,* 2003). However, selected or constructed mutations in *menD* produce the electron transport deficiency-associated phenotypes of SCVs (Bates *et al.,* 2003). Mutations in *menB* were shown to be a possible genetic basis for such a switch in a set of SCV menadione auxotrophic mutants and their revertants recovered from patients (Lannergard *et al.,* 2008).

6.1.2.8 *atl* or *nag* gene (Autolysin gene)

The *S. aureus* autolysin gene, *atl*, encodes a unique 138-kDa protein with amidase and glucosaminidase domains. The atl protein is proposed to undergo proteolytic

processing to generate two extracellular peptidoglycan hydrolases, endo-β-N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase (Komatsuzawa *et al.,* 1997). *S. aureus* contains several types of peptidoglycan hydrolases that can break covalent bonds in their own cell walls (Oshida *et al.,* 1995; Perkins, 1980). The activity of some of these hydrolases is clearly involved in bacterial autolysis (induced by antibiotics or adverse physiological conditions), cell wall turnover, and cell separation (Perkins, 1980; Handwerger & Tomasz, 1985).

6.1.2.9 *folD* gene (coenzyme transport and metabolism)

The *S. aureus folD* gene, bifunctional protein, protein contains two distinct enzymatic activities. Methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase, the latter is a hydrolase and general process is coenzyme transport and metabolism (Gill *et al.,* 2005).

6.1.2.10 *isdA* or *stbA* gene (iron-regulated surface determinant protein A)

isdA gene has broad-spectrum ligand-binding activity, including fibrinogen and fibronectin (Clarke *et al.,* 2004). It's only expressed in iron-limited conditions under the control of iron-regulated surface determinant protein A (Clarke *et al.,* 2008). The *isdA* gene has two domains with distinct functions. The N-terminal NEAT domain of *isdA* binds a broad spectrum of human extracellular matrix and serum proteins, including transferring (Taylor & Heinrichs, 2002; Vermeiren, *et al.,* 2006). The C-terminal domain of *isdA* has recently been shown to decrease the cellular hydrophobicity of *S. aureus* and confer resistance to hydrophobic fatty acids and host antimicrobial peptides and thus aid survival on live human skin (Clarke *et al.,* 2007). Thus in SCVs it is expected to be up regulated.

6.1.2.11 *trmFO* or *gid* gene (methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase)

trmFO encodes for the folate/ FAD-dependent tRNA methyltransferase, has been identified in most Gram-positive and some Gram-negative bacteria, including (Urbonavicius *et al.,* 2005; Urbonavicius *et al.,* 2007).

6.1.2.12 *spl* gene (serine protease)

The *S. aureus spl* gene has been considered as virulence gene as they encode secreted six serine protease-like proteins (Reed *et al.* 2001). The *spl* proteins appear to be synthesized without propeptides. Because of their sequence similarities to serine proteases, the genes encoding these proteins were designated *splA, splB, splC, splD, splE,* and *splF* for serine protease like (Reed *et al.* 2001).

6.1.2.13 *fmtA* gene (autolysis and methicillin resistance related protein)

fmtA gene specifically interacts with β-lactams antibiotics forming covalently bound complexes. *fmtA* has a low binding affinity for β-lactams, and it experiences a slow acylation rate, signifying that this protein is intrinsically resistant to β-lactam inactivation (Fan, 2007). It has been suggested that *fmtA* affects the cell wall structure (Komatsuzawa *et al.,* 1997). The putative *fmt* protein showed a hydropathy pattern similar to that of *S. aureus* penicillin-binding proteins and contained two of the three conserved motifs shared by penicillin-binding proteins and β-lactamases (Komatsuzawa *et al.,* 1997).

6.1.3 Aims

- Annotate the *S. aureus* COL and *S. aureus* N315 SCV genomes.

- Determine the *novel* mutations that produce the SCV phenotype and discriminate these from the *historic* mutations unrelated to SCV formation.

- Comment on and analyse their diversity

- Determine if the forward and reversion mutations that determine the SCV state are random or not.

- Describe the classes of mutations involved

- Determine if the SCV formation cycle is a random or dedicated mutational event.

6.2 Materials and methods

6.2.1 Bacterial strains

The bacterial strains used are listed in Table 6.1. Briefly these were each obtained after 15 cycles of forward (to an SCV form) and reversion (back to a wild type form). For further details refer to Chapter 2.

Table 6.1 Bacterial strains used in this study

Strain	Description	Source/Reference
COL SCV 15-TRI	derived from COL following triclosan selection	(This study chapter 2; section 2.2.1)
N315 SCV 15-TRI	derived from N315 following triclosan selection	(This study chapter 2; section 2.2.1)
COL SCV 15-GEN	derived from COL following gentamicin selection	(This study chapter 2; section 2.2.1)
N315 SCV 15-GEN	derived from N315 following gentamicin selection	(This study chapter 2; section 2.2.1)

6.2.2 Genome sequence analysis

6.2.2.1 Selection of strains for sequencing

Because of time and financial constraints only four of the cycled strains (COL SCV 15-GEN, COL SCV 15-TRI, N315 SCV 15-GEN, N315 SCV 15-TRI) were analysed (Table 6.1). Briefly these four isolates were the result of 15 cycles of selection for SCV by triclosan, combined with 15 cycles of selection for WT by fast growth in non-selective conditions (see chapter 2 for a full explanation). It was hoped that an examination of the genome sequences would provide a clear historical record or evolutionary fingerprint of the mutations that had occurred in the two strains. Although the best strategy would have been to sequence all 60 strains, this was not economically feasible. Thus the final SCV strains isolated (COL-SCV15 and N315-SCV15) were sequenced and compared between themselves and with the published genome sequence of their parental strains. It was recognized that there would be

sequence differences in the genomes of *our* COL and N315 strain parental strains and the published sequences. We thus termed the common mutations arising in the strains after different selection antimicrobials as an indication thus describe these as *historic*. The *novel* mutations are therefore ones not occurring in the same strain undergoing the alternative selection cycles.

6.2.2.2 Rationale of the sequencing strategy

The logic behind the experiment was that the genome would show evidence of the all the mutational changes occurring after 15-cycles. The most mutations expected would be 60 and this would be the case if all forward changes (to SCV) were at different sites and suppressed by reversion at different sites. The least changes would be zero and this would suggest a site specific mutation process. This would deliver a WT revertant with no evidence for mutation.

6.2.2.3 The genome comparison

Sequence comparisons used BLAST. The BLAST software was obtained from the NCBI site. The resulting list of matching tandem repeats was then imported in the database, where it was queried.

6.2.2.4 Electronic genome structure

The original genome map of *S. aureus* was based on the strain 8325, created by Pattee and colleagues, and then by 2000 the whole genome of strain 8325 had been sequenced and annotated. Since then, at least six other *S. aureus* strains have been completed (COL, N315, Mu50, MW2, MRSA252, MSSA476) **(**Patel *et al.,* 1989; Diep *et al.,* 2006). The *S. aureus* strain N315 and COL has been completed circular genome map shows ~2.8-Mb open reading frames. The two strains have 79 and 72 RNA genes, 2614 and 2615 protein coding genes, and 5 complete ribosomal RNA operons in their genome. These strains have ~33% G+C content, utilise ~84% as coding sequence. There is a single plasmid ~25,000 base pairs in length with a G+C content of 28.7% (Kuroda *et al.,* 2001; Gill *et al.* 2005).

6.3 Results

This experiment was designed to determine whether there were a multitude of sites at which the SCV phenotype was conferred. The alternative was that the phenotype was produced from a single site mutational event that was freely revertable, resulting from a site specific mutational change. Thus the genomic sequence of the final revertants in the cycling experiment would provide the evidence for either hypothesis. Numerous changes at various sites would confirm the more random process, whilst no evidence of significant genomic change would suggest the site specific mechanism.

Examining the sequence data in Tables 6.2 and 6.3 show there are numerous changes to the sequence of the cycled strains of COL and N315 when compared to the parental sequenced strains. However the changes highlighted in grey (8 N315and 25 COL) are common to each strain with different repeated selection process. These strains have been cultured in our laboratory for over 10 years and must statistically have acquired spontaneous mutations in their genomes. Thus the mutations highlighted in grey are probably not related to the cycling and selection steps in the experiment, but are *historic*. If this premise is accepted then there are 5 (N315-TRI), 4 (N315-GEN), 10 (COL-TRI) and 21 (COL-GEN) *novel* mutations in the four strains (Table 6.4).

It would be predicted that over the 15 forward and 15 reverse selection cycles that a maximum of 30 independent mutations would be seen. That is if the mutations occurred in different sites. These sites could be in the same gene or in different genes. However there is the possibility that some mutations revert to wild type and so there would be no footprint (sequence change) of their occurrence. Thus logic dictates no more than 30 changes would be seen. So this sequence analysis relies on mutations to wild type that are suppressed and do not occur by true reversion. Thus changes in the number of mutations less than 30 are predicted.

If the mutational event SCV – WT – SCV were a site specific one then there should be no evidence of change other than in genes unrelated to SCV formation. It would also be expected that there would be no sequence change and so the final wildtype would have the same sequence as the initial parental strain. The sequencing data thus suggests that the site specific mutational mechanism is not operating. The finding that there are not about 30 mutational changes in any of the genomes of final revertants implies in many cases the cycled mutant selected was a true revertant. Thus there is no evidence for suppressed mutants.

N315-TRI and N315-GEN share 8 mutations and COL-TRI and COL-GEN share 25 mutations (grey shading in Table 6.2). These indicate *historic* changes unrelated to the cycling experiment. This suggests the rate of spontaneous mutation maybe 3-4-fold higher in COL than in N315. Although this assumes their growth histories are equivalent.

There are just 5 *novel* mutations in N315-TRI and 4 in N315-GEN instead of the predicted 30. There are more in COL-TRI (10) and most in COL-GEN (21). Thus this suggests the mutation rate in COL is higher.

Examining N315 first. Table 6.2 shows some mutations to be greyed out. These are common between the selection cycles and so are predicted to be unrelated to the selection agent and are thus predicted to be historical. Importantly they do not have any obvious sequence (gene function/phenotype) importance, thus inferring this is a reasonable conclusion. Interestingly there are no mutations in common between N315 SCVs selected on triclosan or gentamicin. This implies there are many sites at which SCV formation can occur. Interestingly *fabI* is one gene consistently recognised to be effected when selection is made for triclosan resistance and this was found in both strains (COL and N315) when selection was made on triclosan (Tables 6.2 and 6.3).

An examination of COL (Table 6.3) shows 24 common mutations (greyed out) that are presumed to be *historical*. Only two could potentially be significant, an osmoprotectant transporter (ACOL0781) and an antiporter (SACOL1014) and it is possible that these offer a common phenotypic response to gentamicin and triclosan selection pressure. This remains to be determined and by examining these gene sequences in our parental COL strain by PCR analysis we could determine if they are *historic* or *novel*. Although triclosan selects for a mutation in *fabI*, but this is only one of eight mutations seen in this cycled strain. Thus in this strain there are several putative sites which confer the SCV phenotype.

There are numerous mutations to examine to determine their significance. Without further analysis these can only be examined theoretically to hypothesise their relevance to SCV formation (Tables 6.2 and 6.3). These are examined individually

6.3.1 Genes effected in the *S. aureus* N315 strain

The *S. aureus* N315 SCV-GEN *dnaC* gene has a transition mutation (C→T). The gene encodes a DNA helicase that serves as a single strand DNA template for DNA polymerases (section 6.1.2.1).

S. aureus N315 SCV-GEN (SA0857) *mecA* gene has a transversion mutation (G→C). The gene encodes a penicillin binding protein PBP2A. The *mecA* gene prevents methicillin and other penicillin-like antibiotic binding to the enzymes that help forming the cell wall of the bacterium, and hence the bacteria can replicate normally (section 6.1.2.2).

S. aureus N315 SCV-TRI (SA0323) *mepA* gene has a transition mutation (C→T). This is possibly acted as a multidrug resistance protein and secondary transporter of unknown function. So its impairment affects metabolism resulting in the SCV phenotype.

S. aureus N315-TRI (SA0376) *guaA* gene has a transition mutation (A→G). The enzyme catalyzes the synthesis of GMP (guanine monphosphate synthetase) which converts XMP (xanthosine monophosphate) to guanosine monophosphate (5'-guanidylic acid; Gene bank; NCBI). So its impairment possibly affects energy related metabolism resulting in the SCV phenotype.

S. aureus N315 SCV-GEN (SA0505) *fusA* gene has a transition mutation (A→G). The *fusA* gene encodes the elongation factor G (EF-G; O'Neill *et al.,* 2007). Mutations in the EF-G gene (*fusA*) which prevent binding by fusidic acid protect the staphylococcal translation apparatus from inhibition (O'Neill *et al.,* 2004). *S. aureus* SCV phenotype belongs, on the basis of resistance to aminoglycosides, to either the *fusA*-SCV or *fusE* resistance classes (Norstrom *et al.,* 2007).

S. aureus N315 SCV-TRI (SA1882) *kdpD* gene has a transversion mutation (T→A). KdpD together with KdpE constitutes a two-component signal transduction system, which was first characterized in *E. coli* (Altendorf *et al.,* 1992). In *S. aureus* the proteins KdpD and KdpE regulate the production of Kdp-ATPase, which is an inducible high-affinity K^+ transporter that is synthesized under conditions of severe

K^+ limitation or osmotic upshift (Laimins *et al.,* 1981). So its impairment potentially affects membrane transport/proton transport resulting in the SCV phenotype.

S. aureus N315 SCV-TRI (SA2109) gene has a transversion mutation (A→C). It encodes an amino acid permease (Kuroda *et al.,* 2001). How its impairment affects metabolism resulting in the SCV phenotype remains unknown.

6.3.2 Genes effected in *S. aureus* COL strain

The *S. aureus* COL SCV-TRI (SACOL0129) gene for a hypothetical ABC transporter has a transition mutation (G→A). There are a enormous number of putative ABC transporters in staphylococci, most of which have not yet been characterized. Some have been detected in a signature-tag transposons-mutagenesis approach aiming at finding possible novel drug targets (Coulter *et al.,* 1998). In addition an ABC transporter has been identified as the immunodominant protein during *S. aureus* infections (Burnie *et al.,* 2000), both findings demonstrate than ABC transporters will play important role as target molecules in the search for novel antistaphylococcal therapies (Otto & Gotz, 2001). Thus their removal in SCVs would make them less sensitive to antibody recognition.

The *menD* gene in COL SCV-TRI strain (SACOL1052) had multi-point mutation in different positions gene (substituted A→G, T→C) and one small deletion mutation. Mutations in *menD* are recognised to produce the electron transport deficiency-associated phenotypes of SCVs (Bates *et al.,* 2003). Menaquinone (vitamin K_2) has an important role in transport of electrons in respiratory chain. Mutations which block menadione biosynthesis result in the SCV phenotype (Sendi & Proctor, 2009).

S. aureus COL SCV-GEN (SACOL1058) gene encodes a class 1 aminotransferase. It has a transversion mutation (C→A). How it selects for the SCV phenotype is unknown.

The *atl* gene in COL SCV-TRI strain (SACOL1062) had two different substitution mutations (G→T, G→A) and at one position, a deletion of cytosine. The *atl* gene cluster has several types of peptidoglycan hydrolases that can break covalent bonds in the cell's own cell walls (section 6.1.2.8). How it selects for the SCV phenotype is unknown.

The *folD* gene in COL SCV-TRI (SACOL1072) has a transition mutation (C→T). This gene encodes a protein that possesses two distinct enzymatic activities, dehydrogenase and cyclohydrolase (Gill *et al.*, 2005). How it selects for the SCV phenotype is unknown. It is possible that the dehydrogenase is involved in energy metabolism and maybe thus has some impact in this way.

The *isdA* gene (iron-regulated surface determinant protein A) in COL SCV-TRI strain (SACOL1140) had two deletions in different positions within the gene. *isdA* gene product decreases the cellular hydrophobicity of *S. aureus* and confer resistance to hydrophobic fatty acids and host antimicrobial peptides and thus aid survival on human skin (Clarke *et al.*, 2007). Thus in SCVs it is expected to be up regulated (section 6.1.2.10) and so have a mutation in its regulation, but what impact on its function these internal mutations have to result in the SCV phenotype is unknown.

The *trmFO* gene in COL SCV-GEN (SACOL1428) gene has a transition mutation (G→A). The gene encodes for the folate/ FAD-dependent tRNA methyltransferase. How it selects for the SCV phenotype is unknown.

The *S. aureus* COLSCV-TRI (SACOL2042) *ilvD* gene has a transition mutation (G→A). The i*lvD* gene regulates valine, leucine and isoleucine biosynthesis. How it selects for the SCV phenotype is unknown.

6.3.3 A mutation occurring in both *S. aureus* COL and N315

Both of COL-TRI (SACOL1016; *fabI*) and N315-TRI (SA0869; *fabI*) had mutations in *fabI*. These were C→T and G→T substitutions respectively (Table 6.1 and 6.2). The *fabI* encodes a protein (enoyl acyl carrier protein reductase) involved in lipid transport. The *fabI* mutation reduced the susceptibility of the SCV, compared to the wild type strain, thus selecting for the SCV phenotype (Seaman *et al.*, 2007). This mutation was only observed in SCVs isolated on triclosan.

Table 6.2 Characteristics of the genes associated with SCV formation in N315

Locus	Sequence number	Gene name	Product or putative function	Process	Mutation N315-TRI	Mutation N315-GEN	References and sources
	20766	*dnaC*	Helicase	DNA helicase activity	-	CT	NBCI Gene bank
	36135	UN			AGAAA^{Del}	GAAA^{Del}	
	37605	UN			CT	CT	
	37631	UN			GA	GA	
	41690	UN			CT	CT	
SA0857	64975	*mecA*	Penicillin-binding protein 2a	Cell wall and membrane biogenesis	GC	-	(Kuroda *et al.*, 2001; McAleese *et al.*, 2006)
	81245	UN			T^{Del} + GACA^{Del}	T^{Del} + GACA^{Del}	
SA0323	380993	*mepA*	Multidrug resistance protein	Transport and binding proteins (P-A 165 Multidrug efflux)	CT	-	McAleese *et al.*, 2005; NBCI (Gene bank NCBI)
SA0376	435235	*guaA*	GMP synthase	Nucleotide transport and metabolism	AG	-	(Kuroda *et al.*, 2001; Scherl *et al.*, 2005)
SA0505	589974	*fusA*	Elongation factor G	Translation elongation factor G	-	AG	(Vaezzadeh *et al.*, 2005; O'Neill *et al.*, 2004
SA0869	984367	*fabI*	Enoyl-[acyl-carrier-protein] reductase	Lipid transport and metabolism; (*fabI* G-C 113)	GT	-	Heath *et al.*, 2000
SA1882	2131592	*kdpD*	Sensor histidine kinase *KdpD*	Signal transduction by phosphorylation	TA	-	Kuroda *et al.*, 2001
	2309464	UN			-	GA	
SA2109	2372458	UN	Amino acid permease	Amino acid transport and metabolism	-	AC	Kuroda *et al.*, 2001
	2561821	UN			CT	CT	
	2753061	UN			TA^{Del}	T^{Del}	
Number of *novel* mutations					**5**	**4**	
Differences from reported sequence (*historic* mutations)					**8**		

GEN: N315 SCV 15 gentamicin; TRI: N315 SCV 15 triclosan; UN unknown; ^{Del}: deletion mutant; -: no mutation; GA or AG = guanine substituted by an adenine or *vice versa*; TA or AT = thymine substituted by an adenine or *vice versa*; TG or GT = thymine substituted by a guanine or *vice versa*; CA or AC = cytosine substituted by an adenine or *vice versa*; GC or CG = guanine substituted by a cytosine or *vice versa*; TC or CT = thymine substituted by a cytosine or *vice versa*; bp = base pair.

Table 6.3 Characteristics of the genes associated with SCV formation in COL

Locus	Sequence number	Gene name	Product or putative function	Process	Mutation COL-TRI	Mutation COL-GEN	References
SACOL0085	91185	UN	Peptidase, M20/M25/M40 family	General function prediction only	30bp ^{Del}	30bp ^{Del}	Kuroda *et al.*, 2001;
SACOL0129	145010	UN	Transport ABC V-I 268 putative	Hypothetical	GA	-	Gill *et al.*, 2005
SACOL0132	148085/8	UN	Pseudo	Posttranslational modification	TA, AC	TA, AC	NBCI Gene bank
SACOL0338	363030	UN	Topoisomerase putative	Replication, recombination and repair	GA	-	Kuroda *et al.*, 2001
SACOL0367	375225	UN	Prophage L54a, terminase, large subunit, putative	Miscellaneous-Phage, (M-I)	GA	-	Gill *et al.*, 2005
	465935	UN			TA	TA	
SACOL0511	511017	UN	Hypothetical protein	Hypothetical TM Yib E/F family	-	CA	Gill *et al.*, 2005
	623565	UN			GA	GA	
SACOL0781	805360	UN	Osmoprotectant ABC transporter	Amino acid transport and metabolism	G ^{Del}	G ^{Del}	Gill *et al.*, 2005
SACOL1014	1022865	UN	Na+/H+ antiporter, putative, authentic point mutation	Antiport; hydrogen ion transport; ion transport and sodium transport	T ^{Del}	T ^{Del}	Gill *et al.*, 2005
SACOL1016	1023925	*fabI*	Enoyl-[acyl-carrier-protein] reductase	Lipid transport and metabolism, (*fabI* A-V 95)	CT		Gill *et al.*, 2005; Heath *et al.*, 2000
	1045162	UN			A ^{Del}, T ^{Del}	A ^{Del}, T ^{Del}	
SACOL1052	1058605	*menD*	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid synthase/2-oxoglutarate	Coenzyme transport and metabolism, (Menaquinone biosynthetic process)	-	AG	Gill *et al.*, 2005; Bates *et al.*, 2003
" " "	1058651	" "	" " "	" " "	-	TC	" "
" " "	1058745	" "	" " "	" " "	-	A ^{Del}	" "
" " "	1058775	" "	" " "	" " "	-	AG	" "
" " "	1059135	" "	" " "	" " "	-	TC	" "
	1063050				GT	GT	
SACOL1058	1064925	UN	Aminotransferase, class I	Amino acid transport and metabolism	-	CA	Gill *et al.*, 2005
SACOL1062	1068304	*atl* (*nag*)	Bifunctional autolysin	Cell wall and membrane biogenesis	-	GT	Gill *et al.*, 2005; Komatsuzawa *et al.*, 1997
" " "	1068311	" "	" " "	" " "	-	C ^{Del}	" "
" " "	1070773	" "	" " "	" " "	-	GA	" "

	1081800	UN			-	CT	
SACOL1072	1082805	*folD*	methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	Coenzyme transport and metabolism	-	CT	Gill *et al.*, 2005
SACOL1140	1148845	*isdA* (*stbA*)	Cell wall surface anchor protein; Iron-regulated surface determinant protein A	Cell wall and membrane biogenesis	A ^{Del}	-	Gill *et al.*, 2005; Taylor & Heinrichs, 2002
" " "	1148920	" "	" " "	" " "	G ^{Del}	GC	" "
SACOL1188	1192900	UN	Hydrolase, haloacid dehalogenase-like family	General function prediction only	CT	-	Gill *et al.*, 2005
	1227842	UN			A ^{Del}	A ^{Del}	
	1227906	UN			AG	AG	
SACOL1268	1279941	*gid* (*trmFO*)	Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase *trmFO*	Translation	-	GA	Gill *et al.*, 2005; Urbonavicius *et al.*, 2007
SACOL1359	1365405	UN	Hypothetical protein	Hypothetical	-	CT	Gill *et al.*, 2005
SACOL1428	1440658	*lysC*	Aspartokinase	Amino acid transport and metabolism	T ^{Del}	T ^{Del}	Gill *et al.*, 2005
SACOL1454	1468807	UN			2 bp ^{Del}	-	
SACOL1472	1482135	*ebh*	Extracellular matrix-binding protein	pathogenesis	-	GA	Gill *et al.*, 2005
SACOL1662	1691715	UN	Acetyl carboxylase biotin carrier protein	Lipid transport and metabolism	A ^{Del}	C-A ^{Del}	Gill *et al.*, 2005
SACOL1747	1785033	*accA*	acetyl-CoA carboxylase, carboxyl transferase, alpha subunit	Lipid transport and metabolism	-	GA	Gill *et al.*, 2005
	1830469	UN			CG	CG	
SACOL1866	1918837	*splD*	Serine protease	Posttranslational modification, protein turnover, chaperone	TT ^{Del}	TT ^{Del}	Gill *et al.*, 2005; Reed *et al.* 2001
	2004705	UN			C ^{Del}	AC ^{Del}	
SACOL2042	2102282	*ilvD*	Dihydroxy-acid dehydratase	Valine, leucine and isoleucine biosynthesis, related molecules (*ilvD* R-K 405)	GA	-	Gill *et al.*, 2005
	2123390	UN			-	GA	
	2241656	UN			AG	AG	
	2286720	UN			AT	AT	
	2286770	UN			AT	AT	
SACOL2345	2404465	UN	Esterase putative	Lipid transport and metabolism	-	GA	Gill *et al.*, 2005
SACOL2346	2405685	UN	Hypothetical protein	Hypothetical	-	GA	Gill *et al.*, 2005
	2428185	UN			GC	GC	
	2470605	UN			G ^{Del}	G ^{Del}	

	2482180	UN			TG	TG	
	2492156	UN			TA	TA	
SACOL1066	2504495	*fmtA*	*FmtA*, autolysis and methicillin resistance related protein	Pathogenic factors (toxins and colonization factors)	TA ^{Del}	TA ^{Del}	Komatsuzawa *et al.*, 1997; Komatsuzawa *et al.*, 1999;
	2595460	UN			CA	CA	
SACOL2696	2770880	*hisI* (*hisIE*)	Phosphoribosyl-ATP pyrophosphatase/phosphoribosyl-AMP cyclohydrolase	Amino acid transport and metabolism	A-AG-T ^{Del}	AG ^{Del}	Gill *et al.*, 2005
Number of *novel* mutations					**10**	**21**	
Differences from reported sequence (*historic* mutations)					**25**		

GEN: COL SCV 15 gentamicin; TRI: COL SCV 15 triclosan; UN: Unknown; ^{Del}: deletion mutant; -: no mutation; GA or AG = guanine substituted by an adenine or *vice versa*; TA or AT = thymine substituted by an adenine or *vice versa*; TG or GT = thymine substituted by a guanine or *vice versa*; CA or AC = cytosine substituted by an adenine or *vice versa*; GC or CG = guanine substituted by a cytosine or *vice versa*; TC or CT = thymine substituted by a cytosine or *vice versa*; bp = base pair.

Table 6.4 Total the mutation types and proportion in both strains

Strain	Deletion mutations	Point mutations	Novel mutations	Historic mutations	Agent	Novel deletions mutations	Novel point mutations	Historic deletions mutations	Historic point mutations
S. aureus **N315**	4	13	9	8	**TRI**	0	5	4	4
					GEN	0	4		
S. aureus **COL**	19	37	31	25	**TRI**	4	6	12	13
					GEN	3	18		
Total per mutation	23	50	40	33					
Total to all mutation	73								

6.4 Discussion

General Mutations (novel and historical):

Analysis of the sequences illustrate there are numerous changes to COL and N315 genomes when compared to the parental sequenced strains. However some changes are historic. Thus the mutations highlighted in grey are probably not related to the cycling and selections steps in the experiment, but are *historic*. That is they are present in the strain prior to the experiment. Thus 8 mutations in COL and 25 mutations in N315 have arisen since the strain we obtained was originally sequenced. It would be predicted that over the 15 forward and 15 reverse selection cycles that a maximum of 30 independent mutations would be seen. That is if the mutations occurred in different sites. These sites could be in the same gene or in different genes.

Novel mutation were arose 9 mutations in N315 SCV (5:N315-TRI), (4:N315-GEN) and 31 mutations in COL (10: COL-TRI) and (19: COL-GEN) *novel* mutations in the four strains (Table 6.4).

Exposure of *S. aureus* N315 and COL to triclosan and gentamicin work by inducing point mutations which occurs when a single base pair of a gene is changed, and deletion (loss of DNA bases). N315 and COL strains found point mutations by (13:37) mutations and deletion mutations by (4:19) mutations, while mutations the same in the two strains were presented in *fabI* (Table 6.4).

The genomes of N315 and COL encode 2593 and 2615 protein genes (Kuroda *et al.*, 2001 and Gill et *al.*, 2005). The sequencing data (Table 6.4) shows there are a total of 38 different genes in the two strains which give an SCV phenotype significantly only one is in common (*fabI*) and thus it is probable that there are even more than this. Since there is little difference in the two staphylococci genomes we can add the SCV mutants together and so estimate (33/2615) that 1.3% of the genome (or about 40 Kb if each gene is about 1 kb in size) is concerned with the SCV phenotype. One unexplained aspect of the *historic* mutations column in Table 6.4 is that N315 has more *historic* mutations than COL, but an apparent lower mutation rate, as is evident from the lower number of *novel* mutations. The fact that the rates of SCV formation are not impacted by the selective agent also suggests that they are both generalist. By this it is meant they are not directly selecting for on or a reduced SCV phenotype array. Table 6.5 indicates the known array of genes and phenotypes affected. The importance of the remaining unidentified sequences (genes?) and their potential

phenotypes is unknown. This however suggests there are many other ways to become an SCV. This clearly has implications to understanding the infection success and treatment potential for the organism.

The data in Table 6.5 shows that about a quarter of the mutations occurred in known genes. These are responsible for amino acid metabolism (9 genes), lipid metabolism (5 genes), DNA metabolism (4), pathogenicity (4 genes), transport (3) and cell wall and membranes (2). The remaining mutations were in sequences for which no actual or putative function has been assigned.

N315:

The *S. aureus* N315-GEN *dnaC* gene has a transition mutation (C→T). Kaito and colleagues as identified five mutant strains whose temperature-sensitive colony formation phenotypes were complemented by the *dnaC* gene. DNA replication in these mutants has a fast-stop phenotype (Kaito *et al.,* 2002). How this relates to SCV formation is unknown.

COL:

SACOL1058 in *S. aureus* COLSCV- GEN encodes an aminotransferase and had a point mutation (C-A) conferring gentamicin resistance. There are three types of aminoglycoside modifying enzymes and this type, SACOL1058 N-Acetyltransferases (AAC), catalyzes acetyl CoA-dependent acetylation of an amino group, a class 1 aminotransferase. Enzymatic modification is the most common type of aminoglycoside (gentamicin) resistance (Davies & Wright, 1997). It is hypothesized that the enzyme is derived from organisms that make the aminoglycoside or from the mutation of genes that encode the enzymes involved in cellular respiration (Gilbert, 2000). Gentamicin is member of aminoglycoside family; they inhibit protein synthesis by binding to the 30S ribosomal subunit to prevent the formation of an initiation complex with messenger RNA. They also cause misreading of the messenger RNA message. Another important function of the aminoglycosides is that they increase membrane leakage intracellular contents (White *et al.,* 2005). How it selects for the SCV phenotype is unknown.

The *menD* gene in COL SCV-TRI strain (SACOL1052) had multi-point mutation in different positions within the gene and one deletion mutation. Mutations in *menD* are

recognised to produce the electron transport deficiency-associated phenotypes of SCVs (Bates *et al.,* 2003). Menaquinone (vitamin K_2) has an important role in transport of electrons in respiratory chain. Mutations that block menadione biosynthesis, reduce ATP yield and thus result in the SCV phenotype (Sendi & Proctor, 2009).

N315&COL:

Both mutants COL-TRI (SACOL1016; *fabI*) and N315-TRI (SA0869; *fabI*) arose from a substitution of 2 base pairs (Table 6.2). Seaman *et al.,* (2007) found 5 base pairs substitution mutation in the *fabI* gene of *S. aureus* after exposure to triclosan. The *fabI* gene is one of the key enzymes in the bacterial fatty acid biosynthetic cycle (Bergler *et al.,* 1996; Slater-Radosti *et al.,* 2001). The *fabI* mutation reduced the susceptibility of the SCV, compared to the wild type strain, thus selecting for the SCV phenotype (Seaman *et al.,* 2007).

Thus examining the *historic* mutations we can say they occurred in regions which have no apparent impact on staphylococcal activity. Until we sequence the entire collection we can only assume at present that we have labelled these historic mutations correctly.

The *novel* mutations are more diverse than others have suggested or even considered. It goes to illustrate that there are many individual ways to produce an SCV.
Presumable when these are really understood metabolically and phenotypically an understanding of the individuality of the phenotypes resulting from mutations, their classes and types will become apparent.

Table 6.5 summarises the mutations assigned/associated with SCV formation. Some are hypothetical and others real. Many would not be predicted and have no apparent physiological/biochemical link or prior association with generating the SCV phenotype. So although the basis for SCV formation in the literature seems simple, it is clearly not. The Table also indicates that we have identified a further 77 independent and novel mutations which produce an SCV on unknown basis. Thus with a large number of potential gene target to produce an SCV it is predictable that a microbial cell can acquire the SCV phenotype at a 10-100-fold higher frequency than a normal mutation rate. That is assuming that all SCVs are equal and we know they

are not. However they may be better than the wild type at surviving and that is all they have to do.

Table 6.5 27 genes associated with SCV formation and their metabolic roles

Amino acid metabolism	Lipid metabolism	DNA metabolism	Pathogenicity	Transport	Cell wall/membrane
SA2109	*fabI* (SA0869)	*dnaC*	*fusA*	*KdpD*	*mecA*
SACOL0085	*fabI* (SACOL1016)	*guaA*	*ebh*	SACOL0129[#]	*isdA*
SACOL0781	*accA*	SACOL0338[#]	*splD*	SACOL1014[#]	
SACOL1062	SACOL1662	*gid* (*trmF0*)	SACOL0367[#]		
menD	SACOL2345[#]				
folD					
lysC					
ilvD					
hisI					
9	**5**	**4**	**4**	**3**	**2**

[#] Hypothetical function; 77 others had unknown activities

One of the aims asked if SCV formation cycle was a random or dedicated mutational event. It seems clear from Table 6.4 that finding 6 - 19 mutations in the final wild type sequences is indicative of point mutations as a response to SCV formation and reversion. However finding just 3 *novel* mutations in N315-TRI after 15 cycles suggests this strain (and the other 29 cycle intermediates) ought to be examined in more detail. At the very least the finding of low numbers of mutations after 15 cycles is suggestive of true reversion and not intragenic suppression. It maybe of course that some of the unknown mutations are suppressing intergenically, the original SCV causing mutation.

6.5 Conclusions

- Numerous mutations have occurred in the genomic sequence of COL and N315 when compared to the parental sequenced strains (over 70 mutations; Table 6.4).

- There were 25 historical mutations in COL and 8 in N315.

- *S. aureus* COL and *S. aureus* N315 genomes have acquired spontaneous mutations in their genomes since they have been in our laboratory.

- Novel mutations conferring the SCV phenotype occur in each strain (5: N315-TRI), (4: N315-GEN), (10: COL-TRI) and (21: COL-GEN).

- These novel mutations are not part of a gene a cluster, but effect different components of the cell's metabolic pathways. Thus there is not just one SCV mutation, but a diverse population of them.

- Only one mutational site was found to be common between COL and N315 and this was in the *fab*I gene.

- At least 1.3% of the genome (≥40 Kb) is concerned with the SCV phenotype, but it might well be significantly higher.

- The two strains have shown many different metabolic routes to becoming and SCV.

- Thus SCVs appear to be generated by spontaneous mutations occurring in a range of very different genes.

- The two strains appear to have different rates of mutation.

- There was no evidence for a site specific mechanism.

CHAPTER 7:

DISCUSSION, CONCLUSIONS AND FUTURE WORK

7.1 General discussion

Staphylococcus aureus is recognized as one of the most common organisms causing nosocomial and community-acquired infections in every region of the world. The increasing prevalence of methicillin resistance among staphylococci is an increasing problem (Yilmaz *et al.,* 2007). However under certain conditions *S. aureus* behaves as an opportunistic pathogen and is frequently the causative agent of various human diseases. As such it is of the most intensively studied bacterial species (Plata *et al.*, 2009), and is associated with prolonged hospital stay, increased morbidity and mortality, as well as increased healthcare costs (Que & Moreillon, 2010). Thus the research discussed here should be viewed in this context.

The work in thesis supports and confirms that *S. aureus* SCVs possess many characteristics that are abnormal for wildtype strains, they pose difficulties in identification and isolation in the laboratory ((Proctor *et al.* 1994). Atypical growth rates are clearly shown to be key to their metabolic performance and 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.

These data shows the rate of formation of wild type and SCV strains are within an order of magnitude. Thus the spontaneous mutation rate has not been subject to selection and the remains equal to the original WT strains.

7.1.1 Selection and identification of SCVs

Physiological tests on isolates from the cycling experiment showed all SCV isolates and the wild type revertants (WT15) had a reduction in intracellular ATP levels. They also had a reduction or absence in pigment, catalase, coagulase, haemolytic and DNase activities. The maximum cell yield was also reduced in the COL wild type revertants, but not those from N315. These effects on SCVs have been noted previously (Proctor *et al.*, 2006; Seaman *et al.*, 2007), but no one has examined

revertant physiology or activities before. The fact that N315 wild type revertants reacquire the wild type growth yield and yet the COL revertants do not requires further investigation. It also shows again that not all SCVs are the same and also that not all wild types are either, this metabolic diversity is a key feature of the SCV story.
A comparison of growth rate, pigment and phage yield and maximum cell count showed a relationship for strain COL, while DNAs activity was less well associated, although it was reduced in all SCVs.
Auxotrophy for haemin and menadione has been reported in *S. aureus* SCVs as well as SCVs isolated from several other bacterial species (Sasarman *et al.,* 1970). The failure to produce menaquinone and haemin in the SCVs selected by gentamicin and triclosan results in a disrupted ETC resulting in reduced ATP levels. The reduction in cellular ATP levels results in the characteristic non-pigmented microcolonies observed, thus the ATP concentration was determined in isolates. It was clear that the levels were reduced in all SCV isolates. Thus the evidence suggests that the phenotype changes in SCVs are driven by basic energy insufficiency. This has been suggested by others (Proctor *et al.,* 2006), but none performed an ATP assay to confirm their supposition.
All the SCV isolates were confirmed as *S. aureus* by both RAPD-PCR and Multiplex PCR and were also checked for phage sensitivity.

7.1.2 Transduction and lysogen formation in *S. aureus* and SCVs

Transduction of ciprofloxacin resistance (*grlA*) was observed into COL wildtype at a 5-10-fold higher frequency than into SCV1 and about the same as previously reported (Stiffler *et al.* 1974). Transduction of rifampicin resistance (*rpoB*) into SCVs was reduced almost 10-fold. As transduction was significantly decreased into SCVs maybe this process is influenced by reduced ATP levels too. The data suggests that SCV strains will be less efficient in gene exchange by transduction *in vivo*.
Transduction of GFP (*sarA* P2) by Φ80α-ALC1437 was not observed.

The rate of formation of lysogens was examined. The COL wildtype strain of *S. aureus* and SCV3 both yielded a high number of lysogens (~68%) the remaining being resistant mutants. SCV 1 and SCV 2 provided a much lower proportion of lysogens (4-10%). Although these strains were shown to have different intracellular ATP levels, there was no obvious relationship between cellular ATP levels and

lysogen formation. Consequently the frequency of lysogen formation (or that of resistance mutants) cannot be related to energy status.

7.1.3 Physiological variety in small colony variants

S. aureus has developed various strategies to enable its persistence within the host, particularly when under antimicrobial challenge, and one of those strategies is to generate subpopulations of small colony variants (SCVs) that are auxotrophic metabolic mutants (Proctor et al., 2006). Three SCV strains (SCV1, SCV2 and SCV3) were isolated from the MRSA strain, *S. aureus* COL on triclosan (Norville, 2010). These SCVs showed a reduction of intracellular ATP levels, pigment, catalase, coagulase and DNase activities. Their growth rate and maximum cell yield were also reduced. When examined all showed differences between themselves and the wild type.

Such effects on SCVs have been noted previously (Proctor *et al.*, 2006; Seaman *et al.*, 2007). If 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 (von Eiff *et al.*, 2006). So if this is the case it would explain the differences in carbohydrate utilisation observed in these SCVs.

The original hypothesis was that the SCV 1-3 had mutations in dissimilar loci. We assume these differentially effect cell yield and growth patterns. This we think reflects a difference in ATP metabolism and that this coincidently contributes to the physiological differences between the SCV isolates 1-3 (Day *et al.*, 2012).

A comparison of aerobic growth of the wildtype with the SCV isolates showed these had reduced growth. Interestingly anaerobic growth yields were almost the same. Thus all SCVs appear to be respiring as an anaerobic wild type and this explains why they obtain less energy per glucose molecule.

7.1.4 Phenotypic switching

The ability of *S. aureus* to switch to an alternative phenotype in the presence of antimicrobial agents is clearly favourable if it permits survival. SCVs have many characteristics that are associated with survival in unfavourable conditions. Various

reports of phenotypic switching have been reported in the literature, in the presence of antibiotics (Mitchell *et al.*, 2010a).

Formation of the SCV phenotype in *S. aureus* could be viewed as a phenotypic switching system which appears to be strongly influenced by the alternative sigma factor, σ^B (Mitchell *et al.*, 2010a). This was one hypothesis we discussed early in the work, however the sequencing data on the cycling mutants discussed later suggests strongly that this is not involved

7.1.5 Antibiotic susceptibility

To have raised or lowered sensitivities to these inhibitors suggests that the mutations conferring the SCV phenotype co-incidently impacts on their tolerance or sensitivity to these inhibitors. Therefore to explain this change in sensitivity it is important to consider the potential physiological impact of reduced ATP generation capacity. The process of energy generation is closely tied to the proton pump operation. One inhibitor antimycin effects the Qi site of cytochrome and disrupts the the proton gradient across the inner membrane (Dairaku *et al.*, 2004). The results show that the SCV strains are significantly more sensitive to antimycin for example. Thus it seems the relative lack of energy generation impairs their proton relationships and so makes them more sensitive.

7.1.6 The cycling experiment

This was planned to determine whether there was a single site-specific or multiple sites at which the SCV phenotype arose. Thus the genomic sequence of the final revertants (WT15) in the cycling experiment would provide the evidence for either hypothesis. Numerous changes at various sites would confirm the more random process, whilst no evidence of significant genomic change would suggest the site specific mechanism.

Analysis of the sequences illustrate there are numerous changes to COL and N315 genomes when compared to the parental sequenced strains. However some changes are *historic*. That is they are present in the strain prior to the experiment. Thus 25 mutations in COL and 8 mutations in N315 have arisen since the strain we obtained was originally sequenced. These strains have been cultured in our laboratory for over 10 years and must logically have acquired spontaneous mutations in their genomes. Thus the mutations are probably not related to the cycling and selection steps in the

experiment, and are therefore *historic*. If this premise is accepted then those others are *novel*. There are 5 (N315-TRI), 4 (N315-GEN), 10 (COL-TRI) and 21 (COL-GEN) *novel* mutations in the four strains.

The sequencing data thus suggests that the site specific mutational mechanism is not operating. The finding that there are not about 30 mutational changes in any of the final revertants genomes implies in many cases the cycled mutant selected was a true revertant. Thus not many suppressed mutations were found.

Methicillin resistance was confirmed for both N315 and COL by sequence genome of the *mecA* gene. Just one mutational site was found to be common between COL and N315. The mutation is in the *fab*I gene. It's in the same gene but not at the same site and only occurred when selection was made on triclosan.

7.1.7 How many SCV genes are there?

The genomes of N315 and COL encode 2593 and 2615 protein genes. Since the genomes are much alike in gene content and sequence it was decided they could be seen as just one strain in this analysis, thus we combined the data. The sequencing shows there are a total of 38 different genes in the two strains which give the SCV phenotype. Significantly only one is in common (*fabI*) and thus it is statistically improbable that there are no more.

We determined 33 of the 2615 genes are concerned with SCV formation. This is 1.3% of the genome, a minimum of 40 Kb, is concerned with the SCV phenotype.

Figure 7.1 shows the distribution of the mutations (genes) around the composite chromosome of COL and N315. Figure 7.2 shows the locations of the *historic* mutations and most are located around 91185-2770880. Why they are focus in this region is entirely unclear? One would expect these mutations to be randomly scattered throughout the chromosome and clearly without such a focus.

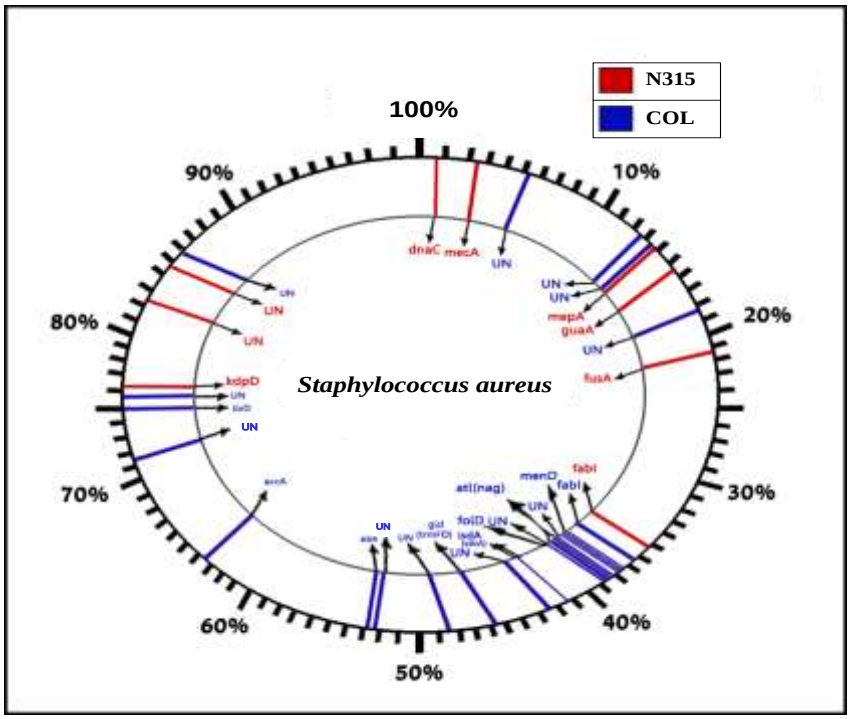


Figure 7.1 A composite of the chromosomes of COL and N315 showing the locations of the mutations conferring the SCV phenotype.

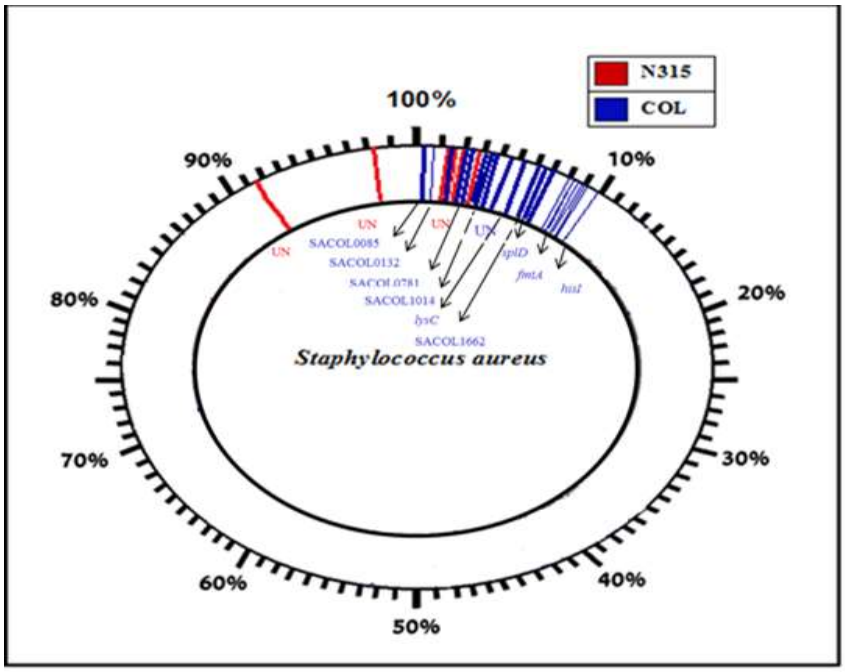


Figure 7.2 Composite chromosomes of COL and N315 showing the locations of the *historic* mutations.

7.2 Conclusions

- SCVs from *S. aureus* COL and N315 share characteristics with other *S. aureus* SCVs including altered growth and biochemical profiles.
- *S. aureus* SCVs acquire and disseminate antimicrobial resistance by transduction with a normal frequency was 1-4 $x10^{-7}$.
- The frequency of phage 80α resistance was significantly different between SCV strains.
- Lysate reproducibility was significantly lower with lysates from SCVs than from SCV lysogens.
- Anaerobic growth was not significant different when *S. aureus* wildtype and SCVs were compared.
- CCCP can select for SCVs in *S. aureus* COL. No SCVs were obtained from antimycin, oligomycin or heavy metal chloride salts.
- The rate of ATP formation is important in SCV phenotype expression.
- There is no evidence for phenotypic switching
- Numerous and diverse mutations occurred in COL and N315 SCVs from the cycling strains (over 100 mutations).
- There were 25 historical mutations in COL and 8 in N315.
- Only one mutational site was found to be common between COL and N315 and this was in the *fab*I gene.

7.3 Future work

- It is clear that the genetic mechanisms for forward to SCV and reversion to wildtype need to be defined by future investigations. The sequencing data shows there is a need to focus on metabolic pathways which are different to those involved with the thymidine biosynthesis. How these deliver the SCV phenotype is a major question.

- The sequencing data demands further analysis of the strains as there are still uncertainties in some of the mutations and how they operate to produce an SCV.

- It is clear that SCVs have electron transport deficiencies. So are these novel and unknown in SCV types that are clinically relevant? There is a requirement to examine clinical strains now and make this determination. Since SCVs are not all the same metabolically there needs to be further study into their biochemistry and genetics.

- What determines the differences in growth rate and physiology of *S. aureus* COL SCVs 1-3 strains has still to be explained fully.

- This approach seems to provide the basis for the development of novel drugs for therapeutic treatment. This is a clinical imperative, since the current drugs are becoming unusable through the development of microbial resistance.

- 4% of the genome (~ 40 Kb) is concerned with the SCV phenotype.

- When I return home I intend to gain funding to further analyse the genomes of these SCV mutants. These organisms have clinical relevance in Saudi Arabia as well as Europe and the ideas developed here may well help their treatment in my country.

References

Abd El-Moez, S. I., Dorgham, S. M. & Abd El-Aziz, E. (2011). Methicillin resistant *Staphylococcus aureus*-Post Surgical in Egyptian hospital. *Life Science journal* **8,** 2.

Abele-Horn, M., Schupfner B., Emmerling, P. Waldner, H. & Goring H. (2000). Persistent wound infection after herniotomy associated with small-colony variants of *Staphylococcus aureus*. *Infection* **28,** 53-54.

Aboshkiwa, M., Rowland, G. & Coleman, G. (1995). Nucleotide sequence of the *Staphylococcus aureus* RNA polymerase *rpoB* gene and comparison of its predicted amino acid sequence with those of other bacteria. *Biochemistry* **1262,** 73-78.

Alderson, P. & Rowland, M. (1995). Making use of Biology. Published by Macmillan press Ltd.

Allegrucci, M. & Sauer, K. (2007). Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *Journal of Bacteriology* **189,** 2030-2038.

al-Masaudi, S. B, Day, M. J. & Russell, A. D. (1991). Antimicrobial resistance and gene transfer in *Staphylococcus aureus*. The *Journal of Applied Bacteriology* **70,** 279-290.

Alonso, A., Sanchez, P. & Martinez, J. L. (2001). Environmental selection of antibiotic resistance genes. *Environmental Microbiology* **3,** 1–9.

Altendorf, K., Siebers, A. & Epstein, W. (1992). The KDP ATPase of *Escherichia coli*. *Annals of the New York Academy of Sciences*. **671,** 228-243.

Amyes, S. G. B., Towner, K. J., Carter, G. I., Thomson, C. J. & Young H-K. (1989). The type VII dihydrofolate reductase: a novel plasmid-encoded trimethoprim-resistant enzyme from Gram-negative bacteria isolated in Britain. *Journal of Antimicrobial Chemotherapy* **24,** 110-119.

Amyes, S. G. B. & Towner, K. J. (1990). Trimethoprim resistance; epidemiology and molecular aspects. *Journal Medical Microbiology* **31,** 1-19.

Andrews, J. M. (2009). BSAC standardized disc susceptibility testing method (version 8). *J Antimicrob Chemother* **64**, 454-489.

Arai, K., Yasuda, S. & Kornberg, A. (1981). Mechanism of *dnaB* protein action. *Journal of Biology Chemistry* **256,** 5247-5252.

Aubry-Damon, H., Soussy, C. J. & Courvalin, P. (1998). Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **42,** 2590-2594.

Anderson, K. L. & Purdom, G. (2009). A creationist perspective of beneficial mutations in bacteria. PhD.

Archer, C. L., Coughter, J. P. & Johnston, J. L. (1986). Plasmid encoded trimethoprim resistance in staphylococci. *Antimicrobial Agents and Chemotherapy* **29,** 735-740.

Archer, G. L. & Armstrong, B. C. (1983). Alteration of staphylococcal flora in cardiac surgery patients receiving antibiotic prophylaxis. *Journal of Infectious Diseases* **147,** 642–649.

Archer, G. L. & Pennell, E. A. (1990). Detection of methicillin resistance in staphylococci by using a DNA probe. *Antimicrobial Agents and Chemotherapy* **34,** 1720-1724.

Arsianti, A., Hanafi, M., Saepudin, E., Morimoto, T. & Kakiuchi, K. (2010). Synthesis and biological activity of 2-hydroxynicotinoyl-serine-butyl esters related to antibiotic UK-3A. *Bioorganic and Medicinal Chemistry Letters*. **20,** 4018-4020.

Avery, O. T., Macloed, C. M. & McCarty, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *Journal of Experimental Medicine* **79,** 137-158.

Ayliffe, G. A. (1997). The progressive intercontinental spread of methicillin-resistant *Staphylococcus aureus*. *Clinical Infection Diseases* **24,** 74-79.

Ayliffe, G. A., Buckles, A., Casewell, M. W., Cookson, B. D., Cox, R. A., Duckworth, G. J., French, G. L., Griffiths-Jones, A., Heathcock, R., Humphreys, H., Keane, C. T., Marples, R. R., Shanson, D. C., Slack, R. & Tebbs. E. (1998). Revised guidelines for the control of methicillin-resistant *Staphylococcus aureus* infection in hospitals. *Journal of Hospital Infection* **39,** 253–290.

Ayliff, G. A. J., Lilly, H. A. & Lowbury, E. J. L. (1979). Decline of the hospital staphylococcus? Incidence of multi-resistant *S. aureus* in three Birmingham hospitals. *Lance* i**,** 538-541.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K. & Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359,** 1819-1827.

Baldock, C., de Boer, G. J., Rafferty, J. B., Stuitje, A. R. & Rice, D. W. (1998). Mechanism of action of diazaborines. *Biochemical Pharmacology* **55,** 1541–1549.

Baldock, C., Rafferty, J. B., Sedelnikova, S. E., Baker, P. J., Stuitje, A. R., Slabas, A. R., Hawkes, T. R. & Rice, D. W. (1996). A mechanism of drug action revealed by structural studies of enoyl reductase. *Science* **274,** 2107–2110.

Balwit, J. M., van Langevelde, P., Vann, J. M. & Proctor, R. A. (1994). Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *Journal Infection Diseases* **170,** 1033-1037.

Bannerman, T. L. & Peacock, S. J. (2007). *Staphylococcus*, *Micrococcus*, and other catalase-positive cocci. In *Manual of Clinical Microbiology*; Murray, P.R.; Baron, E.J.; Jorgensen, J.H.; Landry, M.L.;Pfaller, M.A., Eds.; ASM Press: Washington D.C., USA,; pp. 384–404.

Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S. & Proctor, R. A. (2003). *Staphylococcus aureus menD* and *hemB* mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. *Journal of Infectious Diseases* **187,** 1654–1661.

Bauman, R. W. (2004). Characterizing and classifying viruses, viroids, and prions. Microbiology chapter **13,** C.

Barber, M. (1947). Staphylococcal infection due to penicillin-resistance. *British Medical Journal* **ii,** 863-865.

Barber, M. & Rozwadowska-Dowzenko, M. (1948). Infection by penicillin-resistant staphylococci. *Lancet* **2,** 641-644.

Beck, W. D., Berger-Baichi, B. & Kayser, F. H. (1986). Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of *mec*-specific DNA. *Journal of Bacteriology* **165,** 373-378.

Berger-Bachi, B. & Rohrer, S. (2002). Factors influencing methicillin-resistance in Staphylococci. *Archives of Microbiology* **178,** 165-171.

Bergler, H., Fuchsbichler, S., Hogenauer, G. & Turnowsky, F. (1996). The enoyl-[acyl-carrier-protein] reductase (*FabI*) of *Escherichia coli*, which catalyzes a key regulatory step in fatty acid biosynthesis, accepts NADH and NADPH as cofactors and is inhibited by palmitoyl-CoA. *European Journal of Biochemistry* **242,** 689–694.

Bergoge-Berezin, E. (2000). Resistance and new antibiotic strategies the problem with staphylococcus. *Presse Med* **29,** 2018-2021.

Besier, S., Ludwig, A., Ohlsen, K., Brade, V. & Wichelhaus, T. A. (2007). Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus International journal of Medical Microbiology* **297,** 217-225.

Besier, S., Zander, J., Kahl, B. C., Kraiczy, P., Brade, V. & Wichelhaus, T. A. (2008). The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical *Staphylococcus aureus* isolates. *Antimicrobial Agents Chemotherapy* **52,** 2183-2189.

Betley, M. J. & Mekalanos J. J. (1985). Staphylococcal enterotoxin A is encoded by phage. *Science* **229,** 185–187.

Bhargava, H. N., & P. A. Leonard. (1996). Triclosan: applications and safety. *American Journal of Infection Control* **24**, 209-218.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B. & Projan, S. (2004). Microarray-based analysis of the *Staphylococcus aureus* sigma(B) regulon. *Journal of Bacteriology* **186**, 4085-4099.

Biswas, L., Biswas, R., Schlag, M., Bertram, R. & Gtz, F. (2009). Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. *Applied environmental Microbiology* **75,** 6910-6912.

Bodley, J. W., Zieve, F. J., Lin, L. & Zieve, S. T. (1969). Formation of the ribosome-G factor-GDP complex in the presence of fusidic acid. *Biochemical and Biophysical Research Communications* **37,** 437-443.

Brash, D. E., Rudolph, J. A, Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., Halperin, A. J. & Ponten, J. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. National Academy of Sciences of the USA* **88,** 10124-10128.

Brock, T. D. & Madigan, M. T. (1988). Biology of Microorganisms (5th ed.) *Prentice Hall International*, Inc.

Bronner, S., Monteil, H. & Prevost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *Fems Microbiology Reviews* **28**, 183-200.

Brown, T. A. (1999). Genome. *BIO Scientific Publisher Ltd.* pp. 338-339.

Brussow, H., Canchaya, C. & Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews* **68,** 560–602.

Bryan, L. E. & Kwan, S. (1981). Mechanisms of aminoglycoside resistance of anaerobic bacteria and facultative bacteria grown anaerobically. *Journal of Antimicrobial Chemotherapy* **8,** 1-8.

Bulger, R. J. (1967). In-vitro activity of cephalothin/kanamycin and methicillin/kanamycin combinations against methicillin-resistant *Staphylococcus aureus.* *Lancet* **1,** 17-19.

Bulger, R., J. & Bulger, R., E. (1967). Ultrastructure of small colony variants of a methicillin-resistant *Staphylococcus aureus*. *Journal of Bacteriology* **94,** 1244-1246.

Burnie, J. P., Matthews, R. C., Carter, T., Beaulieu, E., Donohoe, M., Chapman, C., Williamson P. & Hodgetts S. J. (2000). Identification of an immunodominant ABC transporter in methicillin-resistant *Staphylococcus aureus* infections. *Infection and immunity* **68,** 3200–3209.

Burrus, V. & Waldor, M. (2004). Shaping bacterial genomes with integrative and conjugative elements. *Research in Microbiology* **155,** 376–86.

Cafferkey, M. T., Hone, R & Keane, C. T. (1982). Severe staphylococcal infections treated with vancomycin. *Journal of Antimicrobial Chemistry* **9,** 69-74.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A. & Brussow, H. (2003). Prophage Genomics. *Microbiology and Molecular Biology Reviews* **67,** 238–276.

Casman, E.P. & Bennett, R.W. (1963). Culture medium for the production of

Staphylococcal enterotoxin A. *Journal of Bacteriology* **86,** 18-23.

Chambers, H. F. (1988). Methicillin-resistant staphylococci. *Clinical Microbiology Reviews* **1,** 173-186.

Cheeseman, K. E., Williams, G. J., Maillard, J. Y., Denyer, S. P. & Mahenthiralingam, E. (2007). Typing of *Staphylococcus aureus* clinical isolates using random amplification of polymorphic DNA method and comparison with antibiotic susceptibility typing. *Journal of Hospital Infection* **67,** 388-390.

Chen, C. C., Tsuchiya, T., Yamane, Y., Wood, J. M. & Wilson, T. H. (1985). $Na^+(Li^+)$-proline cotransport in *Escherichia coli*. *The Journal of Membrane Biology* **84,** 157–164.

Christon, J. H., Guy, R. K., Michael, J. M., Linda, D. S., Michael, V. W. (1997). Manual of environmental microbiology, ASM, Washington, D.C., USA 349–357.

Clarke, S. R., Wiltshire, M. D. & Foster. S. J. (2004). *IsdA* of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Molecular Microbiology* **51,** 1509-1519.

Clarke, S. R., Mohamed, R., Bian, L. Routh, A. F., Kokai-Kun, J. F., Mond, J. J. Tarkowski, A. & Foster. S. J. (2007). The *Staphylococcus aureus* surface protein *IsdA* mediates resistance to innate defences of human skin. *Cell Host and Microbe* **1,** 199-212.

Clauditz, A., Resch, A., Wieland, K. P., Peschel, A. & Gotz, F. (2006). Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infection and Immunity* **74,** 4950-4953.

Clements, M. O., & Foster S. J. (1999). Stress resistance in *Staphylococcus aureus*. *Trends in Microbiology* **7,** 458–462.

Clements, M. O., Watson, S. P., Poole, R. K. & Foster, S. J. (1999). CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis. *Journal of Bacteriology* **181,** 501–507.

CLSI (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M07-A7. Approved standard, 7th ed. . *Clinical and Laboratory Standards Institute*.

Cheung, A. L., Chien, Y. T. & Bayer, A. S. (1999). Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated *SarA* expression in *Staphylococcus aureus*. *Infection and immunity* **67,** 1331–1337.

Cheung, A. L. & Manna, A. C. (2005). Role of the distal *sarA* promoters in *SarA* expression in *Staphylococcus aureus*. *Infection and immunity* **73,** 4391–4394.

Cheung, A. L., Nast, C. C. & Bayer, A. S. (1998). Selective activation of *sar* promoters with the use of green fluorescent protein transcriptional fusions as the detection system in the rabbit endocarditis model. *Infection and immunity* **66,** 5988–5993.

Christie, G. E., Matthews, A. M., King, D. G., Lane, K. D., Olivarez, N. P., Tallent, S. M., Gill, S. R. & Novick, R. P. (2010). The complete genomes of *Staphylococcus aureus* bacteriophages 80 and 80α– implications for the specificity of *SaPI* mobilization. *Virology* **407,** 381–390.

CLSI (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M07-A7. Approved standard, 7th ed. *Clinical and Laboratory Standards Institute*.

Coates, P. J., Lorimore, S. A. & Wright, E. G. (2004). Damaging and protective cell signalling in the untargeted effects of ionizing radiation. *Mutation Research* **568,** 5–20.

Coia, J. E., Duckworth, G. J., Edwards, D. I., Farrington, M., Fry, C., Humphreys, H., Mallaghan, C. & Tucker, D. R (2006). Guidelines for the control and prevention of methicillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities. *Journal of Hospital Infection* **63,** 1–44.

Collen, D. (1998). Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nature Medicine* **4,** 279-284.

Cookson, B. D. (1990). Mupirocin resistance staphylococci. *Journal of Antimicrobial Chemotherapy* **25,** 497-503.

Cookson, B. D., Schmitz, F-J. & Fluit, A. C. (2003). Introduction to MRSA. *MRSA:Current Perspectives* (Schmitz F-J and Fluit AC, eds) Caister Academic Press, Wymondham.

Coulter, S. N., Schwan, W. R., Ng, E.Y., Langhorne, M. H., Ritchie, H. D., Westbrock-Wadman, S., Hufnagle, W. O., Folger, K. R., Bayer, A. S. & Stover, C. K. (1998). *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Molecular Microbiology* **30,** 393–404.

Cui, L., Neoh, H. M., Iwamoto, A. & Hiramatsu, K. (2012). Coordinated phenotype switching with large-scale chromosome *flip-flop* inversion observed in bacteria. *Proc Natl Acad Sci U S A* **109,** 1647-1656.

Dairaku, N., Kato, K., Honda, K., Koike, T., Iijima, K., Imatani, A., Sekine, H., Ohara, S., Matsui, H. & Shimosegawa, T. (2004). Oligomycin and antimycin A prevent nitric oxide-induced apoptosis by blocking cytochrome C leakage. The *Journal of Laboratory and Clinical Medicine* **143,** 143-151.

Darouiche, R. O. & Hamill, R. J. (1994). Antibiotic penetration of and bactericidal activity within endothelial cells *Antimicrobial Agents and Chemotherapy* **38,** 1059–1064.

Davies, J. & Wright, G. (1997). Bacterial Resistance to Aminoglycoside Antibiotics. *Trends in Microbiology* **5,** 234-239.

Davison, J. (1999). Genetic exchange between bacteria in the environment**.** *Plasmid* **42,** 73–91

Day, M. J., Norville, P. & Plummer, S. (2012). Physiological diversity in small colony variants of *Staphylococcus aureus*. *European Journal of Clinical Microbiology & Infectious Diseases*. In preparation.

Deghorain, M., Bobay, L. M., Smeesters, P. R., Bousbata, S., Vermeersch, M., Perez-Morga, D., Dreze, P. A., Rocha, E. P., Touchon, M. & Van Melderen, L. (2012). Characterization of novel phages isolated in coagulase-negative Staphylococci reveals evolutionary relationships with *S. aureus* phages. *Journal of Bacteriology* **194,** 5829–5839.

de Lencastre, H., de Jonge, B. L., Matthews, P. R. & Tomasz, A. (1994). Molecular aspects of methicillin resistance in *Staphylococcus aureus*. *Journal Antimicrobial and Chemotherapy* **33,** 7-24.

DeLeo, F. R., Kennedy, A. D., Chen, L., Wardenburg, J. B., Kobayashi, S. D., Mathema, B., Braughton, K. R., Whitney, A. R., Villaruz, A. E., Martens, C. A., Porcella, S. F., McGavin, M. J., Otto, M., Musser, J. M. & Kreiswirth, B. N. (2011). Molecular differentiation of historic phage-type 80/81 and contemporary epidemic *Staphylococcus aureus.Proc Natl Acad Sci USA* **108,** 18091–18096.

Denamur, E. & Matic, I. (2006). Evolution of mutation rates in bacteria. *Molecular Microbiology* **60,** 80–87.

Deora, R. & Misra, T. K. (1996). Characterization of the primary sigma factor of *Staphylococcus aureus*. *Journal of Biological Chemistry* **271**, 21828-21834.

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F., Sensabaugh, G. F. & Perdreau-Remington, F. (2006). Complete genome sequence of USA300, an epidemic clone of acquired methicillin-resistant *Staphylococcus aureus*. *Lance*t **367,** 731-739.

Dobrindt, U., Hochhut, B., Hentschel, U., Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews in Microbiology* **2,** 414-424.

Dowd, D., Cafferkey, M. & Dougan, G. (1983). Gentamicin and methicillin resistant *Staphylococcus aureus* in Dublin hospitals: molecular studies. *Journal of Medical Microbiology* **16,** 129–138.

Drlica, K., Zhao & X. L. (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiology and Molecular Biology Reviews* **61,** 377–394.

Duckworth, G. (1998). Report of a combined working party of the British Society for Antimicrobial Chemotherapy, Hospital Infection Society and Infection Control Nurses Association. Revised guidelines for the control of methicillin-resistant *Staphylococcus aureus* infection in hospitals. *Journal of Hospital Infection* **39,** 253–90.

Dufour, P., Jarraud, S., Vandenesch, F., Greenland, T., Novick, R. P., Bes, M., Etienne, J. & Lina, G.(2002). High genetic variability of the *agr* locus in *Staphylococcus* species. *Journal of Bacteriology* **184,** 1180–1186.

Dunman, P. M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, Wu, S., Brown, E. L., Zagursky, R. J., Shlaes, D. & Projan, S. J. (2001). Transcriptional profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *Journal of Bacteriology* **183,** 7341-7353.

Dyke, K. G., Parker, M. T., & Richmond, M. H. (1970). Penicillinase production and metal-ion resistance in *Staphylococcus aureus* cultures isolated from hospital patients. *Journal of Medical Microbiology* **3,** 125-136**.**

Dyke, K. G., Jevons, M. P. & Parker, M. T. (1966). Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* **1,** 835–838.

Ellis, N. A., Ciocci, S. & German, J. (2001). Back mutation can produce phenotype reversion in bloom syndrome somatic cells. *Human Genetics* **108,** 167–73.

El-Sohl, N., Allignet, J., Bismuth, R., Buret, B. & Fouace, J. M. (1986). Conjugative transfer Conjugative transfer of staphylococcal antibiotic resistance markers in the absence of detectable plasmid DNA. *Antimicrobial Agents and Chemotherapy* **30,** 161–169.

Emslie, K. R., Townsend, D. E. & Grubb, W. B. (1985). Two distinct resistance determinants to nucleic acid-binding compounds in *Staphylococcus aureus*. *FEMS Microbiology Letters* **27,** 61-64.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci U S A* **99**, 7687-7692.

Enright, M. C. & Spratt, B. G. (1999). Multilocus sequence typing. *Trends in Microbiology* **7,** 482-487.

Epstein, C. B., Waddle, J. A., Hale, W., Dave, V., Thornton, J. & Macatee, T. L. (2001). Genome-wide responses to mitochondrial dysfunction. *Molecular Biology of the Cell* **12,** 297-308.

Eva, Y., W. Ng, M., Trucksis, T. & Hooper D. C. (1994). Quinolone Resistance Mediated by *norA*: Physiologic characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. *Antimicrobial Agents and Chemotherapy* **38**, 1345-1355.

Fan, F., Yan, K., Wallis, N. G., Reed, S., Moore, T. D, Rittenhouse, S. F., DeWolf, WE, Jr, Huang, J., McDevitt, D., Miller, W. H., Seefeld, M. A., Newlander, K. A., Jakas, D. R., Head, M. S. & Payne, D. J. (2002). Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **46,** 3343 – 3347.

Fan, X., Liu, Y., Smith, D., Konermann, L., Siu, K. W. & Golemi-Kotra, D. (2007). Diversity of penicillin-binding proteins. Resistance factor *FmtA* of *Staphylococcus aureus*. *The Journal of Biological Chemistry* **282,** 35143–35152.

Fasching, C. E., Tenover, F. C., Slama, T. G., Fisher, L. M., Sreedharan, S., Oram, M., Willard, K., Sinn, L. M., Gerding, D. N. & Peterson, L. R. (1991). *gyrA* mutations in ciprofloxacin-resistant, methicillin-resistant *Staphylococcus aureus* from Indiana, Minnesota, and Tennessee. *Journal Infection Diseases* **164,** 976–979.

Ferrero, L., Cameron, B. & Crouzet, J. (1995). Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. *Antimicrobial Agents Chemotherapy* **39,** 1554–1558.

Ferrero, L., Cameron, B., Manse, B., Lagneaux, D., Crouzet, J. F., amechon, A. & Blanche, F. (1994). Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. *Molecular Microbiology* **13,** 641–653.

Finland, M., Frank, P. F. & Wolcox, C. (1950). In-vitro susceptibility of pathogenic staphylococci to seven antibiotics. *American journal of clinical pathology* **20,** 325-334.

Forbes, B. A. & Schaberg, D. R. (1983). Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: Evidence for conjugative exchange of resistance. *Journal of Bacteriology* **153,** 627-634.

Foster, S. J. (1995). Molecular characterization and functional analysis of the major autolysin of *Staphylococcus aureus* 8325/4. *Journal of Bacteriology* **177,** 5723–5725.

Fraise, A. P. (1998). Topical treatment for eradication of MRSA. *CME Bulletin Medical Microbial* **2,** 1-10.

Freifelder, D. (1983). Molecular Biology, A Comprehensive Introduction to Prokaryotes and Eukaryotes. *Science Books International*, Van Nostrand Reinhold Company, U.S.A. pp. 135–154.

Freifelder, D. (1987). Microbial Genetics. Jones and Bartlett Publishers, Boston. pp. 247–280.

Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. (2005). Mobile genetic elements: The agents of open source evolution. *Nature Reviews Microbiology* **3,** 722-732.

Fry, J. C. & Day, M. J. (1992). Release of genetically engineered and other microorganisms. *Cambridge University Press* 54–81.

Furuya, E. Y. & Lowy, F. D. (2006). Antimicrobial-resistant bacteria in the community setting. *Nature Reviews Microbiology* **4,** 36-45.

Gadd, G. M. (1988). Biotechnology-a comprehensive treatise Weinheim; VCH erlagsgesellschaft, 401-433.

Gaetke, L.M., & Chow, C.K. (2003). Copper toxicity, oxidative stress and antioxidant nutrients. *Toxicology* **189,** 147–163.

Ghuysen, J. M. (1991). Serine β-lactamases and penicillin-binding proteins. *Annual Review of Microbiology* **45,** 37–67.

Gilbert, D. (2000). Aminoglycosides. In: Mandell, G. L., Bennett, J. E., Dolin, R., eds. *Principles and Practice of Infectious Diseases*. 5th ed. Philadelphia: Churchill Livingstone 307-336.

Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., Paulsen, I. T., Kolonay, J. F., Brinkac, L., Beanan, M., Dodson, R. J., Daugherty, S. C, Madupu, R., Angiuoli, S. V., Durkin, A. S., Haft, D. H., Vamathevan, J., Khouri, H., Utterback, T., Lee, C., Dimitrov, G., Jiang, L., Qin, H., Weidman, J., Tran, K., Kang, K., Hance, I. R., Nelson, K. E. & Fraser, C. M. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of Bacteriology* **187**, 2426-2438.

Gillespie, M. T. & Skurray, R. A. (1986). Plasmids in multiresistant *Staphylococcus aureus*. *Microbiological Sciences* **3,** 53–58.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J.C., Lina, G., Bes, M., Vandenesch, F., Piémont, Y., Brousse, N., Floret, D., & Etienne, J. (2002). Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* **359,** 753-759.

Gilligan, P. H., Gage, P. A., Welch, D. F., Muszynski, M. J. & Wait. K. R. (**1987).** Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. *Journal of Clinical Microbiology* **25,** 1258-1261.

Godtfredson, W., Roholt, T. K. & Tybring, G, L. (1962). Fusidin: a new orally active antibiotic. *Lancet* **i,** 928.

Goswitz, J. J., Willard, K. E., Fasching, C. E. & Peterson, L. R. (1992). Detection of *gyrA* gene mutations associated with ciprofloxacin resistance in methicillin-resistant *Staphylococcus aureus*: analysis by polymerase chain reaction and automated direct DNA sequencing. *Antimicrobial Agents and Chemotherapy* **36,** 1166–1169.

Greenwood, D., Slack, R. C. B. & Peutherer, J. F. (1995). Medical microbiology. 15th ed. ELST-Churchill Livingstone. 168-174.

Griffiths, A. J. F., Miller, J. H., Suzuki, D. T., Lewontin, R. C. & Gelbart, W. M. (2000). An introduction to genetic analysis. (7th Ed.). New York: W. H. Freeman.

Grohmann, E., Muth, G. & Espinosa, M. (2003). Conjugative plasmid transfer in Gram-positive bacteria. *Microbiol Molecular Biology Reviews* **67,** 277–301.

Guinane, C. M., Ben Zakour, N. L., Tormo-Mas, M. A., Weinert, L. A., Lowder, B. V., Cartwright, R. A., Smyth, D. S., Smyth, C. J., Lindsay, J. A., Gould, K. A., Witney, A., Hinds, J., Bollback, J. P., Rambaut, A., Penades, J. R. & Fitzgerald, J. R. (2010). Evolutionary genomics of *Staphylococcus aureus* reveals insights into the origin and molecular basis of ruminant host adaptation. *Genome Biology and Evolution* **2,** 454-466.

Gutierrez, D., Martinez, B., Rodriguez, A. & Garcia, P. (2012). Genomic characterization of two *Staphylococcus epidermidis* bacteriophages with anti-biofilm potential. *BioMedical Central Genomics* **13,** 228.

Hall, B. M. (1970). Mercury resistance of *Staphylococcus aureus*. *Journal of Hygiene*. **68,** 121-129.

Hamdane, D., Argentini, M., Cornu, D., Myllykallio, H., Skouloubris, S., Hui-Bon-Hoa, G. & Golinelli-Pimpaneau, B. (2011). Insights into folate/FAD-dependent tRNA methyltransferase mechanism: Role of two highly conserved cysteines in catalysis. *The Journal of Biological Chemistry* **286,** 36268–36280.

Handwerger, S. & Tomasz, A. (1985). Antibiotic tolerance among clinical isolates of bacteria. *Review Infection Diseases*. **7,** 368-386.

Hartman, B. & Tomasz, A. (1981). Altered penicillin-binding proteins in methicillin-resistant strains of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **19,** 726–735.

Hartman, B. & Tomasz, A. (1984). Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *Journal of Bacteriology* **158,** 513-516.

Hastings, P., Rosenberg, S. & Slack, A. (2004). Antibiotic-induced lateral transfer of antibiotic resistance. *Trends in Microbiology* **12,** 401–404.

Hayes, W. (1968). The Genetics of Bacteria and their Viruses (2nd ed.) *Blackwell Scientific Publications*, Oxford.

Heath, R. J., Li, J., Roland, G. E. & Rock, C.O. (2000). Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. *The Journal of Biological Chemistry* **18,** 4654–4659,

Heath, R. J. & Rock, C. O. (1995). Enoyl-acyl carrier protein reductase (*fabI*) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*. *The Journal of Biological Chemistry* **270,** 26538–26542.

Heath, R. J. & Rock, C. O. (2000). A triclosan-resistant bacterial enzyme. *Nature* **406,** 145–146.

Heath, R. J., White, S.W. & Rock, C.O. (2001). Lipid biosynthesis as a target for antibacterial agents. *Progress in Lipid Research* **40,** 467–497.

Heath, R. J, Yu, Y. T., Shapiro, M. A., Olson, E. & Rock, C. O. (1998). Broad spectrum antimicrobial biocides target the FabI component of fatty acid **synthesis.** *Journal of Biology and Chemistry* **273,** 30316-20.

Hecker, M., Pane-Farre, J. & Volker, U. (2007). SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. In *Annual Review of Microbiology*, pp. 215-236. Palo Alto: Annual Reviews.

Hendrix, R. W. (2002). Bacteriophages: Evolution of the Majority**.** *Theoretical Population Biology* **61**, 471-480.

Henkin, T. M. (1996). The role of CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. *FEMS Microbiology Letter* **135,** 9-15.

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y. & Kobayashi, I. (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* **350,** 1670–1673.

Hiramatsu, T., Kodama, K., Kuroda, T., Mizushima, T., & Tsuchiya, T. (1998). A putative multisubunit Na^+/H^+ antiporter from *Staphylococcus aureus*. *Journal of Bacteriology* **180,** 6642–6648.

Hirsch, H. M. (1961). Small colony variants of *Escherichia coli:* Mode of Action of copper in variant recovery and population dynamics of cultures containing variants. *Journal of Bacteriology* **81,** 448-458.

Hogberg, L. D., Heddini, A. & Cars, O. (2010). The global need for effective antibiotics: challenges and recent advances. *Trends in Pharmacological Sciences* **31,** 509-515.

Holden, M. T. G., Feil, E. J., Lindsay, J. A. & other authors (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* **101,** 9786-9791.

Holden, M. T. G. & Lindsay, J. A. (2008). Whole genomes: Sequence, microarray and systems biology. In *Staphylococcus molecular gentics* pp. 1-29. Edited by J. A. Lindsay. Norfolk, UK: Caister Academic Press.

Horinouchi, S. & Weinblum, B. (1982). Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible chloramphenicol resistance. *Journal of Bacteriology***150,** 815-825.

Horst, J. P., Wu, T. H., & Marinus, G. M. (1999). *Escherichia coli* mutator genes. *Trends in Microbiology* **7,** 29-36.

Hudson, M. C., Ramp, W. K., Nicholson, N. C., Williams, A. S., Nousiainen, M. T. (1995). Internalization of *Staphylococcus aureus* by cultured osteoblasts. *Microbial Pathogenesis***19,** 409–419.

Inaba, K., Kuroda, T., Shimamoto, T., Kayahara, T., Tsuda, M. & Tsuchiya, T. (1994). Lithium toxicity and $Na^+(Li^+)/H^+$ antiporter in *Escherichia coli*. *Biological and Pharmaceutical Bulletin* **17,** 395–398.

Ito, H., Yoshida, H., Bogaki-Shonai, M., Niga, T., Hattori, H. & Nakamura, S. (1994). Quinolone resistance mutations in the DNA gyrase *gyrA* and *gyrB* genes of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **38,** 2014–2023.

Ito, T., & Hiramatsu, K. (1998). Acquisition of methicillin resistance and progression of multiantibiotic resistance in methicillin-resistant *Staphylococcus aureus*. *Yonsei Medical Journal*. **39,** 526-533.

Ito, T., Katayama, Y. & Hiramatsu, K. (1999). Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. *Antimicrobial Agents and Chemotherapy* **43,** 1449–1458.

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C. & Hiramatsu, K. (2001). Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **45,** 1323-1336.

Ito, T., Okuma, K., Ma, X. X., Yuzawa, H. & Hiramatsu, K. (2003). Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. *Drug Resist Update* **6,** 41-52.

Jackowski, S., Alix, J-H. (1990). Cloning, sequence, and expression of the pantothenate permease (*panF*) gene of *Escherichia coli*. *Journal of Bacteriology* **172,** 3842–3848.

Jaffe, H. W., Sweeney, H. M., Weinstein, R. A., Kabins, S. A., Nathan, C. & Cohen S. (1982). Structural and phenotypic varieties of gentamicin resistance plasmids in hospital strains of *Staphylococcus aureus* and coagulase-negative staphylococci. *Antimicrobial Agents and Chemotherapy*. **21,** 773-779.

Jarolmen, **H., Bondi, A. & Crowell, R. L. (1965).** Transduction of *Staphylococcus aureus* to tetracycline resistance *in vivo*. *Journal of Bacteriology* **89,** 1286–1290.

Jawetz, E., Melnick, J. L. & Adelberg, E. A. (1987). Review of medical microbiology. Lange Medical publication, Losaltos, California. pp 48-53.

Jensen, S. O. & Lyon, B. R. (2009). Genetics of antimicrobial resistance in *Staphylococcus aureus*. *Future Microbiology* **4,** 565-582.

Jevons, M. P. (1961). "Celbenin" resistant staphylococci. *British Medical Journal* **1,** 124-125.

Jonsson, I. M., von Eiff, C., Proctor. R. A., Peters, G., Ryden, C. & Tarkowski, A. (2003). Virulence of a *hemB* mutant displaying the phenotype of *a Staphylococcus aureus* small colony variant in a murine model of septic arthritis. *Microbial Pathogenesis* **34,** 73-79.

Jordan, A. & Reichard, P. (1998). Ribonucleotide reductases. *Annual Review of Biochemistry* **67,** 71–98.

Kaatz, G. W., Seo, S. M. & Ruble, C. A. (1993). Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus. Antimicrobial Agents Chemotherapy* **37,** 1086–1094.

Kaatz, G. W., McAleese, F. & Seo, S. M. (2005). Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. *Antimicrobial Agents and Chemotherapy* **49,** 1857–1864.

Kagawa, Y. & Racker, E. (1966). Oligomycin. The *Journal of Biological Chemistry* **241,** 2461-2466.

Kahl, B. C., Belling, G., Becker, P., Chatterjee, I., Wardecki, K., Hilgert, K., Cheung, A. L., Peters, G., & Herrmann, M. (**2005).** Thymidine-dependent *Staphylococcus aureus* small colony variants are associated with extensive changes in regulator and virulence gene expression profiles. *Infection and Immunity* **73,** 4119-4126.

Kahl, B. C., Herrmann, M., Everding, A. S., Koch, H. G., Becker, K., Proctor, R. A. & Peters, G. (1998). Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *Journal of Infection Diseases **177,*** 1023-1029.

Kahl, B. C., **Duebbers, A**., **Lubritz, G**., **Haeberle, J**., **Koch, H.G**., **Ritzerfeld, B**., **Reilly, M**., **Harms, E**., **Proctor, R.A**., **Herrmann, M**., **& Peters, G. (2003).** Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. *Journal of Clinical Microbiology* **41,** 4424-4427.

Kahl, B., von Eiff, C., Herrmann, M., Peters, G. & Proctor, R. A. (1998). Staphylococcal small colony variants present a challenge to clinicians and clinical microbiologists. *Antibiotics and Infectious Diseases Newsletter* **15,** 59–63.

Kaito, C., Kurokawa, K., Hossain, M. S., Akimitsu, N. & Sekimizu, K. (2002). Isolation and characterization of temperature-sensitive mutants of the *Staphylococcus aureus dnaC* gene. *FEMS Microbiology Letter* **210,** 157-64.

Kayser, F. H., Wust, J. & Corrodi, P. (1972). Transduction and elimination of resistance determinants in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial and Agents Chemotherapy* **2,** 217-223.

Khanna, P. (2008). Cell and Molecular Biology, *I.K. International Publishing House* pp. 339-340.

Khanna, P. (2009). Essentials of genetics, *I.K. International Publishing House* pp. 248-253.

King, K., Brady, I., Thomson, M. & Harkness, J. L. (1982). Antibiotic-resistant staphylococci in a teaching hospital. *The Medical Journal of Australia* **2,** 461–465.

Kipp, F., Becker, K., Peters, G. & von Eiff, C. (2004). Evaluation of different methods to detect methicillin resistance in small-colony variants of *Staphylococcus aureus. Journal of Clinical Microbiology* **42,** 1277–1279.

Kirby, W. M. M., Perry, P.M. & Lane, J. L. (1959). Present status of vancomycin therapy of staphylococcal and streptococcal infections. *Antibiotics Annual* **6,** 580-586.

Kleckner, N., J. Roth, & D. Botstein. (1977). Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics. *Journal of Molecular Biology* **116,** 125-159.

Kloos, W. E. & Schleifer, K. H. (1986). Family I. Micrococcaceae. Genus IV. *Staphylococcus*. *Bergey's manual of systematic bacteriology.* vol 2. (Sneath PHA, Mair NS, Sharpe ME & Holt JG, eds). pp. 1013-1035. Williams & Wilkins, Baltimore.

Klostermeier, D., Sears, P., Wong, C. H., Millar, D. P. & Williamson, J. R. (2004). A three-fluorophore FRET assay for high-throughput screening of small-molecule inhibitors of ribosome assembly. *Nucleic Acids Research* **32**, 2707-2715.

Kock, K. M. L. (1960). In vitro bacterial resistance to chloramphenicol: a six-year study. *Antibiotics and Chemotherapy* **10,** 36-46.

Koch, L. A. (2003). Bacterial Wall as Target for Attack: Past, Present, and Future Research. *Clinical Microbiology Reviews***16,** 673-687.

Kohler, C., von Eiff, C., Peters, G., Proctor, R. A., Hecker, M. & Engelmann, S. (2003). Physiological characterization of a heme-deficient mutant of *Staphylococcus aureus* by a proteomic approach. *Journal of Bacteriology* **185,** 6928-6937.

Kojima, T., Inoue, M. & Mitsuhashi, S. (1990). In vitro activity of AT-4140 against quinolone- and methicillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy* **34,** 1123–1127.

Kokjohn, T. A. & Miller, R. V. (1988). Characterization of the *Pseudomonas aeruginosa recA* gene: the Les-phenotype. *Journal of Bacteriology* **170,** 578-582.

Komatsuzawa, H., Ohta, K., Labischinski H., Sugai, M. & Suginaka, H. (1999). Characterization of *fmtA*, a gene that modulates the expression of methicillin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **43,** 2121-2125.

Komatsuzawa, H., Sugai, M., Nakashima, S., Yamada, S., Matsumoto, A., Oshida, T. & Suginaka, H. (1997). Subcellular localization of the major autolysin, ATL and its processed proteins in *Staphylococcus aureus*. *Microbiology and Immunology* **41,** 469-479.

Komatsuzawa, H., Sugai, M., Ohta, K., Fujiwara, T., Nakashima, S., Suzuki, J., Lee, C.Y. & Suginaka, H. (1997). Cloning and characterization of the *fmt* gene which affects the methicillin resistance level and autolysis in the presence of triton X-100 in methicillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy* **41,** 2355-2361**.**

Konstantinov, Y. M., Subota, I. V. & Arziev, A. S. (1995). Protein synthesis in mitochondria under different redox conditions. *Preprint of the Irkutsk Institute of Plant Physiology and Biochemistry*. **36,** 319-326.

Kovacevic, S., Veal, L. E., Hsiung, H. M. & Miller, J. R. (1985). Secretion of staphylococcal nuclease by *Bacillus subtilis*. *Journal of Bacteriology* **162,** 521–528.

Kozmin, S., Slezak, G., Reynaud-Angelin, A., Elie, C., de Rycke, Y., Boiteux, S. & Sage, E. (2005). UVA radiation is highly mutagenic in cells that are unable to repair 7,8-dihydro-8-oxoguanine in *Saccharomyces* cerevisiae. *Proc. National Academy of Sciences of the USA* **102,** 13538–13543.

Krimmer, V., Merkert, H., von Eiff, C., Frosch, M., Eulert, J., Löhr, J. F., Hacker, J. & Ziebuhr, W. (1999). Detection of *Staphylococcus aureus* and *Staphylococcus epidermidis* in Clinical Samples by 16S rRNA-Directed In Situ Hybridization. *Journal of Clinical Microbiology* **37,** 2667–2673.

Krulwich, T. A. (1983). Na^+/H^+ antiporters. *Biochimica et Biophysica Acta* **726,** 245–264.

Kullik, I., Giachino, P. & Fuchs, T. (1998). Deletion of the alternative sigma factor sigma(B) in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. *Journal of Bacteriology* **180**, 4814-4820.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. & Hiramatsu, K. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357,** 1225-1240.

Lacey, R. W. (1975). Antibiotic resistance plasmids of *Staphylococcus aureus* and their clinical importance. *Bacteriological Reviews* **39,** 1-32.

Lacey, R. W. (1980). Evidence for two mechanisms of plasmid transfer in mixed cultures of *Staphylococcus aureus*. *Journal of General Microbiology* **119,** 423-435.

Lacey, R. W. (1984). Antibiotic resistance in *Staphylococcus aureus* and streptococci. *British Medical Bulletin* **40,** 77-83.

Lacey, R. W. & Kruczenyk, S. C. (1986). Epidemiology of antibiotic resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **18,** 207-214.

Lacey, R. W. & Lord, V. L. (1980). Transfer of gentamicin resistance between cultures of *Staphylococcus aureus* in nutrient broth, serum and urine. *Journal of Medical Microbiology* **13,** 411-421.

Lachica, R. V., Genigeorgis, C. & Hoeprich, P. D. (1971). Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. *Applied Microbiology* **l21,** 585–587.

Lachica, R. V., Hoeprich, P. D. & Riemann, H. P. (1972). Tolerance of staphylococcal thermonuclease to stress. *Applied Microbiology* **23,** 994–997.

Laimins, L. A., Rhoads, D. B. & Epstein, W. (1981). Osmotic control of *kdp* operon expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. U SA* **78,** 464-468.

Larsen, E., Reite, K., Neese, G., Gran, C., Seeberg, E., & Klungland, A. (2006). Repair and mutagenesis at oxidized DNA lesions in the developing brain of wild-type and *Ogg1*$^{-/-}$ mice. *Oncogene* **25,** 2425-2432.

Lannergard, J., von Eiff, C., Sander, G., Cordes, T., Seggewiss, J., Peters, G., Proctor, R. A., Becker, K. & Hughesl, D. (2008). Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus.* *Antimicrobial Agents and Chemotherapy* **52,** 4017–4022.

LeClerc, J. E., Li, B., Payne, W. L., & Cebula, T. A. (1996). High mutation frequencies among *Escherichia coli* and *Salmonella pathogens*. *Science* **274,** 1208–1211.

Lederberg, J., Tatum E. L. (1946). Gene recombination in *E. coli*. *Nature* **158,**558.

Lee, J. H. (2003). Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Applied environmental Microbiology* **69,** 6489-6494.

Levine, M. (1957). Mutations in the temperate phage P22 and lysogeny in *Salmonella*. *Virology* **3,** 22-41.

Lewis, L. A., Li, K. B., Gousse, A., Pereira, F., Pacheco, N., Pierre, S., Kodaman, P. & Lawson, S. (1991). Genetic and molecular analysis of spontaneous respiratory deficient (res-) mutants of *Escherichia-coli* K-12. *Microbiology and Immunology* **35,** 289-301.

Lin, E. C. C. & Kuritzkes, D. R. (1987). Pathways for anaerobic electron transport, p. 202-221. In F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*, vol. 1. *American Society for Microbiology*, Washington, D.C.

Lindberg, M., Sjostrom, J. E. & Johansson, T. (1972). Transformation of chromosomal and plasmid characters in *Staphylococcus aureus. Journal of Bacteriology* **109,** 844-846.

Lindsay, J. A. & Holden, M. T. G. (2004). *Staphylococcus aureus*: superbug, super genome? *Trends in Microbiology* **12**, 378-385.

Lindsay, J. A., Moore, C.E., Day, N.P., Peacock, S.J., Witney, A.A., Stabler. R.A., Hussain, S.E., Butcher, P.D. & Hinds, J. (2006). Microarrays reveal each of the ten dominant lineages of *Staphylococcus aureus* have unique combinations of surface-associated and regulatory genes. *Journal of Bacteriology* **188,** 669-676.

Ling, A. & Cordaux, R. (2010). Insertion sequence inversions mediated by ectopic recombination between terminal inverted repeats. *Plos One* **5,** 12.

Livermore, D. M. (2003). Bacterial resistance: origins, epidemiology, and impact. *Clinical Infectious Diseases* **36,** 11-23.

Long, M., Betran, E., Thornton, K. & Wang, W. (2003). The origin of new genes: glimpses from the young and old. *Nature Reviews Genetics* **4,** 865–75.

Lowbury, E. J. L. & Ayliff, G. A. J. (1974). *Drug Resistance in Antimicrobiological Therapy*. Charles Thomas Illinois USA. Pp. vii

Low, K. B. & Porter, R. D. (1978). Modes of gene transfers and recombination in bacteria. *Annual Review of Genetic* **12,** 259-287.

Lu, P. L., Chin, L. C., Peng, C. F., Chiang, Y. H., Chen., T. P, Ma, L. & Siu, L. K. (2005). Risk factors and molecular analysis of community methicillin-resistant *Staphylococcus aureus* carriage. *Journal of Clinical Microbiology* **43,** 132-139.

Lyon, B. R. & Skurray, R. (1987). Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. *Microbiological Reviews* **51,** 88-135.

Lowy, F. D. (1998). Medical progress-*Staphylococcus aureus* infections. *The New England Journal of Medicine* **339,** 520-532.

Lyon, B. R., Iuorio, J. L., May, J. W. & Skurray, R. A. (1983). Analysis of plasmids in nosocomial strains of multiple antibiotic-resistant *Staphylococcus aureus*. *Antimicrobial Agents and chemotherapy* **23,** 817-826.

Lyon, B .R. & Skurray, R. A. (1987). Antimicrobial resistance of *Staphylococcus aureus*: Genetic basis. *Microbiological Reviews* **51,** 88-135.

Madison, B. M. & Baselski, V. S. (1983). Rapid identification of *Staphylococcus aureus* in blood cultures by thermonuclease testing. *Journal of Clinical Microbiology* **18,** 722–724.

Magnet, S. & Blanchard, J. S. (2005). Molecular insights into aminoglycoside action and resistance. *Chemical Reviews* **105,** 477-498.

Mahenthiralingam, E., Campbell, M. E., Foster, J., Lam, J. S. & Speert, D. P. (1996). Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology* **34,** 1129-1135.

Maiden, M. C. J., Bygraves, J. A., Feil, E. & other authors (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95,** 3140-3145.

Malachowa, N. & DeLeo, F. R. (2010). Mobile genetic elements of *Staphylococcus aureus*. *Cellular and Molecular Life Sciences* **67,** 3057–3071.

Manna, A. C., Bayer, M. G. & Cheung, A. L. (1998). Transcriptional analysis of different promoters in the *sar* locus in *Staphylococcus aureus*. *Journal of Bacteriology* **180,** 3828–3836.

Manna, A. C., & Cheung, A. L. (2001). Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. *Infection and immunity* **69,** 885–896.

Marples, R. R. & Reith, S. (1992). Methicillin-resistant *Staphylococcus aureus* in England and Wales. *Communicable Diseases Report. CDR Review* **2,** 25–29.

Matsuhashi, M., Song, M. D., Ishino, F., Wachi, M., Doi, M., Inoue, M., Ubukata, K., Yamashita, N. & Konno, M. (1986). Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *Journal of Bacteriology* **167,** 975–980.

McAleese, F., Petersen, P., Ruzin, A., Dunman, P.M., Murphy, E., Projan, S.J. & Bradford, P.A. (2005). A novel MATE family efflux pump contributes to the reduced susceptibility of laboratory-derived *Staphylococcus aureus* mutants to tigecycline. *Antimicrobial Agents and Chemotherapy* **49,** 1865-1871.

McDonnell, R. W., Sweeney, H. M. & Cohen, S. (1983). Conjugational transfer of gentamicin resistance plasmids intra- and inter-specifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy* **23,** 151-160.

McNamara, P. J., & Proctor, R. A. (2000). *Staphylococcus aureus* small colony variants, electron transport and persistent infections. *International of Journal Antimicrobial Agents* **14,** 117-122.

Mehndiratta, P. L. & Bhalla, P. (2012). Typing of Methicillin resistant *Staphylococcus aureus*: A technical review. *Review Article* **30,** 16-23.

Menzies, B. E. (2003). The role of fibronectin binding proteins in the pathogenesis of *Staphylococcus aureus* infections. *Current Opinion in Infectious Diseases***16,** 225-229.

Metze, D., Lubritz, G., Hockmann, J., Schwarz, T. & Peters, G. (2001). Intracellular persistence of *Staphylococcus aureus* small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. *Clinical Infection Diseases* **32,** 1643-1647.

Ministry of Health. (1959). Staphylococcal infection in hospitals (report of the subcommitte of the control health services council). London, H.M.S.O.

Mitchell, G., Brouillette, E., Seguin, D. L., Asselin, A. E., Jacob, C. L. & Malouin, F. (2010a). A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides. *Microbial Pathogenesis* **48**, 18-27.

Mitchell, G., Lamontagne, C. A., Brouillette, E., Grondin, G., Talbot, B. G., Grandbois, M. & Malouin, F. (2008). *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. *Molecular Microbiology* **70**, 1540-1555.

Mitsuhashi, S., Morimura, M., Kono, K. & Oshima, H. (1963). Elimination of drug resistance of *Staphylococcus aureus* by treatment with acriflavine. *Journal of Bacteriology*. **86,** 162-164.

Mitsuyama, J., Yamada, H., Maehana, J., Fukuda, Y., Kurose, S., Minami, S., Todo, Y., Wantanabe, Y. & Narita, H. (1997). Characteristics of quinolone-induced small colony variants in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **39,** 697-705.

Moellering, R. C. (2011). Discovering new antimicrobial agents. *International Journal of Antimicrobial Agents* **37**, 2-9.

Montelone, B. A. (1998). Mutation, Mutagens, and DNA Repair, PhD. Division of Biology, Kansas State University.

Moreillon, P., Entenza, J. M., Francioli, P., McDevitt, D., Foster, T. J., Francois, P. & Vaudaux, P. (1995). Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infection and Immunity* **63,** 4738–4743.

Morikawa, K., Inose, Y., Okamura, H., Maruyama, A., Hayashi, H., Takeyasu, K. & Ohta, T. (2003). A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes to Cells* **8**, 699-712.

Mulligan, M. E., Murray-Leisure, K. A., Standiford, H. C., John, J. F., Korvick, J. A., Kauffman, C. & Yu, V. L. (1993). Methicillin-resistant *Staphylococcus aureus:* A 32 Consensus Review of the Microbiology, Pathogenesis, and Epidemiology With implications for prevention and management. *The American Journal of Medicine* **94,** 313-328.

Murchan, S., Aucken, H. M., O'Neill, G. L., Ganner, M. & Cookson, B. D. (2004). Emergence, Spread, and Characterization of Phage Variants of Epidemic Methicillin-Resistant *Staphylococcus aureus* 16 in England and Wales. *Journal of Clinical Microbiology* **42,** 5154 -5160.

Musher, D. M., Baughn, R. E., Templeton, G. B. & Minuth, J. N. (1977). Emergence of variant forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. *Journal of Infection Diseases* **136,** 360-369.

Nagy, Z. & Chandler, M., (2004). Regulation of transposition in bacteria. *Research in Microbiology* **155,** 387–398.

Naidoo, J. (1984). Interspecific co-transfer of antibiotics resistance plasmid in staphylococci *in vivo. Journal of Hygiene Cambridge* **93,** 59-66.

Nakamuraa, A., Hosodaa, M., Katoa, T., Yamadaa, Y., Itoha, M., Kanazawab, K. & Noudab, H. (2000). Combined effects of meropenem and aminoglycosides on *Pseudomonas aeruginosa in vitro*. *Journal of Antimicrobial Chemotherapy* **46,** 901-904.

Neft, N. & Farley T. M. (1972). Conditions influencing antimycin production by a *Streptomyces* species grown in chemically defined medium. *Antimicrobial Agents and Chemotherapy* **3,** 274–276.

Noble, W. C. & Naidoo, J. (1978). Evolution of antibiotic resistance in *Staphylococcus aureus:* the role of the skin. *British Journal of Dermatology* **98,** 481-489.

Norstrom, T., Lannergard, J. & Hughes, D. (2007). Genetic and phenotypic identification of fusidic acid-resistant mutants with the small-colony-variant phenotype in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **51,** 4438-4446.

North, E. A. & Christie, R. (1946). Acquired resistance of staphylococci to the action of penicillin. *Medical Journal of Australia* **1,** 176-179.

Norville, P. H. (2011). Small colony variants in *Staphylococcus aureus* and other species: antibiotic selection, antimicrobial susceptibility, and biofilms formation. PhD, Thesis, Cardiff University.

Novick, R. P. (1963). Analysis by transduction of mutations affecting penicillinase synthesis in *Staphylococcus aureus. Journal of General Microbiology* **33,** 121-1 36.

Novick R. P. (1967). Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology* **33,** 155-166.

Novick, R. P. (1989). Staphylococcal plasmids and their replication. *Annual Review of Microbiology* **43,** 537-565.

Novick, R. P. (2003). Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. *Plasmid* **49**, 93-105.

Novick, R. P., Edelman, I. & Lofdahl, S. (1986). Small *Staphylococcus aureus* plasmids are transduced as linear multimers which are formed and resolved by replicative processes. *Journal of Molecular Biology* **92,** 209-220.

Novick, R. P., Murphy, E., Gryczan, T. J., Baron, E. & Edelman, I. (1979). Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps.*Plasmid* **2,** 109-129.

Novick, R. P., & Roth. C. (1968). Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *Journal of Bacteriology* **95,** 1335-1342.

Novick, R. P., Schlievert, P. & Ruzin, A. (2001). Pathogenicity and resistance islands of staphylococci. *Microbes and Infection* **3,** 585-594.

Nozaki, K., Inaba, K., Kuroda, T., Tsuda, M. & Tsuchiya, T. (1996). Cloning and sequencing of the gene for Na^+/H^+ antiporter of *Vibrio parahaemolyticus*. *Biochemical and Biophysical Research Communications* **222,** 774–779.

Office for National Statistics, (2006). Deaths involving MRSA: England and Wales,2000 to 2004. Health Statistics Quarterly. Vol. 29. (Goldblatt P& Chappell R, eds). Pp63-68. Palgrave Macmillan Ltd, Basingstoke, UK.

Ogino, H., Fuji, M., Satou, W., Suzuki, T., Michishita, E., & Ayusawa, D. (2002). Binding of 5-bromouracil-containing S/MAR DNA to the nuclear matrix. *Journal of DNA Research* **9,** 25-29.

Ogston, A. (1882). Micrococcus poisoning. *Journal of Anatomy* **17,** 24-58.

Ojo, K. K., Striplin, M.J., Ulep, C.C., Close, N.S., Zittle, J., Luis, H., Bernardo, M., Leitao, J. & Roberts, M. (2006). *Staphylococcus* Efflux *msr(A)* gene characterized in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* isolates. *Antimicrobial Agents Chemotherapy* **50,** 1089-1091.

Oliveira, D. C., Tomasz, A. & de Lencastre, H. (2002). Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Lancet Infectious Diseases* **2,** 180-189.

O'Neill, A. J., Larsen, A. R., Henriksen, A. S. & Chopra, I. (2004). A fusidic acid-resistant epidemic strain of *Staphylococcus aureus* carries the *fusB* determinant, whereas *fusA* mutations are prevalent in other resistant isolates. *Antimicrobial Agents and Chemotherapy* **48,** 3594-3597.

O'Riordan, K. & Lee, J. C. (2004). *Staphylococcus aureus* capsular polysaccharides. *Clinical Microbiology Reviews* **17**, 218-234**.**

Oshida, T., Sugai, M., Komatsuzawa, Hong, H., Y. M., Suginaka, H. & Tomasz, A. (1995). A *Staphylococcus aureus* autolysin that has an N-acetylmuramoyl-L-alanine amidase domain and an endo-beta-N-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. *Proc Natl Acad Sci U S A* **92,** 285–289.

Otto, M. & Gotz, F (2001). ABC transporters of staphylococci. *Research in Microbiology* **152,** 351-356.

Ovchinnikov, Y. A., Modyanov, N. N., Grinkevich, V. A., Aldanova, N. A., Trubetskaya, O. E., Nazimov, I. V., Hundal, T. & Ernster, L. (1984). Amino acid sequence of the oligomycin sensitivity-conferring protein (OSCP) of beef-heart mitochondria and its homology with the delta-subunit of the F1-ATPase of *Escherichia coli*. *FEBS Letters* **166,** 19-22.

Padan, E. & Schuldiner, S. (**1994).** Molecular physiology of Na+/H+ antiporters, key transporters in circulation of Na^+ and H^+ in cells. *Biochimica et Biophysica Acta* **1185,** 129–151.

Pane-Farre, J., Jonas, B., Forstner, K., Engelmann, S. & Hecker, M. (2006). The sigma (B) regulon in *Staphylococcus aureus* and its regulation. *International Journal of Medical Microbiology* **296**, 237-258.

Pantucek, R., Doskar, J., Ruzickova, V., Kasparek, P., Oracova, E., Kvardova, V. & Rosypal, S. (2004). Identification of bacteriophage types and their carriage in *Staphylococcus aureus*. *Archives of Virology* **149,** 1689-1703.

Park, J. W., Lee, S. Y., Yang, J. Y., Rho, H. W., Park, B. H., Lim, S. N., Kim, J. S. & Kim H. R. (1997). Effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on the dimerization of lipoprotein lipase. *Biochimica et Biophysica Acta* **1344,** 132–138.

Parker, M. T. & Hewett, J. H. (1970). Methicillin resistance in *Staphylococcus aureus.* *Lancet* **18,** 800-804.

Patel, A. H., Foster T. J. & Pattee, P. A. (1989). Physical and genetic mapping of the protein A gene in the chromosome of *Staphylococcus aureus* 8325-4. *Journal of General Microbiology* **135,** 1799-1807.

Payne, D. J., Warren, P. V., Holmes, D. J., Ji, Y. & Lonsdale, J. T. (2001). Bacterial fatty-acid biosynthesis: a genomics-driven target for antibacterial drug discovery. *Drug Discovery Today* **6,** 537-544.

Peacock, S. J. (2005). *Staphylococcus*. In *Microbiology and Microbial infections*, pp. 771-816. Edited by S.P. Borriello, P.R. Murray & G. Funke. London: Hodder Arnold.

Pechere, J. C. (1992). *In vitro* activity of RP 59500, a semisynthetic streptogramin, against staphylococci and streptococci. *Journal of Antimicrobial Chemotherapy* **30**, 15–18.

Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J. & Schlievert, P. (1988). Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *Journal of Bacteriology* **170,** 4365-4372.

Perkins, H. R. (1980). Microbial cell walls and membranes, eds. Rogers, H. J., Perkins, H. R. & Ward, J. B. (Chapman & Hall, London), pp. 437-456.

Perron, K., Caille, O., Rossier, C., van Delden, C., Dumas, J. L, & Kohler, T. (2004). CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa. The Journal of Biological Chemistry* **279**, 8761–8768.

Peschel, A. (2002). How do bacteria resist human antimicrobial peptides? *Trends Microbiology* **10,** 179–186.

Perry, R. D. & Silver, S. (1982). Cadmium and manganese transport in *Staphylococcus aureus* membrane vesicles. *Journal of Bacteriology* **150,** 973-976**.**

Petersen, A., Chadfield, M. S., Christensen, J. P., Christensen, H. & Bisgaard, M. (2008). Characterization of small-colony variants of *Enterococcus faecalis* isolated from chickens with amyloid arthropathy. *Journal of Clinical Microbiology* **46,** 2686-2691.

Peterson, P. K., Verhoef, J., Sabath, L. D. & Quie, P. G. (1977). Effect of protein A on staphylococcal opsonization. *Infection and Immunity* **15,** 760-764.

Plata, K., Rosato, A. E. & Wegrzyn, G. (2009). *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polonica* **56,** 597–612.

Pohl, K., Francois, P., Stenz, L., Schlink, F., Geiger, T., Herbert, S., Goerke, C., Schrenzel, J. & Wolz, C. (2009). CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. *Journal of Bacteriology* **191,** 2953-2963.

Proctor, R. A. (**1994).** Microbial pathogenic factors: small colony variants. In: Bisno AL, Waldvogel FA, eds. Infections associated with indwelling medical devices. 2nd ed. Washington, DC: *American Society for Microbiology.* pp77–90.

Proctor, R. A. (2008). Role of folate antagonists in the treatment of methicillin-resistant *Staphylococcus aureus* infection. *Clinical Infection Diseases* **46,** 584-593.

Proctor, R. A. (2000). Microbial pathogenic factors: Small colony variants. In: *Infections associated with indwelling medical devices* pp. 41-54. Edited by F. A. Waldvogel & A. L. Bison. Washington D.C.: ASM Press.

Proctor, R. A., Bates, D. M., McNamara, P. J. (2001). In *Emerging Infections*, pp. 95-110. Edited by W. A. Craig. Washington DC: *American Society for Microbiology Press*.

Proctor, R. A., Kahl, B., von Eiff, C., Vaudaux, D., Lew, D.P., & Peters, G. (1998). Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. *Clinical Infection Diseases* **27,** 68-74.

Proctor, R. A. & Peters, G. (1998). Small colony variants in staphylococcal infections: diagnostic and therapeutic implications. *Clinical Infection Diseases **27,*** 419–422.

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M & Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology* **4,** 295-305.

Ptashne, M. (1967). Isolation of the lambda phage repressor. *Proc Natl Acad Sci U S A* **57,** 306–313.

Que, Y. & Moreillon, P. (2010). *Staphylococcus aureus* (Including Staphylococcal Toxic Shock).In Principles and Practice of Infectious Diseases Seventh Edition pp.2543-2589. Edited by G.L. Mandel, J.E Bennett & R. Dolin. Philadelphia: Churchill Livingstone.

Rahman, M. Connolly, S. H., Noble, W. C., Cookson, B. & Phillips, I. (1990). Diversity of staphylococci exhibiting high-level resistance to mupirocin. *Journal of Medical Microbiology* **33,** 97-100.

Rahman, M., Noble, W. C. & Cookson, B. (1987). Mupirocin-resistance *Staphylococcus aureus*. *Lancet* **ii,** 387.

Raygada J, L., Donald, P. & Levine, M.D. (2009). Methicillin-Resistant *Staphylococcus aureus*: A growing risk in the hospital and in the community. *American Health & Drug Benefits* **2,** 86-95.

Reed, S. B., Wesson, C. A., Liou, L. E., Trumble, W. R., Schlievert, P. M., Bohach, G. A. & Bayles, K. W. (2001). Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. *Infection and immunity* **69,** 1521–1527.

Reznikoff, W. (2003). Tn5 as a model for understanding DNA transposition. *Molecular Microbiology* **47,** 1199-1206.

Rice, L. B. (2006). Antimicrobial resistance in Gram-positive bacteria. *The American Journal of Medicine* **119,** 62-70.

Richardson, J. F. & Reith, S. (1993). Characterization of a strain of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) by conventional and molecular methods. *Journal of Hospital Infection* **25,** 45-52.

Richardson, J. F. & Marples, R. R. (1982). Changing resistance to antimicrobial drugs, and resistance typing in clinically significant strains of *Staphylococcus epidermidis.* *Journal of Medical Microbiology* **15,** 475-484.

Richmond, M. H., Parker, M. T., Jevons, M. P. & John, M. (1964). High penicillinase production correlated with multiple antibiotic resistances in *Staphylococcus aureus.* *Lancet* **1,** 293-296.

Rich, P. R. (2003). The molecular machinery of Keilin's respiratory chain. *Biochemical Society Transactions* **31,** 1095–1105.

Ritz, H. L. & Baldwin. J. N. (1958). Induction of penicillinase production in staphylococci by bacteriophage. *Bacteriology Proceedings*, pp 40.

Roggenkamp, A., Sing, A., Hornef, M., Brunner, U., Autenrieth, I. B. & Heesemann J. (1998). Chronic prosthetic hip infection caused by a small-colony variant of *Escherichia coli. Journal of Clinical Microbiology* **36**, 2530-2534.

Romig, W. R. & Brodetsky, A. M. (1961). Isolation and preliminary characterization of bacteriophages for *Bacillus subtilis*. *Journal of Bacteriology* **82,** 135-141.

Ronald, M. A. (1995). Principles of microbiology. 1st ed. St. Louis, MO, USA: Mosby-Year Book, Inc.; pp. 357–360.

Rosdahl, V., T. & Rosendal, K. (1980). Resistance to cadmium, arsenate, and mercury among Danish strains of *Staphylococcus aureus* isolated from cases of bacteraemia. *Journal of Medical Microbiology***, 13,** 383-391.

Rosdahl, V. T. & Rosendal, K. (1983). Correlation of penicillinase production with phage type and susceptibility to antibiotics and heavy metals in *Staphylococcus aureus*. *Journal of Medical Microbiology* **16,** 391-399.

Rosendal, K., & Bulow, P. (1965). Temperate phages influencing lipase production by *Staphylococcus aureus*. *Journal of General Microbiology* **41,** 349–356.

Rosenstein, R., Nerz, C., Biswas, L., Resch, A., Raddatz, G., Schuster, S.C. & Gotz, F. (2009). Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. *Applied Environmental Microbiology* **75,** 811–822.

Rountree, P. M. & Beard. M. A. (1965). The spread of neomycin resistant Staphylococci in a hospital. *Medical Journal of Australia* **1,** 498-502.

Rountree, P. M. (1951). The role of certain electrolytes in the absorption of staphylococcal bacteriophages. *Journal of General Microbiology*. **5,** 673-680.

Rountree, P. M. & Beard. M. A. (1968). Hospital strains of *Staphylococcus aureus* with particular reference to methicillin-resistant strains. *Medical Journal of Australia* **2,** 1163-1168.

Rudin, L., Sjostrom, J. E., Lindberg, M. & Philipson, L. (1974). Factors affecting competence for transformation in *Staphylococcus aureus*. *Journal of Bacteriology* **118,** 155-164.

Russell, A. D. (1991). Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives**.** The *Journal of Applied Bacteriology* **71,** 191-201.

Russell, A. D. (1997). Plasmids and bacterial resistance to biocides. *Journal of Applied Microbiology* **83,** 155-165.

Russell, A. D. (2004). Whither triclosan? *Journal antimicrobial and Chemotherapy* **53,** 693-695.

Sadowska, B., Bonar, A., von Eiff, C., Proctor, R. A., Chmiela, M., Rudnicka, W. & Rozalska, B. (2002). Characteristics of *Staphylococcus aureus* isolated from airways of cystic fibrosis patients, and their small colony variants. *Institute of Microbiology and Immunology* **18,** 191-197.

Saulnier, P., Bourneix, C., Prevost, G. & Andremont, A. (1993). Random amplified polymorphic DNA assay is less discriminant than pulsed-field gel-electrophoresis for typing strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology* **31,** 982-985.

Saunders J. R. (1984). Genetics and evolution of antibiotic resistance. *British Medical Bulletin* **40,** 54-60.

Sawers, G. (1999). The aerobic/anaerobic interface. *Current Opinion in Microbiology* **2,** 181–187.

Saye, D. J., Ogunseitan, O., Sayler, G. S. & Miller, R. V. (1987). Potential for transduction of plasmids in a natural freshwater environment: effect of plasmid donor concentration and a natural microbial community on transduction in *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **53,** 987-995.

Saxild, H. H., Andersen, L. N. & Hammer, K. (1996). *dra-nupCpdp* operon of *Bacillus subtilis*: nucleotide sequence, induction by deoxyribonucleosides, and transcriptional regulation by the *deoR*-encoded DeoR repressor protein. *Journal of Bacteriology* **178,** 424–434.

Schaaff, F., Bierbaum, G., Baumert, N., Bartmann, P. & Sahl, .G. (2003). Mutations are involved in mergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus. International Journal of Medical Microbiology* **293,** 427–435.

Scherl, A., Francois, P., Bento, M., Deshusses, J. M., Charbonnier, Y., Converset, V., Huyghe, A., Walter, N., Hoogland, C., Appel, R. D., Sanchez, J.-C., Zimmermann-Ivol, C. G., Corthals, G. L., Hochstrasser, D. F. & Schrenzel, J. (2005). Correlation of proteomic and transcriptomic profiles of *Staphylococcus aureus* during the post-exponential phase of growth. *Journal of Microbiological Methods* **60,** 247-257.

Schnitzer, R. J., Camagni, L. J. & Buck, M. (1943). Resistance of small colony variants (G Forms) of a *Staphylococcus* towards the bacteriostatic activity of penicillin. *Proceedings of the Society for Experimental Biology and Medicine* **53,** 75-78.

Schroder, A., Kland, R., Peschel, A., von Eiff, C. & Aepfelbacher, M. (2006). Live cell imaging of phagosome maturation in *Staphylococcus aureus* infected human endothelial cells: small colony variants are able to survive in lysosomes. *Medical Microbiology and Immunology* **195,** 185–194.

Seaman, P. F. (2007). Development and horizontal gene transfer of triclosan resistance in *Staphylococcus aureus*. PhD Thesis, Cardiff University.

Seaman, P. F., Ochs, D. & Day, M. J. (2007). Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **59,** 43-50.

Seidl, K., Bischoff, M. & Berger-Bachi. B. (2008). CcpA mediates the catabolite repression of *tst* in *Staphylococcus aureus*. *Infection Immunity* **76,** 5093-5099.

Seidl, K., Muller, S., Francois, P., Kriebitzsch, C., Schrenzel, J., Engelmann, S., Bischoff, M. & Berger-Bachi, B. (2009). Effect of a glucose impulse on the CcpA regulon in *Staphylococcus aureus*. *BMC Microbiology* **9,** 95.

Seidl, K., Stucki, M., Ruegg, M., Goerke, C., Wolz, C., Harris, L., Berger-Bachi, B. & Bischoff, M. (2006). *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance. *Antimicrobial Agents and Chemotherapy* **50,** 1183–1194.

Seifert, H., von Eiff, C. & Fatkenheuer, G. (1999). Fatal case due to methicillin-resistant *Staphylococcus aureus* small colony variants in an AIDS patient. *Emerging Infectious Diseases* **5**, 450-453.

Seligman, S. J. (2006). Small-colony variants of *Staphylococcus aureus*. *Clinical Infection Diseases* **41,** 48-52.

Sendi, P. & Proctor, R. A. (2009). *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends in Microbiology* **17**, 54-58.

Sendi, P., Rohrbach, M., Graber, P., Frei, R., Ochsner, P. E. & Zimmerli, W. (2006). *Staphylococcus aureus* small colony variants in prosthetic joint infection. *Clinical Infection Diseases* **43,** 961-967.

Shakil, S., Khan, R., Zarrilli, R. & Khan, A. U. (2008). Aminoglycosides versus bacteria--a description of the action, resistance mechanism, and nosocomial battleground. *Journal of Biomedical Science* **15,** 5-14.

Shalit, I., Berger, S. A., Gorea, A. & Frimerman, H. (1989). Widespread quinolone resistance among methicillin-resistant *Staphylococcus aureus* isolates in a general hospital. *Antimicrobial Agents and Chemotherapy* **33,** 593–594.

Shanson, C. (1981). Antibiotic-resistant *Staphylococcus aureus. Journal of Hospital Infection* **ii,** 11-36.

Shao, Y. & Wang, I. (2008). Bacteriophage Adsorption Rate and Optimal Lysis Time. *Genetics* **180,** 471–482.

Sitthisak, S., Knutsson, L., Webb, J. W., and Jayaswal, R. K. (2007). Molecular characterization of the copper transport system in *Staphylococcus aureus*. *Microbiology* **153,** 4274–4283.

Sjostrom, J. E., Lofdahl, S. & Philipson, L. (1979). Transformation of *Staphylococcus aureus* by heterologous plasmids. *Plasmid* **2,** 529-535.

Slater-Radosti, C., Van Aller, G., Greenwood, R., Nicholas, R., Keller, P. M., DeWolf, W. E. Jr, Fan, F., Payne, D. J. & Jaworski, D. D. (2001). Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*. *Journal of Antimicrobial and Chemotherapy* **48,** 1–6.

Snyder, L. & Champress, W. (2007). Molecular genetics of bacteria .3rd Ed. *ASM Press*, Washington **3,** 138-141.

Song, M. D., Wachi M., Doi M., Ishino F. & Matsuhashi, M. (1987). Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. *FEBS Letter* **221,**167–171.

Spanu, T., Romano, L., D'Inzeo, T., Masucci, L., Albanese, A., Papacci, F., Marchese, E., Sanguinetti, M. & Fadda, G. (2005). Recurrent ventriculoperitoneal shunt infection caused by small-colony variants of *Staphylococcus aureus*. *Clinical Infectious Diseases* **41**, E48-E52.

Stiffler, P. W., Sweeney, H. M. & Cohen, S. (1974). Co-transduction of plasmids mediating resistance to tetracycline and chloramphenicol in *Staphylococcus aureus*. *Journal of Bacteriology* **120,** 934–934.

Stulke, J., & Hillen, W. (1999). Carbon catabolite repression in bacteria. *Current opinion in Microbiology* **2,** 195-201.

Sun, I. L. (1996). Identification of microorganisms encountered in the upper respiratory tract. Pages 167-184, in tested studies for laboratory teaching, Volume 17 (J. C. Glase, Editor). Proceedings of the 17th Workshop/Conference of the Association for Biology Laboratory Education (ABLE).

Swartz, M.N. (1997). Use of antimicrobial agents and drug resistance. *The New England Journal of Medicine* **337,** 491-492.

Taber, H. (1980). Functions of vitamin K, in microorganisms, p. 177-188. In J. W. Suttie (ed.), Vitamin K metabolism and vitamin K-dependent proteins. *University Park Press*, Baltimore, Md.

Tambic, A., Power, E. G. M., Talsania, H., Anthony, R. M. & French, G. L. (1997). Analysis of an outbreak of non-phagetypable methicillin-resistant *Staphylococcus aureus* by using a randomly amplified polymorphic DNA assay. *Journal of Clinical Microbiology* **35,** 3092–3097.

Tartera, C., Bosch, A. & Jofre, J. (1988). The inactivation of bacteriophages infecting *Bacteroides fragilis* by chlorine treatment and UV-irradiation. *FEMS Microbiology Letters* **56,** 313–316.

Taylor, J. M. & Heinrichs. D. E. (2002). Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Molecular Microbiology* **43,** 1603-1614.

Teitzel, G. M., Geddie, A., De Long, S. K., Kirisits, M. J., Whiteley, M. & Parsek, M.R. (2006). Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *Journal of Bacteriology* **188,** 7242–7256.

Tenover, F. C. & Gorwitz, R. J. (2000). The epidemiology of staphylococcus infection In *Gram positive pathogens* pp. 414-421. Edited by V. A. Fischetti, R. P. Novick, J.J. Ferreti, D. A. Portony & J. I. Rood. Washing D.C.: American Society for Microbiology.

Todar, K. (2005). *Staphylococcus aureus.* In: Todar K, editor. *Todar´s online textbook of bacteriology*; http://www.textbookofbacteriology.net

Tomasz, A. (1984). in Microbial Cell Wall Synthesis and Autolysis, ed. Nombela, C. (Elsevier, Amsterdam), pp. 3-12.

Townsend, D. E., Bolton, S., Ashdown, N., Taheri, S. & Grubb, W. B. (1986). Comparison of phage-mediated and conjugative transfer of staphylococcal plasmids in vitro and in vivo. *Journal of Medical Microbiology* **22,** 107–114.

Tripathi, G. (2010). Cellular biochemical science. I.*K. International Publishing House.* pp. 440-441.

Trucksis, M., Wolfson, J. S. & Hooper, D. C. (1991). A novel locus conferring fluoroquinolone resistance in *Staphylococcus aureus*. *Journal of Bacteriology* **173,** 5854–5860.

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T. & Hiramatsu, K. (2010). Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrobial Agents and Chemotherapy* **54,** 4352–4359.

Tuffnell, D. J, Croton, R. S., Hemingway, D. M., Hartley, M. N., Wake, P. N., Garvey, R. J. (1987). Methicillin resistant *Staphylococcus aureus*; the role of antisepsis in the control of an outbreak. *Journal of Hospital Infection* **10,** 255-259.

Tynecka, Z., Szcześniak, Z., Malm, A. & Los, R. (1999) Energy conservation in aerobically grown *Staphylococcus aureus*. *Research in Microbiology* **150,** 555-566.

Ubukata, K., Nonoguchi, R., Matsuhashi, M. & Konno, M. (1989). Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. *Journal of Bacteriology* **171,** 2882-2885.

Udo, E. E., Grubb, W. B. (1990). A new class of conjugative plasmid in *Staphylococcus aureus*. *Journal of Medical Microbiology* **31,** 207-212.

Udo, E. E. & Grubb, W. B. (1993). Genetic analysis of methicillin-resistant *Staphylococcus aureus* from a Nigerian hospital. *Journal of Medical Microbiology*. **38,** 203-208.

Ulanovskaya, O. A., Janjic, J., Suzuki, M., Sabharwal, S. S., Schumacker, P. T., Kron S. J. & Kozmin S. A. (2008). Synthesis enables identification of the cellular target of leucascandrolide A and neopeltolide *Nature Chemical Biology* **4,** 418-424.

Urban, C. F., Lourido, S. & Zychlinsky, A. (2006). How do microbes evade neutrophil killing? *Cellular Microbiology* **8**, 1687-1696.

Urbonavicius, J., Brochier-Armanet, C., Skouloubris, S., Myllykallio, H. & Grosjean, H. (2007). In vitro detection of the enzymatic activity of folate-dependent tRNA (Uracil-54,-C5)-methyltransferase: evolutionary implications. *Methods in Enzymology* **425,** 103–119.

Urbonavicius, J., Skouloubris, S., Myllykallio, H. & Grosjean, H. (2005). Identification of a novel gene encoding a flavin-dependent tRNA:m^5U methyltransferase in bacteria-evolutionary implications. *Nucleic Acids Research* **33,** 3955–3964.

Urwin, R. & Maiden, M. C. J. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Trends in Microbiology* **11,** 479-487.

Vaezzadeh, A. R., Deshusses, J., Lescuyer, P. & Hochstrasser, D. F. (2007). Shotgun proteomic analysis of total and membrane protein extracts of *S. aureus strain* N315. UniProtKB http://www.uniprot.org/uniprot.

VandenBergh, M. F. Q., Yzerman, E. P. F., van Belkum, A., Boelens, H. A. M., Sijmons, M. & Verbrugh, H. A. (1999). Follow-up of *Staphylococcus aureus* nasal carriage after 8 years: Redefining the persistent carrier state. *Journal of Clinical Microbiology* **37,** 3133-3140.

Vaudaux, P., Francois, P. ,Bisognano, C., Kelley, W. L., Lew, D. P., Schrenzel, J., Proctor, R. A., McNamara, P. J., Peters, G. & Von Eiff, C. (2002). Increased expression of clumping factor and fibro-nectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. *Infection and Immunity* **70,** 5428-5437.

Vaudaux, P., Kelley, W. L. & Lew, D. P. (2006). *Staphylococcus aureus* small colony variants. Difficult to diagnose and difficult to treat. *Clinical Infection Diseases* **43,** 968-970.

van Wamel, W. J., Rooijakkers, S. H., Ruyken, M., van Kessel, K. P., van Strijp, J. A. (2006). The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. *Journal of bacteriology* **188,** 1310–1315.

Vermeiren, C. L., Pluym, M., Mack, J., Heinrichs, D. E. & Stillman, M. J. (2006). Characterization of the heme binding properties of *Staphylococcus aureus IsdA*. *Biochemistry* **45,** 12867-12875.

Vesga, O., Groeschel, M. C., Otten, M. F., Proctor, R. A. & Vann, J. M. (1996). *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. *Clinical Infection Diseases* **173,** 739–742.

von Eiff, C. (**2008).** *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinical. *International Journal of Antimicrobial Agents* **31,** 507-510.

von Eiff, C., Becker, K., Metze, D., Lubritz, G., Hockmann, J., Schwarz, T., Peters, G. (2001). Intracellular persistence of *Staphylococcus aureus* small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. *Clinical Infection Diseases* **32,** 1643-1647.

von Eiff, C., Bettin, D., Proctor, R. A., Rolauffs, B., Lindner, N., Winkelmann, W. & Peters, G. (1997a). Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. *Clinical Infection Diseases* **25,** 1250–1251.

von Eiff, C., McNamara, P., Becker, K., Bates, D., Lei, X. H., Ziman, M., Bochner, B. R., Peters, G., & Proctor, R.A. (2006). Phenotype microarray profiling of *Staphylococcus aureus menD* and *hemB* mutants with the small-colony-variant phenotype. *Journal of Bacteriology* **188,** 687-693.

von Eiff, C., Peters, G., & Proctor, R.A. (2001b). Small colony variants of *Staphylococcus aureus*: Mechanisms for production, biology of infection and clinical significance In *Staphylococcus aureus infection and disease*, pp. 17-34. Edited by A. Honeyman, H. Friedman & M. Bendinelli. New York: Plenum Publishers.

von Eiff, C., **Proctor, R. A.**, **& Peters, G .(2000).** *Staphylococcus aureus* small colony variants: formation and clinical impact. *International Journal of Clinical Practice***15,** 44-49.

von Eiff, C., Vaudaux, P., Kahl, B. C., Lew, D., Emler, S., Schmidt, A., Peters, G. & Proctor, R. A. (1999). Bloodstream infections caused by small-colony variants of coagulase-negative staphylococci following pacemaker implantation. *Clinical Infectious Diseases* **29,** 932-934.

Wadsworth, S. J., Kim, K. H., Satishchandran, V., Axelrod, P., Truant, A. L. & Suh, B. (1992). Development of new antibiotic resistance in methicillin-resistant but not methicillin-susceptible *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **30,** 821-826.

Waisbren, B. A. (1958). The sensitivity of staphylococci to antibiotics. *Archives of Internal Medicine* **101,** 397-407.

Watanakunakorn, C. (1984). Mode of action and *in-vitro* activity of vancomycin. *Journal of Antimicrobial Chemotherapy* **14,** 7-18.

Weische, A. & Leistner, E. (1985). Cell free synthesis of o-succinylbenzoic acid from iso-chorismic acid, the key reaction in vitamin K (menaquinone) biosynthesis. *Tetrahedron Letters* **26,** 1487-1490.

Weiss, A. A., Murphy, S. D. & Silver, S. (1977). Mercury and organomercurial resistances determined by plasmids in *Staphylococcus aureus*. *Journal of Bacteriology* **132,** 197-208.

Weiss, A. A., Silver, S. & Kinscherf, T. G. (1978). Cation transport alteration associated with plasmid-determined resistance to cadmium in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy***. 14,** 856-865.

Weller, T. M. (2000). Methicillin-resistant *Staphylococcus aureus* typing methods: Which should be the international standard? *Journal of Hospital Infection* **44,** 160-172.

Wenzel, R. P., Donowitz, L., & Miller, J. R. (1981). Methicillin-resistant *Staphylococcus aureus* in the United States. *Morbidity and Mortality Weekly Report* **30,** 140–147.

White, D.G., Alekshun, M.N. & McDermott, P.F. (2005). Frontiers in antibiotic resistance. *American Society for Microbiology Press*. pp. 85-88.

Wichelhaus, T. A., Shafer. V., Brade, V. & Boddinghaus, B. (1999). Molecular characterization of *rpoB* mutations conferring cross-resistance to rifamycins on methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **43,** 2813-2816.

Wicker, T., Sabot, F., Hua-Van, A., Bennetzen, J. L., Capy, P., Chalhoub, B., Flavell, A., Leroy, P., Morgante, M., Panaud, O., Paux, E., SanMiguel, P. & Schulman, A. H. (2007). A unified classification system for eukaryotic transposable elements. *Nature Reviews Genetics* **8,** 973-982.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic-markers. *Nucleic Acids Research* **18,** 6531-6535.

Winkler, K. C., de Waart, J. & Grootsen, C. (1965). Lysogenic conversion of staphylococci to loss of beta-toxin. *Journal of General Microbiology* **39,** 321–333.

Wise, E. M. & Park, J. T. (1965). Penicillin; its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proceedings of the National Academy of Sciences of the USA* **54,** 75-81.

Wise, R. (2002). Antimicrobial resistance: priorities for action. *Journal of Antimicrobial Chemotherapy* **49,** 585-586.

Wise, R. (1982). ß-Lactamase inhibitors. *Journal of Antimicrobial Chemotherapy* **9**, 31-40.

Witte, W. (1981). Transfer of drug-resistance plasmids in mixed cultures of Staphylococci. Zenrralblarr fur Bukteriologie Purasitenkunde, InfektionsKrunkheiten und Hygiene, Suppl. **10,** 543-545.

Witte, W., Green, L., Misra, T. K. & Silver, S. (1986). Resistance to mercury and to cadmium in chromosomally resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **29,** 663-669.

Witte, W., Nguyen Van Dip, & Hummel, R. (1980). Resistance against mercury and cadmium in *Staphylococcus aureus* of different ecological origin. Zeitschrift fur Allgemeine Mikrobiologie **20,** 517-521.

Woodford, N. (2005). Biological counterstrike: antibiotic resistance mechanisms of Gram-positive cocci. *Clinical Microbiology and Infection* **11,** 2-21.

World Health Organisation (2011). European strategic action plan on antibiotic resistance. Baku, Azerbaijan. **61** session.

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology* **5, 175-186.**

Wu, S. W., DeLencastre, H. & Tomasz, A. (1996). Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: Molecular cloning and DNA sequencing. *Journal of Bacteriology* **178**, 6036-6042.

Yamagishi, J., Kojima, T., Oyamada, Y., Fujimoto, K., Hattori, H, Nakamura, S. & Inoue, M. (1996). Alterations in the DNA topoisomerase IV *grlA* gene responsible for quinolone resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **40,** 1157–1163.

Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M. & Nakayama, K. (2000). Phage conversion of exfoliative toxin A production in *staphylococcus aureus*. *Molecular Microbiology* **38,** 694–705.

Yarwood, J. M., & Schlievert P. M. (2000). Oxygen and carbon dioxide regulation of toxic shock syndrome toxin 1 production by *Staphylococcus aureus* MN8. *Journal of Clinical Microbiology* **38,** 1797–1803.

Yilmaz, G., Aydin, K., Iskender, S., Caylan, R. & Koksal, I. (2007). Detection and prevalence of inducible clindamycin resistance in Staphylococci. *Journal of Medical Microbiology* **56,** 342–345.

Youmans, G. P., Williston, E. H. & Simon, M. (1945). Production of small colony variants of *Staphylococcus aureus* by the action of penicillin. *Proceedings of the Society for Experimental Biology and Medicine* **58,** 56-57.

Young, H-K., Skurray, R. A. & Amyes, S.G.B. (1987). Plasmid-mediated trimethoprim resistance in *Staphylococcus aureus*: characterization of the first Gram-positive plasmid dihydrofolate reductase (type Sl). *Biochemical Journal* **243,** 309-312.

Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, & Konno, M. (1990). Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. *Journal of Bacteriology* **172,** 6942–6949.

Zafar, A. B., Butler, R. C., Reese, D. J., Gaydos, L. A. & Mennonna, P. A. (1995). Use of 0.3% triclosan (Bacti-Stat) to eradicate an outbreak of methicillin-resistant *Staphylococcus aureus* in a neonatal nursery. *American Journal of Infection Control* **23,** 200–208.

Zhang, K., Sparling, J., Chow, B. L., Elsayed, S., Hussain, Z., Church, D. L. Gregson, D. B., Louise, T. & Conly, J. M. (2004). New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. *Journal of Clinical Microbiology* **42,** 4947-4955.

Appendix

Chapter 2 statistical analysis data - Multiple Comparisons

1- Dependent Variable: ***Cycling*** LSD

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1039555555.4933*	354976756.3767	.004	-1742896495.514	-336214615.473
	11	-118666666.6733	354976756.3767	.739	-822007606.694	584674273.347
	22	-555111111.0933	354976756.3767	.121	-1258452051.114	148229828.927
	111	112222222.2867	354976756.3767	.752	-591118717.734	815563162.307
	222	-2208463644.6067*	354976756.3767	.000	-2911804584.627	-1505122704.586
	1111	-132666666.6067	354976756.3767	.709	-836007606.627	570674273.414
	2222	-1919555555.5400*	354976756.3767	.000	-2622896495.561	-1216214615.519
2	1	1039555555.4933*	354976756.3767	.004	336214615.473	1742896495.514
	11	920888888.8200*	354976756.3767	.011	217547948.799	1624229828.841
	22	484444444.4000	354976756.3767	.175	-218896495.621	1187785384.421
	111	1151777777.7800*	354976756.3767	.002	448436837.759	1855118717.801
	222	-1168908089.1133*	354976756.3767	.001	-1872249029.134	-465567149.093
	1111	906888888.8867*	354976756.3767	.012	203547948.866	1610229828.907
	2222	-880000000.0467*	354976756.3767	.015	-1583340940.067	-176659060.026
11	1	118666666.6733	354976756.3767	.739	-584674273.347	822007606.694
	2	-920888888.8200*	354976756.3767	.011	-1624229828.841	-217547948.799
	22	-436444444.4200	354976756.3767	.221	-1139785384.441	266896495.601
	111	230888888.9600	354976756.3767	.517	-472452051.061	934229828.981
	222	-2089796977.9333*	354976756.3767	.000	-2793137917.954	-1386456037.913
	1111	-13999999.9333	354976756.3767	.969	-717340939.954	689340940.087
	2222	-1800888888.8667*	354976756.3767	.000	-2504229828.887	-1097547948.846
22	1	555111111.0933	354976756.3767	.121	-148229828.927	1258452051.114
	2	-484444444.4000	354976756.3767	.175	-1187785384.421	218896495.621
	11	436444444.4200	354976756.3767	.221	-266896495.601	1139785384.441
	111	667333333.3800	354976756.3767	.063	-36007606.641	1370674273.401
	222	-1653352533.5133*	354976756.3767	.000	-2356693473.534	-950011593.493
	1111	422444444.4867	354976756.3767	.237	-280896495.534	1125785384.507
	2222	-1364444444.4467*	354976756.3767	.000	-2067785384.467	-661103504.426
111	1	-112222222.2867	354976756.3767	.752	-815563162.307	591118717.734

	2	-1151777777.7800*	354976756.3767	.002	-1855118717.801	-448436837.759
	11	-230888888.9600	354976756.3767	.517	-934229828.981	472452051.061
	22	-667333333.3800	354976756.3767	.063	-1370674273.401	36007606.641
	222	-2320685866.8933*	354976756.3767	.000	-3024026806.914	-1617344926.873
	1111	-244888888.8933	354976756.3767	.492	-948229828.914	458452051.127
	2222	-2031777777.8267*	354976756.3767	.000	-2735118717.847	-1328436837.806
	1	2208463644.6067*	354976756.3767	.000	1505122704.586	2911804584.627
	2	1168908089.1133*	354976756.3767	.001	465567149.093	1872249029.134
	11	2089796977.9333*	354976756.3767	.000	1386456037.913	2793137917.954
222	22	1653352533.5133*	354976756.3767	.000	950011593.493	2356693473.534
	111	2320685866.8933*	354976756.3767	.000	1617344926.873	3024026806.914
	1111	2075796978.0000*	354976756.3767	.000	1372456037.979	2779137918.021
	2222	288908089.0667	354976756.3767	.417	-414432850.954	992249029.087
	1	132666666.6067	354976756.3767	.709	-570674273.414	836007606.627
	2	-906888888.8867*	354976756.3767	.012	-1610229828.907	-203547948.866
	11	13999999.9333	354976756.3767	.969	-689340940.087	717340939.954
1111	22	-422444444.4867	354976756.3767	.237	-1125785384.507	280896495.534
	111	244888888.8933	354976756.3767	.492	-458452051.127	948229828.914
	222	-2075796978.0000*	354976756.3767	.000	-2779137918.021	-1372456037.979
	2222	-1786888888.9333*	354976756.3767	.000	-2490229828.954	-1083547948.913
	1	1919555555.5400*	354976756.3767	.000	1216214615.519	2622896495.561
	2	880000000.0467*	354976756.3767	.015	176659060.026	1583340940.067
	11	1800888888.8667*	354976756.3767	.000	1097547948.846	2504229828.887
2222	22	1364444444.4467*	354976756.3767	.000	661103504.426	2067785384.467
	111	2031777777.8267*	354976756.3767	.000	1328436837.806	2735118717.847
	222	-288908089.0667	354976756.3767	.417	-992249029.087	414432850.954
	1111	1786888888.9333*	354976756.3767	.000	1083547948.913	2490229828.954

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	**WT 15 COL-GEN**	**WT 15 COL-TRI**	**SCV 15 COL-GEN**	**SCV 15 COL-TRI**	**N315 WT00**	**WT 15 N315-GEN**	**WT 15 N315-TRI**	**SCV 15 N315-GEN**	**SCV 15N315-TRI**

*. The mean difference is significant at the 0.05 level.

2- Dependent Variable: ***ATP*** LSD

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	2	-1620.333	939.988	.100	-3581.11	340.45
1	11	10498.000*	939.988	.000	8537.22	12458.78
	22	12014.000*	939.988	.000	10053.22	13974.78

	111	12751.000*	939.988	.000	10790.22	14711.78
	222	11691.667*	939.988	.000	9730.89	13652.45
	1111	20395.333*	939.988	.000	18434.55	22356.11
	2222	20966.333*	939.988	.000	19005.55	22927.11
	11111	21206.000*	939.988	.000	19245.22	23166.78
	22222	22815.333*	939.988	.000	20854.55	24776.11
	1	1620.333	939.988	.100	-340.45	3581.11
	11	12118.333*	939.988	.000	10157.55	14079.11
	22	13634.333*	939.988	.000	11673.55	15595.11
	111	14371.333*	939.988	.000	12410.55	16332.11
2	222	13312.000*	939.988	.000	11351.22	15272.78
	1111	22015.667*	939.988	.000	20054.89	23976.45
	2222	22586.667*	939.988	.000	20625.89	24547.45
	11111	22826.333*	939.988	.000	20865.55	24787.11
	22222	24435.667*	939.988	.000	22474.89	26396.45
	1	-10498.000*	939.988	.000	-12458.78	-8537.22
	2	-12118.333*	939.988	.000	-14079.11	-10157.55
	22	1516.000	939.988	.122	-444.78	3476.78
	111	2253.000*	939.988	.026	292.22	4213.78
11	222	1193.667	939.988	.219	-767.11	3154.45
	1111	9897.333*	939.988	.000	7936.55	11858.11
	2222	10468.333*	939.988	.000	8507.55	12429.11
	11111	10708.000*	939.988	.000	8747.22	12668.78
	22222	12317.333*	939.988	.000	10356.55	14278.11
	1	-12014.000*	939.988	.000	-13974.78	-10053.22
	2	-13634.333*	939.988	.000	-15595.11	-11673.55
	11	-1516.000	939.988	.122	-3476.78	444.78
	111	737.000	939.988	.442	-1223.78	2697.78
22	222	-322.333	939.988	.735	-2283.11	1638.45
	1111	8381.333*	939.988	.000	6420.55	10342.11
	2222	8952.333*	939.988	.000	6991.55	10913.11
	11111	9192.000*	939.988	.000	7231.22	11152.78
	22222	10801.333*	939.988	.000	8840.55	12762.11
	1	-12751.000*	939.988	.000	-14711.78	-10790.22
	2	-14371.333*	939.988	.000	-16332.11	-12410.55
	11	-2253.000*	939.988	.026	-4213.78	-292.22
	22	-737.000	939.988	.442	-2697.78	1223.78
111	222	-1059.333	939.988	.273	-3020.11	901.45
	1111	7644.333*	939.988	.000	5683.55	9605.11
	2222	8215.333*	939.988	.000	6254.55	10176.11
	11111	8455.000*	939.988	.000	6494.22	10415.78
	22222	10064.333*	939.988	.000	8103.55	12025.11
	1	-11691.667*	939.988	.000	-13652.45	-9730.89
	2	-13312.000*	939.988	.000	-15272.78	-11351.22
	11	-1193.667	939.988	.219	-3154.45	767.11
	22	322.333	939.988	.735	-1638.45	2283.11
222	111	1059.333	939.988	.273	-901.45	3020.11
	1111	8703.667*	939.988	.000	6742.89	10664.45
	2222	9274.667*	939.988	.000	7313.89	11235.45
	11111	9514.333*	939.988	.000	7553.55	11475.11
	22222	11123.667*	939.988	.000	9162.89	13084.45
	1	-20395.333*	939.988	.000	-22356.11	-18434.55
	2	-22015.667*	939.988	.000	-23976.45	-20054.89
	11	-9897.333*	939.988	.000	-11858.11	-7936.55
	22	-8381.333*	939.988	.000	-10342.11	-6420.55
1111	111	-7644.333*	939.988	.000	-9605.11	-5683.55
	222	-8703.667*	939.988	.000	-10664.45	-6742.89
	2222	571.000	939.988	.550	-1389.78	2531.78
	11111	810.667	939.988	.399	-1150.11	2771.45
	22222	2420.000*	939.988	.018	459.22	4380.78
	1	-20966.333*	939.988	.000	-22927.11	-19005.55
	2	-22586.667*	939.988	.000	-24547.45	-20625.89
	11	-10468.333*	939.988	.000	-12429.11	-8507.55
	22	-8952.333*	939.988	.000	-10913.11	-6991.55
2222	111	-8215.333*	939.988	.000	-10176.11	-6254.55
	222	-9274.667*	939.988	.000	-11235.45	-7313.89
	1111	-571.000	939.988	.550	-2531.78	1389.78
	11111	239.667	939.988	.801	-1721.11	2200.45
	22222	1849.000	939.988	.063	-111.78	3809.78
	1	-21206.000*	939.988	.000	-23166.78	-19245.22
	2	-22826.333*	939.988	.000	-24787.11	-20865.55
	11	-10708.000*	939.988	.000	-12668.78	-8747.22
11111	22	-9192.000*	939.988	.000	-11152.78	-7231.22
	111	-8455.000*	939.988	.000	-10415.78	-6494.22
	222	-9514.333*	939.988	.000	-11475.11	-7553.55
	1111	-810.667	939.988	.399	-2771.45	1150.11

	2222	-239.667	939.988	.801	-2200.45	1721.11
	22222	1609.333	939.988	.102	-351.45	3570.11
	1	-22815.333*	939.988	.000	-24776.11	-20854.55
	2	-24435.667*	939.988	.000	-26396.45	-22474.89
	11	-12317.333*	939.988	.000	-14278.11	-10356.55
	22	-10801.333*	939.988	.000	-12762.11	-8840.55
22222	111	-10064.333*	939.988	.000	-12025.11	-8103.55
	222	-11123.667*	939.988	.000	-13084.45	-9162.89
	1111	-2420.000*	939.988	.018	-4380.78	-459.22
	2222	-1849.000	939.988	.063	-3809.78	111.78
	11111	-1609.333	939.988	.102	-3570.11	351.45

*. The mean difference is significant at the 0.05 level.

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	**WT 15 COL-GEN**	**WT 15 COL-TRI**	**SCV 15 COL-GEN**	**SCV 15 COL-TRI**	**N315 WT00**	**WT 15 N315-GEN**	**WT 15 N315-TRI**	**SCV 15 N315-GEN**	**SCV 15N315-TRI**

3- Dependent Variable: ***DNase*** LSD

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	2	1.1667*	.4472	.017	.234	2.100
	11	3.0000*	.4472	.000	2.067	3.933
	22	4.6667*	.4472	.000	3.734	5.600
	111	2.3333*	.4472	.000	1.400	3.266
1	222	5.1667*	.4472	.000	4.234	6.100
	1111	5.5000*	.4472	.000	4.567	6.433
	2222	5.1667*	.4472	.000	4.234	6.100
	11111	3.3333*	.4472	.000	2.400	4.266
	22222	5.8333*	.4472	.000	4.900	6.766
	1	-1.1667*	.4472	.017	-2.100	-.234
	11	1.8333*	.4472	.001	.900	2.766
	22	3.5000*	.4472	.000	2.567	4.433
	111	1.1667*	.4472	.017	.234	2.100
2	222	4.0000*	.4472	.000	3.067	4.933
	1111	4.3333*	.4472	.000	3.400	5.266
	2222	4.0000*	.4472	.000	3.067	4.933
	11111	2.1667*	.4472	.000	1.234	3.100
	22222	4.6667*	.4472	.000	3.734	5.600
	1	-3.0000*	.4472	.000	-3.933	-2.067
	2	-1.8333*	.4472	.001	-2.766	-.900
	22	1.6667*	.4472	.001	.734	2.600
	111	-.6667	.4472	.152	-1.600	.266
11	222	2.1667*	.4472	.000	1.234	3.100
	1111	2.5000*	.4472	.000	1.567	3.433
	2222	2.1667*	.4472	.000	1.234	3.100
	11111	.3333	.4472	.465	-.600	1.266
	22222	2.8333*	.4472	.000	1.900	3.766
	1	-4.6667*	.4472	.000	-5.600	-3.734
	2	-3.5000*	.4472	.000	-4.433	-2.567
	11	-1.6667*	.4472	.001	-2.600	-.734
	111	-2.3333*	.4472	.000	-3.266	-1.400
22	222	.5000	.4472	.277	-.433	1.433
	1111	.8333	.4472	.077	-.100	1.766
	2222	.5000	.4472	.277	-.433	1.433
	11111	-1.3333*	.4472	.007	-2.266	-.400
	22222	1.1667*	.4472	.017	.234	2.100
	1	-2.3333*	.4472	.000	-3.266	-1.400
	2	-1.1667*	.4472	.017	-2.100	-.234
	11	.6667	.4472	.152	-.266	1.600
	22	2.3333*	.4472	.000	1.400	3.266
111	222	2.8333*	.4472	.000	1.900	3.766
	1111	3.1667*	.4472	.000	2.234	4.100
	2222	2.8333*	.4472	.000	1.900	3.766
	11111	1.0000*	.4472	.037	.067	1.933
	22222	3.5000*	.4472	.000	2.567	4.433
	1	-5.1667*	.4472	.000	-6.100	-4.234
	2	-4.0000*	.4472	.000	-4.933	-3.067
	11	-2.1667*	.4472	.000	-3.100	-1.234
222	22	-.5000	.4472	.277	-1.433	.433
	111	-2.8333*	.4472	.000	-3.766	-1.900
	1111	.3333	.4472	.465	-.600	1.266

	2222	.0000	.4472	1.000	-.933	.933
	11111	-1.8333*	.4472	.001	-2.766	-.900
	22222	.6667	.4472	.152	-.266	1.600
	1	-5.5000*	.4472	.000	-6.433	-4.567
	2	-4.3333*	.4472	.000	-5.266	-3.400
	11	-2.5000*	.4472	.000	-3.433	-1.567
	22	-.8333	.4472	.077	-1.766	.100
1111	111	-3.1667*	.4472	.000	-4.100	-2.234
	222	-.3333	.4472	.465	-1.266	.600
	2222	-.3333	.4472	.465	-1.266	.600
	11111	-2.1667*	.4472	.000	-3.100	-1.234
	22222	.3333	.4472	.465	-.600	1.266
	1	-5.1667*	.4472	.000	-6.100	-4.234
	2	-4.0000*	.4472	.000	-4.933	-3.067
	11	-2.1667*	.4472	.000	-3.100	-1.234
	22	-.5000	.4472	.277	-1.433	.433
2222	111	-2.8333*	.4472	.000	-3.766	-1.900
	222	.0000	.4472	1.000	-.933	.933
	1111	.3333	.4472	.465	-.600	1.266
	11111	-1.8333*	.4472	.001	-2.766	-.900
	22222	.6667	.4472	.152	-.266	1.600
	1	-3.3333*	.4472	.000	-4.266	-2.400
	2	-2.1667*	.4472	.000	-3.100	-1.234
	11	-.3333	.4472	.465	-1.266	.600
	22	1.3333*	.4472	.007	.400	2.266
11111	111	-1.0000*	.4472	.037	-1.933	-.067
	222	1.8333*	.4472	.001	.900	2.766
	1111	2.1667*	.4472	.000	1.234	3.100
	2222	1.8333*	.4472	.001	.900	2.766
	22222	2.5000*	.4472	.000	1.567	3.433
	1	-5.8333*	.4472	.000	-6.766	-4.900
	2	-4.6667*	.4472	.000	-5.600	-3.734
	11	-2.8333*	.4472	.000	-3.766	-1.900
	22	-1.1667*	.4472	.017	-2.100	-.234
22222	111	-3.5000*	.4472	.000	-4.433	-2.567
	222	-.6667	.4472	.152	-1.600	.266
	1111	-.3333	.4472	.465	-1.266	.600
	2222	-.6667	.4472	.152	-1.600	.266
	11111	-2.5000*	.4472	.000	-3.433	-1.567

*. The mean difference is significant at the 0.05 level.

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	**WT 15 COL-GEN**	**WT 15 COL-TRI**	**SCV 15 COL-GEN**	**SCV 15 COL-TRI**	**N315 WT00**	**WT 15 N315-GEN**	**WT 15 N315-TRI**	**SCV 15 N315-GEN**	**SCV 15N315-TRI**

4- Dependent Variable: ***Pigment*** LSD

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	2	-.020000*	.006561	.006	-.03369	-.00631
	11	.041333*	.006561	.000	.02765	.05502
	22	.025000*	.006561	.001	.01131	.03869
	111	.069000*	.006561	.000	.05531	.08269
1	222	.016000*	.006561	.024	.00231	.02969
	1111	.115000*	.006561	.000	.10131	.12869
	2222	.067000*	.006561	.000	.05331	.08069
	11111	.150000*	.006561	.000	.13631	.16369
	22222	.049667*	.006561	.000	.03598	.06335
	1	.020000*	.006561	.006	.00631	.03369
	11	.061333*	.006561	.000	.04765	.07502
	22	.045000*	.006561	.000	.03131	.05869
	111	.089000*	.006561	.000	.07531	.10269
2	222	.036000*	.006561	.000	.02231	.04969
	1111	.135000*	.006561	.000	.12131	.14869
	2222	.087000*	.006561	.000	.07331	.10069
	11111	.170000*	.006561	.000	.15631	.18369
	22222	.069667*	.006561	.000	.05598	.08335
	1	-.041333*	.006561	.000	-.05502	-.02765
	2	-.061333*	.006561	.000	-.07502	-.04765
11	22	-.016333*	.006561	.022	-.03002	-.00265
	111	.027667*	.006561	.000	.01398	.04135

	222	-.025333*	.006561	.001	-.03902	-.01165
	1111	.073667*	.006561	.000	.05998	.08735
	2222	.025667*	.006561	.001	.01198	.03935
	11111	.108667*	.006561	.000	.09498	.12235
	22222	.008333	.006561	.219	-.00535	.02202
	1	-.025000*	.006561	.001	-.03869	-.01131
	2	-.045000*	.006561	.000	-.05869	-.03131
	11	.016333*	.006561	.022	.00265	.03002
	111	.044000*	.006561	.000	.03031	.05769
22	222	-.009000	.006561	.185	-.02269	.00469
	1111	.090000*	.006561	.000	.07631	.10369
	2222	.042000*	.006561	.000	.02831	.05569
	11111	.125000*	.006561	.000	.11131	.13869
	22222	.024667*	.006561	.001	.01098	.03835
	1	-.069000*	.006561	.000	-.08269	-.05531
	2	-.089000*	.006561	.000	-.10269	-.07531
	11	-.027667*	.006561	.000	-.04135	-.01398
	22	-.044000*	.006561	.000	-.05769	-.03031
111	222	-.053000*	.006561	.000	-.06669	-.03931
	1111	.046000*	.006561	.000	.03231	.05969
	2222	-.002000	.006561	.764	-.01569	.01169
	11111	.081000*	.006561	.000	.06731	.09469
	22222	-.019333*	.006561	.008	-.03302	-.00565
	1	-.016000*	.006561	.024	-.02969	-.00231
	2	-.036000*	.006561	.000	-.04969	-.02231
	11	.025333*	.006561	.001	.01165	.03902
	22	.009000	.006561	.185	-.00469	.02269
222	111	.053000*	.006561	.000	.03931	.06669
	1111	.099000*	.006561	.000	.08531	.11269
	2222	.051000*	.006561	.000	.03731	.06469
	11111	.134000*	.006561	.000	.12031	.14769
	22222	.033667*	.006561	.000	.01998	.04735
	1	-.115000*	.006561	.000	-.12869	-.10131
	2	-.135000*	.006561	.000	-.14869	-.12131
	11	-.073667*	.006561	.000	-.08735	-.05998
	22	-.090000*	.006561	.000	-.10369	-.07631
1111	111	-.046000*	.006561	.000	-.05969	-.03231
	222	-.099000*	.006561	.000	-.11269	-.08531
	2222	-.048000*	.006561	.000	-.06169	-.03431
	11111	.035000*	.006561	.000	.02131	.04869
	22222	-.065333*	.006561	.000	-.07902	-.05165
	1	-.067000*	.006561	.000	-.08069	-.05331
	2	-.087000*	.006561	.000	-.10069	-.07331
	11	-.025667*	.006561	.001	-.03935	-.01198
	22	-.042000*	.006561	.000	-.05569	-.02831
2222	111	.002000	.006561	.764	-.01169	.01569
	222	-.051000*	.006561	.000	-.06469	-.03731
	1111	.048000*	.006561	.000	.03431	.06169
	11111	.083000*	.006561	.000	.06931	.09669
	22222	-.017333*	.006561	.016	-.03102	-.00365
	1	-.150000*	.006561	.000	-.16369	-.13631
	2	-.170000*	.006561	.000	-.18369	-.15631
	11	-.108667*	.006561	.000	-.12235	-.09498
	22	-.125000*	.006561	.000	-.13869	-.11131
11111	111	-.081000*	.006561	.000	-.09469	-.06731
	222	-.134000*	.006561	.000	-.14769	-.12031
	1111	-.035000*	.006561	.000	-.04869	-.02131
	2222	-.083000*	.006561	.000	-.09669	-.06931
	22222	-.100333*	.006561	.000	-.11402	-.08665
	1	-.049667*	.006561	.000	-.06335	-.03598
	2	-.069667*	.006561	.000	-.08335	-.05598
	11	-.008333	.006561	.219	-.02202	.00535
	22	-.024667*	.006561	.001	-.03835	-.01098
22222	111	.019333*	.006561	.008	.00565	.03302
	222	-.033667*	.006561	.000	-.04735	-.01998
	1111	.065333*	.006561	.000	.05165	.07902
	2222	.017333*	.006561	.016	.00365	.03102
	11111	.100333*	.006561	.000	.08665	.11402

*. The mean difference is significant at the 0.05 level.

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	WT 15 COL-GEN	WT 15 COL-TRI	SCV 15 COL-GEN	SCV 15 COL-TRI	N315 WT00	WT 15 N315-GEN	WT 15 N315-TRI	SCV 15 N315-GEN	SCV 15N315-TRI

5- Dependent Variable: ***MIC*** LSD

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	2	-.05000000*	.02043473	.031	-.0945234	-.0054766
1	11	.21875000*	.02043473	.000	.1742266	.2632734
	22	.21875000*	.02043473	.000	.1742266	.2632734
	1	.05000000*	.02043473	.031	.0054766	.0945234
2	11	.26875000*	.02043473	.000	.2242266	.3132734
	22	.26875000*	.02043473	.000	.2242266	.3132734
	1	-.21875000*	.02043473	.000	-.2632734	-.1742266
11	2	-.26875000*	.02043473	.000	-.3132734	-.2242266
	22	0E-8	.02043473	1.000	-.0445234	.0445234
	1	-.21875000*	.02043473	.000	-.2632734	-.1742266
22	2	-.26875000*	.02043473	.000	-.3132734	-.2242266
	11	0E-8	.02043473	1.000	-.0445234	.0445234

*. The mean difference is significant at the 0.05 level.

1	11	2	22
COL GEN	COL TRI	N315 GEN	N315 TRI

6- Dependent Variable: ***Phage yield*** LSD

(I) Strains	(J) Strains	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	2	-169999999.99999970*	37505555.144093880	.000	-248235217.09999080	-91764782.90000916
	11	73333333.333333340	37505555.144093880	.065	-4901883.76665747	151568550.43332416
	22	-96666666.666666660*	37505555.144093880	.018	-174901883.76665747	-18431449.56667585
	111	106666666.66666690*	37505555.144093880	.010	28431449.56667588	184901883.76665750
1	222	-136666666.66666660*	37505555.144093880	.002	-214901883.76665747	-58431449.56667585
	1111	136666666.66666700*	37505555.144093880	.002	58431449.56667586	214901883.76665747
	2222	-23333333.333333343	37505555.144093880	.541	-101568550.43332416	54901883.76665746
	11111	103333333.33333340*	37505555.144093880	.012	25098116.23334254	181568550.43332416
	22222	40000000.000000000	37505555.144093880	.299	-38235217.09999081	118235217.09999081
	1	169999999.99999970*	37505555.144093880	.000	91764782.90000916	248235217.09999080
	11	243333333.33333300*	37505555.144093880	.000	165098116.23334250	321568550.43332410
	22	73333333.333333310	37505555.144093880	.065	-4901883.76665750	151568550.43332413
	111	276666666.66666600*	37505555.144093880	.000	198431449.56667584	354901883.76665750
2	222	33333333.333333313	37505555.144093880	.385	-44901883.76665749	111568550.43332413
	1111	306666666.66666600*	37505555.144093880	.000	228431449.56667584	384901883.76665750
	2222	146666666.66666630*	37505555.144093880	.001	68431449.56667581	224901883.76665744
	11111	273333333.33333300*	37505555.144093880	.000	195098116.23334250	351568550.43332410
	22222	209999999.99999970*	37505555.144093880	.000	131764782.90000916	288235217.09999080
11	1	-73333333.333333340	37505555.144093880	.065	-151568550.43332416	4901883.76665747

	2	-243333333.33333330*	37505555.144093880	.000	-321568550.43332410	-165098116.23334250
	22	-170000000.00000000*	37505555.144093880	.000	-248235217.09999081	-91764782.90000919
	111	33333333.333333343	37505555.144093880	.385	-44901883.76665746	111568550.43332416
	222	-210000000.00000000*	37505555.144093880	.000	-288235217.09999080	-131764782.90000919
	1111	63333333.333333330	37505555.144093880	.107	-14901883.76665748	141568550.43332413
	2222	-96666666.666666690*	37505555.144093880	.018	-174901883.76665750	-18431449.56667588
	11111	30000000.000000000	37505555.144093880	.433	-48235217.09999081	108235217.09999081
	22222	-33333333.333333343	37505555.144093880	.385	-111568550.43332416	44901883.76665746
	1	96666666.666666660*	37505555.144093880	.018	18431449.56667585	174901883.76665747
	2	-73333333.333333310	37505555.144093880	.065	-151568550.43332413	4901883.76665750
	11	170000000.00000000*	37505555.144093880	.000	91764782.90000919	248235217.09999081
	111	203333333.33333340*	37505555.144093880	.000	125098116.23334253	281568550.43332416
22	222	-40000000.000000000	37505555.144093880	.299	-118235217.09999081	38235217.09999081
	1111	233333333.33333300*	37505555.144093880	.000	155098116.23334253	311568550.43332416
	2222	73333333.333333310	37505555.144093880	.065	-4901883.76665750	151568550.43332413
	11111	200000000.00000000*	37505555.144093880	.000	121764782.90000919	278235217.09999080
	22222	136666666.66666660*	37505555.144093880	.002	58431449.56667585	214901883.76665747
	1	-106666666.66666690*	37505555.144093880	.010	-184901883.76665750	-28431449.56667588
	2	-276666666.66666600*	37505555.144093880	.000	-354901883.76665750	-198431449.56667584
	11	-33333333.333333343	37505555.144093880	.385	-111568550.43332416	44901883.76665746
	22	-203333333.33333340*	37505555.144093880	.000	-281568550.43332416	-125098116.23334253
111	222	-243333333.33333340*	37505555.144093880	.000	-321568550.43332416	-165098116.23334253
	1111	29999999.999999985	37505555.144093880	.433	-48235217.09999082	108235217.09999080
	2222	-130000000.00000030*	37505555.144093880	.002	-208235217.09999084	-51764782.90000922
	11111	-3333333.333333343	37505555.144093880	.930	-81568550.43332416	74901883.76665747
	22222	-66666666.666666690	37505555.144093880	.091	-144901883.76665750	11568550.43332412
	1	136666666.66666660*	37505555.144093880	.002	58431449.56667585	214901883.76665747
	2	-33333333.333333313	37505555.144093880	.385	-111568550.43332413	44901883.76665749
222	11	210000000.00000000*	37505555.144093880	.000	131764782.90000919	288235217.09999080
	22	40000000.000000000	37505555.144093880	.299	-38235217.09999081	118235217.09999081
	111	243333333.33333340*	37505555.144093880	.000	165098116.23334253	321568550.43332416

	1111	273333333.333333300*	37505555.144093880	.000	195098116.23334253	351568550.43332416
	2222	113333333.333333310*	37505555.144093880	.007	35098116.23334251	191568550.43332413
	11111	240000000.000000000*	37505555.144093880	.000	161764782.90000919	318235217.09999080
	22222	176666666.666666660*	37505555.144093880	.000	98431449.56667584	254901883.76665747
	1	-136666666.666666700*	37505555.144093880	.002	-214901883.76665747	-58431449.56667586
	2	-306666666.666666600*	37505555.144093880	.000	-384901883.76665750	-228431449.56667584
	11	-63333333.333333330	37505555.144093880	.107	-141568550.43332413	-14901883.76665748
	22	-233333333.333333300*	37505555.144093880	.000	-311568550.43332416	-155098116.23334253
1111	111	-29999999.999999985	37505555.144093880	.433	-108235217.09999080	-48235217.09999082
	222	-273333333.333333300*	37505555.144093880	.000	-351568550.43332416	-195098116.23334253
	2222	-160000000.000000000*	37505555.144093880	.000	-238235217.09999081	-81764782.90000920
	11111	-33333333.333333330	37505555.144093880	.385	-111568550.43332414	-44901883.76665748
	22222	-96666666.666666670*	37505555.144093880	.018	-174901883.76665747	-18431449.56667586
	1	23333333.333333343	37505555.144093880	.541	-54901883.76665746	101568550.43332416
	2	-146666666.666666630*	37505555.144093880	.001	-224901883.76665744	-68431449.56667581
	11	96666666.666666690*	37505555.144093880	.018	18431449.56667588	174901883.76665750
	22	-73333333.333333310	37505555.144093880	.065	-151568550.43332413	-4901883.76665750
2222	111	130000000.000000030*	37505555.144093880	.002	51764782.90000922	208235217.09999084
	222	-113333333.333333310*	37505555.144093880	.007	-191568550.43332413	-35098116.23334251
	1111	160000000.000000000*	37505555.144093880	.000	81764782.90000920	238235217.09999081
	11111	126666666.666666690*	37505555.144093880	.003	48431449.56667588	204901883.76665750
	22222	63333333.333333340	37505555.144093880	.107	-14901883.76665746	141568550.43332416
	1	-103333333.333333340*	37505555.144093880	.012	-181568550.43332416	-25098116.23334254
	2	-273333333.333333300*	37505555.144093880	.000	-351568550.43332410	-195098116.23334250
	11	-30000000.000000000	37505555.144093880	.433	-108235217.09999081	-48235217.09999081
11111	22	-200000000.000000000*	37505555.144093880	.000	-278235217.09999080	-121764782.90000919
	111	3333333.333333343	37505555.144093880	.930	-74901883.76665747	-81568550.43332416
	222	-240000000.000000000*	37505555.144093880	.000	-318235217.09999080	-161764782.90000919
	1111	33333333.333333330	37505555.144093880	.385	-44901883.76665748	-111568550.43332414

	2222	-126666666.66666690*	37505555.144093880	.003	-204901883.76665750	-48431449.56667588
	22222	-63333333.333333340	37505555.144093880	.107	-141568550.43332416	14901883.76665746
	1	-40000000.000000000	37505555.144093880	.299	-118235217.09999081	38235217.09999081
	2	-209999999.99999970*	37505555.144093880	.000	-288235217.09999080	-131764782.90000916
	11	33333333.333333343	37505555.144093880	.385	-44901883.76665746	111568550.43332416
	22	-136666666.66666660*	37505555.144093880	.002	-214901883.76665747	-58431449.56667585
22222	111	66666666.666666690	37505555.144093880	.091	-11568550.43332412	144901883.76665750
	222	-176666666.66666660*	37505555.144093880	.000	-254901883.76665747	-98431449.56667584
	1111	96666666.666666670*	37505555.144093880	.018	18431449.56667586	174901883.76665747
	2222	-63333333.333333340	37505555.144093880	.107	-141568550.43332416	14901883.76665746
	11111	63333333.333333340	37505555.144093880	.107	-14901883.76665746	141568550.43332416

*. The mean difference is significant at the 0.05 level.

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	**WT 15 COL-GEN**	**WT 15 COL-TRI**	**SCV 15 COL-GEN**	**SCV 15 COL-TRI**	**N315 WT00**	**WT 15 N315-GEN**	**WT 15 N315-TRI**	**SCV 15 N315-GEN**	**SCV 15N315-TRI**

7- Dependent Variable: ***Growth rate***

LSD

(I) Strains	(J) Strains	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	2	-.0376667	.2138752	.861	-.463292	.387959
	11	.0839778	.2138752	.696	-.341647	.509603
	22	.0873444	.2138752	.684	-.338281	.512970
	111	.0899333	.2138752	.675	-.335692	.515559
1	222	.0402222	.2138752	.851	-.385403	.465847
	1111	.3797778	.2138752	.080	-.045847	.805403
	2222	.3587778	.2138752	.097	-.066847	.784403
	11111	.4028889	.2138752	.063	-.022736	.828514
	22222	.3708889	.2138752	.087	-.054736	.796514
	1	.0376667	.2138752	.861	-.387959	.463292
	11	.1216444	.2138752	.571	-.303981	.547270
	22	.1250111	.2138752	.561	-.300614	.550636
	111	.1276000	.2138752	.552	-.298025	.553225
2	222	.0778889	.2138752	.717	-.347736	.503514
	1111	.4174444	.2138752	.054	-.008181	.843070
	2222	.3964444	.2138752	.067	-.029181	.822070
	11111	.4405556*	.2138752	.043	.014930	.866181
	22222	.4085556	.2138752	.060	-.017070	.834181
	1	-.0839778	.2138752	.696	-.509603	.341647
	2	-.1216444	.2138752	.571	-.547270	.303981
	22	.0033667	.2138752	.987	-.422259	.428992
	111	.0059556	.2138752	.978	-.419670	.431581
11	222	-.0437556	.2138752	.838	-.469381	.381870
	1111	.2958000	.2138752	.170	-.129825	.721425
	2222	.2748000	.2138752	.203	-.150825	.700425
	11111	.3189111	.2138752	.140	-.106714	.744536
	22222	.2869111	.2138752	.184	-.138714	.712536
	1	-.0873444	.2138752	.684	-.512970	.338281
22	2	-.1250111	.2138752	.561	-.550636	.300614
	11	-.0033667	.2138752	.987	-.428992	.422259

	111	.0025889	.2138752	.990	-.423036	.428214
	222	-.0471222	.2138752	.826	-.472747	.378503
	1111	.2924333	.2138752	.175	-.133192	.718059
	2222	.2714333	.2138752	.208	-.154192	.697059
	11111	.3155444	.2138752	.144	-.110081	.741170
	22222	.2835444	.2138752	.189	-.142081	.709170
	1	-.0899333	.2138752	.675	-.515559	.335692
	2	-.1276000	.2138752	.552	-.553225	.298025
	11	-.0059556	.2138752	.978	-.431581	.419670
	22	-.0025889	.2138752	.990	-.428214	.423036
111	222	-.0497111	.2138752	.817	-.475336	.375914
	1111	.2898444	.2138752	.179	-.135781	.715470
	2222	.2688444	.2138752	.212	-.156781	.694470
	11111	.3129556	.2138752	.147	-.112670	.738581
	22222	.2809556	.2138752	.193	-.144670	.706581
	1	-.0402222	.2138752	.851	-.465847	.385403
	2	-.0778889	.2138752	.717	-.503514	.347736
	11	.0437556	.2138752	.838	-.381870	.469381
	22	.0471222	.2138752	.826	-.378503	.472747
222	111	.0497111	.2138752	.817	-.375914	.475336
	1111	.3395556	.2138752	.116	-.086070	.765181
	2222	.3185556	.2138752	.140	-.107070	.744181
	11111	.3626667	.2138752	.094	-.062959	.788292
	22222	.3306667	.2138752	.126	-.094959	.756292
	1	-.3797778	.2138752	.080	-.805403	.045847
	2	-.4174444	.2138752	.054	-.843070	.008181
	11	-.2958000	.2138752	.170	-.721425	.129825
	22	-.2924333	.2138752	.175	-.718059	.133192
1111	111	-.2898444	.2138752	.179	-.715470	.135781
	222	-.3395556	.2138752	.116	-.765181	.086070
	2222	-.0210000	.2138752	.922	-.446625	.404625
	11111	.0231111	.2138752	.914	-.402514	.448736
	22222	-.0088889	.2138752	.967	-.434514	.416736
	1	-.3587778	.2138752	.097	-.784403	.066847
	2	-.3964444	.2138752	.067	-.822070	.029181
	11	-.2748000	.2138752	.203	-.700425	.150825
	22	-.2714333	.2138752	.208	-.697059	.154192
2222	111	-.2688444	.2138752	.212	-.694470	.156781
	222	-.3185556	.2138752	.140	-.744181	.107070
	1111	.0210000	.2138752	.922	-.404625	.446625
	11111	.0441111	.2138752	.837	-.381514	.469736
	22222	.0121111	.2138752	.955	-.413514	.437736
	1	-.4028889	.2138752	.063	-.828514	.022736
	2	-.4405556*	.2138752	.043	-.866181	-.014930
	11	-.3189111	.2138752	.140	-.744536	.106714
	22	-.3155444	.2138752	.144	-.741170	.110081
11111	111	-.3129556	.2138752	.147	-.738581	.112670
	222	-.3626667	.2138752	.094	-.788292	.062959
	1111	-.0231111	.2138752	.914	-.448736	.402514
	2222	-.0441111	.2138752	.837	-.469736	.381514
	22222	-.0320000	.2138752	.881	-.457625	.393625
	1	-.3708889	.2138752	.087	-.796514	.054736
	2	-.4085556	.2138752	.060	-.834181	.017070
	11	-.2869111	.2138752	.184	-.712536	.138714
	22	-.2835444	.2138752	.189	-.709170	.142081
22222	111	-.2809556	.2138752	.193	-.706581	.144670
	222	-.3306667	.2138752	.126	-.756292	.094959
	1111	.0088889	.2138752	.967	-.416736	.434514
	2222	-.0121111	.2138752	.955	-.437736	.413514
	11111	.0320000	.2138752	.881	-.393625	.457625

*. The mean difference is significant at the 0.05 level.

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	**WT 15 COL-GEN**	**WT 15 COL-TRI**	**SCV 15 COL-GEN**	**SCV 15 COL-TRI**	**N315 WT00**	**WT 15 N315-GEN**	**WT 15 N315-TRI**	**SCV 15 N315-GEN**	**SCV 15N315-TRI**

8- Dependent Variable: ***Max cell***

LSD

(I) Strains	(J) Strains	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound

	2	-2333333333.333333500*	371280067.99300295	.000	-3107809983.86509040	-1558856682.80157660
	11	-66666666.666666985	371280067.99300295	.859	-841143317.19842390	-707809983.86508990
	22	300000000.000000000	371280067.99300295	.429	-474476650.53175680	-1074476650.53175690
1	111	466666666.666666500	371280067.99300295	.223	-307809983.86509030	-1241143317.19842340
	222	699999999.999999800	371280067.99300295	.074	-74476650.53175707	-1474476650.53175660
	1111	1933333333.333333000*	371280067.99300295	.000	1158856682.80157640	2707809983.86509000
	2222	1846666666.666666500*	371280067.99300295	.000	1072190016.13490960	2621143317.19842340
	11111	1489999999.999999800*	371280067.99300295	.001	715523349.46824290	2264476650.53175640
	22222	1940000000.000000000*	371280067.99300295	.000	1165523349.46824310	2714476650.53175700
	1	2333333333.333333500*	371280067.99300295	.000	1558856682.80157660	3107809983.86509040
	11	2266666666.666666500*	371280067.99300295	.000	1492190016.13490960	3041143317.19842340
	22	2633333333.333333500*	371280067.99300295	.000	1858856682.80157660	3407809983.86509040
	111	2800000000.000000000*	371280067.99300295	.000	2025523349.46824310	3574476650.53175700
2	222	3033333333.333333000*	371280067.99300295	.000	2258856682.80157660	3807809983.86509000
	1111	4266666666.666666500*	371280067.99300295	.000	3492190016.13490960	5041143317.19842300
	2222	4180000000.000000000*	371280067.99300295	.000	3405523349.46824300	4954476650.53175600
	11111	3823333333.333333000*	371280067.99300295	.000	3048856682.80157660	4597809983.86509000
	22222	4273333333.333333500*	371280067.99300295	.000	3498856682.80157660	5047809983.86509000
	1	66666666.666666985	371280067.99300295	.859	-707809983.86508990	-841143317.19842390
	2	-2266666666.666666500*	371280067.99300295	.000	-3041143317.19842340	-1492190016.13490960
	22	366666666.666667000	371280067.99300295	.335	-407809983.86508983	-1141143317.19842390
11	111	533333333.333333500	371280067.99300295	.166	-241143317.19842336	-1307809983.86509040
	222	766666666.666666700	371280067.99300295	.052	-7809983.86509009	-1541143317.19842360
	1111	2000000000.000000000*	371280067.99300295	.000	1225523349.46824340	2774476650.53175700
	2222	1913333333.333333500*	371280067.99300295	.000	1138856682.80157660	2687809983.86509040
	11111	1556666666.666666700*	371280067.99300295	.000	782190016.13490990	2331143317.19842340
	22222	2006666666.666667000*	371280067.99300295	.000	1232190016.13491000	2781143317.19842400
	1	-300000000.000000000	371280067.99300295	.429	-1074476650.53175690	-474476650.53175680
	2	-2633333333.333333500*	371280067.99300295	.000	-3407809983.86509040	-1858856682.80157660
22	11	-366666666.666667000	371280067.99300295	.335	-1141143317.19842390	-407809983.86508983
	111	166666666.666666500	371280067.99300295	.658	-607809983.86509040	-941143317.19842340

	222	399999999.9999997 60	371280067.9930029 50	.294	- 374476650.5317570 6	- 1174476650.531756 60
	1111	1633333333.333333 000*	371280067.9930029 50	.000	858856682.8015763 0	2407809983.8650900 0
	2222	1546666666.666666 500*	371280067.9930029 50	.000	772190016.1349096 0	2321143317.1984234 0
	11111	1189999999.9999998 00*	371280067.9930029 50	.004	415523349.4682429 4	1964476650.531756 60
	22222	1640000000.0000000 0*	371280067.9930029 50	.000	865523349.4682430 0	2414476650.531757 00
	1	- 466666666.666665 00	371280067.9930029 50	.223	- 1241143317.1984234 0	307809983.8650903 0
	2	- 2800000000.0000000 0*	371280067.9930029 50	.000	- 3574476650.5317570 0	- 2025523349.4682431 0
	11	- 533333333.333333 500	371280067.9930029 50	.166	- 1307809983.8650904 0	241143317.19842336
111	22	- 166666666.666666 500	371280067.9930029 50	.658	- 941143317.19842340	- 607809983.8650904 0
	222	233333333.333333 250	371280067.9930029 50	.537	- 541143317.19842360	1007809983.8650901 0
	1111	1466666666.666666 500*	371280067.9930029 50	.001	692190016.1349097 0	2241143317.1984234 0
	2222	1380000000.0000000 0*	371280067.9930029 50	.001	605523349.4682431 0	2154476650.531757 00
	11111	1023333333.333333 300*	371280067.9930029 50	.012	248856682.8015764 0	1797809983.8650901 0
	22222	1473333333.333333 500*	371280067.9930029 50	.001	698856682.8015765 0	2247809983.8650904 0
	1	- 699999999.9999998 00	371280067.9930029 50	.074	- 1474476650.5317566 0	74476650.53175707
	2	- 3033333333.333333 000*	371280067.9930029 50	.000	- 3807809983.8650900 0	- 2258856682.8015766 0
	11	- 766666666.666666 700	371280067.9930029 50	.052	- 1541143317.1984236 0	7809983.86509009
222	22	- 399999999.9999997 60	371280067.9930029 50	.294	- 1174476650.5317566 0	374476650.5317570 6
	111	- 233333333.333333 250	371280067.9930029 50	.537	- 1007809983.8650901 0	- 541143317.1984236 0
	1111	1233333333.333333 500*	371280067.9930029 50	.003	458856682.8015765 5	2007809983.8650901 0
	2222	1146666666.666666 700*	371280067.9930029 50	.006	372190016.1349099 0	1921143317.1984236 0
	11111	790000000.0000000 00*	371280067.9930029 50	.046	15523349.46824316	1564476650.531756 90
	22222	1240000000.0000000 0*	371280067.9930029 50	.003	465523349.4682433 0	2014476650.531756 90
	1	- 1933333333.333333 000*	371280067.9930029 50	.000	- 2707809983.8650900 0	- 1158856682.8015764 0
	2	- 4266666666.666666 500*	371280067.9930029 50	.000	- 5041143317.1984230 0	- 3492190016.1349096 0
1111	11	- 2000000000.0000000 0*	371280067.9930029 50	.000	- 2774476650.5317570 0	- 1225523349.4682434 0
	22	- 1633333333.333333 000*	371280067.9930029 50	.000	- 2407809983.8650900 0	- 858856682.8015763 0
	111	- 1466666666.666666 500*	371280067.9930029 50	.001	- 2241143317.1984234 0	- 692190016.1349097 0
	222	- 1233333333.333333 500*	371280067.9930029 50	.003	- 2007809983.8650901 0	- 458856682.8015765 5

	2222	-86666666.666666630	371280067.993002950	.818	-861143317.19842350	-687809983.86509030
	11111	-443333333.333333400	371280067.993002950	.246	-1217809983.86509010	-331143317.19842345
	22222	6666666.666666746	371280067.993002950	.986	-767809983.86509010	-781143317.19842360
	1	-1846666666.666666500*	371280067.993002950	.000	-2621143317.19842340	-1072190016.13490960
	2	-4180000000.000000000*	371280067.993002950	.000	-4954476650.53175600	-3405523349.46824300
	11	-1913333333.333333500*	371280067.993002950	.000	-2687809983.86509040	-1138856682.80157660
	22	-1546666666.666666500*	371280067.993002950	.000	-2321143317.19842340	-772190016.13490960
2222	111	-1380000000.000000000*	371280067.993002950	.001	-2154476650.53175700	-605523349.46824310
	222	-1146666666.666666700*	371280067.993002950	.006	-1921143317.19842360	-372190016.13490990
	1111	86666666.666666630	371280067.993002950	.818	-687809983.86509030	-861143317.19842350
	11111	-356666666.666666750	371280067.993002950	.348	-1131143317.19842360	-417809983.86509010
	22222	93333333.333333370	371280067.993002950	.804	-681143317.19842350	-867809983.86509030
	1	-1489999999.999999800*	371280067.993002950	.001	-2264476650.53175640	-715523349.46824290
	2	-3823333333.333333000*	371280067.993002950	.000	-4597809983.86509000	-3048856682.80157660
	11	-1556666666.666666700*	371280067.993002950	.000	-2331143317.19842340	-782190016.13490990
	22	-1189999999.999999800*	371280067.993002950	.004	-1964476650.53175660	-415523349.46824294
11111	111	-1023333333.333333300*	371280067.993002950	.012	-1797809983.86509010	-248856682.80157640
	222	-790000000.000000000*	371280067.993002950	.046	-1564476650.53175690	-15523349.468243116
	1111	443333333.333333400	371280067.993002950	.246	-331143317.19842345	-1217809983.86509010
	2222	356666666.666666750	371280067.993002950	.348	-417809983.86509010	-1131143317.19842360
	22222	450000000.000000100	371280067.993002950	.240	-324476650.53175670	-1224476650.53175690
	1	-1940000000.000000000*	371280067.993002950	.000	-2714476650.53175700	-1165523349.46824310
	2	-4273333333.333333500*	371280067.993002950	.000	-5047809983.86509000	-3498856682.80157660
22222						
	11	-2006666666.666667000*	371280067.993002950	.000	-2781143317.19842400	-1232190016.13491000
	22	-1640000000.000000000*	371280067.993002950	.000	-2414476650.53175700	-865523349.46824300

111	-1473333333.333333500*	371280067.993002950	.001	-2247809983.86509040	-698856682.80157650
222	-1240000000.000000000*	371280067.993002950	.003	-2014476650.53175690	-465523349.46824330
1111	-6666666.666666746	371280067.993002950	.986	-781143317.19842360	767809983.86509010
2222	-93333333.333333370	371280067.993002950	.804	-867809983.86509030	681143317.19842350
11111	-450000000.000000100	371280067.993002950	.240	-1224476650.53175690	324476650.53175670

*. The mean difference is significant at the 0.05 level.

1	11	111	1111	11111	2	22	222	2222	22222
COL-WT 00	**WT 15 COL-GEN**	**WT 15 COL-TRI**	**SCV 15 COL-GEN**	**SCV 15 COL-TRI**	**N315 WT00**	**WT 15 N315-GEN**	**WT 15 N315-TRI**	**SCV 15 N315-GEN**	**SCV 15N315-TRI**