LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

**Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin**

**Copyright statement**

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

**Liability statement**

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

**Access Agreement**

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

# Comparative Genetic Analysis of Nosocomial Methicillin-Resistant *Staphylococcus aureus* Populations in Ireland 1971-2004

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

**Anna Shore**

**April 2005**

**Microbiology Research Unit, Department of Oral Medicine and Pathology, School of Dental Science, Dublin Dental School and Hospital,**

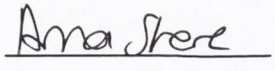
**Trinity College**

**University of Dublin**

TRINITY COLLEGE
13 NOV 2006
LIBRARY DUBLIN
THOS 8
8014

# Declaration

I hereby declare that this thesis has not previously been submitted for a degree at this or any other University and that it represents my own unaided work, except where duly acknowledged in the text. I agree that this thesis may be lent or copied at the discretion of the librarian, Trinity College Dublin.



Anna Shore

*" It is time to close the book on infectious diseases, declare the war against pestilence won..."*

U.S. Surgeon-General William H. Stewart, **1967**

# Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most prevalent pathogen responsible for nosocomial infections worldwide and Ireland has one of the highest rates of nosocomial MRSA infections in Europe. In the past, phenotypic epidemiological typing techniques such as routine antimicrobial susceptibility testing and phage typing, and more recently, pulsed-field gel electrophoresis (PFGE), have been used to monitor the MRSA population in Irish hospitals. The aim of the present study was to perform a comprehensive molecular analysis of an extensive collection of MRSA isolates recovered from patients in Irish hospitals between 1971 and 2004, in order to assess the genetic relationships of nosocomial MRSA in Ireland since its first isolation over thirty years ago. Isolates were also investigated for the prevalence of lysogenic single-, double- and triple- converting serotype F phages, which encode the important virulence factors enterotoxin A (EntA) and staphylokinase (Sak).

A combination of multilocus sequence typing (MLST), which determines the genetic background of bacterial isolates and analysis of the staphylococcal chromosomal cassette (SCC*mec*) element, which carries the genetic determinant for methicillin-resistance *mecA*, was used in the present study to characterise the MRSA isolates. In addition, PFGE was performed on MRSA isolates collected during a surveillance study in 1999 to examine the relationship between isolates exhibiting a newly recognised phenotype (AR43) and isolates with other phenotypes collected during the same study.

MLST revealed that 160/162 isolates tested had the same genotypes as internationally spread MRSA clones including the Hungarian (ST239), Iberian (ST247), Archaic (ST250), New York/Japan (ST5), EMRSA-15 (ST22), EMRSA-16 (ST36) and Irish-1/Irish-2 (ST8) clones. The remaining two isolates exhibited the novel genotypes ST496 and ST609. Using two different SCC*mec* typing methods, approximately half of the isolates (88/177) tested were found to harbour SCC*mec* types I, IA, II, III or IV and two isolates were found to harbour no SCC*mec* element. The remaining 87 isolates (ca. 49%) harboured novel and previously undescribed SCC*mec* variants in three distinct genetic backgrounds; (i) 74/87 isolates had the ST8 genotype and either one of five novel SCC*mec* II variants (IIA, IIB, IIC, IID and IIE) or one of two novel SCC*mec* IV variants (IVE and IVF); (ii) 4/87 isolates had the ST239 genotype and a novel SCC*mec* III variant IIIC; (iii) 9/87 isolates had a novel SCC*mec* I variant associated with ST250.

Nucleotide sequencing revealed that the novel SCC*mec* elements IVE and IVF were similar to SCC*mec* IVc and IVb, respectively, but differed in the region downstream of *mecA*. The five SCC*mec* II variants were similar to SCC*mec* IVb in the region upstream of the *ccr* complex but otherwise were similar to SCC*mec* II except for the following regions: SCC*mec* IIA and IID had a novel *mec* complex, A.4 (Δ*mecI*-IS*1182*-Δ*mecI*-*mecR1*-*mecA*-IS*431mec*); SCC*mec* IIC and IIE had a novel *mec* complex, A.3 (IS*1182*-Δ*mecI*-*mecR1*-*mecA*-*IS431mec*); SCC*mec* IID and IIE lacked pUB110; SCC*mec* IIC and IIE lacked a region of DNA between Tn*554* and the *mec* complex; SCC*mec* IIB lacked Tn*554*. The novel SCC*mec* element IIIC was similar to SCC*mec* III but differed by the absence of a ca. 3kb region found between pI258 and Tn*554* in SCC*mec* III.

PFGE analysis of isolates from 1999 (n=67) revealed that AR43 isolates were possibly related to isolates with the phenotypes AR13 and AR14. This was confirmed by MLST where all AR13, AR14 and AR43 isolates were found to have the ST8 genotype. However, while all AR13 and AR14 isolates harboured SCC*mec* II variants (IIA-IIE) all AR43 isolates harboured novel SCC*mec* IV variants (IVE or IVF).

The majority of MRSA isolates investigated (106/110, 96%) were found to harbour lysogenic serotype F phage. Almost all of these isolates produced the phage encoded virulence factor Sak (103/110, 94%) and in addition over half of these isolates (58/110, 53%) also produced the phage encoded virulence factor and superantigen EntA. Through extensive genetic analysis it was also identified that two multiresistant MRSA isolates recovered from patients in different Irish hospitals in 1999 failed to express Sak due to insertional inactivation of the phage encoded *sak* gene by the insertion sequence element IS*256*. These two isolates harboured serotype F triple-converting phages but the *sak* gene could not be expressed due to the presence of this insertion sequence within *sak*.

The present study demonstrated for the first time that most of the major international and pandemic clones of MRSA have spread to Ireland over the last 30 years and that major changes have occurred in the dominant clonal types of MRSA in Irish hospitals during this time period. In addition, a hitherto unexpected degree of diversity within SCC*mec* was revealed. Nine novel SCC*mec* variants were identified and comprehensively characterised and it was revealed that these variants may be the result of significant recombination and rearrangements between different SCC*mec* elements. The present study has also identified that over half of the Irish MRSA isolates

investigated produced the virulence factor EntA, which is a superantigen known for its role in the pathogenesis of *S. aureus* infections. Finally, the present study identified the first ever case of insertional inactivation of a phage encoded virulence gene by an insertion sequence element.

# Table of Contents


Acknowledgements I
Abbreviations II
Publication V

## Chapter 1 Introduction

**1.1 *Staphylococcus aureus* 2**
**1.2 Methicillin-resistant *Staphylococcus aureus* 2**
1.2.1 The emergence of methicillin resistance in *S. aureus* 2
1.2.1.1 Vancomycin resistant *S. aureus* (VRSA) 4
1.2.1.2 Community acquired MRSA (CAMRSA) 5
1.2.2 The mechanism of methicillin resistance in *S. aureus* 6
1.2.3 Staphylococcal chromosomal cassette *mec* 6
1.2.3.1 The cassette chromosome recombinase (*ccr*) gene complex 7
1.2.3.2 The *mec* gene complex 7
1.2.3.3 SCC*mec I* 9
1.2.3.4 SCC*mec II* 10
1.2.3.5 SCC*mec III* 10
1.2.3.6 SCC*mec IV* 11
1.2.3.7 SCC*mec V* 11
1.2.3.8 The origins and evolution of SCC*mec* 12
1.2.4 Additional factors affecting methicillin resistance 13
1.2.5 Evolution of MRSA 13
1.2.6 Epidemic MRSA (EMRSA) 14
1.2.7 MRSA in Ireland 15
1.2.8 Financial cost of MRSA 16
**1.3 MRSA typing methods 16**
1.3.1 Phenotypic typing methods 17
1.3.1.1 Phage typing 17
1.3.1.2 Antimicrobial susceptibility testing 17
1.3.1.3 Other phenotypic typing methods 18
1.3.2 Genotypic typing methods 19
1.3.2.1 Restriction analysis of chromosomal DNA 19
1.3.2.2 Pulsed-field gel electrophoresis 20
1.3.2.3 Multilocus sequence typing 21
1.3.2.4 SCC*mec* typing 21
1.3.2.5 Protein A and coagulase gene typing 22
1.3.2.6 DNA microarrays 22
**1.4 Bacteriophage 23**
1.4.1 Virulent and temperate bacteriophages 23
1.4.2 Lysogenic conversion in *S. aureus* 23
**1.5 Aims of the present study 24**

## Chapter 2 Materials and Methods


**2.1. Bacterial isolates** **27**
2.1.1 Clinical MRSA isolates 27
2.1.1.1 Hospitals 29
2.1.2 *Escherichia coli* strains 29
**2.2 General microbiological methods** **30**
2.2.1 Bacterial culture media, growth conditions and storage 30
2.2.2 Chemicals, enzymes, radioisotopes and oligonucleotides 30
2.2.3 Buffers and solutions 31
**2.3 Analysis of genomic DNA** **31**
2.3.1 Extraction of genomic DNA from *S. aureus* 31
2.3.2 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis 32
2.3.3 Southern transfer of DNA from agarose gels 33
2.3.4 Random primer labelling of DNA fragments with [$\alpha$-$^{32}$P]dATP 33
2.3.5 Southern hybridisation 33
**2.4 Recombinant DNA techniques** **34**
2.4.1 Extraction of plasmid DNA from *E. coli* 34
2.4.2 Polymerase chain reaction (PCR) 34
2.4.3 Ligation of DNA fragments 35
2.4.4 Transformation of competent *E. coli* prepared using $CaCl_2$ 35
**2.5 DNA sequence analysis** **36**


## Chapter 3 The Evolutionary Epidemiology of Irish Nosocomial MRSA Isolates Recovered Between 1971-2004


**3.1 Introduction** **38**
3.1.1 Irish MRSA 38
3.1.1.1 1971-1987 38
3.1.1.2 1988-1998 39
3.1.1.3 1999-2004 40
3.1.2 Investigating the genetic relatedness of MRSA isolates 41
3.1.3 Aims 42
**3.2 Materials and Methods** **43**
3.2.1 Pulsed-field gel electrophoresis (PFGE) 43
3.2.1.1 Bacterial isolates 43
3.2.1.2 Preparation of *S. aureus* chromosomal DNA in agarose plugs 43
3.2.1.3 Restriction endonuclease digestion of chromosomal DNA embedded in agarose plugs 44
3.2.1.4 Agarose gel electrophoresis 44
3.2.1.5 Analysis and interpretation of DNA restriction patterns produced by PFGE 44
3.2.2 Multilocus Sequence Typing 45
3.2.2.1 Bacterial isolates 45
3.2.2.2 MLST method 46
3.2.3 SCC*mec* typing 47
3.2.3.1 Bacterial isolates 47
3.2.3.2 Simplex SCC*mec* typing 48

3.2.3.3 Multiplex SCC*mec* typing 48
**3.3 Results** **50**
3.3.1 MLST 50
3.3.2 SCC*mec* typing 51
3.3.2.1 Simplex SCC*mec* typing 51
3.3.2.1.1 SCC*mec* I 51
3.3.2.1.2 SCC*mec* II 51
3.3.2.1.3 SCC*mec* III 51
3.3.2.1.4 SCC*mec* IV 52
3.3.2.2 Multiplex SCC*mec* typing 52
3.3.2.2.1 Identification of variant SCC*mec* types 52
3.3.3 MRSA clonal types 54
3.3.4 Pulsed-field gel electrophoresis 54
3.3.4.1 Group I 55
3.3.4.2 Group II 55
3.3.4.3 Group III 55
3.3.4.4 Group IV 55
**3.4. Discussion** **57**

**Chapter 4 Molecular Characterisation of Nine Novel SCC*mec* Variants Present in Irish Nosocomial Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates Recovered Between 1971-2004**

**4.1 Introduction** **68**
4.1.1 Aims 69
**4.2 Materials and Methods** **71**
4.2.1 Bacterial isolates 71
4.2.2 Investigation of the variant SCC*mec* elements harboured by ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns 71
4.2.2.1 Nucleotide sequencing of the entire SCCmec of two ST8 isolates 71
4.2.2.1.1 PCR amplification of the L-C region 73
4.2.2.1.2 PCR amplification of the C-M region 73
4.2.2.1.3 PCR amplification of the M-I and I-R regions 73
4.2.2.2 Investigation of the variant SCC*mec* elements in all other ST8 isolates 74
4.2.3 Investigation of the variant SCC*mec* elements harboured by ST239 isolates with unusual SCC*mec* III multiplex patterns 74
4.2.4 Investigation of the variant SCC*mec* elements harboured by ST250 isolates with unusual SCC*mec* I multiplex patterns 75
4.2.5 Nucleotide sequence submission to the Genbank Database 76
**4.3 Results** **78**
4.3.1 Investigation of the SCC*mec* elements harboured by ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns 78
4.3.3.1 Novel variant of SCC*mec* II identified in ST8 isolate AR13.1/3330.2 78
4.3.3.2 Novel variant of SCC*mec* IV identified in ST8 isolate AR43/3330.1 80

4.3.3.3 Determination of the genomic organisation of the SCC*mec* elements of the remaining Irish MRSA ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns 81
4.3.3.3.1 Additional novel variants of SCC*mec* II 82
4.3.3.3.2 Additional novel variants of SCC*mec* IV 84
4.3.2 Investigation of the SCC*mec* elements harboured by ST239 isolates with unusual multiplex pattern SCC*mec* I –pI258/Tn*554* 84
4.3.3 Investigation of the SCC*mec* elements harboured by ST250 isolates with the unusual multiplex pattern SCC*mec* I – *pls* 85
**4.4 Discussion 88**

**Chapter 5 Prevalence of Serotype F Converting Phages among Irish Nosocomial MRSA Isolates**

**5.1 Introduction 94**
5.1.1 Serotype F phages of *S. aureus* 94
5.1.2 Aims 95
**5.2 Materials and Methods 96**
5.2.1 *Staphylococcus aureus* isolates and strains 96
5.2.1.1 MRSA isolates 96
5.2.1.2 *Staphylococcus aureus* control strains 96
5.2.2 Extracellular protein assays 97
5.2.2.1 Plate assay for β-toxin 97
5.2.2.2 Plate assay for staphylokinase 97
5.2.2.3 Detection of enterotoxin A production 98
5.2.3 Southern hybridisation to detect *entA*, *hlb* and *sak* genes 99
5.2.3.1 *entA* 99
5.2.3.2 *sak* 99
5.2.3.3 *hlb* 99
5.2.4 PCR detection of *sak* and *ent A* genes 100
5.3.5 Cloning of the *sak* gene 100
**5.3 Results 101**
5.3.1 β-toxin (Hlb), staphylokinase (Sak) and enterotoxin A (EntA) production by Irish MRSA isolates 101
5.3.2 Molecular investigation into the $Hlb^-$, $Sak^-$, Ent $A^+$ phenotype expressed by two Irish nosocomial MRSA isolates 101
5.3.2.1 Southern hybridisation analysis of the *entA*, *hlb*, and *sak* structural genes 101
5.3.2.1.1 The entA *structural gene* 101
5.3.2.1.2 *The hlb structural gene* 102
5.3.2.1.3 *The sak structural gene* 102
5.3.2.2 *PCR amplification of the entA and sak genes* 103
5.3.2.3 *Cloning of the sak gene of the two isolates AR14/3448 and AR14/3685* 103
**5.4 Discussion 105**

## Chapter 6 General Discussion


**6.1 General Discussion** **110**
6.1.1 The evolutionary epidemiology of MRSA in Ireland 110
6.1.2 Variants of SCC*mec* in Irish MRSA isolates 111
6.1.3 Controlling the spread of MRSA 113
6.1.4 Treatment of MRSA infections 114
6.1.5 Serotype F bacteriophage harboured by Irish MRSA isolates 116
**6.2. Concluding remarks** 116

**References** **118**

**Publication** **158**

# Index of Figures

Page numbers refer to the text page preceding the figure


| Figure | Title | Page |
|---|---|---|
| 1.1 | Chronological time line of the major events in the emergence of antibiotic resistance in *S. aureus* | 3 |
| 1.2 | Methicillin-resistant and methicillin-susceptible *S. aureus* (MRSA & MSSA, respectively) isolates streaked on Columbia Blood Agar (CBA) plates with methicillin disks | 3 |
| 1.3 | Schematic diagram of the genetic organisation of the *mec* complexes of staphylococci | 7 |
| 1.4 | Schematic diagram showing the genomic organisation of SCC*mec* elements I, II, III, IV and V | 9 |
| 3.1 | Dendrogram showing the percent relatedness of DNA from MRSA isolates representative of the most common AR types recovered from patients in Irish hospitals between 1989 and 1998 | 39 |
| 3.2 | Dendrogram showing the percent relatedness of DNA from MRSA isolates showing AR types AR06, AR07.0, AR07.4, AR13, AR14 and AR43 investigated during the North South study of MRSA in Ireland 1999 | 40 |
| 3.3 | SCC*mec* multiplex patterns and variant multiplex patterns found in Irish MRSA isolates | 52 |
| 3.4 | Fingerprint patterns obtained by PFGE following *Sma*I digestion of total genomic DNA from isolates with AR patterns AR06, AR07.0, AR07.4, AR13, AR14 and AR43 recovered in Irish hospitals in 1999 | 54 |
| 3.5 | Dendrogram generated from the similarity coefficients ($S_{AB}$s) computed for every possible pairwise combination of 67 MRSA isolates exhibiting AR types AR06, AR07.0, AR07.4, AR13, AR14 and AR43 recovered in Irish hospitals during a 2 week period in 1999. | 55 |
| 4.1 | Schematic diagram of the genomic organisation of SCC*mec* II and SCC*mec* IVa showing the approximate location and the direction of primer sequences used in this study to amplify the novel SCC*mec* elements of Irish ST8 isolates | 71 |
| 4.2 | Schematic diagram of the genomic organisation of SCC*mec* III showing the approximate location and direction of primer sequences used in this study to amplify the region from *mer* to Tn*554* of ST239 isolates with the SCC*mec* III - pI258/Tn*554* multiplex pattern | 75 |

| | | |
|---|---|---|
| 4.3 | Schematic diagram of the genomic organisation of SCC*mec* I showing the approximate location and the direction of primer sequences used in this study to attempt to amplify the L-C region of SCC*mec* of ST250 isolates with the SCC*mec* I - *pls* multiplex pattern | 75 |
| 4.4 | Schematic diagram showing the genomic organisation of SCC*mec* II, IIA, IIB, IIC, IID, IIE, IVa, IVb, IVc, IVE and IVF elements | 78 |
| 4.5 | Schematic diagram of the genetic organization of the *mec* complexes of staphylococci | 78 |
| 4.6 | Alignment of the nucleotide sequences of the *mecI* gene (3'-5' direction) of the class A.4 *mec* complex (A.4), class A *mec* (*mecI*) and class A.3 *mec* complex (A.3) generated using the ClustalW sequence alignment program | 79 |
| 4.7 | Alignment of the nucleotide sequences of the hypervariable region (region between *mecA* and IS*431mec* of SCC*mec*) of SCC*mec* II (HVII) and SCC*mec* IIE (HVIIE) generated using the ClustalW sequence alignment program | 79 |
| 5.1 | *Eco*RI and *Hind*III cleavage site maps of the *entA*-, *sak*-containing DNA regions of the triple converting phage ϕ 42 and the *sak* - containing DNA region of the double-converting phage ϕ 13 | 99 |
| 5.2 | Restriction map of the 2.2 kb *Hind*III *hlb*-encoding fragment of plasmid pDC007 and the two *Hind*III fragments generated at the *S. aureus* chromosomal/ bacteriophage DNA junctions following lysogenisation with ϕ13 | 99 |
| 5.3 | Map of the 2.2 kb *sak* encoding *Hind*III/*Eco*RI DNA region and 2.85 kb Hind*III* DNA region of ϕ13 | 100 |
| 5.4 | Southern hybridisation analysis of *Hind*III digested chromosomal DNA of *S. aureus* laboratory strains and the two MRSA isolates AR14/3448 and AR14/3685 with the phenotype $Hlb^-$, $Sak^-$, $EntA^+$ | 101 |
| 5.5 | PCR amplification products obtained using primers to amplify the *sak* gene in MRSA isolates AR14/3448 and AR14/3685 with the $Hlb^-$, $Sak^-$, $EntA^+$ phenotype, electrophoresed on 1% (w/v) agarose gels | 103 |
| 5.6 | Nucleotide sequence of the *sak* gene (5'- 3') cloned from the two MRSA isolates, AR14/3448 and AR14/3685 with the $Hlb^-$, $Sak^-$, $EntA^+$ phenotype | 104 |

5.7 Alignment of the nucleotide sequences of the *sak* gene cloned from the two MRSA isolates with the Hlb$^-$, Sak$^-$, Ent A$^+$ phenotype (clone), the complete *sak* gene (sak) and insertion sequence element IS*256* generated with the CLUSTAL W sequence alignment program 104

5.8 Map of the *sak*-encoding *Hind*III/*Eco*RI DNA region of the two MRSA isolates, AR14/3448 and AR14/3685 with the Hlb$^-$, Sak$^-$, EntA$^+$ phenotype 104

# Index of Tables

Page numbers refer to the text page preceding the table


| Table | Title | Page |
|---|---|---|
| 1.1 | Examples of potential virulence factors of *S. aureus* | 2 |
| 1.2 | Details of the major nosocomial MRSA clones identified within each MRSA genotype | 14 |
| 1.3 | Tenover's guidelines for interpreting PFGE patterns of discrete sets of isolates during potential outbreaks | 20 |
| 1.4 | Examples of lysogenic conversion events reported in *S. aureus* | 23 |
| 2.1 | Clinical MRSA isolates used in this study | 27 |
| 2.2 | Antibiogram-Resistogram (AR) patterns exhibited by MRSA isolates used in this study | 27 |
| 3.1 | The most predominant phenotypes of MRSA identified in Irish hospitals during different time periods since 1971 | 41 |
| 3.2 | Summary of the total number of Irish MRSA isolates investigated from each study period and numbers typed by PFGE, MLST and SCC*mec* typing | 43 |
| 3.3 | MRSA clinical isolates that were not typed by MLST | 45 |
| 3.4 | Primer pairs used for amplification of the seven housekeeping genes used in MLST | 46 |
| 3.5 | Primer pairs used for SCC*mec* typing | 48 |
| 3.6 | Amplimer sizes expected for various SCC*mec* elements using the multiplex SCC*mec* typing method | 49 |
| 3.7 | Multilocus sequence typing and SCC*mec* typing results of nosocomial MRSA isolate recovered in Ireland 1971-2004 | 50 |
| 3.8 | The allelic profiles of the STs identified in Irish nosocomial MRSA isolates recovered between 1971 and 2004 | 50 |
| 3.9 | The CCs, STs, SCC*mec* types, phenotypes and original isolate numbers of those isolates from phenotypic groups that demonstrated more than one ST or SCC*mec* type | 50 |
| 4.1 | Summary of unusual SCC*mec* typing results | 68 |

| | | |
|---|---|---|
| 4.2 | Primers used in this study for SCC*mec* element amplification and sequencing from ST8 isolates | 71 |
| 4.3 | Primers used in this study for amplification and sequencing of part of the SCC*mec* element from ST250 and ST239 isolates with atypical SCC*mec* multiplex patterns | 75 |
| 4.4 | Correlation between SCC*mec* multiplex types and amplimer sizes obtained using overlapping primer pairs to amplify the novel SCC*mec* element variants and comparison to those expected from typical SCC*mec* II and SCC*mec* IV elements | 78 |
| 4.5 | Percentage identities between ORFs of the novel SCC*mec* II variant element of Irish MRSA isolate AR13.1/3330.2 and the conventional SCC*mec* IVb and II elements | 79 |
| 4.6 | Percentage identities between ORFs of the novel SCC*mec* IV variant element IVE of Irish MRSA isolate AR43/3330.1 and the conventional SCC*mec* IVc element | 80 |
| 4.7 | Summary of the variant SCC*mec* elements identified in Irish nosocomial MRSA isolates recovered between 1971-2004 | 82 |
| 4.8 | Percentage identities between ORFs investigated between pI258 and Tn*554* of the novel SCC*mec* III variant element IIIC of Irish MRSA isolate AR09/0065 and the conventional SCC*mec* III element | 84 |
| 5.1 | Clinical MRSA isolates that were investigated for the carriage of serotype F phage | 96 |
| 5.2 | Primers used to amplify *sak* and *entA* structural genes | 100 |
| 5.3 | β-toxin (Hlb), staphylokinase (Sak) and enterotoxin A (EntA) production by Irish nosocomial MRSA isolates recovered between 1971 and 2002 | 101 |
| 5.4 | Correlation between clonal types and β-toxin (Hlb), staphylokinase (Sak) and enterotoxin A (EntA) production of Irish nosocomial MRSA isolates recovered between 1971 and 2002 | 106 |

# Acknowledgements

I would like to thank my supervisor Prof. David Coleman for all the encouragement, expert advice and support that he offered to me throughout my research. I am also very grateful to Dr. Angela Rossney for her constant support, expertise, encouragement and never-ending patience throughout this project. Thanks are also due to Dr. Derek Sullivan for additional guidance and advice.

I acknowledge the financial support received from the Applied Microbiology Fund of the Microbiology Research Unit in the Dublin Dental School and Hospital. I acknowledge the participants and steering groups of both the North/South Study of MRSA in Ireland and EARSS in Ireland for allowing me to use some of the isolates collected during these two studies. I also acknowledge that I was in receipt of a Marie Curie Research Grant to undertake four months of research at the University of Bath. I thank the technical staff of the Moyne prep. room for supplying media and clean glassware.

I am very grateful to both Dr. Mark Enright and Dr. Angus Buckling for giving me the opportunity to work in their respective laboratories over a four month period. Thanks to everyone in the Biology Department at the University of Bath for their kindness during my stay. Thanks to Paul for all his great MLST technical advice.

A special thank you to everyone I have worked with over the past few years in the Microbiology laboratory; Asmaa, Caroline, Cheryl, Claire T, Claire V, Deirdre, Emmanuelle, Gary, Janos, Leanne, Mary, Nicole, Rachael and Tim. Thanks to all of you not only for your technical assistance and advice but also for the friendships we have formed and great laughs that we always have.

Thank you to all my friends who are always a welcome distraction. Thanks to JP for all his encouragement and patience. Finally, I would like to sincerely thank my family especially my parents for everything they have done for me over the years. Thank you for your love and support.

# Abbreviations

| | |
|---|---|
| $A_{260}$ | absorption at 260 nm |
| $A_{600}$ | absorption at 600 nm |
| AR | antibiogram-resistogram |
| | |
| $\phi$ | bacteriophage |
| $\beta$ | beta |
| bp | base pair |
| Bq | becquerel |
| BSA | bovine serum albumin |
| | |
| CAMRSA | community-acquired methicillin-resistant *Staphylococcus aureus* |
| CC | clonal complex |
| CDC | Center for Disease Control and Prevention |
| | |
| *ccr* | cassette chromosome recombinase gene complex |
| Ci | curie |
| CoNS | coagulase negative staphylococci |
| | |
| $\Delta$ | deletion (of part of a gene) |
| dlv | double locus variant |
| DNA | deoxyribonucleic acid |
| dNTP | dideoxynucleoside |
| | |
| EARSS | European Antimicrobial Resistance Surveillance System |
| EDTA | ethylenediamine tetraacetic acid |
| e.g. | for example |
| EMRSA | Epidemic methicillin-resistant *Staphylococcus aureus* |
| EntA | enterotoxin A |
| *et al.* | and others |
| | |
| FDVH | Federated Dublin Voluntary Hospitals |
| | |
| g | gram |
| x *g* | gravity |
| | |
| h | hour(s) |
| | |
| Hlb | beta-toxin |
| hVISA | heterogeneously vancomycin-intermediate *Staphylococcus aureus* |
| | |
| i.e. | that is |
| | |
| JSH | Jervis Street Hospital |
| | |
| kb | kilobase pair |
| kg | kilogram |

| | |
|---|---|
| l | litre(s) |
| L agar | Luria-Bertani agar |
| L broth | Luria-Bertani broth |
| | |
| M | molar |
| *mec* | methicillin resistance gene complex |
| mg | milligram |
| mM | millimolar |
| MIC | minimum inhibitory concentration |
| min | minute |
| ml | millilitre |
| MLEE | multilocus enzyme electrophoresis |
| MLST | multilocus sequence typing |
| MMH | Mater Misericordiae Hospital |
| MRSA | methicillin-resistant *Staphylococcus aureus* |
| | |
| ng | nanogram |
| NMRSARL | National MRSA Reference Laboratory, Ireland |
| No. | number |
| | |
| ORF | open reading frame |
| | |
| PBP | penicillin binding protein |
| PCR | polymerase chain reaction |
| PFGE | pulsed-field gel electrophoresis |
| | |
| r.p.m. | revolution per minute |
| | |
| s | second |
| Sak | staphylokinase |
| SBA | sheep blood agar |
| SCC | staphylococcal chromosomal cassette |
| SCC*mec* | staphylococcal chromosomal cassette *mec* |
| SDS | sodium dodecyl sulphate |
| slv | single locus variant |
| SSSS | staphylococcal scalded skin syndrome |
| ST | sequence type |
| | |
| TBE | tris-borate EDTA |
| Tris | tris (hydroxymethyl)aminomethane |
| TSA | trypticase soy agar |
| TSB | trypticase soy broth |
| TSS | toxic shock syndrome |
| | |
| U | unit |
| $\mu$g | microgram |
| UK | United Kingdom |
| $\mu$l | microlitre |
| USA | United States of America |

| | |
|---|---|
| VISA | vancomycin-intermediate *Staphylococcus aureus* |
| VRSA | vancomycin-resistant *Staphylococcus aureus* |
| v/v | % volume in volume: expresses the number of millilitres of an active constituent in 100 millilitres of solution |
| w/v | % weight in volume: expresses the number of grams of an active constituent in 100 millilitres of solution |
| X-gal | 5-bromo-4-chloro-indoyl-β-D-galactoside |
| > | greater than |
| < | less than |
| ≥ | greater than or equal to |
| ≤ | less than or equal to |

# Publication

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 manuscript are included at the end of the thesis.

**Shore, A., Rossney, A.S., Keane, C.T., Enright, M.C. & Coleman, D.C (2005).** Seven novel variants of the staphylococcal chromosomal cassette *mec* in methicillin-resistant *Staphylococcus aureus* from Ireland. *Antimicrob. Agents Chemother.* **49 (5)**, 2070-2083.

# Chapter 1
# Introduction

## *1.1 Staphylococcus aureus*

The staphylococci are Gram-positive bacteria that belong to the Micrococcaceae family. Although many species of staphylococci have been described, *Staphylococcus aureus* is considered to be the most pathogenic. It is a major human pathogen and one of the leading causes of nosocomial and community acquired infections worldwide. It is a common coloniser of humans, where it can be carried in the anterior nares in up to 40% of healthy individuals (Van Belkum *et al.*, 1997).

*Staphylococcus aureus* expresses many potential virulence factors including both secreted and cell surface proteins (Table 1.1). The secreted proteins include diverse enzymes and toxins that damage host tissue and cells (Projan & Novick, 1997) (Table 1.1). Cell surface proteins include microbial surface components recognising adhesive matrix molecules (MSCRAMMs), which promote host tissue colonisation and factors that aid in establishing infection by allowing the bacteria to evade the host immune system (Projan & Novick, 1997; Foster & Hook, 1998) (Table 1.1).

Due to this plethora of virulence factors, *S. aureus* is capable of causing a wide range of diseases, from superficial skin lesions to severe and life threatening systemic infections (Boyce, 1997). It is a common cause of skin and subcutaneous infections, including foliculitis, furunculosis, mastitis and impetigo. *Staphylococcus aureus* also causes meningitis, pneumonia, urinary tract infections, phlebitis, food poisoning, toxic shock syndrome (TSS) and the blistering skin diseases collectively known as staphylococcal scalded skin syndrome (SSSS). It is the most common hospital acquired organism and is commonly associated with postoperative wound infections, catheter related infections and with persons whose immunity is compromised due to chronic illness, immunosupression, burns or traumatic injury. In these types of individuals it can cause deep-seated infections such as osteomyelitis, endocarditis and overwhelming septicaemia (Lowy, 1998).

## 1.2 Methicllin Resistant *Staphylococcus aureus* (MRSA)

### 1.2.1 The emergence of antibiotic resistance in *S. aureus*

Staphylococcal disease and *S. aureus* were first described in the late 19$^{th}$ century and were associated with high mortality rates (Ogston, 1882; Sheagren 1984a & 1984b; Reacher *et al.*, 2000). It was almost 60 years later, with the development of penicillin

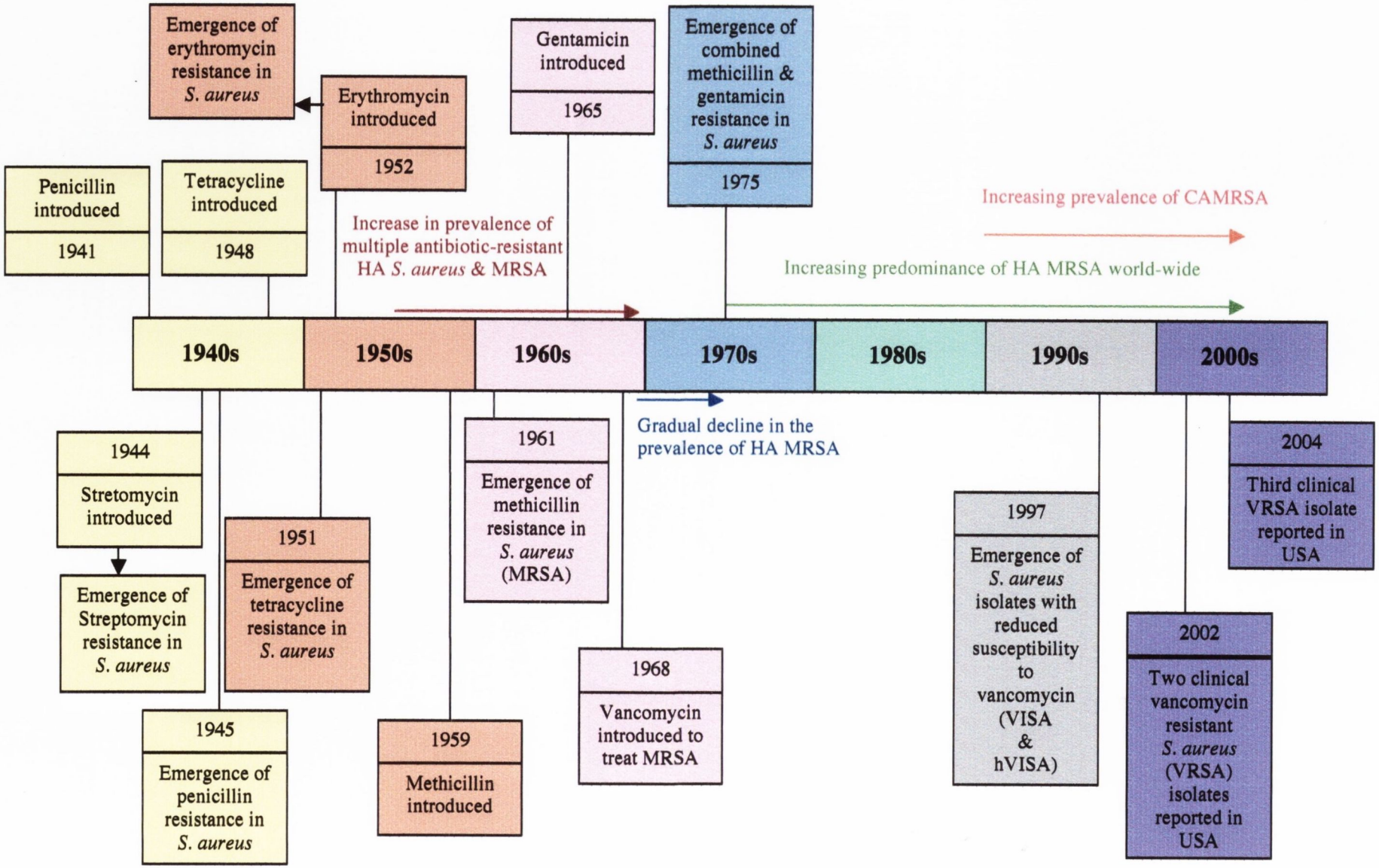
**TABLE 1.1.** Examples of potential virulence factors of *S. aureus*

| **Virulence Factors** | **Biological effect** | **Reference** |
|---|---|---|
| Secreted Proteins | | |
| Staphylokinase | Dissolves fibrin clots, promotes bacterial spread in the tissue by breaking down fibrin barriers surrounding the site of infection, induces secretion of defensins and neutralises their bactericidal activity | (Lahteenmaki *et al.*, 2001; Jin *et al.*, 2004) |
| Lipase | Hydrolyses lipids | (Abdelnour *et al.*, 1993) |
| Hyaluronidase | Hydrolyses hyaluronic acid in connective tissue | (Abdelnour *et al.*, 1993) |
| Cytotoxins α, β, δ, γ & leukocidin | Toxic to erythrocytes, leukocytes, macrophages, fibroblasts & platelets & causes damage to host tissue | (Dinges *et al.*, 2000) |
| Enterotoxins | Superantigens: stimulate mast cells & cause damage to host tissue | (Dinges *et al.*, 2000) |
| Exfoliative toxin | Superantigen: stimulate T cells to release cytokine & cause damage to host tissue | (Dinges *et al.*, 2000) |
| Toxic Shock Syndrome toxin | Superantigen: stimulate T cells to release cytokine & cause damage to host tissue | (Dinges *et al.*, 2000) |
| Surface proteins | | |
| Fibronectin binding protein A & B | Mediate attachment to fibronection & invasion of mammalian cells | (Foster & Hook, 1998) |
| Collagen binding protein | Mediates attachment to collagen | (Foster & Hook, 1998; Rhem *et al.*, 2000) |
| Fibrinogen binding protein (clumping factor) | Mediates attachment to fibrinogen & protect against macrophage phagocytosis | (Foster & Hook, 1998; Palmqvist *et al.*, 2004) |
| Capsular polysacharide | Inhibit phagocytic engulfment & chemotaxis | (Luong & Lee, 2002) |
| Protein A | Inhibits antibody mediated clearance & interacts with platelets | (Forsgren & Sjoquist, 1966; Nguyen *et al.*, 2000; Palmqvist *et al.*, 2002) |

antibiotics for clinical use in the 1940's that a dramatic decrease in morbidity and mortality due to *S. aureus* infections was observed (Brumfitt & Hamilton-Miller, 1989). However, soon after its introduction, resistance to penicillin G and structurally related penicillins emerged in many *S. aureus* isolates, due to the production of β-lactamase, a penicillinase enzyme that degrades the β-lactam ring of this class of antibiotics (North & Christie, 1946) (Fig. 1.1). By the late 1940's over half of nosocomial *S. aureus* strains were β-lactamase producers (Baber & Rozadowska, 1948). Other antibiotics were also discovered and introduced into clinical practice in the 1940's and 1950's including tetracycline, streptomycin and macrolides, but *S. aureus* isolates rapidly developed resistance to these agents (Fig. 1.1).

New semi-synthetic, penicillinase resistant β-lactam antibiotics such as methicillin, oxacillin and flucoxacillin were subsequently developed and rapidly became the drugs of choice for anti-staphylococcal therapy. In 1961, just two years after the introduction of methicillin into clinical practice, the first methicillin-resistant isolates of *S. aureus* were described in England (Jevons, 1961) (Figs. 1.1 & 1.2). Many methicillin-resistant *S. aureus* (MRSA) isolates were resistant to multiple antibiotics and caused severe invasive infections and outbreaks in many hospitals across Europe in the 1960's (Shanson, 1981) (Fig. 1.1). In the early 1970's, the prevalence of MRSA decreased but this was followed by an increase in predominance in the late 1970's and into the 1980's, not only in many European countries but in the USA (Klimek *et al.*, 1976; Haley *et al.*, 1982) and Australia (Turnidge & Bell, 2000) (Fig. 1.1).

Since the 1980's, both the numbers and proportion of MRSA associated with nosocomial infections have increased significantly in many countries worldwide and are a major threat to public health. In the USA the prevalence of MRSA in hospitals increased from 2.4% in 1975, to 29% in 1991, 35% in 1996 and 54.5% in 1999 (Panlilio *et al.*, 1992; Roberts *et al.*, 1998). Similar trends have been reported in many European countries including Ireland and the United Kingdom (Cafferkey *et al.*, 1985; Marples & Cooke, 1985; Speller *et al.*, 1997; Reacher *et al.*, 2000; Rossney & Keane, 2002a). In many Asian countries the levels of MRSA are much worse than in Europe and the USA; in many hospitals in Taiwan and China ca. 60-80% of *S. aureus* isolates are MRSA (Wang *et al.*, 2002; Aires de Sousa *et al.*, 2003b). Today, MRSA is a serious nosocomial problem, causing increased morbidity and mortality in hospitals. One recent study has shown that nosocomial MRSA bloodstream infections result in a two-fold


Emergence of erythromycin resistance in S. aureus
Erythromycin introduced
1952
Gentamicin introduced
1965
Emergence of combined methicillin & gentamicin resistance in S. aureus
1975
Penicillin introduced
1941
Tetracycline introduced
1948
Increase in prevalence of multiple antibiotic-resistant HA S. aureus & MRSA
Increasing prevalence of CAMRSA
Increasing predominance of HA MRSA world-wide
1940s
1950s
1960s
1970s
1980s
1990s
2000s
Gradual decline in the prevalence of HA MRSA
1944
Stretomycin introduced
Emergence of Streptomycin resistance in S. aureus
1945
Emergence of penicillin resistance in S. aureus
1951
Emergence of tetracycline resistance in S. aureus
1959
Methicillin introduced
1961
Emergence of methicillin resistance in S. aureus (MRSA)
1968
Vancomycin introduced to treat MRSA
1997
Emergence of S. aureus isolates with reduced susceptibility to vancomycin (VISA & hVISA)
2002
Two clinical vancomycin resistant S. aureus (VRSA) isolates reported in USA
2004
Third clinical VRSA isolate reported in USA

Abbreviations: HA, hospital acquired; CA, community acquired; MRSA, methicillin-resistant *S. aureus*; VISA, vancomycin intermediate *S. aureus*; hVISA, hetero vancomycin intermediate *S. aureus*; VRSA, vancomycin resistant *S. aureus*.

**FIGURE 1.1.** Chronological time line of the major events in the emergence of antibiotic resistance in *S. aureus*.

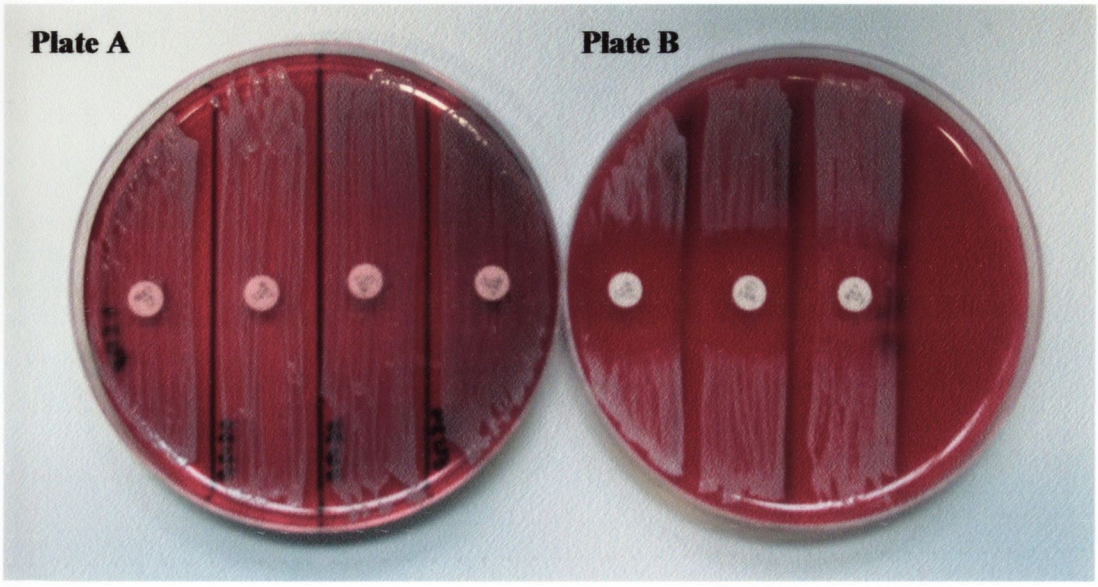



**FIGURE 1.2.** Methicillin-resistant and methicillin-susceptible *S. aureus* (MRSA & MSSA, respectively) isolates streaked on Columbia Blood Agar (CBA) plates with methicillin disks (white disks along the center of each plate). MRSA isolates are capable of growing in the presence of methicillin (Plate A). The growth of MSSA isolates is inhibited by methicillin (Plate B).

increase in attributable mortality when compared to nosocomial methcillin-susceptible *S. aureus* (MSSA) bloodstream infections (Cosgrove *et al.*, 2003).

*1.2.1.1 Vancomycin resistant S. aureus (VRSA)*

Vancomycin is a glycopeptide antibiotic that was introduced into clinical practice in the 1960's and for several decades has been the only drug available to treat infections caused by MRSA isolates that are resistant to multiple antibiotics. The increased prevalence of MRSA led to increased usage of vancomycin and in 1997 the first ever *S. aureus* isolates with reduced susceptibility to vancomycin were reported in Japan (Hiramatsu *et al.*, 1997a & 1997b) (Fig. 1.1). Two types of reduced susceptibility to vancomycin were observed, the so-called vancomycin-intermediate *S. aureus* (VISA) and the hetero- vancomycin-intermediate *S. aureus* (hVISA). Isolates described as VISA have a minimal inhibitory concentration (MIC, the minimum concentration of antibiotic which will inhibit growth of the microorganism) of vancomycin of 8-16 mg/L (Walsh & Howe, 2002). MRSA isolates that are heterogeneously resistant to vancomycin (hVISA) are isolates in which the majority of the bacterial population are susceptible to vancomycin (MIC $\leq$4 mg/L) but a subpopulation of cells are capable of growing in the presence of higher vancomycin concentrations (MIC 8-16mg/L) (Walsh & Howe, 2002). Although the clinical significance of hVISA isolates is unclear, many researchers believe that both hVISA and VISA isolates are associated with treatment failures and that hVISA are precursors of VISA that develop after prolonged exposure to glycopeptides (Kim *et al.*, 2000; Walsh & Howe, 2002).

The reduced susceptibility to vancomycin, seen in both hVISA and VISA isolates, is due to alterations in cell wall biosynthesis in these isolates, resulting in an unusally thickened cell wall capable of binding vancomycin, thus hindering vancomycin reaching its target (Hanaki *et al.*, 1998; Cui *et al.*, 2003). Both hVISA and VISA have been reported in many countries around the world including USA, France, Hong Kong, Ireland, Germany, Korea, Japan, India, Philippines, Singapore, Thailand, Vietnam, South Africa, Belgium and Brazil (Ploy *et al.*, 1998; Sieradzki *et al.*, 1999; Smith *et al.*, 1999; Chesneau *et al.*, 2000; Ferraz *et al.*, 2000; Hood *et al.*, 2000; Kim *et al.*, 2000; Hageman *et al.*, 2001; Oliveira *et al.*, 2001a; Denis *et al.*, 2002; Rossney, 2003a).

Vancomycin-resistant *S. aureus* (VRSA) isolates demonstrate a vancomycin MIC $\geq$32 mg/L and, to date, only three clinical VRSA isolates have been identified (Centre for Disease Control and Prevention (CDC), 2002 & 2004; Chang *et al.*, 2003) (Fig. 1.1).

All three VRSA isolates were identified in the USA but are epidemiologically unrelated and have been found to carry the *vanA* gene which is not found in VISA or hVISA isolates. The *vanA* gene encodes high-level vancomycin resistance in enterococci and although it was demonstrated in 1992 that *vanA* could be transferred from enterococci to *S. aureus* by conjugation *in vitro* (Noble *et al.*, 1992), this resistant determinant was not identified in clinical *S. aureus* isolates until the isolation of the first VRSA isolate in 2002. This first VRSA isolate was recovered from a swab of a catheter exit site of a dialysis patient who had previously received vancomycin treatment. A vancomycin-resistant enterococcus isolate harbouring the *vanA* gene was also recovered from the same swab and further molecular analysis indicated that conjugative transfer of *vanA* very likely had occurred from eneterococcus to *S. aureus*. The emergence of reduced susceptibility and resistance to vancomycin in *S. aureus* is a cause for concern and threatens to compromise further out ability to treat infections caused by *S. aureus*.

*1.2.1.2 Community acquired MRSA (CAMRSA)*

Another worrying aspect in relation to MRSA, is the recent worldwide increase in the frequency of infections due to MRSA acquired in the community (community-acquired MRSA {CAMRSA})(Herold *et al.*, 1998; Maguire *et al.*, 1998; Rings Terry *et al.*, 1998; Stacey *et al.*, 1998; Cookson, 2000; Salmenlinna *et al.*, 2002; Fey *et al.*, 2003; Mongkolrattanothai *et al.*, 2003; Stemper *et al.*, 2004; Rossney *et al.*, 2005) (Fig. 1.1). Community-acquired MRSA are isolates cultured from patients in an outpatient setting or from patients within 48 h of admission to hospital. In addition, in order for MRSA to be defined as CAMRSA it needs to be determined that MRSA was not acquired by the patient as a result of previous exposure to the health care system, such as during previous hospitalisation or nursing home residency or close contact with people who work at, or were recently discharged from, a health care facility. Community-acquired MRSA isolates can be highly virulent and can cause fatal infections in healthy adults and children with no direct ties to healthcare facilities and without the typical risk factors for infections (Herold *et al.*, 1998; CDC, 1999). Recent studies suggest that the majority of CAMRSA strains have not originated in the hospital setting but that they are the result of acquisition of methicillin resistance determinants by MSSA isolates in the community (Okuma *et al.*, 2002; Fey *et al.*, 2003).

### 1.2.2 The mechanism of methicillin resistance in *S. aureus*

The β-lactam antibiotics disrupt bacterial cell wall synthesis and inhibit growth by binding to the enzymatic site of penicillin binding proteins (PBPs), which are cell membrane bound proteins that catalyse peptidoglycan crosslinking in the bacterial cell wall (Georgopapadakou *et al.*, 1986). *Staphylococcus aureus* strains that are susceptible to the β-lactam antibiotics (MSSA) have four PBPs. Methicillin-resistant *S. aureus* strains produce an extra PBP, PBP2a or PBP2', that has decreased β-lactam affinity (Hartman & Tomasz, 1984; Reynolds & Brown, 1985). This PBP2a is only bound at concentrations well above the therapeutic range of β-lactam antibiotics so it can perform all the PBP reactions essential for cell wall assembly when all other β-lactam susceptible PBPs have been inactivated (Hartman & Tomasz, 1984 & 1986).

Penicillin binding protein 2a is encoded by the methicillin resistance gene, *mecA*, that is carried by a novel class of mobile genetic element designated staphylococcal chromosomal cassette *mec* (SCC*mec*), which is not found in MSSA (Beck, 1986; Ito *et al.*, 1999; Katayama *et al.*, 2000). All SCC*mec* elements integrate into the chromosome of MSSA at the same site, $attB_{scc}$ and converts them to MRSA. The $attB_{scc}$ site is at the 3' end of an open reading frame (ORF) of unknown function, designated *orfX*, which is located near the origin of replication of the chromosome, between the genes encoding protein A (*spa*) and a protein involved in purine biosynthesis (*purA*) and is well conserved in *S. aureus* isolates (Kuhl *et al.*, 1978; Ito *et al.*, 2001). When SCC*mec* integrates into the chromosome, it is flanked by direct- and inverted-repeat sequences at either end. The direct repeat is not the result of target duplication but is due to the fact that a 15 bp sequence found in the $attB_{scc}$ is also present at one end of SCC*mec* (Ito *et al.*, 2001). Both ends of SCC*mec* also contain inverted repeats which have been suggested to be recognised by SCC*mec* recombinases responsible for movement of SCC*mec* in and out of the chromosome (Ito *et al.*, 2001).

### 1.2.3 Staphylococcal chromsomal cassette *mec* (SCC*mec*)

To date, five types of the SCC*mec* element (SCC*mec* I, II, III, IV and V) and some variants, have been characterised in MRSA isolates from around the world (Ito *et al.*, 1999 & 2001; Oliveira *et al.*, 2001c; Ma *et al.*, 2002; Oliveira & de Lencastre, 2002a; Ito *et al.*, 2003 & 2004). The fundamental genetic components of each SCC*mec* element are the *mec* gene- and cassette chromosome recombinase (*ccr*) gene-complexes (Ito *et*

*al.*, 2001). It has been suggested previously that the rest of the SCC*mec* element, outside of the *ccr* and *mec* complexes be designated the junkyard (J) region, because it contains genes that are not essential components for SCC*mec* or the bacterial cell in which it resides (Ito *et al.*, 2003). However, this J region can harbour integrated transposons, plasmids and insertion sequences, which can carry resistance genes for various antibiotics and heavy metals. The five SCC*mec* types described to date are defined on the basis of the class of *mec* gene complex and the type of *ccr* complex they possess and variants of each type are defined by the sequences in the J regions.

*1.2.3.1 The cassette chromosome recobinase (ccr) gene complex*

The *ccr* complex consists of the *ccr* genes *ccrA* and *ccrB* in combination (*ccrAB*) or *ccrC* alone, as well as adjacent open reading frames (Katayama *et al.*, 2000; Ito *et al.*, 2004). The *ccrAB* and *ccrC* genes encode polypeptides with homology to the invertase resolvase family and are necessary for orientation specific integration and accurate excision of the SCC*mec* element. Five allotypes of the *ccr* complex have been described to date; *ccrAB*1 (type 1 *ccr*), *ccrAB2* (type 2 *ccr*), *ccrAB3* (type 3 *ccr*), *ccrAB4* (type 4 *ccr*) and *ccrC* (type 5 *ccr*) (Ito *et al.*, 2001 & 2004; Oliveira *et al.*, 2001c).

*1.2.3.2 The mec gene complex*

The *mec* complex consists of an intact copy of the *mecA* gene encoding PBP2a, an integrated copy of insertion sequence element IS*431mec* downstream of *mecA*, and, when present, complete or truncated *mec* regulatory genes *mecI* and *mecR1* upstream of *mecA* (Hiramatsu *et al.*, 2001) (Fig. 1.3). The *mec* gene complex encodes inducible methicillin resistance. The *mecI* gene encodes a *mecA* transcription repressor protein, MecI, and *mecR1* encodes a signal transducer protein with antirepressor activity, MecR1 (Hiramatsu *et al.*, 1992; Hurlimann-Dalei *et al.*, 1992; Zhang *et al.*, 2001). The predicted proteins of both *mecI* and *mecR1* are homologous with those of the *blaZ* regulatory genes, *blaI* and *blaR1*, respectively (Hiramatsu *et al.*, 1992; Kuwahara-Arai *et al.*, 1996). The *blaZ* gene encodes β-lactamase, which is responsible for hydrolysing the β-lactam ring in certain β-lactam antibiotics (Gregory *et al.*, 1997). Both MecI and BlaI are repressors of *mecA* as well as *blaZ* transcription (Lewis & Dyke, 2000; McKinney *et al.*, 2001). The sensor transducer molecules MecR1 and BlaR1 cannot substitute for each other because they are specific for their cognate repressors

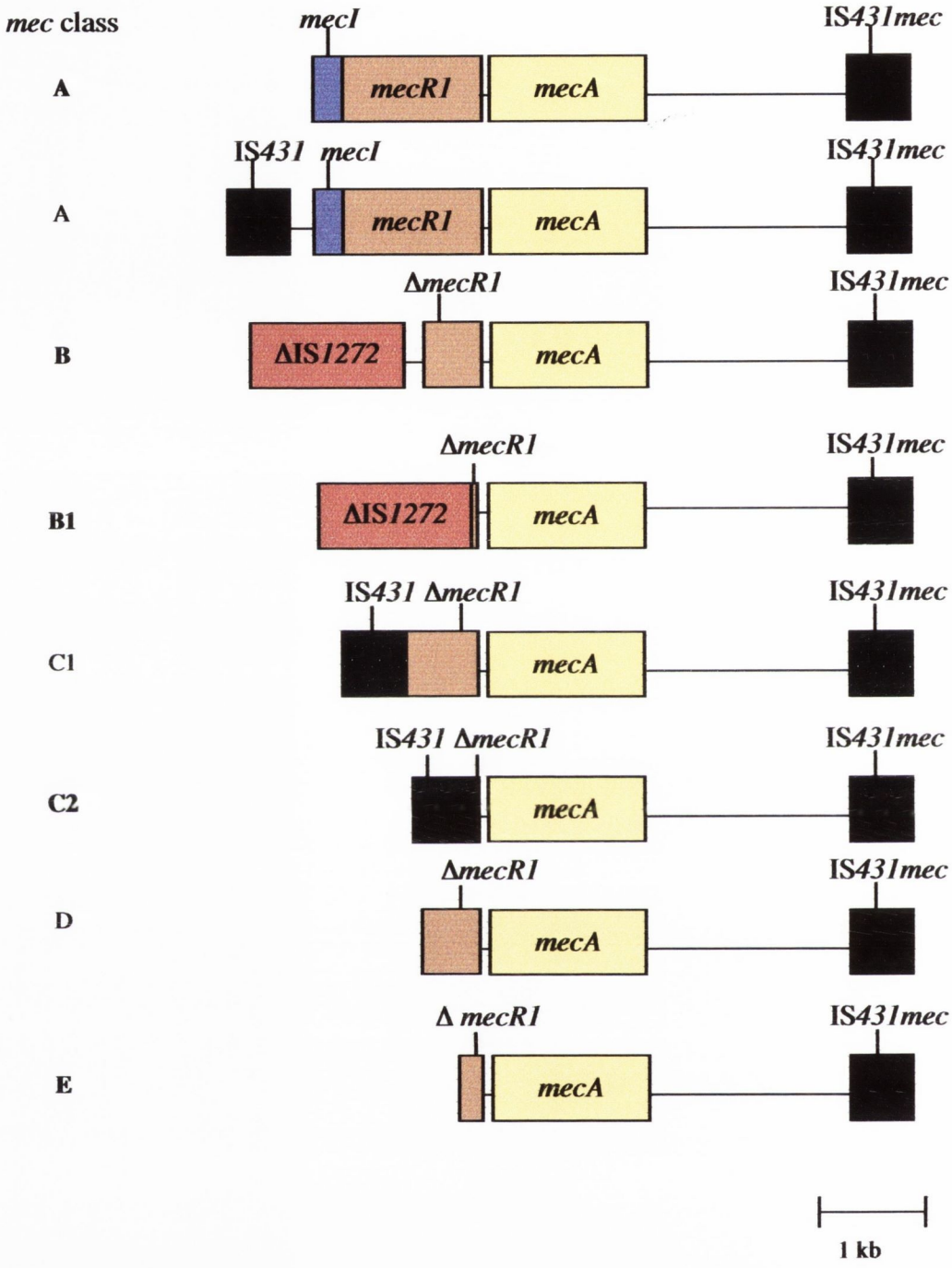

mec class
mecI
IS431mec
A
mecR1
mecA
IS431
mecI
IS431mec
A
mecR1
mecA
ΔmecR1
IS431mec
B
ΔIS1272
mecA
ΔmecR1
IS431mec
B1
ΔIS1272
mecA
IS431 ΔmecR1
IS431mec
C1
mecA
IS431 ΔmecR1
IS431mec
C2
mecA
ΔmecR1
IS431mec
D
mecA
Δ mecR1
IS431mec
E
mecA
1 kb

**FIGURE 1.3.** Schematic diagram of the genetic organisation of the *mec* complexes of staphylococci. The illustrations of *mec* complex A, B, C1, C2 and D are based on studies by Katayama and co-workers (2001) and the illustration of *mec* complex B1 and E are based on studies by Lim and co-workers (2003). The *mec* classes A, B, B1, C2 and E indicated in bold have been previously identified in MRSA (Katayama *et al.*, 2001; Lim *et al.*, 2003; Ito *et al.*, 2004). Class A flanked by a copy of IS*431* upstream of the *mec* complex and class C are mainly found *in S. haemolyticus* while class D has only been found in *S. hominis* (Katayama *et al.*, 2001).

(McKinney *et al.*, 2001). Inducible transcription of *mecA* by MecI and MecR1 is thought to involve the β-lactam inducer binding to the extracellular sensor domain of MecR1 resulting in a transmembrane signal that triggers the clevage of both the sensor transducer and the MecI repressor proteins bound to the operator of the *mecA* region, thus allowing transcription of *mecA* and subsequenct production of PBP2a (Sharma *et al.*, 1998).

The expression of methicillin resistance varies among MRSA strains and is still not completely understood. Some MRSA strains are susceptible to methicillin despite the presence of *mecA*. These isolates are often referred to as pre-MRSA, and PBP2a production is not induced with methicillin because *mecA* transcription is strongly repressed by *mecI* (Kuwahara-Arai *et al.*, 1996; Ito *et al.*, 1999; Katayama *et al*, 2001). Constitutive methicillin-resistance requires alterations in, or the absence of the *mecA* and β-lactamase regulatory genes. Many clinical MRSA isolates have deletions or mutations in *mecI*, or the *mecI* gene is completely deleted and this results in derepression of *mecA* and subsequent production of PBP2a (Kuwahara-Arai *et al.*, 1996; Sharma *et al.*, 1998; Shukla *et al.*, 2004a; Weller, 1999). Ito and colleagues (1999) suggested that the strong repression of *mecA* by *mecI* found in pre-MRSA isolates may be the original form of the *mec* gene complex that was initially transferred from an unknown source to *S. aureus* and that subsequent deletions and mutations in this *mec* complex, which were necessary for PBP2a production to occur in *S. aureus*, may have been selected for by the introduction of methicillin into clinical practice. Methicillin resistance in *S. aureus* can be heterogenously expressed, that is, only a small population subset are resistant to high concentrations of methicillin, or homogeneously expressed where all bacterial cells in the population express high level methicillin-resistance (Hiramatsu, 1995).

Five classes of the *mec* gene complex (A-E) which vary in their genetic structure have been described for to date for staphylococcal species (Katayama *et al.*, 2001; Lim *et al.*, 2003; Ito *et al.*, 2004) (Fig. 1.3). The Class A *mec* complex has intact *mecI* and *mecR1*, as well as *mecA* and IS*431mec* (*mecI-mecR1-mecA*-IS*431mec*) (Katayama *et al.*, 2001) (Fig. 1.3). Class A flanked by a copy of IS*431* upstream of the *mec* complex has also been identified (Katayama *et al.*, 2001) (Fig. 1.3). In the class B *mec* complex, both *mecI* and the penicillin binding domain of *mecR1* are deleted and replaced by a portion of insertion sequence element IS*1272* (ΔIS*1272*-Δ*mecR1-mecA*-IS*431mec*)

(Katayama *et al.*, 2001) (Fig. 1.3). (Lim *et al.*, 2003). A variation of the class B *mec* complex, designated class B1 *mec,* was identified in CAMRSA isolates from Australia, in which the insertion of IS*1272* has deleted *mecI* and both the penicillin binding- and the membrane spanning- domains of *mecR1* (Lim *et al.*, 2003) (Fig. 1.3). Class C *mec* complex has a truncated copy of *mecR1* flanked by IS*431* (IS*431-ΔmecR1-mecA*-IS*431mec*) and two variants, class C1 and C2 have been described differing only in the size of the *mecR1* deletion (Katayama *et al.*, 2001) (Fig. 1.3). The class D *mec* complex has a similar truncated *mecR1* gene to that of the class B *mec* complex but it is not flanked by any insertion sequence (*ΔmecR1-mecA*-IS*431mec)* (Fig. 1.3). The class E *mec* complex was identified in CAMRSA and is characterised by the absence of *mecI* and deletions in *mecR1,* but with no flanking insertion sequences (*ΔmecR1-mecA*-IS*431mec)* (Ito *et al.*, 2004) (Fig. 1.3). Class A, B, B1, C2 and E *mec* have been identified in MRSA isolates to date (Katayama *et al.*, 2001; Lim *et al.*, 2003; Ito *et al.*, 2004) (Fig. 1.3). Class A *mec* flanked by two copies of IS*431* and class C1 *mec* are mainly found *in Staphylococcus haemolyticus*, while class D *mec* has only been found in *Staphylococcus hominis* (Katayama *et al.*, 2001) (Fig. 1.3).

*1.2.3.3 SCCmec type I*

Type I SCC*mec* is 34 kb in size, has type 1 *ccr* genes, class B *mec* complex and apart from *mecA* has no additional antibiotic resistance genes (Fig. 1.4). SCC*mec* I has a large region of DNA located upstream of the *ccr* complex consisting of many ORFs of unknown function and a region which has a high level of homology to a large plasmin-sensitive surface protein of *S. aureus* known as the *pls* gene (Ito *et al.*, 2001) (Fig. 1.4). A region known as the downstream constant (*dcs*) region is located downstream of the *mec* complex in SCC*mec* I (Ito *et al.*, 2001) (Fig. 1.4).

The type I SCC*mec* element was detected in the first MRSA isolate recovered in the UK in 1961 and the majority of the earliest MRSA isolates from the 1960's have been found to harbour this SCC*mec* element (Ito *et al.*, 2001; Enright *et al.*, 2002; Oliveira *et al.*, 2002b). The SCC*mec* I element is not associated with MRSA strains that are current causes of epidemic disease. This may be due, in part, to the fact that isolates harbouring this SCC*mec* element were susceptible to many non β-lactam antimicrobials because SCC*mec* I does not harbour any antibiotic resistance determinants apart from *mecA*. In addition, SCC*mec* I consists of several large sections of DNA such as the 20 kb region

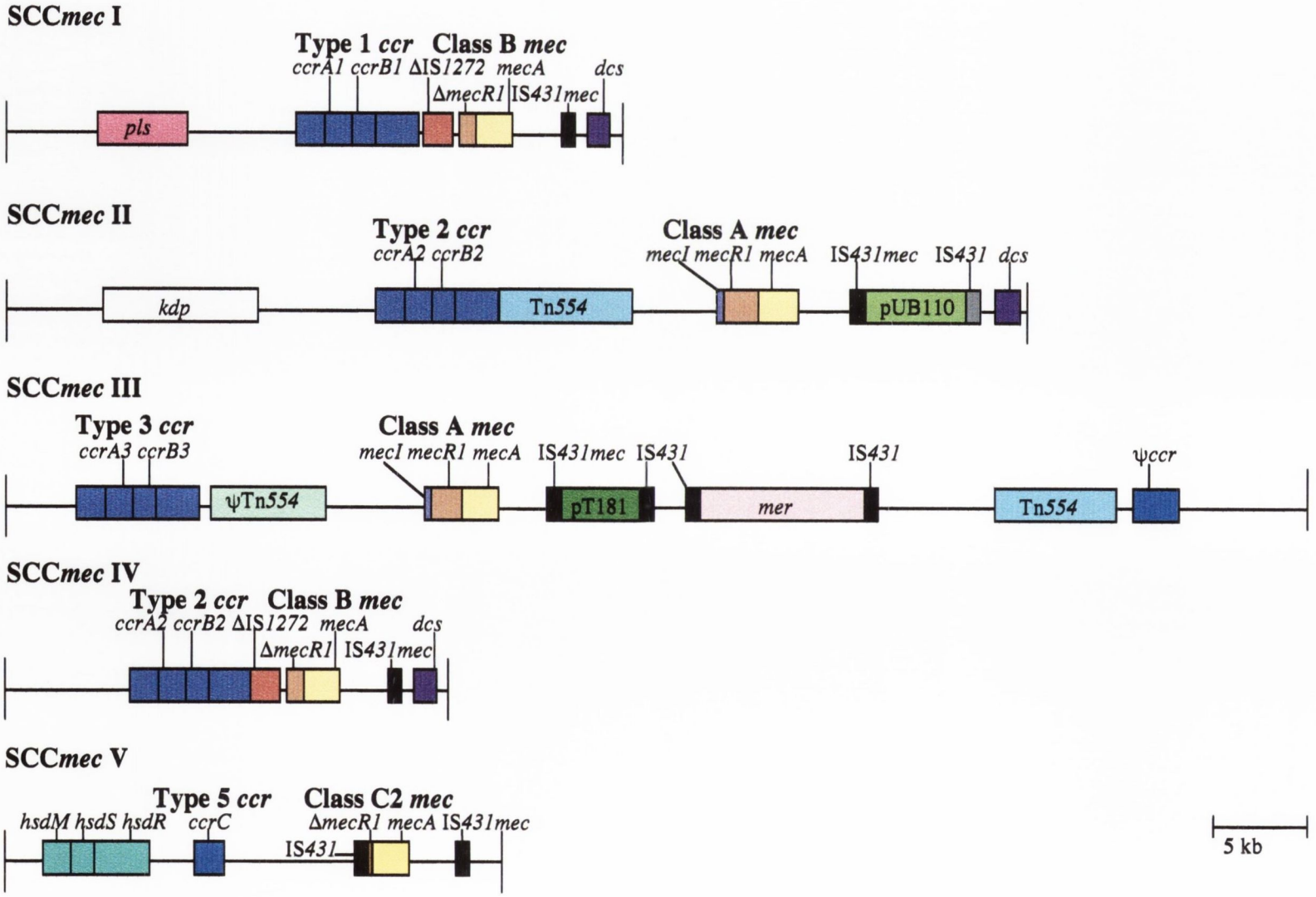

SCCmec I
Type 1 ccr
Class B mec
ccrA1 ccrB1 ΔIS1272 mecA
dcs
ΔmecR1
IS431mec
pls
SCCmec II
Type 2 ccr
ccrA2 ccrB2
Class A mec
mecI mecR1 mecA
IS431mec
IS431
dcs
kdp
Tn554
pUB110
SCCmec III
Type 3 ccr
ccrA3 ccrB3
Class A mec
mecI mecR1 mecA
IS431mec
IS431
IS431
ψccr
ψTn554
pT181
mer
Tn554
SCCmec IV
Type 2 ccr
Class B mec
ccrA2 ccrB2 ΔIS1272 mecA
dcs
ΔmecR1
IS431mec
SCCmec V
Type 5 ccr
Class C2 mec
hsdM hsdS hsdR
ccrC
ΔmecR1 mecA IS431mec
IS431
5 kb

**FIGURE 1.4.** Schematic diagram showing the genomic organisation of SCC*mec* elements I, II, III, IV and V. Diagrams are based on the nucleotide sequences in the GenBank database under accession numbers AB033763, D86934, AB037671, AB063172 and AB121219, respectively.

upstream of *mecA* and the *dcs* region (Fig. 1.4), that do not appear to contribute to methicillin resistance, movement of the SCC*mec* element or to survival of the bacterial cell. These large non-functional regions of DNA contribute to the relatively large size of SCC*mec* I (34 kb), which may compromise the competiveness of the bacterial host without giving any selective advantage except for methicillin resistance.

The variant element SCC*mec* IA, which varies from SCC*mec* I by the presence of an integrated plasmid pUB110 downstream of the *mec* complex (pUB110 encodes resistance to kanamycin, tobramycin and bleomycin) is associated with one of the most widely spread contemporary multidrug resistant MRSA clones (Oliveira *et al.*, 2001c & 2002b).

*1.2.3.4 SCCmec type II*

Type II SCC*mec* is 53 kb in size, has the type 2 *ccr* genes, a class A *mec* complex, contains the *dcs* region, and in addition to *mecA*, has two integrated antibiotic resistant determinants pUB110 and Tn*554*, the latter encoding erythromycin and spectinomycin resistance (Ito *et al.*, 2001) (Fig. 1.4). A copy of a potassium transporter operon *kdp* is also located upstream of the *ccr* complex in type II SCC*mec*, although many of the open reading frames in this *kdp* operon are disrupted (Ito *et al.*, 1999) (Fig. 1.4). This SCC*mec* element was first identified in an MRSA strain recovered in Japan in 1982 and has since been found to be ubiquitous among MRSA isolates worldwide, especially in Japan, Korea and the United States (Ito *et al.*, 1999; Hiramatsu *et al.*, 2001).

*1.2.3.5 SCCmec type III*

The type III SCC*mec* element is 67 kb, has the type 3 *ccr* genes, a class A *mec* complex and contains several antibiotic resistance genes including cadmium resistance encoded by ψTn*554*, tetracycline and mercury resistance encoded by pT181, erythromycin and spectinomycin resistance encoded by Tn*554* and mercury resistance genes located in the *mer* operon between two copies of IS*431* (Ito *et al.*, 2001) (Fig. 1.4). Two variants of the type III SCC*mec* element have been described. The variant element SCC*mec* IIIA differs from SCC*mec* III by the absence of pT181 and its flanking IS*431* elements and the variant element SCC*mec* IIIB lacks the integrated copies of Tn*554*, pT181 and the *mer* operon along with the associated insertion sequences (Oliveira *et al.*, 2001c). Type III SCC*mec* was first identified in a MRSA isolate recovered in New Zealand in 1985. Today, this SCC*mec* element and its variants

are harboured by some of the major multiresistant pandemic clones that are current causes of epidemic MRSA disease especially in Asia, South America and New Zealand (Ito *et al.*, 2001; Oliveira *et al.*, 2001c & 2002b).

*1.2.3.6 SCCmec type IV*

The type IV SCC*mec* element ranges in size from 20-24 kb, has type 2 *ccr* genes and class B *mec* complex and was first identified in two CAMRSA isolates in 2002 (Ma *et al.*, 2002) (Fig. 1.4). Several variants of SCC*mec* IV have been described. Type IVa and IVb SCC*mec* differ in their DNA sequences from the left extremity to the *ccr* complex (L-C region) but both carry *dcs* and encode no additional antibiotic resistance determinants other than *mecA* (Ma *et al.*, 2002). Type IVc SCC*mec* also differs in the L-C region and, upstream of the *mec* complex, carries an integrated copy of transposon Tn*4001* (which encodes aminoglycoside resistance) flanked on either side by IS*256* (Ito *et al.*, 2003). Type IVd SCC*mec* has been partially sequenced from the left junction point to the *ccr* complex and it too has a different L-C region (unpublished, Genbank accession number AB097677). Oliveira and de Lencastre described an SCC*mec* IVA element which has type 2 *ccr* and class B *mec* but harbours the integrated plasmid pUB110 (Oliveira & de Lencastre, 2002a). Another study described a type IV SCC*mec* element with type 4 *ccr* genes instead of type 2 *ccr* genes, which is outside the definition of SCC*mec* IV elements used by other researchers (Oliveira *et al.*, 2001c). The type IV SCC*mec* element (type 2 *ccr* & class A *mec*) is commonly found associated with CAMRSA and many of the major pandemic, non-multiresistant hospital-acquired MRSA clones.

*1.2.3.7 SCCmec V*

The recently discovered type V SCC*mec* element is 28 kb in size, has type 5 *ccr* genes, class C2 *mec* and has no additional antibiotic resistance determinants (Ito *et al.*, 2004) (Fig. 1.4). The type V SCC*mec* element has a restriction-modification system encoded by *hsdS*, *hsdM* and *hsdR*, and it has been suggested that this may help to stabilise this SCC*mec* element in the bacterial chromosome (Ito *et al.*, 2004) (Fig. 1.4). Unlike all other SCC*mec* elements, SCC*mec* V does not have the characteristic inverted repeats at the chromsomal-SCC*mec* junctions and this is thought to be due to the presence of *ccrC* genes in SCC*mec* V rather than the *ccrAB* genes found in SCC*mec* I-IV (Ito *et al.*, 2004). The type V SCC*mec* element has been found to be widely

distributed among CAMRSA in Australia but has yet to be identified in MRSA from other parts of the world (Coombs *et al.*, 2004; O'Brien *et al.*, 2004a).

*1.2.3.8 The origins and evolution of SCCmec*

Staphylococcal chromosomal cassettes (SCCs) are a family of site specific mobile genetic elements that have been proposed to assist in the transport of various genetic markers between staphylococcal species (Ito *et al.*, 2001; Katayama *et al.*, 2003). Thus, SCC*mec* elements, the most prominent members of the SCC family, are SCCs that are specialised for the transfer of methicillin resistance genes (Ito *et al.*, 2001). As the name suggests, SCCs are unique to staphylococci, they have a G-C content similiar to that of the staphylococcal genome, carry *ccr* genes and many SCC elements carry additional determinants such as virulence genes, heavy metal resistance genes, antibiotic resistance genes and restriction modification systems (Luong *et al.*, 2002; Katayama *et al.*, 2003; Mongkolrattanothai *et al*, 2004).

Although the origin of SCC*mec* is unknown, it is believed that the SCC and the *mecA* genes were once individual genetic components that came together in coagulase-negative staphylococci (CoNS) from where they transferred to *S. aureus* (Ito *et al.*, 1999 & 2001). There have been numerous different studies that have provided evidence to support this theory. A homologue of the *mecA* gene found in *S. aureus* has been found to be a native gene that is ubiquitous in independent isolates of the CoNS species *Staphylococcus sciuri*, which may be the evolutionary precursor of the *mecA* gene in MRSA (Couto *et al.*, 1996; Wu *et al.*, 1998). Several different researchers have described SCC elements in CoNS with *ccr* genes and other genes with variable homology to those of SCC*mec* but without *mecA* that may be the evolutionary precursors of SCC*mec* (Luong *et al.*, 2002; Katayama *et al.*, 2003; Mongkolrattanothai *et al.*, 2004).

Wisplinghoff *et al.* provided further evidence for the transfer of SCC*mec* between CoNS and *S. aureus* when they identified SCC*mec* types I-IV in the CoNS species *Staphylococcus epidermidis* and the SCC*mec* IV element of *S. epidermidis* was found to be almost 100% homologous to that of MRSA (Wisplinghoff *et al.*, 2003). This same study also documented that SCC*mec* IV was rarely seen in MRSA isolates recovered before the 1990's but that it was prevalent in *S. epidermidis* isolates from the 1970s, suggesting that the direction of interspecies transfer of SCC*mec* IV may have been from the CoNS to *S. aureus* (Wisplinghoff *et al.*, 2003). Another study found that the

insertion sequence IS*1272*, found in the class B *mec* complex of SCC*mec* I and IV, is common in the CoNS species *S. haemolyticus* and *S. epidermidis* but not in *S. aureus* (Archer *et al.*, 1996). Based on these findings it has been hypothesised that insertion of IS*1272* into SCC*mec* occurred in CoNS, followed by transfer of the IS*1272* containing SCC*mec* element to *S. aureus* (Archer *et al.*, 1996). The insertion junction points of IS*1272* are identical in the SCC*mec* elements of *S. epidermidis* and *S. aureus* (Wisplinghoff *et al.*, 2003). Lastly, the Class C2 *mec* complex, found in SCC*mec* V of CAMRSA, is widely distrubuted among CoNS and it has the same deletion point for *mecR1* and insertion of IS*431* in all species, suggesting that horizontal transfer of the class C2 *mec* gene complex has occurred between staphylococcal species (Ito *et al.*, 2004).

It has been proposed that the first MRSA isolates emerged from an epidemic MSSA lineage upon aquisition of SCC*mec* I from CoNS (Crisostomo *et al.*, 2001; Enright *et al.*, 2002) although the mechanisms and route of transfer of SCC*mec* elements in staphylococci is still unknown (Ito *et al.*, 2001; Ma *et al.*, 2002).

### 1.2.4 Additional factors affecting methicillin resistance

Although *mecR1* and *mecI* largely control the expression of *mecA*, other genetic and environmental factors also have an influence on the expression of methicillin resistance. (Berger-Bachi *et al.*, 1992, Chambers, 1997; Berger-Bachi, 1999; Berger-Bachi & Rohrer, 2002). Several normal staphylococcal genes are necessary for methicllin resistance, such as *fem* (factors essential for methicillin resistance) or *aux* (auxilliary) factors, which are necessary for peptidoglycan precursor formation (Gustafson *et al.*, 1994; Rohrer *et al.*, 1999; Stranden *et al.*, 1997). Two of the major global regulatory systems in *S. aureus*, accessory gene regulator (*agr*) and staphylococcal accessory gene regulator (*sar*), also play a role in maintenance of methicillin resistance by controlling the expression of cell wall and extracellular proteins (Piriz *et al.*, 1996).

### 1.2.5 Evolution of MRSA

Early genetic analysis of MRSA isolates led many researchers to believe that *mecA* entered the *S. aureus* population once, resulting in a single clone that has since spread worldwide (Lacey and Grinsted, 1973; Kreiswirth *et al.*, 1993). However, data from more recent studies has shown that MRSA have evolved multiple independent times by means of horizontal transfer of *mecA* into phylogenetically distinct MSSA isolates

(Musser & Kapur, 1992; Fitzgerald *et al.*, 2001). The majority of nosocomial MRSA infections are caused by relatively few pandemic clones that have evolved by the introduction of SCC*mec* elements into five distinct epidemic MSSA lineages (Enright *et al.*, 2002; Oliveira *et al.*, 2002b). Using Multilocus Sequence Typing (MLST, the sequencing of seven unlinked housekeeping genes) and the programme eBURST the five pandemic MRSA lineages can be visualised as clonal complexes (CCs) which are groups of genotypes/MLST sequence types (STs), that share a recent common ancestor (Feil *et al.*, 2004). Each CC is named after the ST of the ancestral genotype. The CCs of the major nosocomial MRSA lineages are CC5, CC8, CC22, CC30 and CC45 (Enright *et al.*, 2002; Robinson & Enright, 2003) (Table 1.2). In all of these lineages, with the possible exception of CC22, SCC*mec* has been acquired on multiple occasions (Robinson & Enright, 2003).

The names originally assigned to the MRSA clones represent either a unique epidemiological characteristic or signify the geographic area from which they were first isolated (Table 1.2). For example, the Iberian clone was first identified in Spain in 1989, the Brazilian clone in Brazil in 1992, the New York/ Japan clone in metropolitan New York and in Tokyo and the Pediatric clone in a paediatric hospital in Portugal in 1992 (Dominguez *et al.*, 1994; Teixeira *et al.* 1995; Roberts *et al.*, 1998; Aires de Sousa *et al.*, 2001a). This nomenclature is cumbersome and some MRSA clones were given many different names in different parts of the world (Table 1.2). However, a more rational and unambiguous scheme has been proposed based on ST and SCC*mec* type (Enright *et al.*, 2002; Robison & Enright, 2004b). Table 1.2 shows the names given to some of the more common nosocomial MRSA clones using this more rational nomenclature.

### 1.2.6 Epidemic MRSA (EMRSA)

In the United Kingdom (UK) the dominant MRSA strains are termed epidemic MRSA (EMRSA) (Kerr *et al.*, 1990). The first UK EMRSA strain was identified in 1981 and was termed EMRSA-1 (Marples *et al.*, 1986). A survey carried out in hospitals in the UK in the late 1980s identified another 13 EMRSA strains which were designated EMRSA-2 to –14 (Kerr *et al.*, 1990). In the early 1990's EMRSA-15 and –16 emerged (Cox *et al.*, 1995; Richardson & Reith, 1993). Not only did EMRSA-15 and EMRSA-16 become endemic in hundreds of hospitals across the United Kingdom, but they also caused outbreaks in several other countries around the world, including

**TABLE 1.2.** Details of the major nosocomial MRSA clones identified within each MRSA genotype *

| Clonal complex (CC)[1] (ancestral genotype) | MRSA clone (ST/SCC*mec* type)[2] | Allelic profile[3] | Other names used to describe MRSA clone[4] |
|---|---|---|---|
| CC8 | ST8–MRSA-II | 3-3-1-1-4-4-3 | Irish-1 |
| | ST8-MRSA-IV | 3-3-1-1-4-4-3 | EMRSA-2 & -6, clone V, Irish-2 |
| | ST250-MRSA-I | 3-3-1-1-4-4-16 | First MRSA, Archaic |
| | ST239-MRSA-III | 2-3-1-1-4-4-3 | Hungarian, USA clone IV |
| | ST239-MRSA-IIIA | 2-3-1-1-4-4-3 | EMRSA-1 & -4 , Brazilian, Czech |
| | ST247-MRSA-IA | 3-3-1-12-4-4-16 | EMRSA-5 & -17, Iberian |
| CC5 | ST5-MRSA-II | 1-4-1-4-12-1-10 | New York/Japan |
| | ST5-MRSA-IV | 1-4-1-4-12-1-10 | Paediatric |
| CC22 | ST22-MRSA-IV | 7-6-1-5-8-8-6 | EMRSA-15 |
| CC30 | ST36-MRSA-II | 2-2-2-2-2-3-2 | EMRSA-16 |
| CC45 | ST45-MRSA-IV | 10-14-8-6-10-3-2 | Berlin |

*Adapted from Oliveira *et al.*, 2001c; Enright *et al.*, 2002 & Oliverira *et al.*, 2002b.

[1]MRSA lineages can be visualised as clonal complexes (CCs) which are groups of genotypes/MLST sequence types (STs), that share a recent common ancestor (Feil *et al.*, 2004). Each CC is named after the ST of the ancestral genotype.

[2]MRSA clones named using the nomenclature of Enright *et al.*, 2002 based on their ST and SCC*mec* type.

[3]The alleles at each of the seven housekeeping genes used for MLST unambiguously define a strain. Alleles at the seven MLST loci in the order carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (glp*F*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), trioesphosphate isomerase (*tpi*) and acetyl coenzyme A (*yqiL*)

[4]The names originally assigned to the MRSA clones represent either a unique epidemiological characteristic or signify the geographic area from which they were first isolated. All EMRSA clones were first recovered in the United Kingdom.

Abbreviations: ST, sequence type (defined by the allelic profile); EMRSA, Epidemic MRSA.

Australia, New Zealand and Germany (Cookson, 1999; O' Neill *et al.*, 2001). In 2000, a new multiresistant EMRSA strain was reported in the UK and was termed EMRSA-17 (Aucken *et al.*, 2002).

### 1.2.7 MRSA in Ireland

Methicillin-resistant *S. aureus* was first reported in Irish hospitals in 1971, ten years after the initial report of MRSA in England (Hone & Keane, 1974). It rapidly became endemic in Irish hospitals and the increasing prevalence observed in the 1980's and 1990's has continued into the 21st century (Cafferkey *et al.*, 1983 & 1985; Morgan & Harte-Barry, 1989; Murchan & the EARSS Steering group, 2002; Rossney & Keane, 2002a).

The European Antimicrobial Resistance Surveillance System (EARSS), which collates data on invasive *S. aureus* blood stream infections was established in 1998 to monitor antimicrobial resistance across Europe. Data collected by EARSS found that MRSA levels among Irish nosocomial *S. aureus* isolates from blood cultures was one of the highest in Europe with levels similar to those found in the United Kingdom and Southern Europe (Murchan & the EARSS Steering group, 2002). The North/South study of MRSA, which was carried out to review the extent of the MRSA problem in both Northern Ireland (North) and the Republic of Ireland (South) found that in the Republic of Ireland in 1998, 36% *of S. aureus* isolates from blood culture were MRSA (McDonald *et al.*, 2002; Irish Department of Health & Children, 1999). Between 1999 and 2001 the average incidence of MRSA among invasive *S. aureus* blood cultures in the South was 40.2% (Murchan & the EARSS Steering group, 2002). An MRSA level of 42.3% was recorded among cases of *S. aureus* bacteremia in the Republic of Ireland for 2003 (EARSS, 2003a, 2003b, 2003c & 2004a). In 2002 the National MRSA Reference Laboratory (NMRSARL) was opened at St. James's Hospitals in Dublin and carries out extensive antimicrobial susceptibility investigations of MRSA isolates from the diagnostic laboratories that participate in EARSS. In 2003, the first hVISA isolates from an Irish hospital were reported and this reduced susceptibility to vancomycin in MRSA is of great concern because it can potentially result in the loss of the effectiveness of vancomycin to MRSA infections (Rossney, 2003a).

### 1.2.8 Financial cost of MRSA

A major problem associated with MRSA, apart from the increased morbidity and mortality, is the financial cost associated with treating MRSA infections and preventing further spread to other patients. Nosocomial MRSA infections are associated with longer hospital stays and increased hospital costs (Abramson & Sexton, 1999). It has been estimated that treatment of nosocomial MRSA infections can cost between €10,000 and €36,000 per patient (Stone *et al.*, 2002). One outbreak in the UK in the 1990s had an estimated cost of £400,000 (Cox *et al.*, 1995).

## 1.3 MRSA typing methods

Due to the increased frequency of MRSA as the causative agent of hospital and community acquired infection and the emergence of vancomycin resistance in MRSA, typing methods that can characterise isolates and identify clonal spread are essential. Typing of any bacterial pathogen aims at differentiating between strains among groups of isolates. Typing of MRSA isolates is an important tool for detecting and tracking outbreaks in local epidemiological investigations, for studying longer-term or global MRSA epidemiology and for analysis of MRSA population genetics. Bacterial typing is useful in an outbreak situation to determine if there is a common source for all isolates involved and to find out if spread of the outbreak strain has occurred (Pitt, 1999). Typing is also of use in identifying the occurrence of specific strains and in distinguishing between recurrence of the original strain and reinfection with new strains (Pitt, 1999). In longer-term and global epidemiological studies bacterial typing can be used to trace bacterial lineages of different strains or to show an association between certain strains and certain clinical presentations or diseases (Pitt, 1999).

There is an extensive range of both phenotypic and genotypic techniques available for epidemiological typing of MRSA, but the method used depends on the purpose of the analysis. For outbreak investigations, a method that uses a highly discriminatory genetic marker that accumulates variation rapidly is required and many workers consider pulsed field gel electrophoresis (PFGE) to be the "gold standard" for MRSA typing in this situation (Schmitz *et al.*, 1998). For the first stage of an outbreak investigation technically simple methods, which give quick, reproducible and easy to interpret results, such as phage typing or antibiotic susceptibility testing, are commonly favoured. For longer-term or global studies a method that has the ability to distingush a

large number of genotypes but which uses genetic variation that accumulates relatively slowly is required, and in recent years MLST has been effectively used for this purpose. Tenover and colleagues (1994) compared 12 different typing methods and found that no one method was superior. It has been suggested that the best results are obtained when more than one method is used (Tenover *et al.*, 1994; Jorgensen *et al.*, 1996).

### 1.3.1 Phenotypic typing methods

Phenotypic techniques differentiate between isolates by detecting physical or morphological characteristics expressed by bacteria. These typing methods are often inexpensive, rapid, easy to use and are commonly used at the initial stages of an outbreak investigation. However, they may not be reliable for longer-term or global studies because phenotypic traits may be subject to variation, due to loss or gain of plasmids, transposons and bacteriophage.

#### *1.3.1.1 Phage typing*

Phage typing is based on the ability of different bacteriophage to lyse different *S. aureus* strains and is one of the oldest methods for typing *S. aureus* isolates (Parker, 1972). The International Union of Microbiology Societies (IUMS) subcommitee for staphylococcal typing, attempted to coordinate international phage typing techniques by defining a set of experimental phages that would type the majority of MRSA strains that were epidemic at that time (Richardson *et al.*, 1999). Although phage typing is a rapid typing method capable of processing large amounts of isolates, it has become less popular in recent years due to the development of molecular typing techniques and because of the weaknesses and limitations identified for this typing system. These weaknesses and limitations include, the increasing number of isolates that are phage non-typeable, poor reproducibility, and the high level of training and expertise required to employ this typing method routinely (Bannerman *et al.*, 1995). Today, phage typing is mainly used in combination with other typing techniques at the first stage in the investigation of short-term outbreaks.

#### *1.3.1.2 Antimicrobial susceptibility testing*

Antimicrobial susceptibility testing is commonly used at the initial stages of investigating an MRSA population and the many different susceptibility testing methods available are relatively inexpensive, easy to use, and allow large numbers of

isolates to be typed rapidly (Galdbart *et al.*, 2000; Aucken *et al.*, 2002; Denis *et al.*, 2004; Van der Mee-Marquet *et al.*, 2004; Ko *et al.*, 2005). However, there is often variation in the expression of antibiotic resistance markers due to loss or gain of plasmids and transposons encoding antibiotic resistance genes because of selective pressures exerted by widespread antibiotic usage in the hospital environment. An antibiogram-resistogram (AR) typing scheme devised by Rossney and colleagues, overcame this problem, by including an extensive range of antimicrobials and chemicals (mainly heavy metals) not in clinical use (Rossney *et al.*, 1994a). These non-clinical antimicrobials and chemicals are not subject to the same selective pressure exerted by those used in the hospitals environment (Rossney *et al.*, 1994a). This AR typing scheme was devised using isolates that had also been analysed using plasmid screening, enterotoxin profiling, phage typing, PFGE and random amplified polymorphic DNA analysis using PCR (RAPD-PCR) (Rossney *et al.*, 1994a). Using AR typing, isolates are assigned an AR type based on the pattern of resistance to the antimicrobials in the AR typing panel in conjunction with the additional data obtained from a combination of the phenotypic and genotypic investigations and takes variation due to plasmid loss in the MRSA population into account (Rossney *et al.*, 1994a). To date, 44 AR types and a number of subtypes have been recognised. Before isolates are assigned to a new AR type they require full phenotypic and molecular characterisation. Antimicrobial susceptibilty testing is a particularly useful typing method for use in routine laboratory testing of MRSA isolates and in the initial stages of investigating an outbreak situation (Rossney *et al.*, 1994a & 1994b; Tenover *et al.*, 1994; Rossney & Keane, 2002b).

*1.3.1.3 Other phenotypic typing methods*

Other less popular phenotypic typing methods include biotyping, serotyping and multilocus enzyme electrophoresis (MLEE). Biotyping is a simple and quick typing method that involves analysis of the biochemical and cultural characteristics of the test isolate. Serotyping involves detecting type specific cell surface antigens or capsular polysaccharides. Both biotyping and serotyping lack appropriate reproducibility and discriminatory power for typing of MRSA isolates and are mainly used as an adjunct to other typing methods (Fournier *et al.*, 1987; Tenover *et al.*, 1994).

Multilocus enzyme electrophoresis (MLEE) indexes variation within several essential housekeeping genes by analysing the electrophoretic mobilities of the gene products on starch gels (Selander *et al.*, 1986). This typing method analyses genetic

variation that accumulates relatively slowly so its main use is in long-term or global epidemiological investigations. However, MLEE is rarely used today for typing MRSA isolates as it is laborious, somewhat subjective, exhibits poor inter-laboratory reproducibility and thus it has somewhat been outdated by the development of more sophisticated DNA sequencing-based molecular typing techniques.

### 1.3.2 Genotypic typing methods

It has been found that the most effective typing methods for bacterial isolates are those that involve direct DNA-based analysis of chromosomal or extra chromosomal genetic elements, allowing direct comparison of genotypes of different isolates (Hawkey, 1999). Many genotypic typing methods require the interpretation and comparison of complex DNA banding patterns on gels and there is often difficulty in comparing results between laboratories. In more recent years, DNA sequencing-based typing methods have been developed for molecular typing of MRSA isolates and have the advantages of unambigous data interpretation, simplicity of large-scale database creation, standardised methods and speed. Although there are many different genotypic techniques available for epidemiological typing of MRSA isolates, only the methods that are most commonly used today will be discussed here.

#### *1.3.2.1 Restriction analysis of chromosomal DNA*

Informative genetic fingerprints of the whole chromosome of MRSA can be generated by restriction endonuclease digestion of the total genomic DNA followed by fragment separation by agarose gel electrophoresis. The fingerprint patterns obtained can be complex but can be simplified by Southern hybridisation analysis using specific probes (Southern, 1975; Van Belkum, 2000). This approach results in simpler patterns consisting of only those fragments that carry sequences complementary to the probe. Cloned genomic sequences or polymerase chain reaction amplification products of known genes are commonly used as probes with MRSA, including the aminoglycoside resistant determinant encoding transposon Tn*554* and insertion sequences IS*256*, IS*257* and IS*431* which can occur in multiple locations within the chromosome (Monzon-Moreno *et al.*, 1991; Tenover *et al.*, 1994; Kreiswrith *et al.*, 1995).

Southern blot analysis of *Cla*I-digested chromosomal DNA with *mecA* and Tn*554* DNA probes (*Cla*I-*mecA* and *Cla*I-Tn*554* polymorphisms), combined with analysis of *Sma*I-digested genomic DNA banding patterns by PFGE (discussed below in section

1.3.2.2) have been used previously for the molecular characterisation of MRSA clones (Dominquez *et al.*, 1994; Sanches *et al.*, 1995; Teixeira *et al.*, 1995; Oliveira *et al.*, 1998; Roberts *et al.*, 1998; Sa-Leao *et al.*, 1999; Aires de Sousa *et al.*, 2000 & 2001; Crisostomo *et al.*, 2001). These combined methods allowed the detection of widely spread lineages of MRSA but have now been replaced in more recent studies of MRSA populations, by DNA sequenced-based techniques, such as MLST and *spaA* typing (Oliveira *et al.*, 2001c; Enright *et al.*, 2002; Ko *et al.*, 2005).

*1.3.2.2 Pulsed-field gel electrophoresis (PFGE)*

Pulsed-field gel electrophoresis of macrorestriction fragments of bacterial chromosomal DNA, generated by restriction endonucleases that cleave DNA infrequently (such as *Sma*I), often yield informative DNA fingerprint profiles that can be compared between isolates to determine isolate relatedness. For several years now, PFGE has been the most common molecular typing technique for epidemiological typing of MRSA isolates in short term- and local epidemiological studies (Richardson & Reith, 1993; Dominguez *et al.*, 1994; Bannerman *et al.*, 1995; Cox *et al.*, 1995; Schmitz *et al.*, 1998; Von Eiff *et al*, 2001, McDougal *et al.*, 2003). Although PFGE is a highly discriminatory typing method, it is expensive, time consuming and technically complicated. Furthermore, there is often difficulty in comparing results from different laboratories and it is not suitable for screening large numbers of isolates simultaneously.

In 1995, Tenover *et al.* proposed guidelines for interpreting DNA restriction patterns produced by PFGE in order to simplify the interpretation process when PFGE is used in a local study or outbreak situation (Tenover *et al.*, 1995) (Table 1.3). Efforts have also been made in Europe and the USA to standardise certain aspects of the PFGE protocol in order to increase intercenter comparison of pulsed-field gels and to establish a web-based database of MRSA *Sma*I restriction patterns that have been generated using a common protocol (McDougal *et al.*, 2003; Murchan *et al.*, 2003).

PFGE analyses uncharacterised genetic variation that appears to accumulate relatively rapidly and is useful in an outbreak setting (Enright & Spratt, 1999). Although PFGE has been applied to isolates in long term and global epidemiological studies it is generally considered inadequate due to the rapid accumulation of variation that it detects (Blanc *et al.*, 2002). Clones that have arisen decades ago will have undergone a lot of variation making it difficult to discern whetever its descendents are derived from a common ancestor (Enright & Spratt, 1999).

**TABLE 1.3.** Tenover's guidelines for interpreting PFGE patterns of discrete sets of isolates during potential outbreaks*

| **Category** | **No. of genetic differences compared with outbreak strain** | **Typical no. of fragment differences compared to outbreak pattern** | **Epidemiological interpretation** |
|---|---|---|---|
| Indistinguishable | 0 | 0 | Isolate is part of the outbreak |
| Closely related | 1 | 2-3 | Isolate is probably part of the outbreak |
| Possibly related | 2 | 4-6 | Isolate is possibly part of the outbreak |
| Different | ≥3 | ≥7 | Isolates is not part of the outbreak |

*From Tenover *et al.*, 1995

*1.3.2.3 Multilocus sequence typing (MLST)*

Multilocus sequence typing is a modification of MLEE that indexes variation at several unrelated loci by direct DNA sequencing, rather than analysing the electrophoretic mobilities of the gene products. In 2000, Enright and collegues developed and validated a MLST typing scheme for MRSA and MSSA (Enright *et al.*, 2000). The automated nucleotide sequencing used by MLST is fast, allows results to be easily compared between laboratories, is unambiguous and allows far more variation to be detected than with MLEE (Maiden *et al.*, 1998). With MLST, the sequences of internal fragments of seven housekeeping genes (seven alleles) are determined. For each different sequence of the same allele a distinct number is assigned and the allelic profile of the seven genes defines a sequence type (ST). Isolates with the same ST are considered to have the same common ancestor, i.e. belong to the same clone. The accumulation of nucleotide changes in housekeeping genes is considered to be a relatively slow process, so the ST of a bacterial isolate is sufficiently stable over time for this method to be used in long-term and even global epidemiological studies of MRSA (Enright *et al*, 2002). Today, MLST is the most widely used MRSA typing method for grouping evolutionary related isolates in these types of studies (Oliveira *et al.*, 2001c; Enright *et al.*, 2002; Aires de Sousa & de Lencastre, 2003; Melter *et al.*, 2003; Robinson & Enright., 2003; Urwin & Maiden., 2003; Chung *et al.*, 2004; Denis *et al.*, 2004; Velazquez-Meza *et al.*, 2004; Ko *et al.*, 2005). Peacock *et al.* (2002) found that in a micro-epidemiological setting, MLST performed as well as PFGE.

*1.3.2.4 SCCmec typing*

The five SCC*mec* types I-V described to date in MRSA each carry a unique combination of *ccr* and *mec* genes as well as structural variations in the J regions (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004) (Fig. 1.4). This can be exploited by using PCR primers based on sequences that define each SCC*mec* type, which provides useful information in analyzing the relationship between different MRSA isolates. SCC*mec* typing has been shown to be a very useful typing tool when used in conjunction with MLST in long-term and global epidemiological investigations (Oliveira *et al.*, 2001c; Enright *et al.*, 2002; Chung *et al.*, 2004; Perez-Roth *et al.*, 2004; Velazquez-Meza *et al.*, 2004; Ko *et al.*, 2005;).

*1.3.2.5 Protein A and coagulase gene typing*

The protein A gene (*spa*) and the coagulase gene (*coa*) are found in all naturally occurring *S. aureus* isolates. DNA sequencing of a polymorphic 24-bp variable-number tandem repeat (VNTR) within the 3' end of the *spa* gene has been shown to be a highly discriminatory and rapid method of typing *S. aureus* isolates in an outbreak situation (Shopsin *et al.*, 1999). With *spa* typing, sequencing of only a single locus i.e. the *spa* VNTR region, which ranges in size from 200-600 bp is required, so it is less error prone, less time consuming and cheaper than MLST. However, there is still some debate with regard to its use in long-term studies. Oliveira *et al.* (2001b) found that *spa* typing was sufficient for outbreak investigations but that it should not be used alone for long term surveillance or population studies. Recently, Koreen *et al.* (2004) found *spa* typing to be a useful addition to the typing methods of *S. aureus* isolates in global epidemiological studies (Koreen *et al.*, 2004).

The *coa* gene exhibits variation at the 3' end, resulting in numerous allelic forms and has been shown to be useful in typing of MRSA and MSSA in addition to *spa* typing. Both *spa* and *coa* typing have the advantages of only requiring amplification of a single locus, they are rapid methods that give unambiguous results and they are methods that can be standardised easily between laboratories. These methods also allow the creation of large-scale databases for interpretation and comparison of results. Montesinos *et al.* (2002) found that both *spa* and *coa* typing were useful techniques in the initial stages of investigating an outbreak.

*1.3.2.6 DNA microarrays*

In recent years, seven complete *S. aureus* genome sequences have become publically available (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004; Lindsay & Holden, 2004). Using information from these genome sequences, comparative genomics and DNA microarrays are emerging as useful tools to provide extensive genotyping information for *S. aureus* isolates, especially concerning the evolutionary relationships between isolates (Fitzgerald *et al.*, 2001; Dunman *et al.*, 2004; Saunders *et al.*, 2004). Unfortunately, microarray technology is very expensive, time consuming and results require extensive analysis. A similar level of strain discrimination can be achieved using more rapid and cheaper typing methods such as PFGE and MLST. However, micorarrays have the added advantage of providing additional information regarding the gene content of the test isolate and it is thought that in the future, with its increasing

automation, this technology will play an important role in epidemiological studies (Dunman *et al.*, 2004).

## 1.4 Bacteriophage

### 1.4.1 Virulent and temperate bacteriophages

Bacteriophages (phages) are viruses that infect bacterial cells and can be virulent or temperate. Virulent phage can carry out lytic infection only, which results in lysis of the bacterial cell upon release of progeny phage. Temperate phage can either replicate by means of the lytic life-cycle or they can follow the lysogenic life-cycle and integrate into the bacterial chromosome (lysogenisation) and become non-infectious prophage. Bacterial cells with an integrated prophage are known as lysogens. During lysogeny most phage genes are not expressed including the lytic genes and the phage genome is replicated in synchrony with the host chromosome. Lysogenisation can result in the alteration of expression of one or more phenotypic traits by the host organism, a phenomenon termed lysogenic conversion. Lysogenic phage can mediate positive or negative conversion or in some cases both simultaneously. Positive conversion occurs when the temperate bacteriophage that integrates into the bacterial chromosome encodes some bacterial toxins, enzymes or virulence factors that the lysogen then expresses upon lysogenisation. Negative conversion occurs when the temperate bacteriophage integrates into the ORF of a bacterial chromosomal gene resulting in failure of the lysogen to express that gene.

Lysogenic conversion is responsible for controlling the expression of several important bacterial toxins, enzymes and virulence factors such as the botulinum toxin types C1, D and C3 in *Clostridium botulinum* (Eklund *et al.*,1971; Eklund *et al.*, 1974; Popoff *et al.*, 1990), diphtheria toxin in *Corneybacterium diphtheriae* (Freeman, 1951; Groman, 1984), cholera toxin in *Vibrio cholera* (Waldor & Mekalanos, 1996) and Shiga toxins 1 and 2 in *Escherichia coli* (Smith *et al.*, 1983; Strockbine *et al.*, 1986; O'Brien & Holmes, 1987).

### 1.4.2 Lysogenic conversion in *S. aureus*

*Staphylococcus aureus*, including MRSA, very often carry lysogenic phage, the latter of which fall into three main serological groups termed A, B and F. Four categories of lysogenic conversion have been reported in *S. aureus* isolates (Table 1.4). Single

**TABLE 1.4.** Examples of lysogenic conversion events reported in *S. aureus*

| Lysogenic conversion event[a] | Phage involved | Negative conversion[b] | Positive conversion[c] | Biological effect on lysogen | Reference |
|---|---|---|---|---|---|
| **Single** | ϕL54a | Lipase | — | Loss of ability to degrade lipids via lipase activity | Lee & Iandolo, 1986 |
| | Serotype A | β-toxin | — | Loss of ability to lyse erythrocytes via β-toxin activity | Coleman *et al.*, 1986 |
| | Serotype B | — | Staphylokinase (Sak) | Acquisition of ability to degrade fibrin clots via Sak activity | Kondo & Fujise, 1977 |
| | ϕPVL | — | Panton-Valentine Leukocidin (PVL) | Acquisition of ability to degrade leucocytes via PVL activity | Kaneko *et al.*, 1997 |
| | ϕETA | — | Exfoliative toxin A (ETA) | Acquisition of superantigen activity (stimulates T cells to release cytokine) via ETA & the ability to split human skin at the epidermal layer (staphylococcal scalded skin syndrome) | Yamaguchi *et al.*, 2000 |
| **Double** | ϕ13 | β-toxin | Staphylokinase | Loss of ability to lyse erythrocytes via β-toxin activity & acquisition of ability to degrade fibrin clots via Sak activity | Coleman *et al.*, 1989 |
| **Triple** | ϕA1<br>ϕA3 | β-toxin | Staphylokinase & Enterotoxin A (EntA) | Loss of ability to lyse erythrocytes via β-toxin activity, acquisition of ability to degrade fibrin clots via Sak activity, acquisition of superantigen activity via EntA. EntA also increases peristalsis and fluid loss & effects the central nervous system | Coleman *et al.*, 1989 |
| **Quadruple** | ϕ42 | β-toxin | Staphylokinase, Enterotoxin A & *Sau*421 | Loss of ability to lyse erythrocytes via β-toxin activity, acquisition of ability to degrade fibrin clots via Sak activity, acquisition of superantigen activity via EntA. Acquisition of resistance to lysis by a wide variety of other phage via restriction modification system *Sau*421 | Dempsey *et al.*, 2005 |

[a]Lysogenic conversion is the process by which a phage integrates into the bacterial chromosome resulting in the lysogenised bacteria (the lysogen) acquiring or losing the ability to express various phenotypic characteristics. In *S. aureus* lysogenic conversion can be classified as a single-, double-, triple- or quadruple- conversion event , depending on the number of genes affected.

[b]Negative conversion occurs when a lysogen loses the ability to express some phenotypic trait that was previously expressed by the host.

[c]Positive conversion occurs when a lysogen acquires the ability to express some phenotypic trait that was not previously expressed by the host

lysogenic conversion involves either the negative or positive conversion of one phenotypic property only (i.e. the loss or gain of expression of a single phenotypic property). Single conversion events reported in *S. aureus* include the following; the negative conversion of lipase activity by phage L54a (Lee & Iandolo, 1986), the negative conversion of β-toxin activity by serotype A phage (Coleman *et al.*, 1986), the positive conversion of staphylokinase activity by a serotype B phage (Kondo & Fujise, 1977), the positive conversion of Panton-Valentine leukocidin activity by ϕ PVL (Kaneko *et al.*, 1997 & 1998) and the positive conversion of exfoliative toxin A by ϕETA (Yamaguchi *et al.*, 2000) (Table 1.4).

Double lysogenic conversion involves the simultaneous lysogenic conversion of two phenotypic traits. In *S. aureus* a double-converting serotype F phage ϕ13 has been shown to be responsible for the concurrent negative conversion of β-toxin activity and positive conversion of staphylokinase (Coleman *et al.*, 1989) (Table 1.4). Triple lysogenic conversion involves the simultaneous lysogenic conversion of three phenotypic traits. In *S. aureus* the triple-converting serotype F phage ϕA1 and ϕA3 have been shown to be responsible for the simultaneous negative conversion of β-toxin activity and positive conversion of staphylokinase and enterotoxin A (Coleman et al., 1989). For over a decade, the *S. aureus* serotype F phage, ϕ42 was also considered to be a triple converting phage similar to ϕA1 and ϕA3 (Coleman *et al.*, 1989) (Table 1.4). However, recent studies from this laboratory have revealed that ϕ42 is in fact a quadruple converting phage (Dempsey *et al.*, 2005). In addition to the three previously reported conversion events reported for ϕ42, it also encodes a novel restriction modification system, *Sau*421, that confers the ability to resist lysis by a very wide range of other phage (Dempsey *et al.*, 2005).

### 1.5 Aims of the present study

MRSA has been a major cause of serious noscomial infection in Irish hospitals for over 30 years. Both small-scale and surveillance studies of Irish nosocomial MRSA isolates have been carried out at various intervals over this 30 year period, mainly using phenotypic typing tools such as phage typing, plasmid profiling and routine susceptibility typing. These studies provided information at a phenotypic level that suggested that changes in the Irish MRSA population were occurring. Today however, the phenotypic typing tools used in these studies are outdated and it is now widely

accepted that complete characterisation of MRSA isolates requires the use of genotypic typing methods. To date, no comprehensive genotypic analysis of the evolutionary epidemiology of the Irish MRSA population since 1971 has been performed.

It has been found previously that *S. aureus* isolates, including MRSA, commonly harbour serotype F phage, which can encode specific extracellular enzymes and toxins and other factors that can enhance the virulence of a bacterial isolate following lysogenic conversion. A comprehensive analysis of the prevalence of lysogenisation of MRSA isolates recovered in Irish hospitals has never been undertaken.

The aims and objectives of this present study were:

- To perform a comprehensive molecular characterisation of MRSA isolates recovered in Irish hospitals between 1971-2004. Irish MRSA isolates representative of those phenotypes that predominated as causes of nosocomial infections at various different times periods between 1971 and 2002 were selected for use in this present study, as well as some sporadic isolates that were recovered from a small number of hospitalised patients between 1971 and 2004.
- To investigate the occurrence of specific MRSA strains and genotypes among the Irish MRSA population in order to analyse their evolution over time and their genetic relatedness to each other and to internationally spread MRSA clones. The most up to date techniques recommended for these types of epidemiological typing studies were used including, PFGE and MLST as well as an extensive investigation into the types of SCC*mec* elements harboured by Irish MRSA isolates.
- To identify specific MRSA strains and various international MRSA clones such as EMRSA and other major pandemic clones among the Irish MRSA population that may have spread from other countries to Irish hospitals or have originated in Ireland and perhaps are unique Irish MRSA clones.
- To investigate the prevalence of serotype F double- and triple- converting phage among a selection of the dominant phenotypes and genotypes of MRSA isolates recovered in Irish hospitals between 1971 and 2002. Some serotype F phage encode the virulence factor Enterotoxin A that has superantigen activity and may play an important role in the spread and dominance of certain MRSA strains in Irish hospitals.

# Chapter 2
# Materials and Methods

## 2.1 Bacterial isolates

### 2.1.1 Clinical MRSA isolates

One hundred and seventy-two MRSA isolates were investigated in the present study. These isolates were representative of the most prevalent phenotypes of MRSA recovered from patients in Irish hospitals during eight previous study periods between 1971 and 2002 and are listed in Table 2.1. Isolates were chosen to include each type or subtype that had been previously shown to predominate during each of the study periods A-H (Table 2.1). All MRSA isolates used in this study were obtained from the Irish National MRSA Reference Laboratory (NMRSARL).

Isolates recovered prior to 1989 had been stored on individual nutrient agar slopes and stored at room temperature. Isolates from the study periods after and including 1989 had been stored in individual Protect bacterial preserver vials (Technical Services Limited, United Kingdom) at –70°C. The only exception was three isolates from 1989, AR09/0.0064, AR09/0.0065 and AR09/0.0066, that had been stored on nutrient agar slopes at room temperature for approximately nine months but were then transferred to individual Protect bacterial preserver vials and stored at –70°C.

All isolates were reconfirmed as *S. aureus* using the Pastorex Staph Plus identification system which is a rapid slide latex agglutination test that detects clumping factor, capsular polysaccharide and protein A produced by *S. aureus* strains (Sanofi Diagnostics Pasteur, 6565 185$^{th}$ Avenue N.E. Redmond, WA, USA). Isolates were tested for methicillin resistance at 30°C with 25 μg methicillin strips as described previously (Rossney *et al.*, 1994). Prior to 1988 (study periods A, B and C) isolates were classified into four phenotypes (early MRSA and Phenotypes-I, -II and -III, respectively) based on a combination of antimicrobial agent resistance pattern, bacteriophage typing, plasmid screening, location of resistance determinants and Southern hybridisation analysis of plasmid and chromosomal DNA (Coleman *et al.*, 1985; Carroll *et al.*, 1989). Phenotype-I and Phenotype-II isolates (Coleman *et al.*, 1985) were previously identified between 1976-1984 (study period B) but only Phenotype-II isolates were available for this present study.

Isolates recovered after 1988 (study periods D-H) were characterised previously by a combination of techniques including bacteriophage typing, restriction fragment length polmorphism (RFLP) analysis of *Sma*I-digested total cellular DNA using pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA analysis using PCR

**TABLE 2.1.** Clinical MRSA isolates used in this study

| Study period | Year of isolation | Isolate no. | Phenotype/ AR type[1] | Body site/ specimen type [2] | Hospital/source | Reference |
|---|---|---|---|---|---|---|
| A | 1971-75 | 1612 | Early MRSA | NA | FDVH[3], MMH[4] & JSH[5] | Coleman *et al.*, 1985 |
| B | 1976-'84 | 161.2 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 58.2 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 120.1 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 43.2 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 58.1 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 120.2 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 122.2 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 138.1 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 161.1 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 43.11 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 43.21 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 438.1 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 438.2 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 57 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| B | 1976-'84 | 79 | Phenotype-II | NA | FDVH, MMH & JSH | Coleman *et al.*, 1985 |
| C | 1985-'87 | 84 | Phenotype-III | NA | FDVH | Carroll *et al.*, 1989 |
| D | 1989 | 0.1239 | AR01 | Hand | SJH[6] | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1085 | AR01 | Urine | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1118 | AR01 | Catheter site | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1109 | AR01 | Nose | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1206 | AR02 | Calf wound | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1228 | AR02 | Sputum | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1149 | AR02 | Urine | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1345 | AR05 | Leg ulcer | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1264 | AR06 | NA | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1267 | AR07.4 | Sputum | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1342 | AR07.4 | Urine | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1125 | AR07.3 | Groin | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.0064 | AR09 | NA | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.0065 | AR09 | NA | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.0066 | AR09 | NA | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| D | 1989 | 0.1336 | ARNT* | Nose | SJH | Rossney, 1995; Rossney & Keane, 2002a |
| E | 1993 | 0251 | AR06 | NA | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0113 | ARNT* | Urine | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0250 | AR06 | Sputum | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0318 | AR06 | Screening | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0340 | AR06 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0159 | AR07.4 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0237 | AR07.4 | Wound | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0257 | AR07.3 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0202 | AR07.3 | Ear or nose | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0111 | AR07.4 | Sputum | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0220 | AR11 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0101 | AR11 | Screening | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0089 | AR11 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0190 | AR11 | NA | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0242 | ARNT* | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0067 | ARNT* | Wound | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0132 | AR13 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0055 | AR13 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0236 | AR13 | Blood | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0066 | AR13 | Screening | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0246 | AR14 | Sputum | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0298 | AR14 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0225 | AR14 | Sputum | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0078 | AR14 | Screening | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0098 | AR15 | Wound | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0104 | AR15 | Wound | FDVH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0070 | AR22 | Screening | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1993 | 0308 | AR22 | Wound | SJH | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| E | 1992 | 0073 | AR23 | NA | Ext[7] | Rossney *et al*, 1994b; Rossney & Keane, 2002a |
| F | 1998 | 2170 | AR06 | Wound | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2008 | AR06 | Wound | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2110 | AR06 | Sputum | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2024 | AR06 | Screening | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2189 | AR07.0 | Screening | SJH | Rossney & Keane, 2002a |

continued overleaf

**TABLE 2.1.** (continued)

| Study period | Year of isolation | Isolate no | AR type | Body site/ specimen type | Hospital/ source | Reference |
|---|---|---|---|---|---|---|
| F | 1998 | 2039 | AR07.2 | Ulcer swab | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2160 | AR07.3 | Sputum | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2002 | AR07.4 | Screening | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2023 | AR07.4 | Screening | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2179 | AR13 | Screening | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2141 | AR13 | Screening | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2142 | AR13 | Sputum | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2113 | AR13 | Screening | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2027 | AR14 | Swab | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2175 | AR14 | Wound | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2188 | AR14 | Swab | SJH | Rossney & Keane, 2002a |
| F | 1998 | 2080 | AR14 | Burn swab | SJH | Rossney & Keane, 2002a |
| G | 1999 | 3144 | New01* | Nose | N/S Study [8] | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3581 | New02* | Blood | N/S Study(N)[9] | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3442 | New03* | Toe wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3686 | New03* | Throat | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3362 | AR06 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3363 | AR06 | Eye | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3143 | AR06. | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3416 | AR06 | Urine | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3637 | AR06 | Eye | N/S Study(N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3190 | AR06 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3147 | AR06 | Throat | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3222 | AR06 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3591 | AR06 | Wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3430 | AR06 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3594 | AR07.0 | Elbow | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3384 | AR07.0 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3558 | AR07.0 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3142 | AR07.0 | Chest drain | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3366 | AR07.0 | Wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3228 | AR07.0 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3139 | AR07.0 | Blood | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3572 | AR07.0 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3604 | AR07.0 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3188 | AR07.4 | Wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3033 | AR07.4 | Tracheostomy swab | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3137 | AR13 | Hand | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3008 | AR13 | Wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3053 | AR13 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3114 | AR13 | Groin | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3698 | AR13 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3635.2[a] | AR13 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3243.2[b] | AR13 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3596 | AR13 | Hip wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3017 | AR13 | Wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3439 | AR13 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3317.2[c] | AR13 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3636.2[d] | AR13 | Groin | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3330.2[e] | AR13 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3258 | AR13 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3453 | AR13 | Hip wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3045 | AR14 | Throat | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3146 | AR14 | Sputum | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3225 | AR14 | Blood | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3352 | AR14 | Wound | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3132 | AR14 | Sacral ulcer | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3369 | AR14 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3600 | AR14 | Traceostomy swab | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3685 | AR14 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3411 | AR14 | Nose | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3176.1[f] | AR14 | Wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3448 | AR14 | Heel swab | N/S Study | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3623 | AR43 | Nose | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3246 | AR43 | Nose | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3310 | AR43 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3402 | AR43 | Nose | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3488 | AR43 | Catheter site | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3635.1[a] | AR43 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3331 | AR43 | Nose | N/S Study (N) | Irish Dep. of Health & Children, 1999 |

continued overleaf

**TABLE 2.1.** (continued)

| Study period | Year of isolation | Isolate no | AR type | Body site/ specimen type | Hospital/ source | Reference |
|---|---|---|---|---|---|---|
| G | 1999 | 3322 | AR43 | Nose | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3317.1[c] | AR43 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3316 | AR43 | Tongue | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3625 | AR43 | Wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3330.1[e] | AR43 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3337 | AR43 | Wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3665 | AR43 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3243.1[b] | AR43 | Urine | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3328 | AR43 | Wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3636.1[d] | AR43 | Groin | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3176.2[f] | AR43 | Wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3487 | AR43 | Leg wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| G | 1999 | 3094 | AR43 | Wound | N/S Study (N) | Irish Dep. of Health & Children, 1999 |
| H | 2002 | E1164 | AR06 | Blood | EARSS[10] | Rossney *et al.*, 2004 |
| H | 2002 | E1185 | ARNT* | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1098 | AR06 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1095 | AR06 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1174 | AR06 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1113 | ARNT* | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1203 | AR06 | Blood | EARRS | Rossney *et al.*, 2004 |
| H | 2002 | E1115 | AR06 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1139 | ARNT* | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1123 | AR06 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1206 | AR06 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1100 | AR07.0 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1202 | AR07.2 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1140 | AR13 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1168 | AR13 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1154 | AR13 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1134 | AR14 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1119 | AR14 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1121 | AR14 | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E1183 | AR44 | Blood | EARSS | Rossney *et al.*, 2003b & 2004 |
| H | 2002 | E1038 | New03* | Blood | EARSS | Rossney *et al.*, 2004 |
| H | 2002 | E0908 | New03* | Blood | EARSS | Rossney *et al.*, 2004 |
| I | 2003 | M03/0073 | AR44 | Nose | NMRSARL[11] | Rossney *et al.*, 2003b |
| I | 2003 | M03/0068 | Unfamiliar-1* | Burn | NMRSARL | NMRSARL (this study) |
| I | 2003 | M03/0169.2 | Unfamiliar-2* | Blood | NMRSARL | NMRSARL (this study) |
| I | 2003 | E1520 | Unfamiliar-3* | Blood | EARSS | Rossney *et al.*, 2004 |
| J | 2004 | M03/0067 | Unfamiliar-4* | Blood | NMRSARL | NMRSARL (this study) |

[1]AR type, antibiogram-resistigram type which was determined based on the pattern of resistance to a panel of 22 antimicrobials (the panel was extended to 23 in 1998). Forty-four AR types (AR01-AR44) have been recognised to date but only those most commonly encountered during each study period were included in this present study. AR typing was not available for isolates recovered prior to 1988. AR pattern of MRSA isolates used in this study are described in Table 2.2.

[2]NA, information not available.

[3]FDVH, The Federated Dublin Voluntary Hospitals

[4]MMH, The Mater Misericordiae Hospital

[5]JSH, Jervis Street Hospital

[6]SJH, St James's Hospital, Dublin.

[7]Ext, external source, this isolate was recovered from a patient in an Irish hospital who had been previously hospitalised in Bahrain

[8]N/S Study, isolates from the Republic of Ireland, collected during the North/South Study of MRSA in Ireland 1999.

[9]N/S Study (N), isolates from Northern Ireland, collected during the North/South Study of MRSA in Ireland 1999.

[10]EARSS, isolates from unnamed Irish laboratories that participate in the European Antimicrobial Resistance Surveillance System (EARSS).

[11]NMRSARL, the National MRSA Reference Laboratory, St James's Hospital, Dublin.

*Not yet assigned an AR type due to unfamiliar antibiotic resistance patterns. AR pattern designation awaits PFGE, MLST and SCC*mec* analysis.

[a-f]These 12 isolates from 1999 which were recovered in pairs in mixed culture from single samples from individual patients. Each pair is indicated by sharing the same letter suffix.

**TABLE 2.2.** Antibiogram-Resistogram (AR) patterns exhibited by MRSA isolates used in this study

| | Antimicrobial Agent | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Ak** | **Ap** | **Cd** | **Cl** | **Cp** | **Eb** | **Er** | **Fd** | **Gn** | **Kn** | **Ln** | **Mc** | **Mp** | **Nm** | **Pma** | **Rf** | **Sm** | **Sp** | **Su** | **Tb** | **Te** | **Tp** | **Vn** |
| **AR type** | | | | | | | | | | | | | | | | | | | | | | | |
| **Early MRSA** | S | R | R | S | S | R | R | S | S | S | S | R | S | S | R | S | R | R | S | S | R | S | S |
| **Phenotype-II[a]** | S[3] | R | R | S | S | R | R | R | R | R | S[4] | R | S | R[1] | R | S | R | R | R[3] | R[3] | R[1] | R[1] | S |
| **Phenotype-III** | M | R | R | R | S | R | R | S | R | R | S | R | S | R | R | M | R | R | R | R | R | S | S |
| **AR01[a]** | S | R | R | S | S | R[1] | R | S | R[1] | R[1] | R | R | S | S | R | S | R | R | R[2] | M[1] | R | R | S |
| **AR02[a]** | S | R | R | S | S | R | R | R | R | R | R[1] | R | S | R[1] | R | S | R | R | R | R[3] | R[3] | R[1] | S |
| **AR05** | M | R | R | S | S | S | R | R | R | R | S | R | S | R | M | S | R | R | S | R | R | S | S |
| **AR06[a]** | S | R | S[4] | S | S[4] | S | S[4] | S[4] | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **AR07.0[a]** | M | R | R | S | R | S | R | S | S | R | R | S | R[3] | R | S | S | S | R | S | R | S | S | S |
| **AR07.2[a]** | R[1] | R | R | S | R | S | R | S | S | R | S | S | S | R | S | S | S | R | S | R | S | S | S |
| **AR07.3[a]** | R[3] | R | S | S[4] | R | S | R | S | S | R | S | S | S | R | S | S | S | R | S | R | S | S | S |
| **AR07.4[a]** | R[3] | R | S | S | R[1] | S | R | S | S | R | R | S | S | R | S | S | S | R | S | R | S | S | S |
| **AR09** | M | R | R | S | S | R | R | S | R | R | S | R | S | R | R | R | R | R | R | R | R | S | S |
| **AR11** | M | R | R | S | S | R | R | S | S | R | R | R | S | R | R | S | S | R | S | R | S | S | S |
| **AR13[a]** | M[1] | R | R | S | R | R | R | S[4] | R | R | R | R | R[3] | R | R | S | R | R | S | R | S | S | S |
| **AR14[a]** | M[1] | R | R | S | R | R | R | S[2] | R | R | S | R | S[2] | R | R | S | R | R | S | R | S | S[4] | S |
| **AR15** | M | R | R | S | S | R | R | S | R | R | R | R | S | S | R | S | R | R | R | R | R | R | S |
| **AR22** | R | R | R | S | R | R | R | S | R | R | R | R | S | R | R | R | R | R | M | R | R | S | S |
| **AR23** | M | R | R | S | S | R | R | R | R | R | R | R | S | R | R | R | R | S | R | R | R | S | S |
| **AR43[a]** | S | R | R[2] | S | R | S | R | S | S | R | S | S | S[4] | R | S | S | R | R | S | S | S[4] | R | S |
| **AR44[a]** | R | R | R | S | R | R | R | S | R | R | R[1] | R | R[3] | R | R | S | R | R | R | R | R | R | S |
| **ARNT[a]** | S | R | R | S | S[4] | S | S[4] | S[4] | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **New01** | M | R | R | S | R | R | R | S | R | R | R | R | M | S | R | S | S | R | S | R | S | S | S |
| **New02** | R | R | R | S | R | R | S | S | R | R | S | R | S | M | R | R | R | S | R | R | R | S | S |
| **New03** | S | R | R | S | R | R | R | S | R | R | R | R | M | S | R | S | S | R | S | R | S | S | S |
| **Unfamiliar-1** | S | R | R | S | R | S | R | S | S | S | S | R | S | S | R | S | S | R | S | S | S | R | S |
| **Unfamiliar-2** | M | R | R | S | R | S | S | S | R | R | S | R | S | R | S | S | R | S | S | R | R | S | S |
| **Unfamiliar-3** | R | R | R | S | R | R | R | S | R | R | S | R | M | R | R | S | R | R | R | R | R | R | S |
| **Unfamiliar-4** | M | R | R | S | R | S | R | S | R | R | R | S | S | R | S | S | S | S | R | R | R | R | S |

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; Vn, vancomycin; S, susceptible to the indicated antimicrobial; R, resistant to the indicated antimicrobial; M, moderately resistant to the indicated antimicrobial

[a]Some isolates classified as these AR types may demonstrate AR patterns that vary slightly from the pattern indicated in the above table for that AR type. For each AR pattern the variable antimicrobial agent is indicated with a number suffix. The number suffix 1 indicates that some isolates classified as that AR type are susceptible to the indicated antimicrobial agent; the number suffix 2 indicates that some isolates classified as that AR type are susceptible or moderately resistant to the indicated antimicrobial agent; the number suffix 3 indicates that some isolates classified as that AR type are moderately resistant to the indicated antimicrobial agent; the number suffix 4 indicates that some isolates classified as that AR type are resistant to the indicated antimicrobial agent.

(RAPD-PCR), plasmid screening, enterotoxin profiling (to detect enterotoxins A, B, C and D), biotyping (pigment production and hydrolysis of Tween 80 and urea) and antibiogram-resistogram (AR) typing (Rossney *et al.*, 1994a, 1994b & 2004; Rossney, 1995). Isolates were assigned an AR type on the basis of the pattern of resistance to the antimicrobials in the AR typing panel in conjunction with additional data obtained from a combination of the phenotypic and genotypic investigations described above. Forty-four AR types (AR01-AR44) and a number of subtypes have been recognised to date.

Four isolates from 2003 (study period I) and one from 2004 (study period J) were also included in this present study (Table 2.1). Isolate M03/0073 represents a newly recognised AR type (AR44) that was responsible for an outbreak in several Irish hospitals in 2003 (Rossney, 2003b). The four isolates M03/0068, M03/0169.2, E1520 and M03/0067 were included because they gave unfamiliar AR patterns not yet assigned to AR types, and they required further genotypic investigation. Among isolates from 1999, a previously unfamiliar AR pattern, designated AR43, was predominant among isolates from the North of Ireland. AR43 isolates from six patients were recovered in mixed culture with MRSA isolates exhibiting other AR patterns (five AR13 and one AR14 pattern) (Table 2.1). Proportionally more isolates exhibiting AR patterns predominant in 1999 (AR06, AR07, AR13, AR14 and AR43) were included in the present study to investigate the relationship between isolates exhibiting this new AR type, AR43, and isolates exhibiting other AR types commonly recovered during the same study period.

Apart from the four isolates from 2003/2004 described above, no AR type could be assigned to 17 other MRSA isolates included in the present study (Table 2.1). Isolates with AR type "ARNT" (Table 2.1) are those that exhibited the AR06 pattern, but unlike all other AR06 isolates, were positive for the hydrolysis of urea and yielded PFGE patterns unlike those obtained from other AR06 isolates. Hence, assigning urease positive AR06 isolates to AR type AR06 was considered incorrect and these isolates (n=7) were assigned to no AR type (NT) pending PFGE, MLST and SCC*mec* analysis. Isolates designated New01-New03 (n=6) and unfamiliar (n=4) (Table 2.1) were not assigned AR types because they produced unfamiliar AR patterns and AR type designation awaits the results of various phenotypic and genotypic investigations including MLST and SCC*mec* analysis.

All isolate numbers, sources, study periods and phenotypes are summarised in Table 2.1. The resistance patterns of AR types encountered among isolates used in this present study are listed in Table 2.2.

*2.1.1.1 Hospitals*

Isolates recovered between 1971 and 1984 were recovered from 10 Dublin hospitals including the Federated Dublin Voluntary Hospitals (FDVH), the Mater Misericordiae Hospital (MMH) and Jervis St. Hospital (JSH) (Table 2.1). The FDVH was a group of eight Dublin teaching hospitals, which in 1983, accounted for a combined total of 1,231 beds, housed the National Burns Unit and the Plastic Surgery Unit. Isolates recovered between 1985 and 1987 were from the FDVH only (Table 2.1). During the 1980's many of the hospitals that previously constituted the FDVH were closed and their services relocated to St. James's Hospital (SJH), Dublin. In 1988, the National Burns Unit was also transferred to SJH. Isolates recovered between 1989 and 1992-1993 were from either the FDVH of SJH (Table 2.1). Isolates from 1998 were recovered from SJH by which time this hospital catered for admission rates in excess of 20,000 patients per annum, covered all specialties except paediatrics and maternity and housed the National Bone Marrow Transplantation Service. Isolates recovered in 1999 were from hospitals in both the North and South of Ireland that participated in a two-week prevalence study of MRSA (Table 2.1). Ninety-eight percent of Irish hospitals participated in this study (Irish Department of Health & Children, 1999). In 2002, 2003 and 2004 isolates were from blood culture specimens from those Irish hospitals (n=23) that participated in the European Antimicrobial Resistance Surveillance System Study (EARSS) (Table 2.1). This level of participation represented a population cover rate of >90%.

**2.1.2 *Escherichia coli* strains**

The *Escherichia coli* strain DH5α [F- ϕ80d, *lac*ZΔm15, *end*A1, *rec*A1, *hsd*R17, (rK-mK+), *sup*E44, *thi*-1, d-, *gyr*A96, Δ (*lac*ZYA-*arg*F), U169] (Hanahan, 1983) was used as a host strain for plasmid pBluescript II KS (-) (Stratagene, La Jolla, California, USA).

## 2.2 General microbiological methods

### 2.2.1 Bacterial culture media, growth conditions and storage

All *S. aureus* clinical isolates and strains were cultured in Trypticase Soy Broth (TSB), (Oxoid Ltd., Hampshire, England) or on Trypticase Soy Agar (TSA) plates (Oxoid) and incubated overnight at 37°C in a static incubator (Gallenkamp, Leicester, United Kingdom). Liquid cultures were shaken at 200 r.p.m. overnight at 37°C in an orbital incubator (Gallenkamp). Isolates that had been stored in individual Protect bacterial preserver vials at –70°C were cultured by removing a single bead from each vial using a sterile forceps, inoculating it onto a TSA plate followed by overnight incubation at 37°C in a static incubator (Gallenkamp). Isolates that had been stored on individual nutrient agar slopes at room temperature were cultured by removing some colonies from the agar slope using a sterile inoculating loop, streaking these onto a TSA plate followed by overnight incubation at 37°C.

The *Escherichia coli* strain DH5α was routinely cultured on Luria-Bertani agar (LA) pH 7.4 (Sambrook & Russell, 2001) at 37°C overnight in a static incubator (Gallencamp) and, for liquid cultures, in Luria-Bertani broth (LB) pH 7.4 (Sambrook & Russell, 2001) at 37°C in an orbital shaker (Gallencamp) at 200 r.p.m. Derivatives of DH5α harbouring recombinant plasmids were cultured and maintained on LA containing 100 μg/ml ampicillin.

All bacterial strains and isolates were stored at –80°C in individual Protect bacterial preserver vials (Technical Services Limited, United Kingdom). Bacteria were streaked onto the appropriate media plates to obtain confluent growth of the bacterial isolate and grown overnight at 37°C. Using a sterile inoculating loop, all the colonies on the plate were transferred to a Protect bacterial preserver vial and stored at –80°C.

### 2.2.2 Chemicals, enzymes, radioisotopes and oligonucleotides

All chemicals used were of analytical-grade or molecular biology-grade and were purchased from the Sigma-Aldrich Chemical Co. (Tallaght, Dublin, Republic of Ireland), BDH (Poole, Dorset, UK) or Roche Diagnostics Ltd. (Lewes, East Sussex, UK). Enzymes for molecular biology procedures were purchased from the Promega Corporation (Madison, Wisconsin, USA), Roche Diagnostics Ltd. or New England Biolabs Inc. (Beverley, Massachusetts, USA) and were used according to the manufacturers' instructions. DNA molecular weight markers were purchased from

Promega. [$\alpha^{32}$-P]dATP (3,000 Ci/mmol$^{-1}$; 110 TBq/mmol$^{-1}$) was purchased from Perkin Elmer (Life Sciences Inc., Boston, Massachusetts, USA). Oligonucleotide primers were custom synthesised by Sigma-Genosys Biotechnologies Ltd. (Cambridge, United Kingdom).

### 2.2.3 Buffers and solutions

Tris-EDTA (TE) buffer was used routinely in many experiments and consisted of 10 mM Tris-HCl, 1 mM EDTA (tetrasodium), pH 8.

Tris-borate/EDTA electrophoresis (TBE) buffer was prepared at 5x concentration and consisted of 0.45 M Trizma base, 0.45 M boric acid, 0.01 M EDTA. This was diluted in distilled water to 0.5x concentration and was used as buffer for agarose gel electrophoresis.

DNA loading dye was prepared at 10x concentration and consisted of 30% (v/v) glycerol 0.25% (w/v) bromophenol blue and 3.8% (w/v) EDTA.

SSC was prepared at 20x concentration and consisted of 3.0 M NaCl and 0.3 M tri-sodium citrate, pH 7.0.

Proteinase K, lysozyme and lysosptaphin solutions were prepared in sterile distilled water at various concentrations depending on the protocol used and were stored at –20°C.

## 2.3 Analysis of genomic DNA

### 2.3.1 Extraction of genomic DNA from *S. aureus*

Total genomic DNA for use in MLST, SCC*mec* typing and SCC*mec* element amplification was prepared from *S. aureus* strains and isolates using the Qiagen Dneasy kit system (Qiagen, Crawley, Wesr Sussex, UK) according to the manufacturer's instructions.

Total genomic DNA for use in Southern hybridisation experiments was prepared from *S. aureus* isolates using the method of Dempsey (1996). Cells were grown in 5 ml TSB at 37°C in an orbital shaker for 16 h and a 1.5 ml aliquot was harvested by centrifugation for 2 min. The supernatant was decanted and the pellet was washed once in 200 µl chromosomal buffer (CS) (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA). The pellet was then resuspended in 100 µl CS containing a final concentration of 5 µg/ml lysostaphin and incubated at 37°C for 30 min with constant gentle shaking.

Proteinase K, 10 mg/ml, was added to give a final concentration of 0.5 μg/ml and the suspension was incubated for 30 min at 55°C. The cells were then lysed by the addition of 100 μl of 5 % (w/v) SDS (Sigma-Aldrich) in 50% (v/v) ethanol and incubated at room temperature with gentle shaking for 20 min. The DNA was then extracted three times using an equal volume of liquefied phenol washed in Tris-buffer (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) at room temperature with constant gentle shaking, centrifugation at 3,000 x *g* for 30 min and removal of the aqueous layer to a fresh tube each time. The DNA in the aqueous layer was precipitated by the addition of two volumes of ice-cold 100% ethanol. The resulting DNA precipitate was then transferred to a fresh tube, washed twice with 70% (v/v) ethanol, dried briefly at 37°C, resuspended in 100 μl 1x TE pH8 and stored at -20°C.

The concentration of each DNA preparation was determined by measuring their absorbance at 260 nm using a spectrophotometer (Genosys 2, ThermoSpectronic, Thermo Electron Group, Waltham, MA, USA) and calculating the concentration using the following formula; 1 $A_{260}$ unit = 50 μg DNA.

### 2.3.2 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis

Large scale restriction endonuclease digestions of genomic DNA for Southern analysis were carried out with 10 μg of genomic DNA in a 50 μl volume containing 20 U of restriction enzyme (described in the relevant sections) and the appropriate restriction enzyme buffer according to the manufacturer's instructions. Horizontal 0.8% (w/v) agarose (Sigma-Aldrich) gels made up in 0.5x TBE buffer containing 0.5 μg/ml ethidium bromide per ml were cast into horizontal gel trays. DNA loading dye was added to the restriction enzyme-digested DNA samples at a final concentration of 1x and the samples were loaded into the gel wells. A DNA size standard was loaded on each gel. Electrophoresis was carried out at 20 V with constant current until the bromophenol blue tracking dye had reached the end of the gel. DNA in gels was visualised on a High performance UV transilluminator (wavelength 345 nm, Ultra-Violet products Ltd (UVP), San Gabriel, CA, USA) and photographed using the ImageStore 7500 Version 7.22 Gel Documentation System (UVP).

### 2.3.3 Southern transfer of DNA from agarose gels

Following gel electrophoresis, DNA was transferred to BioBond™ Plus nylon membranes (Sigma-Aldrich) by capillary action using the method of Southern (Southern, 1975). The DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for 45 min with gentle agitation, after which the gel was placed in a neutralisation solution (1 M Tris-HCl, pH 7.5, 1.5 M NaCl) for a further 45 min with shaking. DNA fragments were transferred to the nylon membranes using 10x SSC as the transfer buffer. Following transfer the membrane was baked at 70°C for 1 h.

### 2.3.4 Random primer labelling of DNA fragments with [α-$^{32}$P]dATP

For Southern hybridisation experiments, DNA fragments were labelled with [α-$^{32}$P]dATP (6,000 Ci/mmol; 110 TBq/mmol) by random primer labelling using the Prime-a-gene kit purchased from Promega. Probes were routinely labelled to a specific activity of $>10^6$ d.p.m. per μg of DNA. Purified DNA fragments (10-200 ng in a 30 μl volume) were denatured by boiling for 2-3 min. Denatured DNA was added to a reaction mixture containing 1x labelling buffer, which was supplied by the manufacturer and contained a random mixture of hexanucleotides, dNTPs (dCTP, dGTP and dTTP) and bovine serum albumin (BSA). Three microlitres of α-$^{32}$P labelled dATP were added to the reaction mixture before adding 5 U of Klenow DNA polymerase. The reaction was then left to incubate at room temperature for 1 h. Unincorporated nucleotides were removed prior to hybridisation by passing the reaction mixture through a column containing a size exclusion matrix (SigmaSpin™ post-reaction columns, Sigma-Aldrich) following the protocol outlined by the manufacturer.

### 2.3.5 Southern hybridisation

Hybridisation reactions were carried out in a rotary hybridisation oven (Hybaid, Teddington, Middlesex, UK) in 25 x 3.5 cm hybridisation bottles (Hybaid) according to the method of Sambrook and Russell (2001). Nylon membranes on which DNA had been transferred were rinsed in 6x SSC prior to hybridisation to remove excess salt. Membranes were then incubated in the oven at 65°C in 15 ml of prehybridisation solution containing 1x Denhardt's solution [1% (w/v) Ficoll, 1% (w/v)

polyvinylpyrrolidone, 1% (w/v) BSA], 6x SSC, 100 $\mu$g/ml denatured salmon sperm DNA (Sigma-Aldrich) and 0.5 % (w/v) SDS for a minimum of 2 h.

The radiolabelled probe ($\approx$ 2 x $10^6$ d.p.m.) was denatured by boiling for five min followed by incubation on ice and then added to the prehybridisation solution and incubated with the membrane at 65°C for 18 h. Following hybridisation, unbound probe was removed from the membrane by washing the membrane at room temperature for 5 min in a solution consisting of 2x SSC, 0.1% (w/v) SDS. This was followed by a second wash at 37°C in 0.1x SSC, 0.5% (w/v) SDS for 30 min and a final wash at 64°C in 0.1x SSC, 0.5% (w/v) SDS for 30 min. The membrane was then placed in an autoradiography cassette with a Kodak BioMax intensifying screen (Eastman Kodak Company, Rochester, New York, USA) and exposed to Kodak Biomax MS-1 X-ray film for 24 to 72 h at –70°C. Autoradiograms were developed using Kodak GBX developer and fixed in Kodak GBX fixer according to protocols supplied by the manufacturer. In order to reuse membranes in subsequent hybridisation experiments, bound probe was removed from membranes by immersing them in boiling distilled water, followed by a brief rinse in 2x SSC.

## 2.4 Recombinant DNA techniques

### 2.4.1 Extraction of plasmid DNA from *E. coli*

Small scale preparations of plasmid DNA for sequencing were prepared using the GenElute™ plasmid miniprep kit according to the manufacturer's instructions (Sigma-Aldrich).

### 2.4.2 Polymerase chain reaction (PCR)

Specific sequences of *S. aureus* genomic DNA were amplified by PCR and cloned into pBluescript. Oligonucleotide primers were designed to amplify the required region and were stored at a stock concentration of 1 mM in sterile water at –20°C. Amplification reactions were carried out in 50 µl volumes in 0.5 ml microfuge tubes (Eppendorf) containing 2.5 U *Taq* DNA polymerase (Promega), 1x *Taq* reaction buffer, 2 mM MgCl2, 250 µM (each) dATP, dTTP, dCTP, dGTP (Promega), 10 pm of both a forward and reverse primer and 10 ng of genomic DNA template in a Thermo Hybaid Multiblock System (MBS) thermal cycler (Thermo Hybaid, Ashford, Middlesex, England). If the PCR product was to be be cloned into an expression vector or

sequenced, the reactions were carried out using the high fidelity proof reading enzyme mix Expand High Fidelity PCR System (Roche) and the buffers provided by the manufacturer or *pfu* polymerase (Promega). If the expected PCR product was above 5 kb in size the Expand Long Template PCR system (Roche) was used. Amplification conditions and specific primers are described in the relevant sections. Amplification products were separated by electrophoresis through 1% (w/v) agarose gels, unless otherwise stated, containing 0.5 μg/ml ethidium bromide and a DNA molecular weight marker was loaded on each gel. Following electrophoresis at 80V with constant current for 1 h, the amplification products were visualised on a UV transilluminator as described in section 2.3.2.

PCR products were either purified using the GenElute™ PCR DNA purification kit (Sigma-Aldrich) or the Promega SV gel and PCR clean up kit according to the manufacturer's instructions.

### 2.4.3 Ligation of DNA fragments

Purified PCR products were ligated to pBluescript II KS (-) phagemid digested with the appropriate restriction endonuclease. Ligation of DNA fragments to pBluescript were carried out *via* restriction sites which had been designed within the oligonucleotide primers used in the amplification reactions. Ligation was carried out for 18 h at 25°C, in a 20 μl reaction volume, with a 3:1 ratio of insert to vector DNA in 1x ligase buffer, with 1 U of T4 DNA ligase (Promega).

### 2.4.4 Transformation of competent *E. coli* prepared using $CaCl_2$

Transformation of *E. coli* strain DH5α with $CaCl_2$ was carried out by the method of Sambrook and Russell (2001). An overnight broth culture of DH5α was inoculated into 100 ml LB and grown at 37°C for 3 h at 200 rpm in an orbital incubator to an $A_{600}$ of ~0.5. The culture was then decanted in 50 ml volumes into ice-cold 250 ml Sorvall tubes and chilled on ice for 10 min. Cells were then pelleted by centrifugation in a Sorvall SS34 rotor at 5,000 x at 4°C for 10 min. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, and centrifuged as before. Pellets were then resuspended in 2 ml 0.1 M $CaCl_2$ for each 50 ml of original culture.

For each transformation experiment, 0.2 ml of this cell-suspension was transferred to a sterile microfuge tube on ice and plasmid DNA (up to 50 ng) was added to each tube

and incubated on ice for 30 min. A known amount of a standard plasmid preparation was added to a separate tube as a positive control, and a second control tube was also included which contained no plasmid DNA. The tubes were then heat shocked at 42°C for exactly 90 s and rapidly transferred to an ice bath, followed by incubation in a water bath at 37°C in the presence of 0.8 ml LB medium to allow the cells to recover and express the antibiotic resistance marker (ampicillin resistance in the case of pBluescript II KS [-]). A 0.1 ml aliquot of this suspension was then spread on LA plates containing antibiotic (100 μg ampicillin/ml in the case of pBluescript II KS [-]), 1 mM isopropyl-D-thiogalactopyranoside (IPTG, Roche) and 100 μg (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, Roche) and incubated for 20 h at 37°C. Recombinants were identified using blue-white selection as described by Sambrook and Russell (2001).

## 2.5 DNA sequence analysis

DNA sequencing (unless otherwise stated) was performed on a commercial basis by Lark (Takeley, Essex, United Kingdom) using the dideoxy chain termination method of Sanger (Sanger *et al.*, 1977), and an automated Applied Biosystems 373A DNA sequencer (Foster City, California, USA) and dye-labelled terminators. Analysis of chromatograms was carried out using the 373A Data Analysis programme version 1.2.0 (Applied Biosystems) or Chromas version 2.3 (available at hhtp://www.technelysium.com.au/chromas.html). Sequence analysis was carried out using the DNA Strider™ 1.3f11 software package for DNA and protein analysis (CEA/Saclay, Gif-sur-Yvette, France). Searches of the EMBL and GenBank databases for nucleotide and amino acid sequence similarities were performed using the BLAST family of computer programmes (Altschul *et al.*, 1990). Nucleotide and amino acid sequence alignments were carried out using CLUSTAL W sequence alignment computer programme (Higgins & Sharp, 1988).

# Chapter 3

# The Evolutionary Epidemiology of Irish Nosocomial MRSA Isolates Recovered Between 1971-2004.

## 3.1 Introduction

### 3.1.1 Irish MRSA

*3.1.1.1 1971-1987*

Since the first isolation of MRSA in Irish hospitals in 1971, various laboratory studies using mainly phenotypic techniques revealed that the MRSA population in Irish hospitals has continually been changing. In 1971, 5.4 % of *S. aureus* isolates recovered from Dublin hospitals were resistant to methicillin and to the commonly used antimicrobials of that time, except for gentamicin and chloramphenicol (Hone & Keane, 1974). Between 1971 and 1975 these MRSA isolates caused sporadic infections in several Dublin hospitals (Hone & Keane 1974; Cafferkey *et al.*, 1983 & 1985).

Following the first reported case of MRSA bacteremia in a patient in an Irish hospital in 1976, MRSA isolates that were also resistant to gentamcin became endemic in Dublin hospitals (Cafferkey *et al.*, 1983 & 1985; Coleman *et al.*, 1985). Analysis of these methicillin- and gentamcin- resistant *S. aureus* (MGRSA) isolates using plasmid screening and the location of resistance determinants identified two phenotypic groups (Coleman *et al.*, 1985). Phenotype-I isolates expressed high levels of resistance to gentamicin, were susceptible to fusidic acid, predominated as the major cause of MRSA nosocomial infection in Dublin hospitals between 1976 and 1978 and were not recovered after 1982 (Coleman *et al.*, 1985). Phenotype-II isolates predominated in Dublin hospitals between 1978 and 1985, expressed low level gentamicin resistance, were fusidic acid resistant and commonly carried a plasmid that conferred resistance to combinations of ethidium bromide, tetracycline, kanamycin, neomycin and trimethoprim (Coleman *et al.*, 1985).

Between 1985 and 1987 isolates exhibiting a new MGRSA phenotype caused an outbreak in two Dublin hospitals (Carroll *et al.*, 1989; Humphreys *et al.*, 1990). These Phenotype-III isolates were initially recovered from a patient in a Dublin hospital who had previously been hospitalised in Baghdad in Iraq. Using the various techniques available at the time including plasmid screening, hybridisatioin analysis, location of resistance determinants and bacteriophage typing, these Phenotype-III isolates were found to be distinct from both Phenotype-I and Phenotype-II isolates but very similar to MGRSA isolates recovered in 1984 from a Baghdad hospital (Carroll *et al.*, 1989).

*3.1.1.2 1988-1998*

Between 1989 and 1998 a four-fold increase in the number of patients with MRSA was observed in one Irish hospital (Rossney & Keane, 2002a). Routine susceptibility testing indicated that changes in the Irish MRSA population were occurring, with the possible emergence of EMRSA strains and an increasing proportion of isolates demonstrating susceptibility to gentamicin. Between 1988-1989, MRSA isolates exhibiting AR01 (see Table 2.2, for AR patterns) were isolated from 74% of patients carrying MRSA and were similar, but not identical to the UK epidemic strain EMRSA-1 (Rossney, 1995; Rossney & Keane, 2002a). During the same time period, Phenotype-II and Phenotype-III isolates were found to exhibit AR patterns AR02 and AR09 and were recovered from 30% and 4% of patients carrying MRSA, respectively, with 8% of patients carrying more than one AR type (Rossney, 1995). MRSA isolates exhibiting AR01 and AR02 predominated until 1989 but have since been absent from the Irish MRSA population (Rossney, 1995; Rossney & Keane, 2002a).

During the period 1993-1994, 81% of patients carrying MRSA carried isolates exhibiting AR13, AR14, AR11, AR07 or AR06 with 61% exhibiting the multiresistant AR patterns AR13 and AR14 (Rossney & Keane, 2002a). Also, in 1993, AR15 and AR22 isolates occurred frequently in one Dublin hospital with AR22 isolates causing an outbreak in an intensive care unit (Rossney *et al.*, 1994b).

By 1998, although MRSA isolates AR13 and AR14 (multiresistant isolates) predominated, their prevalence had decreased, while the prevalence of MRSA isolates AR06 and AR07 was increasing (Rossney & Keane, 2002a). All AR06 and AR07 isolates are gentamicin susceptible and the gentamicin resistance rates among Irish MRSA isolates has continued to decline up to the present day. In 1999 58.4% of Irish MRSA isolates were gentamicin resistant (EARSS Steering Group, 2003), in 2002 this fell to 27.6% (EARSS Steering Group, 2003) and in the third quarter of 2004 (the latest EARSS report available) less than 10% of Irish MRSA were gentamicin resistant (EARSS, 2004b). This reflects a growing trend in many parts of Europe in which the predominate circulating strains of MRSA are less resistant to multiple antibiotics.

A dendrogram constructed from the analysis of the PFGE patterns of *Sma*I-digested total genomic DNA of MRSA isolates exhibiting the most common AR types recovered from patients in Irish hospitals between 1989 and 1998, showed that the MRSA population could be divided into two main clusters (Fig. 3.1. Rossney & Keane, 2002a). The first cluster consisted of AR06 isolates only, while the second cluster was separated

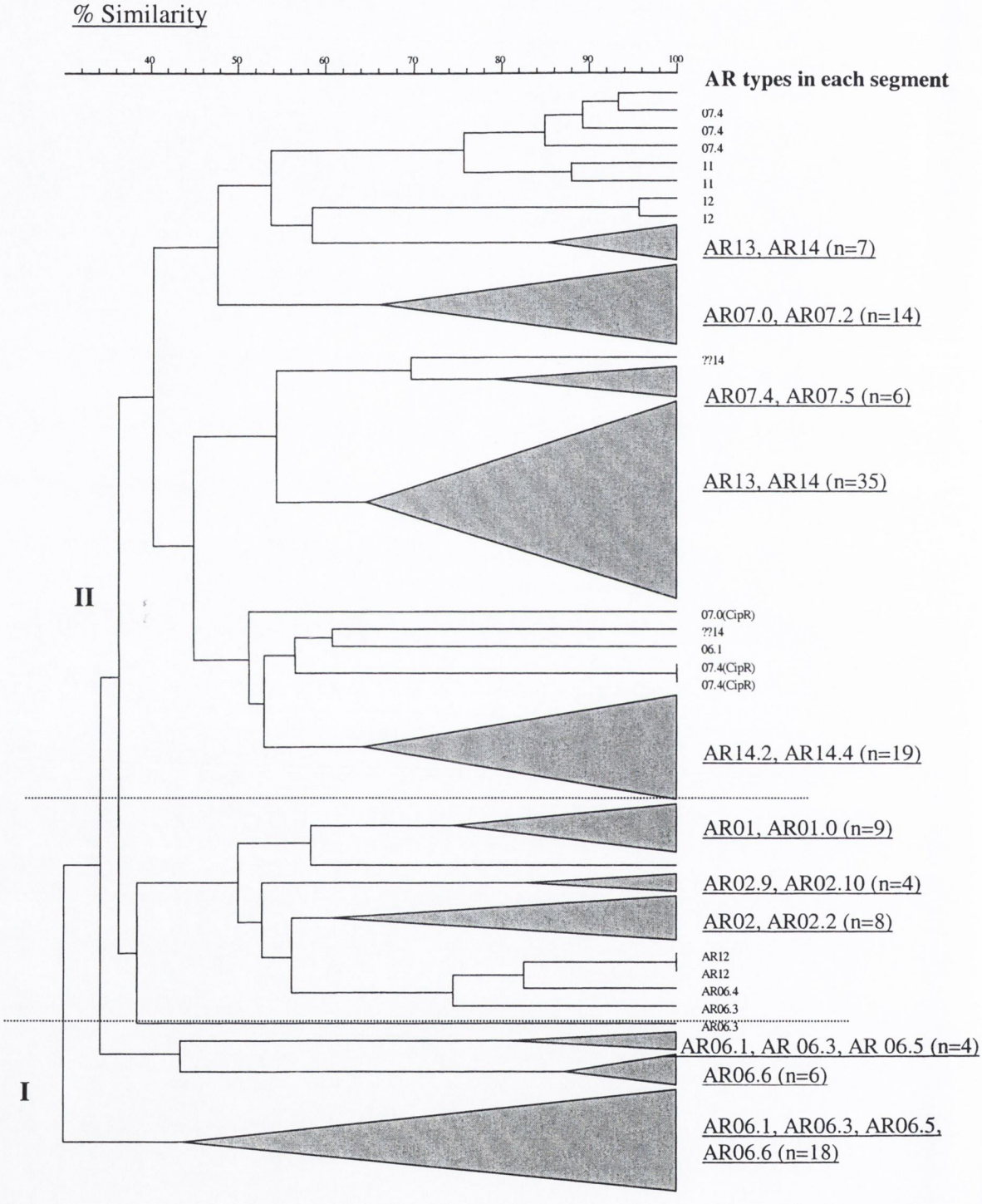

% Similarity
40
50
60
70
80
90
100
AR types in each segment
07.4
07.4
07.4
11
11
12
12
AR13, AR14 (n=7)
AR07.0, AR07.2 (n=14)
??14
AR07.4, AR07.5 (n=6)
AR13, AR14 (n=35)
II
07.0(CipR)
??14
06.1
07.4(CipR)
07.4(CipR)
AR14.2, AR14.4 (n=19)
AR01, AR01.0 (n=9)
AR02.9, AR02.10 (n=4)
AR02, AR02.2 (n=8)
AR12
AR12
AR06.4
AR06.3
AR06.3
AR06.1, AR 06.3, AR 06.5 (n=4)
AR06.6 (n=6)
I
AR06.1, AR06.3, AR06.5, AR06.6 (n=18)

**FIGURE 3.1.** Dendrogram showing the percent relatedness of DNA from MRSA isolates representative of the most common AR types recovered from patients in Irish hospitals between 1989 and 1998 (Rossney & Keane, 2002a). Following *Sma*I restriction endonuclease digestion, DNA fragments were separated by PFGE and the fragment profiles obtained were compared using the fingerprint profile analysis computer software package GelCompar (with a band tolerance of 1%, Dice coefficient and UPGMA clustering) (Rossney & Keane, 2002a). The dendrogram shows that the population of MRSA isolates investigated could be divided into two main clusters. Cluster I consisted of AR06 isolates only, while cluster II could be further separated into two major subgroups, one consisting of AR01 and AR02 isolates and the other containing mainly AR14, AR13 and AR07 isolates. (Rossney & Keane, 2002a).

into two major subgroups, one consisting of AR01 and AR02 isolates and the other containing mainly AR14, AR13 and AR07 isolates (Fig. 3.1. Rossney & Keane, 2002a). In this second cluster of the dendrogram AR13 and AR14 isolates appeared to be closely related, whilst AR07 isolates appeared distinct (Fig. 3.1. Rossney & Keane, 2002a).

*3.1.1.3 1999-2004*

By 1999, isolates exhibiting AR13, AR06, AR14 and AR07 predominated in the Republic of Ireland with prevalence rates of 31%, 26%, 19% and 18%, respectively (Rossney *et al.*, 2003). The proportion of isolates exhibiting AR13 and AR14 had descreased from previous years (Rossney *et al.*, 2003). In Northern Ireland AR43, AR13 and AR06 predominated in 1999, with prevalence rates of 42%, 33% and 15%, respectively (Rossney *et al.*, 2003). This was the first report of isolates exhibiting this new AR pattern, AR43 (Rossney *et al.*, 2003). A dendrogram drawn from the fingerprint patterns obtained following PFGE of *Sma*I-digested total genomic DNA of isolates exhibiting this AR pattern showed that AR43 falls into the same cluster as AR13 and AR14, but the fingerprint patterns showed a great degree of heterogeneity (Fig. 3.2. Irish Dep. of Health and Children, 1999). Additional unfamiliar AR patterns were present in small numbers in the same study and were tentatively called New01, New02 and New03 until further genotypic investigations allowed an AR type to be assigned to these isolates (Irish Dep. of Health and Children, 1999). During this same study of MRSA in Ireland in 1999 it was also observed that the PFGE patterns of AR06 and AR07 isolates appeared closely related to those of UK epidemic strains EMRSA-15 and EMRSA-16, respectively (Irish Dep. of Health and Children, 1999; Rossney *et al.*, 2003).

Between 1999 and 2003, the Irish MRSA population was again shown to be changing. By 2003 isolates exhibiting AR06 (non-multiresistant isolates) accounted for 80% of the MRSA isolates recovered from blood cultures from patients in Irish hospitals, while isolates exhibiting AR14, AR13 and AR07 were also recovered but at low frequencies (Rossney *et al.*, 2004). Between 1999 and 2003 New03 isolates were again identified but accounted for only 2.6% of MRSA isolates recovered (Rossney *et al.*, 2004).

Another new AR pattern was reported by the NMRSARL in 2003 from a patient admitted to an Irish hospital who had previously been hospitalised in Singapore

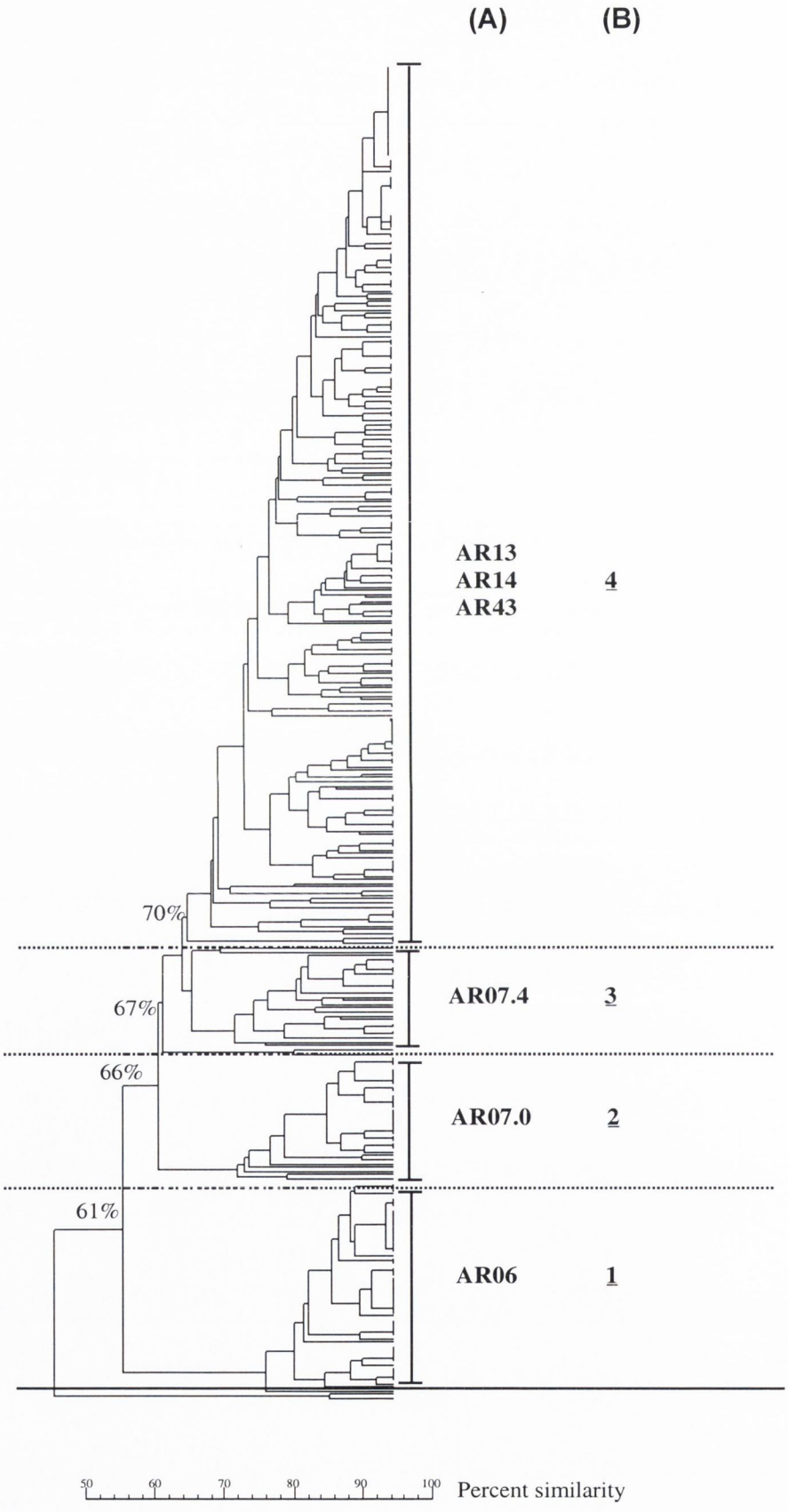

(A)
(B)
AR13
AR14
AR43
4
70%
AR07.4
3
67%
66%
AR07.0
2
61%
AR06
1
50
60
70
80
90
100
Percent similarity

**FIGURE 3.2.** Dendrogram showing the percent relatedness of DNA fragment patterns from MRSA isolates showing AR types AR06, AR07.0, AR07.4, AR13, AR14 and AR43 investigated during the North/South study of MRSA in Ireland 1999 (Irish Dep. Of Health & Children, 1999). Following *Sma*I restriction endonuclease digestion, DNA fragments were separated by PFGE and the fingerprint profiles obtained were compared using a computer the fingerprint profile anlaysis computer package GelCompar (with a band tolerance of 1%, Dice coefficient and UPGMA clustering) (Irish Dep. of Health & Children, 1999). The dendrogram shows that this population of MRSA isolates could be divided into four main groups. The AR types represented by each group are shown under (A) and the name of each group is indicated under (B) (Irish Dep. of Health and Children, 1999).

(Rossney, 2003b). The AR pattern produced by this isolate was unlike any AR pattern held in the NMRSARL's database and was designated, AR44. MRSA isolates exhibiting this AR type were recovered from other patients. In 2003 and 2004, a small number of isolates with unfamiliar AR patterns were again observed among isolates recovered from patients in Irish hospitals. All of these isolates with unfamiliar AR patterns required further genotypic investigations before they could be assigned to AR types.

The most predominant phenotypes of MRSA identified in Irish hospitals during different time periods since 1971 are listed in Table 3.1

### 3.1.2 Investigating the genetic relatedness of MRSA isolates

In recent years, it has become evident from the different studies of the epidemiology and evolutionary relationships of nosocomial MRSA clones from various geographic areas of the world, that in order to understand the genetic relatedness of MRSA isolates, each isolate needs to be analysed for both the genotype of the MSSA isolate into which the SCC*mec* element was introduced and the type of SCC*mec* element it harbours (Hiramatsu *et al.*, 2001; Oliveira *et al.*, 2001c; Enright *et al.*, 2002, Oliveira *et al.*, 2002b; Robinson & Enright., 2003). A highly discriminatory molecular typing technique, such as PFGE, is suitable for analysing the genotypes of MRSA isolates recovered over a relatively short period of time. However, for long-term and global epidemiological studies, MLST has been shown to be the most powerful molecular technique for genotyping MRSA isolates (Enright *et al.*, 2000 & 2002; Oliveira *et al.*, 2002b; Robinson & Enright., 2003; Robinson & Enright., 2004b). Furthermore, computer generated dendrograms, the traditional method used to analyse PFGE patterns and to assess the relationship between the isolates, provide little or no information on the patterns of evolutionary decent among groups of related isolates. In contrast, the relatedness of MLST genotypes and the patterns of evolutionary descent among groups of isolates with similiar MLST genotypes can be discerned using an algorithm known as eBURST (Feil *et al.*, 2004). The eBURST programme (which stands for Based Upon Related Sequence Types), can be used to divide a set of MLST data into groups of related isolates which can be visualised as clonal complexes (CCs) and it can also be used to predict both the ancestral genotype and the patterns of evolutionary decent within each CC. Using MLST and eBURST, Robinson & Enright (2003) have identified the five major clonal complexes for nosocomial MRSA and have predicted the ancestral

**TABLE 3.1.** The most predominant phenotypes of MRSA identified in Irish hospitals during different time periods since 1971

| Year | Predominant phenotypes identified[1] | Reference |
|---|---|---|
| 1971-1975 | Early MRSA | Hone & Keane, 1974; Coleman *et al.*, 1985 |
| 1976-1978 | Phenotype-I[2] | Coleman *et al.*, 1985 |
| 1978-1984 | Phenotype-II | Coleman *et al.*, 1985 |
| 1985-1987 | Phenotype-III | Carroll *et al.*, 1989 |
| 1988-1989 | AR01 & AR02 | Rossney, 1995; Rossney & Keane, 2002a |
| 1993-1994 | AR13 & AR14 | Rossney, 1995; Rossney & Keane, 2002a |
| 1998 | AR06, AR07, AR13 & AR14 | Rossney & Keane, 2002a |
| 1999 | AR06, AR07, AR13, AR14, (AR06, AR13 & AR43) | Irish Dep. Of Health & Children 1999; Rossney *et al.*, 2003 |
| 2002 | AR06 | Rossney *et al.*, 2004 |
| 2003 | AR06 | Rossney *et al.*, 2004 |

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

[2]Phenotype-I isolates were not available for investigation in this present study.

genotypes and patterns of decent of many genotypes within each clonal complex. Genotypes that differ from the ancestral genotype at only one of the seven MLST loci are referred to as single locus variants (slv) (Feil *et al.*, 2004). A SLV that has further diversified resulting in a derivative genotype that differs from that of the ancestral genotype at two of seven MLST loci is known as a double locus variant (dlv) (Feil *et al.*, 2004).

The SCC*mec* type can be investigated using various PCR-based techniques that identify both the *ccr* and *mec* gene type or other sequences in the J regions specific to each SCC*mec* element (Okuma *et al.*, 2002; Oliveira *et al.*, 2002a). Genetic studies of nosocomial MRSA populations in various different countries using MLST and SCC*mec* typing has revealed the majority of MRSA strains identified, have genotypes (i.e. sequence types, STs) within the five previously defined pandemic MRSA lineages (Denis *et al.*, 2004; Perez-Roth *et al.*, 2004; Velazquez-Meza *et al.*, 2004; Ko *et al.*, 2005)

### 3.1.3 Aims

Epidemiological typing of Irish MRSA isolates over the last three decades has mainly depended upon antimicrobial susceptibility testing. The aim of this part of the present study was to comprehensively investigate the evolutionary epidemiology of nosocomial MRSA isolates that have been recovered in Irish hospitals between 1971 and 2004 using a combination of molecular techniques. Multilocus sequence typing combined with SCC*mec* typing was used to identify the major MRSA clonal types causing nosocomial infections during this time period (1971-2004). Furthermore, PFGE was performed in addition to MLST and SCC*mec* typing on isolates recovered during a two-week study of MRSA in Ireland in 1999 in order to examine the relationship between isolates exhibiting a previously unfamiliar AR pattern, AR43, and other isolates also recovered during the two-week study.

## 3.2 Materials and Methods

### 3.2.1 Pulsed-field gel electrophoresis (PFGE)

*3.2.1.1 Bacterial isolates*

Pulsed-field gel electrophoresis was performed on 67 Irish MRSA isolates recovered from hospitalised patients in both the North and South of Ireland, during a two-week surveillance study of MRSA in 1999 (Table 3.2). These isolates were chosen for PFGE in order to investigate the relationship between isolates exhibiting a new AR type, AR43, and isolates exhibiting other AR types (AR06, AR07.0, AR07.4, AR13 and AR14) commonly recovered during the same study period (study period G). Pulsed-field gel electrophoresis was a suitable method for investigating the relationship between these isolates because they were recovered over a relatively short period of time (2 weeks).

3.2.1.2 *Preparation of S. aureus chromosomal DNA in agarose plugs*

A method described previously by Rossney (1995) was used to prepare *S. aureus* DNA for PFGE. This method is a modification of a method described by de Lencastre *et al.* (1994).

Chromosomal DNA was prepared from *S. aureus* cells grown in 5 ml TSB at 37°C for 18 h in an orbital shaker (Gallenkamp). Cells were harvested by centrifugation at 2,500 x *g* at 4°C for 10 min, resuspended in 500 µl PFGE buffer (10 mM Tris pH 8, 0.1 M NaCl) and the centrifugation was repeated. Cells were resuspended in 200 µl PFGE buffer and diluted with an additional 1.8 ml PFGE buffer. A 0.2 ml aliquot of this bacterial suspension was mixed with 2% (w/v) molten InCert agarose (BioWhittaker Molecular Applications, Rockland, Maine, USA) dissolved in PFGE buffer. This suspension was transferred into casting moulds (4 x 9 x 25 mm; Bio-Rad, Hercules, California, USA) and were left to solidify at 4°C for 20 min.

Solidified agarose plugs were then incubated in 1 ml volumes of PFGE EC buffer (6 mM Tris pH 8.0, 1 M NaCl, 0.1 M EDTA pH 8.0, 0.2 % (w/v) sodium deoxycholate, 0.5 % (w/v) sodium lauroyl sarcosine) with lysostaphin (50 mg/l), lysozyme (100 mg/l) and RNase (50 mg/l) (Sigma) with gentle shaking in a waterbath at 37 °C for 3 h. The EC buffer was then decanted off and the plugs were incubated in 1 ml PFGE ES buffer (0.5 M EDTA pH 9.0, 1% (w/v) sodium lauroyl sarcosine) containing proteinase K (100 mg/l) at 50 °C with gentle shaking for 17 h.

**TABLE 3.2.** Summary of the total number of Irish MRSA isolates investigated from each study period and numbers typed by PFGE, MLST and SCC*mec* typing*

| **Study period** | A 1971-1975 | B 1976-1984 | C 1985-1987 | D 1989 | E 1992-1993 | F 1998 | G 1999 | H 2002 | I 2003 | J 2004 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Reference**[a] | 1 | 1 | 2 | 3 & 4 | 4 & 5 | 5 | 6 & 7 | 8 | 8 | 9 | | | |
| **Source hospitals** | **Dublin** | | | | | | **Ireland** | | | | **Numbers typed by** | | |
| | | | | | | | **North & South** | **South** | **South** | **South** | PFGE | MLST | SCC*mec* typing |
| **Isolate Phenotype**[b] | | | | | | | | | | | | | |
| **Early MRSA** | 1 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 1 | 1 |
| **Phenotype-II** | ... | 15 (8)[c] | ... | ... | ... | ... | ... | ... | ... | ... | ... | 8 | 15 |
| **Phenotype-III** | ... | ... | 1 | ... | ... | ... | ... | ... | ... | ... | ... | 1 | 1 |
| **AR01** | ... | ... | ... | 4 | ... | ... | ... | ... | ... | ... | ... | 4 | 4 |
| **AR02** | ... | ... | ... | 3 | ... | ... | ... | ... | ... | ... | ... | 3 | 3 |
| **AR05** | ... | ... | ... | 1 | ... | ... | ... | ... | ... | ... | ... | 1 | 1 |
| **AR06** | ... | ... | ... | 1 | 4 | 4 | 10 (6)[c] | 8 | ... | ... | 10 | 23 | 27 |
| **AR07.0/07.2** | ... | ... | ... | ... | ... | 2 | 9 (5)[c] | 2 | ... | ... | 9 | 9 | 13 |
| **AR07.3/07.4** | ... | ... | ... | 3 | 5 | 3 | 2 | ... | ... | ... | 2 | 13 | 13 |
| **AR09** | ... | ... | ... | 3 | ... | ... | ... | ... | ... | ... | ... | 3 | 3 |
| **AR11** | ... | ... | ... | ... | 4 | ... | ... | ... | ... | ... | ... | 4 | 4 |
| **AR13** | ... | ... | ... | ... | 4 | 4 | 15 | 3 | ... | ... | 15 | 26 | 26 |
| **AR14** | ... | ... | ... | ... | 4 | 4 | 11 | 3 | ... | ... | 11 | 22 | 22 |
| **AR15** | ... | ... | ... | ... | 2 | ... | ... | ... | ... | ... | ... | 2 | 2 |
| **AR22** | ... | ... | ... | ... | 2 | ... | ... | ... | ... | ... | ... | 2 | 2 |
| **AR23** | ... | ... | ... | ... | 1 | ... | ... | ... | ... | ... | ... | 1 | 1 |
| **AR43** | ... | ... | ... | ... | ... | ... | 20 | ... | ... | ... | 20 | 20 | 20 |
| **AR44** | ... | ... | ... | ... | ... | ... | ... | 1 | 1 | ... | ... | 2 | 2 |
| **ARNT**[d] | ... | ... | ... | 1 | 3 | ... | ... | 3 | ... | ... | ... | 7 | 7 |
| **New01**[d] | ... | ... | ... | ... | ... | ... | 1 | ... | ... | ... | ... | 1 | 1 |
| **New02**[d] | ... | ... | ... | ... | ... | ... | 1 | ... | ... | ... | ... | 1 | 1 |
| **New03**[c] | ... | ... | ... | ... | ... | ... | 2 | 2 | ... | ... | ... | 4 | 4 |
| **Unfamiliar 1**[d] | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | ... | 1 | 1 |
| **Unfamiliar 2**[d] | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | ... | 1 | 1 |
| **Unfamiliar 3**[d] | ... | ... | ... | ... | ... | ... | ... | ... | 1 | ... | ... | 1 | 1 |
| **Unfamiliar 4**[d] | ... | ... | ... | ... | ... | ... | ... | ... | | 1 | ... | 1 | 1 |
| **Total in this study** | 1 | 15 | 1 | 15 | 29 | 17 | 71 | 22 | 4 | 1 | 67 | 162 | 177 |

*For more detail on all isolates see Table 2.1., Chapter 2.

..., indicates that isolates exhibiting this phenotype were not recovered during the study period.

[a]References for the original studies involving these isolates: 1, Coleman *et al.*, 1985; 2, Carroll *et al.*, 1989; 3, Rossney, 1995; 4, Rossney & Keane, 2002a; 5, Rossney *et al.*, 1994b; 6, Irish Dep. of Health & Children, 1999; 7, Rossney *et al.*, 2003; 8, Rossney *et al.*, 2004; 9, this study.

[b]The most prevalent phenotypes during each study period are underlined.

[c]In the three cases where not all isolates of a particular AR type from a certain year were MLS typed, the numbers that were MLS typed are indicated in parenthesis adjacent to the number of available isolates (see section 3.2.2.1).

[d]These isolates have not yet been assigned to an AR type due to the unfamiliar antibiotic resistance patterns they produced. AR pattern designation awaits PFGE, MLST and SCC*mec* element analysis.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; SCC*mec*, Staphylococcal cassette chromosome *mec*.

The agarose plugs were then washed five times (for a minimum period of 10 min per wash) in 5 ml TE buffer at 55°C with gentle shaking. Following the five washes, 5 ml of fresh TE buffer was added to each agarose plug, which were then held at 4°C for 2 h. Fresh TE buffer was again added to each plug, followed by incubation at 4°C until required for restriction endonuclease digestion. For long-term storage the plugs were stored in 0.5 M EDTA, pH 8.0 at 4°C and the buffer was replaced at monthly intervals.

*3.2.1.3 Restriction endonuclease digestion of chromosomal DNA embedded in agarose plugs*

An aliquot of each agarose plug containing DNA was incubated in TE buffer at 4°C for a minimum time of 30 min, followed by equilibration in 150 µl of restriction endonuclease buffer at 4°C for a least 30 min. DNA was digested in a total reaction volume of 150 µl using *Sma*I (Promega) (20 units/plug), equilibrated at 4°C for 30 min and incubated at 25°C for 3 h.

*3.2.1.4 Agarose gel electrophoresis*

Agarose plugs containing digested chromosomal DNA were applied to 1.2 % (w/v) ultra-pure DNA grade agarose (Bio-Rad) gels prepared in 0.5 x TBE which were cast in 210 x 127 mm trays. Bacteriophage lambda (λ) DNA fragments ranging in size from 50-1000 kb (Sigma) were included on each gel as molecular size markers. In addition, the *S. aureus* isolate NCTC8325 was included as a control on each gel that was to be analysed using the DENDRON computer package, to allow gel to gel comparisons. The clamped homogenous electrical field (CHEF) electrophoresis system and the CHEF mapper PFGE apparatus (Bio-Rad) were used for PFGE with 0.5 x TBE as the electrophoresis buffer, which was maintained at 4°C during electrophoresis by recirculation through a Bio-Rad minichiller (model 1000).

Gels were run at 6 V/cm with an initial switch time of 1 s and a final switch time of 40 s over a 23 h period. Following electrophoresis, gels were stained for 30 min with 0.5 mg/l ethidium bromide, destained for 15 min in distilled water and examined on a UV transilluminator and photographed.

*3.2.1.5 Analysis and interpretation of DNA restriction patterns produced by PFGE*

The observational criteria described by Tenover *et al.* (1995) and shown in Table 1.3 were used to analyse the PFGE fingerprint profiles produced. In addition, the

DENDRON fingerprint profile anlaysis computer package version 2.0 (Soltech, Iowa City, Iowa, USA) was used to analyse the PFGE fingerprint patterns. In order to use this computer package, the PFGE fingerprint patterns were digitised into the DENDRON software package using an Epson Perfection 1650 scanner (Epson UK Ltd., Hertfordshire, UK). The *S. aureus* isolate NCTC8325 was used as a global standard to link data from different gels. Any distortions in the images were corrected using the straightening and unwarping options of DENDRON. The fingerprint patterns of the various MRSA isolates were compared by computing the similarity coefficient ($S_{AB}$) between every pair. The computation of $S_{AB}$ values was based upon the band positions according to the formula $S_{AB}=2E(2E+a+b)$, where E is the number of bands in patterns A and B sharing the same positions, a is the number of bands in pattern A with no correlation to any bands in pattern B and b is the number of bands in pattern B with no correlation to any bands in pattern A. An $S_{AB}$ of 0.00 indicates that patterns A and B have no bands in common, an $S_{AB}$ of 1.00 indicates that all the bands in patterns A and B match and an $S_{AB}$ ranging from 0.01 to 0.99 represents patterns with increasing numbers of bands at the same position. Based on these $S_{AB}$ values a dendrogram was created using the DENDRON computer package.

### 3.2.2 Multilocus Sequence Typing

*3.2.2.1 Bacterial isolates*

Multilocus sequence typing (MLST) was performed on a total of 162 of the 177 MRSA isolates selected for use in this present study (Table 3.2). These 162 isolates included representatives of all of the predominant phenotypes of MRSA recovered from patients in Irish hospitals during eight study periods (A-H) between 1971 and 2002 (Table 3.2). The only exception was study period B (1976-1984), where Phenotype-I and Phenotype-II isolates predominated but only Phenotype-II isolates were available for use in this present study. The number of isolates typed by MLST also included isolates recovered between 1989 and 2004 (study periods D-J respectively; Table 3.2) to which no AR type could be assigned because of the unusual AR or PFGE patterns they produced (ARNT, New01, New02, New03 and Unfamiliar 1-4; Table 3.2) patterns they produced and isolates from 2002 and 2003 (study periods H and I, respectively) with a relatively newly identified AR type, AR44 (Table 3.2).

In total, 15 isolates were excluded from MLST (Table 3.3); 4 /10 AR06 isolates from 1999 (study period G), 4/9 AR07.0 isolates from 1999 (study period G) and 7/15

**TABLE 3.3.** MRSA clinical isolates that were not typed by MLST

| AR type | Isolate no. | Study period (year isolated) |
|---|---|---|
| Phenotype-II | 58.1 | B (1976-1984) |
| Phenotype-II | 122.2 | B (1976-1984) |
| Phenotype-II | 43.21 | B (1976-1984) |
| Phenotype-II | 438.1 | B (1976-1984) |
| Phenotype-II | 438.2 | B (1976-1984) |
| Phenotype-II | 161.2 | B (1976-1984) |
| Phenotype-II | 120.2 | B (1976-1984) |
| | | |
| AR06 | 3143 | G(1999) |
| AR06 | 3190 | G(1999) |
| AR06 | 3147 | G(1999) |
| AR06 | 3430 | G(1999) |
| | | |
| AR07.0 | 3142 | G(1999) |
| AR07.0 | 3228 | G(1999) |
| AR07.0 | 3572 | G(1999) |
| AR07.0 | 3604 | G(1999) |

Phenotype-II isolates recovered between 1976-1984 (study period B) (Table 3.3). The reasons for excluding these isolates are as follows; firstly as stated in chapter 2 (section 2.1.1), proportionally more isolates recovered in 1999 exhibiting AR patterns AR06 and AR07.0 were included in the present study to investigate their relationship to isolates exhibiting the new AR type, AR43. All these AR06 and AR07.0 isolates were investigated by PFGE. However, 4/10 AR06 and 4/9 AR07.0 isolates from 1999 were excluded from MLST because their PFGE patterns and SCC*mec* types were identical or almost identical to the other AR06 and AR07.0 isolates from 1999 that were MLS typed (see sections 3.3.1 and 3.3.3 for PFGE and SCC*mec* typing results, respectively). Also, all AR06 and AR07.0 isolates that were MLS typed from 1999 yielded the same STs. Hence, it was deemed unnecessary to MLS type all of the AR06 and AR07.0 isolates from 1999. In addition MLST of all 15 Phenotype-II isolates was deemed unnecessary because although two different SCC*mec* typing patterns were identified in these isolates, the MLS type of the eight Phenotype-II isolates that were MLS typed were all the same (see sections 3.3.2 and 3.3.3.for MLST and SCC*mec* typing results, respectively).

*3.2.2.2 MLST method*

MLST typing was carried out as described previously (Enright *et al*, 2000). For each isolate the internal DNA fragments of seven housekeeping genes (carbamate kinase [*arc*], shikimate dehydrogenase [*aro*], glycerol kinase [*glpF*], guanylate kinase [*gmk*], phosphate acetyltransferase [*pta*], trioesphosphate isomerase [*tpi*] and acetyl coenzyme A acetyltransferase [*yqiL*]) were amplified in individual PCR reactions using the primer pairs described in Table 3.4. The PCR reaction was carried out in 96 well plates in a total reaction volume of 50 µl containing approximately 0.5 µg of chromosomal DNA, 0.5 µg of both the forward and reverse primer, 1U of *Taq* DNA polymerase (Promega), 5 µl of 10 X buffer (supplied with the *Taq* polymerase), 5 µl of 25 mM $MgCl_2$ and 0.2 mM deoxynucleoside triphosphates (Promega). PCR reactions were performed with an initial 3 min denaturation step at 95°C, followed by 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min and denaturation at 95°C for 1 min, and a final extension step at 72°C for 10 min. Amplification products were separated by electrophoresis through 1% (w/v) agarose gels and visualised on a UV transillminator as described previously in sections 2.3.2 and 2.4.2, respectively.

**TABLE 3.4.** Primer pairs used for amplification of the seven housekeeping genes used in MLST*

| Primer | Nucleotide sequence 5'-3' | Amplimer size | Region amplified |
|---|---|---|---|
| *arcC*-Up | TTGATTCACCAGCGCGTATTGTC | 456 bp | Internal fragment of carbamate kinase gene(*arcC*) |
| *arcC*-Dn | AGGTATCTGCTTCAATCAGCG | | |
| *aroE*-Up | ATCGGAAATCCTATTTCACATTC | 456 bp | Internal fragment of shikimate dehydrogenase gene (*aroE*) |
| *aroE*-Dn | GGTGTTGTATTAATAACGATATC | | |
| *glpF*-Up | CTAGGAACTGCAATCTTAATCC | 465 bp | Internal fragment of glycerol kinase gene(*glpF*) |
| *glpF*-Dn | TGGTAAAATCGCATGTCCAATTC | | |
| *gmk*-Up | ATCGTTTTATCGGGACCATC | 429 bp | Internal fragment of guanylate kinase gene(*gmk*) |
| *gmk*-Dn | TCATTAACTACAACGTAATCGTA | | |
| *pta*-Up | GTTAAAATCGTATTACCTGAAGG | 474 bp | Internal fragment of phosphate acetyltransferase gene (*gmk*) |
| *pta*-Dn | GACCCTTTTGTTGAAAAGCTTAA | | |
| *tpi*-Up | TCGTTCATTCTGAACGTCGTGAA | 402 bp | Internal fragment of triosephosphate isomerase gene (*tpi*) |
| *tpi*-Dn | TTTGCACCTTCTAACAATTGTAC | | |
| *yqiL*-Up | CAGCATACAGGACACCTATTGGC | 516 bp | Internal fragment of acetyl coenzymeA acetyltransferase (*yqiL*) |
| *yqiL*-Dn | CGTTGAGGAATCGATACTGGAAC | | |

*Table adapted from Enright *et al.*, 2000.

The PCR products in the 96 well plate were purified by the addition of 20% (w/v) polyethylene glycol 6000 (Sigma-Aldrich) and 2.5 M NaCl, followed by centrifugation at 1000 rpm for 30 s, incubation at room temperature for 30 min and centrifugation at 3000 rpm for 1 h. The supernatant was then removed by inverting the 96 well plate on to tissue paper followed by centrifugation at 500 rpm for 20 s. The 96 well plate was again inverted onto clean tissue paper and centrifuged again at 500 rpm for 20 s. The PCR products in the 96 well plate were then reprecipitated by resuspension in 100 µl of cold 70% (v/v) EtOH and centrifugation of the 96 well plate in the upright position at 3500 rpm for 30 min. The 96 well plate was again inverted on to tissue paper, centrifuged at 500 rpm for 20 s, inverted on clean tissue paper and centrifuged at 500 rpm for 20 s. The 96 well plate containing the reprecipitated PCR products was then incubated at 37°C for 2 min to allow any excess EtOH to evaporate. Each PCR product in the 96 well plate was then resuspended in 12 µl of sterile distilled water and mixed throughly by vortexing for 5 min, followed by centrifugation of the 96 well plate at 1000 rpm for 30 s in the upright position and incubation at 4°C for 10 min. This vortexing and centrifugation step were repeated once more. The purified PCR products were detected by electrophoresis of 2 µl of each sample on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide followed by visualisation on a UV transilluminator as described in sections 2.3.2 and 2.4.2.

The sequences of both strands of each PCR product were determined with the primers used in the initial PCR amplification step (Table 3.4) and with the ABI Big-Dye Fluorescent Terminator System using an ABI 3700 automated sequencer (Applied Biosystems, Warrington, UK) at the Genomics facility at the University of Bath (Bath, UK). For each isolate, the alleles at each of the seven housekeeping loci were identified by comparing the sequences obtained from the test isolates with sequences held in the MLST database (http://www.saureus.mlst.net). This database was also used to identify the allelic profile and hence the sequence type (ST) of each isolate.

### 3.2.3 SCC*mec* typing

*3.2.3.1 Bacterial isolates*

The SCC*mec* type of all 177 isolates included in this present study (Table 3.2) was determined using both a simplex and multiplex SCC*mec* typing method.

*3.2.3.2 Simplex SCCmec typing*

A simplex SCC*mec* typing method described by Robinson & Enright (2003) was used in this present study. This method is a modification of the method described previously by Okuma *et al.* (2002). Using this simplex SCC*mec* typing method, SCC*mec* types were assigned to each isolate by PCR detection of the type of *ccr* complex and the class of *mec* gene complex (*ccr-mec* genes) using primers described in Table 3.5. Five separate reactions were performed on each isolate to detect *ccr* types 1, 2 and 3 and *mec* classes A and B (Table 3.5).

PCR amplifications were carried out in 50 µl volumes using *Taq* DNA polymerase (Promega) as described in section 2.4.2. PCR reactions were performed with an initial 3 min denaturation step at 95°C, followed by 34 cycles of annealing at 55°C for 1 min, extension at 72°C for 2 min and denaturation at 95°C for 1 min, and a final extension step at 72°C for 10 min. After PCR amplification, 10 µl of each PCR reaction was electrophoresed on a 1% (w/v) agarose ge1 containing 0.5 µg/ml ethidium bromide and was visualised on a UV transilluminator as described in sections 2.3.2 and 2.4.2. Each isolate was positive for one *ccr* type and one *mec* class only and from this the SCC*mec* type could be deduced because each SCC*mec* type consists of a unique combination of *ccr* and *mec* genes.

*3.2.3.3 Multiplex SCCmec typing*

The multiplex PCR method of Oliveira & de Lencastre (2002a) was also used for SCC*mec* typing. This method utilises nine primer pairs designed based on the nucleotide sequences of several SCC*mec* elements (Table 3.5). One of these primer pairs (MECA P4/P7) is a positive control that detects the presence of *mecA* in all SCC*mec* elements (Table 3.5). The remaining eight primer pairs amplify regions both common and unique to the SCC*mec* elements I, IA, II, III, IIIA, IIIB, IV and IVA, (Oliveira & de Lencastre, 2002) (Table 3.5).

The multiplex PCR reaction was performed in a total reaction volume of 50 µl containing the following: approximately 5 ng of chromosomal DNA of the test isolate; 1.25 U of *Taq* DNA polymerase (Promega); 5 µl of 10 X buffer (supplied with the *Taq* polymerase); 5 µl of 25 mM $MgCl_2$; 0.2 mM of each deoxynucleoside triphosphate (Promega); 200 nM of primers KDP F1, KDP R1, RIF4 F3 and RIF4 R9; 400 nM of primers CIF2 F2, CIF2 R2, MECIP2, MECI P3, RIF5F10, RIF5 R13, pUB110 R1 and

**TABLE 3.5.** Primer pairs used for SCC*mec* typing

| Primer | Nucleotide sequence 5'-3' | Location | Amplimer size | Region amplified | Reference |
|---|---|---|---|---|---|
| **Simplex** | | | | | |
| ***ccr*** | | | | | |
| β2 | ATTGCCTTGATAATAGCCITCT | 25539-25518[a] | 694 bp | Type 1 *ccr* | Ito *et al.*, 2001 |
| α2 | AACCTATATCATCAATCAGTACGT | 24845-24868[a] | | | |
| β2 | ATTGCCTTGATAATAGCCITCT | 25539-25518[b] | 936 bp | Type 2 *ccr* | Ito *et al.*, 2001 |
| α3 | TAAAGGCATCAATGCACAAACACT | 26325-26348[b] | | | |
| β2 | ATTGCCTTGATAATAGCCITCT | 25539-25518[c] | 1791 bp | Type 3 *ccr* | Ito *et al.*, 2001 |
| α4 | AGCTCAAAAGCAAGCAATAGAAT | 5485-5508[c] | | | |
| ***mec*** | | | | | |
| 3490u | ATGATTCAATGCCTAAACCTAATCG | 43760-43736[b] | 650 bp | Class A *mec* | Robinson & Enright, 2003 |
| 4110Bd | CTTTTTGTTTCAAAGTCATACTATTTTCAAC | 43110-43140[b] | | | |
| 3490u | ATGATTCAATGCCTAAACCTAATCG | 16636-16612[d] | 650 bp | Class B *mec* | Robinson & Enright, 2003 |
| 4110Ad | GAATTATAACTGGGAATATTTTAAATCCCA | 15986-16015[d] | | | |
| **Multiplex** | | | | | |
| CIF2F2 | TTCGAGTTGCTGATGAAGAAGG | 18398-18419[a] | 495 bp | Downstream of *pls* of SCC*mec* I & IA | Oliveira & de Lencastre, 2002a |
| CIF2R2 | ATTTACCACAAGGACTACCAGC | 18892-18871[a] | | | |
| KDPF1 | AATCATCTGCCATTGGTGATGC | 10445-10467[b] | 284 bp | Internal region of *kdp* operon unique to SCC*mec* II | Oliveira & de Lencastre, 2002a |
| KDPR1 | CGAATGAAGTGAAAGAAAGTGG | 10728-10707[b] | | | |
| MECIP2 | ATCAAGACTTGCATTCAGGC | 42428-42447[b] | 209 bp | Internal region of *mecI* of SCC*mec* II, III, IIIA & IIIB | Oliveira & de Lencastre, 2002a |
| MECIP3 | GCGGTTTCAATTCACTTGTC | 42636-42617[b] | | | |
| DCSF2 | CATCCTATGATAGCTTGGTC | 38011-37992[a] | 342 bp | Internal region of *dcs* of SCC*mec* I, IA, II, IV, IVA | Oliveira & de Lencastre, 2002a |
| DCSR1 | CTAAATCATAGCCATGACCG | 37670-37689[a] | | | |
| RIF4F3 | GTGATTGTTCGAGATATGTGG | 45587-45607[c] | 243 bp | Region between pI258 & Tn*554* unique to SCC*mec* III | Oliveira & de Lencastre, 2002a |
| RIF4R9 | CGCTTTATCTGTATCTATCGC | 45829-45809[c] | | | |
| RIF5F10 | TTCTTAAGTACACGCTGAATCG | 59573-59594[c] | 414 bp | Region between Tn*554* & *orfX* of SCC*mec* III & IIIA | Oliveira & de Lencastre, 2002a |
| RIF5R13 | GTCACAGTAATTCCATCAATGC | 59986-59965[c] | | | |
| IS431P4 | CAGGTCTCTTCAGATCTACG | 49963-49982[b] | 381 bp | Left junction between IS*431* & pUB110 of SCC*mec* IA, II & IVA | Oliveira & de Lencastre, 2002a |
| pUB110R1 | GAGCCATAAACACCAATAGCC | 50343-50323[b] | | | |
| IS431P4 | CAGGTCTCTTCAGATCTACG | 29654-29673[c] | 303 bp | Left junction between IS*431* & pT181 unique to SCC*mec* III | Oliveira & de Lencastre, 2002a |
| PT181R1 | GAAGAATGGGGAAAGCTTCAC | 29976-29956[c] | | | |
| MECAP4 | TCCAGATTACAACTTCACCAGG | 1190-1211[e] | 162 bp | Internal region of *mecA*, all SCC*mec* types; Control | Oliveira & de Lencastre, 2002a |
| MECAP7 | CCACTTCATATCTTGTAACG | 1351-1332[e] | | | |

Nucleotide coordinates from [a]SCC*mec* I, [b]SCC*mec* II, [c]SCC*mec* III, [d]SCC*mec* IV and [e]*mecA* gene published sequences, Genbank accession numbers AB033763, D86934, AB037671, AB063172 and Y00688, respectively (Song *et al.*, 1987; Ito *et al.*, 2001; Ma *et al.*, 2002).

pT181 R1; 800 nM of primers DCS F2, DCS R2, MECA P4, MECA P7 and IS431 P4. The multiplex PCR reactions were performed with an initial 4 min denaturation step at 94°C, followed by 30 cycles of annealing at 53°C for 30 s, extension at 72°C for 1 min and denaturation at 94°C for 30 s, and a final extension step at 72°C for 4 min. Amplification products were separated by electrophoresis through 2% (w/v) agarose gels and visualised on a UV transillminator as described in sections 2.3.2 and 2.4.2. When multiplex PCR products are electrophoresed on agarose gels, isolates carrying the SCC*mec* elements I, IA, II, III, IIIA, IIIB, IV and IVA, produce characteristic amplification profiles consisting of amplimers of specific sizes and from this the SCC*mec* type harboured by the test isolate can be interpreted (Table 3.6).

**TABLE 3.6.** Amplimer sizes expected for various SCC*mec* elements using the multiplex SCC*mec* typing method*

| SCC*mec* type | Amplimer sizes (bp)[1] |
|---|---|
| I | 495, 342 & 162 |
| IA | 495, 381, 342 & 162 |
| II | 381, 342, 284, 209 & 162 |
| III | 414, 303, 243, 209 & 162 |
| IIIA | 414, 243, 209 & 162 |
| IIIB | 209 & 162 |
| IV | 342& 162 |
| IVA | 381, 342 & 162 |

*Adapted from Oliveira & de Lencastre (2002a).

[1] See Table 3.5 for the corresponding region that was amplified and primers used to produce each amplimer.

## 3.3 Results

### 3.3.1 MLST

A total of 162 MRSA isolates representative of the most predominant phenotypes of MRSA recovered from patients in Irish hospitals between 1971 and 2002 (Table 3.2, study periods A-H) as well as isolates representative of the unusual MRSA phenotypes recovered between 1989 and 2004, were examined using MLST (Table 3.2). Thirteen sequence types (STs) belonging to each of the five major clonal complexes (CC's) described to date for nosocomial MRSA isolates, were identified among the majority of the Irish isolates examined (161/162 isolates) (Tables 3.7, 3.8 and 3.9). The remaining isolate (1/162) had a ST that did not belong to any of the five major CCs and is termed a singleton (Table 3.7). Singletons are STs whose ancestral genotype is undefined.

Isolates recovered in Irish hospitals prior to 1985 exhibited ST250 (early MRSA and Phenotype II isolates, Table 3.7). Between 1985 and 1987, Phenotype-III isolates that caused an outbreak in two Dublin hospitals exhibited ST239 while the STs of the two most common phenotypes in 1989 were ST239 (AR01) and ST250 (AR02) (Table 3.7). In the 1990's, ST8 isolates predominated (AR13, AR14) but by the late 1990s, the prevalence of isolates with ST36 (AR07.0/07.2) and ST22 (AR06) had increased (Table 3.7). By 2002, ST22 had become the major ST (AR06) (Table 3.7). AR43 isolates that predominated in the North of Ireland in 1999 also exhibited ST8.

Interrogation of the MLST database showed that all except two STs were identical to those previously described for MRSA. The first exception was a double locus variant (dlv) of ST5 which differed from ST5 at both the *arcC* and *yqiL* alleles. ST5 has alleles 1 and 10 of *arcC* and *yqiL*, respectively, whereas the dlvST5 had a new allele, termed allele 63 of *arcC* and allele 28 of *yqiL* (Table 3.8). The *arcC* alleles 63 and 1 differ at a single nucleotide site as do alleles 10 and 28 of *yqiL*. This novel ST was coined ST496 and was identified from one isolate recovered in 2002 exhibiting AR type AR07.2 (Table 3.7). The second ST that was not found in the MLST database was a single locus variant (slv) of ST8 that differed from ST8 at the *gmk* allele. ST8 has allele 1 at *gmk* while this new slv of ST8 has allele 58 (Table 3.8). The *gmk* alleles 1 and 58, differ from each other at a single nucleotide site. This novel ST was coined ST609 genotype and was identified from an isolate with an unfamiliar AR type (unfamiliar-4) recovered in 2004 (Table 3.7).

**TABLE 3.7.** Multilocus sequence typing and SCC*mec* typing results of nosocomial MRSA isolate recovered in Ireland 1971-2004

| CC[a] | ST[b] | SCC*mec* typing | | Isolate phenotype[c] | Years | No. of isolates[c] | Other name of MRSA clonal type |
|---|---|---|---|---|---|---|---|
| | | Simplex PCR | Multiplex PCR[b] | | | | |
| **8** | **ST239** | **III** | **III** | **AR01** | **1989** | 4 | Hungarian |
| | | | | AR15 | 1993 | 2 | |
| | | | | AR23 | 1993 | 1 | |
| | | | | AR44 | 2002/'03 | 2 | |
| **8** | **ST239** | **III** | **III – pI258/Tn*554*** | **Phenotype-III** | **1985-'87** | 1 | NA |
| | | | | AR09* | 1989 | 2 | |
| | | | | Unfamiliar-3 | 2003 | 1 | |
| 8 | ST239[d] | negative | negative | AR09* | 1989 | 1 | NA |
| **8** | **ST250** | **I** | **I** | **Early MRSA** | **1971-'74** | 1 | Archaic |
| | | | | **Phenotype-II*** | **1978-'84** | 9(4) | |
| **8** | **ST250** | **I** | **IV or I – *pls*** | **Phenotype-II*** | **1978-'84** | 6(4) | NA |
| | | | | **AR02** | **1989** | 3 | |
| 8 | ST247 | I | Ia | AR22 | 1993 | 2 | Iberian, EMRSA-5, EMRSA-17 |
| | | | | New02 | 1999 | 1 | |
| **8** | **ST8** | **II** | **II – *kdp*** | AR05 | 1989 | 1 | NA |
| | | | | **AR13*** | **1993,'98,'99** | 6 | |
| | | | | **AR14*** | **1993** | 3 | |
| **8** | **ST8** | **II** | **II – *kdp* & *mecI*** | **AR14*** | **1993,'98,'99** | 10 | NA |
| **8** | **ST8** | **II** | **II – *kdp* & pUB110** | **AR13*** | **1998,'99**,'02 | 19 | NA |
| | | | | **AR14*** | **1999** | 3 | |
| | | | | New01 | 1999 | 1 | |
| | | | | New03 | 1999 | 4 | |
| **8** | **ST8** | **II** | **IV or II – *kdp*, *mecI* & pUB110** | **AR13*** | **1999** | 1 | NA |
| | | | | **AR14*** | **1998,'99,**'02 | 6 | |
| **8** | **ST8** | **IV** | **IV – *dcs*** | **AR43** | **1999** | 20 | NA |
| 8 | ST94 | IV | IV | Unfamiliar-2 | 2003 | 1 | NA |
| 8 | ST609 | IV | IVA | Unfamiliar-4 | 2004 | 1 | NA |
| 5 | ST5 | II | II | AR07.3/07.4, | 1989,'93,'98,'99 | 13 | New York/Japan |
| | | | | AR11 | 1993 | 4 | |
| 5 | ST5 | IV | IV | Unfamiliar-1 | 2002 | 1 | Peadiatric |
| 5 | ST496 | II | II | AR07.2* | 2002 | 1 | NA |
| **30** | **ST36** | **II** | **II** | **AR07.0/07.2*** | 1998,**'99**,'02 | 12(8) | EMRSA-16 |
| 30 | ST30 | IV | IV | ARNT* | 1989,'93,'02 | 4 | NA |
| 30 | ST34[d] | negative | negative | ARNT* | 1993 | 1 | NA |
| **22** | **ST22** | **IV** | **IV** | **AR06** | 1989,'93,'98,**'99, '02** | 27(23) | EMRSA-15 |
| 45 | ST45 | IV | IV | ARNT* | 2002 | 1 | Berlin |
| S[e] | ST12 | IV | IV | ARNT* | 2002 | 1 | NA |

The CCs, STs, SCC*mec* types, phenotypes and numbers investigated of those isolates that predominated during various time periods are indicated in bold (the predominant phenotypes at various time periods was discussed previously in section 3.1.1 & Table 3.1). Isolates that were also present but that did not predominate are indicated in plain text.

[a]A clonal complex (CC) is an MRSA lineage that consists of groups of genotypes/MLST sequence types (STs), that share a recent common ancestor (Feil *et al.*, 2004). The name of each CC is derived from the ST of the predicted ancestral genotype.

[b]The sequence type (ST) is the genotype of each isolate as defined by the allelic profile, which is identified by sequencing of the internal fragments of the seven MLST house keeping genes. All novel STs identified in this present study are underlined. The allelic profiles of all STs identified among Irish MRSA isolates are listed in Table 3.8.

[c]Where the total number of isolates that were typed by MLST differed from those that were SCC*mec* typed for any given phenotype, the number in parenthesis indicates the total number of isolates that were typed by MLST and the other number indicates those that were typed by SCC*mec* typing. The MRSA isolate numbers of those isolates that were not MLST typed are listed in Table 3.3

[d]One ARNT isolate ARNT/0113 and one AR09 isolate AR09/0064 were identified as ST34 and ST239, respectively, but were negative for all SCC*mec* types and *mecA* using both SCC*mec* typing methods.

[e]S, a singleton is a ST whose ancestral genotype and thus clonal complex is unknown.

*Isolates representative of these phenotypes exhibited more than one ST or SCC*mec* type. The original MRSA isolate numbers of those isolates with each ST/SCC*mec* type are listed in Table 3.9.

NA, no other name for this clonal type

**TABLE 3.8.** The allelic profiles of the STs identified in Irish nosocomial MRSA isolates recovered between 1971 and 2004

| Clonal Complex | Sequence Type | Alleleic profile[1] |
|---|---|---|
| 8 | 8 | 3-3-1-1-4-4-3 |
| 8 | 239 | 2-3-1-1-4-4-3 |
| 8 | 250 | 3-3-1-1-4-4-16 |
| 8 | 247 | 3-3-1-12-4-4-16 |
| 8 | 94 | 3-3-1-1-4-4-45 |
| 8 | 609 | 3-3-1-58-4-4-3 |
| 5 | 5 | 1-4-1-4-12-1-10 |
| 5 | 496 | 63-4-1-4-12-1-28 |
| 30 | 30 | 2-2-2-2-6-3-2 |
| 30 | 36 | 2-2-2-2-2-3-2 |
| 30 | 34 | 8-2-2-2-6-3-2 |
| 22 | 22 | 7-6-1-5-8-8-6 |
| 45 | 45 | 10-14-8-6-10-3-2 |
| S | 12 | 1-3-1-8-11-5-11 |

[1]The allelic profile is based on the alleles at each of the seven MLST loci in the order carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (glp*F*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), trioesphosphate isomerase (*tpi*) and acetyl coenzyme A (*yqiL*)

**TABLE 3.9.** The CCs, STs, SCC*mec* types, phenotypes and original isolate numbers of those isolates from phenotypic groups that demonstrated more than one ST or SCC*mec* type

| CC | ST | SCC*mec* type | | Phenotype/ AR type | MRSA isolate no. | Year of isolation |
|---|---|---|---|---|---|---|
| | | Simplex | Multiplex | | | |
| 8 | 239 | III | III-pI258/Tn*554* | AR09 | 0.0065 | 1989 |
| 8 | 239 | III | III-pI258/Tn*554* | AR09 | 0.0066 | 1989 |
| 8 | 239 | negative | negative | AR09 | 0064 | 1989 |
| 8 | 250 | I | I | Phenotype-II | 43.2 | 1976-'84 |
| ND | ND | I | I | Phenotype-II | 58.1 | |
| ND | ND | I | I | Phenotype-II | 122.2 | |
| ND | ND | I | I | Phenotype-II | 43.21 | |
| 8 | 250 | I | I | Phenotype-II | 138.1 | 1976-'84 |
| 8 | 250 | I | I | Phenotype-II | 57 | 1976-'84 |
| ND | ND | I | I | Phenotype-II | 438.1 | |
| ND | ND | I | I | Phenotype-II | 438.2 | |
| 8 | 250 | I | I | Phenotype-II | 79 | 1976-'84 |
| ND | ND | I | I - *pls* | Phenotype-II | 161.2 | |
| ND | ND | I | I – *pls* | Phenotype-II | 120.2 | |
| 8 | 250 | I | I - *pls* | Phenotype-II | 58.2 | 1976-'84 |
| 8 | 250 | I | I – *pls* | Phenotype-II | 120.1 | 1976-'84 |
| 8 | 250 | I | I – *pls* | Phenotype-II | 161.1 | 1976-'84 |
| 8 | 250 | I | I – *pls* | Phenotype-II | 43.11 | 1976-'84 |
| 8 | 8 | II | II - *kdp* | AR13 | 0132 | 1993 |
| 8 | 8 | II | II - *kdp* | AR13 | 0055 | 1993 |
| 8 | 8 | II | II - *kdp* | AR13 | 0236 | 1993 |
| 8 | 8 | II | II - *kdp* | AR13 | 0066 | 1993 |
| 8 | 8 | II | II - *kdp* | AR13 | 2141 | 1998 |
| 8 | 8 | II | II - *kdp* | AR13 | 3698 | 1999 |
| 8 | 8 | II | II - *kdp* | AR14 | 0298 | 1993 |
| 8 | 8 | II | II - *kdp* | AR14 | 0225 | 1993 |
| 8 | 8 | II | II - *kdp* | AR14 | 0078 | 1993 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 0246 | 1993 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 2027 | 1998 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 2188 | 1998 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 2080 | 1998 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 3045 | 1999 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 3146 | 1999 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 3369 | 1999 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 3600 | 1999 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 3685 | 1999 |
| 8 | 8 | II | II - *kdp* & *mecI* | AR14 | 3411 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 2179 | 1998 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 2142 | 1998 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 2113 | 1998 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3137 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3008 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3053 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3114 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3635.2 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3243.2 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3596 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3017 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3439 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3636.2 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3317.2 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3258 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 3453 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 1140 | 2002 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 1168 | 2002 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR13 | 1154 | 2002 |

continued overleaf

## Table 3.9 continued

| CC | ST | SCC*mec* type | | Phenotype/ AR type | MRSA isolate no. | Year of isolation |
|---|---|---|---|---|---|---|
| | | Simplex | Multiplex | | | |
| 8 | 8 | II | II - *kdp* & pUB110 | AR14 | 3352 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR14 | 3132 | 1999 |
| 8 | 8 | II | II - *kdp* & pUB110 | AR14 | 3176.1 | 1999 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR13 | 3330.2 | 1999 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR14 | 2175 | 1998 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR14 | 3225 | 1999 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR14 | 3448 | 1999 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR14 | 1140 | 2002 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR14 | 1168 | 2002 |
| 8 | 8 | II | II - *kdp, mecI* & pUB110 | AR14 | 1154 | 2002 |
| 5 | 496 | II | II | AR07.2 | E1202 | 2002 |
| 30 | 30 | IV | IV | ARNT | 0242 | 1993 |
| 30 | 30 | IV | IV | ARNT | 0067 | 1993 |
| 30 | 30 | IV | IV | ARNT | 1113 | 2002 |
| 30 | 34 | negative | negative | ARNT | 0113 | 1993 |
| 45 | 45 | IV | IV | ARNT | 1139 | 2002 |
| S | 12 | IV | IV | ARNT | 1185 | 2002 |

Abbreviations: CC, clonal complex; ST, sequence type; ND, not determined.

### 3.3.2 SCC*mec* typing

Both the simplex and multiplex SCC*mec* typing methods were used to detect the SCC*mec* types of all 177 Irish MRSA isolates used in this present study. The simplex method detects the *ccr-mec* genes of SCC*mec* I-IV and from this the SCC*mec* type can be defined. The multiplex method identifies structural features both unique and common to each SCC*mec* element I-IV as well as some variants, including the region outside of the *ccr* and *mec* complexes.

#### *3.3.2.1 Simplex SCCmec typing*

Using this method, 175/177 isolates (ca. 99 %) were found to carry SCC*mec* types I, II, III or IV (Table 3.7). The SCC*mec* types of two isolates, AR09/0.0064 and ARNT/0113 recovered in 1989 and 1993, respectively, could not be determined using the simplex SCC*mec* typing method because there was no amplification of the *ccr-mec* genes using the primers specific for SCC*mec* types I-IV.

##### *3.3.2.1.1 SCCmec I*

The type I SCC*mec* element was identified in 22 isolates (ca. 12%) including all isolates recovered prior to 1985 (Early MRSA and Phenotype-II), AR02 isolates from 1989, isolates exhibiting AR22 and isolates with the unfamiliar AR pattern termed New02 (Table 3.7).

##### *3.3.2.1.2 SCCmec II*

Eighty-four isolates (ca. 48%) were found to carry SCC*mec* II, including AR13 and AR14 isolates that predominated in the 1990's, AR07.0/07.2 isolates that also predominated in the late 1990's, AR05, AR07.3/07.4 and AR11 isolates, as well as isolates with the unusual AR patterns New01 and New03 (Table 3.7).

##### *3.3.2.1.3 SCCmec III*

Type III SCC*mec* was identified in 13 isolates (ca 7%) including AR01 isolates that predominated in 1989, Phenotype-III, AR15 and AR44 isolates that all caused outbreaks at different time periods in Irish hospitals, as well as isolates with AR23, AR09 and an unfamiliar AR pattern (Unfamiliar-3) (Table 3.7).

*3.3.2.1.4 SCCmec IV*

The type IV SCC*mec* element was identified in 56 isolates (ca. 32%) including isolates that predominated in Northern Ireland in 1999 (AR43), isolates that predominated in the South in 1999 and 2002 (AR06) as well as all isolates classified as ARNT and unfamilar-1,-2 and –4 (Table 3.7).

*3.3.2.2 Multiplex SCCmec typing*

Using the multiplex SCC*mec* typing method, the SCC*mec* types of only 88/177 isolates (ca. 50%) could be directly identified. Of these 88 isolates, 84 were found to have the same SCC*mec* element as identified with the simplex method (Table 3.7). The other four/88 isolates were found to harbour previously described SCC*mec* variants. This included the following; AR22 (n=2) and New02 (n=1) isolates that were found to carry SCC*mec* I by the simplex method but the multiplex method showed that they carried the previously described variant element SCC*mec* IA; the isolate with the unfamiliar AR pattern (n=1) recovered in 2004 that was identified as SCC*mec* IV by the simplex method but was found to carry the previously described variant element SCC*mec* IVA by the multiplex method (Table 3.7).

Of the remaining 89/177 isolates, two (ca. 1%) were found to harbour no SCC*mec* element because there was no amplification of any SCC*mec* associated regions, including *mecA*. These were the same two isolates AR09/0.0064 and ARNT/0113 recovered in 1989 and 1993, respectively that were also SCC*mec* negative using the simplex method (Table 3.7).

*3.3.2.2.1 Identification of variant SCCmec types*

The rest of the isolates investigated in this present study (87/177) (ca.49 %) were found to harbour two different SCC*mec* elements when investigated by the simplex and multiplex SCC*mec* typing methods or the SCC*mec* type could not be inferred because the multiplex patterns produced did not match those of previously described SCC*mec* elements.

Nine isolates {Phenotype-II (six/15), AR02 (3/3)} that were identified as SCC*mec* I by the simplex method, failed to yield one of the bands characteristic of SCC*mec* I elements by the multiplex method. (Table 3.7). The band that was absent was the 495 bp band which results from the amplification of a region downstream of *pls* in SCC*mec* I (Table 3.7; Fig. 3.3., panel A, lanes 2-5). Furthermore, the multiplex pattern obtained

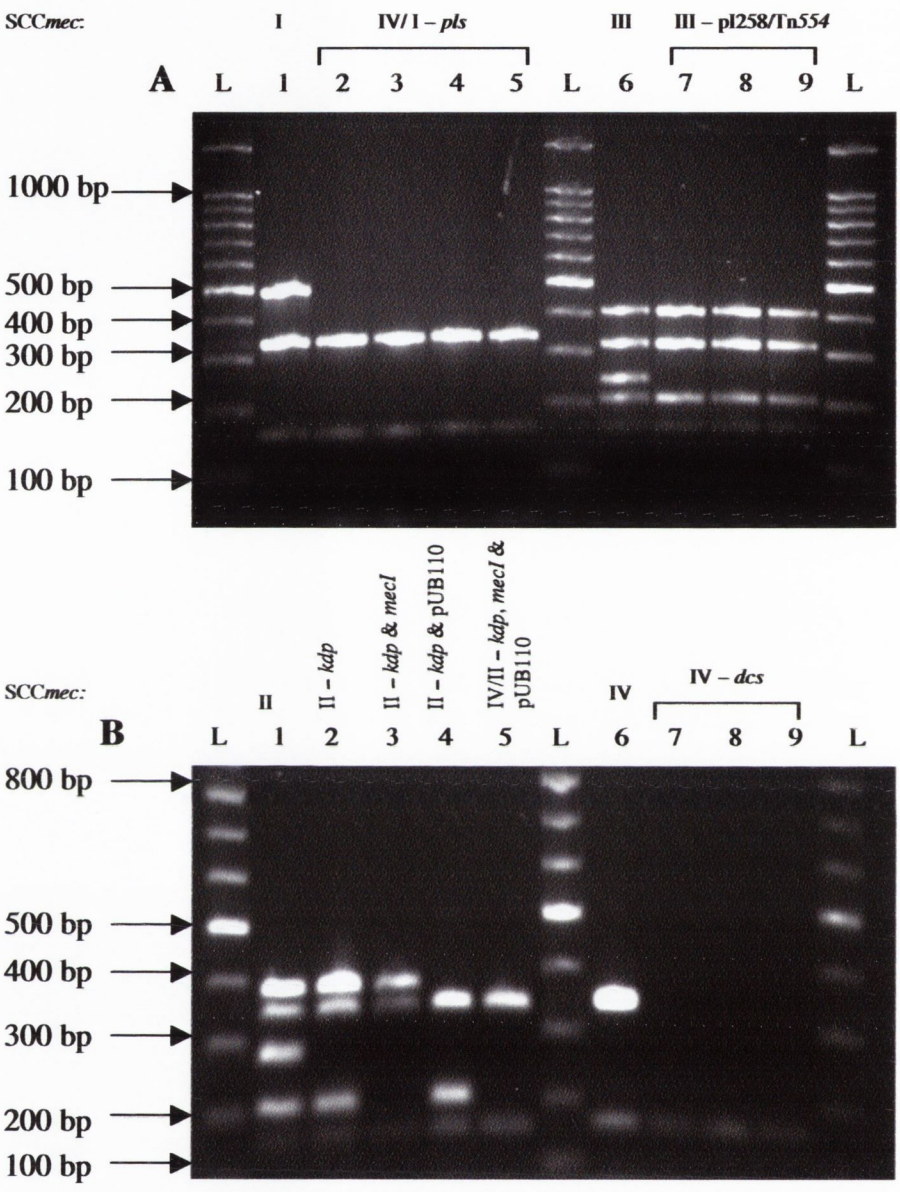

SCCmec:
I
IV/ I – pls
III
III – pI258/Tn554
A
L 1 2 3 4 5 L 6 7 8 9 L
1000 bp
500 bp
400 bp
300 bp
200 bp
100 bp
SCCmec:
II
II – kdp
II – kdp & mecI
II – kdp & pUB110
IV/II – kdp, mecI & pUB110
IV
IV – dcs
B
L 1 2 3 4 5 L 6 7 8 9 L
800 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE 3.3**. SCC*mec* multiplex patterns and variant multiplex patterns found in Irish MRSA isolates.

**Panel A**: Lane 1: SCC*mec* type I multiplex pattern. Lanes 2-5: SCC*mec* IV multiplex pattern produced by AR02 isolates and six Phenotype-II isolates with SCC*mec* type I *ccr-mec* genes by the simplex method. Lane 6: SCC*mec* type III multiplex pattern. Lane 7-9: the variant SCC*mec* III multiplex pattern (III – pI258/Tn*554*) characterised by the absence of the pI258/Tn*554* amplimer at 243 bp, produced by 2/3 AR09, Phenotype-III and unfamiliar-3 isolates with SCC*mec* type III *ccr-mec* genes by the simplex method.

**Panel B**: Lane 1: SCC*mec* type II multiplex pattern. Lanes 2-5: the SCC*mec* multiplex patterns produced by isolates with phenotypes AR05, AR13, AR14 and New01 and New03 with type II *ccr-mec* genes by the simplex method. These multiplex patterns appear to be related to the type II SCC*mec* multiplex pattern but lack the *kdp* amplimer (284 bp) (II – *kdp*, lane 2) or lack the *kdp* and *mecI* (209 bp) amplimers (II – *kdp* and *mecI*, lane 3) or lack the *kdp* and pUB110 (381 bp) amplimers (II – *kdp* and pUB110, lane 4) or lack the *kdp*, *mecI* and pUB110 amplimers (II – *kdp*, *mecI* and pUB110, lane 5). This last pattern is the same as SCC*mec* IV. Lane 6: SCC*mec* type IV multiplex pattern. Lane 7-9: the multiplex pattern produced by isolates with the AR43 phenotype (IV – *dcs*), which consists of the *mecA* control amplimer only. Lane L: 100 bp DNA ladders were used as molecular size reference markers (lane L).

for these nine isolates was indistinguishable from SCC*mec* IV as it displayed amplimers at 342 bp and 162 bp only. This pattern was termed SCC*mec* I minus (-) *pls* (SCC*mec* I - *pls*)

Four isolates that were identified as carrying SCC*mec* III by the simplex method {Phenotype-III (n=1/1), AR09 (n=2/3) and Unfamiliar 3 (n=1/1)} produced multiplex patterns that were similar to SCC*mec* III but lacked one band at 243 bp (Table 3.7; Fig. 3.3., panel A, lanes 7-9). This band is the product of the amplification of the locus between pI258 and Tn*554* found in SCC*mec* III. This pattern was designated SCC*mec* III-pI258/Tn*554*).

All 20 AR43 isolates were classified as SCC*mec* IV by the simplex method but gave a multiplex PCR pattern from which the SCC*mec* type could not be inferred because only the multiplex *mecA* control amplimer found in all SCC*mec* types was obtained (Table 3.7; Fig 3.5., panel B, lanes 7-9). This multiplex pattern could be interpreted as a variant SCC*mec* IV multiplex pattern missing the 342 bp amplimer, which results from the amplification of *dcs* found in SCC*mec* IV. This pattern was called SCC*mec* IV - *dcs*.

The remaining 54 isolates {AR05 (1/1), AR13 (26/26), AR14 (22/22), New01 (1/1), New03 (4/4} were identified as SCC*mec* II by the simplex method but gave variant SCC*mec* II multiplex patterns that lacked one to three of the five amplimers that would identify them as harbouring SCC*mec* II (Table 3.7, Fig. 3.3., panel B, lanes 2-5). Ten isolates lacked a 284 bp band corresponding to the amplification product from a region of the *kdp* operon found in SCC*mec* II (Table 3.7). This pattern was designated SCC*mec* II – *kdp* (Fig. 3.3., panel B, lane 2). Ten isolates lacked both the 284 bp (*kdp* operon) and 209-bp amplimers (Table 3.7). The latter results from amplification of part of the *mecI* gene in SCC*mec* II. This pattern was termed SCC*mec* II – *kdp* and *mecI* (Fig. 3.3., panel B, lane 3). Twenty-seven isolates lacked the 284 bp (*kdp* operon) and the 381 bp amplimers (Table 3.7). This 381 bp amplimer results from amplification of the left junction between IS*431mec* and pUB110 found in SCC*mec* II. This pattern was called SCC*mec* II – *kdp* and pUB110 (Fig. 3.3., panel B, lane 4). Seven isolates produced a multiplex PCR pattern indistinguishable from the pattern of SCC*mec* IV (Table 3.7). This pattern could also be interpreted as a type II SCC*mec* pattern missing the 284 bp (*kdp* operon), the 209 bp (*mecI* gene) and the 381 bp (pUB110) amplimers. This pattern was designated SCC*mec* II – *kdp*, *mecI* and pUB110 (Fig. 3.3., panel B, lane 5).

### 3.3.3 MRSA clonal types

Combining both the MLST and SCC*mec* results using the criteria of Enright *et al.* (2002), where a clonal group is defined as isolates with both the same ST and same SCC*mec* type, 20 MRSA clonal types were identified (Table 3.7). These included the internationally spread MRSA clones, the Hungarian clone (ST239-MRSA-III), the Iberian clone (ST247-MRSA-IA), the Archaic clone or original MRSA clone (ST250-MRSA-I), the New York/Japan clone (ST5-MRSA-II), the Berlin clone (ST45-MRSA-IV), the paediatric clone (ST5-MRSA-IV), and the UK epidemic clones EMRSA-15 (ST22-MRSA-IV) and EMRSA-16 (ST36-MRSA-II) (Table 3.7). Some of the other clonal types identified had the same ST as previously recognised MRSA clones but had different SCC*mec* multiplex patterns which indicated the presence of novel SCC*mec* variants. These included SCC*mec* variants of the Hungarian/Brazilian (ST239-MRSA-III- pI258/Tn*554*), Archaic (ST250-MRSA-I – *pls*), ST8-MRSA-II (ST8-MRSA-II – *kdp*, ST8-MRSA-II – *kdp* & *mecI*, ST8-MRSA-II – *kdp* & pUB110, ST8-MRSA-II – *kdp*, *mecI* & pUB110) and ST8-MRSA-IV (ST8-MRSA-IV - *dcs*) clones (Table 3.7). Some minor MRSA clonal types that were present among the Irish MRSA isolates but that occurred infrequently included ST94-MRSA-IV, ST30-MRSA-IV, ST12-MRSA-IV and the two novel clonal types ST609-MRSA-IVA and ST496-MRSA-II (Table 3.7). Two MSSA clonal types, ST34-MSSA and ST239-MSSA were also identified (Table 3.7).

### 3.3.4 Pulsed-field gel electrophoresis

A total of 67 MRSA isolates, all recovered from hospitalised patients in both the North and South of Ireland during a two-week surveillance study of MRSA in 1999 were examined by PFGE (Table 3.2) in order to examine the relationship between isolates exhibiting a previously unfamiliar AR pattern, AR43, and other isolates exhibiting the AR patterns AR06, AR07.0, AR7.4, AR13 and AR14. The fingerprint profiles obtained following PFGE of *Sma*I-digested total genomic DNA of all 67 isolates (Fig. 3.4) were compared using the observational criteria described by Tenover *et al.* (1995) (Table 1.3). In addition, a dendrogram showing the relationship between all 67 isolates recovered in 1999 was generated using the similarity coefficient ($S_{AB}$) values computed for every possible pairwise combination of fingerprint profiles of these isolates using the DENDRON fingerprint profile analysis computer software package

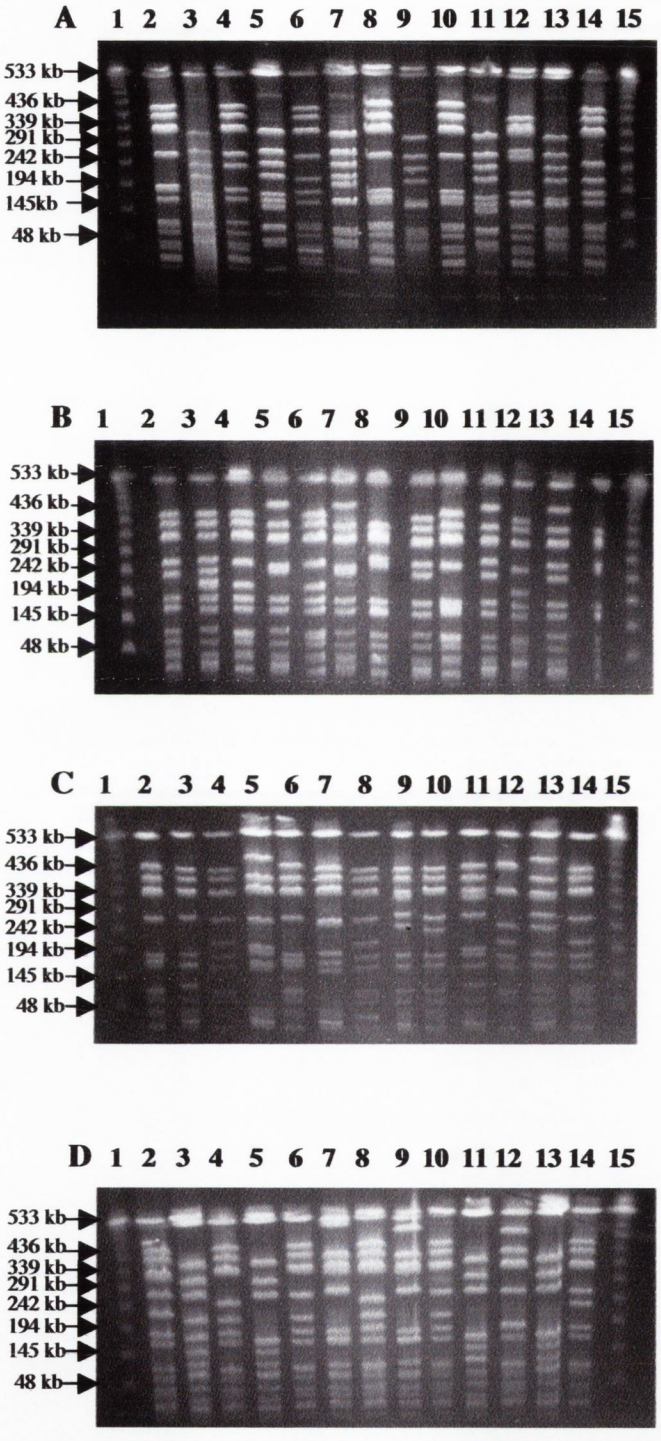

A 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
533 kb
436 kb
339 kb
291 kb
242 kb
194 kb
145kb
48 kb
B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
533 kb
436 kb
339 kb
291 kb
242 kb
194 kb
145 kb
48 kb
C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
533 kb
436 kb
339 kb
291 kb
242 kb
194 kb
145 kb
48 kb
D 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
533 kb
436 kb
339 kb
291 kb
242 kb
194 kb
145 kb
48 kb

**FIGURE 3.4.** Fingerprint patterns obtained by PFGE following *Sma*I digestion of total genomic DNA from MRSA isolates with AR patterns AR06, AR07.0, AR07.4, AR13, AR14 and AR43 recovered in Irish hospitals in 1999.

**Panel A:** Fingerprint patterns obtained from AR06 and AR43 isolates. Lanes 1 and 15, lambda ladder molecular size DNA markers; lanes 2, 4, 6, 8, 10, 12 and 14, AR43 isolates; lanes 3, 5, 7, 9, 11 and 13, AR06 isolates. Isolates exhibiting AR43 do not appear to be closely related to AR06 isolates (greater than seven band differences).

**Panel B:** Fingerprint patterns from AR13 and AR43 isolates. Lanes 1 and 15, lambda ladder molecular size DNA marker; lanes 2, 4, 6, 8, 10, 12 and 14, AR43 isolates; lanes 3, 5, 7, 9, 11 and 13, AR13 isolates. Isolates exhibiting AR43 appear to be related to AR13 isolates (between one and six band differences).

**Panel C:** Fingerprint patterns obtained from AR14 and AR43 isolates. Lanes 1 and 15, lambda ladder molecular size DNA marker; lanes 2, 4, 6, 8, 10, 12 and 14, AR43 isolates; lanes 3, 5, 7, 9, 11 and 13, AR14 isolates. Isolates exhibiting AR43 appear to be related to AR14 isolates (between one and seven band differences).

**Panel D:** Fingerprint patterns obtained from AR07.0, AR07.4 and AR43 isolates. Lanes 1 and 15, lambda ladder molecular size DNA marker; lane 2, *S. aureus* control isolate NCTC8325; lanes 3, 5, 11 and 13, AR07.0 isolates; lanes 4, 6, 8, 10, 12 and 14, AR43 isolates; lanes 7 and 9, AR07.4 isolates. Isolates exhibiting AR43 do not appear to be closely related to AR07.0 isolates (greater than seven band differences). AR07.0 isolates do not appear to be closely related to AR07.4 isolates (greater than seven band differences). AR07.4 isolates do not appear to be closely related to AR43 isolates (seven or more band differences).

(Fig. 3.5). The dendrogram shown in Fig. 3.5 revealed that this MRSA population could be divided into four distinct main populations or groups (I, II, III and IV).

*3.3.4.1 Group I*

AR06 isolates formed a distinct and homogenous group within the population of MRSA isolates studied with an average $S_{AB}$ value of 0.89 (group I, Fig. 3.5). This finding indicated that these AR06 isolates were closely related to each other but clearly distinct from isolates exhibiting all other AR types, including AR43 (Fig. 3.5). An example of some of the PFGE fingerprint patterns of AR43 and AR06 isolates are shown in Fig. 3.4, panel A. The PFGE fingerprint patterns of AR06 isolates differed from the corresponding fingerprint patterns of AR43 isolates by more than seven bands.

*3.3.4.2 Group II*

The second distinct group within this MRSA population from 1999 (group II, Fig. 3.5) consisted of AR13, AR14 and AR43 isolates. Group II isolates were more heterogeneous than groups I (Fig. 3.5) with an average $S_{AB}$ value of 0.73 (Fig. 3.5). Some distinct clusters of isolates were evident in group II, corresponding to subgroups of AR13, AR14 or AR43 isolates. Visual inspection of the fingerprint patterns of AR13, AR14 and AR43 isolates revealed that these isolates displayed a heterogeneous range of fingerprint patterns (Fig. 3.4, panel B and C). Some AR13, AR14 and AR43 isolates had very similar fingerprint patterns (between one and three band differences) while others differed by up to seven bands (Fig. 3.4, panel B and C).

*3.3.4.3 Groups III and IV*

The dendrogram also revealed that AR07.4 and AR07.0 isolates formed two more groups (groups III and IV, respectively, Fig. 3.4) within the population of MRSA isolates studied. The two AR07.4 isolates (group III) examined had an average $S_{AB}$ value of 0.93 (Group 3, Fig. 3.5) which indicated that these isolates were very closely related. AR07.0 isolates (Group IV) also appeared to be very closely related to each other, with an average $S_{AB}$ value of 0.95. AR07.0 and AR07.4 isolates displayed very different fingerprint patterns from each other (Fig. 3.4, panel D) with seven or more band differences between the respective patterns. Furthermore, AR07.0 and AR07.4 isolates exhibited very different fingerprint profiles to AR43 isolates with seven or more band

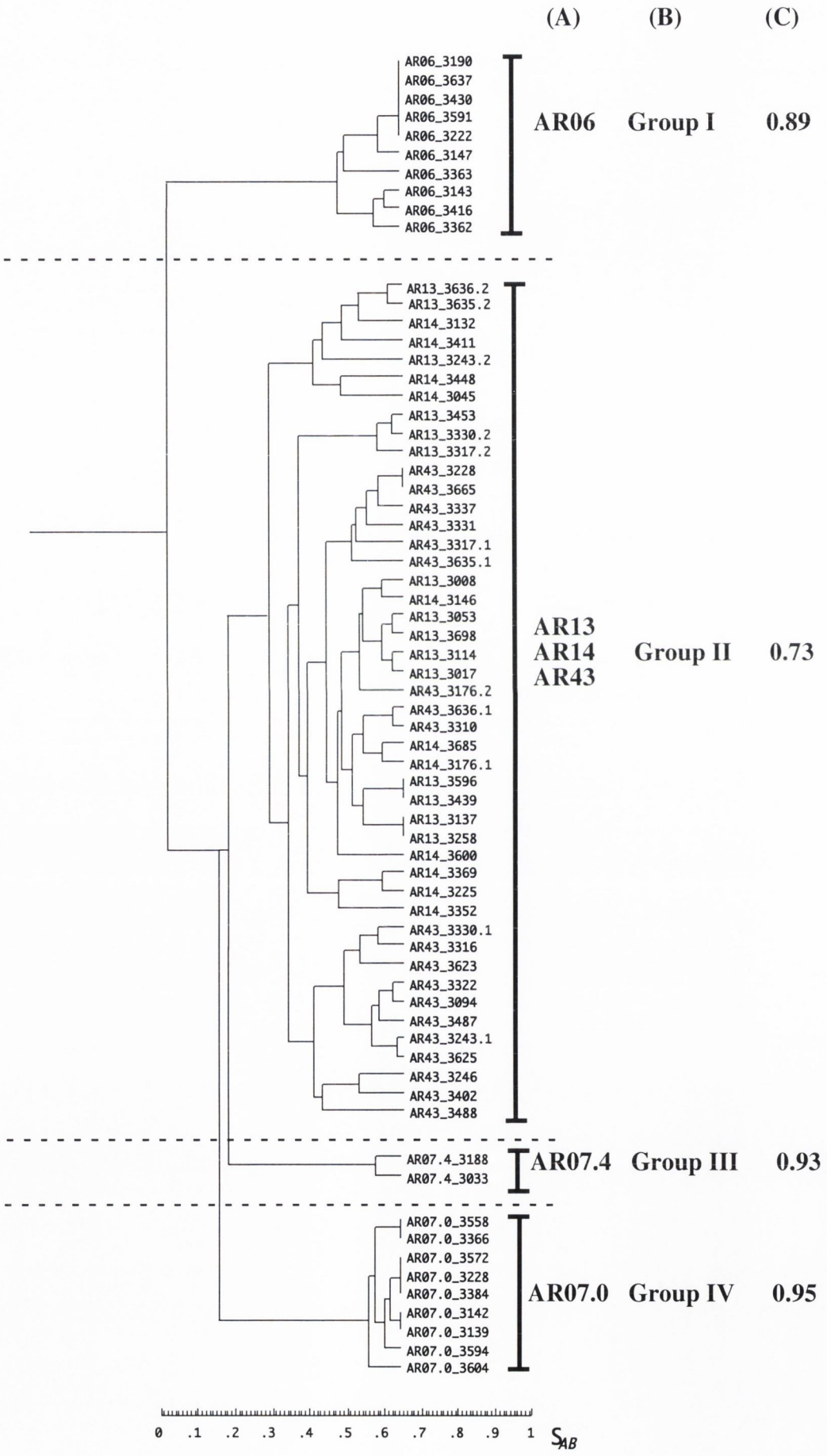

(A)
(B)
(C)
AR06_3190
AR06_3637
AR06_3430
AR06_3591
AR06_3222
AR06_3147
AR06_3363
AR06_3143
AR06_3416
AR06_3362
AR06
Group I
0.89
AR13_3636.2
AR13_3635.2
AR14_3132
AR14_3411
AR13_3243.2
AR14_3448
AR14_3045
AR13_3453
AR13_3330.2
AR13_3317.2
AR43_3228
AR43_3665
AR43_3337
AR43_3331
AR43_3317.1
AR43_3635.1
AR13_3008
AR14_3146
AR13_3053
AR13_3698
AR13_3114
AR13_3017
AR43_3176.2
AR43_3636.1
AR43_3310
AR14_3685
AR14_3176.1
AR13_3596
AR13_3439
AR13_3137
AR13_3258
AR14_3600
AR14_3369
AR14_3225
AR14_3352
AR43_3330.1
AR43_3316
AR43_3623
AR43_3322
AR43_3094
AR43_3487
AR43_3243.1
AR43_3625
AR43_3246
AR43_3402
AR43_3488
AR13
AR14
AR43
Group II
0.73
AR07.4_3188
AR07.4_3033
AR07.4
Group III
0.93
AR07.0_3558
AR07.0_3366
AR07.0_3572
AR07.0_3228
AR07.0_3384
AR07.0_3142
AR07.0_3139
AR07.0_3594
AR07.0_3604
AR07.0
Group IV
0.95
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1
$S_{AB}$

**FIGURE 3.5.** Dendrogram generated from the similiarity coefficients ($S_{AB}$s) computed for every possible pairwise combination of 67 MRSA isolates exhibiting AR types AR06, AR07.0, AR07.4, AR13, AR14 and AR43 recovered in Irish hospitals during a 2 week period in 1999. The dendrogram was generated from the fingerprint patterns obtained following PFGE of *Sma*I-digested total genomic DNA using the DENDRON fingerprint profile analysis computer software program. The dendrogram shows that the population of MRSA isolates studied could be divided into four distinct groups (I, II, III and IV). The AR types represented by each group are shown under (A), the name of each group is indicated under (B) and the average $S_{AB}$ of each group is indicated under (C).

differences between the respective profiles (Fig. 3.4, panel D), indicating that AR07.0 and AR07.4 isolates were not closely related to AR43 isolates.

## 3.4. Discussion

Multilocus sequence typing and SCC*mec* typing of MRSA isolates recovered in Irish hospitals between 1971 and 2004 has shown that clones representative of each of the five major clonal complexes (CCs) and eight major pandemic lineages have been present in hospitals in Ireland at some time over the past 30 years (Table 3.7). This study also confirmed that there have been major changes in the dominant MRSA clones in Irish hospitals over time. The clonal types of the earliest MRSA isolates recovered during the 1970s and early 1980s were ST250-MRSA-I or its novel variant ST250-MRSA-I – *pls*, followed, in the mid to late 1980s, by a new variant of ST239-MRSA-III (ST239-MRSA-III – pI258 & Tn*554*) (Table 3.7). In 1989, ST239-MRSA-III was the predominant clone but during the 1990s, this was displaced by ST8-MRSA exhibiting four novel variant SCC*mec* II multiplex patterns identified in the present study (Table 3.7). In 2002, the dominant clone was ST22-MRSA-IV (Table 3.7). In the North of Ireland in 1999, ST8-MRSA exhibiting the novel variant SCC*mec* IV multiplex pattern identified in the present study predominated. There has been a clear shift from the predominance of multi-antibiotic resistant MRSA in Irish hospitals in the 1980s and 1990s (ST250, ST239 and ST8) to the predominance of a multi-antibiotic susceptible clone in 2002 (ST22).

It is important to note that no Phenotype-I isolates, which predominated in Dublin hospitals from 1976-1978 were available for use in the present study. It is possible that these Phenotype-I isolates, which were previously found to be phenotypically distinct from Phenotype-II and Phenotype-III isolates (Coleman *et al.*, 1985), may represent another MRSA clonal type that also predominated in Dublin hospitals in the 1970s. Equally important to note is the fact that the identification of particular MRSA clonal types prior to 1999 reflects those that were present in a group of Dublin hospitals only whilst those clonal types identified during and after 1999, represent those that were present in Irish hospitals, not just those in Dublin.

All MRSA clonal types identified in this present study belong to one of the five clonal complexes (CC8, CC5, CC22, CC30 and CC45) described for nosocomial MRSA, with the exception of ST12-MRSA-IV (Table 3.7). ST12-MRSA-IV has been described previously as a singleton i.e. a sequence type that does not belong to any previously described CC and whose ancestral genotype has not yet been identified (Enright *et al.*, 2002). ST12-MRSA-IV is a relatively minor MRSA clone with only two

representatives in the MLST database to date. It is more common among MSSA and has been reported as such from the UK and Holland (MLST database).

The four major MRSA lineages ST250, ST239, ST247 and ST8 previously identified within CC8 were identified among the isolates investigated in this present study (Table 3.7). Furthermore, all clones identified in the present study that predominated prior to 1999 had STs within CC8 (Table 3.7). ST8-MSSA is predicted to be the ancestor of CC8 (Enright *et al.*, 2002).

The present study identified that the earliest nosocomial MRSA isolate that was among the first MRSA recovered in a group of Dublin hospitals between 1971 and 1974 (early MRSA, Table 3.7; Coleman *et al.*, 1985) was ST250-MRSA-I, also known as the Archaic clone (Table 3.7). This finding is in line with the results of other studies in which the earliest MRSA isolates recovered in the UK and Denmark in 1961 and 1963, respectively, were found to be ST250-MRSA-I (Crisostomo *et al.*, 2001; Enright *et al.*, 2002). Enright *et al.* (2002) have previously identified that ST250-MRSA-I arose from ST8-MSSA following a point mutation at the *yqiL* allele resulting in ST250-MSSA, which has the *yqiL* allele 16 instead of the *yqiL* allele 3 found in ST8 (Table 3.8). The subsequent acquisition of SCC*mec* I by ST250-MSSA from an unknown source resulted in ST250-MRSA-I (Enright *et al.*, 2002).

ST250-MRSA-I is considered to be the clonal type of the first ever European MRSA isolates (Crisostomo *et al.*, 2001; Enright *et al.*, 2002). Most ST250-MRSA-I isolates reported in other studies were recovered in the 1960's so it is likely that isolates with this MRSA clonal type spread from other countries to hospitals in Dublin in the early 1970s. During the 1970s and early 1980s isolates with the ST250-MRSA-I clonal type, along with ST250-MRSA isolates with a novel variant SCC*mec* element, SCC*mec* I – *pls*, became endemic in Dublin hospitals (Phenotype-II, Table 3.7; Coleman *et al.*, 1985). ST250-MRSA-I – *pls* differs from ST250-MRSA-I by some variation in the *pls* region of the SCC*mec* element. This suggests that changes may have occurred within the SCC*mec* element of ST250-MRSA-I isolates resulting in ST250-MRSA-I – *pls*. Since ST250 isolates with this variant SCC*mec* type have not been reported previously from any other country it is possible to speculate that changes occurred within the SCC*mec* element of the existing ST250-MRSA-I clone endemic in Dublin hospitals resulting in the ST250-MRSA-I – *pls* clone that continued to cause nosocomial infections until 1989 (AR02, Table 3.7). The ST250-MRSA-I and ST250-MRSA-I – *pls* clones were not recovered in Dublin hospitals after 1984 and 1989, respectively. Other

researchers have reported similar findings in which the ST250-MRSA-I clone predominated in the early days of MRSA in several different countries but have rarely been identified among MRSA isolates recovered since the 1990s (Crisostomo *et al.*, 2001; Oliveira *et al.*, 2001c; Enright *et al.*, 2002).

The ST247-MRSA-IA clone, also known as the Iberian clone, is a contemporary pandemic MRSA clone that is believed to have evolved from the ST250-MRSA-I following a point mutation in the *gmk* locus resulting in the *gmk* allele 16 (Table 3.8) and integration of pUB110 into the SCC*mec* I element, resulting in SCC*mec* IA (Robinson & Enright, 2003). The Iberian clone was first reported in 1989 in Spain and has been identified as the dominant clone in hospitals in many European countries especially in Spain and Portugal (Dominguez *et al.*, 1994; Oliveira *et al.*, 1998). While ST247-MRSA-IA did cause an outbreak in an intensive care unit in a Dublin hospital in 1993 (AR22; Table 3.7; Rossney *et al.*, 1994b) and was again recovered in Northern Ireland in 1999, but at low levels (New02, Table 3.7; Irish Dep. Of Health & Children, 1999), this clonal type never predominated in Irish hospitals.

Between 1985 and 1987 a novel variant (ST239-MRSA-III – pI258/Tn*554*) of the major CC8 lineage ST239 caused an outbreak in two Dublin hospitals (Phenotype-III, Table 3.7; Carroll *et al.*, 1989). ST239-MRSA-III/IIIA is a widely spread pandemic MRSA lineage that contains many successful clones such as the UK epidemic clones EMRSA-1, EMRSA-4, EMRSA-7, EMRSA-9, EMRSA-11, the Brazilian, Portuguese, Vienna and Hungarian clones as well as USA clone IV (Oliveira *et al.*, 2001c; Enright *et al.*, 2002). Since the 1980s the ST239-MRSA-III/IIIA clones have predominated in many countries worldwide (Marples & Cooke, 1985; Kerr *et al.*, 1990; Teixeira *et al.*, 1995; de Lencastre *et al.*, 1997; Aires de Sousa *et al.*, 1998, 2001, 2003a & 2003b; Amorim *et al.*, 2002; Melter *et al.*, 2003; MLST database), and although ST239 continues to predominate in many Asian countries today (Ko *et al.*, 2005) it does not do so in Irish hospitals. Following detailed sequence analysis of a large selection of *S. aureus* genes, Robinson and Enright (2004a) discovered that the ST239 lineage arose from a single recombination event between two distinct MRSA lineages, ST30 and ST8, involving > 550 kb of DNA, as well as the acquisition of SCC*mec* III from a unknown source.

Whether the novel clonal type ST239-MRSA-III – pI258/Tn*554* is a derivative of the globally spread ST239-MRSA-III/IIIA clone that arose following insertions, mutations, deletions or recombination within the type III or IIIA SCC*mec* element, or whether the

latter is a derivative that arose from the former, is unclear. It seems most likely that ST239- MRSA-III – pI258/Tn*554* is a derivative of ST239-MRSA-SCC*mec* III/IIIA. Firstly isolates of the ST239–MRSA-III clone were recovered in Australia in 1982 (MLST database & Oliveira *et al.*, 2001c) prior to the emergence of the ST239-MRSA-III – pI258/Tn*554* clone in Ireland (1984), indicating that ST239-MRSA-III is the ancestor. Furthermore, during the 1980s, extensive laboratory studies of Irish Phenotype-III isolates (which this present study has identified as ST239-MRSA-III – pI258/Tn*554* isolates) using the techniques available at the time found that these isolates had spread from Baghdad to Dublin following the transfer of a patient from a Baghdad hospital to a Dublin hospital (Carroll *et al.*, 1989). The SCC*mec* III element is a common SCC*mec* type among Asian MRSA isolates so it seems likely that recombination or rearrangement occurred within SCC*mec* III of ST239 resulting in the derivative ST239-MRSA-III – pI258/Tn*554* that was then transferred to Ireland. The possibility that ST239-MRSA-III is a derivative of the ST239-MRSA-III – pI258/Tn*554* cannot be completely ruled out. Further molecular analysis into this variant SCC*mec* element is required in order to further clarify the relationship between theses two clonal types and is discussed in detail in Chapter 4.

The pandemic ST239-MRSA-III clone was endemic in a Dublin hospital in 1989 (AR01, Table 3.7; Rossney & Keane, 2002a) and may represent the introduction of a new clone into the Irish hospital setting at this time. The ST239-MRSA-III – pI258/Tn*554* was also present in 1989 but at a very low prevalence (AR09, Table 3.7; Rossney, 1995). The ST239-MRSA-III clone occurred frequently in one Dublin hospital between 1992 and 1993 (AR15, Table 3.7; Rossney *et al.*, 1994b) and was also isolated from a single patient who had been hospitalised in Bahrain prior to hospitalisation in a Dublin hospital (AR23). Both the ST239 clonal types were not recovered from the Irish MRSA population again until 2002 when the ST239-MRSA-III clone appears to have been reintroduced from abroad by a patient who had been previously hospitalised in Singapore (AR44, Table 3.7, Rossney, 2003b). ST239-MRSA-III subsequently caused an outbreak in a unit of an Irish hospital (AR44, Rossney, 2003b). The ST239-MRSA-III – pI258/Tn*554* clone was identified in 2003 for the first time since 1989 in a single isolate only (Unfamiliar-3, Table 3.7). It is interesting that some ST239-MRSA-III and ST239-MRSA-III - pI258/Tn*554* isolates such as AR01 and Phenotype-III isolates, respectively, caused major problems in Dublin hospitals while others did not spread (e.g. AR23 and Unfamiliar-3) or were contained within a particular unit within one

hospital (e.g. AR44) (Carroll *et al.*, 1989; Rossney *et al.*, 1994b; Rossney & Kenae, 2002a; Rossney, 2003b).

The ST8-MRSA clone became endemic in various Irish hospitals in the 1990s (AR13, AR14, AR43, Table 3.7; Rossney & Keane, 2002a; Irish Dept. of Health & Children, 1999) and replaced the previously predominant ST239 and ST250 clones (Table 3.7). Enright *et al.* (2002) previously identified all four SCC*mec* types (I-IV) in ST8 and proposed that the ST8-MRSA clones arose from ST8-MSSA by independent insertions of SCC*mec*. ST8-MRSA-II and ST8-MRSA-IV are the major clonal types within ST8 (Enright *et al.*, 2002) and derivatives of both of these clonal types predominated and caused major problems in Irish hospitals during the 1990s (Table 3.7, Rossney *et al.*, 1994b, Rossney & Keane, 2002a). All Irish ST8 isolates identified in this present study produced unusual SCC*mec* multiplex patterns, including four SCC*mec* II variant patterns (Table 3.7; II - *kdp*, II – *kdp* & *mecI*, II – *kdp* & pUB110, II – *kdp*, *mecI* & pUB110) that predominated in the 1990s (AR13 and AR14) and one SCC*mec* IV variant pattern (Table 3.7; IV – *dcs*) that predominated in Northern Ireland in 1999 (AR43). The large number of SCC*mec* variant multiplex patterns among ST8 isolates suggests that significant variation, possibly due to the instability of SCC*mec* in the ST8 genetic background resulting in recombination and rearangments, has occurred within the SCC*mec* element of Irish ST8 isolates. In fact 85% (74/87) of isolates with variant multiplex patterns had the ST8 genotype. It is interesting that all variant SCC*mec* multiplex patterns observed in this study had genotypes within CC8 further supporting the view that CC8 is one of the most diversified and sucessful MRSA lineages (Robinson & Enright, 2003).

Robinson and Enright (2003) have also identified three of these variant SCC*mec* II and IV multiplex patterns in the ST8 genetic background; SCC*mec* II – *kdp* and SCC*mec* II – *kdp*, *mecI* and pUB110 were identified in two isolates described as Irish-1 from the UK and one AR14 phenotype from Ireland and SCC*mec* IV – *dcs* in three isolates (two phenotype AR43 from Ireland and one EMRSA-Irish-2 isolate from Australia. Some of the isolates investigated in that study came from the Irish MRSA collections used in the present study. Furthermore, the similarity between isolates with AR43 and isolates described as EMRSA-Irish-2 in Australia has been noted previously and Irish isolates with phenotypes AR13 and AR14 have been shown to be related to UK isolates described as Irish-1 (Steering Group of the North/ South Study of MRSA in Ireland 1999, 2000; McGechie & Daley, 2000; Rossney *et al.*, 2003). Due to the large

number of these ST8 isolates with variant multiple patterns identified in this present study among Irish MRSA isolates it is possible to predict that these ST8 variant clones evolved in Ireland and spread to the UK and Australia.

One of the aims of this present study was to investigate the relationship between isolates exhibiting AR43 and isolates exhibiting AR13, AR14, AR06, AR07.0 and AR07.4. By analysing PFGE patterns of total cellular DNA of these isolates it was revealed that AR43 isolates were unrelated to AR06, AR07.0 and AR07.4 isolates but were possibly related to isolates exhibiting AR13 and AR14 (Fig. 3.5). This finding was confirmed by MLST/SCC*mec* typing where AR06, AR07.0 and AR07.4 isolates were shown to belong to three separate clonal types from three different CCs (ST22-MRSA-IV (CC22), ST36-MRSA-II (CC30) and ST5-MRSA-II (CC5), respectively) that were completely distinct from the clonal types of AR13, AR14 and AR43 isolates (Table 3.7). All AR13, AR14 and AR43 isolates (not just those recovered in 1999) were found to have the ST8 genotype but they all harboured previously uncharacterised SCC*mec* elements (Table 3.7). Surprisingly, an isolate with the AR05 phenotype from 1989 and isolates classified as New01 and New03 from 1999 were also found to have the ST8 genotype and again produced a variety of SCC*mec* multiplex patterns (Table 3.7). In order to fully comprehend the evolutionary history of the ST8 isolates within the Irish MRSA population it is necessary to extensively characterise at a molecular level, the variant SCC*mec* elements harboured by all ST8 isolates. This is discussed in detail in Chapter 4.

Two other clonal types were also identified within CC8, ST94-MRSA-IV and ST609-MRSA-IVA, in one isolate each recovered in 2003 and 2004, respectively (Unfamiliar-2 and -4, Table 3.7). ST94 and ST609 both differ from ST8 by a single nucleotide change at the *yqiL* and *gmk* loci, respectively. ST94 has allele 45 of *yqiL* instead of allele 3 found in ST8 (Table 3.8) and since the *yqiL* allele 45 is not found in any other allelic profiles, it is considered to be the result of a recent point mutation. There is only one other record of ST94 in the MLST database and that is from an MSSA isolate from Cuba. However, in this present study the ST94 genotype was identified in a MRSA isolate harbouring SCC*mec* IV (Table 3.7).

ST609-MRSA-IVA is a novel clonal type that is probably derived from ST8-MRSA-IV. ST609 is an SLV of ST8 and has allele 58 of *gmk* instead of the ST8 *gmk* allele 1 (Table 3.8). Therefore, it appears that ST609 is a derivative of ST8 that arose by a point

mutation in the *gmk* allele. ST609-MRSA-IVA was recovered from a patient in 2004 who had been hospitalised in USA immediately prior to hospitalisation in Ireland. This novel clonal type may have been imported from the USA, where the ST8-MRSA-IV clone (also called the New York clone V in the USA) has been identified previously (Chung *et al.*, 2004). Introduction of pUB110 into the SCC*mec* IV element would have resulted in the type IVA SCC*mec* element seen in ST609-MRSA-IVA. Both ST94-MRSA-IV and ST609-MRSA-IVA are new clonal types among the Irish MRSA population observed for the first time in 2003 and 2004, respectively and it will be interesting to see if either of these predominates in the Irish hospital environment in the future.

CC5 has been previously identified as the second largest MRSA lineage and contains two more major pandemic clones, ST5-MRSA-II (New York/Japan clone) and ST5-MRSA-IV (Paediatric clone) (Enright *et al.*, 2002; Robinson & Enright, 2003). ST5 is the putative ancestor of this CC and ST5-MRSA-II and ST5-MRSA-IV are believed to have evolved following acquisition of SCC*mec* elements by independent ST5-MSSA isolates (Enright *et al.*, 2002). Although the ST5-MRSA-II clone has formed part of the Irish MRSA population since 1989, it has never predominated (AR07.3/07.4 and AR11, Table 3.7). The ST5-MRSA-IV clone was identified for the first time in an Irish hospital in 2002 and this was from one patient only (Unfamiliar-1, Table 3.7). These pandemic ST5-MRSA clones did not persist in Irish hospitals to the same level that they did in other hospitals worldwide such as in the USA, Mexico, Korea, Japan, Portugal and South America (Corso *et al.*, 1998; Roberts *et al.*, 1998 & 2000; Sa-Leao *et al.*, 1999; Aires de Sousa *et al.*, 2000; Gomes *et al.*, 2001; Chung *et al.*, 2004; Velazquez *et al.*, 2004; Ko *et al.*, 2005).

A novel ST, ST496 was also identified within CC5, from one isolate from 2002 (AR07.2, Table 3.7). This genotype differed from ST5-MRSA-II by single nucleotide substitutions in the *arcC* and *yqiL* alleles and since a base difference between two alleles at a single nucleotide site has previously been suggested to be due to a point mutation in the original allele (Enright *et al.*, 2002), ST496 most likely arose from ST5.

The international MRSA clone ST22-MRSA-IV which belongs to CC22, is the non-multidrug resistant UK epidemic strain EMRSA-15. EMRSA-15 emerged in the UK in 1991 where it displaced most other EMRSA strains and is now endemic in hundreds of hospitals in the UK as well as being identified in Belgium, Australia, Spain, New Zealand, Germany, Finland and Sweden (Richardson & Reith, 1993; O' Neill *et al.*,

2001; Moore & Lindsay, 2002; Witte *et al.*, 2001; Barbagiannakos *et al.*, 2004; Denis *et al.*, 2004; Perez-Roth *et al.*, 2004; MLST database). This present study determined that ST22-MRSA-IV has been present in the Irish MRSA population since 1989 (AR06, Rossney & Keane, 2002a). Its prevalence increased in the mid 1990's and by the late 1990's it was one the most predominant Irish MRSA clonal types (AR06, Table 3.7; Irish Dept. of Health and Children, 1999; Rossney & Keane, 2002a; Rossney *et al.*, 2004). By 2002 it predominated over all other clonal types and had replaced the ST8-MRSA-II derivatives as the most predominant MRSA clones, suggesting that today ST22-MRSA-IV has some selective advantage over ST8 isolates under the current environmental conditions in Irish hospitals.

Three clonal types within CC30 have been identified in this present study, ST36-MRSA-II, ST30-MRSA-IV and ST34-MSSA. ST36-MRSA-II is the UK epidemic strain EMRSA-16 that was first identified in the UK in 1992 and today predominates in hospitals in the UK along with EMRSA-15 (Moore & Lindsay, 2002). ST36-MRSA-II was found to be one of the predominant clones in Irish hospitals in 1998 and 1999 but by 2002 its prevalence had decreased (ARA07.0/07.2, Table 3.7; Irish Dept. of Health & Children, 1999; Rossney & Keane, 2002a).

The ST30-MRSA-IV clonal type was identified in four isolates only (ARNT, Table 3.7) that caused minor problems in Irish hospitals. These four isolates were from a group of seven isolates that gave an AR pattern similar to AR06 but gave different PFGE patterns, and unlike AR06 isolates they were positive for urea hydrolysis and were termed ARNT (see section 2.1.1 Chapter 2). In this present study sporadic isolates of ST30-MRSA-IV were recovered in 1989, 1993 and 2002 (ARNT, Table 3.7). The ST30-MRSA-IV clonal type never predominated in Irish hospitals but it has previously been identified as a widely spread clone in Greece (Aires de Sousa *et al.*, 2003a), in a paediatric hospital in Mexico (Velazquez-Meza *et al.*, 2004), but only among a few isolates from Sweden and Germany (Enright *et al.*, 2002), Spain (Oliveira & de Lencastre, 2002a) and Argentina (Aires de Sousa & de Lencastre., 2003), and in CAMRSA from Australia (O'Brien *et al.*, 2004b).

ST30 is the ancestral genotype of CC30 and it is predicted that ST30-MRSA-IV arose from ST30-MSSA following acquisition of SCC*mec* IV (Enright *et al.*, 2002). The ST36-MRSA-II clonal type is a single locus variant (slv) of ST30 that arose from ST30-MSSA following a point mutation in the *pta* loci resulting in the *pta* allele 2 of ST36 (Table 3.8) and acquisition of SCC*mec* II (Enright *et al.*, 2002).

The ST34 genotype was identified in an ARNT isolate from 1993 that was methicillin-resistant when initially isolated but after storage was found to be methicillin-susceptible and harboured no SCC*mec* elements or *mecA* (Table 3.7). A similar phenomenon was observed in one isolate with the ST239 genotype from 1989 (AR09/0.0064, Table 3.7). It has been reported previously that SCC*mec* can be lost during storage of MRSA isolates (van Griethuysen *et al.*, 2003). Based on the fact that the Irish ST34 isolate referred to above was originally methicillin-resistant but when tested later was methicillin-susceptible and harboured no SCC*mec* element and that in the MLST database the ST34 genotype is only reported from MSSA isolates, it is possible that the isolate lost the SCC*mec* element because the ST34 genotype is not suited to carrying a SCC*mec* element. However, the finding of an ST239-MSSA isolate is unexpected as no other ST239-MSSA isolates have been reported previously and isolates with the combination of the ST239 genotype and SCC*mec* III have been shown to be highly a successful pandemic MRSA clonal type (Enright *et al.*, 2002; MLST database). Due to that fact that the isolate with ST239-MSSA clonal type (AR09/0.0064) was originally stored on a nutrient agar slope for almost nine months prior to storage at –70°C, it may be that the SCC*mec* element was lost from this isolate during storage due to stress conditions such as starvation or elevated temperatures.

Although ST34 only differs from ST30 by a single nucleotide change in the *arcC* allele (Table 3.8), after analysis of a large selection of *S. aureus* genes, Robinson & Enright (2004a) found, that similar to the ST239 genotype, the ST34 genotype was the product of a recombination event between distinct genotypes. However, in the case of ST34 the ancestral genotypes involved were a combination of the ST30/ST39 and the ST10/ST145 genotypes.

Another infrequently occurring clonal type in the Irish MRSA population was ST45-MRSA-IV (Berlin clone, CC45). ST45-MRSA-IV was represented by only a single isolate in this present study (ARNT, 2002, Table 3.7) but has previously been recognised as a widespread clone in many different geographically distinct locations such as Western Europe, Scandinavia, Canada and the northwestern USA (Robinson & Enright, 2003; MLST database).

Other researchers have also reported changes over time in the dominant clonal types in hospitals in different countries. For example, Perez-Roth *et al.* (2004) found that the dominant MRSA clonal type in a Spanish hospital in 1998 was ST247-MRSA-IA but by 2002 this clonal type had been completely replaced by ST36-MRSA-II (EMRSA-16)

as the dominant clone. In a study of the MRSA population in a Mexican hospital, Velazquez-Meza *et al.* (2004) identified that between 1997 and 2000 the ST30-MRSA-IV clone predominated but in 2001 this was replaced by the ST5-MRSA-II clone.

The multiplex SCC*mec* typing method is used by many researchers for routine epidemiological typing of MRSA. However, this present study has shown that if the multiplex method had been used alone as the sole SCC*mec* typing method, almost half the isolates investigated would have either been misclassified as harbouring erroneous SCC*mec* elements or would have yielded SCC*mec* multiplex patterns that could not be interpreted as belonging to any of the currently described SCC*mec* types (Table 3.7). Combining the results of the simplex and multiplex SCC*mec* typing methods allowed correct classification of some of the unusual multiplex patterns and the identification of possible SCC*mec* variants. The lack of amplimers in isolates with incomplete multiplex patterns may be due to deletions, mutations, insertions, rearragments or a combination of events in these SCC*mec* elements and this is investigated further in Chapter 4. Neither the simplex or multiplex methods recognises the SCC*mec* IV variants IVa, IVb IVc or IVd or the recently described type V SCC*mec* element (Ma *et al.*, 2002; Ito *et al.*, 2003 & 2004).

Two of the variant multiplex patterns reported in the present study have also been identified in other studies but in distinctly different genetic backgrounds. These include the multiplex pattern SCC*mec* II - *kdp* & *mecI* in two Japanese isolates with the ST5 and ST89 genotypes (Aires de Sousa & de Lencastre, 2003) and the SCC*mec* IV – *dcs* multiplex pattern in ST30 isolates from Greece (Aires de Sousa *et al.*, 2003a). These isolates may contain the same variant SCC*mec* elements as the Irish MRSA isolates. Alternatively, the similar multiplex patterns in different genotypes may be due to different mutations, deletions, rearrangements or insertions in these highly variable SCC*mec* regions. The fact that some multiplex patterns lack the same amplimer does not automatically mean the same SCC*mec* element is present. Further investigation into the variant SCC*mec* elements observed in the present study and in other studies, is required.

In conclusion, this analysis of Irish MRSA isolates using MLST and SCC*mec* typing has provided data fundamental to understanding the epidemiology and evolutionary history of MRSA in Ireland. It has indicated that there may be a large degree of diversity within the SCC*mec* elements of Irish MRSA isolates that now need to be investigated further.

# Chapter 4

# Molecular Characterisation of Nine Novel SCC*mec* Variants Present in Irish Nosocomial Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates Recovered Between 1971-2004

## 4.1 Introduction

Following the extensive molecular analysis of Irish nosocomial MRSA isolates recovered between 1971 and 2004 using a combination of MLST and both the multiplex and simplex SCC*mec* typing methods during this present study (Chapter 3), it was found that almost half of the isolates investigated harboured novel variant SCC*mec* elements of unknown structure in previously described *S. aureus* genetic backgrounds (Table 4.1). The simplex SCC*mec* typing method identifies the *ccr-mec* gene combination unique to each SCC*mec* type, while the multiplex method detects various other regions of each SCC*mec* element (Oliveira & de Lencastre, 2002a). By combining both the simplex and multiplex SCC*mec* typing results described in Chapter 3, seven different variant multiplex patterns were identified among 87/177 isolates investigated (ca 49%) (Table 4.1).

All ST8 isolates (n=74) produced variant multiplex patterns. Those ST8 isolates identified as having SCC*mec* type II *ccr-mec* genes by the simplex method (n=54) were missing either the *kdp* amplimer (SCC*mec* II – *kdp*) or combinations of the *kdp*, *mecI* and pUB110 amplimers (SCC*mec* II – *kdp* & *mecI*, SCC*mec* II – *kdp* & pUB110, SCC*mec* II – *kdp*, *mecI* & pUB110) (Table 4.1). No variants of the type II SCC*mec* element have been described previously. The *kdp* operon is a copy of a potassium transporter operon and is found in the L-C region of SCC*mec* II. However, the *kdp* operon of SCC*mec* II harbours many deletions and mutations rendering it inactive. The *mecI* gene forms part of the class A *mec* complex that defines a type II SCC*mec* element and pUB110 is found integrated downstream of *mecA* in SCC*mec* II (see Figure 1.4, Chapter 1). All ST8 isolates that were found to have SCC*mec* type IV *ccr-mec* genes by the simplex method (n=20) were missing the *dcs* amplimer typically produced by a type IV SCC*mec* element using the multiplex method (Table 4.1). The *dcs* region is located downstream of *mecA* in SCC*mec* IVa and IVb, but has been found to be absent in the SCC*mec* IV variant element IVc (Ma *et al.*, 2002; Ito *et al.*, 2003).

Another variant multiplex pattern was identified among four Irish MRSA isolates with the ST239 genotype (Table 4.1). These isolates were found to have type III *ccr-mec* genes by the simplex method but were missing the pI258/Tn*554* multiplex amplimer characteristic of SCC*mec* III (SCC*mec* III-pI258/Tn*554*, Table 4.1). Two variants of SCC*mec* III (IIIA and IIIB, see section 1.2.3.5) have been described to date by other researchers (Oliveira *et al.*, 2001c) and are recognised by the multiplex

**TABLE 4.1.** Summary of unusual SCC*mec* typing results*

| ST | Simplex SCC*mec* type[1] | Expected SCC*mec* multiplex amplimers (bp)[2] | Actual SCC*mec* multiplex amplimers (bp) | SCC*mec* multiplex pattern name[3] | AR types[4] | No. of isolates | Years[5] |
|---|---|---|---|---|---|---|---|
| 8 | II | 381, 342, **284**, 209 & 162 | 381, 342, 209 & 162 | II – *kdp* | AR05, AR13, AR14 | 10 | 1989,'93,'98,'99 |
| 8 | II | 381, 342, **284**, **209** & 162 | 381, 342 & 162 | II – *kdp* & *mecI* | AR14 | 10 | 1993,'98,'99 |
| 8 | II | **381**, 342, **284**, 209 & 162 | 342, 209 & 162 | II - *kdp* & pUB110 | AR13, AR14, New01, New03 | 27 | 1998,'99,'02 |
| 8 | II | **381**, 342, **284**, **209** & 162 | 342 & 162 | II – *kdp*, *mecI* & pUB110/ IV | AR13, AR14 | 7 | 1998,'99,'02 |
| 8 | IV | **342** & 162 | 162 | IV - *dcs* | AR43 | 20 | 1999 |
| 239 | III | 414, 303, **243**, 209 & 162 | 414, 303, 209 & 162 | III – pI258/Tn*554* | Phenotype-III, AR09, Unfamiliar-3 | 4 | 1985-'87,'89,'03 |
| 250 | I | **495**, 342 & 162 | 342 & 162 | I – *pls*/IV | AR02, Phenotype-II | 9 | 1978-'84,'89 |

*see Chapter 3 for full details

[1]Simplex SCC*mec* typing based on *ccr-mec* gene amplification (see section 3.2.3.2 for full details).

[2]These are the expected sizes of amplimers when the SCC*mec* multiplex method (Oliveira & de Lencastre, 2002) is used to amplify the SCC*mec* types identified by the simplex method. Amplimers in bold indicate those that are missing in the multiplex patterns of these Irish isolates and correspond to the amplification of the following regions: 284 bp, part of the *kdp* operon found in SCC*mec* II; 209 bp, part of the *mecI* gene; 381 bp, the left junction between IS*431mec* and pUB110; 342 bp, part of the *dcs* region; 243 bp, a region between pI258 and Tn*554* found in SCC*mec* III; 495 bp, a region downstream of *pls* in SCC*mec* I.

[3]SCC*mec* multiplex pattern name designated during this present study

[4]See Table 3.7 & 3.9 (chapter 3) for the isolate numbers of each MRSA phenotype with each variant multiplex pattern.

[5]Years in which isolates with each SCC*mec* variant pattern were identified (see Chapter 3 for full details).

method. However, based on the novel multiplex patterns observed for these four Irish ST239 isolates it was proposed that they harboured a novel variant of the type III SCC*mec* element.

The variant pattern SCC*mec* I - *pls* was identified among nine ST250 isolates. The *pls* gene homologue is found in the L-C region of both SCC*mec* I and its variant element SCC*mec* IA (Ito *et al.*, 1999; Oliveira & de Lencastre, 2001c). The plasmin sensitive surface protein (Pls) that is encoded by *pls* is a surface protein of MRSA shown to reduce both the adhesion to host proteins and invasion of epithelial cells, while also mediating adhesion to cellular lipids and glycolipids and attachment to human desquamated nasal epithelial cells (Savolainen *et al.*, 2001; Huesca *et al.*, 2002; Roche *et al.*, 2003; Juuti *et al.*, 2004). The Pls protein has been identified as a virulence factor for septic arthritis caused by MRSA (Josefsson *et al.*, 2004). This suggests that the *pls* gene found in SCC*mec* I may have contributed to the dissemination of MRSA isolates harbouring the type I SCC*mec* element by mediating their attachment to human nasal epithelial cells and certain cellular components.

There have been many other studies that have reported possible SCC*mec* variants based on SCC*mec* typing results (Aires de Sousa & de Lencastre, 2003; Aires de Sousa *et al.*, 2003a; Robinson & Enright, 2003; Wielders *et al.*, 2003; Chung *et al.*, 2004; Perez-Roth *et al.*, 2004; Shukla *et al.*, 2004b). However, none of these studies has characterised the structures of the SCC*mec* elements that produce these variant typing results. In order to increase the typeability of MRSA isolates during SCC*mec* typing based epidemiological investigations it is vital to elucidate the unknown structures of variant SCC*mec* elements. Furthermore, by clarifying the structure of these novel variant SCC*mec* elements, we may gain a better understanding of the role of SCC*mec* elements in the evolution of MRSA.

### 4.1.1 Aims

The aim of this part of the present study was to investigate comprehensively the variant SCC*mec* elements identified in a large group of Irish MRSA isolates by the unusual SCC*mec* multiplex typing patterns they produced. Based on the fact that ST8 isolates with variant multiplex patterns predominated in Irish hospitals in the 1990s and because the largest number of variants were observed within ST8, the entire SCC*mec* element from two ST8 isolates, one with a variant SCC*mec* II multiplex patttern and one with the variant SCC*mec* IV multiplex pattern were sequenced. The genomic

structure of the SCC*mec* elements of all other ST8 isolates was also determined by a combination of nucleotide sequencing and by observing the mobility of amplimers obtained using different primer pairs in agarose gels. The variant SCC*mec* elements harboured by the ST250 and ST239 isolates were investigated by a combination of nucleotide sequencing of the region corresponding to the absent multiplex amplimers and by observing the mobility of amplimers in agarose gels.

## 4.2 Materials and Methods

### 4.2.1 Bacterial isolates

All isolates that produced variant SCC*mec* multiplex patterns (n=87) were investigated during this part of the present study (Table 4.1). This included all ST8 isolates (n=74; AR05, AR13, AR14, AR43, New01 and New03 isolates), as well as 9/19 ST250 isolates (3/3 AR02 and 6/15 Phenotype-II isolates) and 4/14 ST239 isolates (1/1 Phenotype-III, 2/3 AR09 and 1/1 Unfamiliar-3 isolates) (Table 4.1).

### 4.2.2 Investigation of the variant SCC*mec* elements harboured by ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns

*4.2.2.1 Nucleotide sequencing of the entire SCCmec of two ST8 isolates*

The entire nucleotide sequence of the SCC*mec* element of two ST8 isolates was determined. One isolate (AR13.1/3330.2) from 1999 was chosen because it was representative of isolates with *ccr-mec* genes indicative of SCC*mec* II when analysed by the simplex SCC*mec* typing method but lacked one to three of the five amplimers typical of SCC*mec* II by the multiplex method. Isolate AR13.1/3330.2 exhibited the multiplex pattern II - *kdp*, *mecI* and pUB110. The second isolate (AR43/3330.1) was also from 1999 and was chosen because it was representative of isolates with SCC*mec* IV *ccr-mec* genes by the simplex method but with one of the two amplimers characteristic of SCC*mec* IV missing when isolates were investigated by the multiplex method. These two isolates were recovered in mixed culture from the same clinical specimen.

Overlapping primers were designed to amplify the entire SCC*mec* element from both isolates (Table 4.2) using previously published SCC*mec* type II and SCC*mec* IVa nucleotide sequences (Ito *et al.*, 1999; Ma *et al.*, 2002). These sequences were obtained from the Genbank database website (http://www.ncbi.nlm.gov:80/entrez/guery.fcgi?db=nucleotide) under accession numbers D86934 (SCC*mec* II) and AB063172 (SCC*mec* IVa). The primers used to amplify and sequence the SCC*mec* elements are listed in Table 4.2. The locations and direction of the primers are shown in Figure 4.1. DNA fragments for sequencing and cloning were obtained by PCR amplification of chromosomal DNA using either *Pfu* DNA polymerase (Promega) for primer pairs dcs F-orfX R or the Expand Long Template PCR System (Roche) for all other primer sets (Table 4.2; Fig. 4.1) according to the manufacturers' instructions.

**TABLE 4.2.** Primers used in this study for SCC*mec* element amplification and sequencing from ST8 isolates

| Isolate | Primer pairs | Nucleotide sequence (5'-3')[a] | Restriction site | Nucleotide coordinates | SCC*mec* region amplified |
|---|---|---|---|---|---|
| AR13.1/ 3330.2 | | | | | |
| | IRLII F | CC*CTCGAG*GG**CTCTGCGTATCAGTTAATGA** | *Xho*I | 4684-4703[b] | L-C[d] |
| | ccrA R | ATTT*GCGGCCG*C**GCTTCGATAGCCTGTTTCTG** | *Not*I | 25490-25471[b] | |
| | ccrA F | ACGC*GTCGAC***CAAGTCATAGGCTATTTACG** | *Sal*I | 25417-25436[b] | C-M[e] |
| | Tn554 R | ACGC*GTCGAC***AAGCTATCCACGTTCAATCTCAAC** | *Sal*I | 32442-32419[b] | (*ccr*A2-Tn*554*) |
| | Tn554 F | ATTT*GCGGCCG*C**CTTTAAAGGGTTTCGGAATA** | *Not*I | 32365-32385[b] | C-M[e] |
| | mecR1 R | CC*CTCGAG*GG**CAATGCCTAAACCTAATCG** | *Xho*I | 43754-43736[b] | (Tn*554-mecR1*) |
| | mecR1 F | ATTT*GCGGCCG*C**CGATTAGGTTTAGGCATTG** | *Not*I | 43736-43754[b] | M-I[f] |
| | ugpQ R | CCCT*CGAGGG***GCTTCTGCAGGATCTTGG** | *Xho*I | 47868-47851[b] | (*mecR1-ugpQ*) |
| | ugpQ F | CCCT*CGAGGG***CCAAGATCCTGCAGAAGC** | *Xho*I | 47851-47878[b] | I-R[g] |
| | dcs R | ATTT*GCGGCCG*C**CGGTCATGGCTATGATTTAG** | *Not*I | 56373-56354[b] | (*ugpQ-dcs*) |
| | dcs F | ATTT*GCGGCCG*C**GTCAATGAGATCATCTACAT** | *Not*I | 56109-56128[b] | I-R[g] |
| | orfX R | ATTT*GCGGCCG*C**CCCAAGGGCAAAGCGAC** | *Not*I | 57826-57810[b] | (*dcs*-right SCC*mec* junction) |
| AR43 /3330.1 | | | | | |
| | IRLII F | CC*CTCGAG*GG**CTCTGCGTATCAGTTAATGA** | *Xho*I | 4684-4703[b] | L-C[d] |
| | ccrA R | ATTT*GCGGCCG*C**GCTTCGATAGCCTGTTTCTG** | *Not*I | 25490-25471[b] | |
| | ccrA F | ATTT*GCGGCCG*C**CAAGTCATAGGCTATTTACG** | *Not*I | 9894-9913[c] | C-M[e] |
| | mecA R | ACGC*GTCGAC***CCTATCTCATATGCTG** | *Sal*I | 18160-18145[c] | (*ccr*A2-*mecA*) |
| | mecA F | ATTT*GCGGCCG*C**GATTGGGATCATAGCGTCAT** | *Not*I | 18021-18040[c] | M-I[f] |
| | ISmec R | ACGC*GTCGAC***ACGGTGATCTTGCTCAATGA** | *Sal*I | 22780-22761[c] | (*mecA*-IS*431mec*) |
| | ISmec F | CC*CTCGAG*GG**CCTGACTGTCATTGTAC** | *Xho*I | 22724-22740[c] | I-R[g] |
| | orfX R | ATTT*GCGGCCG*C**CCCCAAGGGCAAAGCGAC** | *Not*I | 25395-25379[c] | (IS*431mec*-right SCC*mec* junction) |

[a]Text in bold indicates primer sequences. Text in italics and underlined indicates restriction sites added to primer sequences and plain text indicates extra bases added to aid with cloning.
[b]Nucleotide coordinates from SCC*mec* type II, Genbank accession number D86934.
[c]Nucleotide coordinates form SCC*mec* type IVa, Genbank accesion number AB063172.
[d]L-C, the region from the left chromosomal/SCC*mec* junction to beginning of the *ccr* complex.
[e]C-M, the region from *ccr* complex to the *mec* complex.
[f]M-I, the region from *mec* complex region to IS*431mec*.
[g]I-R, the region from IS*431mec* to the right extremity of SCC*mec*.

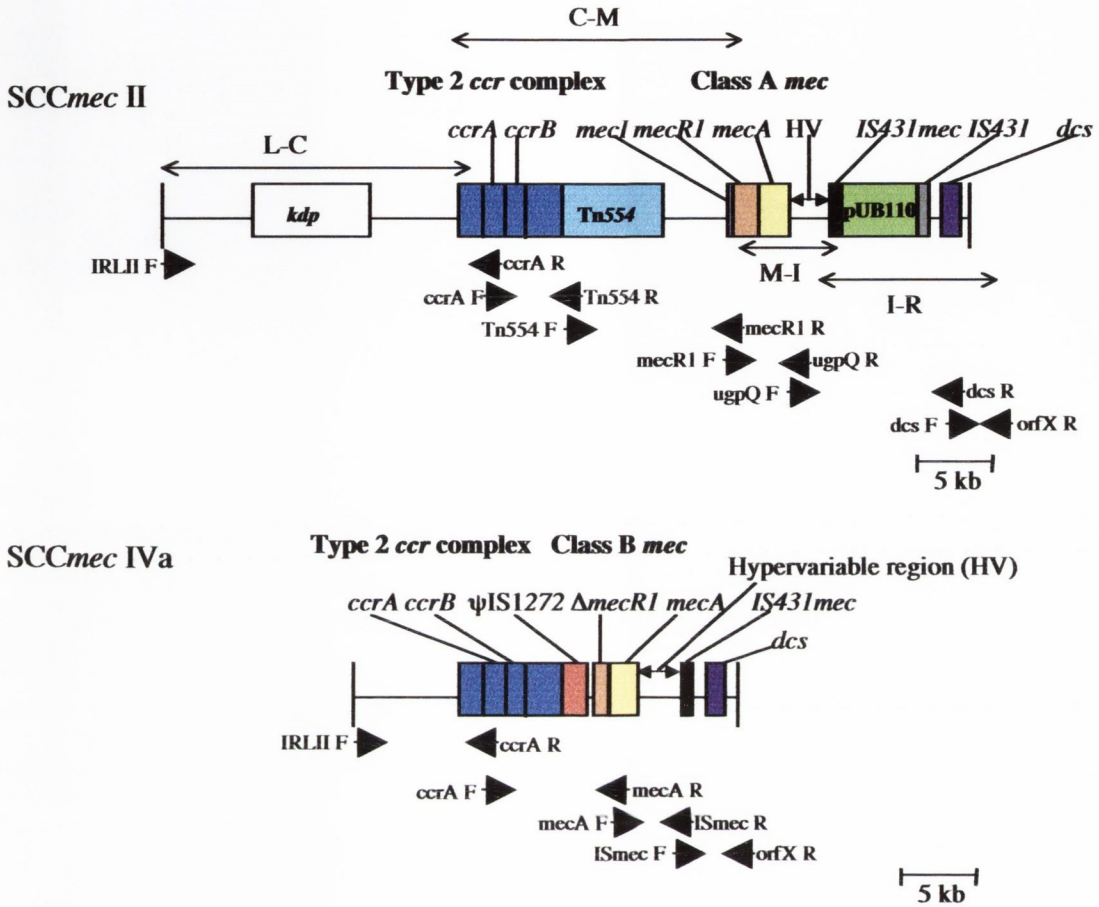



**FIGURE 4.1.** Schematic diagram of the genomic organisation of SCC*mec* II and SCC*mec* IVa showing the approximate location and the direction of primer sequences used in this study to amplify the novel SCC*mec* elements of Irish ST8 isolates. Primer positions and direction are indicated with arrowheads. The different regions amplified are indicated with arrows (L-C, C-M, M-I and I-R regions). The structures of SCC*mec* II and IVa are based on the nucleotide sequences of the SCC*mec* elements in the GenBank database, accession numbers D86934 (SCC*mec* II) and AB063172 (SCC*mec* IVa) (Ito *et al.*, 1999; Ma *et al.*, 2002). Primer sequences are shown in Table 4.2.

Amplifcations using the *Pfu* DNA polymerase system were carried out in 50 µl volumes containing 1.25 U of *Pfu* DNA polymerase enzyme (Promega), 1 X *Pfu* DNA polymerase buffer with $MgSO_4$ (Promega), 200 µM each of dATP, dTTP, dCTP and dGTP (Promega), 1 µM of both forward and reverse primers and approximately 500 ng of genomic DNA template. Reactions were performed using an initial dentauration step of 2 min at 95°C followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and elongation at 72°C for 4 min. A final elongation step was performed for 5 min at 72 °C followed by cooling to 4 °C.

Amplifications using the Expand Long Template PCR System were carried out in 50 µl volumes containing 3.75 U of Expand Long Template enzyme mixture (Roche), 1 X Expand Long Template PCR buffer 3 containing 2.75 mM $MgCl_2$ (Roche), 500 µM each of dATP, dTTP, dCTP and dGTP (Promega), 300 mM of both forward and reverse primers and approximately 500 ng of genomic DNA template. Reactions were carried out with an initial denaturation step for 2 min at 92°C, 10 cycles of denaturation for 10 s at 92°C, primer annealing for 30 s at a temperature between 53°C and 68°C depending on the optimal annealing temperature of the primers being used and elongation at 68°C for between 4 min and 25 min depending on the fragment length. This was followed by 20 cycles of denaturation for 10 s at 92°C, primer annealing for 30 s at a temperature between 53°C and 68°C again depending on the optimal annealing temperature of the primers being used and elongation at 68°C for between 4 min and 25 min depending on the fragment length. A final elongation step was performed at 68°C for 7 min and then each reaction was cooled to 4°C. The annealing temperatures and elongation times used for each primer pair are described in each relevant section below.

When each primer pair was used to amplify DNA from isolate AR13.1/3330.2, template DNA from at least one isolate exhibiting SCC*mec* type II by both SCC*mec* typing methods was included as a control. Similarly for isolate AR43/3330.2, template DNA from at least one control isolate exhibiting SCC*mec* type IV by both methods was included.

Amplified PCR products were observed by agarose gel electrophoresis of 5 µl of each PCR reaction product in 1% (w/v) agarose gels and visualised on a UV transillminator as described previously in sections 2.3.2 and 2.4.2, respectively. PCR products were then purified using either the GenElute PCR Clean-Up kit (Sigma-Aldrich) or the Wizard SV Gel and PCR Clean-Up System (Promega) according to the

manufacturers instructions. Purified PCR products were directly sequenced by Lark Technologies using primer walking or were cloned into pBluescript II KS(-) phagemid and sequenced. Ligations of PCR products to pBluescript were facilitated by the presence of appropriate restriction endonuclease cleavage sites within the primers as indicated in Table 4.2. Ligation reactions were carried out as described in section 2.4.3. Transformation of competent *E. coli* DH5α prepared using $CaCl_2$ and identification of recombinants using blue-white selection was performed as described in section 2.4.4. DNA sequencing was performed on a commercial basis by Lark as described in section 2.5 and analysis of chromatograms and sequences were carried out also as described previously in section 2.5.

*4.2.2.1.1. PCR amplification of the L-C region*

The extreme left region of the SCC*mec* element of isolate AR13.1/3330.2 and AR43/3330.1, spanning from the left chromsomal/SCC*mec* junction to the *ccr* complex (L-C region) was amplified using the primer pair IRLII F-ccrA R (Table 4.2; Fig. 4.1). For isolate AR43/3330.2 primer annealing and elongation using IRLII F-ccrA R were performed for 30 s at 53°C and for 10 min at 68°C, respectively. For isolate AR13.1/3330.2 primer annealing was performed for 30 s at 55°C. Elongation using primer pair IRLII F-ccrA R on isolate AR13.1/3330.2 was initially performed for 25 min at 68°C because the expected product for a type II SCC*mec* element was ca. 20 kb. However, the product for this isolate was found to be only 5.6 kb so the elongation time was reduced from 25 min to 10 min at 68°C.

*4.2.2.1.2 PCR amplification of the C-M region*

The region from the *ccr* complex to the *mec* complex (C-M region) was amplified using primer pairs ccrA F-Tn554 R and Tn554 F-mecR1 R for AR13.1 /3330.2 and ccrA F-mecA R for AR43/3330.1 (Table 4.2; Fig. 4.1). For these three primer pairs, primer annealing and elongation were performed for 30 s at 55°C and for 14 min at 68°C, respectively.

*4.2.2.1.3 PCR amplification of the M-I and I-R regions*

The region from the *mec* complex to IS*431mec* (M-I region) and the extreme right region (I-R) of the SCC*mec* elements were amplified using primer pairs mecR1 F-ugpQ

R, ugpQ F-dcs R, dcs F-orfX R for AR13.1/3330.2 and primer pairs mecA F-ISmec R and ISmec F-orfX R for AR43/3330.1 (Table 4.2; Fig. 4.1). Primer annealing and elongation were performed for 30s at 55°C and 10 min at 68°C, respectively for primer pairs mecR1 F-ugpQ R, mecA F-ISmec R and ISmec F-orfX R. For primer pair ugpQ F-dcs R, annealing and elongation were performed for 30 s at 68°C and 14 min at 68°C, respectively. PCR amplification using primer pair dcs F-orfX R was performed using *Pfu* polymerase as described above in section 4.2.2.1.

*4.2.2.2 Investigation of the variant SCCmec elements in all other ST8 isolates*

The SCC*mec* elements harboured by all other ST8 isolates identified as having SCC*mec* II or IV *ccr-mec* genes by the simplex method but with unusual SCC*mec* multiplex patterns were investigated by PCR amplification. The primers used for amplification and sequencing of the SCC*mec* element from isolate AR13.1/3330.2 (representative of isolates with SCC*mec* II *ccr-mec* genes by the simplex method but lacking one to three of the five SCC*mec* II multiplex amplimers) were used on all atypical SCC*mec* II isolates (Table 4.2; Fig. 4.1). Similarly, the primers for amplification and sequencing of the SCC*mec* element of isolates AR43/3330.1 (representative of isolates with SCC*mec* IV *ccr-mec* genes by the simplex method but lacking one SCC*mec* IV multiplex amplimer) were used on all atypical SCC*mec* IV isolates. The PCR products were separated by electrophoresis in 1% (w/v) agarose gels and the sizes of the PCR products were compared to those obtained by amplification using the same primers on template DNA from AR13.1/3330.2 and AR43/3330.1 and to previously published SCC*mec* element sequences. Amplimers that showed variation from expected sizes were also sequenced.

### 4.2.3 Investigation of the variant SCC*mec* elements harboured by ST239 isolates with unusual SCC*mec* III multiplex patterns

The entire nucleotide sequence of the region between pI258 & Tn*554* of the SCC*mec* element of one ST239 isolate was determined. Isolate AR09/0065 from study period D (1989) was chosen because it was representative of isolates with *ccr-mec* genes indicative of SCC*mec* III when analysed by the simplex SCC*mec* typing method but lacked the pI258/Tn*554* amplimer typical of SCC*mec* III by the multiplex method resulting in the unusual multiplex pattern SCC*mec* III – pI258/Tn*554*. This pI258/Tn*554* multiplex amplimer is the result of the amplification of a region between

integrated plasmid pI258 (also called the *mer* operon) and integrated transposon Tn*554* in SCC*mec* III by the multiplex primers RIF4 F3/RIF4 R9 (Fig. 4.2). As part of this present study primer pair pI258 F-Tn554 R3 was designed to amplify across this region of SCC*mec* AR09/0065 (Table 4.3; Fig. 4.2) using the previously published SCC*mec* III sequence (Ito *et al.*, 1999) that was obtained from the Genbank database website under accession number AB037671. The locations and direction of the primers are shown in Figure 4.2.

DNA fragments for sequencing were obtained by PCR amplification of chromosomal DNA using the Expand Long Template PCR System (Roche) according to the manufacturers' instructions as described in section 4.2.2.1. Primer annealing and elongation were performed for 30 s at 55°C and 14 min at 68°C, respectively. Template DNA from at least one isolate exhibiting SCC*mec* type III by both SCC*mec* typing methods was included as a control. Amplified PCR products were observed by agarose gel electrophoresis of 5 µl of each PCR reaction product in 1% (w/v) agarose gels and visulaised on a UV transillminator as described previously in sections 2.3.2 and 2.4.2, respectively. PCR products were then purified using either the Wizard SV Gel and PCR Clean-Up System (Promega). Purified PCR products were directly sequenced by Lark Technologies using primer walking and analysis of chromatograms and sequences were carried out as described previously in section 2.5.

The pI258 F-Tn*554* R3 primer pair (Table 4.3; Fig. 4.2) were used on all ST239 isolates with the multiplex pattern SCC*mec* III - pI258-Tn*554*. The PCR products were separated by electrophoresis in 1% (w/v) agarose gels and the sizes of the PCR products were compared to those obtained by amplification using the same primers on template DNA from AR09/0065 and to the previously published SCC*mec* III element sequence.

### 4.2.4 Investigation of the variant SCC*mec* elements harboured by ST250 isolates with unusual SCC*mec* I multiplex patterns

Nine ST250 isolates had *ccr-mec* genes indicative of SCC*mec* I when analysed by the simplex SCC*mec* typing method but lacked the *pls* amplimer typical of SCC*mec* I by the multiplex method resulting in the unusual multiplex pattern SCC*mec* I – *pls*. This *pls* multiplex amplimer is the result of the amplification of a region downstream of *pls* in the L-C region of SCC*mec* I using multiplex primers CIF2 F2 and CIF2 R2 (Fig. 4.3). Therefore we decided to design a primer pair that would amplify across the part of

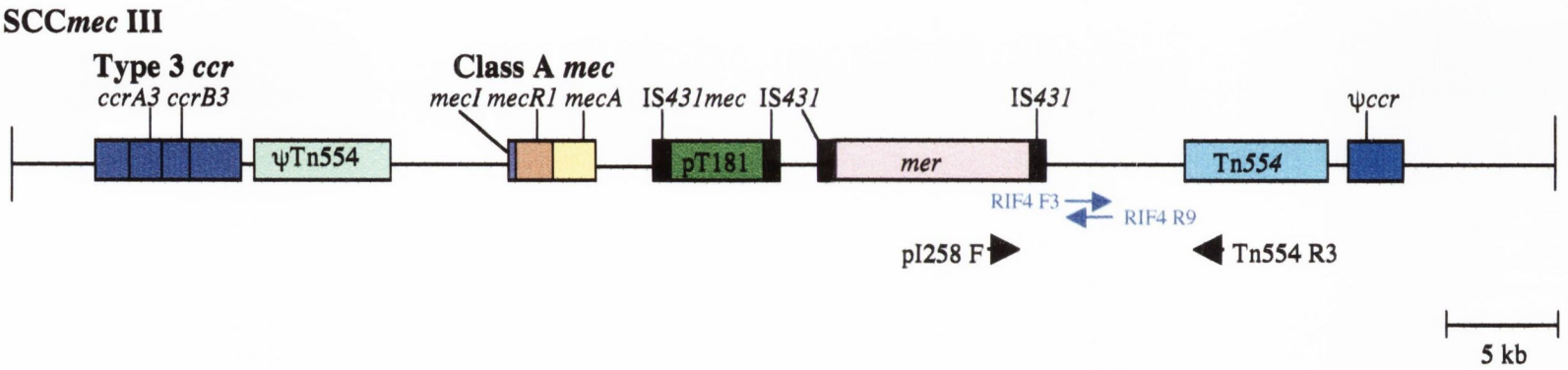



**FIGURE 4.2.** Schematic diagram of the genomic organisation of SCC*mec* III showing the approximate location and direction of primer sequences used in this study to amplify the region from *mer* to Tn*554* of ST239 isolates with the SCC*mec* III - pI258/Tn*554* multiplex pattern. The position and direction of the multiplex primers that failed to produce an amplimer in these isolates are indicated by arrows. Primers designed in this present study to amplify this region are indicated with arrowheads. The structures of SCC*mec* III is based on its nucleotide sequence in the Genbank database under accession numbers AB037671 (SCC*mec* III) (Ito *et al.*, 1999). Primer sequences designed in this present study are shown in Table 4.3 and multiplex primer sequences are shown in Table 3.5.

**TABLE 4.3.** Primers used in this study for amplification and sequencing of part of the SCC*mec* element from ST250 and ST239 isolates with atypical SCC*mec* multiplex patterns

| ST | SCC*mec* multiplex pattern | Primer pairs | Nucleotide sequence (5'-3') | Nucleotide coordinates | SCC*mec* region amplified |
|---|---|---|---|---|---|
| 239 | III – pI258/Tn*554* | pI258 F<br>Tn554 R3 | GACAACTTGATCACTATCCT<br>CAGAGCATTAGATATCGGAT | 42361-42380[a]<br>51750-51731[a] | pI258 (*mer*)-Tn*554* |
| 250 | I - *pls* | EO23F2<br>CEO17 R2 | TACGCTTGTTAGACTCAACG<br>CGGCTATATGAATGTGG | 17706-17725[b]<br>20102-20086[b] | ORF E023-CE017 (region between *pls* and *ccr*) |
| | | CEO15 F<br>EO25 R | CCTTGTATGCGATGAATACG<br>GTACATTTGCACGACGGTT | 17012-17031[b]<br>21560-21541[b] | ORF CEO15-EO25 (region between *pls* to beginning of *ccr*) |
| | | CEO10 F<br>CCRA R3 | GGATTATATCTCCATGCGCA<br>CGTACTGATTGATGATATAGG | 14561-14580[b]<br>24867-24847[b] | *pls* to *ccrA* |
| | | IRLI F1<br>CCRA R4 | CTCTGCGTATCAGATAATGA<br>TTTGAAATAGATGCCAGCGA | 4501-4520[b]<br>24990-24971[b] | DR-L/IR-L of SCC*mec* I-*ccrA* |
| | | IRLI F2<br>CCRA R4 | AACTGTTAGCTACTACTTAAG<br>TTTGAAATAGATGCCAGCGA | 4355-4375[b]<br>24990-24971[b] | Left chromosomal region outside SCC*mec* I- *ccrA* |
| | | plsneg F<br>CCRA R3 | AGTGTACAGAGCATTTAAGA<br>CGTACTGATTGATGATAGG | 38898-38879[b]<br>24867-24847[b] | SCC*mec*/chromosomal junction upstream of *ccr* complex to the *ccr* complex of SCC*mec* of isolates with multiplex pattern SCCmec I - *pls* |

[a]Nucleotide coordinates form SCC*mec* type III, Genbank accession number AB037671.
[b]Nucleotide coordinates from SCC*mec* type I, Genbank accession number AB033763.

the L-C region of SCC*mec* I where the multiplex primers should bind. Template DNA from at least one isolate exhibiting SCC*mec* type I by both SCC*mec* typing methods was included as a control during each PCR reaction.

Primers to amplify the L-C region of SCC*mec* I – *pls* isolates were initially designed based on the L-C and surrounding chromosomal regions of the SCC*mec* I nucleotide sequence obtained from the Genbank database under accession number AB033763 (Ito *et al.*, 1999). Five such primer pairs were designed (1.E023 F2-CEOI7 R2, 2.CEO15 F-EO25F, 3.CEO10-CCRA R3, 4.IRLI F1-CCRA R3, 5.IRLIF2-CCRA R4) one after the other (in the order of 1-5) because each of these primer pairs failed to yield an amplimer in any SCC*mec* I – *pls* isolate but succeeded in producing the expected size amplimer in control isolates (Table 4.3; Fig. 4.3). Finally the primer pair plsneg F-ccrA R3 (Table 4.3; Fig. 4.3) produced an amplimer in one SCC*mec* I – *pls* isolate (Phenotype-II/120.1).

All PCR reactions using these primers designed to amplify part of the SCC*mec* element of SCC*mec* I – *pls* isolates were performed using the Expand Long Template PCR System (Roche) according to the manufacturers' instructions as described in section 4.2.2.1. For all primer pairs (E023 F2-CEOI7 R2, CEO15 F-EO25F, CEO10-CCRA R3, IRLI F1-CCRA R3, IRLIF2-CCRA R4 and plsneg F-CCRA R3) primer annealing was performed at 55°C for 30s. For primer pairs E023 F2-CEOI7 R2, CEO15 F-EO25F and CEO10-CCRA R3, elongation was performed for 10 min at 68 °C. For primers IRLI F1-CCRA R3, IRLIF2-CCRA R4 and plsneg F-CCRA R elongation was performed for 25 min at 68°C. Amplified PCR products were observed by agarose gel electrophoresis of 5 µl of each PCR reaction product in 1% (w/v) agarose gels and visualised on a UV transillminator as described previously in sections 2.3.2 and 2.4.2, respectively. The amplimer obtained from isolate Phenotype II/120.1 using primers plsneg F-CCRA R was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and was directly sequenced by Lark Technologies using primer walking (described previously in section 2.5, chapter 2). Analysis of chromatograms and sequences was carried out as described previously in section 2.5.

### 4.2.5 Nucleotide sequence submission to the Genbank Database

Nucleotide sequences were submitted to the Genbank database using the WEBIN internet tool (http://www.ebi.ac.uk/embl/Submission/webin.html). Accession numbers

**SCC*mec* I**

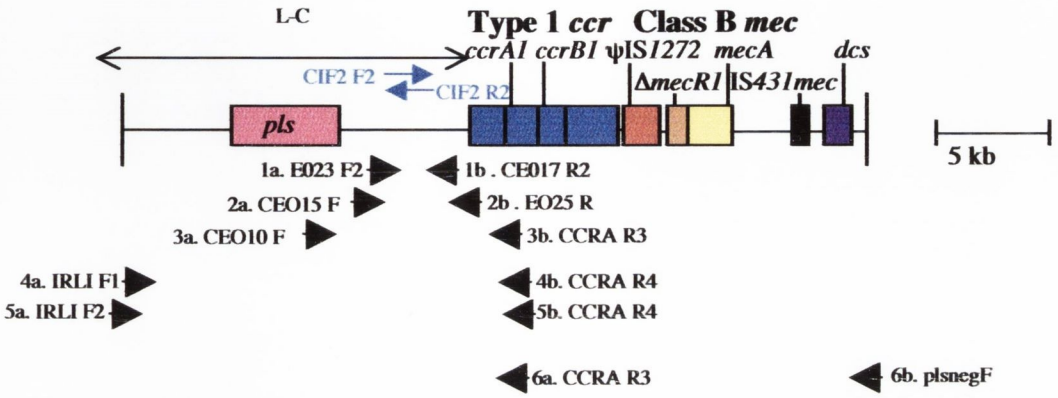



**FIGURE 4.3.** Schematic diagram of the genomic organisation of SCC*mec* I showing the approximate location and the direction of primer sequences used in this study to attempt to amplify the L-C region of SCC*mec* of ST250 isolates with the SCC*mec* I - *pls* multiplex pattern. The position and direction of the multiplex primers that failed to produce an amplimer in these isolates are indicated by arrows (CIF2 F2/CIF2 R2). Primers designed in this present study to amplify this region are indicated with arrowheads. The structure of SCC*mec* I is based on the nucleotide sequence of this SCC*mec* element in the Genbank database, accession number AB033763 (Ito *et al.*, 1999). Primer sequences designed in this present study are shown in Table 4.3 and multiplex primer sequences are shown in Table 3.5.

are AJ810120 (SCCmec IIE from MRSA isolates AR13.1/3330.2), AJ810121 (SCC*mec* IVE from MRSA isolate AR43/3330.2), AJ810122 (partial *mec* complex A.4), AJ810123 (partial SCC*mec* IIB) and AJ888304 (partial sequence of SCC*mec* IIIC).

## 4.3 Results

### 4.3.1 Investigation of the SCC*mec* elements harboured by ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns

Since all ST8 isolates (n=74) exhibited variant multiplex patterns (Table 4.1), we decided to further investigate these SCC*mec* elements. Firstly, the entire SCC*mec* element from two ST8 isolates, AR13.1/3330.2 and AR43/3330.1, with variant SCC*mec* II and SCC*mec* IV multiplex patterns respectively, were sequenced. Isolate AR13.1/3330.2 was representative of isolates with SCC*mec* II by the simplex method but with an SCC*mec* multiplex patterns lacking one to three of the amplimers typical of an SCC*mec* II element. AR 13.1/3330.2 exhibited SCC*mec* multiplex pattern II – *kdp*, *mecI* and pUB110 (i.e. it lacked all three bands, *kdp, mecI* and pUB110). Isolate AR43/3330.1 was representative of isolates with SCC*mec* IV by the simplex method but with an SCC*mec* multiplex pattern lacking the 342 bp *dcs* amplimer (SCC*mec* IV – *dcs*). The genomic organisation of these two variant SCC*mec* elements based on their nucleotide sequence is shown schematically in Fig. 4.4. The genomic structure of the SCC*mec* elements of the remaining 72 ST8 isolates was also determined and are also shown schematically in Fig 4.4.

*4.3.3.1 Novel variant of SCCmec II identified in ST8 isolate AR13.1/3330.2*

The SCC*mec* element in AR13.1/3330.2 consisted of a ca 27 kb sequence containing a combination of regions previously identified in SCC*mec* II and IV and a novel *mec* complex and was designated SCC*mec* IIE (Figs. 4.4 & 4.5). Amplification of the L-C region yielded an amplimer of ca. 5.6 kb which is similar in size to the expected size amplimer for SCC*mec* IVb (Table 4.4). The nucleotide sequence of the L-C region of this 5.6 kb amplimer was found to have 99.7% similarity at the nucleotide sequence level to the L-C region of SCC*mec* IVb and less than 10% similarity to the L-C regions of SCC*mec* types I, II, III, IVa and IVc. This explains why the multiplex primers that amplify part of *kdp* of the L-C region of SCC*mec* II (KDPF1, KDPR1; see chapter 3, Table 3.5) did not produce any amplimer for this isolate as isolate AR13.1/3330.2 harbours an SCC*mec* element that has no *kdp* region and it has a completely different L-C region to that of SCC*mec* II (Fig. 4.4).

Analysis of the sequence of the *ccr* complex of AR13.1/3330.2 identified type 2 *ccr* genes (*ccrA2* and *ccrB2*) with close similarity to those of SCC*mec* II and SCC*mec* IV

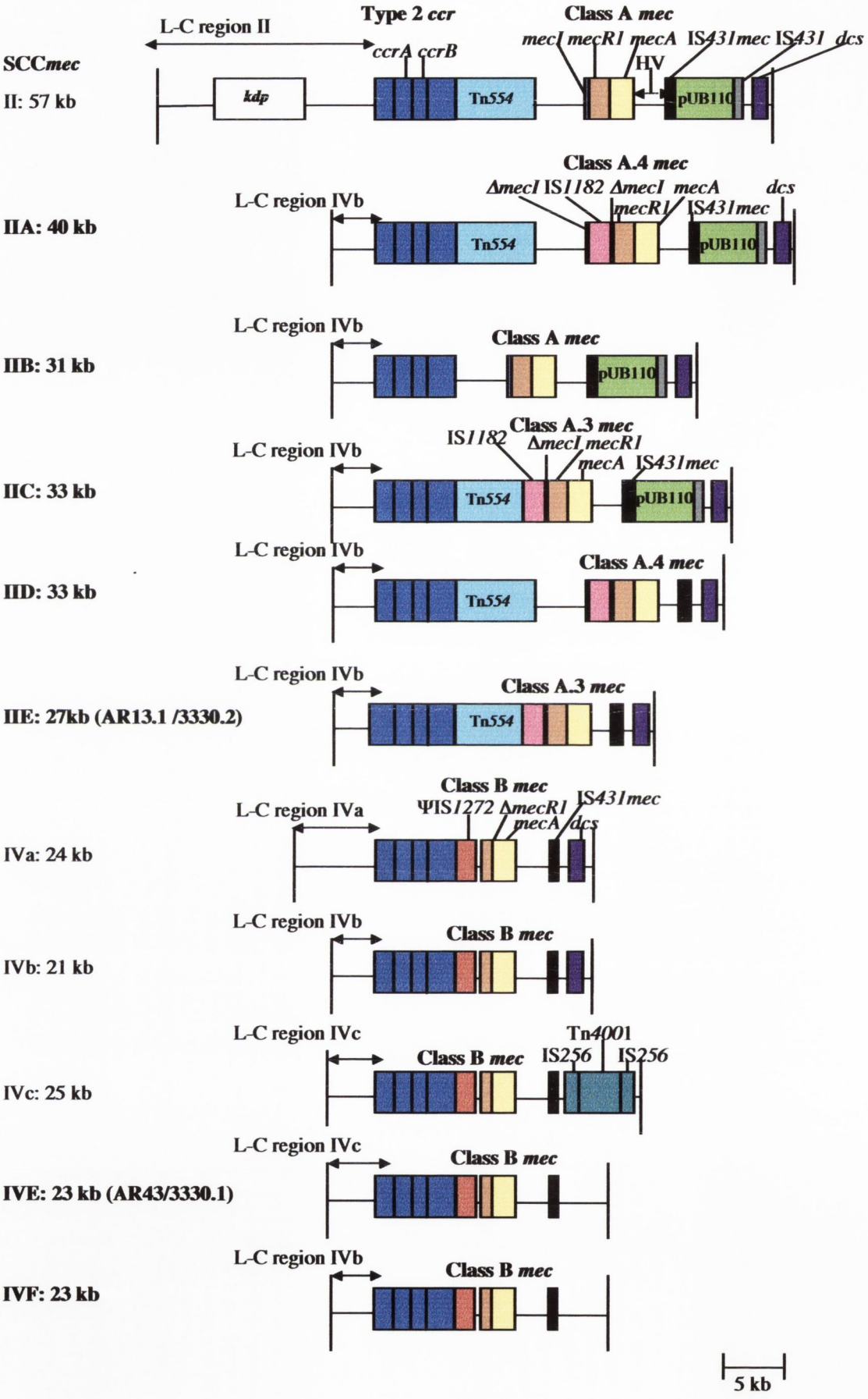

SCCmec
II: 57 kb
L-C region II
Type 2 ccr
ccrA ccrB
Class A mec
mecI mecR1 mecA
IS431mec
IS431
dcs
HV
kdp
Tn554
pUB110
IIA: 40 kb
Class A.4 mec
ΔmecI IS1182 ΔmecI mecA
mecR1
IS431mec
dcs
L-C region IVb
IIB: 31 kb
Class A mec
IIC: 33 kb
Class A.3 mec
IS1182
ΔmecI mecR1
mecA
IS431mec
IID: 33 kb
Class A.4 mec
IIE: 27kb (AR13.1 /3330.2)
Class A.3 mec
IVa: 24 kb
Class B mec
ΨIS1272 ΔmecR1
IS431mec
mecA dcs
L-C region IVa
IVb: 21 kb
Class B mec
IVc: 25 kb
Tn4001
IS256
IS256
L-C region IVc
IVE: 23 kb (AR43/3330.1)
IVF: 23 kb
5 kb

**FIGURE 4.4.** Schematic diagram showing the genomic organisation of SCC*mec* II, IIA, IIB, IIC, IID, IIE, IVa, IVb, IVc, IVE and IVF elements. SCC*mec* IIA, IIB, IIC, IID, IIE, IVE and IVF are novel variants that were identified in this present study. SCC*mec* II, IVa, IVb and IVc were identified previously (Ito *et al.*, 1999, Ma *et al.*, 2002; Ito *et al.*, 2003) and the organisation was determined based on the nucleotide sequences in the Genbank database under accession numbers D86934, AB063172, AB063173, AB096217, respectively. The structure of SCC*mec* IIE and IVE were determined by sequencing of the entire SCC*mec* element of Irish MRSA isolates AR13.1/3330.2 (IIE) (Genbank accession number AJ810120) and AR43/3330.1 (IVE) (Genbank accession number AJ810121). The structures of SCC*mec* IIA, IIB, IIC, IID and IVF were determined using the primers shown in Table 4.2, by observing the mobility of amplimers on agarose gels and in some cases by sequencing.

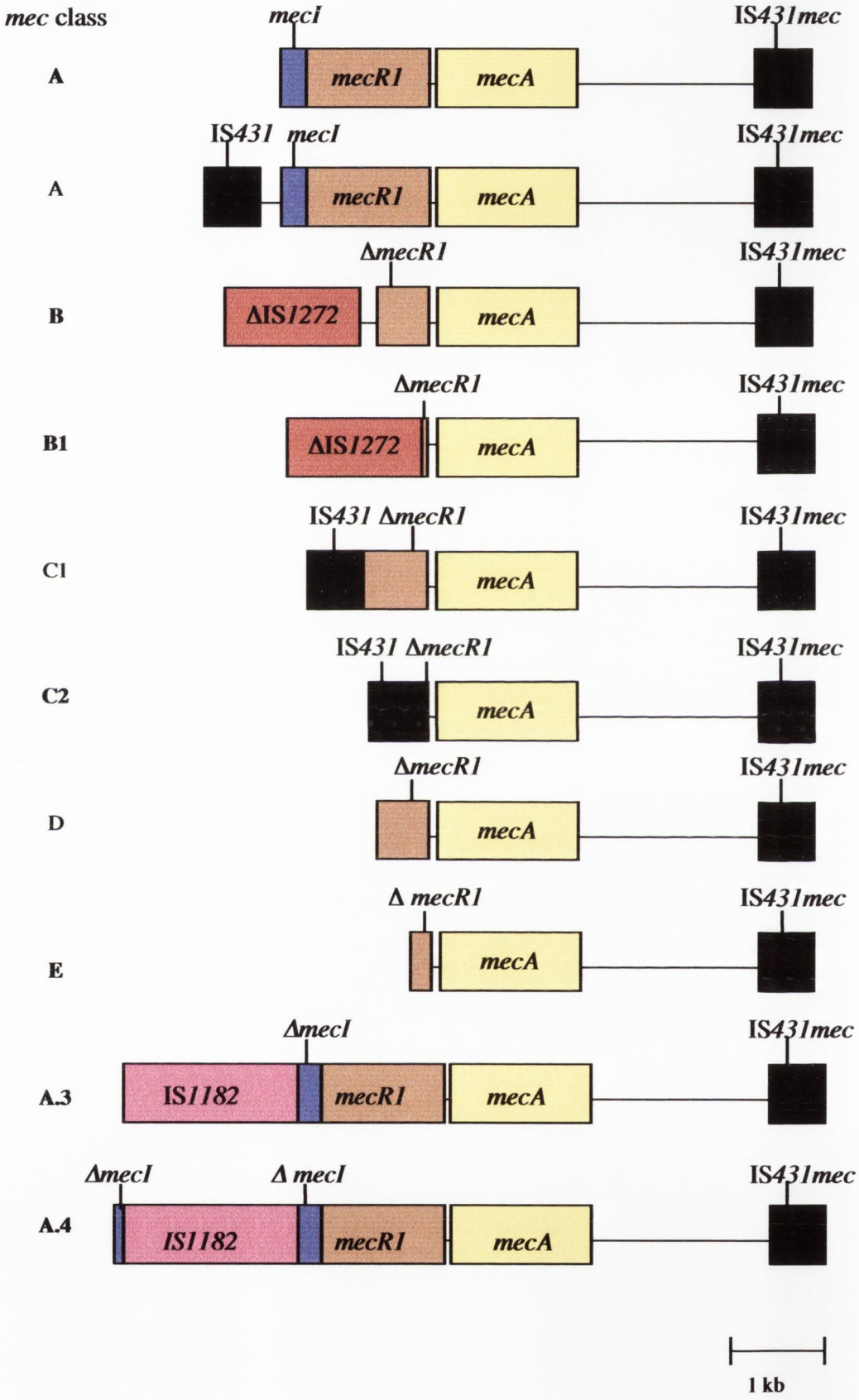

mec class
mecI
IS431mec
A
mecR1
mecA
IS431
mecI
IS431mec
A
mecR1
mecA
ΔmecR1
IS431mec
B
ΔIS1272
mecA
ΔmecR1
IS431mec
B1
ΔIS1272
mecA
IS431 ΔmecR1
IS431mec
C1
mecA
IS431 ΔmecR1
IS431mec
C2
mecA
ΔmecR1
IS431mec
D
mecA
Δ mecR1
IS431mec
E
mecA
ΔmecI
IS431mec
A.3
IS1182
mecR1
mecA
ΔmecI
Δ mecI
IS431mec
A.4
IS1182
mecR1
mecA
1 kb

**FIGURE 4.5.** Schematic diagram of the genetic organization of the *mec* complexes of staphylococci. The *mec* complexes A, B, B1, C1, C2, D and E have been described previously in staphylococci and A, B, C2 and E (indicated in bold) have been identified previously in MRSA (Katayama *et al.*, 2001; Lim *et al.*, 2003; Ito *et al.*, 2004). The novel *mec* classes A.3 and A.4 (indicated in bold) were identified in the present study of Irish MRSA isolates. Class A.3 *mec* differs from Class A *mec* in that it has a copy of *mecI* that is truncated at the 3' end and an upstream copy of IS*1182* (IS*1182*-Δ*mecI*-*mecR1*-*mecA*-IS431mec). The Δ*mecI* is only 253 bp whereas intact *mecI* is 372 bp. In class A.4 *mec* IS*1182* is present within *mecI* and there is a 16 bp deletion of *mecI* immediately upstream of IS*1182* (Δ*mecI*-IS*1182*-Δ*mecI*-*mecR1*-*mecA*-IS*431mec*).

**TABLE 4.4.** Correlation between SCC*mec* multiplex types and amplimer sizes obtained using overlapping primer pairs to amplify the novel SCC*mec* element variants and comparison to those expected from typical SCC*mec* II and SCC*mec* IV elements.

| SCC*mec* multiplex type | SCC*mec* element | Primer Pairs | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **L-C** IRLII F- ccrA R | **C-M** ccrA F- Tn554 R | **C-M** Tn554 F- mecR1 R | **M-I** mecR1 F- ugpQ R | **I-R** ugpQ F- dcs R | **I-R** dcs F- orfX R | **C-M** ccrA F- mecA R | **M-I** mecA F- ISmec R | **I-R** ISmec F- orfX R | |
| II | II | 20 kb | 7 kb | 11 kb | 4.1 kb | 8.5 kb | 1.7 kb | 20 kb | 4.7 kb | 8 kb | Ito *et al.*, 1999 |
| II – *kdp* | IIA | 5.6 kb | 7 kb | 13 kb* | 4.1 kb | 8.5 kb* | 1.7 kb | 22 kb | 4.7 kb | 8 kb | This study |
| II – *kdp* | IIB | 5.6 kb | 7 kb | No amplimer[a]. | 4.1 kb | 8.5 kb | 1.7 kb | 13 kb | 4.7 kb | 8 kb | This study |
| II – *kdp & mecI* | IIC | 5.6 kb | 7 kb | 6.5 kb | 4.1 kb | 8.5 kb | 1.7 kb | 14 kb | 4.7 kb | 8 kb | This study |
| II – *kdp* & pUB110 | IID | 5.6 kb | 7 kb | 13 kb | 4.1 kb | 2.2 kb | 1.7 kb | 22 kb | 3.7 kb | 2.7 kb | This study |
| II – *kdp, mecI* & pUB110 | IIE† | 5.6 kb* | 7 kb* | 6.5 kb* | 4.1 kb* | 2.2 kb* | 1.7 kb* | 14 kb | 3.7 kb | 2.7 kb | This study |
| IV | IVa | 8.9 kb | No amplimer[b] | No amplimer[b] | 4.1 kb | 3.2 kb | 1.7 kb | 8.5 kb | 4.7 kb | 2.7 kb | Ma *et al.*, 2002 |
| IV | IVb | 5.6 kb | No amplimer[b] | No amplimer[b] | 4.1 kb | 3.2 kb | 1.7 kb | 8.5 kb | 4.7 kb | 2.7 kb | Ma *et al.*, 2002 |
| IV | IVc | 6.1 kb | No amplimer[b] | No amplimer[b] | 4.1 kb | No amplimer[c] | No amplimer[c] | 8.5 kb | 4.7 kb | 5.3 kb | Ito *et al.*, 2003 |
| IV – *dcs* | IVE† | 6.1 kb* | No amplimer[b] | No amplimer[b] | 4.1 kb | No amplimer[c] | No amplimer[c] | 8.5 kb* | 4.7 kb* | 4.7 kb* | This study |
| IV – *dcs* | IVF | 5.6 kb* | No amplimer[b] | No amplimer[b] | 4.1 kb | No amplimer[c] | No amplimer[c] | 8.5 kb | 4.7 kb | 4.7 kb* | This study |

Amplicon sizes for SCC*mec* IIA, IIB, IIC, IID, IIE, IVE and IVF were calculated either by sequencing (indicated as *) or based on electrophoretic mobility on agarose gels. Amplicon sizes for SCC*mec* II, IVa, IVb and IVc were calculated from sequences in the Genbank database, accession numbers D86934, AB063172, AB063173 and AB096217, respectively. †SCC*mec* elements IIE and IVE were sequenced in full from isolates AR13.1/3330.2 and AR43/3330.1, respectively. All amplimer sizes are approximate. [a]no amplimer due to no Tn*554*; primers ccrA F- mecR1 R were used instead resulting in a 11.5 kb amplimer which was sequenced. [b]no amplimer due to no Tn*554* in SCC*mec* IVa, IVb, IVc, IVE and IVF. [c]no amplimer due to no *dcs* region in SCC*mec* IVc, IVE and IVF. L-C, C-M, M-I and I-R indicate the region of the SCC*mec* element amplified. See Table 4.2 for primer sequences and figure 4.1 for primer locations and direction.

(Table 4.5). The amino acid identities of the proteins encoded by the *ccrA* and *ccrB* genes of AR13.1/3330.2 and SCC*mec* II were 96.7% and 98%, respectively, and 99.1% and 97.8%, respectively, compared with those of SCC*mec* IVb (Table 4.5).

Amplification and sequencing of the region between the *ccr* and *mec* complexes (C-M) in AR13.1/3330.2 using two primer pairs (ccrA F-Tn554 R and Tn554 F-mecR1 R; Table 4.2; Fig. 4.1), yielded a 7 kb and 6.5 kb product (Table 4.4) and confirmed the presence of a truncated integrated transposon Tn*554* (Table 4.5; Fig. 4.4). Five of the six ORFs (*tnpA, tnpB, tnpC, spc, ermA*) previously identified within Tn*554* of SCC*mec* II were present in the SCC*mec* element of AR13.1/3330.2 and each ORF showed 100% homology at the amino acid level with those of SCC*mec* II (Table 4.5). However, Tn*554* of SCC*mec* of AR13.1/3330.2 was truncated by ca.1000 bp resulting in the absence of an ORF of unknown function (ORF N050) found in Tn*554* of SCC*mec* II. The six ORFs spanning a ca. 5 kb region encoding hypothetical proteins usually found between Tn*554* and the *mec* complex in SCC*mec* II were also absent (Fig. 4.3). Instead of these ORFs, a 1,865 bp sequence was identified, which searches of the Genbank database identified as insertion sequence element IS*1182*. Immediately adjacent to IS*1182*, a 253 bp truncated version of *mecI* (Δ*mecI*) was identified (*mecI* gene is usually 372 bp) (Figs. 4.5 & 4.6). The multiplex primers that amplify part of 3' region of *mecI* in the *mec* complex of SCC*mec* II (MECIP2, MECIP3; see chapter 3, Table 3.5) did not produce any amplimer for this isolate because these multiplex primers bind to a region of the *mecI* gene that is absent in the SCC*mec* element of isolate AR13.1/3330.2. Sequences of *mecR1 and mecA* from AR13.1/3330.2 showed 100% homology to those of SCC*mec* II elements (Table 4.5).

Amplification of the region across IS*431mec* to the *dcs* region downstream of the *mec* complex yielded a product of 2.2 kb which is smaller than that of any previously described SCC*mec* element including SCC*mec* II and IVb (Table 4.4). Sequence data from this product showed that the hypervariable (HV) region (located between *mecA* and IS*431mec*) of AR13.1/3330.2 was shorter than the HV region of previously described SCC*mec* II and SCC*mec* IV elements (Fig. 4.7). The HV region of AR 13.1/3330.2 is 997 bp shorter than that of SCC*mec* II and SCC*mec* IVb (Fig. 4.7). The IS*431mec* region was 100% homologous to that of other SCC*mec* elements but lacked all DNA sequences associated with the integrated plasmid pUB110 or the second IS*431* sequence found in SCC*mec* II (Fig. 4.4). Again this sequencing data explains why the

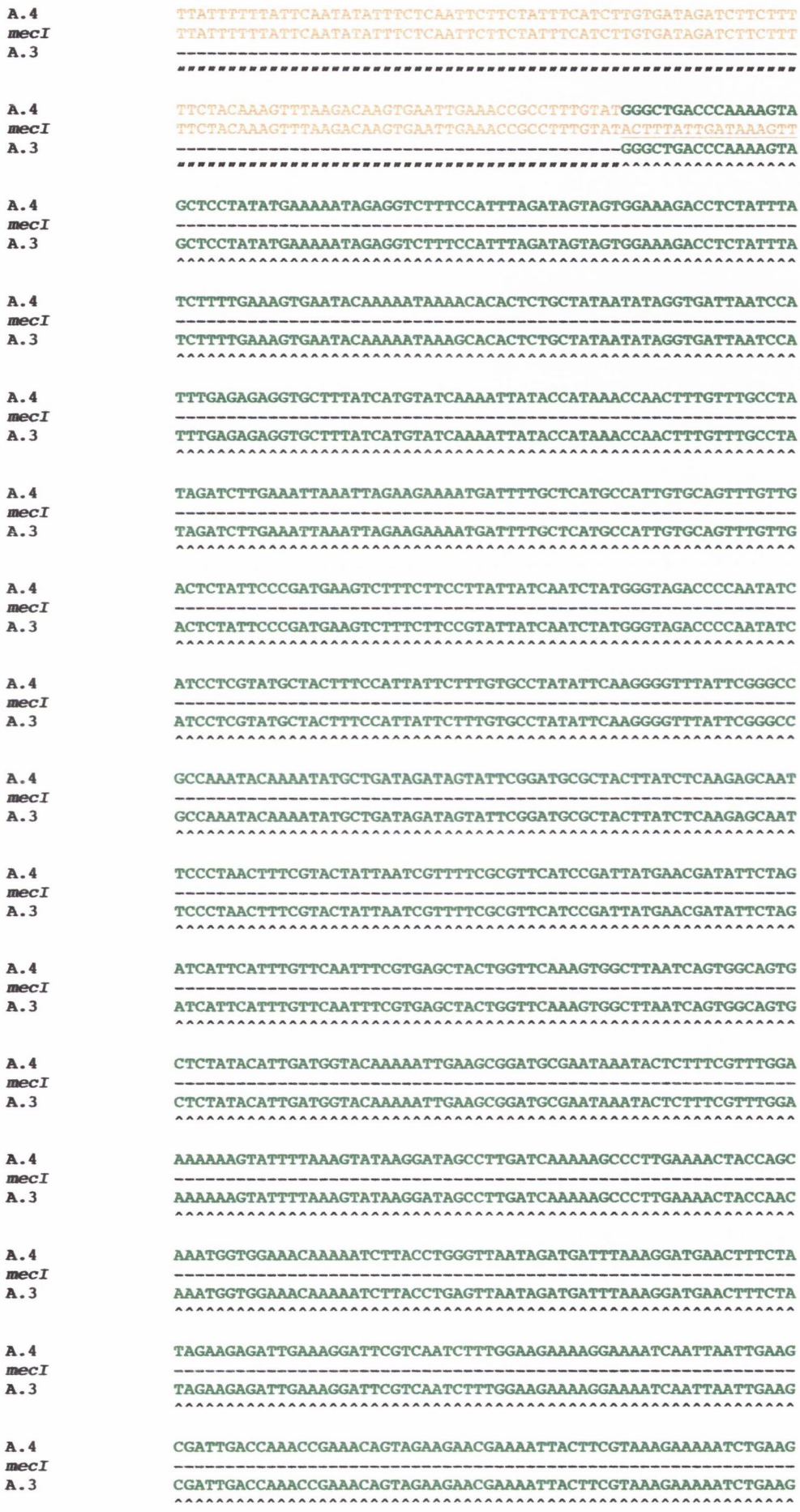
**TABLE 4.5.** Percentage identities between ORFs of the novel SCC*mec* II variant element of Irish MRSA isolate AR13.1/3330.2 and the conventional SCC*mec* IVb and II elements.

| SCC*mec* element and ORFs | Location[a] | Homology to ORFs of SCC*mec* IVb[b] | | | Homology to ORFs of SCC*mec* II[b] | | |
|---|---|---|---|---|---|---|---|
| **AR13.1/3330.2 (IIE)** | | **ORF** | **% homology[c]** | **Gene product** | **ORF** | **% homology[c]** | **Gene product** |
| **L-C region** | | | | | | | |
| IIE01 | 210-1145 (complement) | CM001 | 83.7 % | Hypothetical protein | ..... | ..... | ..... |
| IIE02 | 1123-1893 (complement) | CM002 | 100% | Hypothetical protein | ..... | ..... | ..... |
| IIE03 | 2010-3440 | M001 | 99.6% | Hypothetical protein | ..... | ..... | ..... |
| **C-M region** | | | | | | | |
| ***ccr* complex** | | | | | | | |
| IIE04 | 3560-5350 | M002 | 99.7% | Conserved Hypothetical protein | N031 | 80.3% | Conserved Hypothetical protein |
| IIE05 (*ccr*A2) | 5584-6933 | M004 | 99.1% | Cassette chromosome recombinase A2 | N034 | 96.7% | Cassette chromosome recombinase A2 |
| IIE06 (*ccr*B2) | 6955-8583 | M005 | 97.8% | Cassette chromosome recombinase B2 | N037 | 98% | Cassette chromosome recombinase B2 |
| IIE07 | 9101-9451 | M006 | 88.9% | Conserved Hypothetical protein | N041 | 100% | Conserved Hypothetical protein |
| IIE08 | 9538-9849 | M007 | 89.4% | Conserved Hypothetical protein | N042 | 100% | Conserved Hypothetical protein |
| IIE09 | 9867-10373 | M008 | 91.7% | Conserved Hypothetical protein | N043 | 99.4% | Conserved Hypothetical protein |
| IIE10 | 10394-10711 | ..... | ..... | ..... | N044 | 100% | pseudogene |
| **Tn*554*** | | | | | | | |
| IIE11 (*tnpA*) | 10830-11915 | ..... | ..... | ..... | N045 | 100% | Transpoase A |
| IIE12 (*tnpB*) | 11912-13804 | ..... | ..... | ..... | N046 | 100% | Transpoase B |
| IIE13 (*tnpC*) | 13811-14188 | ..... | ..... | ..... | N047 | 100% | Transpoase C |
| IIE14 (*spc*) | 14339-15121 | ..... | ..... | ..... | N048 | 100% | O-nucleotydiltransferase |
| IIE15 (*ermA*) | 15247-15978 (complement) | ..... | ..... | ..... | CN026 | 100% | rRNA adenine N-6-methyltransferase |
| **M-I region** | | | | | | | |
| ***mec* complex** | | | | | | | |
| IIE16 (*tnp* IS*1182*) | 16499-18103 | ..... | ..... | ..... | ..... | ..... | ..... |
| IIE17 (*ΔmecI*) | 18207-18459 (complement) | ..... | ..... | ..... | CN036 | 67.7% | Methicillin resistance protein MecI |
| IIE18 (*mecR1*) | 18459-20216 (complement) | CM003 | 55.6% | Truncated signal transducer protein MecR1 | CN037 | 100% | signal transducer protein MecR1 |
| IIE19 (*mecA*) | 20316-22322 | M010 | 100% | Penicillin binding protein 2a | N058 | 100% | Penicillin binding protein 2a |
| IIE20 | 22368-22796 (complement) | CM004 | 100% | Conserved Hypothetical protein | CN038 | 100% | Conserved Hypothetical protein |
| IIE21 (*ugpQ*) | 22893-23636 | CM006 | 100% | Glcerophosphoryldiester phosphodiesterase | N039 | 100% | Glcerophosphoryldiester phosphodiesterase |
| IIE22 (*tnp* IS*431mec*) | 23901-24575 | M011 | 100% | Transpoase for IS*431mec* | N062 | 100% | Transpoase for IS*431mec* |
| **I-R region** | | | | | | | |
| IIE23 | 24607-24846 (complement) | CM007 | 100% | Conserved Hypothetical protein | CN049 | 100% | Conserved Hypothetical protein |
| IIE24 | 25261-26556 (complement) | CM008 | 40.7% | Truncated Conserved Hypothetical protein | CN050 | 100% | Truncated Conserved Hypothetical protein |

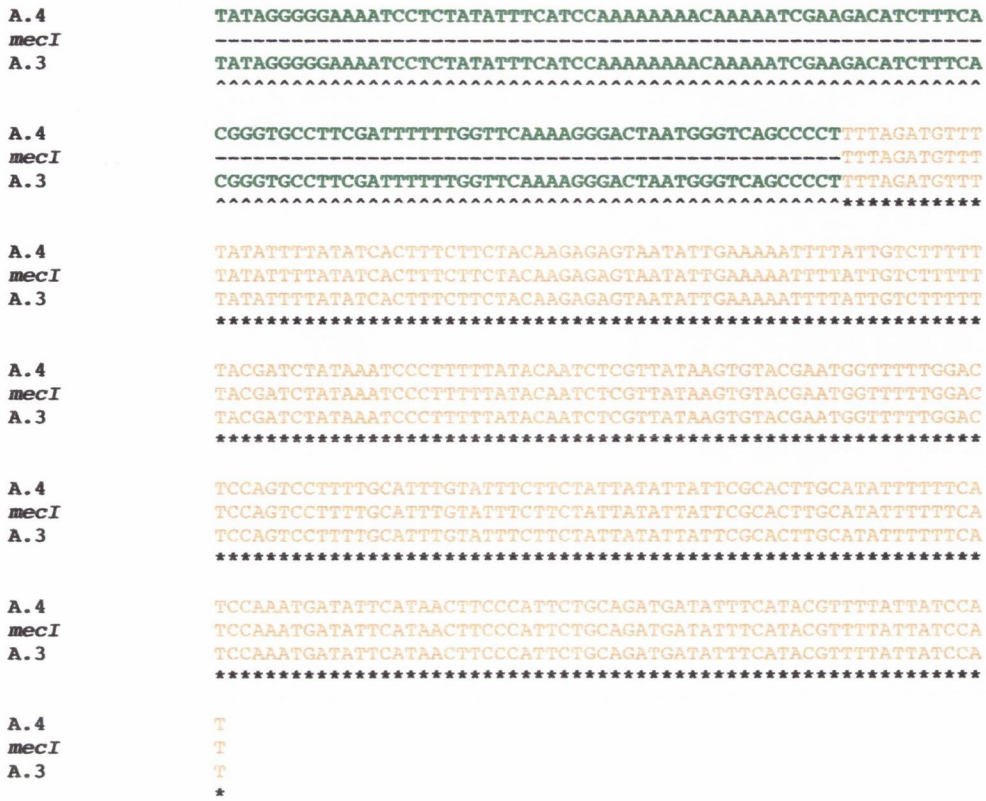
[a]The nucleotide positions were determined based on the sequences for IIE deposited in the Genbank database under acession number AJ810120

[b]ORFs and gene products from SCC*mec* II and SCC*mec* IVb, nucleotide sequences in the Genbank database, accession number D86934 and AB063173, respectively (Ito *et al.*, 1999; Ma *et al.*, 2002).

[c]% homology based on amino acid identity; "....." indicates no significant homology.


A.4
mecI
A.3
TTATTTTTTATTCAATATATTTCTCAATTCTTCTATTTCATCTTGTGATAGATCTTCTTT
TTATTTTTTATTCAATATATTTCTCAATTCTTCTATTTCATCTTGTGATAGATCTTCTTT
TTCTACAAAGTTTAAGACAAGTGAATTGAAACCGCCTTTGTATGGGCTGACCCAAAAGTA
TTCTACAAAGTTTAAGACAAGTGAATTGAAACCGCCTTTGTATACTTTATTGATAAAGTT
GGCTGACCCAAAAGTA
GCTCCTATATGAAAAATAGAGGTCTTTCCATTTAGATAGTAGTGGAAAGACCTCTATTTA
GCTCCTATATGAAAAATAGAGGTCTTTCCATTTAGATAGTAGTGGAAAGACCTCTATTTA
TCTTTTGAAAGTGAATACAAAAATAAAACACACTCTGCTATAATATAGGTGATTAATCCA
TCTTTTGAAAGTGAATACAAAAATAAAGCACACTCTGCTATAATATAGGTGATTAATCCA
TTTGAGAGAGGTGCTTTATCATGTATCAAAATTATACCATAAACCAACTTTGTTTGCCTA
TTTGAGAGAGGTGCTTTATCATGTATCAAAATTATACCATAAACCAACTTTGTTTGCCTA
TAGATCTTGAAATTAAATTAGAAGAAAATGATTTTGCTCATGCCATTGTGCAGTTTGTTG
TAGATCTTGAAATTAAATTAGAAGAAAATGATTTTGCTCATGCCATTGTGCAGTTTGTTG
ACTCTATTCCCGATGAAGTCTTTCTTCCTTATTATCAATCTATGGGTAGACCCCAATATC
ACTCTATTCCCGATGAAGTCTTTCTTCCGTATTATCAATCTATGGGTAGACCCCAATATC
ATCCTCGTATGCTACTTTCCATTATTCTTTGTGCCTATATTCAAGGGGTTTATTCGGGCC
ATCCTCGTATGCTACTTTCCATTATTCTTTGTGCCTATATTCAAGGGGTTTATTCGGGCC
GCCAAATACAAAATATGCTGATAGATAGTATTCGGATGCGCTACTTATCTCAAGAGCAAT
GCCAAATACAAAATATGCTGATAGATAGTATTCGGATGCGCTACTTATCTCAAGAGCAAT
TCCCTAACTTTCGTACTATTAATCGTTTTCGCGTTCATCCGATTATGAACGATATTCTAG
TCCCTAACTTTCGTACTATTAATCGTTTTCGCGTTCATCCGATTATGAACGATATTCTAG
ATCATTCATTTGTTCAATTTCGTGAGCTACTGGTTCAAAGTGGCTTAATCAGTGGCAGTG
ATCATTCATTTGTTCAATTTCGTGAGCTACTGGTTCAAAGTGGCTTAATCAGTGGCAGTG
CTCTATACATTGATGGTACAAAAATTGAAGCGGATGCGAATAAATACTCTTTCGTTTGGA
CTCTATACATTGATGGTACAAAAATTGAAGCGGATGCGAATAAATACTCTTTCGTTTGGA
AAAAAAGTATTTTAAAGTATAAGGATAGCCTTGATCAAAAAGCCCTTGAAAACTACCAGC
AAAAAAGTATTTTAAAGTATAAGGATAGCCTTGATCAAAAAGCCCTTGAAAACTACCAAC
AAATGGTGGAAACAAAAATCTTACCTGGGTTAATAGATGATTTAAAGGATGAACTTTCTA
AAATGGTGGAAACAAAAATCTTACCTGAGTTAATAGATGATTTAAAGGATGAACTTTCTA
TAGAAGAGATTGAAAGGATTCGTCAATCTTTGGAAGAAAAGGAAAATCAATTAATTGAAG
TAGAAGAGATTGAAAGGATTCGTCAATCTTTGGAAGAAAAGGAAAATCAATTAATTGAAG
CGATTGACCAAACCGAAACAGTAGAAGAACGAAAATTACTTCGTAAAGAAAAATCTGAAG
CGATTGACCAAACCGAAACAGTAGAAGAACGAAAATTACTTCGTAAAGAAAAATCTGAAG

```
A.4     TTCATAAGCAGAAGAAGCGATTCGATGATTTTGCCCAAAGAAAAATGAGCTACGAAGAAC
mecI    ------------------------------------------------------------
A.3     TTCATAAGCAGAAGAAGCGATTCGATGATTTTGCCCAAAGAAAAATGAGCTACGAAGAAC
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     AATTAGTGATTATGGGTGTACGCAATAGTTTCTCAAAGACGGATCATGACGCTACTTTTA
mecI    ------------------------------------------------------------
A.3     AATTAGTGATTATGGGTGTACGCAATAGTTTCTCAAAGACGGATCATGACGCTACTTTTA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     TGCGAATGAAAGAAGATCACATGCTTAATGGCCAACTAAAACCAGGTTATAATATTCAAC
mecI    ------------------------------------------------------------
A.3     TGCGAATGAAAGAAGATCACATGCTTAATGGCCAACTAAAACCAGGTTATAATATTCAAC
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     TTGCGACAGAAAATCAATTTGCTCTTGCTTATGATTGTTATCCAAATCCAACAGATACAC
mecI    ------------------------------------------------------------
A.3     TTGCGACAGAAAATCAATTTGCTCTTGCTTATGATTGTTATCCAAATCCAACAGATACAC
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     GAACATTTATCCCATTTCTTGAAAAGATTGAATCAAAAATAGGTTTACCAGAAAATATTG
mecI    ------------------------------------------------------------
A.3     GAACATTTATCCCATTTCTTGAAAAGATTGAATCAAAAATAGGTTTACCAGAAAATATTG
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     TCGCAGATGCCGGTTATGCAAGTGAAGAAAATTATTGTTATGTGTTAGACGAAACAGAAT
mecI    ------------------------------------------------------------
A.3     TCGCAGATGCCGGTTATGCAAGTGAAGAAAATTATTGTTATGTGTTAGACGAAACAGAAT
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     CCACCCCCATCATTCCGCATCAAGGTTACTTAAAAGAGAAGAAACGCGCCCATAAACAAA
mecI    ------------------------------------------------------------
A.3     CCACCCCCATCATTCCGCATCAAGGTTACTTAAAAGAGAAGAAACGCGCCCATAAACAAA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     ATCAGTTTCATCGAGATAATTGGACTTACAACGAGTTGGATGATTACTATATTTGTCCCA
mecI    ------------------------------------------------------------
A.3     ATCAGTTTCATCGAGATAATTGGACTTACAACGAGTTGGATGATTACTATATTTGTCCCA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     ATCAGAAAAGAGTAGTGTTCTCACATTATTCACAACGTAAAGATAGAAATGGATTTATTC
mecI    ------------------------------------------------------------
A.3     ATCAGAAAAGAGTAGTGTTCTCACATTATTCACAACGTAAAGATAGAAATGGATTTATTC
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     GAAACTTCAAAATCTATGAATGTGAAGATTGTACAGGATGTCCATTGAGAGAGAAATGTA
mecI    ------------------------------------------------------------
A.3     GAAACTTCAAAATCTATGAATGTGAAGATTGTACAGGATGTCCATTGAGAGAGAAATGTA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     CCAAAGCAAAAGAAG-GAAAAATCGACGTTTGAATATTAATCCAACCTGGGAATATATAA
mecI    ------------------------------------------------------------
A.3     CCAAAGCAAAAGAAGGAAAAAATCGACGTTTGAATATTAATCCAACCTGGGAATATATAA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     AAATAAAAGCGAATGAAAAGATAGAAAGCAAAGAAACAGCCTCATACTATGCGCAGCGAA
mecI    ------------------------------------------------------------
A.3     AAATAAAAGCGAATGAAAAGATAGAAAGCAAAGAAACAGCCTCATACTATGCGCAGCGAA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     AGATAGATGTCGAAACGGTTTTTGGAAACATTAAACAAAATATGAAATTTAAAAGATTCC
mecI    ------------------------------------------------------------
A.3     AGATAGATGTCGAAACAGTTTTTGGAAACATTAAACAAAATATGAAATTTAAAAGATTCC
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     ATGTCCGAGGAGCTGAAAAAATTTTTAAGGAAATGGGACTTGTATTTCTTGCGCACAATT
mecI    ------------------------------------------------------------
A.3     ATGTCCGAGGAGCTGAAAAAATTTTTAAGGAAATGGGACTTGTATTTCTTGCGCACAATT
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

A.4     TTAGGAAATTAGTAACTAGAGTAAGGAAATATGAGGGAAAAACAATCATACAAAACCAAA
mecI    ------------------------------------------------------------
A.3     TTAGGAAATTAGTAACTAGAGTAAGGAAATATGAGGGAAAAACAATCATACAAAACCAAA
        ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^
```


A.4 TATAGGGGGAAAATCCTCTATATTTCATCCAAAAAAAACAAAAATCGAAGACATCTTTCA
mecI ----------
A.3 TATAGGGGGAAAATCCTCTATATTTCATCCAAAAAAAACAAAAATCGAAGACATCTTTCA
^^^

```
HVII     TTATGAATTATTAATAAGTGCTGTTACTTCTCCCTTAAATACAATTTCTTCATTTTCATT
HVIIE    TTATGAATTATTAATAAGTGCTGTTACTTCTCCCTTAAATACAATTTCTTCATTTTCATT
         ************************************************************

HVII     GTATGTTGAAAGTGACACTGTAACGAGTCCATTTTCTTTTTTTATGGATTTCTTATTTGT
HVIIE    GTATGTTGAAAGTGACACTGTAACGAGTCCATTTTCTTTTTTTATGGATTTCTTATTTGT
         ************************************************************

HVII     AATTTCAGCGATAACGTACAATGTATTACCTGGGTATACAGGTTTAATAAATTTAACGTT
HVIIE    AATTTCAGCGATAACGTACAATGTATTACCTGGGTATACAGGTTTAATAAATTTAACGTT
         ************************************************************

HVII     ATTCATTTGTGTTCCTGCTACAACTTCTTCTCCGTATTTACCTTCTTCTACCCATAATTT
HVIIE    ATTCATTTGTGTTCCTGCTACAACTTCTTCTCCGTATTTACCTTCTTCTACCCATAATTT
         ************************************************************

HVII     AAATGATATTGAAAGTGTATGCATGCCAGATGCAATGATACCTTTAAATCTACTTTGTTC
HVIIE    AAATGATATTGAAAGTGTATGCATGCCAGATGCAATGATACCTTTAAATCTACTTTGTTC
         ************************************************************

HVII     TGCTTTTTCTTTATCTATATGCATATATTGAGGATCAAAAGTTGTTGCAAATTGGATAAT
HVIIE    TGCTTTTTCTTTATCTATATGCATATATTGAGGATCAAAAGTTGTTGCAAATTGGATAAT
         ************************************************************

HVII     TTCTTCTTCTGTAATATGAAGGCTTTTTGTTTTGAATGTTTCTCCTACTATAAAATCATC
HVIIE    TTCTTCTTCTGTAATATGAAGGCTTTTTGTTTTGAATGTTTCTCCTACTATAAAATCATC
         ************************************************************

HVII     GTATTTCATATATGTCTCTCTTTCTTATTCAAATTAATTTTTTAGTATGTAACATGTTAA
HVIIE    GTATTTCATATATGTCTCTCTTTCTTATTCAAATTAATTTTTTAGTATGTAACATGTTAA
         ************************************************************

HVII     AGGTAAGTCTACCGTCACTGAAACGTAAGACTCACCTCTAACTTTCTATTGAGACAAATG
HVIIE    AGGTAAGTCTACCGTCACTGAAACGTAAGACTCACCTCTAACTTTCTATTGAGACAAATG
         ************************************************************

HVII     CACCATTTTATCTGCATTGTCTGTAAAGATACCATCAACTCCCCAATTAGCAAGTTGGTT
HVIIE    CACCATTTTATCTGCATTGTCTGTAAAGATACCATCAACTCCCCAATTAGCAAGTTGGTT
         ************************************************************

HVII     TGCACGTGCTGGTTTGTTTACAGTCCATACGTTCAATTCATAACCCGCTTCTTTTACCAT
HVIIE    TGCACGTGCTGGTTTGTTTACAGTCCATACGTTCAATTCATAACCCGCTTCTTTTACCAT
         ************************************************************

HVII     TTTTACTTTTGCTTTAGTAAGTTTGGCATCTTCAGTGTTTACTATTTTAGCATTACAGTA
HVIIE    TTTTACTTTTGCTTTAGTAAGTTTGGCATCTTCAGTGTTTACTATTTTAGCATTACAGTA
         ************************************************************

HVII     ATCTAAAAGTGTTCTCCAGTCTTCACGAAACGAAGTTGTATGGAATATAACTGCTCTGTT
HVIIE    ATCTAAAAGTGTTCTCCAGTCTTCACGAAACGAAGTTGTATGGAATATAACTGCTCTGTT
         ************************************************************

HVII     ATATTGTGGCATGATTTCTTCTGCAAGTTTAACAAGCACAACATTAAAGCTTGAAATGAG
HVIIE    ATATTGTGGCATGATTTCTTCTGCAAGTTTAACAAGCACAACATTAAAGCTTGAAATGAG
         ************************************************************

HVII     CACTTCTTGATTCTGATTTAAGTTTGTTAATTGTTCTTCCACTTGCTTAACCATACTTTT
HVIIE    CACTTCTTGATTCTGATTTAAGTTTGTTAATTGTTCTTCCACTTGCTTAACCATACTTTT
         ************************************************************

HVII     AGAAAGTGCTAGTCCATTCGGTCCAGTAATACCTTTTAATTCTACATTTAAATTCATATT
HVIIE    AGAAAGTGCTAGTCCATTCGGTCCAGTAATACCTTTTAATTCTACATTTAAATTCATATT
         ************************************************************

HVII     ATATTCATTTGCTATTTTTACTACATCATCGAAAGTTGGCAAATGTTCATCTTTGAATTT
HVIIE    ATATTCATTTGCTATTTTTACTACATCATCGAAAGTTGGCAAATGTTCATCTTTGAATTT
         ************************************************************

HVII     TTCACCAAACCAAGATCCTGCAGAAGCATCTTTAATTTCATCATAATTCAATTCAGTTAT
HVIIE    TTCACCAAACCAAGATCCTGCAGAAGCATCTTTAATTTCATCATAATTCAATTCAGTTAT
         ************************************************************

HVII     TTCCCCGGACATATTTGTAGTCCGTTCTAAATAATCATCATGAATGATAATCAGTTGTTC
HVIIE    TTCCCCGGACATATTTGTAGTCCGTTCTAAATAATCATCATGAATGATAATCAGTTGTTC
         ************************************************************

HVII     ATCTTTTGTAATTGCAACATCTAACTCCAACCAGTTTATACCTTCTACTTCTGAAGCAGC
HVIIE    ATCTTTTGTAATTGCAACATCTAACTCCAACCAGTTTATACCTTCTACTTCTGAAGCAGC
         ************************************************************
```

```
HVII            TTTAAATGATGCAATTGTATTTTCCGGAGCTTTACTAGGTAATCCTCTATGTCCATATAC
HVIIE           TTTAAATGATGCAATTGTATTTTCCGGAGCTTTACTAGGTAATCCTCTATGTCCATATAC
                ************************************************************

HVII            AGTTAGCATATTACCTCTCCTTGCATTTTTATTTTTTTAATTAACGTAACTGTATTATCA
HVIIE           AGTTAGCATATTACCTCTCCTTGCATTTTTATTTTTTTAATTAACGTAACTGTATTATCA
                ************************************************************

HVII            CATTAATCGCACTTTTATTTCCATTAAAAAGAGATGAATATCATAAATAAAGAAGTCGAT
HVIIE           CATTAATCGCACTTTTATTTCCATTAAAAAGAGATGAATATCATAAATAAAGAAGTCGAT
                ************************************************************

HVII            AGATTCGTATTGATTATGGAGTTAATCTACGTCTCATCTCATTTTTAAAAAATCATTTAT
HVIIE           AGATTCGTATTGATTATGGAGTTAATCTACGTCTCATCTCATTTTTAAAAAATCATTTAT
                ************************************************************

HVII            GTCCCAAGCTCCATTTTGTAATCAAGTCTAGTTTTTCTGTACCCCTTATCTGCAATTTTA
HVIIE           GTCCCAAGCTCCATTTTGTAATCAAGTCTAGTTTTT-----------------------
                ************************************

HVII            CTTAGGATTGCTTTTAACTTACCTCTTATCTGCAATTTTACTTAGGATTGCTTTTAACTT
HVIIE           ------------------------------------------------------------

HVII            ACCCCTTATCAGCAATTTTACTGAGAACTGCTTTTAACGCACCTCTTATCTGCAATTTTG
HVIIE           ------------------------------------------------------------

HVII            CCTAGAACTGCTTTTAACGTACCTCTTATCTGCAATTTTACTTAGAACTGCTTTTAACGT
HVIIE           ------------------------------------------------------------

HVII            ACCTCTTATCTGTAATTTTACTGAGAACTGCTTTTAACAAACCTCTTATCTGCAATTTTA
HVIIE           ------------------------------------------------------------

HVII            CTTAGAACTGCTTTTAACAAACCTCTTATCTGCAATTTTACTTAGAACTGCTTTTAACAA
HVIIE           ------------------------------------------------------------

HVII            ACCTCTTATCTGCAATTTTACTTAGAATTGCTTTTACTATTCCTCTTATTAGTATAATCT
HVIIE           ------------------------------------------------------------

HVII            CAGTAAGAATGCGTATAAAAATGAAAATTACAACCGATTTTGTAAGTGCTGACGCCTGAG
HVIIE           ------------------------------------------------------------

HVII            GGAATAGTATGTGCGAGAGACTAATGGCTCGAGCCATACCCCTAGGCAAGCATGCACGTA
HVIIE           ------------------------------------------------------------

HVII            CAAAATCGTAAGATAAAAAAATAAGCATATCACTGTAAACTTTAAAAAATCAGTTTAGTG
HVIIE           ------------------------------------------------------------

HVII            ATATGCTTATTTATTTCGAGTTAGGATTTATGTCCCAAGCTCATCAAGCACAATCGGCCA
HVIIE           ------------------------------------------------------------

HVII            CTAGTTTATTTCTCTATCTTATATGTTCTGATATGGTCTTCTATACTGTATAAGTATACT
HVIIE           ------------------------------------------------------------

HVII            TTTGAATATGGATCTTGTGTCAATTCACGTTCGAAATCAAATTCTTGATTATCAAATCTG
HVIIE           ------------------------------------------------------------

HVII            TTAAAGAATGTTTCGTATTCTTCGACTGATAATTGCTCTCTAGATTCTAGCATATTTAAG
HVIIE           ------------------------------------------------------------

HVII            TGTTTCTCTTTATCTAATGCTTTGTCATATCCTTTAACGATTGAACCACTAAAGATTTCT
HVIIE           ------------------------------------------------------------

HVII            CCTACTGCTCCTGAACCATAACTAAATAGACATACTTTCTCTTCTGGTTGGAATGTGTGG
HVIIE           ------------------------------------------------------------
```

```
HVII        TTCTGTAATAACGAAATTAAACTTAAGTATAATGATCCTGTATAAATGTTACCAACATCT
HVIIE       ------------------------------------------------------------

HVII        CTATTCCATAATA
HVIIE       -------------
```

**FIGURE 4.7.** Alignment of the nucleotide sequences of the hypervariable region (region between *mecA* and IS*431mec* of SCC*mec*) of SCC*mec* II (HVII) and SCC*mec* IIE (HVIIE) generated using the ClustalW sequence alignment program (Higgins & Sharp, 1988). The nucleotide sequence for the HV region of SCC*mec* II was obtained from the SCC*mec* II nucleotide sequence in the Genbank database under accession number D86934 (Ito *et al.*, 1999). The nucleotide sequence of the HV region of SCC*mec* IIE was identified as part of this present study during the complete sequencing of the novel variant SCC*mec* element of MRSA isolate AR13.1/3330.2 and is available from the Genbank database under accession number AJ810120 (SCC*mec* IIE from MRSA isolate AR13.1/3330.2). The HV region SCC*mec* IIE is 997 bp shorter than that of SCC*mec* II.

*indicates identical residues

---indicates no alignment.

multiplex primers that amplify the region between IS*431mec* and pUB110 of SCC*mec* II (IS431P4, pUB110R1; see chapter 3, Table 3.5) did not produce any amplimer for this isolate because the SCC*mec* element of AR13.1/3330.2 does not harbour pUB110 (Fig. 4.3).

The extreme right of the SCC*mec* element of AR13.1/3330.2 from IS*431* to the right chromosomal-SCC*mec* junction (I-R region) showed 100% homology with the region spanning from the second IS*431* sequence to the right junction of SCC*mec* II (Table 4.5). The 15-bp direct repeat sequence characteristic of SCC*mec* elements was identified at the right extremity of SCC*mec* of AR13.1/3330.2 (DR-R) and a 26-bp inverted repeat sequence (IR-R) that were identical to that of SCC*mec* IVb and SCC*mec* II was also found. All SCC*mec* elements identified to date have been found to be integrated at exactly the same nucleotide position of an ORF of unknown function designated *orfX*. This ORF was identified outside the IR-R region and SCC*mec* of AR13.1/3330.2 was integrated at the same nucleotide position of *orfX* as other SCC*mec* elements.

The novel genomic structure of the *mec* complex of the SCC*mec* element of AR13.1/3330.2 with the genomic organisation of IS*1182-ΔmecI-mecR1-mecA*-IS*431mec* was more similar to the class A *mec* complex of MRSA (*mecI-mecR1-mecA*-IS*431mec*) found in SCC*mec* II and SCC*mec* III than to the class B *mec* complex (IS*1272-ΔmecR1-mecA*-IS*431*mec) of SCC*mec* I and IV (Fig. 4.5). Due to its similiarity to the class A *mec* complexes, this novel *mec* complex was designated class A.3 *mec* (Fig. 4.5).

*4.3.3.2 Novel variant of SCCmec IV identified in ST8 isolate AR43/3330.1*

A 23 kb SCC*mec* element was identified in isolate AR43/3330.1, which is within the size range (20-25 kb) of previously described SCC*mec* IV elements, but sequence data indicted that it was distinctly different. This novel SCC*mec* variant was designated SCC*mec* IVE (Fig. 4.3). The genomic structure of this SCC*mec* element showed most similarity to SCC*mec* IVc (Fig. 4.4).

Amplification of the L-C region of SCC*mec* of AR43/3330.1 yielded an amplimer of ca. 6.1 kb which is the expected size for a type IVc SCC*mec* element (Table 4.4). Sequencing revealed that the L-C region of AR43/3330.1 had 100% similiarity with the L-C region of SCC*mec* IVc (Table 4.6). Amplification of the region from the *ccr*

**TABLE 4.6.** Percentage identities between ORFs of the novel SCC*mec* IV variant element IVE of Irish MRSA isolate AR43/3330.1 and the conventional SCC*mec* IVc element

| SCC*mec* element and ORF | Location[a] | Homology to ORFs of SCC*mec* IVc[b] | | |
|---|---|---|---|---|
| **AR43/3330.1 (IVE)** | | **ORF** | **% homology[c]** | **Gene product** |
| **L-C region** | | | | |
| IVE01 | 66-866 (complement) | CR007 | 100% | Hypothetical protein |
| IVE02 | 896-2050 (complement) | CR008 | 100% | Hypothetical protein |
| IVE03 | 2505-3551 | R002 | 100% | Hypothetical protein |
| IVE04 | 3744-4040 | R003 | 100% | Hypothetical protein |
| **C-M region** | | | | |
| **ccr complex** | | | | |
| IVE05 | 4040-5833 | R004 | 100% | Hypothetical protein |
| IVE06 (*ccrA2*) | 6067-7416 | R005 | 99.1% | Cassette chromosome recombinase A2 |
| IVE07 (*ccrB2*) | 7438-9051 | R006 | 99.4% | Cassette chromosome recombinase B2 |
| IVE08 | 9573-9923 | R007 | 100% | Hypothetical protein |
| IVE09 | 10010-10321 | R008 | 100% | Hypothetical protein |
| IVE10 | 10333-10842 | R009 | 100% | Hypothetical protein |
| IVE11 | 10978-12159 | R010 | 100% | Hypothetical protein |
| IVE12 | 12146-12502 | R011 | 100% | Hypothetical protein |
| **M-I region** | | | | |
| ***mec* complex** | | | | |
| IVE13 | 12493-12822 (complement) | CR009 | 100% | Putataive transposase of IS*1272* |
| IVE14 | 12725-13711 (complement) | CR010 (*ΔmecR1*) | 100% | Truncated signal transducer protein MecR1 |
| IVE15 | 13811-15817 | R012 (*mecA*) | 100% | Penicillin binding protein 2a |
| IVE16 | 16388-17131 (complement) | CR011 | 100% | Hypothetical protein |
| IVE17 | 18432-19106 | R013 | 100% | Transpoase of IS*431mec* |
| **I-R region** | | | | |
| IVE18 | 20286-20792 | ..... | ..... | Hypothetical protein |
| IVE19 | 20995-21576 | ..... | ..... | Hypothetical protein |
| IVE20 | 22117-22590 | ..... | ..... | Hypothetical protein |

[a]The nucleotide positions were determined based on the sequences for SCC*mec* IVE deposited in the Genebank database under accession number AJ810121

[b]ORFs and gene products from SCC*mec* IVc nucleotide sequence in the Genebank database, accession number AB096217 (Ito *et al.*, 2001).

[c]% homology based on amino acid identity

..... no homology

complex to IS*431mec* yielded amplimers that showed almost 100% sequence identity with the same regions of SCC*mec* IVc (Table 4.6). The *ccrA and ccrB* genes of AR43/3330.1 showed 99.1% and 99.4% similarity, respectively to those of the type 2 *ccr* complex of SCC*mec* IVc. A class B *mec* complex was identified (Fig. 4.4) consisting of ΨIS*1272-mecR1-mecA*-IS*431mec* as described previously for SCC*mec* I, IVa, IVb and IVc but SCC*mec* of AR43/3330.1 had an I-R region different from that of any previously described SCC*mec* element. An amplimer of 4.7 kb was obtained following amplification of this region which is smaller than what would be observed for SCC*mec* IVc (Table 4.4). Sequence analysis identified that IS*431mec* was present but that a sequence of ca. 4.4 kb was identified between IS*431mec* and the end of *orfX*. Searches of the Genbank database identified that part of this sequence (ca. 1.2 kb) had 100% homology to a sequence of the right extremity of the SCC*mec* element previously identified in two other MRSA isolates (Huletsky *et al.*, 2004). This sequence in the Genbank database consists of *orfX* and the extreme right region of the SCC*mec* element including the direct repeat (DR-R) and inverted repeat (IR-R) regions. The rest of the sequence between IS*431mec* and IR-R identified in AR43/3330.1 were found to have no homology to any sequences in the Genbank database. This explains why the multiplex primers that amplify *dcs* of the I-R region of SCC*mec* IV (DCSF2, DCSR1; see chapter 3, Table 3.5) failed to produce any amplimer for this isolate because the SCC*mec* element of AR43/3330.1 has a novel I-R region that lacks *dcs* (Fig. 4.4; Table 4.6)

*4.3.3.3 Determination of the genomic organisation of the SCCmec elements of the remaining Irish MRSA ST8 isolates with unusual SCCmec II and SCCmec IV multiplex patterns*

In total 74 Irish MRSA isolates had the ST8 genotype and gave variant SCC*mec* II and IV multiplex patterns (Table 4.1). In addition to determining the nucleotide structure of the SCC*mec* elements of two of these isolates as described above (section 4.3.3.1 and 4.3.3.2), the SCC*mec* elements of the remaining 72 isolates were characterized by analysing the mobility of amplimers on agarose gels obtained with the overlapping primers used to amplify and sequence the SCC*mec* elements from isolates AR13.1/3330.2 and AR43/3330.1. In some cases, sequencing was performed on amplimers that differed in size from that expected from previously described SCC*mec*

elements. These results are summarised in Table 4.4 and Table 4.7. This analysis identified five variant SCC*mec* elements, in addition to the two identified above by complete nucleotide sequencing. Of the seven variants recognised, five SCC*mec* II variants (designated SCC*mec* IIA, IIB, IIC, IID and IIE) were identified among the 54 ST8 isolates (AR05, AR13, AR14, New01 and New03) with variant SCC*mec* II multiplex patterns and two novel SCC*mec* IV variants (designated SCC*mec* IVE and IVF) were found among the 20 ST8 isolates (AR43) with the variant SCC*mec* IV multiplex pattern (Table 4.7). A schematic representation of all seven novel SCC*mec* variants is shown in Fig. 4.4.

*4.3.3.3.1 Additional novel variants of SCCmec II*

Significant size variation was observed in the mobility of amplimers obtained from isolates with variant SCC*mec* II elements compared to the sizes expected from isolates with typical SCC*mec* II when the following three regions were amplified: L-C region, Tn*554* to the *mec* complex (part of C-M region), and across plasmid pUB110 (part of I-R region) (Table 4.4). Amplification of other regions showed no significant variation from the expected amplimer size for an SCC*mec* II element (Table 4.4).

The 54 SCC*mec* II variants yielded the 5.6 kb fragment characteristic of the L-C region of SCC*mec* IVb (Table 4.4, Fig.4.4). No amplimer obtained following amplification of the region from Tn*554* to *mecR1* in the SCC*mec* II variants matched the size expected from a typical SCC*mec* II element (Table 4.4). All but one isolate (AR05) with the multiplex pattern SCC*mec* II – *kdp* (n=9) and all isolates with the multiplex pattern SCC*mec* II – *kdp* and pUB110 (n=27) produced amplimers of ca. 13 kb (Table 4.4). This 13 kb region was sequenced from one of these isolates and it was found that IS*1182* was inserted within the *mecI* gene, near the 3' end and that the *mecI* gene harboured a 16 bp deletion (Figs. 4.5 and 4.6). This new class A *mec* complex consisting of *ΔmecI*-IS*1182*-*ΔmecI*-*mecR1*-*mecA*-IS*431mec* was designated *mec* class A.4 (Fig. 4.5)

The Tn*554*-*mecR1* region of one isolate (AR05) with the multiplex pattern SCC*mec* II – *kdp* could not be amplified with the same primers used on the other SCC*mec* II variants (Table 4.4). Instead, a combination of primers ccrA F and mecR1 R were used to amplify the DNA region from the *ccr* complex to *mecR1* resulting in a ca. 11.5 kb amplimer (Table 4.4). Sequencing data revealed that this isolate retained only a few

**TABLE 4.7.** Summary of the variant SCC*mec* elements identified in Irish nosocomial MRSA isolates recovered between 1971-2004

| ST | SCC*mec* typing Simplex | Multiplex | Isolate phenotype[a] | Years | SCC*mec* variant name[b] | SCC*mec* variant description Differs from the indicated SCC*mec* type as follows: |
|---|---|---|---|---|---|---|
| | | | | | | **SCC*mec* II:** |
| 8 | II | II – *kdp* | AR05 | '89 | IIB | SCC*mec* IVb L-C region & lacks Tn*554* |
| 8 | II | II – *kdp* | AR13, AR14 | '93,'98,'99 | IIA | SCC*mec* IVb L-C region; class A.4 *mec*[c] |
| 8 | II | II – *kdp* & *mecI* | AR14 | '93,'98,'99 | IIC | SCC*mec* IVb L-C region; class A.3 *mec*[c]; lacks ORFs between Tn*554* & *mec* complex |
| 8 | II | II - *kdp* & pUB110 | AR13, AR14, New01, New03 | '98,'99,'02 | IID | SCC*mec* IVb L-C region; class A.4 *mec*[c]; lacks pUB110 & second copy of IS*431* |
| 8 | II | II – *kdp*, *mecI* & pUB110/ IV | AR13, AR14 | '98,'99,'02 | IIE | SCC*mec* IVb L-C region; class A.3 *mec*[c]; lacks pUB110, second copy of IS*431* & ORFs between Tn*554* & *mec* complex |
| | | | | | | **SCC*mec* IVc:** |
| 8 | IV | IV - *dcs* | AR43 | '99 | IVE | lacks *dcs*; has an unusual sequence in I-R region |
| | | | | | | **SCC*mec* IVb:** |
| 8 | IV | IV - *dcs* | AR43 | '99 | IVF | lacks *dcs*; has an unusual sequence in I-R region |
| | | | | | | **SCC*mec* III:** |
| 239 | III | III – pI258/Tn*554* | AR09 Phenotype-III Unfamiliar-3 | '85-'87,'89,'03 | IIIC | lacks six ORFs between *mer* & Tn554 |
| 250 | I | I - *pls* | AR02 Phenotype-II | '76-'84,'89 | I - *pls* | ND[d] |

[a]For details of the isolate number of each isolate with each variant SCC*mec* element see Table 3.7 and 3.9 (Chapter 3). Out of the 20 AR43 isolates with the SCC*mec* IV – *dcs* multiplex pattern, three (AR43/3246, AR43/3402 and AR43/3636.1) had the novel SCC*mec* IVF element and the other 17 had the novel SCC*mec* IVE element.

[b]SCC*mec* name assigned during the present study.

[c]These two variants of *mec* complex class A (designated A.3 and A.4) were recognized during the present study (Fig. 4.4 & 4.5).

[d]Not determined

bases of the integrated transposon Tn*554* normally found in SCC*mec* II and that all Tn*554* ORFs were absent. However, the six ORFs usually found between Tn*554* and the *mec* complex of SCC*mec* II were all present (SCC*mec* IIB, Fig. 4.4). This isolate also harboured a class A *mec* complex (Fig. 4.3). Amplification of the same region in isolates with the multiplex pattern SCC*mec* II – *kdp* and *mecI* (n=10) and SCC*mec* II – *kdp*, *mecI* and pUB110/IV (n=7) yielded an amplimer of the same size as the amplimer obtained and sequenced from AR13.1/3330.2 (Table 4.4) in which the region between Tn*554* and the *mec* complex was absent but which carried *mec* class A.3 *mec* complex. All isolates with the SCC*mec* II – *kdp* (n=10) and SCC*mec* II – *kdp* and *mecI* (n=10) harbored the integrated plasmid pUB110 and the second copy of IS*431* found in SCC*mec* II (Table 4.7 and Fig. 4.4). Isolates with multiplex patterns SCC*mec* II – *kdp* and pUB110 (n=27) and II – *kdp*, *mecI* and pUB110/IV (n=7) gave the same size amplimer as that obtained from AR13.1/3330.2 for amplification of the region across pUB110 (Table 4.4) indicating the absence of pUB110 and the second copy of the IS*431* sequence.

In conclusion, five novel SCC*mec* II variants were identified (Table 4.7; Fig. 4.4). All except one SCC*mec* II - *kdp* isolate were found to harbour a novel SCC*mec* variant IIA which has the SCC*mec* IVb L-C region and the novel class A.4 *mec* complex (Table 4.7; Fig. 4.4). The remaining SCC*mec* II - *kdp* isolate (AR05) carried the novel SCC*mec* II variant IIB which also had the SCC*mec* IVb L-C region but had the previously described class A *mec* complex of SCC*mec* II (Table 4.7; Fig. 4.4). All SCC*mec* II – *kdp* & *mecI* isolates harboured the novel variant SCC*mec* IIC, which consists of the SCC*mec* IVb L-C region and the novel class A.3 *mec* complex and lacks ORFs between Tn*554* and the *mec* complex (Table 4.7; Fig. 4.4). The SCC*mec* II – *kdp* & pUB110 isolates carried the novel SCC*mec* IID element which had the SCC*mec* IVb L-C region, the novel class A.4 *mec* complex and the integrated plasmid pUB110 and associated insertion sequence IS*431* are absent (Table 4.7; Fig. 4.4). Finally, all SCC*mec* II – *kdp*, *mecI* & pUB110 isolates harboured the novel SCC*mec* variant IIE which consists of the SCC*mec* IVb L-C region and the novel class A.3 *mec* complex, and lack a ca. 6 kb sequence of DNA between Tn*554* and the *mec* complex and pUB110 and IS*431* (Table 4.7; Fig. 4.4).

*4.3.3.3.2 Additional novel variants of SCCmec IV*

All 20 isolates with the AR43 phenotype, which were found to carry SCC*mec* IV by the simplex method but gave the multiplex pattern SCC*mec* IV - *dcs*, produced the expected size products for a type IVc SCC*mec* element when the primers used on AR43/3330.1 were used except for amplification of the L-C and I-R regions (Table 4.4). Seventeen of the isolates had the same L-C region as SCC*mec* IVc (6.1 kb amplimer) but three isolates (AR43/3246, AR43/3402 and AR43/3636.1) gave a 5.6 kb product (Table 4.4). Sequencing of this amplimer from one AR43 isolate with the 5.6 kb product (AR43/3636.1) confirmed that it contained the SCC*mec* IVb L-C region. As was identified in AR43/3330.1 (section 4.3.3.2), an amplimer that was larger than expected was obtained when the I-R region was amplified in all AR43 isolates (Table 4.4). This 4.7 kb region from an AR43 isolate with an L-C region similar to SCC*mec* IVb (AR43/3636.1) and was found to be 100% identical to that of AR43/3330.1.

In conclusion, two novel SCC*mec* IV variants SCC*mec* IVE and SCC*mec* IVF were identified among isolates with the multiplex pattern SCC*mec* IV – *dcs* and have closest similarity to SCC*mec* IVc and SCC*mec* IVb, respectively, but both have the novel I-R region (Table 4.7; Fig. 4.4).

### 4.3.2 Investigation of the SCC*mec* elements harboured by ST239 isolates with unusual multiplex pattern SCC*mec* I –pI258/Tn*554*

Four isolates with the ST239 genotype exhibited the variant multiplex pattern SCC*mec* III – pI258/Tn*554* (Table 4.1). It was therefore decided that the SCC*mec* element harboured by these isolates should be further investigated. Primers were designed to amplify the region between pI258 to Tn*554* (pI28 F, Tn554 R3; Table 4.3; Fig. 4.2). These primers amplify a ca. 9.3 kb region of SCC*mec* III including partial regions of both of pI258 and Tn*554* and the region between these two integrated elements (Fig. 4.2). Using these primers the region from pI258 to Tn*554* was amplified from all four of these ST239 isolates and they all yielded a ca. 6.3 kb fragment which is 3 kb smaller than the amplimer expected for a type III SCC*mec* element (expected size 9.3 kb). This 6.3 kb amplimer was sequenced from one isolate (09/0065) and it was found to be similar to the same region of SCC*mec* III but a ca. 3 kb region encoding hypothetical proteins that is normally found between pI258 and Tn*554* of SCC*mec* III was absent from the SCC*mec* element of this isolate (Tables 4.7 & 4.8).

**TABLE 4.8.** Percentage identities between ORFs investigated between pI258 and Tn*554* of the novel SCC*mec* III variant element IIIC of Irish MRSA isolate AR09/0065 and the conventional SCC*mec* III element.

| ORFs of SCC*mec* IIID | Location | ORFs of SCC*mec* III | Location | % nucleotide homology | % amino acid homology | Gene product |
|---|---|---|---|---|---|---|
| **pI258 (*mer*)** | | | | | | |
| IIIX40 (partial *merR*)* | 1-1174 (complement) | CZ048 (*mer*R) | 42217-43566 (complement) | 99.8% | 35% | Regulatory protein |
| **IS431** | | | | | | |
| IIIX58 | 1454-2128 | Z058 | 43846-44520 | 100% | 100% | Putative transposase of IS*431* |
| **Region between IS*431* & Tn*554*** | | | | | | |
| ..... | ..... | CZ049 | 44632-44967 (complement) | ..... | ..... | Hypothetical protein |
| ..... | ..... | CZ050 | 45340-45642 (complement) | ..... | ..... | Hypothetical protein |
| ..... | ..... | CZ052 | 46216-46464 (complement) | ..... | ..... | Hypothetical protein |
| ..... | ..... | CZ053 | 46873-47196 (complement) | ..... | ..... | Hypothetical protein |
| ..... | ..... | Z060 | 46888-47190 | ..... | ..... | Hypothetical protein |
| ..... | ..... | CZ054 | 47590-47832 (complement) | ..... | ..... | Hypothetical protein |
| IIID55 (pseudogene) | 2416-2865 | CZ055 | 47760-48248 (complement) | 70% | 69% | Hypothetical protein |
| IIID56 | 2882-3409 | CZ056 | 48263-48792 (complement) | 99% | 98% | Hypothetical protein |
| IIID57 | 3544-4032 | CZ057 | 48927-49415 (complement) | 86% | 95% | Hypothetical protein |
| IIID62 | 3671-3814 | Z062 | 49054-49197 | 64% | 85% | Hypothetical protein |
| IIID58 | 4048-4356 | CZ058 | 49431-49739 (complement) | 95% | 99% | Hypothetical protein |
| IIID59 | 4395-5267 | CZ059 | 49778-50650 (complement) | 97% | 95% | Hypothetical protein |
| **Tn*554*** | | | | | | |
| IIID60* | 5587-6323 | CZ060 | 51198-51767 (complement) | 100% | 100% | Hypothetical protein |

* The forward primer (pI258 F) designed in this present study for amplification and sequencing of the variant region of the SCC*mec* III – pI258/Tn*554* element was within the last ORF (*merR*) of the *mer* operon so only part of *merR* was amplified and sequenced. Similarily, the reverse primer (Tn554 R3) was within the first ORF of Tn*554* so only part of Tn*554* was amplified and sequenced.

"....." indicates no significant homology

Within this 6.3 kb amplimer part of the integrated plasmid pI258 (part of the *merR* gene) of SCC*mec* III and the associated insertion sequence IS*431* were identified (Table 4.8). The part of the *merR* gene that was sequenced was found to have a nucleotide substitution and a base pair deletion that resulted in a premature stop codon when the sequence was analysed at the amino acid level. The IS*431* sequence flanking pI258 was found to have 100% homology to that of SCC*mec* III (Table 4.8). Of the 12 ORFs encoding hypothetical proteins previously identified between pI258 and Tn*554* of SCC*mec* III, the first six were absent in the 6.3 kb fragment amplified from isolate AR09/0065 (Table 4.8). The six ORFs that were identified between pI258 and Tn*554* of SCC*mec* of this isolate showed degrees of homology at the amino acid level ranging from 69% to 99% to other ORFs found between pI258 and Tn*554* of SCC*mec* III (Table 4.8). A portion of Tn*554* was identified adjacent to these six ORFs at the same location as it is found in SCC*mec* III (Table 4.8). The part of Tn*554* that was amplified and sequenced was found to be 100% homologous to that of SCC*mec* III (Table 4.8). This novel variant of SCC*mec* III that was partially sequenced in this present study was termed SCC*mec* IIIC (Table 4.7).

The primer binding sites for the primers used in the multiplex SCC*mec* typing method that amplify the SCC*mec* III region between pI258 and Tn*554* (RIF4F3, RIF4R9; see chapter 3, Table 3.5) are within this 3 kb region of DNA that is absent from the SCC*mec* IIIC element of the four ST239 isolates, thus explaining why these four isolates are missing the multiplex amplimer corresponding to the amplification of this region.

### 4.3.3 Investigation of the SCC*mec* elements harboured by ST250 isolates with the unusual multiplex pattern SCC*mec* I – *pls*

Nine isolates with the ST250 genotype exhibited the variant multiplex pattern SCC*mec* I – *pls* (Table 4.1). It was therefore decided that the SCC*mec* element harboured by these isolates should be further investigated. A primer pair (E023 F2, CEOI7 R2; Table 4.3; Fig. 4.3) was designed to amplify across the part of the L-C region of SCC*mec* I where the multiplex primers failed to bind (Fig. 4.3). Using these primers the expected size product for a type I SCC*mec* element (2.4 kb) was obtained for the control isolate that exhibiting SCC*mec* type I by both SCC*mec* typing methods while the nine SCC*mec* I – *pls* failed to yield any amplimers. Subsequently, primer pair CEO15 F/EO25F was designed further away from the multiplex primer binding sites of

SCC*mec* I than E023 F2/CEOI7 R2 in an attempt to amplify across this region (Table 4.3; Fig. 4.3). This primer pair also failed to produce any amplimers for the SCC*mec* I – *pls* isolates while the control isolate yielded the expected size amplimer of 4.5 kb for a type I SCC*mec* element. Another primer pair (CEO10/CCRA R3) was then designed further away again from the multiplex primer binding sites than either of the first two primer pairs. This primer pair also failed to yield any amplimers for SCC*mec* I – *pls* isolates while the control isolate produced the expected size amplimer for a type I SCC*mec* element (10 kb).

As part of this present study the type I *ccr* complex had been shown to be present in all SCC*mec* I – *pls* isolates using the simplex SCC*mec* typing method (see chapter 3). In all SCC*mec* element described to date in MRSA a contiguous region of DNA consisting of both a direct repeat (DR-L)(part of the bacterial chromosome) and an inverted repeat (IR-L)(part of SCC*mec*), that shows a high level of sequence homology between the different SCC*mec* elements, has been found at the left chromosomal/SCC*mec* junction (Ito *et al.*, 1999; Ma *et al.*, 2002; Ito *et al.*, 2004). Based on this, primer pair IRLI F1/CCRA R3 (Table 4.3; Fig. 4.3) was designed to amplify from within the DR-L/IR-L region of SCC*mec* (Ito *et al.*, 1999; Ma *et al.*, 2002; Ito *et al.*, 2004) to the *ccrA* gene of SCC*mec* I. Surprisingly, all SCC*mec* I - *pls* isolates failed to yield any amplimers using this primer pair while the control isolates again gave the expected size amplimer of 20 kb. Subsequently the primer pair IRLIF2/CCRA R4 was designed to amplify from a region outside of the left junction of SCC*mec* I, common to both SCC*mec* I and SCC*mec* II to the *ccr* complex (Table 4.3; Fig. 4.3). Again an amplimer was only obtained for the control isolate (20 kb).

These unusual findings suggested that although the type I *ccr* complex was present in SCC*mec* I – *pls* isolates it may not be in the same location or direction of the SCC*mec* element as it is in all other SCC*mec* elements. Using this information primers were designed based on the type I *ccr* complex (CCRA R3; Table 4.3; Fig. 4.3) and right chromosomal region of SCC*mec* I (plsneg F; Table 4.3; Fig. 4.3) in order to investigate if some sort of rearrangement events had occurred in the SCC*mec* element in these SCC*mec* I – *pls* isolates resulting in a change in the position and perhaps direction of the *ccr* complex. Using this primer pair an amplimer of ca. 8 kb was obtained from the SCC*mec* I – *pls* isolate Phenotype II/120.1. No amplimers were obtained from the other eight SCC*mec* I – *pls* isolates. Several attempts were made to sequence the 8 kb amplimer from the Phenotype-II 120.1 isolate without success. When primers were

designed to sequence the fragment, they bound in more than one place, which resulted in mixed sequencing reactions and unreadable data. This may be due to the presence of repeat sequences within the 8 kb fragment. In the future, further attempts will be made to sequence this region of the SCC*mec* element and it may require PCR amplification of this region as small over-lapping fragments to isolate the repeats. In addition, the SCC*mec* elements of all other isolates exhibiting SCC*mec* I – *pls* may also require further investigation.

## 4.4 Discussion

In this present study, an extensive molecular analysis of the SCC*mec* elements harboured by MRSA isolates recovered in Irish hospitals between 1971 and 2004 has revealed that at least nine novel variants of the SCC*mec* elements I-IV are present in the chromosome of almost half of the isolates investigated (Table 4.7).

All isolates identified in this study with the ST8 genotype were found to harbour previously undescribed variants of SCC*mec* II (IIA–IIE) or SCC*mec* IV (IVE and IVF). It has been proposed that because both SCC*mec* I and IV have a *mec* complex with IS*1272* inserted at the same junction point, recombination has occurred between SCC*mec* I and other sequences to generate SCC*mec* type IV (Ma *et al.*, 2002). The novel ST8 SCC*mec* variants identified in the present study resemble rearrangements of SCC*mec* II and SCC*mec* IV. SCC*mec* variants IIA-IIE have an L-C region that is almost identical to that of SCC*mec* IVb, but the rest of each element has closest identity with SCC*mec* II including a very similar genomic structure (Fig. 4.4). In addition, the SCC*mec* variants IIA, IIC, IID and IIE have a *mec* complex with IS*1182* inserted at the same junction point (Figs. 4.4, 4.5 & 4.6).

SCC*mec* IIA-IIE appear to be closely related. SCC*mec* IIB was found in an isolate recovered in 1989 (AR05, Table 4.7) making it the earliest SCC*mec* II variant identified in the present study. It harbours the original SCC*mec* II class A *mec* complex but its similarity to SCC*mec* IVb in the L-C region make its provenance unclear (Fig. 4.4). It is unlikely that any of the SCC*mec* elements among the earlier isolates investigated in the present study are its precursor and further study is required to identify a possible ancestor. However, it is likely that SCC*mec* IIB derived from its putative ancestor following loss of part of Tn*554* because SCC*mec* IIB retains ~40 bp of Tn*554*. Acquisition of IS*1182* in the *mec* complex of this putative ancestor may have given rise to the novel variants of class A *mec*, A.3 and A.4 seen in SCC*mec* IIC (AR14 isolates) and IIE (AR13 & AR14 isolates) and SCC*mec* IIA (AR13 & AR14 isolates) and IID (AR13, AR14, New01 & New03 isolates), respectively (Figs. 4.4. & 4.5). Since SCC*mec* IIC and IIA were present in isolates in 1993 and SCC*mec* IIE and IID were not found in isolates until 1998 (Table 4.7), it is likely that the latter derived from the former following loss of pUB110 (Fig. 4.4).

The identification of the two novel *mec* complexes A.3 and A.4 in the novel variant SCC*mec* elements IIA, IIC, IID and IIE was a surprising and significant finding as only four classes of the *mec* complex have been described to date in MRSA isolates from around the world (Katayama *et al.*, 2001; Lim *et al.*, 2003; Ito *et al.*, 2004) (Fig. 4.5). Both class A.3 and A.4 *mec* harbour IS*1182* either adjacent to or within *mecI* and have deletions within *mec*I (Fig. 4.5). Mutations and deletions in *mecI* are common in clinical MRSA isolates, sometimes in association with insertion sequences because the expression of methicillin-resistance in *S. aureus* requires that the *mecA* repressor gene *mecI* is inactivated (Katayama *et al.*, 2001). The class B and class C *mec* complexes previously described in MRSA, have *mecI* and part of *mecR1* deleted and insertions of IS*1272* or IS*431* flanking the truncated end of *mecR1* (Katayama *et al.*, 2001; Fig. 4.4).

The insertion sequence element IS*1182* has been identified previously in *S. aureus* flanking the staphylococcal composite transposon Tn*5405* which encodes an aminogylcoside resistance determinant (Debrise *et al.*, 1996). The present study is the first to report the presence of IS*1182* within a *mec* complex in MRSA. A 16 bp deletion in *mec*I gene has occurred with no flanking target site duplications immediately adjacent to the insertion site of IS*1182* at the 3' end of *mecI* in the class A.4 *mec* complex. Insertion sequences capable of promoting various types of genome rearrangements, including deletions, have been reported previously (Mahillion *et al.*, 1999).

Other workers have reported possible rearrangements in the L-C regions of other SCC*mec* elements. Based on a multiplex SCC*mec* typing method, Wielders *et al.* (2003) reported a type I SCC*mec* element carrying the *kdp* region (normally part of L-C region of SCC*mec* II only) and also an SCC*mec* IV element with *pls* (normally part of L-C region of SCC*mec* I only). Various different SCC*mec* IV elements have been described with diverse L-C regions (Ito *et al.*, 2003; Ma *et al.*, 2002). The integrated plasmid pUB110 normally found in SCC*mec* II encodes resistance to kanamycin, tobramycin and bleomycin (Ito *et al.*, 1999). In the present study all isolates with SCC*mec* IID and IIE were resistant to kanamycin and tobramycin (Rossney & Keane, 2002a; Rossney *et al.*, 2003; Rossney *et al.*, 2004), although they lacked pUB110 in their SCC*mec* elements, indicating that resistance determinants for these aminoglycosides were present elsewhere on the chromosome and pUB110 may have been lost because it no longer benefited the host cell. Similarly Tn*554*, which encodes resistance to erythromycin and spectinomycin, may have been lost from a putative ancestor resulting

in SCC*mec* IIB because it was no longer beneficial for the bacterial cell to retain them, as resistance genes for these antimicrobials were also present elsewhere on the chromosome. Loss or gain of plasmids and transposons such as pUB110 and Tn*554* in SCC*mec* has been documented previously; SCC*mec* IA is believed to have evolved from SCC*mec* I by acquisition of pUB110 or *vice versa* (Oliveira *et al.*, 2001c), SCC*mec* IIIA is thought to have evolved from SCC*mec* following loss of various transposons and plasmids including Tn*554*, SCC*mec* IVA as described by Oliveira and de Lencastre (2002a) harbours pUB110, Wielders *et al.* (2003) described pUB110 associated with SCC*mec* III and SCC*mec* IV and Tn*554* associated with SCC*mec* IV, Shulka *et al.* (2004) reported both a type II SCC*mec* element missing pUB110 and a type II SCC*mec* element missing *mecI* and pUB110.

SCC*mec* IIA-IIE (40-27 kb) are smaller than previously reported SCC*mec* II elements (57 kb) (Ito *et al.*, 1999) and SCC*mec* IIE with a size of 27 kb is more similar in size to SCC*mec* IV (20-25 kb) or SCC*mec* V (28 kb) (Ma *et al.*, 2002; Ito *et al.*, 2004; Fig. 4.4). It has been suggested that due to their relatively small size, SCC*mec* IV and SCC*mec* V are highly competitive mobile genetic elements that are more readily transferred between staphylococci than the larger SCC*mec* elements and once acquired by the host do not compromise its fitness (Ma *et al.*, 2002; Ito *et al.*, 2004). This could also be true for the SCC*mec* II variants described in this present study where the smaller size resulted from loss of regions that are not required for maintenance of methicillin resistance or precise excision and integration of SCC*mec*.

Like SCC*mec* IIA-IIE, the origin of the two ST8-MRSA-IV variants IVE and IVF (AR43 isolates) identified in this present study is unclear. SCC*mec* IVE is almost identical to SCC*mec* IVc except in the region downstream of the *mec* complex (Fig. 4.4) suggesting that SCC*mec* IVE may have arisen by recombination between an SCC*mec* IVc element and another SCC*mec* sequence that was the source of the unusual downstream region. The variant element SCC*mec* IVF has the same L-C region as the ST8 SCC*mec* II variants but the rest of the element was similar to SCC*mec* IVE (Fig. 4.4). SCC*mec* IVF may have evolved from genetic recombination between a type II SCC*mec* variant and SCC*mec* IVE which donated the rest of the element including the unusual downstream region. Alternatively, SCC*mec* IVF may have evolved from recombination between SCC*mec* IVb and SCC*mec* IVE. Recombination events in the *dcs* region appear to be common because other researchers have also reported the absence of *dcs* amplimers following multiplex PCR of SCC*mec* elements in various

genetic backgrounds (ST8 and ST30) (Aires de Sousa *et al.*, 2003a; Robinson & Enright, 2003). In addition, the *dcs* amplimer has been reported in SCC*mec* III although it does not usually carry the *dcs* sequence (Aires de Sousa *et al.*, 2003b; Wielders *et al.*, 2003). The *dcs* region is also absent in the previously described SCC*mec* IV variant IVc (Ito *et al.*, 2003).

Robinson and Enright (2003) constructed evolutionary models based on SCC*mec* typing and the partial sequences of 15 genes in order to predict the evolutionary pathways of decent of many MRSA lineages. Within CC8, ST8 isolates with the multiplex pattern SCC*mec* II – *kdp*, SCC*mec* II – *kdp*, *mecI* & pUB110/IV (n=3) and SCC*mec* IV – *dcs* (n=3) were identified, and because the structures of these SCC*mec* elements were unknown at that time, Robinson and Enright (2003) were unable the elucidate the evolutionary pathways leading to these MRSA clones. However, the present study has clarified the genomic structures of the SCC*mec* elements of isolates with these multiplex patterns and this has allowed us to predict at least part of the possible evolutionary pathways leading to these SCC*mec* variants within the ST8 clone.

The novel SCC*mec* III variant SCC*mec* IIIC was also identified in the present study (Table 4.7). Two variants of SCC*mec* III have been described previously (Oliveira *et al.*, 2001; see section 1.2.3.5). Since the type III SCC*mec* element has been identified from earlier MRSA isolates (1982) (MLST database & Oliveira *et al.*, 2001c) than either SCC*mec* IIIA (1993) (Oliveira *et al.*, 2001c), SCC*mec* IIIB (1993) (Oliveira *et al.*, 2001c) or SCC*mec* IIIC (1985) (this study), it is possible to speculate that SCC*mec* III is the ancestral SCC*mec* type from which the variants arose following loss of regions that were no longer required for survival of the host or SCC*mec*. The SCC*mec* IIIA element (Oliveira *et al.*, 2001c) may have arose from SCC*mec* III following loss of the integrated plasmid pT181 and its associated insertion sequence IS*431*. It is likely that the SCC*mec* IIIC element described in this present study evolved from SCC*mec* III following partial loss of a region of unknown function located between the *mer* operon and Tn*554* in SCC*mec* III, because SCC*mec* IIIC retains both partial and complete ORFs of this region found in SCC*mec* III (Table 4.8). The SCC*mec* IIIB element (Oliveira *et al.*, 2001c) may have evolved from either SCC*mec* III or IIIC by loss of Tn*554*, pT181, the *mer* operon, including the region between *mer* and Tn*554* and all associated insertion sequence elements.

Due to the problems encountered during amplification and sequencing, the variation observed in the SCC*mec* elements of nine isolates with the ST250 genotype and

SCC*mec* I – *pls* multiplex pattern still remains unclear. However, it is interesting to note that the *pls* gene which appears to be absent in these isolates is located within the L-C region of SCC*mec*, which is not thought to be an important part of SCC*mec*. Based on preliminary results it appears that extensive rearrangements may have occurred in the SCC*mec* elements of these isolates. Further work is underway to characterise these novel variants of SCC*mec* I.

In recent years, based on the multiplex SCC*mec* typing method, other researchers have also reported additional novel variants of SCC*mec* III that differ from the SCC*mec* III variant (IIIC) described in the present study. In 2003, Aires de Sousa & de Lencastre described a type III SCC*mec* element with the *dcs* amplimer (normally found in SCC*mec* I, II & IV), Robinson and Enirght described a SCC*mec* element with SCC*mec* III *ccr-mec* genes but with the SCC*mec* II multiplex pattern and Wielders *et al.* described a type III SCC*mec* element with the *pls*, pUB110 and *dcs* amplimers not usually found in SCC*mec* III. In 2004, Shukla *et al.* (2004b) described a multiplex pattern consisting of the *dcs* (normally found in SCC*mec* I, II & IV) and pI258/Tn*554* (normally found in SCC*mec* III only) amplimers only, which does not correspond to the multiplex pattern of any previously described SCC*mec* element.

The present study has shown that PCR amplification and sequencing of SCC*mec* elements was necessary for the complete characterisation of the SCC*mec* elements harboured by isolates with ambiguous multiplex patterns. Such detailed investigations are unsuitable for routine typing purposes. Furthermore, if SCC*mec* is to be used for routine epidemiological typing, some method of standardising nomenclature for the plethora of new and variant SCC*mec* types is needed. However it is clear from this and other studies that SCC*mec* elements can evolve rapidly and that simple PCR based methods for characterising SCC*mec* elements may need to be updated regularly to include important new variants.

# Chapter 5

# Prevalence of Serotype F Converting Phages Among Irish Nosocomial MRSA Isolates

## 5.1 Introduction

### 5.1.1 Serotype F phage of *S. aureus*

The ability of *S. aureus* to cause a range of infections is due in part to its ability to express a vast array of both cell surface-associated and secreted virulence factors. Some of these virulence factors are encoded by accessory genetic elements such as plasmids, transposons, pathogenicity islands and lysogenic bacteriophage. The majority of nosocomial *S. aureus* isolates, including MRSA, have previously been found to harbour serotype F phage which can simultaneously inactivate the chromosomally-located β-toxin gene (*hlb*) and introduce a novel gene encoding staphylokinase (*sak*) production, and sometimes in addition another novel gene, *entA*, encoding enterotoxin A production (Winkler *et al.*, 1965, Betley & Mekalanos, 1985, Coleman *et al.*, 1986, 1989 & 1991).

Beta haemolysin or β-toxin is a membrane-damaging toxin produced by *S. aureus* that degrades sphingomyelin in the outer phospholipid layer of the erythrocyte membrane (Mollby, 1978). Beta-toxin is rarely produced by clinical *S. aureus* isolates due to the carriage of lysogenic prophage that insertionally inactivate the β-toxin structural gene *hlb* during lysogenic conversion (Coleman *et al.*, 1986, 1989 & 1991). The *hlb* structural gene contains the bacterial attachment site, *attB*, for serotype F converting phages. Serotype F phages inactivate the β-toxin gene upon lysogenisation by site- and orientation-specific integration via the phage and bacterial attachment sites *attP* and *attB*, respectively, but they also encode the staphylokinase structural gene (Coleman *et al.*, 1989). Some serotype A phage cause loss of β-toxin activity alone (single-converting phage) upon lysogenisation (Coleman *et al.*, 1986).

Staphylokinase is an exoprotein produced by certain strains of *S. aureus* that activates host plasminogen, which results in degradation of fibrin (fibrinolyis) (Lahteenmaki *et al.*, 2001). This protein is an important factor in the pathogenesis of *S. aureus* as fibrinolysis is thought to aid in the dissemination of the pathogen by breaking down fibrin barriers surrounding the site of infection. Recently, staphylokinase has also been implicated in the neutralisation of defensins, which are peptides with antimicrobial activity produced by polymorphonuclear cells during the early innate immune response (Jin *et al.*, 2004). The genetic determinant for staphylokinase, *sak*, has been found to be carried by lysogenic phages (Kondo & Fujise, 1977; Kondo *et al.*, 1981; Coleman *et al.*, 1989). Some serotype B phage can give rise to a single conversion event following lysogenisation resulting in the acquisition of *sak* (Kondo & Fujise, 1977). Double

conversion of β-toxin (negative conversion) and staphylokinase (positive conversion) has been best characterised in the serotype F phage ϕ13 (Coleman *et al.*, 1989). In addition some serotype F phages can also encode the enterotoxin A structural gene *entA*, with lysogeny resulting in a triple conversion event (Betley & Mekalanos, 1985; Coleman *et al.*, 1989).

Enterotoxin A belongs to a large group of toxins known as superantigens (Betley *et al.*, 1990; Marrack & Kappler, 1990; Huber & Palmer, 1993). These toxins bind to class II major histocompatibility complex molecules on antigen-presenting cells and to specific variable regions of the T-cell antigen receptor (McCormick *et al.*, 2001). As a result of this, T cells are activated at orders of magnitude above antigen-specific activation, causing massive release of cytokines that is believed to be responsible for the most severe features of Toxic Shock Syndrome (TSS). Enterotoxin A can also cause staphylococcal food poisoning (Dinges *et al.*, 2000). The genetic determinant for enterotoxin A, *entA* has also been found to be carried by serotype F lysogenic converting phages, the best characterised of which is ϕ42 (Coleman *et al.*, 1989).

### 5.1.2 Aims

Serotype F phage are commonly found in nosocomial MRSA isolates. The aim of this part of the present study was to investigate the prevalence of double- and triple-converting serotype F phage, that encode the important *S. aureus* virulence factors enterotoxin A and staphylokinase, among MRSA isolates representative of the most predominant MRSA phenotypes and clonal types recovered in Irish hospitals between 1971 and 2002. The presence of double and triple-converting serotype F phage was identified by phenotypic detection of β-toxin, Sak and EntA production. Using these phenotypic protein assays a novel phenotype was identified in two MRSA isolates and in order to further investigate this novel phenotype, southern hybridisation and PCR analysis of the *entA*, *sak* and *hlb* structural genes of these isolates was performed.

## 5.2 Materials and Methods

### 5.2.1 *Staphylococcus aureus* isolates and strains

*5.2.1.1 MRSA isolates*

A total of 110 MRSA isolates were investigated in this part of the present study. All isolates recovered in 1999 were investigated because initially these were the only isolates available (Table 5.1; study period G). When other isolates became available for use in the present study, a single representative of each of the predominant phenotypes of MRSA recovered from patients in Irish hospitals during seven other study periods (A-F and H) between 1971 and 2002 were investigated (Table 5.1). The only exception was study period B (1976-1984), where Phenotype-I and Phenotype-II isolates predominated but only Phenotype-II isolates were available for use in the present study. These 110 isolates also included isolates recovered between 1989 and 2002 (study periods D-H respectively; Table 5.1) to which no AR type could be assigned because of the unusual AR or PFGE patterns they produced (ARNT, New01, New02 & New03) and an isolate from 2002 (study period H) with a relatively newly identified AR type, AR44 (Table 5.1). Where more than one clonal type (i.e. MLST/SCC*mec* type) was identified within a given phenotype (see Chapters 3 & 4 for full details for clonal types identified within each phenotype), an isolate representative of each clonal type within that phenotype was also investigated (Table 5.1).

*5.2.1.2 Staphylococcus aureus control strains*

The *S. aureus* strain 80CR3 is a phage-free β-toxin producing strain (Stobberingh & Winkler, 1977) that was used as a positive control in the extracellular protein assay to detect β-toxin and as a negative control in the extracellular protein assays to detect staphylokinase and enterotoxin A production.

Lysogenic derivatives of 80CR3 (such as 13CR3L) harbouring the double converting phage ϕ13, lose the ability to express β-toxin (negative conversion) by insertional inactivation of the chromosomal β-toxin gene *hlb* during lysogenisation and gain the ability to express the ϕ13 encoded staphylokinase gene *sak* (positive conversion) (Coleman *et al.*, 1989). The *S. aureus* phage ϕ13 was originally isolated from *S. aureus* strain NTCC8325 and then propagated on *S. aureus* strain 80CR3 yielding the lysogenic derivative 13CR3L (Norvick, 1967; Coleman *et al.*, 1986). The *S. aureus* lysogen

**TABLE 5.1. Clinical MRSA isolates that were investigated for the carriage of serotype F phage***

| Study period | Phenotype | Isolate no. | Clonal type[1] |
|---|---|---|---|
| A:1971-'75 | Early MRSA | 1612 | ST250-MRSA-I |
| B:1976-'84 | Phenotype-II | 43.2 | ST250-MRSA-I |
| | Phenotype-II | 161.1 | ST250-MRSA-I - *pls* |
| C:1985-'87 | Phenotype-III | 84 | ST239-MRSA-IIIC |
| D:1989 | AR01 | 0.1239 | ST239-MRSA-III |
| D:1989 | AR02 | 0.1206 | ST250-MRSA-I - *pls* |
| D:1989 | AR05 | 0.1345 | ST8-MRSA-IIB |
| D:1989 | AR06 | 0.1264 | ST22-MRSA-IV |
| D:1989 | AR07.4 | 0.1342 | ST5-MRSA-II |
| D:1989 | AR09 | 0.0064 | ST239-MRSA-IIIC |
| D:1989 | AR09 | 0.0065 | ST239-MSSA |
| D:1989 | ARNT | 0.1336 | ST30-MRSA-IV |
| E:1993 | ARNT | 0113 | ST34-MSSA |
| E:1993 | AR06 | 0251 | ST22-MRSA-IV |
| E:1993 | AR07.4 | 0159 | ST5-MRSA-II |
| E:1993 | AR11 | 0220 | ST5-MRSA-II |
| E:1993 | ARNT | 0242 | ST30-MRSA-IV |
| E:1993 | AR13 | 0132 | ST8-MRSA-IIA |
| E:1993 | AR14 | 0078 | ST8-MRSA-IIA |
| E:1993 | AR14 | 0246 | ST8-MRSA-IIC |
| E:1993 | AR15 | 0104 | ST239-MRSA-III |
| E:1993 | AR22 | 0308 | ST247-MRSA-IA |
| E:1993 | AR23 | 0073 | ST239-MRSA-III |
| F:1998 | AR06 | 2170 | ST22-MRSA-IV |
| F:1998 | AR07 | 2189 | ST36-MRSA-II |
| F:1998 | AR07.3 | 2160 | ST5-MRSA-II |
| F:1998 | AR13 | 2141 | ST8-MRSA-IIA |
| F:1998 | AR13 | 2179 | ST8-MRSA-IID |
| F:1998 | AR14 | 2027 | ST8-MRSA-IIC |
| F:1998 | AR14 | 2175 | ST8-MRSA-IIE |
| G:1999 | New01 | 3144 | ST8-MRSA-IID |
| G:1999 | New02 | 3581 | ST247-MRSA-IA |
| G:1999 | New03 | 3442 | ST8-MRSA-IID |
| G:1999 | AR06 | 3362 | ST22-MRSA-IV |
| G:1999 | AR06 | 3363 | ST22-MRSA-IV |
| G:1999 | AR06 | 3143 | ST22-MRSA-IV |
| G:1999 | AR06 | 3416 | ST22-MRSA-IV |
| G:1999 | AR06 | 3637 | ST22-MRSA-IV |
| G:1999 | AR06 | 3190 | ST22-MRSA-IV |
| G:1999 | AR06 | 3222 | ST22-MRSA-IV |
| G:1999 | AR06 | 3147 | ST22-MRSA-IV |
| G:1999 | AR06 | 3430 | ST22-MRSA-IV |
| G:1999 | AR06 | 3591 | ST22-MRSA-IV |
| G:1999 | AR07 | 3384 | ST36-MRSA-II |
| G:1999 | AR07 | 3558 | ST36-MRSA-II |
| G:1999 | AR07 | 3594 | ST36-MRSA-II |
| G:1999 | AR07 | 3142 | ST36-MRSA-II |
| G:1999 | AR07 | 3366 | ST36-MRSA-II |
| G:1999 | AR07 | 3228 | ST36-MRSA-II |
| G:1999 | AR07 | 3139 | ST36-MRSA-II |
| G:1999 | AR07 | 3572 | ST36-MRSA-II |
| G:1999 | AR07 | 3604 | ST36-MRSA-II |
| G:1999 | AR07.4 | 3188 | ST5-MRSA-II |
| G:1999 | AR07.4 | 3033 | ST5-MRSA-II |
| G:1999 | AR13 | 3137 | ST8-MRSA-IID |
| G:1999 | AR13 | 3008 | ST8-MRSA-IID |
| G:1999 | AR13 | 3053 | ST8-MRSA-IID |
| G:1999 | AR13 | 3114 | ST8-MRSA-IID |
| G:1999 | AR13 | 3698 | ST8-MRSA-IIA |
| G:1999 | AR13 | 3635.2 | ST8-MRSA-IID |
| G:1999 | AR13 | 3243.2 | ST8-MRSA-IID |
| G:1999 | AR13 | 3596 | ST8-MRSA-IID |

continued overleaf

13CR3L was used as a positive control in the staphylokinase assay and as a negative control in the β-toxin and enterotoxin A assays.

Lysogenic derivatives of 80CR3 (such as 42CR3L) harbouring the triple converting phage ϕ42, also lose the ability to express β-toxin (negative conversion) by insertional inactivation of the chromosomal β-toxin gene *hlb* during lysogenisation and gain the ability to express the ϕ42 encoded staphylokinase (*sak*) and enterotoxin A (*entA*) genes (positive conversion) (Coleman *et al.*, 1989). The *S. aureus* phage ϕ42 was originally isolated from *S. aureus* strain PS42D and then propagated on *S. aureus* strain 80CR3 yielding the lysogenic derivative 42CR3L (Coleman *et al.*, 1989). The *S. aureus* lysogen 42CR3L was used as a positive control in the enterotoxin A assay and as a negative control in the β-toxin assay.

### 5.2.2 Extracellular protein assays

*5.2.2.1 Plate assay for β-toxin*

Sheep Blood Agar (SBA) was used to test *S. aureus* isolates and control strains for production of β-toxin. Sheep blood agar consisted of TSA plates supplemented with 2% (v/v) fresh, packed, sheep erythrocytes which had been washed in Tris buffered Saline (TBS), (0.14 M NaCl, 20 mM Tris-HCl, pH 7.2) containing 10 mM $MgCl_2$ (Mollby, 1983). All *S. aureus* isolates and reference strains were streaked on SBA plates and a zone of clearing caused by partial lysis of the red blood cells around culture growth following incubation at 37°C for 18 h, indicated the production of β-toxin. This zone was enhanced in β-toxin positive isolates only when plates are stored at 4°C for 1 h. This was due to the "hot-cold" lysis action of β-toxin on red blood cells with a high sphingomyelin content, such as sheep erythrocytes. The β-toxin positive, phage free *S. aureus* strain 80CR3 (Stobberingh and Winkler, 1977) and the lysogen 13CR3L, a lysogenic derivative of 80CR3 harbouring the double-converting phage ϕ13 (Coleman *et al.*, 1989), were used as the positive and negative control, respectively for this assay (see section 5.2.1.2).

*5.2.2.2 Plate assay for staphylokinase*

Staphylokinase (Sak) is a plasminogen activator that is produced by certain *S. aureus* isolates. This extracellular protein can transform inactive host plasminogen to active plasmin resulting in fibrinolysis (Arvidson, 1983). Sak agar, in which canine

plasminogen is activated by staphylokinase resulting in degradation of fibrinogen, was used to test *S. aureus* isolates and strains for production of staphylokinase. Sak agar was prepared by dissolving 500 mg of fibrinogen (Sigma-Aldrich) slowly in 50 ml of sterile double strength nutrient broth (Oxoid). This was then heated to 55°C for 5 min prior to the addition of 50 ml of molten, sterile Difco TSA agar (3% w/v) (Difco, Becton Dickinson, Microbiology Systems, Sparks, MD, USA). The agar and broth were gently mixed by inversion and cooled to 40°C, followed by the addition of 500 µl of dog serum. The solution was gently mixed to avoid formation of bubbles and was poured into 90 mm diameter petri dishes and allowed to solidify. Sak agar plates without added dog serum were also prepared to distinguish staphylokinase activity from protease activity. *Staphylococcus aureus* isolates and control strains were streaked onto this freshly prepared turbid Sak agar, with and without dog serum, and incubated at 37°C for 18 h. On Sak agar containing dog serum a clear zone around the area of culture growth was seen around each isolate that produces staphylokinase, but this clear zone was absent around these same isolates on Sak agar without added dog serum. The phage free, staphylokinase negative, *S. aureus* strain 80CR3 (Stobberingh and Winkler, 1977) was used a negative control and its lysogenic derivative 13CR3L harbouring the double-converting phage ϕ13 (Coleman *et al.*, 1989) was used as a positive control for this assay (see section 5.2.1.2).

*5.2.2.3 Detection of enterotoxin A production*

Enterotoxin A (EntA) production by *S. aureus* isolates and control strains was detected by Reverse Passive Latex Agglutination (RPLA) using a kit purchased from Oxoid. This kit contains latex-bound antibodies specific to each of the staphylococcal enterotoxins A, B, C and D. In the presence of the corresponding enterotoxin agglutination of these latex particles occurs. A control reagent that consists of non-immune rabbit globulins was provided with the kit. The test was performed in V-well microtitre plates. *Staphylococcus aureus* cells were grown at 37°C for 12-16 h in TSB liquid medium and centrifuged at 900 x *g* for 20 min. Dilutions of the culture filtrate were made in the wells of the microtitre plate and the latex suspension of enterotoxin A was added to each well and the contents mixed. Agglutination occurred if staphylococcal enterotoxin A was present, resulting in the formation of a lattice structure that forms a diffuse layer on the base of the well. If staphylococcal enterotoxin A was absent no such

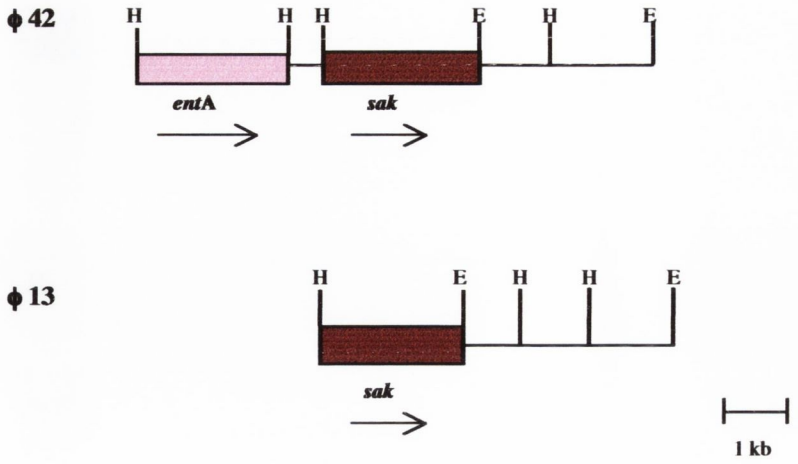



**FIGURE 5.1.** *Eco*RI and *Hind*III cleavage site maps of the *entA*-, *sak*-containing DNA regions of the triple converting phage ϕ 42 and the *sak* -containing DNA region of the double-converting phage ϕ 13 (Coleman *et al.*, 1989).

Arrows indicate the direction of the open reading frame corresponding to *entA* and *sak*.

Restriction endonuclease cleavage sites are as follows; H, *Hind*III; E, *Eco*RI.

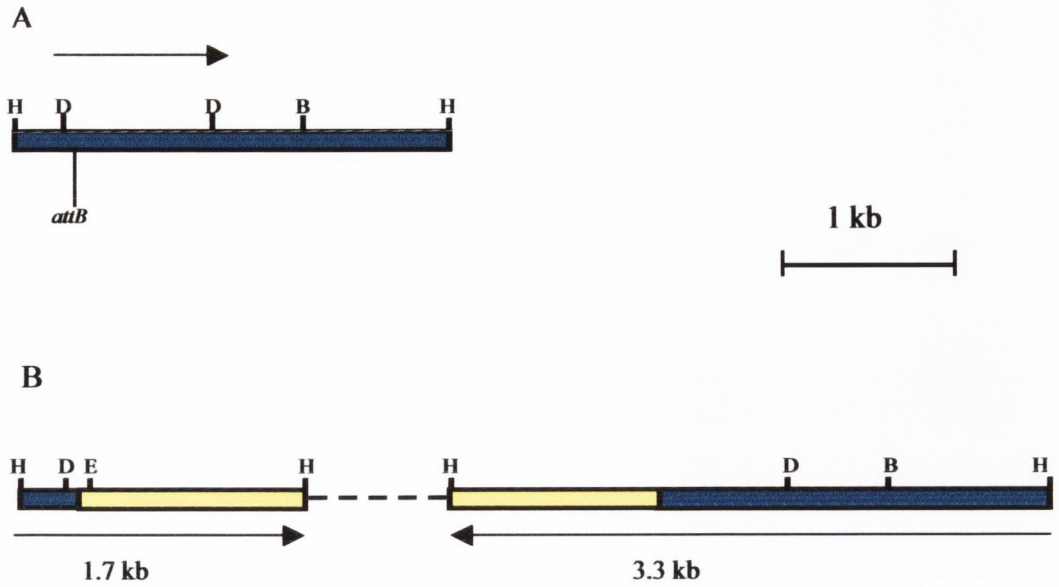



**FIGURE 5.2.** Restriction map of the 2.2 kb *Hind*III *hlb*-encoding fragment of plasmid pDC007 and the two *Hind*III fragments generated at the *S. aureus* chromosomal/ bacteriophage DNA junctions following lysogenisation with ϕ13

A: Restriction map of the 2.2 kb *Hind*III *hlb*-encoding fragment of plasmid pDC007, cloned from *S. aureus* strain CN6708 (Coleman *et al.*, 1986,1989 & 1991) and used as a probe to detect the *hlb* gene in *S. aureus* strains and isolates in this present study. The arrow indicates the position and direction of the open reading frame corresponding to *hlb*. In unlysogenised *S. aureus* strains this gene is intact and the *Hind*III fragment containing the *hlb* gene is 2.2 kb. The attachment site (*attB*) of the phage is located at the 5' end of the *hlb* gene.

B: Restriction map of the two *Hind*III fragments generated at the *S. aureus* chromosomal/ bacteriophage DNA junctions following lysogenisation with ϕ13 (Coleman *et al.*, 1991). The chromosomally located 2.2 kb *hlb*-containing *Hind*III fragment (Panel A) has been split in two by the integration of ϕ13 DNA with the ϕ13 linear genome separating the two disrupted *hlb* portions. Arrows indicate the *Hind*III fragments detected in lysogenised strains when hybridised with the 2.2 kb *Hind*III *hlb*-encoding fragment of plasmid pDC007. The blue portion of the figure indicates the *Hind*III *hlb*-encoding fragment from bacterial DNA and the yellow portion of the figure indicates the bacteriophage DNA that disrupts the *hlb* gene and splits it in two. The linear ϕ13 genome separating the two composite *Hind*III junction fragments is represented by a broken black line.

Restriction endonuclease cleavage sites are as follows; H, *Hind*III; D, *Dde*I; B, *Bam*HI ;E, *Eco*RI.

*hlb* gene sequence (Fig. 5.2). The smaller of the two bands contains only a short sequence homologous with the probe (Fig. 5.2) and therefore produces a weaker hybridisation signal.

### 5.2.4 PCR detection of *sak* and *ent A* genes

The *entA* and *sak* structural genes were detected using PCR with *Taq* DNA polymerase (Promega) as described in section 2.4.2. and the oligonucleotide primers shown in Table 5.2 and Fig. 5.3. For all the primers described, reactions were carried out with 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C and extension for 1 min at 72°C, followed by a final incubation at 72°C for 10 min. Following PCR amplification 5 μl of each reaction product was electrophorsed on 1% (w/v) agarose gels as described in section 2.3.2.

### 5.3.5 Cloning of the *sak* gene

Purified PCR products were cloned into pBluescript II KS(-) phagemid and sequenced. Ligations of PCR products to pBluescript were facilitated by the presence of appropriate restriction endonuclease cleavage sites within the primers as indicated in Table 5.2. Ligation reactions were carried out as described in section 2.4.3. Transformation of competent *E. coli* DH5α prepared using $CaCl_2$ and identification of recombinants using blue-white selection was performed as described in section 2.4.4. DNA sequencing was performed on a commercial basis by Lark as described in section 2.5 and analysis of chromatograms and sequences were carried out also as described previously in section 2.5.

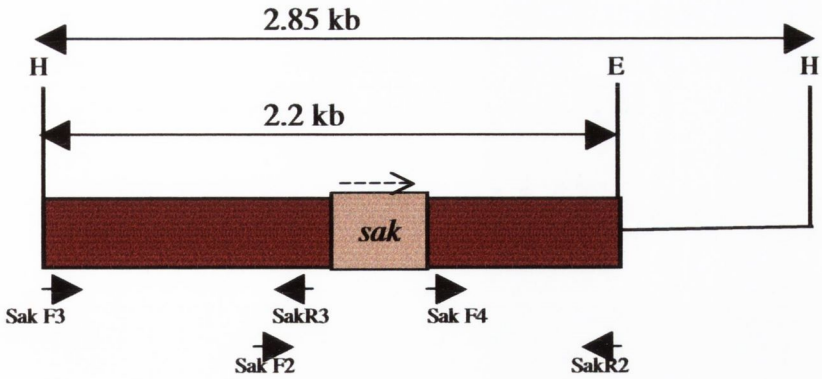



**FIGURE 5.3.** Map of the 2.2 kb *sak* encoding *Hind*III/*Eco*RI DNA region and 2.85 kb *Hind*III DNA region of ϕ13 (Coleman *et al.*, 1989). The lighter colour box represents the *sak* gene and the darker rectangle represents the *Hind*III/*Eco*RI *sak*-encoding fragment. Arrowheads indicate the positions of primer sequences used in this study (see also table 5.2). The broken arrow indicates the direction of the *sak* open reading frame.

Restriction endonuclease cleavage sites are as follows; H, *Hind*III; E, *Eco*RI.

## 5.3 Results

### 5.3.1 β-toxin (Hlb), staphylokinase (Sak) and enterotoxin A (EntA) production by Irish MRSA isolates

A total of 110 MRSA isolates representative of the most predominant phenotypes and clonal types of MRSA recovered from patients in Irish hospitals between 1971 and 2002 (see section 5.2.1.1) were investigated for production of Hlb, Sak and EntA (Table 5.3). The vast majority of isolates, 106/110 (96%), did not produce β-toxin (Table 5.3). Forty-seven isolates (43%) were negative for both Hlb and EntA production but were positive for Sak production (Table 5.3). This Hlb$^-$ Sak$^+$, EntA$^-$ phenotype is considered to be indicative of the presence of lysogenic double-converting serotype F phage (Coleman *et al.*, 1989; Dempsey, 1996). Fifty-six isolates (51%) were negative for Hlb production but were positive for the production of both Sak and EntA (Table 5.3). This Hlb$^-$ Sak$^+$, EntA$^+$ phenotype is considered to be indicative of the presence of lysogenic triple-converting serotype F phage.

One isolate from study period G (1999; Table 5.3) was negative for the production of Hlb, Sak and EntA, a phenotype that is known to be generated by Hlb converting serotype A phage. Two isolates from 1999 were found to be negative for Hlb and Sak production but positive for the production of EntA (Table 5.3). This Hlb$^-$ Sak$^-$, EntA$^+$ phenotype has not previously been reported among clinical MRSA isolates.

### 5.3.2 Molecular investigation into the Hlb$^-$, Sak$^-$, Ent A$^+$ phenotype expressed by two Irish nosocomial MRSA isolates

In the present study two MRSA isolates, AR14/3685 (clonal type ST8-MRSA-IIC) and AR14/3448 (clonal type ST8-MRSA-IIE) from study period G (1999) exhibited the Hlb$^-$ Sak$^-$, EntA$^+$ phenotype (Table 5.3). In order to further investigate this novel phenotype, southern hybridisation and PCR analysis of the *entA*, *sak* and *hlb* structural genes of these isolates was performed.

#### *5.3.2.1 Southern hybridisation analysis of the entA, hlb, and sak structural genes*

##### *5.3.2.1.1 The entA structural gene*

*Hind*III-digested chromosomal DNA from the two MRSA isolates with the Hlb$^-$, Sak$^-$, EntA$^+$ phenotype produced a hybridisation signal at 2.5 kb, when probed with the *Hind*III *entA*-encoding probe (Fig. 5.4, panel A). This is the expected band of homology

**TABLE 5.3. β-toxin (Hlb), staphylokinase (Sak) and enterotoxin A (EntA) production by Irish nosocomial MRSA isolates recovered between 1971 and 2002.**

| Study period | Phenotype | Isolate number | Clonal type[1] | Hlb[2] | Sak[2] | EntA[2] |
|---|---|---|---|---|---|---|
| A:1971-'75 | Early MRSA | 1612 | ST250-MRSA-I | - | + | - |
| B:1976-'84 | Phenotype-II | 43.2 | ST250-MRSA-I | - | + | - |
| | | 161.1 | ST250-MRSA-I - *pls* | - | + | - |
| C:1985-'87 | Phenotype-III | 84 | ST239-MRSA-IIIC | - | + | + |
| D:1989 | AR01 | 0.1239 | ST239-MRSA-III | - | + | + |
| D:1989 | AR02 | 0.1206 | ST250-MRSA-I - *pls* | - | + | - |
| D:1989 | AR05 | 0.1345 | ST8-MRSA-IIB | - | + | + |
| D:1989 | AR06 | 0.1264 | ST22-MRSA-IV | - | + | + |
| D:1989 | AR07.4 | 0.1342 | ST5-MRSA-II | - | + | - |
| D:1989 | AR09 | 0.0064 | ST239-MRSA-IIIC | - | + | + |
| D:1989 | AR09 | 0.0065 | ST239-MSSA | - | + | + |
| D:1989 | ARNT | 0.1336 | ST30-MRSA-IV | - | + | - |
| E:1993 | ARNT | 0113 | ST34-MSSA | - | + | - |
| E:1993 | AR06 | 0251 | ST22-MRSA-IV | - | + | + |
| E:1993 | AR07.4 | 0159 | ST5-MRSA-II | - | + | - |
| E:1993 | AR11 | 0220 | ST5-MRSA-II | - | + | - |
| E:1993 | ARNT | 0242 | ST30-MRSA-IV | - | + | - |
| E:1993 | AR13 | 0132 | ST8-MRSA-IIA | - | + | + |
| E:1993 | AR14 | 0078 | ST8-MRSA-IIA | - | + | + |
| E:1993 | AR14 | 0246 | ST8-MRSA-IIC | - | + | + |
| E:1993 | AR15 | 0104 | ST239-MRSA-III | - | + | + |
| E:1993 | AR22 | 0308 | ST247-MRSA-IA | - | + | - |
| E:1993 | AR23 | 0073 | ST239-MRSA-III | - | + | + |
| F:1998 | AR06 | 2170 | ST22-MRSA-IV | - | + | - |
| F:1998 | AR07 | 2189 | ST36-MRSA-II | - | + | + |
| F:1998 | AR07.3 | 2160 | ST5-MRSA-II | - | + | - |
| F:1998 | AR13 | 2141 | ST8-MRSA-IIA | - | + | + |
| F:1998 | AR13 | 2179 | ST8-MRSA-IID | - | + | + |
| F:1998 | AR14 | 2027 | ST8-MRSA-IIC | - | + | + |
| F:1998 | AR14 | 2175 | ST8-MRSA-IIE | - | + | + |
| G:1999 | New01 | 3144 | ST8-MRSA-IID | - | + | + |
| G:1999 | New02 | 3581 | ST247-MRSA-IA | - | + | + |
| G:1999 | New03 | 3442 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR06 | 3362 | ST22-MRSA-IV | - | + | - |
| G:1999 | AR06 | 3363 | ST22-MRSA-IV | - | + | - |
| G:1999 | AR06 | 3143 | ND | - | + | - |
| G:1999 | AR06 | 3416 | ST22-MRSA-IV | + | - | - |
| G:1999 | AR06 | 3637 | ST22-MRSA-IV | - | + | - |
| G:1999 | AR06 | 3190 | ND | - | + | - |
| G:1999 | AR06 | 3222 | ST22-MRSA-IV | - | + | - |
| G:1999 | AR06 | 3147 | ND | - | + | - |
| G:1999 | AR06 | 3430 | ND | - | + | - |
| G:1999 | AR06 | 3591 | ST22-MRSA-IV | - | + | - |
| G:1999 | AR07 | 3384 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3558 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3594 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3142 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3366 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3228 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3139 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3572 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07 | 3604 | ST36-MRSA-II | - | + | + |
| G:1999 | AR07.4 | 3188 | ST5-MRSA-II | - | + | + |
| G:1999 | AR07.4 | 3033 | ST5-MRSA-II | - | + | + |
| G:1999 | AR13 | 3137 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3008 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3053 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3114 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3698 | ST8-MRSA-IIA | - | + | + |
| G:1999 | AR13 | 3635.2 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3243.2 | ST8-MRSA-IID | - | + | + |

continued overleaf

## TABLE 5.3. continued

| Study period | Phenotype | Isolate no. | Clonal type | Hlb | Sak | EntA |
|---|---|---|---|---|---|---|
| G:1999 | AR13 | 3596 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3017 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3439 | ST8-MRSA-IIA | - | + | + |
| G:1999 | AR13 | 3317.2 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3636.2 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3330.2 | ST8-MRSA-IIE | - | + | + |
| G:1999 | AR13 | 3258 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR13 | 3453 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR14 | 3045 | ST8-MRSA-IIC | + | - | - |
| G:1999 | AR14 | 3146 | ST8-MRSA-IIC | - | + | + |
| G:1999 | AR14 | 3225 | ST8-MRSA-IIE | - | + | + |
| G:1999 | AR14 | 3352 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR14 | 3132 | ST8-MRSA-IID | - | + | + |
| G:1999 | AR14 | 3369 | ST8-MRSA-IIC | + | - | - |
| G:1999 | AR14 | 3600 | ST8-MRSA-IIC | - | - | - |
| **G:1999** | **AR14** | **3685** | **ST8-MRSA-IIC** | **-** | **-** | **+** |
| G:1999 | AR14 | 3411 | ST8-MRSA-IIC | - | + | + |
| G:1999 | AR14 | 3176.1 | ST8-MRSA-IID | - | + | + |
| **G:1999** | **AR14** | **3448** | **ST8-MRSA-IIE** | **-** | **-** | **+** |
| G:1999 | AR43 | 3623 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3246 | ST8-MRSA-IVF | - | + | - |
| G:1999 | AR43 | 3310 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3402 | ST8-MRSA-IVF | - | + | - |
| G:1999 | AR43 | 3488 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3635.1 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3331 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3322 | ST8-MRSA-IVE | + | - | - |
| G:1999 | AR43 | 3317.1 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3316 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3625 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3330.1 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3337 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3665 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3243.1 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3328 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3636.1 | ST8-MRSA-IVF | - | + | - |
| G:1999 | AR43 | 3176.2 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3094 | ST8-MRSA-IVE | - | + | - |
| G:1999 | AR43 | 3487 | ST8-MRSA-IVE | - | + | - |
| | | | | | | |
| H:2002 | AR06 | E1164 | ST22-MRSA-IV | - | + | - |
| H:2002 | ARNT | E1185 | ST12-MRSA-IV | - | + | - |
| H:2002 | ARNT | E1113 | ST30-MRSA-IV | - | + | - |
| H:2002 | ARNT | E1139 | ST45-MRSA-IV | - | + | - |
| H:2002 | AR07 | E1100 | ST36-MRSA-II | - | + | - |
| H:2002 | AR07.2 | E1202 | ST496-MRSA-II | - | + | - |
| H:2002 | AR13 | E1140 | ST8-MRSA-IID | - | + | + |
| H:2002 | AR14 | E1134 | ST8-MRSA-IIE | - | + | + |
| H:2002 | AR44 | E1183 | ST239-MRSA-III | - | + | + |
| H:2002 | New03 | E1038 | ST8-MRSA-IID | - | + | + |

The two isolates from study period G with the novel Hlb$^-$, Sak$^-$, EntA$^+$ phenotype are indicated in bold text.

[1]The clonal types of isolates were identified as part of the present study (see Chapters 3 & 4). ND, clonal type not determined.

[2]-, negative for the production of the indicated protein; +, positive for the production of the indicated protein.

in an EntA$^+$ isolate (Coleman *et al.*, 1989; Fig. 5.4). The *S. aureus* laboratory strain 13CR3L (lysogenic derivative of 80CR3 harbouring the ϕ13 double converting phage) with the Hlb$^-$, Sak$^+$, EntA$^-$ phenotype, which does not produce enterotoxin A, produced a weak hybridisation signal at 2.3 kb when probed with this 2.5 kb *Hind*III *entA*-encoding probe (Fig. 5.4, panel A, lane 2). The nature of this homology has previously been investigated and was found to be due to a high level of sequence identity between sequences flanking the *entA* structural gene in ϕ42 and sequences in ϕ13 (Dempsey, 1996). No *entA* structural gene sequences were found in the homologous ϕ13 region (Dempsey, 1996).

*5.3.2.1.2 The hlb structural gene*

The *hlb* gene was found to be disrupted in both isolates (AR14/3448 and AR14/3685) with the Hlb$^-$, Sak$^-$, EntA$^+$ phenotype. Both isolates produced hybridisation signals at 3.3 kb and 1.7 kb when probed with the *Hind*III *hlb*-encoding probe (Fig. 5.4, panel B). The 1.7 kb band of homology had a characteristically weaker signal (Fig. 5.4, panel B). This is a common phenomenon in *S. aureus* isolates because serotype F phage insert into the *hlb* gene at the 5' end of the 2.2 kb *hlb*-encoding *Hind*III fragment resulting in one band of homology containing most of the *hlb* gene sequence and the other containing only a short sequence homologous with the probe and therefore producing a weaker signal (Coleman *et al.*, 1989 & 1991; Fig. 5.3). If the *hlb* gene was not disrupted and if the isolates contained no serotype F phage then a hybridisation signal at 2.2 kb similar to that observed in the phage-free isolate 80CR3 would be observed (Fig. 5.4).

*5.3.2.1.3 The sak structural gene*

Using the 3.1 kb *Hind*III *sak*-encoding probe the two isolates with the Hlb$^-$, Sak$^-$, Ent A$^+$ phenotype displayed two bands of homology, one band at approximately 3.2 kb and the other at approximately 1.7 kb (Fig. 5.4, panel C). All previously tested *S. aureus* isolates that carry the *sak* gene yielded hybridisation signals at either 3.1 kb or 2.85 kb when probed with the *Hind*III *sak*-encoding probe (Coleman *et al.*, 1989; Fig. 5.4). This result indicates that even though the two isolates, AR14/3448 and AR14/3685 were positive for the production EntA in the absence of Sak production, that the 3.1 kb

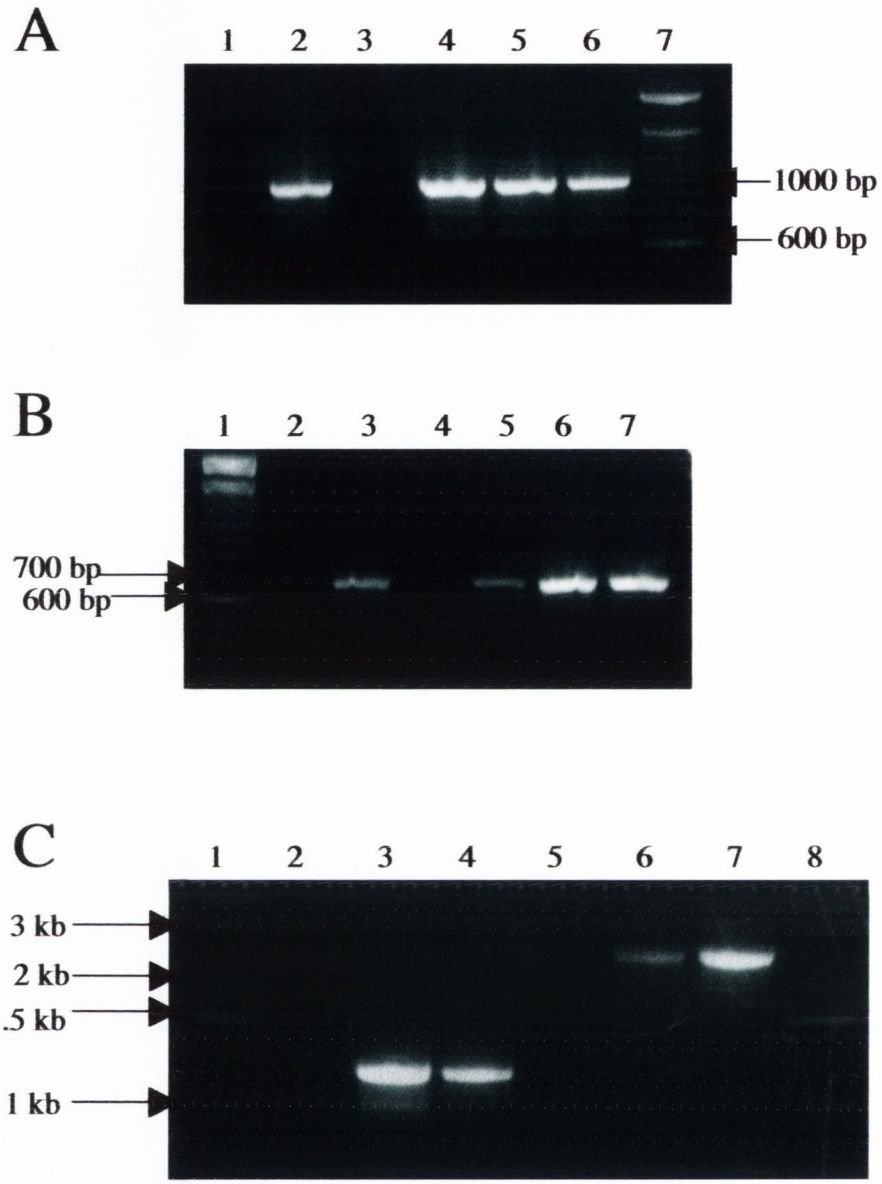

A
1 2 3 4 5 6 7
1000 bp
600 bp
B
1 2 3 4 5 6 7
700 bp
600 bp
C
1 2 3 4 5 6 7 8
3 kb
2 kb
1.5 kb
1 kb

**FIGURE 5.5.** PCR amplification products obtained using primers to amplify the *sak* gene in MRSA isolates AR14/3448 and AR14/3685 with the $Hlb^-$, $Sak^-$, $EntA^+$ phenotype, electrophoresed on 1% (w/v) agarose gels

**Panel A:** PCR amplification products obtained using the oligonuleotide primers SakF3 and SakR3 (Table 5.2 and Fig. 5.3). Lane 1, *S. aureus* phage free laboratory strain 80CR3; lane 2, *S. aureus* laboratory strain 13CR3L (lysogenic derivative of 80CR3 harbouring the double converting phage ϕ13); lane 3, no DNA control reaction; lane 4, MRSA isolate AR14/3448 ($Hlb^-$, $Sak^-$, $EntA^+$), lane 5, MRSA isolate AR14/3685 ($Hlb^-$, $Sak^-$, $EntA^+$); lane 6, MRSA isolate AR13/3137 ($Hlb^-$, $Sak^+$, $EntA^+$); lane 7, 100 bp DNA ladder.

**Panel B:** PCR amplification products obtained using the oligonuleotide primers SakF4 and SakR2 (Table 5.2 and Fig. 5.3). Lane 1, 100 bp DNA ladder; lane 2, *S. aureus* phage free laboratory strain 80CR3; lane 3, *S. aureus* laboratory strain 13CRL3 (lysogenic derivative of 80CR3 harbouring the double converting phage ϕ13); lane 4, no DNA control reaction; lane 5, MRSA isolate AR14/3448 ($Hlb^-$, $Sak^-$, $EntA^+$); lane 6, MRSA isolate AR14/3685 ($Hlb^-$, $Sak^-$, $EntA^+$); lane 7, MRSA isolate AR13/3137 ($Hlb^-$, $Sak^+$, $EntA^+$).

**Panel C:** PCR amplification products obtained using the oligonuleotide primers SakF2 and SakR2 (Table 5.2 and Fig. 5.3). Lane 1, 1 kb DNA ladder; lane 2, *S. aureus* phage free laboratory strain 80CR3: lane 3, *S. aureus* laboratory strain 13CRL3 (lysogenic derivative of 80CR3 harbouring the double converting phage ϕ13); lane 4, *S. aureus* laboratory strain 42CR3L (lysogenic derivative of 80CR3 harbouring the ϕ42 triple converting phage); lane 5, no DNA control reaction; lane 6, MRSA isolate AR14/3448 ($Hlb^-$, $Sak^-$, $EntA^+$); lane 7, MRSA isolate AR14/3685 ($Hlb^-$, $Sak^-$, $EntA^+$); lane 8, 1 kb DNA Ladder.

The nucleotide sequence of this fragment was analysed and it was found that a nucleotide sequence of 1324 bp had inserted within the *sak* structural gene (Figs. 5.6 & 5.7). When a search of the GenBank database was carried out this 1324 bp sequence was found to be 100% homologous to the insertion sequence element IS*256*. Phenotypic detection of Sak in these two isolates was not possible because even though the *sak* structural gene was present, IS*256* had inserted within the 3' end of the *sak* structural gene resulting in insertional inactivation of *sak* (Figs. 5.6 & 5.7). An 8 bp direct repeat sequence was found flanking the IS*256* sequence in these two isolates, one which forms part of the *sak* gene sequence and the other which may have been generated by duplication of this 8 bp sequence of the *sak* gene by IS*256* during transposition (Fig. 5.6).

A *Hind*III restriction endonuclease recognition site was also identified within IS*256* (Fig. 5.6) and this explains the results obtained from Southern analysis (Fig. 5.4. panel C) of *Hind*III digested DNA of the two Hlb$^-$, Sak$^-$, Ent A$^+$ isolates (Fig. 5.8). This *Hind*III site splits the *sak* gene in two (Fig. 5.8) thus two bands of homology, one at 1.7 kb and the other at 3.2 kb, were obtained when the *Hind*III *sak*-encoding DNA probe was used (Fig. 5.4, panel C).

ATGCTCAAAAGAAGTTTATTATTTTTAACTGTTTTATTGTTATTATTCTCATTTTCTTCAATTACTAATGAGGTAAGTGC
ATCAAGTTCATTCGACAAAGGAAAATATAAAAAAAGGCGATGACGCGAGTTATTTTGAACCAACAGGCCCGTATTTGA
TGGTAAATGTGACTGGAGTTGATGGTAAAGGAAATGAATTGCTATCCCCTCATTATGTCGAGTTTCCTATTAAACCTG
GGACTACACTTACAAAAGAAAAAATTGAATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGA
GTAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAATAAGAAAAAAGAAGAAACGAAGT
CTTTCCCTATAACAGAAAAAGGTTTTGTTGTCCcagattta*AGTCAAGTCCAGACTCCTGTGTAAAATGCTATACAATGTTTTT
ACCATTTCTACTTATCAAAATTGATGTATTTTCTTGAAGAATAAATCCATTCATCATGTAGGTCCATAAGAACGG
CTCCAATTAAGCGATTGGCTGATGTTTGATTGGGGAAGATGCGAATAATCTTTTCTCTTCTGCGTACTTCTTGA
TTCAGTCGTTCAATTAGATTGGTACTCTTTAGTCGATTGTGGGAATTTCCTTGTACGGTATATTGAAAGGCGTC
TTCGAATCCATCATCCAATGATGCGCAAGCTTTTGAATATTTTGGTTGATCGATATAATCATGAATCAATCGAT
TTTTAGCCTCACGCGCTAAGTTAATATCTGTGAACTTAAAAATTCCTTTAACAGCTTCTCTGAAAGATTTTGAA
TTTTTTTTAGGAATGGTGGTAAAGATATTTCTTAGGAAGTGAACTTGGCATCTTTGCCAACTTACGTTGGTGAA
GGATTTTCTAATGGCAGAGACTAATCCTTTGTGCGCATCAGAAATAACGAGTTCCGTACCTTGTAAACCGCGT
TCTTTTAGGTATTCAAAAAATGTTGTCCAGGTCTCTTCGCTTTCGCCACTTTGAATCATGAAGCCGATAATTTC
ACGGTCGCCATCTTTGGTTATTCCAATCGCTATATGACAGCTTTTTGAGAGTACTCGATTTTCTTCTCGTACTT
TTATATAGAGTACATCGGTCATTAAGTAAGGATAATTTTTTTCTGATAATAAACGATTCTGCCACTCGTTAACC
ATAGGTTCTAGCTGTTCTGTTAAGCTAGAAACGAAGGACTTAGAGACGGATTTACCACAAAGTTCTTCCACAA
TTTTTGATACTTTACGAGTTGAAACGCCTGATACATACATTTCCAACATTGAAGCCATGAGGGCTTTTTCGTTT
CGTTGATAACGTTCAAACACTGTGGGTGAAAAATGGCCATCACGTGTTCTGGGTACTTTTAATTCTAGCGTGC
CTACACGTGTCGTAAAGCTGCGCTCATAATAGCCATTTCGTTGACTTTGTCGGTTTTCTGTTCGTTCATATTCT
TTTGCTTGAATATATTCTGTTCGTTGATTTTCCATTAGTTGATTAAATACCGTTGTTAAAATATTTTTAGAAACG
TCATCCTTTACAGAATATTCAATAATGCTTTGAATCTCTTCGCTTTTCAGTGTAAAATGTACTTGGGTCATGTAA
AAGTCCTCCTGGGTATGTTTTTGTCGTTAAAAACATTGTACCGTAAAAGGACTGTTATATGGCCTTTTTACTTTTACACA
ATTATACGGACTTTATCcagattta*TCAGAGCATATTAAAAACCCTGGATTCAACTTAATTACAAAGGTTGTTATAGAAAAG
AAATAA

**FIGURE 5.6.** Nucleotide sequence of the *sak* gene (5'- 3') cloned from the two MRSA isolates, AR14/3448 and AR14/3685 with the Hlb$^-$, Sak$^-$, EntA$^+$ phenotype. The complete *sak* gene is present but is split by insertion sequence element IS*256*. Red text indicates the *sak* gene nucleotide sequence and asterisks indicate where the *sak* gene sequence is interrupted. Black uppercase text indicates the non-*sak* nucleotide sequence IS*256*. Lower case letters indicate the 8 bp direct repeat sequence found flanking IS*256*, one which is part of the *sak* gene sequence and the other which may be the result of target duplication by IS*256*. Italicised underlined text indicates the imperfect terminal repeats of IS*256* and bold text indicates the transposase gene of IS*256*. Text highlighted with yellow indicates a *Hind*III restriction site.

```
sak       ATGTCAAGTTCATTCGACAAAGGAAAATATAAAAAAGGCGATGACGCGAGTTATTTTGAA
clone     ATGTCAAGTTCATTCGACAAAGGAAAATATAAAAAAGGCGATGACGCGAGTTATTTTGAA
IS256     ------------------------------------------------------------

sak       CCAACAGGCCCGTATTTGATGGTAAATGTGACTGGAGTTGATAGTAAAGGAAATGAATTG
clone     CCAACAGGCCCGTATTTGATGGTAAATGTGACTGGAGTTGATGGTAAAGGAAATGAATTG
IS256     ------------------------------------------------------------

sak       CTATCCCCTCATTATGTCGAGTTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAA
clone     CTATCCCCTCATTATGTCGAGTTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAA
IS256     ------------------------------------------------------------

sak       ATTGAATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTAGTT
clone     ATTGAATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTAGTT
IS256     ------------------------------------------------------------

sak       GAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAATAAGAAAAAAGAA
clone     GAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAATAAGAAAAAAGAA
IS256     ------------------------------------------------------------

sak       GAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTGTTGTCCCAGATTTA---------
clone     GAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTGTTGTCCCAGATTTAAGTCAAGTC
IS256     ----------------------------------------------------AGTCAAGTC

sak       ------------------------------------------------------------
clone     CAGACTCCTGTGTAAAATGCTATACAATGTTTTTACCATTTCTACTTATCAAAATTGATG
IS256     CAGACTCCTGTGTAAAATGCTATACAATGTTTTTACCATTTCTACTTATCAAAATTGATG

sak       ------------------------------------------------------------
clone     TATTTTCTTGAAGAATAAATCCATTCATCATGTAGGTCCATAAGAACGGCTCCAATTAAG
IS256     TATTTTCTTGAAGAATAAATCCATTCATCATGTAGGTCCATAAGAACGGCTCCAATTAAG

sak       ------------------------------------------------------------
clone     CGATTGGCTGATGTTTGATTGGGGAAGATGCGAATAATCTTTTCTCTTCTGCGTACTTCT
IS256     CGATTGGCTGATGTTTGATTGGGGAAGATGCGAATAATCTTTTCTCTTCTGCGTACTTCT

sak       ------------------------------------------------------------
clone     TGATTCAGTCGTTCAATTAGATTGGTACTCTTTAGTCGATTGTGGGAATTTCCTTGTACG
IS256     TGATTCAGTCGTTCAATTAGATTGGTACTCTTTAGTCGATTGTGGGAATTTCCTTGTACG

sak       ------------------------------------------------------------
clone     GTATATTGAAAGGCGTCTTCGAATCCATCATCCAATGATGCGCAAGCTTTTGAATATTTT
IS256     GTATATTGAAAGGCGTCTTCGAATCCATCATCCAATGATGCGCAAGCTTTTGAATATTTT

sak       ------------------------------------------------------------
clone     GGTTGATCGATATAATCATGAATCAATCGATTTTTAGCCTCACGCGCTAAGTTAATATCT
IS256     GGTTGATCGATATAATCATGAATCAATCGATTTTTAGCCTCACGCGCTAAGTTAATATCT

sak       ------------------------------------------------------------
clone     GTGAACTTAAAAATTCCTTTAACAGCTTCTCTGAAAGATTTTGAATTTTTTTTTAGGAATG
IS256     GTGAACTTAAAAATTCCTTTAACAGCTTCTCTGAAAGATTTTGAATTTTTTTTTAGGAATG

sak       ------------------------------------------------------------
clone     GTGGTAAAGATATTTCTTAGGAAGTGAACTTGGCATCTTTGCCAACTTACGTTGGTGAAG
IS256     GTGGTAAAGATATTTCTTAGGAAGTGAACTTGGCATCTTTGCCAACTTACGTTGGTGAAG

sak       ------------------------------------------------------------
clone     GATTTTCTAATGGCAGAGACTAATCCTTTGTGCGCATCAGAAATAACGAGTTCCGTACCT
IS256     GATTTTCTAATGGCAGAGACTAATCCTTTGTGCGCATCAGAAATAACGAGTTCCGTACCT

sak       ------------------------------------------------------------
clone     TGTAAACCGCGTTCTTTTAGGTATTCAAAAAATGTTGTCCAGGTCTCTTCGCTTTCGCCA
IS256     TGTAAACCGCGTTCTTTTAGGTATTCAAAAAATGTTGTCCAGGTCTCTTCGCTTTCGCCA

sak       ------------------------------------------------------------
clone     CTTTGAATCATGAAGCCGATAATTTCACGGTCGCCATCTTTGGTTATTCCAATCGCTATA
IS256     CTTTGAATCATGAAGCCGATAATTTCACGGTCGCCATCTTTGGTTATTCCAATCGCTATA

sak       ------------------------------------------------------------
clone     TGACAGCTTTTTGAGAGTACTCGATTTTCTTCTCGTACTTTTATATAGAGTACATCGGTC
IS256     TGACAGCTTTTTGAGAGTACTCGATTTTCTTCTCGTACTTTTATATAGAGTACATCGGTC
```

**FIGURE 5.7.** Alignment of the nucleotide sequences of the *sak* gene cloned from the two MRSA isolates with the Hlb$^-$, Sak$^-$, Ent A$^+$ phenotype (clone), the complete *sak* gene (*sak*) and insertion sequence element IS*256* generated with the CLUSTALW sequence alignment program. The nucleotide sequences of *sak* and IS*256* were obtained from the Genbank database, under accession numbers, A17531 and AB049452, respectively. The *sak* gene nucleotide sequence is shown in red and the IS*256* nucleotide sequence is shown in black. The direct repeat sequence flanking IS*256* in the isolates with the Hlb$^-$, Sak$^-$, Ent A$^+$ phenotype is highlighted in green. Hyphens indicate no alignment between the gene indicated and the clone.

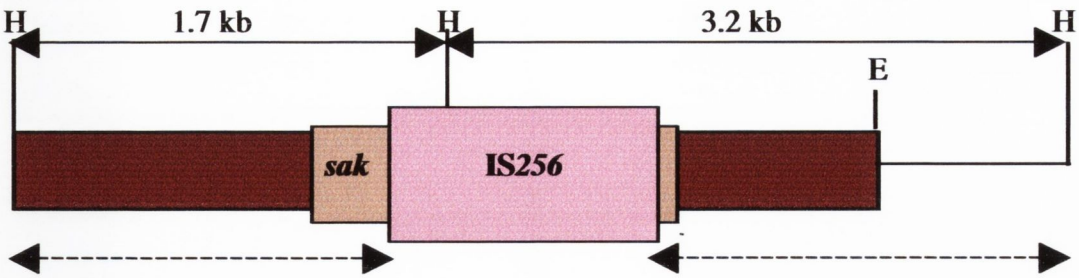



**FIGURE 5.8.** Map of the *sak*-encoding *Hind*III/*Eco*RI DNA region of the two MRSA isolates, AR14/3448 and AR14/3685 with the Hlb$^-$, Sak$^-$, EntA$^+$ phenotype. IS*256* has inserted into the *sak* gene resulting in the introduction of a novel *Hind*III site within the *sak* gene. Arrows indicate the sizes of the *Hind*III fragments. Dotted arrows indicate the region homologous to the *Hind*III *sak*-encoding DNA probe.
Restriction endonuclease cleavage sites are as follows; H, *Hind*III; E, *Eco*RI.

## 5.4 Discussion

In the present study, an analysis of MRSA isolates recovered in Irish hospitals between 1971 and 2002 revealed that the majority of Irish MRSA isolates investigated (96%) did not produce β-toxin and contain serotype F converting phages. In addition, almost 96% of isolates investigated were found to produce either Sak (43%), EntA (2%) or both Sak and EntA (51%) (Table 5.3). Both Sak and EntA have previously been implicated as virulence factors in staphylococcal disease and are encoded by serotype F phage (Coleman *et al.*, 1989; Marrack & Kappler, 1990; Christner & Boyle, 1996: Dinges *et al.*, 2000; Lahteenmaki *et al.*, 2001; Jin *et al.*, 2004).

The majority of Irish nosocomial MRSA isolates investigated (94%) in the present study produced Sak, an important virulence factor in the pathogenesis of *S. aureus*. Jin *et al* (2004) found that Sak can interfere with the early innate immune response and consequently *S. aureus* isolates that produce Sak are potentially more virulent than those that do not produce Sak.

In the present study the prevalence of EntA production among a large collection of Irish nosocomial MRSA isolates recovered between 1971 and 2002 was found to be high, with 53% of isolates testing positive for production of this toxin. In a previous study of enterotoxin production by *S. aureus* isolates, Humphreys *et al.* (1989) found that all of the MRSA isolates examined produced either enterotoxin A, or enterotoxin B, or both. Enterotoxin A is a superantigen that can inhibit the host immune responses to *S. aureus* by causing non-specific T cell activation and induction of gamma interferon, interleukin-I and tumour necrosis factor (Dinges *et al.*, 2000).

In 1992, Coia *et al.* identified two populations of MRSA in Glasgow Royal Infirmary, one that carried aminoglycoside resistance and was largely EntA-negative and a second population that was susceptible to aminoglycosides and largely EntA-positive. The Irish nosocomial MRSA isolates investigated in the present had previously been characterised by determining their resistance to a panel of antimicrobial agents (AR typing) that included five aminoglycosides (see Chapter 2, section 2.1.1 and Table 2.2). In contrast to the results of the above mentioned Scottish study (Coia *et al.*, 1992), all the Irish MRSA isolates investigated in the present study that were found to be EntA positive were resistant to a combination of aminoglycosides (see Table 5.3 for EntA positive isolates and Table 2.2, Chapter 2 for details of the AR patterns of these isolates). However, lack of EntA production was not as clear cut as in the Scottish study

(Coia *et al.*, 1992). In the Scottish study lack of EntA production was associated with aminoglycoside resistant isolates while in the present study lack of EntA production was observed in two groups of isolates, those that were resistant to a combination of aminoglycosides (Phenotype-II, AR02, AR22, AR43, 3/16 AR14 and 4/6 AR07.3/07.4 isolates; Table 5.3) and those that were susceptible to aminoglycosides (AR06, ARNT, early MRSA; Table 5.3).

The phenotypic results obtained in the present study from the investigation into β-toxin, Sak and EntA production by Irish nosocomial MRSA isolates with a variety of MRSA clonal types, indicate that many of the MRSA clonal types identified harboured a specific type of serotype F phage, either double-converting or triple-converting (Table 5.4). For example, all ST250 and ST30 isolates had the $Hlb^-$, $Sak^+$, $EntA^-$ phenotype which is indicative of the presence of double-converting serotype F phage and all ST239 isolates had the $Hlb^-$, $Sak^+$, $EntA^+$ phenotype which is indicative of the presence of triple-converting serotype F phage (Table 5.4). Similarly, the majority of ST8 isolates with the variant SCC*mec* elements IIA-IIE (36/39) and ST36 isolates, were found to have the $Hlb^-$, $Sak^+$, $EntA^+$ phenotype indicating the presence of triple-converting serotype F phage while the majority of ST8 isolates with SCC*mec* IVE and IVF and ST22 isolates had the $Hlb^-$, $Sak^+$, $EntA^-$ phenotype, indicating that they harboured double-converting serotype F phage. Within the ST5 genotype isolates recovered prior 1999 had the $Hlb^-$, $Sak^+$, $EntA^-$ phenotype indicative of the presence of double-converting serotype F phage while those recovered in 1999 had the $Hlb^-$, $Sak^+$, $EntA^+$ phenotype indicative of the presence of triple-converting serotype F phage (Table 5.4). A ST247 MRSA isolate recovered in 1993 had the $Hlb^-$, $Sak^+$, $EntA^-$ phenotype indicating the presence of double-converting serotype F phage while a ST247 MRSA isolate form 1999 had the $Hlb^-$, $Sak^+$, $EntA^+$ phenotype indicative of the presence of a triple-converting serotype F phage. The very few β-toxin positive isolates observed among Irish nosocomial MRSA isolates may be due to loss of a β-toxin inactivating phages from these isolates, which appears to be a rare event because only four β-toxin positive isolates were observed in this study of 110 MRSA isolates (Table 5.4). The ST8-MRSA-IIC isolate (AR14/3600; Table 5.4) with the $Hlb^-$, $Sak^-$, $EntA^-$ phenotype, may harbour a β-toxin inactivating serotype A phage or a serotype F phage with rearrangements.

**TABLE 5.4. Correlation between clonal types and β-toxin (Hlb), staphylokinase (Sak) and enterotoxin A (EntA) production of Irish nosocomial MRSA isolates recovered between 1971 and 2002**

| Clonal type[1] | Years | Hlb[2] | Sak[2] | Ent[2] | Interpretation[3] | Number of isolates |
|---|---|---|---|---|---|---|
| ST250-MRSA-I | | - | + | - | Double-converting serotype F phage | 2 |
| ST250-MRSA-I - *pls* | 1978-'84,'89 | - | + | - | Double-converting serotype F phage | 2 |
| ST239-MRSA-IIIC | 1985-'87,'89 | - | + | + | Triple-converting serotype F phage | 2 |
| ST239-MRSA-III | 1989,'93,'02 | - | + | + | Triple-converting serotype F phage | 4 |
| ST239-MSSA | 1989 | - | + | + | Triple-converting serotype F phage | 1 |
| ST247-MRSA-IA | 1999 | - | + | + | Triple-converting serotype F phage | 1 |
| ST247-MRSA-IA | 1993 | - | + | - | Double-converting serotype F phage | 1 |
| ST8-MRSA-IIA | 1993,'98,'99 | - | + | + | Triple-converting serotype F phage | 5 |
| ST8-MRSA-IIB | 1989 | - | + | + | Triple-converting serotype F phage | 1 |
| ST8-MRSA-IIC | 1993,'98,'99 | - | + | + | Triple-converting serotype F phage | 4 |
| ST8-MRSA-IIC | 1999 | + | - | - | No *hlb* inactivating phage | 2 |
| ST8-MRSA-IIC | 1999 | - | - | - | Single-converting serotype A phage | 1 |
| ST8-MRSA-IIC | 1999 | - | - | + | Triple-converting serotype F phage[4] | 1 |
| ST8-MRSA-IID | 1998,'99,'02 | - | + | + | Triple-converting serotype F phage | 20 |
| ST8-MRSA-IIE | 1998,'99,'02 | - | + | + | Triple-converting serotype F phage | 4 |
| ST8-MRSA-IIE | 1999 | - | - | + | Triple-converting serotype F phage[4] | 1 |
| ST8-MRSA-IVE | 1999 | - | + | - | Double-converting serotype F phage | 16 |
| ST8-MRSA-IVE | 1999 | + | - | - | No *hlb* inactivating phage | 1 |
| ST8-MRSA-IVF | 1999 | - | + | - | Double-converting serotype F phage | 3 |
| ST5-MRSA-II | 1989,'93,'98 | - | + | - | Double-converting serotype F phage | 4 |
| ST5-MRSA-II | '99 | - | + | + | Triple-converting serotype F phage | 2 |
| ST496-MRSA-II | 2002 | - | + | - | Double-converting serotype F phage | 1 |
| ST36-MRSA-II | 1998,'99 | - | + | + | Triple-converting serotype F phage | 10 |
| ST36-MRSA-II | 2002 | - | + | - | Double-converting serotype F phage | 1 |
| ST30-MRSA-IV | 1989,'93,'02 | - | + | - | Double-converting serotype F phage | 3 |
| ST34-MSSA | 1993 | - | + | - | Double-converting serotype F phage | 1 |
| ST22-MRSA-IV | 1989,'93, | - | + | + | Triple-converting serotype F phage | 2 |
| ST22-MRSA-IV | 1998,'99,'02 | - | + | - | Double-converting serotype F phage | 11 |
| ST22-MRSA-IV | 1999 | + | - | - | No *hlb* inactivating phage | 1 |
| ST45-MRSA-IV | 2002 | - | + | - | Double-converting serotype F phage | 1 |
| ST12-MRSA-IV | 2002 | - | + | - | Double-converting serotype F phage | 1 |

[1]The clonal types of isolates were identified as part of the present study (see Chapters 3 & 4).

[2]-, negative for the production of the indicated protein; +, positive for the production of the indicated protein

[3]The $Hlb^+$, $Sak^-$, $EntA^-$ phenotype can be interpreted as indicative of the absence of *hlb* inactivating phage; the $Hlb^-$, $Sak^+$, $EntA^-$ phenotype can be interpreted as indicative of the presence of a double-converting serotype-F phage; the $Hlb^-$, $Sak^+$, $EntA^+$ phenotype can be interpreted as indicative of the presence of a triple-converting serotype-F phage; the $Hlb^-$, $Sak^-$, $EntA^-$ can be interpreted as indicative of the presence of a single-converting serotype A phage.

[4]The two isolates with the $Hlb^-$, $Sak^-$, $EntA^+$ phenotype were both found to harbour triple-converting serotype F phage in which expression of the *sak* gene was prevented by the presence of IS*256* within the ORF of *sak*.

Enterotoxin A production in the absence of both β-toxin and Sak production has never previously been reported in clinical MRSA isolates. This phenotype was identified in the present study in two multi-antimicrobial resistant MRSA isolates with the AR pattern AR14 (Table 5.3). These two isolates were recovered from two different patients in two different Irish hospitals during 1999. Initially, it was suspected that the phenotype observed in these isolates was due to a new phage encoding the *entA* gene in the absence of the *sak* structural gene. However, when they were investigated further using Southern hybridisation analysis, PCR amplification and sequencing, it was found that the lack of Sak production in these two isolates was due to the insertional inactivation of the *sak* structural gene by insertion sequence element IS*256*.

The insertion sequence IS*256* was originally identified in the composite transposon Tn*4001*, where two copies of IS*256* were identified as flanking the central aminoglycoside resistance genes *aac-aph* (Lyon *et al.*, 1984; Byrne *et al.*, 1989). Like all insertion sequences, IS*256* carries only genes necessary for its transposition including the imperfect terminal inverted repeats and the transposase gene and it generates short directly repeated sequences of the target DNA flanking the element upon insertion (Mahillon & Chandler, 1998: Fig. 5.6). IS*256* has been identified in *S. aureus*, *S. epidermidis* and *Enterococcus faecalis* and can occur independently of Tn*4001* or related transposons (Thomas & Archer, 1989; Hodel-Christian and Murray, 1991; Dyke *et al.*, 1992; Rice *et al.*, 1995).

Many studies have shown that IS*256* is widespread in gentamicin-resistant staphylococcal and enterococcal isolates where it was believed to be a relatively stable part of the chromosome and therefore useful as an epidemiological marker in molecular typing (Lyon *et al.*, 1987; Monzon-Moreno *et al.*, 1991; Dyke *et al.*, 1992; Walcher-Salesse *et al.*, 1992; Rice & Marshall, 1994; Morvan *et al.*, 1997). However, more recent data suggests that IS*256* is highly mobile and causes a variety of genetic aberrations such as gene inactivations, DNA rearrangements and large chromosomal deletions that affect the expression of virulence- and antimicrobial resistance-associated genes (Rice *et al.*, 1995; Maki & Murkami, 1997; Rice & Carias, 1998; Ziebuhr *et al.*, 1999; Prudhomme *et al.*, 2002). For example, IS*256* has been shown to cause phase variation of biofilm expression in *S. epidermidis* by inactivation of the *icaADBC* operon which encodes enzymes responsible for biofilm synthesis (Ziebuhr *et al.*, 1999). The transposition of IS*256* has also been shown to be reversible resulting in

restoration of the phenotype it originally disrupted (Ziebuhr *et al*, 1999). This present study found the expression of the phage encoded *sak* gene was disrupted in two isolates due to the presence of IS*256* in the coding region of the gene. Why IS*256* inserted within the *sak* gene is difficult to discern as both the mode of action of IS*256* and the factors that determine target recognition by its transposase enzyme are poorly understood (Mahillon & Chandler, 1998). Although insertion sequences can usually insert into many different sites, target site utilisation is generally not random (Craig, 1997). Most display some degree of target specificity, with some inserting into preferential sites and others avoiding certain sites. What can be concluded from the results obtained here is that this is another example of a naturally occurring insertion sequence IS*256* involved in gene inactivation of an important virulence factor of two multiresistant MRSA isolates. This is the first report of IS*256* disrupting a phage encoded gene.

It is interesting to note that the two Irish MRSA isolates that carry IS*256* within the *sak* gene were recovered from two separate patients in two different hospitals but they have the same antibiotic resistance patterns and have very similar MRSA clonal types, ST8-MRSA-IIC and ST8-MRSA-IIE (Table 5.3). From the extensive sequencing of the SCC*mec* elements IIC and IIE, as described previously in Chapter 4, it was revealed that the SCC*mec* IIE element is a derivative of the SCC*mec* IIC element that arose following loss of pUB110. Based on this information and the fact that these two isolates also have a *sak* gene with IS*256* inserted at the exact same nucleotide position it is probable that these two isolates are indeed closely related and have spread between hospitals.

Recent progress in genomic research has revealed that the genome sequences of bacteria frequently include multiple lysogenic phage that have a major impact on the evolution of the bacterial chromosome (Ohnishi *et al.*, 2001, McClelland *et al.*, 2001; Baba *et al.*, 2002; Smoot *et al.*, 2002; Holden *et al.*, 2004; Dempsey *et al.*, 2005). In the present study the majority of MRSA isolates investigated were found to harbour serotype F lysogenic phage, that carry the important *S. aureus* virulence factors Sak or both Sak and EntA. The presence of these lysogenic phage in the genome of MRSA isolates may enhance their pathogenicity and may contribute to the ability of some MRSA clones to spread and cause disease.

# Chapter 6
# General Discussion

## 6.1 General Discussion

When antibiotics were first introduced into clinical practice in the 1940s to treat bacterial infections they were seen by many as the ultimate weapon that would finally end the battle against infectious disease, including that caused by *S. aureus*. However, sixty years on, the battle against infectious disease is still ongoing due to the ability of bacteria to evolve and adapt to the ever-changing conditions presented by the hospital environment by spontaneous mutation, recombination and to a large extent, by the acquisition of exogenous genes.

### 6.1.1 The evolutionary epidemiology of MRSA in Ireland

Methicillin-resistant *S. aureus* has been a serious problem in Irish hospitals for many years and prior to the present study limited information was available on how the Irish MRSA population had evolved and become a major cause of nosocomial infection since it was first reported from a patient in an Irish hospital in 1971. The ability to accurately determine the genetic relatedness of MRSA isolates is fundamental to understanding their epidemiology and evolutionary history and provides information essential for the establishment of effective infection control measures for combating the spread of MRSA within and between hospitals. It has been proposed that MRSA clones should be defined not only on the basis of their genetic background, as identified by MLST, but also based on their SCC*mec* type. Using both MLST and SCC*mec* element analysis, the present study has comprehensively described the dissemination of the major epidemic MRSA lineages to Ireland. All MRSA isolates investigated from Irish hospitals had the same or closely related genetic backgrounds to the major epidemic MRSA lineages previously identified worldwide. Twenty-one clonal types (MLST-SCC*mec* types) were identified, 10 of which were previously recognised nosocomial MRSA clonal types, nine with previously described nosocomial MRSA genetic backgrounds but with novel SCC*mec* variants (including two new variants of class A *mec*) and two with previously unreported genetic backgrounds that were closely related to those of major epidemic MRSA clones (a double locus variant of ST5 with SCC*mec* II and a single locus variant of ST8 with SCC*mec* IVA). In addition, clonal diversity and temporal shifts were observed in the predominant MRSA clones in Irish hospitals. The most striking temporal shift was the displacement of the multi-resistant ST8 clones harbouring many different SCC*mec* elements, which spread within and between many Irish hospitals

through clonal dissemination in the 1990s, by the multi-antibiotic susceptible EMRSA-15 clone (ST22-MRSA-IV) as the dominant MRSA clone by 2002.

### 6.1.2 Variants of SCC*mec* in Irish MRSA isolates

The SCC*mec* element plays an important role in the evolution of MRSA as it carries the *mecA* gene that encodes resistance to all β-lactam antibiotics and, in addition, can also carry resistance genes to other antimicrobials. However, a striking finding of the present study was the extent of variation within SCC*mec*. A diverse collection of Irish MRSA isolates recovered over a thirty year period were investigated as part of the present study and this allowed a large number of variants SCC*mec* elements to be identified. Based on these findings it is becoming increasingly obvious that SCC*mec* evolves rapidly and that diversity in SCC*mec* elements is more extensive and complex that originally perceived. The presence of a combination of sequences in the novel variant SCC*mec* elements IIA-IIE and IVE and IVF identified in the present study that are also found in other SCC*mec* elements strongly suggests that these SCC*mec* elements are the result of genetic recombination and rearrangement between different SCC*mec* elements. Recently, several other researchers have also reported variant SCC*mec* elements in diverse genetic backgrounds (Aires de Sousa *et al.*, 2003a; Aires de Sousa & de Lencastre, 2003; Robinson & Enright, 2003; Wielders *et al.*, 2003; Chung *et al.*, 2004; Perez-Roth *et al.*, 2004; Shukla *et al.*, 2004b). Therefore, in retrospect, it is not surprising that a large number of variant SCC*mec* elements were identified among the diverse collection of Irish MRSA isolates recovered over a relatively long period of time. It seems likely that novel SCC*mec* elements are constantly being generated in staphylococci through recombination and rearrangement.

The epidemiological significance of variant SCC*mec* elements is unclear. As the SCC*mec* elements of larger collections of isolates are investigated, the epidemiological significance of these variants may become apparent. However, due to the unprecedented numbers of variant SCC*mec* elements that have been identified, not only in the present study but in many others, it appears that the current methods for SCC*mec* typing are inadequate. It is vital that in the future any new SCC*mec* typing scheme includes all currently known SCC*mec* variants. Equally important from an epidemiological point of view is the question of the nomenclature used for SCC*mec* elements. It is widely accepted that SCC*mec* elements should be named using roman numerals and that they

should be defined by the type of *ccr*- and *mec*- gene complexes they harbour. An SCC*mec* variant is an SCC*mec* element with the *ccr*- and *mec*- gene complexes of a previously described SCC*mec* element but which varies from the parent SCC*mec* element in its genetic organisation outside of the *ccr*- and *mec*- gene complexes. Most researchers add letters to the original SCC*mec* element name to define a variant SCC*mec* element. However, with novel SCC*mec* variants constantly being identified, it is important that some web-based database is established so that information on novel SCC*mec* elements and their variants can be regularly updated and readily accessed. At present there is no SCC*mec* database for submission and analysis of SCC*mec* element data, with the result that currently there is considerable confusion regarding what SCC*mec* element variants have been described and what names to assign to novel SCC*mec* variants, with some SCC*mec* variants being confusingly assigned the same names.

In addition, there is also much confusion regarding the nomenclature used for *mec* gene complexes. For example, two different *mec* complexes have previously been described within the class A *mec* complex and both have been termed class A *mec*, even though they differ with respect to the presence or absence of a second copy of insertion sequence element IS*431* (Fig. 4.5). Similarly, within class B *mec*, two different *mec* complexes have also been described previously that differ from each other only by the size of the deletion with *mecR*1, but, in contrast to the class A *mec* complexes that were both termed class A *mec*, these two variants were named class B and B1 *mec* complex (Fig. 4.5). Furthermore, within class C *mec* complex two variants that also differ from each other with respect to the deletion size within *mecR1* have been described previously but in contrast to the nomenclature used for the variants within the class A and B *mec* complexes these variants were named class C1 and C2 *mec* (Fig. 4.5). Based on the fact that SCC*mec* elements and their *mec* complexes appear to be evolving rapidly it seems likely that more novel *mec* complexes will be described in the future and thus there is a need for internationally agreed nomenclature and guidelines for the classification of novel *mec* complexes.

The accumulation of detailed information on the genetic organisation of SCC*mec* elements, their variants and their *mec* complexes will facilitate further development of efficient MRSA typing procedures that provide highly informative epidemiological data by analysing SCC*mec* in combination with MLST analysis of the more stable genomic backbone.

### 6.1.3 Controlling the spread of MRSA

The emergence of antibiotic resistance in MRSA cannot be completely prevented as it is an intrinsic aspect of *S. aureus* evolution. However, it is possible to control the dissemination of resistant strains. On the basis of evidence from countries such as Denmark, Sweden, Finland and Holland, where the prevalence of nosocomial MRSA is low, early detection, implementation of strict infection control measures and rational antibiotic use all decrease the frequency of endemic MRSA strains and limit the transmission of MRSA. A key issue in attempting to control the spread of MRSA in any hospital is that hospital staff at every level must be educated on effective hospital hygiene protocols, antimicrobial prescribing policies, as well as the possible routes of spread of MRSA throughout the hospital.

In response to the increasing prevalence of antibiotic resistant bacteria in Irish hospitals The Strategy for Antimicrobial Resistance in Ireland (SARI) was outlined in 2001. It recommends the improvement of infection control systems, an increase in the surveillance of antimicrobial resistance and education of healthcare workers, patients and the general public regarding antimicrobial resistant bacteria. It also proposes that surveillance of antimicrobial usage and prescribing be increased. Unfortunately, infection control systems in Irish hospitals are, in most cases, inadequate, poorly resourced and ineffectively implemented and fail to prevent the spread of MRSA. Inappropriate prescribing and the misuse and overuse of antibiotics also add to the problem as they favour the selection of resistant MRSA strains. It is vital that all hospital staff wash their hands using an effective procedure between every patient if the spread of MRSA is to be prevented. Unfortunately, this is frequently not complied with due to overcrowding and understaffing in hospitals or due to a lack of knowledge of the importance of this issue in controlling the spread of MRSA. It may be necessary to enforce cross infection control measures for staff who, for whatever reason, fail to comply with hand washing procedures. However, most Irish hospitals participate in EARSS, which is an ongoing surveillance initiative that monitors antimicrobial resistance among different bacteria, including *S. aureus*, across European hospitals. The EARSS project provides Irish hospitals with information on the prevalence of MRSA and identifies priorities for infection control systems and, when necessary, the need for changes to be made to antimicrobial prescribing policies.

### 6.1.4 Treatment of MRSA infections

Methicillin-resistant *S. aureus* strains have developed resistance to practically all antibiotics available today. When a patient becomes infected with a multi-resistant MRSA strain, therapeutic options are limited. For many years vancomycin has been the antibiotic of choice for treating MRSA infections. However, the emeregnce of MRSA isolates with reduced susceptibility to vancomycin as well as vancomycin-resistant MRSA, highlights the need for alternative therapeutic options to treat MRSA infections.

Oxazolidines and cyclic lipopeptides are two recently developed classes of antimicrobial agents that have novel mechanisms of action against multidrug resistant Gram-positive bacteria, including MRSA. Linezolid is an oxazolidinone that has been approved for use in treating infections caused by Gram-positive bacteria and its use in treating multi-resistant staphylococcal infections has been well documented (Shinabarger, 1999; Chien *et al.*, 2000; Cercenado *et al.*, 2001; Dailey *et al.*, 2001; Bassetti *et al.*, 2004). Linezolid is thought to bind to the V domain region of the 23S rRNA gene thus inhibiting bacterial protein synethesis (Kloss *et al.*, 1999; Shinabarger, 1999). Daptomycin is a cyclic lipopeptide that has also recently been approved for the treatment of complicated skin and soft tissue infections caused by Gram-positive bacteria (Thorne & Alder, 2002; Carpenter & Chambers, 2004; Tedesco & Rybak, 2004). It has a distinctive mechanism of action of disrupting plasma membrane function in Gram-positive bacteria (Silverman *et al.*, 2003). Although daptomycin requires further clinical evaluation, studies so far have indicated that it is a promising therapeutic option for treating MRSA infections (Cha & Rybak, 2003; Hermsen *et al.*, 2003; Sader *et al.*, 2004). Unfortunately, although resistance to linezolids is rare, it has been reported in clinical MRSA isolates (Tsiodras *et al.*, 2001; Wilson *et al.*, 2003). It is vital that in order to maintain the efficacy of these new antimicrobial agents against multi-antimicrobial resistant MRSA and to prevent the emergence of widespread linezolid and daptomycin resistance in MRSA isolates, that rational administration of these antimicrobials is observed so they are not over used in uncomplicated infections where other standard antimicrobials would be effective.

Although the prevalence of multi-antimicrobial resistant bacteria continues to increase in many countries worldwide, there has been a dramatic decline in the interest of pharmaceutical companies in the research and development of antibimicrobial agents (Pfizer, 2004). There is an increasing concern that in the future there may be no effective antibiotics left to treat MRSA infections. Recent advances in genome

sequencing projects may help identify novel targets for the design of new drugs and will very likely open new avenues for treating infectious disease. It seems likely that in the future immunotherapy may also play an important role in the prevention of *S. aureus* infections. A *S. aureus* vaccine called StaphVAX™ has been developed and has been shown to confer protection for almost 10 months against *S. aureus* bacteremia in patients at continuous risk (Fattom *et al.*, 2004). StaphVAX™ is a bivalent vaccine composed of *S. aureus* capsular polysaccharides types 5 and 8 bound to mutant non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (Fattom *et al.*, 1993). It is currently not yet available for routine use but it has recently received fast track designation from the Food and Drug Administration (FDA) for use in the prevention of *S. aureus* bloodstream infections in haemodialysis patients.

There is also renewed interest in the possibility of bacteriophage therapy to treat infections involving multi-antimicrobial resistant microbes (Carlton, 1999). This is a method of antibacterial therapy that uses lytic bacteriophage to kill pathogenic microorganisms. Phage therapy showed much promise when it was first developed in the early part of the 20th century. However, since the 1940s treatment of bacterial infections has mainly depended on antibiotics. In some Eastern European countries extensive research and implementation of phage therapy has continued over the last 60 years. In more recent years carefully controlled experimental work with animal models has shown that phage therapy can rescue animals from a variety of potentially fatal infections (Smith & Huggins, 1983 & 1987; Soothill, 1992; Barrow & Soothill, 1997; Barrow *et al.*, 1998; Carlton, 1999). Soothill (1994) found that infections of skin grafts in guinea pigs by *P. aeruginosa* could be prevented by applying phage to the skin grafts and suggested that phage therapy could potentially be used to prevent and treat infections of burns patients. Barrow *et al.* (1998) showed that intramuscular inoculation of chickens and calves with phage was very effective in preventing and treating *E. coli* related septicaemia and meningitis in chickens and calves. Phage therapy could potentially be applied to the treatment of infectious diseases in humans because phage are non-toxic to humans, can survive in blood for several days (Barrow & Soothill, 1997), are lethal to and specific for particular bacteria only and can replicate within the infected host so that only a single dose of the phage should be required. Phage therapy in humans could be directly applied to wounds, administered orally or administered by intramuscular inoculation. However, there is still much scepticism over the use of phage therapy. One major concern is that as with antibiotic resistance, the widespread use of

phage therapy would result in the targeted bacterial species becoming phage resistance and that the phage may not be able to overcome this (Carlton, 1999). If phage therapy is to emerge as a potential alternative to antimicrobials there is a need for renewed research into its practical application for use in the prophylaxis and treatment of a wide range of infectious diseases.

### 6.1.5 Serotype F bacteriophage harboured by Irish MRSA isolates

In addition to the genetic backbone and SCC*mec* element having vital roles to play in the dissemination of epidemic MRSA lineages, lysogenic phage also contribute to bacterial evolution. In the present study the presence of serotype F lysogenic phage was indirectly identified in the majority of Irish nosocomial MRSA isolates by recording various phenotypic characteristics that indicate their presence within the *S. aureus* chromosome. Lysogenic phage have a major impact on bacterial evolution. Firstly, lysogenic conversion resulting in the gain of phage-encoded virulence genes such as *sak* and *entA* increases bacterial pathogenicity. Over half of the Irish nosocomial MRSA isolates investigated in the present study were found to express the superantigen and virulence factor EntA and this may have contributed to virulence of these MRSA isolates within and between Irish hospitals. Secondly, lysogenic phages have been implicated as important vectors for the lateral transfer of bacterial DNA between bacterial strains (Canchaya *et al.*, 2003). It is interesting to note that it has been suggested that SCC*mec* elements may be transferred between *S. aureus* isolates and other staphylococci by lysogenic phages, possibly by the process of transduction (Katayama *et al.*, 2000). Finally, lysogenic phage can confer protection against lytic infection upon the lysogen (Dempsey *et al.*, 2005).

## 6.2 Concluding remarks

The epidemiology of MRSA is constantly changing and needs to be monitored continuously. The surveillance of MRSA in Irish hospitals is ongoing in the National MRSA Reference Laboratory (NMRSARL) at St. James's Hospital in Dublin. In recent years it has become increasingly obvious that antibiogram-resistogram (AR) typing is becoming less useful for epidemiological typing of Irish MRSA isolates because of the increasing prevalence of non-multiresistant MRSA strains that cannot be distinguished by this method but which can be shown to be clearly different based on PFGE (Rossney

*et al.*, 2004). One possible solution to this problem would be to use another highly discriminatory technique, such as a DNA sequencing-based typing method for routine epidemiological typing of MRSA isolates. Although MLST is very useful for long-term epidemiological studies, it is expensive as seven housekeeping genes from each isolate need to be sequenced and may not be suitable for routine investigations. DNA sequencing of the protein A gene (*spa* typing), in which only one gene of ca. 200-600 bp is sequenced, may be a feasible alternative to AR typing. Evaluation of *spa* typing has found it to be a highly discriminatory typing technique that is very easy to implement in a laboratory and one that provides reproducible and unambiguous results (Koreen *et al.*, 2004). In addition, useful epidemiological and evolutionary information would also be obtained by performing MLST and SCC*mec* element analysis of representative Irish MRSA isolates as described in this study on an ongoing basis.

Two of the most alarming recent developments in the evolution of MRSA is the emergence of VRSA isolates in the USA and MRSA as a community pathogen in many countries worldwide, including Ireland (Rossney *et al.*, 2005). Although VRSA isolates are currently rare worldwide, it seems likely that they will become more widespread in the future. Continuing the epidemiological surveillance of both nosocomial and community-acquired MRSA (CAMRSA) is vital in understanding the evolution of these major threats to public health. The extensive characterisation of the genetic backgrounds and SCC*mec* elements of Irish nosocomial MRSA isolates performed in the present study will be useful in the surveillance of CAMRSA by allowing nosocomial MRSA to be readily distinguished from CAMRSA.

Lastly, the pathogenesis of *S. aureus* isolates is enhanced by its ability to express a wide range of virulence factors including several toxins that damage host tissue and cells. These toxins include enterotoxins and exfoliative toxins that have superantigen activity, cytotoxins, and Panton-Valentine Leukocidin toxin that is toxic for leucocytes. An investigation into the various toxins produced by the Irish nosocomial MRSA isolates that have been analysed in the present study would provide much insight the relationship between various MRSA clones and virulence factors and may help explain why certain clones spread to Irish hospitals and dominated over others.

# REFERENCES

**Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C. & Tarkowski, A. (1993).** The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect. Immun.* **61**, 3879-3885.

**Abramson, M. A. & Sexton, D. J. (1999).** Nosocomial methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* primary bacteremia: at what costs? *Infect. Control Hosp. Epidemiol.* **20**, 408-411.

**Aires de Sousa, M., Sanches, I. S., Ferro, M. L., Vaz, M. J., Saraiva, Z., Tendeiro, T., Serra, J. & de Lencastre, H. (1998).** Intercontinental spread of a multidrug-resistant methicillin-resistant *Staphylococcus aureus* clone. *J. Clin. Microbiol.* **36**, 2590-2596.

**Aires de Sousa, M., de Lencastre, H., Santos Sanches, I., Kikuchi, K., Totsuka, K. & Tomasz, A. (2000).** Similarity of antibiotic resistance patterns and molecular typing properties of methicillin-resistant *Staphylococcus aureus* isolates widely spread in hospitals in New York City and in a hospital in Tokyo, Japan. *Microb. Drug Resist.* **6**, 253-258.

**Aires De Sousa, M., Miragaia, M., Sanches, I. S., Avila, S., Adamson, I., Casagrande, S. T., Brandileone, M. C., Palacio, R., Dell'Acqua, L., Hortal, M., Camou, T., Rossi, A., Velazquez-Meza, M. E., Echaniz-Aviles, G., Solorzano-Santos, F., Heitmann, I. & de Lencastre, H. (2001).** Three-year assessment of methicillin-resistant *Staphylococcus aureus* clones in Latin America from 1996 to 1998. *J. Clin. Microbiol.* **39**, 2197-2205.

**Aires de Sousa, M. & de Lencastre, H. (2003).** Evolution of sporadic isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and their similarities to isolates of community-acquired MRSA. *J. Clin. Microbiol.* **41**, 3806-3815.

**Aires de Sousa, M., Bartzavali, C., Spiliopoulou, I., Sanches, I. S., Crisostomo, M. I. & de Lencastre, H. (2003a).** Two international methicillin-resistant *Staphylococcus aureus* clones endemic in a university hospital in Patras, Greece. *J. Clin. Microbiol.* **41**, 2027-2032.

**Aires de Sousa, M., Crisostomo, M. I., Sanches, I. S., Wu, J. S., Fuzhong, J., Tomasz, A. & de Lencastre, H. (2003b).** Frequent recovery of a single clonal type of multidrug-resistant *Staphylococcus aureus* from patients in two hospitals in Taiwan and China. *J. Clin. Microbiol.* **41**, 159-163.

**Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990).** Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

**Amorim, M. L., Aires de Sousa, M., Sanches, I. S., Sa-Leao, R., Cabeda, J. M., Amorim, J. M. & de Lencastre, H. (2002).** Clonal and antibiotic resistance profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) from a Portuguese hospital over time. *Microb. Drug Resist.* **8**, 301-309.

**Archer, G. L., Thanassi, J. A., Niemeyer, D. M. & Pucci, M. J. (1996).** Characterization of IS*1272*, an insertion sequence-like element from *Staphylococcus haemolyticus*. *Antimicrob. Agents Chemother.* **40**, 924-929.

**Arvidson, S. (1983).** Extracellular enzymes from *Staphylococcus aureus*. In *Staphylococcus and Staphylococcal Infections*, pp. 745-808. Edited by C. S. F. Easman & C. Adlam, C. London: Academic Press.

**Aucken, H. M., Ganner, M., Murchan, S., Cookson, B. D. & Johnson, A. P. (2002).** A new UK strain of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA-17) resistant to multiple antibiotics. *J. Antimicrob. Chemother.* **50**, 171-175.

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

**Baber, M. & Rozwadowska-Dowzenko, M. (1948).** Infection by penicillin-resistant staphylococci. *Lancet* **1**, 641-644.

**Bannerman, T. L., Hancock, G. A., Tenover, F. C. & Miller, J. M. (1995).** Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *J. Clin. Microbiol.* **33**, 551-555.

**Barbagiannakos, T., Darbar, A., Prasanna, K., Mercer, J. L., Vickery, A. M. & Gosbell, I. B. (2004).** Clinical and epidemiological survey of MRSA from hospitals in South Western Sydney, New South Wales, Australia. Abstract no. ME01. *The 11th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI)*, 2004. Charleston, South Carolina.

**Barrow, P. A. & Soothill, J. S. (1997).** Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends Microbiol.* **5**, 268-271.

**Barrow, P., Lovell, M. and Berchieri, A. (1998)**. Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and menigitis in chickens and calves. *Clin. Diag. Lab. Immun.* (5) 294-298.

**Bassetti, M., Di Biagio, A., Del Bono, V., Cenderello, G. & Bassetti, D. (2004).** Successful treatment of methicillin-resistant *Staphylococcus aureus* endocarditis with linezolid. *Internat. J. Antimicrob. Agents* **24**, 83-84.

**Beck, W. D., Berger-Bächi, B. and Kayser, F.H. (1986).** Additional DNA in methcillin-resistant *Staphylococcus aureus* and molecular cloning of *mec* specific DNA. *J. Bacteriol.* **165**, 373-378.

**Berger-Bachi, B. (1999).** Genetic basis of methcillin resistance in *Staphylococcus aureus*. *Cell Mol. Life Sci.* **56**, 764-770.

**Berger-Bachi, B. & Rohrer, S. (2002).** Factors influencing methicillin resistance in staphylococci. *Arch. Microbiol.* **178**, 165-171.

**Berger-Bachi, B., Strassle, A., Gustafson, J. E. & Kayser, F. H. (1992).** Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **36**, 1367-1373.

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

**Betley, M. J., Soltis, M.T. & Couch, J.L. (1990).** Molecular biological analysis of staphylococcal enterotoxin genes. In *Molecular biology of staphylococci*. Edited by R. P. Novick. New York: VCH Publishers Inc.

**Blanc, D. S., Francioli, P. & Hauser, P. M. (2002).** Poor value of pulsed-field gel electrophoresis to investigate long-term epidemiology of methicillin-resistant *Staphylococcus aureus*. *Infect. Genet. Evol.* **2**, 145-148.

**Boyce, J. M. (1997).** Epidemiology and prevention of nosocomial infections. In: *The Staphylococci in Human Disease.* pp. 309-329. Edited by G.L. Archer & K. B. Crossley. New York: Churchill Livingstone.

**Brumfitt, W. & Hamilton-Miller, J. (1989).** Methicillin-resistant *Staphylococcus aureus*. *N. Engl. J. Med.* **320**, 1188-1196.

**Byrne, M. E., Duncan, A. R. & Skurray, R. A. (1989).** Nucleotide sequence analysis of IS*256* from the *Staphylococcus aureus* getamicin-tobramycin-kanamycin-resistance transposon Tn*4001*. *Gene* **81**, 361-367.

**Cafferkey, M. T., Hone, R., Falkiner, F. R., Keane, C. T. & Pomeroy, H. (1983).** Gentamicin- and methicillin-resistant *Staphylococcus aureus* in Dublin hospitals: clinical and laboratory studies. *J. Med. Microbiol.* **16**, 117-127.

**Cafferkey, M. T., Hone, R., Coleman, D., Pomeroy, H., McGrath, B., Ruddy, R. & Keane, C. T. (1985).** Methicillin-resistant *Staphylococcus aureus* in Dublin 1971-84. *Lancet* **2**, 705-708.

**Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M. L. & Brussow, H. (2003).** Phage as agents of lateral transfer. *Curr. Opin. Microbiol.* **6**, 4170424.

**Carlton, R. M. (1999).** Phage therapy: past history and future prospects. *Archivum Immunologiae et Therapiae Experimentalis* **47**, 267-274.

**Carpenter, C. F. & Chambers, H. F. (2004).** Daptomycin: Another novel agent for treating infections due to drug-resistant Gram-positive pathogens. *Clin. Infect. Dis.* **38**, 994-1000.

**Carroll, J. D., Pomeroy, H. M., Russell, R. J., Arbuthnott, J. P., Keane, C. T., McCormick, O. M. & Coleman, D. C. (1989).** A new methicillin- and gentamicin-resistant *Staphylococcus aureus* in Dublin: molecular genetic analysis. *J. Med. Microbiol.* **28**, 15-23.

**Center for Disease Cotrol and Prevention (1999).** Four pediatric deaths from community-acquired methicillin-resistant *Staphylococus aureus* -Minnesota and North Dakota, 1997-1999. *Morb. Mortal. Wkly. Rep.* **48**, 707-710.

**Center for Disease Control and Prevention (2002).** Vancomycin-resistant *Staphylococcus aureus*-Pennysylvania, 2002. *Morb. Mortal. Wkly. Rep.* **51**, 902.

**Center for Disease Cotnrol and Prevention (2004).** Vancomycin-resistant *Staphylococcus aureus*-New York. *Morb. Mortal. Wkly. Rep.* **53**, 322-323.

**Cercenado, E., Garcia-Garrote, F. & Bouza, E. (2001).** *In vitro* activity of linezolid against multiply resistant Gram-positive clinical isolates. *J. Antimicrob. Agents Chemother.* **47**, 77-81.

**Cha, R. & Rybak, M. J. (2003).** Daptomycin against multiple drug-resistant staphylococcus and enterococcus isolates in an vitro pharmacodynamic model with simulated endocardial vegetations. *Diagn. Microbiol. Infect. Dis.* **47**, 539-546.

**Chambers, H. F. (1997).** Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *J. Med. Microbiol.* **44**, 125-129.

**Chang, S. C., Sievert, D. M., Hageman, J. C., Boulton, M. L., Tenover, F. C., Downes, F. P., Shah, S., Rudrik, J. T., Pupp, G. R., Brown, W. J. & Cardo, D., Fridkin, S. K; The Vancomysin-Resistant *Staphylococcus aureus* Investigative Team (2003).** Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. *N. Engl. J. Med.* **348**, 1342-1347.

**Chesneau, O., Morvan, A. & Solh, N. E. (2000).** Retrospective screening for heterogenous vancomycin resistance in diverse *Staphylococcus aureus* clones disseminated in French hospitals. *J. Antimicrob. Agents Chemother.* **45**, 887-890.

**Chien, J. W., Kucia, M. L. & Salata, R. A. (2000).** Use of linezolid, an oxazolidinone, in the treatment of multi-drug resistant Gram-positive bacterial infections. *Clin. Infect. Dis.* **30**, 146-151.

**Christner, R. B. & Boyle, M. D. (1996).** Role of staphylokinase in the acquistion of plasmin(ogen)-dependant enzymatic activity by staphylococci. *J. Infect. Dis.* **173**, 104-112.

**Chung, M., Dickinson, G., De Lencastre, H. & Tomasz, A. (2004).** International clones of methicillin-resistant *Staphylococcus aureus* in two hospitals in Miami, Florida. *J. Clin. Microbiol.* **42**, 542-547.

**Coia, J. E., Browning, L., Haines, L., Birkbeck, T. H. & Platt, D. J. (1992).** Comparison of enterotoxins and haemolysins produced by methicillin-resistant (MRSA) and sensitive (MSSA) *Staphylococcus aureus. J. Med. Microbiol.* **36**, 164-171.

**Coleman, D. C., Arbuthnott, J. P., Pomeroy, H. M. & Birkbeck, T. H. (1986).** Cloning and expression in *Escherichia coli* and *Staphylococcus aureus* of the beta-lysin determinant from *Staphylococcus aureus*: evidence that bacteriophage conversion of beta-lysin activity is caused by insertional inactivation of the beta-lysin determinant. *Microb. Pathog.* **1**, 549-564.

**Coleman, D. C., Sullivan, D. J., Russell, R. J., Arbuthnott, J. P., Carey, B. F. & Pomeroy, H. M. (1989).** *Staphylococcus aureus* bacteriophages mediating the simultaneous lysogenic conversion of beta-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. *J. Gen. Microbiol.* **135,** 1679-1697.

**Coleman, D. C., Pomeroy, H., Estridge, J. K., Keane, C. T., Cafferkey, M. T., Hone, R. & Foster, T. J. (1985).** Susceptibility to antimicrobial agents and analysis of plasmids in gentamicin- and methicillin-resistant *Staphylococcus aureus* from Dublin hospitals. *J. Med. Microbiol.* **20**, 157-167.

**Coleman, D. C., Knights, J., Russell, R. J., Shanley, D., Birkbeck, T. H., Dougan, G. & Charles, I. (1991).** Insertional inactivation of the *Staphylococcus aureus* beta-toxin by bacteriophage ϕ13 occurs by site- and orientation-specific integration of the ϕ13 genome. *Mol. Microbiol.* **5**, 933-939.

**Cookson, B. D. (1999).** Nosocomial antimicrobial resistance surveillance. *J. Hosp. Infect.* **43**, S97-103.

**Cookson, B. D. (2000).** Methicillin-resistant *Staphylococcus aureus* in the community: new battlefronts, or are the battles lost? *Infect. Control Hosp. Epidemiol.* **21**, 398-403.

**Coombs, G. W., Nimmo, G. R., Bell, J. M., Huygens, F., O' Brien, F. G., Malkowski, M. J., Pearson, J. C., Stephens, A. J. & Giffard, P. M. (2004).** Genetic diversity among community methicillin-resistant *Staphylococcus aureus* strains causing outpatient infections in Australia. *J. Clin. Microbiol.* **42**, 4735-4743.

**Corso, A., Santos Sanches, I., Aires de Sousa, M., Rossi, A. & de Lencastre, A. (1998).** Spread of a methicillin-resistant and multiresistant epidemic clone of *Staphylococcus aureus* in Argentina. *Microb. Drug Resist.* **4**, 277-288.

**Cosgrove, S. E., Sakoulas, G., Perencevich, E. N., Schwaber, M. J., Karchmer, A. W. & Carmeli, Y. (2003).** Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta analysis. *Clin. Infect. Dis.* **36**, 53-59.

**Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J. A., Hubner, R. J., Sanches, I. S. & Tomasz, A. (1996).** Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. *Microb. Drug Resist.* **2**, 377-391.

**Cox, R. A., Conquest, C., Mallaghan, C. & Marples, R. R. (1995).** A major outbreak of methicillin-resistant *Staphylococcus aureus* caused by a new phage-type (EMRSA-16). *J. Hosp. Infect.* **29**, 87-106.

**Craig, N. L. (1997).** Target site selection in transposition. *Annu. Rev. Biochem.* **66**, 437-474.

**Crisostomo, M. I., Westh, H., Tomasz, A., Chung, M., Oliveira, D. C. & de Lencastre, H. (2001).** The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. *Proc. Natl. Acad. Sci. U S A* **98**, 9865-9870.

**Cui, L., Ma, X., Sato, K., Okuma, K., Tenover, F. C., Mamizuka, E. M., Gemmell, C. G., Kim, M. N., Ploy, M. C., El-Solh, N., Ferraz, V. & Hiramatsu, K. (2003).** Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. *J. Clin. Microbiol.* **41**, 5-14.

**Dailey, C. F., Dileto-Fang, C. L., Buchanan, L. V., Oramas-Shirey, M. P., Batts, D. H., Ford, C. W. & Gibson, J. K. (2001).** Efficacy of linezolid in the treatment of experimental endocarditis caused by methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **45**, 2304-2308.

**De Lencastre, A., Couto, I., Santos, I., Melo-Cristino, J., Torres-Pereira, A. & Tomasz, A. (1994).** Methicillin-resistant *Staphylococcus aureus* disease in a Portuguese hospital: characterisation of clonal types by a combination of DNA typing methods. *Eur. J. Clin. Microbiol. Infect. Dis* **13**, 64-73.

**De Lencastre, H., Severina, E. P., Milch, H., Thege, M. K. & Tomasz, A. (1997).** Wide geographic distribution of a unique methicillin-resistant *Staphylococcus aureus* clone in Hungarian hospitals. *Clin. Microbiol. Infect.* **3**, 289-296.

**Debrise, A., Dyke, K. G. & El Solh, N. (1996).** Characterisation of a *Staphylococcus aureus* transposon, Tn*5405*, located within Tn*5404* and carrying the aminoglycoside resistance genes, *aphA-3* and *aadE*. *Plasmid* **35**, 174-188.

**Dempsey, R. M. (1996).** Molecular genetic analysis of lysogenic conversion by serotype F phages of *Staphylococcus aureus*. Ph.D thesis. *Department of Clinical Microbiology*, University of Dublin, Trinity College, Dublin, Ireland.

**Dempsey, R.M., Carroll, D., Kong, H., Higgins, L., Keane, C.T. & Coleman, D.C. (2005).** *Sau*421, a *Bcg*I-like restriction-modification system encoded by the *Staphylococcus aureus* quadruple-converting phage ϕ42. *Microbiology* **151**,1301-1311.

**Denis, O., Nonhoff, C., Byl, B., Knoop, C., Bobin-Dubreux, S. & Struelens, M. J. (2002).** Emergence of vancomycin-intermediate *Staphylococcus aureus* in a Belgian hospital: microbiological and clinical features. *J. Antimicrob. Chemother.* **50**, 383-391.

**Denis, O., Deplano, A., Nonhoff, C., De Ryck, R., de Mendonca, R., Rottiers, S., Vanhoof, R. & Struelens, M. J. (2004).** National surveillance of methicillin-resistant *Staphylococcus aureus* in Belgian hospitals indicates rapid diversification of epidemic clones. *Antimicrob. Agents Chemother.* **48**, 3625-3629.

**Dinges, M. M., Orwin, P. M. & Schlievert, P. M. (2000).** Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* **13**, 16-34.

**Dominguez, M. A., de Lencastre, H., Linares, J. & Tomasz, A. (1994).** Spread and maintenance of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. *J. Clin. Microbiol.* **32**, 2081-2087.

**Dunman, P. M., Mounts, W., McAleese, F., Immermann, F., Macapagal, D., Marsilio, E., McDougal, L., Tenover, F. C., Bradford, P. A., Petersen, P. J., Projan, S. J. & Murphy, E. (2004).** Use of *Staphylococcus aureus* gene chips in genotyping and genetic composition analysis. *J. Clin. Microbiol.* **42**, 4275-4283.

**Dyke, K. G. H., Aubert, S. & El Solh, N. (1992).** Multiple copies of IS*256* in staphylococci. *Plasmid* **28**, 235-246.

**EARSS (2003a).** EARSS Report Quater 1, 2003. [Online] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/

**EARSS (2003b).** EARSS Report Quater 2, 2003. [Online] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/

**EARSS (2003c).** EARSS Report Quater 3, 2003. [Online] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/

**EARSS (2004a).** EARSS Report Quater 4, 2003. [Online] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/

**EARSS (2004b).** EARSS Report Quater 3, 2004. [Online] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/

**EARSS Steering Group (2003).** Update on the Antimicrobial Resistance Surveillance System (EARSS) in Ireland, 2002. *Epi-Insight* **4**, 2-3. March 2003. [Online] http://www.ndsc.ie/Publications/EPI-Insight/

**Eklund, F. W., Poysky, F. T., Reed, S. & Smith, C. A. (1971).** Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. *Science* **172**, 480-482.

**Eklund, M. W., Poysky, F. T., Meyers, J. A. & Pelroy, G. A. (1974).** Interspecies transfer of *Clostridium botulinum* type C to *Clostridium novyi* type A by bacteriophage. *Science* **186**, 456-458.

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

**Enright, M. C., Day, P. J., Davies, C. E., Peacock, S. J. & Spratt, B. G. (2000).** Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* **38**, 1008-1015.

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

**Fattom, A., Fuller, S., Propst, M., Winston, S., Muenz, L., He, D., Robert, N. & Horwith, G. (2004).** Safety and immunogenicity of a booster dose of *Staphylococcus aureus* types 5 and 8 capsular polysaccharide conjugate vaccine (StaphVAX) in hemodialysis patients. *Vaccine* **23**, 656-663

**Fattom, A., Schneerson, R., Watson, D. C., Karakawa, W. W., Fitsgerald, D., Pastan, I., Li, X., Shiloach, J., Bryla, D. A. & Robbins, J. B. (1993).** Laboratory and clinical evaluation of conjugative vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharide conjugated to *Pseudomonas aeruginosa* recombinant exoprotein A. *Infect. Immun.* **61**, 1023-1032.

**Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P. & Spratt, B. G. (2004).** eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* **186**, 1518-1530.

**Ferraz, V., Duse, A., Kassel, M., Black, A., Ito, T. & Hiramatsu, K. (2000).** Vancomycin-resistant *Staphylococcus aureus* occurs in South Africa. *S. Afr. Med. J.* **90**, 1113.

**Fey, P. D., Said-Salim, B., Rupp, M. E., Hinrichs, S. H., Boxrud, D. J., Davis, C. C., Kreiswirth, B. N. & Schlievert, P. M. (2003).** Comparative molecular analysis of community- or hospital-acquired methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**, 196-203.

**Fitzgerald, J. R., Sturdevant, D. E., Mackie, S. M., Gill, S. R. & Musser, J. M. (2001).** Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc. Natl. Acad. Sci. U S A* **98**, 8821-8826.

**Forsgren, A. & Sjoquist, J. (1966).** Protein A from *Staphylococcus aureus*. Pseudo-immune reaction with human gamma-globulin. *J. Immunology* **97**, 822-827.

**Foster, T. J. & Hook, M. (1998).** Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* **6**, 484-488.

**Fournier, J. M., Bouvet, A., Boutennier, A., Audurier, A., Goldstein, F., Pierre, J., Bure, A., Lebrun, L. & Hochkeppel, H. K. (1987).** Predominance of capsular polysaccharide type 5 among oxacillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* **25**, 1932-1933

**Freeman, V. J. (1951).** Studies in the virulence of bacteriophage-mediated strains of *Corynebacterium diphtheriae*. *J. Bacteriol.* **61**, 675-688.

**Galdbart, J. O., Morvan, A. & El Solh, N. (2000).** Phenotypic and molecular typing of nosocomial methicillin-resistant *Staphylococcus aureus* strains susceptible to gentamicin isolated in France from 1995 to 1997. *J. Clin. Microbiol.* **38**, 185-190.

**Georgopapadakou, N. H., Dix, B. A. & Mauriz, Y. R. (1986).** Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus* and *Streptococcus faecalis:* relation to antibacterial activity. *Antimicrob. Agents Chemother.* **29**, 333-336.

**Gomes, A. R., Sanches, I. S., Aires de Sousa, M., Castaneda, E. & de Lencastre, H. (2001).** Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Columbian hospitals; dominance of a single unique multidrug-resistant clone. *Microb. Drug Resist.* **7**, 23-32.

**Gregory, P. D., Lewis, S. P., Curnock, S. P. & Dyke, K. G. H. (1997).** Studies of the repressor (BlaI) of beta-lactamase synthesis in *Staphylococcus aureus*. *Mol. Microbiol.* **24**, 1025-1037.

**Groman, N. B. (1984).** Conversion by corynephages and its role in the natural history of diphtheria. *J. Hygiene (London)* **93**, 405-417.

**Gustafson, J. E., Strassle, A., Hachler, H., Kayser, F. H. & Bergi-Bachi, B. (1994).** The *femC* locus of *Staphylococcus aureus* required for methicillin resistance includes the glutamine synthetase operon. *J. Bacteriol.* **176**, 1460-1467.

**Hageman, J. C., Pegues, D. A., Jepson, C., Bell, R. L., Guinan, M., Ward, K. W., Cohen, M. D., Hindler, J. A., Tenover, F. C., McAllister, S. K., Kellum, M. E. & Fridkin, S. K. (2001).** Vancomycin-intermediate *Staphylococcus aureus* in a home health-care patient. *Emerg. Infect. Dis.* **7**, 1023-1025.

**Haley, R. W., Hightower, A. W. & Khabbaz, R. F. (1982).** The emergence of methicillin-resistant *Staphylococcus aureus* infections in United States hospitals. *Ann. Intern. Med.* **97**, 297-308.

**Hanahan, D. (1983).** Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557-580.

**Hanaki, H., Kuwahara-Arai, K., Boyle-Vavra, S., Daum, R. S., Labischinski, H. & Hiramatsu, K. (1998).** Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. *J. Antimicrob. Agents Chemother.* **42**, 199-209.

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

**Hartman, B. J. & Tomasz, A. (1986).** Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **29**, 85-92.

**Hawkey, P. M. (1999).** Principles of molecular typing: a guide to the letters. *J. Hosp. Infect.* **43 (supplement)**, S77-S83.

**Hermsen, E. D., Hovde, L. B., Hotchkiss, J. R. & Rotschafer, J. C. (2003).** Increased killing of staphylococci and streptococci by daptomycin compared with cefazolin and vancomycin in an in vitro peritoneal dialysate model. *Antimicrob. Agents Chemother.* **47**, 3764-3765.

**Herold, B. C., Immergluck, L. C., Maranan, M. C., Lauderdale, D. S., Gaskin, R. E., Boyle-Vavra, S., Leitch, C. D. & Daum, R. S. (1998).** Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. *JAMA* **279**, 593-598.

**Higgins, D. G. & Sharp, P. M. (1988).** CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237-244.

**Hiramatsu, K. (1995).** Molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Micrbiol. Immunol.* **39**, 531-543.

**Hiramatsu, K., Cui, L., Kuroda, M. & Ito, T. (2001).** The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol.* **9**, 486-493.

**Hiramatsu, K., Asada, K., Suzuki, E., Okonogi, K. & Yokota, T. (1992).** Molecular cloning and nucleotide sequence determination of the regulator region of *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA). *FEBS lett.* **298**, 133-136.

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

**Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. & Tenover, F. C. (1997b).** Methicillin-resistant *Staphylococcus aureus* clinical isolates with reduced vancomycin susceptibility. *J. Antimicrob. Agents Chemother.* **40**, 135-136.

**Hodel-Christian, S. L. & Murray, B. E. (1991).** Chacterisation of the gentamicin-resistant transposon Tn*5281* from *Enterococcus faecalis* and comparison to staphylococcal transposons Tn*4001* and Tn*4031*. *Antimicrob. Agents Chemother.* **35**, 1147-1152.

**Holden, M. T., Feil, E. J., Lindsay, J. A., Peacock, S. J., Day, N. P., Enright, M. C., Foster, T. J., Moore, C. E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S. D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K. D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B. G., Spratt, B. G. & Parkhill, J. (2004).** Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc. Natl. Acad. Sci. U S A* **101**, 9786-9791.

**Hone, R. & Keane, C. T. (1974).** Characteristics of methicillin-resistant *Staphylococcus aureus*. *Ir. J. Med. Sci.* **143**, 145-154.

**Hood, J., Edwards, G. F. S., Cosgrove, B., Curran, E., Morrison, D. & Gemmell, C. G. (2000).** Vancomycin-intermedicate *Staphylococcus aureus* at a Scottish hospital. *J. Infect.* **40**, A11.

**Huber, B. & Palmer, E. (1993).** Introduction to *Superantigens, a pathogens view of the immune system.*, pp. 1-6. Edited by B. Huber & E. Palmer. New York: Cold Spring Harbour Laboratory Press.

**Huesca, M., Peralta, R., Sauder, D. N., Simor, A. E. & McGavin, M. J. (2002).** Adhesion and virulence properties of epidemic canadian methicillin-resistant *Staphylococcus aureus* strain 1: identification of novel adhesion functions associated with plasmin-sensitive surface protein. *J. Infect. Dis.* **185**, 1285-1296

**Huletsky, A., Giroux, R., Rossbach, V., Gagnon, M., Vaillancourt, M., Bernier, M., Gagnon, F., Truchon, K., Bastien, M., Picard, F. J., van Belkum, A., Ouellette, M., Roy, P. H. & Bergeron, M. G. (2004).** New real-time PCR assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of staphylococci. *J. Clin. Microbiol.* **42**, 1875-1884

**Humphreys, H., Keane, C. T., Hone, R., Pomeroy, H., Russell, R.J. Arbuthnott, J.P. & Coleman, D.C. (1989).** Enterotoxin production by *Staphylococcus aureus* isolates from cases of septicaemia and from healthy carriers. *J. Med. Microbiol.* **28**, 163-172.

**Humphreys, H., Carroll, J. D., Keane, C. T., Cafferkey, M. T., Pomeroy, H. M. & Coleman, D. C. (1990).** Importation of methicillin-resistant *Staphylococcus aureus* from Baghdad to Dublin and subsequent nosocomial spread. *J. Hosp. Infect.* **15**, 127-135.

**Hurlimann-Dalei, R., Ryffel, C., Kayser, F. H. & Berger-Bachi, B. (1992).** Survey of the methicillin-associated genes *mecA*, *mecR1*, *mecI*, *femA* and *femB* in clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **36**, 2617-2621.

**Irish Department of Health and Children (1999).** North/South study of MRSA in Ireland 1999. [Online] http://www.dohc.ie/publications/study_of_mrsa_in_ireland_1999.html

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

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

**Ito, T., Ma, X. X., Takeuchi, F., Okuma, K., Yuzawa, H. & Hiramatsu, K. (2004).** Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrob. Agents Chemother.* **48**, 2637-2651.

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

**Jevons, M. P. (1961).** Celbenin-resistant staphylococci. *Br. Med. J.* **i**, 124-125.

**Jin, T., Bokarewa, M., Foster, T. J., Mitchell, J., Higgins, J. & Tarkowski, A. (2004).** *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J. Immunology* **172**, 1169-1176.

**Jorgensen, M., Givney, R., Pegler, M., Vickery, A. & Funnell, G. (1996).** Typing multidrug-resistant *Staphylococcus aureus*: conflicting epidemiological data produced by genotypic and phenotypic methods clarified by phylogenetic analysis. *J. Clin. Microbiol.* **34**, 398-403.

**Josefsson, E., Juuti, K., Bokarewa, M. & Pentti, K. (2004).** The surface protein Pls of methicillin-resistant *Staphylococcus aureus* is a virulence factor in septic arthritis. Abstract no PA12. *The 11th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI)*, 2004. Charleston, South Carolina.

**Juuti, K. M., SIinha, B., Werbicl, C., Peters, G. & Kuusela, P. I. (2004).** Reduced adherence and host cell invasion by methicillin-resistant *Staphylococcus aureus* expressing the surface protein Pls. *J. Infect. Dis.* **189**, 1574-1584.

**Kaneko, J., Kimura, T., Kawakami, Y., Tomita, T. & Kamio, Y. (1997).** Panton-Valentine Leukocidin genes in a phage-like particle isolated from mitomycin C-treated *Staphylococcus aureus* V8 (ATCC 49775). *Biosci. Biotech. Biochem.* **67**, 1960-1962.

**Kaneko, J., Kimura, T., Narita, S., Tomita, T. & Kamio, Y. (1998).** Complete nucleotide sequence and molecular characterisation of the temperate staphylococcal bacteriophage ϕPVL carrying Panton-Valentine leukocidin genes. *Gene* **215**, 57-67.

**Katayama, Y., Ito, T. & Hiramatsu, K. (2000).** A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus. Antimicrob. Agents Chemother.* **44**, 1549-1555.

**Katayama, Y., Ito, T. & Hiramatsu, K. (2001).** Genetic organization of the chromosome region surrounding *mecA* in clinical staphylococcal strains: role of IS*431*-mediated *mecI* deletion in expression of resistance in *mecA*-carrying, low-level methicillin-resistant *Staphylococcus haemolyticus. Antimicrob. Agents Chemother.* **45**, 1955-1963.

**Katayama, Y., Takeuchi, F., Ito, T., Ma, X. X., Ui-Mizutani, Y., Kobayashi, I. & Hiramatsu, K. (2003).** Identification in methicillin-susceptible *Staphylococcus hominis* of an active primordial mobile genetic element for the staphylococcal cassette chromosome *mec* of methicillin-resistant *Staphylococcus aureus. J. Bacteriol.* **185**, 2711-2722.

**Kerr, S., Kerr, G. E., Mackintosh, C. A. & Marples, R. R. (1990).** A survey of methicillin-resistant *Staphylococcus aureus* affecting patients in England and Wales. *J. Hosp. Infect.* **16**, 35-48.

**Kim, M. N., Pai, C. H., Woo, J. H., Ryu, J. S. & Hiramatsu, K. (2000).** Vancomycin-intermediate *Staphylococcus aureus* in Korea. *J. Clin. Microbiol.* **38**, 3879-3881.

**Klimek, J. J., Marsik, F. J., Bartlett, R. C., Weir, B., Shea, P. & Quintiliani, R. (1976).** Clinical, epidemiological and bacteriological observations of an outbreak of methicillin-resistant *Staphylococcus aureus* at a large community hospital. *Am. J. Med.* **61**, 340-345.

**Kloss, P., Xiong, L., Shinabarger, D. L. & Mankin, A. S. (1999).** Resistance mutations in 23 S rRNA identify the site of action of the protein synthesis inhibitor linezolid in the ribosomal peptidyl transferase center. *J. Mol. Microbiol.* **294**, 93-101.

**Ko, K. S., Lee, J. I., Suh, J. Y., Oh, W. S., Peck, K. R., Lee, N. Y. & Song, J. H. (2005).** Distribution of major genotypes among methicillin-resistant *Staphylococus aureus* clones in Asian countries. *J. Clin. Microbiol.* **43**, 421-426.

**Kondo, I. & Fujise, K. (1977).** Serotype B staphylococcal bacteriophages singly converting staphylokinase. *Infect. Immun.* **18**, 266-272.

**Kondo, I., Itoh, S. & Yoshizawa, Y. (1981).** Staphylococcal phages mediating the lysogenic conversion of staphylokinase. In *Staphylococci and Staphylococcal Infections*, pp. 357-362. Edited by J. Jeljaszewicz. New York, Gustav Fischer Verlag.

**Koreen, L., Ramaswamy, S. V., Graviss, E. A., Naidich, S., Musser, J. M. & Kreiswirth, B. N. (2004).** spa typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macro-variation. *J. Clin. Microbiol.* **42**, 792-799.

**Kreiswirth, B., Kornblum, J., Arbeit, R. D., Eisner, W., Maslow, J. N., McGeer, A., Low, D. E. & Novick, R. P. (1993).** Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* **259**, 227-230.

**Kreiswirth, B. N., Lutwick, S. M., Chapnick, E. K., Gradon, J. D., Lutwick, L. I., Sepkowitz, D. V., Eisner, W. & Levi, M. H. (1995).** Tracing the spread of methicillin-resistant *Staphylococcus aureus* by Southern blot hybridisation using gene-specific probes of *mec* and Tn*554*. *Microb. Drug Resist.* **1**, 307-313.

**Kuhl, S. A., Pattee, P. A. & Baldwin, J. N. (1978).** Chromosomal map location of the methcillin-resistant determinant in *Staphylococcus aureus*. *J. Bacteriol.* **135**, 460-465.

**Kuroda, H., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, Y., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, T. & Hiramatsu, K. (2001).** Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225-1239.

**Kuwahara-Arai, K., Kondo, N., Hori, S., Tateda-Suzuki, E. & Hiramatsu, K. (1996).** Suppression of methicillin resistance in a *mecA*-containing pre-methicillin-resistant *Staphylococcus aureus* strain is caused by the *mecI*-mediated repression of PBP 2' production. *Antimicrob. Agents Chemother.* **40**, 2680-2685.

**Lacey, R. W. & Grinsted, J. (1973).** Genetic analysis of methicllin-resistant strains of *Staphylococcus aureus*: evidence for their evolution from a single clone. *J. Med. Microbiol.* **6**, 511-526.

**Lahteenmaki, K., Kuusela, P. & Korhonen, T. K. (2001).** Bacterial plasminogen activators and receptors. *FEMS Microbiol. Reviews* **25**, 531-552.

**Lee, C. Y. & Iandolo, J. J. (1986).** Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a into the lipase structural gene. *J. Bacteriol.* **166**, 385-391.

**Lewis, R. A. & Dyke, K. G. (2000).** MecI represses synthesis from the beta-lactamase operon of *Staphylococcus aureus*. *J. Antimicrob. Agents Chemother.* **45**, 139-144.

**Lim, T. T., Chong, F. N., O'Brien, F. G. & Grubb, W. B. (2003).** Are all community methicillin-resistant *Staphylococcus aureus* related? A comparison of their *mec* regions. *Pathology* **35**, 336-343.

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

**Lowy, F. D. (1998).** *Staphylococcus aureus* Infections. *N. Engl. J. Med.* **339**, 520-532.

**Luong, T. T. & Lee, C. Y. (2002).** Overproduction of type 8 capsular polysaccharide augments *Staphylococcus aureus* virulence. *Infect. Immun.* **70**, 3389-3395.

**Luong, T. T., Ouyang, S., Bush, K. & Lee, C. Y. (2002).** Type 1 capsule genes of *Staphylococcus aureus* are carried in a staphylococcal cassette chromosome genetic element. *J. Bacteriol.* **184**, 3623-3629.

**Lyon, B. R., May, J. W. & Skurray, R. A. (1984).** Tn*4001*: a gentamicin and kanamycin resistance transposon in *Staphylococcus aureus*. *Mol. Gen. Genet.* **193**, 554-556

**Lyon, B. R., Gillespie, M. T. & Skurray, R. A. (1987).** Detection and charcterisation of IS*256*, an insertion sequence in *Staphylococcus aureus*. *J. Gen. Microbiol.* **133**, 3031-3038.

**Ma, X. X., Ito, T., Tiensasitorn, C., Jamklang, M., Chongtrakool, P., Boyle-Vavra, S., Daum, R. S. & Hiramatsu, K. (2002).** Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob. Agents Chemother.* **46**, 1147-1152.

**Maguire, G. P., Arthur, A. D., Boustead, P. J., Dwyer, B. & Currie, B. J. (1998).** Clinical experiences and outcomes of community-acquired and nosocomial methicillin-resistant *Staphylococcus aureus* in a northern Australian hospital. *J. Hosp. Infect.* **38**, 273-281.

**Mahillon, J. & Chandler, M. (1998).** Insertion sequences. *Microbiol. Molecular Biology Revs.* **62**, 725-774.

**Mahillon, J., Leonard, C. & Chandler, M. (1999).** IS elements as constituents of bacterial genomes. *Res. Microbiol.* **150**, 675-687.

**Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. & Spratt, B. G. (1998).** Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. U S A* **95**, 3140-3145.

**Maki, H. & Murakami, K. (1997).** Formation of potent hybrid promoters of the mutant llm gene by IS*256* transposition in methicillin-resistant *Staphylococcus aureus*. *J. Bacteriol.* **179**, 6944-6948.

**Marples, R. R. & Cooke, E. M. (1985).** Workshop on methicillin-resistant *Staphylococcus aureus*. *J. Hosp. Infect.* **6**, 342-348.

**Marples, R. R., Richardson, J. F. & De Saxe, M. J. (1986).** Bacteriological characters of strains of *Staphylococcus aureus* submitted to a reference laboratory related to methicillin resistance. *J. Hygiene* **96**, 217-223.

**Marrack, P. & Kappler, J. (1990).** The staphylococcal enterotoxins and their relatives. *Science* **248**, 705-711.

**McClelland, M., Sanderson, J., Speith, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, J., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, W., Miller, T., Stoneking, T., Nhan, M., Waterston, R. & Wilson, R. (2001).** Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**, 852-856.

**McCormick, J. K., Yarwood, J. M. & Schlievert, P. M. (2001).** Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol.* **55**, 77-104.

**McDonald, P., Mitchell, E., Johnson, H., Rossney, A., Humphreys, H., Glynn, G., Burd, M. & Doyle, D. M., R. (2002).** MRSA bacteraemia: North/South study of MRSA in Ireland 1999. *J. Hosp. Infect.* **52**, 288-291.

**McDougal, L. K., Steward, C. D., Killgore, G. E., Chaitram, J. M., McAllister, S. K. & Tenover, F. C. (2003).** Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J. Clin. Microbiol.* **41**, 5113-5120.

**McGechie, D. & Daley, D. (2000).** Typing of methicillin-resistant *Staphylococcus aureus* (MRSA) assists in identifying epidemic MRSA (EMRSA) strains and enables control of spread in hospitals. Abstract no. 13. The *9th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI), 2000.* Kolding, Denmark.

**McKinney, T. K., Sharma, V. K., Craig, W. A. & Archer, G. L. (2001).** Transcription of the gene mediating methicillin resistance is *Staphylococcus aureus* (*mecA*) is corepressed but not coinduced by cognate *mecA* and beta-lactamase regulators. *J. Bacteriol.* **183**, 6862-6868.

**Melter, O., Aires de Sousa, M., Urbaskova, P., Jakubu, V., Zemlickova, H. & de Lencastre, H. (2003).** Update on the major clonal types of methicillin-resistant *Staphylococcus aureus* in the Czech Republic. *J. Clin. Microbiol.* **41**, 4998-5005.

**Mollby, R. (1983).** Isolation and properties of membrane damaging toxins. In *Staphylococci and staphylococcal infections*, pp. 619-662. Edited by C. Easmon, & C. Adlam. London, Academic Press.

**Mongkolrattanothai, K., Boyle, S., Kahana, M. D. & Daum, R. S. (2003).** Severe *Staphylococcus aureus* infections caused by clonally related community-acquired methicillin-susceptible and methicillin-resistant isolates. *Clin. Infect. Dis.* **37**, 1050-1058.

**Mongkolrattanothai, K., Boyle, S., Murphy, T. V. & Daum, R. S. (2004).** Novel non-*mecA*-containing staphylococcal chromosomal cassette composite island containing *pbp4* and *tagF* genes in a commensal staphylococcal species: a possible reservoir for antibiotic resistance islands in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **48**, 1823-1836.

**Montesinos, I., Salido, E., Delgado, T., Cuervo, M. & Sierra, A. (2002).** Epidemiological genotyping of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis at a university hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms. *J. Clin. Microbiol.* **40**, 2119-2125.

**Monzon-Moreno, C., Aubert, S., Morvan, A. & El Sohl, N. (1991).** Usefulness of three probes in typing isolates of methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol.* **35**, 80-88.

**Moore, P. C. & Lindsay, J. A. (2002).** Molecular characterisation of the dominant UK methicillin-resistant *Staphylococcus aureus* strains, EMRSA-15 and EMRSA-16. *J. Med. Microbiol.* **51**, 516-521.

**Morgan, M. G. & Harte-Barry, M. J. (1989).** Methicillin-resistant *Staphylococcus aureus*: a ten year survey in a Dublin hospital. *J. Hosp. Infect.* **14**, 357-362.

**Morvan, A., Aubert, S., Godard, S. & El Solh, N. (1997).** Contribution of a typing method based on IS*256* probing of *Sma*I digested cellular DNA for discrimination of European phage type 77 methicillin-resistant *Staphylococcus aureus* strains. *J. Clin. Micro.* **35**, 1415-1423.

**Murchan, S. & the EARSS Steering Group (2002).** Three years of the European Antimicrobial Resistance Surveillance System in Ireland, 1999-2001. [Online] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/d572.PDF

**Murchan, S., Kaufmann, M. E., Deplano, A., de Ryck, R., Struelens, M., Zinn, C. E., Fussing, V., Salmenlinna, S., Vuopio-Varkila, J., El Solh, N., Cuny, C., Witte, W., Tassios, P. T., Legakis, N., van Leeuwen, W., van Belkum, A., Vindel, A., Laconcha, I., Garaizar, J., Haeggman, S., Olsson-Liljequist, B., Ransjo, U., Coombes, G. & Cookson, B. (2003).** Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J. Clin. Microbiol.* **41**, 1574-1585.

**Musser, J. M. & Kapur, C. (1992).** Clonal analysis of methicillin-resistant *Staphylococcus aureus* strains from intercontinental sources: association of the *mec* gene with divergent phylogenetic lineages implies dissemination by horizontal transfer and recombination. *J. Clin. Microbiol.* **30**, 2058-2063.

**Nguyen, T., Ghebrehiwet, B. & Peerschke, E. E. (2000).** *Staphylococcus aureus* protein A recognises platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets. *Infect. Immun.* **68**, 2061-2068.

**Noble, W.C., Virani, Z. & Cree, R.G. (1992).** Co-transfer of vancomycin and other resistant genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol. Lett. 72, 195-198.

**North, E. A. & Christie, R. (1946).** Acquired resistance of staphylococci to the action of penicillin. *Med. J. Aust.* **1**, 176-179.

**Norvick, R. P. (1967).** Properties of a cryptic high-frequency transducing phage in *Staphylococcus* aureus. *Virology* **33**, 155-166.

**O'Brien, A. D. & Holmes, R. K. (1987).** Shiga and shiga-like toxins. *Microb. Reviews* **51**, 206-220.

**O' Brien, F. G., Coombs, G., Christiansen, K. & Grubb, W. B. (2004a).** Distrubition of the staphylococcal cassette chromosome *mec* type V in community methicillin-resistant *Staphylococcus aureus* isolated in Australia. Abstract no. MG07. The *11th international symposium on Staphylococcal and Staphylococci Infections (ISSSI), 2004.* Charleston, South Carolina, USA

**O' Brien, F. G., Lim, T.T., Chong, F.N., Coombs, G.W., Enright, M.C., Robinson, D.A., Monk, A., Said-Salim, Kreiswirth, B.N. and Grubb, W.B. (2004b).** Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia. *J. Clin. Micro.* 42. 3185-3190.

**O'Neill, G. L., Murchan, S., Gil-Setas, A. & Aucken, H. M. (2001).** Identification and chracterisation of phage variants of a strain of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA-15). *J. Clin. Micro.* **39**, 1540-1548.

**Ogston, A. (1882).** Micrococcus poisoning. *J. Anat.* **17**, 24-58.

**Ohnishi, M., Kurokawa, K. & Hayashi, T. (2001).** Diversification of *Escherichia coli* genomes: are bacteriophages the major contributors. *Trends Microbiol.* **9**, 481-485.

**Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Coombs, G. W., Pearman, J. W., Tenover, F. C., Kapi, M., Tiensasitorn, C., Ito, T. & Hiramatsu, K. (2002).** Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J. Clin. Microbiol.* **40**, 4289-4294.

**Oliveira, D., Santos-Sanches, I., Mato, R., Tamayo, M., Ribeiro, G., Costa, D. & de Lencastre, H. (1998).** Virtually all methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the largest Portuguese teaching hospital are caused by two internationally spread multiresistant strains: the 'Iberian' and the 'Brazilian' clones of MRSA. *Clin. Microbiol. Infect.* **4**, 373-384.

**Oliveira, G. A., Aquila, A. M., Masiero, R. A., Levy, C., Gomes, S. G., Cui, L., Hiramatsu, K. & Mamizuka, E. M. (2001a).** Isolation in Brazil of nosocomial *Staphylococcus aureus* with reduced susceptibility to vancomycin. *Infect. Control Hosp. Epidemiol.* **22**, 443-448.

**Oliveira, D. C., Crisostomo, I., Santos-Sanches, I., Major, P., Alves, C. R., Aires-de-Sousa, M., Thege, M. K. & de Lencastre, H. (2001b).** Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* **39**, 574-580.

**Oliveira, D. C., Tomasz, A. & de Lencastre, H. (2001c).** The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated *mec* elements. *Microb. Drug Resist.* **7**, 349-361.

**Oliveira, D. C. & de Lencastre, H. (2002a).** Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **46**, 2155-2161.

**Oliveira, D. C., Tomasz, A. & de Lencastre, H. (2002b).** Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Lancet Infect. Dis.* **2**, 180-189.

**Palmqvist, N., Foster, T. J., Tarkowski, A. & Josefsson, E. (2002).** Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microbial Pathogenesis* **33**, 239-249.

**Palmqvist, N., Patti, J. M., Tarkowski, A. & Josefsson, E. (2004).** Expression of staphylococcal clumping factor A impedes macrophage phagocytosis. *Microbes and Infection* **6**, 188-195.

**Panlilio, A. L., Culver, D. H., Gaynes, R. P., Banerjee, S., Henderson, T. S., Tolson, J. S. & Martone, W. J. (1992).** Methicillin resistant *Staphylococcus aureus* in U.S. hospitals, 1975-1991. *Infect. Control Hosp. Epidemiol.* **13**, 582-586.

**Parker, M. (1972).** Phage-typing of *Staphylococcus aureus*. In Methods in Microbiology vol 7B. pp. 1-28. Edited by J.F. Norris and D.W. Ribbons. London, Academic Press.

**Peacock, S. J., de Silva, G. D., Justice, A., Cowland, A., Moore, C. E., Winearls, C. G. & Day, N. P. (2002).** Comparison of multilocus sequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* isolates in a microepidemiological setting. *J. Clin. Microbiol.* **40**, 3764-3770.

**Perez-Roth, E., Lorenzo-DAiz, F., Batista, N., Moreno, A. & Mendez-Alvarez, S. (2004).** Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998-2002) in a Spanish hospital. *J. Clin. Microbiol.* **42**, 4649-4656.

**Pfizer (2004).** International perspectives on health care and biomedical research; emerging crisis in infectious disease; challenges for the 21st century. *The Pfizer Journal-Global edition* [Online] http://www.thepfizerjournal.com/pdfs/TPJ38.pdf

**Piriz Duran, S., Keyser, F. H. & Berger-Bachi, B. (1996).** Impact of *sar* and *agr* on methicillin resistance in *Staphylococcus aureus*. *FEMS Microbiol. Letts.* **141**, 255-260.

**Pitt, T. L. (1999).** Molecular typing in practice. *J. Hosp. Infect.* **43**, S85-S88.

**Ploy, M. C., Grelaud, C., Martin, C., de Lumley, L. & Denis, F. (1998).** First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. *Lancet* **351**, 1212.

**Popoff, M., Boquet, P., Gill, D. M. & Eklund, M. W. (1990).** DNA sequence of exoenzyme C3, an ADP-ribosyltransferase encoded by *Clostridium botulinum* C and D phages. *Nucleic Acid Res.* **18**, 1291.

**Projan, S. J. & Novick, R. P. (1997).** The molecular basis of pathogenicity. In: The *staphylococci in human diseases.* pp. 55-81. Edited by G. Archer & K. B. Crossley. London, Churchill Livingstone.

**Prudhomme, M., Turlan, C., Claverys, J. P. & Chandler, M. (2002).** Diversity of Tn*4001* transposition products: the flanking IS*256* elements. *J. Bacteriol.* **184**, 433-443.

**Reacher, M. H., Shah, A., Livermore, D. M., Wale, M. C., Graham, C. & Johnson, A. P. (2000).** Bacteraemia and antibiotic resistance of its pathogens reported in England and Wales between 1990-1998: trend analysis. *Br. Med. J.* **320**, 213-216.

**Reynolds, P. E. & Brown, D. G. J. (1985).** Penicillin-binding proteins of beta-lactam resistant strains of *Staphylococcus aureus*: effect of growth conditions. *FEBS Lett.* **192**, 28-32.

**Rhem, M. N., Lech, E. M., Patti, J. M., McDevitt, D., Hook, M., Jones, D. B. & Wilhelmus, K. R. (2000).** The collagen-binding adhesion is a virulence factor in *Staphylococcus aureus* ketatitis. *Infect. Immun.* **68**, 3776-3779.

**Rice, L. B. & Marshall, S. H. (1994).** Insertion of IS*256*-like element flanking the chromosomal beta-lactamase gene of *Enterococcus faecalis* CX19. *Antimicrob. Agents Chemother.* **38**, 693-701.

**Rice, L. B. & Carias, L.L. (1998).** Transfer of Tn*5385*, a composite, multiresistance chromosomal element from *Enterococcus faecalis*. *J. Bacteriol.* **180**, 714-721.

**Rice, L. B., Carias, L.L. and Marshall, S. (1995).** Tn*5384*, a composite enterococcal mobile element conferring resistance to erythromycin and gentamicin whose ends are directly repeated copies of IS*256*. *Antimicrob. Agents Chemother.* **39**, 1147-1153.

**Richardson, J. F. & Reith, S. (1993).** Characterisation of a strain of methicillin-resistant *Staphylococcus aureus* affecting patients in England and Wales. *J. Hosp. Infect.* **22**, 45-52.

**Richardson, J. F., Rosdahl, V. T., van Leeuwen, W. J., Vicekery, A. M., Vindel, A. & Witte, W. (1999).** Phages for methicillin-resistant *Staphylococcus aureus*: an international trial. *Epidemiol. Infect.* **122**, 227-233.

**Rings Terry, R. F. & Lang, S. (1998).** Ethnicity and methicillin-resistant *Staphylococcus aureus* in South Auckland. *N. Z. Med. J.* **24**, 151.

**Roberts, R. B., de Lencastre, A., Eisner, W., Severina, E. P., Shopsin, B., Kreiswirth, B. N. & Tomasz, A. (1998).** Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in 12 New York hospitals. MRSA Collaborative Study Group. *J. Infect. Dis.* **178**, 164-171.

**Roberts, R. B., Chung, M., de Lencastre, H., Hargrave, J., Tomasz, A., Nicolau, D. P., John, J., J.F. & Korzeniowski, O. (2000).** Distribution of methicillin-resistant *Staphylococcus aureus* clones among health care facilities in Connecticut, New Jersey, and Pennsylvania. *Microb. Drug Resist.* **6**, 245-251.

**Robinson, D. A. & Enright, M. C. (2003).** Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**, 3926-3934.

**Robinson, D. A. & Enright, M. C. (2004a).** Evolution of *Staphylococcus aureus* by large chromosomal replacements. *J. Bacteriol.J* **186**, 1060-1064.

**Robinson, D. A. & Enright, M. C. (2004b).** Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect.* **10**, 92-97.

**Roche, F., Meehan, M. & Foster, T. J. (2003).** The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. *Microbiology* **149**, 2759-2767.

**Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H. & Bergi-Bachi, B. (1999).** The essential *Staphylococcus aureus* gene *fmhB* is involved in the first step of peptidoglycan pentaglycine interpeptide formation. *Proc. Natl. Acad. Sci. U S A* **96**, 9351-9356.

**Rossney, A. S., Coleman, D. C. & Keane, C. T. (1994a).** Antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol.* **41**, 430-440.

**Rossney, A. S., Coleman, D. C. & Keane, C. T. (1994b).** Evaluation of an antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol.* **41**, 441-447.

**Rossney, A. S. (1995).** Methicillin-resistant *Staphylococcus aureus* in four Dublin Hospitals (1988-1994). Ph.D thesis. *Department of Clinical Microbiology*, University of Dublin, Trinity College, Dublin, Ireland

**Rossney, A. S. & Keane, C. T. (2002a).** Strain variation in the MRSA population over a 10-year period in one Dublin hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**, 123-126.

**Rossney, A. S. & Keane, C. T. (2002b).** Epidemiological typing of Irish MRSA isolates from blood cultures: 1999 to 2002. Abstract no. 324. *The 10th International Symposium on Staphylococci and Staphylococcal infections (ISSSI)*, 2002. Tsukaba, Japan.

**Rossney, A. (2003a).** Confirmation of hetero-glycopeptide-intermediate *Staphylococcus aureus* among Irish methicillin-resistant *Staphylococcus aureus* blood culture isolates. *Epi-Insight*, **4**, 4. November 2003. [Online] http://www.ndsc.ie/Publications/EPI-Insight/

**Rossney, A. (2003b).** A new strain of MRSA imported from Singapore. *Epi-Insight* **4**, 1. March 2003. [Online] http://www.ndsc.ie/Publications/EPI-Insight/

**Rossney, A. S., McDonald, P., Humphreys, H., Glynn, G. M. & Keane, C. T. (2003).** Antimicrobial resistance and epidemiological typing of methicillin-resistant *Staphylococcus aureus* in Ireland (North and South), 1999. *Eur. J. Clin. Microbiol. Infect. Dis.* **22**, 379-381.

**Rossney, A. S., Lawrence, M. J., Morgan, P. M., Fitzgibbon, M. M. & O' Connell, B. (2004).** Epidemiological Typing of Irish MRSA Isolates from Blood Cultures: 1999 to 2003. Abstract no. ME24. The *11th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI)*, 2004. Charleston, South Carolina, USA.

**Rossney, A. S., Morgan, P. M. & O' Connell, B. (2004).** Community-acquired MRSA and panton-valentine leucocidin in Ireland: a preliminary report. *Epi-Insight*, **6.** 4. March 2005. [Online] http://www.ndsc.ie/Publications/EPI-Insight/

**Sa-Leao, R., Santos Sanches, I., Dias, D., Peres, I., Barros, R. M. & de Lencastre, H. (1999).** Detection of an archaic clone of *Staphylococcus aureus* with low-level resistance to methicillin in a pediatric hospital in Portugal and in international samples: relics of a formerly widely disseminated strain? *J. Clin. Microbiol.* **37**, 1913-1920.

**Sader, H. S., Streit, J. M., Fritsche, T. R. & Jones, R. N. (2004).** Antimicrobial activity of daptomycin against multidrug-resistant Gram-positive strains collected worldwide. *Diagn. Microbiol. Infect. Dis.* **50**, 201-204.

**Salmenlinna, S., Lyytikainen, O. & Vuopio-Varkila, J. (2002).** Community-acquired methicillin-resistant *Staphylococcus aureus*, Finland. *Emerg. Infect. Dis.* **8**, 602-607.

**Sambrook, J. & Russell, D. W. (2001).** *Molecular cloning: a laboratory manual.* 3rd edition. New York: Cold Spring Harbour Laboratory.

**Sanches, I. S., Ramirez, M., Troni, H., Abecassis, M., Padua, M., Tomasz, A. & de Lencastre, H. (1995).** Evidence for the geographic spread of a methicillin-resistant *Staphylococcus aureus* clone between Portugal and Spain. *J. Clin. Microbiol.* **33**, 1243-1246.

**Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467.

**Saunders, N. A., Underwood, A., Kearns, A. M. & Hallas, G. (2004).** A virulence-associated gene microarray: a tool for investigation of the evolution and pathogenic potential of *Staphylococcus aureus*. *Microbiology* **150**, 3763-3771.

**Savolainen, K. L., Paulin, B., Westerlund-Wilkstrom, Foster, T. J., Korhonen, T. K. & Kuusela, P. (2001).** Expression of *pls*, a gene closely associated with the *mecA* gene of methicillin-resistant *Staphylococcus aureus*, prevents bacterial adhesion in vitro. *Infect. Immun.* **69**, 3013-3020.

**Schmitz, F. J., Steiert, M., Tichy, H. V., Hofmann, B., Verhoef, J., Heinz, H. P., Kohrer, K. & Jones, M. E. (1998).** Typing of methicillin-resistant *Staphylococcus aureus* isolates from Dusseldorf by six genotypic methods. *J. Med. Microbiol.* **47**, 341-351.

**Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986).** Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51**, 873-884.

**Shanson, D. C. (1981).** Antibiotic resistant *Staphylococcus aureus*. *J. Hosp. Infect.* **2**, 11-36.

**Sharma, V. K., Hackbarth, C. J., Dickinson, T. M. & Archer, G. L. (1998).** Interactions of native and mutant MecI repressors with sequences that regulate *mecA*, the gene enconding penicillin binding protein 2a in methicillin-resistant *Staphylococcus aureus*. *J. Bacteriol.* **180**, 2160-2166.

**Sheagren, J. N. (1984a).** *Staphylococcus aureus*: the persistent pathogen (first of two parts). *N. Engl. J. Med.* **310**, 1368-1373.

**Sheagren, J. N. (1984b).** *Staphylococcus aureus*: the persistent pathogen (second of two parts). *N. Engl. J. Med.* **310**, 1437-1442.

**Shinabarger, D. L. (1999).** Mechanism of action of oxazolidinone antibacterial agents. *Expert Opin. Investig. Drugs* **8**, 1195-1202.

**Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., Bost, D. A., Riehman, M., Naidich, S. & Kreiswirth, B. N. (1999).** Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **37**, 3556-3563.

**Shukla, S. K., Ramaswamy, S. V., Conradt, J., Stemper, M. E., Reich, R., Reed, K. D. & Graviss, E. A. (2004a).** Novel Polymorphisms in *mec* genes and a new *mec* complex type in methicillin-resistant *Staphylococcus aureus* isolates obtained in rural Wisconsin. *Antimicrob. Agents Chemother.* **48**, 3080-3085.

**Shukla, S. K., Stemper, M. E., Ramaswamy, S. V., Conradt, J. M., Reich, R., Graviss, E. A. & Reed, K. D. (2004b).** Molecular characteristics of nosocomial and native American community-associated methicillin-resistant *Staphylococcus aureus* clones from rural Wisconsin. *J. Clin. Micro* **42**, 3752-3757.

**Sieradzki, K., Pinho, M. G. & Tomasz, A. (1999).** The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. *N. Engl. J. Med.* **340**, 517-523.

**Silverman, J. A., Permutter, N. G. & Shapiro, H. M. (2003).** Correlation of daptomycin bactericidal activity and membrane depolarisation in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**, 2538-2544.

**Smith, H. W. & Huggins, R. B. (1983).** Effectiveness of phages in treating experimental *E. coli* diarrhoea in calves, piglets and lambs. *J. Gen. Microbiol.* **129**, 2659-2675.

**Smith, H. W., Green P. and Parsell, Z. (1983).** Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. *J. Gen. Microbiol.* **129**, 3121-3137.

**Smith, H. W. & Huggins, R.B. (1987).** The control of experimental diarrhea in calves by means of bacteriophage. *J. Gen. Microbiol.* **133**, 1111-1126.

**Smith, T. L., Pearson, M. L., Wilcox, K. R., Cruz, C., Lancaster, M. V., Robinson-Dunn, B., Tenover, F. C., Zervos, M. J., Band, J. D., White, E. & Jarvis, W. R. (1999).** Emergence of vancomycin resistance in *Staphylococcus aureus*. Glycopeptide-Intermediate *Staphylococcus aureus* Working Group. *N. Engl. J. Med.* **340**, 493-501.

**Smoot, J. C., Barbian, K. D., Van Gompel, J. J., Smoot, L. M., Chaussee, M. S., Sylva, G. L., Sturdevant, D. E., Ricklefs, S. M., Porcella, S. F., Parkins, L. D., Beres, S. B., Campbell, D. S., Smith, T. M., Zhang, Q., Kapur, V., Daly, J. A., Veasy, L. G. & Musser, J. M. (2002).** Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. *Proc. Nat. Acad. Sci. U.S.A.* **99**, 4688-4673.

**Song, M. D., Wachi, M., Doi, M., Ishino, F. & Matsuhashi, M. (1987).** Evolution of an inducible penicillin-target protein in methicillin-resiatant *Staphylococcus aureus* by gene fusion. *FEBS lett.s* **221**, 167-171

**Soothill, J. S. (1992).** Treatment of experimental infections of mice with bacteriophages. *Med. Micrbiol.* **37**, 258-261.

**Soothill, J. S. (1994).** Bacteriophage destruction of skin grafts by *Pseudomonas aeruginosa.* Burns **(20) 209-211.**

**Southern, E. M. (1975).** Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Microbiol.* **98**, 503-517.

**Speller, D. C. E., Johnson, A. P., James, D., Marples, R. R. & George, R. C. (1997).** Resistance to methicillin and other antibiotics of *Staphylococcus aureus* from blood and cerebrospinal fluid, England and Wales, 1989-95. *Lancet* **350**, 323-325.

**Stacey, A. R., Endersby, K. E., Chan, P. C. & Marples, R. R. (1998).** An outbreak of methicillin-resistant *Staphylococcus aureus* infection in a rugby football team. *Br. J. Sports. Med.* **32**, 153-154.

**Steering Group of the North/South Survey of MRSA in Ireland (2000).** North/ South Survey of MRSA in Ireland 1999: Recognition of a New Variant Among this MRSA population. Abstract no. 182. *The 9th International Symposium on Staphylococci and Staphylococcal Infections*, 2000. Kolding, Denmark.

**Stemper, M. E., Shukla, S. K. & Reed, K. D. (2004).** Emergence and spread of community-associated methicillin-resistant *Staphylococcus aureus* in rural Wisconsin, 1989 to 1999. *J. Clin. Microbiol.* **42**, 5673-5680.

**Stobberingh, E. E. & Winkler, K. C. (1977).** Restriction-deficient mutants of *Staphylococcus aureus*. *J. Gen. Microbiol.* **99**, 359-367.

**Stone, P. W., Larson, E. & Kawar, L. N. (2002).** A systematic audit of economic evidence linking nosocomial infections and infection control interventions. *Am. J. Infect. Control* **30**, 145-152.

**Stranden, A. M., Ehlert, K., Labischinski, H. & Bergi-Bachi, B. (1997).** Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a *femAB* null mutant of methicillin-resistant *Staphylococcus aureus*. *J. Bacteriol.* **179**, 9-16.

**Strockbine, N. A., Marques, L. R. M., Newland, J. W., Smith, H. W., Holmes, R. K. & O'Brien, A. D. (1986).** Two toxin converting phages from *Escherichia coli* O157 strain 933 encode antigenically distinct toxins with similiar biological activities. *Infect. Immun.* **53**, 135-140.

**Tedesco, K. L. & Rybak, M. J. (2004).** Daptomycin. *Pharmacotherapy* **24**, 41-57.

**Teixeira, L. A., Resende, C. A., Ormonde, L. R., Rosenbaum, R., Figueiredo, A. M., de Lencastre, H. & Tomasz, A. (1995).** Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. *J. Clin. Microbiol.* **33**, 2400-2404.

**Tenover, F. C., Arbeit, R., Archer, G., Biddle, J., Byrne, S., Goering, G., Hancock, G., Hebert, A., Hill, B., Hollis, R., Jarvis, W. R., Kresiwirth, B., Eisner, W., Maslow, J., McDougal, L. K., Miller, J. M., Mulligan, M. & Pfaller, M. A. (1994).** Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J. Clin. Microbiol.* **32**, 407-415.

**Tenover, F. C., Arbeit, R., Goering, R., Mickelsen, P.A., Murray, B.E., Persing , D.H. and Swaminathan, B. (1995).** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**, 2233-2239.

**Thomas, W. D. J. & Archer, G. L. (1989).** Mobility of gentamicin resistance genes in the United States. Identification of Tn*4031*, a gentamicin resistance trasnsposon from *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* **33**, 1335-1341.

**Thorne, G. M. & Alder, J. (2002).** Daptomycin: A novel lipopeptide antibiotic. *Clin. Microbiol. Newsletter* **24**, 33-39.

**Tsiodras, S., Gold, H. A., Sakoulas, G., Eliopoulos, G. M., Wennersten, C., Venkataraman, L., Moellering, R. C. & Ferraro, M. J. (2001).** Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet* **358**, 207-208.

**Turnidge, J. D. & Bell, J. M. (2000).** Methicillin-resistant *Staphylococcus aureus* evolution in Australia over 35 years. *Microb. Drug. Resist.* **6**, 223-229.

**Urwin, R. & Maiden, M. C. (2003).** Multi-locus sequence typing: a tool for global epidemiology. *Trends Microbiol.* **11**, 479-487.

**Van Belkum, A. (2000).** Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* strains: state of affairs and tomorrows possibilities. *Microb. Drug Resist.* **6**, 173-188.

**Van Belkum, A., Riewarts Eriksen, N. H., Sijmons, M., Van Leeuwen, W., Van den Berg, M., Kluytmans, F. & Verbrugh, H. (1997).** Coagulase and protein A polymorphism do not contribute to persistence of nasal colonization by *Staphylococcus aureus*. *J. Med. Microbiol.* **46**, 222-232.

**Van der Mee-Marquet, N., Domelier, A. S., Girard, N., Quentin, R. & The Blodstream Infection Study Group of the Relais d'Hygiene du Centre (2004).** Epidemiology and typing of *Staphylococcus aureus* strains isolated from bloodstream infections. *J. Clin. Microbiol.* **42**, 5650-5657.

**Van Griethuysen, A., Leyten, M., Van Belkum, A., Wannet, W. & Kluytmans, J. (2003).** Instability of *mecA* gene during storage of MRSA strains. Abstraact D-1896. The *43rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)*, 2003. Chicago, Illinois, USA

**Velazquez-Meza, M. E., Aires de Sousa, M., Echaniz-Aviles, G., Solorzano-Santos, F., Miranda-Novales, G., Silva-Sanchez, J. & de Lencastre, H. (2004).** Surveillance of methicillin-resistant *Staphylococcus aureus* in a pediatric hospital in Mexico city during a 7-year period (1997-2003): clonal evolution and impact of infection control. *J. Clin. Microbiol.* **42**, 3877-3880.

**Von Eiff, C. K., Becker, K., Machkam, K., Stammer, H. & Peters, G. (2001).** Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N. Engl. J. Med.* **344**, 11-16.

**Walcher-Salesse, S., Monzon-Moreno, C., Aubert, S. & El Solh, N. (1992).** An epidemiological assesment of coagulase-negative staphylococci (CNS) from an intensive care unit. *J. Med. Microbiol.* **36**, 321-331.

**Waldor, M. K. & Mekalanos, J. J. (1996).** Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910-1914.

**Walsh, T. R. & Howe, R. A. (2002).** The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. *Ann. Rev. Microbio.l* **56**, 657-675.

**Wang, J. T., Chen, Y. C., Yang, T. L. & Chang, S. C. (2002).** Molecular epidemiology and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* in Taiwan. *Diagn. Microbiol. Infect. Dis.* **42**, 199-203.

**Weller, T. M. (1999).** The distribution of *mecA, mecR1* and *mecI* and sequence analysis of *mecI* and the *mec* promoter region in staphylococci expressing resistance to methicillin. *J. Antimicrob. Chemother.* **43**, 15-22.

**Wielders, C., Box, A., Verhoef, J. & Fluit, A. (2003).** Great diversity within the staphylococcal chromosome cassette *mec* (SCC*mec*) types of methicillin-resistant staphylococci. Abstract no. A-068. The 103rd General Meeting of the American Society of Microbiology (ASM), 2003. Washington DC, USA.

**Wilson, P., Andrews, J. A., Charlesworth, R., Walesby, R., Singer, M., Farrell, D. J. & Robbins, M. (2003).** Linezolid resistance in clinical isolates of *Staphylococcus aureus*. *J. Antimicrob. Agents Chemother.* **51**, 186-191

**Winkler, K. C., de Waart, J. & Grootsen, C. (1965).** Lysogenic conversion of staphylococci to loss of beta-toxin. *J. Gen. Microbiol.* **39**, 321-333.

**Wisplinghoff, H., Rosato, A. E., Enright, M. C., Noto, M., Craig, W. & Archer, G. L. (2003).** Related clones containing SCC*mec* type IV predominate among clinically significant *Staphylococcus epidermidis* isolates. *Antimicrob. Agents Chemother.* **47**, 3574-3579.

**Witte, W., Enright, M., Schmitz, F. J., Cuny, C., Braulke, C. & Heuck, D. (2001).** Characteristics of a new epidemic MRSA in Germany ancestral to United Kingdom EMRSA 15. *Int. J. Med. Microbiol.* **290**, 677-682.

**Wu, S., de Lencastre, H. & Tomasz, A. (1998).** Genetic organisation of the *mecA* region in methicillin-susceptible and methicllin-resistant strains of *Staphylococcus sciuri*. *J. Bacteriol.* **180**, 236-242.

**Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M., Nakayama, K., Yamada, S., Hitoshi, K. & Sugai, M. (2000).** Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. *Mol. Microbiol.* **2000**, 694-705.

**Zhang, H. Z., Hackbarth, C. J., Chansky, K. M. & Chambers, H. F. (2001).** A proteolytic transmembrane signalling pathway and resistance to beta-lactams in staphylococci. *Science* **291**, 1962-1965.

**Ziebuhr, W., Krimmer, V., Rachid, S., Lobner, I., Gotz, F. & Hacker, J. (1999).** A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intracellular adhesion synthesis by alternating insertion and excision of the insertion sequence IS*256*. *Mol. Microbiol.* **32**, 345-356.

# Publication

extremity to the *ccr* complex (L-C region) but which both carry the downstream constant region (*dcs*) (25). Both SCC*mec* IVc and IVd also differ in their L-C regions (SCC*mec* IVc carries an integrated copy of Tn*4001* flanked on either side by IS*256*) (19). SCC*mec* IV has a type 2 *ccr* complex and class B *mec*, but Oliveira and colleagues described an SCC*mec* IV element with type 4 *ccr* (25, 32). A type IVA SCC*mec* element has also been described that harbors the integrated plasmid pUB110 (31).

Multilocus sequence typing (MLST), SCC*mec* typing, and other molecular techniques have shown that the majority of nosocomial MRSA infections are caused by relatively few pandemic clones that have evolved by the introduction of SCC*mec* elements into five distinct epidemic MSSA lineages (33, 36). Using MLST and the program eBURST, the five pandemic MRSA lineages can be visualized as clonal complexes (CCs), which are groups of genotypes and MLST sequence types (STs) that share a recent common ancestor (11). CCs are named after the ST of the ancestral genotype. The CCs of the major nosocomial MRSA lineages are CC5, CC8, CC22, CC30, and CC45 (10). In all of these lineages, except possibly CC22, SCC*mec* has been acquired on multiple occasions. All major international nosocomial MRSA clones belong to one of these five CCs. The names originally assigned to the MRSA clones represent either a unique epidemiological characteristic or signify the geographic area from which they were first isolated. A more rational and unambiguous scheme has been proposed based on ST and SCC*mec* type (10, 37). Using this nomenclature, some of the more common MRSA clones are named as follows: CC5, ST5-MRSA-II (New York/Japan) and ST5-MRSA-IV (Pediatric); CC8, ST239-MRSA-IIIA (Brazilian), ST239-MRSA-III (Hungarian), ST247-MRSA-IA (Iberian), ST250-MRSA-I (Archaic), ST8-MRSA-II (Irish-1), and ST8-MRSA-IV (EMRSA-2, EMRSA-6); CC22, ST22-MRSA-IV (EMRSA-15); CC30, ST36-MRSA-II (EMRSA-16); CC45, ST45-MRSA-IV (Berlin) (10, 32, 33).

It has been suggested that genetic relatedness of particular MRSA isolates should be investigated by determining both the genotype of the MSSA isolate into which the SCC*mec* element was introduced and the type of SCC*mec* element it harbors (10). MLST has been shown to be the most powerful molecular technique for genotyping *S. aureus* isolates in long-term and global epidemiological studies (10, 33). The SCC*mec* type can be characterized using various PCR-based techniques that identify both the *ccr* and *mec* gene type or other sequences in the J regions specific to each SCC*mec* element (29, 31).

The objective of the present study was the molecular characterization of representative clinical MRSA isolates recovered in Irish hospitals between 1971 and 2002, examining their genetic relatedness to each other and to internationally recognized MRSA clones using MLST and SCC*mec* element analysis. The results of this investigation identified seven novel SCC*mec* variants and two new *mec* classes, which were further characterized.

## MATERIALS AND METHODS

**Bacterial isolates.** A total of 172 MRSA isolates representative of the most prevalent phenotypes recovered in Irish hospitals during eight study periods between 1971 and 2002 were investigated (Table 1). Isolates were chosen to include at least two isolates of each type or subtype recognized during each study period. Prior to 1988 (study periods A, B, and C), isolates were classified into four phenotypes (termed early MRSA and phenotypes I, II, and III) based on a combination of antimicrobial resistance patterns, bacteriophage typing, plasmid screening, location of resistance determinants, and Southern hybridization analysis of plasmid and chromosomal DNA (4, 5). Phenotype I and phenotype II isolates were previously identified during study period B (1976 to 1984), but only phenotype II isolates were available for the present study. Isolates recovered after 1988 (study periods D through H) were characterized by a combination of techniques that included bacteriophage typing, restriction fragment length polymorphism (RFLP) analysis of SmaI-digested total cellular DNA using pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA analysis using PCR (RAPD-PCR), plasmid screening, enterotoxin profiling, biotyping (pigment production and hydrolysis of Tween 80 and urea), and antibiogram-resistogram (AR) typing (using a panel of 22 antimicrobials [the panel was extended to 23 in 1998]) (39–42, 44). In study periods D and E, isolates were assigned an AR type on the basis of the pattern of resistance to the antimicrobials in the AR typing panel in conjunction with additional data obtained from the phenotypic and genotypic investigations described above (39–41). Forty-four AR types (AR01 to AR44) and a number of subtypes have been recognized to date.

Isolates recovered between 1971 and 1998 were from Dublin hospitals only. Isolates from 1999 were recovered from patients in hospitals that participated in the North/South Study of MRSA in Ireland, a 2-week prevalence study of MRSA in both the Republic of Ireland and the North of Ireland (3, 44). Isolates of each AR type and subtype from both the North and South of Ireland were included. Ninety-eight percent of Irish hospitals participated in the North/South Study. The isolates recovered in 2002 were from blood culture specimens from those Irish hospitals ($n$ = 23) that participated in EARSS (43). This level of participation in EARSS represents a population cover rate of >90%. Details of study isolates are shown in Table 1.

Among isolates from 1999, a previously unfamiliar AR pattern, designated AR43, was predominant among isolates from the North of Ireland. AR43 isolates from six patients were recovered in mixed culture with MRSA isolates exhibiting other AR patterns (five AR13 and one AR14 pattern) (3, 44). Proportionally more isolates exhibiting AR patterns AR43, AR13, and AR14 were included in the present study to investigate this observation.

**Bacterial storage and culture conditions.** MRSA isolates were stored at −80°C in Protect bacterial preserver vials (Technical Services Consultants Ltd., Heywood, United Kingdom) and were routinely cultured onto trypticase soy agar (Oxoid Limited, Basingstoke, United Kingdom) prior to incubation overnight at 37°C.

**DNA isolation and amplification.** Chromosomal DNA was prepared from each isolate using the QIAGEN DNeasy kit system (QIAGEN, Crawley, West Sussex, United Kingdom) according to the manufacturer's instructions. DNA was amplified in a Thermo Hybaid Multiblock system thermal cycler (Thermo Hybaid, Ashford, Middlesex, United Kingdom).

**MLST.** MLST was performed on 130 isolates (Table 1) by PCR amplification of internal fragments of seven housekeeping genes by using a previously described procedure and primers (9). Sequencing of both DNA strands was performed using the ABI Big-Dye Fluorescent Terminator system and an ABI 3700 automated sequencer (Applied Biosystems, Warrington, United Kingdom) at the Genomics Facility at the University of Bath (Bath, United Kingdom). The alleles at each of the seven housekeeping loci were identified by comparing the sequences obtained from the test isolates with sequences held in the MLST database (http://www.saureus.mlst.net). This database was also used to identify the allelic profile and hence the ST of each isolate.

**SCC*mec* typing.** SCC*mec* elements from all 172 isolates included in this study were typed using two methods. Firstly, a previously reported method (29) described in the present study as the simplex method was used to amplify the cassette chromosome recombinase (*ccr*) and *mec* gene complexes (*ccr-mec* genes). The primers of Ito et al. and Robinson and Enright were used for amplification of the *ccr* and *mec* genes, respectively (17, 37). Secondly, the multiplex PCR method of Oliveira and de Lencastre, which utilizes primers designed specifically to distinguish different regions of each SCC*mec* type, was used as described previously (31).

**Nucleotide sequencing of SCC*mec*.** The entire nucleotide sequence of the SCC*mec* element from two isolates was determined. One isolate (AR13.1/3330.2) from study period G (1999) was chosen because it was representative of isolates with *ccr-mec* genes indicative of SCC*mec* II by the simplex PCR method but lacking one to three of the five amplimers typical of SCC*mec* II when analyzed by the multiplex PCR SCC*mec* typing method. The second isolate (AR43/3330.1) from study period G also was chosen because it was representative of isolates with *ccr-mec* genes typical of SCC*mec* IV by the simplex method

TABLE 1. Irish MRSA isolates investigated from each study period and typed by MLST and SCC*mec* typing

| Isolate phenotype[a] | No. of isolates investigated in study period[b]: | | | | | | | | No. of isolates typed by: | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A (1971–75) | B (1976–84) | C (1985–87) | D (1989) | E (1992–93) | F (1998) | G (1999) | H (2002) | MLST | SCC*mec* typing |
| Early MRSA | 1 | — | — | — | — | — | — | — | 1 | 1 |
| Phenotype II | — | 15 | — | — | — | — | — | — | 5 | 15 |
| Phenotype III | — | — | 1 | — | — | — | — | — | 1 | 1 |
| AR01 | — | — | — | 4 | — | — | — | — | 3 | 4 |
| AR02 | — | — | — | 3 | — | — | — | — | 3 | 3 |
| AR05 | — | — | — | 1 | — | — | — | — | 1 | 1 |
| AR06 | — | — | — | 1 | 4 | 4 | 10 | 8 | 11 | 27 |
| AR07.0/07.2 | — | — | — | 2 | 2 | 2 | 9 | 2 | 7 | 17 |
| AR07.3/07.4 | — | — | — | 1 | 3 | 3 | 2 | — | 9 | 9 |
| AR09 | — | — | — | 2 | — | — | — | — | 2 | 2 |
| AR11 | — | — | — | — | 4 | — | — | — | 1 | 4 |
| AR13 | — | — | — | — | 4 | 4 | 15 | 3 | 26 | 26 |
| AR14 | — | — | — | — | 4 | 4 | 11 | 3 | 22 | 22 |
| AR15 | — | — | — | — | 2 | — | — | — | 1 | 2 |
| AR22 | — | — | — | — | 2 | — | — | — | 1 | 2 |
| AR23 | — | — | — | — | 1 | — | — | — | 1 | 1 |
| AR43 | — | — | — | — | — | — | 20 | — | 20 | 20 |
| AR44 | — | — | — | — | — | — | — | 2 | 2 | 2 |
| ARNT (urea +ve)[c] | — | — | — | 1 | 2 | — | — | 3 | 6 | 6 |
| New01[d] | — | — | — | — | — | — | 1 | — | 1 | 1 |
| New02[d] | — | — | — | — | — | — | 1 | — | 1 | 1 |
| New03[d] | — | — | — | — | — | — | 2 | 2 | 4 | 4 |
| Unf01[e] | — | — | — | — | — | — | — | 1 | 1 | 1 |
| Total in this study | 1 | 15 | 1 | 15 | 28 | 17 | 71 | 24 | 130 | 172 |
| Total in original study | 20 | 350 | 67 | 104 | 321 | 220 | 714 | 398 | NA[f] | NA |

[a] The most prevalent phenotypes during each study period are underlined. —, isolates exhibiting this phenotype were not recovered during the study period.
[b] Isolates from groups A to F were from Dublin; isolates in group G were from the North and South of Ireland; isolates in group H were from the South of Ireland. References for the original study with each group are as follows: group A (reference 5); B (5); C (4); D (39, 42); E (39, 42); F (42); G (3, 44); H (38, 43).
[c] No AR type could be assigned. PFGE analysis showed that most urease-positive isolates exhibiting the AR06 pattern yielded PFGE patterns unlike the patterns obtained from other AR06 isolates. Hence, assigning urease-positive AR06 isolates to AR type 06 was considered incorrect, and these isolates were assigned to no type (NT) pending PFGE, MLST, and SCC*mec* analysis.
[d] These isolates were not assigned an AR type because they produced unfamiliar AR patterns; the AR type is pending on the results of plasmid screening, enterotoxin profiling, PFGE, phage typing, biotyping, MLST, and SCC*mec* typing.
[e] This isolate yielded an unfamiliar AR pattern and was not assigned an AR type, pending the outcome of MLST and SCC*mec* typing results in this study.
[f] NA, not applicable.

but with one of the two amplimers characteristic of SCC*mec* IV missing when isolates were investigated by the multiplex method.

Overlapping primers were designed to amplify the entire SCC*mec* element from both isolates using previously published SCC*mec* II and SCC*mec* IVa nucleotide sequences (17, 25) obtained from the Entrez-PubMed Nucleotide database website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide) (accession numbers D86934 [SCC*mec* II] and AB063172 [SCC*mec* IVa]). The primers used to amplify and sequence the SCC*mec* elements are listed in Table 2.

DNA fragments for sequencing and cloning were obtained by PCR amplification of chromosomal DNA using either *Pfu* DNA polymerase (Promega) for primer pairs dcs F and orfX R (Table 2) or the Expand Long Template PCR system (Roche) for all other primer sets (Table 2) according to the manufacturers' instructions. When each primer pair was used to amplify DNA from isolate AR13.1/3330.2, template DNA from at least one isolate exhibiting SCC*mec* II by both SCC*mec* typing methods was included as a control. Similarly, for AR43/3330.2 template DNA from at least one control isolate exhibiting SCC*mec* IV by both methods was included. Amplimers were purified using either the GenElute PCR Clean-Up kit (Sigma-Aldrich) or Wizard SV Gel and PCR Clean-Up system (Promega) prior to direct sequencing (Lark Technologies, Essex, United Kingdom) using primer walking or cloning into pBluescript II KS(−) phagemid and sequencing. Ligations of PCR products to pBluescript were facilitated by the presence of appropriate restriction endonuclease cleavage sites within the primers (Table 2). Transformation of competent *Escherichia coli* DH5α prepared using $CaCl_2$ and identification of recombinants using blue-white selection were performed according to the methods of Sambrook and Russell (45). DNA was sequenced using an Applied Biosystems 373A DNA sequencer (Foster City, Calif.) and dye-labeled terminators. Analysis of chromatograms and sequences were carried out using the 373A Data Analysis software program version 1.2.0 (Applied Biosystems) and DNA Strider 1.3f11 software (CEA/Saclay, Gif-sur-Yvette, France), respectively. Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/index.shtml). The CLUSTAL W sequence alignment computer program was used for alignments of nucleotide sequences (13).

**Investigation of variant SCC*mec* elements.** The SCC*mec* elements harbored by all isolates identified as having SCC*mec* types II and IV *ccr-mec* genes by the simplex method but with unusual SCC*mec* multiplex patterns were investigated by PCR amplification. The primers used for amplification and sequencing of SCC*mec* from isolate AR13.1/3330.2 (representative of isolates with SCC*mec* II *ccr-mec* genes by the simplex method but lacking one to three of the five SCC*mec* II multiplex amplimers) were used on all atypical SCC*mec* II isolates (Table 2). Similarly, the primers for amplification and sequencing of the SCC*mec* element of isolate AR43/3330.1 (representative of isolates with SCCmec IV *ccr-mec* genes by the simplex method but lacking one SCC*mec* IV multiplex amplimer) were used on all atypical SCC*mec* IV isolates (Table 2). PCR products were separated by electrophoresis in 1% (wt/vol) agarose gels, and the sizes of amplimers were compared to those obtained by amplification using the same primers on template DNA from AR13.1/3330.2 and AR43/3330.1 and to previously published SCC*mec* element sequences. Amplimers that showed variation from expected sizes were also sequenced.

**Nucleotide sequence accession numbers.** Nucleotide sequences were submitted to the GenBank database under accession numbers AJ810120 (SCC*mec* IIE from MRSA isolate AR13.1/3330.2), AJ810121 (SCC*mec* IVE from MRSA isolate AR43/3330.2), AJ810122 (partial *mec* complex A.4), and AJ810123 (partial SCC*mec* IIB).

## RESULTS

**MLST.** Of the 172 MRSA isolates investigated in this study, 130 were examined by MLST (Table 1). These 130 isolates

TABLE 2. Primers used in this study for SCC*mec* element amplification and sequencing

| Isolate | Primer pair | Nucleotide sequence[g] | Restriction site | Nucleotide coordinates | SCC*mec* region amplified |
|---|---|---|---|---|---|
| AR13.1/3330.2 | IRLII F | *CCCTCGAGGG***CTCTGCGTATCAGTTAATGA** | XhoI | 4684–4703[a] | L-C[c] |
| | ccrA R | *ATTTGCGGCCGC***GCTTCGATAGCCTGTTTCTG** | NotI | 25490–25471[a] | |
| | ccrA F | *ACGCGTCGAC***CAAGTCATAGGCTATTTACG** | SalI | 25417–25436[a] | C-M[d] (*ccr*A2-Tn*554*) |
| | Tn554 R | *ACGCGTCGAC***AAGCTATCCACGTTCAATCTCAAC** | SalI | 32442–32419[a] | |
| | Tn554 F | *ATTTGCGGCCGC***CTTTAAAGGGTTTCGGAATA** | NotI | 32365–32385[a] | C-M[d] (Tn*554*-*mecR1*) |
| | mecR1 R | *CCCTCGAGGG***CAATGCCTAAACCTAATCG** | XhoI | 43754–43736[a] | |
| | mecR1 F | *ATTTGCGGCCGC***CGATTAGGTTTAGGCATTG** | NotI | 43736–43754[a] | M-I[e] (*mecR1*-*ugpQ*) |
| | ugpQ R | *CCCTCGAGGG***GCTTCTGCAGGATCTTGG** | XhoI | 47868–47851[a] | |
| | ugpQ F | *CCCTCGAGGG***CCAAGATCCTGCAGAAGC** | XhoI | 47851–47878[a] | I-R[f] (*ugpQ*-*dcs*) |
| | dcs R | *ATTTGCGGCCGC***CGGTCATGGCTATGATTTAG** | NotI | 56373–56354[a] | |
| | dcs F | *ATTTGCGGCCGC***GTCAATGAGATCATCTACAT** | NotI | 56109–56128[a] | I-R[f] (*dcs*-right SCC*mec* junction) |
| | orfX R | *ATTTGCGGCCGC***CCCCAAGGGCAAAGCGAC** | NotI | 57826–57810[a] | |
| AR43/3330.1 | IRLII F | *CCCTCGAGGG***CTCTGCGTATCAGTTAATGA** | XhoI | 4684–4703[a] | L-C[c] |
| | ccrA R | *ATTTGCGGCCGC***GCTTCGATAGCCTGTTTCTG** | NotI | 25490–25471[a] | |
| | ccrA F | *ATTTGCGGCCGC***CAAGTCATAGGCTATTTACG** | NotI | 9894–9913[b] | C-M[d] (*ccr*A2-*mecA*) |
| | mecA R | *ACGCGTCGAC***CCTATCTCATATGCTG** | SalI | 18160–18145[b] | |
| | mecA F | *ATTTGCGGCCGC***GATTGGGATCATAGCGTCAT** | NotI | 18021–18040[b] | M-I[e] (*mecA*-IS*431mec*) |
| | ISmec R | *ACGCGTCGAC***ACGGTGATCTTGCTCAATGA** | SalI | 22780–22761[b] | |
| | ISmec F | *CCCTCGAGGG***CCTGACTGTCATTGTAC** | XhoI | 22724–22740[b] | I-R[f] (IS*431mec*-right SCC*mec* junction) |
| | orfX R | *ATTTGCGGCCGC***CCCCAAGGGCAAAGCGAC** | NotI | 25395–25379[b] | |

[a] Nucleotide coordinates from SCC*mec* type II, accession number D86934.
[b] Nucleotide coordinates form SCC*mec* type IVa, accession number AB063172.
[c] L-C, the region from the left chromosome-SCC*mec* junction to the beginning of the *ccr* complex.
[d] C-M, the region from the *ccr* complex to the *mec* complex.
[e] M-I, the region from the *mec* complex region to IS*431mec*.
[f] I-R, the region from IS*431mec* to the right extremity of SCC*mec*.
[g] Text in bold indicates primer sequences, text in italics indicates restriction sites added to primer sequences, and plain text indicates extra bases added to aid with cloning.

included at least one isolate from each of the most common phenotypes identified among MRSA recovered between 1971 and 2002 from Irish hospitals (Table 1, study periods A to H). Multiple isolates were included for the predominant phenotypes during each study period (Table 1), except for study period B (1976 to 1984), for which isolates from only one of the two predominant phenotypes were available. MLST identified 10 STs belonging to each of the five major CCs described to date and one singleton (ST12) among the MRSA isolates investigated (Table 3). Isolates recovered prior to 1985 exhibited ST250, while the STs of the two most prevalent strains in 1989 were ST250 and ST239 (Tables 1 and 3). ST8 predominated in the 1990s, but by the late 1990s the prevalence of isolates with ST36 and ST22 increased (Tables 1 and 3). By 2002, ST22 had become the major ST. All except one ST were identical to those previously described. The exception was a double locus variant of ST5 (ST496), which differed from ST5 at both the *arcC* and *yqiL* alleles. ST5 has alleles 1 and 10 of *arcC* and *yqiL*, respectively, whereas ST496 had a new allele, 63, of *arcC* and allele 28 of *yqiL*. The *arcC* alleles 63 and 1 differ at a single nucleotide site, as do alleles 10 and 28 of *yqiL*. This novel ST was obtained from one isolate from 2002 exhibiting AR type AR07.2.

**SCC*mec* typing.** Using both the simplex and multiplex SCC*mec* typing methods 50% (86/172) of isolates carried SCC*mec* type I, IA, II, III, or IV (Table 3). SCC*mec* I was identified in 10 isolates (5.8%), SCC*mec* II was identified in 30 isolates (17.4%), SCC*mec* III was found in nine isolates (5.2%), and SCC*mec* IV was identified in 34 isolates (19.8%). Three isolates (1.7%) were found to harbor SCC*mec* I by the simplex method, but the multiplex method showed that they carried the previously described variant element SCC*mec* Ia (Table 3).

**Identification of variant SCC*mec* types.** Eighty-six isolates (50%) harbored two apparently different SCC*mec* elements when tested by both SCC*mec* typing methods, or the SCC*mec* type could not be inferred because the multiplex patterns produced did not exactly match those of previously described SCC*mec* elements (Table 3; Fig. 1).

Nine isolates were found to carry SCC*mec* I by the simplex method but failed to yield the 495-bp band characteristic of SCC*mec* I elements by the multiplex method, which results from amplification of a region downstream of the gene encoding a plasmin-sensitive surface protein (*pls*) found in SCC*mec* I (Table 3). Furthermore, the multiplex pattern obtained was indistinguishable from SCC*mec* IV (Fig. 1A, lanes 2 to 5). This pattern was designated SCC*mec* I *–pls*.

Three isolates were identified by the simplex method as carrying SCC*mec* III but their multiplex pattern, although similar to that of SCC*mec* III, was missing a band at 243 bp (Table 3; Fig. 1A, lanes 7 to 9). This band is the product of amplification of the locus between pI258 and Tn*554* and is found in all SCC*mec* III elements characterized to date. This pattern was designated SCC*mec* III –pI258/Tn*554*.

All 20 AR43 isolates were classified as SCC*mec* type IV by the simplex method but gave a multiplex PCR pattern from which the SCC*mec* type could not be inferred because only the multiplex *mecA* control amplimer found in all SCC*mec* types was obtained (Table 3; Fig. 1B, lanes 7 to 9). This multiplex pattern could be interpreted as a variant SCC*mec* IV multiplex pattern missing the 342-bp amplimer, which results from the amplification of *dcs* found in SCC*mec* IV. This pattern was called SCC*mec* IV *–dcs*.

The remaining 54 isolates belonged to phenotypes AR05, AR13, AR14, New01, and New03 (Table 1) and were identi-

TABLE 3. MLST, SCC*mec* type, and variant SCC*mec* elements of MRSA recovered in Ireland, 1971 to 2002

| CC | ST | AR type or phenotype[a] | SCC*mec* type based on: | | | Variant SCC*mec* elements |
|---|---|---|---|---|---|---|
| | | | Simplex PCR | Multiplex PCR[b] | SCC*mec* name[c] | |
| 8 | 239 | AR01, AR15, AR23, AR44 | III | III | | |
| | 239 | PhIII, AR09 | III | III – pI258/Tn*554* | | |
| | 250 | Early MRSA ($n$ = 1), PhII ($n$ = 4) | I | I ($n$ = 10) | | |
| | 250 | PhII ($n$ = 1), AR02 ($n$ = 3) | I | IV or I – *pls* ($n$ = 9) | | |
| | 247 | AR22, New02 | I | Ia | | |
| | 8 | AR05 | II | II – *kdp* | IIB | SCC*mec* IVb L-C region and lacks Tn*554*[f] |
| | 8 | AR13 ($n$ = 6), AR14 ($n$ = 3) | II | II – *kdp* | IIA | SCC*mec* IVb L-C region; class A.4 *mec*[d,f] |
| | 8 | AR14 ($n$ = 10) | II | II – *kdp* & *mecI* | IIC | SCC*mec* IVb L-C region; class A.3 *mec*[d]; lacks ORFs between Tn*554* and *mec* complex[f] |
| | 8 | AR13 ($n$ = 19), AR14 ($n$ = 3), New01, New03 | II | II – *kdp* & pUB110 | IID | SCC*mec* IVb L-C region; class A.4 *mec*[d]; lacks pUB110 and second copy of IS*431*[f] |
| | 8 | AR13 ($n$ = 1), AR14 ($n$ = 6) | II | IV or II – *kdp*, *mecI* & pUB110 | IIE | SCC*mec* IVb L-C region; class A.3 *mec*[d]; lacks pUB110, second copy of IS*431*, and ORFs between Tn*554* and *mec* complex[f] |
| | 8 | AR43 ($n$ = 17) | IV | IV – *dcs* | IVE | SCC*mec* IVc variant, lacks *dcs*; has an unusual sequence in I-R region |
| | 8 | AR43 ($n$ = 3) | IV | IV – *dcs* | IVF | SCC*mec* IVb variant, lacks *dcs*; has an unusual sequence in I-R region |
| 5 | 5 | AR07.3/07.4, AR11 | II | II | | |
| | 5 | Unf01 | IV | IV | | |
| | 496 | AR07.2 ($n$ = 1) | II | II | | |
| 30 | 36 | AR07.0/07.2 ($n$ = 6) | II | II | | |
| | 30 | ARNT ($n$ = 4) | IV | IV | | |
| 22 | 22 | AR06 | IV | IV | | |
| 45 | 45 | ARNT ($n$ = 1) | IV | IV | | |
| S[e] | 12 | ARNT ($n$ = 1) | IV | IV | | |

[a] Where more than one ST/SCC*mec* type was identified within a phenotype (e.g., AR13 or AR07.0/07.2), the numbers represented by each ST or SCC*mec* type are shown in parentheses after the AR type or phenotype. For isolates identified as having genotype ST250, the numbers of isolates analyzed by MLST differed from the numbers subjected to SCC*mec* typing (TABLE 1), and more than one SCC*mec* type was identified within this genotype; therefore, the numbers of isolates identified with each ST and SCC*mec* type are indicated in parentheses after the phenotype and SCC*mec* type, respectively. Otherwise, numbers of isolates are as indicated in TABLE 1.

[b] Some patterns obtained with the multiplex PCR SCC*mec* typing method lacked amplimers corresponding to the amplification of the determinants indicated.

[c] SCC*mec* name assigned during the present study.

[d] These two variants of *mec* complex class A (designated A.3 and A.4) were recognized during the present study (Fig. 2 and 3).

[e] Singleton, no ancestral genotype has been assigned.

[f] SCC*mec* II variant.

fied as SCC*mec* II by the simplex method but gave variant SCC*mec* II multiplex patterns that lacked one to three of the five amplimers that would identify them as harboring SCC*mec* II (Table 3). Ten isolates lacked a 284-bp band corresponding to the amplification product from a region of the *kdp* operon found in SCC*mec* II (Table 3). This pattern was designated SCC*mec* II –*kdp* (Fig. 1B, lane 2). Ten isolates lacked both the 284-bp band (*kdp* operon) and a 209-bp amplimer (Table 3). The latter results from amplification of part of the *mecI* gene in SCC*mec* II. This pattern was termed SCC*mec* II –*kdp* & *mecI* (Fig. 1B, lane 3). Twenty-seven isolates lacked the 284-bp band (*kdp* operon) and the 381-bp amplimer (Table 3). This 381-bp amplimer results from amplification of the left junction between IS*431mec* and pUB110 found in SCC*mec* II. This pattern was called SCC*mec* II –*kdp* & pUB110 (Fig. 1B, lanes 4). Seven isolates produced a multiplex PCR pattern indistinguishable from the pattern of SCC*mec* IV (Table 3). This pattern could also be interpreted as a SCC*mec* II pattern missing the 284-bp (*kdp* operon), 209-bp (*mecI* gene), and 381-bp (pUB110) amplimers. This pattern was designated SCC*mec* II –*kdp*, *mecI* & pUB110 (Fig. 1B, lane 5).

**Investigation of the SCC*mec* elements harbored by ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns.** Since all ST8 isolates ($n$ = 74) exhibited variant multiplex patterns (Table 3), we decided to further investigate these SCC*mec* elements. Firstly, the entire SCC*mec* element from two ST8 isolates, AR 13.1/3330.2 and AR43/3330.1, with variant SCC*mec* II and SCC*mec* IV multiplex patterns, respectively, were sequenced. Isolate AR13.1/3330.2 was representative of isolates with SCC*mec* II by the simplex method but with SCC*mec* multiplex patterns lacking one to three of the amplimers typical of an SCC*mec* II element. AR 13.1/3330.2 exhibited multiplex pattern SCC*mec* II –*kdp*, *mecI* & pUB110 (i.e., it lacked all three bands, *kdp*, *mecI*, and pUB110). Isolate AR43/3330.1 was representative of isolates with SCC*mec* IV by the simplex method but with an SCC*mec* multiplex pattern lacking the 342-bp *dcs* amplimer (SCC*mec* IV –*dcs*). The genomic organizations of these two variant SCC*mec* elements based on their nucleotide sequence are shown schematically in Fig. 2. The genomic structures of the SCC*mec* elements of the remaining 72 ST8 isolates were also determined.

**Novel variant of SCC*mec* II identified in AR13.1/3330.2.** The SCC*mec* element in AR13.1/3330.2 consisted of a ~27-kb sequence containing a combination of regions previously identified in SCC*mec* II and IV and a novel *mec* complex (Fig. 2). Amplification of the L-C region yielded an amplimer of ~5.6 kb, which is similar in size to the expected amplimer for SCC*mec* IVb (Table 4). The nucleotide sequence of this 5.6-kb amplimer was found to have 99.7% similarity to the L-C region

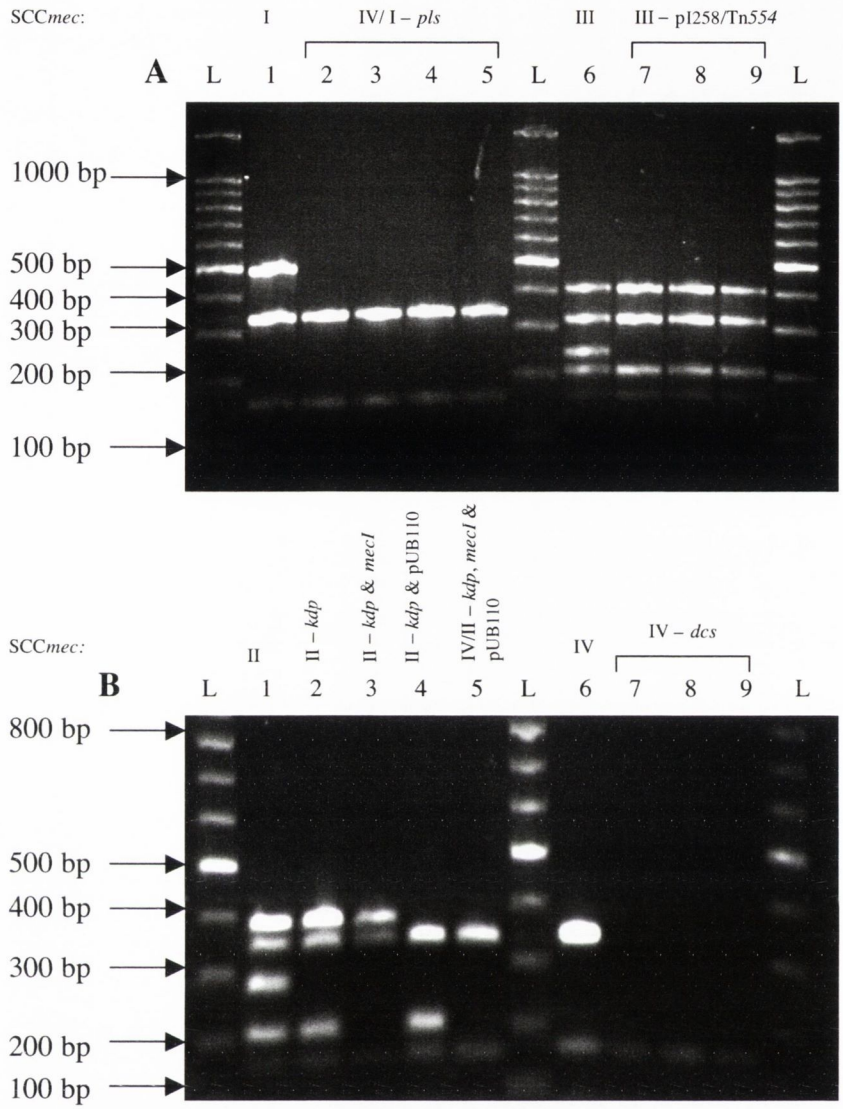



FIG. 1. SCC*mec* multiplex patterns and variant multiplex patterns found in Irish MRSA isolates. (A) Lane 1, SCC*mec* type I multiplex pattern; lanes 2 to 5, SCC*mec* IV multiplex pattern produced by AR02 isolates and six phenotype II isolates with SCC*mec* type I *ccr-mec* genes by the simplex method; lane 6, SCC*mec* type III multiplex pattern; lanes 7 to 9, the variant SCC*mec* III multiplex pattern (III –pI258/Tn*554*) characterized by the absence of the pI258/Tn*554* 243-bp amplimer, produced by AR09 and phenotype III isolates with SCC*mec* type III *ccr-mec* genes by the simplex method. (B) Lane 1, SCC*mec* type II multiplex pattern; lanes 2 to 5, the SCC*mec* multiplex patterns produced by isolates with phenotypes AR05, AR13, AR14, New01, and New03 with type II *ccr-mec* genes by the simplex method. These multiplex patterns appear to be related to the type II SCC*mec* multiplex pattern but lack the *kdp* amplimer (284 bp) (II –*kdp*, lane 2), or lack the *kdp* and *mecI* (209-bp) amplimers (II –*kdp* & *mecI*, lane 3), or lack the *kdp* and pUB110 (381-bp) amplimers (II –*kdp* & pUB110, lane 4), or lack the *kdp*, *mecI*, and pUB110 amplimers (II –*kdp*, *mecI* & pUB110, lane 5). This last pattern is the same as SCC*mec* IV. Lane 6, SCC*mec* type IV multiplex pattern; lanes 7 to 9, multiplex pattern produced by isolates with the AR43 phenotype (IV –*dcs*), which consists of the *mecA* control amplimer only. Lane L, 100-bp DNA ladders used as molecular size reference markers.

of SCC*mec* IVb and less than 10% similarity to the L-C regions of SCC*mec* types I, II, III, IVa, and IVc.

Analysis of the sequence of the *ccr* complex identified type 2 *ccr* genes (*ccrA2* and *ccrB2*) with closest similarity to those of SCC*mec* II and SCC*mec* IV elements (Table 5). Amplification and sequencing of the region between the *ccr* and *mec* complexes (C-M) in AR13.1/3330.2 using two primer pairs yielded a 7-kb and 6.5-kb product (Table 4) and confirmed the presence of a truncated integrated transposon, Tn*554* (Table 5). Five of the six ORFs (*tnpA*, *tnpB*, *tnpC*, *spc*, and *ermA*) previously identified within Tn*554* of SCC*mec* II were present in the SCC*mec* element of AR13.1/3330.2, and each ORF showed 100% homology at the amino acid level with those of SCC*mec* II (Table 5). However, Tn*554* of SCC*mec* of AR13.1/3330.2 was truncated by ~1,000 bp, resulting in the absence of an ORF of unknown function in Tn*554*. The six ORFs spanning a ~5-kb region encoding hypothetical proteins usually found between Tn*554* and the *mec* complex in SCC*mec* II were also absent (Fig. 2). Instead of these ORFs, a 1,865-bp IS*1182* sequence was present. A 253-bp truncated version of *mecI* (Δ*mecI*) was identified (*mecI* gene is usually 372 bp) (Fig. 2). Sequences of *mecR1* and *mecA* from AR13.1/3330.2 showed

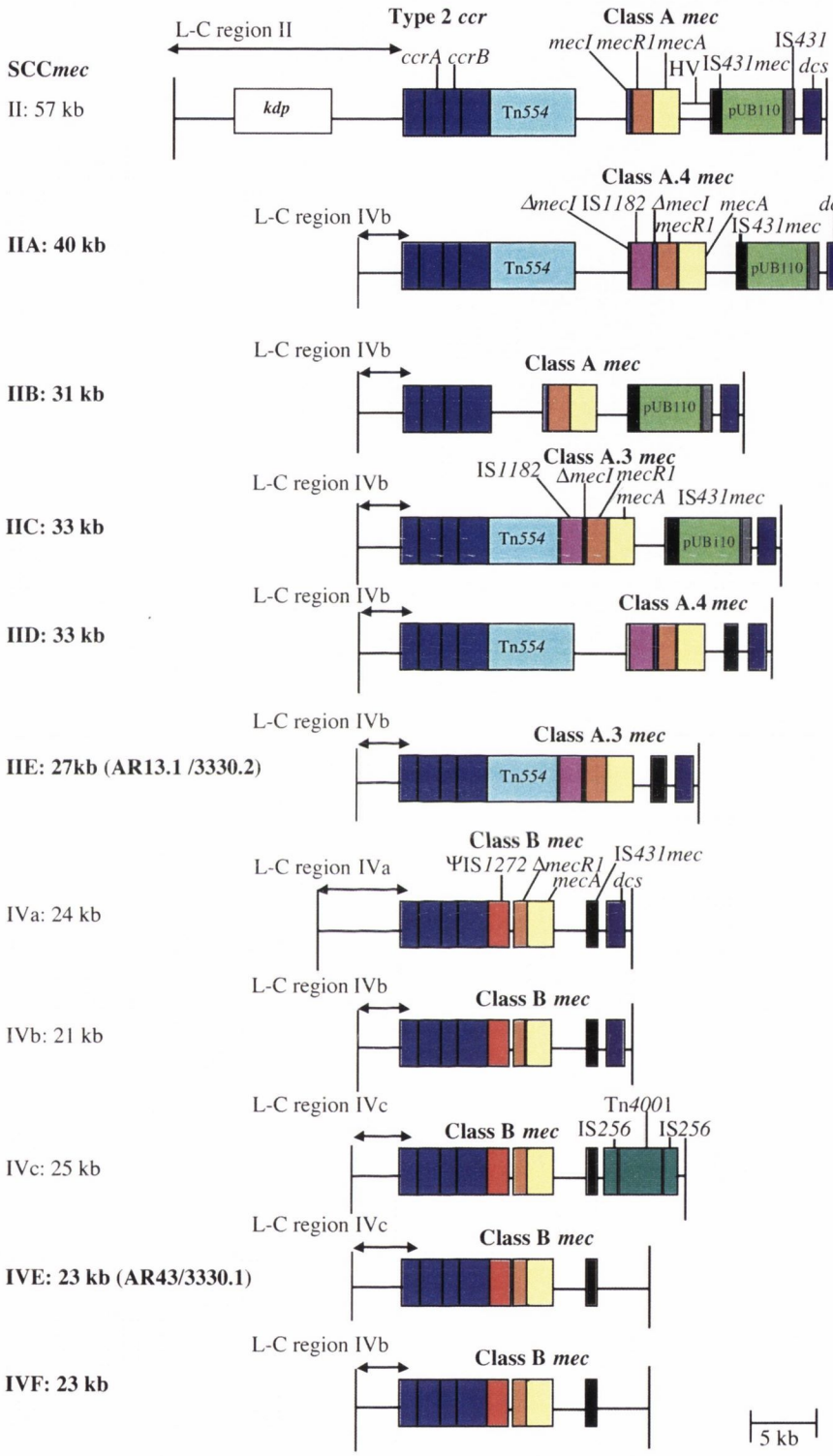



FIG. 2. Schematic diagram showing the genomic organization of SCC*mec* II, IIA, IIB, IIC, IID, IIE, IVa, IVb, IVc, IVE, and IVF elements. SCC*mec* IIA, IIB, IIC, IID, IIE, IVE, and IVF are novel variants that were identified in the present study. SCC*mec* II, IVa, IVb, and IVc were identified previously (19, 25), and the organization was determined based on the nucleotide sequences in the GenBank database under accession numbers D86934, AB063172, AB063173, and AB096217, respectively. The structure of SCC*mec* IIE and IVE were determined by sequencing of the entire SCC*mec* element of Irish MRSA isolates AR13.1/3330.2 (IIE; accession number AJ810120) and AR43/3330.1 (IVE; accession number AJ810121). The structures of SCC*mec* IIA, IIB, IIC, IID, and IVF were determined using the primers shown in Table 3 by observing the mobility of amplimers in agarose gels and in some cases by sequencing.

TABLE 4. Correlation between SCC*mec* multiplex types and amplimer sizes obtained using overlapping primer pairs to amplify the novel SCC*mec* element variants and comparison to those expected from typical SCC*mec* II and SCC*mec* IV elements

| SCC*mec* multiplex type | SCC*mec* element | Amplimer size[a] (kb) with primer pair | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L-C, IRLII F-ccrA R | C-M, ccrA F-Tn*554* R | C-M, Tn*554* F-*mecR1* R | M-I, mecR1 F-ugpQ R | I-R, ugpQ F-dcs R | I-R, dcs F-orfX R | C-M, ccrA F-mecA R | M-I, mecA F-ISmec R | I-R, ISmec F-orfX R | |
| II | II | 20 | 7 | 11 | 4.1 | 8.5 | 1.7 | 20 | 4.7 | 8 | 17 |
| II – *kdp* | IIA | 5.6 | 7 | 13* | 4.1 | 8.5* | 1.7 | 22 | 4.7 | 8 | This study |
| II – *kdp* | IIB | 5.6 | 7 | No amplimer[c] | 4.1 | 8.5 | 1.7 | 13 | 4.7 | 8 | This study |
| II – *kdp* & *mecI* | IIC | 5.6 | 7 | 6.5 | 4.1 | 8.5 | 1.7 | 14 | 4.7 | 8 | This study |
| II – *kdp* & pUB110 | IID | 5.6 | 7 | 13 | 4.1 | 2.2 | 1.7 | 22 | 3.7 | 2.7 | This study |
| II – *kdp*, *mecI* & pUB110 | IIE[b] | 5.6* | 7* | 6.5* | 4.1* | 2.2* | 1.7* | 14 | 3.7 | 2.7 | This study |
| IV | IVa | 8.9 | No amplimer[d] | No amplimer[d] | 4.1 | 3.2 | 1.7 | 8.5 | 4.7 | 2.7 | 25 |
| IV | IVb | 5.6 | No amplimer[d] | No amplimer[d] | 4.1 | 3.2 | 1.7 | 8.5 | 4.7 | 2.7 | 25 |
| IV | IVc | 6.1 | No amplimer[d] | No amplimer[d] | 4.1 | No amplimer[e] | No amplimer[e] | 8.5 | 4.7 | 5.3 | 19 |
| IV – *dcs* | IVE[b] | 6.1* | No amplimer[d] | No amplimer[d] | 4.1 | No amplimer[e] | No amplimer[e] | 8.5* | 4.7* | 4.7* | This study |
| IV – *dcs* | IVF | 5.6* | No amplimer[d] | No amplimer[d] | 4.1 | No amplimer[e] | No amplimer[e] | 8.5 | 4.7 | 4.7* | This study |

[a] Amplimer sizes for SCC*mec* IIA, IIB, IIC, IID, IIE, IVE, and IVF were calculated either by sequencing (indicated by *) or based on electrophoretic mobility in agarose gels. Amplimer sizes for SCC*mec* II, IVa, IVb, and IVc were calculated from sequences in the GenBank database, accession numbers D86934, AB063172, AB063173, and AB096217, respectively. All amplimer sizes are approximate. L-C, C-M, M-I, and I-R indicate the region of the SCC*mec* element amplified.

[b] SCC*mec* elements IIE and IVE were sequenced in full from isolates AR13.1/3330.2 and AR43/3330.1, respectively.

[c] No amplimer due to no Tn*554;* primers ccrA F-mecR1 R were used instead, resulting in an 11.5-kb amplimer, which was sequenced.

[d] No amplimer due to no Tn*554* in SCC*mec* IVa, IVb, IVc, IVE, and IVF.

[e] No amplimer due to no *dcs* region in SCC*mec* IVc, IVE, and IVF.

100% homology to sequences of other SCC*mec* elements (Table 5).

Amplification of the region across IS*431mec* to the *dcs* region downstream of the *mec* complex yielded a product of 2.2 kb, which is smaller than that of any previously described SCC*mec* element including SCC*mec* II and IVb (Table 4). Sequence data from this product showed that the hypervariable (HV) region (located between *mecA* and IS*431mec*) of AR 13.1/3330.2 is 997 bp shorter than that of SCC*mec* II and SCC*mec* IVb. The IS*431mec* region was 100% homologous to that of other SCC*mec* elements but lacked all DNA sequences associated with the integrated plasmid pUB110 or the second IS*431* sequence found in SCC*mec* II (Fig. 2).

The extreme right of the SCC*mec* element of AR13.1/3330.2 from IS*431* to the right chromosomal-SCC*mec* junction (I-R region) showed 100% homology with the same region of SCC*mec* II (Table 5). The 15-bp direct repeat sequence characteristic of SCC*mec* elements was identified at the right extremity of SCC*mec* (DR-R) and a 26-bp inverted repeat sequence (IR-R) that was identical to that of SCC*mec* IVb and SCC*mec* II was also found. All SCC*mec* elements identified to date have been found to be integrated at exactly the same nucleotide position of *orfX*. This ORF was identified outside the IR-R region, and SCC*mec* of AR13.1/3330.2 was integrated at the same nucleotide position in *orfX* as other SCC*mec* elements.

The novel genomic structure of the *mec* complex of the SCC*mec* element of AR13.1/3330.2 (IS*1182*-Δ*mecI*-*mecR1*-*mecA*-IS*431mec*) was more similar to the class A *mec* complex of MRSA (*mecI*-*mecR1*-*mecA*-IS*431mec*) found in SCC*mec* II and SCC*mec* III than to the class B *mec* complex (IS*1272*-Δ*mecR1*-*mecA*-IS*431mec*) of SCC*mec* I and IV (Fig. 3). Due to its similarity to the class A *mec* complexes, this novel *mec* complex was designated class A.3 *mec* (Fig. 3).

**Novel variant of SCC*mec* IV identified in AR43/3330.1.** Isolate AR43/3330.1 carried a 23-kb SCC*mec* element, which is within the size range (20 to 25 kb) of previously described SCC*mec* IV elements, but sequence data indicated that it is distinctly different. The genomic structure of this SCC*mec* element showed most similarity to SCC*mec* IVc (Fig. 2). Amplification of the L-C region of AR43/3330.1 yielded an amplimer of ca 6.1 kb (Table 4), and sequencing revealed that it had 100% similarity with the L-C region of SCC*mec* IVc (Table 6). Amplification of the region from the *ccr* complex to IS*431mec* yielded amplimers that showed almost 100% sequence identity with the same regions of SCC*mec* IVc (Table 6). A class B *mec* complex was identified (Fig. 2), and an I-R region different from any previously described SCC*mec* element was determined. An amplimer of 4.7 kb was obtained following amplification of this region (Table 4). Sequence analysis identified IS*431mec*, but a ~4.4-kb region was identified between it and the end of *orfX*. Searches of the GenBank database showed that ~1.2 kb of this sequence had 100% homology to a sequence of the right extremity of the SCC*mec* element of two other MRSA isolates (15). This sequence in the GenBank database consists of *orfX* and the extreme right region of the SCC*mec* element, including the direct repeat (DR-R) and inverted repeat (IR-R) regions. The rest of the sequence between IS*431mec* and IR-R identified in AR43/3330.1 was found to have no homology to any sequences in the GenBank database.

**Determination of the genomic organization of the SCC*mec* elements of the remaining ST8 isolates with unusual SCC*mec* II and SCC*mec* IV multiplex patterns.** In total, 74 isolates had the ST8 genotype and gave variant SCC*mec* II and IV multiplex patterns (Table 3). In addition to determining the nucleotide structure of the SCC*mec* elements of two of these isolates as described above, the SCC*mec* elements of the remaining 72 isolates were characterized by analyzing the mobility of amplimers on agarose gels obtained with the overlapping primers used to amplify and sequence the SCC*mec* from isolates AR13.1/3330.2 and AR43/3330.1. In some cases, sequencing was performed on amplimers that differed in size from that expected from previously described SCC*mec* elements. These results are summarized in Table 3 and Table 4. This analysis identified five variant SCC*mec* elements, in addition to the two identified above by complete nucleotide sequencing. Of the

TABLE 5. Identities between ORFs of the novel SCC*mec* II variant element of Irish MRSA isolate AR13.1/3330.2 and the conventional SCC*mec* IVb and II elements

| SCC*mec* element and ORF | Location[a] | Homology to ORFs of SCC*mec* IVb[b] | | | Homology to ORFs of SCC*mec* II[b] | | |
|---|---|---|---|---|---|---|---|
| | | ORF | % Homology[c] | Gene product | ORF | % Homology[c] | Gene product |
| IIE01 | 210–1145 (complement) | CM001 | 83.7 | Hypothetical protein | — | — | — |
| IIE02 | 1123–1893 (complement) | CM002 | 100 | Hypothetical protein | — | — | — |
| IIE03 | 2010–3440 | M001 | 99.6 | Hypothetical protein | — | — | — |
| IIE04 | 3560–5350 | M002 | 99.7 | Conserved hypothetical protein | N031 | 80.3 | Conserved hypothetical protein |
| IIE05 (*ccr*A2) | 5584–6933 | M004 | 99.1 | Cassette chromosome recombinase A2 | N034 | 96.7 | Cassette chromosome recombinase A2 |
| IIE06 (*ccr*B2) | 6955–8583 | M005 | 97.8 | Cassette chromosome recombinase B2 | N037 | 98 | Cassette chromosome recombinase B2 |
| IIE07 | 9101–9451 | M006 | 88.9 | Conserved hypothetical protein | N041 | 100 | Conserved hypothetical protein |
| IIE08 | 9538–9849 | M007 | 89.4 | Conserved hypothetical protein | N042 | 100 | Conserved hypothetical protein |
| IIE09 | 9867–10373 | M008 | 91.7 | Conserved hypothetical protein | N043 | 99.4 | Conserved hypothetical protein |
| IIE10 | 10394–10711 | —[d] | — | — | N044 | 100 | Pseudogene |
| IIE11 (*tnpA*) | 10830–11915 | — | — | — | N045 | 100 | Transposase A |
| IIE12 (*tnpB*) | 11912–13804 | — | — | — | N046 | 100 | Transposase B |
| IIE13 (*tnpC*) | 13811–14188 | — | — | — | N047 | 100 | Transposase C |
| IIE14 (*spc*) | 14339–15121 | — | — | — | N048 | 100 | *O*-Nucleotidyltransferase |
| IIE15 (*ermA*) | 15247–15978 (complement) | — | — | — | CN026 | 100 | rRNA adenine *N*-6-methyltransferase |
| IIE16 (*tnp* IS*1182*) | 16499–18103 | — | — | — | — | — | — |
| IIE17 (Δ*mecI*) | 18207–18459 (complement) | — | — | — | CN036 | 67.7 | Methicillin resistance protein MecI |
| IIE18 (*mecR1*) | 18459–20216 (complement) | CM003 | 55.6 | Truncated signal transducer protein MecR1 | CN037 | 100 | Signal transducer protein MecRI |
| IIE19 (*mecA*) | 20316–22322 | M010 | 100 | Penicillin binding protein 2a | N058 | 100 | Penicillin binding protein 2a |
| IIE20 | 22368–22796 (complement) | CM004 | 100 | Conserved hypothetical protein | CN038 | 100 | Conserved hypothetical protein |
| IIE21 (*ugpQ*) | 22893–23636 | CM006 | 100 | Glycerophosphoryldiester phosphodiesterase | N039 | 100 | Glycerophosphoryldiester phosphodiesterase |
| IIE22 (*tnp* IS*431mec*) | 23901–24575 | M011 | 100 | Transposase for IS*431mec* | N062 | 100 | Transposase for IS*431mec* |
| IIE23 | 24607–24846 (complement) | CM007 | 100 | Conserved hypothetical protein | CN049 | 100 | Conserved hypothetical protein |
| IIE24 | 25261–26556 (complement) | CM008 | 40.7 | Truncated conserved hypothetical protein | CN050 | 100 | Truncated conserved hypothetical protein |

[a] The nucleotide positions were determined based on the sequences for SCC*mec* IIE deposited in the GenBank database under accession number AJ8101208.
[b] ORFs and gene products from SCC*mec* II and SCC*mec* IVb, based on nucleotide sequences in the GenBank database, accession number D86934 and AB063173, respectively (17, 25).
[c] Based on amino acid identity.
[d] —, no significant homology.

seven variants recognized, five SCC*mec* II variants (designated SCC*mec* IIA, IIB, IIC, IID, and IIE) were identified among the 54 isolates with variant SCC*mec* II multiplex patterns, and two novel SCC*mec* IV variants (designated SCC*mec* IVE and IVF) were found among the 20 isolates with variant SCC*mec* IV multiplex patterns (Table 3). A schematic representation of all seven SCC*mec* variants is shown in Fig. 2.

**Additional novel variants of SCC*mec* II.** Significant size variation was observed in the mobility of amplimers obtained from variant SCC*mec* II elements compared to the sizes expected from isolates with typical SCC*mec* II when the following three regions were amplified: the L-C region, Tn*554* to the *mec* complex (part of C-M region), and across pUB110 (part of I-R region) (Table 4). The 54 SCC*mec* II variants yielded the 5.6-kb fragment characteristic of the L-C region of SCC*mec* IVb (Table 4; Fig. 2). No amplimer obtained following amplification of the region from Tn*554* to *mecR1* in the SCC*mec* II variants matched the size expected from a typical SCC*mec* II element (Table 4). All but one isolate with the multiplex pattern SCC*mec* II *–kdp* ($n = 9$) and all isolates with the multiplex pattern SCC*mec* II *–kdp* & pUB110 ($n = 27$) produced amplimers of ca. 13 kb. This 13-kb region was sequenced from one isolate, and it was found that IS*1182* was inserted within the *mecI* gene, near the 3′ end, and that *mecI* carried a 16-bp deletion (Fig. 3). This new *mec* class A complex consisting of Δ*mecI*-IS*1182*-Δ*mecI-mecR1-mecA*-IS*431mec* was designated *mec* class A.4 (Fig. 3).

The Tn*554-mecR1* region of one isolate with the multiplex pattern SCC*mec* II *–kdp* could not be amplified with the same primers used on the other SCC*mec* II variants. Instead, a primer pair was used to amplify the DNA region from the *ccr* complex to *mecR1*, resulting in a ca. 11.5-kb amplimer (Table 4). Sequencing data revealed that this isolate retained only a few bases of the transposon Tn*554* normally found in SCC*mec* II and that all Tn*554* ORFs were absent. However, the six ORFs usually found between Tn*554* and the *mec* complex of SCC*mec* II were all present. This isolate also harbored a class A *mec* complex. Amplification of the same region in isolates with the multiplex pattern SCC*mec* II *–kdp* & *mecI* ($n = 10$) and SCC*mec* II *–kdp*, *mecI* & pUB110/IV ($n = 7$) yielded an amplimer of the same size as the amplimer obtained and sequenced from isolate AR13.1/3330.2 (Table 4) in which the region between Tn*554* and the *mec* complex was absent but which carried a *mec* class A.3 *mec* complex. All isolates with

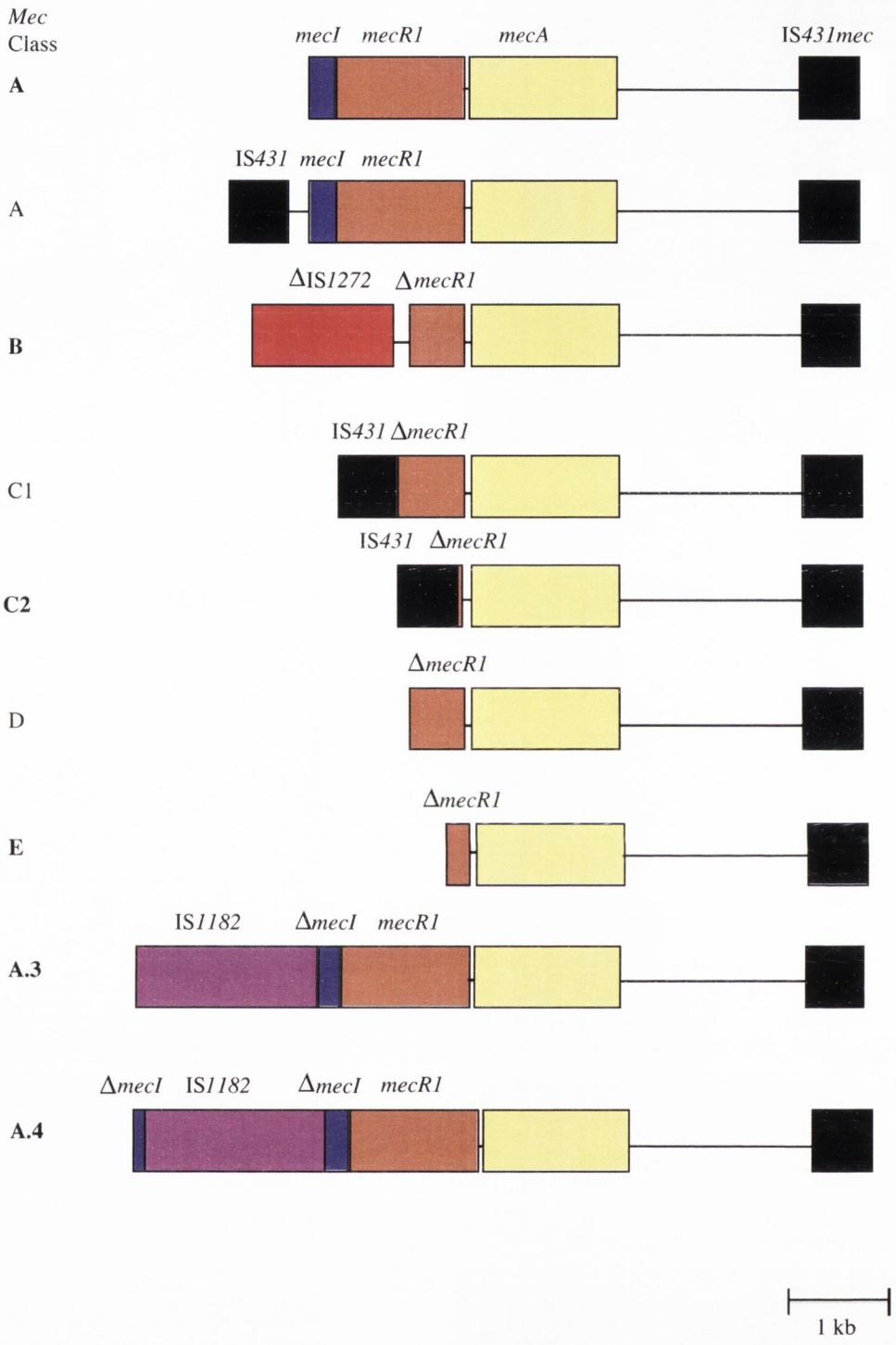



FIG. 3. Schematic diagram of the genetic organization of the *mec* complexes of staphylococci. The illustrations of *mec* complexes A, B, C1, C2, and D are based on studies by Katayama and coworkers (22), and the illustration of *mec* complex E is based on studies by Lim and coworkers (22, 24). The *mec* classes A, B, C2, and E indicated in bold have been previously identified in MRSA (18, 22, 24). The novel *mec* classes A.3 and A.4 were identified in the present study of Irish MRSA isolates. Class A.3 *mec* differs from class A *mec* in that it has a copy of *mecI* that is truncated at the 3′ end and an upstream copy of IS*1182* (IS*1182*-Δ*mecI*-*mecR1*-*mecA*-IS*431mec*). Δ*mecI* is only 253 bp, whereas intact *mecI* is 372 bp. In class A.4, *mec* IS*1182* is present within *mecI* and there is a 16-bp deletion of *mecI* immediately upstream of IS*1182* (Δ*mecI*-IS*1182*-Δ*mecI*-*mecR1*-*mecA*-IS*431mec*).

the SCC*mec* II *–kdp* ($n$ = 10) and SCC*mec* II *–kdp* & *mecI* ($n$ = 10) harbored the integrated plasmid pUB110 and the second copy of IS*431* found in SCC*mec*. Isolates with multiplex patterns SCC*mec* II *–kdp* & pUB110 ($n$ = 27) and SCC*mec* II *–kdp*, *mecI* & pUB110/IV ($n$ = 7) gave the same size amplimer as that obtained from isolate AR13.1/3330.2 for amplification of the region across pUB110 (Table 4), indicating the absence of pUB110 and the second IS*431* sequence.

because it retains only ~40 bp of this element. Acquisition of IS*1182* by a class A *mec* complex may have given rise to the novel variants of class A *mec*, A.3 and A.4, seen in SCC*mec* IIC and IIE and SCC*mec* IIA and IID, respectively (Fig. 2 and 3). Since SCC*mec* IIC and IIA were present in isolates in 1993 and SCC*mec* IIE and IID were not found in isolates until 1998, it is likely that the latter derived from the former following loss of pUB110.

The presence of IS*1182* within *mecI*, although a novel finding, is not surprising. Mutations and deletions in *mecI* are common in MRSA (22), sometimes in association with insertion sequences. Class B and class C *mec* complexes have *mec*I and part of *mec*R1 deleted and insertions of IS*1272* or IS*431* flanking the truncated end of *mec*R1 (22). IS*1182* has been identified in *S. aureus* flanking the staphylococcal composite transposon Tn*5405*, which encodes aminoglycoside resistance determinants (6). Immediately adjacent to the insertion site of IS*1182* at the 3′ end of *mec*I in the class A.4 *mec* complex, a 16-bp deletion in *mecI* gene has occurred with no flanking target site duplications. Insertion sequences capable of promoting various types of genome rearrangements, including deletions, have been reported previously (26).

Other workers have reported possible rearrangements in the L-C regions of other SCC*mec* elements. Based on a multiplex SCC*mec* typing method, Wielders et al. (47) reported a SCC*mec* I element carrying the *kdp* region (normally part of L-C region of SCC*mec* II only) and also an SCC*mec* IV element with *pls* (normally part of L-C region of SCC*mec* I only) (47). Various different SCC*mec* IV elements have been described with diverse L-C regions (19, 25). The integrated plasmid pUB110 normally found in SCC*mec* II encodes resistance to kanamycin, tobramycin, and bleomycin (17). In the present study all isolates with SCC*mec* IID and IIE were resistant to kanamycin and tobramycin (42–44), although they lacked pUB110 in their SCC*mec* elements, indicating that resistance determinants for these aminoglycosides were present elsewhere on the genome and that pUB110 may have been lost. Loss or gain of plasmids such as pUB110 in SCC*mec* has been documented previously; SCC*mec* IA is believed to have evolved from SCC*mec* I by acquisition of pUB110 or vice versa (32). SCC*mec* IVA has been shown to harbor pUB110 (31), and Wielders et al. (47) described pUB110 associated with SCC*mec* III and SCC*mec* IV (47).

SCC*mec* IIA to IIE (40 to 27 kb) are smaller than previously described SCC*mec* II elements (57 kb), and SCC*mec* IIE, with a size of 27 kb, is more similar in size to SCC*mec* IV (20 to 25 kb) or SCC*mec* V (28 kb) (Fig. 2). It has been suggested that due to their relatively small size, SCC*mec* IV and SCC*mec* V are highly competitive mobile genetic elements that are more readily transferred between staphylococci than the larger SCC*mec* elements and, once acquired by the host, do not compromise its fitness (18, 25). This could also be true for the SCC*mec* II variants described in the present study, where the smaller size resulted from loss of regions that are not required for maintenance of methicillin resistance or precise excision and integration of SCC*mec*.

Like SCC*mec* IIA to IIE, the origin of the two ST8-MRSA-IV variants IVE and IVF identified here is unclear. SCC*mec* IVE is almost identical to SCC*mec* IVc, except in the region downstream of the *mec* complex, suggesting that SCC*mec* IVE may have arisen by recombination between an SCC*mec* IVc element and another SCC*mec* sequence. SCC*mec* IVF has the same L-C region as the ST8 SCC*mec* II variants, but the rest of the element is similar to SCC*mec* IVE (Fig. 2). SCC*mec* IVF may have evolved by recombination between a SCC*mec* II variant and SCC*mec* IVE, which donated the rest of the element including the unusual downstream region. Alternatively, SCC*mec* IVF may have evolved by recombination between SCC*mec* IVb and SCC*mec* IVE. Recombination events in the *dcs* region appear to be common, because other researchers have reported the absence of *dcs* amplimers following multiplex PCR of SCC*mec* elements in various genetic backgrounds (ST8 and ST30) (2, 36). In one case, a *dcs* amplimer was reported in SCC*mec* III, although it does not usually carry the *dcs* sequence (47).

Two other SCC*mec* variants were identified in the present study. ST250-MRSA-I *–pls* appears to be a variant of the archaic clone, ST250-MRSA-I. The *pls* gene of SCC*mec* I is located within the L-C region, which is not thought to be an important part of SCC*mec* (16). The variant ST239-MRSA-III –pI258 & Tn*554* may have evolved from ST239-MRSA-III by loss of the integrated plasmid pI258 or transposon Tn*554*, or both. Further work is under way to characterize these novel variants of SCC*mec* I and SCC*mec* III.

The novel variants identified in the present study yielded multiplex PCR patterns similar to those reported in other studies, with the exception of the patterns designated in this study as SCC*mec* II *–kdp* & pUB110 and SCC*mec* III –pI258 & Tn554 (1, 2, 36, 46). However, this is the only study to date that has fully characterized the SCC*mec* elements with some of these unusual SCC*mec* multiplex patterns. Three SCC*mec* variants have been identified previously in the ST8 genetic background: SCC*mec* II *–kdp* and SCC*mec* II *–kdp*, *mecI* & pUB110 were identified in two isolates described as Irish-1 from the United Kingdom and one AR14 phenotype from Ireland, and SCC*mec* IV *–dcs* was determined in three isolates (two phenotype AR43 from Ireland and one isolate from Australia) (36). Some of the isolates investigated in that study came from the Irish MRSA collections used in the present study. The similarity between isolates with phenotype AR43 (carrying SCC*mec* IVE or IVF) and isolates described as EMRSA-Irish 2 in Australia has been noted previously (21, 27). Irish isolates with phenotypes AR13 and AR14 resemble United Kingdom isolates described as Irish-1 (44). Due to the large number of these ST8 variants identified in this present study among Irish isolates, it is possible that these ST8 variant clones evolved in Ireland and spread to the United Kingdom and Australia.

Some of the multiplex patterns reported in the present study have been identified previously in distinctly different genetic backgrounds, including the multiplex pattern SCC*mec* II *–kdp* & *mec*I in two Japanese isolates with ST5 and ST89 genotypes (2), the SCC*mec* IV *–dcs* multiplex pattern in ST30 isolates from Greece (1), and the SCC*mec*I *–pls* multiplex pattern from one ST1 isolate from the United States (46). These patterns may occur if the variant SCC*mec* elements were acquired by isolates with different genetic backgrounds. Alternatively, the similar multiplex patterns in genotypes other than ST8 may be due to different mutations, deletions, or insertions in these highly variable regions. The fact that the multiplex patterns all

lack the same amplimer does not necessarily mean that the same SCC*mec* element is present. Further investigation into these SCC*mec* elements is required.

It has been recommended that the multiplex SCC*mec* typing method should be used with MLST for routine epidemiological typing of MRSA (10). The present study has shown that if the multiplex method had been used alone as the sole SCC*mec* typing method, half the isolates investigated would have been misclassified as harboring an erroneous SCC*mec* element or would have yielded SCC*mec* multiplex patterns that could not be interpreted as belonging to any of the currently described SCC*mec* types. Combining results of the multiplex and simplex SCC*mec* typing methods allowed correct classification of some of the unusual multiplex patterns. However, neither the simplex or multiplex methods recognized a number of SCC*mec* elements recently described in other studies, including SCC*mec* V and the SCC*mec* IV variants SCC*mec* IVa, IVb, IVc, or IVd (18, 19, 25). PCR amplification and sequencing of the entire SCC*mec* element was necessary for the complete characterization of the SCC*mec* elements harbored by isolates with ambiguous multiplex patterns. Such detailed investigations are unsuitable for routine typing purposes. Furthermore, if SCC*mec* is to be used for routine epidemiological typing, some method of standardizing nomenclature for the plethora of new and variant SCC*mec* types is needed. Equally important is the question of the epidemiological significance of variant SCC*mec* elements. As SCC*mec* typing is applied to larger collections of isolates, the epidemiological significance of these variants may become apparent. However, it is clear from this and other studies that SCC*mec* can evolve rapidly, and simple PCR methods for characterizing SCC*mec* elements may need to be updated regularly to include important new variants.

All MRSA clonal types identified in this present study belong to one of the five major lineages of nosocomial MRSA, with the exception of the previously described ST12-MRSA-IV, which is a relatively minor MRSA clone. Within CC5, the two major pandemic clones, ST5-MRSA-II (New York/Japan clone) and ST5-MRSA-IV (Pediatric clone), were identified among Irish MRSA isolates (Table 3). A novel genotype, ST496, was identified in one Irish isolate from 2002. This genotype differs from ST5 by single nucleotide substitutions in both the *arcC* and *yqiL* alleles and most likely arose from ST5 by point mutation. ST5-MRSA-II has been a common clonal type among Irish MRSA isolates since 1992 to 1993 but never predominated.

It is interesting that over the years in Ireland, some MRSA clones have spread and predominated but others that cause major problems in other countries have not dominated following their introduction into Ireland. For example, ST247-MRSA-IA has been identified as the dominant MRSA clone in many hospitals in Europe, especially in Spain and Portugal (7, 30), but never predominated in Irish hospitals. Similarly, some strains of ST239-MRSA-III, such as phenotype AR01, caused major problems in Irish hospitals but other isolates did not spread (e.g., ST239-MRSA-III, phenotype AR23) or were contained within a particular unit within one hospital (e.g., ST239-MRSA-III, phenotype AR44) (38, 40). In contrast, the prevalence of ST22-MRSA-IV (phenotype AR06) has increased and, by 2002, had become the predominant clone among Irish MRSA blood culture isolates investigated under the EARSS project. Interestingly, ST22-MRSA-IV is the most prevalent epidemic strain in the United Kingdom, where it is often referred to as EMRSA-15 (28).

In conclusion, this analysis of MLST and SCC*mec* elements has provided data fundamental to understanding the epidemiology and evolutionary history of MRSA in Ireland. It has revealed a hitherto-unsuspected degree of diversity within SCC*mec* and has raised many questions which need to be addressed.

## ACKNOWLEDGMENTS

This work was supported by the Microbiology Research Unit, Dublin Dental School and Hospital. A.S. was in receipt of a Marie Curie Research grant, number HPMT-CT-2001-00288, for short-term research at the University of Bath.

We thank the Steering Group and participants of the North/South Study of MRSA in Ireland 1999 and the Irish EARSS Steering Group and participant laboratories for allowing us to include some of their MRSA isolates in this study.

## REFERENCES

1. **Aires de Sousa, M., C. Bartzavali, I. Spiliopoulou, I. S. Sanches, M. I. Crisostomo, and H. de Lencastre.** 2003. Two international methicillin-resistant *Staphylococcus aureus* clones endemic in a university hospital in Patras, Greece. J. Clin. Microbiol. **41:**2027–2032.
2. **Aires de Sousa, M., and H. de Lencastre.** 2003. Evolution of sporadic isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and their similarities to isolates of community-acquired MRSA. J. Clin. Microbiol. **41:** 3806–3815.
3. **Anonymous.** 1999. North/South study of MRSA in Ireland 1999. Irish Department of Health and Children. [Online.] http://www.dohc.ie/publications/study_of_mrsa_in_ireland_1999.html.
4. **Carroll, J. D., H. M. Pomeroy, R. J. Russell, J. P. Arbuthnott, C. T. Keane, O. M. McCormick, and D. C. Coleman.** 1989. A new methicillin- and gentamicin-resistant *Staphylococcus aureus* in Dublin: molecular genetic analysis. J. Med. Microbiol. **28:**15–23.
5. **Coleman, D. C., H. Pomeroy, J. K. Estridge, C. T. Keane, M. T. Cafferkey, R. Hone, and T. J. Foster.** 1985. Susceptibility to antimicrobial agents and analysis of plasmids in gentamicin- and methicillin-resistant *Staphylococcus aureus* from Dublin hospitals. J. Med. Microbiol. **20:**157–167.
6. **Debrise, A., K. G. Dyke, and N. El Solh.** 1996. Characterisation of a *Staphylococcus aureus* transposon, Tn*5405*, located within Tn*5404* and carrying the aminoglycoside resistance genes, *aphA-3* and *aadE*. Plasmid **35:**174–188.
7. **Dominguez, M. A., H. de Lencastre, J. Linares, and A. Tomasz.** 1994. Spread and maintenance of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. J. Clin. Microbiol. **32:**2081–2087.
8. **EARSS.** 2002. EARSS annual report 2002. [Online.] http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/.
9. **Enright, M. C., P. J. Day, C. E. Davies, S. J. Peacock, and B. G. Peacock.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
10. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methcillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. USA **99:**7687–7692.
11. **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day.** 2003. How clonal is *Staphylococcus aureus*? J. Bacteriol. **185:** 3307–3316.
12. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158:**513–516.
13. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73:**237–244.
14. **Hone, R., and C. T. Keane.** 1974. Characteristics of methicillin resistant *Staphylococcus aureus*. Ir. J. Med. Sci. **143:**145–154.
15. **Huletsky, A., R. Giroux, V. Rossbach, M. Gagnon, M. Vaillancourt, M. Bernier, F. Gagnon, K. Truchon, M. Bastien, F. J. Picard, A. van Belkum, M. Ouellette, P. H. Roy, and M. G. Bergeron.** 2004. New real-time PCR assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of staphylococci. J. Clin. Microbiol. **42:** 1875–1884.
16. **Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu.** 2001. Structural comparison of the three types of staph-

TABLE 6. Identities between ORFs of the novel SCC*mec* IV variant element IVE of Irish MRSA isolate AR43/3330.1 and the conventional SCC*mec* IVc element

| SCC*mec* element and ORF | Location[a] | Homology to ORFs of SCC*mec* IVc[b] | | |
|---|---|---|---|---|
| | | ORF | % Homology[c] | Gene product |
| IVE01 | 66–866 (complement) | CR007 | 100 | Hypothetical protein |
| IVE02 | 896–2050 (complement) | CR008 | 100 | Hypothetical protein |
| IVE03 | 2505–3551 | R002 | 100 | Hypothetical protein |
| IVE04 | 3744–4040 | R003 | 100 | Hypothetical protein |
| IVE05 | 4040–5833 | R004 | 100 | Hypothetical protein |
| IVE06 (*ccrA2*) | 6067–7416 | R005 | 99.1 | Cassette chromosome recombinase A2 |
| IVE07 (*ccrB2*) | 7438–9051 | R006 | 99.4 | Cassette chromosome recombinase B2 |
| IVE08 | 9573–9923 | R007 | 100 | Hypothetical protein |
| IVE09 | 10010–10321 | R008 | 100 | Hypothetical protein |
| IVE10 | 10333–10842 | R009 | 100 | Hypothetical protein |
| IVE11 | 10978–12159 | R010 | 100 | Hypothetical protein |
| IVE12 | 12146–12502 | R011 | 100 | Hypothetical protein |
| IVE13 | 12493–12822 (complement) | CR009 | 100 | Putative transposase of IS*1272* |
| IVE14 | 12725–13711 (complement) | CR010 (Δ*mecR1*) | 100 | Truncated signal transducer protein MecR1 |
| IVE15 | 13811–15817 | R012 (*mecA*) | 100 | Penicillin binding protein 2a |
| IVE16 | 16388–17131 (complement) | CR011 | 100 | Hypothetical protein |
| IVE17 | 18432–19106 | R013 | 100 | Transposase of IS*431mec* |
| IVE18 | 20286–20792 | —[d] | — | Hypothetical protein |
| IVE19 | 20995–21576 | — | — | Hypothetical protein |
| IVE20 | 22117–22590 | — | — | Hypothetical protein |

[a] The nucleotide positions were determined based on the sequences for SCC*mec* IVE deposited in the GenBank database under accession number AJ810121.
[b] ORFs and gene products from SCC*mec* IVc nucleotide sequence in the GenBank database, accession number AB096217 (19).
[c] Based on amino acid identity.
[d] —, no homology.

**Additional novel variants of SCC*mec* IV.** All 20 isolates with the AR43 phenotype which were found to carry SCC*mec* IV by the simplex method but gave the multiplex pattern SCC*mec* IV *–dcs* produced the expected size products for a type IVc SCC*mec* element when the primers used with isolate AR43/3330.1 were used, but differences were observed following amplification of the L-C and I-R regions (Table 4). Seventeen of the isolates had the same L-C region as SCC*mec* IVc (6.1-kb amplimer), but three isolates yielded a 5.6-kb product (Table 4). Sequencing of this amplimer confirmed it contained the SCC*mec* IVb L-C region. As with isolate AR43/3330.1, an amplimer that was larger than expected was obtained when the I-R region was amplified in all AR43 isolates (Table 4). This 4.7-kb region from an AR43 isolate with an L-C region similar to SCC*mec* IVb was sequenced and was 100% identical to that of isolate AR43/3330.1.

## DISCUSSION

MLST and SCC*mec* element analysis of MRSA isolates recovered in Irish hospitals between 1971 and 2002 showed that clones representative of each of the five major clonal complexes and eight of the major pandemic lineages have been present in Ireland at some time over the past 30 years (Tables 1 and 3). This study also confirmed that there have been major changes in the dominant clonal types. The genotypes of the earliest MRSA during the 1970s and early 1980s were ST250-MRSA-I or its novel variant, ST250-MRSA-I *–pls*, followed in the mid- to late 1980s by a new variant, ST239-MRSA-III –pI258 & Tn*554*. In 1989, ST239-MRSA-III predominated, but during the 1990s this was displaced by ST8-MRSA-II, exhibiting six of the nine novel SCC*mec* variants identified in this study. In 2002, the dominant clone was ST22-MRSA-IV.

Previous studies have proposed that MRSA clones should be defined on the basis of their genetic background as identified by MLST and their SCC*mec* type, but a striking finding of the present study was the extent of variation within SCC*mec*. If clones among the Irish MRSA population are defined on the basis of all SCC*mec* variants recognized, the population includes 20 clonal types consisting of 10 previously recognized clonal types, 9 with previously described genetic backgrounds but with novel SCC*mec* variants (including two new variants of class A *mec*) and one with a previously unreported genetic background (a double locus variant of ST5 in association with SCC*mec* II) (Table 3). The greatest variation was seen among isolates with the ST8 genotype. For example, among the 54 isolates exhibiting SCC*mec* IV by the simplex method, only those with the ST8 genotype (20/54) had variant SCC*mec* elements. In fact, 86% of isolates (74/86) with novel SCC*mec* variants had the ST8 genotype, and all carried previously unreported variants of SCC*mec* II (IIA to IIE) or IV (IVE and IVF).

Because both SCC*mec* I and IV have a *mec* complex with IS*1272* inserted at the same junction point, it has been suggested that recombination has occurred between SCC*mec* I and other sequences to generate SCC*mec* type IV (25). The novel ST8 SCC*mec* variants identified in this study resemble rearrangements of SCC*mec* II and SCC*mec* IV. SCC*mec* variants IIA to IIE have an L-C region that is almost identical to that of SCC*mec* IVb, but the rest of each element has closest identity with SCC*mec* II, including a very similar genomic structure (Fig. 2).

SCC*mec* IIA to IIE appear to be closely related. SCC*mec* IIB was found in isolates from 1989, making it the earliest SCC*mec* II variant identified in this study. It harbors the original class A *mec* complex, but its similarity to SCC*mec* IVb in the L-C region make its provenance unclear. It is likely that SCC*mec* IIB was generated following loss of most of Tn*554*,

ylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **45:** 1323–1336.

17. **Ito, T., Y. Katayama, and K. Hiramatsu.** 1999. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob. Agents Chemother. **43:**1449–1458.
18. **Ito, T., X. X. Ma, F. Takeuchi, K. Okuma, H. Yuzawa, and K. Hiramatsu.** 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob. Agents Chemother. **48:**2637–2651.
19. **Ito, T., K. Okuma, X. X. Ma, H. Yuzawa, and K. Hiramatsu.** 2003. Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. Drug Resist. Update **6:**41–52.
20. **Jevons, M. P.** 1961. Celbenin-resistant staphylococci. Br. Med. J. **i:**124–125.
21. **Johnson, H., Z. Johnson, M. Burd, D. Doyle, G. Glynn, H. Humphreys, P. McDonald, B. McDonnell, E. Mitchell, and A. Rossney.** 2000. 9th Int. Symp. Staphylococci Staphylococcal Infect., abstract no. 182.
22. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2001. Genetic organization of the chromosome region surrounding *mecA* in clinical staphylococcal strains: role of IS*431*-mediated mecI deletion in expression of resistance in *mecA*-carrying, low-level methicillin-resistant *Staphylococcus haemolyticus*. Antimicrob. Agents Chemother. **45:**1955–1963.
23. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **44:**1549–1555.
24. **Lim, T. T., F. N. Chong, F. G. O'Brien, and W. B. Grubb.** 2003. Are all community methicillin-resistant *Staphylococcus aureus* related? A comparison of their *mec* regions. Pathology **35:**336–343.
25. **Ma, X. X., T. Ito, C. Tiensasitorn, M. Jamklang, P. Chongtrakool, S. Boyle-Vavra, R. S. Daum, and K. Hiramatsu.** 2002. Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **46:** 1147–1152.
26. **Mahillon, J., C. Leonard, and M. Chandler.** 1999. IS elements as constituents of bacterial genomes. Res. Microbiol. **150:**675–687.
27. **McGechie, D., and D. Daley.** 2000. 9th Int. Symp. Staphylococci Staphylococcal Infect., abstract no. 13.
28. **Moore, P. C., and J. A. Lindsay.** 2002. Molecular characterisation of the dominant UK methicillin-resistant *Staphylococcus aureus* strains, EMRSA-15 and EMRSA-16. J. Med. Microbiol. **51:**516–521.
29. **Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G. O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu.** 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. J. Clin. Microbiol. **40:**4289–4294.
30. **Oliveira, D., I. Santos-Sanches, R. Mato, M. Tamayo, G. Ribeiro, D. Costa, and H. de Lencastre.** 1998. Virtually all methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the largest Portuguese teaching hospital are caused by two internationally spread multiresistant strains: the "Iberian" and the "Brazilian" clones of MRSA. Clin. Microbiol. Infect. **4:**373–384.
31. **Oliveira, D. C., and H. de Lencastre.** 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **46:** 2155–2161.
32. **Oliveira, D. C., A. Tomasz, and H. de Lencastre.** 2001. The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated *mec* elements. Microb. Drug Resist. **7:**349–361.
33. **Oliveira, D. C., A. Tomasz, and H. de Lencastre.** 2002. Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. Lancet Infect. Dis. **2:**180–189.
34. **Reference deleted.**
35. **Reynolds, P. E., and D. G. J. Brown.** 1985. Penicillin-binding proteins of beta-lactam resistant strains of *Staphylococcus aureus*: effect of growth conditions. FEBS Lett. **192:**28–32.
36. **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**3926–3934.
37. **Robinson, D. A., and M. C. Enright.** 2004. Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect. **10:**92–97.
38. **Rossney, A.** 2003. A new strain of MRSA imported from Singapore. Epi-Insight **4:**1.
39. **Rossney, A. S.** 1995. Methicillin-resistant *Staphylococcus aureus* in four Dublin hospitals (1988–1994). University of Dublin, Trinity College, Dublin, Ireland.
40. **Rossney, A. S., D. C. Coleman, and C. T. Keane.** 1994. Evaluation of an antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*. J. Med. Microbiol. **41:**441–447.
41. **Rossney, A. S., D. C. Coleman, and C. T. Keane.** 1994. Antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*. J. Med. Microbiol. **41:**430–440.
42. **Rossney, A. S., and C. T. Keane.** 2002. Strain variation in the MRSA population over a 10-year period in one Dublin hospital. Eur. J. Clin. Microbiol. Infect. Dis. **21:**123–126.
43. **Rossney, A. S., M. J. Lawrence, P. M. Morgan, M. M. Fitzgibbon, and B. O' Connell.** 2004. 11th Int. Symp. Staphylococci Staphylococcal Infect., abstract no. ME24.
44. **Rossney, A. S., P. McDonald, H. Humphreys, G. M. Glynn, and C. T. Keane.** 2003. Antimicrobial resistance and epidemiological typing of methicillin-resistant *Staphylococcus aureus* in Ireland (North and South), 1999. Eur. J. Clin. Microbiol. Infect. Dis. **22:**379–381.
45. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Shukla, S. K., S. V. Ramaswamy, J. Conradt, M. E. Stemper, R. Reich, K. D. Reed, and E. A. Graviss.** 2004. Novel polymorphisms in *mec* genes and a new *mec* complex type in methicillin-resistant *Staphylococcus aureus* isolates obtained in rural Wisconsin. Antimicrob. Agents Chemother. **48:**3080–3085.
47. **Wielders, C., A. Box, J. Verhoef, and A. Fluit.** 2003. Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. 2003, abstr. A-068.