School of Biomedical Sciences

Molecular Characterisation of Malaysian Methicillin-Resistant *Staphylococcus aureus*

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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.

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ABSTRACT

Seventy-four methicillin-resistant *Staphylococcus aureus* (MRSA) from two Malaysian hospitals were characterised by both phenotypic and genotypic techniques. These isolates were collected over an 18 year time period in the years, 1982, 1989, 1994 and 2000.

All of the Malaysian MRSA isolates were found to be multiresistant and resistant to at least five different antimicrobial agents. Over 30% of them were non-typable by the International Basic Set of bacteriophages. The majority of the typable isolates were susceptible to the group III phages, especially phage 85.

The majority of the isolates carried one to six plasmids. Only two isolates were plasmid free. The plasmid profiles of these isolates, other than the 1982 isolates, were very similar to each other. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was used to examine the genetic relatedness of the isolates. Twenty-six CHEF patterns were found among the isolates. These CHEF patterns were closely related to each other. The predominant CHEF pattern A was found in the 1982, 1989 and 1994 isolates. The CHEF patterns of the year 2000 isolates were different to CHEF pattern A, but still closely related. All of the isolates were found to carry the Allotype III SCC*mec* and have coagulase-gene type 24. Multilocus sequence typing was preformed on the isolates with CHEF pattern A collected in different years. These isolates were found to have either sequence type 239 (ST239), or its single locus variant. The predominant Malaysian clone belongs to the pandemic clone ST239-MRSA-III that is pandemic in Asian countries. (Enright, 2003, Ko *et al.*, 2005)

A 1.5 kb cryptic plasmid found in Malaysian isolates was indistinguishable from a cryptic plasmid found in an Australian isolate. A 3.0 kb cryptic plasmid found in Malaysian isolates was undistinguishable from a 3.0 kb plasmid found in Singaporean isolates. Class II multiresistance plasmids of 28, 30.5 and 35 kb were commonly found together in many Malaysian MRSA isolates. Both the 28 and 30.5 kb plasmids encode resistance to the heavy-metals and nucleic acid-binding (NAB) compounds. The 35 kb plasmid carries heavy-metal and NAB resistance but also encodes β-lactamase. Structurally these three plasmids are almost identical and probably have the same origin. The differences observed between these plasmids is

probably due to excision or partial deletion of the β-lactamase transposon of the original plasmid. The 28 kb plasmid is identical to the 28 kb plasmid of Singaporean and some Australian isolates. A 20 kb plasmid in Indonesian isolates was found to be closely related to these three plasmids. A conjugative plasmid, pWBG707, conferring trimethoprim resistance was found in Malaysian isolates. It did not carry either of the two staphylococcal trimethoprim-resistance genes, *dfrA* and *dfrD*. (Lyon and Skurray, 1987, Dale *et al.*, 1995b) It either encodes a novel resistance gene or the recently discovered *dfrG* gene. (Sekiguchi *et al*., 2005) pWBG707 was also found to mobilise a small 3.0 kb kanamycin-resistance plasmid during conjugation.

The *mecR1* and *mecI* genes regulating the transcription of the methicillin-resistance gene, *mecA*, were also examined in the isolates. The Malaysian isolate, WBG7422, with the predominant CHEF pattern A has a nonsense mutation in its *mecI* gene that disables it. However, its *mecR1* gene is intact. The eastern Australia MRSA (EA MRSA), WBG525, has a CHEF pattern that is closely related to the Malaysian predominant CHEF pattern A and its *mecI* gene has a mutation identical to the Malaysian isolate. Unlike the Malaysian isolate however, its *mecR1* gene has a 166 bp deletion. Both WBG7422 and WBG525 express Class III heterogeneous methicillin resistance. However, WBG525 has more highly resistant cell in its population than WBG7422.

The loss of aminoglycoside resistance, together with *c*. 114 kb of chromosomal DNA, was observed in some Malaysian isolates. The deleted segment was found to carry the *aacA-aphD* gene that encodes a bifunctional aminoglycoside-modifying enzyme conferring resistance to many of the aminoglycosides.

The Malaysian isolates were compared with MRSA from different countries. These MRSA included 18 epidemic MRSA (EMRSA) from the United Kingdom, 15 Australian nosocomial MRSA, five classical MRSA, 22 community-acquired MRSA (CMRSA) from Australia and New Zealand and 46 nosocomial MRSAs from eight Asian-Pacific countries and South Africa. These Asian-Pacific countries were Australia, PR China, Hong Kong, Indonesia, Japan, Philippines, Singapore and Taiwan.

The CHEF patterns of most of the Asian-Pacific and South African isolates were closely related to the Malaysian isolates. Isolates from Singapore, Indonesia and Philippines were found to have an identical CHEF pattern to the Malaysian CHEF patterns A5. The Asian-Pacific and South African isolates, including the Malaysian isolates, were found to be closely related to EMRSA-1, -4 and -7. These EMRSA belong to the ST239-MRSA-III clone and are coagulase-gene type 24. The isolates from Japan were the only Asian-Pacific isolates not related to the other Asian-Pacific isolates and EMRSAs.

EMRSA-1 and EA MRSA have the same 166 bp deletion in their *mecR1* gene. Both of these strains have closely related CHEF patterns, the same sequence type, coagulase-gene type and SCC*mec*. These results indicate that these two strains belongs to the same clone and confirms the international spread of this clone in the early 1980s. However, the Malaysian isolates have CHEF patterns that are more closely related to EMRSA-4 than to EMRSA-1. Similar to the Malaysian isolates EMRSA-4 has an intact *mecR1* gene.

The CMRSA isolates were not related to any of the nosocomial MRSA. They also have very diverse genetic backgrounds but carry less diverse SCC*mec* allotypes. Most of the CMRSA carry either Allotype IV or V SCC*mec*

These results show that the spread of Malaysian MRSA is due to a single clonal expansion. Infection control measures would have to have been more efficient if this clone was to have been contained. The Malaysian epidemic clone is the Asian pandemic clone, ST239-MRSA-III. The Malaysian isolates and EMRSA-4 probably share the same ancestor. The presence of the same MRSA strain in Malaysian hospitals and in the hospitals of neighbouring countries indicates that the inter-hospital spread of an epidemic MRSA has occurred. This observation also suggests that the infection control measures in Malaysian hospitals have not been totally effective. The ineffectiveness of infection control has left Malaysian hospitals vulnerable to the future importation of new pandemic clones and/or highly virulent or resistant clones.

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ABBREVIATIONS

AAC	=	Aminoglycoside *N*-acetyltransferase
AAD	=	Aminoglycoside *O*-adenyltransferase
AFLP	=	Amplified-fragment length polymorphism
Amp	=	Ampicillin
ANT	=	Aminoglycoside *O*-nucleotidyltransferase
APH	=	Aminoglycoside *O*-phosphotransferase
AP-PCR	=	Arbitrarily primed PCR
Asa	=	Sodium arsenate
BHIA	=	Brain heart infusion agar
BHIB	=	Brain heart infusion broth
BHM	=	β-lactamase/heavy metal
BHM/NAB plasmid	=	BHM and NAB resistance plasmid
Bla	=	β-lactamase production
bp	=	Base pair
BURST algorithm	=	Based Upon Related Sequence Types algorithm
c.	=	*circa* about, approximately
CAT	=	Chloramphenicol acetyltransferase
CC	=	Clonal complex
CCC	=	Covalently closed circular
Cd	=	Cadmium acetate
CEM/NET	=	The Centre of Molecular Epidemiology and International Network
CFU	=	Colony-forming units
CG	=	Combined genotypes
CHEF	=	Contour-clamped homogeneous electric field
Cip	=	Ciprofloxacin
Clin	=	Clindamycin
Cm, C	=	Chloramphenicol
CMRSA	=	Community-acquired MRSA
CTAB	=	Cetyltrimethylammonium bromide
DBD	=	DNA-binding domains.
DD	=	Dimerisation domain.
DHFR	=	Dihydrofolate reductase
DHPS	=	Dihydropteroate synthase
DIG	=	Digoxigenin-11-dUTP
DR	=	Direct repeats
EA MRSA	=	eastern Australia MRSA
EDTA	=	Ethylene diamino tetra acetic acid
EF-G	=	Elongation factor G
Em, E	=	Erythromycin
EMRSA	=	Epidemic MRSA
EtBr, Eb	=	Ethidium bromide
Fa	=	Fusidic acid
FCM	=	Ultrasensitive flow cytometer
FIGE	=	Field-inversion gel electrophoresis
GISA	=	Glycopeptide intermediate *Staphylococcus aureus*
Gm, G	=	Gentamicin

ABBREVIATIONS continued

GR-MRSA	=	Gentamicin-resistant MRSA
GS-MRSA	=	Gentamicin-susceptible MRSA
Hg	=	Mercuric chloride
High-Mr PBP	=	High molecular mass PBP
HP water	=	High Pure water
HUKM	=	The Hospital of the University of Kebangsaan Malaysia
HUM	=	The University Hospital of the University of Malaya
IBS	=	International basic set of bacteriophages
IPTG	=	Isopropyl-β-thiogalactopyranoside
IR	=	Inverted repeats
IRD	=	Fluorescent dye
IRS	=	Isoleucyl-tRNA synthetase
IS	=	Insertion sequences
J region	=	Junkyard region
kb	=	Kilobases
Km, K	=	Kanamycin
LB	=	Luria broth
LBA	=	Luria broth agar
Lm, L	=	Lincomycin
Low-Mr PBP	=	Low molecular mass PBP
MCT	=	Mixed-culture transfer
MHA	=	Mueller-Hinton agar
Mi	=	Minocycline
MIC	=	Minimum inhibitory concentration
MLEE	=	Multilocus enzyme electrophoresis
MLS	=	Macrolides, lincosamides and streptogramins
MLS_B	=	Macrolides, lincosamides and streptograminB
MLST	=	Multilocus sequence typing
Mp	=	Mupirocin
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MSSA	=	Methicillin-sensitive *Staphylococcus aureus*
MS	=	Membrane-spanning
NAB	=	Nucleic acid-binding
NCCLS	=	National Committee for Clinical Laboratory Standards
Nm, N	=	Neomycin
nPB	=	Non-penicillin-binding
NSAR	=	The National Surveillance on Antibiotic Resistance program
NSW	=	New South Wales
NT	=	Northern Territory
nt	=	Nucleotide
Nv	=	Novobiocin
NZ	=	New Zealand
OC	=	Open circular
ORF	=	Open reading frame
PABA	=	*p*-aminobenzoic acid
PB	=	Penicillin-binding
PBP	=	Penicillin-binding protein
PCR	=	Polymerase chain reaction

ABBREVIATIONS continued

PEG	=	Polyethylene glycol
PFGE	=	Pulsed-field gel electrophoresis
pfu	=	Plaque-forming units
Pi	=	Propamidine isethionate
Pma	=	Phenyl mercuric acetate
PR China	=	The People's Republic of China
PVL	=	Panton-Valentine leukocidin
QAC	=	Quaternary ammonium compounds
QRDR	=	Quinolone resistance-determining region
RAPD	=	Randomly amplified polymorphic DNA
REAP	=	Restriction-endonuclease analysis of plasmids
Rep-PCR	=	Repetitive element sequence-based PCR
Rf	=	Rifampicin
RFLP	=	Restriction-fragment-length polymorphisms
RNase	=	Ribonuclease A
RTD	=	Routine test dilution
SA	=	South Australia
SCC	=	Staphylococcal cassette chromosome
SCC*mec*	=	Staphylococcal cassette chromosome *mec*
SDS	=	Sodium dodecyl sulphate
SgA	=	Streptogramin type A
SgB	=	Streptogramin type B
SLV	=	Single-locus variants
Sm, S	=	Streptomycin
Sp	=	Spectinomycin
SSTI	=	Skin and soft tissue infections
ST	=	Sequence type
STAR	=	*Staphylococcus aureus* repeats
Su	=	Sulphonamides
superscript *	=	Boderline resistance
superscript I	=	Inducible resistance
Sxt	=	Co-trimoxazole
Tc, T	=	Tetracycline
Tp	=	Trimethoprim
tRNA	=	Peptidyl-transfer RNA
TSA	=	Trypticase soy agar
TSB	=	Trypticase soy broth
UgpQ	=	Glycerophosphoryl diester phosphodiesterase
UK	=	United Kingdom
VIC	=	Victorian
VISA	=	Vancomycin intermediate *S. aureus*
Vm	=	Vancomycin
VRE	=	Vancomycin-resistant enterococci.
VRSA	=	vancomycin-resistant MRSA
WA	=	Western Australia
WA MRSA	=	Western Australia MRSA
WH	=	Winged-helix
WSPP	=	Western Samoan Phage Pattern

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CHAPTER ONE

LITERATURE REVIEW

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a member of the *Micrococcaceae* family. It is morphologically an immobile Gram-positive coccus that forms grape-like clusters due to its mode of cell replication. *S. aureus* can grow in both aerobic and anaerobic conditions, as well as in a high salt environment. It is differentiated from other staphylococci by being positive for coagulase and deoxyribonuclease tests and is able to ferment mannitol. (Murray *et al.*, 2003)

1.1.1 Genomic structure

Seven strains of *S. aureus* have been completely sequenced but only six of them have been published. (Kuroda *et al.*, 2001, Baba *et al.*, 2002, Hiramatsu *et al.*, 2001, Holden *et al.*, 2004, Gill *et al.*, 2005) The *S. aureus* genome is a circular chromosome that is approximately 2.8 Mbp in length and contains roughly 2600 open reading frames (ORFs). It has a low G+C content that ranges from 32.8% to 32.9%. *S. aureus* is phylogenetically related to the *Bacillus* spp based on ribosomal RNA sequences. Over 50% of the predicted proteins encoded in the *S. aureus* genome are highly similar to those encoded in *B. subtilis* and *B. halodurans.* The majority of these proteins encode housekeeping genes involved in metabolic pathways and DNA replication. The published *S. aureus* genomes are highly conserved with similarities ranging from 94.5% to 99%. MRSA252 (EMRSA-16) is the most diverse of the strains and 6% of its genome is unique. The differences in the genomes are due to mobile elements, which are mainly putative exogenous genes in the form of genomic islands. Eight genomic islands have been reported in the *S. aureus* genome. They consist of two groups. First are the pathogenicity islands that carry virulence genes such as those for toxin genes. The other one is the staphylococcal cassette chromosome *mec* (SCC*mec*), which carries antibiotic-resistance genes. In general, the *S. aureus* genome is composed of background

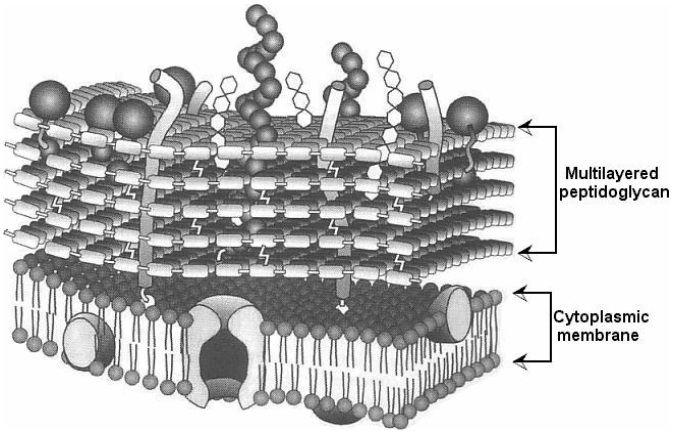
sequences vertically transmitted from a common ancestor with *Bacillus* spp, and exogenous sequences acquired via lateral genetic transfer from other sources. (Kuroda *et al.*, 2001, Baba *et al.*, 2002, Ito *et al.*, 2003a, Holden *et al.*, 2004)

1.1.2 Cell wall

The *S. aureus* cell wall is a homogeneous structure 20 to 40nm thick. The major component of the cell wall is peptidoglycan, which takes up 50% of the cell wall by weight. The peptidoglycan is a heteropolymer composed of murein monomers consisting of a disaccharide backbone, a stem peptide and a pentaglycine bridge. These monomers are synthesised in the cytoplasm and exported out via a lipid transport into the cytoplasmic membrane. (Lowy, 1998, Giesbrecht *et al.*, 1998, Hiramatsu, 2001)

The disaccharide backbone is composed of two amino sugars. They are the alternating β-1,4-linked units of *N*-acetylglucosamine and *N*-acetylmuramic acid. The monomers of the disaccharide backbones are joined together by transglycosylase enzymes to form a peptidoglycan chain. The *N*-acetylmuramic acids carry a tetrapeptide (stem peptide) consisting of L-alanine, D-glutamine, L-lysine and D-alanine. The stem peptides are then cross-linked to another stem peptide through a pentaglycine bridge. The pentaglycine bridge connects the ε-amino group of the L-lysine of one stem peptide to the D-alanine of another one. The cross-linking is catalysed by transpeptidase enzymes which belong to a group of cell-wall proteins known as the penicillin-binding proteins (PBP). (Giesbrecht *et al.*, 1998, Hiramatsu, 2001) Approximately 90% of the stem peptides are cross-linked through pentaglycine bridges. The uncross-linked stem peptides carry an additional D-alanine, which will be cleaved if a subsequent cross-linking reaction occurs. It has been found that approximately 20% of D-alanyl-D-alanine residues remain intact. The pentaglycines are unique to staphylococcal peptidoglycan. (Giesbrecht *et al.*, 1998, Hiramatsu, 2001) The structure and the chemical components of the staphylococcal cell wall are illustrated in Figure 1.1.

A



B

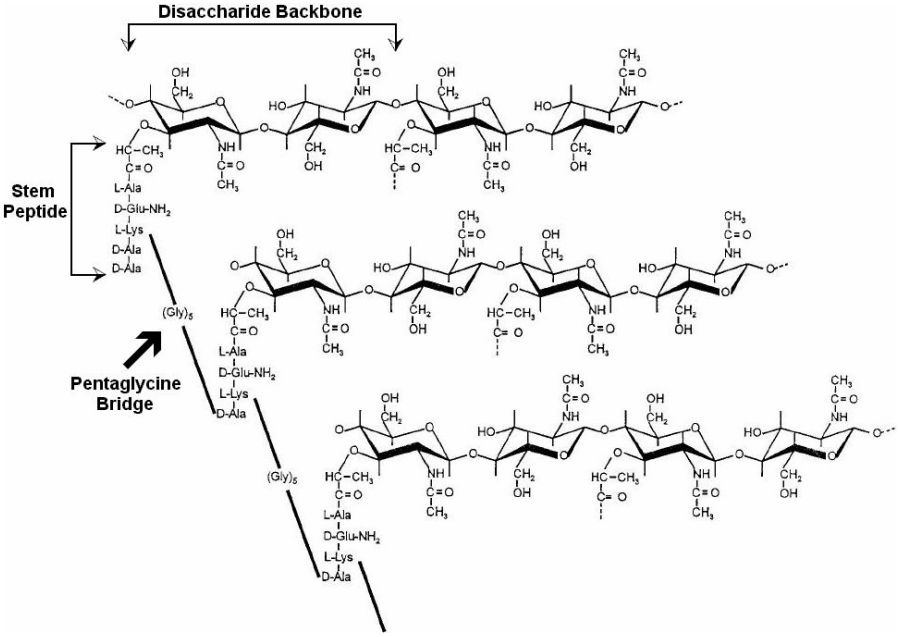


Figure 1.1 Schematic diagrams of a Gram-positive cell wall

A. Model of the Gram-positive cell wall covering the cytoplasmic membrane. Multiple layers of parallel peptidoglycan chains embedded with teichoic acid (rods), polysaccharides (hexagons) and protein (small and large spheres) cover the cytoplasmic membrane. (Coyette and Hancock, 2002)

B. The structure of *S. aureus* peptidoglycan. Three glycan strands of peptidoglycan composed of *N*-acetylglucosamines and *N*-acetylmuramic acids (disaccharide backbone). They are cross-linked with pentaglycine bridges on the stem peptides that branch out from the *N*-acetylmuramic acids. (Giesbrecht *et al.*, 1998)

The other major component of the cell wall is the teichoic acids. Ribitol teichoic acid is a polymer covalently bound to the muramic acid of the peptidoglycan via phosphodiester bonds. Lipoteichoic acid is the other teichoic acid polymer and it is anchored to the cytoplasmic membrane through a glycolipid terminus on the membrane. (Giesbrecht *et al.*, 1998, Lowy, 1998)

1.1.3 Epidemiology and pathogenicity

S. aureus is an extremely versatile pathogen that causes a wide range of infections which vary in their degree of severity. (Archer, 1998, Lowy, 1998) These infections can be generally divided into three types. The first type is those that cause superficial lesions like impetigo bullosa, furuncles, boils and wound infections. The second type causes systemic and life-threatening infections like bacteraemia, osteomyelitis, acute endocarditis, topical myositis and pneumonia. The third type causes the toxin-induced syndromes such as toxic-shock syndrome, scalded-skin syndrome and food-borne gastroenteritis. (Archer, 1998, Novick, 2000) An extensive array of virulent factors contribute to these diverse pathogenic mechanisms. These factors include a protein that interferes with opsonophagocytosis, enzymes that facilitate the spread of infection and toxins that cause specific toxinoses. A list of the known virulent factors is presented in Table 1.1.

S. aureus is carried asymptomatically by people, usually in their anterior nares, on the skin and mucous membranes. (Kluytmans *et al.*, 1997, Archer, 1998) Approximately 20% to 30% of the population are persistent carriers, who almost always carry *S. aureus*. Around 60% of the population intermittently harbour *S. aureus* on different occasions. On the other hand, approximately 20% of the population are noncarriers who almost never carry *S. aureus*. As a result, *S. aureus* is ubiquitously present in the human population, and as a result is found in the hospital environment. (Kluytmans *et al.*, 1997) In the 1997 to 2000 SENTRY nosocomial pathogens' surveillance program conducted worldwide, *S. aureus* was found to be the leading cause of nosocomial pneumonia, skin and soft tissue infections and bloodstream infection. (Bell and Turnidge, 2002, Diekema *et al.*,

2001, Doern *et al.*, 1999, Fluit *et al.*, 2001a, Pfaller *et al.*, 1998, Rennie *et al.*, 2003, Sader *et al.*, 1998, Hoban *et al.*, 2003)

The ability to accumulate antimicrobial resistance is an important factor contributing to the success of *S. aureus* in the hospital environment. *S. aureus* becomes resistant to antibiotics by mutations and by acquiring resistance genes through mobile elements like plasmids, transposons and the antibiotic-resistance genomic island, SCC*mec*. (Firth and Skurray, 2000, Lyon and Skurray, 1987, Skurray *et al.*, 1988) Methicillin-resistant *S. aureus* (MRSA) is usually multiply resistant. This is primarily because SCC*mec* in MRSA often carries more than one antimicrobial resistance genes. MRSA are resistant to all β-lactam antibiotics, but not all MRSA are resistant to other antimicrobial agents (ie. they are not multiply-resistant *S. aureus*). (Hiramatsu *et al.*, 2001, Livermore, 2000, Cookson, 2000, Jevons, 1961)

1.1.4 Methicillin-resistant *S. aureus*

The introduction of penicillin, a β-lactam antibiotic, in 1944 dramatically reduced the morbidity and mortality of severe *S. aureus* infections. However, penicillin resistance emerged quickly after its introduction. By 1948, 50% of hospital isolated *S. aureus* were resistant to penicillin and by the 1980s, more than 80% were resistant. *S. aureus* acquired resistance to penicillin is due to a hydrolysing enzyme, β-lactamase, that inactivates the drug. (Zygmunt *et al.*, 1992, Chambers, 1997) In 1959 a β-lactamase stable, semisynthetic penicillin, methicillin was introduced to treat severe *S. aures* infections. The first resistant strain was reported in 1961. (Jevons, 1961, Livermore, 2000) These MRSA have gradually disseminated in hospitals. By the late 1970s and early 1980s, MRSA became endemic in hospitals worldwide. (Pearman, 1996, Livermore, 2000) In the 1997 to 2000 SENTRY survey, the percentage of *S. aureus* hospital isolates that were MRSA ranged from 26.3% to 73.8%. (Diekema *et al.*, 2001, Bell and Turnidge, 2002, Fluit *et al.*, 2001b) MRSA have become resistant to virtually all-available systemic antibiotics, including the β-lactams, aminoglycosides, tetracyclines, macrolides and lincosamides. The antibiotic vancomycin became the only effective treatment for multiply-resistant MRSA infections. MRSA infections in the hospital significantly prolong the

patient's stay and increase the cost of treatment. In addition, they also increase the morbidity and mortality rate in hospitals. (Rubin *et al.*, 1999, Livermore, 2003)

Recently, MRSA has undergone two worrying developments. First is the emergence of vancomycin resistance in *S. aureus*. MRSA with intermediate resistance to vancomycin (MIC 8 μg/ml) were isolated in Japan in 1997. These strains are known as vancomycin-intermediate *S. aureus*. (VISA). VISAs are now being isolated in hospitals worldwide. (Hiramatsu *et al.*, 1997, Walsh and Howe, 2002, Trakulsomboon *et al.*, 2001, Kim *et al.*, 2000, Hageman *et al.*, 2001, Murray *et al.*, 2004) In 2002, a strain of MRSA with high-level vancomycin resistance (MIC 32 μg/ml) was isolated in a US hospital. This vancomycin-resistant *S. aureus* (VRSA) achieved vancomycin resistance by acquiring a vancomycin-resistance gene from a vancomycin-resistant *Enterococcus* spp. (VRE). (Tenover *et al.*, 2004) To date, VISA and VRSA have not caused any outbreaks or become endemic in hospitals. However, they have the potential to become established in hospitals world wide like MRSA have. (Hiramatsu, 2001, Walsh and Howe, 2002, Tenover *et al.*, 2004)

The other development is the emergence of community-acquired MRSA (CMRSA). Until recently MRSA have always been nosocomial organisms. In the early 1990s MRSA were isolated in community settings. In 1995, a single strain outbreak caused by CMRSA was reported in Royal Perth Hospital, Australia. This change in MRSA epidemiology could have a profound impact on the treatment provided by general practitioners to the community and infection control protocols in the hospitals. (Lim *et al.*, 2003, Okuma *et al.*, 2002, O'Brien *et al.*, 1999, Chambers, 2001, Cookson, 2000)

Table 1.1 Virulence factors of *S. aureus* and their proposed pathogenic mechanisms (Archer, 1998)

- **Thwart Host Defences**

Microcapsule
Protein A
Coagulase
Fatty acid - metabolising enzyme
Leukocidin and/or γ-toxin

- **Invade Tissue**

Proteases
Nucleases
Lipase
Hyaluronate lyase
Staphylokinase

- **Elicit Sepsis Syndrome**

Toxic shock syndrome toxin
Enterotoxins
Cytolytic toxins (α, β, γ, δ)

- **Induced Specific Toxinosis**

Toxic shock syndrome toxin
Enterotoxin
Exfoliative toxin

- **Attach to endothelial cells and basement membrane**

- **Binding proteins for fibrinogen, fibronectin, laminin, collagen, vitronectin, and thrombospondin**

1.2 Genetic basis of antimicrobial resistance in *S. aureus*

Antimicrobial resistance in *S. aureus* is mediated by either acquired exogenous or mutated endogenous genes. Often these exogenous resistance genes are carried and disseminated by mobile genetic elements such as transposons, plasmids and SCC*mec*. As these mobile elements can be readily transferred between staphylococcal species, they are thought to be responsible for the emergence of multiple-resistant *S. aureus* like MRSA. These antimicrobial agents and the corresponding resistance mechanisms are described in the following paragraphs and summarised in Table 1.3 and 1.4. (Lyon and Skurray, 1987, Firth and Skurray, 2000, Skurray and Firth, 1997)

1.2.1 Aminoglycosides

Aminoglycosides are a family of broad spectrum-antimicrobial agents. These large and highly polar molecules require energy dependent membrane transport to enter the cell. Once in the cytoplasm, they bind to the 30S subunit of ribosomal RNA. Although this aminoglycoside-ribosome interaction does not inhibit peptide synthesis, it causes the formation of abnormal proteins resulting in cell death. Some of these abnormal proteins may insert into the cell membrane and further enhance the uptake of aminoglycoside molecules. (Lyon and Skurray, 1987, Mingeot-Leclercq *et al.*, 1999, Berger-Bächi, 2002)

Aminoglycoside *O*-phosphotransferase (APH), aminoglycoside *N*-acetyltransferase (AAC) and aminoglycoside *O*-nucleotidyltransferase (ANT) are the three classes of drug modifying enzymes responsible for aminoglycoside resistance in *S. aureus*. ANT also known as the aminoglycoside *O*-adenylyltransferase, AAD. These enzymes are highly diverse and share little sequence homology among themselves. They reduce the aminoglycoside binding affinity by chemically modifying specific amino or hydroxyl groups on the drug molecules. These modifications also prevent high-level resistance by inhibiting the rapid uptake of the drug. The APH and ANT (AAD) utilise ATP to donate hydroxyl groups to the aminoglycoside in phosphorylation and nucleotidylation respectively. The AAC is an acetylCoA-dependent acetyltransferase and inactivates the drug by acetylation of the amino

groups. (Wright, 1999, Mingeot-Leclercq *et al.*, 1999) These modifying enzymes are often carried by plasmids, transposons as well as the genomic island, SCC*mec*. (Firth and Skurray, 2000, Skurray and Firth, 1997, Lyon and Skurray, 1987, Ito *et al.*, 2003a)

The AAC(6')-APH(2") bifunctional enzyme encoded by the *aacA-aphD* also known as *aac(6')-aph(2")* gene is the most common modifying enzyme responsible for high-level aminoglycoside resistance in *S. aureus*. Although it confers resistance to many aminoglycosides such as gentamicin, kanamycin, tobramycin and amikacin, it has no affects on aminoglycosides like, arbekacin, neomycin and streptomycin. (Wright, 1999, Davies and Wright, 1997, Mingeot-Leclercq *et al.*, 1999, Livermore, 2000, Daigle *et al.*, 1999) The *aacA-aphD* gene is carried by transposon Tn*4001* which is often plasmid borne and has also been found in SCC*mec*. (Rouch *et al.*, 1987, Berg *et al.*, 1998, Ito *et al.*, 2003a, Firth and Skurray, 2000, Skurray and Firth, 1997)

The *aphA-3* or *aph(3')-III* gene encodes APH(3')-III that confers resistance to amikacin, kanamycin and neomycin. The *aadE* or *ant(6*')-I gene encodes AAD(6') that confers high-level resistance against streptomycin. (Lyon and Skurray, 1987, Mingeot-Leclercq *et al.*, 1999, Woodford, 2005) The *sat4* gene encodes streptothricin acetyltransferase that confers streptothricin resistance. (Jacob *et al.*, 1994) These three aminoglycoside resistance genes are often found together as a gene cluster. In some *S. aureus* isolates, the *aadE-sat4-aphA-3* cluster is carried and disseminated by transposon Tn*5405*. (Derbise *et al.*, 1996, Derbise *et al.*, 1997a, Derbise *et al.*, 1997b, Werner *et al.*, 2001) In some instances, these genes have been found to be carried individually by plasmids. The *aphA-3* has been found either individually or with other aminoglycoside resistance genes on some medium-sized multiresistant and large conjugative plasmids. (Lyon and Skurray, 1987) The *str* streptomycin-resistance gene, similar to the *aadE* gene, has been found on the small plasmid pS194. (Projan *et al.*, 1988)

The small plasmid, pUB110, harbours AAD(4')(4") or ANT(4')-I encoded by the *aadD* or *ant(4')-I* gene which confers resistance to amikacin, kanamycin, neomycin and tobramycin. (Lyon and Skurray, 1987, McKenzie *et al.*, 1986, Matsumura *et al.*, 1984, Woodford, 2005) In addition to *aadD*, pUB110 also carries the *ble* gene that

confers resistance to the anticancer glycopeptide, bleomycin. (da Rocha *et al.*, 2001, Sugiyama *et al.*, 2002, Lyon and Skurray, 1987) pUB110 has been found integrated into the SCC*mec* as well as the conjugative plasmid pSK41, probably through the mediation of the insertion sequence IS*431*/*257*. (Ito *et al.*, 2003a, Berg *et al.*, 1998, Stewart *et al.*, 1994, Skurray and Firth, 1997) The *aadD* gene had also been found on other small multicopy and large conjugative plasmids. (Lyon and Skurray, 1987) The *aphC* or *aph(3")-I* gene is also a plasmid-borne gene that confers resistance to streptomycin via the APH(3")-I phosphotransferase. (Vakulenko and Mobashery, 2003, Woodford, 2005, Lyon and Skurray, 1987)

The *spc* or *ant(9)-I* gene which encodes an adenylyltransferase (nucleotidyltransferase) [AAD(9) or ANT(9)-I] confers resistance to spectinomycin. Spectinomycin is an aminocyclitol antibiotic related to the aminoglycosides. The *spc* gene in *S. aureus* is found in transposon, Tn*554*. (See Section 1.3.2.3.2). (Phillips and Novick, 1979, Murphy, 1990, Woodford, 2005) The other spectinomycin resistance gene found in *S. aureus* is *aadA*. The plasmid-borne *aadA* encodes AAD(3")(9) adenylyltransferase [ANT(3")(9) or ANT(3")-I nucleotidyltransferase], which confers resistance to streptomycin and spectinomycin. (Clark *et al.*, 1999, Lyon and Skurray, 1987, Woodford, 2005, Vakulenko and Mobashery, 2003)

In the 1997 European SENTRY antimicrobial-resistance-surveillance program, 68% of the 363 strains of aminoglycoside-resistant staphylococci were found to carry the *aacA-aphD* gene, 48% carried the *aadD* gene and 14% carried the *aphA-3* gene. Aminoglycoside resistance was closely linked with methicillin resistance as 94% of the MRSA carried at least one type of aminoglycoside resistance. In addition, 76% of the 191 MRSA isolates bear the *aacA-aphD* gene. (Schmitz *et al.*, 1999) In a study on Japanese MRSA, most of the 381 isolates carried *aacA-aphD* and *aadD* genes and only a small number of isolates carried *aphA*-3. This study also revealed that most of the isolates carried at least two of these aminoglycoside resistance genes and some even carried all three of these genes. (Ida *et al.*, 2001)

1.2.2 β-lactams

The β-lactams are structural analogues of D-alanyl-D-alanine on the stem peptides, which is the natural substrate of the PBPs. (Chambers, 2003) β-lactams achieve their bactericidal activity by covalently binding with the PBPs. The binding results in interference with the cell wall cross-linking, and leads to the formation of a deformed cell wall. The defective cell wall results in the cells not separating during division and the leaking out of the cytoplasm. These actions subsequently lead to cell death by bacteriolysis. (Giesbrecht *et al.*, 1998)

Two β-lactam resistance mechanisms have been found in *S. aureus*. They are the production of the β-lactam hydrolysing enzyme, β-lactamase, and acquisition of the low affinity PBP, PBP2a/2'. Genetically both of the mechanisms are related to each other. They also can co-exist in the same cell and can influence each others expression. (Lyon and Skurray, 1987, Berger-Bächi, 1999, Berger-Bächi, 2002)

1.2.2.1 β-lactamase

β-lactamase is very common in clinical isolates of *S. aureus*; almost 90% of them are β-lactamase producers. (Livermore, 2000) β-lactamases inactivate β-lactams by hydrolysing the β-lactam ring of the drug, which binds to the PBP. Four serotypes of β-lactamase, with different substrate profiles and amino acid sequences, have been reported in *S. aureus*. In spite of their differences, these β-lactamases are closely related and belong to the Class A serine β-lactamase family. Serotypes A, C and D β-lactamases are plasmid encoded, whereas the serotype B β-lactamase is only encoded on the chromosome. (Waxman and Strominger, 1983, Zygmunt *et al.*, 1992, Bush, 1997, Hall and Barlow, 2004, Voladri and Kernodle, 1998) β-lactamase is encoded by the *blaZ* gene, which is regulated by two upstream regulatory genes, *blaR1* and *blaI*. The region containing these three genes is also known as the *bla* operon and is carried by the transposon, Tn*552*. (Rowland and Dyke, 1989) The β-lactamase in *S. aureus* is often disseminated by plasmids carring Tn*552*. β-lactamase plasmids characteristically carry resistance to heavy metal ions such as mercury and cadmium, and/or, nuclei acid-binding compounds (see 1.2.13) such as antiseptics,

disinfectants and dyes. (Sidhu *et al.*, 2001, Sidhu *et al.*, 2002, Anthonisen *et al.*, 2002, Firth and Skurray, 2000) In some isolates Tn*552* has also been found integrated into the chromosome. (Townsend *et al.*, 1985c, Skurray *et al.*, 1988)

1.2.2.2 Methicillin resistance

Methicillin belongs to a class of β-lactams that is resistant to hydrolysis by β-lactamases. Methicillin resistance is conferred by a novel, low-affinity penicillin-binding protein, PBP2a or PBP2'. It not only confers resistance to methicillin but it also confers blanket resistance to all β-lactams due to its poor binding affinity to β-lactams. (Hartman and Tomasz, 1984, Brown and Reynolds, 1980, Georgopapadakou *et al.*, 1982) PBP2a/2' is encoded by the *mecA* gene which is carried by the SCC*mec* genomic island. Similar to *blaZ*, the *mecA* gene is regulated by two upstream regulatory genes, *mecR1* and *mecI*. The region containing these genes is known as the *mec* complex. (Song *et al.*, 1987, Suzuki *et al.*, 1992, Ito *et al.*, 2003a) The *mec* complex and SCC*mec* will be described in more detail in Section 1.3.4

1.2.2.2.1 Penicillin-binding proteins

PBPs belong to a superfamily of penicilloyl serine transferases, which are found in both Gram-positive and Gram-negative bacteria. (Massova and Mobashery, 1998) All PBPs have three structurally conserved motifs in their penicillin-binding domain that form the active serine-binding site. The active site has a serine-X-X-lysine (SXXK) motif in the centre of the binding cavity, where X is a variable amino acid. The serine residue is where the β-lactam molecule binds. There are two other conserved motifs, a serine or tyrosine-X-asparagine {S(Y)XN} and a lysine or histidine-threonine or serine-glycine {K(H)T(S)G} which are located at opposite sides of the cavity. The majority of PBPs are anchored on the cytoplasmic membrane, and the active serine site or the penicillin-binding domain is exposed and pointing towards the peptidoglycan in the periplasmic space. (Ghuysen, 1991) PBPs have diverse functions which involve the catalysis of cell wall synthesis, such as

transpeptidase, transglycosylase, carboxypeptidase and endopeptidase activities. (Chambers, 2003, Goffin and Ghuysen, 1998)

PBPs can be divided into two groups based on their molecular weight. The high molecular mass PBPs (High-Mr PBP) which have a molecular mass larger than 50kDa, and the low molecular mass PBPs (Low-Mr PBP). Both of the groups can be further divided into three classes based on the similarity of their amino acid sequences. (Chambers, 2003, Goffin and Ghuysen, 1998)

The High-Mr PBPs are multimodular molecules that have a penicillin-binding (PB) domain or transpeptidase domain and a non-penicillin-binding (nPB) domain. The PB domain is located on the amino-terminus (N-terminus) and the nPB domain is located on the carboxy-terminus (C-terminus). The Class A High-Mr PBPs are bifunctional enzymes, the PB domain has transpeptidase activity and is responsible for the cross-linking of the peptidoglycan with pentaglycine bridges. The nPB domain has transglycosylase activity and is responsible for the elongation of the glycan disaccharide backbone. The Class B High-Mr PBP has a transpeptidase PB domain and nPB domain with unknown function. (Chambers, 2003, Goffin and Ghuysen, 1998, Ghuysen, 1991) The Class C High-Mr PBPs are signal-transducing proteins, which have a PB domain and a zinc metalloprotease at the nPB domain that induces site-specific proteolysis of targeted represser proteins. (Zhang *et al.*, 2001, Chambers, 2003, Ghuysen, 1991)

The Low-Mr PBPs are structurally different from the High-Mr PBPs. Their PB domain is located at the N-terminus and they do not have an nPB domain. Their C-terminus has a transmembrane segment anchored to the cytoplasmic membrane. Some of the Low-Mr PBPs are non-membrane bound and do not have a transmembrane segment. (Ghuysen, 1991)

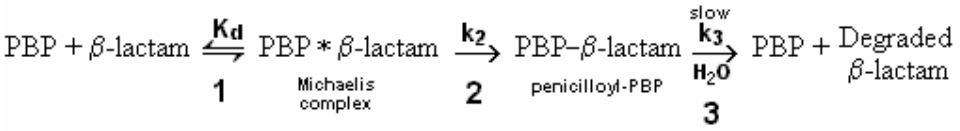
The majority of the Low-Mr PBPs are found to function as carboxypeptidases. This enzyme activity hydrolyses the D-alanyl-D-alanine peptide bonds of the peptidoglycan precursors. This hydrolysis controls the extent of peptidoglycan cross-linking by limiting the number of available stem peptides for transpeptidation. (Goffin and Ghuysen, 1998, Ghuysen, 1991) Some Low-Mr PBPs are found to act

as secondary transpeptidases that facilitate the further cross-linking of the newly incorporated strands. (Waxman and Strominger, 1983) Some of the Low-Mr PBPs in *E. coli* function as endopeptidases and cleave the peptidoglycan chains. They presumedly have a role in peptidoglycan recycling and cell division. (Goffin and Ghuysen, 1998, Massova and Mobashery, 1998, Giesbrecht *et al.*, 1998)

The β-lactamases, like Low-Mr PBPs, do not have an nPB domain. Unlike the other PBPs, they are secreted and are not involved in cell wall synthesis. They do not bind covalently with the β-lactam molecules and are able to hydrolyse the β-lactam molecule. (Ghuysen, 1991, Chambers, 2003)

1.2.2.2.2. Interaction between PBPs and β-lactams

The biochemical reaction between the PBPs and the β-lactam molecules proceeds according to the Michaelis-Menten kinetics in the following formula:



In the first reaction, the PBP non-covalently forms an unstable Michaelis complex with the β-lactam molecule. As the association is unstable, the complex can either dissociate or proceed to the second acylation reaction. The K_d constant is the rate of dissociation that is used to measure the stability of the complex. (Chambers, 2003, Waxman and Strominger, 1983)

In the second reaction, the hydroxyl group of the β-lactam is acylated at the active site serine residue and binds with the PBP active site by an ester bond. The cyclic amide bond of the β-lactam ring is also cleaved in this reaction. The final product is the stable penicilloyl-intermediate of the PBP (penicilloyl-PBP) and the β-lactam is irreversibly bound with the PBP by a covalent bond. The k_2 rate constant is used to indicate the rate of acylation. (Chambers, 2003, Waxman and Strominger, 1983)

In the natural setting, the natural substrate undergoes a rapid deacylation through hydrolysis by the carboxypeptidase or the cross-linking reaction of the transpeptidase. These reactions release the substrate and free the active serine site of PBP for further cell wall synthesis. This reaction is represented in the third reaction. (Chambers, 2003, Waxman and Strominger, 1983)

However, the penicilloyl-PBP blocks the cross-linking reaction and hydrolysis is very slow. This effectively halts the deacylation and inactivates the enzyme. The β-lactamases have high hydrolysis rates (k_3 rate constant) and undergo a rapid deacylation process and release the inactivated β-lactam with a ruptured β-lactam ring. (Chambers, 2003, Waxman and Strominger, 1983, Zygmunt *et al.*, 1992)

1.2.2.2.3 Staphylococcal penicillin-binding proteins

S. aureus has *c.* 1078 PBPs per cell in methicillin-sensitive strains, and *c.* 1935 PBPs per cell in methicillin-resistant strains. This is significantly less than *E. coli*, which has more than double the amount of PBPs per cell (*c.* 2700) of methicillin-sensitive *S. aureus.* (Pucci and Dougherty, 2002)

Six different PBPs have been identified in *S. aureus*, they are PBP1, PBP2, PBP3, PBP4, PBP2a/2' and PBP2B. PBP2a/2' has only been found in MRSA, and this is why the methicillin-resistant strains have more PBPs per cell than the methicillin-sensitive strains. These six PBPs have different molecular masses and DNA sequences and belong to different classes of the PBP superfamily. (Georgopapadakou *et al.*, 1982, Massova and Mobashery, 1998)

PBP1 belongs to the High-Mr Class B monofunctional PBP. It is 82.7kDa in molecular weight and composed of 744 amino acids. PBP1 is encoded by the chromosomal gene *pbpA* which is 2235bp in length. (Wada and Watanabe, 1998, Massova and Mobashery, 1998) PBP1 accounts for 17.1% of total PBPs per cell in methicillin-sensitive *S. aureus* (MSSA) and 8.6% in MRSA. (Pucci and Dougherty, 2002)

In previous studies, PBP1 has been found to be non-essential for cell growth. (Georgopapadakou and Liu, 1980, Waxman and Strominger, 1983) In a recent study, a strain with a disrupted *pbpA* gene could not survive when a vector harbouring a functional *pbpA* gene was removed. This observation has indicated that PBP1 is essential for cell survival. The function of PBP1 is not clear. It is thought to be a transpeptidase due to its amino acid sequence similarity with other Class B PBPs. It might be involved in the septum formation as it has a high degree of homology with *E. coli* PBP3, *B. subtulis* PBP2B and *S. pneumoniae* PBP2X amino acid sequences. (Wada and Watanabe, 1998)

PBP2 is a High-Mr Class A bifunctional PBP. Its PB domain is a transpeptidase and the nPB domain catalyses a transglycosylase reaction. (Massova and Mobashery, 1998, Pinho *et al.*, 2001a) PBP2 has a molecular weight of 81kDa and is composed of 727 amino acids. It is encoded by a 2184bp chromosomal gene, *pbpB* or *pbp2*. (Hackbarth *et al.*, 1995, Murakami *et al.*, 1994) The *pbp2* is found within an operon where a PBP-related factor gene *prfA*-like ORF is located upstream of the *pbp2* gene. The optimal transcription of *pbp2* gene requires both its own promoter and the promoter of *prfA*. (Pinho *et al.*, 1998) PBP2 is 42.8% of the total PBP per cell in MSSA and 23.2% in MRSA. (Pucci and Dougherty, 2002)

Cefotaxime specifically binds to PBP2 and causes cell lysis. This demonstrates that PBP2 is an essential peptidoglycan transpeptidase required for cell growth. (Georgopapadakou *et al.*, 1986) When point mutations were introduced downstream to the active serine site, SXXK motif of PBP2, the β-lactam binding affinity decreased by 2.5 fold (k_2 rate constant) and the deacylation rate (k_3 rate constant) increased one fold. These rate changes increase the amount of penicillin required for 50% PBP2 binding by 40 times. (Hackbarth *et al.*, 1995) Modified PBP2s have been found in borderline-resistant MRSA. (Tomasz *et al.*, 1989) Recently PBP2 was shown to play an important role in both methicillin and vancomycin resistance in *S. aureus.* In MRSA, PBP2 acts as complementary transglycosylase with the methicillin-resistance PBP2a/2'. (See Section 1.2.4) (Pinho *et al.*, 2001b) PBP2 is essential for the expression of vancomycin resistance in *S. aureus.* This is because PBP2, unlike other PBPs, can utilise altered peptidoglycan precursors, generated by

the vancomycin-resistance genes, for cross-linking reactions. (See Section 1.3.5 and 1.4.2.7) (Severin *et al.*, 2004b)

PBP3 is a 75kDa High-Mr Class B monofunctional PBP with 691 amino acid sequences. It comprises 13.7% of the total PBP per cell in MSSA and 10.2% in MRSA. PBP3 is encoded by a 2076bp chromosomal gene, *pbpC*. (Pinho *et al.*, 2000, Massova and Mobashery, 1998) In previous studies, PBP3 was thought to be essential for cell growth. (Georgopapadakou *et al.*, 1986, Georgopapadakou and Liu, 1980) However, recent studies indicate that isolates with mutated *pbpC* show little change in their peptidoglycan composition, morphology and growth rate. The only noticeable change is a small but significant decrease in autolytic rate, which indicates PBP3 is not essential for cell growth. PBP3 has been suggested to be a peptidoglycan transpeptidase involved in septum formation similar to the functions of the essential PBP, PBP1. Cells with inactivated *pbpC* have defective cell separation. When they are grown under sub-methicillin MIC concentrations the cells are enlarged and carry defective septa. It has been suggested that PBP1 probably substituted for PBP3 function in these *pbpC*-inactivated mutants. (Pinho *et al.*, 2000)

PBP4 is the only Low-Mr Class A PBP in *S. aureus* and is 26.4% of the total PBP per cell in MSSA and 15.4% in MRSA. It is 48kDa with 431 amino acid sequences and is encoded by a 1296bp chromosomal gene, *pbpD* or *pbp4*. (Henze and Berger-Bächi, 1995, Domanski and Bayles, 1995, Pucci and Dougherty, 2002) *pbpD* is located 5' upstream to *abcA*, an ATP-binding transporter gene, and probably shares a common regulatory mechanism with it. (Henze and Berger-Bächi, 1996, Domanski *et al.*, 1997, Domanski and Bayles, 1995) PBP4 is a bifunctional protein that acts as a carboxypeptidase and a secondary transpeptidase. The overproduction of the PBP4 gene has been shown to increase the level of peptidoglycan cross-linking and a small increase in β-lactam resistance. (Henze and Berger-Bächi, 1996, Domanski *et al.*, 1997) PBP4 had been found to be not essential for cell growth. (Katayama *et al.*, 2003b, Waxman and Strominger, 1983, Massova and Mobashery, 1998) However, the overproduction of PBP4 is linked to intermediate vancomycin resistance. (Finan *et al.*, 2001)

PBP2B is a 77.2kDa High-Mr Class B PBP comprised of 691 amino acids. It is encoded by the 2037bp *pbpF* gene located on the chromosome. It was found to co-migrate with PBP2 (81kDa) in one-dimensional gels due to their similar molecular weights. PBP2B is a relatively new PBP to be identified in *S. aureus*. The C terminal (PB domain) of the PBP2B is a putative transpeptidase and has been shown to have penicillin-binding ability. The nPB domain of PBP2B, like other High-Mr Class B PBPs, lacks a transglycosylase motif and its function is not known. (Komatsuzawa *et al.*, 1999a)

PBP2a/2' is an additional PBP only found in methicillin-resistant staphylococci and will be describe in the next section.

1.2.2.2.4 Penicillin-binding protein 2a / 2'

PBP2a (Hartman and Tomasz, 1984) or PBP2' (Georgopapadakou *et al.*, 1982) is the additional PBP found only in methicillin-resistant staphylococci and is responsible for their resistance to all β-lactam antibiotics. This is because it has a low binding affinity for β-lactams and it is able to continue cell wall cross-linking in the presence of β-lactam antibiotics. (Sumita *et al.*, 1995, Tonin and Tomasz, 1986, Brown and Reynolds, 1980, Matsuhashi *et al.*, 1986) PBP2a/2' is 42.6% of the total PBP in MRSA, and the percentage of PBP2 decreases from 42.8% in MSSA to 23.2% in MRSA. (Pucci and Dougherty, 2002) PBP2a/2' is encoded by an exogenous gene, *mecA*, which is 2007bp in length and located in the genomic island SCC*mec*. (Ito *et al.*, 1999, Song *et al.*, 1987)

PBP2a/2' was named due to its close proximity to PBP2 in one-dimensional PAGE gels. (Georgopapadakou *et al.*, 1982) However, it is not related to PBP2 at all, either in amino acid composition or in DNA sequences. (Massova and Mobashery, 1998) PBP2a/2' is a 76kDa High-Mr Class B subtype B1 PBP that has only been identified in methicillin-resistant staphylococci. This PBP2a/2' is a multimodular PBP comprised of 668 amino acids. Its PB domain is a transpeptidase and the function of the nPB domain is unknown. (Goffin and Ghuysen, 1998, Song *et al.*,

1987, Pinho *et al.*, 2001a) However, mutations in the nPB domain result in a two-fold increase in the methicillin MIC. (Katayama *et al.*, 2004)

A recent study of PBP2a/2' crystal structure at high-resolution (1.8Å) illustrates the mechanism of its low affinity binding to β-lactam molecules. The PBP2a/2' PB domain shares overall similarities in folding to the PB domains of other PBPs and serine β-lactamases. However, a structural alignment shows that the PB domain of PBP2a/2' has significant differences to the PB domain of β-lactam sensitive PBPs. (Lim and Strynadka, 2002, Chambers, 2003)

The dissociated rate ($K_{d,}$ see section 1.2.2) of PBP2a/2' and bound β-lactams is comparable to other PBPs. This shows that the initial formation of the Michaelis complex (See Section 1.2.2) is not affected by the structural differences. However, the acylation rate (k_2, see section 1.2.2) of PBP2a/2' is significantly lower than that for other PBPs. (Lim and Strynadka, 2002, Chambers, 2003)

The initial binding of PBP2a/2' to β-lactams (Michaelis complex) occurs in the binding cavity but outside of the serine active site. A conformational rearrangement is required to bring the β-lactam molecule to the serine residue for the nucleophilic attack to occur. The nucleophilic attack on the β-lactam's ring is essential for the formation of the irreversible ester covalent bond between the PBP and the β-lactam molecule, ie. acylation. (Lim and Strynadka, 2002, Chambers, 2003)

The active-site serine in the PBP2a/2' PB domain is poorly located in the binding cavity. The conformational change required is energetically expensive and unfavourable. Consequently, the PBP2a/2' PB domain does not have the corresponding conformational change found in penicillin-sensitive PBPs, when it binds with the β-lactam molecule. The distorted active site of the PBP2a/2' results in a slow acylation of the Michaelis complex. (Lim and Strynadka, 2002, Chambers, 2003)

The active site of the PBP2a/2' is located in an extended and narrow groove. The bulky β-lactamase resistant β-lactams, like methicillin, cause a bigger displacement

in the active site than other β-lactams. The bigger displacement in the binding cavity slows the proton transfer in the nucleophilic attack on the β-lactam's ring and reduces the acylation rate. Together with the distorted active site, it results in a net decrease of the acylation rate, hence the low binding affinity toward β-lactam molecules. (Lim and Strynadka, 2002, Chambers, 2003)

PBP2a/2' was originally thought to take over cell-wall synthesis, when the β-lactams inactivated other PBPs in the cell wall. However, PBP2 has been shown to be essential for high-level methicillin resistance in MRSA. The inactivation of PBP2 drastically reduces the methicillin MIC. (Pinho *et al.*, 1997) Under β-lactam selection, the PB domain (transpeptidase) of PBP2 is inactivated by the β-lactam, but the nPB domain (transglycosylase) remains active. The new model suggests that the peptidoglycan cross-linking is catalysed by the transpeptidase activity of the PBP2a/2', and the elongation of the peptidoglycan disaccharide backbone is catalysed by the transglycosylase activity of the nPB domain of PBP2. (Pinho *et al.*, 1998, Pinho *et al.*, 2001a, Pinho *et al.*, 2001b)

PBP2a/2' is an inefficient transpeptidase that forms abnormal cell walls that barely allow the cell to survive in the presence of β-lactams. The cell wall formed by PBP2a/2' is poorly cross-linked, and consists of mainly uncross-linked peptidoglycan monomers and a minority of peptidoglycan dimers and trimers. However, the abnormal peptidoglycan does not affect the cell morphology, physiology and growth rate. (de Jonge and Tomasz, 1993) When the *mecA* gene encoding PBP2a/2' was introduced into methicillin-sensitive hosts (naïve hosts), most of the transferred *mecA* genes were inactivated within the host by spontaneous mutations. When the *mecA* gene was transferred along with its regulatory genes, the host maintained the *mecA* gene but only expressed it under tight regulation and under β-lactam induction. These experiments demonstrated that the production of PBP2a/2' is not well tolerated in methicillin-sensitive hosts. In the same study, host strains with “experienced” backgrounds were generated by excision of the SCC*mec* (ie. *mecA*) from methicillin-resistant strains. The *mecA* gene was introduced into these “experienced” hosts and found to be well maintained and readily expressed. (Katayama *et al.*, 2003c) In

another study, the constitutive production of PBP2a/2' in some strains was shown to reduce the methicillin MIC. (Kondo *et al.*, 2001)

These studies suggest that PBP2a/2' is an exogenous protein to *S. aureus*, and that its production is not well tolerated and requires tight regulation in the "naïve" host. The tolerance and ease of *mecA* expression seen in the "experienced" hosts suggests that some host factors may be involved in optimal expression. This observation also indicates that optimal expression requires further evolution in the host after the acquisition of the *mecA* gene or SCC*mec*. (Hiramatsu, 1995, Katayama *et al.*, 2003c, Finan *et al.*, 2002) Interestingly, even though PBP2a/2' is responsible for β-lactam resistance in MRSA, the amount of PBP2a/2' often does not correlate well with the level of resistance. (Chambers and Hackbarth, 1987, Hartman and Tomasz, 1986, Niemeyer *et al.*, 1996)

1.2.2.2.5 Expression of methicillin resistance

The expression of methicillin resistance in *S. aureus* is typically heterogeneous. In heterogeneous resistance, the majority of the population (99.9%) have low-level resistance, and only a small subpopulation (10^{-8} to 10^{-2} CFU) is highly resistant. Some strains have homogeneous resistance and virtually all of the cells have uniform high-level resistance. (Hartman and Tomasz, 1986, Tomasz *et al.*, 1991, Pfeltz *et al.*, 2001) The level of methicillin resistance is influenced by some external factors like temperature, NaCl concentration in the medium and osmolarity. (Chambers and Hackbarth, 1987, Hartman and Tomasz, 1986) However, the transcription of *mecA* by itself does not influence the methicillin-resistant phenotype. (Niemeyer *et al.*, 1996, Ryffel *et al.*, 1992, Finan *et al.*, 2002) A wide range of genes have been found to influence the level of methicillin resistance. (Berger-Bächi and Rohrer, 2002)

1.2.2.2.5.1 Heterogeneous resistance

The heterotypic expression of methicillin resistance can be classified into three classes, according to their resistance level. (See Figure 1.2) In Class I, the majority of the population are heterotypic-resistant and have a methicillin MIC of 1.5 to 3.0

μg/ml, and the highly-resistant subpopulation occurs at a frequency of 10^{-8} to 10^{-7} (ie. 1 in 10^{7} to 10^{8}) and has a MIC > 25 μg/ml. In Class II heterotypic-resistant strains, the majority of the cells have a methicillin MIC of 6 to 12 μg/ml. The highly resistant subpopulation in Class II occurs at a frequency of 10^{-6} to 10^{-4} and has a methicillin MIC > 25 μg/ml. In Class III heterotypic-resistant strains the majority have a methicillin MIC of 50 to 200 μg/ml and the high-level subpopulation have a MIC > 300μg/ml and occur at a frequency of 10^{-3} to 10^{-2}. (Tomasz *et al.*, 1991)

The Class I and Class II heterotypic strains are clinically difficult to detect with conventional methods because of their low MICs. Both of these heterotypic strains are clinically sensitive to methicillin in the laboratory, as the NCCLS breakpoint for methicillin is equal to or greater than 16μg/ml. Some strains might be classified as sensitive or as intermediate resistant with a methicillin MIC between 8μg/ml and 16μg/ml. However, these strains are still clinically important because of their highly resistant subpopulations. For example, strain N315 has Class I heterotypic expression and appears to be methicillin sensitive in susceptibility tests. (Chambers, 1988, Varaldo, 1993, Hiramatsu, 1995)

All of the heterogeneous-resistant classes are strain specific and stable. They retain their resistance phenotype after consecutive subculturing. However, the methicillin-resistance levels of their high-level resistant subpopulations are not stable. After consecutive subculturing, the homogeneous, highly resistant subpopulations, revert back to the heterogeneous resistance phenotype, the same as their parent strains. (de Lencastre *et al.*, 1993, Tomasz *et al.*, 1991)

1.2.2.2.5.2 Homogeneous resistance

The naturally occurring homogeneous and Class III high-level heterogeneous-resistant strains have been found to have inactivated the *mecA* regulatory elements. (Archer *et al.*, 1994, Hiramatsu, 1995) Inactivation of the *mecA* regulatory elements in N315, a Class I heterotypical-resistant strain, results in a Class III high-level heterotypic strain. Even though the PBP2a/2' is constitutively produced, the homotypic type of resistance is not achieved. This indicates that additional host

factors or genetic backgrounds are essential for homogeneous expression, even when PBP2a/2' is constitutively expressed. (Kuwahara-Arai *et al.*, 1996, Hiramatsu, 1995) A chromosomal mutation, chr*, is needed for the homotypic expression of resistance even though PBP2a/2' is being expressed. (Ryffel *et al.*, 1994) The conversion of the heterotypic to homotypic methicillin resistance is not dependent on the induction of the *mecA* gene, but depends on the occurrence of host chromosomal mutations. This phenotypic conversion was shown to require the occurrence of mutations at a high frequency and increasing level of *mecA* transcription. (Finan *et al.*, 2002)

1.2.2.2.5.3 Eagle-type resistance

Eagle-type resistance is a distinctive type of expression of methicillin resistance found only in experimental mutants. The cell population is susceptible to methicillin at low concentrations (2 to 16μg/ml), but resistant at high concentrations (64 to 512μg/ml). The over expression of high-level methicillin resistance A (*hmr*A) and *hmr*B genes is responsible for this phenomenon. When either of these two genes is introduced into N315, it is converted from Class I heterotypic expression to Eagle-type expression. When these genes are introduced into a N315 mutant, with inactivated *mecA* regulatory elements, the phenotype converts from Class III heterogeneous to homogeneous resistance. It has been proposed that Eagle-type expression is caused by the chr* mutation and results in the over expression of *hmr* genes. (Kondo *et al.*, 2001, Hiramatsu, 1995)

Two different phenotypic conversion pathways have been hypothesised based on the studies of Eagle type expression. The first pathway suggests that under the selection of low-level methicillin concentrations, the inactivation of *mecA* regulatory elements occurs and leads to the conversion of the low-level Class I heterotypic expression to high-level Class III heterotypic expression. The subsequent conversion to the homotypic expression occurs under high-level methicillin selection when over expression of the *hmr* genes is achieved. The second conversion pathway differs to the first pathway in that its initial step occurs under high-level methicillin selection. Under high-level selection, the chr* mutation (ie. over expression of *hmr* genes) occurs and leads to the conversion from Class I expression to Eagle-type expression.

The subsequent conversion to homotypic expression occurs under low-level methicillin selection, when the *mecA* regulatory elements are inactivated. (Kondo *et al.*, 2001, Hiramatsu, 1995)

1.2.2.2.5.4 Borderline resistance

Borderline or low-level methicillin-resistant *S. aureus* has a MIC at or just above the susceptibility breakpoint. The MIC for these borderline-resistant isolates range from 4 to 16μg/ml for methicillin (Varaldo, 1993, McDougal and Thornsberry, 1986, Chambers *et al.*, 1989) and from 4 to 8 μg/ml for oxacillin. (Chambers, 1997) In susceptibility, these strains are very similar to the Class I heterogeneous resistant MRSA. However, these borderline-resistant strains do not produce PBP2a/2' (i.e. They are *mecA* negative) and do not have a highly resistant subpopulation. (Montanari *et al.*, 1990, Tomasz *et al.*, 1989)

Several mechanisms had been suggested to be responsible for borderline resistance. These include the modification, or over expression of endogenous PBPs, over production of β-lactamase and a novel β-lactamase, methicillinase. (Tomasz *et al.*, 1989, McDougal and Thornsberry, 1986, Massidda *et al.*, 1994, Chambers *et al.*, 1989)

β-lactamase-negative, borderline-resistant isolates with modified PBPs are known as MORSA (modified oxacillin-resistant *S. aureus*). (Tomasz *et al.*, 1989) Modified PBP1 and PBP2 and increased amounts of PBP4 were found in MORSA isolates. The penicillin-binding affinity of the modified PBP1 and PBP2 is significantly reduced by point mutations in their penicillin-binding (PB) domains. These point mutations slow the binding (decreased acylation rate) and increase the release of bound β-lactam molecules (increased deacylation rate) to the PB domain. (Tomasz *et al.*, 1989, Hackbarth *et al.*, 1995) The over production of PBP4 causes a significant increase in cell-wall cross-linking and the formation of thicker cell walls and results in low-level methicillin resistance. (Henze and Berger-Bächi, 1996, Henze *et al.*, 1996) The presence of both modified PBPs and over production of

endogenous PBPs causes the increase in methicillin MIC. This is because both mechanisms increase the number of unbound and functional PBPs for cell wall synthesis under β-lactam selection. (Chambers, 1997)

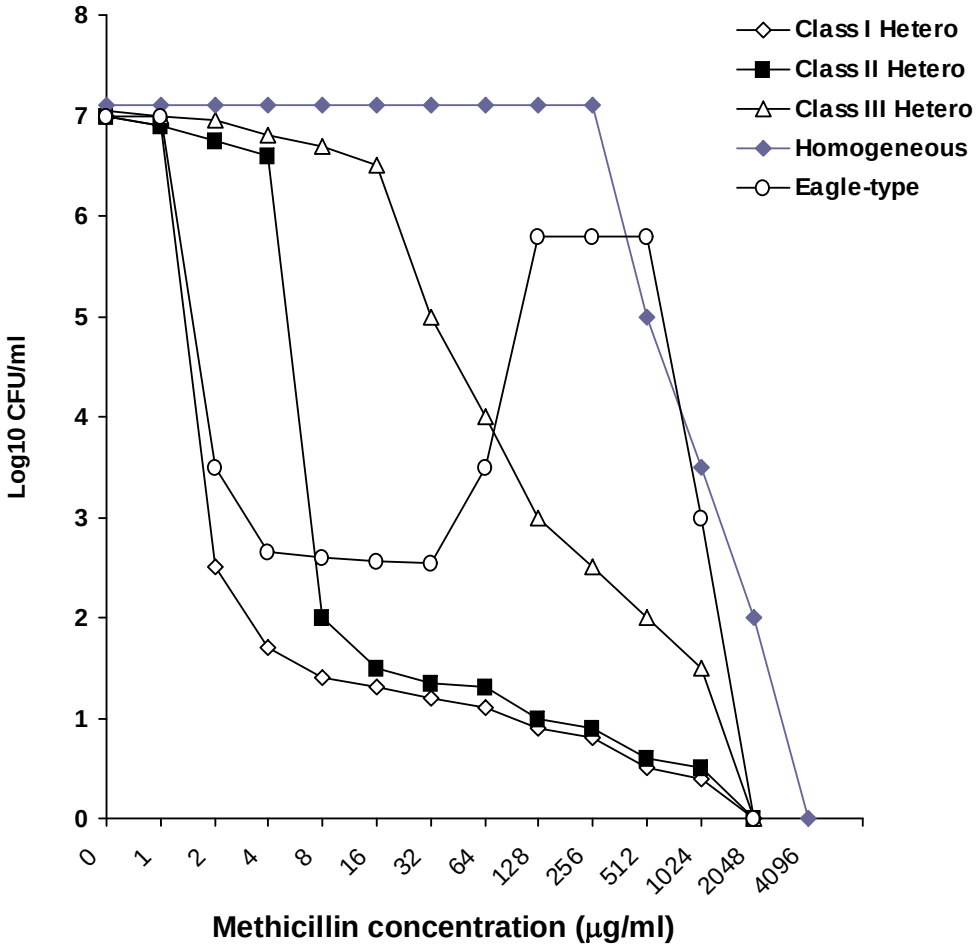


Figure 1.2 Population Analysis of five methicillin-resistant phenotypes

The CFU is the colony-forming unit at different methicillin concentrations. (Hiramatsu *et al.*, 2001, Tomasz *et al.*, 1991)

Borderline-resistant isolates with hyperproduction of β-lactamase are known as BORSA (borderline oxacillin-resistant *S. aureus*). (Tomasz *et al.*, 1989, McDougal and Thornsberry, 1986) Staphylococcal β-lactamase can slowly hydrolyse the β-lactamase resistant β-lactam antibiotics such as methicillin. The hyperproduction of β-lactamase allows the cell to survive in the presence of β-lactams. However, this resistance can be reversed by the removal of β-lactamase genes, and/or the addition of β-lactamase inhibitors such as clavulanic acid and sulbactam. (McDougal and Thornsberry, 1986) The hyperproduction of β-lactamase only leads to borderline resistance in a specific genetic background. (Barg *et al.*, 1991) Borderline-resistant strains were found to be lysed by phage 94 and/or 95 and to hyperproduce type A β-lactamase. (Kernodle *et al.*, 1998) In addition to β-lactamase hyperproduction, some BORSA isolates also carry modified PBP2 with the same mutations as MORSA strains. (Hackbarth *et al.*, 1995, Chambers, 1997)

Methicillinase is a novel β-lactamase found in a β-lactamase hyperproducer. It slowly hydrolyses β-lactamase-resistant β-lactams such as methicillin. However, the gene that encodes for this enzyme has yet to be identified. (Massidda *et al.*, 1994, Varaldo, 1993)

Both MORSA and BORSA in general are not a therapeutic problem but a laboratory issue. They can be effectively treated by conventional treatments such as oxacillin or ampicillin with β-lactamase inhibitors. (Thauvin-Eliopoulos *et al.*, 1990, Pefanis *et al.*, 1993, Chambers *et al.*, 1989) However, it is important to distinguish BORSA from class I heterogeneous MRSA, as the latter will result in treatment failure because of the highly resistant subpopulation. (Chambers, 1997) The strains can be easily and rapidly differentiate by a PCR for *mecA*. (Brakstad *et al.*, 1993)

1.2.2.2.5.5 Factors influencing the level of methicillin resistance

The level of methicillin resistance can be influenced by a group of chromosomal housekeeping genes. Their functions are crucial for the optimal expression of methicillin resistance. These genes are known as *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) factors. So far, more than 20 *fem* or *aux*

factors have been identified. (Berger-Bächi and Rohrer, 2002, Berger-Bächi *et al.*, 1992, de Lencastre *et al.*, 1994) The majority of these genes are either directly or indirectly involved in peptidoglycan synthesis, autolysis or have regulatory functions. None of these genes have been shown to influence PBP2a/2' production. (Berger-Bächi and Rohrer, 2002) The function and effect of these *fem* factors are summarised in Table 1.2.

Several *fem* factors were found to be involved in the formation of peptidoglycan precursors. The *glmM* (*femD*) encodes for a phosphoglucomutase responsible for the formation of the percussor of *N*-acetylglucosamines in the disaccharide backbone. (Jolly *et al.*, 1997, Glanzmann *et al.*, 1999) The *murE* is the UDP-N-acetylmuramyl tripeptide synthetase that catalyses the addition of the L-lysine residue to the stem peptide precursor. (Gardete *et al.*, 2004, Jana *et al.*, 2000) The *femC* and *femF* (*murE*) genes are involved in the formation of stem peptides. (Gustafson *et al.*, 1994, Ornelas-Soares *et al.*, 1994, Gardete *et al.*, 2004, Jana *et al.*, 2000) The *fmhB*, *femA* and *femB* are involved in the formation of the pentaglycine bridge. (Rohrer *et al.*, 1999, Stranden *et al.*, 1997, Henze *et al.*, 1993)

Some *fem* factors are involved in other aspects of cell-wall synthesis. The *dlt* operon is involved in the formation of teichoic acid that is another major component of the Gram-positive cell wall. (Nakao *et al.*, 2000) *pbp2* encodes for PBP2 that complements the PBP2a/2' with its transglycosylase activity. The *fmtA*, *fmtB*(*mrp*) and *fmtC*(*mprF*) genes encode membrane proteins that influence the level of methicillin resistance. (Komatsuzawa *et al.*, 1999b, Wu and De Lencastre, 1999, Komatsuzawa *et al.*, 2000, Komatsuzawa *et al.*, 2001, Peschel *et al.*, 2001)

The *llm* and *lytH* genes are the *fem* factors involved in the autolysis or the turnover of the cells. (Maki *et al.*, 1994, Fujimura and Murakami, 1997) The *sigB*, *hmrA* and *hmrB* genes are the *fem* factors involved in transcription and expression of methicillin resistance. However, their exact roles in these processes have yet to be elucidated. (Wu *et al.*, 1996a, Kondo *et al.*, 2001)

Table 1.2 The *fem* or *aux* factors

Gene (alternate names)	Function and effect on methicillin-resistance levels	References
fmhB (*femX*)	Interpeptide formation; addition of the first glycine to the stem peptide; inactivation is lethal	Rohrer *et al.* (1999)
femA	Interpeptide formation; addition of the 2nd and 3rd glycine to the stem peptide; inactivation abolishes methicillin resistance	Stranden *et al.* (1997)
femB	Interpeptide formation addition of the 4th and 5th glycine to the stem peptide; inactivation reduces methicillin resistance	Henze *et al.* (1993)
femC (*glnR*)	Glutamine synthetase repressor; inactivation reduces amidation of the iD-glutamate of the stem peptide; inactivation reduces methicillin resistance	Gustafson *et al.* (1994)
femD (*femR315*) (*glmM*)	(*glmM*) Phosphoglucosamine mutase; catalyses the interconversion of glucosamine-6-phosphate to glucosamine-1-phosphate; a cytoplasmic peptidoglycan precursor; inactivation reduces methicillin resistance	Jolly *et al.* (1997), Glanzmann *et al.* (1999)
femE	Function unknown; inactivation slightly reduces methicillin resistance	de Lencastre *et al.* (1994)
femF (*murE*)	UDP-N-acetylmuramyl tripeptide synthetase; catalyses incorporation of lysine into peptidoglycan stem peptide; Inactivation of its C-terminal drastically reduces methicillin resistance. Total inactivation is lethal.	Ornelas-Soares *et al.* (1994), Gardete *et al.* (2004), Jana *et al.* (2000)
fmtA	Membrane protein; inactivation decreases cross-linking and amidation of peptidoglycan, and reduces methicillin resistance	Komatsuzawa *et al.* (1999b)
fmtB (*mrp*)	Cell surface protein; function unknown; inactivation reduces pentaglycyl substituted monomer of the cell wall fraction while increasing the amount of unsubstituted pentapeptide and reduces methicillin resistance	Wu and de Lencastre (1999), Komatsuzawa *et al.* (2000)
fmtC (*mprF*)	Membrane-associated protein; inactivation reduces modification of phosphatidyl-glycerol with L-lysine, and reduces methicillin resistance	Komatsuzawa *et al.* (2001), Peschel *et al.* (2001)
llm	Function unknown; inactivation increases Triton-X-100-induced autolysis and reduces methicillin resistance	Maki *et al.* (1994)
lytH	Homologous to lytic enzymes; inactivation increases methicillin resistance	Fujimura and Murakami (1997)
pbp2	Penicillin-binding protein 2; functional transglycosylase domain of PBP2 is needed for methicillin resistance	Pinho *et al.* (2001a)
sigB	Alternate transcription factor; inactivation reduces methicillin resistance	Wu *et al.* (1996a)
hmrA	Putative aminohydrolase; over expression increases methicillin resistance	Kondo *et al.* (2001)
hmrB	Homologue of acyl carrier protein; over expression increases methicillin resistance	Kondo *et al.* (2001)
dlt operon	Transfer of D-alanine into teichoic acids; inactivation increases methicillin resistance	Nakao *et al.* (2000)

This table is according to Berger-Bächi and Rohrer (2002)

1.2.3 Chloramphenicol

Chloramphenicol is a broad spectrum bacteriostatic antibiotic. It binds to the 50S subunit of ribosomal RNA and disrupts the transpeptidation step in protein synthesis. Chloramphenicol resistance in *S. aureus* is conferred by the *cat* gene, which encodes CAT (chloramphenicol acetyltransferase). CAT inactivates chloramphenicol by acetylation using acetylCoA as the donor. Five types of closely related CAT had been reported in *S. aureus.* The *cat* gene is exclusively carried and disseminated by a group of small plasmids in *S. aureus*. (Lyon and Skurray, 1987, Sands and Shaw, 1973, Firth and Skurray, 2000, Gillespie *et al.*, 1984)

1.2.4 Fusidic acid

Fusidic acid inhibits protein synthesis by binding to the complex of elongation factor G and the ribosome. This interaction inhibits the release of elongation factor G (EF-G) after the translocation step of peptide synthesis, blocking further protein synthesis. The *fusA* gene encodes the EF-G in *S. aureus*. The mutations in *fusA* reduce the binding affinity of EF-G with fusidic acid and leads to fusidic acid resistance. The *far1* or *fusB* is a plasmid-borne fusidic acid-resistance gene. It is found on the plasmid pUB101 and the exact resistance mechanism has not been elucidated. (O'Brien *et al.*, 2002, O'Neill *et al.*, 2004) However, it is known that the inducible *far1* gene can prevent fusidic acid from reaching its target, probably by reducing permeability or structurally altering the cell wall. (Lyon and Skurray, 1987, O'Brien *et al.*, 2002, Berger-Bächi, 2002) Recently the *far* gene was found in a unique cassette chromosome SCC_{476} in the community-acquired methicillin-sensitive *S. aureus* 476. (Holden *et al.*, 2004)

1.2.5 Glycopeptides

Glycopeptides are broad-spectrum antibiotics that are often used as the drug of last resort to treat serious MRSA infections. The two glycopeptides used are vancomycin and teicoplanin. (Berger-Bächi, 2002)

Glycopeptides inhibit cell wall cross-linking by binding with the D-alanyl-D-alanine residues in the peptidoglycan chains in the cell wall. When the glycopeptide reaches the cytoplasmic membrane, it blocks the formation of peptidoglycan chains and stops the cell from multiplying. This is because the glycopeptide blocks the transglycosylation of the peptidoglycan chain by binding to the D-alanyl-D-alanine residue of peptidoglycan precursor of the murein monomer. (Hiramatsu, 2001)

Glycopeptide resistance in *S. aureus* is generally in the intermediate level (MIC, 8mg/L). These isolates are known as the glycopeptide or vancomycin intermediate *S. aureus* (GISA or VISA). (Hiramatsu, 2001, Tenover *et al.*, 1998) Although the exact mechanism has yet to be elucidated, the resistance is due to the thickening and poor cross linking of the cell wall. (Hiramatsu, 2001, Cui *et al.*, 2003, Reipert *et al.*, 2003) VISAs have an accelerated peptidoglycan synthesis rate that causes significant thickening of the cell wall. In addition they also synthesise structurally altered non-amidated murein monomers which are poor substrates for cross-linking reactions and this results in less cross-linked cell wall. The thickened and poorly cross-linked cell wall contains significantly more D-alanyl-D-alanine residues than the normal cell wall. (Cui *et al.*, 2000, Hanaki *et al.*, 1998b, Hanaki *et al.*, 1998a, Kuroda *et al.*, 2000, Kuroda *et al.*, 2003) When glycopeptide molecules bind to the residues, they are trapped in the cell wall. The trapped glycopeptide molecules clog up the peptidoglycan mesh of the cell wall and prevent the drugs entry into the cytoplasmic membrane and therefore blocks further uptake of the drug. (Hiramatsu, 2001)

High-level glycopeptide resistance (MIC, 32 mg/L) is encoded by the *vanA, vanB* and *vanD* gene clusters commonly found in VRE. The *van* genes degrade murein monomers and replace it with new murein monomers that contain D-alanyl-D-lactate residues (depsipeptide) instead of the common D-alanyl-D-alanine residue. The glycopeptide binding affinity for new murein monomer depsipeptides is 1000-fold lower than the original D-alanyl-D-alanine residue. (Berger-Bächi, 2002, Severin *et al.*, 2004a) Recently, two strains of high-level resistant *S. aureus* (VRSA) were isolated together with VRE in different US hospitals. Each VRSA carried a large plasmid that harbours the *vanA* gene cluster. The VanA phenotype confers inducible and high-level resistance to both vancomycin and teicoplanin. (Woodford *et al.*,

1998, Tenover *et al.*, 2004, Flannagan *et al.*, 2003) The *vanA* cluster consists of seven *van* genes in the order of *vanR, vanS, vanH, vanA, vanX, vanY* and *vanZ.* The *vanR* and *vanS* encode a two component regulatory system that is responsible for the transcription of *vanH, vanA* and *vanX* genes. These three genes are essential for vancomycin resistance. The *vanH* encodes a dehydrogenase that produce subtract for the formation of depsipeptide, which is catalysed by the ligase encoded by *vanA*. The *vanX* is a polypeptide with unknown function. The *vanY* encodes a carboxypeptidase and the function of *vanZ* is unknown. Both of these genes are not required for vancomycin resistance. (Arthur *et al.*, 1993, Arthur and Quintiliani, 2001)

1.2.6 Macrolides, lincosamides and streptogramins

Macrolides, lincosamides and streptogramins (MLS) are three chemically unrelated antimicrobial agents but with a similar mode of action. These drugs bind to the 23S rRNA of the 50S ribosomal RNA subunit and this stimulate the dissociation of peptidyl-transfer RNA (tRNA) from the ribosome during peptide elongation. This results in chain termination and a reversible stoppage of protein synthesis. (Berger-Bächi, 2002, Lyon and Skurray, 1987)

The macrolide family contains drugs like erythromycin. Lincosamides include lincomycin and clindamycin. Streptogramins consist of A and B types depending on their structure. A streptogramin type A (SgA) is dalfopristin and a streptogramin type B (SgB) is quinupristin. (Lyon and Skurray, 1987, Allignet *et al.*, 1998) The MLS are bacteriostatic compounds, but the mixture of SgA and SgB such as in Synercid (dalfopristin and quinupristin) is bactericidal. (Livermore, 2000, Carbon, 1999, Chambers, 1997) Target modification, an efflux pump and drug modification are the three mechanisms responsible for MLS resistance in *S. aureus.* (Leclercq and Courvalin, 1991b, Leclercq and Courvalin, 1991a, Weisblum, 1995, Roberts *et al.*, 1999)

Target modification is the predominant MLS-resistance mechanism found in *S. aureus.* (Lina *et al.*, 1999) It is a posttranscriptional modification involving methylation or dimethylation of a single adenine residue (A2058) in the 23S rRNA

moiety. The methylation reduces the binding affinity of macrolide molecules to the 23S rRNA, and probably induces a conformational change of the 23S rRNA. Since the binding site of the macrolide overlaps with the binding sites of lincosamides and SgB, this modification of the adenine results in cross-resistance to macrolides, lincosamides and SgB (MLS_B resistance phenotype). The adenine methylation is mediated by a group of adenine-N^6-methyltransferases encoded by the *erm* (erythromycin resistance methylase) genes. There are three related *erm* genes that are regulated by transcriptional or translational regulation and they can be either inducible or constitutively expressed. (Leclercq and Courvalin, 1991a, Weisblum, 1995, Roberts *et al.*, 1999, Werckenthin and Schwarz, 2000, Horinouchi *et al.*, 1983) Three Erm methylases had been reported in *S. aureus*. (Weisblum *et al.*, 1979) The inducible *ermA* is located on the site-specific transposon Tn*554*. (Murphy, 1985, Phillips and Novick, 1979, Roberts *et al.*, 1999) In MRSA, MLS_B resistance is often closely found with methicillin resistance because Tn*554* is carried by many of the *mecA*-bearing SCC*mec* genomic islands. (Ito *et al.*, 2003a, Ito *et al.*, 2001) Strains with this inducible resistance genotype only phenotypically express resistance to 14-membered (eg. erythromycin) and 15-membered (eg. azithromycin) lactone ring macrolides, but remain susceptible to 16-membered lactone ring macrolides (eg. spiramycin), lincosamides and SgB unless induced by inducers such as erythromycin. This is because the 14- and 15-membered lactone ring macrolides are better inducers than the others in this group. (Panagea *et al.*, 1999, Leclercq, 2002) The constitutively expressed *ermB* is harboured by transposon T*n551*. (Wu *et al.*, 1999, Roberts *et al.*, 1999, Lyon and Skurray, 1987) The plasmid-borne *ermC* is inducible. (Horinouchi and Weisblum, 1982, Roberts *et al.*, 1999) The majority of *S. aureus* with the MLS_B phenotype have been found to carry either the *ermA*, or *ermC*, genotypes and the *ermB* gene is relatively uncommon. (Lina *et al.*, 1999, Westh *et al.*, 1995, Nicola *et al.*, 1998, Thakker-Varia *et al.*, 1987)

Active efflux pumps and drug modification of MLS are less prevalent in *S. aureus* compared to the Erm methylases. (Lina *et al.*, 1999) Two different active efflux systems involving ATP-binding transport proteins have been found in *S. aureus*. (Roberts *et al.*, 1999) Macrolides and SgB active efflux is encoded by *msrA*. (Ross *et al.*, 1990) *vgaA* and *vgaB* genes encode two different efflux systems for SgA. (Roberts *et al.*, 1999, Haroche *et al.*, 2002) *vatA*, *vatB* and *vatC* encode

acetyltransferases that inactivate SgA molecules by acetylation. (Allignet and el Solh, 1995, Roberts *et al.*, 1999, Allignet *et al.*, 1998) The hydrolases encoded by *vgbA* and *vgbB* hydrolyse SgB drugs. (Roberts *et al.*, 1999, Allignet *et al.*, 1998) *linA/linA'* or lnuA encode lincomycin nucleotidyltransferases that catalyse nucleotidylation of the hydroxyl group on lincosamides. (Brisson-Noel *et al.*, 1988, Leclercq *et al.*, 1987, Leclercq, 2002) All these efflux systems and drug modification genes are plasmid borne except *vgaA*. (Roberts *et al.*, 1999, Leclercq and Courvalin, 1991b) The *vgaAv* is a variant of *vgaA* and is located on transposon Tn*5406*. (Haroche *et al.*, 2002) Many of these genes are commonly found together on the same plasmid. For example, the *vatB* and *vgaB* were found on Tn*5406*-carrying plasmids. (Haroche *et al.*, 2002) Some staphylococcal plasmids have been found to carry the *vga*, *vgb* and *vat* genes. (Allignet and El Solh, 1999, Lina *et al.*, 1999) Some MLS-resistant isolates have been found to carry both the *erm* genes and the genes for efflux pump and drug modification. (Lina *et al.*, 1999)

1.2.7 Mupirocin

Mupirocin is a bacteriostatic, topical agent, widely used to eliminate *S. aureus* from nasal carriers. (Hitomi *et al.*, 2000, Cookson, 1998) It competitively inhibits the isoleucyl-tRNA synthetase (IRS) and disrupts protein synthesis by preventing the incorporation of isoleucine into nascent peptide chains. (Cookson, 1998) Low-level mupirocin resistance (MIC, 8 to 256 mg/L) is probably due to chromosomal mutations of the native IRS gene. (Gilbart *et al.*, 1993, Cookson, 1998) High-level resistance (MIC > 256 mg/L) is due to an additional mupirocin resistant IRS encoded by the *mupA* gene. (Morton *et al.*, 1995, Cookson, 1998) The *mupA* gene is flanked by a pair of IS*431/257* and is usually plasmid-borne. (Chaves *et al.*, 2004, Morton *et al.*, 1995, Udo and Jacob, 1998, Udo *et al.*, 2001) It is disseminated by conjugative plasmids, but recently it has also been found in chromosome. (Udo and Jacob, 1998, Udo *et al.*, 2003, Bastos *et al.*, 1999)

1.2.8 Quinolones

Quinolones are bactericidal agents that directly inhibit DNA synthesis by interacting with two type 2 topoisomerases, DNA gyrase and topoisomerase IV. They interact

with the enzymes when they are bound to the DNA. The formation of a quinolone-enzyme-DNA complex forms a physical barrier that blocks DNA replication and induces DNA double-stranded breaks that finally lead to cell death. (Hooper, 1999, Drlica and Zhao, 1997, Drlica, 1999) The fluoroquinolones such as ciprofloxacin and ofloxacin were first used in the early 1980s and the former has become widely used as an antistaphylococcal agent. (Lowy, 2003, Hooper, 2001)

Target modification is the predominant quinolone-resistant mechanism found in *S. aureus*. (Hooper, 1999, Drlica and Zhao, 1997) The primary target of quinolones differs according to bacterial species. Topoisomerase IV is the primary target in staphylococci. (Ng *et al.*, 1996) Topoisomerase IV binds to DNA as a tetramer composed of two pairs of GlrA and GlrB subunits that are encoded by *grlA* and *grlB* respectively. The secondary target is DNA gyrase which binds to the DNA in a similar fashion to topoisomerase. Its subunits, GyrA and GyrB, are encoded by *gyrA* and *gyrB* respectively. (Hooper, 1999, Drlica and Zhao, 1997) The mutations of these genes always occur in a region known as the quinolone resistance-determining region (QRDR), but mutations conferring quinolone resistance are known to occur outside the region. (Takahata *et al.*, 1997, Linde *et al.*, 2001, Ince and Hooper, 2000) QRDR is probably the quinolone binding site and mutations reduce the binding affinity of the drug. (Hooper, 2001, Tanaka *et al.*, 1998) High-level resistance is the result of mutations in the primary target (*grlA* and *grlB*) and the secondary target (*gyrA* and *gryB*). However, mutations in the secondary target without a mutation in the primary target do not confer quinolone resistance. (Ng *et al.*, 1996, Schmitz *et al.*, 1998a, Dubin *et al.*, 1999) The most common mutations are in codon 80 of *grlA* when the serine is changed to phenylalanine and codon 84 of *gyrA* when serine is replaced with leucine. (Takahashi *et al.*, 1998, Schmitz *et al.*, 1998b, Wang *et al.*, 1998)

NorA is a proton motive force-dependent, multidrug efflux pump that confers low-level quinolone resistance when over expressed. (Neyfakh *et al.*, 1993, Yoshida *et al.*, 1990, Ng *et al.*, 1994) Although the exact cause of this expression is not known, mutations in its promoter region and the ArlS two-component regulatory system are known to increase *norA* transcription. (Fournier *et al.*, 2000, Kaatz *et al.*, 1999)

Mutations in the *flqB* locus have been linked to the overexpression of the *norA* gene. (Ng *et al.*, 1994)

1.2.9 Rifampicin

Rifampicin is a bactericidal drug that inhibits RNA synthesis. It acts by binding to the β subunit of the RNA polymerase encoded by the *rpoB* gene. (Lyon and Skurray, 1987) Rifampicin resistance is due to mutations in the *rpoB* gene that reduce the binding affinity of rifampcin to its target site. The level of resistance is dependent on the location of the mutations as well as the nature of the new amino acid. (Aubry-Damon *et al.*, 1998) The *rpoB* has been found to have a high mutation frequency (10^{-8}). (O'Neill *et al.*, 2001a) As a result, rifampicin is rarely used as a single agent in treatment even though it is a potent antimicrobial agent. (Chambers, 1997)

1.2.10 Tetracycline

The tetracyclines are a family of broad-spectrum bacteriostatic agents. They gain access to the cytoplasmic membrane as an electroneutral, lipophilic form, by passive diffusion through the membrane pores. The uptake of tetracyclines across the cytoplasmic membrane is energy dependent and is driven by proton-motive force. In the cytoplasm, tetracyclines bind with the 30S rRNA subunit in a reversible fashion. This binding prevents the attachment of the aminoacyl-tRNA to the ribosome, hence they inhibit protein synthesis. To date active efflux and ribosomal protection are the only tetracycline-resistance mechanisms described in *S. aureus*. (Schnappinger and Hillen, 1996, Roberts, 1996, Chopra and Roberts, 2001)

The tetracycline-specific efflux proteins are transmembrane efflux pumps driven by proton-motive force. They exchange protons for tetracycline-divalent metal ion complexes against the concentration gradient. This efflux process reduces the intracellular drug concentration and protects the drug target, the ribosome. (Schnappinger and Hillen, 1996, Chopra and Roberts, 2001) TetK and TetL are the only two tetracycline-specific efflux proteins described in *S. aureus*. Unlike their repressor controlled Gram-negative counterparts, TetK and TetL are regulated by translation attenuation similar to the *ermC* system. (Schwarz *et al.*, 1992, Mojumdar

and Khan, 1988, Chopra and Roberts, 2001, Horinouchi *et al.*, 1983) Their transcription can also been influenced by the elevation of pH and the presence of either Na^+ or K^+ ions.

The TetK and TetL proteins are encoded by the *tetK* and *tetL* genes, respectively. Both of them confer inducible resistance to tetracycline but not to minocycline and glycylcyclines. (Chopra and Roberts, 2001, Schnappinger and Hillen, 1996) Among *S. aureus* strains, the *tetK* is the predominant genotype for tetracycline resistance, whereas the *tetL* genotype is not common. (Trzcinski *et al.*, 2000, Bismuth *et al.*, 1990) Both *tetK* and *tetL* genes are plasmid-borne and often found on small plasmids. For example, pT181 is the *tetK* gene-bearing plasmid commonly found in MRSA isolates. (Mojumdar and Khan, 1988, Chopra and Roberts, 2001, Lyon and Skurray, 1987) This small plasmid has also been found integrated into chromosomal SCC*mec* as well as large conjugative and multiple-resistance plasmids. (Ito *et al.*, 2001, Oliveira *et al.*, 2000, Werckenthin *et al.*, 1996, Skurray and Firth, 1997) The integration of pT181 is believed to be mediated by the insertion sequence IS*431/257*. (See Section 1.3.1.2) (Skurray and Firth, 1997, Chopra and Roberts, 2001)

TetM is the sole ribosomal protecting protein found in *S. aureus*. (Trzcinski *et al.*, 2000, Bismuth *et al.*, 1990) It is an elongation factor-like (EF-like) protein that carries the highly conserved guanosine triphosphate binding motifs of EF-G and Tu. These GTP binding motifs give TetM the ribosome-dependent GTPase activity that generates energy by GTP hydrolysis. (Burdett, 1996, Nesin *et al.*, 1990, Chopra and Roberts, 2001) The binding of TetM to the ribosome induces a conformational change that prevents tetracycline binding but still allows normal protein synthesis to occur. It is also able to mediate the release of tetracycline from ribosome-drug complexes. Both of these protection mechanisms require the energy generated by GTPase activity. (Chopra and Roberts, 2001) TetM confers inducible resistance to both tetracycline and minocycline. It is induced by tetracycline and is probably regulated by transcriptional attenuation. (Trzcinski *et al.*, 2000, Nesin *et al.*, 1990, Roberts, 1996) The *tetM* gene encoding for TetM has been found in many different genera of Gram-positive and -negative bacteria except the enteric Gram-negative bacteria. *tetM* is often associated with, and disseminated by, the conjugative transposons of the Tn*916*-Tn*1545* family. (Chopra and Roberts, 2001) The only *tetM* conjugative transposon found in *S. aureus* is the transposon, Tn*5801*, which is

related to Tn*916*. (Ito *et al.*, 2003a, Nesin *et al.*, 1990) Similar to the *tetK* gene, the *tetM* genotype is common in tetracycline-resistant *S. aureus.* Some *S. aureus* strains were found to carry both *tetK* and *tetM* gene. Their tetracycline and minocycline MICs are significantly higher than the strains carrying only a single *tet* gene. (Trzcinski *et al.*, 2000, Bismuth *et al.*, 1990)

1.2.11 Sulphonamides and Trimethoprim

Sulphonamides and trimethoprim are bacteriostatic agents that block the tetrahydrofolic acid pathway at different stages. The derivatives of this pathway are essential in the formation of several animo acids and nucleotides. In the early stage of the pathway, the dihydropteroate synthase (DHPS) catalyses dihydrofolic acid formation by the condensation of *p*-aminobenzoic acid (PABA) and dihydropteridine. Sulphonamides are analogues of PABA and competitively inhibit the DHPS activity by forming an inactive complex with DHPS. The final stage of the pathway involves reduction of the dihydrofolic acid to form tetrahydrofolic acid by the dihydrofolate reductase (DHFR). Trimethoprim is an analogue of dihydrofolic acid and inhibits DHFR by competitive inhibition. (Amyes and Gemmell, 1992, Lyon and Skurray, 1987) Co-trimoxazole is a mixture of sulphamethoxazole and trimethoprim and is used to treat *S. aureus* infections. (Stein *et al.*, 1998)

Low-level trimethoprim resistance is due to the overproduction of normal DHFR which is probably due to the *sulA* mutation. (Lyon and Skurray, 1987) On the other hand, high-level trimethoprim resistance is conferred by the acquisition of an additional drug-resistant DHFR. (Amyes and Towner, 1990, Lyon and Skurray, 1987) S1 DHFR, S2 DHFR and S3 DHFR encoded separately by the *dfrA, dfrD* and *dfrG* genes, are the three drug-resistant DHFRs found in staphylococcal species. (Lyon and Skurray, 1987, Dale *et al.*, 1995b, Ko *et al.*, 2005) *dfrA* is carried by the transposon, Tn*4003*, which is widely disseminated on staphylococcal plasmids. These plasmids include the pSK639 family of small plasmids, pSK1 family of multiresistance plasmids and large conjugative plasmids such as pGO1. (Skurray *et al.*, 1988, Leelaporn *et al.*, 1996, Skurray and Firth, 1997) *dfrD* was originally found on small plasmids in *S. haemolyticus* and *Listeria monocytogenes*. They can also be

transferred into and express trimethoprim resistance in *S. aureus in vitro*. (Dale *et al.*, 1995b, Charpentier and Courvalin, 1997, Charpentier *et al.*, 1999) The *dfrG* gene was recently identified from nosocomial Thai and Japanese MRSA isolates. Unlike the *dfrA* and *dfrD* genes, it was exclusively found on the chromosome. Even though, the flanking DNA sequences of the *dfrG* gene were identical to an enterococcal conjugative plasmid, it was not transferable by conjugation. (Ko *et al.*, 2005)

1.2.12 Heavy metals

Heavy metal ions are ubiquitous in the environment and are highly toxic to many life forms. They include cadmium, mercury, arsenate, arsenite, antimony, lead, zinc and bismuth ions. Although these substances are rarely used as antimicrobial agents, their resistance genes are widespread in different genera of bacteria and closely linked with the genes for resistance to other antimicrobial agents

1.2.12.1 Cadmium

Cadmium divalent ions (Cd^{2+}) enter the cell via the energy dependent manganese ion transport system. (Perry and Silver, 1982) Cadmium is toxic to respiration, which is probably caused by its binding affinity to the sulphhydryl groups of essential enzymes. (Silver and Laddaga, 1990, Lyon and Skurray, 1987) Cadmium resistance in *S. aureus* is conferred by three different cadmium resistance operons. They are the *cadCA*, *cadBX* and *cadDX* operons. (Silver and Laddaga, 1990, Chaouni *et al.*, 1996, Crupper *et al.*, 1999, Lyon and Skurray, 1987)

The *cadCA* operon is an energy dependent cadmium efflux system that prevents cadmium accumulation in the cytoplasm by pumping it out of the cell. (Tsai *et al.*, 1992, Tynecka *et al.*, 1981) It also confers resistance to lead and zinc ions. (Yoon *et al.*, 1991, Rensing *et al.*, 1998) The CadA efflux pump of this system is the E_1E_2 or P-type cation translocating ATPase, and is encoded by the *cadA* gene. (Nucifora *et al.*, 1989) The CadA ATPase is regulated by the CadC metalloregulatory repressor protein that is encoded by the *cadC* gene and is located upstream of *cadA*. CadC blocks *cadA* transcription by attaching to its operator/promoter DNA sequence as a

homodimer. (Silver and Walderhaug, 1992, Endo and Silver, 1995, Wong *et al.*, 2002, Yoon and Silver, 1991) Lead, cadmium and bismuth ions are able to induce *cadA* transcription by binding with CadC and dislodging it from the DNA. (Yoon *et al.*, 1991, Endo and Silver, 1995, Rensing *et al.*, 1998) Although CadA alone is sufficient to confer cadmium resistant, the presence of CadC is required for full resistance. (Nucifora *et al.*, 1989, Yoon and Silver, 1991) In *S. aureus*, the *cadCA* operon is often carried on β-lactamase/heavy metal-resistance plasmids along with the mercury and arsenate resistance operons. (Firth and Skurray, 2000, Skurray and Firth, 1997, Shalita *et al.*, 1980) However, the earliest known *cadA* gene was found by itself on a plasmid in a strain isolated in 1945. (Udo *et al.*, 2000) Chromosomally it is found in the SCC*mec* carried by a Tn*554*-like element, ψTn*554*. (Dubin *et al.*, 1992, Ito *et al.*, 2003a) The plasmid-borne *cadCA* operon has been found in different staphylococcal species as well as in other Gram-positive and -negative bacteria. This dissemination is believed to be mediated by IS*431/257* because the *cadCA* operon is found either flanked, or in close proximity, to these insertion sequences. (Alonso *et al.*, 2000, Oger *et al.*, 2003, Silver and Phung, 1996)

The *cadBX* operon comprises the *cadB* and *cadX* genes and confers a different resistance mechanism to the *cadCA* operon. (Foster, 1983, Lyon and Skurray, 1987) This operon does not affect the efflux of Cd^{2+} ions, instead it catalyses an intracellular accumulation of cadmium in resistance cells. It is believed that *cadB* encodes a cadmium-binding membrane protein that binds with the Cd^{2+} ions and prevents it from exerting its toxic effects. (Perry and Silver, 1982, Silver and Laddaga, 1990, Crupper *et al.*, 1999) The *cadX* gene is located downstream of the *cadB* gene and shares 40% sequence homology with *cadC*. Similar to the *cadCA* operon, the *cadB* requires the presence of *cadX* to express cadmium resistance fully. (Foster, 1983, Chaouni *et al.*, 1996, Silver and Laddaga, 1990)

The *cadDX* operon consists of the *cadD* and *cadX* genes. It is flanked by a pair of IS*431/257*s, and the *cadX* gene is truncated by one of the flanking IS*431/257*. The *cadD* has 84% sequence homology with a *cadB*-like gene in *S. lugdunensis*. Like the *cadB* gene, the *cadD* requires *cadX* for full cadmium resistance. As a result of the IS*431/257* truncation of *cadX* in the *cadDX* operon, it confers only low-level cadmium resistance. The introduction of an intact *cadX* into a *cadDX* carrying host

leads to a ten-fold increase in cadmium resistance. (Crupper *et al.*, 1999) Both the *cadBX* and *cadDX* operons have only been found on plasmids and are relatively uncommon. (Chaouni *et al.*, 1996, Crupper *et al.*, 1999, O'Brien *et al.*, 2002, Yamaguchi *et al.*, 2001, Lyon and Skurray, 1987, Holden *et al.*, 2004)

1.2.12.2 Mercury

The toxicity of lipid soluble mercury (Hg) and organomercurial compounds is due to their avid binding affinity for the sulphhydryl groups of the membrane proteins and enzymes. This interaction between mercury ions and the enzymes results in the inhibition of macromolecular synthesis and enzyme activity, especially the transcription and translation processes. (Lyon and Skurray, 1987, Foster, 1983) In *S.* aureus, the *mer* operon confers resistance against both mercury ions and organomercurial compounds and includes phenyl- and methylmercury. It carries the *merR*, ORF3, ORF4, *merT*, *merA* and *merB* structural genes that confer the regulatory, transport and detoxification systems of mercury resistance. (Laddaga *et al.*, 1987, Silver and Phung, 1996, Silver and Laddaga, 1990) The *merR* gene encodes for the MerR transcriptional regulator of the *mer* operon and has a unique mode of action. Once bound to the operator/promoter segment of the *mer* operon, the MerR bends the DNA and facilitates the binding of RNA polymerase, forming a nonfunctional protein-DNA complex. In this complex, the -10 promoter is out of reach of the RNA polymerase hence the transcription is repressed. The binding of Hg^{2+} ions with MerR induces a localised distortion that underwinds the operator/promoter DNA by at least 30^0 more than in the repressed state. This underwinding action activates transcription by realigning the -10 promoter so the RNA polymerase can bind to it. (Brown *et al.*, 2003, Silver and Laddaga, 1990, Summer, 1992, O'Halloran, 1993, Skinner *et al.*, 1991) MerR is extremely sensitive to activation by Hg^{2+} ions and is also activated by Cd^{2+} and Zn^{2+} ions but only at high concentrations. (Ralston and O'Halloran, 1990, Chu *et al.*, 1992, Yu *et al.*, 1996) Unlike the cadmium efflux system, the *mer* operon transports toxic inorganic Hg^{2+} ions into the cytoplasm to be detoxified by mercury detoxifying enzymes. The ORF3, ORF4 and *merT* encode for three transmembrane proteins and are believed to be responsible for the mercury transport system in *S. aureus*. Their deletion in strains with inactivated mercury detoxifying enzymes leads to hypersensitivity to,

and hyperaccumulation of, Hg^{2+} ions. (Barkay *et al.*, 2003, Silver and Phung, 1996, Silver and Laddaga, 1990) The organomercurial compounds with their neutralising counter ions, such as acetate and chloride, are believed to be sufficiently lipid soluble to cross the cell wall via diffusion without assistance of any specific transport system. (Barkay *et al.*, 2003) The *merA* gene encodes for MerA, a mercuric reductase, which is the core of *mer* operon. (Laddaga *et al.*, 1987) MerA is a cytoplasmic flavoprotein that is structurally, and in sequence, similar to other oxidoreductases and they all have a highly homologous redox-active disulphide/dithiol active site. MerA functions as a homodimer that has a bound FAD cofactor and utilises NADPH as a source of electrons in the Hg^{2+} ion reduction. In the MerA catalysed reduction, the toxic Hg^{2+} ions are converted into relatively inert elemental Hg^{0}, which is released from the cell as vapour due to its high volatility. (Silver and Laddaga, 1990, Barkay *et al.*, 2003) The *merB* encodes for the MerB organomercurial lyase that is an organomercurial compound-degrading enzyme, with a broad substrate specificity. (Di Lello *et al.*, 2004, Laddaga *et al.*, 1987) MerB acts as a cytoplasmic monomer without disulphide bonds and does not require cofactors. MerB lyase catalyses the protonolytic cleavage of the carbon-mercury bond of the organomercurial substrate and degrades it into a reduced hydrocarbon and Hg^{2+} ion. The reduced hydrocarbon, such as methane from methylmercury, is released but the Hg^{2+} ion is bound to MerB. The bound Hg^{2+} ion is directly transferred by MerB lyase to MerA reductase which reduces Hg^{2+} to less toxic elemental Hg^{0}. (Benison *et al.*, 2004, Barkay *et al.*, 2003, Di Lello *et al.*, 2004)

The *mer* operon is mainly found on the β-lactamase/heavy metal resistant plasmids and the SCC*mec* genomic island. In both cases, the *mer* operon has been found to be flanked by IS*431/257*. (Firth and Skurray, 2000, Ito *et al.*, 2001) As a result, the *mer* operon with the flanking IS*431/257*s has been designated as transposon Tn*4004*. (Lyon and Skurray, 1987, Firth and Skurray, 2000) Even though the transposition of Tn*4004* has not been demonstrated, IS*431/257* is still believed to play a role in the dissemination of the *mer* operon. (Silver and Laddaga, 1990, Skurray and Firth, 1997) The *mer* operon is found widely in both Gram-negative and -positive bacteria and is often found on either plasmids or transposons. (Barkay *et al.*, 2003, Bogdanova *et al.*, 2001) Since the clinical use of mercurial disinfectants was discontinued in the late 1970 (Porter *et al.*, 1982) the selective pressure for mercury

probably comes from the environment such as industrial pollution and the use of mercury in dentistry. (Bogdanova *et al.*, 2001, Stapleton *et al.*, 2004, Narita *et al.*, 2003) Mercury resistance is tightly linked with many other antibiotic resistances which may also contribute to the selection pressure. (Firth and Skurray, 2000, Ito *et al.*, 2001, Barkay *et al.*, 2003)

1.2.12.3 Arsenic and antimony

Arsenate is an analogue of phosphate and enters the cell via the phosphate transport system. Arsenate toxicity is due to its inhibition of enzymes like kinases, as well as interference of the energy transfer in glycolysis. Both arsenite and antimony exert their toxicity by binding to cysteine residues in proteins. The arsenic resistance can arise from chromosomal mutations in the phosphate transport system. However, the most common resistance mechanism is the plasmid mediated arsenate transport system encoded by the *ars* operon. (Lyon and Skurray, 1987, Foster, 1983) Similar to other heavy metal-resistance operons, the *ars* operon is often found together with the cadmium and *mer* operons on β-lactamase/heavy metal resistance plasmids. (Firth and Skurray, 2000, Skurray and Firth, 1997, Lyon and Skurray, 1987, Shalita *et al.*, 1980)

The ars operon consists of three structural genes in the order *arsR*, *arsB* and *arsC*. The *arsR* gene encodes for a repressor protein that regulates the transcription of the *ars* operon. (Ji and Silver, 1992b, Rosenstein *et al.*, 1992) The ArsR belongs to the family of SmtB/ArsR metalloregulatory repressor proteins like the CadC of the *cadCA* operon. (Xiong and Jayaswal, 1998, Busenlehner *et al.*, 2003) It attaches to the *ars* operon operator/promoter DNA as a dimer and blocks transcription. The binding of inducer ions to ArsR significantly weakens its affinity to the DNA and allows the RNA polymerase to be loaded and to transcribe. ArsR is induced by oxyanions of arsenate, arsenite and antimony as well as the bismuth ion. (Ji and Silver, 1992b, Busenlehner *et al.*, 2003, Silver and Laddaga, 1990) The *arsB* gene encodes for a transmembrane chemiosmotic arsenite-transport protein that confers resistance against arsenite and antimonite. (Ji and Silver, 1992b, Bröer *et al.*, 1993, Rosenstein *et al.*, 1992) The ArsB alone has been shown to be driven by proton motive force. (Dey and Rosen, 1995, Kuroda *et al.*, 1997) In *Escherichia coli*, the

ArsB was found to couple with the ArsA anion-translocating ATPase to form an arsenite transport system driven by hydrolysis of ATP. (Dey and Rosen, 1995, Walmsley *et al.*, 2001, Kuroda *et al.*, 1997) ArsA is the primary arsenite pump in *E. coli*, but is absent in *S. aureus* and other Gram-positive bacteria. (Bröer *et al.*, 1993) However, the introduction of ArsA into ArsB carrying *S. aureus* increases the level of arsenate resistance. (Bröer *et al.*, 1993) The ArsC arsenate reductase is a cytoplasmic protein encoded by the *arsC* gene and confers arsenate resistance. ArsC reductase catalysed reduction converts the less toxic arsenate into the more toxic arsenite which is subsequently transported out of the cell by the ArsB transport protein. The energy utilised by ArsC reduction is funnelled from NADPH through the small intracellular thiol compound thioredoxin. (Ji and Silver, 1992b, Silver and Phung, 1996, Ji and Silver, 1992a)

1.2.13 Nucleic acid-binding compounds

The nucleic acid-binding (NAB) compounds are a group of biocidal agents that have been widely used as dyes, antiseptics and disinfectants. They share a common feature of binding with DNA molecules hence the name NAB compounds. These compounds include the quaternary ammonium compounds (QAC), diamidines and DNA intercalating agents. (McDonnell and Russell, 1999, Emslie *et al.*, 1985)

The QACs are cationic surface-active agents like cetyltrimethyl-ammonium bromide and cetrimide. Sometime they have also been described as cationic detergents. They are widely used as antiseptics and disinfectants in both domestic and hospital settings, as well as in the food industry and for veterinary use. (McDonnell and Russell, 1999, Heir *et al.*, 1998) The QACs react with the phospholipid component of the cytoplasmic membrane and cause membrane distortion and lysis due to osmotic stress. They can also interfere with the proton motive force that is linked to the solute transport, flagellar movement and ATP synthesis. The diamidines includes propamidine isethionate and dibromopropamidine isethionate. Although their exact mode of action is not clear, they can inhibit oxygen uptake and induce leakage of amino acids. The diamidines have been used in topical treatment for wounds. (McDonnell and Russell, 1999) The DNA intercalating agents such as ethidium bromide and acridine are biocidal and commonly used as dyes. They damage the

DNA by intercalating between two layers of base pairs in the DNA molecule. (McDonnell and Russell, 1999)

In staphylococci, resistance to NAB compounds is mediated by two groups of multidrug-efflux proteins driven by proton motive force. They belong to the major facilitator superfamily (MFS) and the small multidrug resistance family (SMR). The staphylococcal NAB-resistance, MFS proteins, consist of the QacA and QacB multidrug-efflux proteins. QacA is encoded by the *qacA* gene and is a membrane-transport protein consisting of 14 transmembrane segments. (Putman *et al.*, 2000, Paulsen *et al.*, 1996a, Rouch *et al.*, 1990) QacA confers active efflux of more than 30 toxic lipophilic compounds that belong to 12 distinct chemical classes including the QACs, diamidines and ethidium bromide. All of these compounds are either monovalent or divalent lipophilic organic cations. (Mitchell *et al.*, 1999, Mitchell *et al.*, 1998, Grkovic *et al.*, 2002, McDonnell and Russell, 1999) QacA interacts directly with the substrates and uses two separate binding sites for monovalent and divalent substrates. (Mitchell *et al.*, 1999) The QacB protein encoded by the *qacB* gene is very closely related to the QacA protein. There are only seven base pair differences in separate positions between the *qacA* and *qacB* genes. The most significant difference is in codon 323. QacA has an amino acid with an acid residue whereas QacB does not have the acid residue at the same location. The acid residue at position 323 is critical for efflux of the divalent organic cations. As a result, QacB only confers resistance to monovalent lipophilic organic cations such as the QACs and ethidium bromide, but not the diamidines. (Paulsen *et al.*, 1996a, Mitchell *et al.*, 1999, Grkovic *et al.*, 2002) A plasmid mediated *qacB* gene has been found in a strain isolated in 1951. *qacA* was first found in the 1980s and its emergence coincides with the extensive use of divalent cationic detergents in hospitals. It is believed that *qacA* has probably evolved from *qacB* by mutations. (Paulsen *et al.*, 1998, Mitchell *et al.*, 1999) Both *qacA* and *qacB* are regulated by the *qacR* gene which is located immediately upstream and is transcribed in the opposite direction. *qacR* encodes a QacR transcriptional repressor protein. The QacR repressor inhibits transcription by attaching two of the QacR homodimers onto the operator/promoter region of *qacA* and *qacB*. QacR is different from other transcriptional repressors in that it does not self regulate its own transcription. QacR is dislodged from the DNA by binding with the substrates, and thus allowing the transcription of the *qacA* or *qacB* genes. (Grkovic *et al.*, 1998, Grkovic *et al.*, 2002) *qacA* is disseminated by

medium sized multiple-resistance plasmids of both the pSK1 family and the β-lactamase/heavy metal multiresistance family. The pSK1 family of multiresistance plasmids only carry the *qacA* gene whereas the β-lactamase/heavy metal-multiresistance plasmids can carry either the *qacA* or *qacB* genes. (Skurray and Firth, 1997, Lyon and Skurray, 1987, Firth and Skurray, 2000, Townsend *et al.*, 1985d) Not surprisingly, *qacA* and *qacB* were found to be closely linked with many other antimicrobial-resistance genes, especially the *blaZ* β-lactamase genes. (Sidhu *et al.*, 2001, Sidhu *et al.*, 2002) The insertion sequences IS*256* and IS*431/257* have been found downstream of the *qacA* and *qacB* genes indicating that they may have a role in *qacA/B* gene dissemination. (Alam *et al.*, 2003)

The SMR multidrug efflux protein is the smallest known secondary pump driven by proton-motive force. (Putman *et al.*, 2000) The first SMR gene in *S. aureus* was found on plasmids and was described as the *qacC* gene. (Lyon and Skurray, 1987, Leelaporn *et al.*, 1995) This gene has also been known as the *qacD* (Littlejohn *et al.*, 1991) and *ebr* (Sasatsu *et al.*, 1989) gene, but is now renamed the *smr* gene. (Grinius *et al.*, 1992, Putman *et al.*, 2000, Paulsen *et al.*, 1996b) Three other SMR proteins, encoded by genes other than *smr*, have also been reported. They are the *qacG* (Heir *et al.*, 1999), *qacH* (Heir *et al.*, 1998) and *qacJ* (Bjorland *et al.*, 2003) genes.

All four of these SMR efflux proteins are closely related. They are approximately 70% to 80% identical to each other in both their DNA and amino acids sequences. They are about 107 amino acids in length, and probably function as oligomeric complexes due to their small size. Structurally the SMR efflux proteins are membrane transporter proteins consisting of four transmembrane segments. The SMR, QacG, QacH and QacJ SMR multidrug efflux proteins confer resistance to monovalent organic cations like ethidium bromide and some QACs. Despite their similarity, they are different in substrate specificity and level of resistance. These differences are similar to those seen between QacA and QacB and are probably due to amino acid differences in locations crucial for substrate binding and to differences in the level of gene expression mediated by their promoters. (Heir *et al.*, 1998, Heir *et al.*, 1999, Bjorland *et al.*, 2003, Paulsen *et al.*, 1995, Putman *et al.*, 2000, Paulsen

et al., 1996b) All four staphylococcal SMR genes are carried by small plasmids less than 3 kb that belong to the family of rolling-circle plasmids. (Skurray and Firth, 1997, Bjorland *et al.*, 2003, Heir *et al.*, 1998, Heir *et al.*, 1999, Littlejohn *et al.*, 1991) Small plasmids carrying *smr* have been found integrated into large conjugative plasmids. Similar to the other integrated small plasmids like pT181 and pUB110, the integrated *smr* plasmid is flanked by IS*431/257* indicating that the integration may have mediated by this insertion sequence. (Skurray and Firth, 1997, Berg *et al.*, 1998, Littlejohn *et al.*, 1991)

As a part of the European SENTRY survey study, 497 strains of *S. aureus* were isolated in 24 different hospitals between 1997 and 1999. Out of the 497 strains, 239 were found to carry NAB-compound resistance. The *qacA* and *qacB* genes were the predominant genotype and they were found in 88% (210 strains) of the NAB-resistant strains. The *smr* gene was only found in 12% (29 strains) of the resistant strains. (Mayer *et al.*, 2001, Noguchi *et al.*, 1999) A Japanese study of 522 *S. aureus* clinical isolates also showed that *qacA* and *qacB* were the predominant genotype. (Alam *et al.*, 2003)

The chromosomal *norA* and *mdeA* genes encode for two proton-dependent MFS-efflux proteins that confer NAB-compound resistance. The NorA encoded by *norA* gene is a 42 kDa MFS-efflux protein with 12 transmembrane segments. (Yoshida *et al.*, 1990, Paulsen *et al.*, 1996b) The NorA multidrug-efflux protein is known for its fluoroquinolone resistance, but it also confers active efflux of ethidium bromide, diamidines and some QACs. (Neyfakh *et al.*, 1993, Huang *et al.*, 2004) The *mdeA* gene encodes for a 52 kDa MdeA multidrug-efflux protein that has 14 transmembrane segments. The overexpression of the *mdeA* gene confers resistance to some QACs, ethidium bromide and novobiocin but not the diamidines and fluoroquinolones. (Huang *et al.*, 2004)

Table 1.3 Summary of the mechanism of action of antimicrobial agents

Antimicrobial agent[a]	Target	Mechanism of action
Aminoglycosides	30S ribosomal RNA	Inhibits transcription
β-lactam	Penicillin-binding proteins	Inhibits cell wall cross-linking
Chloramphenicol	50S ribosomal RNA	Inhibits transcription
Fusidic acid	Elongation factor G	Inhibits peptide translocation and protein synthesis
Glycopeptides	D-alanyl-D-alanine residues of peptidoglycan chain and precursor	Inhibits cell wall cross-linking
MLS	50S ribosomal RNA	Inhibits transcription
Mupirocin	isoleucyl-tRNA synthetase	Inhibits incorporation of isoleucine in peptide synthesis
Quinolones	DNA gyrase and topoisomerase IV	Blocks DNA replication
Rifampicin	β subunit of RNA polymerase	Inhibits RNA synthesis
Tetracyclines	30S ribosomal RNA	Inhibits transcription
Spectinomycin	30S ribosomal RNA	Inhibits transcription
Sulphonamides	Dihydropteroate synthase	blocks tetrahydrofolic acid pathway
Trimethoprim	Dihydrofolate reductase	blocks tetrahydrofolic acid pathway
Cadmium	Sulfhydryl groups of essential enzymes	Inactivates essential enzymes
Mercury and organomercurials	Sulfhydryl groups of essential enzymes	Inactivates essential enzymes
Arsenic and antimony	Kinases	Inactivates kinases
QACs[b]	Cytoplasmic membrane	Causes membrane damage
Diamidines[b]	Cytoplasmic membrane	Induces leakage of amino acids
Ethidium bromide[b]	DNA	Intercalating into DNA molecules

[a]Abbreviations: MLS, macrolides, lincosamides and streptogramins; QACs, quaternary ammonium compounds.
[b]QACs, diamidines and ethidium bromide are known as nucleic acid-binding (NAB) compounds.

Table 1.4 Staphylococcal antimicrobial-resistance determinants

Determinant	Resistance(s)[a]	Mechanism	Location[b]
Aminoglycoside			
aacA-aphD[c]	Km ,Gm, Tb	Drug modification	C, T, P
aadA	Sm, Sp	Drug modification	P
aadD[c]	Km, Nm, Tb	Drug modification	P
aadE[c]	Sm	Drug modification	C, T, P
aphA-3[c]	Km, Nm	Drug modification	C, T, P
aphC[c]	Sm	Drug modification	P
str	Sm	Drug modification	P
spc	Sp	Drug modification	C, T
β-lactam			
blaZ	Penicillin	Inactivation	C, T, P
mecA	All β-lactams	Novel PBP2a/2'	C, T?
Chloramphenicol			
cat	Chloramphenicol	Inactivation	P
Fusidic acid			
fusA	Fusidic acid	Target alteration	C
far1	Fusidic acid	Reduced influx?	P
Glycopeptides			
vanA	Vancomycin, Teicoplanin	Target alteration	C, T, P
VISA/GISA[d]	Vancomycin, Teicoplanin	Thicken cell wall	C
ble	Bleomycin	Sequestration	P
Macrolides, lincosamides and streptogramins (MLS)			
ermA	M, L, SgB	Target alteration	C, T, P
ermB	M, L, SgB	Target alteration	C, T, P
ermC	M, L, SgB	Target alteration	P
msrA	M, SgB	Active efflux	P
vgaA	SgA	Active efflux	P, T
vgaB	SgA	Active efflux	P
vatA	SgA	Drug modification	P
vatB	SgA	Drug modification	P
vatC	SgA	Drug modification	P
vgbA	SgB	Inactivation	P
vgbB	SgB	Inactivation	P
linA/linA' (*lnuA*)	Lincomycin	Drug modification	P
Mupirocin			
mupA	Mupirocin	Additional resistant enzyme	C, P
Quinolone			
grlA	Quinolone	Target alteration	C
grlB	Quinolone	Target alteration	C
gryA	Quinolone	Target alteration	C
gryB	Quinolone	Target alteration	C
norA	Quinolone, NAB, Nv	Active efflux	C
Rifampicin			
rpoB	Rifampicin	Target alteration	C

Continued on the following page

Table 1.4 Staphylococcal antimicrobial-resistance determinants cont.

Determinants	Resistance(s)[a]	Mechanism	Location[b]
Streptothricin			
sat4	Streptothricin	Drug modification	C, T, P
Sulphonamide			
sulA	Sulphonamide	Overproduction of target	C
Tetracycline			
tetK	Tetracycline	Active efflux	C, P
tetL	Tetracycline	Active efflux	P
tetM	Tetracycline, Minocycline	Ribosomal protection	C, T
Trimethoprim			
dfrA	Trimethoprim	Additional resistant enzyme	C, T, P
dfrD	Trimethoprim	Additional resistant enzyme	P
dfrG	Trimethoprim	Additional resistant enzyme	C
Cadmium			
cadA	Cadmium	Active transport	P
cadB	Cadmium	Sequestration[e]	P
cadD	Cadmium	Sequestration[e]	P
Mercury and organomercurial compounds			
merA	Hg, OrganoHg	Detoxification	C, T?, P
merB	Hg, OrganoHg	Detoxification	C, T?, P
Arsenic and antimony			
arsB	Arsenite, Antimony	Active efflux	C, P
arsC	Arsenate	Conversion of arsenate into arsenite for *arsB* efflux.	C, P
NAB compounds			
qacA	Divalent and monovalent NAB: QACs, Di, EtBr	Active efflux	P
qacB	Monovalent NAB: QACs, EtBr	Active efflux	P
smr	Monovalent NAB: Some QACs, EtBr	Active efflux	C, P
qacG	Monovalent NAB: Some QACs, EtBr	Active efflux	P
qacH	Monovalent NAB: Some QACs, EtBr	Active efflux	P
qacJ	Monovalent NAB: Some QACs, EtBr	Active efflux	P
norA	EtBr, Di, some QACs, Nv, Fq	Active efflux	C
mdeA	EtBr, some QACs	Active efflux	C

[a]Abbreviations: Di, diamidines; EtBr, ethidium bromide; Fq, fluoroquinolone; Km, kanamycin; Gm, gentamicin; Hg, mercury; L, lincosamides; M, macrolides; NAB, nucleic acid-binding compounds; Nm, neomycin; Nv, novobiocin, OrganoHg, organomercurial compounds; QACs, quaternary ammonium compounds; SgA, streptogramins type A; SgB, streptogramins type B; Sm, streptomycin; Sp, spectinomycin; Tb, tobramycin.

[b]Abbreviations for gene locations: C, chromosomal; P, plasmid, T, transposon; ?, putative

[c]*aacA-aphD* is known as *aac(6')-aph(2'')*; *aadD* as *ant(4')-I*; *aadE* as *ant(6')-I*; *aphA-3* as *aph(3')-III*; and *aphC* as *aph(3'')-I*.

[d]VISA/GISA, vancomycin or glycopeptide intermediate *S. aureus*, determinant(s) is yet to be identified

[e]The mechanisms have been proposed but are yet to be clarified.

1.3 Mobile elements in *S. aureus*

Mobile elements are genetic entities that able to move within and between different genomes. In some instances, these elements insert themselves into the genome and this results in genomic rearrangements. In *S. aureus*, these mobile elements are often found in conjunction with either antimicrobial resistance or toxin genes. Mobile elements enable these genes to transfer during genetic exchanges. As a result, these genetic elements play an important role in the acquisition of antimicrobial resistance and pathogenicity by *S. aureus* as well as the evolution of its genomes. Mobile elements are insertion sequences, transposons, plasmids, genomic islands and bacteriophages. (Baba *et al.*, 2002, Skurray and Firth, 1997, Firth and Skurray, 2000, Novick *et al.*, 2001, Holden *et al.*, 2004)

1.3.1 Insertion sequences

Insertion sequences (IS) are the simplest and smallest transposable elements. They are less than 2.5 kb in length and carry genes that are responsible for their mobility. ISs carry their own transposase gene in one or two ORFs that almost consume the entire length of the element. The transposase gene is flanked by a pair of identical terminal-inverted repeats. These terminal-inverted repeats not only carry the binding and cleavage site for the transposase, in some ISs they also harbour the promoter sequence upstream of the transposase gene. The IS transposases belong to the super family of phosphoryltransferases that carry the conserved DDE motif. The DDE motif is mainly responsible for the coordination of divalent cations, such as Mg^{2+}, and is necessary for catalysing cleavage and the strand transfer activities of the transposase. IS often insert in a staggered fashion and generate a pair of flanking, short direct repeats of the target sequence. (Mahillon and Chandler, 1998, Haren *et al.*, 1999) In *S. aureus*, five insertion sequences have been identified and characterised. They are IS*256*, IS*431*/*257*, IS*1181*, IS*1182* and IS*1272*. (Skurray *et al.*, 1988, Firth and Skurray, 2000, Mahillon and Chandler, 1998)

1.3.1.1 IS*256*

IS*256* is the founding member of the IS*256* insertion sequence family. (Mahillon and Chandler, 1998) It is 1.35 kb in length and flanked by 26 bp imperfect inverted repeats. The IS*256* carries only a single long open reading frame encoding the putative transposase. The entire copy of IS*256* is flanked by a pair of eight bp direct repeats of its target sequence which are generated during transposition. (Lyon *et al.*, 1987b, Byrne *et al.*, 1989, Rouch *et al.*, 1987) IS*256* utilises a novel transpositional mechanism in which it first excises as extrachromosomal circular DNA before the strand transfer stage during transposition. (Prudhomme *et al.*, 2002, Loessner *et al.*, 2002)

IS*256* is non-site specific and has been found in the *S. aureus* chromosome and on plasmids in multiple copies. (Dyke *et al.*, 1992, Wei *et al.*, 1992a) The aminoglycoside resistance transposon, Tn*4001*, bearing the *aacA-aphD* gene is flanked by inverted copies of IS*256*. The flanking IS*256*s are known to facilitate the transposition of Tn*4001*. (Rouch *et al.*, 1987, Lyon *et al.*, 1987b, Prudhomme *et al.*, 2002) IS*256* is associated with a Tn*4001*-like aminoglycoside-resistance transposon and an *erm* macrolide-resistance gene in enterococci and coagulase-negative staphylococci. (Hodel-Christian and Murray, 1991, Rice *et al.*, 1995) The IS*256* is also associated with virulence factors. It has been found in a novel *S. aureus* pathogenicity island carrying the exfoliative toxin. (Yamaguchi *et al.*, 2002) It also plays a role in biofilm formation in *S. epidermidis*. (Ziebuhr *et al.*, 1999, Kozitskaya *et al.*, 2004, Arciola *et al.*, 2004, Conlon *et al.*, 2004)

IS*256* can influence the expression of adjacent genes by forming a hybrid promoter with the native gene or by inducing adjacent deletions. IS*256* forms a potent hybrid promoter with its own promoter and the native promoter that increase the expression of adjacent genes. This IS*256* hybrid promoter influences the transcriptional level of the *aacA-aphD* gene in Tn*4001* and increases the level of methicillin resistance in *S. sciuri* by increasing the transcription of the *mecA* gene. (Lyon *et al.*, 1987b, Firth and Skurray, 2000, Couto *et al.*, 2003) This hybrid promoter can increase the expression of the *llm* gene in MRSA and can change the low-level heterogeneous methicillin-resistance phenotype into the high-level resistance phenotype. (Maki and

Murakami, 1997) IS*256* induced deletion of the *ermA* regulatory segment leads to constitutive expression of the *ermA* macrolide-resistance gene. (Schmitz *et al.*, 2002) A similar IS*256* insertion has been reported in the *mecA* methicillin-resistance gene by deletion of the regulatory genes. (Oliveira *et al.*, 2000) IS*256* insertional inactivation of the *tcaA* gene in *S. aureus* results in an increased level of resistance to the glycopeptide antimicrobial agents. (Maki *et al.*, 2004)

1.3.1.2 IS*431/257*

IS*431* (Barberis-Maino *et al.*, 1987) or IS*257* (Rouch and Skurray, 1989) belong to the IS*6* insertion sequence family. (Mahillon and Chandler, 1998) IS*431/257* is 790 bp in length and composed of two 27 bp terminal inverted repeats and a single ORF encoding its putative transposase. IS*431/257* undergoes non-site specific replicative transposition without resolution and generates eight bp direct repeats of the target sequence. This unresolved transposition results in the fusion of the target and donor replicons involving three copies of IS*431/257*. Of the three IS*431/257*s, two are the original flanking IS*431/257*s of the target replicon and link the target and donor replicons together. The third copy of IS*431/257* is generated by the transposition and is located at the extremity of the donor replicon. (Needham *et al.*, 1995, Skurray and Firth, 1997, Mahillon and Chandler, 1998) IS*431/257* is associated with many genetic determinants on both plasmids and chromosome. In a retrospective studies, IS*257* was found in a NAB-resistance *qacB* plasmid in a *S. aureus* isolated in 1951, (Paulsen *et al.*, 1998) whereas IS*431* was first found in the first MRSA isolated in 1961. (Barberis-Maino *et al.*, 1987) IS*431/257* is more abundant in coagulase-negative staphylococci than in *S. aureus* and in *S. aureus* it is more common in MRSA due to its association with resistance genes. (Kobayashi *et al.*, 2001a)

Many of the small integrated plasmids found on larger plasmids and in the SCC*mec* genomic island are flanked by a pair of IS*431/257* in the same orientation. (Stewart *et al.*, 1994, Firth and Skurray, 2000, Ito *et al.*, 1999, Berg *et al.*, 1998) The genes involved in the replication of the integrated plasmids are often disrupted and inactivated by the insertion of IS*431/257*. The inactivation of the replicative genes would stabilise integrated plasmids in the host. Flanking eight bp target duplications

are often found adjacent to the flanking IS. (Berg *et al.*, 1998, Stewart *et al.*, 1994, Skurray and Firth, 1997, Werckenthin *et al.*, 1996)

IS*431/257* has been found to form putative transposons with resistance genes and are probably a result of transposition. These IS*431/257*-flanked putative transposons, are the *dfrA*-bearing trimethoprim-resistance transposon Tn*4003*, and the *mer* operon bearing, mercury-resistance transposon, Tn*4004*. (Lyon and Skurray, 1987, Firth and Skurray, 2000, Skurray and Firth, 1997, Skurray *et al.*, 1988) Other resistance genes flanked by IS*431/257* include the *cadDX* cadmium-resistance operon, *mupA* mupirocin-resistance gene, the *vat*, *vgb* and *vga* streptogramin-resistance genes and the *aadE-sat4-aphA-3* aminoglycoside-resistance gene cluster. IS*431/257* is thought to be responsible for the mobility of these resistance genes. (Crupper *et al.*, 1999, Morton *et al.*, 1995, Allignet and El Solh, 1999, Derbise *et al.*, 1997a, Firth and Skurray, 2000, Skurray and Firth, 1997) In some cases, IS*431/257* also stabilises the integration of transposon Tn*4001* in its host by truncating the flanking IS*256*s of Tn*4001*. (Berg *et al.*, 1998, Byrne *et al.*, 1990)

IS*431/257* plays a central role in the evolution of multiresistance plasmids because of its association with resistance genes, its transpositional activities and homologous recombination between IS*431/257*s of the host and donor. The large conjugative plasmid pSK41 carries two IS*431/257*-flanked integrated plasmids. They are the aminoglycoside-resistance *aadD* and *ble*-bearing pUB110 and the NAB-resistance, *smr*-carrying, pSK108. pSK41 also harbours the IS*431/257* stabilised Tn*4001*. In total seven copies of IS*431/257* are on pSK41, clearly demonstrating the role of IS*431/257* in the evolution of, and accumulation of, resistance genes by this plasmid. (Berg *et al.*, 1998, Firth and Skurray, 2000, Skurray and Firth, 1997) In the chromosomal SCC*mec* genomic islands, IS*431/257* is partly responsible for the accumulation of resistance genes as the integrated small plasmids and *mer* operon are flanked by IS*431/257*. (See Section 1.4.4.1) (Ito *et al.*, 1999, Ito *et al.*, 2003a, Ito *et al.*, 2004, Ito *et al.*, 2001)

Similar to IS*256*, IS*431/257* can form a potent hybrid promoter with native promoters and influence the transcription and expression of adjacent genes. An IS*431/257* hybrid promoter is responsible for the high-level expression of the *dfrA*

trimethoprim-resistance gene in Tn*4003*. In some instances, IS*431/257* mediates the deletion of the native promoter region resulting in low-level trimethoprim-resistance by Tn*4003*. (Leelaporn *et al.*, 1994a, Skurray and Firth, 1997) A similar situation has been shown for the integrated pT181 where the IS*431/257* hybrid promoter mediates high-level tetracycline resistance. Putative IS*431/257* hybrid promoters have been found and may play a role in the expression of other determinants flanked by IS*431/257*. (Simpson *et al.*, 2000, Skurray and Firth, 1997) The IS*431/257* mediated deletion and arrangement of adjacent genes plays an important role in the regulation of the methicillin-resistance gene, *mecA*. Insertion of IS*431/257* has been found to induce the deletion and rearrangement of the *mecA* regulatory genes and probably affects the expression of *mecA*. (Kobayashi *et al.*, 2001b, Katayama *et al.*, 2001)

1.3.1.3 IS*1272*

IS*1272* is a 1934 bp element composed of two ORFs and 16 bp terminal inverted repeats. (Archer *et al.*, 1996) There are no flanking target site duplications found adjacent to IS*1272*. A truncated copy of IS*1272* (ψIS*1272*) has been found to truncate the *mecA* regulatory region. ψIS*1272* deletes the 3' portion of the *mecR1* signal-transducer gene and the entire *mecI* repressor gene. (Archer *et al.*, 1996, Archer *et al.*, 1994) Although this deletion leads to constitutive expression of *mecA*, it does not necessarily result in high-level methicillin resistance. The expression of high-level methicillin resistance is determined by host factors that affect the level of resistance. (See Section 1.2.5) (Berger-Bächi and Rohrer, 2002) Both IS*1272* and ψIS*1272* have been found in methicillin-resistant and methicillin-susceptible *S. aureus, S. epidermidis* and *S. haemolyticus*. However, IS*1272* is rarely detected in MSSA and some MRSA only carry a single copy of ψIS*1272* associated with the *mec* complex. In contrast, IS*1272* is commonly found in multiple copies in *S. haemolyticus* and *S. epidermidis*. (Archer *et al.*, 1994, Archer *et al.*, 1996, Kobayashi *et al.*, 1999) Complete copies of IS*1272* are found in methicillin-susceptible *S. haemolyticus*, whereas the IS*1272*s found in methicillin-susceptible *S. epidermidis* (MSSE) are often truncated. The ψIS*1272* of MSSE carry a truncation in its ORF2, whereas the ψIS*1272* found in methicillin-resistant staphylococci is

truncated in ORF1. (Archer *et al.*, 1996) In addition, the ψIS*1272* truncated *mec* complex found in MRSA, *S. epidermidis* and *S. haemolyticus* share either identical or highly similar *mecR1*-IS*1272* junction sequences. (Archer *et al.*, 1994, Kobayashi *et al.*, 1999) It has been suggested that *S. haemolyticus* is the natural host of IS*1272* and that the SCC*mec* with *mecI* and part of *mecR1* deleted by ψIS*1272* has been horizontally transferred from either *S. epidermidis* or *S. haemolyticus*. (Archer *et al.*, 1994, Archer *et al.*, 1996, Kobayashi *et al.*, 1999, Ito *et al.*, 2001)

1.3.1.4 IS*1181*

The IS*1181* is a member of the IS*L3* family. It is 1512 bp long and flanked by 23 bp terminal inverted repeats. The transposition of IS*1181* generates a pair of flanking eight bp direct repeats due to target duplication. (Derbise *et al.*, 1994, Chesneau *et al.*, 1999) IS*1181* has only been found in the *S. aureus* chromosome and in multiple copies. However, some isolates, like EMRSA-16, are free of IS*1181*. (Symms *et al.*, 1998, Derbise *et al.*, 1994, Holden *et al.*, 2004, Baba *et al.*, 2002) IS*1181* is unique, because unlike other *S. aureus* insertion sequences, it dose not associate with any resistance genes. Although it has been found in the Tn*5405* aminoglycoside-resistance transposon, it is neither linked with resistance genes nor influences their expression. (Chesneau *et al.*, 1999, Derbise *et al.*, 1994, Derbise *et al.*, 1996, Derbise *et al.*, 1997b)

1.3.1.5 IS*1182*

The 1864 bp long IS*1182* is composed of one ORF flanked by 33 bp imperfect, inverted repeats and generates eight base pairs target duplication. IS*1182* has been found to be related to the insertion sequence IS*1272* with 60% nucleotide similarity. A pair of IS*1182*s, in opposite orientation, are found to flank the transposon Tn*5405*. IS*1182* has been found in multiple copies in both MRSA and in coagulase-negative staphylococci. (Derbise *et al.*, 1996, Derbise *et al.*, 1997a, Derbise *et al.*, 1997b) Recently it was reported in the SCC*mec* resistance island. It was found to truncate the *mecI* gene located in the *mec* complex region. (Shore *et al.*, 2005)

1.3.2 Composite transposons

Composite transposons are either flanked by a pair of ISs or have terminal inverted repeats. They often carry their own transposases which are responsible for their dissemination. In some instances, the transposition of the transposon is mediated by the transposase from the flanking ISs. In addition to their transpositional genes, they have been found to carry others genes, such as those for antimicrobial resistance. (Weaver and Hedrick, 1992)

1.3.2.1 Aminoglycoside resistance transposons

Three different aminoglycoside-resistance transposons had been identified in *S. aureus*. They carry different aminoglycoside-resistance genes that results in different resistance profiles. These transposons are Tn*4001*, Tn*5405* and Tn*3854*. (Lyon *et al.*, 1987a, Derbise *et al.*, 1996, Udo and Grubb, 1991b)

1.3.2.1.1 Tn*4001*

Transposon Tn*4001* carries the AAC(6')-APH(2") bifunctional enzyme that confers resistance to most of the aminoglycosides like gentamicin, kanamycin and tobramycin but not arbekacin. (Wright, 1999, Mingeot-Leclercq *et al.*, 1999) This 4.7 kb transposon carries the *aacA-aphD* resistance gene flanked by a pair of IS*256* in opposite orientation. (Byrne *et al.*, 1989, Lyon and Skurray, 1987, Lyon *et al.*, 1987a, Lyon *et al.*, 1987b) Tn*4001* transposition is mediated by the flanking IS*256* transposases which form extrachromosomal circular DNA before strand transfer. As a result, Tn*4001* transposition generates eight bp direct repeats of the target site sequence. (Loessner *et al.*, 2002, Prudhomme *et al.*, 2002) In some instance, an additional copy of IS*256* has been found next to Tn*4001* due to tandem replication. The presence of this additional IS*256* increases the level of resistance by three to four fold. This is probably due to the IS*256* promoters acting in tandem to increase the level of transcription. (Lyon and Skurray, 1987, Lyon *et al.*, 1987b) Tn*4001* has been found in multiresistance plasmids belonging to the pSK1 family and conjugative plasmids such as pSK41 and pGO1. (Lyon and Skurray, 1987, Firth and Skurray, 2000, Wright *et al.*, 1998, Skurray *et al.*, 1988) Tn*4001*s on the pSK41

plasmid family are different because the flanking IS*256*s are truncated by IS*431/257* in opposite orientation. These Tn*4001*-like elements are immobile due to the inactivation of the IS*256*. Unlike other IS*431*/*257* transpositions, the eight bp direct repeats are not found in the Tn*4001*-like element. This suggests that the flanking IS*431/257*s are not due to direct insertion but due to homologous recombination through IS*431/257*. (Byrne *et al.*, 1990, Berg *et al.*, 1998, Lange *et al.*, 2003, Skurray *et al.*, 1988) Tn*4001*-like elements have also been found in the chromosome and the SCC*mec* genomic island. (Ito *et al.*, 2003a, Wright *et al.*, 1998)

Tn*4001* is typically found on the pSK1 plasmid family in the Australian and English MRSA strains isolated after the 1980s. In contrast, strains isolated before 1980s often carry either Tn*4001* or Tn*4001*-like elements in their chromosomes. (Wright *et al.*, 1998) The Tn*4001*-bearing multiresistance plasmids have been shown to be responsible for the dissemination of aminoglycoside resistance in many hospitals worldwide (Lyon and Skurray, 1987, Udou, 2004, Skurray *et al.*, 1988, Schmitz *et al.*, 1999, Wright *et al.*, 1998, Firth and Skurray, 2000)

Tn*4031* (Thomas and Archer, 1989b, Hodel-Christian and Murray, 1991) and Tn*3851* (Townsend *et al.*, 1984b) are the other two *aacA-aphD* gene-bearing transposons closely related to Tn*4001*. Tn*3851* had been found to be identical to Tn*4001* (Sim, 2003) and Tn*4031* is believed to be either identical or similar to Tn*4001*. (Firth and Skurray, 2000)

1.3.2.1.2 Tn*5405* and Tn*3854*

Tn*5405* carries the *aadE-sat4-aphA-3* gene cluster that confers resistance against kanamycin, neomycin and low-level resistance to streptomycin but not to gentamicin. The *sat4* gene found in Tn*5405* is truncated and therefore does not confer streptothricin resistance. (Derbise *et al.*, 1995, Derbise *et al.*, 1997b)

Structurally, Tn*5405* is flanked by a pair of inactive IS*1182*, in opposite orientation, and the entire copy is flanked by eight bp direct repeats. Both of the inactive IS*1182* carry the same point mutation that prematurely terminates transcription of their transposase genes. In addition to the gene cluster, it carries three putative ORFs with

unknown function and a copy of IS*1181*. This IS*1181* is located downstream of the resistance-gene cluster and adjacent to one of the flanking IS*1182*. (Derbise *et al.*, 1996) In some instances, IS*1181* and some of the ORFs are not present in Tn*5405*. (Derbise *et al.*, 1997a) Tn*5405* was initially found within another transposon Tn*5404*, a 16 kb Tn*552*-like transposon without a β-lactamase operon. (See Section 1.4.2.2) The genes encoding for the putative transposase (*tnpA*), resolvase (*binL*), resolution site and an ATP-binding protein (*tnpB*) in Tn*5404* is identical to their counterparts in Tn*552*. Similar to Tn*552*, Tn*5404* has 116 bp imperfect inverted repeats at both of its extremities and the entire copy is flanked by six bp direct repeats. Tn*5405* found within Tn*5404* is flanked by eight bp target duplication indicating it is directly inserted into Tn*5404*. Since both of the flanking IS*1182* are inactivated, it is believed that the mobility of Tn*5405* is dependent on the host transposon Tn*5404*. (Derbise *et al.*, 1995, Derbise *et al.*, 1996)

Tn*3854* is a 4.5 kb uncharacterised plasmid-borne aminoglycoside-resistance transposon that carries the same resistance profile to the Tn*5405*. It has been shown to undergo recombination independent transposition. (Udo and Grubb, 1991b, Atlas and Grubb, 2002)

1.3.2.2 β-lactamase transposons

Transposons Tn*552* (Rowland and Dyke, 1989) , Tn*4002* (Lyon and Skurray, 1987, Skurray *et al.*, 1988), Tn*3852* (Kigbo *et al.*, 1985) and Tn*4201* (Weber and Goering, 1988) are four closely related β-lactamase transposons found in *S. aureus*. They carry the *bla* operon that encodes for the production and regulation of the β-lactamase. These transposons are often found in the multiresistance staphylococcal plasmids and/or chromosome. (Skurray *et al.*, 1988, Firth and Skurray, 2000)

The 6545 bp β-lactamase transposon Tn*552* consists of two regions and has flanking 120 bp inverted terminal repeats. Tn*552* integration generates six bp flanking direct repeats of the target site sequence. The right half of Tn*552* contains the *bla* operon. (Rowland and Dyke, 1989, Rowland and Dyke, 1990, Lyon and Skurray, 1987) The *bla* operon is composed of the β-lactamase gene *blaZ* and its regulatory genes, *blaR1*

and *blaI*, that encode the signal transducer and repressor, respectively. (Gregory *et al.*, 1997, Kobayashi *et al.*, 1987, Clarke and Dyke, 2001a, Archer and Bosilevac, 2001) The left portion of Tn*552* confers its transposition function. It contains four genetic elements in the order of *resL* (resolution site), *binL* (resolvase/invertase), *tnpA* (transposase gene, previously called *p480*) and *tnpB*, previously known as *p271*. *tnpB* encodes a putative ATP-binding protein that enhances transposition and plays a role in target selection. (Rowland and Dyke, 1989, Rowland and Dyke, 1990, Leschziner *et al.*, 1998) This region, also known as the invertible region, of Tn*552* has been found to be invertible. In some plasmids, this region, together with the host *binR* resolvase/invertase gene, is flanked by the *resR* and *resL* resolution sites. The *resR* and *resL* are the inversely repeated resolution sites of the host plasmid and transposon respectively. This invertible region had been found in either orientation in some Tn*552*-bearing plasmids like pI524. However, in some cases the fragment is locked in one orientation due to deletions in the region. (Weber and Goering, 1988, Lyon and Skurray, 1987, Ito *et al.*, 2003a, Shalita *et al.*, 1980) The orientation of this region was also found to influence the formation of either Tn*552* or Tn*5404* on a plasmid. (Derbise *et al.*, 1995) In addition to its variation in orientation, this region is also highly variable in its components. In some cases, the transposase genes are truncated, and in other cases, the whole region is not detected. (Anthonisen *et al.*, 2002, Sidhu *et al.*, 2002, Ito *et al.*, 2003a)

Tn*4002* (6.7 kb), Tn*3852* (6.7 kb) and Tn*4201* (7.3 kb) are other β-lactamase transposons that are closely related to Tn*552*. Tn*4002* and Tn*4201* have been characterised and their right-hand regions bearing the *bla* operon are identical to Tn*552*. However, their invertible regions containing the transposition genes are different from their Tn*552* counterpart. Tn*4201* does not carry the *tnpB* gene in its invertible region, but is still capable of DNA inversion and transposition. The invertible region of Tn*4002* is almost identical to that in Tn*552*, except the region adjunct to the *tnpB* gene are deleted. The invertible fragment in Tn*4002* has been locked in a negative orientation on plasmid pSK4. (Weber and Goering, 1988, Derbise *et al.*, 1995, Lyon and Skurray, 1987) In spite of the deletion, the chromosomal Tn*4002* has been observed to transpose onto a pSK1 family plasmid. (Skurray *et al.*, 1988) The 6.7 kb Tn*3852* has yet to be characterised, it is presumed

to be identical to Tn*4002* as they were isolated in epidemic strains with a similar background. (Lyon and Skurray, 1987, Firth and Skurray, 2000, Kigbo *et al.*, 1985)

Although Tn*552* transposition appears to be random, it is found to prefer the resolution site of the host resolvases. (Griffin *et al.*, 1999, Rowland *et al.*, 2002) The *sin* resolvase gene is commonly found on the multiresistance plasmids belonging to the pSK1 family and the β-lactamase/heavy metal-resistance plasmid family. (Sidhu *et al.*, 2001, Paulsen *et al.*, 1994) Tn*552* is found inserted within the *resH* resolution site of *sin* resolvase which is 31 bp upstream of the *sin* gene. (Rowland *et al.*, 2002, Berg *et al.*, 1998) pSK1 plasmids are known to be responsible for the dissemination of Tn*552* as they carry the Tn*552* insertion hot spot. (Skurray and Firth, 1997, Lyon and Skurray, 1987, Firth and Skurray, 2000) Tn*552* is sometimes found to be closely linked to the NAB-resistance/*qac* genes due to their close proximity to the *sin* resolvase gene. (Sidhu *et al.*, 2001, Sidhu *et al.*, 2002) The family of pSK41 conjugative plasmids do not harbour the *sin* resolvase gene, but like pSK1, the Tn*552*-like element is found adjacent to a promoter like sequence upstream of its *res* resolvase gene. (Berg *et al.*, 1998)

1.3.2.3 Macrolides, Lincosamides and Streptogramins resistance transposons

Tn*551* (Novick *et al.*, 1979), Tn*554* (Phillips and Novick, 1979) and Tn*5406* (Haroche *et al.*, 2002) are transposons in *S. aureus* that encode macrolide, lincosamide and streptogramin resistance.

1.3.2.3.1 Tn*551*

Transposon Tn*551* carries the *ermB* gene which confers constitutive resistance to macrolides, lincosamides and streptogramin B (MLS_B). (Wu *et al.*, 1999, Novick *et al.*, 1979, Lyon and Skurray, 1987) It is a 5266 bp element that carries 40 bp terminal inverted repeats and is flanked by five bp direct repeats of the target sequence. Tn*551* is almost identical to the enterococcal transposon Tn*917*. They are 99.8% similar in DNA sequences and have the same structural organization. Tn*551* carries four ORFs, a leader peptide of the *erm* gene, the *ermB* rRNA methylase, a

resolvase and a transposase. The constitutive expression of the *ermB* gene was initially thought to be the result of mutations, or deletion in the regulatory leader peptide. However, a comparison of the inducible Tn*917* and the constitutive Tn*551* shows no deletions in the Tn*551* leader peptide. It is the -10 promoter of the *ermB* gene that is responsible for its constitutive expression. (Wu *et al.*, 1999, Lyon and Skurray, 1987) Tn*551* has been found to be able to transpose onto multiple sites on both plasmids and the chromosome. (Novick *et al.*, 1979, Berger-Bächi, 1983) As a result, Tn*551* has been extensively used as a tool for genetic mapping and mutagenesis studies. (Pattee *et al.*, 1990, Berger-Bächi, 1983, de Lencastre and Tomasz, 1994, Gardete *et al*., 2004)

Tn*551* was the first transposon found in *S. aureus.* It was found on the prototype β-lactamase/heavy metal-resistance plasmid pI258 which was isolated in a Japanese *S. aureus* strain in 1964. (Novick *et al.*, 1979, Shalita *et al.*, 1980) It has subsequently been found on plasmids and the chromosome, but is a relative uncommon MLS_B-resistance genotype. (Lina *et al.*, 1999, Lyon and Skurray, 1987)

1.3.2.3.2 Tn*554*

Transposon Tn*554* carries the inducible MLS_B and spectinomycin resistances. This unique transposon differs from other transposons. Its ends are asymmetric because of the lack of terminal inverted repeats. Unlike the other transposons, Tn*554* does not generate direct repeats by target duplication. It is extremely site-specific and transposes at a frequency of almost 100%. (Chikramane *et al.*, 1991, Tillotson *et al.*, 1989, Phillips and Novick, 1979, Murphy, 1990)

Tn*554* is 6691 bp long and consist of six ORFs. Three of the ORFs, *tnpA*, *tnpB* and *tnpC* are responsible for its transposition activities. The *tnpA* and *tnpB* are closely related site-specific recombinase of the *int* integrase family. The *tnpC* is involved in the determination of the orientation specificity of insertion but is not essential for Tn*554* transposition. The two resistance ORFs in Tn*554* are *ermA* and the *spc* genes. The *ermA* gene encodes the rRNA methylase that confers inducible MLS_B resistance. The *spc* gene encodes an AAD(9) nucleotidyltransferase that confers spectinomycin

resistance. (Phillips and Novick, 1979, Lyon and Skurray, 1987) The function of the sixth ORF in Tn*554* is unknown. (Murphy, 1990)

Two closely related Tn*554* preferential insertion sites have been identified in the *S. aureus* chromosome. The insertion site *att554* is located within the *radC* DNA repair gene, whereas the secondary insertion site *att155* is found in a *radC* homologue located in some SCC*mec* genomic islands. Tn*554* is found in, and disseminated by, the Allotype II and III SCC*mec* genomic islands due to this insertion site. (See Section 1.3.4.3) (Kuroda *et al.*, 2001, Ito *et al.*, 2003a, Ito *et al.*, 2001, Chikramane *et al.*, 1991, Tillotson *et al.*, 1989, Holden *et al.*, 2004, Aires de Sousa *et al.*, 1998) MRSA N315 carries five copies of Tn*554*, two of them inserted into the *radC* related insertion sites, while the other three copies interrupt ORFs encoding a probable lytic regulatory protein, a probable extracellular metalloprotease and a protein with unknown function. It has been speculated that carrying multiple copies of Tn*554* increases genome diversity rather than increasing the level of resistance as one copy is sufficient to confer resistance. (Kuroda *et al.*, 2001, Ito *et al.*, 2003a) Tn*554* has been found transposed onto β-lactamase/heavy metal plasmids in close proximity to their replication gene. (Murphy, 1990) In addition to these plasmids, it is known to be carried on conjugative plasmids that encode for diffusible pigment production. (Townsend *et al.*, 1986a, Townsend *et al.*, 1985f) The other inducible MLS_B resistance transposon, Tn*3853*, is probably either similar or identical to Tn*554*. (Townsend *et al.*, 1986a, Firth and Skurray, 2000)

ψTn*554* is a Tn*554*-like element found in the SCC*mec* genomic island. It is located close to the *SCCmec* extremity downstream of the *mecA* gene, whereas Tn*554* is located at the opposite end, upstream of *mecA*. It was originally suggested that Tn*554* and ψTn*554* formed the boundary of SCC*mec* but it has proven to be incorrect in later studies. ψTn*554* carries the *tnpA*, *tnpB* and *tnpC* transpositional genes of Tn*554* in the same orientation but the *tnpA* is truncated. In addition to the transpositional genes, ψTn*554* harbours the *cadCA* cadmium-resistance operon. (Dubin *et al.*, 1992, Dubin, 1993, Ito *et al.*, 2001, Ito *et al.*, 2003a) In some MRSA isolates, probably due an inversion event, the *ermA* and *spc* genes of Tn*554* have transferred into ψTn*554* and the *cadCA* operon of ψTn*554* has transferred into

Tn*554*. Despite this gene swapping event, the organization of the three transpositional genes and the positions of these two elements on SCC*mec* have remained unchanged. (Ito *et al.*, 2003a)

1.3.2.3.3 Tn*5406*

Transposon Tn*5406* confers resistance to the streptogramin A (SgA) drugs. This 5467 bp transposon is closely related to Tn*554*. It carries three transpositional genes that have significant similarity to the three transpositional genes of Tn*554*. Similar to Tn*554*, it does not generate target repeats and transposes into the Tn*554* specific insertion sites in the chromosome and SCC*mec*. Tn*5406* carries the *vgaAv* SgA-resistance gene downstream of the three transpositional genes. The *vgaAv* gene is a variant of the *vgaA* gene that confers resistance to SgA drugs except pristinamycin. It is also found to transpose onto plasmids carrying the SgA-resistance genes, *vgaB* and *vatB*. (Haroche *et al.*, 2000, Haroche *et al.*, 2002)

1.3.2.4 Tn*4003* and Tn*4004*

In Tn*4003*, the trimethoprim-resistance transposon, and Tn*4004*, the mercurial-resistance transposon, the resistance genes are flanked by IS*431/257*. Although in both of these putative transposons the eight bp target direct repeats had been found adjacent to the flanking IS*431/257*, their direct transposition is still yet to be demonstrated. It is believed that the formation of these transposons is a consequence of the unresolved transposition of the IS*431/257*. (See Section 1.3.1.2) (Silver and Laddaga, 1990, Skurray and Firth, 1997, Lyon and Skurray, 1987)

Tn*4003* is 4.7 kb long and carries three ORFs that are flanked by three copies of IS*431/257* in the same orientation. The three ORFs include *thyE* encoding thymidylate synthetase, *dfrA* encoding dihydrofolate reductase and a truncated ORF, *orf140*. Two copies of IS*431/257* are located at the boundary upstream of *dfrA* and the other flanking copy is located at the other end, downstream of *dfrA*. The *dfrA* confers trimethoprim resistance by providing the host with a trimethoprim-resistant dihydrofolate reductase. The *thyE* is not essential for the expression of trimethoprim resistance. However, it is thought to facilitate the non-antimicrobial selection for the

transposon, because *thy* mutations have been shown to moderately increase trimethoprim resistance. (Leelaporn *et al.*, 1994a, Lyon and Skurray, 1987, Skurray and Firth, 1997, Skurray *et al.*, 1988)

It is suggested that the formation of Tn*4003* was mediated by a series of IS*431/257* unresolved replicative transpositions between the *dfrA*-bearing chromosomal region and the IS*431/257*-bearing plasmid of *S. epidermidis*. This transposition has resulted in the Tn*4003*-bearing plasmids such as the pSK639 family of trimethoprim-resistance plasmids found in *S. epidermidis*. Hence, dissemination of Tn*4003* is thought to be mediated by the pSK639 plasmid family via IS*431/257* unresolved replicative transpositions. (Skurray and Firth, 1997, Leelaporn *et al.*, 1996, Dale *et al.*, 1995a) The Tn*4003* found in *S. aureus* plasmids is thought to be the remnant of a cointegrated pSK639-like plasmid. This is because the truncated version of the pSK639 replication gene is found in Tn*4003* and the flanking IS*431/257*s are identical to those in the pSK639 plasmids. In *S. aureus*, Tn*4003* is found on plasmids belonging to the pSK1 multiresistance-plasmid family and the pSK41 conjugative-plasmid family. (Berg *et al.*, 1998, Leelaporn *et al.*, 1994a, Leelaporn *et al.*, 1996, Skurray and Firth, 1997, Firth and Skurray, 2000)

The 7.8 kb long Tn*4004* contains the seven ORFs of the *mer* operon and is flanked by a pair of IS*431/257* in the same orientation. (Silver and Laddaga, 1990, Skurray and Firth, 1997, Lyon and Skurray, 1987) The resistance and regulation mechanisms of the *mer* operon have been described in a previous section. (See Section 1.2.12.2) It confers resistance to both inorganic and organic mercurial compounds through the *merA* and *merB* genes respectively. (Silver and Laddaga, 1990) It is found in, and disseminated by, the β-lactamase/heavy metal resistance plasmids and the Allotype III SCC*mec*. (Ito *et al.*, 2001, Ito *et al.*, 2003a, Firth and Skurray, 2000, Lyon and Skurray, 1987) Tn*4004* is also referred to as the *mer* operon or the integrated pI258 plasmid because it was first isolated in pI258 and its direct transposition has yet to be demonstrated. (Oliveira *et al.*, 2000, Laddaga *et al.*, 1987, Ito *et al.*, 2001, Silver and Laddaga, 1990)

1.3.2.5 Tn*5801*

Tn*5801* is the first putative conjugative transposon found in *S. aureus*. This 28.5 kb transposon carries the *tetM* tetracycline-resistance gene and has been found in VISA Mu50. (Kuroda *et al.*, 2001, Ito *et al.*, 2003a) The *tetM* gene encodes the ribosomal protecting protein that confers resistance to both tetracycline and minocycline. (Trzcinski *et al.*, 2000, Roberts, 1996) Tn*5801* is found to insert into the 3' end of the *gmp* gene encoding GMP synthetase and generates 11 bp target direct repeats at both ends. Tn*5801* has been compared with two other tetM-bearing elements. They are the *tetM* conjugative transposon, Tn*916*, from *E. faecalis* and the chromosomal element, CW459tet(M), from *Clostridium perfringens*. CW459tet(M) is an immobile, chromosomal element, that does not harbour conjugation genes. The conjugative region of Tn*5801* is similar to its counterpart in Tn*916*. However, Tn*5801* carries more ORFs in its conjugative domain compared to Tn*916*, including an ORF that has high homology with the *E. faecalis* transposase. Tn*5801* shares the same insertion site, the *int* integrase gene and regulatory genes with CW459tet(M). It also has the 11 bp target duplication found at the insertion site of CW459tet(M). It has been suggested that the same *tetM* conjugative transposon has been transposed between *S. aureus* and *Clostridium* spp. (Ito *et al.*, 2003a)

1.3.2.6 Tn*4291*

Tn*4291* is a putative transposon that transposes methicillin resistance onto a β-lactamase/heavy metal resistance plasmid. This transposition is not stable and appears to be site specific. The exact composition of Tn*4291* is not known. It was known to be 7.8 kb in length and probably carries the *mecA* gene encoding PBP2a/2'. (Trees and Iandolo, 1988)

1.3.2.7 Tn*1546*

Tn*1546* carries the *vanA* glycopeptide-resistance gene cluster that confers resistance to vancomycin and teicoplanin. (See Section 1.3.5) The 10.8 kb Tn*1546* carries seven *van* genes of the *vanA* gene cluster that are responsible for their own regulation and the modification of murein monomer. In addition to the seven *van* genes, it

carries two putative transposases that are responsible for its mobility. This transposon is flanked by 38 bp imperfect, terminal inverted repeats, and generates five bp direct repeats upon transposition. (Arthur *et al.*, 1993, Woodford *et al.*, 1998) Tn*1546* and Tn*1546*-like elements are found on conjugative plasmids and the chromosome of *Enterococcus* spp. (Jensen *et al.*, 1998, Robredo *et al.*, 2000) Recently two vancomycin-resistant MRSA (i.e. VRSA) isolates carrying large Tn*1546*-bearing plasmids were reported in the United States at two separate hospitals. Although in both case Tn*1546* was located on plasmids, the structure of these two Tn*1546*s are different. The VRSA from Michigan carried a complete copy of Tn*1546*, whereas the VRSA from Pennsylvania carried Tn*1546* type F2. Tn*1546* type F2 is truncated by IS*1216V*-like and IS*1251*-like elements. It also has point mutations in *vanA*, *vanX* and *vanY*. (Flannagan *et al.*, 2003, Weigel *et al.*, 2003, Tenover *et al.*, 2004, Clark *et al.*, 2005)

1.3.3 Plasmids

Plasmids are extrachromosomal genetic elements that are able to replicate independently of the host chromosome. They play a central role in the intra- and inter-genera genetic exchange by acting as a genetic vehicle. (Lederberg, 1998) The plasmids in *S. aureus* play an important role in the evolution of multiply-resistant MRSA by acting as a vehicle for both antimicrobial-resistance determinants and toxin genes for pathogenicity. (Lyon and Skurray, 1987, Skurray *et al.*, 1988, Yamaguchi *et al.*, 2001, de Oliveira *et al.*, 1993) Plasmids can be exchanged between isolates by four possible mechanisms. They are phage-mediated transduction, mixed-culture transfer, conjugation and transformation. (Lacey, 1980, Thomas and Archer, 1989a, Berg *et al.*, 1998, Novick, 1990)

Most of the clinically isolated *S. aureus* carry one or more plasmids. Only a small minority of strains are plasmid free. (Melo-Cristino and Torres-Pereira, 1989, Trindade *et al.*, 2003, Novick, 1990, Skurray *et al.*, 1988) Plasmids carrying antimicrobial-resistance genes have played a role in the rapid emergence of multiresistant *S. aureus* in the past decades. (Skurray *et al.*, 1988, Lyon and Skurray, 1987) Staphylococcal plasmids are approximately 1 kb to 120 kb in size. The majority of them can be put into four classes according to their size, genetic or

physical organization and functional characteristics. (Novick, 1990, Firth and Skurray, 2000, Tenover *et al.*, 2004)

1.3.3.1 Class I small rolling-circle plasmids

Class I staphylococcal plasmids consist of a group of small plasmids that range from 1 kb to 5 kb in size. These high-copy plasmids are found to have 15 to 60 copies per cell. They are often either phenotypically cryptic or carry only one resistance gene. (Novick, 1990, Novick, 1989, Firth and Skurray, 2000, Melo-Cristino and Torres-Pereira, 1989) In some cases, Class I plasmids with two resistance determinants have been reported. (Novick, 1989, Firth and Skurray, 2000, Allignet *et al.*, 1998) Resistance genes include the *aadD* (kanamycin and neomycin resistance), *ble* (bleomycin resistance), *cat* (chloramphenicol resistance), *ermC* (MLS_B resistance), *vatC* and *vgbB* (streptogramin A & B), *str* (streptomycin resistance), *smr*, *qacH* and *qacJ* (NAB resistance), *tetK* (tetracycline resistance), and *cadBX* (the cadmium resistance operons). (Novick, 1989, Khan, 1997, Novick, 1990, Heir *et al.*, 1998, Allignet *et al.*, 1998, Chaouni *et al.*, 1996, Horinouchi and Weisblum, 1982, Bjorland *et al.*, 2003)

Class I plasmids utilise the asymmetric, rolling-circle mechanism for their replication. Rolling-circle replication is commonly found to be mainly utilised by small plasmids of Gram-positive bacteria. Rolling-circle replication involves a single-stranded DNA intermediate and is regulated by antisense RNA. (Novick, 1989, Khan, 1997, del Solar *et al.*, 1998, del Solar and Espinosa, 2000) These small staphylococcal plasmids can be divided into four families according to the homology of their replication region sequences. The four families are pT181, pC194, pE194 and pSN2. Small plasmids from other staphylococcal species and other genera have been found to belong to these four families. The presence of these plasmids in different genera and species is the evidences of inter- and intra-genera horizontal transfer of these Class I plasmids. (Khan, 1997, Firth and Skurray, 2000, Novick, 1989)

In additional to their replication region and/or resistance genes, some Class I plasmids also carry elements responsible for mobilisation and recombination. pC221 carries the *mobA*, *mobB* and *mobC* genes that are involved in mobilisation of small

plasmids during conjugation. (Smith and Thomas, 2004, Caryl *et al.*, 2004, Projan and Archer, 1989, Priebe and Lacks, 1989) pT181 and pE194 carry the *pre* determinant and the RS_A recombination site that are responsible for site-specific recombination. (Gennaro *et al.*, 1987, Firth and Skurray, 2000) All of the Class I plasmids have been found to carry a secondary recombination site, $RS_{B,}$ that is involved in plasmid recombination mediated by a site-specific phage recombination function. (Novick, 1993) Similar to RS_B, RS_A has been shown to be involved in site-specific plasmid recombination mediated by the *pre* gene. (Gennaro *et al.*, 1987, Novick, 1993, Firth and Skurray, 2000) The *pre*-RS_A system may also be involved in the mobilisation of plasmids by conjugation. (Priebe and Lacks, 1989, Firth and Skurray, 2000) The Class I plasmids from different families are known to share highly similar DNA segments or cassettes. Structurally they appear to be a mosaic of different functional cassettes harbouring replication genes, resistance genes, recombination genes and mobilisation genes. (Novick, 1989, Firth and Skurray, 2000) Rolling-circle replication has been suggested to promote the exchange of the DNA segments/cassettes between these plasmids. Site-specific recombination genes, like the *pre* gene, may also be involved in these exchanges. (Firth and Skurray, 2000) Many of the Class I plasmids have been found integrated into either the chromosome or into large conjugative plasmids by transposition or homogeneous recombination through the insertion sequence IS*431/257*. (See Section 1.4.1.2) (Werckenthin *et al.*, 1996, Stewart *et al.*, 1994)

1.3.3.2 Class II multiresistance plasmids

The size of Class II plasmids ranges from 15 to 40 kb and therefore they are present in low-copy numbers from 4 to 6 copies per cell. These plasmids carry several resistance determinants, most of which are encoded by integrated transposons from other plasmids. (Firth and Skurray, 2000, Novick, 1990) Studies on their replication region indicate that they utilise the theta replication system and belong to the Inc1 plasmid incompatibility group. (Firth *et al.*, 2000, Firth and Skurray, 2000) Based on their structural differences, the Class II plasmids can be subdivided into the β-lactamase/heavy metal-resistance and pSK1 multiresistance plasmid families. (Firth and Skurray, 2000, Skurray and Firth, 1997)

1.3.3.2.1 The β-lactamase/heavy metal resistance plasmids

The β-lactamase/heavy metal (BHM) resistance plasmids are commonly found in MRSA isolated in the 1960s and 1970s. Structurally these plasmids often contain the β-lactamase-encoding Tn*552* or Tn*552*-like transposon, Tn*4004* encoding the *mer* mercury-resistance operon, *cadCA*, the cadmium-resistance operon and/or ars, the arsenical-resistance operon. (Shalita *et al.*, 1980, Lyon and Skurray, 1987, Firth and Skurray, 2000, Skurray and Firth, 1997) Other than these elements, some of the BHM plasmids carry the aminoglycoside resistance transposon, Tn*4001*, or the MLS_B resistance transposon, Tn*551*. The *qacA* and *qacB* genes conferring resistance to NAB compounds has also been found in some BHM plasmids. (Lyon and Skurray, 1987, Firth and Skurray, 2000, Novick *et al.*, 1979)

According to their structural homology the BHM plasmids can be subdivided into the alpha (α), beta (β), gamma (γ) and delta (δ) families. This classification is based on restriction patterns generated by *EcoR*I and *Bgl*II restriction enzymes. Some BHM plasmids do not belong to these families and are known as the orphan family. (Shalita *et al.*, 1980) The α and γ families are the most common BHM plasmids. These two families are closely related to each other and most of the genes they carry are identical. The main difference between them is found in the Tn*552* region, especially the invertible fragment of Tn*552* that is involved in transposition. (See Section 1.4.2.2) Most of the plasmids of the α family possess a complete copy of the invertible fragment. In the members of the γ family, the invertible region is truncated and the *bla* operon, Tn*552*, is in the opposite orientation to its counterpart in the α family. These genetic rearrangements, by deletion and sequence inversion, are probably induced by the Tn*552* transpositional genes on the invertible fragment. The invertible fragment of Tn*552* has been found to be the focus of genetic rearrangements. (Shalita *et al.*, 1980, Lyon and Skurray, 1987, Sidhu *et al.*, 2002) The structural variations found in the members of the α plasmid family are due to genetic rearrangements such as deletions, sequence inversions and substitutions in the invertible fragment and in Tn*552*. (Shalita *et al.*, 1980)

Recombinant plasmids of the α and γ plasmids had been isolated. The recombination of these two plasmids is believed to be the consequence of plasmid incompatibility (Inc). Both α and γ plasmids belong to the Inc1 group, and as a result they cannot co-exist in the same host. (Lyon and Skurray, 1987, Novick, 1987, Shalita *et al.*, 1980) Recombination between these two plasmids has been demonstrated in experiments *in vitro*. (Murphy and Novick, 1980) The *sin* recombinase gene commonly found on the BHM plasmids has been shown to be capable of inducing site-specific recombination of two plasmids. (Rowland *et al.*, 2002) The α and γ hybrid plasmids have the general structure of α plasmids and the Tn*552* region of γ plasmids. Hybrids of α and β plasmids have also been reported. (Lyon and Skurray, 1987, Shalita *et al.*, 1980, Firth and Skurray, 2000, Skurray and Firth, 1997) IS*431/257* is believed to play a role in the formation of the BHM-multiresistance plasmids. The *mer* operon, which is a common feature of BHM plasmids, is flanked by two direct copies of IS*431/257*. The IS has also been found located immediately downstream of the right-hand terminal inverted repeat of Tn*552*. (Firth and Skurray, 2000, Ito *et al.*, 2001, Sidhu *et al.*, 2002)

The BHM plasmids are commonly found in MRSA isolated before 1970 and are rarely found in strains after the 1970s. They have been largely replaced by NAB-resistance plasmids such as plasmids from the pSK1 family. (Townsend *et al.*, 1985d, Firth and Skurray, 2000, Skurray *et al.*, 1988, Gillespie *et al.*, 1984, Sidhu *et al.*, 2002) Plasmids commonly isolated in contemporary MRSA from Asian countries typically carry resistance to heavy-metal ions and resistance to NAB compounds with, or without, β-lactamase. It has been proposed that they are recombinants of BHM- and NAB-resistance plasmids. (Grubb and O'Brien, 2004, Grubb, 1990, Sim, 2003, Wei, 1993) BHM plasmids have also been shown to recombine with plasmids of the pSK1 family *in vitro*. (Townsend *et al.*, 1985c)

1.3.3.2.2 The pSK1 plasmid family

The pSK1 plasmid family is prevalent in MRSA isolated after the 1980s. (Firth and Skurray, 2000, Townsend *et al.*, 1985d, Skurray *et al.*, 1988, Wright *et al.*, 1998). These plasmids characteristically carry the NAB-resistance *qacA* gene. The

members may also carry the aminoglycoside resistance transposon Tn*4001*, a Tn*552*-like element, and the remnant of the *dfrA*-bearing integrated plasmid that is also known as Tn*4003*. For example, pSK4 is known to host all of these resistance gene elements. (Skurray *et al.*, 1988, Lyon and Skurray, 1987, Firth and Skurray, 2000, Skurray and Firth, 1997) The pSK1 family plasmids share a 15 kb common region that carries the replication genes. This replication region also shares 89% sequence identity with the replication regions of the BHM plasmids pSX267 and pI9789::Tn*552*. Both pSK1 and pI9789::Tn*552* have the *par* gene that encodes a novel partitioning system. Although the *par* gene is not essential for plasmid replication, it is required for stabilising the plasmids during host cell division. (Skurray *et al.*, 1988, Firth *et al.*, 2000, Simpson *et al.*, 2003)

1.3.3.3 Class III conjugative plasmids

The Class III conjugative-staphylococcal multiresistance plasmids are the largest plasmids found in *S. aureus*. Their sizes range from 30 to 60 kb and they carry their own conjugational transfer system. The members of this family are the only conjugative plasmids that had been studied in detail. (Novick, 1990, Firth and Skurray, 2000) The 46.4 kb pSK41 is the prototype of this family and has been fully sequenced. It carries the theta replication system that is related to its counterpart in the pSK1 and BHM plasmid families. However, it does not belong to the same plasmid incompatibility group as the pSK1 and BHM plasmids and, it has been suggested that this family belongs to a new incompatibility group. (Berg *et al.*, 1998, Firth *et al.*, 2000) The conjugative transfer system of pSK41 consists of 15 genes. Except for two genes located adjacent to the transfer origin, *oriT*, the rest of the determinants are located in a 14 kb IS*432/257* flanked transfer-associated region, *tra*. (Berg *et al.*, 1998, Morton *et al.*, 1993, Climo *et al.*, 1996) Transfer of the pSK41 type plasmids only occurs via cell-to-cell contact on solid surfaces, or in the presence of polyethylene glycol, at a low frequency of 10^{-5} to 10^{-7} transconjugants per donor cell. (Zatyka and Thomas, 1998, Townsend *et al.*, 1986b) They have been shown to be capable of *in vitro* and *in vivo* interspecific transfer (Udo *et al.*, 1997, Flannagan *et al.*, 2003, Archer and Thomas, 1990) and can mobilise co-existing smaller plasmids into the donor during conjugation. (Archer and Thomas, 1990, Udo and Jacob, 1998, Berg *et al.*, 1998, Firth and Skurray, 2000) The mobilisation process

involves the *mob* gene of the smaller plasmids, which is involved in their relaxation. (Smith and Thomas, 2004, Projan and Archer, 1989, Morton *et al.*, 1993) Although the chromosomal integration of the pSK41 type plasmids is uncommon, a pSK41 plasmid has been found integrated into the chromosome of a Japanese MRSA, outside of the SCC*mec* region. (McElgunn *et al.*, 2002)

Members of the pSK41 family such as pSK41, pGO1, pJE1, pUW3626 and pWG14 plasmids were first isolated in the mid-1970s and corresponded to the emergence of gentamicin resistance. (Cohen *et al.*, 1982, Thomas and Archer, 1989b, Archer and Thomas, 1990, Lyon and Skurray, 1987, Firth and Skurray, 2000, Townsend *et al.*, 1985a) These plasmids characteristically carry multiple copies of IS*431/257* that range from seven to nine copies, often in the same orientation. It is thought that IS*431/257* plays an important role in the accumulation of resistance elements in these plasmids. They flank integrated plasmids and transposons such as the aminoglycoside resistance pUB110, a Tn*4001*-like element and the *smr* NAB-resistance plasmid. (See Section 1.4.1.2 and 1.4.3.1) (Skurray and Firth, 1997, Berg *et al.*, 1998, Lyon and Skurray, 1987, Firth and Skurray, 2000) In addition to these elements, pGO1 and pJE1 also carry an IS*431/*257-bound remnant of a *dfrA*-bearing plasmid, also known as Tn*4003*. pUW3626 also harbours a Tn*552*-like element. (Archer and Thomas, 1990, Lyon and Skurray, 1987, Firth and Skurray, 2000, Cohen *et al.*, 1982) IS*431/*257 not only has mediated the integration of these elements, it also inactivates their replication and transposition functions thus stabilising the co-integration. (Skurray and Firth, 1997, Berg *et al.*, 1998) Another member of the pSK41 family is pGO400 which is 34 kb and harbours only the IS*431/257*-flanked *mupA* mupirocin-resistance gene and the *tra* region. (Morton *et al.*, 1995)

The vancomycin-resistance conjugative plasmid, pLW1043, was isolated in the Michigan VRSA. It is 57 kb in length and carries six copies of IS*431/257*. The pLW1043 is closely related to the pSK41 family plasmids as they share 99% similarity in their *tra* region. In addition to the similar *tra* region, pLW1043 also carries the Tn*4001*-like element and the integrated *smr* plasmid that is characteristic of pSK41 family plasmids. However, the integrated pUB110 plasmid, which is a common component of pSK41 family plasmids, was not found on pLW1043. pLW1043 also shares significant homology with the pSK1 family plasmid, pSK4.

Both of them carry the Tn*552*-like element and Tn*4003*, which are also found in the some pSK41 family plasmids. The transposon Tn*1546* carried by pLW1043 is responsible for inducible, high-level, VanA glycopeptide resistance in VRSA. Genetic analysis of the MRSA and the VRE that were isolated together from the VRSA indicates that Tn*1546* transposed *in vivo* from the plasmid of the VRE to the conjugative plasmid of the MRSA. (Weigel *et al.*, 2003)

In addition to the pSK41 family, three other distinct types of conjugative plasmids have also been isolated. The first type is the conjugative plasmids that harbour Tn*554* and the production of diffusible pigment. This group of conjugative plasmids have only been found in Australian isolates and they have not been found to mobilise smaller plasmids during conjugation. (Townsend *et al.*, 1986a, Townsend *et al.*, 1986b, Townsend *et al.*, 1985a) The second group is the cryptic conjugative plasmids found in Nigerian *S. aureus* isolates. Although they do not carry any resistance genes, they are capable of plasmid mobilisation. (Udo *et al.*, 1987, Udo and Grubb, 1990b, Udo and Grubb, 1996) The third type is the trimethoprim-resistance conjugative plasmids found in Malaysian MRSA. Like other conjugative plasmids, they are capable of plasmid mobilisation, the latter two at a high frequency. (Udo *et al.*, 1992b)

The Class III conjugative plasmids play an important role the dissemination of resistance elements that, in turn, have a great impact on the treatment of *S. aureus* infections. These plasmids had been shown to cause the dissemination of aminoglycoside and mupirocin resistances. (Lyon and Skurray, 1987, Udou, 2004, Cookson, 1998, Udo *et al.*, 2001) Recently they have been found to be responsible for the evolution of VRSA by transferring vancomycin-resistance genes. (Weigel *et al.*, 2003, Flannagan *et al.*, 2003, Tenover *et al.*, 2004, Clark *et al.*, 2005)

1.3.3.4 Class IV staphylococcal plasmids

Staphylococcal plasmids that do not belong to the other three well-defined classes are placed in the Class IV plasmids. These plasmids include the fusidic acid-resistance pUB101 and the 120 kb *vanA*-bearing large plasmid found in a VRSA. (Novick, 1989, O'Brien *et al.*, 2002, Tenover *et al.*, 2004) The other group of

plasmids that may belong to the Class IV is the pSK639 family. These plasmids utilise the theta mode of replication and carry the *dfrA* trimethoprim-resistance gene. These small plasmids have only been found in *S. epidermidis*, but remnants of these plasmids are found in Class II and Class III plasmids and are also known as Tn*4003*. (Leelaporn *et al.*, 1996, Apisiridej *et al.*, 1997, Firth and Skurray, 2000)

1.3.4 Staphylococcal cassette chromosome *mec* (SCC*mec*)

The staphylococcal cassette chromosome (SCC) is a novel, mobile genomic island, unique to staphylococcal species. (Ito *et al.*, 2003a) It is one of the largest bacterial mobile elements. (Robinson and Enright, 2004a) Although the exact mobile mechanism is not known, the *ccr* genes found in the SCC have been shown to be responsible for the excision and integration of the genomic island. (Katayama *et al.*, 2000, Luong *et al.*, 2002) SCC*mec* is one of the SCC elements found exclusively in methicillin-resistant staphylococci and carries the *mecA* gene encoding methicillin resistance. The core of SCC*mec* is the *ccr* and *mec* gene complexes that carry the site-specific recombinase and *mecA* genes respectively. The other regions are known as the junkyard (J) regions. These J regions carry mainly nonfunctioning pseudogenes, nonessential genes and genes encoding resistance to non-β-lactam antibiotics and heavy metals. All of these antimicrobial-resistance genes are found on transposons and plasmids that have integrated into the SCC*mec*. (Ito *et al.*, 2001, Hiramatsu *et al.*, 2001)

1.3.4.1 The *mec* complex

The *mec* complex is composed of *mecA*, its regulatory genes, *mecR1* and *mecI*, and the insertion sequence, IS*431/257*. The *mecR1* and *mecI* genes are contiguous and are located immediately upstream of *mecA*, whereas the IS*431/257* is located approximately 2 kb downstream of *mecA*. (Hiramatsu *et al.*, 1992, Ito *et al.*, 1999, Ito *et al.*, 2001, Barberis-Maino *et al.*, 1987) The *mecA* and *mecR1/I* promoters are located in the region between *mecA* and *mecR1/I*. The *mecA* gene is transcribed in the opposite direction to the *mecR1* and *mecI* genes. (Hiramatsu *et al.*, 1992) The intergenic region between IS*431/257* and *mecA* harbours 40bp direct repeat units and

an open reading frame, ORF145, which has 57% amino acid sequence similarity to *E. coli*'s glycerophosphoryl diester phosphodiesterase (UgpQ). (Ryffel *et al.*, 1991)

1.3.4.1.1 *mecA*

mecA is the structural gene that encodes PBP2a/2' which has a low affinity for β-lactams and confers methicillin resistance in staphylococci. (Song *et al.*, 1987, Suzuki *et al.*, 1992) It is 2007 bp in length and is highly conserved among methicillin-resistant staphylococci. A single-base point mutation in *mecA* has been found in some strains that creates a new *Xba*I restriction site. (Song *et al.*, 1987, Ryffel *et al.*, 1990) *mecA* is always found together with its regulatory genes, or their truncated versions, and is only found in the SCC*mec* genomic island. (Kobayashi *et al.*, 1996, Suzuki *et al.*, 1993, Weller, 1999, Ito *et al.*, 2003a)

The only known *mecA* homologues have been found in other staphylococci. In *S. sciuri*, the *mecA* homologue ($mecA_{sciuri}$) has a DNA sequence 79.5% similar to *S. aureus mecA* ($mecA_{aureus}$). The overall deduced amino acid sequences of $mecA_{sciuri}$ and $mecA_{aureus}$ are 79.98% identical and have a similarity of 87.69%. Their penicillin-binding (transpeptidase) domains are almost identical with 95.94% similarity, and the non-penicillin-binding domains have 80.06% similarity in amino acid sequence. (Wu *et al.*, 1996b)

Despite the high similarity to $mecA_{aureus}$, $mecA_{sciuri}$ does not confer methicillin resistance and is ubiquitously found in *S. sciuri*. (Wu *et al.*, 1996b, Kloos *et al.*, 1997) In fact, the majority of methicillin-resistant *S. sciuri* carry copies of both $mecA_{sciuri}$ and $mecA_{aureus}$. The latter has been found to be responsible for methicillin resistance. (Couto *et al.*, 2000) In some methicillin-resistant *S. sciuri*, the $mecA_{sciuri}$ has been found to confer methicillin resistance due to genetic changes in its promoter region. The promoter region of the methicillin-resistance $mecA_{sciuri}$ is either mutated or has an IS*256*, which forms a hybrid promoter that leads to overexpression of $mecA_{sciuri}$. It has been shown that the over expression of $mecA_{sciuri}$ results in the expression of methicillin resistance in *S. sciuri*. (Wu *et al.*, 2001, Couto *et al.*, 2003) The $mecA_{sciuri}$ also differs from the $mecA_{aureus}$ in that it is found by itself without the regulatory genes. The ugpQ-like ORF has been found downstream of $mecA_{aureus}$ and

has also been found downstream of *mecA*$_{sciuri}$. These finding suggest that *mecA*$_{sciuri}$ probably is the precursor of *mecA*$_{aureus}$ and the regulatory genes were acquired by *mecA*$_{aureus}$ in the later stages of evolution. (Couto *et al.*, 2003, Wu *et al.*, 1998, Wu *et al.*, 2001)

1.3.4.1.2 The *mecA* regulatory genes, *mecR1* and *mecI*

The *mecA* regulatory genes, *mecR1* and *mecI*, are located upstream of *mecA* and are transcribed in the opposite direction of *mecA*. These two genes are contiguous and share the same promoter sequences. (Hiramatsu *et al.*, 1992) These promoter sequences are located very close to the *mecA* promoter sequences. The -35 promoter sequence of *mecR1* is only five bp upstream from the *mecA* -10 promoter sequence. (Hiramatsu *et al.*, 1992, Sharma *et al.*, 1998) *mecR1-mecI* is a signal transduction system that regulates *mecA* transcription. It is structurally and functionally closely related to the *blaR1-blaI* system that controls β-lactamase (*blaZ*) production in staphylococci and *B. licheniformis*. (Hiramatsu *et al.*, 1992, Garcia-Castellanos *et al.*, 2004)

The *mecR1* gene is 1758 bp in length and encodes a transmembrane signal sensor/transducer, MecR1. The MecR1 is a Class C High-Mr PBP that is comprised of 585 amino acids with two domains. (Hiramatsu *et al.*, 1992, Ghuysen, 1991, Chambers, 2003) It is 34% homologous to *S. aureus* BlaR1 and 31% homologous to PenJ (BlaR1) of *B. licheniformis*. (Hiramatsu *et al.*, 1992) The membrane-spanning (MS) domain of MecR1, located at the N-terminal, is embedded in the plasma membrane via its transmembrane segment, and carries a zinc metalloprotease motif HEXXH (X stands for any amino acids) in one of its intracellular loops. (Hiramatsu *et al.*, 1992, Zhang *et al.*, 2001, Hardt *et al.*, 1997, Kobayashi *et al.*, 1987) The penicillin-binding (PB) domain is located at the C-terminal and is an extracellular segment carrying a penicillin-binding motif. It crystal structure has been found to resemble the Class D β-lactamases. (Kobayashi *et al.*, 1987, Hiramatsu *et al.*, 1992, Wilke *et al.*, 2004)

The MecR1 protein is activated by the self-proteolytic activity of the zinc metalloprotease at the MS domain. This proteolytic reaction is in turn induced by the

binding of β-lactam molecules to the PB domain. (Zhang *et al.*, 2001, Hardt *et al.*, 1997, Golemi-Kotra *et al.*, 2003) The induction rate of PBP2a/2' (*mecA*) by MecR1 is significantly slower and incomplete compared to the BlaR1 induction of β-lactamase (*blaZ*) production. The MecR1 takes hours to induce the production of PBP2a/2', whereas BlaR1 takes minutes to activate PBP2a/2' production. (McKinney *et al.*, 2001) In addition, MecR1 does not respond well to many β-lactams, including methicillin, except for a small number of β-lactams such as cefitizoxime and cefoxitin. (Kuwahara-Arai *et al.*, 1996, Hiramatsu *et al.*, 2001) Except for the penicillin-binding motif, the PB domain of *mecR1* only has 48% identity to the *blaR1* PB domain. The MS domain of *mecR1* is significantly different from the *blaR1* MS domain. They only have 13% similarity outside the zinc metalloprotease motif. These differences might explain the induction specificity and the slow induction of MecR1. (McKinney *et al.*, 2001) In addition, the expression of MecR1 is tightly controlled because the overexpression of MecR1, in the absence of a β-lactam, retards cell growth. (Rosato *et al.*, 2003b, Garcia-Castellanos *et al.*, 2004)

The 372 bp *mecI* encodes the repressor protein, MecI, comprised of 123 amino acids. The MecI repressor is 61% and 43% homologous to the *S. aureus* BlaI and *B. licheniformis* PenI (BlaI), respectively. (Hiramatsu *et al.*, 1992) MecI belongs to the MecI/BlaI family of closely related transcription repressors found in various genomes. MecI has two domains, the winged-helix (WH) DNA-binding domain at the N-terminal end and the dimerisation domain at the C-terminal end. It exists in the cell as a 14 kDa preformed homodimer. The two WH DNA-binding domains in the dimer carry the helix-turn-helix, DNA-binding motif and do not interact with each other. Two other spiral-staircase dimerisation domains maintain the integrity of the dimer's structure. (Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004)

The MecI protein constitutively represses *mecA*, *mecR1* and its own transcription. (Kuwahara-Arai *et al.*, 1996, Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004) It causes the repression by binding as a dimer to a 25 bp double-stranded DNA that carries the -10 *mecA* and -35 *mecR1* promoters in the intergenic region between *mecA* and *mecR1*. (Hiramatsu *et al.*, 1992, Sharma *et al.*, 1998) The DNA

binding is achieved by the two WH DNA-binding domains of MecI. The specific binding site of the WH DNA-binding domains, is 5'-G-T-A-X-T-3' (X stands for any base) which is present in the inverted repeats of the promoters. The two WH DNA-binding domains, in the MecI repressor, bind to the inverted repeats independently and non-cooperatively from each other. (Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004)

1.3.4.1.3 The regulation of the *mec* complex

The working model for the *mecA-mecR1-mecI* regulatory system is mainly based on the biochemical data of the *blaZ-blaR1-blaI* system and the MecI protein. (See Figure 1.3) (Archer and Bosilevac, 2001, Clarke and Dyke, 2001b, Clarke and Dyke, 2001a, Zhang *et al.*, 2002, Garcia-Castellanos *et al.*, 2004, Garcia-Castellanos *et al.*, 2003, Hanique *et al.*, 2004)

In the native state, two MecI dimers bind to the *mecA* and *mecR1-I* promoter sequences. This binding constitutively blocks the transcription of *mecA*, *mecR1* and *mecI*. (Kuwahara-Arai *et al.*, 1996, Garcia-Castellanos *et al.*, 2004, Garcia-Castellanos *et al.*, 2003) The extracellular PB signal-sensor domain of MecR1, in its native state, is bound to one of the extracellular loops (L2) of its MS signal transduction domain. (Hanique *et al.*, 2004)

The *mecA-mecR1-mecI* system is activated by a unique signal transmitting and activating pathway. In the presence of a β-lactam, the β-lactam molecule binds with the PB domain of MecR1, this breaks the interaction between the PB domain and the L2 loop of the MS domain. (Hanique *et al.*, 2004, Wilke *et al.*, 2004) This movement triggers a conformation change in MecR1 that activates the zinc metalloprotease located in the intracellular loop, L3, of the MS domain. (Hanique *et al.*, 2004, Golemi-Kotra *et al.*, 2003) The activated zinc metalloprotease induces a limited self-proteolytic cleavage in the MS domain. This cleavage occurs at the region connecting the PB and MS domains on the cytosolic side. The metalloprotease cleavage releases a signal across the membrane and reaches the MecI site. (Zhang *et al.*, 2001, Hardt *et al.*, 1997, Hanique *et al.*, 2004) This signal triggers the limited proteolytic cleavage of MecI. The scissile bond of MecI is

located in the middle of the dimerisation domain that is responsible for the integrity of the MecI protein. This eventually leads to the dissociation of MecI and allows the transcription of the *mecA*, *mecR1* and *mecI* genes. (Filee *et al.*, 2002, Lewis *et al.*, 1999, Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004)

As the extracellular β-lactam concentration diminishes, the rate of cleavage of MecR1 will decrease and eventually cease. The intracellular concentration of intact MecI represser will therefore increase and this increase leads to the re-suppression of *mecA*, *mecR1* and *mecI* transcription. (Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004)

The exact mechanisms of signal transmission and proteolytic cleavage from MecR1/BlaR1 to MecI/BlaI are yet to be clarified. BlaR1 mutants with the cleaved MS domain alone are not able induce cleavage of BlaI. (Zhang *et al.*, 2001) Since the MecR1 and MecI cleavage sites are different, it is unlikely that the highly specific zinc metalloprotease of MecR1 will recognise two different sequences. In addition, the scissile bond of MecI is not readily accessible to protease activity and requires the local melting of the protein structures to expose the cleavage site. (Zhang *et al.*, 2001, Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004) Some researchers have suggested that a second or intermediate protease might be involved in the process. The yet to be identified, MecR2/BlaR2 is thought to be the candidate. (Cohen and Sweeney, 1968, Zhang *et al.*, 2001, Zhu *et al.*, 1992, Gregory *et al.*, 1997, Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004)

The *blaZ-blaR1-blaI* β-lactamase system is structurally and functionally very similar to the *mecA-mecR1-mecI* PBP2a/2' system. (Hiramatsu *et al.*, 1992) *blaR1* and *blaI* are able to regulate the expression of PBP2a/2' when the *mecR1* and *mecI* are disabled. (Hackbarth and Chambers, 1993, Hackbarth *et al.*, 1994) The presence of a β-lactamase plasmid also stabilises the unstable SCC*mec* in MRSA. (Hiramatsu *et al.*, 1990) Furthermore, the *blaR1-blaI* regulatory system is better than the native *mecR1-mecI* system in expressing *mecA* because it has a more efficient signal transducer and a poorer repressor. (McKinney *et al.*, 2001, Ryffel *et al.*, 1992) Strains with an intact copy of *mecR1* and *mecI* are methicillin sensitive in the

laboratory because induction of *mecA* is slow and inefficient. (Kuwahara-Arai *et al.*, 1996)

The BlaI and MecI share very similar DNA binding sequences and both can repress the transcription of *mecA* (PBP2a/2') and *blaZ* (β-lactamase). (Clarke and Dyke, 2001b, Lewis and Dyke, 2000, Hackbarth *et al.*, 1994, Garcia-Castellanos *et al.*, 2004) Both have been found to form heterodimers. However, MecI is threefold more effective than BlaI in the repression of *mecA* transcription. (McKinney *et al.*, 2001) Together MecI and BlaI are more effective in *mecA* repression than by themselves alone. (Rosato *et al.*, 2003a)

However, unlike MecI and BlaI, the MecR1 and BlaR1 signal transducers are not interchangeable. The β-lactam induction of BlaR1 was found not to relieve the *mecA* repression by the MecI repressor. (McKinney *et al.*, 2001) In addition, the induction of BlaR1 by β-lactams is ten-fold more efficient and much quicker than MecR1. These differences between MecR1 and BlaR1 in their signal transduction are probably due to their significantly different MS domains. (McKinney *et al.*, 2001)

In a study of nosocomial MRSA, it was found that the majority of isolates that have a dysfunctional *mecI* gene carry a functional *blaI* gene. All of the MRSA in the study were found to have at least one functional copy of either *mecI* or *blaI*, or both. These observations indicate that the expression of *mecA* at least in some isolates, is probably co-regulated by these two systems. (Rosato *et al.*, 2003a, Rosato *et al.*, 2003b)

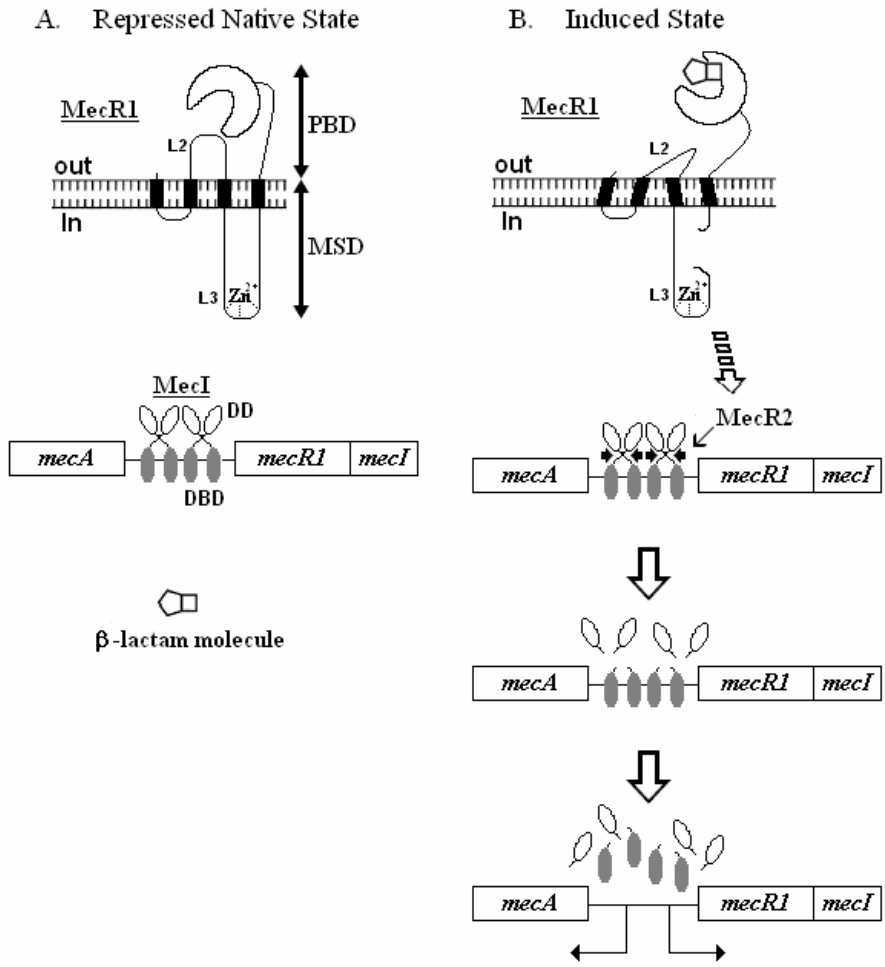


Figure 1.3 A working model of the *mecA* regulatory system

A. In the native state, two MecI repressers block the transcription of *mecA*, *mecR1* and *mecI* by binding to their promoter sequences by their DNA-binding domains (DBD). MecI is a preformed dimer and is held together by its dimerisation domain (DD). The MecR1 penicillin-binding signal sensor domain (PBD) interacts with the extracellular loop L2 of it membrane-spanning signal transducer domain (MSD).

B. In the induced state, the β-lactam molecule binds to the PBD of MecR1 breaking its interaction with the MSD's L2 loop and this triggers the zinc metalloprotease on the MSD's L3 loop. This results in the cleavage of the junction between the PBD and the MSD of MecR1 by the zinc metalloprotease. This cleavage triggers a single bond proteolytic cleavage of MecI possibly via a hypothetical protease MecR2. The scissile bond is located in the middle of the DD of MecI (indicated by small solid arrows). When the cleavage occurs, the two MecI dimers disintegrate and make the *mecA*, *mecR1* and *mecI* available for transcription. The right angle arrows indicate the direction of transcription. This figure is based on the findings of (Hanique *et al.*, 2004, Hardt *et al.*, 1997, Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004, Wilke *et al.*, 2004)

1.3.4.1.4 Diversity of *mec* complexes

PBP2a/2' is an inefficient cell-wall enzyme and over expression of PBP2a/2' retards cell-wall formation and disadvantages the cell's chance of survival. (de Jonge and Tomasz, 1993) When a strain first acquires *mecA* with SCC*mec*, it has to be tightly regulated to prevent the toxic effect of PBP2a/2'. The intact *mecR1-mecI* regulatory genes stabilise the newly acquired *mecA*. The tightly regulated *mecA* expression reduces the occurrence of spontaneous mutations that inactivate *mecA*. (Katayama *et al.*, 2003c)

However, the resistance level generated by this inefficient regulatory system is very low. (Kuwahara-Arai *et al.*, 1996, Hiramatsu, 1995) In the clinical setting, the selective pressure of β-lactams is high and isolates need to be able to express high-level resistance to survive. High-level methicillin resistance is achieved by increasing the *mecA* transcription and generating, or acquiring, compensatory mechanisms to deal with the toxicity of PBP2a/2'. (Finan *et al.*, 2002, Katayama *et al.*, 2003c, Kondo *et al.*, 2001, Rosato *et al.*, 2003a)

Some high-level resistant clinical isolates of MRSA transcribe *mecA* constitutively, and their *mecR1* and *mecI* have been found to be dysfunctional. The *mecR1-mecI* regulatory systems in the majority of clinical-MRSA isolates have been found to be, either truncated by insertion sequences or, disabled by mutations. (Suzuki *et al.*, 1993, Lim *et al.*, 2002, Petinaki *et al.*, 2001, Hiramatsu, 1995, Kobayashi *et al.*, 1995) In some strains, the mutations occur in the promoter sequences and the ribosome-binding sites. (Rosato *et al.*, 2003a, Weller, 1999, Hiramatsu, 1995) These diversities in the *mec* complex can be grouped into different classes. See Figure 1.4. (Katayama *et al.*, 2001, Ito *et al.*, 2001, Lim *et al.*, 2002)

The Class A *mec* complex has intact *mecA*, *mecR1* and *mecI* genes. It is found in the MRSA isolate N315, which appears methicillin sensitive. Its *mecA* transcription is tightly and efficiently controlled by *mecR1* and *mecI*. However, N315 is still able to produce a small, high-level, resistant subpopulation. (Ito *et al.*, 1999, Hiramatsu, 1995, Kuwahara-Arai *et al.*, 1996) The Class A *mec* complex has also been detected in high-level resistant MRSA. (Suzuki *et al.*, 1992, Weller, 1999, Lim *et al.*, 2002)

It has been shown that mutations in the *mecI* gene deactivate the MecI protein. One of the common mutations found is nonsense, single-base mutation, that occurs at nucleotide (nt) 220 and generates a premature termination of MecI translation. (Suzuki *et al.*, 1992, Weller, 1999, Rosato *et al.*, 2003b, Rosato *et al.*, 2003a) This mutation in MecI is in its dimerisation domain and effectively deactivates MecI because it binds to the double stranded DNA as a preformed dimer. (Garcia-Castellanos *et al.*, 2003, Garcia-Castellanos *et al.*, 2004) A subtype of Class A, Class A1, has been found in a high-level, resistant strain. This subtype has a 166 bp deletion in the membrane-spanning domain of *mecR1* and the *mecI* is inactivated by a mutation at nt 202. (Lim *et al.*, 2002, Oliveira *et al.*, 2000) Two Class A variants, Class A.3 and Class A.4, were found in MRSA isolated in Irish hospitals. The Class A.3 has an intact copy of *mecR1*, but 119bp of its *mecI* sequence is truncated by the insertion sequence IS*1182* at its 3′ end. The A.4 is very similar to the A.3, but the IS*1182* has inserted within the *mecI* and caused a 16 bp deletion of *mecI* immediately upstream of the inserted IS*1182*. (Shore *et al.*, 2005)

The Class B *mec* complex is composed of the *mecA*, the membrane-spanning (MS) domain of *mecR1* (975 bp) and a partial copy of the insertion sequence IS*1272* (ψIS*1272*). (Ito *et al.*, 2001, Archer *et al.*, 1996) In the Class B *mec* complex, the whole of the *mecI* gene, the penicillin-binding (PB) domain and 64 bp of the MS domain of *mecR1* are deleted by the insertion of ψIS*1272*. ψIS*1272* is transcribed in the opposite direction of *mecR1* and was first found in the methicillin-resistant *S. haemolyticus* Y176. It has been subsequently found in MRSA isolates, and they have the identical junction sequence between *mecR1* and ψIS*1272* as in *S. haemolyticus* Y176. (Archer *et al.*, 1996, Katayama *et al.*, 2001, Kobayashi *et al.*, 1999)

MRSA with the Class B complex are probably using the *blaR1-blaI* to regulate their *mecA* expression. (Hiramatsu *et al.*, 1990, Hackbarth and Chambers, 1993, Hackbarth *et al.*, 1994) It is typically found in the earlier MRSAs isolated in the 1960s. Some of them have been found to express *mecA* constitutively and are high-level, homogeneous-resistant strains. (Suzuki *et al.*, 1993, Ito *et al.*, 2001, Lim *et al.*, 2002) The Class B *mec* complex is also commonly found in community-acquired

MRSA isolated in the 1990s until the present. These are low-level, heterogeneous-resistant strains. (Ma *et al.*, 2002, Okuma *et al.*, 2002, Lim *et al.*, 2003) A subtype Class B1 has been found in community-acquired MRSA. In this class, the ψIS*1272* truncation extends into almost the whole of the MS domain of *mecR1*. In the Class B *mec* complex only 64 bp of the MS domain of *mecR1* is deleted by ψIS*1272*. (Lim *et al.*, 2003)

Class C *mec* complexes have an incomplete copy of *mecR1* truncated to various extents by a copy of the insertion sequence, IS*431/257*. Like the Class B *mec* complex the *mecI* has been totally deleted. C1 and C2 are two subclasses of Class C *mec* complex that have been reported. In the Class C1 *mec* complex, the PB domain of *mecR1* and the whole of *mecI* are deleted by a copy of IS*431* that is transcribed in the same direction as *mecA*. A 968 bp remnant of the *mecR1* MS domain remains in the C1 *mec* complex. In the C2 *mec* complex, the deletion in *mecR1* is more extensive. Only 91 bp of the *mecR1* MS domain remains and the rest of *mecR1* and the whole of *mecI* are deleted by IS*431*. The IS*431* in the C2 *mec* complex is transcribed in the opposite direction to the *mecA* gene and the IS*431* in the C1 *mec* complex. The Class C *mec* complexes are mainly found in methicillin-resistant *S. haemolyticus* and other coagulase-negative staphylococci and mainly confer a low-level of resistance. (Kobayashi *et al.*, 2001b, Katayama *et al.*, 2001) The Class C2 *mec* complex is also found in community-acquired MRSA. (Ito *et al.*, 2004)

In Class D *mec* complex, like Class B and Class C *mec* complexes, the *mecI* is deleted and the *mecR1* is truncated in the PB domain. However, the truncation of *mecR1* and *mecI* in Class D does not involve any insertion sequences, but a 450 bp deletion is responsible for the truncation. Class D complex has only been found in one high-level resistant *S. caprae*. (Katayama *et al.*, 2001) A similar type of *mec* complex deletion has been found in the community-acquired *S. aureus* isolated from rural Wisconsin in the United States. The deletion starts from nucleotide 785 of the *mecR1* and deletes the remaining 976 bp of the *mecR1* and the entire *mecI* gene. (Shukla *et al.*, 2004)

The Class E *mec* complex has been found in a low-level resistant, community-acquired MRSA. It consists of the *mecA* and a section of the *mecR1* PB domain, with the *mecI* and the MS domain of *mecR1* are deleted. (Lim *et al.*, 2003)

The insertion sequence, IS*256* has also been found to truncate the PB domain of *mecR1* in MRSA isolated sporadically in Poland. (Oliveira *et al.*, 2000)

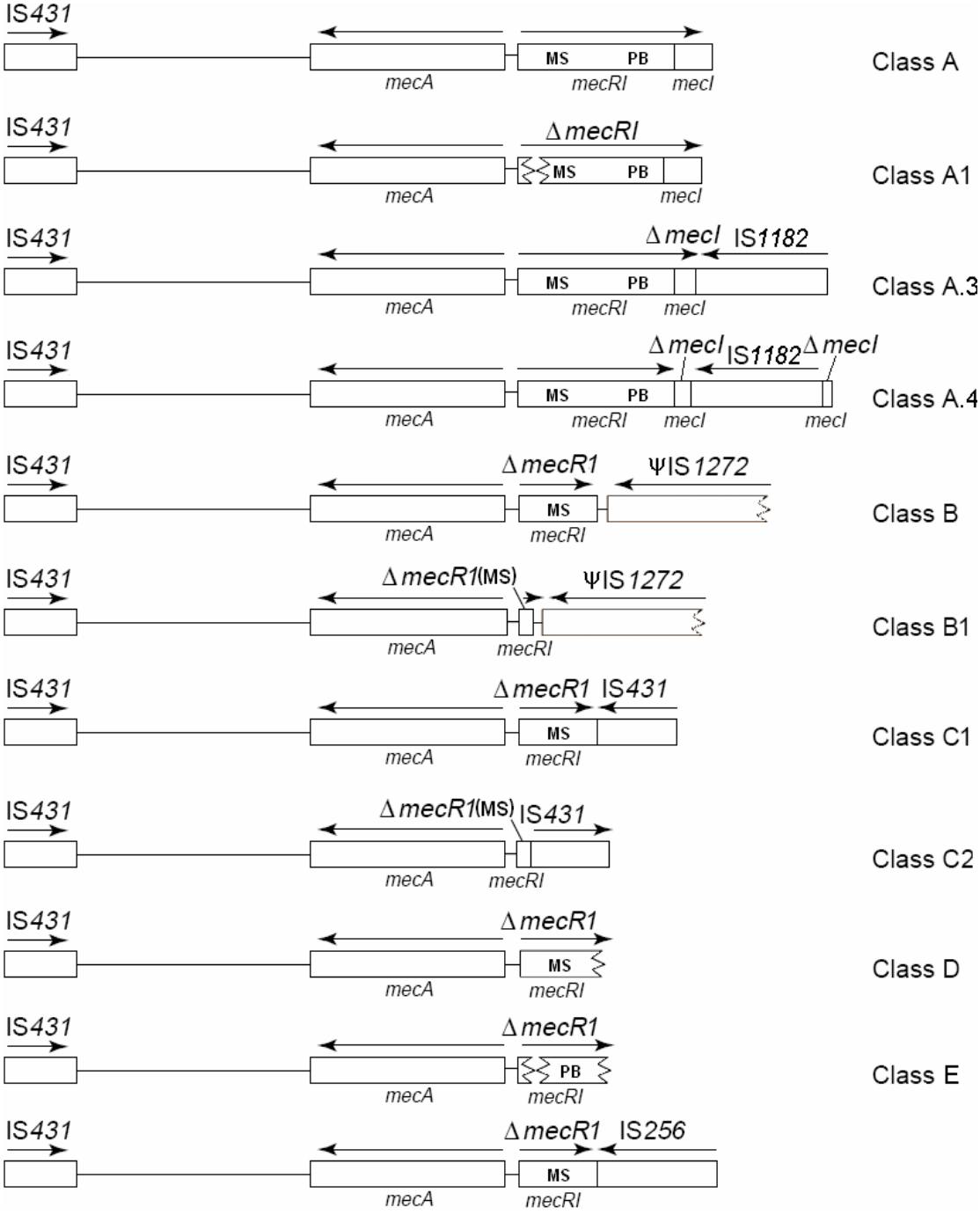


Figure 1.4 Schematic diagrams of the different *mec* complexes

MS: membrane-spanning domain of *mecR1*; PB: penicillin-binding domain of *mecR1*. (Hiramatsu *et al.*, 2001, Oliveira *et al.*, 2000, Lim *et al.*, 2003, Lim *et al.*, 2002)

1.3.4.2 The *ccr* complex

The *ccr* gene complex is another core element of SCC*mec* and is responsible for the mobility of the genomic island. It is composed of *ccr* genes surrounded by open reading frames with unknown functions. (Ito *et al.*, 1999, Ito *et al.*, 2001, Katayama *et al.*, 2000)

The Ccrs are highly basic proteins and have homology with the site-specific recombinases of the invertase/resolvase family. The presumptive active site of the Ccr proteins is located in the N-terminal domain that carries a conserved catalytic motif with a serine residue. It is thought that the serine residue provides hydroxyl groups that attack the DNA molecule during the strand exchange reaction. This catalytic motif is characteristic and well conserved among recombinases in the invertase/resolvase family. The N-terminal domains of all Ccr proteins have substantial homology with the recombinases of this family. However, the C-terminal of the Ccr proteins is different to the recombinases in their amino acid sequences and they are considerably larger. (Ito *et al.*, 1999, Ito *et al.*, 2001, Katayama *et al.*, 2000, Ito *et al.*, 2004)

The Ccrs have been found to catalyse the *in vitro* site and orientation specific integration and excision of SCC*mec*. Three different *ccr* genes have been found among the SCC*mec* and they are the *ccrA*, *ccrB* and *ccrC* genes. *ccrA* and *ccrB* are always found together and need each other to be functional. (Ito *et al.*, 2001, Katayama *et al.*, 2000) The *ccrC* gene has only been found by itself and is able to induce SCC mobility by itself. (Ito *et al.*, 2004) Although these three genes encode for proteins which have the same function, they are only distantly related to each other and to the other site-specific recombinases. The *ccrAB* transcribe in the same direction as *mecA* while *ccrC* transcribes in the opposite direction. (Ito *et al.*, 1999, Ito *et al.*, 2001, Katayama *et al.*, 2000, Ito *et al.*, 2004)

1.3.4.2.1 The *ccr* gene complex allotype

Several different allotypes of the *ccr* gene complex have been reported. The type 1 *ccr* complex consists of *ccrA1* and *ccrB1* genes. The *ccrB1* is truncated by a frame-

shift mutation which results in a dysfunctional *ccr* complex. (Ito *et al.*, 2001) The type 2 *ccr* gene complex contains *ccrA2* and *ccrB2* genes. Both of the type 2 genes have been found to be intact and functional. (Katayama *et al.*, 2000, Ito *et al.*, 2001) The type 3 ccr gene complex has the *ccrA3* and *ccrB3* genes. Like the type 1 complex, they are not functional due to a deletion of one amino acid at the N-terminal of the CcrA3 protein. (Ito *et al.*, 2001) The type 4 ccr complex harbours the *ccrA4* and *ccrB4* genes, and it is not functional due to a 300 bp deletion in the *ccrB4* gene. (Oliveira *et al.*, 2001b) The type 5 *ccr* complex only carries the *ccrC* gene, which is functional. (Ito *et al.*, 2004)

1.3.4.2.2 Site-specific integration and excision of SCC*mec* by *ccr* genes

The SCC*mec* integrates into the staphylococcal chromosome at the 3' end of the highly conserved *orfX* gene. *orfX* encodes for a protein with unknown function and is located near to the origin of replication, between the *purA* and *spa* genes. *orfX* is 10 kb downstream of *purA* and 66 kb to 89 kb upstream of the *spa* gene depending on the size of the integrated SCC*mec*s. (Ito *et al.*, 2003a) The integrated SCC*mec* is flanked by a pair of 15 nucleotide direct repeats, DR-SCC and DR-B. The DR-SCC is found in the SCC*mec* specific DNA, 3' immediately next to *orfX*. DR-B is located at the other end of SCC*mec* and is found in the host specific DNA. In addition to the flanking DRs, the SCC*mec* is also flanked by a pair of 27 bp inverted repeats (IRscc) located at both ends of the SCC*mec* specific DNA. The majority of the DR-SCC is located in, and overlaps, the IR next to *orfX*. These IRs are not found in SCC*mec* that carry *ccrC*. (See Figure 1.5) (Ito *et al.*, 2001, Ito *et al.*, 1999, Ito *et al.*, 2004, Katayama *et al.*, 2000)

A plasmid cloned with *ccrA2* and *ccrB2* has been found to mediate the excision of SCC*mec* after its introduction. The excised SCC*mec* formed an extrachromosomal closed circular DNA. The circular SCC*mec* carried a new attachment site, attSCC, created by the head to tail ligation of SCC*mec*. The attSCC carries both copies of inverted repeats and the DR-SCC. The attSCC of the SCC*mec* carrying *ccrC* gene is different to other SCC*mec*s as it does not carrying flanking IRs. The excision also creates an attachment site on the chromosome, attB, which includes the DR-B which is now located at the 3' end of *orfX*. DR-B had also been found in the *orfX* of non-

SCC strains. These findings indicate that the excision site is at the 5' end of DR-B. (Ito *et al.*, 1999, Katayama *et al.*, 2000, Ito *et al.*, 2004)

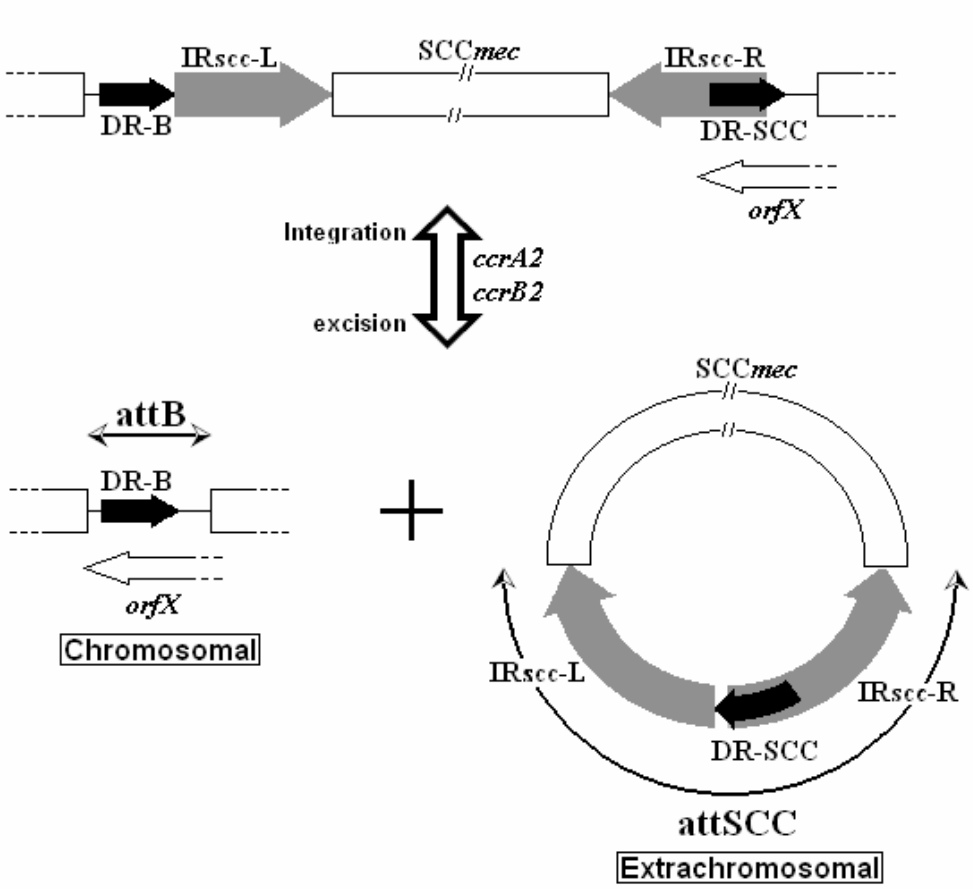


Figure 1.5 The site and orientation specific integration and excision mediated by *ccrA2* and *ccrB2*

The SCC*mec* is flanked by a pair of direct repeats (DR-B and DR-SCC). It also carries a pair of inverted repeats at both ends of the SCC*mec*. The excision of SCC*mec* generates an extrachromosomal circular DNA and two attachment sites, attB and attSCC. The chromosomal attB consists of the highly conserved *orfX* that carries the DR-B. The attSCC, located in the extrachromosomal SCC*mec* circular DNA is comprised of the two IRs and the DR-SCC. The SCC*mec* insertion site is in the 5' end DR-B of the *orfX*. Black arrows: direct repeats (DR); Grey arrows: inverted repeats (IR); att: attachment site. (Katayama *et al.*, 2000, Ito *et al.*, 2004, Mongkolrattanothai *et al.*, 2004)

An experimental plasmid carrying *ccrA2*, *ccrB2* and attSCC, integrates into the chromosome after introduction into a cell. The integration is site and orientation specific, and the integration site is the same as the excision site, the DR-B at the 3'

end of *orfX*. Despite the integration, *orfX* remains functional because both DR-B and DR-SCC carry the stop codon for it. This probably indicates that *orfX* may be functionally important. (Ito *et al.*, 1999, Katayama *et al.*, 2000, Ito *et al.*, 2004)

The other functional *ccr* gene, *ccrC*, has been found to have an identical activity at the same *orfX* site as the *ccrA2* and *ccrB2* genes. However, *ccrC* only excises the SCC*mec* carrying *ccrC*, whereas the *ccrA2* and *ccrB2* can excise SCC*mec*s carrying either type of *ccr* genes. The *ccrC* mediated integration worked more efficiently with the plasmid with *ccrC* attachment sites. This is the same with *ccrA2* and *ccrB2* as well. These specificities are probably due to target-site differences as the SCC*mec* carrying *ccrC* does not have inverted repeats like the SCC*mec* carrying *ccrAB*. (Ito *et al.*, 2004)

The exact mechanism of SCC*mec* mobility mediated by *ccr* genes has yet to be clarified. All the data gathered so far has been collected with *in vitro* experiments using small plasmids. (Katayama *et al.*, 2000) The structure of SCC*mec*, the specific integration/excision site (i.e. attachment sequences), the direct repeat sequences and the putative mobility determinants are very similar to staphylococcal pathogenicity islands. This strongly suggests that the SCC*mec* is mobile. The *in vivo* spontaneous excision and integration of SCC*mec* have also been reported in clinical MRSA strains. (Deplano *et al.*, 2000b, Wielders *et al.*, 2001) However, the size of some SCC*mec*s might prevent them from being moved by bacteriophage-mediated transduction. (Novick *et al.*, 2001) Some researchers have suggested that the SCC*mec* might transfer via conjugation due to its non-species specificity and its capability of moving large-size elements, but this has yet to be demonstrated. (Novick *et al.*, 2001, Robinson and Enright, 2004a) Similar to other integrated genomic islands found in Gram-negative organisms, some SCC*mec* have become immobile due to the truncation and/or mutation of the mobile determinants. (Novick *et al.*, 2001)

1.3.4.3 SCC*mec* allotypes

The SCC*mec* genomic islands found amongst MRSA vary substantially in their sizes, the *ccr* and *mec* complexes and other genes they carry. Consequently, the SCC*mec* are classified into different allotypes according to the *ccr* and *mec* gene complexes they carry. To date six SCC*mec* have been reported and five are completely sequenced. (Ito *et al.*, 2001, Ito *et al.*, 1999, Ma *et al.*, 2002, Oliveira *et al.*, 2001b, Ito *et al.*, 2004, Ito *et al.*, 2003a) The characteristics of the core units of these SCC*mec* are listed in Table 1.5. The SCC*mec* can be roughly divided into three junkyard (J) regions, the *mec* and *ccr* complexes. The J1 region is located between the 5' end of the *ccr* complex and DR-B. The J2 region lies between *mec* and the *ccr* complexes. The J3 region extends from the 3' end of IS*431mec* to the DR-SCC in *orfX*. These regions harbour different mobile elements, genes and dysfunctional pseudogenes, which result in the differences in the lengths and functionality of SCC*mec*. The variation in the J regions may show the evolution and molecular epidemiology of SCC*mec*. (Ito *et al.*, 2003a) See. Figure 1.6

Table 1.5 SCC*mec* allotypes and unique features[a]

SCC*mec* allotype	*ccr* complex types	*mec* complex classes	Sizes (kb)	J1 region	J2 region	J3 region	References
I	**Type 1** (*ccrA1-ccrB1*)	**Class B** (IS*431-mecA*-Δ*mecR1*-ψIS*1272*)	34.4	*pls* gene	-	*mcrB* gene	Ito *et al.* (2001)
II	**Type 2** (*ccrA2-ccrB2*)	**Class A** (IS*431-mecA-mecR1-mecI*)	53	*kdp* operon	Tn554	*mcrB* gene, pUB110	Ito *et al.* (2001), Ito *et al.* (1999)
III	**Type 3** (*ccrA3-ccrB3*)	**Class A** (IS*431-mecA-mecR1-mecI*)	66.9	-	ψTn*554*	*mcrB* gene, pT181, *mer* operon, Tn*554*, Δ*hsdR* gene,CZ072 gene	Ito *et al.* (2001)
IV	**Type 2** (*ccrA2-ccrB2*)	**Class B** (IS*431-mecA*-Δ*mecR1*-ψIS*1272*)	21, 24 to 31	-	-	*mcrB* gene	Ma *et al.*, (2002), Ito *et al.* (2003a)
IVp[b]	**Type 4** (*ccrA4-ccrB4*)	**Class B** (IS*431-mecA*-Δ*mecR1*-ψIS*1272*)	22	-	-	-	Oliveira *et al.* (2001b)
V	**Type 5** (*ccrC*)	**Class C2** (IS*431-mecA*-Δ*mecR1*-IS*431*)	28	-	-	*hsdR*, *hsdS*, *hsdM* genes	Ito *et al.* (2004)

[a] The unique features listed are based on the prototypes of each SCC*mec* allotype. The allotype variants may have different features.

[b]IVp stands for the Type IV SCC*mec* paediatric clone, to distinguish it from the Type IV SCC*mec*.

1.3.4.3.1 Type I SCC*mec* cassette DNA

The Allotype I SCC*mec* carries the Type 1 *ccr* and Class B *mec* complexes. The length of Type I SCC*mec* varies from 34 to 40 kb and typically, it is found in a group of early MRSA isolates. (Ito *et al.*, 2001, Oliveira *et al.*, 2001b) These strains are known as the archaic clone and were isolated in the 1960s and 1970s and includes the first MRSA ever isolated NCTC10442. (Ito *et al.*, 2001, Enright *et al.*, 2002) Type I SCC*mec* is also found in contemporary strains such as the pandemic Iberian clone, which has descended from the archaic clone. (Aires de Sousa and de Lencastre, 2004, Crisostomo *et al.*, 2001, Oliveira *et al.*, 2001b)

Type I SCC*mec* carries the *pls* gene in the J1 region and this is unique to Type I. (Ito *et al.*, 2001, Oliveira *et al.*, 2001b) The *pls* encodes for a 230 kDa large plasmin-sensitive surface protein. The Pls protein inhibits the fibrinogen and fibronectin adhesins, which results in a negative result in the rapid slide identification test for *S. aureus* with IgG and fibrinogen. The Pls protein is sensitive to proteolytic activity and when it is cleaved, organisms show good adherence. It had been suggested that Pls might play a role at one point in the process of infection by inhibiting adherence and allowing the organisms to spread. At a later stage it is cleaved and facilitates the adherence of the bacteria to host tissues. As the *pls* has been found in some very successful pandemic clones, it might be beneficial to the isolates. (Savolainen *et al.*, 2001, Vaudaux *et al.*, 1998, Kuusela *et al.*, 1994) Type I variant IA has an integrated aminoglycoside resistance plasmid pUB110 (*aadD* and *ble*) flanked by two copies of IS*431/257* in the J3 region. The J3 region of Type IA is almost identical to the J3 region of Type II SCC*mec* and is typically found in the pandemic Iberian clone. (Ito *et al.*, 2001, Oliveira *et al.*, 2001b, Aires de Sousa and de Lencastre, 2004, Amorim *et al.*, 2002)

1.3.4.3.2 Type II SCC*mec* cassette DNA

Type II SCC*mec* is characterised by having Type 2 *ccr* and Class A *mec* complexes. It is 53 kb in length and is found in the pre-MRSA N315, epidemic clones Irish-1 and EMRSA-16, as well as in the vancomycin-intermediate *S. aureus*. (Ito *et al.*, 1999, Ito *et al.*, 2001, Enright *et al.*, 2002) In laboratory tests, N315 appears to be

methicillin sensitive due to the repression of *mecA* by *mecR1-mecI* regulation. (Kuwahara-Arai *et al.*, 1996) Irish-1 and EMRSA-16 are epidemic clones from Ireland and the United Kingdom (UK), respectively. EMRSA-16 accounts for 35.4% of all MRSA isolated in 26 UK hospitals between 1998 and 2000. (Johnson *et al.*, 2001)

In addition, to the integrated pUB110 in the J3 region, the J1 region of Type II SCC*mec* carries the *kdp* operon that is unique to Type II. (Ito *et al.*, 2001, Ito *et al.*, 1999) The *kdp* operon encodes a potassium transport ATPase and its regulators. It is very similar to the *Clostridium acetobutylicum kdp* operon except the genes are in a different order. (Treuner-Lange *et al.*, 1997, Ito *et al.*, 2001) In addition, the Type II *kdp* operon has been found to be non-functional as three out of six genes are either mutated or truncated by deletions. (Ito *et al.*, 2001)

The MLS_B and spectinomycin-resistance transposon Tn*554* (*ermA* and *spc*) and a truncated xylose represser have been found in the J2 region. (Phillips and Novick, 1979, Chikramane *et al.*, 1991) Both of these elements are also found in the Type III SCC*mec* but in the J3 region. (Ito *et al.*, 2001)

Five variants of the SCC*mec* II allotype were found among the Irish MRSA strains isolated between 1971 and 2002. These variants are smaller than the SCC*mec* II prototype and range between 40 kb to 31 kb. The *kdp* operon in the J1 region is absent in all five of the variants. The SCC*mec* IIA is very similar to the SCC*mec* II prototype but carries the Class A.4 *mec* complex. The SCC*mec* IIB has the Class A *mec* complex but the Tn*554* in the J2 region is absent. The SCC*mec* IIC carries the Class A.3 *mec* complex and a truncated Tn*554*. The six ORFs typically present between Tn*554* and the *mec* complex in SCC*mec* II allotypes are also absent. SCC*mec* IID has the Class A.4 *mec* complex but lacks the integrated plasmid pUB110 in the J3 region and the additional copy of IS*431* adjunct to the plasmid. The SCC*mec* IIE is almost identical to SCC*mec* IIC. It has the Class A.3 *mec* complex but like SCC*mec* IID does not have the integrated plasmid, pUB110 and the adjunct IS*431*. (Shore *et al.*, 2005)

The SCC*mec* Types I, II and IV carry the *mcrB* restriction enzyme gene in their J1 region close to the *orfX*/DR-SCC junction. The sequences around this junction are also extremely well conserved in these three SCC*mec* types. Type II has a 102 bp unique sequence at the edge of SCC*mec* next to the *orfX* and an additional copy of the direct repeat in the same region. These features have been found in all variants of Type IV SCC*mec* examined. (Ito *et al.*, 2003a, Ma *et al.*, 2002)

1.3.4.3.3 Type III SCC*mec* composite cassette DNA

The 66.9 kb Type III SCC*mec* is the largest SCC*mec* and is commonly found in hospital-acquired strains like the pandemic EMRSA-1 clones. (Ito *et al.*, 2001, Enright *et al.*, 2002) Type III carries a number of antimicrobial resistance genes in its J regions. The J2 region contains the cadmium resistance ψTn*554* (*cadCA* operon). (Ito *et al.*, 2001, Dubin *et al.*, 1992) The J3 region contains the IS*431/257* flanked, integrated tetracycline-resistance plasmid, pT181 and the mercurial resistance, *mer* operon (Tn*4004*). In addition to these two elements, the J3 region also carries a copy of Tn*554*. (Ito *et al.*, 2001, Firth and Skurray, 2000, Oliveira *et al.*, 2001b)

Type III SCC*mec* is significantly different from the other SCC*mec* types, because it has three copies of the direct-repeat sequences and two *ccr* gene complexes, *ccrA3-ccrB3* and CZ072, a *ccrC* homologue. This indicates that Type III SCC*mec* is comprised of two separate SCC elements. (Ito *et al.*, 2003a, Ito *et al.*, 2004) It has a direct repeat on either end of the element. It also has a third copy of the direct repeat sequence located between the pT181 and the *mer* operon in the J3 region. The CZ072 is found in the same region as well as a copy of the truncated *hsdR* gene (Δ*hsdR*). Similar to the Type V junction, no inverted repeats have been found in the J3 region adjacent to the *orfX*/DR-SCC junction. (Ito *et al.*, 2003a, Ito *et al.*, 2001) This suggests that the J3 region from the *mer* operon to *orfX* is a *ccrC* SCC-like element that harbours Tn*554* between the Δ*hsdR* and CZ072 genes. The remaining region is a Type III SCC*mec*. The *ccrC* carrying SCC might have integrated with the Type III SCC*mec* before it become integrated into the chromosome or following its integration. (Ito *et al.*, 2004)

1.3.4.3.4 Type IV SCC*mec* cassette DNA

Type IV SCC*mec* harbours Type 2 *ccr* and Class B *mec* complexes and is the smallest SCC*mec* reported with sizes ranging from 21to 31 kb. This SCC*mec* Type is predominantly found in community-acquired MRSA. (Ma *et al.*, 2002, Ito *et al.*, 2003a, Okuma *et al.*, 2002) This SCC*mec* allotype is also found in coagulase-negative staphylococci. It is the predominant SCC*mec* allotype in clinically significant, methicillin-resistant *S. epidermidis* in patients with prosthetic valve endocarditis. (Wisplinghoff *et al.*, 2003)

Three variants of Type IV SCC*mec* have been reported. The IVa and IVb types are smaller and only carry *ccr* and *mec* complexes, their sizes are 21kb and 24kb, respectively. The differences between these two types are in their J1 regions, which contain genes with unknown function and which are unique to them. (Ma *et al.*, 2002) The IVc SCC*mec* is 31kb in size and like the other two Type IV variants, it has IVc specific DNA and a 5.9 kb putative SCC remnant IE25923 in the J1 region. In addition, the IVc Type also has a copy of the aminoglycoside-resistance transposon, Tn*4001* (*aacA-aphD*) located in its J3 region. (Ito *et al.*, 2003a)

The SCC*mec* IV paediatric (IVp) is only 22 kb and is found in an epidemic paediatric clone. Structurally it is very similar to Type I and IV SCC*mec*s, which only carry *ccr* and *mec* complexes and it does not carry any antibiotic resistances other than *mecA*. Unlike Types I and IV SCC*mec*s it has a Type 4 *ccr* complex together with the Class B *mec* complex. Since it is the only SCC*mec* that has not been completely sequenced, it is not known what other elements it carries. (Oliveira *et al.*, 2001b)

1.3.4.3.5 Type V SCC*mec* cassette DNA

The 28kb Type V SCC*mec* has been found in community-acquired MRSA isolated in Australia. Type V SCC*mec* has also been found in coagulase-negative staphylococci, especially *S. haemolyticus*. It harbours *ccrC* and Class C2 *mec* complexes, which are very different from the other SCC*mec*s found in MRSA. The J1 region contains a Type-1 restriction-modification system. (Ito *et al.*, 2004) It is

composed of three genes, *hsdR*, *hsdS* and *hsdM* genes. The *hsdR* encodes an endonuclease and the other two genes encode for a DNA methyltransferase. (Murray, 2002) These *hsd* genes are found in the pathogenicity islands and are believed to stabilise them by protecting them from the host's restriction systems. (Baba *et al.*, 2002, Yamaguchi *et al.*, 2002) The *hsd* gene cluster might have the same function in Type V SCC*mec*. (Baba *et al.*, 2002)

1.3.4.3.6 Distribution of SCC*mec* allotypes

Compared with the Type IV and V SCC*mec*, the Type II and III SCC*mec* are better adapted to the hospital environment where the antibiotic selection pressure is high. Type II and III SCC*mec* are often found in high-level resistant MRSA, and carry multiple antibiotic-resistance genes in the addition to *mecA*. However, their large size and the array of exogenous genes they carry may slow cell growth. This is a disadvantage in the community setting, where faster growth rates would favour colonisation by non-multiply-resistant strains. (Okuma *et al.*, 2002, Ito *et al.*, 2003a, Ito *et al.*, 2004)

Community MRSA predominantly have low-level methicillin-resistance, probably due to selection by less potent β-lactams. The smaller SCC*mec*s like Types IV and V appear to be well suited to strains in the community setting. Their SCC*mec*s are small and only carry the *mecA* gene. This allows the community strains to confer resistance to β-lactams without being too compromised in their fitness and by having faster growth rates. Types IV and V SCC*mec* have been found in strains with very diverse genetic backgrounds and predominate in methicillin-resistant coagulase-negative staphylococci. These findings not only indicate that Type IV and V SCC*mec* are more mobile than other SCC*mec*s, but also suggests that inter-species transfer of SCC*mec* occurs in the community setting. (Okuma *et al.*, 2002, Ito *et al.*, 2003a, Ito *et al.*, 2004, Wisplinghoff *et al.*, 2003)

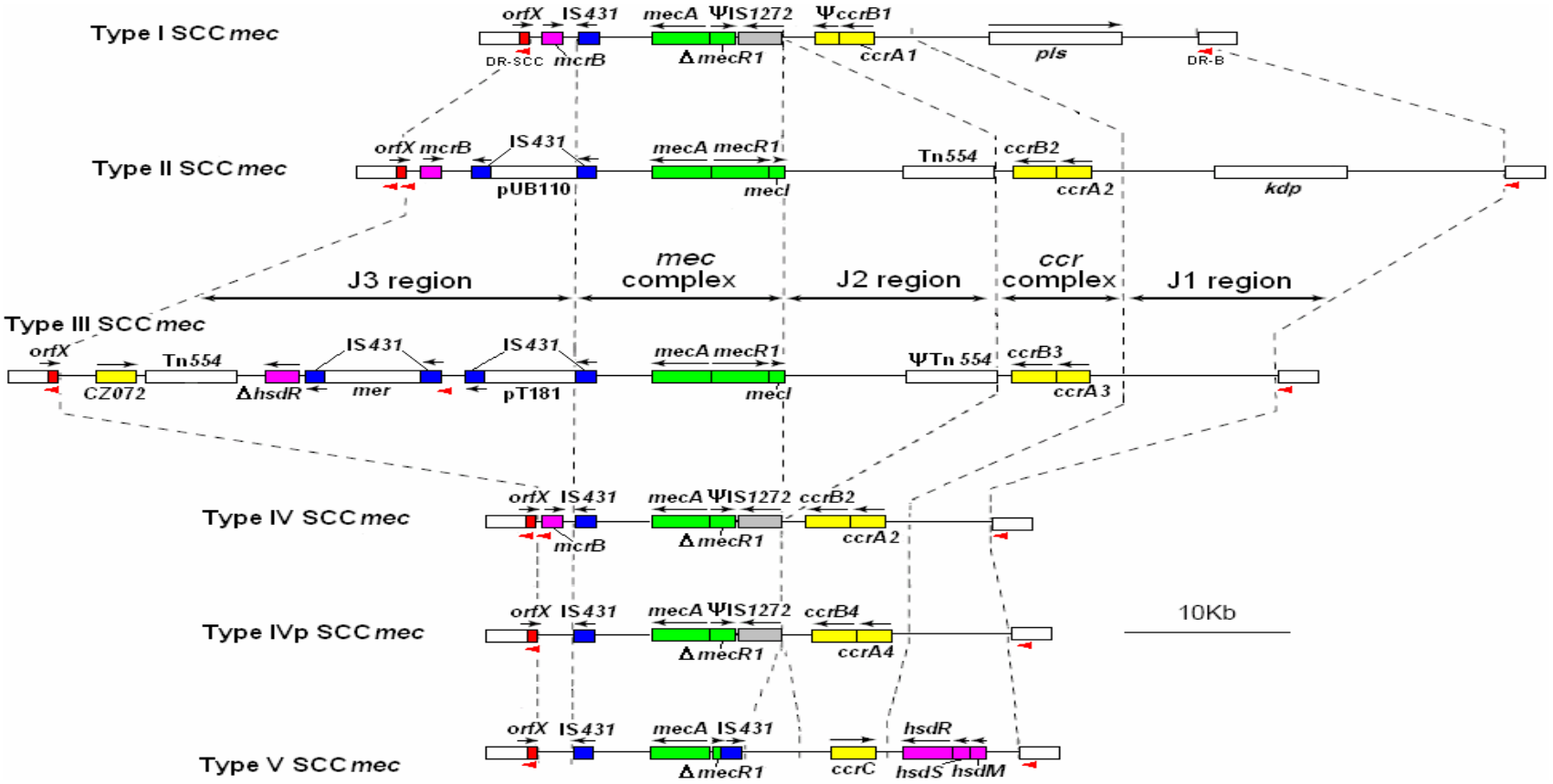


Figure 1.6 Schematic diagrams of different SCC*mec* allotypes

The colours correspond to the different elements, red, *orfX*; blue; IS*431*, also known as IS*257*; green, *mec* genes; yellow, *ccr* genes; magenta, restriction-system genes. The black arrows indicate the direction of transcription; red arrow heads indicate direct repeat sequences. The junkyard (J) regions, *mec* complexes and *ccr* complexes are indicated by double-headed arrows (Ito *et al.*, 2003a, Ito *et al.*, 2004, Okuma *et al.*, 2002)

Reports indicate that some SCC*mec*s in community strains of both *S. aureus* and coagulase-negative staphylococci do not belong to any of the reported SCC*mec*s. This finding indicates that SCC*mec* is more diverse in the community setting. It is possible that these diverse SCC*mec*s originate in coagulase-negative staphylococci and only some of them have been acquired by *S. aureus.* (Ito *et al.*, 2004, Lim *et al.*, 2003, Hanssen *et al.*, 2004)

1.3.4.4 SCC without *mec* complex

The SCC is a site-specific mobile element integrated in the 3' end of *orfX*. It was first discovered as an antibiotic-resistance genomic island with the *mec* complex as its core. Recently, several SCCs without the *mec* complex and carrying mainly structural determinants, have been reported.

The 35 kb SCC*cap* carries the 15 *cap1* genes that encode the Type 1 capsular polysaccharide. It is found in *S. aureus* and contains a truncated *ccrC* recombinase gene. (Luong *et al.*, 2002) The 23 kb SCC_{12263} is found in the *S. hominis* GIFU12263 and carries the DNA methyltransferase *msts1* gene, which protects it from the host-restriction system. It carries a functional Type 1 *ccr* gene complex, whereas the rest of the genes are mainly non-functional pseudogenes. (Katayama *et al.*, 2003a, Takahashi *et al.*, 2002) The 22.8 kb SCC_{476} found in MSSA476 is similar to SCC_{12263} and also carries the Type 1 *ccr* complex. It harbours a putative, fusidic acid-resistance *far* gene and three genes of the Type 1 restriction modification system. (Holden *et al.*, 2004)

The 57 kb SCC composite island found in *S. epidermidis* is comprised of two SCC elements. They are the 19 kb SCC*pbp4* and a 38 kb SCC carrying the Type 4 *ccr* gene complex. This composite SCC is flanked by 28 bp direct repeats, and an additional direct repeat is found at the junction between SCC*pbp4* and the SCC carrying Type 4 *ccr* complex. (Mongkolrattanothai *et al.*, 2004) The SCC*pbp4* is located at the *orfX* end of the composite SCC. It carries the *pbp4* and *tagF* cell-wall synthesis genes, which encode PBP4 and the carboxypeptidase for teichoic acid synthesis, respectively. The SCCpbp4 carries the Type 2 *ccr* gene complex. The 6.9 kb sequence immediately upstream of the *ccrA2* gene in SCC*pbp4* is 99% identical

to the same region in a Type IVc SCC*mec*. This sequence is also found to carry an abortive phage-resistance protein. The SCC*pbp4* is flanked by direct and inverted repeats identical to those of the Type II and Type IV SCC*mec*. The 38 kb SCC with the Type 4 *ccr* gene complex contains the *mer* operon, a *cadA* bearing region and two genes of a restriction-modification system. The *mer* operon and the *cadA* region are located adjacent to each other and are flanked by three copies of IS*431/257*. (Mongkolrattanothai *et al.*, 2004, Ito *et al.*, 2001)

The SCC genomic islands found in both methicillin-susceptible *S. aureus* and coagulase-negative staphylococci are highly diverse. These findings indicate that SCC may be readily transferred within the staphylococcal population. SCC might also serve as a reservoir for the formation of SCC*mec*. (Holden *et al.*, 2004, Ito *et al.*, 2004, Mongkolrattanothai *et al.*, 2004, Ito *et al.*, 2003a, Katayama *et al.*, 2003a)

1.4 Typing techniques for *S. aureus*

The aim of typing is to study the population dynamics and the spread of microorganisms. Typing has been an invaluable tool for clinicians and epidemiologists alike for tracing the spread of particular strains and discovering the route of dissemination and the reservoirs. The object of typing techniques is to reliably differentiate epidemic clones from epidemiologically unrelated strains. The outcomes of these epidemiological investigations are often used to assist the clinical treatment of the patients by selecting suitable antimicrobial agents and differentiating between successive and recurrent infection. Furthermore typing contributes to the understanding of the epidemiology of infection and facilitates infection control measures. (Weller, 2000, Witte, 2000, van Belkum *et al.*, 2001)

Typing techniques can be evaluated on performance (efficacy) and convenience (efficiency) criteria. (Weller, 2000) The performance criteria include the typability, reproducibility, discriminatory power and the agreement between two typing techniques. Typability is the proportion of isolates in a population that can be characterised into a "type" by a typing technique. A high typability of almost all isolates in a population is required for *S. aureus* typing methods. Reproducibility is

the ability of a typing technique to give the same outcome when the same sample is repeatedly tested in the same or different laboratories, as well as during the course of an epidemic. The discriminatory power is the ability of a technique to generate a sufficient number of types and the commonest type should be less than 5% of the number of the isolates of the population investigated. The agreement between two techniques is evaluated by determining if highly related strains demonstrated the same relationship by both techniques. (Witte, 2000, van Belkum *et al.*, 2001, Weller, 2000) The convenience criterion evaluates the practicality of the typing technique. It includes the versatility, rapidity, ease of execution and interpretation. The versatility is the ability of a technique to type any pathogen with modification of its protocol. In addition to the rapidity and ease of performance, the practical evaluation should include the cost and availability of the required equipment and reagents. Currently there are many different typing techniques available. They can be generally separated into phenotypic and genotypic typing methods based on their typing targets. (Weller, 2000)

S. aureus outbreaks are often due to the clonal expansion of a particular strain. Phenotypic techniques have been commonly used to identify staphylococcal epidemic clones. Since advancements in genetic methods, several genotypic techniques have been developed in the past two decades. They were first used in the research laboratories, and have now been increasingly used in clinical laboratories to replace the phenotypic techniques. (Tenover *et al.*, 1994, Witte, 2000, Trindade *et al.*, 2003, Weller, 2000)

1.4.1 Phenotypic techniques

Phenotypic-typing techniques discriminate the isolates according to the physical characteristics they express. These techniques include bacteriophage typing, antimicrobial-resistance profiling (resistogram) and multilocus enzyme electrophoresis.

1.4.1.1 Bacteriophage typing

Bacteriophages or phages are viruses that infect bacterial cells and are either lytic or temperate. Infection with lytic phages results in cell lysis, whereas temperate phages can have either a lytic cycle or a lysogenic cycle. In the lysogenic cycle the phage DNA is inserted into the bacterial chromosome and replicates along with it. It is referred to as prophage at this stage. Bacteria carrying temperate phages are called lysogens. Prophage can be reactivated and undergo a lytic cycle and lyse the host cells. (Campbell, 2003)

Most *S. aureus* strains are multiply lysogenic, carrying several different temperate phages. (Novick, 1990, Baba *et al.*, 2002, Holden *et al.*, 2004) Strains of *S. aureus* that are lysogenised by a phage will not be lysed when exposed to that phage. This is known as lysogenic immunity. *S. aureus* are phage typed on their susceptibility to an International Basic Set (IBS) of 24 phages. These phages are used at a standard dilution known as the routine test dilution (RTD) and at 100 × RTD. (Vickery *et al.*, 1986, Hone, 1992, Richardson *et al.*, 1999) There are four main groups of phages used for typing and strains of *S. aureus* may be lysed by a number of phages within a group. (Novick, 1990)

Bacteriophage typing has been of great epidemiological value in tracing the source and spread of staphylococcal infections in hospitals during the past decades. (Vickery *et al.*, 1986, Richardson *et al.*, 1999) This typing technique was used to distinguish and define the 17 strains of epidemic MRSA that are endemic in England and Wales. (Kerr *et al.*, 1990, Aucken *et al.*, 2002, Marples *et al.*, 1986, Walker *et al.*, 1999) However, the presence of non-typable MRSA and poor reproducibility has greatly reduced its value as an epidemiological tool. (Archer and Mayhall, 1983, Tenover *et al.*, 1994, Murchan *et al.*, 2000) Approximately 20% to 30% of MRSA strains are non-typable, but higher rates have been reported. (Tenover *et al.*, 1994, Richardson *et al.*, 1999) For example, in a study on Malaysian MRSA isolates, 91% of the isolates were non-typable with the IBS phages. The introduction of new sets of supplementary phages has improved its usefulness with some success. (Richardson *et al.*, 1988, Vickery *et al.*, 1986, Richardson *et al.*, 1999) In addition to these inherent problems, phage typing is also time-consuming and technically

demanding. The requirement for maintaining the phage stocks and the propagation of the phages has limited the access of this technique to large laboratories and reference laboratories. Nevertheless, in the pre-genomic era, bacteriophage typing was the standard technique for *S. aureus* typing for many decades. (Tenover *et al.*, 1994, Weller, 2000) Some researchers have now suggested that phage typing has outlived its usefulness and should be replaced by a rigorous genotyping system such as pulse-field gel electrophoresis. This is because phage typing lacks any systematic biological basis and is plagued by non-typable isolates. It is also unpredictable and gives uninterpretable variability. (Tenover *et al.*, 1994, Bannerman *et al.*, 1995, Kreiswirth *et al.*, 1993, Witte, 2000, Walker *et al.*, 1999)

1.4.1.2 Antimicrobial-resistance profiling (Resistogram)

Antimicrobial-resistance profiling or resistograms are a common and cost effective method used for the detection and characterisation of MRSA. It is the resistance profile of an organism to antimicrobial agents and includes antibiotics, heavy metals and disinfectants. It is rapid, simple and routinely available. To be reliable the test must be performed by a standardised method such as that developed by Kirby and Bauer. (Baron *et al.*, 1994) The US NCCLS method is based on this and gives results, which are generally consistent, reproducible and reliable. This test is relatively inexpensive and rapid compared to other conventional methods. (Tenover *et al.*, 1994).

The resistogram can also used to select appropriate antibiotics for the treatment of MRSA infections. The resistogram of the organisms can also be used to characterise isolates because the phenotypic expression of resistance is dependent on the genetic background of the organism. (Tenover *et al.*, 1994, Lyon and Skurray, 1987, Skurray and Firth, 1997)

However, resistograms have their limitations in the characterisation of MRSA. The growth conditions like pH, incubation time, NaCl concentration and temperature are known to influence the expression of methicillin resistance. This is due to the heterogeneous expression of methicillin resistance seen in MRSA isolates, and this

can easily result in MRSA being missed in routine susceptibility testing. (Chambers and Hackbarth, 1987, Hartman and Tomasz, 1986, Tomasz *et al*., 1991)

Many of the resistance genes in MRSA are carried by transposable elements such as plasmids, transposons and SCC genomic islands. (Firth and Skurray, 2000, Ito *et al.*, 2003a) Elements such as plasmids, transposons and SCC*mec* are known to be inherently unstable and can be lost or gained in a strain. (Firth and Skurray, 2000, Deplano *et al*., 2000b, Lawrence *et al.*, 1996) Hence, the antimicrobial-resistance profiles of isolates can be unstable overtime. In general, resistograms work reasonably well in typing strains however, the factors mentioned above should be taken into account when analysing the results. (Tenover *et al.*, 1994, Blanc *et al.*, 1994) Under most circumstances, it cannot be used as the sole typing technique for MRSA. (Weller, 2000)

1.4.1.3 Multilocus enzyme electrophoresis (MLEE)

The MLEE differentiates isolates according to the electrophoretic mobility of individual soluble enzymes that are responsible for primary functions of the cell. These mobility differences are the consequence of non-lethal mutations that affect the charge of the enzymes. Thus, MLEE indirectly reflects the genotypic polymorphism in *S. aureus.* (Musser and Kapur, 1992, Musser and Selander, 1990, Witte, 2000) Around 12 to 20 enzymes are used in this technique, and over 80% of single substitutions can be detected. However, mutations that do not alter the charge of the enzyme will be missed. Isolates are assigned to an electrophoretic type according to their enzyme allelic profiles and the similarity of the enzymes. (Tenover *et al*., 1994, Weller, 2000, Musser and Selander, 1990)

MLEE can apply to all strains and results are readily reproducible. However, the discriminatory power of this technique is dependent on the enzymes selected, because some enzymes are monomorphic in nature. MLEE has not been widely used as a typing technique due to its laborious nature. It has been utilised in several population studies and has proved to be useful. (Tenover *et al.*, 1994, Weller, 2000, Musser and Selander, 1990, van Belkum *et al*., 2001, Witte, 2000)

1.4.2 Genotypic techniques

Genotypic typing techniques target the regions in the genomic and plasmid DNA with a desirable degree of variability. The following sections will describe several genotypic typing methods that have been applied to *S. aureus*.

1.4.2.1 Plasmid profile and plasmid restriction endonuclease analysis

Plasmid profiling was the first genotypic technique utilised for *S. aureus* typing. The plasmids were extracted from the cell and separated by conventional agarose-gel electrophoresis. Plasmid profiling discriminates the isolates by the size and the number of the plasmids they carry. This method is easy to execute and interpret. However, the efficacy of plasmid profiling is limited by the inherent nature of plasmids. (Tenover *et al.*, 1994, Weller, 2000, Trindade *et al.*, 2003) Plasmids are extrachromosomal elements that can be spontaneously lost or gained by an organism. Furthermore, transposons on plasmids can also be readily lost or gained, and this will change the structure of the plasmids. (Novick, 1990, Firth and Skurray, 2000) Thus, plasmid profiling is not stable and may not be reproducible. (Hartstein *et al.*, 1995, Tenover *et al.*, 1994, Trindade *et al.*, 2003) The extracted plasmids occur in three different forms, covalently-closed circular, open circular and linear. All three forms have different electrophoretic motilities. Thus, different bands seen in a gel may represent the same plasmid in different forms. Many *S. aureus* isolates carry only one or two plasmids and this will result in poor strain discrimination. (Weller, 2000, Hartstein *et al.*, 1995)

Restriction-endonuclease analysis of plasmids (REAP) greatly enhances the discrimination power of plasmid profiling. The REAP technique generates restriction-fragment-length polymorphisms (RFLP) when the plasmids are digested with restriction endonucleases. REAP reveals the differences in position and frequency of restriction sites between two unrelated plasmids, even though they might have the same size. REAP still suffers the same instability as plasmid profiling, as the gain or loss of transposons on them will alter their RFLPs. (Liu *et al.*, 1996, Hartstein *et al.*, 1995)

Plasmids can readily be exchanged between two strains. As a result, the same plasmids can be found in two epidemiologically unrelated strains. This can lead to a false interpretation of a clonal outbreak if plasmid typing is the sole method used. The other problem with plasmid typing is that small proportions of *S. aureus* do not carry plasmids and thus would not be typed with this technique. (Tenover *et al.*, 1994, Melo-Cristino and Torres-Pereira, 1989, Novick, 1990) Nevertheless, plasmid-typing techniques are still a useful typing method when used in conjunction with other typing methods. (Tenover *et al.*, 1994, Weller, 2000)

1.4.2.2 Restriction fragment length polymorphism (RFLP)

RFLPs are generated when DNA is digested with a restriction endonuclease. This polymorphism is due to the distributional differences of the restriction endonuclease cleavage sites between individual isolates. The DNA fragments of different length are resolved and the RFLPs are observed by agarose gel electrophoresis. (Tenover *et al.*, 1997, Witte, 2000)

S. aureus is an organism with a low G + C content of 32.8%. (Baba *et al.*, 2002, Kuroda *et al.*, 2001) The restriction endonucleases with GC rich recognition sites, such as *Sma*I (CCCGGG) and *Not*I (GCGGCCGC), are known as the low frequency DNA cutters of *S. aureus*. This is because GC rich sites are relatively uncommon in the *S. aureus* genome. These low frequency cutters generate 15 to 20 large DNA fragments and can be resolved by pulsed-field gel electrophoresis. (Ichiyama *et al.*, 1991, Tenover *et al.*, 1997, Goering, 1993) The high frequency DNA cutters of *S. aureus* are the restriction endonucleases with AT rich recognition sites such as *Eco*RI (GAATTC), *Bgl*II (AGATCT) and *Hin*dIII (AAGCTT). These high frequency DNA cutters generate numerous short fragments that are difficult to resolve by conventional horizontal agarose gel electrophoresis unless followed by subsequent Southern hybridisations. (Jordens and Hall, 1988, Trindade *et al.*, 2003, Witte, 2000)

Typing techniques based on the RFLP of genomic DNA have excellent typability. However, their discriminatory powers and reproducibility are dependent on the choice of restriction endonucleases. (van Belkum *et al.*, 2001, Trindade *et al.*, 2003, Witte, 2000)

1.4.2.2.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a technique used for the separation (resolution) of large DNA fragments over one mega bases. Conventional agarose-gel electrophoresis will not separate DNA fragments bigger than 40 to 50 kb. The large fragments are often in longitudinal orientation relative to the electric field of the agarose-gel matrix, and will not migrate properly according to their size under the constant and unidirectional electric field used in conventional electrophoresis. Unlike conventional electrophoresis, PFGE utilises a multidirectional electric field that is periodically changed (pulsed). The multidirectional-pulsed field allows the large DNA fragments to continuously reorientate their direction of migration and "wiggle" through the gel matrix. Consequently, PFGE has higher resolution of large fragments compared to conventional gel electrophoresis. (Goering, 1993, Tenover *et al.*, 1997, Trindade *et al.*, 2003)

Two variants of PFGE have been used for typing *S. aureus*. They are field-inversion gel electrophoresis (FIGE) and contour-clamped homogeneous electric field (CHEF) gel electrophoresis. (Goering and Duensing, 1990, Wei *et al.*, 1992b, Cookson *et al.*, 1996) The difference between these techniques is the type of apparatus and the arrangements of electrodes. FIGE utilises the conventional chamber with two electrodes and periodically inverts the electric field by 180^{o}. CHEF uses a specially designed chamber where the gel is positioned in the middle of a hexagon formed by three pairs of electrodes. The electric field is first generated from one pair of electrodes for a short period of time, and then consecutively shifted to the second pair and the third pair of electrodes. These sets of electrodes generate a highly uniform electrophoretic field that reorientates the DNA fragments over a 120^{o} angle. FIGE requires a running time of four hours, whereas CHEF is slower and requires a running time up to 24 hours. However, FIGE will not separate fragments larger than 200 kb, and CHEF can separate fragments of megabase sizes. As a result, CHEF has been widely used due to its superior analytical and resolution abilities. (Tenover *et al.*, 1997, Goering, 1993, Trindade *et al.*, 2003) *Sma*I restriction endonuclease has been the most useful for the CHEF typing of *S. aureus*. It digests the *S. aureus* genomic DNA into 15 to 20 fragments that range from 30 to 1500 kb in size. (Ichiyama *et al.*, 1991) The *Sma*I CHEF patterns have been found to be stable during

MRSA outbreaks and after extensive *in vitro* subculturing. (Witte *et al.*, 1994, Blanc *et al.*, 2001b, Bannerman *et al.*, 1995)

The differences between *Sma*I CHEF patterns of individual isolates are derived from genetic events that alter the *Sma*I cleavage sites. For example, a single-base point mutation can cause the loss or gain of a *Sma*I cleavage site, which result in a three-fragment difference between the parental strain and the mutant. A set of criteria has been suggested to interpret *Sma*I CHEF pattern. (See Table 1.6) Under these criteria, isolates with the same *Sma*I CHEF pattern are genetically indistinguishable. Closely related isolates differ by two to three bands because of a single genetic event. Isolates with four to six band differences are possibly related, and result from two genetic events. Unrelated isolates will have more than a seven-band difference as a consequence of more than three genetic events. (Tenover *et al.*, 1995) However, these criteria may be misleading in some instances when more than one *Sma*I cleavage site is involved in the genetic event. (Witte, 2000) In addition, these criteria are only applicable in an outbreak situation during a short time span from one to three months. (Tenover *et al.*, 1995)

PFGE is an excellent typing technique that has high reproducibility and discriminatory power compared with other typing techniques. (van Belkum *et al.*, 2001, Tenover *et al.*, 1994, Bannerman *et al.*, 1995, Kumari *et al.*, 1997, Stranden *et al.*, 2003, Grundmann *et al.*, 2002, Zadoks *et al.*, 2002) In general, the patterns generated by PFGE are not affected by plasmid DNA as the fragments generated from it are too small to affect the pattern. (Struelens *et al.*, 1992) These factors make PFGE an ideal typing technique, and a standard method for many bacterial species including *S. aureus*. (Tenover *et al.*, 1997, Bannerman *et al.*, 1995, Trindade *et al.*, 2003, van Belkum *et al.*, 2001) However, similar to other typing techniques PFGE also carries some drawbacks and limitations. PFGE is a laborious and time-consuming technique, subsequently results are not available for up to four days. The initial cost of setting up PFGE is higher than other typing methods as PFGE requires specialised equipment for its operation. Once the PFGE is in operation, it can be easily applied to a wide range of bacterial species with only minimal modification. (Tenover *et al.*, 1997, van Belkum *et al.*, 2001, Trindade *et al.*, 2003)

Table 1.6 Criteria for interpretation of *Sma*I CHEF patterns

Microbiological interpretation	No. of band differences compared to the outbreak pattern	No. of genetic differences compared with outbreak strain	Epidemiological interpretation
Indistinguishable	0	0	Isolate belongs to the outbreak clonal population
Closely related	2 to 3	1	Isolate probably belongs to the outbreak clonal population
Possibly related	4 to 6	2	Isolate is a subclone of the outbreak clonal population
Unrelated	≥ 7	3	Isolate does not belong to the outbreak population

The comparison of *Sma*I CHEF (PFGE) patterns on multiple gels from a large set of isolates is technically difficult. It is because slight differences in running conditions can alter the distance migrated by each band. The factors that contribute to this variation include the gel volume, concentration of agarose gel, DNA concentration in the gel plug, ionic strength, volume of the running buffer, the running temperature, voltage and the pulse times. (Cookson *et al.*, 1996, van Belkum *et al.*, 1998b, Murchan *et al.*, 2003) Although computer aided analysis with a molecular weight standard or a control strain is used on each gel, mistakes will still occur due to variations in the position of the fragments, normalisation and the matching tolerance algorithms used. (Duck *et al.*, 2003) This problem is especially obvious in the studies of inter-centre comparisons. (van Belkum *et al.*, 1998b, Cookson *et al.*, 1996) This shortcoming of the PFGE technique can be minimised and overcome by standardising PFGE protocols, optimising the computer software settings and inclusion of an internal quality control strain. (Murchan *et al.*, 2003, McDougal *et al.*, 2003, Duck *et al.*, 2003) In a recent study, an ultrasensitive flow cytometer (FCM) has been used for the resolution of large DNA fragments instead of conventional PFGE. It has been shown that the FCM generated patterns are easier to compare than the PFGE generated patterns. (Ferris *et al.*, 2004)

PFGE is not suitable for long-term epidemiological studies that span from years to decades, from country to country and from continent to continent. This is because large restriction fragments are more susceptible to genetic changes (i.e. fast evolution). Although this is advantageous for PFGE by increasing its discriminatory power, it may not reflect long-term genetic evolution. (Witte *et al.*, 1994, Blanc *et al.*, 2002) Despite these limitations, PFGE is still considered the best technique for characterising and defining outbreaks of *S. aureus*. (Weller, 2000, Tenover *et al.*, 1997, Tenover *et al.*, 1994, Trindade *et al.*, 2003, Witte, 2000)

1.4.2.2.2 Southern hybridisation

The RFLPs of chromosomal DNA generated by high-frequency restriction endonucleases have been successfully utilised for distinguishing epidemic *S. aureus* from sporadic isolates. (Cookson and Phillips, 1988, Jordens and Hall, 1988) However, the generated profiles are hard to interpret as it consists of hundreds of indistinct and overlapping small fragments. Subsequently, Southern hybridisation has been introduced to resolve these complex profiles. In this technique, the DNA fragments are transferred onto a membrane and then hybridised with a labelled DNA probe for specific sequences. The DNA probe binds to the complementary sequences on the membrane and generates profiles that have fewer bands and are easier to interpret. (Weller, 2000, Trindade *et al.*, 2003, Tenover *et al.*, 1997)

1.4.2.2.2.1 Ribotyping

The most common Southern hybridisation technique used is ribotyping. The rRNA operon carries the 16S, 23S and 5S rRNA genes that are separated by spacer DNA sequences. (Rouch *et al.*, 1987) *S. aureus* can carry five to eight copies of the rRNA operon and they are relatively conserved. (Baba *et al.*, 2002, van Belkum *et al.*, 1994) Ribotyping generates RFLPs using DNA probes specific for 16S and 23S rRNA genes. (Hiramatsu, 1995, Yoshida *et al.*, 1997, Melter *et al.*, 1999) It is based on the variations in the location of restriction sites that are located within the rRNA gene or in the spacer DNA between the rRNA genes. The rRNA operons also vary in size due to different lengths of the spacer DNA. (Melter *et al.*, 1999, Trindade *et al.*, 2003, Witte, 2000, Tenover *et al.*, 1997) Ribotyping can be applied to all isolates

and the profiles are reproducible and stable. The discriminatory power of this method is lower than PFGE but higher than the phenotypic methods. (Tenover *et al.*, 1994, Prevost *et al.*, 1992, Blanc *et al.*, 1994) Despite these disadvantages, ribotyping has been successfully used for discriminating MRSA isolates from different continents within a long time span. However, it is not as useful when used in studies with short time spans due to its lower discriminatory power. (Hiramatsu, 1995, Yoshida *et al.*, 1997) Nevertheless, ribotyping is still a useful epidemiological technique when PFGE is not available. (Melter *et al.*, 1999) Automated ribotyping system, Riboprinter®, was the primary molecular typing method for SENTRY a large-scale, global antimicrobial-surveillance program. In the same project, it was also used as a complementary method to PFGE. (Deshpande *et al.*, 2004) Although it is less discriminatory than PFGE its ease of performance, high level of sensitivity and rapidity make it a better method for large-scale screening than PFGE. (Hollis *et al.*, 1999, Deshpande *et al.*, 2004, Ito *et al.*, 2003b) Automated ribotyping has been found to have good reproducibility and repeatability, which makes the results suitable for intra- and inter-laboratory comparison. (Lefresne *et al.*, 2004)

1.4.2.2.2.2 Insertion sequence typing

Insertion sequences (IS) have been utilised in Southern hybridisation for the epidemiology of *S. aureus*. The IS used include IS*431/257*, IS*256* and IS*1181*. (Tenover *et al.*, 1994, Yoshida *et al.*, 1997, Wei and Grubb, 1992, Morvan *et al.*, 1997, Symms *et al.*, 1998) These IS-based typing techniques are based on variations in the position and the copy number of specific IS sequences in the chromosome of different strains. (Tenover *et al.*, 1994, Weller, 2000) IS typing possesses several limitation due to the nature of the target markers. In IS typing, some isolates often are non-typable because *S. aureus* isolates do not carry the insertion sequence. (Weller, 2000, Wei *et al.*, 1992a, Symms *et al.*, 1998) Also IS hybridisation patterns can be interfered with by ISs carried on plasmids as this complicates the interpretation of the result. This is because the variations resulting from the interference of plasmid elements do not reflect strain differences. (Tenover *et al.*, 1994) In addition, IS typing is not suitable for typing antibiotic-susceptible isolates. The majority of ISs are associated with antimicrobial-resistance determinants and elements. Antimicrobial-susceptible isolates often do not carry any IS, or only carry

a small number of IS, which makes them not suitable for IS typing because of the low discriminatory power. (Tenover *et al.*, 1994, Weller, 2000) Nevertheless, the hybridisation patterns generated by the IS probes are stable and reproducible. (Symms *et al.*, 1998, Tenover *et al.*, 1994, Yoshida *et al.*, 1997, Morvan *et al.*, 1997) However, their discriminatory power varies and depends on which IS probe is used. The discriminatory power of IS*431/257* in typing MRSA is better than phage typing and ribotyping. However, this IS probe is ineffective in typing methicillin-susceptible *S. aureus* due to the lack of IS*431/257*-associated resistance elements. (Tenover *et al.*, 1994, Yoshida *et al.*, 1997) IS*256* typing is very useful in discriminating gentamicin-resistant isolates due to its association with Tn*4001*. (Wei *et al.*, 1992a, Monzon-Moreno *et al.*, 1991, Soares *et al.*, 2001) IS*1181* is a unique staphylococcal IS that is not directly associated with resistance genes. However, IS*1181* typing had poor discrimination power in typing of some British epidemic MRSA. (Symms *et al.*, 1998) IS typing using IS*431/257* and IS*256* was found to be more discriminating than PFGE in typing MRSA. (Yoshida *et al.*, 1997, Wei *et al.*, 1992a, Tenover *et al.*, 1994, Morvan *et al.*, 1997) However, IS hybridisation patterns often do not correlate with PFGE patterns. This is because ISs can be lost or acquired and can accumulate in isolates over the course of clonal dissemination. As a result, IS typing is suitable for further discriminating or sub-typing isolates that belong to the same outbreak population. It has been suggested that IS typing can be used with PFGE to increase the discriminatory power in epidemiological studies. (Yoshida *et al.*, 1997, Morvan *et al.*, 1997)

1.4.2.2.2.3 *mecA* and Tn*554* typing

The *mecA* and Tn*554* Southern hybridisation method is designed specifically for the typing of MRSA. In this technique, the *mecA* and Tn*554* DNA probes are separately hybridised with *Cla*I digested chromosomal DNA. The hybridisation profiles obtained with the two probes can be used to discriminate MRSA isolates. (Kreiswirth *et al.*, 1993)

The *mecA* gene is a unique feature of all MRSA and a single copy is found in all isolates. The *mecA* gene carries one *Cla*I restriction cleavage site, hence *Cla*I-*mecA* hybridisation patterns consist of only two hybridisation bands. (Matsuhashi *et al.*,

1990, Kreiswirth *et al.*, 1993) Since *mecA* is located in the SCC*mec*, the polymorphisms of *Cla*I-*mecA* hybridisation pattern also represents the genetic variations of the region surrounding the *mecA* gene in SCC*mec*. The smaller band of the *Cla*I-*mecA* pattern, ranges from 1.8kb to 2.4kb. This band represents the upstream region of the *mecA* gene that contains its regulatory regions (i.e. the *mec* complex). (Oliveira *et al.*, 2000, Kreiswirth *et al.*, 1993, Lim *et al.*, 2002) The bigger band in the *Cla*I-*mecA* hybridisation pattern is approximately 3.5kb to 20kb in size. This band represents the downstream area of the *mecA* gene, which includes the J3 region of SCC*mec*. This downstream region consists of the *dru* units, IS*431*/*257*, and the downstream constant segment adjacent to *orfX* and can have small-integrated plasmids, the *mer* operon and IS*256*. The variations in this band are due to the polymorphism of the J3 region in different SCC*mec* allotypes. (Oliveira *et al.*, 2000, Ito *et al.*, 2003a, Kreiswirth *et al.*, 1993) Up to 21 *mecA* polymorphisms have been detected in hospital acquired MRSA from different geographical regions. (Oliveira *et al.*, 2000, Oliveira *et al.*, 2001b, Kreiswirth *et al.*, 1993, Dominguez *et al.*, 1994, Leski *et al.*, 1998, Oliveira *et al.*, 1998, Aires de Sousa and de Lencastre, 2003)

Tn*554* is a site-specific transposon that carries MLS_B and spectinomycin resistance. Although some isolates do not carry Tn*554*, it is commonly found in hospital acquired MRSA and often in multiple copies. (Murphy, 1990, Baba *et al.*, 2002, Kreiswirth *et al.*, 1993) Similar to *mecA*, Tn*554* carries only one *Cla*I restriction site. *Cla*I-Tn*554* hybridisation patterns with multiple bands indicate either the presence of multiple Tn*554* copies or genetic rearrangement within Tn*554*. At least 30 *Cla*I-Tn*554* polymorphisms have been detected in MRSA isolates. Studies have demonstrated that these *Cla*I-Tn*554* patterns are stable over approximately 1000 generations and five days of serial passage in drug-free medium. (Kreiswirth *et al.*, 1993, Sanches *et al.*, 1998)

The *Cla*I-*mecA* and *Cla*I-Tn*554* hybridisations are not used individually due to the lack of their discriminatory power. However, even the combination of the two techniques still lacks adequate discriminatory power. In addition, these probes have limited typability because *mecA* only exists in MRSA and a small minority of isolates do not carry Tn*554*. (Kreiswirth *et al.*, 1993, Tenover *et al.*, 1994, Weller,

2000, Trindade *et al.*, 2003) *mecA*-Tn*554* hybridisation is most useful when utilised in combination with PFGE. This combination of three techniques has a high discriminatory power. It has been successfully use to identify several epidemic and pandemic hospital acquired MRSA clones around the world. (Dominguez *et al.*, 1994, Aires de Sousa *et al.*, 1998, Aires de Sousa and de Lencastre, 2004, Oliveira *et al.*, 2002) This typing scheme is very time consuming and this is its major disadvantage. (Weller, 2000)

1.4.2.2.2.4 Binary typing

Binary typing is a technique based on the hybridisation patterns with 15 DNA probes. (van Leeuwen *et al.*, 1999) These DNA probes are the amplicons generated from randomly amplified polymorphic DNA (RAPD) analysis of 376 *S. aureus* isolates. They were chosen for their ability to differentiate the strains. The 15 DNA probes were immobilised on a nylon strip and hybridised with labelled genomic DNA of *S. aureus* isolates. A binary code was given to the isolates tested based on the presence or absence of hybridisation with individual probes. The results generated by this method were found to be stable and reproducible after 50 serial passages of the isolates in drug-free medium. (van Leeuwen *et al.*, 1999, van Leeuwen *et al.*, 2001) The discriminatory power of the binary typing was also found to be greater than PFGE, *mecA*:Tn*554* probing and RAPD. (van Leeuwen *et al.*, 1999) However, the current set of probes is only useful in isolates originating from humans. Studies of *S. aureus* of bovine origin showed that binary typing is too discriminating and did not correlate well with other epidemiological data. This is because isolates of bovine origin and human isolates belong to two different *S. aureus* populations. (Zadoks *et al.*, 2002, Gilot and van Leeuwen, 2004)

As mentioned previously electrophoretic fingerprinting techniques such as PFGE are not reliable for the inter- and intra-centre exchange of strain information. They generate complex band patterns that are susceptible to variations in electrophoresis conditions. Consequently, the exchange of data between centres faces the limitation related to the subjective analysis of gel images, standardisation and reproducibility. (Murchan *et al.*, 2003, van Belkum *et al.*, 1998b, Cookson *et al.*, 1996, Fry *et al.*, 2000) Probe-based binary typing generates simple binary codes, which allow the

efficient exchange of strain information between centres and the construction of an international database, and this has been demonstrated in inter-centre *S. aureus* studies. (van Leeuwen *et al.*, 2002) Binary typing is a time consuming, laborious and skill demanding technique. These factors are major disadvantages of binary typing. Some researchers have suggested that these disadvantages can be minimised by the adaptation of techniques such as immunosorbent assay-like techniques, DNA microarray and an automated system. (van Leeuwen *et al.*, 2002, van Leeuwen *et al.*, 1999, van Leeuwen *et al.*, 2001, van Leeuwen *et al.*, 1998)

1.4.2.3 Polymerase chain reaction (PCR) based techniques

Typing techniques like PFGE and Southern hybridisation are both time consuming and technically demanding. Unlike these techniques, PCR typing techniques are not only fast and less skill demanding but they are also cheaper to set up and run. PCR can amplify a specific stretch of DNA (i.e. target DNA) to millions of copies from small amount of DNA template. The polymorphism generated by PCR is based on the genetic events that occur within the target regions. Although these PCR techniques are relatively sensitive, their discriminatory power and reproducibility vary from good to poor depending on the primers and target DNA used. PCR techniques can be roughly put into five groups: Arbitrarily primed PCR or randomly amplified polymorphic DNA (AP-PCR/RAPD), repetitive element sequence-based PCR (Rep-PCR), PCR of intergenic regions and amplified-fragment length polymorphism (AFLP) (Weller, 2000, Trindade *et al.*, 2003, Witte, 2000, Olive and Bean, 1999)

1.4.2.3.1 AP-PCR/RAPD

AP-PCR or RAPD discriminates *S. aureus* strains by the size and number of amplicons generated. This technique uses single, short (8 to 10 nt), arbitrary, random primers under low-stringency conditions. (Witte, 2000, Power, 1996, Olive and Bean, 1999)

The discriminatory power of AP-PCR/RAPD varies with different studies. This is due to different kinds and number of primers used in the studies. (Power, 1996, Tambic *et al.*, 1997) Nevertheless, the discriminatory power of AP-PCR/RAPD is

less than PFGE and multilocus sequence typing (MLST). (Saulnier *et al.*, 1993, Grundmann *et al.*, 2002) Its discriminatory power is better than bacteriophage typing and ribotyping. (Struelens *et al.*, 1993, van Belkum *et al.*, 1995, Tambic *et al.*, 1997, Grundmann *et al.*, 2002) Studies revealed that AP-PCR/RAPD was able to differentiate outbreak strains and the results obtained correlated well with epidemiological data. (van Belkum *et al.*, 1993, Struelens *et al.*, 1993, van Belkum *et al.*, 1995, Tambic *et al.*, 1997, Blanc *et al.*, 2001a) A multiprimer RAPD with 10 primers was successfully used to determine the phylogenetic relationship between MRSA isolates. (Blanc *et al.*, 2000, Blanc *et al.*, 2001a) It has been suggested that multiprimer RAPD is a better technique than PFGE for long-term MRSA epidemiological studies. Unlike the short fragments from RAPD, the large fragments of PFGE are more susceptible to genetic events that alter the typing pattern. Hence, PFGE is too discriminatory to be utilised for long-term studies. (Blanc *et al.*, 2002)

The interlaboratory reproducibility of AP-PCR/RAPD results was found to be poor in a multicentre study. (van Belkum *et al.*, 1995) Since this technique is performed under low-stringency conditions, the patterns obtained are susceptible to variations in PCR procedures. Variations such as the quality of the DNA template and the concentration of primers are known to affect the reproducibility. Personnel with different degrees of familiarity with the technique were found to obtain different results with the same primers and DNA templates. (Power, 1996) Standardisation of AP-PCR/RAPD can be difficult as many centres have their own methods for preparing DNA templates and use different equipment. (van Belkum *et al.*, 1995)

AP-PCR/RAPD is one of the cheapest typing methods to set up. (Olive and Bean, 1999) It is quick and technically feasible for many laboratories, even when multiple primers are utilised. This technique is able to type all strains and has relatively good discriminatory power. The other advantage is its flexibility as AP-PCR/RAPD can be readily applied to other microorganisms. The major drawback of this technique is its poor reproducibility, which limits its use in multicentre studies. (Power, 1996, Olive and Bean, 1999, van Belkum *et al.*, 2001, van Belkum *et al.*, 1995)

1.4.2.3.2 Repetitive element sequence-based PCR

The repetitive element sequence-based PCR (Rep-PCR) targets the noncoding repetitive elements that are commonly found in prokaryotic genomes. The molecular profiles obtained reflect the polymorphism of the repetitive elements in their length and location on the genome. (van Belkum *et al.*, 1998a, Trindade *et al.*, 2003, Weller, 2000) Unlike AP-PCR/RAPD, the Rep-PCR is carried out under high stringency conditions and therefore has good reproducibility. Its discriminatory power, although good, is not as good as PFGE. (Trindade *et al.*, 2003, van der Zee *et al.*, 1999) The repetitive elements targeted for *S. aureus* genotyping include the RepMP3-like sequences, the 3' end region of the coagulase gene, the X region of the *spaA* gene, and the intergenic STAR element. (Del Vecchio *et al.*, 1995, Goh *et al.*, 1992, Frenay *et al.*, 1996, Stranden *et al.*, 2003)

1.4.2.3.2.1 RepMP3 PCR

The Rep-PCR primer RW3A is derived from the RepMP3 repetitive sequence from *Mycoplasma pneumoniae.* The molecular profiles obtained are stable after consecutive subculturing and have good reproducibility. Studies have shown that it is as discriminatory as PFGE and RAPD, and able to differentiate epidemic clones. The profiles obtained can be digitally stored, when a fluorescein-labelled primer and polyacrylamide electrophoresis are used. (Del Vecchio *et al.*, 1995, van der Zee *et al.*, 1999) However, a multicentre study found that RepMP3 PCR had less discriminatory power than PFGE and its reproducibility was insufficient for interlaboratory data exchange. (Deplano *et al.*, 2000a) Nevertheless, RepMP3 PCR is still a useful technique for MRSA nosocomial outbreaks. It is cheap to run and quick and offers reasonable discriminatory power and reproducibility. (Trindade *et al.*, 2003, Del Vecchio *et al.*, 1995, van der Zee *et al.*, 1999)

1.4.2.3.2.2 Coagulase-gene typing

The 3' region of the coagulase gene consist of a series of 81 bp tandem repeats which vary in number between *S. aureus* isolates. To demonstrate this polymorphism PCR amplification of the 3' region of the coagulase gene is followed by *Alu*I digestion.

The digestion generates up to four fragments of various lengths depending on the number of the restriction sites present. (Goh *et al.*, 1992, Kobayashi *et al.*, 1995, Nada *et al.*, 1996) Although the sequences of these repeats are relatively conserved, point mutations occur in individual repeats that result in the presence or absence of an *Alu*I restriction site. (Kaida *et al.*, 1987, Kaida *et al.*, 1989, El-Adhami and Stewart, 1998, Schwarzkopf and Karch, 1994)

The patterns generated are stable after continuous subculturing and the results have been found to correlate well with MLEE and PFGE. (Kobayashi *et al.*, 1995, Nada *et al.*, 1996, Goh *et al.*, 1992, Hoefnagels-Schuermans *et al.*, 1997) Coagulase gene typing has been shown to identify a major clone in an outbreak. (Goh *et al.*, 1992) However, coagulase-gene typing only generates a limited number of patterns. In a number of studies, epidemiologically unrelated isolates have been found to share the same pattern with outbreak strains. (Schwarzkopf and Karch, 1994, Nada *et al.*, 1996, Hookey *et al.*, 1998, Chiou *et al.*, 2000) Coagulase gene typing is not suitable for use as a sole typing technique due to its low discriminatory power. (Schwarzkopf and Karch, 1994, Nada *et al.*, 1996, Chiou *et al.*, 2000, Quelle *et al.*, 2003) Nevertheless, it was shown to be able to distinguish three major epidemic MRSA strains in England and Wales. (Hookey *et al.*, 1998)

Coagulase-gene typing is a fast and cost effective method that is best used with other typing methods. Another advantage of this technique is that all the bands generated are multiples of the 81 bp tandem repeat. This is because the polymorphism is based on the presence or absence of the *Alu*I site within the repeat. As a result, it has been possible to devise a numerical system for the coagulase gene type, and this facilitates interlaboratory data exchange and enables results to be archived. (Hoefnagels-Schuermans *et al.*, 1997, Weller, 2000)

1.4.2.3.2.3 Protein A PCR

The *spaA* gene encodes for the surface protein, Protein A, and contains a polymorphic region known as the X region. The X region contains from three to 15 24 bp tandem repeats. (Uhlen *et al.*, 1984, Hoefnagels-Schuermans *et al.*, 1997) The X region is amplified by PCR and the amplicon is digested with the restriction

enzyme *Rsa*I. This digestion generates three fragments from the PCR amplicon. Two of the fragments do not contain the repeats and are constant in size. The third, and the largest fragment, carries the repeats and varies in size depending on the number of repeats it carries. The pattern generated by the Protein A PCR is reproducible and is stable after repetitive subculturing. (Frenay *et al.*, 1996, Frenay *et al.*, 1994) However, the results obtained do not correlate well with the results generated by other typing methods. The discriminatory power of this PCR method is low as it only generates a limited number of types. (Uhlen *et al.*, 1984, Frenay *et al.*, 1994)

1.4.2.3.2.4 STAR elements PCR

The STAR (*S. aureus* repeats) element is a GC rich intergenic repetitive element. Multiple copies of STAR elements have been reported in *S. aureus*. These elements also exhibit sequence diversity at the same locus. (Cramton *et al.*, 2000) In this technique the intergenic STAR element of the *uvrA* and *hprK* genes is amplified and the product digested with *Alu*I and *Tru*9I restriction enzymes. The results obtained are reproducible and correlate well with other typing methods including PFGE, *mecA*-Tn*554* hybridisation, inter-IS*256* PCR, RepMP3 PCR and coagulase-gene typing. The discriminatory power of this method is lower, but still comparable, to PFGE. It was found to be as discriminatory as the inter-IS*256* and RepMP3 PCR methods. (Quelle *et al.*, 2003)

1.4.2.3.2.5 DiversiLab microbial typing system

The DiversiLab system is a recently developed typing method based on Rep-PCR. In this system, the PCR products are separated by the microfluidic DNA chip rather than conventional agarose-gel electrophoresis. The amplicons are detected by a scanner and their sizes digitally stored. This procedure eliminates the variations caused by conventional gel electrophoresis and allows a greater degree of reproducibility. The sizes of the amplicons are digitally recorded as a form of band patterns that allow data archiving and interlaboratory data comparison. It has been shown to have an excellent inter- and intra-laboratory reproducibility in typing *S. aureus* isolates. The results generated also correlate well with the results of PFGE.

It is commercially available in a kit form and is less time consuming than PFGE. (Healy *et al.*, 2005, Shutt *et al.*, 2005)

1.4.2.3.3 PCR of the intragenic regions

PCR had been used to reveal the polymorphisms in the intergenic regions flanked by known sequences. The intergenic regions between the copies of IS*256*, 16S and 23S rRNA genes and the attachment site and ribosomal binding site of Tn*916* have been utilised for *S. aureus* genotyping.

1.4.2.3.3.1 Inter-IS*256* PCR

IS*256* occurs in multiple copies in the *S. aureus* genome. It is found either independently or as a part of the Tn*4001* gentamicin-resistance transposon. (Wei *et al.*, 1992a, Lyon *et al.*, 1987b) Inter-IS*256* PCR discriminates isolates by the length of the polymorphism of the region between IS*256* or IS*256*-like sequences. The band patterns generated are highly reproducible and stable over a period of nearly one year. (Deplano *et al.*, 1997) The discriminatory power of this method is good and better than RepMP3, 16S-23S rRNA PCR and coagulase-gene typing. (Deplano *et al.*, 2000a, Quelle *et al.*, 2003) It is generally less discriminatory than PFGE but in some studies it has been found to be as discriminatory as PFGE. (Wei and Chiou, 2002, Quelle *et al.*, 2003, Deplano *et al.*, 2000a, Deplano *et al.*, 1997) It is particularly useful in typing gentamicin-resistant strains due to the presence of Tn*4001*. (Witte, 2000) In multilaboratory studies, the inter-IS*256* PCR, like other PCR methods, did not generate sufficient reproducibility for interlaboratory data exchange. (Deplano *et al.*, 2000a) Inter-IS*256* PCR has been suggested to be useful as a high-throughput screening system. (Deplano *et al.*, 1997)

1.4.2.3.3.2 rRNA spacer PCR

Many bacteria carry multiple copies of the rRNA operon and *S. aureus* has been found to carry multiple copies of the rRNA operon in its genome. The rRNA operon consists of three, well conserved, ribosomal genes, the 16S, 23S and 5S rRNA genes. In the operons these ribosomal genes are separated by spacer regions that differ

significantly in length and sequences. (Wada *et al.*, 1993, Gürtler, 1999) The length and sequence polymorphism is due to the variation in the number and type of tRNA genes present within the spacer. It has been suggested that this variation is probably due to the intrachromosomal homogeneous recombination between copies of the rRNA operons. (Gürtler *et al.*, 2001) The rRNA spacer PCR amplifies the intergenic spacer between the 16S and 23S rRNA genes, and the length polymorphisms obtained are used for strain discrimination. The rRNA spacer PCR was shown to have a good reproducibility and the resulting band patterns were stable. It has been successfully used for identifying major clones in MRSA outbreaks. (Gürtler and Barrie, 1995, Kumari *et al.*, 1997) However, the rRNA spacer PCR has a much lower discriminatory power than PFGE. In several studies, strains with different PFGE patterns were found to have the same or related rRNA spacer PCR type. The unrelated MRSA strains also clustered together with the outbreak MRSA clone by this technique. (Annemüller *et al.*, 1999, Gürtler and Barrie, 1995, Kumari *et al.*, 1997, Oliveira and Ramos, 2002, Deplano *et al.*, 2000a) In a multicentre MRSA study, the rRNA spacer PCR was found to have less discriminatory power than the inter-IS*256* PCR. (Deplano *et al.*, 2000a) In the same study, the rRNA spacer PCR was found to lack interlaboratory reproducibility and its results did not correlate well with PFGE results. (Deplano *et al.*, 2000a) The rRNA spacer PCR is a technique more suitable for typing MSSA, as it generates a diversity of patterns within MSSA strains. Although it might only have limited value for MRSA genotyping, it is still a good alternative for rapid MRSA screening. (Kumari *et al.*, 1997, Oliveira and Ramos, 2002)

1.4.2.3.3.3 The target 916-Shine-Dalgarno PCR

Several Tn*916* target sites are located in the *S. aureus* genome in different locations. The DNA sequences neighbouring the ribosomal genes are found in nearly all open reading frames and appear to be well conserved. (Novick, 1990) In the right orientation PCR primers designed for Tn*916* and the DNA sequence neighbouring the ribosomal binding site can demonstrate that length polymorphism exists between them. This PCR is known as the target 916-Shine-Dalgarno PCR (or tar 916-shinda PCR). The patterns generated are reproducible and stable. It has been successfully

used to discriminate major MRSA clones and has identified epidemic MRSA from German hospitals. (Witte, 2000, Cuny and Witte, 1996)

1.4.2.3.4 Amplified-fragment length polymorphism (AFLP)

The AFLP typing technique is based on the selective amplification of DNA fragments generated by restriction enzyme digestion of genomic DNA. In this technique, the genomic DNA is digested with two different restriction enzymes, one with an average cutting frequency and the other with a high frequency. The DNA fragments generated are ligated with oligonucleotide adapters that have single stranded ends and are complementary to those on the fragments generated by the restriction enzymes. The adapters are also designed so that the initial restriction site is not restored after the ligation. The fragments ligated with adapters are subjected to selective PCR with adapter-specific primers under high stringency. Only the fragments ligated with adapters are amplified. The addition of one to three nucleotides on the 3' end of the primers that extend into the fragment can greatly increase the selectivity of the PCR. The amplicons are separated by polyacrylamide-gel electrophoresis and the pattern detected by an automatic DNA sequencer. The patterns obtained generally contain 50 to 200 bands. The adapter-specific primer for the average-frequency-cutting restriction site is either radioactively or fluorescent labelled. The AFLP with the fluorescent labelled primer is also known as fluorescent AFLP. (Arnold, 2003, Vos *et al.*, 1995, Savelkoul *et al.*, 1999)

The discriminatory power of AFLP is largely dependent on the restriction enzymes used and the selectivity of the primers. Studies on AFLP typing of *S. aureus* have shown that its discriminatory power is either equal or greater than PFGE. (Hookey *et al.*, 1999b, Grady *et al.*, 1999) However, it was found to have low discriminatory power on typing MRSA isolates with limited strain diversity from a single hospital. (Ip *et al.*, 2003) AFLP has been successfully used to identify major epidemic MRSA clones and the populations of *S. aureus* carried by humans. (Ip *et al.*, 2003, Hookey *et al.*, 1999b, Melles *et al.*, 2004) AFLP generated patterns are stable and highly reproducible. The patterns can be digitally stored and are suitable for data comparison and archiving due to their high reproducibility. (Olive and Bean, 1999, Savelkoul *et al.*, 1999, Hookey *et al.*, 1999b) Although the initial set-up cost of

AFLP is higher than PFGE, the running cost is similar to PFGE. AFLP is also faster and less technically demanding than PFGE. (Olive and Bean, 1999)

1.4.2.3.5 SCC*mec* allotyping

SCC*mec* allotyping differentiates the SCC*mec* allotypes determining their *mec* complex and *ccr* gene complex. The identification of these two gene complexes is done by amplification of these elements with type specific primers. The different gene complexes carried by different SCC*mec* allotypes is listed in Table 1.5. (Ito *et al.*, 2001, Enright *et al.*, 2002) Another technique for SCC*mec* allotyping is done by multiplex PCR. This technique identifies the allotype by amplifying genetic elements other than the *mec* and *ccr* complexes that are carried by specific SCC*mec* allotypes. (Oliveira and Lencastre Hd, 2002) SCC*mec* allotyping is generally used in conjunction with Multilocus sequence typing for evolutionary studies of MRSA. (Robinson and Enright, 2004b, Enright *et al.*, 2002)

1.4.2.4 Typing by DNA sequencing

DNA sequencing is the most recent development in *S. aureus* genotyping. DNA sequencing-based typing techniques differentiate isolates by sequence variations within either a single or in multiple loci. DNA sequencing, unlike other techniques, offers trouble-free data sharing and storage. The typing profiles generated allow a less subjective and unambiguous interpretation, as well as excellent reproducibility. However, the cost of DNA sequencing is higher than other methods and its discriminatory power is comparable but less than PFGE. (Olive and Bean, 1999, Trindade *et al.*, 2003, van Belkum *et al.*, 2001, Shopsin *et al.*, 1999) A number of genes have been selected for typing *S .aureus*. They include the repetitive region of the coagulase gene, the X region of the *spaA* gene, housekeeping genes, surface-protein genes and genes encoding for pathogenicity. (Shopsin *et al.*, 2000, Frenay *et al.*, 1994, Enright *et al.*, 2000, Robinson and Enright, 2003, Gomes *et al.*, 2005) *SpaA* sequencing, targeting the X region of the *spaA* gene, and multilocus sequence typing (MLST) of seven “house-keeping” genes, are the most widely used DNA sequencing technique used for *S. aureus* typing.

1.4.2.4.1 *spaA* sequencing

spaA sequencing targets the X region of the *spaA* gene containing a number of short 24 bp repeat units. In different *S. aureus,* isolates these repeats often have different DNA sequences and the numbers of the unit vary from three to fifteen. (Uhlen *et al.*, 1984, Frenay *et al.*, 1996) The polymorphism is largely due to the duplication and deletion of the repeat units and point mutation. (Shopsin *et al.*, 1999, Kahl *et al.*, 2005, Koreen *et al.*, 2004) In this method, the X region is amplified with primers located in the well-conserved areas flanking this region. The amplicons obtained are sequenced and analysed for the presence of different 24 bp repeat sequences and number of repeat units. Every unique 24 bp repeat sequence is given a code and so far, 38 different repeat sequences have been reported. The *spaA* type of an isolate represents the organization of the X region in terms of the number of repeats and the sequence of each repeat unit. (Frenay *et al.*, 1996, Koreen *et al.*, 2004) The *spaA* type of isolates from a chronic MRSA carrier was found to be stable over a period of five years. (Shopsin *et al.*, 1999) It has also been found to be stable and reproducible during sequential subculturing. (Shopsin *et al.*, 1999, Frenay *et al.*, 1996, Oliveira *et al.*, 2001a)

In several studies, the results generated by *spaA* sequencing correlated well with the results of *mecA*-Tn*554* hybridisation, PFGE and a DNA array of the *S. aureus* genome. Its discriminatory power is comparable but less than PFGE. (Shopsin *et al.*, 1999, Tang *et al.*, 2000, Oliveira *et al.*, 2001a, Koreen *et al.*, 2004, Aires de Sousa *et al.*, 2003a, Harmsen *et al.*, 2003) The *spaA* sequencing can detect clonal grouping of isolates, whereas some other techniques cannot do this when used by themselves. (Shopsin *et al.*, 1999) The repeat units have a slow point mutation rate but faster changes occur in the number of repeats due to duplication and deletion. This enables *spa* typing to have the desired discriminatory power for strain differentiation. At the same time it allows the *spaA* type to detect clonal lineage as the sequences of the repeat units mutate slowly. (Kahl *et al.*, 2005, Koreen *et al.*, 2004) Since only one area of the genomic DNA was target, the *spa* typing does have limited discriminatory and resolution power. (Aires de Sousa and de Lencastre, 2004) The major pandemic MRSA clones carry distinct motifs within their *spaA* type, which correlates well with the clonal groups determined by *mecA*-Tn*554*-PFGE

and MLST. However, the *spaA* sequencing cannot efficiently discriminate individual strains within these major pandemic MRSA clones. (Oliveira *et al.*, 2001b, Tang *et al.*, 2000)

Recently Ridom StaphType software was used for rapid repeat sequence determination, data storage and management. This software also allows internet-based assignment of new *spaA* types and interlaboratory comparison. (Harmsen *et al.*, 2003) *SpaA* sequencing offers reproducible results that are easily interpreted and can be shared between laboratories. This method also has adequate discriminatory power for outbreak investigations. However, *spaA* sequencing is more expensive to setup and run when compared to other gel electrophoresis-based techniques. (Oliveira *et al.*, 2001a, Shopsin *et al.*, 1999, Olive and Bean, 1999, Koreen *et al.*, 2004) In some studies the *spaA* types obtained did not correspond to the PFGE type and other epidemiological data. (Kahl *et al.*, 2005, Tang *et al.*, 2000) *SpaA* sequencing has to be complemented with other techniques in long-term studies as epidemic MRSA clones show a high degree of variability after circulating for a long period of time. (Oliveira *et al.*, 2001a) Modification of the primers is also required for some community-acquired and sporadic MRSA isolates as they may have different flanking sequences. (O'Brien *et al.*, 2004, Aires de Sousa and de Lencastre, 2003) Overall, *spaA* sequencing is a useful screening method that offers fast, unambiguous and interlaboratory comparable results. (Shopsin *et al.*, 1999, Tang *et al.*, 2000)

1.4.2.4.2 Multilocus sequence typing (MLST)

MLST is a technique developed from the MLEE methods. It detects sequence polymorphisms within the *S. aureus* housekeeping genes. (Maiden *et al.*, 1998, Enright *et al.*, 2000) These housekeeping genes are thought to be the core of the genome and are highly conserved. The variations found among these genes are mostly synonymous and neutral. Since these genes accumulate variations in a slow manner, they are considered to be reliable indicators of evolutionary history. (Feil *et al.*, 2003, Feil, 2003) Seven housekeeping genes have been selected for a MLST typing scheme for *S. aureus* because they have the greatest number of alleles. The genes are carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase

(*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), acetyl coenzyme A and acetyltransferase (*yqiL*). (Enright *et al.*, 2000)

Internal fragments of these seven loci, between 402 to 516 bp, are amplified and sequenced. The sequences obtained are compared with the known alleles via an internet-based MLST database. (Enright *et al.*, 2000) All the known alleles of the seven loci are numbered consecutively. The numbering does not reflect their genetic relationship. A distinct allele is assigned a number, even for a single nucleotide difference with the known alleles. The allele profile or the sequence type (ST) of an isolate consists of seven numbers that represent the allele types of the seven loci belonging to the isolate. For example, the ST for isolates of the EMRSA-1 clone is ST239, which is represented by the allele profile of 2-3-1-1-4-4-3 in the order of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* genes. (Enright *et al.*, 2000, Enright *et al.*, 2002, Robinson and Enright, 2004b)

The relationship between the STs is determined by the Based Upon Related Sequence Types (BURST) algorithm. This algorithm clusters STs into groups called clonal complexes. The STs in the same clonal complex have at least five alleles of the seven loci identical to each other. In the other words, these STs are descended from a single ancestral ST and are single or double loci variants of each other. In the early clonal expansion of an ancestral ST, genetic diversification occurs and results in new STs that are only one locus different from the ancestral ST. Thus, STs that have the highest number of single locus variants in a clonal complex, are designated as the putative ancestral ST. The clonal complexes are named according to their putative ancestral STs. For example, CC5 is named after its ancestral ST, ST5. Both ST and clonal complex determination are available from the MLST internet website, http://www.mlst.net/. (Feil *et al.*, 2004, Feil *et al.*, 2003, Enright *et al.*, 2000, Enright *et al.*, 2002, Robinson and Enright, 2004b)

MLST is a highly discriminatory typing technique. Each of the seven loci has an average of 42 alleles, which means that MLST can resolve more than 200 billion STs. (Enright *et al.*, 2002) It is highly unlikely that genetically unrelated strains will have the same ST. (Enright *et al.*, 2000, Enright *et al.*, 2002) The results generated

by MLST correlate well with PFGEs results. (Enright *et al.*, 2000, Peacock *et al.*, 2002, van Leeuwen *et al.*, 2003, Grundmann *et al.*, 2002) Most isolates with the same, or related STs, have PFGE patterns that are either identical or differ by no more than four bands. (Enright *et al.*, 2000, Peacock *et al.*, 2002) Under the Tenover *et al*'s criteria for PFGE pattern interpretation these isolates are genetically related. (Tenover *et al.*, 1995) The isolates with unrelated STs also have unrelated PFGE patterns and have more than six band differences. (Enright *et al.*, 2000, Peacock *et al.*, 2002) In a comparative short-term study, closely related PFGE patterns with less than three band differences, were grouped together as a PFGE type and compared with the MLST type. The PFGE type and MLST were found to have very similar discriminatory power and performed equally well as a typing method. (Peacock *et al.*, 2002) When PFGE patterns are used individually, it is slightly more discriminating than MLST. (Peacock *et al.*, 2002, Enright *et al.*, 2000, Grundmann *et al.*, 2002) This is because the genetic diversity detected by PFGE has a faster evolution time than the seven loci of the MLST. (Peacock *et al.*, 2002, Feil *et al.*, 2003, Enright *et al.*, 2000) However, for epidemiological purposes, MLST has equal value as PFGE, as closely related PFGE patterns are regarded as the same strain. (Peacock *et al.*, 2002, Enright *et al.*, 2000, Tenover *et al.*, 1995) Nevertheless, PFGE is a better tool for outbreak investigations than MLST, as shown in the US PFGE database study. Both of the USA300 and USA500 MRSA strains are ST8, but have very different PFGE type, SCC*mec*, antimicrobial susceptibility patterns and epidemiology. (McDougal *et al.*, 2003) MLST has also been found to have good correlation with AP-PCR/RAPD, the target 916-Shine-Dalgarno PCR, binary typing, bacteriophage typing and *spaA* sequencing. (van Leeuwen *et al.*, 2003, Grundmann *et al.*, 2002, Oliveira *et al.*, 2001b, O'Brien *et al.*, 2004)

MLST provides unambiguous, highly reproducible and discriminatory typing data. Similar to *spaA* sequencing, MLST typing data can be easily shared between laboratories via the MLST internet database. With such good characteristics identical strains can be easily identified by MLST. (Enright and Spratt, 1999, Robinson and Enright, 2004b, Peacock *et al.*, 2002) MLST has been mainly used in long-term epidemiological and population studies of MRSA. (Peacock *et al.*, 2002, Robinson and Enright, 2004b) Evolutionary models and population genetic properties of *S. aureus* have been studied using MLST together with SCC*mec*

allotyping. (Robinson and Enright, 2003, Aires de Sousa and de Lencastre, 2003, Enright *et al.*, 2002, Feil *et al.*, 2004, Feil *et al.*, 2003, Crisostomo *et al.*, 2001) Recently MLST has been successfully used together with other techniques in short-term epidemiological studies of MRSA. (Chung *et al.*, 2004, Aires de Sousa *et al.*, 2003b, Aires de Sousa *et al.*, 2003a, O'Brien *et al.*, 2004)

The shortcomings of MLST are that the setting-up and running costs are much higher than the electrophoretic gel methods. (Olive and Bean, 1999) MLST does not tolerate sequencing mistakes, even a single nucleotide mistake can alter the ST. The chances of this are higher in MLST as seven genes are sequenced. This weakness can be minimised with appropriate quality control protocols. (Peacock *et al.*, 2002) Recently a MLST, high-density DNA array technique, has been developed to change the low-throughput MLST into a high-throughput method used for national reference centres. (van Leeuwen *et al.*, 2003) An attempt has also been made to lower the MLST cost by using the RFLP of the seven MLST loci. (Diep *et al.*, 2003)

1.4.2.5 Combined typing methods

A comparison of 12 typing techniques has been done on three groups of outbreak related *S. aureus* isolates. These typing techniques were analysed in terms of their typability, reproducibility, discriminatory power, ease of use and ease of interpretation. The researchers found that not a single method was clearly superior to the others. They suggested the most effective typing scheme for *S. aureus* is the combination of two techniques, which includes a sensitive, initial screening method, and a second method with high discriminatory power. (Tenover *et al.*, 1994, Tenover *et al.*, 1997) Studies using *spaA* sequencing, *mecA*-Tn*554* hybridisation and PFGE showed that the combination of these techniques was much more discriminatory than the individual techniques. (Shopsin *et al.*, 1999, Roberts *et al.*, 1998) The disadvantages and advantages of these typing techniques are listed in Table 1.7.

The combination of *mecA*-Tn*554* hybridisation and PFGE has been successfully used to identify pandemic MRSA clones in Europe and American. (Oliveira *et al.*, 2001b, Oliveira *et al.*, 2002, Sanches *et al.*, 1998, Roberts *et al.*, 1998) The combination of

MLST and SCC*mec* allotyping is the other combination of methods used to identify pandemic clones in evolutionary studies of *S. aureus*. (Enright *et al.*, 2002, Aires de Sousa and de Lencastre, 2003, Robinson and Enright, 2003) Recently, the combination of MLST, PFGE and SCC*mec* allotyping has been suggested to be the better technique for determining strain relationships of MRSA. (Aires de Sousa and de Lencastre, 2003) MLST provides unambiguous and easily shared data that reveals long-term evolutionary relationships between isolates. PFGE on the other hand, provide better discriminatory power and ability to establish short-term evolutionary relationships. A combination of MLST, PFGE and SCC*mec* allotyping will provide data that reveals evolution and traces the acquisition of SCC*mec* by different MRSA clones. (Aires de Sousa and de Lencastre, 2004)

Table 1.7 Comparison of *S. aureus* typing methods

Typing method[a]	Typeability	Intralaboratory reproducibility	Interlaboratory reproducibility	Discriminatory power	Ease of use	Ease of interpretation	Cost
Bacteriophage typing	Variable	Moderate	Moderate	Moderate	Moderate	Poor	Medium
Resistogram	Excellent	Good	Moderate	Low	Easy	Excellent	Low
MLEE	Excellent	Excellent	Excellent	Moderate	Moderate	Excellent	High
Plasmid profiling	Variable	Good	Moderate	Moderate	Moderate	Moderate	Medium
Plasmid REA	Variable	Good	Moderate	Moderate	Moderate	Excellent	Medium
PFGE	Excellent	Excellent	Moderate	High	Moderate	Moderate	Medium
Southern hybridisation:							
Ribotyping	Excellent	Good	Good	Moderate	Moderate	Good	Medium
IS typing	Variable	Good	Good	Moderate	Moderate	Good	Medium
mecA-Tn*554* typing	Excellent[b]	Good	Good	Moderate	Moderate	Good	Medium
Binary typing	Excellent	Excellent	Excellent	High	Moderate	Excellent	High

Table 1.7 continued on next page

Table 1.7 Comparison of *S. aureus* typing methods continued

Typing method[a]	Typeability	Intralaboratory reproducibility	Interlaboratory reproducibility	Discriminatory power	Ease of use	Ease of interpretation	Cost
PCR-based methods:							
AP-PCR/RAPD	Excellent	Moderate	Poor	High	Easy	Easy	Low
Rep-PCRs	Excellent	Excellent	Moderate	High	Easy	Easy	Low
Intergenic region PCRs	Excellent	Moderate to Excellent	Moderate to Excellent	Moderate	Easy	Easy	Low
AFLP	Excellent	Excellent	Excellent	High	Moderate	Easy	High
SCC*mec* allotyping	Excellent[b]	Excellent	Excellent	Moderate	Easy	Easy	Low
DNA sequencing:							
spaA sequencing	Excellent	Excellent	Excellent	High	Difficult	Easy	High
MLST	Excellent	Excellent	Excellent	High	Difficult	Easy	High

[a]MLEE, multilocus enzyme electrophoresis; REA, restriction endonuclease analysis; PFGE, pulsed-field gel electrophoresis; AP-PCR/RAPD, arbitrarily primed PCR/randomly amplified polymorphic DNA; Rep-PCR, repetitive element sequence-based PCR; AFLP, amplified-fragment length polymorphism; MLST, multilocus sequence typing.
[b]The *mecA*-Tn*554* and SCC*mec* typing are designed specifically for MRSA isolates
This table is based on the following references: (Tenover *et al.*, 1997, Olive and Bean, 1999, Witte, 2000, van Belkum *et al.*, 2001)

1.5 Types of MRSA

1.5.1 Classical MRSA

Methicillin is a semisynthetic β-lactam that is resistant to hydrolysis by penicillinase (a β-lactamase). It was introduced in 1960 for staphylococcal infections due to the increased prevalence of β-lactamase producing *S. aureus* strains. (Livermore, 2000) Resistance to methicillin was first reported in 1961 in a London hospital, soon after the introduction of methicillin. (Jevons, 1961) These methicillin-resistant isolates had acquired the *mecA*-bearing SCC*mec* mobile element. (Ito *et al.*, 1999, Hiramatsu *et al.*, 2001) They are typically resistant to streptomycin, tetracycline and erythromycin in addition to the β-lactams. (Grubb, 1990, Pearman, 1996, Ayliffe, 1997, Livermore, 2000, Oliveira *et al.*, 2002) These isolates were commonly found to carry plasmids belong to the β-lactamase/heavy metal-resistant plasmid family. (Grubb, 1990, Firth and Skurray, 2000) They spread in the hospitals of some European countries, Australia and United States from early 1960s to early 1970s. These isolates were responsible for some sporadic outbreaks but were usually eliminated by infection control measures. (Grubb, 1990, Pearman, 1996, Livermore, 2000, Ayliffe, 1997) Several studies using different typing techniques have shown that these isolates were clonal. (i.e. descendent from a common ancestor) (Musser and Kapur, 1992, Enright *et al.*, 2002, Crisostomo *et al.*, 2001) Collectively these early MRSA isolates are known as the classical MRSA or the archaic clone. (Grubb and O'Brien, 2004, Grubb, 1990, Crisostomo *et al.*, 2001, Oliveira *et al.*, 2002)

1.5.2 Epidemic MRSA

The frequency of MRSA isolations in hospitals started to decline dramatically in the mid 1970s after ten years of steady increase. The reasons for this dramatic decline of MRSA prevalence are unclear. (Grubb, 1990, Pearman, 1996, Livermore, 2000, Ayliffe, 1997) However, some researchers have suggested this declined may be due to several factors or the combination of these factors. The proposed factors include the displacement of the old strain by a newer MRSA strain(s), the increased use of gentamicin and improvement in infection control. (Livermore, 2000, Ayliffe, 1997)

Unfortunately, this decline did not continue and in the late 1970s, early 1980s the prevalence of MRSA started to increase. This coincided with the emergence of gentamicin-resistant MRSA (GR-MRSA). (Pearman, 1996, Ayliffe, 1997, Livermore, 2000) Unlike the early isolates, these strains caused extensive long-lasting outbreaks and involved many hospitals. They were difficult to eliminate and had a high mortality rate. Later, these GR-MRSAs became endemic in many hospitals and spread to other hospitals via patients and staff. (Grubb, 1990, Grubb and O'Brien, 2004, Pearman, 1996, Livermore, 2000, Ayliffe, 1997) The intercontinental spread of these strains has also been reported. (Townsend *et al.*, 1984a) Outbreaks related to these strains were reported in hospitals in Australia, Ireland, United Kingdom and United States. The GR-MRSA carries more antibiotic resistance than the classical MRSA. They are typically resistant to aminoglycosides, chloramphenicol, macrolides, tetracycline, trimethoprim, and the NAB compounds in addition to the β-lactams. (Grubb and O'Brien, 2004, Pearman, 1996, Livermore, 2000, Ayliffe, 1997) Unlike the classical MRSA, they were found to carry plasmids belonging to the pSK1 family with NAB-resistance genes. (Townsend *et al.*, 1985d, Skurray *et al.*, 1988, Firth and Skurray, 2000, Grubb and O'Brien, 2004) These isolates include the first epidemic MRSA (EMRSA) in the UK, EMRSA-1, which was identified in 1981 and was endemic in many English hospitals. (Livermore, 2000) The Eastern Australia MRSA (EA MRSA) also belongs to this group of MRSA. (Townsend *et al.*, 1983b, Grubb, 1990, Grubb and O'Brien, 2004, Pearman, 1996) It was isolated between the late 1970s and the early 1980s and is genetically closely related to EMRSA-1. (Townsend *et al.*, 1984a, Lim *et al.*, 2002, Enright *et al.*, 2002) This period of time in the early 1980s marked a milestone for nosocomial MRSA infections. Even though these GR-MRSA isolates were not more or less virulent than methicillin-susceptible strains and the classical MRSA, they are better hospital organisms due their antibiotic-resistance genes and the ability to spread (epidemic potential). As a result, MRSA became established in many hospitals and presented serious obstacles for infection control and the treatment of staphylococcal infections. (Grubb and O'Brien, 2004, Pearman, 1996, Livermore, 2000, Ayliffe, 1997)

1.5.3 Contemporary EMRSAs

In the late 1980s a further 13 strains of EMRSA were identified in English and Welsh hospitals. The prevalence of EMRSA-1 dropped and it was eventually replaced by other predominant EMRSA, such as EMRSA-3. (Kerr *et al.*, 1990, Livermore, 2000) EMRSA-15 and EMRSA-16 emerged in the early 1990s. By the mid-1990s they became the most predominant EMRSA isolates and accounted for 60 to 70% of the MRSA isolated. (Livermore, 2000, Ayliffe *et al.*, 1998) In one survey, done between 1998 and 2000, EMRSA-15 and EMRSA-16 composed over 95% of the MRSA isolated in 26 participating United Kingdom (UK) hospitals. (Johnson *et al.*, 2001) Unlike the EMRSA-1, both of these strains, and especially EMRSA-15 are not multiply resistant, in fact EMRSA-15 is only resistant to β-lactams, ciprofloxacin and in some instance erythromycin. (Livermore, 2000, Ayliffe, 1997, Ayliffe *et al.*, 1998, Grubb and O'Brien, 2004) EMRSA-1 tends to colonise whereas EMRSA-15 and EMRSA-16 are often involved in invasive infections such as bacteraemia. (Livermore, 2000, Johnson *et al.*, 2001) It is unclear why EMRSA-15 and EMRSA-16 are more successful than the other EMRSAs. It is possible that these strains may have the ability to survive better on the surfaces of inanimate objects or hands. (Moore and Lindsay, 2002, Livermore, 2000) The changing conditions in UK hospitals may also be contributing to their success. As a result of changing medical treatments, the number of debilitated patients with serious underlaying diseases has increased in UK hospitals. In addition, because of better efficiency, more patients are moving through the hospitals. Also, with the ageing of the population there is more movement of patients between long-term healthcare facilities, like nursing homes and hospitals. These changing conditions have provided a fertile ground for the spread of MRSA. The increased use of fluoroquinolones, like ciprofloxacin, may possibly be one of the contributors to the success of EMRSA-15 and EMRSA-16. (Livermore, 2000, Ayliffe, 1997) Fluoroquinolones are excreted in sweat and this creates a selective pressure for both EMRSA-15 and EMRSA-16 which are fluoroquinolone resistant. (Livermore, 2000, Ayliffe, 1997) Both EMRSA-15 and EMRSA-16 have been reported in many European countries, Canada and Australia. (Aires de Sousa and de Lencastre, 2004, Oliveira *et al.*, 2002, Lim *et al.*, 2003, Pearman *et al.*, 2001)

As well as hospitals in the UK, hospitals worldwide have also experienced the same dramatic increase in MRSA since the early 1980s. From the late 1980s to early 1990s, five predominant epidemic MRSA clones have been identified in European, North American, South American and Japanese hospitals. (Aires de Sousa and de Lencastre, 2004, Oliveira *et al.*, 2002, Oliveira *et al.*, 2001b) They are the Iberian clone (Dominguez *et al.*, 1994), the Brazilian clone (Teixeira *et al.*, 1995), the Hungarian clone (de Lencastre *et al.*, 1997), the New York/Japan clone (de Lencastre *et al.*, 1996, Oliveira *et al.*, 2001b) and the Paediatric clone. (Sa-Leao *et al.*, 1999) Similar to the EMRSAs in the UK, these clones have dominated and replaced other MRSA in hospitals. (Aires de Sousa and de Lencastre, 2004, Amorim *et al.*, 2002) The intercontinental spread of these isolates has been reported. The Iberian clone was detected in a New York hospital and the Brazilian clone was found in Portuguese hospitals. (Aires de Sousa *et al.*, 1998, Roberts *et al.*, 1998) The Centre of Molecular Epidemiology and International Network (CEM/NET) initiative has being tracking and identifying MRSA clones in European, Latin American, Asian and North American hospitals since 1995. Under this initiative over 3000 isolates have been tested, and the five epidemic clones were found to have spread into different regions of the world. (Oliveira *et al.*, 2002, Aires de Sousa and de Lencastre, 2004, Oliveira *et al.*, 2001b)

A progressive increase of gentamicin-susceptible MRSA (GS-MRSA) was reported in French hospitals since 1991. (Lelièvre *et al.*, 1999, Lemaître *et al.*, 1998) The emergence of GS-MRSA was linked to the decreased use of gentamicin in these hospitals. (Aubry-Damon *et al.*, 1997) Most of them are derived from GS-MRSA by losing the resistance gene, *aacA-aphD*, via deletion or excision of the *aacA-aphD* bearing transposon, Tn*4001*. (Blanc *et al.*, 2001a, Laurent *et al.*, 2001, Lelièvre *et al.*, 1999, Lemaître *et al.*, 1998) Although most of the GS-MRSA isolates still carry the kanamycin, neomycin and tobramycin resistance gene, *aadD*, they typically carry less antimicrobial-resistance determinants. (Lelièvre *et al.*, 1999, Lemaître *et al.*, 1998) GS-MRSA had become the predominant MRSA in seven French hospitals by 1998 and a Greek hospital in 2000. (Lelièvre *et al.*, 1999, Polyzou *et al.*, 2001, Pournaras *et al.*, 2001)

1.5.4 Community-acquired MRSA

MRSA had been regarded as a nosocomial organism. In the late 1980s and early 1990s, MRSA were isolated from patients who did not have a history of hospitalisation or contact with healthcare workers. These isolates have become known as community-acquired MRSA (CMRSA). (Cookson, 2000, Chambers, 2001) The first report of CMRSA in Australia was the isolation of CMRSA from remote communities of Western Australia in 1989. These isolates were known as the Western Australia MRSA. (Udo *et al.*, 1993, Riley *et al.*, 1995, Riley and Rouse, 1995) CMRSA were also found in New Zealand and were predominantly isolated from people of Polynesian origin. They have been divided into Western Samoan Phage Pattern-1 and -2 based on their bacteriophage types. These strains have subsequently been isolated in eastern Australian states. (Adhikari *et al.*, 2002, Heffernan *et al.*, 1995, Lim *et al.*, 2003, Grubb and O'Brien, 2004) In the US CMRSA started to emerge as an important cause of skin and soft tissue infections (SSTI). The majority of these CMRSA in the US carry the Panton-Valentine leukocidin (PVL) toxin which is rarely found in nosocomial strains. Between 1997 and 1999, four paediatric deaths have resulted from necrotizing pneumonia and severe sepsis caused by CMRSA carrying the PVL toxin. (Centers for Disease Control and Prevention, 1999, Baba *et al.*, 2002) In the period of time between 2000 and 2003 many cases of SSTI caused by CMRSA have been reported among the inmates of correctional facilities in California, Georgia, Tennessee, Texas and Mississippi. (Centers for Disease Control and Prevention, 2003c, Centers for Disease Control and Prevention, 2003b, McDougal *et al.*, 2003) The majority of these CMRSA isolates belong to the USA300 (ST8 and Allotype IV SCC*mec*) clone and carry PVL toxin. (McDougal *et al.*, 2003) The same CMRSA clone was reported to cause SSTIs among athletes participating in close contact sports such as rugby and American Football. (Centers for Disease Control and Prevention, 2003a, Nguyen *et al.*, 2005, Zetola *et al.*, 2005) Similar to the Australian Aboriginal and New Zealand Polynesian communities, the Native American Indian and Pacific Islander communities also have a higher than average isolation rate of CMRSA compared to the general population. (Grubb and O'Brien, 2004, Centers for Disease Control and Prevention, 2004a, Groom *et al.*, 2001) The other major high-risk groups for CMRSA infections are children, the elderly, the gay community and

intravenous drug users. (Fridkin *et al.*, 2005, Lee *et al.*, 2005, Zetola *et al.*, 2005) CMRSA have been reported in other countries such as France and Switzerland (Vandenesch *et al.*, 2003), Netherlands (van der Flier *et al.*, 2003), Finland (Salmenlinna *et al.*, 2002), Hong Kong (O'Donoghue and Boost, 2004), Brazil (Ribeiro *et al.*, 2005) and Canada. (Roman *et al.*, 1997) CMRSA are typically non-multiply resistant. Many of them are only resistant to the β-lactams and sometimes additionally to erythromycin, tetracycline, fusidic acid and ciprofloxacin. (Grubb and O'Brien, 2004, O'Brien *et al.*, 2004, Okuma *et al.*, 2002) Similar to HMRSAs, all CMRSA isolates carry the *mecA*-bearing SCC*mec*. But unlike the HMRSAs, the CMRSAs carry either the types IV or V SCC*mec*. Both of these SCC*mec* are smaller and the *mecA* gene is often the only resistance gene they carry. (O'Brien *et al.*, 2004, Okuma *et al.*, 2002, Ito *et al.*, 2004, Ma *et al.*, 2002) The CMRSA was found to grow two times faster than HMRSA, probably due their small and simple SCC*mec* and lack of antimicrobial-resistance genes. This fast growth rate gives them a competitive advantage in the community setting where antimicrobial selection pressure is not high. (Okuma *et al.*, 2002) The genetic backgrounds of the CMRSA isolates are very diverse and very different from the HMRSA. (O'Brien *et al.*, 2004, Lim *et al.*, 2003, Grubb and O'Brien, 2004, Okuma *et al.*, 2002, Coombs *et al.*, 2004) A small CMRSA outbreak was reported in Royal Perth Hospital, Western Australia, in 1995. (O'Brien *et al.*, 1999) CMRSA have become increasingly common in Australia, in the 2000 and 2002 surveys of MRSA isolated from non-hospitalised patients, approximately 54% of the isolates were non-multiply resistant MRSA and the majority of them were CMRSA. (Coombs *et al.*, 2004) The increasing frequency of CMRSA isolation worldwide is going to have a great impact on the current healthcare system, if this trend is not monitored and controlled. The presence of CMRSA is going to alter the empirical treatment for staphylococcal infections and increase the incidence of severe staphylococcal infections in the community. (Cookson, 2000, Chambers, 2001, Vandenesch *et al.*, 2003, Lim *et al.*, 2003) It has been reported in a Texas hospital that CMRSA encoding the PVL toxin were causing staphylococcal sepsis of increasing severity in previously healthy adolescents without predisposing risk factors. (Gonzalez *et al.*, 2005)

1.5.5 Vancomycin-intermediate and -resistant *S. aureus*

Vancomycin is a glycopeptide and the most effective, and in some cases the only treatment, for serious MRSA infection. Almost all MRSA isolated up to date, have been susceptible to vancomycin and the other glycopeptide, teicoplanin. (Livermore, 2000, Hiramatsu, 1998) In 1996, the first vancomycin or glycopeptide intermediate-resistant *S. aureus* (VISA or GISA) was isolated in Japan. (Hiramatsu *et al.*, 1997) VISA has been subsequently isolated in hospitals worldwide. (Hiramatsu *et al.*, 1997, Walsh and Howe, 2002, Trakulsomboon *et al.*, 2001, Kim *et al.*, 2000, Hageman *et al.*, 2001, Murray *et al.*, 2004) VISAs are also MRSA and have a vancomycin MIC of 8 μg/ml. (Hiramatsu *et al.*, 1997) VISAs achieve their reduced susceptibility by having a thickened cell wall with decreased peptidoglycan cross-linking. (See Section 1.2.5) Due to the narrow therapeutic index of glycopeptides, a moderate rise in the MIC of VISAs has been linked to cases of treatment failure. (Livermore, 2000, Hiramatsu *et al.*, 1997, Ariza *et al.*, 1999) To date, although it has been widely reported, VISA is rarely isolated and no outbreaks have been linked to it. (Hiramatsu, 2001, Walsh and Howe, 2002, Tenover *et al.*, 2004)

Until recently high-level vancomycin resistance (MIC $\geq$ 32 μg/ml) was only observed in VRE. Resistance is mediated by the *van* gene clusters, such as the *vanA* gene cluster, carried by transposon Tn*1546*. (See Section 1.3.2.7) (Arthur *et al.*, 1993) In June 2002, the first high-level, vancomycin-resistant MRSA (VRSA) was isolated from a patient infected with both VRE and MRSA in a Michigan hospital. (Centers for Disease Control and Prevention, 2002a) A second case was soon reported in a Pennsylvanian hospital in September 2002. (Centers for Disease Control and Prevention, 2002b) Both VRSA isolates belong to, or are related to, the New York/Japan clone and carry a large Tn*1546*-bearing plasmid. (Bozdogan *et al.*, 2004, Tenover *et al.*, 2004) However, the Tn*1546*s found in these two isolates are different in their structures, MIC levels and the type of host plasmid. (Clark *et al.*, 2005, Perichon and Courvalin, 2004, Weigel *et al.*, 2003, Tenover *et al.*, 2004) The Michigan VRSA has a MIC of 1028 μg/ml. Its Tn*1546* is complete and located on a 57 kb conjugative plasmid of the pSK41 family. It has been shown that the Michigan VRSA probably acquired the Tn*1546* from a co-isolated VRE via a

conjugative plasmid in the original MRSA. (Clark *et al.*, 2005, Perichon and Courvalin, 2004, Weigel *et al.*, 2003) The Pennsylvania VRSA has a much lower MIC of 32 μg/ml mediated by Tn*1546* type F2. Structurally Tn*1546* type F2 is truncated by insertion sequences and carries mutations in the *van* genes. (See Section 1.3.2.7) It has only been found in VRE from US hospitals. The Pennsylvania VRSA is likely to have acquired the resistance by acquiring a 120 kb plasmid that carries the Tn*1546* type F2. (Clark *et al.*, 2005, Perichon and Courvalin, 2004, Tenover *et al.*, 2004) In both cases, the VRSA infections were successfully treated with antimicrobials that were still effective against them. No transmission of VRSA was detected among the patients and the healthcare workers. (Centers for Disease Control and Prevention, 2002b, Centers for Disease Control and Prevention, 2002a, Gemmell, 2004) A third case of VRSA was isolated from the urine sample of a patient from a New York long-term healthcare facility in March 2004. The vancomycin MIC of this New York VRSA is > 256 μg/ml. It has both *mecA* and *vanA* genes, and appears to be different from the Michigan and Pennsylvania VRSAs. The automated methods for antimicrobial-susceptibility testing were unable to detect the vancomycin resistance in this isolate. To date, little has been reported on its molecular characteristics and transmission. (Centers for Disease Control and Prevention, 2004b) The appearance of VISA and VRSA has highlighted the urgent need of new antimicrobial agents as well as a review of current MRSA control strategies. (Centers for Disease Control and Prevention, 2002a, Livermore, 2000) To date, MRSA with reduced vancomycin susceptibility have not caused serious outbreaks and spread widely. (Gemmell, 2004, Walsh and Howe, 2002) Two of the VRSA strains belong to the pandemic New York/Japan clone, and the VISA isolates are also derived from New York/Japan, Iberian and Brazilian clones. (Enright, 2003, Ariza *et al.*, 1999, Oliveira *et al.*, 2001c) This suggests that they could have the potential to spread throughout the world. The emergence of pandemic VRSA will have serious consequences for the treatment for MRSA infections as we will have lost our most important antibiotic for treating MRSA.

1.5.6 Nomenclature of MRSA

The existing arbitrary systems for designating MRSA clones differ between countries and research groups. These designation systems are either based on the geographical

location or on the typing methods used. The Central Public Health Laboratory, Colindale, London, using bacteriophage typing, antimicrobial susceptibility typing and biochemical tests designated 17 epidemic MRSAs as EMRSA-1 to -17 in England and Wales. (Kerr *et al.*, 1990, Aucken *et al.*, 2002) The joint research groups of Tomasz, at Rockefeller University, New York and de Lencastre at the Instituto de Tecnologia of Universidade Nova de Lisboa, Portugal, used the combined results of PFGE and *mecA*-Tn*554* typing for the designation of five pandemic clones in Europe and North and South America. These pandemic clones were named according to their geographical location and the property of the clones. (Oliveira *et al.*, 2002, Oliveira *et al.*, 2001b) Hiramatsu's group in Juntendo University, Japan, used ribotyping to define five distinct clonal-types among nosocomial MRSA isolates worldwide. (Hiramatsu, 1995, Hiramatsu *et al.*, 2001) The Centres for Disease Control, Atlanta, US, uses PFGE supplemented with MLST and *spaA* typing for the identification of US epidemic MRSA clones like USA400 (McDougal *et al.*, 2003)

The different typing techniques used by these groups make it difficult to compare the MRSA clones from the different centres due to the methods used. PFGE is the most widely used technique for the definition of MRSA clones, but it is plagued by its poor interlaboratory comparability. (Murchan *et al.*, 2003, van Belkum *et al.*, 1998b, Cookson *et al.*, 1996) As a result of this lack of comparison, the same MRSA clones have been named differently by different centres. For example, the EMRSA-1, EA MRSA, the Brazilian clone and the Hungarian clone are closely related strains and should be typed as one group. (Enright *et al.*, 2002, Lim *et al.*, 2002)

In 2002, a subcommittee of the International Union of Microbiology Societies in Tokyo agreed on a new nomenclature system on MRSA clones. The new system named the MRSA clones based on their resistant phenotypes, MLST and SCC*mec* types. For example, EMRSA-1 is a ST239 MRSA with Allotype III SCC*mec* and its designation would be ST239-MRSA-III. (Robinson and Enright, 2004b, Enright *et al.*, 2002) MLST was chosen as the preferred system because it is a systematic and objective method with good discriminatory power. More importantly, as a DNA sequencing typing method, its results can be effortlessly shared and a big internet-based MLST database is available for easy comparison and access. It is hoped that

this system will replace, or at least supplement, the existing system. (Robinson and Enright, 2004b) Table 1.8 lists some epidemic clones with their name in both new and existing nomenclature systems.

1.6 Evolution of MRSA

The *S. aureus* genome has relatively stable core genes. The clonal diversification of *S. aureus* at the initial stage is predominantly driven by point mutations, rather than recombination. The seven core alleles used in the MLST typing are 15 time more likely to change by point mutation than recombination. Despite occurring at a lower rate, homogeneous recombination does contribute to evolution over a long time. The ratio of synonymous and nonsynonymous point mutation rates (d_S/d_N) within the same clonal complex (CC) is close to parity (1.13), whereas the average ratio is larger than 8 when comparing STs from different CCs. This difference in the d_S/d_N indicates that the proportion of nonsynonymous point mutations is significantly higher when comparing STs within the same CC than from different CCs. It also suggests that these non-lethal nonsynonymous point mutations are mainly deleterious and are "purified" by selection. As they survive long enough to be sampled, the rate of purifying selection is relatively slow in *S. aureus*. (See Section 1.4.2.4.2 for the descriptions of ST and CC) (Feil, 2003, Feil *et al.*, 2003)

The evolution of MRSA has been proposed by computing the combined results of MLST, *spaA* sequencing and surface protein gene (*sas*) sequencing with the BURST algorithm. (Robinson and Enright, 2003, Enright *et al.*, 2002) MRSA arise from methicillin-susceptible *S. aureus* by acquisition of SCC*mec*. Several studies have shown that only a number of *S. aureus* genetic backgrounds (i.e. CC) are able to maintain and express the foreign *mecA* gene and its product, PBP2a/2'. (Katayama *et al.*, 2005, Katayama *et al.*, 2003c, Fitzgerald *et al.*, 2001) The majority of nosocomial MRSA are found to belong to five clonal complexes, CC5, CC8, CC22, CC30 and CC45. (See Figure 1.7) (Robinson and Enright, 2004b, Robinson and Enright, 2003, Enright *et al.*, 2002)

CC5 and CC8 are the most diverse CCs and contain the majority of pandemic MRSA clones. They also have the largest number of single-step variants (single-locus and

single-nucleotide variants) of their respective ancestors. The MRSA clones within CC5 evolved from ST5-MSSA by acquiring four different types of SCC*mec*. These CC5 MRSA epidemic clones include the ST-MRSA-II (New York/Japan clone), ST5-MRSA-IV (Paediatric clone) and ST5-MRSA-I (EMRSA-3). (Robinson and Enright, 2003, Enright *et al.*, 2002) The ST5-VISA-II and ST5-VRSA-II clones are believed to have evolved from the ST5-MRSA-II clone. (Enright, 2003)

The ancestor of CC8 is ST8-MSSA, which, by itself, is a successful MSSA clone. Four types of SCC*mec* were found within this CC and it is believed that the CC8 strains acquired them by multiple independent events. Most of the MRSA clones of CC8 evolved from the ancestral ST8-MSSA and two of its single-locus variants (SLV), ST250-MSSA and ST239-MRSA-III. The MRSA clones, ST8-MRSA-IV (EMRSA-2 and -6) and ST8-MRSA-II (Irish-1) evolved from ST8-MSSA by acquisition of their respective SCC*mec*s. The ST8-MRSA-IV is also the predominant CMRSA clone in the US and Norway. The first MRSA, the Archaic clone, and EMRSA-8 belong to ST250-MRSA-I, which is derived from ST250-MSSA. The ST247-MRSA-I (Iberian clone, EMRSA-5 and -17) is a SLV of ST250-MRSA-I and evolved from it. The ST254-MRSA-IV clone contains EMRSA-10 and the Hanover clone, which evolved from ST8-MSSA. (Robinson and Enright, 2003, Enright *et al.*, 2002) ST239-MRSA-III is a distinct branch in CC8. Although it is a SLV of ST8, its *spaA* type and *sas* profiles are not related to ST8, but are similar to the ST30-MSSA clone. (Robinson and Enright, 2003) A study involving 41 chromosomal genes showed that ST239 evolved from an unusual chromosomal replacement in ST8 with ST30 chromosomal DNA. This *c.*557 kb chromosomal replacement with DNA of ST30 origin is located around the origin of replication and accounts for *c.* 20% of the total chromosome. (Robinson and Enright, 2004a) ST239-MRSA-III is a very successful pandemic clone and includes EMRSA-1, -4, -7, -11, EA MRSA, Brazilian, Hungarian and Vienna clones. The ST240-MRSA-III clone contains EMRSA-9 and is derived from ST239-MRSA-III, which is a ST239 SLV. (Robinson and Enright, 2003, Enright *et al.*, 2002)

ST22 is the ancestral ST for CC22. The predominant UK MRSA clone, EMRSA-15 (ST22-MRSA-IV) is derived from it. (Robinson and Enright, 2003, Enright *et al.*, 2002) CC30 contains two MRSA lineages that descended from a ST30-MSSA

ancestor. The ST30-MSSA clone (phage type 80/81) is a successful pandemic clone that is known to have caused numerous nosocomial outbreaks in the 1950s. The nosocomial MRSA lineage of CC30 contains EMRSA-16 (ST36-MRSA-II), which is a SLV of ST30. The community-acquired lineage has the epidemic CMRSA clones WSSP-1 and -2 (ST30-MRSA-IV), which evolved by the acquisition of SCC*mec* and the PVL toxin genes. (Robinson and Enright, 2003, Robinson *et al.*, 2005)

CC45 contains the Berlin epidemic clone (ST45-MRSA-IV), found in Germany and the Netherlands, and the community-acquired WA MRSA-4 (ST45-MRSA-V) found in Western Australia. (Enright *et al.*, 2002, Wannet *et al.*, 2004, Grubb and O'Brien, 2004) ST45-MRSA-II is another MRSA clone that evolved from ST45-MSSA. (Robinson and Enright, 2003, Enright *et al.*, 2002)

CMRSA clones tend to have more diverse genetic backgrounds. They are believed to have arisen by the acquisition of the smallest and simplest Type IV and V SCC*mec*s by ST clones in the community. (Enright, 2003, Aires de Sousa and de Lencastre, 2003, Okuma *et al.*, 2002) The CMRSA clone, ST1-MRSA-IV, from Australia and the United States is derived from the ST1-MSSA. (Okuma *et al.*, 2002, O'Brien *et al.*, 2004, McDougal *et al.*, 2003) The ST1-MSSA is a common community strain and is known to cause invasive nosocomial staphylococcal infections. (Enright *et al.*, 2000, Enright, 2003) The ST80-MRSA-IV clone is a singleton and does not belong to any CC. It is a common CMRSA clone in European countries and it has been isolated in Australia. (Aires de Sousa and de Lencastre, 2003, Hanssen *et al.*, 2005, Holmes *et al.*, 2005, Coombs *et al.*, 2004)

Table 1.8 Details of some epidemic MRSA clones and strains

CC[a]	Clone	MLST profile	Published names[b]
1	ST1-MRSA-IV	1-1-1-1-1-1-1	WA MRSA-1, USA400, MW2
5	ST5-MRSA-I	1-4-1-4-12-1-10	EMRSA-3
5	ST5-MRSA-II	1-4-1-4-12-1-10	New York/Japan, USA100
5	ST5-MRSA-IVp	1-4-1-4-12-1-10	Paediatric
5	ST5-VISA-II	1-4-1-4-12-1-10	Mu50 (VISA)
5	ST5-VRSA-II	1-4-1-4-12-1-10	Michigan VRSA, Pennsylvania VRSA
8	ST8-MRSA-II	3-3-1-1-4-4-3	Irish-1
8	ST8-MRSA-III	3-3-1-1-4-4-3	EMRSA-7
8	ST8-MRSA-IV	3-3-1-1-4-4-3	EMRSA-2, -6, USA300
8	ST8-MRSA-IVp	3-3-1-1-4-4-3	EMRSA-12, -13, Irish-2
8	ST239-MRSA-III	2-3-1-1-4-4-3	EMRSA-1, -4, -11, EA MRSA, Brazilian, Hungarian, Vienna
8	ST240-MRSA-III	2-3-1-1-21-4-3	EMRSA-9
8	ST247-MRSA-I	3-3-1-12-4-4-16	EMRSA-5, -17, Iberian
8	ST250-MRSA-I	3-3-1-1-4-4-16	Archaic, NCTC10442 (First MRSA), COL, EMRSA-8
8	ST254-MRSA-IV	3-32-1-1-4-4-3	EMRSA-10
22	ST22-MRSA-IV	7-6-1-5-8-8-6	EMRSA-15
30	ST30-MRSA-IV	2-2-2-2-6-3-2	WSPP-1, -2
30	ST36-MRSA-II	2-2-2-2-2-3-2	EMRSA-16, USA200
45	ST45-MRSA-IV	10-14-8-6-10-3-2	Berlin
45	ST45-MRSA-V	10-14-8-6-10-3-2	WS1 (WA MRSA-4)

[a]CC, clonal complex, a group of sequence types (ST) with at least 5 identical loci.
[b]EMRSA, epidemic MRSA from the United Kingdom; EA MRSA, eastern Australia MRSA; VISA, vancomycin-intermediate *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; WA MRSA, Western Australia MRSA; WSPP, Western Samoan Phage Pattern.
References for this table: (Coombs *et al.*, 2004, Enright, 2003, Enright *et al.*, 2002, McDougal *et al.*, 2003, O'Brien *et al.*, 2004, Oliveira *et al.*, 2002, Oliveira *et al.*, 2001b, Grubb and O'Brien, 2004, Cassat *et al.*, 2005)

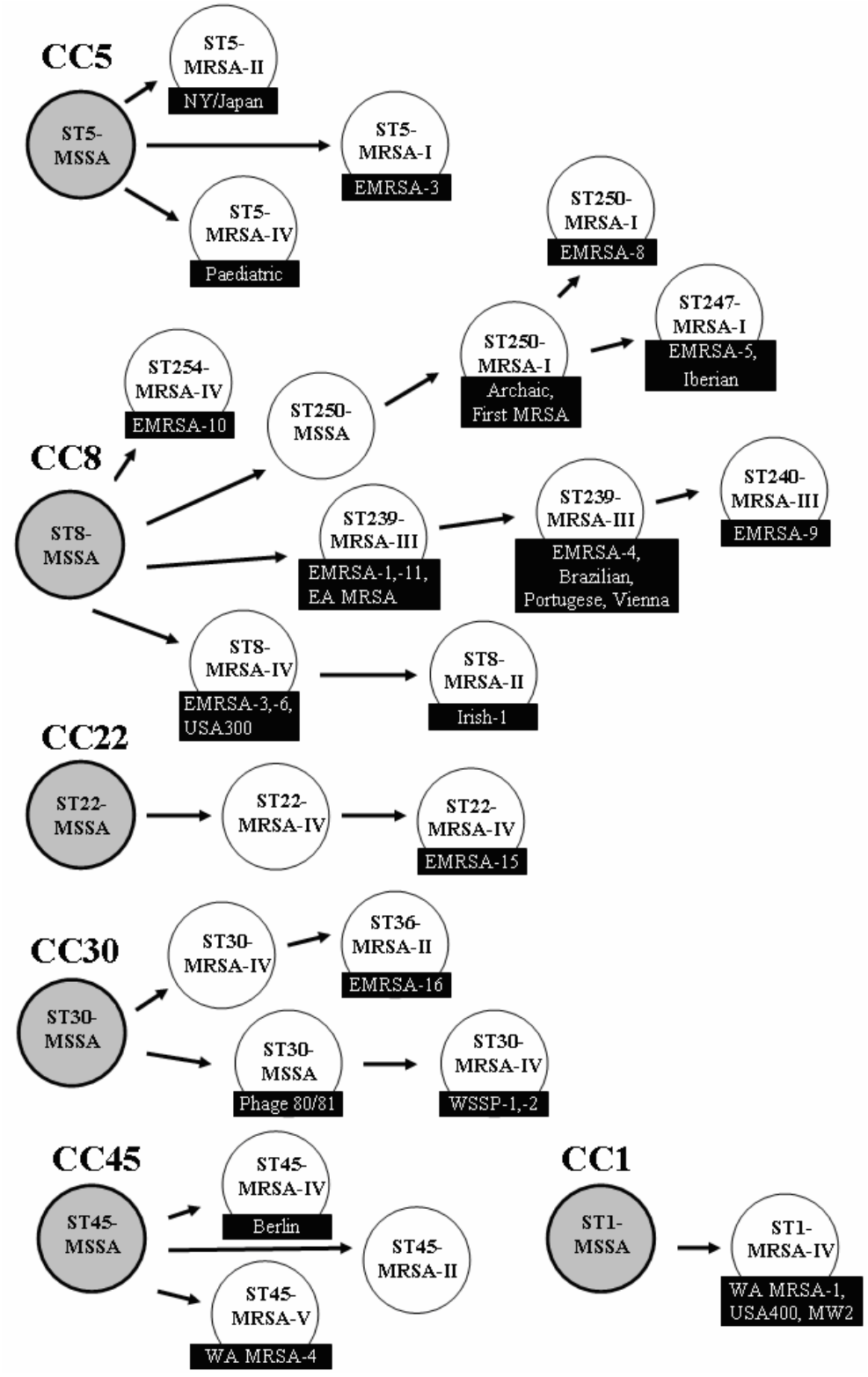


Figure 1.7 Phylogeny of important MRSA clones

Based on MLST, *spaA* and *sas* sequence profiling. Adapted from Robinson and Enright (2003)

1.7 Impact and infection control of MRSA

The colonisation and nosocomial infections of MRSA have had some serious consequences on infected patients and the hospitals. The most obvious impact of MRSA is the increased morbidity and mortality of the infected patients. (Livermore, 2003) The mortality rate of MRSA infections is two times higher than MSSA infections. (Engemann *et al.*, 2003, Cosgrove *et al.*, 2003, Whitby *et al.*, 2001) However, some researchers have argued that this higher rate may be due to other factors. The MRSA infected patients were often seriously ill with underlaying diseases and tended to stay longer in the hospital. It is hard to determine whether the MRSA infections or the underlaying diseases, or the combination of the two, are the real causes of death. (Hurley, 2002, Marshall *et al.*, 2004) Regardless of this argument, MRSA is commonly acknowledged to be associated with a substantial number of fatalities in hospitals. (Ayliffe *et al.*, 1998, Crowcroft and Catchpole, 2002, Rubin *et al.*, 1999)

The presence of MRSA in a hospital environment leads to the increasing failure of empiric regimens for prophylaxis and treatment of infections. Vancomycin is the recommended treatment for MRSA infections. Vancomycin is expensive and more likely to cause drug toxicity than drugs such as flucloxacillin. Vancomycin can only be administrated intravenously and the blood level closely monitored due to its toxicity. (Ayliffe *et al.*, 1998, Livermore, 2003) The use of vancomycin not only creates the selective pressure for VISA and VRSA, but it is associated with the emergence of VRE. (Ayliffe *et al.*, 1998, Gemmell, 2004, Kim and Song, 1998, Cunha, 2002, Rubin *et al.*, 1999) The inclusion of a glycopeptide in the empiric regimens can incur a significant increase in the cost of treatment. (Ayliffe *et al.*, 1998, Vriens *et al.*, 2002) The impact of MRSA on infected patient is relatively intangible. In addition to the pain and the increased risk of mortality, the patient might suffer psychological problems, such as anxiety and depression, and a reduction in the quality of life because of the infection. As the patient often needs additional treatment or more support after discharge, their on going condition affects the family members, friends, and fellow workers. (Ayliffe *et al.*, 1998) Financially, MRSA have become a heavy burden for hospital management. MRSA infected patients often stay longer and require more expensive drugs. As a result, MRSA infections

are more expensive to treat than MSSA infections. (Rubin *et al.*, 1999, Collignon *et al.*, 2005, Inan *et al.*, 2005, Engemann *et al.*, 2003) The treatment of *S. aureus* bacteraemia in Australian hospitals was approximately 150 million Australian dollars over a two year period. Around 26% of these bacteraemias were caused by MRSA. (Collignon *et al.*, 2005) A similar study of New York hospitals in 1995 revealed that the treatment of *S. aureus* infections cost around 434 million US dollars. MRSA contributed to 20% of the infections and cost USD94.5 million to treat. (Rubin *et al.*, 1999)

Around the world, many hospitals have spent substantial amounts of their resources in controlling and eradicating MRSA. (Ayliffe *et al.*, 1998, Vriens *et al.*, 2002) However, only a few countries and regions have been able to prevent MRSA from becoming endemic in their hospitals; they are the Netherlands, Scandinavian countries and Western Australia. These hospitals typically follow strict infection control protocols against MRSA and restrictive antibiotic usage. (Wertheim *et al.*, 2004, Stefani and Varaldo, 2003, Faria *et al.*, 2005, Sorensen and Monnet, 2000, Riley and Rouse, 1995, Ayliffe, 1997) The Dutch "search and destroy" policy is representative of these infection control protocols. Under this policy, all patients suspected to be colonised are isolated until screening cultures for MRSA are negative. This high-risk group includes the patients who have been hospitalised in foreign countries and patients who have contact with MRSA-positive persons. The MRSA-positive patients remain isolated and treated with MRSA eradication therapy. Staffs are also screened for MRSA. Positive staff are given therapy for decolonisation and temporarily discontinue working until negative-screening cultures are obtained. If two consecutive cases of MRSA isolations occur in a ward, the ward is closed to new admissions until negative-screening cultures are obtained. The ward is cleaned and all patients and staff screened for MRSA. The intensive care unit is closed if MRSA isolation is reported. (Wertheim *et al.*, 2004, Vriens *et al.*, 2002) This policy has successfully kept the MRSA rate in Dutch hospitals below 0.5%. (Stefani and Varaldo, 2003, Vos *et al.*, 2005, Vriens *et al.*, 2002) On the other hand, it does come with major financial and logistic consequences. The estimated cost of running this policy over a 10-year period is approximated 2.8 million euro. However, the cost of treating MRSA at an endemic level was estimated to be 8 million euro in a Dutch hospital over the same period of time. This is because the

more expensive vancomycin has to be used in an empirical regimen for infection and prophylaxis. (Vriens *et al.*, 2002) Also the logistic consequences, like temporary ward closure, postponement of surgery, and elective admissions are often unpopular with clinicians and hospital administration. (Vriens *et al.*, 2002, Teare and Barrett, 1997, Barrett *et al.*, 1998) It is important to convince them of the effectiveness of this policy. For the policy to work it requires adequate funding, the toleration of inconveniences and appreciation of the situation by hospital management and clinicians. (Ayliffe *et al.*, 1999, Ayliffe, 1997, Vriens *et al.*, 2002) A simpler policy to the Dutch policy is in place in Western Australia. (Pearman *et al.*, 1985)

The search and destroy method has achieved less success in hospitals where MRSAs have become endemic. The emergence of EMRSA-15 and EMRSA-16 in England overwhelmed the infection control measures, as a result, the rate of MRSA increased significantly and they became endemic. (Farrington *et al.*, 1998, Livermore, 2000) Some clinicians in England have argued that controlling MRSA with search and destroy measures causes more problems than it solves. They describe the consequences of the policy as being detrimental, expensive and ineffective, especially for hospitals with limited resources. They also claim that the over emphasis on MRSA has diverted important resources from the control of other important nosocomial pathogens. (Barrett *et al.*, 1998, van Saene *et al.*, 2004) They believe that MRSA should be treated like other nosocomial pathogens that may not require strict control and that the benefit of MRSA screening in an endemic situation is questionable and should be stopped. (Teare and Barrett, 1997, Barrett *et al.*, 1998) They suggest instead that the resources should be spent on controlling the outbreaks and improving basic infection control measures such as the cleanliness of the hospitals, the compliance of better hand hygiene and infection control education for health care workers. (Corcoran and Kirkwood, 1999, Rahman *et al.*, 2000, Rahman *et al.*, 1999, Barrett and Simmons, 2005, Barrett *et al.*, 1998, van Saene *et al.*, 2004) They advocate high standards of hygiene and the use of parenteral vancomycin regimens for treating and controlling MRSA and claim that this is less intrusive than the search and destroy policy. (Barrett and Simmons, 2005, van Saene *et al.*, 2004, de la Cal *et al.*, 2004, Silvestri *et al.*, 2002)

The different opinions have generated many heated discussions, nevertheless, both sides agree that MRSA is a significant issue for infection control. (Dancer, 2005, Barrett and Simmons, 2005, Cookson, 1997, Marshall *et al.*, 2004, Rahman *et al.*, 1999) It is important to prevent MRSA from becoming endemic in hospitals that are free of MRSA. Also, the improvement of hospital hygiene requires a culture change for better compliance. (Ayliffe *et al.*, 1998, Ayliffe *et al.*, 1999, Marshall *et al.*, 2004)

1.8 Malaysian MRSA

1.8.1 Asian MRSA

The Asia-Pacific SENTRY antimicrobial-resistance-surveillance program between 1999 and 2001 revealed a high percentage of *S. aureus* isolates were MRSA in Asian countries like Japan (67%), Taiwan (60%), Hong Kong (55%) and Singapore (52%). (Christiansen *et al.*, 2004) A high MRSA percentage of 72% was reported in South Korea, which is not a participating country of the SENTRY program. (Chong and Lee, 2000) These rates are significantly higher than the rate reported in Australia and Northern European countries, which range from 0.2% to 33.4% around the same period of time. (Stefani and Varaldo, 2003, Fluit *et al.*, 2001b) However, they are comparable to the MRSA rate in Southern-European hospitals such as Italy (58%) and Portugal (54%), as well as the United States, where the MRSA percentage in the ICU units between 1992 and 2001 was 50.5% and in the non-ICU units 39.9%. (Stefani and Varaldo, 2003, Fluit *et al.*, 2001b, National Nosocomial Infections Surveillance System Report, 2001) On the other hand, a lower MRSA frequency was detected in hospitals from the less developed regions like the Philippines (8%) and mainland China (13%). (Christiansen *et al.*, 2004) The majority of the isolates were resistant to more than three types of antimicrobial agents other than β-lactams. (Bell and Turnidge, 2002, Christiansen *et al.*, 2004) Two MRSA clones were found to be predominant among Asian countries. The ST5-MRSA-II clone (New York/Japan clone) was the predominant MRSA clone in Korean and Japanese hospitals, whereas the ST239-MRSA-III (EMRSA-1, Hungarian/Brazilian clones) and its SLV variants predominated in the hospitals in China, Taiwan, Hong Kong,

Singapore, Thailand, Vietnam, Philippines, India, Sri Lanka and Saudi Arabia. (Ko *et al.*, 2005, Aires de Sousa and de Lencastre, 2004, Enright *et al.*, 2002)

1.8.2 Prevalence of Malaysian MRSA

S. aureus has been reported as one of the most common pathogens in Malaysia causing infections ranging from community-acquired pneumonia to nosocomial infections in surgical patients and neonates in a special care nursery. (Boo and Chor, 1994, Hooi *et al.*, 2001, Lee *et al.*, 1991, Syahrizal *et al.*, 2001) The high rate of MRSA among *S. aureus* would inevitably increase the chances of clinical treatment failure and increase the use of reserved antimicrobial agents. (Lim and Zulkifli, 1987, Livermore, 2003)

MRSA was reported in Malaysian hospitals in the early 1970s. However, it is not know when MRSA was first isolated. (Lim and Zulkifli, 1987, Hanifah *et al.*, 1992) The frequency of methicillin resistance among *S. aureus* strains isolated in the Malaysian hospitals has gradually risen over the years. In 1971, it was reported that only 3% of *S. aureus* were MRSA in the University Hospital of the University of Malaya. (Puthucheary *et al.*, 1972) In the same hospital, the MRSA rate rose to 11.5% in 1979 and by 1985 it was 18.8%. (Lim, 1988, Hanifah *et al.*, 1992) A survey of 22 Malaysian hospitals conducted between 1985 and 1986 revealed that the percentage of MRSA among these hospitals ranged from 10% to 25%. (Lim, 1988) The National Surveillance on Antibiotic Resistance program (NSAR) conducted by the Malaysian Ministry of Health revealed a further increase in the following years. The NSAR results showed that the MRSA rate in 1990 was 20% and rose to 28% in 1992. The MRSA rate rose further to 36% in 1996 before slightly decreasing to 32% in 1998. (Norazah *et al.*, 2003b, Norazah *et al.*, 2001b) The MRSA rate was reported to be higher in some individual hospitals. In a 1996 study of three hospitals located around the capital city, Kuala Lumpur, the average rate was 39.7%. The same study also revealed that the MRSA rate correlated with the size and services provided by the hospitals. The largest tertiary referral hospital of 2500 beds had an MRSA rate of 49.7% compared to 30.1% in an 800-bed referral hospital and 12.9% in a 300-bed hospital. (Rohani *et al.*, 2000) See Figure 1.8.

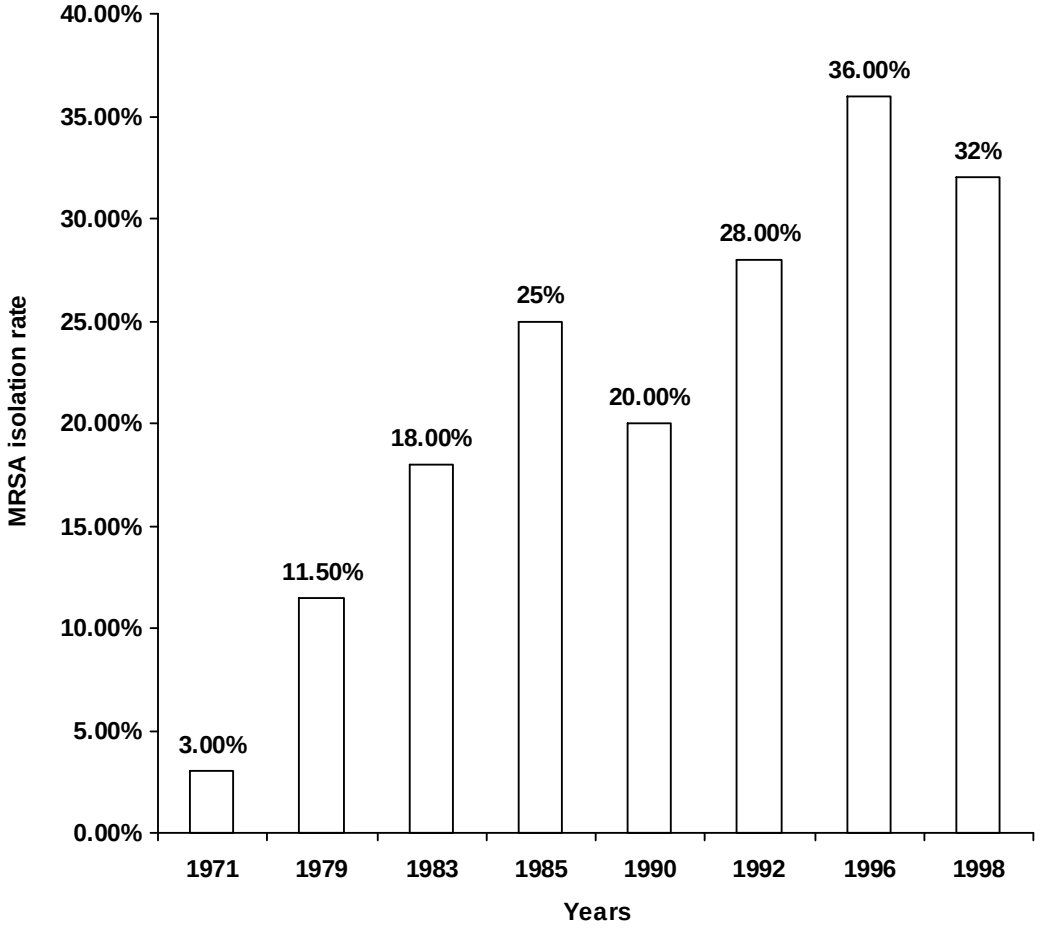


Figure 1.8 The percentage of MRSA among *S. aureus* isolated in Malaysian hospitals from 1971 to 1998.
(Puthucheary *et al.*, 1972, Hanifah *et al.*, 1992, Lim, 1988, Norazah *et al.*, 2003b, Norazah *et al.*, 2001b)

1.8.3 Characterisation of Malaysian MRSA

The majority of MRSA strains isolated in Malaysian hospitals were shown to be mere colonisers and causing only superficial infection. However, these strains did cause serious infections like septicaemia, surgical wound infections and osteomyelitis among immunocompromised patients in intensive care units and in special care nurseries. (Lim and Zulkifli, 1987, Puthucheary *et al.*, 1987, Hanifah *et al.*, 1992) Almost all of the Malaysian MRSA isolates were resistant to more than four classes of antimicrobial agents other than β-lactams. Gentamicin, erythromycin,

tetracycline and ciprofloxacin resistances were the most common resistance phenotype seen in Malaysian MRSA. No isolates with reduced vancomycin susceptibility were detected in Malaysia. (Norazah *et al.*, 2001a, Hanifah *et al.*, 1992, Rohani *et al.*, 2000, Lim and Zulkifli, 1987) Although more than 90% of *S. aureus* isolated in both community and hospitals settings are resistance to penicillin, no MRSA were found in the community. (Cheong *et al.*, 1995, Norazah *et al.*, 2003a, Rohani *et al.*, 2000)

Bacteriophage typing studies done on Malaysian MRSA showed that the majority of the strains were either typable by group III phages, particularly phage 85, or non typable by the IBS set of phages. (Hanifah, 1991, Hanifah *et al.*, 1992) Two distinct MRSA clones were identified from 50 MRSA strains isolated between 1987 and 1989 in the University Hospital of the University of Malaya. (Yoshida *et al.*, 1997, Hanifah *et al.*, 1992) The KL3 clone has a coagulase serotype III and carried the SCC*mec* allotype I. It also had very similar IS*431* hybridisation patterns with NCTC10442, which is the first MRSA, isolated in the UK at 1961. (Yoshida *et al.*, 1997, Hanifah *et al.*, 1992, Ito *et al.*, 2001) The KL3 clone was only moderately resistant to methicillin with an MIC of 12.5 mg/L. (Hanifah *et al.*, 1992) The other clone, KL50, was the predominant clone among the 50 isolates and had a higher level of methicillin resistance (MIC > 50mg/L). (Hanifah *et al.*, 1992) It had a type IV coagulase serotype and carried the SCC*mec* allotype III. (Yoshida *et al.*, 1997, Hanifah *et al.*, 1992, Ito *et al.*, 2001) The IS*431* hybridisation pattern for the KL50 clone was identical to the British strain 86/560 and had the same point mutation in the *mecI* gene. The 86/250 belongs to a group of isolates that have been isolated in European hospitals and countries that have close historical ties to England, such as Hong Kong, Saudi Arabia and New Zealand. (Yoshida *et al.*, 1997, Hanifah *et al.*, 1992, Ito *et al.*, 2001, Hiramatsu, 1995) Both of the clones were found to carry one to four small plasmids ranging from 3.2 kb to 1.6 kb. (Hanifah *et al.*, 1992)

PFGE studies on MRSA isolated between 1997 and 1999 at eight Malaysian hospitals revealed three major PFGE types. One of the PFGE types was found to be predominant and was isolated in all eight hospitals. Along with its subtypes this predominant PFGE type accounted for almost 80% of all MRSA isolated in these eight hospitals. Most of the isolates from this clone were multiply resistant, particularly to erythromycin, gentamicin, tetracycline, co-trimoxazole

(sulphonamides and trimethoprim) and ciprofloxacin. (Norazah *et al.*, 2003b, Norazah *et al.*, 2001b) Another study on MRSA isolated in the Hospital University Kebangsaan Malaysia in 2000 found four distinct PFGE types. Two of these PFGE types were found to predominant and accounted for 84% of all isolates. The isolates with the predominant PFGE type were resistant to erythromycin, gentamicin, and ciprofloxacin. One of the non-predominant PFGE types was gentamicin susceptible. (Alfizah *et al.*, 2002)

1.8.4 Infection control in Malaysian hospitals

Infection control activities in Malaysian hospitals were initiated in the 1970s because of the increasing MRSA problem. However, it was poorly supervised and largely uncoordinated. Almost half of the hospitals failed to meet the standard of hospital infection control in the 1985 quality assurance survey conducted by the Ministry of Health. Responding to the poor standard, a national hospital infection survey was conducted from 1990 to 1993. This survey was to gather data reflecting the situation for hospital infection control practices in Malaysian hospitals. (Lim, 2001) The survey revealed a lack of compliance to the guidelines on antibiotic use issued by the Ministry of Health. (Lim *et al.*, 1993) The incidence of nosocomial infection was grossly under reported. Poor sterilisation and disinfection practices were observed in the six hospitals surveyed. No disinfection and sterilisation policy was available in most of the units. Disinfectant was used as a substitute for sterilisation, items were cleaned and stored in disinfectant solutions, spillages were not decontaminated and the use of diluted disinfectants were a few of the poor practices observed. (Keah *et al.*, 1995) A study, unrelated to the survey, showed that poor disinfection practices were the major cause of neonatal septicaemia in a large Malaysian maternity hospital, from 1986 to 1991, in which MRSA was the major pathogen. (Boo and Chor, 1994) The data gathering by this survey has led to the establishment of the National Antibiotic Policy and Guidelines, an antibiotic resistance-surveillance program and a revised National Policy on Sterilisation and Disinfection. (Lim, 2001)

The progress of the new guidelines and policies was reviewed in 1997. It was found that all state hospitals have established infection control committees and have at least one infection control nurse. The incidence of nosocomial infections was surveyed

and reported in all hospitals. A training program in infection control was conducted in all hospitals, which resulted in an improvement in the compliance to the guidelines and policies on sterilisation and disinfection. Although the situation has been improved, no hospital has the recommended number of infection control nurses and the activities of the infection control committees still require better coordination. (Lim, 2001)

Inadequate infection control, high patient to staff ratios and the lack of resources has been suggested to cause the high nosocomial infection rates (13.95%) observed in a Malaysian university medical centre. MRSA, MSSA and *Pseudomonas aeruginosa* were found to be the most common pathogens. High antibiotic use was also observed in the same study. Although only 36% of antibiotics prescribed were for the treatment of nosocomial infections, they accounted for almost half (47%) of the total antibiotic costs. The antibiotic used to treat nosocomial infection was often broad spectrum and more expensive. The annual cost of antibiotic use for nosocomial infections was estimated to be 1.98 million Malaysian Ringgits (0.68 million Australia dollars). (Hughes *et al*., 2005) An earlier study estimated the annual cost of controlling MRSA in a Malaysian hospital was approximately 250,000 Malaysian Ringgits (114,000 Australian dollars). The additional costs of nosocomial infection are a serious problem for a developing country like Malaysia, which has limited health funding and facilities. (Lee *et al.*, 1991, Hughes *et al.*, 2005)

Malaysian hospitals have achieved major advancements in the field of medicine over the past two decades. As more complex and invasive medical procedures are performed, inevitability the number of immunocompromised inpatients will increase. However, with little advancement in hospital infection control in Malaysia the mortality and morbidity due to nosocomial infections will be expected to increase. (Lim, 2001, Hughes *et al.*, 2005) Infection control in Malaysia is facing a series of challenges. There is a shortage of trained infection control professionals and a general shortage of doctors and nurses. Better awareness of the importance of infection control among administrators, policy makers and healthcare workers is needed. There is a need for better coordination on infection control activities between hospitals in both government and private sectors. Hospital infection control in Malaysia is still in its infancy, it needs more resources, strengthening and

standardising. It is hoped that the establishment of a national surveillance centre will promote better awareness, education and training in infection control. (Lim, 2001)

1.9 Project aims and potential significance

In this study, 74 Malaysian nosocomial MRSA isolates were studied. They were collected in 1982, 1989, 1994 and 2000 at two Malaysian hospitals.

One of the aims of the project was to investigate the genetic relationship of the isolates using both genotypic and phenotypic techniques. The results would give an insight into how Malaysian MRSA have evolved over the 18 years. It would also enable the identification of epidemic strains within the Malaysian hospitals. The project would also examine if there were genetic elements that are unique to Malaysian MRSA.

The other aim was to determine the genetic relationship between Malaysian MRSA and MRSA from different countries. The Malaysian isolates would be compared with UK EMRSAs, classical MRSA, Australian CMRSA and isolates from eight Asian-Pacific countries and South Africa. This would give an insight into the epidemiology of Asian-Pacific MRSA and a better understanding of the evolution of MRSA. It might also enable the detection of inter-hospital spread of MRSA between Malaysian hospitals and other Asian-Pacific hospitals. This study would also enable genotypic and phenotypic typing methods to be evaluated.

MRSA is one of the most common nosocomial pathogens in Malaysian hospitals. It has been endemic in the hospitals since the early 1970s. (Hanifah *et al.*, 1992, Lim, 1988, Norazah *et al.*, 2003b) MRSA cause higher nosocomial infection rates and incur increased costs due to more costly treatment and longer hospital stays. (Lee *et al.*, 1991, Hughes *et al.*, 2005) This study would determine if the dissemination of MRSA in Malaysian hospitals was the result of a single clone spreading or the result of multiple strains spreading. This information would assist in designing suitable infection control measures. The comparison of Malaysian isolates with those from other countries would reveal if any inter-country dissemination of MRSA was occurring. Although this may not help with the current problem it would

demonstrate the need to establish infection control practices to prevent any future importation of potential pandemic and/or highly virulent or resistant MRSA clone. Comparing MRSA from Asian-Pacific countries and South Africa, UK EMRSAs, classical MRSA and CMRSA would also give a better understanding of the epidemiology of MRSA and how they evolve and spread between hospitals and countries."

CHAPTER TWO

MATERIALS AND REAGENTS

2.1 Bacterial strains

The strains used in this study are listed in Table 2.1 and Table 2.2. The WBG prefix indicates that they are from Prof. W.B. Grubb's collection.

Table 2.1 Malaysian isolates

Isolate	Description	Source/Reference
WBG2003 WBG2004 WBG2005 WBG2006 WBG2007 WBG2008 WBG2010 WBG2011 WBG2013 WBG2014 WBG2015	MRSA isolated 1982 - 1983 from the University Hospital of the University of Malaya (HUM), Kuala Lumpur, Malaysia	Prof. S. D. Putchucheary
WBG7409 WBG7410 WBG7411 WBG7412 WBG7413 WBG7414 WBG7417 WBG7419 WBG7420 WBG7422 WBG7424 WBG7425 WBG7426 WBG7427	MRSA isolated in 1989 from the HUM, Kuala Lumpur, Malaysia	Prof. S. D. Putchucheary and Dr. F. Jamal

Continued next page

Table 2.1 continued

Isolate	Description	Source/Reference
WBG7884 WBG7885 WBG7887 WBG7888 WBG7889 WBG7890 WBG7891 WBG7893 WBG7894 WBG7895 WBG7897 WBG7898 WBG7900	MRSA isolate in 1994 from the Hospital of the University of Kebangsaan Malaysia (HUKM), Kuala Lumpur, Malaysia	Dr. F. Moosdeen
WBG7998 WBG7999 WBG8000 WBG8001 WBG8002 WBG8003 WBG8004 WBG8005 WBG8006 WBG8007 WBG8008 WBG8009 WBG8010 WBG8011 WBG8012 WBG8013 WBG8014 WBG8015 WBG8016 WBG8154	1994, HUM, Kuala Lumpur, Malaysia	Prof. S.D. Putchucheary

Continued next page

Table 2.1 continued

Isolate	Description	Source/Reference
WBG8023 WBG8024 WBG8026	MRSA isolate in 1994 from HUKM, Kuala Lumpur, Malaysia	Dr. F. Moosdeen
WBG10358 WBG10359 WBG10360 WBG10361 WBG10362 WBG10363 WBG10364 WBG10365 WBG10366 WBG10367	2000, HUM, Kuala Lumpur, Malaysia	Prof. S.D. Putchucheary

Table 2.2 Other strains used in this study

Isolate	Description	Source/Reference
WBG248	NCTC8325-3	Wei, (1993)
WBG286	Host for phage 85	Wei, (1993)
WBG696	Host for phage 95	Wei, (1993)
WBG512	1968, Royal Perth Hospital, Australia	Townsend *et al*. (1985e)
WBG523	Isolate with chromosomal gentamicin resistance (Tn*3851*) Royal Melbourne Hospital	Townsend *et al*. (1984b)
WBG524 WBG525 WBG526	1983, Royal Melbourne Hospital, Australia	Townsend *et al*. (1983b)
WBG541	WBG248 (NCTC8325-3) mutated to chromosomal fusidic acid and rifampicin resistances	Townsend *et al*. (1983b)
WBG816	1972, Royal Perth Rehabilitation Hospital, Australia	Lim *et al*. (2002)
WBG879	WBG541 lysogenised with phage 85	Townsend *et al*. (1983b)
WBG1161 WBG1163	1983, Austin Hospital, Heidelberg, Australia	Townsend *et al*. (1983b)
WBG1175 WBG1189	1983, Royal Hobart Hospital, Australia	Townsend *et al*. (1983b)
WBG1321	1982, Royal Perth Hospital, Australia	Townsend *et al*. (1985e)
WBG1350	MRSA reference strain, NCTC9468	Townsend *et al*. (1985c)
WBG1434 WBG1438	1972, Royal Perth Hospital, Australia	Townsend *et al*. (1985c)
WBG1876	WBG541 lysogenised with phage J	Townsend *et al*. (1983b)
WBG2713	1983, Royal Free Hospital, London, England	Townsend *et al*. (1984a)
WBG4483	WBG248 transductant and transconjugant carrying plasmids pWBG615, pWBG115, pWBG3 and pE194	Townsend *et al*. (1986b)

Continued next page

Table 2.2 continued

Isolate	Description	Source/Reference
WBG6017	1982, Westmead Hospital, Sydney, Australia	Lim *et al*. (2002)
WBG6070 WBG6083 WBG6085	1986, Repatriation General Hospital, Perth, Australia	Lim *et al*. (2002); Wei (1993)
WBG7483	RN450 transductant carrying pWBG707 trimethroprim resistance conjugative plasmid	Udo *et al*. (1992b)
WBG7583 WBG7597	1993, CMRSA, Kimberley, Western Australia	Udo *et al*. (1993)
WBG8287	1995, CMRSA (WA MRSA), RPH, Western Australia	O'Brien *et al*. (1999)
WBG8400 WBG8404	1995, CMRSA, Eastern Goldfields, Western Australia	O'Brien *et al*. (1999)
WBG8721	*E. coli* DH5α containing pSK636, with a *dfrA* insert	Prof. R.A. Skurray; Leelaporn *et al.* (1994a)
WBG8888	1997, MRSA, South Australia	Dr. P.C. Lee
WBG8873 WBG8897 WBG8900 WBG8918 WBG8952	1997, CMRSA, Adelaide, South Australia	Dr. P.C. Lee
WBG9087 WBG9093 WBG9101	1997, CMRSA, Darwin, Northern Territory	Prof. Bart Currie
WBG9476	*E. coli* DH5α containing C3 probe, 2.5kb *aacA-aphD* insert	Sim (2003)
WBG10035	1997, WSPP1, CMRSA, New Zealand	Ms Helen Heffernan; Adhikari *et al.* (2002)
WBG10049	1999, WSPP2, CMRSA, New Zealand,	Ms Helen Heffernan; Adhikari *et al.* (2002)

Continued next page

Table 2.2 continued

Isolate	Description	Source/Reference
WBG10060 WBG10066	1999, CMRSA, Sydney, New South Wales	Dr. Iain Gosbell
WBG10198 WBG10199 WBG10200 WBG10201 WBG10202	1999, CMRSA, Geelong, Victoria	Dr. Stephen Graves
WBG10265	2000, EMRSA-15, Royal Perth Hospital, Australia	Lim *et al*. (2002)
WBG10267	2000, Irish-2, Royal Perth Hospital, Australia	Lim *et al*. (2002)
81 0532 81 1035 81 1038 91 1201 91 1336 91 1703 91 1827 91 2118 91 2240 91 2652 91 2688	2000, Australia	Ms Paula Lulu Raman, SENTRY program 2000
81 0168 81 0175 81 0295 81 0821 81 0822 91 1310 91 2031 91 2763	2000, Hong Kong	Ms Paula Lulu Raman, SENTRY program 2000
81 0094 81 0131 81 0276 81 0353 81 0766	2000, Japan	Ms Paula Lulu Raman, SENTRY program 2000

Continued next page

Table 2.2 continued

Isolate	Description	Source/Reference
91 1408 91 1423 91 1872 91 2332 91 2337	2000, Japan	Ms Paula Lulu Raman, SENTRY program 2000
91 1497	2000, The People Republic (PR) of China (Mainland China)	Ms Paula Lulu Raman, SENTRY program 2000
81 0090 91 1379 91 1386	2000, Singapore	Ms Paula Lulu Raman, SENTRY program 2000
81 0113 81 1755 91 2441	2000, South Africa	Ms Paula Lulu Raman, SENTRY program 2000
81 0548 81 0552 81 0951 91 3157	2000, Taiwan	Ms Paula Lulu Raman, SENTRY program 2000
BM4293/ WBG10331	*Listeria monocytogenes*, carrying *dfrD* plasmid pIP823.	Charpentier and Courvalin (1997)
COL/ WBG10288	Early MRSA; IS*1272* PCR control	Prof B. Berger-Bächi; Archer *et al*. (1994)
EMRSA-1 EMRSA-2 EMRSA-3 EMRSA-4 EMRSA-5 EMRSA-6 EMRSA-7 EMRSA-8 EMRSA-9 EMRSA-10 EMRSA-11	Epidemic MRSA, England and Wales; WBG10420 to WBG10430	Central Public Health Laboratory, Colindale, England; Lim *et al*. (2002)

Continued next page

Table 2.2 continued

Isolate	Description	Source/Reference
EMRSA-12 EMRSA-13 EMRSA-15 EMRSA-16 EMRSA-17	Epidemic MRSA, England and Wale; WBG10431 to WBG10435	Central Public Health Laboratory, Colindale, England; Lim *et al*. (2002)
IRISH-1 IRISH-2	Epidemic MRSA, Ireland; WBG10436 and WBG10437	Central Public Health Laboratory, Colindale, England; Lim *et al*. (2002)
INDO-1 INDO-2 INDO-3 INDO-4	2000, Dr. Soetomo Hospital, Surabaya, Indonesia; WBG10414 to WBG10417	Dr. Maria Magdalena Padmidewi
NCTC8325	WBG8174/PS47, reference strain	Dr. D.Perret
NCTC10442	1961, London, England, WBG10384. First MRSA	Mr. G.W. Coombs; Ito *et al.* (2001); Enright *et al.* (2002)
DH5α	*E. coli*, host for transformation of pGEM®-T Vector,	Dr. David Groth

2.2 Media

The media used in this study were brain heart infusion agar (BHIA), brain heart infusion broth (BHIB), Luria broth (LB), Luria broth agar (LBA), trypticase soy broth (TSB), trypticase soy agar (TSA) and Mueller-Hinton agar (MHA). These media were purchased from Gibco Diagnostic (Wisconsin, USA).

2.3 "High Pure" (HP) water

The water used in this project was purified through an ELGA Labwater System (Veolia Water System Pty. Ltd.) The conductivity of the water used was approximately 18.2MΩ and will be referred to as HP water in the thesis.

2.4 Antimicrobial agents

Antimicrobial agents and suppliers are listed in Table 2.3

Table 2.3 Antimicrobial agents and suppliers

Antimicrobial	Abbreviations	Supplier
Ampicillin	Amp	Sigma Chemical Company.
Cadmium acetate	Cd	Merck
Chloramphenicol	Cm	Sigma Chemical Company.
Erythromycin	Em	Abbott, Australia
Ethidium bromide	Eb	Sigma Chemical Company.
Fusidic acid	Fa	Leo Laboratories
Gentamicin	Gm	Delta West
Kanamycin	Km	Sigma Chemical Company.
Mercuric chloride	Hg	Ajax Chemicals
Mupirocin	Mp	GlaxoSmithKline
Neomycin	Nm	Sigma Chemical Company.
Novobiocin	Nv	Sigma Chemical Company.
Phenyl mercuric acetate	Pma	Ajax Chemicals
Propamidine isethionate	Pi	May and Baker
Rifampicin	Rf	Ciba Geigy
Sodium arsenate	Asa	Merck
Streptomycin	Sm	GlaxoSmithKline
Tetracycline	Tc	Sigma Chemical Company.
Trimethoprim	Tp	Sigma Chemical Company.

2.4.1 Antimicrobial agents on disc

Commercial antibiotic discs used were supplied by Oxoid and are listed in Table 2.4. Discs that could not be purchased commercially were prepared according to Table 2.5.

Table 2.4 Commercially supplied antibiotic discs

Antibiotics	Abbreviations	Concentration/Disc
Chloramphenicol	Cm	30μg
Ciprofloxacin	Cip	5μg
Erythromycin	Em	15μg
Fusidic acid	Fa	10μg
Gentamicin	Gm	10μg
Kanamycin	Km	30μg
Lincomycin	Lm	2μg
Methicillin	M	5μg
Minocycline	Mi	30μg
Mupirocin	Mup	5μg
Neomycin	Nm	30μg
Novobiocin	Nv	5μg
Penicillin	P	2 units
Rifampicin	Rf	2μg
Spectinomycin	Sp	25μg
Streptomycin	Sm	25μg
Sulphonamide	Su	300 units
Tetracycline	Tc	10μg
Trimethoprim	Tp	25μg
Vancomycin	Vm	30μg

Table 2.5 Preparation of antimicrobial discs

Agent	Concentration	Weight/100ml	Sterile Solvent	Vol. on disc
Asa	0.2μM	3.12g	HP water	20μl
Cd	10^{-2}M	0.54g	HP water	20μl
Eb	15mM	0.59g	HP water	10μl
Hg	0.4μM	1.086g	HP water	10μl
Pi	2%	2.0g	HP water	10μl
Pma	5mM	0.17g	HP water	20μl

The measured volumes indicated in Table 2.5 were dispensed onto the discs and the discs allowed to dry at 35^{o}C overnight. The dried discs were stored in sterile containers containing a sachet of desiccant.

2.4.2 Antimicrobial solutions

Antimicrobial solutions for the preparation of selection and replication media were prepared as indicated in Table 2.6

Table 2.6 Antimicrobial solutions

Antimicrobial	Concentration	Sterile Solvent
Cd	10^{-2}M	HP water
Cm	2.5mg/ml	100% ethanol
Eb	15mM	HP water
Em	1mg/ml	HP water
Fa	1mg/ml	HP water
Gm	2mg/ml	HP water
Km	15mg/ml	HP water
Nv	1mg/ml	HP water
Pi	1%	HP water
Rf	10mg/ml	50% ethanol
Sm	10mg/ml	HP water
Tc	1mg/ml	HP water
Tp	2.5mg/ml	0.5M HCl to 10% of final volume

2.4.3 Antimicrobial selection plates

The recipient strains used in this project were WBG1876 and WBG541, both of which are fusidic acid and rifampicin resistant. All selection plates contained fusidic acid and rifampicin plus one other antibiotic being used to select for plasmid transfer. The concentrations used are shown in Table 2.7. Melted TSA was placed in a 60^{o}C water bath and once the medium had reached this temperature the antimicrobials were added in the volume and concentration described in Table 2.7. The media were poured into 85mm sterile plastic Petri dishes and allowed to set for 15 minutes before they were dried at 35^{o}C with their lids removed and the plates inverted. The plates were then stored at 4^{o}C until required.

Table 2.7 Concentrations of antimicrobial agents in selection media

Antimicrobial	Concentration	Vol. per 100ml TSA
Cd	8×10^{-5}M	0.8ml of 10^{-2}M
Cm	10μg/ml	0.4ml of 2.5mg/ml
Eb	150μM	1ml of 15mM
Em	5μg/ml	0.5ml of 1mg/ml
Fa	5μg/ml	0.5ml of 1mg/ml
Gm	8μg/ml	0.4ml of 2mg/ml
Km	75μg/ml	0.5ml of 15mg/ml
Nv	5μg/ml	0.5ml of 1mg/ml
Pi	0.01%	1ml of 1%
Rf	25μg/ml	0.25ml of 10mg/ml
Sm	50μg/ml	0.5ml of 10mg/ml
Tc	5μg/ml	0.5ml of 1mg/ml
Tp*	10μg/ml	0.4ml of 2.5mg/ml

*Tp was added to MHA instead of TSA because TSA contains thymidine which can be utilised by organisms to bypass the inhibitory mechanism of trimethoprim and thus give a false resistance result.

2.4.4 Antimicrobial replication plates

Transcipients and transconjugants were picked and replicated to media containing antimicrobials at the concentrations listed in Table 2.8. The replication plates were prepared as described in Section 2.4.3. The replication media contained only a single type of antimicrobial agent and were used for screening transcipients and transconjugants after plasmid transfer.

Table 2.8 Concentration of antimicrobial agents in replication media

Antimicrobial	Concentration	Vol. per 100ml TSA
Cd	5×10^{-5}M	0.5ml of 10^{-2}M
Cm	5μg/ml	0.2ml of 2.5mg/ml
Eb	25μM	0.16ml of 15mM
Em	5μg/ml	0.5ml of 1mg/ml
Fa	5μg/ml	0.5ml of 1mg/ml
Gm	2μg/ml	0.1ml of 2mg/ml
Km	50μg/ml	0.33ml of 15mg/ml
Nv	5μg/ml	0.5ml of 1mg/ml
Pi	0.01%	1ml of 1%
Rf	25μg/ml	0.25ml of 10mg/ml
Sm	25μg/ml	0.25ml of 10mg/ml
Tc	5μg/ml	0.5ml of 1mg/ml
Tp*	10μg/ml	0.4ml of 2.5mg/ml

*Tp was added to MHA instead of TSA because TSA contains thymidine, which can be utilised by organisms to bypass the inhibitory mechanism of trimethoprim and thus give a false resistance result.

2.5 Chemicals and reagents

The chemicals and reagents used are listed in Table 2.9. The routine chemicals were laboratory grade reagents.

Table 2.9 Chemicals, reagents, and suppliers

Chemical	Suppliers
Agarose (Chromosomal Grade)	Bio-Rad
Agarose (Molecular Biology Grade)	Promega
Agarose (Pulsed Field Certified)	Bio-Rad
Brij 58 (Polyoxyethelene 20 Cetyl Ether)	Sigma Chemical Company.
Bromophenol blue	TGG, London
CTAB (Cetyltrimethyl ammonium bromide)	Ajax Chemicals
EDTA (Ethylene diamino tetra acetic acid)	Sigma Chemical Company.
Ficol	Sigma Chemical Company.
Isopropanol	BDH Chemicals
IPTG (Isopropyl-β-thiogalactopyranoside)	Promega
Lithium chloride	Sigma Chemical Company.
Lysostaphin	Sigma Chemical Company.
Lysozyme	Sigma Chemical Company.
Polyethylene glycol (PEG) 4000	BDH Chemicals
Proteinase K	Sigma Chemical Company.
RNase	Sigma Chemical Company.
Sarkosyl NL 30 (30% w/v aqueous sodium lauroyl sarcosinate)	BDH Chemicals
SDS (Sodium dodecyl sulphate) ultra pure	ICN (Schwarz/Mann Biotech)
TRIZMA Base (Tris[hydroxymethyl]aminomethane)	Sigma Chemical Company.
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Promega

2.6 Restriction enzyme and buffers

Restriction enzymes and buffers were supplied by Promega. The restriction enzymes and enzyme buffers used are listed in Table 2.10

Table 2.10 Restriction enzymes and buffers

Restriction Enzyme	Buffer
*Cla*I	Buffer C
*Eco*RI	Buffer H
*Hin*dIII	Buffer E
*Pst*I	Buffer D
*Sma*I	Buffer J
*Xba*I	Buffer D

2.7 PCR primers

The primers used for routine PCR (25 primers) and multilocus sequence typing (MLST) PCR (14 primers) were supplied by Fisher Biotech Pty. Ltd., Perth, Australia, and Proligo Australia Pty. Ltd. The six sequencing primers used were supplied by MWG Biotech, Eberberg, Germany. The primers, mI5 and mI6, were designed by using the Hiramatsu *et al.* (1992) *mec* complex sequences. The primers, C1 and C2, were designed according to Oliveira *et al*. (2001b) *ccrA4* and *ccrB4* gene sequences. The primers, D1 and D2, were designed by utilising the published *dfrD* sequences. (Dale *et al.*, 1995b) The ROrf2 primer was designed from the Archer *et al.* (1994) IS*1272* DNA sequence. The primers used are listed in detail in Table 2.11. The location of the primers for the detection of the *mec* complex is shown in Figure 2.1.

The 14 MLST primers used for the multilocus sequence typing (MLST) are listed in Table 2.12. The primers used are according to the DNA sequences published by Enright *et al*. (2000).

Table 2.11 Primers used in this study

Name	Primer sequence (5' to 3')	Nucleotide location	Gene	Accession Numbers	References
***mecA* gene**					
MR1	GTGGAATTGGCCAATACAGG	478-497	*mecA*	Y00688	Song *et al*. (1987), Tokue *et al*. (1992)
MR2	TGAGTTCTGCAGTACCGGAT	1810-1791	*mecA*	Y00688	Song *et al*. (1987), Tokue *et al*. (1992)
***mec* complex**					
mA	ACCAAACCCGACAACTACAAC	69-89	*mecA*	X63598	Archer *et al*. (1994), Niemeyer *et al*. (1996)
mRA1	GTCTCCACGTTAATTCCATT	357-376	*mecR1*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mRA2	GTCGTTCATTAAGATATGACG	676-646	*mecR1*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mRB1	AAGCACCGTTACTATCTGCACA	1211-1230	*mecR1*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mRB2	GAGTAAATTTTGGTCGAATGCC	1445-1424	*mecR1*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mI1	AATGGCGAAAAAGCACAACA	1923-1942	*mecR1*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mI2	GACTTGATTGTTTCCTCTGTT	2403-2383	Region 3' to *mecI*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mI4	TGGACTCCAGTCCTTTTGC	2090-2107	*mecI*	X63598	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mI5	GACTGGAGTCCAAAAACCATTCG	2096-2118	*mecI*	X63598	This study
mI6	AAGACAAGTGAATTGAAACCGCC	2280-2258	*mecI*	X63598	This study
ROrf2	GGACAACTTAAGCCAGGGTA	1074-1055	IS*1272*	U35635	This study
IS-1	ACATTAGATATTTGGTTGCGT	566–596	IS*431*/*257*	X53818	Katayama *et al*. (2001), Barberis-Maino *et al*. (1987)
IS-2	TGAGGTTATTCAGATATTTCGATGT	767–743	IS*431*/*257*	X53818	Katayama *et al*. (2001), Barberis-Maino *et al*. (1987)

Continued next page

Table 2.11 continued

Name	Primer sequence (5' to 3')	Nucleotide location	Gene	Accession Number	Reference
***ccr* gene complex**					
cB	ATTGCCTTGATAATAGCCTTCT	25518-25539	*ccrB*	AB033763	Ito *et al*. (2001)
cA1	AACCTATATCATCAATCAGTACGT	24868-24845	*ccrA1*	AB033763	Ito *et al*. (2001)
cA2	TAAAGGCATCAATGCACAAACACT	26348-26325	*ccrA2*	D86934	Ito *et al*. (2001)
cA3	AGCTCAAAAGCAAGCAATAGAAT	4714-4632	*ccrA3*	AB037671	Ito *et al*. (2001)
C1	TGAAGAAGCACAAGAGCGGC	8548-8567	*ccrA4*	AF411935	This study
C2	CTGCACCACATTTTGGGCAC	10102-10083	*ccrB4*	AF411935	This study
c5F	CGTCTATTACAAGATGTTAAGGATAAT	16321-16347	*ccrC*	AB121219	Ito *et al*. (2004)
c5R	CCTTTATAGACTGGATTATTCAAAATAT	16811-16838	*ccrC*	AB121219	Ito *et al*. (2004)
***dfrD* gene**					
D1	AATCGGCAAGGATAACGACA	135-154	*drfD*	Z50141	This study
D2	ATCTGTTCTCCCCCGAAAAT	395-376	*drfD*	Z50141	This study
Coagulase gene typing					
coag2	CGAGACCAAGATTCAACAAG	1632-1651	3' end region of coagulase gene	D00184	Goh *et al*. (1992), Kaida *et al*. (1987)
coag3	AAAGAAAACCACTCACATCA	2608-2589	3' end region of coagulase gene	D00184	Goh *et al*. (1992, Kaida *et al*. (1987)
Sequencing primers					
M13 forward	GTTTTCCCAGTCACGAC	NA	MCS on pGEM®-T vector	NA	This study
M13 reverse	GTCATAGCTGTTTCCTG	NA	MCS on pGEM®-T vector	NA	This study
T1	GCGGTTTCAATTCACTTGTC	NA	*mecI*	NA	This study
T3	TGTGATATGGAGGTGTAGAAGG	NA	*mecA*	NA	This study
T4	CGAAGACAATGCGAATGG	NA	*mecR1*	NA	This study
T5	ATGATTAAGGCATTCCGAC	NA	*mecR1*	NA	This study

NA, not applicable

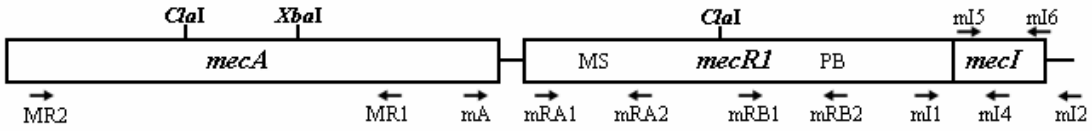


Figure 2.1 The location of the *mec* complex primers

The horizontal arrows indicate the direction of the primers.
MS, membrane-spanning domain of *mecR1*. PB, penicillin-binding domain of *mecRI.*

Table 2.12 MLST primers

Genes	Name	Primer sequence (5' to 3')
arcC	arcC-UP	TTGATTCACCAGCGCGTATTGTC
	arcC-DN	AGGTATCTGCTTCAATCAGCG
aroE	aroE-UP	ATCGGAAATCCTATTTCACATTC
	aroE-DN	GGTGTTGTATTAATAACGATATC
glp	glp-UP	CTAGGAACTGCAATCTTAATCC
	glp-DN	TGGTAAAATCGCATGTCCAATTC
gmk	gmk-UP	ATCGTTTTATCGGGACCATC
	gmk-DN	TCATTAACTACAACGTAATCGTA
pta	pta-UP	GTTAAAATCGTATTACCTGAAGG
	pta-DN	GACCCTTTTGTTGAAAAGCTTAA
tpi	tpi-UP	TCGTTCATTCTGAACGTCGTGAA
	tpi-DN	TTTGCACCTTCTAACAATTGTAC
yqiL	yqiL-UP	CAGCATACAGGACACCTATTGGC
	yqiL-DN	CGTTGAGGAATCGATACTGGAAC

The sequences were published by Enright *et al*. (2000).
arcC : carbamate kinase; *aroE*: shikimate dehydrogenase; *glp*: glycerol kinase; *gmk*: guanylate kinase; *pta*: phosphate acetyltransferase; *tpi*: triosephosphate isomerase; *yqiL*: acetyl coenzyme A acetyltransferase.

2.8 The *dfrA* and *aacA-aphD* DNA probes

The pSK636 plasmid was kindly provided by Prof. R.A. Skurray. The pSK636 plasmid is an 860 bp *Eco*RV/*Eco*RI DNA fragment of the multiple resistance plasmid pSK1 cloned into vector pUC118. (Leelaporn *et al.*, 1994a) This 860 bp fragment contains trimethoprim-resistance gene, *dfrA*. To extract the 574 bp *dfrA* gene specific probe, pSK636 was digested with *Ssp*I. The schematic map of pSK636 is shown in Figure 2.2.

The C3 probe, specific for the *aacA-aphD* genes of Tn*3851* and encoding resistance to gentamicin, kanamycin and tobramycin was kindly provided by Dr. H. C. Sim. (Sim, 2003) It was generated by cloning a 2.5kb *Hin*dIII fragment of Tn*3851* into Bluescript®II pSK+. The 1.3kb internal *aacA-aphD* specific probe was obtained by *Taq*I/*Hin*cII digestion of the clone. The schematic map of the clone is shown in Figure 2.2.

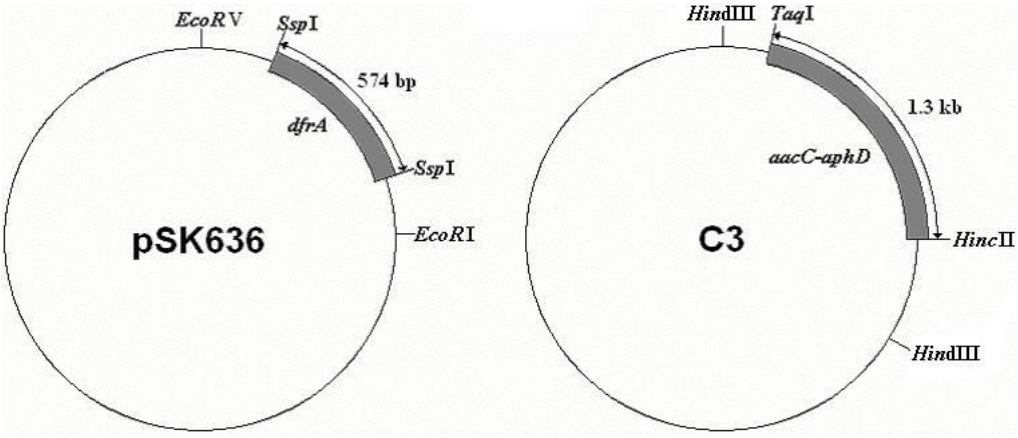


Figure 2.2 Schematic maps of pSK636 and C3

2.9 DNA size markers

Several DNA size markers were used to measure the size of DNA or DNA fragments in gel electrophoresis.

WBG4483 was used as the size marker for measuring the sizes of plasmids. This strain carries four plasmids: pWBG615 (40.3 kb); pWBG115 (22.5 kb); pWBG3 (4.4 kb) and; pE194 (3.5 kb). (Townsend *et al.*, 1986b)

*Sma*I-digested chromosomal DNA of NCTC8325 was used as the size marker for pulsed-field gel electrophoresis (i.e. CHEF). The *Sma*I CHEF pattern of NCTC8325 consists of 16 bands. They are named alphabetically from *Sma*I A band to *Sma*I P band. (Pattee *et al.*, 1990)

The 1 kb Plus DNA ladder (Invitrogen Australia Pty. Ltd.), FN-1 (Fisher Biotech Pty. Ltd.), and *Eco*RI/*Hin*dIII digested Lambda (λ) DNA (Promega), were used as the size markers for DNA fragments and PCR products.

The sizes of the DNA and DNA fragments are shown in Figure 2.3.

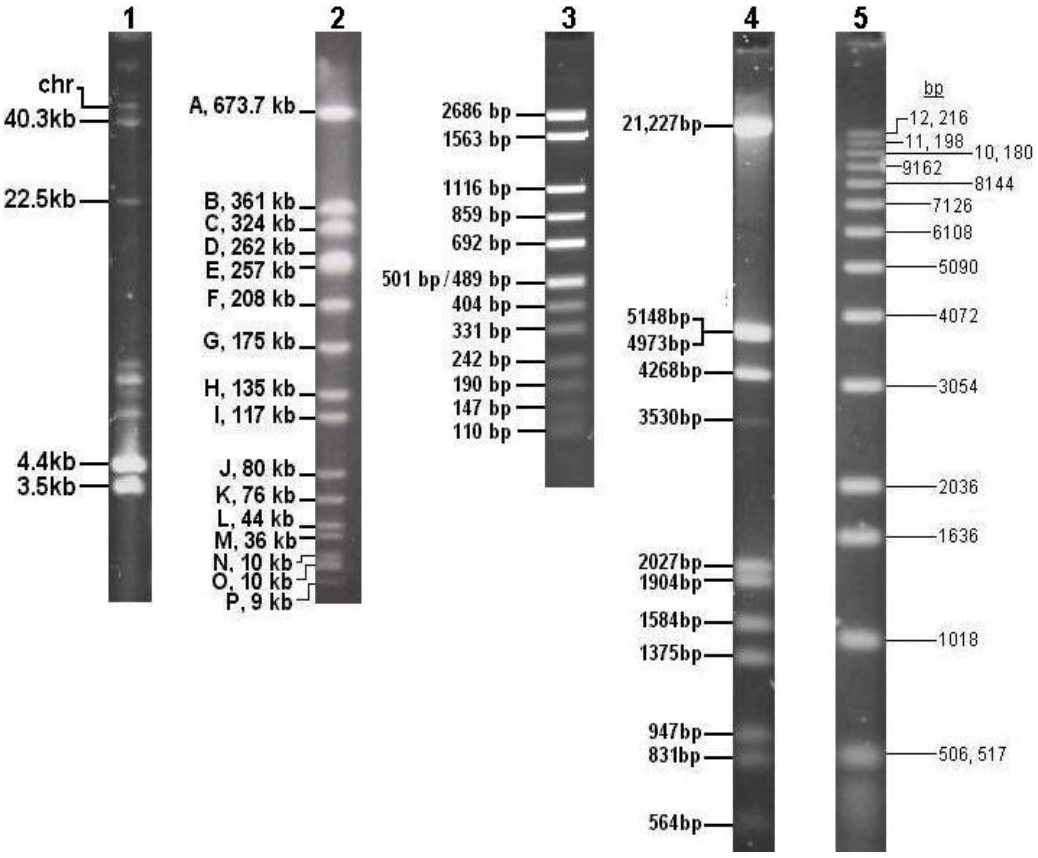


Figure 2.3 DNA size markers

Lane 1 : WBG4483, plasmid size marker

Lane 2 : NCTC8325, CHEF size marker

Lane 3 : FN-1, PCR product and DNA fragment size marker

Lane 4 : *Eco*R1 and *Hin*dIII digested λ DNA, PCR product and DNA fragment size marker

Lane 5 : 1 kb Plus DNA ladder, PCR product and DNA fragment size marker

2.10 Buffers and solutions

2.10.1 Chromosomal DNA and preparation for CHEF

2.10.1.1 EC lysis Buffer:

Brij 58*	0.5%
Sodium deoxycholate*	0.2%
Sarkosyl NL 30*	0.5%

*Dissolved together in HP water at 60^{o}C

EDTA	100mM
NaCl	1M
Tris	6mM

pH adjusted to 7.5 using 10M NaOH.

Do not autoclave

2.10.1.2 50mM EDTA:

EDTA	50mM

HP water

pH adjusted to 8.0 using 10M NaOH.

Autoclaved at 121^{o}C for 20 minutes

2.10.1.3 EST Buffer:

EDTA	0.5M
Tris	5mM
Sarkosyl NL 30	1%

HP water

pH adjusted to 7.5 using 10M NaOH

Autoclaved at 121^{o}C for 20 minutes

2.10.1.4 Lysostaphin:

Lysostaphin	400μg/ml

Dissolved in sterile HP water.

2.10.1.5 Proteinase K:

Proteinase K	2mg/ml

Dissolved in sterile HP water.

2.10.1.6 MacFarland standard for agarose blocks:

A 4 MacFarland standard (1.2×10^9 organisms/ml) was made as follows:

1% Barium chloride	0.4ml
1% Sulphuric acid	9.6ml

2.10.2 Chromosomal DNA isolation

2.10.2.1 Lysis mix:

EDTA	0.1M
NaCl	0.15M
Tris-HCl, pH 8.0	0.1M

Do not autoclave

2.10.2.2 Lysostaphin:

0.1mg/3ml lysis mix

2.10.2.3 SDS/Ethanol:

SDS	5.0%
Ethanol	50%

2.10.2.4 Phenol

Phenol was equilibrated as follows: solid phenol was placed in a sealed container and melted in a 68^{o}C water bath. The melted phenol was then placed in the fume hood and an equal volume of 0.5M Tris-HCl was added and mixed for 20 minutes. The mixture was allowed to set and the aqueous (upper) layer was removed. An equal volume of 0.1M Tris-HCl was added to the remaining liquid and mixed for 20 minutes. It was then allowed to settle and the aqueous phase removed. This was repeated three times until the pH of the phenolic phase was > 7.8 (measured with a

pH paper). Equilibrated phenol can be stored with 0.1M Tris-HCl in a light proof bottle at 4°C for up to 1 month. Phenol is a highly toxic and corrosive chemical. All manipulations were carried out in a fume hood with protective covering.

2.10.2.5 Phenol/Chloroform

Phenol	1 volume
Chloroform	1 volume

Can be stored with 0.1M Tris-HCl in a lightproof bottle at 4°C for 1 month.

2.10.2.6 TE Buffer, pH 8.0:

Tris, pH 8.0	10mM
EDTA, pH 8.0	0.1mM

Autoclaved at 121°C for 20 minutes

2.10.2.7 Ribonuclease (RNase):

Ribonuclease A	10mg/ml

Dissolved in E buffer and steamed for 10 minutes

2.10.3 Plasmid isolation

2.10.3.1 Lysostaphin:

Lysostaphin	150μg/ml

Dissolved in HP water.

2.10.3.2 Ribonuclease (RNase):

See Section 2.10.2.7

2.10.3.3 Lysing solution:

Cethyltrimethylammonium bromide (CTAB)	0.5%
Sodium lauroyl sarcosine (Sarkosyl)	0.5%

HP water

Do not autoclaved.

2.10.3.4 NE Buffer:

NaCl	2.5M
EDTA	10mM

HP water

Autoclaved at 121^{o}C for 20 minutes

2.10.3.5 E Buffer stock (40×):

Tris	40mM
EDTA	2mM

HP water

pH adjusted to 8.0 with glacial acetic acid

Autoclaved at 121^{o}C for 20 minutes

When used, diluted to 2× strength (one in twenty dilution)

2.10.3.6 TE Buffer, pH 7.4:

Tris, pH 7.4	10mM
EDTA, pH 8.0	0.1mM

Autoclaved at 121^{o}C for 20 minutes

2.10.4 Gel electrophoresis

2.10.4.1 Tracking dye:

Bromophenol blue	0.05%
Ficol	20%

2.10.4.2 TAE Buffer (40×):

Tris	1.6M
Sodium acetate	0.8M
EDTA	40mM

HP water

pH adjusted to 7.2 with glacial acetic acid.

Diluted to working strength (1×) when used.

Autoclaved at 121^{o}C for 20 minutes.

2.10.4.3 TBE Buffer (20×):

Tris	1M
Boric acid	1M
EDTA	20mM

HP water

pH adjusted to 8.0 with 10M NaOH.

Diluted to working strength (0.5×) when used.

Autoclaved at 121^{o}C for 20 minutes.

2.10.5 Turbidity standard for NCCLS susceptibility tests

2.10.5.1 0.5 MacFarland standard:

1.175% w/v barium chloride	0.5ml
1% v/v sulphuric acid	99.5ml

2.10.6 Plasmid transfer

2.10.6.1 40% Polyethylene glycol (PEG):

PEG 4000	120g
BHIB	to 300ml

Dissolved in a 56^{o}C water bath.

Dispensed in 100ml aliquots and autoclaved.

2.10.6.2 0.004M Calcium chloride:

0.4M Calcium chloride	1ml
BHIB	99ml

2.10.7 Large scale isolation of *E. coli* Plasmid DNA

2.10.7.1 STE

NaCl	0.1mM
Tris-HCl, pH8.0	10mM
EDTA, pH8.0	1mM

Autoclaved at 121^{o}C for 20 minutes. Stored at 4^{O}C.

2.10.7.2 Solution I

Tris-HCl, pH8.0	25mM
EDTA, pH8.0	10mM

Autoclaved at 121^{o}C for 20 minutes.

Stored at 4^{o}C.

2.10.7.3 Solution II

NaOH	0.2M
SDS	1%

Freshly prepared.

Not autoclaved.

2.10.7.4 Solution III

Potassium acetate	5M
Glacial acetic acid	11.5ml
Sterile water	28.5ml

Autoclaved at 121^{o}C for 20 minutes.

Stored at 4^{o}C.

2.10.7.5 NaCl/PEG

NaCl	1.6M
PEG	13%(w/v)

Autoclaved at 121^{o}C for 20 minutes.

2.10.8 Alkaline Southern blotting

2.10.8.1 5× SSC

NaCl	750mM
Sodium citrate	75mM

pH adjusted to 8.0 with 10M HCl.

Autoclaved at 121^{o}C for 20 minutes.

2.10.9 DNA-DNA Hybridisation

2.10.9.1 Maleic acid Buffer

Maleic acid	0.1M
NaCl	0.15M

pH adjusted to 7.5 with 10M NaOH

Autoclaved at 121^{o}C for 20 minutes.

2.10.9.2 20× SSC

NaCl	3M
Sodium citrate	0.3M

pH adjusted to 7.0 with 10M NaOH

Autoclaved at 121^{o}C for 20 minutes.

2.10.9.3 Blocking reagent stock (10%)

Blocking reagent	10g
Maleic acid buffer	100ml

Autoclaved at 121^{o}C for 20 minutes.

Note: Blocking reagent must be added last or it will not dissolve. Mixture dissolved by constant stirring at 55^{o}C. Blocking reagent should dissolve in maleic acid buffer in about an hour. The solution will appear milky white or cloudy and it should be stored at 4^{o}C for no more than two weeks. Bring to room temperature prior to use. Blocking reagent is commercially supplied by Boehringer Mannheim.

2.10.9.4 Standard prehybridisation solution

Sarkosyl	0.1%
SDS	0.02%
Blocking reagent for nucleic acid hybridisation	1.0% (w/v)

Made up to a total volume of 100ml with 5× SSC and stored at 4^{o}C.

2.10.9.5 Standard hybridisation solution:

The DIG-labelled probe was added to the standard prehybridisation solution until the desired probe concentration was reached. The DIG-labelled probe was heated in boiling water for 10 minutes to denature the DNA before adding to the prehybridisation solution. Stored at -20^{O}C.

2.10.9.6 2× Wash solution:

SSC*	2×
SDS	0.1%

*Diluted from 20× SSC

2.10.9.7 0.5× Wash solution:

SSC*	0.5×
SDS	0.1%

*Diluted from 20× SSC

2.10.10 Chemiluminescent detection

2.10.10.1 Detection Buffer

Tris, pH 7.4	10mM
NaCl	0.1M

pH adjusted to 9.5 with 10M NaOH

Autoclaved at 121^{o}C for 20 minutes.

2.10.10.2 Washing Buffer

Maleic acid	0.3% (w/v)

Not autoclaved.

2.10.11 Probe Stripping

2.10.11.1 Probe-stripping solution

NaOH	0.2M
SDS	0.1%

2.10.11.2 5× SSC

see Section 2.9.8.1 (Alkaline Southern blotting)

2.10.12 Plasmid isolation From *Listeria monocytogenes*

2.10.12.1 Solution 1

50mM Tris/HCl	625µL of 2M Tris/HCl, pH 8.0
1 mM EDTA	125µL of 0.2M EDTA, pH 8.0
6.7% sucrose	1.675g

Add HP H_20 to 25mL

Autoclaved at 121°C for 20 minutes

2.10.12.2 Lysozyme

10mg/ml in 25mM Tris, pH 8.0

2.10.12.3 25mM Tris/HCl

2M Tris/HCl pH 8.0	312.5µL

HP H_20 added to 25mL

Autoclaved at 121°C for 20 minutes

2.10.12.4 Solution 2

0.25M EDTA	1.83g
50mM Tris	0.15g

HP H_20 added to 25mL

pH adjusted to 8.0 using 10M NaOH

Autoclaved at 121°C for 20 minutes

Note: EDTA dissolved first with dilute NaOH (i.e. a few drops of 10M NaOH in HP H_2O) to prevent the EDTA from crystallising (EDTA crystallises around pH 7.0)

2.10.12.5 Solution 3

20% w/v SDS	5g (w/v)
50mM Tris	625µL 2M Tris/HCl pH8.0
20mM EDTA, pH 8.0	250µL, 0.2M EDTA pH8.0

Add HP H_20 to 25mL

Note: Tris and EDTA added first to the HP H_2O, then SDS dissolved in the solution. SDS will take sometime to dissolve as it is a concentrated solution. Do not store at 4^oC as the SDS will precipitate.

2.10.12.6 3M NaOH

10M Na0H 7.5mL

HP H_20 added to 25mL

2.10.12.7 2M Tris/HCl, pH 7.0

24.22g Tris in 100mL of HP H_2O

Adjust pH to 7.0 with HCl

Autoclaved at 121^{o}C for 20 minutes

2.10.12.8 5M NaCI

29.22g in 100mL of HP H_2O

Autoclaved at 121^{o}C for 20 minutes

2.10.12.9 3% NaCl (w/v)

3g NaCl in 100mL of HP H_2O

Autoclaved at 121^{o}C for 20 minutes

2.10.12.10 Phenol saturated with 3% NaCI

Phenol crystals dissolved in a 50^{o}C water bath. An equal volume of 3% NaCl added and stirred for 15 mins. The suspension was allowed to settle until the two phases separated. The aqueous phase (i.e. NaCl) was removed. A new volume of 3% NaCl was added and the process repeated. The saturated phenol was kept in a light proof bottle overlaid with 3% NaCl.

2.10.12.11 Chloroform-isoamyl alcohol (24:1)

24mL of chloroform with 1mL of isoamyl alcohol

Note: The mixing was done in a fume hood.

2.10.13 Freeze-drying

Sodium glutamate	5.0g
Peptone	5.0g
Sterile HP water	to 100ml

2.11 Details of the suppliers

The details of the suppliers of chemicals and reagents used in this study are listed in Table 2.13

Table 2.13 Details of the suppliers

Brand name	Suppliers address
Abbott, Australia	Abbott Australia Pty. Ltd. P.O.Box 101, Cronulla, NSW 2230, Australia
Accelrys Ltd.	Accelrys Ltd. 334 Cambridge Science Park Cambridge, United Kingdom.
Ajax Chemicals	APS Chemicals Ltd. (Formally Ajax Chemicals) 8 Abbott Rd Seven Hills NSW 2147, Australia
Amersham Biosciences	Amersham Biosciences 800 Centennial Avenue P.O. Box 1327 Piscataway, NJ 08855-1327, USA
BBL	Beck Dickson Microbiology System Becton Dickson and Company Cockeysville, Maryland 21030 USA
BDH Chemicals	Merck Pty. Ltd. 207, Colchester Rd, Kilsyth, Victoria 3137, Australia.
Bio-Rad Laboratories	Bio-Rad Laboratories Pty. Ltd. Unit 1 Block Y, 391, Park Road, Regents Park NSW 2143, Australia
Boehringer Mannheim	Boehringer Mannheim Gmbh Sandhofer Strasse 116, D-68305 Mannheim, Germany.

Continued next page

Table 2.13 continued

Brand name	Suppliers address
Ciba Geigy	Novartis International AG (Formally Ciba Geigy) CH-4002 Basel, Switzerland
ELGA Labwater System	Veolia Water System Pty. Ltd. Level 4, 65 Pirrama Rd, Pyrmont, NSW 2009, Australia
Epicentre Technologies	Epicentre Technologies 726 Post Road, Madison, WI 53713 (U.S.A.)
Fisher Biotec Pty. Ltd.	Fisher Biotec Pty. Ltd. 2/64 Thomas Street West Perth WA 6005, Australia.
GeneWorks	GeneWorks Pty. Ltd. PO Box 299, Hindmarsh, SA 5007, Australia
GlaxoSmithKline	GlaxoSmithKline, Pharmaceuticals Division P.O. Box 168, Boronia, Victoria 3155, Australia
ICN (Schwarz/Mann Biotech)	ICN Pharmaceuticals Australia Pty. Ltd. 85 St Hilliers Rd, Auburn 2144, NSW, Australia
Invitrogen Australia Pty. Ltd.	Invitrogen Australia Pty. Ltd. 2A/14 Lionel Rd, Mount Waverley, VIC 3149, Australia
Kodak™	Kodak (Australia) Pty. Ltd. P.O.Box 90, Coburg 3058, Australia
Leo Laboratories	Leo Laboratories, 285, Cashel St., Dublin 12, Ireland.
May and Baker	May and Baker PLC 3/5 Sapara Street Industrial Estate Ikeja Lagos, Nigeria
Merck	Merck Pty. Ltd. 207, Colchester Rd, Kilsyth, Victoria 3137, Australia

Continued next page

Table 2.13 continued

Brand name	Suppliers address
MWG-Biotech GMBH	MWG Biotech AG (Hauptsitz) Anzinger Strasse 7a , D-85560 Ebersberg bei München, Germany
Oxoid	Oxoid Australia Pty. Ltd. 104 Northern Rd, West Heidelberg, Melbourne, VIC 3081, Australia
Proligo	Proligo Australia Pty. Ltd. Level 5, T-Block, Southern Cross University, Military Rd, Lismore NSW 2480, Australia
Promega	Promega Corporation 37 Nelson Street Annandale NSW 2038, Australia
Li-COR	Li-COR Biosciences 4308 Progressive Avenue, P.O.Box 4000, Lincoln, NE 68504, USA
Sigma Chemical Company	Sigma-Aldrich Pty. Ltd. Unit 2, 14 Anella Avenue, Castle Hill, NSW 2154, Australia
Ultra-Violet Products Ltd.	Ultra-Violet Products Ltd. Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge CB4 1TG UK

CHAPTER THREE

METHODS

3.1 Disc diffusion test

Disc diffusion sensitivity testing was performed according to the US NCCLS (**N**ational **C**ommittee for **C**linical **L**aboratory **S**tandards) method. Seventy ml of MHA was added to a 140 mm Petri dish to achieve an agar depth of approximately 4mm. The test strain was plated out onto TSA and incubated overnight at 35^{o}C. Colonies were picked with a sterile loop and emulsified in 5ml of sterile saline to give a MacFarland standard of 0.5. The suspension was then spread onto a MHA plate using a sterile cotton wool swab. The antibiotic discs were aseptically placed onto the inoculated plate using sterile forceps. The plates were then incubated at 35^{o}C for 24 hours. The diameters of the inhibition zones were measured to the nearest millimetre. The interpretation of the sensitivity was determined according to the NCCLS Interpretive Standards Chart. The strains were interpreted as susceptible, moderately susceptible, intermediate resistant, or resistant, to a particular antibiotic according to the diameter of the inhibition zone.

3.2 β-Lactamase Test

β-lactamase production was detected using BBL™ Cefinase discs. (Becton Dickson Microbiology System) The disc containing nitrocefin was moistened with sterile saline and a colony of the test organism from an overnight TSA placed onto the disc with a sterile toothpick. Development of a pink colour after 5 to 60 minutes indicated a positive reaction. The test only detects the presence of β-lactamase and cannot distinguish different classes of β-lactamases.

3.3 Bacteriophage typing

Bacteriophage or phage typing in this study was performed using the International Basic Set of twenty-six bacteriophages listed in Table 3.1. Susceptibility to the

phages was recorded at 100 times the Routine Test Dilution (RTD). (Vickery *et al.*, 1986, Hanifah, 1991)

Table 3.1 International basic set of bacteriophages:

Group I:	29	52	52A	79	80					
Group II:	3A	3C	55	71						
Group III:	6	42E	47	53	54	75	77	83A	84	85
Miscellaneous:	88	187	81	94	95	96	90			

3.4 Staphylococcal plasmid DNA isolation

Plasmid DNA was isolated using a modified version of the CTAB technique described by Townsend *et al*. (1985b) *S. aureus* strains were grown in 5ml of BHIB overnight at 37^{o}C with gentle shaking. One ml of the overnight culture was then transferred into a 10ml TSB and grown overnight at 37^{o}C with gentle shaking.

The culture was then centrifuged in a 10ml Oakridge tube at 4000g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended with 900µl of NE buffer and 100µl of lysostaphin (150µg/ml). The mixture was vortexed and then incubated in a 37^{o}C water bath for 30 minutes. After the incubation, 2ml of pre-warmed lysing solution was added gently to the side of the tube taking care not to mix the lysing solution with the contents of the tube. The contents were then incubated in a 60^{o}C water bath for 10 minutes. It is important to keep the temperature of the contents above the room temperature to prevent premature precipitation of the plasmid DNA. The tubes were then centrifuged at 15,000g for 15 minutes at 30^{o}C.

The supernatant was transferred into a fresh 10ml polyallomer tube containing 5ml of sterile HP water taking care not to mix the supernatant with the water. The tubes were then centrifuged at 4000g for 10 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 600µl of E buffer and 300µl of NE buffer. Twenty µl of RNase was added to the contents, mixed gently and the mixture incubated at 37^{o}C for 30 minutes. After incubation, 750µl of chloroform was added

to the contents and mixed gently by rocking the tubes for 10 to 15 minutes (*c.* 100 times). The tubes were then centrifuged at 4000g for 20 minutes at room temperature.

The 500µl upper aqueous layers were removed into separate 1ml microfuge tubes and 500µl of isopropanol added to each tube and the contents mixed thoroughly by gentle inversion. The contents were then centrifuged at 13,000g for 5 minutes at room temperature for the recovery of plasmid DNA. The supernatants were discarded and the tubes were inverted and left to dry at 37^{o}C for 30 to 40 minutes, or air-dried at room temperature, until the last traces of isopropanol had evaporated. The dried plasmid DNA pellets were then dissolved with 30µl of TE buffer (pH 7.4) and stored at 4^{o}C overnight.

The plasmid profiles of the strains were visualised by using agarose gel electrophoresis. Fifteen µl of plasmid DNA were mixed with 6µl of tracking dye by centrifugation. The mixtures were then loaded into the wells of a 0.6% agarose gel (molecular grade, Bio-Rad Laboratories). The gel was electrophoresed in 0.5 × TAE buffer at 22 volts for 16 hours. After the electrophoresis, the gel was then stained in 600ml of 1.0g/l Eb for 40 minutes. The stained gel was viewed on a UV-transilluminator (Ultra-Violet Products Ltd.) and photographed with a Polaroid 107 camera with a red filter and Polaroid 667 black and white film.

Strain WBG4483 was used as a molecular size standard, and as a control, for plasmid isolation. The sizes of the isolated plasmids were calculated with the Mac Vector 4.0 computer program using the CCC form of the plasmids, except in the case of large relaxed plasmids, which were not isolated in the CCC form.

3.4.1 Interpretation of plasmid profiles on agarose gels

The CTAB method is a very mild extraction method and is particularly useful for detecting the CCC conformer of plasmids that are often only detected in the OC form by other methods. (Townsend *et al.*, 1985b) However, the method also often results in higher yields of linear and OC forms (Townsend *et al.*, 1985b) and this requires

experience in interpreting agarose gels of plasmid isolations. Consequently, WBG4483 is not only used as a molecular weight control but is also used to assist in interpreting the bands on gels. WBG4483 was constructed using known plasmids (Townsend *et al.*, 1986b) and the interpretation of the resulting bands in different preparations has been published. (Townsend *et al.*, 1985b, Udo *et al.*, 1987, Udo and Grubb, 1991a, Udo and Grubb, 1990a) The sizes of the plasmids in WBG4483 are : pWBG615, 40.3 kb; pWBG115, 22.5 kb; pWBG3, 4.4 kb; pE194, 3.5 kb. Only the CCC conformer of these plasmids is included on the gel pictures in the results section. (Townsend *et al.*, 1986b)

The contamination of chromosomal DNA was always observed in the plasmid DNA preparations. The chromosomal DNA was represented as a band slightly above the CCC form of 40.3 kb plasmid of WBG4483. The three different conformers of plasmid DNA travelled at different speeds during agarose gel electrophoresis due to their structural differences. The OC form travelled the slowest and the CCC form travelled the fastest. As a result, the three conformers of plasmid DNA appear in the agarose gel in the order of OC, linear and CCC forms. The majority of the plasmid DNA prepared by the CTAB method was in the CCC form. The CCC form of plasmids is usually the brightest and the strongest of the bands observed in agarose gels. When present the linear and OC forms appear as weak bands above the CCC form. This can be seen in Figure 3.1. For large low copy number plasmids, the linear and OC forms are often too faint to be observed on agarose gels. Also, some large highly relaxed plasmids are often only seen as the OC form. Another factor that has to be taken into account in interpreting plasmid sizes is that the higher the concentration of DNA the faster the CCC form migrates on agarose gels. (Personal communication, Prof. W. B. Grubb and Dr F. G. O'Brien)

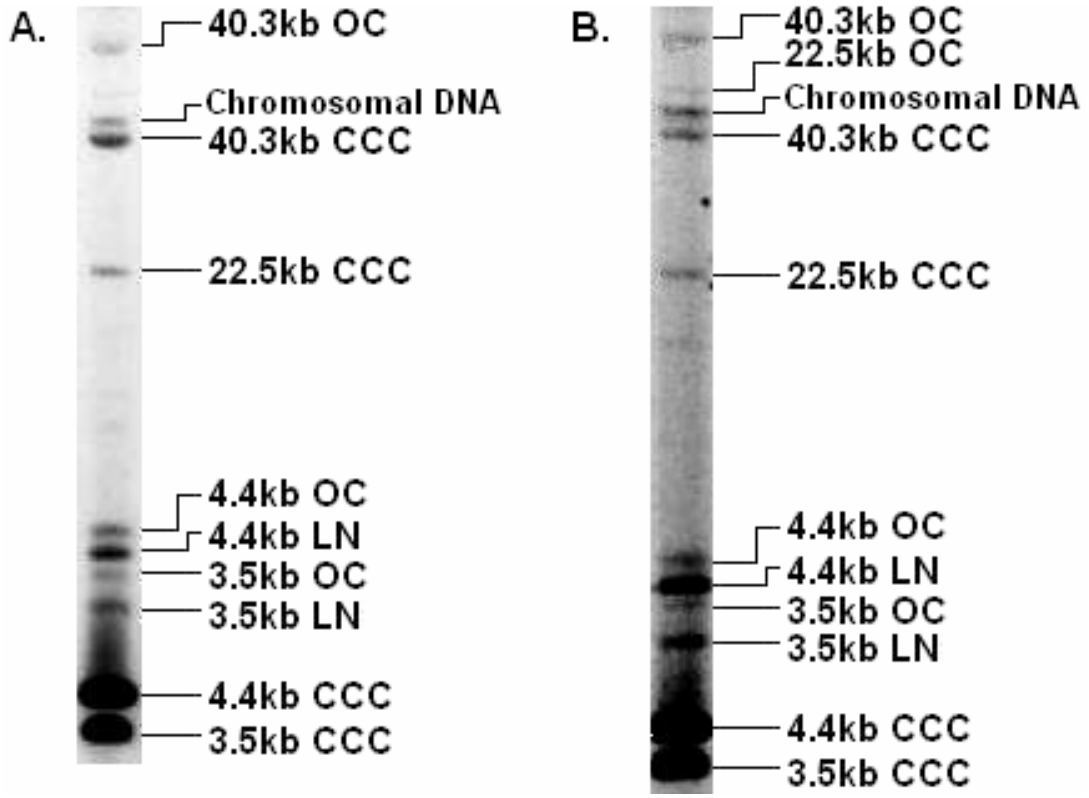


Figure 3.1 WBG4483 plasmid profile

A. and B. are different plasmid isolations of WBG4483 run on agarose gels. Note the different amounts of conformers visible in the different preparations.

LN, linear form; OC, open circular form; CCC, covalently closed circular form

3.5 Restriction endonuclease analysis of plasmids

Assessment of the total amount of DNA was made from the plasmid profile and either 5µl or 10µl DNA was used. To a 1.5ml microfuge tube on ice, either 5µl or 10µl of DNA, 1µl of restriction enzyme and an appropriate amount of reaction buffer as per the instructions of the manufacturer. The volume was adjusted to 20µl with HP water and incubated overnight at 37^{o}C. The reaction was terminated by heat inactivation at 65^{o}C for 15 minutes.

The reaction mixture was mixed with 2µl of tracking dye and then loaded onto a 0.8% agarose gel and electrophoresed at 16 volts for 22 hours in 0.5 × TAE buffer. An *Eco*RI and *Hin*dIII digested lambda marker was used as a molecular weight standard. The sizes of the DNA fragments were determined using the MacVector computer program or by linear regression with DNA size markers.

3.6 Plasmid study

To study the characteristic of individual plasmids they were transferred into a recipient strain with a known genetic background. The transfer was carried out using two methods, mixed-culture transfer and conjugation. The other method used was plasmid curing. This technique, induced plasmid loss and detected the lost phenotype.

3.6.1 Mixed-culture transfer (MCT)

The precise mechanism involved in MCT is not yet understood. It requires cell-to-cell contact, the presence of calcium ions and the presence in the genome of the recipient of a prophage to which the donor is susceptible,. This mechanism is also known as phage-mediated conjugation. (Lacey, 1980) The resulting strain is known as either a transferrant or transcipient.

A recipient was chosen which was lysogenised by a phage to which the donor was susceptible. The bacteriophage susceptibility of the donor was carried out according

to the following procedure. An overnight TSB culture of the strain was diluted (1:100) in saline and spread with a cotton wool swab onto a TSA plate containing 0.04M $CaCl_2$. Bacteriophages were spotted onto the plate and the plate incubated at 30°C for 24 hours. Lysis indicated that the strain was susceptible to the bacteriophage.

Table 3.2 The bacteriophages used in MCT

Bacteriophages	Titre (pfu/ml)	Propagating strain
J	1.2×10^{10}	WBG 248
85	3.1×10^{10}	WBG 286
95	1.4×10^{10}	WBG 696

The matching donor and recipient strains were then grown separately in 5ml of BHIB at 37°C overnight. One hundred µl each of the donor and recipient were then added to 5ml of BHIB containing 0.01M calcium chloride and incubated overnight at 37°C with gentle shaking. The culture was then centrifuged at 1,500g in a Hettich centrifuge for 10 minutes and the pellet inoculated onto a selection plate using a sterile cotton wool swab. The antimicrobials used in the selection media depended on the resistance markers of the recipient and the resistance being selected. All plates were incubated at 37°C for 48 hours before recording the result. Where transfer occurred, transcipients were purified by plating onto selection media. Transcipients were screened for plasmids and for the co-transfer of resistance by the disc diffusion method.

3.6.2 Conjugation

The precise mechanism of plasmid conjugation in staphylococci is yet to be determined. However, it is known that it involves conjugative plasmids (Tra^+) and cell-to-cell contact. (Berg *et al.*, 1998)

Conjugation was carried out by the PEG method. (Townsend *et al.*, 1983a) Donor and recipient strains were grown overnight in 5ml BHIB and 2ml of each cell culture mixed together and pelleted by centrifugation. The supernatant was then discarded

and the pellet was resuspended with 1ml of BHIB. Four ml of 40% PEG dissolved in BHIB was added to the suspension and mixed well. The mixture was then incubated at 37^{o}C overnight with gentle shaking. After incubation, the mixture was pelleted by centrifugation and resuspended in 1ml of BHIB. Three serial 10-fold dilutions were made of the suspension and 0.1ml of each dilution, and the neat mixture, spread separately onto selection plates. Transconjugants were screened for plasmids and for co-transfer of resistance determinants by the disc diffusion method.

3.6.3 Plasmid curing

The strains were grown in 5ml of BHIB overnight at 37^{o}C with gentle shaking. The overnight culture was inoculated into a fresh 5ml of BHIB and inoculated at 43^{o}C overnight. This procedure was repeated three times (i.e. three days). After incubation, the overnight culture was pelleted and inoculated on drug-free BHIA plates. The plates were allowed to growth overnight. Individual colonies were picked with sterile toothpicks and transferred into separate wells of a replicator tray, each well containing one drop of sterile saline. The colonies were then inoculated onto different selection plates and a drug-free plate for control. These plates were incubated overnight at 35^{o}C and examined for the loss of phenotype. The colonies which had lost a phenotype(s) were screened for plasmids and for the loss of additional resistance determinants by the disc diffusion method

3.7 Contour-clamped homogeneous electric field electrophoresis (CHEF)

CHEF procedures were carried out as previously reported. (O'Brien *et al.*, 1999) Bacterial cells were harvested from 5ml overnight culture and resuspended in EC lysis buffer to a turbidity of 0.5 MacFarland standard. The cells were embedded in 0.5% chromosomal-grade agarose (Bio-Rad Laboratories) with 100µg/ml of lysostaphin (Sigma Chemical Company). The gel blocks were incubated in EC lysis buffer for 3 hours for cell lysis. After cell lysis, the gel blocks were treated with 0.2mg/ml proteinase K (Sigma Chemical Company) in EST buffer. The treated gel blocks were washed with four changes of 50mM EDTA and stored in 50mM EDTA until used. The gel blocks were washed with sterile cold water for 30 minutes before

digestion with four units of *Sma*I restriction enzyme (Promega). The digested gel blocks were loaded into a 1% pulsed-field grade agarose gel (Bio-Rad Laboratories) and electrophoresed in a CHEF-DR III™ System (Bio-Rad Laboratories). The initial pulse time was 1 second and the final pulse time was 40 seconds. After electrophoresis, the gel was stained with ethidium bromide and digitised by the Flour-S™ MultiImager system (Bio-Rad Laboratories). The *Sma*I generated CHEF patterns were analysed by the Multi-Analyst®/PC software and a dendrogram of the CHEF patterns generated using the Dice coefficient (Bio-Rad Laboratories). Isolates with more than 80% similarity were considered to be related. This corresponds to the Tenover *et al* (1995) criteria. (Coombs *et al.*, 2006)

NCTC8325, a methicillin-sensitive *S. aureus* which was used as a molecular size marker and used as a reference for the alignment of the gel in the Multi-Analyst®/PC software. (Bio-Rad Laboratories)

3.8 Chromosomal DNA isolation

The technique used was that of Dr. B. Berger-Bächi. (Dr. J. Gustafson, personal communication). The strains were grown in 20ml of BHIB overnight. The culture was then transferred into a 50ml Oakridge tube and the cells pelleted by centrifugation at 15,000g for 1 minute at 4^{o}C. The supernatant was discarded and the cell pellet was resuspended with 3ml of lysis mixture and 750µl of lysostaphin (400µg/ml). The tubes were then incubated in a 37^{o}C water bath for a minimum of 30 minutes.

After the incubation, 300µl of 5% SDS, dissolved in 50% ethanol, was added to the contents. The mixture was then vortexed for 10 seconds and left at room temperature for 5 minutes. One ml of phenol/chloroform (1:1) was added to the mixture, vortexed for 10 seconds, and then centrifuged at 15,000g for 10 minutes.

After the centrifugation, the upper layer was transferred into a sterile 10ml glass bottle and overlaid with 2 volumes of 100% ethanol. Using a Pasteur pipette, or a glass rod, the DNA was spooled out with a gentle constant swirling movement. The

DNA on the Pasteur pipette or rod was then washed with two changes of 4ml of 100% ethanol which were held in two separate 10ml Oakridge tubes. After washing, the DNA was placed in 2ml of TE buffer (pH 8.0) and allowed to dissolve. Twenty µl of RNase were added to the DNA solution and the tube gently shaken overnight at room temperature. The DNA can then be stored at -20^{o}C until needed.

3.9 Determination of isolated DNA concentration

Ten µl of DNA was diluted with 490µl of TE buffer, pH 8.0, and the optical density (OD) measured at 260nm. An OD of one is known to be equal to 50µg/ml of DNA. The concentration of the diluted sample can be calculated as follows:

Concentration of isolated DNA = $OD_{260nm} \times DF \times 50$ µg/ml

DF is the dilution factor, which is 50 in this case.

3.9.1 Restriction analysis of isolated chromosomal DNA

Ten µg of DNA, 1µl of restriction endonuclease and 1µl of restriction enzyme buffer were added to a tube held on ice. Sterile HP water was added to make up a total volume of 10µl. The mixture was incubated at 37^{o}C overnight and the reaction was terminated by heat inactivation at 65^{o}C for 15 minutes. The DNA can be stored at -20^{o}C until used.

Three µl of digested DNA (1.5µg) was mixed with 5µl of tracking dye and loaded onto a 1% agarose gel. The gel was electrophoresed at 50 volts for 3.5 hours. The gel was then stained with ethidium bromide and photographed. After photographing, the DNA in the gel was transferred to a membrane by alkaline Southern blotting. (see Section 3.17)

3.10 Polymerase chain reaction (PCR)

PCR procedures were carried out as described by Frenay *et al*. (1996). The amplification was performed in an iCycler 96 well 0.2ml Reaction Module (Bio-Rad Laboratories). Each 25µl of the amplification mixture contained 2.5 pmol/µl of each primer, 1µl of chromosomal DNA template and final concentrations of 2mM $MgCl_2$, 200µM dNTP, 2.5 unit/µl of *Taq* polymerase (Promega), 5mM KCl, 1 mM Tris-HCl at pH9.0 and 0.01% TritonX-100. Amplification was performed as follows: one cycle at 95^{o}C for 4 min; 25 three step cycles at 95^{o}C for 1 min, 60^{o}C for 1 min and 72^{o}C for 1 min; and one cycle of 72^{o}C for 1 min. After amplification the products were stored at 4^{o}C until used. The PCR products were visualised by agarose-gel electrophoresis.

All the primers used in the study are listed in Table 2.11 and 2.12

3.11 Cloning of PCR products

Four stages were involved in cloning PCR products. The PCR products were first purified. This was done by agarose-gel extraction. The second stage was the cloning of the extracted PCR product into a plasmid vector. The cloned vector was then transferred into competent *E. coli* DH5α.

3.11.1 Extraction of PCR products from agarose gel

The extraction was carried out using the BRESA™clean DNA Purification Kit (GeneWorks Pty. Ltd.). One hundred µl of PCR product was electrophoresed in 1% agarose, dissolved in 1× TAE buffer, for 22 hours at 16 volt in TAE buffer. The gel was cut into half, one half was stained with 1.0g/l Eb and visualised under UV light. The position of the PCR product band was measured, and the corresponding position in the unstained half of the gel cut out. This procedure was used to prevent the UV degradation of the Eb stained PCR product, especially the 3' A (deoxyadenosine nucleotide) overhang which is important for the further cloning procedures. The weight of the excised gel strip was measured and the volume of agarose was

estimated (1g = 1ml). The gel strip was placed in a clean 1.5ml microfuge tube with three volumes of BRESA-SALT™ and incubated at 50°C for 5 minutes, with occasional mixing, until the agarose gel had melted. Seven µl of vortexed BRESA-BIND™ (DNA-binding silica matrix) was added to the tube and incubated at room temperature for 5 minutes, with constant mixing, to keep the BRESA-BIND™ in suspension. After the incubation, the mixture was centrifuged for 5 seconds at 21,637g. The supernatant was removed and the BRESA-BIND™/DNA pellet was resuspended with BRESA-WASH™ (same volume as the BRESA-SALT™). The tube was centrifuged at 21,637g for 20 seconds and the supernatant removed. For the recovery of DNA, the pellet was resuspended in 14µl of Ultrapure water (Fisher Biotec Pty. Ltd.) and incubated at 50°C for 5 minutes. The tube was then centrifuged at 21,637g for 20 seconds and the supernatant containing the DNA was removed to a fresh 1.5ml microfuge tube. The step was repeated once to increase the yield. The recovered DNA was then electrophoresed in a 1% agarose gel in parallel with the original PCR product to determine the efficiency of the recovery.

3.11.2 Ligation

The ligation procedure was carried out using the pGEM®-T Easy Vector System (Promega). The pGEM®-T vector has a 5' T over hang. The components of the ligation mixtures are shown in Table 3.3.

Table 3.3 Formula for ligation reactions

Reagents	Test	Positive Control	Background Control
2 × Rapid ligation buffer	5µl	5µl	5µl
pGEM®-T Easy Vector	1µl	1µl	1µl
Extracted PCR product	3µl	-	-
Control Insert DNA	-	2µl	-
T4 DNA ligase	1µl	1µl	1µl
Sterilized deionised water	-	1µl	3µl

The total volume of the mixture was 10µl and the ligation mixture was mixed gently with pipetting and incubated at 4°C overnight. After incubation, the mixture was ready to be used for transformation with the competent cells.

3.11.3 Preparation of competent cells

The strain used was *E coli* DH5α. DH5α was inoculated into 5ml of LB and incubated overnight. The overnight culture (0.8ml) was inoculated into 40ml of LB and grown for 2 hours until the OD_{600} was between 0.4 and 0.6. The culture was transferred into a sterile 50ml centrifuge tube and the cells were pelleted by centrifugation of 4,274g for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 20ml of ice-cold 0.1M $CaCl_2$. The resuspension was allowed to sit on ice for an hour and the tube was centrifuged at 4,274g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 2ml of ice-cold 0.1M $CaCl_2$. The resuspension was transferred to a 1.5ml centrifuge tube and was ready to be used for the transformation procedure.

3.11.4 Transformation and screening of the clones

The vector plasmid with the inserted PCR product was transformed into competent cells. Ten µl of ligation reaction containing vector and ligated DNA insert was centrifuged at 21,637g for 30 seconds and 2µl of the contents was taken from the bottom of the tube and added to a tube containing 50µl of freshly prepared competent cells. The tube was mixed by gently flicking on the tube and allowed to sit on ice for 20 minutes. The tube was then placed in a 42°C water bath and heat shocked for 1 minute. After the heat shock, the tube was immediately placed on ice for 2 minutes. After the incubation, 950µl of LB was added to the tubes and incubated for 90 minutes at 37°C with gentle shaking. One hundred µl of the mixture was then plated onto a LBA plate containing X-gal, IPTG and ampicillin (XIA). The XIA plate was incubated overnight at 37°C. After the incubation, white colonies were picked from the plate and subcultured onto a fresh XIA plate and incubated overnight at 37°C. Alternatively, the plates may be stored at 4°C for better colour development. The background control should not have any growth and the white colonies on the

positive control should also be subcultured onto a fresh XIA plate. The white colonies were then inoculated into 3ml of LB broth for the small-scale plasmid isolation procedure for *E. coli*. (See Section 3.12.4). The isolated plasmid DNA was then digested with *Eco*RI and electrophoresed in a 1% agarose gel to separate and visualise the insert DNA and the vector DNA.

3.12 Large scale isolation of plasmid DNA from *E coli*

3.12.1 Harvesting

A loopful of *E. coli* DH5α containing a plasmid was grown overnight at 37°C with gentle agitation in 500ml of TSB containing 100μg/ml of ampicillin (the selection marker for the plasmid).

The bacterial cells were harvested by centrifugation at 5000g for 5 minutes at 4°C. The cells were then resuspended and washed with 100ml of STE buffer and recentrifuged at 5000g for 5 minutes at 4°C. The supernatant was discarded.

3.12.2 Lysis by alkali

The washed pellet was resuspended in 18ml of Solution I. Two ml of freshly prepared lysozyme (10mg/ml in 10mM Tris-HCl, pH8.0) followed by 40ml of Solution II were then added to the cell suspension. The contents were mixed thoroughly by gently inverting the bottle several times. The mixture was then incubated at room temperature for 5 to 10 minutes.

After the incubation, 20ml of ice-cold Solution III was added to the mixture and mixed well by shaking the bottle several times until a white precipitate was observed. The bottle was then stored on ice for 10 minutes.

The bacterial lysate was then centrifuged at 4000g for 15 minutes at 4°C. The supernatant was filtered through four layers of clean Kleenex tissue papers into a 50ml centrifuge tube and 0.6 volume of isopropanol added to the filtered supernatant.

This was mixed well by gentle inversions and stored at room temperature for 10 minutes. The precipitated nucleic acids were then harvested by centrifugation at 5000g for 15 minutes at room temperature. The supernatant was discarded and the nucleic acid pellet was then air dried and dissolved in 3ml of TE buffer (pH 8.0).

3.12.3 Purification of plasmid DNA

Three ml of ice-cold 5M lithium chloride was added to the nucleic acid solution, mixed well, and then centrifuged at 10,000g for 10 minutes at 4^{o}C. The supernatant was transferred into a fresh 50ml centrifuge tube and an equal volume of isopropanol was added. The contents were then thoroughly mixed by gentle inversion and the precipitated nucleic acid was recovered by centrifugation at 10,000g for 10 minutes at room temperature. The supernatant was discarded and the pellet was dissolved with 500µl of TE buffer (pH 8.0). The nucleic acid solution was then transferred into a 1ml microfuge tube and 10µl of RNase (20µg/ml) was added to the solution and incubated at room temperature for 30 minutes.

After incubation, 500µl of NaCl/PEG was added to the contents, which were thoroughly mixed by gentle inversion. The precipitated plasmid DNA was recovered by centrifugation at 13,000g for 5 minutes at room temperature. The supernatant was discarded and the plasmid DNA pellet was dissolved with 400µl of TE buffer (pH 8.0).

The contents were then extracted once with 200µl of phenol, once with 200µl of phenol/chloroform and finally once with 200µl of chloroform. The aqueous phase was then transferred into a fresh microfuge tube and 100µl of 10M ammonium acetate was added. The contents were mixed thoroughly and 1ml (2 volumes) of 100% ethanol was added to the contents and mixed well with gentle inversion. The tube was then incubated at room temperature for 10 minutes. The precipitated plasmid DNA was then recovered by centrifugation at 13,000g for 2 minutes at room temperature.

The supernatant was discarded and the pellet was rinsed with 200µl of ice-cold 70% ethanol and then centrifuged at 13,000g for 2 minutes at room temperature. The supernatant was discarded and the plasmid DNA pellet was air dried and then dissolved in 500µl of TE buffer (pH 8.0). The DNA can then be stored at -20^{o}C until used.

3.12.4 Small scale isolation

The small-scale method was similar to the large-scale method. The cells were inoculated in 3 ml LB overnight and the overnight culture was pelleted by centrifugation in a 1.5ml centrifuge tube. The pellet was resuspended in 100µl of ice-cold Solution I and 200µl of Solution II was added to the resuspension. The contents were mixed by gentle inversion of the tube. After the mixing, 150µl of ice-cold Solution III was added to the mixture, vortexed, and stored on ice for 5 minutes. The tube was centrifuged at 12,000g for 5 minutes at 4^{o}C and the supernatant was transferred to a fresh tube. The supernatant was then mixed with 440µl of phenol: chloroform (1:1), vortexed, and centrifuged at 12,000g for 2 minutes at 4^{o}C. The upper layer was transferred to a fresh tube and 900µl of ethanol at room temperature was added for DNA precipitation. The mixture was allowed to stand for 2 minutes at room temperature. The tube was centrifuged at 12000g for 5 minutes at 4^{o}C for DNA recovery. The DNA pellet was washed with 1ml of 70% ethanol at 4^{o}C. The pellet was allowed to air dry at room temperature for 10 minutes. The dried pellet was dissolved in 50µl of TE buffer, pH8.0 and 5µl of 20µg/ml RNase. The plasmid DNA was stored at -20^{o}C.

3.13 DNA sequencing

DNA sequencing was done by using a LI-COR® 4200 Automatic DNA Sequencer (LI-COR). The protocol used was based on the Sanger method, which is a dideoxynucleotide chain-termination method. Dideoxynucleotide is a dNTP analogue that lacks a 3' OH group. The ddNTP will terminate a growing DNA strand when it is incorporated into the strand during DNA polymerisation. The lack of a 3' OH group will not allow additional nucleotides to attach to the DNA strand. This

means that the DNA fragment terminates at the position where the ddNTP is incorporated, which in turn reflects the position of the complementary dNTP on the template sequence.

Thermal cycle sequencing is a modified Sanger sequencing method, which utilises the linear polymerase reaction to amplify DNA fragments complementary to the target DNA sequence. The DNA fragments are synthesised in the presence of fluorescent-labelled primer, dNTP, ddNTP and thermal stable DNA polymerase, like *Taq* or Vent DNA polymerase. The synthesised fluorescent-labelled DNA fragments were electrophoresed in 4% polyacrylamide gel overnight. The laser in the sequencer excites the fluorescent label on the DNA fragments and the signal is picked up and the band position recorded and visualised in the attached computer. The DNA sequences are analysed and interpreted with the help of the software provided by the manufacturer.

3.13.1 Thermal cycle sequencing

The thermal cycle sequencing reaction was carried out with a commercially available kit, SequiTherm EXCEL™ II Long-Read™ DNA Sequencing Kit-LC (Epicentre Technologies). This kit was designed for use with the LI-COR® DNA sequencer. The primers used were labelled with fluorescent dye (IRD) with an excitement wavelength at 700nm. As the IRD700 label is sensitive to light it should be stored in the dark or in a tube wrapped in foil until used. The M13 Forward and M13 Reverse Primers used to start the sequencing were designed for the M13 sequences that flank the DNA insert on the vector plasmid. These two primers were provided by the kit manufacturer (Epicentre Technologies). Unlike PCR (see Section 3.10), only one primer was used in the reaction. As the sequencing reaction may only sequence approximately 400bp to 500bp per reaction, more than two sequencing primers were required. Subsequent primers were designed according to the DNA sequences acquired from the previous reaction using the MacVector™ 6.0 software (Accelrys, Cambridge, UK) and synthesised by MWG-Biotech AG, Ebersberg, Germany. The details of the primers are listed in Table 2.11.

The template DNA used was the cloned PCR product prepared as described in Section 3.10. The Termination Mix provided contained one of the four ddNTPs and

dNTPs. For each DNA template, four reactions were set up for the four different Termination Mixes (i.e. 4 tubes). The tubes were labelled A, G, T and C. Each tube contained 7.2µl of 3.5µl × SequiTherm EXCEL II Sequencing Buffer, 1µl of SequiTherm DNA Polymerase, 2µl of IRD700 labelled primer, 1µl of DNA template, 1µl of sterile deionised water and 2µl of one of the four SequiTherm EXCEL II-LC Long-Read Termination Mixes as per the tube label. For example, Tube labelled A would have 2µl SequiTherm EXCEL II-LC Long-Read Termination Mix A.

Cycling of the sequencing reactions was performed in an iCycler 96 well 0.2ml Reaction Module (Bio-Rad Laboratories). Sequencing reactions were performed as follows: one cycle at 95^{o}C for 5 min and 30 three step cycles at 95^{o}C for 30 seconds, 50^{o}C for 15 seconds and 70^{o}C for 1 min. Three µl of Stop/Loading Buffer were added to each reaction upon completion. The reactions could either be used immediately in polyacrylamide gel electrophoresis or stored at -20^{o}C in the dark for several months until required.

3.13.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel was prepared using the Long Ranger™ Gel Solution (FMC BioProducts). Urea (31.5g) was dissolved in 36ml of deionised water and 9ml of 10× TBE buffer. Six ml of Long Ranger™ 50% was added to the mixture. Before the gel mixture was ready to be poured into the gel apparatus, 500µl of freshly prepared 10% APS and 50µl TEMED were added. The gel mixture was filtered and placed in a 100ml syringe. The gel mixture was then injected into the gel apparatus with constant tapping on the glass plates to avoid the formation of air bubbles. If there were any air bubbles, they could be removed or "hooked out" using a thin wire with a bent end. A shark toothcomb was inserted on top of the gel and the top area was tightened with the casting plate. The polyacrylamide gel was allowed to set at room temperature for at least 2 hours. In the meantime, the DNA sequencer was switched on to warm up for the electrophoresis. The glass plates, containing the polyacrylamide gel, were cleaned with 90% ethanol and placed in the sequencer.

Electrophoresis was carried out in 1× TBE buffer and 1.2µl of each reaction was loaded. The gel was run at 2000V at 45^{o}C overnight.

3.13.3 Interpretation and analysis of DNA sequences

The fluorescent signals from the DNA fragments were detected by the sensor of the DNA sequencer and recorded in the attached computer. The signals were translated into DNA bands in a gel image and DNA sequences were interpreted with the software package Base ImagIR™ version 4 (LI-COR). The fluorescent signals were translated into DNA bands with the Data Collections version 04.01.006 software in the Base ImagIR™ package. The band images were transferred into the Image Analysis version 04.2f and interpreted into DNA sequences. The DNA sequences were transferred to the MacVector version 6.0 package (Accerlrys). The DNA sequences were then analysed and assembled for the whole sequence of the DNA insert using the AssemblyLIGN™ software in the MacVector 6.0 software package. (Accelrys)

3.14 Coagulase-gene typing

The presence of staphylocoagulase is a major phenotypic characteristic of *S. aureus*. It is known to be present in multiple allelic forms, partly due to the genetic variation in the 3' region of the coagulase gene. This region contains a series of 81bp tandem repeats and the number of the repeat units varies from strain to strain. Even though the sequence of the 81bp repeat is conserved, the repeat units may differ from each other by the presence or absence of an *Alu*I restriction site. (Goh *et al.*, 1992)

In the coagulase-gene typing method, the 3' region of the coagulase gene is amplified and digested with *Alu*I. The band patterns (i.e. polymorphisms) generated are used to distinguish *S. aureus* strains. The primers used were coag2 and coag3 (See Table 2.11) and the amplification procedure can be seen in Section 3.10. The amplicons were digested as in Section 3.5, using 4µl of amplicon. The digestion reactions were electrophoresed in 2.5% agarose gels and the sizes of the bands were calculated using the DNA size marker as a reference. Each band was given a primary number

according to its size (Table 3.4) and the sum of the primary numbers would be the coagulase-gene type number for the strain.

Table 3.4 Numbering system for coagulase-gene typing

Band Size (bp)	81	162	243	324	405	486	567	648	729	810
Primary number	1	2	4	8	16	32	64	128	256	512

For example, a strain with 324bp and 405bp *Alu*I fragment would have primary numbers of 8 and 16 respectively. The coagulase-gene type of this strain would be the sum of the two primary numbers, that is, 24.

3.15 Preparation of DNA probes

The DNA probes used were DNA inserts in the vector harboured by DH5α. The vector containing the insert was isolated using the large-scale isolation method described in Section 3.12. The DNA insert was cut out from the vector with appropriate restriction enzymes and the digestion mixture electrophoresed in an agarose gel and the desired band cut out and recovered using the DNA gel extraction kit described in Section 3.11.1. The DNA fragment was then ready for labelling.

3.16 Labelling of probes

Extracted DNA (see Section 3.11.1) was labelled with digoxigenin-11-dUTP (DIG) by random-primed labelling as described by the supplier (Boehringer Mannheim).

Five µl of extracted DNA (template DNA) was diluted with 70µl of sterile HP water. The tube was placed in boiling water for 10 minutes to denature the DNA. After denaturation, the tube was quickly chilled on ice for 30 seconds. Ten µl of hexanucleotide mixture, 10µl of DIG-dNTP labelling mix (containing DIG-dUTP) and 5µl of Klenow enzyme were added to the denatured template DNA. The contents were centrifuged briefly and incubated at 37^{o}C overnight.

The labelling reaction (DNA polymerisation) was terminated after 10 minutes of incubation at 65^{o}C by the addition of 0.1 volume of 200mM EDTA. Glycogen (1µl) was added to facilitate the precipitation of DNA. Eleven µl of 4M LiCl followed by 350µl of ice-cold ethanol were added to tube. The contents were then mixed thoroughly by gentle inversion and incubated at -70^{o}C for at least 30 minutes or at -20^{o}C overnight. The precipitated DNA was recovered by centrifugation at 13,000g for 15 minutes. The supernatant was discarded and the pellet was washed with 100µl of ice-cold 70% ethanol. The 70% ethanol was poured off and the pellet air-dried at 35^{o}C for 30 minutes. The air-dried pellet was dissolved in 50µl of TE buffer (pH 7.9) by incubating at 37^{o}C for 10 minutes with frequent vortexing. The labelled DNA was stored at -20^{o}C until used.

3.17 Alkaline Southern blotting

Restriction endonuclease-digested DNA was electrophoresed in agarose gels and then the DNA fragments transferred onto a positively charged nylon membrane by alkaline Southern blotting.

The edges of an electrophoresed agarose gel were trimmed and the left hand corner cut off for orientation. The trimmed gel was treated with 0.25M HCl for 10 minutes with gentle shaking to depurinate the large DNA fragments and to facilitate the transfer. In the case of larger DNA fragments like the ones in CHEF gels, two changes of HCl for 15 minutes were needed. The acid-treated gel was rinsed with two changes of HP water for 5 minutes for each rinse and with gentle shaking. The gel was then treated with 0.4M NaOH for 30 minutes with gentle shaking.

A sheet of Hybond-N^{+} (positive-charged nylon membrane, Amersham Biosciences) and six sheets of filter paper were cut to exactly the same size of the gel. Three sheets of filter paper were soaked with fresh 0.4M NaOH and placed on a Perspex platform. The NaOH treated gel was inverted and placed on top of the NaOH soaked filter papers. The Hybond-N^{+} membrane was then placed on the gel with three sheets of filter paper soaked with 0.4M NaOH. Two stacks of dry paper towels were

placed on top of the filter papers and a weight of approximately 0.75 kg to 1 kg was placed on top of the paper towels.

The capillary transfer was allowed to occur overnight. After the transfer, the membrane was removed and rinsed with 5 × SSC and air dried at room temperature. The air-dried membrane was then stored in a filter paper envelope until used.

3.18 DNA-DNA hybridisation

The Southern blotted membranes (see Section 3.17) were hybridised with DIG-labelled DNA probes (see Section 3.16). The binding of the probes to the membrane was detected by autoradiography.

3.18.1 Prehybridisation

The membrane was placed on a mesh fabric soaked in 5× SSC. The mesh with the membrane was rolled in a clockwise direction and placed in a clean glass hybridisation tube. Approximately 30ml of prehybridisation solution was then added to the tube. The tube was sealed with a plastic cap and the mesh was unrolled by turning the tube in an anti-clockwise direction. The tube with the unrolled mesh was then placed in a hybridisation oven and incubated at 65^{o}C for at least 2 hours.

3.18.2 Hybridisation

Fifty µl of DIG-labelled DNA probe was added to a microfuge tube and denatured in boiling water for 10 minutes. The tube was then quickly chilled on ice for 30 seconds and the contents removed to a 50ml centrifuge tube and mixed thoroughly with 25ml of prehybridisation solution. The denatured probe can then be stored at -20^{o}C for up to a year.

Before use, the probe was allowed to come to room temperature. During this time, the glass hybridisation tube containing the membrane was removed from the 65^{o}C hybridisation oven and the prehybridisation solution in the tube poured off. The

DNA probe solution was heated in boiling water for 10 minutes and quickly chilled on ice for 30 seconds and poured into the hybridisation tube. The tube was then placed back in the oven and incubated at 60^{o}C for 16 to 18 hours.

3.18.3 Post-hybridisation

After the hybridisation the DNA probe solution was poured back into the 50ml centrifuge tube and stored at -20^{o}C for future use. The membrane was washed twice with the 2 × wash solution at 30^{o}C for 5 minutes. The membrane was then washed with 0.5 × wash solution for 15 minutes at 56^{o}C. All the washing steps were done in the hybridisation oven.

3.18.4 Chemiluminescence detection

The washed membrane was removed from the tube and rinsed in washing buffer for 1 to 5 minutes. After the rinse, the membrane was placed in a fresh container with 100ml of blocking solution. The membrane was then incubated with the blocking reagent for at lease 60 minutes with gentle shaking. At the same time the CDP-STAR™ (Boeheringer Mannheim) solution was removed from cold storage and placed in the dark to come to room temperature. The dilution of the anti-DIG alkaline-phosphatase-conjugated antibody was done before the end of the membrane incubation time. The tube containing the antibody was centrifuged at 13,000g for one minute to precipitate aggregated antibodies. The antibody was diluted 1:20,000 with blocking solution.

After incubation, the blocking solution was poured off and the diluted antibody was added to the membrane and incubated at room temperature with gentle shaking for 30 minutes. After incubation, the excess antibody was poured off and the membrane was then washed twice with washing buffer for 15 minutes for each wash. The washed membrane was rinsed with detection buffer for 1 to 5 minutes. At the same time, the CDP-STAR™ was diluted by adding 1ml of detection buffer to 10µl of CDP-STAR™. As the CDP-STAR™ is light sensitive all subsequent steps were carried out in a dark room under red light.

The membrane was then placed on an open acetate file-folder taped in a light proof cassette. The CDP-STAR™ dilution was distributed over the membrane using a Pasteur pipette. Some detection buffer was also distributed over the membrane. The folder was then sealed with tape and any air bubbles trapped in the folder removed by wiping tissue paper over the top of the folder. The cassette was closed and left in the dark room from 8 hours to overnight to allow the chemiluminescent reaction to take place.

3.18.5 Autoradiography

After incubation, the cassette was opened in the dark room under red light and a sheet of Kodak™ scientific imaging film (X-OMAT) placed on top of the folder. The X-ray film was exposed for at least 1.5 hours and developed after the exposure time. The film was developed with commercially prepared developer and fixer. After development, the film was rinsed with water and air-dried.

3.18.6 Stripping membranes for reprobing

The membrane was removed from the folder and rinsed with deionised water for 1 minute. The rinsed membrane was then treated with two washes of pre-heated stripping solution for 10 minutes per wash. The process was done in the hybridisation oven with the membrane placed on a clean mesh, as described previously in Section 3.18, and incubated at 35^{o}C with pre-heated stripping solution. The stripped membrane was rinsed with 5× SSC and air dried. The dried membrane was then ready for reprobing.

3.19 Plasmid isolation from *Listeria monocytogenes*

The *L. monocytogenes* strain was inoculated into 5ml BHIB and incubated overnight at 37^{o}C. The overnight culture was transferred into a 10ml Oakridge tube and the cells pelleted by centrifugation at 4,000g for 10 minutes. The cell pellet was resuspended with 379µl of Solution 1. The resuspension was transferred into a 1.5ml microfuge tube and incubated at 37^{o}C for 10 minutes. The freshly prepared 96.5µl of

lysozyme (10 mg/ml in 25mM Tris/HCl, pH8.0) and 10µl of Proteinase K (50µg/ml) were added to the cell suspension. The mixture was incubated for 5 minutes at 37°C. A volume of 48.2µl of Solution 2 was added to the mixture and immediately after mixing 27.6µl of Solution 3 was added to the mixture. The mixture was incubated at 37°C for 5 to 10 minutes until the cells were completely lysed. The mixture was vortexed at the highest setting for 30 seconds and 27.6µl of freshly prepared 3M NaOH was added to the mixture. The mixture was then mixed gently by intermittent inversion or swirling for 10 minutes. After the mixing, 49.6µl of 2M Tris/HCl, pH 7.0 and 37.2µl of RNase (50µg/ml) were added and gently mixed for 3 minutes. After the 3 minutes mixing 71.7µl of 5M NaCl was added and mixed thoroughly with the mixture. Seven hundred µl of phenol saturated with 3% NaCl was added and mix thoroughly. The mixture was then centrifuged at 2,968g for 5 minutes. The upper phase was transferred into a fresh 1.5ml microfuge tube and extracted with 700µl of chloroform-isoamyl alcohol (24:1). The mixture was centrifuged at 2,968g for 5 minutes. The upper phase was transferred into a fresh 1.5ml microfuge tube. One volume of isopropanol was added and incubated at 0°C for at least 30 minutes to enhance the DNA precipitation. The DNA was recovered by centrifugation at 7,598g for 5 minutes. The supernatant was removed by aspiration and the DNA pellet was allowed to air dry. The dried DNA pellet was resuspended with 20µl TE buffer, pH8.0. The plasmid DNA was visualised as described in Section 3.4.

3.20 Population analysis for oxacillin resistance

The procedure was carried as previously described. (Pfeltz *et al.*, 2001) The isolate was grown in 5ml of TSB overnight at 37°C. Serial 10-fold dilutions of the overnight culture were made in TSB. The serial dilutions were carried out in 1.5ml microfuge tubes by serially transferring 20µl into 180µl sterile TSB. Each tube was mixed by vigorous vortexing before 20µl of its contents was transferred. The last tube had 20µl discarded from it so that it had the same volume as the other tubes.

BHIA plates were prepared to contain different concentrations of oxacillin. The plates were dried at 37°C for 30 minutes before use to facilitate the drying of the

dilution droplets. Ten µl of each dilution was dispensed onto the plates without spreading and allowed to air dry. The plates were incubated at 35°C overnight and the number of colonies was counted.

The colony-forming units per ml (CFU/ml) for each oxacillin concentration were determined by using the dilution factors and the volumes. The Log_{10} of the CFU/ml for each oxacillin concentration was plotted against the oxacillin concentration used in the plates. The experiments were carried out in triplicate to acquiring accurate values. The graphical presentation of these values is referred to as the population-analysis profile.

3.21 Multilocus sequence typing (MLST)

The MLST typing was carried out as previously reported. (Enright *et al.*, 2000) MLST typing is based on the DNA sequences of seven housekeeping genes. They are the carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*). These genes were amplified by PCR and the products sequenced. The sequences obtained were submitted to an Internet database. The sequences were matched with existing data and given allele numbers. The seven allele numbers of these seven genes are known as the sequence type (ST) of the strain.

3.21.1 PCR with MLST primers

Chromosomal DNA was obtained by the method described in section 3.8. The genes were amplified with the primers listed on Table 2.12. The PCR reactions were carried out in 50µl of reaction mixture. It consisted of 33.5µl of PCR grade water, 5µl of 10X Reaction Buffer (Promega), 4µl of 25mM $MgCl_2$, 1µl of 5.5 unit/µl *Taq* polymerase (Promega), 4µl of 2mM dNTP, 1µl of each primer (0.5µg) and 0.5µl of DNA template (approximately 0.5µg).

The amplification was carried out in an iCycler 96 well 0.2ml Reaction Module (Bio-Rad Laboratories). The reaction mixture was initially denatured at 95^{o}C for 5 minutes, followed by 30 three-step cycles of 1 minute at 95^{o}C (denaturing), 1 minute at 55^{o}C (annealing) and 1 minute at 72^{o}C (extension), and finally a 72^{o}C final extension for 5 minutes. The final amplified product was stored at 4^{o}C until used.

3.21.2 Purification of amplified MLST products

The amplified products were precipitated with 30µl of 20% PEG 8000 in 2.5M NaCl. The mixture was incubated at 37^{o}C for 15 minutes and the DNA pellet was recovered by centrifugation at 15,000g for 10 minutes. The DNA pellet was washed with 500µl of 70% and 100% ethanol and allowed to air dry. The pellet was resuspended in 15µl of TE pH8.0 buffer and stored at -20^{o}C until used.

3.21.3 Sequencing reaction

The sequencing reaction was carried with the IRDye™ 800 Termination Mixes (LI-COR). The 27µl master reaction mix contained 2µl of purified PCR product, 3µl of 1 pmol/µl unlabelled primer, 1.5µl of reaction buffer, 2µl of 4u/µl Thermo Sequenase (LI-COR) and 18.5µl of PCR grade water. From the master mix 6.5ml was then pipetted into 4 different 0.2ml thin-walled microfuge tubes that contained 0.2µl of different labelled terminators (deoxynucleotides).

The tubes were placed in the iCycler (Bio-Rad Laboratories) for the sequencing reaction. The initial denaturing was 2 minutes at 95^{o}C, followed by 30 three-step cycles of 30 seconds at 95^{o}C (denaturing), 30 seconds at 50^{o}C (annealing) and 45 seconds at 72^{o}C (extension). The program finished with a 5 minutes 72^{o}C final extension. The sequencing reactions were stored in lightproof containers at -20^{o}C until used.

The sequencing reaction was denatured at 95^{o}C for 3 minutes and placed on ice before loading on a polyacrylamide gel. The preparation and the procedure for DNA sequencing were as described in section 3.13.

3.21.4 MLST database

The Imperial College, London, maintains the *S. aureus* MLST database. The database can be accessed through its website, http://www.mlst.net/. The DNA sequences obtained were submitted to the database and matched with the existing sequences. An allele number was given to each of the seven genes that formed the sequence type.

CHAPTER FOUR

CHARACTERISATION OF MALAYSIAN MRSA

4.1 Introduction

MRSA first appeared in Malaysian hospitals around the early 1970s however, the first isolation was not reported. (Hanifah *et al.*, 1992, Puthucheary *et al.*, 1987). The percentages of *S. aureus* resistant to methicillin isolated in Malaysian hospitals have gradually increased from 3% in 1971 to 32% in 1998. (Lim, 1988, Puthucheary *et al.*, 1987, Rohani *et al*., 2000). Most of the Malaysian MRSA isolates were reported to be resistant to more than two antibiotics other than β-lactams. (Rohani *et al.*, 2000). Malaysian isolates were reported to carry the Allotype I and Allotype III SCC*mec* cassette and small plasmids. (Hanifah, 1991, Hiramatsu *et al.*, 2001, Ito *et al.*, 2001). *Sma*I CHEF profiling of the Malaysian MRSA found that they were related to two British MRSA, NCTC10442 and 86/560. (Yoshida *et al.*, 1997).

Seventy-four MRSA strains isolated from two Malaysian hospitals from 1982 to 2000 were characterised in this study with phenotypic and molecular typing methods. The phenotypic methods used were antimicrobial-resistance profiling and bacteriophage typing. The molecular methods used were plasmid profiling, *Sma*I CHEF profiling, coagulase-gene typing and SCC*mec* cassette typing.

4.2 Antimicrobial resistance profiling

All seventy-four Malaysian isolates were resistant to more than five different kinds of antimicrobial agents. All of the isolates were susceptible to vancomycin, rifampicin, novobiocin and mupirocin.

The resistance profiles of the isolates from different years were very similar. They have typically high rates of resistance to gentamicin (91%), kanamycin (93%), neomycin (78%), erythromycin (100%), lincomycin (inducible, 100%), streptomycin (97%), tetracycline (100%), trimethoprim (86%) and sulphonamides (100%). The

inducible lincomycin resistance was detected by the D-zone method. An erythromycin disc was placed close to a lincomycin disc (about 15mm). The flattening of the inhibition zone of lincomycin (i.e. D-shape) on the side next to the erythromycin disc was interpreted as inducible lincomycin resistance. Only two of the twelve 1982 isolates (17%) were resistant to trimethoprim, whereas the isolates from the later-years were all resistant to it. Resistance to ciprofloxacin was not seen in the isolates from 1982 to 1994 and by 2000 all isolates were resistant to ciprofloxacin. Chloramphenicol resistance was also common among the isolates, which was higher in 1996 isolates (69%) and relatively low in 1982 isolates (17%). All of the isolates were found to be β-lactamase producers.

A high percentage of the isolates were resistant to heavy metal ions like cadmium and mercury with a resistance rate of more than 90%. However, none of the isolates was resistant to arsenate. The resistance rate of the inorganic NAB compounds, propamidine isethionate and ethidium bromide was 73% and 81% respectively.

Some isolates were found to have lost their gentamicin resistance. The gentamicin resistance variants were labelled “A” and the susceptible variants were labelled “B”. This issue was further studied and the results are shown in Section 4.8.

The percentages of isolates resistant to different antimicrobial agents are listed in Table 4.1 and their resistance profiles are shown in Table 4.8, Table 4.9, Table 4.10, Table 4.11 and Table 4.12 according to the hospital and year of isolation.

Table 4.1 The percentage of resistance for different antimicrobial agents

	1982 HUM	1989 HUM	1994 HUM	1994 HUKM	2000 HUM	All
Arsenate	0%	0%	0%	0%	0%	0%
Cadmium acetate	100%	100%	100%	75%	90%	93%
Chloramphenicol	17%	69%	45%	25%	40%	41%
Ciprofloxacin	0%	0%	30%	0%	100%	22%
Erythromycin	100%	100%	100%	100%	100%	100%
Ethidium bromide	25%	100%	100%	75%	90%	81%
Fusidic acid	0%	0%	15%	0%	0%	4%
Gentamicin	83%	75%	100%	94%	100%	91%
Kanamycin	83%	88%	100%	94%	100%	93%
Lincomycin	100%	100%	100%	100%	100%	100%
Mercuric chloride	100%	100%	100%	94%	100%	99%
Minocycline	100%	100%	100%	100%	100%	100%
Mupirocin	0%	0%	0%	0%	0%	0%
Neomycin	17%	88%	100%	88%	80%	78%
Novobiocin	0%	0%	0%	0%	0%	0%
Phenyl mercuric acetate	100%	100%	100%	94%	100%	99%
Propamidine isethionate	17%	69%	100%	75%	90%	73%
Rifampicin	0%	0%	0%	0%	0%	0%
Spectinomycin	67%	94%	100%	94%	100%	92%
Streptomycin	92%	100%	100%	94%	100%	97%
Sulphonamides	100%	100%	100%	100%	100%	100%
Tetracycline	100%	100%	100%	100%	100%	100%
Trimethoprim	17%	100%	100%	100%	100%	86%
Vancomycin	0%	0%	0%	0%	0%	0%
β-lactamase production	100%	100%	100%	100%	100%	100%

Abbreviations: HUM: University Hospital of the University of Malaya; HUKM: The Hospital of the University Kebangsaan Malaysia

4.3 Bacteriophage typing

All Seventy-four Malaysian isolates were characterised by their susceptibility to the twenty-six bacteriophages belonging to the International Basic Set (IBS).

Eleven phage susceptibility patterns were observed among the Malaysian isolates. These phage patterns consist of susceptibility to one to seven phages. The phages that lysed the Malaysian isolates include 29, 42E, 53, 54, 81, 83A, 85, 88 and 90. Phage 29 belongs to the IBS Group I phages and phages 81, 88 and 90 belong to the miscellaneous supplementary phages. Phage 42E, 53, 54, 83A and 85 are IBS Group III phages. Phage pattern A consisting of only phage 85 was the most common phage type with 22 isolates (29.7%). Phage pattern B (85/90), C (85/88/90), D (83A/85/88/90), G (85/88), I (83A/85/88) and K (29/42E/53/54/81/85/90) are the other phage patterns containing phage 85. Together with phage pattern A, they accounted for 45 isolates of the 74 isolates (60.8%), but phage 85 was not found in the 1982 isolates. Phage pattern B consists of phage 85 and 90 and was found in nine isolates. Phage pattern C (85/88/90) was found in seven isolates and pattern D (83A/85/88/90) was found in four isolates. These four phage patterns were the most common pattern and accounted for 87.7 % (43 out of 49 isolates) of the typable isolates. Phage pattern E to K were all found in single isolates. Twenty-five isolates out of the 74 isolates (33.8%) were not lysed and were non-typable with the IBS phages. (See Table 4.2 and 4.3)

The 1982 isolates from HUM are mainly non-typable (75%) and the typable isolates were either lysed by phage 88, 90, or both phages. The 1989 HUM isolates are either lysed by phage 85 and/or phage 90 and six isolates were non-typable (37.5%). Among the 20 1994 HUM isolates, only two were non-typable (8.6%) and eight isolates were lysed by phage 85 only. The rest of the isolates carry either phage pattern B (85/90) or C (85/88/90). The 1994 isolates from HUKM are all typable by the IBS set of phages and possess diverse phage patterns. Phage pattern A (85) is the most common type. The other phage patterns include pattern B (85/90), C (85/88/90), D (83A/85/88/90), G (85/88), I (83A/85/88), J (88/83A/90) and K (29/42E/53/54/81/85/90). Only two 2000 HUM isolates were typable and both have

the phage pattern A (85). 80% of the 2000 HUM isolates were non typable (See Table 4.4)

Table 4.2 Phage patterns of Malaysian isolates

Phage patterns	Phage susceptibility profiles	Number of isolates	% of the phage types
A	85	22	29.7%
B	85/90	9	12.2%
C	85/88/90	7	9.5%
D	83A/85/88/90	4	5.4%
E	88	1	1.4%
F	90	1	1.4%
G	85/88	1	1.4%
H	88/90	1	1.4%
I	83A/85/88	1	1.4%
J	88/83A/90	1	1.4%
K	29/42E/53/54/81/85/90	1	1.4%

Table 4.3 Phage susceptibility of individual isolates[a]

Phage	Number of isolates	% of isolates susceptible	Phage group
85	45	60.8%	III
88	16	21.6%	Supplement
90	24	34.4%	Supplement
83A	6	8.1%	III
29[b]	1	1.4%	I
42E[b]	1	1.4%	III
53[b]	1	1.4%	III
54[b]	1	1.4%	III
81[b]	1	1.4%	III

[a]33.8% (25 of 74 isolates) were non typable by the IBS set of phages
[b]All found in one isolate with phage pattern K

Table 4.4 Phage susceptibility profiles of Malaysian isolates

Isolate (WBG)	IBS phage pattern	Year of isolation	Location
2003	NT	1982	HUM
2004	88/90	1982	HUM
2005	90	1982	HUM
2006	NT	1982	HUM
2007	NT	1982	HUM
2008	NT	1982	HUM
2010	NT	1982	HUM
2011	88	1982	HUM
2013	NT	1982	HUM
2014	NT	1982	HUM
2015A	NT	1982	HUM
2015B	NT	1982	HUM
7409	NT	1989	HUM
7410A	85	1989	HUM
7410B	85	1989	HUM
7411	85/90	1989	HUM
7412	85	1989	HUM
7413	NT	1989	HUM
7414	NT	1989	HUM
7417	85/90	1989	HUM
7419	85	1989	HUM
7420	85	1989	HUM
7422	85/90	1989	HUM
7424	NT	1989	HUM
7425A	NT	1989	HUM
7425B	NT	1989	HUM
7426	85	1989	HUM
7427	85	1989	HUM

Continued next page

Table 4.4 continued

Isolate (WBG)	IBS phage pattern	Year of isolation	Location
7884	85/90	1994	HUKM
7885	85	1994	HUKM
7887	85	1994	HUKM
7888	83A/85/88	1994	HUKM
7889	85	1994	HUKM
7890	29/42E/53/54/81/85/90	1994	HUKM
7891	85/88/90	1994	HUKM
7893	85/90	1994	HUKM
7894	83A/85/88/90	1994	HUKM
7895	83A/85/88/90	1994	HUKM
7897	85/88	1994	HUKM
7898	85/88/90	1994	HUKM
7900	88/83A/90	1994	HUKM
7998	85/88/90	1994	HUM
7999	85/90	1994	HUM
8000	85	1994	HUM
8001	85/88/90	1994	HUM
8002	NT	1994	HUM
8003	85	1994	HUM
8004	85/88/90	1994	HUM
8005	85/90	1994	HUM
8006	85	1994	HUM
8007	85	1994	HUM
8008	85/90	1994	HUM
8009	85	1994	HUM
8010	85/90	1994	HUM
8011	NT	1994	HUM
8012	85/88/90	1994	HUM

Continued next page

Table 4.4 continued

Isolate (WBG)	IBS phage pattern	Year of isolation	Location
8013	85	1994	HUM
8014	85/88/90	1994	HUM
8015	85	1994	HUM
8016	83A/85/88/90	1994	HUM
8023	85	1994	HUKM
8024	85	1994	HUKM
8026	83A/85/88/90	1994	HUKM
8154	85	1994	HUM
10358	85	2000	HUM
10359	NT	2000	HUM
10360	NT	2000	HUM
10361	NT	2000	HUM
10362	NT	2000	HUM
10363	NT	2000	HUM
10364	85	2000	HUM
10365	NT	2000	HUM
10366	NT	2000	HUM
10367	NT	2000	HUM

HUM, The University Hospital of the University of Malaya; HUKM, The Hospital of the University Kebangsaan Malaysia; NT, non-typable

4.4 Plasmid profiles

Among the 74 isolates, thirteen plasmids of different sizes were detected. These plasmids included four small plasmids, seven medium sized plasmids and two large plasmids. The sizes of the four small plasmids were 1.5, 2.6, 3.0 and 4.4 kb. The sizes of the seven medium sized plasmids in covalently closed circular (CCC) form were 28, 30.5, 31, 32, 35, 37 and 40 kb. The sizes of the two large plasmids were calculated on their open circular (OC) form because their CCC form was too weak, and located too close to the chromosomal band, for proper size estimation. Based on their OC forms their sizes were 40 and 53 kb OC, respectively. Only two out of 74 isolates studied did not carry plasmids. Thirty-five plasmid profiles were observed among the rest of the 72 isolates. The number of plasmids carried by the isolates ranged from one to six. (See Table 4.5)

The plasmid profiles of isolates collected within the same time period were very similar. Most of the 1982 isolates carried a small plasmid ranging in size from 1.5 to 3.0 kb with or without a large OC plasmid. The WBG2015A and WBG2015B were the only isolates that carried the 28 kb with two small plasmids, 2.6 and 3.0 kb.

The 1989 HUM isolates have very similar plasmid profiles. The majority of them carried 1.5 kb, 2.6 kb, 3.0 kb, 28 kb and/or 53 kb OC plasmids. The 1994 HUM and HUKM isolates have similar plasmid profiles. They distinctively carried three medium size plasmids of 28, 30.5 and 35 kb, often with the addition of one to two small plasmids as seen in the 1989 HUM isolates. WBG8000 was the only 1994 isolate that carried a 53 OC plasmid. Most of the 2000 HUM isolates have almost identical plasmid profiles to the 1994 isolates. Unlike the 1994 isolates the 2000 isolates in addition, to the three medium size plasmids, carried only the 3.0 and/or 4.4 kb small plasmids. In general, other than the 1982 isolates, the plasmid profiles of the Malaysian isolates were very similar to each other. More detailed studies of these plasmids are described in Chapter 5. The plasmid profiles of the isolates are shown in Figures 4.1 to 4.9 and summarised in Tables 4.7 to 4.11 by the year and location of isolation.

The contamination of chromosomal DNA in plasmid isolations was often unavoidable. It appeared as a band located immediately above the 40.3 kb sized marker plasmid.

Table 4.5 Plasmid types in Malaysian isolates

Plasmid type	Isolate (WBG)	Plasmid profile	Numbers of plasmids
P01	2006	2.6	
	2008	2.6	1
	2010	2.6	
	2013	2.6	
P02	7888	3.0	1
P03	8012	28	1
P04	2014	1.5, 2.6	2
P05	2007	1.5, 40 OC	2
P06	2003	2.6, 3.0	2
	7885	2.6, 3.0	
P07	7897	2.6, 28	2
	7898	2.6, 28	
P08	10367	3.0, 28	2
P09	10366	3.0, 4.4	2
P10	8014	3.0, 35	2
P11	2011	3.0, 53 OC	2
P12	7900	1.5, 2.6, 3.0	3
P13	7893	1.5, 2.6, 28	3
P14	7419	1.5, 3.0, 28	3
P15	7891	1.5, 3.0, 30.5	3
P16	7413	1.5, 3.0, 31	3
P17	8007	1.5, 3.0, 35	3
	8016	1.5, 3.0, 35	
P18	2015A	2.6, 3.0, 28	
	2015B	2.6, 3.0, 28	3
	7887	2.6, 3.0, 28	
P19	7889	2.6, 3.0, 31	3

Continued on next page

Table 4.5 continued

Plasmid type	Isolate (WBG)	Plasmid profile	Numbers of plasmids
P20	7890	2.6, 3.0, 35	3
	8023	2.6, 3.0, 35	
P21	10363	3.0, 4.4, 28	3
	10361	3.0, 4.4, 28	
P22	8024	3.0, 35, 37	3
P23	7412	1.5, 2.6, 3.0, 28	
	7414	1.5, 2.6, 3.0, 28	
	7417	1.5, 2.6, 3.0, 28	4
	7420	1.5, 2.6, 3.0, 28	
	7895	1.5, 2.6, 3.0, 28	
	7998	1.5, 2.6, 3.0, 28	
P24	8026	1.5, 2.6, 3.0, 35	4
P25	8154	1.5, 3.0, 28, 35	4
P26	7427	1.5, 3.0, 31, 53 OC	4
P27	7409	1.5, 3.0, 28, 53 OC	4
	7426	1.5, 3.0, 28, 53 OC	
P28	7999	2.6, 3.0, 30.5, 31	4
P29	8005	3.0, 28, 30.5, 35	
	8006	3.0, 28, 30.5, 35	
	10358	3.0, 28, 30.5, 35	4
	10359	3.0, 28, 30.5, 35	
	10364	3.0, 28, 30.5, 35	
	10365	3.0, 28, 30.5, 35	
P30	10362	3.0, 28, 30.5, 40	4
P31	7894	1.5, 2.6, 3.0, 28, 35	5
P32	7410A	1.5, 2.6, 3.0, 28, 53 OC	
	7410B	1.5, 2.6, 3.0, 28, 53 OC	
	7411	1.5, 2.6, 3.0, 28, 53 OC	
	7422	1.5, 2.6, 3.0, 28, 53 OC	5
	7424	1.5, 2.6, 3.0, 28, 53 OC	
	7425A	1.5, 2.6, 3.0, 28, 53 OC	
	7425B	1.5, 2.6, 3.0, 28, 53 OC	
P33	7884	2.6, 3.0, 31, 32, 40	5

Continued on next page

Table 4.5 continued

Plasmid type	Isolate (WBG)	Plasmid profile	Numbers of plasmids
P34	8009	2.6, 3.0, 28, 30.5, 35	5
	8015	2.6, 3.0, 28, 30.5, 35	
P35	10360	3.0, 4.4, 28, 30.5, 35	5
P36	8000	1.5,2.6,3.0,30.5,40,53 OC	6
P37	8001	1.5, 2.6, 3.0, 28, 30.5, 35	
	8002	1.5, 2.6, 3.0, 28, 30.5, 35	
	8003	1.5, 2.6, 3.0, 28, 30.5, 35	6
	8004	1.5, 2.6, 3.0, 28, 30.5, 35	
	8008	1.5, 2.6, 3.0, 28, 30.5, 35	
	8010	1.5, 2.6, 3.0, 28, 30.5, 35	
	8011	1.5, 2.6, 3.0, 28, 30.5, 35	
	8013	1.5, 2.6, 3.0, 28, 30.5, 35	

Note: WBG2004 and WBG2005 do not harbour plasmids.

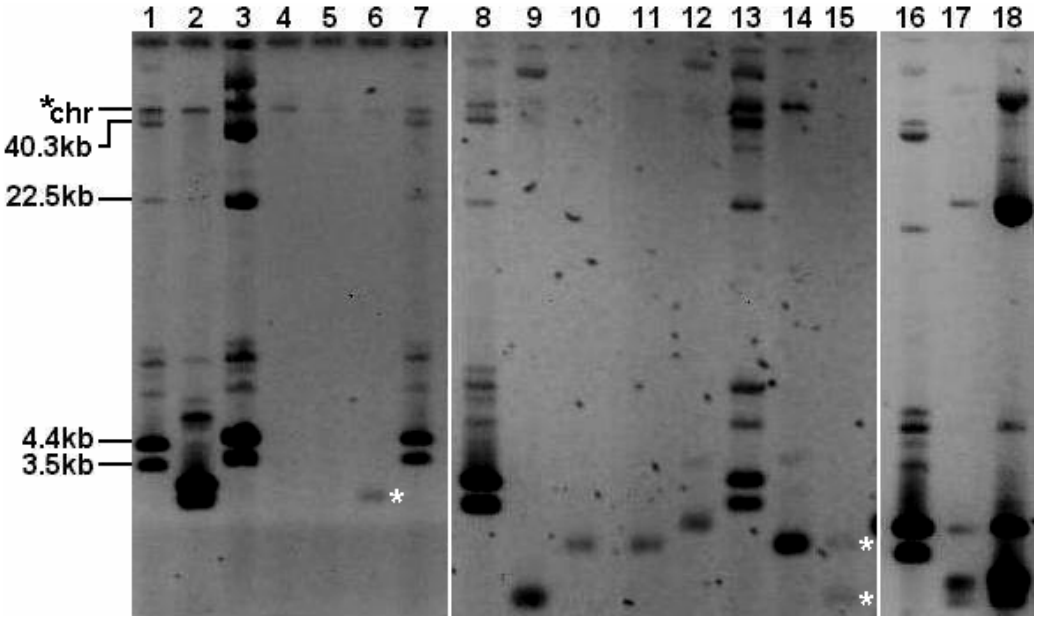


Figure 4.1 Plasmid profiles of 1982 HUM isolates[a]

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb
Lane 2 :	WBG2003	2.6 kb, 3.0 kb
Lane 3 :	WBG4483	As in lane 1
Lane 4 :	WBG2004	None
Lane 5 :	WBG2005	None
Lane 6 :	WBG2006	2.6 kb
Lane 7 :	WBG4483	As in lane 1
Lane 8 :	WBG4483	As in lane 1
Lane 9 :	WBG2007	1.5 kb, 40 kb OC
Lane 10 :	WBG2008	2.6 kb
Lane 11 :	WBG2010	2.6 kb
Lane 12 :	WBG2011	3.0 kb, 53 kb OC
Lane 13 :	WBG4483	As in lane 1
Lane 14 :	WBG2013	2.6 kb
Lane 15 :	WBG2014	1.5 kb, 2.6 kb
Lane 16 :	WBG4483	As in lane 1
Lane 17 :	WBG2015A	2.6 kb, 3.0 kb, 28 kb
Lane 18 :	WBG2015B	2.6 kb, 3.0 kb, 28 kb

*chr, chromosomal DNA; HUM, the University Hospital of the University of Malaya

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

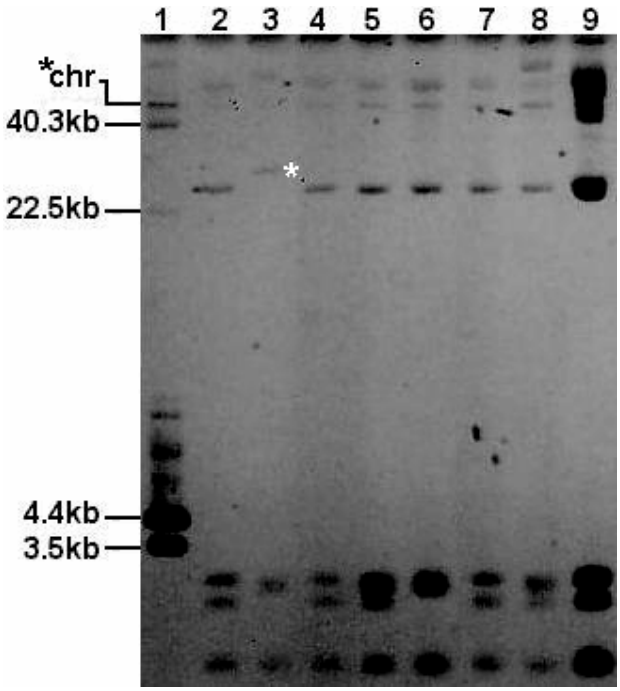


Figure 4.2 Plasmid profiles of 1989 HUM isolates[a]

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 2 :	WBG7412	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 3 :	WBG7413	1.5 kb, 3.0 kb, 31 kb
Lane 4 :	WBG7414	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 5 :	WBG7417	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 6 :	WBG7419	1.5 kb, 3.0 kb, 28 kb
Lane 7 :	WBG7420	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 8 :	WBG7422	1.5 kb, 2.6 kb, 3.0 kb, 28 kb 53 kb OC
Lane 9 :	WBG7424	1.5 kb, 2.6 kb, 3.0 kb, 28 kb 53 kb OC

*chr, chromosomal DNA; HUM, The University Hospital of the University of Malaya

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

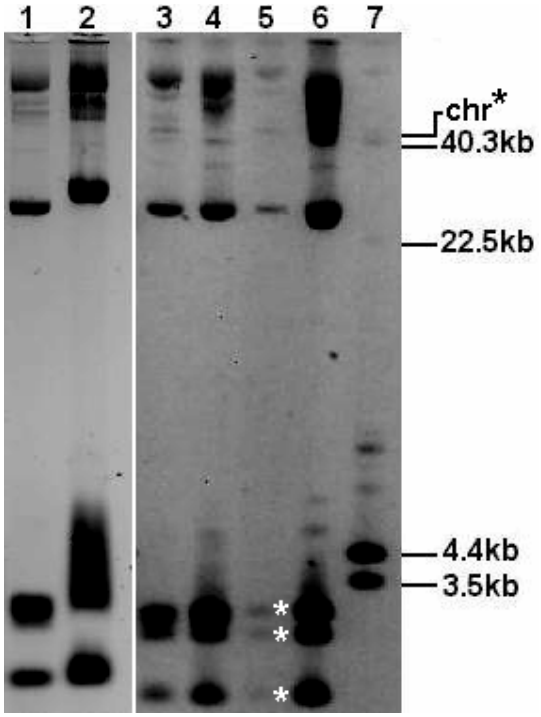


Figure 4.3 Plasmid profiles of 1989 HUM isolates continued[a]

Lane 1 :	WBG7426	1.5 kb, 2.6 kb, 28 kb 53 kb OC
Lane 2 :	WBG7427	1.5 kb, 3.0 kb, 31 kb 53 kb OC
Lane 3 :	WBG7425A	1.5 kb, 2.6 kb, 3.0 kb, 28 kb 53 kb OC
Lane 4 :	WBG7425B	1.5 kb, 2.6 kb, 3.0 kb, 28 kb 53 kb OC
Lane 5 :	WBG7410A	1.5 kb, 2.6 kb, 3.0 kb, 28 kb 53 kb OC
Lane 6 :	WBG7410B	1.5 kb, 2.6 kb, 3.0 kb, 28 kb 53 kb OC
Lane 7 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,

*chr, chromosomal DNA
HUM, The University Hospital of the University of Malaya
[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

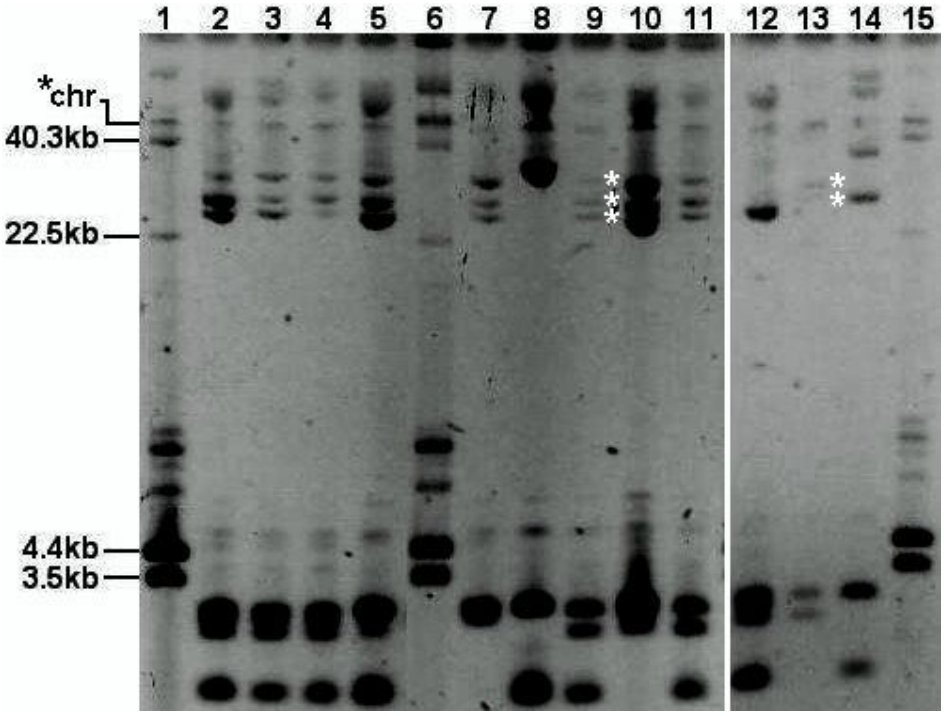


Figure 4.4 Plasmid profiles of 1994 HUM isolates[a]

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 2 :	WBG8001	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 3 :	WBG8002	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 4 :	WBG8003	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 5 :	WBG8004	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 6 :	WBG4483	As in lane 1
Lane 7 :	WBG8006	3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 8 :	WBG8007	1.5 kb, 3.0 kb, 35 kb
Lane 9 :	WBG8008	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 10 :	WBG8009	2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 11 :	WBG8010	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 12 :	WBG7998	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 13 :	WBG7999	2.6 kb, 3.0 kb, 30.5 kb, 31 kb
Lane 14 :	WBG8000	1.5 kb, 3.0 kb, 30.5 kb, 40 kb, 53 kb OC
Lane 15 :	WBG4483	As in lane 1

*chr, chromosomal DNA; HUM, The University Hospital of the University of Malaya.
[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

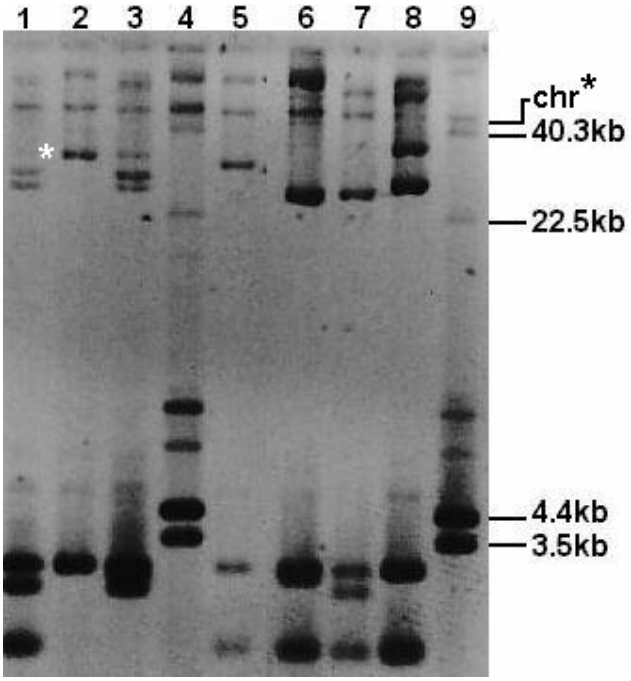


Figure 4.5 Plasmid profiles of 1994 HUM isolates continued[a]

Lane 1 :	WBG8013	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 2 :	WBG8014	3.0 kb, 35 kb
Lane 3 :	WBG8015	2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 4 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 5 :	WBG8016	1.5 kb, 3.0 kb, 35 kb
Lane 6 :	[b]WBG7409	1.5 kb, 3.0 kb, 28 kb, 53 kb OC
Lane 7 :	[b]WBG7411	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 8 :	WBG8154	1.5 kb, 3.0 kb, 28 kb, 35 kb
Lane 9 :	WBG4483	As in lane 4

*chr, chromosomal DNA;
HUM, The University Hospital of the University of Malaya.
[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.
[b]1989 HUM isolates

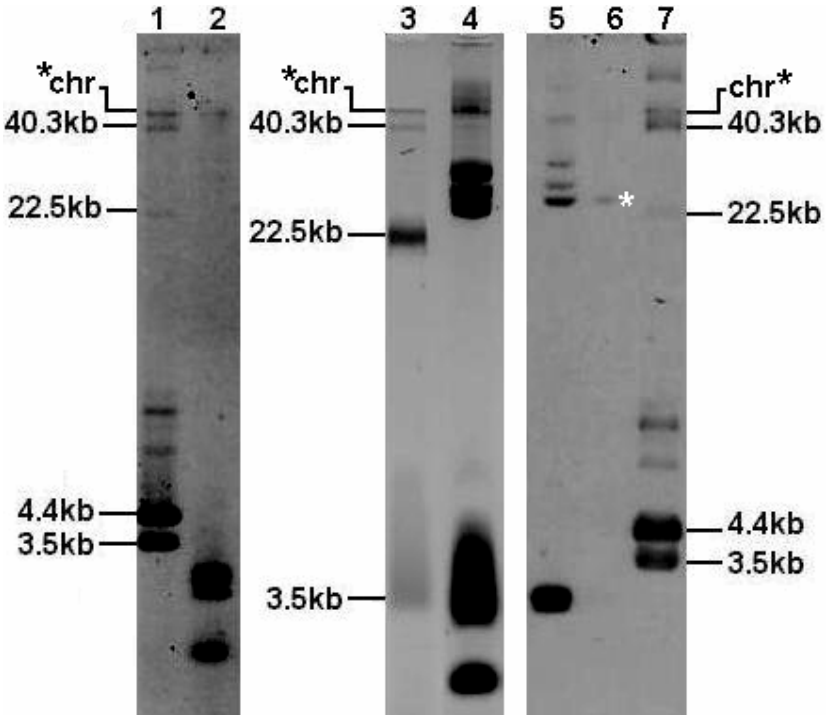


Figure 4.6 Plasmid profiles of 1994 HUM isolates continued[a]

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 2 :	[b]WBG8011	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 3 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 4 :	[c]WBG8011	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 5 :	WBG8005	3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 6 :	WBG8012	28 kb
Lane 7 :	WBG4483	As in lane 1

*chr, chromosomal DNA
HUM, The University Hospital of the University of Malaya
[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.
[b]WBG8011 the 28, 30.5 and 35 kb plasmids were too weak to show on the photograph.
[c]WBG8011 in a subsequent isolation the bigger plasmids were visible but the smaller plasmid region appeared smeary.

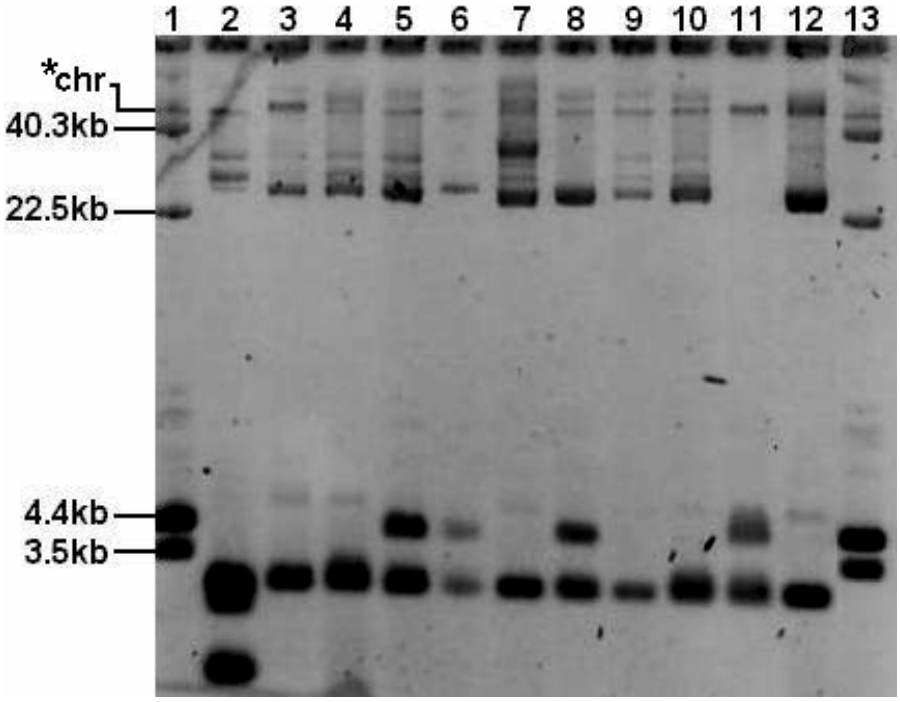


Figure 4.7 Plasmid profiles of 2000 HUM isolates

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 2 :	[a]WBG8003	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 3 :	WBG10358	3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 4 :	WBG10359	3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 5 :	WBG10360	3.0 kb, 4.4 kb, 28 kb, 30.5 kb, 35 kb
Lane 6 :	WBG10361	3.0 kb, 4.4 kb, 28 kb
Lane 7 :	WBG10362	3.0 kb, 28 kb, 30.5, 40 kb
Lane 8 :	WBG10363	3.0 kb, 4.4 kb, 28 kb
Lane 9 :	WBG10364	3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 10 :	WBG10365	3.0 kb, 28 kb, 30.5 kb, 35 kb
Lane 11 :	WBG10366	3.0 kb, 4.4 kb
Lane 12 :	WBG10367	3.0 kb, 28 kb
Lane 13 :	WBG4483	As in lane 1

*chr, chromosomal DNA
[a]1994 HUM isolate
HUM, The University Hospital of the University of Malaya

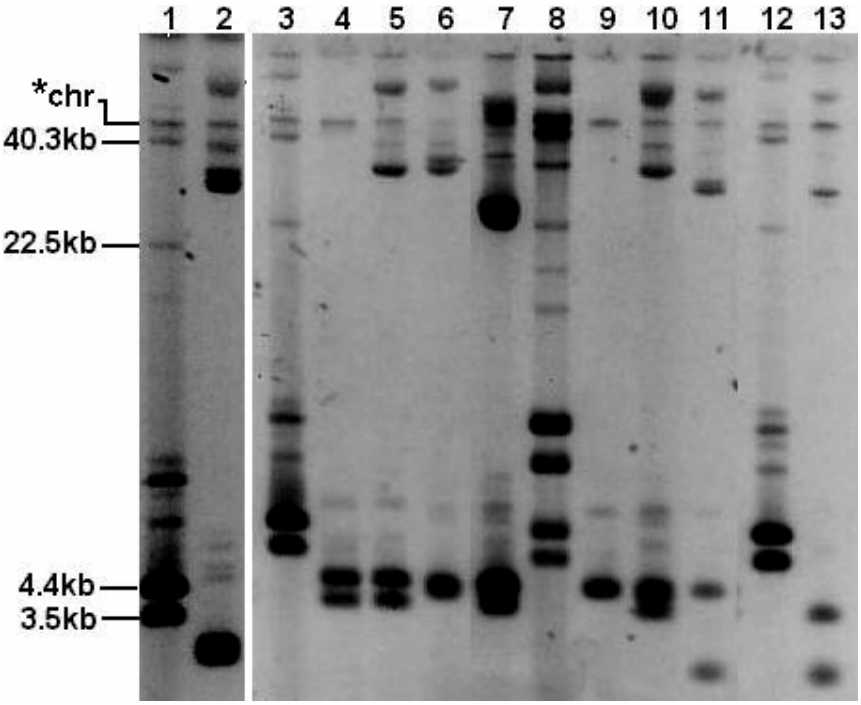


Figure 4.8 Plasmid profiles of 1994 HUKM isolates

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 2 :	WBG7884	2.6 kb, 3.0 kb, 31 kb 32 kb, 40 kb
Lane 3 :	WBG4483	As in lane 1
Lane 4 :	WBG7885	2.6 kb, 3.0 kb
Lane 5 :	WBG8023	2.6 kb, 3.0 kb, 35 kb
Lane 6 :	WBG8024	3.0 kb, 35 kb, 37 kb
Lane 7 :	WBG7887	2.6 kb, 3.0 kb, 28 kb
Lane 8 :	WBG4483	As in lane 1
Lane 9 :	WBG7888	3.0 kb
Lane 10 :	WBG7890	2.6 kb, 3.0 kb, 35 kb
Lane 11 :	WBG7891	1.5 kb, 3.0 kb, 30.5 kb
Lane 12 :	WBG4483	As in lane 1
Lane 13 :	WBG7893	1.5 kb, 2.6 kb, 28 kb

*chr, chromosomal DNA
HUKM, The Hospital of the University Kebangsaan Malaysia

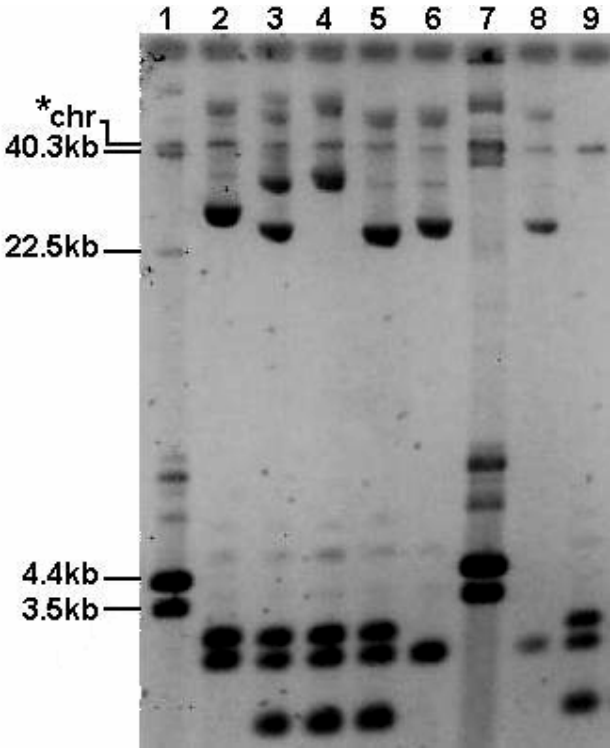


Figure 4.9 Plasmid profiles of 1994 HUKM isolates continued

Lane 1 :	WBG4483	3.5 kb, 4.4 kb, 22.5 kb 40.3 kb,
Lane 2 :	WBG7889	2.6 kb, 3.0 kb, 31 kb
Lane 3 :	WBG7894	1.5 kb, 2.6 kb, 3.0 kb, 28 kb, 35 kb
Lane 4 :	WBG8026	1.5 kb, 2.6 kb, 3.0 kb, 35 kb
Lane 5 :	WBG7895	1.5 kb, 2.6 kb, 3.0 kb, 28 kb
Lane 6 :	WBG7897	2.6 kb, 28 kb
Lane 7 :	WBG4483	As in lane 1
Lane 8 :	WBG7898	2.6 kb, 28 kb
Lane 9 :	WBG7900	1.5 kb, 2.6 kb, 3.0 kb

*chr, chromosomal DNA
HUKM, The Hospital of the University Kebangsaan Malaysia

4.5 CHEF profiles

Twenty-six *Sma*I generated CHEF patterns were found among the 74 Malaysian MRSA. The CHEF patterns are shown in Figure 4.10. A predominant CHEF pattern was found in the 1982, 1989 and 1994 isolates and designated CHEF type A. According to the Tenover *et al.*'s (1995) criteria, CHEF patterns with three or less bands difference are closely related. Based on this criterion, 13 CHEF patterns were closely related to the predominant CHEF pattern A. These 13 CHEF patterns accounted for 79.7% (59 isolates) of all the Malaysian isolates studied. The band differences between the dominant CHEF type A and the other CHEF types are listed in Table 4.6. Although CHEF patterns are not suitable for long-term studies, it is interesting to note that the CHEF patterns have not diverged greatly over the time frame of this study. (Witte *et al.*, 1994, Blanc *et al.*, 2002)

The similarity of the CHEF patterns was analysed with the Dice coefficient using the Molecular Analyst software package (Bio-Rad). The percentage similarity of the different CHEF patterns is shown in the form of a numerical matrix and their relationship demonstrated as a dendrogram. These figures are shown in Figure 4.11 and Figure 4.12 respectively. Type A3 and type A4 CHEF patterns have only one band difference and the bands were too close for the Molecular Analyst software to distinguish them. Therefore, they are shown to be the same in the figures.

All CHEF patterns were related to each other as the overall similarity among these 26 Malaysian CHEF patterns is 81%. All but four CHEF patterns are related to the predominant CHEF pattern A with similarities ranging from 81.3% to 97%. These four CHEF pattern are CHEF patterns C, B, H and I, their similarity with CHEF pattern A ranging from 75.9% to 78.8%. Nevertheless, these four CHEF patterns are still related to other CHEF patterns found among Malaysian MRSA isolates. The majority of the CHEF patterns among the 1982, 1989 and 1994 isolates were closely related to each other except for a few isolates. Their similarities are more than 80% and the close relationship between these CHEF patterns is demonstrated in the dendrogram. The CHEF patterns G, H, I and J of the 2000 isolates are closely related to each other. The similarity of their CHEF patterns ranged from 85.7% to 96.6%. CHEF patterns G, H and J have 80%, 86.7% and 90.3% similarity to CHEF

pattern A, respectively. Although CHEF pattern I is not related to CHEF pattern A, its CHEF pattern is related to CHEF patterns A2, A5 and A9 with similarity ranging from 80% to 82.8%. This indicates that the CHEF patterns of the Malaysian MRSA isolates in this study have a similar ancestry.

The CHEF pattern of an EA MRSA WBG525 was found to have a 90.3% similarity to the CHEF type A patterns. The CHEF results indicate that the Malaysian MRSA isolates studied possibly have a common ancestral origin with the EA MRSA. The EA MRSA is known to be closely related to EMRSA-1 from England. (Townsend *et al.*, 1984a, Wei and Grubb, 1992, Lim and Strynadka, 2002). The distribution of CHEF patterns over the isolation dates is listed in Table 4.7 and collated together with the plasmid profiles and antimicrobial-resistance profiles in Tables 4.8 to 4.12

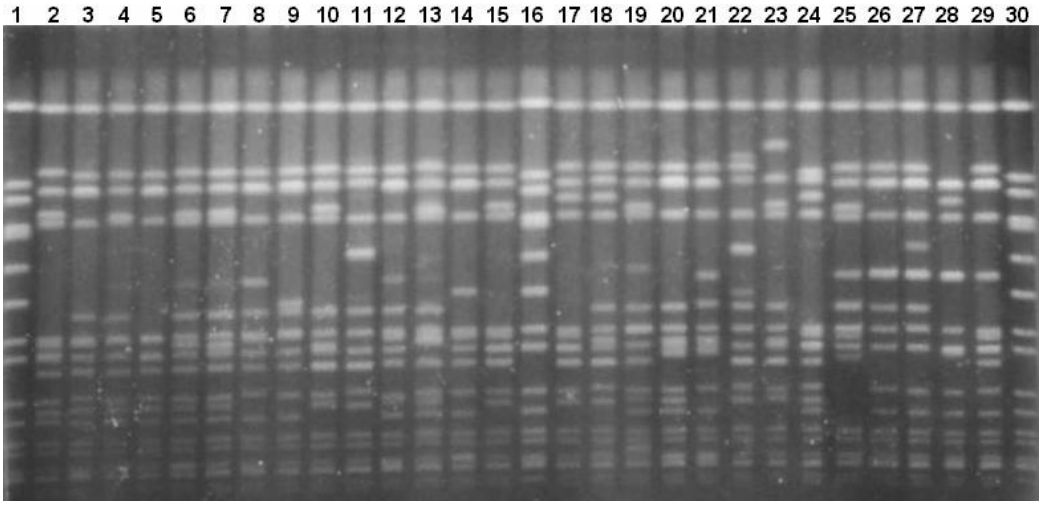


Figure 4.10 CHEF profiles of Malaysian MRSA

Lane 1 :	[a]NCTC8325	REF	**Lane 16 :**	[a]NCTC8325	REF
Lane 2 :	[b]WBG525	Control	**Lane 17 :**	WBG2013	A13
Lane 3 :	WBG7422	A	**Lane 18 :**	WBG2003	A14
Lane 4 :	WBG8009	A1	**Lane 19 :**	WBG8024	A15
Lane 5 :	WBG8003	A2	**Lane 20 :**	WBG7425B	A16
Lane 6 :	WBG8154	A3	**Lane 21 :**	WBG7427	B
Lane 7 :	WBG8010	A4	**Lane 22 :**	WBG2006	C
Lane 8 :	WBG7409	A5	**Lane 23 :**	WBG2007	D
Lane 9 :	WBG8011	A6	**Lane 24 :**	WBG2011	E
Lane 10 :	WBG2005	A7	**Lane 25 :**	WBG7893	F
Lane 11 :	WBG2008	A8	**Lane 26 :**	WBG10358	G
Lane 12 :	WBG7414	A9	**Lane 27 :**	WBG10359	H
Lane 13 :	WBG8007	A10	**Lane 28 :**	WBG10361	I
Lane 14 :	WBG7413	A11	**Lane 29 :**	WBG10364	J
Lane 15 :	WBG2004	A12	**Lane 30 :**	[a]NCTC8325	REF

[a]REF, NCTC8325, size maker reference and alignment control
[b]WBG525, EA MRSA, MRSA control

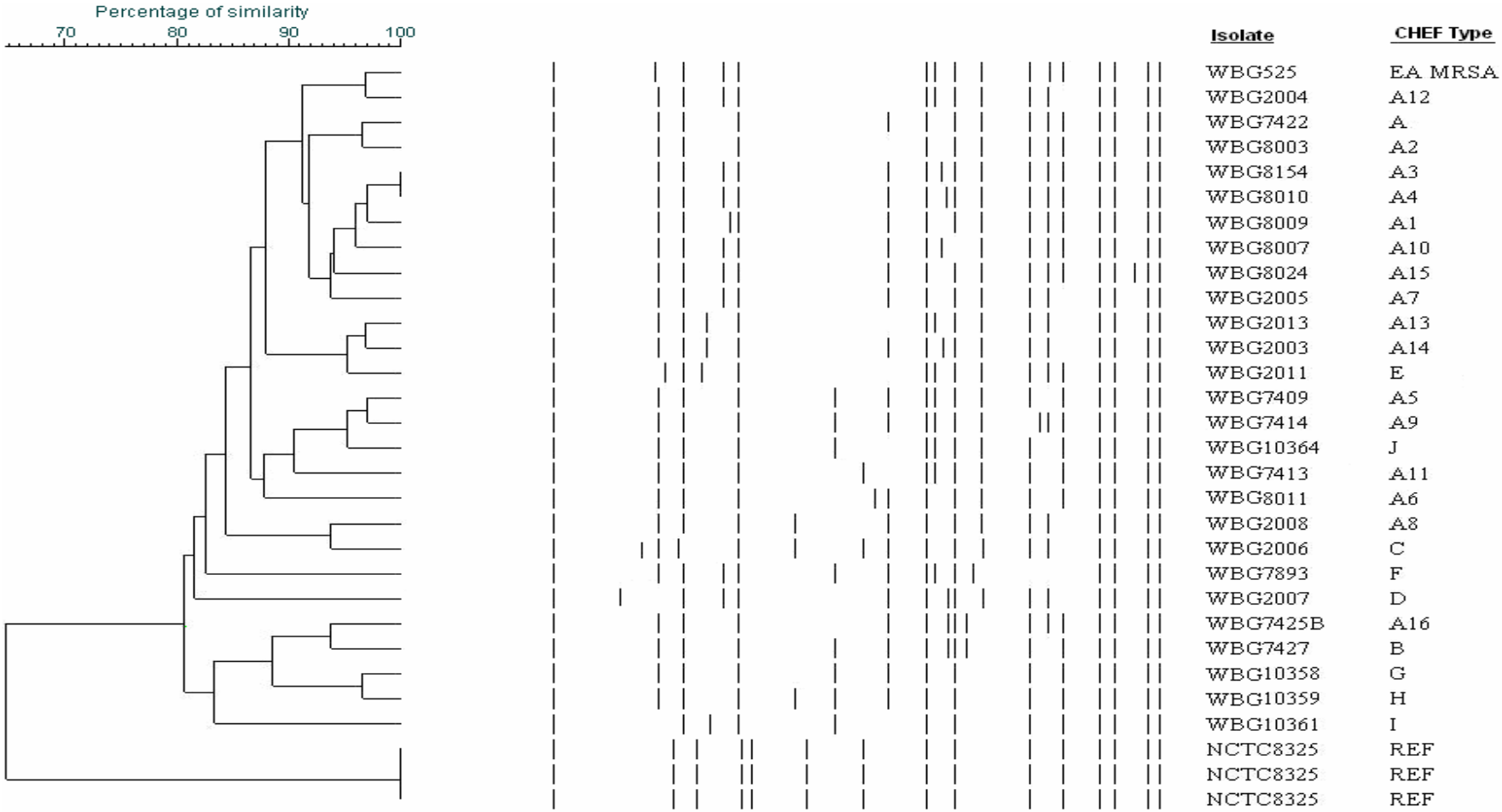


Figure 4.11 Dendrogram of CHEF patterns of Malaysian MRSA.

EA MRSA: eastern Australian MRSA; REF, size reference

Strains	CHEF Types	EA	A12	A	A2	A3	A4	A1	A10	A15	A7	A13	A14	E	A5	A9	J	A11	A6	A8	C	F	D	A16	B	G	H	I	Ref	Ref	Ref
WBG525	EA MRSA	100																													
WBG2004	A12	96.8	100																												
WBG7422	A	90.3	86.7	100																											
WBG8003	A2	93.3	89.7	96.6	100																										
WBG8154	A3	97.0	93.8	93.8	90.3	100																									
WBG8010	A4	90.9	87.5	93.8	90.3	100	100																								
WBG8009	A1	93.8	90.3	96.8	93.3	97.0	97.0	100																							
WBG8007	A10	93.8	90.3	90.3	86.7	97.0	97.0	93.8	100																						
WBG8024	A15	90.9	87.5	93.8	90.3	94.1	94.1	97.0	90.9	100																					
WBG2005	A7	90.3	93.3	93.3	89.7	93.8	93.8	96.8	90.3	93.8	100																				
WBG2013	A13	90.3	93.3	86.7	89.7	87.5	81.3	83.9	83.9	81.3	86.7	100																			
WBG2003	A14	87.5	90.3	90.3	86.7	90.9	90.9	87.5	87.5	84.8	90.3	96.8	100																		
WBG2011	E	93.8	90.3	90.3	93.3	90.9	84.8	87.5	87.5	84.8	83.9	96.8	93.8	100																	
WBG7409	A5	87.5	83.9	90.3	86.7	90.9	84.8	87.5	87.5	84.8	83.9	83.9	87.5	87.5	100																
WBG7414	A9	90.9	87.5	93.8	90.3	88.2	88.2	90.9	84.8	88.2	87.5	87.5	84.8	90.9	97.0	100															
WBG10364	J	90.3	86.7	86.7	89.7	87.5	81.3	83.9	83.9	81.3	80.0	86.7	83.9	90.3	96.8	93.8	100														
WBG7413	A11	90.3	86.7	86.7	89.7	87.5	81.3	83.9	83.9	81.3	80.0	86.7	83.9	90.3	90.3	87.5	93.3	100													
WBG8011	A6	83.9	80.0	93.3	89.7	87.5	87.5	90.3	83.9	87.5	86.7	80.0	83.9	83.9	90.3	87.5	86.7	86.7	100												
WBG2008	A8	83.9	86.7	93.3	89.7	87.5	87.5	90.3	83.9	87.5	93.3	86.7	90.3	83.9	83.9	87.5	80.0	80.0	86.7	100											
WBG2006	C	78.8	81.3	87.5	83.9	82.4	82.4	84.8	78.8	82.4	87.5	81.3	84.8	78.8	78.8	82.4	75.0	81.3	81.3	93.8	100										
WBG7893	F	83.9	86.7	80.0	75.9	87.5	81.3	83.9	83.9	81.3	86.7	80.0	83.9	77.4	90.3	87.5	86.7	80.0	80.0	80.0	75.0	100									
WBG2007	D	81.3	83.9	83.9	80.0	90.9	90.9	87.5	87.5	84.8	90.3	77.4	87.5	75.0	75.0	78.8	71.0	71.0	77.4	83.9	78.8	77.4	100								
WBG7425B	A16	81.3	77.4	90.3	86.7	90.9	90.9	87.5	87.5	84.8	83.9	77.4	87.5	81.3	81.3	84.8	77.4	77.4	83.9	83.9	78.8	77.4	81.3	100							
WBG7427	B	75.0	71.0	83.9	80.0	84.8	84.8	81.3	81.3	78.8	77.4	71.0	81.3	75.0	87.5	84.8	83.9	77.4	83.9	77.4	72.7	83.9	75.0	93.8	100						
WBG10358	G	80.0	75.9	89.7	85.7	83.9	83.9	86.7	80.0	83.9	82.8	75.9	80.0	80.0	93.3	90.3	89.7	82.8	89.7	82.8	77.4	82.8	73.3	86.7	93.3	100					
WBG10359	H	77.4	73.3	86.7	82.8	81.3	81.3	83.9	77.4	81.3	80.0	73.3	77.4	77.4	90.3	87.5	86.7	80.0	86.7	86.7	81.3	80.0	71.0	83.9	90.3	96.6	100				
WBG10361	I	75.9	71.4	78.6	81.5	73.3	73.3	75.9	69.0	73.3	71.4	78.6	75.9	82.8	82.8	80.0	85.7	78.6	78.6	71.4	66.7	71.4	69.0	75.9	82.8	88.9	85.7	100			
NCTC8325	Ref	64.5	60.0	66.7	69.0	62.5	62.5	64.5	58.1	62.5	60.0	66.7	64.5	77.4	64.5	62.5	66.7	73.3	66.7	60.0	68.8	53.3	58.1	64.5	64.5	69.0	66.7	71.4	100		
NCTC8325	Ref	64.5	60.0	66.7	69.0	62.5	62.5	64.5	58.1	62.5	60.0	66.7	64.5	77.4	64.5	62.5	66.7	73.3	66.7	60.0	68.8	53.3	58.1	64.5	64.5	69.0	66.7	71.4	100	100	
NCTC8325	Ref	64.5	60.0	66.7	69.0	62.5	62.5	64.5	58.1	62.5	60.0	66.7	64.5	77.4	64.5	62.5	66.7	73.3	66.7	60.0	68.8	53.3	58.1	64.5	64.5	69.0	66.7	71.4	100	100	100

Figure 4.12 Percentage of similarity of Malaysian MRSA CHEF patterns based on Dice coefficient

Table 4.6 Band differences between the predominant CHEF type A and other CHEF patterns

Strains	CHEF patterns	Band differences to CHEF type A
WBG7422	A	0
WBG8009	A1*	1
WBG8003	A2*	1
WBG8154	A3*	2
WBG8010	A4*	2
WBG7409	A5*	3
WBG8011	A6*	2
WBG2005	A7*	2
WBG2008	A8*	2
WBG7414	A9	4
WBG8007	A10*	3
WBG7413	A11	4
WBG2004	A12	4
WBG2013	A13	4
WBG2003	A14*	3
WBG8024	A15*	2
WBG7425B	A16*	3
WBG7427	B	5
WBG2006	C	6
WBG2007	D	5
WBG2011	E	5
WBG7893	F	8
WBG10358	G*	3
WBG10359	H	4
WBG10361	I	6
WBG10364	J	4
WBG525	EA MRSA	5
NCTC8325	MSSA	12

*CHEF patterns that are closely related to the predominant CHEF pattern A based on Tenover *et al.*'s criteria. (Tenover *et al.*, 1995)

EA MRSA, eastern Australian MRSA.

MSSA, methicillin-sensitive *S. aureus*.

Table 4.7 The distribution of CHEF patterns among Malaysian isolates

***Sma*I CHEF type ( ) number of isolates**	**WBG**	**Origin**	**Year of isolation**
A (15)	2015B	HUM	1982
	7410B	HUM	1989
	7411	HUM	1989
	7412	HUM	1989
	7417	HUM	1989
	7422	HUM	1989
	7424	HUM	1989
	7887	HUKM	1994
	7889	HUKM	1994
	7890	HUKM	1994
	7891	HUKM	1994
	7999	HUM	1994
	8005	HUM	1994
	8006	HUM	1994
A1 (9)	7884	HUKM	1994
	7885	HUKM	1994
	7894	HUKM	1994
	8023	HUKM	1994
	8008	HUM	1994
	8009	HUM	1994
	8013	HUM	1994
	8014	HUM	1994
	8015	HUM	1994

Continued next page

Table 4.7 continued

*Sma*I CHEF type	WBG	Origin	Year of isolation
A2 (12)	7888	HUKM	1994
	7895	HUKM	1994
	7897	HUKM	1994
	7898	HUKM	1994
	7900	HUKM	1994
	8026	HUKM	1994
	7998	HUM	1994
	8001	HUM	1994
	8002	HUM	1994
	8003	HUM	1994
	8004	HUM	1994
	8016	HUM	1994
A3 (2)	8012	HUM	1994
	8154	HUM	1994
A4 (2)	8000	HUM	1994
	8010	HUM	1994
A5 (6)	2015A	HUM	1982
	7409	HUM	1989
	7410A	HUM	1989
	7419	HUM	1989
	7420	HUM	1989
	7426	HUM	1989
A6 (1)	8011	HUM	1994
A7 (3)	2005	HUM	1982
	2010	HUM	1982
	2014	HUM	1982
A8 (1)	2008	HUM	1982
A9 (1)	7414	HUM	1989
A10 (1)	8007	HUM	1994
A11 (1)	7413	HUM	1989

Continued next page

Table 4.7 continued

***Sma*I CHEF type**	**WBG**	**Origin**	**Year of isolation**
A12 (1)	2004	HUM	1982
A13 (1)	2013	HUM	1982
A14 (1)	2003	HUM	1982
A15 (1)	8024	HUKM	1994
A16 (1)	7425B	HUM	1989
B (2)	7425A	HUM	1989
	7427	HUM	1989
C (1)	2006	HUM	1982
D (1)	2007	HUM	1982
E (1)	2011	HUM	1982
F (1)	7893	HUKM	1994
G (4)	10358	HUM	2000
	10360		
	10363		
	10365		
H (3)	10359	HUM	2000
	10366		
	10367		
I (2)	10361	HUM	2000
	10362		
J (1)	10364	HUM	2000

Table 4.8 Profiles of 1982 HUM Isolates

Isolate WBG	Plasmid Profiles Kb	Antimicrobial Resistance Profile	*Sma*I CHEF TYPE
2003	2.6, 3.0	GKSmELTMi*SuCdHgPmaSpBla	A14
2004	---	SmEL^{I}TMi*TpSuCdHgPmaBla	A12
2005	---	GKSmEL^{I}TMi*TpSuCdHgPmaSpBla	A7
2006	2.6	GKEL^{I}TMi*SuCdHgPmaSpBla	C
2007	1.5, 40OC	GKSmEL^{I}TMi*SuCdHgPmaSpBla	D
2008	2.6	GKSmEL^{I}TMi*SuCdHgPmaBla	A8
2010	2.6	GKSmEL^{I}TMi*SuCdHgPmaSpBla	A7
2011	3.0, 53OC	GKNSmEL^{I}TMi*SuCdHgPmaEb*Bla	E
2013	2.6	GKSmEL^{I}TMi*SuCdHgPmaBla	A13
2014	1.5, 2.6	GKSmEL^{I}TMi*SuCdHgPmaSpBla	A7
2015A	2.6, 3.0, 28	GKN*SmEL^{I}CTMi*SuCdHgPmaEb*PiSpBla	A5
2015B	2.6, 3.0, 28	SmEL^{I}CTMi*SuCdHgPmaEb*PiSpBla	A

Abbreviations:

Bla, β-lactamase production; Cd, Cadmium acetate; C, Chloramphenicol; Cip, Ciprofloxacin; E, Erythromycin; Eb, Ethidium bromide; Fa, Fusidic acid; G, Gentamicin; K, Kanamycin; Hg, Mercuric chloride; N, Neomycin; Pma, Phenyl mercuric acetate; Pi, Propamidine isethionate; S, Streptomycin; Sp, Spectinomycin; T, Tetracycline; Tp, Trimethoprim; L, Lincomycin; Mi, Minocycline; Su, Sulphonamides; Cip, Ciprofloxacin. *, refers to low-level resistance; Superscript I, refers to inducible resistance

HUM, The University Hospital of the University of Malaya; HUKM, The hospital of University Kebangsaan Malaysia

Table 4.9 Profiles of 1989 HUM Isolates

Isolate WBG	Plasmid Profiles Kb	Antimicrobial Resistance Profile	*Sma*I CHEF TYPE
7409	1.5, 3.0, 28, 53OC	GKN*SmEL^{I}CTMi*TpSuCdHgPmaEb*Pi*Sp Bla	A5
7410A	1.5, 2.6, 3.0, 28, 53OC	GKNSmEL^{I}TMi*TpSuCdHgPmaEbSpBla	A5
7410B	1.5, 2.6, 3.0, 28, 53OC	KNSmEL^{I}TMi*TpSuCdHgPmaEbSpBla	A
7411	1.5, 2.6, 3.0, 28, 53OC	SmEL^{I}TMi*TpSuCdHgPmaEbPiSpBla	A
7412	1.5, 2.6, 3.0, 28	GKNSmEL^{I}TMi*TpSuCdHgPmaEbPiSpBla	A
7413	1.5, 3.0, 31	SmEL^{I}CTMi*TpSuCdHgPmaEbPiSpBla	A11
7414	1.5, 2.6, 3.0, 28	GKNSmEL^{I}TMi*TpSuCdHgPmaEbSpBla	A9
7417	1.5, 2.6, 3.0, 28	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSpBla	A
7419	1.5, 3.0, 28	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPi*Sp Bla	A5
7420	1.5, 2.6, 3.0, 28	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSpBla	A5
7422	1.5, 2.6, 3.0, 28, 53OC	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSpBla	A
7424	1.5, 2.6, 3.0, 28,53OC	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSpBla	A
7425A	1.5, 2.6, 3.0, 28, 53OC	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*SpBla	B
7425B	1.5, 2.6, 3.0, 28, 53OC	KN*SmEL^{I}CTMi*TpSuCdHgPmaEb*SpBla	A16
7426	1.5, 3.0, 28, 53OC	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiBla	A5
7427	1.5, 3.0, 31, 53OC	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSp Bla	B

Abbreviations: see Table 4.8

Table 4.10 Profiles of 1994 HUKM Isolates

Isolate WBG	Plasmid Profiles Kb	Antimicrobial Resistance Profile	*Sma*I CHEF TYPE
7884	2.6, 3.0, 31, 32, 40	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSp Bla	A1
7885	2.6, 3.0	GKNSmEL^{I}TMi*TpSuHgPmaBla	A1
7887	2.6, 3.0, 28	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSp Bla	A
7888	3.0	GKNSmEL^{I}TMi*TpSuSpBla	A2
7889	2.6, 3.0, 31	GKNSmEL^{I}TMi*TpSuCdHgPmaEbPi*Sp Bla	A
7890	2.6, 3.0, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSp Bla	A
7891	1.5, 3.0, 30.5	SmEL^{I}TMi*TpSuCdHgPmaEbPiSpBla	A
7893	1.5, 2.6, 28	GKNSmEL^{I}TMi*TpSuCdHgPmaEbPiSp Bla	F
7894	1.5, 2.6, 3.0, 28, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEbPiSp Bla	A1
7895	1.5, 2.6, 3.0, 28	GKNSmEL^{I}TMi*TpSuCdHgPmaEbPiSp Bla	A2
7897	2.6, 28	GKNEL^{I}TMi*TpSuCdHgPmaEbPiSpBla	A2
7898	2.6, 28	GKSmEL^{I}TMi*TpSuCdHgPmaEbPiSpBla	A2
7900	1.5, 2.6, 3.0	GKNSmEL^{I}TMi*TpSuHgPmaSpBla	A2
8023	2.6, 3.0, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEbPiSp Bla	A1
8024	3.0, 35, 37	GKNSmEL^{I}CTMi*TpSuCdHgPmaEbPiSp Bla	A15
8026	1.5, 2.6, 3.0, 35	GKNSmEL^{I}TMi*TpSuHgPmaSpBla	A2

Abbreviations: see Table 4.8

Table 4.11 Profiles of 1994 HUM Isolates

Isolate WBG	Plasmid Profiles kb	Antimicrobial Resistance Profile	*Sma*I CHEF TYPE
7998	1.5, 2.6, 3.0, 28	GKNSmELCTMi*TpSuFaCdHgPmaEb*PiSpCip Bla	A2
7999	2.6, 3.0, 30.5, 31	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A
8000	1.5, 2.6, 3.0, 30.5, 40, 53OC	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A4
8001	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuFaCdHgPmaEb*PiSpCi pBla	A2
8002	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSpCip Bla	A2
8003	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSpCip Bla	A2
8004	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuFaCdHgPmaEb*PiSpCi pBla	A2
8005	3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSpBla	A
8006	3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSpBla	A
8007	1.5, 3.0, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A10
8008	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A1
8009	2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSpBla	A1
8010	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A4
8011	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}CTMi*TpSuCdHgPmaEb*PiSpCip Bla	A6
8012	28	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A3
8013	1.5, 2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A1
8014	3.0, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A1
8015	2.6, 3.0, 28, 30.5, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A1
8016	1.5, 3.0, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A2
8154	1.5, 3.0, 28, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*PiSpBla	A3

Abbreviations: see Table 4.8

Table 4.12 Profiles of 2000 HUM Isolates

Isolate WBG	Plasmid Profiles Kb	Antimicrobial Resistance Profile	*Sma*I CHEF TYPE
10358	3.0, 28, 30.5, 35,	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*Pi*SpCip Bla	G
10359	3.0, 28, 30.5, 35,	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*Pi*SpCip Bla	H
10360	3.0, 4.4, 28, 30.5, 35	GKNSmEL^{I}TMi*CTpSuCdHgPmaEb*Pi*Sp CipBla	G
10361	3.0, 4.4, 28	GKSmEL^{I}TMi*CTpSuCdHgPmaEb*Pi*SpCip Bla	I
10362	3.0, 28, 30.5, 40	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*Pi*SpCip Bla	I
10363	3.0, 4.4, 28	GKN*SmEL^{I}TMi*CTpSuCdHgPmaEb*Pi*Sp CipBla	G
10364	3.0, 28, 30.5, 35	GKNSmEL^{I}TMi*TpSuCdHgPmaEb*Pi*SpCip Bla	J
10365	3.0, 28, 30.5, 35	GKN*SmEL^{I}TMi*TpSuCdHgPmaEb*Pi*Sp Cip Bla	G
10366	3.0, 4.4	GKNSmEL^{I}TMi*CTpSuHgPmaSpCipBla	H
10367	3.0, 28	GKSmEL^{I}TMi*TpSuCdHgPmaEb*Pi*SpCip Bla	H

Abbreviations: see Table 4.8

4.6 Coagulase-gene typing

Coagulase-gene typing and the numbering system designed for the interpretation of *Alu*I restricted fragment is described in Section 3.14.

In this study, twenty-six Malaysian isolates representing all of the CHEF patterns were used for the coagulase-gene typing. All 26 of the Malaysian isolates generated two fragments when their amplicons were digested with *Alu*I. The fragments were approximately 405bp and 324bp in length, which give them the primary numbers of 16 and 8 respectively. As the coagulase-gene type is the sum of all primary numbers, all the isolates studied had coagulase-gene type 24.

The *Alu*I DNA fragments of some of the Malaysian isolates are shown in Figure 4.13 and Figure 4.14

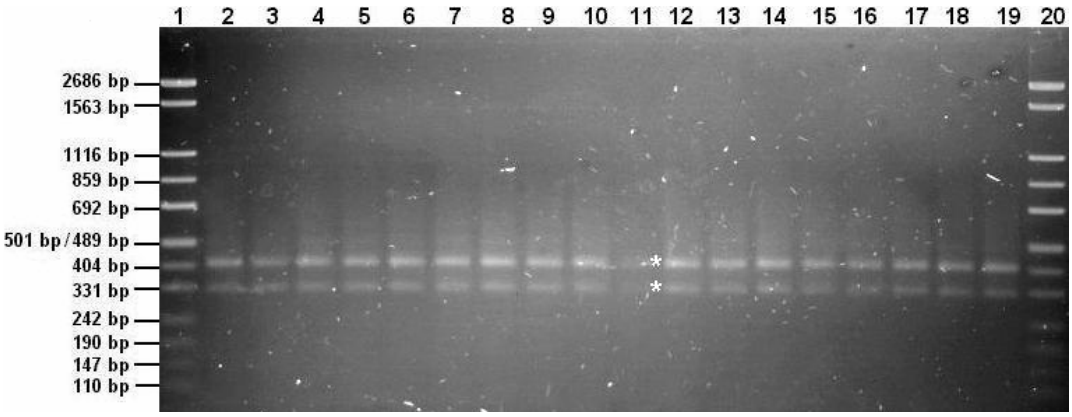


Figure 4.13 Coagulase-gene typing of Malaysian MRSA

Lane 1 :	FN-1	DNA size marker
Lane 2 :	WBG7422	CHEF A
Lane 3 :	WBG8009	CHEF A1
Lane 4 :	WBG8003	CHEF A2
Lane 5 :	WBG8154	CHEF A3
Lane 6 :	WBG8010	CHEF A4
Lane 7 :	WBG7409	CHEF A5
Lane 8 :	WBG8011	CHEF A6
Lane 9 :	WBG2005	CHEF A7
Lane 10 :	WBG2008	CHEF A8
Lane 11 :	[a]WBG7414	CHEF A9
Lane 12 :	WBG8007	CHEF A10
Lane 13 :	WBG7413	CHEF A11
Lane 14 :	WBG2004	CHEF A12
Lane 15 :	WBG2013	CHEF A13
Lane 16 :	WBG2003	CHEF A14
Lane 17 :	WBG8024	CHEF A15
Lane 18 :	WBG7425B	CHEF A16
Lane 19 :	WBG7427	CHEF B
Lane 20:	FN-1	DNA size marker

[a]The white asterisks and lines mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

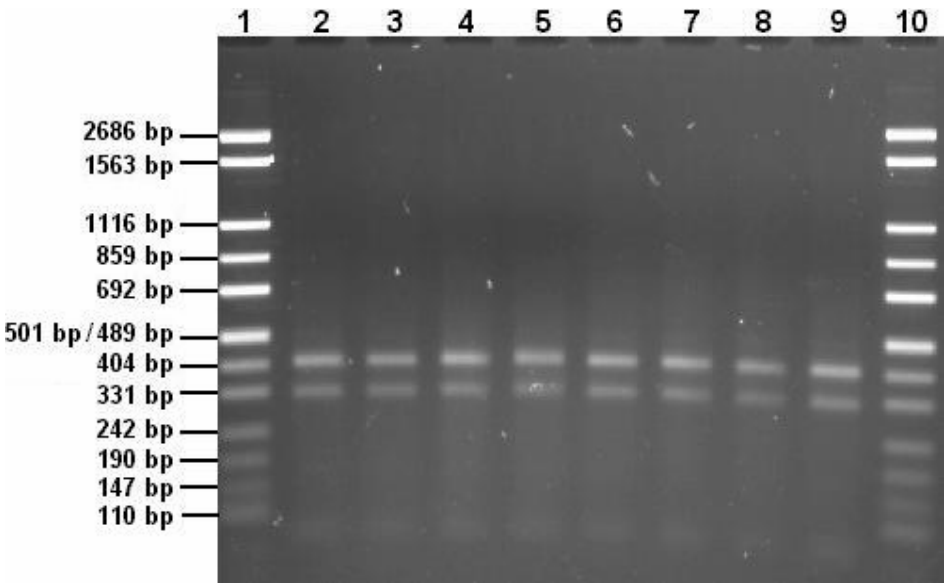


Figure 4.14 Coagulase-gene typing of Malaysian MRSA

Lane 1 :	FN-1	DNA Size Marker
Lane 2 :	WBG2006	CHEF C
Lane 3 :	WBG2007	CHEF D
Lane 4 :	WBG2011	CHEF E
Lane 5 :	WBG7893	CHEF F
Lane 6 :	WBG10358	CHEF G
Lane 7 :	WBG10359	CHEF H
Lane 8 :	WBG10361	CHEF I
Lane 9 :	WBG10364	CHEF J
Lane 10 :	FN-1	DNA Size Marker

4.7 SCC*mec* cassette of Malaysia MRSA

The *mec* complex and *ccr* gene complex of the Malaysian MRSA were determined by PCR. Twenty-six Malaysian isolates representing all the CHEF patterns were used.

All of the isolates amplified with primer mA and mI2 and gave a *c.*2.3 kb amplicon, which is identical to the expected size of 2335 bp. This indicated that they have an intact *mec* complex. The different elements of the *mec* complex were also analysed for their presence. All of the isolates were amplified with primers designed for the membrane-spanning domain, and penicillin-binding domain of the *mecR1* and the *mecI* gene, respectively. These results indicated that all the Malaysian isolates carry the Class A *mec* complex. The PCR results for the *mec* complex are shown in Figure 4.15 and Figure 4.16.

The isolates were amplified with primers designed for the four classes (see Table 2.11) of the *ccr* gene complex. The Malaysian isolates only amplified with primers cB and cA3, indicating that they carry the Class 3 *ccr* gene complex. The results are shown in Figure 4.17

The *mecA* gene was amplified by primers MR1 and MR2 (see Table 2.11) and digested with *Xba*I restriction enzyme to detect the silent single-base mutation. Based on the primers and the published sequences the expected amplicon size is 1336 bp and a *Xba*I cut should give fragments of 1078 and 258 bp. (Ito *et al.*, 2001, Gill *et al.*, 2005) . The *mecA* amplicon obtained was approximately 1300 bp and when digested with *Xba*I gave two fragments of *c.*1000 bp and *c.* 300 bp in size. This indicates the presence of the single-base mutation in the *mec*A gene of the Malaysian isolates. The results are shown in Figure 4.18.

As the Malaysian isolates carry the Class A *mec* complex and Class 3 *ccr* gene complex this indicates that they harbour the Allotype III SCC*mec* cassette.

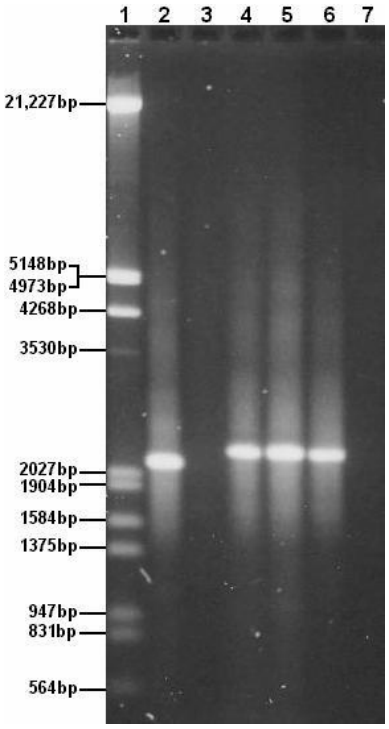


Figure 4.15 Amplification of isolates with mA and mI2 primers[a]

Lane 1 :	Lambda DNA *Eco*RI/*Hin*dIII DNA Marker	
Lane 2 :	[b]WBG525	Positive Control
Lane 3 :	NCTC8325	Negative Control
Lane 4 :	WBG7422	CHEF A
Lane 5 :	WBG8003	CHEF A2
Lane 6 :	WBG2007	CHEF D
Lane 7 :	Reagent Control	

[a]Expected size of the amplicon is 2335bp

[b]WBG525 amplified but with a smaller band due to a 166bp deletion. The *mec* complex of WBG525 is further studied in chapters 6 and 7.

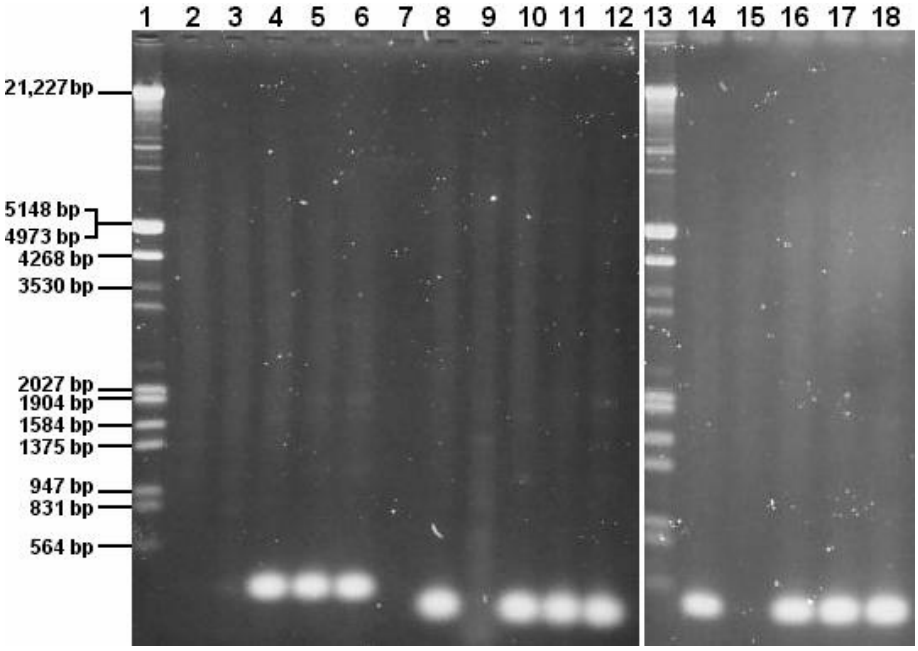


Figure 4.16 PCR of *mec* complex with different primers

Lane 1 :	Lambda DNA *Eco*RI/*Hin*dIII DNA Marker	
Lane 2 :	#WBG525	Positive Control for MS* PCR, mRA1/ mRA2**
Lane 3 :	NCTC8325	Negative Control for MS PCR
Lane 4 :	WBG7422	CHEF A, MS PCR
Lane 5 :	WBG8003	CHEF A2, MS PCR
Lane 6 :	WBG2007	CHEF D, MS PCR
Lane 7 :	Reagent Control for MS PCR	
Lane 8 :	WBG525	Positive Control for PB* PCR, mRB1/ mRB2**
Lane 9 :	NCTC8325	Negative Control for PB PCR
Lane 10	WBG7422	CHEF A, PB PCR
Lane 11	WBG8003	CHEF A2, PB PCR
Lane 12	WBG2007	CHEF D, PB PCR
Lane 13	Lambda DNA *Eco*RI/*Hin*dIII DNA Marker	
Lane 14	WBG525	Positive Control for *mecI* PCR, mI1/ mI2**
Lane 15	NCTC8325	Negative Control for *mecI* PCR
Lane 16	WBG7422	CHEF A, *mecI* PCR
Lane 17	WBG8003	CHEF A2, *mecI* PCR
Lane 18	WBG2007	CHEF D, *mecI* PCR

*MS: membrane-spanning domain of *mecR1*; PB: penicillin-binding domain of *mecR1;*

** Using these primers the expected amplicon sizes are: MS 320bp; PB 235bp; *mecI* 481bp.

#WBG525 did not amplify in the MS PCR due to a 166 bp deletion. Please see Chapter 6 and Chapter 7 for a detailed explanation.

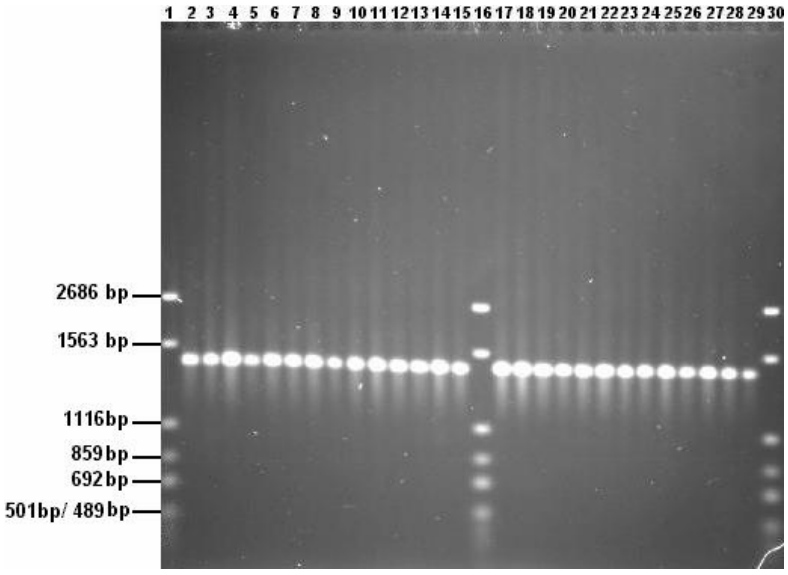


Figure 4.17 PCR of Malaysian MRSA with *ccr* primers cB and cA3[a]

Lane 1 :	FN-1*		**Lane 16 :**	WBG2013	A13
Lane 2 :	#WBG525		**Lane 17 :**	WBG2003	A14
Lane 3 :	WBG7422	A	**Lane 18 :**	FN-1	
Lane 4 :	WBG8009	A1	**Lane 19 :**	WBG8024	A15
Lane 5 :	WBG8003	A2	**Lane 20 :**	WBG7425B	A16
Lane 6 :	WBG8154	A3	**Lane 21 :**	WBG7427	B
Lane 7 :	WBG8010	A4	**Lane 22 :**	WBG2006	C
Lane 8 :	WBG7409	A5	**Lane 23 :**	WBG2007	D
Lane 9 :	WBG8011	A6	**Lane 24 :**	WBG2011	E
Lane 10 :	WBG2005	A7	**Lane 25 :**	WBG7893	F
Lane 11 :	WBG2008	A8	**Lane 26 :**	WBG10358	G
Lane 12 :	WBG7414	A9	**Lane 27 :**	WBG10359	H
Lane 13 :	WBG8007	A10	**Lane 28 :**	WBG10361	I
Lane 14 :	WBG7413	A11	**Lane 29 :**	WBG10364	J
Lane 15 :	WBG2004	A12	**Lane 30 :**	FN-1	

*FN-1, DNA size marker
#WBG525, Positive control
[a]The expected size of the amplicon is 1800 bp. However, the resulted amplicons are 1500 bp indicating that these isolates may have a 300 bp deletion within their *ccr* gene complex.

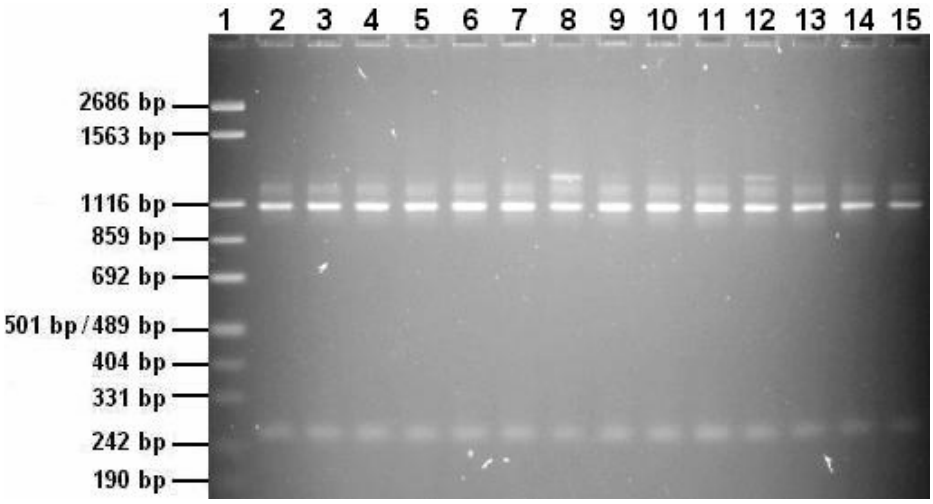


Figure 4.18 *Xba*I digestion of Malaysian *mecA* amplicons*

Lane 1 :	WBG7422	CHEF A
Lane 2 :	WBG8009	CHEF A1
Lane 3 :	WBG8003	CHEF A2
Lane 4 :	WBG8154	CHEF A3
Lane 5 :	WBG8010	CHEF A4
Lane 6 :	WBG7409	CHEF A5
Lane 7 :	WBG8011	CHEF A6
Lane 8 :	WBG2005	CHEF A7
Lane 9 :	WBG2008	CHEF A8
Lane 10 :	WBG7414	CHEF A9
Lane 11 :	WBG8007	CHEF A10
Lane 12 :	WBG7413	CHEF A11
Lane 13 :	WBG2004	CHEF A12
Lane 14 :	WBG2013	CHEF A13
Lane 15 :	WBG2003	CHEF A14

The faint bands bigger than 1116bp are thought to be undigested DNA fragments. The highest faint bands in lanes 8 and 12 are unknown but are thought to be non-specific amplification products.

*See Table 2.11 and Figure 2.1 for *mecA* primers.

4.8 Loss of gentamicin resistance

Four isolates were found to lose their gentamicin resistance after prolonged storage and successive subculturing. The gentamicin-resistant isolates were labelled "A" and their gentamicin susceptible variants were labelled "B". They are WBG2015, WBG7410, WBG7425 and WBG8003 which were isolated in different years.

The CHEF patterns of these four isolates are different but closely related. When the CHEF patterns of the gentamicin-resistant isolates were compared with their corresponding gentamicin susceptible variants, two band differences were found. All the resistant isolates share a *c.*192 kb *Sma*I band which migrates between the *Sma*I F and *Sma*I G bands of NCTC8325. The sensitive variants share a *c.*78 kb band located between *Sma*I K and *Sma*I J bands of NCTC8325. (Pattee *et al.*, 1990) These results indicate that the difference in the bands is probably due to a *c.*114 kb deletion in the *c.*192 kb band that results in the smaller *c.*78 kb band. An identical kind of deletion following the loss of gentamicin resistance has also been seen in Singapore MRSA isolates. (Sim, 2003) See Figure 4.19.

The lost of kanamycin and neomycin resistances was also observed with the loss of gentamicin resistance in WBG2015B and WBG8003B. However, the loss of these two resistance phenotypes was not observed in WBG7410B and WBG7425B.

The C3 DNA probe containing the *aacA-aphD* gene that encodes resistance to gentamicin, kanamycin and tobramycin was hybridised with *Eco*RI chromosomal digests of the gentamicin-resistant isolates and their gentamicin-susceptible variants. Only the resistant strains hybridised with the C3 probe. This indicates that the loss of resistance is due to the loss of the *aacA-aphD* gene. Strain WBG523 was used as the positive control because it has a chromosomal copy of the gentamicin-resistance Tn*3851* transposon. (Townsend *et al.*, 1984b) See Figure 4.20

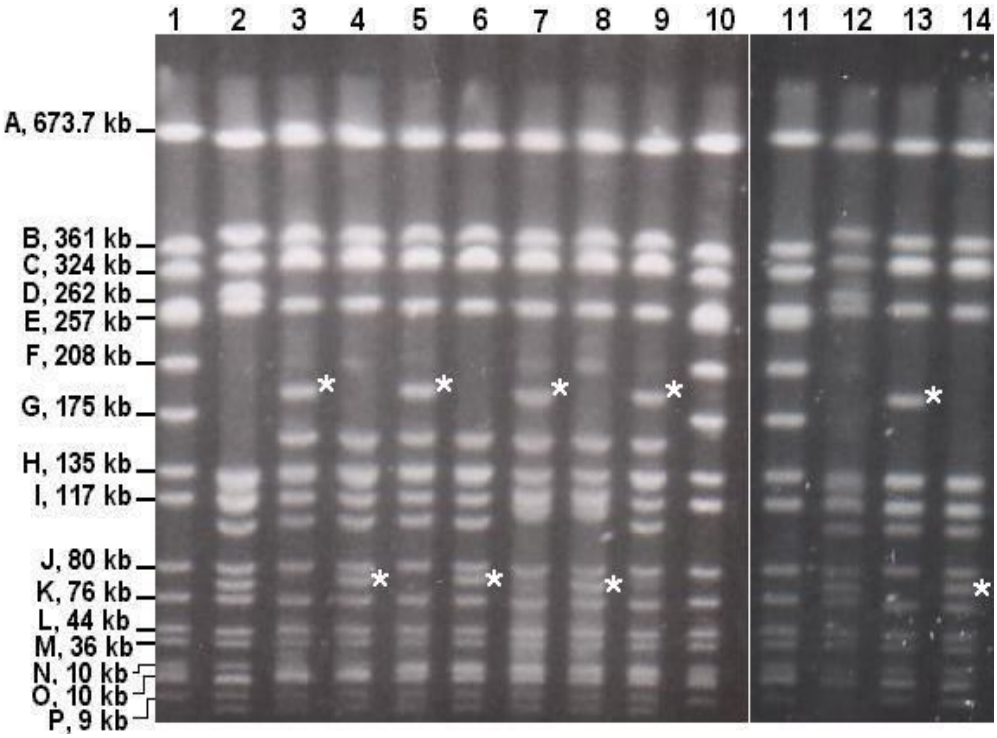


Figure 4.19 CHEF patterns of gentamicin-resistant strains and their susceptible variants

Lane 1 :	NCTC8325	Reference
Lane 2 :	WBG523	Gm^R MRSA control
Lane 3 :	WBG2015A	$Gm^RKm^RNm^R$
Lane 4 :	WBG2015B	$Gm^SKm^SNm^S$
Lane 5 :	WBG7410A	$Gm^RKm^RNm^R$
Lane 6 :	WBG7410B	$Gm^SKm^RNm^R$
Lane 7 :	WBG7425A	$Gm^RKm^RNm^R$
Lane 8 :	WBG7425B	$Gm^SKm^RNm^R$
Lane 9 :	WBG8484	$Gm^RKm^RNm^R$, Singapore MRSA
Lane 10 :	NCTC8325	Reference
Lane 11 :	NCTC8325	Reference
Lane 12 :	WBG523	Gm^R MRSA control
Lane 13 :	WBG8003A	$Gm^RKm^RNm^R$
Lane 14 :	WBG8003B	$Gm^SKm^SNm^S$, also labelled as WBG8003GS2

*The white asterisks indicate the band differences
Gm, gentamicin; Km, kanamycin; Nm, neomycin; Superscript R, resistance; Superscript S, susceptible

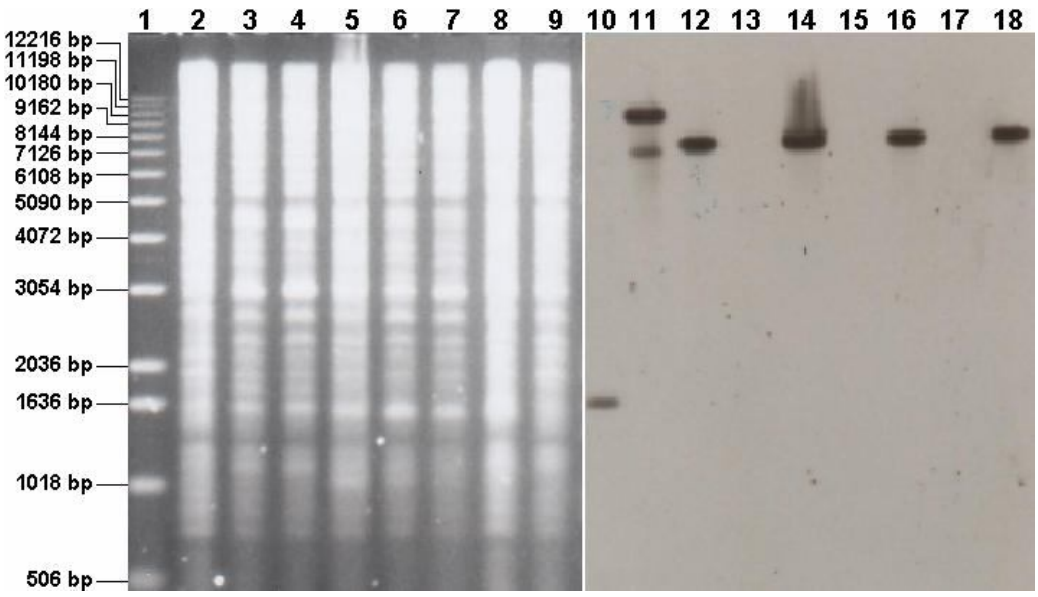


Figure 4.20 Hybridisation with an *aacA-aphD* probe, C3

Lane 1 : 1 kb DNA ladder

Lane 2 : WBG523, *Eco*RI chromosomal digest of the gentamicin-resistant-positive control, Gm^RKm^R

Lane 3 : WBG2015A *Eco*RI chromosomal digests; $Gm^RKm^RNm^R$

Lane 4 : WBG2015B *Eco*RI chromosomal digests; $Gm^SKm^SNm^S$

Lane 5 : WBG7410A *Eco*RI chromosomal digests; $Gm^RKm^RNm^R$

Lane 6 : WBG7410B *Eco*RI chromosomal digests; $Gm^SKm^RNm^R$

Lane 7 : WBG7425A *Eco*RI chromosomal digests; $Gm^RKm^RNm^R$

Lane 8 : WBG7425B *Eco*RI chromosomal digests; $Gm^SKm^RNm^R$

Lane 9 : WBG8484 *Eco*RI chromosomal digests; $Gm^RKm^RNm^R$

Lane 10 : [a]1 kb DNA ladder hybridised with *aacA-aphD* probe

Lane 11 : [b]WBG523 hybridised with *aacA-aphD* probe.

Lane 12 : WBG2015A hybridised with *aacA-aphD* probe

Lane 13 : WBG2015B hybridised with *aacA-aphD* probe

Lane 14 : WBG7410A hybridised with *aacA-aphD* probe

Lane 15 : WBG7410B hybridised with *aacA-aphD* probe

Lane 16 : WBG7425A hybridised with *aacA-aphD* probe

Lane 17 : WBG7425B hybridised with *aacA-aphD* probe

Lane 18 : WBG8484 hybridised with *aacA-aphD* probe

Gm, gentamicin; Km, kanamycin; Nm, neomycin; Superscript R, resistance; Superscript S, susceptible

[a]The 1636bp band of the 1kb DNA ladder hybridised with the C3 probe.

[b]The 2nd weaker band probably is due to a second copy of *aacA-aphD*; or a non-specific hybridisation of the C3 probe similar to the non-specific hybridisation observed with the 1kb DNA ladder

Table 4.13 The molecular characterisation of representative Malaysian MRSA isolates from 1982 to 2000

Strains	Location[a]	Year	*Sma*I CHEF types	Phage type	Plasmid type (kb)	SCC*mec* allotype	*Xba* I in *mecA*	Coagulase-gene type
WBG7422	HUM	1989	A	85/90	P31, 1.5, 2.6, 3.0, 28, 53OC	III	+	24
WBG8009	HUM	1994	A1	85	P32, 2.6, 3.0, 28, 30.5, 35	III	+	24
WBG8003	HUM	1994	A2	85	P35, 1.5, 2.6, 3.0, 28, 30.5, 35	III	+	24
WBG8154	HUM	1994	A3	85	P24, 1.5, 3.0, 28, 35	III	+	24
WBG8010	HUM	1994	A4	85/90	P35, 1.5, 2.6, 3.0, 28, 30.5, 35	III	+	24
WBG7409	HUM	1989	A5	NT	P26, 1.5, 3.0, 28, 53OC	III	+	24
WBG8011	HUM	1994	A6	NT	P35, 1.5, 2.6, 3.0, 28, 30.5, 35	III	+	24
WBG2005	HUM	1982	A7	90	Plasmid free	III	+	24
WBG2008	HUM	1982	A8	NT	P01, 2.6	III	+	24
WBG7414	HUM	1989	A9	NT	P22, 1.5, 2.6, 3.0, 28	III	+	24
WBG8007	HUM	1994	A10	85	P17, 1.5, 3.0, 35	III	+	24
WBG7413	HUM	1989	A11	NT	P16, 1.5, 3.0, 28	III	+	24
WBG2004	HUM	1982	A12	88/90	Plasmid free	III	+	24

[a]HUM, The University Hospital of the University of Malaya; HUKM, The Hospital of the University Kebangsaan Malaysia

Continued next page

Table 4.13 continued

Strains	Location[a]	Year	*Sma*I CHEF types	Phage type	Plasmid type (kb)	SCC*mec* allotype	*Xba* I in *mecA*	Coagulase-gene type
WBG2013	HUM	1982	A13	NT	P01, 2.6	III	+	24
WBG2003	HUM	1982	A14	NT	P06, 2.6, 3.0	III	+	24
WBG8024	HUKM	1994	A15	85	P21, 3.0, 35, 37	III	+	24
WBG7425B	HUM	1989	A16	NT	P31, 1.5, 2.6, 3.0, 28, 53OC	III	+	24
WBG7427	HUM	1989	B	85	P25, 1.5, 3.0, 31, 53OC	III	+	24
WBG2006	HUM	1982	C	NT	P01, 2.6	III	+	24
WBG2007	HUM	1982	D	NT	P05, 1.5, 40OC	III	+	24
WBG2011	HUM	1982	E	88	P11, 3.0, 53OC	III	+	24
WBG7893	HUKM	1994	F	85/90	P13, 1.5, 2.6, 28	III	+	24
WBG10358	HUM	2000	G	85	P28, 3.0, 28, 30.5, 35	III	+	24
WBG10359	HUM	2000	H	NT	P28, 3.0, 28, 30.5, 35	III	+	24
WBG10361	HUM	2000	I	NT	P20, 3.0, 4.4, 28	III	+	24
WBG10364	HUM	2000	J	85	P28, 3.0, 28, 30.5, 35	III	+	24

[a]HUM, The University Hospital of the University of Malaya; HUKM, The Hospital of the University Kebangsaan Malaysia

4.9 Summary

All the Malaysian isolates studied are resistant to at least five of the different antimicrobials tested. They are commonly resistant to gentamicin, kanamycin, erythromycin, lincomycin, tetracycline, trimethoprim and sulphonamide. Ciprofloxacin resistance is not common in strains isolated from 1982 to 1994, but all the isolates in 2000 were resistant to ciprofloxacin.

Over a third of the isolates were not typable by the IBS set of phages. The majority of typable isolates were susceptible to the group III phages, especially phage 85.

The plasmid profiles of Malaysian isolates other than the 1982 isolates were very similar to each other. The majority of 1994 and 2000 isolates carried three medium size plasmids and a number of small plasmids. The genetic characteristics of these plasmids are analysed in Chapter 5.

The seventy-four Malaysian isolates analysed could be separated into 26 groups according to their CHEF patterns. Thirteen of these 26 groups had CHEF patterns that were more than 80% similar and therefore were closely related to each other. A predominant CHEF pattern was found in the 1982, 1989 and 1994 isolates. The CHEF patterns of the 2000 isolates were different from the older isolates, but they were still closely related. All of the strains belonged to the coagulase-gene type 24 and carried the Allotype III SCC*mec* cassette. The Malaysian isolates were closely related to the EA MRSA from Australia. The relationship of Malaysian MRSA and the MRSA isolated from different regions is analysed in Chapter 9

The loss of gentamicin, kanamycin and neomycin resistance seen in Malaysian isolates corresponded to a *c.*114 kb chromosomal deletion. The deleted segment was found to carry the *aacA-aphD* gene that encodes for gentamicin and kanamycin resistance. This deleted region most probably contains the neomycin-resistance gene(s) as this resistance was also lost in the strain with this deletion.

CHAPTER FIVE

PLASMIDS OF MALAYSIAN MRSA

5.1 Introduction

Most of the *S., aureus* isolates carry one or more plasmids. Only a small minority of the isolates are plasmid free. (Melo-Cristino and Torres-Pereira, 1989, Trindade *et al.*, 2003, Novick, 1990, Skurray *et al.*, 1988) Some staphylococcal plasmids can be readily transferred between *S. aureus* isolates by different processes such as by conjugation, phage-mediated transfer (MCT), transduction and transformation. As many plasmids are known to carry antiseptic and antibiotic resistance genes, plasmids are one of the important factors in the dissemination of resistance in *S. aureus.* (Novick, 1991, Firth and Skurray, 2000). Currently, staphylococcal plasmids can be divided into four classes according to their size and features. (Novick, 1991, Firth and Skurray, 2000, Skurray and Firth, 1997)

The MRSA isolates from the Malaysian hospitals carried up to 13 different plasmids and this chapter describes the characterisation of these plasmids and their relationship to each other.

5.2 The small plasmids

Three small plasmids of size 1.5 kb, 2.6 kb and 3.0 kb were found in most of the Malaysian isolates. The number of these small plasmids in the isolates varies from three to one. Representatives of these three plasmids were isolated individually and their relationship examined by restriction enzyme digestions.

The 1.5 kb plasmid was not carried alone in any of the Malaysian isolates. The 1982 strain WBG2007 carried a 40 kb OC plasmid and a 1.5 kb plasmid. The 40 kb OC plasmid was removed by plasmid curing (also see Section 5.4) so that the 1.5 kb plasmid could be isolated. The 2.6 kb and 3.0 kb plasmids were found alone in strain WBG2008 and WBG7888, respectively. These three plasmids were isolated and digested with restriction enzymes. A range of restriction enzymes was used in order

to find ones that gave multiple cuts with the small plasmids. Initially *Alu*I was used but it gave many small faint bands which were difficult to size. So, although it clearly showed that the plasmids were different (See Figure 5.1) it was replaced with other enzymes for further analyses. The 2.6 and 3.0 kb plasmids gave distinct restriction patterns with both AluI and HinfI. (See Figure 5.1) The sum of the restriction enzyme-generated fragments was often less than their corresponding plasmids. This is probably due to the loss of small fragments in the electrophoresis and the cumulative effect of the margins of error in the measurement and calculation of the size of each fragment, together with the inaccuracies of determining whole plasmid sizes in gels.

The 2.6 kb Malaysian plasmid was distinctively different from the other plasmids tested and probably is also a cryptic plasmid. The absence of the 2.6 kb plasmid did not alter any known phenotypes of the host. WBG7419 and WBG7420 have the same plasmid profiles, except WBG7419 does not carry the 2.6 kb plasmid. However, their CHEF and antimicrobial profiles were identical.

The 3.0 kb Malaysian plasmid was compared with a 3.0 kb plasmid carried by a Singaporean MRSA, WBG9018, isolated in 1997. The 3.0 kb plasmid in WBG9018 has been fully sequenced and found to be a cryptic plasmid. (Sim, 2003) The plasmids were digested with *Hin*fI, *Xba*I and *Ssp*I restriction enzymes. Both plasmids gave identical restriction patterns with these restriction enzymes (See Figure 5.3). This suggests that the 3.0 kb Malaysian plasmid is also a cryptic plasmid.

The 3.0 kb plasmid found in the 1989 Malaysian isolates, WBG7410 and WBG7424 was found to carry kanamycin resistance, even though its size is identical to the cryptic 3.0 kb plasmid found in 1994 isolates. This kanamycin-resistance plasmid was only found in the 1989 Malaysian isolates and was mobilised by a large 38.1 kb trimethoprim-resistance conjugative plasmid (See Section 5.4). (Udo *et al.*, 1992b)

The 1.5 kb Malaysian plasmid was compared with a 1.5 kb cryptic plasmid carried by WBG6083, an Australian MRSA isolated in 1986. (Wei, 1993) The two 1.5 kb plasmids were digested with *Eco*RV and *Hin*fI restriction enzymes and the restriction

patterns were found to be identical (see Figure 5.2). This indicates that the two plasmids are very similar and that like the Australian plasmid, like the Malaysian plasmid is most likely also cryptic.

Table 5.1 Restriction enzyme analysis of small plasmids

Plasmid	Carrier	RE[a]	Restriction fragments[b] (bp)
1.5 kb	WBG2007	*Alu*I	1170, 330
		*Hin*fI	1200, 300
		*Eco*RV	1100, 400
1.5 kb	WBG6083	*Hin*fI	1200, 300
		*Eco*RV	1100, 400
2.6 kb	WBG2008	*Alu*I	440, 370, 200, 150
		*Hin*fI	1700, 900
3.0 kb	WBG7888	*Alu*I	831, 540, < 150,< 150, <100, < 100
		*Hin*fI	1160, 700, 698, 400
		*Ssp*I	1900, 1000
		*Xba*I	2850
3.0 kb	WBG9018	*Hin*fI	1160, 700, 698, 400
		*Ssp*I	1900, 1000
		*Xba*I	2850

[a]RE, restriction endonucleases.
[b]Small fragments less than 564 bp were out of the range of the DNA markers used and their sizes were estimated.

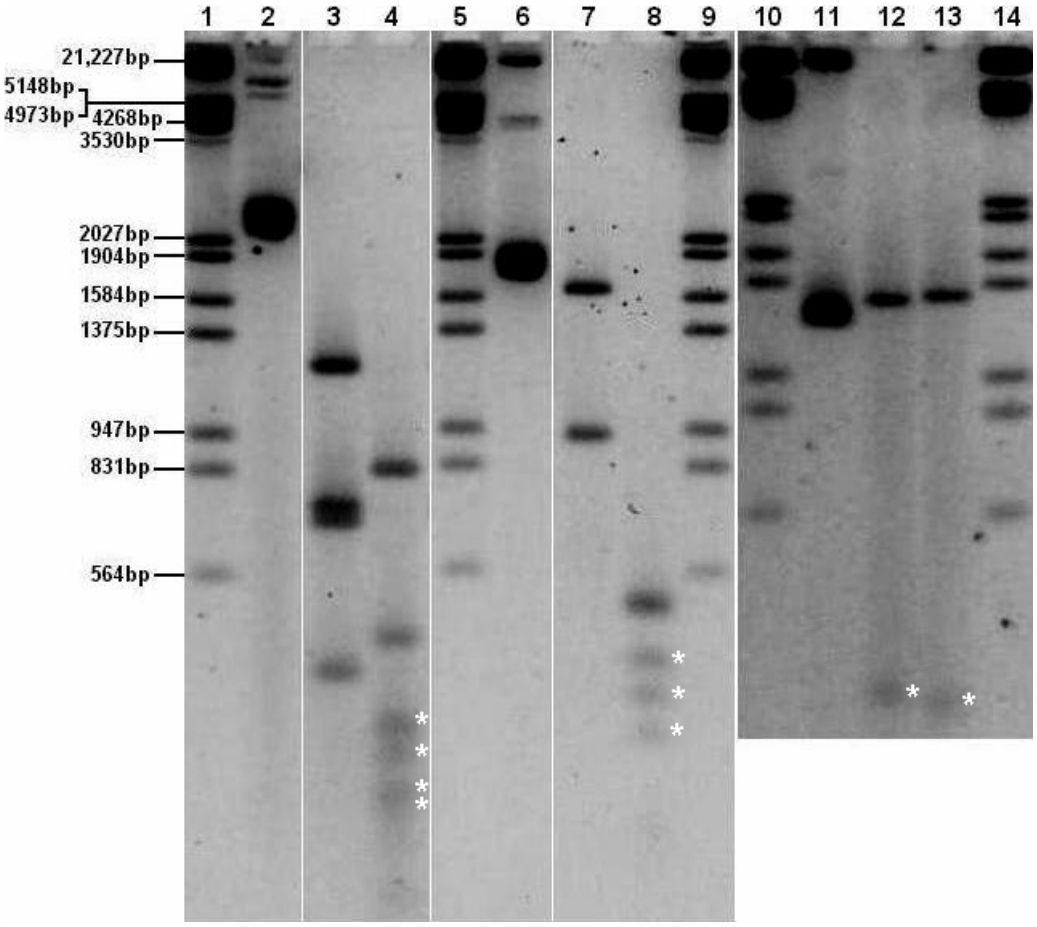


Figure 5.1 Restriction enzyme patterns of small plasmids[a]

Lane 1 : Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 2 : Undigested 3.0 kb plasmid
Lane 3 : *Hin*fI digestion of a 3.0 kb plasmid
Lane 4 : *Alu*I digestion of a 3.0 kb plasmid
Lane 5 : Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 6 : Undigested 2.6 kb plasmid
Lane 7 : *Hin*fI digestion of a 2.6 kb plasmid
Lane 8 : *Alu*I digestion of a 2.6 kb plasmid
Lane 9 : Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 10 : Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 11 : Undigested 1.5 kb plasmid
Lane 12 : *Hin*fI digestion of a 1.5 kb plasmid
Lane 13 : *Alu*I digestion of a 1.5 kb plasmid
Lane 14 : Lambda *Eco*RI/*Hin*dIII DNA marker

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

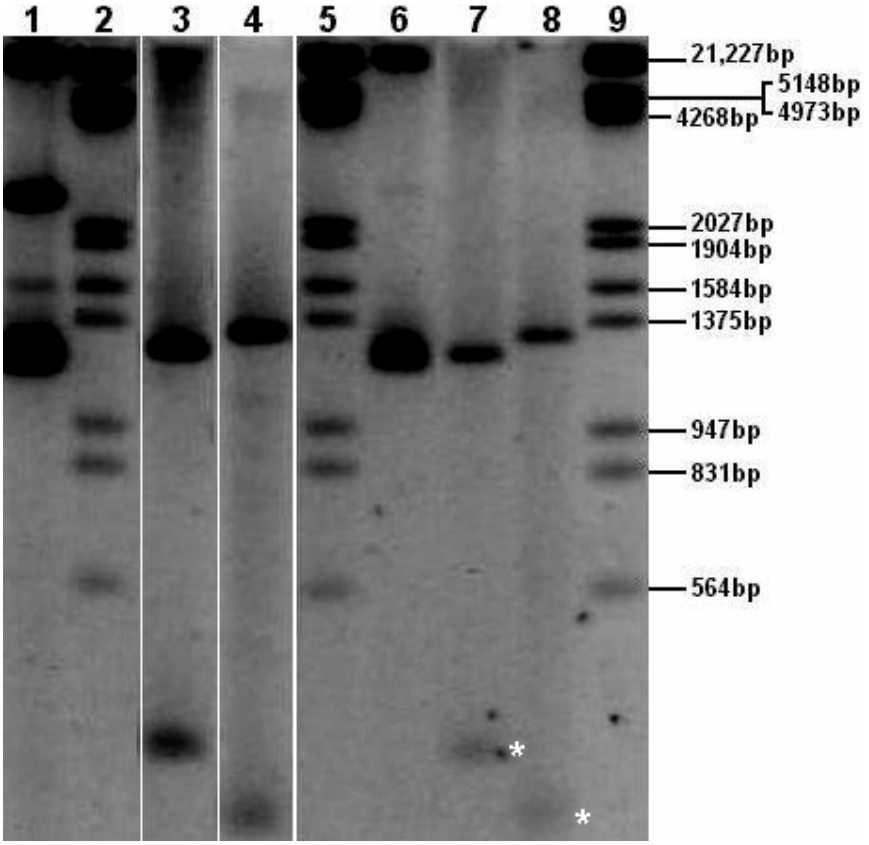


Figure 5.2 Restriction enzyme patterns of 1.5 kb plasmids[a]

Lane 1 : Undigested 1.5 kb plasmid from WBG6083

Lane 2 : Lambda *Eco*RI/*Hin*dIII DNA marker

Lane 3 : [b]*Eco*RV digestion of the 1.5 kb plasmid from WBG6083

Lane 4 : [b]*Hin*fI digestion of the 1.5 kb plasmid from WBG6083

Lane 5 : Lambda *Eco*RI/*Hin*dIII DNA marker

Lane 6 : Undigested 1.5 kb plasmid from WBG2007

Lane 7 : *Eco*RV digestion of the 1.5 kb plasmid from WBG2007

Lane 8 : *Hin*fI digestion of the 1.5 kb plasmid from WBG2007

Lane 9 : Lambda *Eco*RI/*Hin*dIII DNA marker

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

[b]Same gel as lanes 2, 5 to 9

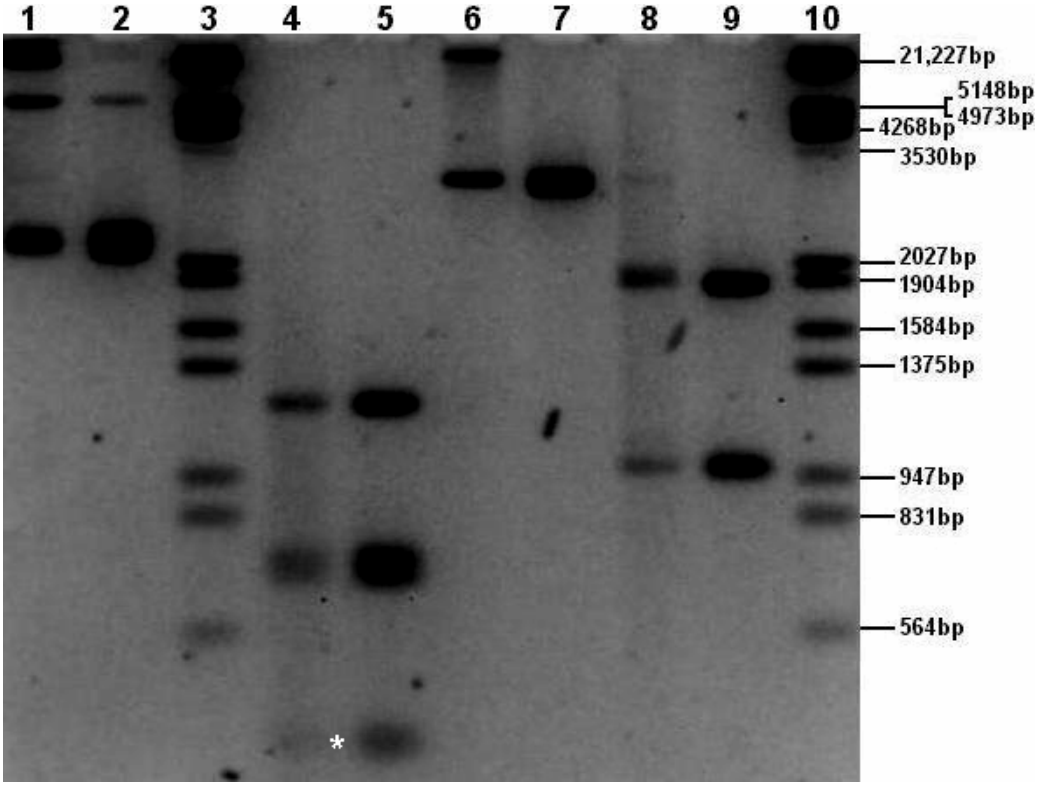


Figure 5.3 Restriction enzyme patterns of 3.0 kb plasmids

Lane 1 : Undigested 3.0 kb plasmid from WBG7888

Lane 2 : Undigested 3.0 kb plasmid from WBG9018

Lane 3 : Lambda *Eco*RI/*Hin*dIII DNA marker

Lane 4 : *Hin*fI digestion of the 3.0 kb plasmid from WBG7888

Lane 5 : *Hin*fI digestion of the 3.0 kb plasmid from WBG9018

Lane 6 : *Xba*I digestion of the 3.0 kb plasmid from WBG7888

Lane 7 : *Xba*I digestion of the 3.0 kb plasmid from WBG9018

Lane 8 : *Ssp*I digestion of the 3.0 kb plasmid from WBG7888

Lane 9 : *Ssp*I digestion of the 3.0 kb plasmid from WBG9018

Lane 10 : Lambda *Eco*RI/*Hin*dIII DNA marker

[a]The white asterisk marks the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

5.3 Plasmid instability

Some isolates were found to lose their plasmids over 10 to 20 years of storage in freeze-dried tubes and during successive subculturing. WBG8003 is one of these isolates. This isolate was initially found to lose its gentamicin (Gm), kanamycin (Km) and neomycin (Nm) resistance together with c.114kb of chromosomal DNA on long-term storage and successive subculturing. (See Section 4.8 and Figure 4.19) The subsequent plasmid profiling of this isolate found three variants, WBG8003GS1, WBG8003GS2 and WBG8003GS3, that had lost some of their plasmids in addition to the loss of Gm, Km and Nm resistances. Their *Sma*I-CHEF patterns are identical indicating that they are the same strain. (See Figure 5.4, Figure 5.5 and Table 5.2)

WBG8003GS1 lost its 28 kb, 30.5 kb and 35 kb plasmids together with resistance to NAB compounds and heavy metals but retained smaller plasmids of 1.5 kb, 2.6 kb and 3.0 kb. Unlike WBG8003GS1, WBG8003GS2 and WBG8003GS3 have each retained one of the larger plasmids. WBG8003GS2 has retained the 35 kb plasmid, whereas WBG8003GS3 has retained the 30.5 kb plasmid. (See Figure 5.4 and Table 5.2) However, unlike WBG8003GS1, WBG8003GS2 and WBG8003GS3 have not lost resistance to the NAB compounds and heavy metals resistance. This indicates that both the 30.5 kb and 35 kb plasmids encode resistance to NAB and heavy metal ions.

Both of these plasmids were isolated and studied in more detail. The results are shown in the following Section, 5.4.

Table 5.2 Plasmids and phenotypes lost from WBG8003

Isolate WBG	Plasmid profiles kb	Plasmids Lost kb	Resistances lost	β-lactamase production
8003	1.5, 2.6, 3.0, 28, 30.5, 35	None	None	Positive
8003GS1	1.5, 2.6, 3.0	28, 30.5, 35	Cd, Eb^{I}, Pi, Gm, Km, Nm	Positive
8003GS2	1.5, 2.6, 3.0, 35	28, 30.5	Gm, Km, Nm	Positive
8003GS3	1.5, 2.6, 3.0. 30.5	28, 35	Gm, Km, Nm	Positive

Abbreviations:

Cd, cadmium acetate; Eb, ethidium bromide; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Nm, neomycin; Pi, propamidine isethionate; Pma, phenyl mercuric acetate; Superscript I, intermediate resistance.

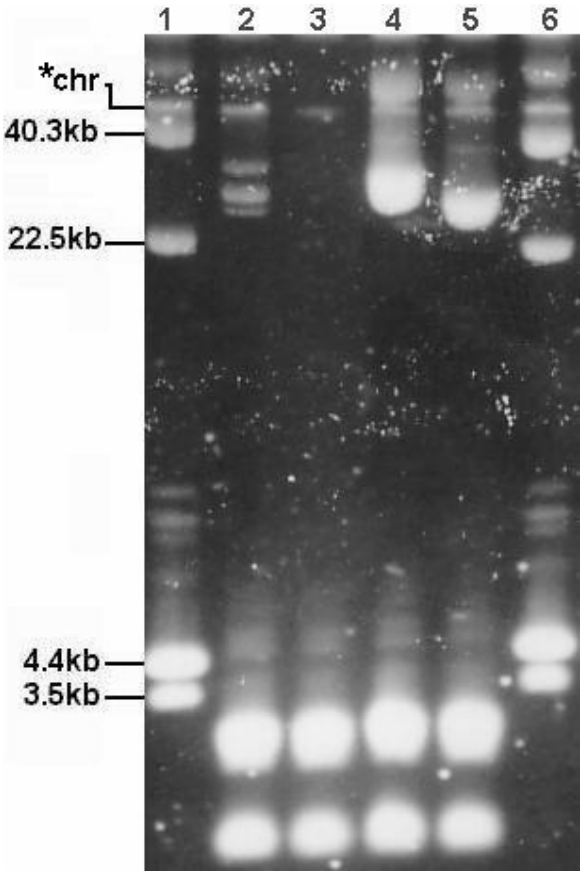


Figure 5.4 Plasmid profiles of WBG8003 variants

Lane 1 : WBG4483, molecular weight marker

Lane 2 : [a]WBG8003 (1.5 kb, [a]2.6 kb, [a]3.0 kb, 28 kb, 30.5 kb, 35 kb)

Lane 3 : WBG8003GS1 (1.5 kb, 2.6 kb, 3.0 kb)

Lane 4 : [b]WBG8003GS2 (1.5 kb, 2.6 kb, 3.0 kb, 35kb)

Lane 5 : [c]WBG8003GS3 (1.5 kb, 2.6 kb, 3.0 kb, 30.5 kb)

Lane 6 : WBG4483, molecular weight marker

*chr, chromosomal DNA

[a]See Figure 4.4 which shows that this large band in comprised of a 2.6 and 3.0 kb plasmids

[b]The restriction cuts, see Figure 5.6 and Table 5.4, show that this is the 35 kb plasmid which has run a lot faster because of the large concentration of DNA in the preparation

[c]The same explanation as above demonstrated that this is the 30.5kb plasmid.

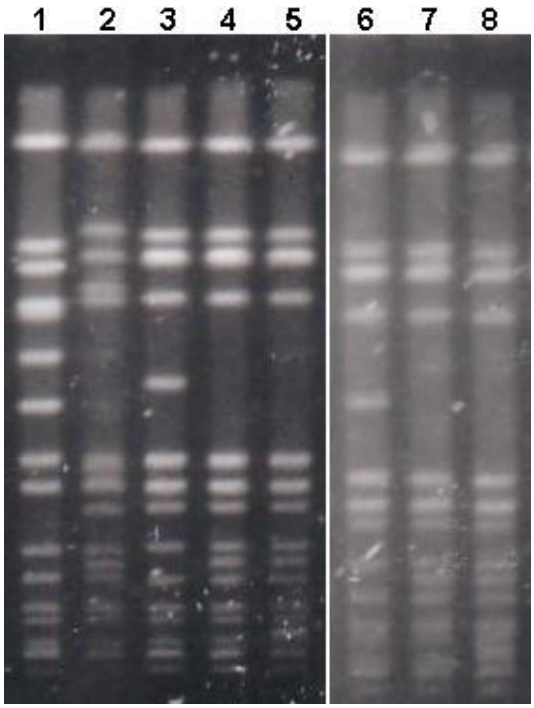


Figure 5.5 CHEF profiles of WBG8003 variants

Lane 1 : NCTC8325, size marker reference and alignment control

Lane 2 : WBG525, EA MRSA, MRSA control

Lane 3 : WBG8003

Lane 4 : WBG8003GS1,

Lane 5 : WBG8003GS2

Lane 6 : WBG8003

Lane 7 : WBG8003GS2

Lane 8 : WBG8003GS3

5.4 The Class II multiresistance plasmids

Many of the Malaysian isolates carried three plasmids of sizes 28 kb, 30.5 kb and 35 kb. (See Tables 4.5 and 4.8 to 4.12) These plasmids were found to carry resistance to the nucleic acid-binding compounds (NAB), which include the quaternary ammonium compounds (QAC), diamidines and DNA intercalating agents. In addition to NAB resistance, they also carry resistance to the heavy metals cadmium and mercury with, or without, the genes for β-lactamase production.

The plasmids were transferred individually into a recipient strain by MCT. (See Section 3.6.1) In the case of WBG7884, three plasmids were transferred together into the recipient. Two WBG8003 variants, WBG8003GS2 and WBG8003GS3 were used as the donors for the transfer of the 35 kb and 30.5 kb plasmids respectively. Isolates carrying a single medium size plasmid ranging from 28 to 35 kb were also used as donors for transfer. The results are shown in Table 5.3, Figure 5.6 and Figure 5.7.

Four 28 kb plasmids, one 30.5 kb plasmid, one 31 kb plasmid and three 35 kb plasmids were transferred into recipient strains. The 28 kb, 30.5 kb, 31 kb and 35 kb plasmids were found to carry the same phenotype with resistances to heavy metals and NAB. The 31 and 35 kb plasmid carried NAB and heavy metal resistances and encoded β-lactamase. (See Table 5.3) The relationship of these plasmids was investigated using restriction fragment length polymorphism. The *Eco*RI restriction patterns of the 28 kb, 30.5 kb and 35 kb plasmids only differed by one or two fragments. (See Figure 5.9) The *Eco*RI RFLPs of the 30.5 kb and 35 kb plasmids were difficult to interpret because some of the fragments have very similar sizes and migrate close together in the agarose gel. (See Figure 5.9) Consequently, *Hin*dIII restriction enzyme was used to study these plasmids. The *Hin*dIII restriction patterns of these three plasmids and the 31 kb plasmid also gave similar results and only had one to two fragment differences. Fragments smaller than 4600 bp were all the same size. This indicates that the four plasmids are closely related to each other. Since these plasmids were found in isolates from different years and hospitals, the

Malaysian MRSA have carried the same family of NAB plasmids for many years. The restriction patterns are shown in Figure 5.8 and Table 5.4

The size of the CCC form of the 28 kb plasmid was determined using the MacVector software and the size of the fragments generated by restriction enzymes was determined by linear regression. The *Eco*RI digestion of the 28 kb plasmid generated six fragments and the sum of the fragments was approximately 28 kb (See Figure 5.9 and Table 5.4). This size correlated well with the size determined by the MacVector Software using the CCC form of this plasmid. However, the *Hin*dIII digestion of the 28 kb plasmid generated nine fragments, the sum of which was approximately 26 kb. This difference is probably due to small fragments not detected by electrophoresis and the cumulative effect of the marginal error in each band size measurement. The sizes of the 30.5 kb, 31 kb and 35 kb plasmids were calculated using their *Hin*dIII and *Eco*RI restriction fragments by linear regression.

A South Australian (SA) MRSA WBG8888 carried a 28 kb plasmid with the same phenotype as the 28 kb and 30.5 kb plasmids in the Malaysian isolates. This plasmid had been previously reported to be identical to a 28 kb plasmid found in a Singaporean MRSA. (Chong, 2003) The 20 kb plasmid isolated from an Indonesian MRSA, INDO-3, possesses a similar phenotype to the 28 kb and 30.5 kb plasmid in Malaysian strains. However, this smaller 20 kb plasmid does not carry the mercury resistance. (Grubb and Williams, personal communication) These plasmids were compared by *Hin*dIII restriction digestion. The 28 kb plasmid from SA had an identical pattern to the 28 kb plasmid from the Malaysian MRSA. The 20 kb plasmid had five *Hin*dIII fragments compared to nine fragments of the 28 kb plasmids. The five fragments of the 20kb plasmid were the same size as five of the 28 kb plasmid fragments (Figure 5.10 and Table 5.4). This indicates that the 28 kb SA MRSA plasmid is similar to its Malaysian counterpart and closely related to the 20 kb plasmid in the Indonesian MRSA.

Table 5.3 Mixed-Culture Transfer Results

Donor Strain WBG	Donor Plasmid Profiles kb	Recipient Strain	Plasmid Transferred kb	Selection	Phenotypes Transferred
7884*	2.6, 3.0, 31, 32, 40	1876	31, 32, 40	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi, Bla
7884*	2.6, 3.0, 31, 32, 40	1876	31, 40	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi, Bla
7419	1.5, 3.0, 28	1876	28	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi
7887	2.6, 3.0, 28	879	28	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi
7895	1.5, 2.6, 3.0, 28	1876	28	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi
8888[a]	28	1876	28	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi
8012	28	1876	28	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi
8003GS3	1.5, 2.6, 3.0, 30.5	1876	30.5	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi
7889	2.6, 3.0, 31	1876	31	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi, Bla
8023	2.6, 3.0, 35	879	35	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi, Bla
8024	3.0, 35, 37	879	35	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi, Bla
8003GS2	1.5, 2.6, 3.0, 35	1876	35	Pi, F, Rf	Cd, Hg, Pma, Eb^{I}, Pi, Bla
INDO-3[b]	2.0, 20	1876	20	Pi, F, Rf	Cd, Eb^{I}, Pi

Bla, β-lactamase production; Resistance to: Cd, cadmium acetate; Eb, ethidium bromide; F, fusidic acid; Hg, mercuric chloride; Pi, propamidine isethionate; Pma, phenyl mercuric acetate; Rf, rifampicin; Superscript I, intermediate resistance,.

[a]The transcipient of WBG8888 x 1876 was kindly provided by Ms F. N. Chong.

[b]The transcipient of INDO-3 x 1876 was kindly provided by Ms Nola Williams.

*Transcipient colonies with different plasmid profiles

Table 5.4 Restriction fragments of Class II multiresistant plasmids from Malaysian, Australian and Indonesian isolates

Transcipients[a] All WBG prefixes	Size kb	No of Fragments	Restriction fragments, bp													
***Eco*RI digestion**																
8012 x 1876	28	6			10200	6617		4400		2700	2400	1200				
8003GS3 x 1876	30.5	6			10200	6617	6616			2700	2400	1200				
8003GS2 x 1876	35	6	11466		10200	6617				2700	2400	1200				
***Hin*dIII digestion**																
8012 x 1876	28	9			9162				4600	3800	3000	1830	1300	1018	800	740
7887 x 879	28	9			9162				4600	3800	3000	1830	1300	1018	800	740
7895 x 1876	28	9			9162				4600	3800	3000	1830	1300	1018	800	740
7419 x 1876	28	9			9162				4600	3800	3000	1830	1300	1018	800	740
8003GS3 x 1876	30.5	9		11700					4600	3800	3000	1830	1300	1018	800	740
7889 x 1876	31	9	12200						4600	3800	3000	1830	1300	1018	800	740
8023 x 879	35	10				8700	7200		4600	3800	3000	1830	1300	1018	800	740
8024 x 879	35	10				8700	7200		4600	3800	3000	1830	1300	1018	800	740
8003GS2 x 1876	35	10				8700	7200		4600	3800	3000	1830	1300	1018	800	740
8888 x 1876[b]	28	9			9162				4600	3800	3000	1830	1300	1018	800	740
INDO-3 x 1876[c]	20	5			9162				4600	3800		1830				740

[a]All transcipients are from Malaysian isolates except the marked transcipients; [b]Singapore isolate; [c]Indonesian isolate

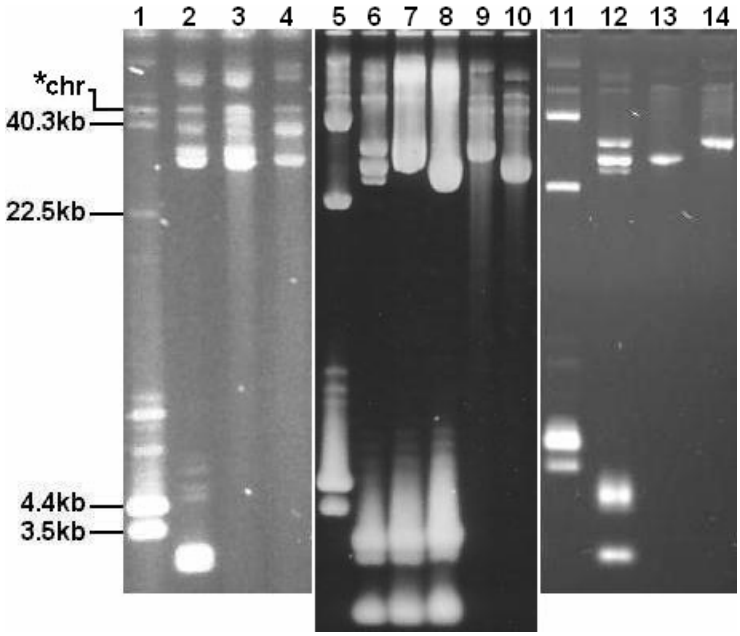


Figure 5.6 Agarose gels of mixed-culture transcipients

Lane 1 : WBG4483, molecular weight marker

Lane 2 : WBG7884, 2.6, 3.0, 31, 32 & 40kb. See also Figure 4.8

Lane 3 : WBG7884 x WBG1876, 31, 32 and 40kb

Lane 4 : WBG7884 x WBG1876, 31 &40 kb

Lane 5 : WBG4483, molecular weight marker

Lane 6 : WBG8003, 1.5, 2.6, 3.0, 28, 30.5 & 35kb

Lane 7 : [a]WBG8003GS2, donor strain, 1.5, 2.6, 3.0 & 35kb

Lane 8 : [b]WBG8003GS3, donor strain, 1.5, 2.6, 3.0 & 30.5kb

Lane 9 : [c]WBG8003GS2 x WBG1876, 35 kb

Lane 10 : [d]WBG8003GS3 x WBG1876, 30.5 kb

Lane 11 : WBG4483, molecular weight marker

Lane 12 : WBG8003, as in lane 6

Lane 13 : [d]WBG8003GS3 x WBG1876, 30.5 kb

Lane 14 : [c]WBG8003GS2 x WBG1876, 35 kb

*chr, chromosomal DNA

[a]The 35 kb has migrated faster than in the parent because of the amount of DNA. Compare transcipients in lane 9 and 14.

[b]The same explaination as in a. Compare rates with the different concentrations of DNA.

[c]Note the difference in migration rates with the different concentrations of DNA.

[d]Note as in c.

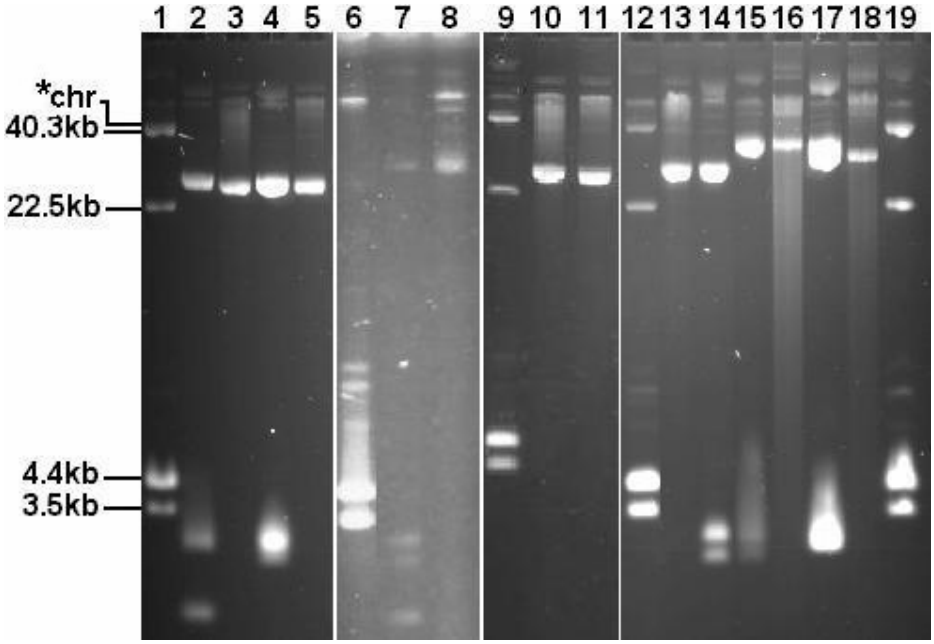


Figure 5.7 Agarose gels of mixed-culture transcipients

Lane 1 : WBG4483, molecular weight marker
Lane 2 : WBG7419, donor strain, 1.5, 3.0 and 28 kb
Lane 3 : WBG7419 x WBG1876, 28 kb
Lane 4 : WBG7887, donor strain, 2.6, 3.0 and 28 kb
Lane 5 : WBG7887 x WBG879, 28 kb
Lane 6 : WBG4483, molecular weight marker
Lane 7 : WBG7895, donor strain, 1.5, 2.6, 3.0 and 28 kb
Lane 8 : WBG7895 x WBG1876, 28 kb
Lane 9 : WBG4483, molecular weight marker
Lane 10 : WBG8012, donor strain, 28 kb
Lane 11 : WBG8012 x WBG1876, 28 kb
Lane 12 : WBG4483, molecular weight marker
Lane 13 : WBG7889 x WBG1876, 31 kb
Lane 14 : WBG7889, donor strain, 2.6, 3.0 and 31 kb
Lane 15 WBG8023, donor strain, 2.6, 3.0 and 35 kb
Lane 16 WBG8023 x WBG879, 35 kb
Lane 17 [a]WBG8024, donor strain, 3.0, 35 and 37 kb
Lane 18 WBG8024 x WBG879, 35 kb
Lane 19 WBG4483, molecular weight marker

*chr, chromosomal DNA

[a]See Figure 4.8 for a clearer gel picture.

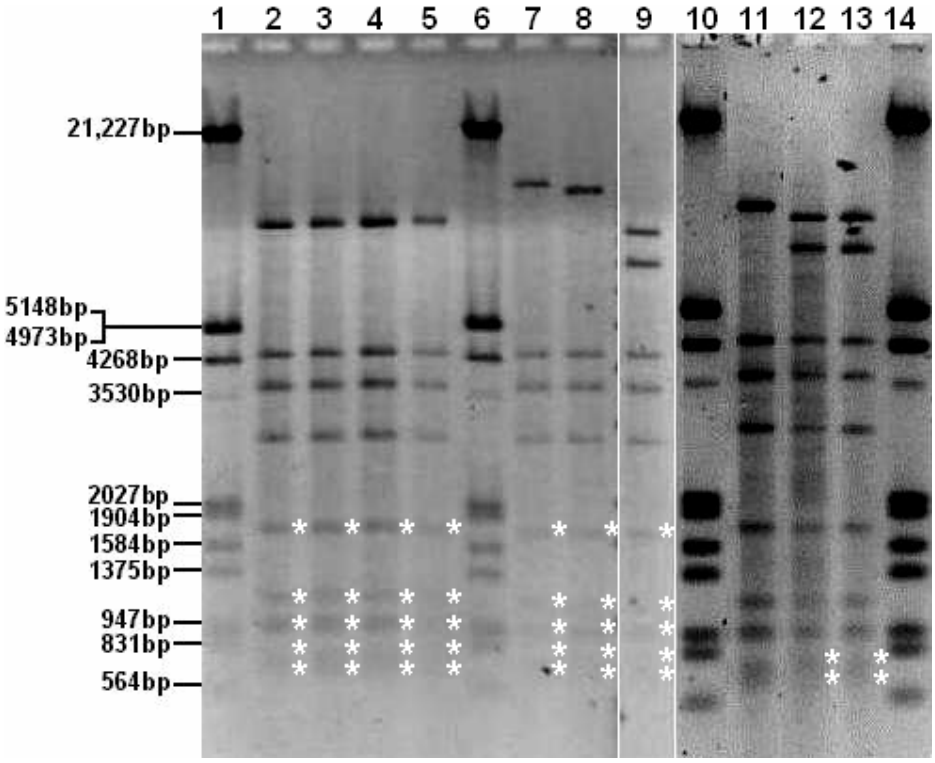


Figure 5.8 *Hin*dIII digests of transferred plasmids[a]

Lane 1 :	Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 2 :	WBG7419 x WBG1876, 28 kb
Lane 3 :	WBG7887 x WBG879, 28 kb
Lane 4 :	WBG7895 x WBG1876, 28 kb
Lane 5 :	WBG8012 x WBG1876, 28 kb
Lane 6 :	Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 7 :	WBG7889 x WBG1876, 31 kb
Lane 8 :	WBG8003GS3 x WBG1876, 30.5 kb
Lane 9 :	1 kb DNA ladder
[b]**Lane 10 :**	WBG8003GS2 x WBG1876, 35 kb
Lane 11 :	Lambda *Eco*RI/*Hin*dIII DNA marker
Lane 12 :	WBG8003GS3 x WBG1876, 30.5 kb
Lane 13 :	WBG8023 x WBG879, 35 kb
Lane 14 :	WBG8024 x WBG879, 35 kb
Lane 15 :	Lambda *Eco*RI/*Hin*dIII DNA marker

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.
[b]Same gel as lanes 1 to 9.

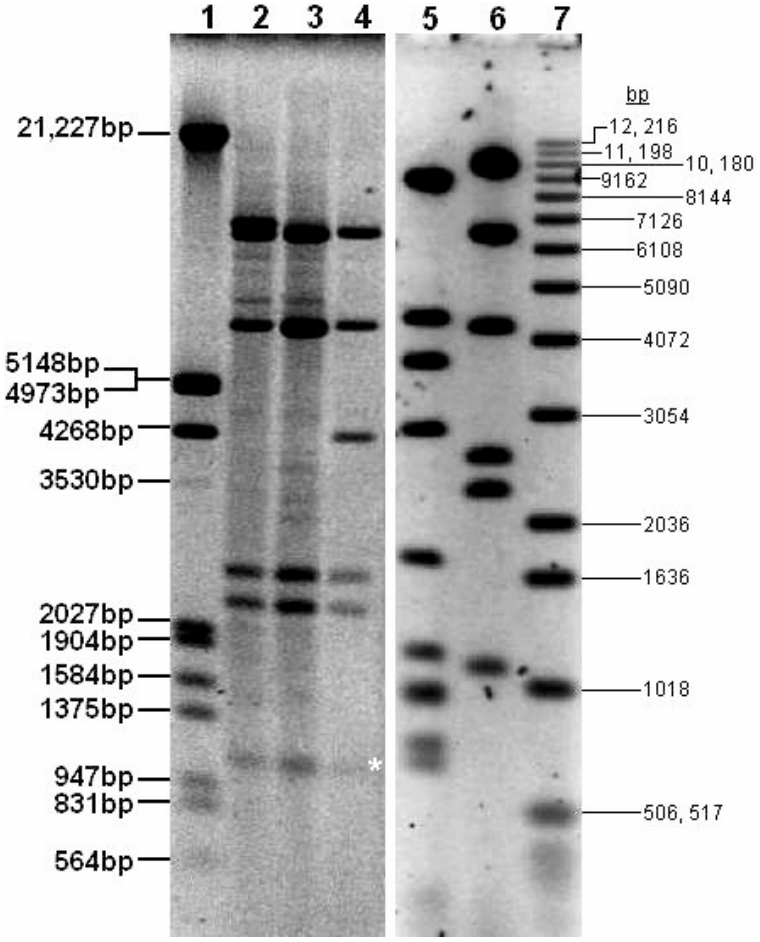


Figure 5.9 *Eco*RI digestion of Malaysian plasmids[a]

Lane 1 : Lambda *Eco*RI/*Hin*dIII DNA marker

Lane 2 : *Eco*RI digestion WBG8003GS2 x WBG1876, 35 kb

Lane 3 : *Eco*RI digestion WBG8003GS3 x WBG1876 , 30.5 kb

Lane 4 : *Eco*RI digestion of WBG8012, 28 kb plasmid

Lane 5 : [b]*Hin*dIII digestion of WBG8012, 28 kb plasmid

Lane 6 : [b]*Eco*RI digestion of WBG8012, 28 kb plasmid

Lane 7 : 1 kb DNA ladder

[a]The white asterisk marks the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.
[b]The *Eco*RI and *Hin*dIII digests of the 28kb plasmid were run with a 1kb DNA ladder for better size determination.
Note, calculation of sizes was done on lanes 5 and 6 using lane 7 as standard because the gel containing lanes 1 to 4 sloped from left to right.

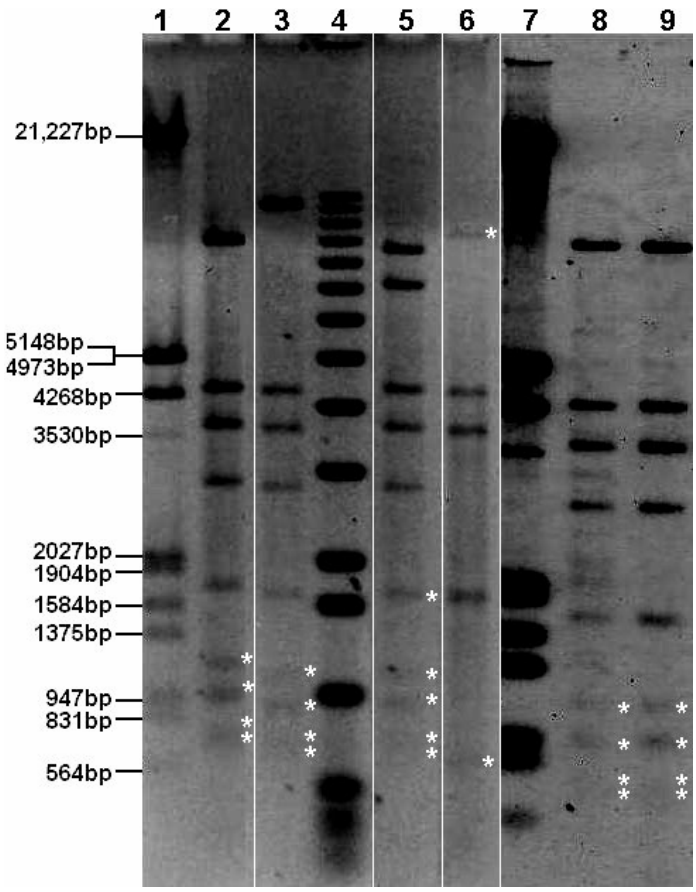


Figure 5.10 *Hin*dIII digests of NAB-resistance plasmids from different countries[a]

Lane 1 : Lambda *Eco*RI/*Hin*dIII DNA marker

Lane 2 : WBG8012 x WBG1876, 28 kb (Malaysian)

Lane 3 : WBG8003GS3 x WBG879, 30.5 kb (Malaysian)

Lane 4 : 1 kb DNA ladder (See Figure 5.9 for detail)

Lane 5 : WBG8003GS2 x WBG879, 35 kb (Malaysian)

Lane 6 : [b]INDO-3 x WBG1876, 20 kb (Indonesian)

Lane 7 : Lambda *Eco*RI/*Hin*dIII DNA marker

Lane 8 : WBG8012 x WBG1876, 28 kb (Malaysian)

Lane 9 : WBG8888 x WBG1876, 28 kb (South Australian)

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

[b] This lane was run on the same gel as lanes 1 to 5.

5.5 Large plasmids

The conjugative plasmid pWBG707 conferring trimethoprim resistance had previously been isolated from a Malaysian MRSA, WBG7410, and found to mobilise a small 3.0 kb kanamycin-resistance plasmid during conjugation. (Udo *et al.*, 1992b). WBG7483 is a transconjugant containing pWBG707 and was used in this study.

Isolate WBG7424 was used as the donor and WBG1876 as the recipient in a mixed-culture transfer. This experiment was initially designed for the isolation of the 28 kb plasmid in WBG7424. However, trimethoprim-resistance and kanamycin-resistance were co-transferred. These trimethoprim- and kanamycin-resistant transcipients could be grouped into two types. The first type of transcipient was resistant to trimethoprim and kanamycin and carried two transferred plasmids 53 kb OC and 3.0 kb. The other transcipient carried only the 53 kb plasmid but was also trimethoprim and kanamycin resistant. (Figure 5.11 and Table 5.5). The 53 kb OC plasmid was digested with *Eco*RI and found to have identical restriction patterns to the trimethoprim-resistance plasmid, pWBG707. As WBG7410 and WBG7424 were isolated in the same hospital during the same survey, it is likely that the 53 kb OC plasmid is identical to pWBG707. Thus, the 3.0 kb plasmid found together with the 53 kb OC plasmid is likely to carry kanamycin resistance. Although the second type of transcipient was kanamycin resistant, it does not carry the 3.0 kb kanamycin-resistance plasmid. It is likely that the small 3.0 kb kanamycin-resistance plasmid has integrated into the chromosome during plasmid transfer. pWBG707 is known to be a conjugative plasmid and able to mobilised a small 3.0 kb kanamycin plasmid. (Udo *et al.*, 1992b) It would appear that the 53 kb (OC) plasmid has the same conjugation and mobilisation ability as pWBG707.

*Eco*RI restriction digestion of the 53 kb OC plasmid generated five fragments which are the same size as the *Eco*RI fragments of pWBG707. (Figure 5.11) The published *Eco*RI pattern for pWBG707 consists of six bands of 23.1, 4.3, 3.6, 2.8, 2.6 and 1.6 kb. (Udo *et al.*, 1992b) However, the results here did not have the 3.6 kb band and the sizes of the other bands were calculated as 23, 4.6, 2.8, 2.6 and 1.56 kb (See Figure 5.11) On this basis the 53 kb OC plasmid is approximately 35 kb. However,

pWBG707 was reported in the published literature to have an additional band of 3.6 kb and a total size of 38.1 kb. (Udo *et al.*, 1992b)

The *dfrA* is the trimethoprim-resistance gene that is exclusively found in *S. aureus*. (Amyes and Towner, 1990). However, the labelled *dfrA* DNA probe failed to hybridise with the transcipient carrying pWBG707. *dfrD* is the trimethoprim-resistance gene found in coagulase-negative staphylococci and *L.monocytogenes*. (Charpentier and Courvalin, 1997, Dale *et al.*, 1995b). The specific primers for the *dfrD* gene also failed to amplify with pWBG707. These results indicate that pWBG707 encodes a novel trimethoprim-resistance gene. The hybridisation and the PCR results are shown in Figure 5.12.

WBG2007 is a 1982 Malaysian isolate that carries a 40 kb OC and a 1.5 kb plasmid. To study the 1.5 kb plasmid in the isolate, the 40 kb OC plasmid was cured from WBG2007 (See Section 5.2 and Figure 5.13). When the 40 kb OC plasmid was cured, no antimicrobial resistance was lost from WBG2007. This indicates that the 40 kb OC plasmid is probably a cryptic plasmid or that it has a phenotype that is also duplicated on the chromosome. If it is a large cryptic plasmid it might be related to other cryptic conjugative plasmids that have been previously described. (Udo *et al.*, 1987)

Table 5.5 Analysis of large plasmids

Donor WBG	Donor Plasmid Profiles, kb	Recipient Strain	Plasmids transferred or cured, kb	Selection	Phenotype transferred or lost
Mixed-culture transfer					
7424	1.5, 2.6, 3.0, 28, 53	1876	53	Km, F, Rf	Km, Tp
7424	1.5, 2.6, 3.0, 28, 53	1876	3.0, 53	Km, F, Rf	Km, Tp
Plasmid curing					
2007	1.5, 40	NA	40	NA	None

Km, kanamycin; Tp, trimethoprim; F, Fusidic acid; Rf, Rifampicin; NA, not applicable.

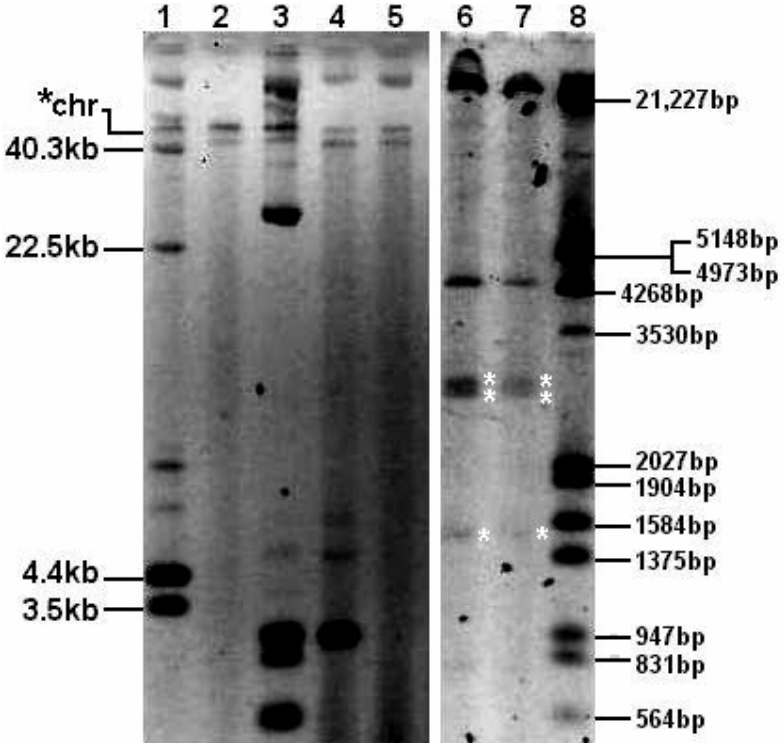


Figure 5.11 Analysis of the 53 kb OC plasmid from WBG7424[a]

Lane 1 : WBG4483, molecular weight marker

Lane 2 : [b]WBG1876, recipient strain

Lane 3 : WBG7424, donor strain, 53 kb OC, 28 kb, 3.0 kb, 2.6 kb, 1.5 kb

Lane 4 : WBG7424 x WBG1876, 53 kb OC, 3.0 kb

Lane 5 : WBG7424 x WBG1876, 53 kb OC

Lane 6 : *Eco*RI digestion of pWBG707 from WBG7483

Lane 7 : *Eco*RI digestion of a WBG7424 x WBG1876, transcipient containing a 53kb OC plasmid

Lane 8 : Lambda *Eco*RI/*Hin*dIII DNA marker

*chr, chromosomal DNA

[a]The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version.

[b]The additional band below the chromosomal band in WBG1876 is thought to be phage. (Personal communication, Prof. W. B. Grubb and Dr F. G. O'Brien)

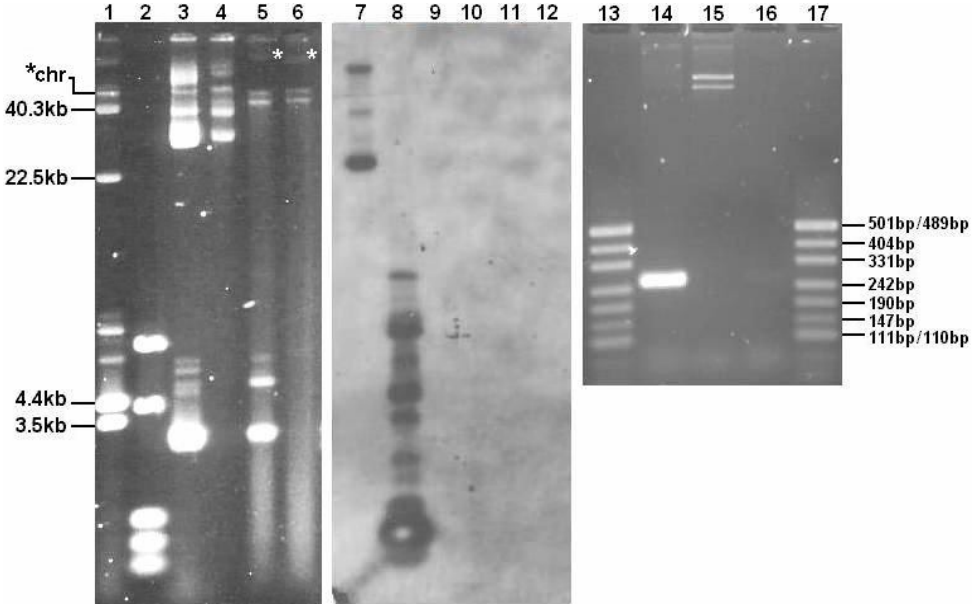


Figure 5.12 Analysis of the trimethoprim-resistance gene on pWBG707

Lane 1 : [a]WBG4483 plasmid preparation. Positive control for *dfrA*. The 22.5kb pWBG115 of WBG4483 carries the *dfrA* gene

Lane 2 : *Ssp*I digestion of pSK636 carrying *dfrA* gene. DNA probe control

Lane 3 : [b]WBG7884, total DNA from a trimethoprim-susceptible strain

Lane 4 : [b]WBG7884 x WBG1876, transcipient containing plasmids of 40 kb and 31 kb

Lane 5 : [b]WBG7424 x WBG1876, transcipients containing plasmids of 53 kb OC and 3.0 kb

Lane 6 : WBG7424 x WBG1876, transcipients containing plasmids of 53 kb OC

Lane 7 : WBG4483. The *dfrA* probe hybridised with pWBG115. The multiple bands are different conformers of pWBG115

Lane 8 : [c]Lane 2 pSK636 hybridised with *dfrA* probe.

Lane 9 : Lane 3 WBG7884 hybridised with *dfrA* probe.

Lane 10: Lane 4 WBG7884 x WBG1876 hybridised with *dfrA* probe.

Lane 11: Lane 5 WBG7424 x WBG1876, 53 kb, 3.0 kb hybridised with *dfrA* probe.

Lane 12: Lane 6 WBG7424 x WBG1876, 53 kb hybridised with *dfrA* probe.

Lane 13: *Hpa*II digested pUC19 size marker

Lane 14: *dfrD* positive control. PCR of pIP823 plasmid of *L. monocytogenes* BM4293 using *dfrD* primers

Lane 15: [d]*dfrD* negative control, PCR of WBG4483 plasmid preparation using *dfrD* primers

Lane 16: PCR of pWBG707 from WBG7483 using *dfrD* primers

Lane 17: *Hpa*II digested pUC19 size marker

*chr, chromosomal DNA; The white asterisks mark the locations of the bands which were present on the original gel picture but were either weak or not visible in the print version

[a]Townsend *et al.* (1986b)

[b]The band below the chromosomal band is believed to be phage DNA. See lane 2 Figure 5.11

[c]It is not known why the probe hybridised with so many bands of the pSK636 digest. It would appear that the probe was contaminated with some of the vector DNA

[d]The two faint bands are believed to be the two smaller plasmids of the WBG4483 plasmid preparation that was used as the template for the PCR.

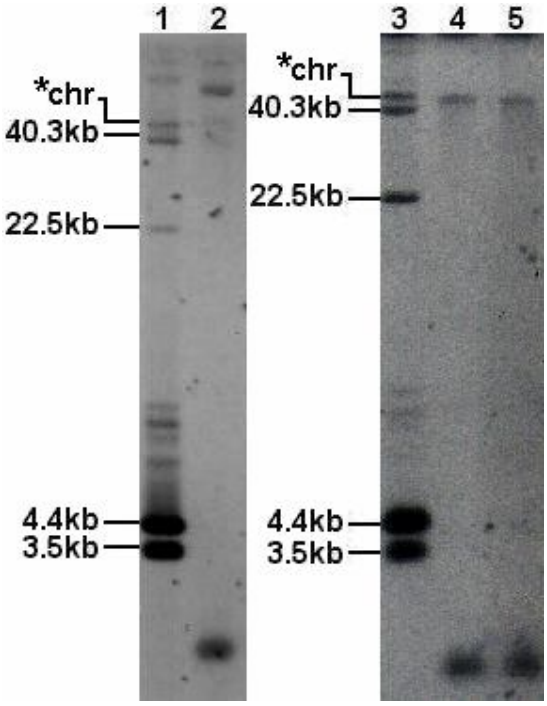


Figure 5.13 Curing of 40 kb OC plasmid from WBG2007

Lane 1 : WBG4483, positive control

Lane 2 : WBG2007, 1.5 kb and 40 kb OC

Lane 3 : WBG4483, positive control

Lane 4 : WBG2007 curing of 40 kb OC plasmid at 43^{o}C

Lane 5 : WBG2007 curing of 40 kb OC plasmid at 43^{o}C

*chr, chromosomal DNA

5.6 Summary

Three small cryptic plasmids of 1.5, 2.6 and 3.0 kb detected in Malaysian MRSA were isolated and studied. Restricted digestion of these plasmids showed that they are not related to each other. The 1.5 kb plasmid was found to be identical to the 1.5 kb cryptic plasmid found in the Australian isolate WBG6083. The 3.0 kb cryptic plasmid was indistinguishable from the 3.0 kb plasmid found in a Singaporean isolate, WBG9018. The 3.0 kb plasmid found exclusively in 1989 Malaysian isolates was found to carry kanamycin resistance and was mobilised by pWBG707 during conjugation.

Three medium size plasmids were commonly found together in the 1994 and 2000 Malaysian isolates. Their sizes were 28 kb, 30.5 kb and 35 kb respectively. The 28 kb and 30.5 kb plasmids were found to have identical phenotypes and conferred NAB and heavy-metal resistances. The 35 kb plasmid also encoded NAB and heavy-metal resistance but also encoded β-lactamase. These three plasmids were found to have closely related restriction-enzyme patterns and were found in isolates from different years and hospitals. The 31 kb plasmid in WBG7889 conferred an identical phenotype and had closely related restriction enzyme patterns to the 28 kb and the 30.5 kb plasmids. A 28 kb plasmid in a South Australian isolate, WBG8888, was indistinguishable from the Malaysian 28 kb plasmid in phenotype and restriction enzyme pattern. WBG8888 has previously been shown to be identical to some Singaporean MRSA. (Chong, 2003). The 20 kb plasmid in an Indonesian MRSA, INDO-3, had the same phenotype as the 28 kb and 30.5 kb plasmids and had closely related restriction-enzyme patterns. These results suggest that the NAB and heavy metal-resistance plasmids are widely spread in Southeast Asian MRSA. The NAB-resistance plasmid (pSK1 plasmid family) found in Eastern Australian MRSA is different from these plasmids and is associated with other antibiotic-resistances, such as gentamicin resistance and trimethoprim resistance. (Grubb, 1990, Firth and Skurray, 2000, Skurray *et al.*, 1988)

The trimethoprim-resistance conjugative plasmid, pWBG707, was found not to have either the *dfrA or dfrD* genes. These two genes are the only trimethoprim-resistance genes that have been found in staphylococci. pWBG707 probably encodes a novel

type of trimethoprim resistance. A 40 kb OC cryptic plasmid was also found in WBG2007. In general, the plasmids found among Malaysian MRSA were very similar to each other.

CHAPTER SIX

THE *mec* COMPLEX OF MALAYSIAN MRSA

6.1 Introduction

The *mec* complex together with the *ccr* complex is the core of the SCC*mec* genomic island. (Ito *et al.*, 1999) It consists of the *mecA* methicillin-resistance gene and its two regulatory genes, *mecR1* and *mecI*. The *mecR1* and *mecI* encode for the MecR1 signal-transducer and MecI repressor, respectively. The MecR1 is a weak signal transducer for β-lactams and the MecI is a strong repressor. Hence, a MRSA with intact *mecR1* and *mecI*, such as N315, only express low-level methicillin resistance that is clinically interpreted as methicillin sensitive. The β-lactamase regulatory genes, *blaR1* and *blaI* are similar to the *mecR1* and *mecI* genes. The BlaR1 signal transducer is a significantly faster transducer than MecR1, whereas the BlaI is a weaker repressor protein than MecI. The BlaR1-BlaI system cannot only regulate the *mecA* transcription but is more effective than the MecR1-MecI system. (Hiramatsu, 1995, McKinney *et al.*, 2001)

The *mec* complexes found among MRSA isolates are highly variable. At the time of writing eleven different classes of *mec* complex in MRSA and methicillin-resistant coagulase negative staphylococci had been published by other workers (Hiramatsu *et al.*, 2001, Oliveira *et al.*, 2000, Lim *et al.*, 2003, Lim *et al.*, 2002) and a further class was found in this study. (See Section 7.3.1) The *mecR1* and *mecI* genes of these *mec* complexes are often either truncated or disabled by mutations. (See Chapter 1, Figure 1.4) (Ito *et al.*, 2001, Lim *et al.*, 2002, Oliveira *et al.*, 2000, Shore *et al.*, 2005) The Class A and Class B *mec* complexes are the only two that had been described in Malaysian MRSA. (Hiramatsu, 1995, Hiramatsu *et al.*, 2001). Although the Class A *mec* complex of Malaysian MRSA has intact *mecR1* and *mecI* genes, a single point mutation was found within the *mecI* gene that prematurely terminated its transcription. The Class B mec complex in the Malaysian MRSA was identical to the prototype where the penicillin-binding domain of *mecR1* and the whole of *mecI* are deleted by a partial copy of IS*1272*. (Archer *et al.*, 1996, Hiramatsu, 1995)

In this dissertation, the Class A complex was the only class of *mec* complex detected among the 74 Malaysian MRSA isolates. (See Chapter 4, Section 4.7) The methicillin expression in MRSA is typically heterogeneous, which means that only a small proportion of the population expresses high-level methicillin resistance. (Hartman and Tomasz, 1986, Tomasz *et al.*, 1991, Pfeltz *et al.*, 2001) Methicillin expression does not correlate with the transcription of the *mecA* gene but to the host genetic background. (Finan *et al.*, 2002, Berger-Bächi and Rohrer, 2002) However, the genetic arrangement within the *mec* complex is thought to play an important role in the conversion of heterogeneous- to homogeneous-methicillin resistance. (Kondo *et al.*, 2001, Hiramatsu, 1995)

Isolate WBG7422 was selected to study the *mec* complex of Malaysian MRSA. It was selected because it belongs to the predominant CHEF group, type A, in the Malaysian isolates. WBG525 an EA MRSA is closely related to WBG7422 and EMRSA-1. WBG525 and WBG7422 were selected to compare *mec* complex of EA MRSA and Malaysian MRSA, respectively. The *mec* complex of these two strains were cloned and sequenced. Also, their population profiles for methicillin resistance were compared.

6.2 Cloning of the *mec* regulatory genes

The *mec* regulatory genes of the isolates were amplified by *Taq* DNA polymerase with primers mA and mI2. The amplified region includes the 3' region of *mecA*, the promoter regions of *mecA*, *mecR1* and *mecI*, the whole *mecR1* and the whole of *mecI*. The sizes of the PCR products for WBG7422 and WBG525 were approximately 2.3kb and 2.2kb, respectively. The PCR products were purified from the gel with the gel extraction kit and cloned into the TA vector pGEM®-T Easy. The ligated recombinant plasmids were transferred into *E. coli* DH5α by transformation. Eight clones were obtained for the WBG7422 *mec* complex and 11 clones for the WBG525 *mec* complex. A recombinant plasmid carrying the WBG7422 *mec* regulatory genes was labelled pLTT1 and a recombinant plasmid carrying the WBG525 *mec* regulatory genes was labelled pLTT2. An *Eco*RI digest

of these two plasmids is shown in Figure 6.1 and schematic diagrams of the plasmids in Figure 6.2.

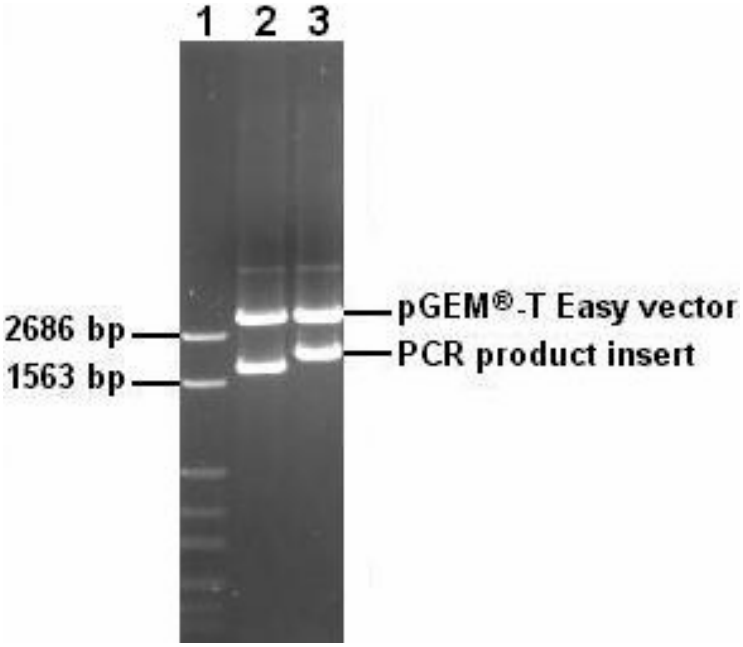


Figure 6.1 *Eco*RI digestion of pLTT1 and pLTT2

Lane 1 : FN-1 DNA marker

Lane 2 : *Eco*RI digestion of pLTT2

Lane 3 : *Eco*RI digestion of pLTT1

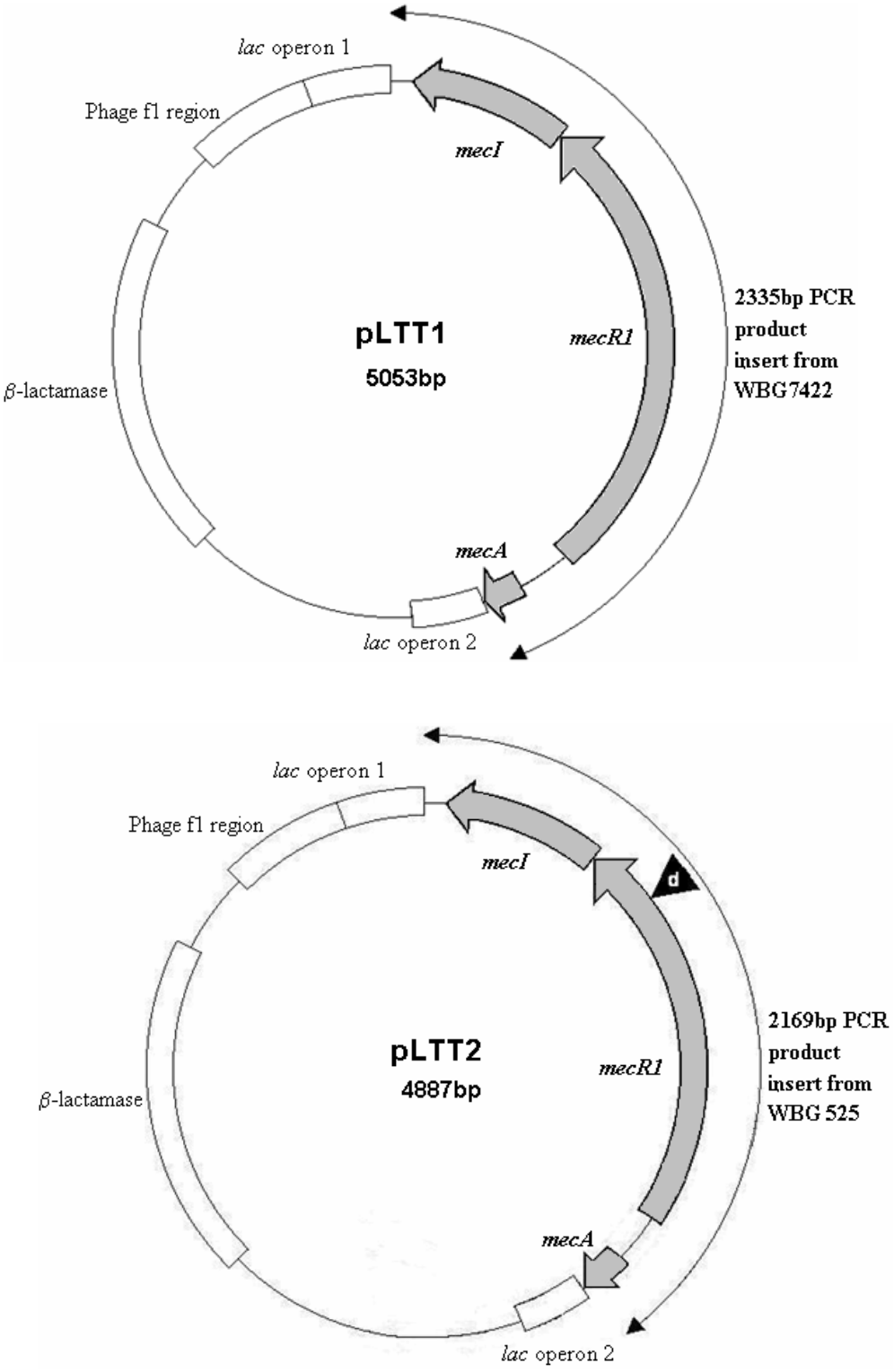


Figure 6.2 Schematic diagrams of pLTT1 and pLTT2

The PCR product was cloned into the pGEM®-T Easy vector. The solid black arrows indicate the location of the PCR insert. The black arrowhead with the letter "d" indicates the 166 bp deletion in the *mecR1* of WBG525.

6.3 Sequencing results

The DNA sequences of the inserts were determined using a LI-COR® 4200 Automatic DNA Sequencer (LI-COR Inc, Lincoln, Nebraska, USA). Three clones were sequenced for each of the PCR inserts. This is to compensate for the possibility of mismatching in the sequence, as the *Taq* DNA polymerase does not have proofreading ability. The final sequence is the consensus of the three clones. The sequences were compared with the published *mec* complex sequence of N315, which has a complete and intact *mec* complex.

The insert of the *mec* complex PCR from WBG7422 is 2335 bp in length. It consists of 57 bp of the 3' region of *mecA*, 1758 bp of the whole *mecR1*, 372 bp of the whole *mecI*, 100 bp region between *mecA* and *mecR1* and 48 bp 5' to the *mecI*. The promoter/operator sequences of *mecA*, *mecR1* and *mecI* are located in the 100 bp between *mecR1* and *mecA*.

The promoter/operator and *mecR1* sequences of the *mec* complexes from WBG7422 were identical to the prototype Class A *mec* complex in N315. The *mecI* gene of WBG7422 has a point mutation at nucleotide 202 that results in a nonsense mutation. This mutation substitutes the cytosine (C) with a thymine (T) and this introduces a translation stop codon (TAA) to replace the glutamine codon (CAA). This premature stop codon deletes the whole dimerisation domain of the *mecI* protein. The amino acid translation of the WBG7422 *mecI* DNA sequence is shown in Fig 6.3.

The insert of the *mec* complex PCR from WBG525 is 2169 bp in length. Similar to the *mec* complex of WBG7422, its sequence was mainly identical to the prototype Class A *mec* complex in N315. The promoter/operator sequences of *mecA* and *mecR1*/*I* in WBG525 were identical to N315 and WBG7422. However, compared with N315, the membrane-spanning domain of its *mecR1* gene has a 166 bp deletion. This deletion starts at nucleotide 98 and ends at nucleotide 263 of the *mecR1* gene. This is shown in Fig 6.4. The sequence of the *mecI* gene in WBG525 was identical to WBG7422 which has a single-base nonsense mutation at nucleotide 202 of the *mecI* gene. The complete sequences of the *mec* complexes of WBG7422 and WBG525 are listed in the appendix.

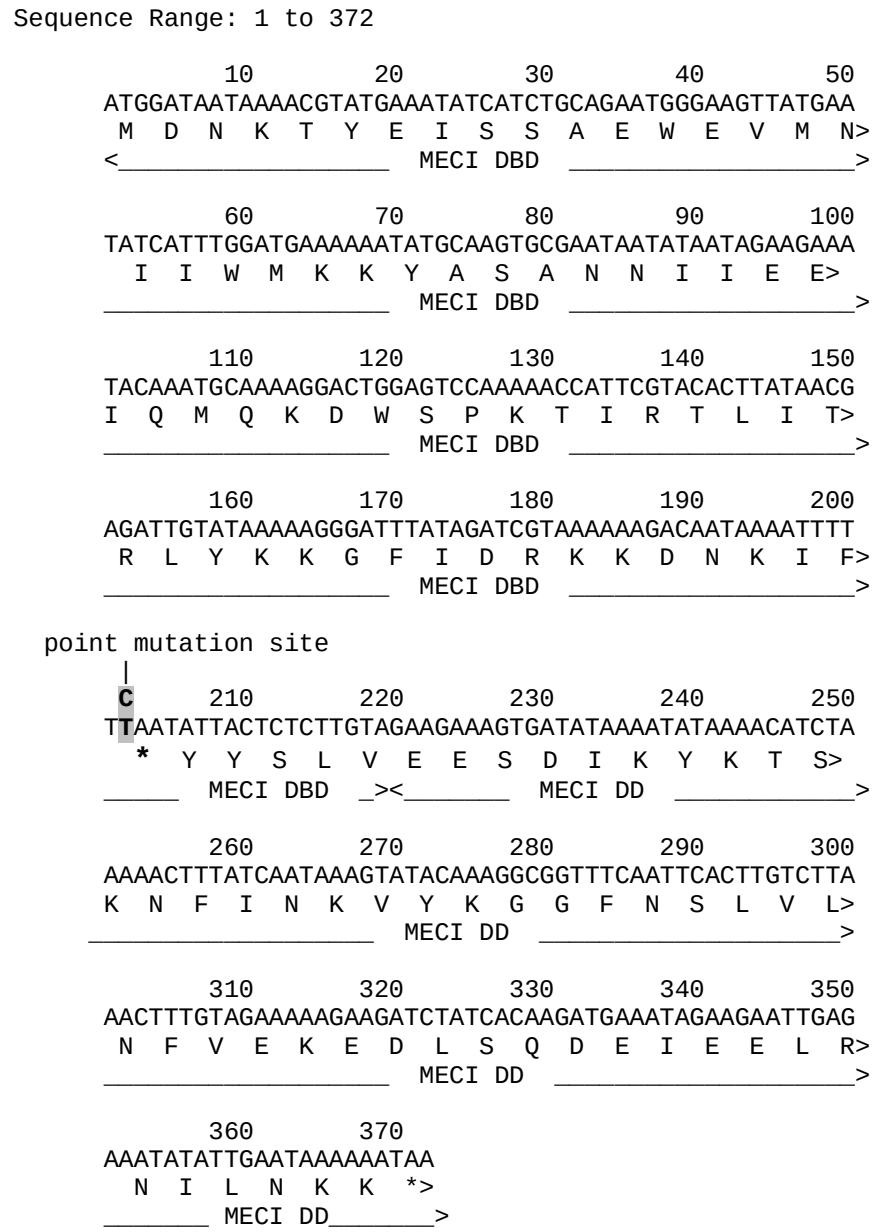
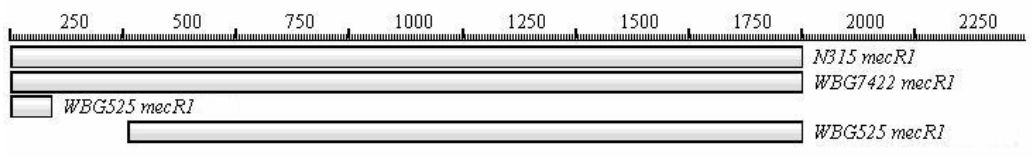


Figure 6.3 The translated sequences of the *mecI* genes of WBG7422 and WBG525

The WBG7422 *mecI* gene is 372 bp in length. The amino acids are represented by letters of the alphabet. The asterisk * indicates a stop codon. DBD, DNA-binding domain; DD, dimerisation domain.

Letters of the alphabet representing amino acids:
A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Iie; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Z, Glx.




```
                  10         20         30         40         50
N315          GTGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCTCACGAT
WBG7422       GTGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCTCACGAT
WBG525        GTGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCTCACGAT

                                                  Deletion start
                                                               |
                   60         70         80         90        |100
N315           ATGTGTAATTTTTTTAGTGAGAATGCTCTATATAAAATATACTCAAAATA
WBG7422        ATGTGTAATTTTTTTAGTGAGAATGCTCTATATAAAATATACTCAAAATA
WBG525         ATGTGTAATTTTTTTAGTGAGAATGCTCTATATAAAATATACTCAAA...

                  110        120        130        140        150
N315           TTATGTCACATAAGATTTGGTTATTAGTGCTCGTCTCCACGTTAATTCCA
WBG7422        TTATGTCACATAAGATTTGGTTATTAGTGCTCGTCTCCACGTTAATTCCA
WBG525         ..................................................

                  160        170        180        190        200
N315           TTAATACCATTTTACAAAATATCGAATTTTACATTTTCAAAAGATATGAT
WBG7422        TTAATACCATTTTACAAAATATCGAATTTTACATTTTCAAAAGATATGAT
WBG525         ..................................................

                  210        220        230        240        250
N315           GAATCGAAATGTATCTGACACGACTTCTTCGGTTAGTCATATGTTAGATG
WBG7422        GAATCGAAATGTATCTGACACGACTTCTTCGGTTAGTCATATGTTAGATG
WBG525         ..................................................

                Deletion end
                        |
                  260   |  270       280        290        300
N315           GTCAACAATCATCTGTTACGAAAGACTTAGCAATTAATGTTAATCAGTTT
WBG7422        GTCAACAATCATCTGTTACGAAAGACTTAGCAATTAATGTTAATCAGTTT
WBG525         ............TGTTACGAAAGACTTAGCAATTAATGTTAATCAGTTT

                  310        320        330        340        350
N315           GAGACCTCAAATATAACGTATATGATTCTTTTGATATGGGTATTTGGTAG
WBG7422        GAGACCTCAAATATAACGTATATGATTCTTTTGATATGGGTATTTGGTAG
WBG525         GAGACCTCAAATATAACGTATATGATTCTTTTGATATGGGTATTTGGTAG
```

Figure 6.4 Comparison of the *mecR1* genes of WBG7422, WBG525 and N315

The schematic diagram at the top compares the *mecR1* genes from the three strains. The bars represent the genes. The parallel bars indicate where the sequences are identical. The *MecR1* sequences of WBG7422 and N315 are identical and so the 166 bp deletion in the *mecR1* of WBG525, indicated by dots, is compared only with WBG7422. The DNA sequences of these three strains, after the deletion in WBG525, are identical and not included in this figure.

6.4 Population analysis of methicillin resistance

Population analysis was carried out on WBG7422 and WBG525 to study whether their dysfunctional *mec* complexes would influence their methicillin expression. (See Section 3.20) The negative control was NCTC8325, a methicillin-sensitive *S. aureus.* (Pattee *et al.*, 1990). WBG9087, an Australian community acquired MRSA from Darwin, was used as the control for heterogeneous-methicillin expression. (Chong, 2003). The COL strain was used as the control for homogeneous-methicillin expression. (Archer *et al.*, 1994). Ten-fold serial dilutions of overnight cultures were added to antibiotic free BHIA and BHIA containing oxacillin. Oxacillin was used because methicillin was not available. The number of colony forming units was counted, calculated and plotted against the oxacillin concentration. The MIC for oxacillin resistance is $\geq$ 2 μg/ml and for oxacillin susceptible is $\leq$ 1 μg/ml. An MIC larger than 16 μg/ml is considered as highly resistant and an MIC between 1 μg/ml and 2 μg/ml as borderline resistant. (Montanari *et al.*, 1990) The population analysis graphs are shown in Figure 6.5. The data for the population analysis is shown in Table 6.1.

In the homogeneous-resistant COL strain almost all the cells were resistant to oxacillin in the concentration from 1.5 to 256 μg/ml. The resistant population decreased to 80.4% of the total population at 512 μg/ml of oxacillin and the MIC for COL was 1024 μg/ml. WBG9087 was know to heterogeneously express oxacillin resistance. (Chong, 2003) Its population profile demonstrated that it had several resistant subpopulations that have different oxacillin MIC levels. The majority of the population (67%) was highly resistant to oxacillin (MIC, 24 μg/ml) and it had an MIC of 256 μg/ml. This indicated that WBG9087 had a profile of Class II heterogeneous-methicillin resistance. Unlike the Class II heterogeneous profile, MRSA with Class I heterogeneous profile only have a small highly resistant population and the MIC of the majority population is only just in the resistance range (2 μg/ml). (Montanari *et al.*, 1990, Tomasz *et al.*, 1991)

The population profile of WBG7422 was slightly different from WBG525. WBG7422 has a homogeneous-resistant population from 1.5 to 24 μg/ml of

oxacillin, whereas WBG525 was able to maintain a homogeneous-resistant population from 1.5 to 96 μg/ml. A great majority of WBG7422 (83%)and WBG525 (81%) populations were resistant up to 128 μg/ml of oxacillin. Both strains have the same very high-level MIC of 1024 μg/ml as the COL strain. Their population profiles were in between the COL Class IV homogeneous and WBG9087 Class II heterogeneous profiles. All subpopulations in WBG7422 and WBG525 possess very high MICs but their MICs were not homogeneous like the Class IV profile. (Tomasz *et al*., 1991, Hiramatsu, 1995) Hence, these results indicate that expression of methicillin resistance in WBG7422 and WBG525 is of the Class III heterogeneous-methicillin resistance.

Table 6.1A Population analysis of WBG7422, WBG525 and control strains[a]

	Oxacillin concentration (μg/ml)												
Strains	**0**	**1.5**	**3**	**6**	**12**	**24**	**48**	**64**	**96**	**128**	**256**	**512**	**1024**
NCTC8325[c]	4.5[b]	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
WBG9087[c]	5.2	4.4	4.2	4.0	3.9	3.5	3.0	3.0	2.8	2.3	NG	NG	NG
COL[c]	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.5	4.5	3.7	NG
WBG525	4.2	4.2	4.1	4.1	4.1	4.1	4.1	4.1	4.0	3.4	3.2	2.7	NG
WBG7422	4.7	4.7	4.7	4.7	4.7	4.6	4.4	4.3	4.0	3.9	3.4	2.6	NG

[a]The values are the average of three separate experiments.
[b]The numbers in the table are the Log_{10} of the CFU/ml values at different concentrations
[c]COL, homogeneous-methicillin resistant control; NCTC8325, methicillin-susceptible control; WBG9087, heterogeneous-methicillin-resistant control; NG, no growth.

Table 6.1B Percentage of oxacillin-resistant cells at different oxacillin concentrations[a]

	Oxacillin concentration (μg/ml)												
Strains	**0**	**1.5**	**3**	**6**	**12**	**24**	**48**	**64**	**96**	**128**	**256**	**512**	**1024**
NCTC8325[b]	100[a]	0	0	0	0	0	0	0	0	0	0	0	0
WBG9087[b]	100	84.6	80.8	76.9	75	67.3	57.7	57.7	57.7	53.8	44.2	0	0
COL[b]	100	100	100	100	100	100	100	100	100	97.8	97.8	80.4	0
WBG525	100	100	97.6	97.6	97.6	97.6	97.6	97.6	95.2	81	76.2	64.3	0
WBG7422	100	100	100	100	100	97.9	93.6	91.5	85.1	83	72.3	55.3	0

[a]The numbers in the table are percentages of oxacillin-resistant populations at different concentrations
[b]COL, homogeneous-methicillin resistant control; NCTC8325, methicillin-susceptible control; WBG9087, heterogeneous-methicillin-resistant control

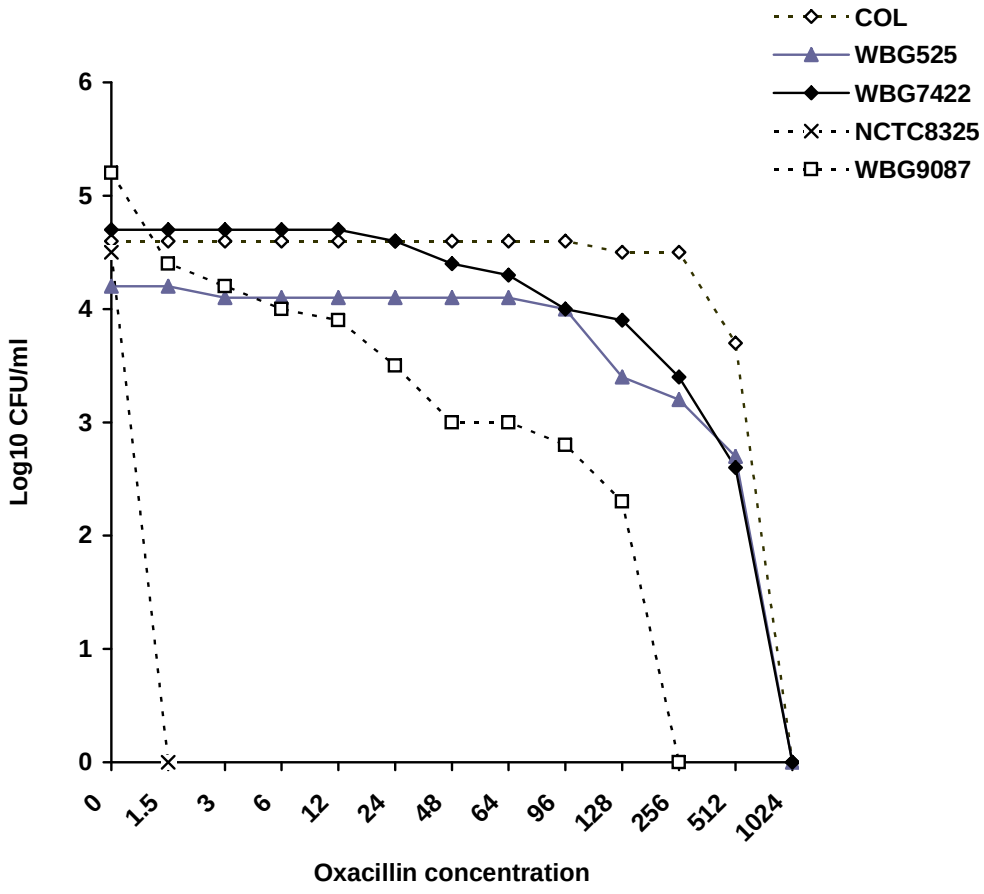


Figure 6.5 Population analysis of oxacillin-resistance in isolates

The oxacillin concentration is in μg/ml. COL, homogeneous-methicillin-resistant control; NCTC8325, methicillin susceptible control; WBG9087, heterogeneous-methicillin-resistant control; See Table 6.1A for the Log_{10} CFU/ml data.

6.5 Summary

The Malaysian strain WBG7422 was found to have a point mutation in the *mecI* gene. This mutation converts a glutamine codon into a stop codon and prematurely terminates *mecI* transcription. The rest of the *mec* complex was found to be identical to the *mec* complex of the pre MRSA N315. This same point mutation has previously been found in MRSAs isolated in Malaysia, Australia, Europe, Japan, New Zealand and Saudi Arabia. (Suzuki *et al.*, 1992, Hiramatsu, 1995)

The EA MRSA strain WBG525 has a closely related CHEF pattern to WBG7422 and was found to have the same *mecI* mutation. However, WBG525 differs from WBG7422 in that is has a 166 bp deletion in the membrane-spanning domain of the *mecR1* gene.

The population analysis showed that WBG7422 and WBG525 have similar population profiles. Both strains were found to express Class III heterogeneous-methicillin resistance. However, the population profile WBG7422 was slightly different from WBG525

CHAPTER SEVEN

CHARACTERISATION OF EPIDEMIC MRSA

7.1 Forward

The EA MRSA, WBG525, has been found to be closely related to the predominant Malaysian strains and EMRSA-1 from England which is known to be related closely to the EA MRSA. (Townsend *et al.*, 1983b, Wei *et al.*, 1992b) To investigate the relationship between Malaysian MRSA, EA MRSA and UK EMRSA, a collection of EA MRSA and EMRSA isolates was investigated using molecular techniques. These results may help us understood the origin and the epidemiology position of Malaysian MRSA on a global scale.

Part of the EA MRSA and EMRSA results were reported in the following publication.

I was the senior author, did all the work, and wrote the paper. My disclaimer is intended to indicate this. This paper in Chapter 7 had the person who supplied me with strains for comparison as second author and my supervisor as the last author.

7.2 Paper

Genetic organisation of *mecA* and *mecA*-regulatory genes in epidemic methicillin-resistant *Staphylococcus aureus* from Australia and England.

By

Lim, T.T., Coombs, G. W. and Grubb, W. B

In

Journal of Antimicrobial Chemotherapy, 2002 December, 50(6): 819-24

7.2.1 Abstract

The *mecA* gene that encodes methicillin resistance in *Staphylococcus aureus* may be regulated by the *mecR1* and *mecI* genes, and this region has been referred to as the

mec complex. An analysis of these regulatory genes in 35 epidemic methicillin-resistant *S. aureus* (MRSA) isolated in England and Australia has found that they contain three classes of *mec* complex. Firstly, the Class A *mec* complex with complete *mecR1* and *mecI* genes. Secondly, a new variant of Class A, the Class A1 *mec* complex, with a 166 bp deletion in the membrane-spanning domain of *mecR1* and a complete *mecI* gene. Thirdly, the Class B *mec* complex, in which the penicillin-binding domain of *mecR1* and the whole *mecI* gene are deleted by the insertion of a partial sequence of IS*1272*. Seven MRSA isolated in England and Australia over different time periods had the Class A *mec* complex. However, the isolates did not have closely related pulsed-field gel electrophoresis (PFGE) patterns. The Class A1 *mec* complex was found in 12 Australian isolates and the UK epidemic MRSA, EMRSA-1. All these organisms were isolated in the early 1980s and had closely related PFGE patterns. The Class B *mec* complex region was found in nine EMRSA and seven Australian MRSAs isolated over the period from the 1970s to 2000. These isolates had related PFGE patterns. The *mecA* region was also compared in the isolates and all but two of the isolates had an *Xba*I restriction site. These results support the global spread of epidemic clones and confirm the close relationship between the Australian and UK MRSA strains.

7.2.2 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported in 1961. (Jevons, 1961) Although there were sporadic outbreaks of MRSA they did not become a major problem until the late 1970s and early 1980s when outbreaks were reported from many parts of the world. (Grubb, 1998) The strains demonstrated an ability to spread within and between hospitals and are known as epidemic MRSA or EMRSA. (Grubb, 1998) This raises the question of the relationship of the earlier and later MRSA. Initial evidence indicated that the early isolates were clonal and that the later isolates were different. (Grubb, 1998) However, there is now conflicting evidence on the relationship of the different isolates. Basically, there are two possibilities. First, *S. aureus* has acquired the genes for methicillin resistance and all subsequent isolates are clonal and differ due to changes brought about by mutation, phages, plasmids and transposons. The other possibility is that different strains of *S. aureus* have acquired the methicillin resistance gene at different times.

Although it may not always be easy to distinguish between these two alternatives, a finding that the genes for methicillin resistance are different in different isolates is more likely to indicate that strains have independently acquired the gene for methicillin resistance. Many different approaches have now been used to compare the overall relatedness of MRSA isolates. (Tenover *et al.*, 1994) Methods that have been particularly useful are contour-clamped homogeneous electric field electrophoresis (CHEF), (Tenover *et al.*, 1994) and more recently *spaA* sequencing, (Crisostomo *et al.*, 2001) multilocus sequence typing (MLST), (Enright *et al.*, 2002) DNA microarray (Fitzgerald *et al.*, 2001) and sequencing of the total MRSA genome. (Kuroda *et al.*, 2001) These techniques all confirm that MRSA have diverse genetic backgrounds. The *mecA* gene in *S. aureu*s confers resistance to methicillin and all other β-lactam antibiotics (Grubb, 1998) *mecA* is also found in other species of staphylococci and is highly conserved except for a single-base mutation in some strains that creates an *Xba*I site. (Ryffel *et al.*, 1990) The *mecA* gene is regulated by two genes, *mecR1* and *mecI*, located upstream of the *mecA* gene, (Hiramatsu, 1995) and this region, together with *mec*A, has been referred to as the *mec* complex. (Katayama *et al.*, 2001) The *mecR1* gene encodes a transmembrane inducer of *mecA* consisting of membrane-spanning (MS) and penicillin-binding (PB) domains. (Hiramatsu, 1995) The *mecI* gene encodes a strong repressor of *mecA* (Hiramatsu, 1995) and consequently strains such as N315 with intact *mecR1* and *mecI* can appear methicillin sensitive in susceptibility tests. (Hiramatsu, 1995) The *mecR1* and *mecI* genes have a high degree of homology to the *blaR1* and *blaI* genes, which regulate β-lactamase production, (Hiramatsu, 1995) and studies have shown that the *blaR*1–*blaI* complex is able to regulate the expression of the *mecA* gene. (Ryffel *et al.*, 1992) MRSA strains that have a dysfunctional regulatory region can either express *mecA* constitutively, or they can use the β-lactamase regulatory genes to optimally express *mecA* because BlaR1 is a strong inducer of *mecA* and BlaI is a weak repressor. (Hiramatsu, 1995, Ryffel *et al.*, 1992) Studies on the *mecR*1–*mecI* region have shown that there is considerable genomic diversity in the *mec* complex. (Katayama *et al.*, 2001, Kobayashi *et al.*, 1996) Currently, five different classes of the *mec* complex have been described. The Class A complex has intact *mecR1* and *mecI* genes. In the Class B complex a partial copy of IS*1272* truncates the PB domain of mecR1 and the complete mecI gene. The Class C complex has two

variants, C1 and C2. In the Class C1 complex, the PB domain of *mecR1* and the whole of *mecI* are truncated by IS*43*1, whereas in the Class C2 complex, both the MS and PB domains of *mecR1* as well as *mecI* are truncated by IS*43*1. In Class D *mec* complex *mecI* is deleted and the PB domain of *mecR1* is truncated. (Katayama *et al.*, 2001) The *mec* complex is part of a larger region known as the staphylococcal cassette chromosome *mec* (SC*Cmec*). (Ito *et al.*, 2001) Four allotypes of SC*Cmec* have been described. (Ito *et al.*, 2001, Ma *et al.*, 2002) The allotypes are different because SC*Cmec* can acquire different genetic elements. Consequently, different SC*Cmec* regions can have the same *mec* complex. Therefore, analysis of the *mec* complex is more likely to reflect the ancestral origin of *mecA* because SC*Cmec* can acquire and/or lose elements, whereas the *mec* complexes are less likely to undergo additional deletions and mutations once *mecA* is being expressed. The *mec* complex would therefore appear to be a useful tool to compare the ancestry of MRSA when combined with other molecular methods.

7.2.3 Materials and methods

Thirty-five isolates, comprising 15 UK EMRSA, 15 Australian EMRSA and five classic MRSA (Grubb, 1998) were analysed. Their *mec* complexes were analysed by PCR (Frenay *et al.*, 1996) using the following primers: MR1 (Tokue *et al.*, 1992) and MR2 (Tokue *et al.*, 1992) for the *mecA* gene; mA (Niemeyer *et al.*, 1996) and mecI2 (Kobayashi *et al.*, 1996) for the Class A *mec* complex; mA (Niemeyer *et al.*, 1996) and ROrf2 (5'-GGACAACTTAAGCCAGGGTA-3') for the Class B *mec* complex; mecRA1 (Kobayashi *et al.*, 1996) and mecRA2 (Kobayashi *et al.*, 1996) for the MS domain of *mecR*1; mecRB1 (Kobayashi *et al.*, 1996) and mecRB2 (Kobayashi *et al.*, 1996) for the PB domain of *mecR*1; and mecI1 (Kobayashi *et al.*, 1996) and mecI2 (Kobayashi *et al.*, 1996) for *mec*I. Primer locations are shown in Figure 7.1. CHEF electrophoresis and Multi-Analyst/PC (Bio-Rad Laboratories, Hercules, CA, USA) were used to determine the overall genetic relatedness of the isolates. (O'Brien *et al.*, 1999)

7.2.4 Results and discussion

A summary of the results and details of the isolates are provided in Table 7.1. All of the isolates amplified with the *mecA* primers, MR1 and MR2. The *mecA* PCR products were digested with *Xba*I to detect the single-base point mutation. Of the 35 isolates, only EMRSA-16 and WBG10267 did not have an *Xba*I site in their *mec*A. Nineteen of the 35 isolates amplified with the Class A primers, mA and mecI2. The products were of two sizes, *c* 2237 and *c.* 2075 bp. PCR for the domains of *mecR*1, followed by *Cla*I digestion of the 2075 bp PCR product revealed a deletion in the MS domain of *mecR*1. Subsequent sequencing of the 2075 bp product (T. T. Lim & W. B. Grubb, unpublished results) showed that the deletion was 166 bp in length. (See Section 6.3) These results indicate a new variant of the Class A *mec* complex, and will be referred to as the Class A1 *mec* complex. The other 16 isolates had a Class B *mec* complex. Although only a few isolates had identical CHEF patterns (See Figure 7.2) many were found to be related based on the Tenover criteria. (Tenover *et al.*, 1995) Two CHEF pattern clusters were found among the isolates (See Figure 7.2). The CHEF A cluster consisted of 13 isolates with 90% similarity and all but one isolate carried the Class A1 *mec* complex. The CHEF B cluster consisted of 12 isolates with 75% similarity and all isolates carried the Class B *mec* complex. The similarity between the two clusters was 66%. Ten isolates that fell outside clusters A and B were not closely related to each other or to the other isolates. The CHEF A cluster isolates, except for one, were all isolated in the early 1980s and have the 166 bp deletion in the MS domain of *mecR*1. This deletion was also reported in an American epidemic clone LHH1 (Oliveira *et al.*, 2000) and the New Zealand MRSA 85/2082. (Ito *et al.*, 2001) Both of these strains were also isolated in the same time period as the CHEF A cluster isolates. These results further support the global spread of an MRSA clone in the early 1980s. (Townsend *et al.*, 1984a) The CHEF B cluster isolates included the five classic MRSA: COL, WBG512, WBG1434, WBG1438 and WBG1350. They had related CHEF patterns and carried the Class B *mec* complex. The first MRSA isolated, NCTC10442, has the Class B *mec* complex. (Ito *et al.*, 2001) This supports the claim that the early MRSA were clonal. (Grubb, 1998) Nine of the UK EMRSA studied carried the Class B *mec* complex but only six were found in the CHEF B cluster. The other three UK EMRSA were only distantly

related to the CHEF B cluster isolates. This is especially so for EMRSA-15, which is only 40% related to the CHEF B cluster isolates. The two most likely explanations of these results are that a strain has acquired the *mec* complex and then some have evolved to give widely different CHEF patterns, or alternatively, strains with different genetic backgrounds have acquired similar *mec* complexes. Recent studies using MLST and DNA microarray techniques have indicated that the latter hypothesis may better explain this phenomenon. (Enright *et al.*, 2002, Fitzgerald *et al.*, 2001) The Class A and Class B *mec* complexes were found in both classic MRSA and later MRSA isolates. Although some of the later isolates were genetically related to the classic isolates and had the same class of *mec* complex, some of them were not related to each other. In a recent study, an Iberian MRSA was found to be a descendant of an early methicillin-susceptible *S. aureu*s. (Crisostomo *et al.*, 2001) In another study, evidence has been presented for at least five horizontal transfers of *mecA* into genetically distinct *S. aureu*s. (Fitzgerald *et al.*, 2001) Both studies have demonstrated the co-existence of descendants of old clones and new clones created by horizontal transfer of the methicillin resistance gene. The analysis of the *mec* complex together with the CHEF patterns of these epidemic strains may support the global spread of epidemic clones and their possible ancestry. However, more comprehensive methods such as using MLST or DNA microarray techniques may give a more definite conclusion.

Table 7.1 Isolate details and summary of results

Isolates	*mecR1*		*mecI*	IS*1272*	*Xba*I site in *mecA*	Date of isolation and origin	References
	MS[a]	PB[a]					
Class A *mec* complex							
WBG526	+	+	+	-	+	1983, Royal Melbourne Hospital, Australia	Townsend *et al.* (1983b)
WBG816	+	+	+	-	+	1972, Royal Perth Rehabilitation Hospital, Australia	c
EMRSA-4	+	+	+	-	+	Epidemic MRSA, England	b
EMRSA-7	+	+	+	-	+	Epidemic MRSA, England	b
EMRSA-9	+	+	+	-	+	Epidemic MRSA, England	b
EMRSA-11	+	+	+	-	+	Epidemic MRSA, England	b
EMRSA-16	+	+	+	-	-	Epidemic MRSA, England	b
Class A1 *mec* complex							
WBG524	-	+	+	-	+	1983, Royal Melbourne Hospital, Australia	Townsend *et al.* (1983b)
WBG525	-	+	+	-	+	1983, Royal Melbourne Hospital, Australia	Townsend *et al.* (1983b)
WBG1161	-	+	+	-	+	1983, Austin Hospital, Heidelberg, Australia	Townsend *et al.* (1983b)
WBG1163	-	+	+	-	+	1983, Austin Hospital, Heidelberg, Australia	Townsend *et al.* (1983b)
WBG1175	-	+	+	-	+	1983, Royal Hobart Hospital, Australia	Townsend *et al.* (1983b)
WBG1189	-	+	+	-	+	1983, Royal Hobart Hospital, Australia	Townsend *et al.* (1983b)
WBG1321	-	+	+	-	+	1982, Royal Perth Hospital, Australia	Townsend *et al.* (1985e)
WBG2713	-	+	+	-	+	1983, Royal Free Hospital, London, England	Townsend *et al.* (1984a)
WBG6017	-	+	+	-	+	1982, Westmead Hospital, Sydney, Australia	C
WBG6070	-	+	+	-	+	1986, Repatriation General Hospital, Perth, Australia	C
WBG6085	-	+	+	-	+	1986, Repatriation General Hospital, Perth, Australia	C
EMRSA-1	-	+	+	-	+	Epidemic MRSA, England	B

[a]MS: membrane-spanning domain; [a]PB: penicillin-binding domain; [b]Provided by Central Public Health Laboratory, Colindale, England; [c]This Study; [d]Provided by Prof. B. Berger-Bächi, University of Zürich, Switzerland; +, amplified; - , not amplified.

Continued on next page

Table 7.1 continued

Isolates	*mecR1* MS[a]	*mecR1* PB[a]	*mecI*	IS*1272*	*Xba*I site in *mecA*	Date of isolation and origin	References
Class B *mec* complex							
COL	+	-	-	+	+	1965, London, England	D
WBG512	+	-	-	+	+	1968, Royal Perth Hospital, Australia	Townsend *et al.* (1985e)
WBG1350	+	-	-	+	+	MRSA reference strain, NCTC9468	Townsend *et al.* (1985c)
WBG1434	+	-	-	+	+	1972, Royal Perth Hospital, Australia	Townsend *et al.* (1985c)
WBG1438	+	-	-	+	+	1972, Royal Perth Hospital, Australia	Townsend *et al.* (1985c)
WBG10265	+	-	-	+	+	2000, EMRSA15, Royal Perth Hospital, Australia	C
WBG10267	+	-	-	+	-	2000, Irish-2, Royal Perth Hospital, Australia	C
EMRSA-2	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-3	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-5	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-6	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-8	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-10	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-12	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-13	+	-	-	+	+	Epidemic MRSA, England	B
EMRSA-15	+	-	-	+	+	Epidemic MRSA, England	B

[a]MS: membrane-spanning domain; [a]PB: penicillin-binding domain; [b]Provided by Central Public Health Laboratory, Colindale, England; [c]This Study; [d]Provided by Prof. B. Berger-Bächi, University of Zürich, Switzerland; +, amplified; - , not amplified.

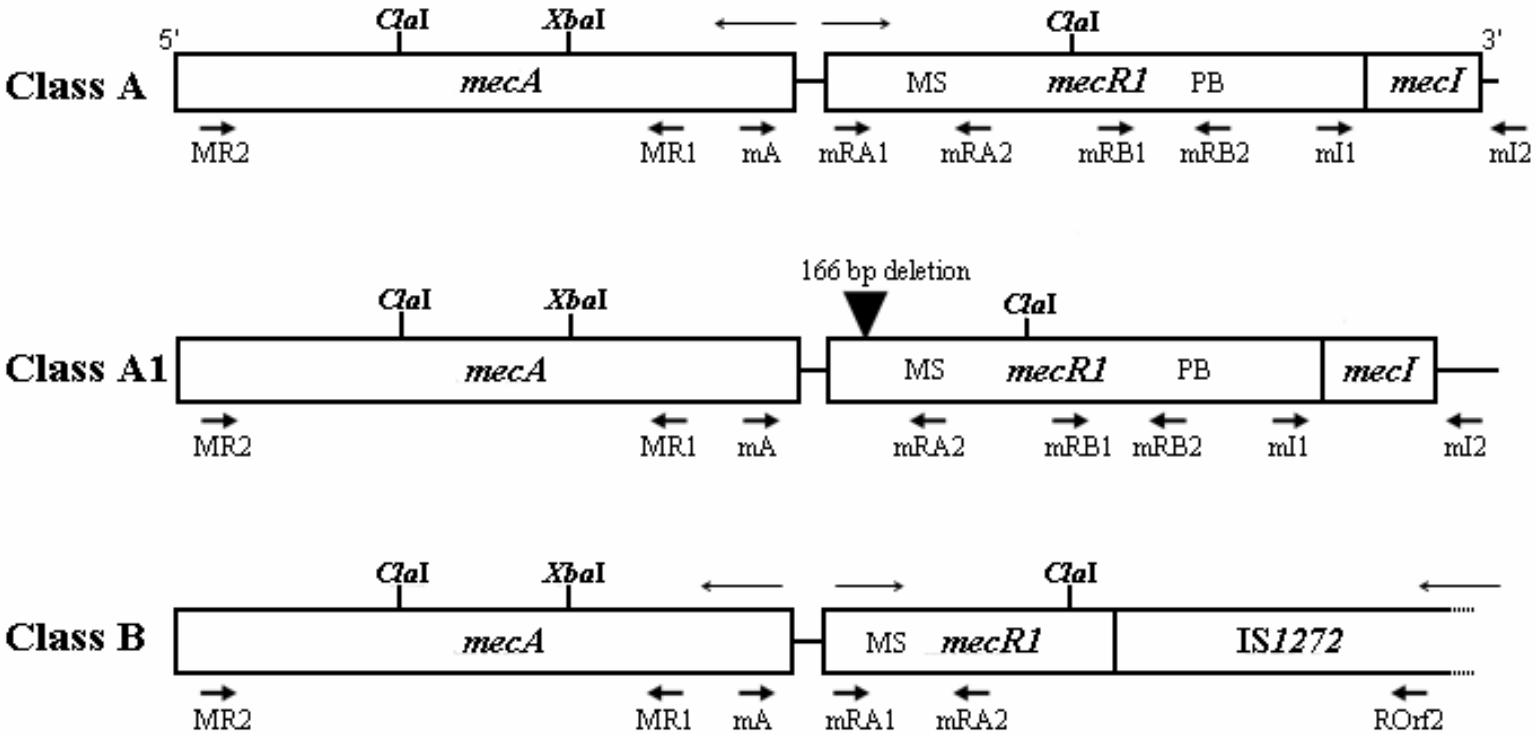


Figure 7.1 Schematic diagram of the three classes of *mec* complex showing the location of primers

After Katamaya et al. (2001) and Kobayashi et al. (1996). The bold arrows indicate the direction of the primers and the thin arrows the direction of transcription. MS, membrane-spanning domain of *mecR1*; PB, penicillin-binding domain of *mecR1*.

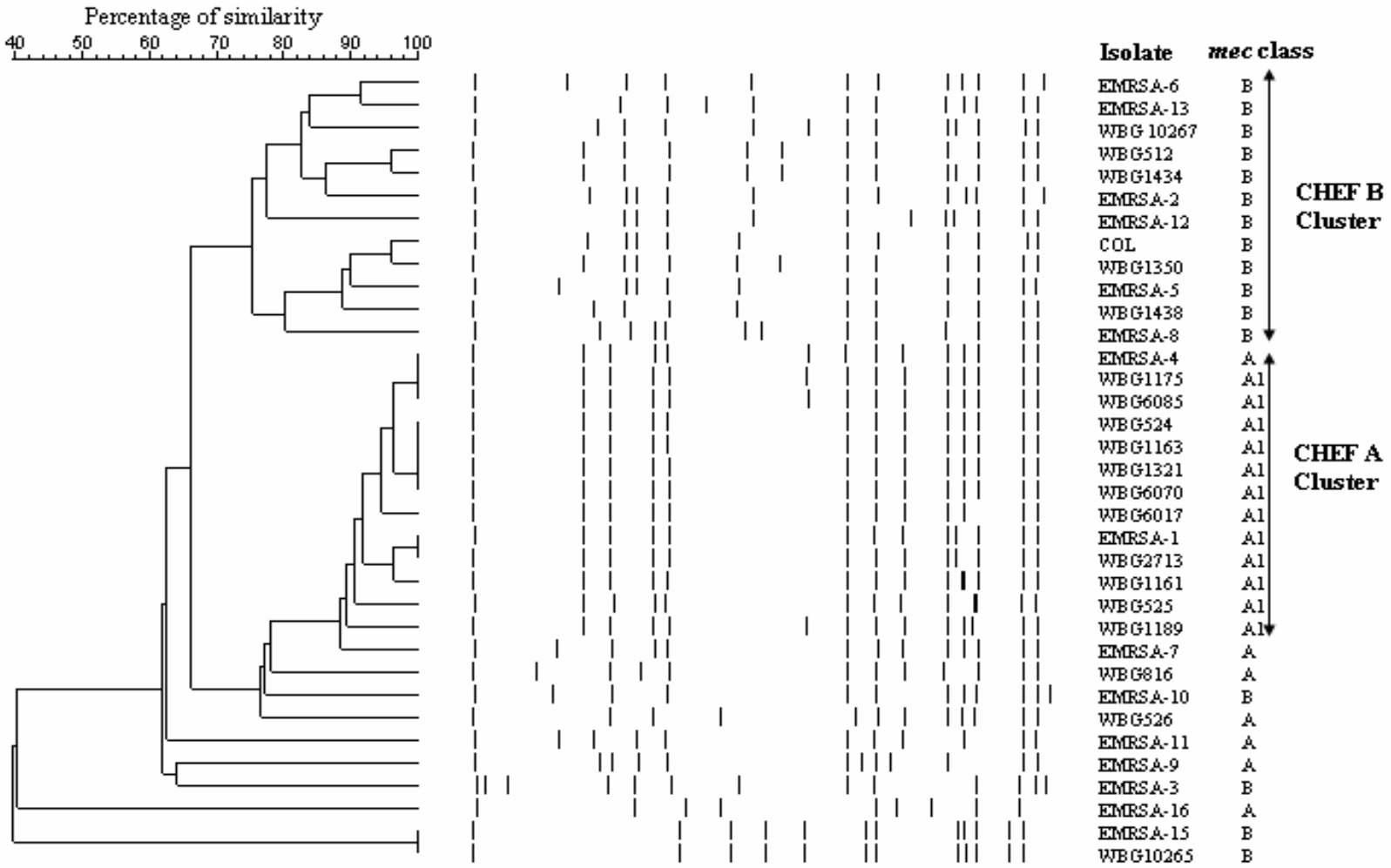


Figure 7.2 Dendrogram of the CHEF patterns of the isolates studied

7.3 Additional studies

After the publication of the paper described in Section 7.2, two additional epidemic strains, EMRSA-17 and IRISH-1 were acquired from the Central Public Health Laboratory, Colindale, London. The SCC*mec* and coagulase-gene typing were performed on these and the results are described in Sections 7.3.1 and 7.3.2. CHEF typing was also done and compared with a predominant Malaysian strain, WBG7422. (See Chapter 4) The dendrogram and the percentage of similarity are shown in Figure 7.4 and 7.5.

7.3.1 The SCC*mec* of EMRSAs

The SCC*mec* allotype is determined by the *mec* and *ccr* gene complexes, as each allotype of SCC*mec* carries a distinct combination of theses gene. Two additional strains were studied, EMRSA-17 and IRISH-1, and these were compared with WBG7422 and NCTC10442. EMRSA-17 and IRISH-1 are epidemic MRSA and NCTC10422 is the earliest MRSA. (Aucken *et al.*, 2002, Enright *et al.*, 2002, Ito *et al.*, 2001) WBG525 was used as the representative strain for the 15 EA MRSA isolates as their CHEF patterns were found to have greater than 88% similarity. (See Figure 7.2 and Section 7.2.4) The widely studied COL strain and the classical MRSA reference strain WBG1350 were used to represent the classical MRSA strains. The results of the SCC*mec* allotyping of these strains is listed in Table 7.2.

Four classes of *mec* complexes were found in the EMRSA isolates. They are the Class A, Class B, Class A1 and Class A2. The first three classes are described in the publication. The Class A2 *mec* complex was only found in the IRISH-1. It is very similar to Class A *mec* complex and has an intact *mecR1* gene, but *c.* 264 bp of the 3' region of *mecI* is truncated. It was amplified by primers mI1and mI4. Unlike other strains, it did not amplify with primer mI6, which is located downstream of mI4 and close to the 3' end of *mecI*. (See Figure 7.3) The primers Rorf2 for IS*1272* and two IS*431* primers, IS-1 and IS-2, failed to amplify with the Class A2 *mec* complex. This indicates that the 264 bp truncation of *mecI* is probably caused by a deletion or the insertion of other sequences such as IS*256* or IS*1182*. (Oliveira *et al.*, 2000, Shore *et*

al., 2005) The schematic diagram of the Class A2 *mec* complex is shown in Figure 7.3. Four types of *ccr* gene complexes were found in these EMRSA. They are the Type 1 *ccrA1-ccrB1*, Type 2 *ccrA2-ccrB2*, Type 3 *ccrA3-ccrB3* and Type 4 *ccrA4-ccrB4* complexes.

Allotype I SCC*mec* is the most common SCC*mec* among the EMRSA isolates, seven out of the 18 UK EMRSA isolates and the two classical MRSA. The Allotype I SCC*mec* has a Class B *mec* complex and Type 1 *ccr* gene complex. The Allotype I isolates have been found over a wide span of time, they include the first MRSA, NCTC10442, isolated in 1961 (Jevons, 1961, Ito *et al.*, 2001) and the latest UK EMRSA, EMRSA-17, isolated in 2000. (Aucken *et al.*, 2002) The CHEF patterns of these Allotype I isolates are closely related to each other. The majority of the isolates have a similarity ranging from 80% to 96.6%. EMRSA-5 and EMRSA-17 belong to this group and only have one band difference in their CHEF patterns. The only exception is EMRSA-3, which is only related to EMRSA-5 and COL with a similarity of 81.5%. (See Figure 7.4 and Figure 7.5).

EMRSA-16 and IRISH-1 were found to carry Allotype II SCC*mec* which has Class A *mec* complex and Type 2 *ccr* gene complex. The IRISH-1 carries Class A2 *mec* complex (a 246bp truncation in *mecI*) whereas the EMRSA-16 carries the Class A *mec* complex (intact *mecI* gene) (See Figure 7.3). Their CHEF patterns only have 61.5% similarity. (See Figure 7.4 and Figure 7.5)

The Allotype III SCC*mec* has Class A *mec* complex and Type 3 *ccr* gene complexes and was found in six strains. EMRSA-1 and WBG525 have the Class A1 *mec* complex (166bp deletion in *mecR1*) instead of Class A *mec* complex (intact *mecR1*). Their CHEF patterns have from 83.9% to 97% similarity with the Allotype III isolates, EMRSA-4, EMRSA-7 and WBG7422. The CHEF patterns of the two remaining Allotype III isolates, EMRSA-9 and EMRSA-11, are not related to the other Allotype III isolates with a similarity ranging from 66.7% to 75.6%. (See Figure 7.4 and Figure 7.5).

Allotype IV SCC*mec* has Class B *mec* complex and Type 2 *ccr* gene complex and was first reported in community acquired MRSAs (Ma *et al.*, 2002, Okuma *et al.*, 2002)

However, the Allotype IV SCC*mec* from the Paediatric clone, HDE288, was found to carry Class B *mec* and Type 4 *ccr* gene complexes. (Oliveira and Lencastre Hd, 2002, Oliveira *et al.*, 2001b) To differentiate these two different types, the SCC*mec* with Type 4 *ccr* gene has been designated here as Allotype IVp SCC*mec*.

Allotype IV SCC*mec* was found in EMRSA-2, EMRSA-6, EMRSA-10 and EMRSA-15. EMRSA-2 and EMRSA-6 are closely related strains that have 90.3% of similarity in their CHEF patterns. However, the other two SCC*mec* IV strains, EMRSA-10 and EMRSA-15 are not closely related to them. The CHEF pattern of EMRSA-10 clusters with strains with SCC*mec* III allotype, it only has less than 76% similarity with the CHEF patterns of EMRSA-2 and EMRA-6. The CHEF pattern of EMRSA-15 is not related to the EMRSA-2, -6 and -10. The CHEF pattern of EMRSA-15 only has less than 52% of similarity with the patterns of these three strains. (See Figure 7.4 & Figure 7.5)

Allotype IVp SCC*mec* was found in EMRSA-12, EMRSA-13 and IRISH-2. The CHEF pattern of IRISH-2 is related to the CHEF pattern of EMRSA-12 (85% similarity) and EMRSA-13 (80% similarity). However, EMRSA-12 and EMRSA-3 are not related to each other, their CHEF pattern similarity is 78.6%. Nevertheless, the CHEF patterns of these three isolates still cluster with the SCC*mec* IV strains, EMRSA-2 and EMRSA-6, and the SCC*mec* II strain IRISH-1. The overall percentage of similarity between these two clusters is 82%. (See Figure 7.4 & Figure 7.5)

Table 7.2 SCC*mec* and coagulase-gene results for different MRSA

Strains	*Xba*I in mecA	*mecR1* MS	*mecR1* PB	*mecI*	IS*1272*	*mec* complex Class	*ccr* complex Type	SCC*mec* Allotype	Coagulase-gene type
EMRSA-3	+	+	-	-	+	B	1	I	66
EMRSA-5	+	+	-	-	+	B	1	I	18
EMRSA-8	+	+	-	-	+	B	1	I	18
EMRSA-17	-	+	-	-	+	B	1	I	18
COL	+	+	-	-	+	B	1	I	18
NCTC10442	+	+	-	-	+	B	1	I	18
WBG1350	+	+	-	-	+	B	1	I	18
EMRSA-16	-	+	+	+	-	A	2	II	18
IRISH-1	-	+	+	-[a]	-	A2	2	II	18
WBG525	+	-	+	+	-	A1	3	III	24
EMRSA-1	+	-	+	+	-	A1	3	III	24
EMRSA-4	+	+	+	+	-	A	3	III	24
EMRSA-7	+	+	+	+	-	A	3	III	24
EMRSA-9	+	+	+	+	-	A	3	III	24
EMRSA-11	+	+	+	+	-	A	3	III	24
EMRSA-2	+	+	-	-	+	B	2	IV	18
EMRSA-6	+	+	-	-	+	B	2	IV	18
EMRSA-10	+	+	-	-	+	B	2	IV	18
EMRSA-15	+	+	-	-	+	B	2	IV	22
EMRSA-12	+	+	-	-	+	B	4	IVp	18
EMRSA-13	+	+	-	-	+	B	4	IVp	18
IRISH-2	-	+	-	-	+	B	4	IVp	18

[a]Partially truncated *mecI*

Abbreviations, MS, membrane-spanning domain; PB, penicillin-binding domain; IVp, Allotype IV SCC*mec* of Paediatric clone

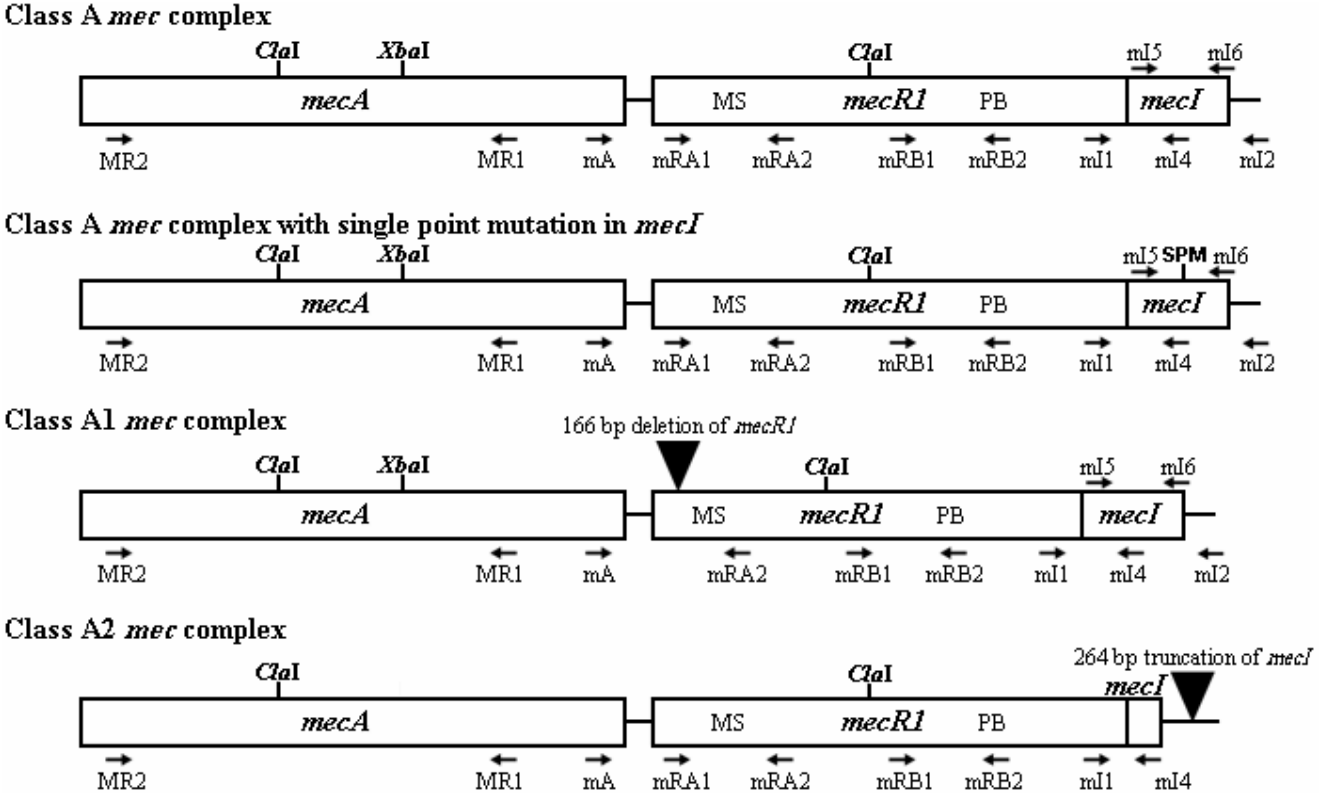


Figure 7.3 Schematic diagram of Class A *mec* complexes

The horizontal arrows indicate the direction of the primers used in the PCR reaction.
MS, membrane-spanning domain of *mecR1*; PB, penicillin-binding domain of *mecR1*; SPM, single-base point mutation

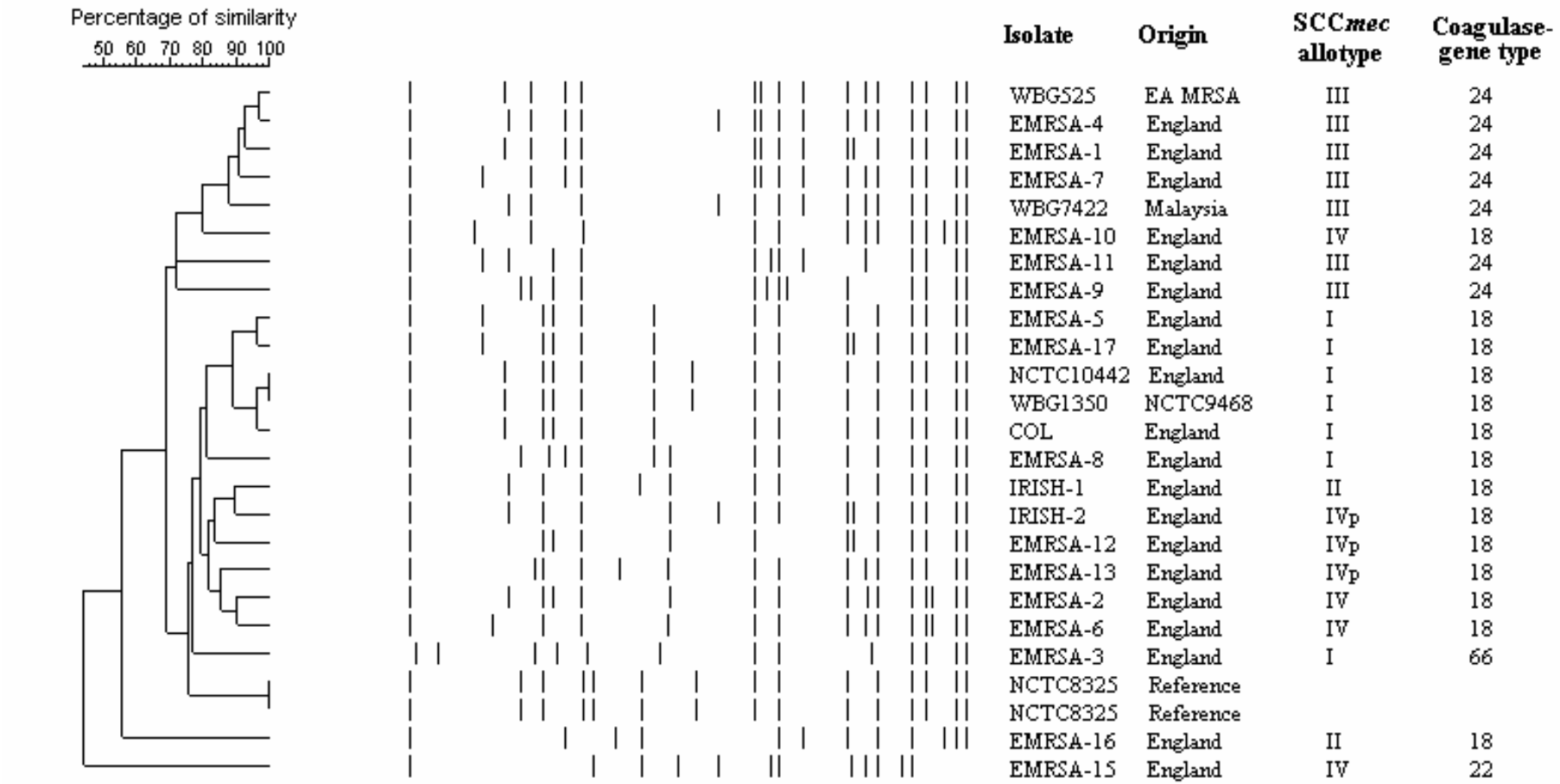


Figure 7.4 Dendrogram of CHEF patterns and SCC*mec* allotypes and coagulase-gene types of classical MRSA and EMRSA strains

Isolate	SCC*mec* allotype	Coagulase-gene type																								
WBG525	III	24	100																							
EMRSA-4	III	24	97.0	100																						
EMRSA-1	III	24	93.8	90.9	100																					
EMRSA-7	III	24	93.8	90.9	87.5	100																				
WBG7422	III	24	90.3	93.8	83.9	83.9	100																			
EMRSA-10	IV	18	80.0	77.4	73.3	86.7	82.8	100																		
EMRSA-11	III	24	73.3	71.0	66.7	73.3	75.9	71.4	100																	
EMRSA-9	III	24	73.3	71.0	73.3	73.3	69.0	71.4	71.4	100																
EMRSA-5	I	18	66.7	64.5	66.7	73.3	69.0	78.6	71.4	71.4	100															
EMRSA-17	I	18	64.5	62.5	71.0	71.0	66.7	75.9	69.0	69.0	96.6	100														
NCTC10442	I	18	71.0	68.8	71.0	64.5	73.3	69.0	69.0	69.0	89.7	86.7	100													
WBG1350	I	18	71.0	68.8	71.0	64.5	73.3	69.0	69.0	69.0	89.7	86.7	100	100												
COL	I	18	73.3	71.0	73.3	66.7	75.9	71.4	71.4	71.4	92.9	89.7	96.6	96.6	100											
EMRSA-8	I	18	71.0	68.8	71.0	71.0	66.7	69.0	62.1	75.9	82.8	80.0	80.0	80.0	82.8	100										
IRISH-1	II	18	73.3	71.0	73.3	66.7	75.9	71.4	64.3	64.3	78.6	75.9	82.8	82.8	85.7	82.8	100									
IRISH-2	IVp	18	71.0	75.0	77.4	64.5	80.0	69.0	62.1	62.1	75.9	80.0	80.0	80.0	82.8	80.0	89.7	100								
EMRSA-12	IVp	18	62.1	60.0	69.0	62.1	64.3	66.7	59.3	66.7	81.5	85.7	78.6	78.6	81.5	78.6	81.5	85.7	100							
EMRSA-13	IVp	18	77.4	75.0	71.0	77.4	80.0	82.8	62.1	69.0	75.9	73.3	73.3	73.3	75.9	80.0	82.8	80.0	78.6	100						
EMRSA-2	IV	18	75.0	72.7	68.8	68.8	77.4	73.3	73.3	66.7	80.0	77.4	83.9	83.9	86.7	77.4	86.7	83.9	82.8	83.9	100					
EMRSA-6	IV	18	71.0	68.8	64.5	77.4	73.3	75.9	69.0	62.1	82.8	80.0	73.3	73.3	75.9	80.0	82.8	80.0	78.6	86.7	90.3	100				
EMRSA-3	I	66	69.0	66.7	69.0	69.0	71.4	74.1	74.1	74.1	81.5	78.6	78.6	78.6	81.5	71.4	74.1	71.4	76.9	71.4	75.9	71.4	100			
NCTC8325			64.5	62.5	64.5	64.5	66.7	69.0	55.2	69.0	75.9	73.3	80.0	80.0	75.9	80.0	82.8	73.3	71.4	73.3	71.0	73.3	71.4	100		
NCTC8325			64.5	62.5	64.5	64.5	66.7	69.0	55.2	69.0	75.9	73.3	80.0	80.0	75.9	80.0	82.8	73.3	71.4	73.3	71.0	73.3	71.4	100	100	
EMRSA-16	II	18	64.3	62.1	64.3	64.3	59.3	61.5	46.2	46.2	53.8	51.9	51.9	51.9	53.8	59.3	61.5	51.9	48.0	59.3	50.0	51.9	48.0	59.3	59.3	100	
EMRSA-15	IV	22	42.9	48.3	35.7	42.9	51.9	46.2	38.5	38.5	38.5	37.0	37.0	37.0	38.5	44.4	53.8	51.9	40.0	51.9	50.0	51.9	40.0	51.9	51.9	50.0	100

Figure 7.5 Matrix of percentage of similarity of classical MRSA and EMRSA CHEF patterns

7.3.2 Coagulase-gene typing of EMRSAs

Coagulase-gene typing is based on the variation in an 81 bp tandem repeat of the coagulase gene. (Goh *et al.*, 1992) The details of the typing method and the determination of coagulase-gene type are described in Section 3.14.

Four coagulase-gene types were found among the EMRSA isolates. Coagulase-gene type 18 is the most common type and was found in 14 isolates. The coagulase-gene type patterns are shown in Figure 7.6 and the results are listed in Table 7.2. Most EMRSA with Allotype I, Allotype IV and all Allotype IVp SCC*mec* are coagulase-gene type 18. The two exceptions are EMRSA-3 with Allotype I SCC*mec* (coagulase-gene type 66) and EMRSA-15 with Allotype IV SCC*mec* (coagulase-gene type 22). The coagulase-gene type 18 isolates have related CHEF patterns, except for EMRSA-10, which is more closely related to the coagulase-gene type 24 isolates and EMRSA-16, which are not closely related to any of the other isolates. (See Figure 7.4).

Coagulase-gene type 24 was only found in the six Allotype III SCC*mec* isolates which have closely related CHEF patterns. Coagulase-gene type 22 was only found in EMRSA-15, which has an Allotype IV SCC*mec* and a CHEF pattern unrelated to the other EMRSA isolates. Coagulase-gene type 66 was found in EMRSA-3 which has an Allotype I SCC*mec* and a CHEF pattern related to isolates with the coagulase-gene type 18 and Allotype I SCC*mec*.

The coagulase-gene types of the EMRSA isolates have a relatively good correlation with the other typing methods such as CHEF and SCC*mec* type.

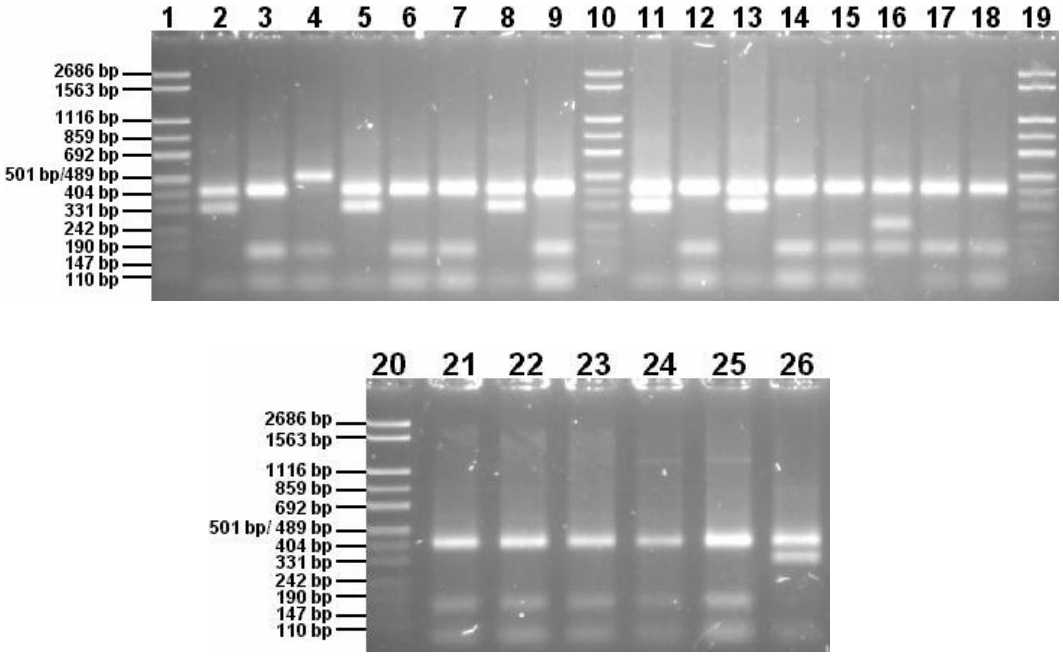


Figure 7.6 Coagulase-gene typing of MRSA

Lane 1 :	FN-1		**Lane 14 :**	EMRSA-12	18
Lane 2 :	EMRSA-1	24	**Lane 15 :**	EMRSA-13	18
Lane 3 :	EMRSA-2	18	**Lane 16 :**	EMRSA-15	22
Lane 4 :	EMRSA-3	66	**Lane 17 :**	EMRSA-16	18
Lane 5 :	EMRSA-4	24	**Lane 18 :**	EMRSA-17	18
Lane 6 :	EMRSA-5	18	**Lane 19 :**	FN-1	
Lane 7 :	EMRSA-6	18	**Lane 20 :**	FN-1	
Lane 8 :	EMRSA-7	24	**Lane 21 :**	IRISH-1	18
Lane 9 :	EMRSA-8	18	**Lane 22 :**	IRISH-2	18
Lane 10 :	FN-1		**Lane 23 :**	COL	18
Lane 11 :	EMRSA-9	24	**Lane 24 :**	NCTC10422	18
Lane 12 :	EMRSA-10	18	**Lane 25 :**	WBG1350	18
Lane 13 :	EMRSA-11	24	**Lane 26 :**	WBG525	24

The numbers following the strain names are the coagulase-gene type number.

The bands smaller than 110 bp are excess primers and dNTPs. (PCR Laboratory, Royal Perth Hospital, personal communication)
FN-1, DNA size marker.

7.4 Summary

EMRSA are a clinically successful and genetically diverse group. They can be separated into nine combined genotypes (CG) according to their CHEF pattern, SCC*mec* and coagulase-gene type. (see Table 7.3). The similarity of the CHEF patterns are shown in Figure 7.4 and Figure 7.5

CG1 contains the strains with Allotype I SCC*mec* and coagulase-gene type 18. These isolates also have closely related CHEF patterns with 96% to 80% similarity. The members include the first MRSA, NCTC10442, the newest EMRSA, EMRSA-17, EMRSA-5, EMRSA-8 and two classical MRSA, COL and WBG1350.

CG2 contains the strains with Allotype I SCC*mec* and coagulase-gene type 66. EMRSA-3 is the sole member of this group. Its CHEF pattern is not related to other isolates except to two CG1 isolates, EMRSA-5 (81.5% similarity) and COL (81.5% similarity). (see Table 7.3).

CG3 contains EMRSA-16 which is SCC*mec* allotype II and coagulase-gene type 18. Its CHEF pattern is not closely related (64.3% to 45% similarity) to any other epidemic MRSA strains in this study. (see Table 7.3).

CG4 contains IRISH-1. It is coagulase-type 18 and has Allotype II SCC*mec*. Its CHEF pattern is closely related to the IRISH-2, EMRSA-12, EMRSA-13, EMRSA-2 and EMRSA-6. Their CHEF patterns have a similarity ranging from 81.5% to 89.7%) However, all these epidemic strains related to IRISH-1 have different SCC*mec* allotypes. (see Table 7.3).

CG5 contains strains with Allotype III SCC*mec* and coagulase-gene type 24. They are a very homogeneous group and their CHEF patterns have a similarity ranging from 83.9% to 97%. The members include WBG7422, WBG525 (EA MRSA), EMRSA-1, EMRSA-4 and EMRSA-7 (see Table 7.3).

CG6 contains strains with Allotype IV SCC*mec* and coagulase-gene type 18. This group includes EMRSA-2 and EMRSA-6 and have closely related CHEF patterns (90.3% similarity).

CG7 contains a single strain, EMRSA-10 which is coagulase-type 18 and has the Allotype IV SCC*mec*. Its CHEF pattern is not related to other strains with the same SCC*mec* and coagulase-gene type, but its pattern is related to WBG525 (80% similarity), WBG7422 (82.8% similarity) and EMRSA-7 (86.7% similarity). All these three strains belong to the CG5 group, which have Allotype III SCC*mec* and coagulase-gene type 24. (see Table 7.3).

CG8 contains strains with Allotype IV SCC*mec* and coagulase-gene type 22. EMRSA-15 is the only member of this group. Its CHEF pattern is distinct from all the strains tested (similarity less than 53.8%). (see Table 7.3).

CG9 contains strains with Allotype IVp SCC*mec* and coagulase-gene type 18 and includes IRISH-2, EMRSA-12 and EMRSA-13. The CHEF patterns of these three isolates are related with similarity ranging from 80% to 89.7%, except the similarity of EMRSA-12 and EMRSA-13 is 78.6%. They were considered as a group as they carry the same SCC*mec* allotype and coagulase-gene type.

CG10 consists of EMRSA-9 and EMRSA-11. Both strains have Allotype III SCC*mec* and coagulase-gene type 24. Their CHEF patterns are not related to each other but have 71.4% similarity. They are grouped together as they have the same SCC*mec* Allotype and coagulase-gene type. Their CHEF patterns are not related to the other isolates, the highest similarity is with the CG5 group isolates with around 70% similarity. The CG5 isolates also have the same SCC*mec* Allotype and coagulase-gene type to these two isolates.

The EMRSA typing results with coagulase-gene, SCC*mec* and CHEF typing correlated well with each other in the majority of cases. This supports earlier work done by others which suggested that EA MRSA (WBG525) was closely related to EMRSA-1 (Townsend *et al.*, 1984a, Wei *et al.*, 1992b) The Malaysian strain, WBG7422, was found to share identical coagulase-gene type, closely related

SCC*mec* type and its CHEF pattern was 83.9% similar to EMRSA-1 and 90.3% to WBG525.

MRSA with similar genetic backgrounds would be expected to have identical, or similar, CHEF patterns. CHEF patterns reflect the general overall similarity of the whole genome, whereas the coagulase-gene typing only represents a small section of the genome. Hence, isolates with similar CHEF patterns may not have the same coagulase-gene types. Also, isolates with similar overall genetic backgrounds may have different SCC*mec* allotypes, as related strains could have acquired different SCC*mec*s on different occasions.

Table 7.3 Combined Genotypes of EMRSA and Classical MRSA

Combined genotype (CG)	**SCC*mec* allotype**	**Coagulase-gene type**	**Strain**
CG1	I	18	COL, NCTC10442, WBG1350, EMRSA-5, EMRSA-8, EMRSA-17,
CG2	I	66	EMRSA-3
CG3[a]	II	18	EMRSA-16
CG4[a]	II[a]	18	IRISH-1
CG5	III	24	EMRSA-1, EMRSA-4, EMRSA-7, WBG525, WBG7422
CG6[b]	IV	18	EMRSA-2, EMRSA-6
CG7[b]	IV	18	EMRSA-10
CG8	IV	22	EMRSA-15
CG9	IVp	18	IRISH-2, EMRSA-12, EMRSA-13
CG10[c]	III	24	EMRSA-9, EMRSA-11

[a]IRISH-1 belongs to CG4 and has Class A2 *mec* complex whereas EMRSA-16, in CG3, has the intact Class A *mec* complex. Their CHEF patterns are also not related.
[b]CG6 and CG7 have the same SCC*mec* allotype and coagulase-gene type but the CHEF patterns of their members are not related.
[c]CG10 and CG5 have the same SCC*mec* allotype and coagulase-gene type but the CHEF patterns of their members are not related

CHAPTER EIGHT

CHARACTERISATION OF COMMUNITY-ACQUIRED MRSA

8.1 Forward

Community acquired MRSA (CMRSA) is an emerging problem in many Western countries. Initially MRSAs were nosocomial organisms however, since the early 1990s MRSA have been isolated in the community. It is important to investigate the molecular characteristics of these CMRSA in order to understand their epidemiology, their origin, and relationship to their hospital counterparts, including the Malaysian isolates.

This study has been published as an original paper in the journal, Pathology in 2003.

I was the senior author, did all the work, and wrote the papers. My disclaimer is intended to indicate this. This paper has my supervisor as the last author and the other two authors provided me with isolates and background information on them.

8.2 Paper

Are all community methicillin-resistant *Staphylococcus aureus* related? A comparison of their *mec* regions

By

Tien Tze Lim, Fuen Nie Chong, Frances G. O'Brien and Warren B. Grubb

In

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8.2.1 Introduction

Although methicillin-resistant *Staphylococcus aureus* (MRSA) were reported in 1961 in England (Jevons, 1961) It wasn't until the late1970s and early 1980s that they became endemic in hospitals around the world. (Cookson, 2000, Chambers, 2001, Grubb, 1998) Some strains appear to spread more readily in, and between hospitals, and these have been referred to as epidemic MRSA (EMRSA). (Cookson and Phillips, 1988) MRSA have characteristically been nosocomial organisms and resistant to multiple antibiotics. (Cookson, 2000, Grubb, 1998) However, MRSA are

now being isolated from people in the community who do not have a history of hospitalisation or contact with health-care workers. (Chambers, 2001, Cookson, 2000) These are referred to as community MRSA (CMRSA). Although they have the *mecA* gene and are resistant to all β-lactam antibiotics, characteristically they are not resistant to multiple antibiotics. (Udo *et al.*, 1993, Turnidge and Bell, 2000, Naimi *et al.*, 2001, Bell and Turnidge, 2002, O'Brien *et al.*, 1999) Consequently, CMRSA have been distinguished from nosocomial MRSA on their resistance to antimicrobials. (Cookson, 2000)

The first Australian CMRSA was isolated in the Kimberley region of WA in 1989 from people living in remote communities and these isolates were referred as WA MRSA. (Riley *et al.*, 1995, Riley and Rouse, 1995, Udo *et al.*, 1993) Non-multi-resistant MRSA were later isolated from patients in the Northern Territory (NT) and because their resistance profiles were similar to the WA MRSA it was though that they may have come from the Kimberley region. (Maguire *et al.*, 1996) However, plasmid analysis and pulsed-field gel electrophoresis failed to shown any similarity between WA MRSA and NT MRSA. (Chong *et al.*, 1999) In 1995 a WA MRSA caused a single-strain outbreak at Royal Perth Hospital. (O'Brien *et al.*, 1999) The index strain for this outbreak was indistinguishable from the predominant CMRSA found in isolated communities in the Goldfields Region of WA. (O'Brien *et al.*, 1999)

CMRSA have also been reported in New Zealand (NZ). (Turnidge and Bell, 2000, Nimmo *et al.*, 2000, Adhikari *et al.*, 2002) These strains appear to have come from Western Samoa and are of two types, referred to as Western Samoan Phage Pattern-1 (WSPP-1) and -2 (WSPP-2). (Adhikari *et al.*, 2002) Strains similar to the NZ strains, but different from the WA MRSA, are now being isolated in hospitals in eastern Australia. (Turnidge and Bell, 2000, Nimmo *et al.*, 2000, Gosbell *et al.*, 2001)

Methicillin resistance in *S. aureus* is due to the production of the low affinity penicillin-binding protein, PBP2a (Chambers, 1988)/2'(Song *et al.*, 1987) which is encoded by the *mecA* gene located in the staphylococcal cassette chromosome *mec* (SCC*mec*), also known as the *mec* region. (Ito *et al.*, 2001) The *mecA* gene is regulated by two genes *mecR1* and *mecI* located upstream to *mecA*. (Hiramatsu *et al.*,

1992) *mecI* encodes a strong repressor of *mecA* and *mecR1* a weak inducer of it. (Hiramatsu, 1995) Consequently, strains such as N315, which have intact *mecI* and *mecR1*, do not express methicillin resistance. Strains, which express methicillin resistance, have various mutations or deletions in *mecI* and/or *mecR1* and *mecA* may be regulated by the β-lactamase regulatory genes *blaR1* and *blaI*. (Ryffel *et al.*, 1992, Hackbarth *et al.*, 1994, Ito *et al.*, 1999) The region surrounding the *mecA* gene is referred to as the *mec* complex, and it is the core unit of the SCC*mec* cassette. Five different classes of the complex have been reported based on variations in the *mecA* regulatory genes. (Katayama *et al.*, 2000, Katayama *et al.*, 2001) In Class A *mec* complex both *mecR1* and *mecI* genes are intact. In Class B *mec* complex the *mecI* gene and the penicillin-binding domain of *mecR1* have been replaced by the insertion of a partial copy of IS*1272*. The Class C *mec* complex has two variants C1 and C2 both of which have all of *mecI* and the penicillin-binding domain of *mecR1* deleted by the insertion of IS*431*/*257*. The only difference is that the *mecR1* deletion in C2 is larger than in C1. In Class D *mec* complex all of *mecI* and the penicillin-binding domain of *mecR1* are also deleted but no IS insertions have been detected. (Katayama *et al.*, 2001) (Please note at the time of writing this paper, there were only five known *mec* complexes)

The other important component of the SCC*mec* cassette is the *ccr* gene complex. The *ccr* gene complex consists of two genes, *ccrA* and *ccrB*. (Katayama *et al.*, 2000) These two recombinase genes facilitate the site-specific excision and integration of the SCC*mec* cassette into the *S. aureus* chromosome. (Katayama *et al.*, 2000) The four reported SCC*mec* cassettes are known to carry different *ccr* gene and *mec* complexes. Allotype I SCC*mec* carries Class B *mec* complex and Type 1 *ccr* gene complex. Allotype II SCC*mec* carries Class A *mec* complex and Type 2 *ccr* gene complex. Allotype III SCC*mec* carries Class A *mec* complex and Type 3 *ccr* gene complex and Allotype IV SCC*mec* has Class B *mec* complex and carries either a Type 4 or Type 2 *ccr* gene complex. (Hiramatsu *et al.*, 2001, Oliveira *et al.*, 2001b) [Please note, at the time of writing this paper, there were only four known *ccr* complexes]

In order to better understand the relationship of CMRSA and their epidemiology, the *mec* complex and *ccr* gene complex in isolates from Australia and New Zealand have been compared.

8.2.2 Material and methods

8.2.2.1 Bacterial strains

A total of 25 MRSA isolates was studied; 22 CMRSA from various locations in Australia and New Zealand, UK epidemic MRSAs, EMRSA-15 and EMRSA-16 (Cookson and Phillips, 1988) and the Eastern Australian (EA) MRSA (Grubb, 1998) WBG525 (See Table 8.1).

The WA CMRSAs were isolated from people living in remote communities who had no history of hospitalisation. The New Zealand and NSW CMRSA isolates have been reported as community MRSA. (Turnidge and Bell, 2000, Nimmo *et al.*, 2000, Adhikari *et al.*, 2002) The SA, VIC and NT CMRSA isolates used were selected because they are non-multiresistant MRSA isolated from community patients.

8.2.2.2 Resistance Profiles

Resistance profiles of the isolates were determined as previously reported. (O'Brien *et al.*, 1999)

8.2.2.3 Chromosomal DNA preparation

Chromosomal DNA was isolated as previously reported. (Maniatis *et al.*, 1989) Bacterial cells were harvested from 20ml overnight culture by centrifugation. The harvested cells were resuspended in 3 ml of lysis buffer (0.1M EDTA, 0.15M NaCl, 0.1M Tris.HCl pH 8.0) containing 100µg/ml of lysostaphin (Sigma Chemical Company, St. Louis, Missouri, USA) and incubated at 37^{o}C for 30 minutes. The lysate was treated with 300µl of 5% sodium dodecyl sulphate dissolved in 50% ethanol and extracted with one ml of phenol/choroform (1:1). The extracted DNA was precipitated with 2 volumes of 100% ethanol and collected by centrifugation.

The DNA pellet was resuspended in one ml of TE buffer (0.1mM EDTA, 10mM Tris.HCl pH8.0) and treated with 20µl of 10mg/ml RNase (Sigma Chemical Company, St. Louis, Missouri, USA). The DNA was then stored at -20^{o}C until used.

8.2.2.4 Primers

The location and details of the primers used to amplify the various regions of the *mec* complex are shown in Figure 8.1 and Table 8.2. The *ccr* gene complexes were amplified with six primers (See Table 8.2). The *ccr* gene primers, cA1, cA2 and cA3 were used as the reverse primers for Type 1, Type 2 and Type 3 *ccr* gene complexes respectively. The primer cB was used as the forward primer for each of the three *ccr* gene types. The Type 4 *ccr* gene complex was amplified with primers C1 and C2 which were designed from the published sequence. (Oliveira *et al.*, 2001b) All primers were supplied by Genset Pacific Pty Ltd, Lismore, New South Wales.

8.2.2.5 PCR amplification

PCR procedures were carried out as described by Frenay *et al*. (1996). The amplification was performed in an iCycler 96 well 0.2ml Reaction Module (Bio-Rad Laboratories, Hercules, California, USA). Each 25µl of the amplification mixture contained 2.5 pmol/µl of each primer, 1µl of chromosomal DNA template and final concentrations of 2mM $MgCl_2$, 200µM dNTP, 2.5 unit/µl of Taq polymerase (Promega, Madison, Wisconsin, USA), 5mM KCl, 1 mM Tris-HCl at pH9.0 and 0.01% TritonX-100 (Promega, Madison, Wisconsin, USA). Amplification was performed as follows: one cycle at 95^{o}C for 4 min; 25 three step cycles at 95^{o}C for 1 min, 60^{o}C for 1 min and 72^{o}C for 1 min; and one cycle of 72^{o}C for 1 min. After amplification the products were stored at 4^{o}C until used. The PCR products were visualized by agarose gel electrophoresis.

8.2.2.6 Contour-clamped homogeneous electric field (CHEF) electrophoresis

CHEF procedures were carried out as previously reported. (O'Brien *et al.*, 1999) Bacterial cells were harvested from 5ml overnight culture and resuspended in EC

lysis buffer (0.5% Brij 58 {Sigma Chemical Company, St. Louis, Missouri, USA}, 0.2% sodium deoxycholate, 0.5% sarkosyl NL30 {BDH Laboratories, Poole, England}, 100mM EDTA, 1M NaCl, 6mM Tris, pH 7.5) to a turbidity of 2.4×10^9 MacFarland standard. The cells were embedded in 0.5% chromosomal grade agarose (Bio-Rad Laboratories, Hercules, California, USA) with 100µg/ml of lysostaphin (Sigma Chemical Company, St. Louis, Missouri, USA). The gel blocks were incubated in EC lysis buffer for 3 hours for cell lysis. After cell lysis, the gel blocks were treated with 0.2mg/ml proteinase K (Sigma Chemical Company, St. Louis, Missouri, USA) in EST buffer (0.5M EDTA, 5mM Tris, 1% sarkosyl NL 30). The treated gel blocks were washed with four changes of 50mM EDTA and stored in 50mM EDTA until used. The gel blocks were washed with sterile cold water for 30 minutes before digestion with four units of *Sma*I restriction enzyme (Promega, Madison, Wisconsin, USA). The digested gel blocks were loaded into a 1% pulsed-field grade agarose gel (Bio-Rad Laboratories, Hercules, California, USA) and electrophoresed in a CHEF-DR III™ System (O'Brien *et al.*, 1999) (Bio-Rad Laboratories, Hercules, California, USA). After electrophoresis, the gel was stained with ethidium bromide and digitised with the Flour-S™ MultiImager system (Bio-Rad Laboratories, Hercules, California, USA). The *Sma*I generated CHEF patterns were analysed by the Multi-Analyst®/PC software and a dendrogram of the CHEF patterns generated using the Dice coefficient (Bio-Rad Laboratories, Hercules, California, USA). (O'Brien *et al.*, 1999)

8.2.3 Results

8.2.3.1 *mec* complex PCR

All of the isolates were amplified by the primers for *mecA* (See Figure 8.1). None of the CMRSAs were amplified by the mA and mI2 primers, which are designed to amplify from *mecA* through to *mecI*. Only the EA MRSA, WBG525 and EMRSA-16 were amplified by these primers.

When the isolates were amplified with primer mA, for *mecA*, and primer ROrf2, specific for IS*1272*, only four of the 22 CMRSA were not amplified (Class E, Table

8.1). Of those that were amplified, 16 CMRSA and EMRSA-15 gave an approximately 2000 bp product (Class B, Table 8.1) and two CMRSA gave an approximately 1000 bp product (Class B1, Table 8.1). Amplification with additional primers (See Figure 8.1) revealed that isolates which produced the 2000 bp product contained the membrane-spanning domain but not the penicillin-binding domain of *mecR1* while those which produced the 1000 bp product lacked both domains (See Table 8.1; Figure 8.1). Both these classes of isolates were negative when amplified for *mecI*. As they gave a positive result with the IS*1272* primer, this indicates that the insertion of IS*1272* produced the Class B and B1 deletions of *mecR1* and *mecI* (See Figure 8.1).

The four CMRSA that did not amplify with the IS*1272* primer (Class E, Table 8.1) were tested with additional primers. They only produced a product with the primers for the penicillin-binding domain of the *mecR1* gene and had a deletion of approximately 976 bp in the membrane-spaning domain of *mecR1* (See Figure 8.1).

WBG525 had the *mecI* gene but had a 166 bp deletion in *mecR1* (See Figure 8.1).

8.2.3.2 *ccr* gene complex PCR

The community MRSA with Class B *mec* complex amplified with the Type 2 *ccr* gene complex primers, cB and cA2. The isolates with Class B1 and Class E *mec* complexes did not amplify with any of the *ccr* gene primers used.

EMRSA-15 and EMRSA-16 were amplified with primers for Type 2 *ccr* gene complex and the EA MRSA, WBG525, was amplified with the Type 3 *ccr* gene primers.

8.2.3.3 CHEF patterns analysis

Twenty CHEF patterns were found in the isolates studied (See Figure 8.2). In general, the similarity of the CHEF patterns of the CMRSA was dependent on the geographical proximity of their origin. Most of the isolates from different geographical areas had unrelated CHEF patterns.

Exceptions were three South Australian (SA) isolates, WBG8873, WBG8952 and WBG8900 and one Victorian (VIC) isolate, WBG10200, which had similarities ranging from 73 to 97% with the WA MRSA WBG8287.

The WA isolates from the Kimberley region (WBG7583 and WBG7597) were closely related to each other but not to the isolates from the Eastern Goldfields of WA or from the NT. None of these isolates were related to the WA MRSA WBG8287.

The CHEF patterns of the NT isolates WBG9087, WBG9101 were identical and not related to other isolates while the NT isolate WBG9093 was related to the WA Eastern Goldfield isolates WBG8400 and WBG8404.

The SA isolates could be separated into two clusters on their CHEF patterns. The first cluster, as mentioned above, was closely related to the WA MRSA WBG8287. The other cluster was closely related to the UK epidemic strain EMRSA-15. One of the SA isolates WBG8897 had 86% similarity with EMRSA-15.

Isolates WBG10198, WBG10199, WBG10202 from VIC had identical CHEF patterns and 96% similarity to EMRSA-16. Of the other VIC isolates, WBG10201 was not closely related to any of the other CMRSA and WBG10200 was closely related to the cluster containing the WA MRSA, WBG8287.

The NSW and NZ isolates were found to have related CHEF patterns. The NSW isolate WBG10060 was indistinguishable from the NZ isolate WBG10049, while the other NSW isolate, WBG10066, was indistinguishable from the NZ isolate WBG10035. This corresponds to results already published. (Gosbell *et al.*, 2001) These two CHEF patterns had 93% similarity with each other and 80% similarity with the cluster containing EMRSA-16.

EA MRSA WBG525 was not closely related to the CMRSA isolates. However, it did have 69% similarity to the CHEF patterns of the WA Kimberley isolates and 73% similarity with the VIC isolate WBG10201.

8.2.4 Discussion

CMRSA were first reported in the early 1990s from people who had no previous history of hospitalisation. (Chambers, 2001, Cookson, 2000, Riley *et al.*, 1995) Since the first reports there has been an increasing number of reports. (Chambers, 2001, Cookson, 2000) There has been some discussion as to the definition of a community MRSA. One definition is that they are from people in the community who have no previous history of hospitalisation. Another definition is that they are isolated from patients within 72 hours of hospitalisation. (Suh *et al.*, 1998) In the present study, the CMRSA either met these criteria or had already been designated as CMRSA by other workers or were non-multiresistant MRSA from patients from the community.

This study compares the overall relationships of a selection of CMRSA from diverse geographical regions using CHEF electrophoresis and PCR analysis of their SCC*mec* (*mec* region).

CHEF has been the gold standard for determining the overall genetic relatedness of strains of *S. aureus.* However, the patterns generated can be affected by mutation or genetic changes caused by phages and transposons. (Tenover *et al.*, 1994) Consequently, isolates with quite different CHEF patterns could have had a clonal origin but have undergone changes with time. A comparison of the SCC*mec* may therefore be useful in determining the relationship of MRSA.

The *mec* and *ccr* gene complexes are the more stable elements in the SCC*mec* cassette. The *mec* complex is the core unit of the SCC*mec*. It is unlikely that different organisms would undergo the same changes in the *mec* complex, as there does not appear to be any evolutionary pressure for any particular change that inactivates *mecI* and *mecR1*. The only pressure appears to be that they do not prevent the expression of *mecA*. The *ccr* gene complex is known to play a role in the integration and excision of SCC*mec* into, and from, the *S. aureus* chromosome (Katayama *et al.*, 2000) It is therefore unlikely the *ccr* gene complex will change from one type of *ccr* complex to another once SCC*mec* has been acquired. As different SCC*mec* cassettes carry distinct combinations of *mec* and *ccr* gene

complexes, the type of SCC*mec* can be determined with these two elements. (Hiramatsu *et al.*, 2001)

Sixteen of the CMRSA and EMRSA-15 were found to have the Class B *mec* complex (See Table 8.1) which has all of *mecI* and the penicillin-binding domain of the *mecR1* deleted by the insertion of a partial copy of IS*1272* (See Figure 8.1). (Katayama *et al.*, 2001) All 16 of these CMRSA were found to carry Type 2 *ccr* gene complex indicating that they are harbouring the Allotype IV SCC*mec* complex (See Table 8.1). CHEF patterns of these isolates fall into five broad unrelated clusters, except forWBG9093 from the NT, and WBG10201 from VIC. The first cluster includes isolates WBG7583 and WBG7597; the second, contains the VIC isolate WBG10200, the WA isolate WBG8287 from RPH and the SA isolates WBG8873, WBG8952 and WBG8900; the third contains EMRSA-15 and the SA isolate WBG8897; the fourth, the NT isolates WBG9087 and WBG9101 and the fifth the NSW and NZ isolates (See Figure 8.2). It has already been reported that the NSW isolates and the WSPP from NZ have similar CHEF patterns. (Gosbell *et al.*, 2001) It is interesting that EMRSA-15 also has the type IV SCC*mec* cassette and that its CHEF pattern has 87% similarity with a CMRSA from SA. This suggests that either EMRSA-15 is in SA or that EMRSA-15 is related to some CMRSA. EMRSA-15 has been found in isolates from eastern Australia (Pearman *et al.*, 2001) and the situation in SA warrants further study.

A variation of Class B *mec* complex is found in two WA isolates, WBG8400 and WBG8404. The insertion of IS*1272* has deleted both the membrane-spanning and the penicillin-binding domains of *mecR1* and all of *mecI*. This type of deletion has been designated Class B1 *mec* complex. (See Figure 8.1) The Class B1 isolates did not amplify with the *ccr* gene primers used indicating that they carry a new type of *ccr* gene complex and hence have a new type of SCC*mec*. These two isolates had similar CHEF patterns to one another but not to any of the other MRSA studied. This together with their different SCC*mec* type suggests that they have acquired their SCC*mec* cassette from a different source and have evolved separately to the other MRSA studied. It has been reported (Okuma *et al.*, 2002) that other CMRSA have a *ccr* complex that did not amplified with primers for known *ccr* complexes and it will

be interesting to see if this *ccr* complex is the same or related to those in the WA CMRSA.

Three CMRSA from VIC and one from SA did not have the class B *mec* complex. They had an approximately 976 bp deletion in the membrane-spanning domain of *mecR1* and although *mecI* was not amplified there was no amplification of IS*1272* (See Figure 8.1 and 8.2). How *mecI* has been deleted is not known. IS*256* and IS*431*/*257* have been reported to cause deletions (Katayama *et al.*, 2001, Ma *et al.*, 2002, Oliveira *et al.*, 2000) but amplification for these was not done. As this type of *mec* complex has not been reported before it has been designated Class E *mec* complex. Like the Class B1 isolates they did not amplify with the *ccr* gene primers and hence their *ccr* gene type could not be determined. The CHEF pattern of the three VIC isolates was closely related to the EMRSA-16. However, EMRSA-16 is known to carry the Allotype II SCC*mec* cassette (Class A *mec* and Type 2 *ccr* complexes) (Enright *et al.*, 2002), which is quite different from the *mec* and *ccr* gene complexes of the VIC isolates. It is possible that the Class E VIC isolates and EMRSA-16 share a common ancestral origin but have acquired a different type of SCC*mec*. The same phenomenon is seen in the SA isolate WBG8918, which has a closely related CHEF pattern to EMRSA-15 but has a different type of SCC*mec*.

WBG525 is typical of EA MRSA and has been endemic in Australian hospitals. (Townsend *et al.*, 1983b, Grubb, 1998) It has a 166 bp deletion in the membrane-spanning domain of *mecR1*. This has been called Class A1 *mec* complex (See Figure 8.1). It was not found in any of the CMRSA and the WBG525 CHEF pattern was not related to any of the CMRSA isolates. These results indicate that the EA MRSA is different from the CMRSA.

CMRSA from different countries are known to carry the Allotype IV SCC*mec*, and most of them do not have related CHEF patterns. (Fey *et al.*, 2003, Okuma *et al.*, 2002) This phenomenon is also seen in this study, as the majority of the CMRSA possess the Allotype IV SCC*mec*, even though they have different CHEF patterns. However, hospital-acquired MRSA with the same type of SCC*mec* are likely to be clonal and have related CHEF patterns. (Enright *et al.*, 2002, Lim *et al.*, 2002, Hiramatsu *et al.*, 2001) These results support the theory that the emergence of

CMRSA is possibly due to the dissemination of the Allotype IV SCC*mec* rather than the spread of a clone(s). (Hiramatsu *et al.*, 2001)

These results further indicate that Allotype IV SCC*mec* is widely distributed in CMRSA from Australia and New Zealand and is also found in EMRSA-15. Although it cannot be determined at this stage whether different strains have acquired the same SCC*mec* or if a strain acquired SCC*mec* and has subsequently undergone changes in CHEF patterns, it nevertheless is tempting to postulate that EMRSA-15 and CMRSA are related. EMRSA-16 on the other hand, although it has a similar CHEF pattern to some of the VIC isolates, has a completely different SCC*mec*. These results demonstrate the importance of using more then one method for typing MRSA (Tenover *et al.*, 1994) and indicate that SCC*mec* typing is potentially very useful.

CMRSA are a challenge to the health care system and will become even more of a challenge if they acquire resistance to additional antibiotics. It is therefore important to understand more about their epidemiology and how they have emerged. This will also help establish a unified definition for CMRSA and assist in establishing appropriate surveillance and control measures. This is vital for the control of this emerging problem. (Cookson, 2000)

Table 8.1 Details of isolates and summary of amplification results

Isolate	*mecR1* MS	*mecR1* PB	*mecI*	IS*1272*	*ccr* gene complex	SCCmec Type	Resistance Profiles	Date of Isolation	Origin	Reference/source
Class B *mec* complex										
WBG 7583	+	-	-	+	2	IV	M, T	1993	Kimberley, WA	Udo *et al*. (1993)
WBG 7597	+	-	-	+	2	IV	M, T	1993	Kimberley, WA	Udo *et al*. (1993)
WBG 8287	+	-	-	+	2	IV	M, E^I	1995	RPH, WA, WA MRSA	O'Brien *et al*. (1999)
WBG 8873	+	-	-	+	2	IV	M, E^I, T, F	1997	Adelaide, SA	a
WBG 8897	+	-	-	+	2	IV	M, C	1997	Adelaide, SA	a
WBG 8900	+	-	-	+	2	IV	M, E^I	1997	Adelaide, SA	a
WBG 8952	+	-	-	+	2	IV	M	1997	Adelaide, SA	a
WBG 9087	+	-	-	+	2	IV	M, E^I	1997	Darwin, NT	b
WBG 9093	+	-	-	+	2	IV	M, E^I	1997	Darwin, NT	b
WBG 9101	+	-	-	+	2	IV	M, E^I	1997	Darwin, NT	b
WBG 10035	+	-	-	+	2	IV	M, T	1999	NZ, WSPP1	d, Adhikari *et al*. (2002)
WBG 10049	+	-	-	+	2	IV	M, Su	1999	NZ, WSPP2	d, Adhikari *et al*. (2002)
WBG 10060	+	-	-	+	2	IV	M	1999	Sydney, NSW	e
WBG 10066	+	-	-	+	2	IV	M	1999	Sydney, NSW	e
WBG 10200	+	-	-	+	2	IV	M	1999	Geelong, VIC	c
WBG 10201	+	-	-	+	2	IV	M	1999	Geelong, VIC	c
EMRSA-15	+	-	-	+	2	IV	M	NA	England, EMRSA	Lim *et al*. (2002)
Class B1 *mec* complex										
WBG 8400	-	-	-	+	UD	UD	M	1995	Eastern Goldfields, WA	O'Brien *et al*. (1999)
WBG 8404	-	-	-	+	UD	UD	M	1995	Eastern Goldfields, WA	O'Brien *et al*. (1999)

Table 8.1 continued on next page

Table 8.1 Continued

Isolate	*mecR1* MS	*mecR1* PB	*mecI*	IS*1272*	*ccr* gene complex	SCCmec Type	Resistance Profiles	Date of Isolation	Origin	Reference/source
Class E *mec* complex										
WBG 8918	-	+	-	-	UD	UD	M, Sm^L	1997	Adelaide, SA	a
WBG 10198	-	+	-	-	UD	UD	M	1999	Geelong, VIC	c
WBG 10199	-	+	-	-	UD	UD	M	1999	Geelong, VIC	c
WBG 10202	-	+	-	-	UD	UD	M	1999	Geelong, VIC	c
Class A *mec* complex										
EMRSA-16	+	+	+	-	2	II	M, C	NA	England, EMRSA	Lim *et al*. (2002)
Class A1 *mec* complex										
WBG525	-	+	+	-	3	III	M, G, Sm, E, Cm, T, Tp, Su	1983	Melbourne, VIC, EA MRSA	Townsend *et al*. (1983b)

Abbreviations:

+, amplified; -, not amplified; UD: unable to determine, MS, membrane-spanning domain of *mecR1*; PB, penicillin-binding domain of *mecR1*; NA, not available; EMRSA, epidemic MRSA; EA MRSA, eastern Australia MRSA; NSW, New South Wales; NT, Northern Territory; NZ, New Zealand; RPH, Royal Perth Hospital; SA, South Australia; VIC, Victoria; WA MRSA, Western Australian MRSA; WSPP, Western Samoa Phage Pattern. [a]Provided by Dr P.C. Lee, Gribbles Laboratories, Adelaide, SA; [b]Provided by Prof Bart Currie, Menzies School of Health Research, Darwin, NT; [c]Provided by Dr. Stephen Graves, Pathcare, Geelong, VIC; [d]provided by Ms Helen Heffernan, Institute of Environmental Science & Research Limited, Wellington, NZ; [e]Provided by Dr Iain Gosbell, South Western Area Pathology Service, NSW; M, methicillin; C, ciprofloxacin; Cm, chloramphenicol; E, erythromycin; F, fusidic acid, G, gentamicin; Sm, streptomycin; Su, sulphamethoxazole; T, tetracycline; Tp, trimethoprim; I, inducible resistance; L, low-level resistance.

Table 8.2 Oligonucleotide primers

Name	Primer sequence (5' to 3')	Nucleotide location	References
***mecA* gene**			
MR1	GTGGAATTGGCCAATACAGG	478-497	Song *et al.* (1987), Tokue *et al*. (1992)
MR2	TGAGTTCTGCAGTACCGGAT	1810-1791	Song *et al.* (1987), Tokue *et al*. (1992)
***mec* complex**			
mA	ACCAAACCCGACAACTACAAC	69-89	Archer *et al.* (1994), Niemeyer *et al*. (1996)
mRA1	GTCTCCACGTTAATTCCATT	357-376	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mRA2	GTCGTTCATTAAGATATGACG	676-646	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mRB1	AAGCACCGTTACTATCTGCACA	1211-1230	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mRB2	GAGTAAATTTTGGTCGAATGCC	1445-1424	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mI1	AATGGCGAAAAAGCACAACA	1923-1942	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
mI2	GACTTGATTGTTTCCTCTGTT	2403-2383	Kobayashi *et al*. (1996), Hiramatsu *et al*. (1992)
ROrf2	GGACAACTTAAGCCAGGGTA	1074-1055	Lim *et al*. (2002)
***ccr* gene complex**			
cB	ATTGCCTTGATAATAGCCTTCT	25518-25539	Ito *et al.* (2001)
cA1	AACCTATATCATCAATCAGTACGT	24868-24845	Ito *et al.* (2001)
cA2	TAAAGGCATCAATGCACAAACACT	26348-26325	Ito *et al.* (2001)
cA3	AGCTCAAAAGCAAGCAATAGAAT	4714-4632	Ito *et al.* (2001)
C1	TGAAGAAGCACAAGAGCGGC	8548-8567	This Study
C2	CTGCACCACATTTTGGGCAC	100102-10083	This Study

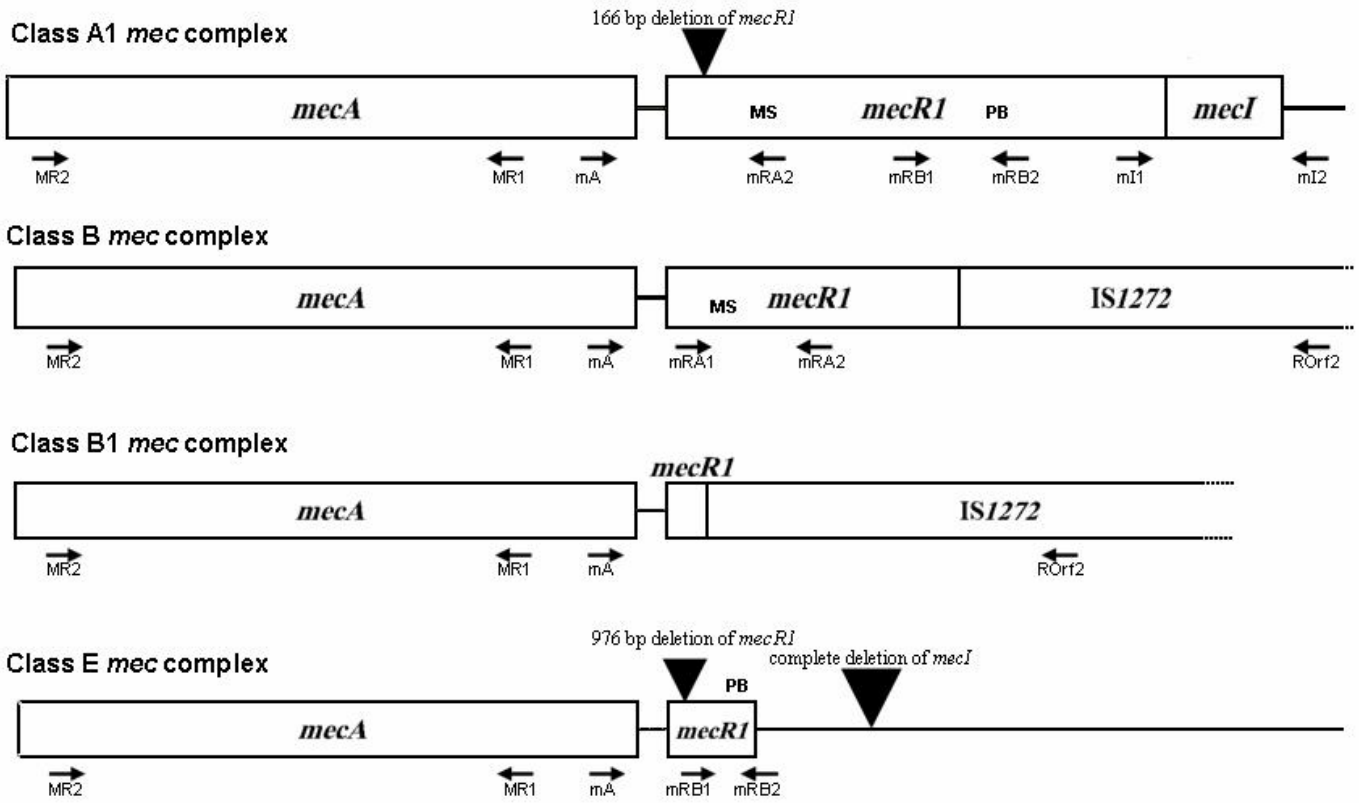

Figure 8.1 Schematic diagram of the *mecA-mecR1-mecI* region showing the location of primers and the classes of *mec* complex

The horizontal arrows indicate the direction of the primers used in the PCR reaction. MS, membrane-spanning domain of *mecR1*. PB, penicillin-binding domain of *mecR1*

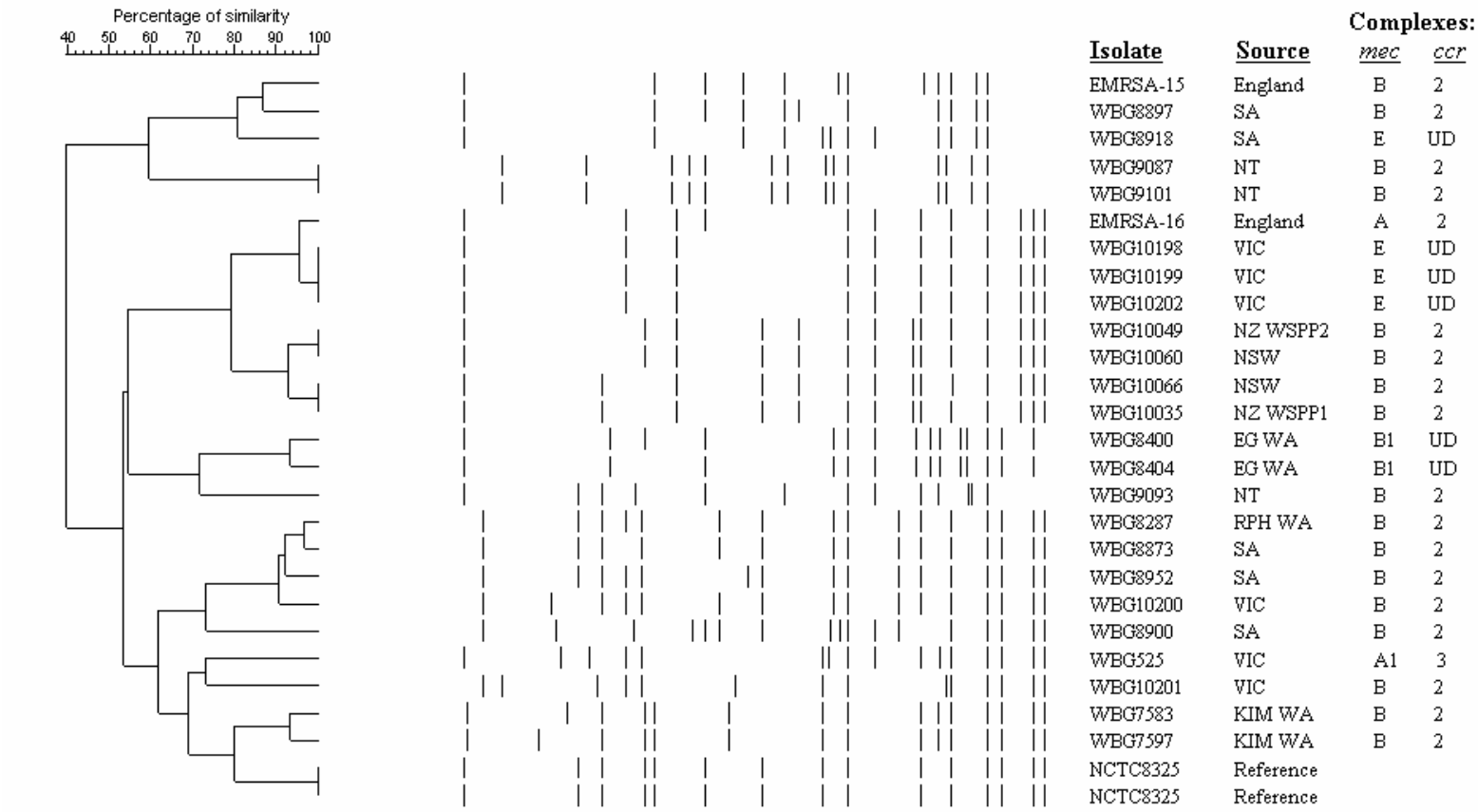

Figure 8.2 CHEF patterns, dendrogram and *mec* and *ccr* complexes of isolates

EG, Eastern Goldfields; KIM, Kimberley region; NSW, New South Wales; NT, Northern Territory; NZ, New Zealand; RPH, Royal Perth Hospital; SA, South Australia; VIC, Victoria; WA, Western Australia; UD, Unable to determine.

8.3 Additional studies

The new SCC*mec* allotype V was recently described in a Western Australian CMRSA strain W1S (WBG8318). (Ito *et al.*, 2004) This novel SCC*mec* has the Class C2 *mec* complex and the novel *ccr* gene, *ccrC*. In allotype V the majority of *mecR1*, and the whole of the *mecI* gene, is truncated by the insertion of IS432/257. (Katayama *et al.*, 2001, Ito *et al.*, 2004) The *ccrC* gene is a novel site-specific recombinase gene. The *ccrC* gene is different from the *ccrA* and *ccrB* genes found in other SCC*mec*s and is functional by itself. (Ito *et al.*, 2004) This gene was found to have more than 90% of sequence similarity with the ORF of Allotype III SCC*mec* CZ072. (Ito *et al.*, 2004)

In the previous study, described in Section 8.2, the *ccr* gene type of WBG8400, WBG8404, WBG8918, WBG10198, WBG10199 and WBG10202 could not be determined, as they did not amplify with the primers specific for *ccr* types 1 to 4. To determine their *ccr* type, these isolates were amplified with the *ccrC* primers, c5F and c5R. (See Table 2.11 and Figure 8.3)

All these CMRSA isolates were found to have the *ccrC* gene, which means they have the Type 5 *ccr* gene complex. Unlike W1S, none of these isolates carry the Class C *mec* complex. WBG8400 and WBG8404 carry the Class B1 *mec* complex and WBG8918, WBG10190, WBG10199 and WBG10202 carry the Class E *mec* complex. It was expected that the Victorian isolates, WBG10198, WBG10199 and WBG10202 would be the same as they had the same resistance profiles, CHEF patterns and carry the same Class E *mec* complex. These results demonstrate that two novel SCC*mec* allotypes are present amongst these isolates.

To determine the presence of CZ072 in the Allotype III SCC*mec* the Malaysian strain, WBG7422, was also included in this study and it was found to be positive for it. (See Figure 8.3)

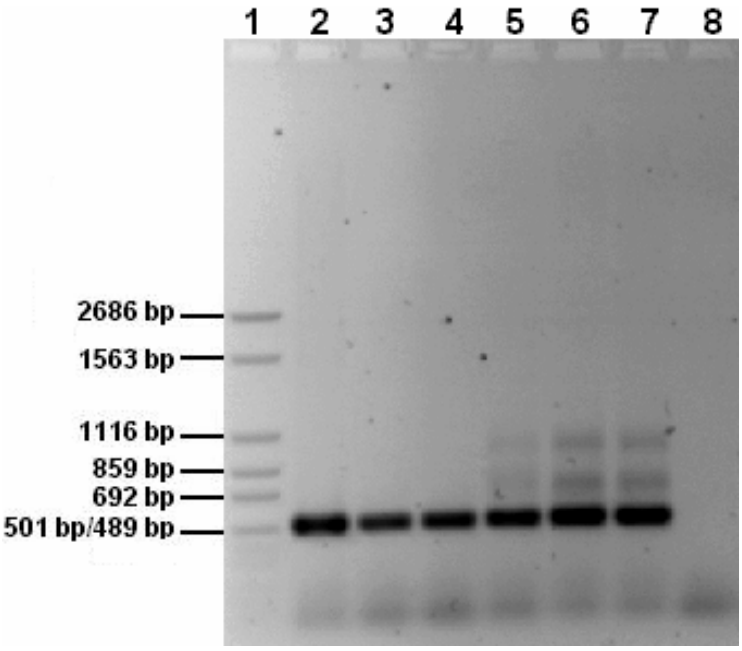

Figure 8.3 PCR of MRSA isolates with *ccrC* primers c5F and c5R[a]

Lane 1 :	FN-1	DNA size marker
Lane 2 :	WBG7422	Malaysian MRSA, SCC*mec* allotype III
Lane 3 :	WBG8400	CMRSA, Class B1 *mec* complex
Lane 4 :	WBG8404	CMRSA, Class B1 *mec* complex
Lane 5 :	[b]WBG8918	CMRSA, Class E *mec* complex
Lane 6 :	[b]WBG10198	CMRSA, Class E *mec* complex
Lane 7 :	[b]WBG10199	CMRSA, Class E *mec* complex
Lane 8 :	EMRSA-16	Negative control, SCC*mec* allotype II[c]

[a]The expected size of the c5F/c5R amplicon is 518 bp
[b]The faint bands above the amplicon are unknown but are thought to be non-specific amplification products.
[c]The faint bands located close to the bottom of the picture are excess primers and dNTPs, as seen in lane 8 negative control.

8.4 Summary

This study investigated the molecular characterisation of twenty-two CMRSA isolated from Western Australia (WA), South Australia (SA), Victoria (VIC), New South Wales (NSW) and New Zealand (NZ) and three hospital-acquired epidemic MRSA (EMRSA). Sixteen community isolates were found to carry Class B *mec* complex and Type 2 *ccr* gene complex. Two WA isolates carried the Class B1 *mec* complex and three VIC and one SA isolate a previously unreported *mec* complex which has been labelled E. The *ccr* gene type of the Class B1 and Class E isolates could not be determined. These isolates may carry previously unreported *ccr* gene complexes. The relatedness of the CHEF patterns of the CMRSA was dependent on their geographical origin. A similar CHEF pattern was found in some WA MRSA, VIC and SA isolates. NSW and NZ CMRSA had the same CHEF patterns and were similar to three VIC isolates and EMRSA-16. Two SA CMRSA isolates had CHEF patterns similar to the UK EMRSA-15 strain. A multiply resistant, nosocomial EMRSA from Australia had a class A *mec* complex, and a CHEF pattern, which was unrelated to any of the CMRSA.

Most of the CMRSA isolated from five Australian states and New Zealand had unrelated CHEF patterns. However, the majority of them carried the Allotype IV SCC*mec* cassette (Class B *mec* and Type 2 *ccr* gene complexes), which indicates that they may have acquired their *mec* complex from the same source or that they have evolved from the same progenitor. Some of the CMRSA had a previously undescribed SCC*mec* cassette.

CHAPTER NINE

RELATIONSHIP BETWEEN MALAYSIAN MRSA AND OTHER MRSA

9.1 Forward

This chapter examines the relationship of Malaysian MRSA with MRSA isolated in different Asian countries and EMRSAs. The CHEF patterns of the Asian MRSAs were provided by Ms Paula Lulu Raman. These isolates were collected as part of the SENTRY programme in 2000. The countries involved were Australia, China, Hong Kong, Indonesia, Japan, Philippines, Singapore, South Africa and Taiwan.

9.2 Malaysian MRSA and EMRSA

In Chapter 4 and Chapter 7 the predominant CHEF pattern A of Malaysian MRSAs was found to be closely related to EMRSA-1 and the EA MRSA WBG525. The CHEF patterns of other Malaysian MRSA were compared with the other EMRSA isolates. The dendrogram and the number matrix of these isolates are shown in Figure 9.1 and Figure. 9.2.

Two major clusters were revealed in the dendrogram of the Malaysian isolates and UK EMRSA strains. All but one Malaysian isolate clustered with EMRSA-1, -4 and -7. The average percentage of similarity within this tightly related cluster was greater than 81%. EMRSA-10, -11 and -9 were the other EMRSA strains that clustered with the Malaysian MRSA but with a low percentage of similarity ranging from 72% to 76%.

EMRSA-4 appeared to have the same CHEF pattern as WBG8154 (Malaysian CHEF A3). However, a two-band difference was detected in the gel pictures. As the two bands involved migrated at very similar distances within the gel, and the differences were very small, the computer software was unable to detect the differences. EMRSA-4 was also found to have a very high percentage of similarity with WBG7422 (Malaysian CHEF A), WBG8009 (Malaysian CHEF A1), WBG8003 (Malaysian CHEF A2), WBG8010 (Malaysian CHEF A4), WBG8011 (Malaysian

CHEF A6), WBG2005 (Malaysian CHEF A7), WBG8007 (Malaysian CHEF A10), WBG2004 (Malaysian CHEF A12), WBG8024 (Malaysian CHEF A15) and WBG525 (EA MRSA). Their percentage of similarity range from 87.5% to 97%

EMRSA-1 clustered together with WBG7409 (Malaysian CHEF A5), WBG7417 (Malaysian CHEF A9), WBG10364 (Malaysian CHEF J) and WBG7413 (Malaysian CHEF A11). The percentage of similarity of these CHEF patterns to EMRSA-1 ranged from 84.8% to 97%. EMRSA-7 was the other EMRSA strain found inside the Malaysian isolate cluster. It clustered with WBG2007 (Malaysian CHEF D) with an 87.5% of similarity.

The Malaysian strain WBG7422 has the predominant Malaysian CHEF A pattern. Similar to other Malaysian isolates, it has a high percentage similarity with the EMRSA strains in the cluster. Its percentage of similarity with EMRSA-1 is 83.9%, EMRSA-4, 93.8%, EMRSA-7, 83.9% and WBG525, EA MRSA, 90.3%. As well as carrying the Allotype III SCC*mec*, EMRSA-1, -4 and -7 also have coagulase-gene type 24 and an *Xba*I site within their *mecA* gene. These molecular characteristics were found in all the Malaysian MRSA isolates. (See Section 4.6, 4.7 and 7.3)

The other major cluster of EMRSA contained the Malaysian isolate WBG10361 which is representative of Malaysian CHEF pattern I. It was the only Malaysian isolate in this cluster of UK EMRSA isolates. It had 85.7% similarity with EMRSA-8 and -13, 84.6% with EMRSA-3, and -12, 82.8% with EMRSA-2, and 81.5% with EMRSA-5, -9, -10, COL and IRISH-1. These EMRSAs carry different SCC*mec* allotypes and coagulase-gene type (See Chapter 7) WBG10361 is not related to EMRSA-1, -4 and -7, their CHEF similarity ranged from 73.3% to 75.9%.

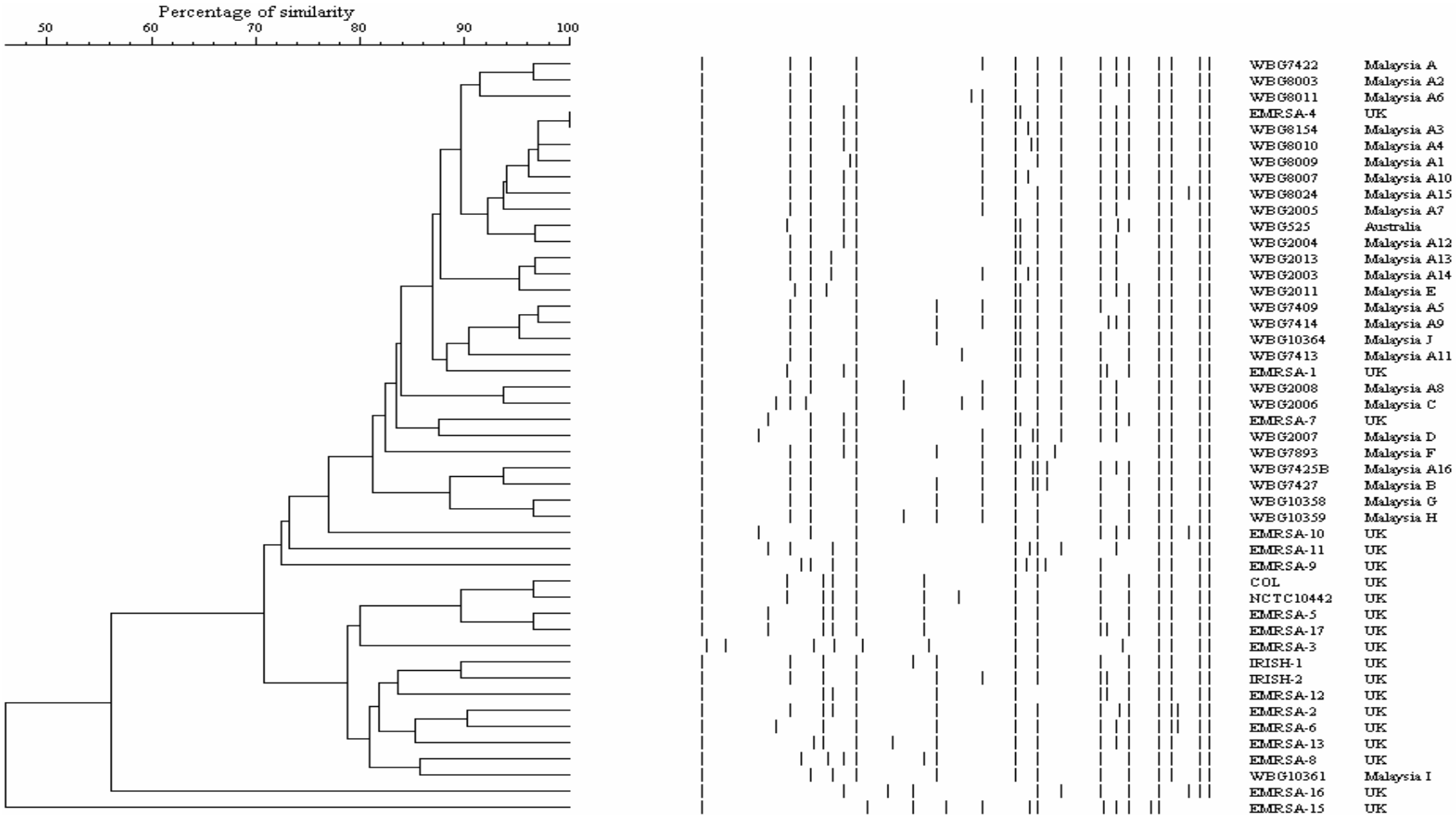

Figure 9.1 Dendrogram comparing Malaysian MRSA and United Kingdom EMRSA isolates

Isolate	Country																																															
WBG7422	Malaysia A	100
WBG8003	Malaysia A2	96.6	100
WBG8011	Malaysia A6	93.3	89.7	100
EMRSA-4	UK	93.8	90.3	87.5	100
WBG8154	Malaysia A3	93.8	90.3	87.5	100	100
WBG8010	Malaysia A4	93.8	90.3	87.5	94.1	100	100
WBG8009	Malaysia A1	96.8	93.3	90.3	97.0	97.0	97.0	100
WBG8007	Malaysia A10	90.3	86.7	83.9	97.0	97.0	97.0	93.8	100
WBG8024	Malaysia A15	93.8	90.3	87.5	94.1	94.1	94.1	97.0	90.9	100
WBG2005	Malaysia A7	93.3	89.7	86.7	93.8	93.8	93.8	96.8	90.3	93.8	100
WBG525	Australia	90.3	93.3	83.9	97.0	97.0	90.9	93.8	93.8	90.9	90.3	100
WBG2004	Malaysia A12	86.7	89.7	80.0	93.8	93.8	87.5	90.3	90.3	87.5	93.3	96.8	100
WBG2013	Malaysia A13	86.7	89.7	80.0	87.5	87.5	81.3	83.9	83.9	81.3	86.7	90.3	93.3	100
WBG2003	Malaysia A14	90.3	86.7	83.9	90.9	90.9	90.9	87.5	87.5	84.8	90.3	87.5	90.3	96.8	100
WBG2011	Malaysia E	90.3	93.3	83.9	90.9	90.9	84.8	87.5	87.5	84.8	83.9	93.8	90.3	96.8	93.8	100
WBG7409	Malaysia A5	90.3	86.7	90.3	90.9	90.9	84.8	87.5	87.5	84.8	83.9	87.5	83.9	83.9	87.5	87.5	100
WBG7414	Malaysia A9	93.8	90.3	87.5	94.1	88.2	88.2	90.9	84.8	88.2	87.5	90.9	87.5	87.5	84.8	90.9	97.0	100
WBG10364	Malaysia J	86.7	89.7	86.7	87.5	87.5	81.3	83.9	83.9	81.3	80.0	90.3	86.7	86.7	83.9	90.3	96.8	93.8	100
WBG7413	Malaysia A11	86.7	89.7	86.7	87.5	87.5	81.3	83.9	83.9	81.3	80.0	90.3	86.7	86.7	83.9	90.3	90.3	87.5	93.3	100
EMRSA-1	UK	83.9	86.7	83.9	90.9	90.9	84.8	87.5	87.5	84.8	83.9	93.8	90.3	83.9	81.3	87.5	87.5	84.8	90.3	90.3	100
WBG2008	Malaysia A8	93.3	89.7	86.7	87.5	87.5	87.5	90.3	83.9	87.5	93.3	83.9	86.7	86.7	90.3	83.9	83.9	87.5	80.0	80.0	77.4	100
WBG2006	Malaysia C	87.5	83.9	81.3	82.4	82.4	82.4	84.8	78.8	82.4	87.5	78.8	81.3	81.3	84.8	78.8	78.8	82.4	75.0	81.3	72.7	93.8	100
EMRSA-7	UK	83.9	86.7	77.4	90.9	90.9	84.8	87.5	87.5	84.8	83.9	93.8	90.3	83.9	81.3	87.5	81.3	84.8	83.9	83.9	87.5	77.4	78.8	100
WBG2007	Malaysia D	83.9	80.0	77.4	84.8	90.9	90.9	87.5	87.5	84.8	90.3	81.3	83.9	77.4	87.5	75.0	75.0	78.8	71.0	71.0	75.0	83.9	78.8	87.5	100
WBG7893	Malaysia F	80.0	75.9	80.0	87.5	87.5	81.3	83.9	83.9	81.3	86.7	83.9	86.7	80.0	83.9	77.4	90.3	87.5	86.7	80.0	83.9	80.0	75.0	77.4	77.4	100
WBG7425B	Malaysia A16	90.3	86.7	83.9	84.8	90.9	90.9	87.5	87.5	84.8	83.9	81.3	77.4	77.4	87.5	81.3	81.3	84.8	77.4	77.4	75.0	83.9	78.8	75.0	81.3	77.4	100
WBG7427	Malaysia B	83.9	80.0	83.9	78.8	84.8	84.8	81.3	81.3	78.8	77.4	75.0	71.0	71.0	81.3	75.0	87.5	84.8	83.9	77.4	75.0	77.4	72.7	68.8	75.0	83.9	93.8	100
WBG10358	Malaysia G	89.7	85.7	89.7	83.9	83.9	83.9	86.7	80.0	83.9	82.8	80.0	75.9	75.9	80.0	80.0	93.3	90.3	89.7	82.8	80.0	82.8	77.4	73.3	73.3	82.8	85.7	93.3	100
WBG10359	Malaysia H	86.7	82.8	86.7	81.3	81.3	81.3	83.9	77.4	81.3	80.0	77.4	73.3	73.3	77.4	77.4	90.3	87.5	86.7	80.0	77.4	86.7	81.3	71.0	71.0	80.0	83.9	90.3	96.6	100
EMRSA-10	UK	82.8	85.7	75.9	77.4	77.4	77.4	80.0	73.3	83.9	75.9	80.0	75.9	75.9	73.3	80.0	73.3	77.4	75.9	75.9	73.3	75.9	71.0	86.7	80.0	62.1	80.0	73.3	78.6	75.9	100
EMRSA-11	UK	75.9	78.6	69.0	71.0	77.4	77.4	73.3	73.3	71.0	75.9	73.3	75.9	82.8	86.7	80.0	86.7	71.0	69.0	69.0	66.7	75.9	77.4	73.3	80.0	69.0	73.3	66.7	64.3	82.1	71.4	100
EMRSA-9	UK	69.0	71.4	69.0	71.0	71.0	71.0	66.7	66.7	64.5	69.0	73.3	75.9	82.8	80.0	86.7	73.3	64.5	75.9	75.9	73.3	69.0	64.5	73.3	73.3	69.0	80.0	80.0	71.4	69.0	71.4	71.4	100
COL	UK	75.9	78.6	75.9	71.0	71.0	71.0	73.3	66.7	71.0	69.0	73.3	69.0	75.9	73.3	80.0	73.3	71.0	75.9	75.9	73.3	69.0	64.5	66.7	60.0	62.1	73.3	73.3	78.6	75.9	71.4	71.4	71.4	100
NCTC10442	UK	73.3	75.9	73.3	68.8	68.8	68.8	71.0	64.5	68.8	66.7	71.0	66.7	73.3	71.0	77.4	71.0	68.8	73.3	80.0	71.0	66.7	68.8	64.5	58.1	60.0	71.0	71.0	75.9	73.3	69.0	69.0	69.0	96.6	100
EMRSA-5	UK	69.0	71.4	69.0	64.5	64.5	64.5	66.7	60.0	64.5	62.1	66.7	62.1	69.0	66.7	73.3	66.7	64.5	69.0	69.0	66.7	62.1	64.5	73.3	66.7	55.2	66.7	66.7	71.4	69.0	78.6	71.4	71.4	92.9	89.7	100
EMRSA-17	UK	66.7	69.0	66.7	62.5	62.5	62.5	64.5	58.1	62.5	60.0	64.5	60.0	66.7	64.5	71.0	64.5	62.5	66.7	66.7	71.0	60.0	62.5	71.0	64.5	53.3	64.5	64.5	69.0	66.7	75.9	69.0	69.0	89.7	86.7	96.6	100
EMRSA-3	UK	71.4	74.1	71.4	66.7	66.7	66.7	69.0	62.1	66.7	71.4	69.0	71.4	78.6	75.9	69.0	69.0	66.7	71.4	71.4	69.0	71.4	66.7	69.0	69.0	64.3	69.0	69.0	74.1	71.4	74.1	74.1	74.1	81.5	78.6	81.5	78.6	100
IRISH-1	UK	75.9	78.6	75.9	71.0	71.0	71.0	73.3	66.7	71.0	69.0	73.3	69.0	75.9	73.3	80.0	80.0	77.4	82.8	75.9	73.3	69.0	64.5	66.7	60.0	69.0	73.3	80.0	85.7	82.8	71.4	64.3	64.3	85.7	82.8	78.6	75.9	74.1	100
IRISH-2	UK	80.0	75.9	80.0	75.0	75.0	75.0	77.4	71.0	75.0	73.3	71.0	66.7	73.3	77.4	77.4	83.9	81.3	80.0	73.3	77.4	73.3	68.8	64.5	64.5	73.3	77.4	83.9	89.7	86.7	69.0	62.1	62.1	82.8	80.0	75.9	80.0	71.4	89.7	100
EMRSA-12	UK	64.3	66.7	64.3	60.0	60.0	60.0	62.1	62.1	60.0	57.1	62.1	57.1	64.3	62.1	69.0	69.0	66.7	71.4	64.3	69.0	57.1	53.3	62.1	55.2	57.1	62.1	69.0	74.1	71.4	66.7	59.3	66.7	81.5	78.6	81.5	85.7	76.9	81.5	85.7	100
EMRSA-2	UK	77.4	80.0	71.0	72.7	72.7	72.7	75.0	68.8	72.7	71.0	75.0	71.0	77.4	75.0	81.3	75.0	78.8	77.4	71.0	68.8	71.0	66.7	68.8	62.5	64.5	75.0	75.0	80.0	77.4	73.3	73.3	66.7	86.7	83.9	80.0	77.4	75.9	86.7	83.9	82.8	100
EMRSA-6	UK	73.3	75.9	66.7	68.8	68.8	68.8	71.0	64.5	68.8	66.7	71.0	66.7	73.3	71.0	77.4	71.0	75.0	73.3	66.7	64.5	66.7	68.8	77.4	64.5	60.0	71.0	71.0	75.9	73.3	75.9	69.0	62.1	75.9	73.3	82.8	80.0	71.4	82.8	80.0	78.6	90.3	100
EMRSA-13	UK	80.0	82.8	73.3	75.0	75.0	75.0	77.4	71.0	75.0	73.3	77.4	73.3	80.0	77.4	83.9	77.4	81.3	80.0	73.3	71.0	73.3	68.8	77.4	71.0	66.7	77.4	77.4	82.8	80.0	82.8	62.1	69.0	75.9	73.3	75.9	73.3	71.4	82.8	80.0	78.6	83.9	86.7	100
EMRSA-8	UK	66.7	69.0	66.7	68.8	68.8	68.8	71.0	64.5	68.8	66.7	71.0	66.7	66.7	64.5	77.4	71.0	68.8	73.3	66.7	71.0	60.0	62.5	71.0	64.5	66.7	64.5	71.0	75.9	73.3	69.0	62.1	75.9	82.8	80.0	82.8	80.0	71.4	82.8	80.0	78.6	77.4	80.0	80.0	100
WBG10361	Malaysia I	78.6	81.5	78.6	73.3	73.3	73.3	75.9	69.0	73.3	71.4	75.9	71.4	78.6	75.9	82.8	82.8	80.0	85.7	78.6	75.9	71.4	66.7	75.9	69.0	71.4	75.9	82.8	88.9	85.7	81.5	68.7	81.5	81.5	78.6	81.5	78.6	84.6	81.5	78.6	84.6	82.8	78.6	85.7	85.7	100
EMRSA-16	UK	59.3	61.5	59.3	62.1	62.1	62.1	64.3	57.1	69.0	59.3	64.3	59.3	51.9	50.0	57.1	57.1	55.2	59.3	59.3	64.3	51.9	48.3	64.3	57.1	51.9	50.0	50.0	53.8	51.9	61.5	46.2	46.2	53.8	51.9	53.8	51.9	48.0	61.5	51.9	48.0	50.0	51.9	59.3	59.3	56.0	100
EMRSA-15	UK	51.9	46.2	44.4	48.3	55.2	55.2	50.0	50.0	48.3	44.4	42.9	37.0	37.0	50.0	42.9	50.0	55.2	44.4	37.0	35.7	44.4	41.4	42.9	50.0	37.0	57.1	57.1	53.8	51.9	46.2	38.5	38.5	38.5	37.0	38.5	37.0	40.0	53.8	51.9	40.0	50.0	51.9	51.9	44.4	48.0	50.0	100

Figure 9.2 The numerical matrix of the dendrogram of the Malaysian MRSA and EMRSA

9.3 Malaysian MRSA, Asian-Pacific MRSA and South African MRSA

The CHEF patterns of MRSA from Malaysia, Asian-Pacific countries and South Africa were compared and the relationship plotted in a dendrogram as shown in Figure 9.3. Their numerical value matrix is shown in Figure 9.4. To reduce the complexity of the dendrogram only a selection of Asian-Pacific MRSA isolates are shown in these two figures. The selection of isolates was based on their CHEF pattern similarities with Malaysian MRSA and each other. The dendrogram and numerical value matrix of all Asian-Pacific strains is shown in appendixes A.3 and A.4.

In the dendrogram, some Asian-Pacific MRSA isolates had CHEF patterns that had an overall percentage of similarity of around 82% with the Malaysian MRSA isolates. The Indonesian isolate INDO-2, Filipino isolate 91 2552 and Singaporean isolate 81 0090 shared an identical CHEF pattern with the Malaysian isolate WBG7409 (Malaysian CHEF A5). They were closely related to the Malaysian isolates WBG7414 (Malaysian CHEF A9) and WBG7413 (Malaysian CHEF A11) with 97% and 90.3% similarity, respectively. The Australian isolate, 81 1035, and Malaysian isolate, WBG10364 (Malaysian CHEF J), were closely related with a similarity of 96.6%. These two strains were also closely related to the WBG7409 (Malaysian CHEF A5) with a similarity of 96.6% and 93.3% respectively.

The Hong Kong isolates 91 1310 and 81 0822 were also found in the Malaysian cluster. They were closely related to Malaysian isolate WBG2004 (Malaysian CHEF A12) with 93.3% and 86.7% similarity, respectively. These three isolates also clustered together with the Australian isolate WBG525 with percentage similarities ranging from 86.7% to 96.6%.

The Taiwanese isolate 81 0951 was the other Asian strain found in the Malaysian cluster. Its CHEF pattern was 96.6% similar to the predominant Malaysian CHEF pattern A (WBG7422). TOGETHER WITH WBG7422, it formed a closely related cluster with Malaysian isolates WBG8154 (Malaysian CHEF A3), WBG8010 (Malaysian CHEF A4), WBG8009 (Malaysian CHEF A1), WBG8007 (Malaysian

CHEF A10), WBG8024 (Malaysian CHEF A15), WBG2005 (Malaysian CHEF A7) and WBG8003 (Malaysian CHEF A2). The percentage similarity of this cluster ranged from 87.5% to 97%.

WBG2007 (Malaysian CHEF D) and WBG10361 (Malaysian CHEF I) were the two Malaysian isolates found outside of the Malaysian cluster. WBG2007 was closely related to the Australian isolates 81 0532 and 81 1038 with similarities of 87.5% and 83.9%, respectively. WBG10361 clustered with Indonesian isolates INDO-1 (96.3% similarity) and INDO-3 (92.9% similarity), Singaporean isolate 91 1379 (92.9% similarity), Hong Kong isolate 81 0821 (85.7% similarity) and PR China isolate 91 1497 (82.8% similarity). INDO-1 was found to be a *mecA* negative, β-lactamase hyper producer, with borderline-methicillin resistance. (Personal communication, Mr Geoff Coombs, 2002). INDO-3 and INDO-2 have similar CHEF patterns (90.3%) and both have Allotype III SCC*mec* and are coagulase-gene type 24, which is identical to the Malaysian isolates and EMRSA isolates with these genotypes. (See Table 9.1 and Section 7.3.2).

Separate to the Malaysian cluster is a closely related cluster formed by the Taiwanese isolates 81 0548, 81 0552 and 91 3157, Hong Kong isolates 81 0295 and 81 0175, Japanese isolate 91 2337, South African isolate 91 1755 and 91 2441, and Australian isolate 91 1703. The percentages of similarity within this cluster are more than 80%, except the similarity between South African isolate 91 2441 and Australia isolate 91 1703 is 75.9%. The overall similarity between this cluster and the Malaysian cluster was around 77%.

The Japanese isolates 81 0766 and 81 0127, and Hong Kong isolate 81 0168 were not related. Their overall similarity with the rest of the isolates studied was less than 60%.

Table 9.1 The SCC*mec* of Indonesian MRSA isolates

Strains	*Xba*I in *mecA*	*mec* complex	*ccr* complex	SCC*mec*	Coagulase-gene Type
		Class	Type	Allotype	
INDO-1	*mecA* negative	n/a	n/a	n/a	24
INDO-2	Present	A	3	III	24
INDO-3	Present	A	3	III	24

n/a, not applicable

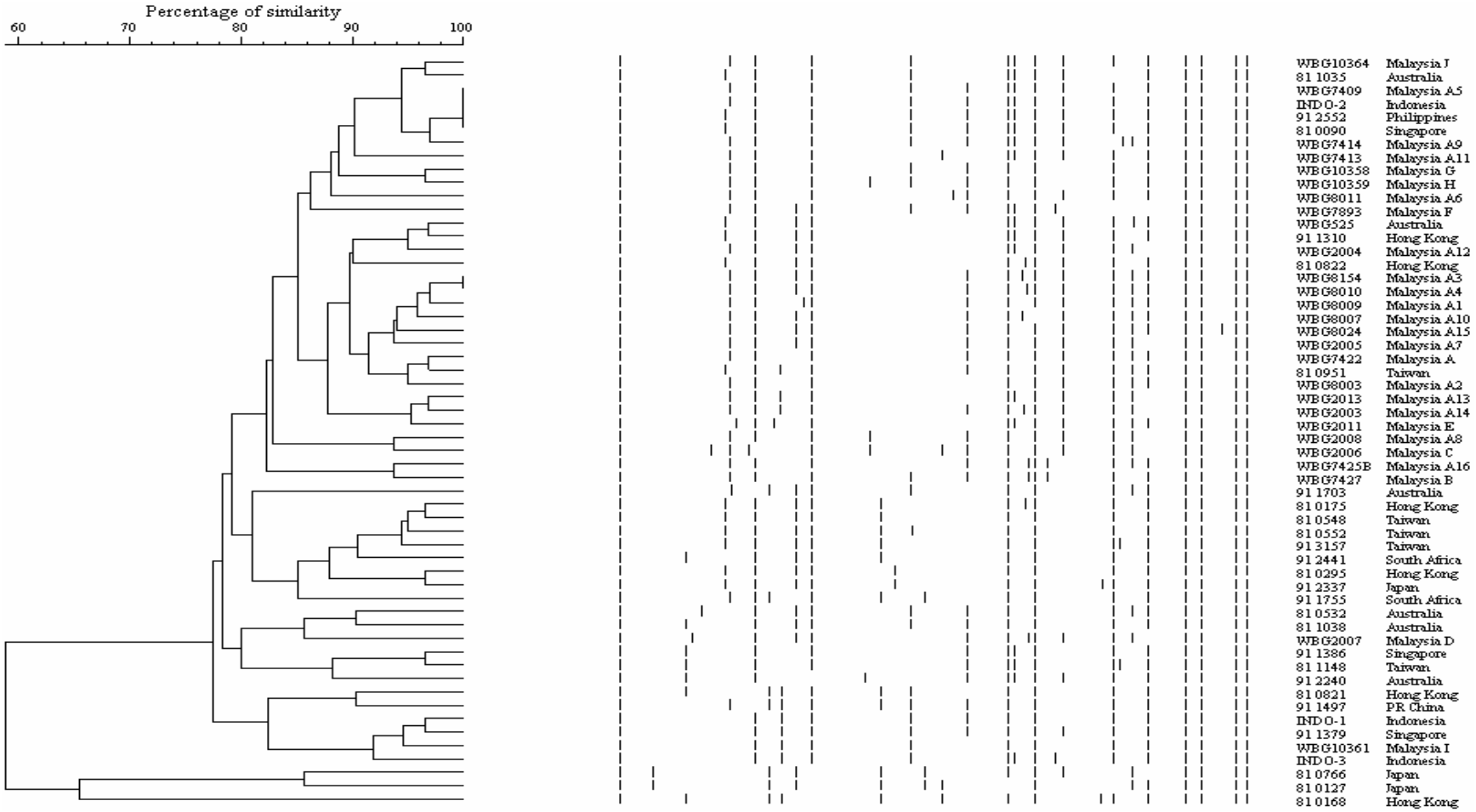

Figure 9.3 Dendrogram of the Malaysian, Asian-Pacific and South African MRSA isolates

Isolate	Country																																																						
WBG10364	Malaysia J	100																																																					
81 1035	Australia	96.6	100																																																			
WBG7409	Malaysia A5	96.6	93.3	100																																																	
INDO-2	Indonesia	96.6	93.3	100	100																																																
91 2552	Philippines	96.6	93.3	100	100	100																																														
81 0090	Singapore	96.6	93.3	100	100	100	100																																												
WBG7414	Malaysia A9	93.8	90.3	97.0	97.0	97.0	97.0	100																																										
WBG7413	Malaysia A11	93.3	89.7	90.3	90.3	90.3	90.3	87.5	100																																								
WBG10358	Malaysia G	89.7	85.7	93.3	93.3	93.3	93.3	90.3	82.8	100																																							
WBG10359	Malaysia H	86.7	82.8	90.3	90.3	90.3	90.3	87.5	80.0	96.6	100																																					
WBG8011	Malaysia A6	86.7	82.8	90.3	90.3	90.3	90.3	87.5	86.7	89.7	86.7	100																																				
WBG7893	Malaysia F	86.7	89.7	90.3	90.3	90.3	90.3	87.5	80.0	82.8	80.0	80.0	100																																		
WBG525	Australia	90.3	86.7	87.5	87.5	87.5	87.5	90.9	90.3	80.0	77.4	83.9	83.9	100																																
91 1310	Hong Kong	93.3	89.7	90.3	90.3	90.3	90.3	87.5	93.3	82.8	80.0	86.7	86.7	96.6	100																														
WBG2004	Malaysia A12	86.7	82.8	83.9	83.9	83.9	83.9	87.5	86.7	75.9	73.3	80.0	86.7	96.6	93.3	100																												
81 0822	Hong Kong	86.7	82.8	83.9	83.9	83.9	83.9	81.3	86.7	82.8	80.0	86.7	80.0	90.3	93.3	86.7	100																											
WBG8154	Malaysia A3	87.5	83.9	90.9	90.9	90.9	90.9	88.2	87.5	83.9	81.3	87.5	87.5	97.0	93.8	93.8	93.8	100																										
WBG8010	Malaysia A4	81.3	77.4	84.8	84.8	84.8	84.8	88.2	81.3	83.9	81.3	87.5	81.3	90.9	87.5	87.5	93.8	100	100																									
WBG8009	Malaysia A1	83.9	80.0	87.5	87.5	87.5	87.5	90.9	83.9	86.7	83.9	90.3	83.9	93.8	90.3	90.3	90.3	97.0	97.0	100																								
WBG8007	Malaysia A10	83.9	80.0	87.5	87.5	87.5	87.5	84.8	83.9	80.0	77.4	83.9	83.9	93.8	90.3	90.3	90.3	97.0	97.0	93.8	100																							
WBG8024	Malaysia A15	81.3	77.4	84.8	84.8	84.8	84.8	88.2	81.3	83.9	81.3	87.5	81.3	90.9	87.5	87.5	87.5	94.1	94.1	97.0	90.9	100																						
WBG2005	Malaysia A7	80.0	75.9	83.9	83.9	83.9	83.9	87.5	80.0	82.8	80.0	86.7	86.7	90.3	86.7	93.3	86.7	93.8	93.8	96.6	90.3	93.8	100																					
WBG7422	Malaysia A	86.7	82.8	90.3	90.3	90.3	90.3	93.6	86.7	89.7	86.7	93.3	80.0	90.3	86.7	86.7	86.7	93.8	93.8	96.6	90.3	93.8	93.3	100																				
81 0951	Taiwan	83.9	80.0	87.5	87.5	87.5	87.5	90.9	83.9	86.7	83.9	90.3	77.4	87.5	83.9	83.9	83.9	90.9	90.9	93.8	87.5	90.9	90.3	96.6	100																			
WBG8003	Malaysia A2	89.7	85.7	86.7	86.7	86.7	86.7	90.3	89.7	85.7	82.8	89.7	75.9	93.3	89.7	89.7	89.7	90.3	90.3	93.3	86.7	90.3	89.7	96.6	93.3	100																		
WBG2013	Malaysia A13	86.7	82.8	83.9	83.9	83.9	83.9	87.5	86.7	75.9	73.3	80.0	80.0	90.3	86.7	93.3	80.0	87.5	81.3	83.9	83.9	81.3	86.7	86.7	90.3	89.7	100																	
WBG2003	Malaysia A14	83.9	80.0	87.5	87.5	87.5	87.5	84.8	83.9	80.0	77.4	83.9	83.9	87.5	83.9	90.3	83.9	90.9	90.9	87.5	87.5	84.8	90.9	90.3	93.8	86.7	96.8	100																
WBG2011	Malaysia E	90.3	86.7	87.5	87.5	87.5	87.5	90.9	90.3	80.0	77.4	83.9	77.4	93.8	90.3	90.3	83.9	90.9	84.8	87.5	87.5	84.8	83.9	90.3	93.8	93.3	96.8	93.8	100															
WBG2008	Malaysia A8	80.0	75.9	83.9	83.9	83.9	83.9	87.5	80.0	82.8	86.7	86.7	80.0	83.9	80.0	86.7	80.0	87.5	87.5	90.3	83.9	87.5	93.3	93.3	90.3	89.7	86.7	90.3	83.9	100														
WBG2006	Malaysia C	75.0	71.0	78.8	78.8	78.8	78.8	82.4	81.3	77.4	81.3	81.3	75.0	78.6	75.0	81.3	75.0	82.4	82.4	84.8	78.8	82.4	87.5	87.5	84.8	83.9	81.3	84.8	78.8	93.8	100													
WBG7425B	Malaysia A16	77.4	73.3	81.3	81.3	81.3	81.3	84.8	77.4	86.7	83.9	83.9	77.4	81.3	77.4	77.4	83.9	90.9	90.9	87.5	87.5	84.8	83.9	90.3	87.5	86.7	77.4	87.5	81.3	83.9	78.8	100												
WBG7427	Malaysia B	83.9	80.0	87.5	87.5	87.5	87.5	84.8	77.4	93.3	90.3	83.9	83.9	75.0	77.4	71.0	83.9	84.8	84.8	81.3	81.3	78.8	77.4	83.9	81.3	80.0	71.0	81.3	75.0	77.4	72.7	93.8	100											
91 1703	Australia	80.0	75.9	77.4	77.4	77.4	77.4	81.3	73.3	82.8	80.0	73.3	73.3	83.9	80.0	80.0	80.0	81.3	81.3	83.9	77.4	81.3	80.0	80.0	83.9	82.8	80.0	77.4	83.9	73.3	68.8	77.4	77.4	100										
81 0175	Hong Kong	80.0	75.9	77.4	77.4	77.4	77.4	75.0	80.0	82.8	80.0	80.0	73.3	83.9	86.7	80.0	93.3	87.5	87.5	83.9	83.9	81.3	80.0	80.0	77.4	82.8	73.3	77.4	77.4	73.3	68.8	83.9	83.9	80.0	100									
81 0548	Taiwan	82.8	78.6	80.0	80.0	80.0	80.0	77.4	82.8	85.7	82.8	82.8	75.9	86.7	89.7	82.8	89.7	83.9	83.9	86.7	80.0	83.9	82.8	82.8	80.0	85.7	75.9	73.3	80.0	75.9	71.0	80.0	80.0	82.8	96.6	100								
81 0552	Taiwan	86.7	82.8	83.9	83.9	83.9	83.9	81.3	80.0	89.7	86.7	80.0	80.0	83.9	86.7	80.0	86.7	81.3	81.3	83.9	77.4	81.3	80.0	80.0	77.4	82.8	73.3	71.0	77.4	73.3	68.8	77.4	83.9	86.7	93.3	96.6	100							
91 3157	Taiwan	80.0	75.9	77.4	77.4	77.4	77.4	75.0	80.0	82.8	80.0	80.0	73.3	83.9	86.7	80.0	86.7	81.3	81.3	83.9	77.4	81.3	80.0	80.0	77.4	82.8	73.3	71.0	77.4	73.3	68.8	77.4	77.4	80.0	93.3	96.6	93.3	100						
91 2441	South Africa	75.9	71.4	73.3	73.3	73.3	73.3	71.0	75.9	78.6	75.9	75.9	69.0	80.0	82.8	75.9	82.8	77.4	77.4	80.0	73.3	77.4	75.9	75.9	73.3	78.6	69.0	66.7	73.3	69.0	64.5	73.3	73.3	75.9	89.7	92.9	89.7	89.7	100					
81 0295	Hong Kong	82.8	78.6	80.0	80.0	80.0	80.0	77.4	82.8	85.7	82.8	82.8	75.9	86.7	89.7	82.8	89.7	83.9	83.9	86.7	80.0	83.9	82.6	82.8	80.0	85.7	75.9	73.3	80.0	75.9	71.0	80.0	80.0	82.8	89.7	92.9	89.7	89.7	85.7	100				
91 2337	Japan	80.0	75.9	77.4	77.4	77.4	77.4	75.0	80.0	82.8	80.0	80.0	73.3	83.9	86.7	80.0	86.7	81.3	81.3	83.9	77.4	81.3	80.0	80.0	77.4	82.8	73.3	71.0	77.4	73.3	68.8	77.4	77.4	80.0	86.7	89.7	86.7	86.7	82.8	96.6	100			
91 1755	South Africa	80.0	75.9	77.4	77.4	77.4	77.4	75.0	80.0	82.8	80.0	80.0	66.7	77.4	80.0	73.3	80.0	75.0	75.0	77.4	71.0	75.0	73.3	80.0	83.9	82.8	80.0	77.4	83.9	73.3	68.8	77.4	77.4	80.0	86.7	89.7	86.7	86.7	82.8	82.8	80.0	100		
81 0532	Australia	77.4	73.3	81.3	81.3	81.3	81.3	84.8	71.0	86.7	83.9	77.4	77.4	81.3	77.4	77.4	77.4	84.8	84.8	87.5	81.3	84.8	83.9	83.9	81.3	80.0	71.0	75.0	75.0	77.4	78.8	81.3	81.3	83.9	77.4	80.0	83.9	77.4	80.0	80.0	77.4	71.0	100	
81 1038	Australia	80.0	75.9	83.9	83.9	83.9	83.9	81.3	73.3	89.7	86.7	80.0	80.0	77.4	80.0	73.3	80.0	81.3	81.3	83.9	77.4	81.3	80.0	80.0	77.4	75.9	66.7	71.0	71.0	73.3	68.8	77.4	83.9	80.0	80.0	82.8	86.7	80.0	89.7	82.8	80.0	73.3	90.3	100													
WBG2007	Malaysia D	71.0	66.7	75.0	75.0	75.0	75.0	78.8	71.0	73.3	71.0	77.4	77.4	81.3	77.4	83.9	83.9	90.9	90.9	87.5	87.5	84.8	90.3	83.9	81.3	80.0	77.4	87.5	75.0	83.9	78.8	81.3	75.0	71.0	77.4	73.3	71.0	71.0	80.0	73.3	71.0	64.5	87.5	83.9	100												
91 1386	Singapore	82.8	78.6	86.7	86.7	86.7	86.7	83.9	82.8	85.7	82.8	82.8	75.9	80.0	82.8	75.9	75.9	83.9	77.4	80.0	80.0	77.4	75.9	82.8	80.0	78.6	75.9	80.0	80.0	75.9	71.0	80.0	80.0	69.0	75.9	78.6	75.9	75.9	85.7	78.6	75.9	75.9	80.0	89.7	80.0	100											
81 1148	Taiwan	80.0	75.9	83.9	83.9	83.9	83.9	81.3	80.0	82.8	80.0	80.0	73.3	77.4	80.0	73.3	73.3	81.3	75.0	77.4	77.4	75.0	73.3	80.0	77.4	75.9	73.3	77.4	77.4	73.3	68.8	77.4	77.4	66.7	73.3	75.9	73.3	80.0	82.8	75.9	73.3	73.3	77.4	86.7	77.4	96.6	100										
91 2240	Australia	80.0	75.9	83.9	83.9	83.9	83.9	81.3	80.0	75.9	80.0	80.0	73.3	77.4	80.0	73.3	73.3	81.3	75.0	77.4	77.4	75.0	73.3	80.0	77.4	75.9	73.3	77.4	77.4	80.0	75.0	71.0	71.0	60.0	86.7	69.0	66.7	66.7	75.9	69.0	66.7	66.7	71.0	80.0	77.4	89.7	86.7	100									
81 0821	Hong Kong	73.3	69.0	71.0	71.0	71.0	71.0	68.8	66.7	75.9	73.3	66.7	60.0	64.5	66.7	60.0	66.7	62.5	62.5	64.5	58.1	62.5	60.0	66.7	71.0	69.0	66.7	64.5	71.0	60.0	56.3	64.5	71.0	80.0	73.3	75.9	80.0	73.3	82.8	69.0	66.7	80.0	71.0	80.0	84.5	75.9	73.3	86.7	100								
91 1497	PR China	77.4	73.3	81.3	81.3	81.3	81.3	78.8	71.0	86.7	83.9	77.4	71.0	68.8	71.0	64.5	71.0	72.7	72.7	75.0	68.6	72.7	71.0	77.4	81.3	73.3	71.0	75.0	75.0	71.0	66.7	75.0	81.3	83.9	77.4	80.0	83.9	77.4	73.3	73.3	71.0	83.9	75.0	77.4	82.5	73.3	71.0	84.5	90.3	100							
INDO-1	Indonesia	82.8	78.6	86.7	86.7	86.7	86.7	83.9	75.9	92.9	89.7	82.8	75.9	73.3	75.9	69.0	75.9	77.4	77.4	80.0	73.3	77.4	75.9	82.8	86.7	78.6	75.9	80.0	80.0	75.9	71.0	80.0	86.7	75.9	75.9	78.6	82.8	75.9	78.6	78.6	75.9	75.9	86.7	89.7	73.3	85.7	82.8	75.9	82.8	86.7	100						
91 1379	Singapore	86.7	82.8	90.3	90.3	90.3	90.3	87.5	80.0	89.7	86.7	86.7	80.0	77.4	80.0	73.3	80.0	81.3	81.3	83.9	77.4	81.3	80.0	86.7	90.3	82.8	80.0	83.9	83.9	80.0	75.0	77.4	83.9	73.3	73.3	75.9	80.0	73.3	75.9	75.9	73.3	73.3	83.9	86.7	77.4	82.8	80.0	80.0	80.0	83.9	96.6	100					
WBG10361	Malaysia I	85.7	81.5	82.8	82.8	82.8	82.8	80.0	78.6	88.9	86.7	78.6	71.4	75.9	78.6	71.4	78.6	73.3	73.3	75.9	69.0	73.3	71.4	78.6	82.8	81.5	78.6	75.9	82.8	71.4	66.7	75.9	82.8	78.6	78.6	81.5	85.7	78.6	81.5	81.5	78.6	78.6	82.8	86.7	69.0	81.5	78.6	71.4	85.7	82.8	96.3	92.9	100				
INDO-3	Indonesia	93.3	89.7	90.3	90.3	90.3	90.3	87.5	86.7	82.8	80.0	80.0	80.0	83.9	86.7	80.0	80.0	81.3	75.0	77.4	77.4	75.0	73.3	80.0	83.9	82.8	86.7	83.9	90.3	73.3	68.8	77.4	83.9	73.3	73.3	75.9	80.0	73.3	75.9	75.9	73.3	73.3	77.4	80.0	71.0	82.8	80.0	80.0	80.0	77.4	89.7	93.3	92.9	100			
81 0766	Japan	55.2	57.1	53.3	53.3	53.3	53.3	58.1	55.2	50.0	48.3	55.2	62.1	66.7	62.1	69.0	62.1	64.5	64.5	66.7	60.0	64.5	69.0	62.1	66.7	64.3	69.0	66.7	66.7	62.1	58.1	53.3	48.7	69.0	62.1	64.3	62.1	62.1	64.3	57.1	55.2	69.0	60.0	55.2	66.7	50.0	48.3	55.2	62.1	60.0	50.0	55.2	51.9	55.2	100		
81 0127	Japan	48.3	50.0	46.7	46.7	46.7	46.7	51.6	55.2	50.0	48.3	48.3	48.3	60.0	55.2	55.2	55.2	58.1	58.1	60.0	53.3	58.1	55.2	55.2	60.0	57.1	55.2	53.3	60.0	48.3	51.6	53.3	48.7	69.0	62.1	64.3	62.1	62.1	64.3	57.1	55.2	69.0	60.0	55.2	53.3	50.0	48.3	48.3	62.1	60.0	50.0	48.3	51.9	48.3	85.7	100	
81 0168	Hong Kong	60.0	55.2	58.1	58.1	58.1	58.1	56.3	66.7	62.1	60.0	60.0	46.7	58.1	60.0	53.3	60.0	56.3	56.3	58.1	51.6	56.3	53.3	60.0	64.5	62.1	60.0	58.1	64.5	53.3	56.3	58.1	58.1	66.7	66.7	69.0	66.7	66.7	75.9	62.1	66.7	73.3	58.1	66.7	58.1	69.0	68.7	66.7	66.7	77.4	69.0	66.7	71.4	66.7	62.1	69.0	100

Figure 9.4 The numerical matrix of the dendrogram of the Malaysian, Asian-Pacific and South African MRSA isolates

9.4 Asian-Pacific MRSA, South African MRSA and EMRSA

The relationship between the Asian-Pacific MRSA, including Malaysian MRSA, South African MRSA isolates and EMRSA strains is shown in Figure 9.5 as a dendrogram. The numerical value matrix of this dendrogram is shown in Figure 9.6. This is similar to the previous section. Only a selection of Asian-Pacific isolates is shown in the two figures. The dendrogram and numerical value matrix of all the Asian-Pacific isolates is shown in appendixes A.3 and A.4.

Four related CHEF clusters with overall similarity of 80% or more were observed in the dendrogram.

The first cluster is mainly composed of Asian-Pacific isolates, from WBG7409 to 91 1755 on the dendrogram in Figure 9.5. The overall similarity within this Asian-Pacific cluster is 80%. Another three subclusters were found within this Asian-Pacific cluster.

The first Asian-Pacific subcluster including four isolates with identical CHEF pattern are described in the previous section 9.3. The isolates with identical CHEF patterns are Malaysian isolate WBG7409 (Malaysian CHEF A5), Indonesian isolate INDO-2, Filipino isolate 91 2552 and Singaporean isolate 81 0090. The other isolates in this subcluster including WBG10361 (Malaysian CHEF I), Singaporean isolates 91 1379, Indonesian isolates INDO-3 and Australian isolate 81 1035. Their CHEF patterns are more than 81.5% similar.

The second Asian-Pacific subcluster is 83% similar to the first subcluster. It includes the WBG7422 (Malaysian CHEF A), WBG525 (EA MRSA), WBG8010 (Malaysian CHEF A4), Taiwanese isolate 81 0951, Hong Kong isolates 81 0822 and 91 1310, EMRSA-1, -4 and -7. The percentage of similarity within these isolates ranges from 81.3% to 97%. EMRSA-1, -7 and -4 have been previously described in Chapter 7 and Section 9.2. They were found to be related to each other, most of the Malaysian isolates and the EA MRSA, WBG525.

The third Asian Pacific subcluster is 80% similar to the first and second subcluster. It comprises of Taiwanese isolates 81 0548, 81 0552 and 91 3157, Hong Kong isolates 81 0295 and 81 0175, Japanese isolate 91 2337, South African isolates 91 1755 and 91 2441. They have a similarity ranging from 80% to 96.6%. Their CHEF patterns similarities have been described in the previous Section 9.3, together with the Australian isolate 91 1703.

The second cluster is not related to the Asian-Pacific cluster (79% similarity). It consists of EMRSA-10, Australian isolates 81 0532, 81 1038 and 91 2240, Malaysian isolate WBG2007 (Malaysian CHEF D), Singaporean isolate 91 1386, and Taiwanese isolate 81 1148. The overall similarity of this cluster is 80%. Within the cluster EMRSA-10 was found to be closely related to the Australian isolates 81 0532 and 81 1038, and the Malaysian isolate WBG2007 (Malaysian CHEF D). The percentage of similarity within this cluster ranged from 80% to 90.3%.

The third cluster consists of mainly UK EMRSA isolates and have an overall 81% similarity in their CHEF patterns. This cluster includes EMRSA-2, -6, -8, -12 and -13, IRISH-1 and -2, PR China isolate 91 1497 and Australian isolate 91 1703. The PR China isolate 91 1497 was related to IRISH-1 (93.3% similarity), IRISH-2 (90.3% similarity), EMRSA-2 (87.5% similarity) and EMRSA-12 (82.8% similarity). Within the cluster, the Australian isolate 91 1703 was found to cluster closely with EMRSA-2, -6, -8 and -13. The percentage of similarity of these isolates ranges from 86.7% to 90.3%. The third cluster is not related to the first Asian-Pacific cluster and nor to the second cluster (72% similarity).

In the fourth cluster, the Hong Kong isolate 91 2031 was closely related to the UK strains COL, NCTC10442 (First MRSA) and EMRSA-5 and -17. The percentage similarity of the organisms in this cluster ranged from 81.3% to 96.8%. This cluster is not related to the other three clusters with a similarity between 70% and 79%.

Some isolates were not found within these four clusters. They were found to be not related to previously described clusters and to each other. The CHEF pattern of EMRSA-9 did not cluster with any of the individual isolates within the clusters, but it

has an 82.8% similarity to the Indonesian isolate INDO-3. The two Hong Kong isolates 81 0168 and 81 0821 did not closely cluster with the other isolates, but they were 86.7% similar to each other. The Japanese isolates, 81 0127, 81 2760 and 91 2332 were not related to each other and to other isolates in this comparison.

Australian isolate 91 1827 was found to have a CHEF pattern identical to EMRSA-15. However, they were not related to the rest of the isolates in this comparison.

9.5 Malaysian MRSA and community-acquired MRSA

The Malaysian MRSA isolates were compared with the CMRSA isolates. The dendrogram is presented in Figure 9.7. The Malaysian MRSA and CMRSA isolates did not clustered with each other. The overall similarity between these two groups of isolates was less than 60%. The CMRSA WBG7583, WBG7597 and WBG10201 were found to have the closest similarity to Malaysian isolates among the CMRSAs with a similarity between 67% to 69%.

The Kimberley Western Australian CMRSA isolates, WBG7583 and WBG7597, are clustered closely with the reference strain NCTC8325 with 80% similarity. Their CHEF patterns also have a 69% overall similarity with the Malaysian isolates

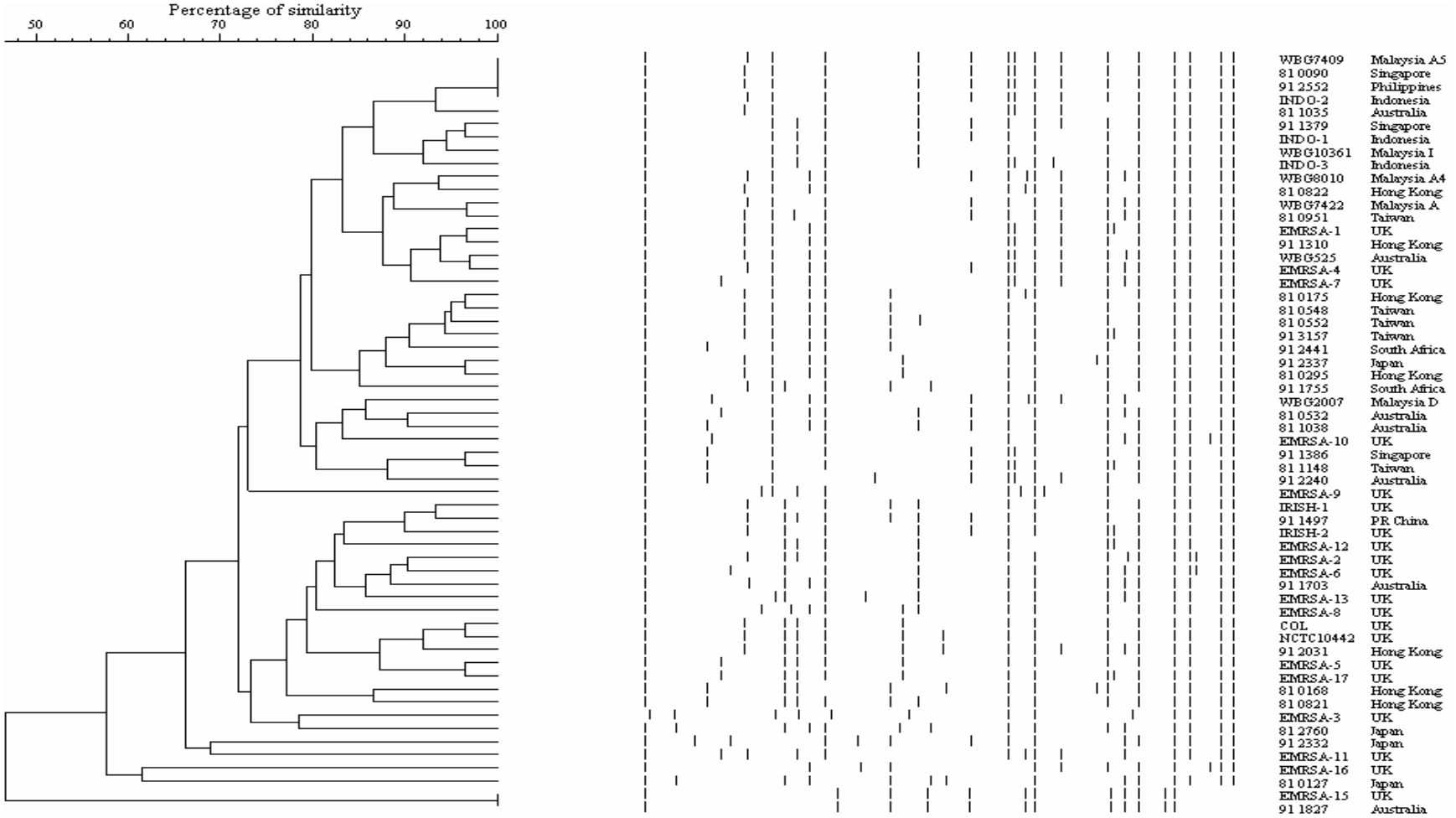

Figure 9.5 Dendrogram of the Asian-Pacific, South African and United Kingdoms EMRSA isolates

Isolate	Country																																																										
WBG7409	Malaysia A5	100
81 0090	Singapore	100	100
91 2552	Philippines	100	100	100
INDO-2	Indonesia	100	100	100	100
81 1035	Australia	93.3	93.3	93.3	93.3	100
91 1379	Singapore	90.3	90.3	90.3	90.3	82.8	100
INDO-1	Indonesia	86.7	86.7	86.7	86.7	78.6	96.6	100
WBG10361	Malaysia I	82.8	82.8	82.8	82.8	81.5	92.9	96.3	100
INDO-3	Indonesia	90.3	90.3	90.3	90.3	89.7	93.3	89.7	92.9	100
WBG8010	Malaysia A4	84.6	84.6	84.6	84.6	77.4	81.3	77.4	73.3	75.0	100
81 0822	Hong Kong	83.9	83.9	83.9	83.9	82.8	80.0	75.9	78.6	80.0	93.8	100
WBG7422	Malaysia A	90.3	90.3	90.3	90.3	82.8	86.7	82.8	78.6	80.0	93.8	86.7	100
81 0951	Taiwan	87.5	87.5	87.5	87.5	80.0	90.3	86.7	82.8	83.9	90.9	83.9	96.8	100
EMRSA-1	UK	87.5	87.5	87.5	87.5	86.7	77.4	73.3	75.9	83.9	84.8	90.3	83.9	81.3	100
91 1310	Hong Kong	90.3	90.3	90.3	90.3	89.7	80.0	75.9	78.6	86.7	87.5	93.3	86.7	83.9	96.8	100
WBG525	Australia	87.5	87.5	87.5	87.5	86.7	77.4	73.3	75.9	83.9	90.9	90.3	90.3	87.5	93.8	96.8	100
EMRSA-4	UK	90.9	90.9	90.9	90.9	83.9	81.3	77.4	73.3	81.3	94.1	87.5	93.8	90.9	90.9	93.8	97.0	100
EMRSA-7	UK	81.3	81.3	81.3	81.3	80.0	77.4	73.3	75.9	83.9	84.8	83.9	83.9	81.3	87.5	90.3	93.8	90.9	100
81 0175	Hong Kong	77.4	77.4	77.4	77.4	75.9	73.3	75.9	78.6	73.3	87.5	93.3	80.0	77.4	83.9	86.7	83.9	81.3	77.4	100
81 0548	Taiwan	80.0	80.0	80.0	80.0	78.6	75.9	78.6	81.5	75.9	83.9	89.7	82.8	80.0	86.7	89.7	86.7	83.9	80.0	96.6	100
81 0552	Taiwan	83.9	83.9	83.9	83.9	82.8	80.0	82.8	85.7	80.0	81.3	86.7	80.0	77.4	83.9	86.7	83.9	81.3	77.4	93.3	96.6	100
91 3157	Taiwan	77.4	77.4	77.4	77.4	75.9	73.3	75.9	78.6	73.3	81.3	86.7	80.0	77.4	90.3	86.7	83.9	81.3	77.4	93.3	96.6	93.3	100
91 2441	South Africa	73.3	73.3	73.3	73.3	71.4	75.9	78.6	81.5	75.9	77.4	82.8	75.9	73.3	80.0	82.8	80.0	77.4	80.0	89.7	92.9	89.7	89.7	100
91 2337	Japan	77.4	77.4	77.4	77.4	75.9	73.3	75.9	78.6	73.3	81.3	86.7	80.0	77.4	83.9	86.7	83.9	81.3	77.4	86.7	89.7	86.7	86.7	82.8	100
81 0295	Hong Kong	80.0	80.0	80.0	80.0	78.6	75.9	78.6	81.5	75.9	83.9	89.7	82.8	80.0	86.7	89.7	86.7	83.9	80.0	89.7	92.9	89.7	89.7	85.7	96.6	100
91 1755	South Africa	77.4	77.4	77.4	77.4	75.9	73.3	75.9	78.6	73.3	75.0	80.0	80.0	83.9	77.4	80.0	77.4	75.0	71.0	86.7	89.7	86.7	86.7	82.8	80.0	82.8	100
WBG2007	Malaysia D	75.0	75.0	75.0	75.0	66.7	77.4	73.3	69.0	71.0	90.9	83.9	83.9	81.3	75.0	77.4	81.3	84.8	87.5	77.4	73.3	71.0	71.0	80.0	71.0	73.3	64.5	100
81 0532	Australia	81.3	81.3	81.3	81.3	73.3	83.9	86.7	82.8	77.4	84.8	77.4	83.9	81.3	75.0	77.4	81.3	84.8	87.5	77.4	80.0	83.9	77.4	80.0	77.4	80.0	71.0	87.5	100
81 1038	Australia	83.9	83.9	83.9	83.9	75.9	86.7	89.7	85.7	80.0	81.3	80.0	80.0	77.4	77.4	80.0	77.4	81.3	77.4	80.0	82.8	86.7	80.0	89.7	80.0	82.8	73.3	83.9	90.3	100
EMRSA-10	UK	73.3	73.3	73.3	73.3	71.4	75.9	78.6	81.5	75.9	77.4	75.9	82.8	80.0	73.3	75.9	80.0	77.4	86.7	75.9	78.6	75.9	75.9	85.7	75.9	78.6	75.9	80.0	86.7	82.8	100
91 1386	Singapore	86.7	86.7	86.7	86.7	78.6	82.6	85.7	81.5	82.8	77.4	75.9	82.8	80.0	80.0	82.8	80.0	83.9	80.0	75.9	78.6	75.9	75.9	85.7	75.9	78.6	75.9	80.0	80.0	89.7	85.7	100
81 1148	Taiwan	83.9	83.9	83.9	83.9	75.9	80.0	82.8	78.6	80.0	75.0	73.3	80.0	77.4	83.9	80.0	77.4	81.3	77.4	73.3	75.9	73.3	80.0	82.8	73.3	75.9	73.3	77.4	77.4	86.7	82.8	96.6	100
91 2240	Australia	83.9	83.9	83.9	83.9	75.9	80.0	75.9	71.4	80.0	75.0	73.3	80.0	77.4	77.4	80.0	77.4	81.3	77.4	66.7	69.0	66.7	66.7	75.9	66.7	69.0	66.7	77.4	71.0	80.0	75.9	89.7	86.7	100
EMRSA-9	UK	73.3	73.3	73.3	73.3	71.4	75.9	78.6	81.5	82.8	71.0	75.9	69.0	73.3	73.3	75.9	73.3	71.0	73.3	75.9	71.4	69.0	69.0	71.4	69.0	71.4	69.0	73.3	66.7	69.0	71.4	78.6	75.9	69.0	100
IRISH-1	UK	80.0	80.0	80.0	80.0	78.6	75.9	78.6	81.5	75.9	71.0	75.9	75.9	80.0	73.3	75.9	73.3	71.0	66.7	82.8	85.7	89.7	82.8	78.6	75.9	78.6	89.7	60.0	73.3	75.9	71.4	71.4	69.0	62.1	64.3	100
91 1497	PR China	81.3	81.3	81.3	81.3	73.3	83.9	86.7	82.8	77.4	72.7	71.0	77.4	81.3	68.8	71.0	68.8	72.7	62.5	77.4	80.0	83.9	77.4	73.3	71.0	73.3	83.9	62.5	75.0	77.4	66.7	73.3	71.0	64.5	66.7	93.3	100
IRISH-2	UK	83.9	83.9	83.9	83.9	75.9	80.0	82.8	78.6	73.3	75.0	73.3	80.0	83.9	77.4	73.3	71.0	75.0	64.5	73.3	75.9	80.0	80.0	69.0	73.3	75.9	80.0	64.5	77.4	80.0	69.0	75.9	80.0	66.7	62.1	89.7	90.3	100
EMRSA-12	UK	69.0	69.0	69.0	69.0	66.7	78.6	81.5	84.6	78.6	60.0	64.3	64.3	69.0	69.0	64.3	62.1	60.0	62.1	64.3	66.7	71.4	71.4	66.7	64.3	66.7	71.4	55.2	69.0	71.4	66.7	66.7	71.4	57.1	66.7	81.5	82.8	85.7	100
EMRSA-2	UK	75.0	75.0	75.0	75.0	73.3	77.4	80.0	82.8	77.4	72.7	71.0	77.4	81.3	68.8	71.0	75.0	72.7	68.8	71.0	73.3	77.4	71.0	66.7	71.0	73.3	77.4	62.5	75.0	71.0	73.3	66.7	64.5	58.1	66.7	86.7	87.5	83.9	82.8	100
EMRSA-6	UK	71.0	71.0	71.0	71.0	69.0	73.3	75.9	78.6	73.3	68.8	66.7	73.3	77.4	64.5	66.7	71.0	68.8	77.4	66.7	69.0	73.3	66.7	69.0	66.7	69.0	73.3	64.5	83.9	73.3	75.9	69.0	66.7	60.0	62.1	82.8	77.4	80.0	78.6	90.3	100
91 1703	Australia	77.4	77.4	77.4	77.4	75.9	73.3	75.9	78.6	73.3	81.3	80.0	80.0	83.9	77.4	80.0	83.9	81.3	77.4	80.0	82.8	86.7	80.0	75.9	80.0	82.8	80.0	71.0	83.9	80.0	75.9	69.0	66.7	60.0	62.1	89.7	83.9	86.7	78.6	90.3	86.7	100
EMRSA-13	UK	77.4	77.4	77.4	77.4	75.9	80.0	82.8	85.7	80.0	75.0	73.3	80.0	83.9	71.0	73.3	77.4	75.0	77.4	73.3	75.9	80.0	73.3	75.9	73.3	75.9	80.0	71.0	83.9	80.0	82.8	75.9	73.3	73.3	69.0	82.8	77.4	80.0	78.6	83.9	86.7	86.7	100
EMRSA-8	UK	71.0	71.0	71.0	71.0	69.0	80.0	82.8	85.7	80.0	68.8	73.3	66.7	71.0	71.0	73.3	71.0	68.8	71.0	73.3	75.9	80.0	73.3	75.9	80.0	82.8	73.3	64.5	77.4	80.0	69.0	69.0	66.7	60.0	75.9	82.8	77.4	80.0	78.6	77.4	80.0	86.7	80.0	100
COL	UK	73.3	73.3	73.3	73.3	71.4	75.9	78.6	81.5	75.9	71.0	75.9	75.9	80.0	73.3	75.9	73.3	71.0	66.7	75.9	78.6	75.9	75.9	71.4	82.8	85.7	82.8	60.0	66.7	69.0	71.4	71.4	69.0	62.1	71.4	85.7	86.7	82.8	81.5	86.7	75.9	82.8	75.9	82.8	100
NCTC10442	UK	71.0	71.0	71.0	71.0	69.0	73.3	75.9	78.6	73.3	68.8	73.3	73.3	77.4	71.0	73.3	71.0	68.8	64.5	73.3	75.9	73.3	73.3	69.0	80.0	82.8	80.0	58.1	64.5	66.7	69.0	69.0	66.7	60.0	69.0	82.8	83.9	80.0	78.6	83.9	73.3	80.0	73.3	80.0	96.6	100
91 2031	Hong Kong	72.7	72.7	72.7	72.7	71.0	75.0	71.0	73.3	75.0	76.5	75.0	81.3	84.8	72.7	75.0	78.8	76.5	72.7	68.8	71.0	68.8	68.8	64.5	75.0	77.4	75.0	66.7	66.7	62.5	71.0	64.5	62.5	62.5	64.5	77.4	78.8	75.0	73.3	84.8	75.0	81.3	75.0	75.0	90.3	93.8	100
EMRSA-5	UK	66.7	66.7	66.7	66.7	64.3	75.9	78.6	81.5	75.9	64.5	69.0	69.0	73.3	66.7	69.0	66.7	64.5	73.3	69.0	71.4	69.0	69.0	71.4	75.9	78.6	75.9	66.7	73.3	69.0	78.6	71.4	69.0	62.1	71.4	78.6	80.0	75.9	81.5	80.0	82.8	75.9	75.9	82.8	92.9	89.7	83.9	100
EMRSA-17	UK	64.5	64.5	64.5	64.5	62.1	73.3	75.9	78.6	73.3	62.5	66.7	66.7	71.0	71.0	66.7	64.5	62.5	71.0	66.7	69.0	66.7	73.3	69.0	73.3	75.9	73.3	64.5	71.0	66.7	75.9	69.0	73.3	60.0	69.0	75.9	77.4	80.0	85.7	77.4	80.0	73.3	73.3	80.0	89.7	86.7	81.3	96.8	100
81 0168	Hong Kong	58.1	58.1	58.1	58.1	55.2	66.7	69.0	74.1	66.7	56.3	60.0	60.0	64.5	58.1	60.0	58.1	56.3	58.1	66.7	69.0	66.7	66.7	75.9	66.7	62.1	73.3	58.1	58.1	66.7	69.0	69.0	66.7	66.7	62.1	75.9	77.4	66.7	71.4	71.0	66.7	66.7	66.7	66.7	75.9	80.0	75.0	75.9	73.3	100
81 0821	Hong Kong	71.0	71.0	71.0	71.0	69.0	80.0	82.8	85.7	80.0	62.5	66.7	66.7	71.0	64.5	66.7	64.5	62.5	64.5	73.3	75.9	80.0	73.3	82.8	66.7	69.0	80.0	64.5	71.0	80.0	75.9	75.9	73.3	66.7	69.0	89.7	90.3	80.0	85.7	83.9	80.0	80.0	80.0	80.0	82.8	80.0	75.0	82.6	80.0	86.7	100
EMRSA-3	UK	69.0	69.0	69.0	69.0	74.1	78.6	81.5	84.6	78.6	66.7	71.4	71.4	75.9	69.0	71.4	69.0	66.7	69.0	71.4	74.1	71.4	71.4	74.1	78.6	81.5	71.4	69.0	69.0	71.4	74.1	74.1	71.4	64.3	74.1	74.1	75.9	71.4	78.9	75.9	71.4	71.4	71.4	71.4	81.5	78.6	73.3	81.5	78.6	71.4	78.6	100
81 2760	Japan	58.1	58.1	58.1	58.1	55.2	60.0	62.1	64.3	60.0	68.8	66.7	66.7	71.0	64.5	66.7	71.0	68.8	71.0	66.7	69.0	66.7	66.7	69.0	73.3	75.9	73.3	71.0	71.0	66.7	69.0	62.1	60.0	53.3	62.1	69.0	64.5	66.7	64.3	71.0	73.3	80.0	73.3	80.0	75.9	73.3	75.0	75.9	73.3	60.0	66.7	78.6	100
91 2332	Japan	64.5	64.5	64.5	64.5	62.1	66.7	69.0	64.3	60.0	68.8	60.0	73.3	71.0	58.1	60.0	64.5	68.8	71.0	66.7	69.0	66.7	66.7	69.0	60.0	62.1	66.7	64.5	77.4	66.7	69.0	69.0	66.7	60.0	55.2	69.0	71.0	66.7	57.1	64.5	73.3	66.7	73.3	60.0	62.1	60.0	62.5	69.0	66.7	60.0	66.7	64.3	60.0	100
EMRSA-11	UK	66.7	66.7	66.7	66.7	71.4	69.0	64.3	66.7	68.0	77.4	75.9	75.9	80.0	66.7	69.0	73.3	71.0	73.3	69.0	64.3	62.1	62.1	57.1	62.1	64.3	62.1	80.0	66.7	55.2	71.4	57.1	55.2	55.2	71.4	64.3	66.7	62.1	59.3	73.3	69.0	69.0	62.1	62.1	71.4	69.0	77.4	71.4	69.0	55.2	62.1	74.1	62.1	69.0	100
EMRSA-16	UK	57.1	57.1	57.1	57.1	53.8	59.3	53.8	56.0	59.3	62.1	66.7	59.3	57.1	64.3	66.7	64.3	62.1	64.3	66.7	69.2	66.7	66.7	69.2	59.3	61.5	59.3	57.1	57.1	59.3	61.5	53.8	51.9	59.3	46.2	61.5	57.1	51.9	48.0	50.0	51.9	59.3	59.3	59.3	53.8	51.9	55.2	53.8	51.9	59.3	59.3	48.0	51.9	59.3	46.2	100
81 0127	Japan	46.7	46.7	46.7	46.7	50.0	48.3	50.0	51.9	48.3	58.1	55.2	55.2	60.0	53.3	55.2	60.0	58.1	60.0	62.1	64.3	62.1	62.1	64.3	55.2	57.1	69.0	53.3	60.0	55.2	57.1	50.0	48.3	48.3	42.9	64.3	60.0	55.2	51.9	60.0	62.1	69.0	62.1	62.1	57.1	62.1	64.5	57.1	55.2	69.0	62.1	66.7	75.9	62.1	50.0	61.5	100
EMRSA-15	UK	50.0	50.0	50.0	50.0	38.5	51.9	53.8	48.0	44.4	55.2	44.4	51.9	50.0	35.7	37.0	42.9	48.3	42.9	51.9	46.2	51.9	44.4	46.2	37.0	38.5	51.9	50.0	57.1	51.9	46.2	46.2	44.4	44.4	38.5	53.8	57.1	51.9	40.0	50.0	51.9	51.9	51.9	44.4	38.5	37.0	41.4	38.5	37.0	44.4	51.9	40.0	44.4	51.9	38.5	50.0	53.8	100
91 1827	Australia	50.0	50.0	50.0	50.0	38.5	51.9	53.8	48.0	44.4	55.2	44.4	51.9	50.0	35.7	37.0	42.9	48.3	42.9	51.9	46.2	51.9	44.4	46.2	37.0	38.5	51.9	50.0	57.1	51.9	46.2	46.2	44.4	44.4	38.5	53.8	57.1	51.9	40.0	50.0	51.9	51.9	51.9	44.4	38.5	37.0	41.4	38.5	37.0	44.4	51.9	40.0	44.4	51.9	38.5	50.0	53.8	100	100

Figure 9.6 The numerical matrix of the dendrogram of the Asian-Pacific, South African and United Kingdoms EMRSA MRSA isolates

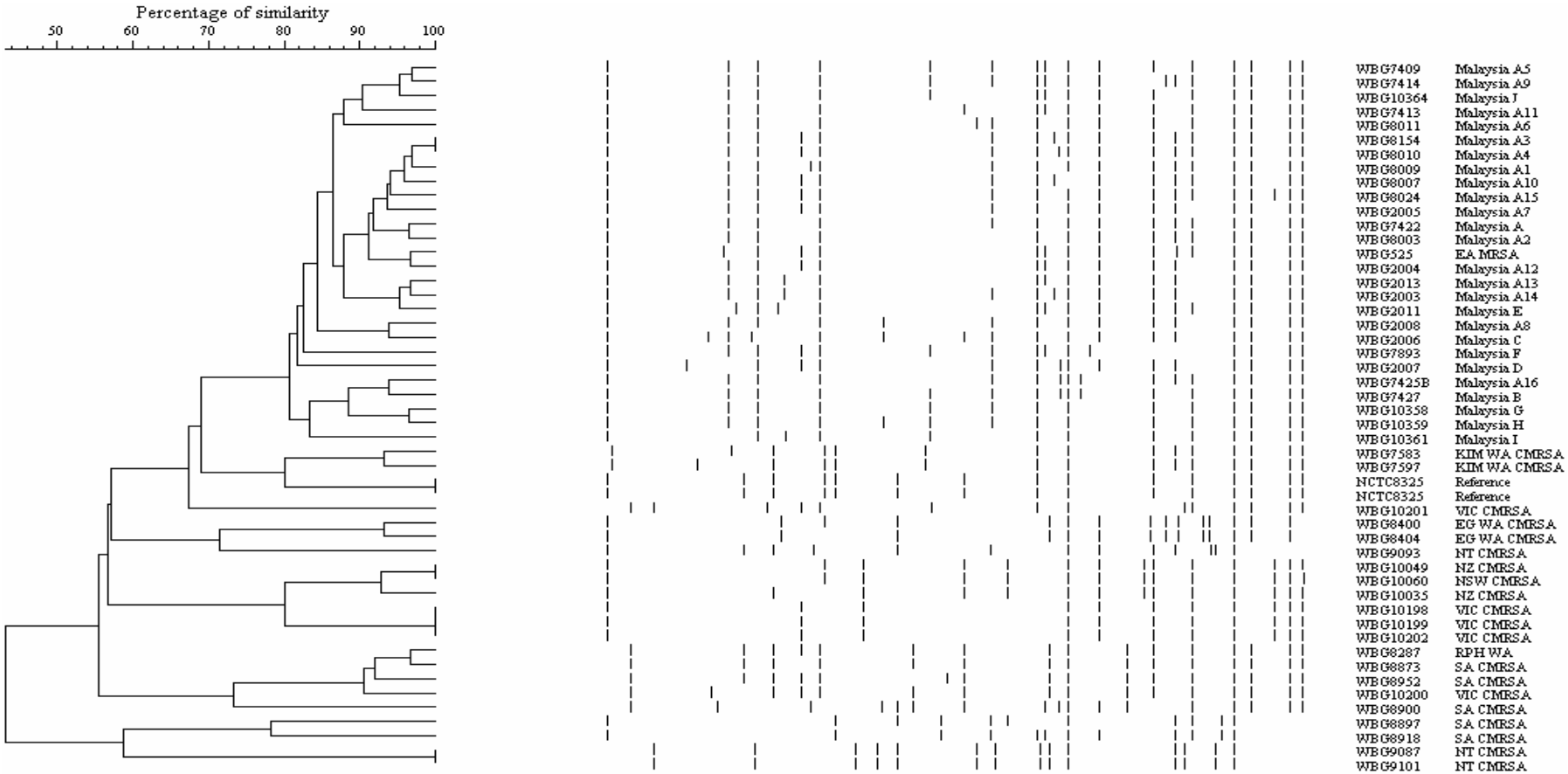

Figure 9.7 Dendrogram of Malaysian MRSA and community-acquired MRSA (CMRSA)

EG, Eastern Goldfields; KIM, Kimberley region; NSW, New South Wales; NT, Northern Territory; NZ, New Zealand; RPH, Royal Perth Hospital; SA, South Australia; VIC, Victoria; WA, Western Australia

9.6 Summary

All but one of the Malaysian MRSA CHEF patterns was found to be closely related to EMRSA-1, -4 and -7, and shared with them SCC*mec* allotype III and coagulase-gene type 24. Malaysian MRSA CHEF pattern I (WBG10361), was the only CHEF pattern that did not cluster closely with those of EMRSA-1, -4 and -7. Nevertheless, WBG10361 is still related to these EMRSAs as it has the same SCC*mec* allotype and coagulase-gene type. However, it was found to be more closely related to EMRSA-2, -3, -5, -8, -12, and -13, IRISH-1 and the COL. Except for EMRSA-3, these EMRSAs have similar genetic backgrounds and belong to the CC8 lineage.

The Malaysian isolate WBG7409 (Malaysian CHEF pattern A5) shares identical CHEF pattern with the Indonesian isolate INDO-2, Filipino isolate 91 2552 and Singaporean isolate 81 0090. The majority of the MRSA isolates from PR China, Taiwan, Hong Kong, Singapore, Indonesia, Australia and South Africa were found to be related to the Malaysian isolates and each other. Only one of the Japanese isolates, 91 2337, was found to be related to Malaysian and other Asian-Pacific MRSA isolates.

The majority of Asian-Pacific MRSA isolates that included Malaysian isolates were closely related to the EMRSA-1, -4, -7 and -10. One Hong Kong isolate was closely related to EMRSA-5, -7, COL and NCTC10442. The Japanese isolate 81 2760 was found to be related to EMRSA-3. The majority of Japanese isolates were not related to the EMRSA and other Asian-Pacific MRSA isolates. One of the Australian isolates, 91 1827, had the same CHEF pattern as EMRSA-15.

The Majority of the CHEF patterns of CMRSA from Australia are not related with Malaysian MRSA, except for WBG7583, WBG7597 and WBG10201. WBG7583 and WBG7597 have 80% and 69% overall CHEF similarity with NCTC8325 and Malaysian isolates, respectively. .

CHAPTER TEN

MULTILOCUS SEQUENCE TYPING (MLST)

10.1 Introduction

MLST typing is based on the DNA sequences of seven housekeeping genes. They are carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*). The differences in these genes are based on single-base point mutations in the genes. The allelic profile of a strain is assigned to a sequence type (ST), according to the Internet database. The ST is then assigned to a clonal complex (CC) with genetically related STs. SCC*mec* allotyping is included for MRSA to increase the resolution of typing. (Enright *et al.*, 2002, Enright *et al.*, 2000)

10.2 MLST results

Six MRSA isolates from Malaysia, Singapore and Indonesia were selected for MLST typing because of their close relationship based on pulsed-field gel electrophoresis typing.

The Malaysian isolates chosen were WBG2015B, WBG7422 and WBG8005 isolated in 1982, 1989 and 1994, respectively. They were isolated in the University Hospital of the University of Malaya (HUM) and have the predominant CHEF pattern A. The other Malaysian isolate chosen was WBG10365 which was isolated in 2000 at HUM and has CHEF pattern G that is closely related to CHEF pattern A. (See Chapter 4)

The Singaporean isolate WBG9032 has been reported to have a similar plasmid profile and the same CHEF pattern as Malaysian CHEF pattern A5. (Sim, 2003) The Indonesian strain INDO-2 was found to have a CHEF pattern either identical, or closely related, to Malaysian and Singaporean isolates. (See Chapter 9) All of the six isolates selected for MLST have the Allotype III SCC*mec*.

All of the DNA sequences were submitted to the *S. aureus* MLST database (http://saureus.mlst.net/) hosted at The Imperial College, London, UK. Numbers were assigned to each sequence according to the matching sequences in the database. Unknown sequences were given the sequence number of the closest. The results are listed in Table 10.1.

Table 10.1 MLST allelic profiles

Isolates	Origin	Year	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
WBG2015B	Malaysia	1982	2	3	1	1	4	4	3
WBG7422	Malaysia	1989	2	3	1	1	4	4	3
WBG8005	Malaysia	1994	2	3	1	1	4	4	novel*
WBG10365	Malaysia	2000	2	3	1	1	4	4	3
WBG9032	Singapore	2000	2	3	1	1	4	4	3
INDO-2	Indonesia	2000	2	3	1	1	4	4	3

*new: closest candidate sequence is *yqiL*30

All but one of the isolates typed has the allelic profile of 2-3-1-1-4-4-3 which is sequence type 239 (ST239). ST239 belongs to the largest MRSA clonal complex, CC8. Since all the ST239 isolates have SCC*mec* allotype III, their MLST type is ST239-MRSA-III. (Enright *et al.*, 2002, Robinson and Enright, 2003) WBG8005 has a different *yqiL* sequence but all the other 6-gene sequences are the same as the other isolates. Even though the ST type of WBG8005 could not be determined, it is a single locus variant of ST239 and would be assigned as a member of CC8.

WBG8005 is the only strain with a different MLST profile. Its *yqiL* gene has a unique mutation at nucleotide 411 that separates it from other *yqiL* sequences in the database. The closest *yqiL* sequence to WBG8005 is *yqiL* 30, which has one mutation different (i.e. one base). Compared to the *yqiL* 3 sequence of the other isolates, the *yqiL* of WBG8005 differs by two mutations (two bases). Compared to the primordial *yqiL* 1 sequence, WBG8005 *yqiL* differs by six mutations (six bases). (See Figure 10.1) The majority of the mutations found in the *yqiL* sequences are silent mutations. The only mutation found to cause an amino acid change is the mutation at nucleotide 268 and this is shared by WBG8005 *yqiL* and *yqiL* 30. This mutation has an adenine (A) replaced by a guanine (G), which results in the ATC, isoleucine codon, changing to GTC, a valine codon. The WBG8005 *yqiL* specific

mutation at nucleotide 411 (T → C) is a silent mutation that does not alter the amino acid sequence. (See Figure 10.2)

10.3 Summary

Three out of four Malaysian, a Singaporean and an Indonesian isolate have the ST239 sequence type. The Malaysian isolate WBG8005 has a novel sequence type that is different from the ST239 sequence type in the *yqiL* gene. The *yqiL* gene of WBG8005 is two bases different from the *yqiL* 3 sequence of the ST239 type sequence. The closest match with this novel *yqiL* sequence is the *yqiL* 30 sequence which is only a single base different.

The MLST results obtained correlated well with the CHEF typing results. It indicates that all the Malaysian isolates tested belong to the same clone, even though they were isolated over an 18-year period. . The Singaporean and Indonesian isolates have an identical MLST type to the Malaysian isolates, which confirms the CHEF typing results that indicated that Singaporean and Indonesian MRSA are closely related to Malaysian isolates. It also indicates that a pandemic clone is present in the hospitals of these three Southeast Asian countries.

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                        10        20        30        40        50
8005 yqiL       GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACA
yqiL 30         GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACA
yqiL 3          GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACA
yqiL 1          GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACA

                        60        70        80        90       100
8005 yqiL       TATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAGTTATCA
yqiL 30         TATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAGTTATCA
yqiL 3          TATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAGTTATCA
yqiL 1          TATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAGTTATCA

                       110       120       130       140       150
8005 yqiL       TCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCT
yqiL 30         TCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCT
yqiL 3          TCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCT
yqiL 1          TCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCT

                       160       170       180       190       200
8005 yqiL       GCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTACGGTGAATAA
yqiL 30         GCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTACGGTGAATAA
yqiL 3          GCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTACGGTGAATAA
yqiL 1          GCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTACAGTGAATAA

                       210       220       230       240       250
8005 yqiL       AGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTG
yqiL 30         AGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTG
yqiL 3          AGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTG
yqiL 1          AGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTG

                       260       270       280       290       300
8005 yqiL       TGACTGGTGAAAATGACGTCGTGCTAGCTGGCGGTATGGAGAATATGTCT
yqiL 30         TGACTGGTGAAAATGACGTCGTGCTAGCTGGCGGTATGGAGAATATGTCT
yqiL 3          TGACTGGTGAAAATGACATCGTGCTAGCTGGCGGTATGGAGAATATGTCT
yqiL 1          TGACTGGTGAAAATGACATCGTGCTAGCTGGCGGTATGGAGAATATGTCT

                       310       320       330       340       350
8005 yqiL       CAATCACCAATGCTTGTCAACAACAGTCGCTTTGGTTTTAAAATGGGACA
yqiL 30         CAATCACCAATGCTTGTCAACAACAGTCGCTTTGGTTTTAAAATGGGACA
yqiL 3          CAATCACCAATGCTTGTCAACAACAGTCGCTTTGGTTTTAAAATGGGACA
yqiL 1          CAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACA

                       360       370       380       390       400
8005 yqiL       TCAATCAATGGTTGATAGCATGGTATATGATGGTTTAACAGATGTATTTA
yqiL 30         TCAATCAATGGTTGATAGCATGGTATATGATGGTTTAACAGATGTATTTA
yqiL 3          TCAATCAATGGTTGATAGCATGGTATATGATGGTTTAACAGATGTATTTA
yqiL 1          TCAATCAATGGTTGATAGCATGGTATATGATGGTTTAACAGATGTATTTA

                       410       420       430       440       450
8005 yqiL       ATCAATATCACATGGGTATTACTGCTGAAAATTTAGTAGAGCAATATGGT
yqiL 30         ATCAATATCATATGGGTATTACTGCTGAAAATTTAGTAGAGCAATATGGT
yqiL 3          ATCAATATCATATGGGTATTACTGCTGAAAATTTAGTAGAGCAATATGGT
yqiL 1          ATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGT

                       460       470       480       490       500
8005 yqiL       ATTTCAAGAGAAGAACAAGATACATTTGCTGTAAACTCACAACAAAAAGC
yqiL 30         ATTTCAAGAGAAGAACAAGATACATTTGCTGTAAACTCACAACAAAAAGC
yqiL 3          ATTTCAAGAGAAGAACAAGATACATTTGCTGTAAACTCACAACAAAAAGC
yqiL 1          ATTTCAAGAGAAGAACAAGATACATTTGCTGTAAACTCACAACAAAAAGC

                       510
8005 yqiL       AGTACGTGCACAGCAA
yqiL 30         AGTACGTGCACAGCAA
yqiL 3          AGTACGTGCACAGCAA
yqiL 1          AGTACGTGCACAGCAA
```

Figure 10.1 Comparison of WBG8005 *yqiL* sequence with other *yqiL* sequences

The single-base differences in the sequences of WBG8005 *yqiL*, *yqiL* 1, *yqiL* 3 and *yqiL* 30 are highlighted in grey.

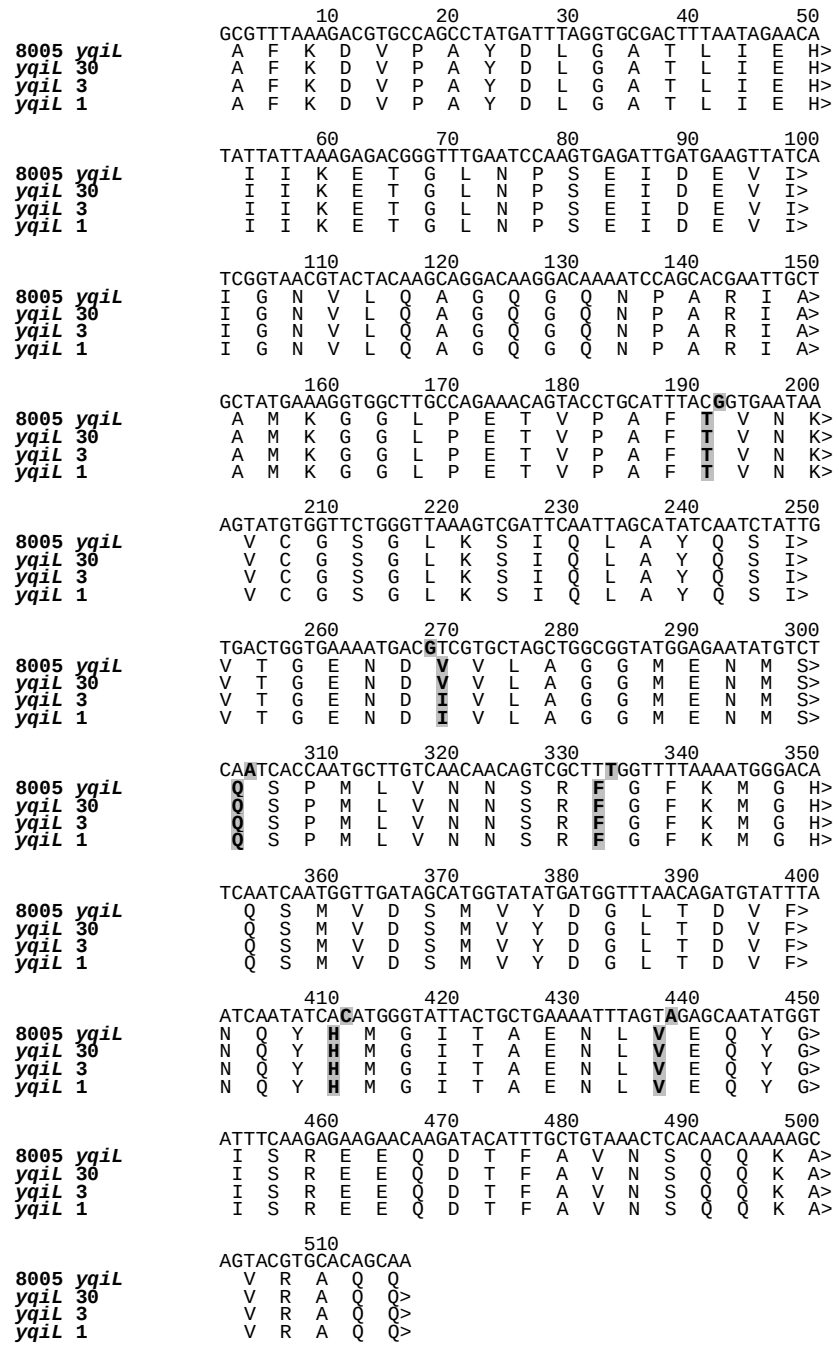


Figure 10.2 Comparison of WBG8005 *yqiL* amino acid sequences with other *yqiL* sequences

The single-base differences in the *yqiL* sequences of WBG8005 *yqiL*, *yqiL* 1, *yqiL* 3 and *yqiL* 30 and their corresponding amino acids are highlighted in grey.

Letters of the alphabet representing amino acids:
A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F. Phe; G, Gly; H, His; I, Iie, K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr, V, Val, W, Trp; Y, Tyr; Z, Glx

CHAPTER ELEVEN

DISCUSSION

11.1 Introduction

MRSA is one of the major nosocomial pathogens in Malaysian hospitals. (Hughes *et al.*, 2005) Although it is not known when MRSA was first isolated in Malaysia it was first reported in the early 1970s. (Lim and Zulkifli, 1987, Hanifah *et al.*, 1992) It has since become prevalent and endemic in many Malaysian hospitals. The prevalence rate of MRSA among *S. aureus* in Malaysian hospitals rose from 3% in 1971 to 32% in 1998. (Puthucheary *et al.*, 1972, Hanifah *et al.*, 1992, Lim, 1988, Norazah *et al.*, 2003b, Norazah *et al.*, 2001b) The larger tertiary referral hospitals are the most affected where the MRSA prevalence rate, in one case, was reported as 49.7%. (Rohani *et al.*, 2000) Similar rates are commonly observed in Asian hospitals. Over half of the *S. aureus* isolated in the hospitals of Japan, Korea, Taiwan, Hong Kong and Singapore were MRSA. (Christiansen *et al.*, 2004, Chong and Lee, 2000)

Over the past two decades, Malaysian hospitals have made significant progress in medicine. More complex and invasive procedures are now carried out in the hospitals and this results in more immunocompromised patients. (Lim, 2001) Under these conditions, the high prevalence MRSA has resulted in a high nosocomial infection rate and increased the cost of treatment. Furthermore, it has also caused increased treatment failure among the patients, which in term raised the rate of mortality and morbidity. (Hughes *et al.*, 2005, Lim, 2001) Infection control measures in Malaysia were first set up in the 1970s to respond to the increasing MRSA problem, but it was poorly coordinated and there was poor compliance. Even though it has been improved over the years, a 2001 report suggested that it still in its infancy and requires more funding and support. (Lim, 2001) Malaysia is a developing country with a limited health budget and the presence of drug-resistant pathogens, like MRSA, places a heavy burden on the health system. (Hughes *et al.*, 2005, Lim, 2001, Lee *et al.*, 1991)

This study has examined the MRSA isolates from the University Hospital of the University of Malaya (HUM) and the Hospital of the University Kebangsaan Malaysia (HUKM). They were isolated in 1982 from HUM, 1989 from HUM, 1994 from HUM and HUKM, and 2000 from HUM. These isolates were characterised by the molecular techniques of CHEF, plasmid profiling, coagulase-gene typing, SCC*mec* allotyping and multilocus sequence typing (MLST). They were also examined with the phenotypic methods of antimicrobial-resistance profiling and bacteriophage typing. To determine the genetic relationship of Malaysian isolates to the isolates from other countries, the isolates were compared with a collection of Asian-Pacific MRSA isolates, United Kingdom epidemic MRSA (UK EMRSA) strains and CMRSA from Australia. The unique plasmids, chromosomal elements and the *mec* regulatory region in these isolates were also studied.

11.2 Characterisation of Malaysian isolates

11.2.1 Resistance profiles of Malaysian isolates

Malaysian MRSAs were reported to be resistant to more than three different types of antibiotics. (Hanifah *et al.*, 1992, Rohani *et al.*, 2000, Norazah *et al.*, 2001a, Norazah *et al.*, 2003b) In this study, all of the MRSA isolates were resistant to at least five different types of antimicrobial agents. (See Chapter 4, Section 4.2) Over 90% of all isolates were resistant to gentamicin, kanamycin, streptomycin, erythromycin, lincomycin, spectinomycin, tetracycline and sulphonamides. Although only 17% of the 1982 isolates were resistance to trimethoprim, all the later strains isolated in 1989, 1994 and 2000 were resistant. The results obtained in this study are compared with the published results in Table 11.1.

Resistance to inorganic compounds was also very common in the isolates. Over 90% of the isolates were resistant to cadmium and inorganic and organic mercurial compounds. The percentage of isolates resistant to propamidine isethionate and ethidium bromide was 73% and 81% respectively. These compounds belong to the family of nucleic acid-binding (NAB) compounds. (McDonnell and Russell, 1999, Heir *et al.*, 1998)

Table 11.1 The MRSA resistance rate in the literature and in this study

	1987 to 1989[a]	1996[a]	1997 to 1999[a]	1982 HUM[b]	1989 HUM[b]	1994 HUM[b]	2000 HUM[b]	1994 HUKM[b]	1982 to 2000[b]
Gm	100%	98.1%	99%	83%	75%	100%	100%	94%	91%
Km	100%	n/a	n/a	83%	88%	100%	100%	94%	93%
Tet	87%	99.3%	99%	100%	100%	100%	100%	100%	100%
Sm	100%	n/a	n/a	92%	100%	100%	100%	94%	97%
Em	91%	98.7%	n/a	100%	100%	100%	100%	100%	100%
Clin[c]	n/a	<5%	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Lin[c,d]	n/a	n/a	n/a	100%	100%	100%	100%	100%	100%
Sp	n/a	n/a	n/a	67%	94%	100%	100%	94%	92%
Cip	n/a	72.9%	84.8%	0%	0%	30%	100%	0%	22%
Fus	n/a	3.2%	6.5%	0%	0%	15%	0%	0%	4%
Tm	n/a	n/a	n/a	17%	100%	100%	100%	100%	86%
Su	n/a	n/a	n/a	100%	100%	100%	100%	100%	100%
Sxt[e]	n/a	n/a	99%	n/a	n/a	n/a	n/a	n/a	n/a
Mup	n/a	8%	1.3%	0%	0%	0%	0%	0%	0%
Chl	62%	19%	25.5%	17%	69%	45%	40%	25%	41%
Rif	n/a	9%	8.5%	0%	0%	0%	0%	0%	0%

Abbreviations: Chl, chloramphenicol; Cip, ciprofloxacin; Clin, clindamycin; Em, erythromycin; Fus, fusidic acid; Gm, gentamicin; Km, kanamycin; Lin, lincomycin; Mup, mupirocin; Rif, rifampicin; Sm, streptomycin; Su, sulphonamides; Sp, spectinomycin; Sxt, co-trimoxazole; Tet, tetracycline; Tm, trimethoprim; Vm, vancomycin; n/a, not available; HUM, The University Hospital of the University of Malaya; HUKM, The Hospital of the University Kebangsaan Malaysia.

[a]References for the published data. The 1987 to 89 result from HUM. (Hanifah *et al.*, 1992) The 1996 survey of three hospitals (Rohani *et al.*, 2000) and the 1997 to 1999 survey of 10 hospitals (Norazah *et al.*, 2001a).

[b]This study

[c]Both Clin and Lin belong to the lincosamide family.

[d]Lin resistance in this study was inducible.

[e]Sxt is a mixture of Su and Tm.

The resistance profiles of isolates from different years were generally very similar. However, compared with MRSA isolated in later years, fewer of the isolates from 1982 were resistant to chloramphenicol, ethidium bromide, neomycin, propamidine isethionate, spectinomycin and trimethoprim. (See Chapter 4, Table 4.1)

The multiple resistance of isolates could be partly explained by the SCC*mec* they carry. Allotype III SCC*mec* was the only type of SCC*mec* detected in the Malaysian isolates in this study. It is the largest SCC*mec* identified so far and carries four genetic elements encoding for antimicrobial resistances. (Ito *et al.*, 2001) Transposon Tn*554* is located in this SCC*mec* and carries two different resistance determinants. One is the inducible *ermA* gene encoding resistance to macrolides (erythromycin), lincosamides (clindamycin and lincomycin) and streptogramin B (MLS_B). The other determinant is the *spc* gene, which confers resistance to spectinomycin. (Ito *et al.*, 2001, Phillips and Novick, 1979, Murphy, 1990, Woodford, 2005) Another element in Allotype III SCC*mec* is the integrated pT181 plasmid, which carries the tetracycline-resistance gene *tetK*. (Ito *et al.*, 2001, Mojumdar and Khan, 1988, Chopra and Roberts, 2001) Allotype III SCC*mec* also carries resistance to mercurial compounds and cadmium via the *mer* operon and *cadCA* operon-bearing ψTn*554*, respectively. (Ito *et al.*, 2001, Silver and Phung, 1996, Dubin *et al.*, 1992) The prevalence of Allotype III SCC*mec* among Malaysian MRSA might partly explain the high percentage of resistance to erythromycin (100%), lincomycin (inducible, 100%), spectinomycin (92%), tetracycline (100%), mercurial compounds (99%) and cadmium (93%).

A 1996 survey of *S. aureus* in three Malaysian hospitals revealed only 2.1% of the methicillin-susceptible *S. aureus* (MSSA) were resistant to clindamycin and 45.9% to erythromycin. Although 98.7% of the MRSA in the same survey were resistant to erythromycin, less than 5% were resistant to clindamycin. (Rohani *et al.*, 2000) This result is very different from the results obtained in this study, which found the same percentage of resistance for both erythromycin and lincomycin (100%). Resistance to lincomycin was inducible as determined by the D-zone test. The D-zone is the flattening of the clindamycin inhibition zone on the side adjacent to an erythromycin disk. This flattening of the inhibition zone for clindamycin is due to the erythromycin diffusing from the neighbouring erythromycin disk and inducing the

bacteria to resistance to clindamycin. (Zelazny *et al.*, 2005) Both lincomycin and clindamycin are members of the lincosamide family. (Leclercq, 2002) The authors of the 1996 survey had suggested that the clindamycin resistance of *S. aureus* in the survey was mainly due to the active efflux pump for macrolides, lincosamides and streptogramins (MLS). The *msrA* gene that encodes for the efflux pump is rare among nosocomial *S. aureus* isolates. In a 1995 study of 32 French hospitals only 2.1% of MLS-resistant *S. aureus* carried the *msrA* gene. The majority of the isolates carried either the *ermA* or *ermC* gene. (Lina *et al.*, 1999) A recent study in Dallas, USA, revealed that the *msrA* gene was more common in community-acquired MRSA than the *erm* genes. (Chavez-Bueno *et al.*, 2005) As the 1996 Malaysian survey was done on nosocomial *S. aureus* and MRSA and Allotype SCC*mec* III has been reported in the literatures, and was observed in this study, in Malaysian MRSA, it is unlikely that the erythromycin resistance reported was due to the MsrA efflux pump. (Ito *et al.*, 2001, Hiramatsu *et al.*, 2001) Recently a multicentre study was carried out on the determination of inducible clindamycin resistance using the CLSI (formerly NCCLS) disc diffusion method. Without an appropriate control, some of the participating laboratories failed to detect inducible clindamycin resistance. The causes of this failure include the failure to recognise the flattening of the clindamycin inhibition zone by laboratories without prior experience with the D-zone test, inadequate inoculum density on the plate and the erythromycin disc being placed too far from the clindamycin disc. (Zelazny *et al.*, 2005) The method used for resistance detection in the 1996 survey was the Kirby-Bauer disc diffusion method. It is possible that in the survey the researchers failed to detect the inducible clindamycin resistance due to the reasons found in the multicentre study.

Ciprofloxacin resistance is due to point mutations on its targets, which are topoisomerase IV, and DNA gyrase. The most common mutations have been found in the *grlA* and *gyrA* genes, which encode a subunit for topoisomerase, IV and DNA gyrase, respectively. (Takahashi *et al.*, 1998, Schmitz *et al.*, 1998b) Ciprofloxacin is a relative new antimicrobial agent. It was first introduced in the early 1980s. (Smith and Eng, 1985) However, resistance quickly appeared and became prevalent, especially amongst MRSA. The high prevalence of ciprofloxacin resistance has limited its usefulness in treating MRSA infections. (Shalit *et al.*, 1989) A similar situation has also been seen in Malaysian hospitals. In 1985 less than 5 % of *S.*

aureus were resistant to ciprofloxacin, but by 1996, 29.2% of the MSSA and 72.9% of MRSA were resistant. The percentage was even higher in the 1997 to 1999 survey with 84.8% of MRSA being resistant. (Lim, 1988, Rohani *et al.*, 2000, Norazah *et al.*, 2001a) This situation is reflected in the results observed in this study. The isolates from HUM isolated in 1982 and 1989 were susceptible to ciprofloxacin. Although all isolates from HKUM in 1994 were susceptible to ciprofloxacin, one third of the isolates from HUM in the same year were resistant. By the year 2000 all of the isolates from HUM were ciprofloxacin resistant.

Resistance to fusidic acid was only seen in the 1994 HUM isolates (15% were resistant) and none of the isolates were resistant to rifampicin. The fusidic acid and rifampicin target and inhibit the elongation factor G and the β subunit of the RNA polymerase, respectively. Similar to ciprofloxacin, resistance results from point mutations in the targets. In this study there was no resistance to rifampicin and no resistance to fusidic acid in isolates at HUM after 1994. In the literature isolates resistant to fusidic acid went from 3.2% in 1996 to 6.5% in 1997-1999 but then dropped to 4% in 2000. In the same isolates resistance to rifampicin went from 9% to 8.5% to 0%. (Lim, 1988, Rohani *et al.*, 2000, Norazah *et al.*, 2001a).

Mupirocin was first registered for use in Malaysia in 1998 and is not widely used. (Norazah *et al.*, 2001a, Rohani *et al.*, 2000) A high-level mupirocin-resistant MRSA was isolated from a burn's patient after prolonged mupirocin use soon after its introduction. (Norazah *et al.*, 2001a) However, the prevalence of mupirocin resistance amongst Malaysian MRSA has remained low at 1.3%. (Norazah *et al.*, 2001a) High-level resistance was not seen in the isolates in this study.

A recent study on antibiotic use in a Malaysian hospital revealed a high use of broad-spectrum antibiotics such as gentamicin, β-lactams with β-lactamase inhibitors and second and third generation cephalosporins. (Hughes *et al.*, 2005) Another study of antibiotic-prescribing patterns in six Malaysian hospitals showed a great variety of antibiotics were used for treatment and prophylaxis. Only 5% of the prophylactic regimens were less than 3 days duration. The lack of compliance to the guidelines for antibiotic use issued by the Ministry of Health was observed. (Lim *et al.*, 1993) These reports indicate that the inappropriate use, or over use, of antibiotics in

Malaysia was common. However, the Malaysian hospitals were not alone, similar misuse, especially in prophylaxis, was also observed in 14 Taiwanese hospitals. (McDonald *et al.*, 2001) Misuse creates selective pressure for multiply resistant pathogens such as MRSA. This might be one of the reasons why most of the isolates in this study have a similar resistance profile, even though they were isolated over a time span of 18 years. This misuse of antibiotics would also reduce the usefulness of antibiotics such as ciprofloxacin where resistance can emerge quickly through point mutations. Hence a strict policy may be required to preserve the effectiveness of antibiotics such as fusidic acid, rifampicin, vancomycin and mupirocin whose prevalence of resistance is still low. (Norazah *et al.*, 2001a, Lim, 2001)

In addition to the lack of compliance to antibiotic guidelines, bad practices involved in the use of antiseptics and disinfectants were common in Malaysian hospitals. (Keah *et al.*, 1995) This might explain the high percentage of NAB resistance in the Malaysian MRSA observed in this dissertation.

The data in this study has shown that Malaysian MRSA have remained resistant to the majority of antimicrobial agents over an 18 years period. This study has also shown that the MRSA were able to acquire resistance to newly introduced drugs such as ciprofloxacin, as well as less popular drugs like chloramphenicol. With the emergence of community-acquired MRSA and vancomycin-resistant MRSA there is an urgent need for the Malaysian authorities to improve infection control measures in their hospitals. This includes a better compliance to the guidelines and education programs for better awareness of infection control and antibiotic use. (Lim, 2001)

11.2.2 Bacteriophage profiles of Malaysian isolates

Bacteriophage typing used to be the gold standard for *S. aureus* typing. (Vickery *et al.*, 1986, Richardson *et al.*, 1999, Bannerman *et al.*, 1995) However, due to the reduced typability of many MRSA and the progress in molecular techniques, it has been gradually replaced by molecular typing methods such as CHEF, one of the PFGE techniques. (Tenover *et al.*, 1994, Murchan *et al.*, 2000, Bannerman *et al.*, 1995) Bacteriophage typing was one of the methods used in reference laboratories in England and Australia to identify EMRSA strains. (Kerr *et al.*, 1990, Cookson and

Phillips, 1988, Givney *et al.*, 1998, Richardson *et al.*, 1999, Vickery *et al.*, 1986) Currently it is still being used for the same purpose but is now used together with PFGE. (Murchan *et al.*, 2004, O'Neill *et al.*, 2001b, Aucken *et al.*, 2002)

In a 1991 study, only 2 out of 21 (9.5%) of Malaysian MRSAs were typable by the IBS set of phages. (Hanifah, 1991) Both of these isolates were only lysed by phage 85. A 1992 study revealed that 95.7% of Malaysian MRSAs were typable. They were only typed by group III phages; the majority only lysed by phages 85 and 83A. (Hanifah *et al.*, 1992) In this dissertation, 66% of MRSA were typable (i.e. lysed) by the IBS set of 26 phages. All but three of the 49 typable isolates were susceptible to group III phages and mostly by phages 85 and phage 83A. The supplementary phages 88 and 90 were found in 9 out of the 11 phage patterns detected among the typable isolates. The most common phage patterns were phage pattern A (85), B (85/90), C (85/88/90) and D (83A/85/88/90) and they accounted for 87.7% of the typable isolates. (See Chapter 4, Section 4.3) The results from this dissertation correlated well with the results from the two published studies as far as the types of phages that lysed the isolates and the similarity of their phage patterns. However, the percentage of typable isolates in this dissertation (66.2%) was greater that the 1991 study (9.5%) but not as high as the 1992 study (95.7%). This difference in typable percentages could be explained by the different years the isolates were collected. The results obtained in this dissertation are more comparable to the previous studies if the percentages for the years are compared instead of the overall percentages. Twenty-five percent of the 1982 and 62.5% of the 1989 HUM isolates were typable which is closer to the 1991 published figure of 9.5% and in 1994 94.4% of isolates from HUM and HUKM were typable, which is very close to the 1992 published figure of 95.7%.

The phage patterns obtained of the Malaysian MRSA were similar to those of EMRSA-1 and Australian isolates. EMRSA-1 is lysed by phage 85 and the experimental or supplementary phages, 88A/932. (Cookson and Phillips, 1988, Kerr *et al.*, 1990) The IBS phage pattern for the Australian isolates from 1976 to 1986 was 83A/85/95/88 and from 1987 to 1996 was 83A/85/88. (Givney *et al.*, 1998) These patterns are very similar to the four major Malaysian Patterns. This similarity in phage patterns indicates that many of Malaysian isolates are closely related to each

other and to both EMRSA-1 and Australian MRSA. The EmRSA-1 and Australian MRSA (Eastern Australian MRSA) have been reported in the literature to be closely related. (Townsend *et al.*, 1984a, Townsend *et al.*, 1987, Lim *et al.*, 2002)

The Malaysian isolates were more typable with the Australian set of nine MRSA phages and the International MRSA set of ten phages. The results obtained were similar to the IBS set of phages. (Personal communication, Mr Geoff Coombs) As the isolates were further typed by molecular methods with better discriminatory power, these results were not included in this dissertation.

11.2.3 Plasmid profiles of Malaysian isolates

The 1992 study showed that the Malaysian MRSA carried one to four small plasmids. The sizes of the plasmids were 1.6 kb, 2.5 kb, 3.0 kb and 3.2 kb, with the 3.0 kb plasmid found to be encoding chloramphenicol resistance. (Hanifah *et al.*, 1992) Other studies have shown that, in addition to these small plasmids, the Malaysian MRSA also carry plasmids ranging from 28 to 34 kb. (Grubb, 1990, Wei, 1993, O'Brien, 1994) Plasmids of this size are commonly found in the South- East Asian MRSA isolates and encode cadmium, mercury and NAB-compound resistance. (Grubb, 1990) The results in this dissertation have shown that the Malaysian MRSA can carry up to six plasmids and that these often include small plasmids from 1.5 kb to 4.4 kb, medium sized plasmids from 28 kb to 40 kb, and large open circular plasmids from 50 to 53 kb.

Although the plasmid profiles obtained in this study are different from the 1992 paper, they could be explained by differences in the techniques used in plasmid isolation. The CTAB method used in this dissertation give a better resolution for plasmid of all sizes as it does not totally lyse the cell and therefore reduces the contamination of chromosomal DNA. (Townsend *et al.*, 1985b) The technique used in the 1992 study totally lyses the cells and this results in heavy contamination of chromosomal DNA and loss of larger plasmids that got trapped with the chromosomal DNA. (Hanifah *et al.*, 1992) In addition, as the chromosomal DNA and the large and OC medium sized plasmids have similar migration distance in agarose gels, these plasmids can be masked by the large amounts of chromosomal

DNA and only the smaller plasmids will be observed in the gel. (Prof W. B. Grubb and Dr. F. G. O'Brien, personal communication)

The plasmid profiles obtained with the Malaysian isolates in this study were similar. Most of the 1982 isolates carried only one to two plasmids except WBG2015A and WBG2015B, which carried three plasmids of 2.6, 3.0 and 28 kb. The 1982 isolates had very similar plasmid profiles to WBG2015s with two additional plasmids of 1.5 and 53 kb OC. Some of the 1982 isolates carried a 31 kb instead of the 28 kb plasmid. The 1994 isolates from both HUM and HUKM have very similar plasmid profiles. Most of the 1994 isolates had plasmid profiles similar to the WBG2015s and 1989 isolates except, they had 30.5 and 35 kb plasmids in addition to the 28 kb in the WBG2015s and 1989 isolates. The 1994 isolates distinctively carried these three medium size plasmids. Although they also carried the three small plasmids found in the 1989 isolates they did not carry the 53 kb OC plasmid. The plasmid profile of the 2000 isolates also varied from that of the 1994 isolates. All 2000 isolates carried a 3.0 kb plasmid with or without a 4.4 kb small plasmid. The distinctive three medium-sized plasmids seen 1994 isolates were also observed in the 2000 isolates. (Table 11.2)

Table 11.2 Plasmid profiles of Malaysian MRSA from different years

Isolates	Plasmid sizes in kb						
1982 isolates (WBG2015s)		2.6	3.0	28			
1989 isolates	1.5	2.6	3.0	28			53 OC
1994 isolates	1.5	2.6	3.0	28	30.5	35	
2000 isolates			3.0	28	30.5	35	

Plasmids of the same size, detected in isolates from the same hospital, are likely to be the same plasmids. However, having identical sizes does not necessarily indicate they are identical. This study showed that the Malaysian isolates had similar plasmids over a period of 18 years (1982-2000). This suggests, together with the phage typing results, that the Malaysian isolates are closely related and could have descended from a common ancestor. However, as plasmids are mobile genetic elements, plasmids carried by the same strains are not necessarily related to each other. Nevertheless, the similarity of the isolates and their related plasmid profiles

indicates that the isolates have probably being under a similar selective pressure over these 18 years. This would suggest that these plasmids probably carry genetic determinants that have been beneficial to the strains over the years.

The other interesting observation was the persistence of the 3.0 and 28 kb plasmids. These two plasmids were detected in the isolates from 1982 to 2000 and they were the most common plasmids detected in all isolates. These two, particularly, may have determinants that have given the isolates a survival advantage under the selective pressure found in Malaysian hospitals. The characteristic of these plasmids will be discussed in more detail in the following sections.

11.2.4 CHEF, SCC*mec*, coagulase-gene and MLST profiles of Malaysian MRSA

The 26 CHEF patterns observed amongst the Malaysian MRSA were closely related. Their overall percentage of similarity was over 80%. CHEF pattern A was the predominant pattern detected in the 1982, 1989 and 1994 isolates. Thirteen CHEF patterns were closely related to CHEF A. Together with CHEF A, they account for the majority of the isolates (79.7%). CHEF pattern A5 detected in the 1982 and 1989 isolates was the only CHEF pattern, other than CHEF pattern A, detected in isolates from different years. The majority of the 1994 HUM isolates (14 out of 20 isolates) and HUKM isolates (14 out of 16 isolates) have CHEF pattern A, A1 and A2. This indicates that closely related strains have spread within hospitals and between hospitals and are therefore by definition epidemic MRSA. (Kerr *et al.*, 1990, Grubb and O'Brien, 2004)

The CHEF patterns of Malaysia isolates collected before 1994 were not found among the 2000 isolates. Four CHEF patterns were detected in the MRSA isolated in 2000, they are CHEF patterns G, H, J and I. Of these only CHEF pattern J clustered closely with CHEF pattern A and CHEF patterns closely related to it. CHEF pattern J was only one and two bands different (96.8% and 93.3% similarity) from CHEF pattern A5 and A11, respectively and four bands different (86.7% similarity) from CHEF pattern A. This indicates that CHEF pattern J was most likely derived from CHEF patterns A5 and A11. Patterns A5 and A11 were only detected in the 1982 and 1989 isolates, but not in the more recent 1994 isolates. The presence of CHEF J

in 2000 suggests that patterns A5 and A11 were present in 1994 but not in sufficient numbers to be detected in the small sample analysed, 16 from each of the two hospitals. The 2000 CHEF patterns G, H and I clustered closely with CHEF pattern B, which was only detected in 1989 isolates. Again it is possible that CHEF pattern B existed in 1994 but was not detected in the isolates analysed. It is interesting that isolates collected in 2000 were carrying CHEF patterns related to the CHEF patterns in the 1982 and 1989 isolates. The predominant CHEF patterns in 1994 were A1 and A2, together these CHEF patterns accounted for more than half of the isolates (20 out of 32). It is possible that the selection pressures in the hospitals up to and during the year 2000 had changed and favoured the isolates with the 1989 CHEF patterns (i.e. genetic backgrounds). Even with these differences, the CHEF patterns are still closely related as shown in the dendrogram (Figure 4.11). Overall, the Malaysian isolates are a related group of isolates that possibly could have descended from a common ancestor that had CHEF A, or patterns related to it.

Recently similar results have been published for Malaysian MRSA. In a survey of eight hospitals between 1997 and 1999 a predominant CHEF pattern and its subtypes were found to account for 212 of 264 MRSA isolates (80.3%). (Norazah *et al.*, 2001b, Norazah *et al.*, 2003b) Another study on HUKM isolates in 2002 also found two predominant CHEF patterns that accounted for over 80% of the isolates studied. (Alfizah *et al.*, 2002)

In a 1992 study the MRSA isolates collected from HUM between 1987 and 1989 were found to be similar to two MRSA clones from United Kingdom and Australia. (Hanifah *et al.*, 1992, Yoshida *et al.*, 1997) The isolates from 1987 tended to be related to NCTC10442, the first MRSA isolated, which was prevalent in the 1960s. The majority of isolates collected after 1987 was related to the British strain 86/560 which was related to EMRSA-1 and the EA MRSA. (Hanifah *et al.*, 1992, Yoshida *et al.*, 1997)

In this study, WBG525, an EA MRSA, was included in the dendrogram for comparison. (Townsend *et al.*, 1983b) The CHEF pattern of WBG525 was closely related to all the Malaysian MRSA CHEF patterns with an overall percentage similarity of more than 81%. The predominant Malaysian CHEF pattern A was

90.3% similar to the CHEF pattern of WBG525. This close relationship in their CHEF patterns correlated well with the bacteriophage typing results, which also indicated a close relationship.

The SCC*mec* allotyping showed that all the representative MRSA studied had Allotype III SCC*mec*, which harbours Class A *mec* and Type 3 *ccr* gene complexes. The EA MRSA, WBG525 also carries Allotype III SCC*mec*, but is different from the Malaysian isolates in that the Class A1 *mec* complex has a 166 bp deletion within its *mecR1* gene. All Malaysian strains have an *Xba*I restriction site, which is generated by a silent point mutation within the *mecA* gene. This mutation is commonly found in the UK EMRSA and EA MRSA. (See Chapter 7)

Four Malaysian isolates were chosen for MLST typing. Three of the isolates have the predominant CHEF pattern A and were isolated at different times, 1982, 1989 and 1994. Malaysian isolates from year 2000 have CHEF pattern G, which is closely related to CHEF pattern A. All but one of the isolates has the allelic profile of 2-3-1-1-4-4-3, which is sequence type 239 (ST239). The only exception is the 1994 isolate, WBG8005, which has an allelic profile different from ST239. It is only one locus different from ST239 and that is in the *yqiL* gene. The Malaysian MRSAs are therefore ST239-MRSA-III, which is identical to the EMRSA-1 and EA MRSA. (Enright *et al.*, 2002, Coombs *et al.*, 2004) ST239-MRSA-III belongs to clonal complex 8 (CC8), which is one of the largest nosocomial MRSA lineages. (Robinson and Enright, 2003) A recent study of nosocomial MRSA from 12 Asian countries, excluding Malaysia, have shown that ST239-MRSA-III was the most common nosocomial MRSA clone in these countries except Japan and Korea. (Ko *et al.*, 2005)

The results of bacteriophage typing, CHEF profiling, coagulase-gene typing, SCC*mec* and MLST all indicate that Malaysian MRSA isolates in this study belong to the same clonal population and are derived from same ancestry. These profiles also indicate the close relationship between Malaysian MRSA, EA MRSA and EMRSA-1. Both EA MRSA and EMRSA-1 were prevalent in the 1980s and have been found around the world, especially in hospitals of the countries that have close historical ties with the United Kingdom or were former colonies. (Yoshida *et al.*,

1997, Ko *et al.*, 2005) The clonal relationship of Malaysian isolates with the other UK EMRSAs and MRSAs from the Asian-Pacific region were further investigated and will be discussed in Section 11.8.

11.3 The characterisation of plasmids in Malaysian MRSA

Malaysian MRSA tended to carry more plasmids than MRSA from Australian hospitals. They could harbour up to six plasmids, whereas most of the Australian isolates carried only up to three plasmids. (Townsend *et al.*, 1983b, O'Brien *et al.*, 1999) The plasmids detected in *S. aureus* could be classified into four classes, mainly based in their sizes and characteristic. They are the Class I small rolling circle replicating plasmids, Class II multiresistance plasmids, Class III conjugative plasmids and Class IV plasmids. (Novick, 1990, Firth and Skurray, 2000)

11.3.1 Class I small rolling circle replicating plasmids in Malaysian MRSA

The majority of Malaysian MRSA carried up to three small plasmids of sizes 1.5, 2.6 and 3.0 kb. These plasmids were isolated individually and compared with plasmids from other isolates and each other.

The restriction enzymes *Alu*I and *Hin*fI were chosen to digest the three plasmids as they cut them all. The RFLP of these three plasmids showed no similarity (i.e. no shared bands). This indicates that these plasmids are not closely related to each other.

The 1.5 kb plasmid of the Malaysian isolate was found to have identical *Eco*RV and *Hin*fI restriction patterns to the 1.5 kb cryptic plasmid isolated in the Australian isolate WBG6083. (Wei, 1993) This indicates that the 1.5 kb plasmid in the Malaysian isolates is most likely cryptic. This finding further supports that the Malaysian isolates are related to the Australian isolates.

The 2.6 kb plasmid in the Malaysian isolates was different from the 1.5 kb and 3.0 kb plasmids. It is not known what phenotype is carried by this small plasmid. However, in an earlier study, a 2.6 kb cryptic plasmid was found in Malaysian MRSA, related isolates from China, Hong Kong and Singapore. (Wei, 1993) In

addition, the absence of the 2.6 kb plasmid did not alter any known phenotypes of the host. WBG7419 and WBG7420 have the same plasmid profiles, except WBG7419 does not carry the 2.6 kb plasmid. However, their CHEF and antimicrobial resistance profiles were identical. Therefore, it is most likely that the 2.6 kb plasmid is cryptic. Sequencing the whole plasmid could have revealed its phenotype. However, due to time and funding constraints of this project, sequencing was not carried out.

The 3.0 kb plasmids in the Malaysian isolates were found to be indistinguishable from the 3.0 kb plasmid in the Singaporean isolate WBG9018. This plasmid has been completely sequenced and was found to have 95% sequence similarity with plasmid pKH3, isolated in a Korean MRSA. (Accession number, AF151117) (Sim, 2003) The 3.0 kb plasmid carries the RepC replication protein and a putative protein with unknown function. These results indicate that it is a cryptic plasmid that undergoes asymmetric rolling-circle replication mediated by RepC. Interestingly, this 3.0 kb plasmid was found in Singaporean isolates collected over a time span of 16 years. (Sim, 2003) This is similar to the Malaysian situation, where this plasmid was detected in isolates collected over a period of 18 years. The long-term persistence of small cryptic plasmid was also reported in *S. aureus* isolated in UK. pOX1000, a small cryptic 1.29 kb plasmid, was detected in *S. aureus* isolated in 1969 and 1980. (Dyke and Curnock, 1989) These cryptic plasmids do not appear to carry any genes that are apparently beneficial to the host. However, they might have carried novel genes that were once beneficial to the host. Alternatively they could have served as a platform for the integration of mobile elements such as insertion sequence or transposons that are beneficial to the host. It is also a possibility that the long-term persistence of these small cryptic plasmids indicates that they are parasitic in nature.

Interestingly, the Malaysian MRSAs often have up to three small plasmids. This was especially common in the 1994 isolates. Plasmids with the same replication modes are incompatible to each other, as their replication systems tend to interfere each other. When two incompatible plasmids occur in the same host, the replication system treats these two different plasmids as a single plasmid. Although both will replicate normally, the final number of their replicates will be limited to the copy

number allowed for a single plasmid instead of two plasmids. As each plasmid will replicate in a random fashion, one plasmid will have more replicates than other. Eventually, the plasmid with the lower copy number will be lost during cell division. (Novick, 1987, Snyder and Champness, 1997) This would suggest that the three small plasmids that occur together in many Malaysian MRSA may have different replication systems.

Although some of the small plasmids found in Malaysian isolates were phenotypically cryptic, two small plasmids in Malaysian isolates were found to carry resistance genes. The 4.4 kb plasmid was only detected in the year 2000 isolates. The isolates with this plasmid only differed from the other isolates in their chloramphenicol resistance; otherwise they share the same resistogram, CHEF, SCC*mec*, and coagulase-gene types. Chloramphenicol resistance in *S. aureus* is exclusively plasmid borne and typically on small plasmids ranging from 2.9 to 5.1 kb. (Gillespie *et al.*, 1984, Lyon and Skurray, 1987) This indicates that this 4.4 kb plasmid is a chloramphenicol-resistance plasmid. Chloramphenicol is not commonly used for staphylococcal infections due to its side affect of causing aplastic anaemia. However, it is still used to treat bacterial meningitidis. (Black, 1993) Chloramphenicol is not a drug of choice for nosocomial MRSA infections in Malaysian hospitals, but it is used in treating cerebral abscesses caused by both Gram-negative and -positive bacteria. (Pit *et al.*, 1993, Hughes *et al.*, 2005) The 40% of Malaysian MRSA that were chloramphenicol resistant indicates that *S. aureus* maintains resistance genes even for the less commonly used antibiotics. This suggests that restricting antibiotic use alone may not control resistance in MRSA.

The 3.0 kb kanamycin-resistance plasmid detected in the 1989 isolates is the other small plasmid that encodes resistance. Its resistance was detected during conjugation and mixed-culture transfer experiments where it was mobilised by a large trimethoprim-resistance conjugative plasmid.

11.3.2 Class II multiresistance plasmids

The Class II multiresistance-staphylococcal plasmids can be divided into two families by the resistance genes they carry. They are the β-lactamase/heavy metal

(BHM) multiresistance plasmids and the pSK1 multiresistance plasmid family. (Firth and Skurray, 2000, Skurray and Firth, 1997) The BHM plasmids were common in isolates collected before the 1970s. This family of plasmids has largely been replaced by plasmids carrying resistance to nucleic acid-binding (NAB) compounds. (Townsend *et al.*, 1985d, Firth and Skurray, 2000, Skurray *et al.*, 1988) The NAB compounds include the quaternary ammonium compounds (QAC), diamidines and DNA intercalating agents. Some of the NAB compounds were commonly used as antiseptic and disinfectants, (McDonnell and Russell, 1999) These NAB resistance plasmids either belong to the pSK1 family or are a putative recombinant plasmid formed between BHM and NAB resistance (BHM/NAB) plasmids. (Firth and Skurray, 2000, Townsend *et al.*, 1985c) The pSK1 family of plasmids was commonly isolated in the EA MRSA and EMRSA-1 isolates. They typically carry *qacA*/NAB-1 resistance, the Tn*4001*/Tn*3851* aminoglycoside-resistance transposon, the *dfrA* trimethoprim-resistance transposon, Tn*4003*, or the β-lactamase transposon, Tn*4002*/Tn*3852*. (Firth and Skurray, 2000, Skurray *et al.*, 1988, Townsend *et al.*, 1987, Grubb and O'Brien, 2004) The BHM/NAB plasmids are commonly found in Asian MRSA and non-epidemic regional UK MRSA isolates. (Townsend *et al.*, 1985c, Grubb and O'Brien, 2004, Grubb, 1990, Sim, 2003, Wei, 1993) They carry *qacA*/NAB resistance gene like the plasmids of the pSK1 family. Similar to the BHM plasmids they carry resistance to mercurial compounds and cadmium with, or without, a β-lactamase. However, the arsenic-resistance (*ars*) operon, commonly found on the BHM plasmids has not found in the BHM/NAB plasmids. It is believed that the *ars* operon was replaced by the NAB-resistance gene during the recombination event. (Gillespie *et al.*, 1986, Lyon and Skurray, 1987, Townsend *et al.*, 1985c)

11.3.2.1 The phenotypes of Malaysian Class II multiresistance plasmids

The medium size Class II plasmids detected among the Malaysian MRSA range from 28 to 40 kb. The 28, 30.5 and 35 kb plasmids were the most common and were often detected together within the same isolate. These plasmids were transferred by mixed-culture transfer to a host with a known background. Their properties were studied and compared with other plasmids isolated from MRSA from different countries.

In general, the resistance profiles of these Malaysian plasmids were very similar to each other. The 28 kb plasmid is the commonest Class II multiresistance plasmid found among the Malaysian isolates. Similar to the 3.0 kb cryptic plasmids, it was detected in the isolates from 1982 to 2000, an 18 year time period. The 28 kb plasmid encodes resistance to NAB compounds, cadmium and mercurial compounds. Even though the 30.5 kb plasmid is larger, its resistance profile is identical to the 28 kb plasmid. Other than encoding a β-lactamase, the resistance profile of the 35 kb plasmid is almost identical to the 28 and 30.5 kb plasmids. Both the 30.5 and 35 kb plasmids were commonly detected together with the 28 kb plasmid in isolates collected in 1994 and 2000. The 31 kb plasmid was a less common plasmid and was only found in the 1989 and 1994 isolates. Although it is only marginally larger than the 30.5 kb plasmid, it encodes a β-lactamase and has a resistance profile identical to the 35 kb plasmid. The size difference between the 30.5 kb plasmid and 31 kb plasmid was determined by the MacVector program and confirmed with *Hin*dIII RFLP. (See Figure 5.8 and Table 5.4)

The resistance profiles of these plasmids are typical of the BHM/NAB plasmids. These plasmids are commonly found in Asian MRSA. (Grubb, 1990, Townsend *et al.*, 1985c, Wei, 1993)

All four plasmid types encode resistance to Pi, a diamidine, and EtBr, indicating they are carrying the *qacA*/NAB-1 resistance gene. The *qacA* gene is unique in its ability to confer resistance to both the monovalent cations like EtBr and divalent cations like Pi. (Paulsen *et al.*, 1996a, Mitchell *et al.*, 1999, McDonnell and Russell, 1999) Resistance to the QAC compounds was not tested for in this study. Although the *qacA* confers a two- to eight-fold increase in the MIC to QACs this is significantly below the concentration used in practice. (Townsend *et al.*, 1984b, Russell, 2000)

11.3.2.2 The structure of Malaysian Class II multiresistance plasmids

The typical structure of a Class II BHM/NAB plasmid, such as pSK57, consists of the replication gene, an IS*257/431* flanked *mer*, a mercurial-resistance operon (Tn*4004*), the *cadCA*, cadmium-resistance operon and a *qacA* or *qacB* NAB-resistance gene and a β-lactamase transposon. (Gillespie *et al.*, 1986, Lyon and

Skurray, 1987, Firth and Skurray, 2000) The BHM/NAB plasmids isolated from Asian MRSA have an identical phenotype to pSK57 except; some of the Asian plasmids do not carry a β-lactamase. The *Eco*RI RFLPs of the Asian plasmids were very similar to that of pSK57. (Grubb, 1990)

Structurally the BHM/NAB plasmids are very similar to the BHM plasmids. Both have the *mer* operon, the *cadCA* operon and the *repA* replication gene. These similarities were reflected in their restriction patterns. An *Eco*RI restriction pattern comparison of pSK57 (BHM/NAB) and pI524, of the α family BHM family, revealed that they share four common *Eco*RI restriction fragments of 6.3, 2.4, 2.1 and 1.0 kb which contain part of the *cadCA* operon, the *mer* operon and the conserved region downstream of the *mer* operon, (See Figure 11.1) (Shalita *et al.*, 1980, Gillespie *et al.*, 1986)

The major difference between pSK57 and pI254 was found in the variable region between the *repA* replication gene and the *mer-cadCA* operon of these plasmids. (See Figure 11.1) (Gillespie *et al.*, 1986) This variable region was found in both BHM and BHM/NAB plasmids. It often consists of a Tn*552* or Tn*552*-like transposon, and has either the *qacA*/NAB-resistance genes or the *ars* operon. (Shalita *et al.*, 1980, Gillespie *et al.*, 1986, Lyon and Skurray, 1987, Sidhu *et al.*, 2002, Anthonisen *et al.*, 2002) The variable region of both pSK57 and pI524 carries a Tn*552* or Tn*552*-like β-lactamase transposon, but their *bla* operons are in the opposite orientation. The invertible region of Tn*552* in pSK57 is truncated whereas the same region is intact in pI524. They also carry different elements adjacent to the Tn*552* in this variable region. The pSK57 has a *qacA* gene adjacent to Tn*552* while in the same region pI524 carries the *ars* operon. This variable region is reflected in the *Eco*RI restriction patterns which differ by two fragments. (Gillespie *et al.*, 1986, Lyon and Skurray, 1987) Among the α and γ family of BHM plasmids the major variation between these plasmids is generated by the variation in the invertible region of their Tn*552*. Similar to pSK57 and pI524, the variation in the BHM plasmids results in one to two fragment differences in their *Eco*RI restriction patterns. (Shalita *et al.*, 1980, Lyon and Skurray, 1987)

The four plasmids of 28, 30.5, 31 and 35 kb from Malaysian MRSA were isolated and analysed with *Eco*RI and *Hin*dIII RFLP. The *Eco*RI RFLPs of the 28, 30.5 and 35 kb Class II plasmids in the Malaysian isolates were compared. *Eco*RI was used as it has been widely used in studies of Class II staphylococcal plasmids. (Lyon and Skurray, 1987, Grubb, 1990). However, the *Eco*RI restriction patterns of the 30.5 and 35 kb plasmids were hard to interpret because some of the fragments have very similar sizes and migrate close together in the agarose gel. The *Hin*dIII restriction patterns of these plasmids were easy to interpret and showed distinctive differences between the plasmids. (See Figures 5.8, 5.9 and Table 5.4) The 28, 30.5 and 35 kb plasmids are commonly found in the same host whereas the 31kb plasmid is often found as the only BHM/NAB plasmid in the host. Its *Hin*dIII RFLP was compared with the *Hin*dIII RFLPs of the other three plasmids. (See Figure 5.8and Table 5.4)

The restriction patterns of these Malaysian Class II plasmids were similar and the majority of the restriction fragments were identical in sizes. Their *Eco*RI restriction patterns shared five common fragments out of six fragments generated. Their *Hin*dIII restriction patterns shared eight common fragments out of nine or ten fragments generated. These observations indicate that these plasmids are structurally similar and share a common conserved region. The structural similarity of these plasmids correlated well with their resistance profiles. All of the four Malaysian Class II plasmids shared the same resistance profiles of heavy metal ions and NAB compounds resistance. They only differed by the presence or absence of the β-lactamase determinant.

The 28, 30.5 and 35 kb plasmids shared five common *Eco*RI fragments of 10.2, 6.6, 2.7, 2.4 and 1.2 kb (See Tables 5.4 and 11.3). As all of these plasmids encode resistance to the heavy metal ions and NAB compounds, it is likely that these common fragments carry the genetic elements that confer resistance to these shared resistance phenotypes. In both BHM/NAB and BHM plasmids resistance to mercurial compounds and cadmium is almost exclusively conferred by the *mer* and *cadCA* operons, respectively. They are found adjacent to each other and form a conserved region with neighbouring sequences. (Shalita *et al.*, 1980, Skurray and Firth, 1997, Gillespie *et al.*, 1986, Lyon and Skurray, 1987) (See Figure 11.1)

The conserved *mer-cadCA* regions in pSK57 and pI524 are located on the four common *Eco*RI fragments of 6.3, 2.4, 2.1 and 1.1 kb (See Figure 11.1). (Gillespie *et al.*, 1986) The 6.3 kb fragment contains part of the *cadCA* operon and most of the *mer* operon. The smaller 2.1 kb fragment contains the rest of the *mer* operon. The 2.1 and 1.1 kb fragments are the conserved region found in BHM/NAB and some BHM plasmids. (Gillespie *et al.*, 1986, Lyon and Skurray, 1987) The four common *Eco*RI fragments (6.6, 2.7. 2.4 and 1.2kb) in the RFLPs of the 28, 30.5 and 35 kb Malaysian BHM/NAB plasmids are very similar to the common *Eco*RI fragments of pSK57 and pI524. Together with their identical phenotype this indicates that these Malaysian plasmids are closely related to the α and γ BHM plasmid families. (Gillespie *et al.*, 1986, Lyon and Skurray, 1987) This hypothesis could be further tested with comparison of Malaysian MRSA plasmids with pSK57 and pI524 plasmids. However, due to the time constraints and the difficulty of obtaining the necessary plasmids, the experiments were not conducted.

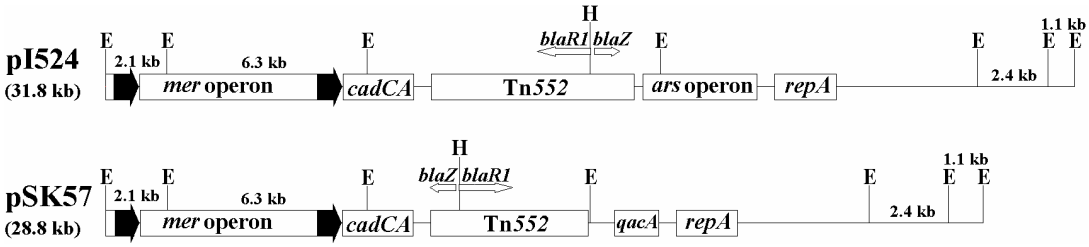


Figure 11.1 Schematic diagrams and restriction maps of pI524 and pSK57

The restriction sites are indicated by E (*Eco*RI) and H (*Hin*dIII). The genetic elements carried by these plasmids are represented by rectangular boxes. The *mer* operon confers resistance to mercurial compounds. The IS431/257s flanking the *mer* operon and their orientation are represented by black solid arrows. The *cadCA* is the cadmium-resistance operon. Tn*552* is the β-lactamase transposon. The open arrows at the *Hin*dIII restriction site show the orientation of the β-lactamase gene, *blaZ*, and its signal transducer gene, *blaR1*, within Tn*552*. The *ars* operon in pI524 confers resistance to arsenical compounds. The *qacA* gene in pSK57 encodes resistance to both monovalent and divalent NAB compounds. *repA* is the replication gene. This diagram is based on (Gillespie *et al.*, 1986) and (Lyon and Skurray, 1987).

The 6.6, 2.7, 2.4 and 1.2 kb common *Eco*RI fragments of the Malaysian plasmids are likely to carry the *mer-cadCA* region. The fragments are similar in size to the four common *Eco*RI fragments of pSK57 and pI524. The differences in the sizes of these

fragments compared to those in pSK57 and pI524 are probably due to variations in the electrophoresis and the methods of sizing the fragments. However, it is also possible that their genetic organizations are slightly different. The Malaysian plasmids also encode resistance to monovalent and divalent NAB compounds. This NAB-resistance phenotype is exclusively conferred by the *qacA* gene. (Paulsen *et al.*, 1996a, Putman *et al.*, 2000) As the *qacA* gene does not possess *Eco*RI restriction sites it is likely to be located on the 10.2 kb *Eco*RI fragment common to all Malaysian BHM/NAB plasmids. (Gillespie *et al.*, 1986, Lyon and Skurray, 1987, Rouch *et al.*, 1990) The *Hin*dIII restriction patterns of the 28, 30.5, 31 and 35 kb Malaysian plasmids showed the same relationship as revealed by the *Eco*RI patterns. They shared eight common *Hin*dIII restriction fragments of 4.6, 3.8, 3.0, 1.8, 1.3, 1.0, 0.8 and 0.7 kb. (See Tables 5.4, 11.3 and 11.4)

The 28 and 35 kb plasmids differ by 7 kb in size and β-lactamase production in phenotype. As mentioned before their RFLPs with *Eco*RI and *Hin*dIII restriction enzymes reveal they are structurally very similar and share most of the restriction fragments (See Tables 5.4 and 11.3). They have only two bands difference in their *Eco*RI RFLP and three bands difference in their *Hin*dIII RFLP. The different *Eco*RI bands are the 4.4 kb band of the 28 kb plasmid and the 11.5 kb of 35 kb plasmid while the different *Hin*dIII bands are the 9.2 kb band of the 28 kb plasmid and the 7.2 and 8.7 kb bands of the 35 kb plasmid. (See Tables 5.4, 11.3 and 11.4). The size difference between the *Eco*RI fragments of the 28 and 35 kb plasmids is 7.1 kb and between their *Hin*dIII fragments is 6.7 kb. These differences in size are within the 6.6 kb to 7.3 kb size range of Tn*552*, or Tn*552*-like, β-lactamase transposons. The difference in the number of *Eco*RI and *Hin*dIII bands is also consistent with Tn*552*-type transposons. The Tn*552* does not have an *Eco*RI restriction site, but it has a *Hin*dIII site at the 3' end of its *blaR1* gene. (Rowland and Dyke, 1989, Lyon and Skurray, 1987, Anthonisen *et al.*, 2002) These results suggest that the differences observed between the 28 and 35 kb plasmids are most likely due to Tn*552*, or a Tn*552*-like, transposon on the 35 kb plasmid.

The 30.5 kb plasmid is 2.5 kb larger than the 28 kb plasmid, but its phenotype is identical to the 28 kb plasmid and it does not express β-lactamase. Its RFLPs are

very similar to the 28 kb and 35 kb plasmids with only two to three bands difference (See Tables 5.4, 11.3 and 11.4). The 30.5 kb plasmid was often found together with the 28 kb and 35 kb plasmids in the same host. It is possible that the 30.5 kb plasmid is derived from the excision of Tn*552* from the 35 kb plasmid. However, the difference in size between the 30.5 and 35 kb plasmids is approximately 4.5 kb which is considerably smaller than the 6.6 kb to 7.3 kb size of Tn*552*, or Tn*552*-like, β-lactamase transposons (Rowland and Dyke, 1989, Anthonisen *et al.*, 2002, Skurray *et al.*, 1988). This difference between the sizes of the 30.5 and the 35 kb plasmids and a Tn*552*-like transposon could have resulted from the incomplete excision of a Tn*552*-like transposon from the 35 kb plasmid. It is also possible that there has been a deletion in a 35 kb plasmid to produce a 30.5 kb plasmid.

The 31 kb plasmid unlike the 28 kb, 30.5 kb and 35 kb plasmids, it is less common and often exists as the only BHM/NAB plasmid in the host. Its RFLP with *Hin*dIII restriction enzyme are very similar to other BHM/NAB plasmids with only one to two bands difference. (See Tables 5.4, 11.3 and 11.4) Although it is 4 kb smaller than the 35 kb plasmid, its phenotype is identical to it, including β-lactamase production. Interestingly the 30.5 kb is only slight smaller than the 31 kb plasmid but it does not expresses β-lactamase. It is possible that the 31 kb plasmid is derived from the 35 kb plasmid through a partial deletion of Tn*552* that does not affect the *blaZ* gene for β-lactamase. An incomplete Tn*552* with only the *blaZ* gene has been reported in other staphylococcal on plasmids and the chromosome. (Sidhu *et al.*, 2001, Sidhu *et al.*, 2002)

The four Malaysian BHM/NAB plasmids of 28 kb, 30.5 kb, 31 kb and 35 kb are closely related to each other. The differences in size and phenotype observed could be mainly due Tn*552*, or Tn*552*-like, transposons. The partial deletion or excision of Tn*552* might also have contributed to these differences. This hypothesis could be further tested with Tn*552*-specific PCR and DNA hybridisation. However, due to the time and funding restraints of this project, these experiments were not conducted.

Table 11.3 *Eco*RI restriction patterns of multiresistance plasmids from MRSA from Malaysia and other countries[a]

Country/plasmid	Size, kb	Resistance profiles[b]	*Eco*RI Restriction fragments, kb									
Malaysia[c]	28	Pi, EtBr, Cd, Hg, Pma		10.2				6.6	4.4	2.8	2.4	1.2
Singapore	26.9	Pi, EtBr, Cd, Hg, Pma		10.3				6.3	4.1	2.4	2.1	1.7
Shanghai	27.7	Pi, EtBr, Cd, Hg, Pma	11.1					6.3	4.1	2.4	2.1	1.7
Australia, pSK57	28.8	Pi. EtBr, Cd, Hg, Pma, Bla			9.4	7.6		6.3		2.4	2.1	1.0
pI524	31.8	Cd, Hg, Pma, Asa, Bla		10.3	9.7			6.3		2.4	2.1	1.0
Malaysia[c]	30.5	Pi, EtBr, Cd, Hg, Pma		10.2			6.6	6.6		2.8	2.4	1.2
Singapore	30.5	Pi, EtBr, Cd, Hg, Pma Bla		10.3		7.7		6.3		2.4	2.1	1.7
UK RMRSA[b]	30.5	Pi, EtBr, Cd, Hg, Pma Bla		10.3		7.7		6.3		2.4	2.4	1.7
Malaysia[c]	35	Pi, EtBr, Cd, Hg, Pma Bla	11.5	10.2				6.6		2.8	2.4	1.2
Singapore	34.5	Pi, EtBr, Cd, Hg, Pma Bla	11.1	10.3				6.3		2.4	2.1	1.7
UK RMRSA[b]	32.3	Pi, EtBr, Cd, Hg, Pma Bla		10.3	9.5			6.3		2.4	2.1	1.7
Hong Kong	23.6	Pi, EtBr, Cd, Hg, Pma Bla	11.1					6.3		2.4	2.1	1.7

[a]The results for plasmids from other countries were obtained from (Grubb, 1990, Wei, 1993, Gillespie *et al.*, 1986, Shalita *et al.*, 1980)

[b]Abbreviations: Asa, arsenate, Bla, β-lactamase production; Cd, cadmium acetate; EtBr, ethidium bromide; Hg, mercury chloride; Pi, propamidine isethionate; Pma, phenyl mercuric acetate; UK RMRSA, United Kingdom regional MRSA.

[c]The Malaysian plasmids are from mixed-culture transferrants of WBG8012 (28kb), WBG8003GS3 (30.5kb) and WBG8003GS2 (35kb)

Table 11.4 *Hin*dIII restriction patterns of multiresistance plasmids from MRSA from Malaysia and other countries

Countries	Size kb	Resistance profiles[a]	*Hin*dIII Restriction fragments, kb											
Malaysia	28	Pi, EtBr, Cd, Hg, Pma		9.2			4.6	3.8	3.0	1.8	1.3	1.0	0.8	0.7
Malaysia	30.5	Pi, EtBr, Cd, Hg, Pma	11.7				4.6	3.8	3.0	1.8	1.3	1.0	0.8	0.7
Malaysia	31	Pi, EtBr, Cd, Hg, Pma Bla	12.2				4.6	3.8	3.0	1.8	1.3	1.0	0.8	0.7
Malaysia	35	Pi, EtBr, Cd, Hg, Pma Bla			8.7	7.2	4.6	3.8	3.0	1.8	1.3	1.0	0.8	0.7
Australia/Singapore[b]	28	Pi, EtBr, Cd, Hg, Pma		9.2			4.6	3.8	3.0	1.8	1.3	1.0	0.8	0.7
Indonesia[c]	20	Pi, EtBr, Cd		9.2			4.6	3.8		1.8				0.7

[a]Abbreviations: Asa, arsenate; Bla, β-lactamase production; Cd, cadmium acetate; EtBr, ethidium bromide; Hg, mercury chloride; Pi, propamidine isethionate; Pma, phenyl mercuric acetate

[b]The Australian plasmid was isolated from a South Australian MRSA, WBG8888, and is indistinguishable from a 28 kb plasmid from a Singaporean MRSA. (Sim, 2003, Chong, 2003)

[c]The plasmid was isolated from an Indonesian MRSA, INDO-3

11.3.2.3 The stability of Malaysian Class II multiresistance plasmids

The Malaysian MRSA isolate, WBG8003 carries the 28 kb, 30.5 kb and 35 kb Class II BHM/NAB multiresistance plasmids and two small cryptic plasmids of 1.5 kb and 2.6 kb. This isolate was found to lose its Class II plasmids together with resistance to gentamicin, kanamycin and neomycin during prolonged storage and successive subculturing. This plasmid instability was also observed in other Malaysian isolates that carried multiple BHM/NAB plasmids. It would appear that these plasmids are not stable over time in some strains in the absence of any selective pressure.

The 28 kb, 30.5 kb and 35 kb plasmids are almost identical in structure and phenotype. It is likely that these plasmids carry the same type of replication gene. The most likely candidate is the *repA* gene which is the only *rep* gene found in Class II staphylococcal multiresistance plasmids. (Berg *et al.*, 1998, Firth *et al.*, 2000) It has been reported that plasmids with the same replication mechanism are not compatible in the same host (See Section 11.3.2.1). (Novick, 1987) The phenotypes of these plasmids are identical except the 35 kb plasmid expresses β-lactamase production. It would not be efficient for the cell to have more than one plasmid. The combination of these two factors might have contributed to the instability of these plasmids in the same host.

WBG8003GS1 is a variant of WBG8003 that has lost the 28 kb, 30.5 kb and 35 kb plasmids. All these plasmids encode resistance to cadmium and mercurial and NAB compounds, while the 35 kb has the additional phenotype of β-lactamase production. The loss of these three plasmids corresponds to the loss of cadmium and NAB resistance. Interestingly, despite losing the plasmids, WBG8003GS1 retains resistance to mercurial compounds and produces β-lactamase. This indicates that WBG8003 carries two copies of the genetic elements that confer resistance to mercurial compounds and β-lactamase production, one on the chromosome and one on a plasmid. WBG8003 has Allotype III SCC*mec*, which characteristically carries the *mer* and *cadCA* (ψTn*554*) operons. (Dubin *et al.*, 1992, Ito *et al.*, 2001) The retention of resistance to mercurial compounds following the loss of the plasmids is consistent with this. The loss of cadmium resistance with the loss of the plasmids

indicates that, either the SCC*mec* of WBG8003 does not have ψTn*554* or, it is non-functional. Genetic rearrangements of Tn*554* and ψTn*554* have been previously reported in SCC*mec* Allotype III, as well as the deletion of genetic components such as Tn*554* in other SCC*mec* allotypes. (Ito *et al.*, 2003a, Shore *et al.*, 2005) Transposon Tn*4002* is a Tn*552*-like β-lactamase transposon. Some MRSA isolates are known to carry Tn*4002* on both the chromosome and on a plasmid. Chromosomal β-lactamase production has been commonly detected in Australian MRSA isolates. (Lyon and Skurray, 1987, Skurray *et al.*, 1988) Despite not carrying any β-lactamase plasmids, many Malaysian MRSA isolates in this study are β-lactamase positive. This indicates that these Malaysian isolates, like the Australian isolates, carry Tn*552*, or Tn*552*-like, transposons on both chromosome and plasmids. (Gillespie *et al.*, 1988, Skurray *et al.*, 1988)

11.3.2.4 Selection pressure for Malaysian Class II multiresistance plasmids

In spite of their proposed incompatibility with each other, the 28 kb, 30.5 kb and 35 kb plasmids were commonly found together in Malaysian MRSA. In terms of biological cost, it is not efficient for the isolates to have plasmids with virtually the same phenotype. The determinants for mercurial-compound resistance and β-lactamase production are already present on the chromosome. The remaining plasmid-borne resistance phenotypes of these plasmids, cadmium and NAB resistance, are more likely to be the selective pressure for these strains maintaining their plasmids.

Cadmium is a naturally occurring and biologically toxic heavy metal that is found ubiquitously in the environment. It is also widely used in different industrial products. Cadmium resistance is widespread among different bacterial species, especially in highly contaminated areas. (Oger *et al.*, 2001, Oger *et al.*, 2003) The *cadCA* cadmium-resistance operon, commonly found in staphylococcal species, also confers resistance to lead and zinc ions. (Yoon and Silver, 1991, Rensing *et al.*, 1998) These heavy metals are not used in treating and controlling MRSA in hospitals. It is hard to see the evolutionary advantage of cadmium resistance in MRSA other than to cope with the presence of environmental cadmium.

Some of the NAB compounds have been widely used as antiseptics and disinfectants in both domestic and hospital settings, as well as in the food industry and in veterinary practices. (Heir *et al.*, 1998, McDonnell and Russell, 1999) The NAB-resistance profile encoded by the Malaysian Class II plasmids is consistent with them having the *qacA* gene. Although the *qacA* gene confers a high-level of resistance to the diamidine compounds, such as Pi, these compounds are not used very much clinically. (Emslie *et al.*, 1985, McDonnell and Russell, 1999) Although the quaternary ammonium compounds have been widely used, *qacA* only confers a two- to eight-folds increase in MIC, which is unlikely to provide much protection to these compounds. (Russell, 2000) Some *qacA* genes also encode low-level resistance to chlorhexidine, an effective disinfectant against *S. aureus*. (Emslie *et al.*, 1985, Cookson *et al.*, 1991) However, once again it is unlikely to confer protection to the organisms at the concentrations at which these compounds are used. (Russell, 2000, Emslie *et al.*, 1985, Cookson *et al.*, 1991) Nevertheless, the elevated MIC to NAB compounds may enhance the survival of these isolates with NAB-resistance plasmids.

The 1995 Malaysian study on disinfection practices in six Malaysian hospitals revealed a number of improper practices in the use of disinfectants. These improper practices included the use of diluted disinfectants, storing sterile items in disinfectants and using disinfectants as a substitute for sterilisation. (Keah *et al.*, 1995) These bad practices could create suitable conditions for the propagation of the NAB-resistance plasmids. Isolates with a NAB-resistance plasmid would more likely survive in lower disinfectant concentrations than those not carrying a NAB-resistance plasmid. The universal use of disinfectants would create a constant selective pressure for *S. aures* isolates with a NAB-resistance plasmid. This selection pressure would also facilitate the transfer and propagation of the NAB-resistance plasmid within the staphylococcal population. Although there is a need to establish the link between disinfectant usage and NAB-resistance plasmids, this discussion does give an insight into possible selective pressures for NAB-resistance. It would appear that resistance to NAB compounds is the most likely selective advantage for the Malaysian MRSA to maintain the BHM/NAB plasmids.

In theory a cell should only need one copy of the *qacA* gene for NAB resistance. It would be interesting to see if there is a gene dosage effect and if isolates with more than one plasmid have higher MICs than those with only one plasmid. This may give isolates with multiple copies of the plasmids a better survival rate. It may be that what exists in the cell is just one plasmid type and what we are seeing is an equilibrium of plasmids that are undergoing continual genetic events such as the acquisition or loss of transposons and insertion sequences. More experiments are needed to establish the relationship of these plasmids.

11.3.2.5 The relationship of Malaysian Class II multiresistance plasmids with those from other countries

Class II BHM/NAB plasmids have been reported in MRSA isolates from Australia, London, Dublin, Singapore and China. (Townsend *et al.*, 1985d, Gillespie *et al.*, 1986, Lyon and Skurray, 1987, Grubb, 1990) Structurally and phenotypically these plasmids are either identical or very similar to one another. (See Table 11.3) (Grubb, 1990)

The Malaysian Class II BHM/NAB plasmids have been found to have a similar structure and phenotype to each other and other BHM/NAB plasmids from different countries. (See Section 11.3.2,2) The 28 kb plasmids from Malaysian isolates and the South Australian MRSA, WBG8888, were found to have identical phenotype and *Hin*dIII RFLP. In a previous study, the 28kb plasmid from WBG8888 was found to be identical to a plasmid from a Singaporean MRSA isolate. (Chong, 2003) The 20kb plasmid from the Indonesian MRSA, INDO-3 was found to have related *Hin*dIII RFLPs and phenotype to these 28 kb plasmids. It shares *Hin*dIII fragments of 9.2, 4.6, 3.8, 1.8 and 0.7 kb and has the same resistance profile except that it does not encode resistance to mercurial compounds. These similarities suggest that the 20 kb is most likely derived from the 28 kb plasmid by the loss of a mercurial-resistance (*mer)* operon. The *mer* operon in the BHM/NAB plasmids is flanked by a pair of IS431/257 insertion sequences and had been described as a putative transposon, Tn*4004*. (Silver and Laddaga, 1990) The spontaneous lost of the *mer* operon was reported in a BHM plasmid of the α-family. (Shalita *et al.*, 1980) A BHM/NAB plasmid without mercury resistance has also been detected in a London MRSA in the

early 1980s. (Townsend *et al.*, 1985d) The transposition of Tn*4004* is yet to be directly demonstrated. However, the *mer* operon is likely to be mobile due to the close proximity of flanking IS*431/257*. (Silver and Laddaga, 1990, Skurray and Firth, 1997) The INDO-3 also has a chromosomal copy of the *mer* operon within its Allotype III SCC*mec*. As the clinical use of mercurial disinfectants has been discontinued since the 1970s it would appear for it to be advantageous for the cell, and the plasmid, to lose the *mer* operon in order to decrease the genetic burden.

The Malaysian Class II BHM/NAB plasmids are either identical or very similar to the BHM/NAB plasmids of MRSA isolates from England and many countries with close historical ties with England. A similar finding was also detected in a chromosomal profiling study of Malaysian MRSA isolates. (Yoshida *et al.*, 1997) The Australian MRSA is an exception, as these isolates often carry the NAB-resistance plasmid belonging to the pSK1 plasmid family. (Skurray *et al.*, 1988, Townsend *et al.*, 1985d, Firth and Skurray, 2000) As plasmids may be lost or acquired plasmids profiles cannot be use as a definitive tool to establish relatedness of isolates. Nevertheless, the fact that MRSAs from different countries carry identical, or similar plasmids, has shown a certain degree of relatedness between these isolates.

11.3.3 Class III conjugative plasmids in Malaysian MRSA

The trimethoprim-resistance plasmid, pWBG707, was isolated from Malaysian MRSAs isolated in 1989 and is able to mobilise smaller plasmids at a high frequency. This plasmid has always been isolated in open-circular form and migrates in an agarose gel equivalent to the position of a 53 kb CCC plasmid. pWBG707 was first isolated in the Malaysian MRSA WBG7410. It was transferred to a recipient strain by conjugation and the transconjugant was designated as WBG7483. (Udo *et al.*, 1992b)

pWBG707-like plasmids from both WBG7424 and WBG7483 have identical *Eco*RI restriction pattern of 23, 4.5, 2.8, 2.78 and 1.5 kb. This pattern is different from the published *Eco*RI pattern of pWBG707. The *Eco*RI pattern published for pWBG707 has an additional 3.6 kb fragment. (Udo *et al.*, 1992b) In another study of

Malaysian MRSA plasmids, a trimethoprim-resistance plasmid from WBG8000 was also found to have no 3.6 kb fragment in its *Eco*RI pattern. (O'Brien, 1994) It is possible that these plasmids have changed during storage.

High-level trimethoprim resistance in *S. aureus* is mediated by a trimethoprim insensitive dihydrofolate reductase (DHFR). The S1, S2 and S3 DHFR are the three drug-resistant DHFRs exclusively found in Gram-positive organisms. (Lyon and Skurray, 1987, Amyes and Towner, 1990, Amyes, 1998, Dale *et al.*, 1995b, Sekiguchi *et al.*, 2005) The S1, S2 and S3 DHFRs are encoded by the *dfrA*, *dfrD* and *dfrG* genes, respectively. (Lyon and Skurray, 1987, Dale *et al.*, 1995b, Sekiguchi *et al.*, 2005) Both *dfrA* and *dfrD* genes are mainly plasmids borne, whereas the *dfrG* gene has only found in the chromosome of Thai and Japanese nosocomial MRSA. (Firth and Skurray, 2000, Dale *et al.*, 1995b, Sekiguchi *et al.*, 2005)

pWBG707 did not hybridise with the *dfrA* DNA probes nor did it amplify with the specific primers for *dfrD*. These observations indicate that the trimethoprim resistance on pWBG707 is encoded by a novel gene(s). pWBG707 is conjugative and confers high-level trimethoprim resistance. Thus, the resistance encoded by pWBG707 is most probably a drug insensitive DHFR. The *dfr* genes of Gram-negative organisms were first detected ten years before the *dfrA* gene. The Gram-positive organisms appear to be unable to either acquire or, express the *dfr* genes of Gram-negative organisms. (Amyes, 1998) Hence, the *dfr* gene on pWBG707 is likely to have originated from a Gram-positive origin.

The novel S3 DHFR was identified in an ST239-MRSA-III clone from Thailand and Japan. (Sekiguchi *et al.*, 2005) This is the dominant MRSA clone in Asian hospitals including Malaysia. (Ko *et al.*, 2005) Thus, the *dfrG* gene is the most likely candidate for the *dfr* gene on pWBG707. Although the flanking DNA sequences of *dfrG* are identical to an enterococcal plasmid it was exclusively found on the chromosome and unable to be transferred by conjugation. (Sekiguchi *et al.*, 2005) If pWBG707 does have the *dfrG* gene, it may indicate that pWBG707 has an enterococcal origin. This plasmid was not found in trimethoprim-resistant Malaysian MRSA collected after 1989. Since the Malaysian isolates from 1982 to 2000 belong to a closely related clone, it is possible that the trimethoprim gene of pWBG707 has

integrated into the chromosome. pWBG707 was isolated from Malaysian MRSAs collected approximately 14 years earlier than the *dfrG*-bearing Thai and Japanese MRSAs. (Udo *et al.*, 1992b, Sekiguchi *et al.*, 2005) pWBG707, or a similar conjugative plasmid, may have transferred *dfrG* into *S. aureus* and it has subsequently integrated into the chromosome of these MRSAs. A *dfrG* gene specific PCR and DNA hybridisation would have confirmed that pWBG707 and trimethoprim-resistant isolates have the *dfrG* gene. However, due to the time constraints of the project, these experiments were not carried out.

11.4 Methicillin resistance in Malaysian MRSA

Methicillin resistance is conferred by PBP2a/2' which is encoded by the *mecA* gene. (Hartman and Tomasz, 1984, Georgopapadakou *et al.*, 1982, Song *et al.*, 1987) This gene is exclusively found within the SCC*mec* genomic island, and together with its regulatory genes, *mecR1* and *mecI*, forms the *mec* complex. (Ito *et al.*, 1999) The expression of methicillin resistance in many isolates is typically heterogeneous. According to the MIC values for the majority of the cell population, methicillin expression can be classified into three classes of heterogeneous resistance. (Hartman and Tomasz, 1986, Tomasz *et al.*, 1991, Pfeltz *et al.*, 2001) The level of methicillin resistance mainly depends on the genetic background of the host and does not correlate with the amount of PBP2a/2' produced. (Hiramatsu, 1995, Berger-Bächi and Rohrer, 2002, Katayama *et al.*, 2003c, Finan *et al.*, 2002, Niemeyer *et al.*, 1996) However, an abundance of PBP2a/2' is required for high-level methicillin resistance. The deactivation of the *mecR1-mecI* regulatory system is thought to be important for the host to achieve high-level methicillin resistance. (Hiramatsu, 1995, Kondo *et al.*, 2001, Finan *et al.*, 2002)

The Malaysian MRSA isolates in this study were found to carry the Class A *mec* complex. Unlike the prototype Class A complex with intact *mecR1* and *mecI* genes, the *mecI* gene in the Malaysian strain, WBG7422, is disabled by a single-base nonsense mutation. This point mutation located at nucleotide 202 results in the conversion of a glutamine codon into a stop codon. An identical mutation was observed within the *mecI* gene of the EA MRSA WBG525. Although they have the

same *mecI* mutation, the *mecR1* gene of WBG525 differs from WBG7422 in that it has a 166 bp deletion within its membrane-spanning domain.

It has been shown that the tight regulation of *mecA* transcription by intact *mecR1-mecI* is needed for the protection of the *mecA* gene after the initial SCC*mec* acquisition in hosts. (Katayama *et al.*, 2003c) Hosts with intact *mecR1-mecI* genes may appear as methicillin susceptible due to this tight regulation. (Hiramatsu, 1995) To achieve higher levels of methicillin resistance the host genes are thought to undergo further evolution for better PBP2a/2' tolerance. The deactivation of the *mecR1-mecI* system was also an important stage in the evolution of high-level methicillin resistance. (Katayama *et al.*, 2003c, Kondo *et al.*, 2001, Finan *et al.*, 2002) It is likely that the mutation of the *mecA* regulatory gene might have occurred after the host had achieved better PBP2a/2' tolerance. In WBG7422 and WBG525, their promoter/operator sequences and the sequences adjunct to the *mecI* gene are identical to the pre-MRSA, N315, which has intact *mecR1* and *mecI* genes. This indicates that the *mec* complexes in these three strains are derived from the same origin, even though they have different SCC*mec* allotypes. This observation suggests that the nonsense mutation within the *mecI* gene might have either occurred after the acquisition of SCC*mec* by *S. aureus*, or the acquisition of the *mec* complex by a SCC. Since WBG525 also has the same *mecI* mutation it is likely that the 166 bp deletion of the *mecR1* occurred after *mecI* mutation.

The 202 nucleotide nonsense mutation of *mecI* has been found to be the most common *mecI* mutation among MRSA isolates. It has typically been found among coagulase serotype IV MRSA isolates with closely related ribotypes. (Suzuki *et al.*, 1993, Hiramatsu, 1995) These MRSA isolates include the MRSA isolates from Malaysia, Hong Kong, Saudi Arabia, New Zealand, the UK and many European countries. This observation once again indicates that the Malaysian MRSA and the EA MRSA have descended from the same clonal population that has been found in countries with close historical ties with the UK. (Hiramatsu, 1995, Yoshida *et al.*, 1997)

blaR1 and *blaI* are the regulatory genes for the *blaZ* β-lactamase gene. They are structurally and functionally very similar to the *mecR1* and *mecI* genes. (Hiramatsu

et al., 1992, Hiramatsu, 1995) As they are similar to the *mecR1* and *mecI* genes, they can also regulate the *mecA* transcription and have been shown to stabilise SCC*mec* in the host. (McKinney *et al.*, 2001, Hiramatsu *et al.*, 1990, Katayama *et al.*, 2003c) Comparatively, the *blaR1-blaI* system is a more efficient regulator of *mecA* than the *mecR1-mecI* system. The *blaR1-blaI* system is significantly faster in signal transduction and weaker in transcriptional repression than the *mecR1-mecI* system. (McKinney *et al.*, 2001, Ryffel *et al.*, 1992, Hiramatsu *et al.*, 1990) β-lactamase production is found in almost 90% of nosocomial *S. aureus* including MRSA. (Livermore, 2000) The majority of nosocomial MRSA have been found to carry inactivated *mecR1-mecI* genes. (Ito *et al.*, 2001, Ito *et al.*, 2003a) It is believed that these isolates use their *blaR1* and *blaI* genes for the control of *mecA* transcription. (Hackbarth and Chambers, 1993, Hackbarth *et al.*, 1994, Rosato *et al.*, 2003b, Rosato *et al.*, 2003a)

In *S. aureus* the β-lactamase genes are on a transposon such as Tn*552*, and can integrate into plasmids or into the chromosome. WBG7422 and WBG525 do not carry plasmids with a β-lactamase, but they are both β-lactamase positive. (Townsend *et al.*, 1983b, Townsend *et al.*, 1984b) This finding indicates that the β-lactamase genes are located in their chromosomes. Many β-lactamase transposons have been found to be truncated by deletions. However, the areas involved are usually in the region of the transposase genes and the *bla* operon (i.e. *blaR1-blaI-blaZ*) usually remains intact. (Lyon and Skurray, 1987, Ito *et al.*, 2003a) It is likely that the *bla* operons in both these isolates are intact. Since their *mecR1-mecI* regulatory system had been deactivated, the intact *bla* regulatory genes might be used to regulate *mecA* transcription in these two strains. The *bla* regulatory system being a less stringent system would allow higher transcription of *mecA* (i.e. higher amounts of PBP2a/2') in the presence of β-lactams. (McKinney *et al.*, 2001) Both isolates belong to the same ST239 genetic background of CC8, which was found to be a suitable host for the *mecA* gene. (Enright *et al.*, 2002, Katayama *et al.*, 2003c, Katayama *et al.*, 2005) The production of higher amounts of PBP2a/2' would contribute to their high oxacillin MICs of 1024 μg/ml.

The oxacillin-resistant population profiles of WBG7422 and WBG525 are similar. Both belong to the heterogeneous-resistance expression Class III as the majority of the population have a high 64 μg/ml oxacillin MIC and a small population have a very high oxacillin MIC of 1024 μg/ml. The major difference between WBG7422 and WBG525 is their homogeneously resistant populations. WBG7422 has a homogeneously resistant population of 1.5 to 24 μg/ml of oxacillin, whereas the homogeneously resistant population in WBG525 is from 1.5 to 96 μg/ml of oxacillin. (See Section 6.4 and Figure 6.5) This means that WBG525 has more highly resistant cells in its population than WBG7422. In WBG7422 and WBG525, the tight control of *mecA* is disabled and replaced by less stringent *bla* regulatory genes that allow higher *mecA* transcription. The increased *mecA* transcription may contribute to their high level of methicillin resistance. However, as *mecA* shares the same regulatory system with the *mecR1* and *mecI* genes, increased *mecA* transcription can result in an increased *mecR1* and *mecI* transcription. (Garcia-Castellanos *et al*., 2004, McKinney *et al.*, 2001) An increased *mecR1* expression may affect cell growth, as the overexpression of *mecR1* is known to retard cell growth. (Rosato *et al.*, 2003b)

Unlike WBG7422 that has an intact *mecR1*, the *mecR1* gene in WBG525 is truncated by a 166 bp deletion. This dysfunctional *mecR1* gene of WBG525 might not retard cell growth and give the host a better survival advantage in the presence of β-lactams. The presence of an intact *mecR1* gene in WBG7422 might affect the expression of methicillin resistance and explain why its highly resistant population has a lower MIC than WBG525. This suggests that, even though the Malaysian MRSA, WBG7422, and EA MRSA, WBG525, are closely related, WBG525 may have evolved to optimise the expression of methicillin resistance.

Similar to WBG525, the COL MRSA strain has both the *mecR1* and *mecI* genes inactivated. In the case of COL, the *mecR1* and *mecI* genes are truncated and deleted by a partial copy of IS*1272*, respectively. Unlike the heterotypic WBG525, COL expresses homogeneous methicillin resistance, even though their genetic backgrounds are related and they both belong to CC8. (Coombs *et al.*, 2004, Enright *et al.*, 2002) This difference in methicillin expression may be because COL does not carry the *bla* operon. (Hackbarth and Chambers, 1993)

The oxacillin MIC of the majority cell population in WBG7422 and WBG525 is 64 μg/ml. This value is greater than the achievable serum concentration of oxacillin, which is *c.* 40 μg/ml. (Chambers, 1997, Reynolds, 1982) This observation suggests that both strains have achieved a sufficient level of resistance to β-lactams, including methicillin and oxacillin, to be untreatable. It may also indicate that clinically there is no additional selection pressure for these strains to become homogeneously methicillin resistant.

11.5 Aminoglycoside resistance in Malaysian MRSA

The Majority of Malaysian MRSAs in this study were resistant to aminoglycosides such as gentamicin, kanamycin, neomycin, streptomycin and spectinomycin. The gentamicin resistance in some isolates was found to be unstable. Four Malaysian isolates lost their gentamicin resistance after long-term storage and consecutive subculturing. These four isolates were collected from different time periods, and only two isolates shared the same CHEF pattern. They are the 1982 isolate WBG2015, 1989 isolates, WBG7410 and WBG7425, and the 1994 isolate WBG8003.

A comparison of the CHEF patterns between gentamicin-resistant and -susceptible variants of these isolates revealed a two-band difference. A larger, *c.* 192 kb *Sma*I, band was observed exclusively in all gentamicin-resistant variants regardless of their CHEF patterns. Similarly, a smaller *c.* 78 kb *Sma*I band was only found in the gentamicin-susceptible variants. Hence, this *c.* 78 kb *Sma*I band observed in the gentamicin-susceptible variants was generated by a *c.* 114 kb chromosomal DNA deletion within the larger *c.* 192 kb *Sma*I band of the resistant variants. The gentamicin-susceptible variants with the *c.* 78 kb *Sma*I band did not hybridise with the *aacA-aphD* probe. On the other hand, the gentamicin-resistant variants hybridised with the probe. This observation clearly suggests that the *c.* 114 kb chromosomal deletion carries the *aacA-aphD* bifunctional aminoglycoside-resistance gene.

The *aacA-aphD* gene, also known as the *aac*(*6*')-*aph*(*2"*) gene, encodes the bifunctional aminoglycoside-modifying enzyme which confers resistance to aminoglycosides including gentamicin, kanamycin and tobramycin. (Schmitz *et al.*, 1999, Ida *et al.*, 2001) In staphylococci, the *aacA-aphD* gene is exclusively carried by Tn*4001* or Tn*4001*-like transposons and the transposons have been reported on plasmids like the pSK1 multiresistance and pSK41 conjugative plasmid families. (Lyon *et al.*, 1987a, Byrne *et al.*, 1990, Firth and Skurray, 2000) However, among the British MRSA isolates collected before the 1980s, the Tn*4001* or Tn*4001*-like transposons were often located on the chromosome. (Wright *et al.*, 1998) The presence of chromosomal *aacA-aphD* gene alone, does not necessarily mean Malaysian MRSA are related to British and Australian MRSA isolated before the 1980s. However, Malaysian MRSA isolates in this study have been shown to have related phage types, CHEF patterns, and identical SCC*mec* allotype and MLST type to UK and Australian MRSA such as EA MRSA and EMRSA-1, isolated in the early 1980s. (See Chapters 4, 7 and 9) This, together with the finding of a chromosomal *aacA-aphD* gene and BHM/NAB plasmids in the Malaysian isolates, further suggests that the Malaysian MRSA are probably related to, or derived from, the British and Australian MRSA collected before the 1980s. This hypothesis could be further tested by comparing early British and Australian isolates with Malaysian MRSA isolates using molecular typing techniques. However, due to the time constraints of this project and the difficulty of obtaining these early isolates, these experiments were not conducted.

Together with their gentamicin resistance isolates WBG2015 and WBG8003 also lost kanamycin and neomycin resistance. As the *aacA-aphD* gene confers both gentamicin and kanamycin resistance, the loss of this gene would naturally result in the loss of resistance to these two aminoglycosides. However, the bifunctional gene does not confer resistance to neomycin. (Lyon and Skurray, 1987, Mingeot-Leclercq *et al.*, 1999) This observation indicates that an additional aminoglycoside-resistance gene(s) was also deleted together with the *aacA-aphD* on the *c*, 114 kb chromosomal DNA deletion.

Unlike WBG2015 and WBG8003, the other two isolates, WBG7410 and WBG7425, only lost their gentamicin resistance but retained their kanamycin and neomycin resistance. Isolate WBG7410 carries a small 3.0 kb kanamycin-resistance plasmid.

(Udo *et al.*, 1992b) Although WBG7410 and WBG7425 have different CHEF patterns they have identical plasmid profiles and both were collected from the same hospital in same time period. In this study the 3.0 kb plasmid from WBG7424 was also found to expresses kanamycin resistance (See Section 5.5 and 11.3.2.1). It also had a significantly reduced susceptibility to neomycin. The resistance profile of this small plasmid was determined by the disc diffusion technique. Its neomycin inhibition zone was 14 mm in diameter which is significantly smaller than the 20 mm zone observed in susceptible strains, and close to the 8 to 12 mm zone for intermediate resistance according to NCCL criteria. These observations suggest that the 3.0 kb kanamycin-resistance plasmid might also confer neomycin resistance. The neomycin-susceptible phenotype observed for this plasmid is probably due to the poor expression of the resistance gene. The presence of this 3.0 kb plasmid could explain the retention of the kanamycin and neomycin resistance observed in the gentamicin-susceptible variants of WBG7410 and WBG7425.

The *aphA-3* and *aadD* genes are also known as the *aph(3')-III* and *ant(4')-I* genes, respectively. Both *aphA-3* and *aadD* confer kanamycin and neomycin resistance, whereas the *aacA-aphD* gene confers resistance to kanamycin and gentamicin. (Lyon and Skurray, 1987, Mingeot-Leclercq *et al.*, 1999, Woodford, 2005) The *aphA*-3, *aadD* and *aacA-aphD* genes are the most common aminoglycoside-resistance genes found in *S. aureus.* Many *S. aureus* isolates have been found to carry two to three of these aminoglycoside-resistance genes. (Schmitz *et al.*, 1999, Ida *et al.*, 2001) Hence, the *aphA-3* and *aadD* genes are the most likely candidates for the kanamycin and neomycin resistance found in these four Malaysian isolates.

Isolates WBG7410 and WBG7425 could carry three copies of the aminoglycoside-resistance genes. One copy of a kanamycin and neomycin resistance gene on the 3.0 kb plasmid, and one copy of the *aacA-aphD* gene as well as a copy of a kanamycin-neomycin resistance gene on the *c.* 114 kb chromosomal DNA which is deleted. As isolates WBG2015 and WBG8003 do not carry the 3.0 kb plasmid they must have the *aacA-aphD* gene and a kanamycin-neomycin resistance gene on the *c.* 114 kb chromosomal DNA which can be deleted.

The loss of the chromosomal *aacA-aphD* gene has been reported in MRSA isolated from French and Greek hospitals. (Lelièvre *et al.*, 1999, Polyzou *et al.*, 2001) The CHEF patterns of some gentamicin-resistant and -susceptible variants of these isolates differed by one to two *Sma*I bands. However, unlike the Malaysian isolates, the fragments involved were larger than *c.* 361 kb. (Blanc *et al.*, 2001a, Polyzou *et al.*, 2001) Many of the gentamicin-susceptible variants of these isolates also retained their kanamycin and neomycin resistance encoded by the *aadD* gene. (Lelièvre *et al.*, 1999) These gentamicin-susceptible MRSA isolates were found to be gradually replacing the gentamicin-resistant isolates and were becoming the predominate clones in these French and Greek hospitals. (Lelièvre *et al.*, 1999, Laurent *et al.*, 2001, Pournaras *et al.*, 2001) The growth rate of these gentamicin-susceptible isolates was found to be significantly faster than the gentamicin-resistant isolates. (Laurent *et al.*, 2001) Although the reasons for the dominance of the gentamicin-susceptible MRSA were not determined, the marked reduction in the use of gentamicin in French hospitals could have been a contributing factor. (Lemaître *et al.*, 1998, Lelièvre *et al.*, 1999, Laurent *et al.*, 2001) Gentamicin has been reported to be the most common aminoglycoside prescribed in Malaysian hospitals. (Hughes *et al.*, 2005) The instability of chromosomal gentamicin resistance in Malaysian MRSA observed in the present study, is similar to those reported in French and Greek hospitals. However, there may not have been a sufficient reduction in gentamicin usage in Malaysian hospitals to enable gentamicin-susceptible MRSA to become the predominant clone as was reported for French and Greek hospitals. (Lelièvre *et al.*, 1999, Laurent *et al.*, 2001, Pournaras *et al.*, 2001)

11.6 The genetic relationship between EMRSA strains

The epidemic MRSA are those that spread to two or more patients in two or more hospitals. (Kerr *et al.*, 1990, Livermore, 2000) Seventeen EMRSA strains have been identified in UK hospitals since 1986 by bacteriophage typing. (Kerr *et al.*, 1990, Ayliffe *et al.*, 1998, Aucken *et al.*, 2002) In this study, the genetic relationship between UK EMRSA strains, EA MRSA and classical MRSA was investigated. Their genetic relationships were examined using CHEF, SCC*mec* allotyping and coagulase-gene typing. The genotypes generated by these techniques were combined as the combined genotype (CG). Ten distinctive combined genotypes were identified

among the 38 organisms. (See Section 7.4 and Figure 11.2) During the time span of this project, the UK EMRSAs were typed by MLST by other researchers and the results have been published. These results are also included in this discussion. (See Figure 11.2) (Robinson and Enright, 2003, Cassat *et al.*, 2005)

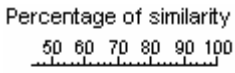
11.6.1 CG1, CG4, CG6 and CG9 genotypes.

Four CGs, CG1, 4, 6 and 9 were found to be related and their CHEF patterns have an overall similarity of 80%. All of the isolates with these CGs have the coagulase-gene type 18. They only differ by their SCC*mec* allotypes. The CG1 strains have the SCC*mec* allotype I. These CG1 strains include the classical MRSA, COL, WBG1350 and NCTC10442 (first MRSA), and EMRSA-5, -8 and -17. The CG4 strain, IRISH-1, has Allotype II SCC*mec* with a truncated *mecI* gene. The CG6 group consists of EMRSA-2 and -6 and they have Allotype IV SCC*mec*. The CG9 group consists of IRISH-2, EMRSA-12 and -13 and they have Allotype IVp. (See Section 7.4 and Figure 11.2) This indicates that these EMRSA strains might have shared the same ancestor with the Archaic MRSA clone that includes the first MRSA, NCTC10442, and they have acquired different SCC*mec*s during their evolution.

The CG types of these strains also correlated well with their MLST sequence type. CG1 strains have either MLST type of ST250 or ST247. ST247 is a single-locus variant (SLV) of ST250 and it is derived from ST250. The strains of the other CG types have the ST8 sequence type of which ST250 is a SLV. All these sequence types belong to the clonal complex CC8 which is one of the two major MRSA genetic lineages reported in hospitals worldwide. It had been suggested that these EMRSA and the classical MRSA belonging to ST8 and its SLVs have been derived from methicillin-sensitive *S. aureus* with the ST8 sequence type. (Enright *et al.*, 2002, Robinson and Enright, 2003)

Interestingly EMRSA-5 and EMRSA-17 both share the same CG type and MLST, but their bacteriophage types are different. (Kerr *et al.*, 1990, Aucken *et al.*, 2002, Enright *et al.*, 2002) However, their CHEF patterns have only one band difference. According to Tenover *et al.*'s (1995) criteria for CHEF pattern interpretation,

EMRSA-17 should be designated as a subtype of EMRSA-5 instead of have its own designation. (Tenover *et al.*, 1995)



Isolate	Origin	SCC*mec* allotype	Coagulase-gene type	Combined genotype	MLST ST	Clonal Complex
WBG525	EA MRSA	III	24	5	ST239	CC8
EMRSA-4	England	III	24	5	ST239	CC8
EMRSA-1	England	III	24	5	ST239	CC8
EMRSA-7	England	III	24	5	ST239	CC8
WBG7422	Malaysia	III	24	5	ST239	CC8
EMRSA-10	England	IV	18	7	ST254	CC8
EMRSA-11	England	III	24	10	ST239	CC8
EMRSA-9	England	III	24	10	ST240	CC8
EMRSA-5	England	I	18	1	ST247	CC8
EMRSA-17	England	I	18	1	ST247	CC8
NCTC10442	England	I	18	1	ST250	CC8
WBG1350	NCTC9468	I	18	1	n/a	n/a
COL	England	I	18	1	ST250	CC8
EMRSA-8	England	I	18	1	ST250	CC8
IRISH-1	England	II	18	4	ST8	CC8
IRISH-2	England	IVp	18	9	ST8	CC8
EMRSA-12	England	IVp	18	9	ST8	CC8
EMRSA-13	England	IVp	18	9	ST8	CC8
EMRSA-2	England	IV	18	6	ST8	CC8
EMRSA-6	England	IV	18	6	ST8	CC8
EMRSA-3	England	I	66	2	ST5	CC5
NCTC8325	Reference	n/a	n/a	n/a	ST8	CC8
NCTC8325	Reference	n/a	n/a	n/a	ST8	CC8
EMRSA-16	England	II	18	3	ST36	CC30
EMRSA-15	England	IV	22	8	ST22	CC22

Figure 11.2 Dendrogram of CHEF patterns and SCC*mec* allotypes, coagulase-gene types, combined genotypes and MLST STs of classical MRSA and EMRSA strains

MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; n/a, not available. The ST and CC of these strains were obtained from Robinson and Enright (2003) and Cassat *et al.* (2005)

11.6.2 CG5, CG7 and CG10 genotypes

Isolates with the CG5 genotype carry SCC*mec* allotype III and are coagulase-gene type 24. They also have related CHEF patterns with a percentage of similarity ranging from 83.9% to 97%. Compared with the larger CHEF clusters formed by CG1, CG4, CG6 and CG9 isolates, CG5 isolates are homogeneous in their genetic backgrounds. They carry an identical SCC*mec* allotype and a distinct coagulase-gene type 24. The strains having this CG type include EMRSA-1, -4, -7, WBG525 and WBG7422. The EMRSA-1 and WBG525 were the predominant epidemic strains of UK and Australian hospitals in the 1980s. (Livermore, 2000, Grubb and O'Brien, 2004) WBG7422 has the predominant CHEF pattern of Malaysian strains. The close relationship observed in this group of isolates suggests an international spread of the CG5 clone.

All CG5 strains have the ST239 MLST sequence type (ST) with SCC*mec* allotype III and coagulase-gene type 24. Although ST239 is a SLV of ST8 and belongs to the CC8 lineage, its *spaA* type motif and *sas* gene profile are distinctively different from other strains in CC8. (Oliveira *et al.*, 2002, Robinson and Enright, 2003) ST239 is derived from a single large chromosomal DNA replacement involving ST8 and ST30. During this genetic event chromosomal DNA of ST30 origin replaced approximately 557 kb, or 20%, of the ST8 genome. ST30 is the putative ancestor of the CC30 lineage. It has different MLST ST, *spaA* type and *sas* gene profiles to ST8 and is not related to ST8. (Robinson and Enright, 2004a) This is possibly the reason why CG5/ST239 strains have a distinct coagulase-gene type 24. The other strains of CC8, CG1, CG4, CG6 and CG9, all have the same coagulase-gene type, 18. The SCC*mec* allotype III is almost exclusively found in ST239 and its SLV strains, and this was observed in this study as well. (Enright *et al.*, 2002, Robinson and Enright, 2003, Robinson and Enright, 2004a)

The CHEF patterns of the EA MRSA WBG525, and the EMRSA-1 have 93.8% similarity. They also share the same CG and MLST types and have a distinct 166 bp deletion in their *mecR1* gene. This observation not only confirms the close relationship between EA MRSA and EMRSA-1 but also agrees with the proposed

inter-national and -continental spread of this pandemic clone in the early 1980s. (Townsend *et al.*, 1987, Grubb and O'Brien, 2004)

Strains with the CG10 type include EMRSA-9 and -11. Their CHEF patterns are not related to each other, nor to any of the other EMRSA or classical MRSA in this study. They are grouped together because they share the same SCC*mec* allotype III and coagulase-gene type 24. EMRSA-9 has the ST240 MLST sequence type, which is the SLV of ST239. (See Section 7.4 and Figure 11.2) EMRSA-9 is thought to be derived from EMRSA-4 and -7 as they have the same *sas* gene profile. (Robinson and Enright, 2003) This might explain why it shared the same coagulase-gene type and SCC*mec* allotypes with the CG5 strains, although its CHEF pattern is not related to them. (See Section 7.4 and Figure 11.2)

EMRSA-11 has the same ST239 sequence type, *spaA* type and *sas* gene profile as EMRSA-1. However, the CHEF patterns of these two strains are not related (66.7% similarity). The same observation was made in a MLST study where strains with the same MLST sequence type were found to have related but different CHEF patterns. (Enright *et al.*, 2002) The CHEF technique is sensitive to short term changes whereas the sequencing based techniques are better for long-term studies. (Witte *et al.*, 1994, Blanc *et al.*, 2002) EMRSA-11 was isolated between 1987 and 1988 whereas EMRSA-1 was isolated in the early 1980s. (Kerr *et al.*, 1990) The CHEF pattern differences observed between these two strains might reflect the genetic changes that occurred in these few years.

The CHEF pattern of the CG7 strain, EMRSA-10, is related to that of the CG5 strains, EMRSA-7, WBG525 and WBG7422. It is also related to EMRSA-13 of the CG1 type. Their CHEF pattern similarity is more than 80%. Interestingly, EMRSA-10 has a different SCC*mec*, coagulase-gene, MLST, *spaA* and *sas* sequence type to these CG5 strains. Its genotype is ST254-MRSA-IV, which is a SLV of ST8, and it is two loci different from the ST239 of those CG5 strains. (Robinson and Enright, 2003) EMRSA-10 is thought to have evolved from the putative ancestor ST8-MSSA of CC8, and then independently to other ST8/ST250-MRSA (CG1, 4, 6 and 9) and ST239-MRSA (CG5) clones. (Robinson and Enright, 2003) Although CHEF pattern on most occasions is a very good guide to the genetic relationship between

isolates, a more accurate genetic relationship requires a comparison of the whole genome of the isolates. The EMRSA-10 might have a similar restriction site distribution to CG5 strains and physically their genomes might have a similar structure. However, their DNA gene sequences show they are not as closely related as the CHEF patterns suggest. (See Section 7.4 and Figure 11.2)

11.6.3 CG2, CG3 and CG8 genotypes

The CG2 strain, EMRSA-3, is a ST5 strain that has Allotype I SCC*mec* and coagulase-gene type 66. (Robinson and Enright, 2003) Its CHEF pattern is not related to strains in this comparison, its CHEF pattern is less than 78% similar to the other strains. (See Section 7.4 and Figure 11.2) EMRSA-3 belongs to the CC5 genetic lineage, which is the major nosocomial MRSA lineage other than CC8. (Robinson and Enright, 2003) This might explained its distinct coagulase-gene type.

The CG3 strain, EMRSA-16, carries Allotype II SCC*mec* and is coagulase-gene type 18. Its CHEF pattern stands out as a separate branch in the CHEF dendrogram and does not relate to other classical MRSA and EMRSA strains. (See Section 7.4 and Figure 11.2) Similarly, the CHEF pattern of the CG8 strain, EMRSA-15, does not relate to other strains and forms a separate branch in the dendrogram. EMRSA-15 has a distinctive coagulase-gene type of type 22 and carries Allotype IV SCC*mec*.

EMRSA-15 is a ST22 strain that belongs to the CC22 genetic lineage, whereas EMRSA-16 has the ST36 MLST sequence type of the CC30 genetic lineage. Both CC22 and CC30 lineages are not related to the CC8 and CC5 lineage or to each other. (Robinson and Enright, 2003) These genetic relationships correlated well with the CHEF dendrogram, as both strains form two unrelated branches. (See Section 7.4 and Figure 11.2)

11.6.4 The overall relationship of CG genotypes

The combined genotypes of these classical and EMRSA strains show that the nosocomial MRSA in UK hospitals only have limited genetic diversity. Six genetic backgrounds were found among these isolates. They are the CG1, 4, 6 and 9 strains

with ST8/250 MLST type, CG5 with ST239, CG2 with ST5, CG7 with ST254, CG3 with ST36 and CG8 with ST22. The majority of the strains either have the ST239 or ST8/250 backgrounds. Even though, both STs belong to the CC8 lineage the isolates with these STs do not have closely related CHEF patterns. The UK EMRSA strains are known to have displaced each other and become predominant strains. The classical MRSA and EMRSA-1 were predominant strains between the 1970s and 1980s and were displaced by EMRSA-3 in the late 1980s. Subsequently EMRSA-3 was displaced by EMRSA-15 and -16 in the late 1990s and early 2000. (Ayliffe *et al.*, 1998, Livermore, 2000, Johnson *et al.*, 2001) This observation, together with their limited diversity in genetic backgrounds, may indicate that the spread of EMRSA strains is due to the clonal expansion of a particularly successful clone.

11.6.5 The SCC*mec* in EMRSAs

The CG4 strain, IRISH-1, is the epidemic strain in the hospitals of Northern Ireland. (Rossney *et al.*, 2003) It carries Allotype II SCC*mec*, which has the Class A *mec* complex and Type 2 *ccr* gene complex. Unlike the typical Class A *mec* complex, the *mecI* gene in IRISH-1 is not intact and is found to have a *c.* 264 bp truncation in its 3' region (Ito *et al.*, 2001). (See Section 7.3.1) The truncation of the *mecI* gene by insertion sequences has been reported in Class B and Class C *mec* complexes. (Katayama *et al.*, 2001) The insertion sequences involved in the truncation of *mecI* include IS*1272*, IS*431/257* and IS*256*. (Ito *et al.*, 2001, Katayama *et al.*, 2001, Oliveira *et al.*, 2000) However, primers designed for IS*431/257* and IS*1272* failed to amplify the *mec* complex of IRISH-1. Recently two novel *mec* complexes, Class A.3 and A.4 *mec* complexes, were identified in Irish MRSA. (Shore *et al.*, 2005) A 119 bp truncation of *mecI* gene by the insertion of IS*1182* was identified in Class A.3 *mec* complex. Similarly, the *mecI* gene of Class A.4 complex was inactivated by IS*1182*. However, in this instance no truncation was detected as the IS*1182* had inserted within the *mecI* gene. (Shore *et al.*, 2005) As IRISH-1 was isolated in the same region, and during the same time period, as these Irish MRSA isolates with IS*1182* in their *mec* complexes, it is likely that the *mecI* truncation observed in IRISH-1 is also due to an IS*1181* insertion. The *mec* complex of IRISH-1 is similar to Class A.3 *mec* complex, as in both cases the 3' region of the *mecI* gene is also not

present. However, the *mecI* deletion in IRISH-1 is larger, indicating that the *mec* complex of IRISH-1 is very similar to the Class A.3 *mec* complex.

Contemporary strains such as EMRSA-15 and -16 carry SCC*mec* allotypes that have a functional Type 2 *ccr* gene complex. (Katayama *et al.*, 2000) The *ccr* genes are responsible for the mobility of SCC*mec*, even though the exact mechanism is yet to be ratified. (Katayama *et al.*, 2000) Comparatively, the majority of the older strains, such as EMRSA-1, EMRSA-3 and EMRSA-5 tend to have immobile SCC*mec* with dysfunctional *ccr* genes. (Ito *et al.*, 2001, Oliveira *et al.*, 2001b) EMRSA-15 and -16 are derived from genetic lineages not related to the older strains, indicating that the acquisition of SCC*mec* in these strains probably is a more recent event than in the older strains. A similar trend has also been observed among the CMRSA where the SCC*mec* allotypes found were all carrying functional *ccr* gene(s) and the SCC*mec* of these CMRSAs is thought to be newly acquired. (Ma *et al.*, 2002, Okuma *et al.*, 2002, O'Brien *et al.*, 2004)

The other interesting observation of SCC*mec* among these strains is the presence, or absence, of a *Xba*I restriction site within their *mecA* gene. The *mecA* gene is a highly conserved gene that encodes the low-affinity PBP2a/2' responsible for blanket resistance to β-lactams. (Song *et al.*, 1987, Ito *et al.*, 1999) The *Xba*I restriction site is a hallmark of the *mecA* gene. (Ryffel *et al.*, 1990) It is created by a single-base point mutation that changes a guanine to an adenine at nucleotide 737 of the *mecA* gene. This mutation leads to a change in the amino acid encoded by the codon from a simple neutral glycine to a more complex acidic glutamic acid. (See Figure 11.3) (Stryer, 1988, Ryffel *et al.*, 1990) However, the single amino acid alteration does not appear to alter the biological function of PBP2a/2'. The mutation occurs within its non-penicillin-binding (nPB) domain and away from its active site in the penicillin-binding domain (transpeptidase). (Ryffel *et al.*, 1990, Lim and Strynadka, 2002) This mutation was originally found in the *mecA* of a nosocomial MRSA and is absent in the *mecA* gene of the methicillin-resistant *S. epidermidis* WT55. (Ryffel *et al.*, 1990)

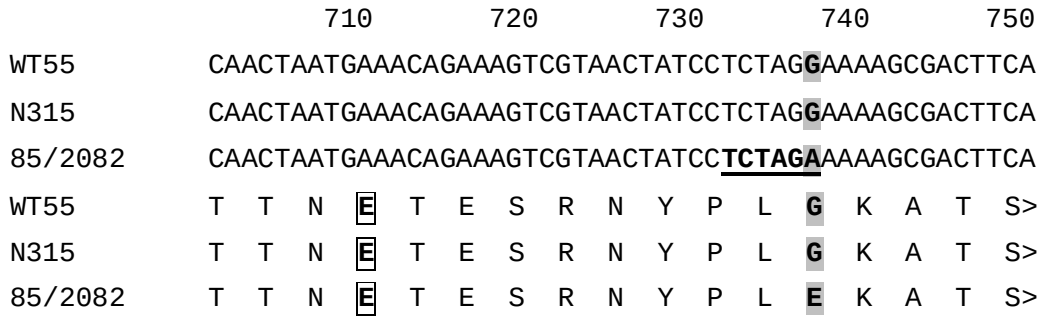


Figure 11.3 The *mecA* DNA and amino acids sequences of the region containing the *Xba*I restriction site.

WT55, methicillin-resistant *S. epidermidis*.
N315, pre-MRSA with *mecA* without a *Xba*I site.
85/2082, nosocomial MRSA with the *mecA* gene with a *Xba*I site.
The highlighted sequences indicate the single-base mutation and the amino acid substitution.
The bolded DNA sequence is the *Xba*I restriction recognition sequence.
The boxed amino acid sequences indicate the amino acid related to high-level β-lactamase resistance. (Katayama *et al.*, 2004)

The classical MRSAs, and the majority of EMRSA strains, were found to have the *Xba*I site in their *mecA* gene. (See Table 7.2) All of these strains except EMRSA-15 were isolated between 1970s and 1980s. (Kerr *et al.*, 1990, Lim *et al.*, 2002) The *mecA* gene with the *Xba*I site has been found in all five SCC*mec* allotypes. Four EMRSA strains were found to have *mecA* without a *Xba*I site. They are EMRSA-16, EMRSA-17, IRISH-1 and IRISH-2. (See Table 7.2) These EMRSAs are contemporary strains isolated in the late 1990s to the present and carry Allotype I, II and IVp SCC*mec*. (Johnson *et al.*, 2001, Aucken *et al.*, 2002) (Lim *et al.*, 2002, Rossney *et al.*, 2003)

A similar result was obtained when 18 published *mecA* sequences were compared. (See Table 11.5) In this comparison *mecA* without a *Xba*I site was commonly found in MRSA isolated in the late 1990s. These MRSA often carry a SCC*mec* with functional *ccr* gene(s) these include SCC*mec* allotypes II, IV, V and their variants. None of the published *mecA* genes from CMRSA strains have this single-base point mutation. (See Table 11.5) The published *mecA* sequences containing the *Xba*I site were typically reported in MRSA isolated before early 1990. These *mecA* sequences were reported in SCC*mec* allotype I, III, IV and their variants. (See Table 11.5)

Allotype I and III SCC*mec* are immobile with dysfunctional *ccr* genes, whereas Allotype IV SCC*mec* is mobile. (Ito *et al.*, 2001, Ma *et al.*, 2002) MRSA strains with this type of *mecA* sequence are often nosocomial strains. (See Table 11.5)

The *mecA* gene is thought to have originated from its methicillin-susceptible homologue found in *S. sciuri*. Methicillin-resistant *S. sciuri* often have two copies of *mecA*, one homologue does not confer methicillin resistance while the other does. (Wu *et al.*, 1996b, Wu *et al.*, 1998, Couto *et al.*, 2000) It has been suggested that these *S. sciuri* might have acquired the methicillin-resistance *mecA* from MRSA or methicillin-resistant coagulase-negative staphylococci (MRCNS). The two published *mecA* sequences from K3 and K8 *S. sciuri* strains both have the *Xba*I site. (Wu *et al.*, 1998) The *mecA* sequence from the MRCNS, *S. epidermidis* WT55, on the other hand, does not carry the restriction site, even though it is a nosocomial strain isolated in 1985. (Tesch *et al.*, 1988, Ryffel *et al.*, 1990) It has been proposed that MRSA acquire the *mecA* or SCC*mec* from MRCNS at some stages of their evolution. (Archer *et al.*, 1994, Hiramatsu, 1995, Hiramatsu *et al.*, 2001, Ito *et al.*, 2004) It is likely that the single-base mutation occurs after the acquisition of *mecA* or SCC*mec*.

It has been shown that the *mecA* gene would be inactivated by spontaneous single-base mutations, if it was introduced into a strain that has never hosted *mecA* before. The *mecA* gene is protected from mutations in these hosts, if its transcription has been strongly suppressed by the regulatory genes. (Katayama *et al.*, 2003c) The pre-MRSA N315 is a nosocomial strain isolated in 1982 in a Japanese hospital. It is clinically methicillin-susceptible as its *mecA* is strongly suppressed by the regulatory genes. (Hiramatsu, 1995, Ito *et al.*, 1999) The *mecA* gene of N315 does not have the *Xba*I site. As this *mecA* DNA sequence is protected by its strong regulatory genes, it is likely that it retains the original sequence it first acquired. In addition, the SCC*mec* of N315 belongs to Allotype II with functional *ccr* genes suggesting that the acquisition of the SCC*mec* occurred not long before N315 was isolated in the hospital. (Katayama *et al.*, 2000, Ito *et al.*, 2001) A similar situation occurs with CMRSA. They have SCC*mec*s with functional *ccr* gene(s) and a *mecA* sequence without a *Xba*I site. As they are derived from different, unrelated genetic lineages, and they have mobile SCC*mec*s, this suggests that their SCC*mec*s are probably

recent acquisitions. This also supports the concept that the original *mecA* gene may not have had a *Xba*I site.

Table 11.5 Published *mecA* gene sequences

Strains	SCC*mec* allotype	MLST ST	Strain Origin	Year	Accession number	Reference
***mecA* gene with *Xba*I site**						
COL	I	ST8	H	1965	CP000046	Gill *et al*. (2005)
NCTC10442	I	ST250	H	1961	AB033763	Ito *et al*. (2001)
85/2082	III	ST239	H	1985	AB037671	Ito *et al*. (2001)
81/108	IVc	n/a	H	1981	AB096217	Ito *et al*. (2001)
M03-68	IVg	ST5	Bovine	2003	DQ106887	Kwon *et al*. (2005)
***S. sciuri* K3**	n/a	n/a	H	n/a	Y13096	Wu *et al*. (1998)
***S. sciuri* K8**	n/a	n/a	Rodent	n/a	Y13095	Wu *et al*. (1998)
***mecA* gene without *Xba*I site**						
***S. epidermidis* WT55**	n/a	n/a	H	1985	X52592	Ryffel *et al*. (1990)
N315	II	ST5	H	1982	D86934	Ito *et al*. (1999)
MRSA525/ EMRSA-16	II	ST36	H	1997	BX571856	Holden *et al*. (2004)
Mu50	II	ST5	H	1997	AP003367	Kuroda *et al*. (2001)
AR13.1/3330.2	IIE	ST8	H	1999	AJ810120	Shore *et al*. (2005)
MW2	IVa	ST1	C	1998	BA000033	Baba *et al*. (2002)
CA05	IVa	n/a	C	1998	AB063172	Ito *et al*. (2003a)
8/6-3P	IVb	n/a	C	1998	AB063173	Ito *et al*. (2003a)
AR43/3330.2	IVE	ST8	H	1999	AJ810121	Shore *et al*. (2005)
W1S/WBG8318	V	ST45	C	1995	AB121219	Ito *et al*. (2004)
TSGH17	V_T	ST59	C	1997-2002	AY894415	Boyle-Vavra *et al*. (2005)

Abbreviations: H, hospital or health-care associated strains; C, community-associated strains; n/a, not available.

The majority of *mecA* sequences with a *Xba*I site have been found in nosocomial isolates. These *mecA* genes are often located in SCC*mec* allotype I, III and IVp. (See Table 7.2 and Table 11.5) These SCC*mec*s are immobile as their *ccr* genes are disabled by either mutations or deletions. (Ito *et al.*, 2001, Oliveira *et al.*, 2001b) These dysfunctional *ccr* genes also indicate that the acquisition of SCC*mec* occurs in the early stages of evolution of these nosocomial strains. The mutation in the *mecA* gene probably occurs after the acquisition and is probably beneficial to the host.

Amino acid substitutions in the nPB domain of PBP2a/2' could significantly increase the level of β-lactamase resistance. One of these substitutions was artificially introduced in the region that contains the *Xba*I site. (Katayama *et al.*, 2004) The induced single-base mutation replaced a guanine with an adenine at nucleotide 710. (See Figure 11.3) This mutation lead to an amino acid substitution of the neutral glycine to a basic lysine at the 237th amino acid of PBP2a/2'. (Katayama *et al.*, 2004) Similarly, the creation of the *Xba*I site involves a single-base mutation of guanine to adenine at nucleotide 737 resulting in glycine being substituted by glutamic acid at the 246th amino acid of PBP2a/2'. (Ryffel *et al.*, 1990) It is not known if this mutation affects the β-lactam resistance level of PBP2a/2'. However, the close proximity of the *Xba*I mutation to the 237th artificial mutation, and their similarity in replacing the neutral glycine with charged amino acids, suggests that it could contribute to increased β-lactam resistance of PBP2a/2'. As the mutation is more common among nosocomial strains, it might have occurred in response to the high-level of β-lactam selection pressure present in hospitals. This mutation has not been found among CMRSA, probably due to the low-level selection pressure present in the community. Nevertheless, as the effects of this mutation on PBP2a/2' are not known, more experiments are needed to test this hypothesis.

Alternatively, the presence, or absence, of the *Xba*I site may simply indicate that the strains have acquired their SCC*mec* or *mecA* gene from different sources. However, it is debatable whether a single-base mutation to produce a *Xba*I site is a basis for ascribing epidemiology or evolution.

11.7 Community-acquired MRSA

CMRSA are MRSA isolated from people without a previous history of hospitalisation. CMRSA are characteristically different from nosocomial MRSA in that they are typically nonmultiresistant. (Okuma *et al.*, 2002, O'Brien *et al.*, 2004, Cookson, 2000) The genetic relationship between twenty-five CMRSA isolates from Australia and New Zealand was investigated with molecular typing techniques. The two contemporary and predominant nosocomial strains, EMRSA-15 and -16 were also included in the study as they also are not resistant to many antimicrobials. Most of the CMRSA isolates were typed by MLST in a later study. (O'Brien *et al.*, 2004) These MLST results are included in this discussion.

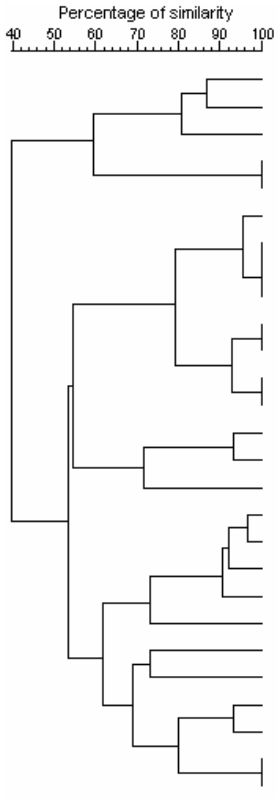
Twenty CHEF patterns were observed among the 25 CMRSA isolates. CHEF patterns of isolates from the same geographic area tended to cluster together in the dendrogram. (See Figure 8.2 and 11.4) In general, isolates from different areas did not have related CHEF patterns. The CHEF patterns correlated well with the MLST sequence types of these CMRSA isolates. Despite their heterogeneous genetic backgrounds the majority of the isolates have Allotype IV SCC*mec* and four isolates have a novel SCC*mec* with the type 5 *ccr* gene complex. (See Section 8.3)

Six clusters were observed in the CHEF dendrogram and most of these clusters, as already mentioned, are confined to specific geographic areas. The only exception is the cluster consisting of WBG8287, WBG8873, WBG8952 and WBG10200, which were collected from Western Australia, South Australia and Victoria. The CHEF patterns of these strains are closely related with a greater than 88% of similarity. Also they all have Allotype IV SCC*mec* and have the ST1 MLST sequence type. (O'Brien *et al.*, 2004) WBG8287 in this cluster caused a small single strain outbreak at the Royal Perth Hospital in Western Australia. (O'Brien *et al.*, 1999) These typing results suggest that there might be a clonal spread of the ST1-MRSA-IV CMRSA clone in Australia.

The CHEF pattern of EMRSA-16 has more than 94% similarity to three CMRSA isolates from Victoria. These three isolates, WBG10198, WBG10199 and WBG10202, have identical CHEF patterns and SCC*mec* allotypes. However,

EMRSA-16 has ST36, which is a SLV of the ST30 of the Victorian isolates. (O'Brien *et al.*, 2004) They also have different SCC*mec* allotypes. EMRSA-16, which has Allotype II SCC*mec* and the Victorian CMRSA have a novel SCC*mec* with Class E *mec* and Type 5 *ccr* gene complexes. The CHEF patterns of EMRSA-16 and the Victorian isolates have a 80% overall similarity to CMRSA from New Zealand (NZ) and New South Wale (NSW). (See Figure 8.2 and 11.4) The NSW and NZ isolates have identical CHEF patterns and belong to the Western Samoan Phage Patterns (WSPP) 1 and 2 clones. WSPP-1 and -2 have the Allotype IV SCC*mec* and ST30 MLST sequence type. (O'Brien *et al.*, 2004, Robinson *et al.*, 2005) All these isolates belong to the same CC30 lineage but have different SCC*mec*s. This indicates that acquisition of SCC*mec* in these isolates occurred on different occasions. Furthermore, there is no evidence of the spread of EMRSA-16 into Australian communities.

These ST30 MRSA isolates belong to the same clonal population of an earlier MSSA pandemic clone, phage type 80/81 clone. This clone caused, among other infections, primary paediatric sepsis and was subsequently controlled by the introduction of methicillin. (Livermore, 2000, Robinson *et al.*, 2005) This highly virulent and transmissible clone carries the phage-borne Panton-Valentine leukocidin (PVL) toxin. (Robinson *et al.*, 2005) This toxin has been shown to cause severe necrotizing pneumonia and skin and soft tissue infections. (Gillet *et al.*, 2002, Diep *et al.*, 2004) The WSSP clones from NSW and NZ were found to carry this toxin. The same observation has also been reported in CMRSA isolates from other countries. The presence of CMRSA, or other methicillin-resistant ST30 strains, with the PVL toxin in a community setting is a worrying sign, and warrants further investigation to establish their prevalence and control measures. CMRSAs with PVL have been responsible for the death of children and immunocompromised young adults in the United States. (Centers for Disease Control and Prevention, 1999, Gillet *et al.*, 2002, Baba *et al.*, 2002)



Isolate	Source	Complexes: *mec*	Complexes: *ccr*	SCC*mec* Allotype	MLST ST	Clonal Complex
EMRSA-15	England	B	2	IV	ST22	CC22
WBG8897	SA	B	2	IV	ST22	CC22
WBG8918	SA	E	5	V	n/a	n/a
WBG9087	NT	B	2	IV	ST75	new
WBG9101	NT	B	2	IV	n/a	n/a
EMRSA-16	England	A	2	II	ST36	CC30
WBG10198	VIC	E	5	V	ST30	CC30
WBG10199	VIC	E	5	V	n/a	n/a
WBG10202	VIC	E	5	V	n/a	n/a
WBG10049	NZ WSPP2	B	2	IV	ST30	CC30
WBG10060	NSW	B	2	IV	ST30	CC30
WBG10066	NSW	B	2	IV	ST30	CC30
WBG10035	NZ WSPP1	B	2	IV	ST30	CC30
WBG8400	EG WA	B1	5	V	n/a	n/a
WBG8404	EG WA	B1	5	V	ST45	CC45
WBG9093	NT	B	2	IV	ST258	new
WBG8287	RPH WA	B	2	IV	ST1	CC1
WBG8873	SA	B	2	IV	ST1	CC1
WBG8952	SA	B	2	IV	n/a	n/a
WBG10200	VIC	B	2	IV	ST1	CC1
WBG8900	SA	B	2	IV	ST257	CC298
WBG525	VIC	A1	3	III	ST239	CC8
WBG10201	VIC	B	2	IV	ST5	CC5
WBG7583	KIM WA	B	2	IV	ST8	CC8
WBG7597	KIM WA	B	2	IV	ST8	CC8
NCTC8325	Reference	n/a	n/a	n/a	ST8	CC8
NCTC8325	Reference	n/a	n/a	n/a	ST8	CC8

Figure 11.4 Dendrogram of CHEF patterns and SCC*mec* allotypes, coagulase-gene types, combined genotypes and MLST STs of community-acquired MRSA

EG, Eastern Goldfields; KIM, Kimberley region; NSW, New South Wale; NT, Northern Territory; NZ, New Zealand; RPH, Royal Perth Hospital; SA, South Australia; VIC, Victoria; WA, Western Australia; MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; n/a, no available; The STs of these strains were obtain from O'Brien *et al.* (2004)

EMRSA-15 is the other EMRSA that clusters closely with the CMRSA isolates. Its CHEF pattern has 86% similarity with the South Australian CMRSA isolate WBG8897. Both isolates have the Allotype IV SCC*mec* and ST22 MLST sequence type. (O'Brien *et al.*, 2004) WBG8897 was the only CMRSA in this study that was resistant to ciprofloxacin, which is typical of EMRSA-15 reported in the United Kingdom. (Livermore, 2000) This suggests that EMRSA-15, or strains derived from EMRSA-15, may have spread into the community.

The Northern Territory isolates WBG9087 and WBG9101 have identical CHEF patterns and Allotype IV SCC*mec*. They have the ST75 MLST sequence type and belong to a clonal complex not related to other isolates. (O'Brien *et al.*, 2004) Similarly, the CMRSA from the Eastern Goldfields of Western Australian, WBG8400 and WBG8404, have ST45, a novel SCC*mec* of Class B1 with Type 5 *ccr* gene complex and related CHEF patterns which are not related to other isolates. (O'Brien *et al.*, 2004) This clearly suggested that these two groups of CMRSA have evolved independently from each other and have acquired the SCC*mec* on different occasions.

WBG7583 and WBG7597 were isolated from the Kimberley region of Western Australia. They have closely related CHEF patterns, identical Allotype IV SCC*mec* and have MLST ST8. (O'Brien *et al.*, 2004) ST8 belongs to one of the largest nosocomial MRSA clonal complexes, CC8. (Enright *et al.*, 2002) ST8 CMRSAs with SCC*mec* IV allotype are also a prevalent CMRSA clone in United States and Norway. (McDougal *et al.*, 2003, Hanssen *et al.*, 2005)

The CMRSA isolates in this study have very diverse genetic backgrounds and within the small population sample of this study, eight non-related clonal complexes were found. (O'Brien *et al.*, 2004) However, their SCC*mec* allotypes are very homogeneous, they are carrying either Allotype IV SCC*mec* or Allotype V or V-like SCC*mec*. This is very different from the nosocomial MRSA isolates that have very homogeneous genetic backgrounds but more diverse but mostly large and immobile SCC*mec* allotypes. Unlike the nosocomial strains, CMRSA SCC*mec*s are much smaller and only encode resistance to β-lactams (the *mecA* gene) and appear to be more mobile. (Ma *et al.*, 2002, Ito *et al.*, 2004) These observations indicate that the

evolution of CMRSA is due to dispersion of Allotype IV and V SCC*mec* among MSSA populations in the community. The different evolutionary path of the nosocomial MRSA and CMRSA probably is due to different selection pressures present in the hospital and community settings

11.8 The genetic relationship between Malaysian MRSA and other MRSA

The Malaysian CHEF patterns were compared with the CHEF patterns of MRSA collected from Asian-Pacific countries and South Africa in 2000. The Asian-Pacific countries were Japan, PR China, Taiwan, Hong Kong, Philippines, Indonesia and Australia. The collections of UK EMRSA, EA MRSA and CMRSA from Western Australia were also included in this investigation.

The majority of the Malaysian, Asian-Pacific and South African MRSA isolates are related to each other and to EMRSA-1, -4, and -7. The overall similarity of their CHEF patterns is more than 80%. (See Section 9.3 and 9.4) The predominant Malaysian CHEF pattern A is 83.9% similar to EMRSA-1 and -7, 93.8% similar to EMRSA-4 and 90.3% similar to WBG525 (EA MRSA). (See Sections 7.3 and 9.2) EMRSA-1, -4 and -7 were reported to have the ST239-MRSA-III genotype that belongs to the CC8 group. (Enright *et al.*, 2002) MLST on four isolates from Malaysia, a Singapore and an Indonesia isolate were found to have either the ST239-MRSA-III genotype or its SLV. (See Chapter 10) These results correlated well with the results from a recent study on MRSA from 12 Asian countries excluding Malaysia. The study found that the majority of the Asian MRSA isolates, except the Japanese and Korean isolates, belong to ST239-MRSA-III or its SLV. (Ko *et al.*, 2005) This indicates that the Asian-Pacific MRSA, including Malaysian and South African MRSA isolates, belong to a single clonal population.

It is interesting that the nations and regions that have the ST239 clone as the predominant nosocomial MRSA are either former British colonies, or have strong historical ties with the United Kingdom. These nations and regions include Australia, Malaysia, Singapore, Hong Kong, India, Saudi Arabia and South Africa. (Ko *et al.*, 2005, Yoshida *et al.*, 1997) The nosocomial spread of the ST239 MRSA

clones in the early 1980s might have been a result of the movement of people, such as medical personnel and patients, between the UK and these countries. This MRSA clone could have subsequently spread into the neighbouring countries, resulting in the ST239 clone being pandemic in Asian-Pacific and South African hospitals.

EMRSAs belonging to the CC8 lineage, especially EMRSA-1, were the predominant MRSA nosocomial strains in the United Kingdom between the 1970s and 1980s. They were replaced by EMRSA-3 from the CC5 lineage in the 90s. (Livermore, 2000) EMRSA-3 was itself displaced by EMRSA-16 of CC30 and EMRSA-15 of CC22 in the late 90s. (Ayliffe *et al.*, 1998, Livermore, 2000, Johnson *et al.*, 2001) It is interesting that the predominant strains of Asian-Pacific and South African hospitals are still ST239-MRSA-III of CC8, a clone that was displaced by other EMRSAs 20 years ago in UK hospitals. (Livermore, 2000, Johnson *et al.*, 2001, Ko *et al.*, 2005) Recently the spread of EMRSA-15 has been reported in Singaporean hospitals and is showing signs of replacing the predominant ST239 clones. (Hsu *et al.*, 2005) It will be interesting to see whether the replacement of ST239-MRSA-III clones by the ST22-MRSA-IV clone (EMRSA-15) will occur in other hospitals in Asian-Pacific and South African countries.

The other interesting observation found in this study is that the isolates belonging to ST239-MRSA-III, or its SLVs, always have the coagulase-gene type 24. (See Sections 4.6 and 7.3) This close relationship can be used as a rapid screening test for the presence of ST239 clone.

Although the Malaysian MRSAs were found to be closely related to EMRSA-1, -4 and -7 and EA MRSA, the Allotype III SCC*mec* of the Malaysian MRSA isolates is slightly different from that of EA MRSA and EMRSA-1. The Allotype III SCC*mec* of EMRSA-1 and EA MRSA carries the Class A1 *mec* complex, which has a 166 bp deletion in its *mecR1* gene. (Lim *et al.*, 2002) This deletion was not found in EMRSA-4, EMRSA-7 and the Malaysian MRSA isolates. Of EMRSA-1, -4, and -7 and EA MRSA, the CHEF pattern of EMRSA-4 has the closest similarity with the Malaysian MRSA. Its CHEF pattern is only two bands different from the Malaysian CHEF pattern A3 (WBG8010). Except for Malaysian CHEF pattern B and I, the rest of the Malaysian CHEF patterns have a similarity with EMRSA-4 ranging from

81.3% to 97%. EMRSA-4, like the Malaysian MRSA isolates, has the Allotype III SCC*mec* with an intact *mecR1* gene. It is likely that these Malaysian isolates have a closer ancestral origin with EMRSA-4 than with EMRSA-1 and EA MRSA. The major disease-causing pandemic clones such as the Brazilian, Portuguese and Vienna clones also have identical MLST sequence type, *spaA* type and *sas* profile to EMRSA-4 and -7. (Robinson and Enright, 2003, Aires de Sousa and de Lencastre, 2004)

The CHEF patterns of Singaporean isolate 81 0090, Indonesian isolate INDO-2 and Filipino isolate 91 2552 were found to be identical to Malaysian CHEF pattern A5. The INDO-2 and the Singaporean isolate were found to have the ST239-MRSA-III genotype and coagulase-gene type 24 (See Chapter 10) indicating that this strain has spread between the two countries. The isolate with Malaysian CHEF pattern A5 might also to be classified as an epidemic MRSA as it had been isolated in more than one hospital. (Kerr *et al.*, 1990) The Malaysian CHEF pattern A5 is related to the CHEF patterns of EMRSA-1, -4 and -7 with a similarity ranging from 81.3% to 90.9%. The Australian isolate 81 1035 was found to be closely related to this Southeast Asian epidemic strain. Their CHEF pattern similarity is 96.6%. This Australian isolates is also closely related to the Malaysian CHEF pattern J with 96.6% similarity. (See Section 9.2)

The PR China isolate 91 1497 is not related to the Malaysian MRSA isolates and the EMRSAs with ST239 genotype. Its CHEF pattern is closely related to IRISH-1 (93.3% similarity) and IRISH-2 (90.3% similarity). IRISH-1 and -2 have the ST8-MRSA-I and ST8-MRSA-II genotypes, respectively. The Hong Kong isolate 91 2031 was closely related to the UK strains COL, NCTC10442 (First MRSA) and EMRSA-5 and -17. All of these MRSA have either the ST250-MRSA-I or its SLV, ST247-MRSA-I, genotypes. This suggests that there are other epidemic clones present in the Asian hospitals other than the ST239.

The Australian isolate 91 1827 is the other exception and it has an identical CHEF pattern to EMRSA-15. EMRSA-15 is ST22-MRSA-IV and belongs to CC22, which is not related to the other CCs of MRSA. This finding correlates well with the report that EMRSA-15 is present in Australian hospitals. (Pearman *et al.*, 2001)

The Japanese isolates are the only exception in the Asian-Pacific and South African group. The Japanese isolate 81 2760 is related EMRSA-3 which is ST5-MRSA-I and belongs to CC5. (Enright, 2003, Robinson and Enright, 2004a) The other Japanese isolates are either loosely related, or not related, to other EMRSA and Asian-Pacific isolates. The majority of Japanese isolates have been found to belong to the New York/Japan clone and are ST5-MRSA-II and belong to CC5. (Robinson and Enright, 2004a, Ko *et al.*, 2005) This explains their relatedness to EMRSA-3 and their distant relationship to the other isolates.

The CHEF patterns of the Malaysian MRSA were compared with the CHEF patterns of CMRSA from Australia and New Zealand. CMRSA generally have a different genetic background to nosocomial MRSA. Consequently, it is not surprising that the CMRSA CHEF patterns were not related to those of the Malaysian MRSA. However, the CMRSA isolates, WBG7583, WBG7597 and WBG10201, did cluster with the CHEF patterns of the Malaysian MRSA. Although not related, their CHEF patterns have a similarity with the Malaysian isolates ranging from 66% to 68%. The CMRSA, WBG7583 and WBG7595, have the genotype ST8, which is like the ST239 of Malaysian isolates. Thus both belong to the CC8 lineage. (Lim *et al.*, 2003, O'Brien *et al.*, 2004) WBG10201 has a genotype of ST5-MRSA-IV, which belongs to the CC5 lineage. CC5 is a large nosocomial lineage that is distantly related to CC8. (Robinson and Enright, 2003) The relationship of their CC lineages explains the minor similarity of their CHEF patterns. This comparison clearly demonstrates that the Malaysian MRSAs have not evolved from *S. aureus* with a community origin.

11.9 Novel MLST sequence type of Malaysia MRSA

MLST sequencing discriminates *S. aureus* isolates by sequence seven housekeeping genes genes. Each distinct sequence (allele) of each housekeeping gene is given a number. The sequence type is composed of a list of numbers representing the allele of the housekeeping genes for an isolate. They are in order, *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*. (Enright *et al.*, 2000)

Four Malaysian isolates were chosen for MLST typing. All but one of the isolates has the allelic profile of 2-3-1-1-4-4-3, which is sequence type 239 (ST239). The only exception is the 1994 isolate, WBG8005, which has an allelic profile different from ST239. It is only one locus different from the ST 239 and that is in the *yqiL* gene.

The sequence of the *yqiL* gene in WBG8005 is novel. It is two bases different from the *yqiL* 3 sequence of ST239. The closest sequence to the *yqiL* of WBG8005 in the current database is the *yqiL* 30 sequence. The *yqiL* 30 sequence is one base different from the sequence of the *yqiL* of WBG8005 and from the *yqiL* 3 sequence. One of the two nucleotide differences between WBG8005 *yqiL* and *yqiL* 3, is the single-base mutation at nucleotide 268. This non-synonymous mutation is also present in the *yqiL* 30 sequence, and is the only difference between *yqiL* 30 and *yqiL* 3. The other single-base difference between WBG8005 *yqiL* and *yqiL* 3 is at nucleotide 411. This synonymous mutation is unique to the *yqiL* of WBG8005. These observations suggest that the *yqiL* of WBG8005 is most possibly derived from *yqiL* 30 by single-base point mutation, rather than from *yqiL* 3 by either recombination or sequential single-base mutations.

yqiL 30 is found in the ST240 sequence type with the allelic profile 2-3-1-1-4-4-30. ST239 is likely to be the ancestral clone of ST240 because it is both a SLV and a single-nucleotide variant of ST239. (Robinson and Enright, 2003) In addition, the ST239-MRSA-III and ST240-MRSA-III clones are the two predominant MRSA clones in Asian hospitals. (Aires de Sousa *et al.*, 2003b, Ko *et al.*, 2005) As suggested, the ancestor of WBG8005 is likely to be an ST240-MRSA-III clone. It is interesting that WBG8005 has the same CHEF pattern as the other Malaysian ST239 isolates. Evidently the mutation that occurred within the housekeeping gene has not altered the distribution of *Sma*I restriction sites in the genome.

ST239-MRSA-III and its SLV clones, such as EMRSA-1, are more often involved in colonisation of patients than in invasive infections. (Livermore, 2000) The same observations have been reported in Malaysian hospitals. (Lim and Zulkifli, 1987, Puthucheary *et al.*, 1987, Hanifah *et al.*, 1992) ST239 has resulted from a large

replacement of the ST8 genome by chromosomal DNA from ST30. (Robinson and Enright, 2004a) Unlike ST239 strains, the ST30 strains are more often associated with primary infection. (Livermore, 2000) Phage type 80/81 and WSSP strains, of the ST30 clone, are involved in devastating skin and wound infections in paediatric patients. (Robinson *et al.*, 2005) The ST239 clone was found to have inherited a number of *sas* genes from its ST30 parent. (Robinson and Enright, 2003) The *sas* genes encode for surface proteins and some of these proteins have been found to be involved in the colonisation process and with disease-causing *S. aureus* strains. (Roche *et al.*, 2003b, Roche *et al.*, 2003a) It is possible that ST239 strains have become efficient colonisers as a result of Sas proteins acquired from ST30 strain(s).

11.10 The efficiency of typing methods used in this study

Several typing methods were utilised in this study for the discrimination of MRSA isolates. These methods include the antimicrobial resistance profiling, bacteriophage typing, plasmid profiling, CHEF, coagulase-gene typing, SCC*mec* allotyping and MLST sequencing typing.

Antimicrobial-resistance profiling is not a useful method for discriminating between Malaysian MRSA isolates, as the resistance profiles of the isolates are very similar. However, antimicrobial-resistance profiling does play an important role in the treatment of *S. aureus* infections. (Tenover *et al.*, 1994, Blanc *et al.*, 1994, Weller, 2000) It was found that the antimicrobial-resistance profile of the Malaysian isolates could be unstable and some isolates were found to lose their aminoglycoside resistance.

The antimicrobial-resistance profile is more useful in the study of CMRSA. Most community isolates are not multiresistant, and often, are only resistant to β-lactams. (Okuma *et al.*, 2002, O'Brien *et al.*, 2004) Being non-multiresistant does not necessarily indicate an MRSA is a CMRSA, as EMSA-15, for example, is usually not multiresistant. However, not being non-multiresistant is a good presumptive screening for CMRSA.

Bacteriophage typing is one of the earliest methods for typing *S. aureus*. However, it was not found to be useful in studying Malaysian MRSA because over 30% of the isolates were not typable with the phages. The majority of the typable isolates (86%) were found in four closely related profiles that did not discriminate sufficiently the isolates. Nevertheless, these results did reflect the homogeneous genetic background of the MRSA isolated over the 18 year time period. The bacteriophage profiles of the Malaysian isolates also showed their close relationship with EMRSA-1 and EA MRSA. (See Section 11.2.2)

Bacteriophage typing was used in the differentiation of the UK EMRSA. (Kerr *et al.*, 1990, Aucken *et al.*, 2002) However, strains like EMRSA-5 and EMRSA-17 have very closely related CHEF patterns and identical MLST genotype and coagulase-gene type and should be grouped together. This observation demonstrates that bacteriophage typing can be used, together with other typing methods, to differentiate isolates.

Plasmid profiling also suffers from the same problem as bacteriophage typing as some isolates do not carry plasmids. As Malaysian isolates have a fairly homogeneous genetic background it is not surprising that their plasmid profiles are closely related. (See Section 11.2.3) Plasmid profiling was better at discriminating the Malaysian isolates than bacteriophage typing. Thirty-five plasmid profiles were identified among the isolates and isolates collected from different time periods had different, although related, plasmid profiles. As plasmids are extrachromosomal, and can be gained or lost, isolates with related plasmid profiles are not necessarily related. Nevertheless, plasmid profiling in the case of the Malaysian MRSA does correlate well with the other typing methods and further demonstrates that these isolates are fairly homogeneous.

Coagulase-gene typing and SCC*mec* allotyping are PCR-based methods. They are not useful in discriminating Malaysian isolates, as they all have the same coagulase-gene and SCC*mec* allotype. However, these methods are fast and able to generate results easily. The coagulase-gene typing method is particularly useful in discriminating the important UK EMRSA strains, as EMRSA-1, -3, -15 and -16 all have different and distinct coagulase-gene types (See Section 7.3.2). (Hookey *et al.*,

1999a) The discriminatory power of SCC*mec* is limited, as only six different SCC*mec* allotypes have been identified. (Ito *et al.*, 2001, Oliveira *et al.*, 2001b, Ma *et al.*, 2002, Ito *et al.*, 2004) However, SCC*mec* allotyping is important in typing MRSA and revealing how they have acquired SCC*mec*. (Ito *et al.*, 2003a, Robinson and Enright, 2004b) This is clearly demonstrated by the nosocomial EMRSA and CMRSA studies that have shown that they have different SCC*mec* allotypes.

CHEF, using *Sma*I restriction enzyme, is regarded as the gold standard for MRSA typing. (Trindade *et al.*, 2003) This highly discriminatory method generated 26 CHEF patterns among the Malaysian MRSA isolates in this study. All these patterns are closely related to each other with different degrees of similarity, indicating a homogeneous genetic background among the isolates. This demonstrates that CHEF has the discriminatory power to resolve a relatively homogeneous group of isolates and reveal their genetic relationship.

CHEF typing was able to determine the genetic relationships between EMRSA, classical MRSA and CMRSA. These genetic relationships generally correlate well with the genetic lineages (clonal complexes) determined by MLST typing. (Lim *et al.*, 2002, Robinson and Enright, 2003, Lim *et al.*, 2003, O'Brien *et al.*, 2004) The CHEF patterns of Malaysian isolates were also compared with the CHEF patterns of Asian-Pacific and South African isolates. The genetic relationship established by this comparison correlated well with the MLST typing of the Asian-Pacific MRSA. (Ko *et al.*, 2005) These results indicate that CHEF typing is an efficient method for discriminating MRSA isolates and determining their genetic relationship. However, CHEF does have its shortcomings, it is difficult to compare inter-laboratory results. (Cookson *et al.*, 1996, van Belkum *et al.*, 1998b, Murchan *et al.*, 2003) In this study, the Malaysian CHEF patterns were compared with other MRSA isolates from different countries and origin. To get around the difficulty of comparing CHEF patterns from different laboratories, all the CHEF typing was done in the same laboratory under the same running conditions. The comparison of such a large number of CHEF patterns is a laborious task and the complicated dendrogram is relatively hard to interpret.

Although the high discriminatory power of CHEF is good for the study of short-term outbreaks it is too discriminatory for long-term studies. The accumulation, over time, of genetic changes that alter the CHEF pattern, can blur the relationship of organisms over the long term. (Witte *et al.*, 1994, Blanc *et al.*, 2002) For example, EMRSA-1 and EMRSA-11 have an identical MLST sequence type, *spaA* sequence type and *sas* gene profile, but their CHEF patterns differ by 10 bands and they are only distantly related with 66.7% similarity. Nevertheless, CHEF still is an important tool for investigating short-term outbreaks of MRSA. (Weller, 2000, Tenover *et al.*, 1994, Trindade *et al.*, 2003)

MLST typing is a relatively new method for typing *S. aureus* by the sequence polymorphism of seven housekeeping genes. (Enright *et al.*, 2000) The Malaysian isolates were found to have either ST239 or its SLV. Similar to other typing methods, this result also shows that the Malaysian isolates have a homogeneous background. However, unlike the other typing methods, MLST can also indicate the evolutionary origin of isolates. This is because the MLST results can be easily compared with other results in the MLST internet database of over 1000 *S. aureus* isolates. (Enright *et al.*, 2000, Robinson and Enright, 2004b) In this study, the similarity between the CHEF patterns of EMRSA, CMRSA, Asian-Pacific and South African MRSA largely correlated well with their MLST sequence type and clonal complexes.

Although MLST is a method with high discriminatory power, it is less discriminatory than CHEF. (Peacock *et al.*, 2002, Enright *et al.*, 2000, Grundmann *et al.*, 2002) This is because sequence polymorphisms arise from neutral mutations in the housekeeping genes and these accumulate more slowly than variations in CHEF patterns. For this reason MLST is more suitable for long-term studies. (Peacock *et al.*, 2002, Oliveira *et al.*, 2001b) For example, EMRSA-1, -4, -7 and -11 have identical sequence type, but their CHEF patterns are different, even though they are related. It is also advantageous to use MLST to study large numbers of isolates, as the results are less complicated, easier to compare and to interpret than a dendrogram generated by CHEF patterns.

Some researchers have suggested the combined use of CHEF, MLST and SCC*mec* allotyping is the optimal method for MRSA typing. CHEF would be able to detect the short-term changes in the isolates. The shortcoming of CHEF patterns in long-term studies and the inter-laboratory comparison of results can be compensated for by MLST. SCC*mec* allotyping will further type MRSA and provide evolutionary information about the acquisition of methicillin resistance by *S. aureus*. (Aires de Sousa and de Lencastre, 2004) In this study, the combined results of these typing methods suggest that most of the nosocomial MRSA have evolved from the clonal expansion of a few MRSA clones, whereas the CMRSAs are likely to have evolved from the dissemination of Allotype IV and V SCC*mec* into different MSSA clones. These results also demonstrate the spread of isolates with Malaysian CHEF A5 pattern in Malaysian, Singaporean and Indonesian hospitals. As they have an identical CHEF pattern, MLST and SCC*mec* allotype these isolates can be linked unambiguously with the Asian pandemic clone ST239-MRSA-III. (Ko *et al.*, 2005)

11.11 Conclusion and future directions

11.11.1 Clonality of Malaysian MRSA

The 74 Malaysian MRSA isolates in this study were found to be a relatively homogeneous group. Among them, 26 related CHEF patterns were identified. The predominant CHEF pattern A was found in isolates collected over the time period of 1982, 1989 and 1994. The isolates collected in year 2000 do not have this predominant pattern but the most common year 2000 CHEF pattern G is closely related to it. The isolates with these two CHEF patterns have the same ST239 MLST sequence type. Representative isolates of the other 24 CHEF patterns were found to have the Allotype III SCC*mec* and coagulase-gene type 24. These two genotypes were almost exclusively found together in MRSA isolates belonging to the ST239-MRSA-III clone or its SLV of CC8 lineage. (Robinson and Enright, 2003, Hookey *et al.*, 1999a) Hence, all the Malaysian isolates in this study most likely belong to the ST239-MRSA-III clone or its SLV. This result correlates well with the studies of Asian MRSA. The ST239-MRSA-III was found to be the predominant clone in the hospitals of the countries neighbouring Malaysia. (Aires de Sousa *et al.*, 2003b, Ko *et al.*, 2005, Hsu *et al.*, 2005) Homogeneity of the Malaysian MRSA demonstrated

in this study was also shown in other surveys of MRSA in Malaysian hospitals where the majority of the isolates belonged to the predominant CHEF pattern reported in the present study. (Norazah *et al.*, 2003b, Norazah *et al.*, 2001b) Together these studies indicate that the spread of MRSA in Malaysian hospitals is likely to be due to a clonal expansion of the ST239-MRSA-III clone.

Interestingly, the predominant MRSA clone of UK hospitals in the 1980s was EMRSA-1, which is ST239-MRSA-III. It was subsequently displaced by EMRSA-3 in the early 1990s and, EMRSA-15 and EMRSA-16 in the late 1990s. (Robinson and Enright, 2003, Livermore, 2000) Strains similar to the classical MRSA were isolated in a Malaysian hospital in 1987. However, they were not isolated 1989 and the predominant strains were similar to the ST239-MRSA-III clone. (Hanifah *et al.*, 1992, Yoshida *et al.*, 1997, Ito *et al.*, 2001) In the present study, MRSA belonging to ST239-MRSA-III were isolated as early as 1982. A possible scenario is that the ST239-MRSA-III clone spread into Malaysia from the UK in the early 1980s. This finding coincides with the emergence in the early 1980s in UK hospitals of EMRSA strains that belong to the ST239 clone. (Kerr *et al.*, 1990, Livermore, 2000) The ST239 strains became the predominant clone in Malaysian hospitals in the late 1980s and expanded until the present time. The displacement of the ST239 clone by clones of the different lineages in UK hospitals has not happened, as yet, in Malaysian hospitals.

Although the Malaysian MRSA isolates in this study were found to be very similar to EMRSA-1 and EA MRSA strains, they lack some of their distinctive genetic features. Their CHEF patterns are more like those of EMRSA-4, which also belong to the ST239 clone. (Robinson and Enright, 2003) Also, unlike EMRSA-1 and the EA MRSA, they do not have the 166 bp deletions in their *mecR1* gene, but have the intact *mecR1* gene like EMRSA-4. (Lim *et al.*, 2002) The Malaysian MRSA isolates typically carry the BHM/NAB multiresistance plasmids, whereas the plasmids in EMRSA-1 and EA MRSA strains typically belong to the pSK1 family of multiresistance plasmid. (Firth and Skurray, 2000, Skurray *et al.*, 1988, Townsend *et al.*, 1987, Grubb and O'Brien, 2004) Further, aminoglycoside resistance in EMRSA-1 and EA MRSA is typically plasmid-borne, whereas aminoglycoside resistance in the Malaysian isolates is chromosomal. (Townsend *et al.*, 1987, Skurray *et al.*, 1988,

Firth and Skurray, 2000) Chromosomal aminoglycoside resistance is typically found in UK MRSA isolates collected before 1980. (Wright *et al.*, 1998) These differences observed between the Malaysian isolates and EMRSA-1/EA MRSA strains indicate that the Malaysian MRSA isolates do not belong to the inter-continental spread of the EMRSA-1/EA MRSA strain in the early 1980s. (Townsend *et al.*, 1984a, Townsend *et al.*, 1987) The Malaysian isolates may share the same ancestor strain with EMRSA-4. As all ST239 strains, and its SLVs, are thought to be derived from an ancestral MRSA, it is possible that the ancestral strain of the Malaysian isolates existed before 1980. (Enright *et al.*, 2002)

11.11.2 Aminoglycoside resistance in Malaysian isolates

The chromosomal aminoglycoside resistance in some isolates was found to be unstable. The loss of aminoglycoside resistance was always accompanied by the loss of *c.* 114 kb of chromosomal DNA. This chromosomal deletion is unlikely to be a random event as it was found in several isolates with different CHEF patterns. The loss of aminoglycoside resistance in these isolates was due to the loss of the *aacA-aphD* gene. The *aacA-aphD* gene encodes the bifunctional aminoglycoside-modifying enzyme that confers resistance to the majority of aminoglycosides used in hospitals. (Mingeot-Leclercq *et al.*, 1999, Daigle *et al.*, 1999) This gene is exclusively found in Tn*4001* or Tn*4001*-like elements and is either chromosomal or plasmid-borne. (Lyon *et al.*, 1987a, Byrne *et al.*, 1990, Firth and Skurray, 2000) It is very likely that the Malaysian MRSA isolates have a chromosomal copy of either Tn*4001* or a Tn*4001*-like element.

However, the Tn*4001* element is only 4.7 kb in size and the chances of its excision resulting in a *c.* 114 kb loss of neighbouring chromosomal DNA is not high. (Lyon and Skurray, 1987) There are probably some other mobile elements also involved in this large chromosomal deletion. Further study of this 114 kb region would be required to find out the exact mechanism. Studies such as sequencing the neighbouring region of the *aacA-aphD* gene and looking for mobile elements in this region by either PCR or Southern hybridisation with DNA probes might give an insight in to how this genetic event occurs.

11.11.3 The Class II multiresistance plasmids

The other unique feature of Malaysian MRSA is that many isolates carry two to three Class II BHM/NAB multiresistance plasmids at the same time. The 28 kb, 30.5 kb and 35 kb plasmids were the most common in the isolates. The 28 and 30.5 kb plasmids have the same resistance profile of resistance to mercury, cadmium, and both the monovalent and divalent NAB compounds. The resistance profile of the 35 kb plasmid is almost the same as the 28 and 30.5 kb plasmids except it also encodes for β-lactamase production. Structurally these plasmids are very similar to each other and their RFLP patterns are only one or two bands different. The differences in phenotype, size and RFLP of these three plasmids are most likely the result of an insertion, excision or truncation of a Tn*552* or Tn*552*-like β-lactamase transposon on these plasmids. (See Section 5.4 and Section 11.3.2)

Plasmids with the same replication system are often incompatible to each other and do not exist in the same host. (Novick, 1987) These three Malaysian multiresistance plasmids are closely related and structurally very similar. The staphylococcal multiresistance plasmids have been found to have very similar replication genes and to utilise the theta replication system. (Firth *et al.*, 2000) It is interesting to find that these three, apparently compatible, plasmids exist in the same host. A study of the genes involved in replication might give an insight into their compatibility. This could be examined by cloning and sequencing their replication (*rep*) regions. This would reveal the organization and sequence of their *rep* genes and neighbouring open reading frames and may explain their compatibility.

Locating the NAB and heavy-metal resistance genes, and detecting the presence or absence of Tn*552*, or Tn*552*-like transposons on these plasmids could be done by PCR, or by Southern hybridisation with DNA probes. However, to analyse the structural differences in these plasmids would require sequencing either the whole plasmids, or the regions that are different between them. This would give a better picture of how these plasmids are related and have evolved.

The Malaysian isolates have a dysfunctional *mecA* regulatory system due to a nonsense mutation in the *mecI* gene. It has been reported that the β-lactamase regulatory system can regulate *mecA* expression and that it is more efficient than the native regulatory system. (McKinney *et al.*, 2001, Ryffel *et al.*, 1992) All of the Malaysian isolates are β-lactamase producers. Many carry a chromosomal copy of the β-lactamase gene as well as plasmid copy. As the *mecA* gene confers blanket β-lactam resistance there is no selective pressure for an isolate to have β-lactamase, other than to regulate the expression of *mecA*. (Hiramatsu *et al.*, 1990, Katayama *et al.*, 2003c, Hackbarth and Chambers, 1993, Hackbarth *et al.*, 1994) It is possible that one of the β-lactamases in isolates with dual β-lactamases has dysfunctional regulatory genes. This could be examined by transferring the β-lactamase plasmid into a penicillin-sensitive donor and analysing its β-lactamase regulatory genes by PCR and DNA sequencing. The chromosomal β-lactamase copy could be studied in the same manner after the strain has been cured of the β-lactamase plasmid. Two copies of the β-lactamase regulatory genes may provide better regulation of *mecA* expression. This hypothesis could be investigated by doing a population analysis of methicillin resistance of an isolate with dual β-lactamases and the same isolate cured of its β-lactamase plasmid. This would give a better understanding of the selection pressure for these Malaysian BHM/NAB plasmids.

Another selective advantage for the BHM/NAB plasmids is their NAB resistance. The NAB compounds are commonly used as disinfectants and antiseptics therefore it could be advantageous for isolates to have NAB resistance. The *qac* gene, conferring NAB resistance, carried by these plasmids only confers a four to eight fold increase in MIC to compounds like the quaternary ammonium compounds and this is unlikely to protect organisms against the concentrations used in practice. (Russell, 2000, Emslie *et al.*, 1986, Leelaporn *et al.*, 1994b) The indiscriminate and improper use of disinfectants and antiseptics has been reported in Malaysian hospitals. (Keah *et al.*, 1995) It is possible that the presence of three NAB-resistance plasmids (i.e. three NAB-resistance genes) in the same host might increase the MIC level and help protect the organisms against the NAB compounds. This hypothesis could be investigated by comparing the resistance of isogenic variants containing one or more of the different sized plasmids. This would determine if

there is a gene dose affect and give an insight into the advantage(s) for a cell of carrying multiple copies of plasmids with identical or similar phenotypes.

11.11.4 The pWBG707 conjugative plasmid

pWBG707 is a large trimethoprim-resistance conjugative plasmid found in Malaysian isolates. (Udo *et al.*, 1992b) Unlike the staphylococcal plasmids of the pSK41 family it is not a multiresistance plasmid and only encodes resistance to trimethoprim. (Berg *et al.*, 1998, Firth and Skurray, 2000) Further more, the trimethoprim-resistance determinant appears to be novel and different from the *dfrA* trimethoprim-resistance gene of the pSK41 plasmid or the *dfrC* gene of coagulase-negative staphylococci. The recently described chromosomal *dfrG* trimethoprim-resistance gene in Thai and Japanese nosocomial MRSA probably is a likely candidate for the trimethoprim-resistance determinant of pWBG707. (Sekiguchi *et al.*, 2005) To determine if pWBG707 is carrying the *dfrG* gene a PCR with *dfrG*-specific primers could be done using pWBG707 as a template. As many of the Malaysian isolates have chromosomal trimethoprim resistance and do not carry the *dfrA* gene they could also be screened by PCR with primers for the *dfrG* gene.

The other distinctive characteristic of pWBG707 is its ability to mobilise a 3.0 kb kanamycin-resistance plasmid at high frequency during conjugation. (Udo *et al.*, 1992b) The mobilisation of small plasmids by large conjugative plasmids has been observed for different conjugative plasmids but not at the frequencies observed for pWBG707. (Udo *et al.*, 1992a, Udo *et al.*, 1997, Projan and Archer, 1989, Udo and Jacob, 1998) Genetic elements on the small plasmids are involved in the relaxation of the supercoiled plasmid DNA and this contributes to the mechanism of mobilisation. (Projan and Archer, 1989, Caryl *et al.*, 2004) To investigate mobilisation of pWBG707 its *tra* region could be cloned and sequenced. This would reveal the organization of the *tra* genes and enable them to be compared with those of the pSK41 family (Berg *et al.*, 1998). The function of these genes could be investigated by introducing Tn*551*, or Tn*917*, insertions into individual *tra* genes and looking for the loss of function and changes in the efficiency of conjugation and mobilisation. Northern blotting to detect of mRNA transcription during conjugation could also be useful. An alternative method to northern blotting that could be used is

real-time PCR. The ability of pWBG707 to mobilise other plasmids could be examined and those that are mobilised at a low frequency compared with the small kanamycin-resistance plasmid by sequencing in order to understand the differences in mobilisation frequencies. The mobilisation of small plasmids by conjugative plasmids is an important pathway of gene transfer. These studies could lead to a better understanding of how genes are transferred between staphylococci.

11.11.5 MRSA epidemiology and Infection control in Malaysian hospitals

This study has shown that most of the Asian-Pacific and South African MRSA are related and likely to belong to the same clonal complex. This observation correlates well with a MLST study that found that the great majority of Asian MRSAs belong to either the ST239-MRSA-III clone or its SLV. (Ko *et al.*, 2005) The Asian-Pacific and South African MRSAs were also found to be closely related to the UK EMRSA and EA MRSA that belong to the same ST239 clone. It is possible that the spread of the ST239 clone in Asian-Pacific countries is a result of the introduction of UK EMRSA strains into Asian-Pacific hospitals. Many of the Asian-Pacific countries have close ties with the United Kingdom as they are either former British colonies or have close historical ties. (Yoshida *et al.*, 1997, Hiramatsu *et al.*, 2001) The difference between Malaysian MRSA and EMRSA-1/EA MRSA strains suggests that the international transmission of the EMRSA between UK and Asian-Pacific countries has occurred on more than one occasion.

This study has also shown that a MRSA strain with the Malaysian CHEF pattern A5 is found in the Malaysian, Singaporean, Indonesian and Filipino hospitals. Similarly, isolates closely related to the Malaysian MRSA CHEF pattern I were found in Malaysian, Singaporean and Indonesian hospitals. All of these MRSA isolates belong to the ST239-MRSA-III clone. These findings suggest that either there were no measures to prevent the inter-hospital, or international, spread of MRSA, or that the measures have failed to prevent the spread of this MRSA strain. This problem was highlighted in a review of infection control in Malaysian hospitals, which found that there was poor inter-hospital coordination, lack or awareness and lack of trained staff. (Lim, 2001)

The ST239-MRSA-III clone, like EMRSA-1, is often a coloniser, and this characteristic was also found in the Malaysian MRSAs that belong to this clone. (Livermore, 2000, Lim and Zulkifli, 1987, Puthucheary *et al.*, 1987, Hanifah *et al.*, 1992) EMRSA-1 has been displaced by other EMRSA strains in UK hospitals. Currently the predominant clones are EMRSA-15 and EMRSA-16. (Livermore, 2000) EMRSA-15 and -16 have overwhelmed infection control measures in UK hospitals, and the prevalence of MRSA has increased significantly. (Farrington *et al.*, 1998, Livermore, 2000) EMRSA-15 and -16 are more often linked to invasive diseases than EMRSA-1. They also cause 95.6% of MRSA bacteraemia. (Livermore, 2000, Johnson *et al.*, 2001) In 2004, EMRSA-15 was found in Singaporean hospitals and shows signs of replacing the ST239 clone and becoming the predominant clone. (Hsu *et al.*, 2005) Although the source of EMRSA-15 in Singaporean hospitals has not been determined it is most likely that patients or medical personnel introduced it from overseas. (Hsu *et al.*, 2005) Without proper infection control measures EMRSA-15 could spread in Malaysian hospitals, like its ST239 predecessor.

EMRSA-15 is very different from EMRSA-1 and other ST239 strains. It has the ST22 MLST sequence type and carries Allotype IV SCC*mec*. (Robinson and Enright, 2003) Similar to other strains with Allotype IV SCC*mec*, it is not multiply resistant. However, strains with SCC*mec* IV allotype grow almost two times faster than ST239 strains with the Allotype III SCC*mec*. (Okuma *et al.*, 2002) This suggests that the EMRSA-15 could easily out compete the ST239 clone and become the predominant clone. The spread EMRSA-15 into Malaysian hospitals would even further stretch the already struggling infection control program in Malaysia. (Lim, 2001) This could result in an increase of invasive diseases caused MRSA, similar to that which has occurred the UK hospitals. (Farrington *et al.*, 1998, Livermore, 2000) Another consequence of the presence of EMRSA-15 in Malaysian hospitals might lead to its Allotype IV SCC*mec* being transferred into the MSSA population in Malaysian hospitals and even in the community as most CMRSA have Allotype IV of V SCC*mec*.

Further, without proper infection control measures, the Malaysian hospitals are vulnerable to the importation of other MRSA clones, such as vancomycin-

intermediate MRSA, vancomycin-resistant MRSA, CMRSA and phage type 80/81 strains with methicillin resistance and PVL toxin. (Walsh and Howe, 2002, Tenover *et al.*, 2004, Okuma *et al.*, 2002, Robinson *et al.*, 2005) There is an urgent need for the Malaysian hospitals to establish more efficient infection control measures. The Dutch "search and destroy" and the Western Australian infection control program might not work in hospitals, like Malaysian hospitals, where MRSA have become endemic. (Pearman *et al.*, 1985, Vriens *et al.*, 2002, Livermore, 2000) However, similar programs with strict infection control measures were found to reduce the MRSA isolate rate in a Portuguese hospital from 40% to 17% within two years. (Sa-Leao *et al.*, 1999) These measures included the control of antibiotic prescribing, improved hand hygiene, barrier precautions, improved staff training and periodic surveys. (Sa-Leao *et al.*, 1999) The Malaysian hospitals appear not to have been in antimicrobial surveillance programs like SENTRY. (Bell and Turnidge, 2002, Christiansen *et al.*, 2004, Song *et al.*, 2004) However, joining these surveillance programs would greatly benefit Malaysian hospitals. They could have access to expertise and resources that are not available to them and at the same time would be able to monitor the different MRSA clones in Malaysia and neighbouring countries. However, infection control in Malaysia is still in its infancy and requires more effort and resources to make it more effective. (Lim, 2001)

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APPENDIX

A.1 The completed sequence of the PCR insert of pLTT1 containing the 57 bp of *mecA*, *mecR1* and *mecI* genes of WBG7422

Sequence Range: 1 to 2335

```
         10         20         30         40         50
ACCAAACCCGACAACTACAACTATTAAAATAAGTGGAACAATTTTTATCT
<_____________________MECA_____________________________

         60         70         80         90        100
TTTTCATCAATATCCTCCTTATATAAGACTACATTTGTAGTATATTACAA
<_______

        110        120        130        140        150
ATGTAGTATTTATGTCAAAATAATGTTATAATTTTTGTGATATGGAGGTG

        160        170        180        190        200
TAGAAGGTGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCT
      _____________________MECR1____________________>

        210        220        230        240        250
CACGATATGTGTAATTTTTTTAGTGAGAATGCTCTATATAAAATATACTC
_____________________MECR1_____________________________>

        260        270        280        290        300
AAAATATTATGTCACATAAGATTTGGTTATTAGTGCTCGTCTCCACGTTA
_____________________MECR1_____________________________>

        310        320        330        340        350
ATTCCATTAATACCATTTTACAAAATATCGAATTTTACATTTTCAAAAGA
_____________________MECR1_____________________________>

        360        370        380        390        400
TATGATGAATCGAAATGTATCTGACACGACTTCTTCGGTTAGTCATATGT
_____________________MECR1_____________________________>

        410        420        430        440        450
TAGATGGTCAACAATCATCTGTTACGAAAGACTTAGCAATTAATGTTAAT
_____________________MECR1_____________________________>

        460        470        480        490        500
CAGTTTGAGACCTCAAATATAACGTATATGATTCTTTTGATATGGGTATT
_____________________MECR1_____________________________>

        510        520        530        540        550
TGGTAGTTTGTTGTGCTTATTTTATATGATTAAGGCATTCCGACAAATTG
_____________________MECR1_____________________________>

        560        570        580        590        600
ATGTTATTAAAAGTTCGTCATTGGAATCGTCATATCTTAATGAACGACTT
_____________________MECR1_____________________________>
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A.1 continued

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       610       620       630       640       650
AAAGTATGTCAAAGTAAGATGCAGTTCTACAAAAAGCATATAACAATTAG
_____________________MECR1_____________________>

       660       670       680       690       700
TTATAGTTCAAACATTGATAATCCGATGGTATTTGGTTTAGTGAAATCCC
_____________________MECR1_____________________>

       710       720       730       740       750
AAATTGTACTACCAACTGTCGTAGTCGAAACCATGAATGACAAAGAAATT
_____________________MECR1_____________________>

       760       770       780       790       800
GAATATATTATTCTACATGAACTATCACATGTGAAAAGTCATGACTTAAT
_____________________MECR1_____________________>

       810       820       830       840       850
ATTCAACCAGCTTTATGTTGTTTTTAAAATGATATTCTGGTTTAATCCTG
_____________________MECR1_____________________>

       860       870       880       890       900
CACTATATATAAGTAAAACAATGATGGACAATGACTGTGAAAAAGTATGT
_____________________MECR1_____________________>

       910       920       930       940       950
GATAGAAACGTTTTAAAAATTTTGAATCGCCATGAACATATACGTTATGG
_____________________MECR1_____________________>

       960       970       980       990      1000
TGAATCGATATTAAAATGCTCTATTTTAAAATCTCAGCACATAAATAATG
_____________________MECR1_____________________>

      1010      1020      1030      1040      1050
TGGCAGCACAATATTTACTAGGTTTTAATTCAAATATTAAAGAACGTGTT
_____________________MECR1_____________________>

      1060      1070      1080      1090      1100
AAGTATATTGCACTTTATGATTCAATGCCTAAACCTAATCGAAACAAGCG
_____________________MECR1_____________________>

      1110      1120      1130      1140      1150
TATTGTTGCGTATATTGTATGTAGTATATCGCTTTTAATACAAGCACCGT
_____________________MECR1_____________________>

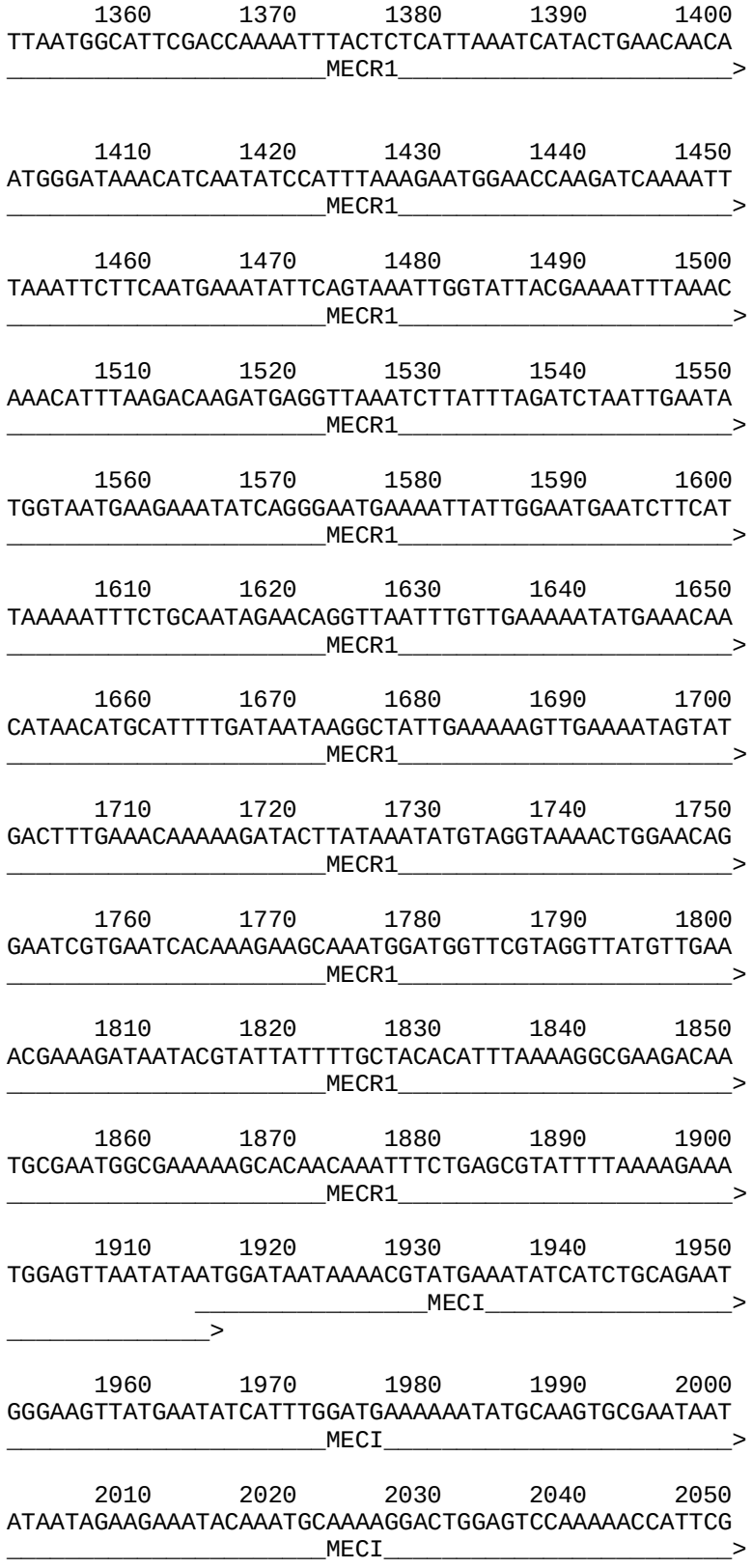
      1160      1170      1180      1190      1200
TACTATCTGCACATGTTCAACAAGACAAATATGAAACAAATGTATCATAT
_____________________MECR1_____________________>

      1210      1220      1230      1240      1250
AAAAAATTAAATCAACTAGCTCCGTATTTCAAAGGATTTGATGGAAGTTT
_____________________MECR1_____________________>

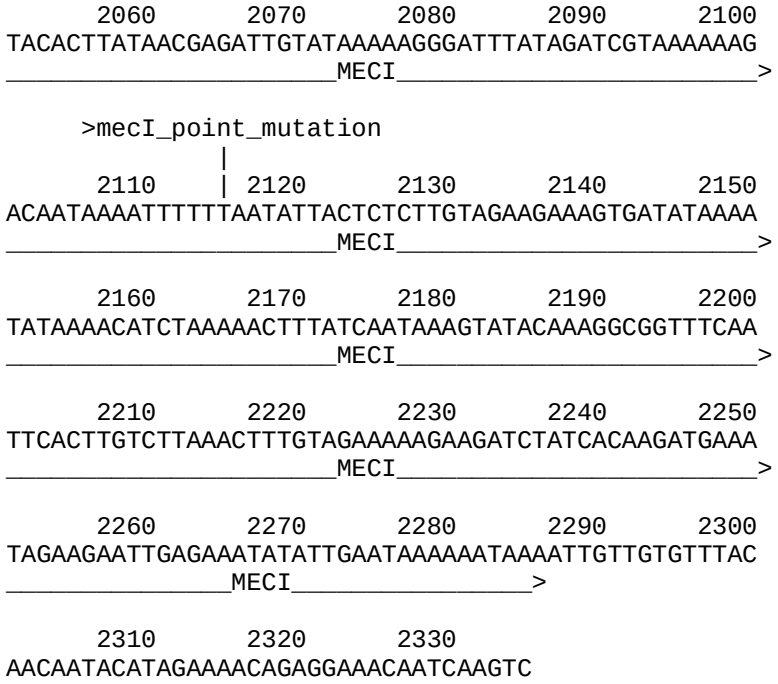
      1260      1270      1280      1290      1300
TGTGCTTTATAATGAACGGGAGCAAGCTTATTCTATTTATAATGAACCAG
_____________________MECR1_____________________>

      1310      1320      1330      1340      1350
AAAGTAAACAACGATATTCACCTAATTCTACTTACAAAATTTATTTAGCG
_____________________MECR1_____________________>
```

A.1 continued



A.1 continued



A.2 The completed sequence of the PCR insert of pLTT2 containing the 57 bp of *mecA*, *mecR1* and *mecI* genes of WBG525

```
Sequence Range: 1 to 2169

                  10         20         30         40         50
               ACCAAACCCGACAACTACAACTATTAAAATAAGTGGAACAATTTTTATCT
              <_________________________MECA_____________________________

                  60         70         80         90        100
               TTTTCATCAATATCCTCCTTATATAAGACTACATTTGTAGTATATTACAA
              <_______

                 110        120        130        140        150
               ATGTAGTATTTATGTCAAAATAATGTTATAATTTTTGTGATATGGAGGTG

                 160        170        180        190        200
               TAGAAGGTGTTATCATCTTTTTTAATGTTAAGTATAATCAGTTCATTGCT
                     _________________________MECR1_____________________>

                 210        220        230        240        250
               CACGATATGTGTAATTTTTTTAGTGAGAATGCTCTATATAAAATATACTC
               _____________________________MECR1_____________________>

>mecR1_166_bp_deletion_point
                 |
                 |   260        270        280        290        300
               AAATGTTACGAAAGACTTAGCAATTAATGTTAATCAGTTTGAGACCTCAA
               _____________________________MECR1_____________________>

                 310        320        330        340        350
               ATATAACGTATATGATTCTTTTGATATGGGTATTTGGTAGTTTGTTGTGC
               _____________________________MECR1_____________________>

                 360        370        380        390        400
               TTATTTTATATGATTAAGGCATTCCGACAAATTGATGTTATTAAAAGTTC
               _____________________________MECR1_____________________>

                 410        420        430        440        450
               GTCATTGGAATCGTCATATCTTAATGAACGACTTAAAGTATGTCAAAGTA
               _____________________________MECR1_____________________>

                 460        470        480        490        500
               AGATGCAGTTCTACAAAAAGCATATAACAATTAGTTATAGTTCAAACATT
               _____________________________MECR1_____________________>

                 510        520        530        540        550
               GATAATCCGATGGTATTTGGTTTAGTGAAATCCCAAATTGTACTACCAAC
               _____________________________MECR1_____________________>

                 560        570        580        590        600
               TGTCGTAGTCGAAACCATGAATGACAAAGAAATTGAATATATTATTCTAC
               _____________________________MECR1_____________________>

                 610        620        630        640        650
               ATGAACTATCACATGTGAAAAGTCATGACTTAATATTCAACCAGCTTTAT
               _____________________________MECR1_____________________>
```

A.2 continued

```
       660       670       680       690       700
GTTGTTTTTAAAATGATATTCTGGTTTAATCCTGCACTATATATAAGTAA
_____________________MECR1_____________________>

       710       720       730       740       750
AACAATGATGGACAATGACTGTGAAAAAGTATGTGATAGAAACGTTTTAA
_____________________MECR1_____________________>

       760       770       780       790       800
AAATTTTGAATCGCCATGAACATATACGTTATGGTGAATCGATATTAAAA
_____________________MECR1_____________________>

       810       820       830       840       850
TGCTCTATTTTAAAATCTCAGCACATAAATAATGTGGCAGCACAATATTT
_____________________MECR1_____________________>

       860       870       880       890       900
ACTAGGTTTTAATTCAAATATTAAAGAACGTGTTAAGTATATTGCACTTT
_____________________MECR1_____________________>

       910       920       930       940       950
ATGATTCAATGCCTAAACCTAATCGAAACAAGCGTATTGTTGCGTATATT
_____________________MECR1_____________________>

       960       970       980       990      1000
GTATGTAGTATATCGCTTTTAATACAAGCACCGTTACTATCTGCACATGT
_____________________MECR1_____________________>

      1010      1020      1030      1040      1050
TCAACAAGACAAATATGAAACAAATGTATCATATAAAAAATTAAATCAAC
_____________________MECR1_____________________>

      1060      1070      1080      1090      1100
TAGCTCCGTATTTCAAAGGATTTGATGGAAGTTTTGTGCTTTATAATGAA
_____________________MECR1_____________________>

      1110      1120      1130      1140      1150
CGGGAGCAAGCTTATTCTATTTATAATGAACCAGAAAGTAAACAACGATA
_____________________MECR1_____________________>

      1160      1170      1180      1190      1200
TTCACCTAATTCTACTTACAAAATTTATTTAGCGTTAATGGCATTCGACC
_____________________MECR1_____________________>

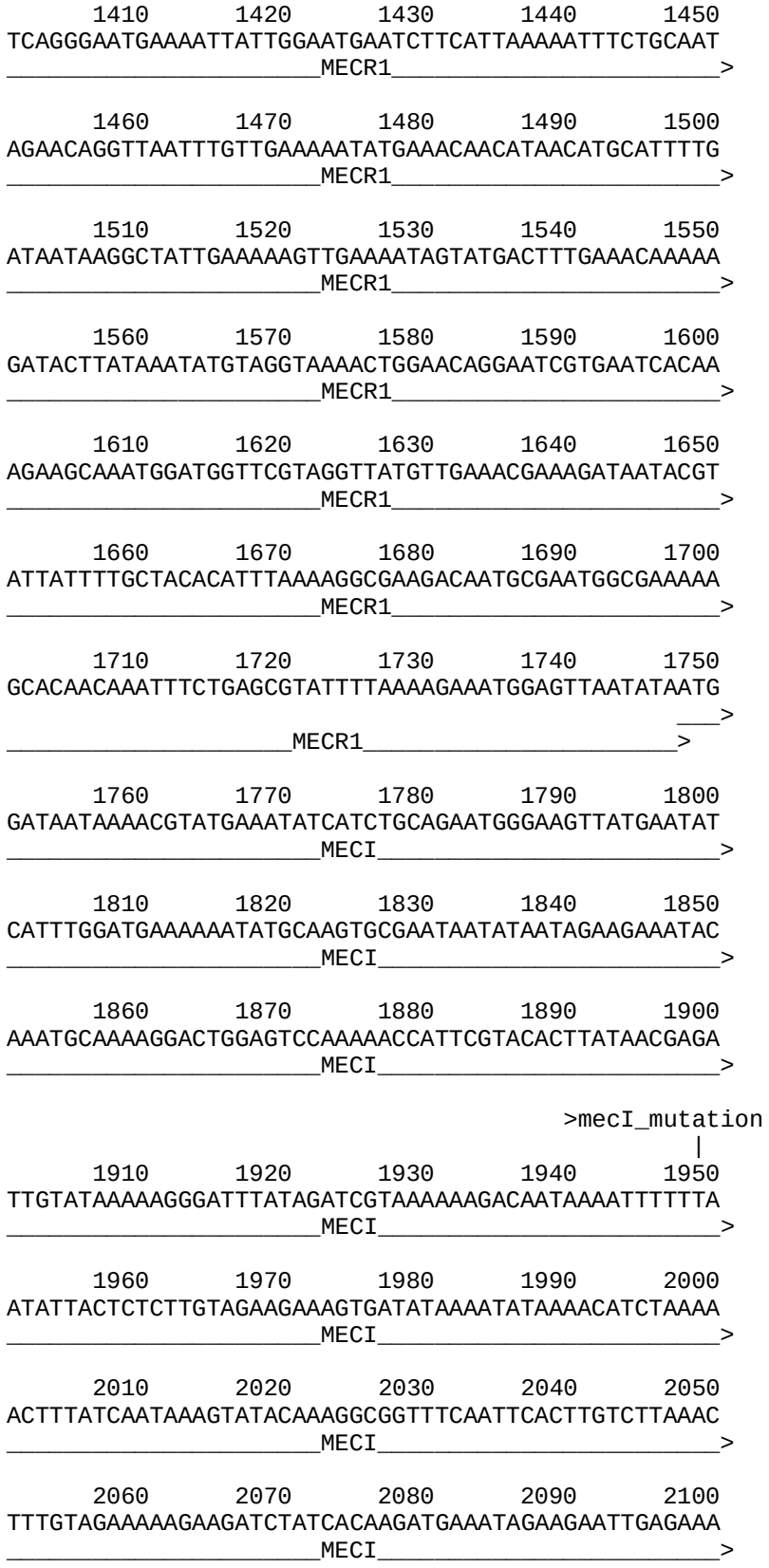
      1210      1220      1230      1240      1250
AAAATTTACTCTCATTAAATCATACTGAACAACAATGGGATAAACATCAA
_____________________MECR1_____________________>

      1260      1270      1280      1290      1300
TATCCATTTAAAGAATGGAACCAAGATCAAAATTTAAATTCTTCAATGAA
_____________________MECR1_____________________>

      1310      1320      1330      1340      1350
ATATTCAGTAAATTGGTATTACGAAAATTTAAACAAACATTTAAGACAAG
_____________________MECR1_____________________>

      1360      1370      1380      1390      1400
ATGAGGTTAAATCTTATTTAGATCTAATTGAATATGGTAATGAAGAAATA
_____________________MECR1_____________________>
```

A.2 continued

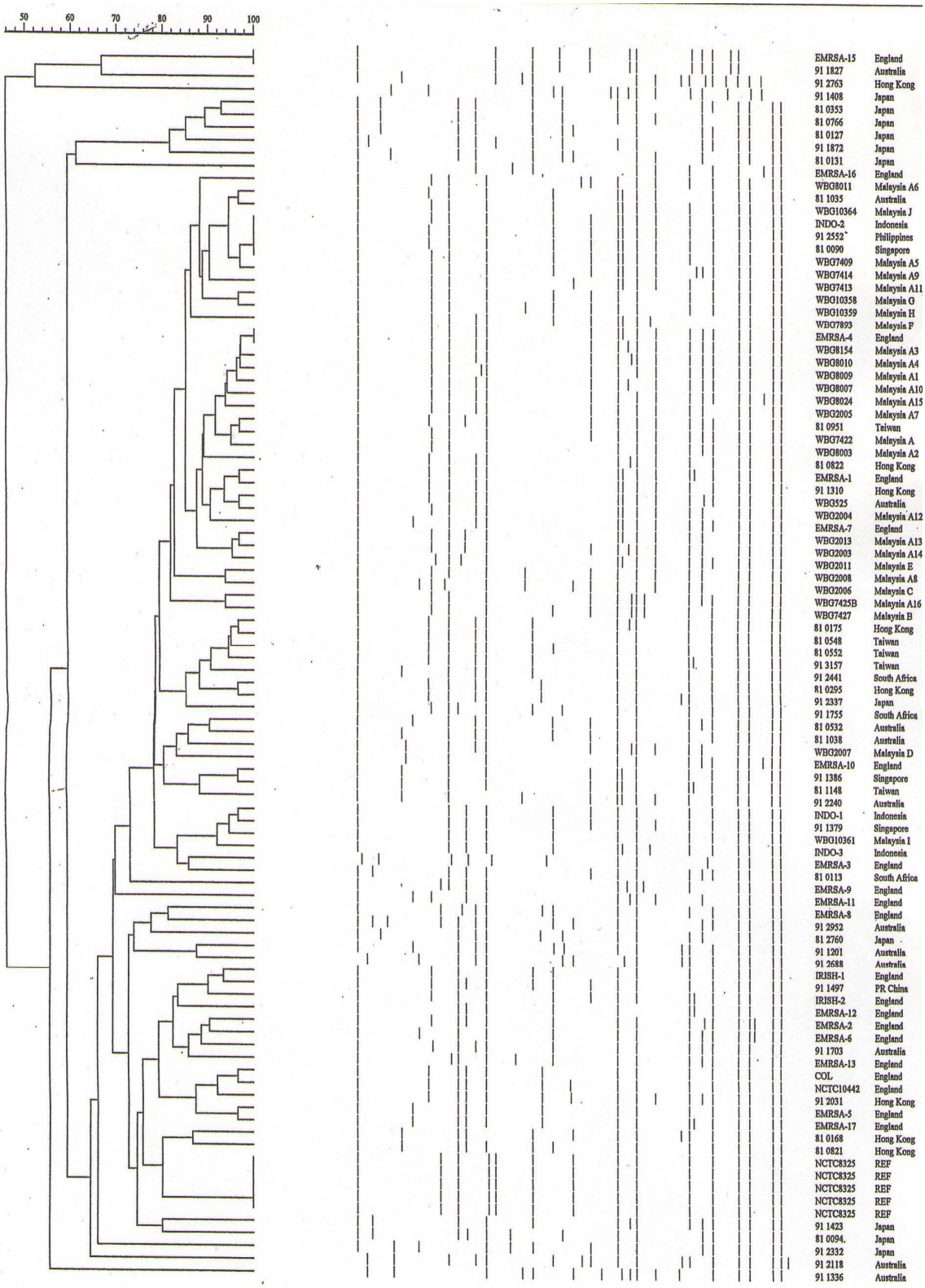


A.2 continued

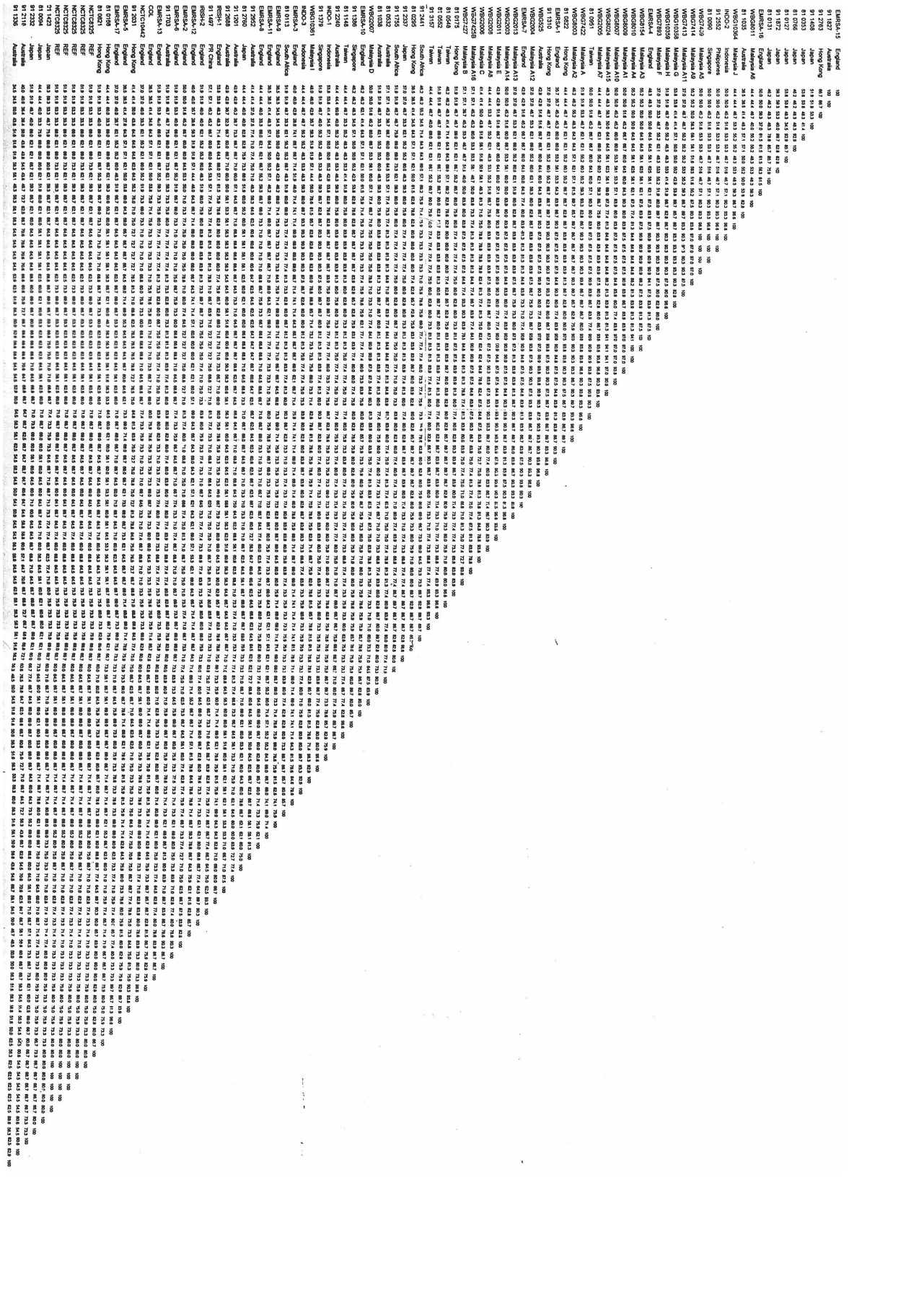
```
      2110      2120      2130      2140      2150
TATATTGAATAAAAAATAAAATTGTTGTGTTTACAACAATACATAGAAAA
_______MECI________>

      2160
CAGAGGAAACAATCAAGTC
```

A.3 Dendrogram of the CHEF patterns of the United Kingdoms (UK) EMRSA, all Asian-Pacific and South African isolates



A.4 The numerical matrix of the CHEF patterns of the United Kingdoms (UK) EMRSA, all Asian-Pacific and South African isolates



A.5 Publications derived from this project

Lim, T. T., Coombs, G. W. and Grubb, W. B. (2002) Genetic organization of *mecA* and *mecA*-regulatory genes in epidemic methicillin-resistant *Staphylococcus aureus* from Australia and England. *Journal of Antimicrobial Chemotherapy,* Vol. 50**,** No. 6, pp. 819-24.

Lim, T. T., Chong, F. N., O'Brien, F. G. and Grubb, W. B. (2003) Are all community methicillin-resistant *Staphylococcus aureus* related? A comparison of their *mec* regions. *Pathology,* Vol. 35**,** No. 4, pp. 336-43.

Murray, R. J., Lim, T. T., Pearson, J. C., Grubb, W. B. and Lum, G. D. (2004) Community-onset methicillin-resistant *Staphylococcus aureus* bacteremia in Northern Australia. *International Journal of Infectious Diseases,* Vol. 8**,** No. 5, pp. 275-83.

O'Brien, F. G., Lim, T. T., Chong, F. N., Coombs, G. W., Enright, M. C., Robinson, D. A., Monk, A., Said-Salim, B., Kreiswirth, B. N. and Grubb, W. B. (2004) Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia. *Journal of Clinical Microbiology,* Vol. 42**,** No. 7, pp. 3185-90.

O'Brien, F. G., Lim, T. T., Winnett, D. C., Coombs, G. W., Pearson, J. C., Delgado, A., Langevin, M. J., Cantore, S. A., Gonzalez, L. and Gustafson, J. E. (2005) Survey of methicillin-resistant *Staphylococcus aureus* strains from two hospitals in El Paso, Texas. *Journal of Clinical Microbiology,* Vol. 43**,** No. 6, pp. 2969–72.