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High-Throughput DNA Microarray Characterisation and Molecular Analysis of Resistance to Mupirocin, Fusidic Acid and Linezolid in Methicillin-Resistant *Staphylococcus aureus* Populations in Irish Hospitals 1971-2010

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy by

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May 2013

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Declaration

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"We need men who can dream of things that never were, and ask why not"

John F. Kennedy, speech to Dáil Éireann

Dublin, Ireland, 28th June, 1963.

Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen of humans and animals and can express multiple virulence and antimicrobial resistance genes. In Ireland, detailed molecular typing showed that different clonal types of nosocomial MRSA prevailed at different time periods over the last 40 years, with highly clonal ST22-MRSA-IV currently predominant. Informative typing plays a vital role in preventing the spread of MRSA. However, typing methods including pulsed-field gel electrophoresis (PFGE), mulitlocus-sequence typing (MLST), staphylococcal cassette chromosome *mec* (SCC*mec*) typing, *spa* and *dru* typing examine only small sections of the genome and often are not sufficiently discriminatory for epidemiological investigations, especially with isolates that exhibit limited genetic diversity. Furthermore, these approaches are not readily ameanble to high-througput screening or to outbreak investigations in real-time and do not detect virulence or resistance genes.

The aims of the present study were three-fold. Firstly to investigate the usefulness of the high-throughput StaphyType DNA microarray (Alere, Germany) for genotyping diverse MRSA strains and for differentiating between ST22-MRSA-IV isolates. This array can detect 334 *S. aureus* genes and alleles, including species-specific, antimicrobial resistance, and virulence-associated genes, as well as markers used for typing. Secondly, due to the increasing prevalence of high-level mupirocin resistance (Hi-MupR) and fusidic acid resistance among Irish MRSA the associated genotypes and mechanisms of resistance were investigated. Thirdly, detailed molecular characterisation was undertaken of the first ST8-MRSA-IVa/USA300 MRSA isolate found by array screening to harbour the multidrug resistance gene *cfr*.

A total of 175 isolates representative of MRSA clones that predominated in Irish hospitals between 1971-2004, previously typed by MLST and SCC*mec* typing, were subject to *spa* typing (175 isolates) and microarray profiling (107 isolates). The isolates belonged to 26 sequence type (ST)-SCC*mec* types and subtypes, and 35 *spa* types. The array assigned all isolates to the correct MLST clonal complex (CC) and 94% (100/107) were assigned an ST, with 98% (98/100) correlating with MLST. The array assigned all isolates to the correct SCC*mec* type but could only subtype some SCC*mec* elements. Additional SCC*mec*/SCC genes or DNA sequence variation not detected by SCC*mec* typing were detected by array profiling, including the SCC-fusidic acid resistance determinant Q6GD50/*fusC*. Apart from fusidic acid and trimethoprim resistance, the correlation between isolate antimicrobial resistance phenotype and specific resistance genes was >97%. The array showed that the majority of isolates harboured one or more superantigen (94%; 100/107) and immune evasion cluster (91%; 97/107) genes.

A total of 168 ST22-MRSA-IV isolates previously subject to PFGE, *spa* and *dru* typing and dendrogram group (DG) assignment underwent microarray profiling. Isolates were differentiated into 13 microarray groups (MGs) based on the presence or absence of the antimicrobial resistance genes *blaZ*, *erm*(C), *aacA-aphD, aadD, lnu*(A) and *ileS2* and the virulence genes and gene clusters *sec* & *sel, egc,* immune evasion cluster genes (IEC) and the arginine catabolic mobile element (ACME). The discriminatory power of the MGs (Simpson's Index of Diversity (SID), 83.60%) was greater than that of *spa* (SID, 66.9%), *dru* (SID, 77.8%) or PFGE (SID, 81.3%) typing. The highest SID was obtained by combining MGs and *dru* typing (SID, 94.5%), while MGs combined with either *spa* or PFGE typing yielded slightly lower SIDs (SID, 91.10% in both cases). The usefulness of the combination of *spa* typing

and MGs for differentiating the ST22-MRSA-IV isolates was analysed further. Combining both typing methods enhanced discrimination of isolates within particular *spa* types e.g. isolates exhibiting the predominant *spa* type t032 were differentiated into nine MGs. Comparison of the *spa*-MGs with the previously assigned DGs and available epidemiological information for a subset of isolates revealed good concordance demonstrating that the less complex approach of *spa*-MGs offers accurate and more detailed differentiation of ST22-MRSA-IV isolates for epidemiological investigations.

Microarray profiling of 110 fusidic acid-resistant Irish MRSA isolates recovered between 1971-2010 assigned the isolates to nine genotypes with ST22-MRSA-IV predominating (75%; 83/110). The fusidic acid resistance determinants *fusB* and *fusC* were detected in 10% (11/110) of isolates (ST59-MRSA-V, ST45-MRSA-IV, ST8-MRSA-IIB, ST1-MRSA-IV and ST30-MRSA-IV), but not among ST22-MRSA-IV isolates. A subset of isolates (n=22), including 20 ST22-MRSA-IV, were investigated for the presence of nucleotide changes in *fusA* by PCR and sequencing. Four combinations of predicted amino acid substitutions in FusA were identified including L461K, L461S, L461F, and A70V & F406K. For four ST22-MRSA-IV isolates, no mechanism of fusidic acid resistance was identified.

Microarray profiling of 109 Hi-MupR MRSA recovered in Irish hospitals between 1998-2009 identified the Hi-MupR gene *ileS2* in five genotypes including ST22-MRSA-IV (56%; 61/109), ST8-MRSA-IIA-IIE & SCC_{M1}/IVE/IVF (36%; 39/109), ST239-MRSA-III & SCC*Hg* (1%; 1/109), ST36-MRSA-II (7%; 8/109) and ST5-MRSA-IV (1%; 1/109). Plasmid analysis of seven of these isolates, representative of the three genotypes with more than one isolate, and the one ST239-MRSA-III & SCC*Hg* isolate revealed seven distinct *ileS2*-encoding conjugative plasmids ranging from ca. 33-45 kb. DNA sequencing of the IS*257*-flanking regions of *ileS2* of each plasmid revealed six distinct configurations.

Microarray profiling identified the staphylococcal *cfr* gene for the first time in a Panton-Valentine Leukocidin (*pvl*)-positive ST8-MRSA-IVa/USA300 MRSA isolate (M05/0060) from an Irish patient. *cfr* has mainly been associated with coagulase-negative staphylococci from animals and only a few *cfr*-positive MRSA have been reported. The *cfr* gene mediates resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A, termed the $PhLOPS_A$ phenotype. M05/0060 exhibited the $PhLOPS_A$ phenotype and *cfr* and a second phenicol resistance gene *fexA* were localised on a novel 45-kb conjugative plasmid, designated pSCFS7. Within pSCFS7, a DNA segment consisting of *cfr*, a truncated copy of IS*21-558* and a region with homology to the DNA invertase gene *bin3* of Tn*552* from *Bacillus mycoides* was integrated into the transposase gene *tnpB* of the *fexA*-encoding transposon Tn*558*.

The results of this study demonstrated the efficacy of microarray profiling for high-throughput genotyping of a diverse range of MRSA and for differentiating between highly clonal ST22-MRSA-IV. The findings of the study also highlight how the array can be used for high-throughput screening of isolates for virulence and resistance genes and to detect emerging strains with enhanced virulence and antimicrobial resistance potential. The identification of multiple conjugative Hi-MupR plasmids among diverse MRSA strains and a multidrug-resistant *cfr*-positive variant of ST8-MRSA-IVa/USA300 requires ongoing surveillance and highlights the potential for spread of such plasmids to other MRSA strains. The identification of multiple mechanisms of fusidic acid resistance among MRSA is also worrying and the mobile genetic elements carrying these determinants warrant detailed investigation.

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Abbreviations

ACME	arginine catabolic mobile element
EARS-Net	European Antimicrobial Resistance Surveillance Network (Formerly EARSS)
EARSS	European Antimicrobial Resistance Surveillance system
AR	typing antibiogram-resistogram typing group
ARI	adjusted rand index
ATP	adenosine triphosphate
BH	Beaumont hospital
BHI	Brain Heart infusion
BLAST	basic local alignment search tool
bp	base pair
BSIs	bloodstream infections
BURST	based upon repeated sequence type
BURP	based upon repeat pattern
C	cluster code
ca	approximately
CAP	community-acquired pneumonia
CBA	columbia blood agar
CA	community -associated
CC	clonal complex
CDC	Centers for Disease Control and Prevention
CHEF	clamped homogenous electrical field
CHIP	chemotaxis inhibitory protein
CIs	composite islands
CI	confidence intervals
CLSI	Clinical and Laboratory Standards Institute

cm^2	centimeter squared
CoNS	coagulase-negative staphylococci
^oC	degrees celsius
DG	dendrogram group
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dr	*dru* repeat
DR	direct repeat
dru	direct repeat unit
dt	*dru* type
dUTP	deoxyuridine Triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	for example
EF-G	elongation factor G
egc	enterotoxin gene cluster
EMRSA	epidemic MRSA
et al.,	and others
etc	et cetera
FdR	fusidic acid-resistant
F Factor	fertility factor
Fig.	figure
g	gravity
h	hours
HA	hospital-associated
hGISA	hetero-glycopeptide intermediate *Staphylococcus aureus*
Hi-MupR	high-level mupirocin resistance
HRP	horseradish peroxidase

I	intermediate
i.e.	that is
ica	polysaccharide intercellular adhesion locus
IEC	immune evasion cluster
IleS	isoleucyl-tRNA synthetase
IR	inverted repeat
IS	insertion sequence
IWG-SCC	International Working Group-Staphylococcal Cassette Chromosome
J	regions junkyard or jigsaw regions
kb	kilobase pair
L	litre
L agar	Luria Bertani agar
LA	livestock-associated
LEADER	Linezolid Experience and Accurate Determination of Resistance
ng	nanogram
nm	wavelength
NMRSARL	National MRSA Reference Laboratory
NaOH	sodium hydroxide
NCTC	National Collection of Type Cultures
NT	non-typeable
M	Molar
mec	methicillin resistance gene complex
mg	milligram
µg	microgram
MG	microarray group
MGEs	mobile genetic elements

MH	Mueller-Hinton agar
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
MLS	macrolide-lincosamide-steptogramin B
MLVF	multilocus variable fingerprinting
MLST	multilocus-sequence typing
MLVA	multilocus VNTR analysis
mM	millimolar
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	microbial surface components recognising adhesive matrix molecules
MSSA	methicillin-susceptible *Staphylococcus aureus*
MST	minimal spanning tree
ORF	open reading frame
PBS	phosphate buffered saline
PBPs/PSP2'	penicillin binding proteins/ penicillin binding protein 2'
PCR	polymerase chain reaction
PFG	pulsed-field group
PFGE	pulsed-field gel electrophoresis
PFT/PFG	pulsed-field type/pulsed-field group
pH	potential hydrogen
$PhLOPS_A$	phenicols, linosamides, oxazolidinones, pleuromutilins and streptogramin A compounds
PMSF	phenylmethysulfonyl fluoride
PSM	phenol-soluble modulin
PSMα	phenol-soluble modulin toxin
PVL	Panton-Valentine leukocidin

R	resistant
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
s	seconds
S	susceptible
SAgs	Staphylococcal superantigens
SARI	The Strategy for the Control of Antimicrobial Resistance in Ireland
SCC	staphylococcal cassette chromosome
SCC-CI	staphylococcal cassette chromosome-composite island
SCC*mec*	staphylococcal cassette chromosome *mec*
SCIN	staphylococcal complement C3 convertase inhibitor
SDS	sodium lauryl sulphate
SE	staphylococcal enterotoxins
SID	Simpson's index of diversity
SNP	single nucleotide polymorphisms
spa	staphylococcal protein A
SSSS	staphylococcal scalded skin syndrome
SSTIs	skin and soft tissue infections
ST	sequence type
23S rRNA	23 subunit ribosomal RNA
TBE	buffer tris-borate/EDTA electrophoresis buffer
TAE	buffer tris acetate/EDTA electrophoresis buffer
TE	buffer tris-EDTA buffer
Tn	Transposon
TMB	tetramethyl benzidine

TRST	tandem-repeat sequence typing
TMP	trimethoprim
TSA	trypticase soy agar
TST	toxic shock toxin
TSST-1	toxic shock syndrome toxin
T4CP	*traG*-like protein called type IV coupling protein
T4SS	type IV secretion system
µg	microgram
UK	United Kingdom
µl	microliter
Unf	unfamiliar
UPGMA	unweighted pair group method with arithmetic averages
USA	United States of America
UV	ultraviolet
v/v %	volume in volume: express the number of millilitres of an active constituent in 100 milliliters of solution
VNTR	variable number of tandem repeat
VRE	vancomycin resistant enterococci
VISA	vancomycin-intermediate Staphylococcus aureus
VRSA	vancomycin-resistant Staphylococcus aureus
W	Wallace coefficient
w/v %	volume in volume: express the number of grams of an active constituent in 100 milliliters of solution
%	percentage
≥	greater than or equal to
≤	less than or equal to
>	greater than
<	less than

Δ	deletion (of part of a gene)
^{o}C	degrees centigrade
n	number
α	alpha
β	beta
γ	gamma
′	prime

Publications

Some of the original work presented in this thesis has been published in a refereed international publication as listed below. Offprints of the proofs of the manuscripts are included at the end of the thesis

Shore AC, Brennan OM, Deasy EC, Rossney AS, Kinnevey PM, Ehricht R, Monecke S, Coleman DC. 2012. DNA microarray profiling of a diverse collection of nosocomial methicillin-resistant *Staphylococcus aureus* isolates assigns the majority to the correct sequence type and staphylococcal cassette chromosome *mec* (SCC*mec*) type and results in the subsequent identification and characterization of novel SCC*mec*-SCC_{MI} composite islands. *Antimicrob. Agents Chemother.* **56**: 5340-55.

Shore AC, Brennan OM, Ehricht R, Monecke S, Schwarz S, Slickers P, Coleman DC. 2011. Identification and characterisation of the multidrug resistance gene *cfr* in a Panton-Valentine leukocidin-positive sequence type 8 methcillin-resistant *Staphylococcus aureus* IVa (USA300) isolate. *Antimicrob. Agents Chemother.* **54**: 49780-85.

Shore AC, Rossney AS, Kinnevey PM, Brennan OM, Creamer E, Sherlock O, Dolan A, Cunney R, Sullivan DJ, Goering RV, Humphreys H, Coleman DC. 2010. Enhanced discrimination of highly clonal ST22-methicillin-resistant *Staphylococcus aureus* IV isolates achieved by combining *spa*, *dru*, and pulsed-field gel electrophoresis typing data. *J. Clin. Microbiol.* **48**: 1839-52.

Chapter 1

General introduction

1.1 *Staphylococcus aureus*

The staphylococci are Gram-positive, catalase positive, facultative anaerobes that belong to the *Micrococcaceae* family. More than 30 species of staphylococci have been described to date they can be differentiated into two large groups based on the production of the protein coagulase that causes the fibrin of blood plasma to clot. The coagulase-negative staphylococci (CoNS) are commensals of animal and human skin and are typically opportunistic pathogens commonly causing infections of indwelling and implanted devices. In contrast, the most pathogenic and clinically relevant staphylococcal species, *Staphylococcus aureus*, belongs to the coagulase-positive staphylococci.

Staphylococcus aureus colonises the anterior nares of animals and humans. Approximately 30% of healthy humans carry *S. aureus* in their anterior nares (van Belkum *et al.*, 2009b). Other sites of carriage include the throat, axilla, perineum, the gastroinstestinal tract and the groin where *S. aureus* can form part of the normal flora. *Staphylococcus aureus* is also a versatile pathogen of animals and humans and can cause a wide range of infections including skin and soft tissue infections (SSTIs), urinary tract infections, food poisoning and toxic shock syndrome. It is commonly associated with serious and life-threatening hospital-associated (HA) infections such as septicaemia, pneumonia, osteomyelitis and endocarditis, particularly in patients whose immunity has been compromised. While it is often the patient's own normal flora that causes the infection, *S. aureus* can be spread from person to person by direct contact and through contact with contaminated surfaces, objects and air (Sexton *et al.*, 2006; Gehanno *et al.*, 2009).

1.2 Antibiotic resistance in *S. aureus*

Antibiotic resistance in *S. aureus* emerged soon after the introduction of penicillin and sulphonamides into clinical practice in the 1940s (Kirby, 1944; Barber and Rozwadowska-Dowzenko, 1948) (Fig. 1.1). Resistance to penicillin in *S. aureus* can be mediated by a pencillinase (β-lactamase) plasmid that encodes a penicillinase enzyme that hydrolyses the β-lactam ring of penicillin, thus inactivating the antimicrobial. Many *S. aureus* infections in hospitals and communities in the 1950s were due to a *S. aureus* clone known as phage-type 80/81. However, this clone disappeared after the introduction of the semisynthetic, penicillinase-resistant β-lactam antibiotic, methicillin, in 1959, to treat infections caused by pencillin-resistant *S. aureus* (Rountree and Freeman, 1955). Just two years later, methicillin-resistant *S. aureus* (MRSA) were first reported in the U.K. (Fig. 1.1) (Jevons, 1961). Subsequently many different antimicrobial agents were discovered and were used to treat MRSA infections but resistance emerged to each of these agents, in some cases very shortly after they were introduced into clinical practice (Fig. 1.1). In the late 1970s, multi-resistant MRSA strains causing serious infections were reported in hospitals across Europe (Shanson, 1981). During the 1980's, the prevalence of MRSA as a cause of HA infections increased significantly worldwide (van Belkum *et al.*, 2009a). Since then HA-MRSA has emerged as a global problem. More recently community-associated (CA) MRSA have emerged as a significant cause of infection among healthy individuals in the community and among animals (Fig. 1.1).

While resistance to antimicrobial agents can arise through spontaneous mutations, many genes encoding antimicrobial resistance in *S. aureus* are carried on mobile genetic elements (MGEs) that play an essential role in the horizontal transfer and dissemination of these genes. This includes plasmids, transposons and

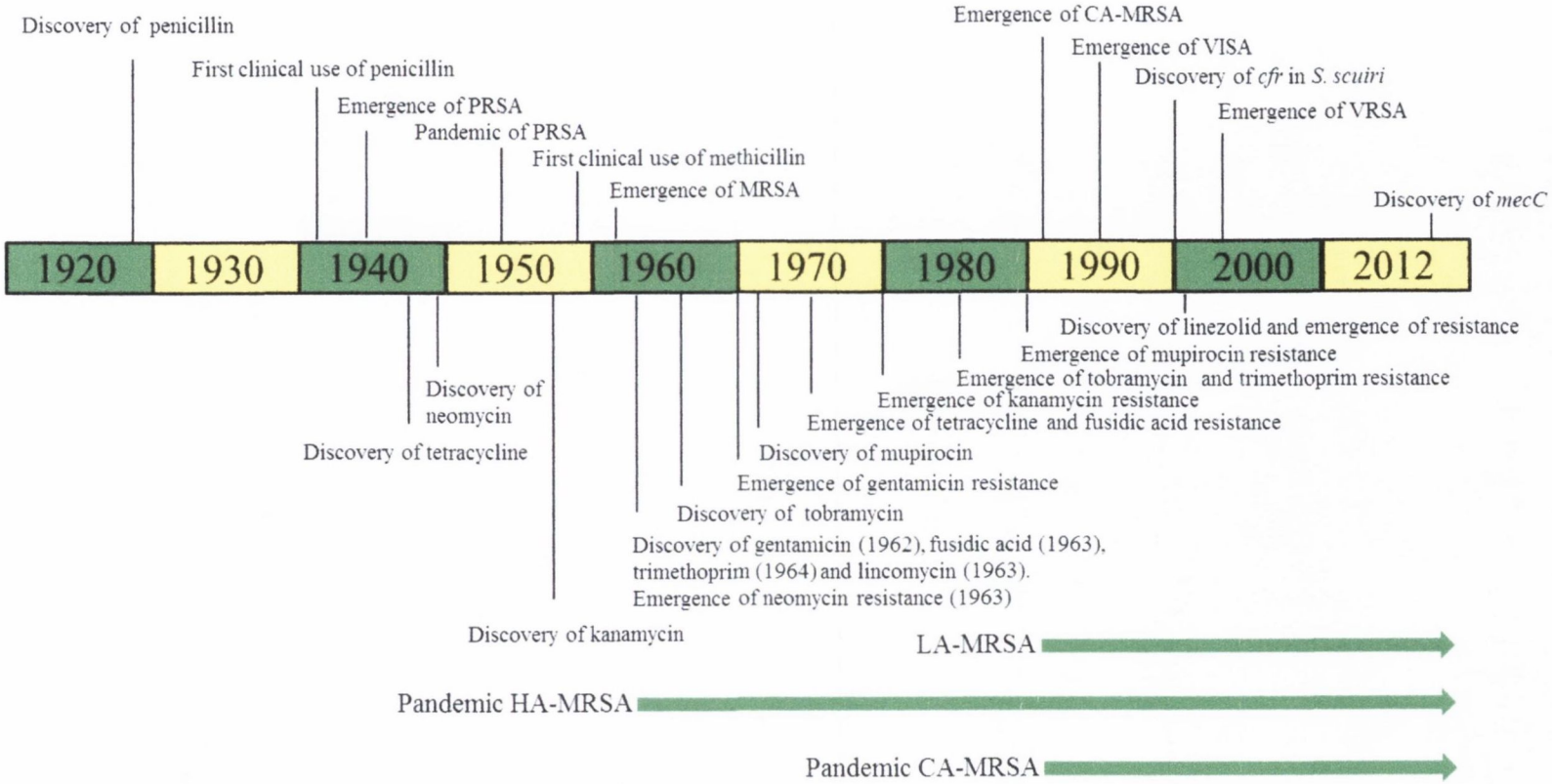


Fig. 1.1 Timeline for the emergence of antibiotic resistance in *S. aureus*. The timeline indicates the year in which an event occurred or was reported. Arrows indicate approximate length of time for each pandemic/epidemic.

Abbreviations: HA, hospital-associated; CA, community-associated; LA, livestock-associated; PRSA, penicilin resistant *S. aureus*; VISA, vancomycin intermediate *S. aureus*; VRSA, vancomycin resistant *S. aureus*.. Adapted from De Leo and Chambers (2009).

staphylococcal cassette chromosome elements [SCC](Malachowa and DeLeo, 2010). These genetic elements can be horizontally transferred from one cell to another by three different methods; (a) transformation, is the naturally uptake of free DNA from the environment by the recipient cell or this process can be replicated articially. By the processes of electrotransformation or protoplast transformation, the recipient cell membrane is damaged by heat shock creating pores in the membrane for free DNA such as plasmids to transfer into the recipient cells; (b) transduction, whereby bacteriophages transduce DNA by infecting and lysing donor cells and subsequently introducing this DNA following infection into recipient cells; (c) conjugation, conjugative plasmids mediate direct contact between donor and recipient cells followed by transfer of plasmid DNA into the recipient (Grohmann *et al.*, 2003). Table 1.1 provides an overview of *S. aureus* antimicrobial resistance genes located on MGEs, in particular those that were detected during the course of the present study, and includes details of their resistance phenotypes, resistance mechanisms, and examples of the various MGEs that have been found to carry these resistance genes. The antimicrobial resistance genes that were investigated in detail in the present study are discussed in detail below (section 1.2.1).

1.2.1 Mechanisms of antibiotic resistance in *S. aureus*

1.2.1.1 Methicillin resistance

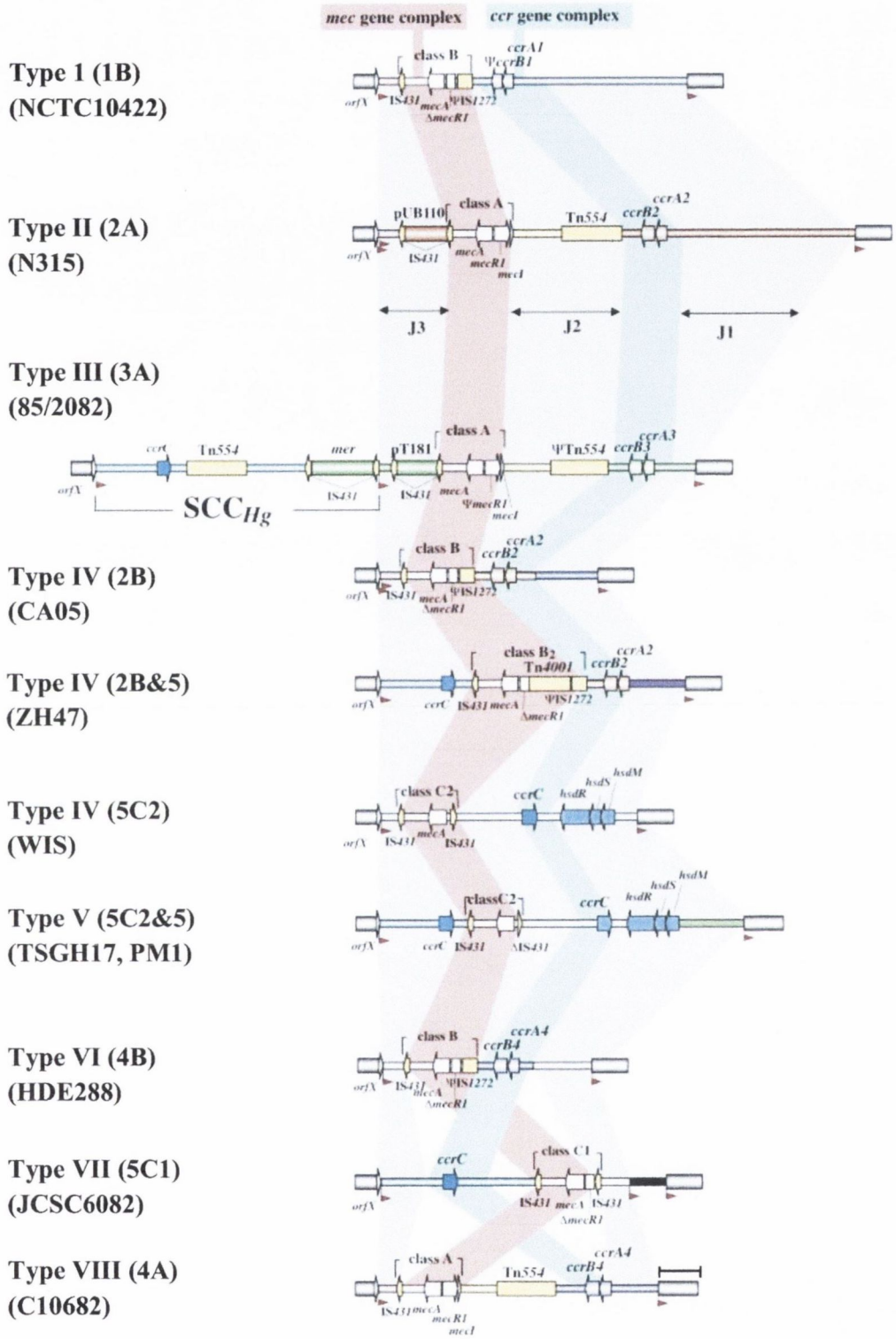
Methicillin is a β-lactam antibiotic that inhibits bacterial growth by binding to the enzymatic site of penicillin binding proteins (PBPs) and inhibiting bacterial cell wall synthesis (Labischinski, 1992). All MRSA express an extra PBP termed PBP2a or PBP2', which can perform all PBP reactions necessary for cell wall synthesis when all other PBPs have been inactivated by β-lactam antibiotics (Hartman and Tomasz, 1984; Reynolds and Brown, 1985). This additional PBP is encoded by the methicillin

resistance gene *mec* (Ito *et al.*, 1999; Antignac and Tomasz, 2009; Shore *et al.*, 2011a).The *mec* gene residues on a large MGE termed staphylococcal chromosomal cassette *mec* (SCC*mec*) which integrates into the *S. aureus* chromosome at the attachment site $attB_{SCC}$ (also termed insertion site sequence ISS), which is located at the 3' end of an open reading frame termed *orfX* encoding an rRNA methyltransferase that methylates *S. aureus* 70S ribosome (Ito *et al.*, 1999; Katayama *et al.*, 2000; Hiramatsu *et al.*, 2001; Boundy *et al.*, 2012). The SCC*mec* element integrates into *orfX* of methicillin susceptible *S. aureus* (MSSA) and thus gives rise to MRSA. These complex genetic elements often contain integrated plasmids and transposons conferring resistance to other antimicrobials.

1.2.1.1.1 SCCmec

To date, eleven SCC*mec* types have been described in MRSA (SCC*mec* I-XI) (Fig. 1.2). Each SCC*mec* type has a different combination of the *mec* and *ccr* gene complexs. The *mec* gene complex consists of the *mec* gene, and, if present, its regulatory genes *mecI* and *mecR1* (Hiramatsu *et al.*, 2001). Two distinct *mec* gene types, *mecA* and *mecC*, have been described to date in MRSA, *mecA* in association with SCC*mec* type I-X and *mecC* with SCC*mec* XI only (Shore *et al.*, 2011a; Ito *et al.*, 2012). Three *mecA* allotypes and two *mecC* allotypes each sharing $\geq$70% but <95% nucleotide sequence identity, have been described in staphylococci, namely *mecA, mecA1, mecA2, mecC* and *mecC1* but only *mecA* and *mecC* have been described in MRSA (Harrison *et al.*, 2012; Ito *et al.*, 2012).

Five classes of the *mec* gene complex (A-E) and several variants have been identified among staphylococcal species and vary in their genetic structure (Katayama *et al.*, 2001; Shore *et al.*, 2005; Kinnevey *et al.*, 2013). In some *mec* gene complexes,


mec gene complex
ccr gene complex
Type 1 (1B)
(NCTC10422)
class B
ccrA1
ΨccrB1
orfX
IS431
mecA
ΨIS1272
ΔmecR1
Type II (2A)
(N315)
pUB110
class A
Tn554
ccrA2
ccrB2
orfX
IS431
mecA
mecR1
mecI
J3
J2
J1
Type III (3A)
(85/2082)
ccrC
Tn554
mer
pT181
class A
ΨTn554
ccrA3
ccrB3
orfX
IS431
IS431
mecA
ΨmecR1
mecI
SCC$_{Hg}$
Type IV (2B)
(CA05)
class B
ccrA2
ccrB2
orfX
IS431
mecA
ΨIS1272
ΔmecR1
Type IV (2B&5)
(ZH47)
class B2
Tn4001
ccrA2
ccrB2
orfX
ccrC
IS431
mecA
ΨIS1272
ΔmecR1
Type IV (5C2)
(WIS)
class C2
ccrC
hsdM
hsdS
hsdR
orfX
IS431
mecA
IS431
Type V (5C2&5)
(TSGH17, PM1)
classC2
ccrC
hsdM
hsdS
hsdR
orfX
ccrC
IS431
ΔIS431
Type VI (4B)
(HDE288)
class B
ccrA4
ccrB4
orfX
IS431
mecA
ΨIS1272
ΔmecR1
Type VII (5C1)
(JCSC6082)
ccrC
class C1
orfX
IS431
mecA
IS431
ΔmecR1
Type VIII (4A)
(C10682)
class A
Tn554
ccrA4
ccrB4
IS431
mecA
mecR1
mecI

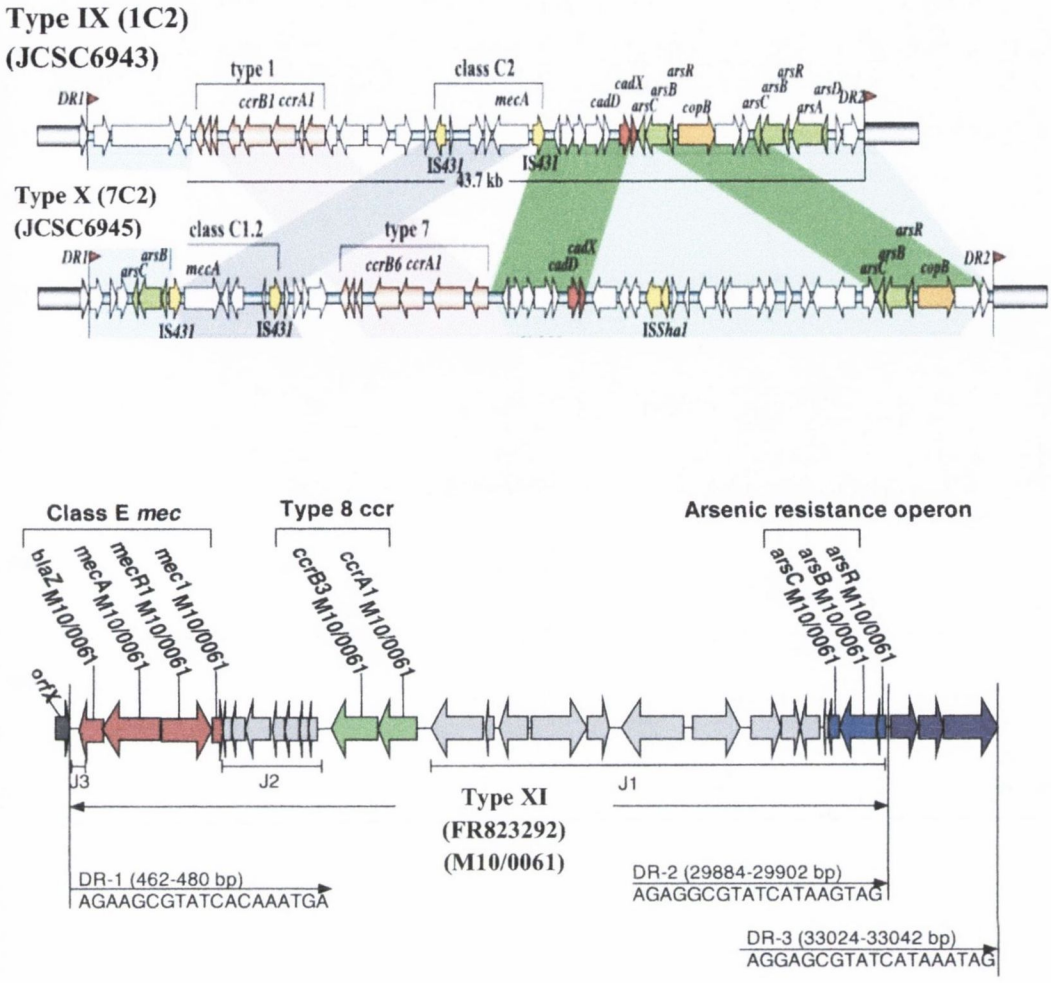


Fig. 1.2 Schematic representation of the genetic organisation of SCC*mec* types I-XI. For each SCC*mec* element, apart from SCC*mec* XI, the *ccr* and *mec* complex type is shown in parenthesis after the SCC*mec* name and the prototype MRSA strain from which it was identified are shown in parenthesis underneath. For SCC*mec* XI, the prototype strain is listed under the SCC*mec* element and the *ccr* and *mec* complex genes are shown in the diagram. Accesion numbers: SCC*mec* I, AB033763; SCC*mec* II, D86934; SCC*mec* type III*mer,* AB037681; SCC*mec* type IV, AB0633172 (CA05) and AM292304 (ZH47); SCC*mec* type VI, AF411935 (HDE288); SCC*mec* type VII, AB373032 (JCSC6082); SCC*mec* type VIII, C10682 (C10682); SCC*mec* type IX, AB505629 (JCSC6943); SCC*mec* X AB505630 (JCSC6945) and SCC*mec* XI FR823292 (M10/0061). Accession numbers are shown in parenthesis beside prototype strains. Adapted from IWG-SCC, (2009), Li *et al.* (2011) and Shore *et al.* (2011).

Table 1.1 *Staphylococcus aureus* antimicrobial resistance determinants carried on mobile genetic elements (MGEs) and investigated in the present study

Resistance gene	Resistance phenotype	MGE	Resistance mechanism	Reference
aphA3	Kanamycin, neomycin, strepthothricin, streptomycin	Tn*5404*/ Tn*5405*	Aminoglycoside phosphotransferase	Derbise *et al.*, 1996
aadD	Kanamycin, neomycin & tobramycin	pUB110	Aminoglycoside adenyltransferase	Byrne *et al.*, 1991
aacA-aphD	Gentamicin, kanamycin & tobramycin	Tn*4001*	Aminoglycoside acetyltransferase	Rouch *et al.*, 1987
blaZ	Pencillin (β-lactam antibiotics)	pI258/Tn*552*	β-lactamase	Wang and Novick, 1876; Sidhu *et al.*, 2002
cat	Chloroamphenicol	*cat*-pC221	Chloroamphenicol acetyltransferase	Projan *et al.*, 1985
cfr	Phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A	pSCFS1, pSCFS3, pSCFS6	Target site modification (rRNA methylation)	Schwarz *et al.*, 2000; Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007; Kehrenberg *et al.*, 2009
dfrS1	Trimethoprim	Tn*4003*	Dihydrofolate reductase	Rouch *et al.*, 1989
dfrD	Trimethoprim	pABU17	Dihydrofolate reductase	Dale *et al.*, 1995
dfrK	Trimethoprim	Tn*559*	Dihydrofolate reductase	Kadlec and Schwarz, 2010
erm(A), *erm*(B), *erm*(C)	Macrolides, lincosamides, streptogramin B	Tn*554*, pI258, pE194	Target site modification (rRNA methylation)	Novick *et al.*, 1979; Horinouchi and Weisblum 1982; Murphy *et al.*, 1985
fexA	Chloramphenicol and florfenicol	Tn*558*	Active efflux	Kehrenberg and Schwarz, 2005
fusB	Fusidic acid	pUB101	Ribosome-protection	O'Brien *et al.*, 2002
fusC	Fusidic acid	SCC476, SCC*mec*$_{N1}$ SCC*mec*-SCC-SCC-*CRISPR*	Ribosome-protection	Kinnevey *et al.*, 2013
fosB	Fosfomycin	pIP1842-like plasmid	Phosphoenolpyruvate synthetase	Etienne *et al.*, 1991
ileS2	Mupirocin	pPR9, pUSA30, pV030 (NC_010279), pGO400	Isoleucyl t-RNA synthetase	Morton *et al.*, 1995; Diep *et al.*, 2006; Perez Roth *et al.*, 2010
lnu(A)	Lincomycin	pUR5425	Lincosamide nucleotidyltransferase	Lozano *et al.*, 2012
mecA & *mecC*	Methicillin	SCC*mec* elements	Pencillin-binding protein, PBP2 or PBP2'	Beck *et al.*, 1986; Shore *et al.*, 2011

Continued overleaf

Table 1.1 continued. *Staphylococcus aureus* antimicrobial resistance determinants carried on mobile genetic elements (MGEs) and investigated in the present study

Resistance gene	Resistance phenotype	MGE	Resistance mechanism	Reference
merA & *merB*	Mercury compounds	SCC/ pI258	Mercuric reductase	Babich *et al.*, 1991; Monecke *et al.*, 2009
msr(A), *mph*(C), *mef*(A)	Macrolides and streptogramin B	pMS97/ Tn*1207.1*	Macrolide active efflux, macrolide phosphotransferase	Matsuoka *et al.*, 1998; Luna *et al.*, 2002
qacA/C	Quaternary ammonium compounds	pSK57, pSK89	Active Efflux	Lyon and Skurray, 1987
tet(K)	Tetracycline	pT181	Active efflux (major facilitator superfamily)	Guay and Rothstein, 1993
tet(L)	Tetracycline	pSTE1	Active efflux (major facilitator superfamily)	Schwarz *et al.*, 1992
tet(M)	Tetracycline	Tn*916*	Ribosomal-protective protein	Nesin *et al.*, 1990
vanA	Vancomycin/teicoplanin A type resistance protein	Tn*1546,* pLW1043	Alternation of disaccharide pentapeptide cell wall	Weigel *et al.*, 2003; Courvalin, 2006
vat(A)	Streptogramin type A	pIP680	Steptogramin acetyltransferases	Allignet and EI Solh,1999
vga(A), *vgb*(A)	Streptogramin type A & B, lincosamides and pleuromutilins	pAMβ1, pSX267	Active efflux, inactivation by virginiamycin B lyase	Allignet and EI Solh,1999

the *mec* regulatory genes *mecR1*, encoding the signal transducer protein MecR1, and *mecI*, encoding the repressor protein MecI, are truncated due to the integration of insertion sequences IS*1182*, IS*1272* or IS*431* (Katayama *et al.*, 2001; Lim *et al.*, 2003; Ito *et al.*, 2004b). Recently, an anti repressor *mecR2* gene has been characterised. The *mecR2* gene was shown in *in vitro* and *in vivo* assays to be co-transcribed with *mecRI-mecI* and was found to be essential for the induction of *mecA* and expression of β-lactam resistance in MRSA strains (Arêde *et al.*, 2012).

The *ccr* gene complex is composed of the *ccr* gene(s) *ccrA* and *ccrB* together (*ccrAB*) or *ccrC*, and an associated open reading frame ORF, termed *ccrAA* located upstream of *ccrC* (Katayama *et al.*, 2000; Ito *et al.*, 2004a; Monecke *et al.*, 2011b). The *ccr* genes encode serine proteases that catalyse excision and integration of SCC*mec* elements, and eight types of the *ccr* gene complex have been reported to date in MRSA, each with a different combination of *ccrA* and *ccrB* allotypes or *ccrC* (www.sccmec.org) (Ito *et al.*, 2001a; IWG-SCC, 2009).

Novel *ccr* genes and subtypes are assigned new designations based upon guidelines published in 2009 (IWG-SCC, 2009). However, the nomenclature is complicated by the fact that not all *ccr* variants reported have been are assigned allelic numbers. Recently, five alleles of the *ccrA4* and *ccrB4* allotypes an along with 10 alleles of the *ccrC1* allotype have been characterised in both MRSA and CoNS (Mongkolrattanothai *et al.*, 2004; Oliveira *et al.*, 2006; Ender *et al.*, 2007; Hanssen and Sollid, 2007; Chen *et al.*, 2009; Zhang *et al.*, 2009; Chlebowicz *et al.*, 2010; Monecke *et al.*, 2011a; Urushibara *et al.*, 2011).

The SCC*mec* regions outside of the *ccr* and *mec* gene complexes are designated the "joining" regions (J) (Fig. 1.2) (IWG-SCC, 2009). These regions can harbour plasmids and transposons that carry additional antimicrobial resistance determinants (Ito

et al., 2003; Holden *et al.*, 2004; Mongkolrattanothai *et al.*, 2004). Each SCC*mec* element can have structural differences in the J regions and are therefore classified further into subtypes based on the presence or absence in the J regions of characteristic genes, pseudogenes, or non-coding regions and MGEs (IWG-SCC, 2009).

SCC*mec* elements are one type of a large group of complex elements known as SCC elements. Others members of this group include; (a) SCC element that lack *mecA* but often encoding additional antimicrobial resistance and virulence genes e.g. SCC*fus*, SCC_{CRISPR}, SCC*cap1* and SCC_{pbp4}; (b) pseudo-SCC*mec* elements that carry *mec* complex genes but lack *ccr* e.g. ψSCC*mec* II.5, $\psi SCCmec_{16691}$ and $\psi SCCmec_{M06/0171}$; (c) pseudo-SCC elements that lack *ccr* and *mec* complex genes but integrate at the ISS e.g. ACME, SCC*mec* remnants and chromosome cassettes and (d) composite islands consisting or two or more elements e.g. SCC_{M1}, ψSCC*arc* & SCC*mec* IVa, ψ $SCCmec_{M06/0171}$, $SCC_{M06/0171}$,SCC_{CRISPR}, and ΔACME II, ΔJ1 SCC*mec* I & SCC*mec* IVh (Luong *et al.*, 2002; Holden *et al.*, 2004; Mongkolrattanothai *et al.*, 2004; Diep *et al.*, 2006; Arakere *et al.*, 2009; Han *et al.*, 2009; Chen *et al.*, 2010c; Bartels *et al.*, 2011; Shore *et al.*, 2011a; Shore *et al.*, 2011b; Lindqvist *et al.*, 2012; Kinnevey *et al.*, 2013).

SCC*mec* elements have also been found in CoNS and the presence of similar, and in some cases the same SCC*mec* elements in *S. aureus* and CoNS, as well as the diversity and abundance of SCC*mec* among CoNS has lead many researchers to speculate that they are a reservoir for SCC*mec* in *S. aureus*. While the mechanism and exact route of SCC*mec* transfer is still unknown several different studies have provided evidence that suggest that *mec* and SCC as individual genetic components originated in CoNS, particularly those associated with animals (Wisplinghoff *et al.*, 2003; Mongkolrattanothai *et al.*, 2004; Takeuchi *et al.*, 2005; Bloemendaal *et al.*, 2010; Zong and Lu, 2010; Yokoi *et al.*, 2011; Monecke *et al.*, 2012b). In particular, the

chromosomally located *mecA1* gene in *Staphylococcus sciuri* with no associated *mec* regulatory or SCC genes, *mecA* and its associated *mec* gene complex in *Staphylococcus fleuretti*, *mecB* with *mec* regulatory genes and *blaZ* adjacent to a SCC element in *Macrococcus caseolyticus*, and *mecC* with *mec* regulatory genes and *blaZ* on SCC*mec* XI, all represent ancestral forms of SCC*mec* (Wu *et al.*, 1998; Tsubakishita *et al.*, 2010a; Tsubakishita *et al.*, 2010b; Shore *et al.*, 2011a; Monecke *et al.*, 2012b).

1.2.1.2 Aminoglycoside resistance

Aminoglycosides such as kanamycin, gentamicin, tobramycin and neomycin are often used individually or in combination with other antimicrobial agents to treat *S. aureus* infections (Graham and Gould, 2002). Three different genes encoding resistance to various combinations of aminoglycosides have been described in *S. aureus*, including *aadD, aacA-aphD* and *aphA3* (Table 1.1). These aminoglycoside resistance genes have been located to various plasmids and transposons and encode various adenyltransferase, acetyltransferase and phosphotransferase enzymes, which interrupt protein synthesis by binding to the bacterial 30S ribosomal subunit (Schwarz *et al.*, 2011) (Table 1.1).

1.2.1.3 Tetracycline resistance

Tetracyclines are broad-spectrum antibiotics which are widely used in the treatment of both human and animal *S. aureus* infections, and resistance to tetracyclines is encoded by numerous resistance determinants. These resistance determinants can be differentiated into two groups based on their mechanism of action; (a) active efflux resulting from the acquisition of the plasmid-mediated genes *tet*(K) and *tet*(L) (Schwarz *et al.*, 1992; Guay and Rothstein, 1993) and (b) ribosomal protection mediated by the transposon- or chromosomally-located genes *tet*(M) and *tet*(O) (Nesin *et al.*, 1990; Schwarz *et al.*, 1998) (Table 1.1).

1.2.1.4 Fusidic acid resistance

Fusidic acid is an antibiotic derived from *Fusidium coccineum*. It has been used in Europe since the 1960's for the treatment of SSTIs as well as for bone infections (Spelman, 1999; Wang *et al.*, 2012a). Fusidic acid interacts with translation elongation factor G (EF-G) on the ribosome, thus inhibiting protein synthesis by halting the release of EF-G (Bodley *et al.*, 1969). However, mutations can occur in the chromosomal gene, *fusA,* encoding EF-G resulting in resistance to fusidic acid. Numerous different mutations resulting in amino acid substitutions within the structural domain of EF-G have been identified in fusidic acid-resistant *S. aureus* isolates (Laurberg *et al.*, 2000; Castanheira *et al.*, 2010b; Farrell *et al.*, 2011a). Resistance to fusidic acid in *S. aureus* small colony variants has been associated with mutations in the *rplF* gene encoding the ribosomal protein L6 FusE (Norstrom *et al.*, 2007).

Resistance to fusidic acid in *S. aureus* also arises as a result of the acquisition of exogeneous genes, namely, the plasmid-located *fusB* (*far1*) gene or the SCC-located *fusC(Q6GD50)* gene (Table 1.1). These genes encode proteins which prevent fusidic acid from interacting with EF-G thus protecting the translocation of the EF-G protein (O'Brien *et al.*, 2002; O'Neill and Chopra, 2006). The *fusB* and *fusC* genes have been identified in *S. aureus*, *Staphylococcus intermedius* and *Staphylococcus epidermidis* (O'Neill *et al.*, 2007; McLaws *et al.*, 2008). While an additional fusidic acid resistance gene *fusD* has been identified in *Staphylococcus saprophyticus* only (O'Neill *et al.*, 2007) (Table 1.1).

1.2.1.5 Trimethoprim resistance

Trimethoprim (TMP) is an antimicrobial agent often used in combination with sulfamethoxazole (co-trimoxazole) to treat *S. aureus* infections. It is an inhibitor of bacterial dihydrofolate reductase (DHFR), a key enzyme in the folate biosynthesis pathway leading to folate levels becoming depleted and subsequent inhibition of bacterial growth (Roccaforte *et al.*, 1988). Many different mechanisms of trimethoprim resistance have been described in *S. aureus*, the most common being due to the presence of an additional plasmid mediated *dfrS1* gene (Rouch *et al.*, 1989) (Table 1.1). Less commonly resistance has been reported to be due to; (a) point mutations in the chromosomally located dihydrofolate reductase B gene, *dfrB*, resulting in low to intermediate resistance (Dale *et al.*, 1993), (b) the chromosomally-located dihydrofolate reductase G (*dfrG*) (Sekiguchi *et al.*, 2005) or (c) a dihydrofolate reductase K gene, *dfrK*, identified on a novel *dfrK*-carrying transposon Tn*559* (Kadlec and Schwarz, 2010b). Various other TMP-resistance genes have been reported in CoNS, such as the chromosomally located dihydrofolate reductase *dfrC*, which was identified in *S. epidermidis* (Dale *et al.*, 1995a) and dihydrofolate reductase D (*dfrD*), which was detected on a plasmid pABU17 in *Staphylococcus haemolyticus* strain MUR313 (Dale *et al.*, 1995b).

1.2.1.6 Resistance to macrolides and lincosamides

Macrolides, such as erythromycin, oleandomycin, spiramycin and lincosamides, such as lincomycin and clindamycin antibiotics are used in human and veterinary practice for the treatment of *S. aureus* infections. Several different genes encoding macrolide resistance have been described in *S. aureus* including the *erm*(A), *erm*(B), *erm*(C) genes encoding for rRNA methylases, which causes target modifications in the 23S rRNA prevent binding of macrolides agents. Other macrolide resistance genes include *msr*(A), *mph*(C) and *mef*(A) which code for proteins mediating macrolide resistance by active macrolide efflux and enzymatic inactivation by phosphorylation. These macrolide genes are located on various plasmids and transposons (Table 1.1) (Schwarz *et al.*, 2011). Resistance to lincosamides is conferred by the *lnu*(A) gene, which encodes a lincosamide nucleotidyltransferase that inactivates lincomycin. The lincomycin resistance gene *lnu*(A) has been localised on two plasmids to date (Table 1.1) (Lozano *et al.*, 2012b).

1.2.1.7 Mupirocin resistance

Mupirocin (pseudomonic acid A) is an antimicrobial agent that is produced by *Pseudomonas fluorescens* and it is commonly used as a topical ointment for effective elimination of MRSA from the colonised nasal passages (Fuller *et al.*, 1971). Mupirocin is a protein synthesis inhibitor that inhibits bacterial isoleucyl-tRNA synthetase (IIeS) (Casewell and Hill, 1985). Two distinct forms of mupirocin resistance have been described in *S. aureus*, low-level and high-level. Low-level mupirocin resistance is due to a mutation in the chromosomally located isoleucyl-tRNA synthetase gene *ileS* and isolates harbouring this mutation exhibit a mupirocin minimum inhibitory concentrations (MIC) of 8 - 256 mg/L (Antonio *et al.*, 2002). In contrast, high-level

mupirocin resistance (Hi-MupR) is due to the acquisition of the plasmid-encoded *ileS2* gene which encodes a second isoleucyl t-RNA synthetase, and isolates harbouring *ileS2* exhibit mupirocin MICs ≥ 256-1024 mg/L (Rahman *et al.*, 1990; Hodgson *et al.*, 1994). To date, *ileS2* has been localised to several large and plasmids, often encoding multiple antibiotic resistance (Morton *et al.*, 1995; Diep *et al.*, 2006; Pérez-Roth *et al.*, 2010) (Table 1.1). Recently a third gene encoding high-level mupirocin resistance has been described and designated *ileS3* or *mupB*. The *ileS3* gene exhibits 65.5% DNA sequence identity with *ileS2* and only 45.5.% identity with *ileS*, and has been localised to a non-conjugative plasmid in three high-level mupirocin resistant ST5-MRSA-IV isolates from Canada (Seah *et al.*, 2012) (Table 1.1).

1.2.1.8 Linezolid resistance

Linezolid was the first oxazolidinone antimicrobial agent licensed by the United States Food and Drug Administration to treat MRSA infections in 2001 (Meka and Gold, 2004). Oxazolidinones bind to the 50S ribosomal subunit and prevent bacterial protein synthesis by inhibiting formation of the 70S ribosomal initiation complex (Bozdogan and Appelbaum, 2004). To date, two mechanisms of linezolid resistance have been described. A number of different mutations affecting the peptidyl transferase domain of 23S rRNA and mutations within the *rplC* and *rplD* genes encoding the 50S ribosomal proteins L3/L4 have been shown to result in linezolid resistance (Tsiodras *et al.*, 2001; Meka *et al.*, 2004; Locke *et al.*, 2009a; Locke *et al.*, 2009b). Linezolid resistance can also emerge as a result of the acquisition of a plasmid carrying the *cfr* gene that confers resistance to five classes of antimicrobial agents including phenicols, linosamides, oxazolidinones, pleuromutilins and streptogramin A compounds, a phenotype that has been termed $PhLOPS_A$ (Long *et al.*, 2006) (Table 1.1). The *cfr* gene was originally identified on the 17.1 kb multiresistance plasmid, pSCFS1, in a bovine *S.*

sciuri isolate (Schwarz *et al.*, 2000), but more recently has also been identified on different plasmids in *S. aureus*, CoNS and other bacterial genera from animals and humans (Toh *et al.*, 2007; Mendes *et al.*, 2008; Mendes *et al.*, 2010a; Morales *et al.*, 2010; Gopegui *et al.*, 2012; Lozano *et al.*, 2012a; Lozano *et al.*, 2012c), but not among any major pandemic MRSA clones. Linezolid resistance does not appear to be very common among *S. aureus*. The most recent results from the Linezolid Experience and Accurate Determination of Resistance (LEADER) program in 2009 reported that 0.34% of 6, 414 *S. aureus* isolates from 56 medical centers in the United States investigated were linezolid resistant (Farrell *et al.*, 2011b).

1.2.2 Plasmids

Plasmids are self-replicating DNA molecules that often carry antibiotic resistance and less commonly, virulence-associated genes. Most staphylococcal isolates carry one of more plasmids (Grohmann *et al.*, 2003). Plasmids can be differentiated into various groupings based on different characteristics including the following; (a) small (3-4 kb) multicopy plasmids, such as cryptic plasmids, or plasmids carrying a single antimicrobial resistance gene, (b) large, low-copy number plasmids (15-30 kb) which can carry more than one antimicrobial resistance determinants, (c) conjugative multiresistance plasmids that are capable of self-transfer via conjugation and can carry several antimicrobial resistance determinants, and (d) non-conjugative plasmids that can be small or large plasmids harbouring antimicrobial resistance genes but lacking the genes required for conjugative transfer. These latter group of plasmids are capable of spread by natural transformation, transduction or can be mobilised by conjugative plasmids allowing them to be transferred to other bacterial cells (Bennett, 2008; Malachowa and DeLeo, 2010). Some conjugative plasmids can transfer between

different staphylococcal species but also to bacteria belonging to other genera and are known as broad-host-range plasmids (Grohmann *et al.*, 2003; Bennett, 2008).

Conjugation is the most common mechanism of plasmid transfer in bacteria and was first discovered in the 1940s in the F (fertility) factor plasmid of *Escherichia coli* K-12 (Lederberg and and Tatum, 1946; Firth *et al.*, 1996). Conjugative transfer involves direct cell-to-cell contact via mating channels also known as conjugation tubes or sex pili (Fig. 1.3). In conjugative plasmids, the *tra* genes are involved in the transfer and the mating between the donor and recipient cells and an relaxase enzyme catalyses the initial and final stages of conjugation. The relaxase cleaves a specific site within the origin of transfer *oriT*, thus initiating conjugation (Fig. 1.3.). The relaxase also interacts with a *traG*-like protein called type IV coupling (T4CP) protein forming a mating channel (pili) that assembles a type IV secretion system (T4SS). The DNA is transported through the mating channel into the recipient cell by ATPase activity of the T4CP. This replication process leaves the donor and the recipient cells with a copy of the plasmid (Grohmann *et al.*, 2003; Smillie *et al.*, 2010) (Fig. 1.3).

Transposons can also encode resistance determinants and can be either chromosomally located or integrated into plasmids. Transposons are commonly flanked by insertion sequences that play a role in their mobility. For example IS*256* and IS*257* flank Tn*4001*, carrying the aminoglycoside resistance gene *aacA-aphD* and Tn*4003*, carrying the trimethoprim resistance gene *dfrS1*, respectively (Rouch *et al.*, 1987; Byrne *et al.*, 1989; Rouch *et al.*, 1989; Malachowa and DeLeo, 2010) (Table 1.1).

1.3 *Staphylococcus aureus* virulence factors

Staphylococcus aureus harbours genes encoding a wide range of virulence factors that play many different roles in the pathogenesis of *S. aureus* infections. Some of these form part of the core genome e.g. genes such as *bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA* and *fnbB* encoding microbial surface components recognising adhesive matrix molecules (MSCRAMMs) elastin, laminin, bone sialoprotein, thrombospondin, and fibrinogen or the polysaccharide intercellular adhesion (*ica*) locus (Foster and Hook, 1998; Fowler *et al.*, 2000; Watkins *et al.*, 2012). However, many virulence genesare encoded on MGEs, namely bacteriophages, pathogenicity islands, plasmids, SCC and pseudo-SCC elements and genomic islands. Examples of *S. aureus* virulence factors encoded on MGEs are shown in Table 1.2, and they include toxins that damage host tissues and cells and virulence factors that help *S. aureus* evade the immune system.

Alpha haemolysin is the most potent membrane damaging toxin expressed by *S. aureus* and can cause septic shock in humans. Human platelets and monocytes are particularly sensitive to the toxin, causing cell death by necrosis or apoptosis depending on toxin concentration (Essmann *et al.*, 2003). The alpha haemolysin gene, *hla*, is located on a pathogenicity island known as νSaγ (Table 1.2) (Highlander *et al.*, 2007). Staphylococcal superantigens (SAgs) include staphylococcal enterotoxins (SEs), and toxic shock syndrome toxin-1 (TSST-1). SAgs can activate increased production of T-lymphocytes (Xu and McCormick, 2012). These toxins can cause staphylococcal food poisoning when ingested or toxic shock syndrome when expressed systemically and the genes encoding these superantigens are located on various bacteriophages, plasmids, genomic islands and pathogenicity islands (Table 1.2). The exfoliative toxins A, B and D are glutamate-specific serine proteases, which cleave a single peptide bond in the extracellular region of human desmoglein-1. This glycoprotein plays a role in

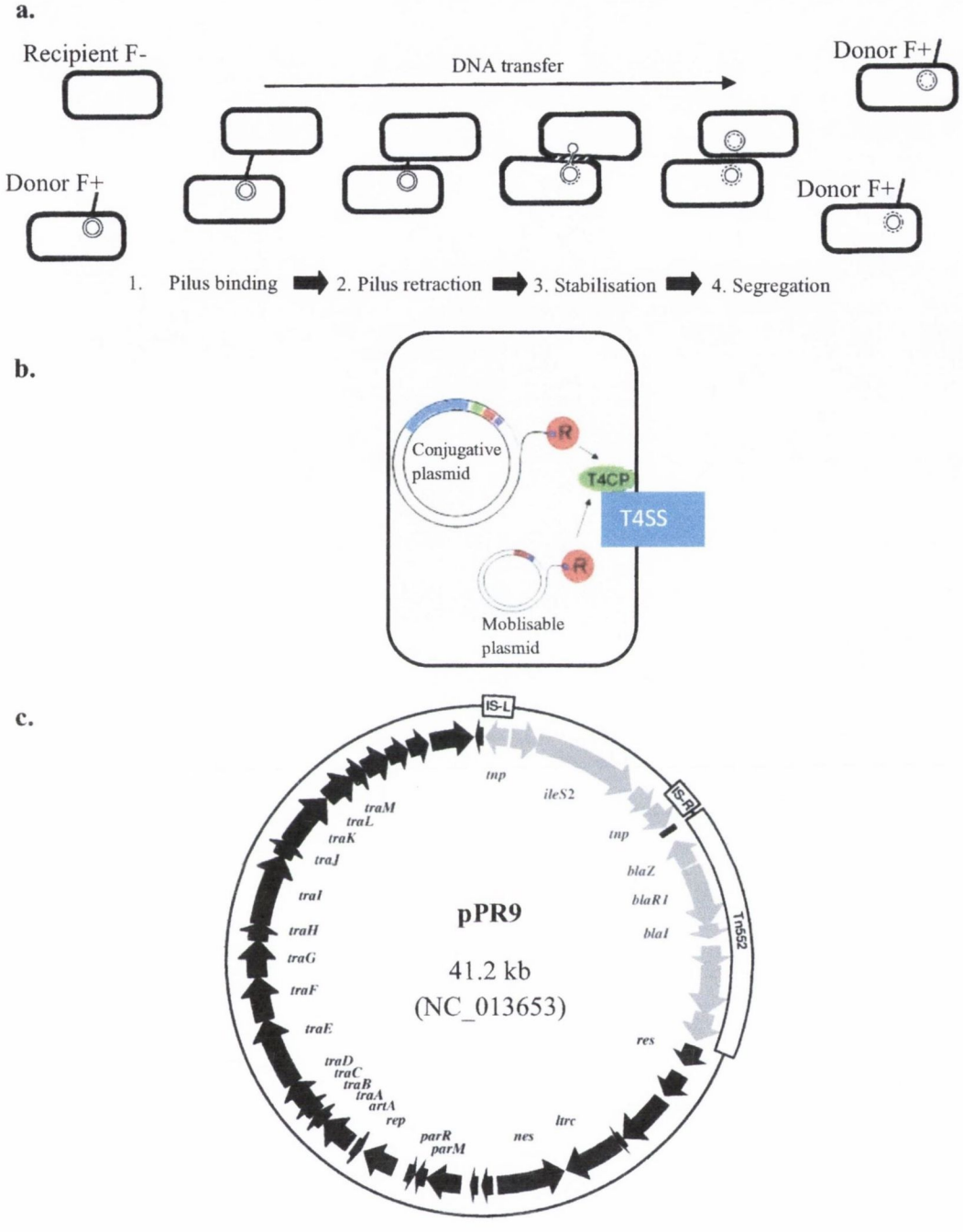


Fig. 1.3 Schematic diagram of the stages of bacterial conjugation. (**a**) Intercellular contact begins when the tip of a special pilus called the sex pilus extends from a F^+ (fertility factor-positive) donor cell and interacts with the F^- recipient cells facilitating direct cell-to-cell contact. The *tra* genes are involved in the transfer of plasmid DNA between the two cells. Adapted from Firth *et al.*, 1996. (**b**) The genetic process of conjugation. The relaxase (R) enzyme cleaves a specific site within the plasmid origin of transfer *ori*T (violet) and starts conjugation. The bacterial strain contains a relaxase protein which interacts with the T4CP (a type IV coupling protein) (green) and then with T4SS (type IV secretion system) (blue) to form the mating channel. As a result, the plasmid is transported to the recipient cell, the plasmid DNA is pumped into the recipient by the ATPase activity of the T4CP. Mobilisable plasmids only contain MOB genes and require the T4SS system of the conjugative plasmids to be mobilised across the mating channel. Adapted from Smillie *et al.*, 2010. (**c**) A genetic map of the *S. aureus* conjugative multiresistance plasmid pPR9 including *tra* genes *traA-M* along with antimicrobial resistance genes *ileS2* and *blaZ* (GenBank entry NC_013653). Adapted from Perez-Roth *et al.*, 2010.

Table 1.2 Examples of *Staphylococcus aureus* virulence factors encoded on mobile genetic elements (MGEs)

Toxin (gene)	MGE	Mechanism of action/disease	References
Chemotaxis inhibitory protein (*chp*)	φ 13, φtp310-3, φ N315, φ252b, φ NM3, φMu3A, φSa3USA300, φSa3JH1, φSa3mw, φSa3 ms,φSa3JH9, φβC-USA300_TCH1516	Blocks C5a and fMLP-induced neutrophil activation and chemotaxis	Van Wamel *et al.*, 2006; Malachowa and DeLeo, 2010
Staphylokinase (*sak*)	φN315, φMu50A, φSa2, φSa3mw, φ6390, φ13, φ252B, φ NM3, φMu3A, φSa3ms, φtp310-3, φβC-USA300_TCH1516, φSa3USA300, φSa3JH1, φSa3JH9	Activates conversion of plasmin; inhibits opsonisation by degradation of IgG and C3b, promotes resistance to defensins	Essmann *et al.*, 2003; Bokarewa and Tarkowski, 2004; Jin *et al.*, 2004; Rooijakkers *et al.*, 2005
Staphylococcal inhibitor of complement (*scn*)	φ13, φtp310-3, φN315, φSa3mw, φ252B, φNM3, φMu3A, φSa3JH1, φSa3ms, φSa3JH9, φMu3A, φSa3USA300, φβC-USA300-TCH1516	Inhibits phagocytosis of *S. aureus* by human neutrophils; blocks formation of C3b	Rooijakkers *et al.*, 2005; van Wamel *et al.*, 2006
Exfoliative toxin A (*etA*)	φETA, φETA2, φETA3	Serine protease that cause separation within the epidermis; causes staphylococcal scalded skin syndrome (SSSS), Ritter's disease, and bullous impetigo in neonates	Kuroda *et al.*, 2001; Yamaguchi *et al.*, 2002; Malachowa and DeLeo, 2010
Exfoliative toxin B (*etB*)	pETB, pRW001	Serine protease that cause separation within the epidermis; causes staphylococcal scalded skin syndrome (SSSS), Ritter's disease, and bullous impetigo in neonates	Jackson and Iandolo, 1986; Yamaguchi *et al.*, 2001; Malachowa and DeLeo, 2010
Exfoliative toxin D (*etD*)	υSAγ	Serine protease that cause separation within the epidermis; causes staphylococcal scalded skin syndrome (SSSS), Ritter's disease, and bullous impetigo in neonates	Yamaguchi *et al.*, 2001; Yamaguchi *et al.*, 2002; Malachowa and DeLeo, 2010
Epidermal cell differentiation inhibitor B (*edinB*)	υSAγ (*etd*PI)	ADP-ribosyltransferase; inhibits morphological differentiation of keratinocytes in vitro and modifies eukaryotic Rho GTPase	Yamaguchi *et al.*, 2002; Malachowa and DeLeo, 2010
Epidermal cell differentiation inhibitor C (*edinC*)	pETB	ADP-ribosyltransferase; inhibits morphological differentiation of keratinocytes *in vitro* and modifies eukaryotic Rho GTPase	Yamaguchi *et al.*, 2002; Malachowa and DeLeo, 2010
α-haemolysin	υSAγ	Pore-forming cytolytic toxin	Essmann *et al.*, 2003; Highlander *et al.*, 2007

Continued overleaf

Table 1.2 continued. Example of *Staphylococcus aureus* virulence factors encoded on mobile genetic elements (MGEs)

Toxin (gene)	MGE	Mechanism of action/disease	References
Panton-Valentine leukocidin (*lukF-PV, lukS-PV*)	φSa2mw, φPVL108, φSa2, φSa2USA300, φSLT, φPVL, φSLT-USA-300_TCH1516, φ tp310-1, φ2958PVL	Pore-forming leukocyte toxin, linked to necrotic infections	Lina *et al.*, 1999; Gillet *et al.*, 2002; Voyich *et al.*, 2006; Tristan *et al.*, 2007
Leukocidin F (*lukF*)	φPV83,	Pore-forming leukocyte toxin	Baba *et al.*, 2002; Barrio *et al.*, 2006
Leukocidin D & E (*lukD & lukE*)	υSAα	Pore-forming leukocyte toxin	Baba *et al.*, 2002; Zou *et al.*, 2000
Enterotoxin A (*sea*)	φSa3ms, φSa3, φSa3mw, φ252B, φNM3, φMu50A	Superantigen (SAg); causes food poisoning & toxic shock	Baba *et al.*, 2002
Enterotoxin B, K & Q (*seb, sek & seq*)	SaPI1, SaPI3, pZA10, φSa3ms, φSa3mw, SaPIbov1, SaPI1, SaPI3, SaPI5	SAg, causes food poisoning & toxic shock	Baba *et al.*, 2002; Yarwood *et al.*, 2002
Enterotoxin C/L (*sec & sel*)	SaPIbov1, SaPI1, SaPIbov1, SaPI3, SaPIn1/m1, SaPI4	SAg, causes food poisoning & toxic shock	Baba *et al.*, 2002; Novick, 2003; Ubeda *et al.*, 2003
Enterotoxin D/J (*sed, sej*)	pIB485, pIB485,	SAg, causes food poisoning & toxic shock	Bayles and Iandolo, 1989; Zhang *et al.*, 1998
Enterotoxin cluster (*egc*: seg, *sei, sem, sen, seo* & *seu*)	φSa3, υSAβ (SaPIn3/m3)	SAg, causes food poisoning & toxic shock	Baba *et al.*, 2002
Enterotoxin P (*sep*)	φN315, φMu50A	SAg, causes food poisoning & toxic shock	Brussow *et al.*, 2004; Chiang *et al.*, 2008
Toxic shock syndrome toxin (*tst*)	SaPIbov1, SaPI1, SaPI2, SaPI3, SaPIn1/m1	Causes toxic shock syndrome	Ubeda *et al.*, 2003; Plano, 2004
ACME (*arcA, arcB, arcC and arcD*)	ΔACME II (ψSCC*arc*), ΔJ1 SCC*mec* I & SCC*mec* IVh	Arginine deiminase, ability to colonise skin and mucous membranes	Diep *et al.*, 2008; Montgomery *et al.*, 2009; Shore *et al.*, 2011

Immune evasion genes *chp*, *sak* and *scn* form the immune evasion cluster (IEC). Six different combinations of IEC types have been defined by van Wamel *et al.* (2006): A= *sea*, *sak*, *chp* and *scn*; B = *sak, chp* and *scn*; C = *chp* and *scn*; D = *sea, sak* and *scn;* E = *sak* and *scn*; F = *sep, sak, chp* and *scn*; G = *sep, sak* and *scn*.

Genomic islands: υSAα, υSAβ, and υSAγ.

Pathogenicity islands: SaPIbov1 and SaPIbov2, SaPI1- SaPI5, SaPIn1/m1, SaPIn3/m3, SaPImw2, SaPIm3 and SaPIm4.

Bacteriophages: φ13, φtp310-3, φN315, φSa3, φSa3mw, φ252B, φNM3, φMu50A, φSa3JH1, φSa3 ms, φSa3JH9, φMu3A, φSa3USA300, φbCUSA300_TCH1516, φETA, φETA2, φETA3, φPV83, φPVL108, φSLT, φPVL, φSLT-USA300_TCH1516, φtp310-1, and φ2958PVL.

Plasmids: pETB, pRW001, pIB485, pIB485.

(ψ) pseudo-element. SCC*mec*: Staphylococcal cassette chromosome *mec* element.

maintaining cell to cell adhesion in the upper epidermis (Ladhani, 2003). They can cause staphylococcal scalded skin syndrome (SSSS), Ritter's disease, and bullous impetigo, which predominantly affect infants (Yamaguchi *et al.*, 2001; Yamaguchi *et al.*, 2002). The exfoliative toxins genes *etA*, *etB*, *etD* are located on plasmids, bacteriophages and genomic islands (Table 1.2). The Panton-Valentine leukocidin (PVL) toxin is a bi-component pore-forming cytolytic toxin that targets leucocytes, particularly neutrophils and is encoded by two genes, *lukF-PV* and *lukS-PV*, which are present in various bacteriophage genomes (Lina *et al.*, 1999a; Gillet *et al.*, 2002; Voyich *et al.*, 2006; Shallcross *et al.*, 2013). The exotoxin PVL is a virulence factor responsible for serious disease such as necrotising pneumonia, fasciitis, septicemia and SSTIs (Govindan *et al.*, 2012; Shallcross *et al.*, 2013). The expression of PVL has been associated with CA-MRSA (see section 1.4.1), but studies have reported conflicting results regarding the pathogenesis of PVL among CA-MRSA. A sepsis and abscess mouse model failed to demonstrate a significant role for PVL as PVL-negative CA-MRSA investigated were just as virulent as the CA-MRSA PVL-positive strains tested (Voyich *et al.*, 2006). In addition, two recent studies using models of SSTIs with PVL-positive CA-MRSA strains failed to produce skin lesions or to detect a role for PVL (Li *et al.*, 2010; Kobayashi *et al.*, 2011). However, a study of PVL production by PVL-positive *S. aureus* strains that caused lysis of human neutrophils supported the theory that PVL is a virulence factor in human CA-MRSA infection (Löffler *et al.*, 2010).

The immune evasion cluster (IEC) proteins are encoded by the *chp*, *sak*, *scn* and *eta* genes located on β-haemolysin (*hlb*)-converting bacteriophages (Table 1.2) (van Wamel *et al.*, 2006; Malachowa and DeLeo, 2010). These bacteriophages integrate into the *S. aureus* chromosome by site- and orientation-specific recombination between an attachment site (*attB*) located within the *hlb* gene and another attachment site (*attP*)

located in the bacteriophage genome (Coleman *et al.*, 1986; Coleman *et al.*, 1991; Carroll *et al.*, 1993). IEC proteins are important virulence factors that protect *S. aureus* from the human innate immune system. CHIP (encoded by the *chp* gene), known as chemotaxis inhibitory protein, is a bacterial chemokine receptor modulator which inhibits human neutrophil chemotaxis and activation (Haas *et al.*, 2004). SCIN (encoded by the *scn* gene), known as staphylococcal complement C3 convertase inhibitor, interferes with the ability of neutrophils to phagocytose *S. aureus* (Rooijakkers *et al.*, 2005a). The staphylokinase *sak* gene is an activator of plasminogen. It inhibits human α-defensins and is a antiopsonic, which reduces the activity of human immunoglobin IgG (Collen, 1998; Rooijakkers *et al.*, 2005b). Enterotoxin A (*sea*) is carried lysogenic bacteriophages such as φ42, φSa3mw, φNW3, and φ252B, and is often co-located with *chp*, *sak*, and *scn* (Table 1.2) (Coleman *et al.*, 1989; van Wamel *et al.*, 2006; Malachowa and DeLeo, 2010). Enterotoxin A is associated with food borne illnesses resulting in gastroenteritis and toxic shock. Enterotoxin A exerts its effects by binding to T cells and antigen presenting cells resulting in the proliferation of T-cells and the release of cytokines causing illness (Betley and Mekalanos, 1985; Coleman *et al.*, 1989; Rasooly *et al.*, 2010).

The arginine catabolic mobile element (ACME)-encoded *arc* genes have also been associated with virulence, particularly among the CA-MRSA clone USA300, ST8-MRSA-IV (Diep *et al.*, 2006). The ACME *arc* genes (*arcA*, *acrB*, *arcC* and *arcD*) encode an arginine deminase pathway which converts L-arginine to carbon dioxide, ATP and ammonia (Table 1.2). Another cluster in ACME is the oligopeptide permease operon (*opp-3A*, *opp-3B*, *opp-3C*, *opp-3D*, and *opp-3E* genes), both the *arc* and *opp* genes are important virulence factors implicated in pathogenesis in other bacteria such as *Streptococcus pyogenes* (Diep *et al.*, 2006). All *S. aureus* isolates encode native *arc*

and *opp* operons. The presence of second ACME-encoded *arc* operon may increase the growth and survival of ST8-MRSA-IVa host. Furthermore, the disruption of the native *opp* operon (*opp-1* and *opp-2*) has been shown to result in disordered growth and decreased virulence in *S. aureus* (Diep *et al.*, 2006). A study using isogenic mutants containing deletions of ACME and SCC*mec* IV in an USA300 CA-MRSA clinical isolates resulted in a change of the membrane-damaging factors associated with CA-MRSA (Diep *et al.*, 2008b; Montgomery *et al.*, 2009). The ACME element appeared to aid fitness and survival of the CA-MRSA whereas the deletion of the SCC*mec* IV element did not change the fitness cost of the strain (Diep *et al.*, 2008b).

1.4 The evolution of MRSA

Methicillin-resistant *S. aureus* have emerged on multiple occasions by independent acquisitions of SCC*mec* elements (Enright *et al.*, 2002; Chambers and Deleo, 2009; Knight *et al.*, 2012; Holden *et al.*, 2013). The majority of HA-MRSA infections are caused by strains belonging to five distinct lineages or clonal complexes (CCs), including CC5, CC8, CC22, CC30 and CC45 (Feil *et al.*, 2003; Chambers and Deleo, 2009; Stefani *et al.*, 2012). While there are also sporadically-occurring HA-MRSA strains belonging to other CCs the most predominant and widely spread HA-MRSA strains belong to these lineages including ST5-MRSA-II (CC5), ST8-MRSA-II (CC8), ST239-MRSA-III (CC8), ST22-MRSA-IV (CC22), ST36-MRSA-II (CC30) and ST45-MRSA-II (CC45). Both CC5 and CC8 are the most prevalent CCs in many countries worldwide such as the USA, South America, Canada, Europe, Africa, Asia and Australia, whereas CC22 is predominantly in Europe, and less frequently dominant in Canada, Australia and Indonesia (Chambers and Deleo, 2009; Stefani *et al.*, 2012). The CC30/ST36 genotype is commonly found in the UK and USA, whereas CC45 is

common more in Europe and the USA (Chambers and Deleo, 2009). The rates of HA-MRSA has declined recently among European countries e.g. Ireland, UK, France, Austria and Greece (EARS-Net, 2012; Stefani *et al.*, 2012). The rate of MRSA are reported to be increasing in East Asia, for example in Thailand (57%), Hong Kong (57%), Taiwan (65%), South Korea (78%), Vietnam (74%) and Sir Lanka (86%) (Song *et al.*, 2011; Stefani *et al.*, 2012). In comparison, the rates of MRSA are low in India (23%) and the Philippines (38%) (Song *et al.*, 2011).

However, CA-MRSA strains belong to different lineages and/or harbour different SCC*mec* elements to HA-MRSA e.g. ST1-MRSA-IV (CC1), ST80-MRSA-IV (CC80) and ST93-MRSA-V (CC75), ST30-MRSA-IV (CC30), ST8-MRSA-IV (CC8) and ST5-MRSA-IV (CC5) (Skov and Jensen, 2009). This data indicates that the emergence of CA-MRSA was not due not to the spread of HA-MRSA into the community but rather through acquisition of SCC*mec* by MSSA lineages in the community (Okuma *et al.*, 2002; Fey *et al.*, 2003). In recent years CA-MRSA strains have been found to be spreading into hospitals and HA-MRSA strains into the community (Maree *et al.*, 2007; D'Agata *et al.*, 2009; DeLeo *et al.*, 2010; Moon *et al.*, 2010; Brennan *et al.*, 2012b).

1.4.1 Community-associated MRSA

Since the mid-1990s, CA-MRSA has been seen with increasing frequency as a cause of SSTIs as well as more serious life threatening conditions such as necrotising pneumonia and fasciitis among healthy children and adults (Fig. 1.1) (Levine *et al.*, 1982; Dammann *et al.*, 1988; Berman *et al.*, 1993; Herold *et al.*, 1998; Vandenesch *et al.*, 2003; Boyle-Vavra and Daum, 2007; DeLeo *et al.*, 2010; Tattevin *et al.*, 2012). Community-associated MRSA was first reported from SSTIs among aborigines in remote areas in Western Australia in 1993 (Fig. 1.1) (Udo *et al.*, 1993). It was first

reported in the USA in 1997 and 1999, when four otherwise healthy children died from CA-MRSA (CDC, 1999). It has become an epidemic in the United States and has also emerged worldwide (Vandenesch *et al.*, 2003; O'Brien *et al.*, 2004; Gastelum *et al.*, 2005; Naas *et al.*, 2005; Huang *et al.*, 2007; Maree *et al.*, 2007; Rossney *et al.*, 2007; Diep *et al.*, 2008b; Diep *et al.*, 2008a; David and Daum, 2010; Khokhlova *et al.*, 2012; Sanchini *et al.*, 2012). Community associated MRSA strains were previously associated with specific continents, however intercontinental spread of CA-MRSA clones such as ST8, ST59, ST30 and ST80 between the USA, Europe, Africa, Asia and Australia have emerged due to travel and immigration (DeLeo *et al.*, 2010). The movement of ST8 from the USA to Europe and ST80 from the Middle East to Europe and Asia, along with the recent emergence of ST772 in Ireland hospitals have been reported (DeLeo *et al.*, 2010; Brennan *et al.*, 2012b).

Community-associated MRSA infections were originally difficult to define precisely. However, in 2000, the Centers for Disease Control (CDC) in the United States published criteria (http://www.cdc.gov/ncidod/dhqp/ar_mrsa_ca.html) defining an CA-MRSA infection as any MRSA infection recovered from a non-hospitalised/outpatient or an inpatient within 48 h of admission to hospital. Patients with suspected CA-MRSA should have no history of healthcare-associated MRSA risk factors such as residence in a long-stay care facility, no previous hospitalisation, or no surgery or haemodialysis during the previous 12 months preceding the onset of the suspect CA-MRSA infection (CDC, 2005; Rossney *et al.*, 2007; David and Daum, 2010). Additionally, the patient should not have any indwelling catheters or medical devices that pass through the skin (David and Daum, 2010).

Community associated-MRSA infections tend to be distinct from HA-MRSA as generally they are more virulent and can cause fatal infection in otherwise healthy

individuals and children without any of risk factors for MRSA (David and Daum, 2010). Community associated-MRSA has been described among specific patients groups such as young children attending crèches, military recruits, sport teams, intravenous drug users and prison inmates (Herold *et al.*, 1998; Zinderman *et al.*, 2004; Nguyen *et al.*, 2005; Diep *et al.*, 2008a). The increased virulence of CA-MRSA has been linked to the expression of virulence genes, in particular *pvl* (Vandenesch *et al.*, 2003; Boyle-Vavra and Daum, 2007), however, *pvl* has been shown not to be reliable as a sole marker for CA-MRSA (Rossney *et al.*, 2007) (see section 1.3). Not all CA-MRSA carry the *pvl* toxin genes, and there is conflicting data on its role in the pathogenesis of CA-MRSA infections (Montgomery *et al.*, 2009) (see section 1.3). The presence of smaller SCC*mec* types such as types IV and V in CA-MRSA may enhance the fitness of the MRSA strains that carry them (David and Daum, 2010). Interestingly, these SCC*mec* elements lack the phenol-soluble modulin (PSM)-*mec* which has been shown to supress colony spreading and exotoxin production, in particular production of the core genome toxin PSMα in *S. aureus* and the absence of PSM-*mec* in SCC*mec* types IV and V may contribute to the increased virulence of these strains (Queck *et al.*, 2009; Kaito *et al.*, 2011; Monecke *et al.*, 2012a).

It has been postulated that the arginine catabolic mobile element (ACME) may play a role in the spread and success of ST8-MRSA-IVa (Diep *et al.*, 2006; Goering *et al.*, 2007; Monecke *et al.*, 2009; Tenover and Goering, 2009). However, it is not widespread among CA-MRSA and apart from USA300 it has been identified among only a small number CA-MRSA and HA-MRSA clones, and is in fact more abundant among CoNS (Diep *et al.*, 2006; Miragaia *et al.*, 2009; Pi *et al.*, 2009; Barbier *et al.*, 2011). The ACME element like PVL is not necessary for the spread of an MRSA clone (Goering *et al.*, 2007). The ACME element was tested for its virulence and pathogenesis

in USA300 infections (Diep *et al.*, 2008b; Montgomery *et al.*, 2009). Both studies showed conflicting results; Montgomery *et al.* 2009 reported no difference between the virulence of the USA300 strain and the ACME knockout strain in a rat model of necrotising pneumonia or skin infection (Montgomery *et al.*, 2009). Whereas, in a rabbit bacteraemia model co-infected with an ACME deletion mutant of USA300, the pathogenicity and fitness of the mutant was significantly reduced compared to the parent (Diep *et al.*, 2008b).

1.4.2 Animal-associated MRSA

The first report of MRSA in animals was in 1971, when MRSA was isolated from milk samples from cows with mastitis in Belgian dairy herds (Devriese *et al.*, 1972; Devriese and Hommez, 1975). The MRSA persisted among the cows on the farms investigated for several months and a possible human origin was identified for the MRSA in the animals. In 1972, two healthy dogs in Nigeria were found to be MRSA-positive (Ola Ojo, 1972). In parallel to the increased prevalence of MRSA in hospitals in the 1980s and 1990s, MRSA also emerged among companion animals such as dogs and cats (Scott *et al.*, 1988; Smith *et al.*, 1989; Cefai *et al.*, 1994). Human HA-MRSA have been shown to be the source of MRSA in pets from the close contact between people and companion animals (Loeffler and Lloyd, 2010).

Animal-associated MRSA have predominantly been associated with SCC*mec* types IV and V, but the novel SCC*mec* types IX and X have also been identified (Fessler *et al.*, 2011a; Vandendriessche *et al.*, 2011; Fessler *et al.*, 2012; Tulinski *et al.*, 2012). In 2005, a livestock-associated (LA)-MRSA clone CC398 emerged among pigs, pig handlers and pigs farms in the Netherlands (Voss *et al.*, 2005), and has since been reported among livestock, including pigs, horses, chickens as well as among humans

with close animal contacts in many counties in Europe, the USA and in Asia (Witte *et al.*, 2007; Kehrenberg *et al.*, 2009; Graveland *et al.*, 2011; Asai *et al.*, 2012; Brennan *et al.*, 2012a; Fluit, 2012; Lim *et al.*, 2012; Pletinckx *et al.*, 2012; Uhlemann *et al.*, 2012). Recent studies have revealed other emerging LA-MRSA clones such as CC5, CC9, CC97, CC151, CC130, and CC131 and the zoonotic spread of some of these strains have been reported (Sung *et al.*, 2008; Guardabassi *et al.*, 2009; Hasman *et al.*, 2010; Cuny *et al.*, 2011; Shore *et al.*, 2011a). In particular, animals have been identified as the likely source of SCC*mec* XI carrying *mecC* in humans because of the lack of IEC genes, due to the absence of lysogenic *hlb*-converting bacteriophages, and the fact that MRSA lineages in which it has been identified in are predominantly animal associated lineages. The *mecC* gene has now being reported in MRSA from both humans and animals, including livestock, companion animals and wild animals (Paterson *et al.*, 2012; Walther *et al.*, 2012).

Due to the extensive use of antimicrobials agents such as β-lactams, trimethoprim, tetracycline and heavy metals in veterinary and husbandry practice it is not surprising that LA-MRSA commonly harbour multiple plasmids, and often novel resistance genes including *fexA*, *cfr*, *amp*(A), *tet*(L), *dfrK*, *erm*(T), *vga*(C) and *vga*(E) (Fessler *et al.*, 2011b; Kadlec *et al.*, 2012). There have also been reports of MRSA in milk for human consumption and among food products such as pork, beef, chicken, fish and processed food (Novak *et al.*, 2000; Kelman *et al.*, 2011; Paludi *et al.*, 2011; Crago *et al.*, 2012; Hammad *et al.*, 2012; Monecke *et al.*, 2012c; O'Brien *et al.*, 2012; Shahraz *et al.*, 2012).

1.5 Typing of MRSA

Typing of MRSA is an important tool for determining the relatedness of isolates and for tracking the spread of strains in both local and global epidemiology investigations. An extensive variety of techniques, both phenotypic and genotypic, have been developed for typing of MRSA and the method used depends on the purpose of the investigation as well on the available resources and expertise. Phenotypic typing methods were traditionally used for typing of MRSA and include antimicrobial susceptibility testing, biotyping and phage typing. These methods have largely been replaced by genotypic methods, both DNA fragment pattern-based methods such as pulsed-field gel electrophoresis (PFGE) (Murchan *et al.*, 2003), and more recently DNA sequencing-based methods such as multilocus sequence typing (MLST) (Enright *et al.*, 2000), staphylococcal protein A (*spa*) typing (Shopsin *et al.*, 1999) and direct repeat unit (*dru*) typing (Goering *et al.*, 2008). DNA sequencing based methods are commonly used in conjunction with SCC*mec* typing (Oliveira and de Lencastre, 2002; Kondo *et al.*, 2007; Milheiriço *et al.*, 2007) as it has been agreed internationally that a MRSA strain should be defined based on its genotype and its SCC*mec* type. Very recently, more advanced typing methods have also emerged for genotyping *S. aureus* isolates such as DNA and RNA microarray analysis (Lindsay *et al.*, 2006; Monecke *et al.*, 2008a) and whole-genome sequencing single nucleotide polymorphisms (SNP) analysis (Feil, 2004; Harris *et al.*, 2012). The most commonly used typing methods for *S. aureus* are described overleaf.

1.5.1 Antibiogram-resistogram (AR) typing

Antibiogram-resistogram typing is a form of antimicrobial susceptibility testing used in the Irish National MRSA Reference Laboratory (NMRSARL) for phenotypic typing of MRSA isolates based on their susceptibility to a panel of 23 antimicrobial agents and is determined by disk diffusion and Clinical and Laboratory Standards Institute, CLSI methodology (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b; Rossney *et al.*, 2007). The AR typing panel includes both clinically used and non-clinical antimicrobial agents and chemicals, thus minimising the effect that antimicrobial selective pressure has on the AR pattern of an isolate. On the basis of the resulting susceptibility pattern, isolates are assigned an AR type e.g. AR06, AR44. AR subtypes are determined based on variations with an AR typing pattern (Rossney *et al.*, 1994a). Isolates exhibiting new patterns are temporarily designated 'New' "Unfamiliar" or "NT" (non-typeable) AR type numbers. Before isolates exhibiting these new AR patterns can be assigned to a new AR type, further molecular typing is required. While AR typing is easy to perform and relatively inexpensive it is of limited value as a typing method due to the loss and/or acquisition of resistance genes and mutations all of which can lead to a change in the resistance phenotype. It is now widely accepted that detailed epidemiological typing requires the use of molecular methods.

1.5.2 Pulsed-field gel electrophoresis (PFGE)

Until recently, PFGE was widely used for typing of MRSA isolates and was regarded by many as the gold standard for typing MRSA in local/outbreak epidemiological investigations (Blanc *et al.*, 2002; McDougal *et al.*, 2003; Murchan *et al.*, 2003). During PFGE, high molecular weight chromosomal DNA is extracted from isolates embedded in agarose plugs and DNA is digested with restriction endonucleases that cleave DNA infrequently, usually *Sma*I for *S. aureus* (MacKenzie *et al.*, 2002). The digested DNA is then separated in agarose gels using alternating clamped homogenous electrical field (CHEF) electrophoresis resulting in characteristic DNA fingerprint profiles. These PFGE patterns can vary based on random genetic events, including insertion, deletions and point mutations in the DNA (Fig. 1.4). Analysis of PFGE banding patterns involves the visual inspection of the patterns using the criteria recommended by Tenover *et al.* (1995) and computer-assisted analysis using various software programs such as the BioNumerics software package (Rossney *et al.*, 2007) (Fig. 1.4). In the NMRSARL in Ireland, PFGE patterns are assigned a five-digit PFGE type (PFT). The first two digits of the PFT are then abbreviated to a pulsed-field group (PFG) (e.g. PFG-00, PFG-01, PFG-02) and isolates that differ by less than or equal to six PFGE bands are assigned to the same PFG. Each isolate is then assigned an AR-PFG group type based on the AR and PFGE typing patterns e.g. isolates exhibiting AR06 and PFG01 are designated AR-PFG 06-01 (Rossney *et al.*, 2006).

PFGE is a highly discriminatory typing method because it detects variation that accumulates rapidly and is therefore useful in an outbreak situation. However, this uncharacterised and rapid accumulation of variation can make it difficult to determine the relatedness of isolates in long-term studies. In addition, PFGE requires the use of expensive equipment is time-consuming and labour intensive, and the comparison of banding patterns between different laboratories can be difficult. In the last decade there

have been major advances in molecular typing, and DNA sequencing-based typing methods, resulting in the development of more rapid and accurate typing methods such as MLST, *spa* and *dru* typing that provide a more reproducible method for typing of MRSA.

1.5.3 Multilocus sequence typing (MLST)

Multilocus sequence typing of *S. aureus* involves sequencing of the internal fragments of seven unlinked housekeeping genes (Fig. 1.5). The different sequences of each gene are assigned as distinct alleles and each different combination of the seven housekeeping loci used is assigned a sequence type (ST) using the MLST database (http://saureus.mlst.net) (Enright *et al.*, 2000). Different STs can be assigned to clonal complexes (CC) based on the evolutionary relatedness using the eBURST algorithm (based upon repeated sequence types) (Feil *et al.*, 2004). Nucleotide changes in housekeeping genes are relatively stable over time and therefore MLST is widely used for typing of MRSA in long-term studies (Saunders and Holmes, 2007; Rijnders *et al.*, 2009; Wagenaar *et al.*, 2009; Chen *et al.*, 2010a; Argudín *et al.*, 2011; Aschbacher *et al.*, 2012). An international nomenclature is widely used for MRSA where each isolate is defined based on its ST and the type of SCC*mec* element that it harbours (Enright *et al.*, 2002; Robinson and Enright, 2004a; Feil *et al.*, 2004; Saunders and Holmes, 2007). In comparison to PFGE, MLST is relatively easy and fast to perform and involves direct DNA sequencing so the results are unambiguous, and can be easily compared between different laboratories using the curated MLST database (Enright and Spratt, 1999; Enright *et al.*, 2002). However, sequencing of seven housekeeping genes per isolate can be expensive, and DNA sequencing-based typing methods, such as *spa* and *dru* typing, that target a single gene only have been developed.

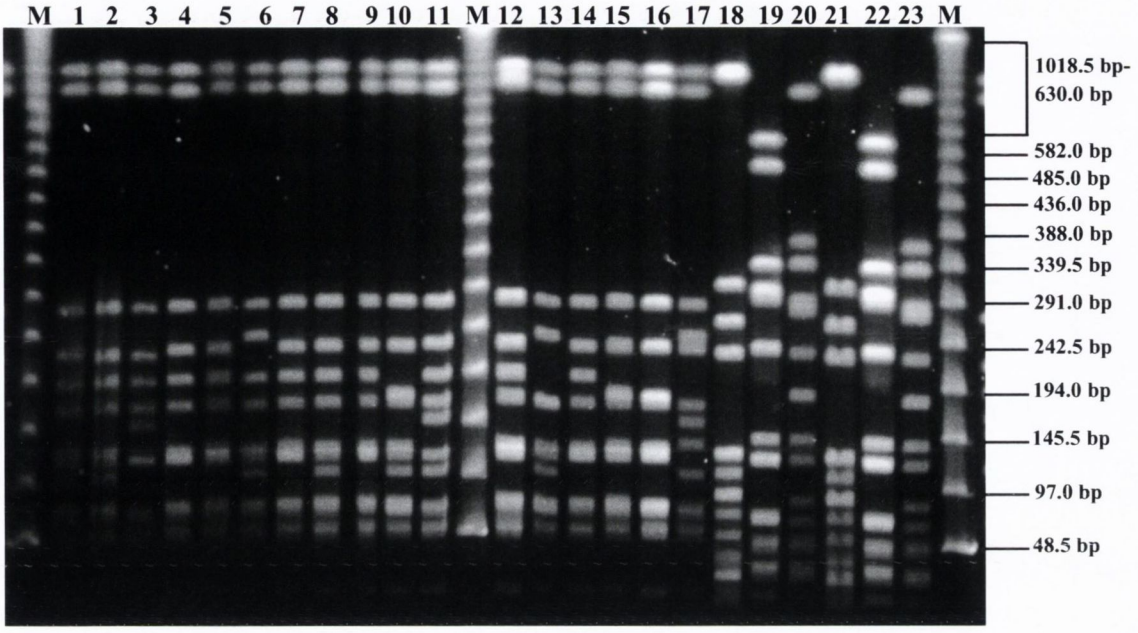


Fig. 1.4 Examples of pulsed-field gel electrophoresis (PFGE) fingerprint patterns obtained by digesting total genomic DNA from MRSA isolates with the restriction endonuclease *Sma*I. DNA bands were stained with ethidium bromide following electrophoresis and subsequently visualised on a UV transilluminator. The gel image was captured using the MultiDocIt (UVP) system. Banding patterns are subject to visual inspection using the guidelines of Tenover (Tenover *et al*., 1995) and computer-assisted analysis using the Gel Compar software option in Bionumerics, Version 5.10 (Applied Maths NV, Belgium). PFGE profiles are assigned 5-digit pulsed field type (PFT) numbers and PFTs that differ by six bands or less are assigned to the same PFT groups (PFG). M lanes Lambda ladder PFG Marker. Lanes 1-11 and 12-17; AR06 isolates. Lanes 18 and 19; AR07 and AR07.3. Lane 20, *S. aureus* NCTC 8325. Lanes 21 and 22 AR07 and AR07.3. Lane 23, *S. aureus* NCTC 8325.

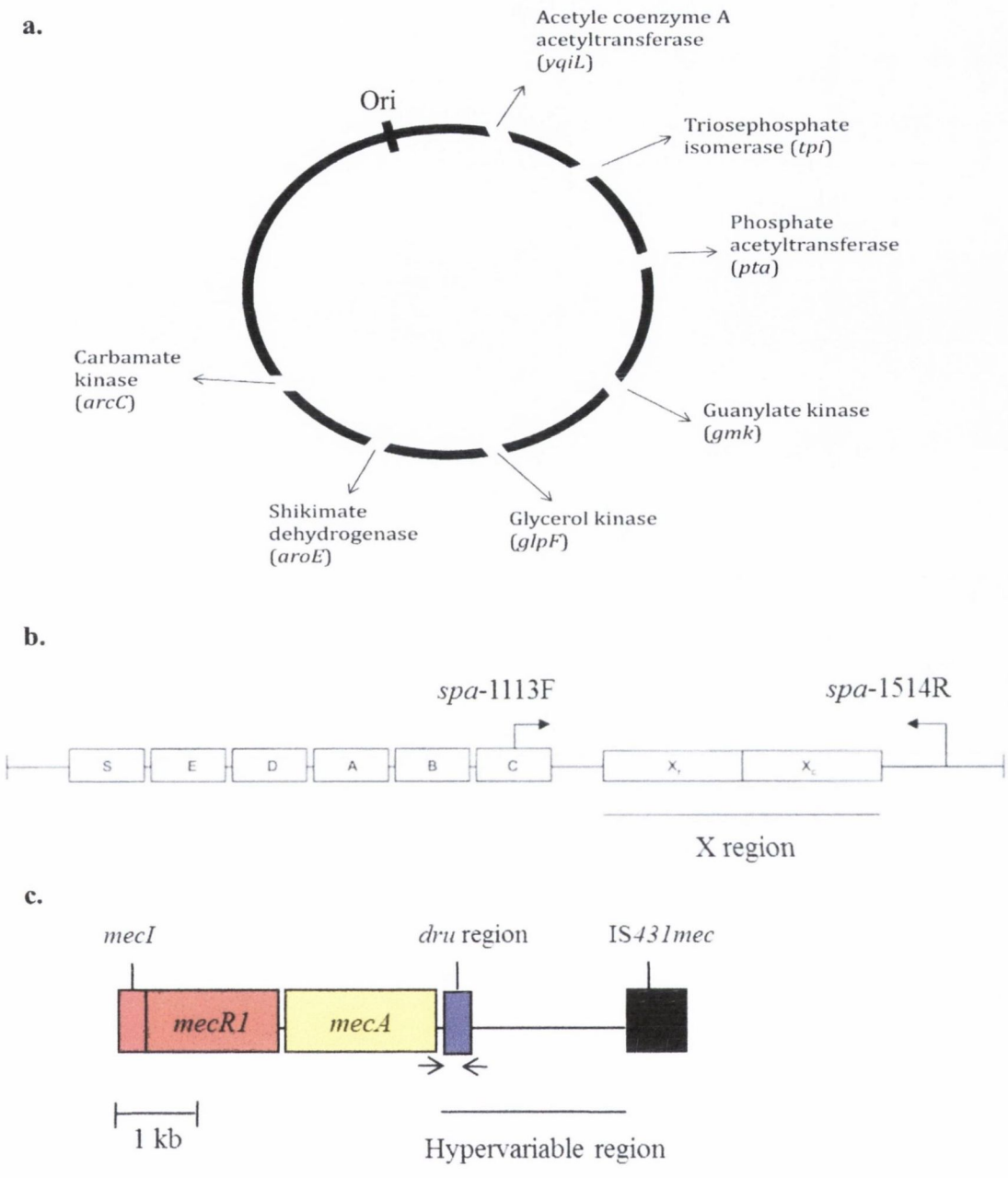


Fig. 1.5 DNA sequence-based typing approaches for *S. aureus* (**a**) Schematic representation of the relative chromosomal location of the seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) used for multilocus sequencing typing (MLST) of MRSA. The seven loci are targets for PCR amplification using published primers and PCR conditions (http://www.mlst.net (Enright *et al.*, 2000). (**b**) Schematic representation of the *S. aureus* protein A (*spa*) gene. The variable X region of the *spa* gene is amplified and sequenced using a forward (*spa* 1113F) and reverse (*spa* 1514R) primer. Each isolate is assigned a *spa* type based on the sequence of the repeat units within the polymorphic X region using a web-based database (SeqNet; www.seqnet.org). Abbreviations: S, gene coding for signal sequence; A-E, immunoglobulin G-binding regions; X, polymorphic X region. Adapted from Shopsin *et al.*, 1999. (**c**) Schematic representation of the SCC*mec*-associated direct repeat unit (*dru*) region. The *dru* region is a non-coding DNA segment consisting of a variable number of 40-bp variable tandem repeat sequences (VNTRs) located in the hypervariable region within the *mec* complex between *mecA* and IS*431mec*. Primer binding regions for amplification of the *dru* region between *mecA* and IS*431mec* of SCC*mec* indicated by arrow heads (Goering *et al.*, 2008).

1.5.4 *S. aureus* protein A gene (*spa*) typing

DNA sequencing of the *S. aureus* protein A gene *spa* is a widely used typing tool for *S. aureus* in both short-term/local and long-term/global epidemiological investigations, and it involves sequencing of a polymorphic variable-number tandem repeat (VNTR) or X region within the 3' end of the *spa* gene (Frénay *et al.*, 1996; Shopsin *et al.*, 1999; Koreen *et al.*, 2004; Strommenger *et al.*, 2006; Mellmann *et al.*, 2007; Strommenger *et al.*, 2008; Grundmann *et al.*, 2010; Hasman *et al.*, 2010; Monaco *et al.*, 2010) (Fig. 1.5). The *spa* region consists of a variable number of 24 bp repeat units, and the variation within this region can be due to deletions, duplications of the repetitive units or point mutations (Shopsin *et al.*, 1999; Kahl *et al.*, 2005). While *spa* typing offers less discrimination than PFGE, it is more discriminatory than MLST and involves DNA amplification, and sequencing of one gene only (Strommenger *et al.*, 2006; Khandavilli *et al.*, 2009; Petersson *et al.*, 2009; Soliman *et al.*, 2009). A web-based *spa* typing database can be used for comparison of *spa* types by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; www.seqnet.org) and an integrated web-based mapping application has also been developed to show the geographical distribution of different *S. aureus spa* types across several European countries was developed in 2007 (http://www.spatialepidemiology.net/srl-maps) (Mellmann *et al.*, 2007). *spa* typing has now replaced PFGE in many centres for routine typing of MRSA, and it is also commonly used instead of MLST, because in many instances an isolates ST and/or CC can be inferred from the *spa* type.

1.5.5 Direct repeat unit (*dru*) typing

The *dru* region is a non-coding DNA segment consisting of imperfect 40 bp VNTRs, and is located between *mecA* and IS*431* in SCC*mec* (Goering *et al.*, 2008; Shore *et al.*, 2010) (Fig. 1.5). There is an internationally agreed *dru* typing nomenclature, and curated web-based *dru* database that contains 51 different *dru*-repeat sequences, and 137 *dru* types (http://www.dru-typing.org) (Goering *et al.*, 2008). However, while *dru* typing has been shown to offer discrimination within, and between particular MRSA strains, particularly those with the same *spa* types. MRSA isolates belonging to different lineages have been identified with the same *dru* types, indicating that *dru* typing cannot be used as a standalone typing method for MRSA (Larsen *et al.*, 2009; Shore *et al.*, 2010; Smyth *et al.*, 2010). Because of its location within SCC*mec*, the *dru* region can only be used for typing of MRSA and not MSSA. Another limitation of *dru* typing, and in fact all typing methods that involve DNA sequencing of a single gene only including *spa* typing, is that it may not detect significant changes that occur elsewhere in the chromosome i.e. they only involve analysis of a very small subset of the whole genome. Recently, more detailed typing methods have been developed for MRSA that involve analysis of both the core and variable genome (see sections 1.5.7 and 1.5.8).

1.5.6 Staphylococcal chromosomal cassette (SCC*mec*) typing

The consensus international nomenclature for MRSA, designates MRSA strains using its CC/ST, and SCC*mec* type e.g. ST22 isolates that harbour SCC*mec* IV are designated ST22-MRSA-IV (IWG-SCC, 2009). Isolates exhibiting the same ST or *spa* type can habour different SCC*mec* types (Enright *et al.*, 2002). In addition, SCC*mec* typing can also provide insights into the evolution of SCC*mec* and MRSA. The essential role of SCC*mec* typing is to define the *ccr* and *mec* complex type of each isolate, which in turn defines each of the 11 SCC*mec* types that have been described to date in MRSA (IWG-SCC, 2009; Li *et al.*, 2011; Shore *et al.*, 2011a). The various SCC*mec* subtypes can also be identified based on structural differences in the J regions. Several SCC*mec* typing schemes involving both real-time and end-point PCR have been developed, the most commonly used of which utilise multiplex PCRs (Oliveira and de Lencastre, 2002; Kondo *et al.*, 2007; Milheirico *et al.*, 2007; Chen *et al.*, 2009). However, accurate SCC*mec* type assignment can require the use of several multiplex PCRs. In addition, these methods constantly require updating to ensure detection of novel SCC*mec* types and subtypes. For example, none of the currently published SCC*mec* typing methods involve detection of *mecC* or SCC*mec* types IX, X or XI (Li *et al.*, 2011; Shore *et al.*, 2011a).

1.5.7 Whole-genome sequencing

Advances in whole-genome sequencing technology in recent years had lead to major advances in research and to the development of next generation sequencing platforms, including single-nucleotide polymorphism (SNP) analysis of whole-genome sequences (Harris *et al.*, 2012) and microarray screening systems (Lindsay *et al.*, 2006; El Garch *et al.*, 2009). A recent study investigated whole-genome sequences for the evolutionary relatedness of strains in an epidemiological study in one hospital (Harris *et al.*, 2012). Whole-genome sequencing has led to the availability of 20 *S. aureus* whole-genome sequences which have been fully annotated, which has allowed the development of DNA microarray systems (Saunders *et al.*, 2004; Monecke *et al.*, 2008b; Monecke *et al.*, 2008a; El Garch *et al.*, 2009). Whole-genome sequencing is still expensive, time-consuming and data analysis is too cumbersome for routine laboratories. In contrast, DNA microarray screening systems offer a valuable alternative as they can rapidly provide a large volume of information concerning an isolate's genome. However, their use at the present time is still restricted to research laboratories, where the research staff have the expertise for data analysis (Witney *et al.*, 2005; Lindsay *et al.*, 2006; Sung *et al.*, 2008; Monecke *et al.*, 2010; Scicluna *et al.*, 2010; Stegger *et al.*, 2010; Sanchini *et al.*, 2011).

1.5.8 DNA microarray profiling of *S. aureus* isolates

Among the DNA microarray platforms that have been developed, one in particular the Staph-Type Kit (Alere, Jena, Germany), offers the potential for high-throughput typing of large numbers of *S. aureus* isolates within a relatively short period of time by simultaneously assigning isolates to a ST and/or CC and a SCC*mec* type, while also detecting other typing markers and an extensive range of clinically relevant virulence and antimicrobial resistance genes (Monecke *et al.*, 2008a; Monecke *et al.*, 2008b; Monecke *et al.*, 2011b). Several studies have found that DNA microarray technology is effective for detailed characterisation of MRSA isolates, including a number of studies from Germany which have involved comparative analysis of *pvl*-positive MRSA isolates and other MRSA strains from both humans and animals (Monecke *et al.*, 2006; Monecke *et al.*, 2007a; Monecke *et al.*, 2007b; Monecke *et al.*, 2008a; Monecke *et al.*, 2008b; Kadlec *et al.*, 2009; Coombs *et al.*, 2010; Scicluna *et al.*, 2010; Fessler *et al.*, 2010). Despite all of these studies, the accuracy of DNA microarray profiling for assigning MRSA isolates to a CC/ST and SCC*mec* type and for predicting an isolate's antimicrobial resistance phenotype has not been fully investigated. Also, the usefulness of the microarray for differentiating between isolates belonging to the same clone has not been investigated in detail.

1.6 MRSA in Ireland

MRSA was first reported in Irish hospitals in 1971, a decade after the first report in the UK (Hone and Keane, 1974). Since the late 1970s and throughout the 1980s and 1990s the incidence of MRSA has increased significantly in Irish hospitals and it has now been endemic for almost four decades (Cafferkey *et al.*, 1985; Coleman *et al.*, 1985; Carroll *et al.*, 1989; Rossney and Keane, 2002; Rossney *et al.*, 2006).

In the 1970s and early 1980s, typing of MRSA in Ireland primarily involved the use of phenotypic typing methods including antimicrobial susceptibility testing, plasmid profiling, bacteriophage typing and the location of resistance determinants because at this time PCR-based techniques for typing *S. aureus* were not available (Coleman *et al.*, 1985; Coleman *et al.*, 1986; Carroll *et al.*, 1989; Humphreys *et al.*, 1989; Rossney *et al.*, 1994a; Rossney *et al.*, 1994b; Rossney *et al.*, 1994c; Carroll *et al.*, 1995). This resulted in the assignment of Irish nosocomial MRSA isolates to three phenotypic groups, Phenotypes-I, II and III (Table 1.3) (Coleman *et al.*, 1985; Carroll *et al.*, 1989). During the late 1980s and the 1990s, Irish MRSA isolates were mainly typed by AR typing (Table 1.3) and PFGE (Carroll *et al.*, 1989; Humphreys *et al.*, 1990; Rossney and Keane, 2002).

With the development of more sophisticated DNA sequencing-based typing methods, a study was carried out in which MRSA isolates recovered in Irish hospitals between 1971 and 2004 underwent MLST and SCC*mec* typing (Shore *et al.*, 2005) (Table 1.3). This work showed that there have been several shifts in the predominant clonal types of MRSA prevalent in Irish hospitals over the time period investigated (Shore *et al.*, 2005). The predominant clone in the 1970s and early 1980s was ST250-MRSA-I/I-*pls*, which was replaced in the mid to late 1980s by ST239-MRSA-III/III-pI258/Tn*554*. This clone was in turn displaced by ST8-MRSA-IIA-IIE in the 1990s

Table 1.3 The predominant phenotypes and genotypes of MRSA identified in Irish hospitals during different time periods between 1971 and 2008

Year	Predominant phenotypes[a]	ST-SCC*mec* type	References
1971-1978	Phenotype-I	ST250-MRSA-I	Hone & Keane, 1974; Coleman *et al.*, 1985; Shore *et al.*, 2005
1978-1984	Phenotype-II	ST250-MRSA-I/I-*pls*	Coleman *et al.*, 1985; Shore *et al.*, 2005
1985-1987	Phenotype-III	ST239-MRSA-III - pI258/Tn*554*	Carroll *et al.*, 1989; Shore *et al.*, 2005
1988-1989	AR01	ST239-MRSA-III	Rossney, 1995; Keane, 2002; Shore *et al.*, 2005
	AR02	ST250-I – *pls*	
1993-1994	AR13	ST8-MRSA-IIA	Rossney, 1995; Rossney & Keane, 2002: Shore *et al.*, 2005
	AR14	ST8-MRSA-IIC	
1998	AR06	ST22-MRSA-IVh	Rossney & Keane, 2002: Shore *et al.*, 2005
	AR07	ST36-MRSA-II	
	AR13	ST8-MRSA-IIA/IID	
	AR14	ST8-MRSA-IIC/IIE	
1999	AR06	ST22-MRSA-IVh	Rossney *et al.*, 2003:
	AR07	ST36-MRSA-II	Shore *et al.*, 2005
	AR13	ST8-MRSA-IIE/IID	
	AR14	ST8-MRSA-IIE/IID/IIC	
	(AR43)	ST8-MRSA-IVE/F	
2002	AR06	ST22-MRSA-IV	Rossney *et al.*, 2006:
			Shore *et al.*, 2005
2003	AR06	ST22-MRSA-IV	Rossney *et al.*, 2006:
			Shore *et al.*, 2005
2007/08	AR06	ST22-MRSA-IV	Shore *et al.*, 2010
	Unfamiliar	ST22-MRSA-IV	

[a] Isolate phenotypes represent those that predominated in certain hospitals in the Republic of Ireland only. Those indicated in parenthesis were recovered in Northern Ireland only.

(Shore *et al.*, 2005). Since 2002, the ST22-MRSA-IV has predominated in Irish hospitals and is known locally as AR-PFG 06-01 (Rossney and Keane, 2002; Rossney *et al.*, 2006), and it now accounts for approximately 80% of bloodstream infection (BSI) isolates recovered in hospitals in the Republic of Ireland each year (Shore *et al.*, 2010).

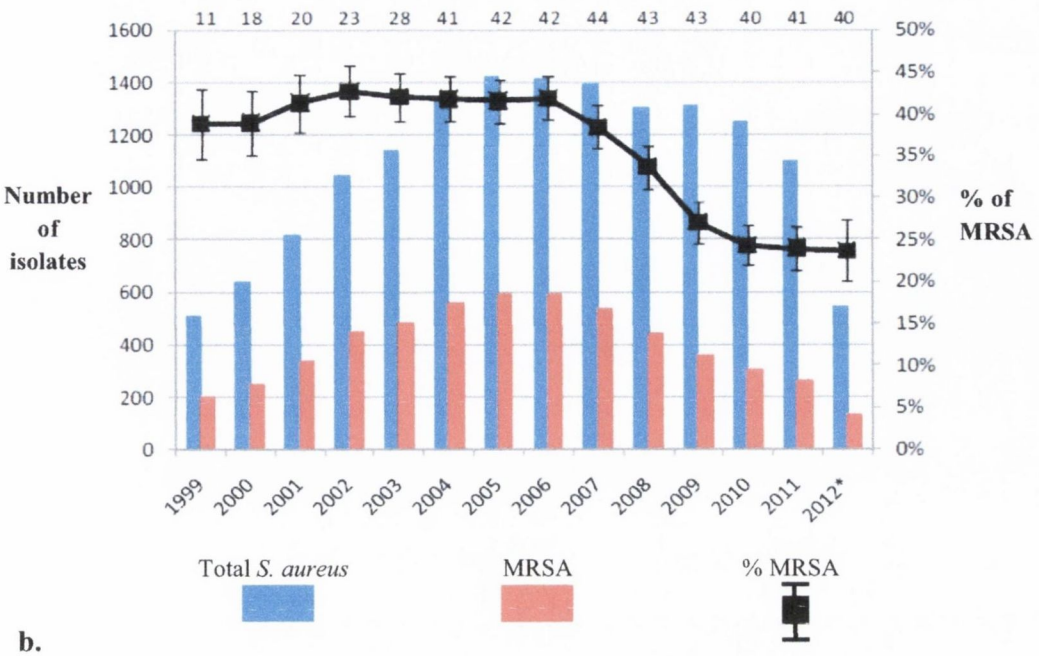
1.6.1 Highly clonal ST22-MRSA-IV in Ireland

Clinical isolates of the ST22-MRSA-IV strain are highly-clonal and are difficult to differentiate making tracking and prevention of spread difficult. Isolates belonging to the CC22 clone exhibit a limited number of PFGE patterns, *spa*, and *dru* types and the majority carry SCC*mec* IVh (Shore *et al.*, 2010). Alternative typing methods to improve discrimination between ST22-MRSA-IV isolates need to be developed. ST22-MRSA-IV was first reported in the UK in 1991 and was termed EMRSA-15 (Richardson and Reith, 1993) and has since become a pandemic nosocomial MRSA clone predominating not only in Ireland and the UK (Johnson *et al.*, 2001), but also in many other countries worldwide including Scotland, Portugal, the Czech Republic, Germany, Netherlands, Malta, Spain, Singapore, New Zealand, Australia and India (Pérez-Roth *et al.*, 2004; Melter *et al.*, 2006; Goering *et al.*, 2008; Monecke *et al.*, 2008a; Nulens *et al.*, 2009; Turnidge *et al.*, 2009; Scicluna *et al.*, 2010; Simoes *et al.*, 2011; Manoharan *et al.*, 2012; Teo *et al.*, 2013). ST22-MRSA-IV has also been recovered from healthcare workers (Amorim *et al.*, 2009; Pinto *et al.*, 2012) and companion animals (Baptiste *et al.*, 2005; O'Mahony *et al.*, 2005; Moodley *et al.*, 2006; Coelho *et al.*, 2011; Wieler *et al.*, 2011). PVL-positive ST22-MRSA-IV have been identified as a cause of CA-MRSA infections in several other countries (Rossney *et al.*, 2007; Coombs *et al.*, 2009; Mollaghan *et al.*, 2010; Monecke *et al.*, 2011b; Wehrhahn *et al.*, 2012).

1.6.2 MRSA surveillance in Ireland

In 1998, the European Antimicrobial Resistance Surveillance System (EARSS), which was recently renamed the European Antimicrobial Resistance Surveillance Network (EARS-Net) was established to monitor antimicrobial resistance among many different bacterial pathogens, including *S. aureus*, responsible for BSIs across Europe. Data collected by EARS-Net indicates that the prevalence of MRSA among BSIs in Ireland reached a peak in 2006 (i.e. 43% of *S. aureus* BSI isolate recovered in 2006 were MRSA) but declined to 23.7% by 2012 (Fig. 1.7) (EARS-Net, 2012). However, it is important to note that these figures include *S. aureus* BSIs only and no other types of *S. aureus* infections such as SSTIs, wound infections, urinary tract infections, pneumonia, osteomyelitis and endocarditis. In addition, while this is the lowest annual proportion of MRSA among *S. aureus* BSIs reported from Ireland since EARS-Net surveillance began, the 2012 prevalence rate of 23.7% is still high compared to many other Northern European countries. The comparative prevalence data of MRSA recovered from BSIs in Norway, Sweden and Estonia is <1%, while that in Denmark, Finland and The Netherlands is between 1 and 5% (Fig. 1.6) (EARS-Net, 2012). Since 2002, the National MRSA reference laboratory (NMRSARL) at St. James's Hospital in Dublin has carried out epidemiological typing of MRSA isolates using AR typing, biotyping, PFGE and PCR detection of *mecA* and *pvl* and more recently, *spa* typing (Rossney *et al.*, 1994a; Rossney *et al.*, 2006; Rossney *et al.*, 2007). Reports from the NMRSARL have revealed the emergence of *pvl*-positive CA-MRSA (Rossney *et al.*, 2005; Rossney *et al.*, 2006; Rossney *et al.*, 2007), heteroglycopeptide intermediate *S. aureus* (hGISA) (Rossney *et al.*, 2005; Fitzgibbon *et al.*, 2007) and high-level mupirocin resistance among MRSA isolates from Ireland (Rossney *et al.*, 2008). A detailed analysis of *pvl*-positive MRSA isolates from Ireland between 1999 and 2005

a.



b.

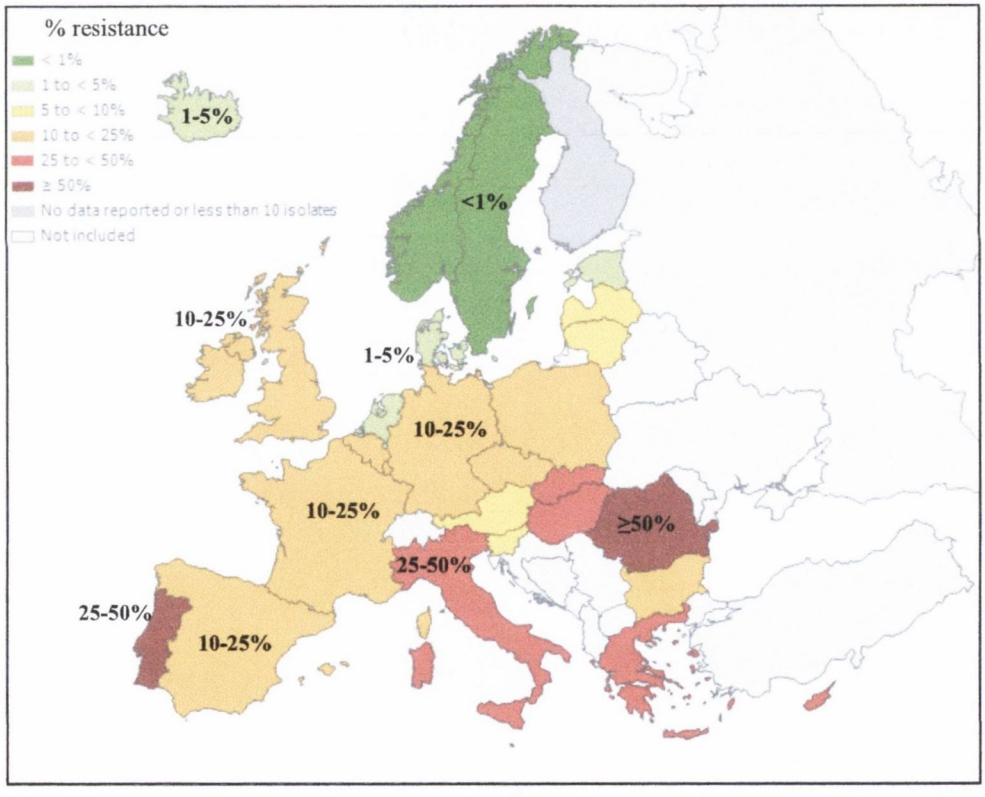


Fig. 1.6 Prevalence of MRSA and MSSA from bloodstream infections in Ireland and Europe. (**a**) Trends for *S. aureus*/MRSA BSIs in Irish hospitals and the proportion of *S. aureus* infections attributed to MRSA including percentages from 1999 to 2012. Adapted from EARS-net report 2012. *Preliminary data January to June 2012. (**b**) Distribution of MRSA among *S. aureus* BSIs in Europe in 2011. Adapted from EARS-net report 2012.

revealed that while the levels of *pvl*-positive MRSA in Ireland were relatively low, *pvl* cannot be used as a sole marker for CA-MRSA in Ireland and that diverse genotypes of *pvl*-positive MRSA are present in Ireland with ST30-MRSA-IV and ST8-MRSA-IV predominating (Rossney *et al.*, 2007). Recent studies of MRSA in Ireland have also revealed the presence of *mecC*-positive MRSA, the increasing prevalence of the *pvl*-positive CA-MRSA clone ST772-MRSA-V in hospitals and communities and the emergence of a novel MRSA clone with a complex pseudo-SCC*mec* CI and highlight the need for on-going surveillance and routine detailed molecular typing of MRSA. In Ireland, the emergence of LA-MRSA and MRSA among companion animals has been reported in cats, dogs, horses and pigs (O'Mahony *et al.*, 2005; Leonard *et al.*, 2006; Moodley *et al.*, 2006; Abbott *et al.*, 2010b; Abbott *et al.*, 2010a; Horgan *et al.*, 2011; Gibbons *et al.*, 2012).

1.6.3 Surveillance of antimicrobial resistance of MRSA in Ireland

On-going surveillance of antimicrobial resistance among MRSA in Ireland is carried out in collaboration with the NMRSARL. This is done using a combination of AR typing of all MRSA isolates received by the NMRSARL and MIC determination for some antimicrobial agent's including mupirocin and linezolid for a selection of MRSA from BSIs.

1.6.3.1 High-level mupirocin resistance

Mupirocin, commercially known as Bactroban, is used as a topical ointment for the effective decolonisation of MRSA from the nares of infected patients. The recommendation dosage should not exceed three times a day for five days to avoid the emergence of mupirocin resistance (SARI, 2005). In spite of these guidelines, the incidence of Hi-MupR among Irish MRSA from BSIs has increased. The rate of Hi-MupR among MRSA from BSIs has increased from 1.4% in 1999 to 3.1% in 2009 (Rossney and O'Connell, 2008)(NMRSARL annual report, 2011). Also, there has been a change in the AR-PFGs associated with Hi-MupR, from AR-PFG 13-00 and AR14-00, indicative of ST8-MRSA-IIA-IIE, which was the most common genotype recovered from Hi-MupR MRSA between 1999 and 2005, to AR-PFG 06-01 indicative of ST22-MRSA-IV, which was the most predominant genotype recovered from Hi-MupR MRSA between 2006 to 2009 (Epi-insight, 2003; Rossney *et al.*, 2006; Rossney and O'Connell, 2008). In addition, during the later period of 2008, 55% of Hi-MupR MRSA also exhibited resistance to aminoglycosides including gentamicin, kanamycin, tobramycin and neomycin (Rossney and O'Connell, 2008). However, little is known about the extent of the genetic diversity of Hi-MupR MRSA in Ireland and also about the virulence genes and additional antimicrobial resistance genes that they harbour.

Researchers in several countries have characterised the genetic elements encoding Hi-MupR among MRSA (Morton *et al.*, 1995; Udo and Jacob, 1998; Diep *et al.*, 2006; Pérez-Roth *et al.*, 2010; Udo and Sarkhoo, 2010; McDougal *et al.*, 2010). However, in Ireland very little is known about the genetic elements encoding Hi-MupR in Irish MRSA.

1.6.3.2 Fusidic acid resistance

Fusidic acid, commercially known as Fucidin, is mostly used as a topical agent for the treatment of SSTIs such as impetigo and infected eczema. Recently in Ireland, the emergence of fusidic acid resistance among MRSA has been reported by the NMRSARL. The rate of fusidic acid resistance among MRSA isolates from BSIs has increased from <10% in 2001 to almost 34% in 2011 (Fig. 1.7) (NMRSARL annual report, 2011). However, the genetic diversity of MRSA isolates exhibiting fusidic acid resistance and the molecular mechanism(s) encoding fusidic acid resistance among MRSA isolates from Ireland has yet to be investigated.

1.6.3.3 Linezolid resistance

Monitoring the development of antimicrobial resistance among newer agents for the treatment of MRSA infections is necessary to prevent the emergence and spread of resistant among MRSA, and to improve antibiotic use to reduce resistance. Linezolid, commercially known as Zyvox, it is used for the treatment of SSTIs and pneumonia caused by MRSA, vancomycin-resistant enterococci and *Streptococcus spp.* (Meka and Gold, 2004). Linzolid resistance in *S. aureus* can be due mutations in 23S rRNA gene and also can be due to the acquisition of a plasmid encoding the *cfr* gene, which confers resistance to $PhLOPS_A$ (above section 1.2.1.8). Since, the introduction of linezolid into clinical practice in Ireland, no linezolid resistance MRSA isolates has

been reported by the NMRSARL to date. Since testing started in 2004, only a selection of BSIs MRSA isolates from EARS-Net (100 isolates) were tested against linezolid (NMRSARL annual reports 2011). Recently, on-going screening of MRSA isolates form the NMRSARL using the Staphy-Type DNA microarray revealed the presence of the *cfr* gene in a CA-MRSA isolate previously found to belong to ST8-MRSA-IVa/USA300 using MLST and SCC*mec* typing (Rossney *et al.*, 2007). Previous studies have identified that *cfr* is often plasmid located but the genetic element carrying *cfr* in this ST8-MRSA-IVa isolates has yet to be investigated. In addition, it has not been determined if this *cfr*-positive isolate exhibits the $PhLOPS_A$ phenotype but this isolate represents the first *cfr*-positive USA300 isolate and potentially the first linezolid resistant MRSA isolate from Ireland.

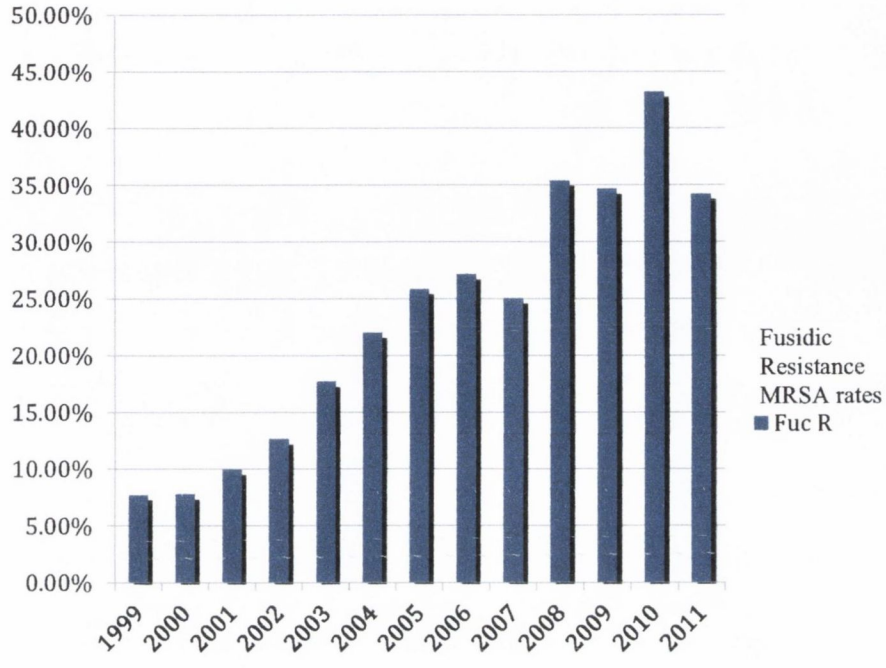


Fig. 1.7 The prevalence of fusidic acid resistance among MRSA isolates from bloodstream infections (BSIs) in Irish hospitals submitted to the National MRSA Reference Laboratory (NMRSARL) between 1999 and 2011. The rate of fusidic acid resistance among MRSA from BSIs increased from <10% between 1999-2001, to 39% in 2010. Adapted from the NMRSARL 2011 annual report.

1.7 Aims of the present study

MRSA have been a major cause of nosocomial infections in Ireland for more than four decades. Different strains representative of distinct pandemic MRSA clones have prevailed in Irish hospitals at different time periods with highly clonal ST22-MRSA-IV currently predominant. Detailed and accurate typing is essential for detecting and tracking and ultimately preventing the spread of MRSA and for understanding the evolution of MRSA. However, typing methods including PFGE, MLST, SCC*mec* typing and *spa* and *dru* typing examine only small sections of the genome and often do not offer sufficient discrimination for epidemiological investigations, especially with isolates that exhibit limited genetic diversity. Furthermore, these approaches are not readily amenable to high-throughput screening or to outbreak investigations in real-time and do not detect virulence or resistance genes.

The Staph-TypeDNA microarray system that can detect 334 *S. aureus* genes and alleles, including species-specific, antimicrobial resistance, and virulence-associated genes, as well as markers used for typing offers an alternative method for high-throughput and informative typing of MRSA. However, the accuracy of this system and its usefulness as a typing method for use either alone or in combination with more commonly used typing methods such as *spa* typing, requires further investigation. In addition, the increasing prevalence of mupirocin and fusidic acid resistance among HA-MRSA in Ireland as well as the detection of a *cfr*-positive CA-MRSA, warrants further investigation.

The aims and objective of this study were:

- To perform an in-depth molecular characterisation of isolates representative of the predominant MRSA clones recovered from patients in Irish hospitals between 1971 and 2004 using *spa* typing and DNA microarray profiling.
- To investigate the usefulness and accuracy of the Staphy-Type DNA microarray for genotyping MRSA isolates representative of major pandemic clones recovered in Irish hospitals that have been previously investigated by MLST and detailed SCC*mec* typing.
- To investigate the correlation between antimicrobial resistance phenotype determined by AR typing and the presence of antimicrobial resistance genes using DNA microarray profiling.
- To investigate the genotypes and molecular mechanism(s) of fusidic acid resistance among fusidic acid-resistant (FdR) MRSA isolates recovered from patients in Irish hospitals between 1971 and 2010.
- To investigate the usefulness of DNA microarray profiling combined with *spa* typing for differentiating isolates belonging to the ST22-MRSA-IV clone, which is currently endemic in Irish hospitals.
- To perform a comprehensive comparative molecular analysis of Hi-MupR MRSA isolates recovered from patients in Irish hospitals, and molecular characterisation of the Hi-MupR plasmids harboured by these isolates.
- To perform detailed molecular characterisation of the genetic environment of the multidrug resistance gene *cfr* in a *pvl*-positive ST8-MRSA-IVa (USA300) CA-MRSA isolate recovered from an Irish patient.

Chapter 2

Materials and Methods

2.1 General microbiological methods

2.1.1 Bacterial culture media, storage and growth conditions

All MRSA isolates, unless otherwise stated, were cultured on Tryticase Soy Agar (TSA), Brain Heart infusion (BHI) agar plates (Oxoid Ltd., Hampshire, UK) or Columbia Blood Agar (CBA) plates (Fannin Ltd., Dublin, Ireland) depending on the method to be used and overnight incubation at 37°C in a static incubator (Gallenkamp, Leicester, UK). Isolates were stored in individual Protect Bacterial Preservation Systemvials (Technical Services Ltd, Lancashire, UK) at -70°C and were cultured by removing a single bead from each vial using a sterile forceps, inoculating it onto the appropriate media plate (see above) followed by overnight incubation at 37°C in a static incubator (Gallenkamp).

2.1.2. Chemicals, enzymes, buffers and oligonucleotides

Unless otherwise stated, all chemicals used were of analytical grade or molecular biology-grade and were purchased from the Sigma-Aldrich Chemical Company (Dublin, Ireland). Enzymes for molecular biology procedures were purchased from the Promega Corporation (Madison, Wisconsin, USA) or Roche Diagnostics Ltd (Burgess Hill, West Sussex, UK) were stored at -20°C and were used according to the manufacturer's instructions. DNA molecular weight markers (100 bp and 1 kb) were purchased from Promega and were used according to the manufacturer's instructions. Oligonucleotide primers were custom synthesised by Sigma and were stored as a stock solution of 10 mM in molecular biology water reagent (Sigma) (section 2.1.3) at -20°C. A 6 x Blue/Orange Loading Dye (Promega) was used for loading DNA samples on agarose gels and for tracking migration during electrophoresis. The loading dye was diluted to 1 x working stock for use. Proteinase K (20 mg/ml), lysozyme (25 mg/ml)

and lysostaphin (1 mg/ml) were prepared using molecular biology reagent water (Sigma).

Tris-EDTA (TE) buffer was used routinely in many experiments and consisted of 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA (tetrasodium), pH 8. Tris-borate/EDTA electrophoresis buffer was prepared at 10 x concentration and consisted of 0.45 M trizma base, 0.45 M boric acid, 0.01 M EDTA, pH 8. This was diluted in ultrapure water (section 2.1.3) to 0.5 x concentration and was used for preparing agarose gels and as the running buffer for agarose gel electrophoresis, except for plasmid DNA analysis, where Tris-acetate-EDTA (TAE) buffer was used.

TAE buffer has a low ionic strength and a low buffering capacity and is suitable for electrophoresis of large (>20 kb) fragments of DNA but has to be replaced frequently or recirculated for longer (>4 h) gel running times. TAE buffer, pH 8.5, was prepared at 50 x concentration, which consisted of 2 M Tris-acetate and 0.5 M EDTA, pH 8. A working stock of 1 x TAE was used for preparing agarose gels and as an electrophoresis buffer for plasmid analysis. All solutions were stored at room temperature unless otherwise stated.

Additional solutions used for plasmid analysis included:

(i) buffer P1 (resuspension buffer), which consisted of 50 mM Tris-Cl pH 8.0, 10 mM EDTA and 100 µg/ml RNase A.

(ii) buffer P2 (lysis buffer), which consisted of 200 mM NaOH and 1% SDS (w/v).

(iii) buffer Minilysat III P3 (neutralisation buffer), was prepared by the addition 3 M sodium acetate pH 4.8, pH was adjusted using acetic acid.

Buffers P1, P2 & P3 were stored at 4°C but buffer P2 was stored at 37°C prior to use.

2.1.3 Water

The Milli Q Biocel system (Millipore Ireland, Carrigtwohill, Ireland) was used to provide ultra-pure water used for the preparation of buffers and agarose gels. Molecular Biology Reagent water was purchased from Sigma and was used in all PCR reactions, DNA elutions and dilutions.

2.1.4 Identification of isolates as *S. aureus*

All isolates were identified as *S. aureus* at the Irish National MRSA Reference Laboratory (NMRSARL, St. James's Hospital, James's Street, Dublin 8, Ireland) by detecting staphylocoagulase, clumping factor production using the tube coagulase test method (Rossney *et al.* 1990) and the Pastorex™ Staph-Plus latex agglutination assay (Bio-Rad, Marnes la Coquette, France), respectively. If an isolate failed to produce staphylocoagulase or clumping factor, identification as *S. aureus* was based on the detection of thermostable deoxyribonuclease and if necessary, biochemical identification using API Staph identification system (bioMérieux, sa, Marcy-l'Etoile, France), all as previously described (Rossney *et al.*, 1994a).

2.1.5. Confirmation of methicillin resistance

Detection of methicillin resistance was also carried out at the NMRSARL, using the oxacillin MIC E-test™ system consisting of antibiotic gradient strips (AB Biodisk, Solna, Sweden) along with 30 µg & 10 µg cefoxitin disks (Oxoid) on BBL Mueller-Hinton II agar (MH) (Becton Dickinson and Company, Sparks, MD, USA) and incubated at 35°C for 18-24 h using Clinical and Laboratory Standards Institute (CLSI) methodology (CLSI, 2006b). Further confirmation of an isolate's susceptibility to methicillin was tested using 1 µg and 5 µg oxacillin disks on Columbia Blood Agar

(CBA) at 30°C for 18-24 h. When an isolate's methicillin susceptibility was anomalous, e.g. any isolate exhibiting an oxacillin MIC of ≤ 4 mg/l, the isolates were tested for the presence of *mecA* or *mecC* by PCR using PCR primers and conditions as described previously (Oliveira *et al.*, 2006; Shore *et al.*, 2011a). Isolates were tested for the production of PBP2' using the Mastalex™-MRSA kit (Mast Diagnostics, Bootle, UK) according to the manufacturer's instructions. The Mastalex™-MRSA kit involves an agglutination reaction between PBP2' and a monoclonal antibody specific for PBP2'. All MRSA isolates were biotyped by testing for the hydrolysis of urea on Christensen's urea agar slopes which contained Urea base agar (Oxoid) with 2% (w/v) urea (Merck, Darmstadt, Germany) followed by incubation at 35°C for 48 h. Urease production was useful for the interpretation of 'no type' (NT), antibiogram-resistogram (AR) type determination see section 2.2.1 below.

2.2 Antibiogram-resistogram (AR) typing and pulsed-fieldgelelectrophoresis (PFGE)

Antibiogram-resistogram and PFGE typing were performed on all isolates in the NMRSARL, all as described previously (Rossney *et al.*, 2007)

2.2.1 AR typing of MRSA isolates

Antibiogram-resistogram typing (AR typing) which is performed by determining the susceptibility of isolates to a panel of 23 antimicrobial agents (Table 2.1), was performed by disk diffusion. For all MRSA isolates recovered since 2005 AR typing was performed by Clinical and Laboratory Standards Institute (CLSI) methodology (CLSI, 2006b; Rossney *et al.*, 2007) (Table 2.1) and for all isolates recovered prior to 2005, the susceptibility was determined using a modified Stokes disk diffusion technique on Diagnostic Sensitivity Test Agar (Oxoid) using *S. aureus* ATCC 25923 as

a susceptible control isolate (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b; Rossney *et al.*, 2003). The concentrations and breakpoints of each of the 23 antimicrobial agents used with the Stokes method are shown in parentheses (μg/disk) in Table 2.1. The Stokes methodology was used for susceptibility testing of isolates recovered before 2005.

Each isolate was assigned an AR type or AR type variant based on the AR pattern. In some instances AR subtypes were also assigned AR type numbers; these were assigned chronologically and 46 AR type numbers have been assigned to date i.e. AR01-AR46. AR subtypes were assigned based on slight variations to an existing AR pattern e.g. if the isolate lost a plasmid encoding a resistant gene, this loss of a resistance phenotype would be assigned a AR subtype by assigning a third numerical number to the AR type e.g. AR14.1. An antimicrobial susceptibility pattern that shares no similarity to any known AR type is classified as unfamiliar (Unf). Isolates assigned to No Type (NT) exhibit particular susceptibility patterns indicative of a known AR type, but also exhibit a urease positive phenotype (see above section 2.1.5) e.g. AR06 is the most prevalent AR types among nosocomial MRSA currently endemic in Irish hospitals. AR06 isolates are usually urease negative, however an MRSA isolate that exhibits a susceptibility pattern related to AR06 but which is urease positive is reported as NT.

Table 2.1 Antimicrobial agents used for antibiogram-resistogram (AR) typing[a] of MRSA clinical isolates

Antimicrobial agent	Concentration (μg/disk)	CLSI breakpoints[b]			Stokes breakpoints[b]		
		R (mm)	I (mm)	S (mm)	R (mm)	M (mm)	S (mm)
Amikacin	30	14	15-19	20	6	≥ 6	≤ 3
Ampicillin	10	28	*	29	6	≥ 6	≤ 3
Cadmium acetate	130	10	11-15	16			
Cadimium nitrate (Stokes only)	20 nmoles	N/A	N/A	N/A	6	≥ 6	≤ 3
Chloramphenicol	30 (CLSI)/ 10 (Stokes)	12	13-17	18	6	≥ 6	≤ 3
Ciprofloxacin	5 (CLSI)/ 1 (Stokes)	15	16-20	21	6	≥ 6	≤ 3
Erythromycin	15 (CLSI) /5 (Stokes)	13	14-22	23	6	≥ 6	≤ 3
Ethidium bromide	60 (CLSI)/ 20 (Stokes)	13	14	15	6	≥ 6	≤ 3
Fusidic acid	10	23	24-26	27	6	≥ 6	≤ 3
Gentamicin	10	12	13-14	15	6	≥ 6	≤ 3
Kanamycin	30	13	14-17	18	6	≥ 6	≤ 3
Lincomycin	2	14	15-16	17	6	≥ 6	≤ 3
Mercuric chloride	10	13	14	15	6	≥ 6	≤ 3
Mupirocin	5	12	13-19	20	6	≥ 6	≤ 3
Mupirocin	200	15	16-29	30	6	≥ 6	≤ 3
Neomycin	30	15	16-17	18	6	≥ 6	≤ 3
Phenyl mercuric acetate	10	24	25-28	29	6	≥ 6	≤ 3
Rifampicin	5 (CLSI)/ 2 (Stokes)	16	17-19	20	6	≥ 6	≤ 3
Spectinomycin	500	13	14-19	20	6	≥ 6	≤ 3
Streptomycin	25	13	14-15	16	6	≥ 6	≤3
Sulfonamide	300	12	13-16	17			
Sulphonamide (Stokes only)	100	N/A	N/A	N/A	6	≥ 6	≤3
Tetracycline	30 (CLSI)/ 10 (Stokes)	14	15-18	19	6	≥ 6	≤3
Tobramycin	10	17	18	19	6	≥ 6	≤ 3
Trimethoprim	5 (CLSI)/ 1.25 (Stokes)	10	11-15	16	6	≥ 6	≤ 3
Vancomycin	30	14	*	15	6	≥ 6	≤ 3

[a] Clinical and Laboratory Standards Institute (CLSI) disk diffusion breakpoints were used for AR typing of isolates recovered since 2005. Stokes disc diffusion and methodology were used for susceptibility testing of isolates recovered prior to 2005.

[b] Zones of growth inhibition (diamater) around antimicrobial agent impregnated discs were recorded in mm.

Abbreviations: R, resistant; I, intermediate resistance; S, susceptible.

* No intermediate breakpoint (CLSI, 2009; Rossney *et al.*, 2007).

2.2.2 PFGE analysis of MRSA isolates

Pulsed-field gel electrophoresis involved the extraction of high molecular weight chromosomal DNA embedded in agarose plugs and subsequent digestion of this chromosomal DNA using the rare-cutting restriction endonuclease *Sma*I (Promega) as described by Rossney *et al.* (2006) for MRSA isolates recovered prior to 2003 and using the achromopeptidase method described by MacKenzie *et al.* (2002) for isolates recovered after 2003. For all PFGE gels, the chromosomal DNA of the *S. aureus* reference strain NCTC 8325 digested with *Sma*I was used as a positive control (Struelens *et al.*, 1993). The digested DNA fragments were separated using 1% (w/v) pulsed-field certified agarose (Bio-Rad, Hertfordshire, UK) in a clamped homogenous electrical field (CHEF) electrophoresis system with 0.5 x TBE chilled and maintained at 4°C by recirculation of the buffer through a BioRad chiller. Following PFGE, the agarose gels were stained for 30 min with 0.5 mg/l ethidium bromide, destained in distilled water for 15 min. Gels were placed on a UV transilluminator (UVP, Cambridge, UK) at 312 nm and gel images were captured using the MultiDocIt system (UVP). The gel images were analysed using a computer-assisted program (Bionumerics, Version 5.10 and Gel Compar II, Applied Maths NV, Belgium). Similarity was calculated using an unweighted paired group method using arithmetic averages (UPGMA) with the Dice coefficient. Patterns were visually inspected to ensure accurate assignment of banding patterns and pattern differences were interpreted according to the criteria described by Tenover *et al.* (1995). PFGE profiles were assigned a 5-digit pulsed field type (PFT) number and related PFTs were assigned to a set of 1000 numbers starting at 00001 to 00999, with the next group assigned to numbers ranging from 01000 to 01999 etc. (Rossney *et al.*, 2006). Isolates exhibiting sporadically occurring patterns were assigned a number within a series from 99,000 to 99,999 in a

block of 100 numbers to cater for further subtypes of these sporadic patterns. Groups of related PFTs that different by less than or equal to six bands were designated the first two digits of the PFT to represent the PFG e.g. PFG-00, PFG-01, PFG-02 etc. PFGs were combined with AR typing results to yield a AR-PFG type e.g. isolates exhibiting AR06 and belonging to PFG01 were designated AR-PFG 06-01 (Rossney *et al.*, 2006).

2.3 Bacterial Isolates

Clinical MRSA isolates

Five groups of MRSA isolates were investigated as part of this study

1. A total of 175 MRSA isolates representative of the most prevalent AR and PFGE types of MRSA recovered from patients in Irish hospitals between 1971 and 2004 (Chapter 3).
2. One hundred and ten fusidic acid-resistant isolates representative of the main AR-PFG types recovered from patients in Irish hospitals from 1971-2010 (Chapter 3)
3. A collection of 173 MRSA isolates recovered from 90 patients and 83 environmental sites in four wards at Beaumont Hospital [BH] Dublin, Ireland (Chapter 4).
4. One hundred and nine high-level mupirocin resistant (Hi-MupR) MRSA isolates recovered from patients in Irish hospitals between 1998 and 2009 (Chapter 5).
5. One PVL-positive CA-MRSA isolate M05/0060, recovered in 2005 from a 26 year-old patient with a scalp abscess that was found to harbour the multidrug resistant gene *cfr* (Chapter 6).

6. The plasmid-free, novobiocin-resistant *S. aureus* reference strain XU21 (Udo and Jacob, 1998) (Chapter 5 & 6) was used as a plasmid recipient in conjugation experiments which are described in detail in section 2.5.2.

2.4 Molecular typing

2.4.1 Whole-genomic DNA extraction

Total genomic DNA was extracted from isolates using the Qiagen DNeasy Blood and Tissue kit, (Qiagen, Crawley, West Sussex, UK) for all PCR applications expect for DNA microarray analysis according to the manufacturer's instructions. Each MRSA isolate was cultured as described in section 2.1.1. A single pure colony was removed from a TSA plate and lawned onto TSA using a sterile loop (Greiner Bio-One GmbH, Solingen, Germany) and incubated overnight at 37°C in a static incubator (Gallenkamp). Following 18 h incubation, cell lysis was performed by removing a 2.5 cm^2 area of culture growth of each isolate separately from lawned TSA plates using a sterile bacteriological loop and resuspending it in a 250 µl lysis solution containing 1 x TE buffer (section 2.1.2), and a final concentration of 0.2 mg/ml lysostaphin (Sigma) and 0.02 mg/ml lysozyme (Sigma). Cell lysis solution was incubated for 2-3 h at 37°C. Proteins and nucleases were then degraded by the addition of 25 µl of proteinase K and 200 µl AL buffer (supplied with Qiagen's DNeasy Blood and Tissue kit) and incubation at 70°C for 30 min. Following protein digestion, released DNA was then bound to a mini-column with a silica-gel membrane that selectively binds DNA. A series of column washes was then undertaken using buffers (supplied with the kit) containing ethanol and salt to wash cellular debris from the column while the DNA remained membrane bound. Following elution of cellular debris, DNA was eluted into 200 µl of elution buffer or molecular biology-grade water (Sigma) and stored at 4°C (for storage

of 4-6 weeks) or at -20°C (for long-term storage, ≥6 weeks). The quality of the extracted genomic DNA was assessed following electrophoresis through 0.8% (w/v) agarose gels. The concentration of genomic DNA (ng/µl) was measured using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc, Massachusetts, USA).

2.4.2 Polymerase chain reaction (PCR) and DNA sequencing

Specific sequences of *S. aureus* DNA were amplified using whole-genomic DNA as a template for PCR. All PCRs were performed using GoTaq DNA polymerase (Promega) according to the manufacturer's instructions. DNA was amplified using a G-storm GSI thermocycler (Life Technologies, corp. California, USA). The resulting PCR products were visualised by agarose gel electrophoresis and were purified using the Genelute PCR cleanup kit (Sigma) or Qiaex II gel extraction kit (Qiagen), or in the case of MLST, PCR products were purified using the Qiaquick 96 PCR purification kit (Qiagen).

Sequencing was performed commercially using the dideoxy chain termination method of Sanger *et al.* (1977) by Cogenics (Takeley, Essex, UK) with an automated Applied Biosystems 373A DNA sequencer (Foster City, California, USA), Geneservice Limited (Source Bioscience, Dublin, Ireland) and GATC Biotech (Cologne, Germany), both using an ABI 3730xl Sanger DNA analyser with the Applied Biosystems and a ABI BigDye terminator v3.1 cycle sequencing kit. Analysis of DNA sequences was carried out using the BioNumerics software package (version 5.1; Applied Maths, Ghent, Belgium), DNA strider™ software package version 1.3f11 for DNA sequence analysis (CEA/Saclay, Gif-sur-Yvette, France) along with the ApE, plasmid Editor software (http://www.biology.utah.edu/jorgensen/wayned/ape/) for analysis of chromatograms or for *spa* typing, using the Ridom StaphType software program,

version 1.3 (Ridom GmbH, Wutzburg, Germany). Homology searches were performed using BLAST software (http://ncbi.nih.gov/BLAST). ClustalW (European Bioinformatics Institute, Saffron Walden, Hinxton, UK) was used for multiple sequence alignment for the comparison of specific protein encoding sequences of genes (Smyth *et al.*, 2007).

2.4.3 *spa* typing

Spa typing was performed as described previously (Shore *et al.*, 2008) using the primers and thermal cycling conditions described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; www.seqnet.org). Primers included forward primer (spa 1113F 5'-TAAAGACGATCCTTCGGTGAGC-3') and a reverse primer (spa 1514R 5'-CAGCAGTAGTGCCGTTTGCTT-3'). *Spa* amplimers were sequenced commercially by Cogencis (Section 2.4.2) and sequence analysis including *spa* repeat determination and *spa* type assignment was performed using the Ridom Staphtype software package version 1.3 beta. The SpaServer website was used to access the clonal relatedness between isolates (http://spaserver2.ridom.de). Clustering of *spa* types was carried out using Based Upon Repeat Patterns (BURP) algorithm, which clustered together *spa* types using default parameters if they contained five or more *spa* repeats and if they had a cost value of less than four, where the cost value accounts for the number of steps of evolution between *spa* types. *Spa* types that had four or less repeats were excluded from the analysis because no evolutionary connection could be inferred. Group founders within BURP clusters is the *spa* type with the highest founder score among at least three different *spa* types (Mellmann *et al.*, 2007).

2.4.4 *dru* typing

The *dru* repeat region was amplified using the primers and conditions and sequence as described previously (Goering *et al.*, 2008). *Dru* sequence analysis and assignment of *dru* types were performed by the Bionumerics Tandem-Repeat Sequence Typing (TRST) plug in tool. An alpha-numeric nomenclature was used to assign *dru* types (Goering *et al.*, 2008). *Dru* repeat (dr) number was designated to a specific 40 bp repeat sequence within the *dru* region, the (dr) number indicates the number of nucleotide change from the consensus sequence (the consensus sequence is available at http://www.dru-typing.org). To define the locations of the nucleotide changes with *dru* repeat (dr), an alphabetic letter is assigned e.g. dr2a and dr2b. The combination of the *dru* type (dt) number which indicates the number of repeat sequences present and an alphabetic designation to indicate the different tandem position of specific repeats e.g. dt10o

2.4.5 DNA microarray analysis

The Staph-Type Kit (Alere, Jena, Germany) consists of individual DNA microarray chips mounted in 8-well microtitre strips. Each chip detects 344 *S. aureus* gene targets and alleles including species-specific, antimicrobial resistance and virulence-associated genes as well as typing markers (Monecke *et al.*, 2008a).

2.4.5.1 Whole-genome DNA preparation for DNA microarray analysis

Genomic DNA for use with the DNA microarray was extracted from MRSA isolates using the Qiagen DNeasy kit and according to the Staphy-Type Kit manufacturer's instructions (Alere Technologies GmbH).For each isolate, a 2.5 cm^2 area of MRSA culture growth was removed from a CBA plate (Oxoid) after 18 h

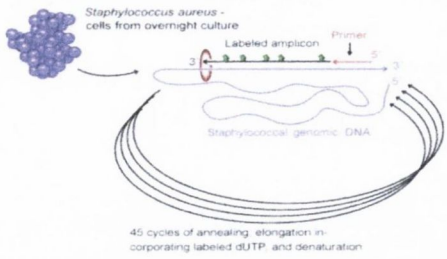
incubation and was resuspended in a lysis solution containing 200 µl buffer A1 and 1.7 µl lysis enhancer A2 (supplied with the Staphy-Type Kit). The cell suspension was vortexed and incubated with shaking (250 rpm) at 37°C for 1 h. Following this, 25 µl of proteinase K and 200 µl of buffer AL (supplied with the DNAeasy Blood and Tissue Kit, Qiagen) were added to each sample and incubated at 70°C for 30 min. The DNA was then purified according to Qiagen's DNeasy Blood and Tissue kit instructions as described in section 2.4.1 above. DNA elution was carried out by the addition of 50 µl of molecular biology-grade water (Sigma) onto the mini-column filter and incubated at room temperature for 10 min followed by centrifugation at 15, 970 x *g* for 1 min using an Eppendorf centrifuge 5417C fitted with an F-45-30-11 rotor (Eppendorf, Hamburg, Germany). The DNA samples were concentrated to remove any residual solvents such as ethanol by an evaporation step in a QB dry heating block (Thomas Scientific, New Jersey, USA) at 70°C for 30 min with the caps of sample microfuge tubes removed. The quality of the DNA samples and the absence of RNA was assessed by running a diluted aliquot (1/50 dilution) of each sample on a 0.8% (w/v) agarose gel. The concentration of genomic DNA (ng/µl) was measured using the Nanodrop 2000c spectrophotometer. Storage of DNA was as described in section 2.4.1

2.4.5.2 Linear PCR amplification and microarray array hybridisation and detection

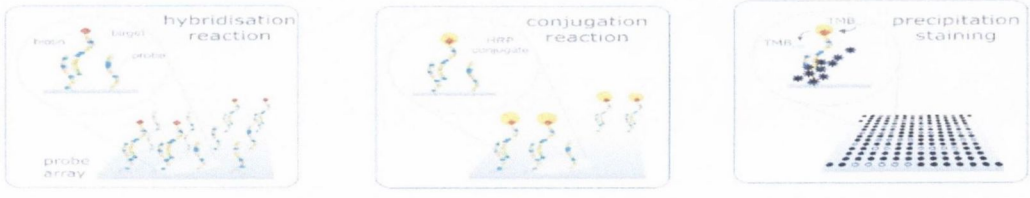
For DNA microarray analysis a linear PCR amplification and labelling reaction was carried out by using thermally synchronised primer elongation reactions. This was performed on all *S. aureus* isolates using a PCR and primer mastermix supplied with the Staphy-Type Kit Alere and using a G-storm GSI thermocycler. This linear PCR reaction allowed all target genes to be amplified and labelled simultaneously using a single primer for all 334 gene targets simultaneously and biotin-16-dUTP, respectively, (Fig.

2.1). Following the linear PCR, amplicons were hybridised to the arrays chips, each with 334 probes. Then the biotin-16-dUTP labelled PCR products were mixed with a hybridisation buffer (buffer C1 supplied with the Staphy-Type Kit) to a total volume of 100 µl and were transferred to each well of the prepared microarray strips, each of which contained a microarray chip integrated into the bottom of each well of the strip (Fig. 2.1). Preparation of the wells included a wash step with ultra-pure water (section 2.1.3) followed by an incubation step at 55°C for two min with shaking using hybridisation buffer C1. Then the capped wells containing the hybridisation mixture (i.e. 90 µl PCR product with 10 µl C1) were incubated for 1 h at 55°C with shaking (550 rpm). After the hybridisation step, the hybridisation mixture was removed completely from the wells and three successive wash steps was performed by the addition of 200 µl of buffer C2 (Staphy-Type kit). This step was followed by the addition of horseradish peroxidase-conjugate (HRP-conjugate), which consisted of reagent C3 containing streptavidin-HRP and Buffer C4 in a 1:100 ratio (Staphy-Type kit). The HRP-conjugate mixture was applied to each of the microarray wells and incubated at 30°C at 550 rpm for 10 min (Fig. 2.1). After the biotin-16-dUTP was bound to the conjugated streptavidin the HRP-conjugate solution was removed and a final wash step was carried out using 200 µl washing buffer C5 (Staphy-Type kit). Once the washing buffer C5 was removed, staining of the bound biotin-HRP conjugate was performed using 100 µl reagent D1 (Staphy-Type kit) containing the substrate tetramethylbenzidine (TMB). A 100 µl volume of TMB was transferred to each well of the microarray and incubated at room temperature for 5 min without agitation. This substrate reacts with the bound HRP-conjugate to produce a blue/green reaction product (Fig. 2.1). The D1 reagent was then discarded completely and the arrays were analysed immediately using the Arraymate reader and IconoClust software (Arraymate, Alere) (Fig. 2.1). The

a.



b.



c.

d.

Fig. 2.1 Schematic diagram illustrating the DNA microarray analysis process. (**a**) Linear PCR amplification reaction of the genomic staphylococcal DNA using a single primer for all 334 gene targets and labelled with biotin-16-dUTP. (**b**) The biotin-16-dUTP labelled amplicons are hybridised to the arrays chips followed by a conjugation reaction with horseradish peroxidase-conjugate (HRP-conjugate) with the final step of precipitation with the substrate tetramethylbenzidine (TMB). (**c**) The 8-well array microarray strip. (**d**) DNA chip images are analysed using the Arraymate reader, which records an image of each DNA microarray chip and the raw data is analysed and interpreted using IconoClust software.

Arraymate reader records an image of each DNA microarray chip and the IconoClust software interprets raw data as "positive", "negative" or "ambiguous", using a previously described algorithm (Monecke *et al.*, 2008a). The breakpoints for the positive, ambiguous or negative signals are defined as an average of each individual signal for the control spots and species markers (*femA*, *gapA*, *katA*, *coa*, *spa*, *sak* and *sbi*). Values < 25% are assigned as ambiguous, values between 25-33% are assigned as negative and values >33% are assigned as positive (Monecke *et al.*, 2007). The IconoClust software can assign *S. aureus* isolates to STs and/or CCs by comparison of the DNA microarray results to those of a collection of previously characterised and MLST-typed reference strains in the Arraymate database (Monecke *et al.*, 2008a).

2.6 Plasmid analysis

2.6.1 Plasmid curing

Plasmid curing was performed by culturing clinical isolates in Brain Heart Infusion (BHI) broth at 43°C for 24 h at 200 rpm. This was followed by subculture by the transfer of 100 µl dilution factor of 10^4 cfu into fresh BHI broth and incubation as before for seven to fourteen consecutive rounds, after which individual colonies obtained following plating on BHI agar were screened by replica plating for loss of resistance to the appropriate antimicrobial agent(s) (see Chapters 5 and 6) using selective and non-selective media. This involved plating a 10^5 dilution of the final overnight culture grown at 43°C in BHI broth so that a countable number of colonies (approximately 50-100 full size colonies per agar plate) could be screened for the loss of particular resistance determinant(s). A 100 µl volume of this 10^5 dilution was plated on BHI agar using sterile plastic spreaders (Greiner Bio-One) and incubated in a static incubator overnight at 37°C. Following incubation, the plates were placed in a refrigerator at approximately 8 °C for 2-3 h, cooling the plates rendered the colonies less

sticky and easier to replica plate. Plates were placed at room temperature for 30 min prior to replica plating. For replica plating, a sterile piece of velvet material (15 cm x 15 cm) was placed on a replica plating device consisting of a circular block of heavy duty plastic with a diameter of 7.6 cm. The velvet was secured in place by a metal ring (Fig. 2.2). The combined diameter of the replica plating block with velvet attached was <8.0 cm in diameter which enabled an agar plate (approx. 90 mm in diameter) with bacterial colonies to be imprinted onto the surface of the velvet. All BHI agar plates that were used for replica plating and the block were marked with a water soluble ink felt pen to provide reference marks so that replica plated colonies could be matched with the corresponding colonies on the master plate. Colonies were replica plated by placing a master BHI agar plate with colonies face down into a sterile velvet secured onto the replica plating block. Gentle pressure was then applied to the bottom of the plate to transfer part of the colony growth onto the velvet. The master plate was then removed and a fresh BHI agar plate supplemented with a specific antimicrobial agent was then placed onto the imprinted velvet and gentle force applied to the back of the plate to transfer bacterial growth onto the agar plate. The process was repeated a second time with an agar plate without antimicrobial supplements to ensure successful transfer of all colonies from the original master plate to the selective plate (Fig. 2.2). Master plates were stored at 4 ^{o}C and replica plated plates were incubated at 37^{o}C for 18 h. Following incubation, the pattern of colonies present on replica plates containing antimicrobial agents was compared visually with the corresponding patterns on master plates. Putative cured derivatives were identified by absence of growth on replica plated media with antimicrobial supplements. These were purified by picking with a sterile toothpick and subculture on fresh BHI agar without antimicrobial supplements and the appropriate antibiotic-containing agar plates. Colonies which grew on non-selective but not on the

Fig. 2.2 Replica plating. Panel (**a**) The replica plating device used in the present study consisting of a circular block of heavy duty plastic with a diameter of 7.6 cm. (**b**) a sterile piece of velvet material (15 cm x 15 cm) on the replica plating device secured in place by a metal ring. (**c**) colonies grown on a BHI agar plate placed face down onto a sterile velvet secured onto the replica plating block. (**d**) imprints of bacterial colonies on the sterile velvet material following removal of the BHI master plate. A fresh BHI agar plate supplemented with a specific antimicrobial agent is placed onto the imprinted velvet and gentle force applied to the back of the plate to transfer bacterial growth onto the agar plate. In this way loss of specific resistance markers by individual colonies can be identified by their inability to grow on selective media following replica plating.

selective BHI agar were subcultured on TSA and stored in individual bacterial preserver vials at -70°C. Putative cured derivatives underwent DNA microarray analysis (section 2.4.4) and antimicrobial susceptibility testing as described in Chapter 5 and 6.

2.6.2 Filter mating

Conjugative transfer of plasmid-encoded resistance genes from donor clinical MRSA isolates to the plasmid-free, novobiocin-resistant *S. aureus* recipient strain XU21 was performed by filter mating (Woodford *et al.*, 1998) (Table 2.2). The donor isolates used are described in detail in Chapters 5 and 6. The donor MRSA isolates and the recipient strain *S. aureus* XU21 were plated separately on BHI agar and incubated overnight at 37°C for 18 h (Udo and Jacob, 1998). A single colony of each isolate was then inoculated into 5 ml of BHI broth and incubated for 18 h at 37°C at 200 rpm. Following incubation, a 50 µl volume each of the overnight culture of the donor isolate and recipient strain were mixed together gently in a sterile 1.5 ml microfuge tube (Eppendorf, Stevenage, UK). This suspension (100 µl) was then applied drop wise to a sterile filter (0.45-µm pore size, Millipore Watford, Hertfordshire, UK) that was placed onto a fresh BHI agar plate using a sterile forceps and incubated overnight at 37°C in a static incubator (Gallenkamp). After incubation, the filter was transferred to a sterile falcon tube (Fisher Scientific) containing 1 ml BHI broth and was very gently agitated to remove the culture lawn from the filter. The filter was removed and the culture suspension was centrifuged at 2,000 x *g* for 5 min using a Hettich universal centrifuge 320 fitted with an swing-out rotor 1624 (Z601527) (Labcare Ltd, Newport Pagnell, Buckinghamshire, UK). The supernatant was decanted, the pellet was resuspended in 1 ml BHI broth and was incubated at 37°C for 1-2 h at 200 rpm in an orbital incubator (Gallenkamp). Resuspended bacterial cells were then plated onto BHI agar containing 100 µg/ml novobiocin, to select for the recipient strain, and the appropriate

antimicrobial agent to select for the donor plasmid marker which was being investigated, followed by incubation at 37°C for 48 h. Donor isolates and the recipient strain were also processed separately using the same filter mating method as negative controls. Any colonies present on the selective media following incubation were deemed to be putative transconjugants, provided that no growth was evident on plates lawned with donor or recipient cells only. Selected putative transconjugants were subject to DNA microarray analysis (section 2.4.4) and antimicrobial susceptibility testing in order to identify true transconjugant derivatives (i.e. XU21 MSSA colonies harbouring conjugative plasmids encoding antimicrobial resistance from donor isolates). The rate of conjugal transfer was expressed as the number of transconjugants per donor cells using filter mating experiments. Selected true transconjugants were stored in individual bacterial preserver vials at -70°C prior to subsequent analysis.

2.6.3 Plasmid DNA extraction

Plasmid DNA was extracted and purified from *S. aureus* isolates using a modified alkaline lysis method as described previously (Schwarz *et al.*, 1989). Each isolate was cultured on BHI agar as described in section 2.1.1. A single pure colony was removed from the BHI agar using a sterile bacteriological loop (Greiner Bio-One) and was inoculated into 3 ml of Luria broth (LB) in a 13 ml sterile plastic tube (Sarstedt, Nümbrecht, Germany) and incubated overnight at 37°C at 200 rpm in an orbital incubator (Gallenkamp). A 1.5 ml volume of overnight culture (i.e. 18 h) was transferred to a sterile 1.5 ml microfuge tube (Eppendorf), and centrifuged at 4,500 x *g* for 10 min at 4°C using an Eppendorf model 5417R centrifuge. After centrifugation all samples were immediately placed on ice, the supernatant was discarded and 100 µl of buffer P1 (see section 2.1.2) was added to each sample and the pellets resupended, then a 5 µl aliquot of lysostaphin (1 µg/ml) was added to the resuspended cells followed by

Table 2.2 Reference *Escherichia coli* and *Staphylococcus aureus* strains used in the study

Strain	Plasmid(s) harboured (kb)	Reference
S. aureus ATCC 25923	Plasmid free	CLSI, 2006; Rossney *et al.*, 2007
S. aureus ATCC 292133	Plasmid free	CLSI, 2006; Rossney *et al.*, 2007
S. aureus XU21	Plasmid free	Udo and Jacob, 1998
E. coli NCTC 50192	150, 64, 36, 7.0	Mendes *et al.*, 2010
E. coli NCTC 50193	49, 7.9, 5.0, 4.6, 3.3, 2.6, 2.3, 1.8	Mendes *et al.*, 2010

incubation at 37°C with gentle shaking for 30 min. Subsequently 200 µl of P2 buffer prewarmed to 37°C was added to each sample and then each microfuge tube was rolled gently from side to side until the solution became viscous, followed by incubation on ice for 5 min. Buffer P3 (150 µl) was then added to each sample and each gently mixed by inverting the tubes manually several times followed by a further incubation period on ice for 10 min. The samples were then centrifuged at 15, 700 x *g* for 30 min at 4°C, and the supernatants transferred to fresh microfuge tubes with the addition of an equal volume (ca. 500 µl) of phenol: chloroform: isoamyl alcohol (24:24:1). The contents of each tube was then mixed by inversion and centrifuged at max speed (15,682 x *g*) for 3 min. Following centrifugation, the aqueous supernatant from each sample as far as the interface was gently removed using a pipette (Gilson Inc, Wisconsin, USA) using a sterile graduated filter tip (Starlab, Milton Keynes, UK) and transferred into a new microfuge tube, and this step was repeated a further two times, followed by a final addition of 500 µl of chloroform: isoamyl alcohol (24:1). The final supernatant containing the plasmid DNA was then transferred to a new microfuge tube 100% (v/v) molecular-biology grade ethanol (Sigma), which was then inverted 14 times and incubated for 1 h at room temperature to allow the DNA to precipitate. DNA was then pelleted by centrifugation at 15, 700 x *g* for 30 min. Following centrifugation, the supernatant was removed and 80% (v/v) ice cold ethanol was added with a further centrifugation step at 15, 700 x *g* at 10 min. The supernatant was gently removed by careful pipetting and the pellet containing the plasmid DNA was resuspended in 40 µl of molecular biology grade water (Sigma). This was centrifuged at 11, 228 x *g* for 30 sec and all residual ethanol was removed by desiccating for 15 min. Plasmid DNA samples (20 µl) were then loaded onto a 0.8% (w/v) TAE agarose gel with 8 µl of DNA loading dye (Promega). Plasmid DNA recovered from the *Escherichia coli* reference

strains NCTC 50192 & 50193 were loaded in gel lanes adjacent to the *S. aureus* plasmid DNA preparations (Mendes *et al.*, 2010b) (Table 2.2). The *E. coli* reference strains NCTC 50192 & 50193 were purchased from the Health Protection Agency (Colindale Ave, London, UK). These strains harboured plasmids of known molecular size and were used to provide plasmid size reference markers. Gels were electrophoresed for 4-5 h and then stained for 30 min in a solution containing 0.5 µg/ml of ethidium bromide (Sigma) in ultrapure water with gentle shaking. Gels were then destained for 2 x 30 min with ultrapure water with gentle shaking. Plasmid DNA was visualised by placing the gel on a UV transilluminator at 312 nm (Alphalmager Mini, AlphaInnotech) and gel images were captured, analysed and stored electronically using the AlphaImager SA software version 3.0.0.0 (AlphaInnotech). Plasmid DNA samples were stored at -20 ^{o}C.

Chapter 3

Detailed molecular characterisation of MRSA isolates recovered in Irish hospitals between 1971 and 2004

3.1 Introduction

MRSA was first reported in Irish hospitals in 1971. In the late 1970s, the incidence of MRSA increased significantly and it has now been endemic in Irish hospitals for almost four decades (Cafferkey *et al.*, 1985; Coleman *et al.*, 1985; Humphreys *et al.*, 1990; Shore *et al.*, 2005; Rossney *et al.*, 2006; Shore *et al.*, 2010). Since 2002, ST22-MRSA-IV has predominated in Irish hospitals and is known locally as AR-PFG 06-01 (Rossney *et al.*, 2006; Cox *et al.*, 2012). ST22-MRSA-IV was first reported in the UK in 1991 and was termed EMRSA-15 (Richardson and Reith, 1993), and has since become a pandemic MRSA clone predominating not only in Ireland and the UK but also in many other countries worldwide including Portugal, the Czech Republic, Germany, Malta, Singapore and New Zealand (Hsu *et al.*, 2005; Smith and Cook, 2005; Melter *et al.*, 2006; Aires-de-Sousa *et al.*, 2008; Monecke *et al.*, 2008a; Scicluna *et al.*, 2010). Over the last decade MRSA strains have also emerged as a significant cause of infections among otherwise healthy individuals in the community and among animals (Witte *et al.*, 2007; DeLeo and Chambers, 2009).

Informative molecular typing is an important tool for epidemiological studies for monitoring emergence, tracking the spread and evolution of MRSA clones in a local geographical area or globally (Faria *et al.*, 2008; Shore *et al.*, 2010). The most common methods used routinely for typing MRSA include MLST, SCC*mec* typing and *spa* typing. MLST is used widely for typing MRSA in long term and global investigations (Enright *et al.*, 2002; Robinson and Enright, 2003; Aires de Sousa and de Lencastre, 2003), but it lacks the discriminatory ability required for local or outbreak studies. It is also labour intensive and expensive due to the requirement for the amplification and sequencing of seven housekeeping genes (Robinson and Enright, 2004b). *spa* typing offers more discrimination than MLST and only involves the DNA sequencing of a

variable number tandem repeat (VNTR) region of the *S. aureus* protein A gene, *spa*. Its usefulness has been demonstrated in both local and global epidemiological investigations (Grundmann *et al.*, 2010).

For the purpose of epidemiological studies, SCC*mec* typing has been shown to be a useful typing tool when used with MLST or *spa* typing, as it allows for greater differentiation between MRSA strains with the same genetic background (Ito *et al.*, 2001b; Ito *et al.*, 2003). Each SCC*mec* type (I-XI) has a unique combination of cassette chromosome recombinase (*ccr*), *mec* complex genes (*mecA*, *mecI* and *mecRI*) and subtypes differ in the joining 'J' regions (Fig. 1.1). SCC*mec* typing involves several multiplex PCRs to detect particular types of *ccr-mec* combinations and variations within the 'J' regions which define SCC*mec* types and subtypes (Oliveira and de Lencastre, 2002; Kondo *et al.*, 2007; Milheiriço *et al.*, 2007). However, the increasing complexity of SCC*mec* and SCC elements and the identification of pseudo SCC*mec* (with *mec* complex and not *ccr* genes) and SCC elements (with *ccr* and no *mec* genes), SCC-like elements and composite islands (CIs) containing multiple pseudo-SCC*mec*/SCC, SCC-likeand SCC*mec* elements, which have been described in *S. aureus* and CoNS, has increased the need for more complex SCC*mec* typing methods to be developed (Ito *et al.*, 2001b; Luong *et al.*, 2002; Katayama *et al.*, 2003; Wisplinghoff *et al.*, 2003; Hanssen *et al.*, 2004; Mongkolrattanothai *et al.*, 2004; Holden *et al.*, 2004; Shore *et al.*, 2011b; Bouchami *et al.*, 2012; Zong *et al.*, 2011; Kinnevey *et al.*, 2013).

Recent advances in high-throughput whole-genome sequencing technologies have allowed more detailed analysis of the whole genome sequence of an MRSA isolate to be undertaken, permitting the analysis of genome-wide single nucleotide polymorphism (SNP) profiles (DeLeo and Chambers, 2009; Harris *et al.*, 2010; Köser *et al.*, 2012b). This approach is highly discriminatory but although whole-genome

sequencing has become considerably cheaper in recent years, it currently is only available for typing MRSA by specialised research laboratories. Data analysis is time consuming, requires considerable technical expertise and expensive computer hardware and software. Whole-genome sequence analysis of MRSA and *S. aureus* isolates has permitted the development of RNA expression arrays (Lindsay and Holden, 2004) and DNA microarrays, the latter which detects the presence or absence of genes in *S. aureus* isolates (Saunders *et al.*, 2004; Witney *et al.*, 2005; Monecke *et al.*, 2008a; Sung *et al.*, 2008; El Garch *et al.*, 2009; Stegger *et al.*, 2010).

The Staphy-Type kit (Alere) based on DNA microarray technology has recently been developed as an alternative typing approach for genotyping *S. aureus* isolates including MRSA. This DNA microarray system permits gene profiles detected in isolates under investigation to be compared to the corresponding profiles of a large database of reference *S. aureus* strains which have been previously characterised by DNA microarray and MLST (Monecke *et al.*, 2007). The characterisation of *S. aureus* using the Staphy-Type Kit is based on the simultaneous detection of 334 *S. aureus* alleles and genes including species specific marker (e.g. *spa*, *nuc*), accessory gene regulator alleles *agr* I-IV, capsule type specific genes (*cap1*, *cap5*and *cap8*) and SCC*mec* genes (*ccr*, *mec* and accessory genes) as well as an extensive range of clinically relevant antimicrobial resistance and virulence genes (Table 3.1). This technology is based on a linear PCR amplification using 334 primers and incorporation of biotin-dUTP into the amplimers. The amplimers are hybridised to the DNA microarray chip (Fig. 2.1) containing the 334 probes followed by the addition of a horseradish-peroxidase (HRP)-streptavidin conjugate which binds to the biotin-16-UTP label, and staining using tetramethyl benzidine (TMB) which reacts with the HRP-

Table 3.1 Molecular typing markers, antimicrobial resistance and virulence-associated genes and MSCRAMM genes detected using the Alere DNA microarray

Gene target	Description	Location
Molecular typing markers		
agr I-IV	Accessory gene regulator genes; alleles I-IV	Core genome
capsule 5	Capsular polysaccharide allele, serotype 5	Genomic island
capsule 8	Capsular polysaccharide allele, serotype 8	Genomic island
ccrA1/ccrA2/ccrA3/ccrA 4 alleles	Cassette chromosome recombinase A alleles	SCC*mec*
ccrB1/ccrB2/ccrB3/ ccrB4 alleles	Cassette chromosome recombinase B alleles	SCC*mec*
*ccr*C	Cassette chromosome recombinase C	SCC*mec*
mecA	Methicillin resistance	SCC*mec*
mecI	Methicillin-resistance regulatory protein	SCC*mec*
mecRI	Signal transducer protein *mec*R1	SCC*mec*
Δ*mecRI*	Truncated *mec*R1	SCC*mec*
Q9XB68-dcs	Hypothetical protein	SCC*mec*
ugpQ	Glycerophosphoryl diester phosphodiesterase	SCC*mec*
*kdp*A-E	Potassium-translocating ATPase A-E	SCC*mec*
xylR	Xylose repressor	SCC*mec*
merA	Mercuric reductase A Hg (II) reductase	SCC*mec*/ pI258
merB	Mercuric reductase B Hg (II) reductase	SCC*mec*/ pI258
plsSCC-COL	Plasmin-sensitive surface protein	SCC*mec*
Gene target	**Description**	**Location**
Antimicrobial resistance genes		
blaZ/blaI/blaR	Beta-lactamase penicillinase	pI*258*/ Tn*552*
erm(A)	Macrolide-lincosamide-streptogramin B	Tn*554*
erm(B)	Macrolide-lincosamide-streptogramin B	pI258
erm(C)	Macrolide-lincosamide-streptogramin B	pE194
lnu(A)	Lincosamide resistance protein	pUR5425
msr(A)	Macrolides and streptogramin B	pMS97
mef(A)	Macrolide efflux protein A	Tn*1207.1*
mph(C)	Macrolides and streptogramin B	pMS97
vat(A)	Virginiamycin A acetyltransferase	pIP680
vat(B)	Acetyltransferase inactivating steptogramin B	pUB110
vga(A)	Steptogramin A, lincosamide, pleuromutilin resistance	pIP680
vgb(A)	Virginiamycin B hydrolase	Plasmid
aacA-aphD	Gentamicin, kanamycin & tobramycin	Tn*4001*
aadD	Kanamycin, neomycin & tobramycin	pUB110
aphA3 & *sat*	Kanamycin, neomycin, strepthothricin, streptomycin resistance	Tn*5404*/Tn*5405*
dfrS1	Trimethoprim resistance	Tn*4003*
far1(*fusB*)	Fusidic acid resistance	pUB101
Q6GD50 (*fusC*)	Fusidic acid resistance	SCC*476*
ileS2	Mupirocin resistance	*IleS2*-plasmids
tet(K)	Tetracycline efflux protein	pT181
tet(M)	Tetracyline resistance	Core genome
sdrM	General efflux protein	Core genome
cat-pC221	Chloramphenicol resistance	pC221
cfr	Chloramphenicol, florfenicol and clindamycin resistance	pSCFS1
fexA	Chloramphenicol and florfenicol resistance	Tn*558*
fosB	Fosfomycin resistance	Core genome
fosB-plasmid	Fosfomycin resistance	pIP1842-like plasmid

Continued overleaf

Table 3.1 continued. Molecular typing markers, antimicrobial resistance and virulence-associated genes and MSCRAMM genes detected using the Alere DNA microarray

Gene target	Description	Location
Antimicrobial resistance genes		
qacA/C	Quaternary ammonium compounds Ethidium Bromide, Antiseptic resistance protein	pSK57, pSK89
vanA/*van*B/*vanZ*	Vancomycin/teicoplanin A- type resistance protein	pLW1043
Gene target	**Description**	**Location**
Virulence factors		
tst	Toxic shock syndrome gene	Pathogenicity island
sea	Enterotoxin A	*hlb* converting phage
sea-320E	Enterotoxin A variation	*hlb* converting phage
sep	Enterotoxin P	*hlb* converting phage
seb, *sek*, *seq*	Enterotoxin B, K, Q	Pathogenicity island
sec & sel	Enterotoxin C & Enterotoxin L	MBE gene cluster
*sec*M14	Enterotoxin C variation	MBE gene cluster
sed, *sej*, *ser*	Enterotoxin D, J R	Plasmid
see	Enterotoxin E	Pathogenicity island
seg, *sei*, *sem*, *sen*, *seo*, and *seu*	Enterotoxin G, I, M, N, O, U	*egc* cluster
seh	Entertoxin H	SCC*mec*
entX	Enterotoxin X	*hlb* converting phage
lukF-PV, *lukS-PV*	Panton-Valentine leucocidin	Prophage
hlgA	Hemolysin gamma	Core genome
lukF/D/E/S/X/Y	Leukocidins	Genomic island
hl	Putative membrane protein	Core genome
hla	Haemolysin alpha toxin	Core genome
hld	Hemolysin delta	Core genome
hlIII	Putative hemolysin III	Core genome
hlb	Hemolysin beta	Core genome
sak	Satphylokinase, *S.aureus* plasminogen	*hlb* converting phage
chp	Chemotaxis-inhibting protein (CHIPS)	*hlb* converting phage
scn	Staphylococcal complement inhibitior	*hlb* converting phage, part of the immune evasion cluster
etA/B/D	Exfoliative toxin A/B/D	Pathogenicity island
edinA	Epidermal cell differentiation inhibitor A	Pathogenicity island
edinB	Epidermal cell differentiation inhibitor B	Pathogenicity island
edinC	Epidermal cell differentiation inhibitor C	Pathogenicity island
arcA/B/C/D	Arginine/ornithine genes	ACME
aur	Aureolysin secreted zinc-metalloproteinase	Core genome
splA	Serine protease A	Genomic island
splB	Serine protease B	Genomic island
splE	Serine protease-like exoprotein E	Genomic island
sspA-	Extracellular serine protease	Core genome
sspB	Cysteine protease	Core genome
sspP	Staphylococcal cysteine proteinase A	Core genome
*ssl*01-11 alleles	Staphylococcal superantigen-like proteins	Genomic Island
setB	Signal peptide	Variable genome

Continued overleaf

Table 3.1 continued. Molecular typing markers, antimicrobial resistance and virulence-associated genes and MSCRAMM genes detected using the Alere DNA microarray

Gene target	Description	Location
MSCRAMMs		
icaA	Intraceullar adhesion protein A	Gene cluster
icaC	Intraceullar adhesion protein B	Gene cluster
icaD	Intraceullar adhesion protein C	Gene cluster
bap	Surface protein involved in biofilm formation	Putative composite transposons
bbp	Bone sialoprotein-binding protein	Core genome
clfA	Clumping factor A	Core genome
clfB	Clumping factor B	Core genome
cna	Collagen-binding adhesion	Genomic island
ebh	Fibronectin-binding protein	Core genome
ebpS	Cell surface elastin binding protein	Core genome
eno	Enolase phosphopyruvate hydratase laminin binding protein	Core genome
fib	Fibronectin-binding protein	Core genome
fnbA	Fibronectin-binding A	Core variable
fnbB	Fibronectin-binding B	Genomic island
map	Major histocompatibility complex class II analog protein	Core variable
sdrC	Serine aspartate repeat protein C	Core genome
sdrD	Serine aspartate repeat protein D	Core genome
vwb	Van Willebrand factor	Core genome
sasG	*Staphylococcus aureus* surface protein G	Genomic island
isaB	Immunodominant antigenB	Core variable genome
mprF	lysylphosphatigylglllycerol synthetase	Core genome
isdA	Heme-binding protein	Gene cluster
lmrP	Putative transporter protein	Core genome
Q2YUB3	Multidrug resistance protein	Core genome
hsdS1	Restriction modification specificity subunit	Core genome

streptavidin conjugate to form a blue precipitate, which is then analysed using a designated reader and the microarray imaging software (IconoClust, Alere).

The genes detected by the DNA microarray array form part of both the core and variable genomes, with many of the antimicrobial resistance and virulence genes being located on MGEs that can be transferred horizontally between staphylococci (Lindsay and Holden, 2004) (Table 3.1). Many different types of MGEs encoding virulence genes have been identified in *S. aureus* including bacteriophages, pathogenicity islands, plasmids, transposons, SCC*mec* elements or SCC-like elements and genomic islands (Carroll *et al.*, 1995; Malachowa and DeLeo, 2010) (Table 3.1). Similarly, antimicrobial resistance genes are also commonly carried on MGEs including plasmids and transposons (Table 3.1), but resistance can also be due to spontaneous mutations. For example, trimethoprim resistance in *S. aureus* has been described to date to be due to either point mutations in the chromosomally located dihydrofolate reductase B (*dfrB*) (Sa DHFR) (Dale *et al.*, 1993), or due to the presence of a trimethoprim resistance gene, the most common being the plasmid mediated gene *dfrS1* (SI DHFR) gene (Rouch *et al.*, 1989). Furthermore, tetracycline resistance genes in *S. aureus* have been identified as being chromosomally-located, as well as on various plasmids and a transposon, while reported mechanisms of fusidic acid resistance in *S. aureus* include mutations in the *fusA* of *fusE* genes and acquisition of the MGE-located *fusB*, *fusC* (Table 3.2).

DNA microarray profiling offers significant potential as a high-throughput system for rapidly screening MRSA isolates and to assign them to an ST and CC without the requirement for MLST and PCR analyses. Furthermore, DNA microarray profiling has potential to predict the antimicrobial susceptibility of an isolate to a wide range of antimicrobial agents and classes. To date the reliability of the Staphy-Type DNA microarray to correctly assign MRSA isolates to the correct sequence type

(ST)/clonal complex (CC) and SCC*mec* type has not yet been investigated with a large collection of well-characterised isolates.

3.1.1 Fusidic acid resistance

In *S. aureus* resistance to fusidic acid was first reported in the 1990s in the UK (Faber and Rosdahl, 1990) and is now common in most European countries. Four mechanisms of fusidic acid resistance have been reported in *S. aureus* (a) mutations in the chromosomal located *fusA* gene encoding elongation factor- G (O'Neill *et al.*, 2007) (b) mutations in the chromosomal located *fusE* (*rplF*) gene encoding fusidic acid resistant in small colony variants in *S. aureus* (c) acquisition of the plasmid encoded *fusB* gene that protects the drug target site, and (d) acquisition of the SCC encoded *fusC* gene that protects the drug target site (Table 3.2).

While the rates of fusidic acid resistance among *S. aureus* in many countries remains low, elevated rates of fusidic acid resistance have been observed in Ireland (Castanheira *et al.*, 2010a)(NMRSARL annual report 2011). In the USA, where fusidic acid has not been used clinically, reports of fusidic acid resistance in *S. aureus* are rare (0.3%). However, in Ireland, the rate of fusidic acid resistance among MRSA from bloodstream infections (BSIs) increased significantly from 1.67% in 1999 to 39% in 2010 (NMRSARL annual report 2011). However little is known about the molecular mechanism(s) of fusidic acid resistance among MRSA isolates from BSIs in Ireland, in particular among the predominant MRSA strain among BSI, namely ST22-MRSA-IV, and this warrants further investigation.

Table 3.2 Fusidic acid, tetracycline and trimethoprim resistance genes found in *Staphylococcal* species and the mechanisms of resistance

Resistance gene	Resistance mechanism	Location	Species	Reference
fusA	Mutations within in the EF-G binding domain	chromosomal EF-G gene *fusA*	*Staphylococcus aureus,* CoNS	Chopra, 1976; Castanheira *et al.*, 2010
fusB	Ribosome-protective protein	pUB101/ chromosomal	*S. aureus,* CoNS	O'Brien *et al.*, 2002; Castanheira *et al.*, 2010
fusC	Ribosome-protective protein	SCC476, SCC*mec*$_{N1}$ SCC*mec*-SCC-SCC-$_{CRISPR}$	*S. aureus,* CoNS	Holden *et al.*, 2004; Ender *et al.*, 2007; Kinnevey *et al.*, 2013
fusD	Intrinsic resistance	chromosomal	*Staphylococcus saprophyticus*	O'Neill *et al.*, 2007
fusE	Mutation within the *rplF* gene encoding for ribosomal protein L6	chromosomal	*S. aureus*	Norstrom *et al.*, 2007
tet(K)	Active efflux (major facilitator superfamily)	pT181, pST 3	*S. aureus,* CoNS	Schwarz *et al.*, 1990; Guay and Rothstein, 1993; Schwarz and Noble, 1994; Schwarz *et al.*, 1998; Hauschild *et al.*, 2007
tet(L)	Active efflux (major facilitator superfamily)	pSTE1, pKKS25, pSTS7	*Staphylococcus hyicus, Staphylococcus sciuri*	Schwarz *et al.*, 1992; Schwarz and Noble, 1994; Hauschild *et al.*, 2007; Kadlec and Schwarz, 2010a; Schwarz *et al.*, 1996
tet(M)	Ribosomal-protective protein	Tn*916*	*S. aureus, S. sciuri Staphylococcus intermedius*	Nesin *et al.*, 1990; Schwarz *et al.*, 1998; Hauschild *et al.*, 2007
tet(O)	Ribosomal-protective protein	chromosomal	*S. intermedius,*	Schwarz *et al.*, 1998
dfrS1	Dihydrofolate reductase	Tn*4003,* pSK1	*S. aureus*	Rouch *et al.*, 1989
dfrD	Dihydrofolate reductase	pABU17	*Staphylococcus heamolyticus*	Dale *et al.*, 1995
dfrG	Dihydrofolate reductase	chromosomal	*S. aureus*	Sekiguchi *et al.*, 2005
dfrK	Dihydrofolate reductase	Tn*559,* pKKS25,	*S. aureus*	Kadlec and Schwarz, 2010b

3.1.2 Aims

(a) The purpose of this part of the current study was to perform an in-depth molecular characterisation of the predominant MRSA clones recovered from patients in Irish hospitals between 1971 and 2004 using *spa* typing and a DNA microarray profiling. DNA microarray profiling was investigated to:

- Determine its effectiveness for genotyping MRSA isolates from Irish hospitals representative of major pandemic clones that have been previously investigated by MLST and detailed SCC*mec* typing.
- Identify the range of virulence and antimicrobial resistance genes harboured by these MRSA clones.
- Investigate the correlation between antimicrobial resistance phenotype determined by AR typing and the presence of antimicrobial resistance gene using DNA microarray profiling.

(b) This part of the study also aimed to investigate the molecular mechanism(s) of fusidic acid resistance among fusidic acid resistant (FdR) MRSA strains recovered from patients in Irish hospitals. To identify additional antimicrobial resistance and virulence factor genes among these isolates.

3.2 Materials and Methods

3.2.1 MRSA isolates

3.2.1.1 MRSA isolates investigated by *spa* typing and DNA microarray profiling

One-hundred and seventy-five MRSA isolates underwent *spa* typing. This included (a) 172 isolates representative of those that predominated in Irish hospitals during eight different study periods between 1971 and 2002 (Shore *et al.*, 2005) (Table 3.3), (b) one isolate representative of an MRSA strain (AR44, ST239-MRSA-III) that caused outbreaks in several Irish hospitals in 2003 (Shore, 2005) and (c) four sporadic MRSA isolates recovered from Irish hospitals in 2004 (Shore, 2005). One hundred and sixty one of these MRSA isolates were previously typed by MLST and all previously underwent SCC*mec* typing using individual PCRs to identify the *mec* complexes A and B, the *ccr* complex type 1, 2, and 3 (Kondo *et al.*, 2007) and analysis of the joining (J) regions by a multiplex PCR method as previously described (Oliveira and de Lencastre, 2002; Shore *et al.*, 2005). Of the one hundred and sixty one, 15 of these MRSA isolates underwent MLST as part of the present study as described previously (Enright *et al.*, 2000; Shore *et al.*, 2008). One hundred and seven of the 175 MRSA isolates investigated underwent DNA microarray analysis, including, where possible, three isolates representative of each AR type, ST, and SCC*mec* type/subtype combination previously identified among the 175 isolates during eight different study periods between 1971 and 2004 (Shore *et al.*, 2005) (Table 3.3).

Table 3.3 Clinical MRSA isolates recovered from Irish hospitals between 1971-2002 investigated in the present study by *spa* typing (n = 174), MLST (n = 15) and DNA microarray profiling (n = 107)

Year of isolation	Isolate no.[a]	Phenotype/ AR type[b]	AR pattern[b]	PFT[b]	ST/SCC *mec* type[c]	Body site/ specimen type
1971-'75[e]	**1612**	**Early MRSA**	**ApCdEbErMcPmaSmSpTe**	**99901**	**ST250-I**	**NA[d]**
1976-'84	161.2	Phenotype-II	ApCdEbErFdGnKnMcSmSpSu^{m}Tb	99910	**ST250-I-*pls***	NA
1976-'84	58.2	Phenotype-II	ApCdEbErFdGnKnMcNmSmSpSuTbTeTp	99912	**ST250-I-*pls***	NA
1976-'84	**120.1**	**Phenotype-II**	**Ak^{m}ApCdEbErFdGnKnMcNmPmaSmSpTbTeTp**	**99910**	**ST250I-*pls***	**NA**
1976-'84	43.2	Phenotype-II	ApCdEbErFd^{m}GnKnMcSmSpSuTbTe		ST250-I	NA
1976-'84	**58.1**	**Phenotype-II**	**ApCdEbErFdGnKnMcNmPmaSmSpSuTbTeTp**	**99912**	**ST250-I**	**NA**
1976-'84	**120.2**	**Phenotype-II**	**ApCdEbErFdGnKnMcNmPmaSmSpTbmTeTp**	**99910**	**ST250-I-*pls***	**NA**
1976-'84	**122.2**	**Phenotype-II**	**ApCdEbErFdGnKnLnMcPmaSmSpSuTbTe**	**99912**	**ST250-I**	**NA**
1976-'84	**138.1**	**Phenotype-II**	**Ak^{m}ApCdEbErFdGnKnLnMcPmaSmSpSu^{m}TbTe**	**99912**	**ST250-I**	**NA**
1976-'84	**161.1**	**Phenotype-II**	**ApCdEbErFdGnKnMcPmaSmSpSu^{m}Tb**	**99910**	**ST250-I-*pls***	**NA**
1976-'84	43.11	Phenotype-II	Ak^{m}ApCdEbErFdKnMcNmSmSpSuTbTe	99911	ST250-I-*pls*	NA
1976-'84	43.21	Phenotype-II	ApCdEbErFdGnKnMcNmSmSpSuTbTe	99912	ST250-I	NA
1976-'84	438.1	Phenotype-II	ApCdEbErFdGnKnMcNmSmSpSuTbTeTp	99914	ST250-I	NA
1976-'84	438.2	Phenotype-II	ApCdEbErFdGnKnLnMcNmPmaSmSpSuTbTeTp	NA	ST250-I	NA
1976-'84	57	Phenotype-II	ApCdEbErFdGnKnLnMcNmSmSpSuTbTeTp	99913	ST250-I	NA
1976-'84	79	Phenotype-II	ApCdEbErFdGnKnLnMcNmPmaSmSpSuTbTeTp		ST250-I	NA
1985-'87	**84**	**Phenotype-III**	**Ak^{m}ApCdClEbErGnKnMcNmPmaRf^{m}SmSpSuTbTe**	**99953**	**ST239-III & SCC*Hg*-pI258/Tn*554***	**NA**
1989	0.1239	AR01	ApCdEbErGnKnLnMcSmSpSuTbTeTp	99925	ST239-III	Hand
1989	**0.1085**	**AR01**	**ApCdEbErGnKnLnMcPmaSmSpSuTb^{m}TeTp**	**99925**	**ST239-III**	**Urine**
1989	**0.1118**	**AR01**	**ApCdErLnMcPmaSmSpTeTp**	**99926**	**ST239-III**	**Catheter site**
1989	**0.1109**	**AR01**	**ApCdErLnMcPmaSmSpSu^{m}TeTp**	**99925**	**ST239-III**	**Nose**
1989	**0.1206**	**AR02**	**ApCdEbErFdGnKnLnMcNmPmaSmSpSuTbTeTp**	**99918**	**ST250-I-*pls***	**Calf wound**
1989	**0.1228**	**AR02**	**ApCdEbErFdGnKnMcPmaSmSpSuTb^{m}**	**99915**	**ST250-I-*pls***	**Sputum**
1989	**0.1149**	**AR02**	**ApCdEbErFdGnKnMcPmaSmSpSuTb^{m}**	**99917**	**ST250-I-*pls***	**Urine**
1989	**0.1345**	**AR05**	**ApCdErFdKnMcNmPmaSmSpTbTe**	**00002**	**ST8-IIB**	**Leg ulcer**
1989	**0.1264**	**AR06**	**ApCdEr**	**01018**	**ST22-IV**	**NA**
1989	**0.1267**	**AR07.4**	**Ak^{m}ApErKnLnNmSpTb**	**03021**	**ST5-II**	**Sputum**
1989	**0.1342**	**AR07.4**	**Ak^{m}ApErKnLnNmSpTb**	**03021**	**ST5-II**	**Urine**
1989	**0.1125**	**AR07.3**	**Ak^{m}ApErKnNmSpTb**	**03021**	**ST5-II**	**Groin**
1989	**0.0065**	**AR09**	**Ak^{m}ApCdErGnKnMcPmaRf^{m}SmSpSuTbTe**	**99922**	**ST239-III**	**NA**
1989	**0.0066**	**AR09**	**Ak^{m}ApCdErGnKnMcNmPmaRfSmSpSuTbTe**	**99923**	**ST239-III**	**NA**
1993	**0.1336**	**ARNT**	**ApCdFd**	**02011**	**ST30-IV**	**Nose**
1993	0251	AR06	ApEb	01033	ST22-IV	NA
1993	**0250**	**AR06**	**ApCd**	**01033**	**ST22-IV**	**Sputum**
1993	**0318**	**AR06**	**ApCdEr**	**01018**	**ST22-IV**	**Screening**
1993	**0340**	**AR06**	**ApCdEr**	**01017**	**ST22-IV**	**Wound**
1993	**0159**	**AR07.4**	**Ak^{m}ApCdErKnLnNmSpTb**	**03043**	**ST5-II**	**Wound**
1993	0237	AR07.4	Ak^{m}ApCdCpEbErKnLnNmTb	03043	ST5-II	Wound
1993	**0257**	**AR07.3**	**Ak^{m}ApCdErKnNmSpTb**	**03012**	**ST5-II**	**Wound**
1993	0202	AR07.3	AkAp^{m}CdClEbErKnNmTb	03021	ST5-II	Ear or nose
1993	**0111**	**AR07.4**	**Ak^{m}ApCdCpErKnLnNmRfSpTb**	**03043**	**ST5-II**	**Sputum**
1993	**0220**	**AR11**	**Ak^{m}ApCdEbErKnLnMcNmPmaSpTb**	**03047**	**ST5-II**	**Wound**
1993	0101	AR11	Ak^{m}ApCdEbErKnLnMcNmSpTb	03047	ST5-II	Screening
1993	**0089**	**AR11**	**Ak^{m}ApCdEbErKnLnMcNmPmaSpTb**	**03004**	**ST5-II**	**Wound**
1993	**0190**	**AR11**	**Ak^{m}ApCdEbErKnLnMcNmPmaSpTb**	**03047**	**ST5-II**	**NA**
1993	**0242**	**ARNT**	**ApCdFd**	**02011**	**ST30-IV**	**Wound**
1993	**0067**	**ARNT**	**ApCdFd**	**02011**	**ST30IV**	**Wound**
1993	0132	AR13	Ak^{m}ApCdCpErGnKnLnMcNmSmSpTb	00002	ST8-IIA	Wound
1993	0055	AR13	Ak^{m}ApCdCpEbErGnKnLnMcNmSmSpTb	00072	ST8-IIA	Wound
1993	**0236**	**AR13**	**Ak^{m}ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb**	**00002**	**ST8-IIA**	**Blood**

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Table 3.3 continued. Clinical MRSA isolates recovered from Irish hospitals between 1971-2002 investigated in the present study by *spa* typing (n = 174), MLST (n = 15) and DNA microarray profiling (n = 107)

Year of isolation	Isolate no.[a]	Phenotype/ AR type[b]	AR pattern[b]	PFT[b]	ST/SCC *mec* type[c]	Body site/ specimen type
1993[e]	0066	AR13	Ak^{m}ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb	00002	ST8-IIA	Screening
1993	0246	AR14	Ak^{m}ApCdCpEbErGnKnMcNmPmaSmSpTb	00002	ST8-IIC	Sputum
1993	0298	AR14	Ak^{m}ApCdCpEbErGnKnMcNmSmSpTb	00002	ST8-IIA	Wound
1993	0225	AR14	Ak_{m}ApCdCpEbErGnKnMcNmSmSpTb	00002	ST8-IIA	Sputum
1993	0078	AR14	Ak^{m}ApCdCpEbErGnKnMcNmPmaSmSpTb	00013	ST8-IIA	Screening
1993	0098	AR15	Ak^{m}ApCdEbErGnKnLnMcPmaSmSpSuTbTeTp	99927	ST239-III	Wound
1993	0104	AR15	Ak^{m}ApCdEbErGnKnLnMcPmaSmSpSuTbTeTp	99927	ST239-III	Wound
1993	0070	AR22	AkApCdCpEbErGnKnLnMcNmPmaRfSmSpSu^{m}TbTe	99930	ST247-Ia	Screening
1993	0308	AR22	AkApCdCpEbErGnKnLnMcNmPmaRfSmSpSu^{m}TbTe	99931	ST247-Ia	Wound
1992	0073	AR23	Ak^{m}ApCdEbErFdGnKnLnMcNmPmaRfSmSuTbTe	99928	ST239-III	NA[c]
1998	2170	AR06	Ap	01000	ST22-IV	Wound
1998	2008	AR06	ApCdCp	01002	ST22-IV	Wound
1998	2110	AR06	ApCdCpEr	01002	ST22-IV	Sputum
1998	2024	AR06	ApCdCpEr	01002	ST22-IV	Screening
1998	2189	AR07.0	Ak^{m}ApCdCpErKnLnNmSpTb	02000	ST36-II	Screening
1998	2039	AR07.2	Ak^{m}ApCdCpErKnNmSpTb	02002	ST36-II	Ulcer swab
1998	2160	AR07.3	Ak^{m}ApCpErKnNmSpTb	03021	ST5-II	Sputum
1998	2002	AR07.4	Ak^{m}ApCpErKnLnNmSpTb	03000	ST5-II	Screening
1998	2023	AR07.4	$Ak^{m}Ap^{m}$CpErKnLnNmSpTb	03000	ST5-II	Screening
1998	2179	AR13	Ak^{m}ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb	00026	ST8-IID	Screening
1998	2141	AR13	Ak^{m}ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb	00001	ST8-IIA	Screening
1998	2142	AR13	ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb	00015	ST8-IID	Sputum
1998	2113	AR13	ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb	00027	ST8-IID	Screening
1998	2027	AR14	Ak^{m}ApCdCpEbErGnKnMcNmPmaSmSpTb	00020	ST8-IIC	Swab
1998	2175	AR14	Ak^{m}ApCdCpEbErGnKnMcMp^{m}NmPmaSmSpTb	00022	ST8-IIE	Wound
1998	2188	AR14	Ak^{m}ApCdCpEbErGnKnMcNmPmaSmSpTbTp	00048	ST8-IIC	Swab
1998	2080	AR14	Ak^{m}ApCdCpEbErFd^{m}GnKnMcMpNmPmaSmSpTbTp	00040	ST8-IIC	Burn swab
1999	3144	New01	Ak^{m}ApCdCpEbErGnKnLnMcMp^{m}PmaSpTb	00052	ST8-IID	Nose
1999	3581	New02	AkApCdCpEbGnKnMcNm^{m}PmaRfSmSuTbTe	00137	ST247-Ia	Blood
1999	3442	New03	ApCdCpEbErGnKnLnMcMp^{m}PmaSpTb	00027	ST8-IID	Toe wound
1999	3686	New03	ApCdCpEbErGnKnLnMcMp^{m}PmaSpTb	00002	ST8-IID	Throat
1999	3362	AR06	ApEbCd	01014	ST22-IV	Nose
1999	3363	AR06	ApEb	01014	ST22-IV	Eye
1999	3143	AR06	ApEb	01014	ST22-IV	Nose
1999	3416	AR06	ApCdCp	01002	ST22-IV	Urine
1999	3637	AR06	ApCdCp	01000	ST22-IV	Eye
1999	3190	AR06	ApEbEr	01000	ST22-IV	Sputum
1999	3147	AR06	ApEbEr	01000	ST22-IV	Throat
1999	3322	AR06	ApCdCpErKnNmSmSpTp	00150	ST22-IV	Throat
1999	3591	AR06	ApCpEbEr	01000	ST22-IV	
1999	3430	AR06	ApCpEbEr	01000	ST22-IV	Sputum
1999	3594	AR07.0	Ak^{m}ApCpEbErKnLnNmTb	02007	ST36-II	Elbow
1999	3384	AR07.0	Ak^{m}ApCdCpErKnLnNmSpTb	02020	ST36-II	Sputum
1999	3558	AR07.0	Ak^{m}ApCpEbErKnLnNmTb	02000	ST36-II	Nose
1999	3142	AR07.0	Ak^{m}ApCpEbErKnLnNmTb	02000	ST36-II	Chest drain

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Table 3.3 continued. Clinical MRSA isolates recovered from Irish hospitals between 1971-2002 investigated in the present study by *spa* typing (n = 174), MLST (n = 15) and DNA microarray profiling (n = 107)

Year of isolation	Isolate no.[a]	Phenotype/ AR type[b]	AR pattern[b]	PFT[b]	ST/SCC *mec* type[c]	Body site/ specimen type
1999	**3366**	**AR07.0**	**Ak^{m}ApCdCpErKnLnNmSpTb**	**02020**	**ST36-II**	**Wound**
1999	3228	AR07.0	Ak^{m}ApCpEbErKnLnNmTb	02000	ST36-II	Sputum
1999[e]	**3139**	**AR07.0**	**Ak^{m}ApCdCpErKnLnNmTb**	**02000**	**ST36-II**	**Blood**
1999	3572	AR07.0	Ak^{m}ApCpEbErKnLnNmTb	02000	ST36-II	Nose
1999	3604	AR07.0	Ak^{m}ApCpEbErKnLnNmTb	02000	ST36-II	Sputum
1999	**3188**	**AR07.4**	**Ak^{m}ApCpErKnLnNmSpTb**	**03036**	**ST5-II**	**Wound**
1999	**3033**	**AR07.4**	**ApCpErFdKnLnNmSpTb**	**03035**	**ST5-II**	**Tracheostomy swab**
1999	3137	AR13	Ak^{m}ApCpEbErGnKnLnMcNmSmSpTb	00049	ST8-IID	Hand
1999	3008	AR13	Ak^{m}ApCpEbErGnKnLnMcNmSmSpTb	00025	ST8-IID	Wound
1999	3053	AR13	Ak^{m}ApCpEbErGnKnLnMcNmSmSpTb	00027	ST8-IID	Nose
1999	**3114**	**AR13**	**Ak^{m}ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb**	**00022**	**ST8-IID**	**Groin**
1999	3698	AR13	Ak^{m}ApCpEbErGnKnLnMcNmSmSpTb	00023	ST8-IIA	Nose
1999	3635.2	AR13	ApCpEbErGnKnLnMcNmSmSpTb		ST8-IID	Urine
1999	3243.2	AR13	ApCpEbErGnKnLnMcNmSmSpTb	00017	ST8-IID	Urine
1999	3596	AR13	ApCpEbErGnKnLnMcNmSmSpTb	00051	ST8-IID	Hip wound
1999	3017	AR13	ApCpErEbGnKnLnMcNmSmSpTb	00027	ST8-IID	wound
1999	3439	AR13	ApCpEbErGnKnLnMcNmSmSpTb	00051	ST8-IID	Nose
1999	**3317.2**	**AR13**	**ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb**	**00071**	**ST8-IIE**	**Urine**
1999	3636.2	AR13	ApCpEbErGnKnLnMcNmSmSpTb	00080	ST8-IID	Groin
1999	**3330.2**	**AR13**	**ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb**	**00002**	**ST8-IIE**	**Urine**
1999	3258	AR13	ApCpEbErGnKnLnMcNmSmSpTb	00002	ST8-IID	Nose
1999	3453	AR13	ApCpEbErGnKnLnMcNmSmSpTb	00004	ST8-IID	Hip wound
1999	**3045**	**AR14**	**Ak^{m}ApCdCpEbErGnKnMcNmPmaSmSpTb**	**00176**	**ST8-IIC**	**Throat**
1999	3146	AR14	Ak^{m}ApCpEbErGnKnMcNmSmSpTb	00083	ST8-IIC	Sputum
1999	**3225**	**AR14**	**Ak^{m}ApCdCpEbErGnKnMcMp^{m}NmPmaSmSpTb**	**00124**	**ST8-IIE**	**Blood**
1999	**3352**	**AR14**	**ApCdCpEbErGnKnMcMp^{m}NmPmaSmSpTb**	**00017**	**ST8-IID**	**Wound**
1999	**3132**	**AR14**	**ApCdCpEbErGnKnMcMp^{m}NmPmaSmSpTb**	**00055**	**ST8-IID**	**Sacral ulcer**
1999	**3369**	**AR14**	**Ak^{m}ApCdCpEbErGnKnMcNmPmaSmSpTbTp**	**00085**	**ST8-IIC**	**Nose**
1999	3600	AR14	Ak^{m}ApCpEbErGnMcKnNmSmSpTbTp	00085	ST8-IIC	Traceostomy swab
1999	3685	AR14	Ak^{m}ApCpEbErGnKnMcNmSmSpTbTp	00001	**ST8**-IIC	Nose
1999	**3411**	**AR14**	**Ak^{m}ApCdCpEbErFd^{m}GnKnMcMpNmPmaSmSpTbTp**	**00040**	**ST8-IIC**	**Nose**
1999	3176.1	AR14	Ak^{m}ApCpEbErFdGnKnMcNmSmSpTbTp	00002	ST8-IID	Wound
1999	3448	AR14	Ak^{m}ApCpEbErGnKnMcMpNmSmSpTbTp	00146	ST8-IIE	Heel
1999	3623	AR43	ApCpEb^{m}ErKnNmSmTp	00143	ST8-IV-*dcs*	Nose
1999	**3246**	**AR43**	**ApCdCpErKnNmSmSpTp**	**00140**	**ST8-IVF**	**Nose**
1999	3310	AR43	ApCpEb^{m}ErKnNmSmTp	00146	ST8-IV-*dcs*	Urine
1999	**3402**	**AR43**	**ApCdCpErKnNmSmSpTp**	**00017**	**ST8-IVF**	**Nose**
1999	3488	AR43	ApCpEb^{m}ErKnNmSmTp	00146	ST8-IV-*dcs*	Catheter site
1999	3635.1	AR43	ApCpEb^{m}ErKnNmSmTp	00151	ST8-IV-*dcs*	Urine
1999	3331	AR43	ApCpEb^{m}ErKnNmSmTp	00067	ST8-IV-*dcs*	Nose
1999	3332	AR43	ApEb		ST8-IV-*dcs*	Nose
1999	3317.1	AR43	ApCpEb^{m}ErKnNmSmTeTp	00141	ST8-IV-*dcs*	Urine
1999	3316	AR43	ApCpEb^{m}ErKnNmSmTeTp	00143	ST8-IV-*dcs*	Tongue
1999	3625	AR43	ApCpErEb^{m}KnNmSmTeTp	00150	ST8-IV-*dcs*	Wound
1999	**3330.1**	**AR43**	**ApCpErKnNmSmSpTeTp**	**00067**	**ST8-IVE**	**Urine**
1999	3337	AR43	ApCpEb^{m}ErKnNmSmTeTp	00070	ST8-IV-*dcs*	Wound
1999	3665	AR43	ApCpEb^{m}ErKnNmSmTeTp	00079	ST8-IV-*dcs*	Urine
1999	3243.1	AR43	ApCpEb^{m}ErKnNmSmTeTp	00138	ST8-IV-*dcs*	Urine
1999	3328	AR43	ApCpEb^{m}ErKnNmSmTeTp	00125	ST8-IV-*dcs*	Wound
1999	**3636.1**	**AR43**	**ApCdCpEbErKnNmSmSpTp**	**00140**	**ST8-IVF**	**Groin**
1999	3176.2	AR43	ApCpErKnNmSmTp	00142	ST8-IV-*dcs*	Wound
1999	**3487**	**AR43**	**ApCdCpErKnNmSmSpTeTp**	00149	**ST8-IVE**	**Leg wound**

Continued overleaf

Table 3.3 continued. Clinical MRSA isolates recovered from Irish hospitals between 1971-2002 investigated in the present study by *spa* typing (n = 174), MLST (n = 15) and DNA microarray profiling (n = 107)

Year of isolation	Isolate no.[a]	Phenotype/ AR type[b]	AR pattern[b]	PFT[b]	ST/SCC *mec* type[c]	Body site/ specimen type
1999	3094	AR43	ApCpErKnNmSmTeTp		ST8-IV-*dcs*	Wound
2002[c]	**E1164**	**AR06**	**ApCp**	**01010**	**ST22-IV**	**Blood**
2002	**E1185**	**ARNT**	**Ap**	**99516**	**ST12-IV**	**Blood**
2002	E1098	AR06	ApCpEb	01010	ST22-IV	Blood
2002	E1095	AR06	ApCpEb	01029	ST22-IV	Blood
2002	**E1174**	**AR06**	**ApCdCpFd**	**01028**	**ST22-IV**	**Blood**
2002	**E1113**	**ARNT**	**ApCdFd**	**02011**	**ST30-IV**	**Blood**
2002	E1203	AR06	ApCpEbEr	01029	ST22-IV	Blood
2002	E1115	AR06	ApCpEbErFdm	01010	ST22-IV	Blood
2002	**E1139**	**ARNT**	**ApCdCpErFdm**	**99513**	**ST45-IV**	**Blood**
2002	E1123	AR06	ApEr	01010	ST22-IV	Blood
2002	**E1206**	**AR06**	**ApCpEr**	**01018**	**ST22-IV**	**Blood**
2002	**E1100**	**AR07.0**	**Ak^{m}ApCdCpErKnLnNmSpTb**	**02017**	**ST36-II**	**Blood**
2002	**E1202**	**AR07.2**	**ApCdCpErKnNmSpTb**	**03029**	**ST496-II**	**Blood**
2002	**E1140**	**AR13**	**ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb**	**00098**	**ST8-IID**	**Blood**
2002	**E1168**	**AR13**	**ApCdCpEbErGnKnLnMcMp^{m}NmPmaSmSpTb**	**00099**	**ST8-IID**	**Blood**
2002	**E1154**	**AR13**	**ApCdCpEbErFdGnKnLnMcMp^{m}NmPmaSmSpTb**	**00079**	**ST8-IID**	**Blood**
2002	**E1134**	**AR14**	**ApCdCpEbErGnKnMcMp^{m}NmPmaSmSpTb**	**00103**	**ST8-IIE**	**Blood**
2002	**E1119**	**AR14**	**ApCdCpEbErGnKnMcMp^{m}NmPmaSmSpTbTp**	**00071**	**ST8-IIE**	**Blood**
2002	**E1121**	**AR14**	**ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp**	**00070**	**ST8-IIE**	**Blood**
2002	**E1183**	**AR44**	**AkApCdCpEbErGnKnLnMcMpNmPmaSmSpSuTbTeTp**	**99400**	**ST239-III**	**Blood**
2002	**E1038**	**New03**	**ApCdCpEbErGnKnLnMcMp^{m}PmaSpTb**	**00052**	**ST8-IID**	**Blood**
2002	**E0908**	**New03**	**ApCdCpEbErGnKnLnMcMp^{m}PmaSpTb**	**00074**	**ST8-IID**	**Blood**
2003	**M03/ 0073**	**AR44**	**AkApCdCpEbErGnKnLnMcNmPmaSmSpSuTbTeTp**	**99401**	**ST239-III**	**Nose**
2003	**M03/ 0068**	**Unfamiliar-1**	**ApCdCpErMcPmaSpTp**	**99932**	**ST5-IV**	**Burn**
2003	**M03/ 0169.2**	**Unfamiliar-2**	**Ak^{m}ApCdGnKnMcNmSmTbTe**	**99940**	**ST94-IV**	**Blood**
2003	**E1520**	**Unfamiliar-3**	**AkApCdCpEbErGnKnMcMp^{m}NmPmaSmSpSuTbTeTp**	**99404**	**ST239-III**	**Blood**
2004	**M04/ 0067**	**Unfamiliar-4**	**Ak^{m}ApCpErGnKnLnNmSuTbTeTp**	**99014**	**ST609-IVA**	**Blood**

[a] Isolate were recovered from patients in the following Irish hospitals or studies; FDVH, The Federated Dublin Voluntary Hospitals; MMH, The Mater Misericordiae Hospital; JSH, Jervis Street Hospital; SJH, St James's Hospital, Dublin; N/S Study, isolates from the Republic of Ireland, collected during the North/South of MRSA in Ireland 1999. N/S Study (N); isolates from Northern Ireland, collected during the North/South Study of MRSA in Ireland 1999; EARS-Net isolates from Irish laboratories that participate in European Antimicrobial Resistance Surveillance Network (EARS-Net), NMRSARL, and the National MRSA Reference Laboratory, St James's Hospital, Dublin.

[b] AR type, antibiogram-resistogram type; Unf, unfamiliar, these isolates exhibited a hitherto unfamiliar AR pattern; NT nontypeable, these isolates displayed an AR pattern associated with AR06 but were urease positive; New, AR patterns identified in 1999 that were assigned the AR types, New01, New02 and New03. Abbreviations: Ak, amikacin; Ap, ampicillin; Cd, cadmium nitrate/cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenylmercuric acetate; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; Tb, tobramycin; Te, tetracycline; Tp, trimethoprim. Isolates were assigned pulsed field types (PFTs) and AR types were determined by the NMRSARL. PFGE profiles were assigned a 5-digit pulsed field type (PFT) number. AR type; antibiogram-resistogram type; Unf, unfamiliar, these isolates exhibited a hitherto unfamiliar AR pattern; NT nontypeable, these isolates displayed an AR pattern associated with AR06 but were urease positive.

[c] Isolates assigned to ST and SCC*mec* types underwent MLST typing and SCC*mec* characterisation as part of a previous study (Shore *et al.*, 2005) or, for 15 isolates, as part of the present study are shown in red.

[d] NA, information not available, ND not determined.

[e] The 107 isolates shown in bold underwent DNA microarray analysis as part of the present study.

Abbreviations: ST (sequence type), SCC*mec* (staphylococcal cassette chromosome *mec*).

3.2.1.2 Fusidic acid resistant MRSA isolates investigated using DNA microarray profiling

A total of 110 fusidic acid resistant (FdR) MRSA isolates were investigated by DNA microarray profiling as part of this study to help identify the mechanism(s) of fusidic acid resistance in these isolates (Table 3.4). This included (a) 21 FdR MRSA isolates recovered from patients in Irish hospitals between 1976 and 2002 and representative of diverse genotypes and SCC*mec* types (Shore, 2005) (b) 47 FdR ST22-MRSA-IV isolates recovered from patients and environmental sites in a large 700-bed acute care hospital in Dublin in 2007 and 2008 (Shore *et al.*, 2010) and (c) 42 FdR MRSA isolates recovered from patients with BSIs in Irish hospitals between 2009 and 2010 as part of EARS-Net, including 31 AR-PFT 06-01 MRSA isolates indicative of ST22-MRSA-IV, and 11 other MRSA isolate with unfamiliar AR pattern or non-typeable NT-AR patterns.

3.2.1.3 MRSA isolate investigated for the presence of a novel *mec* complex

MRSA isolate 05540 was investigated in the present study for the presence of a novel *mec* complex due to the unexpected size amplimer obtained for the *mec* complex region using a multiplex PCR for class A, B and C *mec* (Shore *et al.*, 2010). Isolate 05540 exhibited *spa* type t190 indicative of ST8 and apart from the unusual size *mec* complex amplimer, it was previously found to harbour *ccrAB2*, *ccrAB4*, *dcs* and a SCC*mec* IVb J1 region (Shore *et al.*, 2010).

3.2.2 MLST typing

A total of 161/176 of the isolates investigated in this part of the present study that were recovered between 1971 and 2002 were previously typed by MLST (Shore *et al.*, 2005). The remaining 15 isolates underwent MLST as part of the present study as described previously (Enright *et al.*, 2000; Shore *et al.*, 2008). The MLST scheme relies on sequencing internal fragments of seven housekeeping genes, namely carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). Amplimers were sequenced commercially by Geneservice Ltd. (Source Bioscience) as described in Chapter 2, section 2.4.2. Sequences were analysed using the BioNumerics software package. Assignment of sequence types (STs) was carried out by comparing the allele sequences to ST alleles using the MLST website (http://saureus.mlst.net). The assignment of STs to a clonal complex (CC) was undertaken using the rule that 6/7 alleles should be identical to the allelic profile of at least one ST within a CC to be assigned to that CC. The STs for all FdR isolates were assigned using DNA microarray profiling.

3.2.3 *spa* typing

All 175 isolates underwent *spa* typing. The amplification of the *spa* gene was performed as described previously (Shore *et al.*, 2008) using *spa* primers *spa*-1113F and *spa*-1514R (primer sequences see section 2.4.3) and thermal cycling conditions outlined by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; www.seqnet.org). *Spa* amplimers were sequenced commercially by Cogencis (Essex, United Kingdom) (Chapter 2, section 2.4.2) and sequence analysis including *spa* repeat determination and *spa* type assignment was performed using the

Table 3.4 Fusidic acid resistant MRSA isolates (n = 110) recovered from patients in Irish hospitals between 1976 and 2010 investigated in the present study

Year of isolation	Isolate no.[a]	Phenotype/ AR type[b]	AR pattern[b]	PFT[b]	ST/ SCC*mec*[c]	Body site/ specimen[c]
1976-'84	138.1	Phenotype-II	Ak^{m}ApCdEbErFdGnKnLnMcPmaSmSpSu^m TbTe	99912	ST250- I	NA
1976-'84	120.1	Phenotype-II	Ak^{m}ApCdEbErFdGnKnMcNmPmaSmSpTb TeTp	99910	ST250-I-*pls*	NA
1976-'84	122.2	Phenotype-II	ApCdEbErFdGnKnLnMcPmaSmSpSuTbTe	99912	ST250-I	NA
1976-'84	58.1	Phenotype-II	ApCdEbErFdGnKnMcNmPmaSmSpSuTbT eTp	99912	I	NA
1976-'84	120.2	Phenotype-II	ApCdEbErFdGnKnMcNmPmaSmSpTb^{m}Te Tp	99910	I-*pls*	NA
1976-'84	161.1	Phenotype-II	ApCdEbErFdGnKnMcPmaSmSpSu^{m}Tb	99910	ST250-I-*pls*	NA
1989	0.1206	AR02	ApCdEbErFdGnKnLnMcNmPmaSmSpSuT bTeTp	99918	ST250-I-*pls*	Calf wound
1989	0.1149	AR02	ApCdEbErFdGnKnMcPmaSmSpSuTb^m	99917	ST250-I-*pls*	Urine
1989	0.1228	AR02	ApCdEbErFdGnKnMcPmaSmSpSuTb^m	99915	ST250-I-*pls*	Sputum
1989	0.1345	AR05	ApCdErFdKnMcNmPmaSmSpTbTe	00002	ST8- II-*kdp*	Leg ulcer
1992	0073	AR23	Ak^{m}ApCdEbErFdGnKnLnMcNmPmaRfSm SuTbTe	99928	ST239-III	NA
1993	0.1336	ARNT	ApCdFd	02011	ST30-IV	Nose
1993	0067	ARNT	ApCdFd	02011	ST30-IV	Wound
1993	0242	ARNT	ApCdFd	02011	ST30-IV	Wound
1998	2080	AR14	Ak^{m}ApCdCpEbErFd^{m}GnKnMcMpNmPmaS mSpTbTp	00040	ST8-IIC	Burn swab
1999	3033	AR07.4	ApCpErFdKnLnNmSpTb	03035	ST5-II	T swab[e]
1999	3411	AR14	Ak^{m}ApCdCpEbErFd^{m}GnKnMcMpNmPmaS mSpTbTp	00040	ST8-IIC	Nose
2002	E1113	ARNT	ApCdFd	02011	ST30-IV	Blood
2002	E1139	ARNT	ApCdCpErFd^m	99513	ST45-IV	Blood
2002	E1174	AR06	ApCdCpFd	01028	ST22-IV	Blood
2002	E1154	AR13	ApCdCpEbErFdGnKnLnMcMp^{m}NmPmaSm SpTb	00079	ST8-IID	Blood
2007	01629	AR06.5	ApCdCpErFd	01032	IVh	P
2007	01669	AR06.5	ApCdCpErFd	01075	IVh	P
2007[d]	**00365**	**AR06.5**	**ApCdCpErFd**	**01151**	**IVh**	**P**
2007	01266	AR06.5	ApCdCpErFd	01042	NA	P
2007	01569	AR06.3	ApCdCpFd	01018	IVh	P
2007	173	NT	ApCpFdLn	01039	IVh	E
2007	298	AR06.6	ApCpErFd	01002	IVh	E
2007	00226	AR06.5	ApCdCpEr	01002	IVh	E
2007	**00878**	**AR06.5**	**ApCdCpErFd**	**01039**	**IVh**	**E**
2007	245	AR06.1	ApCdCpFd	01018	NA	P
2007	00513	AR06.1	ApCpFdMp^m	01024	IVh	P
2007	01625	AR06.5	ApCdCpErFd	01024	IVh	P
2007	01207	NT	ApCdCpErFdKn	01042	IVh	P
2007	**00851**	**AR06.5**	**ApCdCpErFd**	**01018**	**IVh**	**P**
2007	00871	AR06.5	ApCdCpErFd	01018	NA	E
2007	00887	AR06.5	ApCdCpErFd	01018	IVh	E
2007	00953	AR06.5	ApCdCpErFd	01018	IVh	P
2007	01829	AR06.5	ApCdCpErFd	01018	IVh	P
2007	300	AR06.6	ApCpErFd	01018	IVh	E
2007	00667	AR06.5	ApCdCpErFd	01024	IVh	P
2007	01844	AR06.5	ApCdCpErFd	01018	IVh	P
2007	163	AR06.5	ApCdCpErFd	01006	IVh	E
2007	01395	AR06.6	ApCpErFd	01024	IVh	P
2007	01961	AR06.5	ApCdCpErFd	01018	IVh	E
2008	05245	AR06.6	ApCpErFd	01039	IVh	P
2008	05295	AR06.6	ApCpErFd	01039	NA	E
2008	05332	AR06.6	ApCpErFd	01039	NA	E
2008	05331	AR06.6	ApCpErFd	01039	IVh	E
2008	05002	AR06.5	ApCdCpErFd	01042	IVh	P
2008	02386	AR06.5	ApCdCpErFd	01075	NA	P
2008	03035	AR06.5	ApCdCpErFd	01039	NA	P
2008	03764	AR06.6	ApCpErFd	01018	NA	P
2008	**02355**	**AR06.3**	**ApCdCpFd**	**01114**	**NA**	**P**
2008	**05962**	**AR06.5**	**ApCdCpErFd**	**01039**	**NA**	**P**
2008	02783	AR06.6	ApCpErFd	01002	IVh	P
2008	**05239**	**AR06.5**	**ApCdErFd**	**01077**	**ST22**	**P**
2008	05006	AR06.6	ApCpErFd	01049	NA	P
2008	05328	AR06.6	ApCpErFd	01039	NA	E

Continued overleaf

Table 3.4 continued. Fusidic acid resistant MRSA isolates (n = 110) recovered from patients in Irish Hospitals between 1976 and 2010 investigated in the present study

Year of isolation	Isolate no.[a]	Phenotype/ AR type[b]	AR pattern[b]	PFT[b]	ST/ SCC*mec*[c]	Body site/ specimen[c]
2008	02317	AR06.5	ApCdCpErFd	01018	IVh	P
2008	06382	AR06.5	ApCdCpErFd	01018	NA	P
2008	05996	AR06.5	ApCdCpErFd	01018	NA	P
2008	04294	AR06.5	ApCdCpErFd	01018	NA	E
2008	04038	AR06.5	ApCdCpErFd	01018	NA	P
2008	02391	AR06.5	ApCdCpErFd	01006	IVh	P
2008	05068	AR06.5	ApCdCpErFd	01024	NA	E
2008	06484	AR06.5	ApCdCpErFd	01047	NA	E
2008[d]	**06470**	**AR06.5**	**ApCdCpErFd**	**01047**	**IVh**	**P**
2009	E4154	AR06.6	ApCpErFd	01018	ND	BSI
2009	E4160	AR06.6	ApCpErFd	01006	ND	BSI
2009	E4161	AR06.1	ApCpFd	01039	ND	BSI
2009	**E4162**	**AR06.3**	**ApCdCpFd**	**01130**	**ND**	**BSI**
2009	E4171	AR06.3	ApCdCpFd	01018	ND	BSI
2009	E4184	Unfamiliar	ApCpFdSm	01018	ND	BSI
2009	**E4226**	**Unfamiliar**	**ApCdCpFdGnKnLnMpTb**	**01006**	**ND**	**BSI**
2009	E4229	AR06.6	ApCpErFd	01006	ND	BSI
2009	**E4277**	**AR06**	**ApCdErFd**	**99081**	**ND**	**BSI**
2009	E4283	AR06.3	ApCdCpFd	01006	ND	BSI
2009	**E4292**	**AR06**	**ApCdCpErFd**	**01042**	**ND**	**BSI**
2009	E4329	AR06.1	ApCpFd	01130	ND	BSI
2009	**E4347**	**AR06.5**	**ApCdCpErFd**	**01075**	**ND**	**BSI**
2009	**E4372**	**AR06.6**	**ApCpErFd**	**01002**	**ND**	**BSI**
2009	E4378	NT	ApCpErFd	99513	ND	BSI
2009	**E4383**	**AR06.6**	**ApCpErFd**	**01006**	**ND**	**BSI**
2009	E4413	AR06.5	ApCdCpErFd	99513	ND	BSI
2009	E4415	AR06.6	ApCpErFd	01002	ND	BSI
2009	**E4434**	**AR06.5**	**ApCdCpErFd**	**99597**	**ND**	**BSI**
2009	**E4455**	**AR06.5**	**ApCdCpErFd**	**01063**	**ND**	**BSI**
2009	E4462	AR06.5	ApCdCpErFd	01063	ND	BSI
2010	E4483	Unfamiliar	ApErFdNmTb	99531	ND	BSI
2010	**E4509**	**NT**	**ApCdCpErFdLn**	**01024**	**ND**	**BSI**
2010	E4512	Unfamiliar	ApCdCpErFd	99102	ND	BSI
2010	E4514	AR06.6	ApCpErFd	01018	ND	BSI
2010	E4515	AR06	ApCdCpErFd	01024	ND	BSI
2010	E4524	AR06.6	ApErFd	99535	ND	BSI
2010	E4525	AR06.1	ApCpFd	01039	ND	BSI
2010	E4527	AR06.3	ApCdCpFd	01018	ND	BSI
2010	**E4528**	**AR06.1**	**ApCpFd**	**01049**	**ND**	**BSI**
2010	E4559	AR06	ApCpFd	01018	ND	BSI
2010	**E4562**	**AR06.6**	**ApCpErFd**	**01017**	**ND**	**BSI**
2010	**E4577**	**AR06.5**	**ApCdCpErFd**	**01076**	**ND**	**BSI**
2010	E4578	AR06.3	ApCdCpFd	01018	ND	BSI
2010	E4581	AR06.3	ApCdCpFd	01018	ND	BSI
2010	E4594	AR06.3	ApCdCpFd	01006	ND	BSI
2010	E4597	AR06	ApCdCpErFdMp	01039	ND	BSI
2010	E4627	AR06.5	ApCdCpErFd	01075	ND	BSI
2010	E4641	AR06.1	ApCdCpFd	01039	ND	BSI
2010	E4644	AR06.5	ApCdCpErFd	99604	ND	BSI
2010	E4646	Unfamiliar	ApErCpFdGnKnMpTb	01059	ND	BSI
2010	**E4676**	**NT**	**ApCdCpErFdLn**	**01029**	**ND**	**BSI**

[a] Isolate were recovered from patients in the following Irish hospitals or studies; FDVH, The Federated Dublin Voluntary Hospitals; MMH, The Mater Misericordiae Hospital; JSH, Jervis Street Hospital; SJH, St James's Hospital, Dublin; N/S Study, isolates from the Republic of Ireland, collected during the North/South of MRSA in Ireland 1999. N/S Study (N); isolates from Northern Ireland, collected during the North/South Study of MRSA in Ireland 1999; EARS-Net isolates from Irish laboratories that participate in European Antimicrobial Resistance Surveillance Network (EARS-Net), NMRSARL, and the National MRSA Reference Laboratory, St James's Hospital, Dublin. Isolates recovered from patients and environmental sites in four wards at Beaumont Hospital (BH) Dublin. The isolates were recovered over two six-week study periods between May 2007 and September 2008 as part of a previously described study (Shore *et al.*, 2010). Isolates recovered from patients with bloodstream infections (BSIs) in Irish hospitals between 2009 and 2010 as part of the European Antimicrobial Resistance Surveillance Network (EARS-Net).

[b] Abbreviations: Ak, amikacin; Ap, ampicillin; Cd, cadmium nitrate/cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn,

gentamicin; Kn, kanamycin; Ln, lincomycin; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenylmercuric acetate; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; ; Tb, tobramycin; Te, tetracycline; Tp, trimethoprim. Isolates were assigned pulsed field types (PFTs) and AR types were determined by the NMRSARL. PFGE profiles were assigned a 5-digit pulsed field type (PFT) number. AR type; antibiogram-resistogram type; Unf, unfamiliar, these isolates exhibited a hitherto unfamiliar AR pattern; NT nontypeable, these isolates displayed an AR pattern associated with AR06 but were urease positive.

[c] NA, information not available; ND, information not determined

[d] Isolates shown in bold underwent DNA microarray analysis as part of the present study, Isolates assigned to ST and SCC*mec* types by DNA microarray analysis.

[e] Tracheotomy swab

Abbreviations: E; environment, P: patient (Nasal, groin or non-intact skin sites); ST (sequence type), SCC*mec* (staphylococcal cassette chromosome *mec*)

Ridom Staphtype software package version 1.3 beta (Ridom GmbH) and *spa* types were assigned using the SpaServer website (http://spaserver2.ridom.de) (see section 2.4.3).

3.2.4 DNA microarray analysis

One hundred seven of the 175 MRSA isolates investigated underwent DNA microarray analysis, including where possible three isolates representative of each antibiogram-resistogram (AR) type, sequence type (ST), and SCC*mec* type/subtype combination previously identified among the 175 isolates during eight different study periods between 1971 and 2004 (Shore *et al.*, 2005) (Table 3.3). One hundred and ten FdR isolates were analysed by the DNA microarray to assign STs and SCC*mec* types, and identify the antimicrobial resistance genes associated with fusidic acid resistance, along with the identification of virulence factors (Table 3.4). The Staphy-Type kit (Alere) was used for DNA microarray analysis according to the manufacturer's instructions, which have been described in detail previously (Monecke *et al.*, 2008a; Monecke *et al.*, 2008b). The Staphy-Type kit consists of individual DNA microarrays adhered to each well of an 8-well microtiter strip; each chip detects 334 *S. aureus* target genes including species-specific, antimicrobial resistance and virulence-associated genes, genes involved in adhesion, attachment, and biofilm formation as well as typing markers (see section 2.4.5) (Table 3.1).

3.2.5 PCR detection of antimicrobial resistance determinants

For the 110 isolates recovered between 1971 and 2004 that were used to investigate the usefulness of the DNA microarray for ST and CC assignment. Isolates that exhibited phenotypic resistance to a particular antimicrobial agent by disk diffusion for which associated resistance genes were not detected using the DNA microarray were further investigated using previously described PCR primers and conditions for additional resistance genes (Table 3.5). This included PCR amplification and sequencing of the native *fusA* gene to detect mutations previously associated with fusidic acid resistance and PCR detection of the fusidic acid resistance gene *fusD*, the trimethoprim resistance genes *dfrD*, *dfrG* and *dfrK* and the tetracycline resistance gene *tet*(L) and *tet*(O) (Table 3.5). *E. coli* pB*2187dfrK* was used as a positive control for *dfrK* and *tet*(L) (Kadlec and Schwarz, 2009a), and *S. aureus* CM.S2 (IMCJ1454) was used as a positive control for *dfrG* (Sekiguchi *et al.*, 2005). No positive controls for *fusD*, *dfrD*,and *tet*(O) were available for this study. PCR products were purified using the Genelute PCR cleanup kit (Sigma), sequenced commercially by Geneservice, and analysed using DNA Strider and Bionumerics software.

Of the 110 FdR isolates analysed using the DNA microarray, 22 isolates representative of the different AR-PFG types recovered between 2007 and 2010 which were negative for *fusB* or *fusC* using the DNA microarray underwent *fusA* PCR and sequencing. The *fusA* gene sequence of MRSA reference strain N315 (GenBank accession number NC_002745) was used as a reference sequence for analysis of *fusA* gene sequences. In addition, ten of the 110 FdR isolates investigated that were not found to harbour either of the FdR genes encoded by the DNA microarray (*fusB* and *fusC*) were also investigated for the presence of *fusD* by PCR as described above.

Table 3.5 Primers and thermal cycling conditions used for PCR detection of additional resistance determinants in MRSA not detected using the DNA microarray

Primer Name	Primer sequence (5′-3′)	Thermal cycling conditions	Expected amplimer size	Reference
fusA-F2	CTCGTAAYATCGGTATCATG	94°C for 5 min; 35 cycles of 94°C for 20s, 53°C for 45s and 72°C for 30s; and a final extension for 10 min at 72°C	2002 bp	Castanheira *et al*., 2010
fusA-R2	GCATAGTGATCGAAGTAC			
fusD-1F	TGCTTATAATTCGGTCAACG	94°C for 2 min; 35 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 5 min; and a final extension for 10 min at 72°C	525 bp	Castanheira *et al*., 2010
fusD-1R	TGGTTACATAATGTGCTATC			
tet(L)-F	ATAAATTGTTTCGGGTCGGTAAT	94°C for 2 min; 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 30 s; and a final extension for 5 min at 72°C	1077 bp	Trzcinski *et al*., 2000
tet(L)-R	AACCAGCCAACTAATGACAATGAT			
tet(O)-F	AGCGTCAAAGGGGAATCACTATCC	94°C for 2 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 30 s; and a final extension for 5 min at 72°C	1723 bp	Trzcinski *et al*., 2000
tet(O)-R	CGGCGGGGTTGGCAAATA			
dfrD-F	AATCGGCAAGGATAACGACA	94°C for 2 min; 30 cycles of 94°C for 30 sec, 55°C for 30 min, and 72°C for 1 min; and a final extension for 5 min at 72°C	221 bp	Dale *et al*., 1995
dfrD-R	ATCTGTTCTCCCCCGAAAAT			
dfrG-F	TCCCAAGGACTGGGAATATG	94°C for 2 min; 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min; and a final extension for 5 min at 72°C	315 bp	Sekiguchi *et al*., 2005
dfrG-R	CCCTTTTTGGGCAAATACCT			
dfrK-F	GCTGCGATGGATAAGAACAG	94°C for 2 min; 35 cycles of 94°C for 30s, 50°C for 30s, and 72°C for 30 s; and a final extension for 5 min at 72°C	170 bp	Kadlec and Schwarz, 2010
dfrK-R	GGACGATTTCACAACCATTAAAGC			

3.2.6 Investigation of the *mec* complex of MRSA isolates 05540

Due to the unexpected size amplimer obtained for the *mec* complex region of MRSA isolate 05540 using a multiplex PCR for class A, B and C *mec* (Shore *et al.*, 2010), three individuals PCRs were performed for class A, B and C *mec* using the same primers used for the multiplex PCR (Kondo *et al.*, 2007). The Expand Long Template PCR System (Roche) was used for amplification and was used according to the manufacturer's instructions. An unexpected size amplimer was obtained using primers for the class A *mec* complex and this amplicon was purified and sequenced using primer walking as described in Chapter 2, section 2.4.2. The region upstream of the *mec* complex gene *mecR1*, extending from *mecR1* to Tn*554*, was also amplified by PCR and sequenced using the *erm*(A)-f (5'-GAACAATCAATACAGAGTCTAC-3') and *mec*R1-r (5'-GCGTAACAGAAGCGGT-3') primers and sequencing to confirm the structure of the SCC*mec* element in this isolate.

3.3 Results

3.3.1 Molecular characterisation of MRSA isolates recovered in Irish hospitals between 1971 and 2004

3.3.1.1 *spa* typing

The 175 MRSA isolates were differentiated into 35 *spa* types (Table 3.5). The *spa* type t190 predominated (41%; 73/175) and accounted for 99% (73/74) of ST8-MRSA-IIA-IIE and ST8-MRSA-IVE/IVF isolates (Table 3.6). Single *spa* types accounted for the majority of isolates within four other clones: t008 in ST250-MRSA-I/I-*pls* (95%; 18/19); t045 in ST5-MRSA-II (100%; 17/17); t037 in ST239-MRSAIII/III-p1258/Tn*554* (92%; 12/13), and t018 in ST36-MRSA-II (75%; 9/12) (Table 3.6). In contrast, the 27 ST22-MRSA-IV isolates exhibited the greatest number of *spa* types (16 *spa* types), with *spa* type t032 (33%; 9/27) predominating, followed by t005 (11%; 3/27) and t002 (7%; 2/27) with all other ST22 *spa* types being represented by single isolates (Table 3.6). In no instance was the same *spa* type identified among isolates belonging to different CCs, while only one *spa* type (t012) was represented by isolates belonging to different STs, ST36 and ST30, which both belong to CC30 and differ in one of the MLST alleles only (allele *pta*) (Table 3.6).

Four cluster groups were identified among 26/35 *spa* types using the BURP algorithm (Fig. 3.1). Cluster 1 (*spa*-CC032/022) consisted of 11/16 *spa* types exhibited by ST22-MRSA-IV isolates. This included the cluster co-founders t022 and t032 as well as t3505, t790, t531, t005, t515, t2945, t3501, t4902, t1467 and t005 with cost values ranging from 1-4 (Fig. 3.1). Cluster 2 (*spa*-CC012) consisted of *spa* types exhibited by CC30 isolates and the CC8/ST239-MRSA-III *spa* types t037 and t138. This was not unexpected because the ST239 genotype arose a result of a large chromosomal replacement between ST8 and ST30 isolates encompassing the *spa* locus (Robinson and

Table 3.6 MLST clonal complexes (CC), sequence types (ST), SCC*mec* types and *spa* types of 175 MRSA isolates representative of different antibiogram-resistogram, ST and SCC*mec* type combinations recovered from patients in Irish hospitals between 1971 and 2004 and the corresponding CCs/STs and SCC*mec* types deduced from DNA microarray profiles of 107 representative isolates

			DNA microarray[a]		
CC	MLST and SCC*mec* typing strain assignment (No. isolates tested)	*spa* types (No. isolates tested)	CC/ST-SCC*mec* type assignment by microarray (No. isolates tested)	SCC*mec*-associated genes detected by microarray	SCC*mec* type/subtype assignment following manual inspection of microarray profiles
8	ST250-MRSA-I (10)	t008 (10)	ST250-MRSA-I (4)	*mecA, ugpQ, ΔmecR1, ccrAB1, dcs, pls*	I
8	ST250-MRSA-I – *pls* (9)	t008 (8), t121 (1)	ST250-MRSA-I (6)	*mecA, ugpQ, ΔmecR1, ccrAB1, dcs*	I - *pls*
8	ST239-MRSA-III*mer* (9)	t037 (8), t138 (1)	ST239-MRSA-III (8)	*mecA, ugpQ, ΔmecR1*(5 *ΔmecR1* negative), *mecR1, mecI, xylR, ccrAB3, ccrC, merA&B*	III*mer*
8	ST239-MRSA-III*mer* – p1258/Tn554 (4)	t037 (4)	ST239-MRSA-III (4)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB3, ccrC, merA&B*	III*mer*
8	ST247-MRSA-Ia (3)	t3503 (2), t052 (1)	ST247-MRSA-I (3)	*mecA, ugpQ, ΔmecR1, ccrAB1, dcs, pls, aadD*	Ia
8	ST8-MRSA-IIA & *ccrAB4* (9)	t190 (9)	ST8-MRSA-II & CI (4)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, ccrB4, dcs* (1 *dcs* negative), *aadD*	IIA or IIB & *ccrAB4*
8	ST8-MRSA-IIB & *ccrAB4* (1)	t190 (1)	ST8-MRSA-II & CI (1)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, ccrB4, dcs, aadD*	IIA or IIB & *ccrAB4*
8	ST8-MRSA-IIC & *ccrAB4* (9)	t190 (9)	ST8-MRSA-II & CI (6)	*mecA, ugpQ, ΔmecR1, mecR1, ccrAB2, ccrB4, dcs* (1 *dcs* negative), *aadD*	IIC & *ccrB4*
8	ST8-MRSA-IIC (1)	t190 (1)	ST8-MRSA-II (1)	*mecA, ugpQ, ΔmecR1, mecR1, ccrAB2, dcs, aadD*	IIC
8	ST8-MRSA-IID & *ccrAB4* (24)	t190 (23), t2196 (1)	ST8-MRSA-II & CI (12)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, ccrB4, dcs* (4 *dcs* negative)	IID & *ccrAB4*
8	ST8-MRSA-IID (3)	t190 (3)	ST8-MRSA-II (3)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, dcs*	IID
8	ST8-MRSA-IIE & *ccrAB4* (6)	t190 (6)	ST8-MRSA-II & CI (5)	*mecA, ugpQ, ΔmecR1, mecR1, ccrAB2, ccrB4, dcs*	IIE & *ccrAB4*

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Table 3.6 continued. MLST clonal complexes (CC), sequence types (ST), SCC*mec* types and *spa* types of 175 MRSA isolates representative of different antibiogram-resistogram, ST and SCC*mec* type combinations recovered from patients in Irish hospitals between 1971 and 2004 and the corresponding CCs/STs and SCC*mec* types deduced from DNA microarray profiles of 107 representative isolates

			DNA microarray[a]		
CC	MLST and SCC*mec* typing strain assignment (No. isolates tested)	*spa* types (No. isolates tested)	CC/ST-SCC*mec* type assignment by microarray (No. isolates tested)	SCC*mec*-associated genes detected by microarray	SCC*mec* type/subtype assignment following manual inspection of microarray profiles
8	ST8-MRSA-IIE (1)	t190 (1)	ST8-MRSA-II & CI (1)	*mecA, ugpQ, ΔmecR1, mecR1, ccrAB2*	IIE
8	ST8-MRSA-IVE & *ccrAB4* (17)	t190 (17)	ST8-MRSA-IV & CI (2) CC8-MRSA-VI (1)	*mecA, ugpQ, ΔmecR1, ccrAB2, ccrAB4* (1 *ccrA4* negative) *mecA, ugpQ, ΔmecR1, ccrAB4*	IVE or IVF & *ccrAB4* VI
8	ST8-MRSA-IVF & *ccrAB4* (3)	t190 (3)	CC8-MRSA-VI (3)	*mecA, ugpQ, ΔmecR1, ccrAB4 (1 ccrA4* negative*)*	VI
8	ST94-MRSA-IVg & *ccrAB4* (1)	t4691 (1)	ST8-MRSA-IV & CI (1)	*mecA, ugpQ, ΔmecR1, ccrAB2, ccrB4, dcs*	IV & *ccrAB4*
8	ST609-MRSA-IVA (1)	t064 (1)	CC8-MRSA-IV (1)	*mecA, ugpQ, ΔmecR1, ccrAB2, dcs, aadD*	IVA
22	ST22-MRSA-IVh (22)	t032 (9), t022 (2), t3505 (1), t1467(1), t531(1), t3506(1), t3504(1), t790(1), t515(1), t3501(1), t1802(1), t2945(1), t4253(1)	ST22-MRSA-IV (11)	*mecA, ugpQ, ΔmecR1, ccrAB2, dcs*	IV

Continued overleaf

Table 3.6 continued. MLST clonal complexes (CC), sequence types (ST), SCC*mec* types and *spa* types of 175 MRSA isolates representative of different antibiogram-resistogram, ST and SCC*mec* type combinations recovered from patients in Irish hospitals between 1971 and 2004 and the corresponding CCs/STs and SCC*mec* types deduced from DNA microarray profiles of 107 representative isolates

			DNA microarray[a]		
CC	MLST and SCC*mec* typing strain assignment (No. isolates tested)	*spa* types (No. isolates tested)	CC/ST-SCC*mec* type assignment by microarray (No. isolates tested)	SCC*mec*-associated genes detected by microarray	SCC*mec* type/subtype assignment following manual inspection of microarray profiles
22	ST22-MRSA-IVa (5)	t005 (3), t902(1), t4902(1)	ST22-MRSA-IV (2)	*mecA, ugpQ, ΔmecR1, ccrAB2, dcs*	IV
5	ST5-MRSA-II (17)	t045 (17)	ST5-MRSA-II (14)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, dcs* (2 *dcs* negative), *kdp, aadD*	II
5	ST5-MRSA-IV non subtypeable (1)	t001 (1)	CC5-MRSA-atypical SCC*mec* element (1)	*mecA, ugpQ, ΔmecR1, dcs*	Class B *mec* & *dcs*, no *ccr* genes detected
5	ST496-MRSA-II (1)	t002(1)	ST5-MRSA-II (1)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, dcs, kdp, aadD*	II
30	ST36-MRSA-II (12)	t018 (9), t012 (3)	ST36/39-MRSA-II (6)	*mecA, ugpQ, ΔmecR1, mecR1, mecI, xylR, ccrAB2, dcs, kdp, aadD*	II
30	ST30-MRSA-IV non subtypeable (4)	t5093(1), t012(2), t021(1)	ST30-MRSA-IV (4)	*mecA, ugpQ, ΔmecR1, ccrAB2, dcs* (2 *dcs* negative), *Q6GD50* (*fusC*)	IV & *Q6GD50/fusC*
45	ST45-MRSA-IVa (1)	t727(1)	ST45-MRSA-IV (1)	*mecA, ugpQ, ΔmecR1, ccrAB2, dcs*	IV
12	ST12-MRSA-IVc (1)	t160(1)	CC12-MRSA-IV (1)	*mecA, ugpQ, ΔmecR1, ccrAB2, dcs*	IV

[a] The StaphyType Kit (Alere Technologies) was used for DNA microarray analysis.

Abbreviations: CI, composite island; ST, sequence type; CC, clonal complex.

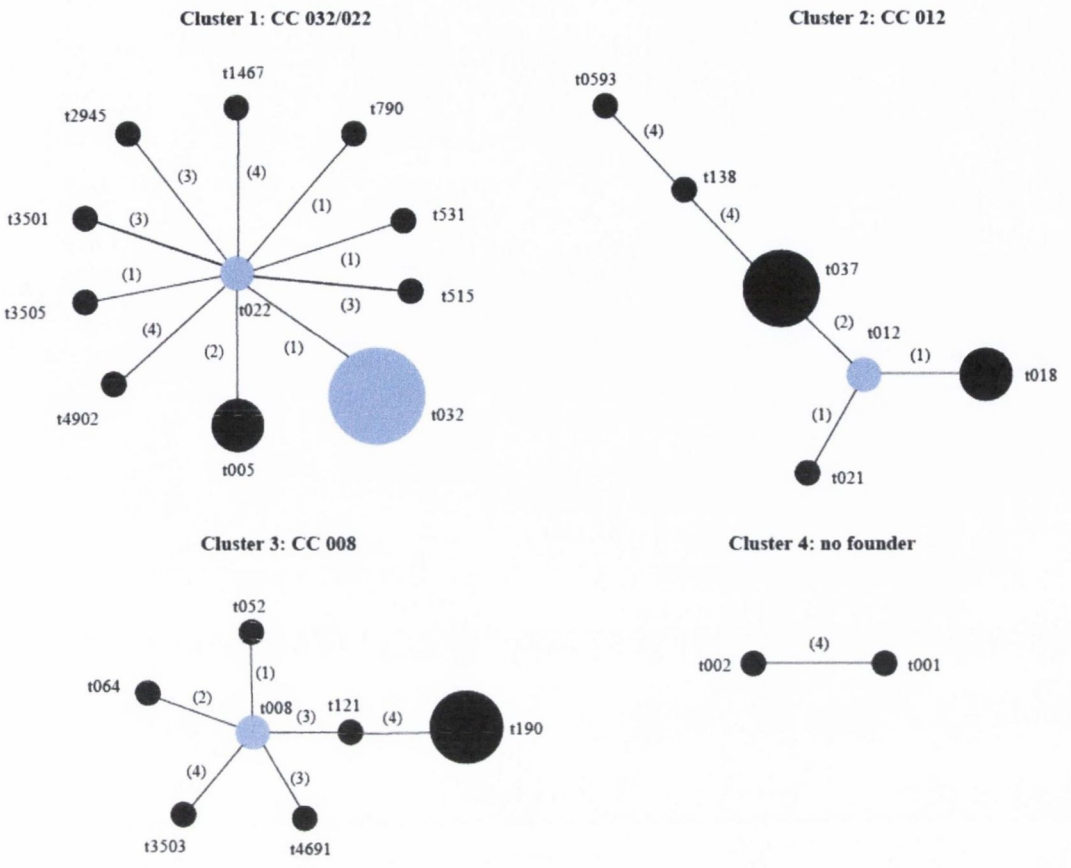

Fig. 3.1 Based Upon Repeat Pattern (BURP) analysis of the *spa* types identified among 176 MRSA isolates representative of the different AR, sequence type and SCC*mec* type combinations recovered from patients in Irish hospitals between 1971and 2004. Twenty-six of the 35 *spa* types were grouped into four cluster groups using the BURP algorithm. *spa* types were clustered together if they contained five or more *spa* repeats and if they had a cost value ≤4 where cost accounts for the number of steps of evolution between *spa* types. A low cost value indicates close evolutionary relatedness between two *spa* types. The following six *spa* types were defined as singletons by BURP (t045, t160, t902, t3504, t3506 and t4253) as they could not be clustered with any other *spa* type i.e. cost value >5. The *spa* types t727, t1802 and t2196 were excluded as they consisted of four repeat units only. Cost values are shown in parentheses. Group founders and co-founders (*spa* types with the second highest group founder score) are shown in blue and are determined based on the *spa* type that shares the highest sequence identity with the greatest number of *spa* types within that cluster as determined by the cost values. No founder was assigned to Cluster 4.

Enright, 2004a). The *spa* types within this cluster included the cluster founder t012 as well as t018, t021, t138 and t0593 with cost values ranging from 1-4. Cluster 3 (*spa*-CC008) consisted of all except one of the remaining *spa* types exhibited by CC8 isolates including t008 (cluster founder), t052, t064, t121, t4691, t190 and t3503 all with cost values ranging from 1-4. Cluster 4 consisted of two of the three *spa* types (t002 and t001) found among CC5 isolates with a cost value of 4. The remaining *spa* types were not assigned to any cluster because they consisted of four repeats only, which are considered to be too few data to deduce evolutionary history, or they differed from all other *spa* types by a cost of ≥ 5 (where cost indicates the steps of evolution between two *spa* types) (Fig 3.1). A cost value of <5 between two *spa* types indicates close evolutionary relatedness between the isolates exhibiting these *spa* types.

3.3.1.2 DNA microarray profiling

3.3.1.2.1 Correlation between the DNA microarray and MLST

The DNA microarray assigned the majority of the MRSA isolates investigated to the correct ST (92%; 98/107) (Table 3.6). Seven isolates were not assigned to any ST but were assigned to the correct CC (7%; 7/107), while two isolates, exhibiting ST496 and ST94, were assigned to incorrect STs, but these incorrect STs were within the correct CCs, CC5 and CC8, respectively (Table 3.6). One isolate with ST496 (which is in CC5) was assigned to ST5, and one isolate belonging to ST94 (which is in CC8) was assigned to ST8 (Table 3.6). ST496 is a double-locus variant of ST5, while ST94 is a single-locus variant of ST8.

3.3.1.2.2 Correlation between SCCmec typing and the SCCmec types deduced by DNA microarray analysis

Following DNA microarray analysis the majority of the MRSA isolates were assigned to the correct SCC*mec* type (i.e., to SCC*mec* types I, II, III or IV) (95%; 102/107) compared to results obtained previously by conventional SCC*mec* typing (Table 3.6). The main characteristics of the SCC*mec* types and subtypes investigated are shown in Table 3.7 in the present study. Four ST8/t190 isolates that were previously assigned by SCC*mec* typing to SCC*mec* types IVE or IVF (J1 regions IVc and IVb, respectively, as well as class B *mec* and *ccrAB2* and lacking *dcs*) with an adjacent *ccrAB4* were assigned to SCC*mec* VI by the DNA microarray (Table 3.6). In these isolates, the class B *mec* complex and *ccrAB4* genes were detected using the DNA microarray, but *ccrAB2* was not detected (Table 3.6). One MRSA isolate belonging to CC5 with *spa* type t001, which was previously found to harbour SCC*mec* IV by SCC*mec* typing, was designated as an atypical SCC*mec* element by the DNA microarray (Table 3.6). Manual inspection of the SCC*mec*-associated DNA microarray signals of this isolate revealed the presence of a class B *mec* complex and *dcs* but the absence of *ccrAB2*. Lastly, five of the eight ST239-MRSA-III*Hg* isolates did not yield a signal for the truncated *mecR1* gene (Δ*mecR1*) (Table 3.6).

The DNA microarray did not automatically assign SCC*mec* subtypes to isolates, but manual inspection of the DNA microarray profiles allowed subtyping of SCC*mec* I, II and III*Hg* (Table 3.6). The SCC*mec* subtypes Ia and I – *pls* were differentiated based on the presence of a plasmid (pUB110, which is located in the J2 region of SCC*mec* Ia) encoding the aminoglycoside resistance gene *aadD* in SCC*mec* Ia and the absence of *pls* in SCC*mec* I – *pls* (Table 3.6). The SCC*mec* II subtypes IIA, IIB, IIC, IID and IIE all lack *kdp*, which is commonly associated with SCC*mec* II elements (Table 3.6). While

Table 3.7 The main characteristics of the SCC*mec* types and subtypes investigated during the present study[a]

SCC*mec* type	*ccr* type	*mec* class	Major characteristics of J regions[b]			Reference
			J1	J2	J3	
I	1	B	*pls*	SCC*mec* I specific J2 region	*dcs*	IWG-SCC, 2009; Oliveira *et al.*, 2001; Shore *et al.*, 2005
I – *pls*	1	B	Lacks *pls*	ND	*dcs*	Shore *et al.*, 2005
Ia			*pls*	SCC*mec* I specific J2 region	pUB110 (*aadD*) & *dcs*	Shore *et al.*, 2005
II			*kdp*	Tn*554* (*erm*(A) & *spc*)	pUB110 & *dcs*	Shore *et al.*, 2005
IIA	2	A.4[c]	SCC*mec* IVb specific J1 region	Tn*554*	pUB110 & *dcs*	Shore *et al.*, 2005
IIB	2	A	SCC*mec* IVb specific J1 region	Lacks Tn*554*	pUB110 & *dcs*	Shore *et al.*, 2005
IIC	2	A.3[d]	SCC*mec* IVb specific J1 region	Tn*554*, lacks ORFs between Tn*554* & *mec* complex in SCC*mec* II	pUB110 & *dcs*	Shore *et al.*, 2005
IID	2	A.4[c]	SCC*mec* IVb specific J1 region	Tn554	*dcs*	Shore *et al.*, 2005
IIE[e]	2	A.3[d]	SCC*mec* IVb specific J1 region	Tn*554*; lacks ORFs between Tn*554* & *mec* complex	*dcs*	Shore *et al.*, 2005

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Table 3.7 continued. The main characteristics of the SCC*mec* types and subtypes investigated during the present study[a]

SCC*mec* type	*ccr* type	*mec* class	Major characteristics of J regions[b]			Reference
			J1	J2	J3	
III & SCC*Hg*	3	A	SCC*mec* III*Hg*-specific J1 region	ΨTn554	pT181 (*tet*(K)) & SCC*Hg* with p1258 (*mer*), Tn554 & *ccrC*	IWG-SCC, 2009; Oliveira and de Lencastre, 2002
III & SCC *Hg* – p1258/Tn554	3	A	ND	ND	pT181 & SCC*Hg* with p1258, Tn554 & *ccrC* but lacking ORFs between p1258 & Tn554	Shore *et al.*, 2005
IVA	2	B	SCC*mec* IVc-specific J1 region	No J2 region	pUB110 & *dcs*	IWG-SCC, 2009
IVa	2	B	SCC*mec* IVa-specific J1 region	No J2 region	*dcs*	IWG-SCC, 2009; Ma *et al.*, 2002
IVc	2	B	SCC*mec* IVc-specific J1 region	No J2 region	*dcs*	IWG-SCC, 2009
IVE	2	B	SCC*mec* IVc-specific J1 region	ND	Lacks *dcs*	Shore *et al.*, 2005
IVF	2	B	SCC*mec* IVb-specific J1 region	ND	Lacks *dcs*	Shore *et al.*, 2005
IVg	2	B	SCC*mec* IVg-specific J1 region	No J2 region	*dcs*	IWG-SCC, 2009; Kwon *et al.*, 2005

[a] This table was adapted from the Guidelines on the Classification of SCC*mec* elements . For the main characteristics of additional SCC*mec* types and subtypes not investigated in the present study see reference or http://www.sccmec.org/Pages/SCC_HomeEN.html.
[b] The antimicrobial resistance genes carried on plasmids and transposons integrated within the SCC*mec* elements are written in parenthesis after the first time the relevant plasmid or transposon name is listed.
[c] class A.4 *mec* complex differs from class A *mec* due to the presence of IS1182 within *mecI*
[d] class A.3 *mec* complex differs from class A *mec* due to the presence of IS1182 within *mecI* and deletion of the 3′ end of *mec* regulatory gene *mecI* .
ND, not determined.

the SCC*mec* subtypes IIA and IIB cannot be further differentiated using the array, SCC*mec* IIC is characterised by the absence of *xylR* and *mecI* due to the presence of a variant *mec* complex termed class A.3 *mec*. SCC*mec* subtype IID is characterised by the absence of *aadD* while *xylR*, *mecI* and *aadD* are absent in SCC*mec* IIE (Table 3.6, 3.7). SCC*mec* type III*Hg* was identified by the presence of the mercury resistance genes *merA* and *merB* and by *ccrC*.While the DNA microarray was unable to subtype SCC*mec* IVa, IVb, IVc, IVd, IVE, IVF, IVg, IVh and IV non-subtypeable, the recognition of SCC*mec* IVA was possible due to the presence of the pUB110-encoding gene *aadD* (Table 3.6, 3.7). The microarray detected the *fusC* gene (designated *Q6GD50* on the array) indicative of the presence of the SCC*fus* element (Holden *et al.*, 2004) in four ST30 isolates, but additional *ccr* genes (*ccrAB1*) normally associated with the presence of this SCC element were not detected. The *dcs* gene, located between *mecA* and *orfX* associated with SCC*mec* II and IV elements, was not detected in eight isolates harbouring SCC*mec* II elements (seven ST8-MRSA-II and one ST5-MRSA-II isolates). The lack of detection of *dcs* was also noted in eight isolates with SCC*mec* IV elements (ST8-MRSA-IVE, *n*=3; ST8-MRSA-IVF, *n*=3; ST30-MRSA-IV non subtypeable, *n*=2), although this has been reported previously in SCC*mec* elements IVE and IVF (Shore *et al.*, 2005). The DNA microarray also allowed the identification the presence of a second set of *ccr* genes, known as *S. epidermidis ccrAB4*, in ST8-MRSA isolates (*spa* type t190) a phenomenon that has been reported previously in ST8 isolates (Shore *et al.*, 2008) (Table 3.6). The array yielded positive or ambiguous signals for *ccrA4* and/or *ccrB4* in 36 CC8-MRSA isolates, including ST8-MRSA-IIA-IIE isolates (29/33), ST8-MRSA-IVE-IVF isolates (6/6), and the single ST94-MRSA-IVg isolate investigated using the array. The presence of *S. epidermidis ccrAB4* was confirmed in these isolates by PCR, indicating the possible presence of an SCC element in addition to

SCC*mec* in these isolates. The presence of *S. epidermidis ccrAB4* was also investigated by PCR in the ST8-MRSA-IIA-IIE (n=21), and ST8-MRSA-IVE-IVF (n=14) isolates from the present study that did not undergo DNA microarray analysis; the presence of this gene was confirmed in 95% (20/21) and 100% (14/14) of these isolates, respectively.

3.3.1.2.3 Antimicrobial resistance, virulence-associated and adhesion genes detected using the DNA microarray

The *agr* capsule and immune evasion cluster (IEC) types, as well as the antimicrobial resistance, virulence-associated, MSCRAMM, adhesion and biofilm formation genes identified among the 107 MRSA isolates representative of the different ST and SCC*mec* type combinations investigated by the DNA microarray analysis are shown in Table 3.8. All isolates lacked the PVL locus genes, *lukF-PV* and *lukS-PV*, the exfoliative toxin genes *etA*, *etB* and *etD* and the arginine catabolic mobile element (ACME). The most common enterotoxin and antimicrobial resistance genes (apart from *mecA*) identified among the isolates were *blaZ* (97%; 104/107) and enterotoxin A (48%; 51/107), respectively.

The majority of isolates belonged to *agr* type I (75%; 80/107) and included all CC8and CC22 isolates (n= 66 and 13, respectively). All CC5 (n=16) and the single CC12 isolate belonged to *agr* type II, while isolates within *agr* III were CC30 (n=10). The single CC45 isolate belonged to *agr* type IV. Most isolates belonged to capsule type 5(78%; 83/107), with the remainder belonging to capsule type 8 (22%; 24/107). The immune evasion genes (*sak*, *chp*, *scn*) IEC genes were detected in the majority of isolates (91%; 97/107), the most common occurring IEC type was type D (39%; 42/107), which was in the CC8 lineages ST239-MRSA-III, ST8-MRSA-IIA–IIE, and

Table 3.8 *agr*, capsule and IEC types, antimicrobial resistance, virulence-associated, MSCRAMM, adhesion and biofilm formation genes identified using the DNA microarray[a] among isolates representative of each antibiogram-resistogram, ST and SCC*mec* type combination recovered from patients in Irish hospitals between 1971 and 2004

CC/ST-SCC*mec* type (*n*)	Additional Typing Markers			Antimicrobial resistance genes		Virulence-associated genes		MSCRAMM, adhesion & biofilm genes	
	agr	*cap*	IEC (*n*)[b]	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)
CC8/ST250-MRSA-I/I-pls (10)	I	5	B (10)	*blaZ*, *merA* & *B*, *erm*(A), *sdrM*, *qacA*, *fosB*	*tet*(K) (70%), *aacA-aphD* (90%), *aphA3* & *sat* (40%), *dfrS1* (40%)	*sak*, *chp*, *scn*	*seb* (90%), *sek* & *seq* (90%)	*bbp*, *clfA*, *clfb*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC* & *icaD*	…..
CC8/ST239-MRSA-III & SCC*Hg*/ III & SCC*Hg*–p1258/Tn554 (12)	I	8	D (12)	*blaZ*, *merA* & *B*, *erm*(A), *tet*(M), *sdrM*, *fosB*	*tet*(K) (92%), *qacA* (58%), *qacC* (8%), *aacA-aphD* (83%), *aadD* (8%), *aphA3* & *sat* (50%), *dfrS1* (25%), *ileS2* (8%), *cat*–pC194 (pMC524) (8%)	*sea*, *sak*, *scn*	*sek* & *seq* (83%)	*clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC* & *icaD*	*bbp* (58%)
CC8/ST247-MRSA-Ia (3)	I	5	A (1) B (2)	*blaZ*, *merA* & *B* *tet*(M), *sdrM*, *qacA*, *aacA-aphD*, *aadD*, *fosB*	*erm*(A) (67%)	*sak*, *chp*, *scn*	*sea* (33%)	*clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC* & *icaD*	…..
CC8/ST8-MRSA-IIA-IIE (33)	I	5	D (29) NT (1)[c] Neg (3)	*blaZ*, *erm*(A), *aacA-aphD*, *sdrM*, *fosB*, *merA* & *B*	*erm*(C) (6%), *tet*(K) (3%), *qacA* (97%), *qacC* (3%), *aadD* (36%), *aphA3* & *sat* (85%), *dfrS1* (3%), *far1*/*fusB* (3%), *ileS2* (12%)	…..	*sea* (91%), *sak* (91%), *scn* (88%)	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fnbA*, *fnbB*, *sdrD*, *vwb*, *icaA*, *icaC* & *icaD*	*fib* (97%), *map* (97%), *sdrC* (3%)

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Table 3.8 continued. *agr*, capsule and IEC types, antimicrobial resistance, virulence-associated, MSCRAMM, adhesion and biofilm formation genes identified using the DNA microarray[a] among isolates representative of each antibiogram-resistogram, ST and SCC*mec* type combination recovered from patients in Irish hospitals between 1971 and 2004

CC/ST-SCC*mec* type (*n*)	Additional Typing Markers			Antimicrobial resistance genes		Virulence-associated genes		MSCRAMM, adhesion & biofilm genes	
	agr	*cap*	IEC (*n*)[b]	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)
CC8/ST8-MRSA-IVE/F (6)	I	5	E (38) Neg (1)	*blaZ*, *erm*(A), *sdrM*, *aphA3* & *sat*, *fosB*	*tet*(M) (33%), *qacA* (17%),	…..	*sak* (83%), *scn* (83%)	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *vwb*, *sasG*, *icaA*, *icaC* & *icaD*	*sdrD* (83%)
CC8/ST94-MRSA-IVg (1)	I	5	Neg (1)	*blaZ*, *tet*(M), *sdrM*, *aacA-aphD*, *aphA3* & *sat*, *fosB*	…..	…..	…..	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC* & *icaD*	…..
CC8/ST609-MRSA-IVA (1)	I	5	D (1)	*blaZ*, *erm*(C), *tet(M)*, *sdrM*, *aacA-aphD*, *aadD*, *dfrS1*, *fosB*	…..	*sea*, *seb*, *sek* & *seq*, *sak*, *scn*	…..	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC* & *icaD*	…..
CC22/ST22-MRSA-IVh/IVa (13)	I	5	B (8) Neg (38)	*blaZ*	*erm*(C) (46%), *qacC* (8%)	*egc*	*sec* & *sel* (85%), *sak* (62%), *chp* (62%), *scn* (62%)	*clfA*, *clfB*, *cna*, *ebpS*, *eno*, *fib*, *fnbA*, *sdrC*, *vwb*, *sasG*, *icaA*, *icaD*	*fnbB* (15%), *icaC* (85%), *bbp* (85%), *ebh* (15%), *map* (92%), *sdrD* (85%)

Continued overleaf

Table 3.8 continued. *agr*, capsule and IEC types, antimicrobial resistance, virulence-associated, MSCRAMM, adhesion and biofilm formation genes identified using the DNA microarray[a] among isolates representative of each antibiogram-resistogram, ST and SCC*mec* type combination recovered from patients in Irish hospitals between 1971 and 2004

CC/ST-SCC*mec* type (*n*)	Additional Typing Markers			Antimicrobial resistance genes		Virulence-associated genes		MSCRAMM, adhesion & biofilm genes	
	agr	*cap*	IEC (*n*)[b]	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)
CC5/ST5-MRSA-IV non subtypeable (1)	II	5	B (1)	*merA &B, blaZ, erm*(A), *sdrM, fosB*	…..	*egc, sak, chp & scn*	…..	*bbp, clfA, clfB, ebh, ebpS, eno, fib, fnbA, map, sdrC, vwb, sasG, icaA, icaC, icaD*	…..
CC5/ST496-MRSA-II (1)	II	5	B (1)	*erm*(A), *erm*(C), *aadD*, *sdrM, fosB*	…..	*egc, sak, chp & scn*	…..	*bbp, clfA, clfB, ebh, ebpS, eno, fib, fnbA, fnbB, map, sdrC, sdrD, vwb, sasG*, *icaA, icaC, icaD*	…..
CC30/ST36-MRSA-II (6)	III	8	A (6)	*blaZ*, *erm*(A), *sdrM*, *aadD, fosB*	…..	*sea, egc, sak, chp, scn*	*tst* (67%)	*bbp, clfA, clfB, cna, ebh, ebpS, eno, fnbA, fib, map, sdrC, sdrD*, *vwb*, *icaA, icaC, icaD*	…..
CC30/ST30-MRSA-IV non subtypeable (4)	III	8	B (4)	*blaZ*, Q6GD50/*fusC*, *fosB*	*sdrM* (75%)	*tst, egc, sak, chp, scn*	…..	*clfA, clfB, cna, ebh, ebpS, eno, fib, fnbA, map, sdrC, sdrD*, *vwb*, *icaA, icaC, icaD*	*bbp* (75%)

Continued overleaf

Table 3.8 continued. *agr*, capsule and IEC types, antimicrobial resistance, virulence-associated, MSCRAMM, adhesion and biofilm formation genes identified using the DNA microarray[a] among isolates representative of each antibiogram-resistogram, ST and SCC*mec* type combination recovered from patients in Irish hospitals between 1971 and 2004

CC/ST-SCC*mec* type (*n*)	Additional Typing Markers			Antimicrobial resistance genes		Virulence-associated genes		MSCRAMM, adhesion & biofilm genes	
	agr	*cap*	IEC (*n*)[b]	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)
CC45/ST45-MRSA-IVa (1)	IV	8	B (1)	*blaZ, merA* & *B, erm*(C), *far1/fusB, sdrM*	…..	*egc, sak, chp, scn*	…..	*bbp, clfA, clfB, cna, ebh, ebpS, eno, fib, fnbA, fnbB, map, sdrC, sdrD, vwb, sasG, icaA, icaC, icaD*	…..
CC12/ ST12-MRSA-IVc (1)	II	8	G (1)	*blaZ, sdrM, fosB*	…..	*sep, seb, sec* (M14), *sak, scn*	…..	*clfA, clfB, cna, ebh, ebpS, eno, fib, fnbA, fnbB, map, sdrC, vwb, icaA, icaC, icaD*	…..

[a] The StaphyType Kit (Alere Technologies) was used for DNA microarray analysis.

[b] Immune evasion cluster (IEC) type as defined by van Wamel *et al*. (2006): A= *sea*, *sak*, *chp* and *scn*; B = *sak, chp* and *scn*; C = *chp* and *scn*; D = *sea, sak* and *scn;* E = *sak* and *scn*; F = *sep, sak, chp* and *scn*; G = *sep, sak* and *scn*; NT, novel IEC type consisting of *sak* and *sea* only; neg (negative), no IEC genes detected.

[c] *seg* and sei were not detected in one and two ST5-MRSA-II isolates, respectively, found to carry the enterotoxin gene cluster (*egc*) genes seg/*sei, sem, sen, seo* and *seu.*

Abbreviations: n, number of isolates; *agr*, genes encoding accessory gene regulators; *cap*, genes encoding capsular proteins; CC, clonal complex; ST, sequence type; MSCRAMM, microbial surface components recognizing adhesive matrix molecules.

'…..', no genes detected.

ST609-MRSA-IVA harboured the enterotoxin A gene *sea* and the gene encoding staphylokinase (*sak*) and the staphylococcal complement inhibitor (*scn*). Other IEC types included IEC types A (*n*=8 isolates), B (*n*=28 isolates), E (*n*=5 isolates), F (*n*=12 isolates) and G (*n*=1 isolate). The main antimicrobial resistance and virulence-associated genes identified among the most predominant MRSA clones recovered in Irish hospitals in the 1970s and 1980s (ST250-MRSA-I/I–*pls* and ST239-MRSA-III & SCC*Hg*/III & SCC*Hg*-p*I258*/Tn*554*) and in the 1990s (ST8-MRSA-IIA-IIE, ST22-MRSA-IV and ST36-MRSA-II) are discussed below in more detail (Table 3.8).

ST250-MRSA-I

All ST250-MRSA-I/I-*pls* isolates (*n*=10) were assigned to capsule type 5 and *agr* type I and carried the antimicrobial resistance genes *erm*(A) encoding macrolide, lincosamide and streptogramin B (MLS_B) resistance, *sdrM* encoding a general efflux pump, *fosB* encoding fosfomycin resistance, *qacA* encoding resistance to quaternary ammonium compounds and *merA* (mercuric reductase) and *merB* (alkylmercury lyase). The presence of the aminoglycoside resistance genes *aacA-aphD* (90%; 9/10 isolates), *aphA3* (40%; 4/10), the trimethoprim resistance gene *dfrS1* (40%; 4/10) and the tetracycline resistance gene *tet*(K) (70%; 7/10) were variable (Table 3.6). The majority of these isolates harboured the enterotoxin genes *seb*, *sek*, and *seq, entB*, *entK* and *entQ* (90%, 9/10). All ST250-MRSA-I//I-*pls* isolates harboured IEC type B (*sak*, *chp* and *scn*) as well as the following MSCRAMM, adhesion and biofilm genes: *bbp* (bone sialoprotein-binding protein), *sdrC* (serineaspartate repeat protein C), *sdrD* (serine aspartate repeat protein D), *ebh* (cell wall associated fibronectin binding protein), *fib* (fibrinogen binding protein), *fnbB* (fibronectin binding protein B), *map* (MHC class II

analog protein), *sasG* (*S. aureus* surface protein) and *icaC* (intercellular adhesion protein A) (Table 3.8).

ST239-MRSA-III & SCC*Hg*/ III & SCC*Hg*-pI258/Tn*554*

Isolates belonging to the ST239-MRSA-III & SCC*Hg*/III-pI258/Tn*554* clone (*n* =12) exhibited capsule type 8 and *agr* type I. Antimicrobial resistance genes harboured by all of these isolates included *erm*(A), *fosB*, *sdrM*, the tetracycline resistance gene *tet*(M) and *merA* & *merB*. Carriage of *tet*(K) (92%; 11/12), the mupirocin resistance gene *ileS2* (8%; 1/12), the aminoglycoside resistance genes *aacA-aphD* (83%; 10/12), *aphA-3* (50%; 6/12) and *aadD* (8%; 1/12), *dfrS1* (25%; 3/12), the chloramphenicol resistance gene *cat*-pC194(PMC524) (8%; 1/12), *qacC* (8%; 1/12) and *qacA* (58%; 7/12) were variable (Table 3.6). All ST239-MRSA-III & SCC*Hg*/III-pI258/Tn*554* isolates harboured the enterotoxin genes *sek* and *seq* (83%; 10/12) (Table 3.6).All isolates harboured IEC type D (*sea*, *sak* and *scn*). In addition, they carried the same MSCRAMM genes as ST250-MRSA-I/I-*pls* isolates except that 7/12 isolates (58%) lacked *bbp* and all ST239-MRSA-III & SCC*Hg*/III-pI258/Tn*554* isolates harboured the collagen binding adhesion gene *cna* (Table 3.8).

ST8-MRSA-IIA-IIE

All ST8-MRSA-IIA-IIE (*n*=33) isolates belonged to capsule type 5 and *agr* type I. CC8 harboured the highest number of resistant determinants, included the antimicrobial resistance genes *erm*(A), *aac-aphD*, *sdrM, fosB*, *merA* and *merB*. Carriage of other antimicrobial resistance genes was variable, namely *qacA* (97%; 32/33) *aphA3* & *sat* (85%; 28/33), *aadD* (36%; 12/33), *ileS2* (12%; 4/33), *erm*(C) (6%; 2/33), *dfrS1* (3%; 1/33), *tet*(K) (3%; 1/33) and *qacC* (3%; 1/33). In addition, the majority of ST8-MRSA-IIA-IIE isolates harboured the enterotoxin gene *sea* (91%; 30/33). The majority

of ST8-MRSA-IIA-IIE isolates harboured IEC type D (*sea*, *sak* and *scn*) (88%; 29/33) but a novel IEC type with *sea* and *sak* but lacking *scn* and *chp*, was detected in one isolate. All ST8-MRSA-IIA-IIE isolates lacked *cna* but were positive for *bbp*, *clfA*, *clfB*, *ebh, ebpS, eno, fnbA, fnbB, sdrD, vwb,* and *icaA/C/D*, while carriage of *fib* (97%; 32/33), *sdrC* (3%; 1/33) and *map* (97%; 32/33) were variable (Table 3.8).

ST22-MRSA-IV

Isolates representative of the ST22-MRSA-IV clone (*n*=13) exhibited capsule type 5 and *agr* type I. The ST22-MRSA-IV isolates only harboured genes encoding resistance to three classes of antimicrobial agents, including *erm*(C) (46%; 6/13) and *qacC* (8%; 1/13). All ST22-MRSA-IV isolates carried the enterotoxin gene cluster (*egc; seg, sei, sem, sen, seo* and *seu/sey*), while carriage of *sec* and *sel* (85%; 11/13) were variable. Only 62% (8/13) of isolates harboured IEC (IEC type B, *sak*, *chp* and *scn*), while the genes *clfA*, *clfB*, *cna, ebpS, eno, fib, fnbA sdrC, vwb, sasG, icaA,* and *icaD* were always present. The presence of *map* (92%; 12/13), *icaC* (85%; 11/13), *bbp* (85%; 11/13), *sdrD* (85%; 11/13), *ebh* (15%, 2/13), *fnbB* (15%; 2/13) were variable.

ST36-MRSA-II

The ST36-MRSA-II isolates (*n*=6) exhibited capsule type 8 and *agr* type III and harboured *erm*(A), *sdrM, aadD*, *fosB* and *egc*, while carriage of the toxic shock syndrome toxin (*tst*) (67%; 4/6) was variable. All ST36-MRSA-II isolates harboured the IEC genes *sak*, *chp* and *scn* (IEC type B) as well as *bbp*, *clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fnbA*, *fib*, *map*, *sdrC*, *sdrD, vwb* and *icaA/C/D.*

3.3.1.3 Correlation between antimicrobial resistance phenotype determined by AR typing, and antimicrobial resistance genotype determined using the DNA microarray

The correlation between the antimicrobial resistance phenotype (determined by disk diffusion) and genotype for the 107 MRSA isolates (determined by DNA microarray profiling), are shown in Table 3.9. for antimicrobial resistance genes yielding positive results only, i.e., all isolates yielded negative results for the genes encoding resistance to antimicrobial in the following antimicrobial classes, and results are not included in Table 3.9: chloramphenicol (*cfr* and *fexA*, encoding chloramphenicol and florfenicol resistance); glycopeptides (*vanA*, *vanB* and *vanZ*, encoding vancomycin and/or teicoplanin resistance); macrolides lincosamides, or streptogramins (MLS) encoding genes [(*lnu*(A), *mef*(A), *mph*(C), *msr*(A), *vat*(A), *vat*(B), *vga*(A), encoding resistance to a range of MLS antimicrobial compounds].

Eighty-three of the 107 MRSA isolates exhibited phenotypic and genotypic resistance to aminoglycosides. The three aminoglycoside genes detected by the DNA microarray are *aacA-aphD* (encoding resistance to amikacin, gentamicin, kanamycin and tobramycin), *aadD* (encoding resistance to amikacin, kanamycin, neomycin and tobramycin) and *aphA3* (encoding resistance to kanamycin and neomycin). The level of resistance to amikacin encoded by *aacA-aphD* is low and may be difficult to detect by disk diffusion (Coombs *et al.*, 2011). Hence, many isolates carrying *aacA-aphD* or *aadD* exhibited resistance in the intermediate category or were phenotypically susceptible to amikacin (Table 3.9). The relative frequencies of occurrence of the various aminoglycoside resistance gene combinations was as follow; *aacA-aphD* and *aphA3*,31% (26/83); *aadD*, 24% (20/83); *aacA-aphD*, 17% (14/83); *aacA-aphD*, *aadD*, and *aphA3*, 16% (13/83); and *aphA3*, 7% (6/83). The phenotypic resistance profiles for each isolate harbouring these gene combinations are shown in Table 3.9. In general

Table 3.9 Correlation between antimicrobial resistance gene detection by array profiling and phenotypic detection of resistance by disk diffusion

Gene Detection by DNA Array			Phenotypic Resistance Detection			
			Antimicrobial(s) tested		Correlation between genotype and phenotype	
	Conferring resistance to	Isolates with gene profile (*n*)	Resistance profile obtained	Isolates Resistant (*n*)	Discrepancies (*n*)	Correlation % (*n*/*n*)
Aminoglycoside resistance genes			**AMI, GEN, KAN, NEO, TOB**	83	1	99 (105/106)
aacA-aphD	AMI, GEN, KAN, TOB	14	AMI, GEN, KAN, ..., TOB	5	0	
			....., GEN, KAN, ..., TOB	9[a]	0[a]	
aadD	AMI, KAN, NEO, TOB	20	AMI, ..., KAN, NEO, TOB	18	0	
			....., ..., KAN, NEO, TOB	2[a]	0[a]	
aphA3	KAN, NEO	6	....., ..., KAN, NEO, ...	6	0	
aacA-aphD & aadD & aphA3	AMI, GEN, KAN, NEO, TOB	13	AMI, GEN, KAN, NEO, TOB	12	0	
			....., ..., KAN, NEO, TOB	1	1	
aacA-aphD & aphA3	AMI, GEN, KAN, NEO, TOB	26	AMI, GEN, KAN, NEO, TOB	11	0	
			....., GEN, KAN, NEO, TOB	15[a]	0[a]	
aacA-aphD & aadD	AMI, GEN, KAN, NEO, TOB	4	AMI, GEN, KAN, NEO, TOB	4	0	
None		24	Susceptible to all (*n* = 24)	0	0	
Antiseptics, disinfectants, intercalating dyes resistance genes			**Ethidium bromide**	58	0	100 (107/107)
qacA	QACs[b], Ethidium bromide, Chlorhexidine	55	Ethidium bromide	55	0	
qacC	QACs[b], Ethidium bromide	2	Ethidium bromide	2	0	
qacA & qacC	QACs, Ethidium bromide, Chlorhexidine	1	Ethidium bromide	1	0	
None		49	Susceptible (*n* = 49)	0	0	

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Table 3.9 continued. Correlation between antimicrobial resistance gene detection by array profiling and phenotypic detection of resistance by disk diffusion

Gene Detection by DNA Array			Phenotypic Resistance Detection			
			Antimicrobial(s) tested		Correlation between genotype and phenotype	
	Conferring resistance to	Isolates with gene profile (*n*)	Resistance profile obtained	Isolates Resistant (*n*)	Discrepancies (*n*)	Correlation % (*n*/*n*)
Beta-Lactam (excluding methicillin) resistance gene			**Ampicillin**	107	3[c]	97 (104/107)
blaZ	Beta-lactams (not methicillin)	104	Ampicillin	104	0	
None		3	Susceptible (*n* = 0)	3[c]	3[c]	
Chloramphenicol resistance gene			**Chloramphenicol**	1	0	100 (107/107)
cat-pC194 (pMC524)	Chloramphenicol	1	Chloramphenicol	1	0	
None		106	Susceptible (*n* = 106)	0	0	
Fusidic acid resistance genes			**Fusidic acid**	21	15	86 (92/107)
far1 (fusB)	Fusidic acid	2	Fusidic acid	2	0	
Q6GD50 (fusC)	Fusidic acid	4	Fusidic acid	4	0	
None		101	Susceptible (*n* = 86)	15	15	
Heavy metal ions resistance genes			**MER, PMA**	63	2	98 (105/107)
merA	Mercury ions	0	Mercuric chloride	0	0	
merB	Organomercurial ions	0	Phenylmercuric acetate	0	0	
merA & *merB*	Mercury & Organomercurial ions	63	MER, PMA	62	1	
None		44	Susceptible (*n* = 43)	1	1	

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Table 3.9 continued. Correlation between antimicrobial resistance gene detection by array profiling and phenotypic detection of resistance by disk diffusion

Gene Detection by DNA Array			Phenotypic Resistance Detection			
			Antimicrobial(s) tested		Correlation between genotype and phenotype	
	Conferring resistance to	Isolates with gene profile (*n*)	Resistance profile obtained	Isolates Resistant (*n*)	Discrepancies (*n*)	Correlation % (*n*/*n*)
Macrolides, lincoamides, streptogramins (MLS) resistance genes			**Erythromycin, [Lincomycin]**	93 [47][d]	0	100 (107/107)
erm(A)	Erythromycin, Clindamycin	82	Erythromycin [Lincomycin]	82 [46][d]	0	
erm(C)	Erythromycin, Clindamycin	8	Erythromycin [Lincomycin]	8 [1][d]	0	
erm(A) & *erm*(C)		3	Erythromycin [Lincomycin[	3 [0][d]	0	
None		14	Susceptible (*n* = 14)	0	0	
Mupirocin resistance gene			**Mupirocin (high-level)**	5	0	100 (107/107)
mupA/ileS2	Mupirocin (high-level)	5	Mupirocin (high-level)	5	0	
None		102	Susceptible (*n* = 102)	0	0	
Tetracycline resistance genes			**Tetracycline**	29[e]	2[e]	98 (105/107)[e]
tet(K)	Tetracycline	8	Tetracycline	8	0	
tet(M)	Tetracycline	8	Tetracycline	8	0	
tet(K) & *tet*(M)	Tetracycline	11	Tetracycline	11	0	
None		80	Susceptible (*n* = 78[e])	2[e]	2[e]	
Trimethoprim resistance gene			**Trimethoprim**	26	17	84 (90/107)
dfrS1	Trimethoprim	9	Trimethoprim	9	0	
	None	98		Susceptible (*n* = 81)	17	17

[a] Variations in expression of phenotypic resistance to amikacin are not considered to be discrepant results because the reduction in amikacin susceptibility encoded by *aacA-aphD* may be very slight (Lelievre *et al.*, 1999) .
[b] QAC, quaternary ammonium compound. *qacA* encodes resistance to QACs, divalent cations and intercalating dyes (such as ethidium bromide); *qacC* encodes resistance to QAC and intercalating dyes.
[c] One of the three ampicillin resistant isolates exhibited intermediate resistance to ampicillin.
[d] Numbers in square brackets indicate numbers of isolates exhibiting resistance to lincomycin.
[e] Two of 29 tetracycline-resistant isolates exhibited intermediate resistance and no tetracycline resistance genes were detected.
Abbreviations: AMI, amikacin; GEN, gentamicin; KAN, kanamycin; MER, mercuric chloride; NEO, neomycin; PMA, phenylmercuric acetate; TOB, tobramycin.

(aside from the problem of amikacin resistance with isolates carrying *aacA-aphD* and/or *aadD*), correlation between phenotypic and genotypicexpression of resistance was excellent (99%), with just one isolate carrying *aacA-aphD*, *aadD*, and *aphA3* exhibiting phenotypicsusceptibility to gentamicin (Table 3.9).

For all other antimicrobials tested, apart from fusidic acid and trimethoprim, the correlation between phenotype and genotype was ≥97%. A 100% correlation between the antimicrobial resistance phenotype and the presence of a particular gene or genes was observed for high-level mupirocin resistance *ileS2*, ethidium bromide resistance and *qacA* and/or *qacC*, erythromycin resistance and *erm*(A) and/or *erm*(C), and chloramphenicol resistance and *cat* (Table 3.9). All beta-lactam-resistant isolates harboured *mecA*, while 97% (104/107) also harboured the beta-lactam resistance gene *blaZ*. One isolate that lacked *blaZ* exhibited intermediate resistance to ampicillin. Twenty nine isolates were phenotypically resistant to tetracycline, 27% (8/29) and 27%(8/29) of these harboured *tet*(K) and *tet*(M), respectively, whereas 38% (11/29) harboured both *tet*(K) and *tet*(M). No tetracycline resistant genes were detected in the remaining two isolates exhibited intermediate resistance to tetracycline using the DNA microarray and tested negative for *tet*(L) and *tet*(O) using specific PCR assays. The mercury resistance genes *merA* and *merB* were detected in all but one isolate exhibiting resistance to mercuric chloride, but one isolate exhibiting susceptibility to mercuric chloride harboured *merA* and *merB*. One isolate (ST239-MRSA-III*Hg*) exhibited phenotypic resistance to chloramphenicol and harboured the chloramphenicol resistance gene *cat* (Table 3.9).

There were major discrepancies with fusidic acid and trimethoprim resistance. Only 29% of fusidic acid-resistant isolates (6/21) (Table 3.9). The fusidic acid resistance gene *far1/fusB* was detected in one ST45-MRSA-IV and one ST8-MRSA-IIB

isolate, while the Q6GD50/*fusC* gene was detected in four ST30-MRSA-IV isolates. None of the remaining 15 fusidic acid-resistant isolates were found to harbour the alternative fusidic acid resistance gene *fusD* by *fusD*-specific PCRs (*fusD* is not detected by the DNA microarray). The *fusA* gene in each of these 15 fusidic acid-resistant isolates was amplified by PCR followed by amplimer sequencing, as mutations in *fusA* have previously been shown to be associated with fusidic acid resistance in *S. aureus*. Mutations in *fusA* were identified in all15 isolates. Three different combinations of mutations were identified in these isolates, including (a) an amino acid substitution from leucine to lysine at position 461 in the protein sequence (L461K) in eight isolates (seven CC8 isolates: one ST8-MRSA-IID,one ST250-MRSA-I-*pls*, and five ST250-MRSA-I/I-*pls* isolates; one ST22-MRSA-IVh isolate), (b) an amino acid substitution from leucine to serine at position 461 in the protein sequence (L461S) in five isolates (one ST5-MRSA-II, one ST239-MRSA-III, and three ST250-MRSA-I-*pls* isolates), and (c) an amino acid substitution from alanine to valine and phenylalanine to leucine at positions 70 (A70V) and 406 (F406K), respectively, in the protein sequence of two isolates (two ST8-MRSA-IIC isolates).

Twenty-six isolates exhibited phenotypic resistance to trimethoprim but only 9/26 (35%) harboured *dfrS1*. The *dfrS1* gene is the only trimethoprim resistance gene detected by the DNA microarray. All nine isolates positive for *dfrS1* gene were CC8 isolates as follows: ST609-MRSA-IVA (*n*=1), ST8-MRSA-IIE (*n*=1), ST239-MRSA-III (*n*=3) and ST250-MRSA-I/I-*pls* (*n*=4). The remaining 17 trimethoprim-resistant isolates were investigated by a variety of specific PCR assays designed to detect other genes encoding trimethoprim resistance, including *dfrD*, *dfrG*, and *dfrK* (Dale *et al.*, 1995b; Sekiguchi *et al.*, 2005; Kadlec and Schwarz, 2010b). None of the 17 trimethoprim-resistant isolates harboured *dfrD* or *dfrK*, whereas three isolates harboured *dfrG* (all

ST239-MRSA-III). The 14 isolates in which no *dfr* genes were identified belonged to ST8-MRSA-IIC (*n*=4), ST8-MRSA-IIE (*n*=2), ST8-MRSA-IVE (*n*=2), ST8-MRSA-IVF (*n*=3), ST5-MRSA-IV (*n*=1), and ST239-MRSA-III (*n*=2).

3.1.2 Molecular characterisation of fusidic acid resistance MRSA isolates recovered in Ireland between 1971 and 2010

3.1.2.1 Genotyping of fusidic acid resistant MRSA isolates by the DNA microarray

The 110 fusidic acid resistant MRSA isolates recovered from patients in Irish hospitals between 1971 and 2010 were assigned to nine genotypes within seven CCs (Table 3.9). The highest number of fusidic acid resistant isolates were assigned to CC22/ST22-MRSA-IV (75%; 83/110) followed by ST250-MRSA-I/I-*pls* (5%; 6/110), ST45-MRSA-IV (5%; 5/110), ST8-MRSA-IIB/D (4%; 4/110), ST30-MRSA-IV non subtypable (4%; 4/110), ST5-MRSA-IV (2%; 2/110), while the remaining genotypes, namely ST239-MRSA-III & SCC*Hg*, CC1-MRSA-IV and ST59-MRSA-V, were exhibited by one isolate each (Table 3.10).

3.1.2.2 Additional typing markers, antimicrobial resistance and virulence associated genes detected by the DNA microarray analysis

The antimicrobial resistance, virulence-associated, MSCRAMM, adhesion and biofilm formation genes along with the *agr* capsule and immune evasion cluster (IEC) types identified among the 110 fusidic MRSA isolates representative of the different ST and SCC*mec* type combinations investigated by the DNA microarray profiling are shown in Table 3.10. All isolates lacked the PVL locus genes, *lukF-PV* and *lukS-PV*, the exfoliative toxin genes *etA*, *etB* and *etD* and the arginine catabolic mobile element (ACME).

3.1.2.2.1 agr, capsule and IEC types

The majority of the isolates belonged to *agr* type I (91%; 100/110) and included all CC22, CC8 and CC59 isolates (*n*=81, 14 and 1, respectively) and 2/5 CC45 isolates. The remaining three CC45 isolates were assigned to *agr* type IV. The single CC1 isolate and all CC30 isolates (*n*=4) were assigned to *agr* type III and the two CC5 isolates to *agr* type II. Most isolates belonged to capsule type 5 (90%; 99/110), including CC22, CC8, CC5 isolates (*n*=83, 6, 4, 1 and 2, respectively) and 1/5 CC45 isolates. The remainder of isolates (CC8/ST239-MRSA-III & SCC*Hg*, CC30/ST30-MRSA-IV non-subtypable, CC1-MRSA-IV, CC45/ST45-MRSA-IV (4/5), CC59/ST59-MRSA-V) belonging to capsule type 8 (10%; 11/110). The IEC genes were detected in the majority of isolates (91%; 100/110). The IEC type B (*sak*, *chp* & *scn*) was the most common type detected (84%, 92/110) and was found among all isolates belonging to CC22, the CC8 lineages (ST250), CC30, CC45 and CC59 as well as one of the two CC5 isolates. The remainder of isolates either carried no IEC genes (*n* = 10) (CC22/ST22-MRSA-IV) or IEC type D (*n*=7) (*sea*, *sak* & *scn*), belonging to CC8 (5/5 isolates), CC1 (1/1 isolate), CC5 (1/2 isolates), while one isolate harboured the IEC type E (*sak* and *scn*) (Table 3.10).

3.1.2.2.2 Virulence-associated genes

The most common virulence genes detected *egc* (85%; 94/110), *sec* and *sel* (48%; 53/110), *seb* and *sek* (11%; 12/110) and *sea* (6%; 7/110). The toxic shock toxin gene *tst* was detected in seven isolates belonging to ST22-MRSA-IV (*n*=1), ST5-MRSA-II (*n*=2), and ST30-MRSA-IV (*n*=4) (Table 3.10).

Table 3.10 Genotypes and antimicrobial resistance and virulence-associated genes detected using a DNA microarray[a] among the 110 fusidic acid resistant MRSA isolates recovered from patients in Irish hospitals between 1971 and 2010

CC/ST-SCC*mec* type (*n*)	Additional typing markers (% indicated when not 100%)			Antimicrobial resistance genes (% indicated when not 100%)	*fusA* mutations for 36 isolates tested[d]	Virulence associated genes (% indicated when not 100%)	MSCRAMM, adhesion, & biofilm genes
	agr	*cap*	IEC (*n*)[b]				
CC22/ST22-MRSA-IV (83)	I	5	B (72), E (1)	*blaZ*, *erm*(C) (74%), *ileS2* (4%), *aacA-aphD* (2%), *tet*(K) (1%) *lnu*(A) (1%)	L461K (16/21), L461S (1/21), no mutation (4/21)	*egc, sec & sel* (61%), *seb* (2%), *sak, chp, scn, tst* (1%)	*bbp, clfA, clfB, cna, ebpS, eno, fib, fnbA, map* (91%), *sdrC*, *sdrD* (91%), *sasG*, *vwb*, *icaA*, *icaC* (91%), *icaD*
CC8/ST250-MRSA-I (3)/I-*pls* (6)	I	5	B	*merA & merB, blaZ, erm*(A), *aacA-aphD, sdrM, fosB, qacA, tet*(K) (67%), *aphA3* (44%), *sat* (44%), *dfrA* (44%)	L461K (6/9), L461S (3/9)	*seb, sek & seq, sak, chp, scn*	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vmb*, *sasG*, *icaA*, *icaC*, *icaD*
CC8/ST8-MRSA-IIB-IIC (+*S.epi ccrAB4*)-IID (+*S.epi ccrAB4*) (4)	I	5	D	***farI (fusB)* (25%)**, *merA & merB, blaZ, erm*(A), *aacA-aphD, aphA3 & sat, sdrM, fosB, erm*(C) (50%), *aadD* (75%), *ileS2* (50%), *tet*(K) *(25%), qacA* (75%)	L461K (1/3), A70V & F406K (2/3)	*sea, sak, scn*	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC* (25%), *sdrD*, *vmb*, *icaA*, *icaC*, *icaD*
CC8/ST239-MRSA-III & SCC*Hg* (1)	I	8	D	*merA & merB, blaZ, erm*(A), *aacA-aphD, aphA3, sat, tet*(K), *tet*(M), *sdrM, fosB, qacC*	L461S (1)	*sea, sek, sak, scn*	*bbp, clfA, clfB, cna, ebh, ebpS, eno, fib, fnbA, fnbB, map, sdrC, sdrD, vwb, sasG, icaA, icaC, icaD*
CC30/ST30-MRSA-IV non-subtypable (4)	III	8	B	***Q6GD50 (fusC)***, *blaZ, fosB, sdrM* (75%)	.....	*egc*[c], *sak, chp, scn, tst*	*bbp* (75%), *clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *map*, *sdrC*, *sdrD*, *vwb*, *icaA*, *icaC*, *icaD*

Continued overleaf

Table 3.10 continued. Genotypes and antimicrobial resistance and virulence-associated genes detected using a DNA microarray[a] among the 110 fusidic acid resistant MRSA isolates recovered from patients in Irish hospitals between 1971 and 2010

CC/ST-SCC*mec* type (*n*)	Additional typing markers			Antimicrobial resistance genes (% indicated when not 100%)	*fusA* mutations for 36 isolates tested[d]	Virulence associated genes (% indicated when not 100%)	MSCRAMM, adhesion, & biofilm genes
	agr	*cap*	IEC (*n*)[b]				
CC1/ST1-MRSA-IV (1)	III	8	D	***Q6GD50 (fusC)***, *blaZ*, *erm*(C), *sdrM*	.....	*sea, seh, sek & seq, sak, scn*	*bbp, clfA, clfB, cna, ebh, ebpS, eno, fib, fnbA, fnbB, map, sdrC, sdrD, vwb, sasG, icaA, icaC, icaD*
CC45/ST45-MRSA-IV (5)	I (2) & IV (3)	5 (1) & 8 (4)	B	***farI (fusB)* (60%)**, *blaZ*, *erm*(C), *merA & merB* (60%), *ileS2* (20%), *tet*(M) (20%), *sdrM* (80%)	L461F (1)	*sec & sel* (20%), *egc*[c], *sak, chp, scn*	*bbp, clfA, clfB, cna, ebh* (80%), *ebpS, eno, fib, fnbA, map, sdrC, sdrD, vwb, icaA, icaC, icaD*
CC59/ST59-MRSA-V (1)	I	8	B	***farI (fusB)***, *blaZ, msrA, ileS2, sdrM, fosB*	.....	*seb, sek, seq, sak, chp & scn*	*bbp, clfA, clfB, cna, ebh, ebpS, eno, fib fnbA, map, sdrC, sdrD, vwb, icaA, icaC, icaD*
CC5/ST5-MRSA-II (2)	II	5	B (1) D (1)	***Q6GD50 (fusC)***, *blaZ*, *erm*(C), *aadD, sdrM, fosB*	L461S (1)	*sea, sec & sel, sed, egc* (*sei* 50%)[c], *tst, sak, chp* (50%), *scn*	*bbp, clfA, clfB, ebh, ebpS, eno, fib, fnbA, fnbB, map, sdrC, sdrD, vwb, sasG, icaA, icaC, icaD*

[a] The StaphyType Kit (Alere Technologies) was used for DNA microarray profiling.
[b] Immune evasion cluster (IEC) type as defined by van Wamel *et al.* (2006): A= *sea*, *sak*, *chp* and *scn*; B = *sak, chp* and *scn*; C = *chp* and *scn*; D = *sea, sak* and *scn;* E = *sak* and *scn*; F = *sep, sak, chp* and *scn*; G = *sep, sak* and *scn*; NT, novel IEC type consisting of *sak* and *sea* only; Neg (negative), no IEC genes detected.
[c] The enterotoxin gene cluster (*egc*) genes are seg, *sei, sem, sen, seo* and *seu;* numbers in parenthesis refer to the numbers of isolates positive for IEC types.Abbreviations: n, number of isolates; *agr*, genes encoding accessory gene regulators; *cap*, genes encoding capsular proteins; CC, clonal complex; ST, sequence type; MSCRAMM, microbial surface components recognising adhesive matrix molecules. [d]Numbers in parenthesis refer to the numbers of isolates underwent *fusA* mutation PCR detection; ....., no genes detected.

3.1.2.2.3 Antimicrobial resistance genes

Only 10% of isolates (11/110) were found to habour the fusidic acid resistance genes *far1* (*fusB*) (5/110, 4.5%) or *Q6GD50* (*fusC*) (5.4%; 6/110) (Table 3.10). All isolates harboured *mecA* and *blaZ* and the following resistance genes were also detected; *sdrM* (23%; 25/110); *fosB* (19%; 21/110), *merA* & *merB* (15%; 17/110), *aacA-aphD* (15%; 16/110), *aadD* (6%; 5/110), *aphA3* & *sat* (7%; 9/110), *erm*(A) (14%; 15/110), *erm*(C) (64%; 70/110), *tet*(K) (7%; 9/110), *tet*(M) (2%; 2/110), *lnu*(A) (2%; 2/110), *msrA* (1%; 1/110), *dfrS1* (4%; 4/110), *far1* (*fusB*) (4.5%; 5/110), *Q6GD50* (*fusC*) (5.4%; 6/110), *ileS2* (6%; 7/110), *qacA* (11%; 12/110), *qacC* (1%; 1/110) (Table 3.10).

3.1.2.3 Investigation of the mechanism of fusidic acid resistance among fusidic acid resistant isolates that did not harbour *fusB* or *fusC*

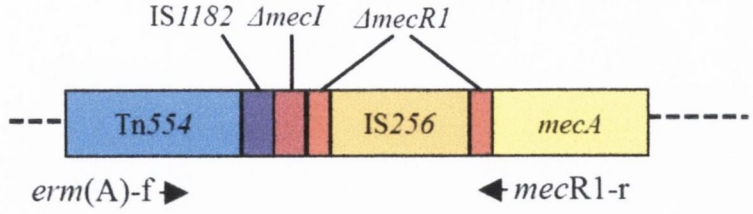
Only 10% of isolates (11/110) were found to habour the fusidic acid resistance genes *far1* (*fusB*) (4.5%; 5/110) among isolates belonging to ST59-MRSA-V, ST45-MRSA-IV, and ST8-MRSA-IIB or *Q6GD50* (*fusC*) (6/110, 5.4%) among isolates belonging to ST1-MRSA-IV, and ST30-MRSA-IV. Of the 110, twenty two isolates, representative of different AR-PFG type combinations recovered between 2007 and 2010 were selected were selected for further investigation of the possible mechanism(s) of fusidic acid resistance by PCR and sequencing of *fusA*. Of the 22 isolates, 21 isolates belonged to ST22-MRSA-IV from 2007-2010, with only one isolates belonging to ST45-MRSA-IV from 2009, previously described mutations in *fusA* were detected in 18 of the 22 fusidic acid resistant isolates (Table 3.10). Three different combinations of amino acid changes were detected (a) an amino acid substitution from leucine to lysine at position 461 in the protein sequence (L461K) in 72% (15/22) of isolates (ST22-

MRSA-IVh) (b) an amino acid substitution from leucine to serine at position 461 in the protein sequence (L461S) in one ST22-MRSA-IVh isolate and (c) an amino acid substitution from leucine to phenylalaine at position 461 in the protein sequence (L461F) in one ST45-MRSA-IV isolate and for the remaining 4 isolates (ST22-MRSA-IVh) no amino acid change was detected (Table 3.10).

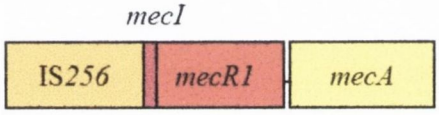
3.1.3 Molecular characterisation of a novel *mec* complex variant in ST8-MRSA-IIA-IIE and *spa* type t190, isolate 05540

Isolate 05540 underwent detailed SCC*mec* analysis due to the larger than expected size amplimer obtained using a *mec* complex multiplex PCR. Three individuals PCRs were performed using template DNA from this isolate and the primers for class A, B and C *mec* complexes. An unexpected size amplimer (ca. 3500 bp compared to the expected size of 1942 bp) was obtained using primers for the class A *mec* complex. Sequence analysis revealed a novel *mec* complex variant consisting of IS*256* within the entire *mecR1* of a class A.3 *mec* complex (Fig. 4.3). Class A.3 *mec* has previously been described in SCC*mec* IIC and IIE in ST8/*spa* type t190 isolates. This novel *mec* complex variant was tentatively designated class A.6 *mec* as it differed from A.3 due to the presence of IS*256* within the class A.3 *mec* complex (Fig. 3.2). Analysis of results obtained previously (Shore *et al.*, 2010) for typing of this SCC*mec* element revealed that is had most similarity to SCC*mec* IIE as it carried *ccrAB2* and *dcs*, but lacked pUB110 (differentiates IIE from IIC). Primers were also designed to amplify from Tn*544* to *mec*RI and a 7.5 kb amplimer was obtained, similar to what is expected from a SCC*mec* IIE element from Tn*554*. Schematic diagrams showing the structure of the SCC*mec* element from this isolate extending from Tn*554* to *mecA* together with an illustration of the novel class A.6 *mec* complex, and class A.3 *mec* complex and the

SCC*mec* IIE: 05540 novel class A.6 *mec* complex variant



class A *mec* complex with adjacent IS*256*



class A.3 *mec*

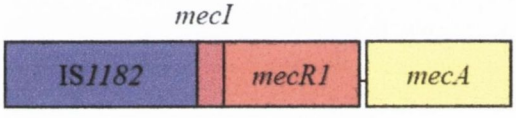


Fig. 3.2 Schematic diagram showing the genetic organisation of the novel class A.6 *mec* complex identified in ST8/t190-MRSA-IIE MRSA isolate 05540 and previously described class A *mec* complexes from SCC*mec* IIb (class A *mec* with adjacent IS*256,* Hisata *et al.*, 2005) and SCC*mec* IIC/IIE from ST8/t190 MRSA isolates (class A.3 *mec*, Shore *et al.*, 2005). An unexpected size amplimer (ca. 3500 bp compared to the expected size of 1942 bp) was obtained using primers mI6 (nucleotide coordinates 42866 bp- 42888 bp with *mec*I of the class A *mec* complex of SCC*mec* II, Genbank accession number D86934) and mA7 (nucleotide coordinates 44830 bp-44808 bp within *mec*A of the class A *mec* complex of SCC*mec* II Genbank accession number D86934) (Kondo *et al.*, 2007). The region upstream of the *mec* complex gene *mecR1*, extending from *mecR1* to Tn*554*, was also amplified using primer walking and sequenced using PCR using primers *erm*(A)-f based on the Tn*554* of (SCC*mec* II Genbank accession number D86934) and *mec*RI-r based on *mec*RI sequence obtained from mI6 and mA7 reaction.

class A *mec* complex from SCC*mec* IIb, which is the only previously described *mec* complex with IS*256* (Hisata *et al.*, 2005), are shown in Fig. 3.2.

3.4 Discussion

3.4.1 DNA microarray profiling of a diverse collection of nosocomial methicillin-resistant *Staphylococcus aureus* isolates assigns the majority to the correct sequence type and staphylococcal cassette chromosome *mec* (SCC*mec*) type

The application of *spa* typing and DNA microarray profiling permitted extensive molecular characterisation of a diverse collection of nosocomial MRSA isolates representative of the predominating strains in Irish hospitals between 1971 and 2004 and revealed extensive genetic diversity among the 175 isolates investigated. A total of 35 *spa* types were identified among these 175 isolates which were previously assigned to 13 STs representing six CCs by MLST. Isolates were assigned to 26 ST-SCC*mec* type combinations representative of the main pandemic and epidemic MRSA lineages by MLST and SCC*mec* typing. The greatest numbers of *spa* types (46%, 16/35) were exhibited by ST22-MRSA-IV isolates which represented only 15% of isolates investigated (27/175). In contrast, within the majority of other clonal lineages, isolates were assigned to one or two *spa* types only. In particular, the majority of ST8 isolates (42% of isolates investigated) were assigned to a single *spa* type (t190), suggesting that the *spa* types of ST8 isolates are more stable over time than those among ST22-MRSA-IV isolates. This finding was also reported by Strommenger *et al.* (2006) who described isolates within different CC8 clones recovered from different geographic regions at different time periods with the same *spa* types (Strommenger *et al.*, 2006). A possible explanation for the greater variation in *spa* types among ST22-MRSA-IV isolates may lie in the higher number of repeat sequences in the VNTR region of the *spa* gene in this

clone. The average number of repeats in the 16 ST22 *spa* types in the present study was 13 (range, 4 to 17), with the most frequently occurring *spa* type (t032) carrying 16 repeats. In contrast, the *spa* type t190 to which most ST8-MRSA-IIA-IIE and ST8-MRSA-IVE/IVF isolates were assigned and consisted of only seven repeats.

DNA microarray analysis allowed for effective genotyping of MRSA isolates by assigning isolates to the correct CCs and/or STs and SCC*mec* types, indicating that this technology has the potential to replace MLST and SCC*mec* typing for genotyping and for investigating the evolutionary relatedness of MRSA. The advantages of using the DNA microarray for genotyping MRSA includes the fact that the DNA microarray also detects clinically relevant virulence and antimicrobial resistance genes and only requires a single multiplex PCR with a 2- to 3- h test procedure per isolate to generate all of the above data that would require multiple multiplex PCRs and agarose gel electrophoresis to assign an SCC*mec* type/subtype and commercial sequencing of 7 housekeeping genes to assign an ST by conventional methods. In a hospital setting, and particularly in an outbreak investigation, greater discrimination than that provided by ST and SCC*mec* type assignment is required to effectively type and track MRSA. While each of the MRSA clones in the present study was found to have a characteristic combination of antimicrobial and virulence genes, there were also differences observed in the carriage of these genes within the clones e.g., the carriage of *sec*, *sel*, *erm*(C), and IEC were variable in ST22-MRSA-IV. Whether these differences could be used to provide enhanced discrimination and tracking of MRSA in hospitals either using the array as a standalone typing tool or in combination with other methods, such as *spa* typing was investigated and is described in Chapter 4.

It is important to note that the microarray encountered difficulties differentiating some sporadically occurring STs that are single locus variants of more common STs and

with subtyping of some SCC*mec* elements. While the identification of some SCC*mec* I, II, and IV subtypes was possible following manual inspection of array profiles, in some cases the assignment relied on the presence of a particular resistance determinant associated with a particular SCC*mec* type, such as *aadD* located on plasmid pUB110 in SCC*mec* types IA, IIA, IIB, IIC and IVA. However, while the presence of an SCC*mec*-associated resistance determinants may aid the identification of a particular SCC*mec* subtype, i.e., *erm*(A), *tet*(K), and *mer*, which can be located on Tn*554* in SCC*mec* II and III, pT181 in SCC*mec* III, and p1258 in SCC*mec* III with SCC*Hg*, respectively, the location of any of these resistance determinants to within the SCC*mec* element would need to be confirmed with PCR. Array profiling facilitated the identification of possible DNA sequence variation in *mecR1* in some SCC*mec* III elements and in the *dcs* gene in some SCC*mec* II and IV elements that were not detected by conventional SCC*mec* typing. In addition, the fusidic acid resistance determinant Q6GD50/*fusC* was detected in four ST30-MRSA-IV isolates using the array, indicating the possible presence of a SCC*fus* element in these isolates.

Array profiling also indicated that five isolates harbouring SCC*mec* IV may have lost *ccrAB2*, which was subsequently confirmed by PCR. Four of these isolates were assigned to ST8-MRSA-IVE/F, and also harboured a second set of *ccr* genes (*ccrAB4*) outside SCC*mec*, suggesting that the loss of the *ccrAB2* genes in these isolates was not essential for excision or integration of the SCC*mec* element due to the presence of secondary *ccr* genes. The ability to accurately identify SCC*mec* types is essential to the use of this method for genotyping MRSA, and isolates such as these that may have lost *ccr* genes may be wrongly classified using SCC*mec* typing because of the presence of the second set of *ccr* genes outside the SCC*mec* element.

DNA microarray profiling allowed for the detection of characteristic combinations of antimicrobial resistance and virulence genes among major MRSA clones. Only nine percent of isolates investigated lacked the bacteriophage-encoded IEC genes, and half of these belonged to ST22-MRSA-IV. Ninety-four percent of isolates investigated carried one or more superantigen gene. The ST22-MRSA-IV isolates were the only isolates to harbour *sec* and *sel*, which have been reported previously to be co-located on a pathogenicity island (Malachowa and DeLeo, 2010). The toxic shock toxin *tst* gene, which has also been found to be located on various pathogenicity islands (Malachowa and DeLeo, 2010), was identified in CC30 and CC5 isolates only. The enterotoxin genes *sek* and *seq* were identified only in the CC8 lineages ST239 and ST250 and have been reported to be co-located on various pathogenicity islands and bacteriophages, while *seb*, which has also been found to be located either on a pathogenicity island with *sek* and *seq* or on a plasmid, was also detected in ST250 isolates (Malachowa and DeLeo, 2010). Isolates belonging to ST22-MRSA-IV harboured the fewest antimicrobial resistance genes, with approximately half of the isolates harbouring *blaZ* only. In contrast, isolates belonging to CC8 lineages (ST8-MRSA-IIA-E and ST239-MRSA-III/III-pI258/Tn*554*) harboured the greatest number of antimicrobial resistance genes (up to 17 in each case).

The present study revealed that the presence of a particular antimicrobial resistance gene correlated with phenotypic resistance to a specific type of antimicrobial agent(s), but the absence of the gene was not always indicative of susceptibility. For example, the presence of genes such as *cat* (chloramphenicol resistance), *erm*(A) and/or *erm*(C) (encoding MLS resistance), *qacA* and/or *qacC* (ethidium bromide resistance), *far1/fusB* or *Q6GD50/fusC* (fusidic acid resistance), *merA* and *merB* (mercuric chloride and phenylmercuric acetate resistance), *ileS2* (high-level mupirocin resistance), *tet*(K)

and/or *tet*(M) (tetracycline resistance) and *dfrS1* (trimethoprim resistance) almost always correlated with phenotypic resistance to the associated antimicrobial agent(s). However, some isolates exhibited a particular resistance phenotype where no corresponding resistance gene was detected using the DNA microarray. Mutations were identified in the *fusA* gene in the 15 fusidic acid-resistant isolates that lacked *far1/fusB* or *Q6GD50/fusC*. Only 35% of trimethoprim-resistant isolates (9/26) were found to harbour *dfrS1* by DNA microarray profiling and while 11% (3/29) were subsequently shown to carry *dfrG* (ST239 isolates), none of the previously reported trimethoprim resistance genes were detected in the remaining 14 trimethoprim-resistant isolates. Similarly, one isolate exhibited resistance to mercury and two isolates exhibited moderate resistance to tetracycline but none of the associated resistance genes were detected using the DNA microarray. This suggests the possible presence of alternative or novel trimethoprim, mercury and tetracycline resistance genes and/or resistance mechanisms in these isolates. This highlights the fact that currently array profiling cannot replace antimicrobial susceptibility testing but can be used in conjunction with it to help identify the mechanism of resistance. The inclusion of alternative or novel antimicrobial resistance genes on the array such as tetracycline [*tet*(L) and *tet*(O)], trimethoprim (*dfrG* and *dfrK*) and fusidic acid (*fusD*) resistance genes would greatly enhance the ability of the array to predict a resistance phenotype.

The presence of aminoglycoside resistance gene(s) detected by array profiling could be used to accurately predict an isolate's phenotypic aminoglycoside resistance pattern. However, the phenotypic expression of aminoglycoside resistance could not always be used to infer which aminoglycoside resistance gene(s) was present. This was partly due to the fact that different aminoglycoside resistance genes encode resistance to the same antimicrobial agents and also because approximately half of the

aminoglycoside resistant isolates (52%, 43/83), carried two or more aminoglycoside resistance genes. For example, all isolates found to harbour a single aminoglycoside resistance gene exhibited resistance to the expected combinations of aminoglycoside and all isolates exhibiting these phenotypes harboured the corresponding resistance genes (Table 3.9). However, for isolates harbouring two or three aminoglycoside resistance genes, the phenotype could be predicted from the aminoglycoside resistance genes detected but the presence of a gene could not be reliably inferred from the phenotype. An advantage of the use of the array was confirmation of reduced susceptibility to amikacin in isolates carrying the *aacA-aphD* gene (Lelievre *et al.*, 1999) (Table 3.9). This gene encodes low-level amikacin resistance that may not be detected using the standard 30 µg susceptibility testing disk. Among 14 isolates carrying *aacA-aphD* only, nine showed reduced amikacin susceptibility; the remainder were phenotypically susceptible. Inclusion on the array of genes encoding resistance to the aminocyclitol spectinomycin including *spc* (carried on Tn*554*) and *aadA* would be helpful to further differentiate among this MRSA population (Lyon and Skurray, 1987). The addition of the Tn*554*-encoded *spc* gene would aid differentiation of SCC*mec* IIA and IIB and the addition of the *apmA* gene conferring resistance to apramycin and gentamicin (Fessler *et al.*, 2011b) and the recently described *mecC* gene (Shore *et al.*, 2011a) would further enhance the usefulness of the array.

In conclusion, the present study showed that DNA microarray profiling can accurately assign isolates representative of major pandemic MRSA clones to the correct MLST and SCC*mec* types. Microarray profiling allows rapid, high-throughput genotyping and detection of clinically relevant staphylococcal virulence and antimicrobial resistance genes, and can, in most cases, be used to predict the antimicrobial agent resistance phenotype of an isolate.

3.4.2 Molecular characterisation of fusidic acid resistant MRSA isolates recovered in Ireland reveals three different mechanisms of fusidic acid resistance

Of the 110 fusidic acid resistant MRSA isolates investigated, only 10% harboured either *fusB* (4.5%; 5/110) or *fusC* (5.4%; 6/110), but interestingly the *fusC*- and *fusB*-positive isolates belonged to several different genotypes, namely ST59-MRSA-V, CC45-MRSA-IV, ST8-MRSA-IIB, ST5-MRSA-I, CC1-MRSA-IV and ST30-MRSA-IV. Of the ninety-nine isolates that did not harbour *fusB* or *fusC,* 36 isolates representative of different ST and AR type combinations were investigated further and 89% (32/36) were found to have amino acid changes in *fusA*. The majority of these were ST22-MRSA-IV (47%; 17/36). More than 30 *fusA* amino acid substitutions have been described to date (Lannergård *et al.*, 2009), but it is important to note that not all of these have been investigated using functional studies *in vitro* to demonstrate a link with fusidic acid resistance. Five different *fusA* amino acid changes were identified among fusidic acid resistant MRSA in the present study, the most common being L461K, which was detected in 56% (20/36) of isolates, followed by the L461S, which was detected in 17% (6/36) of isolates. The L461K amino acid change, is common among different lineages of *S. aureus* and is associated with a higher fusidic acid MIC (>256 mg/L), than the L461S amino acid change which is associated with a lower fusidic acid MIC (8 mg/L) (Besier *et al.*, 2003). However, no fusidic acid MICs were determined in the present study. The three remaining *fusA* amino acid changes in the present study, were A70V, which has previously been reported among MSSA in Europe (Castanheira *et al.*, 2010b), L461F, which was identified in a previous study among ST239-MRSA-III isolates in Taiwan (Chen *et al.*, 2010b) and F406K, which has not been reported previously. No amino acid change was detected in four ST22-MRSA-IV fusidic acid resistant isolates tested and two amino acid changes were identified

(A70V & F406K) in the fusidic acid resistant ST8-MRSA-IIB isolate investigated. Isolates belonging to ST8-MRSA-IIB-IID, ST45-MRSA-IV and ST5-MRSA-IV harboured *fusB* or *fusC* together with amino acid changes.

A range of fusidic acid resistance mechanisms have previously been reported among *S. aureus* isolates but only a limited number of studies have involved MRSA. Among these studies of MRSA some have reported *fusA* mutations as the predominant mechanism of fusidic acid resistance while others have reported *fusB* or *fusC* as the predominant mechanism (Chen *et al.*, 2010b; Chen *et al.*, 2011; McLaws *et al.*, 2011). In addition, some of these studies also identified different mechanisms of fusidic acid resistance among isolates of the same clone, with a small number of isolates harbouring both *fusA* mutations and either *fusB* or *fusC* (Chen *et al.*, 2011; McLaws *et al.*, 2011). Very few studies have characterised the MGEs carrying *fusB* and *fusC*, the former being reported previously solely on a plasmid and the latter on SCC and SCC*mec* elements (O'Brien *et al.*, 2002; Holden *et al.*, 2004; Yazdankhah *et al.*, 2006; Ender *et al.*, 2007; Kinnevey *et al.*, 2012). It remains to be determined if the *fusB* and *fusC* resistance determinants found in the current study are on the same or different genetic elements to those which have been previously described.

The majority of reports of the mechanism of fusidic acid resistance among *S. aureus* from Ireland, UK and Europe have involved MSSA. For example, a 2008 European study of fusidic acid resistant MSSA isolates from patients with SSTIs in France, the UK and Ireland found that *fusB* was the most common fusidic acid resistance determinant among Irish MSSA from diverse clonal lineages including CC121, CC8, CC5, CC1, CC15, CC22 and CC80 (Larsen *et al.*, 2008). This is in contrast to the present study, where mutations in *fusA* were the most common mechanism among MRSA. In 2010, a study involving 13 European countries including

Ireland, the UK, France, Germany, Greece, and Italy, investigated *S. aureus* isolates from BSIs, respiratory tract infections and SSTIs for the mechanism of fusidic acid resistance (Castanheira *et al.*, 2010b). The study revealed a higher prevalence of fusidic acid resistance among MSSA from Greece (62.4%) and Ireland (19.9%), compared to other countries. Similar to the MRSA isolates in the present study, the majority of Irish MSSA isolates investigated in the 2010 European study were found to harbour *fusA* mutations and the amino acid change L461K with just 6% of isolates harbouring the *fusB* or *fusC*.

In the present study, no mechanism of fusidic acid resistance was identified in 79% of the fusidic acid resistant isolates investigated (all ST22-MRSA-IV). However these isolates were not tested for *fusE* mutations i.e. mutations within the L6 ribosomal located gene *rplF*, due to the fact that this mutation has only been reported previously in small colony variants (Norstrom *et al.*, 2007). These isolates were also not tested for the presence of *fusD*, which has only been reported in *S. saprophyticus* (O'Neill *et al.*, 2007). It has been suggested by McLaw *et al.* (2011), who identified two fusidic acid resistant MRSA isolates lacking *fusB*, *fusC* and *fusD* as well as *fusA* and *fusE* mutations, that it is possible that there are other mechanisms or additional resistance determinants yet to be identified in *S. aureus* that confer fusidic acid resistance (McLaws *et al.*, 2011). The mechanism(s) of fusidic acid resistance in the ST22-MRSA-IV isolates in the present study warrant further investigation.

A previous study of fusidic acid resistant MSSA from SSTIs in Ireland reported the presence of exfoliative toxin genes among isolates of a particular clone (Larsen *et al.*, 2008). No exfoliative genes were detected among the fusidic acid MRSA isolates in the present study and overall, no virulence or resistance gene was common to all isolates. However, a range of virulence and antimicrobial resistance genes were

identified among the isolates including enterotoxin genes, IEC genes and the toxic shock toxin gene as well as genes encoding resistance to macrolides, aminoglycosides and quaternary ammonium compounds. Resistance to these additional antimicrobial agents could further compromise our ability to treat infections caused by fusidic acid resistant MRSA and the presence of additional virulence determinants could enhance the pathogenicity of these isolates.

In conclusion, the present study found that the most common mechanism of fusidic acid resistance identified among the MRSA isolates investigated, particularly among ST22-MRSA-IV, was mutations in *fusA*. It is possible that the high prevalence of *fusA* mutations among Irish MRSA isolates, in particular among ST22-MRSA-IV, is caused by the over use and misuse of fusidic acid as a topical antimicrobial agent, which has selected for resistant strains. This requires close monitoring and a more prudent use of this antimicrobial agent. While the MGE-located *fusB* and *fusC* genes were only detected among a small number of MRSA isolates, these belonged to six different lineages, indicating that the acquisition of these genes, possibly from CoNS, occurred on multiple independent occasions and the potential for these genes to spread. CoNS could act as a possible reservoir for the fusidic acid resistance genes *fusB* and *fusC*. A study carried out in 2010, reported that *fusB* is more prevalent among CoNS (65%) than *S. aureus* (17.4%), whereas *fusC* was detected at similar rates in both CoNS (29.0%) and *S. aureus* (32.6%) in North America, Canada, and Australia (Castanheira *et al.*, 2010a). Similarity within Europe, fusidic acid resistance rates were higher among CoNS, with *fusB* (26.5%) being the predominant fusidic acid gene, followed by *fusC* (11.3%) (Castanheira *et al.*, 2010b). Several species of staphylococci have been reported to harbour the *fusB*-pUB101 plasmid located gene including *S. epidermidis* (78%; 18/23) (McLaws *et al.*, 2008), *Staphylococcus lugdunensis* (100%; 4/4) (O'Neill

et al., 2007). However *fusB*- pUB101 plasmid located in *S. haemolyticus* (33%; 1/3) with the remaining *fusB* being chromosomal located (66%; 2/3) (Yazdankhah *et al.*, 2006).

A detailed investigation of additional MRSA isolates belonging to lineages apart from ST22-MRSA-IV would further enhance our understanding of the mechanism of fusidic acid resistance among MRSA. In addition, characterisation of the MGEs carried by these isolates would augment our understanding of the evolution of these strains.

3.4.3 Identification of a novel *mec* complex variant

Finally, this part of the present study also resulted in the identification of a novel *mec* complex variant in a ST8/*spa* type t190 MRSA isolates and a novel SCC*mec* subtype similar to SCC*mec* IIE previously identified among ST8/t190 MRSA isolates from Ireland (Shore *et al.*, 2005; Shore *et al.*, 2008). The newly identified *mec* complex differed from class A.3 *mec* due to the presence of IS*256* within the *mec* regulatory gene *mecR1*. Five different class A *mec* complexes have been reported to date including (a) the prototype class A *mec* complex consisting of *mecA* and complete *mecI* and *mecR1* genes as well as a IS*431* element (Katayama *et al.*, 2001); (b) a class A *mec* complex, similar to the prototype class A *mec* complex but flanked by two IS*431* elements (Katayama *et al.*, 2001); (c) class A.3 *mec* complex with IS*1182* inserted within *mecI*(Shore *et al.*, 2005); (d) class A.4 *mec*, similar to A.3 but with a large deletion of *mecI* (Shore *et al.*, 2005); (e) the class A *mec* complex described by Hisata *et al* (2005) similar to the prototype class A *mec* complex but has a IS*256* element located upstream of *mecI*. Because the class A *mec* complex variant described in the present study is the sixth described to date, it was tentatively designated class A.6 *mec* complex.

The insertion sequence IS*256* has previously been reported adjacent to *mecI* in the *mec* complex of a SCC*mec* IIb element (Hisata *et al.*, 2005) but has not been previously been reported within *mecR1* (Fig. 3.2). The SCC*mec* IIb element is the predominant SCC*mec* type amongst MRSA from healthy children from various regions of Japan and in these isolates IS*256* is inserted upstream of *mecI* and downstream of the integrated transposon Tn*554* (Hisata *et al.*, 2005). Insertion sequences can be found at multiple sites on the *S. aureus* chromosome, on different plasmids and flanking transposons encoding antimicrobial resistance genes (Lyon and Skurray, 1987; Bennett, 2008). IS*256* has been reported to flank both sides of Tn*4001*, which aid in the transcription of the adjacent genes. These insertion sequences aid in the intraspecific transfer by a homologous recombination process of antibiotic resistance determinants in a new genetic location (Lyon *et al.*, 1987; Lyon and Skurray, 1987). This isolate (05540) also harboured *ccrAB* which has been previously reported as part of a novel SCC*mec*-SCC-CI known as SCC_{M1}. This SCC_{M1} element has been recently reported among five MRSA isolates (ST8-MRSA-IIA, ST8-MRSAIIB, ST8-MRSA-IIC, ST8-MRSA-IID, and ST94-MRSA-IVg) (Shore *et al.*, 2012). The SCC_{M1} has only been identified in MRSA belonging to CC8 (Bartels *et al.*, 2011; Coombs *et al.*, 2011; Zhang *et al.*, 2009). Bartels *et al.* 2011 reported the SCC_{M1} element in an ST8-MRSA which has DNA sequence identity to the SCC-CI in *S. epidermidis* ATCC 12228. He suggested that this element evolved from the recombination events between SCC*mec* element and SCC-CI elements in MRSA and *S. epidermidis* (Bartels *et al.*, 2011). This novel class A.6 *mec* complex variant could not be identified by routine multiplex SCC*mec* typing PCR as it resulted in an unexpected size amplimer. Simplex SCC*mec* PCR detected it was a class A *mec* complex but complete nucleotide sequencing was required to fully characterise this novel *mec* complex variant. The emergence of

diversity among SCC*mec* and SCC elements in *S. aureus* and other staphylococcal poses as a challenge to current SCC*mec* typing such as DNA microarray profiling and conventional SCC*mec* typing PCRs. Therefore routine typing methods need to be constantly updated and there is a necessity for a more robust SCC*mec* typing method as SCC*mec* typing is essential for genotyping MRSA isolates and for MRSA strain differentiation.

Chapter 4

Enhanced discrimination of highly-clonal ST22-MRSA-IV isolates achieved by combining DNA microarray analysis and *spa* typing data

4.1 Introduction

Since MRSA first emerged in Irish hospitals in 1971 (Hone and Keane, 1974) and became endemic in the 1980s, different clones have emerged and predominated during different time periods. ST22-MRSA-IV (known locally as AR06-PFG-01), a major pandemic MRSA clone similar to the UK epidemic strain EMRSA-15, has predominated in Irish hospitals since 2002, where it accounts for approximately 80% of MRSA isolates recovered from BSIs each year (Johnson *et al.*, 2005; Shore *et al.*, 2005; Shore *et al.*, 2010; NMRSARL and report., 2011) (Fig. 4.1). Due to the endemic nature and limited genetic diversity of ST22-MRSA-IV in Irish hospitals, tracking and controlling the spread of this clone is particularly difficult using many commonly used typing methods (Tenover *et al.*, 2006; Goering *et al.*, 2008). In a recent Irish study, ST22-MRSA-IV isolates yielded indistinguishable or closely related PFGE patterns, designated as PFG-01, exhibited antibiogram-resistogram (AR) type AR06 or closely related AR patterns (Rossney *et al.*, 2006) and exhibited 17 *dru* types and 17 *spa* types with the majority of isolates (55.4%), belonging to *spa* type t032 (Shore *et al.*, 2010). Difficulties in sub-typing highly clonal ST22-MRSA-IV MRSA isolates have been also reported in other European studies (Witte *et al.*, 2001; Strommenger *et al.*, 2006; Ghebremedhin *et al.*, 2007; Goering *et al.*, 2008; Khandavilli *et al.*, 2009; Soliman *et al.*, 2009).

A combination of PFGE and the sequence based *spa* and *dru* typing methods have been shown previously to enhance the discrimination of ST22-MRSA-IV isolates (Shore *et al.*, 2010). When all three methods were combined it resulted in the highest discriminatory power (Simpsons index of diversity (SID) of 96.53% versus 81.34%, 66.90%, and 77.83%, for each of the individual methods PFGE, *spa* and *dru* typing) for the ST22-MRSA-IV isolates investigated. However, some of the differences between

the various type combinations were due to changes consistent with a single genetic event, so in order not to over interpret the findings such isolates were deemed to very closely related, and assigned to the same dendrogram group (DG) if they shared ≥ 98.5% similarity, while those with ≤ 98.5% similarity were deemed to be distinguishable and were assigned to different DGs (Shore *et al.*, 2010) (Fig. 4.2).

While Shore *et al.*, (2010) found that enhanced tracking of ST22-MRSA-IV was achieved using the combination of PFGE, *spa* and *dru* typing, on a routine basis such detailed typing may not be practical. The use of PFGE is time consuming, technically demanding, requires specialised equipment and analysis of banding patterns can be difficult and subjective even with the use of dedicated software programs. In addition, the routine use of PFGE has been replaced in many laboratories by sequence based typing methods in particular *spa* typing, which is becoming the most widely used typing method for *S. aureus* (Grundmann *et al.*, 2010). The use of *dru* typing is not as common as *spa* typing for MRSA, and can result in isolates from different lineages being assigned to the same *dru* type (Goering *et al.*, 2008; Larsen *et al.*, 2009; Smyth *et al.*, 2010). While *spa* typing is less discriminatory than PFGE, but more discriminatory than MLST, it is based on sequencing of a variable repeat region within the protein A gene *spa*, which forms part of the core genome of *S. aureus*. In many instances the MLST of an isolate can be accurately inferred from the *spa* type. However, with ST22-MRSA-IV, *spa* typing alone does not offer sufficient discrimination, particularly in an endemic setting.

Another molecular method used for typing of *S. aureus* is multilocus variable number tandem repeat (VNTR) fingerprinting (MLVF) (Sabat *et al.*, 2003) and multilocus VNTR analysis (MLVA) (Holmes *et al.*, 2010). Holmes *et al.* reported that MLVF was more discriminatory than MLVA but comparable to PFGE for

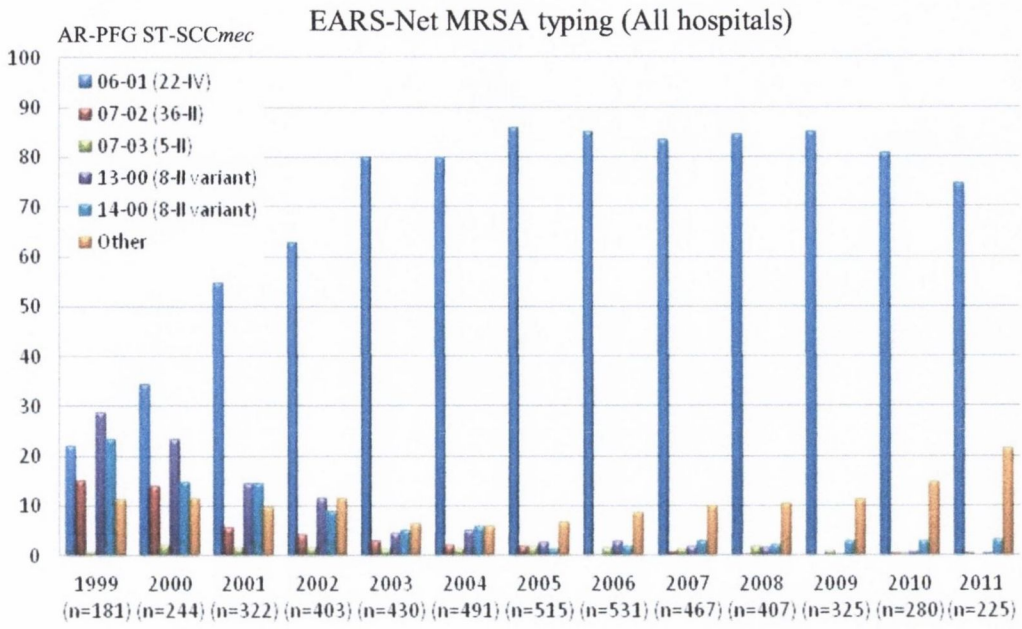


Fig. 4.1 Trends for the different AR-PFG and ST-SCC*mec* types identified among MRSA from Irish hospitals collected by the NMRSARL from 1999 to 2011. Adapted from the NMRSARL 2011 annual report.

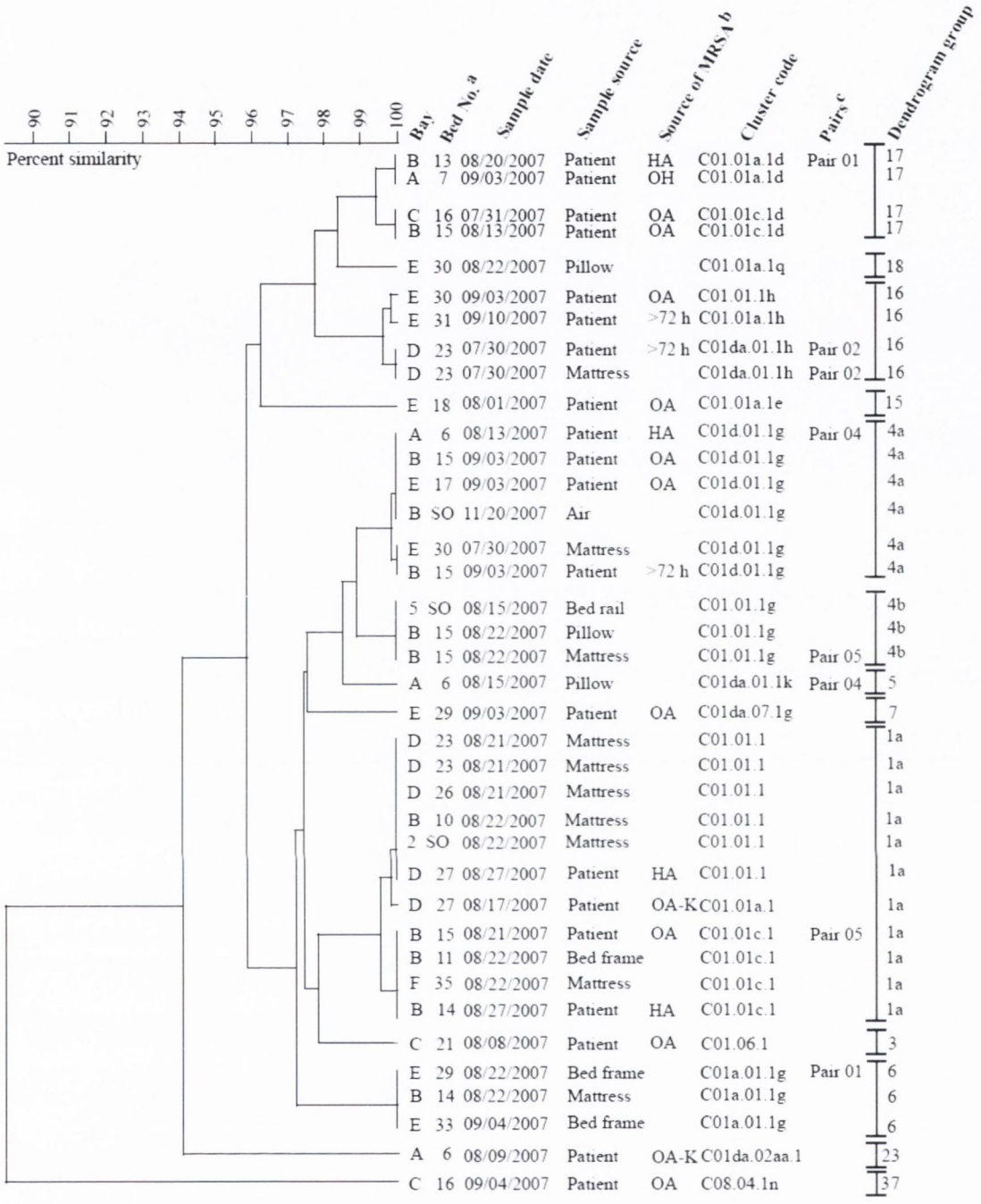


Fig. 4.2a. Composite dendrogram generated using UPGMA clustering and the averages of the similarity matrices from *spa*, *dru* and PFGE typing for 38 PFG-01 MRSA isolates investigated during Study Period I. Available epidemiological information for each isolate, as well as cluster codes and dendrogram groups (DGs) are indicated. DGs were assigned to the different cluster codes determined from the dendrogram depicting all PFG-01 isolates identified as part of a previous study (Shore *et al.*, 2010). The dendrogram shows that the PFG-01 isolates from Study Period I (n=38) were into 13 DGs, respectively. [a] The abbreviation 'SO' instead of a bed number indicates a single occupancy room. [b] HA, hospital-acquired; OA, MRSA-positive on admission; OA-K, MRSA-positive status known at time of admission to ward; > 72 h, MRSA status determined 72 h after admission to ward. [c] The pairs or triplets of isolates recovered during Study Periods I and II are indicated and represent isolates recovered from different patients and from their immediate environment during the same study period. Each pair consists of one isolates from a patient and at least one environmental isolate. Adapted from Shore *et al.* (2010).

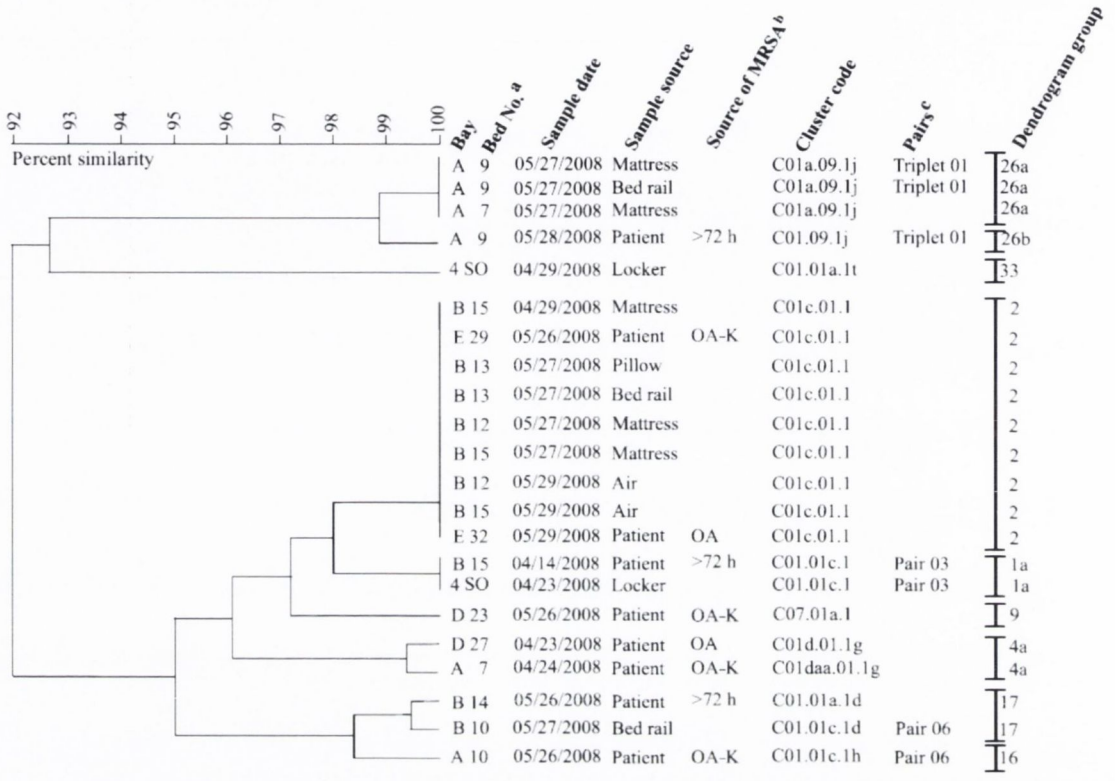


Fig. 4.2b. Composite dendrogram generated using UPGMA clustering and the averages of the similarity matrices from *spa*, *dru* and PFGE typing for 22 PFG-01 MRSA isolates investigated during Study Period II. Available epidemiological information for each isolate, as well as cluster codes and dendrogram groups (DGs) are indicated. DGs were assigned to the different cluster codes determined from the dendrogram depicting all PFG-01 isolates identified as part of a previous study (Shore *et al.*, 2010). The dendrogram shows that the PFG-01 isolates from Study period II (n=22) were into eight DGs, respectively. [a] The abbreviation 'SO' instead of a bed number indicates a single occupancy room. [b] HA, hospital-acquired; OA, MRSA-positive on admission; OA-K, MRSA-positive status known at time of admission to ward; > 72 h, MRSA status determined 72 h after admission to ward. [c] The pairs or triplets of isolates recovered during Study Periods I and II are indicated and represent isolates recovered from different patients and from their immediate environment during the same study period. Each pair consists of one isolates from a patient and at least one environmental isolate. Adapted from Shore *et al.* (2010).

differentiating EMRSA-15 (ST22-MRSA-IV) (Holmes *et al.*, 2010). MLVA is used to genotype MRSA based on the length of tandem DNA repeats present in genes such as *clfA*, *clfB*, *sdrC*, *sdrD*, *sdrE*, *sspA*, *spa*, *mecA*, *fnbA*, *fnbB*, and *can* (Lindstedt, 2005; Vergnaud and Pourcel, 2009). However, the traditional MLVA scheme relies on the sizing of PCR products separated by agarose gel electrophoresis making it difficult to interpret. Recent improvements to the MLVA scheme has seen the introduction of MLVA assays such as MLVA-8, MLVA-14, and MLVA-16 based on multiplex PCR amplimer profiles resolved by capillary electrophoresis (Pourcel *et al.*, 2009; Schouls *et al.*, 2009; Sobral *et al.*, 2012). A recent report of the improved MLVA-14 assay for typing MRSA was shown to have a higher discriminatory power than MLST and *spa* typing (Pourcel *et al.*, 2009), whereas the MLVA-16 assay was shown to assign animal and human *S. aureus* isolates to the same clonal complex as MLST (Sobral *et al.*, 2012).

In recent years, single nucleotide polymorphism (SNP) analysis of whole bacterial genomes or whole genome sequencing (WGS) has been shown to be useful for typing of MRSA isolates (Harris *et al.*, 2010; Köser *et al.*, 2012a). It has recently been described as a rapid, and accurate method for tracking MRSA isolates in an outbreak investigation in a UK hospital and the authors of that study indicated that the WGS of the four isolates took one day, data analysis took less than one hour, and the cost was less than £95 per MRSA isolate (Harris *et al.*, 2012). While this technology has become cheaper in recent years, commercial WGS for SNP analysis for most laboratories is still costly and the data analysis can take several weeks or months. A recent study applied a TaqMan real time PCR assay on three SNPs to differentiate among ST125 MRSA isolates resulting in 98% typeability (Nubel *et al.*, 2012).

The Staphy-Type DNA microarray assay (Alere) has recently been shown to accurately assign MRSA isolates to MLST and SCC*mec* types and to detect additional

typing and species-specific markers and an extensive range of antimicrobial resistance and virulence-associated genes (Monecke *et al.*, 2008b; Monecke *et al.*, 2008a; Monecke *et al.*, 2011b; Shore *et al.*, 2012). However, its usefulness for differentiating between isolates of a particular clone based on the presence of absence of antimicrobial resistance and virulence-associated genes has not been investigated. Since many of these genes are located on MGEs the use of microarray profiling in combination with a typing method such as *spa* typing could potentially provide enhanced and accurate discrimination of MRSA.

4.1.1 Aims

In order to effectively track the spread of the ST22-MRSA-IV clone currently endemic in Irish hospitals, and ultimately to inform infection prevention and control procedures that can prevent its spread, there is a need for alternative, high-throughput, rapid and accurate approaches that can be used for typing of this clone.

The objectives of this part of the present study were to:

- Investigate the usefulness of DNA microarray profiling in combination with *spa* typing for differentiating between ST22-MRSA-IV isolates from Irish hospitals to facilitate epidemiological tracking of this MRSA clone.
- Investigate the stability of DNA microarray profiles, *spa* types and *dru* types of ST22-MRSA-IV clinical isolates recovered sequentially from the same patients over time periods at Beaumont hospital.
- Investigate the stability of DNA microarray profiles, *spa* and *dru* types of two ST22-MRSA-IV isolates recovered from one patient and one environment site and their derivatives recovered following repeated sub-culturing at elevated temperatures and following freezing and thawing.

4.2 Materials and Methods

4.2.1 Investigation of the usefulness of the combination of DNA microarray profiling and *spa* typing for enhanced discrimination of ST22-MRSA-IV

4.2.1.1 MRSA isolates and experimental design

A total of 168 ST22-MRSA-IV isolates recovered from patients and environmental sites in four wards at Beaumont Hospital (BH) Dublin were investigated using a DNA microarray. The isolates were recovered over two six-week study periods between May 2007 and September 2008 as part of a previously described study (Shore *et al.*, 2010). All isolates had previously underwent AR, PFGE, *spa*, *dru* and SCC*mec* typing and had been assigned to dendrogram groups (Table 4.1). In the previous study isolates were either assigned to ST22-MRSA-IV by MLST or the ST was inferred from the *spa* type (Table 4.1) (Shore *et al.*, 2010). In the present study all isolates underwent DNA microarray profiling and this was combined with the *spa* types to enhance differentiation of isolates. The inferences drawn from the combined DNA microarray and *spa* typing data were confirmed with epidemiological evidence from a pilot study in one ward and were compared to the inferences drawn from the previously published dendrogram groupings (Fig. 4.2) (Shore *et al.*, 2010).

4.2.1.2 DNA microarray profiling

The Staphy-Type kit (Alere) was used for DNA microarray profiling of isolates according to the manufacturer's instructions, which have been described in detail in Chapter 2, section 2.4.5. The StaphyType kit consists of individual DNA microarray chips adhered to each well of an 8-well microtiter strip. Each chip detects 334 *S. aureus* target genes and alleles including species-specific and typing markers, antimicrobial resistance and virulence-associated genes and genes involved in adhesion, attachment

and biofilm formation. The array also allows accurate assignment of *S. aureus* isolates to a ST and SCC*mec* type (Chapter 2, section 2.4.5).

4.2.1.3. Assignment of isolates to *spa* type and microarray group (MG) combinations

Each isolate had previously been assigned a *spa* type as part of a previous study (Shore *et al.*, 2010). In the present study isolates within each *spa* type were assigned a microarray group (MG) number. Each MG had a characteristic combination of virulence and antimicrobial resistance genes. Some of the MGs differed by just one or two genes only, so in order not to over-interpret the results guidelines were devised to interpret the difference between the MGs within each *spa* type (Table 4.2). Isolates assigned to the same MG within the same *spa* type were deemed to be indistinguishable from each another, while isolates assigned to the same MG but distinct *spa* types were deemed to be different. Isolates within MGs that differed by the presence or absence of one virulence or antimicrobial resistance gene were deemed closely related as this is very likely to be due to a single genetic resulting in the loss or gain of virulence or antimicrobial resistance genes (Table 4.2). Isolates within MGs that differed by the presence or absence of two antimicrobial and/or virulence genes, were deemed possibly related because the loss or gain of two such determinants, could indicate a single genetic event if the determinants are collocated but could also be due to more than one genetic event. Isolates assigned to MGs that differed by the presence or absence of three or more antimicrobial and/or virulence genes were considered unrelated (Table 4.2). In this latter group careful consideration of the determinants lost or gained was also required to definitely assign isolates as distinct, because although three gene differences may represent more than one genetic event, it is possible, that all three determinants could be co-located on a single MGE.

Table 4.1 ST22-MRSA-IV isolates recovered from four wards of Beaumont Hospital between March 2007 and September 2008 (*n*=168)

Date of isolation	Isolate no.	Source	AR pattern[a]	ST/ SCC*mec* [a, b]	*spa*[a]	*dru*[a]	(PFT)[a]	DG[a]	MG[c]
28/05/2007	7	P	ApCp	IVh	t1865	dt10j	01018	9	5
20/06/2007	298	E	ApCpErFd	IVh	t032	dt10j	01002	24	9
20/06/2007	300	E	ApCpErFd	IVh	t032	dt10n	01018	1a	11
20/06/2007	303	E	ApCpEr	IVh	t515	dt10a	01039	4a	11
20/06/2007	312	E	ApCpErRf	IVh	t1865	dt10j	01002	10	9
20/06/2007	315	E	ApCp	IVh	t1865	dt10j	01018	9	5
02/07/2007	00145	P	ApCdCp	IVh	t032	dt10o	01039	4b	2
11/07/2007	00349	P	ApCdCpEr	IVh	t032	dt10a	01049	23	4
13/07/2007	00365	P	ApCdCpErFd	IVh	t032	dt10a	01151	32	6
11/06/2007	141	P	ApCpEr	IVh	t4765	dt10a	01063	30	6
12/06/2007	183	P	ApCp	ST22-IVh	t025	dt10j	01018	1a	5
19/06/2007	245	P	ApCdCpFd	IVh	t032	dt10n	01018	1a	5
11/06/2007	163	E	ApCdCpErFd	IVh	t1214	dt10a	01006	14	11
11/06/2007	173	E	ApCpFdLn	IVh	t032	dt10a	01039	4b	2
02/07/2007	00152	P	Ap	ST22-IVa	t4267	dt10a	01039	29	3
02/07/2007	00154	P	ApCdCpEr	IVh	t032	dt10j	01002	24	9
03/07/2007	00183	P	ApCpEr	IVh	t032	dt10a	01018	1a	11
04/07/2007	00208	P	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01024	17	12
04/07/2007	00224	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
04/07/2007	00223	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
04/07/2007	00226	E	ApCdCpEr	IVh	t032	dt10j	01002	1a	9
04/07/2007	00238	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
04/07/2007	00240	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
09/07/2007	00303	P	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
09/07/2007	00312	P	ApCp	IVh	t032	dt10j	01018	1a	5
06/07/2007	00262	P	ApCp	IVh	t032	dt7g	01018	3	5
13/07/2007	00379	P	ApCdCpEr	IVh	t032	dt11a	01126	34	11
30/07/2007	00489	P	ApCp	IVh	t515	dt10a	01042	16	2
30/07/2007	00494	E	ApCp	IVh	t515	dt10a	01042	16	2
30/07/2007	00460	E	ApCpEr	IVh	t022	dt10p	01039	6	6
30/07/2007	00467	P	ApCpEr	IVh	t022	dt10p	01039	6	6
30/07/2007	00503	P	ApCpErLn	ST22-IVh	t515	dt10p	01039	4a	6
1/08/2007	00542	P	ApCpEr	IVh	t032	dt10j	01030	15	6
31/07/2007	00513	E	ApCpFdMpM	IVh	t032	dt10n	01024	17	5
08/08/2007	00597	P	ApCp	IVh	t032	dt7g	01018	3	5
09/08/2007	00638	P	ApCdCpEr	IVh	t515	dt11j	01049	23	4
13/08/2007	00667	P	ApCdCpErFd	IVh	t032	dt10n	01024	17	11
15/08/2007	00684	P	ApCp	IVh	t1214	dt10a	01039	4a	2
15/08/2007	00725	E	ApCdCpEr	IVh	t032	dt10a	01039	4b	6
17/08/2007	00726	E	ApCp	IVh	t515	dt10a	01063	5	2
20/08/2007	00745	P	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01018	1a	12
21/08/2007	00777	P	ApCdCpErLn	IVh	t032	dt10j	01024	17	11
21/08/2007	00795	E	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
21/08/2007	00801	E	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
21/08/2007	00804	E	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
22/08/2007	00831	E	ApCdCpEr	ST22-IVh	t022	dt10a	01039	6	6
22/08/2007	00833	E	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01146	18	12
21/08/2007	00851	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
22/08/2007	00868	E	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
22/08/2007	00871	E	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
22/08/2007	00873	E	ApCdCpEr	IVh	t032	dt10a	01039	4b	6
22/08/2007	00875	E	ApCdCpEr	IVh	t022	dt10a	01039	6	6
22/08/2007	00878	E	ApCdCpErFd	IVh	t032	dt10a	01039	4b	11
22/08/2007	00887	E	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
22/08/2007	00893	E	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
27/08/2007	00953	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
28/08/2007	00992	P	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
03/09/2007	01003	P	ApCpM	IVh	t032	dt10j	01024	17	5
03/09/2007	01010	P	ApCpEr	IVh	t515	dt10a	01039	4a	6
03/09/2007	01019	P	ApCp	IVh	t1214	dt10a	01039	4a	2
03/09/2007	01025	P	ApCpEr	IVh	t1214	dt10a	01039	4a	6
03/09/2007	01027	P	ApCpEr	IVh	t032	dt10a	01042	16	6
03/09/2007	01042	P	ApCdCpEr	IVh	t515	dt7i	01039	7	6
04/09/2007	01088	E	ApCdCpEr	IVh	t022	dt10a	01039	6	6
10/09/2007	01207	P	ApCdCpErFdLn	IVh	t032	dt10j	01042	16	6
29/08/2007	01531	P	ApCp	ST22-IVh	t2951	dt6e	01088	37	2
04/10/2007	01375	P	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01024	17	12

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Table 4.1 continued. MRSA isolates recovered from four wards of Beaumont Hospital from March 2007- September 2008 (*n*=168)

Date of isolation	Isolate no.	Source	AR pattern[a]	ST/ SCC*mec* [a, b]	*spa*[a]	*dru*[a]	(PFT)[a]	DG[a]	MG[c]
20/11/2007	02014	E	ApCdCpEr	IVh	t1214	dt10a	01039	4a	6
05/10/2007	01258	P	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01024	17	12
05/10/2007	01277	P	ApCpErMpM	IVh	t515	dt10a	01039	4a	6
08/10/2007	01322	P	ApCpEr	IVh	t1802	dt10a	01039	35	6
10/10/2007	01329	E	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01024	17	12
10/10/2007	01395	P	ApCpErFd	IVh	t515	dt10j	01018	1a	11
07/11/2007	01569	P	ApCdCpFd	IVh	t032	dt10n	01018	1a	2
07/11/2007	01438	E	ApCdCpEr	IVh	t032	dt5b	01018	21	11
07/11/2007	01573	E	ApCdCpEr	IVh	t032	dt5b	01018	21	11
29/10/2007	01623	P	ApCdCp	IVh	t032	dt10j	01039	4b	6
29/10/2007	01625	P	ApCdCpErFd	IVh	t032	dt10n	01024	17	5
29/10/2007	01629	P	ApCdCpErFd	IVh	t032	dt10a	01032	31	6
31/10/2007	01647	P	ApCdCpEr	IVh	t3213	dt10a	01042	28	6
01/11/2007	01669	P	ApCdCpErFd	IVh	t032	dt10a	01075	20	6
12/11/2007	01844	P	ApCdCpErFd	IVh	t032	dt9j	01018	1a	11
15/11/2007	01905	P	ApCdCpEr	ST22-IVh	t3213	dt10a	01039	27	6
13/11/2007	01894	P	ApCpEr	IVh	t515	dt11o	01039	4a	6
19/11/2007	01959	E	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01018	1a	12
19/11/2007	01961	E	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
19/11/2007	01978	E	ApCdCpEr	IVh	t032	dt9p	01049	25	4
01/10/2007	01266	P	ApCdCpErFd	IVh	t515	dt10a	01042	16	6
08/11/2007	01829	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
04/01/2008	02355	P	ApCdCpFd	IVh	t032	dt10a	01114	36	1
04/01/2008	02386	P	ApCdCpErFd	IVh	t032	dt11j	01075	20	6
04/01/2008	02391	P	ApCdCpErFd	IVh	t032	dt10n	01006	13	11
28/01/2008	02783	P	ApCpErFd	IVh	t515	dt10a	01002	24	9
28/01/2008	02815	P	ApCp	IVh	t1214	dt10a	01039	4a	2
05/02/2008	02881	P	ApCdCp	IVh	t4122	dt10j	01039	4b	2
05/02/2008	02911	P	ApCdCpEr	IVh	t1214	dt10a	01039	4a	4
07/02/2008	03002	E	ApCdCpEr	IVh	t032	dt10a	01018	1a	11
18/02/2008	03032	P	ApCdCpEr	IVh	t515	dt10a	01022	19	9
18/03/2008	03746	E	ApCpEr	IVh	t515	dt8a	01039	8	6
18/03/2008	03764	P	ApCpErFd	IVh	t032	dt10j	01018	1a	11
19/03/2008	03940	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
18/02/2008	03035	P	ApCdCpErFd	IVh	t022	dt10a	01039	6	6
12/02/2008	03091	P	ApCpEr	IVh	t1214	dt10a	01042	16	6
25/02/2008	03123	P	ApCdCpErKnLnMpTb	IVh	t3185	dt10o	01154	22	13
28/02/2008	03201	E	ApCdCpEr	IVh-non subtypeable	t3185	dt10a	01022	19	9
28/02/2008	03213	E	ApCdCpErKnLnMpTb	IVh	t3185	dt10o	01154	22	13
28/02/2008	03219	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
28/02/2008	03222	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
28/02/2008	03225	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
04/03/2008	03288	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
04/03/2008	03300	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
04/03/2008	03304	E	ApCdCpEr	IVh	t032	dt10j	01018	1a	11
04/03/2008	03308	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	7
11/03/2008	03376	E	ApCdCpErGnKnLnMpTb	IVh	t515	dt10o	01154	22	13
10/03/2008	03626	P	ApCpErLn	IVh	t032	dt10j	01039	4b	6
11/03/2008	03638	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
11/03/2008	03642	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
11/03/2008	03646	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
11/03/2008	03651	E	ApCdCpEr	ST22-IVh	t3185	dt10o	01154	22	10
11/03/2008	03663	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
11/03/2008	03693	E	ApCdCpEr	IVh	t3185	dt10o	01154	22	10
10/04/2008	03832	E	ApCdCpErGnKnLnMpTb	IVh	t3185	dt10o	01154	22	13
10/04/2008	03886	E	ApCpEr	IVh	t022	dt10j	01018	1b	11
18/02/2008	03064	P	ApCpErGnKnTb	IVh	t032	dt10j	01018	1a	12
14/04/2008	04038	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
23/04/2008	04485	P	ApCdCpEr	IVh	t1214	dt10a	01039	4a	6
24/04/2008	04497	P	ApCdCpM	ST22-IVh	t4622	dt10a	01039	4a	2
29/04/2008	04747	E	ApCdCp	ST22-IVh	t628	dt10a	01018	2	5
29/04/2008	04607	E	ApCdCpEr	IVh	t032	dt10j	01156	33	8
22/04/2008	04294	E	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
26/05/2008	05002	P	ApCdCpErFd	IVh	t032	dt10n	01042	16	6
26/05/2008	05006	P	ApCpErFd	IVh	t032	dt10j	01024	17	11

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Table 4.1 continued. MRSA isolates recovered from four wards of Beaumont Hospital from March 2007- September 2008 (*n*=168)

Date of isolation	Isolate no.	Source	AR pattern[a]	ST/ SCC*mec* [a, b]	*spa*[a]	*dru*[a]	(PFT)[a]	DG[a]	MG[c]
26/05/2008	05018	P	ApCp	ST22-IVh	t1865	dt10j	01018	9	11
27/05/2008	05087	E	ApCpEr	IVh	t022	dt8p	01049	26a	6
27/05/2008	05060	E	ApCpEr	IVh	t022	dt8p	01049	26a	4
27/05/2008	05058	E	ApCpEr	IVh	t022	dt8p	01049	26a	4
27/05/2008	05068	E	ApCdCpErFd	IVh	t032	dt10n	01024	2	11
27/05/2008	05075	E	ApCdCp	IVh	t628	dt10a	01018	26b	5
28/05/2008	05117	P	ApCpEr	IVh	t032	dt8p	01049	2	4
29/05/2008	05149	E	ApCdCp	IVh	t628	dt10a	01018	2	5
29/05/2008	05158	E	ApCdCp	IVh	t628	dt10a	01018	2	5
27/05/2008	05094	E	ApCdCp	IVh	t628	dt10a	01018	2	5
27/05/2008	05091	E	ApCdCp	IVh	t628	dt10a	01018	2	5
26/05/2008	05023	P	ApCdCp	IVh	t628	dt10a	01018	2	5
27/05/2008	05076	E	ApCdCp	IVh	t628	dt10a	01018	2	5
29/05/2008	05177	P	ApCdCp	IVh	t628	dt10a	01018	2	5
02/01/2008	02317	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
25/06/1008	05219	P	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01024	17	12
26/06/2008	05239	P	ApCdErFd	ST22-IVh	t4623	dt10a	01077	12	9
30/06/2008	05245	P	ApCpErFd	IVh	t032	dt10af	01039	4b	6
01/07/2008	05327	E	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01024	17	12
01/07/2008	05328	E	ApCpErFd	IVh	t032	dt10af	01039	4b	6
01/07/2008	05331	E	ApCpErFd	IVh	t032	dt10af	01039	4b	6
01/07/2008	05332	E	ApCpErFd	IVh	t032	dt10af	01039	4b	6
01/07/2008	05336	E	ApCpErFd	IVh	t032	dt10af	01039	4b	9
01/07/2008	05354	P	ApCdCpErGnKnMpTb	IVh	t032	dt10j	01006	13	12
09/07/2008	05295	E	ApCpErFd	IVh	t032	dt10af	01039	4b	6
03/07/2008	05412	E	ApCpErKnMpTb	IVh	t032	dt10j	01024	17	12
14/07/2008	05599	P	ApCpErGnKnTb	IVh	t032	dt10j	01018	1a	12
15/07/2008	05656	E	ApCpErGnKnTb	IVh	t032	dt10j	01018	1a	12
07/08/2008	06314	P	ApCdCpEr	ST22-IVh	t578	dt10a	01049	23	4
05/08/2008	05996	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
17/07/2008	05840	E	ApCpErGnKnMpTb	IVh	t032	dt10a	01006	13	12
21/07/2008	05864	P	ApCpEr	IVh	t1214	dt11o	01039	4a	6
31/07/2008	05962	P	ApCdCpErFd	IVh	t032	dt10a	01049	23	4
18/08/2008	06382	P	ApCdCpErFd	IVh	t032	dt10n	01018	1a	11
25/08/2008	06470	P	ApCdCpErFd	IVh	t032	dt10n	01047	11	11
01/09/2008	06484	E	ApCdCpErFd	IVh	t032	dt10n	01047	11	11

[a] Isolate antbiogram-resistogram (AR) types and patterns, SCC*mec*, *spa* and *dru* types, pulsed field types (PFTs) and dendrogram groups (DGs) were determined as part of a previous study (Shore *et al.*, 2010).

[b] STs are indicated where MLST was performed as part of a previous study (Shore *et al.*, 2010). MLST was performed in that study on one isolate representative of each *spa* type identified. For all other isolates the ST was inferred to be ST22 based on the *spa* type (Shore *et al.*, 2010).

[c] Microarray groups (MGs) were determined as part of the present study based on the presence or absence of virulence and resistance genes detected using the StaphyType DNA microarray (Chapter 3, Table 3.1).

Abbreviations: Unf, unfamiliar AR pattern or variant of a known AR pattern, NT, AR type pattern indicative of AR06 but urease positive (AR06 isolates are usually urease-negative). Ak, amikacin; Ap, ampicillin; Cd, cadmium nitrate/cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenylmercuric acetate; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; Tb, tobramycin; Te, tetracycline; Tp, trimethoprim.

Table 4.2 Proposed guidelines for interpreting microarray groups (MGs) for ST22-MRSA-IV isolates

No. of gene differences[a]	Category	Comment
0	Indistinguishable	.....
1	Closely related	.....
≥2	Possibly related[b]	If evidence suggests that genes that differ are co-located
≥2	Different[c]	If evidence suggests that genes that differ are not co-located

[a] Gene differences as determined based on the presence or absence of virulence and/or antimicrobial resistance genes using the StaphyType DNA microarray (Alere).

[b] Isolates that differed by the presence or absence of two or more antimicrobial and/or virulence genes were deemed possibly related if evidence suggested that the determinants were co-located.

[c] Isolates that differed by the presence or absence of two or more antimicrobial and/or virulence genes were considered unrelated if there was no evidence to suggest colocation of the variable genes.

4.2.1.4 Calculation of discriminatory powers

The ability of the different typing methods to discriminate among the ST22-MRSA-IV isolates was assessed quantitatively by calculating the SID for each typing method and various combinations of methods with 95% confidence intervals (CI) using an online tool developed by Faria *et al.* (available at www.comparingpartitions.info) (Faria *et al.*, 2008). The SID for *spa*, *dru* and PFGE alone, in pairwise combinations and with all three methods had been calculated previously for these isolates by Shore *et al.* (2010). As part of the present study the SID was calculated for (a) MGs and *spa* typing, (b) MGs and *dru* typing, (c) MGs and PFGE typing, (d) dendrogram groups (DGs) and (e) MGs and DGs.

4.2.2. Investigation of the stability of DNA microarray profiles, *spa* types and *dru* types of MRSA isolates

4.2.2.1 MRSA isolates and experimental design

A total of 14 MRSA isolates were investigated in order to determine the stability of DNA microarray profiles and, in some instances, the stability of the *spa* and *dru* types. Details of isolates investigated are shown in Table 4.3. All isolates were recovered from Beaumont Hospital and included (a) six pairs of ST22-MRSA-IV isolates, each pair recovered from a different patient (patients 1-6, Table 4.3) in Beaumont Hospital at two different time points (Table 4.3) (Shore *et al.*, 2010). This group of isolates were investigated for the stability of *spa*, *dru* types and DNA microarray profiles over time and each pair of isolates was recovered between 6 days and 19 months apart (Table 4.3); (b) two ST22-MRSA-IV isolates (00145 and 03300) (Shore *et al.*, 2010) recovered from one patient and one environmental site in Beaumont Hospital which following multiple cycles of either freezing and thawing or exposure to

elevated temperature were investigated for the stability of their DNA microarray profiles, *spa* and *dru* types only.

4.2.2.2 *spa* typing

spa typing was performed on isolates as described in Chapter 2, section 2.4.3.

4.2.2.3. *dru* typing

dru typing was performed on isolates as described in Chapter 2, section 2.4.4.

4.2.2.4 Stability experiments

The two ST22-MRSA-IV isolates, 00145 and 03300, that underwent repeated cycles of freezing and thawing and exposure to elevated temperatures were cultured on brain heart infusion (BHI) agar and incubated at 37°C for 24 h. Four separate colonies of each primary clinical isolate were selected and were re-inoculated onto fresh BHI agar and incubated at 37°C for 24 h. Following incubation, each of the four single colony isolates was subjected to freezing and thawing by the transfer of culture growth using a sterile loop into individual Protect Bacterial Preservation System vials and was stored at -70°C overnight. Thawing was carried out by removing each set of Protect vials from -70°C, and placing at room temperature for 1-2 h.

Isolates were re-cultured by removing a single bead from each vial using a sterile bacteriological loop, inoculating it onto BHI agar and incubating at 37°C for 24 h. Each of the four MRSA derivatives was then transferred once again, using a sterile loop, to individual Protect vials and stored at -70°C overnight. This procedure was repeated for a minimum of 10 subcultures. On the final round of freeze-thawing, four separate colonies of each of the four MRSA derivatives were analysed using the DNA microarray, *spa* and *dru* typing.

Table 4.3 Details of the 14 MRSA isolates and derivatives investigated for the stability of their DNA microarray profiles and in some instances for the stability of their *spa* and *dru* types[a]

Isolate/ derivative[a]	Patient number	Date of isolation	Time period between recovery of each isolate	Sample source	ST-SCC*mec* type	*spa* type	*dru* type	Microarray virulence and antimicrobial resistance genes detected[b,c]	No. of DNA microarray gene/gene cluster differences[d]
Isolate									
01277	1	2/10/2007	6 days	Nasal	22-IV	t515	dt10a	*blaZ*, *erm*(C), *egc*, IEC type B	2
01399	1	8/10/2007		Nasal	22-IV	t515	dt10j	blaZ, *erm*(C)**, *erm*(B)**, ***tst***, *egc*, IEC type B	
00489	2	30/07/2007	21 days	Groin	22-IV	t515	dt10a	*blaZ*, *egc*, IEC type B	4
00788	2	20/08/2007		Groin	22-IV	t032	dt10a	*blaZ*, ***erm*(C)**, ***aacA-aphD***, ***ileS2***, ***sec/sel***, *egc*, IEC type B	
00638	3	09/08/2007	3 weeks	Nasal	22-IV	t515	dt11j	*blaZ*, *erm*(C), *egc*	2
01052	3	03/09/2006		Groin	22-IV	t032	dt10j	*blaZ*, *erm*(C), ***sec/sel***, *egc*, **IEC type B**	
10757	4	01/02/2010	5 months	Nasal	22-IV	t032	dt10j	*blaZ*, *erm*(C), *egc*, IEC type B	0
12323	4	28/06/2012		Nasal	22-IV	t557	dt10n	*blaZ*, *erm*(C), *egc*, IEC type B	
08484	5	15/06/2009	9 months	Nasal	8-IID	t190	NT	*blaZ*, *aacA-aphD*, *aphA3* & *sat*, *sdrM*, *fosB*, *qacA*, IEC type A	0
11268	5	08/03/2010		Nasal	8-IID	t190	NT	*blaZ*, *aacA-aphD*, *aphA3* & *sat*, *sdrM*, *fosB*, *qacA*, IEC type A	
01647	6	31/10/2007	19 months	Nasal	22-IV	t3213	dt10a	*blaZ*, *sec/sel*, IEC type B	0
08481	6	15/06/2009		Groin	22-IV	t3213	dt10a	*blaZ*, *sec/sel*, IEC type B	
00145	7	02/07/2007	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
03300	E	04/03/2008	NA	Mattress	22-IV	t3185	dt10o	*blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, *egc*, IEC B, ACME	0

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Table 4.3 continued. Details of the 14 MRSA isolates and derivatives investigated for the stability of their DNA microarray profiles and in some instances for the stability of their *spa* and *dru* types[a]

Isolate/ derivative[a]	Patient number	Date of isolation	Time period between recovery of each isolate	Sample source	ST-SCC*mec* type	*spa* type	*dru* type	Microarray virulence and antimicrobial resistance genes detected[b,c]	No. of DNA microarray gene/gene cluster differences[d]
Derivative									
D00145 F/T1	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 F/T2	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 F/T3	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 F/T4	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 S/C1	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 S/C2	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 S/C3	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D00145 S/C4	N/A	N/A	NA	N/A	22-IV	t032	dt10o	*blaZ*, *egc*, IEC type B	0
D03300 F/T1	N/A	N/A	NA	N/A	22-IV	t3185	dt10o	*blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, *egc*, IEC B, ACME	0
D03300 F/T2	N/A	N/A	NA	N/A	22-IV	t3185	dt10o	*blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, *egc*, IEC B, ACME	0

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Table 4.3 continued. Details of the 14 MRSA isolates and derivatives investigated for the stability of their DNA microarray profiles, and, in some instances for the stability of their *spa* and *dru* types[a]

Isolate/ derivative[a]	Patient number	Date of isolation	Time period between recovery of each isolate	Sample source	ST-SCC*mec* type	*spa* type	*dru* type	Microarray virulence and antimicrobial resistance genes detected[b,c]	No. of DNA microarray gene/gene cluster differences[d]
D03300 F/T3	N/A	N/A	NA	N/A	22-IV	t3185	dt10o	*blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, *egc*, IEC B, ACME	0
D03300 S/C1	N/A	N/A	NA	N/A	22-IV	t3185	dt10o	*blaZ*, *erm*(C), *lnu*(A), *aacA-aphD, aadD*, *ileS2*, *egc*, IEC B, ACME	0
D03300 S/C2	N/A	N/A	NA	N/A	22-IV	t3185	dt10o	*blaZ, erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, *egc*, IEC B, ACME	0
D03300 S/C3	N/A	N/A	NA	N/A	22-IV	t3185	dt10o	*blaZ, erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, *egc*, IEC B, ACME	0

[a] Sequential ST22-IV MRSA clinical isolates from seven separate patients and derivatives generated from one clinical MRSA isolate (00145) and one environmental MRSA isolate (03300) were investigated for the stability of their DNA microarray profiles, *spa* and *dru* types. Derivatives were obtained following 10 consecutive rounds of freezing and thawing (F/T) and following 14 consecutive rounds of subculturing (S/C) and growth at elevated temperatures (43°C).

[b] Microarray virulence and antimicrobial resistance genes were detected using the StaphyType DNA microarray profiling kit (Alere). DNA microarray genes that were detected in one patient isolate but not in the other are indicated in bold.

[c] Immune evasion cluster (IEC) type as defined by van Wamel *et al*. (2006): A= *sea*, *sak*, *chp* and *scn*; B = *sak, chp* and *scn*; C = *chp* and *scn*; D = *sea, sak* and *scn;* E = *sak* and *scn*; F = *sep, sak, chp* and *scn*; G = *sep, sak* and *scn.*

[d] For isolates from patients 1 to 6 the number of gene/gene cluster differences were determined by comparing the DNA microarray profiles of the isolates within each pair. For the isolates recovered from 7 patients and the environmental isolate the number of gene/gene cluster differences were determined by comparing the DNA microarray profiles of each isolate before and after 10 consecutive rounds of freezing and thawing and multiple cycles of exposure to elevated temperatures.

Abbreviations: NT, non typeable; NA, not applicable; E, environmental isolate.

Three individual colonies of the two ST22-MRSA-IV isolates 00145 and 03300 were subjected to growth at elevated temperatures by culturing into BHI broth, incubated at 43°C for 24 h with shaking at 200 rpm and subcultured into fresh BHI broth for 14 consecutive rounds. Following the final round of subculturing, three separate colonies derived from each parental isolate were analysed by DNA profiling, *spa* and *dru* typing.

4.3.1 Combined DNA microarray profiling and *spa* typing of ST22-MRSA-IV isolates

4.3.1.1 DNA microarray profiling

Analysis of the DNA microarray profiles of the 168 ST22-MRSA-IV isolates recovered from patient and environmental sites in Beaumont hospital in 2007 and 2008 identified 10 variable genes or gene clusters among the isolates. This included the antimicrobial resistance genes *blaZ*, *erm*(C), *aacA-aphD, aadD, lnu*(A) and *ileS2*, and the virulence genes and gene clusters *sec/sel, egc,* IEC and ACME (Table 4.4). Each different combination of these genes was assigned a microarray group (MG) number, and a total of 13 MGs based on the presence or absence of these genes were identified among the ST22-MRSA-IV isolates (Table 4.4). The most common MGs, namely MGs 11 and 6, accounted for 27.4% (46/168) and 23.8% (40/168) of isolates, respectively. Isolates within these two MGs harboured *blaZ*, *erm*(*C*), *egc* and IEC type B (*sak*, *chp* and *scn*) with MG6 isolates also harbouring *sec* & *sel* (Table 4.4). Microarray group 1 harboured the least number of these genes (n=2), while MG13 harboured the greatest number of these genes (n=9). The antimicrobial resistance and virulence genes and gene cluster differences between each MG are shown in a matrix in Table 4.5. The MGs differed by between one and eight genes or gene clusters. Despite encoding several

genes the IEC-genes were considered to represent one difference only since these genes are usually linked on lysogenic bacteriophage genomes integrated in the disrupted chromosomal *hlb* gene (Bokarewa and Tarkowski, 2004; Postma *et al.*, 2004; Rooijakkers *et al.*, 2005a; van Wamel *et al.*, 2006). Similarly, *sec/sel* are known to be co-located on a pathogenicity island SaPIbov1 (Avena and Bergdoll, 1967; Baba *et al.*, 2002). Others possibly co-located gene difference detected by the array include *egc,* which are encoded by genomic island υSAβ, plasmid located *aacA-aphD* & *ileS2* (SAP082A) (Accession number NC013344), and plasmid located *ileS2* & *lnu*(A) (SAP015B, GQ900502).

All except three of the MGs identified were represented by isolates belonging to more than one *spa* type and six were represented by isolates belonging to more than one dendrogram group (DG) (Table 4.4). Conversely, isolates belonging to six *spa* types and 9/37 DGs were assigned to more than one MG indicating that in these instances the microarray was able to further differentiate between isolates that were assigned to the same *spa* type and DG, respectively (Table 4.4).

4.3.1.2 Combining the microarray groups (MGs) and *spa* types of isolates

Seventeen *spa* types were previously identified among the ST22-MRSA-IV investigated (Table 4.6). Six of these *spa* types, representing 88% of isolates (148/168), were further differentiated into multiple MGs (Table 4.6). Six of the eight *spa* types that consisted of more than one isolate were differentiated into multiple MGs (Table 4.6). This included the following: the most predominant *spa* type, t032, which accounted for 55.4% of isolates (93/168), and was differentiated into nine MGs (Table 4.6); *spa* type t515, representing 9.5% of isolates (16/168) which were assigned to six MGs; *spa* types t3185 and t1214, representing 9% and 6% of isolates, respectively and which were each

Table 4.4 Characteristic antimicrobial resistance and virulence genes, *spa* types and dendrogram groups (DGs) among each microarray groups (MGs) identified among the 168 ST22-MRSA-IV isolates recovered from Beaumont Hospital in 2007 and 2008

Microarray group no. (*n*)[a]	Characteristic antimicrobial resistance and virulence genes[b]	*spa* type (*n*)[c]	Dendrogram group no. (*n*)[d]
MG1 (1)	*blaZ, egc*	t032 (1)	36
MG2 (12)	*blaZ, egc*, IEC type B	t032 (3), t515 (3), t2144 (3), t2951 (1), t4122 (1), t4622 (1)	**1a (1)**, **4a (4)**, **4b (3)**, 5 (1), **16 (2)**, 37 (1)
MG3 (1)	*blaZ, egc*, IEC type F	t4267 (1)	29
MG4 (9)	*blaZ, erm*(C), *egc*	t032 (4), t022 (2), t515 (1), t578 (1), t1214 (1)	**4a (1)**, 23 (4), 25 (1), **26a (2)**, 26b (1)
MG5 (19)	*blaZ, sec/sel, egc,* IEC type B	t1865 (2), t025 (1), t032 (7), t628 (9)	**1a (3)**, 2 (9), 3 (2), **9 (2)**, **17 (3)**
MG6 (40)	*blaZ, erm*(C), *egc,* IEC type B	t022 (7), t032 (17), t1214 (5), t1802 (1), t3213 (2), t4765 (1), t515 (7)	**4a (8)**, **4b (9)**, 6 (6), 7 (1), 8 (1), 15 (1), **16 (5)**, 20 (2), **26a (1)**, 27 (1), 28 (1), 30 (1), 32 (1), 31 (1), 35 (1)
MG7 (1)	*erm*(C), *egc,* IEC type B, ACME	t3185 (1)	**22 (1)**
MG8 (1)	*blaZ, erm*(C), *egc,* ACME	t032 (1)	33 (1)
MG9 (9)	*blaZ, erm*(C), *sec/sel, egc*	t032 (4), t515 (2), t3185 (2), t4623 (1)	**4b (1),** 10 (1), 12 (1), 19 (2), 24 (4),
MG10 (2)	*blaZ, erm*(C), *egc* ACME, IEC type B	t3185 (2)	**22 (2)**
MG11 (46)	*blaZ, erm*(C), *sec/sel, egc,* IEC type B	t022 (1), t032 (41), t515 (2) t1214 (1), t1865 (1)	1b (1), **1a (31)**, **4a (1), 4b (1)**, **9 (1)**, 11 (2), **13 (1)**, 14 (1), **17(4)**, 21 (2), 34 (1)
MG12 (15)	*blaZ, erm*(C), *aacA-aphD, ileS2, sec/sel, egc,* IEC type B	t032 (15)	**1a (5), 13 (2), 17 (7)**, 18 (1)
MG13 (12)	*blaZ, erm*(C), *lnu*(A), *aacA-aphD, aadD, ileS2, egc,* IEC type B, ACME	t3185 (11), t515 (1)	**22 (12)**

[a] Each ST22-MRSA-IV isolate was assigned a microarray group (MG) number based on the different combinations of antimicrobial resistance and virulence-associated genes identified. MGs shown in bold were assigned to more than one dendrogram group.

[b] Immune evasion cluster (IEC) type as defined by van Wamel *et al.* (2006): B = *sak, chp* and *scn*; F = *sep, sak, chp* and *scn*;

[c] *spa* types were assigned to isolates as part of a previous study (Shore *et al.*, 2010). *spa* types indicated in bold were assigned to more than one MG.

[d] Dendrogram groups (DGs) were assigned previously by constructing a dendrogram based on the average of the similarity matrices for *spa*, *dru* and PFGE typing data for these isolates and using a cut-off of 98.5% similarity for DG assignment i.e. isolates that shared greater than 98.5% similarity were assigned to the same DG and those with less than 98.5% similarity were assigned to different DGs . DGs shown in bold were assigned to more than one MG.

n, number of isolates.

Table 4.5 Matrix showing the antimicrobial resistance and virulence gene differences between each microarray group (*n*)[a]

Microarray group (MG)	MG1	MG2	MG3	MG4	MG5	MG6	MG7	MG8	MG9	MG10	MG11	MG12
MG1	....	....	....	....	....	....	....	....	....	....	....	....
MG2	IEC type B[b] (**1**)	....	....	....	....	....	....	....	....	....	....	....
MG3	IEC type F[b] (**1**)	IEC B, IEC type F (**2**)	....	....	....	....	....	....	....	....	....	....
MG4	*erm*(C) (**1**)	*erm*(C), IEC type B (**2**)	*erm*(C), IEC type F (**2**)	....	....	....	....	....	....	....	....	....
MG5	*sec/sel*, IEC type B (**2**)	*sec/sel* (**1**)	*sec/sel*, IEC type B, IEC type F (**3**)	*erm*(C), *sec/sel*, IEC type B (**3**)	....	....	....	....	....	....	....	....
MG6	*erm*(C), IEC type B (**2**)	*erm*(C) (**1**)	*erm*(C), IEC type B, IEC type F (**3**)	IEC type B (**1**)	*erm*(C), *sec/sel* (**2**)	....	....	....	....	....	....	....
MG7	*blaZ*, *erm*(C), IEC type B, ACME (**4**)	*blaZ*, *erm*(C), ACME (**3**)	*blaZ*, *erm*(C), IEC type F, IEC type B, ACME (**5**)	*blaZ*, IEC type B, ACME (**3**)	*blaZ*, *erm*(C), *sec/sel*, ACME (**4**)	*blaZ*, ACME (**2**)	....	....	....	....	....	....
MG8	*erm*(C), ACME (**2**)	*erm*(C), IEC type B, ACME (**3**)	*erm*(C), IEC type F, ACME (**3**)	ACME (**1**)	*erm*(C), *sec/sel*, ACME, IEC type B (**4**)	IEC type B, ACME (**2**)	*blaZ*, IEC type B (**2**)	....	....	....	....	....
MG9	*erm*(C), *sec/sel* (**2**)	*erm*(C), *sec/sel*, ACME (**3**)	*erm*(C), *sec/sel*, IEC type F (**3**)	*sec/sel* (**1**)	*erm*(C), IEC type B (**2**)	*sec/sel*, IEC type B (**2**)	*blaZ*, *sec/sel*, IEC type B, ACME (**4**)	*sec/sel*, ACME (**2**)	....	....	....	....

Continued overleaf

Table 4.5 continued. Matrix showing the antimicrobial resistance and virulence associated genes differences between each microarray group (*n*)[a]

Microarray group (MG)	MG1	MG2	MG3	MG4	MG5	MG6	MG7	MG8	MG9	MG10	MG11	MG12
MG10	*erm*(C), IEC type B, ACME (**3**)	*erm*(C), ACME (**2**)	*erm*(C), IEC type B, IEC type F, ACME (**4**)	ACME, IEC type B (**2**)	*erm*(C), *sec/sel*, ACME (**3**)	ACME (**1**)	*blaZ* (**1**)	IEC type B (**1**)	*sec/sel*, IEC type B, ACME (**3**)	....	....	....
MG11	*erm*(C), *sec/sel*, IEC type B (**3**)	*erm*(C), *sec/sel* (**2**)	*erm*(C), *sec/sel* IEC type B, IEC type F (**4**)	*sec/sel*, IEC type B (**2**)	*erm*(C) (**1**)	*sec/sel*(**1**)	*blaZ*, *sec/sel*, ACME (**3**)	*sec/sel*, IEC type B, ACME (**3**)	IEC type B (**1**)	*sec/sel*, ACME (**2**)	....	....
MG12	*erm*(C), *aacA-aphD*, *ileS2*, *sec/sel*, IEC type B (**5**)	*erm*(C), *aacA-aphD*, *ileS2*, *sec/sel* (**4**)	*erm*(C), IEC type B, IEC type F *aacA-aphD*, *ileS2*, *sec/sel* (**6**)	*aacA-aphD*, *ileS2*, *sec/sel*, IEC type B (**4**)	*erm*(C), *aacA-aphD*, *ileS2* (**3**)	*lnu*(A), *aacA-aphD*, *aadD*, *ileS2*(**5**)	*blaZ*, *sec/sel*, *aacA-aphD*, *ileS2*, ACME (**5**)	*sec/sel*, *aacA-aphD*, *ileS2*, ACME, IEC type B (**5**)	*aacA-aphD*, *ileS2*, IEC type B (**3**)	*sec/sel*, *aacA-aphD*, *ileS2*, ACME (**4**)	*aacA-aphD*, *ileS2* (**2**)	....
MG13	*erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, IEC type B, ACME (**7**)	*erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, ACME (**6**)	*erm*(C), IEC type B, IEC type F, *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, ACME (**8**)	*lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, IEC type B, ACME (**6**)	*sec/sel*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, ACME (**7**)	*lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, ACME (**5**)	*blaZ*, *lnu*(A), *aacA-aphD*, *aadD*, *ileS2* (**5**)	*lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, IEC type B (**5**)	*sec/sel*, *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, IEC type B, ACME (**7**)	*lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, ACME (**5**)	*sec/sel*, *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*, ACME (**6**)	*sec/sel*, *lnu*(A), *aadD*, ACME (**4**)

[a] Each ST22-MRSA-IV isolate was assigned a microarray group (MG) number based on the different combinations of antimicrobial resistance and virulence genes identified. Numbers in parenthesis refer to the numbers of gene differences between each MG.

[b] Immune evasion cluster (IEC) type as defined by van Wamel *et al*. (2006): IEC type B = *sak*, *chp*, and *scn*; IEC type F = *sep*, *sak*, *chp*, and *scn*.

Numbers shown in bold in parenthesis indicate the numbers of gene differences when comparing two microarray groups MGs to one another, for example MG1 and MG5 differ two gene differences (*sec/sel* and IEC type B).

Table 4.6 The different microarray groups (MGs) identified among each *spa* type for the 168 ST22-MRSA-IV isolates recovered from Beaumont Hospital in 2007 and 2008

spa type (*n*)[a]	Microarray group (MG) (*n*)[b]
t025 (1)	MG5
t578 (1)	MG4
t1802 (1)	MG6
t2951 (1)	MG2
t4122 (1)	MG2
t4267 (1)	MG3
t4622 (1)	MG2
t4623 (1)	MG9
t4765 (1)	MG6
t3213 (2)	MG6
t1865 (4)	MG5 (2), MG9 (1), MG11 (1)
t628 (9)	MG5
t022 (10)	MG6 (7), MG4 (2), MG11 (1)
t1214 (10)	MG2 (3), MG4 (1), MG6 (5), MG11 (1)
t3185 (15)	MG7 (1), MG9 (1), MG10 (2), MG13 (11)
t515 (16)	MG2, MG6, MG4, MG9 MG11, MG13 (6)
t032 (93)	MG1 (1), MG2 (3), MG4 (4), MG5 (7), MG6 (17), MG8 (1), MG9 (4), MG11 (41), MG12 (15)

[a] Isolates were *spa* typed previously (Shore *et al.*, 2010). Numbers in parenthesis refer to the numbers of isolates with the particular *spa* type indicated.

[b] Each ST22-MRSA-IV isolate was assigned a microarray group (MG) number based on the different combinations of antimicrobial resistance and virulence-associated genes identified. Numbers in parenthesis indicate the numbers of isolates with the particular MG indicated when isolates within a *spa* type were assigned to more than one MG.

n, number of isolates.

assigned to four MGs; *spa* types t1865 and t022, representing 2% and 6% of isolates, respectively, and which were each assigned to three MGs (Table 4.6). All other *spa* types were represented by single MGs only (Table 4.6).

4.3.1.3 Interpreting the differences between MGs within each *spa* type

For each *spa* type that was differentiated into more than one MG, the guidelines outlined in Table 4.2 were used in order not to over-interpret differences between isolates i.e. if there was only one gene difference isolates were deemed to be closely related but >1 gene difference they were deemed to be different, unless available evidence suggests they may be co-located e.g. multiple resistance genes previously described in some instance on the same plasmid, where they were then considered to be possibly related. Using this criteria 10 of the 36 possible pairwise combinations of MGs within *spa* type t032, the most common *spa* type identified, were deemed to be closely related as they differed by one gene only (Table 4.7). Of the other 25 pairwise combinations of MGs within t032, only one pair, MG11 and MG12, were deemed to be possibly related because although they exhibited two gene differences, the genes have been shown elsewhere in this study to be co-located on a plasmid (*ileS2* and *aacA-aphD*) (Chapter 5, Table 5.5). All other pairwise combinations of the MGs within *spa* type t032 were deemed to be different (Table 4.7).

None of the MGs within the other five *spa* types represented by more than one MG were considered to be possibly related, but 13 pairwise combinations of MGs were considered to be closely related within *spa* types t515, t3185, t1214, t022, and t1865 (Table 4.7). Also among these five *spa* types, 20 pairwise combinations of MGs within *spa* types t515, t3185, t1214, t022 and t1865 were considered to be different (Table 4.7). Overall, of the MGs differing by just two or more genes, only MG11 and MG12

were deemed to be "possibly related" as all others exhibited differences indicative of loss or acquisition of multiple resistance, and/or virulence genes.

4.3.1.4 Discriminatory power of the MGs alone and in combination with other typing methods

The discriminatory power of *spa*, *dru* and PFGE typing, DGs, and MGs and all combinations of these methods were determined quantitatively by calculating SID values for each (Table 4.8). Of the individual methods, *spa*, *dru* and PFGE typing and DNA microarray profiling, the latter yielded the greatest discriminatory power (SID 83.60%) among the 168 ST22-MRSA-IV isolates investigated. Although the combination of *spa*, *dru* and PFGE typing yielded the largest number of type combinations (65), and the greatest discriminatory power (SID 96.53%) among the isolates, this does not take into account the fact that some of type combinations generated using this method only exhibit minor differences (Shore *et al.*, 2010). However, this is taken into account using the DGs, which had a very similar discriminatory power to the combination of *spa* typing and MG profiling (SID 91.0% for DGs and 91.1% for *spa* and MGs), as well as to the MGs combined with PFGE (SID 91.1%). An even higher discriminatory power was obtained when the MGs were combined with *dru* typing (SID 94.5%) or the DGs (SID 95.8%) (Table 4.8).

Table 4.7 The relatedness of MRSA isolates within each microarray group (MG) of *spa* types that were represented by more than one MG

spa type[a]	Relatedness[b]		
	Closely related[b]	Possibly related[b]	Different[b]
t032 (93)	MG1 & MG2	MG11 & MG12	MG1 & MG5
	MG1 & MG4		MG1 & MG6
	MG2 & MG5		MG1 & MG8
	MG2 & MG6		MG1 & MG9
	MG4 & MG6		MG1 & MG11
	MG4 & MG8		MG1 & MG12
	MG4 & MG9		MG2 & MG4
	MG5 & MG11		MG2 & MG8
	MG6 & MG11		MG2 & MG9
	MG9 & MG11		MG2 & MG11
			MG2 & MG12
			MG4 & MG5
			MG4 & MG11
			MG4 & MG12
			MG5 & MG6
			MG5 & MG8
			MG5 & MG9
			MG5 & MG12
			MG6 & MG8
			MG6 & MG9
			MG 6 & MG12
			MG8 & MG9
			MG8 & MG11
			MG8 & MG12
			MG9 & MG12
t515 (16)	MG2 & MG6		MG2 & MG4
	MG4 & MG6		MG2 & MG9
	MG4 & MG9		MG2 & MG11
	MG6 & MG11		MG2 & MG13
	MG9 & MG11		MG4 & MG11
			MG4 & MG13
			MG6 & MG9
			MG6 & MG13
			MG9 & MG13
			MG11 & MG13
t3185 (15)	MG7 & MG10		MG7 & MG9
			MG7 & MG13
			MG9 & MG10
			MG9 & MG13
			MG10 & MG13
t1214 (10)	MG2 & MG6		MG2 & MG4
	MG4 & MG6		MG2 & MG11
	MG6 & MG11		MG4 & MG11
t022 (10	MG6 & MG4		MG4 & MG11
	MG6 & MG11		
t1865 (4)	MG5 & MG11		MG5 & MG9
	MG9 & MG11		

[a] Numbers in parenthesis refer to the numbers of isolates with the *spa* type indicated.

[b] The relatedness of each MG is indicated for *spa* types with isolates that were assigned to more than one MG. MGs were deemed to be closely related if they differed by one antimicrobial or virulence gene only. MGs were deemed to be possibly related if they differed by the presence or absence of two or more antimicrobial and/or virulence genes if evidence suggested that the determinants were co-located. MGs were deemed unrelated if they differed by the presence or absence of two or more antimicrobial and/or virulence genes and if there was no evidence to suggest colocation of the variable genes.

Table 4.8 Discriminatory powers of *spa*, *dru*, PFGE typing, and DNA microarray profiling[a]

Typing method(s)	No. of types	SID[b]	95% CI
spa	17	66.90	59.36 – 74.44
dru	17	77.83	73.86 – 81.80
PFGE	21	81.34	77.38 – 85.31
Microarray groups[c]	13	83.60	80.70 – 86.50
Dendrogram groups[d]	40	91.00	88.40 – 93.70
spa & *dru*	37	90.84	88.66 – 93.02
spa & PFGE	44	91.00	88.12 – 93.89
dru & PFGE	43	93.57	92.08 – 95.66
Microarray & *spa*	40	91.10	88.30 – 93.80
Microarray & *dru*	40	94.50	93.30 – 95.70
Microarray & PFGE	37	91.10	88.70 – 93.40
Microarray group & dendrogram group	58	94.80	92.80 – 96.80
spa, *dru* & PFGE	65	96.53	95.53 – 97.52

[a] The discriminatory powers of the methods calculated using Simpson's Index of Diversity (with 95% confidence intervals, CI) for ST22-MRSA-IV isolates recovered from BH (n=168)[a]

[b] SIDs for *spa*, *dru* and PFGE and all different combinations of these methods were determined previously by Shore *et al.* (2010). SIDs for microarray groups (MGs), dendrogram groups (DGs) and all combinations involving these, were determined in the present study.

[c] Microarray groups were assigned in the present study based on combinations of antimicrobial resistance and virulence genes identified among the isolates using a DNA microarray (Alere).

[d] Isolates were previously assigned to dendrogram groups (DGs) following construction of a dendrogram based on the averages of the similarity matrices from *spa*, *dru* and PFGE typing data for these isolates and using a cut-off of 98.5% similarity for DG assignment i.e. isolates that shared >98.5% similarity were assigned to the same DG and those with <98.5% similarity were assigned to different DGs (Shore *et al.*, 2010).

4.3.1.5 Comparing the *spa* type and microarray group combinations with dendrogram groups and epidemiological evidence for one ward during two six-week study periods

The combined *spa* typing and microarray groupings for isolates recovered from one ward during two six-week study periods were compared to previously assigned dendrogram groups (Shore *et al.*, 2010), and available epidemiological data (Table 4.9). While many isolates that were assigned to the same DG were also assigned to the same *spa*-MG, differences were also identified between the assignment of isolates to DGs, and to *spa*-MGs (Table 4.9).

4.3.1.5.1. Study period I

The 38 isolates recovered during study period I, were differentiated into 12 DGs, and 10 *spa*-MGs. The majority of isolates (10/11) within the largest DG (DG1a) were assigned to *spa*-MG t032-MG11 with just one isolate belonging to t032-MG12. Using the criteria described in section 4.2.1.3 and Table 4.2, within *spa* type t032 MGs 11, and 12 were considered to be possibly related. However, four additional t032-MG11 or t032-MG12 isolates were also identified but within different DGs (DG-17 and DG-4b for MG11, and DG18 for MG12). The isolates from these DGs differ from DG1a in their PFGE patterns only by one or two PFGE bands (data not shown) which represents a single genetic event (Tenover *et al.*, 1995). Therefore, it is possible that the *spa*-MG correctly assigned the isolates within the same group as the DGs may have resulted in over-interpretation of the differences between PFGE patterns. While the DG assignments showed that the earliest DG1a isolate was recovered from a patient who was MRSA-positive on admission to the ward on the 17/08/07 using the *spa*-MGs the earliest t032-MG1a isolate was recovered from a patient who was found to be MRSA-positive on admission to the ward on the 13/08/07 (Table 4.9). The t032-MG11/-12

isolates were subsequently recovered over the next 15 days from nine environmental sites and four patients, with MRSA from three patients deemed to be hospital associated (HA).

Within the second largest study period I DG (DG-4), which consisted of the DG subgroups DG4a (six isolates) and DG4b (three isolates), a total of four *spa*-MGs were identified (Table 4.9). All DG4a isolates were assigned to either *spa*-MG t1214-MG02 or t1214-MG06, which differ by one gene only and are considered to be closely related (Tale 4.9). However, unlike the DG assignment, DG4b isolates were found to be distinct from DG4a isolates as they were assigned to distinct *spa* types, t1214 and t032, respectively. Within DG4b, the isolates were also assigned to two closely related *spa*-MGs, t032-MG06 and t032-MG11, which are considered to be closely related. No other isolates with these *spa*-MGs were identified in study period I. The closely related t1214-MG02 or t1214-MG06 isolates were recovered from four patients and two environmental sites with the earliest isolate recovered from a bed mattress while the next isolate was recovered two weeks later from a patient whose MRSA was HA (Table 4.9).

Of the three remaining DGs in study period I represented by multiple isolates (DG-17, -16 and-6), isolates were deemed to be indistinguishable using DGs and *spa*-MGs (DG-06 isolates all assigned to t022-MG06) or closely related isolates within DG-17 and DG-16, were each assigned to one of two *spa*-MGs but these were considered to be closely related due to one MG gene difference (Table 4.9).

Of the four pairs of isolates recovered during study period I, only one pair (pair 02) was assigned to the same DG (DG16). This pair was also assigned to the *spa*-MG (t515-02), but an additional pair (pair 05), that was differentiated into two DGs (DG4b

Table 4.9 Dendrogram groups, *spa* types, microarray groups, and epidemiological data for ST22-MRSA-IV MRSA isolates recovered from one ward in Beaumont Hospital in study periods I (2007, *n*=38), and II (2008, *n*=22)

Study period	Dendro-gram grouping[d]	*spa* type-Micro array grouping[c]	Bay	Bed No.	Date of isolation	Sample source	Source of MRSA[a]	Pair No.[b]
I	17	t032-11	B	13	20/08/2007	Patient	HA	Pair 01
I	17	t032-05	A	7	03/09/2007	Patient	OH	n/a
I	17	t032-05	C	16	31/07/2007	Patient	OA	
I	17	t032-11	B	15	13/08/2007	Patient	OA	
I	18	t032-12	E	30	22/08/2007	Pillow	n/a	
I	16	t032-06	E	30	03/09/2007	Patient	OA	
I	16	t032-06	E	31	10/09/2007	Patient	>72 h	
I	16	t515-02	D	23	30/07/2007	Patient	>72 h	Pair 02
I	16	t515-02	D	23	30/07/2007	Mattress		Pair 02
I	15	t032-06	E	18	01/08/2007	Patient	OA	
I	4a	t1214-02	A	6	13/08/2007	Patient	HA	Pair 04
I	4a	t1214-02	B	15	03/09/2007	Patient	OA	
I	4a	t1214-06	E	17	03/09/2007	Patient	OA	
I	4a	t1214-06	B	SO	20/11/2007	Air		
I	4a	t1214-06	E	30	30/07/2007	Mattress		
I	4a	t1214-06	B	15	03/09/2007	Patient	>72 h	
I	4b	t032-06	5	SO	15/08/2007	Bed rail		
I	4b	t032-06	B	15	22/08/2007	Pillow		
I	4b	t032-11	B	15	22/08/2007	Mattress		Pair 05
I	5	t515-02	A	6	15/08/2007	Pillow		Pair 04
I	7	t515-06	E	29	03/09/2007	Patient	OA	
I	1a	t032-11	D	23	21/08/2007	Mattress		
I	1a	t032-11	D	23	21/08/2007	Mattress		
I	1a	t032-11	D	26	21/08/2007	Mattress		
I	1a	t032-11	B	10	22/08/2007	Mattress		
I	1a	t032-11	2	SO	22/08/2007	Mattress		
I	1a	t032-11	D	27	28/08/2007	Patient	HA	
I	1a	t032-12	D	27	17/08/2007	Patient	OA-K	
I	1a	t032-11	B	15	21/08/2007	Patient	OA	Pair 05
I	1a	t032-11	B	11	22/08/2007	Bed frame		
I	1a	t032-11	F	35	22/08/2007	Mattress		
I	1a	t032-11	B	14	27/08/2007	Patient	HA	
I	3	t032-05	C	21	08/08/2007	Patient	OA	
I	6	t022-06	E	29	22/08/2007	Bed frame		Pair 01
I	6	t022-06	B	14	22/08/2007	Mattress		
I	6	t022-06	E	33	04/09/2007	Bed frame		
I	23	t515-04	A	6	08/08/2007	Patient	OA-K	

Continued overleaf

Table 4.9 continued. Dendrogram groups, *spa* types, microarray groups, and epidemiological data for ST22-MRSA-IV MRSA isolates recovered from one ward in Beaumont Hospital in study periods I (2007, *n*=38), and II (2008, *n*=22)

Study period	Dendrogram grouping[d]	*spa* type-Microarray grouping[c]	Bay	Bed No.	Date of isolation	Sample source	Source of MRSA[a]	Pair No.[b]
II	26a	t022-04	A	9	27/05/2008	Mattress		Triplet 01
II	26a	t022-04	A	9	27/05/2008	Bed rail		Triplet 01
II	26a	t022-06	A	7	27/05/2008	Mattress		
II	26b	t032-04	A	9	28/05/2008	Patient	>72 h	Triplet 01
II	33	t032-08	4	SO	29/04/2008	Locker		
II	2	t628-05	B	15	29/04/2008	Mattress		
II	2	t628-05	E	29	26/04/2008	Patient	OA-K	
II	2	t628-05	B	13	27/05/2008	Pillow		
II	2	t628-05	B	13	27/05/2008	Bed rail		
II	2	t628-05	B	12	27/05/2008	Mattress		
II	2	t628-05	B	15	27/05/2008	Mattress		
II	2	t628-05	B	12	29/05/2008	Air		
II	2	t628-05	B	15	29/05/2008	Air		
II	2	t628-05	E	32	29/05/2008	Patient	OA	
II	1a	t032-05	B	15	04/14/2008	Patient	>72 h	Pair 03
II	1a	t032-11	4	SO	22/04/2008	Locker		Pair 03
II	9	t1865-11	D	23	26/05/2008	Patient	OA-K	
II	4a	t1214-06	D	27	23/04/2008	Patient	OA	
II	4a	t4622-02	A	7	24/04/2008	Patient	OA-K	
II	17	t032-11	B	14	26/05/2008	Patient	>72 h	
II	17	t032-11	B	10	27/05/2008	Bed		Pair 06
II	16	t032-06	A	10	26/05/2008	Patient	OA-K	Pair 06

[a] OA, the patient was MRSA positive on admission; OA-K, the patients MRSA-positive status was known at the time of admission to the ward; > 72 h, the patient's MRSA status was determined 72 h after admission to the ward.

[b] The pairs of triplets of isolates recovered during study periods I and II are indicated and include isolates recovered from patients and from their immediate environments during the same study period. Each pair or triplet consists of one isolate from a patients and at least one environmental isolate.

[c] Microarray groups (MGs) were assigned in the present study based on combinations of antimicrobial resistance and virulence genes identified among these isolates using a DNA microarray (Alere) (see section 4.2.1.3).

[d] Isolates were previously assigned to dendrogram groups (DGs) following the construction of a dendrogram based on the averages of the similarity matrices from *spa*, *dru* and PFGE typing data for these isolates and using a cut-off of 98.5% similarity for DG assignment i.e. isolates that shared greater than 98.5% similarity were assigned to the same DG and those with less than 98.5% similarity were assigned to different DGs (Shore *et al.*, 2010). Three DGs (DG-1, DG-4, and DG-26) were divided into subgroups because they included isolates that showed between 98.5 and 99% similarity. Each pair or triplet consists of one isolate from a patient and at least one environmental isolate.

Abbreviations: SO, a single-occupancy room; n/a not applicable.

and DG1a), were assigned to the same *spa*-MG (t032-MG11). These two DGs differ by one PFGE band only (data no shown) and were recovered from the patient and their mattress a day apart, supporting the assignment of these isolates to the same *spa*-MG (Table 4.7). Isolates within pair 01, and pair 04 were assigned to different DGs and *spa*-MGs and were therefore deemed to be different (Table 4.9).

4.3.1.5.2. Study period II

The 22 isolates recovered during study period II were differentiated into nine DGs and 11 *spa*-MGs. All isolates (nine isolates) within the largest DG (DG2) were assigned to *spa*-MG t628-MG05 (Table 4.9) and no other isolates with this *spa*-MG were identified during that study period. The earliest t628-MG05 isolate was recovered from a mattress and isolates within this group were subsequently recovered from five environmental sites and two patients, both of whom were MRSA positive on admission to the ward. Also during study period II, four DG26 isolates, including a triplet of isolates recovered from a patient, their mattress and bed rail, were identified. These were subdivided into two DG subgroups, DG26a and DG26b. The isolates within DG26a and within DG26b were assigned to distinct *spa*-MGs with one of the triplet isolates being assigned as distinct using *spa*-MGs. While the two remaining isolates of the triplet were assigned to the same *spa*-MG within DG26a, the other DG26a isolate was assigned a different but closely related *spa*-MG that differed by one gene only (t032-MG06 and –MG-4) (Table 4.9). Of the remaining two DGs consisting of more than one isolate, the two isolates in one DG, DG17, were assigned to one *spa*-MG (t032-MG11), while isolate in the other, DG4a, were assigned to different *spa*-MGs (t1214-MG06 and t4622-MG02). While the latter isolates were assigned to different *spa* types, the MGs differ by just one gene, and the isolates were recovered within a day of each other, both patients were positive on admission to the ward (Table 4.9).

For the two pairs of isolates recovered during study period II, isolates within pair 03, were assigned to the same DG and to closely related *spa*-MGs, with only one gene difference while isolates within pair 06 were assigned to different DGs but to *spa*-MGs with only one gene difference indicating that they are closely related (Table 4.9). The PFGE patterns of the DGs of the pair 06 isolates differ by <3 bands indicating that they are in fact closely related according to the Tenover criteria (Tenover *et al.*, 1995).

4.3.2 The stability of DNA microarray profiles, *spa* types, and *dru* types of MRSA isolates and derivatives

4.3.2.1 The stability of DNA microarray profiles, *spa* types and *dru* types of MRSA isolates and derivatives were maintained after consecutive rounds of freezing and thawing and after culture at elevated temperatures

No differences were detected in the DNA microarray profiles, *spa*, and *dru* types of the two separate ST22-MRSA-IV isolates (00145 and 03300) following either 10 consecutive rounds of freezing and thawing, or 14 consecutive rounds of growth at elevated temperature (42°C). The parental isolates and all their derivatives exhibited the same DNA microarray profile, *spa* (t032 and t3185, respectively), and *dru* (both dt10o) type (Table 4.3).

4.3.2.2 The stability of *spa*, *dru* and DNA microarray of six pairs of patient isolates

Six pairs of isolates recovered from six patients were investigated for the stability of *spa* and *dru* types and DNA microarray profiles. The time between recovery of the different isolates from the same patient varied from between six days to 19 months (Table 4.3). For two pairs of isolates recovered from patients 5 and 6, no difference was seen between the isolates, which were recovered nine and 19 months apart, respectively. However, differences were seen among the pairs of isolates from the

other patients. The isolates from patient 1 were isolated six days apart, and while both exhibited the same *spa* type (t515), they yielded different *dru* types (*dru* type 10a and 10j, which differ by a single *dru* repeat substitution only), and yielded DNA microarray profiles that differed by two genes (*erm*(B) and *tst*) (Table 4.3). Using the MG interpretation guidelines outlined in Table 4.2, these microarray profiles would be considered to be different.

The isolates from patient 2 (00489 and 00788) and from patient 3 (00638 and 01052) were both recovered 3 weeks apart and differences were detected between the isolate pairs (Table 4.3). The isolates from patient 2 exhibited different *spa* types (t515 and t032, which differ by the presence of two *spa* repeats in t032), and microarray array profiles (four additional genes detected in 00788 and not in 00489, namely *erm*(C), *aacA-aphD*, *ileS2*, and *sec/sel*). Using the MG interpretation guidelines outlined in Table 4.2, these microarray profiles would be considered to be different. The isolates from patient 3 (00638 and 01052) differed using all three typing methods. The *dru* types of these isolates, dt11j and dt10j, differ by the addition of a *dru* repeat in dt11j, the *spa*, t515 and t032, differ by the addition of a *spa* repeat, and a *spa* repeat substitution while there were two gene/gene cluster differences using the microarray (*sec/sel* and IEC type B), all indicating that these isolates were different (Table 4.3).

Lastly, the pair of MRSA isolates from patient 4, 10757 and 12323, were recovered five months apart and these isolates yielded indistinguishable DNA microarray profiles, they exhibited different *spa* types (*spa* type t032 and t052, which differ by the addition of one *spa* repeat), and *dru* types (*dru* type 10j and 10n, which differ by a *dru* repeat substitution) (Table 4.3).

4.4. Discussion

4.4.1 DNA microarray profiling combined with *spa* typing provides enhanced discrimination of highly clonal ST22-MRSA-IV isolates

Improved discrimination of ST22-MRSA-IV isolates is required due to the highly clonal nature of this strain, which has been endemic in Irish hospitals for the last decade. While *spa* typing is one of the most widely used typing methods for differentiating MRSA, its usefulness as a standalone typing method for ST22-MRSA-IV is limited because in many studies the majority of isolates exhibit a small number of *spa* types, with many exhibiting *spa* type t032 (Ghebremedhin *et al.*, 2007; Khandavilli *et al.*, 2009; Soliman *et al.*, 2009; Shore *et al.*, 2010; Simoes *et al.*, 2011). A previous study from this laboratory demonstrated that enhanced discrimination of ST22-MRSA-IV isolates could be achieved by combining *spa*, *dru* and PFGE typing and subsequently assigning isolates to dendrogram groups (DGs). In the present study, the use of a combination of *spa* typing with DNA microarray profiling was investigated as an alternative approach to improving the discrimination of ST22-MRSA-IV isolates. DNA microarray analysis has the advantage that it simultaneously detects a range of genes and alleles from the core and accessory genome, including virulence and antimicrobial resistance genes. The results of the study showed that the combination of *spa* typing, and array profiling yielded a similar discriminatory power to DG assignment as reported by Shore *et al.*, 2010 (SIDs, 91.10 and 91.00, respectively). However, the use of the combination of *spa* typing and DNA microarray profiling has several advantages over the DG assignment approach. Firstly, the assignment of isolates to DGs requires the analysis of complex PFGE banding patterns and composite dendrograms. In contrast, this is not a feature of *spa* typing and microarray profiling where the presence or absence of array genes are automatically detected using specialised software.

Secondly, PFGE is no longer routinely used in many laboratories for typing of MRSA but has been replaced by *spa* typing in many centres. In addition, the use of the Staphy-Type DNA microarray has increased in recent years (Walther *et al.*, 2009; Fessler *et al.*, 2010; Coombs *et al.*, 2010; Küpfer *et al.*, 2010; Luedicke *et al.*, 2010; Monecke *et al.*, 2010; Scicluna *et al.*, 2010; Akpaka *et al.*, 2011; Sanchini *et al.*, 2011; Wolf *et al.*, 2011; Aamot *et al.*, 2012; Coombs *et al.*, 2012; Vincze *et al.*, 2012). Lastly, the combined use of *spa* typing and DNA microarray profiling requires the use of just two typing methods compared to the use of three methods for DG assignment, which can be expensive and time consuming and DNA microarray profiling has the added advantage over DG assignment of accurately predicting an isolates CC/ST, SCC*mec* type as well as detecting an extensive rage of virulence and antimicrobial resistance genes.

The higher SID determined for array groupings (MGs) relative to the corresponding SIDs determined for PFGE, *spa* and *dru* typing as individual typing methods was surprising since each of these methods assigned the isolates to a greater number of types than the microarray (21, 17, 17 and 13 types for PFGE, *spa*, *dru* and the microarray, respectively). This can be explained by the fact that the isolates were more evenly assigned to MGs than to PFTs, *spa* or *dru* types e.g. >50% of isolates were assigned to just two PFTs, a single *spa* type and just two *dru* types. Furthermore, nine *spa* types and six *dru* types consisted of one isolate each, whereas in contrast six MGs consisted of one isolate each with the remaining seven MGs consisting of >5 isolates each. Interestingly, when the MGs were combined with either *dru* types, *spa* types or PFTs, the combination of MGs and *dru* typing yielded the highest SID (94.50) followed by MG and *spa*, and MG and PFGE, the latter two exhibiting the same SID (91.10%), which is comparable with the SID obtained for the assignment of isolates to DGs. This

indicates that the combination of *dru* and MGs also has potential for enhancing discrimination of ST22-MRSA-IV isolates, and warrants further investigation.

The 168 ST22-MRSA-IV isolates were assigned to 13 microarray groups (MGs) based on the presence or absence of 11 antimicrobial resistance and virulence genes/gene clusters, whose presence was found to be variable among the isolates, namely the antimicrobial resistance genes *blaZ*, *erm*(C), *aadD, aacA-aphD, ileS2 lnu*(A), and the virulence genes *sec/sel, egc,* IEC type B (*sak, chp* and *scn*), IEC type F (*entP*, *sak*, *chp*, and *scn*) and ACME. Using the same DNA microarray platform, Monecke *et al.* (2007, 2008a) previously described the variability of *erm*(C), *sec/sel*, *fnbB* and *bbp* among ST22 MRSA, and MSSA isolates from eastern Saxony and the UK as did Scicluna *et al.* (2010) among ST22-MRSA-IV from Malta. However, in the present study the presence or absence of MSCRAMM genes such as *fnbB* and *bbp* were not used for differentiating isolates into MGs because on-going work in this laboratory has indicated that these genes are, in many instances, always present in ST22-MRSA-IV isolates, and can be detected using PCR, and different primers. While this phenomenon requires further investigation, the variability of these genes may be due to nucleotide changes in the DNA microarray primer or probe binding sites for these genes, and these genes are not suitable for differentiating among the ST22-MRSA-IV highly clonal strain.

While isolates with different *spa* types were in some instances assigned to the same MG the combination of *spa* typing and MGs significantly enhanced discrimination of the isolates compared to using *spa* typing or MGs alone (SIDs for *spa*, 66.90, MGs, 83.60 and *spa*-MG, 91.10). For example, six *spa* types were further differentiated by assignment to multiple MGs, including the predominant *spa* type, t032 which accounted for 55.4% of isolates, and were assigned to nine MGs. However, within the six *spa*

types that were represented by more than one MG, there were instances where MGs differed by one gene/gene cluster only and were therefore considered to be closely related as this could represent a single genetic event. For example, within *spa* type t032-MG1/MG2 combinations exhibited just one gene difference. In addition, while many of the MG combinations with a particular *spa* type differed by >1 gene and were mostly considered to be different, there were instances where two MGs were considered to be possibly related because the genes may be linked. For example, MGs 11 and 12, differ by the presence of two genes, *ileS2* and *aacA-aphD*, in MG12 only. These two resistance determinants have been shown elsewhere in this study (Chapter 5, Table 5.5) to be co-located in some isolates.

Comparison of the *spa*-MGs with the previously assigned DGs, and available epidemiological information for the isolates recovered from one ward during two six-week study periods revealed good concordance with DG assignment. For example, during these two study periods, DGs consisting of single isolates only were assigned unique *spa*-MGs, while within the majority of DGs consisting of multiple isolates; isolates were assigned to the same *spa* type and to closely- or possibly-related MGs. There was only one exception to this where the two isolates recovered during study period II belonged to DG4a but to different *spa*-MGs. However, these isolates were assigned to different *spa* types as well as to different MGs indicating that they are not closely related as the DG assignment suggests. In addition, there was no epidemiological evidence to support the DG assignment of these isolates as closely related as they were recovered from two patients who were MRSA-positive on admission to the ward.

There were instances where isolates were assigned to different DGs but to the same *spa*-MG but in each instance the difference between the DGs were found to

represent a single genetic event i.e. <3 PFGE band differences, and therefore should not be considered to be distinct. The *spa*-MGs also highlighted the differences within some of the DGs that were assigned as subgroups i.e. DGs 4a, and 4b and DGs 26a, and 26b, as isolates within each subgroup were assigned to distinct *spa*-MGs. In addition, the *spa*-MGs identified additional isolates involved in the largest cluster during study period I, and identified an earlier isolate as the first isolate of this *spa*-MG type compared to the DG assignments. Lastly, the assignment of isolates to *spa*-MGs also identified an additional pair of isolates as indistinguishable as they were assigned to the same *spa*-MG but to different DGs (DG16, *spa*-MG t032-MG06, and DG15, *spa*-MG t032-MG06). However, the DGs of these isolates only differ by one PFGE band, and were recovered from a patient and the patient's mattress a day apart, supporting the *spa*-MG assignment of these isolates. This data highlight the importance of not over interpreting results from DGs, and that the *spa*-MG assignments are more accurate.

Recent studies have demonstrated the potential of whole genome sequencing and SNP analysis for genotyping and tracking MRSA isolates. However, this technology is still relatively expensive and data analysis is particularly challenging for use on a routine basis and for the foreseeable future remains a research tool rather than a routine typing tool. While it has also been suggested that MLVA may enhance discrimination of certain MRSA clones its use for differentiating ST22-MRSA-IV isolates requires further investigation (Schouls *et al.*, 2009). Another recent study described a novel 19-target binary typing system for MRSA using a multiplex-PCR/reverse line blot hybridisation platform (Cai *et al.*, 2009; O'Sullivan *et al.*, 2012b), which consists of the detection of many similar genes to the DNA microarray used in the present study (toxin, phage and SCC*mec* subtyping). They showed that this combined method yielded a higher discriminatory power (SID 99.40%) than PFGE

(98.70%) or *spa* typing (92.60%) tested on 42 isolates from 15 genotypes including ST239, ST8, ST250, ST1, ST59, ST36, ST45, ST36, and with two isolates belonging to ST22 (O'Sullivan *et al.*, 2012b). However, the higher SIDs obtained for all typing methods used in the O'Sullivan *et al.* 2012 study compared to the results of the present study may be due to the larger sample group (n=168 in the present study; n=42 in O'Sullivan *et al.* (2012)), and the highly clonal nature of the isolates, all ST22-MRSA-IV, investigated in the present study.

In conclusion, the present study has provided convincing evidence of the usefulness of the combination of *spa* typing and DNA microarray profiling for enhanced epidemiological tracking of ST22-MRSA-IV. The use of DNA microarray profiling in combination with *spa* typing not only provides data that can be used for typing isolates but also detects clinically relevant virulence and antimicrobial resistance genes.

4.4.2. The stability of DNA microarray profiles, *spa* and *dru* types

While the results of the present study have shown that the combination of *spa* and DNA microarray profiling can be used to differentiate ST22-MRSA-IV isolates, there is no published data about the stability of the virulence and antimicrobial resistance genes used for differentiating isolates using the DNA microarray. However, a recent study that used a similar DNA microarray scheme reported that MRSA transmission could be identified based on the presence or absence of MGEs (McCarthy *et al.*, 2012). Another study reported that *spa* typing was stable after six months using *in vivo* Kaplan Meier survival experiments (O'Sullivan *et al.*, 2012a). The majority of the variable genes used to differentiate ST22-MRSA-IV in this study have been shown previously to be located on MGEs i.e. transposons, plasmids, pathogenicity islands and

bacteriophages, which can potentially be lost or gained, emphasising the importance of the need to determine the stability of these genes. The present study also showed that *dru* typing combined with DNA microarray profiling could potentially provide even greater discrimination of ST22-MRSA-IV isolates. The *dru* types of two MRSA isolates have been shown previously to be stable following 10 sequential subcultures (Shore *et al.*, 2010). This finding was supported in the present study where no changes were identified in the *dru* types of two ST22-MRSA-IV isolates following repeated freezing and thawing and following sequential subculture at elevated temperatures. Similarly, exposure of the isolates to these conditions did not result in a change in *spa* type or DNA microarray profiles highlighting the stability of these regions and their usefulness for typing. The DNA microarray genes used for differentiating the ST22-MRSA-IV isolates which were found to be stable over time included the IEC genes encoded on lysogenic bacteriophages. Verkaik *et al.* (2011) also identified no change in the IEC type among MSSA following a three-month period of subculture (Verkaik *et al.*, 2011).

Prior to the present study there have been no reports that have investigated how often changes occur in *spa* and *dru* types and in DNA microarray profiles in patients *in vivo*. In the present study six pairs of MRSA isolates, each recovered from the same patient over different time periods, were investigated to determine the stability of the *spa* and *dru* types and DNA microarray profiles. For the two patients with the longest time period between recovery of isolates (nine and 19 months, respectively), no changes were identified indicating the stability of these regions. For the pair of isolates from one patient (patient 5) no *dru* type was assigned to either isolate as no *dru* consensus region was detected. This could be due to a deletion or mutation in the *dru* region. Similar findings were described previously by Witte *et al.* (Witte *et al.*, 2001) who reported on 10 epidemic MRSA isolates from Northern Germany, which yielded a negative *dru*

Southern hybridisation result indicating the absence of the *dru* sequence region. For the four remaining patients, differences were detected in the isolates of each pair using two or three typing methods. This suggested that either (a) there had been a change in the *spa*, *dru* and/or DNA microarray profile of each isolate over time, or (b) that these patients harboured two different MRSA strains. Sakwinska *et al.* (2010) reported that hospitalised patients exchange strains more frequently than healthy carriers (Sakwinska *et al.*, 2010). In the case of each of the four pairs of isolates in the present study the evidence suggested that the patients harboured two different strains. For example, in patient 1 the two isolates were recovered just six days apart but exhibited different *dru* types and different microarray patterns. However, while the *dru* types differed by one repeat substitution only, a change consistent with a single genetic event, the DNA microarray patterns differed by the presence of two additional genes, *erm*(B) (MLS_B resistance determinant) and the toxic shock toxin gene *tst*, in the later isolate. The *erm*(B) gene has been reported previously to be plasmid located (Duran *et al.*, 2012), while *tst* has been found on various pathogenicity islands (Lindsay *et al.*, 1998), but these genes have never been found to be co-located on the same MGE. Using the microarray criteria proposed in the current study (Table 4.2), these two isolates are considered to be different suggesting that the patient became infected with a second strain after acquiring the first strain. However, since the isolate that harboured the additional genes was the isolate that was recovered on the later date and due to the isolates exhibiting the same *spa* type and closely related *dru* types, it is possible that the original strain may have acquired MGEs carrying the genes.

Similarly, differences were identified between the isolates recovered from patient 2, using two of the typing methods and the isolates were recovered three weeks apart. Again these isolates could represent the same strain that had undergone changes

but evidence suggested that they represented two distinct strains. While the isolates shared the same *dru* type, they exhibited different *spa* types, t515 and t032, that differed by two *spa* repeat duplications (additional *spa* repeat 17 and 25 in t032) and their DNA microarray profiles differed by four genes, namely three antibiotic resistance determinants *erm*(C), *aacA-aphD*, *ileS2* and the enterotoxin genes *sec/l*. While *aacA-aphD*, and *ileS2* have been reported in some instances on the one plasmid, there have be no reports of the co-location of these genes with *erm*(C) and/or *sec/l*.

The two isolates recovered from patient 3 and 4, also appeared to be different. The isolates from patient 3 differed by all three typing methods, and were recovered within 3 weeks of each other. They exhibited *spa* types t515 and t032 that differed by two *spa* repeat duplications, *dru* types dt10j and dt11j that differed by the addition of a *dru* repeat and a *dru* repeat substitution and microarray profiles that differed by two distinct gene cluster, IEC and *sec/l* which are located on two distinct MGEs. Lastly, the isolates from patient 4 differed in their *spa* types, (t032 & t557) by the duplication of a *spa* repeat but also in their *dru* types, dt10j and dt10n that differed by a *dru* repeat substitution.

The results of the stability experiments involving exposure to elevated temperatures, and repeated freezing, and thawing indicated that changes in *spa* and *dru* types and DNA microarray profiles is a rare event. Frénay *et al.* (1996) also found the repeat region of the *spa* gene to be stable following repeated subculture (Frénay *et al.*, 1996). However, it cannot be ruled out that in the case of these four pairs of isolates changes may have occurred. The differences detected between the *spa* types among the pairs of isolates in the present study were due to duplications rather than nucleotide changes, and it has been reported previously that deletions and duplications in *spa* types

are more common than point mutations (Brígido Mde *et al.*, 1991; Koreen *et al.*, 2004; Kahl *et al.*, 2005).

It has also been reported that hospitalised patients exchange strains more frequently than healthy individuals (Wertheim *et al.*, 2004). It has yet to be elucidated whether or not host characteristics have a role to play in the acquisition and loss of certain strains. One study found no evidence that hospitalisation, antibiotic use, age or gender were associated with a particular genotype of *S. aureus* (Sakwinska *et al.*, 2009). Interestingly, this study showed that two of the six pairs of isolates investigated were indistinguishable using three different typing methods despite been recovered from the patients months apart indicating persistent carriage of a single strain by these patients. Precise longitudinal sampling of more patients together with detailed molecular epidemiological typing is required in order to further elucidate the stability of the *S. aureus* genome *in vivo*.

Chapter 5

Molecular analysis of high-level mupirocin-resistant MRSA recovered in Ireland between 1998 and 2009

5.1 Introduction

High-level mupirocin resistant (Hi-MupR) MRSA (MIC >1024 mg/L) have emerged in many countries worldwide and among many different MRSA clones (Cookson, 1990; Needham *et al.*, 1994; Udo *et al.*, 2001b; Upton *et al.*, 2003; Pérez-Roth *et al.*, 2004; Simor *et al.*, 2010). In many studies the Hi-MupR gene *ileS2* has been localised to a plasmid, which in most cases has been shown to be cable of spreading between *S. aureus* strains by conjugation. The conjugative *ileS2*-encoding plasmids that have been reported to date vary in size but all belong to the pSK41/pGO1 family of multiresistance plasmids (Firth and Skurray, 1998) (Fig. 5.1). This includes the fully sequenced and annotated plasmids pPR9 (41.7 kb, GenBank entry NC_013653) (Pérez-Roth *et al.*, 2010) and pUSA30 (37 kb, NC_007792) (Diep *et al.*, 2006), and the partially annotated sequence of the two plasmids pV030 (39 kb; NC_010279) and pGO400 (34 kb, no Genbank entry) (Morton *et al.*, 1995) (Fig. 5.1). In each of these four plasmids, *ileS2* is flanked by insertion sequence IS*257* but the direction of transcription of the flanking IS*257* transpoase genes is variable as well as the intergenic region between *ileS2* and IS*257* (Fig. 5.2). The intergenic regions between IS*257* and *ileS2*, including the upstream (L) *orf*2 and downstream (R) *orf*4 in pPR9 encode hypothetical proteins including a complete methyltransferase domain and a truncated fructosamine kinase family of proteins, respectively. The *orf*2 region is truncated in pV030-8, pUSA03 and pGO400, with pVO30-8 encoding the largest downstream region encompassing *orfA* and *orfB* (Pérez-Roth *et al.*, 2010) (Fig. 5.2).

It has been suggested previously that the variability in the regions flanking *ileS2* can be used to assign *ileS2*-encoding plasmids to plasmid types (Pérez-Roth *et al.*, 2011). Using this method a total of nine *ileS2* plasmid types have been assigned to Hi-MupR plasmids from strains belonging to clonal complexes CC30, CC22, CC8 and

CC5 from patients in Spanish hospitals but many plasmids with unfamiliar plasmid types were also identified. In addition to carrying *ileS2*, Hi-MupR plasmids often carry additional resistance determinants. For example, plasmid pPR9, a 41.7 kb conjugative plasmid from a ST36-MRSA-II isolate recovered from a patient in a Spanish hospital, has been sequenced and was found to harbour *ileS2* together with an integrated copy of a Tn*552*-like β-lactamase transposon. The CA-MRSA USA300/ST8-MRSA-IV strain found to harbour plasmid pUSA03 also encoded *erm*(C) encoding resistance to macrolides, lincosamides and streptogramins (MLS). Other Hi-MupR plasmids carrying additional resistance determinants have been reported including those carrying *tetK/tetL* (encoding resistance to tetracycline), *cadA* (cadmium resistance), *aacA-aphD* (resistance to amikacin, gentamicin, kanamycin and tobramycin), *aadD* (resistance to amikacin, kanamycin, neomycin and tobramycin), *qacA*, *qacB* and *smr* (resistance to quaternary ammonium compounds), *dfrS1* (resistance to trimethoprim), *bleO* (resistance to bleomycin) and *lnu*(A) (resistance to lincosamide; Genbank entry number GQ900502.1) (Udo *et al.*, 1994; McDougal *et al.*, 2010).

Recently, a second gene encoding high-level mupirocin resistance has been described, known as *ileS3* or *mupB*. This Hi-MupR gene (*ileS3*) have been identified on a non-conjugative plasmid in three Hi-MupR ST5-MRSA-IV isolates in Canada (Seah *et al.*, 2012). Another form of mupirocin resistance is low-level mupirocin resistance (MIC 8 - 256 mg/L), which is due to mutations in the native chromosomally located isoleucyl-tRNA synthetase gene *ileS*. The most common mutation exhibited by low level mupirocin resistance isolates is the isoleucyl-tRNA synthetase mutation V588F affecting the Rossman fold of the *ileS* protein. This fold is important is critical to allow mupirocin to bind to isoleucyl-tRNA synthetase (Antonio *et al.*, 2002).

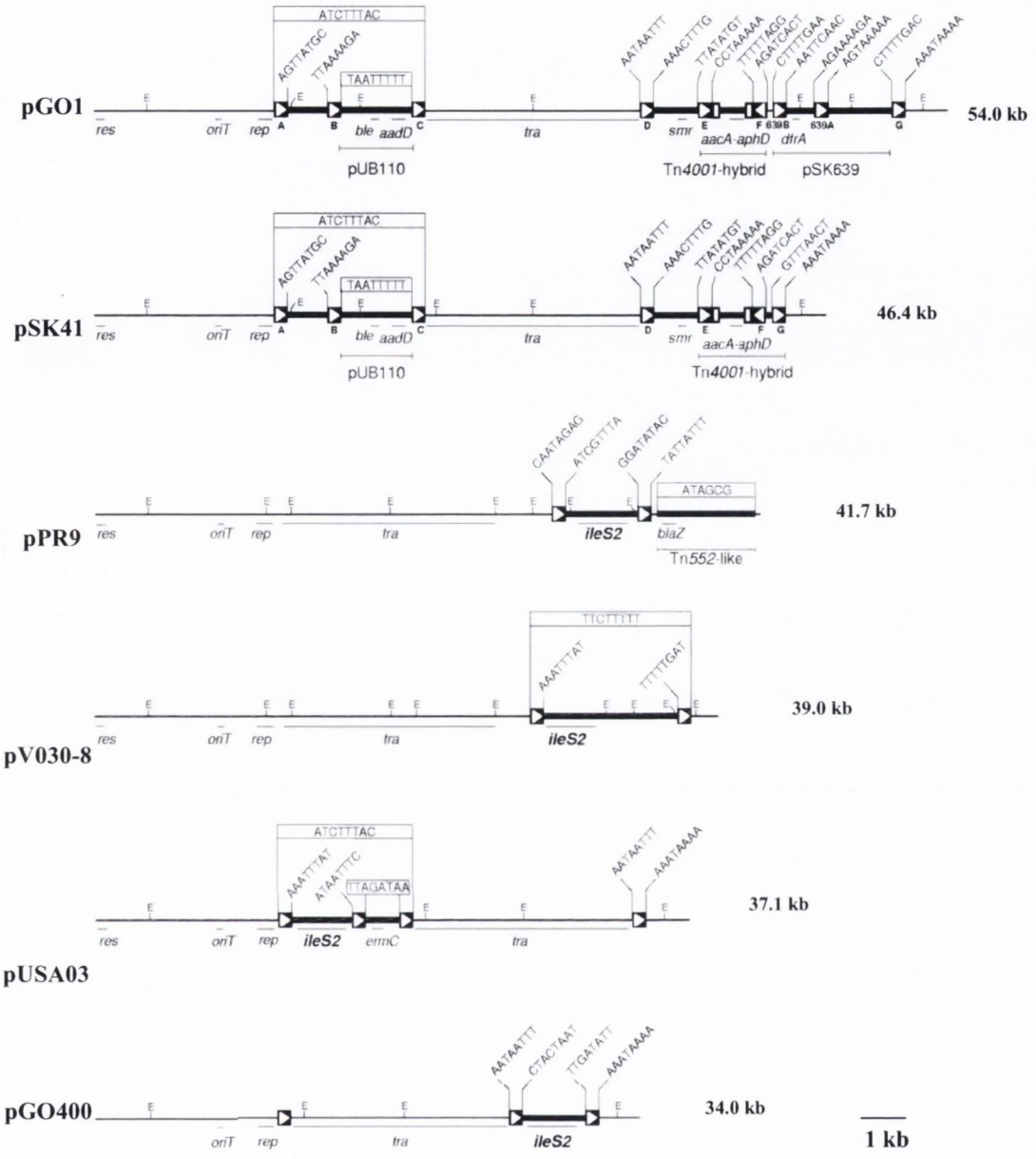


Fig. 5.1 Schematic organisation of previously described high-level mupirocin resistant staphylococcal conjugative plasmids pGO1 (Caryl and O'Neill, 2009), pSK41 (Berg *et al.*, 1998), pPR9 (Perez-Roth *et al.*, 2010), pV030-8, pUSA03 (Diep *et al.*, 2006), and pGO400 (Morton *et al.*, 1995). Plasmid names are shown on the left and plasmid sizes are shown on the right. The locations of various antimicrobial genes are indicated including *aacA-aphD* (gentamicin-tobramycin-kanamycin resistance), *aadD* (kanamycin-neomycin-paromomycin-tobramycin resistance), *ble* (bleomycin resistance), *dfrS1* (trimethoprim resistance), *smr* (antiseptic and disinfectant resistance), *ileS2* (high-level mupirocin resistance), *blaZ* (penicillin resistance), *erm*(C) (macrolide, lincosamide, and streptogramin B resistance). Plasmid mobilisation and replication genes are also indicated; *tra* (conjugative transfer functions), *rep* (replication initiation), *res* (resolvase), and *oriT* (origin of conjugative DNA transfer). The direction of transposase transcription for IS*257* flanking regions are indicated by solid boxes with internal white arrows. Abbreviations: E, *Eco*RI cleavage site. Adapted from Perez-Roth *et al.*, 2010.

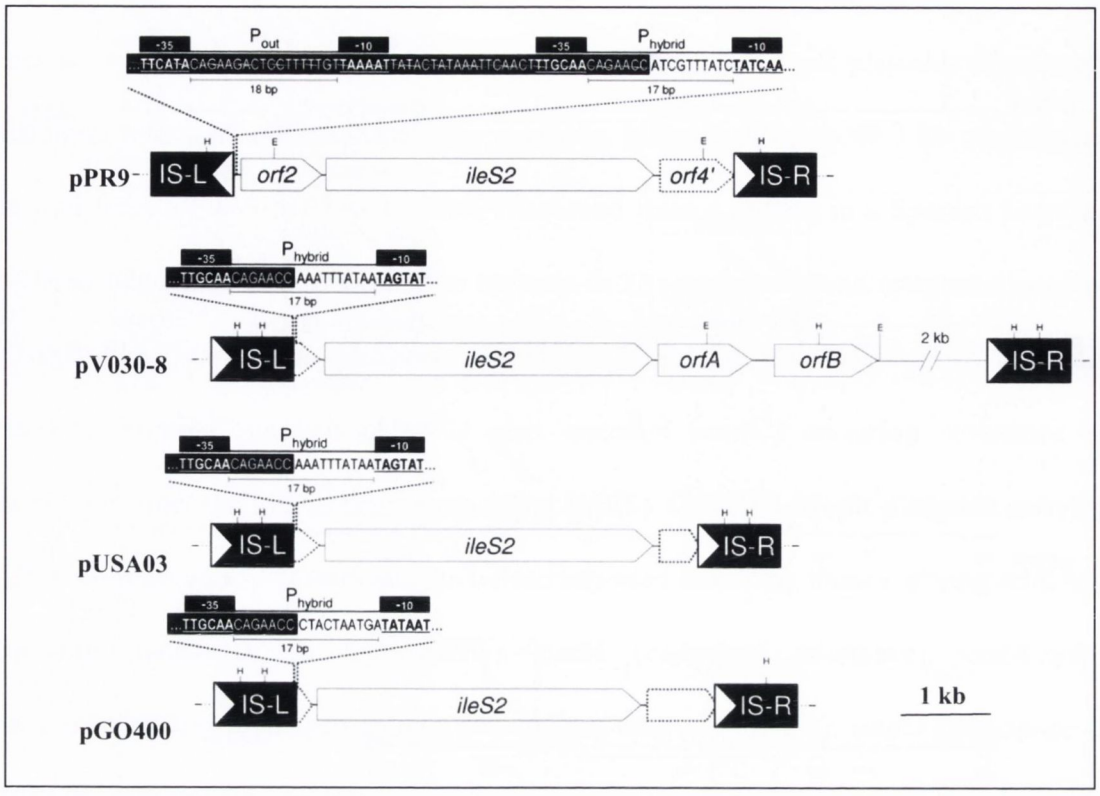


Fig. 5.2 Schematic representation of the genetic organisation of the high-level mupirocin resistance gene *ileS2* flanked by insertion sequence IS*257* harboured by plasmids pPR9, pV030-8, pUSA03, and pGO400. The respective plasmid names are shown on the left. IS*257* regions (IS-L and IS-R) flanking *ileS2* are represented by solid black boxes; the white arrows indicate the direction of IS*257* transposase transcription. Abbreviations: E, and H, *Eco*RI and *Hin*dIII cleavage sites, respectively; *orf*, open reading frame which are represented by arrowed solid white boxes indicating their orientation. A distance scale in kb is given below the figure. Adapted from Perez-Roth *et al.*, 2010.

Previous studies have found evidence to suggest that CoNS are a reservoir for resistance genes in *S. aureus*. Many studies have revealed the increasing prevalence of Hi-MupR among CoNS (Leski *et al.*, 1999; Yun *et al.*, 2003; Cavdar *et al.*, 2004; Petinaki *et al.*, 2004; Wisniewska *et al.*, 2004; Rotger *et al.*, 2005) in particular among *S. epidermidis*, *S. haemolyticus*, *Staphylococcus warneri*, *Staphylococcus hominis*, *Staphylococcus xylosus*, and *Staphylococcus captitis* (Rahman *et al.*, 1990; Leski *et al.*, 1999; Ferreira *et al.*, 2002; Petinaki *et al.*, 2004).

High-level mupirocin resistance and aminoglycoside resistance has been reported in CA-MRSA such as in USA300/ST8-MRSA-IVa, the predominant MRSA clone in the USA, where it is circulating among CA- and HA-MRSA backgrounds (Diep *et al.*, 2006; Tenover and Goering, 2009; Cadilla *et al.*, 2011). Recently High-level mupirocin resistance has been reported in a CA-MRSA clone ST30-MRSA-IV in Japan (Takizawa *et al.*, 2005). The first reports of Hi-MupR plasmids (26 kb and 21 kb) among the community acquired ST80-MRSA-IV clone was in Kuwait where it was identified on two non-conjugative plasmids (Udo and Sarkhoo, 2010). To date there has been no reports of Hi-MupR in CA-MRSA in Ireland (Rossney *et al.*, 2007), however the presence of *ileS2* in our current endemic clone ST22-MRSA-IV, suggests that it may only be a matter of time before further clonal spread of this gene occurs(Rossney and O'Connell, 2008) (NMRSARL annual report 2009). Currently, Irish guidelines recommend that the use of mupirocin topical ointment treatment for MRSA decolonisation should not exceed a dosage of three times a day for five days in order to avert the emergence of mupirocin resistance (SARI, 2005). Despite this, the proportion of Hi-MupR among Irish MRSA isolates from BSIs more than doubled between 1999 and 2009, from 1.4% to 3.1%(Rossney and O'Connell, 2008) (NMRSARL annual report 2009). There has also been a change in the epidemiological types associated with Hi-

MupR, with Hi-MupR MRSA recovered during 2006 and 2007 more commonly exhibiting an AR and PFG pattern indicative of ST22-MRSA-IV (AR-PFG 06-01 and Unfamiliar-01), compared to ST8-MRSA-IIA-IIE (AR-PFG 13-00 and 14-00), which was the more common type among Hi-MupR MRSA recovered in Ireland between 1999 and 2005 (Epi-insight, 2003; Shore *et al.*, 2005). This finding also reflects the increasing prevalence of ST22-MRSA-IV among MRSA from patients with BSIs in Ireland in the last decade and the decreasing prevalence of ST8-MRSA-IIA-IIE since the late 1990s. In addition, more than half of the Hi-MupR ST22-MRSA-IV isolates recovered in Ireland also exhibit resistance to aminoglycoside antimicrobial agents. Despite its increasing prevalence, very little is known about the genetic diversity of Hi-MupR MRSA in Ireland and the *ileS2*-encoding genetic elements that they harbour.

5.1.1 Aims

The principal objective of this part of the present study was to undertake comparative molecular analysis of Hi-MupR MRSA recovered from patients in Irish hospitals between 1998 and 2009. This was achieved by:

- DNA microarray profiling to investigate the genetic background of isolates and to detect virulence-associated and antimicrobial resistance genes including the mupirocin resistance genes *ileS2*.
- Comparative molecular analysis of Hi-MupR-conferring plasmids from MRSA isolates representative of the main genotypes identified among the Hi-MupR isolates.

5.2 Materials and Methods

5.2.1 MRSA isolates

5.2.1.1 High-level mupirocin resistant MRSA isolates investigated by DNA microarray analysis

A total of 109 MRSA isolates recovered from patients in Irish hospitals between 1998 and 2009 were investigated (Table 5.1). This included (a) 77 Hi-MupR MRSA isolates recovered from patients with BSIs in Irish hospitals between 1999 and 2009 as part of EARS-Net, (b) 28 MRSA isolates recovered from patients in a 770-bed acute care hospital in Dublin between 2007 and 2008 as part of a previously described study that were found to harbour *ileS2* following DNA microarray profiling (Shore *et al.*, 2010), and (c) four Hi-MupR MRSA isolates recovered between 1998 and 2002 also as part of a previous study (Shore *et al.*, 2005; Rossney *et al.*, 2006) (Table 5.1). Antibiogram-resistogram and PFGE typing were performed on all isolates in the NMRSARL, all as described previously (Table 5.1) (Rossney *et al.*, 2007) (Chapter 2, section 2.2).

5.2.1.2 High-level mupirocin resistant MRSA isolates selected for plasmid analysis

Seven Hi-MupR MRSA isolates were selected for detailed plasmid analysis and included at least one isolate representative of four of the five main genotypes of Hi-MupR MRSA identified (Tables 5.1). The plasmid-free novobiocin-resistant *S. aureus* strain XU21 was used as a plasmid recipient in filter mating experiments (Udo and Jacob, 1998) (Chapter 2, Table 2.2). In addition, four mupirocin-susceptible MRSA isolates 03693 (ST22-MRSA-IV), AR07/2189 (ST36-MRSA-II), AR13/0066 (ST8-MRSA-II) and M03/0073 (ST239-MRSA-III) were also selected for plasmid analysis for comparison with the plasmid profiles from the Hi-MupR isolates.

5.2.2 Mupirocin powder

Mupirocin powder was provided as a kind gift by GlaxoSmithKline (Great West Road, Brentford, UK).

5.2.3 Antimicrobial susceptibility testing

Isolates recovered prior to 2005 were previously investigated for mupirocin resistance using Stoke's disc diffusion methodology (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b). Isolates were recorded as Hi-MupR if they exhibited no zones of inhibition using both 5 μg and 200 μg mupirocin disks (Oxoid), intermediately resistant to mupirocin if they exhibited a zone of inhibition to the 5 μg disk only and were recorded as mupirocin susceptible if the zone of inhibition around both the 5 μg and 200 μg mupirocin disks were equal to or no more than 3 mm less than the zone obtained with the mupirocin-susceptible control isolate *S. aureus* ATCC 29213 (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b) (Chapter 2, Table 2.2). MRSA isolates recovered from 2005 onwards were investigated for mupirocin susceptibility using CLSI methodology (Chapter 2, section 2.2.1) (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b; Rossney *et al.*, 2006; Rossney *et al.*, 2007).

All isolates also underwent AR typing to a panel of 23 antimicrobial agents using disk diffusion as described previously (Rossney *et al.*, 2007). Comparative antimicrobial susceptibility testing of plasmid-cured derivatives, the plasmid-free *S. aureus* recipient strain XU21 and its transconjugant derivatives were performed by AR typing (Chapter 2, section 2.2.1) and using the mupirocin E-test system (bioMérieux) according to the manufacturer's instructions.

Table 5.1 High-level mupirocin resistant (Hi-MupR) MRSA isolates recovered from patients with bloodstream infections in Irish hospitals between 1999 and 2009 investigated in the present study (*n*=109)

Isolate no.[a]	Year of isolation	AR type[b]	AR pattern	PFT[c]	Reference/ Source
2080	1998	AR14	ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp	00040	Shore *et al.*, 2005
3411	1999	AR14	ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp	00040	Shore *et al.*, 2005
3317.2	1999	AR13	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00071	Shore *et al.*, 2005
E0014	1999	AR14.2	ApCdCpEbErGnKnMcMpNmPmaRfSmSpTb	00123	EARS-Net
E0042	1999	AR14.4	ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp	00040	EARS-Net
E0075	1999	AR07.2	ApCdCpErKnMpNmTb	02029	EARS-Net
E0103[a]	**1999**	**AR14.4**	**ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp**	**00080**	EARS-Net
E0161	1999	AR14.4	ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp	00011	EARS-Net
E0197	1999	AR14.4	ApCpEbErGnKnMpNmSmSpTbTp	00071	EARS-Net
E0273	2000	AR07.0	ApCdCpErLnMpNmSpTb	02017	EARS-Net
E0354	2000	AR14.2	ApCdCpEbErGnKnMcMpNmPmaSmSpTb	00118	EARS-Net
E0477.1	2001	AR13.1	ApCdClCpEbErGnKnLnMcMpNmPmaSmSpTb	00066	EARS-Net
E0491.2	2000	AR13.1	ApAkCdClCpEbErGnKnLnMcMpNmPmaSmSpTb	00133	EARS-Net
E0542	2001	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00029	EARS-Net
E0565	2001	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00030	EARS-Net
E0592[a]	**2001**	**AR13.1**	**ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb**	**00077**	EARS-Net
E0593	2001	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00030	EARS-Net
E0716	2001	AR14	ApCdCpEbErGnKnMpNmSmSpTbTp	00071	EARS-Net
E1121	2002	AR14	ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp	00070	EARS-Net
E1183[a]	**2002**	**AR44**	**AkApCdCpEbErGnKnLnMcMpNmPmaSmSpSuTbTeTp**	**99400**	EARS-Net
E0881	2002	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00001	EARS-Net
E0882.1	2002	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00084	EARS-Net
E1183	2002	AR44	AkApCdCpEbErGnKnLnMcMpNmPmaSmSuTbTeTpSp	99400	EARS-Net
E1350	2003	AR14.4	ApCdClCpEbErGnKnMcMpNmPmaSmSpTbTp	00001	EARS-Net
E1426	2003	AR14.4	ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp	00046	EARS-Net
E1443	2003	AR14.2	ApCdCpEbErGnKnMcMpNmPmaSmSpTb	00062	EARS-Net
E1567	2003	AR43	ApCdCpErKnMpNmSmSpTp	00175	EARS-Net
E1794	2004	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00182	EARS-Net
E1797.2[a]	**2004**	**AR7.0**	**ApCdCpErKnLnMpNmSpTb**	**02017**	EARS-Net
E1890	2004	Unf	ApCdCpErGnKnMpTb	01002	EARS-Net
E2237	2005	New03	ApCdCpErGnKnLnMcMpPmaSpTb	00202	EARS-Net
E2510	2005	New03	ApCdCpEbErGnKnLnMcMpPmaSpTb	00202	EARS-Net
E2554	2005	AR07	ApCdCpErGnKnLnMpNmSpTb	02038	EARS-Net
E2595	2005	Unf	ApCdCpErGnKnLnMpNmSpTb	02020	EARS-Net
E2597	2005	NT	ApCdCpErFdMp	01018	EARS-Net
E2625	2005	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTbTp	00209	EARS-Net
E2632	2005	Unf	ApCdCpErGnKnLnMpNmSpTb	02020	EARS-Net
E2675	2006	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTbTp	00071	EARS-Net
E2696	2006	AR06	ApCpEr	01018	EARS-Net
E2794	2006	Unf	ApCdChCpErGnKnLnMpNmTb	01117	EARS-Net
E2829	2006	NT	ApCdCpErFdLnMpTp	01042	EARS-Net
E2986	2006	Unf	ApCdChCpGnKnMpLnTb	01072	EARS-Net
E2989	2006	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb	00019	EARS-Net
E2908	2006	AR13.1	ApCdCpEbEr GnKnLnMcMpNmPmaSmSpTp	00071	EARS-Net
E3024	2006	AR06	ApCdCpErFdMpTp	01024	EARS-Net
E3118	2006	Unf	ApCpErKnLnMpNmTb	01018	EARS-Net
E3130	2006	AR14.4	ApCdCpErGnKnMpNmSmSpTbTp	00165	EARS-Net
E3148	2006	Unf	ApCdCpGnKnMpTb	01002	EARS-Net
E3188	2006	AR06	ApCdCpErMp	01039	EARS-Net
E3201	2006	Unf	ApCdCpErGnKnMpTb	01018	EARS-Net
E3257	2007	Unf	ApCdCpErGnKnMpTb	01024	EARS-Net
E3295	2007	Unf	ApCdCpGnKnMpTb	01022	EARS-Net
E3335	2007	Unf	ApCdCpEbErGnKnLnMcMpPmaTb	03025	EARS-Net
E3361	2007	Unf	ApCpErGnKnMpRfTb	01134	EARS-Net
E3367	2007	AR13	ApCdCpEbErKnNmMcMpPmaSmSpTp	00064	EARS-Net
E3387	2007	Unf	ApCdCpErGnKnMpTb	01133	EARS-Net
E3388	2007	Unf	ApCdCpErGnKnMpTb	01022	EARS-Net
E3411	2007	Unf	ApCdCpErGnKnLnMpTb	01018	EARS-Net
E3428	2007	Unf	ApCdCpErGnKnLnMpTb	01042	EARS-Net
E3435	2007	Unf	ApCpErGnKnErMpTb	01133	EARS-Net
E3487	2007	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTpTb	00071	EARS-Net

Continued overleaf

Table 5.1 continued. High-level mupirocin resistant (Hi-MupR) MRSA isolates recovered from patients with bloodstream infections in Irish hospitals between 1999 and 2009 in the present study (*n*=109)

Isolate no.[a]	Year of isolation	AR type[b]	AR pattern	PFT[c]	Reference/ Source
E3553	2007	Unf	ApCdCpErGnKnMpTb	01018	EARS-Net
01258	2007	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
00833	2007	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
00745	2007	Unf	ApCdCpEyGnKnMpTb	01018	Shore *et al.*, 2010
00208	2007	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
01329	2007	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
01375	2007	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
01959	2007	Unf	ApCdCpErGnKnMpTb	01018	Shore *et al.*, 2010
E3833	2008	Unf	ApCdCpErGnKnLnMpTb	01054	EARS-Net
E3835	2008	Unf	ApCdCpErFdLnMpTb	01024	EARS-Net
E3848	2008	AR13	ApCdCpEbErGnKnMcMpNmPmaSmSpTb	00155	EARS-Net
E3860	2008	AR14.4	ApCdCpEbErGnKnMcMpNmPmaSmSpTpTb	00070	EARS-Net
E3868	2008	Unf	ApCdCpErLnMpTb	01039	EARS-Net
E3886[a]	**2008**	**AR06**	**ApCdCpErMpTp**	**01042**	EARS-Net
E3901	2008	AR13.1	ApCdCpEbGnKnLnMcMpNmPmaSmSpTpTb	00001	EARS-Net
E3911	2008	AR13.1	ApCdCpEbErGnKnLnNmMcMpPmaSmSpTb	00025	EARS-Net
E3942	2008	NT	ApCdCpErFdLnMp	01024	EARS-Net
E3948	2008	AR14	ApCdCpEbErGnKnNmMpSmSpTbTp	00165	EARS-Net
E3988	2008	Unf	ApCdCpErGnKnMpTb	01024	EARS-Net
E4088	2008	AR14	ApCdCpEbErGnKnNmMpSmSpTpTb	99085	EARS-Net
E4134	2009	AR06	ApCpErMpTp	01042	EARS-Net
E4137	2009	AR13.1	ApCdCpEbErGnKnLnMcMpNmPmaSmSpTpTb	00013	EARS-Net
E4139	2008	Unf	ApCdCpErGnKnMpTb	01018	EARS-Net
05412	2008	Unf	ApCpErKnMpTb	01024	Shore *et al.*, 2010
05327	2008	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
05219	2008	Unf	ApCdCpErGnKnMpTb	01024	Shore *et al.*, 2010
05599	2008	Unf	ApCpErGnKnTb	01018	Shore *et al.*, 2010
05840	2008	Unf	ApCpErGnKnMpTb	01006	Shore *et al.*, 2010
05656	2008	Unf	ApCpErGnKnTb	01018	Shore *et al.*, 2010
03064	2008	Unf	ApCpErGnKnTb	01018	Shore *et al.*, 2010
05354	2008	Unf	ApCdCpErGnKnTb	01006	Shore *et al.*, 2010
03123	2008	Unf	ApCdCpErKnLnMpTb	01154	Shore *et al.*, 2010
03219	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03300	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03213[a]	**2008**	**Unf**	**ApCdCpErKnLnMpTb**	**01154**	Shore *et al.*, 2010
03222	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03225	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03376	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03636	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03638	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03642	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03663	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03832	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
03646	2008	Unf	ApCdCpErGnKnLnMpTb	01154	Shore *et al.*, 2010
E4175	2009	Unf	ApCdCpErGnKnMpTb	01165	EARS-Net
E4226[a]	**2009**	**Unf**	**ApCdCpFdGnKnLnMpTb**	**01006**	EARS-Net
E4258	2009	AR14.4	ApCdCpErGnKnNmMpSmSpTpTb	00165	EARS-Net
E4322	2009	Unf	ApCdCpErGnKnLnMpTb	01154	EARS-Net
E4327	2009	Unf	ApCdCpErGnKnMpTb	01024	EARS-Net

[a] Isolates shown in bold underwent plasmid analysis.

[b] Antibiogram-resistogram (AR) type; Unf, unfamiliar, these isolates exhibited a hitherto unfamiliar AR pattern; NT, nontypeable, these isolates displayed an AR pattern associated with AR06 but were urease positive. New; AR patterns identified in 1999 that were assigned the AR types, New01, New02 and New03.

[c] PFT, pulsed-field type.

Abbreviations: Ak, amikacin; Ap, ampicillin; Cd, cadmium nitrate/cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenylmercuric acetate; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; Tb, tobramycin; Te, tetracycline; Tp, trimethoprim.

5.2.4 Mupirocin minimum inhibitory concentration (MIC) determination

All isolates underwent mupirocin MIC determination using the E-test system (bioMérieux) according to the manufacturer's instructions using *S. aureus* strain ATCC292133 as a mupirocin-susceptible control reference strain (Table 2.2). MIC determination was undertaken by sub-culturing the isolates onto CBA and incubated at 35°C for 24 h. Briefly, an inoculum of each isolate was prepared separately in sterile PBS (Dulbecco A, Oxoid) in glass bijou bottles to a density equivalent to a 0.5 McFarland turbidity standard (bioMérieux). Inoculum suspensions are only stable for 15 min. Bacterial suspensions were lawned onto Müller Hinton (MH) agar (Oxoid) plates by using a sterile swab dipped into the inoculum suspension. Prior to lawning, excess inoculum was removed from the swab by pressing firmly against the wall of the bijou. The MH agar plate was lawned by swabbing downwards from side to side to half down the MH agar plate, and this process was repeated on the other half of the plate. The mupirocin E-test (bioMérieux) was used according to manufacturer's instructions by applying the strip to the plate carefully while holding the area labelled "E" on the E-test strip with a forceps. Plates were inverted and incubated at 35°C for 18 h. After incubation, the plates were examined closely, using a magnifying glass if necessary, to look for the presence of colonies within the elliptical zone of inhibition. If growth occurred along the entire strip i.e. no inhibition ellipse present, the MIC was recorded as greater than the highest value on the reading scale. When the inhibition ellipse zone was below the strip i.e. the zone edge did not intersect the strip, the MIC was recorded as less than the lowest value on the reading scale. The mupirocin E-test zone of inhibition was read at 80% as this antibiotic is bacteriostatic. MIC values are reported as the nearest two fold dilution, before making an interpretation of susceptible (S) < 4 mg/L,

intermediate (I) between 8.0-128.0 mg/L, or resistant (R) > 256 mg/L to mupirocin (Fig. 5.3).

5.2.5DNA microarray profiling

The 109 Hi-MupR MRSA isolates, several plasmid-cured derivatives, the plasmid-free *S. aureus* recipient strain XU21 and its transconjugants were subject to DNA microarray profiling using the Staphy-Type Kit (Alere) according to the manufacturer's instructions as previously described in Chapter 2, section 2.4.5.

5.2.6 PCR amplification of mupirocin resistance determinants and flanking regions

5.2.6.1 PCR detection of *ileS2*, *mupB* and *ileS*

Three isolates that were previously shown to be susceptible to mupirocin by AR typing were identified as positive for the *ileS2* gene by DNA microarray profiling. These isolates were investigated for the presence of the entire Hi-MupR gene *ileS2* by PCR using the primers Mup1A-F and Mup1A-R (Table 5.2). Thirty-two isolates representative of the different ST and SCC*mec* type combinations identified among the Hi-MupR isolates, including the seven Hi-MupR MRSA isolates selected for plasmid analysis, were also investigated for the presence of the recently described Hi-MupR gene *mupB* (*ileS3*) by PCR as described previously (Seah *et al.*, 2012) (Table 5.2). In addition, mutations in *ileS* that have been found to be associated with low-level mupirocin resistance were investigated by PCR and amplimer sequencing as described previously (Antonio *et al.*, 2002) in the seven Hi-MupR isolates selected for plasmid analysis (Table 5.2). PCR amplifications, amplimer purification and DNA sequencing of the *ileS* gene using primer walking was performed as described in Chapter 2, section 2.4.2.

Table 5.2 Primers and thermal cycling conditions used for PCR detection of mupirocin resistance determinants

Primer Name	Primer sequence (5'-3')	Thermal cycling conditions	Primer application	Expected amplimer size	Reference
MupA MupB	TATATTATGCGATGGAAGGTTGG AATAAAATCAGCTGGAAAGTGTTG	94°C for 5 min; 10 cycles of 94°C for 30s, 64°C for 30s and 72°C for 45s; 20 cycles of 94°C for 45s, 50°C for 45s and 72°C for 1 min; and a final extension for 10 min at 72°C	*ileS2* probe for Southern blot analysis	458 bp	Perez-Roth *et al.*, 2001
mupB-F mupB-R	CTAGAAGTCGATTTTGGAGTAG AGTGTCTAAAATGATAAGACGATC	94°C for 1 min; 35 cycles of 94°C for 30s, 53°C for 30s, and 68°C for 4 min; and a final extension for 10 min at 72°C	Amplification of the *mupB*	674 bp	Seah *et al.*, 2012
*ileS*54L *ileS*2798U	GAGCAATCGTCCCTTTTA TTCATCAACAGCACCAAG	94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension for 10 min at 72°C	Amplification and sequencing of the *ileS*	2726 bp	Antonio *et al.*, 2002
Mup1A-F Mup1A-R	CAATAGAAAAGGACAGGAAAG GATTCTAATAATTCCCCAGTTACA	94°C for 3 min; 32 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 5 min; and a final extension for 10 min at 72°C	Amplification and sequencing of the *ileS2*	412 bp	Present study
*ileS*2-5 *ileS*2-3 IS257F IS257R	CCATGTCAACCCAGTATCC TCGGTGTAACTGGGGAATTA GGCATGGCGAAAATCCGTAG TGGCGTATTGATGAGACGTACATC	94°C for 5 min; 30 cycles of 94°C for 30s, 60°C for 40s, and 72°C for 1 min; and a final extension for 10 min at 72°C	Amplification and sequencing of the *ileS2*-IS*257*(L) and *ileS2*-IS*257*(R)	957-4447 bp	Perez-Roth *et al.*, 2011

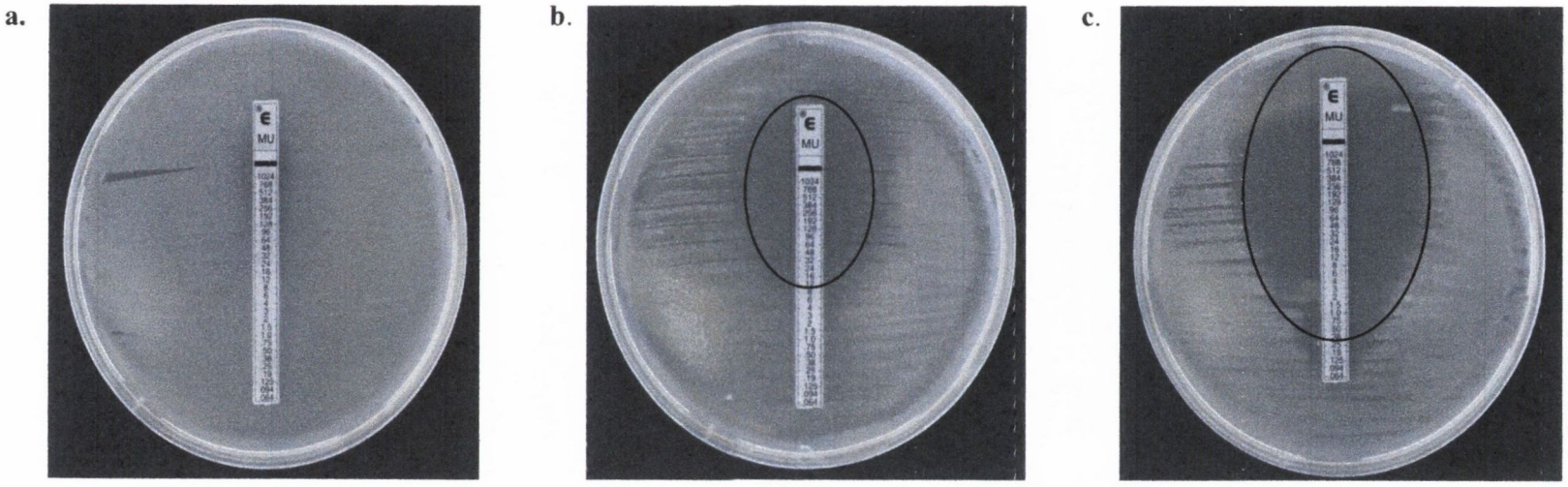


Fig. 5.3 Mupirocin minimum inhibitory concentration (MIC) determination using the E-test system (bioMérieux). If growth occurs along the entire strip (i.e. no inhibition ellipse is observed), then the MIC is recorded as greater than the highest value on the reading scale (> 1024 mg/L) (panel a). When the inhibition ellipse zone is between 8.0-128.0 mg/L (i.e. the zone intersects at 8.0-128.0 mg/L), then the MIC is recorded to the nearest two-fold dilution (panel b). When the inhibition ellipse zone is below the strip (i.e. the zone edge does not intersect the strip), then the MIC is recorded as less than the lowest value on the reading scale (<4 mg/L) (panel c). When interpreting the mupirocin MIC, the mupirocin E-test inhibition is read at 80% as it is a bacteriostatic drug. The MIC values are reported as the nearest two-fold dilution, before making an interpretation of susceptible (S) < 4 mg/L, intermediate (I) between 8.0-128.0 mg/L, or resistant (R) > 256 mg/L to mupirocin. *S. aureus* strain ATCC292133 was used as a mupirocin-susceptible control strain for mupirocin E-test determinations.

5.2.6.2 Characterisation of the *ileS2* flanking region

Characterisation of the *ileS2* flanking sequences in transconjugant derivatives of XU21 harbouring *ileS2*-encoding plasmids from the seven Hi-MupR MRSA isolates selected for detailed plasmid analysis (Table 5.1) was performed by PCR and sequencing from the left IS*257* junction to *ileS2* and from *ileS2* to the right IS*257* junction as previously described (Perez-Roth *et al.*, 2010). PCR amplifications, amplimer purification and DNA sequencing with the *ileS2*-IS*257* PCR primers was performed as described in Chapter 2, section 2.4.2 (Table 5.2). Each new distinct configuration of *ileS2* and its flanking IS*257* regions were assigned a new roman numeral a *ileS2*-IS*257* designation previously described by Perez-Roth *et al.* 2011, i.e. XVI to XX.

5.2.7 Plasmid analysis

5.2.7.1 Plasmid curing

Plasmid curing was performed on the three isolates selected for plasmid analysis due to the presence of multi-plasmids (Table 5.1).To enhance the curing process the Hi-MupR isolates were first incubated under nutrient-depleted conditions. Each isolate was cultured in 5 ml LB (Oxoid) at 37°C for 18 h at 200 rpm. One millilitre of the overnight culture was centrifuged at 15, 970 x *g* for 2 min using an Eppendorf centrifuge (Eppendorf model 5417C fitted with an F-45-30-11 rotor), the supernatant was removed and the pellet was resuspended in 1 ml phosphate buffered saline (PBS) (Oxoid) and centrifuged again and the process was repeated three times. The final pellet was again resuspended in 1 ml PBS and 100 µl of this solution was added to six serial dilutions (doubling dilutions) of LB and were incubated at 37°C for 18 h at 200 rpm. Following incubation, the dilution showing visible growth (end-point) was selected and

the process was repeated as described above for seven consecutive days. For curing experiments, the highest doubling dilution showing visible growth from the final round of nutrient depletion was subcultured into BHI broth (Oxoid), incubated at 43°C for 24 h at 200 rpm and subcultured into fresh BHI broth for five consecutive rounds. Individual colonies obtained following plating on BHI agar were screened for loss of resistance to mupirocin (100 mg/L) by replica plating. Putative cured derivatives underwent AR typing, mupirocin MIC determination and DNA microarray as described above (sections 5.2.3, 5.2.4 and 5.2.5).

Plasmid curing rates were calculated as the number of mupirocin-susceptible cells (i.e. individual cells that grow up to form a colony) on BHI agar supplemented with mupirocin (100 mg/L) proportional to the the number of resistant cells after the curing regimen also grown on BHI agar supplemented with mupirocin (100 mg/L).

5.2.7.2 Filter mating

Conjugative transfer of mupirocin resistance from the seven selected Hi-MupR isolates to the plasmid-free, novobiocin-resistant *S. aureus* recipient strain XU21 was performed by filter mating (Woodford *et al.*, 1998) (Chapter, section 2.6.2).Putative transconjugants were plated onto BHI agar containing 100 mg/L novobiocin to select for the recipient strain and 100 mg/L mupirocin to select for putative *ileS2*-encoding plasmids. Putative transconjugants underwent AR typing, mupirocin MIC determination and DNA microarray analysis as described above (sections 5.2.3, 5.2.4 and 5.2.5). The rate of conjugal transfer was expressed as the number of transconjugants grown on BHI supplemented with novobiocin (100 mg/L) and mupirocin (100 mg/L) per donor cells grown on non-selective BHI agar.

5.2.7.3 Plasmid DNA extraction

Plasmid DNA from parental Hi-MupR MRSA isolates, plasmid-cured derivatives and transconjugant derivatives of XU21 was extracted and purified using a modified alkaline lysis method (Schwarz *et al.*, 1989) (Chapter 2, section 2.6.3). Various Molecular markers were used as plasmid molecular size reference markers for plasmid profiling as follows; M1, pVA517A (German colocation of microorganisms and cell culture (DSMZ), Braunschweig, Germany), M2 (*E. coli*NCTC 50193), M3 (*E. coli* NCTC 50192) (Health Protection Agency Culture Collections,Salisbury, UK) and M4 supercoiled DNA ladder (Biolabs Inc., Ipswich, USA).

5.2.8 Size determination of Hi-MupR plasmids

To estimate the size of the *ileS2*-encoding plasmids, whole-cell DNA of transconjugant derivatives were digested with the endonuclease S1 nuclease Thermo Fisher Scientific) in agarose gel plugs and then separated by PFGE as described previously (Barton *et al.*, 1995; Rosvoll *et al.*, 2010; Liu *et al.*, 2012b). This procedure involves the lysis of the bacterial cells and the S1 nuclease treatment converts plasmid into a linear form. A 10 µl aliquot of whole-cell culture from overnight BHI broth at 37°C, was resuspend in 500 µl EC buffer (1 M NaCl, 100 mM EDTA, 6 mM Tris-HCL (pH 7.6) 0.5% (w/v) Brij 58 (Polyoxyethylene 20 cetyl ether detergent) 0.2% (w/v) deoxycholate, 0.5% (w/v) N-lauroylsarcosine). This suspension was added to an equal volume of molten 1% (w/v) InCert agarose (BioRad, Marnes La Coquette, France) and maintained at 45°C, followed by the addition of RNase (20 µg/ml) (Sigma), lysozyme (1 µg/ml) (Sigma), and lysostaphin (50 µg/ml) (Sigma). One hundred microliter volumes of this suspension was transferred into casting moulds (4 x 9 x 25 mm;

BioRad) and allowed to cool for 10 min at 4°C. Solidified plugs were incubated for 1 h at 37°C in 1 ml of EC lysis buffer with RNase, lysozyme, and lysostaphin at the same concentration as described above. Each plug was then incubated overnight at 50°C in 100 µl 0.5 M EDTA (pH 8.0), 1% *N*-lauroylsarcosine (Sigma) and 1 mg/ml proteinase K (Sigma). The agarose plugs were then washed twice for 2 h each with 1 ml of freshly prepared 1.0 mM phenylmethysulfonyl fluoride (PMSF, Sigma) in TE (10 mM Tris, pH 7.5 and, 1 mM EDTA) at 37°C to inactivate the proteinase K, followed by 2 x 1 h incubations at 37°C in 1 ml volumes of TE buffer to remove the PMSF. Agarose plugs were used immediately or stored in ES buffer (0.5 M EDTA (pH 8.0), 1% N-lauroylsarcosine) at 4°C. Agarose plugs were cut into 2-3 mm sections and placed into an 1.5 ml Eppendorf tube with 300 µl 1 x S1 nuclease buffer (200 mM sodium acetate (pH 4.5 at 25°C), 1.5 M sodium chloride (NaCl) and 10 mM zinc sulfate ($ZnSO_4$) (Thermo Scientific) for 20 min. The buffer was discarded and replaced by 100 µl fresh 1 x S1 nuclease buffer along with 100 µl 8U S1 nuclease (Thermo Scientific) and incubated at 37°C for 45 min. This reaction was stopped by the addition of 10 µl of 0.5 M EDTA (pH 8) for ca. 5 min, and the buffer was then replaced with 10 µl of 0.5 M TBE and incubated for 20 min at 4°C. The S1 nuclease-digested agarose plugs were loaded into 1% (w/v) ultra-pure DNA grade agarose gels (BioRad) and electrophoresed in a CHEF-DR III PFGE device (Bio-Rad) at 4°C, with a pulse time of 1–12 s, at 6 V cm on a 120° angle in 0.5 x TBE buffer 17 h. The *S. aureus* reference strain NCTC8325 digested with *Sma*I (In silico, website; insilico.ehu.es) was used as a molecular weight ladder (M5) (Bikandi *et al.*, 2004). The S1 nuclease digestion was carried by our collaborators at the Institute of Farm Animal Genetics, Friedrich-Löffler-Institut, FLI, Neustadt-Mariensee, Germany.

5.2.9 Southern blot analysis

The restriction fragment carrying *ileS2* was detected in transconjugant derivatives based on the sum of the fragment sizes obtained following digestion of plasmid DNA with the restriction endonucleases *Eco*RI and *Hin*dIII (Promega). Southern hybridisation analysis of the *Hin*dIII-digested plasmid DNA from the seven Hi-MupR isolates was performed using a *Hin*dIII-digested 458 bp *ileS2* PCR product generated using previously described primers (Pérez-Roth *et al.*, 2001) (Table 5.2). The probe was labelled using digoxigenin-11-dUTP and the PCR DIG probe synthesis kit according to the manufacturer's instructions (Roche). The *Hin*dIII-digested plasmid DNA from the seven Hi-MupR isolates was transferred to a nylon membrane (Roche) and Southern blot analysis was carried out by DNA hybridisation. Post-hybridisation washes and detection of the hybridised nylon membrane were performed according to DIG labelling manufacturer's instructions (Roche) (Sambrook, 2001). DNA Molecular Weight Marker II, DIG-labelled (M6) (Roche) was used as a molecular size reference marker for Southern blot analysis. Southern blot analysis results were confirmed by our collaborators at the Institute of Farm Animal Genetics, Friedrich-Löffler-Institut, FLI, Neustadt-Mariensee, Germany.

5.3 Results

One-hundred and nine Hi-MupR MRSA isolates recovered from patients in Irish hospitals between 1998 and 2009 underwent DNA microarray profiling to determine their genetic backgrounds and to investigate carriage of virulence-associated and antimicrobial resistance genes including the high-level mupirocin resistance gene *ileS2*. In addition, all isolates underwent mupirocin MIC determination and seven isolates, representative of the different AR/genotypes identified among Hi-MupR isolates from Ireland (shown in bold type in Table 5.1) were also subjected to plasmid analysis in order to characterise and locate the genetic element(s) encoding high-level mupirocin resistance among Irish MRSA.

5.3.1. Mupirocin susceptibility testing

The majority of the Hi-MupR MRSA (96%; 105/109) exhibited a mupirocin MIC indicative of high-level mupirocin resistance i.e. MIC > 1024 mg/L during the present study. The remaining four isolates exhibited a mupirocin MIC < 4 mg/L, indicative of susceptibility to mupirocin. Three isolates (05656, ST22-MRSA-IV; 05599, ST22-MRSA-IV; 03064, ST22-MRSA-IV) were found to harbour *ileS2* by the DNA microarray profiling, but were susceptible to mupirocin by mupirocin disk susceptibility testing using the Mup5 (5 mg/L mupirocin) and Mup200 (200 mg/L) disks respectively. The entire *ileS2* gene in each of these three isolates was amplified by PCR and sequenced. The same mutations were identified in all three isolates, namely a base pair (adenine) deletion at position 272 and a base pair (adenine to guanine) substitution at position 1752 (A1752G) resulting in an earlier stop codon at lysine position 93. The remaining isolate, which was previously identified as phenotypically resistant to mupirocin by AR typing and by MIC determination by the NMRSARL, was

found in the present study to be negative for *ileS2* gene by microarray profiling. Antimicrobial susceptibility testing and *ileS2* gene PCR analysis were repeated on this isolate (E0491.2; ST8-MRSA-IID and SCC_{M1}), which revealed that this isolate did not harbour the *ileS2* gene and was mupirocin susceptible (Table 5.3).

5.3.2. Genotyping of the Hi-MupR isolates by DNA microarray profiling

The 109 Hi-MupR MRSA isolates recovered in Irish hospitals between 1998 and 2009 were assigned to five genotypes within four CCs (Table 5.4). The ST22-MRSA-IV clone predominated (56%), followed by ST8-MRSA-IIA-E with or without SCC_{M1} or non-typeable SCC*mec* (36%), ST36-MRSA-II (7%), ST239-MRSA-III & SCC*Hg* (1%) and ST5-MRSA-IV (1%) (Tables 5.3 and 5.4).

5.3.2.1 Antimicrobial resistance and virulence-associated genes detected by DNA microarray profiling

Of the 109 isolates investigated for the presence of *ileS2* by the DNA microarray, 108 (99%) were found to harbour *ileS2*. One isolate lacked *ileS2* (isolate E0491.2; ST8-MRSA-IID & SCC_{M1}) (section 5.3.1). The *agr*, capsule and immune evasion cluster (IEC) types as well as the antimicrobial resistance and virulence-associated genes identified among the 109 Hi-MupR MRSA isolates are shown in Table 5.4. The majority of the Hi-MupR isolates belonged to *agr* type I (92%; 101/109) and capsule type 5 (92%; 101/109). The most common antimicrobial resistance genes apart from *mecA*, *blaZ* and *ileS2* were *aacA-aphD* (encoding resistance to amikacin, gentamicin, kanamycin and tobramycin; 85%; 93/109), *erm*(C) (encoding MLS resistance including resistance to erythromycin, lincomycin and clindamycin; 56%; 61/109), *aadD* (encoding resistance to amikacin, kanamycin, neomycin and tobramycin; 45%; 49/109) *aphA3* and *sat* (encoding resistance to kanamycin and neomycin; 36%;

39/109), *sdrM* and *fosB* (encoding resistance to norofloxacin, acriflavine and fosfomycin, respectively; 44%; 48/109), *qacA* and *qacC* (encoding resistance to quaternary ammonium compounds, ethidium bromide, respectively; 37%; 40/109 and 13%; 14/109, respectively), *lnu*(A) (encoding resistance to lincomycin; 34%; 37/109), *merA&merB* (encoding resistance to mercury; 32%; 35/109), *cat*-pC1221 (encoding resistance to chloroamphenicol; 4%; 4/109), *dfrS1* (encoding resistance to trimethoprim; 6%; 7/109) and *tet*(K) and *tet*(M) (both encoding resistance to tetracycline; 2%; 2/109 and 1%; 1/109, respectively) (Table 5.4).

Superantigen genes detected included the enterotoxin gene cluster (*egc*, 62%; 68/109), enterotoxin A (*sea*, 38%; 42/109), enterotoxins C and L (*sec*& *sel*, 34%; 37/109) and the toxic shock toxin (*tst*, 6%; 7/109). The IEC genes detected included *sak* (92%; 100/109), *scn* (90%; 98/109), *chp* (58%; 63/109) and *sep* (2%; 2/109), and the most common IEC type was B (*sak*, *chp* & *scn*, 50%; 55/110). Fourteen percent of the ST22-MRSA-IV isolates harboured the arginine catabolic mobile element (ACME)-*arc* which has been reported previously for these isolates (Shore *et al.*, 2011b) (Table 5.4).

5.3.3 Investigation of the presence of *ileS3* and low-level mupirocin resistance mutations in *ileS*

Thirty isolates were investigated for the presence of the Hi-MupR-encoding *ileS3* gene including isolates belonging to ST22-MRSA-IV (*n*=12) isolates, ST8-MRSA-IIA-IIE (*n*=13), ST5-MRSA-IV (*n*=1), ST36-MRSA-II (*n*=3) and ST239-MRSA-III & SCC*Hg* (*n*=1). This included the seven Hi-MupR isolates selected for plasmid analysis. All thirty Hi-MupR isolates were found to be negative by *mupB* PCR.

Mutations were detected in the chromosomal located isoleucyl t-RNA synthetase-encoding gene *ileS* in all seven isolates selected for plasmid analysis. The

Table 5.3 High-level mupirocin resistant MRSA isolates strain assignment and mupirocin susceptibility testing

Isolate no.	Year of isolation	AR type	DNA microarray assignment			Disc Diffusion		Mupirocin MIC[a]
			CC	ST	SCC*mec*	Mup5	Mup200	
2080	1998	AR14	CC8	ST8	II	R	R	>1024 mg/L
3411	1999	AR14	CC8	ST8	II	R	R	>1024 mg/L
3317.2	1999	AR13	CC8	ST8	II	R	R	>1024 mg/L
E0014	1999	AR14.2	CC8	ST8	II	R	I	>1024 mg/L
E0042	1999	AR14.4	CC8	ST8	II	R	R	>1024 mg/L
E0075	1999	AR07.2	CC30	ST36	II	R	R	>1024 mg/L
E0103[b]	**1999**	**AR14.4**	**CC8**	**ST8**	**II**	**R**	**R**	**>1024 mg/L**
E0161	1999	AR14.4	CC8	ST8	II	R	I	>1024 mg/L
E0197	1999	AR14.4	CC8	ST8	II	R	R	>1024 mg/L
E0273	2000	AR07.0	CC30	ST36	II	R	R	>1024 mg/L
E0354	2000	AR14.2	CC8	ST8	II	R	R	>256 mg/L
E0477.1	2001	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E0491.2	2000	AR13.1	CC8	ST8	II	I	S	4 mg/L
E0542	2001	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E0565	2001	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E0592[b]	**2001**	**AR13.1**	**CC8**	**ST8**	**II**	**R**	**R**	**>1024 mg/L**
E0593	2001	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E0716	2001	AR14	CC8	ST8	II	R	R	>1024 mg/L
E1183[b]	**2002**	**AR44**	**CC8**	**ST239**	**III**	**R**	**R**	**>1024 mg/L**
E0881	2002	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E0882.1	2002	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E1121	2002	AR14	CC8	ST8	II	R	R	>1024 mg/L
E1350	2003	AR14.4	CC8	ST8	II	R	I	>1024 mg/L
E1426	2003	AR14.4	CC8	ST8	II	R	I	>1024 mg/L
E1443	2003	AR14.2	CC8	ST8	II	R	I	>1024 mg/L
E1567	2003	AR43	CC8	ST8	II	R	R	>1024 mg/L
E1794	2004	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E1797.2[b]	**2004**	**AR7.0**	**CC30**	**ST36**	**II**	**R**	**R**	**>1024 mg/L**
E1890	2004	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E2237	2005	New03	CC8	ST8	II	R	R	>1024 mg/L
E2510	2005	New03	CC22	ST22	IV	R	R	>1024 mg/L
E2554	2005	AR07	CC30	ST36	II	R	R	>1024 mg/L
E2595	2005	Unf	CC30	ST36	II	R	R	>1024 mg/L
E2597	2005	NT	CC22	ST22	IV	R	R	>1024 mg/L
E2625	2005	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E2632	2005	Unf	CC30	ST36	II	R	R	>256 mg/L
E2675	2006	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E2696	2006	AR06	CC8	ST8	II	R	R	>1024 mg/L
E2794	2006	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E2829	2006	NT	CC22	ST22	IV	R	R	>1024 mg/L
E2986	2006	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E2989	2006	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E2908	2006	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E3024	2006	AR06	CC22	ST22	IV	R	R	>1024 mg/L
E3065	2006	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3118	2006	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3130	2006	AR14.4	CC8	ST8	II	I	I	>1024 mg/L
E3148	2006	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3188	2006	AR06	CC22	ST22	IV	R	R	>1024 mg/L
E3201	2006	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3257	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3295	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3335	2007	Unf	CC5	ST5	IV	R	R	>1024 mg/L
E3361	2007	Unf	CC22	ST22	IV	R	R	>256 mg/L
E3367	2007	AR13	CC8	ST8	II	R	I	>256 mg/L
E3387	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3388	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3411	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3428	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3435	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3487	2007	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E3553	2007	Unf	CC22	ST22	IV	R	R	>1024 mg/L
01258	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
00833	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
00745	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
00208	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L

Continued overleaf

Table 5.3 continued. High-level mupirocin resistant MRSA isolates strain assignment and mupirocin susceptibility testing

Isolate no.	Year of isolation	AR type	DNA microarray assignment			Disc Diffusion		Mupirocin MIC[a]
			CC	ST	SCC*mec*	Mup5	Mup200	
01375	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
01329	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
01959	2007	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
E3833	2008	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3835	2008	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3848	2008	AR13	CC8	ST8	II	R	R	>1024 mg/L
E3860	2008	AR14.4	CC8	ST8	II	R	R	>1024 mg/L
E3868	2008	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E3886[b]	**2008**	**AR06**	**CC22**	**ST22**	**IV**	**R**	**R**	**>1024 mg/L**
E3901	2008	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E3911	2008	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E3942	2008	NT	CC22	ST22	IV	R	R	>1024 mg/L
E3948	2008	AR14	CC8	ST8	II	R	I	>256 mg/L
E3988	2008	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E4088	2008	AR14	CC8	ST8	II	R	R	>1024 mg/L
E4134	2009	AR06	CC22	ST22	IV	R	R	>256 mg/L
E4137	2009	AR13.1	CC8	ST8	II	R	R	>1024 mg/L
E4139	2008	Unf	CC22	ST22	IV	R	R	>1024 mg/L
05412	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
05327	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
05219	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
05599	2008	Unf	CC22	ST22	IV	S	S	<4 mg/L
05840	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
05656	2008	Unf	CC22	ST22	IV	S	S	<4 mg/L
03064	2008	Unf	CC22	ST22	IV	S	S	<4 mg/L
05354	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03123	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03219	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03300[b]	**2008**	**Unf**	**CC22**	**ST22**	**IV**	**R**	**ND**	**>1024 mg/L**
03213[b]	**2008**	**Unf**	**CC22**	**ST22**	**IV**	**R**	**ND**	**>1024 mg/L**
03222	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03225	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03376	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03638	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03642	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03663	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03832	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
03646	2008	Unf	CC22	ST22	IV	R	ND	>1024 mg/L
E4175	2009	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E4226[b]	**2009**	**Unf**	**CC22**	**ST22**	**IV**	**R**	**R**	**>1024 mg/L**
E4258	2009	AR14.4	CC8	ST8	II	R	R	>1024 mg/L
E4322	2009	Unf	CC22	ST22	IV	R	R	>1024 mg/L
E4327	2009	Unf	CC22	ST22	IV	R	R	>1024 mg/L

[a] Mupirocin MIC, mupirocin minimum inhibitory concentration determined by E-test (bioMérieux). The mupirocin MIC values are reported as the susceptible (S) < 4 mg/L, intermediate (I) between 8.0-128.0 mg/L, or resistant (R) > 256 mg/L to mupirocin.

[b] Isolates shown in bold underwent plasmid analysis.

Abbreviations: Ak, amikacin; Ap, ampicillin; Cd, cadmium nitrate/cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenylmercuric acetate; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamide; ; Tb, tobramycin; Te, tetracycline; Tp, trimethoprim; AR type, antibiogram-resistogram type; Unf, unfamiliar, these isolates exhibited a hitherto unfamiliar AR pattern; NT, non-typeable, these isolates displayed an AR pattern associated with AR06 but were urease-positive. New, AR patterns identified in 1999 that were assigned the AR types, New01, New02 and New03. PFT, pulsed-field type; Mup 5 and Mup 200, 5 µg and 200 µg mupirocin disks, respectively. ND, not determined.

Table 5.4 Genotypes and antimicrobial resistance and virulence-associated genes detected by DNA microarray profiling[a] of the 109 high-level mupirocin resistant (Hi-MupR) MRSA isolates recovered from patients in Irish hospitals between 1998 and 2009

CC/ST-SCC*mec* type (*n*)	PFG-AR type	Additional Typing Markers			Antimicrobial resistance genes (% indicated when not 100%)	Virulence associated genes (% indicated when not 100%)
		agr	*cap*	IEC (*n*)[e]		
CC22/ST22-MRSA-IV (61)	01-06, 01-Unfamiliar, 01-New03, 01-NT	I	5	B (55) Neg (6)	*blaZ*, *ileS2*, *erm*(C) (93%), *lnu*(A) (41%), *aacA-aphD* (82%), *aadD* (34%), *dfrS1* (5%), *cat* pC221 (3%), *qacC* (5%)	*egc* (2 *sei* negative & 2 *sen* negative)[f], *sec* & *sel* (62%), *sak* (90%), *chp* (90%), *scn* (90%), ACME (25%)
CC8/ ST8-MRSA-IIA-E & SCC_{MI} (31)[b], IIC-E (2)[c], or NT (6)[d]	00-13, 00-13.1, 00-14, 00-14.2, 00-14.4, 00-43	I	5	D (32) E (1) G (1) Novel (1) Neg (3)	*merA* & *merB* (85%), *blaZ*, *erm*(A), *erm(C)* (8%), *lnu*(A) (26%), *aacA-aphD* (97%), *aadD* (51%), *aphA3* & *sat* (97%), *dfrS1* (10%), *ileS2* (97%), *tet*(K) (3%), *sdrM*, *cat*-pC221 (5%), *fosB*, *qacA* (97%), *qacC* (23%)	*sea* (90%), *sep* (3%), *sak* (92%), *scn* (90%)
CC8/ST239-MRSA-III & SCC*Hg* (1)	99-44	I	8	D (1)	*merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aadD*, *aphA3* & *sat*, *ileS2*, *tet*(K), *tet*(M), *sdrM*, *fosB*, *qacA*	*sea*, *sak*, *scn*
CC30/ST36-MRSA-II (7)	02-7.0, 02-7.2, 02-Unfamiliar, 02-New03	III	8	A (7)	*blaZ*, *erm*(A), *lnu*(A) (14%), *aacA-aphD* (43%), *aadD*, *ileS2*, *sdrM*, *fosB*, *qacC* (14%)	*tst*, *sea*, *egc*[f], *sak*, *chp*, *scn*
CC5/ST5-MRSA-IV & *ccrB4* (1)	03-Unfamiliar	II	5	F (1)	*merA* & *merB*, *blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *ileS2*, *sdrM*, *fosB*, *qacA*, *qacC*	*sep*, *egc*[f], *sak*, *chp*, *scn*

[a] The StaphyType Kit (Alere Technologies) was used for DNA microarray profiling.
[b] ST8-MRSA-IIA-E & SCC_{MI}.
[c] ST8-MRSA-IIC-E. One isolate exhibited class A *mec* complex with *ccrAB4* but without *ccrAB2*. Four isolates were ST8-MRSA-IIC (2) and ST-MRSA-IIE (2).

[d] NT (non typeable), class B *mec*, *ccr*B4 and *ccr*C (1 isolate), class A *mec* & *ccr*B4 (1 isolate) (possibly SCC*mec* VIII), class A.3 *mec* & ccrB4 (3 isolates), class A.3 *mec* only (1 isolate)

[e] Immune evasion cluster (IEC) type as defined by van Wamel *et al.* (2006): A= *sea*, *sak*, *chp* and *scn*; B = *sak, chp* and *scn*; C = *chp* and *scn*; D = *sea, sak* and *scn;* E = *sak* and *scn*; F = *sep, sak, chp* and *scn*; G = *sep, sak* and *scn*; NT, novel IEC type consisting of *sak* and *sea* only; neg (negative), no IEC genes detected.

[f] The enterotoxin gene cluster (*egc*) genes are seg, *sei, sem, sen, seo* and *seu.*

Abbreviations: n, number of isolates; *agr*, genes encoding accessory gene regulators; *cap*, genes encoding capsular proteins; CC, clonal complex; ST, sequence type PFG-AR type; PFGs were combined with AR typing results to yield a AR-PFG type i.e. AR-PFG 06-01 (Rossney *et al.*, 2006).

following three Hi-MupR isolates E0103 (ST8-MRSA-IIE) E0592 (ST8-MRSA-IID) and E4226 (ST22-MRSA-IV) were found to harbour a commonly known point mutation (V588F) affecting the Rossman fold within *ileS* protein (Antonio *et al.*, 2002). A novel mutation was detected in *ileS* in one isolate (E1183, ST239-MRSA-III & SCC*Hg*) resulting in an amino acid substitution from phenylalanine to valine at nucleotide position 563. Additional mutations in *ileS* were identified in three remaining Hi-MupR isolates (E1797.2, ST36-MRSA-II; E3886, ST22-MRSA-IV; 03123, ST22-MRSA-IV) at nucleotide position 147 and 150 resulting in an amino acid substitution from aspartic acid to asparagine in the predicted protein.

5.3.4 Molecular characterisation of *ileS2*-encoding plasmids

The seven isolates selected for plasmid analysis included at least one isolate representative of four of the five main genotypes identified among the Hi-MupR isolates namely (a) three ST22-MRSA-IV isolates (E3886, 03123 and E4226), (b) two ST8-MRSA-IIE & IID isolates (E0103 and E0592), (c) one ST239-MRSA-III & SCC*Hg* isolate (E1183) and (d) one ST36-MRSA-II isolate (E1797.2) (Table 5.5). Each of these isolates harboured *ileS2*, had a mupirocin MIC >1024 mg/L, exhibited resistance to a range of antimicrobial agents and harboured a range of additional resistance determinants including those encoding resistance to aminoglycosides (*aadD*, and *aacA-aphD* and *aphA3*), MLS (*erm*(A), *erm*(C), *lnu*(A)) tetracycline (*tet*(K) and *tet*(M)) and trimethoprim (*dfrS1*) (Table 5.5).

Analysis of plasmid DNA from the seven Hi-MupR isolates revealed two Hi-MupR isolates, E3886 (Fig. 5.4, lane 13) and 03123 (Fig. 5.4, lane 10) appeared to harbour four plasmids. Three Hi-MupR isolates yielded plasmid profiles indicative of the presence of three plasmids namely E0103 (Fig. 5.4, lane 2) E0592 (Fig. 5.4, lane 3)

and E1183 (Fig 5.4, lane 6). One Hi-MupR isolates (E4226) appeared to harbour two plasmids (Fig. 5.4, lane 8) and one isolate (E1797.2) appeared to harbour a single plasmid (Fig. 5.4; lane 5). Plasmid analysis revealed that mupirocin susceptible isolates AR13/0066 (*n*=2), M03/0073 (*n*=2), 03663 (*n*=4) and AR07/2189 (*n*=1) haboured one to four plasmids (Fig. 5.4, lanes 4, 7 and 11, respectively).

5.3.4.1 Conjugative transfer of *ileS2* from Hi-MupR isolates to the plasmid free recipient strain XU21 and analysis of plasmids

The plasmid location of *ileS2* in the seven Hi-MupR isolates was confirmed in conjugation experiments by filter mating using the mupirocin-susceptible recipient *S. aureus* strain XU21. All seven Hi-MupR transconjugant derivatives of *S. aureus* strain XU21 harboured *ileS2* by DNA microarray profiling (Table 5.5). Also, five of the seven Hi-MupR transconjugant derivatives were found to harbour additional resistance determinants including *tet*(K), *aadD, dfrS1*, *aacA-aphD* and *lnu*(A) in addition to *ileS2* (Table 5.5). Plasmid profiling of selected Hi-MupR transconjugant derivatives generated from each clinical Hi-MupR isolate revealed the presence of a single plasmid in 5/7 cases (Fig. 5.5 and Table 5.5). Hi-MupR transconjugant derivatives generated from the two remaining Hi-MupR clinical isolates (E0592 and E3886) harboured two plasmids (Fig. 5.5 and Table 5.5).

Table 5.5 Phenotypic and molecular characteristics of the seven high-level mupirocin-resistant clinical MRSA isolates selected for detailed plasmid analysis and their mupirocin-resistant transconjugant derivatives of *S. aureus* XU21

Parental MRSA isolates					Transconjugant derivatives of XU21				
Isolate no. (year of isolation)	CC/ST-SCC*mec* type	AR pattern[a]	Antimicrobial resistance genes detected[b]	Mupirocin MIC[c]	Transconjugnat no.	AR pattern[a]	Antimicrobial resistance genes transferred[b, d]	Mupirocin MIC[b]	Rate of transfer[e]
E0103 (1999)	CC8/ST8-IIE	ApCdCdEbGnKnMcMpN mPmaSmSpTbTp	*merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aphA3* & *sat*, *ileS2*, *sdrM*, *fosB*, *qacA*	>1024 mg/L	E0103-XU21-T1	Rf, Mp	*ileS2*	>1024 mg/L	2×10^{-4}
E0592 (2001)	CC8/ST8-IID	ApCdCpEbErGnKnLnMc MpNmPmaSmSpTb	*merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aphA3* & *sat*, *dfrS1*, *ileS2*, *tet*(K), *sdrM*, *fosB*, *qacA*	>1024 mg/L	E0592-XU21-T1	Rf, Mp	*ileS2*, *tet*(K)	>1024 mg/L	4×10^{-6}
E1183[d] (2002)	CC8/ST239-III- SCC*Hg*	AkApCdCpEbErGnKnLn McMpNmPmaSmSpSuTb TeTp	*merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aadD*, *aphA3* & *sat*, *ileS2*, *tet*(K), *tet*(M), *sdrM*, *fosB*, *qacA*	>1024 mg/L	E1183-XU21-T1	Nm[d] Rf, Mp	*ileS2*, *aadD*	>1024 mg/L	1.3×10^{-7}
E1797.2 (2004)	CC30/ST36-II	ApCdCpErKnLnMpNmSp Tb	*blaZ*, *erm*(C), *ileS2*	>1024 mg/L	E1797.2-XU21-T1	Rf	*ileS2*	0.125 mg/L	4.6×10^{-4}
E3886 (2008)	CC22/ST22-IV	ApCdCpErMpTp	*blaZ*, *erm*(C), *dfrS1*, *ileS2*	>1024 mg/L	E3886-XU21-T1	Rf, Mp, Tp	*ileS2*, *dfrS1*	>1024 mg/L	1.5×10^{-7}
03123 (2008)	C22/ST22-IV	ApCdCpErKnLnMpTb	*blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD*, *ileS2*	>1024 mg/L	03123-XU21-T1	Rf, Mp	*ileS2*	>1024 mg/L	5.0×10^{-7}
E4226[d] (2009)	C22/ST22-IV	ApCdCpFdGnKnLnMpTb	*blaZ*, *lnu*(A), *aacA-aphD*, *ileS2*	>1024 mg/L	E4226-XU21-T1	Gn[d], Kn, Tb, Ln, Rf	*ileS2*, *aacA-aphD*, *lnu*(A)	0.125 mg/L	1.2×10^{-7}
XU21[f]	CC8-MSSA	NmRf	*fosB*	N/A	N/A	N/A	N/A	N/A	NA

[a] Antimicrobial resistance was determined by antibiogram-resistogram (AR) typing against a panel of 23 antimicrobial agents including Ak, amikacin; Ap, ampicillin; Cd, cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Ld, linezolid; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenyl mercuric acetate; Rf, rifampicin; Sp, spectinomycin; Sm, streptomycin; Su, sulphonamide; Te, tetracycline; Tb, tobramycin; Tp, trimethoprim.

[b] The StaphyType Kit (Alere Technologies, Germany) was used to detect the presence or absence of antimicrobial resistance genes.
[c] The mupirocin minimum inhibitory concentration (MIC) was determined using the E-test system (bioMérieux, Nuertlingen, Germany). The MIC values are reported as susceptible (S) < 4 mg/L, intermediate (I) between 8.0-128.0 mg/L, or resistant (R) > 256 mg/L to mupirocin.
[d] Isolates exhibited intermediate resistance to the indicated antimicrobials
[e] Rate of conjugal transfer of *ileS2* for the seven clinical isolates to the recipient strain XU21 was calculated on the number of transconjugants per per donor cell.
[f] The XU21 recipient is resistant to novobiocin and rifampicin (Udo and Jacob, 1998). N/A not applicable
UDO, E. E. & JACOB, L. E. 1998. Conjugative transfer of high-level mupirocin resistance and the mobilization of non-conjugative plasmids in Staphylococcus aureus. *Microb Drug Resist,* **4**, 185-93.

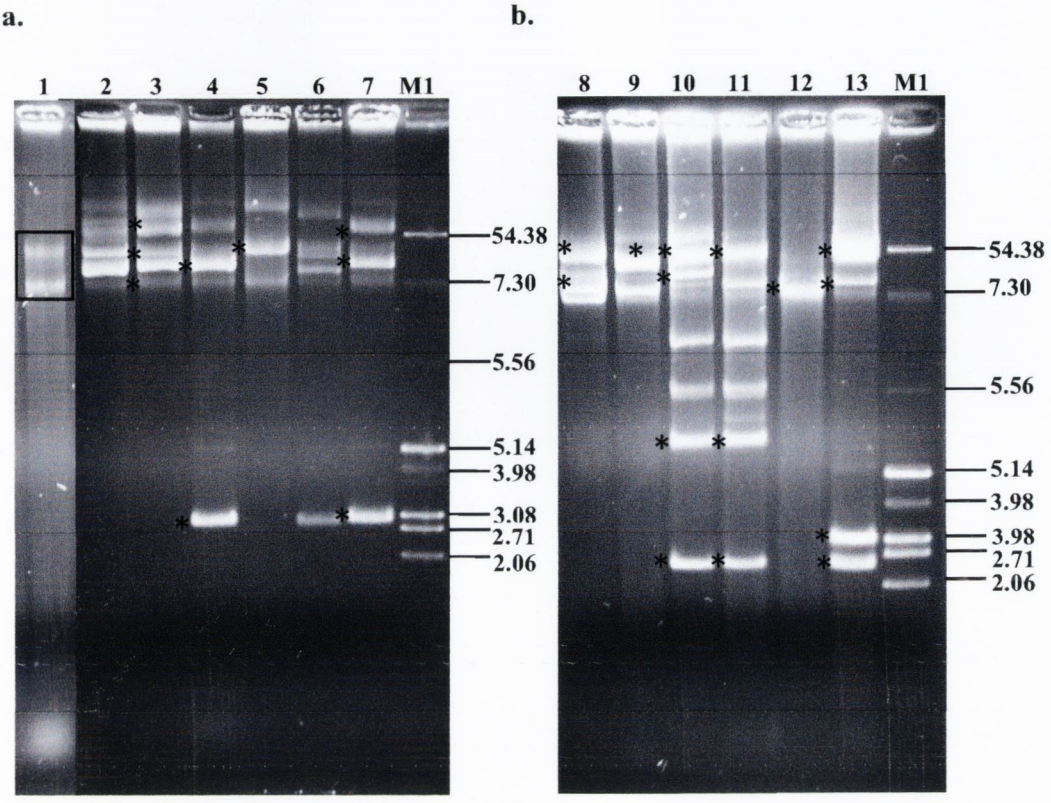


Fig. 5.4 Plasmid profiles of eight Hi-MupR MRSA isolates, the transconjugant derivative E4226-XU21-T1 and four mupirocin-susceptible MRSA isolates. Panel (**a**) shows an agarose gel showing: plasmid free-recipient control strain *S. aureus* strain XU21 (lane 1); Hi-MupR MRSA parental isolates; E0103 (**3**) (lane 2), E0592 (**3**) (lane 3), mupirocin-susceptible MRSA isolate AR13/0066 (**2**) (lane 4), Hi-MupR MRSA parental isolate E1797.2 (**1**) (lane 5), E1183 (**3**) (lane 6), mupirocin-susceptible MRSA isolate M03/0073 (**3**) (lane 7). Panel (**b**) shows an agarose gel showing: Hi-MupR MRSA parental isolate E4226 (**2**) (lane 8), its transconjugant derivative E4226-XU21-T1 (**1**) (lane 9), Hi-MupR MRSA parental isolates 03123 (**4**) (lane 10), E3886 (**4**) (lane 13), mupirocin susceptible MRSA isolates 03693 (**3**) (lane 11), AR07/2189 (**1**) (lane 12). Plasmids are indicated by asterisk *. Numbers in bold type in parenthesis refer to the numbers of plasmid in each isolate or derivative. Lanes: M1, pVA517A, plasmid molecular size reference markers with band sizes shown in kb. The presence of chromosomal DNA is indicated by a square box.

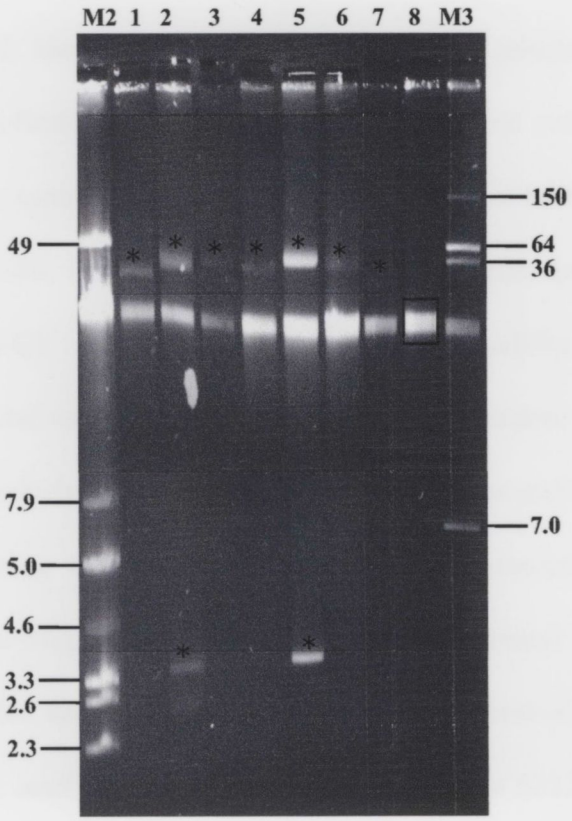


Fig. 5.5 Plasmid profiles DNA isolated from seven Hi-MupR transconjugant derivatives of *S. aureus* strain XU21 harbouring Hi-MupR-encoding plasmids. Panel (**a**) shows an agarose gel showing: transconjugant derivatives of *S. aureus* XU21 harbouring Hi-MupR-encoding plasmids: E1797.2-XU21-T1 (**1**) (lane 1), E0592-XU21-T1 (**2**) (lane 2), E0103-XU21-T1 (**1**) (lane 3), E1183-XU21-T1 (**1**) (lane 4), E3886-XU21-T1 (**2**) (lane 5), 03123-XU21-T1 (**1**) (lane 6), and E4266-XU21-T1 (**1**) (lane 7) and plasmid free-recipient control strain *S. aureus* strain XU21 (lane 8). Plasmids are indicated by asterisk *. Numbers in bold type in parenthesis refer to the numbers of plasmid in each derivative. Lanes: M2, (*E. coli* NCTC 50193), M3 (*E. coli* NCTC 50192), plasmid molecular size reference markers with band sizes shown in kb. The presence of chromosomal DNA is indicated by a square box.

5.3.4.2 Curing of *ileS2* from Hi-MupR isolates

The plasmid location of *ileS2* in the seven selected Hi-MupR isolates investigated was confirmed by a combination of curing and conjugation experiments. Curing experiments were undertaken on three isolates that harboured more than one plasmid (03123, E3886, E1183, Fig. 5.6) and mupirocin-susceptible cured derivatives (03123-C1, E3886-C1 and E1183-C1) were successfully generated. Several independent cured derivatives of each parental clinical isolate were investigated by DNA microarray analysis for the loss of *ileS2*. In all cases *ileS2* was absent from the cured derivatives along with combinations of various antimicrobial resistance genes (Table 5.6). The Hi-MupR ST22-MRSA-IV (03123) parental isolate exhibited four plasmids and encoded resistance to the following antimicrobial genes: *blaZ*, *erm*(C), *lnu*(A), *aacA-aphD*, *aadD* and *ileS2*. The cured derivative 03123-C1 harboured three plasmids and encoded resistance to *blaZ*, *erm*(C) and *lnu*(A). The Hi-MupR ST36-MRSA-IV (E3886) parental isolate harboured four plasmids and encoded resistance to the following antimicrobial agents: *blaZ*, *dfrS1*, *erm*(C) and *ileS2*. The cured derivative E3886-C1 harboured three plasmids and encoded resistance to *blaZ*, *dfrS1* and *erm*(C). The Hi-MupR ST239-MRSA-III-SCC*Hg* (E1183) harboured three plasmids and encoded resistance to the following antimicrobial agents: *merA*& *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aadD*, *aphA2 & sat*, *ileS2*, *tet*(K), *tet*(M), *sdrM*, *fosB* and *qacA*. Cured derivatives harboured two plasmids and encoded resistance to *merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aphA2 & sat*, *tet*(K), *tet*(M), *sdrM*, *fosB* and *qacA*. The rate of loss of the Hi-MupR phenotype by curing expressed as the number of colonies susceptible to mupirocin per donor cells ranged from 1 in 5.0×10^{-7}- 9.0×10^{-7} (Table 5.6).

5.3.4.2.1 Phenotypic characterisation of seven transconjugant derivatives

Comparative antimicrobial susceptibility testing revealed that 5/7 transconjugants (E0103-XU21-T1, E0592-XU21-T1, E1183-XU21-T1, E3886-XU21-T1 & 03123-XU21-T1) exhibited a high-level mupirocin MIC (>1024 mg/L) phenotype. However, two transconjugant derivatives (E1797.2-XU21-T1 and 03123-XU21-T1), exhibited a MIC of 0.125 mg/L indicative of a mupirocin-susceptible phenotype (Table 5.7). The high-level mupirocin resistance phenotype was absent in the parental strain XU21 and in three of the cured derivatives (E0592-C1, E1183-C1 and E3886-C1) (Table 5.6).

5.3.4.2.2 S1 nuclease digestion analysis resulted in seven distinct ileS2-encoding plasmids

S1 nuclease PFGE macrorestriction digestion analysis of whole chromosomal DNA purified from selected transconjugant derivatives resulted in the linearisation of each *ileS2*-encoding plasmid, which yielded seven distinct plasmid patterns (Fig 5.7). S1 nuclease digestion revealed seven distinct plasmid sizes in the following transconjugant derivatives; E4226-XU21-T1 (33 kb), E1797.2-XU21-T1 (36 kb), E1183-XU21-T1 (39 kb), E0103-XU21-T1 (44 kb) and 03123-XU21-T1 (45 kb) (Table 5.7; Fig. 5.7). Transconjugants generated from the two remaining clinical isolates (E0592 and E3886) harboured two plasmids of approximately 41 kb and 3 kb (E0592) and 42 kb and 3 kb (E3886) (Table 5.7; Fig. 5.6 and 5.7). Each different plasmid profile was assigned a different alphabetic designation A-G (Table 5.7). Overall, the rate of conjugal transfer of *ileS2* for the seven clinical isolates to the recipient strain XU21 ranged from 1.0×10^{-4} to 1.5×10^{-7} transconjugants per donor cell (Table 5.7).

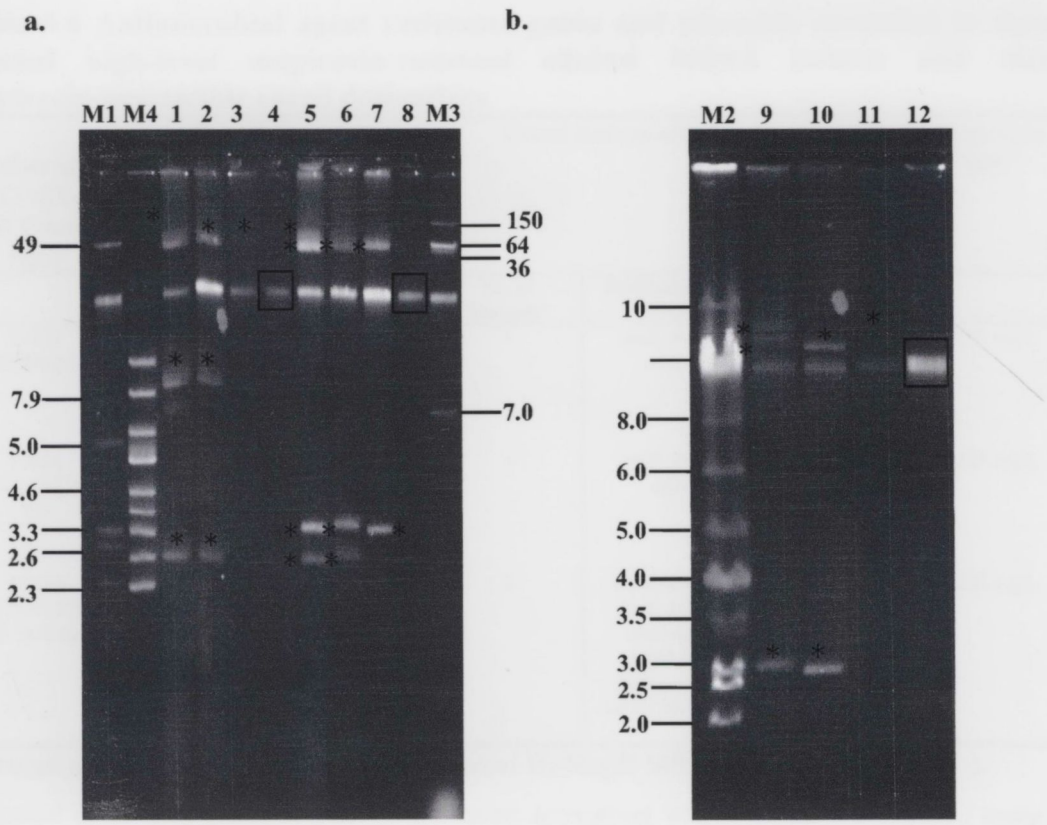


Fig. 5.6 Plasmid profiles of four Hi-MupR MRSA isolates, mupirocin-susceptible cured derivatives and Hi-MupR transconjugant derivatives of *S. aureus* strain XU21. Panel (**a**) shows an agarose gel showing: parental Hi-MupR MRSA isolate 03123 (**4**) (lane 1), mupirocin-susceptible cured derivative 03123-C1 (**3**) (lane 2), Hi-MupR transconjugant derivative 03123-XU21-T1 (**1**) (lane 3) and plasmid free-recipient control strain *S. aureus* strain XU21 (lane 4), parental Hi-MupR MRSA isolate E3886 (**4**) (lane 5), mupirocin-susceptible cured derivative E3886-C1 (**3**) (lane 6), Hi-MupR transconjugant derivative E3886-XU21-T1 (**2**) (lane 7), plasmid free-recipient control strain *S. aureus* strain XU21 (lane 8). Panel (**b**) shows an agarose gel showing: parental Hi-MupR MRSA isolate E1183 (**3**) (lane 9), mupirocin-susceptible cured derivative E1183-C1 (**3**) (lane 10), Hi-MupR transconjugant derivative E1183-XU21-T1 (**1**) (lane 11) and plasmid free-recipient control strain *S. aureus* strain XU21 (lane 12). Plasmids are indicated by asterisk *. Numbers in bold type in parenthesis refer to the numbers of plasmid in each derivative. Lanes: M2, (*E. coli* NCTC 50193), M3 (*E. coli* NCTC 50192), M4, (supercoiled DNA ladder), plasmid molecular size reference markers with band sizes shown in kb. The presence of chromosomal DNA is indicated by a square box.

Table 5.6 Antimicrobial agent resistance genes and plasmids identified in three selected high-level mupirocin-resistant clinical MRSA isolates and their mupirocin-susceptible cured derivatives

Isolate no. (CC/ST-SCC*mec* type)	Parental isolate		Cured derivatives[c]		Rate of curing[d]	Mupirocin MIC[e]
	No. of plasmids[b]	Resistance genes detected[c]	No. of plasmids[b]	Resistance genes detected[c]		
03123 (C22/ST22-IV)	4	*blaZ*, *erm*(C), ***lnu*(A)**, ***aacA-aphD***, ***aadD***, ***ileS2***	3	*blaZ*, *lnu*(A). *erm*(C)	1 in 5.0×10^{-7}	0.125 mg/L
E3886 (CC22/ST22-IV)	4	*blaZ*, *erm*(C), *dfrS1*, ***ileS2***	3	*blaZ*, *erm*(C) *dfrS1*	1 in 9.0×10^{-7}	0.125 mg/L
E1183 (CC8/ST239-III- SCC*Hg)*	3	*merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, ***aadD***, *aphA3* & *sat*, ***ileS2***, *tet*(K), *tet*(M), *sdrM*, *fosB*, *qacA*	2	*merA* & *merB*, *blaZ*, *erm*(A), *aacA-aphD*, *aphA3* & *sat*, *tet*(K), *tet*(M), *sdrM*, *fosB*, *qacA*	1 in 6.0×10^{-7}	0.125 mg/L

[a] Plasmid curing was performed on three selected Hi-MupR MRSA (see section 5.2.5.1.).

[b] Plasmid DNA from parental isolates and cured derivatives was extracted and purified using a modified alkaline lysis method (Schwarz *et al.*, 1989).

[c] The StaphyType Kit (Alere Technologies) was used to detect the presence or absence of antimicrobial resistance genes. Antimicrobial resistance genes present in parental isolates but absent in cured derivatives are highlighted in bold.

[d] Plasmid curing rates were calculated as the number of mupirocin-susceptible cells grown on BHI agar supplemented with mupirocin (100 mg/L) proportional to the the number of resistant cells after the curing regimen grown also on BHI agar supplemented with mupirocin (100 mg/L).

[e] The mupirocin minimum inhibitory concentration (MIC) was determined using the E-test system (bioMérieux). The MIC values are reported as susceptible (S) < 4 mg/L, intermediate (I) between 8.0-128.0 mg/L, or resistant (R) > 256 mg/L to mupirocin.

Table 5.7 Phenotypic and molecular characteristics and plasmid analysis of high-level mupirocin resistant transconjugants of *S. aureus* XU21 derived harbouring *ileS2*-encoding plasmids from high-level mupirocin resistant MRSA isolates

Parent	Transconjugant derivatives of XU21				Plasmid analysis					
Isolate no. (CC/ST-SCC*mec* type)	Transconjugnat No.	Antimicrobial resistance genes transferred[a]	AR pattern[b]	Mupirocin MIC[c]	No. of plasmids	Approximate plasmid(s) size(s)[d]	Plasmid Type[e]	IS*257-ileS2* amplification configuration[f]	Hybridised *Eco*RI fragment size[g]	Hybridised *Hin*dII fragment size[g]
E0103 (CC8/ST8-IIE)	E0103-XU21-T1	*ileS2*	Rf, Mp	>1024 mg/L	1	44 kb	A	XVI UpL849-DnR716	4 kb	6.6 kb
E0592 (CC8/ST8-IID)	E0592-XU21-T1	*ileS2*, *tet*(K)	Rf, Mp	>1024 mg/L	2	41 kb & 3kb	B	XVII UpL849-DnR148	5 kb	5.5 kb
E1183 (CC8/ST239-III- SCC*Hg)*	E1183-XU21-T1	*ileS2*, *aadD*	Nm[m], Rf, Mp	>1024 mg/L	1	39 kb	C	XVII UpL849-DnR148	5 kb	5.6 kb
E1797.2 (CC30/ST36-II)	E1797.2-XU21-T1	*ileS2*	Rf	0.125 mg/L	1	36 kb	D	XXI UpL1849-DnR593	4 kb	6 kb
E3886 (CC22/ST22-IV)	E3886-XU21-T1	*ileS2*, *dfrS1*	Rf, Mp, Tp	>1024 mg/L	2	42 kb & 3 kb	E	XVIII UpL849-DnR694	4 kb	6 kb
03123 (C22/ST22-IV)	03123-XU21-T1	*ileS2*	Rf, Mp	>1024 mg/L	1	45 kb	F	XIX UpL849-DnR973	4 kb	6.6 kb
E4226 (C22/ST22-IV)	E4226-XU21-T1	*ileS2*, *aacA-aphD*, *lnu*(A)	Gn[m], Kn, Tb, Ln, Rf	0.125 mg/L	1	33 kb	G	XX UpL247-Dn3699	15 kb	10 kb

[a] The StaphyType Kit (Alere Technologies, Germany) was used to detect the presence or absence of antimicrobial resistance genes

[b] Antimicrobial resistance was determined by antibiogram-resistogram (AR) typing against a panel of 23 antimicrobial agents including Ak, amikacin; Ap, ampicillin; Cd, cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Eb, ethidium bromide; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Ld, linezolid; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenyl mercuric acetate; Rf, rifampicin; Sp, spectinomycin; Sm, streptomycin; Su, sulphonamide; Te, tetracycline; Tb, tobramycin; Tp, trimethoprim.

[c] The mupirocin minimum inhibitory concentration (MIC) was determined using the E-test system (bioMérieux, Nuertlingen, Germany). The MIC values are reported as susceptible (S) < 4 mg/L, intermediate (I) between 8.0-128.0 mg/L, or resistant (R) > 256 mg/L to mupirocin.
[d] Plasmid sizes were determined by using S1 nuclease PFGE macrorestriction digestion analysis (Barton *et al.*, 1995).
[e] Each different plasmid profile was assigned a different alphabetic designation.
[f] IS*257*-*ileS2* distinct configurations was assigned a new roman numeral as described previously by Perez-Roth *et al.* (2011).
[g] Southern blot hybridisation analysis of *Eco*RI- or *Hin*dIII-digested plasmid DNA isolated from transconjugant derivatives of *S. aureus* XU21 harbouring Hi-MupR-encoding plasmids from MRSA probed with a DIG-labelled *ileS2*-specific probe

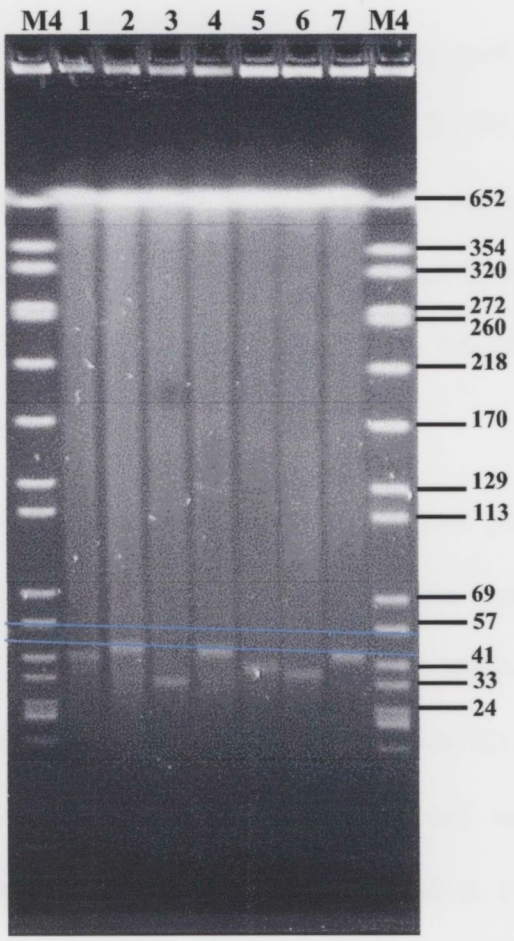


Fig. 5.7 S1-generated macrorestriction endonuclease digests of whole-cell DNA from transconjugant derivatives of *S. aureus* XU21 harbouring Hi-MupR-encoding plasmids from MRSA. S1 nuclease digestion of transconjugant derivative whole-cell DNA was used to estimate the size of the *ileS2*-encoding plasmids. Following S1-digestion of whole-cell DNA fragments were separated by PFGE. Panel A shows an agarose gel showing: S1-digested whole-cell DNA from transconjugant derivatives: E0592-XU21-T1 (41 kb) (lane 1), 03123-XU21-T1 (45 kb) (lane 2), E4226-XU21-T1 (33 kb) (lane 3), E0103-XU21-T1 (44 kb) (lane 4), E1183-XU21-T1 (39 kb) (lane 5), E1797.2-XU21-T1 (36 kb) (lane 6), E3886-XU21-T1 (42 kb) (lane 7). Lane M5 (whole-cell DNA from *S. aureus* reference strain NCTC8325 digested with *Sma*I) was used as a DNA molecular size references marker with band sizes shown in kb.

5.3.4.2.3 Hybridisation analysis of restriction fragments carrying ileS2 in the seven transconjugant derivatives using Southern blot analysis

Plasmid DNA of all seven *ileS2*-positive transconjugant derivatives of XU21 (E0103-XU21-T1, E0592-XU21-T1, E1183-XU21-T1, E1797.2-XU21-T1, E3886-XU21-T1, 03123-XU21-T1, and E4226-XU21-T1) were digested with *Eco*RI and *Hin*dIII separately. Digested plasmid DNA was then probed by Southern blot analysis with a DIG-labelled *ileS2* fragment generated using the MupA and MupB primers (Table 5.2) (Pérez-Roth *et al.*, 2001).

Seven *Eco*RI restriction patterns were generated from plasmid DNA from the seven transconjugant derivatives of XU21. Hybridisation analysis of the *Eco*RI restriction fragments with the *ileS2* probe identified a 4 kb *ileS2*-encoding fragment in four transconjugant derivatives (E3886-XU21-T1, E0103-XU21-T1, 03123-XU21-T1 and E1797.2-XU21-T1), a 5 kb *ileS2*-encoding fragment in two transconjugant derivatives (E0592-XU21-T1 and E1183-XU21-T1) and a 15 kb *ileS2*-encoding fragment in the remaining transconjugant derivative (E4226-XU21-T1) (Fig. 5.8, Table 5.7). Similarly, seven *Hind*III restriction patterns were generated from plasmid DNA from the seven transconjugant derivatives of XU21. Hybridisation analysis using the *ileS2* probe identified five *ileS2*-encoding *Hind*III fragments ranging in size from 5.5-10 kb (Fig. 5.8, Table 5.7).

5.3.5 Molecular characterisation of IS*257*-*ileS2* flanking regions on *ileS2*-encoding plasmids

Six distinct configurations of *ileS2* and its flanking IS*257* regions were detected among the seven Hi-MupR isolates (Table 5.7). Each distinct configuration was assigned a new roman numeral as described previously by Perez-Roth *et al.* (2011) i.e. XVI to XXI (Fig. 5.9; Table 5.7).

5.3.5.1 E0103 (ST8-MRSA-IIE) & E1797.2 (ST36-MRSA-II)

The IS*257*-*ileS2* configuration type XVI and XXI was identified on type A & D *ileS2*-encoding plasmids in isolates E0103 (ST8-MRSA-IIE) & E1797.2 (ST36-MRSA-II), respectively. This IS*257*-*ileS2* type XVI & XXI showed 99% DNA sequence identity to previously described IS*257*-*ileS2* regions, consisting of IS*257*L, IS*257*R, the methyltransferase domain, the fructosamine kinase family protein and *ileS2*, previously identified in in pPR9 and the direction of transcription of IS*257* were indistinguishable to pPR9 (Perez-Roth *et al.*, 2010) (Fig. 5.9; Table 5.7).

5.3.5.2 E0592 (ST8-MRSA-IID) & E1183 (ST239-MRSA-III-SCC*Hg*)

The IS*257*-*ileS2* configuration type XVII was identified on type B and C *ileS2*-encoding plasmid in two Hi-MupR isolates E0592 (ST8-MRSA-IID) & E1183 (ST239-MRSA-III-SCC*Hg*) (Fig. 5.9; Table 5.7). The type XVII, IS*257*R-*ileS2* orientation was in the same direction in E0592 and E1183 and exhibited 99% DNA sequence identity to the IS*257*R-*ileS2* in the Korean *S. aureus* plasmid (Accession number DG102365.1) which harbours no additional predicted ORFs apart from *ileS2* and IS*257*. While the left IS*257*L-*ileS2* junction in both E0592 and E1183 had 99% DNA sequence identity to left IS*257*L and fructcosamine-3-kinase OFR2 junction in pPR9, the IS*257*L was

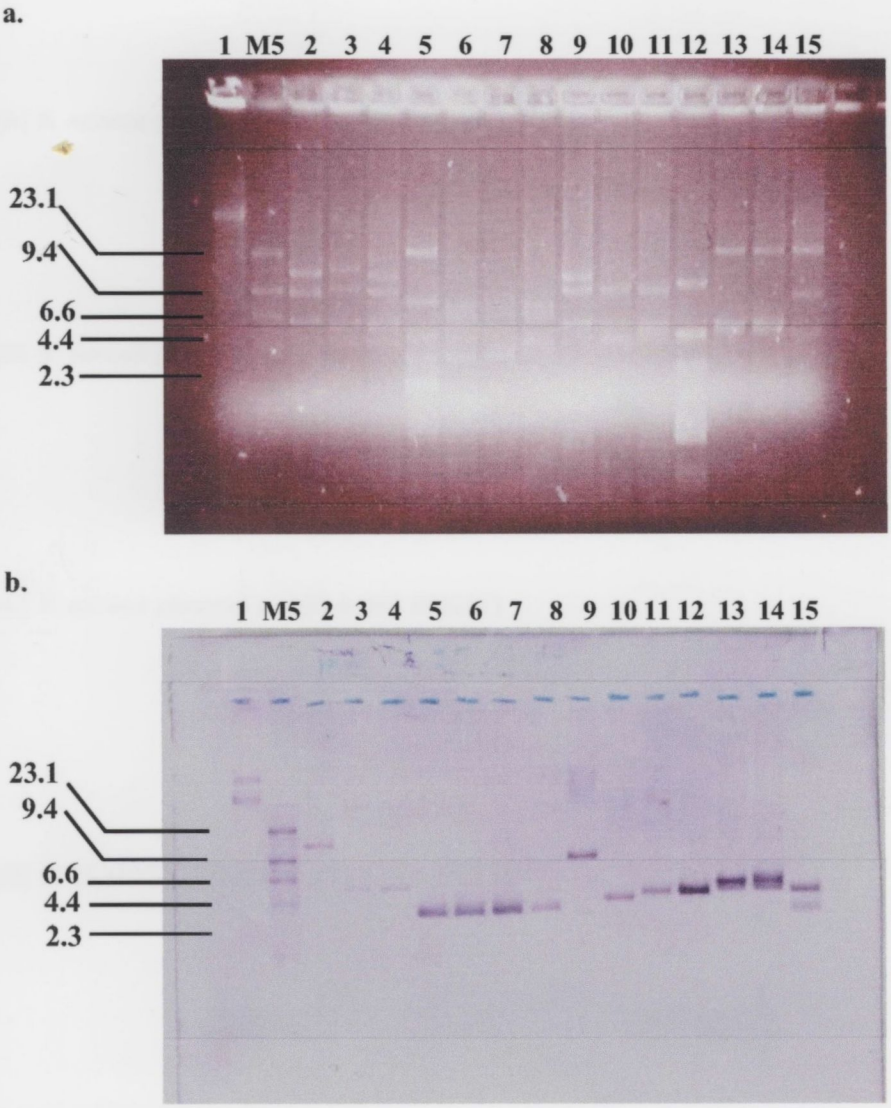
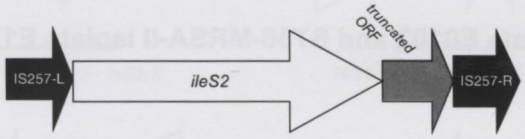
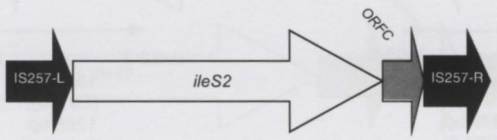


Fig. 5.8 Southern blot hybridisation analysis of *Eco*RI- or *Hin*dIII-digested **plasmid DNA isolated from transconjugant derivatives of *S. aureus* XU21 harbouring** Hi-MupR-encoding plasmids from MRSA probed with a DIG-labelled *ileS2*-specific probe. Panel (**a**) shows an agarose gel showing: *Eco*RI-digested plasmid DNA from transconjugant derivatives E4226-XU21-T1 (lane 2), E0592-XU21-T1 (lane 3), E1183-XU21-T1 (lane 4), E3886-XU21-T1 (lane 5), E0103-XU21-T1 (lane 6), 03123-XU21-T1 (lane 7), E1797.2-XU21-T1 (lane 8) and *Hin*dIII-digested plasmid from transconjugant derivatives E4226-XU21-T1 (lane 9), E0592-XU21-T1 (lane 10), E1183-XU21-T1 (lane 11), E3886-XU21-T1 (lane 12), E0103-XU21-T1 (lane 13), 03123-XU21-T1 (lane 14), E1797.2-XU21-T1 (lane 15). Lane M6 (DNA Molecular Weight Marker II, DIG-labelled, Roche Diagnostics). Panel (**b**) shows a Southern blot of the gel shown in panel A following hybridisation with the *ileS2*-specific probe.

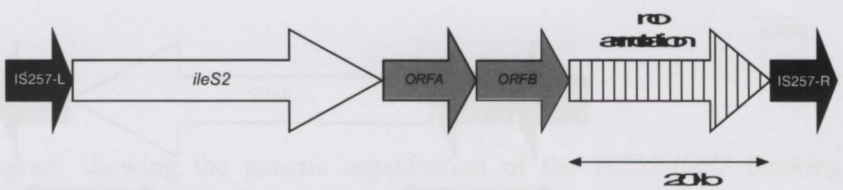
(A) *S. aureus* plasmid pG0400 (Morton *et al.*, 1990)



(B) *S. aureus* plasmid pUSA03 (Accession number NC 00792)



(C) *S. aureus* plasmid pV030-8 (NC 010297)



(D) ST36-MRSA-II plasmid pPR9 (GU 237136.1)

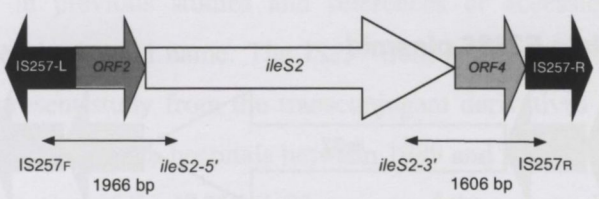
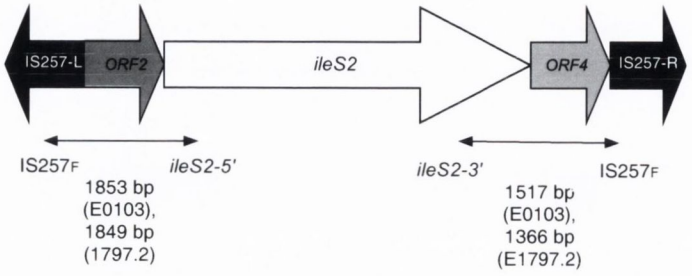
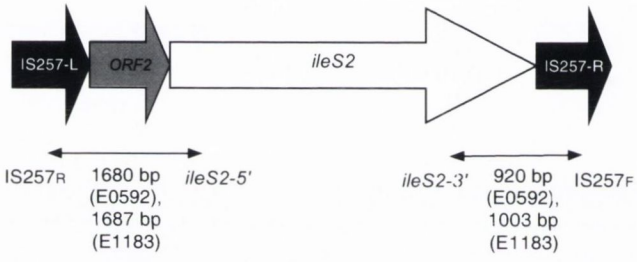


Fig. 5.9 Schematic diagram showing the genetic organisation of the IS*257-ileS2* flanking regions of Hi-MupR plasmids from MRSA. Continued overleaf

(E) ST8-MRSA-IIE isolate E0103 and ST36-MRSA-II isolate E1797.2 plasmid



(F) ST8-MRSA-IID isolate E0592 and ST239-MRSA-III&SCC*Hg* isolate E1183 plasmid



(G) ST22-MRSA-IV isolate E3886 plasmid

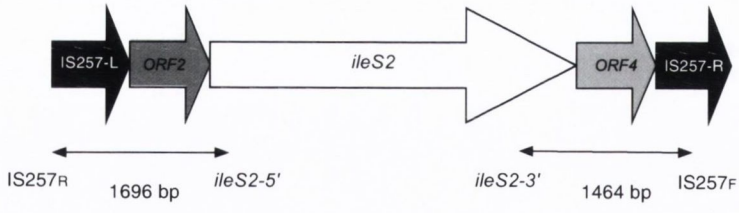
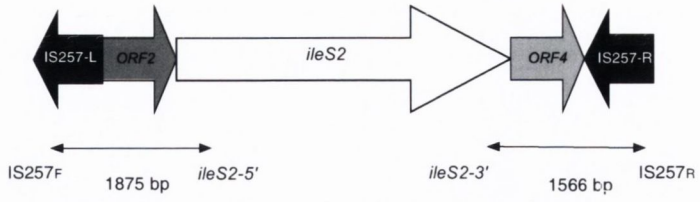


Fig. 5.9 Schematic diagram showing the genetic organisation of the IS*257-ileS2* flanking regions of Hi-MupR plasmids from MRSA. Continued overleaf

(H) ST22-MRSA-IV isolate 03123 plasmid



(I) ST22-MRSA-IV isolate E4226 plasmid

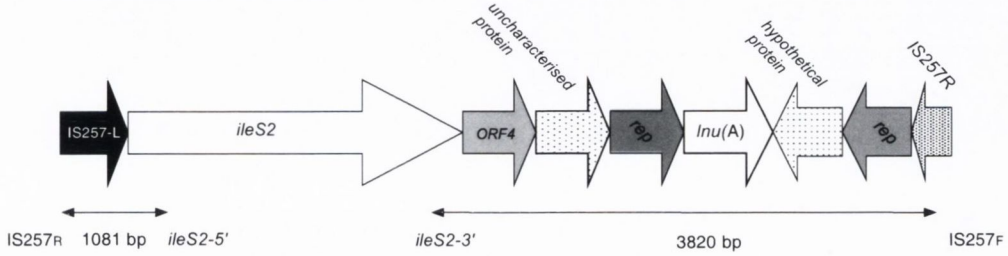


Fig. 5.9 Schematic diagram showing the genetic organisation of the IS*257-ileS2* flanking regions of Hi-MupR plasmids from MRSA. The genetic organisation of the IS*257-ileS2* regions of plasmids pGO400, pUSA03, pV030-8, and pPR9, shown in panels (A) to (D), respectively, were determined in previous studies and references or accession numbers are shown in parenthesis after each plasmid name. The IS*257-ileS2* regions of the *ileS2*-encoding plasmids identified in the present study from the transconjugant derivatives of seven Hi-MupR MRSA recovered from patients in Irish hospitals between 1999 and 2009 are shown in panels (E) to (I). The genetic organisation of the IS*257-ileS2* regions of the transconjugants in the present study were determined by PCR and sequencing of the IS*257-ileS2* regions using the previously described primers indicated under the double-headed arrows (Perez-Roth *et al.*, 2011); the sizes of amplimers obtained are indicated under the double headed arrows. The different open reading frames (ORFs) and genes are indicated with different coloured arrowed boxes with the arrowheads indicating the direction of transcription. A distance scale in kb is given. Abbreviations: ORF, open reading frame; *rep*, replication gene; *lnu*(A), lincosamide resistance gene.

transcribed in the reverse orientation to that in pPR9 but was similar to the direction of transcription in pPR3 and pPR7 (Pérez-Roth *et al.*, 2011) (Fig. 5.9; Table 5.7).

5.3.5.3 E3886 (ST22-MRSA-IV)

IS*257-ileS2* configuration type XVIII was identified in E3886 (ST22-MRSA-IV). The IS*257*L-*ileS2* exhibited 99% DNA sequence identity to pPR9. The transcription of IS*257*L was in same direction to plasmids pPR3 & pPR7, previously described in an ST36-MRSA-IV. However XVIII *ileS2*-IS*257*R junction exhibited 99% DNA sequence identity to the fructosamine kinase family protein and *ileS2*, previously identified in in pPR9 and the direction of the IS*257*R sequence were indistinguishable to pPR9 (Perez-Roth *et al.*, 2010) (Fig. 5.9; Table 5.7).

5.3.5.4 03123 (ST22-MRSA-IV)

The IS257-*ileS2* type XIX was assigned to plasmid type F (45 kb) found in one ST22-MRSA-II (03123). The IS*257*R & IS*257*L-*ileS2* orientation shared 99% DNA sequence identity to IS*257*L-*ileS2* and IS*257*R-*ileS2* junctions in plasmid pPR9, however the direction of transcription of both IS*257*R & IS*257*L-*ileS2* were sequences were similar to plasmid group S4 (pPR2, pPR5, pPR6) (Pérez-Roth *et al.*, 2011) (Fig. 5.9; Table 5.7).

5.3.5.5 E4226 (ST22-MRSA-IV)

The final IS*257-ileS2* configuration type XX identified in plasmid type G (33 kb) was found in one ST22-MRSA-IV (E4226). The left junction IS*257*L exhibited 99% DNA sequence identity to the IS*257*L in pPR9 (Fig. 5.9; Table 5.7). The IS*257*R-*ileS2* showed 98% DNA sequence identity to *S. aureus* plasmid SAP015B (Accession number GQ9005022) in which a fructosamine-3-kinase, an uncharacterised protein in

ileS2-5'region, a replication gene (*rep*) and a lincosamide resistance gene was identified downstream of the *ileS2*. Downstream of *lnu*(A), a region encoding ca. 139 DNA nucleotides exhibited 87 % DNA sequence identity to a unknown region to *S. aureus* strain WBG4364 plasmid pWBG1773 (Accession number EF537646). This region was followed by a gene encoding hypothetical protein sharing 87% DNA sequence identity to *S. aureus* plasmid SAP082A (Accession number GQ900434). This region was flanked by a transpoase for IS-like element a possible IS*257* with 87% identity to *S. aureus* plasmid SAP082A (Fig. 5.9; Table 5.7).

5.4 Discussion

DNA microarray profiling of 109 Hi-MupR MRSA isolates recovered from patients in Irish hospitals between 1998 and 2009 revealed the presence of the Hi-MupR gene *ileS2* among isolates belonging to five MRSA clones including ST22-MRSA-IV, ST8-MRSA-IIA-IIE, ST239-MRSA-III-SCC*Hg*, ST36-MRSA-II and ST5-MRSA-II. The most predominant Hi-MupR clone identified was ST22-MRSA-IV, which accounted for (55%; 60/109) of the isolates, and is the MRSA clone currently endemic in Irish hospitals (Shore *et al.*, 2010).

Numerous Hi-MupR-encoding plasmids have been reported worldwide (Rahman *et al.*, 1989; Needham *et al.*, 1994; Udo *et al.*, 1994; Udo and Jacob, 1998; Woodford *et al.*, 1998; Schmitz *et al.*, 1998; Pawa *et al.*, 2000; Udo *et al.*, 2001a; Pérez-Roth *et al.*, 2006; Simor *et al.*, 2007; Udo and Sarkhoo, 2010; Yoo *et al.*, 2010; Abimanyu *et al.*, 2012). However, the Hi-MupR gene *ileS2* has only been fully characterised on four different sized plasmids flanked by the insertion sequence IS*257* (Morton *et al.*, 1995; Diep *et al.*, 2006; Yoo, 2008; Pérez-Roth *et al.*, 2010). In the present study detailed plasmid analysis of seven MRSA isolates representative of three of the five clones harbouring *ileS2* in Irish MRSA revealed the presence of seven distinct large conjugative *ileS2*-encoding plasmids, ranging in size from 33-45 kb. Based on PCR and sequencing of the region extending from *ileS2* to the left and right IS*257* flanking regions IS*257*(L) and IS*257*(R), respectively, six different combinations of IS*257*(L)-*ileS2* and IS*257*(R)-*ileS2* were identified among these seven *ileS2*-encoding plasmids. Two of the seven *ileS2*-encoding plasmids identified were also found to carry additional resistance determinants. This included one *ileS2*-encoding plasmid that also harboured *aadD* (encoding amikacin, kanamycin, neomycin and tobramycin resistance) in a ST239-MRSA-III-SCC*Hg* isolate, and one carrying *aacA-aphD* and *lnu*(A) (encoding

amikacin, gentamicin, kanamycin and tobramycin resistance and lincosamide resistance, respectively) in a ST22-MRSA-IV isolate. The finding of a variety of large conjugative plasmids encoding *ileS2*, in some cases along with other resistance determinants, among MRSA from Ireland belonging to several distinct genetic backgrounds indicates the potential for this gene to spread and to compromise out ability to successfully decolonise nasal MRSA carriers.

Three distinct *ileS2*-plasmid types were identified among ST22-MRSA-IV isolates suggesting that acquisition of *ileS2* plasmids among ST22-MRSA-IV isolates in Ireland has either occurred on several occasions, or that evolution of these plasmids has occurred within this clone. One of these *ileS2*-encoding plasmids was 33 kb in size and encoded *aacA-aphD* and *lnu*(A) as well as *ileS2*. The *lnu*(A) gene was localised downstream of *ileS2* within the IS*257*(R) flanking region, while the location of *aacA-aphD* within the plasmid was not determined but was not detectable within the flanking IS*257* regions by PCR and sequencing. Interestingly, these three genes, *aacA-aphD*, *lnu*(A) and *ileS2*, have not been reported previously on a single plasmid. *lnu*(A) has been previously localised upstream of *ileS2* (Accession number SAP015B) flanked by a *tnpB* (transposase B).The IS*257*(L) flanking region of this plasmid (E4226) exhibited 99% DNA sequence identity to the IS*257*(L) flanking region of the Spanish *ileS2*-encoding plasmid pPR9, but because of the presence of *lnu*(A) in the right flanking region it was designated as novel IS*257-ileS2* type XX (Fig. 5.9 and Table 5.7). The second *ileS2*-encoding plasmid from an ST22-MRSA-IV isolate (03123) was 45 kb in size and did not encode additional antimicrobial resistance determinants as determined from microarray profiling of transconjugant derivatives harbouring this plasmid (Table 5.7). This plasmid was assigned to IS*257-ileS2* type XIX which has been described previously among Spanish ST22-MRSA-IV isolates (Pérez-Roth *et al.*, 2011) (Fig. 5.9).

In the final ST22-MRSA-IV isolate (E3886) two plasmids were isolated from *ileS2*-positive transconjugant derivatives (Table 5.7). Many independent transconjugants generated in separate conjugation experiments were all found to harbour both plasmids. One plasmid was 42 kb and the other 3 kb and both *ileS2* and the trimethoprim resistance gene *dfrS1* were detected following microarray profiling of a selected transconjugant. Which of these plasmids harboured the individual resistance determinants was not determined during the course of this study. However Hi-MupR plasmids are generally large plasmids (Pérez-Roth *et al.*, 2010) and large conjugative plasmids can mobilise smaller non-conjugative plasmids resulting in the transfer of both plasmids between bacterial cells (Smillie *et al.*, 2010). Characterisation of *ileS2* flanking regions in this final ST22-MRSA-IV revealed that it exhibited IS*257*-*ileS2* type XVIII and shared DNA sequence identity to the both IS*257*(R)-*ileS2* and IS*257*(L)-*ileS2* region of pPR9. However, the direction of transcription of both IS*257*(R)-*ileS2* and IS*257*(L)-*ileS2* transposase gene was similar to that of IS*257* harboured by the previously characterised *ileS2*-encoding plasmids pPR3, pPR4, pPR7, and pPR8, which have only been identified previously among ST36-MRSA-IV, ST146-MRSA-IVa, and NT (SCC*mec* and ST type not determined) MRSA isolates (Pérez-Roth *et al.*, 2011).

Two distinct plasmids encoding *ileS2* were identified among transconjugant derivatives of Hi-MupR ST8-MRSA-IID and ST8-MRSA-IIE isolates, which were 44 kb and 41 kb in size, respectively. A ST8-MRSA-IID transconjugant (E0592-XU21-T1, Table 5.7) carried *ileS2* and *tet*(K) and harboured two plasmids, one 44 kb in size and a second smaller 3 kb plasmid. Again, which of these plasmids harboured the individual resistance determinants was not determined during the course of this study but it seems more likely that the larger plasmid carries *ileS2*. Only one plasmid of 41 kb was identified in a ST8-MRSA-IIE transconjugant (E0103-XU21-T1, Table 5.7) and apart

from *ileS2* no additional resistance determinants were identified. Distinct IS*257-ileS2* types were identified in the *ileS2*-encoding plasmids from the ST8 isolates. The *ileS2* plasmid from the ST8-MRSA-IID transconjugant was assigned IS*257-ileS2* type XVII which has been reported previously in ST36-MRSA-II (*n*=2) and NT (SCC*mec* and ST type not determined) (*n*=5) (Pérez-Roth *et al.*, 2011). Interestingly, in the present study the IS*257-ileS2* type XVII type was also identified on a large 39 kb *ileS2*-encoding plasmid in the transconjugant derivative of ST239-MRSA-III & SCC*Hg* (E1183-XU21-T1, Table 5.7), but unlike that in the ST8 isolate this plasmid also harboured *aadD* encoding amikacin, kanamycin, neomycin and tobramycin resistance. The *ileS2* plasmid from the ST8-MRSA-IIE transconjugant was designated IS*257-ileS2* type XVI which has been identified previously among ST247-MRSA-Ia (*n*=9) and ST36-MRSA-II (*n*=4) isolates (Pérez-Roth *et al.*, 2011). IS*257-ileS2* type XXI was also identified in the present study in the *ileS2*-encoding plasmid from the transconjugant derivative of ST36-MRSA-II (E1797.2-XU21-T1, Table 5.7). However, the ST36-MRSA-II *ileS2* plasmid was smaller (*ca.* 36 kb) than that from the ST8-MRSA-IIE isolate.

Similar to the findings of the present study, Perez-Roth *et al.*, 2011 identified diverse plasmids encoding *ileS2* among MRSA from Spanish hospitals (1998-2004) During that study 19 different plasmid types (pPR1-pPR9 and 10 unknown plasmids) were identified among isolates belonging to five different clones (ST22-MRSA-IV, ST36-MRSA-II, ST147-MRSA-IVA, ST247-MRSA-IA, ST125-MRSA-IVA) and each one hybridised with distinct *Hin*dIII fragments and exhibited unique *ileS2*-IS*257* configurations (Pérez-Roth *et al.*, 2011). McDougal *et al.*, (2010) also reported *ileS2* on five different *ileS2*-encoding plasmids harboured by USA300 isolates recovered from invasive MRSA infections as part of the CDC collection from 2005 and 2008. Five plasmids were found to harboured *ileS2* along with various other antimicrobial

resistance determinants as follows:plasmid pUSA03 (37 kb) was found to harbour *ileS2* and *erm*(C) (Accession numberNC_007792), pV030-8 plasmid (39 kb) harboured just *ileS2* (Accession number NC_010279), plasmid pTN147 (43.9 kb) harboured *aacA-aphD*, *aadD*, *dfrS1*, *bleO* and *ileS2* (Accession number GQ900433.1), plasmid pGA672 (47.3 kb) encoded *aacA-aphD*, *aadD*, *qacA*, *qacC*, *smr* and *ileS2* (Accession number GQ900432.1). The final plasmid pPAnicu (44.1 kb) harboured *aacA-aphD*, *aadD*, *qacA, qacB*, *smr* and *ileS2* (Accession number GQ900434.1).

In the present study seven plasmids were found among seven isolates from three different genotypes recovered over a 10-year period (1999-2009). It has been suggested that IS*257* may play a role in acquisition of *ileS2* and other resistance determinants (Pérez-Roth *et al.*, 2010). The *ileS2* gene has also been found to be chromosomally located however the mechanism of *ileS2* transfer was not determined, but could lead to increase clonal spread of this gene (Udo *et al.*, 2003).

All except one of the 109 MRSA isolates investigated in this part of the present study were found to harbour *ileS2* using DNA microarray profiling. The single isolate that lacked *ileS2* was originally phenotypically resistant to mupirocin however on repeat analysis it was susceptible, suggesting that it may have lost an *ileS2*-encoding plasmid. This isolate was first recovered in 2000 but did not undergo DNA microarray analysis until 2009. All Hi-MupR MRSA isolates were also found to harbour various combinations of additional antimicrobial resistance genes. In particular, aminoglycoside resistance genes were identified in 83% of isolates and these isolates belonged to all five STs. Overall, the greatest number of antimicrobial resistance genes were identified among the CC8 lineage (*merA & merB*, *blaZ*, *erm*(A), *erm(C)*, *lnu*(A), *aacA-aphD*, *aadD*, *aphA3* & *sat*, *dfrS1*, *ileS2*, *tet*(K), *sdrM*, *cat*-pC221, *fosB*, *qacA* and *qacC*), which supports the findings presented in Chapter 3, section 3.3.1.2.3.

Each different Hi-MupR MRSA clone was also found to harbour a characteristic combination of virulence-associated genes e.g. the ST22-MRSA-IV isolates were the only isolates to harbour *sec* and *sel* and ACME and *tst* were only identified in CC30 isolates. This also supports the findings presented in Chapter 3, section 3.3.1.2.3 and in Chapter 4, section 4.3.1.1. Hi-MupR isolates belonging to CC8 (ST8-MRSA-IIA-E & SCC_{M1}, IIC-E, or IVE/IVF) and the CC30 clone (ST36-MRSA-II) represented by more then one isolate harboured the least number of virulence genes, enterotoxin genes *sea*, *sep* and *egc* cluster and IEC (immune evasion cluster genes) *sak*, *chp* and *scn*, *tst*. In contrast, Hi-MupR isolates from China, including both ST239-MRSA-*agr*1 (no SCC*mec* type indicated) and ST5-MRSA-*agr*1, were found to harbour different combinations of virulence genes; ST239-MRSA-*agr*1 (*sea*, *tst*, *seg*, *sei*, *sec*) and ST5-MRSA-*agr*1 (*sea*, *seg*, *sei*, *sej* and *tst*) (Liu *et al.*, 2012a).

In the present study, three isolates exhibiting a mupirocin-susceptible phenotype (MICs < 4 mg/L) harboured *ileS2* and in each case DNA sequencing of amplified *ileS2* amplimers revealed a single base deletion that resulted in a premature stop codon. This mutation in *ileS2* has also been reported elsewhere recently (Swenson *et al.*, 2010; McNeil *et al.*, 2011). The seven Hi-MupR isolates selected for plasmid analysis were also investigated for the presence of low-level *ileS* mutations. Three of these isolates were found to contain a previously described point mutation in the chromosomal *ileS* gene that results in an amino acid substitution (V588F). This V588F mutation affects the Rossman fold within IleS and has also been described previously in MRSA harbouring *ileS2* (Antonio *et al.*, 2002). However, studies have shown that this mutation does not affect the *in vitro* fitness or virulence of glycopeptide-resistant *S. aureus* Mu50 or laboratory-derived mupirocin-resistant mutants of Mu50 (Hurdle *et al.*, 2004). Novel mutations were detected in *ileS* in one isolate resulting in an amino acid substitution

F563V, while in the remaining three Hi-MupR isolates the amino acid substitutions R147N and R150N was detected. The impact of these novel mutations, if any, on the IleS protein function warrants further investigation. The recently identified *mupB* (*ileS3*) gene was not identified among isolates in the present study. The *ileS3* gene has so far only been described among three ST5-MRSA-IV CA-MRSA isolates in Canada and it was found to be located on a non-conjugative plasmid suggesting that the spread of this new high-level mupirocin resistance gene may be limited (Seah *et al.*, 2012).

Certain limitations were encountered with plasmid analysis methodology used in the present study. Determining the size of plasmids and determining the plasmid content of isolates was difficult using the minilysate plasmid extraction method. The sizing of *ileS2*-encoding plasmids was improved as part of this study by S1 nuclease digestion of total cellular DNA in agarose gel plugs followed by PFGE. However, PFGE is laborious, time-consuming and requires expensive equipment and consumables. Ideally, whole-plasmid sequencing is needed for the full characterisation of these multiresistance plasmids. However, purification of large plasmids for whole-plasmid sequence analysis is currently difficult with only one reasonably effective commercial Hi-Speed maxi-plasmid purification kit (Qiagen, California, USA) being described (Pérez-Roth *et al.*, 2010).

Since the plasmids identified among the different MRSA isolates were all different it is possible that the sources of these plasmids are CoNS. Previous studies have found evidence to suggest that CoNS are a reservoir for resistance genes in *S. aureus*. (Leski *et al.*, 1999; Yun *et al.*, 2003; Cavdar *et al.*, 2004; Petinaki *et al.*, 2004; Wisniewska *et al.*, 2004; Rotger *et al.*, 2005). The *ileS2* gene has been identified in CoNS and since many CoNS species are also commonly found in the anterior nares, the use of mupirocin for MRSA nasal decolonisation may also select for mupirocin-

resistant CoNS and the resistance determinant could spread to MRSA. A five-year study from the Netherlands reported an increase from 8% in 2006 to 22% in 2011 of *ileS2* among CoNS from BSIs (Bathoorn *et al.*, 2012). A European study reported that 5.6% of CoNS exhibit Hi-MupR compared to 1.6% of *S. aureus* isolates from BSIs, hospital-acquired pneumonia and SSTIs in Belgium, France, Germany, Spain and the UK (Schmitz *et al.*, 1998). *In vivo* transfer of an *ileS2*-encoding plasmids ranging in sizes from 31.5 - 57 kb from *S. epidermidis, S. haemolyticus, S. xylosus, S. saprophyticus* and *S. capitis* to *S. aureus* has been shown, supporting the conjugative transfer of the *ileS2* gene between the species (Connolly *et al.*, 1993; Udo *et al.*, 1997; Hurdle *et al.*, 2005; do Carmo Ferreira *et al.*, 2011). Preliminary investigations from this laboratory have identified *ileS2* among CoNS from hospitalised patients but analysis is on-going (Emily Deasy, Microbiology Research Unit, Dublin Dental University Hospital, personal communication). A detailed investigation of the prevalence of *ileS2* among CoNS isolates in Ireland and detailed comparative analysis of the plasmids encoding Hi-MupR in CoNS and MRSA would further enhance our understanding of the evolution and reservoir of these plasmids in staphylococci.

The present study revealed a high prevalence of *ileS2* among ST22-MRSA-IV isolates which is a worrying development that may impact on the success of mupirocin for nasal decolonisation and should be closely monitored as this MRSA strain accounts for >80% of MRSA isolates recovered from patients in Irish hospitals. The localisation of *ileS2* on seven distinct conjugative plasmids among diverse MRSA genotypes is also alarming and indicates that *ileS2* has the potential to spread to other MRSA isolates. The results of the present study revealed the potential for these plasmids to either incorporate or mobilise additional resistance determinants such as those encoding resistance to aminoglycosides and lincosamides.

Chapter 6

Investigation of linezolid resistance in MRSA and MSSA isolates recovered from patients in Irish hospitals in 2005

6.1 Introduction

The ST22-MRSA-IV clone has predominated in Irish hospitals since 2002, while the ST30-MRSA-IVc and ST8-MRSA-IVa clones were the most common genotypes reported among *pvl*-positive CA-MRSA in Ireland (Rossney *et al.*, 2007). The *pvl*-positive ST8-MRSA-IVa, also known as USA300, was first reported in 2000 as a cause of SSTIs in the USA and it has since become the prevalent CA-MRSA strain in the USA and has also spread into hospitals (Diep *et al.*, 2006; Tenover *et al.*, 2006; David and Daum, 2010). Originally, ST8-MRSA-IVa isolates harboured only the methicillin (*mecA*) and erythromycin [*msr*(A)] resistance genes. However, they have since spread worldwide and acquired additional resistance determinants, mostly plasmid encoded, including *erm*(A) and *erm*(C), *tet*(M) and *tet*(K), and *ileS2*, which encode resistance to macrolides-lincosamides-streptogramin B (MLS_B), tetracyclines, and mupirocin (Tenover *et al.*, 2006; Han *et al.*, 2007; Monecke *et al.*, 2007; Diep *et al.*, 2008a; Tenover and Goering, 2009). In addition, resistance to fluoroquinolones (levofloxacin), gentamicin, and trimethoprim-sulfamethoxazole and reduced susceptibility to vancomycin and daptomycin have also been observed among some ST8-MRSA-IVa/USA300 isolates (Moran *et al.*, 2006; Graber *et al.*, 2007; Hageman *et al.*, 2008; Tenover and Goering, 2009).

Linezolid was the first oxazolidinone antimicrobial agent approved for clinical use by the FDA in the USA. It was licensed for the treatment of SSTIs and pneumonia caused by MRSA, *Streptococcus spp.* and vancomycin-resistant enterococci in 2001 (Meka and Gold, 2004). Linezolid inhibits protein synthesis by binding to the peptidyltransferase centre of the 50S ribosomal subunit (Bozdogan and Appelbaum, 2004). Linezolid resistance in *S. aureus* was reported just one year after it received FDA approval and since then there have been numerous reports of it in both MRSA and

MSSA (Kaatz and Seo, 1996; Tsiodras *et al.*, 2001). Two different mechanism of linezolid resistance have been reported. Firstly, several different mutations have been associated with linezolid resistance including mutations in the V domain region of 23S rRNA genes, with G2576T and T2500A being the most common amino acid substitutions identified in clinical *S. aureus* isolates (Tsiodras *et al.*, 2001; Meka *et al.*, 2004), and mutations in *rplC* and *rplD* encoding the 50S ribosomal proteins L3/L4 in *Streptococcus pneumonia* (Wolter *et al.*, 2005) and in *S. aureus* (Locke *et al.*, 2009b; Locke *et al.*, 2009a). The second mechanism of linezolid resistance is mediated by the *cfr* gene which encodes an rRNA methyltransferase that methylates the adenosine at position 2503 (*E. coli* numbering) in 23S rRNA within the overlapping binding site for linezolid and also for some other antimicrobial agents (Kehrenberg *et al.*, 2005). Recently the co-occurrence of the *cfr* gene and L3 mutations have been reported in linezolid resistant *S. aureus* (Locke *et al.*, 2010).

The staphylococcal *cfr* gene can be plasmid or chromosomally-located and encodes resistance to five classes of antimicrobial agents including phenicols, linosamides, oxazolidinones, pleuromutilins and streptogramin A compounds, a phenotype that has been termed $PhLOPS_A$ (Long *et al.*, 2006). Each of these antimicrobial classes include antimicrobial agents that are used in the treatment of staphylococcal infections in either human or veterinary medicine, including chloramphenicol, florfenicol, clindamycin, lincomycin, pirlimycin, linezolid, retapamulin, tiamulin, valnemulin and quinupristin/dalfopristin (Long *et al.*, 2006).

The *cfr* gene was originally identified in CoNS from animals (Schwarz *et al.*, 2000), but more recently it has also been found in a small number of *S. aureus* and CoNS isolates from humans (Mendes *et al.*, 2008; Gopegui *et al.*, 2012). It is usually plasmid located and nine *cfr*-carrying plasmids have been described to date in

staphylococci (Table 6.1). Plasmid pSCFS1 (17.1 kb) was the first *cfr* plasmid to be described and it was isolated from a bovine *S. sciuri* isolate (Schwarz *et al.*, 2000). This plasmid also harbours additional antimicrobial resistance genes including *erm*(33), encoding MLS_B macrolide, lincosamide and streptogramin B resistance, and *lsa*(B) encoding reduced susceptibility to lincosamides (Kehrenberg *et al.*, 2004). The larger *cfr*-encoding plasmids pSCFS3 (36 kb) and pSCFS6 (43 kb) were reported soon after pSCFS1 and were identified in staphylococci from bovine and porcine. These plasmids carry the Tn*558*-associated phenicol resistance gene *fexA* in addition to *cfr*. In pSCFS3 and pSCFS6, *cfr* is integrated at different positions within the transposase genes *tnpA-tnpB* or *tnpC* of Tn*558* (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007). The insertion sequence IS*21-558* is present upstream of the *cfr* gene in pSCFS3, but it is present both upstream and downstream of the *cfr* gene in pSCFS6 (Table 6.1).

The first report of a *cfr*-encoding plasmid in a human clinical MSSA isolate was in 2008 from a female patient in a long-term healthcare facility in the USA (Mendes *et al.*, 2008). The *cfr* and *erm*(A) genes were found to be located to a large ca. 55 kb plasmid, termed p004-737X, in this *S. aureus* isolate together with the insertion sequences *istAS* and *istBS*. In the same study *cfr* was also identified in a *S. epidermidis* isolate from a different patient and in was found to be located on a large ca. 175 kb plasmid termed p426-314L, although it was not reported whether the plasmid was conjugative (Mendes *et al.*, 2008) (Fig. 6.1). Recently, a large ca. 50 kb conjugative plasmid, pERGB, was identified in two CC125/ST125-MRSA-IVc isolates from two patients with respiratory tract infections in a Spanish hospital (Gopegui *et al.*, 2012). This plasmid carried *cfr*, *ant*(4)-*Ia* (encoding resistance to tobramycin), *tet*(L) (encoding resistance to tetracycline) and *dfrK* (encoding resistance to trimethoprim) (Fig. 6.1). An entire copy of insertion sequence IS*21-558* with a 100% homology to that in pSCFS3

Table 6.1 *cfr*-carrying plasmids that have been described to date in staphylococci and other genera

Plasmid designation[a]	Antimicrobial resistance genes	Size (kb)	Host bacteria	Source	Reference
pSCFS1	*cfr*, *erm*(33), *spc* & *lsa*(B)	17.1	*S. sciuri*	Bovine	Schwarz *et al.*, 2000
pSCFS3	*cfr* & *fexA*	35.7	*S. lentus, S. aureus* (ST9-MSSA & ST398-MRSA-V)	Bovine & Porcine	Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2009
pSCFS6	*cfr*, *fexA* & *lsa*(B)	43	*S. warnei, S. simulans*	Porcine	Kehrenberg *et al.*, 2007
p004-737X	*cfr* & *erm*(A)	55	*S. aureus*	Human	Mendes *et al.*, 2008
p426-314L	*cfr*	175	*S. epidermidis*	Human	Mendes *et al.*, 2008
pERGB	*cfr* & *tet*(L), *ant*(4')-*Ia*, *dfrK*	50	ST125-MRSA-IVc	Human	Gopegui *et al.*, 2012
pSS-01	*cfr*, *fexA* & *aacA-aphD*	40	*S. saprophyticus* *S. cohnii*	Porcine	Wang *et al.*, 2012d
pSS-02	*cfr*	35.4	*S. saprophyticus* *S. sciuri*	Porcine	Wang *et al.*, 2012d
pSS-03	*cfr* & *erm*(C)	7.1	*S. sciuri*	Porcine	Wang *et al.*, 2012d
pBS-01	*cfr*, *fexA* & *erm*(B)	16.5	*Bacillus spp.*	Porcine	Dai *et al.*, 2010
pBS-02	*cfr* & *fexA*	16.5	*Bacillus spp.*	Porcine	Zhang *et al.*, 2011
pBS-03	*cfr* & *aadY*	7.4	*Bacillus sphaericus*	Porcine	Wang *et al.*, 2012b
pHOU-*cfr*	*cfr*	97	*E. faecalis*	Human	Diaz *et al.*, 2012
pEF-01	*cfr* & *fexB*	32.4	*E. faecalis*	Bovine	Liu *et al.*, 2012b
pW3	*cfr*, *fexB* &*erm*(B)	75	*E. thailandicus*	Porcine sewage	Liu *et al.*, 2012a
p3-38	*cfr* & *erm*(A)	72	*E. thailandicus*	Porcine	Liu *et al.*, 2012a
pW9-2	*cfr*, *fexA* & *erm*(B)	55	*E. faecalis*	Porcine sewage	Liu *et al.*, 2012a
pJP1	*cfr*, *ble*, *aadD*, *aacA-aphD*, *erm*(C)	53	*Jeotgalicoccus pinnipedialis* & *M. caseolyticus*	Porcine	Wang *et al.*, 2012c
pEC-01	*cfr*	110	*E. coli*	Porcine	Wang *et al.*, 2012a

[a] Plasmids shown in bold were confirmed as conjugative plasmids.
Abbreviations: S. *Staphylococcus*; E, *Enterococococcus;* M, *Macrococcus* E. *Escherichia*

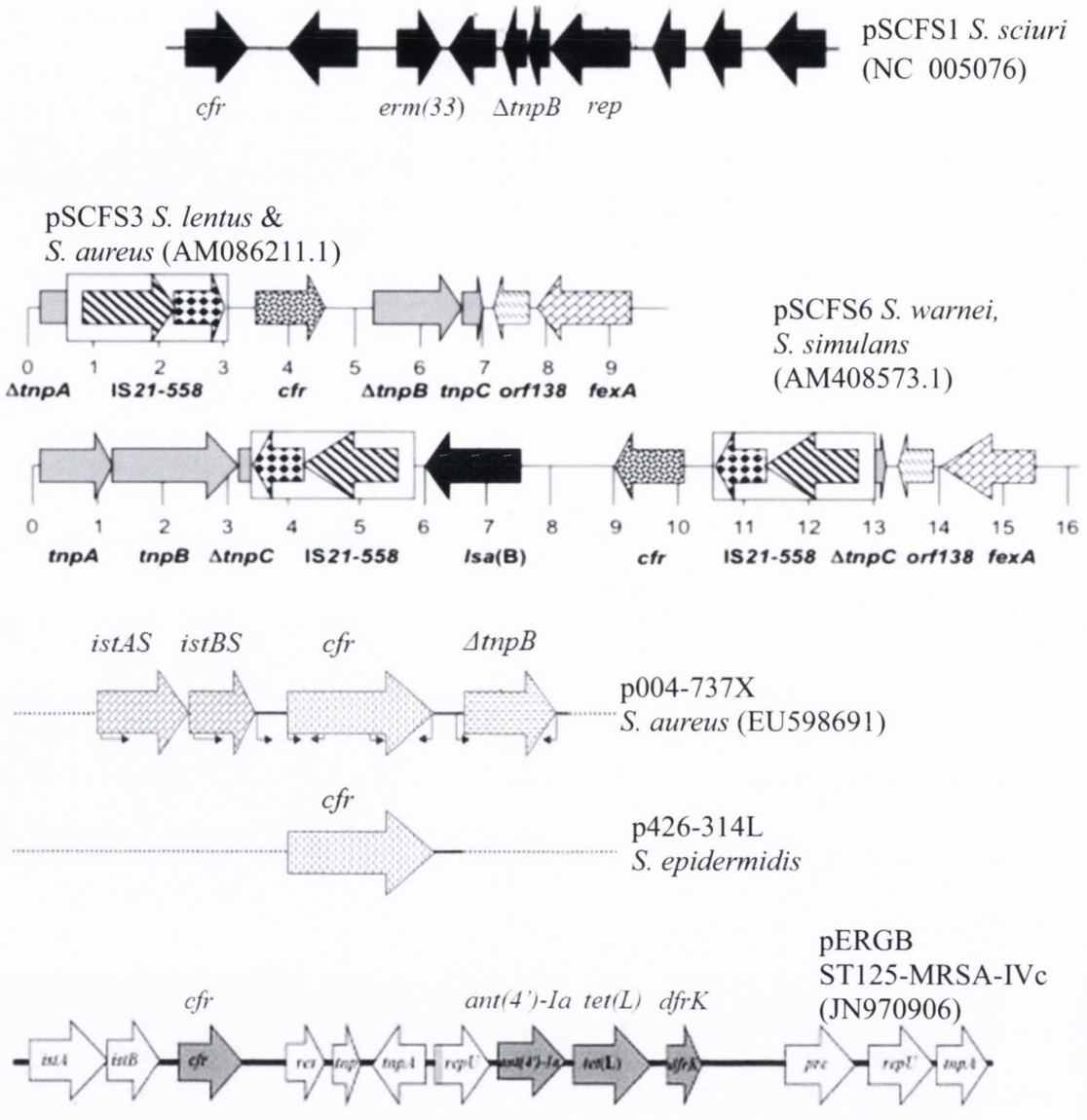


Fig. 6.1 Schematic diagram showing the organisation of *cfr*-carrying plasmids described to date in staphylococci and other genera

Continued overleaf

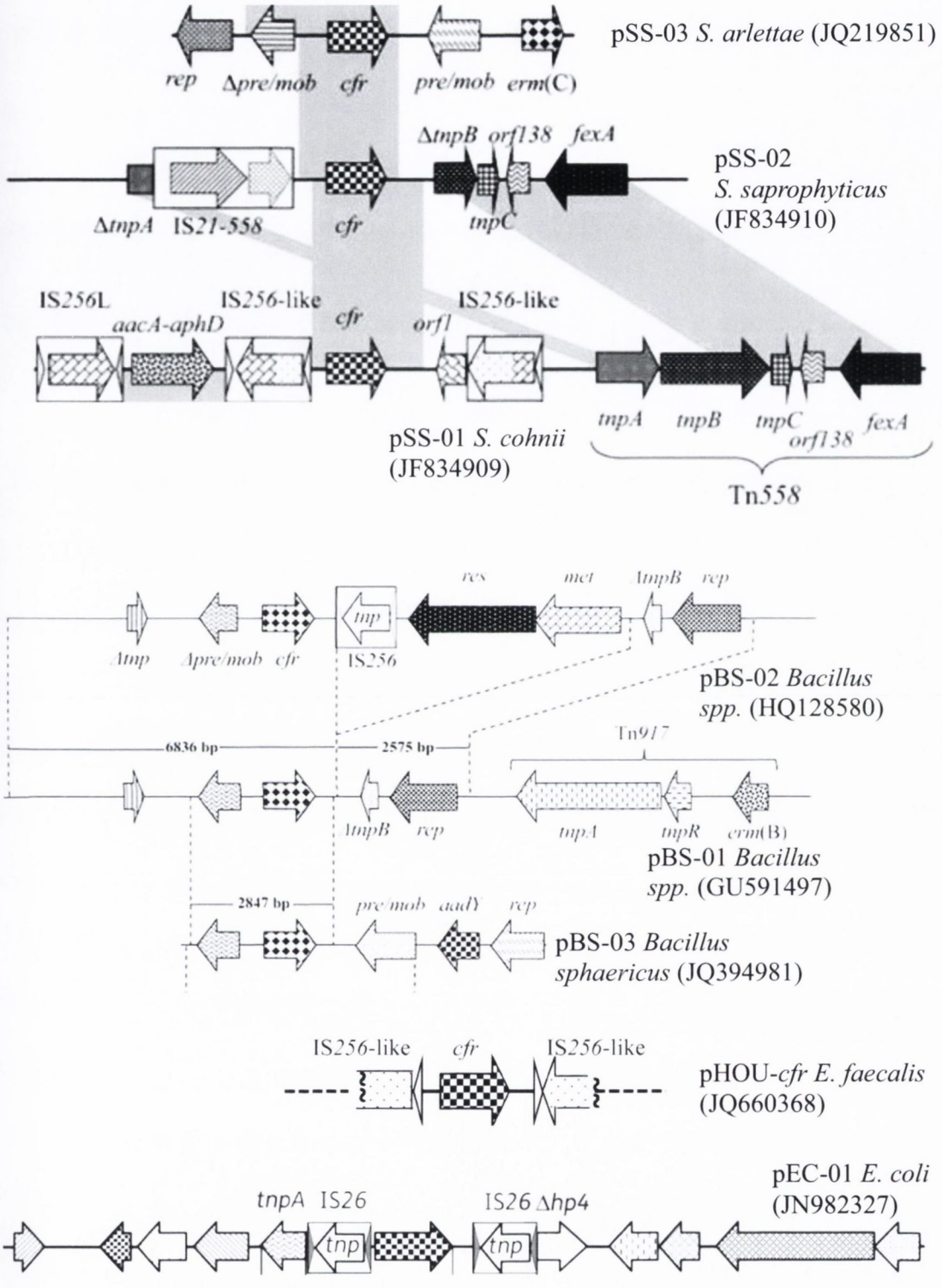


Fig. 6.1 Schematic diagram showing the organisation of *cfr*-carrying plasmids described to date in staphylococci and other genera

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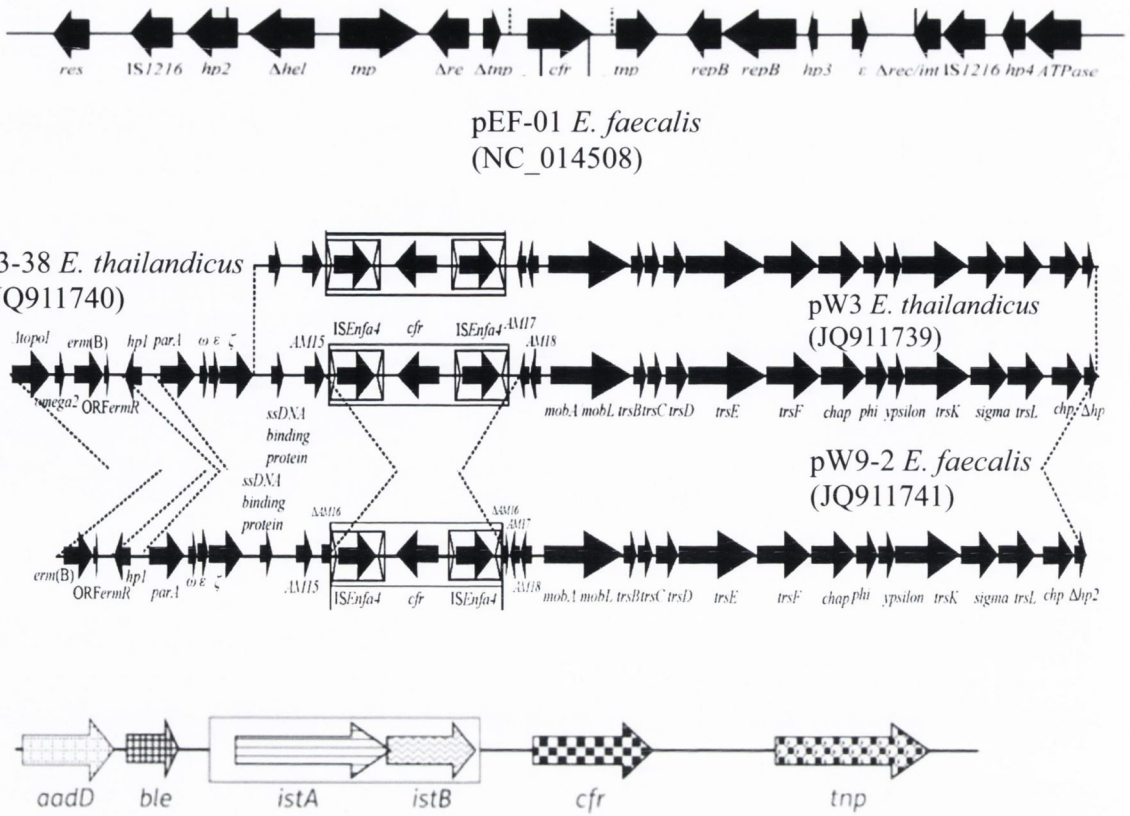

Fig. 6.1 Schematic diagram showing the organisation of *cfr*-carrying plasmids described to date in staphylococci and other genera

(Kehrenberg *et al.*, 2007) was identified upstream of *cfr*, and *ant*(4')-*Ia*, *tet*(L), and *dfrK* were located downstream of *cfr*, with each of these resistance genes exhibiting high DNA sequence identity with the corresponding genes carried by the pKKS825 multiresistance plasmid from a porcine ST398-MRSA strain (Kadlec and Schwarz, 2009b; Gopegui *et al.*, 2012). The co-location of *cfr* on conjugative plasmids with genes encoding resistance to additional antimicrobial agents may select for the carriage and spread of the *cfr* gene.

The most recently described *cfr* plasmids, namely pSS-01 (40 kb), pSS-02 (35.4 kb) and pSS-03 (7.1 kb), were reported among CoNS recovered from pigs in China in 2012 (Wang *et al.*, 2012e) (Fig. 6.1). In pSS-01, a Tn*4001*-like transposon encoding *aacA-aphD* is located upstream of *cfr* and the *fexA*-carrying transposon Tn*558* is located downstream. In contrast in pSS-02, IS*21-558* is located upstream of *cfr* and truncated Δ*tnpA* and Δ*tnpB* genes are located downstream (Wang *et al.*, 2012e). In the smaller plasmid pSS-03, *pre*/*mob* genes that are involved in plasmid mobilisation, are located upstream of *cfr* and *erm*(C) is located downstream (Wang *et al.*, 2012e) (Fig. 6.1).

The *cfr* gene is not unique to staphylococci and recently it has been reported on 10 different plasmids, mainly in *Bacillus* and *Enterococcus* spp. and mostly from animals (Table 6.1). In 2010, *cfr* was reported for the first time in a non-staphylococcal species in *Bacillus spp.* strain BS-01 from swine faeces in China (Dai *et al.*, 2010b). In this *Bacillus spp.* isolate *cfr* was detected on a novel 16.5 kb plasmid, pBS-01, together with the transposon Tn*917*, which carries the MLS_B resistance gene *erm*(B) (Dai *et al.*, 2010a) (Fig. 6.1). One year later, a 16 kb plasmid, pBS-02, carrying *cfr* and *fexA* was identified in *Bacillus spp.* from pigs (Zhang *et al.*, 2011). In pBS-02, an IS*256* sequence was identified upstream of *cfr* together with *nes*, encoding a restriction endonuclease, and *met*, encoding a DNA methylase, which the authors speculated may aid in the

horizontal transfer of *cfr* (Zhang *et al.*, 2011) (Fig. 6.1). In 2012, a smaller plasmid, pBS-03 (7.4 kb), was identified in a *Bacillus sphaericus* isolate from China (Wang *et al.*, 2012c). The *cfr* gene in pBS-03 exhibited 99% DNA sequence homology to *cfr* in pBS-01 and pBS-02. The pBS-03 plasmid also carried the streptomycin resistance gene *aadY*, together with the plasmid mobilisation genes *pre*/*mob* (Wang *et al.*, 2012c) (Fig. 6.1).

The *cfr* gene was first reported in enterococci (*Enterococcus faecalis*) from a patient in Thailand in 2012 (Diaz *et al.*, 2012a). In this case, the *cfr* gene was localised on a 97 kb conjugative plasmid, pHOU-*cfr*, and *cfr* exhibited 100% nucleotide sequence identity to *cfr* found in *S. sciuri* pSCFS1 (Kehrenberg *et al.*, 2004). Interestingly, *cfr* on pHOU-*cfr* is flanked on both sides by IS*256*-like sequences which showed 99% nucleotide sequence identity to IS*256* previously identified in *Staphylococcus cohnii* (Diaz *et al.*, 2012a; Wang *et al.*, 2012e) (Fig. 6.1).A second *cfr*-positive *E. faecalis* isolate was recovered from porcine sewage on a pig farm in China and was located to another novel plasmid, pEF-01 (Liu *et al.*, 2012c) (Table 6.1 and Fig. 6.1). The *cfr* gene has also been detected on various different plasmids in additional enterococcus spp. as well as in *M. caseolyticus*, *Jeotgalicoccus pinnipedialis* and *E. coli* from animals (Wang *et al.*, 2012b; Wang *et al.*, 2012d). Of the 17 different *cfr* plasmids that have been reported to date, only two from staphylococci, pSCFS7 and pERGB, and four from enterococci, pHOU-*cfr*, pW3, p3-38, and pW9, have been shown to be conjugative (Table 6.1 and Fig. 6.1).

The *cfr* gene has also been found to be chromosomally located in one MRSA isolate and one *Proteus vulgaris* isolate. The MRSA isolate was recovered from a patient in a Columbian hospital and *cfr* was found to be located on a 15.5 kb plasmid, integrated within the chromosomal 23S rRNA allele 4 (Toh *et al.*, 2007; Locke *et al.*,

2012). This element also harboured *erm*(B) and IS*21-558*. The CM05 *cfr* plasmid-like element can excise from the chromosome by *erm*(B) recombination creating minicircles which has the potential to promote dissemination of *cfr* (Locke *et al.*, 2012). The *cfr*-positive *P. vulgaris* isolates was from a pig farm in China and *cfr* was flanked by IS*26* and inserted into the chromosomally located *fimD* (encoding a putative fimbiral biogenesis usher protein) gene (Wang *et al.*, 2011).

A recent survey investigated the rates of linezolid resistance among staphylococci recovered in the USA, Brazil, Columbia, Spain, the UK, Germany, Ireland, Korea and Japan (Gu *et al.*, 2012). Rates of linezolid resistance were higher among CoNS (LRCoNS; 1.4%) compared to *S. aureus* (LRSA; 0.5%). Linezolid resistance was found to be due to either chromosomal mutations (60.2% of LRCoNS and 63.5% of LRSA) or the presence of *cfr* (18.5% of LRCoNS and 40% of LRSA). The most common amino acid substitution in 23S rRNA in both LRSA and LRCoNS was G2576T, which was found among 60.2% of LRCoNS and 63.5% of LRSA, but other mutations identified in LASA include G2534T, G2603T, T2504A, C2190T, G2474T, G2447T, C2461T and G2447U (Gu *et al.*, 2012). Unfortunately this study did not specify which countries the *cfr*-positive LRCoNS and LRSA originated from and to what species the one isolate from Ireland belonged to (Gu *et al.*, 2012). A study from 2008 identified linezolid resistant *S. epidermidis* isolates from 16 colonised patients in a hospital in Ireland. In all 16 isolates the 23S rRNA gene G2576T amino acid substitution was identified but isolates were not investigated for the presence of *cfr* (Kelly *et al.*, 2008).

6.1.1 Aims

As part of ongoing surveillance and detailed characterisation of HA- and CA-MRSA in Ireland by this laboratory, a previously described *pvl*-positive ST8-MRSA-IVa (USA300) isolate recovered in 2005 in Ireland, was found to harbour *cfr*. In addition, as part of ongoing surveillance of linezolid resistance among *S. aureus* in Ireland, two MSSA isolates exhibiting intermediate resistance to linezolid were identified by the NMRSARL in 2005.

The purpose of this part of the study was to comprehensively characterise the genetic environment of *cfr* in the ST8-MRSA-IVa/USA300 isolate and to genotype and determine the mechanism(s) of linezolid resistance of the two MSSA isolates.

6.2 Materials and Methods

6.2.1 Bacterial isolates

A previously described *pvl*-positive CA-MRSA isolate, M05/0060, recovered in Ireland as part of a study of *pvl*-positive MRSA in Ireland (Rossney *et al.*, 2007) was found to harbour the multidrug resistance gene *cfr* using DNA microarray profiling. Isolate M05/0060 was previously found to belong to the USA300 clone ST8-MRSA-IVa and was recovered from a 26 year-old patient with a superficial scalp abscess in 2005 (Rossney *et al.*, 2007). Two ST8-MRSA-IVa/USA300 reference strains, for which whole genome sequences are available (USA300-FPR3757 [GenBank accession no.CP000255] and USA300-TCH1516 [GenBank accession no. CP000730]) were included for comparison of their DNA microarray hybridisation profiles. The plasmid-free, novobiocin-resistant *S. aureus* strain XU21 (Udo and Jacob, 1998) was used as a recipient in filter mating experiments (Chapter 2, section 2.6.2).

Two MSSA isolates, M05/0209 and M05/0210 recovered from a 17-year male in an Irish hospital that exhibited intermediate resistance to linezolid, underwent DNA microarray profiling. The *cfr* gene was not identified and the isolates were investigated for the presence of mutations in the V domain region of 23S rRNA genes which have been previously shown to be associated with linezolid resistance.

6.2.2 Antimicrobial susceptibility testing

The three *S. aureus* isolates underwent AR typing as described in Chapter 2, section 2.2.1. Comparative antimicrobial susceptibility testing of the *cfr*-positive MRSA isolate M05/0060, its plasmid-cured derivative M05/0060-C1, the plasmid-free *S. aureus* recipient strain XU21, and its *cfr*-positive transconjugant derivative XU21-T1 was performed by broth microdilution as described in CLSI documents M07-A8 (CLSI, 2009) and M100-S20 (CLSI, 2010). Further susceptibility tests on M05/0060 and the transconjugant derivative XU21-T1 were performed using Vitek-2 (AST P580 panel, susceptibility tests for Gram-positive bacteria, BioMérieux, Germany) and E-test (chloramphenicol, clindamycin, linezolid and quinopristin-dalfopristin, BioMérieux, Germany) according to manufacturers' protocols and were performed by collaborators at the Technical University of Dresden, Germany.

6.2.3 DNA microarray profiling

All isolates underwent DNA microarray profiling using the Staph-Type Kit (Alere) as described in Chapter 2, section 2.4.5.

6.2.4 Molecular typing

The *pvl*-positive MRSA isolate M05/0060 was previously typed by PFGE and SCC*mec* typing as well as by MLST (Rossney *et al.*, 2007). M05/0060 was also previously subject to toxin gene typing, which involved several multiplex PCRs to detect the staphylococcal enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*; the exfoliative toxin genes *eta*, *etb*, and *etd*; the toxic shock toxin (TST) gene *tst* and *pvl*(Rossney *et al.*, 2007). As part of the present study M05/0060 underwent *spa* typing

and *dru* typing, as previously described in Chapter 2, sections 2.4.3, and 2.4.4 respectively.

6.2.5 PCR amplification and sequencing of domain V of the five 23S rRNA alelles

The *pvl*-positive MRSA isolate M05/0060 along with MSSA isolates M05/0209 and M05/0210 underwent PCR amplification of five of the 23S rRNA alleles as described previously (Meka *et al.*, 2004). Each 23S rRNA allele (*rrn1-rrn5*) was amplified from each isolate using the primers listed in Table 6.2, the long template PCR system (Roche) and the following PCR conditions; 94°C for 1 min followed by 32 cycles of 94°C for 30s, 55°C for 30 s and 68°C for 7 min, with a final extension at 72°C for 10 min (Meka *et al.*, 2004). PCR products were visualised in agarose gels and purified as described in Chapter 2, section 2.4.2. For each isolate, the purified PCR products for each allele were used as a template to amplify the domain V region of each allele using primers 23s rRNA-F and 23s rRNA-R (Table 6.2) (Pillai *et al.*, 2002) and the following PCR conditions; 94°C for 5 min followed by 32 cycles of 94°C for 30s, 55°C for 30 s and 71°C for 1 min, with a final extension at 72°C for 10 min. PCR products were visualised, purified and sequenced as described in Chapter 2, section 2.4.2. The domain V sequence of each allele from each isolate was compared to the corresponding domain V consensus sequences of the *S. aureus* reference strain N315 (Genbank Accession number NC_002745.2) in order to identify any mutations.

6.2.6 Plasmid analysis

Plasmid analysis was carried out on isolate M05/0060 only.

6.2.6.1 Plasmid curing

For plasmid curing, isolate M05/0060 was cultured in BHIB at 43°C for 24 h at 200 rpm, subcultured into fresh BHIB and incubated as before for five consecutive rounds, after which individual colonies obtained following plating on BHIA were screened for loss of resistance to chloramphenicol (30 mg/L) by replica plating (Chapter 2, section 2.6.1). Putative cured derivatives underwent DNA microarray analysis (Chapter 2, section 2.4.5) and antimicrobial susceptibility testing as described above in section 6.2.2.

6.2.6.2 Filter Mating

Conjugative transfer of *cfr* from MRSA isolate M05/0060 to the plasmid-free, novobiocin-resistant *S. aureus* recipient strain XU21 was undertaken by filter mating (Woodford *et al.*, 1998) as described in Chapter 2, section 2.6.2. Putative transconjugants were plated onto BHIA containing 100 mg/L novobiocin to select for the recipient strain and chloramphenicol (30 mg/L) to select for a putative *cfr*-carrying plasmid (Chapter 2, section 2.6.2). Putative transconjugants underwent DNA microarray profiling and comparative antimicrobial susceptibility testing as described in Chapter 2, section 2.4.5 and in section 6.2.2 above, respectively.

Table 6.2 PCR primers used in the study

Gene or region amplified	Primer pair	Nucleotide sequence (5'-3')	Nucleotide coordinates
cfr	cfrF1	GACTTTCGGCACCGGTAAT	3419 – 3437[a]
	cfrR3	CAGTAGTCCATTCATATTTG	4681 – 4662[a]
radC	radC-fw01	AGAGAAAATGCCAATGCAATCA	1736892–173687[c]
	radC-rv02	TCACCGATTATAATATGATCCAATAAATC T	1736773–1736744[c]
fexA	fexAF2	GCGTGATTCCTAAGCTATT	7146-7164[a]
	fexAR1	GAGAACCGAATCTTTAATCA	8800-8781[a]
pSCFS6 *fexA-cfr*	fexA-02	TTGCCTTAATAGCTGGATTAGGT	7268-7246[a]
	cfr-01	ACCTTCATATCCTCAAATCGACT	10941-10963[a]
pSCFS3 *fexA-cfr*	fexA-02	TTGCCTTAATAGCTGGATTAGGT	7268-7246[a]
	cfr-02	GCTGCTTGTGGTCAATTATATGG	4517-4539[b]
tnpA	tnpA-fw	GGTTCAGAAAGTAATTGTGGAGGA	1159-1182[b]
tnpA	tnpA-rv	TGTGGCATGCGTATGACGTA	2089-2070[b]
tnpB	tnpB-fw	GGAACAGTGATAAGCCGGATG	2536-2556[b]
tnpB	tnpB-rv	ACATGGTCCTGCAAT TACTGGTA	3954-3932[b]
tnpC	tnpC-fw	CAGCTAGCTAAAGACAAGTCGGA	6019-6041[a]
tnpC	tnpC-rv	GTTCATTCTCTTCTTCTAACGCCTT	6307-6283[a]
rrn1	RRN1-F	GCGGTGTTTTGAGAGATTATTTA	505658–505680[d]
rrn 1	RRN1-R	GCTTCATGATATACGCTTCCTTT	512162–512184[d]
rrn2	RRN2-F	GCAGACGCACAGGACTTA	550100–550117[d]
rrn2	RRN2-R	GATACCGTCTTACTGCTCTTCTC	555948–555970[d]
rrn3	RRN3-F	AGGCCGGCAATATGTAAG	1918612–1918629[d]
rrn3	RRN3-R	GTCGTCAAACGGCACTAATA	1924170–1924189[d]
rrn4	RRN4-F	TGTGGACGGTGCATCTGTAG	2107748–2107767[d]
rrn4	RRN4-R	ATCACCCGCTCCATAGATAAT	2114263–2114283[d]
rrn5	RRN5-F	GCCGATAGCTCTACCACTG	2230678–2230696[d]
rrn5	RRN5-F	AGGTGCGATGGCAAAACA	2236573–2236590[d]
23S rRNA	23s rRNA-F	GCGGTCGCCTCCTAAAAG	2280–2297[e]
23S rRNA	23s rRNA-R	ATCCCGGTCCTCTCGTACTA	2680–2699[e]

[a] Nucleotide coordinates based on the nucleotide sequence of plasmid pSCFS3 (Genbank accession number AM086211)

[b] Nucleotide coordinates based on the nucleotide sequence of plasmid pSCFS6 (Genbank accession number AM408573.1)

[c] Nucleotide coordinates based on the nucleotide sequence of MRSA strain COL (GenBank accession number CP000046.1).

[d] Nucleotide coordinates based on the nucleotide sequence of *S.aureus* strain N315 (GenBank accession number NC002745.2)

[e] Nucleotide coordinates based on the nucleotide sequence of *S. aureus* gene for 23S rRNA (GenBank accession number X68425.1).

6.2.6.3 Plasmid DNA Extraction

Plasmid DNA from the *cfr*-positive MRSA isolate M05/0060, its plasmid-cured derivative M05/0060-C1 and a transconjugant derivative of XU21 (XU21-T1) were extracted and purified using a modified alkaline lysis method as described previously (Schwarz *et al.*, 1989) (Chapter 2, section 2.6.3).

6.2.6.4 Restriction enzyme analysis of plasmid DNA

The approximate molecular size of the *cfr*-plasmid detected in the *cfr*-positive transconjugant derivative XU21-T1 was determined based on the sum of the fragment sizes obtained following digestion of plasmid DNA with the restriction endonuclease *Blg*II, *Eco*RI, and *Hin*dIII (Roche Diagnostics Ltd) according to the manufacturer's instructions. A 1 kb DNA ladder (Invitrogen) was used as a molecular reference marker (M7).

6.2.7 Molecular characterisation of the novel *cfr*- and *fexA*- encoding plasmid pSCFS7

The *cfr* and *fexA* genes of M05/0060 were amplified and sequenced using primers cfrF1/cfrR3 and fexAF2/fexAR1, respectively (Table 6.2), using GoTaq DNA polymerase (Promega) according to the manufacturer's instructions and the following conditions: 94°C for 2 min, followed by 34 cycles of 95°C for 30 s, 50°C for 2 min and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were purified and sequenced as described in Chapter 2, section 2.4.2.

In order to investigate if *cfr* was associated with the *fexA*-encoding transposon Tn*558* in the *cfr*-encoding plasmid pSCFS7 identified in M05/0060 in the present study, total cellular DNA from M05/0060 and the transconjugant derivative XU21-T1

harbouring pSCFS7, were subjected to PCR analysis for the different regions of Tn*558*. The transposase genes *tnpA*, *tnpB* and *tnpC* and the region from *tnpB-fexA* were amplified from M05/0060 and XU21-T1 using previously described primers and conditions (Kehrenberg and Schwarz, 2006) (Table 6.2). Since the *tnpB* amplicon obtained was larger than expected for Tn*558* in both M05/0060 and XU21-T1, PCRs were also performed using the gel purified *tnpB* amplification product obtained with template DNA from both organisms using previously described *cfr*-specific primers (Kehrenberg and Schwarz, 2006) (Table 6.2). The resulting amplicon from M05/0060 was cloned, as described previously (Kehrenberg and Schwarz, 2006) and sequenced by primer walking (MWG, Ebersberg, Germany) by collaborators at the Institute of Farm Animal Genetics, Friedrich-Löeffler-Institut FLI, Neustadt-Mariensee, Germany.

6.2.8 Nucleotide sequence accession numbers

The nucleotide sequences of *cfr*, *fexA*, and the 4,043-bp *tnpB* amplicon of M05/0060 have been deposited in GenBank under accession numbers FN995111, FN995110 and FR675942, respectively.

6.3 Results

6.3.1 Identification and characterisation of the novel *cfr* and *fexA* encoding plasmid pSCFS7 identified in the ST8-MRSA-IVa/USA300 isolate M05/0060

The *pvl*-positive MRSA isolate M05/0060 was found to harbour the multidrug resistance gene *cfr* following DNA microarray screening. This isolate was further characterised using DNA microarray profiling and the genetic environment of the *cfr* gene was also investigated.

6.3.1.1 Genotypic and phenotypic characterisation of M05/0060

Isolate M05/0060 exhibited the same MLST (ST8), *spa* (t008), *dru* (dt9g) and SCC*mec* type (IVa) as the ST8-MRSA-IVa/USA300 clone (Tenover *et al.*, 2006). DNA microarray profiling of M05/0060 and comparison with DNA microarray profile data for two whole-genome sequenced ST8-MRSA-IVa/USA300 reference strains (FPR3757 and TCH1516) (Diep *et al.*, 2006; Highlander *et al.*, 2007), revealed that M05/0060 was indistinguishable from ST8-MRSA-IVa/USA300, apart from carriage of *cfr* and *fexA* (Table 6.3). M06/0060 harboured *blaZ*, *msr*(A) & *mph*(C) (encoding MLS resistance including erythromycin, lincomycin and clindamycin), *sdrM* (encoding resistance to norofloxacin and acriflavine), *fosB* (encoding fosfomycin resistance), *egc* (*seg, sei, sem, sen, seo and seu*) and IEC type B (*sak*, *chp* and *scn*) (Table 6.4). No mutations were identified in the domain V of the five 23S rRNA alleles of M05/0060. Antimicrobial susceptibility testing revealed that M05/0060 exhibited the *cfr*-associated resistance phenotype ($PhLOPS_A$), including resistance to chloramphenicol and florfenicol (phenicols), clindamycin, lincomycin and pirlimycin (lincosamides), linezolid (oxazolidone), ratapamulin, tiamulin and valnemulin (pleuromutilins) and virginamycin and quinupristin/dalfopristin (streptogramin A compounds) (Table 6.4).

6.3.1.2 Confirmation of the presence of *cfr* and *fexA*

The presence of *cfr* and *fexA* in M05/0060 was confirmed by PCR and sequencing of the entire *cfr* and *fexA* genes. The *cfr* sequence of M05/0060 exhibited 100% identity with *cfr* of pSCFS1 (GenBank accession number NC_005076.1)(Kehrenberg *et al.*, 2004) and pSCFS6 (AM408573.1) (Kehrenberg *et al.*, 2007) and 99.9% identity with *cfr* of pSCFS3 (AM086211.1) (Kehrenberg and Schwarz, 2006) due to a single nucleotide difference at nucleotide position 761 of *cfr* with no resulting amino acid change. The *fexA* sequence of M05/0060 exhibited 100% identity with *fexA* of *Bacillus sp.* BS-01 (GU591496.1) and 99.9% identity with *fexA* of pSCFS3(AM086211.1), *Staphylococcus lentus* (AJ549214.1 and AJ715531.1), *Staphylococcus warneri* (AM408573.1) and *Staphylococcus simulans* (AM086400.1) due to two nucleotide differences at nucleotide positions 391 and 913, respectively, which resulted in two amino acid changes, from isoleucine to valine and valine to isoleucine, respectively.

6.3.1.3. Localisation of *cfr* and *fexA* in M05/0060 on the novel plasmid pSCFS7

The plasmid location of *cfr* and *fexA* in M05/0060 was confirmed by curing and conjugation experiments. Plasmid profiling revealed the presence of a ca. 45 kb plasmid which was absent in the cured derivative M05/0060-CI and present in the transconjugant XU21-T1 (Fig. 6.2). Comparative susceptibility testing revealed the presence of the *cfr*-associated resistance phenotype in XU21-T1, but its absence in the XU21 parental strain and the plasmid cured derivative M05/0060-C1 (Table 6.5). Moreover, microarray analysis confirmed that M05/0060-C1 and XU21-T1 were indistinguishable from M05/0060 and XU21, except for the absence and the presence, respectively, of *cfr* and *fexA*, (Table 6.2). Following the purification of plasmid DNA

Table 6.3 Comparative DNA microarray[a] hybridisation profiles of the *cfr*-positive M05/0060 *pvl*- MRSA isolate, derivatives and reference strains

Class	Genes	USA300-FPR3757[b]	USA300-TCH1516[b]	M05/0060[b]	M05/0060-CI[b]	XU21[b]	XU21-T1
Species markers	*katA, coa, nuc, spa*	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**
***agr* group**	*agr* group I	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**
SCC*mec*-associated markers	*mecA*	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	Δ mecR	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	mecR/I	NEG	NEG	NEG	NEG	NEG	NEG
	ccrA/B-1	NEG	NEG	NEG	NEG	NEG	NEG
	Q9XB68-dcs	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	ccrA/B-2	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	kdp-SCC locus	NEG	NEG	NEG	NEG	NEG	NEG
	ccrA/B-3	NEG	NEG	NEG	NEG	NEG	NEG
	ccrC, ccrA/B-4	NEG	NEG	NEG	NEG	NEG	NEG
Antimicrobial resistance genes	*blaZ/I/R*	NEG	**POS**	**POS**	**POS**	NEG	NEG
	erm(A), *erm*(B)	NEG	NEG	NEG	NEG	NEG	NEG
	erm(C)	**POS**	NEG	NEG	NEG	NEG	NEG
	lnu(A)	NEG	NEG	NEG	NEG	NEG	NEG
	msr(A), *mph*(C)	NEG	**POS**	**POS**	**POS**	NEG	NEG
	aacA-aphD	NEG	NEG	NEG	NEG	NEG	NEG
	aadD	NEG	NEG	NEG	NEG	NEG	NEG
	aphA3/sat	NEG	**POS**	**POS**	**POS**	NEG	NEG
	dfrS1	NEG	NEG	NEG	NEG	NEG	NEG
	far1	NEG	NEG	NEG	NEG	NEG	NEG
	mupA	**POS**	NEG	NEG	NEG	NEG	NEG
	tet(K)	**POS**	NEG	NEG	NEG	NEG	NEG
	tet(M)	NEG	NEG	NEG	NEG	NEG	NEG
	cat	NEG	NEG	NEG	NEG	NEG	NEG
	cfr	NEG	NEG	**POS**	NEG	NEG	**POS**
	fexA	NEG	NEG	**POS**	NEG	NEG	**POS**
Virulence-associated genes	*tst1*	NEG	NEG	NEG	NEG	NEG	NEG
	sea, seb, see, seh	NEG	NEG	NEG	NEG	NEG	NEG
	sec/l, sed/j/r	NEG	NEG	NEG	NEG	NEG	NEG
	seg/i/m/n/o/u	NEG	NEG	NEG	NEG	NEG	NEG
	sek/q	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	lukF/S-PV	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	lukF/S-hlg, hlgA	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**
	sak/chp/scn	**POS**	**POS**	**POS**	**POS**	NEG	NEG
	etA/B/C	NEG	NEG	NEG	NEG	NEG	NEG
	edinA/B/C	NEG	NEG	NEG	NEG	NEG	NEG
	arcA/B/C/D	**POS**	**POS**	**POS**	**POS**	NEG	NEG
Capsule type	capsule type 5	**POS**	**POS**	**POS**	**POS**	**POS**	**POS**
	capsule type 8	NEG	NEG	NEG	NEG	NEG	NEG

[a]The StaphyType Kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis of isolates, reference strains and derivatives.

[b] FPR3757 and TCH1516 are two USA300 reference strains for which full-genome sequences are available (accession numbers CP000255 and CP000730, respectively). M05/0060 is the *cfr*-positive ST8-MRSA-IVa/USA300 isolate and M05/0060-CI is the cured derivative. XU21 is the plasmid-free novobiocin-resistant *S. aureus* recipient strain (Udo *et al.*, 1998) and XU21-T1 is the transconjugant harbouring the *cfr* plasmid from M05/0060.

Abbreviations : POS, positive i.e. yielded a hybridisation signal corresponding to the gene indicated; NEG, negative i.e. failed to yield a hybridisation signal corresponding to the gene indicated.

Table 6.4 Antimicrobial resistance profiles of M05/0060 harbouring the *cfr*-encoding conjugative plasmid pSCFS7, its *cfr*-negative cured derivative M05/0060-C1 lacking pSCFS7, *S. aureus* recipient strain XU21 and its *cfr*-positive transconjugant derivative XU21-T1 harbouring pSCFS7

Isolate or derivative	*cfr* and *fexA* carriage	Antimicrobial agent[a] MICs (mg/mL)									
		TIA*	VIR M_1*	Q-D*	LZD*	CLI*	CHL*	FFC*	ERY	OXA	VAN
MRSA M05/0060	*cfr*, *fexA*	≥128	32	2	8	≥128	256	256	≥64	≥32	1
MRSA M05/0060-C1	—	1	1	0.5	1	0.25	8	4	≥64	≥32	1
S. aureus XU21	—	1	1	0.25	1	0.25	8	4	0.5	0.12	1
S. aureus XU21-T1	*cfr*, *fexA*	≥128	32	2	4	≥128	128	256	0.5	0.12	1

[a] Antimicrobial agents are abbreviated as follows: TIA (tiamulin), VIR M_1 (virginamycin M_1), Q-D (quinupristin/dalfopristin), LZD (linezolid), CLI (clindamycin), CHL (chloramphenicol), FFC (florfenicol), ERY (erythromycin), OXA (oxacillin), VAN (vancomycin). Resistance to antimicrobial agents marked with an astericks (*) are indicative of the $PhLOPS_A$ phenotype.

a.

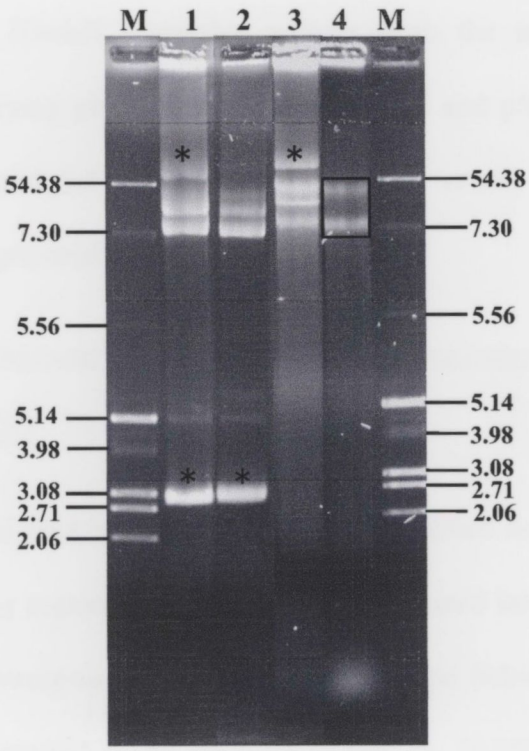

Fig. 6.2 Plasmid profile from *cfr*-parental strain M05/0060, cured derivative M05/0060-C1, and its transconjugant derivative M05/0060-XU21-T1, and the plasmid-free recipient *S. aureus* XU21. Panel (**a**) shows an agarose gel showing: *cfr*-parental strain M05/0060 (lane 1), cured derivative M05/0060-C1 (lane 2), its transconjugant derivative M05/0060-XU21-T1 (lane 3) and the plasmid-free recipient *S. aureus* XU21 (lane 4). Lanes: M, plasmid molecular size reference marker (pVA517A, DSMZ) with band sizes shown in kb. The presence of chromosomal DNA is indicated by a square box.

from the trasnsconjugant XU21-T1, this ca. 45 kb plasmid was found to differ in its *Blg*II, *Eco*RI, and *Hin*dIII restriction pattern from the previously described non-conjugative, *cfr*-carrying plasmids pSCFS1, pSCFS3 and pSCFS6 (Kehrenberg *et al.*, 2004; Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007) and was therefore designated as novel plasmid pSCSF7 (Fig. 6.3).

6.3.1.4 Molecular characterisation of the genetic environment of *cfr* and *fexA* on the novel plasmid pSCFS7

When *cfr* and *fexA* have previously been identified on the same plasmid, the *cfr* gene and its flanking regions were found to be integrated into the transposase genes of the *fexA*-encoding transposon Tn*558* (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007). Tn*558*-targeted PCRs were performed on Tn*558* and amplicons for *tnpA, tnpC* and *tnpB-fexA* obtained using M05/0060 and tranconjugant XU21-T1 template DNA corresponded in size with those obtained for a complete Tn*558*. However the *tnpB* amplicon obtained from both M05/0060 and XU21-T1 was approximately 2.5 kb larger than expected. PCR detection of *cfr* using this gel purified larger *tnpB* amplicon obtained from both M05/0060 and the transconjugant XU21-T1 as template and primers cfr-fw and cfr-rv (Kehrenberg and Schwarz, 2006) yielded the expected size amplicon for both strains, which suggested that a *cfr*-encoding DNA segment had been integrated into the *tnpB* reading frame in the novel pSCFS7. This *tnpB* amplicon from M05/0060 was cloned and sequenced to confirm this hypothesis of *cfr* gene integration. The entire *tnpB* amplicon of pSCFS7 comprised 4,043 bp and consisted of initial (positions 1-859) and terminal (positions 3826-4043) *tnpB* segments (Fig. 6.4). A largely truncated IS*21-558* element was detected (positions 846-1131) of which only the 3' terminus of the *istBS*, and the downstream region including the terminal inverted repeat, were present. The region downstream of this IS*21-558* relic (positions 1132-3254) including the entire

cfr gene (positions 1622-2671) exhibited 99.9% nucleotide sequence identity to the corresponding *cfr* sequences of pSCFS3 and pSCFS6 (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007). The adjacent 187 bp did not reveal significant homology to sequences deposited in the databases, but were followed by the 5' end of a reading frame (positions 3442-3825) whose 127 amino acid product revealed 78% identity and 90% similarity to the transposon Tn*552* DNA-invertase Bin3 from *Bacillus mycoides* Rock3-17 (Fig. 6.5). Analysis of the boundaries of this integrated DNA segment revealed two potential recombination sites, one of 14 bp between *tnpB* and *istBS* and another of 13 bp between *bin3* and *tnpB* (Fig. 6.5). These putative recombination sites are assumed to play a role in the integration of this 2,980 bp segment and the concomitant 433 bp deletion of *tnpB*.

6.3.2 Detection of mutations in the V domain of the 23S rRNA gene of the MSSA isolates exhibiting intermediate resistance to linezolid

Sequencing the domain V region of the five individual copies of the 23S rRNA gene of the MSSA isolates, M05/0209 and M05/0210, revealed a G2603T amino acid substitution (*S. aureus* N315 numbering) in the five copies of the rRNA gene (*rrn1-rrn5*) in both isolates. Sequencing of the five individual copies of the 23S rRNA gene in the *cfr* positive M05/0060 isolate revealed no amino acid substitutions.

As well as exhibiting intermediate resistance to linezolid, both isolates exhibited resistance to ampicillin and erythromycin and were indistinguishable by DNA microarray profiling. Both isolates were assigned to CC9 and harboured *blaZ*, *erm*(A) (encoding MLS resistance including erythromycin, lincomycin and clindamycin), *sdrM* (encoding resistance to norofloxacin and acriflavine), *fosB* (encoding fosfomycin

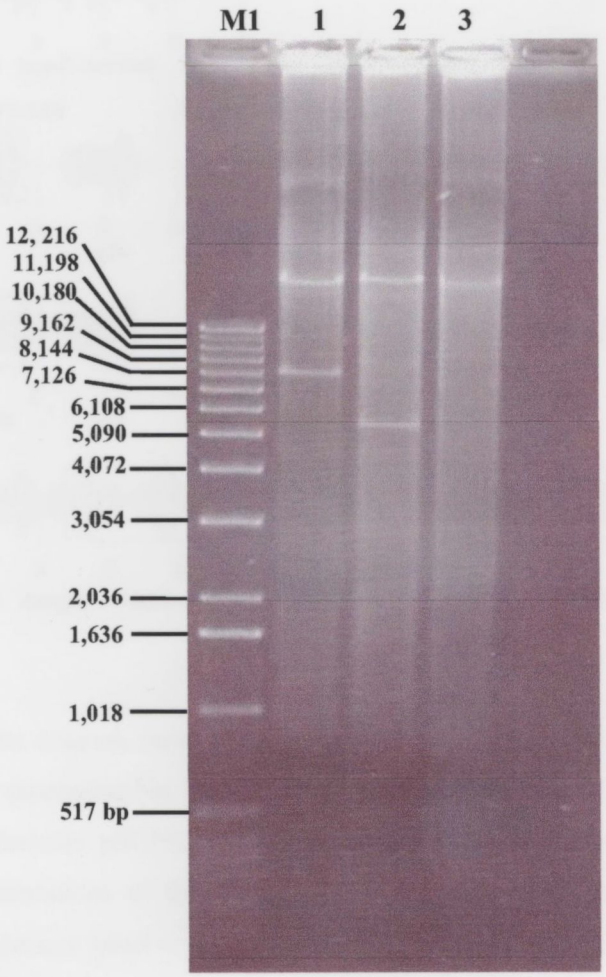

Fig. 6.3 Agarose gel showing DNA fragement patterns of purified plasmid DNA from the *cfr*-positive transconjugant derivative XU21-T1 following digestion with the restriction endonucleases *Blg*II, *Eco*RI, and *Hin*dIII (lane 1- 3). M7, 1 kb DNA ladder with bands sizes shown in kb. The *cfr*-containing plasmid harboured by the transconjugant derivative XU21-T1 was estimated to be ca. 45 kb in size based on the sum of the fragments obtained.

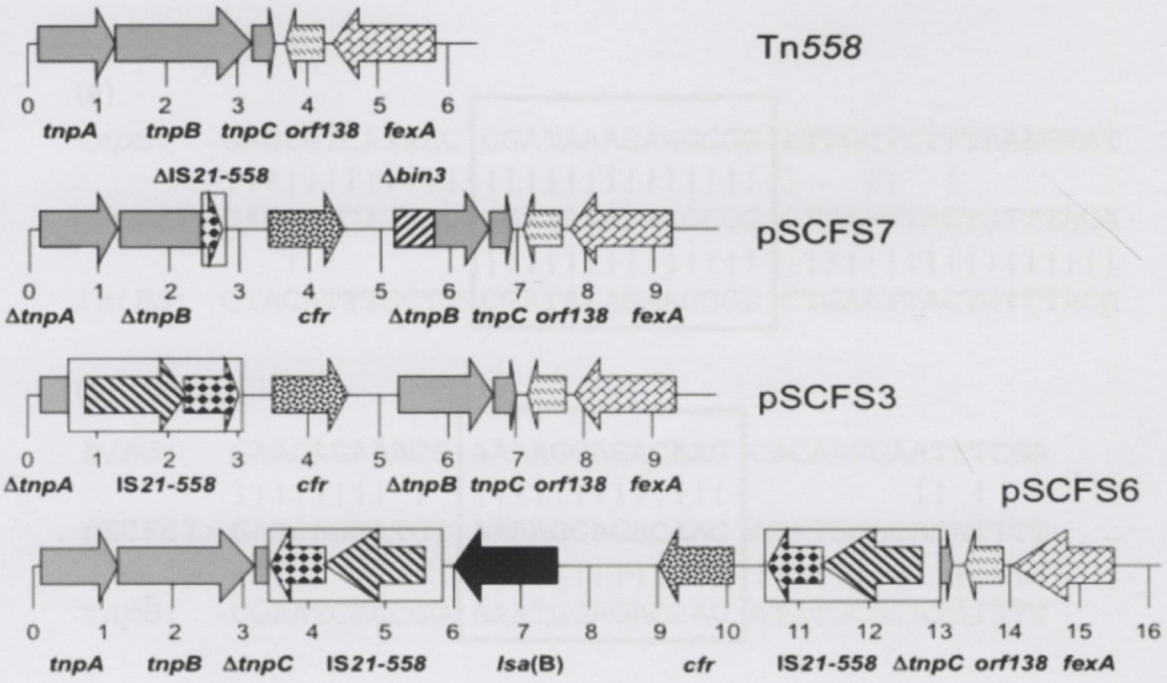

Fig. 6.4 Schematic diagram showing the organisation of the novel variant of transposon Tn*558* of plasmid pSCFS7 (accession No. FR675942), relative to Tn*558* (AJ715531) and the Tn*558* variants harboured by plasmids pSCFS3 (AM086211) and pSCFS6 (accession No. AM408573). The positions and orientations of the genes coding for transposition functions (*tnpA*, *tnpB*, *tnpC*), antimicrobial resistance [*fexA* – resistance to florfenicol and chloramphenicol; *cfr* – resistance to phenicols, lincosamides, oxazolidinones, pleuromutilines, and streptogramin A antibiotics; *lsa*(B) – reduced susceptibility to lincosamides], or unknown functions [*orf138*] are indicated by arrows. The delta symbol (Δ) indicates a truncated gene. A distance scale in kb is shown below each map.

Fig. 6.5 Potential sites used for the integration of the 2,966 *cfr*-containing segment into the *tnpB* reading frame thereby causing a 433-bp deletion of *tnpB*. (**a**) Recombination site between the 5' end of *tnpB* and IS*21-558*. Displayed are the *tnpB* sequence at positions 3369-3411 of plasmid pSCFS6 (accession No. AM408573), the *istBS* sequence at positions 4724-4682 of plasmid pSCFS6 and the sequence of pSCFS7 at positions 834-878. (**b**) Recombination site between the *bin3* and the 3' end of *tnpB*. Displayed are the *bin3* sequence of *B. mycoides* at positions 4550-4512 (ACMW01000216), the *tnpB* sequence at positions 3726-3764 of plasmid pSCFS6 and the sequence of pSCFS7 at positions 3811-3853. Nucleotides identical to the sequence of pSCFS7 are indicated by vertical bars. The recombination sites, where crossover is believed to have occurred, are boxed.

resistance), *egc* (*seg, sei, sem, sen, seo and seu*) and IEC type B (*sak*, *chp* and *scn*) (Table 6.3).

6.4 Discussion

6.4.1 A novel *cfr* and *fexA* encoding plasmid pSCFS7 in a *pvl*-positive ST8-MRSA-IVa (USA300) isolate

This is the first report of a human infection with the major pandemic MRSA clone ST8-MRSA-IVa/USA300 harbouring the multidrug-resistance gene *cfr*. Isolates of this *pvl*-positive MRSA clone are commonly associated with SSTIs but also with life-threatening necrotising pneumonia. Therapy options for the latter condition are very limited since glycopeptide antibiotics tend to have a poor penetration into lung tissue and reduced susceptibility to vancomycin has been observed among MRSA isolates including USA300 (Graber *et al.*, 2007; Hageman *et al.*, 2008). Daptomycin cannot be used in cases of pneumonia as it reacts with lung surfactant (Baltz, 2009). While rifampicin, levofloxacin and fosfomycin are viable options, resistance to these antimicrobial agents is common and can emerge during therapy. The synergistic effect of sulfonamide and trimethoprim in co-trimoxazol is in dispute in *in-vivo* conditions, which limits its use in life-threatening situations. Ceftobiprole is not yet available in most countries, and for tigecycline, clinical experience is still lacking. Thus, treatment of necrotising pneumonia and of other serious infections caused by PVL-positive MRSA primarily relies on oxazolidinones, such as linezolid.

The *cfr* gene identified in the ST8-MRSA-IVa isolate M05/0060 in the present study was located, along with *fexA*, on a novel 45 kb conjugative plasmid designated pSCFS7. This plasmid (pSCFS7) is most similar to a number of previously described plasmids including pSCFS3 (35.7 kb) and pSCFS6 (43 kb), which encode both *cfr* and *fexA* in staphylococci, and a recently described 40 kb plasmid, pSS-01, encoding *cfr*, *fexA* and *aacA-aphD* (Table 6.1). All of these plasmids have only been detected in

staphylococcal isolates from animals (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007; Kehrenberg *et al.*, 2009; Wang *et al.*, 2012e), however in both pSCFS3, pSCFS6 *cfr* is integrated into the *fexA*-encoding transposon Tn*558,* whereas *cfr* is located upstream of the Tn*558* transposon inpSS-01 (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007; Wang *et al.*, 2012e). In pSCFS3 and pSCFS6 the Tn*558* elements are disrupted and in part truncated by the insertion of DNA segments of 4,674 bp and 9,594 bp, respectively, which comprised the *cfr* gene along with one or two copies of the insertion sequence IS*21-558* and, in the case of pSCFS6, the clindamycin resistance gene *lsa(B)* (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007). In contrast, in the novel plasmid pSCFS7 described in the present study, the *tnpB* gene of Tn*558* was disrupted and in part deleted by the insertion via recombination of a DNA segment of 2,980 bp encoding *cfr*, a truncated IS*21-558* (upstream of *cfr*) and *Δbin3* (downstream of *cfr*) (Fig. 6.4). The *bin3* gene encodes a DNA invertase and has previously only been reported in the transposon Tn*552* from *B. mycoides*. These observations, coupled with the report of *cfr* in several different bacterial species and genera (Dai *et al.*, 2010a; Zhang *et al.*, 2011; Diaz *et al.*, 2012b; Liu *et al.*, 2012b; Liu *et al.*, 2012c; Wang *et al.*, 2012b; Wang *et al.*, 2012c; Wang *et al.*, 2012d), point towards the mobility of *cfr*, its ability to spread to different bacterial genera and the role that recombination processes play in the stable fixation of *cfr* in new vector plasmids.

The *cfr*-positive ST8-MRSA-IVa isolate M05/0060 harbouring *cfr* on the novel plasmid pSCFS7 differs significantly from at least three of the four previously reported cases of *cfr*-positive *S. aureus* isolates recovered from humans and to those from animals. One report of a *cfr*-positive MRSA isolate from Columbia reported that *cfr* was chromosomally located on 15.5 kb sequence inserted within the 23S rRNA allele 4, however, no details of the sequence type (MLST/SCC*mec*) of the isolate were provided

(Toh *et al.*, 2007; Locke *et al.*, 2012). Another study reported a *cfr*-positive MSSA isolate from the USA in which the *cfr* gene and *erm*(A) were located on a 55-kb plasmid, p004-737X (Mendes *et al.*, 2008). A ca. 50 kb conjugative *cfr* plasmid (pERGB) was recently reported from Spain from two ST125-MRSA-IVc isolates from two different patients with chronic obstructive pulmonary disease (COPD) admitted in the same unit of the main tertiary hospital in Majorica, Spain. Plasmid pERGB also carried genes encoding resistance to tobramycin (*ant*(4')-*Ia*), tetracycline *tet*(L) and trimethoprim (*dfrK*) (Gopegui *et al.*, 2012). In a separate report from Spain, the *cfr*-positive MRSA isolates reported from an outbreak in a Spanish hospital were not investigated for the location of *cfr* and while these isolates appeared to be closely related by PFGE, no details of MLST or SCC*mec* types were provided so comparison to international MRSA strains is not possible (Morales *et al.*, 2010).

Reports of *cfr* among *S. aureus* isolates from animals have all been from pigs, including isolates belonging to ST9-MSSA and ST398-MRSA-V and all these isolates harboured *cfr* on plasmid pSCFS3 (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2009). While *cfr* is still rare among *S. aureus* isolates, it appears to be more abundant among CoNS and it is evident that CoNS from both humans and animals may act a reservoir for *cfr* and other resistance genes in *S. aureus*. The *cfr* gene was first reported in 2000 from a bovine *S. sciuri* isolate and has since been identified among additional *S. sciuri* isolates as well as in isolates of *S. warneri*, *S. simulans, Staphylococcus hyicus, S. lentus, S. saprophyticus* and *Staphylococcus cohnii* all from animal sources (Schwarz *et al.*, 2000; Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007; Wang *et al.*, 2012e). These CoNS from animals harboured *cfr* on a variety of plasmids and often carried additional resistance determinants (Table 6.1) (Kehrenberg *et al.*, 2004; Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007; Wang *et al.*, 2012e). In addition, there have

been reports of CoNS from humans harbouring *cfr*, including two *S. epidermidis* isolates from Mexico, in which the location of *cfr* was not determined (Mendes *et al.*, 2010a) and the USA., in which *cfr* was localised on a 175 kb plasmid (Mendes *et al.*, 2008). The recent identification of *cfr* in many different staphylococcal species and in other bacterial genera, often located on novel plasmids frequently carrying multiple resistance determinants, in animals and humans is worrying and highlights the ability of these plasmids to spread and to compromise our ability to treat infections (Dai *et al.*, 2010b; Zhang *et al.*, 2011; Wang *et al.*, 2012b; Wang *et al.*, 2012c; Wang *et al.*, 2012d).

While there have been no reports of *cfr*-positive staphylococci from Ireland prior to the present study, linezolid resistance has been reported among sixteen clinical *S. epidermidis* isolates due to the presence of a mutation in the 23S rRNA gene (Kelly *et al.*, 2008). Interestingly, during the present study, problems were encountered interpreting linezolid MICs using the VITEK-2 software which may hamper the detection of linezolid resistance isolates. The VITEK-2 software reported a linezolid MIC of 8 mg/mL for the *cfr*-positive MRSA isolate M05/0060, which is above the resistance breakpoint for linezolid (Chapter 3, Table 3.1). However, the VITEK-2 software recommended that the linezolid susceptibilities should be manually changed to 4 mg/L and that the isolate should be reported as susceptible to linezolid.

In the present study, the localisation of *cfr* to a novel conjugative plasmid and the identification of a potential novel integration site within Tn*558* indicate that *cfr* has the ability to spread to other MRSA strains. Our finding of *cfr* among ST8-MRSA-IVa/USA300 would almost certainly not have occurred if we had not used the DNA microarray to screen for the presence of an extensive range of virulence and antimicrobial resistance genes among large numbers of MRSA isolates. Our findings highlight the effectiveness of such high throughput systems for ongoing surveillance of

MRSA. Recently, another MRSA isolate, representative of a different pandemic MRSA strain, ST22-MRSA-IV, was recently identified in this laboratory as carrying *cfr* and *fexA*. Further work is underway to determine the location (i.e. plasmid or chromosomal) and the genetic environment of *cfr* in this isolate. Since ST8-MRSA-IVa/USA300 and ST22-MRSA-IV are both successful clones capable of pandemic spread, the emergence of a *cfr*-positive variant of these strains is cause for significant concern and warrants close surveillance.

6.4.2 Identification and characterisation of 23S rRNA mutations in two CC9-MSSA isolates

The present study also reports for the first time intermediate resistance to linezolid among *S. aureus* isolates in Ireland and identified mutations in 23S rRNA alleles as the likely mechanism of resistance. Linezolid resistance was first reported in *S. aureus* in 2001 in the USA just one year after the introduction of linezolid into clinical practice. Point mutations in 23S rRNA alleles constitute the most common underlying mechanism of linezolid resistance with subsequent amino acid substitutions in the 23S protein. Various amino acid substitutions in the 23S rRNA alleles have been reported worldwide in both LRSA and LRCoNS the most common being G2576T (Jones *et al.*, 2009; Katopodis *et al.*, 2010; Farrell *et al.*, 2011b; Benefield *et al.*, 2012; Gupta *et al.*, 2012). In the current study the less common G2603T 23S rRNA allele amino acid substitution was identified in the two CC9-MSSA isolates from a single patient. To date this mutation has been reported as a cause of linezolid resistance in clinical isolates of CoNS (Sorlozano *et al.*, 2010; Seral *et al.*, 2011; Lozano *et al.*, 2012a) with only a single report in *S. aureus* in isolates from cystic fibrosis patients in Cleveland, Ohio (Endimiani *et al.*, 2011). Mutations associated with linezolid resistance appear to be spontaneous and are not clonally related. For example, the linezolid

resistance isolates in the present study and in the Endimiani *et al.* (2011) study belonged to distinct lineages, CC9 and CC5, respectively, but harboured the same amino acid substitution. In addition this same amino acid substitution has been identified previously among *S. epidermidis*, *S. haemolyticus* and *S. hominis* (Sorlozano *et al.*, 2010; Seral *et al.*, 2011; Lozano *et al.*, 2012a).

It is also interesting to note that in the present study the G2603T amino acid substitution was in five of the six copies of the 23S rRNA gene while in the Endimiani *et al.* study the amino acid substitutions occurred in one to four copies. This suggests that the emergence of intermediate linezolid resistance in the CC9-MSSA isolates in the present study may be due to prolonged exposure to the antimicrobial agent.

Currently, the frequency of linezolid resistance worldwide appears to be low, less than 0.1% among *S. aureus* and less than 2% among CoNS (Jones *et al.*, 2009; Flamm *et al.*, 2012). In Ireland, 6,573 *S. aureus* isolates (6,048 MRSA and 525 MSSA) have been investigated for resistance to linezolid by the NMRSARL since 2002 and only 0.06% (4/6,573) have been found to exhibit resistance or intermediate resistance (Grainne Brennan NMRSARL, personal communication). This indicates that linezolid resistance among *S. aureus* is not a common problem in Ireland but the use of this antimicrobial agent should be restricted to avoid the widespread emergence of resistance.

Prior to the present study, linezolid resistance had only been reported among *S. epidermidis* isolates from Ireland and the G2576T amino acid substitution was detected in all 16 isolates tested from 16 patients in an intensive therapy unit (Kelly *et al.*, 2008). Interestingly, following restrictions on the use of linezolid, the disappearance of the linezolid resistant *S. epidermidis* strain was noted by Kelly *et al.* (2008) who concluded

that the spread of this single *S. epidermidis* strain over a 6 month period was caused by the increase in the linezolid usage in the institution (Kelly *et al.*, 2008). However the *S. epidermidis* isolates were not investigated for the presence of *cfr* (Kelly *et al.*, 2008). The co-occurrence of mutations with the presence of the *cfr* gene have been reported previously in a linezolid resistant *S. aureus* isolate from a patient in a Spanish hospital (Locke *et al.*, 2010). It is therefore essential that all studies investigating the mechanism of linezolid resistance investigate isolates not just for the presence of mutations but also for *cfr*.

Linezolid is often the drug of last resort to treat complicated MRSA infections. It has also been recommended for the treatment of pulmonary tuberculosis. Resistance to this relatively novel antimicrobial agent has been detected not just among staphylococci, including MRSA, but also among *Mycobacterium tuberculosis* isolates (Huang *et al.*, 2008; Beckert *et al.*, 2012), *Enterococci*, *Bacillus* and *Steptococcus spp* (Feng *et al.*, 2009; Afsar *et al.*, 2012; Cai *et al.*, 2012). The use of this antimicrobial agent requires close monitoring and its overuse and/or misuse will select for resistant strains.

Chapter 7

General Discussion

7.1 General Discussion

Methicillin resistant *S. aureus* is one of the leading causes of hospital- and community-associated infections in humans and of infections among animals worldwide. The success of MRSA as a pathogen is due to its ability to express a diverse range of virulence factors and antimicrobial resistance determinants. In order to prevent and control the spread of MRSA and to gain insights into its origins and evolutionary history it is vital that informative, rapid, high-throughput and reproducible genotyping methods are used for screening MRSA isolates from individuals, institutions and wider geographic areas for epidemiological purposes, but also to provide a better understanding of the virulence and antimicrobial resistance potential of isolates and populations.

7.1.1. Advances in genotyping of MRSA

PFGE and *spa* typing are commonly used for routine typing of MRSA (MacKenzie *et al.*, 2002). However, PFGE is time consuming, subjective and laborious and both of these methods only allow very limited differentiation of high-clonal MRSA strains such as the ST22-MRSA-IV clone currently endemic in Irish hospitals (Shore *et al.*, 2010). MLST and SCC*mec* typing are often used for long-term studies but can be very expensive and time consuming as MLST requires sequencing of seven genes and SCC*mec* typing commonly requires the use of multiple multiplex PCRs (Enright *et al.*, 2002; Robinson and Enright, 2004b; Kondo *et al.*, 2007). In addition, none of these methods provide any information on the virulence or antimicrobial resistance potential of an isolate.

Recently, there have been major advances in whole-genome sequencing technologies that have led to the development of more comprehensive typing methods that allow unprecedented characterisation of the core and variable genome of MRSA

isolates. While whole-genome sequencing as a typing tool is still very much restricted to specialised research laboratories it has permitted the development of DNA microarray profiling platforms that allow rapid genotyping of isolates together with the detection of clinically important virulence and resistance genes (Monecke *et al.*, 2008a; Harris *et al.*, 2010).

7.1.1.1 DNA microarray profiling assigns the majority of MRSA isolates to the correct ST and SCC*mec* type and provides enhanced discrimination highly-clonal ST22-MRSA-IVwhen combined with *spa* typing

The results of the present study demonstrate that the StaphyType DNA microarray platform allows high-throughput and accurate assignment of MRSA isolates to CCs/STs and SCC*mec* types and, in the majority of cases, can accurately predict the resistance phenotype of an isolate (Chapter 3, section 3.3.1.2; Table 3.6 and Table 3.7). This technology has significant potential to replace conventional MLST and SCC*mec* typing methods and traditional antimicrobial susceptibility testing. However, some sporadically-occurring STs, some SCC*mec* subtypes, more recently identified SCC*mec* elements and *mec* variants and some antimicrobial resistance determinants are not detected using the DNA microarray. These current limitations will be soon be eliminated following the recent development of an upgraded version of the StaphyType DNA microarray that incorporates detection of many of these determinants including *mecC*, SCC*mec* IX, X and XI and many additional resistance genes. The updated array platform will soon be commercially available (Stefan Monecke, Alere Technologies, personal communication).

The results of the present study also showed that the combination of *spa* typing and DNA microarray profiling (based on the presence or absence of both antimicrobial

and virulence genes) enhances discrimination of highly clonal ST22-MRSA-IV permitting tracking of isolates (Chapter 4, section 4.3.1.2; Table 4.8). These two typing methods could be routinely use in both research and clinical laboratories, however the DNA microarray technology is still relatively expensive and the determination of *spa* types for large collections of MRSA isolates is also relatively expensive and laborious. However, *spa* typing is amenable to automation, which may render it more cost effective for clinical laboratories in the future.

7.1.1.2 Whole-genome sequencing

In addition to facilitating the development of microarray platforms, advances in whole-genome sequencing technology have resulted in its application for typing of MRSA (Harris *et al.*, 2012). The first generation sequencing platforms were slow and expensive, often taking years to sequence the genome of single bacterium at a cost of millions of Euro. In 2004, several second- or next-generation high-throughput sequencing platforms became available including (a) the Illumina Platform (Illumina, San Diego, CA, USA), (b) the 454 genome sequencer (Roche, Applied Science, Rotkreuz, Switerland), and (c) the Sequencing by Oligo Ligation and Detection (SOLiD) platform (Life Technologies, Applied Biosystems, Carlsbad, CA, USA). Very recently, third generation bench-top sequencing platforms have been introduced, namely the MiSeq (lllumina) and Ion Torrent platforms (Life Technologies) and a new GridION platform (Oxford Nanopore, Oxford, UK) is due to be released soon (Branton *et al.*, 2008; Price *et al.*, 2013). These bench-top sequencers are becoming more affordable, accessible and allow for a rapid turn-around of isolate analysis (Eyre *et al.*, 2012; Köser *et al.*, 2012b). Recently, this technology has been shown to have huge potential for typing of MRSA. It has been shown to provide data on MRSA lineage diversity over time, in various continents and within healthcare settings (Harris *et al.*, 2010; Harris *et*

al., 2012). Two recent studies have revealed the very significant potential for this technology for routine typing of MRSA in an outbreak situation to track MRSA transmission within two neonatal units (Harris *et al.*, 2012; Köser *et al.*, 2012b). While whole-genome sequencing does appear to be the ultimate typing tool, currently there are limitations that restrict its widespread use for routine typing including the fact that it is costly, time-consuming and data analysis requires extensive bioinformatics expertise and access to sophisticated sequence analysis software. For whole-genome sequencing to become a routine typing method, it will have to overcome these issues which will involve the development of fully-automated equipment and analysis systems. This is likely to take at least another decade.

7.1.2 Antimicrobial resistance among MRSA in Ireland

The increase in the rate of fusidic acid and high-level mupirocin resistance among Irish MRSA isolates from BSIs since 1999 and the identification of an isolate in the present study harbouring the *cfr* gene encoding resistance to multiple antimicrobial agents, including linezolid, are worrying developments. Mupirocin (commercial name Bactroban) is widely used for nasal decolonisation of MRSA. Fusidic acid (commercial name Fucidin) is mostly used as a topical agent for impetigo and infected eczema but can also be used orally or parenterally as an additional agent to treat bone infections and endocarditis (Bertrand *et al.*, 2010; Wang *et al.*, 2012a). Linezolid is often the drug of last resort to treat serious MRSA infections such as necrotising pneumonia and other serious infections caused by *pvl*-positive MRSA (Meka and Gold, 2004). Resistance of MRSA isolates to these antimicrobial agents threatens our ability to successfully prevent and treat MRSA infections. It is therefore vital that we have an understanding of the mechanisms and evolution of resistance to these antimicrobial agents in MRSA.

7.1.2.1 Extensive diversity in the genotypes and *ileS2*-encoding plasmids of Hi-MupR MRSA in Ireland

The present study revealed that the Hi-MupR gene *ileS2* is located on a diverse range of large conjugative and sometimes multiresistance plasmids in MRSA isolates in Ireland belonging to several distinct lineages (Chapter 5, section 5.3.4; Table 5.6 and Table 5.7). This study highlighted the potential for these *ileS2*-plasmids to spread via conjugation and for the presence of multiple resistance determinants, including genes encoding aminoglycoside and lincomycin resistance. The location of these additional antimicrobial resistance genes on these plasmids or on smaller mobilisable plasmids, can allow selection for the spread of multiple resistance determinants in the presence of just one of these antimicrobial agents. Different Hi-MupR plasmids were identified among isolates of the same clone, particularly among the currently predominant clone ST22-MRSA-IV (Chapter 5, section 5.3.4; Table 5.6 and Table 5.7). Whether these represent different plasmids that have been acquired by isolates of the same clone or whether evolution of Hi-MupR plasmids within these MRSA strains has occurred remains to be seen. For the future comparison of large multiresistance plasmids, whole plasmid sequencing would be the ultimate method of investigating their evolution. However currently, whole plasmid sequencing is only carried on *S. aureus* plasmids following cloning of overlapping restriction fragments in *E. coli* (Morton *et al.*, 1995). Purifying large plasmids from *S. aureus* suitable for DNA sequencing is technically demanding as the plasmid preparations are frequently subject to contamination with chromosomal DNA. To date, only commercial Hi-Speed maxi-plasmid purification kit (Qiagen, California, USA) is available for the extraction and purification of large *S. aureus* plasmids (Pérez-Roth *et al.*, 2010). However, many commercially available plasmid purification kits provide insufficient plasmid yields and purity for DNA

sequencing. The use of caesium chloride-ethidium bromide buoyant density centrifugation to purify large *S. aureus* plasmids from bulk cultures may very well provide a solution to these technical problems (Coleman *et al.*, 1985). However, this approach requires the use of expensive ultracentrifuge equipment, which may be available in research laboratories but unlikely in routine clinical laboratories.

7.1.2.2 Mutations in *fusA* is the predominant mechanism of fusidic acid resistance identified among Irish ST22-MRSA-IV isolates

While the fusidic acid resistant MRSA investigated in the present study (1971-2010) belonged to diverse genotypes, the most common clone was ST22-MRSA-IV. Isolates of this clone account for approximately 80% of MRSA recovered from BSIs in Ireland each year. The prevalence rate of fusidic acid resistance among MRSA isolates recovered from BSIs in Ireland has increased three-fold to 34% in 2011 (NMRSARL annual report 2011). Various amino changes in the *fusA* gene were the most common mechanism of fusidic acid resistance identified (O'Neill *et al.*, 2007). These findings are supported by the results of the present study which indicate that within ST22-MRSA-IV, it is predominantly random mutations and subsequent selective pressure that is responsible for the increasing prevalence of fusidic acid resistance among MRSA from BSIs rather than the acquisition of MGEs encoding fusidic acid resistance (Chapter 3, section 3.1.2; Table 3.10). However, a small percentage (10%) of fusidic acid-resistant MRSA isolates investigated in the present study harboured *fusB* or *fusC* which have been shown previously to be located on plasmids and SCC/SCC*mec* elements, respectively (Chapter 3, section 3.1.2; Table 3.1) (O'Brien *et al.*, 2002; Holden *et al.*, 2004; Ender *et al.*, 2007; Kinnevey *et al.*, 2013). Interestingly, very recent studies from this laboratory have identified an increasing prevalence of *fusC* among non-BSI MRSA in Ireland (Grainne Brennan, NMRSARL, personal communication). Therefore, a

detailed and more extensive molecular characterisation of the resistance determinants encoding fusidic acid resistance in MRSA from both BSIs and non-BSIs and of the MGEs carrying these determinants is warranted. The emergence of widespread fusidic acid resistance among MRSA in Ireland will reduce the effectiveness of fusidic acid therapy for SSTIs, which in turn could result in an increased prevalence of more severe SSTI conditions among adults and children both in the community and hospitals. Surveillance involving longitudinal studies and the close monitoring of the molecular mechanisms for fusidic acid resistance is essential to combat such a detrimental development.

7.1.2.3 Identification of a novel conjugative plasmid carrying *cfr* in *pvl*-positive USA300 MRSA

In the present study the finding of the staphylococcal *cfr* gene encoded on a conjugative plasmid in a community acquired *pvl*-positive ST8-MRSA-IVa/USA300 isolate is of great concern (Chapter 6, section 6.3.1; Fig. 6.4). Firstly, this virulent CA-MRSA strain can cause serious necrotising pneumonia and linezolid resistance to which is mediated by *cfr*, is often the drug of last resort to treat such serious MRSA infections (Bozdogan and Appelbaum, 2004). Secondly, *cfr* also encodes resistance to several other classes of antimicrobial agents used in veterinary and human medicine (Kehrenberg *et al.*, 2005). The emergence of this resistance gene, which previously was predominantly associated with animal staphylococci, in a pandemic MRSA clone, is worrying as the findings of this study highlight the potential not just for clonal spread of *cfr* but also for its conjugative spread between different MRSA clones. In this regard, it is important to note that very recently, studies from this laboratory have identified *cfr* in an isolate of the current endemic clone ST22-MRSA-IV. Preliminary analysis of this isolate has indicated that *cfr* is located on a large conjugative plasmid distinct from the

plasmid harboured by the ST8-MRSA-IVa isolate. Work on this *cfr*-encoding ST22-MRSA-IV plasmid is on-going in this laboratory. The source of these *cfr*-encoding plasmids in MRSA is unknown but due to the numbers of reports of *cfr* among animal staphylococci, in particular among CoNS, they may be a reservoir for these and other resistance determinants in *S. aureus* (Kehrenberg and Schwarz, 2006; Kehrenberg *et al.*, 2007; Mendes *et al.*, 2008; Kehrenberg *et al.*, 2009; Wang *et al.*, 2012e). Other bacterial genera may also be a potential source of these staphylococcal resistance genes as *cfr* has recently been identified in many different bacterial genera in animals indicating *Bacillus spp*, *Bacillus sphaericus*, *E. faecalis*, *Enterococcus thailandicus*, *J. pinnipedialis*, *P. vulgaris*, *M. caseolyticus* and *E. coli*. (Dai *et al.*, 2010b; Wang *et al.*, 2011; Zhang *et al.*, 2011; Diaz *et al.*, 2012b; Liu *et al.*, 2012b; Liu *et al.*, 2012c; Wang *et al.*, 2012b; Wang *et al.*, 2012c; Wang *et al.*, 2012d).

7.1.2.4 CoNS as a reservior for antimicrobial resistance genes in *S. aureus*

For effective infection prevention and control, in-depth epidemiological studies on the spread of MGEs encoding emerging antimicrobial resistance genes are essential, as are studies on the sources (e.g. human, animal, or environment) and bacterial species in which the antimicrobial resistance genes emerged. Staphylococci and CoNS are natural commensals on the skin and mucous membranes of both human and animals. Due to the higher prevalence of many resistance genes among CoNS, several studies have highlighted that CoNS are a potential reservoir for resistance genes in *S. aureus*. However, apart from *mecA*, direct evidence on the possible mechanisms of gene transfer between CoNS and *S. aureus* is lacking and detailed comparative genetic analysis of resistance determinants and their associated MGEs in *S. aureus* and CoNS would provide valuable insights into the role of CoNS in the emergence of antimicrobial resistance in MRSA. Also, an investigation of specific CoNS species would further

enhance our understanding of the role that the different CoNS play in this phenomenon, particularly CoNS from farmed animals. Preliminary investigations from this laboratory have identified the presence of *ileS2* and *fusC* among CoNS in Ireland but detailed analysis of these organisms and their MGEs is pending.

While resistance to antimicrobial agents in bacteria is a natural occurrence the widespread overuse and misuse of these agents and certain disinfectants such as triclosan selects for, and enhances the spread of resistant strains. A key factor in preventing the widespread emergence of antimicrobial resistance is the prudent use of antimicrobial agents both in human and veterinary medicine and the use of effective infection prevention and control measures within healthcare settings and animal husbandry. However, the on-going development of novel antimicrobial agents is essential and should be a priority for pharmaceutical companies and healthcare professionals, although commercial factors and the significant financial investment required are important limiting factors.

7.1.2.5 Novel antimicrobial agents and vaccines for treating MRSA infections

Since the 1980's very few antimicrobial agents have been successfully developed and in the last decade, just two, daptomycin and tigecycline, have been approved for the treatment of MRSA infections. Daptomycin is produced by *Streptomyces roseosporus* and is a cyclic lipopetide antimicrobial agent first approved by the FDA in 2003 for the treatment of MRSA bacteraemia and SSTIs. Just two years later in 2005, the first two cases of daptomycin-resistant MRSA were reported (Mangili *et al.*, 2005; Steenbergen *et al.*, 2005). Recently, daptomycin resistance has also been reported among hVISA and VISA strains (Kelley *et al.*, 2011; van Hal *et al.*, 2011). The combination of daptomycin with other antimicrobial agents such as oxacillin,

rifampicin, gentamicin, and ampicillin are currently being investigated to increase efficacy and delay the development of daptomycin resistance after prolonged therapy (Credito *et al.*, 2007; Entenza *et al.*, 2010; Berti *et al.*, 2012). Daptomycin resistance has occurred due to point mutations in the gene encoding the chromosomally-located *S. aureus* membrane protein MprF (Slavetinsky *et al.*, 2012). Recently two additional genetic mechanisms of daptomycin resistance due to mutations in the phospholipid biosynthesis genes, *cls2* and *pgsA*, in *S. aureus* have also been described (Peleg *et al.*, 2012). Tigecycline is a glycylcycline antibiotic and is structurally related to tetracycline and monocyline and which prevents the incorporation of amino acids into growing peptide chains within the 30S ribosomal subunit (Tong *et al.*, 2012). Tigecycline has a broad spectrum activity against Gram-positive and Gram-negative bacteria (Chen *et al.*, 2012; Tong *et al.*, 2012). This antimicrobial agent was approved for use by the FDA in 2005 to treat complicated intra-abdominal infections, SSTIs and community-acquired respiratory infections (Cai *et al.*, 2011). To date, no tigecycline-resistant MRSA isolates have been reported (Chen *et al.*, 2012). Additional novel antimicrobial agents that are under development for treating MRSA include lipoglycopeptides such as telavancin, dalbavancin and ortavancin, which are in the late phase of clinical trials (Tong *et al.*, 2012), and new broad-spectrum antibiotics such as cephaolosporin which has yet not received FDA approval (Bazan *et al.*, 2011). Ceftobiprole and ceftaroline are new beta-lactam (β-lactam) antibiotics which have been recently approved by the FDA for the treatment of SSTIs and community-acquired pneumonia (CAP) caused by MRSA (Bazan *et al.*, 2011; Lim *et al.*, 2011). These new anti-staphylococcal antibiotics are the newest antimicrobial agents for the future treatment for *S. aureus* infections.

Over the past 10 years different staphylococcal vaccines have been developed as an alternative to antimicrobial therapy. However, to date, the results from clinical trials

have been disappointing. StaphVAX ® is a polysaccharide conjugate vaccine which has shown no efficacy in phase III in haemodialysis patients (Broughan *et al.*, 2011; Tong *et al.*, 2012). Merek's V710 ® vaccine contains the MSCRAMMs protein IsdB (Iron surface determinant B). On phase II/III Merek's V710 ® failed to eradicate an *S. aureus* infection after cardiothoracic surgery and trails have been terminated over insignificant clinical benefit and safety concerns (Harro *et al.*, 2010; Tong *et al.*, 2012). Other vaccines such as Altastaph® (polyclonal immune globulin), Tefibazumab® (monoclonal antibodies to ClfA), Pagibaximab ® (chimeric monoclonal antibody lipoteichoic acid) and Aurograb ® (antibiotics to the *S. aureus* ATP-binding transporters) are in clinical phase II trials (Tong *et al.*, 2012). A successful vaccine against *S. aureus* has yet to be developed. A range of vaccines being investigated in clinical phase II trials show promise but difficulties remain concerning the best combination of antigens to use and which patient groups to target for clinical trials.

7.2 Concluding Remarks

MRSA has evolved and adapted to become a well-established pandemic pathogen in hospitals, healthcare facilities, in the community and among animals. The emergence of further multi-resistant strains and strains with enhanced virulence will cause clinical and socio-economic difficulties in the treatment of human and animal infections. Recent advances in whole-genome sequencing and DNA microarray technology will permit the tracking of established and novel MRSA clones in real-time. The application of DNA microarray profiling is an effective platform for the high-throughput typing of a large collection of isolates using in-depth analysis. This technology will eventually ultimately replace current typing methods e.g. PCR-based methods. It has permitting a reasonable discrimination of the highly ST22-MRSA-IV clone as part of this present study. Future improvements and further automation very

likely will facilitate the routine application of DNA microarray technology in clinical laboratories, where it can be used to generate comprehensive data in real-time that can be used to minimise spread of MRSA and inform patient treatment. The present study highlights some of the mechanisms involved in the emergence of antimicrobial resistance among MRSA and the potential reservoir for these genes among CoNS or other bacterial genera from human and zoonotic sources. On-going surveillance and an in-depth understanding of the genetic relatedness of MRSA and of the resistance determinants that they and CoNS harbour will aid in tracking, preventing and controlling the spread of MRSA.

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DNA Microarray Profiling of a Diverse Collection of Nosocomial Methicillin-Resistant *Staphylococcus aureus* Isolates Assigns the Majority to the Correct Sequence Type and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Type and Results in the Subsequent Identification and Characterization of Novel SCC*mec*-SCC_{M1} Composite Islands

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One hundred seventy-five isolates representative of methicillin-resistant *Staphylococcus aureus* (MRSA) clones that predominated in Irish hospitals between 1971 and 2004 and that previously underwent multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing were characterized by *spa* typing (175 isolates) and DNA microarray profiling (107 isolates). The isolates belonged to 26 sequence type (ST)-SCC*mec* types and subtypes and 35 *spa* types. The array assigned all isolates to the correct MLST clonal complex (CC), and 94% (100/107) were assigned an ST, with 98% (98/100) correlating with MLST. The array assigned all isolates to the correct SCC*mec* type, but subtyping of only some SCC*mec* elements was possible. Additional SCC*mec*/SCC genes or DNA sequence variation not detected by SCC*mec* typing was detected by array profiling, including the SCC-fusidic acid resistance determinant Q6GD50/*fusC*. Novel SCC*mec*/SCC composite islands (CIs) were detected among CC8 isolates and comprised SCC*mec* IIA-IIE, IVE, IVF, or IVg and a *ccrAB4*-SCC element with 99% DNA sequence identity to SCC_{M1} from ST8/t024-MRSA, SCC*mec* VIII, and SCC-CI in *Staphylococcus epidermidis*. The array showed that the majority of isolates harbored one or more superantigen (94%; 100/107) and immune evasion cluster (91%; 97/107) genes. Apart from fusidic acid and trimethoprim resistance, the correlation between isolate antimicrobial resistance phenotype and the presence of specific resistance genes was ≥97%. Array profiling allowed high-throughput, accurate assignment of MRSA to CCs/STs and SCC*mec* types and provided further evidence of the diversity of SCC*mec*/SCC. In most cases, array profiling can accurately predict the resistance phenotype of an isolate.

Staphylococcus aureus is a versatile pathogen responsible for a wide range of infections, reflecting its ability to express an extensive array of virulence factors and antimicrobial resistance genes. Methicillin-resistant *S. aureus* (MRSA) was first reported in 1961 in the United Kingdom, and subsequently a variety of different MRSA clones that exhibited resistance to numerous antimicrobial agents emerged (6, 22). Different epidemic and pandemic clones have spread worldwide since the 1970s (6). Today, MRSA strains are a major nosocomial problem worldwide and have also emerged as a significant cause of infections among otherwise healthy individuals in the community and among animals (10, 55).

While many virulence-associated genes in *S. aureus* are part of the core genome (such as those encoding microbial surface components recognizing adhesive matrix molecules [MSCRAMMs] and the polysaccharide intercellular adhesion [*ica*] locus), many are encoded on mobile genetic elements (MGEs) that can be horizontally transferred between *S. aureus* isolates and from coagulase-negative staphylococci (CoNS) (29). Many different types of MGEs encoding virulence genes have been identified in *S. aureus*, including bacteriophages, pathogenicity islands, plasmids, staphylococcal cassette chromosome (SCC) and SCC-like elements, and genomic islands (3, 32).

Antimicrobial resistance in *S. aureus* can be due to spontaneous chromosomal mutations, but resistance is more commonly due to the acquisition of MGEs, including plasmids, transposons, and SCC elements harboring antimicrobial resistance genes (32). SCC elements include SCC*mec*, which harbors the methicillin resistance gene *mecA*. Two divergent *mecA* alleles have been described in MRSA to date (20, 46). A search of GenBank revealed an additional 32 variants that differ at the nucleotide sequence level and 28 protein variants. Eleven different SCC*mec* types, as well as numerous subtypes, have been identified in MRSA, and each type has a unique combination of cassette chromosome recombinase (*ccr*) genes which are involved in the integration and excision of

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the element, as well as *mec* complex genes, including *mecA* and, when present, the *mec* regulatory genes *mecI* and *mecR1* (15, 20, 28, 46). Each different SCC*mec* type has been assigned a Roman-numeral name in the order in which it was identified (20). SCC*mec* subtypes differ from SCC*mec* types mainly in the joining or "J" regions, i.e., the regions outside the *ccr* and *mec* gene complexes (20). Subtypes are designated by the use of either lower- or uppercase alphabetic suffixes after the relevant Roman-numeral SCC*mec*-type name (20). Lowercase alphabetic suffixes (e.g., IVa, IVb, or IVc) are used to indicate DNA sequence variation in the J regions of SCC*mec* subtypes, while uppercase alphabetic suffixes (e.g., IVA, IIA, IA) indicate variation due to the presence or absence of mobile genetic elements (20). SCC elements without *mecA* but with *ccr* genes and sometimes with additional antimicrobial resistance or virulence-associated determinants, as well as composite islands (CIs) consisting of a combination of two or more SCC, SCC-like, or SCC*mec* elements, have also been described in *S. aureus* and CoNS (2, 11, 18, 20, 21, 25, 30, 37, 47). Evidence suggests that CoNS are reservoirs for SCC*mec* in *S. aureus*. Due to the diversity and frequency of identification of novel SCC and SCC*mec* types and subtypes, some reported novel elements do not follow any standardized or internationally agreed rules of nomenclature. While it is now recommended that nomenclature of novel SCC*mec* types and SCC types and subtypes should be undertaken in conjunction with the International Working Group on the Classification of SCC elements, this does not always happen, and there are ambiguities in the classification of SCC and SCC*mec* elements in the published literature. A binary system is currently in development to simplify this confusing SCC*mec* subtype nomenclature (20).

Effective and informative molecular typing plays an important role in monitoring the emergence, spread, and evolution of different MRSA clones. Multilocus sequence typing (MLST) is commonly used for MRSA typing, and while it has provided invaluable insights into its evolution, it lacks the discriminatory ability required for local or outbreak investigations and is relatively expensive and labor-intensive, as it involves amplification and sequencing of internal fragments of seven housekeeping genes (40). In contrast, *spa* typing involves DNA sequencing of a variable number tandem repeat (VNTR) region of just one gene (the *S. aureus* protein A *spa* gene), provides greater discrimination than MLST, and is widely used internationally for local and global epidemiological studies (16). Accurate identification of the type and subtype of the SCC*mec* element harbored by a MRSA isolate enhances understanding of the genetic relatedness of MRSA isolates. SCC*mec* typing commonly involves the use of several multiplex PCR assays to identify the *ccr* and *mec* complex genes which are used to define the SCC*mec* type and to identify the main characteristics of the J regions for SCC*mec* subtype determination and which can harbor integrated transposons and/or plasmids with additional antimicrobial resistance determinants. In the case of novel SCC*mec* elements, complete nucleotide sequencing is commonly carried out. The combination of MLST and SCC*mec* typing data is used to describe MRSA clones (14), and for the purpose of the present study, clones are defined as MRSA isolates with the same sequence type (ST) and the same or very closely related SCC*mec* type. Strains are defined as isolates not just with the same ST and SCC*mec* type but also with additional shared traits, such as specific combinations of virulence or antimicrobial resistance genes.

In recent years, major advances in high-throughput whole-genome sequencing technologies have resulted in the development of more in-depth typing methods, including single-nucleotide polymorphism (SNP) analysis of whole-genome sequences (17) and different microarray platforms (29). A recent study demonstrated the potential of SNP analysis of whole-genome sequences for investigating evolutionary relatedness and for epidemiological tracking of MRSA (17). However, this approach is currently expensive and time-consuming, and data analysis is too complicated for routine typing. DNA microarray systems have been developed based on the whole-genome sequences that are available for *S. aureus* and allow simultaneous hybridization of an isolate's genome against the entire gene content of multiple *S. aureus* genomes. While these DNA microarray systems provide a large amount of information about the isolate's genome, their use is restricted mainly to specialized research laboratories, as data analysis is complicated (29, 50, 52, 56). In contrast, several groups have recently developed more focused DNA microarrays (12, 35, 36, 43), with one system in particular offering simultaneous high-throughput genotyping of *S. aureus* isolates by assigning isolates to an MLST clonal complex (CC) or ST and to an SCC*mec* type and detection of species-specific markers, accessory gene regulator (*agr*) alleles, capsule types, MSCRAMMs, and a range of clinically relevant antimicrobial resistance and virulence-associated genes (34–36).

Like in many other countries, MRSA has been a major problem in Irish hospitals since the 1970s (4, 7, 19, 45). Previous studies of nosocomial MRSA isolates recovered in Irish hospitals over a 33-year period using MLST and detailed SCC*mec* typing revealed that the predominant MRSA clone was replaced after a certain period of years (45). Dominant clones identified included ST250-MRSA-I/I-*pls* in the 1970s and the early 1980s, the ST239-MRSA-IIIHg/IIIHg-p1258/Tn*554* clone in the 1980s, and the ST8-MRSA-IIA-IIE, ST36-MRSA-II, and ST22-MRSA-IV clones in the 1990s and early 2000s. The ST22-MRSA-IV clone has continued to predominate in Irish hospitals to the present day (45).

The purpose of the current study was to perform an in-depth molecular characterization of the predominant MRSA clones recovered from patients in Irish hospitals between 1971 and 2004 using *spa* typing and a DNA microarray system. The DNA microarray was investigated to (i) determine its value for genotyping MRSA isolates representative of major pandemic clones that have been previously investigated by MLST and detailed SCC*mec* typing, (ii) identify the range of virulence and antimicrobial resistance genes harbored by these MRSA clones, and (iii) study the correlation between antimicrobial resistance phenotype and the presence of antimicrobial resistance genes.

MATERIALS AND METHODS

MRSA isolates. A total of 175 MRSA isolates, representative of the most prevalent antibiogram-resistogram (AR) and pulsed-field gel electrophoresis (PFGE) types of MRSA isolates recovered among patients in Irish hospitals at different time periods between 1971 and 2004, were investigated (45).

Molecular typing. SCC*mec* typing of all isolates was undertaken previously using individual PCR assays to detect *mec* complexes A and B and *ccr* complexes 1, 2, and 3 and analysis of the joining (J) regions by a previously described multiplex PCR method (38, 45). For all ST8 isolates previously identified as harboring novel SCC*mec* II and IV variant elements, in-depth molecular characterization of the SCC*mec* elements was performed using long-range PCR amplification and sequencing (45). As

part of the present study, all isolates harboring SCC*mec* IV were subtyped using a multiplex PCR method (33) which detects the SCC*mec* IV subtypes IVA, IVa, IVb, IVc, IVd, IVg, and IVh. Previously described *S. aureus* reference strains were used as positive controls for SCC*mec* IV subtyping (48). One hundred thirty of the 175 isolates were previously typed by multilocus sequence typing (MLST) (45). MLST was performed as described previously on the remaining 45 isolates as part of the present study (13, 49). All 175 isolates underwent *spa* typing as described previously (49).

DNA microarray analysis. One hundred seven of the 175 MRSA isolates investigated underwent DNA microarray analysis. These included (where possible) three isolates representative of each AR type, sequence type (ST), and SCC*mec* type/subtype combination previously identified among the 175 isolates during different time periods between 1971 and 2004 (45). The StaphyType kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis according to the manufacturer's instructions, which have been described in detail elsewhere (35, 36). The StaphyType kit consists of a DNA microarray chip adhered to each well of a microtiter strip; each chip consists of 334 *S. aureus* target sequences, including species-specific, antimicrobial resistance, and virulence-associated genes, genes involved in attachment, adhesion, and biofilm formation, as well as markers used for typing. Data generated by StaphyType arrays were analyzed for the presence or absence of these genes using Arraymate software (Alere Technologies), which can assign *S. aureus* isolates to STs and/or clonal complexes (CCs) by comparing each isolate's DNA microarray results to those of a diverse reference collection of previously characterized strains in the Arraymate database (36).

PCR to confirm the absence of *ccrAB2* genes. For five isolates that were previously identified as harboring *ccrAB2* and a class B *mec* complex by SCC*mec* typing PCR, the *ccrAB2* genes were not detected using the DNA microarray. PCR detection of *ccrAB2* in these isolates was repeated using a previously described multiplex *ccr* PCR assay (26) and previously described reference strains (49).

PCR amplification of the *S. epidermidis ccrAB4* gene. The presence of *S. epidermidis ccrAB4* was investigated by PCR in all isolates that yielded positive or ambiguous signals for *ccrA4* and/or *ccrB4* genes using the DNA microarray (which has primers and probes specific for *S. aureus ccrA4* and *ccrB4*) using previously described conditions and primers specific for the *S. epidermidis ccrAB4* genes (49). Since the majority of isolates that were positive for these *ccrAB4* genes exhibited the ST8-MRSA-IIA-IIE or IVE/IVF and *spa* type t190 genotype, all isolates belonging to these genotypes that underwent *spa* typing but not DNA microarray analysis were also investigated by PCR for the presence of the *S. epidermidis ccrAB4* genes.

Whole-genome sequencing. The whole genome of one MRSA isolate (AR13.1/3330.2) representative of the most frequently occurring ST-SCC*mec* type identified as harboring the *S. epidermidis ccrAB4* genes was sequenced to further investigate the location and adjacent sequences of these genes. High-throughput *de novo* sequencing was undertaken commercially by Geneservice (Source BioScience plc, Nottingham, United Kingdom) using the Illumina Genome Analyzer system (Illumina HiSeq 2000 platform; Illumina, Essex, United Kingdom). The average coverage across the genome was 40×. The reads were assembled into contigs using a Velvet *de novo* genome assembler (version 1.0.15; Illumina). Contigs were analyzed using the Artemis DNA sequence viewer and annotation tool (42) and BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (1). Contigs identified as containing SCC-associated DNA sequences were aligned using the BioNumerics (version 5.1) (Applied Maths, Ghent, Belgium) and DNA Strider (version 1.3f11) (CEA Saclay, Gif-sur-Yvette, France) software packages. Any gaps identified between the SCC-related contigs in the whole-genome sequence of AR13.1/3330.2 were closed by primer walking using PCR with primers based on the surrounding contigs and the Expand long-template PCR system (Roche Diagnostics Ltd., West Sussex, United Kingdom) followed by amplimer sequencing by Geneservice (SourceBioscience, Guinness Enterprise Centre, Dublin). Data were analyzed, and overlapping sequences were assembled using the BLAST, Bionumerics, and DNA Strider software packages.

Confirmation of the genetic organization and location of *ccrAB4*. Having determined the location and genetic organization of the *ccrAB4* region of an SCC element adjacent to SCC*mec* in AR13.1/3330.2 using whole-genome sequencing, the genetic organization of this element was confirmed by PCR and amplimer sequencing using four overlapping primer pairs to amplify from the SCC*mec* element to the end of the *ccrAB4*-carrying element of AR13.1/3330.2. The primers used are listed in Table S1 in the supplemental material. These PCR assays were performed using chromosomal template DNA with GoTaq DNA polymerase (Promega Corporation, Madison, WI). PCR products were visualized by agarose gel electrophoresis, and the sizes of amplicons obtained were compared to the expected sizes of amplicons based on the whole-genome sequence (see Table S1).

The presence of the *ccrAB4*-carrying SCC element and its location adjacent to SCC*mec* were investigated in one isolate representative of each ST and SCC*mec* type combination identified as harboring the *S. epidermidis ccrAB4* genes, including ST8-MRSA-IIA (AR13/0132), ST8-MRSA-IIB (AR05/1345), ST8-MRSA-IIC (AR14/0246), ST8-MRSA-IID (AR13/3698), ST8-MRSA-IVE (AR43/3330.1), ST8-MRSA-IVF (AR43/3246), and ST94-MRSA-IVg (M03/0169.2), using GoTaq DNA polymerase (Promega). The primers described above for AR13.1/3330.2 were used for these PCR amplifications, except that primers J1_IVc_F2, J1_IVb_F2, and J1_IVg_F1 were used instead of primer J1_IIE_F3 for isolates harboring SCC*mec* types IVE, IVF, and IVg, respectively (see Table S1 in the supplemental material). PCR products were visualized by agarose gel electrophoresis, and the sizes of amplicons were compared to those obtained with template DNA from AR13.1/3330.2 and to the expected size of amplicons based on the whole-genome sequence of this isolate.

PCR detection of antimicrobial resistance determinants. Phenotypic resistance to selected antimicrobial agents where corresponding antimicrobial resistance genes were included on the DNA microarray was determined by disk diffusion susceptibility testing as described previously (41). Isolates that exhibited phenotypic resistance to particular antimicrobial agents for which associated resistance genes were not detected by the DNA microarray were further investigated using PCR assays and previously described primers and thermal cycling conditions specific for additional resistance genes. These investigations included PCR detection of the fusidic acid resistance gene *fusD* (5), the trimethoprim resistance genes *dfrD* (9), *dfrG* (44), and *dfrK* (24), the tetracycline resistance gene *tet*(L) and *tet*(O) (53), and PCR DNA amplification and sequencing to detect further mutations in *fusA* encoding elongation factor G (EF-G) (5). *Escherichia coli* pBS2187*dfrK* (23) and *S. aureus* CM.S2 (44) were used as positive controls for *dfrK* and *dfrG*, respectively. Positive controls for *fusD* and *dfrD* were not available for use in the present study. Following PCR amplification of the *fusA* gene, PCR products were purified using the Genelute PCR cleanup kit (Sigma-Aldrich, Tallaght, Dublin, Ireland), sequenced commercially by Geneservice, and analyzed using DNA Strider and Bionumerics software using the *fusA* gene consensus sequence of MRSA reference strain N315 (GenBank accession number NC_002745) as a reference sequence.

Nucleotide sequence accession number. The nucleotide sequence of the *ccrAB4*-carrying element from MRSA isolate AR13.1/3330.2 was deposited in the GenBank database under accession number HE858191.

RESULTS

***spa* typing.** The 175 MRSA isolates recovered in Irish hospitals between 1971 and 2004 were previously assigned to 26 ST-SCC*mec* types and subtypes (Table 1) (45, 49). In the present study, these 175 isolates were assigned to 35 *spa* types (Table 1). The *spa* type t190 predominated (41%; 73/175) and accounted for 99% (73/74) of ST8-MRSA-IIA-IIE and ST8-MRSA-IVE/IVF isolates (Table 1). Single *spa* types also accounted for the majority of isolates within four other clones: t008 in ST250-MRSA-I/I-*pls* (95%; 18/

TABLE 1 MLST CCs, STs, SCC*mec* types, and *spa* types of 175 MRSA isolates representative of different antibiogram-resistogram, ST, and SCC*mec* type combinations recovered from patients in Irish hospitals between 1971 and 2004 and the corresponding CCs/STs and SCC*mec* types deduced from DNA microarray profiles of 107 representative isolates

			DNA microarray[a]		
CC	MLST and SCC*mec* typing assignment for 175 isolates tested[b]	*spa* types for 175 isolates tested[c]	CC/ST-SCC*mec* type assignment by microarray for 107 isolates tested[d]	SCC*mec*-associated genes detected by microarray[e]	SCC*mec* type/subtype assignment following manual inspection of microarray profiles
8	ST250-MRSA-I (10)	t008 (10)	ST250-MRSA-I (4)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB1*, *dcs*, *pls*	I
8	ST250-MRSA-I-*pls* (9)	t008 (8), t121 (1)	ST250-MRSA-I (6)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB1*, *dcs*	I-*pls*
8	ST239-MRSA-III and SCC*Hg* (9)	t037 (8), t138 (1)	ST239-MRSA-III (8)	*mecA*, *ugpQ*, Δ*mecR1*(5 Δ*mecR1* negative), *mecR1*, *mecI*, *xylR*, *ccrAB3*, *ccrC*, *merA/B*, *erm*(A), *tet*(K) (1 negative)	III and SCC*Hg*
8	ST239-MRSA- III and SCC*Hg* -p1258/Tn*554* (4)	t037 (4)	ST239-MRSA-III (4)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB3*, *ccrC*, *merA/B*, *erm*(A), *tet*(K)	III and SCC*Hg*
8	ST247-MRSA-Ia (3)	t3503 (2), t052 (1)	ST247-MRSA-I (3)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB1*, *dcs*, *pls*, *aadD*	Ia
8	ST8-MRSA-IIA and *ccrAB4* (9)	t190 (9)	ST8-MRSA-II and CI (4)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *ccrB4*, *dcs* (1 *dcs* negative), *aadD*, *erm*(A)	IIA or IIB and *ccrAB4*
8	ST8-MRSA-IIB and *ccrAB4* (1)	t190 (1)	ST8-MRSA-II and CI (1)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *ccrB4*, *dcs*, *aadD*, *erm*(A)	IIA or IIB and *ccrAB4*
8	ST8-MRSA-IIC and *ccrAB4* (9)	t190 (9)	ST8-MRSA-II and CI (6)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *ccrAB2*, *ccrB4*, *dcs* (1 *dcs* negative), *aadD*, *erm*(A)	IIC and *ccrB4*
8	ST8-MRSA-IIC (1)	t190 (1)	ST8-MRSA-II (1)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *ccrAB2*, *dcs*, *aadD*, *erm*(A)	IIC
8	ST8-MRSA-IID and *ccrAB4* (24)	t190 (23), t2196 (1)	ST8-MRSA-II and CI (12)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *ccrB4*, *dcs* (4 *dcs* negative), *erm*(A)	IID and *ccrAB4*
8	ST8-MRSA-IID (3)	t190 (3)	ST8-MRSA-II (3)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *dcs*, *erm*(A)	IID
8	ST8-MRSA-IIE and *ccrAB4* (6)	t190 (6)	ST8-MRSA-II and CI (5)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *ccrAB2*, *ccrB4*, *dcs*, *erm*(A)	IIE and *ccrAB4*
8	ST8-MRSA-IIE (1)	t190 (1)	ST8-MRSA-II and CI (1)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *ccrAB2*, *erm*(A)	IIE
8	ST8-MRSA-IVE and *ccrAB4* (17)	t190 (17)	ST8-MRSA-IV and CI (2)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *ccrAB4* (1 *ccrA4* negative)	IVE or IVF and *ccrAB4*

(Continued on following page)

TABLE 1 (Continued)

CC	MLST and SCC*mec* typing assignment for 175 isolates tested[b]	*spa* types for 175 isolates tested[c]	DNA microarray[a]		
			CC/ST-SCC*mec* type assignment by microarray for 107 isolates tested[d]	SCC*mec*-associated genes detected by microarray[e]	SCC*mec* type/subtype assignment following manual inspection of microarray profiles
			CC8-MRSA-VI (1)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB4*	VI
8	ST8-MRSA-IVF and *ccrAB4* (3)	t190 (3)	CC8-MRSA-VI (3)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB4* (*1 ccrA4* negative)	VI
8	ST94-MRSA-IVg and *ccrAB4* (1)	t4691 (1)	ST8-MRSA-IV and CI (1)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *ccrB4*, *dcs*	IV and *ccrAB4*
8	ST609-MRSA-IVA (1)	t064 (1)	CC8-MRSA-IV (1)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *dcs*, *aadD*	IVA
22	ST22-MRSA-IVh (22)	t032 (9), t022 (2), t3505 (1), t1467 (1), t531 (1), t3506 (1), t3504 (1), t790 (1), t515 (1), t3501 (1), t1802 (1), t2945 (1), t4253 (1)	ST22-MRSA-IV (11)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *dcs*	IV
22	ST22-MRSA-IVa (5)	t005 (3), t902 (1), t4902 (1)	ST22-MRSA-IV (2)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *dcs*	IV
5	ST5-MRSA-II (17)	t045 (17)	ST5-MRSA-II (14)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *dcs* (2 *dcs* negative), *kdp*, *aadD*, *erm*(A)	II
5	ST5-MRSA-IV nonsubtypeable (1)	t001 (1)	CC5-MRSA-atypical SCC*mec* element (1)	*mecA*, *ugpQ*, Δ*mecR1*, *dcs*	Class B *mec* and *dcs*, no *ccr* genes detected
5	ST496-MRSA-II (1)	t002 (1)	ST5-MRSA-II (1)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *dcs*, *kdp*, *aadD*, *erm*(A)	II
30	ST36-MRSA-II (12)	t018 (9), t012 (3)	ST36/39-MRSA-II (6)	*mecA*, *ugpQ*, Δ*mecR1*, *mecR1*, *mecI*, *xylR*, *ccrAB2*, *dcs*, *kdp*, *aadD*, *erm*(A)	II
30	ST30-MRSA-IV nonsubtypeable (4)	t5093 (1), t012 (2), t021 (1)	ST30-MRSA-IV (4)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *dcs* (2 *dcs* negative), *Q6GD50* (*fusC*)	IV and Q6GD50/*fusC*
45	ST45-MRSA-IVa (1)	t727 (1)	ST45-MRSA-IV (1)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *dcs*	IV
12	ST12-MRSA-IVc (1)	t160 (1)	CC12-MRSA-IV (1)	*mecA*, *ugpQ*, Δ*mecR1*, *ccrAB2*, *dcs*	IV

[a] The StaphyType kit (Alere Technologies) was used for DNA microarray analysis. CI, composite island.
[b] Numbers in parenthesis refer to the numbers of isolates with the particular MLST and SCC*mec* type assignment indicated.
[c] Numbers in parenthesis refer to the numbers of isolates with the particular *spa* type assignment indicated.
[d] Numbers in parenthesis refer to the numbers of isolates with the MLST and SCC*mec* type assignment indicated as deduced from DNA microarray data.
[e] It is not possible to localize the antimicrobial resistance determinants *aadD*, *tet*(K), *erm*(A), *merA*, and *merB* to within the SCC*mec* elements using the array, but those that were detected and are commonly associated with a particular SCC*mec* type or subtype are listed.

19); t045 in ST5-MRSA-II (100%; 17/17); t037 in ST239-MRSA-III/III-p1258/Tn*554* (92%; 12/13), and t018 in ST36-MRSA-II (75%; 9/12) (Table 1). In contrast, the 27 ST22-MRSA-IV isolates exhibited 16 *spa* types, with *spa* type t032 predominating (33%; 9/27), followed by t005 (11%, 3/27) and t022 (7%, 2/27), with all other ST22 *spa* types being represented by single isolates (Table 1). In no instance was the same *spa* type identified among isolates belonging to different CCs, while only one *spa* type (t012) was detected among isolates belonging to different STs, ST36 and ST30, but to the same CC, CC30 (Table 1).

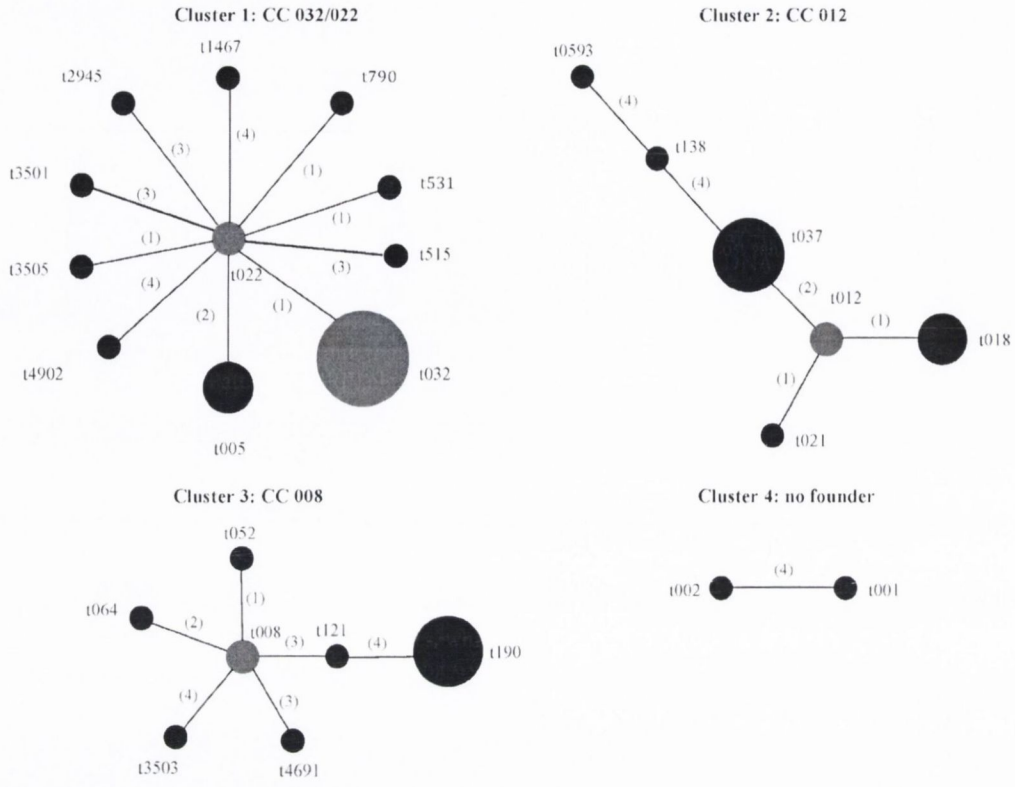

FIG 1 Based Upon Repeat Pattern (BURP) analysis of the *spa* types identified among 175 MRSA isolates representative of the different antibiogram-resistogram, sequence type, and SCC*mec* type combinations recovered from patients in Irish hospitals between 1971 and 2004. Twenty-six of the 35 *spa* types were grouped into four cluster groups using the BURP algorithm. *spa* types were clustered together if they contained five or more *spa* repeats and if they had a cost value of ≤4, where cost accounts for the number of steps of evolution between *spa* types. A low cost value indicates close evolutionary relatedness between two *spa* types. The following six *spa* types were defined as singletons by BURP (t045, t160, t902, t3504, t3506, and t4253), as they could not be clustered with any other *spa* type, i.e., cost value of ≥5. The *spa* types t727, t1802, and t2196 were excluded, as they consisted of four repeat units only. Cost values are shown in parentheses. Group founders and cofounders (*spa* types with the second-highest group founder score) are shown in blue and are determined based on the *spa* type that shares the highest sequence identity with the greatest number of *spa* types within that cluster as determined by the cost values. No founder was assigned to cluster 4.

Analysis of the 35 *spa* types represented among the study isolates using the Based Upon Repeat Pattern (BURP) algorithm assigned 26 of the 35 *spa* types to four clusters (Fig. 1). Cluster 1 (*spa*-CC 032/022) consisted of 11 of the 16 *spa* types identified among ST22-MRSA-IV isolates. Cluster 2 (*spa*-CC 012) consisted of all *spa* types exhibited by CC30 isolates and the CC8/ST239-MRSA-III *spa* types t037 and t138. This was not unexpected, because the ST239 genotype arose as a result of a large chromosomal replacement between ST8 and ST30 isolates encompassing the *spa* locus (39). Cluster 3 (*spa*-CC 008) consisted of all except one of the remaining *spa* types identified among the CC8 isolates, while cluster 4 consisted of two of three *spa* types found among CC5 isolates. The remaining *spa* types were not assigned to any cluster, because they consisted of four repeats only, which is considered to be too few data to deduce evolutionary history, or they differed from all other *spa* types by a cost of ≥5 (where cost indicates the steps of evolution between two *spa* types).

Correlation between DNA microarray analysis and MLST. The DNA microarray assigned the majority of the MRSA isolates investigated to the correct ST (92%; 98/107) (Table 1). Seven isolates were not assigned to any ST but were assigned to the correct CC (7%; 7/107), while two isolates were assigned to incorrect STs (2%; 2/107); but these incorrect STs were within the correct CCs. One isolate with ST496 (which is in CC5) was assigned to ST5, and one isolate belonging to ST94 (which is in CC8) was assigned to ST8 (Table 1). ST496 is a double-locus variant of ST5, while ST94 is a single-locus variant of ST8.

Correlation between SCC*mec* typing and SCC*mec* types deduced by DNA microarray analysis. The majority of MRSA isolates subjected to DNA microarray analysis were assigned to the correct SCC*mec* type (i.e., to SCC*mec* types I, II, III, or IV) (95%; 102/107) compared to results obtained previously by conventional SCC*mec* typing (Table 1). Table S2 in the supplemental material provides an overview of the main characteristics of the SCC*mec* types and subtypes investigated in the present study. Four ST8/t190 isolates that were previously assigned by SCC*mec* typing to SCC*mec* types IVE or IVF (J1 regions IVc and IVb, respectively, as well as class B *mec* and *ccrAB2* and lacking *dcs*) with an adjacent *ccrAB4* were assigned to SCC*mec* VI by the DNA microarray (Table 1). In these isolates, the class B *mec* complex and *ccrAB4* genes were detected using the DNA microarray, but *ccrAB2* was not detected (Table 1). One MRSA isolate belonging to CC5 with *spa* type t001, which was previously found to harbor SCC*mec* IV by SCC*mec* typing, was designated as an atypical SCC*mec* element by the DNA microarray (Table 1). Manual inspection of the SCC*mec*-associated DNA microarray signals of this isolate re-

vealed the presence of a class B *mec* complex and *dcs* but the absence of *ccrAB2*. These five isolates with anomalous SCC*mec* types, as deduced from their DNA microarray profiles because they lacked *ccrAB2*, were retyped using conventional SCC*mec* typing by PCR, which confirmed the absence of *ccrAB2* and suggested that these isolates may have lost the *ccr* genes. Lastly, manual inspection of the DNA microarray profiles indicated that while the eight ST239-MRSA-III*Hg* isolates yielded signals for *mecR1*, the probe for the truncated *mecR1* gene (Δ*mecR1*) did not yield a signal for five of these eight isolates (Table 1).

The DNA microarray did not automatically assign SCC*mec* subtypes to isolates, but manual inspection of the DNA microarray profiles allowed subtyping of SCC*mec* types I, II, and III*Hg* (Table 1). The differentiation of SCC*mec* subtypes Ia and I-*pls* is based on the presence of plasmid pUB110 carrying the aminoglycoside resistance gene *aadD* in SCC*mec* Ia and the absence of *pls* in SCC*mec* I-*pls* (Table 1). The SCC*mec* II subtypes IIA, IIB, IIC, IID, and IIE all lack *kdp*, which is normally associated with SCC*mec* II elements (Table 1). While the SCC*mec* subtypes IIA and IIB cannot be further differentiated using the array, SCC*mec* IIC lacks *xylR* and *mecI* due to the presence of a variant *mec* complex termed class A.3 *mec*. SCC*mec* subtype IID is characterized by the absence of *aadD*, while *xylR*, *mecI*, and *aadD* are absent in SCC*mec* IIE (Table 1). SCC*mec* type III*Hg* was identified by the presence of the mercury resistance genes *merA* and *merB* and by *ccrC* (Table 1). While the DNA microarray was unable to subtype the majority of SCC*mec* IV elements, recognition of SCC*mec* IVA was possible due to the presence of the pUB110-encoding gene *aadD* (Table 1). The array detected the *fusC* gene (designated Q6GD50 on the array) indicative of the presence of the SCC*fus* element (18) in the four ST30 isolates, but additional *ccr* genes (*ccrAB1*) normally associated with the presence of this SCC element were not detected. The *dcs* gene, located between *mecA* and *orfX* and normally found in SCC*mec* II and IV elements, was not detected in eight isolates harboring SCC*mec* II elements (ST8 and ST5) and eight isolates with SCC*mec* IV elements (ST8 and ST30), although this has been reported previously for the ST8 SCC*mec* IVE and IVF elements (45).

The DNA microarray also allowed the identification of the presence of *S. epidermidis ccrAB4* in ST8-MRSA isolates (Table 1). The presence of *S. epidermidis ccrAB4* has been reported previously in ST8 isolates (49). The array yielded positive or ambiguous signals for *ccrA4* and/or *ccrB4* in 36 CC8-MRSA isolates, including ST8-MRSA-IIA-IIE isolates (29/33), ST8-MRSA-IVE-IVF isolates (6/6), and the single ST94-MRSA-IVg isolate investigated using the array. The presence of *S. epidermidis ccrAB4* was confirmed in these isolates by PCR, indicating the possible presence of an SCC element in addition to SCC*mec* in these isolates. The presence of *S. epidermidis ccrAB4* was also investigated by PCR in the ST8-MRSA-IIA-IIE ($n = 21$) and ST8-MRSA-IVE-IVF ($n = 14$) isolates from the present study that did not undergo DNA microarray analysis; the presence of this gene was confirmed in 95% (20/21) and 100% (14/14) of these isolates, respectively.

Detection of a novel SCC*mec*-SCC composite island in ST8-MRSA-IIE isolate AR13.1/3330.2. Whole-genome sequencing was performed on one isolate (AR13.1/3330.2) representative of isolates harboring the *S. epidermidis ccrAB4* gene to determine the genetic organization of the region surrounding *ccrAB4*. Whole-genome sequencing of AR13.1/3330.2 yielded 344 contigs ranging in size from ca. 113 bp to 137 kb. The SCC-associated DNA sequences were identified in three contigs (13 kb, 11 kb, and 764 bp in size), and the gaps between these contigs were closed using long-range PCR amplification and sequencing with primers based on the surrounding contigs. The order of the contigs was confirmed by PCR. This analysis revealed the presence of an approximately 14-kb SCC element downstream and immediately adjacent to SCC*mec* IIE in AR13.1/3330.2 (Fig. 2a). This SCC element exhibited 99% DNA sequence identity to SCC_{M1} (Fig. 2b) previously identified in the Danish ST8-MRSA isolate M1 (*spa* type t024) (2).

In MRSA M1, the SCC_{M1} element exists as part of a composite island and is located directly adjacent to *orfX*, upstream of a truncated arginine catabolic mobile element (ACME) and SCC*mec* IVa (Fig. 2b). In contrast, in AR13/3330.2, the SCC*mec* IIE element was located adjacent to *orfX*, whereas SCC_{M1} was located downstream of SCC*mec* IIE (Fig. 2a). No ACME-*arc* genes were detected in AR13.1/3330.2, either in the whole-genome sequence or by DNA microarray analysis. The SCC_{M1} element comprises 18 ORFs that have been described previously (2), most significantly those encoding *ccrAB4*, a truncated copy of *ccrA* and a gene encoding a copper-transporting ATPase (*copA*) (Fig. 2a and b). SCC_{M1} is similar in its genetic organization to the part of SCC-CI element in *S. epidermidis* ATCC 12228 outside SCC*pbp* (99% DNA sequence identity) but significantly smaller in size and lacking the mercury and cadmium resistance genes (Fig. 2c). SCC_{M1} also shows 99% DNA sequence identity to a region downstream of *mecA* in SCC*mec* VIII (Fig. 2d). The composite island (CI) detected in AR13.1/3330.2 was designated SCC*mec* IIE-SCC_{M1} (Fig. 2a).

Confirmation of the presence of SCC_{M1} in representative CC8 isolates harboring the *S. epidermidis ccrAB4* genes. The presence of the SCC_{M1} element and its location downstream of SCC*mec* was investigated by PCR in seven additional isolates representative of other ST and SCC*mec* type combinations identified as harboring the *S. epidermidis ccrAB4*. Five of the seven isolates, including those representative of ST8-MRSA-IIA, ST8-MRSA-IIB, ST8-MRSA-IIC, ST8-MRSA-IID, and ST94-MRSA-IVg, yielded amplicons of the expected size for SCC_{M1}, and its location was downstream of SCC*mec*, as found in isolate AR13.1/3330.2 (Fig. 2a). These SCC*mec*-SCC CIs were assigned the following designations: SCC*mec* IIA-SCC_{M1}, SCC*mec* IIB-SCC_{M1}, SCC*mec* IIC-SCC_{M1}, SCC*mec* IID-SCC_{M1}, and SCC*mec* IVg-SCC_{M1}. For the remaining two isolates (ST8-MRSA-IVE and ST8-MRSA-IVF), the primer pairs located within the SCC_{M1} element (primer pairs 2, 3, and 4; see Table S1 in the supplemental material) yielded amplicons of the expected size, but those amplifying from the respective SCC*mec* elements to within the SCC_{M1} element (primer pairs 5 and 6; see Table S1 in the supplemental material) failed to yield any amplicons. These findings indicate that while the SCC_{M1} element is present in these ST8-MRSA isolates harboring SCC*mec* IVE and IVF, further investigations are required to determine the genetic organization of the SCC*mec* and SCC_{M1} elements in these isolates.

Additional typing markers and antimicrobial resistance, virulence-associated, and adhesion genes detected using the DNA microarray. The *agr* capsule and immune evasion cluster (IEC) types, as well as the antimicrobial resistance, virulence-associated, MSCRAMM, adhesion, and biofilm formation genes identified among the 107 MRSA isolates representative of the different ST and SCC*mec* type combinations investigated by DNA microarray

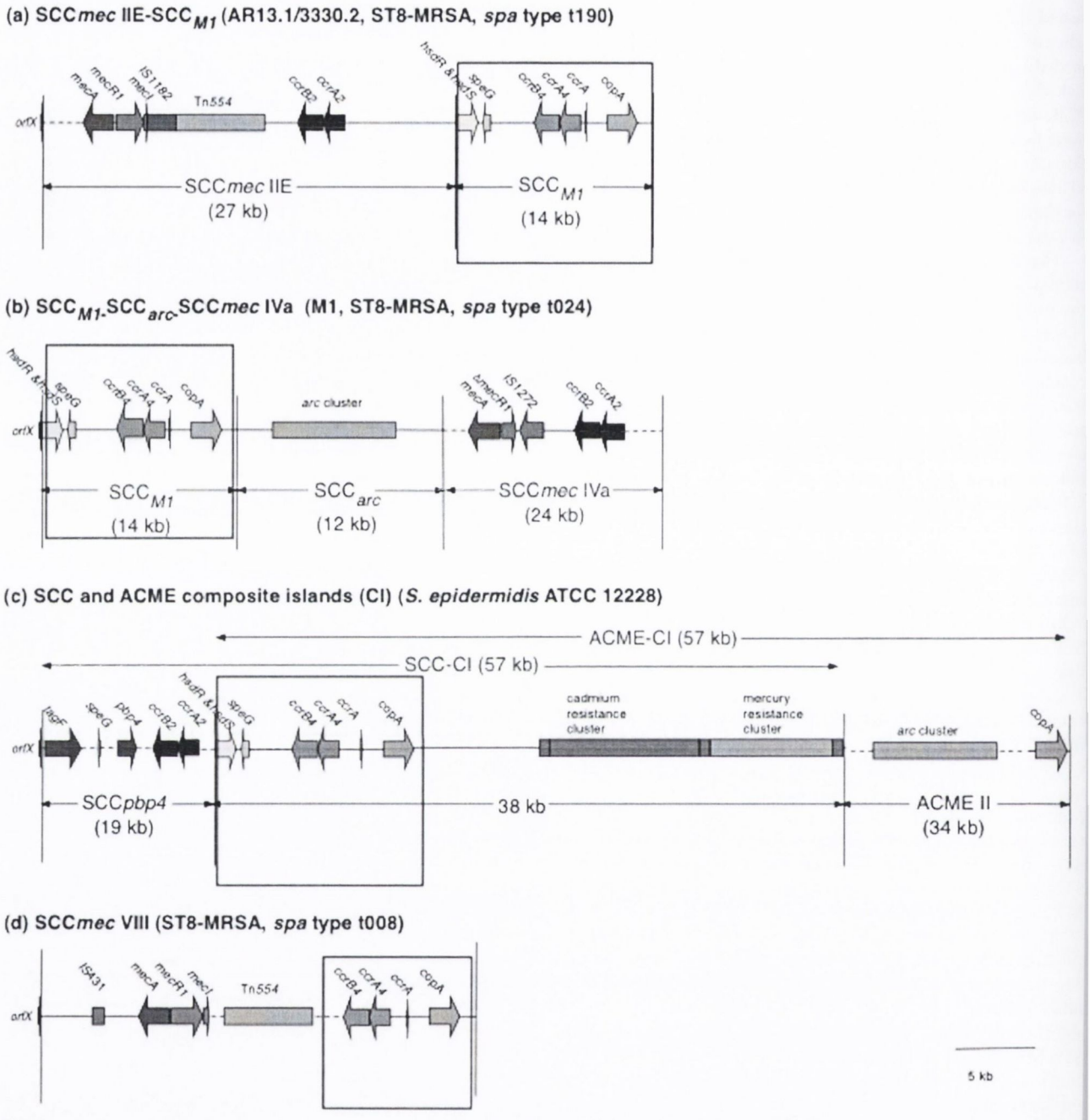

FIG 2 Schematic diagram showing the genomic organization of the novel SCC*mec*-SCC composite island (CI) SCC*mec* IIE-SCC$_{M1}$ identified in the ST8-MRSA *spa* type t190 isolate AR13.1/3330.2 (GenBank accession number HE858191) (a), the SCC$_{M1}$-SCC$_{arc}$-SCC*mec* element previously reported in ST8-MRSA *spa* type t024 isolate M1 (HM030720) (b), the ACME- and SCC-CIs previously reported in *S. epidermidis* ATCC 12228 (NC004461) (c), and SCC*mec* VIII previously described in ST8-MRSA *spa* type t008 isolate C10682 (FJ390057) (d). The structure of the novel SCC*mec* IIE-SCC$_{M1}$ element was determined by high-throughput whole-genome sequencing of AR13.1/3330.2 and was confirmed using primers spanning the SCC*mec*/SCC region. The areas surrounded by the red square indicate the regions of high similarity between SCC$_{M1}$ identified in isolate AR13.1/3330.2 and the other SCC*mec*/SCC elements.

analysis in the present study, are shown in Table 2. The majority of isolates belonged to *agr* type I (75%; 80/107) and included all CC8 and CC22 isolates (n = 66 and 13, respectively). All CC5 (n = 16) and the single CC12 isolate belonged to *agr* type II, while isolates within *agr* III were CC30 (n = 10). The single CC45 isolate belonged to *agr* type IV. Most isolates belonged to capsule type 5 (78%; 83/107), with the remainder belonging to capsule type 8 (22%; 24/107). Various combinations of IEC genes were detected in the majority of isolates (91%; 97/107), with the most frequently occurring IEC type being type D (39%; 42/107). IEC type D was detected in the CC8 lineages ST239-MRSA-III, ST8-MRSA-IIA–IIE, and ST609-MRSA-IVA and consisted of the enterotoxin A

TABLE 2 *agr*, capsule and IEC types, antimicrobial resistance, virulence-associated, MSCRAMM, adhesion, and biofilm formation genes identified using the DNA microarray among isolates representative of each antibiogram-resistogram, ST, and SCC*mec* type combination recovered from patients in Irish hospitals between 1971 and 2004[a]

	Additional typing marker			Antimicrobial resistance genes		Virulence-associated genes		MSCRAMM, adhesion, and biofilm genes	
CC/ST-SCC*mec* type (*n*)	*agr*	*cap*	IEC (*n*)[b]	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)
CC8/ST250-MRSA-I/I-*pls* (10)	I	5	B (10)	*blaZ*, *merA/B*, *erm*(A), *sdrM*, *qacA*, *fosB*	*tet*(K) (70), *aacA-aphD* (90), *aphA3* and *sat* (40), *dfrS1* (40)	*sak*, *chp*, *scn*	*seb* (90), *sek* and *seq* (90)	*bbp*, *clfA*, *clfb*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....
CC8/ST239-MRSA-III and SCC*Hg*/III and SCC*Hg*-p1258/Tn554 (12)	I	8	D (12)	*blaZ*, *merA/B*, *erm*(A), *tet*(M), *sdrM*, *fosB*	*tet*(K) (92), *qacA* (58), *qacC* (8), *aacA-aphD* (83), *aadD* (8), *aphA3* and *sat* (50), *dfrS1* (25), *mupA* (8), *cat*-pC194 (pMC524) (8)	*sea*, *sak*, *scn*	*sek* and *seq* (83)	*clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	*bbp* (58)
CC8/ST247-MRSA-Ia (3)	I	5	A (1), B (2)	*blaZ*, *merA/B* *tet*(M), *sdrM*, *qacA*, *aacA-aphD*, *aadD*, *fosB*	*erm*(A) (67)	*sak*, *chp*, *scn*	*sea* (33)	*clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....
CC8/ST8-MRSA-IIA-IIE (33)	I	5	D (29), NT (1),[c] Neg (3)	*blaZ*, *erm*(A), *aacA-aphD*, *sdrM*, *fosB*, *merA/B*	*erm*(C) (6), *tet*(K) (3), *qacA* (97), *qacC* (3), *aadD* (36), *aphA3* and *sat* (85), *dfrS1* (3), *far1/fusB* (3), *mupA* (12)	.....	*sea* (91), *sak* (91), *scn* (88)	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fnbA*, *fnbB*, *sdrD*, *vwb*, *icaA*, *icaC*, *icaD*	*fib* (97), *map* (97), *sdrC* (3)
CC8/ST8-MRSA-IVE/F (6)	I	5	E (5), Neg (1)	*blaZ*, *erm*(A), *sdrM*, *aphA3* and *sat*, *fosB*	*tet*(M) (33), *qacA* (17)	.....	*sak* (83), *scn* (83)	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	*sdrD* (83)
CC8/ST94-MRSA-IVg (1)	I	5	Neg (1)	*blaZ*, *tet*(M), *sdrM*, *aacA-aphD*, *aphA3* and *sat*, *fosB*	.....	.....	.....	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....
CC8/ST609-MRSA-IVA (1)	I	5	D (1)	*blaZ*, *erm*(C), *tet*(M), *sdrM*, *aacA-aphD*, *aadD*, *dfrS1*, *fosB*	.....	*sea*, *seb*, *sek* and *seq*, *sak*, *scn*	.....	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC* *icaD*	.....
CC22/ST22-MRSA-IVh/IVa (13)	I	5	B (8) Neg (5)	*blaZ*	*erm*(C) (46), *qacC* (8)	*egc*	*sec* and *sel* (85), *sak* (62), *chp* (62), *scn* (62)	*clfA*, *clfB*, *cna*, *ebpS*, *eno*, *fib*, *fnbA*, *sdrC*, *vwb*, *sasG*, *icaA*, *icaD*	*fnbB* (15), *icaC* (85), *bbp* (85), *ebh* (15), *map* (92), *sdrD* (85)
CC5/ST5-MRSA-II (14)	II	5	F (12) B (1) A (1)	*erm*(A), *sdrM*, *aadD*, *fosB*	*blaZ* (86), *qacA* (21), *merA/B* (21)	*tst*, *egc*,[c] *sak*, *chp*, *scn*	*sea* (7), *sep* (86), *sed*, *j* and *r* (71)	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....

(Continued on following page)

TABLE 2 (Continued)

CC/ST-SCC*mec* type (*n*)	Additional typing marker			Antimicrobial resistance genes		Virulence-associated genes		MSCRAMM, adhesion, and biofilm genes	
	agr	*cap*	IEC (*n*)[b]	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)	Always present	Variably present (% isolates positive)
CC5/ST5-MRSA-IV nonsubtypeable (1)	II	5	B (1)	*merA* and *B*, *blaZ*, *erm*(A), *sdrM*, *fosB*	.....	*egc*, *sak*, *chp*, *scn*	.....	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *map*, *sdrC*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....
CC5/ST496-MRSA-II (1)	II	5	B (1)	*erm*(A), *erm*(C), *aadD*, *sdrM*, *fosB*	.....	*egc*, *sak*, *chp*, *scn*	.....	*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....
CC30/ST36-MRSA-II (6)	III	8	A (6)	*blaZ*, *erm*(A), *sdrM*, *aadD*, *fosB*	.....	*sea*, *egc*, *sak*, *chp*, *scn*	*tst* (67)	*bbp*, *clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fnbA*, *fib*, *map*, *sdrC*, *sdrD*, *vwb*, *icaA*, *icaC*, *icaD*	.....
CC30/ST30-MRSA-IV nonsubtypeable (4)	III	8	B (4)	*blaZ*, Q6GD50/*fusC*, *fosB*	*sdrM* (75)	*tst*, *egc*, *sak*, *chp*, *scn*	.....	*clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *map*, *sdrC*, *sdrD*, *vwb*, *icaA*, *icaC*, *icaD*	*bbp* (75)
CC45/ST45-MRSA-IVa (1)	IV	8	B (1)	*blaZ*, *merA* and *B*, *erm*(C), *far1*/*fusB*, *sdrM*	.....	*egc*, *sak*, *chp*, *scn*	.....	*bbp*, *clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*, *sasG*, *icaA*, *icaC*, *icaD*	.....
CC12/ST12-MRSA-IVc (1)	II	8	G (1)	*blaZ*, *sdrM*, *fosB*	.....	*sep*, *seb*, *sec* (M14), *sak*, *scn*	.....	*clfA*, *clfB*, *cna*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *vwb*, *icaA*, *icaC*, *icaD*	.....

[a] The StaphyType kit (Alere Technologies) was used for DNA microarray analysis. *n*, number of isolates; *agr*, genes encoding accessory gene regulators; *cap*, genes encoding capsular proteins; CC, clonal complex; ST, sequence type; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; ....., no genes detected.

[b] Immune evasion cluster (IEC) type as defined by van Wamel et al. (54): A = *sea*, *sak*, *chp*, and *scn*; B = *sak*, *chp*, and *scn*; C = *chp* and *scn*; D = *sea*, *sak*, and *scn*; E = *sak* and *scn*; F = *sep*, *sak*, *chp*, and *scn*; G = *sep*, *sak*, and *scn*; NT, novel IEC type consisting of *sak* and *sea* only; Neg (negative), no IEC genes detected.

[c] *seg* and *sei* were not detected in one and two ST5-MRSA-II isolates, respectively, found to carry the enterotoxin gene cluster (*egc*) genes *seg*/*sei*, *sem*, *sen*, *seo*, and *seu*.

gene *sea* and the genes encoding staphylokinase (*sak*) and the staphylococcal complement inhibitor (*scn*). Other IEC types represented included IEC types A ($n = 8$ isolates), B ($n = 28$ isolates), E ($n = 5$ isolates), F ($n = 12$ isolates), and G ($n = 1$ isolate).

The main virulence-associated genes identified among the MRSA isolates representative of the different STs and CCs are shown in Table 2. Ninety-four percent of isolates carried one or more superantigen genes with enterotoxin A (*sea*) (48%; 51/107) predominating, followed by *egc* (37%; 40/107), *tst* (21%; 22/107), and *sek* and *seq* (19%; 20/107). All isolates lacked the PVL locus genes *lukF-PV* and *lukS-PV*; the exfoliative toxin genes *etA*, *etB*, and *etD*; and ACME. All isolates harbored the MSCRAMM, adhesion, and biofilm genes *fnbA* (fibronectin binding protein A), *clfA* and *clfB* (clumping factor A and B), *ebpS* (elastin binding protein), *eno* (laminin binding protein), *icaA* (intracellular adhesion protein A), *icaD* (intracellular adhesion protein A), and *vwb* (van Willebrand factor binding protein). The majority of isolates also harbored *fib* (fibrinogen binding protein) (98%; 105/107) and *map* (major histocompatibility compound class II analog protein) (97%; 104/107).

The most common antimicrobial resistance genes (apart from *mecA*, which was detected in all isolates tested) were *blaZ* (beta-lactamase resistance gene) (97%; 104/107), *sdrM* (encoding a multidrug efflux pump conferring resistance to norofloxacin, acriflavine, and possibly ethidium bromide [57]) (87%; 93/107), *fosB* (fosfomycin resistance gene) (87%; 93/107), *erm*(A) (resistance to macrolides, lincosamides, and streptogramins) (79%; 85/107), *merA* and *merB* (mercury resistance genes) (59%; 63/107),

qacA (resistance to quaternary ammonium compounds, ethidium bromide) (52%; 56/107), and *aacA-aphD* (resistance to amikacin, gentamicin, kanamycin, and tobramycin) (53%; 57/107). CC8 isolates harbored the highest number of resistance determinants, with ST8-MRSA-IIA-IIE and ST239-MRSA-III carrying genes encoding resistance to 10 classes of antimicrobials, whereas ST22-MRSA-IV carried resistance to three classes of antimicrobials.

Correlation between antimicrobial resistance phenotype and genotype. The correlation between antimicrobial agent resistance phenotype (determined by disk diffusion) and genotype for the 107 MRSA isolates (determined by DNA microarray profiling) are shown in Table 3 for antimicrobial resistance genes yielding positive results only, i.e., all isolates yielded negative results for the genes encoding resistance to antimicrobials in the following antimicrobial classes, and results are not included in Table 3: chloramphenicol (*cfr* and *fexA*, encoding chloramphenical and florfenicol resistance); glycopeptides (*vanA*, *vanB*, and *vanZ*, encoding vancomycin and/or teicoplanin resistance); macrolides, lincosamides, or streptogramins (MLS) [*lnu*(A), *mef*(A), *mph*(C), *msr*(A), *vat*(A), *vat*(B), *vga*(A), *vgb*(A), encoding resistance to a range of MLS antimicrobial compounds].

Eighty-three of the 107 MRSA isolates exhibited both phenotypic and genotypic resistance to aminoglycosides. The three aminoglycoside resistance genes detected by the DNA microarray are *aacA-aphD*, encoding resistance to amikacin, gentamicin, kanamycin, and tobramycin; *aadD*, encoding resistance to amikacin, kanamycin, neomycin, and tobramycin; and *aphA3*, encoding resistance to kanamycin and neomycin. The level of resistance to amikacin encoded by *aacA-aphD* is low (27) and may be difficult to detect by disk diffusion. Hence, many isolates carrying *aacA-aphD* or *aadD* exhibited resistance in the intermediate category or were phenotypically susceptible to amikacin (Table 3). The relative frequencies of occurrence of the various aminoglycoside resistance gene combinations was as follows: *aacA-aphD* and *aphA3*, 31% (26/83); *aadD*, 24% (20/83); *aacA-aphD*, 17% (14/83); *aacA-aphD*, *aadD*, and *aphA3*, 16% (13/83); and *aphA3*, 7% (6/83). The phenotypic resistance profiles for each isolate harboring these gene combinations are shown in Table 3. In general (aside from the problem of amikacin resistance with isolates carrying *aacA-aphD* and/or *aadD*), correlation between phenotypic and genotypic expression of resistance was excellent (99%), with just one isolate carrying *aacA-aphD*, *aadD*, and *aphA3* exhibiting phenotypic susceptibility to gentamicin (Table 3).

For all other antimicrobials tested, apart from fusidic acid and trimethoprim, the correlation between phenotype and genotype was ≥97%. A 100% correlation between the antimicrobial resistance phenotype and the presence of a particular gene or genes was observed for high-level mupirocin resistance and *mupA*, ethidium bromide resistance and *qacA* and/or *qacC*, erythromycin resistance and *erm*(A) and/or *erm*(C), and chloramphenicol resistance and *cat* (Table 3). All beta-lactam-resistant isolates harbored *mecA*, while 97% (104/107) also harbored the beta-lactam resistance gene *blaZ*. One isolate that lacked *blaZ* exhibited intermediate resistance to ampicillin. All isolates harboring *tet*(K), *tet*(M), or both of these genes exhibited tetracycline resistance. Two isolates exhibited intermediate resistance to tetracycline, lacked *tet*(K) and *tet*(M) in their DNA microarray profile, and tested negative for *tet*(L) and *tet*(O) when investigated using conventional PCR assays. The mercury resistance genes *merA* and *merB* were detected in all but one isolate exhibiting resistance to mercuric chloride, but one isolate exhibiting susceptibility to mercuric chloride harbored *merA* and *merB*.

There were major discrepancies with fusidic acid and trimethoprim resistance. Only 29% of fusidic acid-resistant isolates (6/21) and 35% of trimethoprim-resistant isolates (9/26) were DNA microarray gene positive for genes encoding resistance to these agents (Table 3).

The fusidic acid resistance genes *far1/fusB* were detected in one ST45-MRSA-IV and one ST8-MRSA-IIB isolate, while the Q6GD50/*fusC* gene was detected in four ST30-MRSA-IV isolates. None of the remaining 15 fusidic acid-resistant isolates were found to harbor the alternative fusidic acid resistance gene *fusD* by *fusD*-specific PCRs (*fusD* is not detected by the DNA microarray). The *fusA* gene in each of these 15 fusidic acid-resistant isolates was amplified by PCR followed by amplimer sequencing, as mutations in *fusA* have previously been shown to be associated with fusidic acid resistance in *S. aureus*. Mutations in *fusA* were identified in all 15 isolates. Three different combinations of mutations were identified in these isolates, including (i) an amino acid substitution from leucine to lysine at position 461 in the protein sequence (L461K) in eight isolates (seven CC8 isolates: one ST8-MRSA-IID, one ST250-MRSA-I-*pls*, and five ST250-MRSA-I/I-*pls* isolates; one ST22-MRSA-IVh isolate), (ii) an amino acid substitution from leucine to serine at position 461 in the protein sequence (L461S) in five isolates (one ST5-MRSA-II, one ST239-MRSA-III, and three ST250-MRSA-I-*pls* isolates), and (iii) an amino acid substitution from alanine to valine and phenylalanine to leucine at positions 70 (A70V) and 406 (F406K), respectively, in the protein sequence of two isolates (two ST8-MRSA-IIC isolates).

Twenty-six isolates exhibited phenotypic resistance to trimethoprim, and only 35% (9/26) of these harbored the *dfrS1* gene, as determined by DNA microarray profiling (all were CC8 isolates as follows: ST609-MRSA-IVA [$n = 1$], ST8-MRSA-IIE [$n = 1$], ST239-MRSA-III [$n = 3$], and ST250-MRSA-I/I-*pls* [$n = 4$]). The *dfrS1* gene is the only trimethoprim resistance gene detected by the microarray. The remaining 17 trimethoprim-resistant isolates were investigated by a variety of specific PCR assays designed to detect other genes encoding trimethoprim resistance, including *dfrD*, *dfrG*, and *dfrK*. None of the 17 trimethoprim-resistant isolates harbored *dfrD* or *dfrK*, whereas three isolates harbored *dfrG* (all ST239-MRSA-III). The 14 isolates in which no *dfr* genes were identified belonged to ST8-MRSA-IIC ($n = 4$), ST8-MRSA-IIE ($n = 2$), ST8-MRSA-IVE ($n = 2$), ST8-MRSA-IVF ($n = 3$), ST5-MRSA-IV ($n = 1$), and ST239-MRSA-III ($n = 2$).

DISCUSSION

The application of *spa* typing and DNA microarray profiling for in-depth molecular characterization of a diverse collection of nosocomial MRSA isolates representative of clones that predominated in Irish hospitals between 1971 and 2004 revealed extensive genetic diversity among the 175 isolates investigated. A total of 35 *spa* types were identified among these 175 isolates that had previously been assigned to 13 STs representing six CCs by MLST and to 26 ST-SCC*mec* type combinations representative of pandemic and epidemic MRSA lineages by MLST and conventional SCC*mec* typing. Almost half of all *spa* types (46%; 16/35) were identified among isolates of the ST22-MRSA-IV clonal lineage, which represented only 15% of isolates investigated (27/175). In contrast, within the majority of other clonal lineages, isolates were assigned to one or two *spa* types only. In particular, the majority of ST8

TABLE 3 Correlation between antimicrobial resistance gene detection by array profiling and phenotypic detection of resistance by disk diffusion[f]

Gene(s)	Conferring resistance to:	No. of isolates with gene profile	Antimicrobial(s) tested (resistance profile obtained)[h]	No. of isolates resistant	Correlation between genotype and phenotype	
					No. of discrepancies	% correlation[g]
Aminoglycoside resistance genes			AMI, GEN, KAN, NEO, TOB	83	1	99 (106/107)
aacA-aphD	AMI, GEN, KAN, TOB	14	AMI, GEN, KAN, ..., TOB	5	0	
			....., GEN, KAN, ..., TOB	9[a]	0[a]	
aadD	AMI, KAN, NEO, TOB	20	AMI, ..., KAN, NEO, TOB	18	0	
			....., ..., KAN, NEO, TOB	2[a]	0[a]	
aphA3	KAN, NEO	6	....., ..., KAN, NEO, ...	6	0	
aacA-aphD and *aadD* and *aphA3*	AMI, GEN, KAN, NEO, TOB	13	AMI, GEN, KAN, NEO, TOB	12	0	
			....., ..., KAN, NEO, TOB	1	1	
aacA-aphD and *aphA3*	AMI, GEN, KAN, NEO, TOB	26	AMI, GEN, KAN, NEO, TOB	11	0	
			....., GEN, KAN, NEO, TOB	15[a]	0[a]	
aacA-aphD and *aadD*	AMI, GEN, KAN, NEO, TOB	4	AMI, GEN, KAN, NEO, TOB	4	0	
None		24	Susceptible to all (n = 24)	0	0	
Antiseptics, disinfectants, intercalating dye resistance genes			Ethidium bromide	58	0	100 (107/107)
qacA	QACs,[b] ethidium bromide, chlorhexidine	55	Ethidium bromide	55	0	
qacC	QACs,[b] ethidium bromide	2	Ethidium bromide	2	0	
qacA and *qacC*	QACs, ethidium bromide, chlorhexidine	1	Ethidium bromide	1	0	
None		49	Susceptible (n = 49)	0	0	
Beta-lactam (excluding methicillin) resistance gene			Ampicillin	107	3[c]	97 (104/107)
blaZ	Beta-lactams (not methicillin)	104	Ampicillin	104	0	
None		3	Susceptible (n = 0)	3[c]	3[c]	
Chloramphenicol resistance gene			Chloramphenicol	1	0	100 (107/107)
cat-pC194 (pMC524)	Chloramphenicol	1	Chloramphenicol	1	0	
None		106	Susceptible (n = 106)	0	0	
Fusidic acid resistance genes			Fusidic acid	21	15	86 (92/107)
farI (fusB)	Fusidic acid	2	Fusidic acid	2	0	
Q6GD50 *(fusC)*	Fusidic acid	4	Fusidic acid	4	0	
None		101	Susceptible (n = 86)	15	15	
Heavy metal ions resistance genes			MER, PMA	63	2	98 (105/107)
merA	Mercury ions	0	Mercuric chloride	0	0	
merB	Organomercurial ions	0	Phenylmercuric acetate	0	0	
merA and *merB*	Mercury and organomercurial ions	63	MER, PMA	62	1	
None		44	Susceptible (n = 43)	1	1	
Macrolides, lincoamides, streptogramins (MLS) resistance genes			Erythromycin, (lincomycin)	93 (47)[d]	0	100 (107/107)
erm(A)	Erythromycin, Clindamycin	82	Erythromycin (lincomycin)	82 (46)[d]	0	
erm(C)	Erythromycin, Clindamycin	8	Erythromycin (lincomycin)	8 (1)[d]	0	
erm(A) and *erm*(C)		3	Erythromycin (lincomycin)	3 (0)[d]	0	
None		14	Susceptible (n = 14)	0	0	
Mupirocin resistance gene			Mupirocin (high level)	5	0	100 (107/107)
mupA-ileS2	Mupirocin (high level)	5	Mupirocin (high level)	5	0	
None		102	Susceptible (n = 102)	0	0	

(Continued on following page)

TABLE 3 (Continued)

Gene(s)	Conferring resistance to:	No. of isolates with gene profile	Antimicrobial(s) tested (resistance profile obtained)[h]	No. of isolates resistant	Correlation between genotype and phenotype	
					No. of discrepancies	% correlation[g]
Tetracycline resistance genes			Tetracycline	29[e]	2[e]	98 (105/107)[e]
tet(K)	Tetracycline	8	Tetracycline	8	0	
tet(M)	Tetracycline	8	Tetracycline	8	0	
tet(K) and *tet*(M)	Tetracycline	11	Tetracycline	11	0	
None		80	Susceptible (n = 78[e])	2[e]	2[e]	
Trimethoprim resistance gene			Trimethoprim	26	17	84 (90/107)
dfrS1	Trimethoprim	9	Trimethoprim	9	0	
None		98	Susceptible (n = 81)	17	17	

[a] Variations in expression of phenotypic resistance to amikacin are not considered to be discrepant results because the reduction in amikacin susceptibility encoded by *aacA-aphD* may be very slight (27).

[b] QAC, quaternary ammonium compound. *qacA* encodes resistance to QACs, divalent cations and intercalating dyes (such as ethidium bromide); *qacC* encodes resistance to QAC and intercalating dyes.

[c] One of the three ampicillin resistant isolates exhibited intermediate resistance to ampicillin.

[d] Numbers in square brackets indicate numbers of isolates exhibiting resistance to lincomycin.

[e] Two of 29 tetracycline-resistant isolates exhibited intermediate resistance, and no tetracycline resistance genes were detected.

[f] AMI, amikacin; GEN, gentamicin; KAN, kanamycin; MER, mercuric chloride; NEO, neomycin; PMA, phenylmercuric acetate; TOB, tobramycin.

[g] The numbers in parentheses refer to the total number of isolates for which the antimicrobial phenotype correlated with the genotype as determined by array profiling over the total number of isolates investigated.

[h] ....., no phenotypic resistance detected to the relevant aminoglycoside antimicrobial.

isolates (42% of total isolates investigated) were assigned to a single *spa* type, suggesting that the *spa* types of ST8 isolates may be more stable over time than those among ST22-MRSA-IV isolates. In support of this suggestion, Strommenger et al. previously reported that isolates within the different CC8 clones recovered at different time periods and from different geographic locations also exhibited the same *spa* types (51). A possible explanation for the greater variation in *spa* types among ST22-MRSA-IV isolates may lie in the higher number of repeat sequences in the VNTR region of the *spa* gene in this clone. The average number of repeats in the 16 *spa* types identified among ST22-MRSA-IV isolates in the present study was 13 (range, 4 to 17), with the most frequently occurring *spa* type (t032) carrying 16 repeats. In contrast, the *spa* type t190, to which all the ST8-MRSA-IIA-IIE and ST8-MRSA-IVE/IVF isolates were assigned, consists of only seven repeats.

DNA microarray profiling was found to be effective for genotyping MRSA isolates by assigning them to the correct CCs and/or STs and SCC*mec* types, indicating that this approach has the potential to replace MLST and SCC*mec* typing for genotyping and for investigating the evolutionary relatedness of MRSA. The advantages of using the DNA microarray for MLST and SCC*mec* typing include the fact that the DNA microarray also detects clinical relevant virulence and antimicrobial resistance genes and requires only a single multiplex PCR and a 2- to 3-h test procedure per isolate to generate data that require multiple multiplex PCRs and agarose gel electrophoresis to assign an SCC*mec* type/subtype and at least seven PCRs and subsequent sequencing to assign an ST by conventional methods. In a hospital setting, and particularly in an outbreak investigation, greater discrimination than that provided by ST and SCC*mec* type assignment is required to effectively type and track MRSA. While each clone included in the present study was found to have a characteristic combination of virulence and antimicrobial resistance genes, there were also differences observed in the carriage of these genes within the clones, e.g., the carriages of *sec*, *sel*, *erm*(C), and IEC were variable in ST22-MRSA-IV. Whether these differences could be used to provide enhanced discrimination and tracking of MRSA in hospitals either using the array as a standalone typing tool or in combination with other methods, such as *spa* typing, requires further investigation.

It is important to note that the array had difficulty differentiating between some sporadically occurring STs that are single-locus variants of more common STs and with subtyping of some SCC*mec* elements. While identification of some SCC*mec* I, II, and IV subtypes was possible following manual inspection of array profiles, in some cases this identification relied on the detection of the presence of a particular resistance determinant associated with a particular SCC*mec* type, such as *aadD* located on plasmid pUB110 in SCC*mec* types IA, IIA, IIB, IIC, and IVA. However, while this may aid in the identification of a particular SCC*mec* subtype, it is not possible to definitively localize this or any of the other possibly SCC*mec*-associated resistance determinants, i.e., *erm*(A), *tet*(K), and *mer*, which can be located on Tn*554* in SCC*mec* II and III, pT181 in SCC*mec* III, and p1258 in SCC*mec* III with SCCHg, respectively, to within the SCC*mec* element using the array, and results need to be confirmed by PCR. Array profiling facilitated the identification of possible DNA sequence variation in *mecR1* in some SCC*mec* III elements and in the *dcs* gene in some SCC*mec* II and IV elements that were not detected using conventional SCC*mec* typing PCR assays. In addition, the fusidic acid resistance determinant Q6GD50/*fusC* was detected in four ST30-MRSA-IV isolates using the array, indicating the possible presence of an SCC*fus* element in these isolates. Array profiling also indicated that five isolates harboring SCC*mec* IV may have lost *ccrAB2*, which was subsequently confirmed by PCR. Four of these isolates were assigned to ST8-MRSA-IVE/F, which also harbored a second set of *ccr* genes (*ccrAB4*) outside SCC*mec*, suggesting that these isolates may have lost *ccrAB2* genes because they were not essential for excision or integration of the SCC*mec* element due to the presence of alternative *ccr* genes. The ability to accurately identify SCC*mec* types is essential to the use of this

method for genotyping MRSA, and isolates such as these that may have lost *ccr* genes may be wrongly classified using SCC*mec* typing because of the presence of the second set of *ccr* genes outside the SCC*mec* element.

Several novel SCC*mec*-SCC-CIs were identified in the present study, each consisting of an SCC*mec* II or IV element (IIA-IIE, IVE, IVF, or IVg) and SCC_{M1} harboring *ccrAB4*. In *S. aureus*, the DNA sequence corresponding to the SCC_{M1} element has been identified only in MRSA belonging to CC8 and mainly to ST8 (2, 8, 58). CC8 is one of the largest MRSA lineages, consisting of a substantial number of MRSA clones with extensive diversity in SCC*mec* (14, 34, 45), and evidence suggests that the diversity in the SCC*mec* elements of MRSA isolates is due to horizontal transfer and recombination events between SCC*mec* and SCC elements from *S. aureus* isolates and CoNS. DNA sequence identity between SCC_{M1} in ST8-MRSA and the SCC-CI in *S. epidermidis* ATCC 12228 led Bartels et al. to speculate that the former may have evolved from the latter following recombination events between SCC*mec* and SCC-CI elements in MRSA and *S. epidermidis* (2). We previously reported that DNA sequence analysis of the SCC*mec* elements identified in ST8-MRSA isolates recovered in Ireland revealed the presence of SCC*mec* elements that may have been generated following recombination and genetic rearrangements between different SCC*mec* elements (45). The finding of the additional SCC_{M1} element in these ST8 isolates in the present study suggests that they have acquired the SCC_{M1} element either from another *S. aureus* or from CoNS. Only 8% (6/75) of the ST8/t190 or t2196 and ST94/t4691 isolates investigated in the present study did not carry the *ccrAB4* element, and while they may have lost SCC_{M1}, it is also possible that they never harbored it. Thus, these ST8 isolates carrying the SCC*mec* region only may be precursors of isolates harboring the SCC*mec*-SCC_{M1} elements.

Whether the acquisition of the SCC_{M1} element within the ST8 isolates occurred on one or multiple occasions remains to be determined. However, the presence of SCC_{M1} in association with three distinct groups of SCC*mec* elements (SCC*mec* IIA-IIE, SCC*mec* IVE/IVF, and SCC*mec* IVg) suggests at least three possible independent acquisitions of SCC_{M1}. Interestingly, the ST8-MRSA-IIA–IIE isolates which harbored the SCC*mec*-SCC_{M1} element predominated in Irish hospitals during the 1990s but since 2002 have been recovered only sporadically. This may be due in part to a fitness cost associated with harboring such a large genetic element. While the presence of an SCC element in addition to an SCC*mec* element in MRSA can complicate SCC*mec* typing, the presence of SCC elements, such as SCC_{M1}, in MSSA could also compromise the accuracy of rapid MRSA detection assays, such as the GeneXpert real-time PCR assay (49).

DNA microarray profiling allowed the detection of characteristic combinations of antimicrobial resistance and virulence genes among major MRSA clones. Only nine percent of isolates investigated lacked the bacteriophage-encoded IEC genes, and half of these belonged to ST22-MRSA-IV. Ninety-four percent of isolates carried one or more superantigen gene. The ST22-MRSA-IV isolates were the only isolates found to harbor *sec* and *sel*, which have been reported previously to be colocated on a pathogenicity island (32). The toxic shock toxin *tst* gene, which has also been found to be located on various pathogenicity islands (32), was identified in CC30 and CC5 isolates only. The enterotoxin genes *sek* and *seq* were identified only in the CC8 lineages ST239 and ST250 and have been reported to be colocated on various pathogenicity islands and bacteriophages, while *seb*, which has also been found to be located either on a pathogenicity island with *sek* and *seq* or on a plasmid, was also detected in ST250 isolates (32). Isolates belonging to ST22-MRSA-IV harbored the fewest antimicrobial resistance genes, with approximately half of the isolates harboring *blaZ* only. In contrast, isolates belonging to CC8 lineages (ST8-MRSA-IIA-E and ST239-MRSA-III/III-pI258/Tn*554*) harbored the greatest number of antimicrobial resistance genes (up to 17 in each case).

The present study revealed that, in most cases, the presence of a particular antimicrobial resistance gene correlated with phenotypic resistance to a specific type of antimicrobial agent(s), but absence of the gene was not always indicative of susceptibility. The latter was due in most cases either to the absence of additional genes encoding a particular resistance phenotype on the array (e.g., some trimethoprim-resistant isolates did not harbor the trimethoprim resistance gene *dfrS1* that can be detected using the DNA microarray) or to the fact that resistance was due to the presence of mutations in a specific gene which the DNA microarray does not detect (e.g., *fusA* gene mutations resulting in fusidic acid resistance). This highlights the fact that array profiling cannot replace antimicrobial susceptibility testing but can be used in conjunction with it to help identify the mechanism of resistance. The inclusion of alternative antimicrobial resistance genes on the array, such as additional tetracycline [*tet*(L) and *tet*(O)] and trimethoprim (*dfrG* and *dfrK*) resistance genes, would enhance the ability of the array to predict a resistance phenotype.

The presence of an aminoglycoside resistance gene(s) detected by array profiling could be used to accurately predict an isolate's phenotypic aminoglycoside resistance pattern. However, the phenotypic expression of aminoglycoside resistance could not always be used to infer which aminoglycoside resistance gene(s) was present. This was partly because of the fact that different aminoglycoside resistance genes encode resistance to the same antimicrobial agents and also because approximately half of the aminoglycoside-resistant isolates (52%; 43/83) carried two or more aminoglycoside resistance genes. For example, all isolates found to harbor a single aminoglycoside resistance gene exhibited resistance to the expected combinations of aminoglycosides, and all isolates exhibiting these phenotypes harbored the corresponding resistance genes. However, for isolates harboring two or three aminoglycoside resistance genes, the phenotype could be predicted from the aminoglycoside resistance genes detected, but the presence of a gene could not be reliably inferred from the phenotype. An advantage of the use of the array was confirmation of reduced susceptibility to amikacin in isolates carrying the *aacA-aphD* gene (27). This gene encodes low-level amikacin resistance that may not be detected using the standard 30-μg susceptibility testing disk. Among 14 isolates carrying *aacA-aphD* only, nine showed reduced amikacin susceptibility; the remainder were phenotypically susceptible. Inclusion on the array of genes encoding resistance to the aminocyclitol spectinomycin, including *spc* (carried on Tn*554*) and *aadA*, would be helpful to further differentiate among this MRSA population (31).

In conclusion, the present study showed that DNA microarray profiling can accurately assign isolates representative of major pandemic MRSA clones to the correct MLST and SCC*mec* types. Microarray profiling allows rapid, high-throughput genotyping and detection of clinically relevant staphylococcal virulence and antimicrobial resistance genes, and can, in most cases, be used to

predict the antimicrobial agent resistance phenotype of an isolate. The inclusion of additional antimicrobial resistance genes, including the recently described novel *mecA* gene (46), in future updated versions of the array would further enhance the usefulness of this system. The present study also provided further evidence of the diversity of SCC*mec* and SCC elements and the need for complete nucleotide sequencing to determine the genetic organization of these complex SCC*mec* elements and composite islands.

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Identification and Characterization of the Multidrug Resistance Gene *cfr* in a Panton-Valentine Leukocidin-Positive Sequence Type 8 Methicillin-Resistant *Staphylococcus aureus* IVa (USA300) Isolate▽

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The staphylococcal *cfr* gene mediates resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, a phenotype that has been termed PhLOPS$_A$. The *cfr* gene has mainly been associated with coagulase-negative staphylococcal isolates from animals, and only a few *cfr*-positive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates have been described so far. This study reports the first description of a *cfr*-positive MRSA isolate (M05/0060) belonging to the pandemic Panton-Valentine leukocidin (PVL)-positive sequence type 8 MRSA IVa/USA300 (ST8-MRSA-IVa/USA300) clone. The *cfr* gene was detected in M05/0060 using a DNA microarray which was used to screen PVL-positive MRSA isolates for the presence of virulence genes, typing markers, and antimicrobial resistance genes. Antimicrobial susceptibility testing revealed that M05/0060 exhibited the *cfr*-associated resistance phenotype. Molecular analysis identified the presence of *cfr* and a second phenicol resistance gene, *fexA*, on a novel 45-kb conjugative plasmid, which was designated pSCFS7. Within pSCFS7, a DNA segment consisting of *cfr*, a truncated copy of insertion sequence IS*21-558*, and a region with homology to the DNA invertase gene *bin3* of transposon Tn*552* from *Bacillus mycoides* was integrated into the transposase gene *tnpB* of the *fexA*-carrying transposon Tn*558*. The emergence of a multidrug-resistant *cfr*-positive variant of ST8-MRSA-IVa/USA300 is alarming and requires ongoing surveillance. Moreover, the identification of a novel conjugative plasmid carrying the *cfr* gene indicates the ability of *cfr* to spread to other MRSA strains.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen and a significant cause of hospital-acquired infection and, more recently, community-acquired infection worldwide. Globally, different MRSA strains predominate in different geographical locations. In Ireland, a diverse range of MRSA strains have prevailed at different time periods (40), but for the last decade sequence type 22 MRSA IV (ST22-MRSA-IV) isolates have dominated in Irish hospitals (41), while ST30-MRSA-IVc and ST8-MRSA-IVa have predominated among community-acquired MRSA (CA-MRSA) isolates (37). Since ST8-MRSA-IVa, also known as USA300, was first reported in 2000 as a cause of skin and soft tissue infections in the United States, it has become the prevalent CA-MRSA strain in the United States, and evidence suggests that it is also emerging as a major nosocomial MRSA strain in the United States (6, 43). Originally, ST8-MRSA-IVa isolates harbored only the methicillin (*mecA*) and erythromycin [*msr*(A)] resistance genes, but they have since spread worldwide while also acquiring additional resistance determinants, including *erm*(A) and *erm*(C), *tet*(M) and *tet*(K), and *mupA*, which encode resistance to macrolides-lincosamides-streptogramin B (MLS$_B$), tetracyclines, and mupirocin, respectively; most of these resistance determinants are located on plasmids (8, 12, 27, 43, 44). In addition, resistance to fluoroquinolones (levofloxacin), gentamicin, and trimethoprim-sulfamethoxazole and reduced susceptibility to vancomycin and daptomycin have also been observed among some ST8-MRSA-IVa/USA300 isolates (10, 11, 32, 43).

Mobile genetic elements, including plasmids, bacteriophages, pathogenicity islands, transposons, and chromosomal cassettes, play a vital role in the dissemination of virulence and antimicrobial resistance genes in *S. aureus* (3, 14, 24, 34–36, 47). The *cfr* gene can be plasmid or chromosomally located and encodes resistance to five classes of antimicrobial agents, i.e., phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, a phenotype that has been termed PhLOPS$_A$ (23). Each of these antimicrobial classes contains antimicrobial agents that are used in the treatment of staphylococcal infections in either human or veterinary medicine, including chloramphenicol, florfenicol, clindamycin, lincomycin, pirlimycin, linezolid, retapamulin, tiamulin, valnemulin, and quinupristin-dalfopristin (23). The gene *cfr* encodes an rRNA methyltransferase that methylates the adenosine at position 2503 in 23S

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TABLE 1. Novel PCR primers used in the study

Gene or region amplified	Primer	Nucleotide sequence (5′ → 3′)	Nucleotide coordinates
cfr	cfrF1	GACTTTCGGCACCGGTAAT	3419–3437[a]
	cfrR3	CAGTAGTCCATTCATATTTG	4681–4662[a]
fexA	fexAF2	GCGTGATTCCTAAGCTATT	7146–7164[a]
	fexAR1	GAGAACCGAATCTTTAATCA	8800–8781[a]
radC	radC-fw01	AGAGAAAATGCCAATGCAATCA	1736892–173687[b]
	radC-rv02	TCACCGATTATAATATGATCCAATAAATCT	1736773–1736744[b]

[a] Nucleotide coordinates based on the nucleotide sequence of plasmid pSCFS3 (GenBank accession number AM086211).
[b] Nucleotide coordinates based on the nucleotide sequence of MRSA strain COL (GenBank accession number CP000046.1).

rRNA (*Escherichia coli* 23S rRNA gene numbering) within the overlapping binding sites for these antimicrobial agents (20).

The *cfr* gene was originally identified in coagulase-negative staphylococci (CoNS) from animals (18, 39) but more recently has also been found in a very limited number of *Staphylococcus aureus* isolates and CoNS from humans (25, 26, 31, 45) and animals (16, 18). It is usually plasmid borne, and three plasmids, pSCFS1, pSCFS3, and pSCFS6 (15, 17, 18), have been characterized in detail. Plasmid pSCFS1 (17.1 kb) also harbors additional antimicrobial resistance genes, including *erm*(33), encoding MLS_B resistance, or *lsa*(B), encoding reduced susceptibility to lincosamides (17). The larger plasmids pSCFS3 (36 kb) and pSCFS6 (43 kb) also carry the Tn*558*-associated phenicol resistance gene *fexA*. In pSCFS3 and pSCFS6, *cfr* is integrated at different positions within the transposase genes *tnpA-tnpB* or *tnpC* of Tn*558* (15, 18). In some staphylococcal isolates, *cfr* is located in the chromosomal DNA (15, 45). Recently, *cfr* has also been reported for the first time in a non-staphylococcal strain, *Bacillus* sp. strain BS-01 from swine feces in China (7). The *cfr* gene was detected on a novel 16.5-kb plasmid, pBS-01, in this *Bacillus* strain, along with transposon Tn*917* harboring the MLS_B resistance gene *erm*(B) (7).

Here we report the first case of human clinical infection with an MRSA isolate belonging to the pandemic Panton-Valentine leukocidin (PVL)-positive ST8-MRSA-IVa/USA300 strain harboring the multidrug resistance gene *cfr*. The aim of the present study was to comprehensively characterize the *cfr*-positive MRSA isolate and the genetic environment of the *cfr* gene.

MATERIALS AND METHODS

Bacterial isolates. A previously described collection (37) of Panton-Valentine leukocidin (PVL)-positive MRSA isolates, recovered in Ireland between 1999 and 2005, was characterized using DNA microarrays (see below). One isolate (M05/0060) was found to harbor the multidrug resistance gene *cfr* and was characterized in detail in the present study. This isolate was originally recovered from a 26-year-old Irish male with a superficial scalp abscess (37).

Two ST8-MRSA-IVa/USA300 reference strains, for which full genome sequences are available (USA300-FPR3757 [GenBank accession no. CP000255] and USA300-TCH1516 [GenBank accession no. CP000730]), were included for comparison of DNA microarray hybridization profiles. The plasmid-free novobiocin-resistant *S. aureus* strain XU21 was used as a plasmid recipient in filter mating experiments (48).

Antimicrobial susceptibility testing. All PVL-positive MRSA isolates previously underwent antibiogram-resistogram (AR) typing using disk diffusion (37). Comparative antimicrobial susceptibility testing of M05/0060, its plasmid-cured derivative M05/0060-C1, the plasmid-free *S. aureus* recipient strain XU21, and its transconjugant derivative XU21-T1 (see below) was performed by broth microdilution as described in CLSI documents M07-A8 (4) and M100-S20 (5). In addition, further susceptibility tests on M05/0060 and the transconjugant derivative XU21-T1 were performed using Vitek-2 (AST P580 panel, susceptibility tests for Gram-positive bacteria; bioMérieux, Nürtingen, Germany) and Etest (chloramphenicol, clindamycin, linezolid, and quinopristin-dalfopristin; bioMérieux) according to the manufacturer's protocols.

DNA microarray analysis. The StaphyType kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis of isolates, reference strains, and derivatives. The StaphyType kit consists of individual DNA microarrays mounted in eight-well microtiter strips which detect 334 *S. aureus* gene sequences, including species-specific, antimicrobial resistance, and virulence-associated genes, as well as typing markers (29, 30). Genomic DNA for use with the DNA microarray was extracted from all isolates by enzymatic lysis and using the Qiagen DNeasy kit (Qiagen, Crawley, West Sussex, United Kingdom) as described previously (29). The DNA microarray procedures were performed according to the manufacturer's instructions and have been described previously in detail (28, 29).

Molecular typing. Pulsed-field gel electrophoresis (PFGE); *spa*, staphylococcal cassette chromosome *mec* (SCC*mec*), and toxin gene typing; and multilocus sequence typing (MLST) analysis of the PVL-positive MRSA isolates were performed as described previously (37, 42). The *cfr*-positive MRSA isolate M05/0060 also underwent direct repeat unit (*dru*) typing, which was performed as described previously (41).

Plasmid analysis. Plasmid curing was performed by culturing M05/0060 in brain heart infusion (BHI) (Oxoid Ltd., Hampshire, United Kingdom) broth at 43°C for 24 h at 200 rpm. This was followed by subculture into fresh BHI broth and incubation as before for five consecutive rounds, after which individual colonies obtained following plating on BHI agar were screened for loss of resistance to chloramphenicol (30 μg/ml) by replica plating. Conjugative transfer of *cfr* to the plasmid-free, novobiocin-resistant *S. aureus* recipient strain XU21 was conducted by filter mating (49). Putative transconjugants were plated onto BHI agar containing 100 μg/ml novobiocin to select for the recipient strain and 30 μg/ml chloramphenicol to select for a putative *cfr*-carrying plasmid. Putative cured derivatives and transconjugants underwent DNA microarray analysis and comparative antimicrobial susceptibility testing as described above. Plasmid DNAs from M05/0060, its plasmid-cured derivative M05/0060-C1, and a transconjugant derivative of XU21 (XU21-T1) were extracted and purified using a modified alkaline lysis method (38). The size of the *cfr*-carrying plasmid detected in the transconjugant derivative XU21-T1 was determined based on the sum of the fragment sizes obtained following digestion of plasmid DNA with the restriction endonuclease BglII (Roche Diagnostics GmbH, Mannheim, Germany).

Molecular characterization of the novel *cfr*- and *fexA*-carrying plasmid pSCFS7. The *cfr* and *fexA* genes of M05/0060 were amplified and sequenced using primers cfrF1/cfrR3 and fexAF2/fexAR1, respectively (Table 1), using GoTaq DNA polymerase (Promega, Madison, Wisconsin) according to the manufacturer's instructions and the following conditions: 94°C for 2 min; 34 cycles of 95°C for 30 s, 50°C for 2 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were purified using the Genelute PCR cleanup kit (Sigma-Aldrich Chemical Co. Tallaght, Dublin, Ireland). DNA sequencing of amplimers was performed commercially by Geneservice (Source Bioscience, St. James's Hospital, Dublin, Ireland).

To investigate whether *cfr* was associated with the *fexA*-carrying transposon Tn*558* in the *cfr*-carrying plasmid pSCFS7 identified in M05/0060, plasmid DNAs from M05/0060 and the transconjugant derivative XU21-T1 harboring pSCFS7 were subjected to PCR analysis for the different regions of Tn*558*. The transposase genes *tnpA*, *tnpB*, and *tnpC* and the region from *tnpB-fexA* were amplified from M05/0060 and XU21-T1 using previously described primers and conditions (18). Since the *tnpB* amplicon obtained was larger than expected for Tn*558* in

both strains. PCRs were also performed using the gel-purified *tnpB* amplification product obtained with template DNAs from both organisms using previously described *cfr*-specific primers (18). Gel purification was performed using the QiaexII PCR cleanup kit (Qiagen). The resulting amplicon from M05/0060 was cloned as described previously (18) and sequenced by primer walking (MWG, Ebersberg, Germany).

Primers radC-fw01 and radC-rv02 (Table 1) were designed and used to amplify the *radC* gene covering the potential chromosomally located *cfr* integration site in M05/0060, using *Taq* DNA polymerase and the following conditions: 95°C for 2 min; 35 cycles of 95°C for 30 s, 64°C for 20 s, and 72°C for 2 min; and a final extension at 72°C for 5 min.

Nucleotide sequence accession numbers. The nucleotide sequences of *cfr*, *fexA*, and the 4,043-bp *tnpB* amplicon of M05/0060 have been deposited in GenBank under accession numbers FN995111, FN995110, and FR675942, respectively.

RESULTS

Phenotypic and genotypic characteristics of M05/0060. M05/0060 exhibited the same MLST (ST8) and *spa* (t008), *dru* (dt9g), and SCC*mec* (IVa) types as the ST8-MRSA-IVa/USA300 clone (44). In the present study, DNA microarray analysis of M05/0060 and comparison with DNA microarray data for two whole-genome-sequenced ST8-MRSA-IVa/USA300 reference strains (FPR3757 and TCH1516) (9, 13), revealed that M05/0060 was indistinguishable from the ST8-MRSA-IVa/USA300 strain TCH1516, apart from carriage of *cfr* and *fexA* (Table 2). Antimicrobial susceptibility testing revealed that M05/0060 exhibited the *cfr*-associated resistance phenotype ($PhLOPS_A$), including resistance to chloramphenicol, florfenicol, clindamycin, linezolid, tiamulin, virginamycin M_1, and quinupristin-dalfopristin (Table 3).

Confirmation of the presence of *cfr* and *fexA*. The presence of *cfr* and *fexA* in M05/0060 was confirmed by PCR and sequencing of the entire *cfr* and *fexA* genes. The *cfr* sequence of M05/0060 exhibited 100% identity with *cfr* of pSCFS1 (GenBank accession number NC_005076.1) (17) and pSCFS6 (AM408573.1) (15) and 99.9% identity with *cfr* of pSCFS3 (AM086211.1) (18) due to a single nucleotide difference at nucleotide position 761 of *cfr* with no resulting amino acid change. The *fexA* sequence of M05/0060 exhibited 100% identity with *fexA* of *Bacillus* sp. strain BS-01 (GU591496.1) and 99.9% identity with *fexA* of pSCFS3 (AM086211.1), *Staphylococcus lentus* (AJ549214.1 and AJ715531.1), *Staphylococcus warneri* (AM408573.1), and *Staphylococcus simulans* (AM086400.1) due to two nucleotide differences at nucleotide positions 391 and 913, which resulted in two amino acid changes, from isoleucine to valine and valine to isoleucine, respectively.

Localization of *cfr* and *fexA* in M05/0060 on the novel plasmid pSCFS7. The *radC* integration site in M05/0060 was examined using primers spanning this integration site, because to date *cfr* has been reported to be colocated with *tnpB* on transposon Tn*558* and this transposon as well as other members of the Tn*554* transposon family are known to integrate specifically into *radC* (19, 33). This can, for instance, be observed in the published genome sequence for *S. aureus* Mu50 (GenBank accession number BA000017) or in other *S. aureus* strains which carry Tn*554* (22). As PCR using primers spanning this integration site yielded a product of the expected length (approximately 150 nucleotides), it was assumed that (i) *cfr* was not located there and (ii) it was not localized on a chromosomally integrated Tn*554*/Tn*558*-like transposon.

Dilution series of unfragmented and RNA-free M05/0060 total cellular DNA were subjected to DNA microarray analysis, and signals for *fexA* and *cfr* were significantly more intense in stepwise-diluted samples than all other species markers, including 23S rRNA. Since the microarray method uses a linear labeling and amplification procedure, the higher signal intensity observed for *fexA* and *cfr* relative to housekeeping genes suggested that M05/0060 harbored multiple, most likely plasmid-borne copies of these genes.

The plasmid location of *cfr* and *fexA* in M05/0060 was confirmed by curing and conjugation experiments. Plasmid profiling identified the presence of a ca. 45-kb plasmid in M05/0060, which was absent in its cured derivative M05/0060-C1 and present in the transconjugant XU21-T1. Comparative susceptibility testing revealed the presence of the *cfr*-associated resistance phenotype in XU21-T1 but its absence in the XU21 parental strain and the plasmid-cured derivative M05/0060-C1 (Table 3). Moreover, microarray analysis confirmed that M05/0060-C1 and XU21-T1 were indistinguishable from M05/0060 and XU21 except for the absence and the presence of *cfr* and *fexA*, respectively (Table 2). Following the purification of plasmid DNA from transconjugant XU21-T1, this ca. 45-kb conjugative plasmid was found to differ in its BglII restriction pattern from the previously described nonconjugative, *cfr*-carrying plasmids pSCFS1, pSCFS3, and pSCFS6 (15, 17, 18), and it was therefore designated novel plasmid pSCSF7.

Molecular characterization of the genetic environment of *cfr* on plasmid pSCFS7. When *cfr* and *fexA* have previously been identified on the same plasmid, the *cfr* gene and its flanking regions were found to be integrated into the transposase genes of the *fexA*-carrying transposon Tn*558* (15, 18). Tn*558*-directed PCRs revealed that while the amplicons for *tnpA*, *tnpC*, and *tnpB-fexA* obtained using M05/0060 and transconjugant XU21-T1 template DNAs corresponded in size to those obtained for a complete Tn*558*, the *tnpB* amplicon obtained with both M05/0060 and XU21-T1 was approximately 2.5 kb larger than expected. PCR detection of *cfr* using this gel-purified larger *tnpB* amplicon obtained from both M05/0060 and the transconjugant XU21-T1 as templates and primers cfr-fw and cfr-rv (18) yielded an amplicon of the expected size for both strains, which suggested that a *cfr*-carrying DNA segment had been integrated into the *tnpB* reading frame in pSCFS7. To confirm this hypothesis, the *tnpB* amplicon from M05/0060 was cloned and sequenced. The entire *tnpB* amplicon of pSCFS7 comprised 4,043 bp and consisted of initial (positions 1 to 859) and terminal (positions 3826 to 4043) *tnpB* segments (Fig. 1). A largely truncated IS*21-558* element was detected (positions 846 to 1131), of which only the 3′ terminus of *istBS* and the downstream region, including the terminal inverted repeat, were present. The region downstream of this IS*21-558* relic (positions 1132 to 3254), including the entire *cfr* gene (positions 1622 to 2671), exhibited 99.9% nucleotide sequence identity to the corresponding *cfr* sequences of pSCFS3 and pSCFS6 (15, 18). The adjacent 187 bp did not reveal significant homology to sequences deposited in the databases but were followed by the 5′ end of a reading frame (positions 3442 to 3825) whose 127-amino-acid product revealed 78% identity and 90% similarity to the transposon Tn*552* DNA-invertase Bin3 from *Bacillus mycoides* Rock3-17 (ACMW01000216) (Fig. 1). Analysis of the boundaries of this integrated DNA segment revealed

TABLE 2. DNA microarray hybridization profiles of the two USA300 reference strains FPR3757 and TCH1516, M05/0060, its cured derivative M05/0060-C1, the *S. aureus* recipient strain XU21, and its transconjugant derivative XU21-T1[a]

Class	Gene(s)	Hybridization result for[b]:					
		USA300-FPR3757	USA300-TCH1516	M05/0060	M05/0060-CI	XU21	XU21-T1
Species markers	*katA*, *coa*, *nuc*, *spa*	POS	POS	POS	POS	POS	POS
agr group	*agr* group I	POS	POS	POS	POS	POS	POS
SCC*mec*-associated markers	*mecA*	POS	POS	POS	POS	NEG	NEG
	Δ*mecR*	POS	POS	POS	POS	NEG	NEG
	mecR1	NEG	NEG	NEG	NEG	NEG	NEG
	ccrA/B-1	NEG	NEG	NEG	NEG	NEG	NEG
	Q9XB68-dcs	POS	POS	POS	POS	NEG	NEG
	ccrA/B-2	POS	POS	POS	POS	NEG	NEG
	kdp-SCC locus	NEG	NEG	NEG	NEG	NEG	NEG
	ccrA/B-3	NEG	NEG	NEG	NEG	NEG	NEG
	ccrC, *ccrA/B-4*	NEG	NEG	NEG	NEG	NEG	NEG
Antimicrobial resistance genes	*blaZ/I/R*	NEG	POS	POS	POS	NEG	NEG
	erm(A), *erm*(B)	NEG	NEG	NEG	NEG	NEG	NEG
	erm(C)	POS	NEG	NEG	NEG	NEG	NEG
	lnu(A)	NEG	NEG	NEG	NEG	NEG	NEG
	msr(A), *mph*(C)	NEG	POS	POS	POS	NEG	NEG
	aacA-aphD	NEG	NEG	NEG	NEG	NEG	NEG
	aadD	NEG	NEG	NEG	NEG	NEG	NEG
	aphA3/sat	NEG	POS	POS	POS	NEG	NEG
	dfrS1	NEG	NEG	NEG	NEG	NEG	NEG
	far1	NEG	NEG	NEG	NEG	NEG	NEG
	mupA	POS	NEG	NEG	NEG	NEG	NEG
	tet(K)	POS	NEG	NEG	NEG	NEG	NEG
	tet(M)	NEG	NEG	NEG	NEG	NEG	NEG
	cat	NEG	NEG	NEG	NEG	NEG	NEG
	cfr	NEG	NEG	POS	NEG	NEG	POS
	fexA	NEG	NEG	POS	NEG	NEG	POS
Virulence-associated genes	*tst1*	NEG	NEG	NEG	NEG	NEG	NEG
	sea, *seb*, *see*, *seh*	NEG	NEG	NEG	NEG	NEG	NEG
	sec/l, *sed/j/r*	NEG	NEG	NEG	NEG	NEG	NEG
	seg/i/m/n/o/u	NEG	NEG	NEG	NEG	NEG	NEG
	sek/q	POS	POS	POS	POS	NEG	NEG
	lukF/S-PV	POS	POS	POS	POS	NEG	NEG
	lukF/S-hlg, *hlgA*	POS	POS	POS	POS	POS	POS
	sak/chp/scn	POS	POS	POS	POS	NEG	NEG
	etA/B/C	NEG	NEG	NEG	NEG	NEG	NEG
	edinA/B/C	NEG	NEG	NEG	NEG	NEG	NEG
	arcA/B/C/D	POS	POS	POS	POS	NEG	NEG
Capsule type	Capsule type 5	POS	POS	POS	POS	POS	POS
	Capsule type 8	NEG	NEG	NEG	NEG	NEG	NEG

[a] The StaphyType kit (Alere Technologies, Jena, Germany) was used for DNA microarray analysis of isolates, reference strains, and derivatives. Full data sets are available upon request.

[b] POS, positive (i.e., yielded a hybridization signal corresponding to the gene indicated); NEG, negative (i.e., failed to yield a hybridization signal corresponding to the gene indicated). FPR3757 and TCH1516 are two USA300 reference strains for which full-genome sequences are available (accession numbers CP000255 and CP000730, respectively).

two potential recombination sites, one of 14 bp between *tnpB* and *istBS* and another of 13 bp between *bin3* and *tnpB* (Fig. 2). These putative recombination sites are assumed to play a role in the integration of this 2,980-bp segment and the concomitant 433-bp deletion of *tnpB*.

DISCUSSION

This is, to our best knowledge, the first report of a human infection with the major pandemic MRSA clone ST8-MRSA-IVa/USA300 harboring the multidrug resistance gene *cfr*. Isolates of this PVL-positive MRSA clone are commonly associated with skin and soft tissue infections but also with life-threatening necrotizing pneumonia. Therapy options for the latter condition are very limited, since glycopeptide antibiotics tend to have poor penetration into lung tissue and reduced susceptibility to vancomycin has been observed among MRSA isolates, including USA300 (10, 11). Daptomycin cannot be used in cases of pneumonia, as it reacts with lung surfactant (2). While rifampin, levofloxacin, and fosfomycin are viable options, resistance to these antimicrobial agents is common and can emerge during therapy. The synergistic effect of sul-

TABLE 3. Antimicrobial resistance profiles of M05/0060 harboring the *cfr*-encoding conjugative plasmid pSCFS7, its cured derivative M05/0060-C1 lacking pSCFS7, *S. aureus* recipient strain XU21, and its transconjugant derivative XU21-T1 harboring pSCFS7

Isolate or derivative	*cfr* and *fexA* carriage	MIC (μg/ml)[a]									
		TIA	VIR M_1	Q-D	LZD	CLI	CHL	FFC	ERY	OXA	VAN
MRSA M05/0060	*cfr*, *fexA*	≥128	32	2	8	≥128	256	256	≥64	≥32	1
MRSA M05/0060-C1		1	1	0.5	1	0.25	8	4	≥64	≥32	1
S. aureus XU21		1	1	0.25	1	0.25	8	4	0.5	0.12	1
S. aureus XU21-T1	*cfr*, *fexA*	≥128	32	2	4	≥128	128	256	0.5	0.12	1

[a] TIA, tiamulin; VIR M_1, virginamycin M_1; Q-D, quinupristin-dalfopristin; LZD, linezolid; CLI, clindamycin; CHL, chloramphenicol; FFC, florfenicol; ERY, erythromycin; OXA, oxacillin; VAN, vancomycin. Resistance to tiamulin, virginamycin M_1, quinupristin-dalfopristin, linezolid, clindamycin, chloramphenicol, and florfenicol is indicative of the $PhLOPS_A$ phenotype.

fonamide and trimethoprim under *in vivo* conditions is in dispute, which limits its use in life-threatening conditions. Ceftobiprole is not yet available in most countries, and for tigecycline, clinical experience is still lacking. Thus, treatment of necrotizing pneumonia and of other serious infections caused by PVL-positive MRSA relies primarily on vancomycin and oxazolidinones, such as linezolid, and resistance to the latter is encoded by *cfr* or can be due to mutations in the domain V region in the 23S rRNA gene (46).

The *cfr* gene identified in the ST8-MRSA-IVa isolate M05/0060 in the present study was located, along with *fexA*, on a novel 45-kb conjugative plasmid designated pSCFS7. There have been two previous reports of plasmids that carry both *cfr* and *fexA* in staphylococci, including pSCFS3 and pSCFS6. Both of these plasmids have been detected only in staphylococcal isolates from animals (15, 16, 18), and in each plasmid *cfr* is integrated into the *fexA*-carrying transposon Tn*558* (15, 18). In pSCFS3 and pSCFS6, the Tn*558* elements are disrupted and in part truncated by the insertion of DNA segments of 4,674 bp and 9,594 bp, respectively, which comprise the *cfr* gene along with one or two copies of the insertion sequence IS*21-558* and, in the case of pSCFS6, the clindamycin resistance gene *lsa*(B) (15, 18). In contrast, in the novel plasmid pSCFS7, the *tnpB* gene of Tn*558* was disrupted and in part deleted by the insertion via recombination of a DNA segment of 2,980 bp carrying *cfr*, a truncated IS*21-558* (upstream of *cfr*), and Δ*bin3* (downstream of *cfr*) (Fig. 1). The *bin3* gene encodes a DNA invertase and has previously been reported only in the transposon Tn*552* from *Bacillus mycoides*. These observations, coupled with the recent report of *cfr* in a *Bacillus* sp. isolate (7), point toward the mobility of *cfr*, its ability to spread to different bacterial species, and the role that recombination processes play in the stable fixation of *cfr* in new vector plasmids.

The *cfr*-positive ST8-MRSA-IVa isolate M05/0060 harboring *cfr* on the novel plasmid pSCFS7 differs significantly from at least two of the three previously reported cases of *cfr*-positive *S. aureus* isolates recovered from humans and from those from animals. A *cfr*-positive MRSA isolate from a Colombian patient belonged to ST5/ST221-MRSA-I, and *cfr* was chromosomally located (1, 45). A *cfr*-positive methicillin-susceptible *S.*

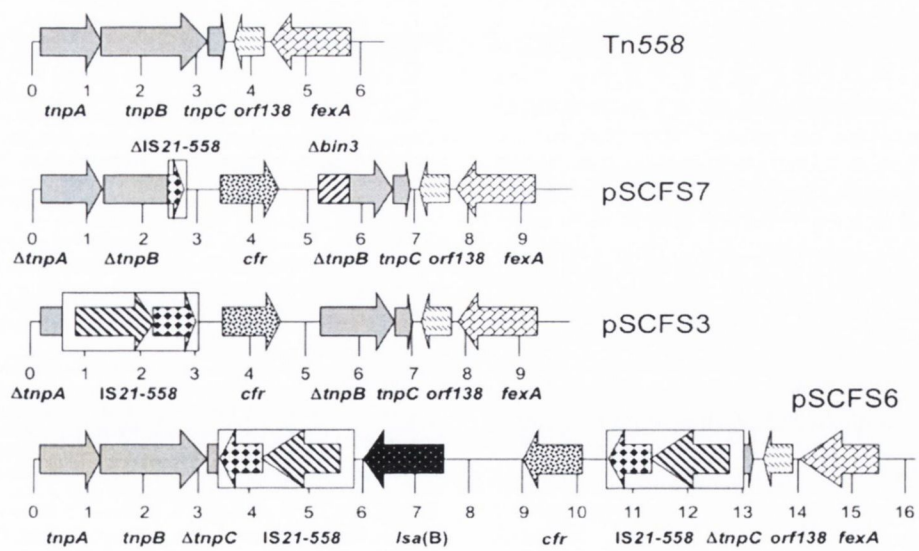

FIG. 1. Schematic diagram showing the organization of the novel variant of transposon Tn*558* of plasmid pSCFS7 (accession no. FR675942) relative to Tn*558* (AJ715531) and the Tn*558* variants harbored by plasmids pSCFS3 (AM086211) and pSCFS6 (AM408573). The positions and orientations of the genes coding for transposition functions (*tnpA*, *tnpB*, and *tnpC*), antimicrobial resistance [*fexA*, resistance to florfenicol and chloramphenicol; *cfr*, resistance to phenicols, lincosamides, oxazolidinones, pleuromutilines, and streptogramin A antibiotics; *lsa*(B), reduced susceptibility to lincosamides], or unknown functions (*orf138*) are indicated by arrows. Δ, truncated gene. A distance scale in kb is shown below each map.

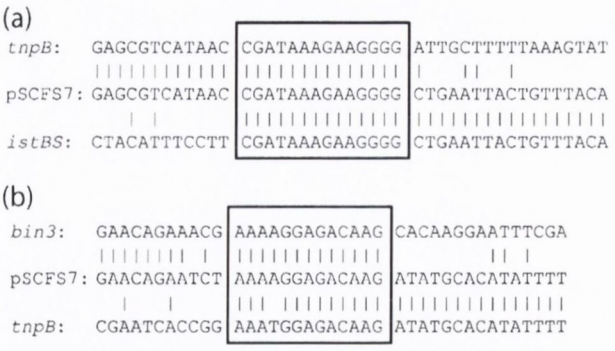

FIG. 2. Potential sites used for the integration of the 2,966-bp *cfr*-containing segment into the *tnpB* reading frame, thereby causing a 433-bp deletion of *tnpB*. (a) Recombination site between the 5′ end of *tnpB* and IS*21-558*. Displayed are the *tnpB* sequence at positions 3369 to 3411 of plasmid pSCFS6 (accession no. AM408573), the *istBS* sequence at positions 4724 to 4682 of plasmid pSCFS6, and the sequence of pSCFS7 at positions 834 to 878. (b) Recombination site between *bin3* and the 3′ end of *tnpB*. Displayed are the *bin3* sequence of *B. mycoides* at positions 4550 to 4512 (ACMW01000216), the *tnpB* sequence at positions 3726 to 3764 of plasmid pSCFS6, and the sequence of pSCFS7 at positions 3811 to 3853. Nucleotides identical to the sequence of pSCFS7 are indicated by vertical bars. The recombination sites, where crossover is believed to have occurred, are boxed.

aureus (MSSA) isolate from a patient in a hospital in the United States was a sporadic strain, and *cfr* was located on a 55-kb plasmid that was not further characterized (26). However, the *cfr*-positive MRSA isolates reported from an outbreak in a Spanish hospital were not investigated for the location of *cfr* (31), and while these isolates appeared to be closely related by PFGE, no details of MLST or SCC*mec* types were provided, so comparison to international MRSA strains is not possible (31). Reports of *cfr* among *S. aureus* isolates from animals have all involved pigs, including isolates belonging to ST9-MSSA and ST398-MRSA-V, and all these isolates harbored *cfr* on plasmid pSCFS3 (16, 18).

While *cfr* is still rare among *S. aureus* isolates, it appears to be more abundant among CoNS, and it is evident that CoNS from both humans and animals may act as a reservoir for *cfr* and other resistance genes in *S. aureus*. The *cfr* gene was first reported in 2000 from a bovine *Staphylococcus sciuri* isolate and has since been identified among additional *S. sciuri* isolates as well as in isolates of *S. warneri*, *S. simulans*, *S. hyicus* and *S. lentus*, all from animal sources (15, 18, 39). These CoNS from animals all harbored *cfr* on plasmid pSCFS1, pSCFS3, or pSCFS6 (15, 17, 18). In addition, there have been two recent reports of CoNS from humans harboring *cfr*, including two *Staphylococcus epidermidis* isolates from Mexico, in which the location of *cfr* was not determined (25), and the United States, in which *cfr* was localized on a 175-kb plasmid (26). While there have been no reports of *cfr*-positive staphylococci from Ireland prior to the present study, linezolid resistance has been reported among three clinical *S. epidermidis* isolates (21). However, it was not determined if *cfr* was present in these *S. epidermidis* isolates, and the linezolid resistance phenotype in these isolates may alternatively have been due to the presence of a mutation in the 23S rRNA gene. Interestingly, during the present study, problems in interpreting linezolid MICs using the Vitek-2 software were encountered. The Vitek-2 software reported a linezolid MIC of 8 μg/ml for the *cfr*-positive MRSA isolate M05/0060, which is above the resistance breakpoint for linezolid (Table 3). However, the Vitek-2 software recommended that the linezolid susceptibilities should be manually changed to 4 μg/ml and that the isolate should be reported as susceptible to linezolid. While the authors of a 2008 study (1) did not encounter problems using the Vitek system, they did report problems with detecting *cfr*-mediated linezolid resistance using disk diffusion and Etest. The authors found that a *cfr*-positive MRSA isolate was reported as linezolid susceptible after 24 h of incubation with the Etest and suggested that a longer incubation period was required to detect this resistance phenotype (1).

Since ST8-MRSA-IVa/USA300 has proved to be a successful clone capable of epidemic spread, the emergence of a *cfr*-positive variant of this strain is cause for significant concern and warrants close surveillance. The localization of *cfr* on a novel conjugative plasmid and the identification of a potential novel integration site within Tn*558* indicate that *cfr* has the ability to spread to other MRSA strains. Our finding of *cfr* in ST8-MRSA-IVa/USA300 would almost certainly not have occurred if we had not used the DNA microarray to screen for the presence of an extensive range of virulence and antimicrobial resistance genes among large numbers of MRSA isolates. Our findings highlight the effectiveness of such high-throughput systems for ongoing surveillance of MRSA.

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Enhanced Discrimination of Highly Clonal ST22-Methicillin-Resistant *Staphylococcus aureus* IV Isolates Achieved by Combining *spa*, *dru*, and Pulsed-Field Gel Electrophoresis Typing Data[▽†]

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ST22-methicillin-resistant *Staphylococcus aureus* type IV (ST22-MRSA-IV) is endemic in Irish hospitals and is designated antibiogram-resistogram type-pulsed-field group (AR-PFG) 06-01. Isolates of this highly clonal strain exhibit limited numbers of pulsed-field gel electrophoresis (PFGE) patterns and *spa* types. This study investigated whether combining PFGE and *spa* typing with DNA sequencing of the staphylococcal cassette chromosome *mec* element (SCC*mec*)-associated direct repeat unit (*dru* typing) would improve isolate discrimination. A total of 173 MRSA isolates recovered in one Irish hospital during periods in 2007 and 2008 were investigated using antibiogram-resistogram (AR), PFGE, *spa*, *dru*, and SCC*mec* typing. Isolates representative of each of the 17 pulsed-field group 01 (PFG-01) *spa* types identified underwent multilocus sequence typing, and all isolates were ST22. Ninety-seven percent of isolates (168 of 173) exhibited AR-PFG 06-01 or closely related AR patterns, and 163 of these isolates harbored SCC*mec* type IVh. The combination of PFGE, *spa*, and *dru* typing methods significantly improved discrimination of the 168 PFG-01 isolates, yielding 65 type combinations with a Simpson's index of diversity (SID) of 96.53, compared to (i) pairwise combinations of *spa* and *dru* typing, *spa* and PFGE typing, and *dru* and PFGE typing, which yielded 37, 44, and 43 type combinations with SIDs of 90.84, 91.00, and 93.57, respectively, or (ii) individual *spa*, *dru*, and PFGE typing methods, which yielded 17, 17, and 21 types with SIDs of 66.9, 77.83, and 81.34, respectively. Analysis of epidemiological information for a subset of PFG-01 isolates validated the relationships inferred using combined PFGE, *spa*, and *dru* typing data. This approach significantly enhances discrimination of ST22-MRSA-IV isolates and could be applied to epidemiological investigations of other highly clonal MRSA strains.

Staphylococcus aureus is an important human pathogen, due largely to its ability to express a wide variety of virulence factors and antimicrobial resistance determinants which are often encoded by mobile genetic elements (7, 10, 21, 24, 25, 39, 56). Methicillin-resistant *S. aureus* (MRSA) infections are a major public health problem worldwide, both in hospitals and in the community, although the incidence varies. Ireland has one of the highest prevalence rates of nosocomial MRSA infection in Europe and also has an emerging problem with community-acquired MRSA (CA-MRSA) infections (http://www.rivm.nl/earss/result/Monitoring_reports/Annual_reports.jsp) (49).

MRSA first emerged in Irish hospitals in 1971 (22) and, following a major increase in prevalence in the late 1970s, 1980s, and 1990s, has now been endemic in Ireland for three decades (8, 9, 45–48). Molecular typing showed that each decade since the 1970s has been associated with a major shift in the predominant MRSA clonal type in Irish hospitals (51). The clone that predominated in the 1970s and early 1980s, ST250-MRSA-I (or staphylococcal cassette chromosome *mec* element I [SCC*mec* I variant]), was replaced by the ST239-MRSA-III (or SCC*mec* III variant) clone in the mid-1980s, and this clone was in turn displaced by the ST8-MRSA-II clone (harboring SCC*mec* IIA to IIE) in the 1990s (51). Since the late 1990s, a strain designated locally as antibiogram-resistogram type-pulsed field group (AR-PFG) 06-01, belonging to the international MRSA clone classification ST22-MRSA-IV, which is similar to the United Kingdom epidemic strain EMRSA-15, has predominated in Irish hospitals, and its incidence increased from 22% in 1999 to 80% in 2003 (46, 47).

EMRSA-15 (ST22-MRSA-IV) was first reported in England in 1991 (44) and has since been described as a pandemic MRSA strain due to the predominance of ST22-MRSA-IV among nosocomial MRSA strains in many countries (1, 17, 23, 26, 33, 35, 47, 50, 53). ST22-MRSA-IV has also been identified among patients with hospital-acquired (HA) MRSA infections

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(11, 42, 43, 60, 63) and CA-MRSA infections (5, 15, 32, 49) in several countries, among health care workers (2, 55), and among companion animals (3, 18, 36, 41).

Informative molecular typing is essential for investigating MRSA strains and populations in individual institutions, countries, and wider geographic areas. This approach permits the genetic relatedness of isolates to be determined, which in turn allows the spread of different MRSA strains to be monitored both locally and globally. However, differentiating among isolates of some MRSA strains is very difficult, particularly in a setting where a single strain is endemic, due to the limited genetic diversity exhibited by MRSA strains such as ST22-MRSA-IV (17, 58). ST22-MRSA-IV isolates yield indistinguishable or closely related pulsed-field gel electrophoresis (PFGE) patterns (17). In Ireland, ST22-MRSA-IV isolates belong to a PFGE group described as pulsed-field group 01 (PFG-01) and exhibit the non-multiantibiotic-resistant antibiogram-resistogram (AR) type AR06 or closely related AR patterns (47). ST22-MRSA-IV isolates also yield a limited number of *spa* types following DNA sequencing of the protein A (*spa*) gene (27, 55).

Sequencing of the SCC*mec*-associated direct repeat unit (*dru*) of MRSA isolates has shown potential for differentiating MRSA isolates exhibiting limited diversity in PFGE analyses, including EMRSA-15 isolates from Scotland (17, 62). The *dru* region is a noncoding DNA segment consisting of imperfect 40-bp variable-number tandem repeats (VNTRs) located in the hypervariable region between *mecA* and IS*431mec* of SCC*mec* (17, 38). The majority of MRSA isolates investigated harbor the *dru* region, which ranges in size from 1 to 15 repeat units (17, 38, 58, 59, 62) (http://www.dru-typing.org). The *dru* region has been shown to be stable over time by *dru* typing of individual MRSA isolates following repeated subculture (59), and there is now an internationally agreed-upon *dru* typing nomenclature and a Web-based *dru* database (17) (http://www.dru-typing.org).

Currently, there is no effective method for subtyping of ST22-MRSA-IV isolates. The objective of the present study was to investigate the efficacy of *dru* typing in combination with PFGE and *spa* typing to discriminate among the highly clonal ST22-MRSA-IV (PFG-01) isolates in an Irish hospital where ST22-MRSA-IV is endemic and to investigate the potential of the combined integrated typing approach to facilitate epidemiological tracking of this MRSA strain.

MATERIALS AND METHODS

Isolates and experimental design. MRSA isolates (n = 173) from 90 patients and 83 environmental sites in four wards in a 700-bed acute care hospital in Dublin, Ireland, were investigated. The isolates were recovered over two 6-week study periods in each of the four wards between May 2007 and September 2008. Isolates recovered from individual patients and their immediate ward environments during the same 6-week study period are referred to as pairs or triplets of isolates. In the majority of cases, one isolate per patient or patient-associated environmental site was investigated.

The validity of inferences drawn from the typing data was confirmed with epidemiological evidence during a pilot study in one ward. Epidemiological data collected included the numbers for the bed and bed bay corresponding to the patient or the environmental site from whom/which the sample was taken and the sample date and source (i.e., a patient or an environmental site). For patient isolates only, the probable source of the patient's MRSA (whether it was HA or whether the patient was MRSA positive on admission or had a previously known MRSA-positive status) was also recorded. An isolate was deemed to be HA if the patient was negative for MRSA upon admission screening but upon subsequent screening was found to be positive for MRSA.

Isolates were identified as *S. aureus* and stored in bacterial preserver vials at −70°C and methicillin resistance was confirmed, all as described previously (49). All isolates were typed by AR typing against a panel of 23 antimicrobial agents as described previously (47, 49).

Molecular typing. All isolates were typed by DNA macrorestriction digestion analysis using SmaI and PFGE, *spa*, *dru*, and SCC*mec* typing. One representative isolate of each *spa* type identified among the 173 MRSA isolates investigated was typed by multilocus sequence typing (MLST). PFGE was performed as described previously (47). Each PFGE pattern was assigned a 5-digit pulsed-field type (PFT) to allow for future variation in PFGE patterns, and related 5-digit PFTs that differed by ≤6 bands were abbreviated to 2-digit PFGs (47). PFGs were combined with AR typing results to give AR-PFGs (47).

Genomic DNA for use in *spa*, *dru*, and SCC*mec* typing and MLST was extracted using a DNeasy kit according to the instructions of the manufacturer (Qiagen, Crawley, United Kingdom). *spa* typing was performed using the primers and thermal cycling conditions described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet [http://www.seqnet.org]). Analysis of *spa* sequences and assignment of *spa* types were performed using the Spa typing plug-in tool of the BioNumerics software package (version 5.1; Applied Maths, Ghent, Belgium). For *dru* typing, the *dru* region was amplified and sequenced as described previously (17). The BioNumerics tandem-repeat sequence typing (TRST) plug-in tool was used for *dru* sequence analysis and assignment of *dru* types. *dru* types were assigned using an alphanumeric nomenclature (17). SCC*mec* typing was performed using four multiplex PCR assays to identify (i) the *mec* complex type (class A, B, or C) (28), (ii) the *ccr* complex type (*ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4*, or *ccrC*) (28), (iii) the various J regions and *mecI* (40), and (iv) the SCC*mec* IV subtype (34). Previously described MRSA control strains were used as positive controls for multiplex PCR assays i to iii (52). The following *S. aureus* reference strains and clinical isolates were used as positive controls for SCC*mec* IV subtyping: CA05 (SCC*mec* IV.1/IVa) (31), 8/63P (SCC*mec* IV.2/IVb) (31), JCSC4788 (SCC*mec* IV.3/IVc) (30), JCSC4469 (SCC*mec* IV.4/IVd) (30), M04/0177 (SCC*mec* IV.5/IVg) (52), and E1749 (SCC*mec* IV.6/IVh) (52). MLST was performed and sequences were analyzed as described previously (13, 52).

Investigating the stability of *dru* types. The stability of the *dru* region was investigated using three MRSA isolates that had previously been subjected to *dru* typing. These comprised two Irish ST8-MRSA-IV isolates, M05/0028 (49) and M06/0376, both of which exhibited *dru* type dt9g, and one EMRSA-15 isolate from the Harmony collection with *dru* type dt10h (17, 37). Each isolate was cultured on brain heart infusion (BHI) agar (Becton Dickinson and Company, Sparks, MD) and incubated at 37°C for 24 to 48 h. Several colonies from each isolate were subsequently subcultured on fresh BHI agar plates and incubated at 37°C for 24 to 48 h. This procedure was repeated for a minimum of 10 subcultures over a 14-day period. For each isolate, several colonies from the original and final subculture plates were analyzed by *dru* typing as described above.

Cluster analyses of *spa* and *dru* types. The BioNumerics Spa typing and TRST plug-in tools were used for cluster analyses of *spa* and *dru* types, respectively. With both of these plug-ins, sequences are compared and aligned using an algorithm based on the DSI (duplication, substitution, and indels) model for pairwise alignment of repeats, which considers that modification of sequences can occur through duplication of tandem repeats, substitutions, insertions, and deletions (the latter two events are collectively termed indels) (4). A similarity matrix is generated based on the DSI model and used to construct a minimum spanning tree (MST); the type with the greatest number of related types is assigned as the root node, and the other types derive from this node. In the present study, the default parameters were used for alignment of sequences. The software creates groups of certain distance intervals or similarity values (which BioNumerics terms bins) and converts the data into distance units. Because of the highly clonal nature of the MRSA isolates investigated in the present study, the bin distance was set to 0.5%, i.e., the distance between two entries with >99.5% similarity was 0 (a distance interval of 99.5 to 100% similarity equals a distance of 0) on the MST, and the distance between two entries with 99 to 99.5% similarity was 1 (a distance interval of 99 to 99.5% similarity equals a distance of 1). Using the MSTs, the following criteria were established for clustering of *dru* types and for clustering of *spa* types: *spa* types and *dru* types were deemed to belong to different clusters if they were separated by an MST distance of >2 (i.e., if they showed <98.5% similarity). Therefore, if two *spa* types or two *dru* types were at an MST distance of ≤2, they were considered to be closely related (i.e., they formed a subgroup).

Clustering of isolates. Each isolate was assigned a 3-digit cluster code with the first number representing the *spa* type, the second representing the *dru* type, and

TABLE 1. Cluster code nomenclature used to describe clusters identified by *spa*, *dru*, and PFGE typing[a]

Method	Type	Repeat succession	Type cluster code	Type subcluster code
spa typing	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	01	Founder
	t022	26-23-13-23-31-29-17-31-29-17-25-17-25-16-28	01	01a
	t557[b]	26-23-23-13-23-31-31-29-17-31-29-17-25-17-25-16-28	01	01b[c]
	t628	26-23-23-13-23-31-29-17-31-29-17-31-29-17-25-17-25-16-28	01	01c
	t1214	26-23-23-13-23-31-29-17-31-29-17-25-16-28	01	01d
	t515	26-23-23-13-23-31-29-17-31-29-17-25-16-16-28	01	01da
	t4622	26-23-23-13-23-31-31-29-17-31-29-17-25-16-16-28	01	01daa
	t018	15-12-16-02-16-02-25-17-24-24-24	02	NSC
	t1802	26-16-16-28	03	NSC
	t025	26-23-23-13-23-29-17-31-29-17-25-17-25-16-28	04	NSC
	t578	26-23-23-13-23-31-29-17-31-29-17-25-17-25-28	05	NSC
	t4623	26-23-13-23-31-29-132-17-31-29-17-25-17-25-16-28	06	NSC
	t1865	26-23-23-17-31-29-17-25-17-25-16-28	07	NSC
	t2951	26-16-31-29-17-25-17-25-16-28	08	NSC
	t2978	04-20-17-20-17-31-16-16-34	09	NSC
	t3185	26-23-23-20-31-29-17-31-29-17-25-17-25-16-28	10	NSC
	t3213	26-23-23-13-23-31-29-17-31	11	NSC
	t4122	26-23-23-13-23-31-29-23-31-29-17-25-17-16-28	12	NSC
	t4267	26-23-13-23-31-36-25-28	13	NSC
	t4765	26-23-23-13-23-31-29-17-31-17-25-16-28	14	NSC
	t190	11-17-34-24-34-22-25	15	NSC
dru typing	dt10a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e	01	NSC
	dt10j	5a-2d-4a-0-2d-7a-3a-2g-3b-4e	01	01a
	dt10af	5a-2d-4a-0-2d-2c-3a-2g-3b-4e	01	01b
	dt10n	5a-2d-4a-0-2d-3b-3a-2g-3b-4e	01	01c
	dt10i	5a-2d-4a-0-2d-4f-3a-2g-3b-4e	01	01d
	dt10o	5a-2d-4a-0-2d-4f-3a-2g-2c-4e	01	01da
	dt10p	5a-2d-4a-1b-2d-7a-3a-2g-3b-4e	01	01aa
	dt11a	5a-2d-4a-0-2d-5b-3a-2g-3b-4e-3e	02	NSC
	dt11o	5a-2d-4a-0-2d-5b-3a-2g-3b-4e-4e	02	02a
	dt11j	5a-2d-2d-4a-0-2d-5b-3a-2g-3b-4e	02	02aa
	dt5b	5a-2d-4a-5b-3a	03	NSC
	dt6e	5a-7a-3a-2g-3b-4e	04	NSC
	dt7c	5a-2d-2d-4a-0-3e-3e	05	NSC
	dt7g	5a-2d-7a-3a-2g-3b-4e	06	NSC
	dt7i	5a-2d-4a-0-2d-3b-4e	07	NSC
	dt8a	5a-2d-4a-0-2d-2g-3b-4e	08	NSC
	dt8p	6d-0-2d-7a-3a-2g-3b-4e	09	NSC
	dt9j	5a-2d-4a-0-2d-5b-3a-2g-3b	10	NSC
	dt9p	5a-2d-4a-0-7a-3a-2g-3b-4e	11	NSC
PFGE	01018		1	Founder
	01002		1	1a
	01006		1	1b
	01022		1	1c
	01024		1	1d
	01030		1	1e
	01032		1	1f
	01039		1	1g
	01042		1	1h
	01047		1	1i
	01049		1	1j
	01063		1	1k
	01075		1	1l
	01077		1	1m
	01088		1	1n
	01114		1	1o
	01126		1	1p
	01146		1	1q
	01151		1	1r
	01154		1	1s
	01156		1	1t
	00041		2	Founder
	00080		2	2a
	00216		2	2b
	02017		3	NSC
	99083		4	NSC

[a] *spa* and *dru* types at a distance of >2 on the MSTs (i.e., types that showed <98.5% similarity) were assigned distinct cluster codes (Fig. 1). *spa* and *dru* types that showed >98.5% similarity on the MSTs (i.e., types at an MST distance of ≤2) were assigned *spa* subcluster codes. Subclusters were assigned alphabetic suffixes following the relevant numerical element of the cluster code. *spa* types and *dru* types that were identified as subgroups of *spa*/*dru* types that were already assigned to *spa*/*dru* subclusters were assigned additional alphabetic suffixes, e.g., *spa* type t515 was assigned the *spa* cluster code 01da, as it is a subgroup of t1214 (*spa* cluster code 01d), which is a subgroup of *spa* type t032 (*spa* cluster code 01). For each cluster that consisted of more than one *spa* or *dru* type, the type that was assigned as the founder by using the MSTs was not assigned a subcluster code but retained the original cluster code designation. PFTs were designated with distinct cluster codes if they differed by >6 bands. PFTs that differed by ≤6 bands were assigned subcluster codes. For PFT clusters that were represented by more than one PFT, the most frequently occurring PFT was assigned the numerical value for that cluster code and all other PFTs were assigned alphabetic suffixes. NSC, no subcluster code.

[b] Isolates exhibiting *spa* type t557 were recovered from staff members only during a wider investigation and were assigned *spa* cluster code 01b but were not included in the present study.

TABLE 2. DGs, cluster codes, data from *spa*, *dru*, PFGE, SCC*mec* typing, MLST, and AR-PFGs for 173 MRSA isolates

DG	Cluster code[a]	*spa* type	*dru* type	PFT	AR-PFG[b] (no. of isolates)	SCC*mec* type	ST[c]
1a	01.01.1	t032	dt10a	01018	06-01 (8)	IVh	ST22
1a	01.01c.1	t032	dt10n	01018	06-01 (14)	IVh	ND
1a	01.01a.1	t032	dt10j	01018	06-01 (10)	IVh	ND
1a	01.01a.1	t032	dt10j	01018	Unf-01 (5)	IVh	ND
1a	01da.01a.1	t515	dt10j	01018	06-01 (1)	IVh	ND
1a	04.01a.1	t025	dt10j	01018	06-01 (1)	IVh	ST22
1a	01.10.1	t032	dt9j	01018	06-01 (1)	IVh	ND
1b	01a.01a.1	t022	dt10j	01018	06-01 (1)	IVh	ND
2	01c.01.1	t628	dt10a	01018	06-01 (9)	IVh	ST22
3	01.06.1	t032	dt7g	01018	06-01 (2)	IVh	ND
4a	01d.01.1g	t1214	dt10a	01039	06-01 (6)	IVh	ST22
4a	01daa.01.1g	t4622	dt10a	01039	06-01 (1)	IVh	ST22
4a	01da.01.1g	t515	dt10a	01039	06-01 (4)	IVh	ST22
4a	01da.01.1g	t515	dt10a	01039	NT-01 (1)	IVh	ND
4a	01d.02a.1g	t1214	dt11o	01039	06-01 (1)	IVh	ND
4a	01da.02a.1g	t515	dt11o	01039	06-01 (1)	IVh	ND
4b	01.01a.1g	t032	dt10j	01039	06-01 (1)	IVh	ND
4b	01.01a.1g	t032	dt10j	01039	NT-01 (1)	IVh	ND
4b	01.01.1g	t032	dt10a	01039	06-01 (3)	IVh	ND
4b	01.01.1g	t032	dt10a	01039	NT-01 (1)	IVh	ND
4b	01.01b.1g	t032	dt10af	01039	06-01 (6)	IVh	ND
4b	01.01da.1g	t032	dt10o	01039	06-01 (1)	IVh without *dcs*	ND
4b	12.01a.1g	t4122	dt10j	01039	06-01 (1)	IVh	ST22
5	01da.01.1k	t515	dt10a	01063	06-01 (1)	IVh	ND
6	01a.01.1g	t022	dt10a	01039	06-01 (4)	IVh	ST22
6	01a.01aa.1g	t022	dt10p	01039	06-01 (2)	IVh	ND
7	01da.07.1g	t515	dt7i	01039	06-01 (1)	IVh	ND
8	01da.08.1g	t515	dt8a	01039	06-01 (1)	IVh	ND
9	07.01a.1	t1865	dt10j	01018	06-01 (3)	IVh	ST22
10	07.01a.1a	t1865	dt10j	01002	06-01 (1)	IVh	ND
11	01.01c.1i	t032	dt10n	01047	06-01 (2)	IVh	ND
12	06.01.1 m	t4623	dt10a	01077	06-01 (1)	IVh	ST22
13	01.01.1b	t032	dt10a	01006	Unf-01 (1)	IVh	ND
13	01.01c.1b	t032	dt10n	01006	06-01 (1)	IVh	ND
13	01.01a.1b	t032	dt10j	01006	Unf-01 (1)	IVh	ND
14	01d.01.1b	t1214	dt10a	01006	06-01 (1)	IVh	ND
15	01.01a.1e	t032	dt10j	01030	06-01 (1)	IVh	ND
16	01.01a.1h	t032	dt10j	01042	NT-01 (1)	IVh	ND
16	01da.01.1h	t515	dt10a	01042	06-01 (3)	IVh	ND
16	01.01.1h	t032	dt10a	01042	06-01 (1)	IVh	ND
16	01.01c.1h	t032	dt10n	01042	06-01 (1)	IVh	ND
16	01d.01.1h	t1214	dt10a	01042	06-01 (1)	IVh	ND
17	01.01a.1d	t032	dt10j	01024	06-01 (2)	IVh	ND
17	01.01a.1d	t032	dt10j	01024	NT-01 (1)	IVh	ND
17	01.01a.1d	t032	dt10j	01024	Unf-01 (7)	IVh	ND
17	01.01c.1d	t032	dt10n	01024	06-01 (4)	IVh	ND
18	01.01a.1q	t032	dt10j	01146	Unf-01 (1)	IVh	ND
19	01da.01.1c	t515	dt10a	01022	06-01 (2)	IVh (n = 1)	ND
						IV, nonsubtypeable (n = 1)	ND
20	01.01.1l	t032	dt10a	01075	06-01 (1)	IVh	ND
20	01.02aa.1l	t032	dt11j	01075	06-01 (1)	IVh	ND
21	01.03.1	t032	dt5b	01018	06-01 (2)	IVh	ND
22	10.01da.1s	t3185	dt10o	01154	06-01 (2)	IVh	ND
22	10.01da.1s	t3185	dt10o	01154	Unf-01 (13)	IVh	ST22
23	01.01.1j	t032	dt10a	01049	06-01 (2)	IVh	ND
23	05.01.1j	t578	dt10a	01049	06-01 (1)	IVh	ST22
23	01da.02aa.1j	t515	dt11j	01049	06-01 (1)	IVh	ND
24	01.01a.1a	t032	dt10j	01002	06-01 (3)	IVh	ND
24	01da.01.1a	t515	dt10a	01002	06-01 (1)	IVh	ND
25	01.11.1j	t032	dt9p	01049	06-01 (1)	IVh	ND
26a	01a.09.1j	t022	dt8p	01049	06-01 (3)	IVh	ND
26b	01.09.1j	t032	dt8p	01049	06-01 (1)	IVh	ND
27	11.01.1g	t3213	dt10a	01039	06-01 (1)	IVh	ST22
28	11.01.1h	t3213	dt10a	01042	06-01 (1)	IVh	ND
29	13.01.1g	t4267	dt10a	01039	06-01 (1)	IVa	ST22
30	14.01.1k	t4765	dt10a	01063	06-01 (1)	IVh	ST22

Continued on following page

TABLE 2—*Continued*

DG	Cluster code[a]	*spa* type	*dru* type	PFT	AR-PFG[b] (no. of isolates)	SCC*mec* type	ST[c]
31	01.01.1f	t032	dt10a	01032	06-01 (1)	IVh	ND
32	01.01.1r	t032	dt10a	01151	06-01 (1)	IVh	ND
33	01.01a.1t	t032	dt10j	01156	06-01 (1)	IVh with *ccrAB4*	ND
34	01.02.1p	t032	dt11a	01126	06-01 (1)	IVh with *ccrC* and Tn*554* to *orfX*	ND
35	03.01.1g	t1802	dt10a	01039	06-01 (1)	IVh	ST22
36	01.01.1o	t032	dt10a	01114	06-01 (1)	IVh	ND
37	08.04.1n	t2951	dt6e	01088	06-01 (1)	IVh	ST22
38	02.05.3	t018	dt7c	02017	NT-02 (1)	II without p*UB110*	ST36
39	15.01a.2	t190	dt10j	00041	New03-00 (1)	Characterized by *ccrAB4*, class A *mec*, *mecI*, and *dcs*	ST8
40	15.01.02a	t190	dt10a	00080	14-00 (1)	Characterized by *ccrAB2*, *ccrAB4*, J1 type IVb, *dcs*, and novel *mec* complex	ND
41	15.01.2b	t190	dt10j	00216	13-00 (1)	IIE	ND
42	09.01d.4	t2978	dt10i	99083	Unf-99 (1)	IVb	ST87

[a] Based on the nomenclature presented in Table 1, each isolate was assigned a 3-digit cluster code in which the first number represents the *spa* type, the second represents the *dru* type, and the third represents the PFT.

[b] Unf, unfamiliar (these isolates exhibited a hitherto unfamiliar AR pattern); NT, nontypeable (these isolates exhibited AR patterns that differed from the AR06 group of patterns only with regard to resistance to lincomycin [see Table S1 in the supplemental material for further details]).

[c] One isolate representative of each *spa* type identified in the present study underwent MLST. ND, not determined.

the third representing the PFT (Table 1). For example, *spa* type t032, *dru* type dt10a, and PFT 01018 were assigned the codes 01, 01 and 1, respectively, and isolates with this *spa*, *dru*, and PFGE type combination were assigned the 3-digit cluster code 01.01.1. Subtypes recognized by each typing method were designated by alphabetic suffixes after the relevant numerical element of the cluster code (Table 1). To investigate the overall relatedness of isolates, a composite dendrogram for all PFG-01 (ST22-MRSA-IV) isolates identified during the present study was constructed in BioNumerics by using the averages of the similarity matrices from the individual experiments (PFGE, *spa*, and *dru* typing) and clustering by the unweighted-pair group method using average linkages (UPGMA).

Discriminatory powers of and concordance of data from *spa*, *dru*, and PFGE typing methods. The abilities of PFGE, *spa*, and *dru* typing methods alone and in every combination to discriminate among the PFG-01 (ST22-MRSA-IV) isolates investigated were assessed quantitatively by calculating Simpson's indices of diversity (SIDs) with 95% confidence intervals (CI) using an online tool developed by Faria et al. (14) (available at http://www.comparingpartitions.info). SID provides an objective assessment of the discriminatory power of a typing method (14, 20).

The concordance among the data from the typing methods was determined by calculating the adjusted Rand index (ARI) using the online tool mentioned above (14). The ARI indicates the overall concordance between data from two typing methods and includes a correction factor to take into account the possibility that concordance may have arisen by chance. The online tool was also used to calculate the Wallace (W) coefficient (14), which indicates the probability that two isolates classified as the same type by one method will also be classified as the same type by another method. Hence, the W coefficient gives a quantitative estimate of the value of including additional typing methods. A high W coefficient suggests that including a particular additional method does not yield further information. The W coefficient also provides directional information about the concordance of data from typing methods in that it quantifies the probability that isolates clustered by one typing method (e.g., PFGE) will be assigned to the same cluster by a second typing method (e.g., *spa* typing) and vice versa (14). Where the value of the W coefficient is low when comparing one method to another and results are similar in both directions (e.g., *spa* to *dru* versus *dru* to spa), the inference is that isolates clustered by one typing method may be subdivided by the other typing method (6).

RESULTS

MRSA isolates (n = 173) were recovered from one Dublin hospital during two 6-week study periods in four different wards. The PFTs and AR-PFGs as well as the *spa*, *dru*, and SCC*mec* typing and MLST results for the 173 MRSA isolates are shown in Table 2.

PFGE and AR typing. Twenty-six PFTs representing four PFGs were identified among the 173 isolates (Table 2). PFG-01 predominated, accounting for 97% of isolates (168 of 173). The 168 PFG-01 isolates exhibited 21 highly similar PFTs, with the two most predominant patterns (PFT 01018 [n = 57] and PFT 01039 [n = 39]), which accounted for 57.1% of all PFG-01 isolates (96 of 168), differing by only a single band.

The majority of PFG-01 isolates (135 of 168 [80.4%]) exhibited AR type AR06 and were assigned to AR-PFG 06-01 (Table 2). The AR types and subtypes and the antimicrobial resistance patterns for all isolates investigated are shown in Table S1 in the supplemental material.

***spa* typing.** Seventeen *spa* types were identified among the 168 isolates classified into PFG-01, but 55.4% of these isolates (93 of 168) belonged to *spa* type t032. The proportions of isolates of other *spa* types among the PFG-01 isolates were as follows: t515, 16 of 168 (9.5%); t3185, 15 of 168 (8.9%); t1214, 10 of 168 (6%); t022, 10 of 168 (6%); t628, 9 of 168 (5.4%); t1865, 4 of 168 (20.4%); and t3213, 2 of 168 (1.2%). *spa* types t025, t578, t1802, t2951, t4122, t4267, t4622, t4623, and t4765 were exhibited by single isolates only (Table 2).

***dru* typing.** The stability of the *dru* region in three MRSA isolates was confirmed by the finding that several colonies from original cultures of each isolate on BHI agar plates and from growth following a minimum of 10 sequential subcultures exhibited the same *dru* types originally assigned in earlier studies (i.e., dt9g for M06/0376 and M05/0028 and dt10h for the Harmony EMRSA-15 isolate).

Seventeen *dru* types were identified among the 168 PFG-01 isolates, with dt10a isolates (61 of 168 [36.3%]) predominating. Proportions of isolates of other types were as follows: dt10j, 43 of 168 (25.6%); dt10n, 22 of 168 (13%); dt10o, 16 of 168 (9.5%); dt10af, 6 of 168 (3.6%); dt8p, 4 of 168 (2.4%); dt5b, 2

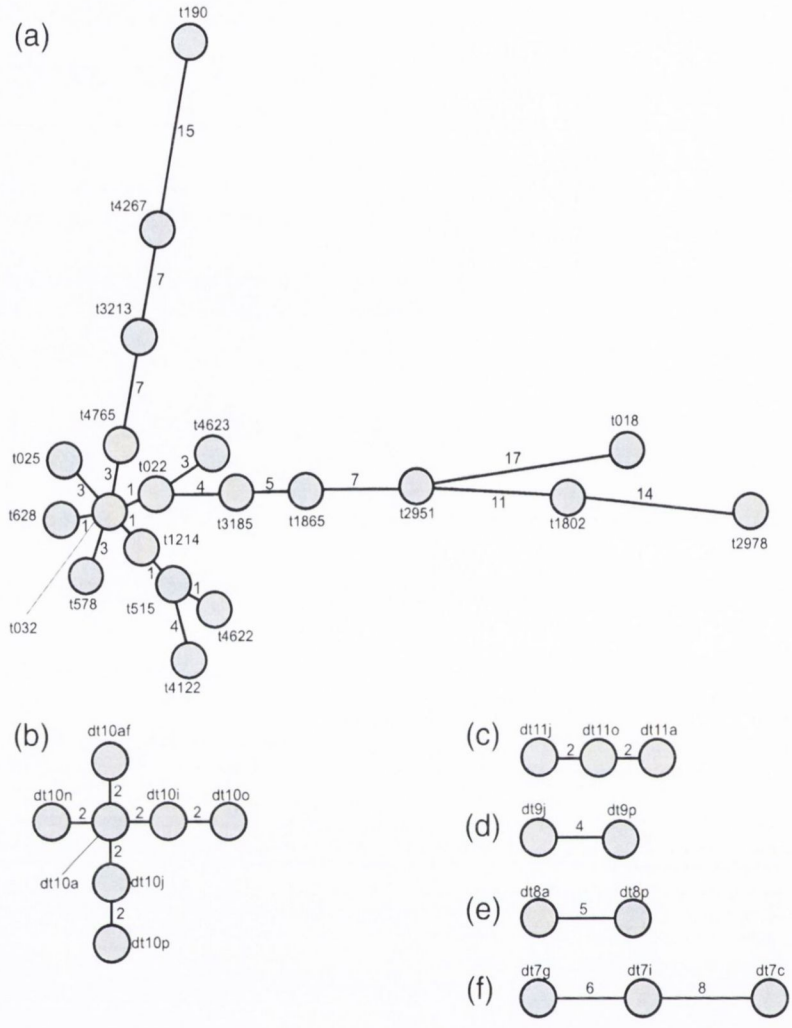


FIG. 1. MSTs generated using the BioNumerics software program representing the 20 *spa* types (a) and the *dru* types with 10 (b), 11 (c), 9 (d), 8 (e), and 7 (f) repeat units identified among the 173 MRSA isolates investigated. Each individual circle represents a different *spa* or *dru* type, and the numerical values on the branches represent the similarity (expressed as the MST distance) between two *spa* or two *dru* types. The BioNumerics software creates groups of certain distance intervals or similarity values (termed bins) and converts these data into distance units. The bin distance was set to 0.5% (i.e., two entries at a distance of 1 on the MST have between 99 and 99.5% similarity, and two entries at a distance of 2 have between 98.5 and 99% similarity, etc.). *spa* types and *dru* types were assigned the same cluster code if they were separated by an MST distance of <2 (i.e., if they showed >98.5% similarity) (Table 1).

of 168 (1.2%); dt7g, 2 of 168 (1.2%); dt10p, 2 of 168 (1.2%); dt11j, 2 of 168 (1.2%); and dt11o, 2 of 168 (1.2%). The *dru* types dt6e, dt7i, dt8a, dt9j, dt9p, and dt11a were exhibited by single isolates among the remaining PFG-01 isolates (Table 2).

MLST and SCC*mec* typing. By MLST, four distinct sequence types (STs) were detected among 20 isolates representative of each of the 20 *spa* types identified (Table 2). Isolates exhibiting the 17 *spa* types found among the 168 PFG-01 isolates were all identified as ST22 (i.e., MLST allelic profile 7-6-1-5-8-8-6) and belonged to clonal complex 22 (i.e., CC22).

SCC*mec* typing revealed that the majority of PFG-01 isolates (163 of 168 [97%]) harbored SCC*mec* IVh (with *ccrAB2*, class B *mec*, *dcs*, and J1 region type IVh). Of the remaining five PFG-01 isolates, one harbored SCC*mec* IVa (with *ccrAB2*, class B *mec*, *dcs*, and J1 region type IVa) and four harbored novel SCC*mec* IV variants (Table 2).

Clustering of isolates. The 173 MRSA isolates were divided into clusters based on *spa*, *dru*, and PFGE typing data. Each isolate was assigned a 3-digit cluster code with the first number representing the *spa* type, the second representing the *dru* type, and the third representing the PFT (Tables 1 and 2). Subgrouping among *spa* and *dru* types was investigated by constructing MSTs (Fig. 1), and subgrouping among PFGE types was based on the numbers of band differences, as described below.

Cluster analysis of *spa* types. An MST constructed from all *spa* types identified is shown in Fig. 1a. *spa* types were deemed to be distinct if they differed from all others identified by an

TABLE 3. Discriminatory powers of *spa*, *dru*, and PFGE typing methods[a]

Typing method(s)	No. of types	SID	95% CI
spa typing	17	66.90	59.36–74.44
dru typing	17	77.83	73.86–81.80
PFGE	21	81.34	77.38–85.31
spa and *dru* typing	37	90.84	88.66–93.02
spa typing and PFGE	44	91.00	88.12–93.89
dru typing and PFGE	43	93.57	92.08–95.66
spa, *dru*, and PFGE typing	65	96.53	95.53–97.52

[a] Discriminatory powers of the methods used individually and in combination for the 168 PFG-01 (ST22-MRSA-IV) isolates investigated were measured by using SID (with 95% CIs).

MST distance of >2 (corresponding to <98.5% similarity) and were assigned different *spa* cluster codes with numerical values ranging from 01 to 15 (Fig. 1a and Table 1). *spa* types that showed >98.5% similarity on the MST (i.e., those at an MST distance of ≤2) were assigned *spa* subcluster codes (Table 1). Each *spa* type within a subcluster, apart from the founder of a subgroup, was assigned an additional alphabetic suffix (Table 1). The MST showed that some *spa* type subgroups contained further subgroups, so additional alphabetic suffixes were added to the alphanumeric *spa* subcluster codes (Table 1).

Cluster analysis of *dru* types. For *dru* typing, individual MSTs were generated for all groups of distinct *dru* types harboring the same number of *dru* repeat units. MSTs for *dru* types with 11 (dt11a, dt11j, and dt11o), 10 (dt10a, dt10i, dt10j, dt10n, dt10o, dt10p, and dt10af), 9 (dt9j and dt9p), 8 (dt8a and dt8p), and 7 (dt7c, dt7g, and dt7i) repeat units are shown in Fig. 1c, b, d, e, and f, respectively.

dru type cluster codes were based on the number of *dru* repeat units present (e.g., *dru* types with 10 repeats were assigned *dru* cluster code 01) (Table 1). Thereafter, subgroups of closely related *dru* types were identified using the same criteria used for subgrouping of *spa* types (Table 1).

Cluster analysis of PFTs. For cluster analysis, each of the four PFGs was assigned a PFGE cluster code consisting of a number ranging from 1 to 4. Where PFGs were represented by more than one PFT, the most frequently occurring PFT was assigned the numerical value for that PFG and all other PFTs were assigned additional alphabetic suffixes (Table 1).

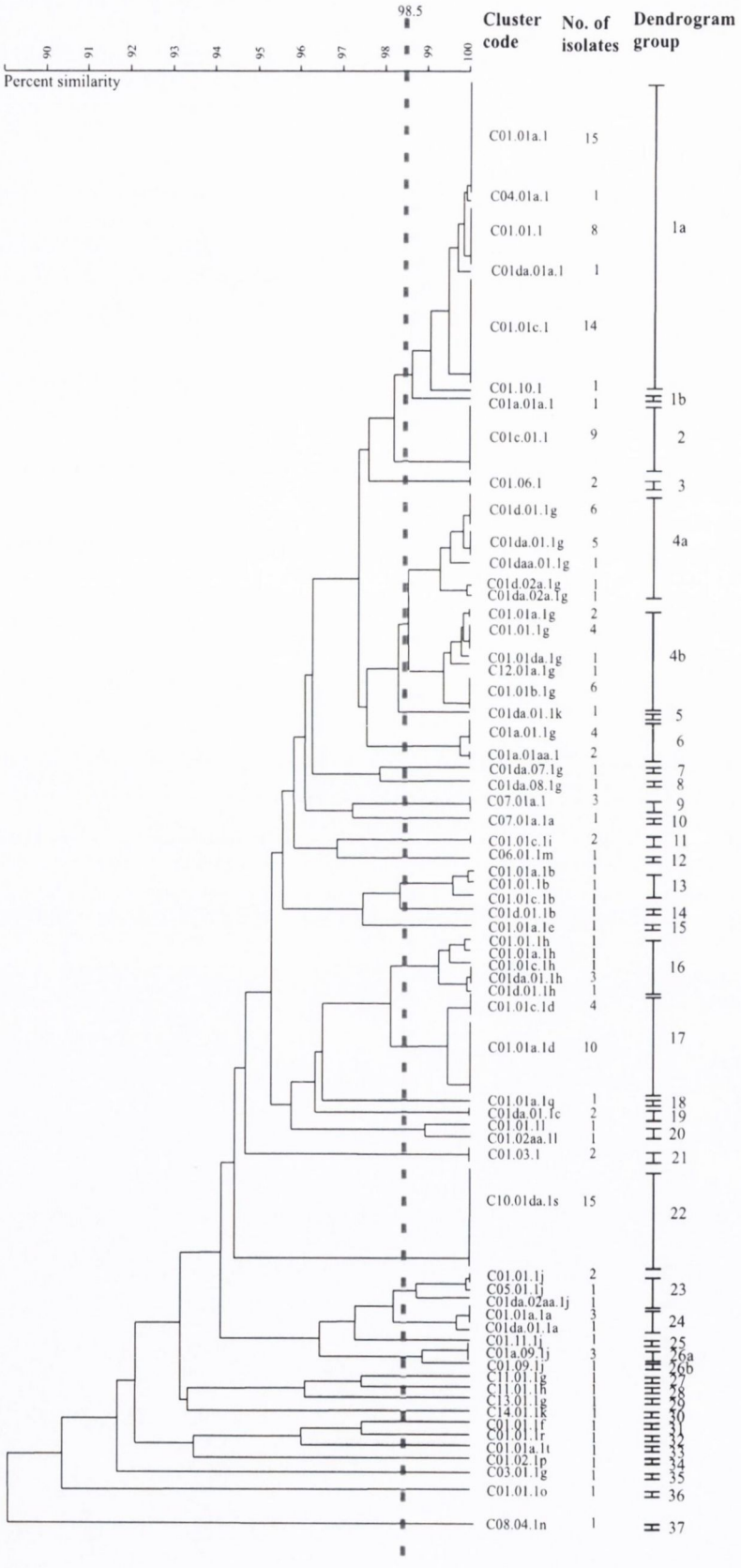
Discriminatory powers of and concordance of data from *spa*, *dru*, and PFGE typing methods. The abilities of PFGE, *spa*, and *dru* typing methods to discriminate among the 168 PFG-01 (ST22-MRSA-IV) isolates were determined quantitatively using SID for each individual typing method and for all combinations of the three methods (Table 3). The combination of *spa*, *dru*, and PFGE typing yielded the largest number of type combinations (65 types) and the greatest discriminatory power (SID, 96.53) with the narrowest 95% CI (Table 3). Of the three individual methods, PFGE was the most discriminatory (SID, 81.34) (Table 3).

The enhanced discrimination obtained by combining all three typing methods was confirmed by the ARI and W coefficient values (Table 4). Based on the ARI, the probability that the isolate clustering patterns obtained using the combination of *spa*, *dru*, and PFGE typing methods would be similar to those obtained using any one of the typing methods individually or pairwise combinations of the methods was <69% (range, ca. 13 to 69%) (Table 4). In addition, the low W coefficients obtained for the comparison of individual methods suggest that no method is redundant and that each method contributes additional information. The highest value for the comparison between a pair of individual methods was obtained for PFGE and *spa* typing (W coefficient, 0.482), but the value for the comparison between *spa* typing and PFGE was much lower (W coefficient, 0.272) (Table 4). Hence, the PFGE type could predict the *spa* type with 48% probability whereas the probability with which the *spa* type predicted the PFGE type was only 27%. High W coefficients were obtained for comparisons between a combination of two or three methods and one of the methods individually (e.g., the combination of *spa* typing and PFGE and *spa* typing alone) (Table 4).

Cluster analysis of isolates based on the combination of *spa*, *dru*, and PFGE typing results. Seventy cluster codes representative of each different combination of *spa*, *dru*, and PFGE types were identified for the 173 isolates investigated (Table 2). For the 168 PFG-01 isolates, 65 cluster codes were identified (Table 2). Many of the isolates with different cluster codes exhibited only minor differences by combinations of *spa*, *dru*, and/or PFGE typing methods. To further investigate the relationship among PFG-01 isolates, a dendrogram was generated from the averages of the similarity matrices for *spa*, *dru*, and PFGE typing data for all PFG-01 isolates (Fig. 2). Isolates that

TABLE 4. Concordance of data from *spa*, *dru*, and PFGE typing methods used individually and in combination for the 168 PFG-01 (ST22 MRSA-IV) isolates

Typing method(s)	ARI for comparison with:						W coefficient for comparison with:						
	spa typing	*dru* typing	PFGE	*spa* and *dru* typing	*spa* typing and PFGE	*dru* typing and PFGE	*spa* typing	*dru* typing	PFGE	*spa* and *dru* typing	*spa* typing and PFGE	*dru* typing and PFGE	*spa*, *dru*, and PFGE typing
spa typing								0.277	0.272	0.277	0.272	0.105	0.105
dru typing	0.090						0.413		0.290	0.413	0.157	0.290	0.157
PFGE	0.143	0.141					0.482	0.345		0.186	0.482	0.345	0.186
spa and *dru* typing	0.339	0.523	0.144				1.000	1.000	0.379		0.379	0.379	0.379
spa typing and PFGE	0.333	0.109	0.602	0.321			1.000	0.386	1.000	0.386		0.386	0.386
dru typing and PFGE	0.076	0.389	0.461	0.400	0.406		0.540	1.000	1.000	0.540	0.540		0.540
spa, *dru*, and PFGE typing	0.136	0.224	0.271	0.526	0.534	0.687	1.000	1.000	1.000	1.000	1.000	1.000	


98.5
90
91
92
93
94
95
96
97
98
99
100
Percent similarity
Cluster code
No. of isolates
Dendrogram group
C01.01a.1 15
C04.01a.1 1
C01.01.1 8
C01da.01a.1 1
C01.01c.1 14
C01.10.1 1
C01a.01a.1 1
C01c.01.1 9
C01.06.1 2
C01d.01.1g 6
C01da.01.1g 5
C01daa.01.1g 1
C01d.02a.1g 1
C01da.02a.1g 1
C01.01a.1g 2
C01.01.1g 4
C01.01da.1g 1
C12.01a.1g 1
C01.01b.1g 6
C01da.01.1k 1
C01a.01.1g 4
C01a.01aa.1 2
C01da.07.1g 1
C01da.08.1g 1
C07.01a.1 3
C07.01a.1a 1
C01.01c.1i 2
C06.01.1m 1
C01.01a.1b 1
C01.01.1b 1
C01.01c.1b 1
C01d.01.1b 1
C01.01a.1e 1
C01.01.1h 1
C01.01a.1h 1
C01.01c.1h 1
C01da.01.1h 3
C01d.01.1h 1
C01.01c.1d 4
C01.01a.1d 10
C01.01a.1q 1
C01da.01.1c 2
C01.01.1l 1
C01.02aa.1l 1
C01.03.1 2
C10.01da.1s 15
C01.01.1j 2
C05.01.1j 1
C01da.02aa.1j 1
C01.01a.1a 3
C01da.01.1a 1
C01.11.1j 1
C01a.09.1j 3
C01.09.1j 1
C11.01.1g 1
C11.01.1h 1
C13.01.1g 1
C14.01.1k 1
C01.01.1f 1
C01.01.1r 1
C01.01a.1t 1
C01.02.1p 1
C03.01.1g 1
C01.01.1o 1
C08.04.1n 1
1a
1b
2
3
4a
4b
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26a
26b
27
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36
37

showed ≥98.5% similarity on the dendrogram were deemed to be very closely related and were assigned to the same dendrogram group (DG), while those with <98.5% similarity were deemed to be distinguishable and were assigned to different DGs (Fig. 2). By using these criteria, a total of 37 DGs were identified among the 168 PFG-01 isolates (Table 2 and Fig. 2). Three of these DGs (DG-1, DG-4, and DG-26) were divided into subgroups because they included isolates that showed between 98.5 and 99% similarity (Fig. 2). DG-1 and DG-4 were the largest groups identified and consisted of 41 of 168 isolates (24.4%) and 28 of 168 isolates (16.7%), respectively (Fig. 2).

Five distinct cluster codes were identified among the five non-PFG-01 isolates; these isolates showed <90% similarity to one another and to all other isolates investigated according to a dendrogram generated from the averages of the similarity matrices for *spa*, *dru*, and PFGE typing data for all isolates investigated in the present study (data not shown). Therefore, these five isolates were deemed to be distinguishable and were assigned to distinct DGs (DG-38 to DG-42) (Table 2).

Combining the dendrogram groupings with epidemiological evidence. The dendrogram groupings for one hospital ward during two 6-week study periods were analyzed in the context of available epidemiological data. Dendrograms were generated from the averages of the similarity matrices for *spa*, *dru*, and PFGE typing data for all PFG-01 isolates recovered from patients and environmental sites in one ward (ward 1) from (i) July to September 2007, with one additional isolate recovered in November 2007 (study period I; $n = 38$) (Fig. 3, top), and (ii) April to May 2008 (study period II; $n = 22$) (Fig. 3, bottom).

Twelve DGs were identified among the 38 PFG-01 isolates recovered during study period I (Fig. 3, top). The largest DG recognized was DG-1a, consisting of 11 isolates. The earliest DG-1a isolate was recovered from a patient who was MRSA positive upon admission to the ward (Fig. 3, top). Over the next 10 days, DG-1a isolates were recovered from three patients and seven environmental sites; MRSA isolates from two of these patients were deemed to have been HA (Fig. 3, top).

The second largest DG (DG-4) consisted of nine isolates belonging to DG-4a ($n = 6$) or DG-4b ($n = 3$) (Fig. 3, top). The earliest DG-4 isolate was recovered from a bed mattress and belonged to DG-4a (Fig. 3, top). Two weeks later, a second DG-4a isolate was recovered from a patient whose MRSA infection was considered to have been HA. Subsequently, DG-4a and DG-4b isolates were recovered from additional patients and environmental sites, but none of the MRSA isolates from the patients were deemed to have been HA (Fig. 3, top). Four pairs of isolates were recovered during study period I, but isolates from one pair only (pair 02) were assigned to the same DG (DG-16) (Fig. 3, top).

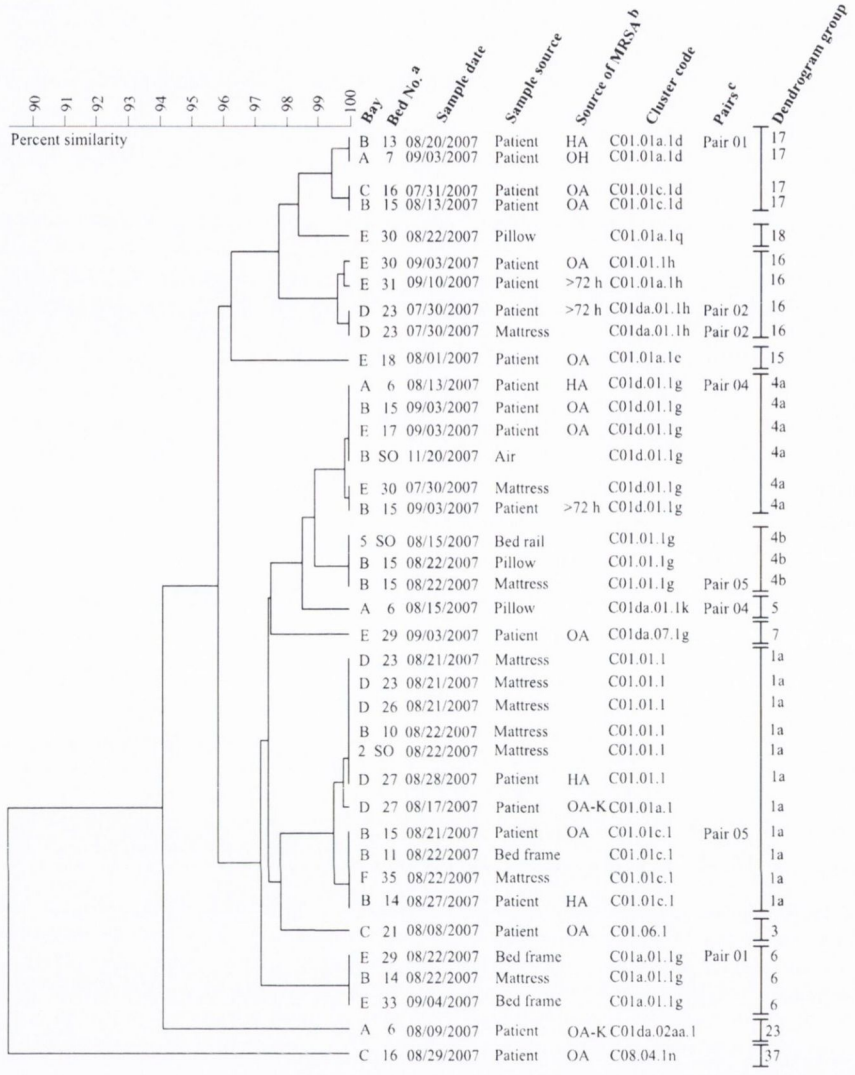
During study period II, eight DGs were identified among the 22 PFG-01 isolates recovered, with DG-2 isolates (9 of 22 [40.9%]) predominating (Fig. 3, bottom). The earliest DG-2 isolate came from a bed mattress. Subsequently, DG-2 isolates were recovered from six environmental sites and from two patients, both of whom were MRSA positive upon admission (Fig. 3, bottom). Four DG-26 isolates, including a triplet of isolates (triplet 01) recovered from a patient and the patient's mattress and bed rail, were identified during study period II. Two DG-1a, two DG-4a, and two DG-17 isolates were also identified during study period II (Fig. 3, bottom). The two DG-1a isolates (pair 03) were recovered from a patient and, 8 days later, from that patient's locker, while the DG-4a isolates were recovered from two patients within 24 h of each other. The first DG-17 isolate was from a patient, and 1 day later, the second isolate was recovered from a bed rail of a different bed (bed 10) in the same bed bay occupied by the patient (Fig. 3, bottom). A DG-16 isolate had been recovered the previous day from the patient in bed 10. This DG-16 isolate and the DG-17 isolate recovered from the rail of bed 10 (pair 06) differ by one PFGE band only, and while they showed 98.1% similarity on the dendrogram generated for all PFG-01 isolates (Fig. 2), they showed ca. 98.4% similarity on the dendrogram for PFG-01 isolates recovered in ward 1 during study period II (Fig. 3, bottom).

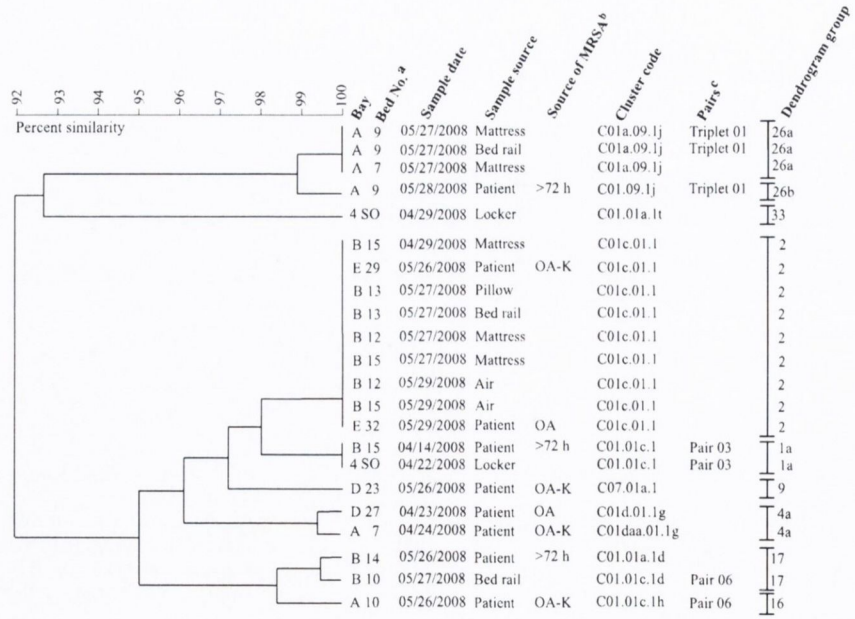
DISCUSSION

Epidemiological tracking of ST22-MRSA-IV isolates is a major challenge, as they exhibit limited diversity by PFGE and *spa* typing, the most frequently used epidemiological typing methods available for MRSA. The present study investigated whether integration of PFGE, *spa*, and *dru* typing data would provide improved discrimination among ST22-MRSA-IV isolates recovered in a large tertiary-referral hospital in Ireland.

A total of 168 ST22-MRSA-IV isolates were investigated using the three typing methods. The combined use of the PFGE, *spa*, and *dru* typing data yielded the highest number of type combinations (65 types) and the greatest discriminatory power (SID, 96.53) with the narrowest 95% CI. Faria et al. (14) compared the abilities of several typing methods, including PFGE and *spa* typing, to discriminate among a diverse collection of MRSA and methicillin-susceptible *S. aureus* isolates. They reported that *spa* typing and PFGE differentiated their MRSA isolates with SIDs of 95.85 and 94.27, respectively. In contrast, the SIDs for *spa* and PFGE typing of the ST22-MRSA-IV isolates obtained in the present study were 66.9 and 81.34, respectively. Faria et al. (14) also found that the combination of PFGE and *spa* typing had a discriminatory power yielding a SID of 98.32, whereas the SID for this combination of typing methods in the present study was 91.00. These findings indicate that while the combination of PFGE and *spa*

FIG. 2. Composite dendrogram generated using UPGMA clustering and the averages of the similarity matrices from *spa*, *dru*, and PFGE typing data for the 168 PFG-01 MRSA (ST22 MRSA-IV) isolates investigated during the present study. Isolates were assigned 3-digit cluster codes (C) with the first number representing the *spa* type, the second representing the *dru* type, and the third representing the PFT. Isolate cluster codes were then assigned to DGs as follows: isolates with cluster codes that showed ≥98.5% similarity on the dendrogram were deemed to be very closely related and were assigned to the same DG. Those isolates with cluster codes showing <98.5% similarity were deemed to be distinguishable and were assigned to different DGs. The dendrogram demonstrates that the PFG-01 isolates were assigned to 65 cluster codes that were divided into 37 DGs. The red, dashed vertical line marks 98.5% similarity.


Percent similarity
90 91 92 93 94 95 96 97 98 99 100
Bay
Bed No. a
Sample date
Sample source
Source of MRSA b
Cluster code
Pairs c
Dendrogram group
B 13 08/20/2007 Patient HA C01.01a.1d Pair 01 17
A 7 09/03/2007 Patient OH C01.01a.1d 17
C 16 07/31/2007 Patient OA C01.01c.1d 17
B 15 08/13/2007 Patient OA C01.01c.1d 17
E 30 08/22/2007 Pillow C01.01a.1q 18
E 30 09/03/2007 Patient OA C01.01.1h 16
E 31 09/10/2007 Patient >72 h C01.01a.1h 16
D 23 07/30/2007 Patient >72 h C01da.01.1h Pair 02 16
D 23 07/30/2007 Mattress C01da.01.1h Pair 02 16
E 18 08/01/2007 Patient OA C01.01a.1e 15
A 6 08/13/2007 Patient HA C01d.01.1g Pair 04 4a
B 15 09/03/2007 Patient OA C01d.01.1g 4a
E 17 09/03/2007 Patient OA C01d.01.1g 4a
B SO 11/20/2007 Air C01d.01.1g 4a
E 30 07/30/2007 Mattress C01d.01.1g 4a
B 15 09/03/2007 Patient >72 h C01d.01.1g 4a
5 SO 08/15/2007 Bed rail C01.01.1g 4b
B 15 08/22/2007 Pillow C01.01.1g 4b
B 15 08/22/2007 Mattress C01.01.1g Pair 05 4b
A 6 08/15/2007 Pillow C01da.01.1k Pair 04 5
E 29 09/03/2007 Patient OA C01da.07.1g 7
D 23 08/21/2007 Mattress C01.01.1 1a
D 23 08/21/2007 Mattress C01.01.1 1a
D 26 08/21/2007 Mattress C01.01.1 1a
B 10 08/22/2007 Mattress C01.01.1 1a
2 SO 08/22/2007 Mattress C01.01.1 1a
D 27 08/28/2007 Patient HA C01.01.1 1a
D 27 08/17/2007 Patient OA-K C01.01a.1 1a
B 15 08/21/2007 Patient OA C01.01c.1 Pair 05 1a
B 11 08/22/2007 Bed frame C01.01c.1 1a
F 35 08/22/2007 Mattress C01.01c.1 1a
B 14 08/27/2007 Patient HA C01.01c.1 1a
C 21 08/08/2007 Patient OA C01.06.1 3
E 29 08/22/2007 Bed frame C01a.01.1g Pair 01 6
B 14 08/22/2007 Mattress C01a.01.1g 6
E 33 09/04/2007 Bed frame C01a.01.1g 6
A 6 08/09/2007 Patient OA-K C01da.02aa.1 23
C 16 08/29/2007 Patient OA C08.04.1n 37


Percent similarity
92 93 94 95 96 97 98 99 100
Bay
Bed No. a
Sample date
Sample source
Source of MRSA b
Cluster code
Pairs c
Dendrogram group
A 9 05/27/2008 Mattress C01a.09.1j Triplet 01 26a
A 9 05/27/2008 Bed rail C01a.09.1j Triplet 01 26a
A 7 05/27/2008 Mattress C01a.09.1j 26a
A 9 05/28/2008 Patient >72 h C01.09.1j Triplet 01 26b
4 SO 04/29/2008 Locker C01.01a.1t 33
B 15 04/29/2008 Mattress C01c.01.1 2
E 29 05/26/2008 Patient OA-K C01c.01.1 2
B 13 05/27/2008 Pillow C01c.01.1 2
B 13 05/27/2008 Bed rail C01c.01.1 2
B 12 05/27/2008 Mattress C01c.01.1 2
B 15 05/27/2008 Mattress C01c.01.1 2
B 12 05/29/2008 Air C01c.01.1 2
B 15 05/29/2008 Air C01c.01.1 2
E 32 05/29/2008 Patient OA C01c.01.1 2
B 15 04/14/2008 Patient >72 h C01.01c.1 Pair 03 1a
4 SO 04/22/2008 Locker C01.01c.1 Pair 03 1a
D 23 05/26/2008 Patient OA-K C07.01a.1 9
D 27 04/23/2008 Patient OA C01d.01.1g 4a
A 7 04/24/2008 Patient OA-K C01daa.01.1g 4a
B 14 05/26/2008 Patient >72 h C01.01a.1d 17
B 10 05/27/2008 Bed rail C01.01c.1d Pair 06 17
A 10 05/26/2008 Patient OA-K C01.01c.1h Pair 06 16

typing is highly discriminatory for collections of diverse MRSA isolates, it is inadequate in local epidemiological studies where strain diversity is limited, as with ST22-MRSA-IV. While the 168 ST22-MRSA-IV isolates investigated here were differentiated into 65 type combinations by integrating *spa*, *dru*, and PFGE typing data, some of the types identified using each individual method exhibited only minor differences and were assigned to subgroups. All 21 PFTs identified among the 168 ST22-MRSA-IV isolates were assigned to a single group (PFG-01) and were deemed to be possibly related according to the criteria of Tenover et al. (57), as they all differed by ≤6 bands. These criteria were originally devised for a range of bacterial species, including *S. aureus*, but can present problems with clonal populations of MRSA exhibiting limited genetic diversity (16). A cutoff of 80% similarity for grouping clusters of MRSA isolates, with a cutoff of 95% similarity for recognition of subtypes, has been proposed previously (14, 37). In the present study, dendrogram clustering of PFGE data showed >80% similarity among all 168 ST22-MRSA-IV isolates. The most frequently occurring PFTs (PFT 01018 and PFT 01039) differed from each other by a single band and showed 98% similarity to each other (data not shown). With PFGE, undue weight cannot be placed on a single band difference, so for the majority of the isolates in the study population, PFGE alone could not provide reliable differentiation. Unlike PFGE, for which there are agreed-upon interpretive criteria (57), *spa* and *dru* typing currently have no criteria defined by international consensus for interpreting the significance of differences in results. Consequently, isolates with different *spa* types or *dru* types are deemed to be distinct even though they may be closely related if the types differ by changes consistent with a single genetic event, such as duplication of a tandem repeat, a point mutation, or a base insertion or deletion. To investigate the significance of such differences objectively, *spa* and *dru* types were assigned as subgroups by using MSTs if they showed ≥98.5% similarity. This cutoff value grouped 7 of the 17 *spa* types identified among the 168 ST22-MRSA-IV isolates investigated into six *spa* subgroups (Table 1). Each *spa* type within each subgroup differed from the other members of the subgroup by the presence or absence of one to three tandem repeats. With *dru* typing, 10 of the 17 *dru* types identified were grouped into eight subgroups (Table 1). The *dru* types within each subgroup differed by nucleotide changes in one or two repeat units only.

While *spa* typing and PFGE are well established methods for typing of MRSA isolates, few studies have investigated the usefulness of *dru* typing for MRSA. Smyth et al. (54) identified 42 *dru* types among 111 isolates of the pandemic nosocomial strain ST239-MRSA-III. Goering et al. (17) identified 13 and 12 *dru* types among 47 EMRSA-15 (ST22-MRSA-IV) and 57 EMRSA-16 (ST36-MRSA-II) isolates, respectively. In contrast, the majority of CA-MRSA USA300 (ST8-MRSA-IV) and CC80 isolates from patients from various geographical locations exhibited dt9g and dt10a, respectively (58, 29). The results of these studies indicate that CA-MRSA strains exhibit less genetic diversity within the *dru* region than nosocomial MRSA strains. This difference may reflect the fact that the nosocomial strains have been extant longer than CA-MRSA strains (12, 44, 54, 61). In the present study, 17 *spa* types were identified among 168 ST22-MRSA-IV isolates, of which 4, t032, t515, t022, and t1214, were further differentiated into 12, 6, 4, and 2 *dru* types, respectively. Nevertheless, *dru* typing cannot be used as a stand-alone method for typing MRSA isolates, as the two predominant types recognized among the ST22-MRSA-IV isolates (dt10a and dt10j) were also identified among the three ST8-MRSA-II variant isolates in the present study and among CC80-MRSA-IV isolates described in a previous study (29). Three *dru* types identified among ST22-MRSA-IV isolates in the present study (dt10a, dt11a, and dt8a) were also identified previously among ST239-MRSA-III isolates (54), while dt10i has been identified in unrelated MRSA lineages, including EMRSA-15 (17) and the ST87-MRSA-IV isolate in the present study. These data indicate that an isolate's *dru* type is not lineage or SCC*mec* type specific. However, unrelated MRSA lineages sharing indistinguishable *dru* types may reflect the presence of related SCC*mec* elements in diverse genetic backgrounds.

The composite dendrogram generated from the combined *spa*, *dru*, and PFGE typing data for all 168 ST22-MRSA-IV isolates provides a visual representation of the overall relatedness of isolates. Using a cutoff of 98.5% similarity, isolates were differentiated into 37 DGs, 17 (46%) of which contained more than one isolate and 10 of which contained isolates representing more than one cluster code, further indicating the close relatedness of the isolates. Of the 10 DGs consisting of isolates belonging to different cluster codes, 4 contained isolates with *spa* and/or *dru* types that were not assigned as subgroups (Fig. 2 and Table 2), including DG-1a (t025 and dt9j), DG-4 (for DG-4b, t4122, and for DG-4a, dt11o), DG-23 (t578 and dt11j), and DG-20 (dt11j). In each case, the PFGE patterns were indistinguishable from those of other isolates within that DG. In addition, where the *spa* type was distinct, the *dru* type was indistinguishable from or closely related to that of other isolates within that DG; where the *dru* type was distinct, the *spa* type was indistinguishable from or closely related to that of other isolates within that DG (Table 2). These findings

FIG. 3. Composite dendrogram generated using UPGMA clustering and the averages of the similarity matrices from *spa*, *dru*, and PFGE typing data for 38 PFG-01 MRSA isolates investigated during study period I (top) and 22 PFG-01 MRSA isolates investigated during study period II (bottom). Available epidemiological information for each isolate, as well as the cluster code and DG, is indicated. DGs were assigned to the different cluster codes determined from the dendrogram depicting all PFG-01 isolates identified in the present study (Fig. 2). The dendrogram shows that the PFG-01 isolates from study period I (n = 38) and study period II (n = 22) were differentiated into 12 and 8 DGs, respectively.[a], the abbreviation SO in place of a bed number indicates a single-occupancy room.[b], OA, the patient was MRSA positive on admission; OA-K, the patient's MRSA-positive status was known at the time of admission to the ward; >72 h, the patient's MRSA status was determined 72 h after admission to the ward.[c], the pairs or triplets of isolates recovered during study periods I and II are indicated and include isolates recovered from patients and from their immediate environments during the same study period. Each pair or triplet consists of one isolate from a patient and at least one environmental isolate.

highlight the need for caution when interpreting data from individual typing methods and show how combining data from the three typing methods permits a more informative evaluation of the relationship among isolates.

In the present study, analysis of available epidemiological information for a selected subset of ST22-MRSA-IV isolates was used to confirm the validity of the relationships inferred from the combined PFGE, *spa*, and *dru* typing data. Six pairs and one triplet of isolates were recovered from individual patients and their immediate ward environments during the same time periods, and by using the combination of all three typing methods, isolates in four of these pairs (pairs 01, 04, 05, and 06) were differentiated into distinct DGs. Isolates in pair 01 differed by all three typing methods and exhibited <96% similarity on the composite dendrogram (Fig. 3, top). Interestingly, the environmental isolate in pair 01 was recovered from a bed bay 2 days after the patient isolate was obtained while the patient was in a different bed bay. Pair 04 isolates belonged to DG-4a and DG-5 and differed in their *spa* type and PFT, and the composite dendrogram showed that these isolates had <98.5% similarity (Fig. 3, top). Pair 05 isolates belonged to DG-1a and DG-4b, and although they shared the same *spa* type and belonged to *dru* types and PFTs that were assigned as subtypes, the composite dendrogram showed that they had <98% similarity (Fig. 3, top), suggesting that they are distinguishable. However, pair 06 isolates belonged to DG-16 and DG-17, differed only with regard to the PFGE patterns (exhibiting a one-band difference), and showed ca. 98.4% similarity (Fig. 3, bottom), suggesting that these isolates should be considered to be very closely related. Isolates in each of the two remaining pairs (pair 02, consisting of DG-16 isolates, and pair 03, consisting of DG-1a isolates) and the triplet (triplet 01, consisting of DG-26 isolates) were indistinguishable from each other (Fig. 3). This analysis revealed that certain patient and environmental ST22-MRSA-IV isolates could be differentiated while others remained indistinguishable and showed that the combination of typing methods used in the present study significantly improves isolate discrimination and therefore can be used for epidemiological tracking of isolates of this highly clonal strain.

Different DGs predominated among isolates from the two study periods (DG-1a in study period I and DG-2 in study period II). Particular isolates within DG-1a and DG-2 differed in the *spa* type only, with DG-1a isolates exhibiting *spa* type t032 while DG-2 isolates belonged to *spa* type t628. While these *spa* types were assigned to the same subgroup, only nine isolates exhibiting *spa* type t628 were recovered during the study and all nine were recovered from ward 1 during study period II, suggesting that the difference between t032 and t628 is significant (Fig. 3).

Based on the results of this study, it is recommended that in performing epidemiological investigations of a highly clonal MRSA strain, such as ST22-MRSA-IV, in a hospital setting where the strain is endemic, optimal tracking can be achieved by combining *spa* and PFGE typing data with *dru* typing data. This approach has revealed a previously unrecognized level of diversity among ST22-MRSA-IV isolates that can be used to provide data fundamental to epidemiological tracking of isolates of this pandemic MRSA strain. PFGE and *spa* typing are routinely used for typing of MRSA isolates (19, 37), and while *dru* typing may not be as well established or widely used for typing of MRSA isolates, it involves the same techniques and principles as *spa* typing (DNA sequencing of a VNTR unit). Therefore, use of *dru* typing by a laboratory that currently uses DNA-based sequencing methods for routine epidemiological typing of MRSA isolates should not require additional expertise or result in a major increase in costs. In addition, all *spa*, *dru*, and PFGE data analyses can be performed with a commonly used software package (i.e., BioNumerics), and if required, statistical analysis of results can be readily and easily attained using a previously published free online tool (http://www.comparingpartitions.info). However, the ability of *dru* typing in combination with PFGE and *spa* typing to discriminate among isolates of highly clonal strains of MRSA other than ST22-MRSA-IV remains to be determined.

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