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NATIONAL HEALTH LABORATORY SERVICE

DIVISION OF MEDICAL MICROBIOLOGY

UNIVERSITY OF CAPE TOWN
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

THE IDENTIFICATION AND CHARACTERIZATION OF A CLINICAL ISOLATE OF A METHICILLIN SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* ST612

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Declaration

I, Malefu Moleleki, hereby declare that the work on which this dissertation/ thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Thank you and May God bless.

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List of Abreviations

CA-MRSA	Community-associated MRSA
CC	Clonal Complex
CFU	Colony-forming-units
CLSI	Clinical Laboratory Standard Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EtBr	Ethidium bromide
GSH	Groote Schuur Hospital
$H_{481}N$	Histidine (H) at position 481 substituted by Asparagine (N)
HA-MRSA	Hospital-associated MRSA
$I_{527}M$	Isoleucine (I) at position 527 substituted by Methionine (M)
KZN	KwaZulu Natal
MDR	Multi-drug resistant
$MgCl_2$	Magnesium chloride
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMS	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NHLS	National Health Laboratory Services
OD	Optical Density

University of Cape Town

PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine Leukocidin
RNA	Ribonucleic acid
RRDR	Rifampicin resistance determining region
rRNA	ribosomal Ribonucleic acid
SCC*mec*	Staphylococcus Cassette Chromosome *mec*
ST	Sequence Type
UV	Ultraviolet
v/v	volume per volume
w/v	Weight per volume

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Overall Abstract

A previous study of 100 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, collected between January 2007 and December 2008 from five Cape Town hospitals, identified ST612-MRSA-IV as the predominant MRSA. This raised the question of whether methicillin-susceptible *S. aureus* (MSSA) isolates with the same sequence type (ST) were present in hospitals in this city. Nineteen MSSA isolates, collected during the same period, and from four of the hospitals in Cape Town, as the previously characterized MRSA isolates, were screened for the presence of ST612-MSSA. *spa* typing and multi-locus sequence typing (MLST) identified one isolate, MS14, as ST612-MSSA, *spa* type t064.

MS14 was resistant to multiple antibiotics, including rifampicin, as was observed in ST612-MRSA-IV. Characterization of MS14 identified remnants of SCC*mec,* within the *attB* site, which included the *dcs* region 1.7kb downstream of *orfX*. An investigation of the mechanism of rifampicin resistance in MS14 showed that the resistant isolates and MS14 share an uncommon *rpoB* genotype, and a unique single nucleotide mutation within this gene, suggesting that MS14 resulted from a partial loss of SCC*mec* from a corresponding ST612-MRSA strain.

As studies have shown that the carriage of SCC*mec* may impose a fitness cost on its host, MS14 was competed against four representative ST612-MRSA-IV isolates in 24-hour and five-day pairwise assays. Three of the resistant strains were outcompeted by MS14, suggesting that the carriage of SCC*mec* IV may impose a fitness cost on these isolates. On the other hand, the remaining resistant isolate competed successfully with MS14, suggesting that it may have evolved to accommodate the presence of SCC*mec* IV in its genome.

University of Cape Town

Table of Contents

University of Cape Town

University of Cape Town

University of Cape Town

University of Cape Town

University of Cape Town

List of Figures

University of Cape Town

Figure 3.2: Representations of imprecise excision of SCC*mec,* adapted from Shore, et al. (2008). **1**, 33kb SCC*mec* IID prototype; **2**, 26kb remnant of SCC*mec* IID from GrMSSA isolate M06/0075 after loss of the *mec* complex and neighboring genes from the parent strain; **3**, 1.7kb remnant, *dcs-orfX* fragment of the SCC*mec*, from the GrMSSA isolate M04/0269; **4**, 7.5kb remnant, *ccr4-orfX* fragment of SCC*mec*, from the GrMSSA isolates M02/0021, M05/0146, M06/0004, M06/0288.

Figure 3.3: A schematic of SCC*mec* IV outlining the genes that were targeted for amplification. SCC*mec* is enclosed between *attL* and *attR* which are the left and right junctions respectively, between SCC*mec* and the chromosome. The conserved region, *orfX*, is indicated downstream of *attR* on the chromosome. The primer pairs for the amplification of each region are represented by the small black and grey arrows.

Figure 3.4: Gel electrophoresis of the *attB* amplicons. H1 and H4, molecular weight markers, Hyperladder 1^{TM} and HyperLadder IV^{TM} respectively (Celtic Molecular Diagnostics, Cape Town, South Africa). H_2O, the water control which was used as a DNA negative control for the reaction.

Figure 3.5: Gel electrophoresis of the PCR products for the detection of remnants of SCC*mec* in MS14. The internal loci (*mecA*, *dru*, *ccrB2* and *dcs*) of SCC*mec* were targeted for amplification. ST612-MRSA-IV was included as a positive control for the amplification of *mecA*, *ccrB2* and *dcs* while MRSA COL was included as a positive control for *dru*. MSSA NCTC 8325 was included in the PCR assays as a negative control for all four loci. H1 and H4, molecular weight markers, Hyperladder 1^{TM} and HyperLadder IV^{TM} respectively (Celtic Molecular Diagnostics, Cape Town, South Africa). H_2O, the water control which was used as a DNA negative control for the reaction.

Figure 3.6: Gel electrophoresis of the amplicons for the determination of the location of *dcs* in MS14. ST612-MRSA-IV (SCC*mec* IVd) and MRSA MW2 (SCC*mec* IVa) were used as positive controls while MSSA NCTC 8325 was used as a negative control. H1, molecular weight marker, Hyperladder 1^{TM} (Celtic Molecular Diagnostics, Cape Town, South Africa). H_2O, the water control which was used as a DNA negative control for the reaction.

Figure 3.7: Alignment of the internal fragment of the rifampicin-resistance determining region of *rpoB* nucleotide sequences and the corresponding amino acid sequences of MS14, ST612-MRSA-IV and a *rpoB* reference sequence (X64172 from a rifampicin susceptible *S. aureus* RN4220). Clusters I and II are sequences internal to the RRDR where most mutations associated with rifampicin resistance occur**.**

Figure 4.1: The dilution series for the quantification of the viable cell count. The glycerol stocks were diluted six fold at a 1: 10 ratio in PBS solution on a 96-well round bottom micro-titre plate (Adcock Ingram, Johannesburg, South Africa), represented by the blue vials. 10 µl of the 10^4 to 10^6 dilutions was plated in duplicate on 2YT agar, and then incubated overnight at 37°C.

Figure 4.2: The serial dilution of the glycerol stocks for each strain. The stocks were initially diluted to 10^5 CFUml^{-1} in 1 XPBS then 25 µl transferred into 225 µl of 2YT broth to a final concentration of 10^4 CFUml^{-1}. Concurrently with the comparison of growth rates, the

University of Cape Town

viability of the cells was monitored following storage at -80°C. Broken arrow indicates a series of steps which included the dilution and subsequent plating of the samples to monitor the viable cell count.

Figure 4.3: Flow diagram depicting the 24-hour pairwise competition assays with MS14 and the ST612-MRSA-IV strains. 100 µl of 10^6 $CFUml^{-1}$ each of the resistant and susceptible strains was inoculated into 10 ml 2YT broth in a 100 ml Erlenmeyer flask, to give 10^5CFUs, then incubated on a shaker (100 rpm) at 37°C. At two hour intervals, 20 µl of the culture was removed, diluted and 10 µl of the respective dilutions plated, in parallel, on non-selective 2YT agar and on selective 2YT agar media containing 4 µg/ml cefoxitin (as per CLSI guidelines (CLSI 2009)). 10^x, 10^y and 10^z, the dilution factors for the respective dilutions plated. All the experiments were carried out in duplicate.

Figure 4.4: The flow diagram depicting the five-day pairwise competition assays with MS14 and the ST612-MRSA-IV strains. 100 µl of 10^6 $CFUml^{-1}$ each of the resistant and susceptible strains was inoculated into 10 ml 2YT broth in a 100 ml Erlenmeyer flask, to give 10^5CFUs, then incubated on a shaker (100 rpm) at 37°C. At 24-hour intervals, 20 µl of the culture was removed, diluted and 10 µl of the respective dilutions plated, in parallel, on non-selective 2YT agar and on selective 2YT agar media containing 4 µg/ml cefoxitin (as per CLSI guidelines (CLSI 2009)). 100 µl of the 10^4 dilution of the 24-hour culture was transferred into 10 ml fresh 2YT broth in a 100 ml Erlenmeyer flask, incubated under the same conditions as before, in a serial passage over a four day period. 10^x, 10^y and 10^z, the dilution factors for the respective dilutions plated. All the experiments were carried out in duplicate.

Figure 4.5: The growth parameters of the individual strains, based on 0D 600 nm measurements over time. For all strains, the exponential growth occured between 2 and 3.5 hours, as indicated by the grey bar on the graph.

Figure 4.6: Comparative growth rates between ST612-MRSA-IV strains and MS14 over a 24-hour period. The exponential phase of the strains is indicated in grey on the graph, the values of which were used to compare the growth rates of the resistant strains relative to MS14.

Figure 4.7: The average growth rates of the strains. Average growth rates, with error bars (mean ± standard deviation), were calculated based on growth of the strains at exponential phase (OD 0.2 – OD 0.6) as depicted in Fig 4.6. Each value represents the mean of six independent cultures per isolate.

Figure 4.8: The pairwise competition assays between MS14 and ST612-MRSA-IV strains, carried out over a 24 hour period. The viable cell count of the susceptible strain in each assay was calculated by subtracting the viable cell count of the resistant strain from the total viable cell count in the mixed culture. Each assay was carried out in duplicate and the data represented as an average of the two experiments.

Figure 4.9: The growth curves for pairwise competition assays between ST612-MRSA-IV strains and MS14 during five-day serial passage experiments. The viable cell count of the susceptible strain in each assay was calculated by subtracting the viable cell count of the

University of Cape Town

resistant strain from the total viable cell count in the mixed culture. Each assay was carried out in duplicate and the data represented as an average of the two experiments.

Figure 4.10: The relative fitness of MS14 in comparison to the resistant strains, D4, E3, E8 and S5. Relative fitness was calculated, every 24 hours, as the ratio between the average number of generations of the individual resistant strains and MS14 for the duration of the five-day competition assays (Table A3.2.3). Each assay was carried out in duplicate.

Figure 4.11: *Sma*I restriction profiles of MS14 and ST612-MRSA-IV isolates, D4, E3, E8 and S5. Yellow arrow shows a fragment present in D4 but absent in MS14.

University of Cape Town

List of Tables

University of Cape Town

Literature review

University of Cape Town

1.1 Staphylococci: Bacteriology

Staphylococci are Gram positive, facultative anaerobic cocci which form golden or white colonies on nutrient agar. Gram stain microscopy reveals singles, pairs, tetrads, short chains or irregular 'grape-like' clusters of the cocci. Members of this genus are part of the normal microflora inhabiting skin, skin glands and mucous membranes of mammals, although they have occasionally been isolated from birds as well. Staphylococci are traditionally classified based on their ability to produce coagulase, an extracellular protein that facilitates clotting of blood plasma. Of over 32 species belonging to this genus, at least five species including *Staphylococcus aureus* and *Staphylococcus intermedius* have been identified as coagulase-positive while the rest are coagulase-negative (Kloos 1998).

S. aureus is an opportunistic pathogen causing infections in both immuno-competent and immuno-compromised individuals. This organism is carried mainly in the nasal cavity but can also be present on the skin, in the skin glands, in the oral cavity as well as in the gastrointestinal tract. It has been suggested that *S. aureus* may be carried asymptomatically in 20% of individuals at any given time, and transiently in 60% of the population, while the remaining 20% is rarely or never colonised (von Eiff, Becker et al. 2001; Pynnonen, Stephenson et al. 2011).

Colonisation by *S. aureus* provides a reservoir for subsequent infection if the host's defences are breached. Colonisation is a complex process that involves the host's interaction with *S. aureus* and the ability of the bacteria to adhere to the host's cells and to evade immune detection. A break in the skin or the mucous barrier predisposes to simple skin infections or serious life threatening infections such as pneumonia, meningitis, osteomyelitis,

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endocarditis, toxic shock syndrome (TSS), bacteremia and sepsis, both in the community and hospitals (Kloos 1998; Noble 1998; Gordon and Lowy 2008).

Coagulase-negative staphylococci (CNS) are generally less pathogenic than *S. aureus* however, some species are potentially pathogenic particularly to immuno-compromised individuals. Minor infections such as *Staphylococcus saprophyticus* urinary tract infections (UTIs) in males and females have been described. On the other hand, *Staphylococcus epidermidis* can be the cause of life threatening infections such as endocarditis which is often associated with implantation of foreign devices (Noble 1998; Petti, Simmon et al. 2008).

1.2 Virulence factors of *S. aureus* and their role in pathogenesis

Pathogenesis of infection is facilitated by a host of virulence factors which can be both structural and secreted products of *S. aureus*. In establishing infection, *S. aureus* utilises its surface proteins called "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) which mediate adherence to host tissues (Foster and Hook 1998; Gordon and Lowy 2008). These recognise and bind several host factors such as fibrinogen, fibronectin, and collagen while different MSCRAMMs may adhere to the same host-tissue component.

MSCRAMMs are expressed early in infection in order to initiate colonisation of specific tissue sites with *S. aureus*. To facilitate spread, a host of toxins are released, including enzymes such as proteases, lipases and elastases which are useful in host cell invasion and damage as well as the spread of *S. aureus.* Also critical in pathogenesis is the release of proteins that inhibit chemotaxis, resulting in impairment of the mechanisms of recruitment of macrophages as well as neutrophils to the site of infection (Fig1.1). Another role player in

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immune evasion is the release of leukocidins such as the Panton-Valentine leukocidin (PVL), which punctures holes in the membranes of leukocytes (Gordon and Lowy 2008).

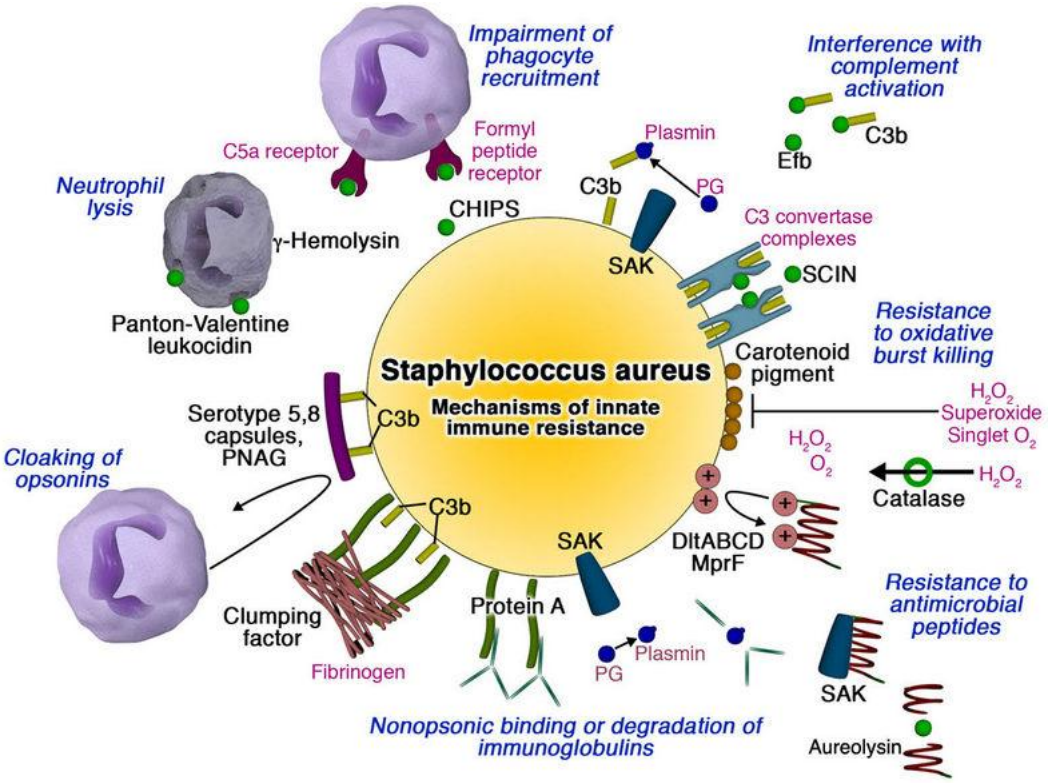

Figure 1.1: A diagrammatic representation, adopted from Nizet (2007), of *S .aureus* mechanisms of innate immune resistance by production of adherence factors that adhere to the host tissues and release of virulence factors that interfere with the host's defences.

To avoid metabolic stress in the organism, expression and release of virulence factors is controlled by cell density, energy availability and environmental signals (Novick 2003). Expression of MSCRAMMs occurs during the exponential growth phase while toxins are released during the stationary phase (Gordon and Lowy 2008). There are several mechanisms that *S. aureus* uses for regulation of the expression of its virulence factors. For instance, the accessory gene regulator (*agr*), which works in conjunction with, or antagonistically to, two-component systems, such as *sae*, *arlRS* and *srrAB*/*srhSR,* plays a key role in a complex regulatory network involved in the signalling pathway for the regulation of the virulence factors (Novick 2003). *agr* is a quorum-sensing system which responds to signals from the external environment and coupled with the energy metabolism of the cell,

facilitates global regulation of a subset of the virulence factors in a time and quantity dependent manner (Novick 2003).

It is important to note that not all *S. aureus* strains have the same repertoire of virulence genes; in fact, certain clones are associated with particular virulence genes (Gordon and Lowy 2008). For instance, PVL, encoded by *luk*F-PV and *luk*S-PV genes located on at least eight different phages (Zhang, Ito et al. 2011), has been associated with hypervirulent community associated methicillin-resistant *S. aureus* (CA-MRSA) strains. These hypervirulent CA-MRSA, such as USA400, are associated with life threatening infections such as necrotising pneumonia even in previously healthy individuals (Vandenesch, Naimi et al. 2003; Monecke, Coombs et al. 2011).

1.3 Overview of antibiotic resistance in *S. aureus*

Over the past few decades, there has been an increase in the occurrence of multi-drug resistant *S. aureus* strains, identified both in the community and in health care settings (Lyon and Skurray 1987; Enright, Robinson et al. 2002; Deurenberg and Stobberingh 2008; Boucher and Corey 2008). Emergence of antibiotic resistance in bacteria is attributed to the selective pressure imposed by antibiotic therapy (Lyon and Skurray 1987). This is evidenced by the emergence of resistance following the introduction of an antibiotic into clinical use (Clatworthy, Pierson et al. 2007).

One of the factors which has made *S. aureus* a successful human pathogen is the ability of the bacteria to adapt to a wide range of antibiotics (Nübel, Dordel et al. 2010). Table 1.1 outlines mechanisms of resistance of *S. aureus* to some clinically useful antibiotics.

University of Cape Town

Table 1.1: Mechanisms of resistance to some clinically useful antibiotics in *S. aureus*.

Antibiotic	**Class**	**Target**	**Resistance mechanism**	**Location**
Benzyl- penicillins (penicillin) Semi-synthetic penicillins (methicillin)	β-lactams	PBPs	Production of β-lactamase (BlaZ) which hydrolyses the β-lactam nucleus	Plasmid: Transposon
			Synthesis of acquired PBP2a (MecA) which has reduced affinity for semi-synthetic β-lactams	Chromosome : SCC*mec*
Erythromycin Clindamycin Streptogramin B	Macrolide Lincosamide Streptogramins	23SrRNA	Methylation of the 23SrRNA ribosomal subunit by erythromycin ribosomal methylases (ErmA, B, C) reduces its affinity for erythromycin, clindamycin and streptogramin B.	Plasmid, Chromosome
Rifampicin	Rifamycins	RpoB	Modification of the RNA polymerase due to amino acid substitutions in the β-subunit reduces its affinity for rifamycins	Chromosome
Ciprofloxacin	Fluoroquinolone	Gyrases (GyrA, B) Topoisomerase IV (GrlA)	Mutations in the targets reduce their affinity for fluoroquinolones	Chromosome
		Target independent	Overexpression of NorA results in active efflux of fluoroquinolones.	Chromosome
Vancomycin	Glycopeptide	D-Ala-D-Ala	Altered cross-linking of peptidoglycan results in an increase in the quantities of D-Ala-D-Ala residues exposed to vancomycin. Excess dipeptides bind and trap vancomycin in the cell wall but do not interfere with cell wall synthesis.	Chromosome
			Synthesis of peptidoglycan precursors terminating in D-Ala-D-Lac (*vanA* operon), which have reduced affinity for vancomycin.	Plasmid: Transposon
Linezolid	Oxazolidine	23SrRNA	Modifications in domain V of the 23SrRNA of the 50S ribosomal subunit, either by mutations or by products of *cfr*, result in decreased affinity for the antibiotic.	Plasmid, Chromosome

Prepared from data derived from Walsh (2003), Lowy (2003), Lyon and Skurray (1987), Kaatz, Seo et al. (1993) Markham and Neyfakh (1996), Mrales, Picazo et al. (2010) and LaMarre, Locke et al. (2011) .

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As the studies described in this thesis relate to methicillin-resistant *S. aureus* (MRSA), resistance to β-lactams, and methicillin in particular, will be described more fully.

1.3.1 Resistance to β-lactams in *S. aureus*

Resistance to β-lactams in *S. aureus* was reported in 1942, two years after the introduction of penicillin into clinical use. It has since been reported that 80% of all *S. aureus* strains are resistant to penicillin (Deurenberg and Stobberingh 2008). β-lactams inhibit cell wall synthesis by binding to penicillin binding proteins, PBPs, transpeptidases and carboxypeptidases which catalyse cross-linking of peptidoglycan during cell wall synthesis (Essack 2001).

β-lactams have a four-atom ring, the β-lactam ring, common to all β-lactams including penicillins and cephalosporins (Fig 1.2).

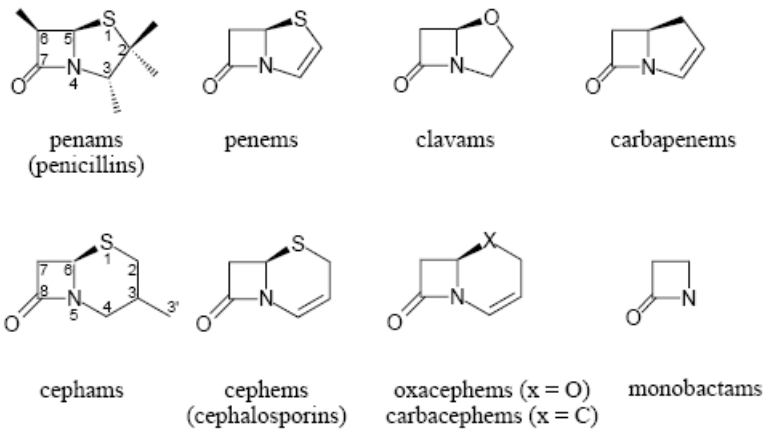

Figure 1.2: The core structure of some major classes of β-lactam antibiotics. Adopted from Andersson, et al. (2001).

This β-lactam ring is the basic structure of monobactams and is the nucleus of all β-lactams, where it can be fused to a five-membered thiazolidine ring as in the case of penicillins, or to a six-membered dihydrothiazine ring as in cephalosporins (Fig 1.2) (Andersson, Terwisscha

van Scheltinga et al. 2001). The β-lactam ring is the chemically reactive moiety of the antibiotic as it acylates the serine side chains in the active sites of PBPs. As β-lactams are pseudo-substrates for PBPs, the hydrolysis of the β-lactam ring is partial, blocking the active site of the PBPs, thus inhibiting activity of the enzymes in the cross-linking of peptidoglycan (Walsh 2003).

One mechanism of resistance to penicillin in *S. aureus* is through the production of an extracellular penicillin-specific β-lactamase, penicillinase, which hydrolyses the β-lactam ring of benzyl-penicillins. Penicillinase is a serine β-lactamase, so called due to the serine-dependent catalytic mechanism of the enzyme. Genes encoding these enzymes in *S. aureus* can be either plasmid (*blaZ*) or chromosomally (*PC1*) located (Lyon and Skurray 1987; Hall and Barlow 2004).

Expression of BlaZ is regulated by a repressor BlaI and a transmemberane transducer BlaR1. In the absence of penicillin, BlaI represses the transcription of *blaZ* and *blaR1-blaI*. In the presence of penicillin, BlaR1 is autoactivated and cleaves BlaI thereby releasing it from the *blaZ* operator region. With the cleavage of BlaI, *blaZ* is transcribed, resulting in the hydrolysis of the antibiotic in the extracellular space (Lyon and Skurray 1987; Lowy 2003).

In the early 1960s, penicillinase resistant semi-synthetic penicillins, such as methicillin, were introduced for the treatment of *S. aureus* infections (Noble 1998). Semi-synthetic penicillins are structural analogues of the benzyl-penicillins with modifications to the core β-lactam structure (Fig 1.3).

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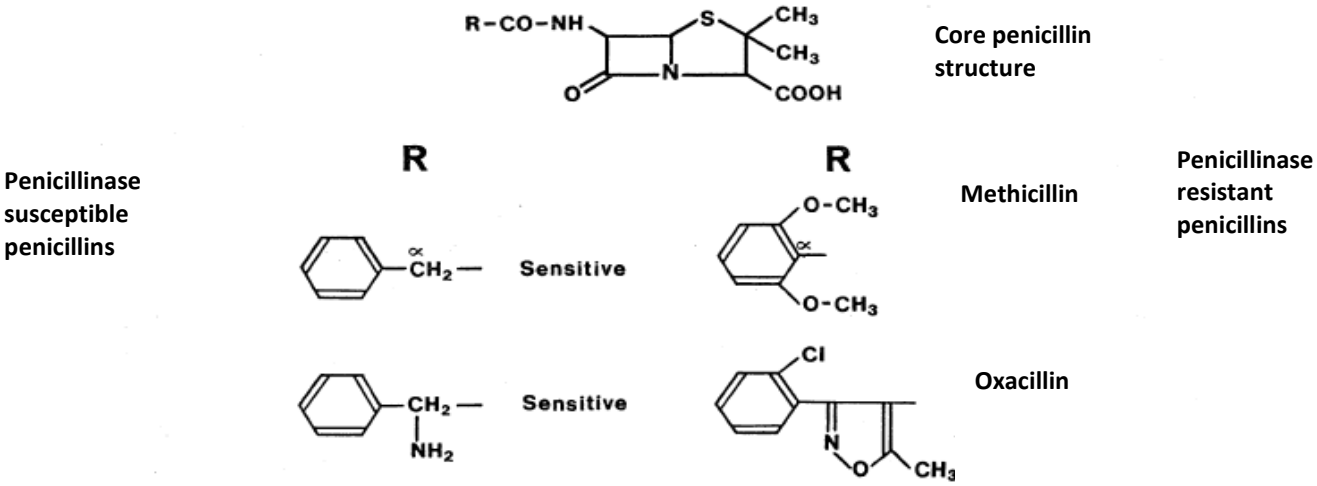

Figure 1.3: The differences in the structural modifications on the side chains of penicillins which determine susceptibility of the antibiotics to β-lacatamses. Adapted from O'Callaghan (1980).

These modifications to the β-lactam nucleus, such as, direct linking of a bulky side chain to the carbon (Fig 1.3), replacing the sulphur by a carbon or, the enlargement of the fused ring to a six-membered heterocyclic ring, as in the case of caphalosporins, increase sterric hindrance that inhibits binding of the penicillinase, which the less bulky penicillinase susceptible penicillins are unable to prevent (Walsh 2003; O'Callaghan 1980).

1.3.1.1 Resistance to methicillin

Soon after the introduction of methicillin into clinical use, resistance to this antibiotic was reported in some clinical isolates of *S. aureus* (Rolinson 1961; Çetin and Ang 1962; Noble 1998; Enright, Robinson et al. 2002; Deurenberg and Stobberingh 2008). Resistance to methicillin follows the acquisition of *mecA*, which encodes PBP2a (PBP2'), a penicillin binding protein that has a low affinity for methicillin, enabling cell-wall synthesis to continue. The *mecA* gene is embedded in a large genetic element (20kb to 64kb) called the Staphylococcal cassette chromosome *mec* (SCC*mec*). This element is integrated at *attB*, an integration site at the 3'end of an open reading frame, *orfX,* a conserved region that encodes a 23s rRNA methyltransferase in *S. aureus* (Shore, Deasy et al. 2011).

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1.3.1.1.1 Integration of SCC*mec*

Integration of SCC*mec* at the *attB* site is mediated by site specific recombinase enzymes, CcrA, CcrB and CcrC, encoded by the cassette chromosome recombinase gene, *ccr*. CcrA and CcrB are members of the serine resolvase and invertase family and contain catalytic and DNA-binding domains; the former can act as both an integrase and an excisase. For integration, the recombinase enzymes recognize a core 15bp nucleotide sequence, present in *attB*, which is then recombined with a homologous sequence (*attS*) on SCC*mec* (Fig 1.4). After recombination, the attachment sites are duplicated on either side of the SCC*mec* to create left (*attL*) and right (*attR*) junctions between SCC*mec* and the chromosome (Katayama, Ito et al. 2000; Noto and Archer 2006; Noto, Kreiswirth et al. 2008; Wang and Archer 2010).

Cape Town

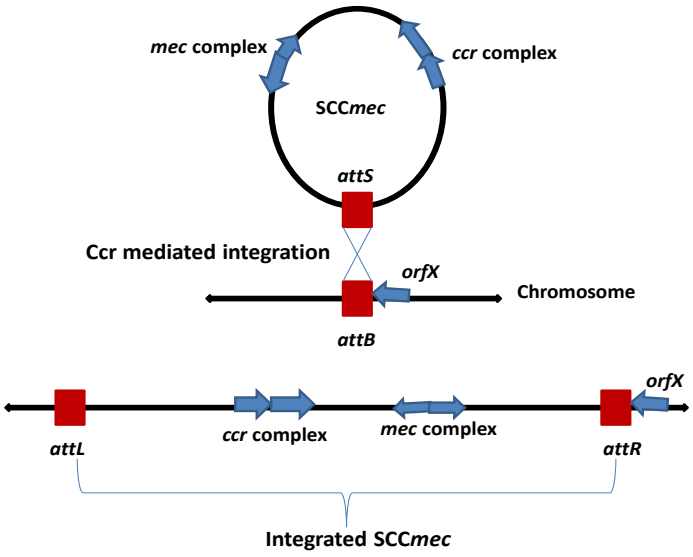

Figure 1.4: Integration of SCC*mec* at the *attB* site on the *S. aureus* chromosome. The recombinase enzymes encoded by the *ccr* recognize the homologous 15bp sequences on the SCC*mec* (*attS*) and the chromosome (*attB*). Following recombination, the 15bp sequences, *attR* and *attL* flank the SCC*mec* to create left and right junctions between SCC*mec* and the chromosome.

While the 15bp nucleotide core sequence of *attB* plays a key role in the integration of SCC*mec*, Noto, et al. (2008) considered the importance of DNA sequences outside of *attB*, in

the non-*orfX* side, in recombinase functionality. DNA sequences, contiguous with *attB* in the non-*orfX* region, from MSSA NCTC 8325 and five MRSA strains (MRSA COL, N315, NRS384, J39 and MRSA252) known to respond positively to Ccr-mediated recombinations, and from MRSA MW2, which contains SCC*mec* that cannot be excised, were used as comparators (Noto, Kreiswirth et al. 2008). The sequences (102bp) of MSSA NCTC 8325 and four of the MRSA were identical. A portion of this sequence (12bp), next to *attB*, is present in MRSA252; MRSA MW2 does not contain the conserved sequence. Of the 11 MSSA included in the comparison, only one shared the sequence (12bp) conserved in MRSA252. The authors concluded that the *attB* flanking sequence in the non-*orfX* region may play a role in the functionality of Ccr and therefore the acquisition of SCC*mec*. Important, also, was the observation that *attB* was not only a frequent site for the acquisition of foreign DNA, other than SCC*mec*, but also a 'hot spot' for recombination events and rearrangements of acquired sequences (Noto, Kreiswirth et al. 2008).

1.3.1.1.2 SCC*mec* types

To the best of the author's knowledge, eleven SCC*mec* types (I-XI) have been identified and extensively characterized in *S. aureus* (Ito, Katayama et al. 2001; Ito, Ma et al. 2004; Oliveira, Milheirico et al. 2006; Higuchi, Takano et al. 2008; Zhang, McClure et al. 2009; IWG-SCC 2009; Shore, Deasy et al. 2011; Garcia-Alvarez, Holden et al. 2011). The diversity in the genetic organisation of the different SCC*mec* types is classified based on the different classes of the *mec* gene complex (A,B, D, C1, C2, D and E), allotypes of the *ccr* gene complex (1, 2, 3-8) and the components of the junkyard regions (*J1-3*) (Ito, Katayama et al. 2001; Oliveira and de Lencastre 2002; Zhang, McClure et al. 2009; Shore, Deasy et al. 2011; Garcia-Alvarez, Holden et al. 2011).

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1.4 Epidemiology of *S. aureus*

S. aureus has been reported as a leading cause of hospital-acquired infections, resulting in disease in 2% of all newly admitted patients (Nubel, Roumagnac et al. 2008). It has been reported that 400 000 patients contract *S. aureus* infections in American health-care settings every year (Boucher and Corey 2008). In recent years, MRSA has also established itself as a common cause of community-acquired infections in persons who do not have health-care associated risks (Grundmann, Aires-de-Sousa et al. 2006).

1.4.1 Molecular tools to study the epidemiology of *S. aureus*

Molecular epidemiological analyses of *S. aureus* have played a key role in the understanding of both the local and global epidemiology of MRSA (Bannerman, Hancock et al. 1995; Enright, Day et al. 2000; Oliveira and de Lencastre 2002; Enright, Robinson et al. 2002; Harmsen, Claus et al. 2003; Strommenger, Kettlitz et al. 2006; Grundmann, Aires-de-Sousa et al. 2006; Goering, Shawar et al. 2008). Several typing techniques have been developed for the molecular characterisation of *S. aureus*. These techniques are generally based on either DNA sequences or gel electrophoresis profiles. The choice of typing technique depends on the discriminative power of the technique and the information required. In order to study the molecular evolution of *S. aureus* on a global level, DNA sequence based tests, including multi-locus sequence typing (MLST), are ideal. This approach facilitates standardisation of test protocols which further enables the adoption of a common nomenclature. The data generated can therefore be synchronised to a central database and be shared between laboratories (Harmsen, Claus et al. 2003). For the investigation of hospital outbreaks or hospital to hospital transmission of *S. aureus*, a highly discriminative tool, such as pulsed field gel electrophoresis (PFGE), is required. This technique has been

University of Cape Town

considered the gold standard for such studies (Koreen, Ramaswamy et al. 2004; Deurenberg and Stobberingh 2008).

1.4.1.1 Pulsed field gel electrophoresis (PFGE)

In PFGE, following the digestion of *S. aureus* intact DNA with *Sma*I*,* which recognises and cleaves at a few sites on the chromosome, the resulting fragments, being too large to be separated by conventional agarose gel electrophoresis, are separated by agarose gel electrophoresis in an electric field with an alternating voltage gradient (Bannerman, Hancock et al. 1995; Deurenberg and Stobberingh 2008). The banding patterns obtained are then analysed using a variety of dedicated software packages to determine the relatedness between strains. Alternatively, visual inspection of the PFGE patterns, as described by Tenover, Arbeit et al (1994), has been used to analyse profiles. Based on the differerences in the number of bands, isolates are determined to be either related or unrelated. The fact that PFGE is based on the comparison of whole genomes of the isolates makes it invaluable in indexing genotypic variations that accumulate rapidly (Bannerman, Hancock et al. 1995; Koreen, Ramaswamy et al. 2004). The limitations of the technique include the lack of standardisation of protocols that prevents inter-laboratory comparison of data (Bannerman, Hancock et al. 1995; Enright, Day et al. 2000; Deurenberg and Stobberingh 2008), limiting the usefulness of the technique to local outbreak investigations.

1.4.1.2 Multi-locus sequence typing (MLST)

MLST is based on the DNA sequences of internal fragments of seven highly conserved housekeeping genes which are listed on Table 1.2 (Enright, Day et al. 2000; Enright, Robinson et al. 2002; Deurenberg and Stobberingh 2008).

University of Cape Town

Table 1.2 The seven loci that are used to define the sequence type (ST) of *S. aureus* strains

Gene	*Protein product*
arcC	Carbamate kinase
aroE	Shikimate dehydrogenase
glpF	Glycerol kinase
gmk	Guanylate kinase
pta	Phosphate acetyltransferase
tpi	Triosephosphate isomerase
yqiL	Acetyl coenzyme A acetyltransferase

MLST is a three step process: 1) PCR amplification of the internal fragments of each of the seven loci; 2) sequencing of the amplicons; and 3) analysis of the sequencing data on the MLST website (http://saureus.mlst.net/). For each locus, the sequence is assigned a unique allele number. For each isolate, the seven allele numbers for the seven loci generate an allelic profile which defines the sequence type (ST) for the strain (Enright, Day et al. 2000; Enright, Robinson et al. 2002). To establish evolutionary relatedness between different strains, the STs are further organised into clonal complexes (CCs). This is achieved by using the algorithm 'based upon related sequences (BURST)', which is linked to the MLST website (www.eburst.mlst.net). BURST is a user-defined algorithm in which the user defines the similarity threshold that clusters related STs into a CC. The most widely accepted threshold clusters any strains with ST of single or double locus variants of a founding member of a CC in the same CC. This then allows for further understanding of the evolutionary patterns within a particular *S. aureus* population (Enright, Robinson et al. 2002; Deurenberg and Stobberingh 2008).

University of Cape Town

1.4.1.3 *spa* typing

A strain typing technique that is efficient for the study of both local outbreaks and the molecular evolution of strains of *S. aureus* would be invaluable. It has been reported that *spa* typing, based on amplification and sequencing of the variable region of the *spa* gene encoding protein A, has the potential to index both the micro- and macro-variations required for long-term and short-term epidemiological studies respectively (Koreen, Ramaswamy et al. 2004; Deurenberg and Stobberingh 2008).

The gene (*spa*) encoding protein A consists of a hypervariable region flanked by conserved sequences. The variable region is comprised of variable repeats where variation results from deletions, substitutions, duplications or point mutations (Shopsin, Gomez et al. 1999; Strommenger, Kettlitz et al. 2006). *spa* typing involves the amplification of the variable region and subsequent sequencing of the amplicons generated. Each repeat is assigned an alphanumeric number, the sequence of which can be analyzed using specialized mathematical models which, in reference to the central database, can map the relatedness between different *spa* sequences (Enright, Robinson et al. 2002; Strommenger, Kettlitz et al. 2006). This analysis of the *spa* sequences is conducted using the specialised software package Ridom StaphType. The software consists of three modules: a sequence editor; a database; and a report generator module. For analysis, a *spa* sequence is uploaded onto the sequence editor where signature sequences flanking the variable region are detected by the software, thus allowing assignment of the corresponding *spa* type. The epidemiological data pertaining to that strain is added to the report generator module and the information is then saved onto a user database. The user database can subsequently be synchronised with a central database on the *spa* website, Spaserver (http://www.spaserver.ridom.de).

University of Cape Town

Synchronisation with the Spaserver allows for adoption of a common nomenclature and exchange of epidemiological data.

By further classifying the *spa* types into *spa* complexes with respect to the similarity of the short tandem repeats, it is possible to use this technique both for short-term and long-term epidemiological studies (Ruppitsch, Indra et al. 2006). The application that allows assignment of *spa* types into clonal complexes is called 'Based Upon Repeat Pattern (BURP)' and is an additional application on the Ridom StaphType programme. A defined algorithm assigns *spa* types into complexes based on the relatedness of the repeats. Studies have shown very good correlation between *spa* complexes and MLST as well as PFGE complexes (Grundmann, Aires-de-Sousa et al. 2006; Mellmann, Weniger et al. 2008).

Discordance between MLST and *spa* complexes can occur where a similar *spa* type can be mapped onto different MLST complexes. The ambiguity also applies to STs within the same CC where a *spa* type can exhibit different STs. This is attributed to convergent or parallel evolution of strains originally belonging to the same clonal lineage (Strommenger, Braulke et al. 2008). A combination of *spa* typing with another technique is therefore recommended especially in studies which require high levels of discrimination.

University of Cape Town

1.4.1.4 SCC*mec* typing

Knowledge of the SCC*mec* content of MRSA is important to an understanding of the epidemiology and evolutionary relationships of MRSA clones (Oliveira and de Lencastre 2002). Multiplex PCR strategies have been developed for SCC*mec* typing (Oliveira and de Lencastre 2002; Milheirico, Oliveira et al. 2007; Kondo, Ito et al. 2007; Zhang, McClure et al. 2009) where assignment of SCC*mec* type is based on the agarose gel electrophoresis profiles of the amplicons. Additionally, Oliveira and de Lencastre (2002) have developed a multiplex

PCR for the detection of SCC*mec* types I-IV, but as novel SCC*mec* types and subtypes were identified, these assays have been modified to accommodate the genetic diversity of the cassettes (Zhang, McClure et al. 2009; Milheirico, Oliveira et al. 2007).

1.5 Overview of the distribution and population structure of *S. aureus*

Application of the techniques described in the previous section has provided insight into the population structure and distribution of *S. aureus*. These techniques have revealed a population structure of *S. aureus* that is highly clonal when compared to other pathogenic bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Helicobacter pylori* (Feil, Holmes et al. 2001; Feil, Cooper et al. 2003). The clonality of *S. aureus* is attributed to a low rate of recombination in the core genome which results in strains that are slow to diversify and are therefore highly related (Ruimy, Maiga et al. 2008).

Studies comparing the global structure of MSSA with that of MRSA have shown that MSSA are more heterogenous, comprising strains which are genetically related to successful MRSA lineages as well as lineages which occur infrequently, if at all, in MRSA (Katayama, Robinson et al. 2005; de Sousa, Conceição et al. 2005; Vivoni, Diep et al. 2006; Hallin, Denis et al. 2007; Goering, Shawar et al. 2008; Deurenberg and Stobberingh 2008; Ruimy, Maiga et al. 2008; Baranovich, Zaraket et al. 2010; Wu, Wang et al. 2010; Breurec, Fall et al. 2011; Schaumburg, Kock et al. 2011). One factor likely to have contributed to the heterogeneity in MSSA is time; on the evolutionary timescale MSSA are much older and therefore have had more time to diversify (Grundmann, Aanensen et al. 2010).

A number of MSSA clones have been identified worldwide; however, some clones may be restricted to specific regions (Deurenberg and Stobberingh 2008; Grundmann, Aanensen et al. 2010). Our understanding of why some MSSA and MRSA clones are more prevalent in

University of Cape Town

local populations yet rare globally, is in its infancy. However, this phenomenon may be due to selection imposed by factors such as the environment and the host population, as well as the competitive advantages of certain strains against other clones (Ruimy, Maiga et al. 2008; Fan, Shu et al. 2009).

The lack of heterogeneity in MRSA populations is attributed mainly to the acquisition of SCC*mec* by specific lineages, suggesting that acquisition and stability of SCC*mec* in *S. aureus* strains is highly dependent on the genetic backgrounds of the recipient MSSA isolates (Katayama, Robinson et al. 2005). Six major pandemic MRSA clones belonging to MLST CC1, CC5, CC8, CC22, CC30 and CC45 have been described (Enright, Robinson et al. 2002). Other lineages of MRSA have been identified but are less common (Monecke, Coombs et al. 2011). Undoubtedly, geographical spread of MRSA, following the acquisition of SCC*mec*, has played the major role in the world-wide prevalence of MRSA. Nevertheless, a report on the evolutionary history of MRSA ST5 (Nubel, Roumagnac et al. 2008) suggested that SCC*mec* acquisition events may be more frequent than was previously thought. In this context, the majority of MRSA ST5 investigated clustered according to geographic region, suggesting local acquisition of SCC*mec* (Nubel, Roumagnac et al. 2008).

Similarly, Grundmann, et al. (2010) reported that MRSA populations tended to cluster geographically. In a study of 967 MRSA isolates, associated with invasive infections, from hospitals across Europe, 13 of the 15 major *spa* types identified showed restricted distribution (Grundmann, Aanensen et al. 2010). Furthermore, the study reported limited overlapping of no more than three clusters in the same region, suggesting that competitive advantages of existing strains over intruding or incoming clones may be a key factor in dissemination of clones into new environments. This is in agreement with Nubel, et al.

University of Cape Town

(2008) who suggested that MRSA clones are likely to predominate in environments for which they are best fitted.

Indisputably, some clones, such as ST239-MRSA-III and ST22-MRSA-IV, have spread worldwide and have become predominant in new environments (Smith and Cook 2005; Cha, Moon et al. 2005; Aires-de-Sousa, Correia et al. 2008; Smyth, McDougal et al. 2010; Monecke, Coombs et al. 2011; Breurec, Fall et al. 2011). ST239-MRSA-III (CC8), also known as the Czech, Vienna, Hungarian, Portuguese or Brazillian clone, UK EMRSA-1, -4, -7, -9, -11, AUS-EMRSA-2, -3, Irish Phenotype III, Irish AR-01, -09, -15, -23, or Canadian MRSA-3, -6, is reported to be possibly the oldest pandemic clone and has been isolated from every continent. It was reported to be the predominant MRSA clone in mainland Asia, accounting for 90% of hospital associated MRSA (HA-MRSA) (Chongtrakool, Ito et al. 2006; Feil, Nickerson et al. 2008) and was the second most prevalent strain in Western Australia between 2000 and 2004 (Coombs, Pearson et al. 2006).

A study on the evolutionary history of ST239-MRSA-III, based on whole-genome sequencing of a global collection of 63 isolates, revealed three clades with this genetic background namely, South American, Asian and European clades. Clones and their variants from each of these clades have expanded intra-continentally (Harris, Feil et al. 2010). Evidence of intercontinental spread comes from reports of the identification and dominance of South American variants in Portugal between 1994 and 1997 (Aires-de-Sousa, Correia et al. 2008; Harris, Feil et al. 2010), while an Asian strain was responsible for a two year outbreak in a London hospital between 2002 and 2004 (Edgeworth, Yadegarfar et al. 2007). Other examples include the close relatedness of isolates from USA with their counterparts in

University of Cape Town

Europe, and a similar relatedness between isolates from Egypt and Asian strains (Smyth, McDougal et al. 2010).

A number of monikers, UK-EMRSA-15, Irish ARO6, Barnim Epidemic Strain, Spanish PFGE type E13 or Canadian MRSA-8 (Monecke, Coombs et al. 2011), have been assigned to ST22-MRSA-IV which is also disseminated worldwide. Indeed this strain has displaced ST239-MRSA-III in India (D'Souza, Rodrigues et al. 2010). Also, from the mid to late 1980s, ST239-MRSA-III predominated in Irish hospitals, afterwhich, it was displaced by ST8-MRSA-IIA-E. Towards the end of the 1990s ST22-MRSA-IV emerged and by 2002 the strain was the predominant MRSA (Rossney and Keane 2002; Rossney, Lawrence et al. 2006). ST22-MRSA-IV was first detected in both the Midlands and south-east of England in the early 1990s (Richardson and Reith 1993; Duckworth 1998; Johnson, Aucken et al. 2001; Moore and Lindsay 2002) but has since been reported to account for 85% of all MRSA bacteraemia cases across the UK (Monecke, Coombs et al. 2011). Further, in Western Australia, as a percentage of the annual number of persons colonised or infected with MRSA, the prevalence of ST22-MRSA-IV increased from 2% to 21% between 1997 and 2004 (Coombs, Pearson et al. 2006).

Based on PFGE and *spa* typing, ST22-MRSA-IV is highly clonal, making it difficult to distinguish between strains (Goering, Shawar et al. 2008; Shore, Rossney et al. 2010). Carriage of virulence markers has been used to distinguish between ST22-MRSA-IV from different regions; for example, the Dublin clone is distinguished by carriage of the ACME-locus while carriage of PVL and *tst1* distinguishes the Indian clone (Monecke, Coombs et al. 2011). On the other hand, the Maltese clone can be distinguished by carriage of the fusidic acid resistance marker, Q6GD50 (Monecke, Coombs et al. 2011). Characterization of

University of Cape Town

virulence markers has identified cases of intra- and inter-continental transmission of ST22-MRSA-IV clones; for example Indian clonal strains carrying the virulence marker, *tst1*, have also been identified in Abu Dhabi, Egypt, England (Monecke, Coombs et al. 2011) and USA (Wolter, Chatterjee et al. 2008). Shore, et al. (2010) reported an increased efficiency in the epidemiological characterisation of ST22-MRSA-IV in an endemic hospital setting by combining *dru* typing, which indexes the hypervariable *dru* region located between *mecA* and IS*431* on the *mec* cassette, with *spa* and PFGE typing. The usefulness of *dru* typing in global epidemiological studies to distinguish between highly clonal strains such as ST22-MRSA-IV, has not, to the best of the author's knowledge, been reported.

1.5.1 Health-care associated MRSA (HA- MRSA)

MRSA was first identified in hospitals and has since been increasing in prevalence causing infections such as bacteraemia, pneumonia and surgical site infections, among others (Noble 1998). Studies have suggested an association of particular lineages of MRSA with the health-care setting; for example, the major clones of CC5, CC8, CC22, CC30, and CC45, are likely to be identified in connection with health-care predisposing factors such as recent hospitalization or invasive procedures such as implantation of foreign devices (Enright, Robinson et al. 2002; Deurenberg and Stobberingh 2008). Also, due to the less prudent use of antimicrobials, typical of health-care settings, health-care associated MRSA (HA-MRSA) are more likely to, but do not exclusively, carry SCC*mec* types I-III. These *mec* cassettes generally carry antibiotic resistance determinants, useful in a hospital setting, in addition to *mecA* (Enright, Robinson et al. 2002; Deurenberg and Stobberingh 2008; Monecke, Coombs et al. 2011).

University of Cape Town

1.5.2 Community-associated MRSA (CA-MRSA)

CA-MRSA can cause minor infections such as skin and soft tissue infections. However, some CA-MRSA strains, such as MW2/USA400, have been reported to carry the Panton-Valentine leukocidin (PVL) toxin, a virulence factor often associated with rapidly progressive life-threatening infections such as necrotising pneumonia occurring even among young healthy people (Boyle-Vavra and Daum 2007; Monecke, Coombs et al. 2011).

It has been suggested that the emergence of CA-MRSA coincided with the emergence of the smaller SCC*mec* types, IV and V, which had been described mostly in MRSA identified outside of health care settings (Grundmann, Aires-de-Sousa et al. 2006; Monecke, Coombs et al. 2011). The literature indicates that MRSA carrying these SCC*mec* types are now present in hospitals in many countries, blurring the distinction between CA-MRSA and HA-MRSA (Moore and Lindsay 2002; Huang, Tseng et al. 2007; Deurenberg and Stobberingh 2008; Reinert, McCulloch et al. 2008; Basset, Senn et al. 2010; Jansen van Rensburg, Eliya Madikane et al. 2011; Chen, Wang et al. 2010).

1.6 MRSA in South Africa

Prevalence rates of MRSA circulating in South African hospitals vary throughout the country with Gauteng and Kwa-Zulu Natal (KZN) provinces reporting rates of 23% (Perovic, Koornhof et al. 2006) and 27% (Shittu and Lin 2006), respectively. Current information from three Cape Town hospitals suggests MRSA prevalence rates ranging between 26% and 35% for *S. aureus* isolated from blood cultures between January and December 2009 (NHLS Public Sector Susceptibility Data, Dr Colleen Bamford).

There have been few studies on the molecular epidemiology of *S. aureus* in South Africa (Shittu, Nubel et al. 2009; Makgotlho, Kock et al. 2009; Moodley, Oosthuysen et al. 2010; Jansen van Rensburg, Eliya Madikane et al. 2011). In their study, Moodley, et al. (2010)

University of Cape Town

characterized 320 MRSA isolates, collected between August 2005 and November 2006, from 15 state and eight private diagnostic microbiology laboratories, to describe the population structure of MRSA in the nine provinces South Africa. The study reported five major clonal types, PFGE A-SCC*mec* I-*spa*CC5-ST5 (*n*=21), PFGE D-SCC*mec* III-*spa*CC12-ST239 (*n*=67), PFGE F-SCC*mec* IV-*spa*CC64-ST612 (*n*=20), PFGE K-SCC*mec* IV-*spa*CC64-ST612 (*n*=61), and PFGE T-SCC*mec* II-*spa*CC12-ST36 (*n*=76), which were distributed throughout the country (Fig 1.5).

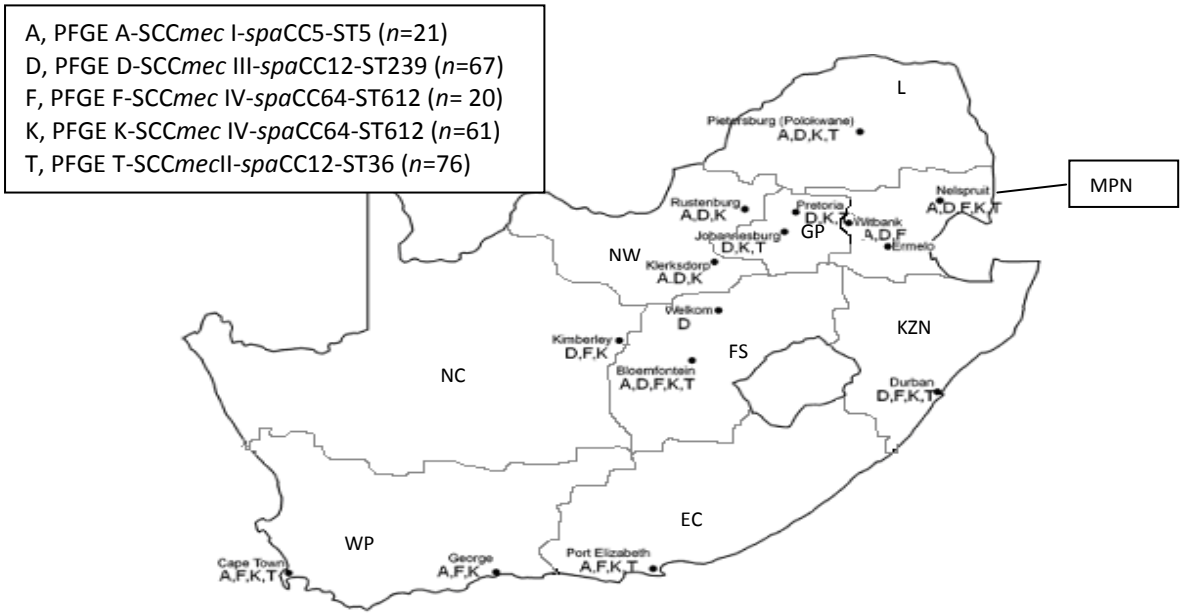

Figure 1.5: The distribution and frequencies of the five major MRSA clonal types identified in the study by Moodley, et al. (2010), in South Africa. L, Limpopo; GP, Gauteng province; MPN, Mpumalanga; NW, North West; FS, Free State; KZN, Kwa-Zulu Natal; EC, Eastern Cape; NC, Northern Cape; WP, Western Province. Adapted from Moodley, et al. (2010).

As the number of isolates from the regional centres in each of the provinces is unknown, it is difficult to draw conclusions about geographical clustering or restriction of clones. Nevertheless, based on the data presented in Fig 1.5, PFGE D-SCC*mec* III-*spa*CC12-ST239 may be more prevalent in provinces north of the Western Province and the Eastern Cape. Although ST612-MRSA-IV was isolated from all provinces, PFGE F-SCC*mec* IV-*spa*CC64-ST612 was not identified in three northern provinces: Gauteng, North West and Limpopo (Moodley, Oosthuysen et al. 2010). While this is the first study that describes the clonal

types of MRSA throughout South Africa, other studies describing MRSA from individual provinces have been reported. For instance, Shittu, et al. (2009) identified *spa*CC12-ST239-SCC*mec* III in KZN between March 2001 and August 2003, a finding subsequently corroborated by Moodley, et al. In Shittu's study, *spa*CC64-ST1338-SCC*mec* IV and *spa*CC64-ST1173-SCC*mec* IV, single and double locus variants of *spa*CC64-ST612-SCC*mec* IV, together constituted the dominant cluster.

In a recent study by Jansen van Rensburg, et al. (2011) in which 100 MRSA isolates, collected, froma variety of infections, between January 2007 and December 2008 from five Cape Town hospitals, were characterized, ST612-MRSA-IV was identified as the predominant MRSA followed by ST5-MRSA-I and then ST36-MRSA-II. Interestingly, in agreement with the findings of Moodley, et al. (2010), ST239-MRSA-III was not common in Cape Town hospitals (Fig 1.6) (Jansen van Rensburg, Eliya Madikane et al. 2011).

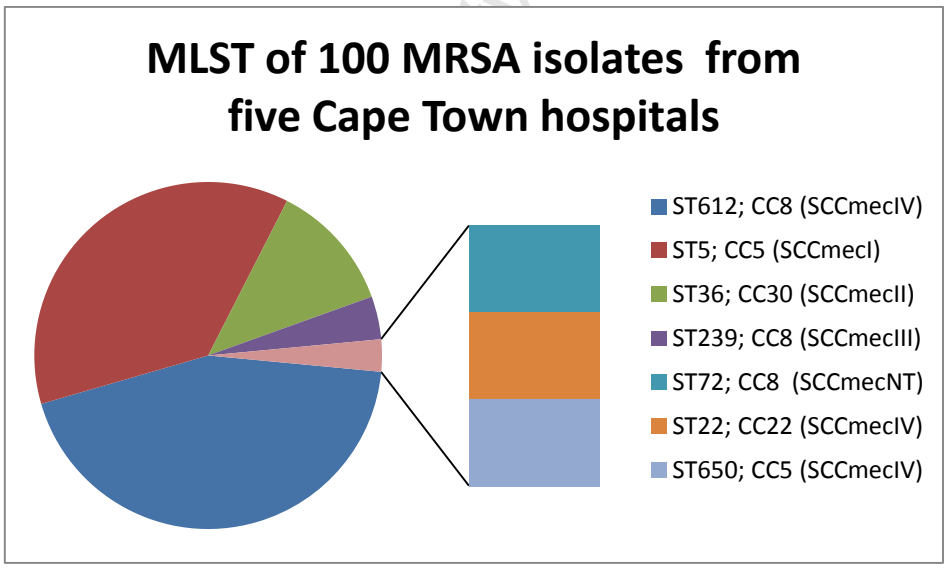

Figure 1.6: MLSTs of 100 MRSA. The isolates were collected between January 2007 and December 2008 from five Cape Town hospitals (Jansen van Rensburg, Eliya Madikane et al. 2011).
NT, not typable.

The high frequency of ST612-MRSA-IV across South Africa (Moodley, Oosthuysen et al. 2010; Jansen van Rensburg, Eliya Madikane et al. 2011) as well as the high prevalence of its

variants, *spa*CC64-ST1173-SCC*mec* IV and *spa*CC64-ST1338-SCC*mec* IV, in KZN (Shittu, Nubel et al. 2009) make it possible to suggest that this clone may be best fitted to South Africa. To date, Australia is the only other country that has reported ST612-MRSA-IV, and then only two isolates. The studies carried out by Jansen van Rensburg, et al which included molecular characterisation of the two Australian isolates led to the suggestion that ST612-MRSA-IVd may have arisen locally (Jansen van Rensburg, Eliya Madikane et al. 2011).

University of Cape Town

1.7 The aims and objectives of the study

The aims of the study were: 1) to determine whether ST612-MSSA was present in hospitals in Cape Town and 2) to characterize any ST612-MSSA identified.

The objectives of the study are:

- To screen a small sample collection of MSSA isolates, collected from four hospitals in Cape Town between February 2007 and December 2008, using *spa* typing and MLST to determine the presence of MSSA ST612.

- To describe ST612-MSSA isolates identified.

- To understand the relatedness of ST612-MSSA isolates to their MRSA counterparts.

- To determine whether the presence of SCC*mec* imposes a fitness cost on ST612-MRSA-IV isolates.

University of Cape Town

Screening of 19 MSSA isolates from Cape Town hospitals using *spa* typing and MLST for the identification of ST612-MSSA.

University of Cape Town

2.1 Abstract

A previous study characterizing 100 MRSA isolates, collected between January 2007 and December 2008 from five Cape Town hospitals, identified ST612-MRSA-IV as the predominant MRSA. Interestingly, Australia is the only other country which has reported this genetic background and then for only two isolates. To the best of the author's knowledge there have been no reports of the identification of ST612-MSSA. Nineteen MSSA isolates, which were collected during the same period, and from four of the hospitals in Cape Town, as the previously characterized MRSA isolates, were screened for the presence of ST612-MSSA.

Initially, *spa* typing was used to detect *spa* types associated with ST612-MRSA-IV. Based on these results, MLST was conducted to determine the STs of the relevant MSSA isolates.

A total of 10 *spa* types were detected among the 19 isolates. *spa* type t891, associated with ST22, was detected in nine isolates but only one isolate had a *spa* type (t064) associated with ST612. MLST defined these genetic backgrounds for the respective isolates. This study, therefore, reports the first identification of ST612-MSSA.

University of Cape Town

2.2 Introduction

A previous study which characterized 100 MRSA isolates, collected over a 24 month period between January 2007 and December 2008 from five Cape Town hospitals, identified ST612-MRSA-IV as the predominant strain. To date, Australia is the only other country which has reported clinical MRSA with this genetic background, and then for only two isolates (Jansen van Rensburg, Eliya Madikane et al. 2011). Subsequently ST612-MRSA was isolated from horses in Australia (Axon, Carrick et al. 2011). However, the SCCmec type (SCC*mec* IVd) is different from that of t he equine isolates (SCC*mec* IVa) (Axon, Carrick et al. 2011; Jansen van Rensburg, Whitelaw et al. 2012)

Recent studies have suggested the geographic restriction of some MRSA populations. It is supposed that *S.aureus* strains are likely to emerge and predominate in environments for which they are best fitted (Nubel, Roumagnac et al. 2008). In this context, it was suggested that ST612-MRSA-IV originated locally and expanded following the acquisition of SCC*mec* by a methicillin susceptible ST612 or a closely related strain (Jansen van Rensburg, Eliya Madikane et al. 2011).

The best strategy to determine whether ST612-MRSA-IV arose locally would have been to analyse a longitudinal collection of MSSA isolates that preceded the emergence of ST612-MRSA-IV; regrettably, no such collection was available. However, MSSA isolates contemporaneous with the previously characterized local MRSA were available. Although not ideal, considering the possibility that ST612-MRSA-IV may have emerged some time ago, these MSSA isolates were screened for the presence of ST612 strains.

A number of studies to determine the relatedness between the genetic backgrounds of MRSA and MSSA populations from the same settings have been carried out. A study

University of Cape Town

involving 450 hospitals in 26 European countries identified geographical clustering of MRSA *spa* types whereas the *spa* types in MSSA were more geographically diverse than their MRSA counterparts (Grundmann, Aanensen et al. 2010). Studies involving contemporaneous MRSA and MSSA populations from the same settings have also been carried out (de Sousa, Conceição et al. 2005; Vivoni, Diep et al. 2006; Hallin, Denis et al. 2007; Goering, Shawar et al. 2008; Baranovich, Zaraket et al. 2010; Wu, Wang et al. 2010; Breurec, Fall et al. 2011). Most of these studies have reported some incongruence between the genetic backgrounds of the predominant MRSA and MSSA isolates. The study by Baranovich, et al. (2010), which compared the genetic relatedness between 30 MRSA and 33 MSSA isolates collected between August 2006 and April 2007 in four hospitals in Russia, reported ST239 as predominant in and exclusive to the MRSA, while the dominant MSSA was ST121 which was also exclusive to the MSSA. This is not surprising as ST239 has only been identified in MRSA populations while ST121 is extremely rare among MRSA populations (Smyth, McDougal et al. 2010; Monecke, Coombs et al. 2011). The same results were reported in a study by Breurec, et al. (2011) where the genetic relatedness between 99 MRSA and 456 MSSA isolates, collected between January 2007 and March 2008 from seven hospitals in five African countries (Madagascar, Morocco, Senegal, Niger and Cameroon), was investigated. Similarly, ST239 was identified as one of the predominant MRSA which also included ST88 and ST5; while ST121, ST15 and ST152 were the leading MSSA. Only 7% of the MSSA had genetic backgrounds corresponding to the major MRSA clones identified in the study (Breurec, Fall et al. 2011).

With few exceptions, the study by de Sousa, et al. (2005) supports the premise that global spread of MRSA has played the more important role in the expansion of MRSA populations.

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A comparison of the STs of MSSA and MRSA, coincident with the emergence of dominant MRSA clones ST247 (1992-1993), ST239 (1995) and ST22 (2001), obtained from three Portuguese hospitals and the community served by these hospitals, did not identify or rarely detected MSSA isolates corresponding to these genetic backgrounds. Nevertheless there was some overlap between the backgrounds of MSSA and MRSA; ST5, which was identified as one of the dominant MSSA, was also identified among the MRSA (ST5-MRSA-IV/ Paediatric clone). Although it was not a major MRSA clone during the period of investigation, as ST5-MRSA-IV was first described in a paediatric Portuguese hospital (Sa-Leao, Santos Sanches et al. 1999), de Sousa, et al. (2005) suggested that the clone may have emerged within Portugal following acquisition of SCC*mec* IV by ST5-MSSA.

In contrast, a study by Hallin, et al. (2007), which involved the molecular characterisation of contemporaneous collections of 103 MSSA and 511 MRSA strains from a national survey of patients admitted to 112 Belgian hospitals in 2003, identified predominant MSSA lineages which were also common among the successful MRSA clones in the population. The most common MSSA lineages identified were ST5 and ST45 accounting for 18% and 14% of the isolates, respectively. ST45 was the most common genetic background (49%) among the MRSA populations while ST5-MRSA was third, accounting for 10% of the MRSA. Further characterisation of the MSSA and MRSA isolates using PFGE identified common PFGE types between the populations for both ST5 and ST45 which led the authors to suggest that the MRSA clones may have emerged in Belgium.

In this context, the objective of the work described in this chapter was to determine whether ST612 was present in MSSA collected simultaneously with the previously characterized local MRSA. In the study of ST612-MRSA-IV, four *spa* types t064, t1443, t1257,

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and t2196 were identified in the 44 ST612-MRSA-IV isolates identified (Jansen van Rensburg, Eliya Madikane et al. 2011). In the current study, *spa* typing was used for the initial molecular characterisation of the MSSA isolates and, based on the results, MLST was carried out to determine the STs of relevant isolates.

University of Cape Town

2.3 Materials and Methods

2.3.1 MSSA isolates included in the study

The MRSA which were characterized by Jansen van Rensburg, et al. (2011) in the previous project and the MSSA characterized in the current project are contemporaneous collections. The MRSA collections were obtained from paediatric and adult patients from five hospitals in Cape Town, South Africa, namely Groote Schuur hospital (GSH); Red Cross Children's hospital (RCCH); Mowbray Maternity hospital (MMH); UCT Private Hospital (UCTPH) and Victoria hospital (VH) between January 2007 and December 2008. The MSSA samples were collected from the same hospitals with the exception of VH and between February 2007 and December 2008. A single colony for each sample was sub-cultured in broth then stored at -80°C in 50% glycerol.

Antibiotic susceptibility testing was carried out at the National Health Laboratories Service laboratory (NHLS), GSH, using disc diffusion method or the VITEK 2 system (Card #) and the results re presented in Table 2.1. Zone sizes or VITEK 2 MIC results were interpreted using the Clinical Laboratory Standards Institute (CLSI) breakpoints, as well as the Advanced Expert System of the VITEK 2.

PFGE of the MRSA isolates (Jansen van Rensburg, Eliya Madikane et al. 2011) segregated ST612-MRSA-IV strains into two clusters, D and E and four singletons. Antimicrobial susceptibility patterns of representative strains from each cluster as well as those of the singletons were used as guidelines in the selection of the MSSA isolates in the current study (Table 2.1).

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Table 2.1: Antibiotic resistance patterns of ST612-MRSA-IV strains.

PFGE cluster*	Representative strains	Antibiogram										
		P	Clo	E	Cli	Rif	Co	Ci	G	F	V	L
D	D4	R	R	R	R	R	R	S	R	S	S	S
E	E3	R	R	S	S	R	R	R	R	S	S	S
	E8	R	R	R	R	R	R	R	R	S	S	S
Singleton	S5	R	R	S	S	R	R	S	R	S	S	S

***** (Jansen van Rensburg, Eliya Madikane et al. 2011).

P, penicillin; **Clo,** cloxacillin; **E,** erythromycin; **Cli,** clindamycin; **Rif,** rifampicin; **Co,** co-trimoxazole; **Ci,** ciprofloxazin; **G,** gentamicin; **F,** fusidic acid; **V,** vancomycin; **L,** linezolid. R, resistant; S, susceptible.

Nineteen MSSA isolates were selected for the investigation (Table 2.2), 14 (MS1-MS14) of which had antibiotic susceptibility profiles, with the exception of resistance to fusidic acid, similar to those of ST612-MRSA-IV (Table 2.1). An additional five MSSA strains (MS15-MS19) were included so as to be inclusive of the susceptibility profiles of the MSSA collection.

Table 2.2: MSSA isolates selected for strain typing and their corresponding antibiograms

MSSA isolates	Date of isolation	Antibiogram										
		P	Clo	E	Cli	Rif	Co	Ci	G	F	V	L
MS1	10.02.2007	R	S	S	R	S	R	R	S	S	S	NT
MS2	26.02.2007	R	S	S	S	S	R	S	R	S	S	NT
MS3	26.02.2007	R	S	S	S	S	R	S	R	S	S	NT
MS4	11.06.2007	R	S	R	R	S	R	S	S	S	S	NT
MS5	12.06.2007	S	S	R	R	S	R	S	R	R	S	S
MS6	12.12.2007	R	S	S	S	R	R	S	S	S	S	S
MS7	30.12.2007	R	S	S	S	S	R	S	R	S	S	S
MS8	19.02.2008	R	S	S	S	S	R	S	R	S	S	S
MS9	19.02.2008	S	S	R	R	S	S	S	R	R	S	S
MS10	07.04.2008	R	S	S	S	S	R	S	R	S	S	S
MS11	27.04.2008	R	S	S	S	S	R	S	R	S	S	S
MS12	22.08.2008	R	S	S	S	S	R	S	R	S	S	S
MS13	01.09.2008	R	S	S	S	S	R	S	R	S	S	S
MS14	14.12.2008	R	S	R	R	R	R	S	R	S	S	S
MS15	05.07.2007	R	S	S	S	S	S	S	R	S	S	NT
MS16	16.01.2008	S	S	S	S	S	S	S	S	S	S	S
MS17	22.02.2008	R	S	S	S	R	S	S	S	S	S	NT
MS18	28.02.2008	R	S	R	S	S	S	S	S	S	S	NT
MS19	10.03.2008	R	S	S	S	S	S	S	R	S	S	S

P, penicillin; **Clo**, cloxacillin; **E**, erythromycin; **Cli**, clindamycin; **Rif**, rifampicin; **Co**, co-trimoxazole; **Ci**, ciprofloxazin; **G**, gentamicin; **F**, fusidic acid; **V**, vancomycin; **L**, linezolid. R, resistant; S, susceptible; NT, not tested.

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2.3.2 Extraction of whole-genomic DNA

Each MSSA strain was individually cultured overnight on a boiled blood agar plate (Green point NHLS laboratories, Cape Town) at 37°C in preparation for extraction of DNA (Hal S. Larsen 1995). Extraction of DNA from the isolates was carried out using the QIAamp DNA mini and Blood mini kit (Qiagen, South Africa). The extraction process included pre-treatment of the isolates with 25 µg/ml lysostaphin, an antistaphylococcal lytic enzyme, in order to lyse the cells. After cell lysis, the DNA was then isolated from the cell debris, RNA and proteins according to the instructions from the kit. This process starts with the addition of 20 µl proteinase K provided in the kit, which is a broad spectrum proteolytic enzyme aimed at digestion of proteins from the cell lysate that may include nucleases that may otherwise degrade the DNA. The subsequent steps included treatment of the cell lysate with the buffers included in the kit; precipitation of the DNA following the addition of absolute ethanol (EtOH); adhesion of the genomic DNA onto a silica membrane in the QIAmp spin columns provided in the kit; and the subsequent elution of the DNA using the elution buffer also provided in the kit. Centrifugation and vortexing were carried out where stipulated in the protocol. The genomic DNA was suspended in 50 µl of the elution buffer.

To determine the integrity of the genomic DNA, 10 µl of the suspension was electrophoresed on a 1% (w/v) agarose gel with 5 µl/100 ml (v/v) ethidium bromide (EtBr) (10 mg/ml) at 90V for 60 minutes and visualised under UV light.

The genomic DNA was quantified using the Nanodrop spectrophotometer, ND-1000 UV/Vis (Thermo Fisher Scientific Inc, Delaware, USA).The remaining genomic DNA suspensions were stored at 4°C until further use.

University of Cape Town

2.3.3 Molecular typing

2.3.3.1 *spa* typing

This technique involves the amplification and subsequent sequencing of an internal fragment of the *spa* hypervariable region for each of the isolates. The PCR protocol included the primers, *spa-1113f* (5'-TAA AGA CGA TCC TTC GGT GAG C-3') and *spa-1514r* (5'-CAG CAG TAG TGC CGT TTG CTT-3'), described by Strommenger, et al. (2008) to produce amplicons of variable sizes depending on the number and type of the repeats. All primers used in the current and subsequent studies were synthesised at the DNA synthesis laboratory, Department of Molecular and Cellular Biology, University of Cape Town, South Africa.

The PCR assay consisted of 1X PCR buffer; 1.5 mM $MgCl_2$; 400 µM dNTPs; 1.25 U SuperTherm *Taq* polymerase (all four items were purchased from Southern Cross Biotechnology, Cape Town, South Africa); 0.2 µM of each primer; and 5 µl of the genomic DNA extracts to a final volume of 50 µl. The PCR was carried out on the 2720 thermal cycler (Applied Biosystems, South Africa) with the cycling conditions as follows: initial denaturation at 80°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 90 s with a final extension at 72°C for 10 min.

The products were electrophoresed on a 2% (w/v) agarose gel with 5 µl/100 ml (v/v) EtBr (10 mg/ml) at 80V for 95 min and then viewed under UV light. The MRSA strain COL (generously provided by Prof. Hermínia de Lencastre, Professor and Head, Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal) was used as a positive control for the amplification process but was not included for further experiments.

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A MinElute gel extraction kit (Qiagen) was used to purify the PCR products following the excision of the respective bands after gel electrophoresis (60V for 3 h) of the PCR products. The purified PCR products were then sent for automated sequencing at the Central Analytical Facility in Stellenbosch. The same primer pairs used for PCR were also used for sequencing.

Sequence analysis was carried out using the Ridom StaphType software package version 1.4 (Ridom GmbH, Würzburg, Germany). This process was preceded by an initial analysis of the forward and reverse sequences using the sequence analyser, BioEdit v7.0.5 (Ibis Biosciences, California, USA), to ensure that the sequences did not contain mismatched bases and that both contained the *spa* signature sequences provided on the SpaServer (http://spaserver.ridom.de) which are short sequences which flank the *spa* repeats. Both the reverse and forward sequences were then uploaded onto the Ridom StaphType programme and then subsequently assigned the corresponding *spa* type (Harmsen, Claus et al. 2003).

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2.3.3.2 Multilocus sequence typing (MLST)

MSSA isolates with *spa* types associated with ST612-MRSA-IV were characterized further using MLST, a technique that involved the amplification of internal fragments of seven loci, *arcC, aroE*, *glpF, gmk, pta, tpi* and *yqiL,* described in Chapter 1, and the subsequent sequencing of the amplicons. Individual PCR assays were performed using previously described primer pairs and PCR protocols (Table 2.3) (Enright, Day et al. 2000). All of the reagents used in the assay, with the exception of the primers, were purchased from Southern Cross Biotechnology.

Table 2.3: PCR primers and PCR protocols for MLST of the MSSA isolates

Gene	Primer pairs[1]	Primer sequences (5'-3')	Components in individual PCR assays	PCR Amplification
arcC	*arcC* UP *arcC* DN	TTGATTCACCAGCGCGTATTGTC AGGTATCTGCTTCAATCAGCG	1X PCR buffer; 1.5 mM $MgCl_2$; 200 µM dNTPs; 1U SuperTherm *Taq* polymerase; 0.5 µM of each primer; and 100 ng of the genomic DNA.	Initial denaturation at 95°C for 5 min. 30 cycles of denaturation at 95°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min. Final extension at 72°C for 5 min.
aroE	*aroE* UP *aroE* DN	ATCGGAAATCCTATTTCACATTC GGTGTTGTATTAATAACGATATC		
glpF	*glpF* UP *glpF* DN	CTAGGAACTGCAATCTTAATCC TGGTAAAATCGCATGTCCAATTC		
gmk	*gmk* UP *gmk* DN	ATCGTTTTATCGGGACCATC TCATTAACTACAACGTAATCGTA		
pta	*pta* UP *pta* DN	GTTAAAATCGTATTACCTGAAGG GACCCTTTTGTTGAAAAGCTTAA		
yqil	*yqil* UP *yqil* DN	CAGCATACAGGACACCTATTGGC CGTTGAGGAATCGATACTGGAAC		
tpi	*tpi* UP *tpi* DN	TCGTTCATTCTGAACGTCGTGAA TTTGCACCTTCTAACAATTGTAC	Same as above but increased dNTP concentration (400 µM)	

[1]As described by Enright, Day et al. (2000).

The PCR products were separated by gel electrophoresis and purified [2.3.3.1]. The purified PCR products were sequenced at the Central Analytical Facility in Stellenbosch. The same primers used for PCR (Table 2.3) were also used for sequencing.

Sequence analysis began with the alignment of the generated sequences to a reference sequence, provided on the MLST website (http://saureus.mlst.net/), for each of the genes using the sequence alignment editor and analysis program, BioEdit v7.0.5. The purpose of the alignment to a reference sequence was to ensure that the sequence is the correct length for the respective internal fragment of the gene. The edited sequence was then uploaded onto the MLST website. On the website, each sequence was synchronised with a global database and subsequently assigned an appropriate allelic number. The seven allelic numbers for the seven loci then generated an allelic profile which defined the sequence type (ST) for the particular isolate (Enright, Day et al. 2000).

University of Cape Town

2.4 RESULTS

2.4.1 *spa* typing

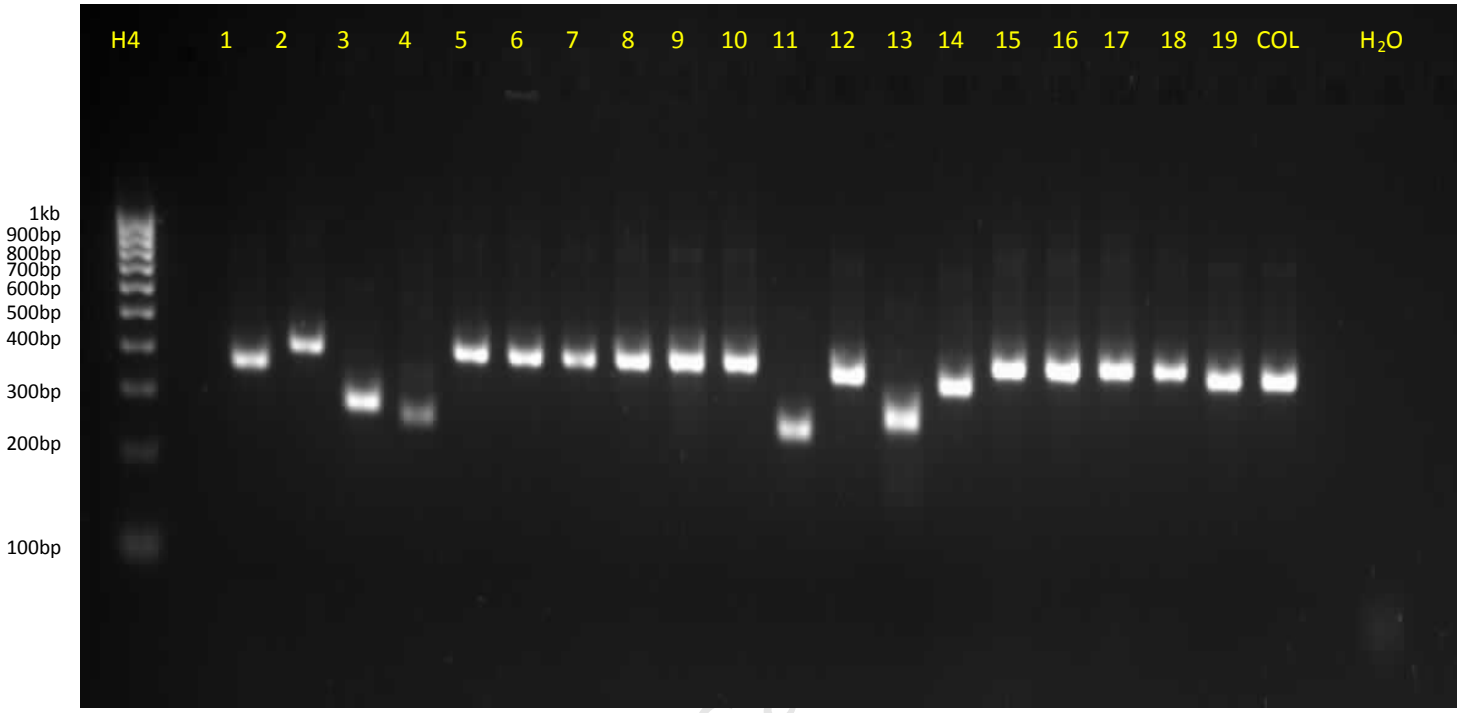

Figure 2.1: Gel electrophoresis of the *spa* amplicons for the 19 MSSA isolates. Lanes 1-5, MS1-MS5; Lane 6, MS15; Lanes 7-8, MS6-MS7; Lane 9, MS16; Lanes 10-11, MS8-MS9; Lanes 12-14, MS17-MS19; Lanes 15-19, MS10-MS14. MRSA COL was used as a positive control for the reaction. H_2O, the water control, containing no DNA, was used as a negative control for the reaction. H4, HyperLadder IV™ molecular weight marker (Celtic Molecular Diagnostics, Cape Town, South Africa).

A product of the expected size (~380bp) was obtained from the MRSA COL strain as well as ampicons of varying sizes from each of the 19 MSSA isolates (Figure 2.1). Following the extraction, purification and sequencing of the amplicons, the data were analysed using the Ridom StaphType [2.3.3.1] to assign the corresponding *spa* types.

Of the 19 isolates, only one had a *spa* type (t064) which was identified in ST612-MRSA-IV (Jansen van Rensburg, Eliya Madikane et al. 2011) (Table 2.4). For the remaining isolates, the association of each *spa* type to a ST was established on the MLST mapping technique described on the SpaServer (http://spaserver.ridom.de). One isolate had a novel *spa* sequence (26-23-25-17-25-28) and could therefore not be associated with a ST.

Interestingly, nine of the isolates had a *spa* type, t891, associated with ST22 (Table 2.4). A variety of *spa* types were identified in the remaining MSSA strains (Table 2.4).

Table 2.4: *spa* types of the 19 MSSA isolates and the associated STs.

MSSA isolate	Date of isolation	Antibiogram											*spa* type	Associated ST*
		P	Clo	E	Cli	Rif	Co	Ci	G	F	V	L		
MS1	10.02.2007	R	S	S	R	S	R	R	S	S	S	NT	t345	ST5
MS2	26.02.2007	R	S	S	S	S	R	S	R	S	S	NT	t891	ST22
MS3	26.02.2007	R	S	S	S	S	R	S	R	S	S	NT	novel	unknown
MS4	11.06.2007	R	S	R	R	S	R	S	S	S	S	NT	t1835	ST9
MS5	12.06.2007	S	S	R	R	S	R	S	R	R	S	S	t1805	ST45
MS6	12.12.2007	R	S	S	S	R	R	S	S	S	S	S	t891	ST22
MS7	30.12.2007	R	S	S	S	S	R	S	R	S	S	S	t891	ST22
MS8	19.02.2008	R	S	S	S	S	R	S	R	S	S	S	t891	ST22
MS9	19.02.2008	S	S	R	R	S	S	S	R	R	S	S	t1805	ST45
MS10	07.04.2008	R	S	S	S	S	R	S	R	S	S	S	t891	ST22
MS11	27.04.2008	R	S	S	S	S	R	S	R	S	S	S	t891	ST22
MS12	22.08.2008	R	S	S	S	S	R	S	R	S	S	S	t891	ST22
MS13	01.09.2008	R	S	S	S	S	R	S	R	S	S	S	t891	ST22
MS14	14.12.2008	R	S	R	R	R	R	S	R	S	S	S	t064	ST612
MS15	05.07.2007	R	S	S	S	S	S	S	R	S	S	NT	t891	ST22
MS16	16.01.2008	S	S	S	S	S	S	S	S	S	S	S	t127	ST1
MS17	22.02.2008	R	S	S	S	R	S	S	S	S	S	NT	t2360	ST6
MS18	28.02.2008	R	S	R	S	S	S	S	S	S	S	NT	t888	ST12
MS19	10.03.2008	R	S	S	S	S	S	S	R	S	S	S	t071	ST5

*Identified using the MLST mapping application on the SpaServer (http://spaserver.ridom.de).

P, penicillin; **Clo**, cloxacillin; **E**, erythromycin; **Cli**, clindamycin; **Rif**, rifampicin; **Co**, co-trimoxazole; **Ci**, ciprofloxazin; **G**, gentamicin; **F**, fusidic acid; **V**, vancomycin; **L**, linezolid. R, resistant; S, susceptible; NT, not tested.

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2.4.2 MLST

The ST of MS14, which was *spa* t064, previously shown to be associated with ST612-MRSA-IV, was determined. Although not an objective of this work, the STs of the nine isolates associated with ST22 were also determined. In addition, the ST of the isolate with the novel *spa* sequence was also defined.

Following amplification and subsequent agarose gel electrophoresis of the amplicons [2.3.3.2] as shown in Fig 2.2, the sequencing data obtained from purified products [2.3.3.2] were analysed [2.3.3.2].

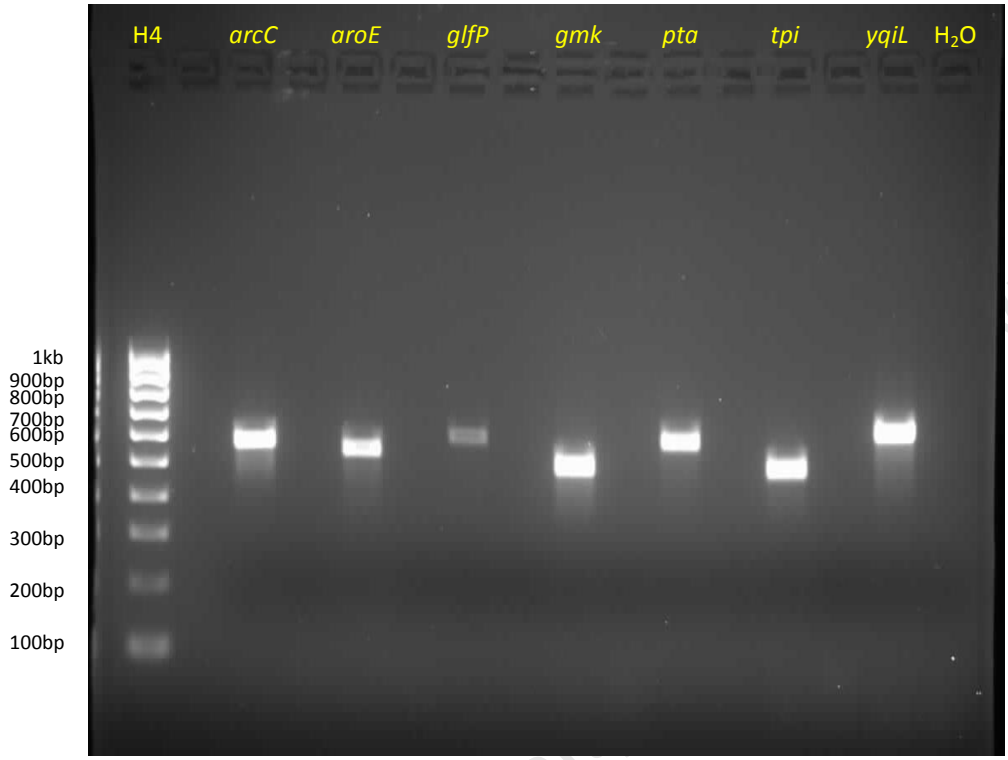

Figure 2.2: Gel electrophoresis of *arcC*, *aroE*, *glfP*, *gmk*, *pta*, *tpi* and *yqiL* amplicons for MLST of one of the MSSA isolates. H_2O, the water control, containing no DNA, used as a negative control for the reaction. H4, HyperLadder IV™ molecular weight marker (Celtic Molecular Diagnostics, Cape Town, South Africa).

The strain that was *spa* t064 was defined as ST612, while the strains which were *spa* t891 were defined as ST22. Additionally, the strain that had the novel *spa* sequence was also defined as ST22.

2.5 Discussion

ST612-MRSA-IV was predominant among a collection of 100 MRSA isolates, accumulated over a 24 month period between 2007 and December 2008, from paediatric and adult patients from five hospitals in Cape Town (Jansen van Rensburg, Eliya Madikane et al. 2011). To date, Australia is the only other country that has reported ST612-MRSA-IV, and then only two isolates (Jansen van Rensburg, Whitelaw et al. 2012). Molecular characterisation of the local strains, including two isolates previously isolated in South Africa (Goering, Shawar et al. 2008), and the two Australian isolates led to the suggestion that ST612-MRSA-IV may have arisen locally (Jansen van Rensburg, Eliya Madikane et al. 2011).

Based on studies which have demonstrated congruency in the genetic relatedness between contemporaneous MSSA and MRSA populations from the same setting as described in the introduction of this chapter, the objective of the current study was to determine if ST612-MSSA was present among MSSA isolates collected simultaneously with the previously characterized MRSA.

Only one of the 19 isolates had a *spa* type (t064) associated with ST612. MLST identified this background for the strain. This strain was resistant to a number of antibiotics including rifampicin and co-trimoxazole, similar to the profile of D4 (Table 2.1). It was suggested by Jansen van Rensburg, et al. (2011) that the frequent use of rifampicin and co-trimoxazole in the treatment of *Mycobaterium tuberculosis* and *Pneumocystis* infections in HIV infected individuals in South Africa may have selected for the emergence of *S. aureus* resistant to these antibiotics. That ST612-MSSA strain also demonstrated resistance to the same antibiotics is interesting and raised the question as to whether MSSA isolates with this genetic background may also be best fitted to the local environment.

University of Cape Town

MLST defined 10 MSSA as ST22; the same *spa* type (t891) was detected in nine of the isolates while a novel *spa* sequence was detected in the remaining isolate. This is not the first report of ST22-MSSA from South Africa; out of 70 MSSA isolates collected between 2004 and 2005 and characterized by Goering, et al. (2008) during five global clinical trials of the antibiotic, repatamulin, 13 isolates with this genetic background were identified. Interestingly, only one ST22-MRSA isolate was identified in the 100 MRSA isolates characterized by Jansen van Rensburg, et al. (2011). Also, in a study that characterized 320 MRSA, collected between August 2005 and November 2006 at 15 state and eight private diagnostic microbiology laboratories in the nine provinces in South Africa, only four ST22-MRSA isolates were identified, thus suggesting the scarcity of this genetic background among MRSA in the local environment (Moodley, Oosthuysen et al. 2010).

In contrast, ST22-MRSA-IV, which corresponds to the endemic UK-EMRSA 15 strain, is one of the major international clones of HA-MRSA prevalent in Europe and some parts of South East Asia (Deurenberg and Stobberingh 2008; D'Souza, Rodrigues et al. 2010). The scarcity of ST22-MRSA-IV in South Africa could suggest a recent emergence of this ST either through local acquisition of SCC*mec* into ST22-MSSA isolates or a recent importation of the resistant strain.

A variety of *spa* types was identified in the remaining MSSA isolates which were equally associated with a variety of STs. Two of the isolates carried *spa* types t071 and t345, which are both associated with ST5. Interestingly, ST5-MRSA-I was the second most prevalent clone in the contemporaneous MRSA collection. Jansen van Rensburg, et al. (2011) suggested a possible local origin of ST5-MRSA based on the theory of multiple acquisitions of SCC*mec* into this genetic background (Nubel, Roumagnac et al. 2008). While the

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identification of MSSA isolates associated with ST5 in the same hospitals as ST5-MRSA–I, albeit with different *spa* types, does not unequivocally prove that ST5-MRSA arose locally, it does provide evidence of a presence of a suitable MSSA background for such an event.

An investigation of the ST612-MSSA isolate recovered from strain typing of 19 MSSA isolates from five Cape Town hospitals

University of Cape Town

3.1 Abstract

MS14 was identified as ST612-MSSA-t064 as described in the previous chapter. MS14 was resistant to penicillin, erythromycin, clindamycin, rifampicin, co-trimoxazole and gentamicin; and was sensitive to cloxacillin, ciprofloxacin, fusidic acid, vancomycin and linezolid. This antibiotic susceptibility pattern was similar to that seen in ST612-MRSA-IV isolated from hospitals in Cape Town.

Recent studies have identified remnants of SCC*mec* in multi-drug resistant MSSA from hospitals, suggesting partial loss of these elements from the corresponding MRSA. PCR to detect remnants of SCC*mec* in MS14 was conducted; subsequent sequencing of the amplicons identified the *dcs* region of SCC*mec*. Additional PCR assays indicated that this region is located 1.7kb upstream of the *orfX* region.

To better understand the relatedness of MS14 to the ST612-MRSA-IV strains, the nucleotide sequence of the internal fragment of the rifampicin-resistance determining region of the *rpoB* gene in MS14 was compared with the corresponding region in ST612-MRSA-IV. The amino acid substitutions ($H_{481}N$, $I_{527}M$) previously identified in ST612-MRSA-IV were also present in the *rpoB* in MS14.

These data support the suggestion that MS14 resulted from a partial loss of SCC*mec* from a ST612-MRSA strain.

University of Cape Town

3.2 Introduction

MS14, isolated from a patient with septic arthritis at the Red Cross Children's Hospital in December 2008, was identified as ST612-MSSA-t064 as described in the previous chapter. MS14 was resistant to penicillin, erythromycin, clindamycin, rifampicin, co-trimoxazole and gentamicin; and was sensitive to cloxacillin, ciprofloxacin, fusidic acid, vancomycin and linezolid. This antibiotic susceptibility pattern was similar to that seen in ST612-MRSA-IV isolates (Jansen van Rensburg, Eliya Madikane et al. 2011). Recently a number of studies have shown that MSSA, particularly multi-drug resistant strains, defined as strains resistant to at least one agent in three classes of antimicrobials (Magiorakos, Srinivasan et al. (2011), frequently contain remnants of SCC*mec*, suggesting imprecise excision of SCC*mec* from the chromosome of the corresponding MRSA isolate (Donnio, Louvet et al. 2002; Donnio, Oliveira et al. 2005; Donnio, Fevrier et al. 2007; Shore, Rossney et al. 2008).

As described in Chapter 1, SCC*mec* is a mobile genetic element that can either be integrated at or excised from the attachment site (*attB*) on the chromosome of S. *aureus* (Fig 3.1). This integration onto and excision of SCC*mec* from the chromosome is mediated by site specific recombinase enzymes encoded by the *ccr* complex on SCC*mec*. For integration, the recombinase enzymes recognize a 15bp nucleotide sequence, *attB*, upstream of *orfX,* which is recombined with a homologous sequence (*attS*) on SCC*mec.* After recombination, the attachment sites are duplicated on either side of the SCC*mec* to create left (*attL*) and right (*attR*) junctions between SCC*mec* and the chromosome. For excision, *attL* and *attR* are recombined by the recombinase enzymes to result in reconstituted *attB* and *attS* on the chromosome and the SCC*mec*, respectively. As *attS* is reconstituted, it forms a non-replicating circular version of SCC*mec* (Noto and Archer 2006; Noto, Kreiswirth et al. 2008; Wang and Archer 2010).

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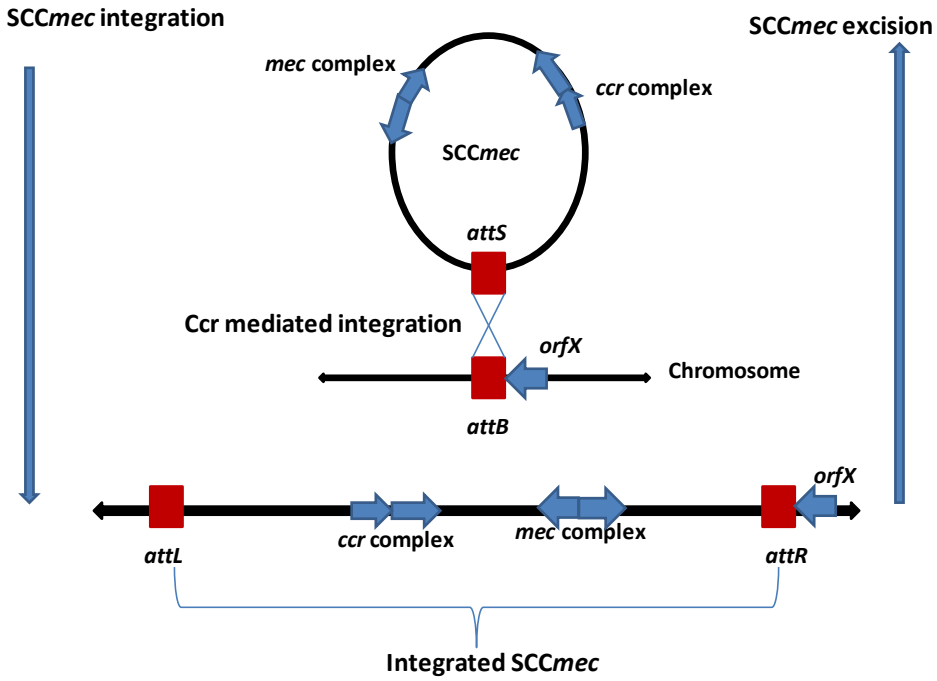

Figure 3.1: Integration and excision of SCC*mec* (Noto, Kreiswirth et al. 2008). For integration, the recombinase enzymes encoded by the *ccr* recognize the homologous 15bp sequence on SCC*mec* (*attS*) and *S. aureus* chromosome (*attB*). Following recombination, the 15bp sequences, *attL* and *attR*, flank SCC*mec* creating left and right junctions between SCC*mec* and the chromosome. For excision, *attL* and *attR* are recombined by the recombinase enzymes resulting in reconstituted *attB* and *attS.* As *attS* is reconstituted, it forms a non-replicating circular SCC*mec* element.

Occasionally, although the mechanism is not well understood, the excision of SCC*mec* from the chromosome can be imprecise, leaving some remnants of SCC*mec* within the *attB* site. Figure 3.2, adapted from Shore, et al. (2008), shows examples of imprecise excision of SCC*mec* from corresponding MRSA isolates which results in the presence of remnants of SCC*mec* in the resulting MSSA isolates.

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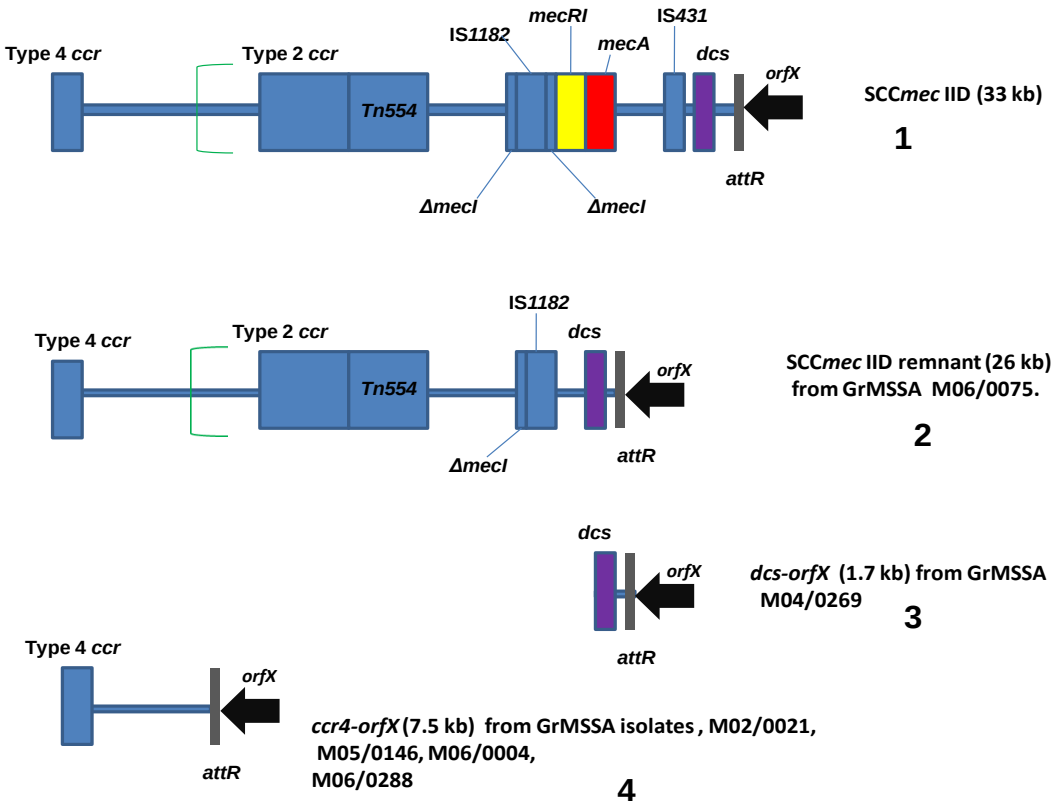

Figure 3.2: Representations of imprecise excision of SCC*mec,* adapted from Shore, et al. (2008). **1**, 33kb SCC*mec* IID prototype; **2**, 26kb remnant of SCC*mec* IID from GrMSSA isolate M06/0075 after loss of the fragment enclosed within IS*1182* and *dcs* i.e. IS*431, mecA, mecRI* and one copy of the truncated *mecI,* from the parent strain; **3**, 1.7kb remnant, *dcs-orfX* fragment of the SCC*mec*, from the GrMSSA isolate M04/0269; **4**, 7.5kb remnant, *ccr4-orfX* fragment of SCC*mec*, from the GrMSSA isolates M02/0021, M05/0146, M06/0004, M06/0288. Green line represents the approximate location of the SCCmec/SCC junction marking the divide between SCCmec IID and the ccrAB4 carrying element.

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The remnants (Fig 3.2), all showing similarity to elements of SCC*mec* IID, range between 1.7kb to 26 kb in size, suggesting multiple independent losses of portions of SCC*mec* from the MRSA strains (Shore, Rossney et al. 2008). Table 3.1, adapted from Shore, et al. (2008), summarizes the reports of various SCC*mec* remnants in MSSA isolates from various studies.

Table 3.1: Summary of reports, adapted from Shore, et al. (2008), of SCC*mec* remnants in MSSA isolates from various studies reported between 2004 and 2011.

No. of MSSA isolates with SCC*mec* regions (source; date)	**SCC*mec* remnants found**	**MLST/*spa* genotype (n*)**	**Reference**
7 (four Irish hospitals; 2002-2006)	*ccrAB2* *ccrAB4* *mecI*; d*cs*	ST8/t190 (4) ST8/t3209 (1) ST5/t088 (2)	(Shore, Rossney et al. 2008)
2 (i.v. drug users, UK; 2002-2003)	*ccrAB1* *dcs*	Not done	(Corkill, Anson et al. 2004)
26 (regions worldwide)	Right SCC*mec*/*orfX* junction	Not done	(Huletsky, Giroux et al. 2004)
6 (French hospitals; 1998-2002)	IS*431*-p*UB110*, *dcs* IS*431*-p*UB110*-IS*431-dcs*	ST8/t008 (1)	(Donnio, Oliveira et al. 2005)
1 (nasal swab, HCW, Germany)	Right SCC*mec*/*orfX* junction	t498	(Rupp, Fenner et al. 2006)
169 (60 French Hospitals; 2002-2004)	Right SCC*mec*/*orfX* junction *dcs* only HVR-IS*431*-p*UB110*-IS*431-dcs*	ST8/t008 (1) ST8/t024 (1)	(Donnio, Fevrier et al. 2007)
51 (Östergötland County, Sweden; 2004-2007)	Right SCC*mec*/*orfX* junction 12kb remnant of SCC*mec* II (1)	CC5/t002 (49)	(Lindqvist, Isaksson et al. 2011)
7 (USA and Cadanadian hospitals)	SCC*mec* II specific remnants (*kdp* and *J1* region) *dcs* p*UB110* *ccrAB2*	ST5	(Wong, Louie et al. 2010)

*number of isolates characterized using *spa* typing and/or MLST.

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The array of remnants of SCC*mec* in the MSSA isolates is inconsistent with the site-specific excision that is typical of Ccr mediated excision and therefore suggests the possibility of alternative mechanism(s) of SCC*mec* excision (Shore, Rossney et al. 2008).

The instability of SCC*mec* in some MRSA lineages is common and has been reported *in vitro* as well as in some clinical isolates (Donnio, Louvet et al. 2002; Donnio, Oliveira et al. 2005; Donnio, Fevrier et al. 2007; Shore, Rossney et al. 2008; Noto, Fox et al. 2008; Lindqvist, Isaksson et al. 2011). *In vitro* loss of SCC*mec* has been attributed to a number of stressful conditions. Noto, Fox et al. (2008) reported *in vitro* loss of SCC*mec* from MRSA strains, following prolonged exposure to increasing levels of vancomycin, as a mechanism to compensate for chromosomal mutations that confer resistance to vancomycin which would otherwise add a cost to fitness to the strains. In some of the staphylococci, SCC*mec* was excised precisely while in others, remnants of SCC*mec* were retained in the *attB* site indicating partial excision of SCC*mec* (Noto, Fox et al. 2008).

While factors that may contribute to partial excision of SCC*mec in vivo* may not be well understood, several studies have reported the occurrence of clinical isolates of MSSA with remnants of SCC*mec* in the *attB* site. As most MRSA are multi-drug resistant, the loss of SCC*mec*, may result in MSSA strains with resistance to a number of antibiotics other than β-lactams (Donnio, Fevrier et al. 2007; Shore, Rossney et al. 2008; Lindqvist, Isaksson et al. 2011). A recent study (Donnio, et al. 2007) investigated 247 multi-drug resistant MSSA isolates, collected from 60 French hospitals between 2002 and 2004, to determine if the isolates resulted from a loss of SCC*mec* from corresponding MRSA strains, and to also monitor the spread of these strains with respect to the incidence of multi-drug resistant MSSA in the hospitals. Molecular analyses based on PFGE and Southern hybridization revealed evidence of MRSA background in 92% of the isolates. Furthermore, amplification of

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various regions associated with SCC*mec* revealed evidence of remnants of this element in 68% of the isolates. Moreover, 87.5% of the isolates which showed evidence of remnants of SCC*mec* were members of a single PFGE cluster and were associated with clonal complex (CC) 8. Most of these CC8 strains, following loss of SCC*mec*, showed clonal expansion and spread into high incidence hospitals as opposed to the other strains which showed independent loss of SCC*mec* in hospitals with a low incidence of these strains (Donnio, Fevrier et al. 2007).

Another example of a multi-drug resistant (MDR) MSSA clone that became prevalent after loss of SCC*mec* was identified in a study by Lindqvist, et al. (2011), where 54 MDR-MSSA, 49 of which were *spa* type t002 (MLST CC5) and were part of a clonal outbreak, were investigated for remnants of SCC*mec* in the *attB* site. The samples were collected from patients in Östergötland County, Sweden between January 2004 and October 2007. The 49 isolates from the clonal outbreak, as well as two isolates from the additional collection, were positive for remnants of SCC*mec* thus accounting for 93% of the total isolates tested (Lindqvist, Isaksson et al. 2011). *spa* t002 was very common among MRSA isolates in Sweden during the period when the MSSA strains were isolated, demonstrating instability of SCC*mec* in a major MRSA clone.

Monitoring of the epidemiology of MRSA populations in Irish hospitals between 1999 and 2003, identified a change in the epidemicity of a multi-drug resistant clone ST8-MRSA-IIA-E (CC8), which accounted for 50% of the MRSA population in Irish hospitals in 1999, but was shown to be slowly being displaced by the non-multi-drug resistant ST22-MRSA-IV strain in the period between 1999 and 2003 (Shore, Rossney et al. 2008). Based on the suggestion that loss of SCC*mec* from epidemic strains may lead to changes in the evolution of MRSA in hospitals (Wagenvoort, Toenbreker et al. 2000; Donnio, Louvet et al. 2002), Shore, et al.

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(2008) investigated for SCC*mec* components present in multi-drug resistant MSSA isolates recovered in Irish hospitals, and compared their genotypes to those of MRSA isolates previously identified in Ireland. Twenty five MDR-MSSA isolates, collected from six hospitals between 2004 and 2006, were investigated. The study reported the presence of SCC*mec* remnants in only seven of the isolates, five of which were CC8 while the remaining two were CC5. A variety of SCC*mec* remnants were identified in the CC8 isolates thus suggesting independent loss of SCC*mec* in the strains instead of clonal expansion of the strains following SCC*mec* excision. Interestingly, the SCC*mec* remnants in all five CC8 isolates showed similarity to components of SCC*mec* II (Fig 3.2) which suggested that the strains may have been derived from the previously predominant ST8-MRSA-II (Shore, Rossney et al. 2008).

In this context, MS14 may have resulted from a loss of SCC*mec* from a corresponding MRSA ST612 isolate while retaining the multi-drug resistance profile that is typical of this MRSA background.

In addition, the relatedness of MS14 to ST612-MRSA-IV was investigated based on similarities in the nucleotide sequences of the rifampicin-resistance determining region (RRDR) of the *rpoB* gene. Rifampicin inhibits transcription following its binding to the β-subunit of the RNA polymerase encoded by *rpoB*. Several mutations in an internal fragment of the *rpoB* which result in changes in the amino acid sequence of the β-subunit have been reported to confer resistance to rifampicin (Aubry-Damon, Soussy et al. 1998). This mechanism of resistance to rifampicin has been reported in several bacterial species including *Escherichia coli*, *Mycobacterium tuberculosis* and *S. aureus* (Aubry-Damon, Soussy et al. 1998; O'Neill, Huovinen et al. 2006). All the ST612-MRSA-IV isolates characterized by Jansen van Rensburg, et al. (2011) as well as the two ST612-MRSA-IV strains from Australia

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and two additional ST612-MRSA-IV strains from South Africa, were shown to have identical amino acid substitutions ($H_{481}N$, $I_{527}M$) in the RRDR of the β-subunit (Jansen van Rensburg, Eliya Madikane et al. 2011). These amino acid substitutions are associated with high level rifampicin resistance and were not identified in any of the other rifampicin resistant strains characterized by Jansen van Rensburg, et al. (2011). In addition, all the ST612-MRSA-IV strains characterized (including the two isolates from Australia and the two from South Africa) had identical silent mutations in the RRDR thus supporting the suggestion of a common ancestor for the ST612-MRSA-IV strains. In this light, the relatedness of MS14 to ST612-MRSA-IV strains was investigated using the RRDR of the β-subunit of the isolates as a marker of genetic relatedness.

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3.3 Materials and Methods

3.3.1 *S. aureus* isolates

MS14 (ST612-MSSA, *spa* type t064), isolated from a patient with septic arthritis at the Red Cross Children's Hospital (Cape Town) in December 2008, was investigated in the current study. MS14 was resistant to penicillin, erythromycin, clindamycin, rifampicin, co-trimoxazole and gentamicin. The control strains included MSSA NCTC 8325, MRSA COL and ST1-MRSA-IV (MW2) strains (all generously provided by Prof. Hermínia de Lencastre, Professor and Head, Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal) and MRSA SCH3304764 (ST612-MRSA-IV) (Jansen van Rensburg, Eliya Madikane et al. 2011).

3.3.2 Extraction of whole-genomic DNA

Genomic DNA was extracted from MS14 and the control strains as described [2.3.2].

3.3.3 Amplification of the *attB* site in MS14

The *attB* site, as shown in Fig 3.3, was amplified using the primer pair *CL1* (5'-ATTTAATGTCCACCATTTAACA-3') and *CR1* (5'-AAGAATTGAACCAACGCATGA-3') and PCR protocol described by Katayama, et al. (1999), to produce a 275bp amplicon.

The PCR assay consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1U SuperTherm *Taq* polymerase (all four items purchased from Southern Cross Biotechnology, Cape Town, South Africa), 0.5 µM of each primer; and 200 ng of the genomic DNA extracts to a final volume of 50 µl. The PCR was carried out on the 2720 thermal cycler (Applied Biosystems, South Africa) with the cycling conditions as follows: initial denaturation at 95°C for 5 min

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followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min.

MSSA NCTC 8325 strain was used as the positive control while MRSA COL strain was used as the negative control for the amplification. The PCR products were electrophoresed on 2% (w/v) agarose gel with 5 µl/100 ml (v/v) EtBr (10 mg/ml) at 80V for 95 minutes and then viewed under UV light.

3.3.4 Detection of SCCmec remnants in MS14

The detection of SCC*mec* elements involved amplification of various genes, normally associated with SCC*mec* cassettes (Fig 3.3), in individual assays.

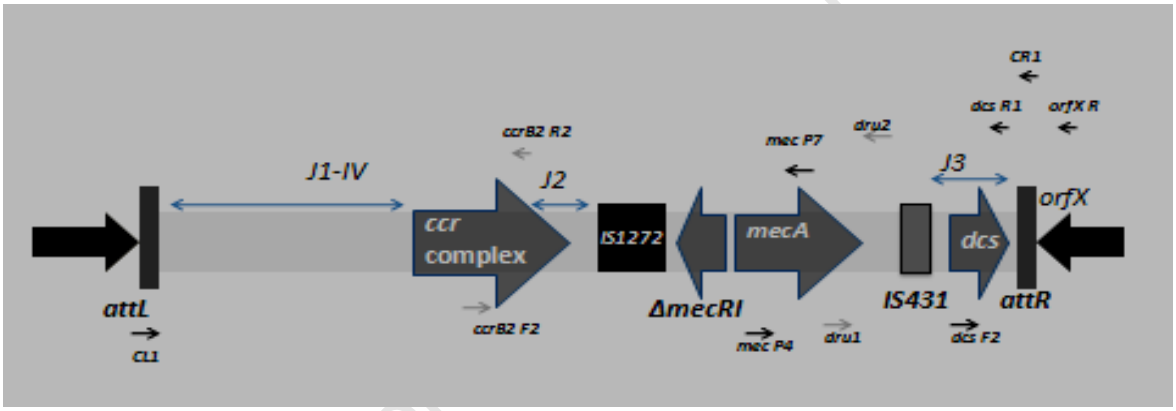

Figure 3.3: A schematic of SCC*mec* IV outlining the genes that were targeted for amplification. SCC*mec* is enclosed between *attL* and *attR* which are the left and right junctions respectively, between SCC*mec* and the chromosome. The conserved region, *orfX*, is indicated downstream of *attR* on the chromosome. The primer pairs for the amplification of each region are represented by the small black and grey arrows.

The primer pairs and PCR protocols for the amplification of the different target regions are presented in Table 3.2.

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Table 3.2: Primer pairs and PCR protocols for the amplification of the remnants of SCC*mec* in MS14.

Primer pairs	Primer nucleotide sequence (5'-3')	Gene	Amplicon size	Components in the PCR assays	Amplification conditions	Reference
mecA P4	CCAGATTACAACTTCACCAGG	*mecA*	162bp	1X PCR buffer; 1.5 mM $MgCl_2$; 200 µM dNTPs; 1.2 U SuperTherm *Taq* polymerase; 0.8 µM of each primer; and 5 ng of the genomic DNA	Initial denaturation at 94°C for 4 min. 30 cycles of denaturation at 94°C for 30 s; annealing at 53°C for 30 s; extension at 72°C for 1 min; and final extension at 72°C for 4 min.	(Milheirico, Oliveira et al. 2007)
mecA P7	CCACTTCATATCTTGTAACG					
ccrB2 F2	AGTTTCTCAGAATTCGAACG	*ccrB2*	311bp			
ccrB2 R2	CCGATATAGAAWGGGTTAGC					
dcs F2	CATCCTATGATAGCTTGGTC	*dcs*	342bp			
dcs R1	CTAAATCATAGCCATGACCG					
dru1	GTTAGCATATTACCTCTCCTTGC	*dru* hyper-variable region	variable	1X PCR buffer; 1.5 mM $MgCl_2$; 400 µM dNTPs; 1.25 U SuperTherm *Taq* polymerase; 0.5 µM of each primer; and 100 ng of the genomic DNA	Initial denaturation at 94°C for 2 min. 30 cycles of denaturation at 94°C for 1 min; annealing at 52°C for 1 min; extension at 72°C for 1 min; and final extension at 72°C for 3 min.	(Goering, Shawar et al. 2008)
dru2	GCCGATTGTGCTTGATGAG					

The positive controls were ST612-MRSA-IV for the amplification of *mecA*, *ccrB2* and *dcs* regions and MRSA COL for *dru*. MSSA NCTC 8325 was used as a negative control for all assays. The PCR products were electrophoresed as described [3.2.3].

3.3.4.1 Determination of the location of dcs in MS14

Following the amplification of *dcs* in MS14, the location of the locus in MS14 was determined by amplification of the right junction/*orfX* region of the SCC*mec*. The primer pair, *dcs F2* (5'-GTCAATGAGATCATCTACAT-3') and *orfX R* (5'-CCCAAGGGCAAGCGAC-3'), and the PCR protocol for the amplification of the 1701bp amplicon, were described by Shore, et al. (2008).

The PCR assay consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.25U SuperTherm *Taq* polymerase (all four items purchased from Southern Cross Biotechnology, Cape Town, South Africa), 0.8 µM of each primer; and 5 ng of the genomic DNA extracts to a final

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volume of 50 µl. The PCR was carried out on the 2720 thermal cycler (Applied Biosystems, South Africa) with the cycling conditions as follows: initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min with a final extension at 72°C for 4 min.

For the amplification, ST612-MRSA-IV and MW2 were used as positive controls while MSSA NCTC 8325 was used as a negative control. The PCR products were electrophoresed as described [3.2.3].

Amplicons were purified and sequenced as described [2.3.3.1]. The primers used for the PCR assay were also used for the sequencing of the amplicons. Sequence analysis was carried out using the sequence alignment editor and analysis program, BioEdit v7.0.5 (Ibis Biosciences, California, USA).

3.3.5 PCR amplification and sequencing of rifampicin-resistance determining region of *rpoB* of MS14

The rifampicin-resistance determining region of the *rpoB* gene of MS14 was amplified using the primer pair previously described by Aubry-Damon, et al. (1998), *rpoB F3* (5'-AGTCTATCACACCTCAACAA-3'), and *rpoB F4* (5'-TAATAGCCGCACCAGAATCA-3') to produce a 702bp amplicon.

The PCR assay consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 400 µM dNTPs, 1U SuperTherm *Taq* polymerase (all four items purchased from Southern Cross Biotechnology, Cape Town, South Africa), 40 pmol of each primer; and 100 ng of the genomic DNA extracts to a final volume of 100 µl. The PCR was carried out on the 2720 thermal cycler (Applied Biosystems, South Africa) with the cycling conditions as follows: initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s and extension at 72°C for 45 s with a final extension at 72°C for 3 min.

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The PCR products were separated by gel electrophoresis and purified [2.3.3.1]. The purified products were sequenced, using the same primer pairs used for PCR, at the Central Analytical Facility in Stellenbosch.

Sequence analysis began with alignment of the generated sequences to a reference sequence of the *rpoB* (X64172 from a rifampicin susceptible *S. aureus* RN4220), retrieved from GenBank (http://ncbi.nlm.nih.gov), using the sequence alignment editor and analysis programme, BioEdit v7.0.5 (Ibis Biosciences, California, USA).The aligned sequences were then translated into the corresponding amino acid sequences. The amino acid sequences were also aligned to ensure that the query sequence was in the correct frame as the reference sequence so as to determine the type and location of any changes in the query sequence.

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3.4 Results

3.4.1 Amplification of the *attB* site in MS14

As a first step to understanding the genetic arrangement at the *attB* site in MS14, a PCR assay was carried out using the primer pairs [3.2.3] which anneal to *attL* and *attR* respectively (Fig 3.3). As shown in Fig 3.4, a product of the expected size (275bp) was obtained from MSSA NCTC 8325. Also as expected, no product was obtained for MRSA COL containing SCC*mec* I, which is too large for PCR amplification. Similarly no product was obtained with the MS14 indicating that it contains an insert in the *attB* site.

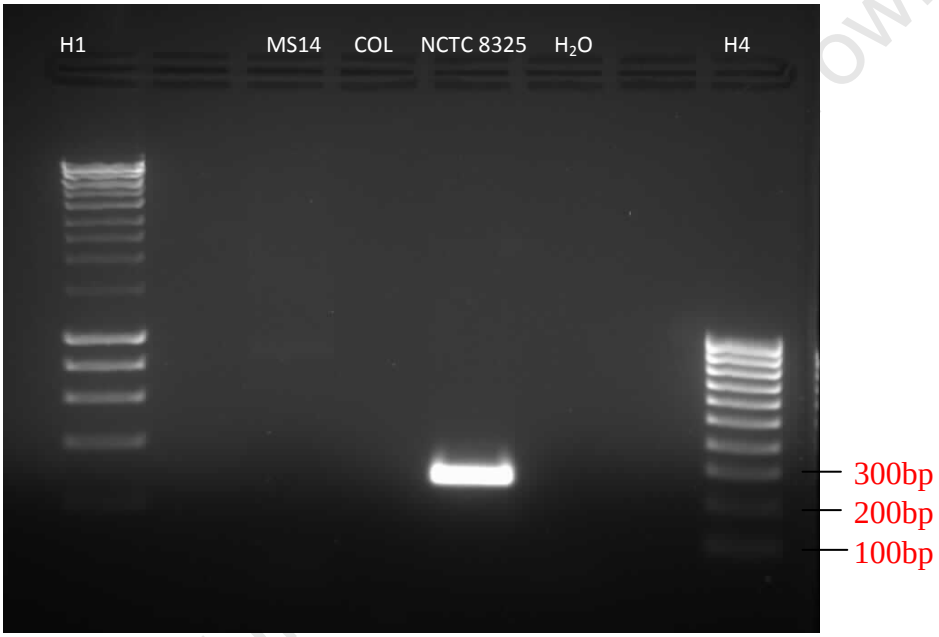

Figure 3.4: Gel electrophoresis of the *attB* amplicons. H1 and H4, molecular weight markers, Hyperladder 1™ and HyperLadder IV™ respectively (Celtic Molecular Diagnostics, Cape Town, South Africa). H_2O, the water control which was used as a DNA negative control for the reaction.

3.4.2 PCR for the detection of remnants of SCC*mec*

To determine the identity of the insert at the *attB* site in MS14, PCR assays were carried out for the detection of SCC*mec* components using the primer pairs described [3.2.4]. Of the different loci (*mecA*, *dru*, *ccrB2* and *dcs*) targeted on SCC*mec*, a product was obtained only for *dcs* (Fig 3.5).

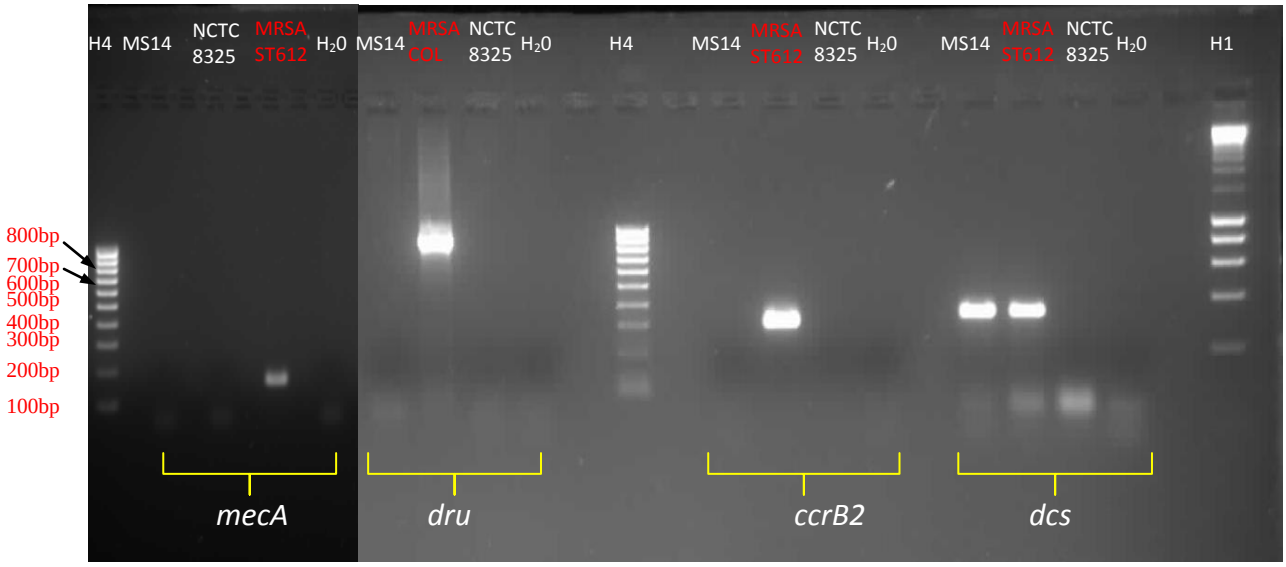

Figure 3.5: Gel electrophoresis of the PCR products for the detection of remnants of SCC*mec* in MS14. The internal loci (*mecA*, *dru*, *ccrB2* and *dcs*) of SCC*mec* were targeted for amplification. ST612-MRSA-IV was included as a positive control for the amplification of *mecA*, *ccrB2* and *dcs* while MRSA COL was included as a positive control for *dru*. MSSA NCTC 8325 was included in the PCR assays as a negative control for all four loci. H1 and H4, molecular weight markers, Hyperladder 1™ and HyperLadder IV™ respectively (Celtic Molecular Diagnostics, Cape Town, South Africa). H_2O, the water control which was used as a DNA negative control for the reaction.

3.4.2.1 Determination of the location of *dcs* in MS14

To ascertain whether *dcs* is associated with *attB* site in MS14, a PCR assay was performed using primers *dcs F2* and *orfX R* [3.2.4.1].

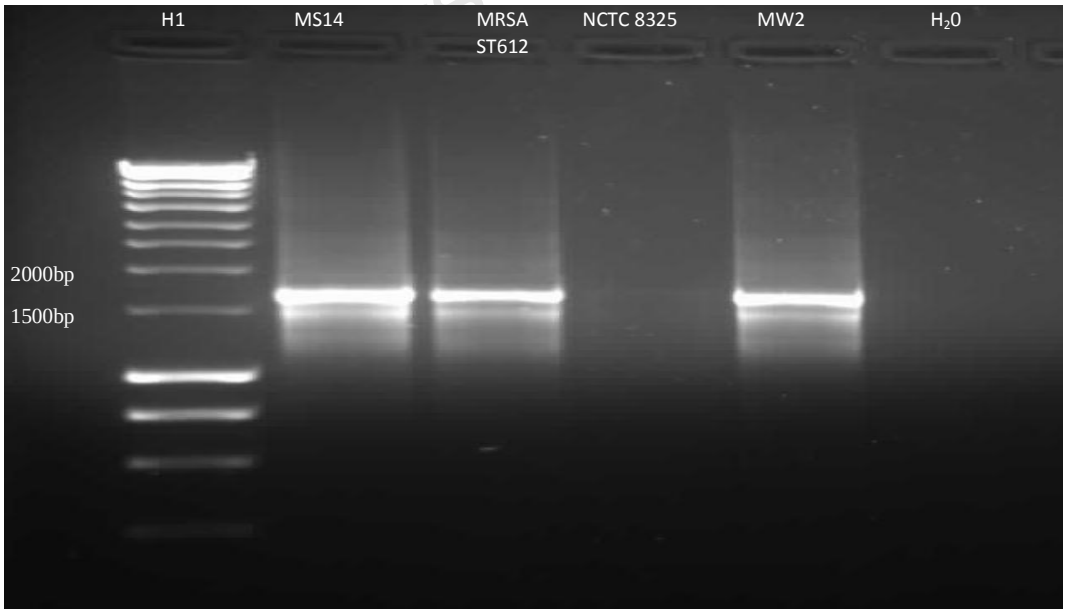

Figure 3.6: Gel electrophoresis of the amplicons for the determination of the location of *dcs* in MS14. ST612-MRSA-IV (SCC*mec* IVd) and MW2 (SCC*mec* IVa) were used as positive controls while MSSA NCTC 8325 was used as a negative control. H1, molecular weight marker, Hyperladder 1™ (Celtic Molecular Diagnostics, Cape Town, South Africa). H_2O, the water control which was used as a DNA negative control for the reaction.

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Amplicons (1.7kb) were obtained with the ST612-MRSA-IV and MW2 (SCC*mec* IVa) strains as well as with MS14. The product from MS14 was purified and sequenced [2.3.3.1]. Sequences were obtained with the primers, *dcs F2* (1077bp) and *orfX R* (1256bp), respectively. Alignment of the data with the corresponding sequences of SCC*mec* IVa of MRSA JCSC 4469 (AB097677), acquired from Genbank (http://www.ncbi.nlm.nih.gov/genbank/), showed 97% and 92% sequence similarity with *dcs* and *orfX*, respectively.

3.4.3 PCR and Characterization of the rifampicin-resistance determining region (RRDR) of MS14

Following PCR of the RRDR of *rpoB* of MS14, the 702bp product was sequenced [3.2.5]. Alignment of the sequencing data with the corresponding region from a rifampicin susceptible *S. aureus* (X64172 from *S. aureus* RN4220) as well as from ST612-MRSA-IV, revealed the mutations: C→A, G→T, T→C and T→G at nucleotide positions 2662, 2715, 2757, and 2802 respectively, present in both ST612-MRSA-IV and MS14 (Fig 3.7). Translation of the *rpoB* nucleotide sequence into an amino acid sequence revealed amino acid substitutions $H_{481}N$ and $I_{527}M$ corresponding to C→A (2662) and T→G (2802) mutations respectively, located in the RRDR of MS14 and ST612-MRSA-IV. The G→T (2715) and T→C (2757) mutations did not result in changes in the corresponding amino acids.

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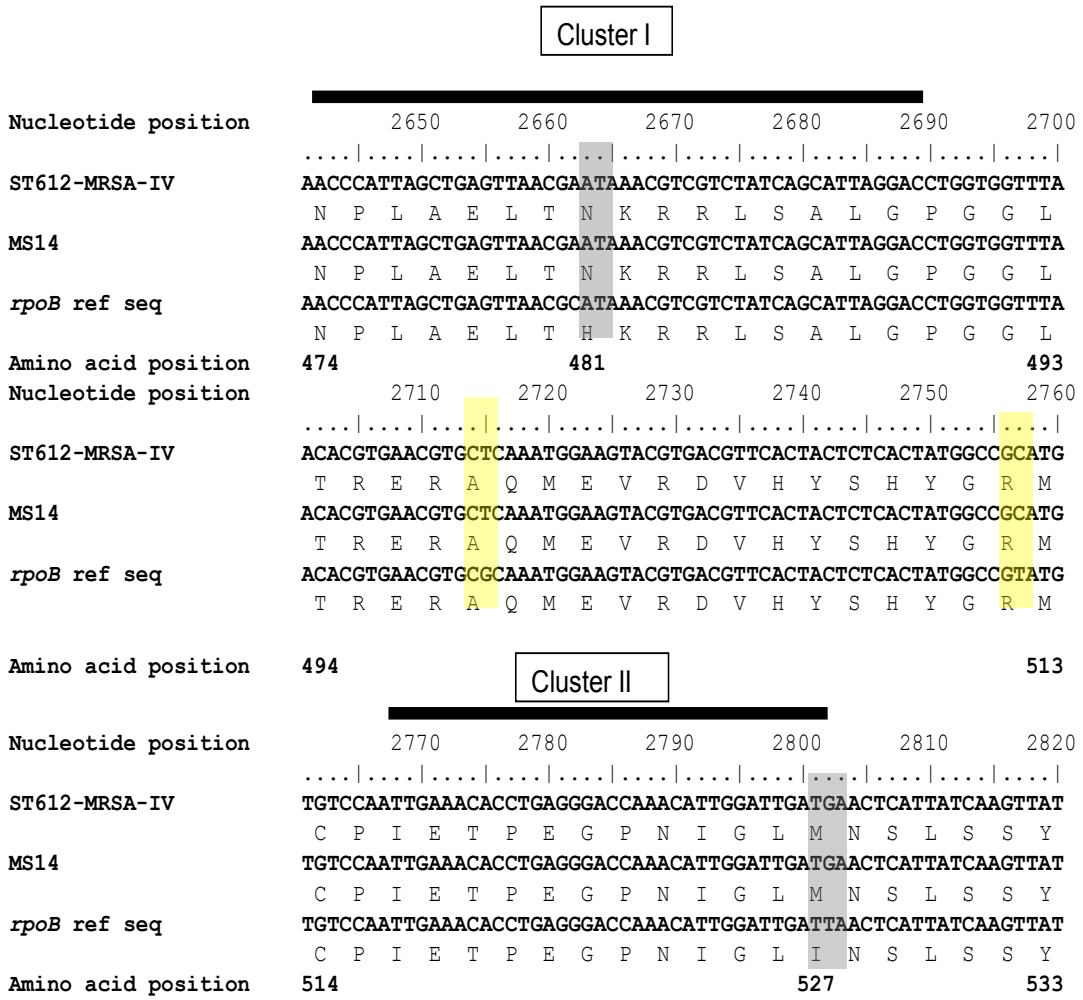

Figure 3.7: Alignment of the internal fragment of the rifampicin-resistance determining region of *rpoB* nucleotide sequences and the corresponding amino acid sequences of MS14, ST612-MRSA-IV and a *rpoB* reference sequence (X64172 from a rifampicin susceptible *S. aureus* RN4220). Clusters I and II are sequences internal to the RRDR where most mutations associated with rifampicin resistance occur**.**

3.5 Discussion

MS14, isolated from a patient with septic arthritis at the Red Cross Children's Hospital in December 2008, was identified as ST612-MSSA-t064. It was resistant to penicillin, erythromycin, clindamycin, rifampicin, co-trimoxazole and gentamicin and was sensitive to cloxacillin, ciprofloxacin, fusidic acid, vancomycin and linezolid. This antibiotic susceptibility pattern was similar to that seen in ST612-MRSA-IV isolates (Jansen van Rensburg, Eliya Madikane et al. 2011).

Recent studies have suggested that multi-drug resistant MSSA isolates in hospitals frequently result from either precise or imprecise excision of SCC*mec* from corresponding MRSA (Donnio, Louvet et al. 2002; Donnio, Oliveira et al. 2005; Donnio, Fevrier et al. 2007; Shore, Rossney et al. 2008; Lindqvist, Isaksson et al. 2011). This raised the question of whether MS14 arose as a result of a loss of SCC*mec* from a corresponding ST612-MRSA strain.

Of the different SCC*mec* components investigated, only *dcs* was detected. This gene is the common downstream sequence associated with SCC*mec* I, II and IV and is located immediately upstream of the right junction/*orfX* region on these elements as shown for SCCmec II and IV in Fig 3.2 and Fig 3.3, respectively.

Even though *dcs* has been classified as SCC*mec*-related, Wong, et al. (2010) have reported the presence of this gene immediately downstream of *orfX* in some MSSA strains. In the same study, some isolates of MRSA were shown to contain a second copy of *dcs* outside of SCC*mec*, adjacent to *attL*. In MS14, a PCR assay and sequencing of the amplicons confirmed the presence of *dcs* within SCC*mec* related sequences, suggesting that this strain arose due to partial excision of SCC*mec* from ST612-MRSA.

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Support for this suggestion comes from the investigation of the relatedness of MS14 to ST612-MRSA-IV using the nucleotide sequences of the RRDR of *rpoB* as a marker of relatedness. Jansen van Rensburg, et al. (2011) identified two amino acid substitutions ($H_{481}N$, $I_{527}M$) in the RRDR of the β-subunit of the RNA polymerase in local ST612-MRSA-IV strains as well as in the two Australian strains but not in any of the other rifampicin resistant strains in the study. Further, all of the isolates investigated contained the single nucleotide polymorphism (SNP), CGT → CGC, at amino acid position 512. This silent SNP and the amino acid substitutions identified in ST612-MRSA-IV isolates were also identified in MS14, suggesting the strains are related.

As shown in Table 3.1, the presence of *dcs* in MSSA strains, shown to be the result of a partial loss of SCC*mec*, has been identified previously. As described in [3.2], of the seven MSSA isolates investigated by Shore, et al. (2008) one isolate contained *dcs* 1.7kb downstream of *orfX* in the right junction/*orfX* region. Similarly, in the study by Lindqvist, et al. (2011), elaborated in [3.2], 51 of the 54 samples, which were investigated for remnants of SCC*mec*, carried *dcs* at the right junction/*orfX* region. Forty nine of the isolates which carried *dcs* were from a clonal outbreak, suggesting expansion of a clone following partial excision of SCC*mec*.

The *dcs* region has also been identified in combination with other remnants of SCC*mec* in MSSA. In the study by Donnio, et al. (2007) (expanded in [3.2]), 169 of the 247 MDR-MSSA isolates investigated contained remnants of SCC*mec*. Of the 169 isolates, 141 had *spa* types (t008 (*n=104*), t024 (*n=7*), other (n=30)) associated with CC8. A representative strain from *spa* t008 was shown to have a *HVR-IS431-pUB110-IS431-dcs* fragment associated with the right junction on SCC*mec* IA, II, and IVA.

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For the purpose of the current study, the identification of the *dcs* region in the *attB* site in MS14 confirmed the presence of a SCC*mec* remnant in the strain and therefore further characterization of the insert was not conducted. While the findings were insufficient to determine whether *dcs* in MS14 emanated from SCC*mec* IV, the fact that no other SCC*mec* was identified in association with ST612-MRSA (Jansen van Rensburg, Eliya Madikane et al. 2011) supports such a suggestion.

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Which is fitter: MS14 or ST612-MRSA-IV?

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4.1 Abstract

Evidence presented in the previous chapters suggested that MS14 resulted from a partial loss of SCC*mec* from a corresponding ST612-MRSA strain. Studies have associated fitness advantage with the loss of this element. The study described in this chapter compared fitness between MS14 and ST612-MRSA-IV strains (D4, E3, E8 and S5).

Exponential phase growth rates of the resistant strains, relative to MS14, revealed a slower growth rate for S5 while E3, E8 and D4 grew at similar rates to MS14.

Competition between the resistant strains and MS14 was investigated with 24-hour and five-day pairwise competition assays. Collectively these assays revealed a reduction in the fitness of the resistant strains, with the exception of D4, relative to MS14.

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4.2 Introduction

Evidence presented in the previous chapters suggested that MS14 resulted from a partial loss of SCC*mec* from a corresponding ST612-MRSA strain. Loss of SCC*mec* has been shown to occur spontaneously; studies have associated this loss with an increase in fitness (Ender, McCallum et al. 2004; Lee, Ender et al. 2007; Noto, Fox et al. 2008) where fitness is defined as the ability of bacteria to grow and survive in a specific environment. Nübel, et al. (2008) suggested that bacterial strains are likely to predominate in environments for which they are best fitted. When fitness is compromised the bacterial population may be vulnerable to being displaced by a stronger competitor in the same environment.

Cost to fitness in bacteria may be due to a number of factors, including the acquisition of extra-chromosomal elements, which carry antibiotic resistance genes, as well as chromosomal mutations which alter the target of an antibiotic, thus reducing its affinity for the antibiotic (Levin, Lipsitch et al 1997; Andersson and Levin 1999). For example, amino acid substitutions in the RNA polymerase, the target for rifampicin, confers resistance to this antibiotic in a number of organisms, but frequently at a fitness cost to the host (Billington, McHugh et al. 1999; Reynolds 2000; Wichelhaus, Boddinghaus et al. 2002; Mariam, Mengistu et al. 2004; O'Neill, Huovinen et al. 2006). A study by Wilchelhaus, et al. (2002) compared fitness of a rifampicin susceptible *S. aureus* strain with a number of its isogenic resistant counterparts, each with a different mutation associated with rifampicin resistance. Compared to the parent, pairwise competition assays revealed that with the exception of $H_{481}N$, all the substitutions imposed a fitness cost, to varying degrees, on the host. In a similar assay involving a rifampicin susceptible reference *S. aureus* strain and its resistant mutants, O'Neill, et al. (2006) reported a correlation between relative fitness of the isogenic test strain and its rifampicin MIC. Strains with mutations associated with high rifampicin

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MICs were less fit than their counterparts with low MICs. Interestingly, unlike in the study of Wilchelhaus, et al, O'Neill, et al observed a fitness cost in a strain carrying the $H_{481}N$ substitution, even though this strain had a MIC of only 2 µg/ml, which is the equivalent of the MIC interpretative standard for intermediate resistance to rifampicin in *S. aureus* (CLSI 2009).

It is often the acquisition, as well as the maintenance of extra-chromosomal elements, which impose fitness cost on the host (Andersson and Levin 1999; Foucault, Courvalin et al. 2009; LaMarre, Locke et al. 2011). A good example is *cfr*, which encodes a rRNA methyltransferase that modifies a conserved adenine in the 23SrRNA, thus conferring resistance to antibiotics such as linezolid (LaMarre, Locke et al. 2011). In 2005, this gene, often plasmid borne in staphylococcal isolates from animals, was identified located on the chromosome in a linezolid resistant MRSA isolate from a patient in Medellin, Columbia (Toh, Xiong et al. 2007). In this MRSA, *cfr* was located downstream of *ermB* (*erm* (B)), a rRNA methyltransferase that modifies another adenine in the same region as *cfr.* Both genes are co-expressed in the *mlr* operon under the P_{erm} promoter (Toh, Xiong et al. 2007). In their study, LaMarre, et al. (2011) reported a fitness cost, albeit low at 4.6% per generation, associated with basal expression of *cfr* when pairwise competition assays were carried out between two isogenic MRSA strains; one expressing *cfr* on a plasmid vector and the other carrying the empty vector. Interestingly, in similar experiments, competition between two isogenic MRSA strains carrying plasmid borne *cfr::ermB*, with or without an active *cfr*, revealed a significant increase of 10.6% in the cost to fitness of *cfr* with the co-expression of *ermB* (LaMarre, Locke et al. 2011).

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Foucault, et al. (2009) also reported similar findings in which the acquisition and basal expression of the *vanA* operon, which encodes VanA-type glycopeptide resistance in *S. aureus* strains, was suggested to impose fitness cost on its MRSA host. In the study, the authors compared fitness between a *vanA* naïve MRSA strain, HIP11713, and its *vanA* transconjugant MRSA strain, VRSA-1. The *vanA* operon, in VRSA-1, is carried on a transposon, Tn*1546*, which in turn is carried on a multi-copy resident plasmid. Pairwise competition assays between the isogenic strains revealed a slight decrease in the fitness of the transconjugant strain in comparison to the susceptible strain, in the absence of vancomycin, suggesting a fitness disadvantage to the acquisition and basal expression of the *vanA* operon.

Similarly, the acquisition and basal expression of *mecA* has been reported to impose fitness cost on *S. aureus* strains (Noto, Fox et al. 2008). In their study, Noto, Fox et al. (2008) identified *S. aureus* strains which recovered their fitness, when serial passage in increasing concentrations of vancomycin promoted partial loss of SCC*mec*. To investigate if the loss of *mecA* contributed to the recovery in fitness of the vancomycin resistant strains, the authors compared fitness between MRSA 3130V32 and its *mecA* inactivated variant, 3130V32Δ*mec*A, which were recovered following serial passage in increasing concentrations of vancomycin. An eight-day pairwise competition assay between the two strains revealed poor survival of 3130V32, suggesting a fitness advantage to the inactivation of *mecA.* Further experiments, in which the authors compared fitness between vancomycin susceptible MRSA and their *mecA* inactivated MSSA counterparts, revealed that the fitness cost imposed by the carriage of *mecA* was not limited to vancomycin resistant strains, suggesting that cost to fitness is a consequence of the expression of *mecA* in *S. aureus* strains (Noto, Fox et al. 2008).

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Other studies have suggested that when compared to MRSA-IV strains, cost to fitness is increased in MRSA carrying SCC*mec* types I and II which frequently contain additional resistance genes (Lee, Ender et al. 2007; Collins, Rudkin et al. 2010). Relevant to the current study, Collins, Rudkin et al. (2010) compared fitness of clinical MRSA (CC8-MRSA-IV, CC22-MRSA-IV and CC30-MRSA-II) and related MSSA against a marker strain, MSSA 466. A cost to fitness on the clinical strains was associated with the presence of both *mec* types with a lower fitness cost associated with SCC*mec* IV while SCC*mec* II exerted a significantly higher cost (Collins, Rudkin et al. 2010). In this context, the author considered the possibility that MS14 could be fitter than ST612-MRSA-IV strains.

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4.3 Materials and Methods

4.3.1 *S. aureus* isolates used in this study

The MRSA and MSSA strains investigated as part of this study were presented in Tables 2.1 and 2.2, but for clarity of reading, the strains are further described in Table 4.1.

Table 4.1: *S. aureus* strains used in the current study

Isolate	Phenotype	MLST	*spa* type	PFGE	Antibiogram										
					P	**Clo**	**E**	**Cli**	**R**	**Co**	**Ci**	**G**	**F**	**V**	**L**
MS14	MSSA	ST612	t064	D*	R	S	R	R	R	R	S	R	S	S	S
D4	MRSA	ST612	t064	D	R	R	R	R	R	R	S	R	S	S	S
E3	MRSA	ST612	t1443	E	R	R	S	S	R	R	R	R	S	S	S
E8	MRSA	ST612	t064	E	R	R	R	R	R	R	R	R	S	S	S
S5	MRSA	ST612	t064	Sporadic	R	R	S	S	R	R	S	R	S	S	S

*typed in the current study
P, penicillin; **Clo**, cloxacillin; **E**, erythromycin; **Cli**, clindamycin; **Rif**, rifampicin; **Co**, co-trimoxazole; **Ci**, ciprofloxazin; **G**, gentamicin; **F**, fusidic acid; **V**, vancomycin; **L**, linezolid. R, resistant; S, susceptible.

4.3.2 Synchronization of the initial inocula

The initial inocula for the comparative assays were synchronized to exponential phase. This involved determining duration to exponential phase and the OD 600 nm values at these time points in the growth of the individual strains.

4.3.2.1 Determining duration to exponential phase and the OD 600 nm values at these time points

From the 50% glycerol stocks [2.3.1], each isolate was streaked for single colonies on boiled blood agar plates (Green point NHLS laboratories, Cape Town) and incubated overnight at 37°C. For each strain, a single colony from the overnight culture was inoculated into 5 ml 2YT broth [Appendix (A) 1.1]. Using a plastic loop, the cells were homogenized in the broth and then three 300 µl aliquots of each of the broth cultures were transferred onto a 100 honeycomb micro-well plate. The plate was transferred onto the Bioscreen C Microbiological Reader (Thermo Electron Corporation, USA), an automated incubator and

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OD reader system for monitoring the growth of microorganisms [A2.1]. This system consists of three components: the incubator, OD reader and the automated data analysis application (Research Express Software, Transgalatic, USA).

The system was programmed to incubate the bacterial cultures at 37°C for 24 hours, automatically recording OD 600 nm readings every 30 minutes. Using the software provided, growth curves were generated which were used to establish duration to exponential phase and the OD 600 nm value at this time point for each strain.

4.3.2.2 Cultivation of exponential phase cells

Single colonies for each strain were obtained and the cells were then grown to exponential phase as described [4.3.2.1]. Based on the data obtained from 4.3.2.1, incubation was terminated at exponential phase. A 250 µl aliquot of the exponential phase culture from each of four individual wells, for each strain, was added to 250 µl of 50% glycerol solution [A1.3].

For each strain, a representative glycerol stock vial was used to determine the number of CFUs prior to storage at -80°C. Initially, 10^6–fold serial dilutions of the stocks were prepared in 1X PBS, on a 96-well round bottom micro-titre plate (Adcock Ingram, Johannesburg, South Africa). Then 10 µl of the 10^4 to 10^6 dilutions was plated in duplicate on 2YT agar [A1.2], and incubated at 37 °C overnight (Fig 4.1).

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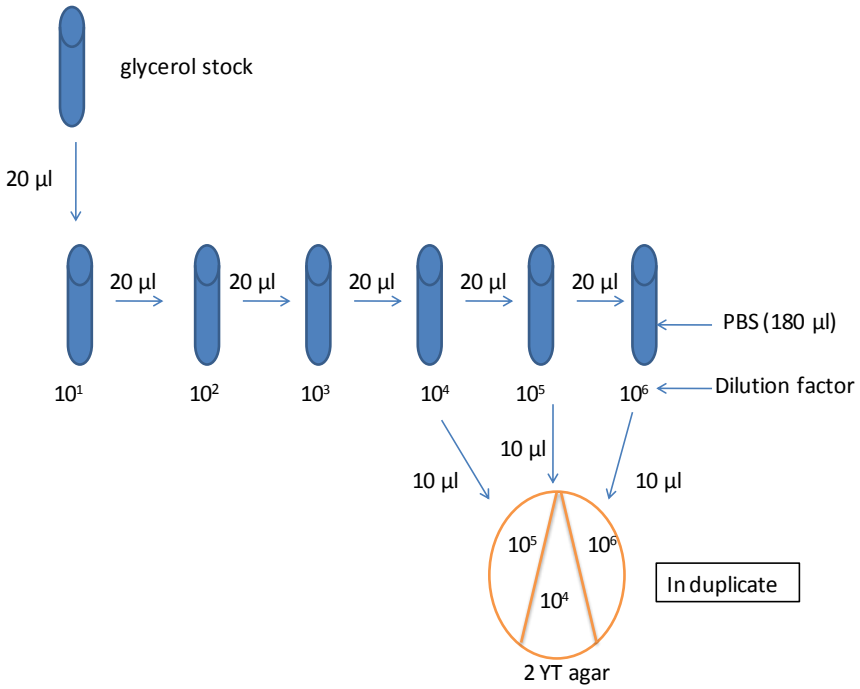

Figure 4.1: The dilution series for the quantification of the viable cell count. The glycerol stocks were diluted six fold at a 1: 10 ratio in PBS solution on a 96-well round bottom micro-titre plate (Adcock Ingram, Johannesburg, South Africa), represented by the blue vials. An aliquot of 10 µl of the 10^4 to 10^6 dilutions was plated in duplicate on 2YT agar, and then incubated overnight at 37°C.

The CFUs in the glycerol stocks were calculated as follows:

Average nō. of colonies X Total dilution factor = CFUml^{-1}

The dilution yielding between 10 to ≤ 100 colonies was acceptable for the experiments, taking into account the congruence in the dilutions. Following quantification, the cells were stored at -80°C. To confirm that the viability of the cells was not compromised during storage, quantification of CFUs was repeated after 24 hours of storage.

4.3.3 Comparison of the growth kinetics of the strains

The protocol described by Lindqvist, R (2006) for the comparison of individual strain growth rates, based on turbidity measurements, was adapted. Initially, the glycerol stocks of the individual strains were diluted, in a 96-well round bottom micro-titre plate, to 10^5 CFUml^{-1} in 1X PBS and then to 10^4 CFUml^{-1} in 2YT broth (Fig 4.2).

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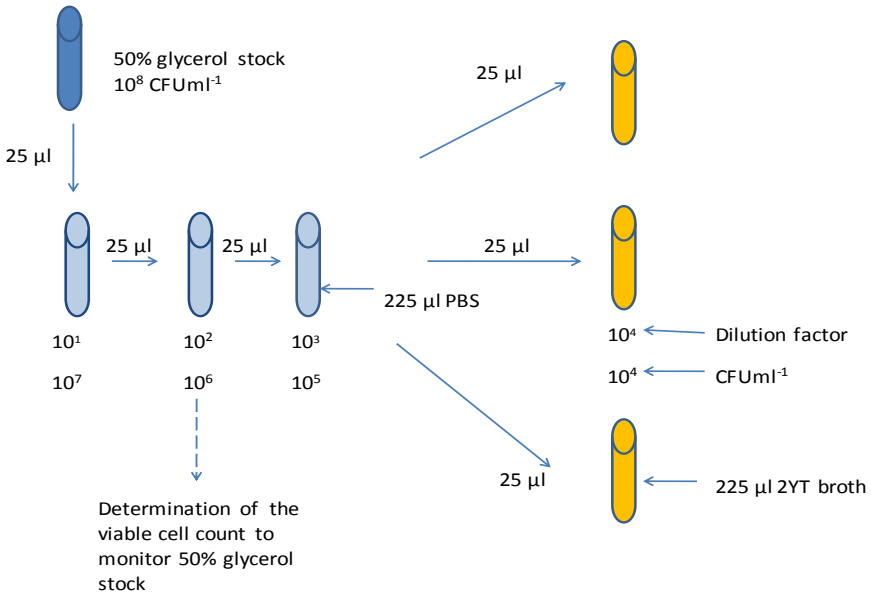

Figure 4.2: The serial dilution of the glycerol stocks for each strain. The stocks were initially diluted to 10^5 $CFUml^{-1}$ in 1 XPBS then 25 µl transferred into 225 µl of 2YT broth to a final concentration of 10^4 $CFUml^{-1}$. Concurrently with the comparison of growth rates, the viability of the cells was monitored following storage at -80°C. Broken arrow indicates a series of steps which included the dilution and subsequent plating of the samples to determine the viable cell count.

Triplicate aliquots of 250 µl of the 10^4 $CFUml^{-1}$ broth cultures were transferred onto a honeycomb plate, and cultured for 24 hours using the Bioscreen C system [4.3.2.1]. Concurrently, the viability of the cells was monitored following storage at -80°C as a control [4.3.2.3].

The growth rates (μ) per independent culture were calculated based on the equation described below (Foucault, Courvalin et al. 2009):

$\mu = [\ln N_t - \ln N_0] / (t - t_0)$; where N represents the OD 600 nm values at two time points (t) over which growth increased exponentially.

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4.3.4 Pairwise competition assays between *S. aureus* strains

Twenty four-hour and five-day pairwise competition experiments, adapted from the protocols previously described by Laurent, Lelievre et al. (2001) and Ender, McCallum et al. (2004), respectively, were carried out between MS14 and representative ST612-MRSA-IV strains in individual assays. In both experiments, cefoxitin (4 µg/ml), a surrogate for methicillin as per CLSI guidelines (CLSI 2009), was added to 2YT agar to select for the resistant strains.

4.3.4.1 Twenty four-hour pairwise competition assays between MS14 and ST612-MRSA-IV

Initially, 24-hour pairwise competition experiments, adapted from the protocol described by Laurent, Lelievre et al. (2001), were carried out, in duplicate. Serial dilutions of the glycerol stocks were prepared as shown in Fig 4.2. Then, 100 µl of 10^6 CFUml^{-1} each of the resistant and susceptible strains was inoculated into 10 ml 2YT broth in a 100 ml Erlenmeyer flask, to give initial inocula of 10^5 CFUs each (Fig 4.3).

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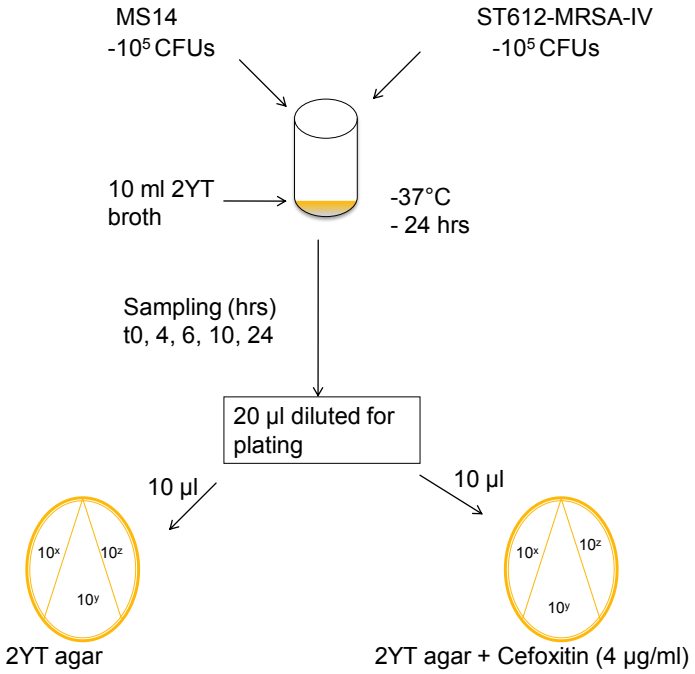

Figure 4.3: Flow diagram depicting the 24-hour pairwise competition assays with MS14 and the ST612-MRSA-IV strains. A volume of 100 µl of 10^6 CFUml^{-1} of each of the resistant and susceptible strains was inoculated into 10 ml 2YT broth in a 100 ml Erlenmeyer flask, to give 10^5 CFUs, and incubated at 37°C with shaking (100 rpm). At specific time points as indicated, 20 µl of the culture was removed, diluted and 10 µl of the respective dilutions plated, in parallel, on non-selective 2YT agar and on selective 2YT agar media containing 4 µg/ml cefoxitin (as per CLSI guidelines (CLSI 2009)). 10^x, 10^y and 10^z, the dilution factors for the respective dilutions plated. All the experiments were carried out in duplicate.

For the calculation of the CFUs for the susceptible and resistant strains in the individual assays, the total cell count was determined by plating the mixed cultures on non-selective 2YT agar media. The CFUs for the resistant strain, determined by plating on selective 2YT agar media containing 4 µg/ml cefoxitin, were subtracted from the total cell count to give the CFUs for the susceptible strain.

4.3.4.2 Five-day mixed culture competition assays between MS14 and ST612-MRSA-IV

Five-day pairwise competition experiments, adapted from the protocol described by Enders, et al. (2004), were carried out. Serial dilutions of the glycerol stocks were conducted [4.3.2.3] to obtain 10^4 CFUs as initial inocula for individual strains. The assays proceeded as depicted in Fig 4.4.

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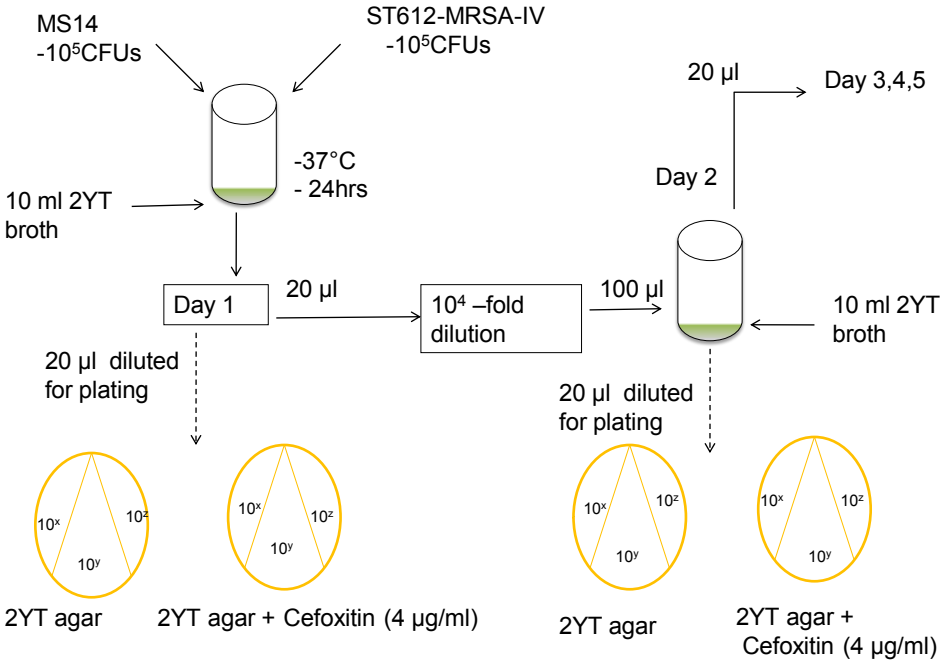

Figure 4.4: The flow diagram depicting the five-day pairwise competition assays with MS14 and the ST612-MRSA-IV strains. A volume of 100 µl of 10^6 CFUml^{-1} of each of the resistant and susceptible strains was inoculated into 10 ml 2YT broth in a 100 ml Erlenmeyer flask, to give 10^5CFUs, and incubated at 37°C with shaking (100 rpm). At 24-hour intervals, 20 µl of the culture was removed, diluted and 10 µl of the respective dilutions plated, in parallel, on non-selective 2YT agar and on selective 2YT agar media containing 4 µg/ml cefoxitin (as per CLSI guidelines (CLSI 2009)). 100 µl of the 10^4 dilution of the 24-hour culture was transferred into 10 ml fresh 2YT broth in a 100 ml Erlenmeyer flask, incubated under the same conditions as before, in a serial passage over a four day period. 10^x, 10^y and 10^z, the dilution factors for the respective dilutions plated. Broken arrows indicate a series of steps in the serial dilution of the cultures. All the experiments were carried out in duplicate.

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4.3.4.3 Data analyses for the pairwise competition assays

For the investigation of the competition between MS14 and the ST612-MRSA-IV strains, two methods were used: 1) end point analyses which compared the ratio of the colony forming units for each individual strain at the beginning (t_0) and the end (t_n) of the assay (Laurent, Lelièvre et al. 2001); 2) calculation of the relative fitness of the resistant strains in comparison to MS14 which was based on the equations below (Foucault, Courvalin et al. 2009; Billington, McHugh et al. 1999):

Generation number (g) = (log$B$ – log$A$)/ log2; where A and B were colony forming units for each individual strain at the beginning and the end of the assay, respectively.

Relative fitness (RF) = g_R/g_S; where g_R is the average generation number of the resistant strain and g_S the average generation number of the susceptible strain.

4.3.5 PFGE for the relatedness of MS14 to D4

The results of the competition assays indicated that it was necessary to carry out PFGE to better understand the relatedness of MS14 to the resistant isolates, particularly D4. PFGE [1.4.1.1] was carried out based on the protocol described by Reed, Stemper et al. (2007). The strains used in this experiment included, MS14, and the comparators, D4, E3, E8 and S5. From the glycerol stocks [4.3.2.2], individual strains were plated on 2YT agar and incubated overnight at 37°C. For each strain, a sweep of colonies was suspended in 2 ml TEN buffer [A4.1] in a sterile universal bottle and the turbidity adjusted to between OD 600 nm 0.825 - 0.875 using the Spectronic Biomate 5 UV-Vis (Thermo Electron Corporation, USA).

For the preparation of the plugs, 250 µl of the cell suspension of each of the isolates was transferred into a sterile 2 ml tube, to which 5 µl of lysostaphin was added, followed by 250 µl of molten 1.8% (w/v) Seakem Gold Agarose gel (Cambrex BioScience, Rockland, USA), prepared as outlined in A4.2. The mixture was immediately homogenised by pipetting up and down and 250 µl aliquoted into plug moulds to form two plugs per suspension, which were then allowed to solidify at 4°C for 10 min.

To obtain intact genomic DNA each of the isolates was lysed *in situ* by submerging the agarose plugs in 2 ml EC buffer [A4.3], which had been pre-warmed to 37°C, and incubating the plugs at 37°C for 4 hours to facilitate digestion of the cell wall and membrane. Subsequently, the EC buffer was decanted and replaced with 2 ml ESP buffer [A4.4]. The

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plugs were then incubated at 55°C for 16 hours; after which the plugs were transferred to sterile universal bottles and submerged in 10 ml TE buffer [A4.5].

The universal bottles were then placed on a variable speed rotor at 30 rpm. After 30 min, the buffer was decanted, replaced with 10 ml fresh TE buffer, and then placed on the rotor for a further 30 min. These washes were repeated three times with fresh TE buffer.

The genomic DNA obtained was digested with *Sma*I. Using a blade, a 1 x 5 mm slice of a plug was transferred into the *Sma*I restriction digest mixture which consisted of 1 µl BSA (BioLabs, New England), 30U *Sma*I (Roche Diagnostics, Germany) and 1X *Sma*I restriction buffer in a final volume of 128 µl. The plugs were then incubated at 25°C for 3 hours.

As outlined in A4.6, 150 ml of 1% (w/v) Biorad Ultrapure Molecular Grade Agarose gel (Bio-Rad Laboratories, California, USA) was prepared and equilibrated at 60°C for 10 min. The agarose was poured into the gel tray and allowed to set for 2.5 hours. Two and a half litres of 0.5X TBE [A4.7] was transferred into the cooling unit of the PFGE apparatus (Gene Navigator™ System, Amersham Biosciences AB, Sweden) and allowed to chill at 4°C for 2.5 hours.

Following the restriction digest, the plugs were carefully loaded on the gel and the wells sealed with 1% (w/v) Seakem Gold Agarose gel [A4.2]. The PFGE components were assembled and the programme parameters set as follows: 5 s initial switch time, 30 s final switch time, 20-hour duration run, 200V and chiller at 14°C.

For visualisation of the restriction digest profiles, the gel was placed in a container to which 500 ml of the running buffer with 1 mg/ ml EtBr had been added. The container was placed on a rotor set at 30 rpm for 30 min. The gel was destained in 400 ml distilled water using the rotor set at 30 rpm for 15 min, and then viewed under UV light.

Analysis of the PFGE profiles was carried out visually.

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4.4 Results

4.4.1 Synchronization of the initial inocula

The initial inocula for the test strains were synchronized to exponential phase based on OD 600 nm measurements over time [4.3.2.1]. Initially the cells were cultured to stationary phase revealing exponential growth for all strains between two and three and a half hours, as indicated in Fig 4.5, at OD values ranging between 0.3 and 0.8.

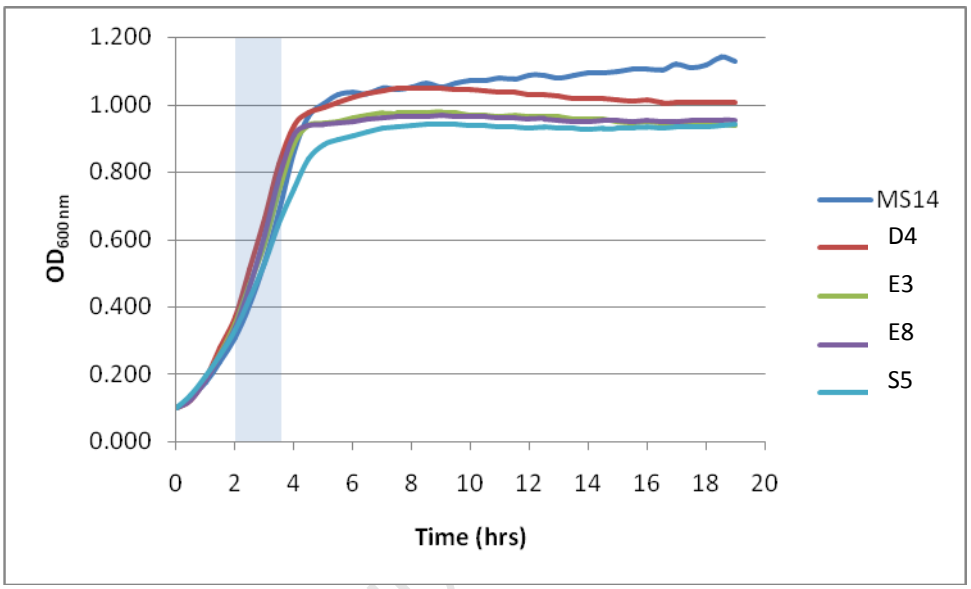

Figure 4.5: The growth parameters of the individual strains, based on 0D 600 nm measurements over time. For all strains, the exponential growth occured between 2 and 3.5 hours, as indicated by the grey bar on the graph.

The second step of synchronisation involved culturing the cells for three hours [4.3.2.2], after which, the experiment was stopped at the OD values indicated in Table 4.2. Based on the average of duplicate cultures (Fig 4.1) viable cell counts [A3.1] of the exponential phase cells were determined (Table 4.2).

Table 4.2: OD 600 nm measurements and the viable cell count at exponential phase (3 hours).

***S. aureus* isolates**		**Viable cell count (3 hours)**			
		OD 600 nm	**Average number of colonies**	**Total dilution factor**	**CFUml^{-1}**
MS14		0.609	57	10^7	5.7×10^8
ST612-MRSA-IV	D4	0.441	23	10^7	2.3×10^8
	E3	0.479	35	10^7	3.5×10^8
	E8	0.473	54	10^6	5.4×10^7
	S5	0.451	21	10^7	2.1×10^8

4.4.2 Comparison of the growth kinetics of the resistant strains relative to MS14

The growth kinetics of the individual strains were compared based on turbidity at OD 600 nm over a 24-hour period. As depicted in Fig 4.6, while the resistant strains had a slightly longer lag phase (six to eight hours) in comparison to MS14 (five hours), the exponential phase for all strains was in the range OD 0.2 – OD 0.6.

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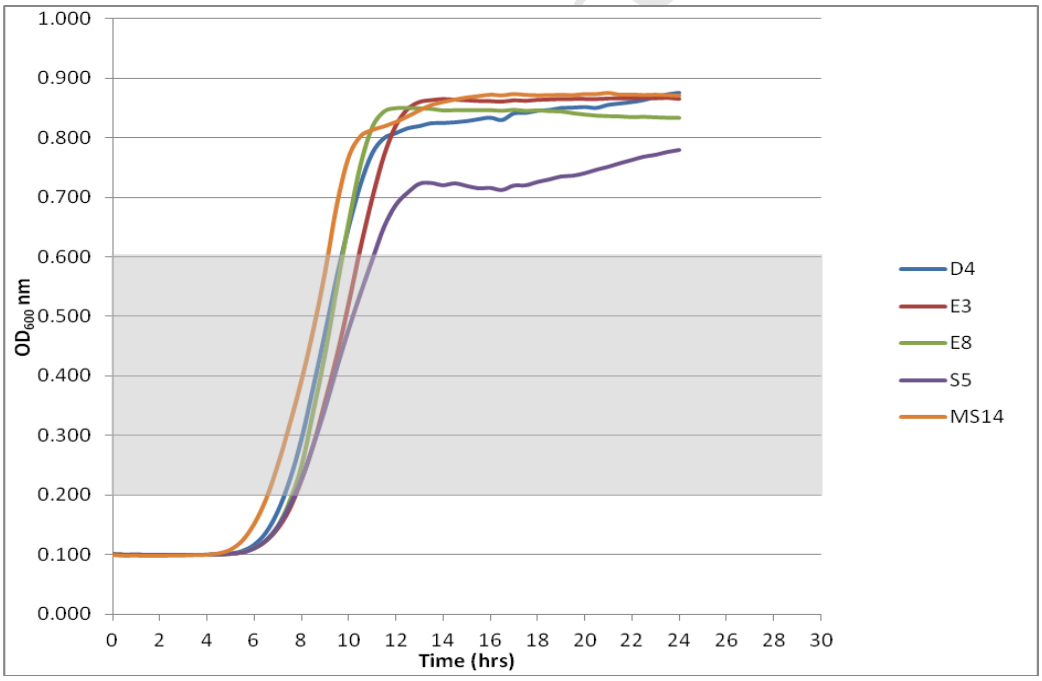

Figure 4.6: Comparative growth rates between ST612-MRSA-IV strains and MS14 over a 24-hour period. The exponential phase of the strains is indicated in grey on the graph, the values of which were used to compare the growth rates of the resistant strains relative to MS14.

Using the OD 600 nm values, 0.2 - 0.6, which represent exponential growth of the strains as shown in Fig 4.6, the growth rates (μ) of the individual strains were calculated using the formula, $\mu = [\ln N_t - \ln N_0]/ (t - t_0)$ (Table 4.3).

Table 4.3: The growth rates calculated based on OD 600 nm measurements at exponential phase (0.2 – 0.6) of the individual strains.

Isolate	**$\mu = [\ln N_t - \ln N_0]/ (t - t_0)$**						**Average growth rate ± SD[a]**
	A	B	C	D	E	F	
MS14	0.414	0.524	0.434	0.429	0.408	0.443	0.442 ± 0.042
D4	0.494	0.415	0.493	0.397	0.543	0.586	0.488 ± 0.073
E3	0.402	0.392	0.437	0.423	0.479	0.466	0.433 ± 0.035
E8	0.494	0.508	0.503	0.593	0.603	0.478	0.530 ± 0.054
S5	0.102	0.323	0.385	0.262	0.294	0.336	0.284 ± 0.098

$\mu = [\ln N_t - \ln N_0]/ (t - t_0)$; where N represents OD 600 nm values at two time points (t) over which growth increased exponentially. A, B, C, D, E, F, growth rates of the strains calculated per individual culture.
[a] Exponential growth rate of individual strains represented as a mean of six independent cultures per isolate ± standard deviation.

A bar graph of these data (Fig 4.7) indicates that there was no significant difference in the growth rates between MS14, D4, E3 and E8. S5, on the other hand, grew more slowly.

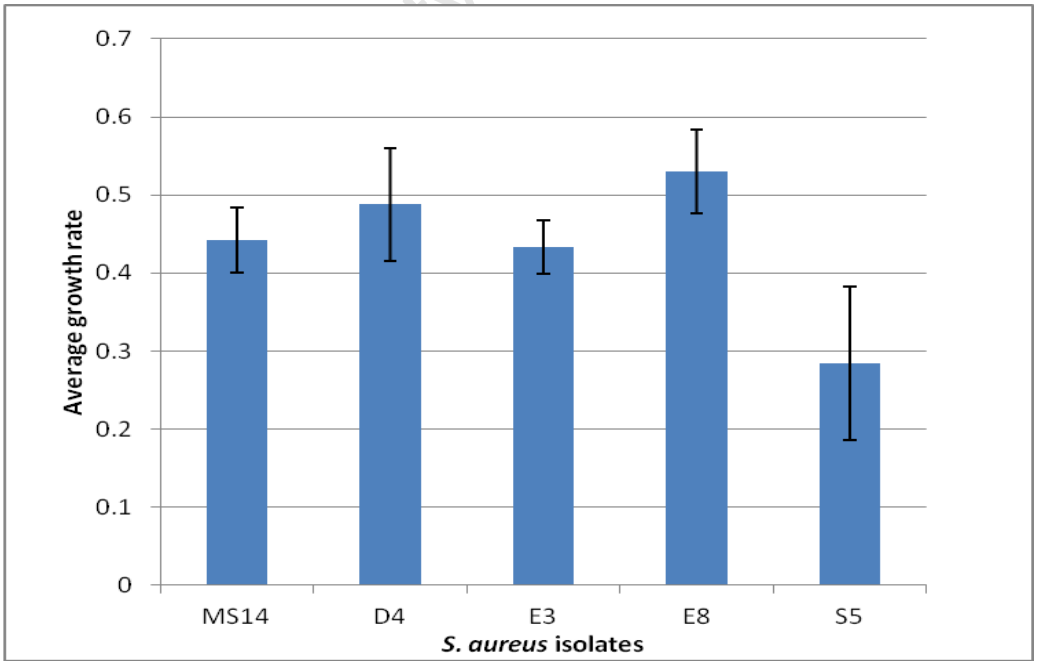

Figure 4.7: The average growth rates of the strains. Average growth rates, with error bars (mean ± standard deviation), were calculated based on growth of the strains at exponential phase (OD 0.2 – OD 0.6) as depicted in Fig 4.6. Each value represents the mean of six independent cultures per isolate.

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4.4.3 Pairwise competition assays between *S. aureus* strains

4.4.3.1 Twenty four-hour pairwise competition assays between MS14 and ST612-MRSA-IV

Pairwise competition assays between MS14 and each of the resistant strains were carried out, over a 24-hour period [4.3.4.1]. As shown in Fig 4.8, there was no difference in the bacterial cell count between MS14 and each of the resistant strains for the first 10 hours of growth, with the bacterial cell count in the individual paired experiments increasing from the initial inocula (Table 4.4) to a maximum of 10^9 CFUml^{-1} for all the strains (Table A3.2.1).

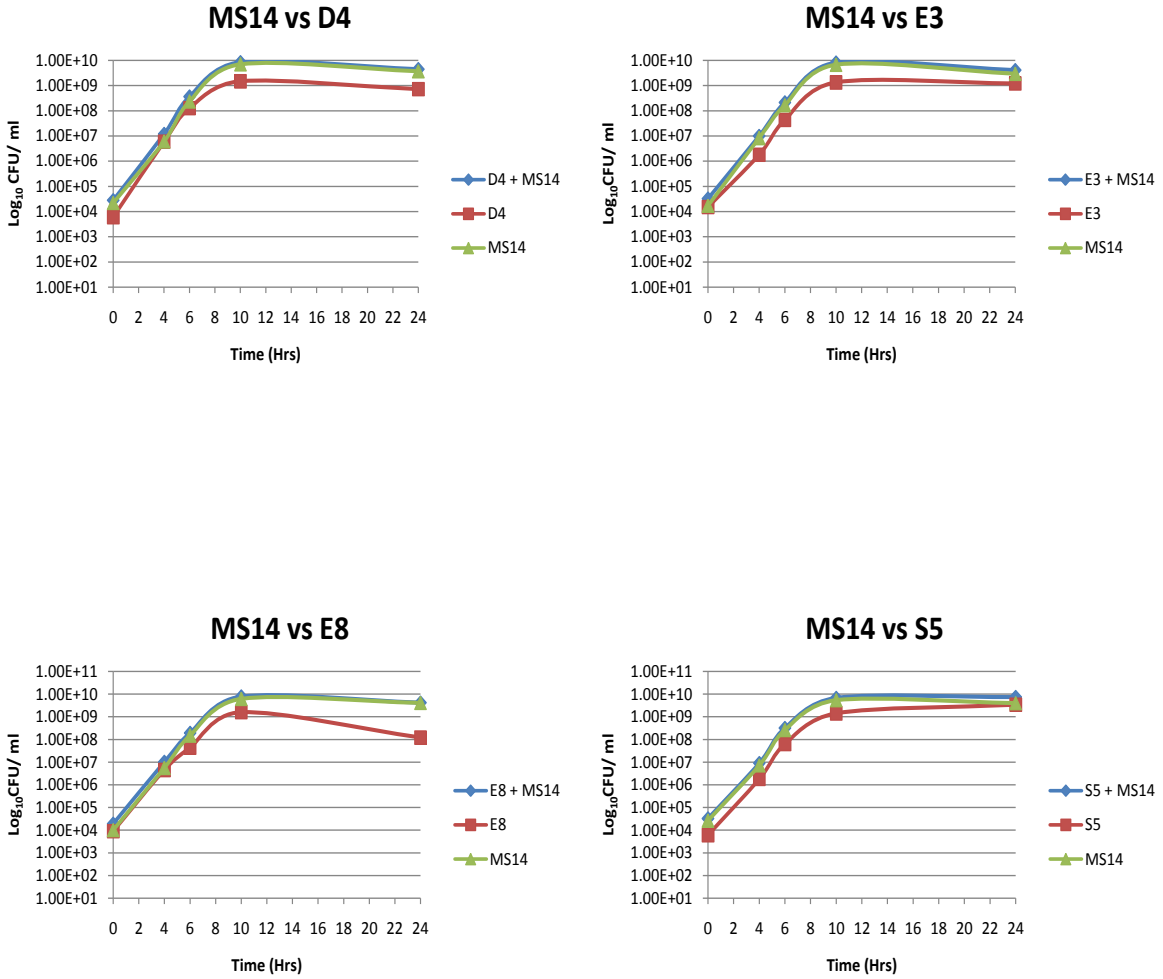

Figure 4.8: The pairwise competition assays between MS14 and ST612-MRSA-IV strains, carried out over a 24-hour period. The viable cell count of the susceptible strain in each assay was calculated by subtracting the viable cell count of the resistant strain from the total viable cell count in the mixed culture. Each assay was carried out in duplicate and the data represented as an average of the two experiments.

During the subsequent 14 hours of growth, the bacterial cell count for MS14, E3 and S5 remained at 10^9 CFUml^{-1} while D4 and E8 cell counts decreased to 10^8 CFUml^{-1} (Fig 4.8). Similar results were obtained when the ratio of CFUs for each individual strain was obtained at the beginning (t_0) and the end (t_{24}) of the assay (Table 4.4).

Table 4.4: Ratio of ST612-MRSA-IV and MS14 in the 24-hour pairwise competition assays

Isolate	**ST612-MRSA-IV CFUml^{-1}**		**MS14 CFUml^{-1}**		**Ratio ST612-MRSA-IV/MS14 (CFUml^{-1})**	
	Initial CFUml^{-1} (t_0)	**Final CFUml^{-1} (t_{24})**	**Initial CFUml^{-1} (t_0)**	**Final CFUml^{-1} (t_{24})**	**Initial ratio (t_0)**	**Final ratio (t_{24})**
D4	6.0 x 10^3	7.2 x 10^8	2.2 x 10^4	3.7 x 10^9	0.27	0.19
E3	1.5 x 10^4	1.2 x 10^9	1.7 x 10^4	2.9 x 10^9	0.88	0.41
E8	9.0 x 10^3	1.2 x 10^8	1.0 x 10^4	4.1 x 10^9	0.90	0.03
S5	6.0 x 10^3	3.4 x 10^9	2.6 x 10^4	4.0 x 10^9	0.23	0.85

t, time, in hours, of sampling following incubation at 37°C. CFUml^{-1} values presented as a mean of two separate experiments.

When the cell counts obtained at t_0 and t_{24} for the resistant isolates were divided by the corresponding counts obtained for MS14, the ratio between D4 and MS14 remained relatively constant after 24 hours. On the other hand, there were respective 2-fold and 30-fold decreases in E3 and E8, and a 4-fold increase in the cell count of S5, when compared to MS14 (Table 4.4).

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4.4.3.2 Five-day pairwise competition assays between MS14 and ST612-MRSA-IV

Five-day serial passages of the pairwise competition assays were carried out [4.3.4.2]. The bacterial cell count for all the resistant strains remained relatively similar to the respective count of MS14 in the paired assays for the first 24 hours, growing from the initial inocula (Table 4.5) to the final count of 10^9 CFUml^{-1} for all strains (Fig 4.9).

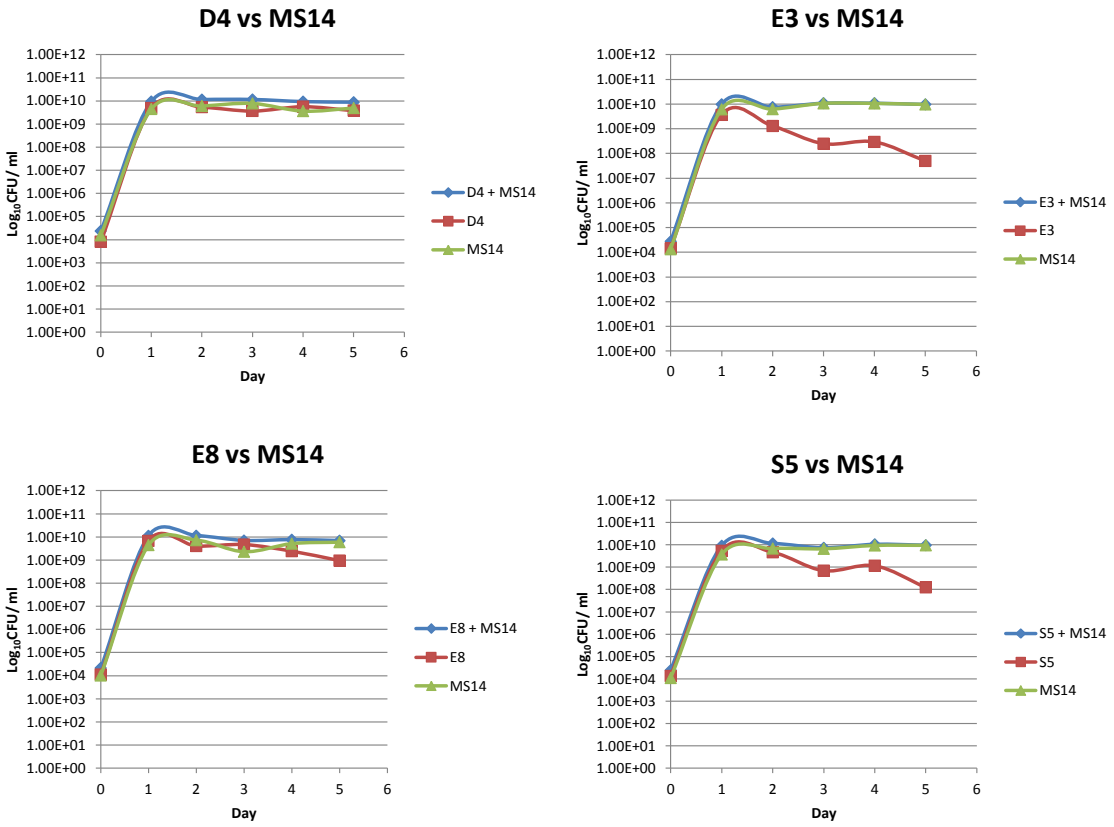

Figure 4.9: The growth curves for pairwise competition assays between ST612-MRSA-IV strains and MS14 during five-day serial passage experiments. The viable cell count of the susceptible strain in each assay was calculated by subtracting the viable cell count of the resistant strain from the total viable cell count in the mixed culture. Each assay was carried out in duplicate and the data represented as an average of the two experiments.

However, the cell count for three of the resistant strains (E3, E8 and S5) began to decrease from between day two and day four in comparison to MS14 (Table A3.2.2). The colony count of D4 remained similar to MS14 at 10^9 CFUml^{-1} (Fig 4.9). By day five of the experiments, E8 and S5 had each decreased to 10^8 CFUml^{-1} while the cell count for E3 decreased to 10^7 CFUml^{-1}. In each of these assays, MS14 remained constant at 10^9 CFUml^{-1} but increased to 10^{10} CFUml^{-1} when paired with E3. These findings were reflected in the end-point analyses of the competition assays (Table 4.5), in which the ratio of D4 to MS14 remained the same throughout the five-day assay, while significant differences were reflected in the ratios of E3, E8 and S5 to MS14 in the respective assays.

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Table 4.5: Ratio of ST612-MRSA-IV to MS14 in the five-day pairwise competition assays

Isolate	ST612-MRSA-IV CFUml^{-1}		MS14 CFUml^{-1}		Ratio ST612-MRSA-IV/MS14 (CFUml^{-1})	
	Initial CFUml^{-1} (t_0)	Final CFUml^{-1} (t_5)	Initial CFUml^{-1} (t_0)	Final CFUml^{-1} (t_5)	Initial ratio (t_0)	Final ratio (t_5)
D4	8.25×10^3	4.35×10^9	1.55×10^4	4.45×10^9	0.53	0.977
E3	1.44×10^4	5.00×10^7	1.34×10^4	9.70×10^9	1.07	0.005
E8	1.13×10^4	9.50×10^8	1.02×10^4	5.95×10^9	1.11	0.160
S5	1.35×10^4	1.25×10^8	1.09×10^4	9.53×10^9	1.24	0.013

t, time, in days, of sampling following incubation at 37°C. CFUml^{-1} values presented as a mean of two separate experiments.

At the end of the five-day assays, there was a seven-fold decrease in E8 cell numbers in comparison to MS14 while E3 decreased by 200-fold (Table 4.5). A 95-fold decrease in the cell count of S5 was significantly different from the four-fold increase observed for this isolate for the 24-hour assay.

4.4.3.2.1 Relative fitness of MS14 in comparison to ST612-MRSA-IV

The relative fitness of MS14, in comparison to its resistant counterparts, was calculated based on the five-day pairwise competition assays. Using the formula, relative fitness (RF) = average generation number of resistant strains (g_R) / average generation number of susceptible strains (g_S) [4.3.4.3], there were no significant differences in the relative fitness of D4 and MS14 over the five days (Fig 4.10). After the first 24 hours, a similar result applied to E3, E8, and S5. However, relative to MS14, the fitness of E3 and S5 decreased daily over the five-day period to approximately 0.6 (Fig 4.10). Although the relative fitness of E8 remained constant for four days, it decreased to 0.8 by day five (Fig 4.10).

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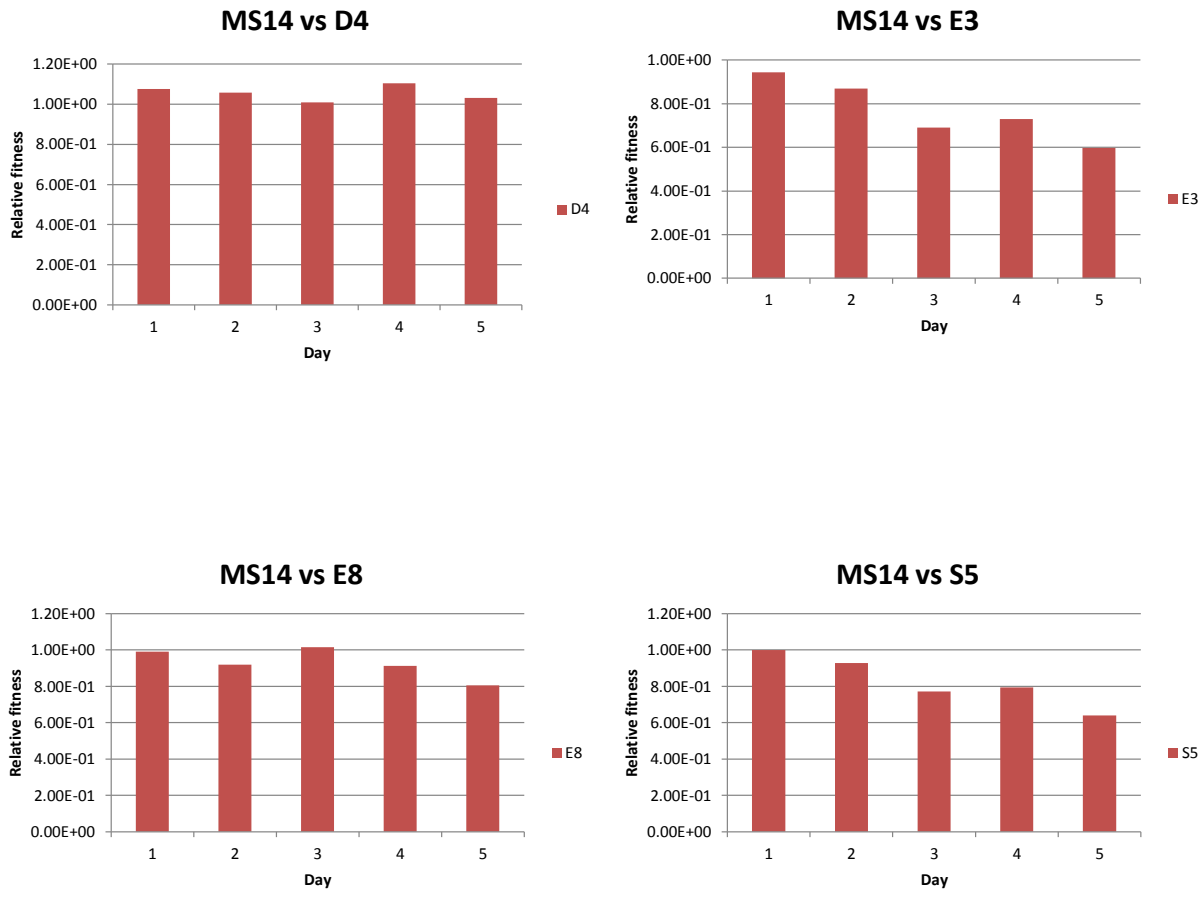

Figure 4.10: The relative fitness of MS14 in comparison to the resistant strains, D4, E3, E8 and S5. Relative fitness was calculated, every 24 hours, as the ratio between the average number of generations of the individual resistant strains and MS14 for the duration of the five-day competition assays (Table A3.2.3). Each assay was carried out in duplicate.

For the duration of the assay, there were no differences in the relative fitness of D4 and MS14 (Fig 4.10). On the other hand, the relative fitness of E3 and S5 decreased over the five day period, to approximately 0.6 on day five, for both strains, relative to MS14. Although the relative fitness of E8 remained relatively constant for four days, it decreased to 0.8 by day five (Fig 4.10).

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4.4.4 PFGE profiles of MS14, D4, E3, E8 and S5

Analysis of the PFGE profiles of MS14 and the resistant isolates indicated that MS14 is more closely related to D4 than to E3, E8 and S5 (Fig 4.11). Isolate D4 contains a *Sma*I fragment which is absent in MS14, while the latter contains a doublet immediately below the missing fragment (Fig 4.11). It is assumed these differences result from the partial excision of SCC*mec* IV; this could be confirmed by Southern blot hybridisation using *mecA* as a probe.

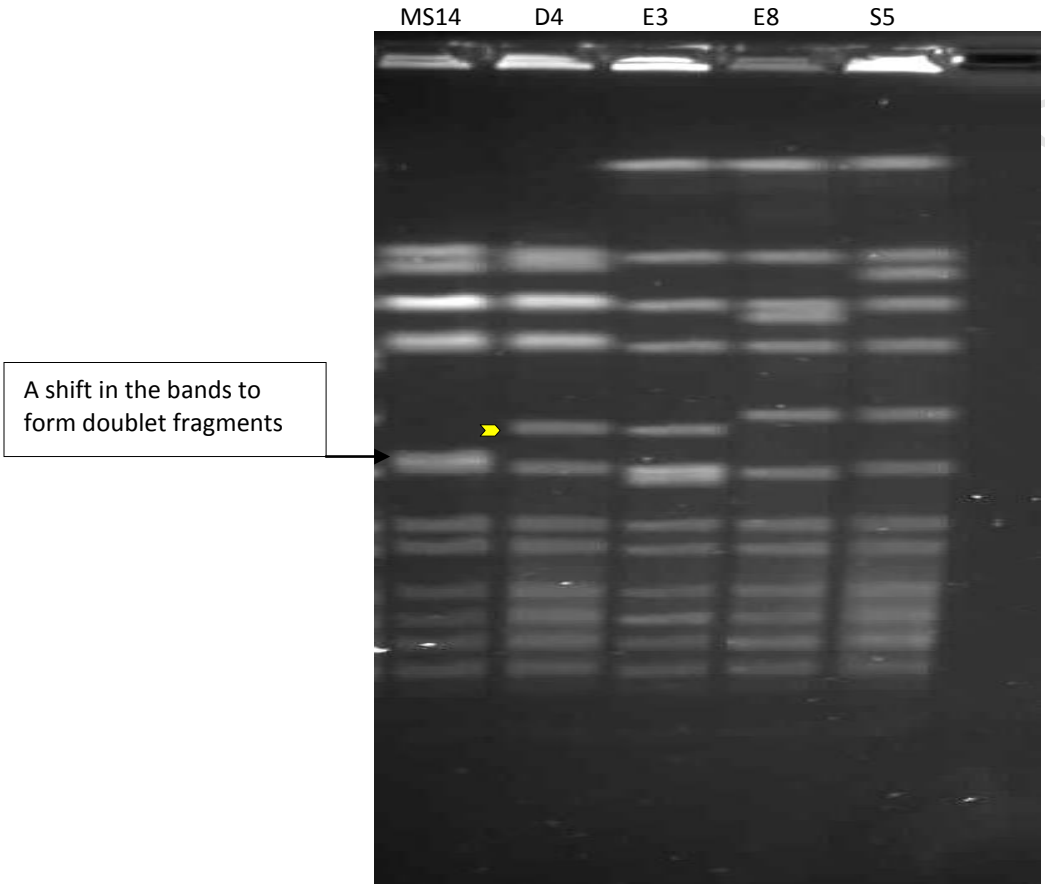

Figure 4.11: *Sma*I restriction profiles of MS14 and ST612-MRSA-IV isolates, D4, E3, E8 and S5. Yellow arrow shows a fragment present in D4 but absent in MS14.

4.5 Discussion

Acquisition and basal expression of *mecA* imposed a fitness cost on *S. aureus* strains in the studies by Ender, McCallum et al. (2004), Lee, Ender et al. (2007), Collins, Rudkin et al. (2010) and Noto, Fox et al. (2008). Partial deletion of SCC*mec*, notably the deletion of *mecA*, has been associated with fitness advantage in MSSA relative to MRSA and was first described by Noto, Fox et al. (2008).

The relative growth rates of the individual strains were compared based on turbidity measurements at OD 600 nm. Compared with the growth rate of MS14, all of the resistant strains had longer lag phases; however, the exponential growth rates of D4, E3 and E8 were the same as MS14 (Fig 4.7). On the other hand, the growth rate of S5 was slower than the corresponding rate of MS14. A lack of consensus in exponential growth rates of resistant strains compared to their susceptible counterparts was also observed in a study by Noto, et al. (2008) in which one of three MSSA strains grew faster than its MRSA counterpart. In their study, the authors compared exponential growth phase doubling times between three vancomycin resistant MSSA strains, two with partial SCC*mec* II and one (5836VR) with site-specifically excised SCC*mec* II, and their vancomycin resistant MRSA counterparts. A non-significant difference in the doubling times of the MSSA strains and their MRSA counterparts was observed; on the other hand, complete excision of SCC*mec* resulted in improvement in the growth rates of 5836VR. The reasons for the differences in the growth rates within D4, E3, E8 and S5, and between the individual resistant isolates, and MS14 are unknown and may be unrelated to the presence or absence of *mecA*. As a measure of fitness of strains, the comparison of exponential growth rates alone is a poor yardstick as it fails to index the global fitness of the strains throughout their growth cycle (Noto, Fox et al. 2008).

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Based on the exponential phase growth rates of the individual strains, in which S5 was dividing at a slower rate than MS14 while D4, E3 and E8 grew at similar rates as the susceptible counterpart, MS14 could be expected to outcompete S5 but compete successfully with D4, E3 and E8. However when the individual resistant strains and MS14 were competed over 24 hours, S5, with a four-fold increase in cell numbers at 24 hours revealed a slight competitive advantage over MS14 (Fig 4.8). On the other hand, respective two-fold and 30-fold decreases in E3 and E8 suggest that MS14 is more competitive with respect to these resistant strains. No fitness differences were observed between MS14 and D4 (Fig 4.8).

With the exception of D4, differences in the viable cell counts, only detected after 24 hours, between MS14 and the resistant strains, were mirrored in a study by Ender, McCallum et al. (2004) in which MRSA SCC*mec* type I strains and their isogenic MSSA counterparts were competed. After the 24 hours there was no significant difference in the cell counts of the strains investigated (Ender, McCallum et al. 2004). By extending the experiment, Ender, et al. were able to determine differences in viable cell counts of the competing strains on day six. In this light, pairwise competition assays were carried out to investigate competition between MS14 and the resistant strains over five days, during which time cell cultures were passaged every 24 hours. After five days MS14 outcompeted E3 and S5 as indicated by the respective 200-fold and 95-fold decreases in the final cell counts of the resistant strains. Although at 24 hours there was no significant difference in the fitness of both strains relative to MS14, there was steady decrease in the fitness of these strains by day five (Fig 4.10). In contrast, E8 was the outlier; relative to MS14, it remained fit for four days before it was outcompeted by MS14 based on the seven-fold decrease in E8. Ender, McCallum et al. (2004) reported similar findings in which susceptible *S. aureus* strains outcompeted their

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isogenic MRSA SCC*mec* type I counterparts, but at different rates. Although it cannot be assumed that the presence of SCC*mec* IV was responsible for the poor survival of the ST612-MRSA-IV strains, it is possible to suggest that the *mec* imposes a fitness cost on these strains.

As was observed in the growth rates of D4 in both the 24-hour and five-day competition assays (Fig 4.8 and Fig 4.9), there was no difference in the relative fitness of this isolate compared to MS14. Thus the presence of SCC*mec* IV in D4 did not appear to impose a fitness cost on this resistant strain and it may be that D4 has evolved, possibly by the acquisition of a fitness-compensatory mutation, to accommodate SCC*mec* IV in its genome. In bacterial populations, expansion of a particular clone in a specific environment is preceded by a population bottleneck which selects for the persistence of strains which are best fitted for a particular environment. Often, this is evidenced by a reduction in the genetic diversity of the population (Spratt and Maiden 1999). The study of Jansen van Rensburg, Madikane et al. (2011) showed that, based on PFGE and *spa* typing, cluster E was more genetically diverse than cluster D. On the evolutionary scale, cluster E may be more recent than D. As PFGE indicated that MS14 is more closely related to D4 than to the other resistant counterparts, it is perhaps not surprising that this isolate competed successfully with D4.

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5. CONCLUDING COMMENTS

The studies carried out by Jansen van Rensburg, Madikane et al. (2011) led to the suggestion that ST612-MRSA-IV may have arisen locally. To the best of the author's knowledge, there have not been any reports of ST612-MSSA. The best strategy to determine whether ST612-MRSA-IV may have arisen locally would have been to analyse a longitudinal collection of MSSA isolates that preceded the emergence of ST612-MRSA-IV; regrettably no such collection was available and therefore, MSSA isolates contemporaneous with the previously characterized MRSA were screened for the presence of ST612-MSSA. Using *spa* typing and MLST, one ST612-MSSA-t064 isolate, MS14, was identified among 19 MSSA isolates.

Interestingly, this isolate contained remnants of SCC*mec*, including *dcs*, within the *attB* site, suggesting that it may have resulted from a CcrABC independent and partial excision of SCC*mec* from a corresponding ST612-MRSA strain. This partial deletion of SCC*mec* has recently been described in clinical isolates of *S. aureus* (Donnio, Louvet et al. 2002; Donnio, Oliveira et al. 2005; Donnio, Fevrier et al. 2007; Noto, Fox et al. 2008; Shore, Rossney et al. 2008). The detection of uncommon amino acid substitutions, associated with rifampicin resistance, in RpoB, as well as a unique single nucleotide polymorphism in the RRDR of MS14, previously identified in ST612-MRSA-IV isolates (Jansen van Rensburg, Eliya Madikane et al. 2011) suggested that MS14 resulted from a partial loss of SCC*mec* from a corresponding ST612-MRSA.

Noto, Fox et al. (2008) associated partial loss of SCC*mec* with an increase in fitness in the resulting strains. This led the author to consider the possibility that MS14 could be fitter than ST612-MRSA-IV. Comparison of the exponential growth rate of MS14 with those of representative ST612-MRSA-IV isolates, D4, E3, E8 and S5, proved to be a poor measure of fitness when compared to the results of the competition assays. This accords with the data

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of Noto, Fox et al. (2008) who noted the importance of indexing the global fitness of strains throughout their growth cycle when ascertaining their fitness. Competition between the resistant strains and MS14 was investigated with 24-hour and five-day assays. The 24-hour assays also proved to be poor yardsticks to determine the comparative fitness of the strains, as they revealed minor differences between MS14 and the resistant strains and, only during the stationary phase. As was observed by Ender, et al. (2004), extending the competition assays to five days proved more informative. By day five the cell numbers of E3, E8 and S5, had decreased 200-fold, seven-fold and 95-fold, respectively, indicating poor survival of these resistant isolates when compared to the survival of MS14. Since cost to fitness has been described for strains containing SCC*mec* (Ender, McCallum et al. 2004; Lee, Ender et al. 2007; Collins, Rudkin et al. 2010; Noto, Fox et al. 2008), it may be that the carriage of SCC*mec* IV by E3, E8 and S5 exerts a similar cost. The possibility of other factors, besides carriage of SCC*mec* IV, contributing to differences in the relative fitness of the resistant strains cannot be excluded.

However, the survival of D4 in comparison to MS14 suggests that the two isolates are equally fit. Evidence from PFGE experiments carried out in the current study suggest a closer relatedness of MS14 to D4 than to its E counterparts and S5, suggesting that D4 may have evolved, possibly through acquisition of fitness-compensatory mutations, to accommodate the *mec*.

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Appendices

A1

A1.1 Preparation of 2YT broth

To make 1L broth:

- Measure out about 900ml RO water
- Add 16g Bacto Tryptone
- Add 10g Bacto Yeast Extract
- Add 5g NaCl
- Adjust to pH7 with NaOH
- Make up to 1000ml with RO water
- Autoclave

A1.2 Preparation of 2YT agar

To make 1L agar:

- Measure out about 900ml RO water
- Add 16g Bacto Tryptone
- Add 10g Bacto Yeast Extract
- Add 5g NaCl
- Add 15g Bacteriological agar
- Adjust to pH7 with NaOH
- Make up to 1000ml with RO water
- Autoclave

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A1.3 Preparation of 50% glycerol stocks

To make 1ml stock:

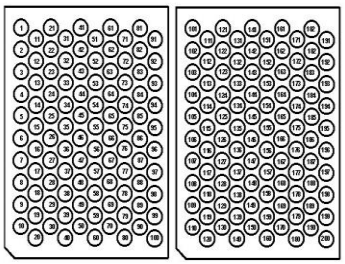
- Add 250 µl glycerol to 250 µl RO water, autoclave solution then store at room temperature.
- Streak the sample onto agar, to separate into single colonies, then incubate sample at 37°C overnight.
- Inoculate 1 colony into broth media and culture at 37°C overnight.

- Aliquot 500 µl of the overnight culture onto the 50% glycerol solution (warmed to 37°C), mix thoroughly and then store at -80 °C.

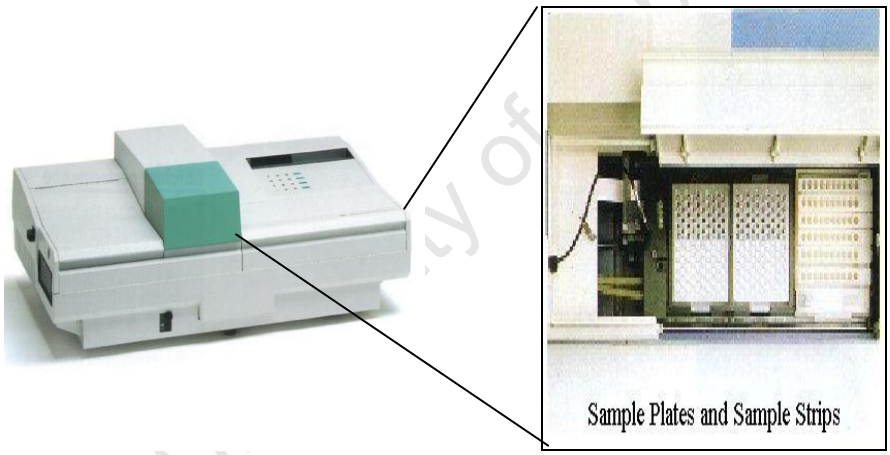
A2

A2.1 Microbial growth analysis using Bioscreen C Microbiological Reader

1. Load samples onto 100-well Honeycomb plate



2. Transfer plates into the incubation chamber on the Bioscreen C reader



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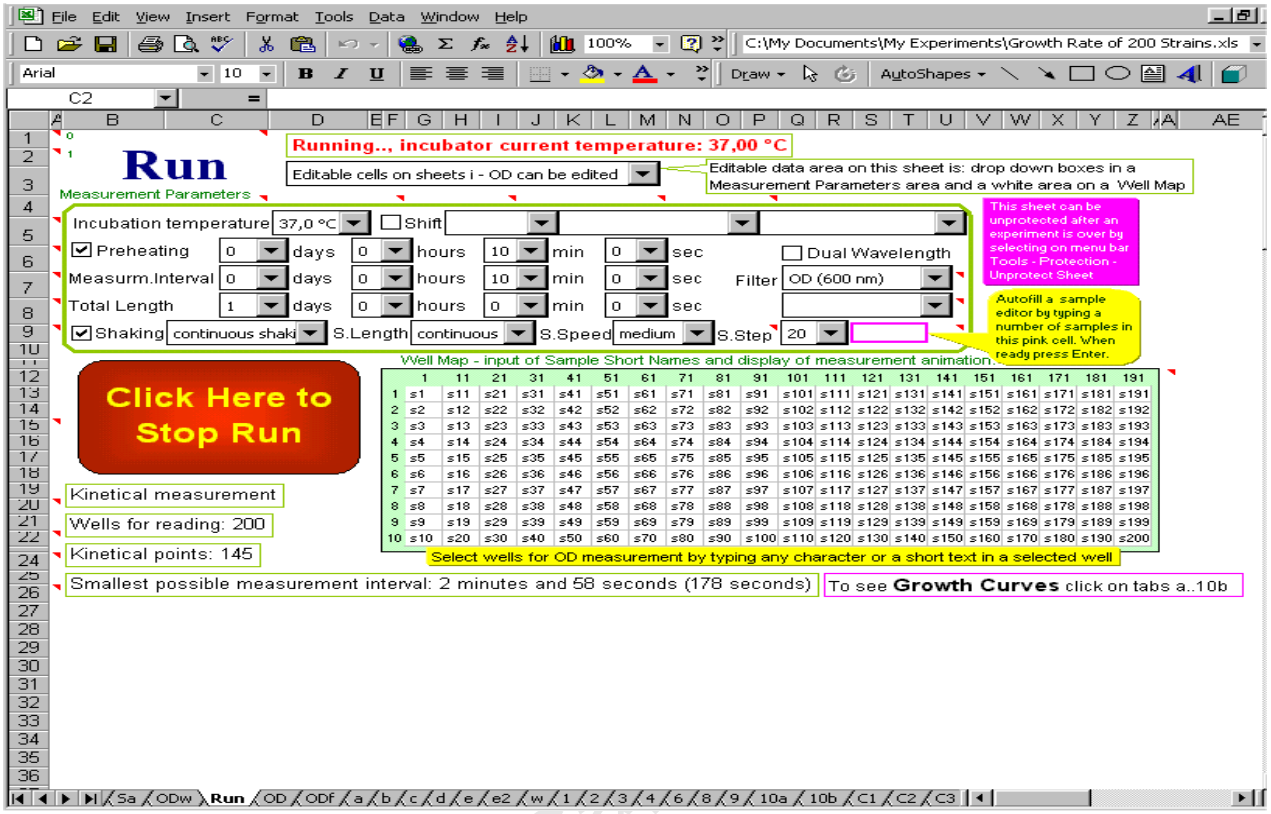
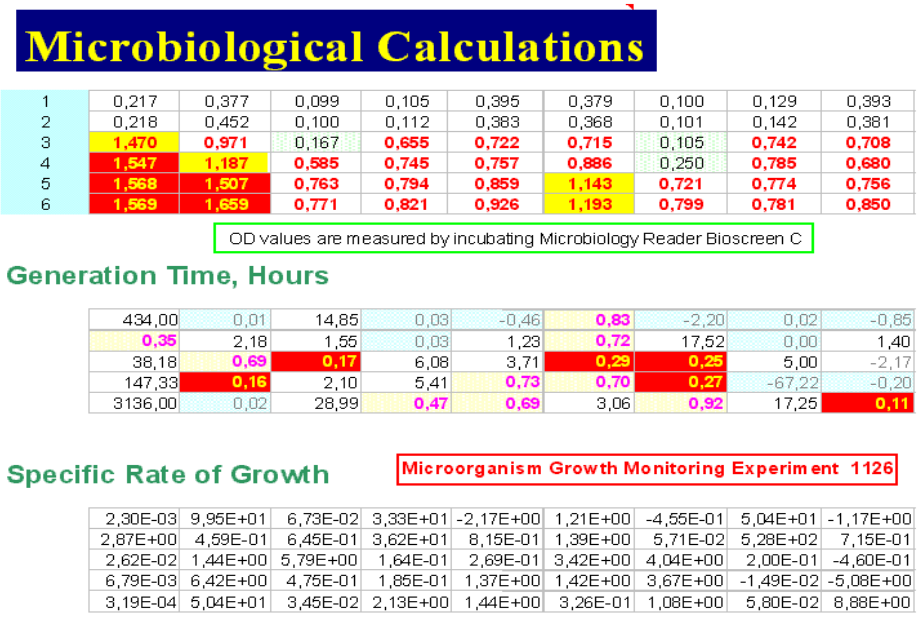
3. Activate the Bioscreen C reader using the Research Express software



4. View microbiological calculations in progress using the Research Express software



1	0,217	0,377	0,099	0,105	0,395	0,379	0,100	0,129	0,393
2	0,218	0,452	0,100	0,112	0,383	0,368	0,101	0,142	0,381
3	1,470	0,971	0,167	0,655	0,722	0,715	0,105	0,742	0,708
4	1,547	1,187	0,585	0,745	0,757	0,886	0,250	0,785	0,680
5	1,568	1,507	0,763	0,794	0,859	1,143	0,721	0,774	0,756
6	1,569	1,659	0,771	0,821	0,926	1,193	0,799	0,781	0,850

434,00	0,01	14,85	0,03	-0,46	0,83	-2,20	0,02	-0,85
0,35	2,18	1,55	0,03	1,23	0,72	17,52	0,00	1,40
38,18	0,69	0,17	6,08	3,71	0,29	0,25	5,00	-2,17
147,33	0,16	2,10	5,41	0,73	0,70	0,27	-67,22	-0,20
3136,00	0,02	28,99	0,47	0,69	3,06	0,92	17,25	0,11

2,30E-03	9,95E+01	6,73E-02	3,33E+01	-2,17E+00	1,21E+00	-4,55E-01	5,04E+01	-1,17E+00
2,87E+00	4,59E-01	6,45E-01	3,62E+01	8,15E-01	1,39E+00	5,71E-02	5,28E+02	7,15E-01
2,62E-02	1,44E+00	5,79E+00	1,64E-01	2,69E-01	3,42E+00	4,04E+00	2,00E-01	-4,60E-01
6,79E-03	6,42E+00	4,75E-01	1,85E-01	1,37E+00	1,42E+00	3,67E+00	-1,49E-02	-5,08E+00
3,19E-04	5,04E+01	3,45E-02	2,13E+00	1,44E+00	3,26E-01	1,08E+00	5,80E-02	8,88E+00

A3

A3.1 Calculation of $CFUml^{-1}$

Sample			Number of colonies/ dilution		
			10^4	10^5	10^6
MS14		Plate 1	TNTC	57	7
		Plate 2	TNTC	TNTC	14
	Average number of colonies		**TNTC**	**57**	**11**
D4		Plate 1	214	14	2
		Plate 2	TNTC	32	6
	Average number of colonies		**214**	**23**	**4**
E3		Plate 1	236	23	6
		Plate 2	TNTC	46	4
	Average number of colonies		**236**	**35**	**3**
E8		Plate 1	41	1	1
		Plate 2	66	18	0
	Average number of colonies		**54**	**10**	**1**
S5		Plate 1	194	22	2
		Plate 2	210	19	1
	Average number of colonies		**202**	**21**	**2**

Sample	Average # colonies	Initial dilution factor	Total dilution factor	CFU/ml
MS14	57	10^5	10^7	5.7×10^8
D4	23	10^5	10^7	2.3×10^8
E3	35	10^5	10^7	3.5×10^8
E8	54	10^4	10^6	5.4×10^7
S5	21	10^5	10^7	2.1×10^8

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A3.2 Results of the competition assays

Table A3.2.1 Results of the 24 h assays, presented as an average of two experiments

hours	D4 + MS14	E3 + MS14	E8 + MS14	S5 + MS14	D4	E3	E8	S5	MS14(D4)	MS14(E3)	MS14(E8)	MS14(S5)
0	2.80E+04	3.20E+04	1.90E+04	3.20E+04	6.00E+03	1.50E+04	9.00E+03	6.00E+03	2.20E+04	1.70E+04	1.00E+04	2.60E+04
4	1.20E+07	1.00E+07	1.00E+07	9.00E+06	5.90E+06	1.80E+06	4.50E+06	1.80E+06	6.10E+06	8.20E+06	5.50E+06	7.20E+06
6	3.60E+08	2.10E+08	1.90E+08	3.20E+08	1.27E+08	4.30E+07	4.30E+07	6.20E+07	2.33E+08	1.67E+08	1.47E+08	2.58E+08
10	8.40E+09	8.00E+09	7.70E+09	6.80E+09	1.47E+09	1.33E+09	1.58E+09	1.40E+09	6.93E+09	6.67E+09	6.12E+09	5.40E+09
24	4.40E+09	4.10E+09	4.20E+09	7.40E+09	7.20E+08	1.20E+09	1.20E+08	3.40E+09	3.68E+09	2.90E+09	4.08E+09	4.00E+09

Table A3.2.2 Results of the 5 day assays, presented as an average of two experiments

day	D4 + MS14	E3 + MS14	E8 + MS14	S5 + MS14	D4	E3	E8	S5	MS14(D4)	MS14(E3)	MS14(E8)	MS14(S5)
0	2.37E+04	2.78E+04	2.15E+04	2.44E+04	8.25E+03	1.44E+04	1.13E+04	1.35E+04	1.55E+04	1.34E+04	1.02E+04	1.09E+04
1	9.45E+09	9.80E+09	1.12E+10	9.10E+09	4.95E+09	3.65E+09	6.75E+09	5.40E+09	4.50E+09	6.15E+09	4.45E+09	3.70E+09
2	1.15E+10	7.55E+09	1.13E+10	1.15E+10	5.40E+09	1.30E+09	4.05E+09	4.65E+09	6.05E+09	6.25E+09	7.25E+09	6.85E+09
3	1.16E+10	1.10E+10	7.00E+09	7.35E+09	3.70E+09	2.50E+08	4.70E+09	7.00E+08	7.85E+09	1.07E+10	2.30E+09	6.65E+09
4	9.35E+09	1.10E+10	7.58E+09	1.06E+10	5.70E+09	2.95E+08	2.43E+09	1.17E+09	3.65E+09	1.07E+10	5.15E+09	9.38E+09
5	8.80E+09	9.75E+09	6.90E+09	9.65E+09	4.35E+09	5.00E+07	9.50E+08	1.25E+08	4.45E+09	9.70E+09	5.95E+09	9.53E+09

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Table A3.2.3 Average generation numbers of the strains for the 5 day assays

	Average Generation number		
day	D4 + MS14	D4	MS14
0	0	0	0
1	19.15	19.98	18.58
2	19.42	20.11	19.02
3	19.44	19.57	19.40
4	19.14	20.17	18.25
5	18.86	19.16	18.56

	Average Generation number		
day	E3 + MS14	E3	MS14
0	0	0	0
1	19.15	19.98	18.58
2	19.42	20.11	19.02
3	19.44	19.57	19.40
4	19.14	20.17	18.25
5	18.86	19.16	18.56

	Average Generation number		
day	E8 + MS14	E8	MS14
0	0	0	0
1	19.51	19.49	19.69
2	19.53	18.67	20.29
3	18.82	18.97	18.70
4	18.92	18.02	19.76
5	18.83	16.23	20.13

	Average Generation number		
day	S5 + MS14	S5	MS14
0	0	0	0
1	18.91	18.89	18.88
2	19.30	18.64	20.10
3	18.57	15.43	20.01
4	19.17	16.32	20.57
5	18.96	13.13	20.51

A4

A4.1 Preparation of TEN buffer

- 20 ml 1M Tris
- 40 ml 0.5M EDTA
- 6 ml 5M NaCl
- Make up to 200 ml with H_2O
- Adjust to pH7.5 and then autoclave

A4.2 Preparation of Seakem Gold AGarose gel

To make 1.8%:

- 1.8 g Seakem Gold Agarose
- 100 ml TE buffer
- Homogenise and then equilibrate at 60°C

A4.3 Preparation of EC buffer

- 0.5% Sarkosyl
- 0.5% Brij
- 0.1M EDTA
- 1M NaCl
- 0.006M Tris
- Use H_2O as diluent, adjust to pH7.5 and then autoclave

A4.4 Preparation of ESP buffer

- 10mM Tris-HCl
- 1mM EDTA
- 1% SDS
- 1 mg/ml Proteinase K
- Use H_2O as diluent, adjust to pH8.0 and store at -20°C

A4.5 Preparation of TE buffer

- 10 ml 1M Tris
- 2 ml 0.5M EDTA
- Make up to 1000ml with H_2O

A4.6 Preparation of 1% Biorad Ultrapure Molecular Grade Agarose gel

- 1.5 g Biorad Biorad Ultrapure Molecular Grade Agarose
- 150 ml 0.5X TBE buffer

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A4.7 Preparation of TBE

To make 10X TBE:

- 108 g Tris Base
- 55 g Boric Acid
- 40 ml 0.5M EDTA (pH8.0)

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