The dissemination among *Staphylococcus aureus* of the Staphylococcal Chromosome Cassette *mec* (SCC*mec*) which confers multiresistance

De verspreiding onder staphylococcen van de Staphylococcal Chromosome Cassette *mec* (SCC*mec*) die multiresistentie veroorzaakt

(met samenvatting in het nederlands)

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Camiel Lambert Christiaan Wielders geboren op 12 mei 1971 te Weert

Promotor:

Prof. J. Verhoef

Co-promotor:

Dr. A.C. Fluit

Werkzaam bij het Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation.

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SAMENVATTING

De meeste staphylococcen zijn commensalen die weinig klinische problemen veroorzaken. *Staphylococcus aureus* is daarentegen een van de voornaamste pathogenen, verantwoordelijk voor het grootste deel van de infecties van ziekenhuispatiënten. Naar schatting een derde van alle mensen draagt deze grampositieve bacterie, die ernstige long-, bloed- of wondinfecties kan veroorzaken bij personen met gecompromitteerde huid of immuunfunctie, met zich mee. In Nederlandse ziekenhuizen worden deze in den regel behandeld met meticilline of gelijksoortige ß-lactam antibiotica. In ons land is minder dan 1% van de stammen ongevoelig, maar in buitenlandse ziekenhuizen nemen methiciline-resistente *S. aureus* (MRSA) reeds meer dan 30% van alle *S. aureus* infecties voor hun rekening. De meeste MRSA vertonen multiresistentie en in de nabije toekomst zijn infecties die nauwelijks reageren op de beschikbare therapieën niet ondenkbaar. In deze studie werd onderzocht hoe dergelijke resistente *S. aureus* stammen kunnen ontstaan.

In een steekproef onder 3012 Europese isolaten bleek meer dan een kwart van de *S. aureus* isolaten in voornamelijk Zuid-Europese ziekenhuizen ongevoelig voor meticillineof analogen. Methiciline-resistentie wordt veroorzaakt door een aangepast Peneciline Binding Protein, PBP2a, dat wordt gecodeerd door het *mec*A gen en een lage affiniteit vertoond voor de familie van ß-lactam antibiotica. Hierdoor is de bacterie in staat de peptydoglycanen in de celwand intact te houden ondanks aanwezigheid van deze antibiotica. Praktisch alle (99%) MRSA isolaten bleken tegelijkertijd ook ongevoelig voor verschillende klassen van antibiotica en omgekeerd werd met behulp van PCR aangetoond dat alle isolaten die multiresistentie vertonen het *mec*A gen bezitten. Het *mec*A gen maakt deel uit van een grotere DNA regio, genaamd Staphylococcal Chromosome Cassette *mec* (SCC*mec*), dat allerhande combinaties van insertie sequenties, transposons en plasmides heeft opgenomen. Hierdoor zijn veel genen die resistentie tegen verschillende soorten antibiotica veroorzaken, waaronder naast ß-lactam antibiotica ook aminoglycosides, tetracyclines, macrolides en streptogramines, zijn in SCC*mec* terechtgekomen. Daarnaast zijn MRSA, die immers middels verschillende alternatieve therapieën worden behandeld, ook eerder geneigd resistentie kenmerken tegen verschillende middelen te verzamelen in de rest van hun genoom. Twee typeringstechnieken werden gebruikt om de populatie structuur van *S. aureus*

te onderzoeken en vast te kunnen stellen in hoeverre SCC*mec* verspreid is onder de *S. aureus* stammen. Analyse van restrictie fragmenten van zowel het gehele genoom als van de evolutionair gezien stabielere ribosomale genen toonde aan dat de populatie bestond uit 10 omvangrijke clonale lijnen, *S. aureus* Types I-IX, waarvan het merendeel was verspreid over verschillende continenten. Acht hiervan hadden een of meerdere keren SCC *mec* opgenomen, met daarin de genen voor resistentie tegen verschillende combinaties van antibiotica. Dit bevestigt dat SCC *mec* een mobiel genetisch element is, in staat is zich vanuit het genoom van de ene stam naar het andere te verplaatsen, om zo de resistentiekenmerken door te geven. Door herhaalde overdracht van SCC*mec* zijn talrijke MRSA klonen gevormd, waaronder een klein aantal wijdverbreide multiresistente stammen die de ziekenhuispopulatie in het buitenland domineren, maar ook minder resistente klonen die slechts sporadisch werden geïsoleerd. Hoewel na typering van de *S. aureus* stammen die gedurende twee jaar infecties veroorzaakten in een perifeer ziekenhuis bleek dat van kruisbesmetting door meticilline-gevoelige *S. aureus* stammen nauwelijks sprake is, kunnen multiresistente MRSA stammen zich juist makkelijk door het ziekenhuis verspreiden dankzij het selectieve voordeel dat ze ondervinden van het alleen door hen te overleven antibioticagebruik.

Na analyse van restrictie fragmenten van het SCC *mec* DNA, na electroforese zichtbaar gemaakt middels een gelabelde specifieke probe die meer dan 20kb van SCC*mec* beslaat, werden vier veelvoorkomende types *mec* onderscheiden. Deze werden allen waargenomen in meerdere staphylococcen soorten, wat aantoont dat deze chromosomale DNA sequentie tussen hen kan worden uitgewisseld. Nadat SCC*mec* vermoedelijk is ontstaan in één van de coagulase negatieve staphylococcen, heeft het zich snel verspreid onder de andere soorten. De overdracht van SCC*mec* tussen twee verschillende soorten staphylococcen werd waargenomen in een patiënt. Na toediening van antibiotica ter behandeling van een oorspronkelijk gevoelige *S. aureus* infectie verscheen SCC *mec* in een veelvoorkomende *S. aureus* stam, aanwezig in geheel Europa en Noord-Amerika, waardoor deze resistent werd voor methiciline. Deze specifieke variant van SCC *mec* vonden we buiten het zeldzame MRSA isolaat van deze patiënt verder in geen enkele andere *S. aureus* stam, maar wel in een *S. epidermidis* stam die op dezelfde patiënt aanwezig was. Dit toont aan dat het mobiele SCC *mec* tijdens de behandeling werd doorgegeven tussen deze twee soorten staphylococcen. We vermoeden dat uitwisseling van dergelijke chromosoom

cassettes een belangrijke rol heeft gespeeld tijdens de evolutie van de genomen van staphylococcen. In dit licht vormt het grote aandeel meticillineresistente CNS stammen op patiënten een potentieel probleem. Een recent type SCC*mec*, veelvuldig aangetoond in *S. epidermidis*, wordt gemakkelijk doorgegeven aan *S. aureus* en veroorzaakt momenteel een golf van nieuwe MRSA stammen. Herhaalde overdracht van SCC*mec* naar *S. aureus* verklaart een groot deel van de patiënten die tijdens behandeling met ß-lactam antibiotica in Nederlandse ziekenhuizen onverwacht MRSA infecties ontwikkelen en waarom in veel andere landen MRSA stammen reeds zijn doorgedrongen tot de in de gemeenschap aanwezige *S. aureus* populatie. Meestal betreft dit stammen die wel nog gevoelig zijn voor andere soorten antibiotica.

De Nederlandse aanpak omtrent MRSA infecties blijkt vooralsnog succesvol in het voorkomen van de verspreiding van resistente bacteriën in ziekenhuizen. Hierbij wordt aan de poort bij risicopatiënten uit het buitenland gescreend en na constatering van MRSA middels drastische hygiënische maatregels, waaronder strikte isolatie en zonodig sluiting van hele afdelingen, verdere verspreiding zoveel mogelijk voorkomen. Wanneer deze maatregelen ook worden toegepast wanneer zich onverwachte infecties voordoen kan ook de verspreiding van nieuwe stammen worden voorkomen, die vaak minder selectie voordeel ondervinden dan hun multiresistente tegenhangers.

GENERAL INTRODUCTION

Staphylococci are gram-positive bacteria present on the skin of most healthy humans. Although majority of coagulase-negative staphylococci (CNS) are commensals that generally do not cause severe clinical problems, some, including *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, may cause clinically relevant infections and bactereamia related to indwelling devices (1). In addition, one third of healthy humans carry *Staphylococcus aureus*, which may cause severe invasive disease (2). *S. aureus* accounts for the majority of nosocomial infections around the world (3), causing high mortality when untreated (4). Several types of antibiotics, directed to different targets in bacterial metabolism, were employed to fight infections caused by *S. aureus*. However, *S. aureus* has acquired resistance to most extant antibiotics, including vancomycin (5), and evolved to be one of the most difficult-to-treat pathogens in hospitals. This thesis describes the essential role of horizontal gene transfer between the chromosomes of different staphylococci in the dissemination of resistance genes.

History of *S. aureus* treatment.

During world-war II, ß-lactam antibiotics were introduced, which block synthesis of the cell wall by targeting Penicillin-Binding-Proteins (PBP). Within a few years, the *S. aureus* population had been taken over by resistant strains, which possessed plasmid encoded ß-lactamases to destroy the ß-lactam ring in penicillin and later derivatives (6). In 1960, the semi-synthetic ß-lactam methicillin, that is less sensitive to ß-lactamase, became the drug of choice to fight staphylococcal infections. Soon afterwards, however, methicillin-resistant *S. aureus* (MRSA) isolates were reported (7), which carried the *mec*A gene encoding an alternative PBP insensitive to all ß-lactams, including the carbapenems and cephalosporines. In addition, resistance to other drugs, including the aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, chloramphenicol, which target ribosomes to inhibit protein synthesis, and fluoroquinolones, which target topoisomerase and gyrase to inhibit bacterial DNA metabolism, developed quickly. During the 1950s, many of

the genes causing resistance were located on small plasmids. In the 1980s, however, they had formed clusters on large molecular weight conjugative plasmids or on the chromosomal DNA (8) and multiresistant *S. aureus* infections were reported around the world by that time (9). These were treated with the glycopeptide antibiotic vancomycin, which was long considered the last remaining reliable therapeutic option (10). The glycopeptides bind to the D-alanyl-D-alanine side chains of peptidoglycan or its precursors, thereby preventing cross-linking of the peptidoglycan.

By now, multiresistant *S. aureus* represents a major cause of nosocomial infections worldwide (3). After the use of vancomycin has increased, resistance among *S. aureus* emerged, causing infections that are hardly responsive to treatment and that may compromise medical practice in the near future (11). New treatment options, including quinupristin/dalfopristin (Synercid) and oxazolidinones (Linezolid) have recently become available, but resistance has already emerged (3, 12-14).

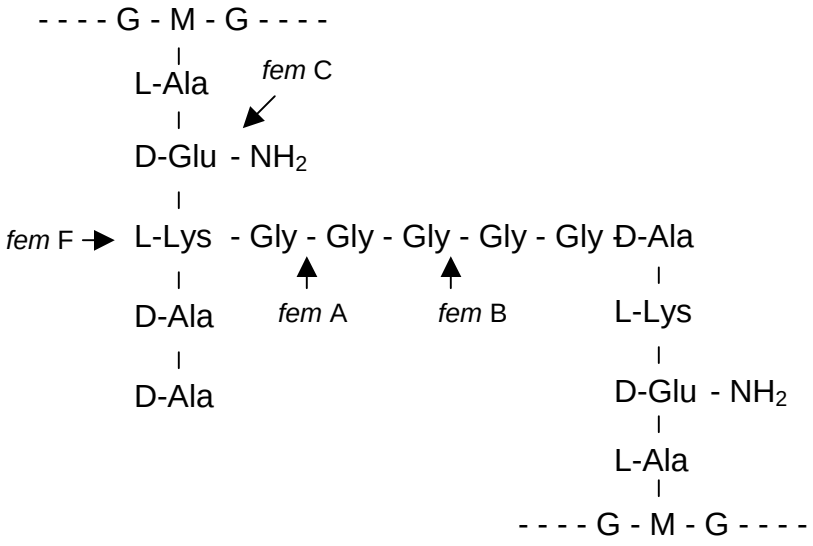

Fig. 1. PBPs are involved in peptidoglycan synthesis. The polysaccharide backbone of peptidoglycan is a polymer of N-acetylmuramic acid (M) and N-acetylglucosamine (G) residues. Oligopeptides are attached to each N-acetylmuramic acid residue, which are cross-linked by a chain of glycine residues by the transpeptidation reaction catalyzed by PBPs. The sites in the peptidoglycan synthesis that are affected by different *fem* factors are indicated.

Antimicrobial action of methicillin and other β-lactam antibiotics

ß-lactam antibiotics, such as penicillin, ampicillin, methicillin and cephalosporines, are very effective against staphylococci, and were always considered the drug of choice to fight infections. Gram-positive bacteria produce a thick peptidoglycan outer cell wall, which protects the cell against the actions of the complement system and osmotic damage. The characteristic ring structure of ß-lactam antibiotics binds to and acetylates the PBP's involved in peptydoglycan synthesis, preventing synthesis of the cell wall (15). PBPs are membrane-bound D,D-transpeptidases that cross-link the peptidoglycan in the bacterial cell wall by forming a pentaglycin bridge (16, 17). Other factors, including *fem* A-F, cooperate in the peptidoglycan pentaglycine interpeptide bridge formation (Fig.1). Several PBPs occur in staphylococci, with varying affinities for the different ß-lactams (18).

The genetic basis for resistance to methicillin and other β-lactam antibiotics

β-lactam degradation by β-lactamases. Resistance to ß-lactams may result from enzymatic degradation of the ß-lactam ring by ß-lactamases or expression of a low-affinity PBP. After the introduction of penicillin, transmittable plasmids carrying the *bla*Z gene, that encodes a ß-lactamase, appeared in *S. aureus* isolates around the world. Expression of the *bla*Z gene is upregulated in response to penicillin by the products of the *bla*I and *bla*R1 genes. Later, ß-lactamases evolved to inactivate later generations of ß-lactam antibiotics, including eventually methicillin. This resulted in "borderline" MRSA, which express high amounts of β-lactamase and display low-level resistance (19).

A novel penicillin binding protein, PBP2a. Starting from a ß-lactamase operon, an alternative PBP evolved, PBP2a, which is added to the set of normal PBPs present in cells, and shows low affinity to the family of ß-lactams. While other PBPs are inactivated by low concentrations of β-lactams, PBP2a retains its transpeptidation activity even at high concentrations, ensuring continued cell wall synthesis and causing resistance (17, 20). PBP2a is encoded by the *mec*A gene, that was formed by recombination of an unknown PBP and a ß-lactamase gene in another species (21). The PBP having the greatest similarity to PBP2a so far was identified in *S. sciuri* (87.8% amino acid identity) (22). The *mec*A gene,

that is found in many staphylococcal species, but not in most methicillin susceptible isolates, was acquired by *S. aureus* by horizontal transfer (23) (24).

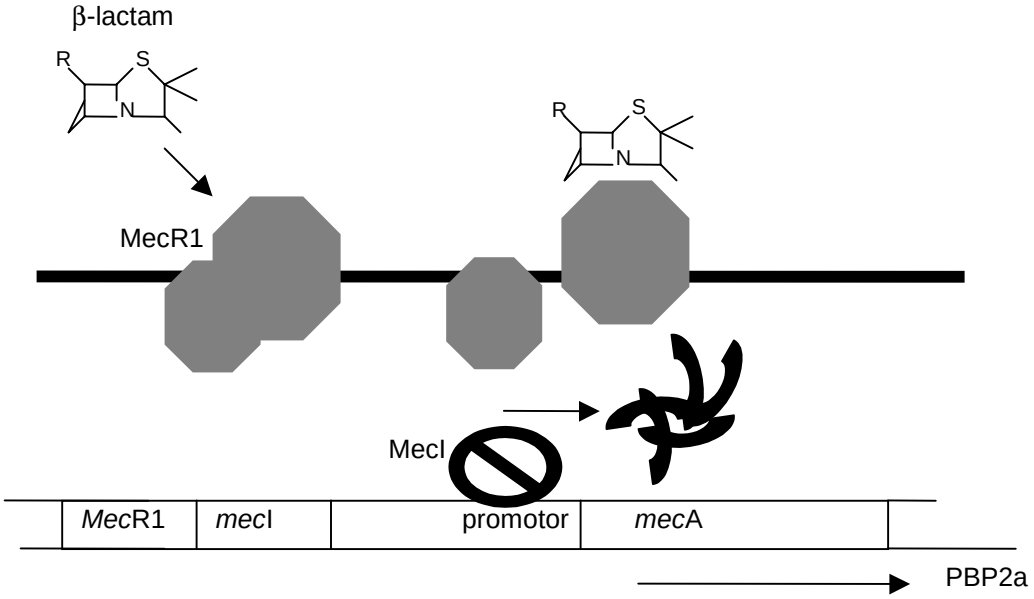

Fig. 2. Regulation of PBP2a expression. The *mec*R1 gene encodes a receptor in the cytoplasmic membrane that senses certain β-lactams. Upon binding, mecR1 is cleaved and free to inactivate mecI, causing derepression of PBP2a by releasing the *mec*I repressor protein from the operator region of the *mec*A gene. Redrawn from Archer et al. (25).

Expression of methicillin resistance caused by PBP2a. Analogous to the ß-lactamase operon, PBP2a may be expressed in response to β-lactams. The *mec*I and *mec*R1 genes allow expression of PBP2a when certain ß-lactam antibiotics, such as penicillin or cephalosporins, are present (25). The *mec*R1 gene encodes a receptor in the cytoplasmic membrane that senses their presence, and transmits a signal causing derepression of PBP2a by releasing the *mec*I repressor protein from the operator region of the *mec*A gene (25) (Fig. 2). However, most ß-lactams are poor inducers of PBP2a, because of their low affinity for the *mec*R1 sensor (26). Therefore, strains carrying the intact regulatory sequences (5) appear susceptible to methicillin due to the strong repressive function of the *mec*I gene. When exposed to methicillin, such pre-MRSA may become heterogeneous resistant (27) due to inactivation the *mec*I repression by deletion of the regulatory sequences, or mutations in *mec*I, *mec*R1, and the *mec*A operator, thus allowing constitutive expression of PBP2a (28, 29). Because

unrestricted PBP2a production alone does not confer full resistance to ß-lactams, such strains comprise cells with various (or heterogeneous) levels of methicillin resistance (5, 30). Upon selection, subpopulations that homogeneously express high-level methicillin-resistance can be isolated. In Japan, heterogeneous resistant MRSA have been dominant during the 1980s, but were later replaced by the clonal dissemination of homogeneous resistant MRSA strains (5). To become homogeneously resistant to methicillin, adaptations of other genes, such as the *fem*A-F genes involved in peptidoglycan metabolism (Fig. 1), are required (31). In addition, two genes of unknown function were recently cloned which, when over-expressed, resulted in homogenous methicillin resistance (32). However, the exact mechanisms underlying the transition from hetero- to homogeneous resistant MRSA are poorly understood (5, 30).

The staphylococcal chromosome cassette *mec* (SCC*mec*)

Structure of SCCmec. The *mec*A gene is found within a chromosomal DNA region called Staphylococcal Chromosome Cassette *mec* (SCC*mec*) (33). The size of SCC*mec* ranges from approximately 21 to 67 kb, or 1%-2% of the entire *S. aureus* genome (34). In addition to the *mec* locus, SCC*mec* may contain several additional resistance genes, adhesion factors and numerous open reading frames (33, 35, 36). Due to the presence of insertion sequences like IS*431* or IS*257* (37), various combinations of genetic elements are found integrated in the cassette (Fig. 3). These include transposon Tn*554*, which encodes resistance macrolides, streptogranin B and clindamycin, plasmid pUB110, which encodes resistance to aminoglycosides, plasmid pT181, encoding resistance to tetracyclines (27, 38), or plasmid p1258 (39). In addition, a gene segment encoding mercury resistance, possibly derived from p1258 (40) and pseudo Tn*554*, a partial copy of Tn*554* encoding cadmium resistance, were detected in SCC*mec* (41).

SCCmec is a mobile genetic element. Evidence was provided that the SCC*mec*, which contains no phage related genes, no transposases and no *tra* genes, constitutes a new class of mobile genetic element encoding recombinases of the invertase/resolvase family (33). These enzymes, called cassette chromosome recombinase A and B (*ccrA* and *ccrB*), catalyze precise excision of the SCC*mec* from the *S. aureus* chromosome and site-specific as

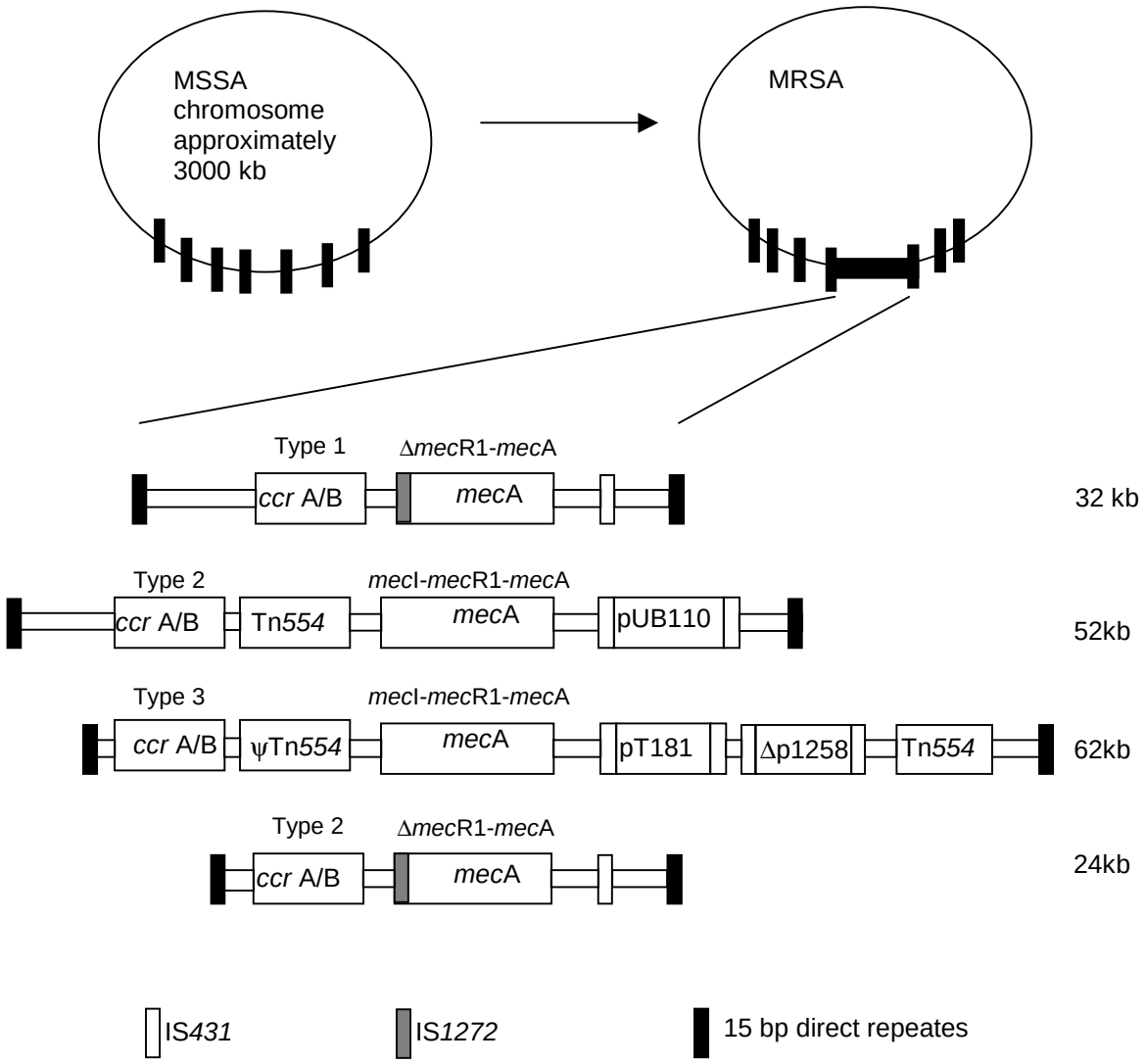

Fig 3. MRSA are formed by insertion of SCC*mec* in to the MSSA chromosome. Untill now, 4 types of SCC*mec* have been recognized based on polymorphisms of the *ccr* genes and the *mec*A locus. Various plasmids and transposons may be integrated in SCC*mec*. Redrawn from Ito and Hiramatsu (27).

well as orientation-specific integration of the SCC*mec* into the chromosome when introduced into the cells as a recombinant multicopy plasmid (42). Early on, it was shown that the *mec*A gene can be transferred between a ß-lactamase plasmid and a specific chromosomal location *in vitro* (43).

SCC*mec* is integrated at a unique site (*att*Bscc), located between *spa* and *pur* on the *S. aureus* genome near the origin of replication (44). The *att*B site is well conserved in different *S. aureus* strains (36). When SCC*mec* is integrated in the chromosome, *att*BSCC sequences are reconstituted at both chromosome-SCC*mec* junctions, forming 15 bp direct

repeats (36). Such repeats were repeatedly found in one sextant of the *S. aureus* genome, which was proposed to have been formed by repetitive integration of Staphylococcal chromosome cassettes during evolution (44).

Types of SCCmec. Structural analysis revealed different types of SCC*mec*, which are considered to comprise the SCC*mec* family of staphylococcal mobile genetic elements (Fig.3). Although there were substantial differences in the size and nucleotide sequences between different SCC*mec* types, they share the chromosomal integration site, conserved terminal inverted repeats and direct repeats at the integration junction points, conserved genetic organization around the *mec*A gene, and the recombinase genes responsible for the movements (5, 36). Three homologous for each of the *ccr* genes have been described (36). Two polymorphisms around the *mec* locus exist in *S. aureus*: the complete structure (IS*431mec*I-*mec*R1-*mec*A-IS*431*) or with a deletion of *mec*I and the 3' region of *mec*R1 and integrated insertion sequence (IS*1272Δ*-*mec*R1-*mec*A-IS*431*). Based on this, at least four types of SCC*mec* can be distinguished in *S. aureus*, which may differ in their repertoire of antibiotic determinants (5).

Dissemination of MRSA

The first MRSA were reported during the 1960s in Africa and Europe, soon after the introduction of methicillin (45, 46). Over the next ten years, an increasing number of outbreaks occurred in mainly European countries, including the United Kingdom, Denmark, France an Switzerland (7, 47, 48). In addition, there were occasional reports from other countries, including Australia, Poland, India and Turkey (49-52). The first important MRSA outbreak in the USA was reported in 1968 (53), but major inter-hospital spread did apparently not occur for another 5-10 years (9, 54). While some of the initial MRSA isolates were only resistant to β-lactams, others were resistant to multiple drugs, including tetracycline, and sometimes streptomycin, erythromycin, lincomycin, neomycin, tobramycin, and novobiocin (5, 9). During the 1970s, a decline in the prevalence of MRSA occurred in European countries, possibly due to hygienic measures or reduced use of antibiotics, particularly tetracycline (9, 55, 56). Although sporadic strains were still isolated, outbreaks were rarely reported until the 1980s.

A second wave of MRSA emerged in the late 1970s, causing large outbreaks in North America and Australia (57, 58). During the 1980s, the prevalence of MRSA increased dramatically around the world. In the US, the frequency of MRSA rose from 2.4% in 1975 to 29% in 1991(59), and similar reports came from Italy (60), France (61), Japan (62). In the UK, at least 16 epidemic strains appeared during the 1980s (63-66). In a recent survey among isolates from worldwide locations, MRSA was found to be a major cause of nosocomial- bloodstream infection, -skin and –soft tissue infection in most areas. The share of methicillin-resistant isolates varied greatly by region, site of infection, and whether the infection was nosocomial or community-acquired (3). In Europe, prevalence ranges from over 50% in Portugal and Italy to below 2% in Switzerland and the Netherlands, where infection control measures apply (67). Overall, several studies found that about 30% of the *S. aureus* isolates in the U.S., Latin America, Australia and Europe are resistant to methicillin (3, 68) (69). In Asia, the prevalence lies around 50%, with extremely high rates among *S. aureus* isolates from centers in Hong Kong (75%) and Japan (72%) (3). Most MRSA strains are also resistant to other antibiotics than the ß-lactams. High levels of erythromycin, clindamycin and ciprofloxacin resistance were found among all MRSA, while resistance to chloramphenicol, tetracyclines, rifampicin, and gentamicin depended on the region (3). Uniformly high levels of methicillin resistance were observed among CNS isolates, the prevalence lying over 70 % around the world (3).

Traditionally, MRSA infections have been acquired almost exclusively in hospitals or long-term care facilities (70). However, a notable increase of MRSA in the community was recently observed, particularily in the US where 28% of community-acquired *S. aureus* strains may be resistant to methicillin (3, 71). In 1999, 4 children lacking risk factors for MRSA infection, died of community-acquired MRSA infection in the US (72), indicating that we might face a new wave of MRSA originating in the community (73). Like β-lactamase mediated resistance to penicillin decades ago, MRSA has got foothold in the community. Such community-acquired MRSA are generally susceptible to multiple antibiotics (71, 74), in contrast to the typical multidrug-resistant hospital isolate.

The rise of high level resistant MRSA is thought to coincide with expansion of few multiresistant clonal types that are found around the world and account for most of the clinical problems (75, 76). When the hybridisation patterns obtained from world-wide collected isolates using *mecA*- and Tn*554*- specific probes were shown to form a temporally

ordered tree, it was concluded that MRSA represent a single clone (46). However, multilocus enzyme electrophoresis studies later showed that the *mec*A gene was harbored by different genotypes (77), suggesting transfer of SCC*mec* occurred more frequently. Based on PFGE typing of MRSA from Germany, revealing 14 epidemic strains among 39 different genotypes, it was proposed that sporadic genotypes had arisen by horizontal transfer of SSC*mec* from epidemic strains frequently found in close proximity (78). In two other studies, analysis of the *mec*I and *mec*R1 region, which lies 5' of the *mec*A gene showed that older isolates lacked part of this region, whereas the organization of this region in more recent MRSA isolates is similar to CNS isolates (23, 34), suggesting that SSC *mec* transfer from CNS into *S. aureus* might have taken place more often.

Purpose of this study

This study clarifies the role of repeated transfer of SCC*mec* between different species of staphylococci in the rapid world-wide dissemination of MRSA and gives insight in the dynamics of resistance genes in the *S. aureus* population.

To study the dissemination of SCC*mec* among *S. aureus,* antibiograms were first taken from European isolates, and the occurrence of multi-drug resistance among MSSA and MRSA was examined (chapter 2). The structure of the *S. aureus* population was established using pulsed-field gel electrophoresis (PFGE) of chromosomal *Sma*I fragments and automated ribotyping, and the presence of the SCC*mec* in the different *S. aureus* lineages was determined (chapter 3). In addition, the transmission of MSSA strains between patients in a teaching hospital was studied (chapter 4). To track its evolution, the SCC*mec* residing in different *S. aureus* lineages and other staphylococcal species was compared by generating restriction fingerprints (Chapter 5). In a case study, the molecular epidemiology of a MSSA, a MRSA and a *mec*A$^+$ CNS isolate taken from one patient are examined, because transfer of SCC*mec* was suspected (Chapter 6).

To win the continuing war against *S. aureus*, *g*lobal strategies are needed to control the emergence and prevent the spread of multiresistant infections. A better knowledge of the dissemination of resistance genes in the *S. aureus* population is required to win the next battle.

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Epidemiology and Susceptibility of 3,051 *Staphylococcus aureus* Isolates from 25 University Hospitals Participating in the European SENTRY Study

A. C. FLUIT,[1]*† C. L. C. WIELDERS,[1] J. VERHOEF,[1]† AND F.-J. SCHMITZ, [1,2]†

Eijkman-Winkler Institute for Microbiology, University Medical Center, Utrecht, The Netherlands,[1] and Institute for Medical Microbiology, Heinrich-Heine University, Düsseldorf, Germany[2]

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A total of 3,051 methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates and methicillin-resistant *S. aureus* (MRSA) isolates in Europe were compared. MRSA isolates constituted 25% of all isolates and were more prevalent in southern Europe. MRSA isolates appeared to be more prevalent in intensive care units than in outpatient departments. Only a small minority of MSSA isolates were multidrug resistant, whereas the majority of MRSA isolates were multidrug resistant.

Methicillin resistance in *Staphylococcus aureus* is now common in many areas of the world. The frequencies of infections and outbreaks due to methicillin-resistant *S. aureus* (MRSA) have continued to increase (7, 11, 12). It is noteworthy that the prevalence of MRSA varies from one geographic region to another and between different institutions in a given area. The prevalence of MRSA differs markedly among European countries (18). MRSA is an increasingly important clinical problem since MRSA is often multidrug resistant and therapeutic options are limited.

The aim of the present study was to analyze recent data on the epidemiologies and susceptibilities of 3,051 *S. aureus* isolates from 25 university hospitals participating in the European SENTRY Antimicrobial Surveillance Program from April 1997 through February 1999 (6). The epidemiologies of methicillin-susceptible *S. aureus* (MSSA) and MRSA isolates were studied by determining their prevalences in different specimens, on various wards, and in different age groups. The in vitro activities of 21 various antibiotic compounds were tested, and additionally, the percentage of multidrug-resistant isolates was determined for MSSA and MRSA isolates.

The species of the isolates (only one isolate per patient was allowed) were determined at the source and when deemed clinically significant by local criteria and were sent to the Eijkman-Winkler Institute (the European reference center for the SENTRY Antimicrobial Surveillance Program), together with relevant information for the isolate. The MICs of a range of antibiotics were determined by a broth microdilution (Sensititre, Westlake, Ohio) method by standard methods defined by the National Committee for Clinical Laboratory Standards (10). The origins of the *S. aureus* isolates tested are shown in Table 1. The presence of the *mecA* gene was determined by PCR with primers whose sequences were 5′-GTTGTAGTTGTCG GGTTTGG and 5′-CTTCCACATACCATCTTCTTTAAC.

Twenty-five percent of the isolates were methicillin resistant. The prevalence of MRSA is comparable to that found in recent U.S. studies (7, 12), but the percentage of MRSA isolates is less than half of the percentage reported from Japan (4). The prevalence of MRSA was confirmed to vary considerably between different European countries and also between hospitals

TABLE 1. Origins of *S. aureus* isolates

City	Country	No. of isolates	% MRSA
London	England	131	28
Utrecht	The Netherlands	147	2
Brussels	Belgium	82	25
Düsseldorf	Germany	215	5
Freiburg	Germany	132	4
Lausanne	Switzerland	114	2
Linz	Autstria	117	9
Paris I	France	219	25
Paris II	France	119	20
Lille	France	188	12
Lyon	France	192	18
Warsaw	Poland	58	33
Cracow	Poland	101	23
Coimbra	Portugal	318	54
Madrid	Spain	113	12
Seville	Spain	132	34
Barcelona	Spain	107	9
Rome	Italy	145	58
Genoa	Italy	152	43
Tirana	Albania	23	17
Athens	Greece	128	34
Ankara I	Turkey	24	21
Ankara II	Turkey	77	44
Istanbul	Turkey	4	0
Hash Homer	Israel	13	31

* Corresponding author. Mailing address: Eijkman-Winkler Institute, University Medical Center, Room G04.614, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Phone: 31 30 2507630. Fax: 31 30 2541770. E-mail: A.C.Fluit@azu.lab.nl.

† This author is a member of the European SENTRY Participants Group, which includes H. Mittermayer, Linz, Austria; M. Struelens, Brussels, Belgium; F. Goldstein and V. Jarlier, Paris, and J. Etienne and P. R. Courcol, Lille, France; F. Daschner, Freiburg, and U. Hadding, Düsseldorf, Germany; N. Legakis, Athens, Greece; G.-C. Schito, Genoa, and G. Raponi, Rome, Italy; P. Heczko, Cracow, and W. Hyrniewicz, Warsaw, Poland; D. Costa, Coimbra, Portugal; E. Perea, Seville, F. Baquero, Madrid, and R. Martin Alvarez, Barcelona, Spain; J. Bille, Lausanne, Switzerland; G. French, London, United Kingdom; R. Andoni, Tirana, Albania; V. Korten, Istanbul, and S. Unal and D. Gür, Ankara, Turkey; and N. Keller, Hash Homer, Israel.

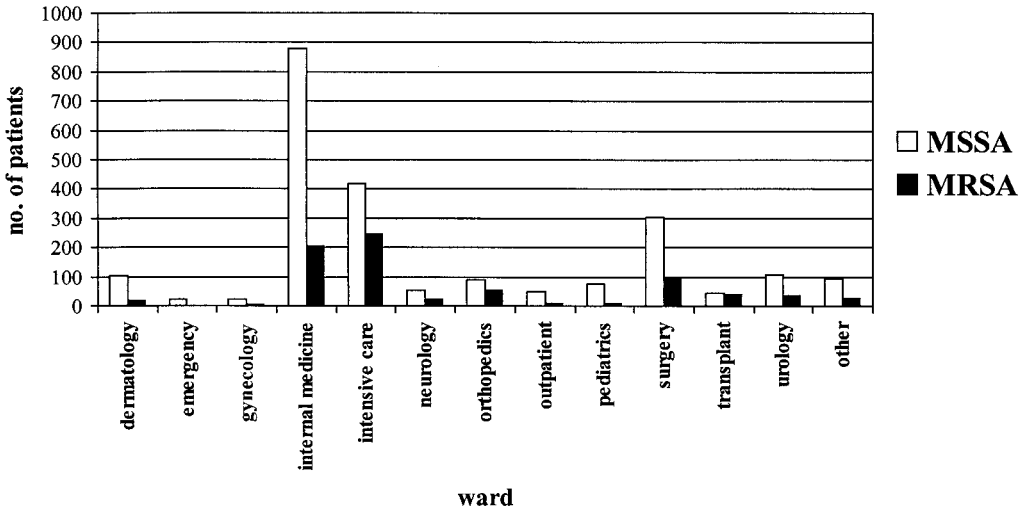

FIG. 1. Distributions of MSSA and MRSA isolates for different wards within the hospital.

within a country (Table 1) (18). In general, the highest prevalence of MRSA isolates was seen in hospitals in Portugal (54%) and Italy (43 to 58%). In contrast, the prevalence of MRSA was lowest in participating hospitals in Switzerland and The Netherlands (2%). However, only a few hospitals per country participated in the European SENTRY Antimicrobial Surveillance Program study. In addition, large differences in a country may occur; e.g., the proportion of MRSA isolates was 34% for the hospital in Seville, Spain, whereas it was 9% for the hospital in Barcelona, Spain. Similar observations were reported in recent U.S. studies of the prevalence of MRSA (2). The reason for the low prevalence in some university hospitals may be related to the rapid identification and strict policies of isolation of patients with MRSA colonization or infection, combined with the restricted use of antibiotics.

The prevalence of methicillin resistance was highest among *S. aureus* isolates deemed responsible for nosocomial pneumonia (34.4%); the prevalence of methicillin resistance was 28.3% among urinary tract infection isolates and 23.8% among blood isolates and was lowest among isolates associated with skin and

TABLE 2. MIC distributions, antimicrobial susceptibilities, and spectra of activity of the different antimicrobial agents tested for the MRSA isolates tested[a]

Antimicrobial agent	No. of isolates for which MIC (mg/liter) is:															MIC_{50}/MIC_{90}[b]	% Susceptible
	<0.03	0.06	≤0.12	0.12	0.25	≤0.5	0.5	1	2	>2	≤4	4	>4	8	>8		
Erythromycin				7	28		40	13	17			7		8	644	>8/>8	4.8
Clindamycin				103	60		5	4	1			2		7	572	>8/>8	23.3
Gentamicin				21	58		75	6	5			9		16	574	>16/>16	22.8
Tetracycline											309			11	444	>8/>8	42.9
Doxycycline						300		5	22			150	287			4/8	85.2
Minocycline						132		16	103			74	27			2/4	92.3
Ciprofloxacin	1	9		16	23		9	12	9	605						>2/>2	9.2
Gatifloxacin[c]	18	23		19	5		11	79	376	233						2/4	
Trovafloxacin[c]	43	14		7	9		97	255	195	144						1/>4	
Rifampin			33		240		9	20	78	334						2/>2	46.1
Chloramphenicol			1		1		2	2	10			180		438	130	8/16	83.0
Quinupristin-dalfopristin			24		167		427	111	14			3		12	6	0.5/1	99.5
Linezolid[c,d]			0		6		7	166	220			13		0	0	2/2	
Teicoplanin			5		35		158	260	236			63		5	2	1/2	99.7
Vancomycin			1		1		65	493	200			4		0	0	1/2	100

[a] A total of 764 MRSA isolates were tested.
[b] MIC_{50}/MIC_{90}, MICs at which 50%/90% of isolates are inhibited.
[c] Investigational drug. No susceptibility breakpoints are available (10).
[d] Only 412 MRSA isolates were tested, unless indicated otherwise.

TABLE 3. MIC distributions, antimicrobial susceptibilities, and spectra of activity of the different antimicrobial agents tested for the MSSA isolates tested[a]

Antimicrobial agent	No. of isolates for which (mg/liter) is:																MIC_{50}/MIC_{90}	% Susceptible
	≤0.03	0.06	≤0.12	0.12	0.25	≤0.5	0.5	1	2	>2	≤4	4	>4	8	>8	16	>16	
Penicillin			351		28		33	61	93			130		182		297	1,463	16/>32	15.4
Ampicillin			323		44		47	87	137			150		177		289	1,033	16/>16	16.1
Amoxicillin-clavulanate			315		181		462	805	326			80		30		31	57	1/2	94.8
Ceftriaxone			0		4		13	146	1,604			372		47		22	79[c]	2/4	95.6
Cefepime			3		5		20	431	1,268			886		34		12	68	2/4	96.5
Imipenem			1,974		196		27	9	6			11		14		50[d]		0.12/0.25	97.2
Erythromycin			21		716		1,011	149	6			13		10	361			0.5/>8	77.5
Clindamycin			1,661		460		20	6	2			0		0	138			0.12/0.25	93.7
Gentamicin			222		56		1,034	293	47			7		7	117			0.5/1	94.6
Tetracycline											2,023			20	244			≤4/8	89.7
Doxycycline						2,019		41	94			62	71					≤0.5/1	97.7
Minocycline						1,145		9	11			23	21					≤0.25/≤0.25	92.3
Ciprofloxacin	19	169		868	793		185	37	13	203								0.25/1	90.6
Gatifloxacin[e]	471	1,110		468	34		25	33	83	63								0.06/0.25	
Trovafloxacin[e]	1,470	504		90	28		37	82	46	30								≤0.03/0.12	
Rifampin			1,840		366		13	6	10	52								0.03/0.25	97.4
Chloramphenicol			0		3		5	12	41			637		1,474	115			8/8	96.3
Quinupristin-dalfopristin			185		1,372		656	61	0			6		0	0			0.25/0.5	95.3
Linezolid[e,f]			2		1		11	139	838			86		0	0			2/2	
Teicoplanin			10		327		1,574	313	55			7		1	0			0.5/1	100
Vancomycin			0		3		260	1,954	67			3		0	0			0.5/1	100

[a] A total of 2,287 MSSA isolates were tested, unless indicated otherwise.
[b] See footnote *b* of Table 2.
[c] For 9 isolates the MIC was 32 mg/liter, and for 70 isolates the MIC was >32 mg/liter.
[d] MIC, >8 mg/liter.
[e] Investigational drug. No susceptibility breakpoints are available for this drug (10).
[f] Only 1,075 MSSA isolates were tested.

soft tissue infections (22.4%). These differences might be due to prolonged antibiotic treatment of severely sick patients, which generally have longer hospital stays, resulting in enhanced selection pressure. However, U.S. SENTRY Antimicrobial Surveillance Program staphylococcal isolates from different sources displayed rates of resistance comparable to those described above (12).

Considerable differences were observed when the distributions of MRSA isolates in different wards were compared (Fig. 1). Almost 38% of the *S. aureus* isolates from intensive care units (ICUs) and 22.6% of the isolates from internal medicine wards were MRSA, whereas 0% of the isolates from emergency rooms and 1% of the isolates from outpatient departments were MRSA. This partly reflects the relative sizes of some specialties, but it also reflects the fact that some patients, e.g., critically ill patients in ICUs, have a greater chance of becoming colonized or infected. Our results concerning the prevalence of MRSA in different wards are largely in accordance with recent data from the United States. However, we were not able to confirm the extremely high prevalence of MRSA in ICUs described in the European Prevalence of Infection in Intensive Care study (17). The low prevalence of MRSA in emergency rooms and outpatient departments suggests that the level of MRSA in the community is still lower than that in hospitals (5, 9).

The distributions of both MSSA and MRSA among different age groups were similar. However, with the exception of newborns, *S. aureus* infections were more often found with increasing age, but their prevalence declined after 75 years of age. Compared to the age distribution for all infections with other organisms, no significant differences in the age distributions of individuals with MRSA infections were observed.

The distributions of the MICs for the isolates were as follows: ≤0.06 μg/ml, 18.5% (n = 565); 0.12 μg/ml, 21.1% (n = 645); 0.25 μg/ml, 12.7% (n = 388); 0.5 μg/ml, 16.4% (n = 501); 1 μg/ml, 4.5% (n = 137); 2 μg/ml, 1.7% (n = 51); 4 μg/ml, 1.1% (n = 35); 8 μg/ml, 1.7% (n = 51); and >8 μg/ml, 22.2% (n = 678). In 5% of the MRSA isolates, for all of which the oxacillin MIC was 4 μg/ml, the *mecA* gene could not be detected by PCR (data not shown). Oxacillin resistance in these isolates may be explained by undetected penicillin-binding protein alterations or the production of large amounts of β-lactamase (1, 8, 16).

The comparative in vitro activities of 21 antimicrobial agents

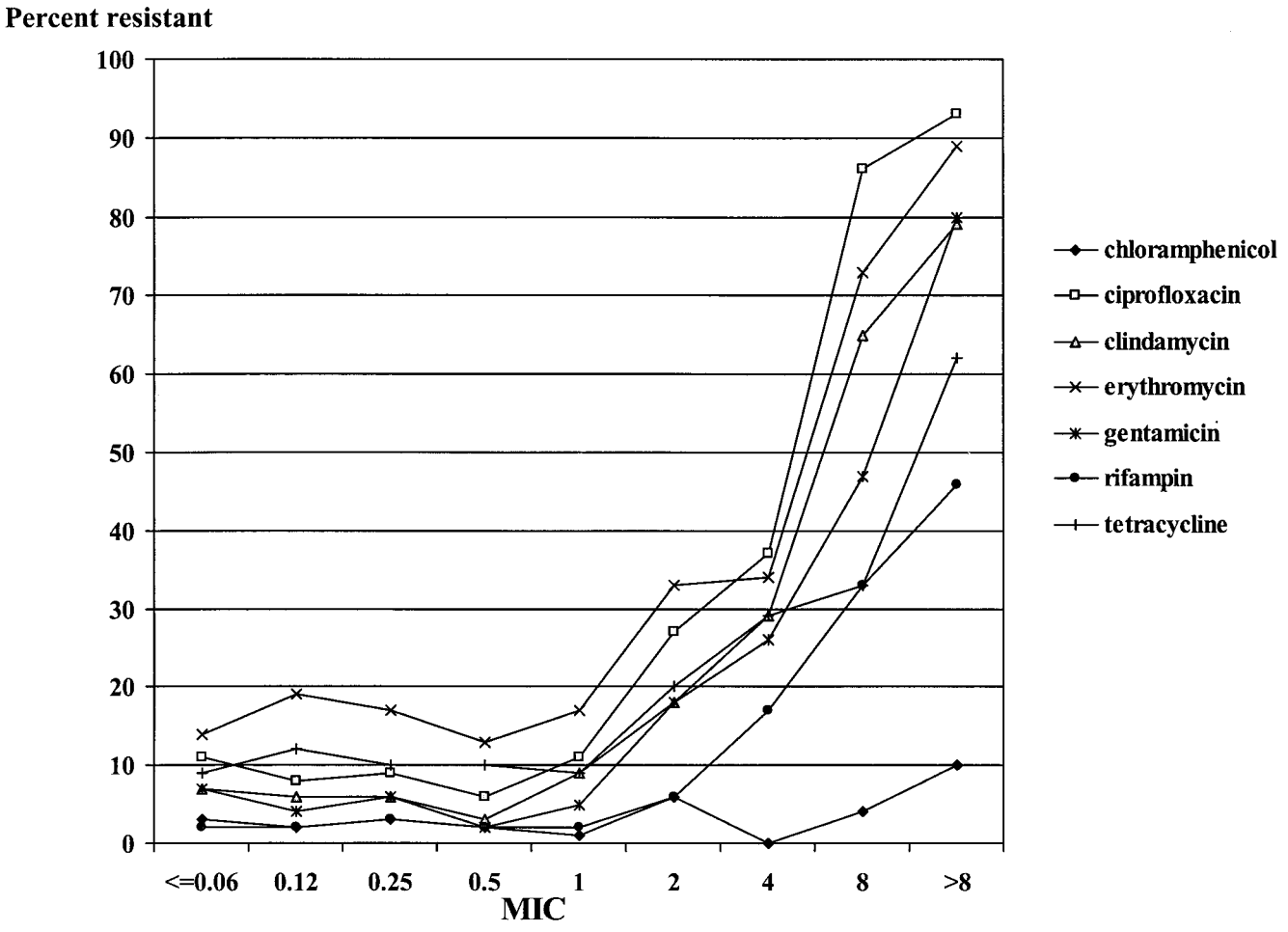

FIG. 2. Percent resistance to selected antibiotics for *S. aureus* isolates for which oxacillin MICs varied.

against MSSA and MRSA isolates are listed in Tables 2 and 3, respectively. Of the MSSA isolates tested, 84.7% were resistant to penicillin, while MRSA isolates are, by definition, resistant to all β-lactam antibiotics. There is an obvious relationship between oxacillin resistance and resistance to other antibiotics (Table 2). The percentage of MSSA isolates which were susceptible to erythromycin (77.5%) was more than eightfold higher than the percentage of MRSA isolates which were susceptible to erythromycin. While 94% of the MSSA isolates were susceptible to clindamycin, only 23% of the MRSA isolates exhibited susceptibility. Eighty-eight percent of the erythromycin-resistant MRSA isolates and 37% of the erythromycin-resistant MSSA isolates displayed a constitutive macrolide-lincosamide-streptogramin B (MLS) resistance phenotype on the basis of the MICs. The other erythromycin-resistant *S. aureus* isolates had an inducible MLS resistance phenotype. The percentage of MRSA isolates showing susceptibility to gentamicin (22.8%) was more than fourfold lower than that of MSSA isolates. While susceptibility to tetracyclines fell from 88.5% among MSSA isolates to 40.5% among MRSA isolates, this decrease was far less pronounced for the structurally related compounds minocycline and doxycycline, to which some 90% of the MRSA showed in vitro susceptibility. More then 90% of all MSSA isolates were susceptible to ciprofloxacin, whereas less than 10% of all MRSA isolates tested were susceptible to ciprofloxacin (Table 2).

While 99.5% of the MSSA isolates were susceptible to quinupristin-dalfopristin, this rate was slightly decreased to 95.3% for the MRSA isolates. Vancomycin and linezolid were the only compounds tested to which reduced susceptibility was not recognized for any of the *S. aureus* isolates tested. One MRSA isolate was resistant to teicoplanin, whereas a second one was intermediate resistant.

The percentage of isolates resistant to all of the antibiotics listed in Fig. 2 with the exception of chloramphenicol was quite stable among the population of *S. aureus* isolates for which oxacillin MICs were ≤0.06 to 1 μg/ml, but the percentage increased significantly with an increase in the oxacillin MIC to >2 μg/ml.

Isolates were considered to be multidrug resistant when they displayed resistance to five (or more) of the following antibiotics, which represented different antibiotic classes: oxacillin, penicillin, erythromycin, clindamycin, gentamicin, ciprofloxacin, tetracycline, rifampin, and chloramphenicol. MRSA is, by definition, also resistant to penicillin (10). Thus, all MRSA isolates were resistant to at least two classes of antibiotics. The

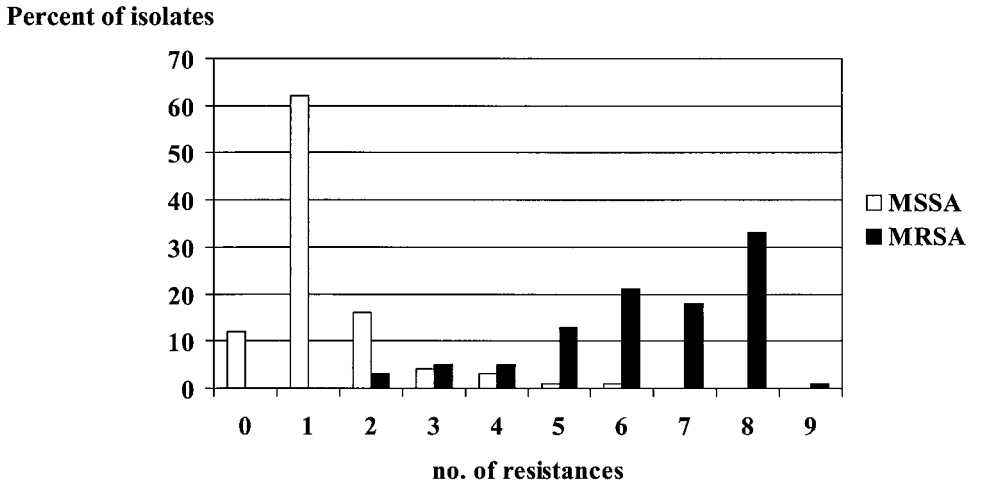

FIG. 3. Number of drugs to which *S. aureus* isolates were resistant for selected antibiotics (oxacillin, penicillin, gentamicin, erythromycin, clindamycin, ciprofloxacin, tetracycline, rifampin, and chloramphenicol) (number of resistances). Note that MRSA isolates were always resistant to two of these antimicrobial agents.

results are shown in Fig. 3. Only 2% of the MSSA isolates were multidrug resistant. However, 87% of the MRSA isolates were multidrug resistant and only 3% of the MRSA isolates were resistant to β-lactam antibiotics only.

The rates of susceptibility of the European *S. aureus* population were comparable to those determined from the data of Voss et al. (18). The results from the SENTRY Antimicrobial Surveillance Program for blood isolates from the United States, Canada, and Latin America generally showed higher percentages of susceptibility for MSSA isolates to most antimicrobial agents with the exception of erythromycin, chloramphenicol, and rifampin (13). This pattern was also observed for MRSA isolates from the United States and Latin America. A similar result was obtained when the European data were compared to the data from the SCOPE program (7), which investigated the susceptibilities of *S. aureus* isolates implicated in nosocomial bloodstream infections in the United States.

The glycopeptide agent vancomycin is still the drug of choice for the treatment of life-threatening infections caused by multidrug-resistant MRSA strains. Recent studies have suggested that treatment of infections with staphylococci currently considered susceptible according to the standards of the National Committee for Clinical Laboratory Standards but for which vancomycin MICs are 4 μg/ml might lead to therapeutic failures and that such isolates might be precursors of vancomycin-resistant *S. aureus* strains (14). Although we did not find MRSA isolates with reduced susceptibility to vancomycin in the European *S. aureus* population, emerging vancomycin resistance is a constant threat since the first glycopeptide-intermediate-resistant *S. aureus* (GISA) isolates and hetero-GISA isolates have also been detected in Europe (3). For seven strains (0.23%) in the present European collection, vancomycin MICs were 4 μg/ml. Recently, we investigated the seven strains for which the vancomycin MIC was 4 μg/ml for their hetero-glycopeptide-intermediate resistance status. However, neither GISA nor hetero-GISA was detected (15). Nevertheless, it is important to carefully monitor the prevalence of (hetero-)GISA, especially in MRSA populations because of the almost invariable multidrug-resistant nature of MRSA.

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THE STAPYLOCOCCAL CHROMOSOME CASSETTE *mec* (SCC*mec*) ASSOCIATED WITH MULTIRESISTANCE IS WIDELY DISSEMINATED IN THE *STAPHYLOCOCCUS AUREUS* POPULATION

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important causes of hospital infections worldwide. The *mec*A gene causing resistance was acquired by *S. aureus* from another species. To determine the clonal relationships between methicillin-susceptible *S. aureus* (MSSA) and MRSA, 1067 *Staphylococcus aureus* isolates (521 MSSA and 546 MRSA), collected mainly in North American and European hospitals between the 1960s and the year 2000, were typed using pulsed-field gel electrophoresis and ribotyping. Of ten widespread *S. aureus* lineages recognized, eight had corresponding *mec*A^+ strains. This supports the hypothesis that, in the *S. aureus* population, horizontal transfer of the *mec*A gene plays an important role.

Introduction

Staphylococcus aureus strains resistant to methicillin (MRSA) and many other antibiotics are a major cause of nosocomial infections worldwide [1] . Resistance to methicillin is caused by the *mec*A gene, which encodes the low-affinity penicillin-binding protein 2A [2] . The *mec*A gene is part of a 40- to 60-kb staphylococcal chromosome cassette *mec* (SCC *mec*), a mobile genetic element that may also contain genetic structures such as Tn*554*, pUB110, and pT181, which encode resistance to non-ß-lactam antibiotics [3] . Two hypotheses have been raised to explain the evolutionary origin of MRSA strains. The „single clone hypothesis“, based on early analyses of the restriction fragment length polymorphisms obtained from MRSA isolates collected worldwide using probes for *mec*A and Tn*554*, suggests that the *mec*A gene entered the *S. aureus* population on one occasion and formed a single MRSA clone that has since spread around the world [3, 4] . The second

hypothesis, based on the detection of the *mec*A gene in diverse *S. aureus* multilocus enzyme electrophoresis types, proposes that MRSA strains evolved a number of times by means of the horizontal transfer of the *mec*A gene into phylogenetically distinct methicillin-susceptible *S. aureus* (MSSA) precursor strains [5] . Using DNA microarray technology in 11 MRSA strains, the *mec*A gene has been detected in at least five divergent lineages, implying that horizontal *mec*A transfer has played a fundamental role in the evolution of MRSA [6] . The transfer of *mec*A from *S. epidermidis* to *S. aureus* was recently witnessed *in vivo*, suggesting that *mec*A may transfer frequently to MSSA [7] .

We presents molecular typing data that support the theory of *mec*A gene transfer into resident lineages of *S. aureus*. Pulse-field gel electrophoresis (PFGE) and subsequent ribotyping of MSSA and MRSA isolates collected between the 1960s and 2000 in Europe and North America revealed ten major lineages of varying size. Both *mec*A^- MSSA and *mec*A^+ MRSA were detected in eight of these lineages, while the remaining two lineages consisted of only MSSA.

Materials and Methods

Bacterial isolates. The clonal relationships and susceptibility of 546 MRSA and 521 MSSA isolates were determined. These isolates were selected from different sources in order to study isolates from different geographic backgrounds and time periods. The origins of the isolates were as follows: 367 MRSA and 290 MSSA isolates had been collected between April 1997 and December 1998 in 20 university hospitals in 12 European countries as part of the SENTRY Antimicrobial Surveillance Program [1] . They included isolates from Athens (8 MSSA/19 MRSA), Düsseldorf (10/9), Freiburg (14/2), Lausanne (56/1), Linz (7/5), Paris 1 (17/21), Paris 2 (10/33), Lille (13/22), Lyon (12/14), Coimbra (16/70), Warsaw (7/17), Krakow (5/3), Madrid (14/2), Seville (27/29), Barcelona (7/6), Rome (10/32), Genoa (9/34), Brussels (10/5), London (26/36), and Istanbul (12/7). An additional 181 MSSA and 54 MRSA isolates had been collected between 1996 and 1999 in the University Medical Center (UMCU), Utrecht, the Netherlands. These MRSA isolates, detected during 12 MRSA episodes, had evaded the hospital's 'Search and Destroy' procedure and could not be linked epidemiologically to any foreign hospitals. One hundred and three more MRSA isolates

were selected to represent the genetic diversity of the MRSA collections of Drs. Kreiswirth et al. [4] , Roberts et al. [8] , Lencastre et al. [9, 10] , and Witte et al. [11] . They included the earliest isolates from Europe and Africa collected during the 1960s, North American isolates collected from the 1970s to the 1990s, and European reference strains like the Iberian clone [9] , the Brazilian clone [9] , the North [11] and South German clone [11] , the Berlin clone [11] , the Hannover clone [11] , the Portuguese clone [9] , the pediatric clone [10] , EMRSA 15 [12] , and EMRSA 16 [12] . Another 12 MRSA isolates were studied that had been collected in South Africa during 1998. Finally, 10 MRSA and 50 MSSA isolates were included that had been taken from colonized patients who had no clinical signs of *S. aureus* infection within 2 hours after admission to the Cook County Hospital (Chicago, IL, USA).

The isolates were identified as *S. aureus* by routine microbiological methods. Only one isolate per patient was included.

Susceptibility testing. Susceptibility to oxacillin, erythromycin, clindamycin, rifampicin, chloramphenicol, ciprofloxacin, gentamicin, and tetracycline was determined using the broth microdilution method defined by the National Committee for Clinical Laboratory Standards. Isolates were considered multiresistant when they displayed a decreased susceptibility to at least four of the eight antimcrobial agents tested.

Detection of the mec*A *gene by PCR. The *mec*A gene was detected by PCR using the primers 5'GTT GTA GTT GTC GGG TTT GG 3' and 5'CTT CCA CAT ACC ATC TTC TTT AAC 3'.

PFGE analysis. Genomic DNA was digested with *Sma*1 and resolved using the CHEF-DRII system (Bio-Rad laboratories, Hercules, Ca, USA), as described by the manufacturer.

Ribotyping. Ribotypes were determined using an automated riboprinter system (Qualicon, Wilmington, DE, USA), using *Eco*RI as described by the manufacturer.

Analysis of the restriction patterns. The restriction patterns were compared by calculating a similarity index using the UPGMA cluster algorithm and Dice coefficient provided by the Bionumerics software (Applied Mathematics, Kortrijk, Belgium).

Results

Clonal relationships among the S. aureus isolates. When the PFGE patterns of 546 MRSA and 521 MSSA were compared in a dendrogram, ten major clusters of varying size were discerned. To confirm the clonal relatedness of isolates within these clusters, 330 isolates were selected for ribotyping, which covered the chromosomal diversity of the isolates (Fig. 1). Compared to the diversity of PFGE patterns, the riboprints were much more conserved during evolution. Ten clusters were distinguished at the 80% similarity level, which define clonal lineages called *S. aureus* Types I-X (Fig. 2). A very good correlation between PFGE typing and ribotyping was observed: except for Type IV isolates, 99% of the double-typed isolates clustered in corresponding branches of both the riboprint and PFGE dendrograms. The PFGE patterns obtained from the Type IV isolates were often found not only in a cluster of their own, but in other clusters as well (Fig. 1). Further, both Type II and Type III isolates could be divided into two subtypes (a and b) that formed subclusters in the ribotyping dendrogram and had different susceptibility patterns.

Pandemic strains, yielding identical PFGE patterns, were collected in many hospitals on both continents (Fig. 3). Isolates of the Type I (40% of all samples), Type II (20%), and Type III lineages (12%) were present in nearly all hospitals studied. The Type IV (8%), Type VI (8%), Type IX (7%), and Type VIII (2%) lineages were also detected in Europe and North America, although less often. Isolates of the remaining lineages (Type V (2%), Type VII (0.5%), and Type X (0.5%)) were only referred from European countries.

Dissemination of the mec*A *gene in the different S. aureus lineages. Using PCR, the *mec*A gene was detected in all but the Type IX and X lineages. Some pandemic *mec*A^- MSSA isolates come with *mec*A^+ MRSA counterparts that share the identical ribotype, while their PFGE patterns differ by a single bandshift, due to insertion of a fragment containing *mec*A (Fig 4). However, we found no *mec*A^- counterparts among Type IIb and IIIb isolates which, with no exception, all contained *mec*A.

More than 60% of the *mec*A^+ isolates belonged to the Type I lineage. These isolates predominated in North America, Africa, and Europe. The Type I MRSA included all MRSA isolates from the 1960s, the Brazilian clone [9] , the North German clone [11] , the Hannover clone [11] , the Iberian clone [9] , and the Portuguese clone [9] . One quarter of the *mec*A^+

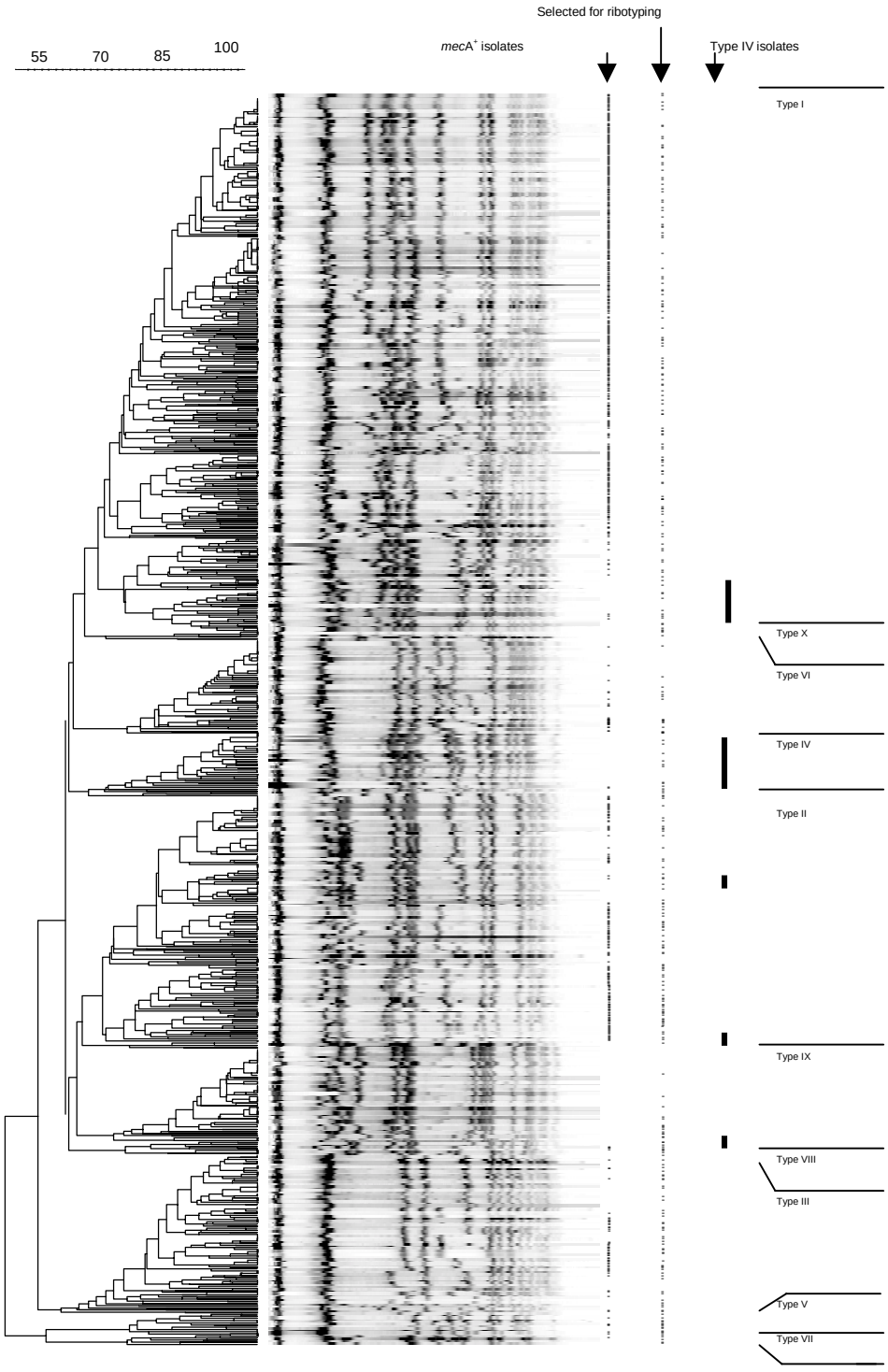

Fig. 1. The PFGE patterns obtained from European and North American MSSA (n=521) and MRSA (n=546) isolates form ten clusters. Isolates containing *mec*A are indicated by hyphens. In order to confirm the clonal relatedness of isolates within the ten clusters, a total of 330 isolates (hyphens) representing the variability of PFGE types were selected for ribotyping (Fig. 2).

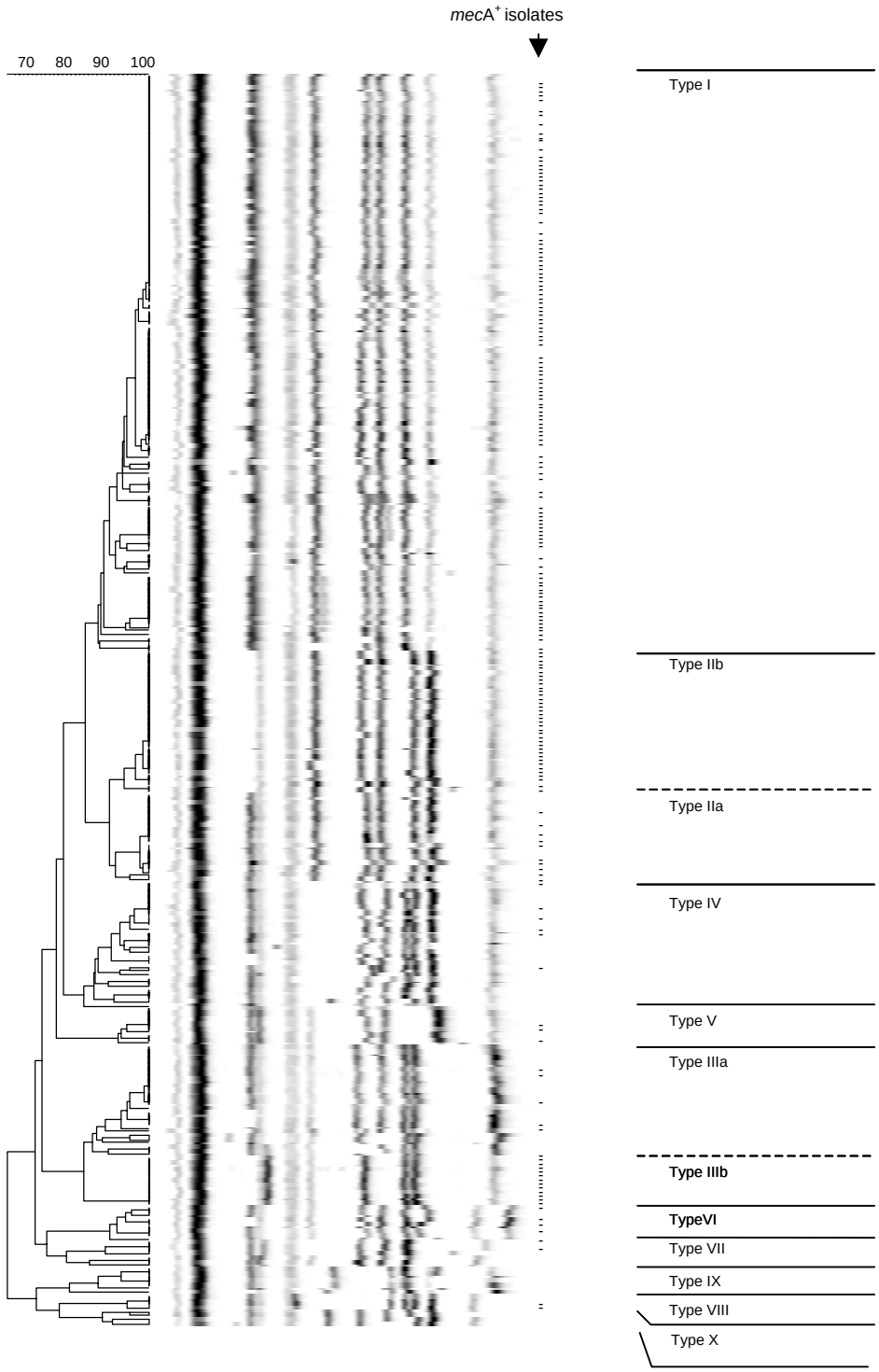

Fig. 2. Ten clusters were again found by ribotyping of 330 isolates. These *S. aureus* lineages were called Types I-X. There was an excellent correlation between the PFGE patterns and ribotyping. Except for the Type IV isolates, 99% of the double-typed isolates were found in corresponding PFGE and ribotype clusters. The position of the Type IV isolates in the PFGE dendrogram is indicated by a black line (panel A). The isolates containing *mec*A are indicated by hyphens.

samples were of the Type II lineage, which dominated the North American samples from the 1980s were later isolated Europe and Africa. Type IIa MRSA isolates (14% of *mec*A$^+$ samples) included the pediatric clone [10] . Type IIb MRSA isolates (11% of *mec*A$^+$ isolates) included the South German clone [11] .

Type III MRSA were found among samples isolated since the 1980s in Africa, Europe, and North America. Type IIIb MRSA isolates (6% of *mec*A$^+$ isolates) included EMRSA 16 [12] . In contrast, Type IIIa, Type IV (the Berlin clone [11]), Type V (EMRSA 15 [12]), Type VI, Type VII, and Type VIII MRSA strains, which appeared during the 1990s, were only isolated sporadically (<1% of *mec*A$^+$ isolates). Interestingly, these sporadic MRSA types were relatively abundant among the *mec*A$^+$ isolates from Chicago (20% Type IV) and the UMC (33% Type IIa, Type IV, and Type VI), hospitals where patients and staff coming from foreign hospitals are screened for MRSA carriership.

Multiresistance and dissemination of MRSA in Europe. The *mec*A gene was present in all isolates resistant to four or more antibiotics. Moreover, this multiresistance was displayed by the most prevalent and geographically widespread MRSA types (I, IIa, IIb, and IIIa), which together represent 99% of the tested *mec*A$^+$ population in Europe.

Most of the Type I MRSA isolates, representing 68% of the recent European MRSA population and being present in 17 of 20 SENTRY hospitals, were resistant to erythromycin (97%), gentamicin (98%), and clindamycin (89%) and showed decreased susceptibility to ciprofloxacin (98%), tetracycline (98%), and rifampicin (98%). Although resistant to erythromicin (98%), clindamicin (88%), ciprofloxacin (98%), and gentamicin (100%), most of the Type IIb MRSA isolates, representing 16% of the recent European samples found in 5 of 20 SENTRY hospitals, remained susceptible to tetracycline (98%) and rifampicin (100%). Type IIIb MRSA isolates, representing 8% of the recent European population and found in 2 of 21 SENTRY hospitals, were resistant to erythromicin (100%), ciprofloxacin (100%) and clindamicin (84%), but remained susceptible to rifampicin (100%) tetracycline (100%) and gentamicin (84%). Also the Type IIa MRSA isolates, representing 7% of population found in 6 of 20 SENTRY hospitals, were mostly resistant to erythromicin (75%), clindamicin (69%), ciprofloxacin (90%), but remained susceptible to rifampicin (100%), tetracycline (100%), and gentamicin (100%). In contrast, Type IIIa, Type IV, Type V, Type VI, Type VII, and Type VIII MRSA, which were only isolated sporadically (<1% of recent European *mec*A$^+$ isolates),

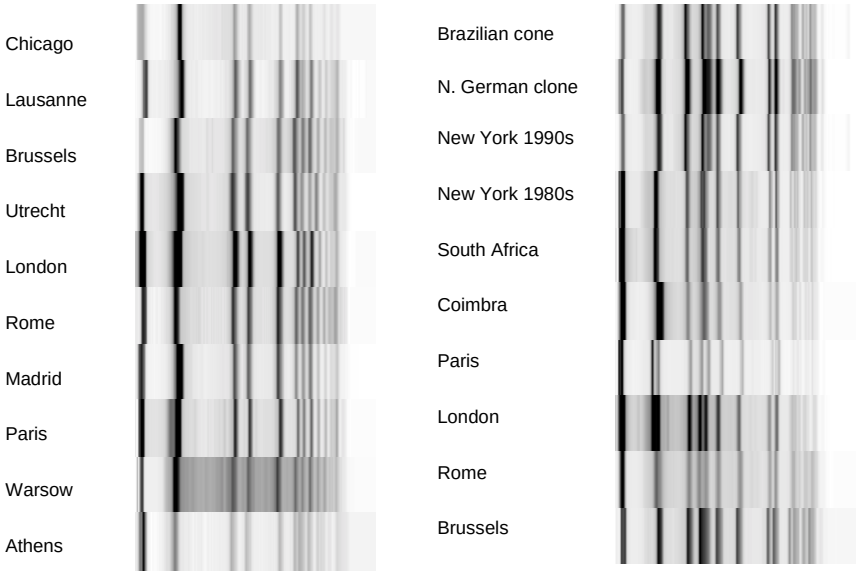

Fig. 3. Examples of Type III MSSA (left) and Type I MRSA (right) isolates referred from different locations, but yielding identical PFGE patterns.

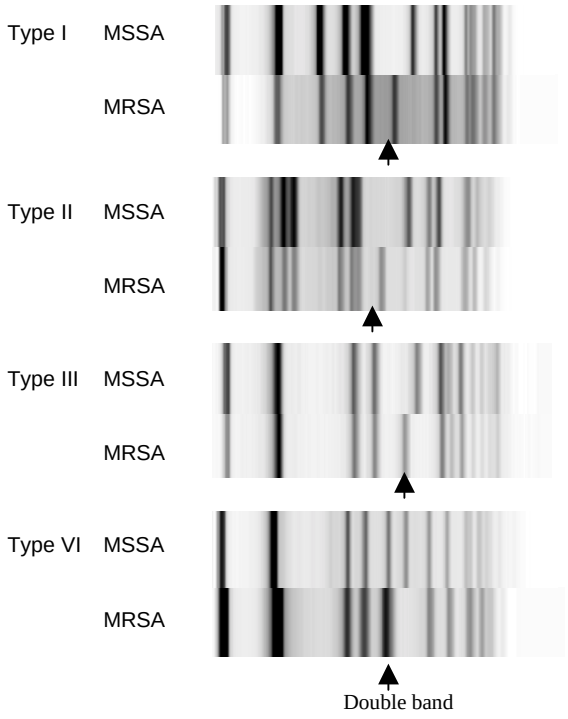

Fig. 4. Most *mec*A^{+} MRSA isolates have *mec*A^{-} MSSA counterparts that differ by a single bandshift (arrow) due to the insertion of a fragment that hybridizes with a *mec*A probe (data not shown).

mostly remained susceptible to all but the ß-lactam antibiotics (clindamycin, 100%; tetracycline, 100%; gentamicin, 100%; rifampicin, 100%; erythromycin, 60%; ciprofloxacin, 60%).

Discussion

This study aimed to examine the dissemination of the *mec*A gene in the *S. aureus* population. Two methods were applied to determine the clonal relationships between *mec*A^{+} MRSA (n=546) and *mec*A^{-} MSSA (n=521) isolates collected between 1960 and 2000 from over 50 locations in the western world. The overall chromosomal organization of the isolates was first compared using *Sma*I-generated PFGE patterns, which provide a relatively quickly evolving genotypic marker. Because there is little evolutionary pressure to conserve the *Sma*I restriction sites *per se*, this technique has high discriminatory power and highlights the differences between the strains. Ribotyping was then used to combine evolutionary closely related PFGE types into clonal lineages. The genes that encode ribosomal DNA are more conserved during evolution and provide a relatively slowly evolving marker. Therefore, automated ribotyping results in fewer ribotypes compared to the diversity of PFGE types.

S. aureus isolates of ten different lineages, called Types I-X, were present in the hospitals studied, and eight of these have acquired the *mec*A DNA. From the major lineages, pandemic MSSA and MRSA clones yielding identical PFGE patterns were collected in many European and North American hospitals and the community. Several pandemic *mec*A^{-} MSSA have *mec*A^{+} counterparts that share the identical ribotype, while their PFGE patterns differ by a single bandshift due to acquisition of the element containing *mec*A. The worldwide appearance of specific MRSA clones has been shown before [9] , but the existence of widespread MSSA counterparts was not described before in detail. In line with this observation, a comparison between MRSA and MSSA samples isolated in the UK and Denmark in the early 1960s suggests that contemporary MSSA isolates served as an early recipient of the *mec*A gene in Europe [13] .

The dissemination of particular MRSA lineages is correlated with their resistance profile. The majority of the multiresistant Type I MRSA isolates, predominant on both continents, lacked susceptibility to tetracycline, erythromycin, clindamycin, gentamicin,

ciprofloxacin, and rifampicin. A second multiresistant lineage (Type IIb), found in North America and several European countries, was susceptible only to tetracycline and rifampicin. Smaller pandemics were caused by isolates susceptible to gentamicin, tetracycline, and rifampicin (Type IIa and Type IIIb). Although antibiotic selection pressure by itself provides a reasonable explanation for the widespread dissemination of such multiresistant strains, additional factors, e.g. modifications in expression of virulence factors and binding capacities, may add to their high prevalence. In contrast, the sporadically isolated *mec*A^{+} MRSA Types IIIa, IV-VIII (1% of recent European isolates) generally remained susceptible to all except the ß-lactam antibiotics. Such sporadic MRSA types were over-represented in the samples from the Chicago community (20%) and the UMC (30%), where the 'Search and Destroy' procedure prevents epidemic MRSA strains from entering the hospital and results in a MRSA prevalence below 1%. Many of the rare MRSA infections that do occur at the UMCU may be formed *de novo* by the horizontal transfer of the *mec*A gene to all resident MSSA lineages, as was witnessed recently *in vivo* when the *mec*A gene was transferred from *Staphylococcus epidermidis* to *S. aureus* during antibiotic treatment [7] . The role of such sporadic isolates in the evolutionary epidemiology of MRSA, however, is not clear.

Although the number of times that the *mec*A gene has been transferred to *S. aureus* remains unknown, the hypothesis that the *mec*A gene entered *S. aureus* on only one occasion has been questioned by several authors [5-7, 14] . The data presented here indicate the repeated horizontal transfer of *mec*A DNA to at least eight resident *S. aureus* lineages and the spread of more resistant clones favored most by antibiotic selection pressure. When DNA microarray technology was used to characterize the genetic diversity of 11 MRSA isolates, the *mec*A gene was detected in at least five highly divergent chromosomal genetic groups [6] . In addition, MLEE data showed that MRSA constitute 15 electrophoretic types forming six clusters or lineages, implicating multiple MRSA lineages arose by horizontal transfer between *S. aureus* strains [5] . Analysis of the *mecI* and *mecR1* region, which lies 5' of the *mecA* gene, revealed that older isolates lacked part of this region, whereas more recent isolates showed a organization at this position similar to coagulase-negative staphylococci [14] . Because these data cannot easily be explained by the hierarchy of rearrangements within a single clone, it was concluded that MRSA strains have independently arisen twice by the horizontal transfer of the *mec*A gene to MSSA.

It has been proposed that coagulase negative staphylococci (CNS) serve as donors for the transfer of the *mec*A gene to *S. aureus* [14] . The *in-vitro* transfer of the *mec*A gene from CNS to *S. aureus* supports this theory [15] . In this context, it is important to note that 70-75% of all CNS worldwide are resistant to methicillin [1] , thus representing a huge potential reservoir of resistance. The mechanism of transfer, however, remains unclear. There is evidence that the *mec*A DNA resides within a mobile genetic element, SSC *mec*, that encodes recombinases for its excision from and integration into the staphylococcal chromosome [3] . This element may also contain other genetic elements, like Tn*554*, pUB110, and pT181, which encode resistance to non-ß-lactam antibiotics, causing multiresistance [3] . Thus, while new MRSA strains continue to emerge by the horizontal transfer of *mec*A, those strains possessing additional resistance traits are favored most by antibiotic selection pressure and may disseminate widely.

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Genotyping of Clinical Methicillin-Susceptible *Staphylococcus aureus* Isolates in a Dutch Teaching Hospital

Yvonne Van Dijk,[1] Camiel L. C. Wielders,[2] Ad C. Fluit,[2] Armand Paauw,[2] Rob J. A. Diepersloot,[1] and Ellen M. Mascini[1,2]*

Departments of Infection Control and Microbiology and Immunology, Diakonessenhuis,[1] and Eijkman-Winkler Institute for Microbiology, Infectious Diseases, and Inflammation, Department of Hospital Hygiene and Infection Prevention, University Medical Center,[2] Utrecht, The Netherlands

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Methicillin-susceptible *Staphylococcus aureus* isolates, recovered from 204 patients in our hospital in a 22-month period, were characterized by pulsed-field gel electrophoresis. Among the multiple *S. aureus* types six clonal lineages dominated, comprising isolates from 158 patients. Despite the limited genetic variation, cross-transmission was made plausible only sporadically.

Staphylococcus aureus is an important causative agent of nosocomial infections, including surgical site infections and catheter-related bacteremia (5, 12). Consequently, microbiologists are frequently asked to determine the relatedness of staphylococcal isolates collected during the investigation of an outbreak or as part of an ongoing surveillance system.

From earlier studies it has been concluded that pulsed-field gel electrophoresis (PFGE) is well suited for genetic analysis and monitoring of nosocomial spread of *S. aureus* (1, 10). Most attention has been focused on the characterization of methicillin-resistant *S. aureus* (MRSA), but not much is known about the structure of methicillin-susceptible *S. aureus* (MSSA). Due to the search-and-destroy policy, MRSA is not endemic in The Netherlands (11). In order to increase the understanding of the molecular epidemiology of MSSA strains in our hospital, we collected all *S. aureus* isolates recovered from clinical specimens between November 1997 and September 1999 and subjected them to PFGE.

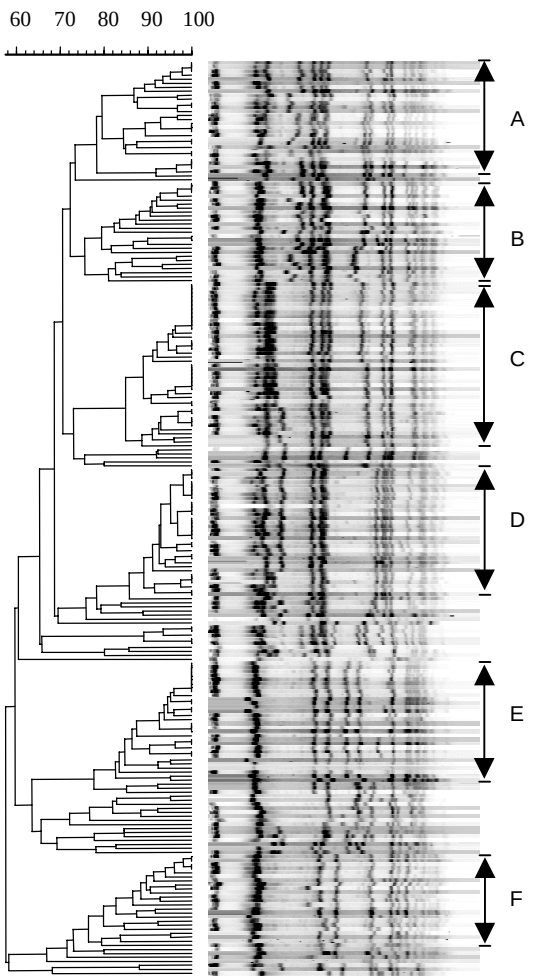
The Diakonessenhuis is a 378-bed teaching hospital in Utrecht, The Netherlands, with approximately 11,500 admissions and 7,000 clinical surgical procedures each year. Between November 1997 and September 1999, the ward, date, and site of isolation were recorded for each hospitalized patient with a positive *S. aureus* culture. *S. aureus* isolates were defined as catalase-producing gram-positive cocci which were positive for coagulase. Antibiograms were determined by disk diffusion on Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards (NCCLS) (6). The antimicrobial agents tested included penicillin, oxacillin, gentamicin, clindamycin, erythromycin, and vancomycin. PFGE typing by *Sma*I macrorestriction was performed as described previously (10). Patterns were not subjected to the guidelines for interpretation of PFGE based on differences in banding patterns posed by Tenover et al., because these are intended to be used to examine relatively small sets of isolates (<30). For larger collections of isolates, equipment to perform computer-based image acquisition and analysis is recommended (9). The PFGE patterns were analyzed by Gel-Compar (Applied Maths, Kortrijk, Belgium). A cutoff value of 70% of genetic similarity was chosen for discrimination between distinct clusters of strains, while confirmation of genetic similarity or difference was performed by visual interpretation of the gels.

Two hundred and twenty-six *S. aureus* isolates recovered from 204 hospitalized patients were available for antimicrobial susceptibility tests and PFGE typing. Most staphylococci were recovered from wounds, pus, drains, and indwelling catheters; 16 isolates were derived from blood and 36 were derived from respiratory specimens. Isolates from 35 patients were susceptible to all antibiotics tested, while all isolates showed in vitro susceptibility to oxacillin and vancomycin. High-level resistance against gentamicin was not observed. The percentages of isolates resistant to penicillin, erythromycin, and clindamycin amounted to 72, 11, and 7%, respectively.

After analysis of PFGE patterns we were able to discriminate 19 main types dominated by 6 clusters comprising 177 isolates from 158 patients, which were designated A through F (Fig. 1). From 22 patients of whom more than one isolate was available for typing, successive isolates were identical in all cases. The genetic diversity observed is in agreement with the limited data available in the literature (2–3). Blumberg et al. identified 15 different ribotypes among a selected collection of 13 MSSA and 37 MRSA isolates (2), while Couto et al. found 23 distinct main types among 54 MRSA and 93 MSSA isolates from a Portuguese hospital by using PFGE (3). Within the six clusters, small variations in genetic profile could be distinguished. We are reluctant to ascribe small variations in profile for isolates to different genetic background or to variations within one strain, since it is not possible to make certain whether isolates with two to six fragment differences are related or not (9).

In agreement with other studies, no consistent correlation between antibiograms and genotype patterns was seen (2–3). Antibiograms would have erroneously identified a large number of MSSA isolates with distinct PFGE types as homogeneous strains. Similar findings have been reported previously for MRSA isolates (3).

* Corresponding author. Mailing address: Eijkman-Winkler Institute for Microbiology, Infectious Diseases, and Inflammation, University Medical Center G04.614, P.O. Box 85500, NL-3508 GA Utrecht, The Netherlands. Phone: 31-302-508784. Fax: 31-302-541770. E-mail: e.m.mascini@lab.azu.nl.



	Patients (isolates)	Wards	Cross-transmission
A	23 (29)	11	1 episode: 3 patients in the cardiology ward.
B	23 (25)	13	1 episode: 3 patients in ward with miscellaneous patients.
C	33 (40)	11	3 episodes: 4,2 and 2 patientsresp. in same surgical wardepsisode: 3 patients ICU.
D	30 (32)	15	
E	27 (29)	13	
F	22 (22)	11	1 episode: 4 patients in the internal ward.

FIG. 1. Dendrogram containing PFGE patterns from clinical MSSA isolates of 204 patients collected between November 1997 and September 1999. Six clusters designated A through F are distinguished, for which numbers of infected patients and affected wards are given. To indicate possible dissemination, episodes in which two or more patients on the same ward were infected with MSSA with identical PFGE types within 1 month of each other are presented. resp., respectively.

The clusters comprised the most frequently encountered types in the hospital, accounting for 158 out of 204 patients (77%). These six clusters were all identified during a prolonged period of at least 15 months. The number of wards where isolates belonging to one of these clusters were recovered varied from 11 to 15. The fact that six clusters were identified next to unique genotypes suggests that staphylococcal strains may vary considerably in epidemiological potential. It seems likely that, among the different PFGE types, certain clusters of *S. aureus* spread easily and remain genotypically relatively con-stant. The minor differences in PFGE patterns among the isolates belonging to the same strain might thus be clarified.

Obviously, the occurrence of cross-infection in our hospital was of minor importance, since outbreaks with an epidemic *S. aureus* strain were not elicited. In addition, clear epidemiolog-ical linkages between patients with an isolate belonging to one of the clustered pulsotypes could generally not be demonstrated. There were seven episodes of repetitive isolation of staphylococci belonging to the same cluster in patients on the same ward within a 1-month period at five wards, involving a total of only 21 patients (Fig. 1). In these cases, cross-trans-mission between hospitalized patients could not be excluded and had possibly occurred via the hands of health care workers (8). Moreover, medical equipment, such as surgical instruments, catheters, ventilators, stethoscopes, and ultrasound in-struments, can be reservoirs for *S. aureus* (7).

Otherwise, it might be speculated that the genetic variation between *S. aureus* isolates among non-hospitalized individuals in the population is limited. Thus, the six clustering genotypes could be highly endemic in the Utrecht area and subsequently be represented in our hospital. Accordingly, the PFGE results suggest that most staphylococcal infections arise endogenously. Correspondingly, several epidemiological studies indicated that nasal carriers of *S. aureus* have an increased risk for the

development of surgical site infection (5, 11). Whereas the majority of MSSA cases in this study could not be explained by cross-infection or a common source, MRSA infections are acquired predominantly by hospital cross-infection (2, 4).

In conclusion, this study was designed to gain insight into the population characteristics of the resident MSSA strains and nosocomial transmission in our hospital. A considerable variation in the genetic background was detected, but six clusters were found to be dominant. PFGE patterns suggest that nosocomial MSSA infections differ from MRSA infections in that most arise endogenously. Cross-transmission which may occur now and then had not resulted in the dissemination of an epidemic MSSA strain in our hospital.

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FREQUENT TRANSFER OF DIFFERENT STAPHYLOCOCCAL CHROMOSOME CASSETTE *mec* TYPES BETWEEN COAGULASE-NEGATIVE STAPHYLOCOCCI AND *STAPHYLOCOCCUS AUREUS*

Abstract

The Staphylococcal Chromosome Cassette *mec* (SCC*mec*) found in *Staphylococcus aureus* was acquired from coagulase-negative staphylococci (CNS). The purpose of this investigation was to determine whether *S. aureus* acquired SCC*mec* just once or on multiple occasions. Therefore, the relationships were studied between SCC*mec* from different clonal lineages of MRSA collected in North America and Europe from the 1960s until 2000 and that from various species of CNS. A total of 146 MRSA and 36 CNS isolates belonging to 7 species were analyzed. Ribotyping showed eight clonal lineages, called MRSA Types I-VIII. Restriction Fragment Length Polymorphism (RFLP) analysis of the SCC*mec* element, using a probe covering approximately 20 kb of this DNA region, revealed four clusters of closely related RFLP patterns, SCC*mec* Types A-D, which were found in 93% of the isolates. Eleven isolates yielded miscellaneous patterns. SSC *mec* TypeA was present in MRSA Type I-II, and in 2 CNS species since the 1960s. SCC*mec* Type B was first observed among MRSA Type II isolates from the 1970s. It was also found in MRSA Types I, III, VI and VIII collected later and in isolates of *S. epidermidis*. SCC*mec* Type C first appeared during the 1980s and was detected in isolates of MRSA Types I and III and 4 CNS species. SCC*mec* Type D was detected in isolates from the 1990s, including MRSA Types I-VII and 3 CNS

species. These results indicate that SCC*mec* transfer from CNS to *S. aureus* took place at least four times and most likely occurred much more often.

Inleiding

The introduction of methicillin for treatment of staphylococcal infections in the early 1960s has resulted in the rapid development of resistance (1). In fact, methicillin-resistant *Staphylococcus aureus* (MRSA) strains currently represent a major cause of nosocomial infections around the world and most coagulase-negative staphylococci (CNS) have become resistant to the drug (2). In some countries, MRSA constitutes up to 80% of all *S. aureus* isolates found in intensive care units (3). It is thought that staphylococci have become resistant to methicillin and other β-lactam antibiotics by acquiring the Staphylococcal Chromosome Cassette *mec* (SCC*mec*) (4). This genetic element contains the *mec*A gene, which encodes the low affinity pencillin-binding protein PBP2a. SCC *mec*, which is of extraspecies origin (5), ranges in size from 30 to more than 60 kb (6). The size difference of the SCC *mec* element can be partly explained by integration of various additional genetic elements, including Tn*554* (encodes resistance to macrolides, lincosamides, and streptogramin B), plasmid pUB110 (encodes resistance to aminoglycosides), and/or plasmid pT181 (encodes resistance to tetracycline) (7). The number of times *S. aureus* has acquired SCC*mec* is as yet unknown.

The purpose of the present study was to determine whether *S. aureus* acquired SCC*mec* on one or multiple occasions. SCC *mec* in different staphylococci was characterised by comparing the SCC *mec* fingerprints obtained from different lineages of North American

and European MRSA isolates collected since the 1960s and from various species of coagulase-negative staphylococci (CNS).

Methods

Bacterial isolates. A total of 146 MRSA isolates were selected from the collections of Kreiswirth et al. (8), Roberts et al. (9), de Sousa et al. (10), Witte et al. (11), Hookey et al. (12), and the SENTRY antimicrobial surveillance program (13). This selection was based on the isolates' PFGE type and the place and year of isolation (Table 1). In addition to recent European MRSA isolates, this selection included the earliest isolates from Europe and Africa collected during the 1960s, North American isolates collected from the 1970s until the present, and earlier described reference strains like the Iberian clone, the Brazilian clone, the North and the South German clones, the Berlin clone, the Hannover clone, EMRSA 15 and 16, the Portuguese clone, and the pediatric clone (8-12, 14, 15).

Further, 36 methicillin-resistant CNS isolates from 15 different hospitals in 11 European countries were randomly selected to represent different genotypes. These isolates included 23 *Staphylococcus epidermidis*, 6 *Staphylococcus haemolyticus*, 3 *Staphylococcus xylosus*, 1 *Staphylococcus lentus*, 1 *Staphylococcus warneri*, 1 *Staphylococcus simulans*, and 1 *Staphylococcus auricularis*. The origin of the MRSA and CNS isolates is shown in Table 1. The isolates were identified using standard microbiological methods.

For comparison of ribotypes, we selected 10 MSSA isolates, which represent 10 widespread MSSA lineages identified among 521 MSSA isolates during a previous epidemiological study (submitted).

Tabel 1. Place and time of isolation of the MRSA and CNS isolates.

collection	time	location	MRSA	CNS
SENTRY	90s	France	10	5
		Italy	10	7
		UK	10	2
		Portugal	6	1
		Turkey	5	1
		Spain	5	1
		Austria	3	
		Greece	2	7
		S. Africa	6	
		Germany	1	1
		Belgium	1	1
		Poland		3
UMC	90s	Netherlands	14	7
Dr Hookey	90s	Europe	16	
Dr Roberts	90s	New York	7	
Dr Lencastre	90s	Portugal	4	
Dr Witte	90s	Germany	3	
Dr Kreiswirth	60s	Europe	6	
	60s	Africa	2	
	70s	N. America	1	
	80s	N. America	27	
	80s	Europe	7	

Ribotyping of isolates. Ribotyping was performed with an automated riboprinter system (Qualicon, Wilmington, DE, USA), using *Eco*RI as described previously (16).

Construction of the SCC *mec*-specific probe. The isolation of a *mec*A^{+} MRSA and its *mec*A^{-} MSSA counterpart has already been described (17). Both of these isolates were shown to be suceptible to macrolides, lincosamides, gentamicin, and tetracycline. They differed by a single bandshift in their *Sma*I-generated PFGE patterns. This bandshift was caused by the presence of approximately 40 kb additional DNA in the MRSA isolate. Moreover, the DNA fragment from the MRSA isolate hybridized with a *mec*A-specific DNA probe. In this study, the *Sma*I-generated DNA fragments with and without the additional

DNA were isolated from agarose gel and used for subtractive hybridization in order to generate a DNA probe specific for SCC*mec*. Subtractive hybridization was carried out using the PCR Select Subtraction kit as recommended by the manufacturer (Clontech Laboratories, Palo Alto, Ca, USA). The differential DNA fragments were labeled by subsequent amplification in the presence of digoxigenin-labeled nucleotides using the PCR DIG probe kit as described by the manufacturer (Roche, Basel, Switzerland).

SCC*mec* fingerprinting. One μg genomic DNA isolated from the staphylococcal isolates was simultaneously digested with 1 U each of *Eco*RI, *Cla*I, and *Hin*DIII. The restriction fragments were then separated in a 1.5% agarose gel, transferred to a nylon membrane, and hybridized with the SCC*mec*-specific DNA probe. Hybridization was visualized using the DIG Luminescent Detection kit (Roche).

Analysis of riboprints and SCC*mec* fingerprints. The riboprints and SSC *mec* fingerprints were compared by calculating a similarity index using the UPGMA cluster algorithm and Dice coefficient using Bionumerics software (Applied Mathematics, Kortrijk, Belgium) scoring individual bands for comparison.

Detection of Tn*554*. Using the primers CTTGGTTCCTGAATTTGTCC and TAGGCAAAGAATCGAATC (26) Tn*554* was amplified in the presence of digoxigenin-labeled nucleotides using the PCR DIG probe kit as described by the manufacturer (Roche). Spot-blots of the MRSA isolates were hybridized with this probe and hybridization was visualized using the DIG Luminescent Detection kit (Roche).

Results

Ribotyping of MRSA. Analysis of the riboprints of 146 MRSA isolates from different cities and collections in Europe, North America, and Africa revealed eight different clonal lineages, called MRSA Types I-VIII (Fig. 1). Both MRSA Type II and Type III could be divided into two closely related subtypes (a and b), based on slight differences in their riboprint (Fig. 1) and resistance patterns (data not shown). All European and African isolates obtained during the 1960s belonged to MRSA Type I, a type that now predominates around the world. MRSA Types II and III first appeared during the 1970s and 1980s, respectively, among the North American isolates of the Kreiswirth collection. Later, they were also isolated in Europe and Africa. The other five MRSA types (IV-VIII) have been isolated sporadically in Europe and North America since the end of the 1980s. When the MRSA riboprints were compared to 10 different MSSA ribotypes identified previously, we found MSSA counterparts with identical riboprints for each of the 8 MRSA types (Fig. 2).

Characterization of the SCC*mec*-specific probe. In order to fingerprint SCC*mec* from different staphylococci, a specific probe was constructed from a *mec*A^{+} MRSA isolate and its *mec*A^{-} MSSA counterpart using subtractive hybridization. Hybridization of the probe with *Eco*RI-, *Cla*I-, and *Hin*DIII-digested DNA from the isolates showed that the probe recognized approximately 20 kb of MRSA DNA. It did not hybridize with the DNA obtained from the MSSA isolate (Fig. 3).

SCC*mec* fingerprints from different staphylococci. Analysis of the fingerprints obtained from the hybridization of *Eco*RI-, *Cla*I-, and *Hin*DIII-digested DNA from 146 MRSA and 36 CNS isolates showed that 93% of the patterns formed four large clusters, called SCC*mec* Types A-D (Fig. 4).

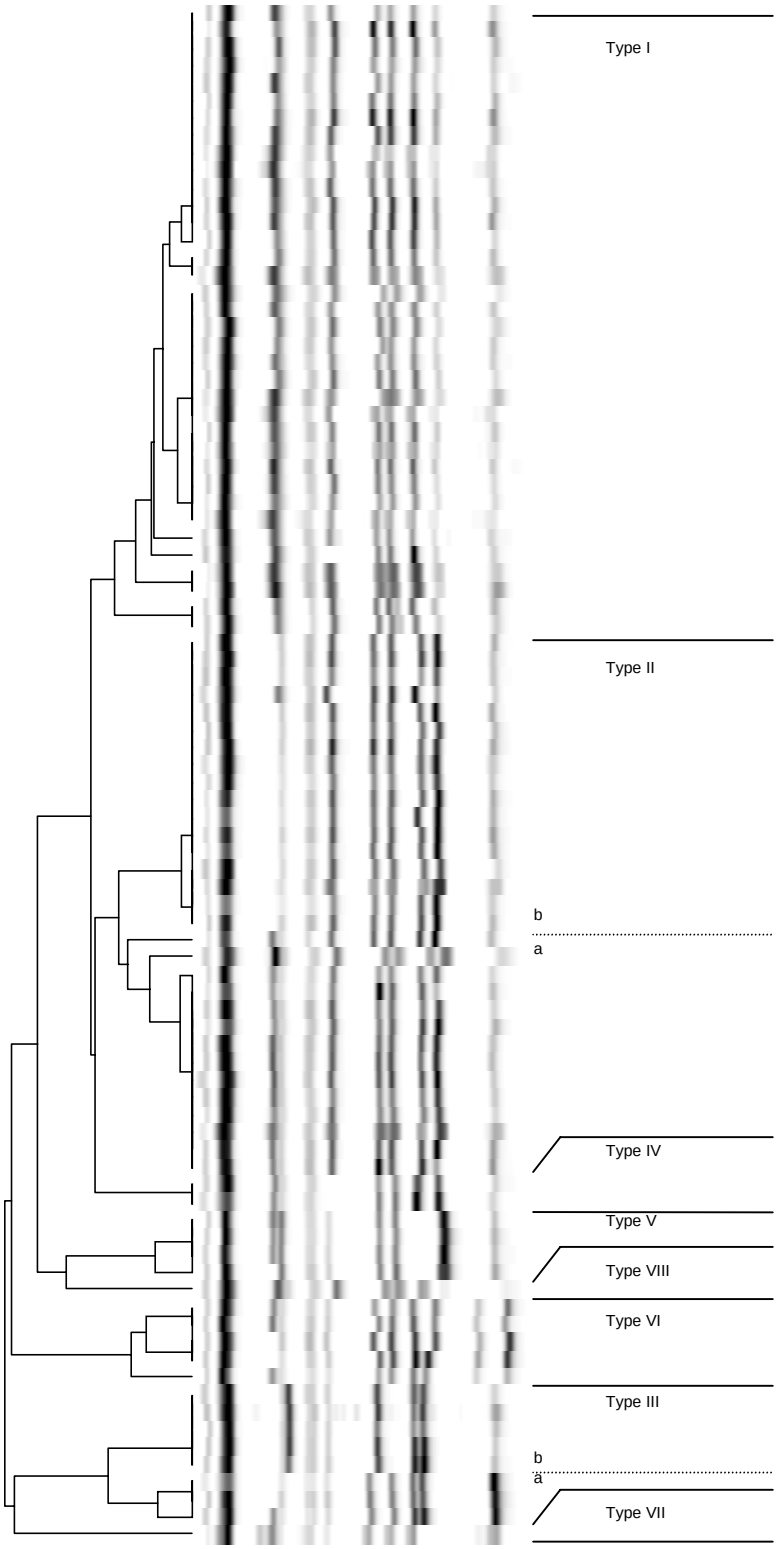

Fig. 1. Dendrogram containing the riboprints of 146 *mec*A^{+} MRSA isolates. Eight branches were recognized, called MRSA Types I-VIII. Types II and III could be divided into subtypes (a & b; dotted line).

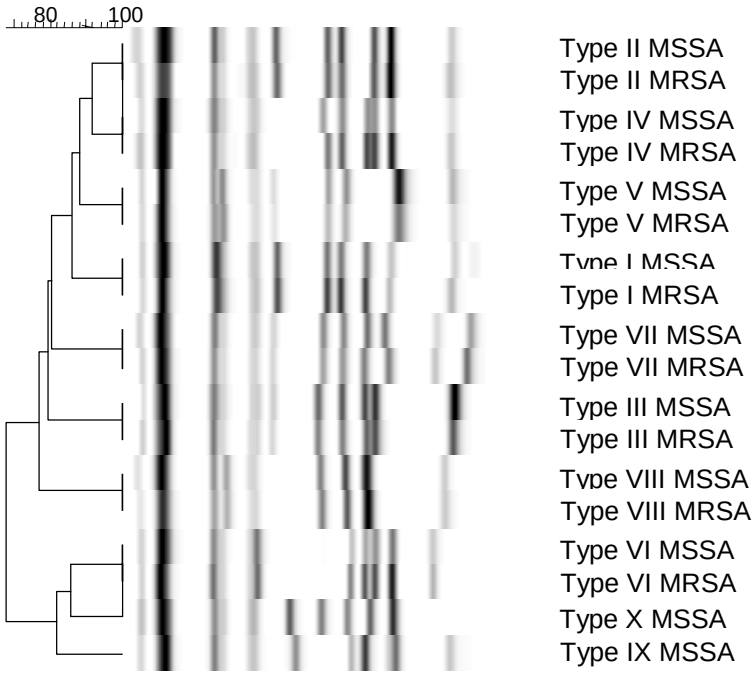

Fig. 2. Comparison of the riboprints of the 8 MRSA types to those of 10 widespread MSSA types showed they were part of the same lineages.

Different staphylococcal species and MRSA types were represented within each of the four clusters. Sometimes, the fingerprints obtained from different MRSA types and staphylococcal species were identical. Eleven isolates yielded patterns that shared less homology with the four clusters (Fig. 4).

SCC*mec* Type A was present in MRSA Type I isolates collected since the 1960s, in Type II isolates collected since the 1980s (including all Subtype IIb MRSA), and in *S. haemolyticus*, and *S. warneri* isolates. SCC *mec* Type B was first found in Type IIa MRSA isolates collected at the end of the 1970s. These isolates predominated the North American isolates from the 1980s in the Kreiswirth collection, and are now found worldwide. Type B SCC *mec* was also detected in isolates of MRSA Types I, III, VI, and VIII from that collection and in *S. epidermidis* isolates. MRSA isolates carrying the Type C SCC *mec* were first collected during the 1980s and included MRSA Type I and all Type IIIb isolates.

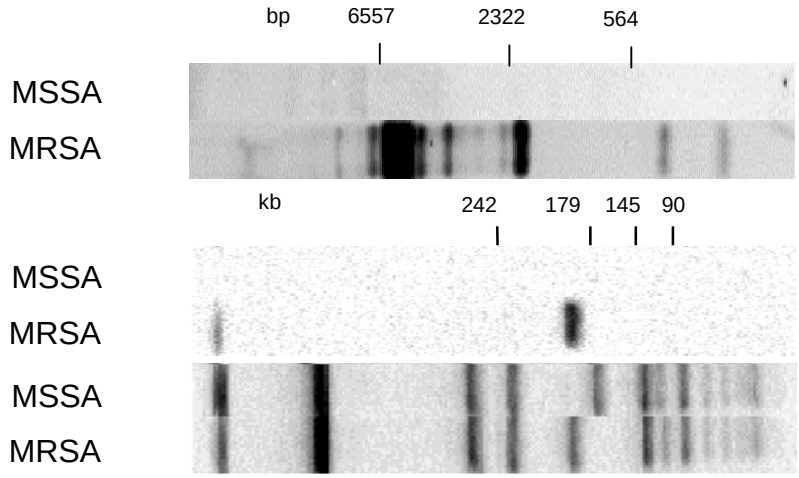

Fig. 3. MSSA and MRSA counterparts yielding identical PFGE patterns, except for a single bandshift (bottom lanes) caused by the insertion of a fragment that hybridized to a *mec*A probe (middle lanes), were used to isolate SCC *mec* by subtractive hybridization. The different fragments obtained after labeling recognized approximately 20 kb of additional DNA in the MRSA isolate. No hybridization was observed in the MSSA isolate (upper lanes).

Nine isolates of *S. epidermidis*, 2 *S. haemolyticus*, 1 *S. lentus*, and 1 *S. simulans* also carried SCC*mec* Type C. SCC*mec* Type D was present in seven different MRSA types and three CNS species. Most of the MRSA isolates with SCC*mec* Type D belonged to smaller MRSA lineages, i.e. Types IV, V, VI, and VII and Subtype IIIa, but few were Type I or Type IIa MRSA. All but one of these isolates had been isolated during the 1990s. Nine isolates of *S. epidermidis*, 2 *S. haemolyticus*, and 1 *S. xylosus* also contained SCC*mec* Type D. The SCC*mec* fingerprints of 5 isolates of Type I and Type II MRSA, 9 *S. epidermidis*, 2 *S. xylosus*, 1 *S. haemolyticus*, and 1 *S. auricularis* showed little homology with the patterns seen in the four major clusters.

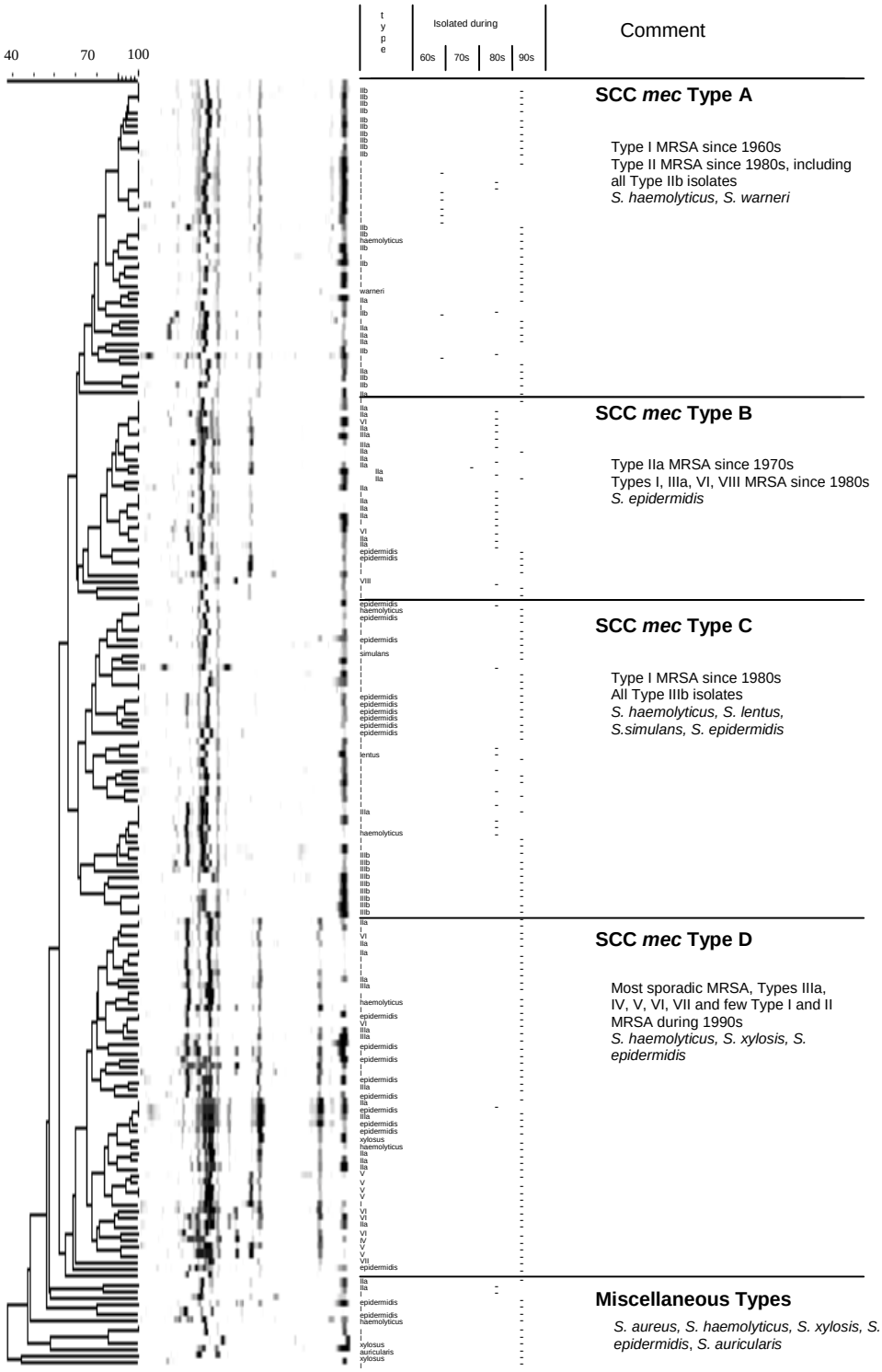

Fig. 4. Dendrogram containing the restriction fingerprints of SCC*mec* obtained from 182 isolates belonging to 8 staphylococcal species and including different MRSA types. Based on the presence of short hybridizing sequences, four clusters could be discerned. Each cluster contained several different species. A few isolates showed little homology with the patterns seen in the four major clusters.

Detection of *Tn554*. *Tn554* was detected in all MRSA types and CNS species using a probe (data not shown). Nearly all of the isolates harboring SCC *mec* Types B and C carried Tn*554*, while only half the isolates with SCC *mec* Type A and a quarter of those with SCC *mec* Type D carried this transposon.

Discussion

Although the SCC*mec* in *S. aureus* is believed to be acquired by horizontal transfer from a CNS species (5), the frequency of this transfer, the evolution of SCC*mec*, and the mechanism of transfer are still a matter of debate. An early study concluded that MRSA has a clonal origin (8). This was based on Southern blots of isolates collected worldwide using *mec*A- and Tn*554*-specific probes, but our data show that only part of the MRSA and CNS isolates carry Tn*554*. Furthermore, the "clonal orgin" theory of MRSA was challenged by a MLEE study, which used many of the same isolates (18). It showed that MRSA constitutes 15 electrophoretic types that form six clusters or lineages and suggested that the multiple MRSA lineages arose by the horizontal transfer of *SCCmec* between *S. aureus* strains. Recently, based on the PFGE typing of 378 MRSA isolates from Germany, revealing 14 epidemic strains among 39 different genotypes, it was proposed that the sporadic genotypes had arisen by the horizontal transfer of SCC*mec* from epidemic strains that were frequently found nearby (19). The hypothesis that MRSA forms a clonal lineage was further challenged by the recent analysis of 11 MRSA isolates using micro array technology: these isolates belonged to at least five different lineages and there was an extensive diversity in SCC *mec* between the lineages (20). Two studies analyzing the *mec*I / *mec*R1 regions, which lies 5' of

the *mec*A gene, showed that older isolates lacked part of this region, while in more recent MRSA isolates that region was similar to that of CNS isolates (6, 21). Because this cannot be easily explained by a hierarchy of rearrangements within a single clone, it was suggested that SCC*mec* transfer from CNS into *S. aureus* might have taken place at least two times.

The purpose of the present study was to determine whether SCC*mec* was acquired by *S. aureus* on one or multiple occasions. To do this, we studied the relationships between SCC*mec* from different clonal lineages of MRSA obtained from North America and Europe from the 1960s until 2000 and from various species of CNS. Ribotyping 146 MRSA isolates revealed eight lineages, called MRSA Types I-VIII. These eight MRSA lineages all had widespread MSSA counterparts having the identical ribotype, some sharing identical PFGE patterns except for the fragment carrying the *mec*A gene (17). These data extend the identification of several historically early MSSA strains, whose genetic backgrounds match those of contemporary epidemic clones of MRSA (22).

The majority of the isolates collected between 1960 and 2000 from various geographic locations contained one of four common SCC*mec* fingerprints, Types A-D. Most importantly, the isolates carrying a particular SCC*mec* type belonged not only to different ribotypes, but even to different staphylococcal species. SCC*mec* Type A, observed first in isolates collected during the 1960s, was detected in MRSA Types I and II, *S. haemolyticus* and *S. warneri*. This type is characterized by the absence of regulatory genes (6, 21), which allows the constitutive expression of PBP2a (6, 23). SCC*mec* Type B was observed in epidemic North American MRSA Type II isolates collected in the 1970s, in isolates of MRSA Types I, III, IV, and VIII collected later, and in *S. epidermidis* isolates. They carried the regulatory genes that were missing from earlier isolates (6, 21). SCC*mec* Type C first appeared in isolates from the 1980s. It was observed in isolates of MRSA Types I and III

and *S. epidermidis*, *S. haemolyticus*, *S. lentus*, and *S. simulans*. SCC*mec* Type D was detected in isolates from the 1990s, including MRSA Types I-VII, and in isolates of *S. epidermidis*, *S. haemolyticus*, and *S. xylosus*. In addition, a few miscellaneous fingerprints, showing little homology with the others were also observed among different MRSA types and CNS species. It is not clear whether the miscellaneous SCC*mec* fingerprints in these MRSA isolates were obtained from CNS by horizontal transfer or whether they evolved by rearrangement. At any rate, SCC*mec* seems to be maintained stably in all Type IIb and Type IIIb isolates. The presence of at least four SCC*mec* types in different CNS species and in eight different *S. aureus* ribotypes is difficult to explain by the separate evolution of SCC*mec* in *S. aureus* and CNS.

Our data implicate that SCC*mec* transfer from CNS to *S. aureus* took place at least four times, but most likely occurred even more often. This latter hypothesis is compatible with and extends the results obtained by other investigators. Frequent horizontal transfer of SCC *mec* between staphylococcal species is further supported by the fact that a novel *mec*A^{+} MRSA and its *mec*A^{-} MSSA counterpart were isolated from a single patient together with an *S. epidermidis* isolate that yielded an SCC*mec* fingerprint identical to that of the MRSA strain (17).

Other occasions of horizontal transfer of other genetic material between different species of staphylococci has already been described, e.g. the transfer of plasmids encoding resistance to several antibiotics (24) (25). Our data provide evidence that the horizontal transfer of SCCs, including integrated elements like the *mec* region and possibly TN*554*, has occurred frequently between different species of staphylococci.

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Evidence for *in-vivo* transfer of *mec*A DNA between staphylococci

C L C Wielders, M R Vriens, S Brisse, L A M de Graaf-Miltenburg, A Troelstra, A Fleer, F J Schmitz, J Verhoef, A C Fluit

Staphylococcus aureus is thought to have acquired *mec*A DNA by horizontal transfer. DNA fingerprints made by restriction nucleases that cut certain sequences of DNA can be used to compare complete genomes or particular genes between bacteria. We isolated an epidemic *mec*A$^-$ methicillin-susceptible *S. aureus* genotype and, subsequently, a rare isogeneic *mec*A$^+$ meticillin-resistant S aureus (MRSA) genotype from a neonate who had never been in contact with MRSA. This MRSA contained *mec*A DNA that was identical to that in a coagulase-negative staphylococcal strain isolated from this patient, but different from other MRSA genotypes. We believe that this MRSA was formed *in vivo* by horizontal transfer of the *mec*A DNA between two staphylococcal species.

Meticillin-resistant *Staphylococcus aureus* causes nosocomial infections worldwide. Its strong resistance is a result of its penicillin-binding protein 2a, which has a low affinity for ß–lactam antibiotics[1]. Penicillin-binding protein 2a is encoded by the *mec*A gene, which is in the chromosome of meticillin-resistant strains of many staphylococcal species. Horizontal *mec*A transfer could contribute to the worldwide dissemination of MRSA[2]. The *mec*A DNA has been identified in a 40 kb mobile genetic element that encodes recombinases that can catalyse its excision from, and integration into, the *S aureus* chromosome[3]. We isolated a successive pair of isogeneic *mec*A$^-$ and *mec*A$^+$ *S aureus* strains from a patient whose initial meticillin-susceptible infection had just been treated with ß-lactam antibiotics. We analysed the molecular epidemiology of the isolates to attempt to answer whether *mec*A DNA was acquired by a pre-existing *mec*A$^-$ variant during treatment or lost by a pre-existing *mec*A$^+$ counterpart. A male infant with Pierre Robin syndrome was delivered by forceps after a 40-week pregnancy. He was intubated and *mec*hanically ventilated from 4 days after birth because of respiratory problems. Amoxicillin and clavulanic acid was used to treat a suspected respiratory tract infection. When he became bacteraemic 3 days later, the treatment was changed

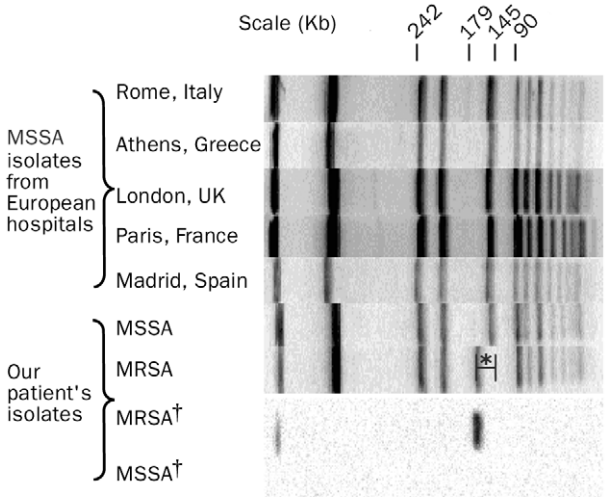

Figure 1: **Pulsed-field gel electrophoresis patterns of *S aureus* isolates from our patient and from isolates in European hospitals**
*Bandshift of about 40 kb from addition of *mec*A DNA (bottom two lanes). †hybrised with *mec*A probe only.

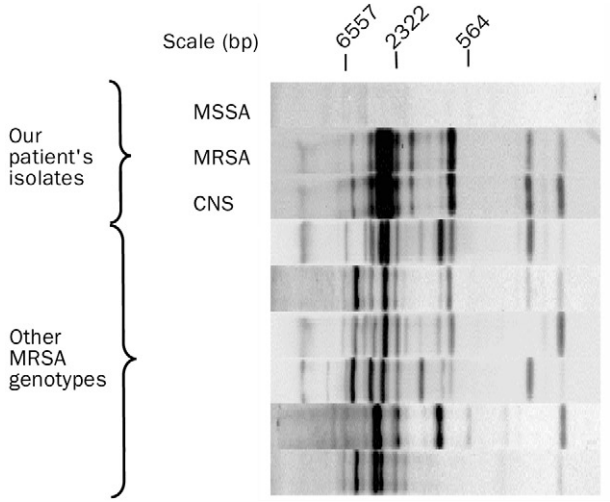

Figure 2: **Restriction patterns of *mecA* DNA from our patient's MRSA and coagulase-negative *S epidermidis*, and from other MRSA isolates**
Hybridised to probe specific for *mec*A region. MRSA=meticillin-resistant *S aureus*. MSSA=meticillin-susceptible *S aureus*. CNS=coagulase-negative staphylococci.

to amoxicillin and cefotaxime. Eventually, when his blood cultures grew a *mec*A⁻ meticillin-susceptible *S aureus* (MSSA), treatment was changed to flucloxacillin. He then seemed to recover, but amoxicillin and clavulanic acid were restarted on day 32 for 10 more days because his respiratory tract infection recurred. On day 56, routine cultures of nasal swabs unexpectedly showed a *mec*A⁺ MRSA that was resistant to β-lactam antibiotics, but susceptible to other antibiotics. Several strains of *mec*A⁺ coagulase-negative staphylococci were also identified. The patient was put in strict isolation to prevent the spread of this MRSA strain. However, because he had improved, no further antibiotic treatment was required for the remainder of his stay in hospital.

We digested DNA from the isolates with SmaI and separated the fragments by pulse-field gel electrophoresis. The *mec*A⁻ MSSA and *mec*A⁺ MRSA isolates differed by only one bandshift, which was caused by about 40 kb of DNA that hybridised with a *mec*A probe in the MRSA isolate (figure 1). Moreover, the MSSA and MRSA isolates had identical ribotypes and a rare phage type that had not previously been recorded by the Dutch reference centre (data not shown).

The *mec*A⁺ MRSA genotype from our patient was unique among a collection of 312 European MRSA isolates (data not shown). Its phagetype, antibiogram, pulsed-field gel electrophoresis pattern, and ribotype did not seem to be related to any MRSA strains in western and central Europe. However, the isogeneic *mec*A⁻ MSSA genotype from our patient is frequently isolated in Europe (figure 1), and from patients and staff in our hospital.

*Mec*A⁺ coagulase-negative staphylococci might be a source of *mec*A DNA in horizontal transfer.[2] The week after we isolated the MRSA, three *mec*A⁺ coagulase-negative staphylococci strains were isolated from the patient. *Mec*A DNA from an *S epidermidis* strain had a hypervariable region,[4] *Tn*554, and *mec*I profile that was identical to the *mec*A DNA in the MRSA isolate (data not shown). We compared the restriction patterns (simultaneous *Cla*I, *Eco*RI, and *Hind*III digestion) of the *mec*A DNA in both these strains more closely, with a specific probe that recognises 20 kb of the 40 kb *mec*A DNA region. The *mec*A restriction patterns were identical in the two strains, whereas those from all the other MRSA genotypes that we assessed were substantially different (figure 2). Therefore, a potential donor strain for *mec*A transfer was present in the patient.

Our results show that, except for the *mec*A DNA, the MSSA and MRSA isolates were isogeneic—ie, they evolved either by the loss or acquisition of *mec*A DNA. The order in which we isolated the strains (*mec*A⁻ before *mec*A⁺) supports the horizontal gene transfer of *mec*A DNA; if the *mec*A⁺ MRSA had been present early on, it seems unlikely that it would be seen only after, and not during, antibiotic treatment. Furthermore, if the *mec*A⁺ MRSA existed before the *mec*A⁻ MRSA, *mec*A⁺ would probably have been more, rather than less, prevalent than the isogeneic *mec*A⁻ variant because of its apparent selective advantage.

Because of the search and destroy policy in the Netherlands,[5] the MRSA prevalence in our hospital remains less than 1%. The new *mec*A⁺ MRSA genotype is difficult to explain since we isolated it from an infant younger than 2 months, who was neither transferred from a foreign hospital nor in contact with an MRSA carrier. We conclude that this MRSA isolate was formed in vivo, during treatment, by the horizontal transfer of *mec*A DNA from an *S epidermidis* strain. Many *mec*A⁺ MRSA might be formed in this way, followed by the spread of multiresistant clones that are favoured most by antibiotic selection pressure.

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Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, University Medical Center, Heidelberglaan 100, 3584 CX Utrecht, Netherlands (C L C Wielders, M R Vriens MD, S Brisse PhD, L A M de Graaf-Miltenburg, A Troelstra MD, A Fleer MD, F J Schmitz MD, Prof J Verhoef MD, A C Fluit PhD)

Correspondence to: Dr C L C Wielders (e-mail: c.wielders@lab.azu.nl)

GENERAL DISCUSSION

The introduction of methicillin into medical practice in the early 1960s has resulted in the continuous selection of resistant staphylococci in hospitals. By now, most commensal coagulase-negative staphylococci (CNS) have become resistant to methicillin, while methicillin-resistant *Staphylococcus aureus* (MRSA) strains represent a major cause of nosocomial infections around the world (1). While low-level methicillin-resistance usually results from ß-lactamases or alterations of intrinsic PBPs, high level resistance is due to expression of the *mec*A gene, that is present in 90% of all MRSA. *Mec*A$^+$ MRSA has become endemic throughout most of Europe: about 25% of the *S. aureus* isolates are resistant to methicillin (Chapter 2). However, the prevalence of MRSA varies considerably between different European countries, but also between hospitals within a country. In some Swiss and Dutch hospitals where strict infection control measures are applied, including screening and isolation of patients, the MRSA prevalence remains below 1% (2).

Multiresistance and SCC*mec*

Due to resistance genes present both inside and outside of the Staphylococcal Chromosome Cassette *mec*, most MRSA strains are also resistant to at least three additional generically different antibiotics. It was shown that, while only 5% of the European MSSA displayed multi-drug resistance, 99% of the European *mec*A$^+$ MRSA were multiresistant (Chapter 2). In fact, the *mec*A gene was detected in all multiresistant isolates, including the latter 5% of multiresistant isolates remaining susceptible to methicillin in the phenotypic test (Chapter 3). This may be explained by the inadequate expression of the PBP2a protein in these isolates. Although separate resistance traits may evolve in any strain independent of SCC*mec*, multirestance appears to be exclusively associated with SCC*mec* in *S. aureus* (Chapter 3). Until now, *mec*A, encoding resistance to β-lactams, *erm*A, encoding resistance to erythromycin, *aad*D, encoding resistance to tobramycin, *ble*, encoding resistance to bleomycin, *spc*, encoding resistance to spectomycin, *tet*K, encoding resistance to tetracycline, *cad*A/C, encoding resistance to cadmium, and *mer*A/B/T, encoding resistance to mercurium, have been detected in SCC*mec* (3).

Clonal relationships of MRSA isolates

Different genotyping techniques were applied to study the clonal relationships among *S. aureus* isolates. MLEE studies showed that the *mec*A gene was transferred to at least five different *S. aureus* chromosomal backgrounds (4, 5). When automated ribotyping and PFGE were combined to determine the clonal relationships of European and N. American *S. aureus* isolates, ten different lineages, called Types I-X, were distinguished, and eight of these had acquired the SCC*mec*. Several pandemic *mec*A$^-$ MSSA were identified, that have *mec*A$^+$ counterparts that share the identical ribotype, while their PFGE patterns differ by a single bandshift due to acquisition of SCC*mec* (Chapter 3). SCC*mec* is transferred to the resident *S. aureus* lineages repeatedly, forming numerous different *mec*A$^+$ MRSA clones. Several widespread multiresistant MRSA clones have been reported (6, 7), and the dissemination of particular clones correlated strongly with their resistance profiles (Chapter 3).

The earliest MRSA encountered in Europe and Africa during the 1960s were Type I clones, which are now present in nearly all hospitals on all continents studied (1). We estimate that a single multiresistant Type I MRSA clone, which acquired resistance to tetracycline, erythromycin, clindamycin, gentamicin, ciprofloxacin and rifampicin, causes over 10% of all nosocomial *S. aureus* infections around the world. During the 1970s, Type II MRSA appeared, while Type III MRSA arose during the 1980s, and both became widespread. A multiresistant Type II clone, which remains susceptible to tetracycline and rifampicin, is present in several European countries and North America. Smaller pandemics are caused by multiresistant Type II and III MRSA clones susceptible to gentamicin, tetracycline and rifampicin. In addition to such widely disseminated multiresistant MRSA, there were sporadic MRSA clones (Types I-VIII), the majority of which arose during the 1990s. These generally remained susceptible to all but ß-lactam antibiotics, and were cultured from only few patients.

In general, MSSA strains causing infections in hospitals are derived from the community, and the occurrence of cross-infection between hospitalized patients is of minor importance (Chapter 4). It was shown that virulence correlates with colonization of the host (8) and that nasal carriers of *S. aureus* have increased risk to develop surgical site infections (9, 10). Whereas most MSSA infections arise endogenously, most MRSA strains are acquired in the hospital (11). The *S. aureus* population in many hospitals is taken over by epidemic multiresistant SCC*mec* harboring clones, which were transmitted to hospitals around the world (Chapter 3). Due to antibiotic selection pressure in hospitals, multiresistant MRSA strains may replace the community-derived MSSA strains, and subsequently cause disease. Although antibiotic selection pressure by itself provides a

reasonable explanation for the wide spread dissemination of such multiresistant strains, other factors, including adhesion factors (3, 12), may add to their prevalence. In addition, some strains may have optimized the expression of the *mec*A gene or have undergone secondary alterations supporting the action of PBP2a. The global dissemination of few multiresistant clones implies that, since identical strains pose identical threats to all hospitals, the differences in MRSA prevalence among hospitals result from differences in infection control policies and use of antibiotics.

Dissemination of SCC*mec* among staphylococci

The evolution of SCC*mec* in staphylococci was studied by comparing the restriction fragment length polymorphisms of this DNA element from different MRSA types and different CNS species. A common origin for the SCC*mec* in staphylococci is suggested by the homogenous restriction fingerpints obtained from majority of isolates from all species, that share many restriction fragments and form a temporary ordered tree (13)(Chapter 5). After SCC*mec* presumably evolved in CNS to protect against the older ß-lactams, it was passed on to several staphylococcal species by horizontal transfer. It has gained access to an increasing number of resident *S. aureus* lineages during four major waves, that are characterized by Type A-D restriction patterns also obtained from CNS (Chapter 5). These types, that problably correspond to the SCC*mec* Types I-IV described by Ito and Hiramatsu (3, 14), have all entered several *S. aureus* lineages and each formed multresistant MRSA. Indeed, MRSA isolates yielding identical riboprints, showing they are part of one and the same lineage, may contain different types of SCC*mec*: they had been formed on separate occasions by the repeated horizontal transfer events.

SCC*mec* Type A (SCC*mec* Type I) sequences found first in European and African Type I MRSA isolated soon after the introduction of methicillin are characterized by deleted regulatory genes (15), allowing constitutive expression of PBP2a (16). It was also found in *S. haemolyticus*, and *S. warneri* and, since the 1980s, in Type II MRSA. Although some of the earliest isolates carrying SCC*mec* Type A were only resistant to methicillin, other strains displayed multi-drug resistance.

After a decline in the prevalence of MRSA at the end of the 1960s (17), a new SCC*mec* type appeared in Type II MRSA during the 1970's, which caused major outbreaks in North America. Because the SCC*mec* Type B (SCC*mec* Type II or III) did contain the regulatory genes that were missing from earlier MRSA, it must have originated in CNS, like *S. epidermidis,* which carried these regulatory sequences as well (15). SCC*mec* Type B rapidly proved capable of entering additional *S.*

aureus lineages, forming mostly multiresistant clones.

The Type C SCC*mec* (SCC*mec* Type II or III) was detected in five staphylococcal species, including mostly multiresistant Type I and III MRSA since the 1980's.

During the 1990's, SCC*mec* Type D (SCC*mec* Type IV) is found in four staphylococcal species and a wide variety of North American and European *S. aureus* lineages, including Type I-VII. These include many of the sporadic MRSA types, which are often only resistant to methicillin, but also some muliresistant strains.

MRSA in the community

MRSA is emerging in the community in some areas. (1, 18). The PFGE patterns riboprints and susceptibility patterns of community-acquired strains often differ from typical nosocomial isolates (19). Many are sporadic MRSA types that hardly found in hospitals, and these isolates are often only resistant to β-lactams (Chapter 3). Community-acquired MRSA are not descendants of hospital strains, but arise by the dissemination of a successful SCC*mec* type by horizontal transfer to resident MSSA (14). The rise of MRSA infections without predisposing risk factors such as previous hospitalization coincides with the dissemination of SCC*mec* Type D in the *S. aureus* population seen during the 1990s. In this context, it is important to note that 70-75% of all CNS worldwide are resistant to methicillin (1), thus representing a huge potential reservoir of resistance. More than other types, SCC*mec* Type D, which is found in one third of CNS including *S. simulans*, *S. haemolyticus*, *S. lentus*, and mainly *S. epidermidis*, proved capable of entering most MSSA linages. Recently, transfer of SCC*mec* Type D from *S. epidermidis* to *S. aureus* was witnessed *in vivo* when a patient was treated with ß-lactam antibiotics (Chapter 5). It remains unclear, however, whether selection pressure is required for efficient transfer to occur.

Prospects

While new MRSA strains will continue to emerge, some may acquire additional resistance traits during dissemination, and become more favored by antibiotic selection pressure. Given the increasing multidrug resistance among staphylococci, including the emergence of vancomycin-resistant strains, the terror of unresponsive *S. aureus* infections may be closer than we think. Without measures, single multiresistant clones may spread to every corner the world within a decade. This is

very fast, considering the time needed to design and test new antimicrobial compounds. Therefore, global strategies are needed to control the spread of multiply resistant staphylococci. It should be clear that, due to the genetic linkage of different combinations of antibiotic resistance traits in mobile cassettes, the use of old antibiotics may also select for resistance to newer antibiotic compounds. Unnecessary use of all antibiotics, particularly in household items and agriculture, should therefore be avoided.

It may prove more successful to think of new strategies to fight MRSA infections. The adaptation of the cell-wall caused by PBP2a confers methicillin resistance, but may also expose a weak spot to attack the bacterium. It has been shown that PBP2a is immunogenic when the gene is expressed in *Escherichia coli* (20). Using antibodies that recognize the altered cell wall, we may be able to fight MRSA infections. Alternatively, insight in the mechanisms causing the transition of heterogeneously-resistant MRSA to homogeneously-resistant MRSA may provide a strategy to fight MRSA.

Enclaves of low MRSA prevalence are maintained by several countries or even by single hospitals surrounded by endemic MRSA. This suggests that, by taking the appropriate infection control measures, particular *S. aureus* clones can be banned from hospitals. If we wish to enjoy the benefits of effective antibiotics, we must not allow multiply resistant clones to spread.

Conclusion

The structure of the MRSA population shows frequent formation of SCC*mec*$^{+}$ MRSA genotypes by horizontal transfer from other staphylococcal species. While new MRSA strains will continue to emerge by the horizontal transfer of *mec*A, those strains possessing additional resistance traits are favored most by antibiotic selection pressure and may disseminate widely. SCC*mec* is packed with genes reflecting the most relevant change in the bacterial environment during the last 50 years: the introduction of antibiotics. Due to insertion sequences, the structures which carry these genes, including Tn*554*, pUB110, and pT181 were captured (21-23). Within years after their formation, many resistance genes ended up concentrated in mobile chromosome cassettes, which are transferred between many staphylococcal species. Multiple copies of the insertion sequences characteristic for Staphylococcal Chromosome Cassettes are present in one sextant of the *S. aureus* chromosome, suggesting that, during evolution, related cassettes have been frequently shuttled between the genomes of staphylococci, allowing rapid exchange of essential genes and forming a large part of the *S. aureus* genome (24).

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Curriculum Vitae C.L.C. Wielders

Camiel Lambert ChristiaanWielders, geboren te weert op 12 mei 1971, begon na voltooiing van de middelbare school in België in 1989 aan een universitaire opleiding tot Medisch Bioloog in Utrecht. De auteur volgde aanvullende Organische Chemie en Thermodynamica vakken uit het curriculum van Farmacie en behaalde certificaten voor omgang met micro-organismen, radionucleïden en proefdieren en voltooide twee lange stages. Het onderwerp van de eerste stage (12 maanden), bij de vakgroep Biochemie van de faculteit Diergeneeskunde, was de biogenese van lipiden tijdens de celdeling. De tweede stage (10 maanden), die werd gevolgd bij de Amerikaanse Universiteit UCSD, bestudeerde de invloed van DNA schade op celcyclusprogressie. Na voltooiing van een celbiologische scriptie over de celdeling werd in 1996 het doctoraal examen (met genoegen) gehaald. Tijdens en na zijn studie verzorgde de auteur de Nederlandse vertaling van de boeken “Immunology” van Roit en “Histology” van Stevens. Dit promotie onderzoek begon in 1998 bij de onderzoeksschool Infectie en Immuniteit in het UMC. Daarnaast verzorgde de auteur gedurende drie jaar het Immunologie onderwijs bij het HLO te Utrecht en bekwaamde hij zich als infectioloog/immunoloog.

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