School of Biomedical Sciences

The Investigation of the Staphylococcal Cassette Chromosome Elements and Ciprofloxacin Resistance in Community Methicillin-Resistant *Staphylococcus aureus* Strains Isolated in Western Australia

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Lynne Wilson
June 2012

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List of Abbreviations

A	adenine
AAC	acetyltransferase
AAD	adenytransferase
aap	accumulation-associated protein
ACCESS	Australian Collaborating Centre for Enterococcus and Staphylococcus Species
ACME	arginine catabolic mobile element
AGAR	Australian Group for Antimicrobial Resistance
ANT	nucleotidyltransferase
APH	phosphotransferase
Approx	approximately
Asa	sodium arsenate
Asn	asparagine
*att*B	attachment site of SCC element
*att*R	right hand SCC*mec* junction
aux	auxiliary factors
bp	base pair
BA	blood agar
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
C	cytosine
C	chromosome
ºC	degrees Celsius
CA-MRSA	community-associated methicillin-resistant *Staphylococcus aureus*
CC	clonal complex
ccr	cassette chromosome recombinase
Cd	cadmium acetate
Cip	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
COG	cluster of orthologous groups of proteins
CoNS	coagulase-negative staphylococci
D	direct
dcs	downstream constant segment
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DNAmp	did not amplify
DNC	did not cut

List of Abbreviations (Continued)

dNTP	deoxyribonucleotide
DR	direct repeat
dru	direct repeat unit
DUF	domain of Unknown Function
Eb	ethidium bromide
EDTA	ethylenediamino-tetra-acetic acid
EF-G	encoding elongation factor-G
Ery	erythromycin
EMRSA	epidemic methicillin resistant *staphylococcus aureus*
Fem	factors essential for the expression of methicillin resistance
Fully S	fully sensitive
Fus	fusidic Acid
g	grams
G	guanine
G+C	guanine and cytocine
Gent	gentamicin
GI	genomic island
GISA	glycopeptide-intermediate *S. aureus*
Glu	glutamine
Gly	glycine
GPBTRU	Gram Positive Bacteria Typing and Research Unit
HA-MRSA	hospital-associated methicillin-resistant *Staphylococcus aureus*
Hg	mercury
hGISA	heterogeneous glycopeptide-intermediate *S. aureus*
HP water	high pure water
HP	hypothetical protein
hVISA	heterogeneous vancomycin-intermediate *S. aureus*
HVR	hypervariable Region
I	inverted
IR	inverted repeat
IS	insertion sequence
ISS	integration site sequence
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
J region	joining or junkyard region
kb	kilo bases
kDa	kilo Dalton
kdp	potassium-dependent ATPase and its regulators
l, L	litre
L	left

List of Abbreviations (Continued)

LH	left hand
Leu	leucine
Mc	mercuric chloride
MFS	major facilitator superfamily
$MgCl_2$	magnesium chloride
MHA	Mueller-Hinton agar
MHB	Mueller Hinton broth
MLS_B	macrolide-lincosamide-streptogramin B group of antibiotics
MLST	multi locus sequence typing
MIC	minimum inhibitory concentration
mRNA	messenger ribonucleic acid
µ	micro
m	milli
M	molar
nM	nanomolar
MΩ	megohm
Min	minutes
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *Staphylococcus aureus*
MS	membrane-spanning domain
Mup	mupirocin
n	number
NA	not available
NAB	nucleic-acid binding compounds
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NCCLS	National Committee for Clinical Laboratory Standards
Neg	negative
ng	nanograms
nt	nucleotide
NT	not typable
NoTest	not tested
ORF	open reading frame
P	plasmid
PB	penicillin-binding domain
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
Phe	phenylalanine
Pi	propamidine isethionate

List of Abbreviations (Continued)

Pls	plasmin-sensitive protein
Pma	phenylmercuric acetate
Pos	positive
PSM	phenol-soluble modulin
PVL	Panton-Valentine leukocidins
QACs	quaternary ammonium compounds
Qld clone	Queensland clone
R	right
®	registered trade mark
RFLP	restriction fragment length polymorphism
RH	right hand
Rif	rifampicin
RNase	ribonuclease A
rRNA	ribosomal ribonucleic acid
sasG	*Staphylococcus aureus* surface protein G
SCC	staphylococcal cassette chromosome
SCC-CI	staphylococcal cassette chromosome-composite island
SCC*mec*	staphylococcal cassette chromosome *mec*
SCV	small-colony variant
SD	serine-aspartic acid repeat
Sec	seconds
Ser	serine
spa	staphylococcal protein A
Spc	spectinomycin
SSR	short-sequence repeat
T	thymidine
Tn	transposon
Tet	tetracycline
Thr	threonine
™	trade mark
Tobra	tobramycin
Tra	transposable-resistance determinants
Trim	trimethoprim
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TSB	trypticase soy broth
Tyr	tyrosine
U	Units
ugpQ	glycerophosphoryl diester phosphodiesterase
UK	United Kingdom

List of Abbreviations (Continued)

USA	United States of America
Val	valine
VISA	vancomycin-intermediate *S. aureus*
VRE	vancomycin-resistant entercocci
WA	Western Australia
Wc	non-repeats
Wr	repeats
WSPP	Western Samoan phage pattern
w/v	weight by volume
Ψ	pseudo
Δ	truncated
Ω	ohm
≥	greater than or equal to
>	greater than
≤	less than or equal to
<	less than

Abstract

In Western Australia, community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) continues to be a public health concern. Antibiotic resistance places additional burdens on the community and health-care systems. Updates in MRSA typing and molecular characterisation techniques have benefits in furthering understanding of the evolution and emergence of MRSA and ultimately result in improvements in the control and prevention of its spread. Molecular studies not only give an understanding of how MRSA evolve but enable typing to be improved so that isolates can be better identified and improvements made to their control and prevention of spread.

Forty-five prototypes of CA-MRSA lineages isolated in Western Australia (WA) between 2003 and 2006 were used in this study and were provided by the Australian Collaborating Centre for Enterococcus and Staphylococcus Species (ACCESS) Typing and Research Unit, situated at PathWest Laboratory Medicine, Royal Perth Hospital, Perth, WA, which is also associated with Curtin University, School of Biomedical Sciences, Molecular Genetics Research Unit in a joint collaboration.

The polymorphic region of the staphylococcal protein A (*spa*) gene is considered a useful typing tool for differentiation of MRSA. A *spa* typing protocol was trialled in this study. To assess the method's suitability for typing MRSA, all 45 isolates were tested to determine their *spa* type. Significant diversity was revealed with 28 different *spa* types identified. The *spa* type t002 was the most prevalent (7/45). Except for one isolate, which could not be typed, *spa* types correlated with the multi locus sequence typing (MLST) and clonal complex designations of the 45 CA-MRSA. A major outcome of this study has been the introduction of a standardised protocol for *spa* typing as a routine typing technique by the ACCESS Typing and Research Unit.

Ciprofloxacin and other fluoroquinolones have restricted use in the community with their availability more accessible in hospitals and at the initiation of this study it was unusual to find ciprofloxacin resistance in CA-MRSA, however, eight of the prototype CA-MRSA possessed high-level ciprofloxacin resistance. This study therefore included an investigation of genetic mutations that result in resistance to ciprofloxacin in *S. aureus*. The subset of eight isolates were tested for mutations in the genes *grlA, grlB, gyrA* and *gyrB*. Sequence analysis revealed that all eight isolates contained point mutations resulting in amino acid changes at codon 80 in *grlA* and at codon 84 in *gyrA*. Additional mutations were seen in three of the eight isolates analysed. One isolate contained previously unreported mutations that may be associated with ciprofloxacin resistance. It was concluded that the CA-MRSA isolates could have developed resistance to ciprofloxacin when the patients may have received fluoroquinolones as a course of treatment for infection caused by other organisms such as Gram-negative bacteria.

The staphylococcal cassette chromosome *mec* (SCC*mec*) is a genomic resistance island found in MRSA and a primary typing target for their classification. The SCC*mec*s of a subset of eight of the 45 prototype CA-MRSA isolates that had novel classifications were analysed as part of this study. The novel classifications for four of the eight were resolved into already classified SCC*mec* types, three of them had novel sub-types, two remained novel and the SCC region of one, WA MRSA-40 was completely sequenced. The J regions of the SCC*mec* elements were found to be structurally heterogeneous leading to the conclusion that they are "hot spots" for recombination, rearrangement and acquisition of genetic information that enables adaptation for survival of the host cell in changing environments.

The SCC region of WA MRSA-40 was found to be a composite genomic island named SCC*mec*$_{\text{WA MRSA-40}}$-CI (GenBank Accession No JQ746621). The 72,522 bp SCC*mec*$_{\text{WA MRSA-40}}$-CI, was found to be comprised of three elements, ΨSCC*pls*, SCCsorbitol and SCC*mec*V_T (5C2&5) integrated in tandem into the *att*B site of the conserved hypothetical protein gene *orfX*. Each element was delineated by direct and inverted repeats. ΨSCC*pls* is 11,736 bp and was homologous to the ΔJ1

region of SCC*mec* type I which encodes a *pls* gene. SCCsorbitol is 19,497 bp, and encodes a sorbitol operon, type 1 restriction modification genes, and a *ccrA2B2* gene complex together with seven open reading frames. This is the first time that a sorbitol operon has been reported in a SCC element and only the second time that a sorbitol operon has been reported in a staphylococcus, with it previously being found in *S. carnosus.* Acquisition of a sorbitol operon has been linked to dietary sorbitol usage. The SCC*mec* of WA MRSA-40 is 41,289 bp and is highly homologous with the SCC*mec* V_T of the Taiwan strain PM1; it carries a class C2 *mec* complex and two *ccrC1* gene complexes with *ccrC1,* alleles 8 and 2. An insertion sequence IS*Sau4*-like was found in the J2 region. Polymorphisms that generated premature stop codons that would prevent transposase activity and stabilise the *mec* complex were detected in the sequences of the transposases of IS*431*L and IS*431*R that flank the *mec* region.

In conclusion this study has provided important and relevant information on the utilisation of *spa* typing for routine typing, the genetics and emergence of ciprofloxacin resistance in CA-MRSA and the analysis of the SCC*mec* region and SCC elements of CA-MRSA, particularly WA MRSA-40.

Chapter One

Literature Review

1.1 *Staphylococcus aureus*

Staphylococci were first described by Pasteur and Koch. The genus *Staphylococcus aureus* was named by Ogstan in 1881, after describing grape-like clusters of bacteria in pus from human abscesses. *S. aureus* was observed by Rosenbach and named because of the yellow to orange pigmented appearance of their colonies in culture (Fluit and Schmitz 2003).

In the pre-antibiotic era, *S. aureus* was a serious life threatening pathogen often responsible for wound infections, post-surgical infections, fatal pneumonia and meningitis. Staphylococcal infections can be superficial skin infections or deep tissue and systemic infections. Superficial skin infections are commonly seen as furuncles or boils usually on the neck and buttocks, while deep tissue infections are seen in conditions such as endocarditis, osteomyelitis, arthritis and breast abscesses. *S. aureus* can also cause pneumonia as a secondary infection frequently following a viral infection (Fey et al. 2002). Some *S. aureus* produce enterotoxins which cause food poisoning if contaminated food is eaten (Livermore 2000).

1.2 History of MRSA

1.2.1 History of Penicillin Resistance

The antibiotic effect of the fungus *Penicillium notatum* against *S. aureus* was observed by Alexander Fleming in 1928. Fleming named a culture filtrate of the *Penicillium* mould, "Penicillin". In 1939, Howard Florey and colleagues demonstrated the *in vivo* bactericidal action of penicillin (Hoephrich 1968).

By 1940, the effectiveness of treating staphylococcal and other Gram-positive infections with penicillin was evident. Mass production of penicillin was soon required and went from harvesting penicillin from mouldy cantaloupes to large scale production by deep tank fermentation using the strain *P. chrysogenum* (Stefaniak et al. 1947; Hoephrich 1968). The use of penicillin during World War II greatly aided in the treatment of infected wounds and reduced the mortality rate (Hoephrich 1968).

By 1942, however, the occurrence of *S. aureus* strains resistant to penicillin were being reported, first in hospitals and then in the community. This resistance resulted from the acquisition of a plasmid encoding the penicillin-hydrolysing enzyme penicillinase or β-lactamase. By 1948 about 60% of *S. aureus* in the United Kingdom (UK) were resistant to penicillin and by the late 1960s, more than 80% of *S. aureus* in the United States of America (USA) were resistant (Lowy 2003). By 1957 a strain known as the hospital staphylococcus was responsible for a large number of infections in the USA and the UK. This strain was termed 80/81 according to the phage typing method. The incidence of the 80/81 strain declined in the 1960s following the introduction of methicillin (Rountree and Beard 1958; Robinson et al. 2005).

1.2.2 History of Methicillin Resistance

Methicillin is a narrow spectrum β-lactamase stable, semi-synthetic β-lactam antibiotic that was developed in 1959 to treat infections caused by bacteria that had acquired resistance to penicillin. By 1961, however, the first cases of *S. aureus* resistance to methicillin were recorded in a British hospital (Stewart and Holt 1963). These resistant strains were called methicillin-resistant *Staphylococcus aureus* (MRSA) and they became known as the classic MRSA (Townsend et al. 1985; Casewell 1986).

Once the first case of MRSA was reported in 1961 in a UK hospital, the strain rapidly spread. During the 1960s, MRSA were identified in other European hospitals. Soon after, MRSA were also identified from countries around the world. By the mid 1970s, the incidence of MRSA in Europe declined, possibly due to changes in prescribing antibiotics and the introduction of infection control policies (Rosdahl and Knudsen 1991; Westh et al. 1992).

In the 1980s, a new strain of MRSA that was gentamicin resistant was reported in several countries including Australia (King et al. 1982; Pavillard et al. 1982), the UK (Duckworth, Lothian, and Williams 1988) and the USA (Boyce, White, and Spruill 1983). This strain was unlike the classic MRSA previously seen, as it was found to be multi-resistant (resistant to three or more antibiotics) (Pearman et al. 1985), and to carry plasmids that variably encoded resistance to aminoglycosides, chloramphenicol, macrolides, tetracycline, trimethoprim, quaternary ammonium compounds (QACs) and nucleic-acid binding (NAB) compounds (Firth and Skurray 1998; Grubb, Coombs, and O'Brien 2004). This strain, now known as EMRSA-1 (epidemic methicillin resistant *staphylococcus aureus*) , is thought to have originated in Australia and then imported into the UK by an Australian health-care worker (Grubb, Coombs, and O'Brien 2004). EMRSA-1 spread readily within and between hospitals and tertiary care institutions by person to person contact between patients and staff causing extensive outbreaks. Serious infections with high mortality rates were reported. This strain was considered to have a high epidemic potential due to its ability to spread among patients in hospitals (Ayliffe

1997; Livermore 2000). EMRSA-1 was followed by other epidemic stains with multi-resistance such as EMRSA-3 (Livermore 2000). In England 17 epidemic strains (EMRSA-1 to EMRSA-17) have been identified to date (Grubb, Coombs, and O'Brien 2004).

The mid-1990s saw the emergence of EMRSA-15 and EMRSA-16 in the UK, with EMRSA-15 resistant to ciprofloxacin with variable resistance to erythromycin and EMRSA-16 usually resistant to ciprofloxacin, erythromycin, kanamycin, neomycin and occasionally gentamicin. It has been suggested that these strains are successful due to their ability to survive on hands and inanimate objects (Livermore 2000).

Over the years several strains of MRSA have emerged, become dominant and displaced other strains in health-care environments. The more successful strains have spread pandemically. The dominant hospital-associated MRSA (HA-MRSA) are presented in Table 1.1. HA-MRSA of the clonal complex eight lineage predominate and include the Archaic, Brazilian/Hungarian, Iberian, Irish-1, UK EMRSA-2 and UK EMRSA-6 clones. HA-MRSA of clonal complex five includes the New York/Japan, Paediatric, Southern German and UK EMRSA-3 clones. Other HA-MRSA include clonal complex 45, 22 and 36, being the Berlin, UK EMRSA-15 and UK EMRSA-16 clones respectively (Deurenberg and Stobberingh 2008).

Table 1.1 Major HA-MRSA Worldwide

HA-MRSA Strain	ST/SCC*mec*	Clonal Complex	Geographic Spread
Archaic	ST250-MRSA-I	8	Aust, UK, Europe, Canada, USA, China
Berlin	ST45-MRSA-IV	45	Aust, UK, Europe, USA, China
Brazilian/Hungarian	ST239-MRSA-III	8	Aust, UK, Europe, USA, Canada, China, Korea
Iberian	ST247-MRSA-I	8	Aust, UK, Europe, USA
Irish-1	ST8-MRSA-II	8	Ireland, UK, Austria, Canada, USA
New York/Japan	ST5-MRSA-II	5	Japan, USA, UK, Europe, China, Korea
Paediatric	ST5-MRSA-VI	5	Aust, Europe, UK, USA
Southern German	ST228-MRSA-I	5	Aust, Europe
UK EMRSA-2/-6	ST8-MRSA-IV	8	Aust, Europe, UK, USA, Canada, China, Japan
UK EMRSA-3	ST5-MRSA-I	5	Europe, UK, Japan
UK EMRSA-15	ST22-MRSA-IV	22	Aust, Europe, UK, New Zealand
UK EMRSA-16	ST36-MRSA-II	36	Aust, Europe, UK, Canada

1.2.3 Community MRSA – Global

Up until the late 1980s MRSA were largely hospital-associated, however from the 1990s, community-associated MRSA (CA-MRSA) have emerged worldwide (Deurenberg and Stobberingh 2008). CA-MRSA infections are defined as those occurring either in the community or within 48 hours of admission to a hospital.

CA-MRSA infections can also be classified as CA-MRSA with healthcare associated risk factors and those without. Healthcare associated risk factors include recent contact with hospitals, health-care facilities or their staff. CA-MRSA in this category may be multi-resistant. CA-MRSA infected patients with no health-care associated risk factors have little or no contact with hospitals, health-care facilities or their staff. CA-MRSA in this category are generally non-multi-resistant (Herold et al. 1998; O'Brien et al. 2004).

CA-MRSA and HA-MRSA differ from each other both phenotypically and genotypically with CA-MRSA tending to be non-multi-resistant and susceptible to most non-β-lactam antibiotics. Pulsed Field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST) analysis of MRSA shows that there is far more genetic diversity among CA-MRSA strains than HA-MRSA. It is not characteristic of CA-MRSA to spread in a hospital or health-care environment, possibly because they lack the ability to survive on hands and inanimate objects as epidemic strains do (Livermore 2000; Fey et al. 2002; Naimi et al. 2003). The CA-MRSA strain ST8-MRSA-IV, commonly known as USA300, is a recent exception however, with the clone causing a cluster of infections in patients, health-care workers and their families in a neonatal intensive care unit in 2005. Extensive infection control practices were required to prevent further infections (McAdams et al. 2008).

In 2006 it was revealed that USA300, a Panton-Valentine leukocidin (PVL) positive CA-MRSA strain in USA, had disseminated into tertiary institutions. The study, based on isolates from a tertiary care Veterans Affairs Medical Center, showed 100% of nosocomial infections were associated with USA300 (Gonzalez et al. 2006).

The first reported CA-MRSA were in Detroit USA during the early 1980s, following an outbreak in a large urban hospital. The majority of those infected were drug abusers though occurrence in patients with serious underlying illnesses, previous antimicrobial therapy and previous hospitalisation were also noted. Infection control measures were effective in preventing ongoing transmission (Saravolatz et al. 1982; Saravolatz, Pohlod, and Arking 1982). Another early

report of CA-MRSA in the 1980s was in Canada involving seven intravenous drug abusers over a 15 month period. Infections included bacterial endocarditis, septic thrombophlebitis and soft tissue infections. All isolates were of the same phage type that was distinct from previous HA-MRSA. The likely source of the transmission was determined to be the drug "shooting gallery" patronised by the drug addicts (Craven et al. 1986). Both the outbreaks in Detroit and Canada were in the category of CA-MRSA infections with the added risk factor of drug abuse (O'Brien et al. 2004).

The first genetically defined CA-MRSA were from remote regions in Western Australia (WA) in 1993 (Udo, Pearman, and Grubb 1993). It was found among hospitalised people from remote Aboriginal communities with no previous health-care exposure or other risk factors for MRSA infections.

In 1997-1999, four cases were recorded in the US states of Minnesota and North Dakota where four children died of severe infections caused by a PVL positive CA-MRSA, designated as MW2. With the development of MLST and SCC*mec* typing MW2 was found to belong to the ST1-MRSA-IVa (2B) lineage (CDC 1999; Fey et al. 2002). The PVL determinant was found to be associated with CA-MRSA in some countries (Vandenesch et al. 2003).

A study in the Saxony region of Germany found there was a 30% occurrence of the PVL gene in MSSA (methicillin sensitive *staphylococcus aureus*) from skin and soft tissue infections. This study further suggested the possibility that the PVL genes may spread to other *S. aureus* by phages or that a PVL positive MSSA acquired a SCC*mec* element to become a PVL positive CA-MRSA (Monecke et al. 2007).

Spain has also seen an increase in the occurrence of a ST8 strain related to USA300, primarily among immigrants from South America (Manzur et al. 2008).

There have been CA-MRSA clones that have emerged in defined geographical regions. This was seen with the Western Samoan Phage Pattern (WSPP) ST30-MRSA-IV (2B) from the South Pacific region (Collignon et al. 1998), the

Queensland clone ST93-MRSA-IV (2B) from Ipswich, Queensland, Australia (Munckhof et al. 2003), the European clone ST80-MRSA-IV (2B) (Vandenesch et al. 2003) from Europe, USA300 ST8-MRSA-IV (2B) from USA (Tenover et al. 2006), the Taiwan clone ST59-MRSA-V_T (5C2&5) from Taiwan (Boyle-Vavra et al. 2005) and the Bengal Bay clone ST772-MRSA-V (5C2) from Bangladesh (Afroz et al. 2008). These clones have appeared initially in distinct regions and then disseminated to neighbouring areas and countries, and now each can be found worldwide. Common features among these CA-MRSA are their ability to cause severe infections and the presence of PVL genes in the strains (Coombs et al. 2011).

Apart from USA300, CA-MRSA do not generally cause hospital outbreaks, however some CA-MRSA have also been associated with hospital outbreaks. In 1995 an outbreak occurred in a major teaching hospital in WA, following the admission of an infected patient from a remote community (O'Brien et al. 1999). Another occurrence of a CA-MRSA outbreak occurred in a New York hospital where eight post-partum women were infected with the PVL positive strain MW2. Extensive screening was conducted of staff, patients, family members and hospital equipment to find the source to transmission but without success (Saiman et al. 2003)

1.2.3.1 MRSA in Animals

There have been increasing reports of MRSA in companion or domestic animals, where MRSA can colonize skin, nasal and oral mucosa in animals. A case was reported in 1994 where two nurses from the same household were found to be nasal carriers of the strain EMRSA-1. De-colonisation treatment with mupirocin and triclosan washes was successful. Follow up screenings six months later were again positive for EMRSA-1. It was discovered that their dog had an eye infection, with eye and nose swabs from the dog also growing EMRSA-1. All were successfully treated with nasal mupirocin and triclosan washes and the EMRSA was eliminated. It is suggested that animals can act as a reservoir for MRSA carriage (Cefai, Ashurst, and Owens 1994). A study in Hong Kong

reported the occurrence of ten MRSA strains from healthy dogs and their owners. Between the dogs and humans were found five HA-MRSA SCC*mec* type III and five CA-MRSA SCC*mec* type IV, one of which was PVL positive. This study also suggested dogs as a possible reservoir for MRSA in the community (Boost, O'Donoghue, and Sui 2007).

Horses and staff at a veterinary teaching hospital in Michigan USA were investigated following the occurrence of post-procedural infections in 11 horses over a 13 month period. Five staff volunteered nasal swabs. Three staff and all 11 horses were found to have identical MRSA. It was suggested that members of the veterinary teaching hospital staff were the source of the infections in the horses (Seguin et al. 1999).

In Europe, the emergence of a MRSA strain among pigs and pig farmers in the Netherlands has been observed. Following intensive surveillance, the occurrence of this clone has been reported to have increased from 0% in 2002 to >21% in 2006 with a finding of 25% in Dutch hospitals. Molecular typing showed it belonged to a new clonal complex, CC398. The study concluded that MRSA from an animal reservoir had recently entered the human population (van Loo et al. 2007). ST398 has also been reported in other European countries, Canada and China (Wulf et al. 2007; Yu et al. 2008) and has now been isolated in Australia (GPBTRU 2009).

1.2.4 Community MRSA – Australia

The first community MRSAs in Australia were reported in the early 1990s. These MRSAs were isolated from people in remote Indigenous communities in the far north of WA who had no previous hospitalisation or other risk factors. These MRSA isolates were unusual in that they were resistant to very few antibiotics other than the β-lactams. The MRSA were analysed by plasmid characterisation and PFGE; two MRSA types were identified and determined to be genetically

different to the previously studied Australian EMRSA (Udo, Pearman, and Grubb 1993). In 2004, five types of WA CA-MRSA, ST1-MRSA-IVa (2B), ST78-MRSA-IVa (2B), ST5-MRSA-IVa (2B), ST45-MRSA-V (5C2), and ST8-MRSA-IVa (2B) were identified in WA communities (O'Brien et al. 2004).

In the eastern states of Australia, in the 1990s, community strains were found that were indistinguishable by phage typing and PFGE from CA-MRSA reported in New Zealand (Nimmo et al. 2000; Gosbell et al. 2001). This strain, which was subsequently found to be ST30-MRSA-IV (2B), became known as the Western Samoan Phage Pattern (WSPP). WSPP had a high incidence among people of Polynesian decent (Collignon et al. 1998). The WSPP clone was thought to have originated in Western Samoa after it was detected in New Zealand in individuals who came from, or had visited Western Samoa. WSPP has a MLST of ST30 and appears related to a non-typable MSSA strain isolated in Denmark in 1962 and 17 MSSA isolates from a UK study, and also closely related to EMRSA-16 (Enright et al. 2000; Crisóstomo et al. 2001; Smith and Cook 2005). WSPP encodes the PVL determinant and is responsible for cutaneous and systemic infections. WSPP has also been isolated in California, the USA, from members of a Samoan family found to be infected, and or, carriers of WSPP (Gross-Schulman, Dassey, and Mascola 1998).

A second clone emerged at this time in Queensland (Qld) Australia, known as the Qld or Oceanic Clone. It was subsequently found to be of the ST93-MRSA-IVa (2B) genetic lineage (Nimmo et al. 2000). In a retrospective study in 1993 of isolates from 44 patients from a Qld hospital, the Qld clone was revealed to be responsible for superficial abscesses, or boils, as well as four serious life-threatening infections. The Qld clone was assigned pulsotype “R” by PFGE and, like WSPP, was PVL positive (Munckhof et al. 2003). The first fatal case of necrotising pneumonia due to a CA-MRSA in Australia resulted from an infection by the Qld clone with the pulsotype “R” (Peleg and Munckhof 2004).

A study by the Australian Group for Antimicrobial Resistance (AGAR) surveyed *S. aureus* isolates collected Australia-wide between 2004-2005 and compared results with a similar survey from 2000-2002 (Nimmo et al. 2006). The major CA-

MRSA clones within Australia were determined to be WA-MRSA-1, WSPP and the Qld clone. In 2004 14.9% of 2,652 *S. aureus* isolates surveyed were MRSA, with the number of CA-MRSA increasing from 4.7% in 2000 to 7.3% in 2004. WA MRSA-1 decreased from 18% in 2000 to 16% in 2004. The Qld clone increased from 5% in 2000 to 15% in 2004. WSPP decreased from 13% in 2000 to 7% in 2004. PVL analysis in 2004 showed that 46% of CA-MRSA were PVL positive with 8% WA MRSA-1, 97% Qld clone and 96% WSPP. These results are presented in Table 1.2. The major HA-MRSA isolated were Aus-2 and Aus-3 (both ST239-MRSA-III) and ST22-MRSA-IV or UK EMRSA-15. In 2000, 35% of MRSA identified were Aus-2, 7% Aus-3 and 12% UK-15. In 2004 Aus-2 had decreased to 23%, Aus-3 to 6% while UK-15 had increased to 16%. Conclusions were that CA-MRSA were spreading throughout Australia and the PVL determinant was present in two of the most common CA-MRSA (Qld clone and WSPP) (Nimmo et al. 2006).

Table 1.2 Major CA-MRSA in Australia 2000-2004

Strain	**2000**	**2004**
Total CA-MRSA	4.7%	7.3%
WA MRSA-1	18%	16%
Qld Clone	5%	15%
WSPP	13%	7%
PVL positive CA-MRSA	NA	46%

Abbreviations: NA, Not Available

By 2006, WA MRSA-1 (ST1-MRSA-IV) was the most prevalent CA-MRSA isolated throughout Australia, and was seen in higher numbers in WA. The Qld clone was the second most common CA-MRSA with WSPP third. Both Qld Clone and WSPP predominated in Brisbane and Sydney (Nimmo et al. 2006)

By 2008, there was a significant rise in the incidence of MRSA in Australia due mainly to the emergence of CA-MRSA. Of 3,075 *S. aureus* isolates, 18% were classified as MRSA, with 62.2% CA-MRSA and 37.8% HA-MRSA. The HA-MRSA were ST22-MRSA-IV, the most predominant at 54.1%, followed by ST239-MRSA-III at 44.9% (Aus-2 and Aus-3 combined), ST5-MRSA-II (New York/Japan) 0.5% and ST36-MRSA-II (EMRSA-16) 0.5% (AGAR 2009).

Twenty two CA-MRSA clones were identified with the Qld clone being the most prevalent. The top six, which comprise 90.6% of total CA-MRSA studied in 2008, are presented in Table 1.3.

Table 1.3 The Most Prevalent CA-MRSA in Australia 2008

Clone	Alternative Name	% of CA-MRSA
ST93-MRSA-IV (2B)	Qld CA-MRSA	44.1%
ST1-MRSA-IV (2B)	WA MRSA-1	18.5%
ST30-MRSA-IV (2B)	WSPP CA-MRSA	13.8%
ST5-MRSA-IV (2B)	WA MRSA-3	5.9%
ST78-MRSA-IV (2B)	WA MRSA-2	5.3%
ST8-MRSA-IV (2B)	USA 300	2.9%

PVL positive clones made up 64.7% of CA-MRSA. The top five are presented in Table 1.4.

Table 1.4 The Most Prevalent PVL Positive CA-MRSA in Austalia 2008

Clone	Alternative Name	No. of Isolates
ST93-MRSA-IV (2B)	Qld CA-MRSA	149
ST30-MRSA-IV (2B)	WSPP CA-MRSA	46
ST8-MRSA-IV (2B)	USA 300	10
ST80-MRSA-IV (2B)	European CA-MRSA	2
ST59-MRSA-V_T (5C2&5)	Taiwan CA-MRSA	1

By 2009 there had been a 136% increase in the number of CA-MRSA Australia-wide, with the PVL positive Qld clone, being the most prevalent (AGAR 2009).

1.2.5 Community MRSA - Western Australia

In 1982, an infection control policy was introduced in WA, which required all new staff and patients who had been in a hospital in the previous year, outside WA, to be screened for MRSA and the isolates be sent to a central laboratory for epidemiological typing and storage. Positive patients were decolonised and were not admitted to general wards until they returned three negative swabs (Pearman et al. 1985). This was in response to an incident in which a patient who was infected with an MRSA was transferred from another state into Royal Perth Hospital. An outbreak resulted with numerous patients and staff being infected or colonised. The policy has been very successful in preventing serious outbreaks of MRSA within WA hospitals and also in maintaining a low incidence of HA-MRSA (Pearman et al. 1985; Nimmo et al. 2006). It has not, however, prevented the emergence of CA-MRSA in the wider WA community (Coombs et al. 2006).

While the first community MRSA reported in Australia, were isolated in remote Indigenous communities in the north of Western Australia (Udo, Pearman, and

Grubb 1993; O'Brien et al. 2004) the same clones were later identified in Perth, the state capital (Coombs et al. 2006). These clones are now known as WA MRSA-1 ST1-MRSA-IVa (2B), WA MRSA-2 ST78-MRSA-IVa (2B), WA MRSA-3 ST5-MRSA-IVa (2B), WA MRSA-4 ST45-MRSA-V (5C2) and WA MRSA-5 ST8 MRSA-IVa (2B).

Between the years 1983-1992 a total of 631 notifications of MRSA were recorded, ranging from 36 in 1988 and increasing to 117 in 1992. It was noticed that there was a significant increase in numbers after 1989 of WA MRSA, now known as CA-MRSA, especially from the remote Kimberley region. Strains were classified as either EMRSA or WA MRSA solely according to their antimicrobial resistance profile. A panel of eight antibiotics; Gentamicin, Erythromycin, Tetracycline, Trimethoprim, Rifampicin, Fusidic acid, Ciprofloxacin and Mupirocin, was used to distinguish CA-MRSA from HA-MRSA. Strains resistant to two or less of the antibiotics were regarded as being non-multi-resistant, strains resistant to three or more of the antibiotics were regarded as being multi-resistant (Riley and Rouse 1995).

MRSA notification data 1994-1997 showed that numbers of EMRSA remained quite stable during this period while WA MRSA (CA-MRSA) increased. In 1994 there was a total of 327 MRSA notifications, with 86% being WA MRSA. By 1997, there were 998 notifications of MRSA with 93% being WA MRSA. There was an increase in fusidic acid resistance from 4.6% in 1994 to 12.4% in 1997, due to the availability of topical fusidic acid treatment. Conversely, mupirocin resistance decreased from 6.4% in 1994 to 0.3% in 1997, due to a change in policy which restricted the use of mupirocin. This study also differentiated EMRSA and WA MRSA according to their antibiotic resistance profiles (Torvaldsen, Roberts, and Riley 1999).

In 1995, a single-strain CA-MRSA outbreak occurred in a major Perth hospital in WA in which the index case was a patient from a remote WA community. Screening two communities in the region where the patient had come from revealed that 39% of people in one community and 17% in the second community were carriers of a CA-MRSA identical to the outbreak strain. The MRSA was

named WA-MRSA-1 (O'Brien et al. 1999) and subsequently was shown to be of the ST1-MRSA-IVa (2B) genetic lineage (O'Brien et al. 2004).

By 2004, 26 different CA-MRSA types had been identified in WA, 25 were designated as WA-MRSA with the 26th being ST30-MRSA-IVa (2B) or WSPP and it appeared that resistance was emerging in CA-MRSA. The Gram Positive Bacteria Typing and Research Unit (GPBTRU) analysed 4,099 MRSA isolates from July 2003 to December 2004 with 77.5% being CA-MRSA. A sub-group of 22 CA-MRSA were characterised further by MLST, staphylococcal cassette chromosome *mec* (SCC*mec*) typing and PFGE. MLST determined 10 clonal complexes and two singleton groups. The two predominant CA-MRSA clones identified were ST1-MRSA-IV (WA MRSA-1), 55.3% and ST78-MRSA-IV (WA MRSA-2), 29.8%. Five PVL positive strains were identified. 44% of CA-MRSA were resistant to β-lactam antibiotics only, 54.5% were non-multi-resistant and 1.5% were multi-resistant. Conclusions were that some CA-MRSA have become multi-resistant. Of concern was the number of PVL positive clones, with the apparent emergence of PVL in a previously PVL negative CA-MRSA clone (Coombs et al. 2006).

By mid 2006, 45 CA-MRSA clones had been identified. These were characterised by phenotype and genotype with analysis including SCC*mec,* MLST, PFGE and staphylococcal protein A (*spa*) type. In 2007, an increase in ST93-MRSA-IV (Qld clone) was seen in WA, with surveillance studies showing the Qld clone currently the third most prevalent CA-MRSA in WA following ST1-MRSA-IV (WA MRSA-1) and ST78-MRSA-IV (WA MRSA-2) (GPBTRU 2008).

In 2010 the GPBTRU was renamed the Australian Collaborating Centre for Enterococcus and Staphylococcus Species (ACCESS) Typing and Research.

At the end of 2011, 108 CA-MRSA clones had been identified (ACCESS Typing and Research unpublished data).

1.3 Antibiotic Resistance

Antibiotic-resistant organisms cause an increasing healthcare burden with increasing healthcare costs, morbidity and mortality (Jones and Pfaller 1998). The spread of antibacterial-resistant organisms can be related to selective pressure caused by the overuse of antibiotics in medicine, agriculture, fisheries and animal feed (Trzcinski et al. 2000). The use of invasive devices and procedures and less than adequate infection control procedures in healthcare facilities leads to the transmission of resistant organisms (Jones and Pfaller 1998).

S. aureus is an adaptable pathogen that gains resistance and virulence factors as a result of horizontal gene transfer due to the selective pressures caused by the miss-use and over-use of antimicrobials in hospital and health care environments. These organisms have then found their way into the community (Chambers and DeLeo 2009). Controlling the spread of MRSA requires effective polices for both infection control and antibiotic usage (Trzcinski et al. 2000).

CA-MRSA are usually resistant to β-lactam antibiotics only. Multi-resistance, associated with HA-MRSA, such as ST239-MRSA-III (3A), commonly known in Australia as Aus-3, is not often seen in CA-MRSA. There is, however, concern that multi-resistance may develop among CA-MRSA and place an additional burden on community and health-care systems. Although most CA-MRSA currently do not spread readily in hospitals, the combination of multi-resistance and virulence in CA-MRSA would be a serious situation should they become endemic in hospitals (Grundmann et al. 2006). PVL positive CA-MRSA such as the Taiwan clone and USA300 are currently regarded as non-multi-resistant strains however, there is great concern should these clones become multi-resistant. A multi-resistant PVL positive CA-MRSA strain ST772-MRSA-V (5C2) has been reported with its origin thought to be India or Bangladesh (Afroz et al. 2008). This strain has been reported in Europe, USA and Australia (D'Souza, Rodrigues, and Mehta 2010). In WA it was first identified in 2007 as WA MRSA-60 and in 2010 it was designated as the Bengal Bay clone (ACCESS 2011).

In bacteria, antibiotic resistance is mediated via three main mechanisms:

1. Inactivation of the antibiotic as seen with resistance to β-lactam and aminoglycoside antibiotics.
2. Exclusion of the antibiotic from the target by efflux as seen with resistance to ciprofloxacin, erythromycin and tetracycline.
3. Target modification as seen with resistance to ciprofloxacin, erythromycin, mupirocin, rifampicin, fusidic acid and tetracycline (McCallum, Berger-Bächi, and Senn 2009).

Acquisition of resistance determinants may be associated with a physiological fitness cost or burden on the bacterial cell. Bacterial clones may compensate with other chromosomal functions to alleviate this fitness loss which may reduce the cell's viability, however, the cell is able to survive, become multi-resistant and cause further disease. Bacterial strains may also be able to regulate the expression of the resistance determinants in the absence of the antibiotic to conserve fitness to the cell (McCallum, Berger-Bächi, and Senn 2009).

Antimicrobial agents, mechanisms of action, mechanisms of resistance, genetic determinants of resistants and genetic locations are summarised in Table 1.5.

1.3.1 Penicillin

Penicillin is a naturally occurring antimicrobial agent initially obtained from the fungus *Penicillium notatum* and commercially from *P. chrysogenum* and has been in use as an antibiotic since the 1940s (Hoephrich 1968; Stefaniak et al. 1947). Penicillin contains a β-lactam ring structure consisting of four atoms and belongs to the β-lactam group consisting of penicillin and derivatives such as methicillin and cephalosporins. Penicillin is a generic term for benzylpenicillin (Penicillin G), procainepenicillin (procaine penicillin), benzathine benzylpenicillin (benzathine penicillin) and phenoxymethylpenicillin (Penicillin V) (Hoephrich 1968).

1.3.1.1 Mechanism of Action

Penicillin has a bactericidal activity by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall. The β-lactam ring structure of penicillin mimics the D-alanyl-D-alanine terminal amino acid residue of the pepdidoglycan layer and will bind in its place to the active site of penicillin-binding proteins (PBPs). This prevents the final cross-linking or transpeptidation of the peptidoglycan layer and disrupts cell wall synthesis, which results in a build up in peptidoglycan precursors without new peptidoglycan being made which then leads to the eventual death of the cell (Waxman, Yocum, and Strominger 1980).

1.3.1.2 Mechanism of Resistance

Resistance to penicillin is due to the production of the enzyme β-lactamase or penicillinase, which hydrolyses the β-lactam ring of the antibiotic, deactivating its antibacterial properties (Geronimus and Cohen 1958; Imsande 1978). β-lactamase is the product of the *blaZ* gene, which is regulated by *blaR1-blaI* inducer-repressors (Wang, Projan, and Novick 1991). The genetic determinant for β-lactamase production is mostly located on plasmids and often transposes into the chromosome (Novick 1989).

1.3.2 Methicillin

Methicillin is a semi-synthetic, β-lactam antibiotic with narrow spectrum activity. Methicillin was first developed in 1959 to treat infections caused by penicillin-resistant *S. aureus* (Stewart and Holt 1963). Methicillin is structurally different from benzylpenillicin with the addition of methoxy groups to the phenol group. This produces a steric hindrance around the amide bond and reduces the affinity for staphylococcal β-lactamases (Stapleton and Taylor 2002).

Methicillin is no longer used as a therapeutic agent and has been replaced by other semi-synthetic penicillins such as flucloxacillin and dicloxacillin (Fluit and

Schmitz 2003). For clinical antimicrobial susceptibility testing, oxacillin is more stable than methicillin and more likely to detect heterogeneous strains, however cefoxitin resistance tests are easier to read than oxacillin as it is a better inducer of the *mecA* gene, and will give a clearer endpoint with disc diffusion testing (Brown 2001; Skov et al. 2003; Felton et al. 2002).

1.3.2.1 Penicillin-Binding Proteins

The cell wall of *S. aureus* forms a protective barrier against the environment, contributes to colonisation, virulence and resistance and maintains the bacterial shape, especially against high internal osmotic pressure. Structurally the cell wall is composed of multi-layers of highly cross-linked peptidoglycan, with teichoic acids covalently linked to the peptidoglycan. Also found are membrane-associated proteins, peptidoglycan-anchored surface proteins and extracellular-polysaccharide matrices, all of which contribute to the properties of the bacterial cell surface (Georgopapadakou, Dix, and Mauriz 1986; Pinho, De Lencastre, and Tomasz 2001). The peptidoglygan, which is the major component, is a macromolecular network of glycan strands cross-linked through short peptides. The glycan strands are comprised of chains of alternating amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) connected by a β-(1,4)-glycosidic bond. A short amino acid chain is attached to the NAM amino sugar residue, containing L-Ala-iD-Glu-γ-L-Lys-D-Ala peptide units (Giesbrecht et al. 1998; Berger-Bächi and Tschierske 1998). A pentaglycine side-chain is found at the lysine amino group, that allows three-dimensional cross-linking of the multi-layered peptidoglycan, and can also be an attachment site for cell wall anchored surface proteins, such as the plasmin-sensitive protein, Pls (Schneewind, Fowler, and Faull 1995; Foster and Höök 1998). See Figure 1.1.

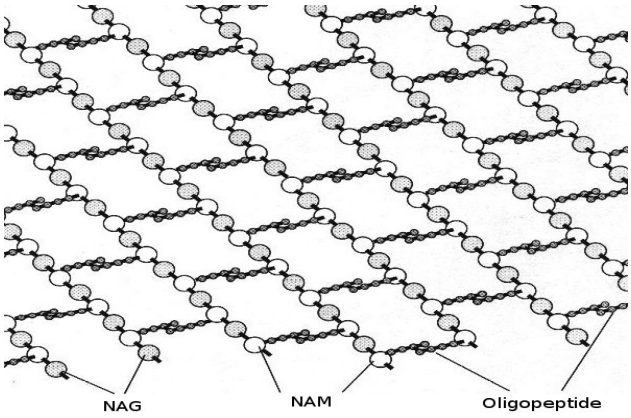

Figure 1.1 The Peptidoglycan Layer
Abbreviations: NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid
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Continual production and processing of peptidoglycan components are essential for cell-wall synthesis, with PBPs essential in the last extracellular enzymatic reactions. Five different PBPs have been found in *S. aureus*, PBP1, PBP2, PBP3, PBP4 and PBP2a/2' (Georgopapadakou, Dix, and Mauriz 1986).

PBP1 is a protein of 85 kDa encoded by the *pbpA* gene. The function of PBP1 is unclear, but it is thought to be essential for cell wall growth with a partial function in the synthesis of the peptidoglycan septum (Wada and Watanabe 1998; Komatsuzawa et al. 1999).

PBP2 is a protein of 81 kDa encoded by the *pbpB* gene. PBP2 is bifunctional with transpeptidase and transglycosylase activities. Transpeptidase is essential for the cross-linkage formation in the peptidoglycan layer (Wada and Watanabe 1998; Pinho, De Lencastre, and Tomasz 2001).

PBP3 is a protein of 75 kDa encoded by the *pbpC* gene and is thought to be non-essential for cell growth. PBP3 is thought to be a peptidoglycan transpeptidase involved in septum formation similar to PBP1 (Wada and Watanabe 1998; Pinho, De Lencastre, and Tomasz 2001).

PBP4 is a protein of 45 kDa encoded by the *pbpD* gene, which is associated with an ATP-binding transporter gene. PBP4 is bifunctional with carboxypeptidase and transpeptidase activities. While considered not essential for cell growth, overproduction of PBP4 has been shown to increase β-lactamase resistance (Komatsuzawa et al. 1999; Domanski and Bayles 1995).

PBP2a/2' is a protein of 78 kDa encoded by the *mecA* gene found in the genomic-resistance island, the staphylococcal cassette chromosome *mec* (SCC*mec*). PBP2a/2' confers intrinsic resistance to all β-lactams and their derivatives. PBP2a/2' has a low affinity for binding β-lactam antibiotics, due to an altered active site and is able to continue transpeptidation activity of PBP2 at concentrations of antibiotics that might otherwise be lethal (Chambers 1997; Berger-Bächi and Rohrer 2002).

1.3.2.2 Mechanism of Action

Methicillin and other β-lactam antibiotics act by inhibiting the synthesis of the cell wall by preventing cross-linkages between the peptidoglycan polymer chains. They are structural analogues of *D-alanyl-alanine* that competively bind and inhibit the transpeptidase enzyme PBPs ability, by acylation, to cross-link the *D-alanyl-alanine* peptide used in peptidoglycan synthesis (Chambers 1997).

1.3.2.3 Expression of Resistance

Resistance to methicillin occurs with the acquisition of the genomic resistance island SCC*mec* which contains the *mecA* gene, encoding the protein PBP2a/2'. PBP2a/2' is able to continue with cell wall synthesis in the presence of methicillin. The expression of methicillin resistance has been seen to be heterogeneous, homogeneous or borderline with factors both genetic and environmental affecting and influencing methicillin resistance (Berger-Bächi and Rohrer 2002).

1.3.2.3.1 Homogeneous Resistance

Homogeneous resistance to methicillin occurs where all the cells in a population are uniformly resistant to high concentrations of methicillin (>128 mg/L). Homogeneous resistance appears not to be influenced by the level of PBP2a/2' produced indicating that other factors are involved. A chromosomal mutation(s) chr* that occurs outside the SCC*mec* is thought to be responsible for the conversion of a heterogeneous cell population to a homogenous cell population (Ryffel et al. 1994; Chambers 1997).

1.3.2.3.2 Heterogeneous Resistance

The heterogeneous resistance of methicillin is seen when the majority of cells within a population (typically 99% or more) express a wide variation of resistance levels (1-5 μg/ml methicillin), with a smaller population resistant to methicillin at higher concentrations (> 50 μg/ml). The *in vivo* degree of heterogeneity varies according to the sub-clone of the strain, culture conditions and the β-lactam antibiotic being used (Matthews and Stewart 1984; Hartman and Tomasz 1986)

1.3.2.3.3 Borderline Resistance

Borderline or low-level methicillin resistance is characterised by a minimum inhibitory concentration (MIC) at, or just above, the susceptibility breakpoint (oxacillin MIC 4-8 μg/ml). Borderline strains may or may not have *mecA* present. Borderline strains that contain *mecA* are heterogeneous and will have a small sub-population that can grow at high antibiotic levels. Borderline strains that do not contain *mecA* are thought to result from alterations in PBP genes or an over-production of β-lactamase (McDougal and Thornsberry 1986; Hackbarth et al. 1995; Chambers 1997).

1.3.2.3.4 Eagle-Type Resistance

Eagle-type methicillin resistance was found only in experimental mutants of strain N315 (h4), ST5-MRSA-II (2A). The eagle-type resistance characteristically showed resistance to high concentrations of methicillin (64-512 μg/ml) and susceptibility at lower concentrations of methicillin (2-16 μg/ml). Two novel genes *hmrA* and *hmrB* were detected, which conferred homogeneous-methicillin

resistance. A homogeneous to heterogeneous resistance conversion occurred when these genes were transferred to strain LR5 via multi-copy plasmids, strongly indicating that genetic alteration is responsible for heterogeneous to homogeneous conversion of methicillin resistance in *S. aureus* (Kondo et al. 2001).

1.3.2.3.5 Environmental Factors Affecting *In Vitro* Resistance

The degree of methicillin resistance is influenced by environmental factors and cultural conditions such as temperature, osmolality, pH, light, anaerobiosis, chelating agents, metal ions and previous exposure to β-lactam antibiotics (Matthews and Stewart 1984). Discrepant results may be seen between MICs conducted at 30ºC and 37ºC. Changes in osmolality of media with sodium chloride, ammonium sulphate or sucrose can have an effect on the expression of methicillin resistance of MRSA strains. Increases in sucrose and these salts will increase the resistance of MRSA strains. The pH has a major effect on methicillin resistance with MICs in broths at pH5.2 being reduced by as much as 128-fold. Visible light affects methicillin resistance in those strains that don't produce pigment, with highly pigmented strains not affected. Chelating agents/metal ions have been shown to have varying effects on resistance to methicillin. Chelating agents such as EDTA reduce MICs while addition of metal ions such as magnesium will increase the MIC. Anaerobic conditions result in an increase in methicillin MICs.

1.3.2.3.6 Genetic Factors Affecting Resistance

Chromosomal genes outside of the SCC*mec* have been found to be associated with the expression of methicillin resistance. These genes have been identified by transposon insertional mutagenesis producing susceptible mutants. Factors essential for methicillin resistance (*fem*) or auxiliary (*aux*) factors have been found in both methicillin-sensitive and -resistant strains. Over 20 *fem* or *aux* factors have been found (Chambers 1997; Berger-Bächi and Rohrer 2002).

1.3.2.3.7 *fem AB* operon

The *femAB* operon has the greatest impact on the methicillin-resistance levels. The *femAB* operon is thought to be a duplication of sequences that code for two

similar cytoplasmic proteins each of 49 kDa, which are required for the formation of the pentaglycine interpeptide bridge cross-link of peptidoglycan. *femA* is responsible for glycines Gly2-Gly3 and FemB for Gly4-Gly5. *femB* mutants produce cross-links with only three glycines, while *femA* mutants only produce a monoglycine interpeptide bridge and do not incorporate the second and third glycines into the bridge. The resulting poor peptidoglycan cross-linking and reduced cell wall turnover results in the cell becoming hyper-susceptible to methicillin and other β-lactams, with PBP2a/2' losing its function even though it is still being produced (Henze et al. 1993; Strandén et al. 1997).

1.3.2.3.8 *femX*

femX or *fmhB,* like *femAB,* is involved with pentaglycine interpeptide bridge formation, being responsible for the attachment of the first glycine residue Gly1 to the stem peptide (Rohrer et al. 1999).

1.3.2.3.9 *femC*

femC was identified by a Tn*551* insert in the gene *glnR* which encodes a glutamine synthetase repressor and is found in the *glnRA* operon. This insertion results in a reduction in glutamine synthesis due to a polar effect in *glnA* transcription, leading to reduced glutamine availability and reduced amidation of iD-glutamate of the stem peptide in the peptidoglycan pentapeptide. The cell wall will have reduced peptidoglycan cross-linking and reduced methicillin resistance (Gustafson et al. 1994).

1.3.2.3.10 *femD*

femD (*femR* or *glmM*) was identified by a Tn*551* insert in the gene *glmM*, which encodes phosphoglucosamine mutase and functions by catalysing the inter-conversion of glucosamine-6-phosphate to glucosamine-1-phosphate, a cytoplasmic peptidoglycan precursor. Inactivation of *femD* results in reduced methicillin resistance (Jolly et al. 1997).

1.3.2.3.11 *femE*

The function of *femE* is unknown with its inactivation only slightly reducing methicillin resistance. *femE* was identified by a Tn*551* insert (De Lencastre et al. 1994).

1.3.2.3.12 *femF*

femF (*murE*) mutation results in an abnormality in peptidoglycan composition with the absence of the unsubstituted pentapeptide and incorporation of alanylglutamate and alanylisoglutamate into the muropeptide, before the lysine addition step. This results in reduced methicillin resistance (Ornelas-Soares et al. 1994).

1.3.2.3.13 *fmtA*

fmtA encodes a cell wall associated protein containing two of three conserved motifs typically found in PBPs and β-lactamases. Mutants of *fmtA* show reduced amidation of glutamate residues and reduced cross-linking in the peptidoglycan, and reduce methicillin resistance (Komatsuzawa et al. 1999).

1.3.2.3.14 *fmtB*

fmtB (*mrp*) encodes a cell surface protein, that when inactivated affects the monomer section of the cell wall muropeptide and the disaccharide-pentapeptide-pentaglycyl-monomer is converted to an unsubstituted pentapeptide. This reduces methicillin resistance (Wu and De Lencastre 1999).

1.3.2.3.15 *fmtC*

fmtC (*mprF*) encodes a hydrophobic membrane-associated protein, that when inactivated reduces the modification of the membrane lipid phosphatidyl-glycerol with L-lysine, resulting in a reduced negative charge on the membrane surface. This reduces methicillin resistance (Peschel et al. 2001).

1.3.2.3.16 *llm*

llm encodes a 38 kDa hydrophobic membrane-associated protein, TarO (TagO) which catalyzes the first step in teichoic acid synthesis (Swoboda et al. 2010). *llm*

was identified by insertional inactivation by Tn*918* carrying a tetracycline-resistance marker. Inactivation of *llm* reduces growth and changes homogeneous methicillin resistance to the heterogeneous phenotype. In some strains inactivation of *llm* increases autolysis and reduces resistance (Maki, Yamaguchi, and Murakami 1994).

1.3.2.3.17 *lytH*

The *lytH* gene encodes a lytic enzyme that has homology to a cell-wall hydrolysing enzyme. Deletion or inactivation of *lytH* results in high levels of methicillin resistance (Fujimura and Murakami 1997). A recent study showed the inactivation of the *lytH* gene by the insertion of IS*1182* (Fujimura and Murakami 2008).

1.3.3 Gentamicin

Gentamicin is a broad spectrum antibiotic with bactericidal activity first discovered in 1944. The chemical structure of gentamicin is comprised of aminated sugars joined to a dibasic cyclitol. Gentamicin belongs to the aminoglycoside group of antibiotics along with tobramycin, kanamycin and streptomycin (Kotra, Haddad, and Mobashery 2000) and often acts in synergy when used in combination with another antibiotic.

1.3.3.1 Mechanism of Action

Gentamicin, along with all aminoglycosides, acts by inhibiting protein synthesis by binding to the 30S ribosomal subunit at the 16S rRNA component. This action results in a misreading of mRNA codons which is thought to result in the production of faulty proteins, or disruption of the cell membrane in some way (Hancock 1981; Lyon and Skurray 1987).

1.3.3.2 Mechanism of Resistance

The mechanism of resistance to aminoglycosides is via inactivation by aminoglycoside-modifying enzymes, acetyltransferase (AAC), phosphotransferase (APH) and nucleotidyltransferase (ANT) or adenytransferase (AAD) either singly or in combinations. Resistance to gentamicin is mediated by AAC(6′) – APH(2″) which is encoded by the *aac(6')+aph(2")* genes (Lyon and Skurray 1987). The genes encoding the aminoglycoside-modifying enzymes for gentamicin are commonly located on transposon Tn*4001,* which is found on the chromosome and on many plasmid types (Wood, Carter, and Best 1977; Kayser, Homberger, and Devaud 1981; Lyon and Skurray 1987). It has been shown that Tn*4001* is found inserted at various chromosomal locations including within the SCC*mec* (Gillespie et al. 1987; Heusser et al. 2007). The plasmid types where Tn*4001* may be found include the pSK1 family, conjugative plasmids such as pSK41 and occasionally on β-lactamase/heavymetal resistance plasmids (Gillespie et al. 1987).

1.3.4 Tetracycline

Tetracyclines are a broad spectrum class of antibiotic that have a bacteriostatic action against bacteria. Tetracycline is derived from the soil bacterium, streptomycetes, and was discovered in the late 1940s. The originally developed tetracyclines are known as first generation tetracyclines. Later, second generation tetracyclines were developed, such as doxycycline and minocycline (Chopra and Roberts 2001). Third generation tetracyclines, now being developed, are known are glycylcyclines, such as tigecycline (Chopra 2001).

1.3.4.1 Mechanism of Action

Tetracycline inhibits bacterial protein synthesis by reversibly binding to a high affinity site on the 30S subunit of the bacterial ribosome. This action blocks the

binding of aminoacyl-tRNA to the acceptor site on the ribosome, preventing protein synthesis (Buck and Cooperman 1990).

1.3.4.2 Mechanisms of Resistance

There are two mechanisms of resistance: active efflux of the antibiotic from the cell and ribosomal protection.

1.3.4.2.1 Active Efflux

Resistance to tetracyclines by active efflux occurs when efflux genes code for membrane-bound proteins to export tetracycline out of the bacterial cell. The third generation glycylcyclines are not affected by these proteins (Trzcinski et al. 2000; Chopra and Roberts 2001). These membrane-bound proteins are encoded by genes *tet*(K) and *tet*(L) and belong to the major facilitator superfamily (MFS). These genes are often found on small plasmids such as pT181 (Khan and Novick 1983; Guay, Khan, and Rothstein 1993). The *tet* genes may also become integrated into larger staphylococcal plasmids or into the bacterial chromosome (Chopra and Roberts 2001). Plasmid pT181 also integrates into the chromosome and is found in the SCC*mec* (Gillespie, May, and Skurray 1986).

1.3.4.2.2 Ribosomal Protection

Tetracycline resistance occurs when ribosomal protection proteins bind to the bacterial ribosome thereby removing any bound tetracycline by the hydrolysis of GTP. This action allows continuing protein synthesis and results in resistance to tetracycline (Lyon and Skurray 1987). The genes that encode the ribosomal protection proteins in *S. aureus* are *tet*(M) and *tet*(O), and they are located on a transposon or on the chromosome (Khan and Novick 1983; Nesin et al. 1990). The *tet*(M) gene is found on the putative conjugative transposon Tn*5801* which is found on the *S.aureus* chromosome (Kuroda et al. 2001).

S. aureus with the *tet*(K) gene only have been found to be resistant to tetracycline but sensitive to minocycline, while the *tet*(M) gene confers resistance to all the drugs in the group (Bismuth et al. 1990). The presence of both *tet*(K) and *tet*(M)

has been shown to result in a higher MIC for tetracycline resistance than in strains carrying just one of the genes (Schmitz et al. 2001).

1.3.5 Erythromycin

Erythromycin is a naturally occurring antimicrobial agent obtained from *Streptomyces erythreus* and has been in use as an antibiotic since the early 1950s (Fluit and Schmitz 2003). Erythromycin is a macrolide with a 14 membered-ring-structure, and belongs to the macrolide-lincosamide-streptogramin B group (MLS_B) of antibiotics (Douthwaite and Champney 2001).

1.3.5.1 Mechanism of Action

Macrolides such as erythromycin have a bacteriostatic action on bacteria, by binding reversibly to the 50S bacterial ribosome subunit at the peptidyl transferase region resulting in the inhibition of protein synthesis (Champney and Tober 2000). A second action of macrolides prevents the assembly of functionally active 50S subunits. This results in the accumulation of the 50S subunit precursor particles which are then degraded by cellular ribonucleases (Champney and Burdine 1998).

1.3.5.2 Mechanism of Resistance

There are four mechanisms of bacterial resistance to erythromycin: target site modification, antibiotic modification, mutation and active efflux of the antibiotic from the cell (Weisblum 1995).

1.3.5.2.1 Target Site Modification

Target site modification occurs with the production of a resistance determinant known as erythromycin ribosome methylase encoded by the *erm* gene. This protein methylates the adenine residue A2058 in the peptidyltransferase region in

domain V of 23S rRNA. This prevents the binding of erythromycin to the bacterial ribosome and results in high-level resistance (Weisblum 1995).

Expression of methylase genes can be either constitutive or inducible. Strains with constitutive resistance to erythromycin can show a cross-resistance to MLS_B antibiotics. This occurs due to overlapping binding sites on the 50S subunit of the individual antimicrobial agents (Garrod 1957; Champney and Tober 2000). Strains with inducible MLS_B resistance will be resistant to erythromycin, which is an inducer antibiotic, while appearing susceptible to a non-inducer antibiotic such as clindamycin. Exposure to erythromycin induces cross-resistance to all the MLS_B antibiotics (Drinkovic et al. 2001; Daurel et al. 2008). This can be detected *in vitro* using a D-zone test.

In *S. aureus* the most commonly found methylase genes are *ermA* and *ermC* (Westh et al. 1994). The *ermA* gene is found on the transposon Tn*554* located on the staphylococcal cassette chromosome *mec* (SCC*mec*) and plasmids, while the *ermC* gene is found on plasmids (Thakker-Varia et al. 1987).

1.3.5.2.2 Antibiotic Modification

Resistance to erythromycin due to antibiotic modification or inactivation results with the production of phosphotransferases encoded by *mph*(C) (Leclercq 2002), or *mph*(BM) (Matsuoka et al. 1998).

1.3.5.2.3 Target Site Mutation

Resistance to erythromycin due to mutations has been recorded, although rare. This was seen with erythromycin resistance and mutations in *rrl* and *rplV* genes on 23S rRNA in *S. aureus* from cystic fibrosis patients (Prunier et al. 2002).

1.3.5.2.4 Antibiotic Efflux

Active efflux of the antibiotic from the cell is another mode of resistance. The efflux proteins known as ABC transporters are encoded by *msr*A genes found on plasmids. ABC transporters require ATP to function and are comprised of two

membrane-spanning domains and two ATP-binding domains (Ross et al. 1990; Leclercq 2002).

1.3.6 Ciprofloxacin

Ciprofloxacin is a synthetic antimicrobial from the fluoroquinolone group, with its mode of action directed against bacterial DNA replication (McCallum, Berger-Bächi, and Senn 2009). Ciprofloxacin is a broad spectrum antimicrobial with good activity towards Gram-positive bacteria such as *S. aureus* (Fluit and Schmitz 2003). Fluoroquinolones were developed in the mid 1980s with the addition of a fluorine atom to a quinolone. This resulted in the improvement of its antibacterial spectrum and pharmokinetics (Fluit and Schmitz 2003). Soon after, however, resistances were reported (Hooper 2002).

1.3.6.1 Mechanism of action

Ciprofloxacin targets two essential enzymes of the Type II DNA topoisomerase group, DNA gyrase and topoisomerase IV (Drlica and Zhao 1997). The action of ciprofloxacin blocks the replication fork, resulting in the breakage of the DNA strand, inhibition of DNA synthesis and death of the cell.

1.3.6.1.1 DNA gyrase

DNA gyrase is an enzyme that controls DNA supercoiling and relieves stresses during transcription and replication. This is achieved when the enzyme cuts strands of the DNA double helix, passing a strand through the resulting gap before the DNA is resealed. DNA gyrase is comprised of two subunits GyrA and GyrB encoded by the genes *gyrA* and *gyrB* respectively. The main function of DNA gyrase is to maintain the correct level of negative supercoiling in preparation for DNA replication, to remove knotted forms from the DNA and to help bend and fold DNA (Drlica and Zhao 1997; Fluit and Schmitz 2003).

1.3.6.1.2 Topoisomerase IV

Topoisomerase IV is an enzyme that has a decatenating or separating action. Topoisomerase IV is comprised of two subunits, GrlA and GrlB encoded by the genes *grlA* and *grlB* respectively. The main feature of topoisomerase IV is its ability to decatenate DNA, by the breakdown of ATP, before replication is complete. This occurs where one DNA molecule passes through a cut made in a separate DNA molecule (Drlica and Zhao 1997; Fluit and Schmitz 2003).

1.3.6.2 Mechanisms of Resistance

1.3.6.2.1 Chromosomal Mutations

The primary mechanism of resistance to ciprofloxacin and fluoroquinolones in *S. aureus* involves chromosomal mutations in the DNA gyrase genes (*gyrA* and *gyrB*) and/or topoisomerase IV genes (*grlA* and *grlB*). Studies have shown that ciprofloxacin resistance occurs as a stepwise selection, with higher-level resistance resulting with the combination of single point mutations within the *grlA* gene and *grlB* genes (Ferrero, Cameron, and Crouzet 1995; Schmitz, Jones, et al. 1998). Furthermore, combinations of single point mutations with the *gyrA* gene show a higher-level resistance than a single point mutation within that gene (Takenouchi et al. 1995). This is also seen within the *grlA* gene (Schmitz, Jones, et al. 1998). This study by Schmitz et al. 1998, from 116 isolates, found 9 combinations of amino acid changes resulting from single point mutations within *grlA, grlB* and *gyrA*. These changes were all associated with ciprofloxacin resistance.

Ciprofloxacin, at low concentrations, is reported to be mutagenic due to its ability to damage bacterial DNA during transcription of the SOS and methyl-mismatch repair (MMR) genes (Mesak, Miao, and Davies 2008).

Resistance to newer fluoroquinolones is at a much lower rate because they are less susceptible to the dual mutations within the gyrase and topoisomerase IV genes

than the older fluoroquinolones such as ciprofloxacin (McCallum, Berger-Bächi, and Senn 2009).

1.3.6.2.2 Efflux Mediated Resistance

Another process that causes ciprofloxacin resistance in *S. aureus* is via a chromosomally encoded efflux pump protein, NorA (Fluit and Schmitz 2003). NorA protects the cell from lipophilic and monocationic compounds such as ethidium bromide, centrimide, chloramphenicol as well as ciprofloxacin. The *S. aureus* NorA protein is a multi-drug transporter in the MFS (Truong-Bolduc, Zhang, and Hooper 2003) that depends on a transmembrane protein gradient for active efflux (Fluit and Schmitz 2003), which is essential to maintain a homeostatic environment in the bacterial cell.

Efflux mediated resistance is usually seen as a lower-level resistance when compared with topoisomerase mutations. Resistance results from point mutation within a regulatory region 89 base pairs (bp) upstream of the *norA* gene rather than within the *norA* gene itself. The mutation increases the stability of *norA* mRNA by altering its structure causing an overexpression of the *norA* mRNA, resulting in the reduction of ciprofloxacin accumulation within the bacterial cell (Fluit and Schmitz 2003).

1.3.6.2.3 Plasmid-Mediated Resistance

To date, resistance of ciprofloxacin in *S. aureus* has been due to chromosomal mutation. No evidence has been shown to suggest plasmid-mediated resistance. There has previously been some suggestion of plasmid-mediated quinolone resistance in Gram-negative bacteria, *Shigella dysentieriae* (Ambler et al. 1993) and also *Klebsiella pneumoniae* (Jacoby, Chow, and Waites 2002). A possible suggestion for this is that in Gram-negative bacteria, the mode of resistance is mostly due to the action of DNA gyrase while in staphylococci, the mode of action is mostly due to topoisomerase IV (Drlica and Zhao 1997). Perhaps the dynamics between these two enzymes and their encoding genes lends itself to a predisposition to being either plasmid mediated as with Gram-negative bacteria or chromosomal, as with staphylococci.

1.3.7 Fusidic Acid

Fusidic acid is a steroid-like antibiotic that is derived from the fungus *Fusidium coccineum* (Fluit and Schmitz 2003).

1.3.7.1 Mechanism of Action

Fusidic acid antibacterial action inhibits bacterial protein synthesis. This occurs when fusidic acid binds to the ribosome-elongation factor-G-GDP complex and prevents its dissociation from the ribosome. This prevents the next stage of protein synthesis, the entry of aminoacyl-tRNA species into the ribosomal A site (Willie et al. 1975; Besier et al. 2003).

1.3.7.2 Mechanism of Resistance

There are two mechanisms of resistance to fusidic acid: alteration of the antibiotic target site and protection of the antibiotic target site.

1.3.7.2.1 Alteration of the Antibiotic Target Site

Alterations of the antibiotic target site occur due to mutations in genes encoding elongation factor-G (EF-G) This category includes FusA, FusA-SCV and FusE classes (Besier et al. 2003).

The *fusA* gene encoding EF-G has a chromosomal location that has been shown to have a possible 30 different amino acid substitution mutations that result in fusidic acid resistance (Besier et al. 2003). These mutations occur mostly in domain III of EF-G and occasionally in domains I and V (Laurberg et al. 2000).

FusA-SCV class resistance is a subset of the mutations in *fusA*, with most mutations located in domain V of EF-G. This is a small-colony variant (SCV) phenotype (Norstrom, Lannergard, and Hughes 2007).

FusE class resistance may also express the SCV phenotype. Fusidic acid resistance results following mutations in the *rplf* gene which encodes the ribosomal protein L6. Protein L6 interacts with EF-G (Norstrom, Lannergard, and Hughes 2007).

1.3.7.2.2 Protection of the Antibiotic Target Site

This mechanism of resistance to fusidic acid prevents the antibiotic from interacting with EF-G. The exact mechanism of the interaction is still unknown (O'Brien et al. 2002; O'Neill and Chopra 2006). This category includes FusB and FusC Classes of resistance.

The *fusB* gene is found on a plasmid, pUB101, a 22kb class II staphylococcal replicon that also encodes penicillinase production and cadmium resistance (O'Neill and Chopra 2006). *fusB* has been reported in SCC*mec*$_{N1}$, which is a 45.7 kb SCC element consisting of a class B *mec* complex and a truncated Tn*4003* that encodes resistance to trimethoprim (*dfr*) (Ender, Berger-Bächi, and McCallum 2007). *fusB* has also been reported on a chromosomal location on a 16.6 kb genomic island designated SaRI$_{fusB}$ (*S. aureus* resistance island carrying *fusB*) (O'Neill et al. 2007).

The gene *fusC* was found following the complete genome analysis of a *S. aureus* strain that revealed the existence of a novel staphylococcal cassette chromosome (SCC) designated SCC$_{476}$ encoding *fusC* (Holden et al. 2004).

1.3.8 Rifampicin

Rifampicin is a broad spectrum antibiotic used mostly to treat Gram-positive bacteria. Rifampicin is a semi-synthetic antibiotic belonging to the family of rifamycins produced by the actinomycete *Amycolatopsis mediterranea* (Floss and Tin-Wein 2005). Rifampicin has strong activity, being most effective when used in combination with other antibiotics such as ciprofloxacin (Robertson et al.

2008), especially when treating a deep-tissue infection (Kunin 1996). Rifampicin is also effective when combined with fusidic acid for difficult to treat MRSA infections (Howden and Grayson 2006; Aboltins et al. 2007).

1.3.8.1 Mechanism of Action

Rifampicin binds to the bacterial RNA polymerase β subunit and prevents transcription. The RNA polymerase is encoded by the *rpoB* gene (Aboshkiwa, Rowland, and Coleman 1995).

1.3.8.2 Mechanism of Resistance

Resistance to rifampicin results after an alteration in the β subunit of the RNA polymerase that reduces the affinity of the antibiotic to bind to it. These alterations result from base pair mutations in the *rpoB* gene (Morrow and Harmon 1979). A study in 1998, showed that rifampicin resistance levels are dependent on both the location and nature of the amino acid substitution (Aubrey-Damon, Soussy, and Courvalin 1998).

1.3.9 Mupirocin

Mupirocin is a topical antibiotic and commonly used for MRSA decolonisation practices. Originally known as pseudomonic acid, mupirocin is derived from *Pseudomonas fluorenscens* and is unrelated to any other antibiotics (Eltringham 1997).

1.3.9.1 Mechanism of Action

Mupirocin is bacteriostatic but may be bactericidal at lower pH (Cookson 1998). Mupirocin acts as an inhibitor of protein synthesis by binding irreversibly at the

isoleucyl-tRNA synthetase (Iles) at a site near an ATP-binding sub-site (Yanagisawa et al. 1994).

1.3.9.2 Mechanism of Resistance

Resistance to mupirocin is expressed according to its MIC, and falls into two distinct categories of low-level resistance and high-level resistance (Eltringham 1997).

1.3.9.3 Low-Level Resistance

Low-level resistance is defined by an MIC of ≥4 mg/L to <256 mg/L. Low-level resistance is not considered of clinical importance (Cookson 1998). The mechanism of resistance for low-level resistance to mupirocin is due to a chromosomal base change in the isoleuyl-tRNA sythetase gene *iles* (Antonio, McFerran, and Pallen 2002).

1.3.9.4 High-Level Resistance

High-level resistance is defined by an MIC of ≥256 mg/L. High-level mupirocin resistance has been found to be plasmid-borne via the *mupA* gene which codes for an alternative isoleucyl-tRNA sythetase that does not bind mupirocin (Udo, Jacob, and Mathew 2001).

The occurrence of the genetic locations of these resistance determinants resulting in low-level or high-level resistance to mupirocin have also seen some exceptions. The gene for *mupA* has been detected on the chromosome expressing low-level mupirocin resistance (Ramsey et al. 1996) and a study in 2005, suggested that there could be a clonal transmission of the mupirocin *mupA* resistance determinant (Yoo et al. 2006).

Table 1.5 Antibiotic Resistance in *S. aureus*

Antimicrobial Agent	Mechanism of Action	Mechanism of Resistance	Genetic Determinant	Location
Methicillin	Cell wall	PBP2a/2'	*mecA*	C
Gentamicin	Inhibits protein synthesis	Drug modification	*aacA-aphD*	C, Tn, P
Tetracycline	Inhibits protein synthesis	Active efflux Active efflux Ribosomal protection	*tet*(K) *tet*(L) *tet*(M)	C, P P C, Tn
Erythromycin	Inhibits Protein Synthesis	Target alteration	*ermA, ermB, ermC* *msrA*	C, Tn, P P
Ciprofloxacin	Inhibits DNA replication	Target alteration	*grlA, grlB, gryA, grlB* *norA*	C C C
Fusidic Acid	Inhibits protein synthesis	Target alteration	*fusA* *far1*	C P
Rifampicin	Inhibits protein synthesis	Target alteration	*rpoB*	C
Mupirocin	Inhibits protein synthesis	Target alteration	*mupA*	C, P
Trimethoprim	Inhibits folate synthesis	Target alteration	*dfrA* *dfrC*	C, Tn, P P
Vancomycin	Cell wall	Target alteration Thicken cell wall	*vanA* VISA/GISA	C, Tp C

Abbreviations: C, Chromosome; P, Plasmid; Tn, Transposon

1.3.10 Trimethoprim

Trimethoprim is a synthetic antibacterial agent that was first used in the early 1960s. It is a broad spectrum antimicrobial, being effective for both Gram-positive and Gram-negative micro-organisms. Trimethoprim is most effectively used in combination with sulphonamides due to a synergistic effect (Bushby and Hitchings 1968; Huovinen et al. 1995).

1.3.10.1 Mechanism of Action

Trimethoprim affects bacterial folic acid synthesis by inhibiting dihydrofolate reductase (DHFR). This prevents the formation of tetrahydrofolate from dihydrofolate and blocks folic acid synthesis (Huovinen 2001).

1.3.10.2 Mechanism of Resistance

The mechanism of resistance to trimethoprim is via the production of a type S1 trimethoprim-resistant DHFR, which is encoded by the *dfrA* gene (Dale et al. 1995). This gene is located on transposon Tn*4003,* either on a plasmid, or on the chromosome (Lyon and Skurray 1987). Trimethoprim resistance can be high-level or low-level depending on the level of transcription of *dfrA*. A study has suggested the involvement of insertion sequence IS*257*, which flanks Tn*4003*, in variations in trimethoprim-resistance levels (Leelaporn et al. 1994). Trimethoprim resistance is a consequence of a point mutation in the *dfrA* gene resulting in an amino acid substitution of Phe to Tyr at position 98 of the DfrA molecule (Dale et al. 1997).

A novel trimethoprim-resistance gene has been identified and designated *dfrK*. This gene was found on a plasmid that also contained the gene for tetracycline resistance in the porcine MRSA ST398 (Kadlec and Schwarz 2009).

1.3.11 Vancomycin

Vancomycin was first discovered in 1956. It is produced by the actinomycete *Streptomyces orientalis* that was cultivated from soil collected in Borneo. Vancomycin was introduced into clinical use in 1958 with the intention of treatment for penicillin-resistant *S. aureus*. Even though staphylococci did not develop significant resistance, use of vancomycin was limited for the next two decades, due to vancomycin needing to be administered intravenously, other β-lactamase resistant antimicrobials such as methicillin and its successors being

developed, and early impure forms of vancomycin being found to be toxic (Barna and Williams 1984).

Vancomycin is a glycopeptide antibiotic that is comprised of large, rigid molecules containing glycosylated cyclic or polycyclic nonribosomal peptides. Other glycopeptide antibiotics include teicoplanin, telavancin, bleomycin, ramoplanin and decaplanin. Glycopeptides are narrow-spectrum antibiotics with activity against Gram-positive bacteria. Vancomycin is reserved as a last resort treatment for *S. aureus*, used when other antibiotics cannot be used due to multi-resistance. Vancomycin-resistant organisms may be treated with newer antimicrobials, linezolid and daptomycin (Barna and Williams 1984).

1.3.11.1 Mechanism of Action

Vancomycin, like all glycopeptides acts by inhibiting a late step in cell wall peptidoglycan synthesis and cross-linking. Vancomycin acts by binding to the D-alanyl-D-alanine terminus of the N-acetyl-muramyl-pentapeptide subunit and sterically hindering enzymatic reactions necessary for the formation of peptidoglycan chains (Barna and Williams 1984; Hiramatsu et al. 1997).

1.3.11.2 Mechanism of Resistance

Intermediate resistance to vancomycin occurs following acquired mutations resulting in thickening of the cell wall due to accumulation of excess amounts of peptidoglycan (Hiramatsu et al. 1997).

Generally this type of vancomycin resistance is seen as intermediate, with a MIC of 8 mg/L, and is known as VISA for vancomycin-intermediate *S. aureus* or GISA for glycopeptide-intermediate *S. aureus* (Hiramatsu et al. 1997). VISA/GISA resistance is associated with thickening and poorly cross-linked cell wall peptidoglycan, with increased D-alanyl-D-alanine dipeptide targets able to bind glycopeptide molecules. This form of glycopeptide resistance is due to multiple genetic alterations including genomic mutations and regulatory changes that may

also burden the cell with high fitness costs, causing slower growth and decreased expression of some virulence factors (Cui et al. 2003; McCallum et al. 2006).

VISA/GISA organisms partially overcome the fitness costs by expressing heterogeneous resistance. Heterogeneous VISA/GISA (hVISA/hGISA) have a low resistance levels with MICs of 1-4 mg/L but are able to segregate sub-populations of higher resistant organisms in the presence of glycopeptides, leading to vancomycin therapy failure (Hiramatsu et al. 1997).

In enterococci high-level vancomycin resistance is seen with a MIC ≥ 32 mg/L, and is encoded by the *vanA, vanB* and *vanD* gene clusters. The first high-level vancomycin resistant *S. aureus* was recorded in 2002 from a dialysis patient in Michigan USA, who also harboured a vancomycin-resistant *Enterococcus faecalis* (VRE) with *vanA* (Chang et al. 2003). This first case of high-level vancomycin resistance had a recorded MIC of 1024 mg/L with the *vanA* operon found on a Tn*1546*-like element that was carried by a pSK41-like multi-resistance conjugative plasmid (57.9 kb) (Weigel et al. 2003). The *van* operon encodes enzymes VanH and VanA that synthesise an altered stem peptide D-alanyl-D-lactate that replaces the D-alanyl-D-alanine residue reducing the affinity for glycopeptides 1000 fold (Bugg et al. 1991). The *van* operon contains seven *van* genes, *vanR, vanS, vanH, vanA, vanX, vanY* and *vanZ*. The *vanR* and *vanS* genes encode a two-component regulatory system that activates transcription of *vanH, vanA* and *vanX*. The genes *vanH, vanA* and *vanX* are essential for vancomycin resistance, with *vanA* encoding a ligase that acts on the D-2-hydroxyacid substrate which is produced by the dehydrogenase encoded by *vanH*, while *vanX* encodes a polypeptide with unknown function. The genes *vanY* and *vanZ* are not required for resistance (Arthur et al. 1993).

1.4 Vectors / Mobile Genetic Elements

The acquisition of antibacterial resistance in *S. aureus* can be classified into two main mechanisms. The mutation of a bacterial gene on the chromosome or the acquisition of resistance-encoding genetic material, transferred from other bacteria, also known as horizontal gene transfer (Ito et al. 2003b).

Mutations in a bacterial gene can result in antibacterial resistance. This is seen with mutations in DNA gyrase (*gyr*) and/or topoisomerase IV (*grl*) genes, resulting in resistance to ciprofloxacin (Drlica and Zhao 1997).

The acquisition of resistance-encoding genetic material occurs by conjugation, transposition, transduction or transformation of mobile genetic elements such as plasmids, insertion sequences (IS), transposons or genomic islands (GIs) (Ito et al. 2003b). These mobile genetic elements are also known as vectors.

1.4.1 Genomic Islands

Genomic islands are regions of chromosomal DNA usually >10 kb, that mostly contain genes that confer pathogenicity. Genomic islands are flanked by characteristic repeat sequences and can contain integrated mobile-genetic elements such as transposons and plasmids. Some genomic islands have the ability to excise spontaneously from the chromosome due to the action of intrinsically encoded recombinase/integrase genes. In others, these genes are truncated and non-functional (Baba et al. 2002; Ito et al. 2003b).

Genomic islands that confer virulence can contain genes for leukocidins, staphylococcal superantigens and superantigen-like toxins (entertoxins and exotoxins). Genomic islands may also carry genes for antibacterial resistance. These resistance genes are found mostly on the SCC*mec* (Ito et al. 2003b).

1.4.2 Staphylococcal Cassette Chromosome *mec*

The Staphylococcal cassette chromosome *mec* (SCC*mec*), is a genomic island that can range in size between 21 kb – 67 kb depending on the SCC*mec* type (Okuma et al. 2002). It is demarcated by flanking direct and inverted repeat sequences and contains recombinase genes for incision and excision from the chromosome. The SCC*mec* is the site for the methicillin-resistance gene *mecA* which is contained in the *mec* complex region (Katayama, Ito, and Hiramatsu 2001). Other resistance determinants may be found in the J regions of the SCC*mec* (Oliveira and De Lencastre 2002).

CA-MRSA are not known to carry antibiotic-resistance genes other than the *mecA* gene on the SCC*mec*. An exception is ST100-MRSA$_{ZH47}$ which carries genes for aminoglycoside resistance, *aac(2')-aph(6")* on the integrated transposon Tn*4001*, which truncates the *mec* complex region of its SCC*mec* (Heusser et al. 2007). The SCC*mec*s of HA-MRSA, however, contain multiple resistance genes. Type II SCC*mec* contains plasmid pUB110 in the J3 region, encoding resistance to aminoglycosides and transposon Tn*554* in the J2 region encoding resistance to erythromycin (Ito, Katayama, and Hiramatsu 1999). Type III SCC*mec* contains plasmid pT181, transposon Tn*554* and pseudo Tn*554*, that encode resistance to tetracycline, erythromycin and cadmium, respectively (Ito et al. 2001). Integrated beside the type III SCC*mec* in some strains is the SCCHg. This genomic island contains a *mer* operon, transposon Tn*544*, a *ccrC* recombinase gene and confers resistance to the heavy metal Hg and erythromycin (Ito et al. 2003b).

1.4.3 Insertion Sequences

The properties of *S. aureus* insertion sequences (IS) are summarised in Table 1.6. IS are small transposable elements, being less than 2.5 kb and can be found flanking antibacterial-resistance genes within transposons as well as on their own at random sites on the bacterial chromosome. IS are themselves flanked by

inverted repeat (IR) sequences (10-40 bp) and contain only the genes needed for transposition and also a regulatory gene that stimulates or inhibits transposition activity (Lupski 1987). There are five ISs associated with *S. aureus,* IS*256,* IS*431/257,* IS*1181,* IS*1182,* IS*1272* (Firth and Skurray 1998)

ISs can exert an influence by insertion directly into the gene or by a polar effect, inactivating nearby genes. Conversely, ISs may contain promoters that activate nearby genes, as cited by Ito et al. 2003.

1.4.3.1 IS*256*

IS*256* is 1.3 kb in length, consists of a single ORF (open reading frame), that encodes a 45.6 kDa protein and is flanked by 26 bp terminal IR (Byrne, Rouch, and Skurray 1989). IS*256* is not site specific and can be found at numerous sites on the *S. aureus* chromosome and on plasmids (Dyke, Aubert, and el Solh 1992). IS*256* contains promoter sequences located at each end which act as promoters for neighbouring genes (Byrne, Rouch, and Skurray 1989). This is seen in Tn*4001,* which contains the resistance genes *aacA-aphD* which encode resistance to the aminoglycosides, gentamicin, kanamycin and tobramycin. The promoter action of IS*256* increases the level of aminoglycoside resistance (Rouch et al. 1987).

1.4.3.2 IS*431/257*

IS*431/257* is comprised of one ORF of 675 bp which has a 40% homology with IS*26* of *Proteus vulgaris* and is flanked by terminal IR of 22 bp. IS*431/257* is non-site specific and can be found in numerous sites on the *S. aureus* chromosome and on plasmids (Barberis-Maino et al. 1987). It has been shown that IS*431/257* undergoes non-resolved replicative transposition by mediating replicon fusion. This results in the cointegration of various DNA segments such as plasmids, with each junction containing directly repeated copies of IS*431/257* (Leelaporn et al. 1996). IS*431/257* plays a role in the evolution of, and accumulation of resistance genes on plasmids (Firth and Skurray 1998). In the transposon Tn*4003,* IS*431/257* has been found to have a controlling effect by acting as a promoter on the level of

transcription of *dfrA,* which results in variations of trimethoprim resistance levels (Leelaporn et al. 1994).

1.4.3.3 IS*1272*

IS*1272* is 1,934 kb in length and is comprised of 2 ORFs of 819 and 687 bp with flanking terminal IR of 16 bp and no flanking target-site duplications. A truncated IS*1272* has been found in the *mecA* complex of MRSA replacing *mecI* and a truncating *mecR1* (Archer et al. 1996). The presence of truncated IS*1272* classifies the *mec* complex as class B, which is a component of SCC*mec* types I, IV and VI (Ma et al. 2002; Oliveira, Milheirico, and de Lencastre 2006). IS*1272* is also found in *S. haemolyticus* and *S. epidermidis* in multiple copies. IS*1272* is rarely seen in MSSA with only truncated single copies found in the *mec* complex of MRSA. It has been suggested that *S. haemolyticus* is the natural host for IS*1272* and that *S. aureus* has acquired it by horizontal transfer (Archer et al. 1996; Ito et al. 2001).

1.4.3.4 IS*1181*

IS*1181* is 1,985 bp in length and is comprised of a single ORF of 1,317 bp with flanking terminal IR of 23 bp. IS*1181* is found only in *S. aureus* with up to eight copies found on both the chromosome and on plasmids (Derbise, Dyke, and El Solh 1993).

1.4.3.5 IS*1182*

IS*1182* is 1,864 bp in length and is comprised of either one ORF or two ORFs separated by a stop codon, with flanking terminal IR of 33 bp. IS*1182* has been found flanking the transposon Tn*5405* which is contained in the transposon Tn*5404* and contains genes that encode resistance to aminoglycosides. IS*1182*R of Tn*5405* contains a copy of IS*1181* flanked by 8 direct repeats (DR) (Derbise, Dyke, and el Solh 1996). IS*1182* has also been found in the *mec* complex of SCC*mec* type II subtypes. IS*1182* has been found to have inserted into *mecI* of

SCC*mec* IIA and SCC*mec* IID (IS*431mec-mecA-mecR1-*Δ*mecI*-IS*1182*) and truncated the *mecI* of SCC*mec* IIC and SCC*mec* IIE (IS*431mec-mecA-mecR1-*Δ*mecI*-IS*1182*) (Shore et al. 2005). IS*1182* has also been shown to influence the chromosomal lytic gene *lytH* of MRSA by insertional inactivation (*lytH::*IS*1182*) resulting in an increase in the methicillin resistance level (Fujimura and Murakami 2008).

Table 1.6 Insertion Sequences in *S. aureus*

Element	Size (kb)	Associated Resistance	Resistance Genes	Location
IS*256*	1.3	Gentamicin,Kanamycin, Tobramycin	*aacA-aphA*	C,P
IS*431*/IS*257*	0.8	Antiseptics,disinfectants	*smr*	P
		Bleomycin	*ble*	C,P
		Gentamicin, Kanamycin, Tobramycin	*aacA-aphA*	C,P
		Kanamycin, Neomycin, Paramycin, Tobramycin	*aadD*	C,P
		Mercury	*merA,merB*	C,P
		Mupirocin	*mupA*	P
		Tetracycline	*tetA(K)*	C,P
		Trimethoprim	*dfrA*	P
		Virginiamycin	*vgb*	P
IS*1181*	2.0	Kanamycin, Neomycin, Streptomycin	*aphA-3*	C
			aadE	C
IS*1182*	1.9	Kanamycin, Neomycin	*aphA-3*	C
		Streptomycin	*aadE*	C
IS*1272*	1.9	Methicillin	*mecA*	C

Abbreviations: C chromosome; P plasmid.

This table is according to Firth, N and Skurry, R (1998)

1.4.4 Composite Transposons

The properties of *S. aureus* composite transposons are summarised in Table 1.7. Composite transposons are mobile genetic elements varying in size from 2.5 – 23 kb that are only able to replicate by integrating into a chromosome or plasmid by transposition. Transposons are usually characterised by flanking IR and contain

genes that encode a transposase to enable site specific incision and excision of the element. Transposons may also contain insertion sequences, which are capable of independent movement by themselves (Lupski 1987). Transposons are classified according to their components and complexity (Brown and Evans 1991).

1.4.4.1 Aminoglycoside Resistance Transposons

There are three transposons described, that confer resistance to aminoglycosides in *S. aureus*, Tn*4001,* Tn*3854* and Tn*5404* (Lyon, May, and Skurray 1984; Udo and Grubb 1991; Derbise, Dyke, and el Solh 1996).

1.4.4.1.1 Tn*4001*

Transposon Tn*4001* is 4.7 kb in length, carries the *aacA-aphD* gene which encodes the 56.9-kDa bifunctional modifying enzyme AAC(6′)-APH(2″) and confers resistance to gentamicin, kanamycin and tobramycin. Tn*4001* is found on the chromosome and on plasmids and is flanked by two copies of IS*256* reading in opposite directions (Lyon, May, and Skurray 1984; Rouch et al. 1987; Byrne, Rouch, and Skurray 1989). The central region of Tn*4001*, 1918 bp in length, is comprised of two ORFs. The larger ORF, 1,437 bp in length, contains the coding region for the *aacA-aphD* genes (Rouch et al. 1987). The second ORF (ORF 132) is truncated by IS*256*(R) and does not possess signals for the initiation of translation (Byrne, Rouch, and Skurray 1989).

Transposition of Tn*4001* generates 8 bp repeats of the target sequence. Additional copies of IS*256* have been found increasing the level of resistance due to promoter action (Byrne, Rouch, and Skurray 1989). Tn*4001* may also be flanked by IS*431/257* (Byrne, Gillespie, and Skurray 1990). Tn*4001* has been found in the SCC*mec* of ST100-MRSA$_{ZH47}$ (Heusser et al. 2007). Tn*4001* is also associated with multi-resistant plasmids of the pSK1 family and conjugative plasmid family pSK41/pGO1 (Firth and Skurray 1998).

The rapid emergence of resistance to gentamicin, kanamycin and tobramycin in the early 1980s is thought to have been caused by the transposition of Tn*4001* from the chromosome to a readily disseminating plasmid (Gillespie et al. 1987).

1.4.4.1.2 Tn*3854*

The transposon Tn*3854* is 4.5 kb in length and carries the genes *aphA-3* and *aadE* which encodes the enzyme APH(3′)-III, conferring resistance to kanamycin, neomycin and streptomycin, but not gentamicin (Gray and Fitch 1983). Tn*3854* is found on plasmid pWBG628 isolated from a Nigerian *S. aureus* (Udo and Grubb 1991).

1.4.4.1.3 Tn*5404* and Tn*5405*

The transposon Tn*5404* is 16 kb in length and, like Tn*3854,* also carries the genes *aphA-3* and *aadE* conferring resistance to kanamycin, neomycin and streptomycin (Gray and Fitch 1983). Tn*5404* is comprised of a transposase and contains the insertion sequence IS*1181*. Tn*5404* is structurally related to the transposon Tn*552* with similar transposition and resolution systems, and is flanked with terminal IR of 116 bp and DR of 6 bp. The insertion of this transposon into the plasmid pIP1066 carrying β-lactamase genes generates a 3.5 kb invertible segment flanked by inversely repeated resolution sites (resR, resL). This invertible segment carries *p480* encoding a probable transposase, *p271* encoding a putative ATP-binding protein, and *binL* encoding a resolvase (Rowland and Dyke 1990; Derbise, Dyke, and el Solh 1995).

Contained within Tn*5404* is the transposon Tn*5405,* which is 12 kb in length and also carries the aminoglycoside genes *aph-3* and *aadE,* which confer resistance to kanamycin, neomycin and streptomycin. Also found is the *sat4* gene which encodes resistance to streptothricin and three ORFs *orfx, orfy* and *orfz* of unknown functions (Derbise, Aubert, and El Solh 1997). Tn*5405* is flanked by IS*1182,* with IS*1182*R truncated by IS*1181* (Derbise, Dyke, and el Solh 1996).

1.4.4.2 β-Lactamase Resistance Transposons

There are four closely related β-lactamase transposons found in *S. aureus* that carry the *bla* gene complex (*blaZ, blaR1* and *blaI*) which encodes the production and regulation of β-lactamase. These are Tn*552*, Tn*4002,* Tn*3852* and Tn*4201* which are found on the chromosome and on plasmids (Rowland and Dyke 1989; Lyon and Skurray 1987; Kigbo et al. 1985; Weber and Goering 1988).

1.4.4.2.1 Tn*552*

The transposon Tn*552* is 6.5 kb in length and is flanked by DR of 6 bp (Rowland and Dyke 1989). Tn*552* carries the gene *blaZ* which encodes β-lactamase production and the regulatory genes *blaRI* and *blaI* which encode a signal transducer and repressor (Gregory et al. 1997). Tn*552* is found on plasmids such as pI258 and pI9789 (Rowland and Dyke 1989, 1990). Tn*552* is structurally similar to Tn*5404* (Derbise, Dyke, and el Solh 1996).

1.4.4.2.2 Tn*4002*

The transposon Tn*4002* is 6.7 kb in length and is structurally similar to Tn*552* except with flanking DR of 7 bp. A *bla* gene complex is found on the transposon and it is carried by the pSK1 family of plasmids (Gillespie, Lyon, and Skurray 1988).

1.4.4.2.3 Tn*3852*

The transposon Tn*3852* is 7.3 kb in length and is closely related to Tn*552* and Tn*4002*. Tn*3852* is found on the plasmid pWBG53 (Kigbo et al. 1985; Gillespie, Lyon, and Skurray 1988).

1.4.4.2.4 Tn*4201*

The transposon is 6.7 kb in length and is also structurally similar to the other β-lactamase transposons. Tn*4201* is able to be expressed equally well in either orientation, as it can be found in the inverted orientation, and is capable of movement between plasmid and chromosome sites (Weber and Goering 1988).

1.4.4.3 Macrolide, Lincosamide and Streptogramin B Resistance Transposons

Macrolide, Lincosamide and Streptogramin B (MLS_B) resistance in *S. aureus* is encoded on the transposons Tn*551* and Tn*554* (Novick, Edelman, Schwesinger, et al. 1979; Murphy 1983; Haroche, Allignet, and El Solh 2002).

1.4.4.3.1 Tn*551*

The transposon Tn*551* is 5.2 kb in length, with terminal IR of 40 bp and is flanked by 5 bp DR. Tn*551* has 99.8% homology with Tn*917*, found in enterococci. Tn*551* consists of four ORFs including the *ermB* gene, which confers constitutive resistance to erythromycin, a resolvase and a transposase. Tn*551* is found on the chromosome and on plasmids (Novick, Edelman, Latta, et al. 1979; Lyon and Skurray 1987).

1.4.4.3.2 Tn*554*

The transposon Tn*554* is 6.7 kb in length and lacks terminal repeats. Tn*554* carries six ORFs with two containing the genes *ermA* and *spc* that confer inducible MLS_B (rRNA methylase) and spectinomycin (AAD(9) nucleotidyltransferase) resistance respectively (Murphy 1983; Lyon and Skurray 1987). Tn*554* is site specific and can be found on the chromosome and on plasmids. One of these sites in the chromosome is within the SCC*mec* of types II and III. A Tn*554*-like element known as pseudo Tn*554* (ΨTn*554*) is found in SCC*mec* type III, carrying the *cadCA* determinant which confers resistance to cadmium (Ito et al. 2001).

1.4.4.4 Other Resistance Transposons

1.4.4.4.1 Tn*4003*

The transposon Tn*4003* is 4.7 kb in length, has imperfect terminal repeats of 27-28 bp and is flanked by DR of 8 bp. Tn*4003* contains three copies of IS*431/257* and the genes *dfrA*, which encodes the SI dihydrofolate reductase (DHFR) which confers trimethoprim resistance, and *thyE* which encodes thymidylate synthetase. Tn*4003* is found on the pSK1 family of plasmids (Rouch et al. 1989) and pSK41 (Leelaporn et al. 1996). Tn*4003* is also found in SCC*mec*$_{N1}$ (Ender, Berger-Bächi, and McCallum 2007).

1.4.4.4.2 Tn*4004*

The transposon Tn*4004* is 6.4 kb in length contains seven ORFs, and is flanked by IS*431/257*. Tn*4004* contains the gene *merA*, which encodes organomercurial lyase, conferring resistance to the heavy metal mercury. Tn*4004* is found on the plasmid pI258 (Laddaga et al. 1987).

1.4.4.4.3 Tn*5801*

The transposon Tn*5801* is 28.5 kb in length with flanking DR of 11 bp. Tn*5801* contains the gene *tet*(M) which confers resistance to tetracycline (Ito et al. 2003b).

1.4.4.4.4 Tn*4291*

The transposon Tn*4291* is 7.8 kb in length. Tn*4291* is thought to carry the *mecA* gene, which encodes PBP2a/2′ conferring resistance to methicillin. Tn*4291* was found on the β-lactamase plasmid pI524 (Trees and Iandolo 1988). These findings are considered controversial as they have never been confirmed (Chambers 1997).

1.4.4.4.5 Tn*1546*

The transposon Tn*1546* originated in *E. faecalis,* is 10.8 kb in length with imperfect terminal IR of 38 bp and flanking DR of 5 bp. Tn*1546* contains seven *van* genes and two transposases. The *van* gene cluster contains the genes required for the production and regulation of resistance to vancomycin and teicoplanin (Arthur et al. 1993). Tn*1546* and Tn*1546*-like elements are found on conjugative plasmids of the pSK41/pGO1 family (Zhu et al. 2008).

1.4.4.4.6 Tn*5406*

The transposon Tn*5406* is 5.4 kb in length with terminal repeats absent. Tn*5406* has 61-73% homology with Tn*554*, carries three transposase genes, the gene *ermA,* which encodes resistance to $MLS_{B,}$ *spc,* which encodes resistance to spectinomycin and *vgaAv,* which confers resistance to streptogramin A drugs except pristinamycin. Tn*5406* is found on the chromosome, in the Tn*554* specific insertion sites, and on plasmids (Haroche, Allignet, and El Solh 2002).

Table 1.7 Antimicrobial Resistance Transposons in *S. aureus*

Element	Size (kb)	Associated Resistance	Flanking IS	Resistance Genes	Location
Tn*551*	5.2	Macrolides, Lincomides, Streptogramin B (MLS_B)	None	*ermB*	P
Tn*552,* Tn*3852,* Tn*4002,* Tn*4201*	6.5-7.3	Penicillins	None	*blaZ*	C,P
Tn*554*	6.7	Macrolides,Lincosamides, Streptogramin B (MLS_B)	None	*spc*	C
Tn*3854*	4.5	Kanamycin, Neomycin, Streptomycin		*aphA-3 aadE*	P
Tn*4001*	4.7	Gentamicin, Kanamycin, Tobramycin	IS*256* (I)	*aacA-aphD*	C,P
Tn*4003*	4.7	Trimethoprim	IS*431* (D)	*dfrA*	C,P
Tn*4004*	6.4	Mercury	IS*431* (D)	*merA, merB*	P
Tn*4291*	7.8	Methicillin		*mecA*	C
Tn*5404*	16	Kanamycin, Neomycin, Streptomycin	none	*aphA-3 aadE*	C
Tn*5405*	12	Kanamycin, Neomycin Streptomycin	IS*1182* (I)	*aphA-3 aadE*	C
Tn*5406*	5.4	Streptogramin B (MLS_B) Spectinomycin		*ermA, spc vgaAv*	C,P
Tn*5801*	28.5	Tetracycline		*tet*(M)	C
Tn*1546*	10.8	Vancomycin, Teicoplanin		*vanA*	P

Abbreviations: C, chromosome; (D), direct; (I), inverted; IS, insertion sequence; P, plasmid.

This table is based on Firth, N and Skurry, R. (1998)

1.4.5 Plasmids

Plasmids are extra-chromosomal self replicating genetic elements that frequently carry antibiotic resistant genes. Plasmids are categorised into four classes according to their size and components. Classes II and III are comprised of integrated class I plasmids, transposons and transposon remnants. Class I plasmids

appear to be resistant to transposon insertion and encode single antibiotic resistance genes. Class IV plasmids are comprised of plasmids that don't fit in the other three classes. There is a strong tendency for certain resistance markers to be associated with particular plasmid classes (Novick 1989).

1.4.5.1 Class I Plasmids

Class I plasmids are 1 – 5 kb, have a high copy number of 15 - 50 per cell, and replicate by the rolling circle mechanism (Novick 1989). Only a single antibiotic resistance is encoded on class I plasmids such as the plasmid pUB110, which encodes resistance to aminoglycosides. Class I plasmids are able to integrate into the chromosome or into large plasmids, by transposition or recombination, mediated by IS*431/257* (Werckenthin, Schwarz, and Roberts 1996; Ito et al. 2003b). Some class I plasmids can be mobilised by class III plasmids (Forbes and Schaberg 1983; Lyon and Skurray 1987). Common antibiotic resistances encoded by class I plasmids include tetracycline, neomycin, streptomycin, chloramphenicol and erythromycin (Novick 1989). Cryptic plasmids are class 1 plasmids with no known phenotype and they frequently contain only the genes for their own replication. Cryptic plasmids are less than 2 kb in size.

1.4.5.2 Class II Plasmids

Class II plasmids were the first *S. aureus* plasmids identified and characterised in the 1960s. Class II plasmids are 15 – 30 kb in size and have a low copy number of 4 - 6 per cell. They contain resistance determinants integrated on transposable elements and replicate by the theta mechanism (Novick 1989; Ito et al. 2003b). Class II plasmids can be subdivided into the β-lactamase/heavy metal resistances and pSK1 plasmid families (Firth and Skurray 1998). Plasmids often contain β-lactamase genes (*blaZ, blaR1* and *blaI*) on Tn*552*-like transposons. Examples include plasmids pMW2 and pN315 which encode β-lactamase on transposon Tn*552* (Rowland and Dyke 1990; Ito et al. 2003b). The plasmid pMU50, while not encoding β-lactamase, encodes an efflux-mediated antiseptic-resistance determinant (*qacA*) on transposon Tn*4001*. Tn*4001* also encodes aminoglycoside resistance (*aacA-aphD*) and can be found on pSK1 related plasmids (Gillespie,

May, and Skurray 1986; Gillespie et al. 1987). Resistance determinants to trimethoprim have also being found on class II plasmids (Novick 1989).

1.4.5.3 Class III Plasmids

Class III plasmids or conjugative plasmids are much larger, 40 – 60 kb, and carry determinants for conjugative transfer (*tra*), transposable-resistance determinants and a number of IS-like sequences (Novick 1989; Ito et al. 2003b). Class III plasmids are the only class of plasmids that are self transmissible by conjugative transfer and they can mobilise some class I plasmids to facilitate horizontal gene transfer (Forbes and Schaberg 1983; Lyon and Skurray 1987). Class III plasmids replicate by the theta mechanism and can be subdivided by phenotypic and restriction patterns and by conjugative proficiency (Novick 1989). Class III plasmids can be multi-resistance plasmids and commonly carry resistance determinants for aminoglycosides and trimethoprim (Novick 1989). The insertion sequence IS*431/*257 has an important role in the accumulation and integration of genes, carried by transposons and smaller plasmids, into Class III plasmids which can contain as many as seven to nine copies of IS*431/257* (Werckenthin, Schwarz, and Roberts 1996). An example of a class III plasmid is pJE1 which contains the integrated class I plasmid pUB110 (confers resistance to bleomycin), a Tn*4003*-like transposon (confers resistance to trimethoprim), Tn*4001*-hybrid transposon (confers resistance to aminoglycosides) and genes for antiseptic and disinfectant resistance (*smr*) (Evans and Dyke 1988; Firth and Skurray 1998).

1.4.5.4 Class IV Plasmids

Class IV plasmids are classified if a plasmid doesn't fit into the previous three well defined classes (Ito et al. 2003b). Class IV plasmids commonly encode resistance to fusidic acid (Novick 1989). An example of a class IV plasmid is pUB101 which confers resistance to fusidic acid, β-lactamase and cadmium (O'Brien et al. 1998).

1.5 Staphylococcal Protein A

Staphylococcal protein A was the first surface protein identified in *S. aureus* (Jensen 1958). The expression of surface proteins influences the adhesive and serum binding properties of staphylococci to human tissues causing abscesses at many anatomical sites (Flock 1999).

The staphylococcal protein A spans the bacterial cell wall and is covalently linked to the peptidoglycan layer. It is encoded by the *spa* gene which is 2,150 bp in size and its structure is shown in Figure 1.2. It is comprised of distinct regions characteristic of surface proteins –

1. NH_2-terminal signal sequence encoding a YSIRK type signal peptide that is cleaved during secretion across the cytoplasmic membrane.
2. COOH-terminal region contains a LPXTG motif (Leu-Pro-X-Thr-Gly) which precedes a hydrophobic membrane region containing 15-22 amino acids followed by 5-12 amino acid residues that form a positively charged tail. During cell wall anchoring, the LPXTG sorting signal is proteolytically cleaved between the threonine and glycine residues with an amide bond forming between threonine and the pentaglycine crossbridge before final peptidoglycan cross-linking. The staphylococcal protein A is orientated with the NH_2-terminus at the bacterial cell surface and the COOH-terminus anchoring the protein in the cell wall or membrane (Schneewind, Fowler, and Faull 1995).
3. The Fc-Immunoglobulin binding region consists of five 160 bp domains displayed on the bacterial surface that interact with IgG antibodies and cause precipitation of the immunoglobulin (Jensen 1958; Mazmanian, Ton-That, and Schneewind 2001).
4. The X region, situated between the Fc binding region and the LPXTG motif, is highly polymorphic and consists of a variable number of 24 bp repeats which have been used as a sequence-based marker in *spa* typing (Koreen et al. 2003).

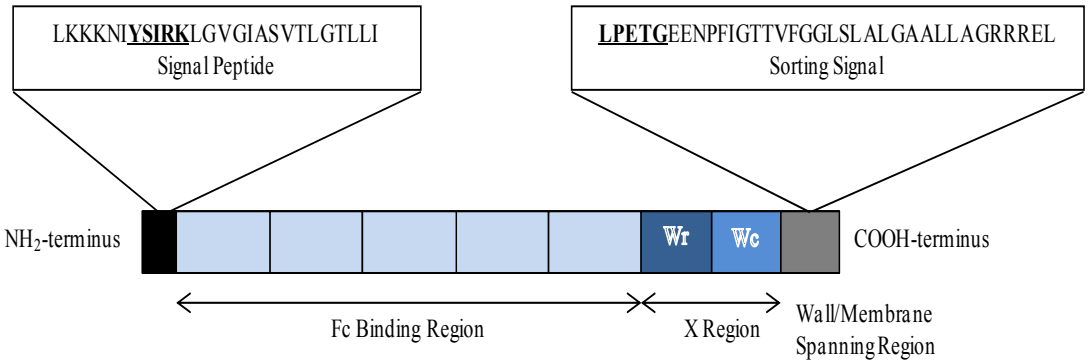

Figure 1.2 Diagrammatic Representation of the *spa* Gene
Abbreviations: Wr, repeats; Wc, non-repeats

1.5.1 *spa* Typing

Sequencing and analysis of the polymorphic X region to determine the *spa* type is a valuable typing tool to characterise *S. aureus* lineages and is complimentary to MLST and PFGE in epidemiological studies (Koreen et al. 2003).

1.6 Staphylococcal Cassette Chromosome *mec*

1.6.1 Definition

Methicillin resistance occurs with the chromosomal acquisition of a site-specific genomic resistance island known as the staphylococcal cassette chromosome *mec* or SCC*mec* (Ito, Katayama, and Hiramatsu 1999; Katayama, Ito, and Hiramatsu 2000). The *mecA* gene, which confers resistance to methicillin, was found to be located on the chromosome in 1975 (Sjöström, Löfdahl, and Philipson 1975).

Soon after, in 1978, the *mecA* gene was mapped between the *purA* gene, which encodes a protein involved in the biosynthesis of purines, and the staphylococcus protein A (*spa*) gene (Kuhl, Pattee, and Baldwin 1978). The SCC*mec* consists of the *mec* complex (Ito, Katayama, and Hiramatsu 1999), the cassette chromosome recombinase (*ccr*) genes (Katayama, Ito, and Hiramatsu 2000) and three variable regions known as the J or joining regions located at the ends of the SCC*mec* and between the *mec* complex and *ccr* genes (Ma et al. 2002; Oliveira and De Lencastre 2002). SCC*mec*(s) are typed according to the class of the *mec* complex and the type of *ccr* genes they encode. Eleven SCC*mec* types have been described, including types I, II, III (Ito et al. 2001) and type VI (Oliveira, Milheirico, and de Lencastre 2006), which are primarily associated with HA-MRSA, types IV (Ma et al. 2002) and V (Ito et al. 2004) primarily associated with CA-MRSA and, recently, types VII (Berglund et al. 2008), type VIII (Zhang et al. 2008), types IX and X (Li et al. 2011) and type XI (Garcia-Álvarez et al. 2011).

1.6.2 SCC*mec* Elements

The SCC*mec* is a large heterogeneous mobile, or once mobile, genetic element defined by the organisation of the *mec* complex and the *ccr* genes. Sub-types are defined by the genetic content of the J regions. Overtime the SCC*mec* structure is becoming more diverse between the various classes and sub-types, with elements and regions occurring in other SCC*mec* classes. The SCC*mec* structure is suggested to have been assembled outside the species of *S. aureus* and integrated into the chromosome. Environmental and adaptive pressure have given rise to the SCC*mec* allotypes that have been described to date, with sub-types and variants emerging through recombination and insertion events (Oliveira, Tomasz, and De Lencastre 2001; Milheiriço, Oliveira, and De Lencastre 2007).

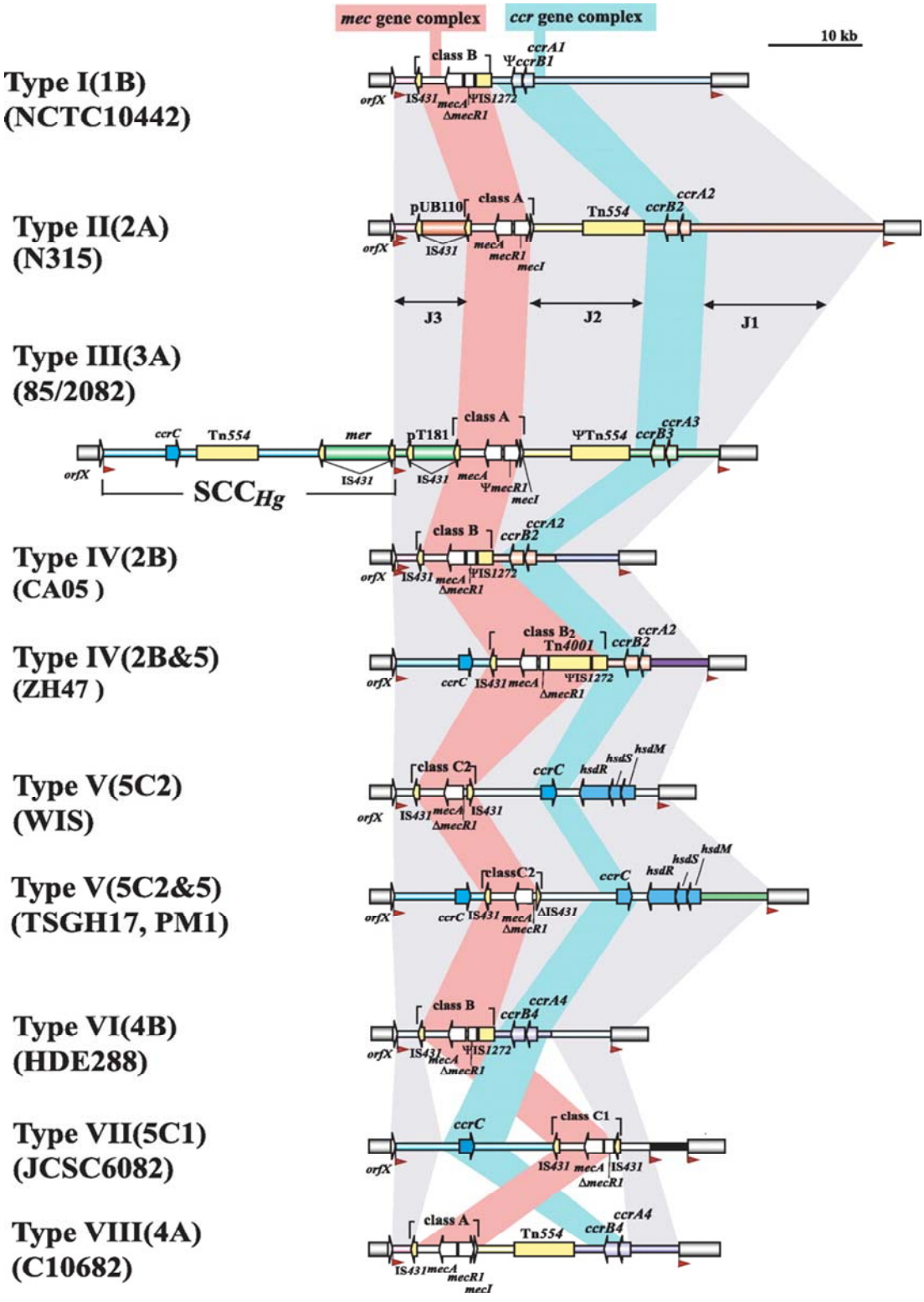

Figure 1.3 Basic Structures of SCC*mec* Types

The figure is according to the International Working Group on the Classification of Staphylococcal Cassette Chromosome elements (IWG-SCC) from www.sccmec.org.

1.6.2.1 The *mec* Complex Regions

The *mec* complex region is composed of the structural resistance gene, *mecA,* with intact or truncated sets of the regulatory genes *mecR1* and *mecI* and starts with the insertion sequence IS*431* (IS*431mec*) at its left end (Deurenberg and Stobberingh 2008; Ito, Katayama, and Hiramatsu 1999). See Figure 1.4.

1.6.2.2 The *mecA* Gene

The *mecA* gene is 2.1 kb in length and encodes the modified penicillin-binding protein 2a/2' (PBP2a/2'), which has a low affinity for β-lactams due to a distorted active site (Deurenberg and Stobberingh 2008; Song et al. 1987). This confers resistance to methicillin and most β-lactam antibiotics. *S. aureus* possess four PBPs with PBP2 responsible for peptidoglycan synthesis with transpeptidase and transglycosylase activity (Pinho, De Lencastre, and Tomasz 2001). PBP2a/2' has a molecular mass of 78 kDa and contains a transpeptidase domain and a non-penicillin-binding (n-PB) domain of unknown function (Berger-Bächi and Rohrer 2002). Resistance to methicillin and the other β-lactam antibiotics occurs because the acylation action of β-lactams on the active sites of PBP2a/2' is reduced, thus enabling the synthesis of the bacterial cell wall in the presence of β-lactam antibiotics. Methicillin/β-lactam sensitive bacteria would bind to these sites and prevent acylation, interrupting cell-wall construction, resulting in the death of the cell (Goffin and Ghuysen 1998; Lim and Strynadka 2002)

1.6.2.3 The *mecA* Regulatory Genes

The *mecA* regulatory genes *mecR1* and *mecI* are located upstream of *mecA* and are transcribed in the opposite direction to *mecA* (Tesch et al. 1990; Hiramatsu et al. 1992). The molecular organisation, structure, function and mechanism of regulation by *mecR1* and *mecI* are similar to that of the β-lactamase regulatory elements *blaR1* and *blaI* (Tesch et al. 1990).

The *mecR1* gene is 1,758 bp in length, is comprised of 585 amino acids and has 34% homology to *blaR1* in *S. aureus* (Hiramatsu et al. 1992). The *mecR1* gene is

comprised of two domains. The membrane-spanning domain, which is embedded in the plasma membrane, has zinc metalloprotease activity. The second domain is the penicillin-binding domain, which protrudes through the cell wall and contains site-specific receptors for the binding of β-lactam antimicrobials (Zhang et al. 2001). *mecR1* acts as a co-inducer of *mecA* (Hiramatsu et al. 1992).

The *mecI* gene is 372 bp in length, is comprised of 123 amino acids and has 61% homology to *blaI* in *S. aureus* (Hiramatsu et al. 1992). The MecI protein represses the production of PBP2a/2' and the transcription of *mecR1* when it binds as a homodimer to palindromic sites within the *mecA* promoter region (Zhang et al. 2001).

The MecR1 protein has a sensor-transducing function. When β-lactam antimicrobials bind to the penicillin-binding domain of MecR1, MecR1 is autocatalytically cleaved, activating the intracellular zinc metalloprotease of the membrane-spanning domain of *mecR1*. This protease cleaves the repressor MecI and enables transcription of the *mecA* gene (Zhang et al. 2001; Berger-Bächi and Rohrer 2002).

The *blaZ-blaR1-blaI* β-lactamase complex is structurally and functionally very similar to the *mecA-mecR1-mecI* PBP2a/2' complex system (Hiramatsu et al. 1992), with *blaR1* and *blaI* able to regulate *mecA,* in cases when *mecR1* and *mecI* are non-functioning (Hackbarth and Chambers 1993). The *blaR1-blaI* regulatory complex has been shown to be more efficient and faster than that of *mecR1-mecI*, with *mecR1*, although inducible by cefoxatin, less efficient and slower in the presence of methicillin and oxacillin (McKinney et al. 2001). *blaI* and *mecI* are both able to repress the transcription of *mecA* and *blaZ*, while their action together forms a more effective repression of *mecA* than when acting alone (Rosato et al. 2003).

1.6.2.4 The *mec* Complex Classes

The *mec* complex classes are illustrated in Figure 1.4. The *mec* complex is structurally diverse and the expression of *mecA* is affected by this diversity. The

regulatory genes *mecR1* and *mecI* in some classes have been found to be dysfunctional due to truncation with insertion sequences, or disabling mutations (Archer et al. 1994; Katayama, Ito, and Hiramatsu 2001). The *mec* complex is classified into classes according to these diversities (Ito et al. 2001).

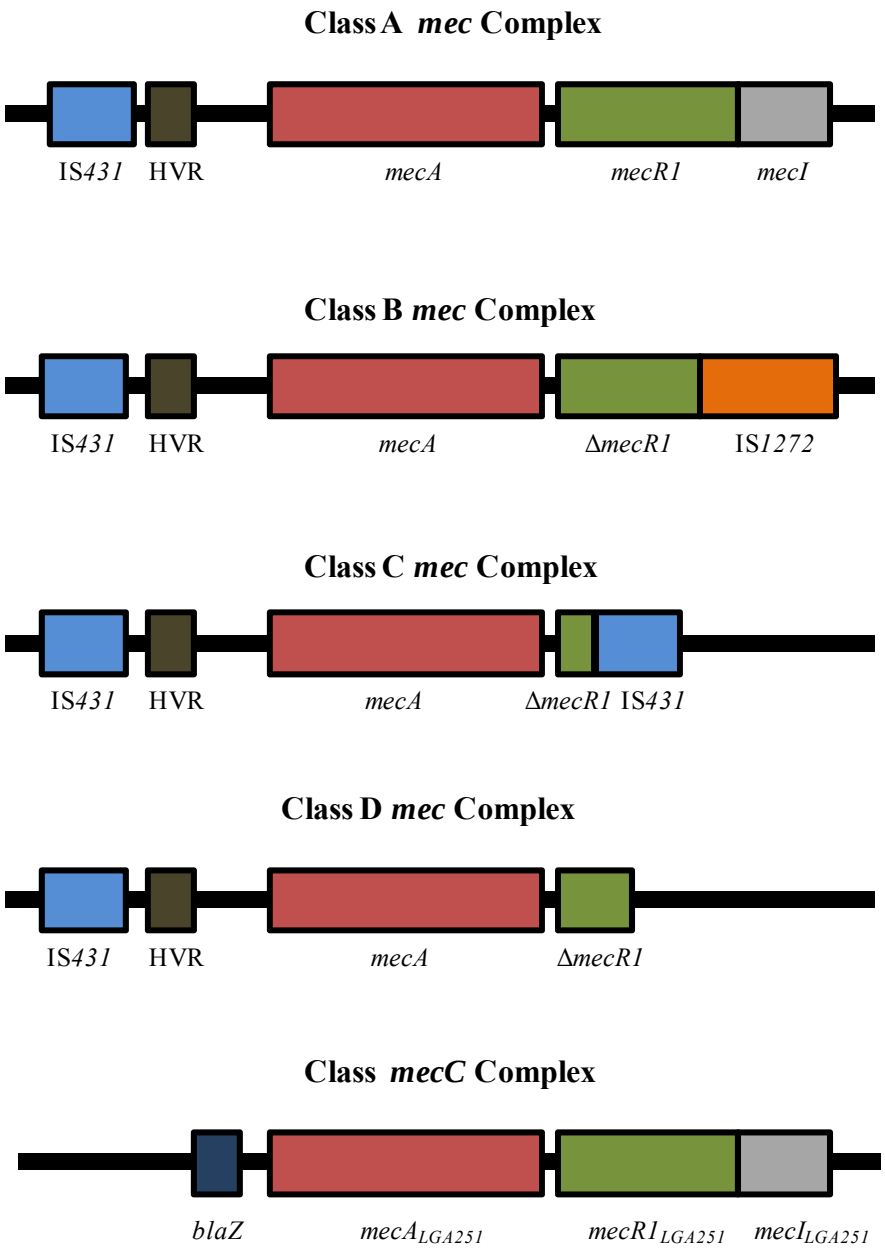

Figure 1.4 The *mec* Complex Regions

Abbreviations: HVR, Hypervariable Region

1.6.2.4.1 Class A *mec* Complex

The class A *mec* complex was first characterised in the MRSA isolate N315 and is comprised of *mecA, mecR1* and *mecI,* with *mecR1* containing both membrane-spanning and penicillin-binding domains (Ito et al. 2001).

Mutations and deletions in *mecI* have been described, with *blaI* taking its place functionally due to its high homology with *mecI* (Rosato et al. 2003).

Subtypes of class A have been described with subtype A.3 found to have *mecI* truncated by IS*1182*. Subtype A.4 has IS*1182* inserted into *mecI* (Shore et al. 2005). See Table 1.8.

Table 1.8 Descriptive Designations of Class A *mec* Complex Sub-Types

Sub-Type	Descriptive Designation
Class A	IS*431-mecA-mecR1-mecI*
Class A.3	IS*431-mecA-mecR1-*Δ*mecI*-IS*1182*
Class A.4	IS*431-mecA-mecR1-*Δ*mecI*-IS*1182-*Δ*mecI*

1.6.2.4.2 Class B *mec* Complex

Class B *mec* complex is commonly found in CA-MRSA (Okuma et al. 2002), however, it is also found in the epidemic UK EMRSA-15 clone (Oliveira and De Lencastre 2002). The class B *mec* complex is comprised of *mecA*, *mecR1* truncated by a partial copy of insertion sequence IS*1272* and *mecI* completely absent. The remaining portion of *mecR1* contains only the membrane-spanning domain (Ito et al. 2001; Katayama, Ito, and Hiramatsu 2001; Lim, Coombs, and Grubb 2002).

A Class B *mec* complex subtype, B1 has been described containing a larger truncation of IS*1272* into Δ*mecR1* (Lim et al. 2003). The class B *mec* complex subtype B2 contains *mecR1* truncated by the aminoglycoside-resistance transposon Tn*4001* (Heusser et al. 2007). The B3 *mec* complex contains *mecR1*

truncated by the integration of a 1,014 bp IS*Sep1*-like transposase (Berglund et al. 2009). See Table 1.9.

Table 1.9 Descriptive Designations of Class B *mec* Complex Sub-Types

Sub-Type	Descriptive Designation
Class B	IS*431*-*mecA*-Δ*mecR1*-IS*1272*
Class B1	IS*431*-*mecA*-Δ*mecR1*-IS*1272*
Class B2	IS*431*-*mecA*-Δ*mecR1*-Tn*4001*
Class B3	IS431-*mecA*-Δ*mecR1*-IS*Sep1*-ΔIS*1272*

1.6.2.4.3 Class C *mec* Complex

Class C2 *mec* complexes are found in CA-MRSA and consist of *mecA* with the penicillin-binding domain of *mecR1* and *mecI* truncated by IS*431*. (Ito et al. 2004). There are two subtypes of class C *mec* complex. Class C1 *mec* complex consists of Δ*mecR1* containing only the membrane-spanning domain with IS*431* transcribed in the same direction as *mecA*. Class C2 *mec* complex consists of *mecR1* with a greater truncation and contains only a small remnant of the membrane-spanning domain and IS*431* is transcribed in the opposite direction to *mecA* (Katayama, Ito, and Hiramatsu 2000; Berglund et al. 2008). See Table 1.10.

Table 1.10 Descriptive Designations of Class C *mec* Complex Sub-Types

Sub-Type	Descriptive Designation
Class C1	IS*431*-*mecA*-Δ*mecR1*-IS*431*
Class C2	IS*431*-*mecA*-Δ*mecR1*-IS*431**

Note: IS*431** transcribes in the opposite direction to *mecA*

1.6.2.4.4 Class D *mec* Complex

The class D *mec* complex consists of *mecA* and a truncated *mecR1*. The truncation occurs in the penicillin-binding domain. Neither IS*431* nor IS*1272* are present at this deletion site. This class of *mec* complex has been described in *S. caprae* (Katayama, Ito, and Hiramatsu 2001). See Table 1.11.

Table 1.11 Descriptive Designation of the Class D *mec* Complex

Sub-Type	Descriptive Designation
Class D	IS*431*-*mecA*-Δ*mecR1*

1.6.2.4.5 *mecC* Complex

The *mecC* complex, which was formally class E, consists of a *mecA* homologue, $mecA_{LGA251}$, found in the *S. aureus* strain LGA251(Garcia-Álvarez et al. 2011). See Table 1.12

Table 1.12 Descriptive Designation of the *mecC* Complex

Sub-Type	Descriptive Designation
mecC	*blaZ*-$mecA_{LGA251}$-$mecR1_{LGA251}$-$mecI_{LGA251}$

1.6.2.5 Cassette Chromosome Recombinase Genes

The *ccr* genes control the precise excision, the orientation and the integration of the SCC*mec* element. The *ccr* genes encode proteins of the invertase-resolvase family (Ito et al. 2001; Deurenberg and Stobberingh 2008). They have been classified as *ccrA, ccrB* and *ccrC*. *ccrA* and *ccrB* are usually found as a pair together with surrounding ORFs of unknown function to form the *ccrAB* gene complex. The *ccrC* gene forms a *ccr* gene complex alone with three upstream and three downstream surrounding ORFs (IWG-SCC 2009). The *ccrAB* genes

transcribe in the same direction as *mecA* while *ccrC* transcribes in the opposite direction (Katayama, Ito, and Hiramatsu 2000; Ito et al. 2001; Ito et al. 2004).

Occurrences of multiple *ccr* genes within one SCC*mec* have been reported in *S. aureus* (Boyle-Vavra et al. 2005). The occurrence of three *ccr* gene complexes has been reported in one *S. epidermidis* isolate studied (Hanssen and Sollid 2007). In these instances it is unclear as to which *ccr* gene(s) controls the SCC*mec* integration (Boyle-Vavra et al. 2005).

Diversity exists between the *ccr* genes. Four allotypes have been described for the *ccrAB* genes (Ito et al. 2001; Oliveira, Tomasz, and De Lencastre 2001) with 60-82% homology between the allotypes (Ito et al. 2004; IWG-SCC 2009). The *ccrC* gene consists of nine different alleles of ≥87% homology (Boyle-Vavra et al. 2005; Higuchi et al. 2008; IWG-SCC 2009). ST59-MRSA-V (5C2&5), commonly known as the Taiwan Clone, contains two *ccrC* gene complexes of alleles 2 and 8. The *ccrC1,* allele 8 gene complex is found downstream of the *mec* complex, while the *ccrC1,* allele 2 gene complex is found upstream. Both these gene complexes consist of the *ccrC* gene and three accompanying ORFs flanking each side. Allele 2 and allele 8 are 93% homologous with each other while the gene complexes with accompanying ORFs are 85% homologous. The gene complex of allele 8 is 6.5 kb in size and found to have ≥97% homology with the *ccrC* gene complexes in SCC*mec*$_{ZH47}$, which carries *ccrC1,* allele 9, and in SCC*mercury*, which carries *ccrC,* allele 3. The *ccrC1* gene complexes of allele 3, 8 and 9 are located downstream of the *mec* complex and have a homology of ≥97%. This gene complex with its accompanying ORFs is termed the *ccrC* carrying unit (Higuchi et al. 2008).

The *ccr* gene classes are summarised in Table 1.13 (Garcia-Álvarez et al. 2011; Ito et al. 2001; Ito et al. 2004; Oliveira et al. 2006; Li et al. 2011).

Table 1.13 *ccr* Gene Classes

***ccr* Gene Complex**	**Gene**
Class1	*ccrA1 ccrB1*
Class 2	*ccrA2 ccrB2*
Class 3	*ccrA3 ccrB3*
Class 4	*ccrA4 ccrB4*
Class 5	*ccrC1*
Class 6	*ccrA5ccrB3*
Class 7	*ccrA1ccrB6*
Class 8	*ccrA1ccrB3*

1.6.2.5.1 The Function of the *ccr* Gene Complex

The site-specific excision and integration of the SCC*mec* element is dependent on a recombinase of the invertase/resolvase family, which is encoded by the *ccrAB* or *ccrC* genes located within the SCC*mec* element. The SCC*mec* integrates into the chromosome at a site-specific location, *orfX*. The *orfX* is located near the origin of replication, has an unknown function and is well conserved among clinical strains of *S. aureus*. The integration of SCC*mec* occurs specifically at the site designated *att*BSCC with the SCC*mec* element inserting into *orfX* resulting in a larger portion of *orfX* at the 5′ end of SCC*mec* and a smaller remnant of *orfX* at the 3′ end of SCC*mec*.

The integration site is flanked by a pair of DR (DR-B and DR-SCC) 15 bp in length, and a pair of inverted terminal repeats 27 bp in length (IR-L and IR-R) (Ito, Katayama, and Hiramatsu 1999; Katayama, Ito, and Hiramatsu 2000). The inverted terminal repeats are absent in SCC*mec* type V, which carries the *ccrC* gene. The *ccrC* can only mediate integration of SCC*mec* carrying *ccrC* (Ito et al. 2004).

1.6.2.6 The J Regions

The J or joining regions of the SCC*mec* constitute the non-essential components of the SCC*mec*, but may carry additional antimicrobial-resistance determinants. The J regions are demonstrated (shaded in gray) in Figure 1.3. The J regions were previously termed junkyard regions and are comprised of three parts. The J1 region is between *ccr* and the right hand end of the SCC*mec*, the J2 region is between *mec* and *ccr* and the J3 region is between *orfX* and *mec*. The J1 region is believed to reflect the original form of SCC into which the *mec* complex was integrated (Kondo et al. 2007). Plasmids, such as pUB110 and pT181, encoding antimicrobial-resistance genes may integrate into the J3 region due to the activity of the insertion sequence IS*431*. The occurrence of two copies of IS*431* flanking integrated plasmids suggests they were accumulated by homologous recombination events (Ito et al. 2001). The presence or absence of resistance genes, polymorphisms, or variations in the J regions are used as markers to classify SCC*mec* elements into subtypes or variants (Oliveira, Tomasz, and De Lencastre 2001; Oliveira and De Lencastre 2002; Shore et al. 2005; Kondo et al. 2007).

1.6.3 The Direct Repeat Region

The direct repeat (*dru*) region is a HVR (hyper variable region) that is located downstream of *mecA* within the *mec* region between IS*431* and *mecA*, and contains direct repeat units (Ryffel et al. 1991). See Figure 1.4. These units are comprised of 40 bp repeated 2 to 11 times within 500 bp. Variations within the *dru* region result from deletion or addition of repeats and point mutations within a repeat. These variations allow for the determination of the *dru* region to be used in the typing of MRSA for epidemiological studies (Ryffel et al. 1991; Witte, Werner, and Cuny 2001).

1.6.4 Direct and Inverted Repeats

Direct repeat (DR) and inverted repeat (IR) sequences are found flanking SCC and other transposable elements such as transposons. DR sequences are in the same direction while IR are opposite to each other.

DR play a role in the replication process with one DR found at the left hand end of the integrated element and the other, found immediately outside the element at the right hand end (Recchia and Hall 1995; Stokes et al. 1997). DR sequences are site specific and are associated with an attachment site such as the attachment site *att*B of the SCC*mec* (Ito, Katayama, and Hiramatsu 1999).

IR have a role in mobility and integration of DNA elements (Recchia and Hall 1995; Stokes et al. 1997; Ito, Katayama, and Hiramatsu 1999). They can serve as primers in the replication process acting as assembly points of DNA (Mathews 1995). They are important for genetic instability by promoting DNA re-arrangement and prevent degradation of damaged DNA (Lin et al. 2001).

1.6.5 The SCC*mec* and Virulence

S. aureus can contain virulence factors that affect its ability to cause disease. The SCC*mec* is not normally associated with virulence, but is the location for the known virulence determinants encoded by the *pls* and *pms* genes (Savolainen et al. 2001; Queck et al. 2009).

1.6.5.1 The *pls* Gene

The *pls* gene encodes a surface-protein molecule Pls (plasmin sensitive), of 230 kDa. The function of the Pls protein is to reduce adhesion to host proteins and cellular invasiveness, which may allow the spread of cells, but once the protein is cleaved by receptor-bound plasminogen on the bacterial surface, this may then aid

in adhesion to tissues and structures of the host (Savolainen et al. 2001). The *pls* gene is a characteristic marker on SCC*mec* type I, although it has also been reported on SCC*mec* types II, III and IV (Werbick et al. 2007) and has recently been found encoded on *S. aureus* plasmid pWBG761 (GenBank Accession No. GQ900474). The *pls* gene is comprised of a number of regions typical of other surface proteins, beginning with a signal sequence, a constant region, three different regions of repeat sequences (R1, R2 and R3) and ending with a LPXTG cell wall attachment sequence. The size of the *pls* gene is approx. 4 kb however, it varies according to the size of the repeat regions (Savolainen et al. 2001). The *pls* surface protein covalently anchors to the cell wall peptidoglycan when sortase, the membrane-bound transpeptidase, cleaves the link between the threonine and glycine residues of the LPXTG motif (Ton-That et al. 1999). The preceding serine-aspartic acid SD repeat region (R3) acts as an arm spanning the peptidoglycan layer (Hartford et al. 1997). Pls is a member of the SD repeat containing family (SDr) of surface proteins with regions homologous to the accumulation-associated protein (Aap) of *S. epidermidis*, which is associated with biofilm formation (Savolainen et al. 2001; Hussain et al. 1997). Pls is cleaved in the presence of receptor-bound plasminogen on the *S.aureus* surface into 175 and 68 kDa segments by proteolysis (Kuusela and Saksela 1990). Because of it size, Pls produces a steric hindrance to the adherence and invasiveness of MRSA to the host, but may allow the bacteria to multiply or be of another advantage to the host cell (Hussain et al. 2009).

1.6.5.2 The *psm* Gene

A recent study has shown a previously unknown occurrence of virulence determinants within the SCC*mec*. The staphylococcal cytolytic toxins, phenol-soluble modulins (PSMs) encoded by the *psm* gene, contribute to neutrophil lysis, immune evasion and disease. A *psm-mec* gene was found encoded within the SCC*mec* rather than on the core genome suggesting for the first time a link with methicillin resistance and virulence factors. The *psm-mec* gene was found adjacent to the *mec* complex of SCC*mec* II and SCC*mec* III isolates (Queck et al. 2009).

1.6.6 SCC*mec* Types

The SCC*mec* is classified into sub-types or variants according to the content of the J regions and in some cases variations of the *mec* complexes and *ccr* gene complexes (Ito et al. 2001; Oliveira, Tomasz, and De Lencastre 2001). SCC*mec* types or allotypes are summarised in Table 1.14.

1.6.6.1 SCC*mec* Type I

Isolates containing SCC*mec* type I were found in the 1960s and 1970s and are known as the archaic clone (Ito et al. 2001). SCC*mec* type I carries a class B *mec* complex and type 1 *ccr* gene complex and is 34 kb in length. See Figure 1.3. The SCC*mec* type I carries the *pls* gene in the J1 region and the downstream constant segment (*dcs*) in the J3 region (Oliveira, Wu, and De Lencastre 2000). See Table 1.14 for sub-types of SCC*mec* type I.

Table 1.14 SCC*mec* Allotypes

Feature	SCC*mec* Allotypes										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**
Size (kb)	34	53	67	21-24	28	24	32	32	44	51	29
***mec* complex**	B	A	A	B	C2	B	C1	A	C2	C1	*mecC*
***ccr* complex**	*A1B1*	*A2B2*	*A3B3*	*A2B2*	*C*	*A4B4*	*C*	*A4B4*	*A1B1*	*A1B6*	*A1B3*
IS*431* (n)	1	2	4	1	2	1	2	1	2	2	0
Tn*554* (n)	-	1	2	-	-	-	-	1	-	-	-
pUB110	-	+	-	-	-	-	-	-	-	-	-
pT181	-	-	+	-	-	-	-	-	-	-	-
p1258	-	-	+	-	-	-	-	-	-	-	-
Other Resistance Genes	None	*ermA*	*ermA,tet*(K) *mer*	None	None	None	None	*ermA, spc*	*cadDX, arsRBC, arsDARBC*	*cadDX, arsRBC*	*arsRBC, blaZ*

Abbreviations: n, number; kb, kilobases

1.6.6.2 SCC*mec* Type II

SCC*mec* type II was first found in the strain N315 ST5-MRSA-II (2A) commonly known as New York/Japan (Ito, Katayama, and Hiramatsu 1999; Ito et al. 2001). It carries a class A *mec* complex and type 2 *ccr* gene complex, and is 53 kb in length. See Figure 1.3. The antimicrobial-resistance plasmid pUB110 is found integrated and flanked by IS*431* in the J3 region together with the downstream constant sequence *dcs*. The *kdp* operon is characteristically found in the J1 region. The *kdp* operon encodes a potassium-dependent ATPase and its regulators. The J2 region carries the transposon Tn*554* (*ermA* and *spc*) encoding resistance to erythromycin and spectinomycin (Ito et al. 2001). See Table 1.15 for sub-types of SCC*mec* type II.

1.6.6.3 SCC*mec* Type III

The SCC*mec* type III was first described in HA-MRSA strain 85/2082 and carries a class A *mec* complex and type 3 *ccr* gene complex (Ito et al. 2001). See Figure 1.3. SCC*mec* type III was originally considered to be the largest SCC*mec* type at 66 kb but is actually comprised of a SCC*mec* of 34 kb and an adjacent SCC element, SCC*mercury* (SCCHg) of 32 kb. The SCC*mec* type III carries in the J2 region, the pseudo-transposon ψTn*554* containing *cadA,* which confers cadmium resistance. It has an integrated tetracycline-resistance plasmid, pT181, flanked by two copies of IS*431* in the J3 region. The separate SCC element, SCCHg, found adjacent to the J3 region, contains the *mer* operon, encoding mercury resistance, flanked by two copies of IS*431/257*, a *ccrC* gene complex, a truncated *hsdR* gene (Δ*hsdR*) and Tn*554,* which encodes resistance to macrolide, lincosamide and streptogramin B (MLS_B). DR sequences are found at each end of the SCC*mec* element with a third copy found between the plasmid pT181 and the *mer* operon of SCCHg (Ito et al. 2001; Ito et al. 2003a). See Table 1.15 for sub-types of SCC*mec* type III.

1.6.6.4 SCC*mec* Type IV

SCC*mec* type IV was first described in CA-MRSA MW2 and carries a class B *mec* complex and a type 2 *ccr* gene complex (Ma et al. 2002). See Figure 1.3. At 21-31 kb it is the smallest SCC*mec* type. The SCC*mec* type IV is most commonly found in CA-MRSA, however it has also been described in the epidemic HA-MRSA, EMRSA-15 (Ma et al. 2002; Ito et al. 2003a). See Table 1.15 for sub-types of SCC*mec* type IV.

1.6.6.5 SCCmec Type V

The first SCC*mec* type V was described in an Australian CA-MRSA strain WIS, ST45-MRSA-V (WBG 8318) (Ito et al. 2004). SCC*mec* type V carries a class C2 *mec* complex, a type 5 or *ccrC* gene complex (5C2) and is 28.6 kb in size. See Figure 1.3. This SCC*mec* is commonly found in CA-MRSA. See Table 1.15 for sub-types of SCC*mec* type V.

1.6.6.6 SCC*mec* Type VI

SCC*mec* type VI was first detected in a MRSA from a paediatric hospital in Portugal in 1992 which became known as the "paediatric clone". It carries a class B *mec* complex and type 4 *ccr* gene complex and is 23.3 kb in length (Oliveira, Milheirico, and de Lencastre 2006). See Figure 1.3 and Table 1.15 for SCC*mec* types.

1.6.6.7 SCC*mec* Type VII

SCC*mec* type VII (26.7 kb) was first detected in strain JCSC6082 from Sweden and carries a class C1 *mec* complex characterised by IS*431* transcribing in the same direction as *mecA* (opposite to class C2). See Figure 1.3. An SCC-like cassette (CC6082) is found adjacent to the J1 region. The J3 region has 99% homology to regions of the SCCHg of SCC*mec* III strain 85/2082 but with the *mer* operon and Tn*554* absent (Berglund et al. 2008). See Table 1.15 for SCC*mec* types.

Table 1.15 SCC*mec* Types and Sub-Types

SCC*mec* Type	Sub-Type	Alternative Nomenclature	*mec* Complex Class	*ccr* Gene Complex	J1 Region	J2 Region	J3 Region	References
I (1B)	I	1B.1.1 (I.I.I)	B	*ccrA*1-*ccrB*1	*pls*		*dcs*	Ito et al. 2001
	IA	1B.1.2 (I.I.2)					pUB110 *dcs*	
	IB	1B.2.1 (I.2.1)			*pls absent* I.2 J1		pUB110 absent two IS*431* present	Han et al. 2009 Bergland et al. 2009
II (2A)	IIa	2A.1.1 (II.I.I)	A	*ccrA*2-*ccrB*2	*kdp*	Tn*554*	pUB110 *dcs*	Ito et al. 2001
	Variant	2A.1.2 (II.1.2)					pUB110 absent	Cha et al. 2005
	IIb	2A.2 (II.2)			*kdp* absent II.2 J1		*dcs*	Hisata et al. 2005
	IIA	2A.3.1 (II.3.1)	A.4 IS*1182*		*kdp* absent IVb J1		pUB110 *dcs*	Shore et al. 2005
	IIB	2A.3.2 (II.3.2)	A			Tn*554* absent		
	IIC	2A.3.3 (II.3.3)	A.3 IS*1182*			ΔTn*554* 6 orfs absent short J2		
	IID	2A.3.4 (II.3.4)	A.4 IS*1182*			Tn*554*	pUB110 absent	
	IIE	2A.3.5 (II.3.5)	A.3 IS*1182*			Tn*554* short J2		
		2A.4.1 (II.4.1)	A		*kdp* absent	Tn*554*	pUB110 *dcs*	Kwon et al.2005
		2A.5.1 (II.5.1)			I.2 J1		pUB110	Han et al. 2009
		2A.5.2 (II.5.2)				Tn6012 present Tn554 absent		
III (3A)	III	3A.1.1 (III.1.1)	A	*ccrA*3-*ccrB*3	Unique III	ψTn*554*	pT181, SCCHg-p1258(*mer* operon), Tn*554*, *ΔhsdR ccrC*	Ito et al. 2001
	IIIA	3A.1.2 (III.1.2)			III J1		pT181 and region between pT181 and *mer* absent	Oliveria & De Lencastre 2002
	IIIB	3A.1.3 (III.1.3)					pT181 absent	
	IIIC	3A.1.4 (III.1.4)						Chongtrakool et al. 2006
	novel	3A.1.5 (III.1.5)					Elements of SCCHg absent including *ccrC*, Δ*hsdR*, Tn*554*	Arakere et al. 2009
	Unclassified (Aus2)	3A.N.N (III.N.N)			Undetermined	Undetermined	*mer* operon absent	Unpublished

Table 1.15 SCC*mec* Types and Sub-Types (Continued)

SCC*mec* Type	Sub-Type	Alternative Nomenclature	*mec* Complex Class	*ccr* Gene Complex	J1 Region	J2 Region	J3 Region	References
IV (2B)	IVa	2B.1 (IV.1)	B	*ccrA2-ccrB-2*	Unique IV		*dcs*	Ma et al. 2002
	IVA	2B.N.2 (IV.N.2)			IVc J1	Undetermined	pUB110 *dcs*	Oliveria & De Lencastre 2002 Park et al. 2009
	IVA variant (Korean)	2B.3	B variant Tn*20* with HVR and *ugpQ* absent		IVc J1		pUB110	Mongkolrattanothai et al.2004 Park et al. 2009
	IVb	2B.2.1 (IV.2.1)	B		Unique IVb			Shore et al. 2005
	IVc	2B.3.1 (IV.3.1)			Unique IVc		Tn*4001* with flanking IS*256* *dcs*	
	IVc	2B.3.2 (IV.3.2)			IVc J1		*dcs*	
	IVd	2B.4 (IV.4)			Unique IVd		*dcs* absent	
	IVE	2B.3.3 (IV.3.3)			IVc J1			
	IVF	2B.2.2 (IV.2.2)			IVb J1			
	IVg	2B.5 (IV.5)			Unique IVg		*dcs*	Kwon et al. 2005
	IVh	2B.6 (IV.6)			Unique IVh			Shore et al. 2005
	IVi	2B.7 (IV.7)	B.3 IS*Sep1*-like		II.2 J1	II.2 J2 Tn*554* absent		Berglund et al 2009
	IVj	2B.8 (IV.8)	B		I.2 J1			
	ZH47	2B&5	B2 Tn*4001*	*ccrA2-ccrB2* & *ccrC*1 allele 9	IVc J1	IVc J2	SCC carrying *ccrC*	Heusser et al. 2007
V (5C)	V	5C.1 (V.1)	C2	*ccrC*1	Unique V	Unique V	Unique V	Ito et al. 2004
	V_T (Taiwan)	5C2&5 (V.2.2)		*ccrC*1 alleles 2&8	Unique V_T	ISSau-4 like Transposase	SCC carrying *ccrC*	Boyle-Vavra et al. 2005
VI (4B)	Paediatric	4B (VI)	B	*ccrA4-ccrB4*	IVa J1		*dcs*	Oliveira et al. 2006
VII (5C1)	VII	5C1 (VII)	C1	*ccrC*1	SCC-like cassette (CC6082)		Homologous to SCCHg *mer,* Tn*554* absent	Berglund et al. 2008
VIII (4A)	VIII	4A (VIII)	A	*ccrA4-ccrB4*		Tn*554*		Zhang et al. 2009

Table 1.15 SCC*mec* Types and Sub-Types (Continued)

SCC*mec* Type	Sub-Type	Alternative Nomenclature	*mec* Complex Class	*ccr* Gene Complex	J1 Region	J2 Region	J3 Region	References
IX(1C2)	XI	1C2(IX)	C2	*ccrA1-ccrB1*	*cadDX, copB, arsRBC,arsDARBC*			Li et al. 2011
X(7C1)	X	7C1(X)	C1.2	Type 7 *ccrA1-ccrB6*	*cadDX, copB, arsRBC,* IS*Sha1*		*arsRBC*	Li et al. 2011
XI(8E)	XI	(XI) SCC*mec*$_{LGA251}$	*mecC blaZ*	Type 8 *ccrA1-ccrB3*	*arsRBC*		Absent	Garcia-Álvarez et al. 2011

1.6.6.8 SCC*mec* Type VIII

SCC*mec* type VIII (32.1 kb) was first described in 2003 in MRSA from a hospital in Canada and carries a class A *mec* complex and type 4 *ccr* gene complex (Zhang et al. 2008). See Figure 1.3. The J1 region has 100% homology to a region between DR-SCC*pbp4* and IS*431* of the SCC-CI element in ATCC 12228 (Zhang et al. 2003). The J2 region contains 19 ORFs with seven belonging to Tn*554* (Zhang et al. 2008). See Table 1.15 for SCC*mec* types.

1.6.6.9 SCC*mec* Type IX

SCC*mec* type IX ((43.7 kb) was first detected in strain JCSC6943 and carries a class C2 *mec* complex and type 1 *ccr* gene complex. The J1 region contains a *cadDX* operon encoding cadmium resistance, a *copB* gene and two operons, *arsRBC* and *arsDARBC*, encoding arsenate resistance (Li et al. 2011).

1.6.6.10 SCC*mec* Type X

SCC*mec* type X (50.8 kb) was first detected in strain JCSC6945 and carries a novel class C1-like *mec* complex (C1.2) and a type 7 *ccr* gene complex consisting of *ccrA1-ccrB6* genes. The J1 region carries a *cadDX* operon, a *copB* gene, an *arsRBC* operon and a IS*Sha1* insertion sequence. The J3 region carries an *arsRBC* operon (Li et al. 2011).

1.6.6.11 SCC*mec* Type XI

SCC*mec* type XI (29.4 kb) was first detected in *S. aureus* strain LGA251 that was isolated from bulk milk, and found to be phenotypically resistant to methicillin but negative for *mecA*. Type XI was determined to carry a novel *mecA* homologue, ($mecA_{LGA251}$) *blaZ-*$mecA_{LGA251}$*-*$mecR1_{LGA251}$*-*$mecI_{LGA251,}$ with the same organisation to that found in the plasmid, pMCCL2 (Baba et al. 2009), and a SCC-like element found in *Macrococcus caseolyticus* (Tsubakishita et al. 2010; Garcia-Álvarez et al. 2011). The *mec* region of the SCC*mec* XI has been designated *mecC* by the International Working Group (IWG) for the Classification of SCC*mec* (Peterson et al. 2012). SCC*mec* XI carries a type 8 *ccr* gene complex with *ccrA1-ccrB3* genes. The J1 region carries an *arsRBC*, membrane proteins, a lipase gene fragment, ABC transport genes, and an exported protein. The J2 region carries genes that encode exported and membrane proteins, while the J3 region is absent (Garcia-Álvarez et al. 2011).

1.6.7 SCC Elements Without *mecA*

SCC elements have been described that do not carry *mecA*. These elements, like SCC*mec,* carry a *ccr* gene complex, integrate into the staphylococcal chromosome at integration site sequence (ISS) and possess flanking DR sequences containing the ISS (IWG-SCC 2009).

1.6.7.1 SCC-Composite Island (SCC-CI)

The SCC-CI (57 kb) has been described in *S. epidermidis* strain ATCC12228 and is flanked by 28 bp SCC DR sequences. The SCC-CI contains a 19 kb SCC element SCC*pbp4,* together with a 38 kb element in tandem. The SCC*pbp4* carries the *pbp4* gene that encodes cell-wall biosynthesis proteins, the *tagF* gene, which encodes a teichoic acid biosynthesis protein, a type 2 *ccr* gene complex and a phage-resistance protein. SCC*pbp4* is flanked by DR and IR sequences. Adjacent to SCC*pbp4*, the 38 kb element that completes the composite island contains a

second *ccr* gene complex (type 4), three IS*431* elements, type 1 restriction modification genes (*hsdR, hsdS*), a CadR region encoding cadmium resistance and a MerR region encoding mercury resistance. This region is also flanked by DR and IR sequences (Mongkolrattanothai et al. 2004). This 38 kb element also contains the arginine-deiminase pathway gene cluster (*arc*) and is classed as an ACME II (arginine catabolic mobile element) as the oligopeptide-permease gene cluster (*opp-3*) which is present in ACME I, is absent (An Diep et al. 2006).

1.6.7.2 SCC*cap1*

SCC*cap1* (27.4 kb) encodes 27 ORFs of which 15 are *cap1* genes making up the *cap1* operon (15.5 kb), which encodes the type 1 capsular polysaccharide. SCC*cap1* carries a *ccrB* homologue with a nonsense mutation which renders the *ccr* incapable of an excision function. SCC*cap1* has been found inserted into *orfX* at the same *att* site as SCC*mec*. A novel enterotoxin was found adjacent to the SCC*cap1* site (Luong et al. 2002).

1.6.7.3 SCC*far* or SCC_{476}

SCC*far* or SCC_{476} (22.8 kb) was first found in the strain MSSA476. It encodes 18 ORFs including the resistance determinant *far1*, which encodes resistance to fusidic acid. SCC*far* also carries a type 1 *ccr* gene complex and a type 1 restriction modification system of *hsdM, hsdS* and *hsdR* (Holden et al. 2004).

1.6.7.4 SCC_{12263}

SCC_{12263} (21.6 kb) found in *S. hominis* strain GIFU 12263 encodes 26 ORFs. SCC_{12263} carries a type 1 *ccr* gene complex and a cluster of restriction-modification enzyme gene homologues in the right end. No antibiotic resistance genes or mobile elements were found in SCC_{12263} (Katayama et al. 2003).

1.6.7.5 SCCmercury or SCCHg

SCCHg is found inserted adjacent to the J3 region of SCC*mec* type III, is flanked by DR sequences and contains the *mer* operon, which encodes resistance to mercury, a *ccrC* gene complex, a truncated restriction-modification gene (Δ*hsdR*) and Tn*554* (Ito et al. 2001). See Figure 1.3.

1.6.8 SCC-like Cassette Chromosome Elements

SCC-like elements do not carry *mecA* or a *ccr* gene complex and are reliant on neighbouring *ccr* gene complexes for their incision and excision. These elements are flanked by DR sequences and are often found at the 3' end of a SCC*mec* element (Ito et al. 2003a). It is thought that SCC-like elements are remnants of something that was previously active (Ito et al. 2003a; Berglund et al. 2008).

1.6.8.1 Arginine Catabolic Mobile Element (ACME)

The arginine catabolic mobile element (ACME) was first described in the *S. aureus* strain USA300. This mobile element is 30.9 kb in size and carries a characteristic cluster of six genes including the *arc* gene cluster, which encodes an arginine-deiminase pathway and an Opp-3 operon, which encodes a putative oligopeptide-permease system. ACME, like other SCC elements integrates into *orfX* and is flanked by characteristic DR (15 bp) and IR (26 bp). ACME does not have *ccrA/ccrB* recombinase genes and relies on adjacent recombinase genes to catalyse integration and excision. The presence of ACME possibly has a role enhancing the capacity of USA300 to grow and survive in its host (An Diep et al. 2008). ACME is classified into two allotypes with allotype I being found in USA300 and allotype II, differing by the absence of the Opp-3 operon found in *S. epidermidis* ATCC12228 (An Diep et al. 2006).

1.6.8.2 CC6082

CC6082 (5.6 kb) is found at the right extremity of the SCC*mec* adjacent to the J1 region of SCC*mec* type VII strain JCSC6082, is flanked by DR sequences, typical of SCC-like cassettes, and contains five ORFs, three encoding hypothetical proteins, a *hsdM* gene encoding a type 1 restriction-modification system DNA methylase, a membrane protein homologue gene and a gene encoding a hypothetical protein similar to a reverse transcriptase (Berglund et al. 2008).

1.6.8.3 CC_{V14}

CC_{V14} is found at the right extremity of the SCC*mec* adjacent to the J1 region of a novel SCC*mec* type III strain, V14, isolated in India. CC_{V14} is flanked by DR sequences (Arakere et al. 2009).

1.6.8.4 IE25932

IE25932 (5.8kb) was first described in the MSSA strain ATCC25932 which was isolated in 1945. The IE25932 element was found in *orfX,* at the same site utilised by SCC*mec,* and is flanked by incomplete IR and DR (15bp) sequences. IE25932 carries no drug-resistance genes or any ORFs with inferable function or *ccr* genes, suggesting it is a remnant of SCC or SCC*mec* (Ito et al. 2001). The IE25932 SCC-like element has more recently been described in the SCC*mec* subtype IVc strain 81/108 where it is found at the right extremity adjacent to the J1 region (Ito et al. 2003a).

These SCC-like elements CC6082, CC_{V14}, and IE25932 have been found at the right extremity of SCC*mec* suggesting that they previously existed in MSSA strains and that a mobile SCC*mec* element has inserted itself between *orfX* and the SCC-like element at the same *att* site.

Chapter Two

Materials and Methods

Materials

2.1 Bacterial Strains

The strains used in this study were prototypes of CA-MRSA lineages isolated in WA between the years 2003-2006. They were provided by ACCESS (formally known as GPBTRU) at PathWest laboratories, Royal Perth Hospital (RPH), Perth, WA. ACCESS Typing and Research Unit is a joint collaboration with Curtin University, School of Biomedical Sciences, Molecular Genetics Research Unit, Medical Research Foundation, RPH, Perth, WA. The strains were partially characterised and are listed in Table 2.1.

2.2 Media

The media used in this project included trypticase soy broth (TSB), Mueller-Hinton Agar (MHA), Mueller-Hinton broth (MHB), blood agar (BA), brain-heart infusion agar (BHIA) and, brain-heart infusion broth (BHIB). All media were supplied by Oxoid Ltd (Hampshire, UK) and prepared by PathWest Laboratory Medicine WA, Media (Mt Claremont, WA).

Table 2.1 Panel of WA CA-MRSA

Name	Resistance Profile	Urea	COAG PCR-RFLP	PFGE	MLST	SCC*mec*	*ccr*	*mec* com-plex	Clonal Complex
WA MRSA-1	Ery, Fus	POS	20	WA 1	ST1	IVa	2	B	1
WA MRSA-2	Ery	POS	258	8	ST255	IVa	2	B	88
WA MRSA-3	Ery	POS	36	12sub	ST5	IVa	2	B	5
WA MRSA-4	Fully S	POS	DNC	9sub	ST45	V	5	C2	45
WA MRSA-5	Tet	POS	18	1sub	ST8	IVa	2	B	8
WA MRSA-6	Tet	POS	18	WA 6	ST8	IVa	2	B	8
WA MRSA-7 Qld Clone	Fully S	POS	32	nmMRSA A	ST93	IVa	2	B	Singleton
WA MRSA-8	Fully S	POS	DNAmp	nmMRSA F	ST75	IVa	2	B	75
WA MRSA-9 Taiwan Clone	Ery, Tet	POS	40	WA 9	ST59	V_T	5	C2	59
WA MRSA-10	Trim	POS	34	WA 10	ST573	V	5	C2	1
WA MRSA-11	Gent	POS	34	26	ST5	V	5	C2	5
WA MRSA-12/ USA300	Tet	POS	18	nmMRSA D	ST8	IVc	2	B	8
WA MRSA-13	Ery	POS	32	WA 13	ST834	IVc	2	B	9
WA MRSA-14	Tet, Trim, Fus	POS	40	WA 14	ST5	V	5	C2	5
WA MRSA-15	Fully S	POS	40	WA 15	ST59	IVa	2	B	59
WA MRSA-16	Gent, Ery, Cip, Mup	NEG	18	WA 16	ST8	Novel	4	A	8
WA MRSA-17	Tet, Fus	POS	DNC	WA 17	ST583	IVc	2	B	80
WA MRSA-18	Fully S	POS	36	WA 18	ST5	Novel	NT	B	5
WA MRSA-19	Ery, Gent, Cip, Fus, Mup	NEG	18	WA 19	ST609	IVb	2	B	8
WA MRSA-20	Gent, Tet, Trim, Rif	POS	DNC	WA 20	ST612	IVd	2	B	8
WA MRSA-21	Fully S	POS	34	WA 21	ST5	Novel	NT	B	5
WA MRSA-22	Ery	POS	42	WA 22	ST577	V	5	C2	121
WA MRSA-23	Fully S	POS/ NEG	22	WA 23	ST45	IVc	2	B	45

Table 2.1 Panel of WA CA-MRSA (Cont.)

Name	Resistance Profile	Urea	COAG PCR-RFLP	PFGE	MLST	SCC*mec*	*ccr*	*mec* com-plex	Clonal Complex
WA MRSA-24	Ery	POS	40	WA 24	ST87	IVb	2	B	59
WA MRSA-25	Fully S	POS	DNC	WA 25	ST575	IVa	2	B	5
WA MRSA-26	Fully S	POS	40	WA 26	ST59	Novel	5	B	59
WA MRSA-27	Ery, Cip	POS	36	WA 27	ST5	Novel	2	C2	5
WA MRSA-28	Tet	POS	18	WA 28	ST361	Novel	4	C2	361
WA MRSA-29	Cip	POS	18	WA 29	ST672	IVa	2	B	672
WA MRSA-30	Fully S	POS	DNC	WA 30	ST728	IVc	2	B	80
WA MRSA-31	Fully S	POS	18	WA 31	ST576	IVv	2	B	8
European Clone	Tet, Fus	POS	DNC	WA 32	ST80	IVc	2	B	80
WA MRSA-33	Ery	POS	32	WA 33	ST733	IV	2	B	Singleton
WA MRSA-34	Fully S	NEG	36	WA 34	ST5	V	5	C2	5
WA MRSA-35	Ery, Tet, Cip, Fus	POS	36	WA 35	ST5	V	5	C2	5
WA MRSA-36	Ery, Cip	NEG	17	WA 36	ST8	Novel	NT	B	8
WA MRSA-37	Fully S	POS	40	WA 37	ST732	IV	2	B	Un-determined
WA MRSA-38	Gent, Ery, Cip, Trim, Rif	POS	22	WA 38	ST188	IVa	2	B	188
WA MRSA-39	Ery, Fus	POS	18	WA 39	ST526	IVv	2	B	5
WA MRSA-40	Cip	POS	36	WA 40	ST835	Novel	5&2	C2	5
WA MRSA-41	Fully S	POS	32	WA 41	ST834	IV	2	B	9
WA MRSA-42	Ery	POS	40	WA 42	ST87	Novel	NT	B	59
WA MRSA-43	Cip	POS	36	WA 43	ST835	V	5	C2	5
WA MRSA-44	Trim	POS	40	WA 44	ST72	IVa	2	B	72
WA MRSA-45	Ery, Fus, Trim, Mup	POS	DNAmp	WA 45	ST872	IVa	2	B	1

Abbreviations for Table 2.1: Cip, Ciprofloxacin; DNAmp, Did Not Amplify; DNC, Did Not Cut; Ery, Erythromycin; Fully S, Fully sensitive; Fus, Fusidic Acid; Gent, Gentamicin; NT, Not Typable; Tet, Tetracycline; Mup, Mupirocin; RFLP, restriction fragment length polymorphism; Rif, Rifampicin; Trim, Trimethoprim

The bacterial strains were stored at -20ºC in 15% glycerol in BHIB.

2.3 Antimicrobial Agents

Antimicrobial agents and concentrations are presented in Table 2.2. All antibiotic discs were supplied by Oxoid. The heavy metal discs were prepared as previously published (Townsend, Grubb, and Ashdown 1983).

Table 2.2 Antimicrobial Agents

Antimicrobial Agent	Antimicrobial Abbreviation	Concentration/Disc
Gentamicin	Gent	10µg
Erythromycin	Ery	15µg
Tetracycline	Tet	10µg
Ciprofloxacin	Cip	5µg
Fusidic Acid	Fus	10µg
Rifampicin	Rif	2µg
Mupirocin	Mup	5µg
Trimethoprim	Trim	25µg

Table 2.2 Antimicrobial Agents (Cont.)

Antimicrobial Agent	Antimicrobial Abbreviation	Concentration/Disc
Heavy Metals		
Cadmium acetate	Cd	0.1M
Phenylmercuric acetate	Pma	0.17% w/v
Mercuric chloride	Mc	0.4g
Propamidine isethionate	Pi	1.0% w/v
Sodium arsenate	Asa	0.2µM
Ethidium bromide	Eb	15µg

2.4 Buffers, Reagents and Chemicals

2.4.1 High Pure (HP) Water

Water was purified with an ELGA Labwater System (Veolia Water System Pty Ltd), with conductivity at 18.2 MΩ.

2.4.2 Injectable Water

Injectable water was supplied by Pfizer Australia in 20ml Steriamp® ampoules.

2.4.3 Lysis Solution

Lysozyme	250U
Lysostaphin	25U
EDTA	1mM
Tris pH 8	10mM
Triton X	1.2%

2.4.4 Gel Electrophoresis

Ethidium Bromide	1mg/ml

2.4.4.1 TAE Buffer (40x)

Tris	1.6M
Sodium acetate	0.8M
EDTA	40mM

pH to 7.2 with glacial acetic acid.

2.4.4.2 Tracking Dye

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Ficol	15%

2.4.5 PCR Primers

The primers generated in this study were designed using MacVector 7.2 software (Accelrys, Cambridge UK).

Table 2.3 Primers for *spa* Typing

Primer Name	Primer Sequence (5' to 3')	Gene	Reference
spaF	TAAAGACGATCCTTCGGTGAGC	*spa*	Shopsin et al. 1999
spaF2	GAACAACGTAACGGCTTCATCC		Koreen et al. 2004
spaF3	CTAAAAGATGACCCAAGCC		This Study
spaR	CAGCAGTAGTGCCGTTTGCTT		Shopsin et al. 1999

Table 2.4 Primers for Sequencing the Regions of Ciprofloxacin Resistance (*grlAB* and *gyrAB*)

Primer Name	Primer Sequence (5' to 3')	Gene	Reference
grlAF	ACTTGAAGATGTTTTAGGTGAT	*grl*	Schmitz F-J et al. 1998
grlAR	TTAGGAAATCTTGATGGCAA		
grlBF	CGATTAAAGCACAACAAGCAAG		
grlBR	CATCAGTCATAATAATTACTC		
gyrAF	AATGAAGAAGGTATGACACC	*gyr*	
gyrAR	TACGCGCTTCAGTATAACGC		
gyrBF	CAGCGTTAGATGTAGCAAGC		
gyrBR	CCGATTCCTGTACCAAATGC		

Table 2.5 Primers for Typing the SCC*mec*

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
De Lencastre Multiplex Primer Set	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	SCC*mec* I	Oliveira & de Lencastre 2002
	CIF2 R2	ATTTACCACAAGGACTACCAGA		
	KDP F1	AATCATCTGCCATTGGTGATGC	SCC*mec* II	
	KDP R1	CGAATGAAGTGAAAGAAAGTGG		
	MEC1 P2	ATCAAGACTTGCATTCAGGC	SCC*mec* II, III	
	MEC1 P3	GCGGTTTCAATTCACTTGTC		
	DCS F2	CATCCTATGATAGCTTGGTC	SCC*mec* I, II, IV	
	DCS R1	CTAAATCATAGCCATGACCG		
	RIF4 F3	GTGATTGTTCGAGATATGTGG	SCC*mec* III	
	RIF4 R9	CGCTTTATCTGTATCTATCGC		
	RIF5 F10	TTCTTAAGTACACGCTGAATCG	SCC*mec* III	
	RIF5 R13	GTCACAGTAATTCCATCAATGC		
	IS431 P4	CAGGTCTCTTCAGATCTACG	SCC*mec* IA	
	pUB110 R1	GAGCCATAAACACCAATAGCC		
	IS431 P4	CAGGTCTCTTCAGATCTACG	SCC*mec* IIIA	
	pT181 R1	GAAGAATGGGGAAAGCTTCAC		
	MECA P4	TCCAGATTACAACTTCACCAGG	*mecA*	
	MECA P7	CCACTTCATATCTTGTAACG		
mec Complex	MRA1	GTCTCCACGTTAATTCCATT	*mecR1* MS domain	Hiramatsu et al.1992
	MRA2	GTCGTTCATTAAGATATGACG		
	MRB1	AAGCACCGTTACTATCTGCACA	*mecR1* PB	
	MRB2	GAGTAAATTTTGGTCGAATGCC	*mecR1* PB domain	

Table 2.5 Primers for Typing the SCC*mec* (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
mec Complex	mA	ACCAAACCCGACAACTACAAC	*mecA*	Archer et al. 1994
	Rorf2	GGACAACTTAAGCCAGGGTA	IS*1272*	Lim et al. 2003
	IS-1	ACATTAGATATTTGGTTGCGT	IS*431/257*	Barbaris-Maino et al. 1987
	IS-2	TGAGGTTATTCAGATATTTCGATGT		
	m15	GACTGGAGTCCAAAAACCATTCG	*mecI*	Lim et al. 2003
	m16	AAGACAAGTGAATTGAAACCGCC		
ccr Gene Complex	CB	ATTGCCTTGATAATAGCCTTCT	*ccrB*	Ito et al. 2001
	cA1	AACCTATATCATCAATCAGTACGT	*ccrA1*	
	cA2	TAAAGGCATCAATGCACAAACACT	*ccrA2*	
	cA3	AGCTCAAAAGCAAGCAATAGAAT	*ccrA3*	
	C1	TGAAGAAGCACAAGAAGAGCGGC	*ccrA4*	Lim et al. 2003
	C2	CTGCACCACATTTTGGGCAC	*ccrB4*	Lim et al. 2003
	c5 F	CGTCTATTACAAGATGTTAAG	*ccrC*	Ito et al. 2004
	c5 R	CCTTTATAGACTGGATTATTC		
Zhang Multiplex Primer Set	Type I F	GCTTTAAAGAGTGTCGTTACAGG	SCC*mec* I	Zhang et al. 2005
	Type I R	GTTCTCTCATAGTATGACGTCC		
	Type II F	CGTTGAAGATGATGAAGCG	SCC*mec* II	
	Type II R	CGAAATCAATGGTTAATGGACC		
	Type III F	CCATATTGTGTACGATGCG	SCC*mec* III	
	Type III R	CCTTAGTTGTCGTAACAGATCG		
	Type IVa F	GCCTTATTCGAAGAAACCG	SCC*mec* IVa	

Table 2.5 Primers for Typing the SCC*mec* (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
Zhang Multiplex Primer Set	Type IVa R	CTACTCTTCTGAAAAGCGTCG	SCC*mec* IVa	Zhang et al. 2005
	Type IVb F	TCTGGAATTACTTCAGCTGC	SCC*mec* IVb	
	Type IVb R	AAACAATATTGCTCTCCCTC		
	Type IVc F	ACAATATTTGTATTATCGGAGAGC	SCC*mec* IVc	
	Type IVc R	TTGGTATGAGGTATTGCTGG		
	Type IVd F5	CTCAAAATACGGACCCCAATACA	SCC*mec* IVd	
	Type IVd R6	TGCTCCAGTAATTGCTAAAG		
	Type V F	GAACATTGTTACTTAAATGAGCG	SCC*mec* V	
	Type V R	TGAAAGTTGTACCCTTGACACC		
	MecA147 F	GTGAGGATATACCAAGTGATT	*mecA*	
	MecA147 R	ATGCGCTATAGATTGAAAGGAT		

Table 2.6 Primers for Sequencing the SCC*mec* of WA MRSA-40

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
SCC*mec* junction-*ccrC1* Allele 8	P127 (orf3orf51F)	CGTGTTATTTTAGAAGCGACTATG	orf3-orf5	This Study
	P133 (orf3orf52F)	TGTCTACCAAGAAGGAGAG		
	P140 (orf3orf53F)	GCGTTTATTGGGCTTTCAG		
	P134 (orf3orf52R)	CGTTTCTCACTACCATTTGGAC		
	P128 (orf3orf51R)	CGTATGTTGGACTGCTTTAGG		
	P109 (TCccrC1F)	GACAAAACACAGCAATGGC	orf5-*ccrC*	
	P129 (orf5ccrC1F)	TACAATGGTTGGATGCGGTCAGCC		

Table 2.6 Primers for Sequencing the SCC*mec* of WA MRSA-40 (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
SCC*mec* junction-*ccrC1* Allele 8	P135 (orf5ccrC2F)	CGTTAGATGCGTTTGGAGG	orf5-*ccrC*	This Study
	P141 (orf5ccrC3F)	GCTTGAAGGTCGTTTTGAC		
	P130 (orf5ccrC1R)	TTAGATTATTGCTACAGACTATCGG		
	P89 (ccrC8R)	GGTTGTAATGGCTTTGAGG	*ccrC1* Allele 8	Higuchi et al. 2008
	P10 (RIF5 R13)	GTCACAGTAATTCCATCAATGC		Oliveira & De Lencastre 2002
ccrC1 Allele8-*mecA*	P17 (Fdru1F)	AATCTGCCAAGGGACGAAG	*ccrC-dru*	This Study
	P23 (Fdru2F)	CCGCATTATGATGGCATTC		
	P24 (Fdru2R)	GATGTTATCACTGTAGCCGTTG		
	P18 (Fdru1R)	GGGAATAGTATGTGCGAGAGAC		
	P9 (dru F)	GTTAGCATATTACCTCTCCTTGC	*dru*	Larsen et al. 2008
	P11 (dru R)	GCCGATTGTGCTTGATGAG		
	P1 (drumec1F)	CTCCATAATCAATACGAATC	*dru-mecA*	This Study
	P2 (drumec1R)	GCTACAATCCCAAAATCTCAGG		
	P3 (MECA P4)	TCCAGATTACAACTTCACCAGG	*mecA*	Oliveira & De Lencastre 2002
	P7 (MECA P7)	CCACTTCATATCTTGTAACG		
mecA-ccrC1 Allele 2	P15 (mecF1F)	CAACGATTGTGACACGATAG	*mecA*-Zhang V	This Study
	P21 (mecF2F)	CCCAATCTAACTTCCACATACC		
	P80 (mecZh4R)	TCTGAAGAGACCTGCGGTTC		
	P72 (mecZh3F)	AGAACCGCAGGTCTCTTCAG		
	P68 (mecZh3R)	ACCACCATTTTTCCCTGG		

Table 2.6 Primers for Sequencing the SCC*mec* of WA MRSA-40 (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
mecA-*ccrC1*-Allele 2	P65 (mecFZh2R)	CACTCCACAGGTAGCCTTTC	*mecA*-Zhang V	This Study
	P37 (mecFZh1R)	CATAGAAAAAACACCTCCGC		
	P13 (Zhang VF)	GAACATTGTTACTTAAATGAGCG	Zhang V	Zhang et al. 2005
	P19 (ZhF1F)	GACGACAAGGATAGGTAAACC	Zhang V-*ccrC1* Allele 2	This Study
	P25 (ZhF2F)	TCATTCCTCGCTAAAGTCTG		
	P32 (ZhF3F)	GCATCCATTACCAAAGTCAG		
	P34 (ZhF4F)	TTGGGAGCAGATTAGCGAG		
	P49 (ZhF5F)	GGAAGAGAGCAACATTATCCTG		
	P60 (ZhF6F)	TTGGCACACGAACACATCGG		
	P50 (ZhF5R)	GCTGTGCTACATTTTGTGC		
	P35 (ZhF4R)	CCGTTGATTTCTGTATGCCATC		
	P33 (ZhF3F)	ACACGCTTTCTGATGTGC		
	P26 (ZhF2R)	AGTTGTTCCTGCTCCTTTTC		
	P20 (ZhF1R)	TTACCACTTCTTCGCTGAAC		
	P14 (RIF5 F10)	TTCTTAAGTACACGCTGAATCG	*ccrC1* Allele 2	Oliveira & De Lencastre 2002
ccrC1 Allele 2-*hsd*	P66 (Zh49hsd2F)	GCGTCAAGTGTGATAGAAGAACTG	*ccrC1* Allele2-*hsd*	This Study
	P78 (Zh49hsd3F)	GTGGCTGAAGATGTATTACCTG		
	P79 (Zh49hsd3R)	TCGCATAAGCACCATAGGC		
	P67 (Zh49hsd2R)	GGTATCTAAGCGTTTCTCTGAG		
	P61 (Zh49hsd1R)	GATAAACCGATGAAAGGGC		

Table 2.6 Primers for Sequencing the SCC*mec* of WA MRSA-40 (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
hsd	P54 (VO21R)	GAACCTGAACAAAACGAAACGACC	*hsdR*	This Study
	P52 (VO21F)	CCGCTTTATCTGTATCTATTGCCG		
	P70 (hsdRM2F)	GCATTCGGTAAAGCATCTC	*hsdR-hsdM*	
	P76 (hsdRM3F)	CAATCACTACGATAGGCAAGC		
	P77 (hsdRM3R)	TCTACAGGGAGTGCTAATCAAG		
	P69 (hsdRM2R)	GGACAACTCTTTTACTCAACCC	*hsdR-hsdM*	
	P64 (hsdRM1R)	TGTGTTAGGTCGTGTGTATGAG		
	P53 (VO23R)	TTGAGAGGAAGTATGGATGCTGC	*hsdM*	
	VO22F	ATCCTGCTTTTCCTCCAG	*hsdS*	
	VO22R	GCGGCATTTCTAACGATG		
	P55 (VO23F)	TACCCCCTGAACCACAACAAGG	*hsdM*	
hsd-Right hand end	P81 (hsdorfY1F)	GGTGGATGGTGTTACAAATG	*hsd-orfY*	
	P83 (hsdorfY2F)	GTCAATAAAATCTGGGTGGG		
	P87 (hsdorfY3F)	GGCTCATCTACATCCTACTATGC		
	P91 (hsdorfY4F)	CACAAATGACAGTTGGAATG		
	P94 (hsdorfY5F)	GATTACCCATTCATTCTCAC		
	P96 (hsdorfY6F)	TCAGGGTTTAGAAAGGGGGC		
	P104 (hsdorfY7F)	CGTATCACACCAGCAAAAC		
	P97 (hsdorfY6R)	GGAAGTGCCGAATAAAGG		
	P95 (hsdorfY5R)	GCTCCAGCGTCATTTTCTAC		

Table 2.6 Primers for Sequencing the SCC*mec* of WA MRSA-40 (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
hsd-Right hand end	P92 (hsdorfY4R)	ACTTCGCTTTCAGTCGTATG	hsd-*orfY*	This Study
	P88 (hsdorfY3R)	TTGTTCTTTTGAAACTTC		
	P84 (hsdorfY2R)	TTCCTGTGGATGTCCTTGG		
	P82 (hsdorfY1R)	GCCAACTCTATTCCATTTCC		
	P62 (orfY)	GCGAACACACAAGCAAAG	Taiwan Clone *orfY*	
ccrC	ccrC8F	GCATGGGTACTCAATCCA	*ccrC8*	Higuchi et al.2008
	ccrC8R	GGTTGTAATGGCTTTGAGG		
	ccrC2F	ATAAGTTAAAAGCACGACTCA	*ccrC2*	
	ccrC2R	TTCAATCCTATTTTTCTTTGTG		

Table 2.7 Primers for Sequencing ΨSCC*pls*

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
ΨSCC*pls* *orfX-hsd*	P158 (OSorfXF)	CCAGCAGCGATGTTTGTATTAG	*orfX*	This Study
	P57 (orfXF)	CCGCCAATGACGAATACAAAGTC		
	P28 (orfXR)	GTTCCAGACGAAAAAGCACCAG	*orfX-pls*	
	P71 (orfXdru1F)	GCGATGTAACGAAGATGCTAAC		
	P74 (orfXdru2F)	GTTCCATTTTCTCGCCTC		
	P137 (orfXpls1R)	CGCCTTTAGTTCCTGTATC		
	P126 (plsR)	ACAATAACATCAGGTGAGCC		
	P138 (plsHP1F)	GGTCAAAATGCCCTAAATAC	*pls*-HP	
	P144 (plsHP2F)	AAAAGCAGGCGAACCAGAGG		

Table 2.7 Primers for Sequencing ΨSCC*pls* (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
ΨSCC*pls* *orfX-hsd*	P160 (pls1F)	AGGCTCACCTGATGTTATTG	*pls*-HP	This Study
	P161 (pls2F)	GGAAGTAACGAAAGAACCAATAGAC		
	P162 (pls2R)	TCAAGCCATCTTTACCTGG		
	P148 (plsHP3R)	GAGCATCATTACCAGTATCC		
	P145 (plsHP2R)	CTCTTTAGTAGTATTATCTGGTCG		
	P139 (plsHP1R)	CCTTTAGCCACTTTCTGAGC	HP-*hsd*	
	P146 (HPF1)	GACACTCTTCTTCTACTTATGC		
	P151 (HPhsdF1)	AAGCGAAAGATACGGGTC		
	P153 (HPhsd2F)	AGCATCTTGAATCTCAGCAC		
	P156 (HPhsd3F)	CCTTCAGGAGTGTAATAATGTTCAC		
	P159 (HPhsd4F)	GTCGTCAACAGTGAAGTTTTG		
	P157 (HPhsd3R)	CGCCAAACAAAATACAAG		
	P154 (HPhsd2R)	GCTGAAGGTAAAGAGGACAATG		
	P152 (HPhsd1R)	GCCGTTCCATTAGCAGTAAC		
	P117 (orfXhsd2R)	ACATACTCATTATTATTAGG		
hsd/ SCCsorbitol	P111 (orfXhsd1R)	TAACTCAATCTGGCGGTCG		

Table 2.8 Primers for Sequencing SCCsorbitol

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
hsd-SCC*mec* junction	P28 (orfXR)	GTTCCAGACGAAAAAGCACCAG	*hsd-ccrA2B2*	This Study
	P98 (CIccr21F)	ATAGCAGGTATGACACAG		
	P102 (CIccr22F)	ATGAAAGCGAGCCCAAGAC		
	P105 (CIccr23F)	CTCTCTGATTCATCTAATAAATG		
	P107 (CIccr24F)	CATCATAGGGACAACTGGG		
	P114 (CIccr26F)	ATTAAAGCATCCCTCACCG		
	P110 (CIccr25R)	ATGTGATGCTTTTGCCAG		
	P108 (CIccr24R)	CACCAACATTTATCACGGG		
	P106 (CIccr23R)	CGACTCAAAGCATTGTATCTATCAG		
	P103 (CIccr22R)	CAAGTCAGTCAAAAACCCC		
	P99 (CIccr21R)	GGTAAATCTATGAACAGACCTGAG		
	P41 (CA2)	TAAAGGCATCAATGCACAAACACT	*ccrA2B2*	Ito et al. 2001
	P40 (CB)	ATTGCCTTGATAATAGCCTTCT		
	P112 (ccr2orf31F)	TGATAAAAGTCTCGGCACAC	*ccrA2B2*-orf3	This Study
	P115 (ccr2orf32F)	CCACTACGATTATCTTTGCG		
	P118 (ccr2orf33F)	TAGTTGTTTGGCGTCCACGGTCAG		
	P120 (ccr2orf34F)	AACCTTCAAGTGCGTTACG		
	P124 (ccr2orf35F)	AGCAATCACCTCCTTTCG		
	P131 (ccr2orf36F)	GAAAAGAGGTAAATAAAGTG		
	P142 (ccr2orf37F)	TTGTAAGTGAGAACCAAAAG		
	P149 (ccr2orf38F)	GTGATTTTCATAAGCGTG		

Table 2.8 Primers for Sequencing SCCsorbitol (Continued)

PCR Name	Primer Name	Primer Sequence (5' to 3')	Target	Reference
hsd-SCC*mec* junction	P155 (ccr2orf39F)	CATCTTTGAATGCCTCTGC	*ccrA2B2*-orf3	This Study
	P150 (ccr2orf38R)	CGGATGGGTAACTGATTTCACAAC		
	P143 (ccr2orf37R)	GAAAAAAATACCATCGCC		
	P132 (ccr2orf36R)	GATTTTGTTACAGAAAGTG		
	P125 (ccr2orf35R)	GAGCAACATTTCTTGATG		
	P121 (ccr2orf34R)	TTCTATGTCAGTAAAGGATGTC		
	P119 (ccr2orf33R)	TTCCAAAGAAGAACATCCTG		
	P116 (ccr2orf32R)	ATGACGCAGAATGTAAGAGC		
	P113 (ccr2orf31R)	TCATAATCTGGTAAGGACGG		
	P51 (TC1R)	CCTTACAACTTTTACCGTCG		

2.4.6 DNA Size Marker

The 1 kb Plus DNA ladder (Invitrogen™ Australia Pty Ltd) was used to estimate the size of PCR products. See Figure 2.1.

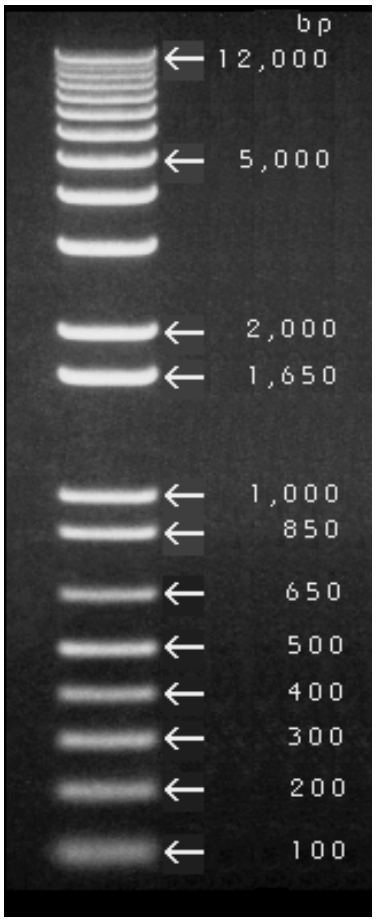

Figure 2.1 The Increments of the 1 kb Plus DNA Ladder. Figure from Invitrogen™ 1kb Plus DNA ladder catalogue documentation.

2.5 Suppliers of Reagents and Chemicals

Table 2.9 Suppliers of PCR Primers, Reagents and Chemicals

Reagents/Chemicals	Suppliers
Abbott	Abbott Australia Pty Ltd Cronulla, NSW, Australia
Ajax Chemicals	APS Chemicals Ltd Seven Hills, NSW, Australia
Applied Biosystems/Life Technologies	Applied Biosystems/Life Technologies Mulgrave Vic, Australia
BBL®	Becton, Dickinson and Company Macquarie University, Research Park, North Ryde, NSW, Australia
BDH Chemicals	Merck Pyt Ltd Kilsyth, Vic, Australia
Bio-Rad Laboratories	Bio-Rad Laboratories Reagents Park, NSW, Australia
ELGA Labwater System	Veolia Water System Pty Ltd Pyrmont, NSW, Australia
GeneWorks	GeneWorks Ltd, Thebarton, SA, Australia
GlaxoSmithKline	GlaxoSmithKline Pharmaceuticals Division Baronia, Vic, Australia
Invitrogen™ Australia	Invitrogen™ Australia Pty Ltd Mt Waverley, Vic, Australia
Kodak™	Kodak Australia Coburg, Vic, Australia
Merck	Merck Pty Ltd Kilsyth, Vic, Australia
Mo Bio Laboratories	GeneWorks Ltd Thebarton, SA, Australia
Oxoid	Oxoid Australia Pty Ltd Melbourne, Vic, Australia
Pfizer	Pfizer Australia West Ryde, NSW, Australia
Promega	Promega Corp Annandale, NSW, Australia
QBiogene	MP Biomedicals Australasia Seven Hills, NSW, Australia
Roche Diagnostics	Roche Diagnostics Australia Pty Ltd Castle Hill, NSW, Australia
Sigma Chemical Company	Sigma-Aldrich Pty Ltd Castle Hill, NSW, Australia
Ultra-Violet Products Ltd	Ultra-Violet Products Ltd Cambridge, UK

Methods

2.6 Antimicrobial Sensitivity Testing

Antimicrobial sensitivity testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2006). CLSI was formally the National Committee for Clinical Laboratory Standards (NCCLS)

2.7 Chromosomal DNA Extraction

DNA was extracted using the Invitrogen PureLink™ Genomic DNA Purification Kit, as recommended by the supplier, with the addition of lysostaphin for lysis of the *S. aureus* cell wall.

The strains were grown in 5ml TSB overnight at 35ºC. 700µl of culture was transferred into a 1.5ml Eppendorf tube and centrifuged at 13,000g for 3 min. The supernatant was discarded and the cell pellet was resuspended with 180µl lysis buffer and 20µl proteinase K. The tubes were then incubated for 30 min at 37ºC. After incubation 20µl RNAse was added. The tubes were left to stand for 2 min at room temperature. After mixing with a vortex mixer, 200µl binding buffer was added before further vortexing and incubated for 30 min at 70ºC. After incubation, 200µl ethanol was added followed by further mixing by vortexing. The contents of each tube were then transferred to an extraction column and centrifuged for 1 min at 12,000g. After centrifugation, 500µl wash buffer was added to the column which was centrifuged for 30 sec at 12,000g. This was followed by the addition of 500µl wash buffer with ethanol and a further 30 sec centrifugation at 12,000g. The flow through and tube were discarded as required and replaced with a sterile tube for elution. DNA was eluted following the addition of 200µl sterile HP water

into the column, incubation for 1 min at room temperature and then centrifugation for 1.5 min at 12,000g. The DNA extracts were stored at -20ºC for future use.

2.8 Agarose Gel Electrophoresis

Amplified DNA from PCR products were viewed following gel electrophoresis. A 1% PCR grade agarose gel made in TAE buffer was used. See Table 2.10 for electrophoresis times and voltages.

Table 2.10 Gel Electrophoresis Times and Voltages

PCR Reaction	Voltage	Time (Min)
spa	60	40
Ciprofloxacin *gyrAB/grlAB*	60	40
dru	60	40
SCC*mec*	22	960
	60	40
Long Range PCR	22	960
	80	150

The ethidium bromide (1µg/ml) was either mixed directly (5µl) with the 1% gel during preparation or the gel was stained post-electrophoresis in an ethidium bromide bath, consisting of 300µl ethidium bromide and 600ml HP water. DNA in the agarose gels was visualised using the Bio-Rad Scanner Fluor-S™ MultiImager and Bio-Rad Quantity One 4.6.3 1-D Analysis Software.

2.9 PCR Clean-Up

PCR products required a purification or clean-up process prior to DNA sequencing to remove any unwanted components of the PCR reaction such as primers, dNTPs, polymerase and buffer constituents. One of two methods was used according to the size of the PCR product. For a product size of ≤ 5 kb, the UltraClean™ PCR Clean-up Kit (MO BIO Laboratories, Inc) was used, and for a product size of > 5 kb the GENECLEAN® *Turbo* Kit (QBIOgene) was used. Both methods were performed according to the manufacturers recommendations with the final elution using injectable water according to the DNA concentration estimated from the band intensity on gel electrophoresis. If required, samples were further diluted with injectable water before sequencing to attain the required DNA concentration for PCR products of 50-10,000 bp length of approximately 0.02ng/µl/bp of sequence.

2.10 DNA Sequencing

DNA sequencing was performed by capillary electrophoresis on a 3730 (48 capillary) DNA Analyser (Applied Biosystems/Life Technologies) by the Department of Clinical Immunology and Immunogenetics, Royal Perth Hospital, Perth WA

2.11 Bioinformatic Analysis

DNA sequence analysis for relevant sections of this project was performed using the following software programmes:

AssemblyLIGN™ 1.0.9 (Oxford Molecular Group)

MacVector 7.2 (Accelrys, Cambridge UK)

BioEdit Sequence Alignment Editor 7.0.9 –

www.mbio.ncsu.edu/bioedit.bioedit.html

RidomTraceEdit - www.ridom.de

spa analysis – www.spaserver.ridom.de

dru analysis – druID from www.dru-typing.org

2.12 Staphylococcal Protein A (*spa*) Typing

Staphylococcal protein A (*spa*) region is a polymorphic region of the *S. aureus* chromosome characterised by a variable number of 24 bp repeats that are used for strain typing.

PCR for amplification of *spa* repeats was performed as previously published (Koreen et al. 2003).

Amplification was performed using an iCycler, version 1.259, 96 well 0.2 ml Reaction Module (Bio-Rad Laboratories). The cycler programme consisted of one cycle at 95ºC for 10 min; 30 cycles of 30 sec at 95ºC, 30 sec at 60ºC and 45 sec at 72ºC; final extension at 72ºC for 10 min.

PCR products were viewed following gel electrophoresis in a 1% agarose gel containing ethidium bromide. PCR products were then sequenced following PCR clean-up.

spa sequences were analysed for repeat segments, 24 bp in length, that were then each assigned a Kreiswirth ID code (Koreen et al. 2003) and a Ridom Repeat Succession ID code (Harmsen et al. 2003). The web-site www.spaserver.ridom.de was used for the designation of the *spa* type. Sequences of unknown *spa* types were submitted online at this web-site following the instructions provided.

spa sequences can be further analysed using the BioNumerics software program (version 6.5; Applied Maths, Ghent, Belgium), to generate a minimum spanning tree with *spa* types that are ≥97% similar being considered closely related and assigned to the same lineage (Benson 1997; Tenover et al. 2012).

2.13 Investigation of Ciprofloxacin Resistance

Ciprofloxacin resistance in *S. aureus* results from base pair mutation in the gyrase (*gyrA/B)* and topoisomerase (*grlA/B)* genes. The genes were amplified by PCR and sequenced to detect any mutations.

PCR for *S. aureus gyrA/B* and *grlA/B* genes amplifications were performed as previously published (Schmitz, Jones, et al. 1998).

Amplification was performed using an iCycler, version 1.259, 96 well 0.2 ml Reaction Module (Bio-Rad Laboratories). The cycler programme consisted of one cycle at 94ºC for 10 min; 25 cycles of 20 sec at 94ºC, 20 sec at 55ºC and 50 sec at 72ºC; final extension at 72ºC for 5 min.

PCR products were viewed following gel electrophoresis in a 1% agarose gel containing ethidium bromide. PCR products were then sequenced following PCR clean-up

2.14 SCC*mec* Typing

2.14.1 *mec* Complex

Classes of the *mec* complex were detected using the following primer pairs in seven separate PCR reactions: MRA1 and MRA2 (class A-membrane-spanning domain), MRB1 and MRB2 (class A-penicillin-binding domain), mA and RORF2 (class B), M15 and M16 (class A-*mecI*), mA and IS-2 (class C2) and mA and IS-1 (class C1).

PCR amplification was performed as previously published (Lim et al. 2003).

PCR amplification was performed using an iCycler (Bio-Rad Laboratories). The iCycler program consisted of one cycle of 94ºC for 5 min; 10 cycles of 94ºC for 45 sec, 65ºC for 45 sec and 72ºC for 1 min 30 sec; 25 cycles 94ºC for 45 sec, 55ºC for 45 sec and 72ºC for 1 min 30 sec; final extension at 72ºC for 10 min.

2.14.2 *ccr* Gene Complex

2.14.2.1 Multiplex PCR for *ccr* Types 1, 2 and 3

A multiplex was performed to determine *ccr* gene complexes 1-3. The final reaction volume was 25µl with 2µl chromosomal DNA used as the template. PCR amplification was performed as previously published (Lim et al. 2003).

The iCycler program (Bio-Rad Laboratories) consisted of one cycle of 94ºC for 5 min; 10 cycles of 94ºC for 45 sec, 65ºC for 45 sec and 72ºC for 1 min 30 sec; 25 cycles 94ºC for 45 sec, 55ºC for 45 sec and 72ºC for 1 min 30 sec; final extension at 72ºC for 10 min (Lim et al. 2003).

2.14.2.2 PCR for *ccr* Types 4 and 5

PCR was performed to determine *ccr* gene complexes *ccr4* and 5 (*ccrC*). The final reaction for each was 25µl using 1µl chromosomal DNA and was performed as previously published (Lim et al. 2003).

The iCycler program (Bio-Rad Laboratories) consisted of one cycle of 95ºC for 5 min; 25 cycles of 95ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min; final extension 72ºC for 5 min (Lim et al. 2003).

2.14.2.3 Multiplex PCR for *ccr* Types 1, 2, 3, 4 and 5

A multiplex PCR was also used to confirm the *ccr* gene complex. This enabled the determination of *ccr* 1-5 in one PCR reaction. The final reaction volume was 50µl with 2µl chromosomal DNA used in the template and was performed as previously published (Kondo et al. 2007).

PCR amplification was performed using an iCycler (Bio-Rad Laboratories). The iCycler program consisted of one cycle of 94ºC for 5 min; 30 cycles of 94ºC for 2 min, 57ºC for 1 min, 72ºC for 2 min; final extension 72ºC for 7 min (Kondo et al. 2007).

2.14.3 Structural Elements

2.14.3.1 SCC*mec* Typing Utilising the Method of Oliveira, D. and de Lencastre, H. (2002)

The multiplex PCR was performed using a final volume of 50µl and 2 µl DNA.

PCR amplification was performed using an iCycler (Bio-Rad Laboratories). The iCycler program consisted of one cycle of 94ºC for 4 min; 30 cycles of 94ºC for 30 sec, 53ºC for 30 sec and 72ºC for 1 min; final extension at 72ºC for 1 min

(Oliveira and De Lencastre 2002). See Table 2.11 for the location and size (bp) of loci A-H and the *mecA* control used in the multiplex PCR.

Table 2.11 Loci Locations and Amplicon Sizes for the Multiplex PCR of the SCC*mec* (Oliveira, D and de Lencastre, H. 2002)

Locus	Amplicon Size (bp)	Location	SCC*mec* Type
A	495	Downstream of *pls* gene	I
B	284	Within *kdp* operon	II
C	209	Within *mecI*	II, III
D	342	Within *dcs*	I, II, III
E	243	Between plasmid pI258 and Tn*554*	III
F	414	Between Tn*554* and *orfX*	III
G	381	Between IS*431* and pUB110	IA
H	303	Between IS*431* and pT181	IIIA
mecA	162	*mecA*	All SCC*mec* Types

2.14.3.2 SCC*mec* Typing Utilising the Method of Zhang, K. et al. (2005)

This multiplex PCR was performed to detect structural components in the J regions of the SCC*mec*. The final reaction volume of the reaction mix was 25µl with 1µl chromosomal DNA and PCR amplification was performed using an iCycler (Bio-Rad Laboratories). The iCycler program consisted of one cycle of 94ºC for 5 min; 10 cycles of 94ºC for 45 sec, 65ºC for 45 sec, 75ºC for 1 min 30 sec; 25 cycles 94ºC for 45 sec, 55ºC for 45 sec, 75ºC for 1 min 30 sec; final extension at 72ºC for 10 min (Zhang et al. 2005). See Table 2.12 for the primer names and amplicons sizes (bp) of the SCC*mec* types detected.

Table 2.12 Amplicon Sizes of the Multiplex PCR Utilising the Method of Zhang, K. et al. (2005)

SCC*mec* Type/ Primer Name	**Amplicon Size (bp)**
I	613
II	398
III	280
IVa	776
IVb	493
IVc	200
IVd	881
V	325
mecA Control	146

2.15 Long Range PCR

Long Range PCR was performed using the Expand Long Range dNTPack (Roche) according to recommendations of the manufacturer. The final reaction volume was 50µl with 4µl chromosomal DNA as the template. The reaction mix contained 1x Expand Long Range buffer with 12.5 mM $MgCl_2$, 500µM PCR Nucleotide mix (10nM each dNTP), 0.3 µM primers, 2.6% DMSO and 3.5U/µl Expand Long Range Enzyme mix. PCR amplification was performed using an iCycler (Bio-Rad Laboratories). The iCycler program consisted of one cycle of 92ºC for 2 min; 10 cycles of 92ºC for 10 sec, 52ºC for 15 sec, 68ºC for 60 sec/kb expected product size; 20 cycles of 92ºC for 10 sec, 52ºC for 15 sec, 68ºC for 60 sec/kb expected product size + 20 sec for each successive cycle; final extension of 68ºC for 7 min. PCR products were viewed by gel electrophoresis using 1%

agarose gel and stained with ethidium bromide (1mg/ml). PCR products were sequenced following PCR clean-up using Ultra Clean DNA PCR Clean Up Kit (Mo Bio Laboratories) for PCR products < 5 kb. For PCR products ≥5 kb, GeneClean PCR Clean-Up Kit (QBiogene) was used.

2.16 *dru* Typing

The *dru* region was amplified as previously published (Larsen et al. 2008) and typed according to Goering, et al (2008). The final reaction volume was 25µl with 1µl of chromosomal DNA used in the template.

PCR amplification was performed using an iCycler (Bio-Rad Laboratories). The iCycler program consisted of one cycle of 94ºC for 5 min; 10 cycles of 94ºC for 45 sec, 65ºC for 45 sec and 72ºC for 1 min 30 sec; 25 cycles 94ºC for 45 sec, 55ºC for 45 sec and 72ºC for 1 min 30 sec; final extension at 72ºC for 10 min (Larsen et al. 2008). *dru* sequences were analysed using the druID software from www.dru-typing.org.

Chapter Three

Results

3.1 Staphylococcal Protein A Typing

To determine the usefulness of staphylococcal protein A typing, or *spa* typing, as a typing tool for CA-MRSA, *spa* typing was performed on all CA-MRSA in this study. The results are presented in Table 3.1.

Table 3.1 *spa* Types of CA-MRSA in this Study

Isolate	*spa* Type	Ridom Repeat Succession ID	Kreiswirth ID
WA MRSA-1	t127	07-23-21-16-34-33-13	UJFKBPE
WA MRSA-2	t186	07-12-21-17-13-13-34-34-33-34	UGFMEEBBPB
WA MRSA-3	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-4	t123	09-02-16-34-13-16-34-16-34	A2AKBEKBKB
WA MRSA-5	t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO
WA MRSA-6	t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO
WA MRSA-7 (Qld Clone)	t202	11-17-23-17-17-16-16-25	YMJMMKKO
WA MRSA-8	NT	259-31-17-17-17-23-17-17-23-17-22	[r259]NMMMJMMJML
WA MRSA-9 (Taiwan Clone)	t437	04-20-17-20-17-25-34	ZDMDMOB
WA MRSA-10	t5073	26-23-13-21-17-34-34-34-33-02-02-34	TJEFMBBBPAAB
WA MRSA-11	t045	26-17-20-17-12-17-16	TMDMGMK
WA MRSA-12 (USA300)	t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO

Table 3.1 *spa* Types of CA-MRSA in this Study (Continued)

Isolate	*spa* Type	Ridom Repeat Succession ID	Kreiswirth ID
WA MRSA-13	t3029	15-12-17-20-17-12-17-16	WGMDMGMK
WA MRSA-14	t442	35-17-34-17-20-17-12-17-16	C3MBMDMGMK
WA MRSA-15	t976	04-20-17-20-31-16-34	ZDMDNKB
WA MRSA-16	t024	11-12-21-17-34-24-34-22-25-28	YGFMBQBLO
WA MRSA-17	t044	07-23-12-34-34-33-34	UJGBBPB
WA MRSA-18	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-19	t064	11-19-12-05-17-34-24-34-22-25	YHGCMBQBLO
WA MRSA-20	t064	11-19-12-05-17-34-24-34-22-25	YHGCMBQBLO
WA MRSA-21	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-22	t3025	14-51-12-17-23-18-17	I2P2GMJH2M
WA MRSA-23	t1575	08-16-02-16-34-17-34	XKAKBMB
WA MRSA-24	t216	04-20-17-20-17-31-16-34	ZDMDMNKB
WA MRSA-25	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-26	t976	04-20-17-20-31-16-34	ZDMDNKB
WA MRSA-27	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-28	t315	26-22-17-20-17-12-17-17-16	TLMDMGMMK
WA MRSA-29	t1309	26-22-17-20-17-12-17-17-16-16	TLMDMGMMKK
WA MRSA-30	t044	07-23-12-34-34-33-34	UJGBBPB
WA MRSA-31	t334	11-12-21-17-34-22-25	YGFMBLO
European cMRSA	t044	07-23-12-34-34-33-34	UJGBBPB
WA MRSA-33	t3029	15-12-17-20-17-12-17-16	WGMDMGMK
WA MRSA-34	t458	26	T1

Table 3.1 *spa* Types of CA-MRSA in this Study (Continued)

Isolate	*spa* Type	Ridom Repeat Succession ID	Kreiswirth ID
WA MRSA-35	t688	26-23-17-34-17-16	TJMBMK
WA MRSA-36	t190	11-17-34-24-34-22-25	YMBQBLO
WA MRSA-37	t528	4	Z1
WA MRSA-38	t189	07-23-12-21-17-34	UJGFMB
WA MRSA-39	t4065	26-23-23-17-20-17-12-17-16	TJJMDMGMK
WA MRSA-40	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-41	t3029	15-17-20-17-12-17-16	WMDMGMK
WA MRSA-42	t216	04-20-17-20-17-31-16-34	ZDMDMNKB
WA MRSA-43	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
WA MRSA-44	t791	07-23-12-21-12-17-20-17-12-17-17	UJGFGMDMGMM
WA MRSA-45	t127	07-23-21-16-34-33-13	UJFKBPE

Abbreviations: NT, not typable

spa types are also arranged according to their clonal complex to indicate the association between *spa* type and MLST/clonal complex. These results are presented in Table 3.2.

WA MRSA-8 was not typable. This isolate did not amplify by the standard or modified PCR protocols. A new forward primer (*spa* F3), was designed upstream of the existing *spa* F and the alternative *spa* F2 primer sites. *spa* F3 primer yielded a PCR product, however, the resulting sequence was not considered of high enough quality to be assigned a new spa type by the Ridom database curator. Analysis of a consensus sequence however, enabled the designation of a Ridom repeat succession ID and a Kreiswirth ID. The Ridom number for the repeat sequence, r259 was used as the start number for the Kreiswirth ID for WA MRSA-8 as no Kreiswirth nomenclature has been assigned for this repeat sequence. See Table 3.1.

Table 3.2 CA-MRSA Grouped into Clonal Complexes

Clonal Complex	Isolate	*spa* Type	Ridom Repeat Succession ID	Kreiswirth ID
CC1	WA MRSA-1	t127	07-23-21-16-34-33-13	UJFKBPE
	WA MRSA-10	t5073	26-23-13-21-17-34-34-34-33-02-02-34	TJEFMBBBPAAB
	WA MRSA-45	t127	07-23-21-16-34-33-13	UJFKBPE
CC5	WA MRSA-3	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
	WA MRSA-11	t045	26-17-20-17-12-17-16	TMDMGMK
	WA MRSA-14	t442	35-17-34-17-20-17-12-17-16	C3MBMDMGMK
	WA MRSA-18	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
	WA MRSA-21	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
	WA MRSA-25	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
	WA MRSA-27	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
	WA MRSA-34	t458	26	T1
	WA MRSA-35	t688	26-23-17-34-17-16	TJMBMK
	WA MRSA-39	t4065	26-23-23-17-20-17-12-17-16	TJJMDMGMK
	WA MRSA-40	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
	WA MRSA-43	t002	26-23-17-34-17-20-17-12-17-16	TJMBMDMGMK
CC8	WA MRSA-5	t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO
	WA MRSA-6	t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO
	WA MRSA-12 (USA300)	t008	11-19-12-21-17-34-24-34-22-25	YHGFMBQBLO
	WA MRSA-16	t024	11-12-21-17-34-24-34-22-25-28	YGFMBQBLO
	WA MRSA-19	t064	11-19-12-05-17-34-24-34-22-25	YHGCMBQBLO
	WA MRSA-20	t064	11-19-12-05-17-34-24-34-22-25	YHGCMBQBLO
	WA MRSA-31	t334	11-12-21-17-34-22-25	YGFMBLO
	WA MRSA-36	t190	11-17-34-24-34-22-25	YMBQBLO

Table 3.2 CA-MRSA Grouped into Clonal Complexes (Continued)

Clonal Complex	Isolate	*spa* Type	Ridom Repeat Succession ID	Kreiswirth ID
CC9	WA MRSA-13	t3029	15-12-17-20-17-12-17-16	WGMDMGMK
	WA MRSA-41	t3029	15-17-20-17-12-17-16	WMDMGMK
CC45	WA MRSA-4	t123	09-02-16-34-13-16-34-16-34	A2AKBEKBKB
	WA MRSA-23	t1575	08-16-02-16-34-17-34	XKAKBMB
CC59	WA MRSA-15	t976	04-20-17-20-31-16-34	ZDMDNKB
	WA MRSA-24	t216	04-20-17-20-17-31-16-34	ZDMDMNKB
	WA MRSA-26	t976	04-20-17-20-31-16-34	ZDMDNKB
	WA MRSA-42	t216	04-20-17-20-17-31-16-34	ZDMDMNKB
	WA MRSA-9 (Taiwan Clone)	t437	04-20-17-20-17-25-34	ZDMDMOB
CC72	WA MRSA-44	t791	07-23-12-21-12-17-20-17-12-17-17	UJGFGMDMGMM
CC75	WA MRSA-8	NT	259-31-17-17-17-23-17-17-23-17-22	[r259]NMMMJMMJML
CC80	WA MRSA-17	t044	07-23-12-34-34-33-34	UJGBBPB
	WA MRSA-30	t044	07-23-12-34-34-33-34	UJGBBPB
	European cMRSA	t044	07-23-12-34-34-33-34	UJGBBPB
CC88	WA MRSA-2	t186	07-12-21-17-13-13-34-34-33-34	UGFMEEBBPB
CC93	WA MRSA-7 (Qld Clone)	t202	11-17-23-17-17-16-16-25	YMJMMKKO
CC121	WA MRSA-22	t3025	14-51-12-17-23-18-17	I2P2GMJH2M
CC188	WA MRSA-38	t189	07-23-12-21-17-34	UJGFMB
CC361	WA MRSA-28	t315	26-22-17-20-17-12-17-17-16	TLMDMGMMK
CC672	WA MRSA-29	t1309	26-22-17-20-17-12-17-17-16-16	TLMDMGMMKK
Singleton	WA MRSA-33	t3029	15-12-17-20-17-12-17-16	WGMDMGMK
Undetermined	WA MRSA-37	t528	4	Z1

Abbreviations: NT, not typable

3.2 Ciprofloxacin Resistance in WA CA-MRSA

A subset of ciprofloxacin-resistant CA-MRSA isolates was analysed for chromosomal mutations within the DNA gyrase and topoisomerase IV genes. The isolates analysed were WA MRSA-16, WA MRSA-19, WA MRSA-27, WA MRSA-29, WA MRSA-35, WA MRSA-38, WA MRSA-40 and WA MRSA-43.

Previously defined regions of the ciprofloxacin-resistance associated genes, *gyrA, gyrB, grlA* and *grlB* were amplified by PCR and sequenced. The sequences were then compared to the ciprofloxacin sensitive reference strain *S. aureus* NCTC 8325, GenBank Accession No. CP000253, and analysed for single point mutations using BioEdit 7.0.9 Sequence Alignment Editor. The corresponding codon locations for the detected mutations and the resulting amino acid changes are displayed in Table 3.3.

Table 3.3 Mutations and Amino Acid Changes Within the *grlA, grlB, gyrA* and *gyrB* Genes in Eight Ciprofloxacin Resistant WA CA-MRSA

Isolate	***grlA***			***grlB***	***gyrA***	***gyrB***
	Codon 80 nt 239	**Codon 84 nt 251**	**Codon 126 nt 376**	**Codon 471 nt 1412**	**Codon 84 nt 251**	
WA MRSA-16	Ser→Phe TCC→TTC	Glu→Val GAA→GTA			Ser→Leu TCA→TTA	
WA MRSA-19	Ser→Phe TCC→TTC			Glu→Lys GAA→GGA	Ser→Leu TCA→TTA	
WA MRSA-27	Ser→Tyr TCC→TAC	Glu→Gly GAA→GGA	Leu→Leu TTA→CTA		Ser→Leu TCA→TTA	
WA MRSA-29	Ser→Phe TCC→TTC				Ser→Leu TCA→TTA	
WA MRSA-35	Ser→Phe TCC→TTC				Ser→Leu TCA→TTA	
WA MRSA-38	Ser→Phe TCC→TTC				Ser→Leu TCA→TTA	
WA MRSA-40	Ser→Phe TCC→TTC				Ser→Leu TCA→TTA	
WA MRSA-43	Ser→Phe TCC→TTC				Ser→Leu TCA→TTA	

Abbreviations (for Table 3.3): Glu, Glutamine; Gly, Glycine; Leu, Leucine; nt, nucleotide; Phe, Phenylalanine; Ser, Serine; Tyr, Tyrosine; Val, Valine
All isolates possessed mutations in the *grlA* and *gyrA* genes.

WA MRSA-16 possessed two resistance mutations in *grlA,* one at codon 80 of C→T resulting in a Ser→Phe amino acid change and one at codon 84 of A→T resulting in a Glu→Val amino acid change. In the *gyrA* gene a C→T change was detected at codon 84 resulting in an amino acid change of Ser→Leu. No mutations were detected in *grlB* or *gyrB* genes.

WA MRSA-19 possessed a C→T mutation in the *grlA* gene at codon 80 resulting in an amino acid change of Ser→Phe. The *grlB* gene showed a mutation at codon 471 of A→G resulting in an amino acid change of Glu→Lys. The *gyrA* gene showed a mutation at codon 84 of C→T resulting in an amino acid change of Ser→Leu, while no mutations were detected in the *gyrB* gene.

WA MRSA-27 possessed four mutations. The *gyrA* gene contained three mutations, with the first at codon 80 of C→T resulting in an amino acid change of Ser→Tyr. The second mutation was at codon 84 with a change of A→G resulting in an amino acid change of Glu→Gly with the third mutation at codon 126 of T→C, not resulting in an amino acid change (Leu→Leu). The *gyrA* gene showed a mutation at codon 84 of C→T resulting in an amino acid change of Ser→Leu. No mutations were detected in *grlB* or *gyrB*.

WA MRSA-29, WA MRSA-35, WA MRSA-38, WA MRSA-40 and WA MRSA-43 all showed the same combination of mutations, with a C→T mutation in the *grlA* gene at codon 80 resulting in an amino acid change of Ser→Phe and a mutation in the *gyrA* gene at codon 84 of C→T resulting in an amino acid change of Ser→Leu. No mutations were detected in *grlB* or *gyrB*.

In this study, three previously unreported amino acid changes in the *S. aureus* ciprofloxacin-resistance region were reported. The WA MRSA-19 mutation in *grlB* at codon 471, the WA MRSA-27 mutation at codon 84 of *grlA* and the silent mutation at codon 126 (Leu→Leu).

3.3 Novel SCC*mec*s

Another subset of eight isolates was selected for SCC*mec* investigation in this study because their SCC*mec*s were classed as novel following previous testing (See Table 2.1). The class of *mec* complex and type of *ccr* gene complex was determined together with relevant structural elements present in the J regions.

3.3.1 The *mec* Complex

Results are presented in Table 3.4.

WA MRSA-16, -27 and -28 were found to possess the membrane-spanning and penicillin-binding domains of *mecR1* indicating an intact *mecR1* gene. The presence of *mecI* and absence of IS*1272* resulted in the SCC*mec*s of WA MRSA-16, -27 and -28 being classified with class A *mec* complexes.

The SCC*mec*s of WA MRSA-18, -21, -26 and -42 were found to possess only the membrane-spanning domain of *mecR1,* with the penicillin-binding domain and *mecI* truncated by the insertion of IS*1272*. The *mec* complexes of these MRSA were therefore classified as class B.

The SCC*mec* of WA MRSA-40 was found to have a class C2 *mec* complex with *mecI* and the membrane-spanning domain of *mecR1* truncated by IS*431*.

Class A is positive for both the membrane-spanning and penicillin-binding domains of *mecR1* and positive for *mecI*.

Class B is positive for the membrane-spanning domain and negative for the penicillin-binding domain of *mecR1* and *mecI*. Class B is positive for IS*1272* while Class A is negative for IS*1272*.

Table 3.4 Components of the *mec* Complexes of CA-MRSA with Novel SCC*mec*s

Isolates	Components					***mec*** **Complex**
	***mecR1*(MS)**	***mecR1*(PB)**	***mecI***	**IS*1272***	**IS*431***	
WA MRSA-16	**POS**	**POS**	**POS**	neg	NoTest	A
WA MRSA-18	**POS**	neg	neg	**POS**	NoTest	B
WA MRSA-21	**POS**	neg	neg	**POS**	NoTest	B
WA MRSA-26	**POS**	neg	neg	**POS**	NoTest	B
WA MRSA-27	**POS**	**POS**	**POS**	neg	neg	A
WA MRSA-28	**POS**	**POS**	**POS**	neg	neg	A
WA MRSA-40	**POS**	neg	neg	neg	**POS**	C2
WA MRSA-42	**POS**	neg	neg	**POS**	neg	B

Abbreviations: MS, membrane-spanning domain; neg, negative; NoTest, not tested; PB, penicillin-binding domain; POS, positive.

3.3.2 The *ccr* Gene Complex

Results are presented in Table 3.5.

The SCC*mec*s of WA MRSA-16 and -28 encoded the *ccrA4B4* gene complex.

The SCC*mec*s of WA MRSA-26, -27 and -42 encoded the *ccrA2B2* gene complex.

The SCC*mec* of WA MRSA-40 encoded both a *ccrA2B2* and *ccr5* (*ccrC*).

The SCC*mec*s of WA MRSA-18 and -21 were both negative for all *ccr* PCR reactions, and were classified as not typable.

Table 3.5 Results for the *ccr* Gene Complexes

Isolates	*ccr* Gene Complex					*ccr* Result
	1	**2**	**3**	**4**	**5/C**	
WA MRSA-16	neg	neg	neg	**POS**	neg	4
WA MRSA-18	neg	neg	neg	neg	neg	Not Typable
WA MRSA-21	neg	neg	neg	neg	neg	Not Typable
WA MRSA-26	neg	**POS**	neg	neg	neg	2
WA MRSA-27	neg	**POS**	neg	neg	neg	2
WA MRSA-28	neg	neg	neg	**POS**	neg	4
WA MRSA-40	neg	**POS**	neg	neg	**WEAK POS**	2 and 5/C
WA MRSA-42	neg	**POS**	neg	neg	neg	2

3.3.3 Structural Elements

Two multiplex strategies were employed to determine the structural sub-types of the CA-MRSA.

3.3.3.1 SCC*mec* Typing Utilising the Multiplex PCR Method of Oliveira, D and de Lencastre, H (2002)

This multiplex PCR was used to differentiate structural elements within the SCC*mec*. Nine loci (A to H and *mecA*) were targeted. See Table 2.11 in Chapter 2. Structural heterogeneity of the J regions of the SCC*mec*s was demonstrated. While the reaction was useful to identify regions of sequence it was not informative as a SCC*mec* typing technique for the novel SCC*mec*s in this study.

This multiplex PCR was developed in 2002 and designed to detect SCC*mec* type's I-IV. SCC*mec* type V, however, is not detected with this method (Oliveira and De Lencastre 2002). An update of this method has now been described with improved characterisation for SCC*mec* type IV subtypes and also including the detection of SCC*mec* type V (Milheiriço, Oliveira, and De Lencastre 2007).

Results are presented in Figure 3.1 and Table 3.6. All isolates were positive for *mecA*.

The SCC*mec* of WA MRSA-16 encoded locus A indicating a region with homology with SCC*mec* type I, locus C, which indicates the presence of *mecI* and thus a class A *mec* complex, and locus D, indicating the presence of the *dcs* region.

The SCC*mec* of WA MRSA-18 was not positive for any of the loci detected by this multiplex PCR.

The SCC*mec* of WA MRSA-21 was positive for locus A, indicating a region with homology to the SCC*mec* type I J1 region.

The SCC*mec* of WA MRSA-26 was positive for locus D, indicating the presence of the *dcs* region, locus E, indicating homology for the region between pI258 and Tn*554* of the type III *SCCmec* and locus F, indicating a region with homology with the region between Tn*554* and *orfX* for SCC*mec* type III.

The SCC*mec* of WA MRSA-27 was positive for locus B, indicating the presence of the *kdp* operon specific for SCC*mec* type II, locus C, which indicated the presence of *mecI* and locus G, which indicated a region with homology to the region between IS*431* and pUB110 of SCC*mec* variant IA.

The SCC*mec* of WA MRSA-28 was positive for locus A, indicating a region with homology with SCC*mec* type I and locus C, indicating the presence of *mecI* and therefore a class A *mec* complex.

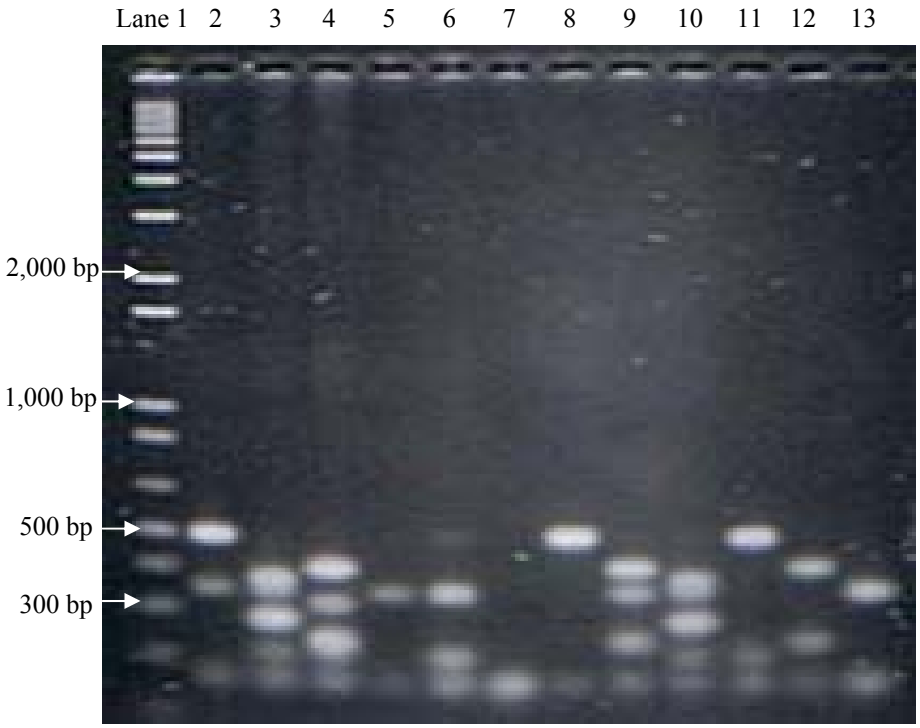

Figure 3.1 Gel of Multiplex PCR SCC*mec* Typing Utilising the Method of Oliveira, D and de Lencastre, H (2002)

Lane 1	1 Kb Plus DNA Ladder
Lane 2	Control SCC*mec* I
Lane 3	Control SCC*mec* II
Lane 4	Control SCC*mec* III
Lane 5	Control SCC*mec* IV
Lane 6	WA MRSA-16
Lane 7	WA MRSA-18
Lane 8	WA MRSA-21
Lane 9	WA MRSA-26
Lane 10	WA MRSA-27
Lane 11	WA MRSA-28
Lane 12	WA MRSA-40
Lane 13	WA MRSA-42

The SCC*mec* of WA MRSA-40 was positive for locus E, indicating a region with homology with the region between pI258 and Tn*554,* and locus F, indicating a region with similar specificity for the region between Tn*554* and *orfX*. Both loci E and F were previously considered to be specific for SCC*mec* III.

The SCC*mec* of WA MRSA-42 was found to contain locus D only, indicating the presence of the *dcs* region found in SCC*mec* types I, II and IV.

Table 3.6 SCC*mec* Typing Utilising the Multiplex PCR Method of Oliveira, D and de Lencastre, H (2002)

Isolates	**PCR Loci**									**SCC*mec* Type**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	***mecA***	
WA MRSA-16	**POS**	neg	**POS**	**POS**	neg	neg	neg	neg	**POS**	I, II
WA MRSA-18	neg	neg	neg	neg	neg	neg	neg	neg	**POS**	Not Typable
WA MRSA-21	**POS**	neg	neg	neg	neg	neg	neg	neg	**POS**	I
WA MRSA-26	neg	neg	neg	**POS**	**POS**	**POS**	neg	neg	**POS**	IV, III
WA MRSA-27	neg	**POS**	**POS**	neg	neg	neg	**POS**	neg	**POS**	I, II
WA MRSA-28	**POS**	neg	**POS**	neg	neg	neg	neg	neg	**POS**	I, II
WA MRSA-40	neg	neg	neg	neg	**POS**	**POS**	neg	neg	**POS**	III
WA MRSA-42	neg	neg	neg	**POS**	neg	neg	neg	neg	**POS**	I, II, IV

3.3.3.2 SCC*mec* Typing Utilising the Multiplex PCR Method of Zhang, K et al. (2005)

This PCR Multiplex detects structural elements of SCC*mec* types I to V and allows for the differentiation of SCC*mec* IV subtypes a-d (Zhang et al. 2005). Further sub-typing of SCC*mec* type IV has been described with subtypes IVg and

IVh now classified (Berglund et al. 2008). Following primer analysis during this study it was determined that the sub-type IVc primers published for this reaction were not amplifying any region within the subtype IVc (Accession No. AB096217) but actually an element adjacent to the SCC*mec* (IE25923).

Differentiation of SCC*mec* types by this method is determined by amplicon size. Results are presented in Figure 3.2 and Table 3.7.

The SCC*mec* of WA MRSA-16 encoded structural elements for SCC*mec* types IVa and V, with bands of 776 and 325 bp respectively.

The SCC*mec*s of WA MRSA-18, -26, -28 and -42 encoded structural elements for SCC*mec* type IVa, with a single band of 776 bp.

The SCC*mec* of WA MRSA 21 encoded structural elements for SCC*mec* types IVa and III with bands of 776 and 280 bp respectively.

The SCC*mec* of WA MRSA-27 encoded structural elements for SCC*mec* type II, with a band of 398 bp.

The SCC*mec* of WA MRSA-40 amplified a band of 1600 bp, which didn't correspond to the guideline of this multiplex. Sequencing of the product revealed the presence of an IS*Sau4*-like element inserted into the SCC*mec* type V specific ORF (V011) (GenBank Accession No. AB121219) (results not shown).

The multiplex PCR utilising the method of Zhang, K et al, (2005) also gave anomalous typing results, probably due to the heterogeneity of the J regions. Eventually the SCC*mec*s were typed according to the class of *mec* complex and type of *ccr* complex.

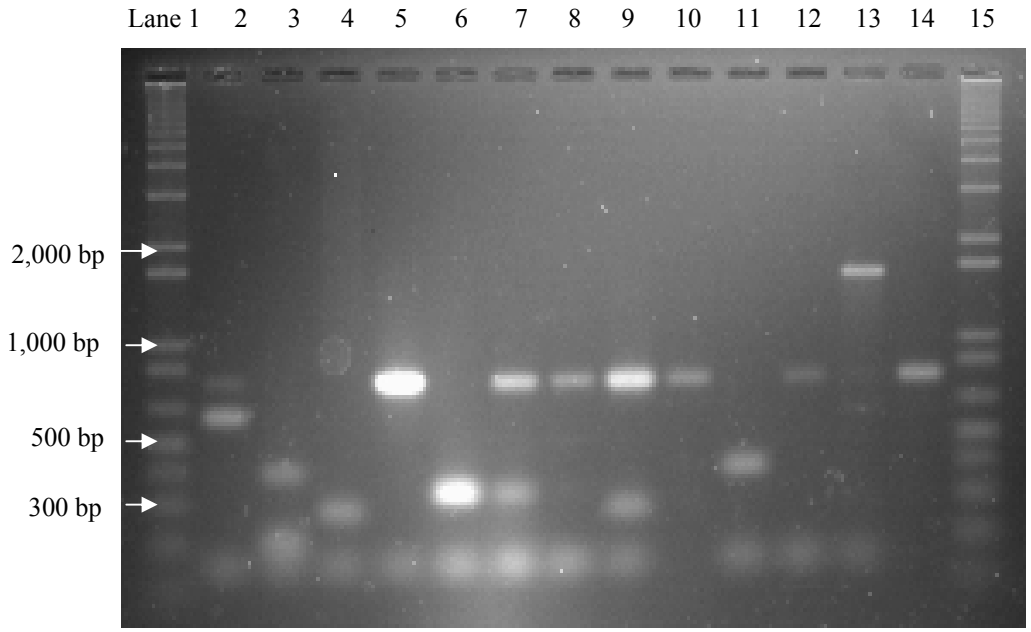

Figure 3.2 Gel of Multiplex PCR of the J Regions for SCC*mec* Typing (Zhang, K et al. 2005)

Lane 1 1 Kb Plus DNA Ladder
Lane 2 Control SCC*mec* type I (613 bp)
Lane 3 Control SCC*mec* type II (398 bp)
Lane 4 Control SCC*mec* type III (280 bp)
Lane 5 Control SCC*mec* type IVa (776 bp)
Lane 6 Control SCC*mec* type V (325 bp)
Lane 7 WA MRSA-16
Lane 8 WA MRSA-18
Lane 9 WA MRSA-21
Lane 10 WA MRSA-26
Lane 11 WA MRSA-27
Lane 12 WA MRSA-28
Lane 13 WA MRSA-40 (1600 bp)
Lane 14 WA MRSA-42
Lane 15 1 Kb Plus DNA Ladder

Table 3.7 Multiplex PCR of the J Regions for SCC*mec* Typing (Zhang, K et al. 2005)

Isolates	PCR Loci								Preliminary SCC*mec* Results
	I (613bp)	**II (398bp)**	**III (280bp)**	**IVa (776bp)**	**IVb (493bp)**	**IVc (200bp)**	**IVd (881bp)**	**V (325bp)**	
WA MRSA-16	neg	neg	neg	**POS**	neg	neg	neg	**POS**	IVa, V
WA MRSA-18	neg	neg	neg	**POS**	neg	neg	neg	neg	IVa
WA MRSA-21	neg	neg	**POS**	**POS**	neg	neg	neg	neg	III, IVa
WA MRSA-26	neg	neg	neg	**POS**	neg	neg	neg	neg	IVa
WA MRSA-27	neg	**POS**	neg	neg	neg	neg	neg	neg	II
WA MRSA-28	neg	neg	neg	**POS**	neg	neg	neg	neg	IVa
WA MRSA-40	neg	neg	neg	neg	neg	neg	neg	**POS (1600bp)**	V variant
WA MRSA-42	neg	neg	neg	**POS**	neg	neg	neg	neg	IVa

The SCC*mec* results of the eight CA-MRSA with novel SCC*mec*s are summarised in Table 3.8

Table 3.8 A Summary of the SCC*mec* Results of the Eight CA-MRSA with Previously Novel SCC*mec*s

Isolate	*mec* Complex	*ccr* Gene Complex	SCC*mec* Type
WA MRSA-16	A	4	VIII
WA MRSA-18	B	Not Typable	Novel
WA MRSA-21	B	Not Typable	Novel
WA MRSA-26	B	2	IV variant
WA MRSA-27	A	2	II

Table 3.8 A Summary of the SCC*mec* Results of the Eight CA-MRSA with Previously Novel SCC*mec*s (Continued)

Isolate	*mec* Complex	*ccr* Gene Complex	SCC*mec* Type
WA MRSA-28	A	4	VIII
WA MRSA-40	C2	2 and C1	Novel
WA MRSA-42	B	2	IVa

3.4 SCC*mec* Region of WA MRSA-40

WA MRSA-40 remained unclassified following SCC*mec* typing, due to the presence of two different *ccr* gene complexes, *ccrC* and *ccrA2B2*. To classify the SCC*mec* of WA MRSA-40, the entire SCC*mec* element was sequenced and analysed.

A summary of SCC*mec* results of WA MRSA-40 is presented in Table 3.9.

Table 3.9 SCC*mec* Results for WA MRSA-40

PCR Reaction	Result
mec Complex	Class C2
ccr Gene Complex	*ccrA2B2* and *ccrC*
Oliveira, D and de Lancastre, H. (2002) Multiplex PCR	Loci E and F
Zhang, K et al. (2005) Multiplex PCR	*Type V (1600bp product)

*The multiplex PCR utilising the method of Zhang, K et al. 2005, produced a PCR product of 1,600 bp due to the presence of an IS*sau4*-like element within the target ORF.

The multiplex PCR utilising the method of Oliveira, D and de Lencastre, H. (2002), which determines structural elements of SCC*mec* was positive for loci E and F, which are present in SCC*mec* III. Locus E was designed to detect the region between the integrated plasmid pI258 and transposon Tn*554,* while Locus F was designed to detect the region between Tn*554* and the chromosomal junction *orfX*. PCR amplicons of WA MRSA-40 of these locus points were sequenced and showed homology with a restriction modification system (*hsd*) at locus E and homology with a *ccrC* gene at locus F. These results demonstrate that in this instance, these two locus points are not specific for only SCC*mec* III.

3.4.1 Strategy for Sequencing the SCC*mec* of WA MRSA-40

A long range PCR kit (Roche Diagnostics) capable of amplifying a product up to 25,000 bp was used to amplify PCR products between known loci as presented in Figure 3.3. These loci were based on previous PCR typing results and were used as terminals to amplify larger products using the long range PCR kit. These loci were F locus/*ccrC*, *dru*, *mecA,* Zhang V primer site, *hsdR*, *hsdM* with the right hand end located using the locus *orfY*, which is located outside the SCC*mec*.

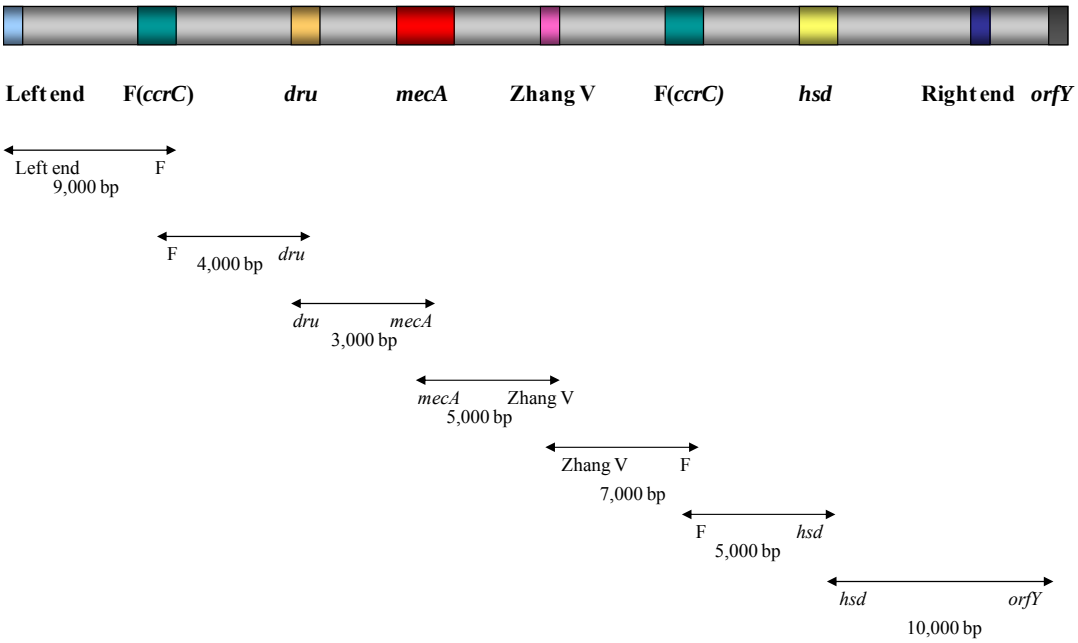

Figure 3.3 Long Range PCR Products Covering the SCC*mec* of WA MRSA-40 (Not drawn to scale)

A technique of primer walking was then used to fully sequence the SCC*mec* of WA MRSA-40, because the resultant amplicons from the long range PCR reactions were too large for a single sequence read.

The primer walking technique can be summarised as follows and is presented in Figure 3.4

- Long Range PCR reaction, with a product that is too large to be sequenced in a single sequence read.
- New forward and reverse primers were designed toward the ends of each of the forward and reverse sequences respectively.
- The resultant PCR product was sequenced again, this time with the new primers.
- The sequences were analysed with sequence assembly software.
- New forward and reverse primers were again designed toward the ends of the sequences.
- The PCR product was sequenced again with the next set of primers.
- The sequences were analysed with sequence assembly software.

This process was continued until these short-sequence segments overlapped and the DNA fragment was completely sequenced.

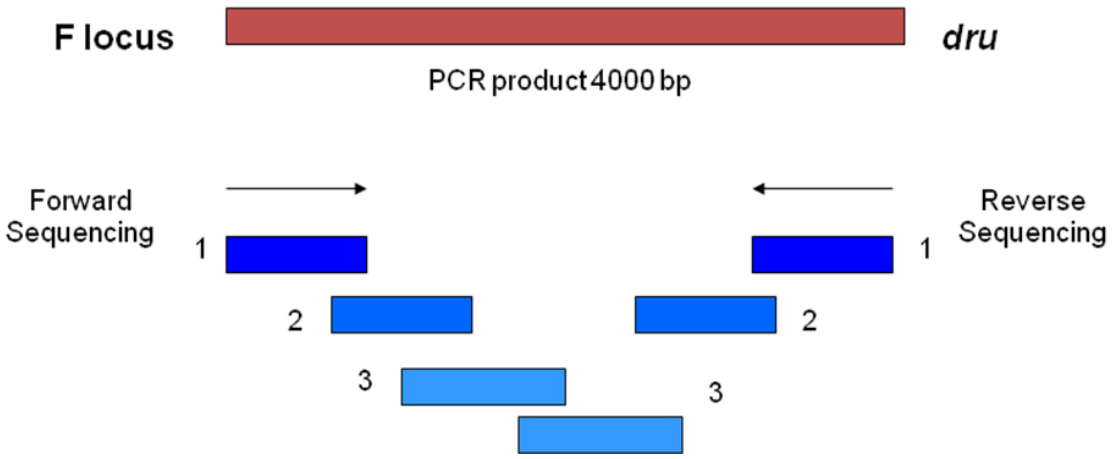

Figure 3.4 Primer Walking Strategy

Figure 3.5 presents an example of the long range PCR reactions. The PCR products of this long range PCR reaction of WA MRSA-40 used the primers for *mecA* (MECA P4 and MECA P7) and the F locus (RIF5 F10 and RIF5 R13). The forward and reverse primers for each of these loci were used in all possible combinations to ensure that the correct orientation of these genes could be determined.

The PCR reactions revealed three products (see Figure 3.5), a 6,000 bp product in lane 3, a 12,000 bp product in lane 4 and a 4,000 bp product in lane 5. This lead to the postulation, that WA MRSA-40 possibly encoded multiple copies of the F locus, which is specific for the *ccrC* gene complex. One copy approx. 6,000 bp downstream of *mecA* and another copy approx. 12,000 bp upstream of *mecA*. The gel picture indicated a third location of a *ccrC* gene complex, also upstream of the *mecA* gene, with a 4,000 bp product (lane 5). This was later found to be misleading as sequencing revealed that this product was generated by non-specific primer binding.

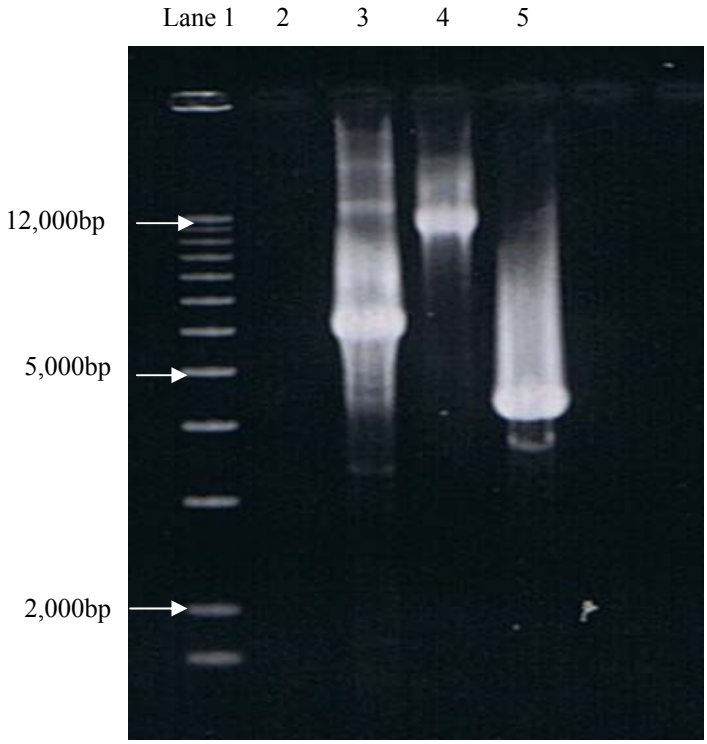

Figure 3.5 WA MRSA-40 Long Range PCR using *mecA* – F Locus Primers

Lane 1	1 Kb DNA Ladder
Lane 2	*mecA*R (MECA P4) – F Locus R (RIF F10)
Lane 3	*mecA*R (MECA P4) – F Locus F (RIF R13)
Lane 4	*mecA*F (MECA P7) – F Locus R (RIF F10)
Lane 5	*mecA*F (MECA P7) – F Locus F (RIF R13)

3.4.2 The Structure of the SCC*mec* of WA MRSA-40

The nucleotide sequence of the SCC*mec* of WA MRSA-40 was determined to be 41,289 bp flanked by imperfect DR sequences of 15 bp and imperfect IR of 24 bp in length. See Table 3.14 and Figure 3.18. The SCC*mec* of WA MRSA-40 has a G+C content of 30.7% which is lower than the 32.8-32.9% for *S. aureus* (Kuroda et al. 2001), indicating that it originally came from a species other than *S. aureus*.

The SCC*mec* of WA MRSA-40 contained 36 ORFs, which could be divided into the regions of *mec* and *ccr* gene complexes and their surrounding regions J1, J2 and J3. See Table 3.13 and Figure 3.12.

The right hand (RH) end was integrated into the SCC*mec* insertion site in the *orfX* gene however, the left hand (LH) insertion site could not initially be determined. Further sequencing revealed two SCC-like elements inserted at the *orfX* integration site adjacent to the left hand end of the SCC*mec* of WA MRSA-40. The three elements formed a SCC*mec*-Composite Island (SCC*mec*-CI) with a total size of 72,522 bp. See Figure 3.18.

The sequence of the SCC*mec*-CI of WA MRSA-40 has been named as SCC$mec_{\text{WA MRSA-40}}$-CI and has been deposited in the NCBI GenBank public database under Accession number JQ746621.

3.4.3 The *mec* Complex of WA MRSA-40

WA MRSA-40 was found to have a class C2 *mec* complex consisting of a truncated *mecR1*, and the *mecI* gene completely deleted due to the insertion of IS*431* at nucleotide (nt) 93 of *mecR1*. Sequences of IS*431*L (left/downstream of *mecA*) and IS*431*R (right/upstream of *mecA*) truncating *mecR1,* were analysed and compared with equivalent IS*431* elements of two SCC*mec* type V isolates, WIS CA-MRSA (V[5C2]) and the Taiwan CA-MRSA (V_T[5C2&5]) strain PM1 with

GenBank Accession Nos AB121219 and AB462393 respectively. See Table 3.10 and Figures 3.6 and 3.7.

Table 3.10 Polymorphisms with Base-Pair Substitutions in IS*431*L and IS*431*R of the SCC*mec* of WA MRSA-40

Nucleotide Position IS*431*	**IS*431*L**	**IS*431*R**	**Stop Codon**
73	A→G		
93	A→G		
134	C→T	C→T	
144	C→G	C→G	TAG
150	G→A	G→A	TGA
168	A→G	A→G	
174	G→A	G→A	
260	G→A	G→A	
283	G→A	G→A	
344	C→T	C→T	
345	G→A	G→A	
400	C→T	C→T	TAG
420	A→C		
435	A→C		
457	G→C		

ClustalW alignment using MacVector™ 7.2 (Accelrys Inc) of the IS*431* gene, consisting of 481 nucleotides, revealed WA MRSA-40 to have 15 nucleotide polymorphisms in IS*431*L and 10 nucleotide polymorphisms in IS*431*R when compared with IS*431*L and IS*431*R of the prototypical type V SCC*mec* from WIS. See Table 3.10 for the nucleotide positions, base-pair substitutions and consequent stop codon formations.

A further comparison of IS*431*L and IS*431*R sequences of the three isolates analysed revealed IS*431*L and IS*431*R for WA MRSA-40 contained the same premature stop codons, resulting in truncated IS*431* left and right transposase genes which would inactivate both transposases and stabilise the *mec* complex. The analysis showed the IS*431*L transposase gene for Taiwan CA-MRSA to be intact with no premature stop codons, however the IS*431*R transposase gene was identical to that of WA MRSA-40, IS*431*L and IS*431*R; IS*431* transposase genes for WIS CA-MRSA were both intact. See Figures 3.6 and 3.7.

```
WA 40      1 ATGAACTATTTCAGATATAAACAATTTAACAAGGATGTTATCACTGTAGC  50
Taiwan     1 ATGAACTATTTCAGATATAAACAATTTAACAAGGATGTTATCACTGTAGC  50
WIS        1 ATGAACTATTTCAGATATAAACAATTTAACAAGGATGTTATCACTGTAGC  50
             **************************************************

WA 40     51 CGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATGTCTGAAA 100
Taiwan    51 CGTTGGCTACTATCTAAGATATACATTGAGTTATCGTGATATATCTGAAA 100
WIS       51 CGTTGGCTACTATCTAAGATATACATTGAGTTATCGTGATATATCTGAAA 100
             ********************** ****************** *******

WA 40    101 TATTAAGGGAACGTGGTGTAAACGTTCATCATTTAACGGTCTAGCGTTGA 150
Taiwan   101 TATTAAGGGAACGTGGTGTAAACGTTCATCATTCAACGGTCTACCGTTGG 150
WIS      101 TATTAAGGGAACGTGGTGTAAACGTTCATCATTCAACGGTCTACCGTTGG 150
             ********************************* ********* *****

WA 40    151 GTTCAAGAATATGCCCCGATTTTATATCAAATTTGGAAGAAAAAGCATAA 200
Taiwan   151 GTTCAAGAATATGCCCCAATTTTGTATCAAATTTGGAAGAAAAAGCATAA 200
WIS      151 GTTCAAGAATATGCCCCAATTTTGTATCAAATTTGGAAGAAAAAGCATAA 200
             ***************** ***** *************************

WA 40    201 AAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAG 250
Taiwan   201 AAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAG 250
WIS      201 AAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAG 250
             **************************************************

WA 40    251 GAAAATGGAACTATTTATATCGTGCCATTGATACAGAGGGACATACATTA 300
Taiwan   251 GAAAATGGAGCTATTTATATCGTGCCATTGATGCAGAGGGACATACATTA 300
WIS      251 GAAAATGGAGCTATTTATATCGTGCCATTGATGCAGAGGGACATACATTA 300
             ********* ********************** ****************

WA 40    301 GATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGTATTTAT 350
Taiwan   301 GATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGCGTTTAT 350
WIS      301 GATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGCGTTTAT 350
             ****************************************  *****
```

```
WA 40   351 CAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATT 400
Taiwan  351 CAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATC 400
WIS     351 CAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATC 400
            **************************************************

WA 40   401 AGGCACCTTCAACGAAGGTCGCAATGGCTAAAGTCATTAAAGCTTTTAAA 450
Taiwan  401 AGGCACCTTCAACGAAGGTAGCAATGGCTAAAGTAATTAAAGCTTTTAAA 450
WIS     401 AGGCACCTTCAACGAAGGTAGCAATGGCTAAAGTAATTAAAGCTTTTAAA 450
            ****************** ************** ***************

WA 40   451 CTTAAACCTGACTGTCATTGTACATCGAAAT 481
Taiwan  451 CTTAAACCTGACTGTCATTGTACATCGAAAT 481
WIS     451 CTTAAAGCTGACTGTCATTGTACATCGAAAT 481
            ****** ************************
```

Figure 3.6 ClustalW Alignment of 481 Nucleotides of IS*431*L Gene, of WA MRSA-40, the Taiwan clone and WIS CA-MRSA. Nucleotide positions are numbered on the ends of the lines and identical nucleotides are indicated by asterisks beneath the alignment. Premature stop codons are boxed.

```
WA 40     1 ATGAACTATTTCAGATATAAACAATTTAACAAGGATGTTATCACTGTAGC  50
Taiwan    1 ATGAACTATTTCAGATATAAACAATTTAACAAGGATGTTATCACTGTAGC  50
WIS       1 ATGAACTATTTCAGATATAAACAATTTAACAAGGATGTTATCACTGTAGC  50
            **************************************************

WA 40    51 CGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATGTCTGAAA 100
Taiwan   51 CGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATGTCTGAAA 100
WIS      51 CGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATGTCTGAAA 100
            **************************************************

WA 40   101 TATTAAGGGAACGTGGTGTAAACGTTCATCATTTAACGGTCTAGCGTTGA 150
Taiwan  101 TATTAAGGGAACGTGGTGTAAACGTTCATCATTTAACGGTCTAGCGTTGA 150
WIS     101 TATTAAGGGAACGTGGTGTAAACGTTCATCATTCAACGGTCTACCGTTGG 150
            ********************************* ********* *****

WA 40   151 GTTCAAGAATATGCCCCGATTTTATATCAAATTTGGAAGAAAAAGCATAA 200
Taiwan  151 GTTCAAGAATATGCCCCGATTTTATATCAAATTTGGAAGAAAAAGCATAA 200
WIS     151 GTTCAAGAATATGCCCCAATTTTGTATCAAATTTGGAAGAAAAAGCATAA 200
            ***************** ***** *************************

WA 40   201 AAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAG 250
Taiwan  201 AAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAG 250
WIS     201 AAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAG 250
            **************************************************
```

```
WA 40   251 GAAAATGGAACTATTTATATCGTGCCATTGATACAGAGGGACATACATTA 300
Taiwan  251 GAAAATGGAACTATTTATATCGTGCCATTGATACAGAGGGACATACATTA 300
WIS     251 GAAAATGGAGCTATTTATATCGTGCCATTGATGCAGAGGGACATACATTA 300
            ********* ********************** *****************

WA 40   301 GATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGTATTTAT 350
Taiwan  301 GATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGTATTTAT 350
WIS     301 GATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGCGTTTAT 350
            ****************************************  *****

WA 40   351 CAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATT 400
Taiwan  351 CAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATT 400
WIS     351 CAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATC 400
            ************************************************

WA 40   401 AGGCACCTTCAACGAAGGTCGCAATGGCTAAAGTCATTAAAGCTTTTAAA 450
Taiwan  401 AGGCACCTTCAACGAAGGTCGCAATGGCTAAAGTCATTAAAGCTTTTAAA 450
WIS     401 AGGCACCTTCAACGAAGGTCGCAATGGCTAAAGTCATTAAAGCTTTTAAA 450
            ************************************************

WA 40   451 CTTAAACCTGACTGTCATTGTACATCGAAAT 481
Taiwan  451 CTTAAACCTGACTGTCATTGTACATCGAAAT 481
WIS     451 CTTAAACCTGACTGTCATTGTACATCGAAAT 481
            ******************************
```

Figure 3.7 ClustalW Alignment of 481 Nucleotides of IS*431*R Gene of WA MRSA-40, the Taiwan clone and WIS. Nucleotide positions are numbered on the ends of the lines and identical nucleotides are indicated by asterisks beneath the alignment. Premature stop codons are boxed.

3.4.4 *dru* Typing

The direct repeat unit (*dru*) region is the hyper-variable region (HVR) of the SCC*mec* adjacent to IS*431*L within the *mec* region (Figure 1.4), and is useful for typing purposes when further differentiation is required for SCC*mec* evolutionary studies. The repeat sequences are 40 bp in length. The *dru* type designations are assigned according to the number of repeat sequences present and indicate the different arrangements of specific repeats with regard to divergence from the consensus (Ryffel et al. 1991; Goering et al. 2008). Figure 3.8 presents the

sequence of the *dru* repeats of WA MRSA-40 including 5' and 3' signature flanking sequences. The sequence was submitted to http://www.dru-typing.org where it was analysed by druID software.

5' TCTTACTGAGATTATACTA
ATAAGAGGAA TAGTAAAAGC AATTCTAAGT AAAATTGCAG
ATAAGAGGTT TGTTAAAAGC AGTTCTAAGT AAAATTGCAG
ATAAGAGGTT TGTTAAAAGC AGTTCTCAGT AAAATTACAG
ATAAGAGGTA CGTTAAAAGC AGTTCTAAGT AAAATTGCAG
ATAAGAGGTT TGTTAAAAGC AGTTCTAAGT AAAATTGCAG
ATAAGAGGTA CGTTAAAAGC AATTCCATGC AAAATTGCTG
ATAAGGGGTA AGTTAAAAGC AGTTCTCAGT AAAATTGCAG
ATAAGAGGTA CGTTAAAAGC AGTTCTAGGC AAAATTGCAG
ATAAGAGGTG CGTTAAAAGC AGTTCTAAGT AAAATTGCTG
ATAAGGGGTA AGTTAAAAGC AATCCTAAGT AAAATTGCAG
ATAAGAGGTA AGTTAAAAGC AATCCTAAGT AAAATTGCAG
ATAAGGGGTACAGAAAAAC 3'

Figure 3.8 Sequence of the *dru* Region of WA MRSA-40
Note: Flanking sequences are in bold type

The *dru* repeats were designated: 5a-2d-4a-0-2d-5b-3a-2g-2h-4e-3e which is characteristic of *dru* type: **dt 11p** (Goering et al. 2008**)**.

3.4.5 *ccrC* Analysis of WA MRSA-40

WA MRSA-40 was found to carry a *ccrC* gene complex by PCR typing. See Table 3.5. Sequencing the SCC*mec* of WA MRSA-40 however, actually revealed the presence of two *ccrC* gene complexes, one situated upstream of *mecA*, initially designated *ccrC*L (left) and the other situated downstream of *mecA*, designated *ccrC*R (right). See Figure 3.9.

The sequences of the two *ccrC* genes of WA MRSA-40 were classified by phylogenetic analysis using MacVector™. *ccrC* reference sequences from GenBank that were used in the comparison are presented in Table 3.11.

WA MRSA-40 *ccrC*L showed 100% homology with *ccrC8* by phylogenetic analysis, which was confirmed by nucleotide blast search (http://blast.ncbi.nlm.nih.gov). WA MRSA-40 *ccrC*R showed 100% homology with *ccrC2* by phylogenetic analysis which was confirmed by nucleotide blast search. See Figure 3.9.

The International Working Group on the Classification of Staphylococcal Cassette Chromosome elements (IWG-SCC 2009) currently recognises one *ccrC* allotype with all *ccrC* variants to date shown to have ≥87% nucleotide similarity. These variants are designated as alleles of the *ccrC1* allotype (IWG-SCC 2009). Consequently the *ccrC* alleles of WA MRSA-40 were designated as *ccrC1,* allele 8 and *ccrC1*, allele 2.

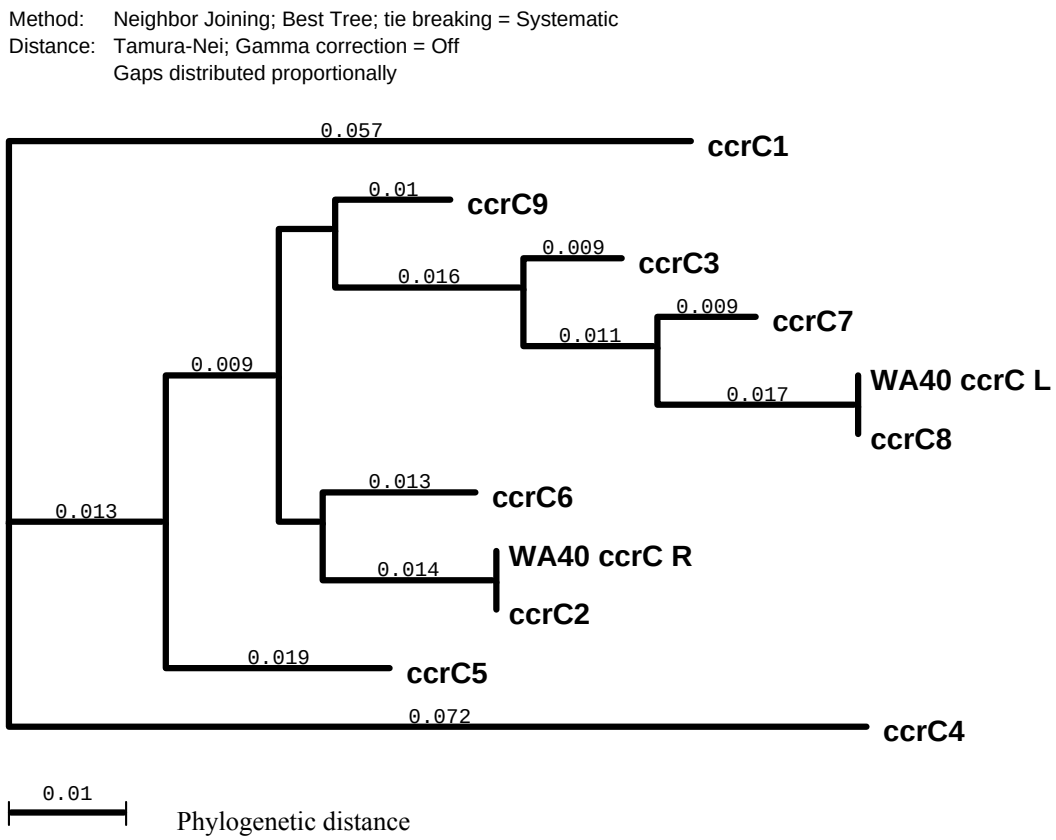

Figure 3.9 Phylogenetic Analysis of *ccrC* Allelic Gene Sequences
Note: Phylogenetic distance is indicated below the figure.

A multiplex PCR using primers ccrC2F, ccrC2R, ccrC8F and ccrC8R which differentiate *ccrC1,* alleles 2 and 8 was devised and performed. Figure 3.10 presents PCR confirmation of WA MRSA-40 containing *ccrC1,* allele 8 and *ccrC1,* allele 2 gene complexes. The Taiwan CA-MRSA was the positive control for both *ccrC* gene complexes, while SCC*mec*$_{ZH47}$ was the negative control because it carries *ccrC1,* allele 9 (Higuchi et al. 2008). See Figure 3.10.

Table 3.11 Reference *ccrC* Sequences Used for Phylogenetic Analysis of the *ccrC* of WA MRSA-40

Alleles of *ccrC1*	**Accession No.**	**SCC element/ Organism**	**Strain Name**
1	AB121219	SCC*mec* V	(WIS) JCSC3624
2	AY894416	SCC*mec* V	(Taiwan)
3	AB037671	SCC*mercury*	85/2082
4	U10927	SCC*cap*	*S. aureus* M Type 1 capsular gene cluster
5	AP006716	*S. haemolyticus*	JCSC1435
6	EF190467	*S. haemolyticus*	25-60
7	EF190468	*S. epidermidis*	13-48
8	AB462393	SCC*mec* V	(Taiwan) PM1
9	AM292304	SCCmec_{ZH47}	ZH47

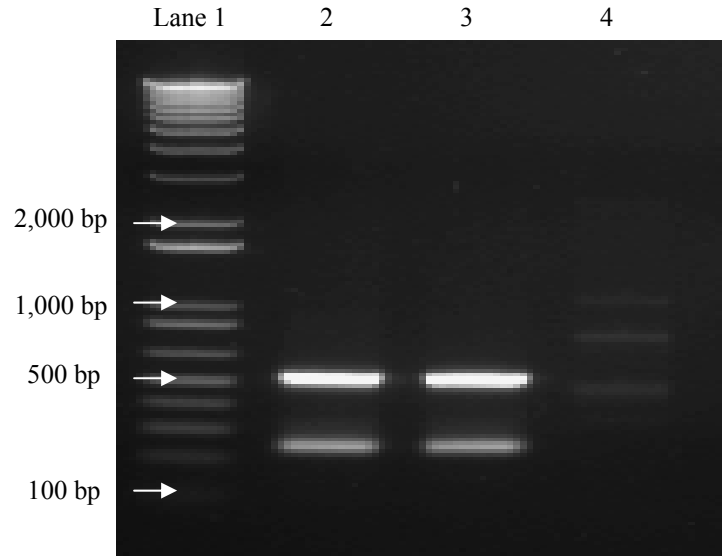

Figure 3.10 *ccrC* Multiplex PCR to Detect *ccrC1,* Alleles 2 and 8

Lane 1 1 Kb DNA Ladder
Lane 2 Taiwan CA-MRSA
Lane 3 WA MRSA-40
Lane 4 SCC*mec*$_{ZH47}$

The *ccrC1*, allele 8 gene complex of WA MRSA-40 was found to consist of the *ccrC* gene together with three flanking ORFs on each side. Upstream of *ccrC1,* allele 8 the three ORFs are identified as hypothetical proteins with ORF M04 a homologue of the DNA polymerase family A and ORF M06 a homologue of a primase of the P4 family, C-terminal domain. Downstream, three ORFs were identified as encoding hypothetical proteins with domains of unknown functions (DUF) of 950, 960 and 1643 bp respectively. See Table 3.13. This conserved *ccrC* region, designated the *ccrC*-carrying unit, downstream of *mecA* has been described in Taiwan CA-MRSA and SCCmercury and SCC*mec*$_{ZH47}$ (Higuchi et al. 2008). See Figure 3.11.

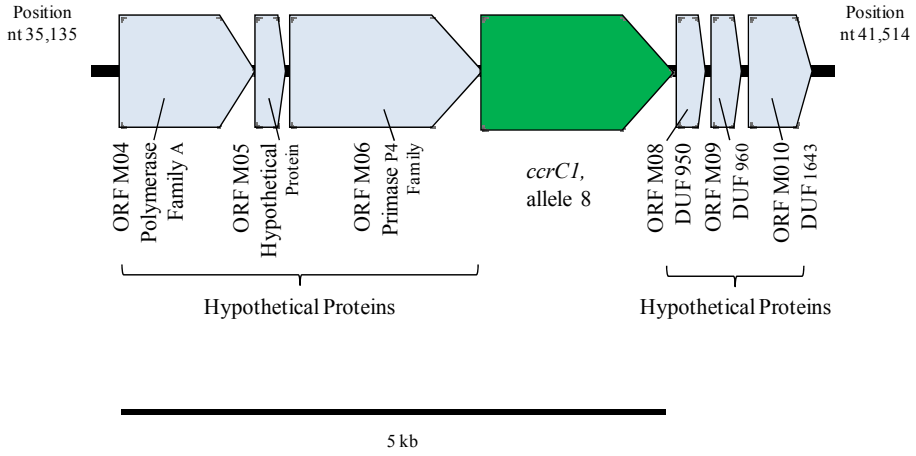

Figure 3.11 Diagram of the *ccrC1,* Allele 8 Carrying Unit of WA MRSA-40
Abbreviations: DUF, Domain of Unknown Function; nt, nucleotide.

The PCR, sequencing and phylogenetic analysis of the two *ccr* gene complexes of WA MRSA-40 has characterised it as carrying *ccrC* gene complexes of two different alleles, *ccrC1,* allele 8 and *ccrC1,* allele 2.

3.4.6 J Regions of WA MRSA-40

3.4.6.1 J1 Region

The J1 Region of WA MRSA-40 is located between the right hand end (*att*R) of the SCC*mec* and the *ccrC1,* allele 2 gene complex. It is 13,602 bp in size and encodes 7 ORFs. See Table 3.13. Contained in this region is a type 1 restriction-modification system (*hsdR, hsdS,* and *hsdM*). The J1 region of WA MRSA-40 was 100% homologous with the J1 region of the Taiwan CA-MRSA, except for three nucleotide polymorphisms. These three polymorphisms occurred in a non-coding region between ORF M29 and *hsdR*. See Table 3.12.

Table 3.12 Polymorphisms with Base Pair Changes in the J1 Region of WA MRSA-40 when Compared with the J1 Region of the Taiwan CA-MRSA

Position (nt)	Base Pair Change
59,408	C→T
59,410	T→A
59,414	C→A

3.4.6.2 J2 Region

The J2 region of WA MRSA-40 is located between the *mec* region and the *ccrC1,* allele 2 gene complex. This region is 5,056 bp in size and encodes 6 ORFs. This region also contains the insertion sequence IS*Sau4*-like element inserted into ORF M20. See Figure 3.12. The IS*Sau4*-like sequence is 1,269 bp in size and 97% homologous with IS*Sau4* (Accession No. DQ680163). It contains two ORFs, an ORF encoding a hypothetical protein and a transposase gene of 843 bp. See Table 3.13. The IS*Sau4*-like element is flanked by IR of 38 bp. These repeats indicate that the transposase inserted via an active transposition event. See Table 3.14 for IR sequences.

Homology between WA MRSA-40 and the Taiwan CA-MRSA in the J2 region is 99-100% with a polymorphism seen in ORF M23 of WA MRSA-40 at nt 53,982 with the addition of an extra A causing the amino acid change from threonine (Thr) to asparagine (Asn) and a premature stop codon to the ORF at nt 987 (of the ORF), whereas the equivalent ORF of the Taiwan CA-MRSA ends at nt 1110 and the ORF is presumably intact.

3.4.6.3 J3 Region

The J3 region is located between the SCC*mec* left hand end and the *mec* region, and includes the *ccrC1,* allele8 gene complex. This region is 13,602 bp in size and encodes 10 ORFs. See Table 3.13. The J3 region of WA MRSA-40 is 100% homologous with the J3 region of the Taiwan CA-MRSA. See Figure 3.12.

Table 3.13 The Open Reading Frames of the SCC*mec* of WA MRSA-40

Genetic Region	ORF Name	Position (nt)	Size (bp)	Identity (%)	Homology	Information
Repeat Region	DR-3	31,695-31,709	15	93%	NCTC 8325	Direct Repeat SCC*mec*L
	IR-5	31,700-31,722	23			Inverted Repeat SCC*mec*L
J3	ORF M01	31,959-32,264	306	100	ORF2 Taiwan (PM1)	Hypothetical protein, predicted restriction endonuclease (COG 3183)
	ORF M02	32,463-33,326	864	100	ORF3 Taiwan (PM1)	Hypothetical protein (COG 3680)
	ORF M03	33,410-34,909	1500	100	ORF4 Taiwan (PM1)	Hypothetical protein
ccrC Gene Complex	ORF M04	35,135-36,235	1101	100	ORF5 Taiwan (PM1)	Hypothetical protein, DNA polymerase family A
	ORF M05	36,228-36,599	372	100	ORF6 Taiwan (PM1)	Hypothetical protein
	ORF M06	36,596-38,239	1644	100	ORF7 Taiwan (PM1)	Hypothetical protein, primase-Cterm: phage/plasmid primase, P4 family, C-terminal domain
	ORFM07/ *ccrC*	38,465-40,141	1677	100	*ccrC8* Taiwan (PM1)	Cassette chromosome recombinase C1, Allele 8
	ORF M08	40,247-40,585	339	100	ORF9 Taiwan (PM1)	Hypothetical protein (DUF 950)
	ORF M09	40,681-40,992	312	100	ORF10 Taiwan (PM1)	Hypothetical protein (DUF 960)
	ORF M10	41,008-41,514	507	100	ORF11 Taiwan (PM1)	Hypothetical protein (DUF 1643)

Table 3.13 The Open Reading Frames of the SCC*mec* WA MRSA-40 (Continued)

Genetic Region	ORF Name	Position (nt)	Size (bp)	Identity (%)	Homology	Information
mec Region	IS*431*	41,604-42,393	790	99	Taiwan (PM1)	Mobile Element: IS*431*L Insertion sequence
	IR_{IS431}	41,604-41,619	15	100	Taiwan (PM1)	Inverted repeat of IS*431*L
	ORF M11/ IS*431*	Complement 42,194-42,337	144	99	Taiwan (PM1)	Truncated transposase for IS*431*
	IR_{IS431}	42,378-42,393	15	100	Taiwan (PM1)	Inverted repeat of IS*431*L
	ORF M12/ *ugpQ*	43,679-44,422	744	99-100	All SCC*mec* types	Glycerophosphoryl diester phosphodiesterase
	ORF M13	44,519-44,947	429	99	ORF15 Taiwan (PM1)	MaoC domain protein dehydratase
	ORF M14/ *mecA*	Complement 44,993-47,002	2010	99-100	All SCC*mec* types	Penicillin-binding protein PBP2a/2'
	ORF M15/ Δ*mecR1*	47,099-47,209	111	100	ORF17 Taiwan (PM1)	Truncated signal transducer protein MecR1
	IS*431*	47,191-47,981	790	100	Taiwan (PM1)	Mobile Element: IS*431*R Insertion sequence
	IR_{IS431}	47,191-47,206	15	100	Taiwan (PM1)	Inverted repeat of IS*431*R
	ORF M16/ IS*431*	47,247-47,390	144	100	ORF 18 Taiwan (PM1)	Truncated transposase for IS*431*
	IR_{IS431}	47,966-47,981	15	100	Taiwan(PM1)	Inverted repeat of IS*431*R
J2	ORF M17	Complement 47,981-48,409	429	100	ORF19 Taiwan (PM1)	Hypothetical protein
	ORF M18	48,457-49419	963	100	ORF20 Taiwan (PM1)	Hypothetical protein
	ORF M19	49,581-50,063	483	100	ORF21 Taiwan (PM1)	Hypothetical protein
	IS*Sau4*-like Element	50,046-51,315	1269	100	Taiwan (PM1)	Mobile element: IS*Sau4*-like Element
	$IR_{IS\,Sau4\text{-like}}$ L	50,047-50,084	38	100	Taiwan (PM1)	Inverted repeat of IS*Sau4*-like Element L
	ORF M20	50,131-50,445	315	100	ORF22 Taiwan (PM1)	Hypothetical protein
	ORF M21	50,430-51,272	843	100	ORF23 Taiwan (PM1)	Transposase for IS*Sau4*-like Element
	$IR_{IS\,Sau-4\ like}$ R	51,278-51,315	38	100	Taiwan (PM1)	Inverted repeat of IS*Sau4*-like Element R
	ORF M22	51,279-52,844	1566	100	ORF24 Taiwan (PM1)	Hypothetical protein

Table 3.13 The Open Reading Frames of the SCC*mec* WA MRSA-40 (Continued)

Genetic Region	ORF Name	Position (nt)	Size (bp)	Identity (%)	Homology	Information
ccrC Gene Complex	ORF M23	53,039-54,025	987	99	ORF25 Taiwan (PM1)	Hypothetical protein, DNA polymerase family A
	ORF M24	54,142-54,510	369	100	ORF26 Taiwan(PM1)	Hypothetical protein
	ORF M25	54,510-56,126	1617	100	ORF27 Taiwan (PM1)	Hypothetical protein, primase-Cterm: phage/plasmid primase, P4 family, C-terminal domain
	ORF M26/ *ccrC*	56,351-58,030	1680	100	ORF28 *ccrC2* Taiwan (PM1)	Cassette chromosome recombinase C1, Allele 2
	ORF M27	58,083-58,457	375	100	ORF29 Taiwan (PM1)	Hypothetical protein (DUF 950)
	ORF M28	58,536-58,862	327	100	ORF30 Taiwan (PM1)	Hypothetical protein (DUF 960)
	ORF M29	58,868-59,380	513	100	ORF31 Taiwan (PM1)	Hypothetical protein (DUF 1643)
J1	*hsdR*	Complement 59,690-62,812	3123	100	ORF32 Taiwan (PM1)	Type 1 restriction-modification system endonuclease homologue
	hsdS	Complement 62,793-64,094	1302	100	ORF33 Taiwan (PM1)	Type 1 restriction-modification system specificity subunit
	hsdM	Complement 64,084-65,607	1524	100	ORF34 Taiwan (PM1)	Type 1 restriction-modification system DNA methylase
	ORF M33	66,007-68,103	2097	100	ORF35 Taiwan (PM1)	Hypothetical protein, predicted ATP-dependent endonuclease of the OLD family (COG 3593)
	ORF M34	68,078-69,853	1776	100	ORF36 Taiwan (PM1)	Hypothetical protein, UvrD Superfamily I DNA and RNA helicases (COG 0210)
	ORF M35	Complement 70,313-71,737	1425	100	ORF37 Taiwan (PM1)	Hypothetical protein, DOPA decarboxylase family
	ORF M36	Complement 71,820-72,146	327	100	ORF38 Taiwan (PM1)	Hypothetical protein (DUF 1958)
Repeat Region	IR-6	72,960-72,982	23			Inverted Repeat
	DR-4	72,984-72,998	15	100	NCTC 8325	Direct Repeat SCC*mec*R

Abbreviations: COG, Cluster of orthologous groups of proteins; DUF, Domain of Unknown Function; L, left repeat; R, right repeat; SCC*mec*L, SCC*mec* left end; SCC*mec*R, SCC*mec* right end. Accession Nos: Taiwain CA-MRSA PM1, AB462393; IS*Sau4*-like Element, DQ680163; NCTC 8325, CP000253

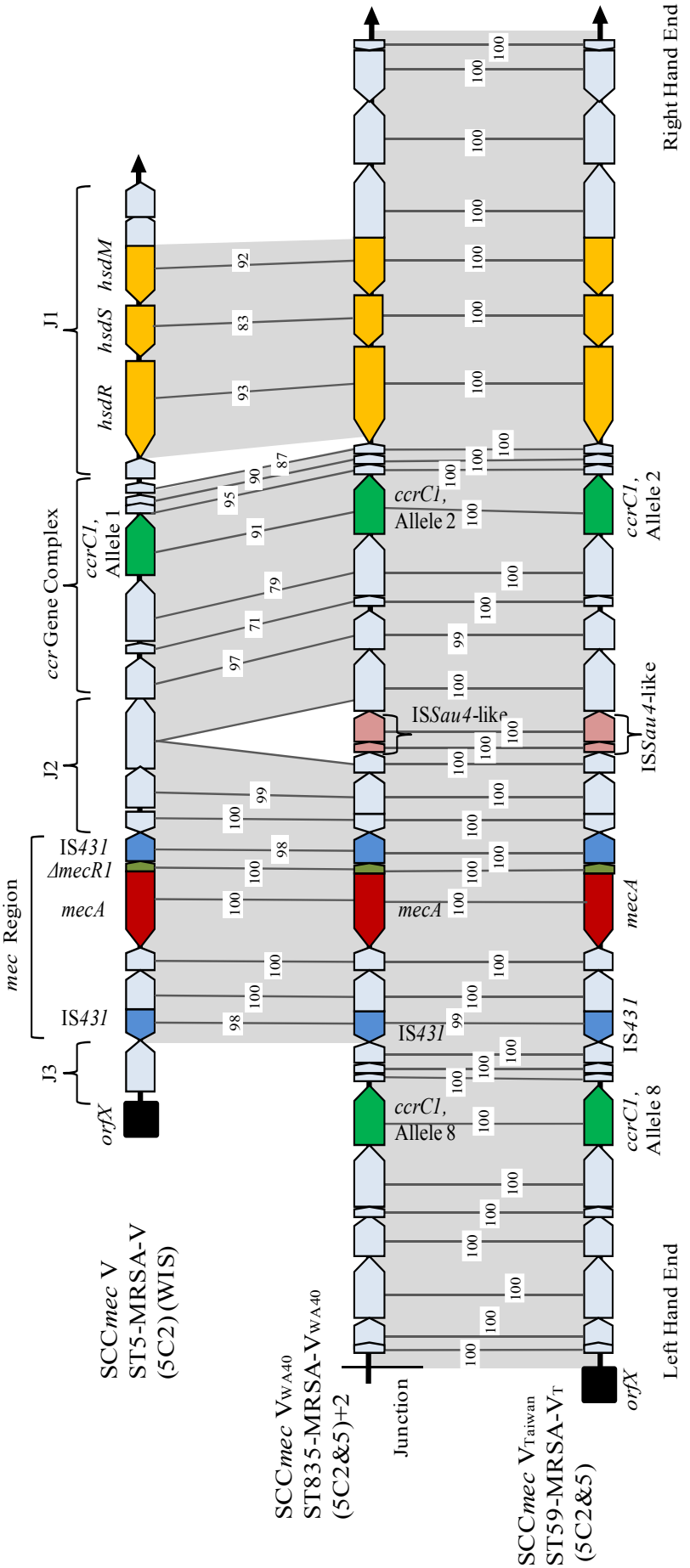

Figure 3.12 Comparison of the SCC*mec* of WA MRSA-40 with the SCC*mec*s of the Taiwan CA-MRSA and WIS CA-MRSA. Shading indicates regions of homology. Homologous genes are joined by a vertical line with % nucleotide identity indicated.

3.4.7 Repeat Sequences of the SCC*mec* of WA MRSA-40

Repeat regions of sequence flanking genetic elements indicate incision, excision and genetic recombination events. The following table (Table 3.14) presents the DR and IR sequences of the SCC*mec* of WA MRSA-40.

Table 3.14 Repeat Sequences of the SCC*mec* of WA MRSA-40

Genetic Region	Repeat Type	Sequence	Size bp	Position
SCC*mec* Left end	Direct DR-3	AGAGGC**t**TATCATAA	15	31,695-31,709
SCC*mec* Right end	Direct DR-4	AGAGGC**g**TATCATAA	15	72,984-72,998
SCC*mec* Left end	Inverted IR-5	C**t**TATCA**taa**ATa**aaa**CTAAAAA	23	31,700-31,722
SCC*mec* Right end	Inverted IR-6	TTTTTAG**caaa**AT**cac**TGATA**g**G	23	72,960-72,982
IS*431*L	Inverted	GGTTCTGTTGCAAAGT	15	41,604-41,619
	Inverted	ACTTTGCAACAGAACC	15	42,378-42,393
IS*431*R	Inverted	GGTTCTG**t**TGCAAAGT	15	47,191-47,206
	Inverted	ACTTTGCA**g**CAGAACC	15	47,966-47,981
IS*Sau4*-like	Inverted	G**g**ACTGACCCC**aa**T**t**A**gt**GGGAA**t**T**at**ATAAAAACACT	38	50,047-50,084
	Inverted	AGTGTTTTTAT**ta**A**g**TTCCC**gt**T**t**A**ag**GGGGTCAGT**a**C	38	51,278-51,315

Figure 3.14 demonstrates the similarities between the DR and the flanking sequences at the SCC*mec* junctions of reference SCC*mec* types I, II, III, IV, V and V_T and relevant SCC-like elements. Note: Non-complementary nt are in lower case and bold type

3.5 SCC and SCC-like Elements

Previous PCR results indicated the presence of a *ccrA2B2* gene complex but not the location. Several failed PCR reactions at the left hand end of the SCC*mec* of WA MRSA-40 led to the possibility of extra insertions between *orfX* and the SCC*mec* which were too large to amplify with the PCR conditions. A long range PCR reaction between *orfX* and *ccrA2B2* amplified a 7,000 bp product between an *hsd* gene and *ccrA2B2* gene complex with the *orfX* primer (P28) non-specifically binding to a semi-homologous region in an *hsd* gene. The P28 primer was homologous for 14 of its 22 bases with sequence of the *hsdS* gene at nt 12,699 to nt 12,720. An otherwise technical error was fortuitous. The primer walking technique was then used to sequence this SCC-like element. The following locus points were used for primer walking: *orfX, pls,* HP (hypothetical protein), *hsd*, *ccrA2B2* and the SCC*mec* junction of WA MRSA-40. See Figure 3.13.

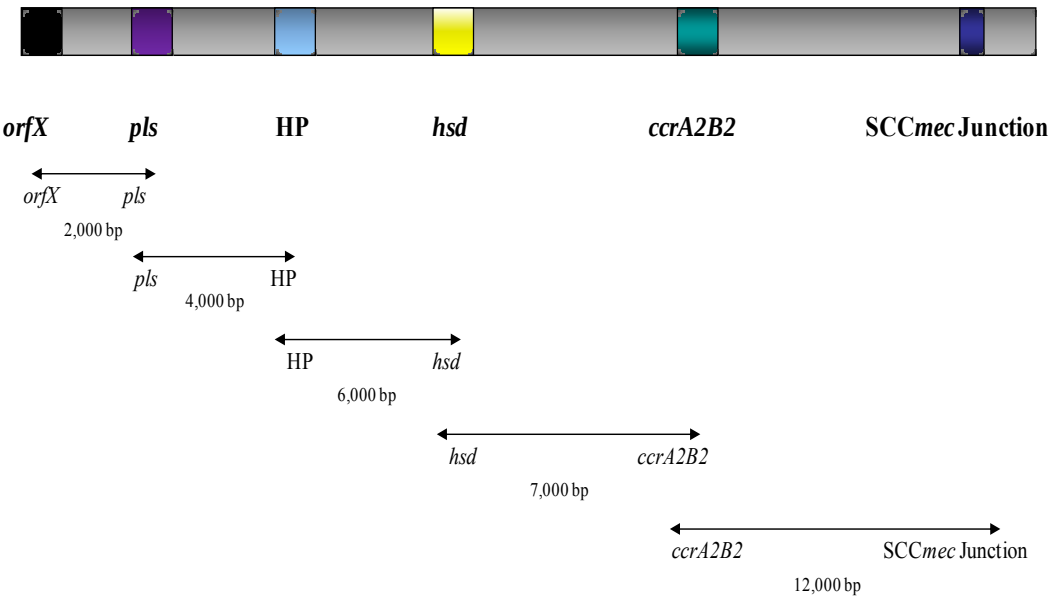

Figure 3.13 Sites Along the SCC-like Element of WA MRSA-40 Used for Long Range PCR (Not drawn to scale).

The SCC-like element was 31,233 bp in length. Identification of DR and IR revealed that this region was actually comprised of two elements that have been named according to the IWG Classification of SCC elements that do not carry *mecA* (IWG-SCC 2009). The first element was homologous with the ΔJ1 region of SCC*mec* type I, contained a *pls* gene and was designated pseudo SCC*pls* (ΨSCC*pls*). The second element was designated SCCsorbitol or SCC*srl* and it contained a sorbitol operon, a *ccrA2B2* complex and *hsd* genes. See Figure 3.18 and Table 3.17. The entire region of DNA from the left hand end of the SCC-like element to the right hand end of the SCC*mec* has been designated as SCC*mec*$_{\text{WA MRSA-40}}$-Composite Island (SCC*mec*$_{\text{WA MRSA-40}}$-CI).

DR and IR are presented in Table 3.15 and illustrated in Figures 3.14 and 3.18. The SCC*mec*$_{\text{WA MRSA-40}}$-CI was flanked by 15 bp imperfect DR sequences, one at the *orfX*-CI (composite island) junction, designated DR-1, and the other at the right hand end of the SCC*mec*-chromosome junction, designated DR-4. Two similar 15 bp DRs were found within the composite island, with DR-2 found at the junction of ΨSCC*pls* and SCCsorbitol and DR-3 found at the junction of SCCsorbitol and the left hand end of the SCC*mec*. The DR at the right end of the SCC*mec* (DR-4) was 100% homologous with the DR sequence at the *att*B site in the *orfX* of the of *S. aureus* strain NCTC8325. The DR at the left end of the SCC*mec* (DR-3) had two base pairs dissimilar with those of NCTC8325. The DR at the junction of ΨSCC*pls* and SCCsorbitol (DR-2) had two base pairs dissimilar, while the left end of the ΨSCC*pls* element (DR-1) had three base pairs dissimilar. A maximum ambiguity of four base pairs is required for integration (Ito, Katayama, and Hiramatsu 1999). A unique occurrence in the SCC*mec*$_{\text{WA MRSA-40}}$-CI was identified with two additional DR sequences at the junction of the ΨSCC*pls* and SCCsorbitol (DR-5) and the junction of SCCsorbitol and the SCC*mec* (DR-6). These DR sequences, which flank the SCCsorbitol element, were imperfect (3 base pair differences from each other), 39 bp in size and contained the 15 bp SCC*mec* DR.

Imperfect IR sequences of 7 bp, were identified within DR-1 (IR-1), and immediately preceding DR-2 (IR-2). Imperfect IR of 24 bp were identified starting from within DR-2 at the ΨSCC*pls*/SCCsorbitol junction (IR-3) and

immediately preceding DR-3 at the SCCsorbitol/SCC*mec* junction (IR-4), and starting from within DR-3 at the left junction (IR-5) and immediately preceding the DR, DR-4 at the SCC*mec* right end (IR-6). At the junction between the ΨSCC*pls* and the SCCsorbitol, a large IR was found as previously described in the SCC-composite island of the *S. epidermidis* ATCC 12228 with a 13 bp perfect complementary IR separated by 29 nucleotides (including 13 bp DR) AAAAACCGCATCA-29nt-TGATGCGGTTTTT (IR-7 and IR-8) (Mongkolrattanothai et al. 2004).

Table 3.15 Direct and Inverted Repeat Sequences of SCC*mec*$_{\text{WA MRSA-40}}$-CI

Repeat	Sequence	Size (bp)	Location	nt Position
DR-1	AGAAGC**g**TA**c**CA**c**AA	15	*orfX*-ΨSCC*pls* (ΨSCC*pls*L)	462-476
DR-2	AGAAGC**a**TA**t**CA**t**AA	15	ΨSCC*pls*-SCCsorbitol (ΨSCC*pls*R)	12,198-12,212
DR-3	AGAGGC**t**TATCATAA	15	SCCsorbitol-SCC*mec* (SCC*mec*L)	31,695-31,709
DR-4	AGAGGC**g**TATCATAA	15	SCC*mec*-chromosome (SCC*mec*R)	72,984-72,998
DR-5	AAACCGCATCATTA**t**C**t**GATACGC AGA**a**GC**a**TATCATAA	39	ΨSCC*pls*-SCCsorbitol (SCCsorbitolL)	12,174-12,212
DR-6	AAACCGCATCATTA**a**C**c**GATACGC AGA**g**GC**t**TATCATAA	39	SCCsorbitol-SCC*mec* (SCCsorbitolR)	31,671-31,709
IR-1	GCGTA**c**C	7	*orfX*-ΨSCC*pls* (ΨSCC*pls*L)	466-472
IR-2	G**a**TACGC	7	ΨSCC*pls*-SCCsorbitol (ΨSCC*pls*R)	12,191-12,197
IR-3	GC**a**TATC**a**T**a**A**g**TGATGCGGTTT	23	ΨSCC*pls*-SCCsorbitol (SCCsorbitolL)	12,202-12,224
IR-4	AAACCGCATCA**t**T**a**A**cc**GATA**c**GC	24	SCCsorbitol-SCC*mec* (SCCsorbitolR)	31,671-31,694
IR-5	C**t**TATCA**taa**AT**aaaa**CTAAAAA	23	SCCsorbitol-SCC*mec* (SCC*mec*L)	31,700-31,722
IR-6	TTTTTAG**caaa**AT**cac**TGATA**g**G	23	SCC*mec*-chromosome (SCC*mec*R)	72,960-72,982

Table 3.15 Direct and Inverted Repeat Sequences of SCC*mec*$_{\text{WA MRSA-40}}$-CI (Continued)

Repeat	Sequence	Size (bp)	Location	nt Position
IR-7	AAAAACCGCATCA	13	ΨSCC*pls*-SCCsorbitol (SCCsorbitolL)	12,172-12,184
IR-8	TGATGCGGTTTTT	13	ΨSCC*pls*-SCCsorbitol (SCCsorbitolL)	12,214-12,226
IR-9	AG**t**TTTT	6	Sorbitol Operon L	25,085-25,091
IR-10	AAAA**c**CT	6	Sorbitol Operon R	30,216-30,222

Note: Non-complementary nucleotides are in lower case and bold type.

Figure 3.14 (on the following page), compares the boundaries of SCC*mec* direct and inverted repeats of the SCC elements of WA MRSA-40, SCC*mec* types I-V and relevant SCC elements with MSSA strain NCTC 8325.

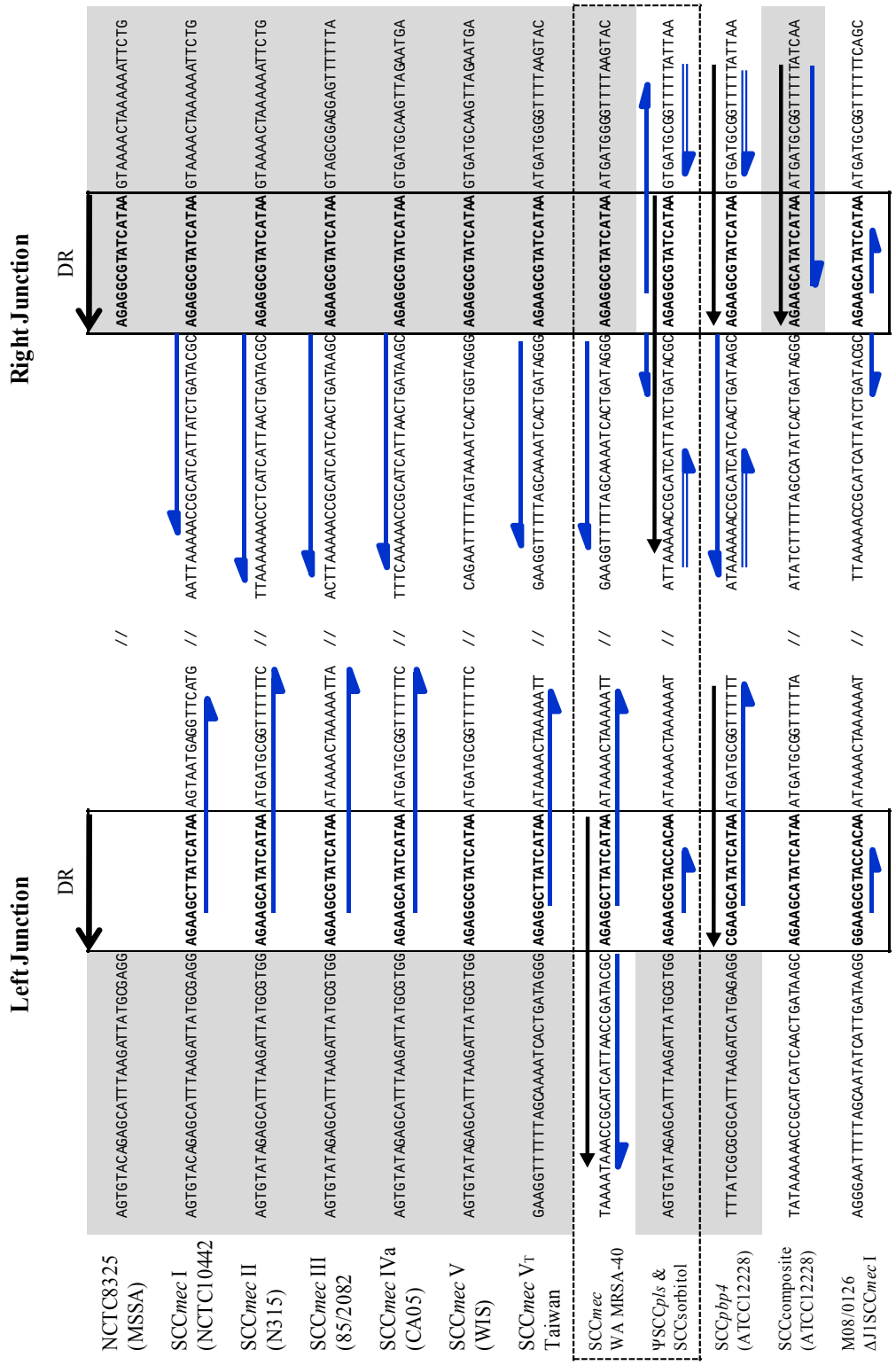

Figure 3.14 Boundaries of the SCC*mec* Types and SCC Elements

The direct repeat sequences are in bold type and surrounded by a box. The inverted repeats are indicated by half arrows. IR-7 and IR-8 at the junction of the SCCsorbitol element are indicated as parallel lines with half arrows. WA MRSA-40-Composite Island elements are surrounded by a dashed box.

3.5.1 ΨSCC*pls*

ΨSCC*pls* was 11,736 bp in length and comprised of eight ORFs. See Table 3.17. It is flanked by 15 bp imperfect DR (DR-1 and DR-2) that have three base pairs dissimilar and 7 bp imperfect IR (IR-1 and IR-2) that have one base pair difference. ΨSCC*pls* has high homology with a truncated J1 region of SCC*mec* type I. It contains a 4,473 bp gene designated ORF P03, homologous to the *pls* (plasmin-sensitive protein) gene, which is ORF CE010 of SCC*mec* I strain NCTC 10442 (Accession No. AB033763.2) and ORF SACOL0051 of SCC*mec* I strain COL (Accession No CP000046.1). The *pls* genes of strains NCTC 10442 and COL are 5,097and 4,646 bp, respectively.

The *pls* gene of WA MRSA-40 contained three regions of repeat sequences, R1, R2 and R3. See Figure 3.15. R3 was found to encode SD-dipeptide (serine-aspartate) repeats that are characteristic of the Sdr protein family. The N-terminus encoded a characteristic YSIRK type signal peptide of 27 amino acid residues from nt 1,533 to nt 1,613 while the C-terminus encoded a wall spanning domain carrying the characteristic cell-wall sorting signal, the LPXTG motif from nt 5,850 to nt 5,972 which was followed by hydrophobic amino acids and then positively charged residues. See Figure 3.15.

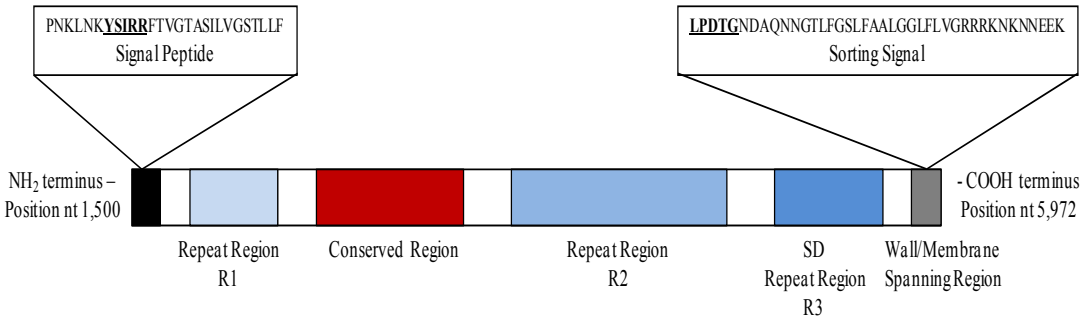

Figure 3.15 Diagrammatic Representation of the *pls* Gene of WA MRSA-40

Note: The YSIRK region of the signal peptide and LPXTG motif of the sorting signal are underlined and in bold type.

A conserved region, located between repeat regions R1 and R2 (see Figure 3.15) is homologous with the equivalent region of other surface proteins, such as the *pls* of SCC*mec* I, the *aap* (accumulation-associated protein) gene of *S. epidermidis* and *sasG* (*Staphylococcus aureus* surface protein G) gene (Hussain et al. 2009).

The sequence from DR-1 at the integration site of nt 462 of *orfX* to the start of the *pls* gene at nt 1,500 has homology with several SCC and SCC*mec* entries in the NCBI database. This region was 100% homologous with a SCC*mec*-ACME-composite island (CI) region of *S. epidermidis* strain BCM-HMP0060 from nt 204,545 to nt 205,591 (Accession No. NZ_GG696727.1), 99% homologous with the truncated J1 SCC*mec* I element of the ACME-composite island strain M08/0126 at nt 13,026 to nt 14,313 (Accession No. FR753166.1), 100% homologous from nt 462 to nt 1,292 (the first 830 bp of ΨSCC*pls*) with SCC*mec* type II strain CCRI-9583 at nt 453 to nt 1,282 (Accession No. AY267384.1) and 100% homologous from nt 462 to nt 903 (the first 441 bp of ΨSCC*pls*) with SCC*mec* type III strain V14 from nt 587 to nt 1,027 (Accession No. AB425427.1). See Figure 3.16.

The sequence from the end of the *pls* gene at nt 5,973 to DR-2 at nt 12,198 was 99% homologous with the sequences of SCC*mec* type I strain NCTC 10442 (nt 4,485 to nt 10,730), SCC*mec* type I strain COL (nt 61,859 to nt 68,100), the truncated J1 SCC*mec* I element of the ACME-CI strain M08/0126 from nt 17,765-24,006 and *S. epidermidis* strain BCM-HMP0060, from nt 207,630 to nt 213,869.

The closest similarity was seen with the *S. epidermidis* strain BCM-HMP0060 with only one base pair polymorphism in this region, resulting in both WA MRSA-40 and BCM-HMP0060 having the same ORF positions (start and stop) and sizes. See Table 3.17. A few more polymorphisms were seen in this region between the sequence of WA MRSA-40 and NCTC 10442, COL and ACME-CI M08/0126, which resulted in differences in ORF positions and sizes.

3.5.2 The Direct Repeats of ΨSCC*pls*

Interestingly, it was noted that flanking the BCM-HMP0060 region, was a 15 bp sequence highly homologous to the SCC DR (*att*B site) within *orfX*. A nucleotide search of the NCBI database of the entire BCM-HMP0060 genome (Accession No. NZ_GG696727.1) revealed 15 matches of 11 bp or more, (including the DR sequences that flank the SCC*mec*-ACME-CI of BCM-HMP0060) with the 15 bp DR sequence of NCTC 8325 at the *att*B site. If a maximum ambiguity of four bases is required for integration (Ito, Katayama, and Hiramatsu 1999), these 15 sites are potential DR and potential "hot spots" or recombination sites for SCC-element formation.

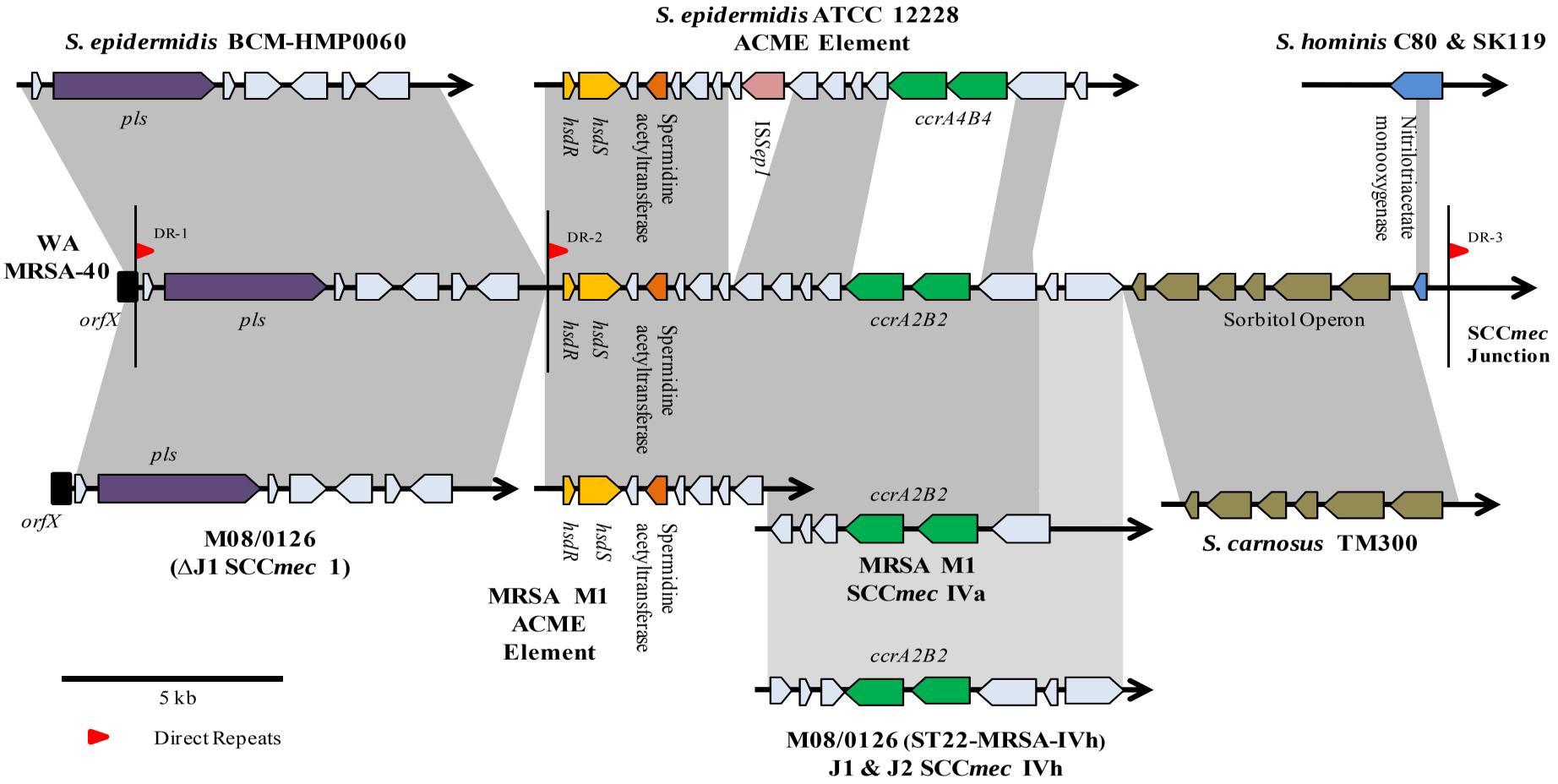

Figure 3.16 Comparison Diagram of the SCC and SCC-like Elements of WA MRSA-40. Shading in gray indicates region of homology

3.5.3 SCCsorbitol

The second SCC element was SCCsorbitol which contains a sorbitol operon, a *ccrA2B2 ccr* complex and a truncated type 1 restriction modification system. SCCsorbitol is 19,497 bp in length, encodes 23 ORFs (see Table 3.17) and is flanked by 39 bp imperfect DR (DR-5 and DR-6) that have four base pair differences and 23 bp IR (IR-3 and IR-4) that have 5 base pair differences. See Table 3.15 and Figure 3.18.

The type 1 restriction modification region of SCCsorbitol (nt 12,317 to nt 13,655) consists of an *hsdS* gene with a truncated *hsdR* gene of 144 bp. An *hsdM* component is absent. Type 1 restriction modification systems require three genes *hsdR* (restriction), *hsdS* (specificity) and *hsdM* (modification). Restriction modification systems encode endonucleases that cleave and then further degrade the double stranded DNA of foreign organisms such as bacteria or bacteriophages (Murray 2000). The *hsdM* of SCCsorbitol has probably been deleted by the insertion of a transposase, ORF S03 which immediately follows it. This transposase, 252 bp in size (at nt 13,659-13,910) is truncated and has 80% homology to IS*1272* in *S. haemolyticus* strain Y176 (GenBank accession No. U35835.1). A copy of spermidine acetyltransferase follows (at nt 13,998-14,126), which is thought to have a role in the restriction modification system (Kaminska and Bujnicki 2008). See Table 3.17.

SCCsorbitiol carries a *ccrA2B2* gene complex. The IWG classify *ccr* genes with nucleotide identities of more than 85% as being assigned to the same allotype. Phylogenetic analysis shows the *ccrAB* complex of SCCsorbitol of WA MRSA-40 to be allotype A2B2 (IWG-SCC 2009). The reference strains used in the phylogenetic analysis are listed in Table 3.16. See Figure 3.17.

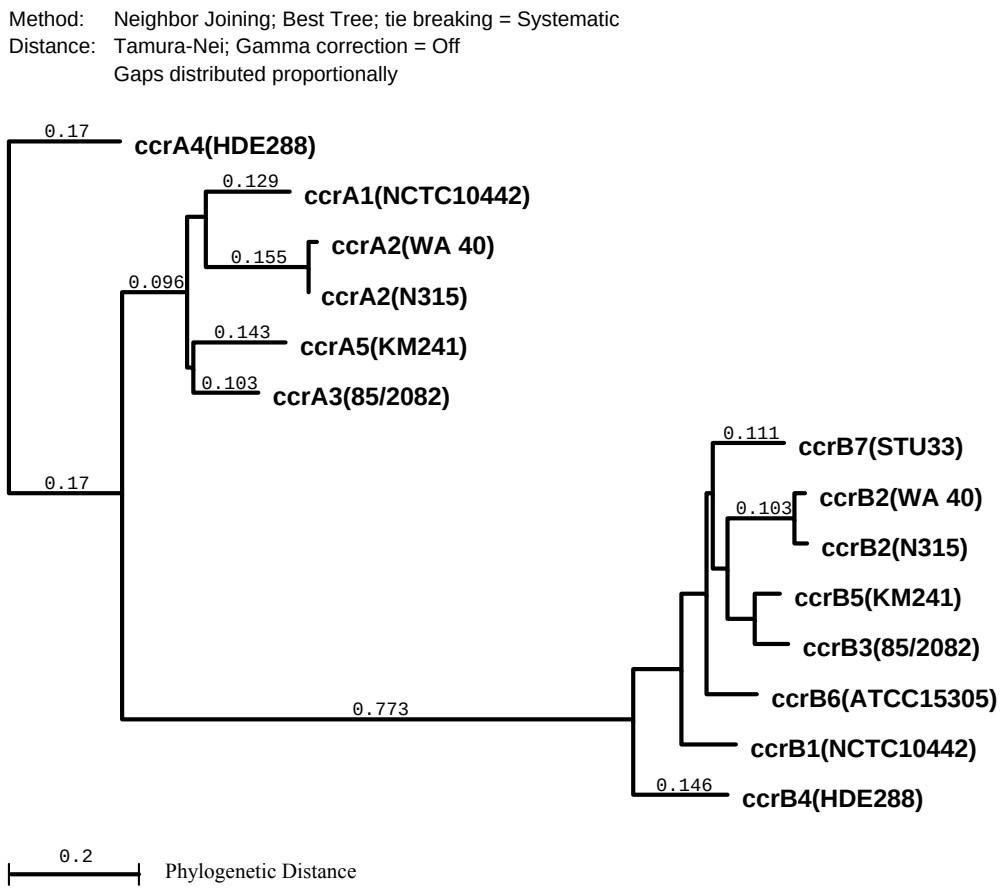

Figure 3.17 Phylogenetic Analysis of the *ccrAB* Gene Sequence of SCCsorbitol. Note: The *ccrA2B2* in SCCsorbitol of WA MRSA-40 is denoted as WA40 in the figure. Phylogenetic distance is indicated below the figure.

SCCsorbitol is a mosaic structure that possesses homologies with regions found in the SCC-Composite Island (SCC-CI) of *S.epidermidis* ATCC 12228 (Accession No. AE015929), the ACME and SCC*mec* regions of *S. aureus* M1 type IV SCC*mec* (Accession No. HM030720), the ACME-CI of *S. aureus* M08/0126 (Accession No. FR753166), and chromosomal regions of *S. carnosus* TM300 (Accession No. AM295250.1), *S. hominis* C80 (Accession No. ACRM1000005.1) and *S. hominis* SK119 (Accession No. (Accession No. NZ_ACLP01000020.1) (Sebastian et al. 2010; Mongkolrattanothai et al. 2004; Bartels et al. 2011; Rosenstein et al. 2009; Ward et al. 2010). Regions of homology are presented diagrammatically in Figure 3.16. Genetic regions, ORFs, positions, homologies and putative proteins are presented in Table 3.17.

Table 3.16 Reference Strains used in the Phylogenetic Tree Analysis of *ccrA2B2* of WA MRSA-40

***ccrAB* Allotype**	**Accession No.**	**Strain**	**Strain Name**
A1B1	AB033763	*S. aureus*	NCTC10442
A2B2	D56934	*S. aureus*	N315
A3B3	AB014436	*S. aureus*	85/2082
A4B4	AF411935	*S. aureus*	HDE288
A5B5	AM904731	*S. pseudintermedius*	KM241
A1B6	NC_007350	*S. saprophyticus*	ATCC15305
A1B7	AB353724	*S. saprophyticus*	STU33

WA MRSA-40 SCCsorbitol possessed the most extensive homology with the *S. epidermidis* ATCC 12228 SCC-CI. There was 100% homology between SCCsorbitol nt 12,198 to nt 15,955 and ATCC 12228 SCC-CI up to their respective *ccr* complexes of *ccrA2B2* and *ccrA4B4* respectively. ATCC 12228 carries an IS*Sep1*-like transposase of 1014 bp at ORFSE51, which belongs to the Transposase 20 superfamily, although it is not annotated as such in the GenBank database. This transposase is 99.6% homologous to Tn*20* found in the *mec* complex of ST72 MRSA-IV commonly known as the Korean clone (Park et al. 2009) (not shown). The SCCsorbitol is absent of this IS*Sep1*-like transposase. Upstream of the *ccr* complexes, the homology resumes with 90% homology between nt 20,979 to nt 22,772 of SCCsorbitol and nt 61,855 to nt 62,396 of ATCC 12228 SCC-CI. This region contains the 5' terminal of the SCC*pbp4* element of the ATCC 12228 SCC-CI.

The *ccr* downstream region of SCCsorbitol, nt 12,198 to nt 17,745, also possessed between 100% and 87% homology with nt 1 to nt 5,024 of the ACME region of *S. aureus* ACME-CI strain M1. From nt 15,955 to nt 22,808 of SCCsorbitol there was 87-97% homology with the J1 and J2 regions of the SCC *mec* IVa (2B) of M1

at nt 40,175 to nt 47,028, with this region containing a *ccrA2B2* gene complex. See Table 3.17 and Figure 3.16.

There was 84-98% homology with the *S. aureus* ACME-composite island strain M08/0126 nt 34,551 to nt 43,406 (ORF C10-17) and nt 15,955 to nt 24,772 of SCCsorbitol. This region comprises the J1 and J2 regions of the SCC*mec* IVh subtype that is included in the composite island of M08/0126, and includes a *ccrA2B2* gene complex. See Figure 3.16 and Table 3.17.

Upstream of the *ccrA2B2* complex of SCCsorbitol (nt 25,092 to nt 30,212) is a sorbitol operon. This region is 5,120 bp in length and encodes six ORFs (ORF S17-ORF S22) that are flanked by 6 bp imperfect IR, IR9 and IR10. See Tables 3.15 and 3.17. The sorbitol operon is a phosphotransferase system which transfers sorbitol into the bacterial cell. The operon consists of *srlD, srlM, srlR, srlA, srlE* and *srlB* genes. It was most homologous with the sorbitol operon of *S. carnosus* strain TM300, which encodes six ORFs (ORFSca2317-ORFSca2322) on a chromosomal region (not a SCC element), with nucleotide homology ranging between 81% and 69%. See Figure 3.16.

Adjacent to the right hand end of the SCCsorbitol was a 276 bp ORF S23, with homologies of 83% (nt 30,918 to nt 31,659) and 91% (nt 30,749 to nt 30,906) with the nitrilotriacetate mono-oxidase component A genes of *S. hominis* C80 and *S. hominis* SK119 respectively. Given that the nitrilotriacetate mono-oxidase component A gene of *S. hominis* SK119 is 1,301 bp in size, ORF S23 probably represents a truncated form of the gene.

Table 3.17 The Open Reading Frames of ΨSCC*pls* and SCCsorbitol

Genetic Region	ORF Name	Position (nt)	Size (bp)	Identity (%)	Homology	Information
orfX	ORF P01	1-480	480	99%	*S.aureus*	Conserved Hypothetical Protein *orfX*
Repeat Region	DR-1	462-476	15	80% 100%	NCTC8325; M08/0126 (ΔJ1SCC*mec*I)	Direct Repeat ΨSCC*pls*L
	IR-1	466-472	7	100%	M08/0126 (ΔJ1SCC*mec*I)	Inverted Repeat ΨSCC*pls*L
ΨSCC*pls*	ORF P02	Complement 1,030-1,263	234	100%	BCM-HMP0060; M08/0126	Hypothetical protein
	ORF P03/ *pls*	1,500-5,972	4473	98% 95% 93%	BCM-HMP0060 ORF1721; M08/0126; NCTC10442/COL	*pls* gene
	ORF P04	6,057-7,712	1656	100% 100% 99%	BCM-HMP0060 ORF1722; M08/0126; NCTC10442/COL	Hypothetical Protein
	ORF P05	Complement 7,872-9,383	1512	100% 99% 99%	BCM-HMP0060 ORF1723; M08/0126; NCTC10442/COL	Hypothetical Protein Glycosyltransferase (DUF1975); pfam09318
	ORF P06	Complement 9,406-11,064	1659	100% 100% 99%	BCM-HMP0060 ORF1724; M08/0126; NCTC10442/COL	Hypothetical Protein Mur ligase middle domain; pfam08245
	ORF P07	Complement 11,058-11,456	399	100% 99% 99%	BCM-HMP0060 ORF1725; M08/0126; NCTC10442/COL	Hypothetical Protein PRK11929
	ORF P08	11,744-11,866	123	100%	BCM-HMP0060 ORF1726; M08/0126; NCTC10442/COL	Hypothetical Protein
	ORF P09	Complement 11,932-12,060	129	100%	BCM-HMP0060 ORF1727; M08/0126; NCTC10442/COL	Hypothetical Protein
Repeat Region	IR-2	12,191-12,197	7			Inverted Repeat ΨSCC*pls*R
	IR-7	12,172-12,184	13	100%	ATCC 12228	Inverted Repeat SCCsorbitol
	DR-5	12,174-12,212	39			Direct Repeat SCCsorbitolL
	DR-2	12,198-12,212	15	87%	NCTC8325	Direct Repeat ΨSCC*pls*R
	IR-3	12,202-12,224	23			Inverted Repeat SCCsorbitolL
	IR-8	12,214-12,226	13	100%	ATCC 12228	Inverted Repeat SCCsorbitol
SCCsorbitol	ORF S01/ *hsdR*	12,317-12,460	144	100%	ATCC 12228 SE0043; M1	Type I Restriction Enzyme R Protein
	ORF S02/ *hsdS*	12,453-13,655	1203	100%	ATCC 12228 SE0044; M1	Type I Restriction Enzyme S subunit
	ORF S03	Complement 13,659-13,910	252	100%	ATCC 12228 SE0045; M1	Truncated transposase (80% homology with IS*1272* of *S. haemolyticus*)
	ORF S04	Complement 13,978-14,475	498	100%	ATCC 12228 SE0046	Spermidine acetyltransferase

Table 3.17 The Open Reading Frames of ΨSCC*pls* and SCCsorbitol (Continued)

Genetic Region	ORF Name	Position (nt)	Size (bp)	Identity (%)	Homology	Information
SCCsorbitol	ORF S05	Complement 14,754-14,882	129	100% 100% 98%	ATCC 12228 SE0047; M1; ATCC 12228 SE0027	Hypothetical Protein
	ORF S06	Complement 15,147-15,557	411	100%	ATCC 12228 SE0048; M1	Hypothetical Protein pfam 11070 DUF 2871
	ORF S07	Complement 15,865-15970	108	100%	ATCC 12228 SE0049; M1	Hypothetical Protein
	ORF S08	Complement 15,955-16,458	504	99% 99% 99% 88%	ATCC 12228 SE0030; ATCC 12228 SE0052; M1; M08/0126 ORFC10	Hypothetical Protein
ccr Gene Complex	ORF S09	Complement 16,474-16,785	312	100% 95% 95% 91%	M1; ATCC 12228 SE0031; ATCC 12228 SE0053; M08/0126 ORFC11	Hypothetical Protein
	ORF S10	Complement 16,787-16,879	93	100% 86% 86%	M1; ATCC 12228 SE0032; ATCC 12228 SE0054	Hypothetical Protein
	ORF S11	Complement 16,872-17,222	351	87% 85% 85% 84%	M1; ATCC 12228 SE0033; ATCC12228 SE0055; M08/126 ORFC12	Hypothetical Protein pfam 06106 DUF 950
	ORF S12/ *ccrB2*	Complement 17,746-19,395	1650	95-99%	Various type II & IV SCC*mec*s	Cassette chromosome recombinase B *ccrB2*
	ORF S13/ *ccrA2*	Complement 19,396-20,763	1368	95-99%	Various type II & IV SCC*mec*s	Cassette chromosome recombinase A *ccrA2*
	ORF S14	Complement 20,979-22,772	1794	96% 90% 90% 90%	M1; M08/0126 ORF C15; ATCC 12228 SE0038; ATCC 12228 SE0058	Hypothetical Protein DUF 927
	ORF S15	Complement 22,766-23,077	312	98% 84%	M08/0126 ORFC16; ATCC 12228 SE0039	Hypothetical Protein
	ORF S16	Complement 23,234-24,772	1538	95% 92%	SCC*mec* I COL M08/0126 ORFC17	Hypothetical Protein
Repeat Region	IR-9	25,085-25,091	6			Inverted Repeat Sorbitol Operon L
Sorbitol operon	ORF S17 *srlB*	Complement 25,092-25,445	354	69%	TM300 Sca2322	Putative sorbitol phosphotransferase system EIIA
	ORF S18 *srlE*	Complement 25,461-26,486	1026	77%	TM300 Sca2321	Putative sorbitol phosphotransferase system EIIBC
	ORF S19 *srlA*	Complement 26,498-27,145	648	80%	TM300 Sca2320	Putative sorbitol phosphotransferase system EIIC

Table 3.17 The Open Reading Frames of ΨSCC*pls* and SCCsorbitol (Continued)

Genetic Region	ORF Name	Position (nt)	Size (bp)	Identity (%)	Homology	Information
Sorbitol operonl	ORF S20 *srlR*	Complement 27,090-27,515	426	73%	TM300 Sca2319	Sorbitol operon activator
	ORF S21 *srlM*	Complement 27,508-29,052	1545	74%	TM300 Sca2318	Putative regulator of sorbitol operon
	ORF S22 *srlD*	Complement 29,418-30,212	795	73%	TM300 Sca2317	Putative sorbitol-6-phosphate 2- dehydrogenase
Repeat Region	IR-10	30,216-30,222	6			Inverted Repeat Sorbitol Operon R
SCCsorbitol	ORF S23	Complement 30,667-30,942	276	81%	C80 SK119 ORF0518	Nitrilotriacetate monooxygenase
Repeat Region	IR-4	31,671-31,694	24			Inverted Repeats SCCsorbitolR
	DR-6	31,671-31,709	39			Direct Repeat SCCsorbitolR
	DR-4	72,984-72,998	15			Direct Repeat SCC*mec*R
	DR-3	31,695-31,709	15	93%	NCTC8325	Direct Repeat SCC*mec*L
	IR-5	31,700-31,722	23			Inverted Repeat SCC*mec*L

Abbreviations: DUF, Domain of unknown function; L, left repeat; R, right repeat; Accession Numbers: *S. epidermidis* BCM-HMP0060, NZ_GG696727.1; *S. epidermidis* ATCC 12228, AE015929; ACME composite island SCC*mec* IVh strain M08/0126, FR753166.1; ACME-SCC*mec* IVa strain M1, HM030720.1; *S. carnosus* strain TM300, AM295250.1; *S. hominis* strain C80, ACRM1000005.1; *S. hominis* strain SK119, NZ_ACLP01000020.1.

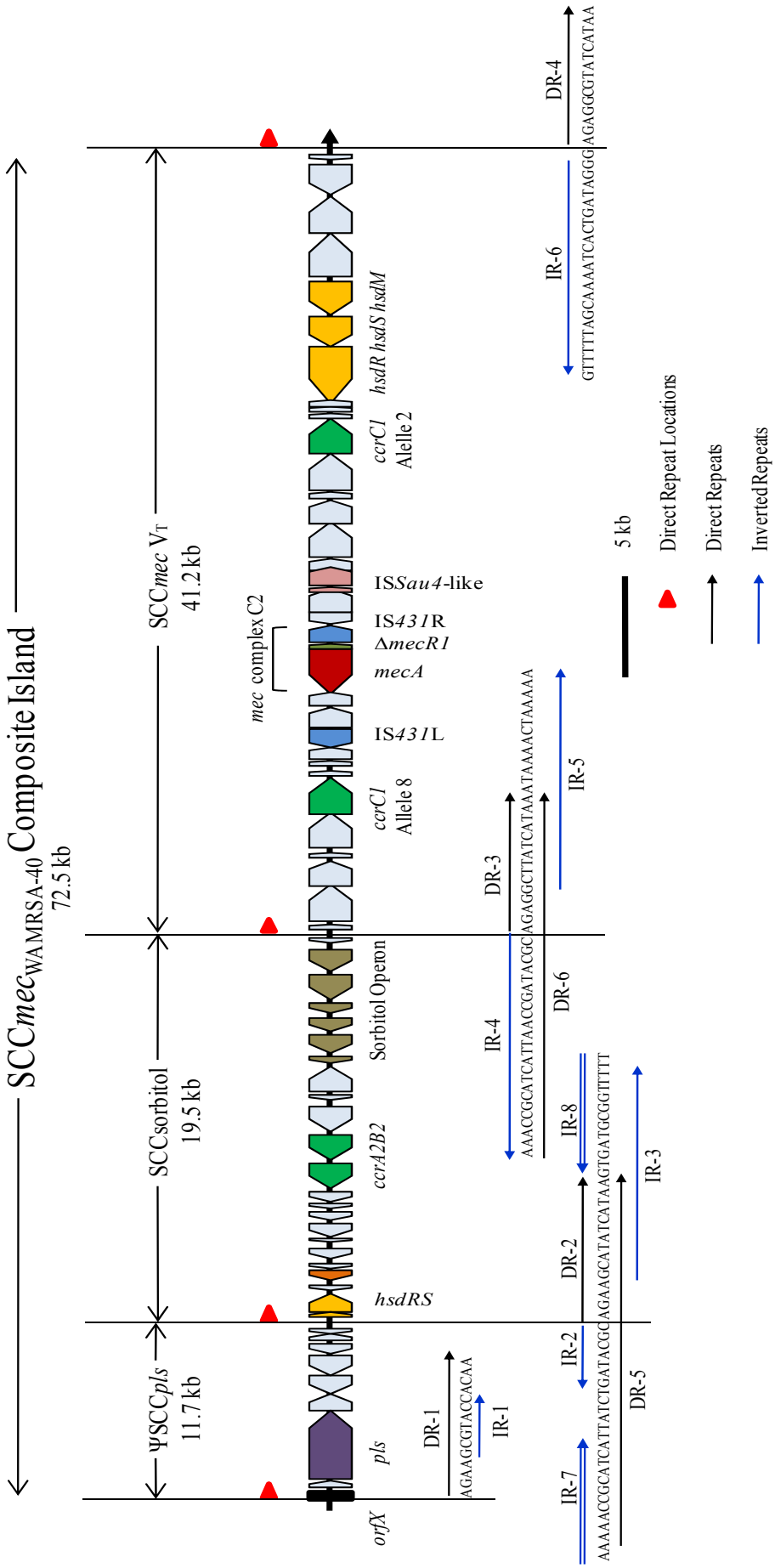

Figure 3.18 The SCC*mec*-Composite Island of WA MRSA-40

Chapter Four

Discussion

4.1 Staphylococcal Protein A Typing

Staphylococcal protein A or, *spa,* typing was found to be useful for typing WA CA-MRSA and has consequently been established as a routine typing technique by the ACCESS Typing and Research Unit.

The *spa* gene is 2,150 bp in length and is comprised of distinct regions characteristic of surface proteins, including a starting signal peptide, regions of repeats and a LPXTG motif in the membrane-spanning region of the C-terminus. The polymorphic X region, which is embedded in the cell wall, consists of a variable number of 24 bp repeats. The diversity of this short-sequence repeat (SSR) region occurs as a result of deletion or duplication of repetitive units and by point mutation. Sequence analysis of the repeat region of the *spa* gene is considered a valuable typing technique and has been found to be comparable to the traditional methods of MLST and PFGE (Shopsin et al. 1999).

The *spa* typing results for the CA-MRSA in this study revealed significant diversity with 28 different *spa* types seen among the 45 clones. *spa* type t002 was the most prevalent (7/45). One isolate, WA MRSA-8 (ST75), was considered not typable due to poor sequence quality. This isolate belongs to the clonal complex 75 lineage and is believed to have its origins in the remote Indigenous communities in the northern regions of the Northern Territory of Australia. The strain is considered to be a phylogenetically distinct subspecies of *S. aureus* and is awaiting formal classification under taxonomic guidelines (Ng et al. 2009). MLST of CC75 strains has revealed a high degree of sequence divergence of the MLST

loci (Moneke, Slickers, and Ehricht 2008; Monecke et al. 2010) and this study now shows that the *spa* locus is also divergent.

An association with *spa* type and lineage was demonstrated and is presented in Table 3.2, where MLST types and clonal complex groups coincided with spa identities. This is especially apparent when comparing the *spa* types using the Kreiswirth letter codes or the Ridom Number codes.

Typing the *spa* gene provided useful information about the *S. aureus* lineages and possessed significant advantages over existing molecular typing procedures, such as MLST and PFGE. These advantages include increased speed, ease of performance, ease of interpretation, ability to create a database, ability to classify isolates into clonal complexes because of a correlation with MLST, and a high degree of reproducibility. Detection of the micro-variation provided by *spa* typing could be useful in outbreak situations, as with PFGE, and could be useful in phylogenetic population analysis, as with MLST (Koreen et al. 2003).

4.2 Ciprofloxacin Resistance in WA CA-MRSA

At the initiation of this study it was unusual to find ciprofloxacin resistance in CA-MRSA. Ciprofloxacin is a fluoroquinolone with broad-spectrum activity. Resistance to ciprofloxacin in *S. aureus* thus far has been due to chromosomal mutations within the DNA gyrase genes (*gyrA* and *gyrB*) and/or topoisomerase IV genes (*grlA* and *grlB*). This resistance occurs in a stepwise manner resulting from the combination of single point mutations within the *grlA, grlB* and *gyrA* genes (Schmitz, Jones, et al. 1998).

A subset of eight isolates that were tested in this study were phenotypically high-level ciprofloxacin resistant. Sequence analysis of mutations in the four genes

associated with ciprofloxacin resistance revealed that all eight isolates contained the amino acid changes Ser→Phe (C→T) or Ser→Tyr (C→A) at codon 80 in *grlA* and Ser→Leu (C→T) at codon 84 in *gyrA*. This combination of chromosomal mutations is known to result in high-level ciprofloxacin resistance (Schmitz, Jones, et al. 1998). Additional mutations seen in WA MRSA-19 at codon 471 (Glu→Lys) and WA MRSA-27 at codon 84 (Glu→Gly) have not been reported to be responsible for high-level ciprofloxacin resistance but could possibly result in low-level resistance when not in combination with the *grlA* codon 80 and *gyrA* codon 84 base pair mutations. WA MRSA-19 and -27, are probably showing evidence of the stepwise process to ciprofloxacin resistance that results when an isolate first develops low-level resistance and then, high-level resistance due to continuing selective pressures or burdens (Schmitz, Hofmann, et al. 1998).

In this study three previously unreported point mutations resulting in putative amino acid changes were found, two of which are possibly associated with ciprofloxacin resistance. These were a mutation of Glu→Lys at codon 471 in *grlB* and Glu→Gly at codon 84 in WA MRSA-19. The silent mutation of Leu→Leu at codon 126 of *grlA* of WA MRSA-27 is unlikely to be associated with ciprofloxacin resistance.

WA MRSA-27 contained the most mutations with a total of four. This isolate was subsequently found to be the HA-MRSA, ST5-MRSA-II New York/Japan clone. The increased number of mutations found in this isolate may be due to frequent exposure to ciprofloxacin or other fluoroquinolones.

Use of ciprofloxacin and other antimicrobials of the fluoroquinolone group are restricted in the community with their usage generally limited to hospitals. CA-MRSA isolates are therefore more likely to develop resistance to ciprofloxacin when patients are hospitalised or been previously treated for other infections involving organisms such as Gram-negative bacteria where a fluoroquinolone was used in the course of treatment (Schmitz, Hofmann, et al. 1998; Schmitz, Jones, et al. 1998).

4.3 Novel SCC*mec*s

The SCC*mec* is classified according to the class of *mec* complex and the type of *ccr* gene complex and sub-typed according to the elements present in the J regions. Several PCR methods have been developed to determine these components of the SCC*mec* (Ito et al. 2001; Lim et al. 2003; Oliveira and De Lencastre 2002; Zhang et al. 2005; Kondo et al. 2007). Throughout the time period of this study it has become evident that the SCC*mec* is evolving rapidly in WA and new heterogeneous structures are appearing (Coombs et al. 2011).

The SCC*mec*s of a subset of eight WA CA-MRSA isolates that had novel classifications were analysed. The SCC*mec*s of four isolates, WA MRSA-16, -27, -28 and -42 were resolved into existing SCC*mec* types. WA MRSA-26 encoded a type IV (2B) structural variant. WA MRSA-18 and -21 remain novel and the SCC region of WA MRSA-40 was completely sequenced.

The SCC*mec* of WA MRSA-16 contained a class A *mec* complex and a *ccrA4B4* gene complex, enabling it to be classified as the newly described SCC*mec* type VIII (Zhang et al. 2008). The presence of various structural elements in the J regions, possibly originating from other SCC*mec* types, emphasised the heterogeneous nature of the SCC*mec*. Regions of homology with SCC*mec* types I (1B) and III (3A) were detected which may indicate that WA MRSA-16 is a sub-type of SCC*mec* type VIII that has not yet been described.

The SCC*mec* of WA MRSA-18 contained a class B *mec* complex. The *ccr* gene complex was not typable and the multiplex PCR utilising the method of Zhang, K et al. 2005, detected elements of a type IVa SCC*mec*, while the multiplex PCR utilising the method of Oliveira, D and de Lencastre, H. 2002, indicated that the characteristic *dcs* region was absent. This is a novel SCC*mec* that requires complete sequencing.

The SCC*mec* of WA MRSA-21 contained a class B *mec* complex. The *ccr* gene complex was not typable although structural elements were detected that indicated

some regions of homology with SCC*mec* types I and III. This also is a novel SCC*mec* that requires sequencing.

WA MRSA-26 contained a class B *mec* complex and *ccrA2B2* gene complex, which classified it as SCC*mec* type IV. The inability to sub-type the type IV with existing primers, appears to be due to the presence of structural elements with regions of homology with SCC*mec* type III. As these have not been found in already characterised type IVs, this indicates that WA MRSA-26 is a SCC*mec* IV variant.

The SCC*mec* of WA MRSA-27 contained a class A *mec* complex and *ccrA2B2* gene complex, which classified it as SCC*mec* type II. The presence of type II structural elements also confirmed this finding. SCC*mec* II is generally considered to be associated with HA-MRSA and not CA-MRSA. Due to this finding, WA MRSA-27 was removed from the ACCESS database of CA-MRSA panel organisms and renamed ST5-MRSA-II (2A), New York/Japan epidemic clone. In May 2005 an outbreak of the New York/Japan MRSA clone occurred in Western Australia involving 31 isolates, with 25 epidemiologically linked to a colonised healthcare worker who had previously been hospitalised in the USA (Coombs et al. 2007). The isolate that was previously designated WA MRSA-27 was collected at the time of the outbreak, May 2005, and would have been included in the study if it had been recognised as a New York/Japan MRSA clone at that time. Although molecular typing revealed that WA MRSA-27 was the New York/Japan clone, an epidemiological link to the outbreak strain carried by the health care worker was not apparent.

The SCC*mec* of WA MRSA-28 contained a class A *mec* complex and *ccrA4B4* gene complex, which classified it into a SCC*mec* type VIII. Structural elements possessed homology with regions of SCC*mec* types I and IVa. These features indicate that this SCC*mec* could also be a sub-type of VIII. Our results suggest that WA MRSA-28 and WA MRSA-16 encode novel, but different sub-types of the type VIII SCC*mec*.

The SCC*mec* of WA MRSA-40 could not be classified. It contained a class C2 *mec* complex with both *ccrA2B2* and *ccrC* gene complexes detected and structural elements showed homology with regions of SCC*mec* type III.

The SCC*mec* of WA MRSA-42 contained a class B *mec* complex with a *ccrA2B2* gene complex which classified it as SCC*mec* type IV. The presence of the structural element *dcs* confirmed that WA MRSA-42 carried a SCC*mec* IVa.

Analysis of the SCC*mec*s of WA CA-MRSA isolates with untypable SCC*mec*s by routine PCR primers revealed that while in most cases the core elements could be defined the composition of the J regions was heterogeneous. It is apparent that these regions readily incorporate genetic material from other sources allowing for variants of SCC*mec* and that this probably facilitates the ability of CA-MRSA to adapt to different conditions in the community environment.

4.4 SCC*mec* Region of WA MRSA-40

WA MRSA-40 was isolated from a foot wound of an 89 year old male in a nursing home. The isolate was characterised as ST835, clonal complex 5, spa type t002, and was resistant to ciprofloxacin. SCC*mec* typing revealed that the SCC*mec* was type V_T (5C2&5) with an extra *ccrA2B2* gene complex. Because several of these isolates had been found and they appeared to be epidemiologically significant in WA, it was decided to completely sequence the SCC*mec* region. To date, four clones with a SCC*mec* type V_T (5C2&5) have been isolated in WA ST835, ST59, ST952 and ST5 (ACCESS 2011) suggesting that this SCC*mec* is quite mobile.

4.4.1 SCC and SCC-like Elements of WA MRSA-40

Sequencing the entire SCC*mec* Composite Island of WA MRSA-40 has enabled the full characterisation of its SCC and SCC*mec* components. The location of the *ccrA2B2* gene complex was found to be in an SCC-like element outside the SCC*mec*. It has been reported that *ccr* genes are also located on non-*mec* containing SCC elements where they have a role in the incision and excision of the elements. SCC elements and the SCC*mec* integrate into *orfX* at the same attachment site (Mongkolrattanothai et al. 2004; Holden et al. 2004) and SCC elements have been found integrated in tandem with the SCC*mec* in MRSA, upstream as with the element SCC*far,* and downstream of the SCC*mec* in USA300 with the ACME element (Holden et al. 2004; An Diep et al. 2006). The ACME element has recently also been reported integrated into *orfX* upstream of the SCC*mec* (Bartels et al. 2011; Shore et al. 2011). SCC elements have also been found in other staphylococcal species. *S. epidermidis* strain ATCC12228, carries an SCC-composite island that contains a SCC*pbp4* encoding *pbp4* (penicillin-binding protein 4) and *tagF* (teichoic acid biosynthesis protein) genes and a *ccrA2B2* gene complex. Adjacent to this is another SCC element that encodes *ccrA4B4*, restriction modification genes, genes mediating resistance to heavy metals and a type II ACME complex (Mongkolrattanothai et al. 2004).

The SCC-like element of WA MRSA-40 contained a region homologous to the ΔJ1 region of SCC*mec* type I, it encodes a *pls* gene and was designated ΨSCC*pls*. The gene ORF P03 (Table 3.17), which encodes a surface-membrane protein, was characterised as *pls* due to 100% homology with the LPXTG motif sorting signal and its following translated amino acid residues (Mazmanian, Ton-That, and Schneewind 2001). See Figure 3.15.

The *pls* gene of WA MRSA-40 was 100% homologous with ORF 1721 of *S. epidermidis* strain BCM-HMP0060. Homology was also found with the ACME-Composite Island strain M08/0126 which contains the same ΔJ1 SCC*mec* I region. While most of the *pls* gene of WA MRSA-40 showed high homology with

strain M08/0126, there were polymorphisms present in the repeat regions especially in the second and third repeat regions (R2 and R3).

The *pls* gene has been found on mobile elements including the SCC*mec* and plasmids of both animal and human origin (Accession Nos. GQ900485 and GQ900474 respectively) in several lineages of MRSA.

Adjacent to the right hand end of the ΨSCC*pls* was SCCsorbitol, which encoded a sorbitol operon, restriction modification genes and a *ccrA2B2* gene complex along with seven ORFs (Table 3.17). SCCsorbitol was flanked by unique DR sequences (DR-5 and DR-6). Following the IWG guidelines of Staphylococcal Chromosomal Cassette classification this element has been named SCCsorbitol (IWG-SCC 2009).

The sorbitol operon of the SCCsorbitol of WA MRSA-40 is comprised of six ORFs (*srlD, M, R, A, E, B*) that encode a sugar phosphotransferase system which transports sorbitol into the cell and mediates its phosphorylation. This is a multi-component system for the uptake of sorbitol into the bacterium with phosphoenolpyruvate as the source of energy. Once sorbitol is imported a phosphoryl group from phosphoenolpyruvate is transferred via a series of proteins encoded by the sorbitol operon. A concentration gradient is maintained that enables the further import of sorbitol through the transporter (Boyd et al. 2000; Saier and Reizer 1994). The first gene of the operon, *srlD*, encodes a protein that functions to convert D-sorbitol-diphosphate to D-fructose-6-phosphate. The *srlM* gene is a putative regulator of the sorbitol operon while the *srlR* encodes an activator protein. The *srlA, srlE* and *srlB* genes encode putative phosphotransferase permease enzymes (EII) (Boyd et al. 2000; Rosenstein et al. 2009). This is the first time that a sorbitol operon has been reported in a SCC element and only the second time that a sorbitol operon has been reported in a staphylococcus, with it previously being found in *S. carnosus* (Rosenstein et al. 2009). See Table 3.17. The sorbitol operon has a G+C content of 27.7% which is lower than the G+C content of the whole SCC*mec*$_{\text{WA MRSA-40}}$-CI, 30.9%. This low G+C content of the sorbitol operon suggests a possible interspecies gene transfer (Gill et al. 2005).

The SCCsorbitol could mediate an advantage to the bacterial cell by enabling it to utilise sorbitol as an energy source. Sorbitol is a common dietary sweetener which is used 3.7 times more frequently with elderly individuals as compared to 20-29 year olds (Boyd et al. 2000; Zanni Rde, Araújo, and Matinez-Mesa 2011). It is therefore probable that the index patient of WA MRSA-40, who was an elderly individual in a nursing home, could have had an exposure to dietary sorbitol. Also, increased production of sorbitol may occur as a complication of diabetes, where excess glucose is converted to sorbitol via an aldose reductase pathway (Nishikawa et al. 2000). This has been associated with cataract formation in the elderly (Lee, Chung, and Chung 1995).

The SCCsorbitol contained a truncated ORF (ORF S24) with 83-91% homology with a 276 bp portion of nitrilotriacetate monooxygenase component A of *S. hominis* strains C80 and SK119. Nitrilotriacetate is a synthetic chelator used in various domestic and industrial processing and decontamination procedures, such as textiles, paper and pulp processing and water treatment. Nitrilotriacetate is a non-phosphate alternative used in detergents, and is degradable by microbial processes (biodegradable) and the carbon, nitrogen and energy used by the bacteria (Cripps and Noble 1973). Nitrilotriacetate monooxygenase requires two component proteins, component A and B. Component A oxidises nitrilotriacetate using oxygen and component B is an oxidoreductase (Knobel, Egli, and Meer 1996). Component B was completely absent from SCCsorbitol.

4.4.2 SCC*mec* of WA MRSA-40

The SCC*mec* of WA MRSA-40 was thought to be novel due to the presence of both *ccrC* and *ccrA2B2* gene complexes. Complete sequencing of the SCC*mec* of WA MRSA-40 revealed that it carried a class C2 *mec* complex and two *ccrC1* gene complexes with *ccrC1,* alleles 8 and 2. Classification of the SCC*mec* of WA MRSA-40 according to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements designated it as SCC*mec*V_T (T

for Taiwan) or SCC*mec* V (5C2&5). When compared with the SCC*mec*V_T (5C2&5) sequence, the SCC*mec* of WA MRSA-40 was 99% homologous, with most of the polymorphisms occurring in the IS*431/257* elements.

The SCC*mec* of WA MRSA-40 was found to carry a class C2 *mec* complex with *mecI* deleted and *mecR1* truncated by insertion sequence IS*431* (IS*431*R). Deletion of *mecI* de-represses *mecA* and allows the expression of methicillin resistance (Katayama, Ito, and Hiramatsu 2001).

Analysis of the IS*431* elements within the *mec* region of WA MRSA-40 and comparison with the IS*431* elements of two other type V MRSA, the Taiwan CA-MRSA and WIS CA-MRSA, revealed that there is diversity among SCC*mec* type V isolates and the IS*431* element. Nonsense mutations at nucleotide positions 144, 150 and 400 of the WA MRSA-40 IS*431*R transposase gene created premature stop codons TAG, TGA and TAG respectively. The presence of these premature stop codons would prevent further transposase activity of IS*431*R, thus stabilising the *mec* complex (O'Brien et al. 2005). Both IS*431* elements in the *mec* region of WA MRSA-40 contained non-functioning transposases, with the same nonsense mutations in both IS*431*L and IS*431*R. When compared with the sequence of WIS CA-MRSA, a total of 15 polymorphisms were detected in the WA MRSA-40 transposase of IS*431*L, and 10 in IS*431*R. When compared with the Taiwan clone, the transposase of IS*431*R of both WA MRSA-40 and the Taiwan clone possessed the same polymorphisms that generated the same premature stop codons. Base pair and amino acid changes for WA MRSA-40 are recorded on Table 3.10.

The transposase of IS*431*L of the Taiwan CA-MRSA encoded an intact transposase even though a polymorphism was detected when compared with WIS CA-MRSA at nt 457 (G→C), but as this did not generate a stop codon the transposase would probably remain functional. See Figure 3.6.

IS*431* has been associated with transposition of genetic information amongst staphylococci, promoter activity and gene regulation (Leelaporn et al. 1994; Leelaporn et al. 1996). IS*431* is implicated in the transposition of plasmids and genes such as antimicrobial-resistance and virulence. Genes and plasmids that

have transposed are found to be flanked by *IS431* (Lyon and Skurray 1987; Kobayashi, Alam, and Urasawa 2001). This study now shows that it also possesses polymorphic variations. These variations demonstrate a genetic flexibility that probably enhances its utility as an important contributor to the evolutionary adaptability of *S. aureus*.

Two *ccrC1* allotype gene complexes were identified in the SCC*mec* of WA MRSA-40. The *ccrC1,* allele 8, found downstream or to the left of *mecA* and *ccrC1,* allele 2 found upstream of *mecA*. These *ccrC* alleles are in the same positions and orientation as those found in the SCC*mec* of the Taiwan CA-MRSA. The three ORFs flanking each side of the *ccrC1,* allele 8 gene, have a conserved *ccrC* region, designated the *ccrC*-carrying unit. The *ccrC1,* allele 3, of SCCmercury, the *ccrC1,* allele 9 of SCC*mec*$_{ZH47,}$ and the *ccrC1,* allele 8 of the Taiwan clone are also encoded in a similar *ccrC1*-carrying unit. The entire region from *att*B in *orfX* up to, and including, the carrying unit at ORF M10 (9,819 bp) of *ccrC1,* allele 8 of WA MRSA-40 was homologous with the Taiwan clone V_T (5C2&5), SCCmercury and SCC*mec*$_{ZH47}$ (Higuchi et al. 2008).

Although a *ccrA2B2* locus was found during PCR testing the sequence of the entire SCC*mec* did not reveal the presence of a *ccrA2B2* gene complex or homologue associated with the SCC*mec* of WA MRSA-40. Furthermore a junction between the left-hand end and *orfX* could not be found. This suggested that the *ccrA2B2* gene complex may exist in a SCC element adjacent to the SCC*mec*.

The insertion sequence IS*Sau4*-like was found in the J2 region inserted into ORF M19 which encodes a hypothetical protein of unknown function. This insertion sequence has no known function and the impact of this insertion is not known. It is 1,269 bp in size and had 100% homology with the insertion sequence found in V_T (5C2&5) of the Taiwan CA-MRSA. Frequently insertion sequences are found inserted into regulatory genes where they influence the expression of other genes and that may be its function in WA MRSA-40.

The left and right extremities of the SCC*mec* of WA MRSA-40 are flanked by characteristic DR sequences of 15 bp in length (DR-3 and DR-4) and inverted repeats of 23/24 bp in length (IR-5 and IR-6). See Table 3.15. The DR at the left hand end were identical to those of V_T (5C2&5) of the Taiwan CA-MRSA and there was one base pair difference in the DR (G→A) at the right hand end. The IR, IR-5 and IR-6 were identical to the IR of V_T (5C2&5) of the Taiwan clone at the corresponding ends. The junction at the left hand extremity of WA MRSA-40 was found to be adjacent to another SCC element.

Contained within the SCC*mec* are three mobile elements IS*431*L, IS*431*R and IS*Sau4*-like, that are flanked by IR. See Table 3.14. IS*431*L and IS*431*R are flanked by 15 bp IR while IS*Sau4*-like is flanked by 38 bp IR. IR define the boundary of a mobile element, such as an insertion sequence or transposon, and have a role in DNA recombination, rearrangement and gene amplification (Lin et al. 2001).

Overall, the SCC*mec* of WA MRSA-40 possessed 99-100% homology with the SCC*mec* of the Taiwan CA-MRSA, even though the strains are of different genetic lineages. WA MRSA-40 is clonal complex 5 and PVL negative while the Taiwan CA-MRSA is clonal complex 59 and PVL positive. As the same SCC*mec* type is in a different clonal complex type this indicates that the integration of the SCC*mec* into WA MRSA-40 was a separate genetic event.

4.4.3 The Formation of a SCC Element

The integration site of SCC*mec* and SCC-like elements into the bacterial chromosome is at *att*B in the 3' end of the hypothetical protein gene *orfX*. The boundaries of the SCC elements are demarcated by direct and, in most cases, also IR. Upon integration, the 15 bp DR of the integrating element attaches in *orfX* with the corresponding DR of *orfX* subsequently found at the right hand end of the

inserted element. Integration is mediated by the *ccr* gene complex (Ito, Katayama, and Hiramatsu 1999; Ito et al. 2003a).

The 11,736 bp ΨSCC*pls* element of WA MRSA-40, flanked by and including, DR-1 and DR-2, is highly homologous with a region within a possible SCC*mec*-ACME-CI of the *S. epidermidis* strain BCM-HMP0060. At the left hand end of the BCM-HMP0060 region, is a 15 bp DR sequence, with 12 bp similar to the DR at the integration site *att*B of *orfX*. It is possible that genetic DNA containing ΨSCC*pls* has been transferred from *S. epidermidis* BCM-HMP0060, or a related *S. epidermidis,* into WA MRSA-40 resulting in a recombination event with ΨSCC*pls* inserting into the *att*B of *orfX* to form the SCC element.

A nucleotide search of the genome of the strain BCM-HMP0060 found 15 matches of 11 bp or more which matched the 15 bp sequence of the *att*B site of the *orfX* gene. With a maximum ambiguity of four base pairs required for integration (Ito, Katayama, and Hiramatsu 1999), these 15 matches of DR homologues could also serve as *orfX att*B attachment sites and potentially form SCC elements. Further analysis revealed that of these 15 matches, nine were together with the flanking sequence that contained the characteristic IR sequences found at the SCC junctions. Some of these DR and IR occurrences could in fact be flanking already existing elements on the BCM-HMP0060 genome. This finding has further supported the hypothesis that *S. epidermidis* is a reservoir for genetic information for *S. aureus*.

The genetics of the ΨSCC*pls* and SCCsorbitol elements and their possible origin from coagulase-negative staphylococci (CoNS) such as *S. epidermidis* can offer an explanation as to the origin and formation of other SCC elements and the SCC*mec* and the heterogeneous nature of its sub-types. The SCC*mec*-composite island of WA MRSA-40 would have certainly resulted from a number of recombination events.

The SCC*mec* has been termed a “hot spot” for recombination with genetic information moving in and out of the element, as a survival strategy for the host cell. Coagulase-negative staphylococci are considered to be potential reservoirs of

antibiotic-resistance genes and genetic elements that are being shuttled between staphylococci by horizontal transfer (An Diep et al. 2006; Hanssen and Ericson Sollid 2006). Transfers resulting in recombination and rearrangement of the staphylococcal genome may provide the necessary genetic material for survival in a changing environment.

4.5 Achievements from this study

The following have been accomplished as a result of this study.

1. *spa* typing has been established as a useful molecular typing technique for MRSA and is now used routinely by the ACCESS Typing and Research Unit.
2. It has been revealed that ciprofloxacin resistance in WA CA-MRSA is due to chromosomal mutations and probably emerged in response to the exposure of CA-MRSA to ciprofloxacin or other fluoroquinolones in hospitals.
3. Four novel SCC*mec*s or SCC regions have been identified.
4. A new SCC*mec* composite island, SCC*mec*$_{WA\ MRSA\text{-}40}$-CI, has been sequenced.
5. A new SCC element, SCCsorbitol, has been described.
6. A possible mechanism for the formation of SCC elements and their distribution amongst staphylococcal species has been presented.

4.6 Further Studies

The results from this study have yielded valuable information and opened the possibility to explain one way that genetic transfer occurs. However nothing is known about how the SCC elements are transferred horizontally. Known methods

of genetic transfer in staphylococci need to be investigated to see if transfer can occur between candidate organisms in the laboratory.

spa typing was shown to be a valuable typing tool for MRSA. One isolate remained non-typable with the existing *spa* method and will require further investigation to determine if it is possible to improve the PCR reaction conditions to enable a typable sequence of WA MRSA-8, ST75-MRSA-IV to be produced. Other clones of the ST75 lineage also remain non-typable by the current *spa* typing method. These could also be possibly typable by adjusting PCR conditions and/or designing different primers.

The SCC*mec*s of WA MRSA-18 and WA MRSA-21 remain novel due to non-typable *ccr* gene complexes. Investigation of these regions will involve designing primers in the flanking ORFs either side of the *ccr* genes to attain a PCR product for sequencing of the entire *ccr* region. This would be followed by phylogenetic analysis with reference known *ccr* sequences to determine if they are related to known types or are new types of *ccr* sequences. Ultimately these SCC*mec*s require complete sequencing.

The formation of a SCC element and the rearrangement of the SCC*mec* to form sub-types require more study. An investigation of the role of DR and IR in SCC*mec* and SCC elements in the movement and arrangement of elements needs to be explored, involving genome scanning using the GenBank database and site directed mutagenesis directed at the repeat region. The movement of J1 regions among SCC*mec* types to form sub-types also may be related to a function of the flanking DR and/or IR sequences. Such as the J1 region of SCC*mec* IVb that occurs in SCC*mec* IIA. The role of CoNS, such as *S. epidermidis* and their contribution as a reservoir for potential SCC elements needs to be investigated, also using genome scanning to search for the occurrence of regions of sequence with homology with direct and inverted SCC-like repeats within genomes of CoNS and their potential role in the formation of SCC elements.

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