2007

Characterization of the Factors Associated with SCCmec Mobility in Staphylococcus Aureus

Michael James Noto
Virginia Commonwealth University

CHARACTERIZATION OF THE FACTORS ASSOCIATED WITH SCC*MEC* MOBILITY IN *STAPHYLOCOCCUS AUREUS*

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

MICHAEL JAMES NOTO
B.S., James Madison University, 2000

Director: Gordon L. Archer
Associate Dean for Research
Professor, Division of Infectious Diseases
Departments of Medicine and Microbiology and Immunology

Virginia Commonwealth University
Richmond, Virginia
February, 2007

Acknowledgements

I would like to thank my advisor, Dr. Gordon Archer, for the opportunity to conduct my graduate research in his laboratory and for the challenging and stimulating project I was assigned. Dr. Archer has proven to be an excellent scientific advisor and a great mentor to an aspiring physician-scientist. His guidance and support have been extremely valuable. I would also like to thank all the members of the Archer lab, past and present. Dr. Jon Finan introduced me to the Archer lab. Dr. Qixun Zhao and Dr. Adriana Rosato have been great resources throughout my training. Dr. Alastair Monk and Paige Fox were not only helpful scientifically, but were instrumental in creating a productive yet reasonably enjoyable work environment. I would also like to acknowledge Bill Craig for his assistance along the way. The advice and suggestions I received from my committee members – Dr. Gail Christie, Dr. Frank Macrina, Dr. Rich Marconi, and Dr. Darryl Peterson – were vital to my success as a student. I would also like to thank members of the neighboring Marconi and Cornelissen labs for all of their help, particularly Kelley Hovis, Beth Rogers, Dr. Greg Price, and Dr. Tracey Hagen. Also, Dr. Barry Kreiswirth provided much assistance during our collaboration.

Above all, I would like to thank my family because without their support I would never have made it this far. My parents, Susan and James, and my stepmother Diane have provided me with loving homes, an education, and every opportunity to succeed in life. For this I am eternally grateful. My brother Chris, and sisters Jess and Michole have

provided love and support throughout my life. Lastly, I would like to thank Jennifer McMillan for brightening my every day.

Table of Contents

Page

List of Tables

Page

List of Figures

Page

Abstract

CHARACTERIZATION OF THE FACTORS ASSOCIATED WITH SCC*MEC*

MOBILITY IN *STAPHYLOCOCCUS AUREUS*

By Michael James Noto

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

Major Director: Gordon L. Archer, M.D.
Professor, Division of Infectious Diseases
Departments of Medicine and Microbiology and Immunology

The gene encoding resistance to β-lactam antibiotics in the staphylococci is found on the chromosome in a genomic island designated Staphylococcal Chromosome Cassette *mec*, or SCC*mec*. In addition to the resistance gene, *mecA*, SCC*mec* also contains site specific recombinase genes, *ccrAB*, that are capable of catalyzing the chromosomal excision and integration of SCC*mec*. The increasing prevalence of methicillin-resistant *Staphylococcus aureus* infections may be due, in part, to the transfer of SCC*mec* into successful methicillin-sensitive *S. aureus* lineages. In this work we attempt to better

characterize the factors associated with SCC*mec* transfer, beginning with CcrAB-mediated SCC*mec* excision in a collection of MRSA containing type IV SCC*mec*. CcrAB-mediated excision of type IV SCC*mec* was not demonstrated for all strains tested, as a subset of *S. aureus* strains with type IV SCC*mec* did not excise their element. These strains are all highly related and represent a lineage of successful community associated pathogens. In addition, the inability to excise SCC*mec* in these strains is associated with the presence of a presumptive mobile element containing the gene for staphylococcal enterotoxin H (*seh*) immediately outside of SCC*mec* on the chromosome. *Staphylococcus aureus* strain USA300 contains SCC*mec* type IV and a genomic island containing an arginine deiminase pathway, known as ACME, inserted adjacent to one another in the SCC*mec* chromosomal attachment site. Each element was site-specifically excised from the chromosome by CcrAB, resulting in two independent, extra-chromosomal, circularized elements. Therefore the presence of ACME did not disrupt SCC*mec* excision.

Next, we describe three MRSA strains that became resistant to vancomycin during passage on increasing concentrations of the drug. In each case, *mecA* was lost during passage. Strain 5836VR lost *mecA* by the site-specific chromosomal excision of SCC*mec* while the other two strains (3130VR and VP32) deleted portions of their SCC*mec* elements in a manner that appears to involve IS431. Conversion to vancomycin resistance caused a decrease in growth rate that was partially compensated for by deletion of *mecA*. In mixed culture competition experiments, vancomycin resistant strains that lacked *mecA* readily out-competed their *mecA*-containing counterparts, suggesting that the loss of *mecA* during conversion to vancomycin resistance was advantageous to the organism.

Finally, we examined the genetic structure surrounding the SCC*mec* attachment site in a diverse collection of methicillin-sensitive *S. aureus* isolates. This region of the chromosome varies greatly from strain to strain and appears to be prone to recombination. Open reading frames found in this region were homologous to enterotoxins, restriction-modification enzymes, and transposases. Several open reading frames that have not been previously reported in staphylococci were also present in this region. 28 out of the 42 isolates examined did not contain the attachment site sequence found in *S. aureus* isolates known to be capable of CcrAB-mediated SCC*mec* integration or excision. This may indicate that these strains do not contain a functional attachment site and therefore may not have the potential to acquire SCC*mec* by CcrAB-mediated recombination.

Chapter 1 Introduction

General characteristics

The genus *Staphylococcus* consists of more than 30 species of Gram-positive cocci that are approximately 1μm in diameter. Because cell division takes place in more than one plane, the cocci form clusters that, when viewed by Gram stain, look like bunches of grapes. Staphylococci are most frequently found in association with the skin, skin glands, and mucus membranes of warm-blooded animals but they can also be found in other environments, such as soil. Staphylococci are facultative anaerobes and are capable of generating energy by both respiratory and fermentative processes. Most members of the genus are catalase-positive, oxidase-negative, and have complex nutritional needs, requiring many amino acids and B vitamins for growth. Also, they are tolerant of high concentrations of NaCl and are capable of growth in the presence of up to 1.7 M NaCl (150).

Staphylococcal DNA has a G + C content of 30–38% making it a member of the low G + C group of Gram-positive bacteria from the order Bacillales, which also contains the genera *Enterococcus*, *Listeria*, and *Bacillus* (56, 133). *Staphylococcus aureus* is the most well studied of the staphylococci because of its frequent involvement in human disease. *S. aureus* has the ability to cause blood plasma to clot or coagulate. This is used to distinguish *S. aureus* from the other pathogenic members of the genus, which are

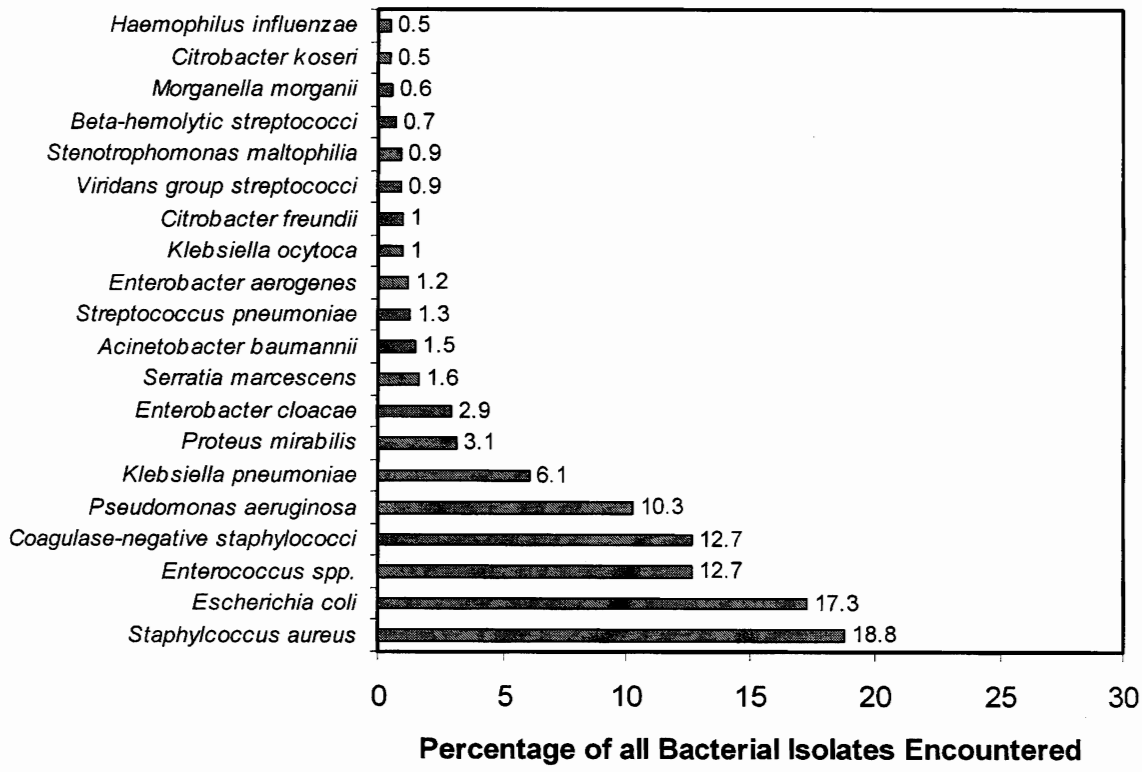
collectively known as coagulase-negative staphylococci. *S. epidermidis* is the most well studied of the coagulase-negative staphylococci and is a commensal of the human skin as well as an opportunistic human pathogen.

Staphylococcus aureus infections

The anterior nares are the ecological niche of *S. aureus* and the major site of colonization in humans. Nasal carriers of *S. aureus* can be divided into three groups. Individuals whose nares are always colonized with one strain of *S. aureus* are called persistent carriers and make up approximately 20% of the population (83). Persistent carriers seem to be protected from the acquisition of other strains during hospitalization, but this barrier is reduced when patients receive antibiotic therapy (102). Nearly 60% of the population are intermittent carriers and are transiently colonized by varying strains of *S. aureus*. People that are never colonized by *S. aureus* are called noncarriers and make up 20% of the population. The incidence of *S. aureus* nasal carriage rate among the general population is 37.2% (83). A positive *S. aureus* carrier state is a predictor of infection in surgical patients and is also associated with an increased risk of disease in other hospitalized patients (83). Treatment of the *S. aureus* carrier state decreases the rate of overall *S. aureus* infections in these patients (113).

S. aureus is a prominent human pathogen capable of causing a wide range of disease in the community as well as in healthcare settings. Figure 1 shows the frequency of bacterial species encountered in inpatient settings in the United States during a seven year period (138). *S. aureus* was the most frequent cause of nosocomial disease in this

Figure 1. Relative frequency of bacterial species/groups encountered in clinical specimens from inpatients. Data is cumulative from 1998- 2005 and based on a total of 3,209,413 bacterial isolates. Taken from Styers, D. et al. (138).

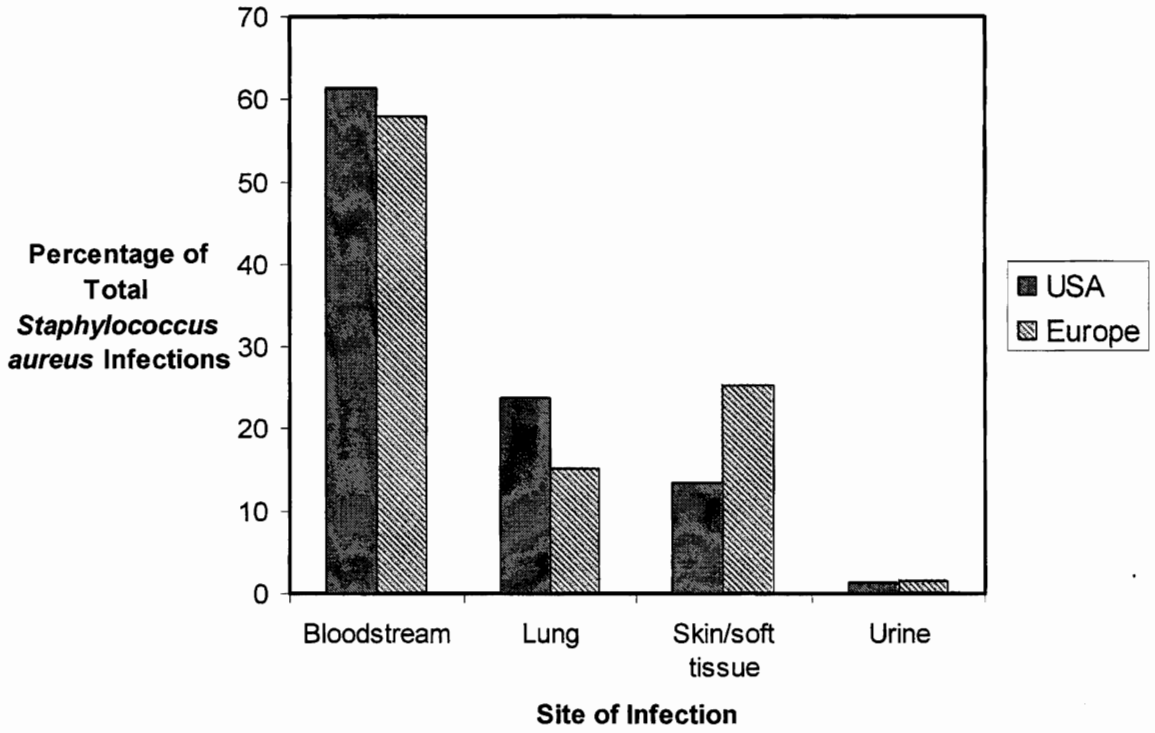

Haemophilus influenzae 0.5
Citrobacter koseri 0.5
Morganella morganii 0.6
Beta-hemolytic streptococci 0.7
Stenotrophomonas maltophilia 0.9
Viridans group streptococci 0.9
Citrobacter freundii 1
Klebsiella ocytoca 1
Enterobacter aerogenes 1.2
Streptococcus pneumoniae 1.3
Acinetobacter baumannii 1.5
Serratia marcescens 1.6
Enterobacter cloacae 2.9
Proteus mirabilis 3.1
Klebsiella pneumoniae 6.1
Pseudomonas aeruginosa 10.3
Coagulase-negative staphylococci 12.7
Enterococcus spp. 12.7
Escherichia coli 17.3
Staphylcoccus aureus 18.8
0
5
10
15
20
25
30
Percentage of all Bacterial Isolates Encountered

study, comprising 18.8% of all isolates encountered. A retrospective study of *Staphylococcus aureus* disease in the New York City metropolitan area during 1995 examined the incidence of disease and modeled the economic cost associated with disease (122). *S. aureus* disease had an incidence of 13,550 (6,300 nosocomial, and 7,250 community-associated infections) and an estimated total cost of $435.5 million. In addition, 1,400 deaths were attributed to *S. aureus* infections. *S. aureus* is capable of, but not limited to, causing the following infections: skin and soft-tissue infections including furuncles (boils), empitigo (superficial blisters), and cellulitis (infectious inflammation of subcutaneous tissue), bacteremia, both native and prosthetic-valve endocarditis, osteomyelitis (infection of the bone), pneumonia, surgical-site infections, diabetic ulcers, dialysis-related infections, enterocolitis, scalded skin syndrome, and toxic shock syndrome (77, 124). Figure 2 shows the relative frequency of sites infected by *S. aureus* as reported by SENTRY-participating hospitals in the United States and Europe during a two year period (38). Bloodstream infections were the most common in both the USA and Europe, while lung and skin/soft-tissue infections were also prevalent. It is clear that *S. aureus* is among the most frequent causes of bacterial infectious disease and these infections place a large burden on the healthcare system. These infections are further complicated by the organism's ability to develop resistance to chemotherapeutic agents.

Virulence determinants

The wide range of *S. aureus* disease manifestations is due to the organism's ability

Figure 2. Frequency of *Staphylococcus aureus* infections from the United States and Europe by site of infection. Data was collected by SENTRY-participating hospitals between 1997 and 1999. Adapted from Diekema, D. J. et al. (38).


Percentage of Total *Staphylococcus aureus* Infections
70
60
50
40
30
20
10
0
Bloodstream
Lung
Skin/soft tissue
Urine
Site of Infection
USA
Europe

to survive in many different environments, which is aided by a number of different virulence determinants.

Attachment

S. aureus produces many proteins involved in attachment to human cellular and acellular components that are thought to play a role in virulence. These proteins either remain associated with the surface of the bacteria or are released extracellularly. The former are known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) while the latter are known as SERAMs (secretable expanded repertoire adhesive molecules). Fibronectin binding proteins A and B (FnbpA and FnbpB) are MSCRAMMs that are cell wall anchored and bind the extra-cellular matrix constituent, fibronectin. Additional cell wall anchored proteins can bind fibrinogen and collagen. These are known as clumping factor A and B (ClfA and ClfB) and collagen binding protein (Cna), respectively. In addition, plasmin-sensitive surface protein (Pls) plays a role in binding to both fibrinogen and fibronectin once it is cleaved by plasmin. *S. aureus* also produces a membrane bound elastin binding protein, EbpS (68). Protein A, encoded by the *spa* gene, is a cell wall anchored protein that is capable of binding to the Fc portion of IgG molecules, which may have a role in circumventing phagocytosis (112). Protein A has also exhibited the ability to bind to platelets and von Willebrand factor (66). *S. aureus* also produces SERAMs that bind to fibrinogen, fibronectin, prothrombin, and Von Willebrand factor (68).

Device-related *S. aureus* infections are dependent on the bacterium's ability to adhere to the surface and then form a mucoid biofilm. Biofilms are complex communities of surface-attached bacteria that are embedded in an exopolymeric matrix composed primarily of poly-N-acetylglucosamine (PNAG) (51, 92). PNAG synthesis is directed by proteins encoded by the *ica* (intercellular adhesion) operon. Unlike their planktonic counterparts, bacteria in a biofilm are typically resistant to a large variety of antimicrobial agents, which can complicate treatment (30).

Iron acquisition

The ability to acquire iron is essential for virulence, as iron is sequestered by host proteins such as lactoferrin and transferrin in vivo. *S. aureus* produce several low molecular weight siderophores known as aereochelin and staphyloferrin A and B that aid in iron acquisition. *S. aureus* also have the ability to bind and utilize human transferrin (86).

Exotoxins

S. aureus produces several cytolytic toxins, including α-toxin, β-toxin, and the bicomponent exotoxins such as LukF-PV/LukS-PV and γ-toxin. α-toxin is a secreted protein, encoded by *hla*, that is hemolytic, cytotoxic, and dermonecrotic. α-toxin monomers insert into the eukaryotic membrane and oligomerize into a β-barrel that forms a membrane pore, which causes osmotic cytolysis (97). LukF-PV/LukS-PV is a bicomponent cytolysin that is also known as the Panton-Valentine Leukocydin (PVL). The

bicomponent toxins are dependent on two secreted proteins that insert into the eukaryotic membrane and hetero-oligomerize to form a pore (78). PVL has a high cell specificity for leukocytes and is strongly associated with highly virulent community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections (24, 58, 88). Despite the epidemiological correlation between PVL and highly virulent CA-MRSA infections, PVL is not a key virulence determinant in mouse models of infection (145).

Superantigen toxins

S. aureus produce an assortment of staphylococcal enterotoxins (SEs) that are heat stable and a major cause of gastroenteritis as well as toxic shock-like syndromes (10). SEs can act as potent gastrointestinal toxins as well as superantigens that stimulate T-cell proliferation in a non-specific manner. Toxins SEA through SEJ (excluding SEF) are typically involved in gastroenteritis, while TSST-1 is the prototypical cause of toxic shock syndrome (10, 107). The superantigen activity of these toxins is based on the toxin's ability to cross-link the T-cell receptor with the MHC class II molecule found on antigen-presenting cells, in an antigen-independent manner. Thus, general T-cell proliferation occurs instead of the activation of an antigen-specific subset of the T-cell population (10, 86). These toxins are typically found on mobile genetic elements, some of which will be discussed in a later section.

Virulence gene regulation

The production of virulence factors is controlled in response to cell density, energy availability, and environmental signals so that they are only produced when needed. Genes encoding surface proteins are down regulated early in the growth phase while secreted proteins are up regulated after exponential phase in a cell density-dependent manner (41). This regulation is due, in large part, to the accessory gene regulator (*agr*) two-component regulatory system. The *agr* system is encoded by an approximately 3 kb operon driven by two promoters, P2 and P3. P2 controls transcription of *agrBDCA*, while RNAIII is transcribed from P3. AgrC (sensor) and ArgA (response regulator) comprise the two component system that responds to AIP (auto-inducing peptide) in the extracellular environment and serves to drive transcription from P2 and P3. *agrD* encodes AIP, which is processed and exported by AgrB. The RNAIII transcript is the effector of *agr*, responsible for the regulation of wide variety of accessory genes (106). Virulence gene expression is also controlled by the alternate sigma factor, σ^B, as well as transcription factors, including SarA and its homologues (7).

Mechanisms of antibiotic resistance

One of the most difficult aspects of *S. aureus* disease is the selection of effective therapeutic agents. This difficulty arises because *S. aureus* have developed resistance to every major class of antibiotics. The following sections describe the mechanisms of action for some prominent classes of antibiotics as well as the resistance mechanisms employed by *S. aureus*.

B-lactams and glycopeptides

Resistance to the β-lactam and glycopeptide classes of antibiotics will be discussed in later sections.

Aminoglycosides

Aminoglycosides are bactericidal agents that potently inhibit protein synthesis by binding to the 30S ribosomal unit. Gentamicin and tobramycin are the most active against staphylococci, but they should not be used as single therapy because they select for resistant small colony variants that have defective transport of aminoglycosides into the cell. Resistance also occurs through the acquisition of genes encoding aminoglycoside modifying enzymes (AMEs). These include acetyltransferases, phosphotransferases, and nucleotidyltransferases that inactivate the drug (23, 153).

Resistance to macrolides, lincosamides, and streptogramin-B

Macrolides, lincosamides, and streptogramins are structurally unrelated but all act to inhibit the translocation step of protein synthesis by binding to the 50S ribosomal subunit. MLS_B resistance (for macrolide, lincosamide, and streptogramin-B) occurs by methylation of the common binding site on the ribosome. The methylases responsible for this action are encoded by *ermA*, *ermB*, and *ermC*. Expression of these genes is either inducible or constitutive. The latter results in resistance to all three antibiotic classes whereas only macrolides can induce expression of the *erm* genes. Macrolide resistance can

also be mediated by an efflux protein encoded by *msrA*. However, efflux does not produce cross-resistance to lincosamides or streptogramins (23).

Tetracyclines

Tetracyclines are a class of bacteriostatic antibiotics that inhibit protein synthesis by binding to the 30S ribosomal subunit and blocking the tRNA from entering the acceptor site. Staphylococci develop resistance in two ways; drug efflux and ribosomal protection. The tetracycline efflux system is encoded by *tetK* while *tetM* is the determinant of ribosomal protection (153).

Fluoroquinolones

Fluoroquinolones are bactericidal antibiotics that inhibit two essential bacterial enzymes, DNA gyrase and topoisomerase IV. Inhibition of either target is sufficient to inhibit bacterial growth. Fluoroquinolone resistance is a multi-step process, where a series of mutations in the gene for DNA gyrase (*gyrA*) and the gene for topoisomerase IV (*grlA*) lead to increasing levels of resistance (23, 153).

Linezolid

Linezolid is an oxazolidinone antibiotic that binds to the 50S ribosomal subunit and inhibits formation of the 70S ribosomal initiation complex, thereby inhibiting protein synthesis. Linezolid is one of the few antibiotics effective in treating infections by methicillin-resistant *S. aureus* (MRSA), and resistance to linezolid is rare. When

resistance does occur, it is achieved through mutation of the chromosomal gene encoding the 23S rRNA (153).

Rifampin

Rifampin inhibits transcription, and therefore protein synthesis, by binding to RNA polymerase. Resistance occurs through mutation of *rpoB*, which encodes the β subunit of RNA polymerase (23).

Mupirocin

Mupirocin is a topical agent that acts by inhibiting isoleucyl tRNA synthetase, thereby preventing protein synthesis. Clinically relevant resistance is achieved by acquiring *mupA*, which encodes an insensitive tRNA synthetase (153).

Chloramphenicol

Chloramphenicol is a protein synthesis inhibitor that functions by binding to the 50S ribosomal subunit and blocking the action of peptidyltransferases. Staphylococci become resistant through the action of chloramphenicol acetyl transferases (encoded by *cat*), which inactivate the drug (153).

Genome organization and mobile genetic elements

The first genome sequences of *Staphylococcus aureus* strains N315 and Mu50 were determined in 2001 (84). Currently, the complete genome sequences of eight *S. aureus*

strains are known and the genomes of several other strains have been partially determined (8, 39, 57, 70, 84). *S. aureus* contains a circular chromosome that is 2.8-2.9 Mbp in size with a G + C composition of 32.8%. The *S. aureus* chromosome can be divided into a core component, which includes gene regions shared by all sequenced strains, and an accessory component, which includes gene regions not found in all strains. The organization of the core component is well conserved and variations from strain to strain are largely due to single nucleotide polymorphisms. The accessory component of the chromosome is predominantly contained in large genetic elements, such as prophage and genomic islands, which carry known virulence determinants (described below).

Genomic islands

A series of 15-20 kb chromosomal elements carrying superantigen toxins exist in *S. aureus*. These are collectively known as staphylococcal pathogenicity islands (SaPIs) (107). The prototypical member of this family, SaPI1, carries the gene encoding TSST-1 (*tst*) and is transduced at a high frequency by phage 80α. SaPI1 is chromosomally excised, replicates extra chromosomally, and is packaged into smaller versions of 80α phage heads for transfer (89). SaPI2 has a similar relationship with phage 80, and SaPIbov1 with phages 80α, 11, and 147 (123, 144). Members of the SaPI family are present in most of the genomes of sequenced *S. aureus* strains. In addition to members of the SaPI family, the genome sequenced strains contain genomic islands νSaα and νSaβ, which do not contain recombinase genes, but do contain a class I restriction–modification system (84).

Prophage

Staphylococcal phages can be divided into three classes based on the size of their genomes; less than 20 kb (class I), approximately 40 kb (class II), and greater than 125 kb (class III) (85). These phages play an important role in the evolution and virulence of *S. aureus* and offer a means for the horizontal transfer of genetic information. Each of the genome sequenced strains contain between one and three lysogenic class II phages, most of which carry known virulence determinants (staphylococcal enterotoxins a, g, k, staphylokinase, exfoliative toxin, and Panton-Valentine Leukocidin) (8, 39, 57, 70, 73, 84).

Insertion sequences and transposons

IS elements (insertion sequences) contain at least one gene encoding a transposase, which carries out the recombination necessary for transposition. Most IS elements also contain short, terminal, inverted repeats that act as the transposase binding sites. Transposons are larger transposable elements that contain additional genes, which are often antibiotic resistance determinants. *S. aureus* is known to contain more than ten transposons, the majority of which carry antibiotic resistance determinants (9). In addition *S. aureus* contain several insertion sequences, including IS431/IS257. IS431/IS257 is found flanking antibiotic resistance determinants on many staphylococcal plasmids and is universally found in association with the methicillin-resistance determinant, *mecA* (12, 101).

Plasmids

Staphylococcal plasmids can be divided into three classes. Class I plasmids are small (1-5 kb), have a high copy number (15-50/cell), and usually encode a single antibiotic resistance determinant. Class II plasmids are of intermediate size and copy number, and they encode β-lactamase and resistance to inorganic ions. A number of larger plasmids (40-60 kb) that are conjugatively proficient comprise class III plasmids. These plasmids contain multiple resistance determinants including resistance to gentamicin, trimethoprim, ethidium bromide, and quaternary amines (108). Staphylococcal plasmids are an important means by which antibiotic resistance is spread and the conjugative plasmids also offer a means for the horizontal transfer of non-plasmid genetic information. Staphylococcal plasmids are prevalent, with each of the genome sequenced *S. aureus* strains containing between one and three plasmids (8, 39, 57, 70, 84).

SCCmec elements

The methicillin resistance determinant is carried by a family of genomic islands known as Staphylococcal Chromosome Cassette *mec* (SCC*mec*) (80). These elements are the subject of detailed discussion below.

Strain typing

Several genome based means of determining the evolutionary relatedness of *S. aureus* strains exist. These include pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and *spa* typing. PFGE is based on restriction fragment length polymorphism banding patterns on pulsed-field agarose gels by cleavage of chromosomal

DNA with the SmaI endonuclease (139). MLST is a sequence-based technique where evolutionary relatedness is determined based on the variability in the DNA sequences of seven house keeping genes (45). *spa* typing is another sequence based technique that compares the variable-number of tandem repeat sequences present within the 3' end of the *spa* gene to determine the relatedness between strains (129). Enright, et al. have used MLST to analyze the population structure of *S. aureus*, and found that the population is largely clonal (46). They examined a collection of 359 MRSA and compared them to 553 MSSA isolates based on their MLST pattern. They found that the strains clustered into 162 different multi-locus sequence types (STs). However, MRSA were found in only 38 of the 162 STs, indicating that only a minority of *S. aureus* lineages have acquired the methicillin resistance determinant. Additionally, Feil, et al. found that point mutations give rise to new MLST alleles at least 15-fold more frequently than does recombination, although recombination is an important driving factor in the long term evolution of *S. aureus* (48).

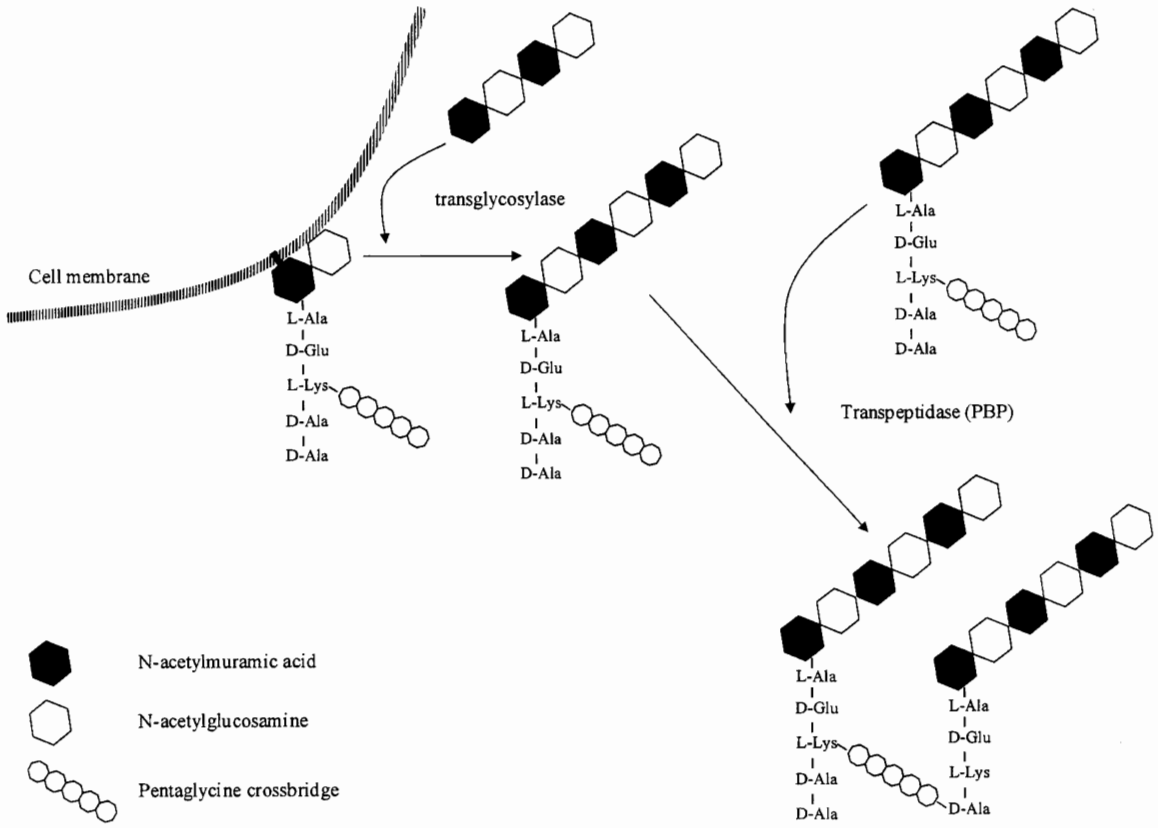
Cell wall structure and biosynthesis

The staphylococcal cell wall consists of three components; peptidoglycan, teichoic acid, and cell surface proteins. Of these, peptidoglycan is the most abundant. Peptidoglycan is composed of repeating disaccharide units of β-1-4 linked N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). The average glycan chain length is 12–16 disaccharide units and these polymers form the rigid backbone of peptidoglycan. Extending from the carboxyl residue of each NAM moiety is an oligomeric

stem peptide with the sequence L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine (137). A series of five L-glycine residues is added to the L-lysine of the stem peptide (94). In a transpeptidation reaction catalyzed by penicillin-binding proteins (PBPs), the terminal L-glycine is attached to the penultimate D-alanine residue of another stem peptide. The terminal D-alanine of this stem peptide is cleaved during this reaction. Thus, a flexible pentaglycine cross-bridge is formed between two peptidoglycan moieties, resulting in a strong yet flexible, three-dimensional cell wall structure. The flexibility of this cross bridge allows for the highly cross-linked nature of the *S. aureus* cell wall, where nearly 90% of peptidoglycan moieties are cross-linked (55). Figure 3 diagrams the cell wall structure as well as the transglycosylation and transpeptidation reactions involved in its synthesis.

Synthesis of peptidoglycan monomeric units takes place in the cytoplasm, where it is linked to a lipid carrier, undecaprenyl phosphate, for assembly and transport to the cell surface (16). Once outside of the cell, peptidoglycan monomeric units are incorporated into the growing cell wall. This involves both transglycosylation and transpeptidation reactions. Transglycosylation is carried out by the multi-modular PBPs, specifically PBP2 in *S. aureus*, as well as by the monofunctional glycosyltransferase, *mgt*, and results in the incorporation of the monomeric unit into the glycan polymer (13, 147). The transpeptidation, or cross-linking, of cell wall described above is catalyzed by PBPs, of which *S. aureus* typically contain four. PBPs 1, 2, and 3 are high molecular weight

Figure 3. Staphylococcal cell wall structure. The structure of staphylococcal cell wall is shown including the transglycosylation and transpeptidation reactions involved in cell wall synthesis. Stem peptides and cross-bridges are not shown on all N-acetylmuramic acid residues of the glycan chains.


Cell membrane
transglycosylase
L-Ala
D-Glu
L-Lys
D-Ala
D-Ala
Transpeptidase (PBP)
N-acetylmuramic acid
N-acetylglucosamine
Pentaglycine crossbridge

proteins (87, 80, and 75 kDa, respectively) while PBP 4 is a low molecular weight protein (41 kDa) (150).

Another key component of the *S. aureus* cell wall is teichoic acid, which is a polymer of ribitol residues linked by phosphodiester bridges. Teichoic acid imparts a negative charge on the cell surface, which plays a role in the acquisition of metal ions, and has been implicated as a component of the *S. aureus* phage receptor (29, 150). *S. aureus* MSCRAMM cell surface proteins are another component of the cell wall. These proteins contain a signal sequence required for protein secretion and an LPXTG motif. The LPXTG motif is cleaved by sortase and then covalently attached to peptidoglycan. Protein A, Cna, FnBP, and ClfA are anchored in this manner (52).

Vancomycin resistance

Mechanism of vancomycin action

Vancomycin is a bactericidal, cell wall active antibiotic that inhibits the transpeptidation of peptidoglycan by a mechanism unique from that of β-lactams. Vancomycin binds with high affinity to the C-terminal D-ala-D-ala of the peptidoglycan stem pentapeptide, thereby preventing the interaction between PBPs and their substrate, D-ala-D-ala. Vancomycin does not penetrate into the cytoplasm and can therefore only interact with D-ala-D-ala on the outer surface of the cell membrane (32).

Resistance mediated by the van genes

VanA-mediated resistance is characterized by inducible high levels of resistance to vancomycin and is carried by Tn1546 and elements closely related to it. This type of resistance has been well characterized in *Enterococcus*. VanH is a dehydrogenase that reduces pyruvate to D-lactate (6). The VanA ligase links D-ala and D-lac by esterification, and the resulting depsipeptide replaces the terminal D-ala-D-ala of the stem pentapeptide. Vancomycin has a low affinity for the terminal D-ala-D-lac, and therefore does not inhibit the incorporation of this substrate into the cell wall (17). However, the simultaneous production of D-ala-D-ala and D-ala-D-lac stem pentapeptides is not sufficient for vancomycin resistance. VanX is a D, D-dipeptidase that hydrolyzes the D-ala-D-ala dipeptide synthesized by the host (116). VanY is a D, D-carboxypeptidase that removes the C-terminal D-ala residues of stem pentapeptide when VanX hydrolysis is incomplete (5). Thus, VanA-mediated resistance results from the production of an altered, low affinity, target molecule, D-ala-D-lac, with the simultaneous cleavage of any existing D-ala-D-ala in the cell wall. The *van* genes have been detected on conjugative plasmids in *S. aureus* on only five occasions, but they are more commonly found in *Enterococcus* (20-22, 149).

Resistance due to thickened cell wall

S. aureus with intermediate levels of resistance to vancomycin are more frequently encountered in the clinic than highly resistant isolates (136). These strains do not contain the *van* genes nor do they contain any other known determinant of vancomycin resistance. Instead, they share a common phenotype of a thickened cell wall, and a high ratio of cell

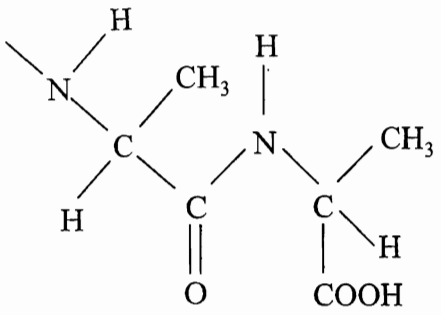
wall to total cell volume (35, 36, 131). Another feature of these cell walls is a low level of peptidoglycan cross-linking as compared to the typical staphylococcal cell wall (36, 131, 132). Cui, et al. proposed that the common cell wall phenotype is responsible for vancomycin resistance in these isolates. The combination of a thickened cell wall and reduced levels of peptidoglycan cross-linking result in increased D-ala-D-ala stem peptides in the periphery of the cell wall. Vancomycin binds these targets and therefore cannot access the interior of the cell wall where peptidoglycan transpeptidation occurs (36). This hypothesis is supported by evidence showing a concordant decrease in cell wall thickness as vancomycin intermediately resistant cells (VISA) become increasingly vancomycin sensitive (35). Also, the thickened cell wall of VISA could protect ongoing peptidoglycan biosynthesis in the cytoplasmic membrane from vancomycin inhibition (34).

B-lactam resistance

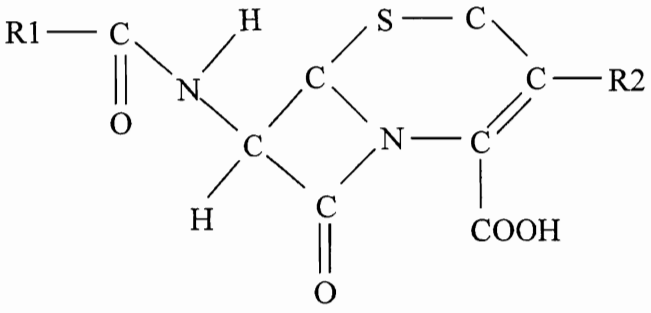
Mechanism of β-lactam action

β-lactam antibiotics consist of penicillins, cephalosporin, and the penicillinase-resistant β-lactams, such as methicillin and oxacillin. They are bactericidal, cell wall active antibiotics that function by inhibiting the transpeptidation step of peptidoglycan synthesis. This is accomplished by binding to and inactivating PBPs in the cell wall (23). β-lactams mimic the natural substrate of PBPs, which is the terminal D-ala-D-ala of the peptidoglycan stem peptide. Figure 4 shows the basic structure of β-lactam antibiotics and that of the natural PBP substrate, D-ala-D-ala. The biochemical reaction between a PBP and its substrate, whether D-ala-D-ala or β-lactam, begins with a non-covalent association

Figure 4. The structures of D-alanyl-D-alanine and β-lactam antibiotic. D-ala-D-ala and β-lactams have a similar structure that allow them both to be bound by PBPs. R1 and R2 are groups that vary among different β-lactams.



D-Ala-D-Ala



B-lactam

between the two. This is followed by an irreversible acylation reaction during which, the active site serine of the PBP covalently binds the substrate. When the substrate is D-ala-D-ala, there is a rapid deacylation reaction that frees the PBP for more cell wall synthesis. However, when the substrate is a β-lactam, deacylation is a much slower process and so, the PBP is effectively killed (26). Without functioning PBPs, cell wall synthesis cannot continue and cell death ensues.

Mechanisms of resistance – β-lactamase

β-lactamases are PBPs that have no role in cell wall synthesis. Instead, they serve to inactivate many β-lactam antibiotics in a reaction similar to the one β-lactams use to inhibit the cell wall synthetic PBPs. The β-lactamase binds a β-lactam and the formation of an acylated intermediate ensues. Unlike what takes place with the cell wall synthetic PBPs, resolution of the acylated intermediate results in the cleavage of the amide bond of the β-lactam. The now inactive β-lactam and functional β-lactamase are released (54). Four different types of *S. aureus* β-lactamases have been described that differ in substrate specificity (156). The gene encoding β-lactamase (*blaZ*) in *S. aureus* is usually contained on a plasmid. *blaZ* transcription is regulated by the products of two divergently transcribed genes, *blaI* and *blaRI*. BlaI dimers repress transcription by binding to regions of dyad symmetry in the *blaZ–blaRI* intergenic region (60, 126). BlaRI is a membrane-spanning sensor transducer that consists of an external PBP domain as well as a zinc-metalloprotease domain. β-lactam binding to the PBP domain of BlaRI results in

autocleavage and the subsequent cleavage of the BlaI repressor, resulting in the derepression of *blaZ* transcription (154).

Mechanisms of resistance – methicillin resistance

Methicillin, oxacillin, and nafcillin are semi-synthetic β-lactams that are not inactivated by β-lactamases. Strains develop resistance to these agents by acquiring *mecA* and are known as methicillin-resistant *S. aureus* (MRSA), although they are actually resistant to all β-lactam antibiotics (14, 25, 26, 67). *mecA* encodes an alternate penicillin binding protein, PBP2a, that has a low affinity for β-lactams. PBP2a is a high molecular weight PBP (78 kDa) that is unlike the other *S. aureus* PBPs (<21% sequence identity with other PBPs) (59). The crystal structure of PBP2a has been solved to 1.8 Å resolution (87). The protein consists of an N-terminal transmembrane anchor, a bilobal nonpenicillin-binding domain, and a C-terminal transpeptidase domain. The nonpenicillin-binding domain appears to play a structural role, while the transpeptidase domain shares a similar overall fold structure with other transpeptidases and the serine β-lactamases. Active site serine 403 is responsible for nucleophilic attack of either the β-lactam ring or D-ala-D-ala substrate and is found in an extended narrow groove of the transpeptidase domain. This groove mediates noncovalent interactions with the β-lactam that position the β-lactam unfavorably for interaction with serine 403. Therefore, acylation between the β-lactam and the active site does not occur. PBP2a effectively balances the retention of essential transpeptidase activity with a reduction in β-lactam affinity. Thus, it is capable of active

cell wall synthesis at what would otherwise be lethal concentrations of β-lactams (26, 87, 114).

mecA is regulated in a manner highly similar to that of the β-lactamase gene, *blaZ*. Like *blaZ*, *mecA* is transcribed divergently from its two regulatory genes, *mecI* and *mecRI*. Dimeric MecI constitutively represses transcription of the operon by binding to two palindromic sequences in the promoter region (125). Derepression is achieved through cleavage of MecI by the activated metalloprotease component of the MecRI sensor transducer. MecRI spans the membrane and has an extracellular penicillin-binding domain that, once bound by β-lactams, undergoes a conformational change resulting in autocleavage of the protease domain. The now active MecRI is able to cleave MecI, which allows for the transcription of *mecA* (93). MecI and BlaI are both capable of binding to and repressing transcription from the *mecA* promoter. However, MecI or BlaI-mediated repression is only relieved by induction through homologous, and not heterologous, sensor-transducer proteins, demonstrating the repressor specificity of induction. Induction through the MecRI-MecI system is much slower than that of BlaRI-BlaI and never leads to maximal *mecA* transcription (96). Rosato, et al. found that the majority of clinical *S. aureus* isolates contain mutations in, or deletions of *mecI*. However, in the vast majority of these cases *mecA* transcription was regulated by BlaI, suggesting that *S. aureus* must have at least one of the two functional *mecA* regulators (119). Given the more rapid rate of induction through BlaRI–BlaI, this may be the preferred means of *mecA* regulation. Katayama, et al. have shown that naïve methicillin-sensitive *S. aureus* (MSSA) do not tolerate the introduction of plasmid-borne, unregulated *mecA*, as *mecA* is often mutated or

deleted to circumvent expression of PBP2a. When either *mec* or *bla* regulatory genes are introduced along with *mecA*, the system is tolerated (82). These findings highlight the importance of the tight regulation of *mecA* expression – it seems that expression of PBP2a is only favorable during times of β-lactam stress.

While expression of *mecA* and PBP2a is necessary for high level methicillin resistance, it is not sufficient. The majority of MRSA are heterotypically resistant to methicillin with only a small subpopulation (≤0.1%) of cells expressing high level resistance, while the majority of the population is killed by low concentrations of the antibiotic. This is in contrast to homotypic methicillin resistance, where the entire population expresses high level resistance (143). It is possible to select homotypic resistance from a heterotypic population by passing the strains in the presence of a β-lactam. The conversion to homotypic resistance is due to the selection of a highly resistant mutant population and these mutants are stable when passed in antibiotic-free media (50). These mutational events are not well understood but arise in genes other than *mecA*.

MRSA infections now have a higher incidence than methicillin-susceptible *S. aureus* (MSSA) infections in some settings. MRSA were responsible for 59% of skin and soft tissue infections seen in 11 US Emergency Departments, and MRSA made up 59.5% of all *S. aureus* infections in ICU patients in 2004 (100, 117). In addition, the incidence of MRSA disease in the community has more than doubled from 2002 to 2004 (33). The increasing rate of MRSA infections has shifted chemotherapy away from β-lactam antibiotics and toward drugs effective against MRSA, such as vancomycin.

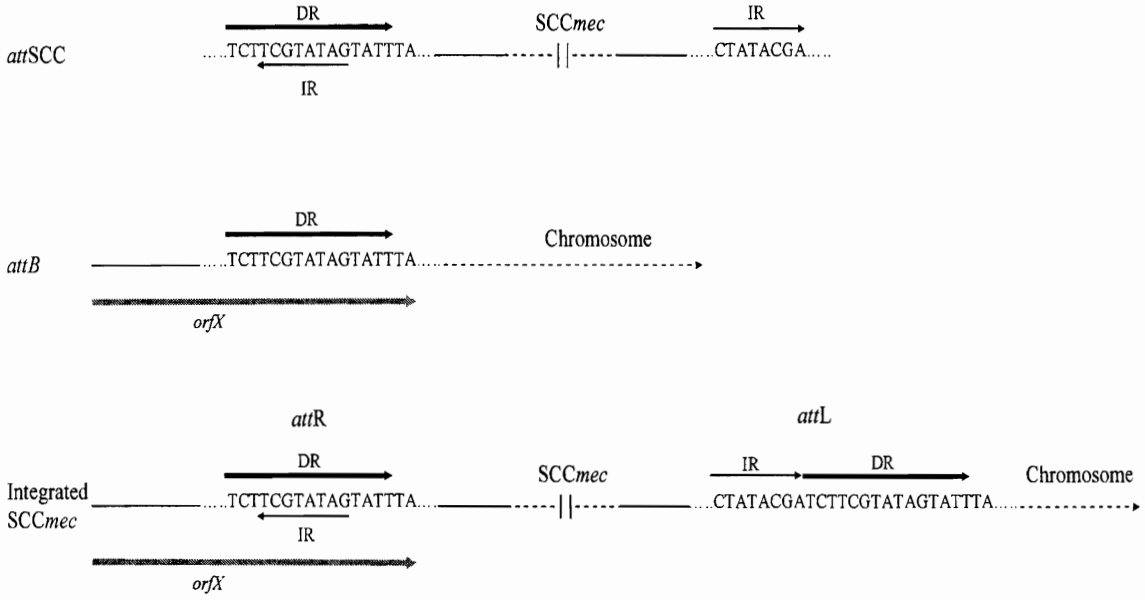
SCC*mec*

mecA is located in a genomic island known as Staphylococcal Chromosome Cassette *mec* (SCC*mec*) (80). SCC*mec* is universally found in the chromosome of MRSA near the origin of replication, inserted into an open reading frame of unknown function known as *orfX*. The site into which SCC*mec* inserts, *att*B, is marked by a 15 bp core sequence encoding the last five amino acids in the C-terminal of *orfX*. Upon SCC*mec* insertion, the core sequence of *attB* is duplicated and found flanking the integrated SCC*mec* element to form the direct repeats of the left and right SCC*mec* attachment sites (*att*L and *att*R). *attL* and *attR* of SCC*mec* are also marked by degenerated inverted repeat. *att*SCC (circular, extrachromosomal SCC*mec* element), *attB*, *attL*, and *attR* are shown in Figure 5.

SCC*mec* is a variable element with some conserved features. Among the conserved features, SCC*mec* contain the *mec* operon, which consists of *mecA* and its regulatory genes, as well as the cassette chromosome recombinase locus (*ccr*) (8, 39, 57, 70, 74-76, 80, 84, 91). The *ccr* locus encodes the recombinase genes, *ccrAB* or *ccrC*, that are involved in SCC*mec* mobility (74, 76, 80, 104). The variable regions of SCC*mec* often contain inserted genetic elements such as integrated plasmids (pUB110, pT181, and p1258), transposons (Tn554), and insertion sequences (IS431, IS1272, and IS256) (64).

The *mec* operon consists of *mecA* and the regulatory genes, *mecRI* and *mecI*. This operon is the hallmark of SCC*mec*, but is found in several varieties as a component of different SCC*mec* elements. The variations of *mec* operons can be divided into two main categories: those with both regulatory genes intact and those where portions of one or both

Figure 5. Attachment sites for SCC*mec* integration. The SCC*mec* attachment site (*att*SCC) as it is found on the circular extrachromosomal SCC*mec* element, chromosomal attachment site (*att*B), and SCC*mec* integrated into the chromosome with hybrid attachment sites at either end (*att*L and *attR*) are shown. Thick black arrows represent direct repeat sequences, thin black arrows represent inverted repeat sequences, and gray arrows represent the coding region of *orfX*.


attSCC
DR
TCTTCGTATAGTATTTA
IR
SCCmec
IR
CTATACGA
attB
DR
TCTTCGTATAGTATTTA
Chromosome
orfX
attR
attL
Integrated SCCmec
DR
TCTTCGTATAGTATTTA
IR
SCCmec
IR
DR
CTATACGATCTTCGTATAGTATTTA
Chromosome
orfX

regulatory genes are deleted. The former group is known as the class A *mec* operon, while the latter is known as classes B-E (64, 65, 74, 75, 91, 110, 111). Figure 6 depicts the different structures of the known varieties of *mec* operons (64). All classes contain an IS431 that is found associated with *mecA*. Classes B-E contain deletions of *mecI* that may or may not include partial deletion of *mecRI*. In most cases, these deletions are coincident with the insertion of IS elements. Similar to the *mec* operon, the *ccr* locus of SCC*mec* exists in several varieties. The *ccr* locus is composed of either *ccrA* and *ccrB*, or *ccrC*. *ccrAB* are divided into types I-IV based on differences in their nucleotide sequences (74, 76, 79, 91, 110).

SCC*mec* elements are classified into five main types based on the variety of *mec* operon and *ccr* genes present. Table 1 lists the characteristics of the five main SCC*mec* types. Two PCR-based SCC*mec* typing strategies exist. The first was developed by Ito, et al. and uses PCR to amplify portions of *mecRI-mecI* as well as specific portions of the different *ccr* genes. Elements are typed according to the combination of *mec* operon and *ccr* genes detected (74). Oliveira, et al. developed a multiplex PCR strategy that detects other regions of SCC*mec* in addition to the *mec* and *ccr* loci. This strategy allows for the detection of structural variants of known SCC*mec* elements (111). Neither strategy is entirely satisfactory due to the rapidly evolving nature of SCC*mec* elements and the frequent detection of novel variants (130).

MRSA arose soon after the introduction of methicillin and were predominantly nosocomial pathogens. These strains were resistant to multiple antibiotics in addition to β-

lactams, and it was thought that they were selected for by the frequent use of antibiotics in healthcare settings (117). These isolates tend to contain SCC*mec* types I-III, which vary in

Figure 6. Structural variations of the mec operon. Different structural variants of the *mec* operon are shown with the direction of transcription indicated by arrows beneath each gene. The designation of each variant is shown on the left. This figure was adapted from Hanssen, et al., 2006 (64).

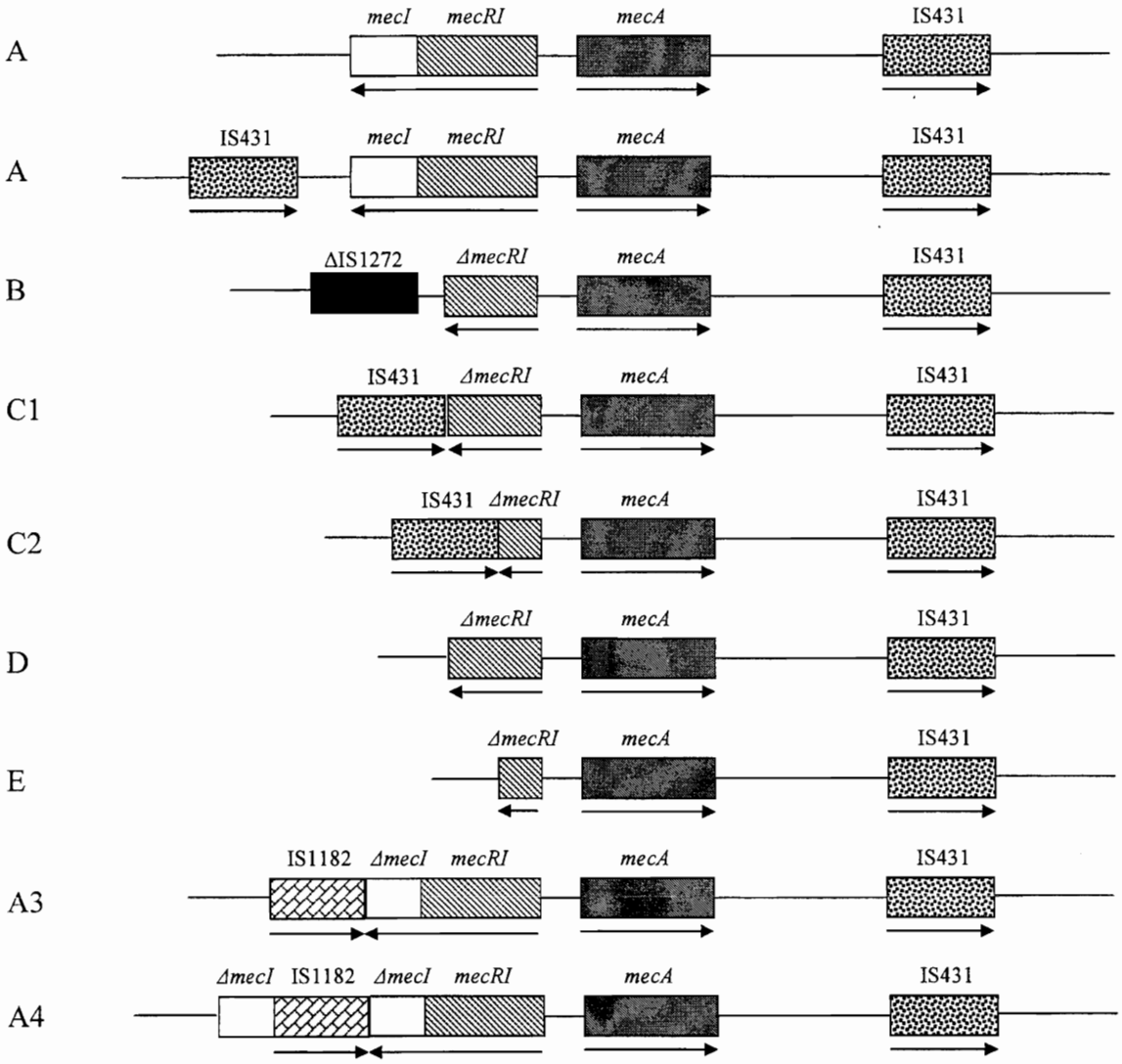

A
mecI
mecRI
mecA
IS431
A
IS431
mecI
mecRI
mecA
IS431
B
ΔIS1272
ΔmecRI
mecA
IS431
C1
IS431
ΔmecRI
mecA
IS431
C2
IS431
ΔmecRI
mecA
IS431
D
ΔmecRI
mecA
IS431
E
ΔmecRI
mecA
IS431
A3
IS1182
ΔmecI
mecRI
mecA
IS431
A4
ΔmecI
IS1182
ΔmecI
mecRI
mecA
IS431

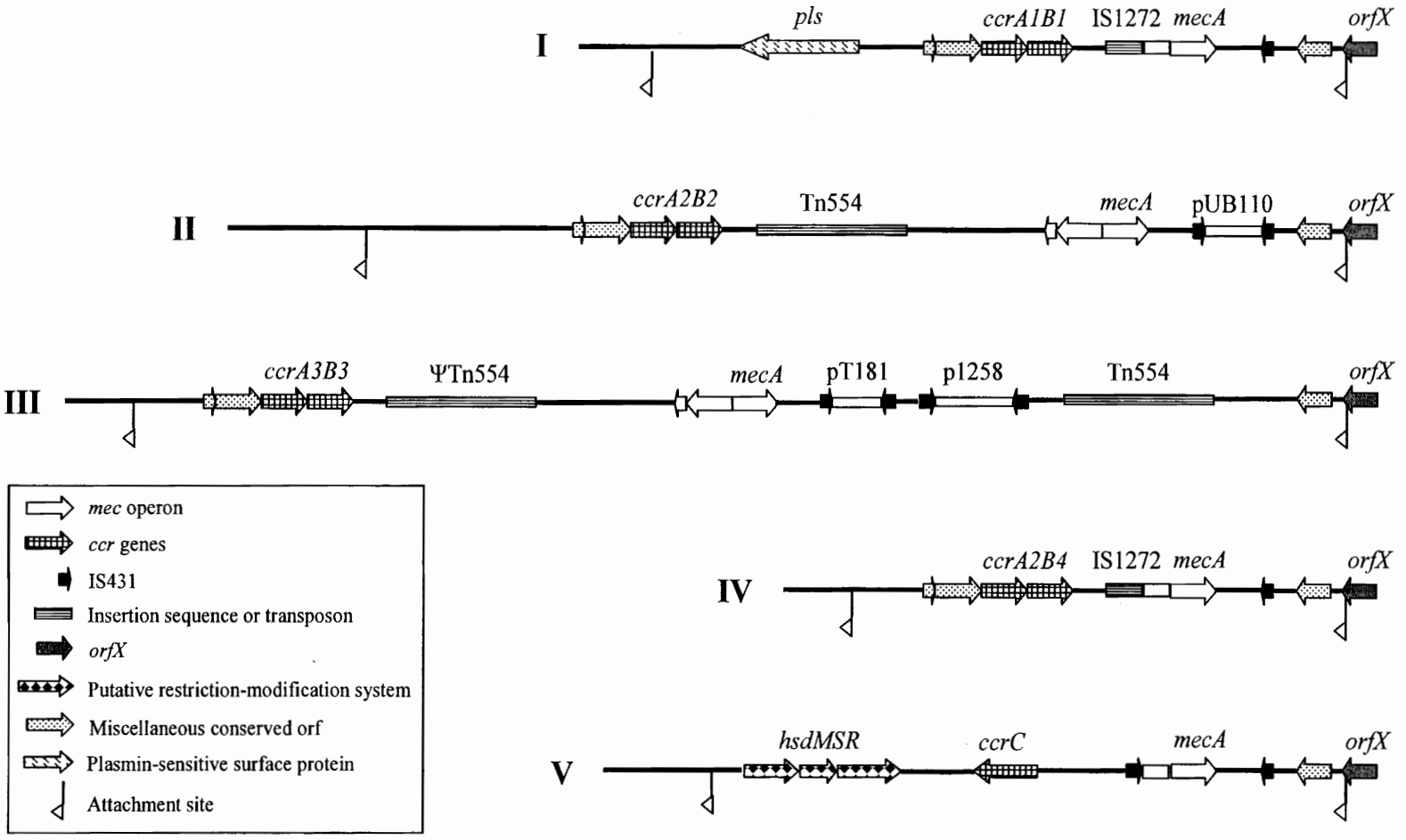
Table 1. Characteristics of the five different types of SCC*mec* elements.

SCC*mec* Type	*mec* operon	*ccr* genes	Size	Other resistance determinants
I	Class B-E	*ccrA1B1*	34 kb	None
II	Class A	*ccrA2B2*	52-58 kb	Erythromycin, spectinomycin, tobramycin, bleomycin
III	Class A	*ccrA3B3*	67 kb	Cadmium, mercury, tetracycline, erythromycin, spectinomycin
IV	Class B-E	*ccrA2B2 or ccrA4B4*	20-25 kb	None
V	Class B-E	*ccrC*	28 kb	None

size from 34 - 67 kb. This variation is due, in part, to the insertion of transposons and integrated plasmids that carry additional resistance determinants (Table 1). Thus, the multi-resistant phenotype of these strains was largely due to the presence of SCC*mec* (74, 80). In the mid-1990's, several MRSA were isolated as the cause of severe disease in the community. These strains caused disease in a patient population with none of the typical risk factors for MRSA infection, such as recent hospitalization, residence in a long-term care facility, or preexisting health problems. These MRSA were not resistant to antibiotics other than β-lactams and were found to contain the smaller SCC*mec* type IV (20 – 25 kb) (19, 49). MRSA containing SCC*mec* type V were isolated more recently and these strains are not known to be a frequent cause of human disease (76). Schematics of the five SCC*mec* types are shown in Figure 7.

Okuma, et al. found that SCC*mec* type IV was the dominant SCC*mec* type found in community-associated MRSA (CA-MRSA) infections. Wisplinghoff, et al. found SCC*mec* type IV in *S. epidermidis* isolates that predate the earliest known MRSA containing SCC*mec* type IV (110, 152). These findings suggested that SCC*mec* type IV originated in *S. epidermidis* and was subsequently transferred to *S. aureus*. The rapid emergence and spread of CA-MRSA containing SCC*mec* type IV also led to the idea that *S. epidermidis* may act as a reservoir of SCC*mec* type IV in the community and the repeated transfer of this element into MSSA is partially responsible for the increased incidence of CA-MRSA disease (152). Enright et al., examined a collection of 359 MRSA isolates by MLST and SCC*mec* typing. They found strains with distantly related sequence types containing the same SCC*mec* element, and strains with identical sequence types

Figure 7. Schematic of SCC*mec* types I–V. Depicted are the structures of SCC*mec* types I-V (74, 76, 80, 91). Select open reading frames are depicted by block arrows indicating the direction of transcription. Labels indicate the names of genes, insertion sequences, and transposons. Dotted block arrows are open reading frames of unknown function that are conserved in different SCC*mec* elements. pUB110, p1258, and pT181 are integrated plasmids.


I
pls
ccrA1B1
IS1272
mecA
orfX
II
ccrA2B2
Tn554
mecA
pUB110
orfX
III
ccrA3B3
ΨTn554
mecA
pT181
p1258
Tn554
orfX
IV
ccrA2B4
IS1272
mecA
orfX
V
hsdMSR
ccrC
mecA
orfX
mec operon
ccr genes
IS431
Insertion sequence or transposon
orfX
Putative restriction-modification system
Miscellaneous conserved orf
Plasmin-sensitive surface protein
Attachment site

containing different SCC*mec* elements (46). This suggested that SCC*mec* elements had been independently acquired by *S. aureus* on several occasions. Also, two of the genome sequenced *S. aureus* strains (MW2 and MSSA476) are highly similar (285 single nucleotide polymorphisims in their chromosomes), yet differ in that MW2 has acquired SCC*mec* type IV, while MSSA476 has not (70). Taken together, these data support the idea that SCC*mec* elements are mobile in the environment and their transfer into MSSA backgrounds contributes to the spread of MRSA. Since CA-MRSA containing SCC*mec* type IV were first described, there has been a dramatic increase in the incidence of CA-MRSA disease (33). During this time, there have also been rising rates of CA-MRSA lineages causing disease in the hospitals and these strains are becoming resistant to multiple antibiotics in addition to β-lactams (24, 100). To better understand the spread and increasing burden of MRSA, it is necessary to better characterize the factors associated with SCC*mec* transfer.

Dissertation goal

The goal of this dissertation is to better characterize the factors associated with SCC*mec* transfer. Specifically, we examine CcrAB-mediated SCC*mec* excision in CA-MRSA, the mechanism of spontaneous *mecA* loss during passage to vancomycin resistance, and the structure of *attB* and the surrounding region in a diverse collection of MSSA. We also attempt the intraspecies transfer of *mecA*/SCC*mec* by bacteriophage.

The mechanism of SCC*mec* transfer is unknown. However, it is hypothesized that the SCC*mec* element is site-specifically excised from the chromosome by the CcrAB

recombinases contained in the element. Once excised, the circularized SCC*mec* element may be packaged in a phage head and transferred to an MSSA recipient strain. Once inside the recipient cell, CcrAB would catalyze the site-specific chromosomal integration of the element. Alternatively, the excised, circular SCC*mec* element may integrate into a conjugative plasmid and than be conjugatively transferred to an MSSA recipient. Staphylococcal conjugative plasmids and SCC*mec* each contain copies of IS431 that may mediate this integration. Once inside the recipient MSSA, CcrAB may excise SCC*mec* from the plasmid and integrate it into the host chromosome.

This dissertation begins with an examination of SCC*mec* excision in CA-MRSA strains containing SCC*mec* type IV. We are interested in CA-MRSA strains containing SCC*mec* type IV because of the evidence suggesting that this element is mobile in the environment and because the relatively small size of the element makes its packaging in phage heads feasible. We then examine the spontaneous deletion of *mecA* as strains are passaged to increasing vancomycin resistance. It is possible that the mechanism used by *S. aureus* to delete *mecA* is similar to that used for SCC*mec* transfer. Successful transfer of SCC*mec* is dependent upon the recipient having an intact *att*B for chromosomal integration of SCC*mec*. To address this issue, we examined the structure surrounding *att*B in a genetically and temporally diverse collection of MSSA. Lastly, we attempt to transfer SCC*mec* type IV using both endogenous and exogenous bacteriophage.

CHAPTER 2 Materials and Methods

General protocols

Strains and plasmids

Lists of strains and plasmids appear in Tables 2 and 3, respectively.

Restriction enzyme digestions and ligations

All restriction digestions and ligations were performed according to the manufacturers' specifications (New England Biolabs, Ipswich, MA).

Transformation

Plasmid was mixed with chemically competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA) and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for one minute before continued incubation on ice. 1 ml of rich media was added and the cells were allowed to recover at 37°C with shaking for 1 hour. Cells were then plated onto selective agar.

Electroporation

S. aureus strain RN4220 was made electrocompetent by the following procedure. A culture was grown to mid-logarithmic phase, pelleted, and washed with dH_2O, pelleted,

Table 2. Strain list

Strain	Relevant Characteristics	Remarks and References
E. coli strain		
TOP10	*recA*1 *lacZ*ΔM15	Host for *lacZ*-containing cloning vectors (Invitrogen)
S. aureus strains		
RN4220	Mc^s	Restriction deficient mutagenized RN450
RN450	Mc^s	8325-4 (105)
450M	Mc^r (he), SCC*mec* type I	RN450 transformed with COL *mec* region DNA
450MHomo	Mc^r (ho), SCC*mec* type I	450M passaged on oxacillin until homotypic resistance was achieved
450MHomoΔ*mecA*	Mc^s, SCC*mec* type I	450MHomo with *mecA* insertionally inactivated by *tetM*
N315	Mc^r (he), SCC*mec* type II	Clinical isolate, Japan 1982 (84)
MW2	Mc^r (he), SCC*mec* type IV	CA-MRSA, North Dakota 1998 (19)
MW2Δ*mecA*	Mc^s, SCC*mec* type IV	MW2 with *mecA* insertionally inactivated by *tetM*
COL	Mc^r (ho), SCC*mec* type I	England, 1965 (4)
C98-370	Mc^r (he), SCC*mec* type IV	CA-MRSA, USA 1998 (19, 49)
C99-529	Mc^r (he), SCC*mec* type IV	CA-MRSA, USA 1998 (19, 49)
J28	Mc^r, SCC*mec* type IV	CA-MRSA (27)
J28Δ*mecA*	Mc^s, SCC*mec* type IV	J28 with *mecA* insertionally inactivated by *tetM*
J35	Mc^r, SCC*mec* type IV	CA-MRSA (27)
J35Δ*mecA*	Mc^s, SCC*mec* type IV	J28 with *mecA* insertionally inactivated by *tetM*
J39	Mc^r, SCC*mec* type IV	CA-MRSA (27)
J39Δ*mecA*	Mc^s, SCC*mec* type IV	J28 with *mecA* insertionally inactivated by *tetM*
J52	Mc^r, SCC*mec* type IV	CA-MRSA (27)
J52Δ*mecA*	Mc^s, SCC*mec* type IV	J28 with *mecA* insertionally inactivated by *tetM*
USA300	Mc^r, SCC*mec* type IV	San Francisco (39)
3130	Mc^r, SCC*mec* type II	Clinical isolate, SCOPE surveillance study (151)
3130VR	Mc^s, SCC*mec* type II, Vm^r	3130 passaged to vancomycin resistance
3130V32	Mc^r, SCC*mec* type II, Vm^r	3130 passaged to vancomycin resistance
5827	Mc^r, SCC*mec* type II	Clinical isolate, Michigan, 1997 (140)

Strain	Relevant characteristics	Remarks and References
VP32	Mc^s, SCC*mec* type II, Vm^r	5827 passaged to vancomycin resistance (99)
5827V32	Mc^r, SCC*mec* type II, Vm^r	5827 passaged to vancomycin resistance
5836	Mc^r, SCC*mec* type II	Clinical isolate, New Jersey, 1997 (140)
5836VR	Mc^s, SCC*mec* type II, Vm^r	5836 passaged to vancomycin resistance
5836V32	Mc^r, SCC*mec* type II, Vm^r	5836 passaged to vancomycin resistance
15578	Mc^s	Isolates from the *S. aureus* collection at PHRI provided by Barry Kreiswirth*
15607	Mc^s	
15673	Mc^s	
15649	Mc^s	
15680	Mc^s	
15594	Mc^s	
15668	Mc^s	
15634	Mc^s	
15575	Mc^s	
15666	Mc^s	
15576	Mc^s	
15591	Mc^s	
15604	Mc^s	
15639	Mc^s	
15653	Mc^s	
15679	Mc^s	
15602	Mc^s	
15601	Mc^s	
15584	Mc^s	
15658	Mc^s	
15671	Mc^s	
15677	Mc^s	
15580	Mc^s	
15651	Mc^s	
15630	Mc^s	
15637	Mc^s	
15647	Mc^s	
15611	Mc^s	
15681	Mc^s	
15589	Mc^s	
15579	Mc^s	
15585	Mc^s	

Strain	Relevant characteristics	Remarks and References
15682	Mc^s	Isolates from the *S. aureus* collection
3294	Mc^s	at PHRI provided by Barry
3298	Mc^s	Kreiswirth*
3304	Mc^s	
NRS104	Mc^s	Cowan I, 1935
NRS198	Mc^s	United Kingdom, 1949
NRS199	Mc^s	United Kingdom, 1947
NRS204	Mc^s	NCTC6571, United Kingdom, 1940
6520	Mc^s	Centers for Disease Control and Prevention
6881	Mc^s	Centers for Disease Control and Prevention
S. epidermidis strains		
SE5	Mc^r, SCC*mec* type IV	PVE, Richmond, VA (152)
SE7	Mc^r, SCC*mec* type IV	
SE50	Mc^r, SCC*mec* type IV	
SE63	Mc^r, SCC*mec* type IV	

*Barry Kreiswirth at The Public Health Research Institute Center at the International Center for Public Health, New Jersey Medical School – UMDNJ. Mc = methicillin, Vm = vancomycin, r = resistant, s = sensitive, he = heterotypic, ho = homotypic, PVE = prosthetic valve endocarditis isolate.

Table 3. Plasmid list

Plasmid	Relevant Characteristics	Remarks and References
E. coli plasmids		
TOPO pCR2.1	Ap^r, Km^r	Cloning vector (Invitrogen)
S. aureus plasmids		
pRN5543	Cm^r	High copy number *S. aureus* plasmid (31)
pCN36	Tc^r	*E. coli-S. aureus* shuttle vector (28)
p*ΔmecA*	Tc^r, Em^r	*mecA::tetM* with a temperature sensitive origin of replication for allelic replacement (50)
pMJN51	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* MW2 (104)
pMJN60	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. epidermidis* SE5 (104)
pMJN61	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. epidermidis* SE7 (104)
pMJN62	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. epidermidis* SE50 (104)
pMJN63	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. epidermidis* SE63 (104)
pMJN64	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* C98-370 (104)
pMJN65	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* C99-529 (104)
pMJN66	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* J35 (104)
pMJN68	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* J52 (104)
pMJN69	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* J39 (104)
pMJN70	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* J28 (104)
pMJN71	Ap^r, Km^r, Cm^r	pCR2.1 fused with pRN5543 containing the *ccrAB* coding region from *S. aureus* N315 (104)

Ap = ampicillin, Km = kanamycin, Cm = chloramphenicol, Tc = tetracycline, Em = erythromycin

and washed with 0.5 M sucrose. The cells were pelleted again and resuspended in 0.5 M sucrose and stored at -70°C until needed. Electroporation into RN4220 was performed using 2 mm cuvettes in a Gene Pulser (Bio Rad, Richmond, CA) at settings of 100 Ω, 25 μF, and 2.5 kV. 10 μl of plasmid was mixed with 100 μl of electrocompetent cells. The mixture was cooled on ice, transferred to a chilled cuvette, and electroporated. 1 ml of rich media was added and the cells were allowed to recover at 37°C for 1-2 hours before plating onto selective agar.

Transduction

The generalized transducing phage 80α was used to transfer plasmids from RN4220 to other *S. aureus* strains using the method of Thompson and Pattee (141). Briefly, RN4220 containing the plasmid of interest was mixed with serial dilutions of phage 80α and soft agar. The mixture was poured over tryptic soy agar (TSA) (Beckton Dickinson, Cockeysville, MD) plates containing $CaCl_2$. Plates were incubated upright at 30°C or 37°C overnight. The soft agar from the plates with high levels of bacterial lysis was taken and homogenized by vortexing with 5 ml of tryptic soy broth (TSB) (Beckton Dickenson, Cockeysville, MD). The agarose was pelleted and the supernatant was filtered through a 0.22 μm filter. This transducing lysate was then mixed with the strain of interest and shaken at 37°C for 20 minutes. One milliliter of ice cold 0.02 M sodium citrate was added to the mixture and the cells were pelleted, and resuspended in 0.02 M sodium citrate. This mixture was then plated on TSA containing selective antibiotic and sodium citrate.

Polymerase chain reaction (PCR)

PCR primers were synthesized by the Nucleic Acid Research facility at Virginia Commonwealth University. Amplification using Taq Master Mix (Qiagen, Valencia, CA) was performed in a MiniCycler (MJ Research, Watertown, MA) according to standard protocol (47).

DNA isolation and purification

Total cellular DNA was isolated and purified using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' recommended parameters. Plasmid DNA was extracted and purified using the QIAprep Spin Mini Prep Kit (Qiagen, Valencia, CA) according to the manufacturers' parameters. When extracting DNA from staphylococci, the cells were incubated with lysostaphin (Biosynexus, Gaithersburg, MD) before proceeding with the manufacturers' protocol. Gel extraction and PCR purification were done using the QIAquick Gel Extraction Kit and QIAquick PCR Purification Kit (Qiagen, Valencia, CA), respectively, according to the provided protocols.

DNA sequencing

Sequencing was performed on PCR amplified products by the Nucleic Acid Research Facility at Virginia Commonwealth University.

Pulsed field gel electrophoresis

Preparation of genomic DNA and separation by PFGE was adapted from the method of Bannerman, T.L., et al.(11). Briefly, a 250 µl aliquot of an overnight culture was pelleted and resuspended in 300 µl PIV buffer (10mM Tris-HCl, 1M NaCl). The cell suspension was mixed with 2% molten Seaplaque agarose (Cambrex, Rockland, ME) and added to the plug molds. The plugs were treated with lysostaphin (Biosynexus, Gaithersburg, MD) and proteinase K (Fisher, Fair Lawn, NJ). Plugs were then washed in 5 ml autoclaved, deionized water for 8 hours with frequent water changes before digestion with SmaI (Promega, Madison, WI). Each plug was placed into a well of a 1% agarose gel prepared with 0.5x TBE buffer (45mM Tris, 45mM Boric acid, 1.3mM EDTA). The gel was run in 0.5x TBE under the following parameters: 6 Volts/cm, initial switching time - 1 second, final switching time -30 second, run time - 22 hours, run temperature -14°C. Bands were visualized with UV light after ethidium bromide staining.

Chapter 3 specific protocols

ccrAB cloning

ccrAB was PCR amplified from the genomic DNA of *S. aureus* strains N315, MW2, C98-370, C99-529, J28, J35, J52 and *S. epidermidis* strains SE5, SE7, SE50, and SE63 using primers ccuprev and ccdwnfor and the Taq PCR Master Mix Kit (Qiagen, Valencia CA). PCR products were purified using the QIAquick Gel Extraction Kit and ligated into the ligase-independent cloning site of the pCR2.1-TOPO vector (Invitrogen, Carlsbad CA) and transformed into chemically competent TOP10 *E. coli* (Invitrogen, Carlsbad CA). A staphylococcal origin of replication was introduced by cloning plasmid

pRN5543 into the unique XbaI site on pCR2.1-TOPO, and the constructs were moved into *S. aureus* RN4220 by electroporation. Phage 80α was used to transduce the constructs from RN4220 into all other *S. aureus* strains used in this study.

Sequence determination and alignments

ccrAB sequencing was performed on overlapping 600-700 bp PCR fragments generated using the sequencing primers listed in Table 4. All sequencing was performed by the Nucleic Acid Research Facility at Virginia Commonwealth University (Richmond, VA). *ccrAB* open reading frames were translated using Vector NTI software (InforMax, Frederick MD). Amino acid alignments were generated using ClustalX, and CloureD software was used to generate the dot alignments.

PCR-based SCC*mec* excision

Briefly, *ccrAB* constructs were transduced into the appropriate *S. aureus* strain. Brain Heart Infusion (BHI) broth cultures were started from isolated colonies on the transductant plates and grown overnight. The presence of the construct was confirmed by restriction analysis of plasmid DNA (prepared using the QIAprep Spin Miniprep kit), and total genomic DNA was isolated using the QIAamp DNA Mini kit (Qiagen, Valencia CA) to serve as template for PCR reactions. Detection of SCC*mec* excision from the chromosome was done using primer sets designed to amplify across the chromosomal junction and a second set designed to detect the excised, circular SCC*mec* element. Primer

Table 4: Primers used in Chapter 3

Name	Sequence (5'-3')	Description
ccdwnfor	CGAGATATTAGCCGATTTGG	*ccrAB* cloning. Amplifies *ccrAB* and upstream region
ccuprev	CCTTCTGTTTCTTCGAATCTGC	
cc1for	CGTACCATGTTCATATCTTAAGC	*ccrAB* sequencing
cc2for	GCAACACTTACAAATATGACC	
cc3for	CGTGTCGGTATCTATGTACG	
cc4for	GCTATGTCACTAAAAAGGGTAAACC	
cc5for	GGACTTAACATCAGTAATCAGACC	
cc1rev	CGTAATGTCATTGAGTTGC	
cc2rev	CGATATAGAATGGGTTAGC	
cc3rev	CGATACTATAACCTTCTGTGC	
cc4rev	GCATTCATGTTTTTAGGACAGACG	
cc5rev	GAGTCGGTCGAAAGCTTGATCC	
cL1	ATTTAATGTCCACCATTTAACA	Excised chromosomal junction in N315 (80)
cR1	AAGAATTGAACCAACGCATGA	
mL1	GAATCTTCAGCATGTGATTTA	Circular SCC*mec* in N315 (80)
mR8	ATGAAAGACTGCGGAGGCTAACT	
cL1	ATTTAATGTCCACCATTTAACA	Excised chromosomal junction in COL (74)
cR2	AAACGACATGAAAATCACCAT	
colA	GTTCCCAGTAGCAACCTTCC	Circular SCC*mec* in COL
colB	CAATGAAAGCTTGGAAGAAGGGC	
jxn1	ATAGACATCATAGAAGTTACAGACGA	Excised chromosomal junction in MW2, C98-370, C99-529, J28 and MSSA476
jxn2	ATAGGCAACAATACATGGCAACTCAGA	
mw2A	GTCTATCATTTGAAATTCCCTCC	Circular SCC*mec* in MW2, C98-370, C99-529, and J28
mw2B	GATAGACAACTTTAAACAGGTCC	
mR8	ATGAAAGACTGCGGAGGCTAACT	Circular SCC*mec* in J35 and J52 (80)
mwattRfor	GATCCTCGAGCATCCTCCACGTTATGGA GGTGC	
Mw2B	GATAGACAACTTTAAACAGGTCC	Detects MW0048
Imw2R	GCTTTTAATAGAGAACAACCAATGG	
Trafor	GGTACTTCTTGGTATTTTTAGGAC	Detects region from putative transposase to *seh*
sehrev	CTGCTTTCGCATATGATGTG	
I1-F	GTTCCAGACGAAAAAGCACCAG	Bind outside of either end of SCC*mec* in numerous strains
I1-R	CATTTTATGAGTCTCGCAAATTGTCAG	

sets used were strain specific and are listed in Table 4. PCR was performed using the Taq PCR Master Mix Kit, annealing temperature was generally 50°C, and extensions were at 72°C for 1.5 minutes/kb of amplicons. All PCR products were confirmed by nucleotide sequence determination.

Accesssion numbers

ccrAB sequences have been submitted to Genbank and the accession numbers are as follows: DQ514327 for *S. aureus* C98-370, DQ514328 for *S. aureus* C99-529, DQ514329 for *S. aureus* J28, DQ514330 for *S. aureus* J35, DQ514331 for *S. aureus* J52, DQ514332 for *S. epidermidis* SE5, DQ514333 for *S. epidermidis* SE7, DQ514334 for *S. epidermidis* SE50, and DQ514335 for *S. epidermidis* SE63.

Chapter 4 specific protocols

Bacterial strains and primers

All bacterial strains are listed in Table 2. All primers used for PCR and sequencing are listed in Table 5. Bacteria were grown in Brain Heart Infusion (BHI) broth or agar (Becton Dickinson, Sparks, MD) at 37°C with shaking at 220 rpm, unless otherwise noted. Strain 450MHomo is a derivative of 450M that was passaged on 6 μg/ml of oxacillin until the strain was homotypically resistant as determined by population analysis profiling. 450MHomoΔ*mecA* is strain 450MHomo with *mecA* insertionally inactivated by *tetM*. This was accomplished by transducing plasmid pΔ*mecA* into 450MHomo. This plasmid contains *mecA::tetM* and a temperature sensitive origin of replication (50). Transductant

Table 5. Primers used in Chapter 4

Primers	Sequence	Description	Reference
I1F	GTTCCAGACGAAAAAGCACCAG	Amplify	(104)
I1R	CATTTTATGAGTCTCGCAAATTGTCAG	across the junction when SCC*mec* is excised	(104)
mecAF	CTCATATAGCTCATCATACACTTTACC	Detect *mecA*	This study
mecAR	CACTTATTTTAATAGTTGTAGTTGTCGG		This study
unirev	GCACAGTGGGAATTAATCGAAGC	Long-range	This study
252rev	CCACTATTTAACTGACTTGATATACC	PCR primers	This study
Map1F	GGAAGATCTGATTGCTTAACTGC	Map SCC*mec*	This study
Map1R	CTCTCTTGGTCGTCAGACTGATGG	deletion	This study
Map2F	GCATGCTGCTTGCCTTAGG	Map SCC*mec*	This study
Map2R	CACACAGCCAAAGCAATCAGC	deletion	This study
Map3F	CCATTTGGCAGTTCTAAAAATCCG	Map SCC*mec*	This study
Map3R	CGTAATACATTCGGTCATTGGGAAGC	deletion	This study
Map4F	GGTTTCATGTTTGTGCTTCAGG	Map SCC*mec*	This study
Map4R	CACGATACAAATCAAAAAAAGGTTGG	deletion	This study
Map6F	CGTATCCTTTACAGGATATTTTGC	Map SCC*mec*	This study
Map6R	CTGCATATTCTTGAATTTAAAAAGG	deletion	This study
Map7F	GTTTCAGACTTTAGCGAGGAATGG	Map SCC*mec*	This study
Map7R	CTATGTTGTATTTATCTTCGATAATGG	deletion	This study
Map8F	GTGTTGCATTTGGTAGCC	Map SCC*mec*	This study
Map8R	CGATGAGTTAAGAGCACGTATC	deletion	This study
Map9F	CCGTTCGTTATAAATACTGCC	Map SCC*mec*	This study
Map9R	CATGGAAAGTACATATAAAAAAAGAGG	deletion	This study

colonies were picked and grown on TSA + tetracycline at 42°C – the non-permissive temperature for plasmid replication. Any colonies that grew were then screened for tetracycline resistance, oxacillin sensitivity, and erythromycin sensitivity. An erythromycin resistance cassette is present on the plasmid backbone and erythromycin sensitivity indicates secondary recombination and loss of the plasmid backbone. Bacteria with the correct resistance profile were confirmed to contain *mecA::tetM* by PCR.

Vancomycin passage technique

Parental strains 3130, 5827, and 5836 were grown in broth with increasing levels of vancomycin (Sigma Aldrich, St. Louis, MO) until a thick culture was grown (up to 48 hours). Strains were plated on BHI agar with the same concentration of vancomycin as their most recent growth in broth to check stability and resistance. A single colony was then selected for continued passage. Passage was continued until strains grew reliably on BHI agar containing 32 μg/ml of vancomycin. Colonies were picked from the vancomycin 32 μg/ml plate and streaked onto plates containg 6 μg/ml of oxacillin. Isolate pairs were saved that could (3130V32, 5827V32, and 5836V32; oxacillin resistant) and could not (3130VR, VP32, and 5836VR; oxacillin susceptible) grow on oxacillin (Table 2).

Growth analysis

Overnight cultures were diluted to $OD_{600} < 0.1$ and 150 μl aliquots were placed in each well of a 96-well plate. Plates were incubated at 37°C with shaking for 18 hours in a Multiskan Ascent (Thermo Lab Systems, Franklin, MA) and OD_{595} was measured every 15

minutes. Doubling times were determined by averaging the time required for OD_{595} doubling at ≥2 time intervals within log phase growth.

Competition experiments

Overnight cultures of strain pairs to be competed were diluted back and 10^5 cells of each were inoculated into one 5 ml broth culture and grown at 37°C until thick cultures were achieved (20-26 hours). At this point cultures were serially diluted and plated onto selective media containing 30 μg/ml of chloramphenicol, 10 μg/ml of tetracycline, or 6 μg/ml of oxacillin (Sigma Aldrich, St. Louis, MO). At the same time each culture was diluted 1:50,000 in fresh media. Serial plating, back dilution, and regrowth was continued daily for up to nine days. Strains 3130VR and 3130V32, VP32 and 5827V32, and 5836VR and 5836V32 do not contain differential selectable markers, and so markers were introduced in order to identify each member of a competed pair. Plasmid pRN5543 (chloramphenicol resistance) and pCN36 (tetracycline resistance) were introduced into each of the above strains and growth competition was carried out in the following manner to control for differential fitness costs associated with the different plasmids. 3130VR/pRN5543 was competed against 3130V32/pCN36 and also 3130VR/pCN36 was competed against 3130V32/pRN5543. A similar approach was taken for the other strain pairs. The stability of each plasmid was monitored, and there was no significant plasmid loss during the time needed to complete competition.

Chapter 5 specific protocols

Bacterial strains and primers

Table 2 lists the strains used in this study as well as their relevant characteristics. PCR primers are listed in Table 6. Strains NRS104, NRS198, NRS199, and NRS204 were provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). Strains 6520 and 6881 were kindly provided by Fred Tenover at the Centers for Disease Control and Prevention. All other MSSA isolates were kindly provided by Barry Kreiswirth at The Public Health Research Institute Center at the International Center for Public Health, New Jersey Medical School – UMDNJ.

PCR and Southern analysis

Primers used for PCR and sequencing are listed in Table 6. PCR reactions were preformed using the PCR Master Mix Kit (Qiagen, Valencia CA) according to the manufacturers suggested parameters on total cellular DNA isolated using the QIAamp DNA Mini kit (Qiagen, Valencia CA). Southern analysis was performed on DNA separated by pulsed-field gel electrophoresis (described above). Southern analysis was preformed as described by Hovis, et al. (71). DNA was transferred onto a Hybond-N membrane by using the VacuGene vacuum blotting system (Pharmacia) and fixed to a membrane by UV irradiation using the Stratagene UV cross-linker. The membrane was then incubated for 2 hours in hybridization buffer (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidine, 0.2% Ficoll, 50 mM Tris-HCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate [SDS], 10% dextran sulfate, 100 μg of herring sperm DNA μl^{-1},

Table 6. Primers used in Chapter 5

Primer	Sequence	Description
orfXfor	GAGAAATATTGGAAGCAAGCC	Detect *orfX*
orfXrev	CGCATAATCTTAAATGCTCTG	
mecAfor	CTCATATAGCTCATCATACACTTTACC	Detect *mecA*
mecArev	CACTTATTTTAATAGTTGTAGTTGTCGG	
cc1rev	CGTAATGTCATTGAGTTGC	Detect *ccrB*
cc4for	GCTATGTCACTAAAAAGGGTAAACC	
cc4rev	GCATTCATGTTTTTAGGACAGACG	Detect *ccrA*
cc1for	CGTACCATGTTCATATCTTAAGC	
ccrCF	CAGTAATGTCAAGATGTCGATGAATGC	Detect *ccrC*
ccrCR	CCGTCGACATACCATATTATTGCCG	
I1-F	GTTCCAGACGAAAAAGCACCAG	Amplify *attB*
I1-R	CATTTTATGAGTCTCGCAAATTGTCAG	
unirev	GCACAGTGGGAATTAATCGAAGC	Reverse primer for LR-PCR
252R	CCACTATTTAACTGACTTGATATACC	Alternate reverse primer for LR-PCR
uniF	GCTTCAAATTCATCTAGTAGTGC	Amplify unirev binding site
uniR	GATAAAAGATTTAATGCCACTGATG	
a252F	CCTTCAAAAATAAATGTATGGTC	Amplify 252R binding site
a252R	CATCATAAATACAATTAATACGTTGAC	
LE1F	CTAATGCTCAATGCATTTTCTTCAG	PCR primer walking the region outside of SCCmec type II in strain MRSA252
LE1R	GCATAGCGAAGCCATTTAATAGCG	
LE2F	CGTAGTCATCAAAGTTTGATTCAGC	
LE2R	GGGAGGCGTCAAAATTTGAGG	
LE3F	CATTTCGAAAGCGCCAGCTAATCTC	
LE3R	CATATGTAGGTAGTAAAATTTTTAAAAGC	
LE4F	GCAATATGCCATAATGCTATCTCC	
LE4R	GATAGATTATAATGATACAACATTGG	
LE5F	GTAATACAAACTGAAAGCAAGGG	
LE5R	GATGATGTTACAACAAGCTCTGG	

50% formamide) at 50°C, before adding fresh buffer containing the radioactively labeled probe. The probe was generated by PCR amplification and labeled using the Prime-A-Gene labeling system (Promega, Madison, WI) with [α-^{32}P]dATP (6,000 Ci mmol^{-1}). The probe was incubated with the membrane overnight at 42°C before washing twice with 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS for 10 minutes at 37°C and then once with 0.2 x SSC-0.1% SDS for 2 h at 37°C. The membranes were wrapped in saran and exposed to film at –70°C before developing.

Long range PCR and primer walk sequencing

Long range PCR was performed using Platinum PCR Supermix High Fidelity (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. Amplification products were gel extracted and purified using the Qiagen Gel Extraction kit. Both strands of the DNA were sequenced using a primer walking approach by The DNA Facility of the Iowa State University Office of Biotechnology. Initial sequencing of strain 15580 revealed a structure identical to a portion of the MRSA252 genome. This structure was confirmed by PCR walking this region of 15580 and was found to contain the same structure as MRSA252.

Sequence analysis

Sequence data was assembled using the Contig Express portion of the Invitrogen VectorNTI software. Nucleotide homology searches were performed using Blastn available on the NCBI website (http://www.ncbi.nlm.nih.gov/). Open reading frames were

predicted and translated using the VectorNTI software default parameters. Protein homology searches were performed using Blastp available on the NCBI website.

Chapter 6 specific protocols

Induction of prophage by UV light

An overnight culture was diluted 1:100 in 15 ml fresh BHI broth (Beckton Dickinson, Cockeysville, MD) and grown at 37°C with shaking to an OD_{600} = 0.6. Cells were pelleted at 5,000 x G for 15 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml phage buffer (100mM NaCl, 10 mM $MgCl_2$, 50 mM Tris HCl, pH 7.5). This suspension was transferred to a sterile Petri dish and was exposed to a germicidal UV lamp for 25 seconds at a distance of 25 cm. At this point, the cell suspension was transferred to a flask, 10 ml of BHI broth was added, and the culture was incubated at 37°C with shaking for four hours. The cells were pelleted, and the supernatant was filter sterilized.

Induction of prophage by mitomycin C

This protocol was adapted from those of Maniatis and Eggers and Samuels (42, 127). Briefly, overnight cultures were diluted 1:200 in fresh BHI broth and grown at 37°C with shaking to an OD_{600} = 0.6. Mitomycin C (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 2 μg/ml and the cultures continued their incubation for an additional six hours. At this point the cells were pelleted and the supernatant was filter sterilized.

PEG precipitation of transducing lysates

Briefly, overnight cultures were diluted 1:200 in 1 L fresh BHI broth and grown at 37°C with shaking to an $OD_{600} = 0.6$. Mitomycin C was added to a final concentration of 2 μg/ml and the cultures continued their incubation for an additional 6 hours. At this point, cells were pelleted and the supernatant was cooled to 4°C. NaCl was added to a final concentration of 1M and the mixture was left standing for one hour at 4°C before centrifugation at 8,000 RPM for 15 minutes. 10% w/v PEG 8000 (Sigma-Aldrich, St. Louis, MO) was added to the supernatant and allowed to dissolve at room temperature. The mixture was allowed to stand for ≥ 1 hour at 4°C before centrifugation at 8,000 RPM for 15 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml phage buffer (100mM NaCl, 10 mM $MgCl_2$, 50 mM Tris HCl, pH 7.5). This lysate was then filter sterilized.

Insertional inactivation of *mecA*

Plasmid p*ΔmecA* contains *mecA* insertionally inactivated by *tetM* with a temperature sensitive origin of replication (50). A phage 80α transducing lysate of RN4220/p*ΔmecA* was used to infect strains MW2, J35, and J52. Transductants were selected on TSA containing tetracycline after overnight incubation at 30°C. Transductant colonies were picked and grown on TSA + tetracycline at 42°C the non-permissive temperature for plasmid replication. Any colonies that grew were then screened for tetracycline resistance, oxacillin sensitivity, and erythromycin sensitivity. An

erythromycin resistance cassette is present on the plasmid backbone and erythromycin sensitivity indicates secondary recombination and loss of the plasmid backbone. Bacteria with the correct resistance profile were confirmed to contain *mecA::tetM* by PCR.

CHAPTER 3 CcrAB-Mediated Excision of SCC*mec* Type IV in *Staphylococcus aureus*

The horizontal mobility of SCC*mec* is presumed to involve the site-specific recombinase genes found in each element, either *ccrAB* or *ccrC*. These proteins are serine recombinases of the invertase/resolvase family and resemble the large phage integrases (62, 134). Serine recombinases are thought to function as dimers and, in the case of CcrAB, integrate SCC*mec* into the staphylococcal chromosome by binding to two core recognition sequences, or attachment sites; one found on SCC*mec* and the other on the staphylococcal chromosome (*att*SCC and *attB*, respectively). Following SCC*mec* integration, two hybrid attachment sites are formed at either end of the inserted SCC*mec* element, known as *attL* and *attR*. SCC*mec* excision is the reverse of this process. It has been shown that both CcrA and CcrB are necessary for and capable of catalyzing the site-specific chromosomal excision of SCC*mec* types I and II (74, 80). Similarly, CcrC has been shown to catalyze the site-specific excision and reintegration of SCC*mec* type V (76). Spontaneous chromosomal excision, presumably mediated by CcrAB, has also been detected for SCC*mec* type III (74).

There is considerable sequence variation among the *ccr* genes of the same type. For example, the *ccrAB* genes found in SCC*mec* types II and IV are the same isotype, but can vary up to five percent at the nucleotide level (65). This is in contrast to *mecA* which

is known to be highly conserved (99-100% nucleotide identity) (63). It is possible that some of the mutations in CcrAB cause a loss of recombinase function, thereby stabilizing SCC*mec* in the chromosome. In this study we investigate the sequence diversity and recombinase ability of CcrAB from a panel of strains containing SCC*mec* type IV, and test for site-specific excision of the SCC*mec* type IV element.

CcrAB sequence

The nucleotide sequences of *ccrA* and *ccrB* from 9 strains carrying type IV SCC*mec* were determined and their amino acid sequences were aligned with that of strain N315 (Figure 8). N315 was used in these comparisons because it harbors SCC*mec* type II and CcrAB from N315 are known to catalyze SCC*mec* excision (80). CcrA from the type IV strains varied from 0.7-5.1% from the amino acid sequence of N315. The CcrA sequence of *S. aureus* strain MW2 was most divergent from N315. *S. aureus* strains C98-370, C99-529, J35, and J52 all shared the identical CcrA sequence. When the CcrB amino acid sequences of these strains were compared to N315, less variability was seen. *S. epidermidis* strain SE50 showed the greatest divergence from N315 (3.1%). The CcrB sequence of strain J28 was identical to N315. C98-370, C99-529, and J35 also have identical CcrB sequences.

SCC*mec* excision

ccrAB contained on a multi-copy plasmid has been shown to lead to the excision of SCC*mec* types I and II by using a PCR-based method of detecting SCC*mec* excision. This

Figure 8. CcrA and CcrB amino acid sequence alignments. A. Aligned CcrA amino acid sequences from representative *S. aureus* and *S. epidermidis* strains. B. Aligned CcrB amino acid sequences from representative *S. aureus* and *S. epidermidis* strains. Dots represent amino acids shared with the amino acid sequence shown along the top, while amino acids that differ from this sequence are shown.

A.

```
SE7         VKGGADMKRVIGYLRQSTMKQQSLAAQKQAIEAIAEKHHIQHINFYSDKQSGRKDNRSGY
SE63        ........Q.......................................................
SE5         ........Q.......................................................
C98-370     ........Q.......................................................
MW2         ........Q.......................................................
SE50        ........Q.......................................................
N315        ........Q.......................................................
J28         ........Q.......................................................

SE7         RQITQLIQQGQCDILCCYRLNRLHRNLKNALKLIKLCQTYHVHILSAHDGYFDMDQAFDR
SE63        ..............................................V.................
SE5         ..............................................V.................
C98-370     ..............................................V.................
MW2         ..............................................V.................
SE50        ..M...........................................R....V.............
N315        ..M...........................................V.................
J28         ..M...........................................V.................

SE7         LKLNIFISLAELESDNIGEQVRNGLQEKAKQGRLITTHAPFGYEYHNGTFIINQNESPTV
SE63        ............................................................
SE5         ............................................................
C98-370     ............................................................
MW2         ............................................................
SE50        ..........................................D.................
N315        F.........................................A...S.......
J28         F.........................................A...S.......

SE7         KAVFNYYIKGHGYKKIAQLLEEDNTYINRQPYQVRNIIINPNYCGRVNNQYGQFDNMFPS
SE63        ............................................................
SE5         ............................................................
C98-370     ............................................................
MW2         ............................................................
SE50        ............................................................
N315        ............................................................
J28         ............................................................

SE7         IVSTSIYEQAQRLRLQKQTKQTPSDNQLKQKIKCPCCNATLTNMTIRKKNHTLRYYVCPK
SE63        ............................................................
SE5         ............................................................
C98-370     ............................................................
MW2         ............................................................
SE50        ......................S.....................................
N315        .........S....S...I...S..............T......V..............
J28         .........S....S...I...S..............T......V..............
```

```
SE7         NMNASRFVCDFKGINAQTLEDKVLEVCRDFYQNQRIYTKIKSAIDKRIKRQRNIEKHHTL
SE63        ............................................................
SE5         ............................................................
C98-370     ............................................................
MW2         ............................................................
SE50        ..........................................G.................I.
N315        ..........................K.............G...................
J28         ..........................K.............G...................

SE7         TQEQLIEKLAQGIIDAETFREQTQSLRQQPQRTTSINGHQIQHIIQNIIQKRFTLNILYP
SE63        ............................................................
SE5         ............................................................
C98-370     ............................................................
MW2         ..K.....................................NT............M...
SE50        ..........................................T.................
N315        ..........................................TV................
J28         ..........................................TV................

SE7         YIETIHITKDKNLIGIYFKNEPLNIVNQTMQSSIA
SE63        ....................................
SE5         ....................................
C98-370     ....................................
MW2         ..DE.L...S.T.M..............T.....
SE50        ............................A.......
N315        ....................................
J28         ....................................
```

B.

```
MW2         MQQLKTKRVGIYVRVSTEMQSTEGYSIDGQINQIKEYCDFHHFEVKDIYADRGISGKSMN
N315        ............................................................
SE5         ............................................................
SE7         .....................................C......................
SE63        ............................................................
J52         ......E.....................................................
C98-370     ............................................................
SE50        ....................D.......................................

MW2         RPELQRILKDAKDGYIDCVMVYKTNRLARNTSDLLKIVEDLHKQNVEFFSLSERMEVNTS
N315        ............E...............................................
SE5         ......M.....E.N.............................................
SE7         ......M.....E.N.............................................
SE63        ......M.....E.N.................................L...........
J52         ......M.....E.N.............................................
C98-370     ......M.....E.N.............................................
SE50        ......M.....E.N....TI.......................................

MW2         SGKLMLQILASFSEFERNNIVENVFMGQTRRAQEGYYQGNLPLGYDKIPNSKHELMINQH
N315        ............................................................
SE5         ...........................................................D..
SE7         ...........................................................D..
SE63        ..........................................D.....D..........
J52         ...........................................................D..
C98-370     ...........................................................D..
SE50        ...........................................................D..

MW2         EANIVKYIFESYAKGHGYRKIANALNHKGYVTKKGKPFSISSITYILANPFYIGKIQFAK
N315        ............................................................
SE5         ..........C.................................................
SE7         ..........C.................................................
SE63        ..........C.................................................
J52         ..........C.................................................
C98-370     ..........C.................................................
SE50        ............................................................

MW2         YKDWSEKRRKGLNDKPVIAEGKHSPIINQDLWDKVQMRKKQVSQKPQVHGKGTNLLTGII
N315        ............................................................
SE5         ............................................................
SE7         ............................................................
SE63        ............................................................
J52         ............................................................
C98-370     ............................................................
SE50        ....................E.......................................
```

```
MW2         HCPQCGAPMAASNTTNTLKDGTKKRIRYYSCSNFRNKGSKVCSANSVRANVIEDYVMKQI
N315        ..................................................D.........
SE5         ..................................................D.........
SE7         ..................................................D.........
SE63        ..................................................D.........
J52         ..................................................D.........
C98-370     ..................................................D.........
SE50        ..................................................D.........

MW2         LEIVKSDKIIERVVARVNQENQVDCASLHHDIAYKQQQYDEVQIKLNNLIKTIEDNPDLT
N315        ........V.Q...TH........G.A.....................................
SE5         ........V.Q...TH........G.A...................I.................
SE7         ........V.Q...TH........G.A...................I.................
SE63        ........V.Q...TH........G.A...................I.................
J52         ........V.Q...TH......A.G.A...................I.................
C98-370     ........V.Q...TH........G.A...................I.................
SE50        ........V.Q..........K..G.A.................T...................

MW2         SVIRPSIQKYEKQLNDITYQINQLKNQQNEDKTLFDAKEISKLLQHIFHDIKHIEKSRLK
N315        ...................N.............P.............................
SE5         ...................N.............................................
SE7         ...................N.............................................
SE63        ...................N.............................................
J52         ...................N.............................................
C98-370     ...................N.............................................
SE50        ...................N.............S.....Q.........Q....M......

MW2         ALYLSVIDRIDIKKDGNHKKQFYVTLKLNNEIIKQLFNNKQLDEVHLSTSSLFLPQTLYL
N315        ............................................................
SE5         .S.................E.........................................
SE7         ............................................................
SE63        ............................................................
J52         ............................................................
C98-370     ............................................................
SE50        ..........N.R.....................................................

MW2         TI
N315        ..
SE5         ..
SE7         ..
SE63        ..
J52         ..
C98-370     ..
SE50        ..
```

PCR-based approach amplifies the chromosomal junction (*attB*) where SCC*mec* has excised, as well as the circularized, extra-chromosomal SCC*mec* (*att*SCC) (74, 80). In a similar manner, we investigated the recombinase ability of *ccrAB* from type IV SCC*mec*. *ccrAB* from ten strains containing type IV SCC*mec* (six *S. aureus*, and four *S. epidermidis*) were cloned onto multi-copy plasmids and moved into *S. aureus* strains N315 (SCC*mec* type II) and COL (SCC*mec* type I). The bacteria were grown overnight and total cellular DNA was extracted. Figure 9A and 9B show the results of PCR-based detection of SCC*mec* excision in strain N315. An amplification product corresponding to the chromosomal junction was detected when *ccrAB* from nine of the ten type IV strains was present; and an amplicon corresponding to the excised, circular form of SCC*mec* was detected when *ccrAB* from eight of the ten type IV strains was present. *ccrAB* from strain SE50 did not lead to detectable excision in N315, while CcrAB from SE63 led to amplification of the chromosomal junction but not the circular form of SCC*mec*. However, following 48 hours of growth, *ccrAB* from SE50 and SE63 yielded excision in N315 (Figure 9C and 9D). Figure 10A and 10B show the results of PCR-based detection of SCC*mec* excision in strain COL. *ccrB* found on the COL *SCCmec* has a premature stop codon, which is believed to produce a non-functional recombinase. Therefore all excision in this strain is presumably due to *ccrAB* contained in trans. All *ccrAB* genes from type IV SCC*mec* were capable of catalyzing the excision of SCC*mec* in COL except for *ccrAB* from the *S. epidermidis* strain SE63. Again, after 48 hours, SCC*mec* was excised by SE63 *ccrAB* (Figure 10C and 10D). Based on these results, we conclude that the *ccrAB* genes contained in the type IV SCC*mec* element encode functional proteins capable of the site-

Figure 9. PCR based excision in strain N315. A. PCR based detection of the chromosomal junction when SCC*mec* is excised (*att*B) using primers cL1 and cR1 (80). B. PCR based detection of the excised circular form of SCC*mec* (*att*SCC) using primers mR8 and mL1 (80). These PCR reactions were performed using genomic DNA as a template obtained after strains containing *ccrAB* constructs were grown for 24 hours. C. PCR-based detection of the chromosomal junction. D. PCR-based detection of the excised circular form of SCC*mec* using genomic DNA isolated after the strains containing *ccrAB* constructs were grown for 48 hours. Labels represent the strains from which *ccrAB* originated.

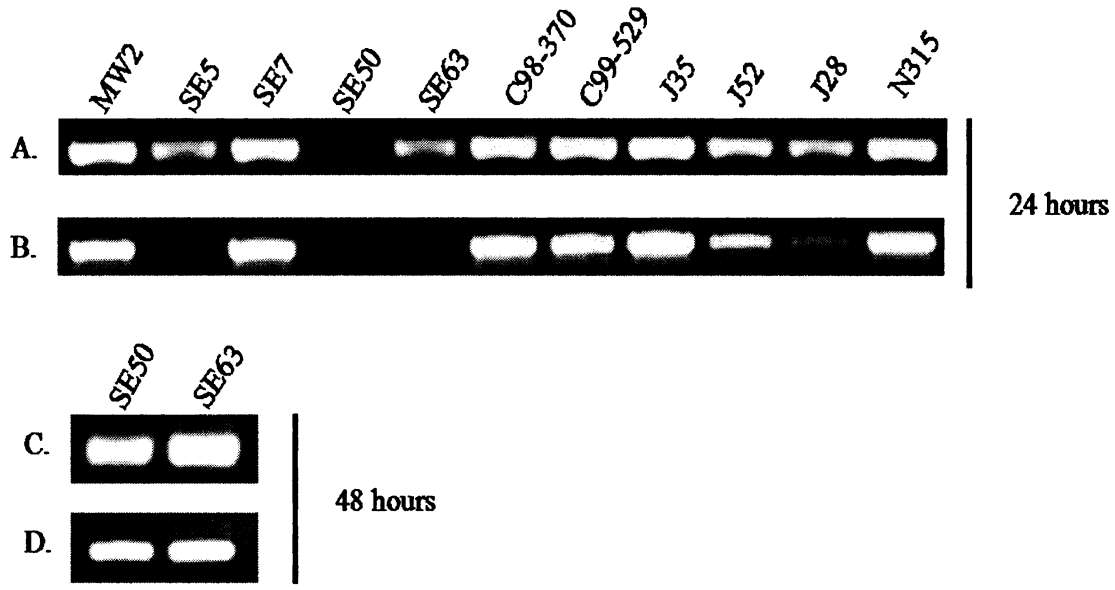
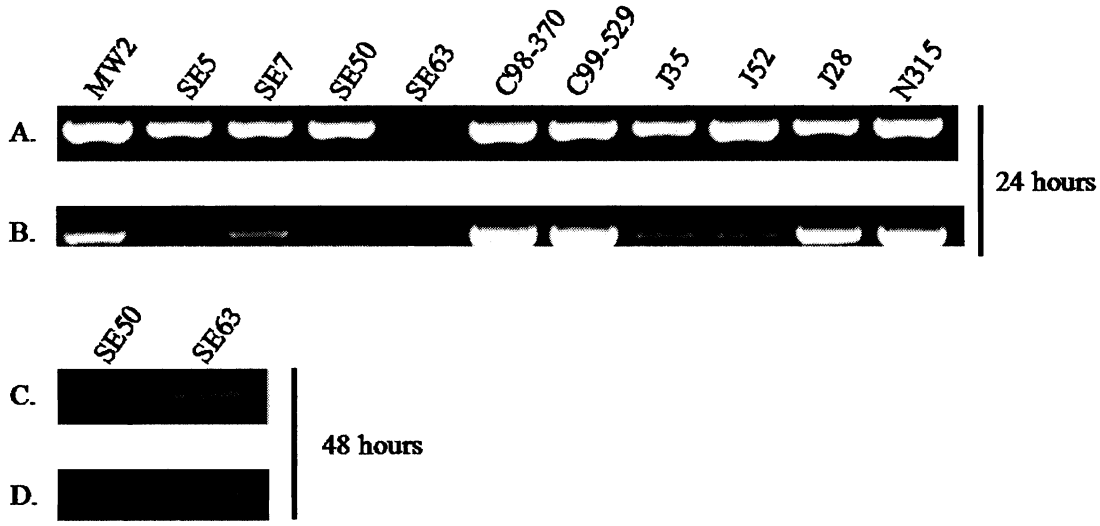

MW2
SE5
SE7
SE50
SE63
C98-370
C99-529
J35
J52
J28
N315
A.
B.
24 hours
SE50
SE63
C.
D.
48 hours

Figure 10. PCR based excision in strain COL. A. PCR based detection of the chromosomal junction when SCC*mec* is excised (*att*B) using primers cL1 and cR2 (80). B. PCR based detection of the excised circular form of SCC*mec* (*att*SCC) using primers colA and colB. These PCR reactions were performed using genomic DNA as a template obtained after strains containing *ccrAB* constructs were grown for 24 hours. C. PCR-based detection of the chromosomal junction. D. PCR-based detection of the excised circular form of SCC*mec* using genomic DNA isolated after the strains containing *ccrAB* constructs were grown for 48 hours. Labels represent the strains from which *ccrAB* originated.


MW2
SE5
SE7
SE50
SE63
C98-370
C99-529
J35
J52
J28
N315
A.
B.
24 hours
SE50
SE63
C.
D.
48 hours

specific excision of SCC*mec* despite the differences in amino acid sequence.

Excision of SCC*mec* type IV in strain MW2

We next sought to test the ability of these *ccrAB* constructs to cause the excision of type IV SCC*mec* in *S. aureus* strain MW2. Primers were designed to detect the excision of type IV SCC*mec* in an analogous fashion to that employed for the detection of SCC*mec* types I and II above (Jxn1/Jxn2 detect the chromosomal junction while mw2A/mw2B detect the circular, excised SCC*mec*). All ten *ccrAB* constructs were moved into strain MW2 and PCR-based detection of SCC*mec* was performed. Using this approach, excision of SCC*mec* type IV was not detected in MW2 containing any of the *ccrAB* constructs (data not shown). Based on these results, we hypothesize that *S.* aureus strain MW2 is somehow defective in SCC*mec* excision.

Structure of *attL* and surrounding sequence in MW2

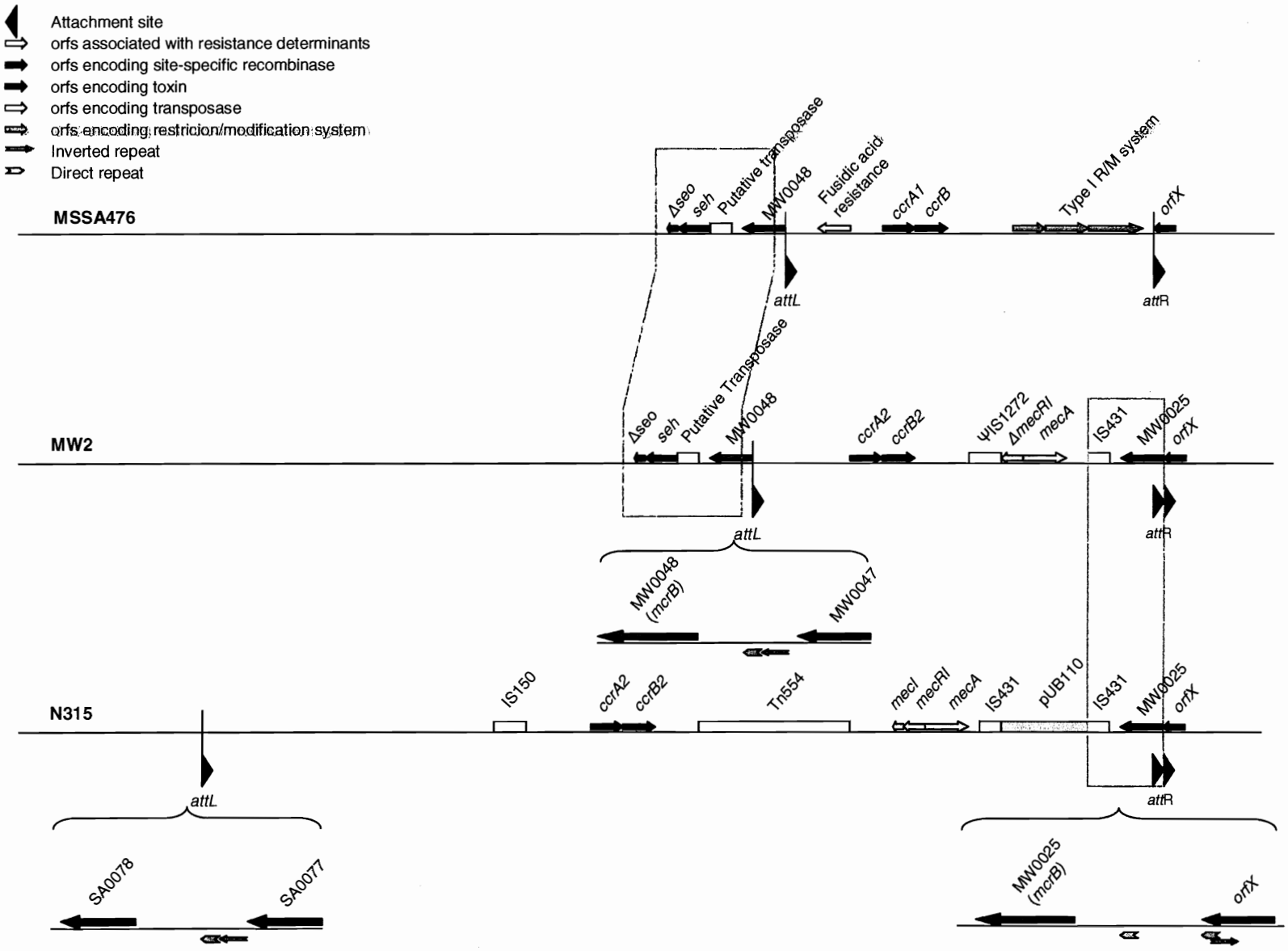
Since we were unable to detect chromosomal excision of SCC*mec* type IV in MW2 under conditions known to catalyze excision of other SCC*mec* elements, we sought to determine if there was any abnormality with the left or right attachment sites of this type IV SCC*mec*. These attachment sites are composed of a 15 bp direct repeat as well as degenerated inverted repeats (8, 74, 76, 80). By examining the genome sequence of strain MW2 (accession number NC_003923) and comparing it to that of N315 and COL (accession numbers NC_002745 and NC_002951, respectively), we found that the structural composition of the left and right attachment sites was very similar, and therefore

not a likely explanation for the lack of SCC*mec* excision in MW2 (8, 57, 84). However, an open reading frame designated MW0048 on the NCBI website is found immediately preceding *attL* in MW2. This open reading frame is not found in this position in either N315 or COL. An open reading frame with 97% nucleotide identity is also found within *SCCmec*, 326 bp upstream of *attR* in MW2, N315, and COL (designated MW0025 on the NCBI website). Figure 11 diagrams the structure of the SCC*mec* elements, including the region upstream of *attL*, in strains N315, MW2, and the non-*mecA* containing SCC element found in MSSA476. We therefore feel that the presence of MW0048 outside of *attL* in MW2 somehow prevents CcrAB-mediated recombination at this site.

Structure and excision of type IV SCC*mec* from other *S. aureus* strains

We next investigated whether the findings in MW2 were also true of other *S. aureus* strains harboring SCC*mec* type IV. SCC*mec* excision could not be demonstrated in strains C98-370, C99-529, and J28 by overexpressing *ccrAB* (data not shown) but could be shown for J35 and J52. Figure 12 shows amplification of the circular SCC*mec* element in J35 and J52 as well as amplification of *att*R. However, we could not detect the SCC*mec*-excised chromosomal junction or *attL* because our primer sets did not bind to the unknown sequence downstream of SCC*mec* in these strains. *attB*, corresponding to the SCC*mec* excised chromosomal junction was amplified from strain J39, indicating SCC*mec* excision in this strain (data not shown). A 2048 bp region extending from MW0048 to the downstream putative transposase, with sequence identical to that found in MW2 and MSSA476, could be PCR amplified in strains C98-370, C99-529, and J28 (Figure 13A).

Figure 11. Comparison of SCC*476* found in *S. aureus* strain MSSA476, SCC*mec* type IV found in *S. aureus* strain MW2, and SCC*mec* type II found in *S. aureus* strain N315. Diagram is based on the sequenced genomes of each strain: accession numbers NC_002953, NC_003923, and NC-002745, respectively (8, 70, 84). SCC*476* from strain MSSA476 contains genes encoding a type I restriction/modification system and a fusidic acid resistance cassette. MW0025 and MW0048 are duplicated genes as annotated on the MW2 genome the MW2 designations are used for the homologues in MSSA476 and N315. Flanking the left SCC*mec* boundary in MW2 is a gene cluster containing staphylococcal enterotoxin H (*seh*), a truncated staphylococcal enterotoxin O (*Δseo*), and a putative transposase gene. This gene cluster is also found flanking the left boundary of SCC*476*. *att*L – left SCC attachment site. *att*R – right SCC attachment site.


Attachment site
orfs associated with resistance determinants
orfs encoding site-specific recombinase
orfs encoding toxin
orfs encoding transposase
orfs encoding restricion/modification system
Inverted repeat
Direct repeat
MSSA476
Δseo
seh
Putative transposase
MW0048
Fusidic acid resistance
ccrA1
ccrB
Type I R/M system
orfX
attL
attR
MW2
Δseo
seh
Putative Transposase
MW0048
ccrA2
ccrB2
ΨIS1272
ΔmecRI
mecA
IS431
MW0025
orfX
attL
attR
MW0048 (mcrB)
MW0047
N315
IS150
ccrA2
ccrB2
Tn554
mecI
mecRI
mecA
IS431
pUB110
IS431
MW0025
orfX
attL
attR
SA0078
SA0077
MW0025 (mcrB)
orfX

Preceding the putative transposase in MW2 and MSSA476 are *seh* and a truncated *seo*, which may comprise a once mobile genetic element. Figure 13B shows amplification of a 725 bp region from the putative transposase to *seh* in all strains containing MW0048. Therefore, all of the strains in which SCC*mec* excision was not detected contain a structure outside of the left boundary of SCC*mec* that is very similar to that of MW2. Those strains lacking this structure are capable of SCC*mec* excision.

Phenotypic and genotypic confirmation of SCC*mec* excision

To confirm the PCR-based SCC*mec* excision results described above, we passaged each of these strains containing *ccrAB* in multiple copies for seven days. We then plated the cultures and picked isolated colonies. These colonies were used to inoculate overnight cultures from which 100 µl of each culture was plated onto BHI agar containing 6 µg/ml of oxacillin and growth was assessed after 72 hours. Also, total genomic DNA was isolated from each of these cultures and used as template in PCR reactions designed to detect *mecA*. In this manner, N315, J35, and J52 produced oxacillin-sensitive colonies that were negative for *mecA* by PCR, indicating that these strains had excised and subsequently lost SCC*mec* during passage. In contrast MW2, C98-370, C99-529, and J28 maintained oxacillin resistance and the *mecA* positive genotype after passage, indicating that these strains did not excise SCC*mec* (data not shown). These data are in agreement with the PCR-based means of detecting SCC*mec* excision employed above.

Discussion

Figure 12. Detection of *att*B, *att*SCC, *att*L, and *att*R in strains J35 and J52. Attempts to detect the SCC*mec* excised chromosomal junction (*att*B) using primers I1-R and I1-F, the excised-circular SCC*mec* element (*att*SCC) using primers mR8 and MW2attRfor, the right chromosomal-SCC*mec* junction (*att*R) using primers I1-R and MW2attRfor, and the left chromosomal-SCC*mec* junction (*att*L) using primers I1-F and mR8.

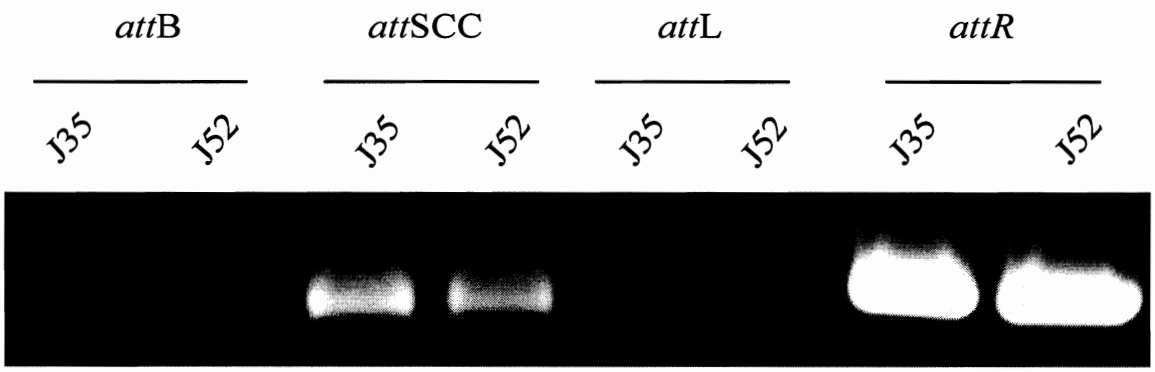
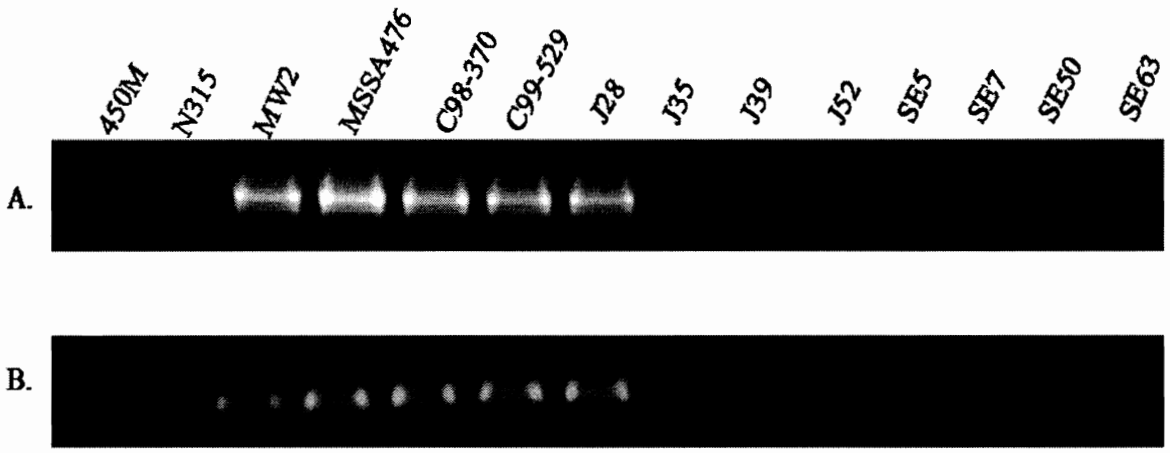

attB
attSCC
attL
attR
J35
J52
J35
J52
J35
J52
J35
J52

Figure 13. Detection of MW0048, putative transposase, and *seh* using PCR. A. PCR-based detection of a 2048 bp region from MW0048 to the downstream putative transposase ORF using primers Imw2R and mw2B. B. PCR-based detection of a 725 bp region from the putative transposase ORF to *seh* using primers trafor and sehrev. PCR was performed on total genomic DNA from the strains listed.


450M
N315
MW2
MSSA476
C98-370
C99-529
J28
J35
J39
J52
SE5
SE7
SE50
SE63
A.
B.

The variability seen in CcrAB from this collection of strains indicates that *ccrAB* is much less conserved than the other key component of SCC*mec*, *mecA*. PBP2a from *S. aureus*, *S. epidermidis*, and *S. sciuri* differ by only one or two amino acids, whereas the CcrAB sequences described here can vary by 5% within the same species (63). We hypothesized that the changes in *ccrAB* may result in a loss of recombinase function and therefore may be advantageous to the organism by stabilizing the methicillin resistance gene in the chromosome. However, this was not the case, since all of the *ccrAB* genes investigated in this study were capable of excising SCC*mec*.

We have identified a different mechanism by which *S. aureus* has stabilized the chromosomal integration of SCC*mec*. Several *S. aureus* isolates, representing a successful community-associated clonal type (ST-1), have a structure preceding the left SCC*mec* attachment site that prevents CcrAB-catalyzed excision of the element. These results are not likely due to problems with PCR primers because the primers used are capable of amplifying the left and right chromosomal-SCC*mec* junctions in each of these strains (data not shown), and the PCR-based results were confirmed by phenotypic and genotypic detection of *mecA* following *ccrAB* overexpression.

The stable integration of SCC*mec* in these strains may have implications for the presumed mobility of type IV SCC*mec*. Multi-locus sequence typing has shown that SCC*mec* type IV is found in several unrelated *S. aureus* clonal types, suggesting horizontal movement of the element (44, 46, 118). Because of its small size and its presence in distantly related *S. aureus* clonal types, type IV SCC*mec* is thought to be a promiscuous

genetic element. It is also thought that this mobility is at least partially responsible for the increasing recognition of MRSA in the community. It is interesting that all of the *S. aureus* strains that are deficient in SCC*mec* excision belong to sequence type 1. If *ccrAB* mediated SCC*mec* excision is truly necessary for the subsequent horizontal mobility of the methicillin resistance gene, then it is very unlikely that strains from ST-1 are contributing to the spread of methicillin resistance. ST-8 is emerging as the dominant MRSA type in the community, replacing ST-1. The sole representative of ST-8 in this study, strain J39, was capable of SCC*mec* excision, and so SCC*mec* mobility may play a role in the success of this lineage.

The genome sequences of MW2 and MSSA476 (accession numbers NC_002953 and NC_003923, respectively) reveal that downstream of MW0048 (and its MSSA476 homologue) is a putative transposase followed by Staphylococcal enterotoxin H and a truncated enterotoxin O (*seh* and *seo*). Staphylococcal enterotoxins (SEs) are a family of heat stable enterotoxins that are responsible for staphylococcal food poisoning. In addition to their gastrointestinal effects, SEs are superantigen toxins capable of causing toxic shock like diseases (10, 107). SEs are almost exclusively found on mobile genetic elements including plasmid, prophage, and pathogenicity islands (107). The gene cluster located downstream of SCC*mec* may, in itself, represent a once mobile element whose insertion has disrupted the mobility of SCC*mec*. We have shown here that strains C98-370, C99-529, and J28 contain MW0048 but J35, J39, and J52 do not. We have also amplified the region from the putative transposase to *seh* in each of these strains, confirming that all of the strains that do not excise SCC*mec* have this entire gene cluster located downstream.

This suggests that the acquisition of an additional virulence factor has resulted in maintenance of the methicillin-resistant phenotype.

These data strongly suggest that DNA sequence outside of the core recognition regions (direct repeat) is necessary for proper CcrAB mediated recombination. This observation is not without precedent. The serine recombinases, Tn3 resolvase and Sin, are both dependent upon accessory DNA binding sites that lie outside of the core recognition sequence where strand exchange occurs. Two subunits of Tn3 resolvase bind to the core recognition site but catalysis is dependent upon the binding of another four subunits to accessory binding sites located nearly 100 bp outside of the strand exchange site (18, 61). In the case of Sin recombinase, four subunits of Sin bind to the core recognition sequence but catalysis is dependent upon the binding of the non-specific DNA-bending protein Hbsu to a site 70 bp outside of the strand cleavage site (120, 121). Although the strand exchange site for CcrAB mediated recombination has been inferred by the results of SCC*mec* excision and chromosomal integration, the precise DNA elements necessary for this process are not yet known. It is clear from this study that CcrAB-mediated strand exchange is dependent upon sequence outside of the core recognition sites and that this sequence is absent or not functional in MRSA belonging to sequence type 1.

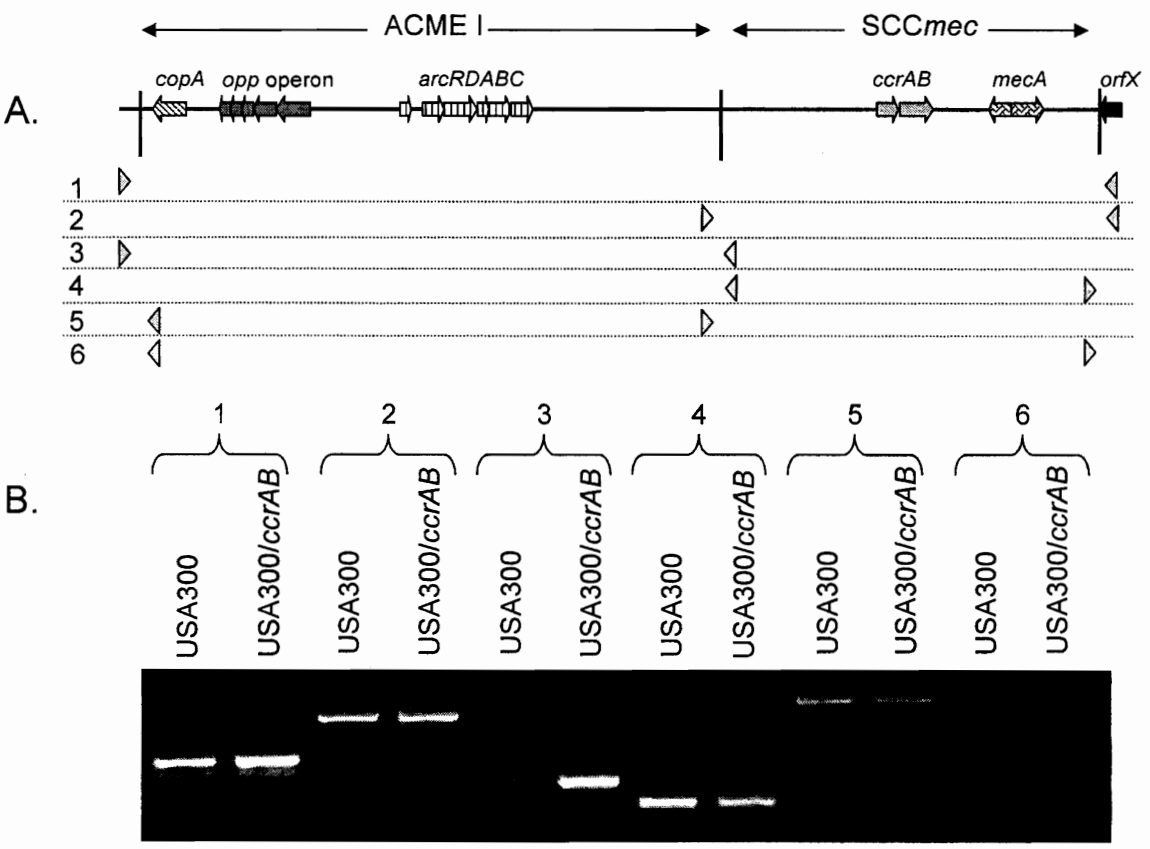
USA300

S. aureus strain USA300 (MLST sequence type [ST] 8) is among the most frequent causes of staphylococcal disease in the USA, replacing ST1 as the dominant cause of *S. aureus* disease in the community. The entire nucleotide sequence of this strain was

recently determined (39, 100). This work revealed the presence of a genomic island, referred to as ACME, which encodes an arginine deiminase pathway. ACME is inserted into the USA300 chromosome adjacent to SCC*mec* in the SCC*mec* chromosomal attachment site (Figure 1A). Here we examine the site-specific excision of SCC*mec* in USA300 to better understand the impact of adjacent DNA insertions, such as ACME, on this process.

Briefly, primers were designed to amplify across the chromosomal junction once SCC*mec* has excised and second set of primers was used to detect the excised, circular SCC*mec* element, as previously described. A third set of primers was used to detect the excised, circular ACME element (cACMEfor 5'- GAGG GTGAATTTAAAGTGGGAG-3' and cACMErev 5'- GGTGACAAATAAAAGGCTACC-3'). PCR reactions were performed on total cellular DNA from USA300 and from USA300 containing *ccrAB* on a multi-copy plasmid. A schematic of SCC*mec* and ACME in USA300 is shown in Figure 14A along with the primer binding sites. Figure 14B shows the results of the PCR-based excision experiments. PCR detected the empty chromosomal attachment site (1), the chromosomal junction when SCC*mec* is excised but ACME remains integrated (2), the chromosomal junction when ACME is excised but SCC*mec* remains integrated (3), extra-chromosomal, circular SCC*mec* (4), and extra-chromosomal, circular ACME. However, a circular element containing both SCC*mec* and ACME was not detected (6). Taken together, these results indicate that SCC*mec* and ACME are independently excised from the chromosome and form separate, circular elements once excised. It is not necessary for excision that *ccrAB* be contained in multiple copies, as spontaneous excision, presumably

Figure 14. Excision of SCC*mec* and ACME in *S. aureus* USA300. A. Schematic representation of the region of the USA300 chromosome containing SCC*mec* and ACME, including select open reading frames (depicted as block arrows) as reference points. Vertical lines represent the SCC*mec* and ACME attachment sites. The positions of primer binding sites (triangles) are shown below the schematic and the numbers correspond to the PCR reactions shown in B. B. PCR-based excision experiments performed on total cellular DNA extracted from USA300 or USA300 containing *ccrAB* in multiple copies. PCR reactions labeled 1 detect the chromosomal junction when both SCC*mec* and ACME are excised, 2 detect the junction when SCC*mec* is excised but ACME remains integrated, 3 detect the junction when ACME is integrated and SCC*mec* is excised, 4 detect the circular SCC*mec* element, 5 detect the circular ACME element, 6 detect circularized SCC*mec* and ACME.


A.
ACME I
SCCmec
copA
opp operon
arcRDABC
ccrAB
mecA
orfX
1
2
3
4
5
6
B.
1
2
3
4
5
6
USA300
USA300/ccrAB
USA300
USA300/ccrAB
USA300
USA300/ccrAB
USA300
USA300/ccrAB
USA300
USA300/ccrAB
USA300
USA300/ccrAB

mediated by chromosomal *ccrAB*, was detected in most cases. The nucleotide sequence of each of the PCR amplification products was determined and the results corresponded to the expected excision events.

In an effort to visualize the excision and loss of SCC*mec* and ACME, SmaI restriction digestion followed by pulse-field gel electrophoresis was performed on USA300 containing *ccrAB* in multiple copies after 0-4 days of passage. We would expect to see a shift in the SmaI fragment containing SCC*mec* and ACME corresponding to excision of these elements. However, no band shift was seen during passage despite detecting excision of both SCC*mec* and ACME by PCR (data not shown). This suggests that, even when *ccrAB* is present in multiple copies, excision is a rare event and the majority of cells contain both SCC*mec* and ACME integrated into the chromosome. It should be noted that plasmid-borne *ccrAB* is under the control of the native promoter, and that excision and integration may be a dynamic process. These factors may contribute to the small population of SCC*mec* and ACME excised bacteria in these experiments.

In addition to USA300, ACME is present in the genome sequence of *S. epidermidis* ATCC12228 and previous reports note a high prevalence of ACME in *S. epidermidis* isolates (39, 155). We have also detected the ACME element in *S. epidermidis* as well as the spontaneously excised, circular ACME element in these isolates (data not shown). This further supports the notion that *S. epidermidis* acts as a reservoir of mobile elements that are then acquired by *S. aureus*.

It is interesting that, from the standpoint of excision, SCC*mec* and ACME act independently and do not form one circular element upon chromosomal excision. ACME

does not encode a known recombinase and so its mobility is dependent upon *ccrAB* found in SCC*mec*. Since ACME and SCC*mec* are not excised as one, it seems that transfer of ACME would require that both donor and recipient strains contain *ccrAB*, and therefore, SCC*mec*. Thus, the virulence determinants found on ACME are limited to MRSA. Members of ST1 were among the first described community-associated MRSA isolates but the USA300 lineage has since become the dominant MRSA in the community (19, 100). Members of ST1 are not capable of CcrAB-mediated SCC*mec* excision and therefore are not likely to acquire ACME in a CcrAB-dependent fashion. It is possible that the added degree of genome plasticity that allowed for the acquisition of ACME played a role in the success of USA300.

CHAPTER 4 Spontaneous Deletion of *mecA* Partially Compensates for the Fitness Cost Associated with High Level Vancomycin Resistance

S. aureus become resistant to vancomycin in two ways. The first mechanism involves the acquisition of the *van* operon contained on Tn1546 (or other similar elements), which is typically present on a conjugative plasmid (20-22). The second mechanism of vancomycin resistance involves alterations in cell wall structure, presumably the result of mutations. This type of resistance is thought to be mediated by a thickened, poorly cross-linked cell wall that contains sufficient D-Ala-D-Ala targets in the periphery to bind vancomycin. This prevents the drug from accessing more lethal targets at the interior of the cell wall where cell wall synthesis occurs (34-36).

There are several lines of evidence suggesting that there is an incompatibility between simultaneous expression of high level vancomycin resistance and high level methicillin resistance in *S. aureus*. PBP2a is not capable of cross-linking peptidoglycan containing stem peptide modified by the *van* genes (53, 128). There are also observations that MRSA passaged on increasing concentrations of vancomycin have a decrease in oxacillin resistance or have deleted *mecA* (2, 115, 132). Here we investigate genetic and fitness alterations that occurred in three MRSA strains that became oxacillin susceptible during passage to high level vancomycin resistance, in comparison with isogenic strains that retained oxacillin resistance during the same vancomycin passage experiments.

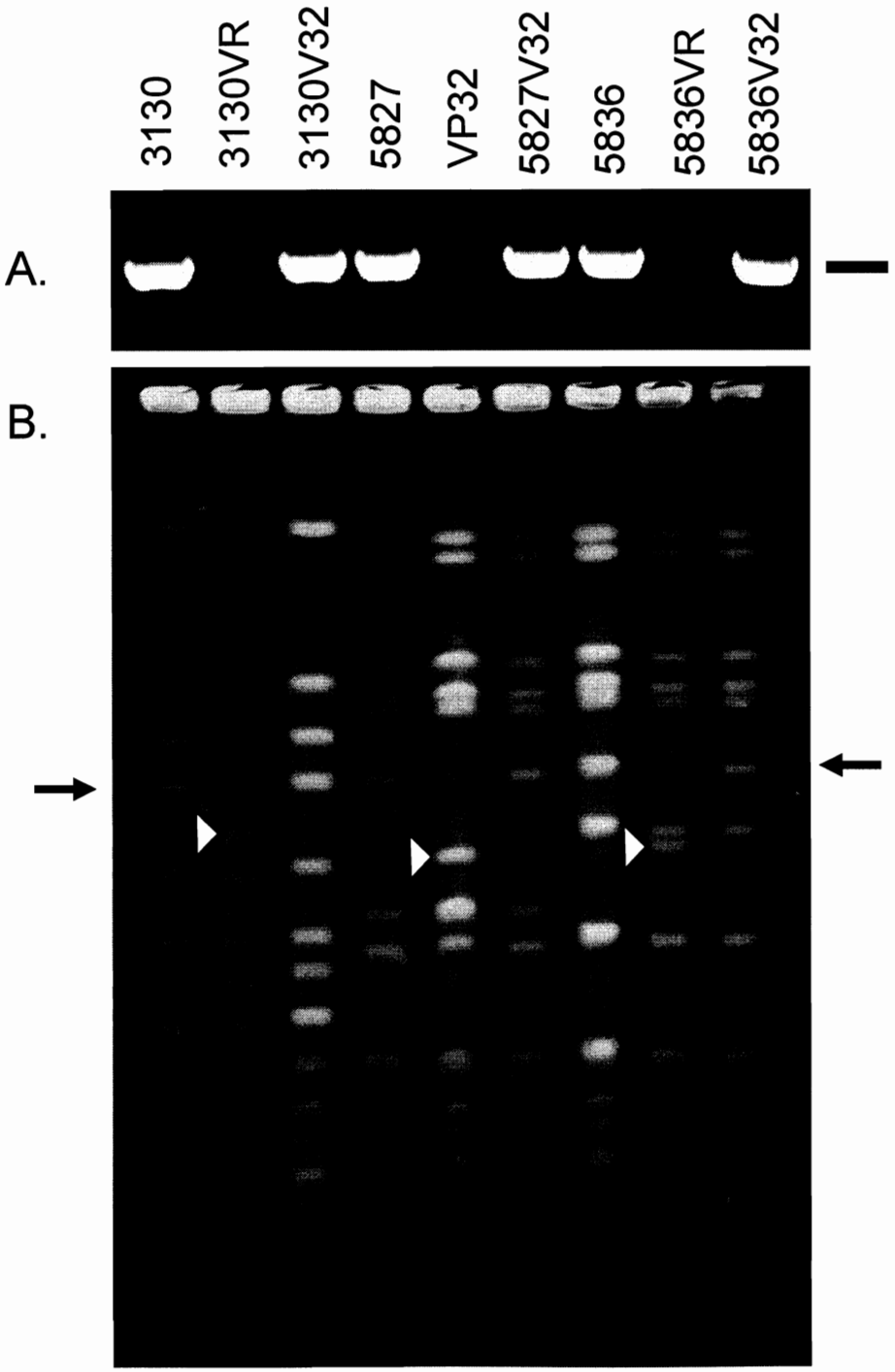
Strains

All strains are shown in Table 2. Parental MRSA strains 3130, 5827, and 5836 were passaged to vancomycin resistance. The resulting strains, 3130VR, VP32, and 5836VR, respectively, were resistant to vancomycin but sensitive to oxacillin. Vancomycin passage of the parental strains was repeated and the resulting strains 3130V32, 5827V32, and 5836V32 were resistant to vancomycin and oxacillin. Parental strain 5836 was heterotypically resistant to methicillin, whereas both 3130 and 5827 were homotypically resistant to methicillin (determined by oxacillin gradient plates and population analysis – data not shown). During passage to high level vancomycin resistance, strain 5836V32 converted to homotypic methicillin resistance (data not shown).

***mecA* genotype**

PCR amplification of *mecA* was performed on all nine strains to gain an understanding of the methicillin-sensitive phenotype of 3130VR, VP32, and 5836VR (Figure 15A). *mecA* was not detected in strains 3130VR, VP32, or 5836VR, while it was present in their parental strains and methicillin-resistant, vancomycin-resistant derivatives. SmaI restriction digestion of total cellular DNA and pulsed-field gel electrophoresis was used to confirm the identity of each strain as well as to visualize a chromosomal deletion encompassing *mecA* (Figure 15B). 3130VR, VP32, and 5836VR differ from their parental strains by band-shifts, suggesting that, in each case, the loss of *mecA* is coincident with the loss of a rather large fragment of the chromosome.

Figure 15. Detection of *mecA*. A. PCR for the detection of *mecA* from the strains indicated along the top. B. SmaI digested total cellular DNA separated by pulsed-field gel electrophoresis. Black arrows show a band present in the parental strains and the vancomycin-resistant, *mecA*-positive strains that shifts (white arrows) in the vancomycin-resistant, *mecA*-negative strains indicating a loss of DNA.


3130
3130VR
3130V32
5827
VP32
5827V32
5836
5836VR
5836V32
A.
B.

Mapping SCC*mec* deletions

CcrAB have been shown to catalyze the precise chromosomal excision of SCC*mec* and so a likely explanation for this loss of DNA is that the SCC*mec* element was site-specifically excised in these strains (74, 80, 104). Excision of SCC*mec* from the chromosome can be detected by PCR amplification across the chromosomal junction from which SCC*mec* was excised using primers I1F and I1R as previously described (104). Using this approach, 5836VR yielded a positive amplification product and DNA sequencing of this product revealed that the loss of *mecA* in this strain was due to site-specific SCC*mec* excision (data not shown). However, site-specific SCC*mec* excision from 3130VR and VP32 was not detected.

orfX, the open reading frame into which SCC*mec* inserts, was detected by PCR in both 3130VR and VP32 as was a region of the staphylococcal chromosome present outside of SCC*mec*. Primers were designed in these areas for long-range PCR amplification across the SCC*mec* insertion site in each of these strains (I1F and unirev for strain VP32 and I1F and 252rev for strain 3130VR). A long-range PCR amplification product of 6 kb was generated from VP32 but there was no amplification product from 3130VR (data not shown). The long-range PCR fragment from VP32 was sequenced to determine the precise deletion. 5836, 5827, and 3130 contained SCC*mec* type II, as determined by the method described by Ito, et al. (data not shown) (74). Primers were designed to amplify various regions along the type II SCC*mec* element in an effort to map the *mecA* deletion in 3130VR. Using this approach, an approximately 32 kb deletion was mapped to a 2 kb

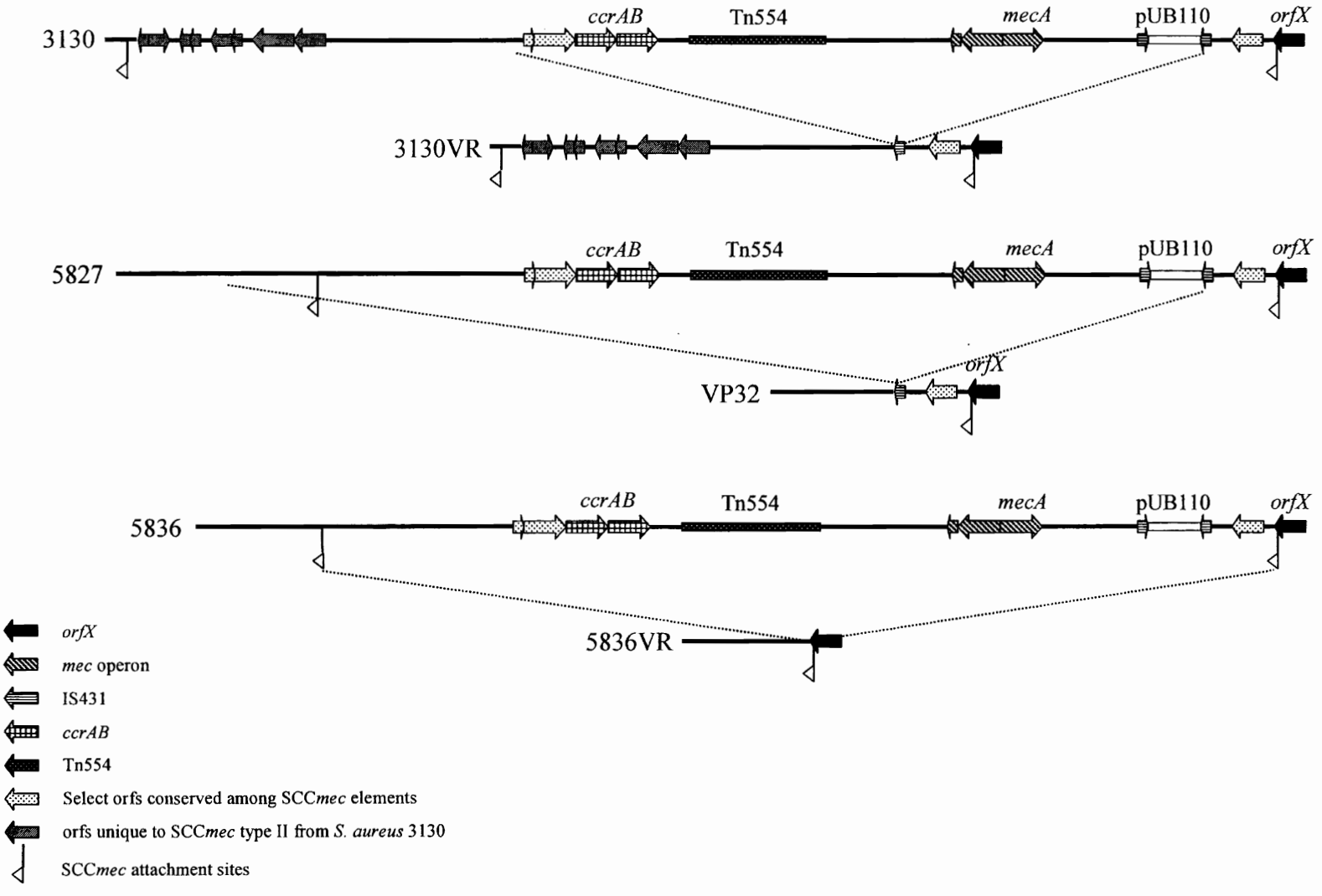
region of the SCC*mec* remnant in 3130VR. This 2 kb region was amplified and sequenced to precisely map the deletion. Figure 16 shows a schematic of the SCC*mec* elements present in the parental strains as well as the exact deletions from the vancomycin-resistant derivatives. Strain 3130VR has a 32 kb deletion of SCC*mec* spanning from the IS431 that flanks the integrated pUB110 to a region just beyond *ccrA*. VP32 deleted a larger portion of SCC*mec,* spanning from the IS431 flanking pUB110 to a region nearly 7 kb outside the left end of SCC*mec*. As indicated above, strain 5836VR site-specifically excised the entire SCC*mec* element.

CcrAB-mediated SCC*mec* excision from strains 5827 and 3130

SCC*mec* elements contain recombinase genes (*ccrAB*) responsible for their mobility, so it was surprising that CcrAB-mediated recombination appeared to be responsible for the loss of *mecA* in only one of the three strains. To determine if CcrAB-mediated SCC*mec* excision is possible in strains 3130 and 5827, *ccrAB* was introduced on a multi-copy plasmid into each strain and excision of SCC*mec* was monitored by PCR as previously described (104). This method has been very sensitive for detecting SCC*mec* excision, but the excision of SCC*mec* was not detected from strains 3130 and 5827, suggesting that CcrAB-mediated SCC*mec* excision does not occur in these strains (data not shown).

Growth analysis

Figure 16. Schematic of the type II SCC*mec* elements present in parental strains 3130, 5827, and 5836 as well as the SCC*mec* deletions in strains 3130VR, VP32, and 5836VR. Block arrows represent select open reading frames. pUB110 is an integrated plasmid flanked by copies of IS431. Dotted lines indicate the SCC*mec* region deleted in each of the vancomycin resistant derivatives. The boundaries of SCC*mec* are marked by flags representing the two hybrid SCC*mec* attachment sites.


3130
ccrAB
Tn554
mecA
pUB110
orfX
3130VR
5827
ccrAB
Tn554
mecA
pUB110
orfX
orfX
VP32
5836
ccrAB
Tn554
mecA
pUB110
orfX
5836VR
orfX
mec operon
IS431
ccrAB
Tn554
Select orfs conserved among SCCmec elements
orfs unique to SCCmec type II from S. aureus 3130
SCCmec attachment sites

The fact that three strains deleted portions of SCC*mec* during passage to vancomycin resistance suggests that the loss of this DNA is somehow advantageous to the bacterium. The growth rate of each strain was monitored and the doubling times during the exponential growth phase are shown in Figure 17. In each case, passage to vancomycin resistance caused a decrease in the growth rate when compared to the parent. This decrease in growth rate was partially compensated for by the deletion of SCC*mec*, as 3130VR, VP32, and 5836VR each grew faster than their SCC*mec* containing counterparts 3130V32, 5827V32, and 5836V32, respectively (differences are not statistically significant).

Competition

To further assess the fitness advantage of deleting portions of SCC*mec*, mixed culture competition experiments were employed. In order to compete 3130VR against 3130V32, VP32 against 5827V32, and 5836VR against 5836V32, plasmids with differential selectable markers were introduced into each strain. To control for fitness costs associated with each plasmid, each pair of strains was competed twice so the plasmids could be switched. For instance, 3130VR/pRN5543 was competed against 3130V32/pCN36 and 3130VR/pCN36 was competed against 3130V32/pRN5543. Representative results of three independent competition experiments are shown in Figure 18. After three days of competition, 3130V32 was not recovered from the mixed cultures. Similar results were seen with 5827V32 after five days of competition, and 5836V32 after three days of competition. These data reveal that, in each case, deletion of SCC*mec*

Figure 17. Growth analysis. The doubling time in minutes is shown for each strain listed. Doubling times were calculated from the exponential growth phase of seven independent growth curves.

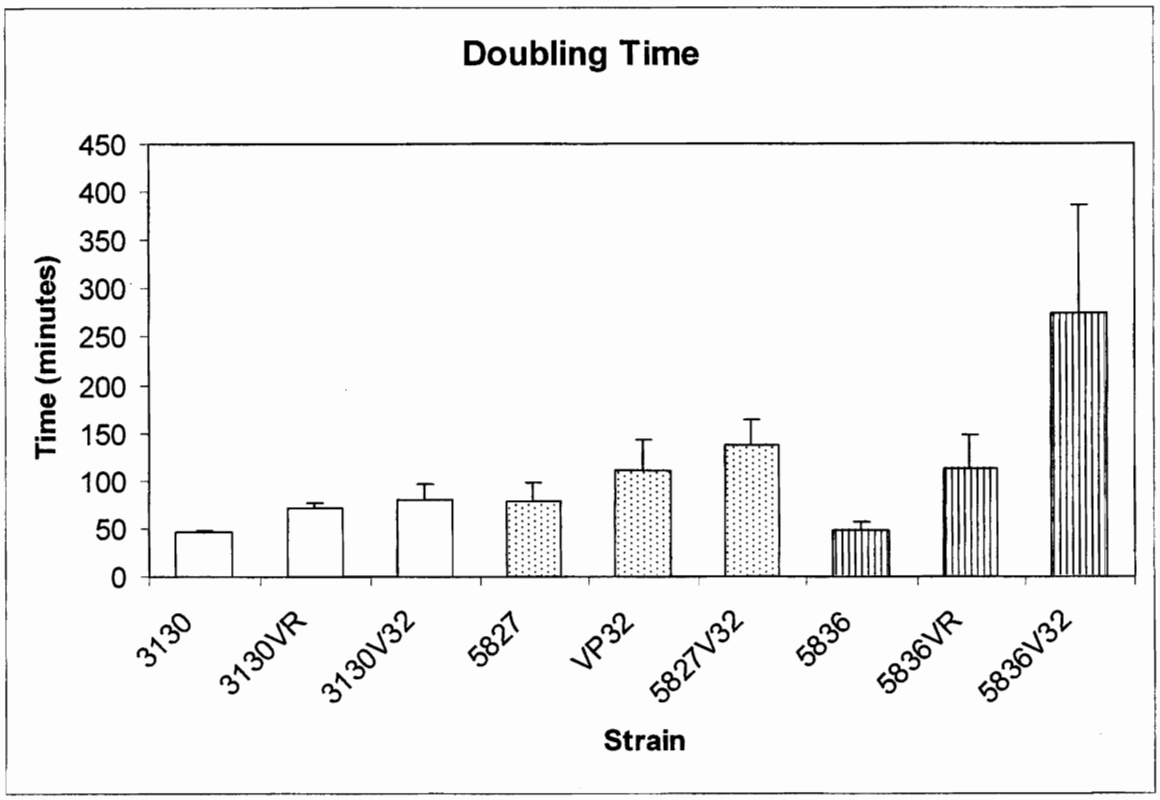
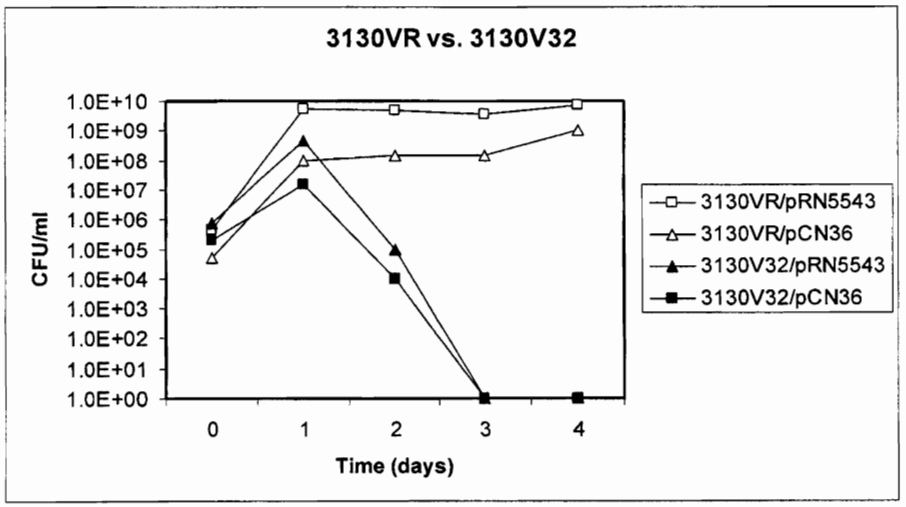
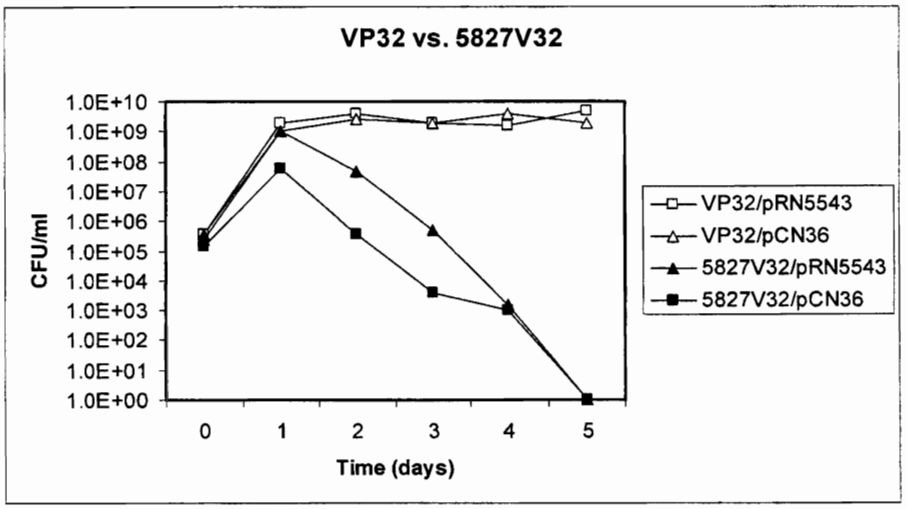
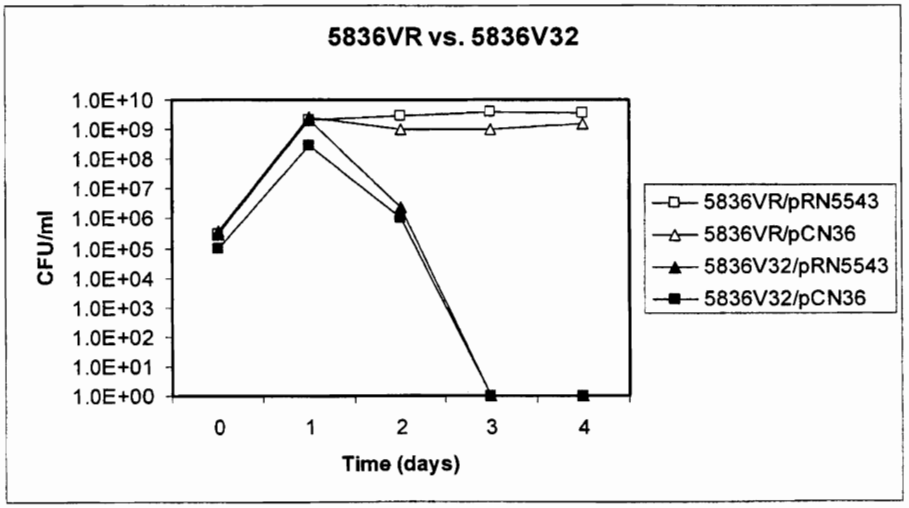

Doubling Time
Time (minutes)
450
400
350
300
250
200
150
100
50
0
3130
3130VR
3130V32
5827
VP32
5827V32
5836
5836VR
5836V32
Strain

Figure 18. Mixed culture competition experiments. Equal numbers of two different strains were mixed in liquid culture and passed for up to five days. Bacteria were enumerated by plating on differential selective media. 3130VR/pRN5543 was competed against 3130V32/pCN36 and 3130VR/pCN36 was competed against 3130V32/pRN5543 in the top, left panel. The top, right panel shows the results of competition of VP32/pRN5543 against 5827V32/pCN36 and VP32/pCN36 against 5827V32/pRN5543. The competition of 5836VR/pRN5543 against 5836V32/pCN36 and 5836VR/pCN36 against 5836V32/pRN5543 is shown in the bottom panel.


3130VR vs. 3130V32
CFU/ml
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
4
Time (days)
3130VR/pRN5543
3130VR/pCN36
3130V32/pRN5543
3130V32/pCN36


VP32 vs. 5827V32
CFU/ml
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
4
5
Time (days)
VP32/pRN5543
VP32/pCN36
5827V32/pRN5543
5827V32/pCN36


5836VR vs. 5836V32
CFU/ml
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
4
Time (days)
5836VR/pRN5543
5836VR/pCN36
5836V32/pRN5543
5836V32/pCN36

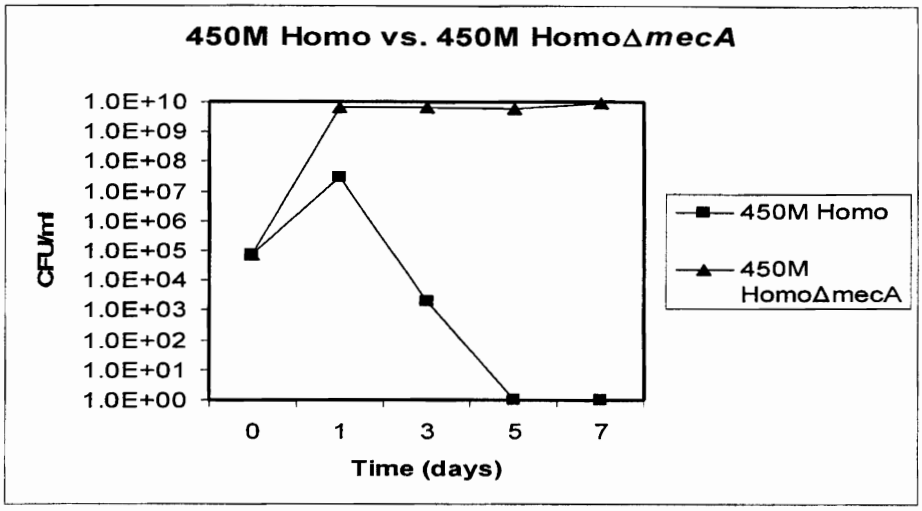
provided a significant fitness advantage that allowed the strains to completely out-compete their SCC*mec* containing counterparts in fewer than five days.

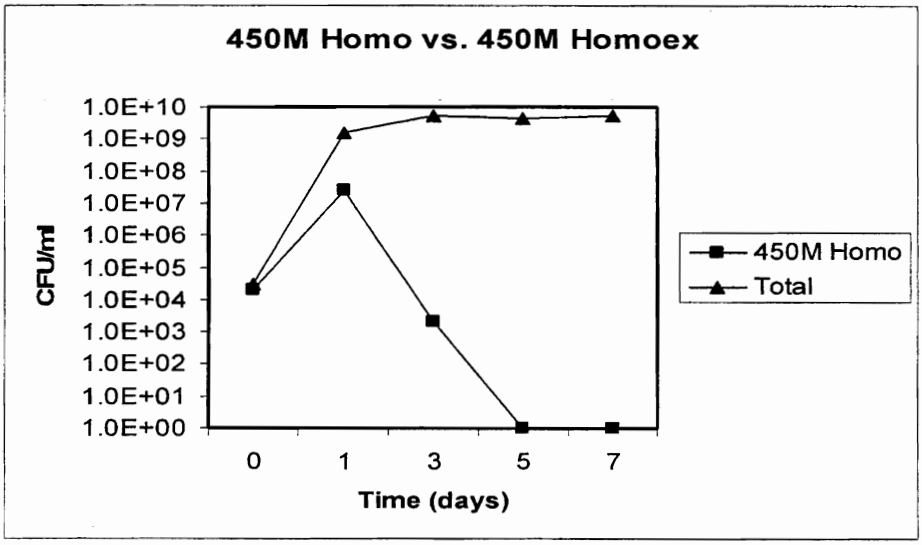
3130VR, VP32, and 5836VR each lost a significant amount of DNA containing the *mec* operon, including the identified regions: *mecA*, *ccrAB*, Tn554, and pUB110. It was not clear exactly which portion of this deleted DNA was responsible for the gain in fitness. To further assess the role of *mecA* in fitness, we examined strains 450M Homo, 450M Homoex, and 450M HomoΔ*mecA* by competition. 450M Homo contains the intact type I SCC*mec* and is homotypically oxacillin resistant, 450M Homoex is strain 450M Homo with the SCC*mec* element site-specifically excised, and 450M HomoΔ*mecA* is 450M Homo with *mecA* insertionally inactivated by *tetM*. Representative results of these competition experiments are shown in Figure 19. 450M Homo was readily out-competed by 450M HomoΔ*mecA* and 450M Homoex (Figure 19 top). However, there is no detectable decline in 450M HomoΔ*mecA* when it is competed with 450M Homoex (Figure 19 bottom), suggesting that these two strains have similar fitness levels. Taken together, these data show that there is not an appreciable difference in fitness between a strain that has excised SCC*mec* and a strain in which *mecA* is inactivated, although both show a fitness advantage over a strain containing *mecA*. Therefore, *mecA* is the gene responsible for the fitness cost associated with SCC*mec* in 450M.

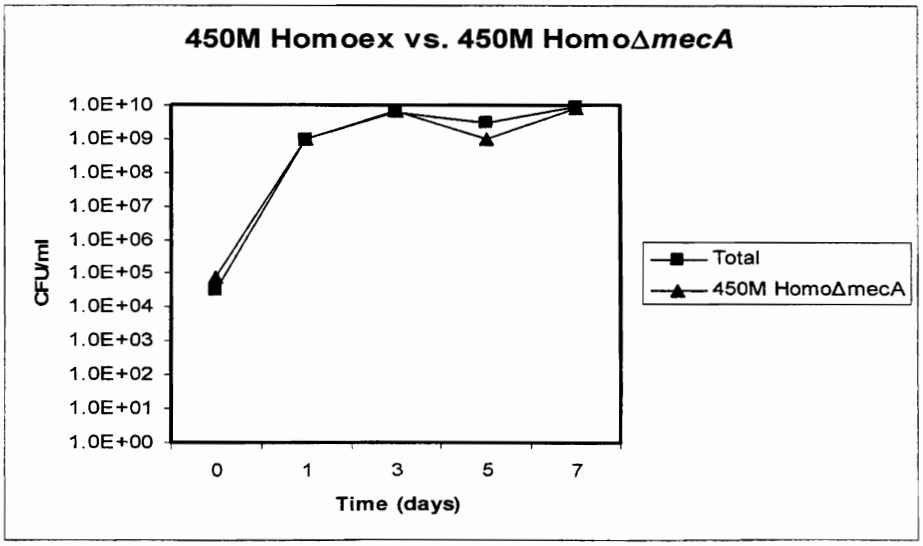
Discussion

Passage of *Staphylococcus aureus* on increasing concentrations of vancomycin leads to vancomycin resistance mediated by changes in cell wall structure. Although this

Figure 19. Mixed culture competition experiments. Equal numbers of two different strains were mixed in liquid culture and passed for up to five days. Bacteria were enumerated by plating on differential selective media. The top panel shows competition between 450M Homo and 450M HomoΔ*mecA*. Competition between 450M Homo and 450M Homoex is seen in the middle panel. The result of 450M Homoex competed against 450M HomoΔ*mecA* is seen in the bottom panel.


450M Homo vs. 450M HomoΔmecA
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
1
3
5
7
Time (days)
450M Homo
450M HomoΔmecA


450M Homo vs. 450M Homoex
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
1
3
5
7
Time (days)
450M Homo
Total


450M Homoex vs. 450M HomoΔmecA
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
1
3
5
7
Time (days)
Total
450M HomoΔmecA

is a laboratory phenomenon, it is thought to mimic the mechanism of resistance seen in vancomycin-intermediate sensitivity *S. aureus* (VISA) clinical isolates. Here we show that this mechanism of vancomycin resistance is associated with a decrease in growth rate, which is an observation also made by others (132). It is of note that parental strain 5836 was heterogeneously resistant to methicillin and had the fastest growth rate of the three parental strains. The vancomycin-resistant derivative, 5836V32, became homogenously methicillin-resistant and was the slowest growing of the vancomycin-resistant strains examined. It is likely that the conversion from heterotypic to homotypic methicillin resistance placed an additional burden on the bacterium that resulted in a decrease in fitness greater than that resulting from vancomycin resistance alone.

Loss of *mecA* or a decrease in methicillin resistance during passage to vancomycin resistance has been previously reported (2, 115, 132). Here we describe three strains that deleted portions of SCC*mec*, including *mecA*, during passage to vancomycin resistance. These deletions resulted in strains with a faster growth rate and an ability to out-compete their *mecA*-containing, vancomycin-resistant counterparts in mixed culture competition experiments (Figures 17 and 18). These data indicate that deletion of this DNA is not just coincident with passage on vancomycin but advantageous to the bacterium during the gradual conversion to vancomycin-resistance. We also provide evidence suggesting that it was the loss of *mecA*, and not other regions of SCC*mec*, that was responsible for the gain in fitness since, in mixed culture competition experiments using a laboratory strain unrelated to the clinical isolates examined in the study, excision of SCC*mec* provided no fitness advantage over inactivating *mecA* (Figure 19). It seems that *Staphylococcus aureus*

has difficulty simultaneously expressing high level resistance to methicillin and high level resistance to vancomycin by this mechanism. When high level resistance to both is achieved it comes at a great fitness cost to the bacterium. It remains to be determined if this decrease in fitness would impact the organism's ability to sustain an infection when both a β-lactam and vancomycin are used in therapy.

Several lines of evidence suggest that expression of methicillin resistance or *mecA* is deleterious to the staphylococcal cell. Ender, et al. found an inverse correlation between oxacillin resistance levels and growth rate by competing strains with heterotypic and homotypic expression of oxacillin resistance (43). Also, Katayama, et al. have shown that naïve methicillin-sensitive *S. aureus* (MSSA) did not tolerate the introduction of plasmid-borne, unregulated *mecA*, as *mecA* was often mutated or deleted to circumvent expression of PBP2a. When either the *mec* or *bla* regulatory genes were introduced along with *mecA*, the system was tolerated (82). The fitness cost associated with *mecA* in this study is puzzling because transcription of *mecA* should be tightly regulated by the MecI or BlaI repressors, as 3130V32, 5827V32, and 5836V32 each contain an intact copy of *mecI* or *blaI* (data not shown). It is difficult to conceive of a mechanism by which *mecA* is harmful to the cell when its expression is minimal. However, McAleese, et al. found that *mecA* transcription was down-regulated 2.9 fold in a VISA clinical isolate when compared to its vancomycin-sensitive parent (95). These strains contained *mecI*, yet *mecA* transcription was reduced below the MecI-repressed levels as vancomycin resistance increased. This suggests that the MecI-repressed levels of *mecA* transcription in the parent were in excess of what could be tolerated by the VISA derivative. It is possible, therefore, that the

MecI/BlaI-repressed levels of *mecA* transcription place an additional burden on the bacterium during passage to vancomycin resistance and result in the fitness costs seen in this study.

Strain 5836VR lost *mecA* by site-specific excision of SCC*mec* while strains 3130VR and VP32 both deleted portions of SCC*mec* in a manner inconsistent with CcrAB-mediated SCC*mec* excision. The introduction of *ccrAB* in trans did not lead to excision of SCC*mec* from the chromosomes of parental strains 3130 and 5827, suggesting that these strains are not capable of CcrAB-mediated SCC*mec* excision. We have previously reported that some MRSA containing SCC*mec* type IV are deficient in CcrAB-mediated recombination (104). This may indicate that, in some cases, SCC*mec* mobility is accomplished by CcrAB-independent mechanisms. Despite deleting different size fragments, the DNA deleted in both 3130VR and VP32 maps precisely to an IS431element located adjacent to pUB110 in SCC*mec*. Reipert, et al. also noted a chromosomal deletion encompassing *mecA* in a vancomycin-resistant *S. aureus* strain, and this deletion also mapped to the IS431 present upstream of *mecA* (115). IS431 is present in all known SCC*mec* elements just upstream of the *mec* operon and, in some cases, the *mec* operon is flanked by IS431 on either side (110, 130). These findings suggest a role for IS431 in the deletion of portions of SCC*mec*, and therefore it is possible that IS431 transposition/recombination may also play a role in the mobility and transfer of *mecA*.

CHAPTER 5 Structure of the SCC*mec* Attachment Site, *attB*, in Methicillin-Sensitive *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) arose from methicillin-susceptible *Staphylococcus aureus* (MSSA) upon acquisition of a genomic island known as Staphylococcal Chromosome Cassette *mec* (SCC*mec*) (80). The burden of MRSA disease is increasing. This is likely due to two factors: clonal expansion of existing MRSA lineages and the conversion of successful MSSA lineages to MRSA by the transfer of SCC*mec* into these backgrounds. SCC*mec* elements exist in five major isotypes (SCC*mec* I-V) and several variations of each type have been recognized (74, 76, 80, 91, 110). In addition, several non-*mecA* containing SCC elements have been discovered. These elements are precisely integrated into the same chromosomal attachment site as SCC*mec* elements (*att*B), contain homologues of the SCC*mec* recombinase genes, *ccrA* and *ccrB*, and are flanked by the 15 bp directly repeated sequences characteristic of SCC*mec* elements (70, 81, 90, 98).

Using multi-locus sequence typing in conjunction with SCC*mec* typing, Enright, et al. have found that MRSA are present in 38 of the 162 *S. aureus* genetic backgrounds (46). The presence of MRSA in only a subset of possible *S. aureus* genetic lineages suggests that only select lineages are capable of acquiring SCC*mec*. This may be due to several factors, one of which is that some lineages of *S. aureus* do not contain the genetic region

necessary for the chromosomal integration of SCC*mec*. More specifically, these lineages may not contain an intact *attB*, or accessory regions necessary for CcrAB-mediated integration of SCC*mec*. It is also possible that the *attB* of some lineages is otherwise occupied with non-*mecA* containing SCC elements. In the present study, we examine the genetic structure surrounding *attB* from a collection of 42 methicillin-susceptible *S. aureus* isolates, representing diverse genetic backgrounds, to gain a better understanding of these isolates' potential for acquiring SCC*mec*.

Detection of genes associated with SCC*mec*

This collection consists of 42 *S. aureus* isolates, shown in Table 7, that are methicillin-sensitive (as determined by failure to grow on 6 μg/ml of oxacillin). To better characterize the SCC*mec* chromosomal region, PCR and Southern blot analysis were used to detect *mecA*, *ccrAB*, *ccrC*, and *orfX*. None of the strains contained *mecA*, *ccrAB*, or *ccrC*. The absence of *ccrAB*, *ccrC*, or their remnants suggests that SCC*mec*-like elements are not present in this collection, as all known SCC*mec*-like elements contain recombinase genes. Every isolate in this collection was found to contain the *orfX* gene. The nucleotides encoding the C-terminal five amino acids of *orfX* comprise the directly repeated core sequence of the SCC*mec attB*. Therefore each of these strains may have the potential to acquire SCC*mec*.

attB

S. aureus 8325 can be thought of as the prototypical MSSA in terms of its SCC*mec* attachment site. This is because an 8325 derivative, 450M, was transduced with the type I SCC*mec* DNA and contains this SCC*mec* element site specifically integrated into the SCC*mec attB* (15). In addition, *ccrAB,* contained on a multi-copy plasmid, caused the site specific excision of SCC*mec* from the 450M chromosome (data not shown), indicating that this attachment site is functional for both integration and excision of SCC*mec*. PCR primers (I1-F and I1-R) were designed to amplify the SCC*mec attB* from strain 8325. These primers were used to amplify *attB* from this collection of MSSA and 9 of the 42 isolates yielded amplification products (Table 7). The DNA sequence of each of these amplification products was determined and each was found to be identical or nearly identical to the sequence of 8325.

Structure surrounding *attB* in other MSSA isolates

To characterize the *attB* genetic region of the 33 MSSA isolates that do not contain the 8325 *attB*, long range PCR amplification of this region was performed. One of the long range PCR primers was designed to bind in *orfX* (orfXfor). When examining the genome sequenced *S. aureus* strains, there is genetic diversity on the non-*orfX* side of *attB* (or the non-*orfX* side of SCC*mec* for the sequenced MRSA strains) (8, 39, 57, 70, 84). By aligning these sequences, a genetic region containing an open reading frame with homology to a tRNA dihydrouridine synthase was common to all sequenced strains. This open reading frame is present nearly 10 kb outside of SCC*mec* in the genome of MRSA252. A reverse primer (unirev) was designed to bind in this common region. Long

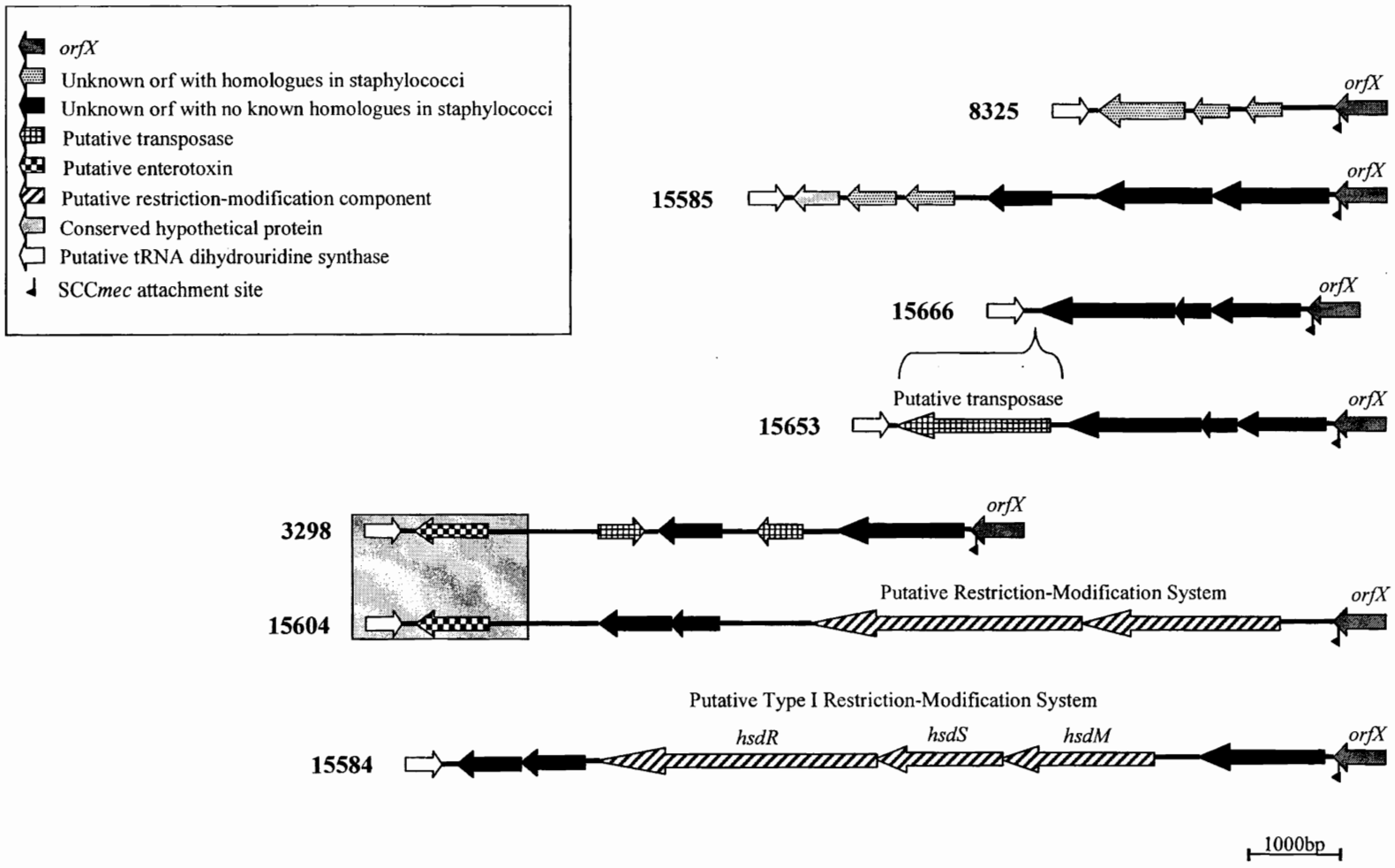
Table 7. Characteristics of MSSA isolates.

Strain	*spa* type	*spa* profile	MLST type	*attB* structure
15634	514	T1-K1-B1-M1-D1-M1-G1-M1-K1	1	8325
15575	515	U1	1	15575
15666	152	U1-J1-F1-G1-M1-D1-M1-G1-G1-M1	1	15666
15576	35	U1-J1-F1-K1-B1-P1-E1	1	15575
15591	175	U1-J1-F1-K1-P1-E1	12	15575
15604	141	U1-J1-F1-Q1-P1-L1-M1	12	15604
15668	2	T1-J1-M1-B1-M1-D1-M1-G1-M1-K1	5	8325
15658	529	W1-A1-K1-A1-O1-M1-Q1-Q1-Q1	30	15580
15671	18	W1-F1-K1-A1-O1-M1-Q1	30	15580
15677	33	W1-G1-K1-A1-K1-A1-O1-M1-Q1-Q1	30	15580
15580	468	X1-K1-A1-K1-A1-O1-M1-Q1-Q1	30	15580
15679	105	U1-J1-G1-F1-M1-B1-B1-B1-P1-B1	97	15584
15601	526	U1-M1-B1	188	3298
15584	527	U1-M1-F1-M1-B1-B1-P1-B1	ND	15584
15579	46	Y1-M1-B1-Q1-B1-L1-O1	195	8325
15594	509	T1-J1-A1-I3-J1-A1-B1-B1-B1	9	8325
15611	139	Y1-G1-F1-M1-B1-L1-O1	8	8325
15681	7	Y1-H1-G1-C1-M1-B1-Q1-B1-L1-O1	8	8325
15589	1	Y1-H1-G1-F1-M1-B1-Q1-B1-L1-O1	8	8325
15602	37	U1-K1-G1-J1-B1	109	8325
15637	208	X1-K1-B1-Q1-B1-B1-M1-M1	291	15580
15682	549	Z1-G1-F1-G1-U2-D1-M1-G1-M1	25	15682
15680	507	T1-G2-M1-F1-B1-B1-B1	20	ND
15649	466	I2-Z2-E1-M1-M1-J1-H2-M1	121	3298
15630	93	X1-K1-B1	47	ND
15578	42	A2-A1-K1-B1-E1-M1-B1-K1-B1	45	ND
15651	220	X1-K1-A1-K1-E1-M1-B1-K1-B1	45	ND
15647	154	Y1-C2-F1-M1-B1-Q1-B1-L1-O1-O1	6	15580
15673	151	I2-G1-B1-B1-G1-G1-J1-A1-G1-J1	15	15666
15639	519	U1-J1-G1-B1-B1-G1-G1-B1-B1-G1-G1-J1-A1-G1-J1	15	ND
15653	21	U1-J1-G1-B1-B1-G1-G1-J1-A1-G1-J1	15	15653
15585	17	Z1-D1-M1-D1-M1-N1-K1-B1	59	15585
15607	505	I2-F1-K1-B1-P1-E1	ND	15575
3294	118	U1-J1-G1-B1-B1-G1-E1-G1-J1-A1-G1-J1	ND	15666
3298	411	I2-Z2-E1-G1-M1-J1-H2-M1	ND	3298
3304	558	I2-Z2-E1-G1-M1-M1-M1-J1-H2-M1	ND	3298
NRS104	99	X1-K1-K1-A1-K1-A1-O1-M1-Q1-Q1	ND	15580
NRS198	1	Y1-H1-G1-F1-M1-B1-Q1-B1-L1-O1	ND	8325
NRS199		Z1-O3-M1-O1-M1-O1-M1	ND	NRS199
NRS204	251	W1-G1-K1-K1-A1-K1-A1-O1-M1-Q1	ND	15580
6520	43	W1-G1-K1-A1-K1-A1-O1-M1-Q1	30	15580
6881	43	W1-G1-K1-A1-K1-A1-O1-M1-Q1	30	15580

range PCR amplification of the *attB* genetic region yielded products, ranging in size from 3.7 to over 10 kb, in 28 of the 33 uncharacterized MSSA isolates. Four out of the five isolates for which long range PCR failed to amplify were found to lack the chromosomal regions to which the reverse primer binds, while the fifth of these may have too much intervening sequence for long range PCR amplification. Amplification products of the same size were analyzed by restriction fragment length, which revealed that 10 of the 28 amplification products were unique (Table 7). The nucleotide sequences of these ten amplification products were determined.

A schematic of the *attB* region of *S. aureus* 8325 is shown in Figure 20. This region is approximately 3 kb and contains three predicted open reading frames between *orfX* and the reverse primer (unirev) binding site. These three open reading frames were not shared with any of the ten MSSA for which the *attB* region was sequenced. A schematic of the *attB* genetic regions from isolates 15585, 15666, 15653, 3298, 15604, and 15594 are also shown in Figure 20. These regions vary in size (3.7 up to 10 kb) and, with some exceptions, do not contain sequence in common. The *attB* region of strain 15585 is composed of three predicted open reading frames of unknown function with homologues in staphylococci, as well as three unknown open reading frames with no homology to the staphylococci. The *attB* regions of strains 15666 and 15653 contain three open reading frames with weak homology to three hypothetical proteins from *Bacillus sp.* These two regions differ only by a putative transposase insertion into 15653 that is not present in 15666. Two predicted open reading frames with no homologues in staphylococci are

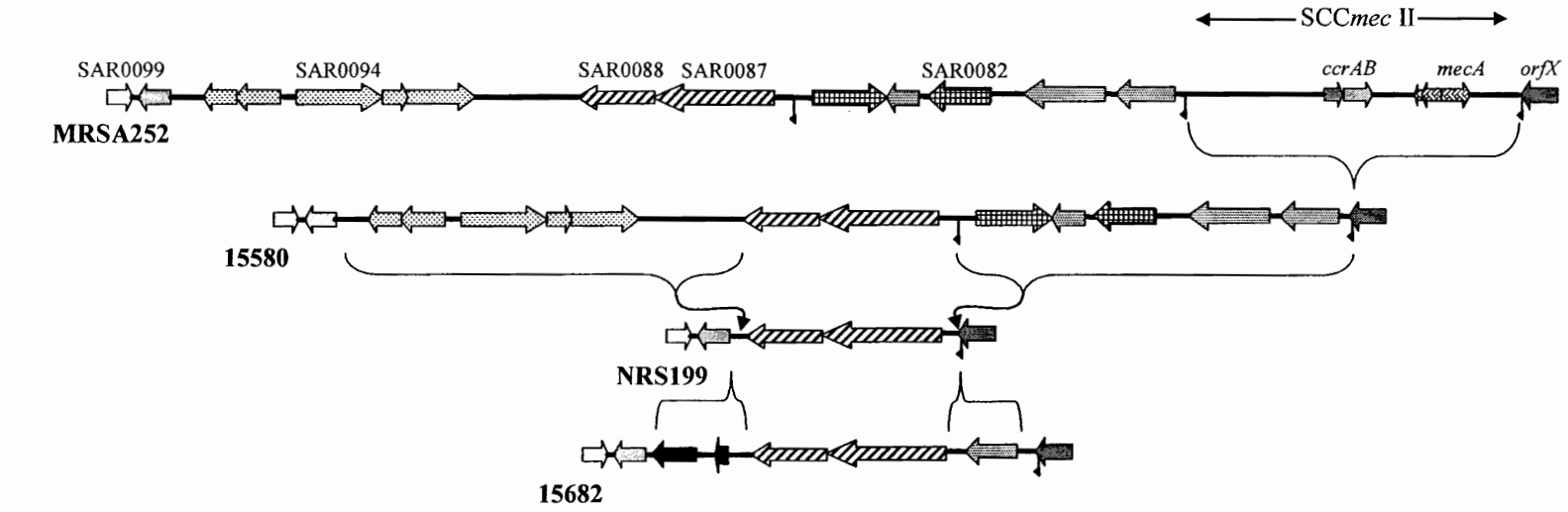
Figure 20. Schematic of the genetic regions surrounding *attB* in seven MSSA. The nucleotide sequences surrounding *attB* in strains 8325, 15585, 15666, 15653, 3298, 15604, and 15584 are depicted. Block arrows represent predicted open reading frames and their fill pattern indicates their putative function as shown in the legend. Each region is flanked by *orfX* (gray arrow) and a conserved predicted open reading (white arrow). 15566 and 15653 differ only by the insertion of a putative transposase into 15653. 3298 and 15604 share a region at the left end that encodes a putative enterotoxin. Open reading frames labeled *hsdS*, *hsdR*, and *hsdM* are predicted components of a type 1 restriction-modification system.


orfX
Unknown orf with homologues in staphylococci
Unknown orf with no known homologues in staphylococci
Putative transposase
Putative enterotoxin
Putative restriction-modification component
Conserved hypothetical protein
Putative tRNA dihydrouridine synthase
SCCmec attachment site
8325
orfX
15585
orfX
15666
orfX
Putative transposase
15653
orfX
3298
orfX
15604
Putative Restriction-Modification System
orfX
Putative Type I Restriction-Modification System
15584
hsdR
hsdS
hsdM
orfX
1000bp

present in strain 3298, along with two putative truncated transposases. This region also contains a 259 amino acid open reading frame with 99% identity to a probable enterotoxin from *S. aureus* RF122. This probable enterotoxin is also present in strain 15604 (boxed in gray in Figure 20). In addition, strain 15604 contains two unknown open reading frames and two open reading frames with homology to a restriction-modification system from *Lactococcus lactis*. Three open reading frames with high homology to a type 1 restriction-modification system from *S. haemolyticus* JCSC1435 are present in strain 15584.

Isolates 15580, NRS199, and 15682 have structures outside of *attB* that are also found in *S. aureus* MRSA252 genome sequence (70). MRSA252 contains SCC*mec* type II. This element has a nearly 6 kb region at the non-*orfX* end that is not shared by other type II SCC*mec* elements. This region contains several hypothetical open reading frames, two of which show homology to transposases. Although not noted in the genome sequence, this 6 kb region is flanked by 15 bp directly repeated sequences characteristic of SCC*mec* attachment sites. This suggests that MRSA252 has two elements, SCC*mec* and the 6 kb element, inserted adjacent to each other in the SCC*mec* attachment site. Outside of these elements is a region of the MRSA252 chromosome that contains, among other things, two open reading frames with homology to restriction-modification components. Figure 21 shows a schematic of this region of the MRSA252 chromosome, along with schematics of the regions from strains 15580, NRS199, and 15682 (SCC*mec* is not drawn to scale in Figure 21). Isolate 15580 has the same structure as MRSA252 except that it is missing SCC*mec*. NRS199 contains a portion of the sequence present in strain 15580 but it is missing the 6 kb element and an additional region. However, NRS199 does contain

Figure 21. Comparison of the chromosomal regions of MRSA252, 15580, NRS199, and 15682. The chromosomal region containing SCC*mec* and the surrounding sequence of MRSA252 is shown along with the similar regions from MSSA strains 15580, NRS199, and 15682. MRSA252 contains SCC*mec* type II and a 6 kb region encoding two regions homologous to transposases inserted into *attB* (SAR0082 and SAR0084 as annotated on the MRSA252 genome). The left end of this chromosomal region contains two open reading frames with homology to a restriction and modification genes (SAR0087 and SAR0088). Strain 15580 differs from MRSA252 by the absence of SCC*mec*. Strains NRS199 and 15682 contain the two open reading frames with homology to a restriction and modification genes, but the other regions are absent in these strains. Each region is flanked by *orfX* (dark gray arrow) and a conserved predicted open reading (white arrow). SCC*mec* type II is not drawn to scale.

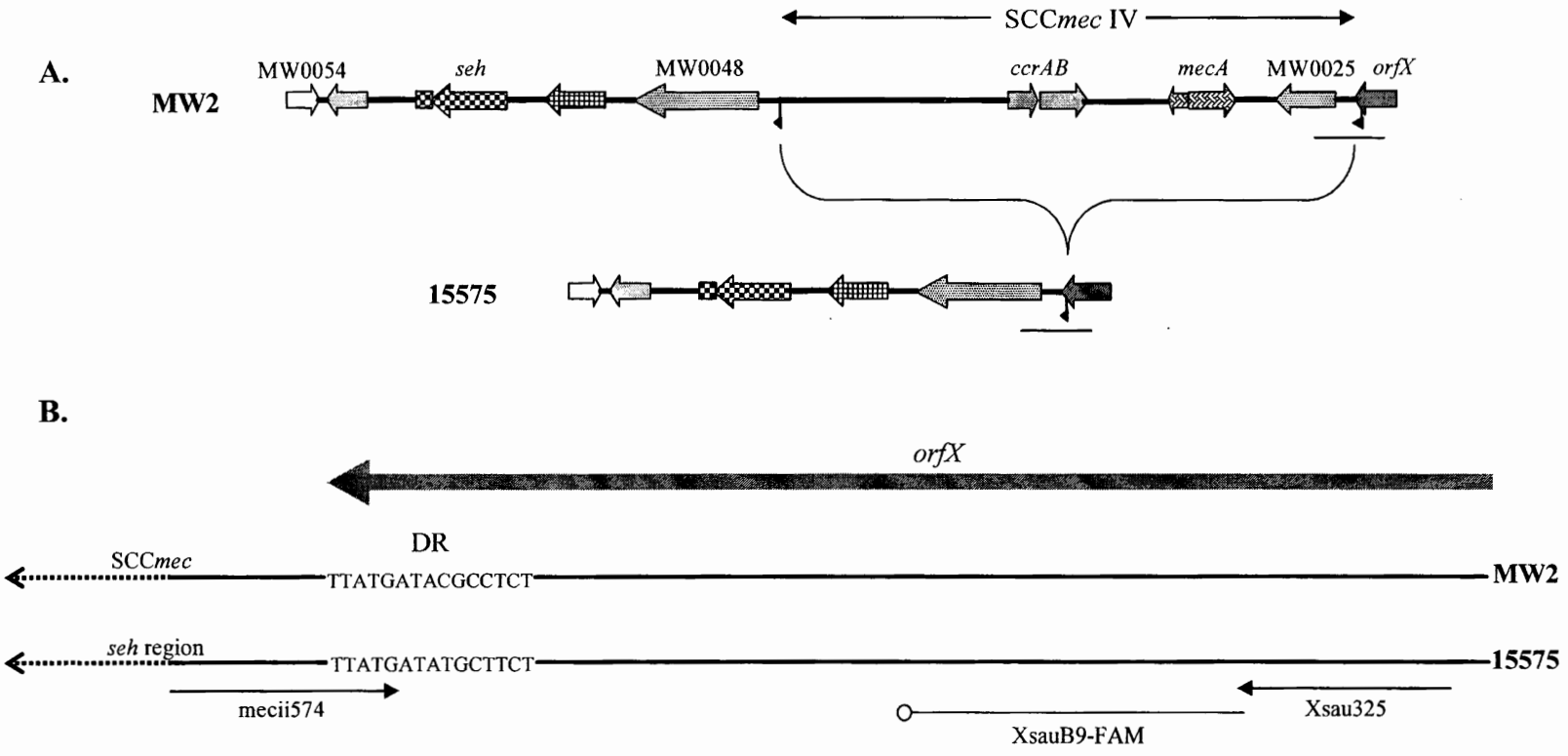

SCCmec II
SAR0099
SAR0094
SAR0088
SAR0087
SAR0082
ccrAB
mecA
orfX
MRSA252
15580
NRS199
15682
orfX
Unknown orf with homologues in staphylococci
Unknown orf with no known homologues in staphylococci
Putative transposase
Putative restriction-modification component
Conserved hypothetical protein
Putative tRNA dihydrouridine synthase
SCCmec attachment site
1 kb

the restriction-modification homologues. The sequence from strain 15682 is very similar to that of strain 15580 except for the insertion of two regions containing open reading frames of unknown function.

Adjacent to *orfX* in strain 15575 is a region found outside of SCC*mec* in the genome of *S. aureus* strain MW2. This region consists of an open reading frame (MW0048) with high nucleotide identity to an open reading frame (MW0025) present just inside the *orfX* end of SCC*mec* in MW2. It seems that the region containing this open reading frame has duplicated in strain MW2 and is now present inside SCC*mec* and on the chromosome outside of SCC*mec*. Following this is an open reading frame predicted to encode a truncated transposase, staphylococcal enterotoxin H (*seh*), and a truncated staphylococcal enterotoxin O (*seo*). A schematic of this region of the MW2 chromosome as well as strain 15575 chromosome is depicted in Figure 22A (SCC*mec* is not drawn to scale in Figure 22A). The region found in strain 15575 was also found in three other MSSA isolates, that of 15666 was found in two other MSSA, 15580 was present in an additional 9 isolates, 3298 was found in three other isolates, and the structures of 15682, 15653, 15585, NRS199, and 15604 were only found in single isolates.

A real-time PCR-based assay for detecting MRSA directly from clinical samples has been developed by Infectio Diagnostic, Inc. and is known as IDI-MRSA (148). This approach uses a *S. aureus* specific *orfX* primer with a series of primers that bind just inside the right end of SCC*mec* elements in combination with a target-specific, fluorogenic-molecular beacon probe to detect MRSA (72). Figure 22B depicts the combinations of primers and probe that would detect the type IV SCC*mec* element from strain MW2 using

Figure 22. Comparison of the chromosomal regions of MW2 and 15575. A. The chromosomal region containing SCC*mec* and surrounding sequence in MW2 is shown along with a similar region from MSSA 15575. Each region is flanked by *orfX* (dark gray arrow) and a conserved predicted open reading (white arrow). MW2 contains SCC*mec* type IV inserted into *attB*. The region outside of SCC*mec* contains an open reading frame also present within the right end of SCC*mec* (MW0048 and MW0025, respectively as annotated on the MW2 genome). This region also contains a putative transposase, staphylococcal enterotoxin H (*seh*), and a small, truncated region with homology to staphylococcal enterotoxin O (*seo*). 15575 differs from MW2 by the absence of SCC*mec* type IV. SCC*mec* type IV is not drawn to scale. B. An enlarged view of the regions underlined in A is shown. The thick arrow depicts the coding region of *orfX*, and the nucleotide sequence of the 15 bp direct repeat is shown. Also shown are the binding sites for primers mecii574 and Xsau325, and the probe, XsauB9-FAM, used by the IDI-MRSA detection system.

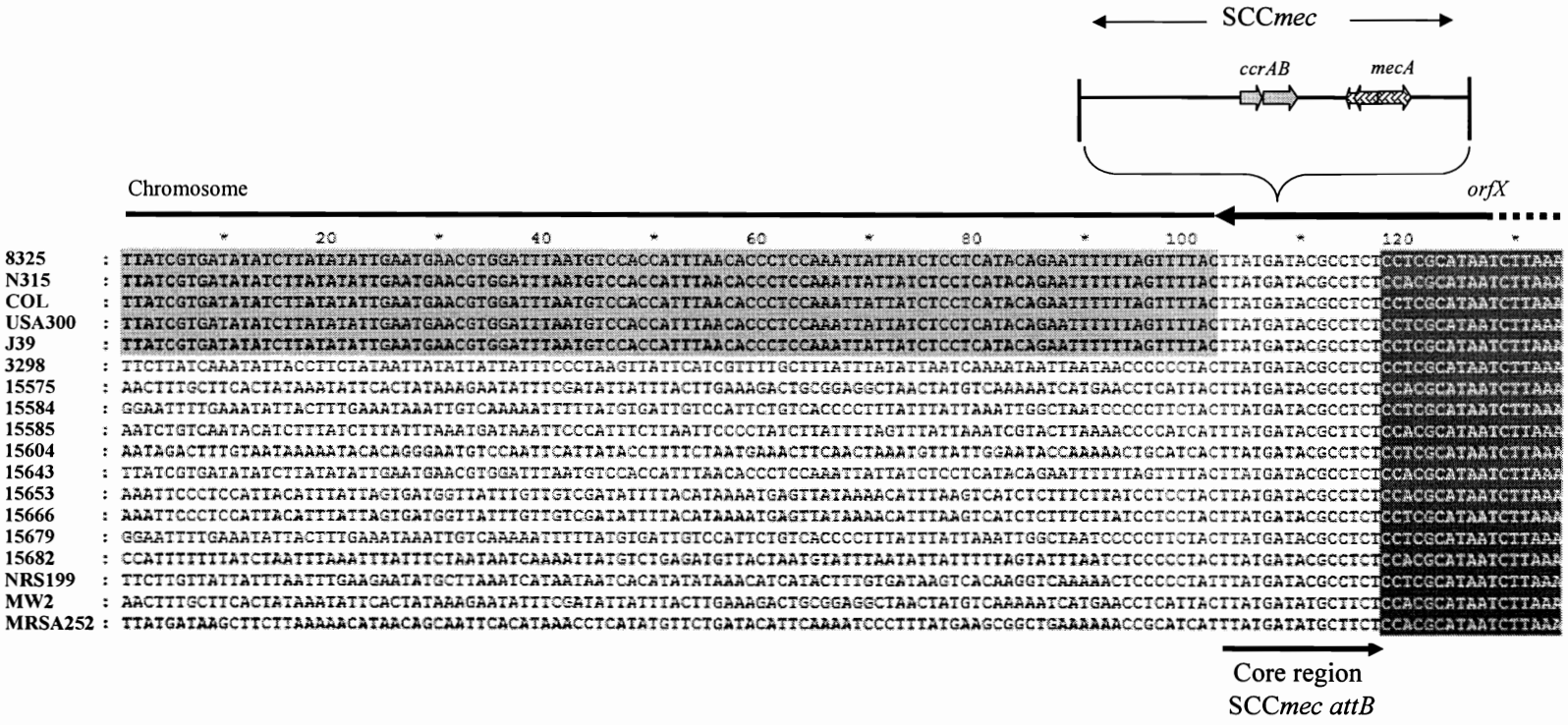

A.
SCCmec IV
MW2
MW0054
seh
MW0048
ccrAB
mecA
MW0025
orfX
15575
B.
orfX
DR
SCCmec
TTATGATACGCCTCT
MW2
seh region
TTATGATATGCTTCT
15575
mecii574
Xsau325
XsauB9-FAM

the IDI-MRSA diagnostic system. The primer and probe binding sites utilized by this system are also present in strain 15575 (and three additional MSSA isolates from this study) (Figure 22B, bottom). Therefore, this system would erroneously categorize *S. aureus* strains with the genetic region found in 15575 as MRSA when they are methicillin-sensitive.

Comparison of *attB* sequences

The CcrAB or CcrC-mediated recombination events that result in the chromosomal integration or excision of SCC*mec* are dependent on the nucleotide composition of the 15 bp core region of the SCC*mec* attachment site, as this is the site of DNA strand exchange. However, it is likely that the recombinases also bind to DNA regions outside of the direct repeat. Therefore, these regions may also be important for integration or excision of SCC*mec*. Figure 23 shows the nucleotide sequence alignment of *attB* and surrounding regions from 18 *S. aureus* strains. *attB* from *S. aureus* 8325 and 15643 represent the nine MSSA isolates with an 8325-like *attB*. SCC*mec* can be excised from strains N315, COL, USA300, and J39 by introducing plasmid-borne *ccrAB*. Following SCC*mec* excision, the *attB* region was PCR amplified from these strains, and the nucleotide sequences are shown in Figure 23. Strain MW2 is not capable of CcrAB-mediated SCC*mec* excision (104). The sequence of *attB* for MW2 and MRSA252 has been inferred from their published genomes and included in Figure 22 (8, 70). The sequence surrounding *attB* from the ten sequenced MSSA isolates are also included in Figure 23. The sequence of *orfX* and the 15 bp direct repeat was highly conserved among all *S. aureus* isolates.

Figure 23. Nucleotide alignment of *attB* and surrounding regions from 18 *S. aureus* strains. The thick black arrow indicates the portion of the *orfX* coding region shown. Thin black line marks the 15 bp core region of the SCC*mec* attachment site. SCC*mec* and its point of chromosomal insertion is depicted above the alignment. The region shaded in light gray represents a conserved sequence present outside of *attB* in all *S. aureus* strains known to undergo CcrAB-mediated SCC*mec* integration or excsision. The conserved sequence of *orfX* is shaded in dark gray and strains are indicated at the left of the figure. The *attB* regions of strains MW2 and MRSA252 were inferred based on the genome sequences of *attL* and *attR* and may not correctly represent the *attB* found if SCC*mec* was excised.


SCCmec
ccrAB
mecA
Chromosome
orfX
20
40
60
80
100
120
8325
N315
COL
USA300
J39
3298
15575
15584
15585
15604
15643
15653
15666
15679
15682
NRS199
MW2
MRSA252
Core region
SCCmec attB

However, the region on the non-*orfX* side of the direct repeat varies greatly. This region is nearly identical in 8325, N315, COL, USA300, and J39 – all the strains in which integration or excision of SCC*mec* has been demonstrated. MW2 does not share this sequence and was not capable of CcrAB-mediated SCC*mec* excision. The ten sequenced MSSA isolates also do not share this region and their ability to acquire SCC*mec* is unknown.

Discussion

The chromosomal integration site for the SCC*mec* element, *attB*, has been studied in MRSA as part of investigations examining the structure and evolution of the various SCC*mec* elements. However, this region in MSSA has been largely neglected. Here we report on the nucleotide sequence surrounding *attB* in a genetically and temporally diverse collection of MSSA isolates. These studies were undertaken to better understand the sequence diversity in this region, as it is possible that differences in *attB* may account for the exclusion of SCC*mec* from certain *S. aureus* lineages. Also, these studies may provide insight into the prevalence of non-*mecA* containing SCC elements. Such elements have been reported, but because of the bias towards studying MRSA, there is uncertainty as to whether these elements are outliers or commonplace among MSSA in the environment.

The *attB* region was not amplified from five of the 42 MSSA isolates due to the lack of primer binding sites or because there was too much intervening sequence for PCR amplification. Nine of the remaining 37 isolates contained an *attB* sequence identical to that of *S. aureus* 8325. SCC*mec* is known to precisely integrate into the 8325 (RN450 –

450M) *attB* sequence as well as to be precisely excised in a CcrAB-dependent manner (15, 103). Therefore *S. aureus* strains containing this *attB* sequence are likely capable of acquiring SCC*mec* in a CcrAB-dependent fashion. Of the nine MSSA isolates with an 8325-like *attB* sequence, three belong to multi-locus sequence type 8 (ST8), one to ST1, and one to ST5. Although multi-locus sequence typing was not performed on strain NRS198, this strain was spa type 1, which corresponds to ST8. STs 1, 5, and 8 are among the prominent MRSA lineages, indicating that isolates with these genetic backgrounds have acquired SCC*mec* in the past (44, 46).

CcrA and CcrB belong to the group of large serine recombinases (80). This is a diverse group of recombinases that are capable of both excision and integration functions. These enzymes catalyze strand exchange between core regions of the attachment sites (15 bp direct repeat), but sequences outside of the core region are usually required for recombinase binding and catalysis (18, 62, 134). CcrAB-mediated SCC*mec* excision has been demonstrated for strains N315 and COL, which indicates that these strains contain functional SCC*mec* attachment sites (74, 80). We have also demonstrated SCC*mec* excision from strain J39 and USA300 (103, 104). However, *ccrAB* present on a high copy plasmid did not lead to the excision of SCC*mec* from strain MW2 (104). The lack of SCC*mec* excision in MW2 was attributed to sequence differences outside of the direct repeat in the left SCC*mec* attachment site (*attL*). Figure 23 shows the sequence alignments of *attB* from these strains as well as the sequence of *attB* regions from MSSA in this study. All of the *S. aureus* strains that are known to be proficient in CcrAB-mediated recombination contain 102 nucleotides of conserved sequence on the non-*orfX* side of the

direct repeat. Beyond these 102 nucleotides the sequences of each strain diverge. MW2 does not contain this conserved sequence, which is a plausible explanation for the lack of SCC*mec* excision in this strain. None of the ten of the MSSA isolates for which long-range PCR and sequencing were used to identify the structure surrounding *attB* contain this conserved sequence (these ten sequences are representative of 28 isolates in this study). It is possible that the absence of this conserved sequence renders *attB* non-functional in these strains. Therefore, these MSSA isolates may not be capable of acquiring SCC*mec* in a CcrAB-dependent manner.

Long range PCR was used to amplify across the chromosomal *attB* site in MSSA isolates for which *attB* could not be amplified by the standard approach. Using this approach, ten unique sequences were amplified from the 28 MSSA isolates. None of these sequences corresponded to the described SCC*mec*-like elements, which suggests that the non-*mecA* containing SCC elements are rare in the environment. It is therefore unlikely that MSSA containing SCC*mec*-like elements somehow acquire the *mec* operon to become MRSA at a high frequency. It seems more likely that the intact SCC*mec* elements are transferred from MRSA to MSSA with functional *attB* sites.

However, these isolates did reveal large amounts of diversity in the chromosomal region surrounding *attB*. The diversity appears to be due to the insertion of DNA into this region of the *S. aureus* chromosome, as these sequences vary in size and were entirely different than that found in strain 8325. This region of the chromosome was found to contain putative transposases, open reading frames with homology to enterotoxin genes, and several different open reading frames with homology to restriction-modification

systems. Restriction-modification systems limit the acquisition of foreign DNA, thereby protecting the cell from carrying potentially harmful genes or preventing the burden of maintaining superfluous genetic regions. Waldron and Lindsay have recently shown that the SauI restriction-modification system is lineage specific and limits the ability of *S. aureus* to acquire foreign DNA by conjugation or transduction (146). It is possible that the restriction-modification homologues found in these MSSA isolates play a role in limiting further acquisition of foreign DNA.

Also found in this region of the chromosome were a number of predicted open reading frames not previously detected in the staphylococci, to which no function could be attributed by homology searches. This finding furthers the idea that this region of the chromosome is a hot spot for the acquisition of foreign DNA. The frequent recombination in this region of the chromosome can be seen by comparing the sequences present in strains MRSA252, 15580, NRS199, and 15682 (Figure 21). Each of these strains share a common genetic region including the open reading frames labeled SAR0088 and SAR0087. However, blocks of sequence have been lost or gained in each of the strains. These events are likely the result of both site-specific and homologous recombination.

One of the more interesting findings from this study is the genetic structure surrounding *attB* in strain 15575. This region is nearly identical to the region outside of SCC*mec* in the genome sequence of MW2 (Figure 22A). Since SCC*mec* in MW2 is not excised in a CcrAB-dependent fashion, it is likely that CcrAB did not catalyze the integration of SCC*mec* in this strain. The genetic structure surrounding *attB* in 15575 reveals another possible means of SCC*mec* integration into MW2. 15575 contains an open

reading frame (MW0048) that is found outside of *attL* in MW2. MW0048 is highly homologous to an open reading frame (MW0025) present within SCC*mec* type IV of MW2. Therefore, if a circular type IV SCC*mec* element was present in a strain with the genetic structure of 15575, the chromosomal insertion of SCC*mec* could occur by homologous recombination between MW0025 present on SCC*mec* and MW0048 present on the chromosome. This integration would result in the chromosomal structure surrounding SCC*mec* in MW2.

The genetic structure of strain 15575 also uncovered a flaw in the IDI-MRSA diagnostic system. According to their website, the IDI-MRSA assay was approved by the FDA for the detection of MRSA in hospital clinical microbiology labs and has appeal because results are obtained directly from clinical specimens in less than two hours (www.cephied.com). In addition, IDI-MRSA is able to distinguish between MRSA and methicillin-resistant coagulase-negative staphylococci (148). However, the primers and probe used by this real-time PCR assay also bind the sequence of the methicillin-sensitive strain 15575. The sequence found in 15575 is present in three additional MSSA isolates from this study for a total of four out of 42 isolates, which would cause a false positive rate of 9.5%. This false positive rate does not take into account the potential for the clonal expansion or potential for outbreaks of one strain in a given hospital. This finding could explain the report of high levels of false-positive test results when the IDI-MRSA assay is used clinically (37).

CHAPTER 6 Phage-based SCC*mec* Transfer

On a molecular level, the mobility of SCC*mec* results from the recombinase genes, *ccrAB* or *ccrC*, contained within SCC*mec*. However, the mobility of SCC*mec* is also evident when examining the staphylococcal population as a whole. SCC*mec* elements of the same type are found in *S. aureus* from diverse genetic backgrounds and *S. aureus* with identical genetic backgrounds have been found to contain different SCC*mec* types (44, 46). It is therefore unlikely that all MRSA have descended from a common ancestor. A more feasible explanation for these observations is that MSSA of different genetic backgrounds have independently acquired SCC*mec* elements on multiple occasions. In these cases, SCC*mec* acquisition would result from the transfer of the element from an MRSA donor cell to an MSSA recipient cell. However, the mechanism by which SCC*mec* is transferred from cell to cell remains unknown.

There are three basic mechanisms of genetic exchange in bacteria: transformation, conjugation, and transduction (135). Transformation, or the uptake of DNA from the environment by competent bacteria, is not a known means of genetic exchange in staphylococci and therefore not a likely mechanism by which SCC*mec* is transferred. Conjugal transfer is a known means of genetic exchange in *S. aureus*, and is the only known mechanism by which genes are transferred between staphylococcal species (3). Transfer of SCC*mec* by conjugation would depend on the element's ability to integrate

onto a conjugative or mobilizable plasmid. This may occur by recombination between identical insertion sequences (IS431) present on SCC*mec* and most staphylococcal conjugative plasmids (64, 101). However, a conjugative plasmid containing SCC*mec* has never been found. Transduction is a known means of intraspecies genetic exchange in *S. aureus*, and a possible mechanism by which SCC*mec* is transferred.

Bacteriophage are responsible for the transfer of important virulence determinants in *S. aureus*. The genes encoding the Panton-Valentine leukocidin (PVL) are contained on a staphylococcal phage and *S. aureus* lysogenized with this phage are associated with severe disease in the community (8, 88). In addition, toxins and other virulence determinants contained on staphylococcal pathogenicity islands (SaPIs) are transferred at high frequency by staphylococcal phages (107). SaPIs each interact with a specific phage and SaPI transduction is highly specialized. These elements are chromosomally excised, replicated, and packaged in smaller phage heads in a process that interferes with the production of normal phage particles (89, 144). In addition, some staphylococcal phages are capable of the generalized transduction of plasmid or chromosomal DNA (109).

Lysogenic phage are widespread in *S. aureus* with each of the genome sequenced *S. aureus* strains containing at least one prophage (8, 39, 57, 70, 84). The genomes of these phages are approximately 40 kb in size. Because of their size, these phages are not likely to be involved in the transfer of the larger SCC*mec* type II (52-58 kb) or SCC*mec* type III (67 kb) elements (74). SCC*mec* type IV is a smaller element (20-25 kb) that may be transferred by phage in a specialized or generalized manner (91, 110). Here we attempt the

transfer of SCC*mec* type IV using the generalized transducing phage 80α and prophage contained within various *S. aureus* strains.

S. aureus strains MW2, J35, and J52 were used to examine the potential for SCC*mec* transfer. These strains are from different genetic backgrounds (ST1, ST12, and ST59, respectively) and all contain SCC*mec* type IV. MW2, J35, and J52 were first tested for the ability to be productively infected by the phage 80α. Phage 80α is capable of generalized transduction of plasmid and chromosomal DNA and thus may also transfer SCC*mec* at a low frequency (141). Spotting 10 µl of a 10^{10} pfu/ml 80α lysate onto confluent bacterial plates leads to visible bacterial lysis if the strains are productively infected. None of the three strains exhibited the clearing characteristic of bacterial lysis and therefore were not productively infected by 80α. However, plasmid DNA had been successfully transduced into each of these strains using phage 80α. This suggests that 80α was capable of binding to these strains and the plasmid DNA was not restricted once inside the cell. The lack of a productive infection may be due to the exclusion of 80α by prophage resident in these *S. aureus* strains.

The genome of strain MW2 has been sequenced and revealed the presence of two prophage, φSa2mw and φSa3mw (8). φSa2mw is a 45,919 bp phage that contains the genes for Panton-Valentine leukocidin. φSa3mw is a 42,615 bp phage that carries the genes for enterotoxin G and K. It is not known if strains J35 and J52 contain prophage, although the exclusion of phage 80α suggests that these strains are lysogenized. Lysogenic phage enter the lytic cycle in response to DNA damage (135). The addition of the DNA damaging agent, such as mitomycin C, to a culture of lysogenic bacteria will lead to

repeated rounds of infection and phage-mediated bacterial lysis, and the eventual clearing of the culture. Treating cultures of strains MW2, J35, and J52 with mitomycin C did not lead to the visible clearing of the culture during continued incubation at 37°C with shaking. Even when the cultures were stored overnight at 4°C after mitomycin C addition, there was no visible bacterial lysis. Similarly, when UV light was used to induce phage in these strains, no visible bacterial lysis was observed.

Although there was no visible bacterial lysis, it is possible that there are still lytic phage particles present, but infection and bacterial lysis was not robust enough to cause clearing of the culture. To test this hypothesis, transducing lysates were prepared from MW2, J35, and J52 after induction with mitomycin C. Strain RN4220 was then infected with each of the three transducing lysates. RN4220 is a restriction-deficient *S. aureus* strain that lacks SCC*mec* and is the propagating strain for several *S. aureus* bacteriophages. Infection of RN4220 with lysates from J35 and J52 did not lead to plaque formation. However, the MW2 lysate did produce plaques on RN4220, indicating that this culture contains phage capable of infecting RN4220. The titer of this MW2 lysate was approximately 10^5 pfu/ml and these plaques were very small and turbid in appearance. To test if this phage is capable of generalized transduction, transfer of the high copy plasmid pRN5543 was attempted. Generalized transduction of this plasmid using phage 80α occurs at a frequency of 1.7×10^{-7}, and so demonstrating generalized transduction requires a phage titer of 10^8 to 10^9 pfu/ml. In an effort to achieve these concentrations of phage, 1 L cultures of MW2 containing pRN5543 were grown, induced with mitomycin C, and the transducing lysate was precipitated using polyethylene glycol 8000 (PEG) and resuspended

in 10 ml of phage buffer. The highest titer achieved using this approach was 7 x 10^7 pfu/ml. When RN4220 was infected with this lysate, no pRN5543 transductants were found. The lack of transductants does not eliminate the possibility that this phage is capable of generalized transduction because the phage titers were too low to be conclusive.

Although generalized transduction using the MW2 lysate was not demonstrated, it is possible that SCC*mec* is transduced by this phage at a higher frequency. To test this hypothesis, *mecA* was insertionally inactivated with *tetM* in MW2 to provide a more reliable selectable marker for transduction. A 1 L culture of MW2 *mecA::tetM* was grown, induced with mitomycin C, and the lysate was PEG precipitated and resuspended in 10 ml of phage buffer. When RN4220 was infected with this lysate, no tetracycline resistant transductants were recovered, indicating that SCC*mec* was not transduced into RN4220.

We have found that non-phage DNA, such as plasmids, can be transduced into strains that are not productively infected by the transducing phage. Therefore transfer of SCC*mec* to RN4220 by transducing lysates prepared from strains J35 and J52 may be possible. The selectable marker, *tetM*, was introduced into these strains by the insertional inactivation of *mecA*. Transducing lysates of J35 *mecA::tetM* and J52 *mecA::tetM* were prepared in the same manner as that of MW2 *mecA::tetM*. When RN4220 was infected with either of these lysates, no tetracycline resistant transductants were recovered. This indicates that SCC*mec* was not transduced into RN4220 under these circumstances.

The fact that none of the strains examined in this study were productively infected with the generalized transducing phage 80α prevented the demonstration of SCC*mec* transfer by exogenous bacteriophage. It is possible that these *S. aureus* strains are

productively infected by other generalized transducing phages, however those experiments were not attempted. MW2 is lysogenic for a phage that is also capable of infecting *S. aureus* RN4220. Induction of MW2 with mitomycin C or UV light did not produce a lysate of sufficient phage concentration to truly evaluate this phage's capability for generalized transduction of plasmid DNA. However, all attempts to transfer pRN5543 were unsuccessful. Given the precedent for the high frequency transfer of other staphylococcal genomic islands (SaPIs) by bacteriophage, it was thought that the transfer of SCC*mec* occurs in a similar manner and would not require the high phage titers necessary to demonstrate generalized transduction (107). However, this was not the case as attempts to transfer SCC*mec* following the induction of prophage in strains MW2 *mecA::tetM*, J35 *mecA::tetM*, and J52 *mecA::tetM* were not successful. Although we cannot exclude the possibility that SCC*mec* is transduced in a generalized manner by lysogenic phage in these strains, we can exclude the possibility that SCC*mec* is transduced at a high frequency by lysogenic phage residing in these strains.

CHAPTER 7 Conclusions

Staphylococcus aureus is among the most frequent causes of bacterial infections in this country and, as a result, places a tremendous burden on our healthcare system. *S. aureus* is a notorious cause of nosocomial infections but also a frequent cause of disease in healthy individuals in the community (33, 38, 100, 122). The pathogenic abilities of *S. aureus* are compounded by the bacterium's ability to resist chemotherapy. Through mutation or gene acquisition events, *S. aureus* has developed resistance to every major class of antibiotic, making the treatment of these infections particularly challenging (23). Infections caused by methicillin-resistant *S. aureus* (MRSA) are among the most problematic. MRSA are resistant to the entire class of β-lactam antibiotics, which eliminates a group of effective, bactericidal agents from the physician's treatment options. In addition, the element on which *mecA* resides, SCC*mec*, may also carry other resistance determinants, which further limits the treatment options (74). Thereby, the acquisition of one element, SCC*mec*, may render the bacterium resistant to several important classes of antibiotics. The rates of MRSA disease continue to increase and infections caused by MRSA outnumber those caused by methicillin-sensitive *S. aureus* (MSSA) in some settings (1, 100). Because the same SCC*mec* elements are found in unrelated *S. aureus* lineages, and different SCC*mec* elements can be found in the same *S. aureus* lineage,

SCC*mec* is believed to have been acquired by different MSSA isolates on more than one occasion (44, 46, 118). The conversion of successful MSSA lineages to MRSA upon the acquisition of SCC*mec* may play an important role in the increasing incidence of MRSA disease. The studies undertaken in this dissertation aim to provide a better understanding of the factors associated with SCC*mec* mobility and transfer.

These investigations began by examining the CcrAB-mediated excision of the type IV SCC*mec* element. The type IV SCC*mec* element was associated with an emerging subset of MRSA–those that caused disease in the community. *S. aureus* containing this element were first isolated in the late 1990s, but their rapid spread was thought to be aided by this element's mobility (19, 91, 110). We found that CcrAB from ten strains carrying SCC*mec* type IV were functional recombinases despite their amino acid variation. In addition, we demonstrated the site-specific chromosomal excision of SCC*mec* type IV. CcrAB had previously been shown to catalyze the excision of SCC*mec* types I, II, and III, and CcrC was found to catalyze the excision of SCC*mec* type V (74, 76, 80). These results show that all varieties of SCC*mec* are mobile genetic elements and the excision and integration functions of CcrAB and CcrC may be important factors in the transfer of these elements.

We also provide evidence that the type IV SCC*mec* element could not be excised from MRSA belonging to the ST1 lineage, including MW2. These isolates each contained a common genetic region outside of the left SCC*mec* attachment site. This region included an open reading frame (MW0048 as annotated on the MW2 genome) with high homology to an open reading frame contained within SCC*mec* type IV (MW0025) (8). In addition, a

putative truncated transposase, staphylococcal enterotoxin H, and a truncated portion of staphylococcal enterotoxin O were also present. The presence of a putative transposase in this region led us to suggest that this region of the chromosome was a mobile element whose insertion disrupted CcrAB-mediated SCC*mec* excision and therefore stabilized the integration of SCC*mec* in these strains. We later found an MSSA isolate that carried the genetic region present outside of SCC*mec* in the ST1 strains, but did not contain the SCC*mec* element. The presence of this genetic region in MSSA provided a better rationale for the mechanism by which SCC*mec* was acquired by this lineage. An extra-chromosomal, circular type IV SCC*mec* element present in an MSSA with this genetic structure could insert into the chromosome by homologous recombination between the chromosomal MW0048 and the SCC*mec* encoded MW0025. SCC*mec* insertion by homologous recombination would not generate the hybrid SCC*mec* attachment sites, *attL* and *attR*, and therefore CcrAB-mediated excision could not occur.

Strain USA300 contains type IV SCC*mec* and ACME integrated adjacent to one another in the SCC*mec* *attB* (39). SCC*mec* and ACME are site specifically excised from the chromosome independent of one another in a CcrAB-dependent manner. Upon excision, two circular, extra-chromosomal elements were detected. The presence of two extra-chromosomal elements may indicate that SCC*mec* and ACME are not transferred together. Since ACME does not encode its own recombinase, it is dependent on *ccrAB* for chromosomal excision and integration. Therefore, ACME is likely to only be transferred among strains containing *ccrAB* and SCC*mec*, and so may represent an MRSA specific virulence determinant.

During passage to vancomycin resistance, three MRSA became methicillin-sensitive. In each case, this was due to the loss of a large portion of SCC*mec*, including *mecA* and *ccrAB*. In one case (5836VR), CcrAB-mediated SCC*mec* excision was responsible for the lost DNA. However, 3130VR and VP32 deleted portions of SCC*mec* in a process that appears to involve IS431-mediated recombination. IS431 is found on all SCC*mec* elements in proximity to *mecA*. Loss of portions of SCC*mec* in a manner involving IS431 may provide insight into a potential mechanism for SCC*mec* transfer as the staphylococcal conjugative plasmids also contain copies of IS431. These plasmids appear to have been formed in a cassette-like fashion, with each cassette flanked by copies of IS431 (3, 31). If SCC*mec* could be removed from the chromosome by an IS431 transposition/recombination event, it is possible that it could then insert into a conjugative plasmid and be mobilized to a recipient MSSA. These data suggest that chromosomal integration and excision of SCC*mec* was not universally mediated by CcrAB. Instead, it appears that SCC*mec* type IV entered the chromosome of the *S. aureus* ST1 lineage by homologous recombination, not CcrAB-mediated integration. Also, IS431 was shown to play a role in the deletion of the majority of SCC*mec* during passage on increasing concentrations of vancomycin, not CcrAB.

We have also provided evidence that the region surrounding the SCC*mec* attachment site in MSSA is highly variable and appears to be a hot spot for the insertion of foreign DNA. This region was found to contain putative transposases, enterotoxins, and restriction-modification systems, along with predicted open reading frames that have not previously been reported in *Staphylococcus*. The various types of SCC*mec* elements also

appear to be an insertion point for foreign DNA, as many of them contain IS elements, transposons, and integrated plasmids. The majority of the DNA sequence of SCC*mec* elements is composed of open reading frames with little or no homology to known genes, which has prompted some to refer to these regions as junkyard DNA (70, 74, 76, 80, 91). These findings suggest that this region of the chromosome, in both MSSA and MRSA, is prone to frequent recombination and is therefore rapidly changing. The rapidly changing nature of this region may offer an explanation to the frequent detection of structural variants of the known SCC*mec* types (40, 130).

Not all *S. aureus* lineages have shown the ability to acquire SCC*mec* as MRSA have only been found in 38 of a total of 162 *S. aureus* MLST types (46). The limited distribution of SCC*mec* may be due to several factors. It may be that SCC*mec* transfer is relatively rare in the environment and it has not yet been transferred into all lineages. It may also be that differential restriction-modification systems prevent certain lineages from acquiring DNA from other lineages. It is also possible that the SCC*mec* attachment site is not intact in all MSSA lineages. To address the latter issue, *attB* from 37 MSSA isolates was examined. *attB* sequences from *S. aureus* strains known to be proficient in CcrAB-mediated SCC*mec* integration or excision were highly conserved. The conserved region in these strains consists of the *orfX* coding region, the 15 bp core region of the SCC*mec* attachment site, and 102 bp of sequence following the direct repeat. This conserved *attB* sequence was present in nine of the 37 MSSA isolates. The remaining 28 isolates contained the conserved *orfX* sequence and the conserved core region, but did not contain the 102 bp of conserved sequence following the direct repeat. CcrA and CcrB are serine

recombinases. Other members of this family include Sin, a resolvase from *S. aureus* plasmid pI9789, Tn3 resolvase, and the Streptomyces phage C31 integrase. Each of these recombinases is dependent upon DNA sequence outside of the core sequence where strand exchange occurs (18, 120, 121, 142). Therefore, it is likely that portions of the 102 bp region are essential for CcrAB binding or catalysis, and the absence of this region in some of the MSSA may explain the limited distribution of MRSA among all *S. aureus* lineages.

The prevalence of MRSA has forced a shift in antibiotic therapy towards drugs effective against MRSA, such as vancomycin. As a result of the increased use of vancomycin, vancomycin-resistant *S. aureus* (VRSA) and vancomycin intermediate sensitivity *S. aureus* (VISA) have arisen. These isolates become resistant either by acquiring the *vanA* genes or by mutational events that lead to a thickened cell wall (20-22, 34-36, 69). Three MRSA strains were passaged to vancomycin resistance mediated by the latter mechanism. In each case, the vancomycin-resistant derivatives had a growth defect as compared to the parent. This growth defect was partially compensated for by the deletion of *mecA*, as each vancomycin-resistant, methicillin-sensitive derivative was able to out compete its vancomycin-resistant, methicillin-resistant counterpart. Fox, et al. have shown that treatment of infections by MRSA expressing *vanA*-mediated vancomycin resistance with a combination of a β-lactam and vancomycin was effective in a rabbit model of endocarditis (53). This finding is likely due to the inability of PBP2a to effectively cross link D-ala-D-lac containing stem peptide. These data suggest that when *S. aureus* expresses high level resistance to both vancomycin and β-lactams, there is a

severe defect in growth. This growth defect may render these strains sufficiently less virulent and prevent them from sustaining an infection in vivo.

The deletion of *mecA* in vancomycin-resistant strains provided a fitness advantage over isogenic vancomycin-resistant strains containing *mecA*. *mecA* also caused a fitness cost in homotypic methicillin-resistant strains that were not resistant to vancomycin. Other groups have also found a fitness cost associated with methicillin resistance and that unregulated *mecA* is not well tolerated by naïve hosts (43, 82). These findings may reveal a partial barrier to *mecA* acquisition. *mecA* is likely to be expressed when it is first introduced into a naïve cell, unless the β-lactamase repressor, BlaI, is present in that cell. If *mecA* expression is not well tolerated, the gene may be mutated or deleted, thereby preventing the conversion of an MSSA to MRSA. If *mecA* is transferred by integrating onto a conjugative plasmid, it will likely be present in more than one copy per cell. This increase in copy number may increase the burden placed on the cell by *mecA*. This problem could be circumvented through mutation or deletion of *mecA*, or by the integration of *mecA* into the chromosome where its copy number would be fewer. This may explain why SCC*mec* or *mecA* have never been found on a conjugative plasmid.

Finally, we have also attempted to transfer SCC*mec* type IV using phage. Although unsuccessful, these experiments did improve our understanding of SCC*mec* transfer. It is now clear that the SCC*mec* element present in MW2 is not transferred in a specialized manner by the lysogenic phage present in MW2. This is in contrast to the SaPI elements, which are transferred at a high frequency by specific phage. It also seems that

the limited host range of the *S. aureus* phages investigated poses an added challenge to the demonstration of phage-based SCC*mec* transfer.

These studies further our understanding of the factors associated with the mobility of SCC*mec* and suggest a possible limitation to the spread of these elements. Increased knowledge of the means by which SCC*mec* is transferred may provide insight into the epidemiology of MRSA disease and may lead to better strategies for MRSA control.

References

References

1. 2004. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. Am J Infect Control **32:**470-85.

2. **Adhikari, R. P., G. C. Scales, K. Kobayashi, J. M. Smith, B. Berger-Bachi, and G. M. Cook.** 2004. Vancomycin-induced deletion of the methicillin resistance gene mecA in Staphylococcus aureus. J Antimicrob Chemother **54:**360-3.

3. **Archer, G. L., J. P. Coughter, and J. L. Johnston.** 1986. Plasmid-encoded trimethoprim resistance in staphylococci. Antimicrob Agents Chemother **29:**733-40.

4. **Archer, G. L., D. M. Niemeyer, J. A. Thanassi, and M. J. Pucci.** 1994. Dissemination among staphylococci of DNA sequences associated with methicillin resistance. Antimicrob Agents Chemother **38:**447-54.

5. **Arthur, M., F. Depardieu, L. Cabanie, P. Reynolds, and P. Courvalin.** 1998. Requirement of the VanY and VanX D,D-peptidases for glycopeptide resistance in enterococci. Mol Microbiol **30:**819-30.

6. **Arthur, M., P. Reynolds, and P. Courvalin.** 1996. Glycopeptide resistance in enterococci. Trends Microbiol **4:**401-7.

7. **Arvidson, S.** 2004. Virulence Gene Regulation and Their Role in Pathogenesis of Disease. *In* D. a. H. Ala'aldeen, K. (ed.), *Staphylococcus aureus* Molecular and Clinical Aspects. Horwood Publishing Limited, West Sussex, England.

8. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu.** 2002. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet **359:**1819-27.

9. **Baba, T., Takeuchi, F., Kuroda, M., Ito, T., Yuzawa, H., and Hiramatsu, K.** 2004. The *Staphylococcus aureus* Genome. *In* D. a. H. Ala'aldeen, K. (ed.), *Staphylococcus aureus* Molecular and Clinical Aspects. Horwood Publishing Limited, West Sussex, England.

10. **Balaban, N., and A. Rasooly.** 2000. Staphylococcal enterotoxins. Int J Food Microbiol **61:**1-10.

11. **Bannerman, T. L., G. A. Hancock, F. C. Tenover, and J. M. Miller.** 1995. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of Staphylococcus aureus. J Clin Microbiol **33:**551-5.

12. **Barberis-Maino, L., B. Berger-Bachi, H. Weber, W. D. Beck, and F. H. Kayser.** 1987. IS431, a staphylococcal insertion sequence-like element related to IS26 from Proteus vulgaris. Gene **59:**107-13.

13. **Barrett, D., C. Leimkuhler, L. Chen, D. Walker, D. Kahne, and S. Walker.** 2005. Kinetic characterization of the glycosyltransferase module of Staphylococcus aureus PBP2. J Bacteriol **187:**2215-7.

14. **Berger-Bachi, B.** 1994. Expression of resistance to methicillin. Trends Microbiol **2:**389-93.

15. **Berger-Bachi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by Tn551. J Bacteriol **154:**479-87.

16. **Bugg, T. D., and P. E. Brandish.** 1994. From peptidoglycan to glycoproteins: common features of lipid-linked oligosaccharide biosynthesis. FEMS Microbiol Lett **119:**255-62.

17. **Bugg, T. D., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408-15.

18. **Burke, M. E., P. H. Arnold, J. He, S. V. Wenwieser, S. J. Rowland, M. R. Boocock, and W. M. Stark.** 2004. Activating mutations of Tn3 resolvase marking interfaces important in recombination catalysis and its regulation. Mol Microbiol **51:**937-48.

19. **CDC.** 1997-1999. Four Pediatric Deaths from community-acquired meticillin-resistant *Staphylococcus aureus*: Minnesota and North Dakota. MMWR Morbidity and Mortality Weekly Report **48:**707-710.

20. **CDC, C. f. D. C. a. P.** 2002. *Staphylococcus aureus* resistant to vancomycin - United States, 2002. Morb Mortal Wkly Rep **51:**565-7.

21. **CDC, C. f. D. C. a. P.** 2004. Vancomycin-resistant *Staphylococcus aureus* - New York, 2004. Morb. Mortal. Wkly. Rep. **53:**322-3.

22. **CDC, C. f. D. C. a. P.** 2002. Vancomycin-resistant Staphylococcus aureus - Pennsylvania, 2002. Morb. Mortal. Wkly. Rep. **51:**902.

23. **Chambers, H. F.** 2004. Antimicrobial Resistance and Therapy. *In* D. a. H. Ala'aldeen, K. (ed.), *Staphylococcus aureus* Molecular and Clinical Aspects. Horwood Publishing Limited, West Sussex, England.

24. **Chambers, H. F.** 2005. Community-associated MRSA--resistance and virulence converge. N Engl J Med **352:**1485-7.

25. **Chambers, H. F.** 1997. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev **10:**781-91.

26. **Chambers, H. F.** 2003. Solving staphylococcal resistance to beta-lactams. Trends Microbiol **11:**145-8.

27. **Charlebois, E. D., F. Perdreau-Remington, B. Kreiswirth, D. R. Bangsberg, D. Ciccarone, B. A. Diep, V. L. Ng, K. Chansky, B. R. Edlin, and H. F. Chambers.** 2004. Origins of community strains of methicillin-resistant Staphylococcus aureus. Clin Infect Dis **39:**47-54.

28. **Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang, and R. P. Novick.** 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl Environ Microbiol **70:**6076-85.

29. **Chatterjee, A. N.** 1969. Use of bacteriophage-resistant mutants to study the nature of the bacteriophage receptor site of Staphylococcus aureus. J Bacteriol **98:**519-27.

30. **Chuard, C., P. E. Vaudaux, R. A. Proctor, and D. P. Lew.** 1997. Decreased susceptibility to antibiotic killing of a stable small colony variant of Staphylococcus aureus in fluid phase and on fibronectin-coated surfaces. J Antimicrob Chemother **39:**603-8.

31. **Climo, M. W., V. K. Sharma, and G. L. Archer.** 1996. Identification and characterization of the origin of conjugative transfer (oriT) and a gene (nes) encoding a single-stranded endonuclease on the staphylococcal plasmid pGO1. J Bacteriol **178:**4975-83.

32. **Courvalin, P.** 2006. Vancomycin resistance in gram-positive cocci. Clin Infect Dis **42 Suppl 1:**S25-34.

33. **Crum, N. F., R. U. Lee, S. A. Thornton, O. C. Stine, M. R. Wallace, C. Barrozo, A. Keefer-Norris, S. Judd, and K. L. Russell.** 2006. Fifteen-year study of the changing epidemiology of methicillin-resistant Staphylococcus aureus. Am J Med **119:**943-51.

34. **Cui, L., A. Iwamoto, J. Q. Lian, H. M. Neoh, T. Maruyama, Y. Horikawa, and K. Hiramatsu.** 2006. Novel mechanism of antibiotic resistance originating in

vancomycin-intermediate Staphylococcus aureus. Antimicrob Agents Chemother **50:**428-38.

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

36. **Cui, L., H. Murakami, K. Kuwahara-Arai, H. Hanaki, and K. Hiramatsu.** 2000. Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by Staphylococcus aureus Mu50. Antimicrob Agents Chemother **44:**2276-85.

37. **Desjardins, M., C. Guibord, B. Lalonde, B. Toye, and K. Ramotar.** 2006. Evaluation of the IDI-MRSA assay for detection of methicillin-resistant staphylococcus aureus from nasal and rectal specimens pooled in a selective broth. J Clin Microbiol **44:**1219-23.

38. **Diekema, D. J., M. A. Pfaller, F. J. Schmitz, J. Smayevsky, J. Bell, R. N. Jones, and M. Beach.** 2001. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin Infect Dis **32 Suppl 2:**S114-32.

39. **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. Lancet **367:**731-9.

40. **Donnio, P. Y., D. C. Oliveira, N. A. Faria, N. Wilhelm, A. Le Coustumier, and H. de Lencastre.** 2005. Partial excision of the chromosomal cassette containing the methicillin resistance determinant results in methicillin-susceptible Staphylococcus aureus. J Clin Microbiol **43:**4191-3.

41. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of Staphylococcus aureus genes regulated by the agr and/or sarA loci. J Bacteriol **183:**7341-53.

42. **Eggers, C. H., and D. S. Samuels.** 1999. Molecular evidence for a new bacteriophage of Borrelia burgdorferi. J Bacteriol **181:**7308-13.

43. **Ender, M., N. McCallum, R. Adhikari, and B. Berger-Bachi.** 2004. Fitness cost of SCCmec and methicillin resistance levels in Staphylococcus aureus. Antimicrob Agents Chemother **48:**2295-7.

44. **Enright, M. C.** 2003. The evolution of a resistant pathogen--the case of MRSA. Curr Opin Pharmacol **3:**474-9.

45. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. J Clin Microbiol **38:**1008-15.

46. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc Natl Acad Sci U S A **99:**7687-92.

47. **F. M. Ausubel, R. B., R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 2002. The Polymerase Chain Reaction, p. 15-1 to 15-40, Short Protocols in Molecular Biology, Fifth ed, vol. 2. John Wiley and Sons, Inc., USA.

48. **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:**3307-16.

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

50. **Finan, J. E., A. E. Rosato, T. M. Dickinson, D. Ko, and G. L. Archer.** 2002. Conversion of oxacillin-resistant staphylococci from heterotypic to homotypic resistance expression. Antimicrob Agents Chemother **46:**24-30.

51. **Fitzpatrick, F., H. Humphreys, and J. P. O'Gara.** 2005. The genetics of staphylococcal biofilm formation--will a greater understanding of pathogenesis lead to better management of device-related infection? Clin Microbiol Infect **11:**967-73.

52. **Foster, T. J., and M. Hook.** 1998. Surface protein adhesins of Staphylococcus aureus. Trends Microbiol **6:**484-8.

53. **Fox, P. M., R. J. Lampen, K. S. Stumpf, G. L. Archer, and M. W. Climo.** 2006. Successful therapy of experimental endocarditis caused by vancomycin-resistant

Staphylococcus aureus with a combination of vancomycin and beta-lactam antibiotics. Antimicrob Agents Chemother **50:**2951-6.

54. **Frere, J. M.** 1995. Beta-lactamases and bacterial resistance to antibiotics. Mol Microbiol **16:**385-95.

55. **Gally, D., and A. R. Archibald.** 1993. Cell wall assembly in Staphylococcus aureus: proposed absence of secondary crosslinking reactions. J Gen Microbiol **139:**1907-13.

56. **Garrity, G. M., Bell J. A., and Lilburn, T. G.** 2004. Taxonomic Outline of the Prokaryotes Bergey's Manual of Systematic Bacteriology, Second Edition. Bergey's Manual Trust.

57. **Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser.** 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. J Bacteriol **187:**2426-38.

58. **Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, N. Brousse, D. Floret, and J. Etienne.** 2002. Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet **359:**753-9.

59. **Goffin, C., and J. M. Ghuysen.** 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol Mol Biol Rev **62:**1079-93.

60. **Gregory, P. D., R. A. Lewis, S. P. Curnock, and K. G. Dyke.** 1997. Studies of the repressor (BlaI) of beta-lactamase synthesis in Staphylococcus aureus. Mol Microbiol **24:**1025-37.

61. **Grindley, N. D.** 1993. Analysis of a nucleoprotein complex: the synaptosome of gamma delta resolvase. Science **262:**738-40.

62. **Groth, A. C., and M. P. Calos.** 2004. Phage integrases: biology and applications. J Mol Biol **335:**667-78.

63. **Gurtler, V., and B. C. Mayall.** 2001. Genetic transfer and genome evolution in MRSA. Microbiology **147:**3195-7.

64. **Hanssen, A. M., and J. U. Ericson Sollid.** 2006. SCCmec in staphylococci: genes on the move. FEMS Immunol Med Microbiol **46:**8-20.

65. **Hanssen, A. M., G. Kjeldsen, and J. U. Sollid.** 2004. Local variants of Staphylococcal cassette chromosome mec in sporadic methicillin-resistant Staphylococcus aureus and methicillin-resistant coagulase-negative Staphylococci: evidence of horizontal gene transfer? Antimicrob Agents Chemother **48:**285-96.

66. **Hartleib, J., N. Kohler, R. B. Dickinson, G. S. Chhatwal, J. J. Sixma, O. M. Hartford, T. J. Foster, G. Peters, B. E. Kehrel, and M. Herrmann.** 2000. Protein A is the von Willebrand factor binding protein on Staphylococcus aureus. Blood **96:**2149-56.

67. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in Staphylococcus aureus. J Bacteriol **158:**513-6.

68. **Hauck, C. R., and K. Ohlsen.** 2006. Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by Staphylococcus aureus. Curr Opin Microbiol **9:**5-11.

69. **Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover.** 1997. Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother **40:**135-6.

70. **Holden, M. T., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill.** 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:**9786-91.

71. **Hovis, K. M., J. V. McDowell, L. Griffin, and R. T. Marconi.** 2004. Identification and characterization of a linear-plasmid-encoded factor H-binding protein (FhbA) of the relapsing fever spirochete Borrelia hermsii. J Bacteriol **186:**2612-8.

72. **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:**1875-84.

73. **Iandolo, J. J., V. Worrell, K. H. Groicher, Y. Qian, R. Tian, S. Kenton, A. Dorman, H. Ji, S. Lin, P. Loh, S. Qi, H. Zhu, and B. A. Roe.** 2002. Comparative analysis of the genomes of the temperate bacteriophages phi 11, phi 12 and phi 13 of Staphylococcus aureus 8325. Gene **289:**109-18.

74. **Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu.** 2001. Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother **45:**1323-36.

75. **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:**1449-58.

76. **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:**2637-51.

77. **Joseph F. John, J.** 2004. Staphylococcal Infection: Emergin Clinical Syndromes. *In* D. A. a. a. K. Hiramatsu (ed.), *Staphylococcus aureus* Molecular and Clinical Aspects. Horwood Publishing Limited, West Sussex, England.

78. **Kaneko, J., and Y. Kamio.** 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem **68:**981-1003.

79. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2001. Genetic organization of the chromosome region surrounding mecA in clinical staphylococcal strains: role of IS431-mediated mecI deletion in expression of resistance in mecA-carrying, low-level methicillin-resistant Staphylococcus haemolyticus. Antimicrob Agents Chemother **45:**1955-63.

80. **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:**1549-55.

81. **Katayama, Y., F. Takeuchi, T. Ito, X. X. Ma, Y. Ui-Mizutani, I. Kobayashi, and K. Hiramatsu.** 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:**2711-22.

82. **Katayama, Y., H. Z. Zhang, D. Hong, and H. F. Chambers.** 2003. Jumping the barrier to beta-lactam resistance in Staphylococcus aureus. J Bacteriol **185:**5465-72.

83. **Kluytmans, J., A. van Belkum, and H. Verbrugh.** 1997. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev **10:**505-20.

84. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet **357:**1225-40.

85. **Kwan, T., J. Liu, M. DuBow, P. Gros, and J. Pelletier.** 2005. The complete genomes and proteomes of 27 Staphylococcus aureus bacteriophages. Proc Natl Acad Sci U S A **102:**5174-9.

86. **Lee, J. C., and Bohach, G. A.** 2004. Pathogenesis of Disease. *In* D. A. a. a. K. Hiramatsu (ed.), *Staphylococcus aureus* Molecular and Clinical Aspects. Horwood Publishing Limited, West Sussex, England.

87. **Lim, D., and N. C. Strynadka.** 2002. Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant Staphylococcus aureus. Nat Struct Biol **9:**870-6.

88. **Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne.** 1999. Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Clin Infect Dis **29:**1128-32.

89. **Lindsay, J. A., A. Ruzin, H. F. Ross, N. Kurepina, and R. P. Novick.** 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in Staphylococcus aureus. Mol Microbiol **29:**527-43.

90. **Luong, T. T., S. Ouyang, K. Bush, and C. Y. Lee.** 2002. Type 1 capsule genes of Staphylococcus aureus are carried in a staphylococcal cassette chromosome genetic element. J Bacteriol **184:**3623-9.

91. **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:**1147-52.

92. **Mack, D., P. Becker, I. Chatterjee, S. Dobinsky, J. K. Knobloch, G. Peters, H. Rohde, and M. Herrmann.** 2004. Mechanisms of biofilm formation in Staphylococcus epidermidis and Staphylococcus aureus: functional molecules, regulatory circuits, and adaptive responses. Int J Med Microbiol **294:**203-12.

93. **Mallorqui-Fernandez, G., A. Marrero, S. Garcia-Pique, R. Garcia-Castellanos, and F. X. Gomis-Ruth.** 2004. Staphylococcal methicillin resistance: fine focus on folds and functions. FEMS Microbiol Lett **235:**1-8.

94. **Matsuhashi, M., Dietrich, C. P., and Strominger, J. L.** 1967. Biosynthesis of the Peptidoglycan of Bacterial Cell Walls. III. The Role of Soluble Ribonucleic Acid and of Lipid Intermediates in Glycine Incorportation in *Staphylococcus aureus*. Journal of Biologica Chemistry **242:**3191-3206.

95. **McAleese, F., S. W. Wu, K. Sieradzki, P. Dunman, E. Murphy, S. Projan, and A. Tomasz.** 2006. Overexpression of genes of the cell wall stimulon in clinical isolates of Staphylococcus aureus exhibiting vancomycin-intermediate- S. aureus-type resistance to vancomycin. J Bacteriol **188:**1120-33.

96. **McKinney, T. K., V. K. Sharma, W. A. Craig, and G. L. Archer.** 2001. Transcription of the gene mediating methicillin resistance in Staphylococcus aureus (mecA) is corepressed but not coinduced by cognate mecA and beta-lactamase regulators. J Bacteriol **183:**6862-8.

97. **Menestrina, G., M. D. Serra, and G. Prevost.** 2001. Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. Toxicon **39:**1661-72.

98. **Mongkolrattanothai, K., S. Boyle, T. V. Murphy, and R. S. Daum.** 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:**1823-36.

99. **Mongodin, E., J. Finan, M. W. Climo, A. Rosato, S. Gill, and G. L. Archer.** 2003. Microarray transcription analysis of clinical Staphylococcus aureus isolates resistant to vancomycin. J Bacteriol **185:**4638-43.

100. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, and D. A. Talan.** 2006. Methicillin-resistant S. aureus infections among patients in the emergency department. N Engl J Med **355:**666-74.

101. **Morton, T. M., J. L. Johnston, J. Patterson, and G. L. Archer.** 1995. Characterization of a conjugative staphylococcal mupirocin resistance plasmid. Antimicrob Agents Chemother **39:**1272-80.

102. **Noble, W. C., R. E. Williams, M. P. Jevons, and R. A. Shooter.** 1964. Some Aspects Of Nasal Carriage Of Staphylococci. J Clin Pathol **17:**79-83.

103. **Noto, M. J., and Archer, G. L.** 2005. Unpublished data.

104. **Noto, M. J., and G. L. Archer.** 2006. A subset of Staphylococcus aureus strains harboring staphylococcal cassette chromosome mec (SCCmec) type IV is deficient in CcrAB-mediated SCCmec excision. Antimicrob Agents Chemother **50:**2782-8.

105. **Novick, R.** 1967. Properties of a cryptic high-frequency transducing phage in Staphylococcus aureus. Virology **33:**155-66.

106. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol **48:**1429-49.

107. **Novick, R. P.** 2003. Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of Staphylococcus aureus. Plasmid **49:**93-105.

108. **Novick, R. P.** 1989. Staphylococcal plasmids and their replication. Annu Rev Microbiol **43:**537-65.

109. **Novick, R. P., I. Edelman, and S. Lofdahl.** 1986. Small Staphylococcus aureus plasmids are transduced as linear multimers that are formed and resolved by replicative processes. J Mol Biol **192:**209-20.

110. **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:**4289-94.

111. **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:**2155-61.

112. **Patel, A. H., P. Nowlan, E. D. Weavers, and T. Foster.** 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of Staphylococcus aureus isolated by allele replacement. Infect Immun **55:**3103-10.

113. **Perl, T. M., J. J. Cullen, R. P. Wenzel, M. B. Zimmerman, M. A. Pfaller, D. Sheppard, J. Twombley, P. P. French, and L. A. Herwaldt.** 2002. Intranasal mupirocin to prevent postoperative Staphylococcus aureus infections. N Engl J Med **346:**1871-7.

114. **Pinho, M. G., S. R. Filipe, H. de Lencastre, and A. Tomasz.** 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphylococcus aureus. J Bacteriol **183:**6525-31.

115. **Reipert, A., K. Ehlert, T. Kast, and G. Bierbaum.** 2003. Morphological and genetic differences in two isogenic Staphylococcus aureus strains with decreased susceptibilities to vancomycin. Antimicrob Agents Chemother **47:**568-76.

116. **Reynolds, P. E., F. Depardieu, S. Dutka-Malen, M. Arthur, and P. Courvalin.** 1994. Glycopeptide resistance mediated by enterococcal transposon Tn1546 requires production of VanX for hydrolysis of D-alanyl-D-alanine. Mol Microbiol **13:**1065-70.

117. **Rice, L. B.** 2006. Antimicrobial resistance in gram-positive bacteria. Am J Infect Control **34:**S11-9; discussion S64-73.

118. **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother **47:**3926-34.

119. **Rosato, A. E., B. N. Kreiswirth, W. A. Craig, W. Eisner, M. W. Climo, and G. L. Archer.** 2003. mecA-blaZ corepressors in clinical Staphylococcus aureus isolates. Antimicrob Agents Chemother **47:**1460-3.

120. **Rowland, S. J., M. R. Boocock, and W. M. Stark.** 2005. Regulation of Sin recombinase by accessory proteins. Mol Microbiol **56:**371-82.

121. **Rowland, S. J., W. M. Stark, and M. R. Boocock.** 2002. Sin recombinase from Staphylococcus aureus: synaptic complex architecture and transposon targeting. Mol Microbiol **44:**607-19.

122. **Rubin, R. J., C. A. Harrington, A. Poon, K. Dietrich, J. A. Greene, and A. Moiduddin.** 1999. The economic impact of Staphylococcus aureus infection in New York City hospitals. Emerg Infect Dis **5:**9-17.

123. **Ruzin, A., J. Lindsay, and R. P. Novick.** 2001. Molecular genetics of SaPI1--a mobile pathogenicity island in Staphylococcus aureus. Mol Microbiol **41:**365-77.

124. **Ryan, K. J.** 2004. Staphylococci. *In* J. C. Sherris (ed.), Sherris Medical Microbiology. An Introduction to Infectious Disease. The McGraw-Hill Companies, Inc., United States of America.

125. **Safo, M. K., T. P. Ko, F. N. Musayev, Q. Zhao, A. H. Wang, and G. L. Archer.** 2006. Structure of the MecI repressor from Staphylococcus aureus in complex with the cognate DNA operator of mec. Acta Crystallograph Sect F Struct Biol Cryst Commun **62:**320-4.

126. **Safo, M. K., Q. Zhao, T. P. Ko, F. N. Musayev, H. Robinson, N. Scarsdale, A. H. Wang, and G. L. Archer.** 2005. Crystal structures of the BlaI repressor from Staphylococcus aureus and its complex with DNA: insights into transcriptional regulation of the bla and mec operons. J Bacteriol **187:**1833-44.

127. **Sambrook, J., Fritsch, E. F., and Maniatis, T.** 1989. Molcular Cloning A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, USA.

128. **Severin, A., S. W. Wu, K. Tabei, and A. Tomasz.** 2004. Penicillin-binding protein 2 is essential for expression of high-level vancomycin resistance and cell wall synthesis in vancomycin-resistant Staphylococcus aureus carrying the enterococcal vanA gene complex. Antimicrob Agents Chemother **48:**4566-73.

129. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, D. E. Dodge, D. A. Bost, M. Riehman, S. Naidich, and B. N. Kreiswirth.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of Staphylococcus aureus strains. J Clin Microbiol **37:**3556-63.

130. **Shore, A., A. S. Rossney, C. T. Keane, M. C. Enright, and D. C. Coleman.** 2005. Seven novel variants of the staphylococcal chromosomal cassette mec in methicillin-resistant Staphylococcus aureus isolates from Ireland. Antimicrob Agents Chemother **49:**2070-83.

131. **Sieradzki, K., and A. Tomasz.** 2003. Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of Staphylococcus aureus. J Bacteriol **185:**7103-10.

132. **Sieradzki, K., and A. Tomasz.** 1999. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of Staphylococcus aureus. J Bacteriol **181:**7566-70.

133. **Silvestri, L. G., and Hill, L. R.** 1965. Agreement Between Deoxyribonucleic Acid Base Composition and Taxometric Classification of Gram-Positive Cocci. Journal of Bacteriology **90:**136-140.

134. **Smith, M. C., and H. M. Thorpe.** 2002. Diversity in the serine recombinases. Mol Microbiol **44:**299-307.

135. **Snyder, L. a. C., W.** 2003. Molecular Genetics of Bacteria. ASM Press, Washington, DC.

136. **Srinivasan, A., J. D. Dick, and T. M. Perl.** 2002. Vancomycin resistance in staphylococci. Clin Microbiol Rev **15:**430-8.

137. **Strominger, J. L., and J. M. Ghuysen.** 1967. Mechanisms of enzymatic bacteriaolysis. Cell walls of bacteri are solubilized by action of either specific carbohydrases or specific peptidases. Science **156:**213-21.

138. **Styers, D., D. J. Sheehan, P. Hogan, and D. F. Sahm.** 2006. Laboratory-based surveillance of current antimicrobial resistance patterns and trends among Staphylococcus aureus: 2005 status in the United States. Ann Clin Microbiol Antimicrob **5:**2.

139. **Tenover, F. C., R. Arbeit, G. Archer, J. Biddle, S. Byrne, R. Goering, G. Hancock, G. A. Hebert, B. Hill, R. Hollis, and et al.** 1994. Comparison of traditional and molecular methods of typing isolates of Staphylococcus aureus. J Clin Microbiol **32:**407-15.

140. **Tenover, F. C., M. V. Lancaster, B. C. Hill, C. D. Steward, S. A. Stocker, G. A. Hancock, C. M. O'Hara, S. K. McAllister, N. C. Clark, and K. Hiramatsu.** 1998. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J Clin Microbiol **36:**1020-7.

141. **Thompson, N. E., and P. A. Pattee.** 1981. Genetic transformation in Staphylococcus aureus: demonstration of a competence-conferring factor of bacteriophage origin in bacteriophage 80 alpha lysates. J Bacteriol **148:**294-300.

142. **Thorpe, H. M., S. E. Wilson, and M. C. Smith.** 2000. Control of directionality in the site-specific recombination system of the Streptomyces phage phiC31. Mol Microbiol **38:**232-41.

143. **Tomasz, A., S. Nachman, and H. Leaf.** 1991. Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrob Agents Chemother **35:**124-9.

144. **Ubeda, C., E. Maiques, E. Knecht, I. Lasa, R. P. Novick, and J. R. Penades.** 2005. Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. Mol Microbiol **56:**836-44.

145. **Voyich, J. M., M. Otto, B. Mathema, K. R. Braughton, A. R. Whitney, D. Welty, R. D. Long, D. W. Dorward, D. J. Gardner, G. Lina, B. N. Kreiswirth, and F. R. Deleo.** 2006. Is Panton-Valentine Leukocidin the Major Virulence Determinant in Community-Associated Methicillin-Resistant Staphylococcus aureus Disease? J Infect Dis **194:**1761-70.

146. **Waldron, D. E., and J. A. Lindsay.** 2006. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into Staphylococcus aureus and between S. aureus isolates of different lineages. J Bacteriol **188:**5578-85.

147. **Wang, Q. M., R. B. Peery, R. B. Johnson, W. E. Alborn, W. K. Yeh, and P. L. Skatrud.** 2001. Identification and characterization of a monofunctional glycosyltransferase from Staphylococcus aureus. J Bacteriol **183:**4779-85.

148. **Warren, D. K., R. S. Liao, L. R. Merz, M. Eveland, and W. M. Dunne, Jr.** 2004. Detection of methicillin-resistant Staphylococcus aureus directly from nasal swab specimens by a real-time PCR assay. J Clin Microbiol **42:**5578-81.

149. **Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore, and F. C. Tenover.** 2003. Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus. Science **302:**1569-71.

150. **Wilkinson, B. J.** 1997. Biology. *In* K. B. C. a. G. L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone Inc., New York.

151. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis **39:**309-17.

152. **Wisplinghoff, H., A. E. Rosato, M. C. Enright, M. Noto, W. Craig, and G. L. Archer.** 2003. Related clones containing SCCmec type IV predominate among clinically significant Staphylococcus epidermidis isolates. Antimicrob Agents Chemother **47:**3574-9.

153. **Woodford, N.** 2005. Biological counterstrike: antibiotic resistance mechanisms of Gram-positive cocci. Clin Microbiol Infect **11 Suppl 3:**2-21.

154. **Zhang, H. Z., C. J. Hackbarth, K. M. Chansky, and H. F. Chambers.** 2001. A proteolytic transmembrane signaling pathway and resistance to beta-lactams in staphylococci. Science **291:**1962-5.

155. **Zhang, Y. Q., S. X. Ren, H. L. Li, Y. X. Wang, G. Fu, J. Yang, Z. Q. Qin, Y. G. Miao, W. Y. Wang, R. S. Chen, Y. Shen, Z. Chen, Z. H. Yuan, G. P. Zhao, D. Qu, A. Danchin, and Y. M. Wen.** 2003. Genome-based analysis of virulence genes in a non-biofilm-forming Staphylococcus epidermidis strain (ATCC 12228). Mol Microbiol **49:**1577-93.

156. **Zygmunt, D. J., C. W. Stratton, and D. S. Kernodle.** 1992. Characterization of four beta-lactamases produced by Staphylococcus aureus. Antimicrob Agents Chemother **36:**440-5.

VITA

Michael James Noto was born in Port Jefferson, New York and was raised in Port Jefferson Station. He graduated from Comsewogue Senior High School in 1996. In May of 2000 he graduated from James Madison University with a Bachelor of Science in Biology. He then spent the 2000-2001 academic year conducting research at the University of Virginia. Michael entered the Virginia Commonwealth University School of Medicine in August of 2001 and joined the MD/PhD program shortly after that.

Publications:

Noto, M. J., and G. L. Archer. 2006. A subset of *Staphylococcus aureus* strains harboring staphylococcal cassette chromosome *mec* (SCC*mec*) type IV is deficient in CcrAB-mediated SCC*mec* excision. Antimicrob Agents Chemother **50:**2782-8.

McDowell, J. V., J. Wolfgang, L. Senty, C. M. Sundy, M. J. Noto, and R. T. Marconi. 2004. Demonstration of the involvement of outer surface protein E coiled coil structural domains and higher order structural elements in the binding of infection-induced antibody and the complement-regulatory protein, factor H. J Immunol **173:**7471-80.

Wisplinghoff, H., A. E. Rosato, M. C. Enright, M. Noto, W. Craig, and G. L. Archer. 2003. Related clones containing SCCmec type IV predominate among clinically significant Staphylococcus epidermidis isolates. Antimicrob Agents Chemother **47:**3574-9.

Priebe, G. P., C. R. Dean, T. Zaidi, G. J. Meluleni, F. T. Coleman, Y. S. Coutinho, M. J. Noto, T. A. Urban, G. B. Pier, and J. B. Goldberg. 2004. The galU Gene of Pseudomonas aeruginosa is required for corneal infection and efficient systemic

spread following pneumonia but not for infection confined to the lung. Infect Immun **72:**4224-32.

Wiggins, B. A., P. W. Cash, W. S. Creamer, S. E. Dart, P. P. Garcia, T. M. Gerecke, J. Han, B. L. Henry, K. B. Hoover, E. L. Johnson, K. C. Jones, J. G. McCarthy, J. A. McDonough, S. A. Mercer, M. J. Noto, H. Park, M. S. Phillips, S. M. Purner, B. M. Smith, E. N. Stevens, and A. K. Varner. 2003. Use of antibiotic resistance analysis for representativeness testing of multiwatershed libraries. Appl Environ Microbiol **69:**3399-405.