Natural Population Dynamics and Carriage of *Staphylococcus aureus*

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Natural Population Dynamics and Carriage of *Staphylococcus aureus*

Populatiestructuur en dragerschap van *Staphylococcus aureus*

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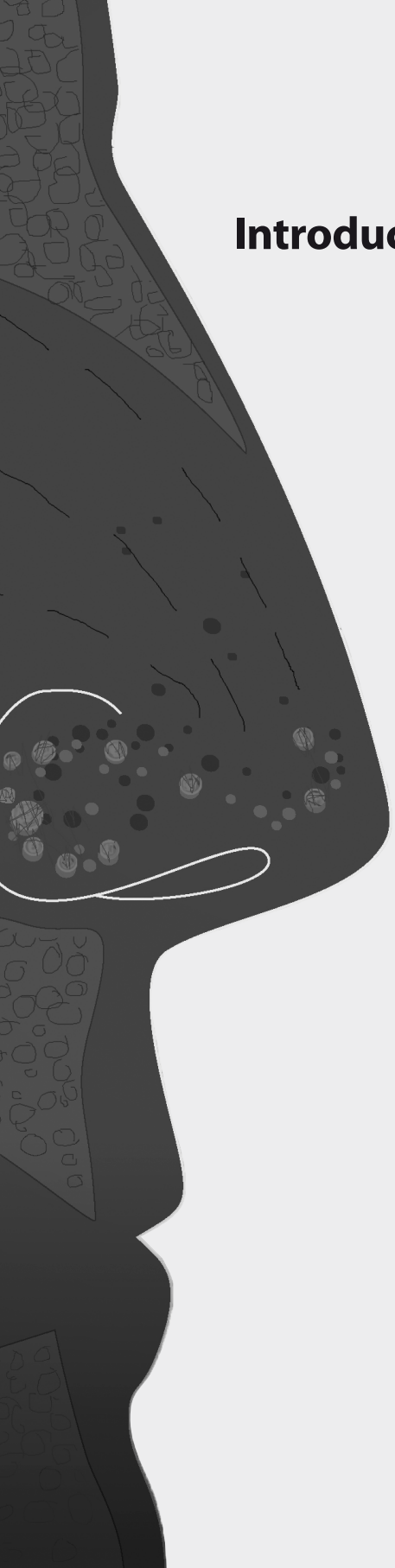

Chapter 1

Introduction and outline of the thesis

Damian C. Melles

GENERAL INTRODUCTION

Staphylococcus aureus is a major human pathogen capable of causing a wide range of infections, from relatively mild skin infections such as folliculitis and furunculosis to life-threatening conditions, including sepsis, deep abscesses, pneumonia, osteomyelitis, and infective endocarditis (1, 2). *S. aureus* belongs to the genus *Staphylococcus*, which contains more than 30 species. In human beings, the most clinically relevant species are *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, and *S. saprophyticus*, of which *S. aureus* is by far the most virulent. In contrast to most *Staphylococcus* species, *S. aureus* is capable of being pathogenic in the absence of predisposing host conditions such as immunosuppression or the presence of foreign body material (2).

S. aureus was discovered in Aberdeen, Scotland, in 1880 by the surgeon Sir Alexander Ogston in pus from human abscesses. He viewed pus under a microscope and observed grape-like clusters of bacteria, which he therefore named *Staphylococcus* from the Greek expression staphylé (a "bunch of grapes") (3). In 1884, Rosenbach was able to isolate and grow these bacteria from abscesses and called them *Staphyloccocus aureus* because of the yellow-orange or "gold" pigmented appearance of the colonies (aureus means "golden" in Latin) (4).

Next to an important pathogen, *S. aureus* is a ubiquitous commensal and colonizer of the skin and mucosa of humans and several animal species (1). Although multiple body sites can be colonized in human beings, the anterior nares (of the nose) is the most frequent carriage site for *S. aureus*. Nasal carriage appears to play a key role in the epidemiology and pathogenesis of infection (5, 6). The association between *S. aureus* nasal carriage and staphylococcal disease was first reported by Danbolt in 1931, who studied furunculosis (7). Several studies have confirmed that the majority of the (nosocomial) *S. aureus* infections is of endogenous origin (i.e. the nasal *S. aureus* strain and the infecting strain share the same phage type or genotype) (8-10). Furthermore, nasal application of an antistaphylococcal drug temporarily decolonizes the nose and other body sites, which prevents infection (11). The mechanisms leading to *S. aureus* nasal carriage appear to be multifactorial. Bacterial factors (e.g. staphylococcal toxins and cell wall-associated proteins) (1, 12), environmental factors (e.g. hospitalization and crowding) (13-15) as well as host factors (e.g. male sex, age) play an important role (14, 16). Nasal colonization of *S. aureus* in human beings can be seen as the net result of repellent and attracting forces. An optimal fit between host and bacteria seems to be essential.

The numbers of both community-acquired and hospital-acquired staphylococcal infections have increased in the past 25 years (1, 17, 18). *S. aureus* ranks second as the cause of nosocomial blood stream infections that leads to increased morbidity, mortality, hospital stay, and costs (19-22). *S. aureus* has shown a high adaptability and developed resistance mechanisms to almost all antibiotics that were introduced over the last few decades. Epi-

demic methicillin-resistant *S. aureus* (EMRSA) strains have been and currently still are a major problem in hospitals all over the world for many years. More recently, highly pathogenic, methicillin resistant strains have emerged in the community outside the hospital setting causing serious infections in otherwise healthy individuals (23-25).

It is obvious that effective measures to prevent *S. aureus* infections are urgently needed. To be able to develop preventive strategies we must elucidate the predisposing factors and mechanisms behind *S. aureus* nasal carriage and subsequent infection.

OUTLINE OF THE THESIS

The main aim of the research described in this thesis is to obtain a better understanding of the natural population structure and its relevance to nasal carriage of *S. aureus* and the relation with subsequent disease. In **Chapter 2** we summarize the latest insights into the determinants of *S. aureus* nasal carriage and the risks of infection associated with *S. aureus* nasal carriage. The subsequent chapters in this thesis have been divided into three sections.

Part I: Population dynamics of *Staphylococcus aureus*

To understand *S. aureus* nasal carriage and the relation with subsequent disease, we need to define the population structure of *S. aureus*. To be able to assess differences in the virulence potential of various strains of *S. aureus*, insight into the natural (non-clinical) population structure is essential. In **Chapter 3** we for the first time describe the detailed population structure of naturally occurring methicillin-susceptible *S. aureus* (MSSA) isolated from the nose of persons living in the community. To this aim we collected over 2,000 non-clinical *S. aureus* isolates from various groups of healthy individuals in the Dutch community over an extended period of time, creating a unique population-based strain collection. For genetic typing of the *S. aureus* isolates we used high-throughput amplified fragment length polymorphism (ht-AFLP), which documents nucleotide sequence variation, insertions, and deletions across genomes. To investigate genomic differences between these carriage strains and invasive isolates, contemporary isolates from blood, deep-seated, and soft-tissue infections from the same geographical area were included. In **Chapter 4** we used comparative genomics to assess genetic differences between *S. aureus* strains derived from infected animals versus colonized or infected humans.

Another important question we addressed is whether the distribution of *S. aureus* genotypes from various geographical locales differs significantly or, alternatively, is rather similar (**Chapter 5**). This chapter describes the first high throughput genotyping effort for large numbers of non-clinical isolates of *S. aureus* from healthy volunteers living in the USA and The Netherlands.

A hot topic currently in the Netherlands is the "pig" associated methicillin-resistant *S. aureus* (MRSA). MRSA has been identified in large numbers among Dutch pigs and pig farmers, although the overall MRSA prevalence in the Netherlands is one of the lowest in the world. The Dutch Working Group on Infection Prevention (WIP) has recently adjusted its guidelines to halt further spread of this strain, and advises that the population at risk (pig breeders, slaughterhouse personnel and veterinarians) be held in isolation when hospitalized until MRSA colonization has been excluded. In **Chapter 6**, we establish the position of this "pig-associated" MRSA in the overall population structure of Dutch *S. aureus*.

Part II: Determinants of nasal carriage and infection with *Staphylococcus aureus*

Bacterial interference between *S. aureus* and *Streptococcus pneumoniae* in the nasopharynx has been observed during colonization in humans. The study described in **Chapter 7** aimed to determine whether the capacity of *S. aureus* to compete with *S. pneumoniae* (in the nasopharynx) is dependent on bacterial genotype. Furthermore, we studied demographic and microbiological determinants of carriage of specific genotypes of *S. aureus* in children. In addition, *S. aureus* nasal carriage and subsequent infections are supposedly more common in patients infected with the human immune deficiency virus (HIV). The reason for the higher carriage rates in HIV-positive patients is unclear. In **Chapter 8** we investigated the host-microbe interplay of persistent *S. aureus* nasal carriage in HIV-infected patients by studying host determinants of persistent carriage as well as the population structure of these *S. aureus* strains. We integrated this population structure with the one previously determined for *S. aureus* isolates obtained from healthy individuals.

The pathogenicity of *S. aureus* depends on various bacterial surface components, such as peptidoglycans and lipoteichoid acid, and exotoxins, such as superantigens. Recent studies suggest that the exotoxin Panton-Valentine leukocidin (PVL) has a major role in pathogenesis of *S. aureus*. This toxin has been associated with skin infections (furuncles and abscesses), community-acquired MRSA infections, and necrotizing pneumonia. Despite the presumed importance of PVL as a virulence factor in *S. aureus*, few data are available on its prevalence among *S. aureus* isolates from the nares of healthy persons compared with strains isolated from infections. This led us to investigate the frequency of PVL gene-positive *S. aureus* strains obtained from the nares of healthy carriers in the community (**Chapter 9**).

Another set of important virulence factors are the staphylococcal enterotoxins (SEs), which are responsible for food-associated outbreaks of diarrhoea among humans. Many of the enterotoxins display superantigen characteristics and are obvious targets for antistaphylococcal therapies. Genes encoding several of the enterotoxins are physically clustered in the *S. aureus* genome. The locus encoding the enterotoxins SEG, SEI, SEM, SEN, and SEO is currently known as *egc* (enterotoxin gene cluster). The prevalence of *egc* genes in isolates of *S. aureus* appears to be negatively correlated with the severity of infection. In **Chapter 10** we investigated whether strains isolated during Dutch *S. aureus* screening studies of carriage

per se and *S. aureus* strains causing bacteremia can be differentiated on the basis of the absence or presence of the *egc* locus.

Infections caused by *S. aureus* involve bacterial adhesion to the host extra-cellular matrix. These *S. aureus* adhesins are mostly cell wall-anchored proteins and are grouped into a single family named microbial surface components recognizing adhesive matrix molecules (MSCRAMM). The serine-aspartate repeat (Sdr) proteins are members of the MSCRAMM family that are encoded by the tandemly arrayed *sdrC*, *sdrD*, and *sdrE* genes. In **Chapter 11** we studied the distribution of these serine-aspartate repeat protein-encoding (*sdr*) genes among nasal carriage and invasive *S. aureus* strains.

A new approach to combat the highly versatile and adaptable *S. aureus* should address its virulence factors that are associated with disease. Immunotherapeutic strategies for prevention and treatment of *S. aureus* infections need to be designed. To study the potential relevance of a putative trivalent *S. aureus* polysaccharide-conjugate vaccine, we aimed to determine the surface and/or capsular polysaccharide serotype of 162 well-characterized *S. aureus* strains from both carriage and clinically relevant infection. In addition, we determined if there is an association between capsular type and strain genotype (**Chapter 12**).

Part III: Technical aspects of defining genomic variability in *Staphylococcus aureus*

Many different pheno- and genotyping methods have been used to distinguish *S. aureus* strains. All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility. Having access to an accurate strain typing method is critical for understanding the changing epidemiology of MSSA and MRSA infections and for evaluating the efficacy of MRSA outbreak intervention and prevention strategies (26). The aim of the study, described in **Chapter 13**, was to compare three well-known and frequently used staphylococcal typing methods, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and ht-AFLP. All three methods have been used to map outbreaks and to study the natural population structure of both MSSA- and MRSA-isolates. In **Chapter 14** we have compared whole genome polymorphism (as defined by ht-AFLP) and short sequence repeat length variation (using multi-locus variable number of tandem repeat analysis [MLVA]) assessed for 994 *S. aureus* strains from both carriage and infection. Data obtained by these different genome-screening methods, focusing on constraint versus hypermutabily regions, have rarely been linked in order to compare the assignment of genetic types. Finally, in **Chapter 15** we used ht-AFLP to characterize molecular markers associated with bacterial phenotypes. Methicillin-resistant and -susceptible isolates of *S. aureus* have been used for this model study in conjunction with the available *S. aureus* genome sequences. The aim of the analysis was to cover a significant fraction of the staphylococcal genome by ht-AFLP and thereby enabling the identification of phenotype-specific molecular markers on this collection of MSSA and MRSA strains.

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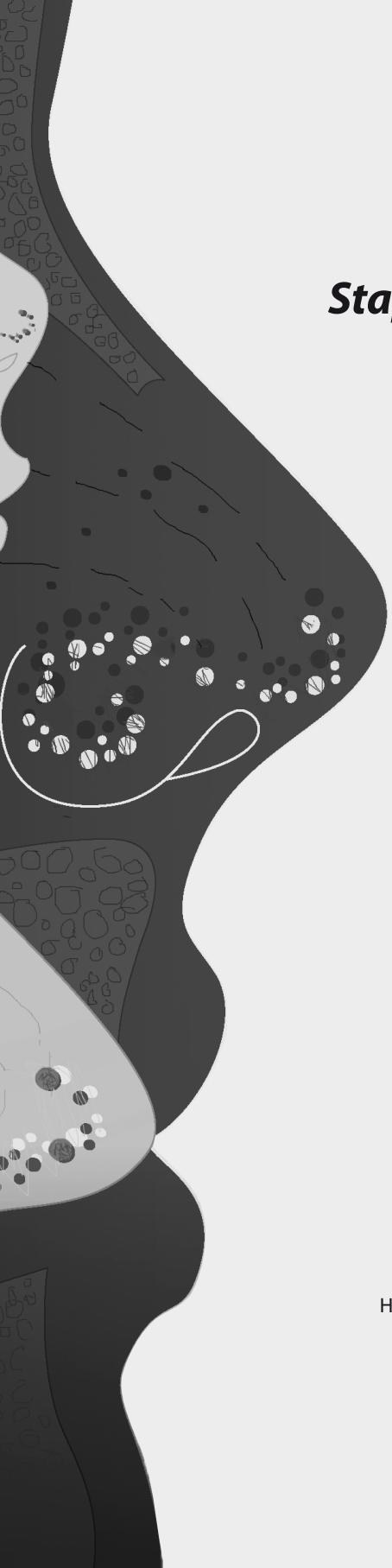

Chapter 2

The role of nasal carriage in *Staphylococcus aureus* infections

Damian C. Melles

Adapted from:
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ABSTRACT

Staphylococcus aureus is a frequent cause of infections in both the community and hospital. Worldwide, the increasing resistance of this pathogen to various antibiotics complicates treatment of *S. aureus* infections. Effective measures to prevent *S. aureus* infections are therefore urgently needed. It has been shown that nasal carriers of *S. aureus* have an increased risk of acquiring an infection with this pathogen. The nose is the main ecological niche where *S. aureus* resides in human beings, but the determinants of the carrier state are incompletely understood. Eradication of *S. aureus* from nasal carriers prevents infection in specific patient categories, e.g. hemodialysis and general surgery patients. However, recent randomized clinical trials in orthopedic and non-surgical patients failed to show the efficacy of eliminating *S. aureus* from the nose to prevent subsequent infection. Thus we must elucidate the mechanisms behind *S. aureus* nasal carriage and infection to be able to develop new preventive strategies. We present an overview of the current knowledge of the determinants (both human and bacterial) and risks of *S. aureus* nasal carriage.

INTRODUCTION

Staphylococcus aureus is both a human commensal and a frequent cause of clinically important infections (Figure 1) (1). Although the prevalence of methicillin-resistant *S. aureus* (MRSA) is still very low in northern European countries (2), there is a worldwide increase in the number of infections caused by MRSA. Vancomycin is one of the last therapeutic options

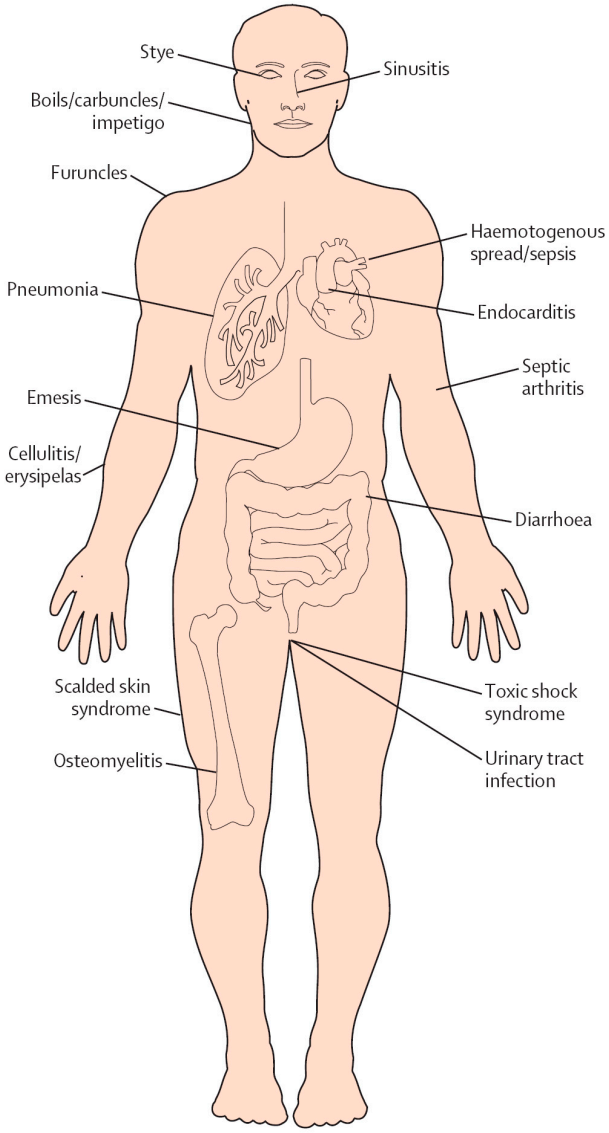

Figure 1. Large diversity in *S. aureus* infections.

Table1. Major events in *S. aureus* research

Year	Event
1880	Alexander Ogsten identifies micrococci in purulent infections (12)
1931	Association between nasal colonization and furunculosis discovered (4)
1934	Popularization of the coagulase test for the identification of *S. aureus* (5)
1944	Introduction of phage typing (13)
1947	Penicillin-resistant *S. aureus* reported (14)
1952	Association between nasal colonization of *S. aureus* and infection with the same strain determined by phage typing (10, 15)
1961	Methicillin-resistant *S. aureus* (MRSA) reported (16)
1991	Pulsed field gel electrophoresis used for genotyping *S. aureus* (17)
1994	Identification of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (18)
2000	Multilocus sequence typing developed for studying clonality of *S. aureus* (19)
2001	Whole genome of *S. aureus* sequenced (20)
2001	80% of bacteremic *S. aureus* isolates are endogenous (8)
2001	Increase in community-onset MRSA infections (21)
2002	Vancomycin-resistant *S. aureus* reported (22)

available for MRSA infections. The recent isolation of vancomycin-resistant MRSA strains in the USA is a major cause for concern (3). Therefore, the prevention of staphylococcal infections and reduction of the spread and emergence of MRSA are essential.

The association between *S. aureus* nasal carriage and staphylococcal disease was first reported by Danbolt in 1931, who studied furunculosis (4). The increasing incidence of penicillin-resistant *S. aureus* hospital infections since 1947 emphasized the need for a better understanding of the pathogenesis of staphylococcal disease. Subsequently, numerous studies confirmed Danbolt's finding (5-9). A causal relation between *S. aureus* nasal carriage and infection is supported by the fact that the nasal *S. aureus* strain and the infecting strain share the same phage type or genotype (8, 10). Furthermore, nasal application of an anti-staphylococcal drug temporarily decolonizes the nose and other body sites, which prevents infection (11).

Our knowledge of the mechanisms, risks, and treatment of *S. aureus* nasal carriage has greatly expanded over the past decade. Table 1 presents an overview of major events in *S. aureus* research. Here, we focus on the latest insights into the determinants of *S. aureus* nasal carriage and the risks of infection associated with *S. aureus* nasal carriage. Most studies were done in western countries, so conclusions drawn can not always be generalized.

DETERMINANTS OF NASAL CARRIAGE OF *S. AUREUS*

S. aureus nasal carriage patterns

S. aureus colonizes the skin and mucosae of human beings and several animal species (5). Although multiple body sites can be colonized in human beings, the anterior nares of the nose is the most frequent carriage site for *S. aureus* (5). Extra-nasal sites that typically harbor the organism include the skin, perineum, and pharynx (5, 23-25). Other carriage sites including the gastrointestinal tract (5, 26), vagina (27), and axillae (5, 25, 28) harbor *S. aureus* less frequently (Figure 2).

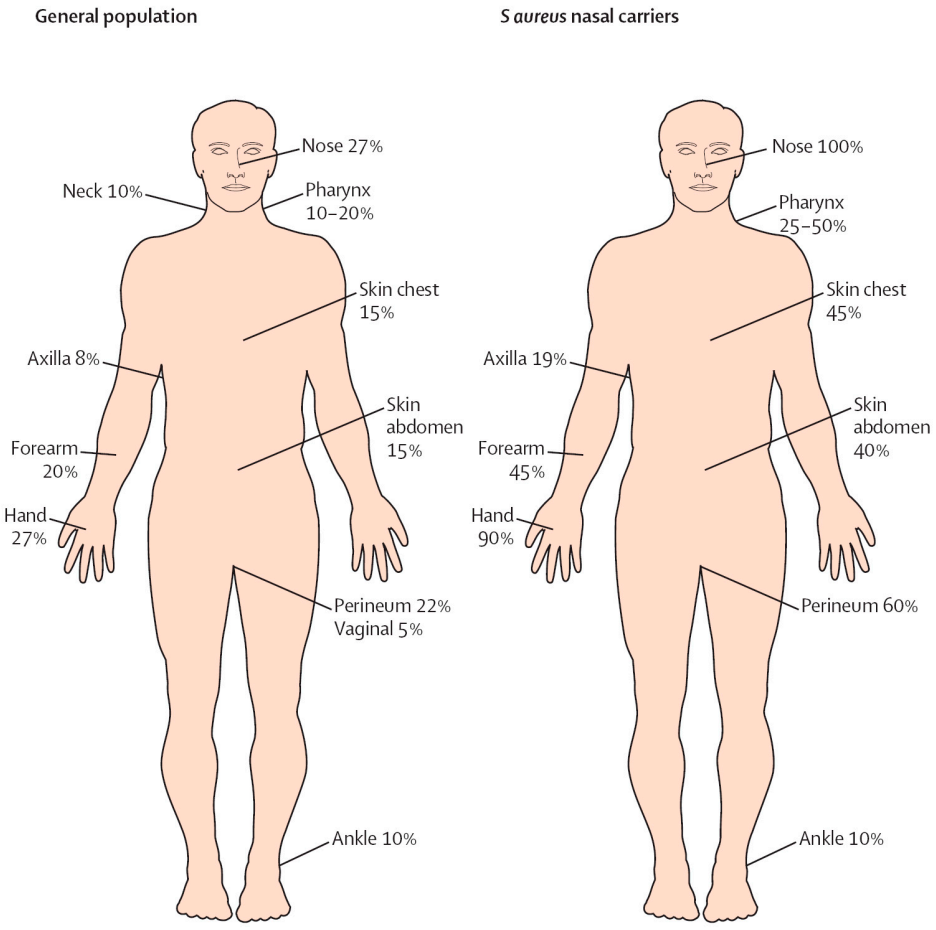

Figure 2. *S. aureus* carriage rates per body site in adults.
There is an increase in carriage rates at extra-nasal sites within nasal *S. aureus* carriers. The mentioned rates are approximations using data from the literature cited in the text.

Most studies on *S. aureus* nasal carriage have used a cross-sectional design with a single nasal culture to classify an individual as a carrier or not. However, longitudinal studies distinguish at least three *S. aureus* nasal carriage patterns in healthy individuals: persistent carriage, intermittent carriage, and non-carriage (5, 6, 23, 29, 30). Some studies make a further distinction between occasional and intermittent carriers (29, 31). Therefore, a patient classified as a carrier in cross-sectional studies could either be a persistent or an intermittent carrier. This distinction is important because persistent carriers have higher *S. aureus* loads and a higher risk of acquiring *S. aureus* infection (32, 33). Likewise, non-carriers in a cross-sectional study may actually be intermittent carriers.

The definition of persistent carriage varies from study to study. There is no general consensus on how many cultures should be taken and how many cultures should be positive to define persistence. One study concludes that a "culture rule" that combines qualitative and quantitative results of two nasal swabs taken with a week interval can accurately classify *S. aureus* nasal carriage (34). Since adequate, internationally accepted definitions are needed, the so-called culture rule is an improvement for those studying determinants and risks of *S. aureus* nasal carriage.

Longitudinal studies show that about 20% (range 12-30%) of individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers (range 16-70%), and about 50% (range 16-69%) noncarriers (6, 29, 34, 35). The very wide ranges found in the proportions of intermittent and non-carriers are the result of the use of different culture techniques, different populations being studied, and the use of different interpretation guidelines (30). Although at least seven nasal swab cultures are necessary to segregate non-carriers from in-

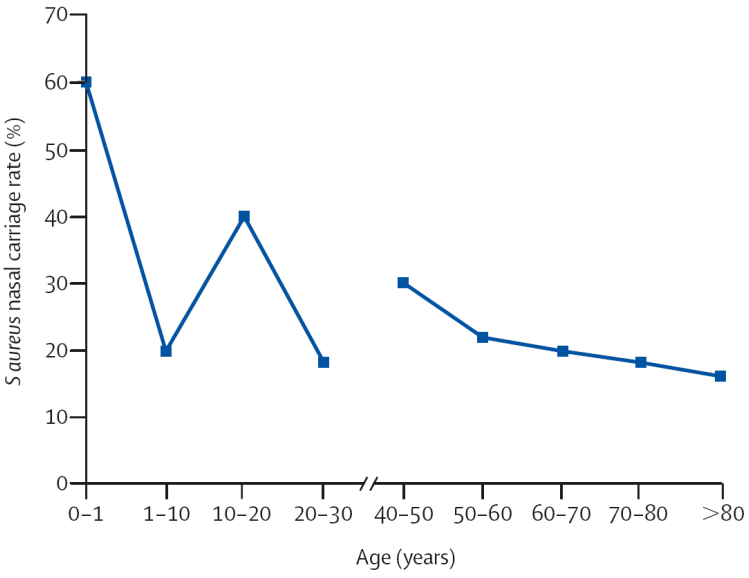

Figure 3. Rates of *S. aureus* nasal carriage according to age.

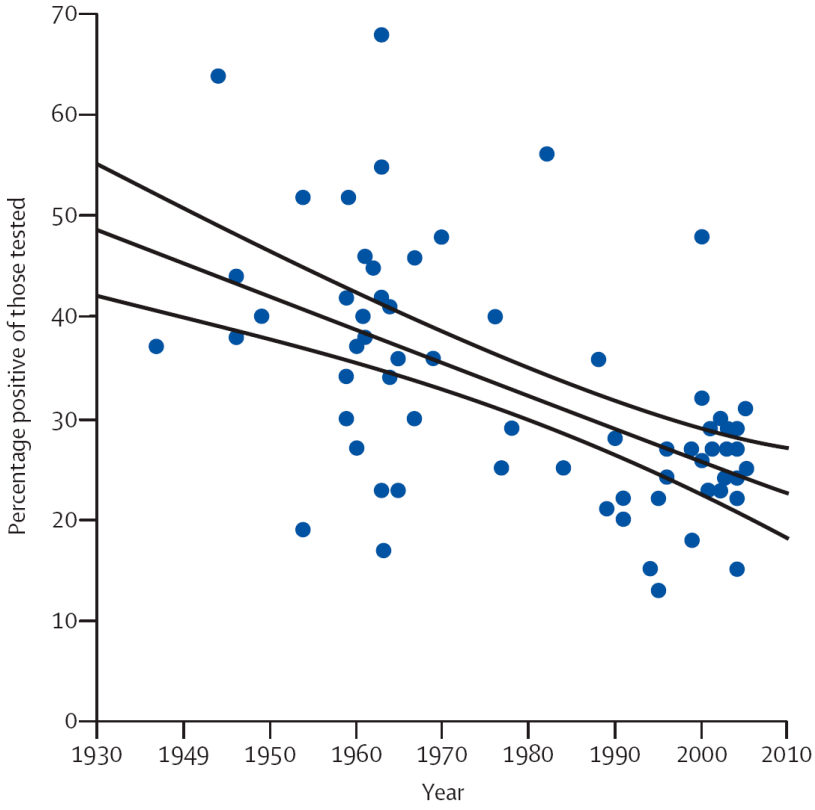

Figure 4. Reported *S. aureus* nasal carriage rates through the years.
There is a significant negative correlation between the year of reporting and the reported carriage rate (correlation coefficient -0.55; $P < 0.001$).

termittent carriers, the more nasal cultures are analyzed, the higher the chance of identifying an intermittent carrier (34).

Children have higher persistent carriage rates than adults (23, 36, 37). Rates vary substantially with age, falling from approximately 45% during the first 8 weeks to 21% by 6 months (38). More than 70% of newborn babies have at least one positive nasal culture with *S. aureus* (38). There is a transition from persistent carriage to intermittent or noncarriage states during adolescence (Figure 3) (5, 23). Cross-sectional surveys of healthy adult populations have reported *S. aureus* nasal carriage rates of approximately 27% since 2000 (7, 9, 39-46). This rate is much lower than the earlier reported prevalence of 35%, which included studies since 1934 (6). Plotting the carriage rates of either healthy populations or a general hospital population clearly illustrates a substantial decline in the *S. aureus* nasal carriage rate in time (Figure 4, patient categories with known higher *S. aureus* nasal carriage rates, like dialysis patients, were excluded). Explanations for this decline include improved personal hygiene, changes in socioeconomic class (47), and smaller families (48).

Determinants of *S. aureus* nasal carriage

Although the reasons remain unknown, the basic determinants of persistent and intermittent carriage are thought to be different. Persistent carriers are often colonized by a single strain of *S. aureus* over long time periods, whereas intermittent carriers may carry different strains over time (29, 30, 35). Furthermore, the load of *S. aureus* is higher in persistent carriers, resulting in increased dispersal and a higher risk of infection (33, 34). Nasal carriers who are also perineal carriers have higher *S. aureus* loads and disperse more *S. aureus* (4, 25, 49).

The mechanisms leading to *S. aureus* nasal carriage are multifactorial. A recent study in which volunteers (noncarriers and persistent carriers) were artificially inoculated with a mixture of *S. aureus* strains showed that noncarriers quickly eliminated the inoculated *S. aureus* strains, whereas most persistent carriers selected their original resident *S. aureus* strain from the inoculation mixture (50). The investigators concluded that host characteristics substantially co-determine the *S. aureus* carrier state and that an optimal fit between host and bacteria seems to be essential (50).

This view is further supported by the fact that *S. aureus* carriage rates vary between different ethnic groups, with higher rates in white people (5, 40) and in men (5, 29, 51), and depend on age (23, 38, 52). Patients with diabetes mellitus (both insulin dependent and non-insulin dependent) (53), patients undergoing hemodialysis (54 ,55) or continuous peritoneal dialysis for end stage renal disease (56), patients with end stage liver disease (57, 58) patients with HIV (59, 60), patients with *S. aureus* skin infections and skin disease (e.g. eczema or psoriasis) (61-63), and obesity and a history of cerebrovascular accident (51) have been shown to have higher *S. aureus* nasal carriage rates. Most studies are hospital or outpatient clinic based and need confirmation from community-based surveys. In one community-based study, Boyko and co-workers (64) found similar *S. aureus* carriage rates in diabetics and non-diabetics, by contrast with an earlier clinic-based study (53).

Nasal colonization of *S. aureus* can be seen as the net result of repellent and attracting forces. There are four prerequisites to becoming a nasal carrier of *S. aureus*. First, the nose has to come in contact with *S. aureus*. Second, *S. aureus* needs to adhere to certain receptors in the nasal niche. Third, *S. aureus* needs to overcome the host defences. Finally, *S. aureus* should be able to propagate in the nose. We will discuss these issues separately (Table 2).

How does *S. aureus* reach the nose?

S. aureus cells can survive for months on any type of surface (65). Hands are the main vector for transmitting *S. aureus* from surfaces to the nasal niche, e.g. nose picking (66). *S. aureus* cells are principally found in the anterior nares (vestibulum nasi or "nose picking area"), and *S. aureus* nasal carriage and hand carriage are strongly correlated (4). Some studies find higher carriage rates more proximal in the nose, but these studies are rare and probably reflect a chance finding (67). *S. aureus* may also reach the nose directly through the air, but

Table 2. Overview of mechanisms associated with *S. aureus* nasal carriage

Mechanism	Host	*S. aureus*
General	Age, sex, ethnicity	Virulence
	Socioeconomic class	
	Antibiotic use	Antibiotic resistance
	Underlying disease (insulin-dependent diabetes mellitus, HIV, liver disease, eczema, nasal abnormalities, and others)	
	HLA type	
	Immune status	
Exposure	(Heavily) colonized partner	
	Hospital environment	
	Nose picking	
Adherence	Receptors	Adhesins
	(Extracellular) matrix proteins	MSCRAMMs [a]
	Cytokeratin type 10	Clumping factor B
	Epithelial membrane	(Lipo)teichoic acid
		Capsule
	Mucins	Capsular polysaccharides
	Surface charge	Surface charge
	Hydrophobicity	Hydrophobicity
(Evading) immune response	Mucosal/skin barrier	Proteases, lipases
	Clearance in mucus by microvilli	Host cell internalization
	Immunoglobulins	Protein A (binds Fc of IgG)
	Lysozyme, lactoferrin, antimicrobial peptides	Resistance to antimicrobial peptides
	Opsonization	Capsule

[a] MSCRAMMs = microbial surface components recognizing adhesive matrix molecules

this probably occurs less frequently (68). However, airborne transmission is important for the dispersal of staphylococci to many different reservoirs, from where, via the hands, they can reach the nose. *S. aureus* nasal carriers with rhinitis can disperse high loads of *S. aureus* into the environment and may be the source of an outbreak of *S. aureus* infections-the so called "cloud" individual (69).

Environmental factors can also influence the *S. aureus* nasal carriage state. Hospitalisation, for example, has been shown to be an important risk factor (70). Furthermore, it seems that *S. aureus* carriers can "impose" their carrier state upon other household members. Recently, Peacock and colleagues (38) found concordant carrier states between mothers and their children. Also, Bogaert and co-workers (48) found large households (≥ five members) to be positively associated with *S. aureus* nasal carriage. Most mothers carry the

same strain as their children, indicating that carriage strains are transmitted to close contacts (38). A study among an elderly population demonstrated that not only persistent but also non-carriage or intermittent *S. aureus* nasal carrier states are shared among household members (71). Up to 65% of people with positive cultures living within one household shared genotypically identical strains (71). Intrafamilial spread of MRSA from and to health-care workers has also been shown to be an important risk factor for the re- introduction of MRSA into hospitals (72). Furthermore, Herwaldt and colleagues (73) demonstrated that in 21% of patients receiving continuous peritoneal dialysis, the source of newly acquired nasal *S. aureus* strains were their respective family members.

Activities leading to skin lesions are also correlated with higher *S. aureus* nasal carriage rates. These include river rafting (74), football (75), and (pig-)farming (76). Repeated skin punctures in drug users and diabetics were thought to explain higher *S. aureus* nasal carriage rates (6). However, recent studies do not support this theory: intravenous drug users have a lower prevalence of *S. aureus* nasal carriage compared with drug users on an oral methadone programme (77), and *S. aureus* nasal carriage rates are not different between diabetic patients injecting insulin and those using oral glucose-lowering medication (53, 64).

There is no relation between carriage rate and seasonality, temperature, or relative humidity (5, 78, 79). A population-based cohort of children and adolescents showed that active cigarette smoking is associated with a lower *S. aureus* nasal carriage rate, whereas passive smoking is associated with a higher *S. aureus* nasal carriage rate (48). The aetiological basis of this observation is unknown.

How does *S. aureus* withstand and evade the host immune response?

Nasal secretions have a prominent role in the innate host defence. Components of nasal secretions that contribute to the innate immune response include immunoglobulin A and G, lysozyme, lactoferrin, and antimicrobial peptides (80). *S. aureus* nasal carriers may have a dysregulation of these innate humoral factors in their nasal secretions (81). Such people have raised concentrations of the alpha-defensins (e.g. human neutrophil peptide [HNP] 1, 2, and 3) and human beta-defensin 2 (HBD2), indicative of the presence of both neutrophil-mediated and epithelial-mediated inflammation (81). Lipoteichoic acid, present in the *S. aureus* cell wall, is a strong stimulus for neutrophil recruitment (82). Therefore, this inflammatory response could be induced by *S. aureus* colonization. However, studies have shown that HNP1, 2, and 3, and HBD2 are not microbicidal against *S. aureus* in vitro, suggesting that the host response is ineffective and insufficient to prevent *S. aureus* nasal carriage (40). The role of the cellular response is unclear. The previously established relation between glycaemic control and *S. aureus* carriage rate in diabetics (53) could be seen as the result of hyperglycaemia-related reduced phagocytic activation (83).

Several studies have found that certain antimicrobial peptides have no or little activity against *S. aureus* or that other peptides are needed to enhance their activity (84, 85). The

inability of nasal antimicrobial peptides to clear *S. aureus* from the nose may be explained by (1) the anatomy of the nose in relation to *S. aureus* nasal carriage and (2) resistance of *S. aureus* to many antimicrobial peptides (40, 86). *S. aureus* predominantly colonizes an area in the vestibulum nasi that is devoid of cilia and relatively free from nasal mucous secretions that contain antimicrobial peptides and immunoglobulins (40). It is nevertheless possible that the innate immune response prevents *S. aureus* from invading the mucosa and causing more extensive forms of colonization or even infection.

In-vitro studies have shown that *S. aureus* is able to resist certain cationic antimicrobial peptides by reducing the net negative charge of its cell wall and cell membrane, or perhaps by using efflux pumps or by releasing proteases (86). *S. aureus* has several mechanisms-including staphylokinase (87) and membrane lipid modification (88)-through which it can withstand an attack by cationic antimicrobial peptides, including defensins and cathelicidins, which are present in nasal secretions (86, 89). Whether the resistance of *S. aureus* to defensins and other cationic antimicrobial peptides is a determinant of *S. aureus* nasal carriage is currently not known. Cathelicidin can synergistically work with defensins to exert a bactericidal effect on *S. aureus* (84). Furthermore, all *S. aureus* strains are lysozyme resistant since they possess the peptidoglycan-specific O-acetyltransferase (90).

The presence of *S. aureus* in the nose elicits a subclinical immune response, as shown in a study where seroconversion occurred after carriage was established (91). *S. aureus* produces protein A that binds the Fc region of immunoglobulins, thereby inactivating them (65). It is clear that *S. aureus* has a wide arsenal of strategies to evade the host immune response. Further studies are needed to identify all the components of the immune response towards *S. aureus* in the nose.

How does *S. aureus* adhere to, and propagate in, the anterior nares?

The vestibulum nasi is limited laterally by the interior of the wing of a nostril and medially by a mucous fold (limen nasi), behind which the nasal cavity with mucosal lining begins (Figure 5) (92). The epithelial inner wall of a nostril is fully keratinised and includes apocrine sweat glands, sebaceous glands, and hair follicles of the vibrissae (92). Most studies on determinants of *S. aureus* nasal carriage focus on mucosal and mucin binding (93-95). Considering the anatomy of the vestibulum nasi, this focus should be changed.

Bibel and colleagues (96) demonstrated the importance of keratinised epithelial cells in binding *S. aureus*. In addition to the nose, *S. aureus* can also multiply independently in the area of the perineum (97). Both the vestibulum nasi and the perineum contain large apocrine sweat glands, which is an important clue in studying determinants of *S. aureus* nasal carriage, but has not been studied thoroughly (25). Since *S. aureus* binding to mucosa or mucin probably has a transient nature, we propose that: (1) intermittent carriers are actually "mucosal carriers" and (2) persistent carriers use a special niche, such as an apocrine gland, where *S. aureus* cells can multiply to high numbers.

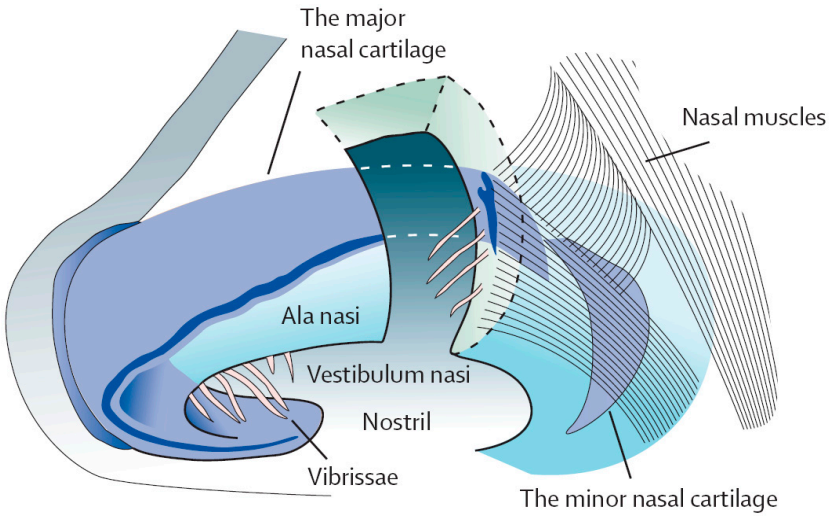

Figure 5. Anatomy of the nostril.
Adapted from reference 92.

S. aureus adherence may also be non-specifically mediated via physicochemical forces, including hydrophobic interactions (6). Alternatively, adherence may be more specifically accomplished through binding of certain bacterial cell surface moieties (adhesins) to defined structural receptors in the membranes of the host cells (6). *S. aureus* has a greater affinity for nasal epithelial cells sampled from carriers than from non-carriers (94), and the bacterium adheres better to nasal epithelial cells from patients with eczema than to cells from patients without eczema (6).

Recent experiments have shown that clumping factor B (ClfB) and the *S. aureus* surface protein G (SasG) bind to nasal epithelial cells (98, 99). ClfB specifically binds human cytokeratin type 10 and SasG to an unknown ligand of desquamated nasal epithelial cells (98). Also, cell wall teichoic acid is essential for *S. aureus* nasal carriage (95, 100). Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) can bind to fibronectin, fibrinogen, and collagen related polysaccharides (18). MSCRAMMs probably have a role in the binding of staphylococci to sites where the mucosal lining is breached, exposing these matrix molecules (66). Differences in the expression of genes coding for these factors, depending on the ecological niche, and other putative adhesins and receptors may provide clues to the true determinants of *S. aureus* nasal carriage or non-carriage.

Bacterial interference has been postulated to be a major determinant of the *S. aureus* carrier state, or rather, noncarrier state. When an ecological niche is already occupied by certain bacteria, other bacteria do not seem to have the means to replace this resident bacterial population (101). The resident flora must be reduced or eliminated before other bacteria can successfully "interfere" with the resident bacterial population (102). Cross-inhibition of the

expression of various virulence factors by the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*) may be one mechanism by which one strain excludes others from colonizing sites including the anterior nares (103), although a large *S. aureus* population genetic analysis failed to confirm this suggestion (104). Still, bacterial interference can be seen as a determinant of *S. aureus* nasal carriage, although it does not appear to be the ultimate determinant (38).

Bacterial interference by active colonization using a nonpathogenic *S. aureus* strain (502A) was successful in nurseries during outbreaks of *S. aureus* infections in the 1960s and for treatment of patients with recurrent furunculosis (102, 105). The early practice of artificial inoculation with *S. aureus* 502A was abandoned after alleged complications (106) and the advent of newer antistaphylococcal antibiotics in the early 1970s.

RISKS OF *S. AUREUS* NASAL CARRIAGE

Community-acquired infections

Most studies regarding the risks of acquiring *S. aureus* infections in the community concern skin and soft tissue infections. Several, mostly older, studies investigated the relation between *S. aureus* nasal carriage and skin infections (108), including furunculosis (109, 110), impetigo (111), sycosis barbae (10, 109, 112), and stye (113). On average, 80% (range 42-100%) of those with skin lesions were *S. aureus* nasal carriers, and 65% (range 29-88%) had the same phage type in the nose and lesion.

In one large prospective population-based study among elderly people there was no relation between persistent *S. aureus* nasal carriage and all-cause mortality, a surrogate endpoint for serious staphylococcal disease (71). Earlier retrospective cohort or case-control studies have demonstrated increasing age, male sex, alcoholism, lung disease, cancer, diabetes mellitus, end stage renal failure, and dialysis to be risk factors for community-acquired *S. aureus* infections necessitating hospital admission (114-116). These factors have also been identified earlier as determinants of *S. aureus* nasal carriage in case-control or cross-sectional studies (6).

The spectrum of community *S. aureus* disease is rapidly changing with the advent and spread of community-onset MRSA strains (75, 107, 117, 118). Overall MRSA carriage rates in the community are still low (2, 42, 119), but seem to be rising rapidly in certain parts of the world (117, 120). In the only prospective study done so far on nasal carriage of community-onset MRSA and risk of infections in soldiers, Ellis and coworkers (121) found a relative risk of 3.1 (95% CI 1.5-6.5) for nasal MRSA carriers to acquire a MRSA infection (e.g. cellulitis, abscesses) in the community. In a retrospective study concerning community-onset MRSA skin infections among professional football players, Kazakova and colleagues (75) did not find any MRSA in nasal swabs or environmental cultures, although 42% were nasal

carriers of MSSA strains. Apart from these highly selected populations, it remains questionable whether the results from these studies can be extrapolated to the general population (121). We need more community-based studies to better understand the ecology, pathophysiology, and epidemiology of *S. aureus* nasal carriage and infections in the community and to develop and target preventive measures.

Nosocomial infections

S. aureus (MSSA as well as MRSA) ranks as the second most common cause of hospital acquired (nosocomial) bloodstream infections. About 20% of patients undergoing surgery acquire at least one nosocomial infection, leading to increased morbidity, mortality, hospital stay, and costs (122-126). Hospital treatment usually requires that first line barriers for pathogens (of which the skin is an important one) are intentionally breached, resulting in an increased risk of infection. Most of these nosocomial *S. aureus* infections are caused by the patient's own *S. aureus* cells, which were already present on the skin or mucosal membranes before hospital admission in at least 80% of the cases (7, 8). It could well be that more infections are of endogenous origin, since 10% of the nasal *S. aureus* carriers have more than one genotype or phage type in their nose (5, 127).

S. aureus nasal carriage has been identified as a risk factor for the development of nosocomial infections in general hospital populations (128), surgical patients (general (5, 6, 9), orthopaedic (129), thoracic surgery (130), and children (131)), patients on hemodialysis or continuous peritoneal dialysis (6, 33, 54, 132, 133), patients with liver cirrhosis and after liver transplantation (58, 134-136), HIV-infected patients (59, 60), and patients admitted to intensive care units (137-139). In a recent study there was a threefold increased risk for nonsurgical patients who were *S. aureus* nasal carriers to acquire a nosocomial *S. aureus* bacteremia versus noncarriers (7). Also nasal carriers among surgical patients have a higher risk (OR 4.0) for nosocomial *S. aureus* bacteremia compared with controls (140).

Second to coagulase-negative staphylococci, *S. aureus* is the most prevalent organism causing intravascular device-associated bacteremia (6, 124, 141). Pujol and colleagues (137) looked at bacteremia in an intensive care unit. Most of the *S. aureus* bacteremias had an intravascular device as a source. In this study, carriers of *S. aureus* had a relative risk of 12.4 for the development of *S. aureus* bacteremia (137). In a study by Wertheim and co-workers (7), the source of bacteremia was device related in more than 50% of the cases. Interestingly, the mortality rate from *S. aureus* bacteremia is higher in non-carriers compared with carriers (7). Since bacteremia is usually endogenous in carriers, partial immunity may have an important role here. This finding needs confirmation and the underlying mechanism resolved.

In HIV-positive patients, increased rates of *S. aureus* bacteremia and deep soft tissue infections have been observed, which frequently recur. Even higher infection rates are found in patients with AIDS compared with HIV-positive asymptomatic patients. Nguyen and colleagues (59) found that nasal carriage is an important risk factor in this patient population

(OR 5.1). Other risk factors for infection in this study were presence of a vascular catheter (OR 4.9), low CD4 cell count (< 100 cells/μL; OR 3.5), and neutropenia. The risk for developing an *S. aureus* infection was approximately 10% for every 6 months in patients who were nasal carriers of *S. aureus* and had CD4 cell counts of less than 100 cells/μL. It should be noted that *S. aureus* nasal carriage was more common in patients who were not receiving cotrimoxazole prophylaxis for prevention of *Pneumocystis jiroveci* pneumonia.

In hemodialysis patients, *S. aureus* is the most frequently found pathogen in infections at the vascular access site and in bacteremia. The infection rate is higher in carriers on hemodialysis, with relative risks varying from 1.8 to 4.7 (6, 54, 132, 133, 142). *S. aureus* isolates are usually identical to the one previously isolated from the patient's nose (143). In a study by Nielsen and colleagues (142), the relative risk for *S. aureus* bacteremia was 26.2 (6.1-113) when *S. aureus* was colonizing the insertion site, and 3.3 (0.74–15.1), in the case of only *S. aureus* nasal carriage. However, multiple studies have demonstrated that long-term eradication of *S. aureus* nasal carriage by (repeated) application of mupirocin effectively prevents *S. aureus* infections among patients who are receiving dialysis, thereby decreasing complications and costs (144-147). Additional application of a local antibiotic ointment to exit sites is also important in preventing infections (148).

In patients on continuous peritoneal dialysis, *S. aureus* is the leading cause of continuous peritoneal dialysis-related infections, often leading to catheter loss. *S. aureus* nasal carriage has been found to be a major risk factor for infections in patients on continuous peritoneal dialysis, mainly associated with exit site and tunnel infections (33, 56, 149-153). Intervention studies consistently demonstrated a substantial reduction in the incidence of exit site infections, but not a consistent reduction in the incidence of continuous peritoneal dialysis-related peritonitis (54, 153-157). Two studies did not find a correlation between *S. aureus* nasal carriage and the development of *S. aureus* exit site infections (158, 159). In a recent study it was demonstrated that only continuous peritoneal dialysis patients who are persistent *S. aureus* nasal carriers are at increased risk of acquiring continuous peritoneal dialysis- related *S. aureus* infections (33). Intermittent nasal carriers of *S. aureus* have the same risk of *S. aureus* infection as noncarriers (33). Targeting interventions to prevent continuous peritoneal dialysis-related infections is thus possible, thereby eliminating unnecessary prophylactic and therapeutic antibiotic use and resistance development (160). The nasal strain and the infectious strain are clonally related in most patients on continuous peritoneal dialysis with *S. aureus* infection (6, 33, 56).

Studies in the 1950s and 1960s show that with increasing numbers of staphylococcal bacteria in the nose, as in persistent carriers, *S. aureus* skin carriage rates increase proportionally, in parallel with a rise in risk of *S. aureus* surgical site infections (4, 32, 161, 162). The more recent observation that patients carrying *S. aureus* in their nose as well as perineal (or rectal) skin are at a higher risk for subsequent *S. aureus* infections when compared with only perineal or nasal carriers can probably also be explained by a higher *S. aureus* load

(49). Presumably people who carry *S. aureus* in their nose contaminate their hands, then transferring the organism to other sites on their bodies (66). The number of staphylococcal cells needed to cause infection decreases dramatically at the site of a suture, compared with healthy skin (163).

Although *S. aureus* nasal carriage is unanimously accepted as one of the most important risk factors for nosocomial and surgical site infections today and studies using historical controls have reported substantial reductions of surgical site infections among patients receiving mupirocin (136, 164-166), randomised controlled trials uniformly failed to confirm these results (9, 167, 168). Perl and colleagues (9) could only demonstrate a significant effect (48% risk reduction, $P = 0.02$) on the rate of nosocomial *S. aureus* infections after surgery among *S. aureus* nasal carriers before surgery. The 37% reduction in *S. aureus* surgical site infections was not statistically significant ($P = 0.15$) (9). Wertheim and colleagues (167) and Kalmeijer and co-workers (168) did not find a significant effect of eradication of *S. aureus* nasal carriage in a general hospital and orthopaedic patient population, respectively. In the study of Perl and co-workers (9), 53% of *S. aureus* surgical site infections occurred in the non-carrier group, and 15% of the *S. aureus* surgical infections in carriers was caused by a strain other than their resident strain. These infections probably result from exogenous transmissions from the hospital environment or undetected extra nasal *S. aureus* carriage sites. Health-care workers can be important sources of transmission of *S. aureus* and cross-infection (169).

CONCLUSIONS

Many studies have been published on *S. aureus* nasal carriage; a Pubmed search with the terms "*Staphylococcus aureus*" and "nasal" gives 1675 hits. Based on these studies and the results of contradicting twin studies (170, 171) a simple Mendelian trait probably does not explain the different *S. aureus* nasal carrier states (38, 48). The repeated exposure to *S. aureus* in the (household) environment is considered to be an important determinant of *S. aureus* nasal carriage, probably more important than the genetic background of individuals. In general, a multifactorial genesis underlies *S. aureus* nasal carriage.

We now need to identify which factors of *S. aureus* and the nasal niche are of importance in adherence. Recent in-vitro and in-vivo studies in rats have begun to elucidate these factors, which is an important step forward (98-100). Furthermore, we may need to change the focus from mucosal adherence to adherence to more prevalent epitopes present in the anterior nares. The real importance of these factors needs to be confirmed in a human colonization model. Only then may we find new, effective ways of decolonizing the nares and other body sites. So far there is limited evidence that decolonization of the anterior nares to prevent staphylococcal disease is only effective in dialysis and surgical patients. Recent

clinical trials in non-surgical and orthopaedic patients did not show any positive effect (167, 168). Focusing only on at-risk patients, e.g. persistent carriers, may improve the outcome of an intervention. Also the decolonization of extra-nasal sites needs to be improved (24).

So far, there has been concern only for the increased risk of *S. aureus* nasal carriers for acquiring *S. aureus* infections. However, studies have shown that non-carriers who acquire exogenous *S. aureus* bacteremia have a fourfold increased mortality rate compared with *S. aureus* nasal carriers (7). Thus, the immunological mechanisms of *S. aureus* nasal carriage need to be resolved. In noncarriers, preventing the acquisition of *S. aureus* strains deserves more attention.

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Part I

Population dynamics of *Staphylococcus aureus*

Chapter 3

Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*

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ABSTRACT

The population structure of *Staphylococcus aureus* carried by healthy humans was determined using a large strain collection of nonclinical origin (n = 829). High-throughput amplified fragment length polymorphism (AFLP) analysis revealed 3 major and 2 minor genetic clusters of *S. aureus*, which were corroborated by multilocus sequence typing. Major AFLP cluster I comprised 44.4% of the carriage isolates and showed additional heterogeneity whereas major AFLP groups II and III presented 2 homogeneous clusters, including 47.3% of all carriage isolates. Coanalysis of invasive *S. aureus* strains and epidemic methicillin-resistant *S. aureus* (MRSA) revealed that all major clusters contained invasive and multiresistant isolates. However, clusters and subclusters with overrepresentation of invasive isolates were also identified. Bacteremia in elderly adults, for instance, was caused by a IVa cluster-derived strain significantly more often than by strains from other AFLP clusters. Furthermore, expansion of multiresistant clones or clones associated with skin disease (impetigo) was detected, which suggests that epidemic potential is present in pathogenic strains of *S. aureus*. In addition, the virulence gene encoding Panton-Valentine leukocidin was significantly enriched in *S. aureus* strains causing abscesses and arthritis in comparison with the carriage group. We provide evidence that essentially any *S. aureus* genotype carried by humans can transform into a life-threatening human pathogen but that certain clones are more virulent than others.

INTRODUCTION

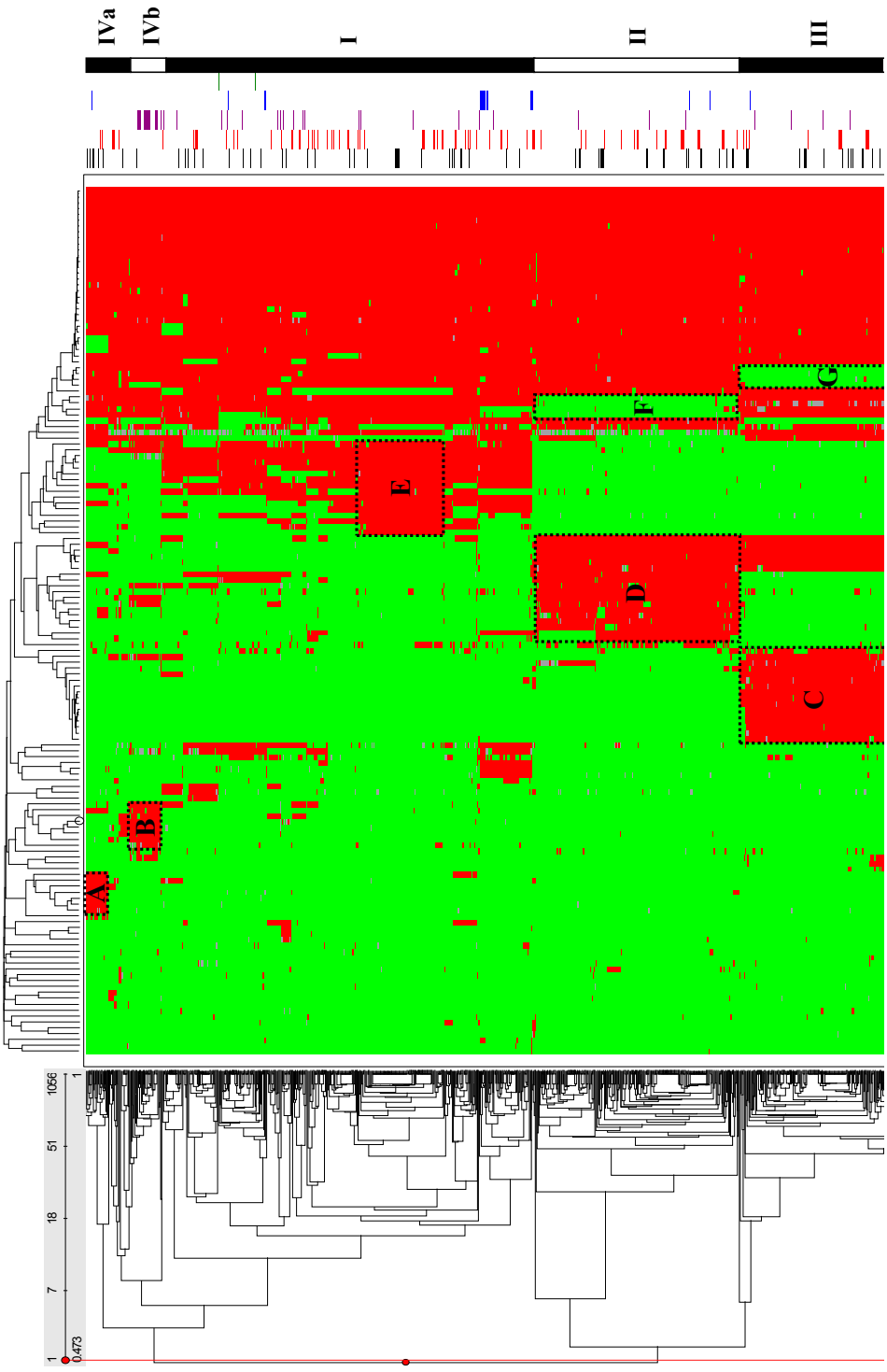
Staphylococcus aureus is a major human pathogen capable of causing a wide range of infections. Over the last 25 years, the incidence of both community-acquired and hospital-acquired *S. aureus* infections has increased (1, 2). It is assumed that most of the infections derive from nasal carriage (3-7) with the nose acting as the primary ecological reservoir of *S. aureus* in humans (8).

In order to perform detailed comparative genomics and population genetics for a bacterial species, the limited availability of adequate strain collections needs to be resolved. Although the general population structure of *S. aureus* has been studied previously (9-11), these studies were biased because of the use of mostly clinical isolates and collections of nosocomial-acquired methicillin-resistant *S. aureus* (MRSA). The population structure of naturally occurring methicillin-susceptible *S. aureus* isolated from the nose of persons living in the community has never been described in detail.

There is controversy over whether all strains of *S. aureus* have equal disease-invoking potential or whether invasive disease is associated with particularly virulent genotypes. Day et al. had to rescind their conclusion that clonal *S. aureus* strains that are most successful in colonizing humans also show increased virulence potential (9, 12). They finally concluded that there is no significant difference in population structure between *S. aureus* carriage and disease-associated strains. When Peacock et al. focused on the presence of putative virulence determinants rather than overall genome polymorphism, it was concluded that 7 of these determinants were significantly more present in invasive isolates of *S. aureus* (13). Whether this increased virulence gene density may be specific for certain phylogenetic branches or lineages of *S. aureus*, however, remains unclear.

Most recent studies have assessed the population structure of *S. aureus* using multilocus sequence typing (MLST) (9, 14, 15). This molecular typing method characterizes bacterial isolates on the basis of the sequence of internal fragments of 7 housekeeping genes, representing the stable "core" of the bacterial genome. For each gene fragment, the different sequences are translated into distinct alleles, and each isolate is defined by the combination of alleles of the 7 housekeeping loci (the allelic profile or sequence type [ST]) (14). In contrast, whole genome typing methods, including amplified fragment length polymorphism (AFLP) (16, 17), document the contribution of accessory genetic elements as well as genome-core polymorphisms. AFLP is a method that scans for polymorphism in actual restriction sites but also among the nucleotides bordering these sites. As such, it documents nucleotide sequence variation, insertions, and deletions across genomes (16). This may be a more comprehensive approach for coming to a full understanding of staphylococcal genome diversity and evolution.

We collected over 2,000 nonclinical *S. aureus* isolates from various groups of healthy individuals in the Dutch community over an extended period of time, creating a unique


IVa
IVb
I
II
III
A
B
C
D
E
F
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1056
51
18
7
1
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population-based strain collection. To assess differences in the virulence potential of various strains of *S. aureus*, insight into the natural (nonclinical) population structure is essential. We here present whole genome scanning by high-throughput AFLP (ht-AFLP) of a random selection of these *S. aureus* strains (n = 829). Strains were derived from the anterior nares of healthy children (1–18 years) and healthy elderly adults (>55 years) from the Rotterdam area (The Netherlands) (18, 19). To investigate genomic differences between these carriage strains and invasive isolates, contemporary isolates from blood, deep-seated, and soft-tissue infections from the same geographical area were included (n = 164). Furthermore, a collection of international epidemic MRSA strains (n = 21) (20) and *S. aureus* isolates from Rotterdam children with nonbullous impetigo (n = 40) were included (21). The prevalence of the clinically relevant *mecA* and Panton-Valentine leukocidin (PVL) genes was determined for all strains as well.

RESULTS

Genetic diversity of *S. aureus*

Using the set of 1,056 *S. aureus* strains, a total of 155,232 AFLP fragments were generated, covering 147 different marker fragments per strain. These outcomes are visualized in Figure 1. The dendrogram on the left (y axis) reveals bacterial clustering. The bar on the right of this figure delineates the presence of 3 major (I, II, III) and 2 minor (IVa, IVb) branches, as identified by principal component analysis (PCA) (Figure 2, A and B). Unsupervised cluster analysis of the 1,056 strains (Figure 3) clearly demonstrates that the AFLP data represent 2

Figure 1. Two-dimensional hierarchical clustering of the 1,056 *S. aureus* strains.
The green/red figure represents 155,232 binary outcomes generated by ht-AFLP with 147 marker fragments. Marker-absence corresponds with green and marker presence with red. The dendrogram on the y-axis represents the phylogenetic clustering of the 1,056 strains. The dendrogram on the x-axis shows the clustering of the 147 AFLP-markers, many of which segregate in specific groups. These groups are cluster specific, and some of these groups are shown as boxes in the figure (A through G). The colored, striped bars on the right represent the distribution of the invasive strains (children and elderly adults), the impetigo isolates, the MRSA strains, and the reference strains, respectively. The carriage strains (n = 829) are not pointed out separately. In conjunction with principle component analysis 3 major (I, II, III) and 2 minor (IVa, IVb) branches were identified; these are represented by the black and white bar on the right of the figure.

Legend of colored, striped bars

- Invasive isolates children
- Invasive isolates elderly
- Impetigo
- MRSA
- Reference strains

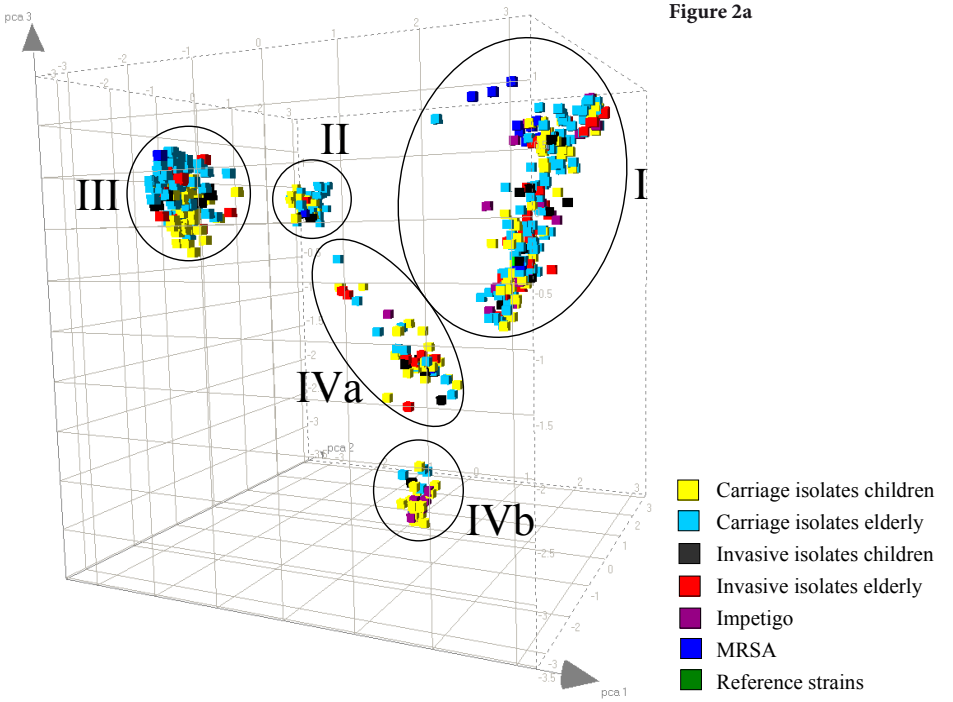
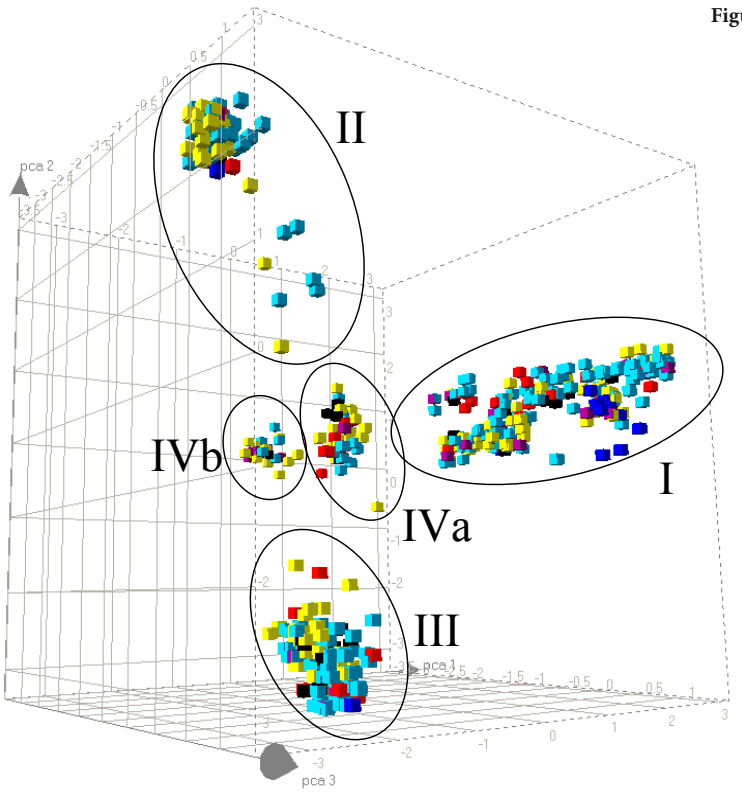


Figure 2. Principle component analysis of the 1,056 *S. aureus* strains.
The different cubes (plotted in 3-dimensional space), colored according to the source, represent every strain in the study. Each axis represents the score calculated for that strain on each principle component. The distribution of the strains is shown from two different angles (a and b). The 5 circles indicate the different phylogenetic AFLP-clusters.

distinct, homogenous major clusters (II and III) and, conversely, several other smaller subclusters that could be divided into 1 major and 2 minor AFLP clusters by PCA (I, IVa, IVb). MLST analysis indeed revealed additional heterogeneity in AFLP group I, identifying different clonal complexes (CCs) [CC5, CC8, CC15 (Figure 4)]. CCs are defined as clusters of closely related STs where single differences in the allelic profile are tolerated (9). In contrast, AFLP clusters II and III harbor single CCs, CC30 and CC45, respectively. These 2 major CCs embrace almost half (47.3%) of all carriage isolates. Clusters IVa and IVb are associated with CC22 and CC121, respectively (Figure 4). The unsupervised correlation-analysis showed that minor AFLP group IVa also consists of different subclusters (Figure 3).

The horizontal dendrogram in Figure 1 shows the clustering of the 147 AFLP markers, many of which segregate in separate groups. These groups are cluster specific; some of these are shown as boxes A-G (Figure 1). The colored bars on the right represent the distribution of the invasive strains (in children and elderly adults), the impetigo isolates, the MRSA strains, and the reference strains across the dendrogram. The central area of this figure best defines the 5 phylogenetic lineages. The clustered green and red boxes show components of

Figure 2b



genomic diversity within the 5 *S. aureus* subpopulations. The distributions of the carriage isolates as well as the invasive isolates are also visualized in Figure 3.

Carriage versus disease-causing strains of *S. aureus*

Among the *S. aureus* isolates from healthy individuals with nasal carriage, 3 major genetic clusters (I, II, III) could be identified, comprising 760 of the 829 (91.7%) carriage strains. Two minor clusters (IVa, IVb) embrace the remaining 69 (8.3%) carriage strains. All 5 clusters coherently contain carriage strains isolated from children as well as elderly adults (Table 1). The distribution of the pediatric and geriatric strains across the 5 AFLP clusters was overlapping (Figure 3). However, healthy children more often carried strains from cluster IVa+b than did healthy elderly adults (Fisher's exact test, $P < 0.0001$), whereas cluster I strains were slightly overrepresented in elderly adults (Fisher's exact test, $P = 0.03$). Although carriage isolates from children and elderly adults were equally divided in AFLP cluster II (Table 1), a clone strongly associated with carriage isolates in children could be identified (Fisher's exact test, $P < 0.0001$) (area between the dotted lines in Figure 3).

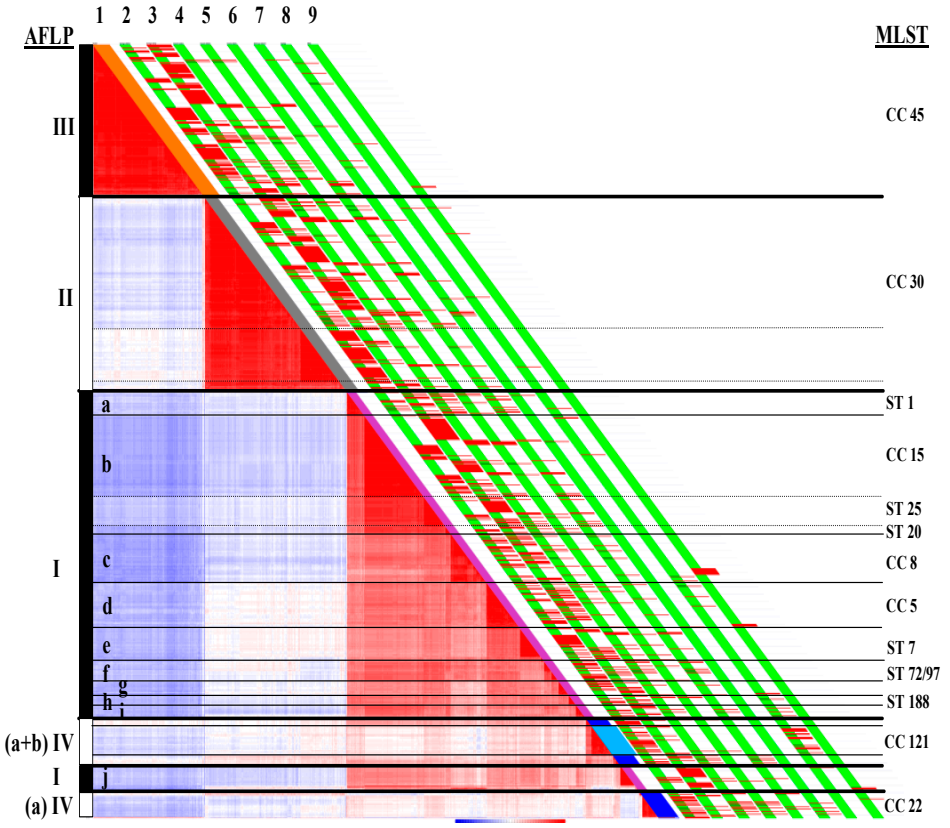


Figure 3. Cluster analysis of the 1,056 *S. aureus* strains using OmniViz.

The cells in the correlation visualization are colored by Pearson's correlation coefficient values with deeper colors indicating higher positive (red) or negative (blue) correlations. The small scale bar (underneath the figure) indicates 100% correlation (red) toward 100% anti-correlation (blue). In order to reveal correlation patterns, a matrix-ordering method was applied to rearrange the samples. The OmniViz correlation view generated with 1,056 strains was adapted so that descriptive (clinical) parameters could be plotted directly adjacent to the original diagonal. The black-and-white bar on the left indicates the 5 AFLP groups based on PCA. This figure shows additional subclustering in major group I (a-j) as well as in minor group IVa, indicated by several lines. The dotted lines identify blocks of minimal changes in one cluster or subcluster. The corresponding MLST data (see also Figure 4) are shown on the right side of the figure. The distributions of the strains from different origins are visualized as red lines in the diagonal red and green bars of the figure (numbered 2 – 9). Variable **1** indicates the different AFLP clusters based on PCA (orange = AFLP-cluster III; grey = cluster II; purple = cluster I; dark blue = cluster IVa; light blue = cluster IVb.)

Other variables: **2**, carriage isolates, children (n = 400); **3**, carriage isolates, elderly adults (n = 429); **4**, invasive isolates, total (n = 164); **5**, invasive isolates, children (n = 74); **6**, invasive isolates, elderly adults (n = 90); **7**, invasive isolates, children (deep-seated and soft-tissue infections) (n = 18); **8**, impetigo isolates (n = 40); **9**, MRSA (n = 21).

Contemporary invasive *S. aureus* strains (n = 164) from children and elderly adults were distributed across all phylogenetic branches and widely scattered across the AFLP dendrogram (Figures 1 and 3). The population structure of contemporary carriage isolates and in-

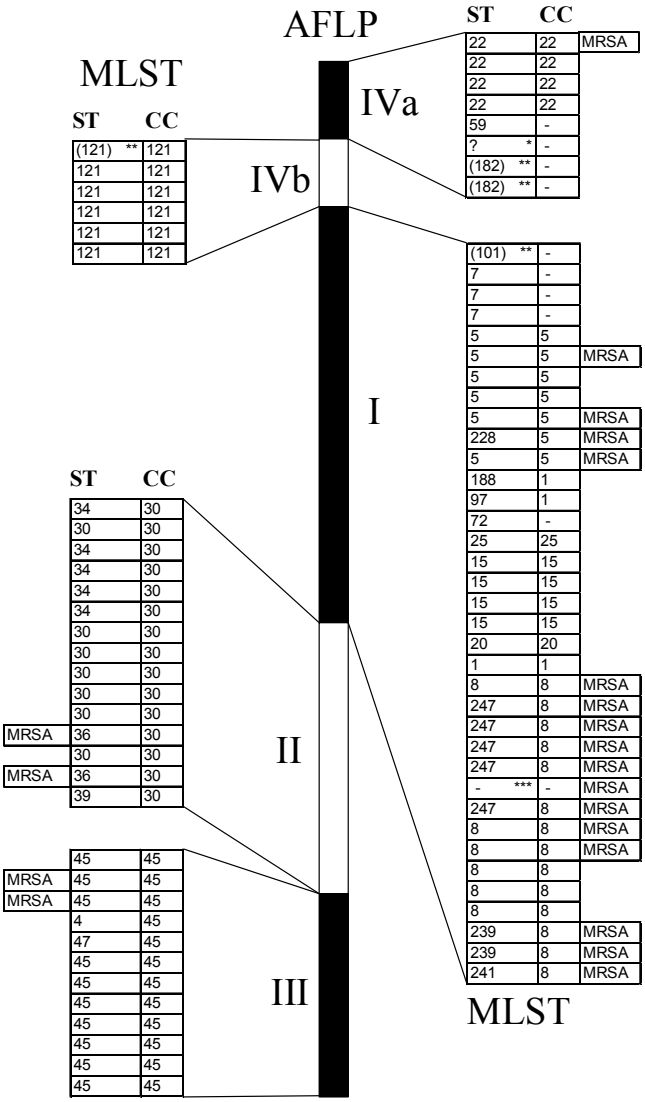


Figure 4. AFLP analysis versus MLST analysis of *S. aureus*.

The bar in the center of the figure represents the 1,056 strains divided into the five phylogenetic AFLP clusters (similar to those defined at the right side of Figure 1). MLST data is shown for 77 *S. aureus* strains, which are spread over the different AFLP clusters. The order of the MLST sequence types in this figure is determined by the location of the strain in the AFLP dendrogram (Figure 1). * Unknown ST; ** 6 of the 7 loci are similar to the particular ST; *** data not available.

vasive isolates in the same geographical area appeared to be strongly overlapping (Table 1). However, bacteremia in elderly adults was significantly more often caused by a IVa cluster–derived strain (Fisher's exact test, $P = 0.0095$). Additional analysis of AFLP cluster IVa showed that this group consists of distinct subclusters (Figure 3). Therefore it would be incorrect to define this minor cluster as a single invasive clone. Apparently, several subclusters, plotted next to each other by 3D (AFLP cluster IVa), contain proportionately more bacteremia isolates from elderly adults than carriage isolates from the same group. Statistical analysis of major cluster I revealed 2 subclusters potentially associated with invasive disease. Subcluster If (Figure 3) contains proportionately more bacteremia isolates from elderly adults ($n = 5$; 5.6%) in comparison with carriage isolates from elderly adults ($n = 6$; 1.4%) (Fisher's exact

Table 1. Distribution of *S. aureus* strains in the five phylogenetic branches

	AFLP-cluster (*n* (%))					
	I	II	III	IVa	IVb	
Carriage						
Children ($n = 400$)	162 (41) [a]	103 (26)	86 (22)	31 (8) [b]	18 (5) [c]	
Elderly ($n = 429$)	206 (48) [a]	113 (26)	90 (21)	14 (3) [b]	6 (1) [c]	
Invasive isolates						
Children ($n = 74$)	30 (41)	20 (27)	16 (22)	7 (9)	1 (1)	
Elderly ($n = 90$)	47 (52)	22 (24)	2 (13)	9 (10) [d]	0 (0)	
Impetigo ($n = 40$)	17 (43)	3 (8) [e]	4 (10)	1 (3)	15 (38) [f]	
MRSA ($n = 21$)	16 (76) [g]	2 (10)	2 (10)	1 (5)	0 (0)	

[a] Overrepresentation of carriage in the elderly adults (Fisher's exact test; $P = 0.03$); [b+c] overrepresentation of carriage in children (Fisher's exact test; $P < 0.0001$); [d] proportionally more bacteremia-associated strains from elderly adults as compared to carriage strains from the same group (3 vs. 10%; Fisher's exact test; $P = 0.0095$); [e] proportionally fewer impetigo-associated strains as compared to carriage in children (8 vs. 26%; Fisher's exact test; $P = 0.01$); [f] overrepresentation of impetigo-associated strains as compared to carriage in children (38 vs. 5%; Fisher's exact test; $P < 0.0001$); [g] proportionally more MRSA strains as compared to all carriage isolates (76 vs. 44%; Fisher's exact test; $P = 0.006$).

test, $P = 0.027$). Subcluster Ia shows overrepresentation of invasive isolates from both children and elderly adults ($n = 11$; 6.7%) in comparison with carriage isolates from both groups ($n = 20$; 2.4%) (Fisher's exact test; $P = 0.01$) (Figure 3).

The invasive strains from children in major cluster I are associated with hospital-acquired disease (Fisher's exact test, $P = 0.01$) (data not shown). No significant difference was found in the distribution of isolates from individuals with invasive community-acquired disease versus invasive hospital-acquired disease in the other clusters.

All 5 clusters contain *S. aureus* strains isolated from children with community-acquired nonbullous impetigo. The distribution is shown in Figures 1 and Table 1. Compared to pediatric carriage strains, impetigo isolates were more frequently found in cluster IVb (Fisher's exact test, $P < 0.0001$) and less frequently in cluster II (Fisher's exact test, $P = 0.01$) (Table 1), suggesting clonal expansion of a certain genotype associated with impetigo (22).

MecA- and PVL-positive strains

The 21 international epidemic MRSA strains are present in several clusters and subclusters of *S. aureus* (Ic, Id, Ij, II, III, IVa). This strain collection comprises epidemic MRSA from Belgium, Finland, France, Greece, Spain, Germany, and the United Kingdom (20). AFLP cluster I contained disproportionately more MRSA isolates as compared to the other clusters (Fisher's exact test, $P = 0.006$) (Table 1), suggesting that many of these epidemic MRSA strains are derived from a common cluster I ancestor. Notably, most of the 21 MRSA strains are located in subcluster Ic ($n = 9$; 42,9%; Fisher's exact test, $P < 0.0001$)].

None of the Dutch carriage and clinical *S. aureus* isolates ($n = 1033$) included in this study harbor the *mecA* gene, which is consistent with the reported low MRSA prevalence in the Dutch population (23). Four PVL-positive *S. aureus* strains (1.0%) were found in the pediatric carriage group ($n = 400$) and 1 (0.2%) in the elderly adults carriage group ($n = 429$). Three of 146 (2.1%) blood-culture isolates carried the PVL gene, 2 of which derived from pediatric patients. Seven of the 18 (38.9%) invasive strains isolated from deep-seated or soft-tissue infections in children were PVL positive. There was no significant difference in the presence of PVL when comparing the carriage isolates and invasive blood-culture isolates. In contrast, *S. aureus* strains causing abscesses and arthritis were significantly enriched in the presence of PVL (38.9%) in comparison with the pediatric carriage group (1.0%) (Fisher's exact test, $P < 0.0001$) and in comparison with the pediatric bacteremia isolates (3.6%) (Fisher's exact test, $P = 0.0005$). All impetigo strains ($n = 40$) were PVL negative.

Sequence assessment of AFLP markers

To determine the origin of genetic polymorphism, the nucleotide sequence of a set of 81 AFLP markers was established, 60 of which were located in cluster-specific marker boxes (Figure 1, boxes A-G). Nineteen of the remaining 21 markers were not cluster specific and were present in almost all 1,056 *S. aureus* strains (red area on the right side of box G, Figure

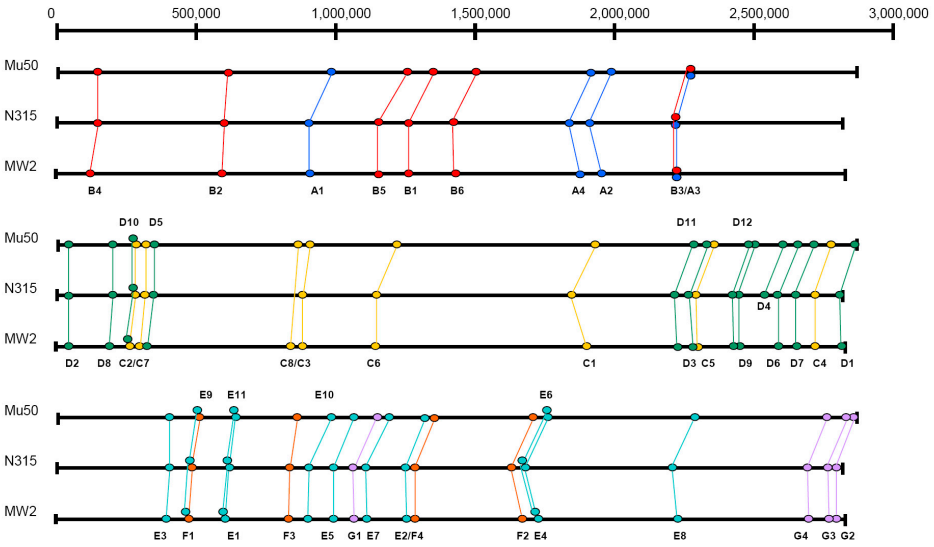


Figure 5. The localization is shown for 48 AFLP-markers on the three completed *S. aureus* genomes (Mu50, N315 and MW2). The different characters (A through G) correspond with the different cluster-defining blocks in Figure 1. The numbering of the characters (markers) corresponds with the order of the markers in the horizontal dendrogram in Figure 1. (For color see the online version of this thesis.)

1). Interestingly, 66 (81.5%) of the 81 markers showed homology to all 3 completed (MW2, Mu50, N315) and 4 unfinished (252, 476, COL, NCTC 8325) genome sequences of *S. aureus*. Conversely, 7 (8.6%) of the 81 markers appear to be absent in all 3 completed genomes (MW2, Mu50, N315) of *S. aureus* (see Supplemental Table S1; supplemental material available at http://www.jci.org/cgi/content/full/114/12/1732/DC1). Of these 7 marker fragments, 3 showed homology to the *S. aureus* strain 252 epidemic MRSA-16 (EMRSA-16), 1 to bacteriophage Φ exfoliative toxin A (ETA) DNA, and 1 to *S. aureus* TY4 exfoliative toxin B (ETB) plasmid DNA. Only 2 did not match with any GenBank entry and may represent novel (or hypermutable) *S. aureus* genome segments. The 15 markers that appear to be absent in at least 1 of the known genome sequences of *S. aureus* may be associated with (larger) deletions, the existence of which has been described before (24).

The cluster-specific marker boxes are associated with different AFLP clusters. Box A is associated with a part of cluster IVa, B with cluster IVb, C (present) and G (absent) with cluster III, D (present) and F (absent) with cluster II, and box E with cluster I (Figure 1). The majority (78.0%) of the markers present in boxes A-D (not present in cluster I) showed homology to the reference strains Mu50 and N315, which are located in AFLP cluster I. This suggests that these markers become cluster specific by point mutations rather than genomic rearrangement (deletions or insertions). This is also supported by additional data (Figure 5),

which show that cluster-defining markers in each box are widely spread across the *S. aureus* chromosome.

Cluster-specific box B, associated with cluster IVb (with overrepresentation of impetigo-isolates), comprises both virulence genes bacteriophage Φ ETA DNA and ETB plasmid DNA (Supplemental Table S1).

DISCUSSION

The incidence of *S. aureus* bacteremia is rising and has more than doubled over the past 25 years in some Western European countries. This increase coincides with a growing rate of community-acquired disease (in proportion to hospital-acquired disease) and the epidemic emergence of nosocomial MRSA strains (2). Elderly adults are most frequently affected, particularly those with additional predisposing risk factors. Although a part of this effect may be explained by host susceptibility and population aging, this does not fully explain the current and drastic rise in the number of infections. However, little is known about possible bacterial determinants and whether or not these are associated with changes in the virulence of *S. aureus*.

We previously showed that AFLP analysis using optimal enzyme and primer combinations is an excellent tool for assessing genetic polymorphism in the clonal microorganism *M. tuberculosis* (17). For *S. aureus* we used the enzyme combination *Mbo*I-*Csp*6I, which resulted in a fingerprint of about 70 polymorphic AFLP fragments well distributed within the size range of 100 bp to 600 bp in a single AFLP reaction. However, a potential limitation of this AFLP approach is in the randomness of the restriction sites for *Mbo*I and *Csp*6I. For instance, genomic islands with underrepresentation of these restriction sites will not be fully scanned for polymorphism. However, if currently known genomic sequences of *S. aureus* (Mu50 and N315) are analyzed by computer for the occurrence of these sites, the average number of fragments (useful for AFLP) generated per genome is 4.373, and the average length of the fragments is 200 bp. This suggests that coverage is indeed random and, in this respect, AFLP provides more of a whole genome–scanning approach than MLST, for instance.

In the present study, the population structure of *S. aureus*, isolated from the nose of healthy individuals in the Rotterdam area (The Netherlands), has been determined. Using ht-AFLP, we analyzed 147 polymorphic markers for 1,056 *S. aureus* strains. Two large unbiased strain collections of a nonclinical origin were used. These collections were obtained from children (<19 years) and elderly adults (>55 years) with nasal carriage of *S. aureus*. ht-AFLP analysis revealed the existence of 3 major (I, II, III) and 2 minor (IVa, IVb) phylogenetic branches. Major AFLP group I could be subdivided into 10 different subclusters, indicating its heterogeneity. In contrast, major clusters II and III showed a very homogenous nature.

Carriage isolates were not completely randomly distributed over these clusters. AFLP cluster IVa+b represents more carriage strains from children (isolated in 2002) than carriage strains from elderly adults (isolated from 1997–1999). A clonal expansion associated with carriage isolates in children was also observed in AFLP group II. Conversely, AFLP group I embraces more carriage strains from elderly adults. Apparently, a certain degree of bacterial population heterogeneity exists between the 2 groups included. Whether the different sampling moments or the different age categories are fundamental to the observed differences is currently unclear. Danish studies from the late 1970s demonstrated that waves of phage types of *S. aureus* go through human populations (25, 26). Such a phenomenon may explain our current observations. However, the proportionate distribution of the major phylogenetic branches (I, II, III) within the overall population of *S. aureus*, isolated from humans, appears to be fairly stable over time and comparable for children and elderly adults.

The 5 AFLP clusters identified in this study match with the major CCs as defined by MLST (http://www.mlst.net/). These MLST-based CCs have been defined by studying carriage, invasive, and MRSA isolates mainly from the United Kingdom. The top 5 CCs in the MLST database are CC8, CC30, CC5, CC22, and CC45 (9, 15, 27, 28). We studied more than 1,000 strains isolated in the Rotterdam region (The Netherlands), and we identified essentially the same CCs. Apparently, these clonal clusters have spread successfully in the United Kingdom and The Netherlands and probably worldwide. All large-scale molecular typing studies of nonclinical isolates of *S. aureus* have been performed using geographically biased strain collections, including our present analysis. However, considering the overlap in MLST types and the similarity in prevalence of certain major clonal clusters, it is supposed that geographical bias is not a confounding factor. An ongoing analysis of strains derived from Indonesian carriers corroborated this hypothesis. The Indonesian strains clustered in the same groups (I to IV), although there was a difference in the relative numbers of isolates per cluster. No new AFLP clusters were identified (Melles et al., unpublished data).

ht-AFLP clusters II and III, identical to MLST CCs 30 and 45, respectively, account for almost half (47%) of all carriage isolates in our population, which suggests that these 2 CCs have evolved to be very successful in colonizing humans.

The above-mentioned observations suggest that virulent and nonvirulent strains are probably not fundamentally different from each other: clinical isolates and MRSA from international sources fall into the same main clusters as carriage isolates. In addition, invasive *S. aureus* strains, mainly blood-culture isolates (89%), were found to be widely distributed across all 5 AFLP groups in this study. This suggests that strains from each of the genetic clusters are essentially able to cause invasive disease. On the other hand, 1 minor AFLP cluster (IVa) and 2 AFLP subclusters (Ia and If) contained proportionately more invasive isolates. Direct cross-infection is not a likely explanation for this finding because these epidemiologically nonrelated invasive strains were isolated from patients in different medical departments over a period of several years. There have been controversial reports on the

existence of hypervirulent lineages of *S. aureus* (9, 12), but our data suggest that not all *S. aureus* strains share the same invasive potential. This is not in agreement with recent findings from the Oxford (United Kingdom) region where MLST data suggest that virulence may not be associated with clonal lineages (15). Rather, a relationship between genetic background and disease type is thought to be primarily dependent on the presence of certain toxin genes only (29).

S. aureus strains isolated in the Rotterdam area from patients with nonbullous impetigo showed less clonal diversity than bacteremia-associated strains. Although each of the 5 clusters was found to contain impetigo-derived isolates, AFLP cluster IVb clearly represented significantly more isolates causing impetigo. An explanation for this clonal expansion in impetigo could be the facile spread of this disease. A recent study by Koning et al. concerning nonbullous *S. aureus* impetigo concluded that a combination of staphylococcal virulence and resistance genes determines the development and course of nonbullous impetigo (22).

The 21 international epidemic MRSA strains included in this study were spread across several lineages, indicating that methicillin resistance has developed in distinct phylogenetic subpopulations of *S. aureus*, which has been described before (10, 30). MLST studies have placed most of the MRSA in 5 major clusters: CC5, CC8, CC22, CC30, and CC45 (10, 27, 31, 32). Figure 4 shows similar dissemination for strains from a nonclinical origin as well. Notably, computer algorithms used to solve the *S. aureus* population structure based on MLST data (eBURST; ref. 28) generate clustering identical to that found in the AFLP Spotfire and OmniViz analyses. This provides a solid experimental and mathematical framework for all conclusions drawn from our AFLP data.

We have also assessed the prevalence of *mecA* and the PVL genes in the carriage and disease-causing populations of *S. aureus*. All carriage isolates (n = 829) were *mecA* negative, corroborating data showing the insignificant spread of MRSA in the Dutch community (23). Also, all clinical isolates (n = 204) were *mecA* negative, as opposed to proportions of above 10% in many of the other European countries, including those sharing borders with The Netherlands (33). PVL is a toxin associated with skin infections (furuncles), community-acquired MRSA infections, and necrotizing pneumonia (31, 34, 35). PVL prevalence in a *S. aureus* population of nonclinical isolates has never been studied accurately. We found a very low prevalence of 0.6% in a large (n = 829) carriage collection. In this study PVL was carried in 2.1% of blood-culture isolates. However, a significantly higher prevalence of PVL (38.9%) was found in *S. aureus* strains causing abscesses and arthritis. This is in agreement with the proposed involvement of PVL in severe and invasive (soft-tissue) staphylococcal infections (31, 35).

Our sequence analysis for the clustered AFLP markers suggested that genetic diversity among clusters is primarily caused by point mutation rather than by large-scale deletions or insertions (15). Ultimate proof for this hypothesis should be provided by detailed physical mapping and large-scale sequencing studies, however. Furthermore, we provide indirect

proof that 7 genome sequences quite accurately represent the genetic potential of *S. aureus* as a species; only 4 of 81 marker sequences did not match with the 7 known *S. aureus* whole genome sequences.

In conclusion, we have solved the population structure of *S. aureus* of nonclinical origin. Three major and 2 minor phylogenetic branches were identified in our geographically restricted group. Inclusion of invasive *S. aureus* strains and international-epidemic MRSA revealed that within all major clusters, invasive and multiresistant isolates could be identified. However, clusters and subclusters with overrepresentation of bacteremia-associated isolates were identified. Expansion of multiresistant clones or clones associated with skin disease (impetigo) was observed as well. We suggest that essentially any *S. aureus* genotype that is carried by humans can transform into a life-threatening human pathogen, but strains from some clonal lineages are more virulent than others.

METHODS

Bacterial strains

Two strain collections provided nonclinical *S. aureus* carriage isolates from healthy individuals. These collections were obtained from 2 study cohorts involving children and elderly adults. In addition, various clinical isolates were included. Contemporary invasive *S. aureus* strains, isolated from children and elderly adults from the same geographic region, were cultured from normally sterile sites in hospitalized patients with clinical signs of *S. aureus* infection. Community-acquired invasive disease was defined as isolation of *S. aureus* from patients within 48 hours of admission; hospital-acquired was defined as isolation of *S. aureus* 48 hours or longer after admission. The different subcollections are described in more detail below.

In total, 3,198 children from Rotterdam (The Netherlands), aged between 1 and 19 years and participating in the national 2002 Meningococcal Vaccination Campaign, were enrolled (19). A team of 10 research nurses and medical doctors obtained a single nasopharyngeal swab per child at the time of vaccination. *S. aureus* was isolated from 1,116 children. All isolates were stored at –80°C in broth containing glycerol. A random sample of 400 *S. aureus* carriage isolates was drawn.

The second collection originated from a community-based prospective study of elderly adults in Rotterdam (The Netherlands) (18). From 3,851 persons aged over 55 years, nasal swab cultures were obtained between April 1, 1997, and December 31, 1999. *S. aureus* strains were isolated from 1,043 elderly adults. All isolates were stored at –80°C in glycerol containing broth. A random sample of 429 carriage isolates was drawn.

Seventy-four clinical *S. aureus* isolates were retrospectively collected from children with invasive *S. aureus* disease identified in Sophia Children's University Hospital (Rotterdam,

Table 2. Number of *S. aureus* strains included in this study

	No. (%) of isolates		
	Children	**Elderly**	**Total**
Carriage	400	429	829
Invasive – blood-culture			
Hospital-acquired	43	68	111
Community-acquired	13	22	35
Invasive – deep-seated or soft-tissue infection			
Hospital-acquired	4	-	4
Community-acquired	12	-	12
Unknown	2	-	2
Impetigo	40	-	40
MRSA	-	-	21
Reference strains	-	-	2
Total	514	519	1056

The Netherlands) (2000–2002). Fifty-six isolates derived from blood cultures and 18 isolates were obtained from deep-seated (arthritis; $n = 4$) or soft-tissue (abscess; $n = 14$) infections. Ninety clinical isolates from elderly adults (>55 years) were obtained from persons with *S. aureus* bacteremia identified in Erasmus MC (Rotterdam, The Netherlands) (1997–1999). Forty *S. aureus* strains obtained from lesions of children suffering from impetigo were randomly drawn from a collection described by Koning et al. (21).

Twenty-one international epidemic MRSA strains were obtained from the HARMONY collection (http://www.harmony-microbe.net), described by Murchan et al. (20). Finally, we included 2 reference strains. N315 is an MRSA strain isolated in 1982, and Mu50 is an MRSA strain with reduced susceptibility to vancomycin isolated in 1997. For both strains genome sequences have been determined (36). In total, 1,056 *S. aureus* strains were included (Table 2).

Cultures, DNA isolation, and detection of *mecA* and PVL genes

Bacteria were grown overnight at 37°C on Columbia III agar (BD) supplemented with 5% sheep blood. Three to 5 colonies were suspended in TEG buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose) containing lysostaphin (50 µg/ml) and incubated at 37°C for 1 hour. DNA was extracted with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) using the MagNA Pure LC Instrument (Roche Diagnostics) and stored at –20°C. We determined the presence of the *mecA* and PVL genes by PCR (34, 37).

AFLP

AFLP analysis has been performed as described by Van den Braak et al. (17). Using the predictive software package Recomb (Keygene NV) (38), the optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *Mbo*I and *Csp*6I, and

the linker oligonucleotide pair for *Mbo*I (5′-CTCGTAGACTGCGTACC-3′ and 5′-GATCGGTACGCAGTCTAC-3′) and for *Csp*6I (5′-GACGATGAGTCCTGAC-3′ and 5′-TAGTCAGGACTCAT-3′) were ligated. Subsequently, a nonselective preamplification was performed using the *Mbo*I primer (5′-GTAGACTGCGTACCGATC-3′) and *Csp*6I primer (5′-GACGATGAGTCCTGACTAC-3′). In the final amplification, a 33P-labeled *Mbo*I primer containing 1 selective nucleotide (either +C or +G) and a *Csp*6I primer containing 2 selective nucleotides (+TA) were used. Amplified material was analyzed using standard polyacrylamide slabgels and subsequent autoradiography. Marker fragments were scored and a binary table scoring marker fragment absence (0) or presence (1) was constructed.

After excision of some selected AFLP fragments from dried gels, reamplification followed by double-strand sequence analysis was performed (17, 38). The sequence of several additional fragments was determined by computer analysis. The size of the fragments in combination with the selective nucleotides of the AFLP primers facilitated adequate mapping of the fragments on the staphylococcal genome sequence. These fragments were further analyzed by BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/) (39) against the 3 completed (MW2, NC_003923.1; Mu50, NC_002758.1; N315, NC_002745.2) and 4 unfinished (252, NC_002952; 476, NC_002953; COL, NC_002951; NCTC 8325, NC_002954) genomic sequences of *S. aureus*. The BLAST results enabled computer-mediated genomic localization and gene annotations of the AFLP fragments.

MLST

MLST was carried out for 56 *S. aureus* strains using DNA arrays (40). The selected strains were equally distributed across the AFLP dendrogram by selecting 1 out of 10 carriage or invasive strains isolated from children, going from top to bottom through the AFLP dendrogram (Figure 1). MLST data for the 21 epidemic MRSA strains are available at the MLST home page (http://www.mlst.net/) (20).

Data analysis

The method used for 2D clustering of the AFLP data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic mean (UPGMA). The similarity metric used was Tanimoto (Spotfire DecisionSite 7.2; Spotfire), which defines similarity for binary data (0 and 1) based on the number of positive attributes that 2 records have in common. The resulting dendrogram was ordered by average value.

PCA is a standard multivariate method used to reduce the dimensional space of the data to its principal components (PCs) (41, 42). PCA aims to reduce a large number of variables that explain most of the variation in the data (43). It is basically a rotation of axes after centering data to the means of the variables, the rotated axes being the PCs, which are linear combinations of the original variables. The PC computation is displayed as a 3D scatter plot in which the position along the axes shows the PCA score of the strain. PCA was used to

identify subgroups of AFLP clusters as hidden by 2D representation of hierarchical clustering. The distribution of the strains in the 5 phylogenetic branches was defined on the basis of PCA. Hierarchical cluster analysis and PCA were performed using Spotfire DecisionSite 7.2 software.

The OmniViz package (OmniViz Inc.) was used to perform and visualize the results of unsupervised cluster analysis in a correlation visualization. This correlation visualization tool displays pairwise correlations among the samples calculated by Pearson's correlation coefficient (44). In order to reveal correlation patterns, a matrix-ordering method is applied to rearrange the samples. The ordering algorithm starts with the most correlated sample pair and, through an iterative process, sorts all the samples into correlated blocks. Each sample is joined to a block in an ordered manner so that a correlation trend is formed within a block with the most correlated samples at the center. The blocks are then positioned along the diagonal of the plot in a similar ordered manner. As the resultant visualization is symmetrical about the diagonal, half the matrix display is replaced by appropriate clinical data.

To compare the distribution of strain categories in different phylogenetic lineages, Fisher's exact test was used. A 2-sided *P* value of less than 0.05 was considered significant.

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Nonstandard abbreviations used: AFLP, amplified fragment length polymorphism; CC, clonal complex; ETA, exfoliative toxin A; ht-AFLP, high-throughput AFLP; MLST, multilocus sequence typing; MRSA, methicillin-resistant Staphylococcus aureus; PC, principal component; PCA, principal component analysis; PVL, Panton-Valentine leukocidin; ST, sequence type.

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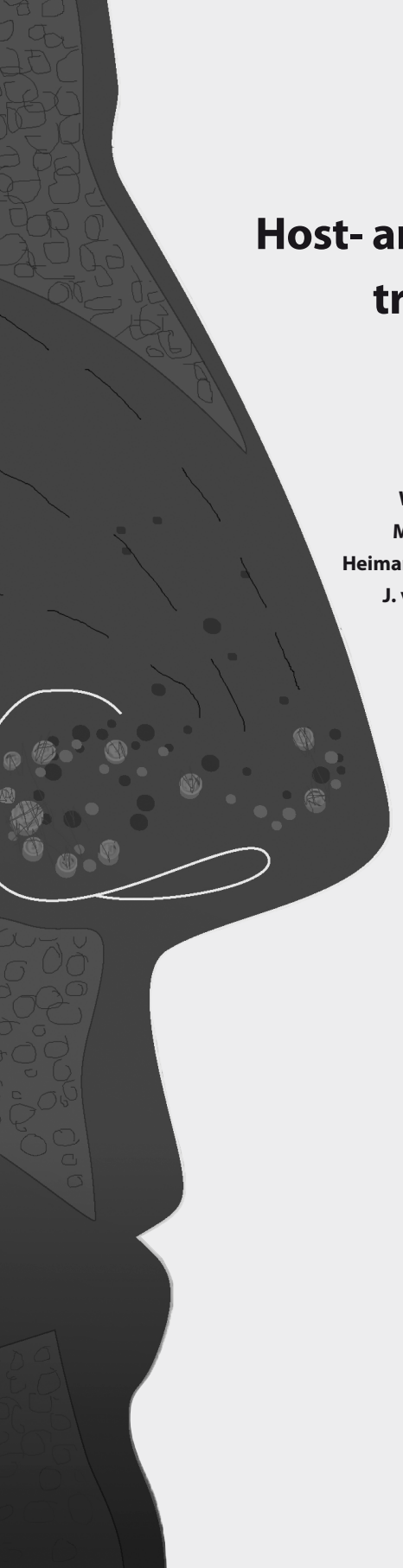

Chapter 4

Host- and tissue-specific pathogenic traits of *Staphylococcus aureus*

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ABSTRACT

Comparative genomics were used to assess genetic differences between *Staphylococcus aureus* strains derived from infected animals versus colonized or infected humans. A total of 77 veterinary isolates were genetically characterized by high-throughput amplified fragment length polymorphism (AFLP). Bacterial genotypes were introduced in a large AFLP database containing similar information for 1,056 human *S. aureus* strains. All *S. aureus* strains isolated from animals in close contact with humans (e.g. pet animals) were predominantly classified in one of the five main clusters of the AFLP database (cluster I). In essence, mastitis-associated strains from animals were categorized separately (cluster IVa) and cosegregated with bacteremia-associated strains from humans. Distribution of only 2 out of 10 different virulence genes differed across the clusters. The gene encoding the toxic shock syndrome protein (*tst*) was more often encountered among veterinary strains ($P < 0.0001$) and even more in the mastitis-related strains ($P < 0.0001$) compared to human isolate results. The gene encoding the collagen binding protein (*cna*) was rarely detected among invasive human strains. The virulence potential, as indicated by the number of virulence genes per strain, did not differ significantly between the human- and animal-related strains. Our data show that invasive infections in pets and humans are usually due to *S. aureus* strains with the same genetic background. Mastitis-associated *S. aureus* isolated in diverse farm animal species form a distinct genetic cluster, characterized by an overrepresentation of the toxic shock syndrome toxin superantigen-encoding gene.

INTRODUCTION

Staphylococcus aureus can colonize and infect a variety of members of the animal kingdom, including mammals, reptiles, and birds. Infection models for this clinically highly relevant bacterial species in lower organisms, such as insects (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*), have been described as well (1, 2). Various studies address the molecular basis of the apparent host specificity or diversity of *S. aureus* strains. This is usually approached by determining genome polymorphism with multilocus enzyme electrophoresis (3, 4), pulsed-field gel electrophoresis (PFGE) (5), binary typing (6), or shotgun sequencing (7). In addition, animal studies often involve *S. aureus* strains with specific mutations in defined virulence factors (8). These studies have demonstrated that strains from humans can cross-infect domestic animals (9) and poultry (10) and vice versa (11). It has been determined that separate host-specific lineages of *S. aureus* do exist, but the question with regard to cross-species pathogenicity of these lineages still remains unanswered (4).

Genomic studies as mentioned above have revealed extensive genetic variation in natural populations of *S. aureus*. Pathogenic strains may harbor complete pathogenicity islands (12) or specific "accessory" genes, such as the one encoding the Panton-Valentine leucocidin (13). The evolutionary processes in *S. aureus* restricting or expanding invasiveness in different hosts are ill defined (14), although, for instance, numbers and combinations of certain virulence genes may be important contributors to pathogenic potential (15). We studied *S. aureus* host specificity and virulence by comparative genomics (high-throughput amplified fragment length polymorphism [ht-AFLP]). In addition, the distribution and number of virulence genes were compared between clinical and nonclinical strains isolated from different host species.

MATERIALS AND METHODS

Bacterial strains

Two strain collections were used; the first strain collection comprised 77 *S. aureus* strains isolated from different infection sites in a variety of animal species, including dogs ($n = 8$), monkeys and apes ($n = 4$; different species), pigs ($n = 6$), birds ($n = 5$; different species), cats ($n = 12$), sheep ($n = 6$), seals ($n = 4$), goats ($n = 8$), rabbits ($n = 3$), cows ($n = 7$), and horses ($n = 10$), a rat, a chinchilla, a guinea pig, and an iguana (one isolate each). The second strain collection consisted of 168 human *S. aureus* strains, which were classified as invasive ($n = 56$) or colonizing ($n = 112$). Strains were obtained and selected from a previous study (16). An invasive strain was derived from a patient with a positive nasal culture upon admission to the hospital while suffering from a manifest *S. aureus* infection by the same strain at a normally sterile site more than 2 days after admission. A colonizing strain was derived from

a positive nasal culture from a matched control patient for whom no *S. aureus* infection was documented during hospitalization. For each invasive isolate, two matched controls were selected. The risk factors for both patient groups were matched as well (same hospital, ward, age, gender, and period of hospitalization) (16). Prior to genomic analysis, bacteria from glycerol stocks, stored at −80°C, were inoculated on Columbia III agar supplemented with 5% sheep blood (Becton Dickinson, Etten-Leur, The Netherlands) and incubated at 37°C for 24 h. All strains were identified as *S. aureus* by accepted microbiological methods (17).

DNA extraction

Chromosomal DNA was extracted from bacterial cells with a MagnaPure LC DNA system (DNA isolation kit III; Roche, Almere, The Netherlands) according to the manufacturer's instructions, with the modification that 50 μg/ml lysostaphine (Sigma, Zwijndrecht, The Netherlands) was added during the cell lysis step. The DNA concentration was measured by UV spectroscopy, samples were diluted with distilled water to a final concentration of 10 ng/μl, and DNA was stored at −20°C until use.

Ht-AFLP

The genomes of the animal-associated *S. aureus* strains (n = 77) were compared by ht-AFLP. Individual AFLP-PCRs were performed essentially as described before in the presence of radioactive nucleotides for the visualization of the fingerprints (18). Ht-AFLP was performed using the restriction enzyme combination *Mbo*I and *Csp*6I (New England Biolabs, Westburg, Leusden, The Netherlands). Each restriction site was filled in by ligation with specific linker oligonucleotide pairs (for *Mbo*I, 5′-CTCGTAGACTGCGTACC-3′ and 5′-ATCGGTACGCAGTCTAC-3′; for *Csp*6I, 5′-ACGATGAGTCCTGAC-3′ and 5′-TAGTCAGGACTCAT-3′). Subsequently, a nonselective preamplification was performed using the *Mbo*I primer (5′-GTAGACTGCGTACCGATC-3′) and *Csp*6I primer (5′-ACGATGAGTCCTGACTAC-3′). In the final amplification, a ^{33}P-labeled *Mbo*I primer containing one selective nucleotide (either +C or +G) and a *Csp*6I primer containing two selective nucleotides (+TA) were used.

AFLP data analysis

Agglomerative hierarchical cluster analysis was used for two-dimensional data clustering of the AFLP patterns. The unweighted pair group arithmetic mean method was performed for cluster analysis. After inclusion of the AFLP patterns in the AFLP database, the Tanimoto method was used to calculate the similarity matrix. The resulting dendrogram was ordered by average value. Spotfire DecisionSite 7.2 software has been applied to perform statistical analyses.

AFLP database

This database consists of ht-AFLP fingerprints obtained for 1,056 *S. aureus* strains from human origin (18) and comprises 829 nonclinical carriage strains from healthy individuals, 74 strains from children with invasive *S. aureus* disease (bacteremia, arthritis, and abscess), 90 isolates from elderly (>55 years) individuals with *S. aureus* bacteremia, 40 isolates obtained from lesions of children suffering from impetigo, 21 international epidemic methicillin-resistant *S. aureus* strains (19), and 2 reference strains (12).

MLST

A selection of 13 of the animal-associated strains were analyzed by the microarray-mediated multilocus sequence typing (MLST) protocol as described previously (20). Briefly, allele types of seven *S. aureus* housekeeping genes were defined by specific hybridization of amplified gene fragments to short oligonucleotide probes, synthesized on a Gene Chip array (Affymetrix, Santa Clara, Calif.; bioMérieux, Marcy l'Etoile, France). The resulting allelic sequences were matched with those from the MLST database (www.MLST.net), and an allelic profile with the corresponding sequence type (ST) and clonal complex (CC) could be determined.

PCR of putative virulence factors

For both strain collections, the genes coding for adhesins *fnbA*, *clfA*, *clfB*, *cna*, *sdrE*, and *ebpS* and for the exoprotein toxins *tst*, *eta*, *etb*, and *pvl* were amplified using the PCR primers and amplification conditions outlined in Table 1 (15, 21-31). For the confirmation of the PCR results, a second PCR, targeting a different domain of the same genes, was done. PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). A positive control (Table 1) and a negative control (PCR mixture excluding DNA) were included in each PCR run.

Statistical analysis

The distribution of putative virulence factors across *S. aureus* strains originating from different hosts were compared using Fisher's exact test. The odds ratio was defined as the cross-product ratio of the numbers shown in a two-by-two contingency table. Confidence intervals of 95% were used throughout. *P* values less than 0.05 were considered significant.

RESULTS

Genotyping of *S. aureus* strains

Ht-AFLP was used to determine the genomic variability within the veterinary *S. aureus* strain collection. AFLP results are summarized in Fig. 1. Four clusters, I, IVa, II, and III (corresponding to the previously defined AFLP clusters for human strains included in the

Table 1. PCR primers and conditions for identification of potential virulence genes

PCR product	Oligonucleotide sequences (5'-3')	GenBank accession no.	Position in gene	Positive control	PCR conditions	Reference
fnbA	Fw, CAC AAC CAG CAA ATA TAG	AJ629121	424-441	8325-4	1 min at 94°C, 1 min at 50°C, 2 min	15
	Rv, CTG TGT GGT AAT CAA TGT C		1785-1667		at 72°C	
	Fw, GGT AAT CAT TCA TTC GAG		2355-2372	8325-4		31
	Rv, TGG CAG ACT GTC GAA GTC		2561-2544			
clfA	Fw, GTA GGT ACG TTA ATC GGT T	Z18852	368-386	Newman	1 min at 94°C, 1 min at 45°C, 2 min	15
	Rv, CTC ATC AGG TTG TTC AGG		1951-1934		at 72°C	
	Fw, GAT TAA GCT TTA CGT TCA AC		1805-1824	Newman		24
	Rv, GAT TGG TAC CAT TTT TAG GTG		2948-2928			
clfB	Fw, TGC AAG ATC AAA CTG TTC CT	AJ224764	425-444	Newman	1 min at 94°C, 1 min at 45°C, 2 min	15
	Rv, TCG GTC TGT AAA TAA AGG TA		1020-1001		at 72°C	
	Fw, AGG ACA ATC GAA CGA TAC AAC G		162-183	Newman	1 min at 94°C, 1 min at 52°C, 1 min	29
	Rv, ACT ACG TAC AGC TCT CGT TCT AAC ACT		618-592		at 72°C	
cna	Fw, AGT GGT TAC TAA TAC TG	M81736	1719-1735	Philips	1 min at 94°C, 1 min at 55°C, 2 min	15
	Rv, CAG GAT AGA TTG GTT TA		3457-3441		at 72°C	
	Fw, ATG GTA CCA AGA AGA TAC G		688-705	Philips		28
	Rv, TCT TGA TAC CAA GCT TGT G		1052-1034			
sdrE	Fw, CAG TAA ATG TGT CAA AAG A	AJ005647	650-668	Isolate 476	1 min at 94°C, 1 min at 45°C, 1 min	15
	Rv, TTG ACT ACC AGC TAT ATC		1416-1399		at 72°C	
	Fw, CTG AAA ACA CTA GTA CAG AAA ATG CA		158-182	Isolate 476		23
	Rv, GGT ACT GTT AAA CCT GAA GAA AAG		1795-1818			
ebpS	Fw, CAA TCG ATA GAC ACA AAT TC	U48826	40-59	Isolate 252	1 min at 94°C, 1 min at 50°C, 1 min	15
	Rv, CAG TTA CAT CAT CAT GTT TA		565-546		at 72°C	
	Fw, CGT CAA TCG ATA GAC ACA AAT		37-57	Isolate 252		27
	Rv, CTG TAC CAG CAC CAA TT		638-621			

PCR product	Oligonucleotide sequences (5'-3')	GenBank accession no.	Position in gene	Positive control	PCR conditions	Reference
tst	Fw, AAG CCC TTT GTT GCT TGC G	AY074881	36-54	Mu50	1 min at 94°C, 1 min at 55°C, 2 min	21
	Rv, ATC GAA CTT TGG CCC ATA CTT T		480-459		at 72°C	
	Fw, ACC CCT GTT CCC TTA TCA TC		88-107	Mu50		25
	Rv, TTT TCA GTA TTT GTA ACG CC		394-375			
eta	Fw, GCA GGT GTT GAT TTA GCA TT	M17357	719-738	Isolate D72	2 min at 94°C, 2 min at 57°C, 2 min	25
	Rv, AGA TGT CCC TAT TTT TGC TG		811-792		at 72°C	
	Fw, ACT GTA GGA GCT AGT GCA TTT GT		308-330	Isolate D72		22
	Rv, TGG ATA CTT TTG TCT ATC TTT TTC ATC AAC		496-467			
etb	Fw, ACA AGC AAA AGA ATA CAG CG	M17348	509-528	Isolate I 128	2 min at 94°C, 2 min at 57°C, 2 min	25
	Rv, GTT TTT GGC TGC TTC TCT TG		734-719		at 72°C	
	Fw, CAG ATA AAG AGC TTT ATA CAC ACA TTA C		574-661	Isolate I 128		22
	Rv, AGT GAA CTT ATC TTT CTA TTG AAA AAC ACT C		1183-1153			
pvl (lukF)	Fw, ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	AB186917	579-609	Isolate D48	1 min at 94°C, 1 min at 55°C, 2 min	30
	Rv, GCA TCA AST GTA TTG GAT AGC AAA AGC		2072-2046		at 72°C	
pvl (lukS)	Fw, GCA AGG TTT TAT CAA TTC AAA GAC TAC TT		234-262	Isolate D48		26
	Rv, GGG TCA TTT GTT TTG AGA CCA ATA T		344-320			

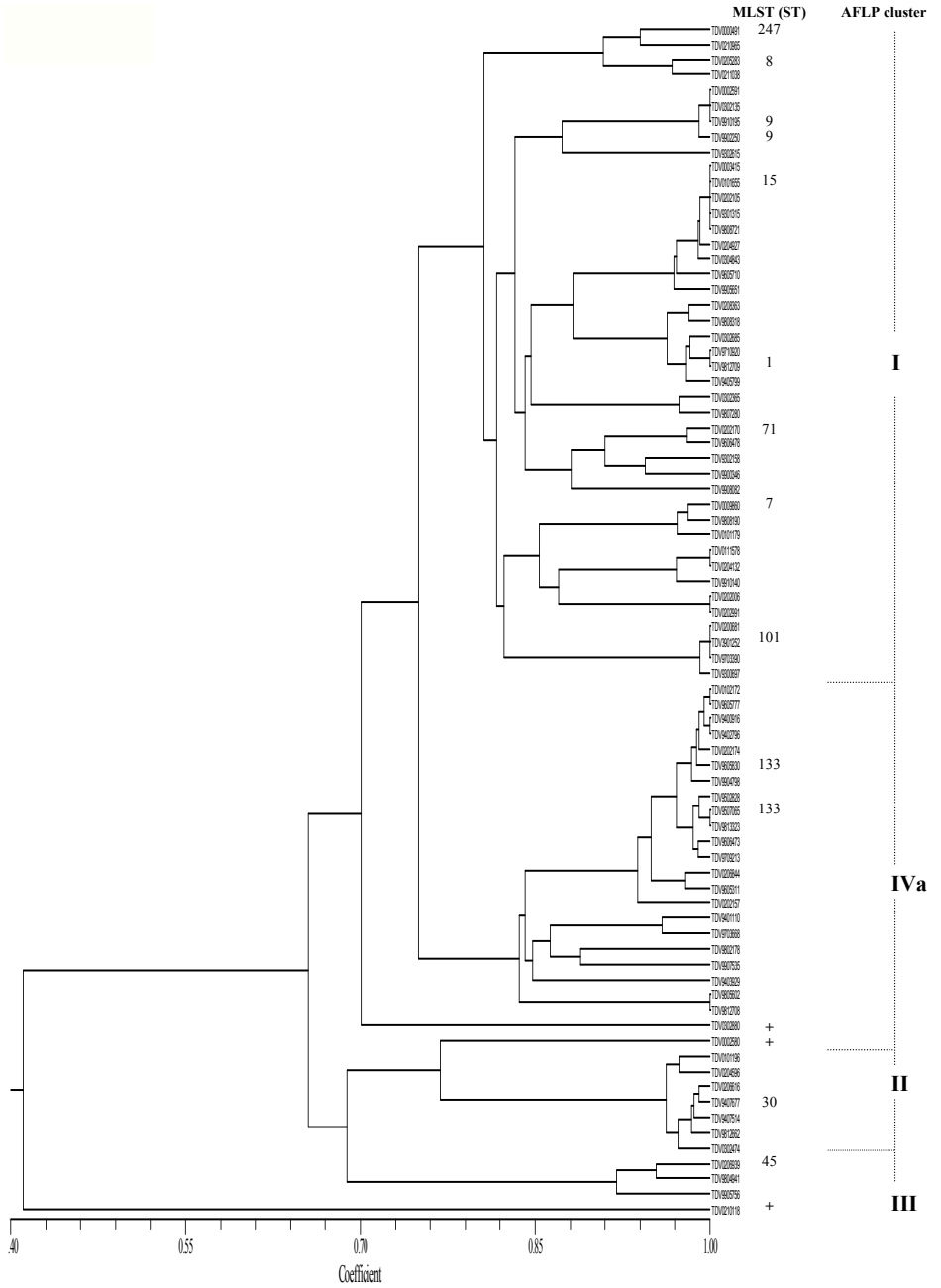

Figure 1. The dendrogram shows the level of similarity, expressed by the similarity coefficient, between AFLP patterns from the animal strains (n = 77). AFLP cluster identification is indicated on the right side of the figure. MLST was performed on a selection of 13 animal strains, representing the different AFLP (sub-)clusters (defined by an arbitrarily chosen similarity-coefficient level ≥ 0.8). Plus signs represent three unique AFLP patterns that were found for 0302880 (horse), 0002580 (ape [gorilla]), and 0210118 (seal).

database (18)), representing 54.5% (n = 42), 28.6% (n = 22), 9.1% (n = 7), and 3.9% (n = 3) of the veterinary collection, respectively, could be identified. Strains causing infections in pet animals (21 out of 24; cat, dog, guinea pig, and rabbit) were overrepresented in AFLP cluster I (Fisher's exact test; $P < 0.0001$). AFLP cluster IVa mainly comprised geographically unrelated isolates obtained from farm animals (goats, sheep, and cows) and caused mastitis (14/22 versus 3/55, Fisher's exact test; $P < 0.0001$), which suggested tissue specificity.

Integration of the veterinary strains in the AFLP database

The resulting AFLP patterns obtained for the animal-associated strains were introduced in the AFLP database (18), and the analysis is summarized in Fig. 2. The different AFLP clusters of the animal-related strains matched with the different AFLP clusters of the database. In essence, the strains causing mastitis in the farm animals formed a homogenous subcluster in IVa (see red bars in Fig. 2). In comparison to the human strains (63/1,056), the animal strains (22/77) were significantly overrepresented in AFLP cluster IVa (Fisher's exact test; $P < 0.0001$) (Table 2). The human isolates in this cluster were associated with bacteremia (Fisher's exact test; $P = 0.0095$) (18). None of the animal-related strains were classified in AFLP cluster IVb. *S. aureus* strains (21 out of 24) isolated from pet animals were predominantly

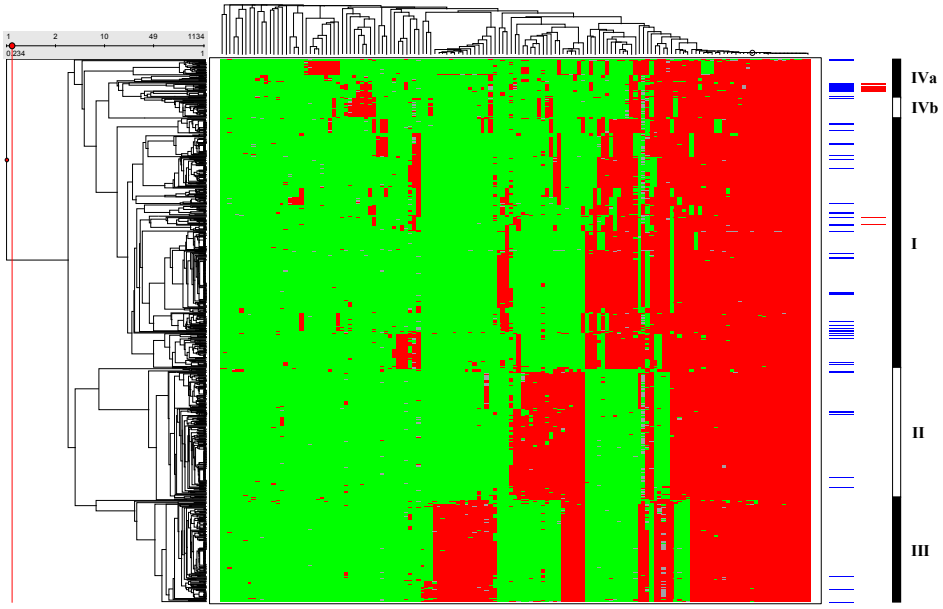

Figure 2. Agglomerative two-dimensional clustering of all strains (n = 1,133) and AFLP markers.
AFLP fingerprints of the animal strains (n = 77) are included. The red fields in the figure represent the presence of AFLP markers; green indicates absence. Blue bars at the right side of the figure indicate the position of each animal strain in the analysis; the red bars represent the mastitis-associated strains. The AFLP database is divided in 3 major strain clusters (indicated as I, II, III at the far right of the figure) and 2 minor clusters (indicated as IVa and IVb) (18). (For color see the online version of this thesis.)

Table 2. Distribution of lineages (AFLP clusters) across the human strains and the veterinary strains

AFLP cluster	No. (%) of animal strains	No. (%) of human strains	*P*	Odds ratio	95% Confidence interval
I	42 (54.5)	480 (45.5)	0.1256	1.44	0.9-2.3
II	7 (9.1)	263 (24.9)	0.0012	0.3	0.14-0.67
III	3 (3.9)	210 (19.9)	0.0001	0.16	0.05-0.52
IVa	25 (32.5)	63 (6.0)	< 0.0001	7.3	4.28-12.6
IVb	0	40 (3.8)			
Total	77	1,056			

found in major AFLP cluster I (87.5%) but were scattered over its different subclusters (Fig. 2). Strains belonging to AFLP clusters II and III were significantly underrepresented among the animal strains (7/77 and 3/77, respectively) compared to the human strains (263/1,056 and 210/1,056; $P = 0.0012$ and $P = 0.0001$, respectively) (Table 2).

AFLP versus MLST

MLST analysis was performed for a selection of the animal strains, representing most of the subclusters in Fig. 1. The animal-related strains classified in AFLP cluster I revealed several different sequence types (including ST1, ST7, ST8, ST9, and ST15). In contrast, AFLP clusters II and III were more homogenous, showing limited marker variability within each cluster (Fig. 2), and only harbored a single clonal complex as shown before (CC30 and CC45, respectively) (18). The animal isolates in cluster IVa were associated with ST133. Notably, the human-associated strains in cluster IVa were more genetically heterogeneous and the majority of these human isolates belonged to CC22. MLST results agreed with the AFLP classification and were concordant with existing MLST sequence types within each AFLP cluster in the database as determined before (18).

Comparison of the virulence gene distribution between human and veterinary *S. aureus* isolates

The presence or absence of 10 genes associated with pathogenicity were measured for the animal ($n = 77$) and human ($n = 168$) strain collections. Results obtained with both PCR strategies were fully concordant. Virulence gene incidence among the animal-related strains was compared to those of the invasive ($n = 56$) and colonizing ($n = 112$) strains. Significant differences in distribution of virulence genes between the groups are outlined in Fig. 3. No significant variation in virulence gene distribution within the different subgroups of the animal strains (mastitis associated, pet animal, and rest group) was measured (data not shown). Most of the genes coding for adhesion proteins were evenly distributed among the human and veterinary strains. The gene encoding collagen binding protein, *cna*, was underrepresented in the human invasive *S. aureus* strains (6/56) compared to the human colonizers

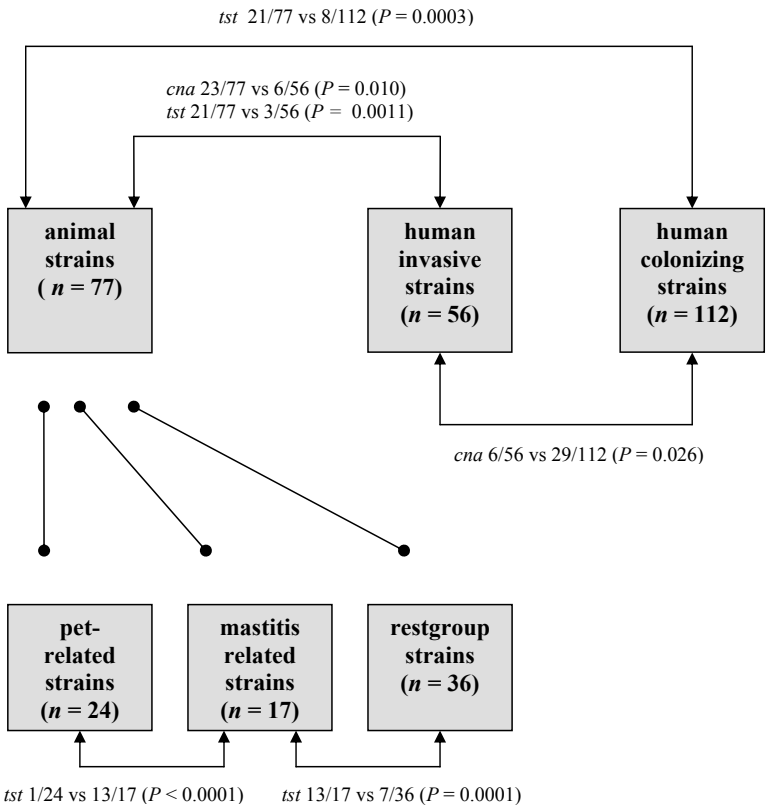

Figure 3. A flow diagram of the virulence gene distribution in the 4 different strain clusters. Only significant differences are noted.

(29/112, Fisher's exact test; $P = 0.026$) and the animal strains (23/77, Fisher's exact test; $P = 0.01$). This gene was not equally distributed among the different genetic lineages of *S. aureus*, since all animal strains from AFLP cluster II harbored the *cna* gene ($P = 0.0001$; data not shown). The exotoxin gene, *tst*, was found significantly more often in the animal-associated strains (21/77 versus 11/168 in the human strains [$P < 0.0001$] or versus 3/56 in the human-invasive strains [$P = 0.0011$]). Strains causing mastitis in animals contribute strongly to *tst* overrepresentation. The *tst* gene was present in 13 out of the 17 mastitis-associated strains, which significantly differs from results observed among strains isolated from pets (1/24, $P < 0.0001$) and among the rest group animals (7/39, $P = 0.0001$) (data not shown). The bicomponent leucocidin gene, *pvl*, was only detected in two human-related strains and not at all in the animal isolates. The association between the number of virulence genes and the proportion of animal and human *S. aureus* strains is stated in Table 3. No difference in the number of virulence genes of the strains causing a variety of infections in the animal host ($n = 77$) and in the human host ($n = 168$) was found.

Table 3. Association of the number of virulence genes and the proportion of animal and human *S. aureus* strains

No. of virulence genes	No. of animal strains (n = 77)	No. of human strains (n = 168)	*P*	Odds ratio	95% Confidence interval
2	0	5	0.33	0.19	0.01-3.5
3	3	14	0.28	0.45	0.1-1.6
4	17	41	0.75	0.9	0.5-1.7
5	39	80	0.68	1.1	0.7-1.9
6	15	27	0.58	1.3	0.6-2.5
7	3	1	0.09	6.77	0.7-66.2

DISCUSSION

Host specificity of *S. aureus*

Previous studies have compared host specificity of *S. aureus* strains isolated from humans and animals. Determinants such as antimicrobial resistance could not discriminate between human and veterinary isolates (32). Other studies identified a certain genetic schism between human and veterinary isolates of *S. aureus*, based on variability in PFGE (5, 6) or multilocus enzyme electrophoresis patterns (3, 4). In general, these studies compared bovine mastitis-associated isolates with strains of human origin. It was concluded that the overall genetic constitution of these *S. aureus* strains seemed to indicate that the majority of cases of bovine mastitis are caused by a few "specialized" clones (3, 4, 33, 34). This suggests the existence of bacterial host-specificity factors (7). The aim of the present study was to identify genetic polymorphism of *S. aureus* associated with host specificity. Moreover, the pathogenic potential, defined by the presence, absence, or number of selected bacterial virulence determinants, was determined.

Genome comparison

Comparative genomics of the strains was initially done by PFGE. This was not useful for effective determination of the population structure of veterinary *S. aureus* strains. No host specificity could be determined except for the apparent clonality of goat-specific strains. Ht-AFLP was the prime method used to compare the animal-associated *S. aureus* strains. The veterinary strains integrated conveniently into the AFLP database, comprising strains isolated from humans. Animals in close contact with humans for reasons of care and treatment (pet animals and animals in contact with humans in a children's farm, riding school, or seal sanctuary or through caretakers in a zoo) were infected with these human-associated strains. Notably, strains isolated from cats and dogs were mainly classified in the heterogeneous AFLP cluster I, scattered over its subclusters that consist of human-related *S. aureus*

strains. The mastitis-related strains, isolated from diverse host species (sheep, goat, and cow) were genetically clustered, signifying tissue specificity.

Cross-infection with *S. aureus* between humans and domestic animals in the household has been described previously (9). Cats were involved in an outbreak of an epidemic methicillin-resistant *S. aureus* in a geriatric ward (11). Dogs and children's farm cattle are potential reservoirs for the transmission of *S. aureus* strains to humans, causing diverse skin infections (35). Transmission of strains from humans to animals has also been observed (10, 36). We here clearly confirm these earlier findings on the basis of an integrated comparison between human and animal isolates and show that many of these veterinary isolates from various animal hosts fall within the same genomic classes.

Host-specific virulence potential

The presence of certain virulence determinants and subsequent pathogenicity in humans has been observed previously (13, 15, 21, 37, 38). Identical virulence determinants were found in *S. aureus* strains causing animal infections. Bovine mammary isolates harbor genes encoding superantigens, such as *tst* (39, 40), exfoliative toxins (41), and enterotoxins (42). These virulence factors were also identified in *S. aureus* strains causing pneumonia in horses (43-45) or in poultry (46). However, these studies concern small numbers of strains or single isolation sites. We analyzed 10 different virulence genes and determined their distribution in various *S. aureus* populations. The *tst* gene, encoding the exotoxin with superantigen activity, was found significantly more often in the mastitis-associated *S. aureus* strains. Whether these genes are all actively expressed and whether this signifies tissue specificity is not known but is considered quite likely. The *cna* gene was not evenly distributed between the different AFLP clusters of the animal-related strains. The prevalence of exotoxin-encoding genes (*pvl*, *eta*, and *etb*) in both strain collections is low. The numbers of virulence genes in the animal- and human-related *S. aureus* strains were similar on a per strain basis. Apparently, the nature of the virulence genes encountered in an *S. aureus* strain is primarily an important determinant for host specificity. It has to be noted, however, that the virulence genes selected for the present study were based on those found for human pathogens. Most likely, veterinary pathogens may contain other host-specific virulence genes that are currently unknown.

In conclusion, many *S. aureus* clones have disseminated widely among humans, colonizing more than 30% of the population and causing a wide variety of severe infections. These same clones have the potency to colonize and infect many different host species. On the other hand, we here identified a tissue-specific clone (udder) responsible for causing disease in diverse host species. The presence of (combinations of) virulence factors plays an important role in host or even tissue specificity in *S. aureus* infections.

ACKNOWLEDGEMENTS

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AFLP is a registered trademark of Keygene N.V., and the AFLP technology is subject to patents and patent applications owned by Keygene N.V.

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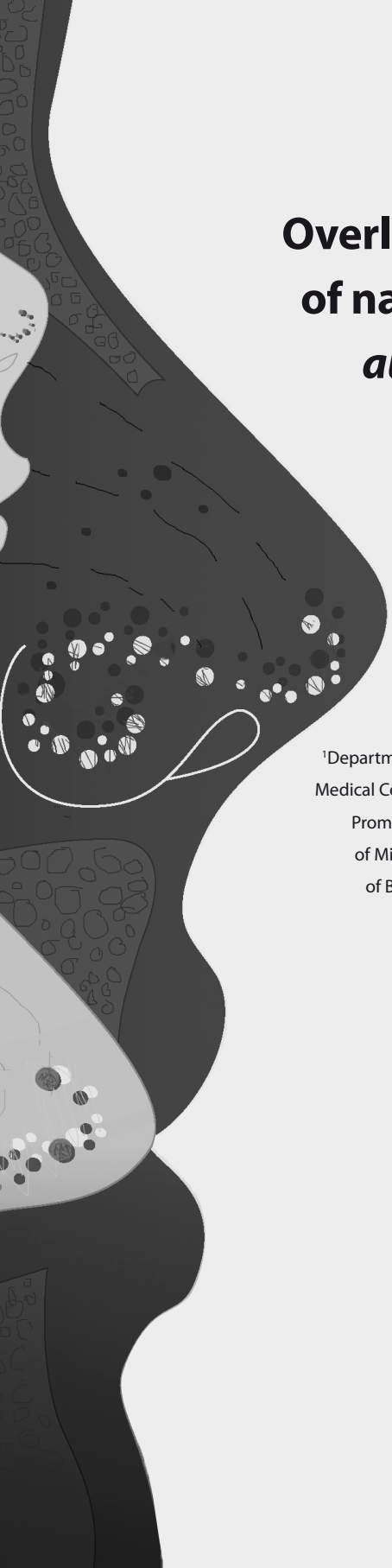

Chapter 5

Overlapping population structures of nasal isolates of *Staphylococcus aureus* from healthy Dutch and American individuals

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ABSTRACT

To understand *Staphylococcus aureus* nasal carriage and its relation with subsequent disease, insight into the natural (non-clinical) bacterial population structure is essential. This study aimed to investigate whether the distribution of *S. aureus* genotypes that cause colonization differs by geographic locales. High-throughput amplified fragment length polymorphism (AFLP) analysis was performed on nasal isolates of *S. aureus* from healthy American (n = 391) and Dutch (n = 829) volunteers. In total, 164,970 binary outcomes, covering 135 different markers per isolate, were scored. Methicillin-resistance was defined for all strains; pulsed-field gel electrophoresis (PFGE) typing was performed for the American isolates. The overall population structures of the American and Dutch *S. aureus* isolates were comparable. The same four major AFLP clusters (I - IV) and subclusters were identified in both collections. However, the Dutch methicillin-susceptible *S. aureus* (MSSA) isolates were overrepresented in AFLP cluster III (P = 0.0016). Furthermore, the majority of the American MRSA isolates (90.5%) were located in AFLP cluster I (P < 0.0001). This identifies differences in the local prevalence of certain *S. aureus* genotypes. AFLP clusters II and III, which represent MLST clonal complexes 30 and 45, respectively, account for 46.4% of all MSSA isolates in the study, suggesting that these two lineages have evolved as extremely successful pandemic colonizers of humans. In conclusion, the overall population structure of American and Dutch nasal carriage isolates of *S. aureus* is surprisingly similar, despite subtle geographic differences in prevalence of certain *S. aureus* genotypes.

INTRODUCTION

Since many *Staphylococcus aureus* infections occur in persons with prior bacterial colonization of the anterior nares, knowledge of the human nasal colonization state is of clinical importance (1). In addition, since some strains of *S. aureus* appear to have a higher potential to cause disease than others, it is essential that studies of nasal carriage include bacterial strain typing information (1, 2). Studies of *S. aureus* nasal carriage have been performed in various geographic regions, including the United States (3), and The Netherlands (4) and some of these included epidemiological typing of the bacterial isolates obtained (2, 3). However, whether the distribution of *S. aureus* genotypes from various geographical locales differs significantly or, alternatively, is rather similar, remains unclear. We here describe the first high throughput genotyping effort for large numbers of non-clinical isolates of *S. aureus* from healthy volunteers living in the USA and The Netherlands.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains from the United States that were included in this study were collected from civilian, non-institutionalized U.S. citizens during the National Health and Nutrition Examination Survey (NHANES) cohort in 2001-2002. In total, nasal samples from 9,622 persons (≥ 1 year old) were obtained as previously described (3). *S. aureus* was isolated from 2,964 individuals over the two year period. Every tenth methicillin-suscpetible *S. aureus* (MSSA) isolate (n = 307) and all methicillin-resistant *S. aureus* (MRSA) isolates (n = 84) were included in the study. Nine additional MRSA isolates that were excluded from the study by Kuehnert et al. (3), were included in the present study (these isolates were earlier excluded, because they were obtained from persons outside of the target cohort). Strain characteristics of the American isolates are presented in Table 1. Forty-two isolates demonstrated PFGE patterns that did not fit within established USA lineages (5).

The Dutch strains (n = 829) were collected during two different carriage surveys among children and the elderly. In total, 3,198 children from the city of Rotterdam, aged 1 to 19 years that participated in a national Meningococcal Vaccination Campaign (in 2002), were enrolled. *S. aureus* was isolated from 1,116 of these children (6). All isolates were stored at –80°C in broth containing glycerol. A random sample of 400 *S. aureus* carriage isolates was drawn for this cohort (2). The second collection originated from a community-based prospective study of elderly in Rotterdam (7). Nasal swabs were obtained from 3,851 persons over 55 years of age, between April 1st 1997 and December 31st 1999. *S. aureus* was isolated from 1,043 elderly (8). All isolates were stored at -80°C in glycerol containing broth. A random sample of 429 carriage isolates was drawn (2). The genotyping data and demographic

characteristics for the 829 Dutch strains have been described previously (2). All Dutch isolates were methicillin-susceptible (MSSA); the absence of *mecA* was confirmed by PCR assay (9). Two *S. aureus* reference strains, N315 and Mu50, both of which have been completely sequenced, were included (10). In total, 1,222 *S. aureus* strains were typed by AFLP.

Amplified fragment length polymorphism (AFLP)

AFLP analysis was performed on purified DNA (MagNA Pure LC Instrument, Roche Diagnostics, Almere, The Netherlands) from all 1,222 *S. aureus* strains as described by Melles et al (2). Using the predictive software package Recomb (11), optimal enzyme and primer combinations were selected. DNA was digested with *Mbo*I and *Csp*6I and the linker oligonucleotide pair for *Mbo*I (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGCAGTCTAC-3') and for *Csp*6I (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3') were ligated. Subsequently, non-selective pre-amplification was performed. In the final amplification, a labeled *Mbo*I primer containing a single selective nucleotide (either +C or +G) and a *Csp*6I primer containing two selective nucleotides (+TA) were used. Amplified material was analyzed using polyacrylamide slabgels and autoradiography. Marker fragments were scored and a binary table of fragment absence (0) or presence (1) was constructed.

Table 1. Microbiological characteristics of the 391 American *S. aureus* isolates.

PFGE type	Total	MSSA	MRSA
	n (% of total)	*n* (% of PFGE type)	*n* (% of PFGE type)
USA100	46 (11.8)	2 (4.3)	44 (95.7)
USA200	89 (22.8)	88 (98.9)	1 (1.1)
USA300	20 (5.1)	13 (65.0)	7 (35.0)
USA400	6 (1.5)	5 (83.3)	1 (16.7)
USA500	4 (1.0)	3 (75.0)	1 (25.0)
USA600	39 (10.0)	38 (97.4)	1 (2.6)
USA700	13 (3.3)	7 (53.8)	6 (46.1)
USA800	38 (9.7)	21 (55.3)	17 (44.7)
USA900	28 (7.2)	28 (100)	0
USA1000	10 (2.6)	7 (70.0)	3 (30.0)
USA1200	6 (1.5)	6 (100)	0
Group A	18 (4.6)	18 (100)	0
Group B	10 (2.6)	10 (100)	0
Group C	6 (1.5)	6 (100)	0
Group D	11 (2.8)	11 (100)	0
Group E	4 (1.0)	4 (100)	0
Iberian	1 (0.3)	0	1 (100)
Unique	42 (11.0)	40 (93.0)	2 (4.7)
Total	391 (100)	307 (78.3)	84 (21.4)

AFLP data analysis

Analysis of the AFLP data was performed as described previously (2). The method used for two-dimensional clustering of the data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic mean (UPGMA), and the similarity metric was Tanimoto. This defines similarity for binary data (0 and 1) based on the number of positive attributes that two records have in common. The resulting dendrogram (Figure 1) was ordered by average value.

Principle component analysis (PCA) is a standard multivariate method to reduce the multi-dimensional space of the data to its principle components (PCs). The PC computation is displayed as a 3-dimensional scatter plot in which the position along the axes shows the PCA score of the strain. PCA was used to identify subgroups of AFLP clusters as hidden by a 2-dimensional representation of hierarchical clustering. The distribution of the strains in four branches was defined on the basis of PCA. Both hierarchical cluster analysis and PCA were performed using Spotfire DecisionSite 7.2 software (www.spotfire.com). To compare the distribution of strain categories in different phylogenetic lineages, Fisher's exact test was used. A 2-sided *P*-value of less than 0.05 was considered significant.

Other laboratory methods

The 391 American *S. aureus* isolates were screened for oxacillin resistance using the Clinical and Laboratory Standards Institute (CLSI; formerly known as the NCCLS) disk diffusion method as previously described (12). Pulsed-field gel electrophoresis (PFGE) was performed for the 391 American *S. aureus* isolates using *Sma*I. The PFGE patterns were analyzed using BioNumerics (Applied Maths), and isolates were grouped into PFGE clonal types using Dice coefficients and a value of > 80% relatedness (3, 5).

RESULTS

Using the set of 1,222 *S. aureus* isolates, a total of 164,970 binary outcomes were generated, covering 135 scorable AFLP marker fragments per isolate. The AFLP data of the 829 Dutch isolates, the 391 American isolates, and the two reference strains (Mu50 and N315) were subjected to two-dimensional hierarchical cluster analysis of which the graphical output is shown in Figure 1. The blue-striped bar on the right of this figure indicates the presence of the American isolates, and clearly shows that the overall population structures of the American and Dutch isolates are rather comparable: the grouping of the strains into four major AFLP clusters as defined before for the Dutch isolates remains valid (Figure 2) (2). The American isolates do not segregate from the Dutch isolates and no additional major *S. aureus* clones were identified. Most importantly, homogeneous clusters II (clonal complex 30 by multilocus sequence typing) and III (clonal complex 45) strains seem to be impor-

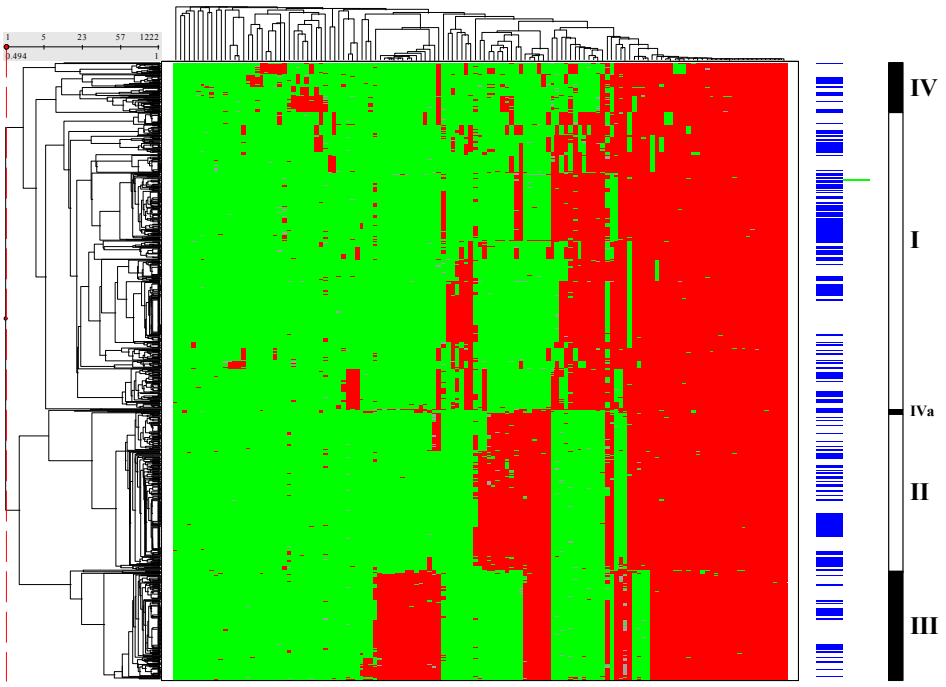

Figure 1. Two-dimensional hierarchical clustering of the 1,222 *S. aureus* strains.
The green/red figure represents 164,970 binary outcomes generated by ht-AFLP with 135 marker fragments per strain. Marker absence corresponds with green and marker presence with red (grey represents ambiguous positions [i.e. weak bands] which are scored as marker absence in the mathematical analyses). The dendrogram on the *y*-axis represents the phylogenetic clustering of the 1,222 strains. The dendrogram on the *x*-axis shows the clustering of the 135 AFLP markers, many of which segregate in specific groups. The colored, striped bars on the right represent the distribution of the USA strains (blue) and the reference strains Mu50 and N-315 (light green). The Dutch carriage strains (n = 829) are not pointed out separately. In conjunction with principle component analysis (PCA) 4 major (I, II, III and IV) branches were identified; these are represented by the black and white bar on the right of the figure. AFLP cluster IVa has been annotated separately (between AFLP cluster I and II), because these *S. aureus* strains were assigned (arbitrarily) to cluster IV, based on principle component analysis (see Figures 2 - 4).

tant "*S. aureus* carriage lineages" in both The Netherlands and the USA. However, there are some differences in the distribution of the Dutch and American isolates across the major AFLP clusters (Table 2). Compared to the distribution among the USA collection of strains, relatively more isolates of the Dutch collection belonged to AFLP cluster III (P = 0.0016). Additionally, all 829 Dutch strains were methicillin-susceptible whereas in the original USA collection 75/2,964 (2.5%) isolates were resistant to methicillin (P < 0.0001), with a weighted prevalence of MRSA colonization among the US population of 0.8% (75/9,622) MRSA isolates in the USA cohort (3). Table 2 shows that the majority of the (American) MRSA isolates (90.5%) are located in AFLP cluster I, which is a genetically heterogeneous cluster

Table 2. Distribution of *S. aureus* strains (*n* = 1,220) in the 4 phylogenetic branches.

	Number of strains (%) per AFLP-cluster			
AFLP-cluster	**Netherlands** [a]	**USA (MSSA)**	**USA (MRSA)**	**Total**
I	367 (44.3)	140 (45.6)	76 (90.5) [b]	583 (47.8)
II (CC30)	216 (26.1)	95 (30.9)	2 (2.4) [c]	313 (25.7)
III (CC45)	176 (21.2) [d]	40 (13.0) [d]	1 (1.2) [e]	217 (17.8)
IV	70 (8.4)	32 (10.4)	5 (6.0)	107 (8.8)
Total	829 (100)	307 (100)	84 (100)	1220 (100)

[a] all Dutch strains were methicillin-susceptible (MSSA)

[b] Proportionally more MRSA strains in the USA cohort as compared to all strains in Dutch cohort (Fisher's exact test; $P < 0.0001$) or MSSA strains in the USA cohort (Fisher's exact test; $P < 0.0001$)

[c] Proportionately fewer MRSA strains in the USA cohort as compared to all strains in Dutch cohort (Fisher's exact test; $P < 0.0001$) or MSSA strains in the USA cohort (Fisher's exact test; $P < 0.0001$)

[d] Proportionally more carriage strains in the Dutch cohort as compared to MSSA strains in USA cohort (Fisher's exact test; $P = 0.0016$)

[e] Proportionately fewer MRSA strains in the USA cohort as compared to all strains in Dutch cohort (Fisher's exact test; $P < 0.0001$) or MSSA strains in the USA cohort (Fisher's exact test; $P = 0.0005$)

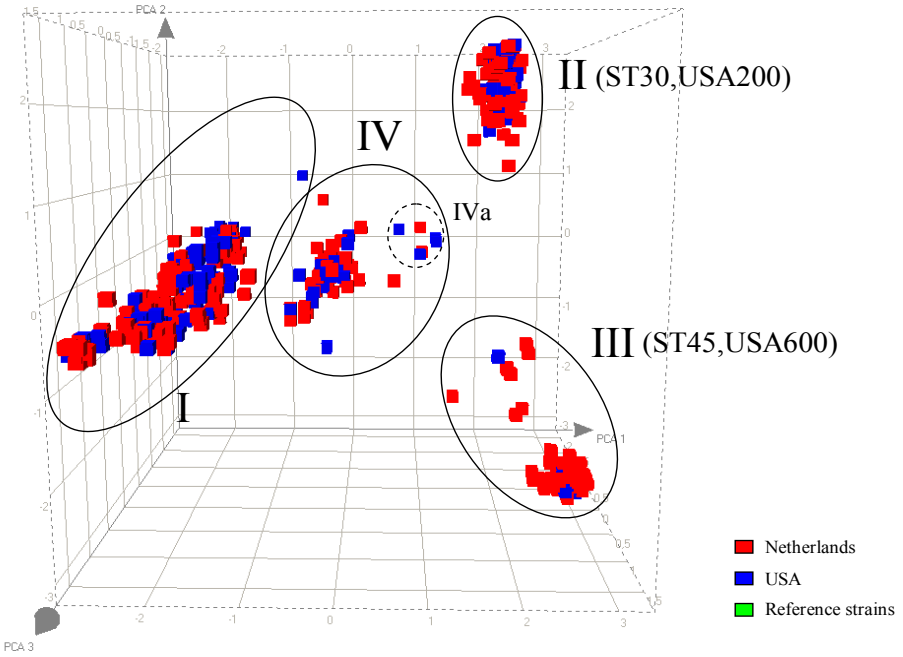

Figure 2. Principal component analysis of the AFLP data of all 1,222 *S. aureus* isolates

The different cubes (plotted in 3-dimensional space), colored according to the source, represent every strain in the study. Each axis represents the score calculated for that strain on each principle component. The 4 circles indicate the different phylogenetic AFLP clusters. The two reference strains (N315 and Mu50) are not visible as they are hidden by other strains.

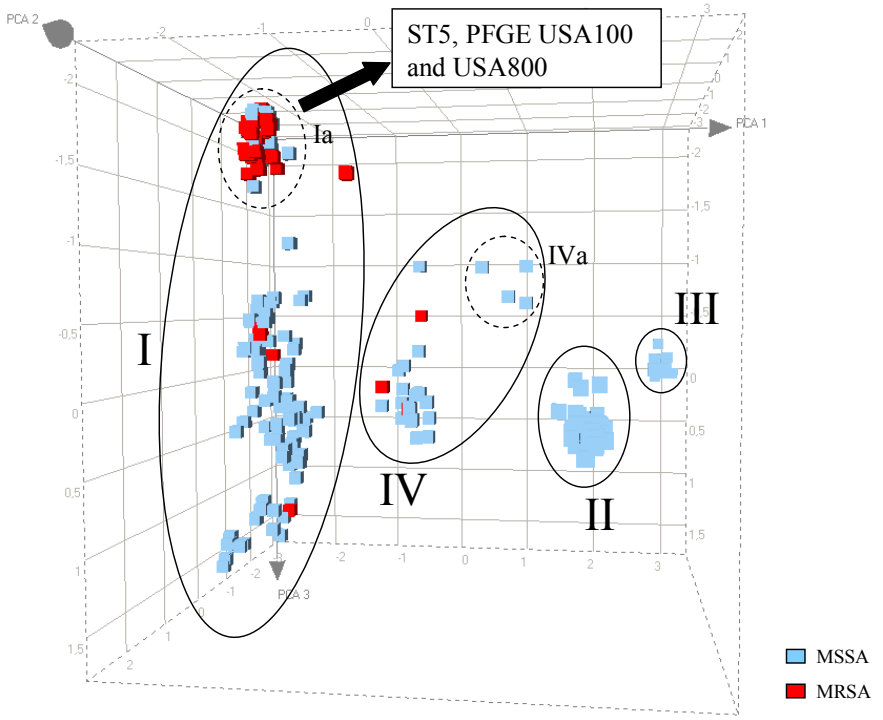

Figure 3. Distribution of the American MSSA (*n* = 307) and the American MRSA (*n* = 84) isolates
Principal component analysis of the AFLP data of all strains (*n* = 1,222) with only showing the 391 American *S. aureus* isolates with the distribution of the MSSA versus MRSA strains.

(overrepresentation of MRSA isolates in cluster I compared to the other AFLP clusters; $P < 0.0001$). Furthermore, Figure 3 shows that most MRSA strains in AFLP genetic lineage I are clustered separately in a small subcluster, named Ia (embracing two PFGE lineages, USA100 and USA800, as shown in Figure 4). This subcluster Ia embraces 24 American MSSA isolates (7.8% of all American MSSA strains included in this study) compared to 60 American MRSA isolates (71.4% of all American MRSA strains included in this study; $P < 0.0001$).

Figure 4 shows the comparison of AFLP and PFGE typing of the American *S. aureus* carriage isolates. PFGE typing confirms the genetic homogeneity in AFLP clusters II and III, as well as the relatively high genetic heterogeneity in AFLP clusters I and IV. The different PFGE clonal types (e.g. USA700, USA900, group A, and group B,) in AFLP cluster I are clustered separately, indicating the similarity between the two typing methods. Furthermore, as shown in Figure 4, AFLP analysis still identifies genetic heterogeneity among several clonal PFGE types, indicating its high discriminatory power. As described earlier, MLST analyses of strains assigned to AFLP cluster I and IV also identified different clonal lineages (including CC5, CC8, CC15, CC20, CC25 in AFLP cluster I and CC22 and CC121 in cluster IV) (2). In contrast, AFLP cluster II and III are genetically homogeneous lineages, which has previ-

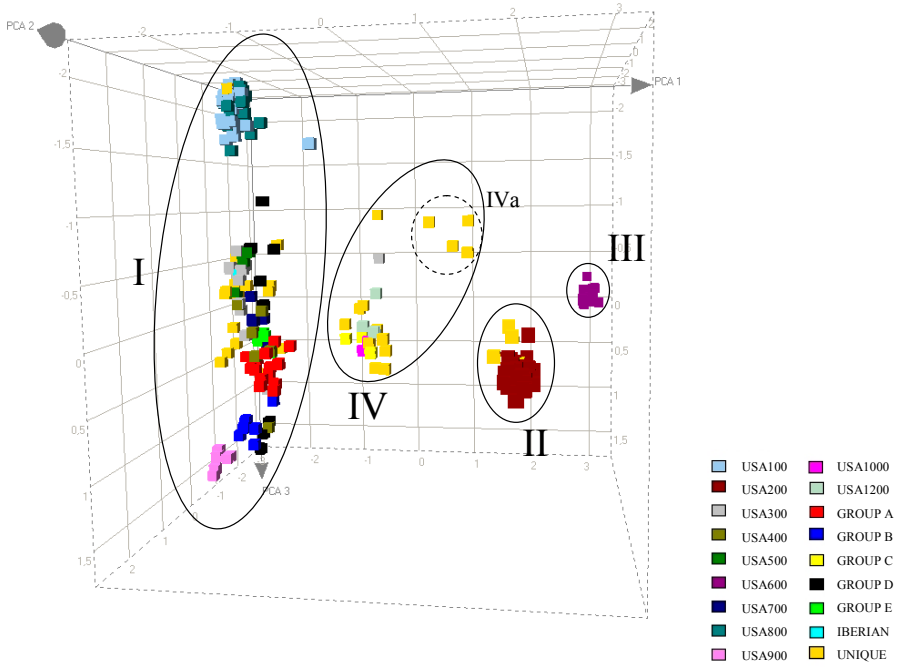

Figure 4. Distribution of PFGE types of the 391 American *S. aureus* isolates over the 4 AFLP clusters
Principal component analysis of the AFLP data of all strains (n = 1,222) with only showing the 391 American *S. aureus* isolates (which is including the American isolates that did not fit within the established USA PFGE lineages: "unique"). The cubes are colored according to the PFGE-type.

ously been corroborated by MLST and now confirmed by PFGE analysis. Strains in AFLP cluster II all belong to CC30 (and PFGE USA200), and strains in AFLP cluster III all belong to CC45 (and PFGE USA600).

DISCUSSION

In the United States, the overall nasal *S. aureus* colonization rate among humans was 32.4% (2001-2002) (3). This is significantly higher than the prevalence for patients admitted to Dutch hospitals (24.4%; $P < 0.0001$) (13). Whether this is due to the difference between the groups of volunteers from whom the strains were isolated or different sampling methods is unknown. However, despite these differences in colonization rate and origin of the strains, the population structures of American and Dutch isolates are surprisingly similar.

The same major AFLP clusters (I, II, III, and IV) are identified in both Dutch (n = 829) and American (n = 391) community-based nasal isolates of *S. aureus*. Genetically heterogeneous AFLP cluster I was the largest cluster found, comprising nearly half of all MSSA isolates and the majority of the MRSA isolates. However this cluster could be subdivided

in several subclusters corresponding with different MLST clonal complexes. Notably, genetically homogeneous AFLP clusters II and III, which represent MLST clonal complexes 30 and 45, respectively, account for 46.4% of all carriage MSSA isolates in the current study population (47.3% of the Dutch carriage isolates and 44.0% of the American MSSA carriage isolates), suggesting that these two clonal complexes have evolved to be extremely successful in colonizing humans (2). It has been shown before that these two clonal types (CC30 and 45) as found in The Netherlands have a seemingly different potential to cause invasive infection (14). It would be worthwhile to seek confirmation of this observation in the American setting. This would contribute to our understanding of staphylococcal virulence traits in relation to their clonal background. Understanding this relation may lead to optimized prophylactic and treatment options for the increasing burden of staphylococcal infections.

Despite the strong similarity between both *S. aureus* population structures, there are also some differences. Dutch cluster III strains are overrepresented compared to their American counterparts ($P = 0.0016$). Furthermore, none of the Dutch nasal isolates included in the current study carried the *mecA* gene. In contrast, the MRSA prevalence in the American collection was approximately 2.5% ($P < 0.0001$) (3). However, it should be emphasized that methicillin-resistance for the American isolates was tested on the total original collection, comprising approximately 3,000 *S. aureus* isolates. Methicillin-resistance in the Dutch isolates was only tested on the 829 isolates included in the current study by *mecA* PCR, as described earlier (2). All MRSA strains isolated from the American cohort were included in the current study, in contrast to the American MSSA isolates, which were selected at random for the current study (by 1 out of 10). Therefore, we have compared the Dutch *S. aureus* isolates with the American MSSA and MRSA isolates, separately. The American MRSA isolates were clearly overrepresented in major AFLP phylogenetic lineage I, and, additionally, most of them clustered separately in a sub-cluster, named Ia. Our data clearly show that the American MRSA isolates (obtained in the open population) show significantly less genetic diversity in comparison with the MSSA carriage isolates included in this study, which is in agreement with the assumption that MRSA primarily consists of a limited number of highly successful pandemic clones.

PFGE typing showed that the American MSSA and MRSA isolates in AFLP sub-cluster Ia are PFGE USA100 and USA800 strains. It is known that these clonal PFGE types share the same MLST sequence type (ST 5) and *spa* motif (MDMGMK). Apparently, these PFGE types share the same genetic background, which is corroborated by MLST and AFLP analysis. However, McDougal et al. earlier concluded that the two clonal PFGE types carry different SCC*mec* structures and had different susceptibility profiles (5). USA100 isolates cluster with representatives of the multi-resistant New York/Japan clone containing SSC*mec* II, while isolates from USA800 cluster with representatives of the Pediatric clone containing SSC*mec* IV (5). Thus, while these two lineages have diverged over time, they both appear to have retained the ability to colonize the nares of humans.

Finally, it should be emphasized, that there are several differences in the demographics of the two populations surveyed, which could influence the results of this study. The Dutch *S. aureus* isolates were obtained from two separate cohorts, i.e. children under 19 years of age and elderly adults over 55 years of age, and all volunteers were living in the Rotterdam area (local region in The Netherlands). In contrast, the American *S. aureus* isolates were obtained from individuals covering all age groups and living all over the country.

In conclusion, the population structures of nasal *S. aureus* isolates from humans are congruent on both sides of the Atlantic. The same successful clones are present although their relative frequency may vary with geographic origin. Apparently, both MRSA and MSSA show similar epidemic behaviour with some clones being notably more successful colonizers of the human vestibulum nasi.

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AFLP is a registered trademark of Keygene N.V. and the AFLP® technology is covered by patents (US006045994A, EP0534858B1) and patent applications owned by Keygene N.V. The REcomb software is covered by patent applications (WO 00/44937) of Keygene N.V.

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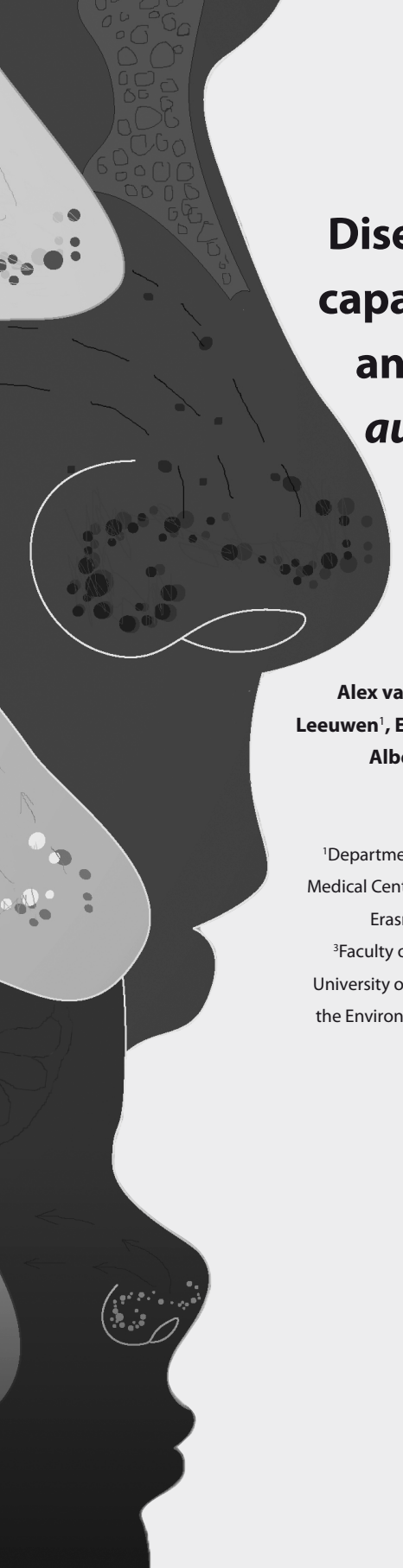

Chapter 6

Disease-invoking and colonizing capacities of methicillin-resistant and susceptible *Staphylococcus aureus* of sequence type 398 in pigs and humans

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ABSTRACT

MRSA of sequence type 398 (ST398) has been identified among Dutch pigs and pig farmers. Using high-throughput amplified fragment length polymorphism (AFLP) analysis, SCC*mec* typing, and *spa* sequencing we defined the clonal structure of the ST398 MRSA population. We established the position of the ST398 MRSA in the overall phylogenetic tree for *S. aureus*. ST398 MRSA is clonal and segregates from the general *S. aureus* population. s*pa* typing of the ST398 strains resulted in the discovery of primarily types t011 and t108 which seemed to be co-evolving with SCC*mec* types. In addition, we show that methicillin-susceptible ST398 (MSSA) circulates among humans in a low frequency. Despite this low incidence among carriage isolates (0.2%), ST398 MSSA was isolated in three cases of bacteremia in humans (2.1%; $P = 0.026$). Our findings show that although the most optimal host for ST398 MRSA is probably porcine, it is capable of causing serious infections in humans.

INTRODUCTION

The risk of being a nasal *Staphylococcus aureus* carrier is significantly increased among pig farmers and specific lineages of *S. aureus* are shared by farmers and their animals (1-3). Nasal carriage of methicillin-resistant *S. aureus* (MRSA) by veterinary personnel, especially those working with pigs, was recently documented to be high (4-6). These "pig-associated" or porcine MRSA isolates appeared to be highly clonal and were all identified by multi-locus sequence typing (MLST) as sequence type 398 (7-9). The prevalence of nasal MRSA carriage appeared to be significantly higher in pig breeders than in the open human population in regions with low "pig densities" (10). In the vicinity of bio-industrial animal facilities, environmental dissemination of drug-susceptible but also drug- resistant micro-organisms through plumes has been documented. This suggests that resistant bacterial strains can spread from animals to the environment, which may further facilitate the colonization of human individuals not directly involved in animal husbandry (11-13). More importantly, the pig-associated MRSA strain has been isolated from humans with invasive infections: a case of life-threatening endocarditis and cases of nosocomial ventilator-associated pneumonia were recently documented (3, 7). Additionally, familial outbreaks of (cross-)colonization with this particular strain, also involving superficial infections, such as mastitis, have been documented (8). One important question arising from the observations listed above is whether additional experimental support for the colonizing and infectious capacity of the porcine MRSA strain can be derived from the existence of genetically homologous, methicillin-susceptible *S. aureus* (MSSA) strains among healthy people and individuals suffering from infections with this type.

In order to identify human MSSA's similar to the porcine MRSA ST398 strains, we performed molecular typing of pig-associated MRSA strains in comparison with large numbers of clinical and carriage isolates of MSSA. The international MLST database hosted at the University of Oxford (www.mlst.net) (14) revealed that only a single human ST398 MSSA strain had been identified before. This strain was a nasal carriage isolate from Cape Verde, cultured in 1997. However, detailed demographic data on the strain was lacking, so that source tracing could not be performed. In addition, a single ST398 MRSA strain was described, isolated in 2003 from a woman living in Groningen (The Netherlands) for whom no clinical and epidemiological data were available. We here describe the detailed population genetic analysis of Dutch community-based and nosocomial MSSA isolates in comparison with pig- and pig farmer-derived ST398 MRSA isolates. The comparison was performed using *spa*-sequencing and amplified fragment length polymorphism (AFLP) analysis (15, 16).

MATERIALS AND METHODS

ST398 MRSA isolates

The majority of the ST398 MRSA strains studied were collected at the Dutch Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Twenty strains were isolated directly from pig nares in slaughterhouses (RIVM 21-40) (17), whereas eighteen additional strains were detected during screenings for MRSA carriage in Dutch hospitals and infections among Dutch farmers from independent farms (RIVM 1-8, 10-12 and 14-20). In addition, eight strains were obtained from the Veterinary Medical Diagnostic Centre in Utrecht (VMDC strains). Isolates represented both clinical and carriage isolates. Additional demographic and clinical data (including sampling site, geographic origin and several molecular characteristics) are summarized in Table 1.

AFLP-mediated typing of ST398 MRSA and sequencing of marker DNA fragments

AFLP analysis was performed as described by Melles *et al* (15). The optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *Mbo*I and *Csp*6I, and linker oligonucleotide pairs for *Mbo*I and for *Csp*6I were ligated. Subsequently, a non-selective pre-amplification was performed using an *Mbo*I-specific and a *Csp*6I-specific primer. In the final amplification, a 33P-labeled *Mbo*I primer containing 1 selective nucleotide (either +C or +G) and a *Csp*6I primer containing 2 selective nucleotides (+TA) were used. Amplified material was analyzed using standard polyacrylamide slabgels and autoradiography. Marker fragments were scored and a binary table with marker fragment absence (0) or presence (1) was constructed. Thirty fragments with differential occurrence, when genetically heterogenous MSSA ("other strains; not-pig-related") and ST398 MRSA fingerprints were compared, were isolated from the AFLP gels (see Fig. 3a and 3b). These fragments were re-amplified using the homologous AFLP primers and sequenced using ABI technology and a capillary sequencer. Fragments were sequenced in triplicate (from three independent strains) and the consensus sequence was analyzed using BLAST. Database hits were collected and correlated with the host specificity of the various strains.

AFLP fingerprint database of *S. aureus* carriage isolates from healthy and infected individuals

We embedded the genetic fingerprints of the 46 pig-related MRSA isolates in the population structure of *S. aureus* as obtained before. Previously, 1052 *S. aureus* (1050 [99.8%] MSSA) strains were collected by nasal culture from healthy individuals and invasive isolates from both humans and animals, described by Melles et al. and Van Leeuwen et al. (15, 16). These studies include ht-AFLP fingerprints of 829 non-clinical *S. aureus* human carriage isolates and 146 and 77 (including 2 MRSA isolates) clinical isolates of human and animal origin, respectively. All carriage strains were isolated from volunteers that live in the Rotterdam

region, where pig farms are absent. AFLP patterns for the current set of 46 MRSA isolates were added to this database.

spa sequencing

Sequencing of the repetitive region of the protein A gene *spa* was performed for all MRSA isolates (18). Data were analyzed using the Ridom Staphtype software version 1.4 (Ridom GmbH, Wurzburg, Germany).

Bio-informatics approaches

Analysis of the AFLP data was performed as described previously (15). The method used for two-dimensional clustering of the data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic mean (UPGMA), and the similarity metric was Tanimoto. This defines similarity for binary data (0 and 1) based on the number of positive attributes that two records have in common. The resulting dendrogram (Figure 1a and b) was ordered by average value.

Principle component analysis (PCA) is a standard multivariate method to reduce the multi-dimensional space of the data to its principle components (PCs). The PC computation is displayed as a 3-dimensional scatter plot in which the position along the axes shows the PCA score of the strain. Both hierarchical cluster analysis and PCA were performed using Spotfire DecisionSite 7.2 software (www.spotfire.com). To compare the distribution of strain categories in different phylogenetic lineages, Fisher's exact test was used. A 2-sided *P*-value of less than 0.05 was considered significant.

RESULTS

The AFLP analysis of the ST398 MRSA strains derived from human and animal sources (n = 46) indicated that these strains are highly clonal. When the AFLP patterns for the ST398 strains are included in the overall population analysis for Dutch MSSA strains from carriage and infection, the distinct cluster was still observed (Fig. 1a and b). Surprisingly, very few Dutch MSSA strains from the Rotterdam region co-cluster with the ST398 pig-related MRSA isolates (Figure 1b). In total, 6 MSSA (0.6%) isolates co-clustered with the ST398 MRSA isolates, of which 2 were nasal carriage isolates from healthy individuals (Table 1). Three of these six strains were blood culture isolates derived from three elderly patients. All three patients suffered from nosocomial bacteremia, one after inflammatory aneurism of the aorta, the second during Fournier's Gangrene and the last one after a primary ventricular fibrillation. In addition, a single infectious strain from an animal source (gorilla) falls within the ST398 cluster. It is unclear whether this strain was particular to this primate or whether it

Table 1: Survey of demographic and clinical data of the ST398 pig-associated MRSA (*n* = 46) and MSSA (*n* = 6) strains.

Isolate number	Isolated from	Infection	Site of sampling	City (in NL)	*mecA*	PVL	PFGE	MLST	*spa*- type	Sc*cmec*
RIVM-1	human	no	nose	Putten	pos	neg	nt	398	t011	IVa
RIVM-2	human	yes	urine	Rosmalen	pos	neg	nt	398	t108	V
RIVM-3	human	no	nose	Gendringen	pos	neg	nt	398	t108	V
RIVM-4	human	yes	wound/abscess	Helden	pos	neg	nt	398	t108	V
RIVM-5	human	no	sputum	Amsterdam	pos	neg	nt	398	t011	V
RIVM-6	human	yes	urine	Zeeland	pos	neg	nt	398	t108	V
RIVM-7	human	no	throat/nose	Bennekom	pos	neg	nt	398	t898	III
RIVM-8	human	no	sputum	Kootwijkerbroek	pos	neg	nt	398	t011	V
RIVM-10	human	yes	wound/abscess	Galder	pos	neg	nt	398	t567	III
RIVM-11	human	yes	wound/abscess	Olburgen	pos	neg	nt	398	t108	V
RIVM-12	human	no	sputum	Sas van Gent	pos	neg	nt	398	t011	IVa
RIVM-14	human	yes	nose	Siebengewald	pos	neg	nt	398	t108	V
RIVM-15	human	no	sputum	Grijpskerke	pos	neg	nt	398	t034	V
RIVM-16	human	no	sputum	Harskamp	pos	neg	nt	398	t108	V
RIVM-17	human	yes	wound/abscess	Haarlem	pos	pos	nt	398	t034	III
RIVM-18	human	yes	sputum	Heeswijk dinther	pos	neg	nt	398	t108	III
RIVM-19	human	yes	perineum	Veenoord	pos	neg	nt	398	t571	III
RIVM-20	human	yes	wound/abscess	Roggel	pos	neg	nt	398	t108	V
RIVM-21	pig	no	nose	Venray	pos	neg	nt	398	t108	V
RIVM-22	pig	no	nose	Heeze-Leende	pos	neg	nt	398	t108	V
RIVM-23	pig	no	nose	Barneveld	pos	neg	nt	398	t011	IVa
RIVM-24	pig	no	nose	Dalfsen	pos	neg	nt	398	t108	V
RIVM-25	pig	no	nose	Gemert-Bakel	pos	neg	nt	398	t108	V
RIVM-26	pig	no	nose	Venray	pos	neg	nt	398	t108	V
RIVM-27	pig	no	nose	Venray	pos	neg	nt	398	t011	V

Isolate number	Isolated from	Infection	Site of sampling	City (in NL)	*mecA*	PVL	PFGE	MLST	*spa*- type	Scc*mec*
RIVM-28	pig	no	nose	Alphen-Chaam	pos	neg	nt	398	t011	IVa
RIVM-29	pig	no	nose	Hoogeveen	pos	neg	nt	398	t1254	IVa
RIVM-30	pig	no	nose	Skarsterlân	pos	neg	nt	398	t1254	IVa
RIVM-31	pig	no	nose	Someren	pos	neg	nt	398	t011	V
RIVM-32	pig	no	nose	Someren	pos	neg	nt	398	t1255	V
RIVM-33	pig	no	nose	Zundert	pos	neg	nt	398	t108	V
RIVM-34	pig	no	nose	Baarle-Nassau	pos	neg	nt	398	t108	V
RIVM-35	pig	no	nose	HofvanTwente	pos	neg	nt	398	t011	IVa
RIVM-36	pig	no	nose	Zwolle	pos	neg	nt	398	t011	IVa
RIVM-37	pig	no	nose	Alphen-Chaam	pos	neg	nt	398	t011	V
RIVM-38	pig	no	nose	IJselstein	pos	neg	nt	398	t011	IVa
RIVM-39	pig	no	nose	IJselstein	pos	neg	nt	398	t011	IVa
RIVM-40	pig	no	nose	Dalfsen	pos	neg	nt	398	t567	III
6302/3	pig	yes	skin	Utrecht	pos	neg	nt	398	t011	IV
6303/6	pig	yes	nose	Utrecht	pos	neg	nt	398	t011	IV
6303/7	pig	yes	nose	Utrecht	pos	neg	nt	398	t011	IV
6303/9	pig	no	nose	Utrecht	pos	neg	nt	398	t011	IV
6303/11	pig	no	nose	Utrecht	pos	neg	nt	398	t011	IV
6303/1 MRSA1	human	no	nose	Utrecht	pos	neg	nt	398	t011	IV
6303/1 MRSA2	human	no	nose	Utrecht	pos	neg	nt	398	t011	IV
V0606303/8	pig	yes	nose	Utrecht	pos	neg	nt	398	t011	IV
T0976	human	no	nose	Rotterdam	neg	neg	nt	398	t571	-
TE5029	human	no	nose	Rotterdam	neg	neg	nt	398	t571	-
TB27855	human	yes	blood	Rotterdam	neg	neg	nt	398	t571	-
TB28395	human	yes	blood	Rotterdam	neg	neg	nt	398	t571	-
TB29854	human	yes	blood	Rotterdam	neg	neg	nt	398	t571	-
TDV0002580	gorilla	yes	ear	Velp	neg	neg	nt	398	t034	-

nt = non-typeable

was indirectly obtained from one of the care-takers or other sources in the animal's environment. These 6 strains were all *mec*A PCR negative and flucloxacillin-susceptible.

When the AFLP data are subjected to PCA analysis, the ST398 MRSA strains still cluster as a clearly separate group (Figure 2). The AFLP analysis as such does not distinguish those derived from pigs or pig farmers and a limited number of polymorphic fragments is seen upon marker scoring (see Figures 3a and 3b for a survey).

The ST398 MRSA cluster could thus be clearly distinguished from most of the MSSA strains from the Rotterdam region. This involved AFLP markers that were positive for the ST398 MRSA's and absent from the other strains or vice versa. These fragments were selected from the gels and sequenced (see Figures 3a and 3b). Out of the 30 fragments analyzed, 9 were (close to) 100% specific for the pig-associated strains. Another three fragments were present in a subset of the pig-associated strains only. Out of these 12 fragments four did not show any homology with any other entry in the GenBank sequence database including the ten currently known *S. aureus* full genome sequences. Eight of the 12 pig-specific markers show homology with known sequences, which suggests that these markers become pig-specific by point mutations in the AFLP primer annealing site(s) rather than by genomic rearrangement (deletions or insertions). The nature of the homologies highlighted by the 30 sequence searches is given underneath Fig. 3a and 3b. Several of the sequences are encoding factors associated with membranes or transport.

Sequencing of the repetitive regions in the *spa* gene confirmed the preponderance of types t011 and t108 (17). These comprised over 80% of all cases and all of the other types were found incidentally only. However, these types all belonged to the same family of *spa* types, suggesting recent drift in the sequence motifs. There are some specifics in the associations between region of origin and the *spa* type, but numbers do not allow statistical evaluation. It is interesting to note, however, that the t011 types are primarily associated with SCC*mec* IV and IVa, whereas the t108 type is nearly fully associated with SCC*mec* V. This suggests that ST398 MRSA have arisen independently upon at least two occasions. Finally, SCC*mec* III is found in association with t108, t898, t567, t034, and t571. This suggests rather promiscuous dissemination of this *mec* cassette among the ST398 MRSA.

Figure 1a. Meta-analysis of the AFLP data obtained for the pig-associated ST398 MRSA and its closely related MSSA strains, carriage MSSA isolates from healthy children and elderly, invasive MSSA from hospitalized children and elderly and invasive animals *S. aureus* isolates (including 2 MRSA isolates) (15, 16). The green/red figure represents 161,700 binary outcomes generated by ht-AFLP with 147 marker fragments. Marker absence corresponds with green and marker presence with red (grey represents ambiguous positions [i.e. weak bands] which are scored as marker absence in the mathematical analyses). The dendrogram on the *y*-axis shows the phylogenetic strain clustering, the dendrogram on the *x*-axis highlights marker clustering. Marker groups are cluster specific. The colored and striped bars on the right represent the distribution of specific strain types as specified below. The ST398 MRSA strains are boxed in red.

Figure 1a. Legend of colored, striped bars

Carriage isolates (n = 829) Animal isolates (n = 77) Reference strains (Mu50/N315)

Bacteremia isolates (n = 146) ST398 MRSA isolates (n = 46)

Figure 1a

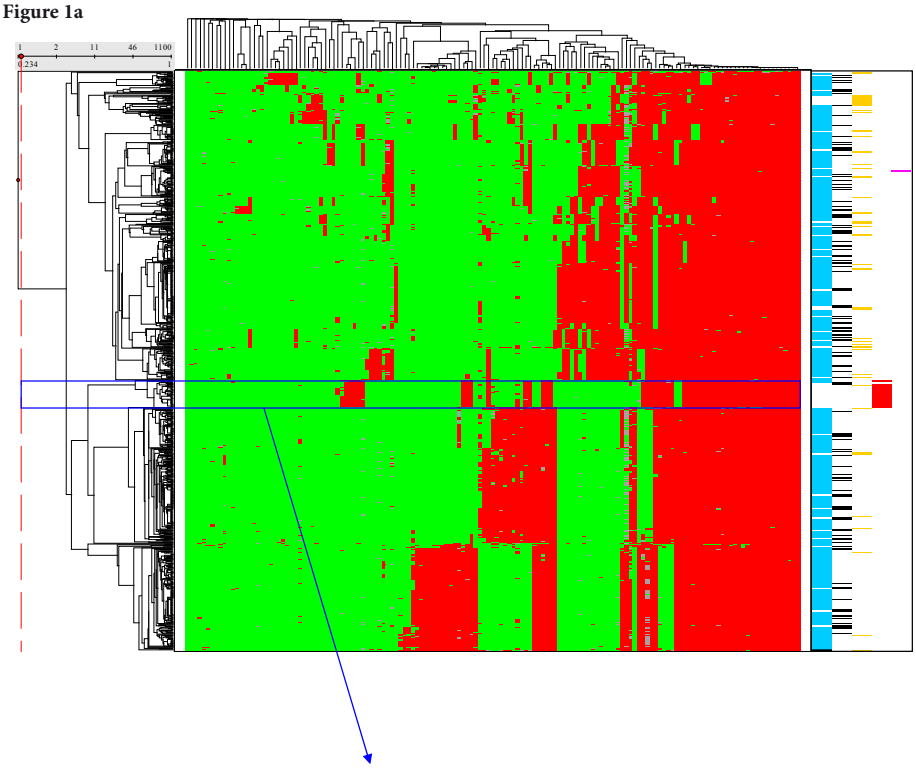


Figure 1b

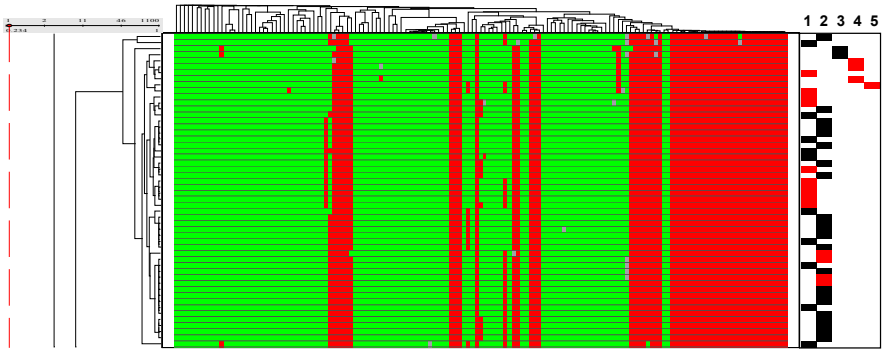


Figure 1b. Detail of Figure 1a, highlighting the ST398 isolates. The specific nature of the strains is stated below.

Legend of bars (black represents a carriage isolate, red represents a clinical isolate):

1) ST398 M**R**SA isolated from humans

2) ST398 M**R**SA isolated from pigs

3) ST398 M**S**SA human carriage isolates

4) ST398 M**S**SA human bacteremia isolates

5) ST398 M**S**SA animal clinical isolate

Figure 2a

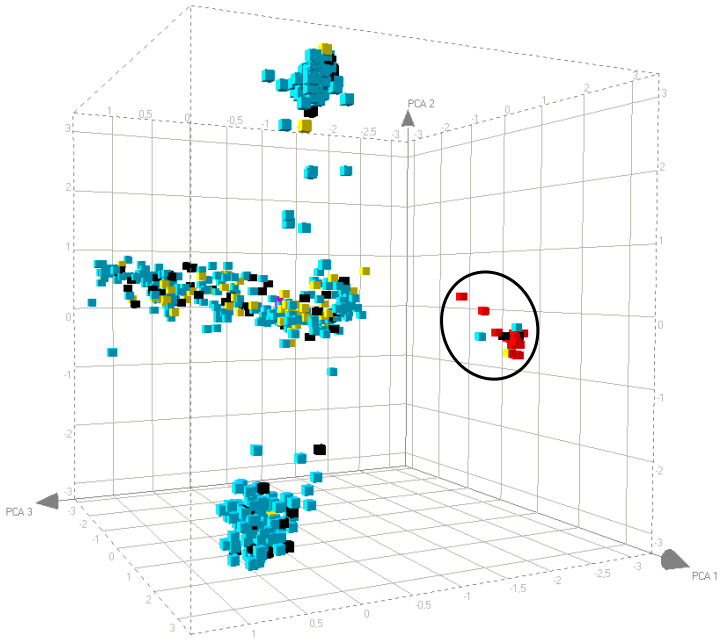


Figure 2. PCA analysis of the AFLP data obtained for the pig-associated ST398 MRSA and its closely related MSSA strains, carriage MSSA isolates from healthy children and elderly, invasive MSSA from hospitalized children and elderly and invasive animals *S. aureus* isolates (including 2 MRSA isolates). The different cubes, plotted in 3D space, represent all of the strains displayed in Figure 1a. Each axis represents the score calculated for that strain on each principal component. The distribution is shown from two different angles. The ST398 strains are circled.

Surprisingly, strain RIVM-17 harbored the PVL genes. Apparently, the bacteriophage carrying these genes found it way into the porcine ST398 MRSA lineage, but has not yet become highly prevalent.

DISCUSSION

Cattle, chickens and pigs are natural carriers of MSSA and form zoonotic reservoirs (1, 19, 20). However, the massive colonization of Dutch pigs with a single sequence type of MRSA came as a sudden and unpleasant surprise and molecular searches for their origin were immediately initiated (17). Unfortunately, molecular typing of the ST398 MRSA was initially compromised, because standard MRSA typing by PFGE failed. This appeared to be due to DNA methylation of the CCNGG sequence motif, which disqualified the restriction enzyme *Sma*I through modification of its recognition site (21). *Spa* gene sequencing (18) gave rise to initial characterisations that indicated that one or two new MRSA lineages had been discov-

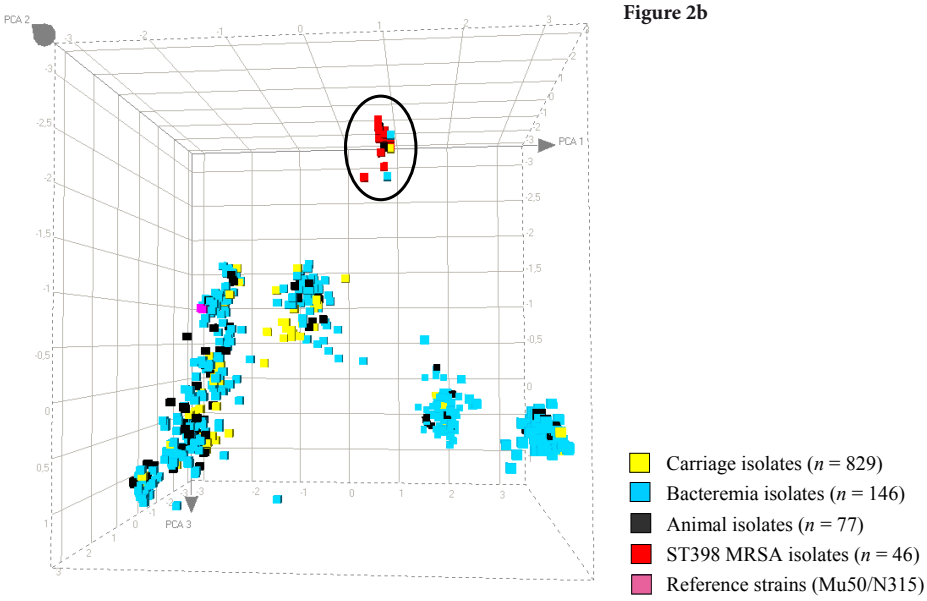
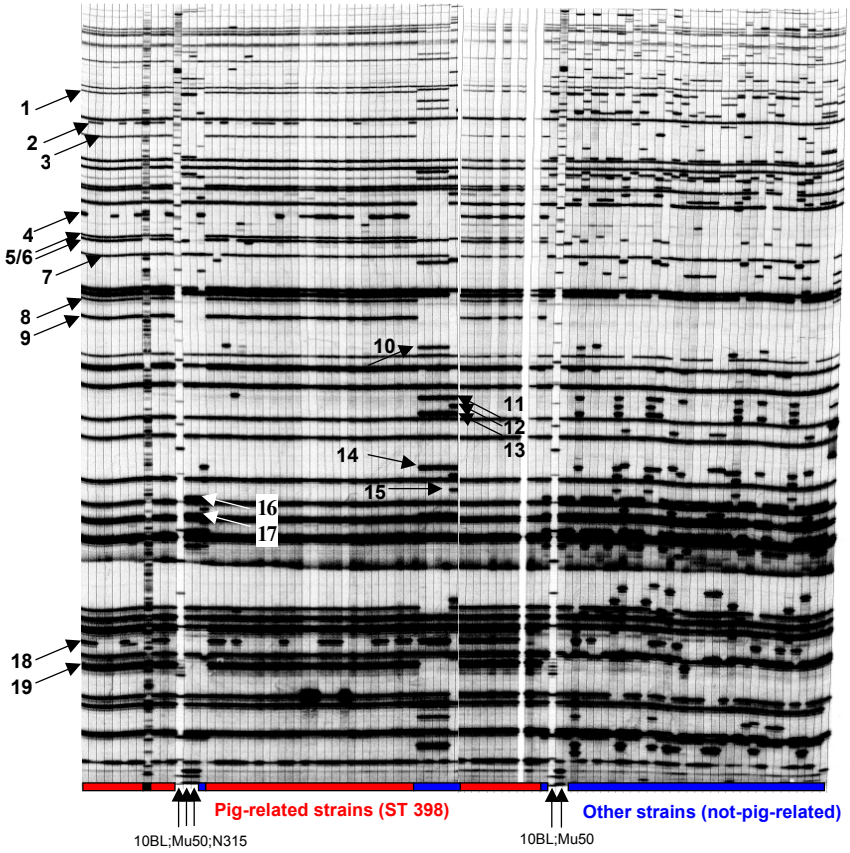


Figure 2b

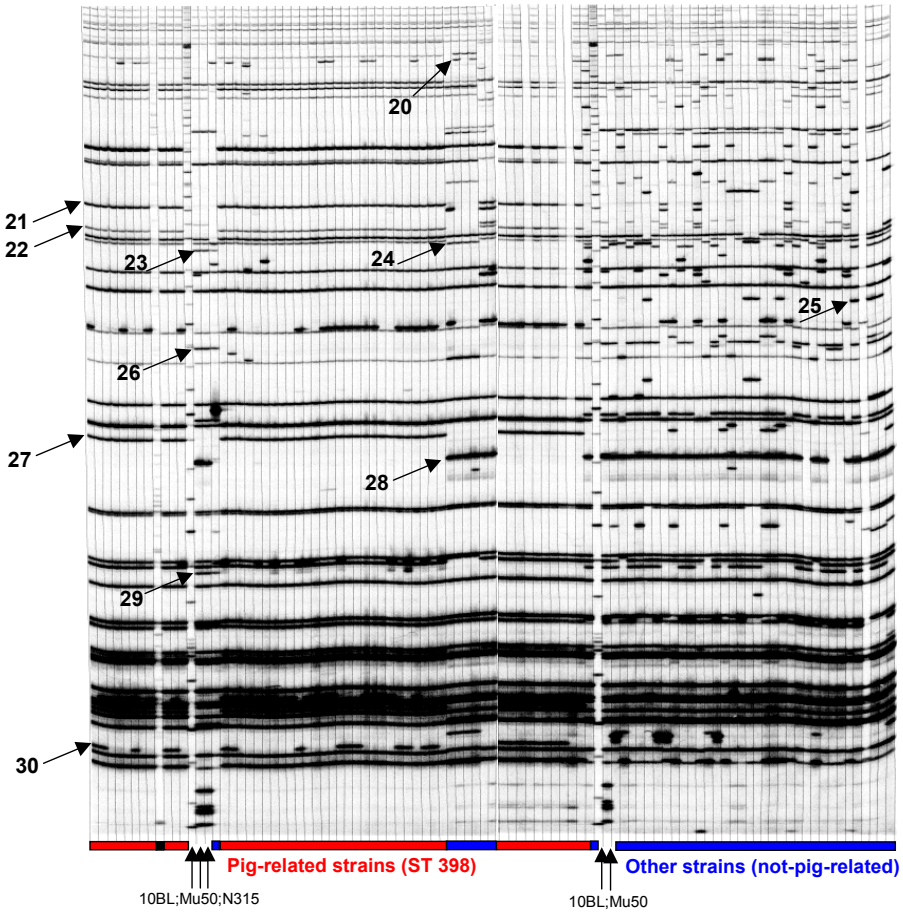
ered. *Spa* typing revealed heterogeneity in the ST398 MRSA lineage with types t011 and t108 (which are closely related) covering over 80% of all isolates. We here show a certain degree of genetic association between *spa* types and the presence of certain SCC*mec* cassettes. This highlights bacterial evolution and horizontal exchange of genetic material in the zoonotic reservoir.

Whether or not the ST398 sequence type was prevalent among Dutch MSSA strains from an urban region was not clear prior to the present study. We here show that ST398, as judged by our AFLP analysis, is very rare among Dutch MSSA strains that are colonizing healthy individuals (only two out of 829 strains isolated from nasal swabs of healthy individuals [0.2%]). However, a relatively high number of MSSA isolates homologous to the ST398 MRSA was derived from bacteremic patients (3 out of 146 [2.1%; $P = 0.026$]). These 3 bacteremia isolates were epidemiologically non-related, isolated from different patients in different medical departments over an extended period of time. This suggests that these MSSA strains are quite virulent. The strict segregation of ST398 strains from the other *S. aureus* strains (Figure 1a and 2) corroborates that the strains might belong to a separate biotype associated with pigs (22, 23). Most likely the pig-associated strains have evolved separately from their human counterparts.

Our findings pose a warning to public health surveillance: if the ST398 MSSA virulence towards humans would be maintained within the ST398 MRSA lineage from pigs, care should be taken not to introduce this strain into humans. The Dutch Working Group on Infection Prevention (WIP) has recently adjusted its guidelines to halt further spread of this strain, and advises that the population at risk (pig breeders, slaughterhouse personnel and



1) hyaluronate lyase precursor 1 (E-value = 0.0; Pig-specific fragment)
2) chaperone protein (E-value = 0.0; Pig-specific fragment; variable)
3) **No homology** (Pig-specific fragment)
4) **No homology** (Pig-specific fragment; variable)
5) 30S ribosomal protein S4, conserved hypothetical protein (E-value = 1e-98; Pig-specific fragment)
6) oligopeptide transport system permease protein (E-value = 1e-58; Pig-specific fragment)
7) Drp35 (E-value = 1e-113; general fragment)
8) conserved hypothetical protein (E-value = 8e-112; Pig-specific fragment)
9) **No homology** (Pig-specific fragment)
10) putative membrane protein (pseudogene); putative transposase (pseudogene) (E-value = 4e-95; general fragment, not in pig-strains)
11) conserved hypothetical protein (E-value = 4e-76); general fragment, not in pig-strains)
12) **No homology** (general fragment, not in pig-strains)
13) PTS system, mannitol-specific IIBC component (E-value = 6e-78; general fragment, not in pig-strains)
14) putative lipoprotein (E-value = 7e-68; general fragment, not in pig-strains)
15) iron compound ABC transporter, permease protein (E-value = 1e-32; general fragment, not in pig-strains)
16) glutamine synthetase, type I (E-value = 5e-53; general fragment, not in pig-strains)
17) threonyl-tRNA synthetase (E-value = 3e-57; general fragment, not in pig-strains)
18) Bacteriophage phi ETA3 DNA (E-value = 9e-19; general fragment, variable)
19) conserved hypothetical protein (E-value = 1e-24; Pig-specific fragment)



20) SLT orf 488-like protein (E-value = 0.0; general fragment, variable)
21) putative membrane protein (E-value = 6e-116; Pig-specific fragment)
22) glycine betaine transporter (E-value = 5e-79; Pig-specific fragment)
23) putative membrane protein (E-value = 2e-80; general fragment)
24) putative oligopeptide transport ATP-binding protein (E-value = 1e-110; general fragment)
25) nitrite reductase [NAD(P)H], large subunit (E-value = 3e-89; general fragment)
26) sodium-dependent transporter (E-value = 2e-78; general fragment, variable)
27) hypothetical protein, similar to transcription antiterminator BglG family (E-value = 1e-66; Pig-specific fragment)
28) transcriptional regulator, TetR family (E-value = 4e-63; general fragment, not in pig-strains)
29) chorismate synthase (E-value = 3e-41; general fragment)
30) **No homology** (Pig-specific fragment; variable)

Figure 3 a and b: AFLP fingerprinting data and identification of the fragments subjected to reamplification and DNA sequencing. Shown are autoradiographs generated during the AFLP process, which were used for marker scoring. Below the gel picture the GenBank BLAST hits for the sequence motifs as determined for the numbered fragments are identified. Reference strain fingerprints are identified by arrows underneath the gel pictures (Mu50 and N315). Two lanes harbor molecular weight markers only (10BL). The "other strains" are formed from a random selection of the non-ST398 strains as also shown in figure 1a.

veterinarians) be held in isolation when hospitalised until MRSA colonization has been excluded. We consider it to be likely that ST398 MRSA from pigs is capable of causing serious infection in humans even though its primary host seems to be porcine.

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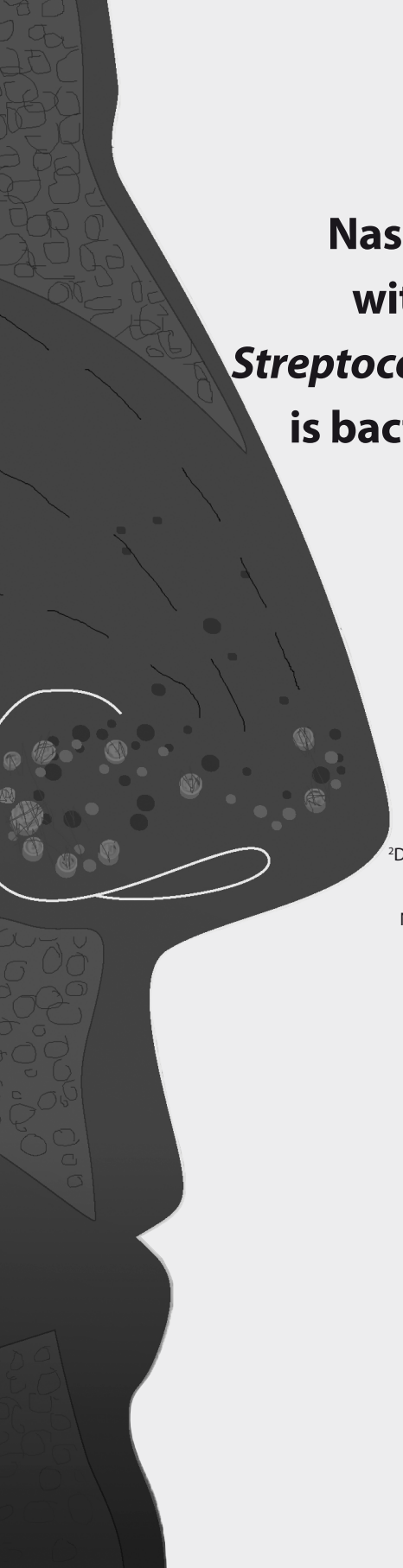
Part II

Determinants of nasal carriage and infection with *Staphylococcus aureus*



Chapter 7

Nasopharyngeal co-colonization with *Staphylococcus aureus* and *Streptococcus pneumoniae* in children is bacterial genotype independent

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ABSTRACT

Bacterial interference between *Staphylococcus aureus* and *Streptococcus pneumoniae* in the nasopharynx has been observed during colonization, which might have important clinical implications for the widespread use of pneumococcal conjugate vaccine in young children. This study aimed to determine whether the capacity of *S. aureus* to compete with *S. pneumoniae* is dependent on bacterial genotype. Demographic and microbiological determinants of carriage of specific genotypes of *S. aureus* in children were also studied. Children (n = 3,198) were sampled in the nasopharynx to detect carriage of *S. aureus*, *S. pneumoniae* and *Neisseria meningitidis*. *S. aureus* genotypes and pneumococcal sero- and genotypes were determined. Age, gender, zip code, active smoking and co-colonization with *N. meningitidis* or *S. pneumoniae*, both vaccine- and non-vaccine types, were not associated with colonization by specific *S. aureus* genotypes. Based on the whole-genome typing data obtained, there was no obvious correlation between staphylococcal and pneumococcal genotypes during co-colonization. Passive smoking showed a significant association (P = 0.003) with carriage of a specific *S. aureus* cluster. This study suggests that there are no major differences between *S. aureus* clones (with different disease-invoking potential) in their capacity to compete with *S. pneumoniae* subtypes. Further studies should demonstrate whether differences in bacterial interference are due to more subtle genetic changes.

INTRODUCTION

Staphylococcus aureus causes a wide range of human infections and the incidence of these infections is steadily increasing, both in the community and in hospitals (1, 2). The anterior nares are the primary ecological reservoir of *S. aureus* in humans (3), and it has been determined that most infections result from endogenous nasal carriage (4-8). The molecular basis of nasal colonization with *S. aureus* is not understood, but environmental factors (9, 10) as well as host factors (11, 12) play an important role (7, 13). Age, sex, fasting glucose levels, diabetes mellitus and smoking were recently demonstrated to be independent determinants of *S. aureus* nasal carriage in adults (14). Furthermore, two recent large-scale population studies revealed an inverse relationship between nasopharyngeal *S. aureus* colonization and vaccine-type strains of *Streptococcus pneumoniae* (9, 15), suggesting natural competition between these bacterial species in the nasopharyngeal niche. This could not be confirmed in HIVinfected children (16). A recent trial with the 7-valent pneumococcal-conjugate vaccine (PCV7) in children with recurrent acute otitis media (AOM) showed a shift in pneumococcal colonization towards non-vaccine serotypes and an increase in *S. aureus*-related AOM after vaccination (17). A similar trend was recently observed for bacteremia in young children (18). Hence, bacterial co-colonization is considered to have important clinical implications for the widespread use of PCV7 in young children. It has become obvious that the nasopharyngeal microbial ecology is complex, and microbial inter-species interactions, as well as host–pathogen interactions, may define whether or not potential infectious pathogens can persist locally.

We aimed to determine whether the capacity of *S. aureus* to compete with *S. pneumoniae* is dependent on bacterial genotype, the main question being whether certain successful staphylococcal clones are better equipped to compete with pneumococci. We also studied demographic and bacteriological determinants of carriage of specific genotypes of *S. aureus* in children.

METHODS

Study cohort and cultures

In total, 3198 healthy children from the Rotterdam area, The Netherlands, between 12 months and 19 years of age, were enrolled during a meningococcal vaccination campaign (9). Informed consent was obtained from the child or the accompanying parent. A single nasopharyngeal sample was obtained per child with rayon-tipped pernasal swabs (Copan, Brescia, Italy). Swabs were transported in Amies medium and plated within 6 h on gentamicin blood agar and Thayer–Martin medium, and finally submerged in phenyl mannitol broth for selective isolation of *S. pneumoniae*, *Neisseria meningitidis* and *S. aureus*, respectively.

Bacterial identification was performed according to standard procedures (19). *S. aureus* and *S. pneumoniae* strains were isolated from 1117 and 598 children, respectively. In this study cohort a negative correlation for co-colonization of *S. aureus* and vaccine-type pneumococci (odds ratio 0.68, condfidence interval 0.48-0.94) was identified (9). All isolates were stored at -80 ˚C in broth containing glycerol. A random sample of 400 *S. aureus* isolates was drawn for the present study.

Demographics

Demographic information was obtained from each child through a standardized questionnaire which was completed under supervision of an instructed interviewer. Questions addressed gender, date of birth, zip code, and active and passive smoking habits. In our analysis we grouped zip codes into four categories (north, south, centre and suburbs of Rotterdam).

DNA isolation and bacterial genotyping

The 400 *S. aureus* isolates were grown on Columbia III agar (Becton Dickinson) supplemented with 5% sheep blood. Three to five colonies were suspended in 25 mM Tris, 10 mM EDTA, 50 mM glucose containing lysostaphin (50 μg ml^{-1}) and incubated at 37 ˚C for 1 h. DNA was extracted with the MagNA Pure LC DNA Isolation Kit III using the MagNA Pure LC robot (Roche Diagnostics) and stored at -20 ˚C. High-throughput amplified fragment length polymorphism (ht-AFLP) analysis was performed as described by Melles et al. (20). AFLP is a whole-genome typing method that scans for polymorphism in actual restriction sites but also among the nucleotides bordering these sites. As such it documents nucleotide sequence variation, insertions and deletions across genomes. Briefly, using the predictive software package Recomb, the optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *Mbo*I and *Csp*6I, and the linker oligonucleotide pairs for *Mbo*I (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGCAGTCTAC-3') and for *Csp*6I (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3') were ligated. Subsequently, a nonselectivepre-amplification was performed using the *Mbo*I primer (5'-GTAGACTGCGTACCGATC-3') and *Csp*6I primer (5'-GACGATGAGTCCTGACTAC-3'). In the final amplification, a ^{33}P-labelled *Mbo*I primer containing one selective nucleotide (either +C or +G) and a *Csp*6I primer containing two selective nucleotides (+TA) were used. Amplified material was analyzed using standard polyacrylamide slab gels and subsequent autoradiography. Marker fragments were scored, and a binary table, scoring marker fragment absence (0) or presence (1), was compiled.

Pneumococcal DNA was extracted (from 578 of the 598 pneumococcal isolates) and analyzed by restriction fragment end labelling (RFEL) as described before (21, 22). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end-labelled at 72 ˚C with [α-^{32}P]dATP usingDNApolymerase (Goldstar; Eurogentec). After the radiolabelled fragments had been denatured and separated

electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY, USA), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories).

Multilocus sequence typing (MLST)

MLST was carried out for a selection of 45 of the *S. aureus* strains using DNA arrays (23). The selected strains were equally distributed across an AFLP dendrogram by selecting approximately 1 out of 10 carriage strains isolated from healthy children, going from top to bottom through the AFLP dendrogram (20).

Data analysis

Analysis of the AFLP data was performed as described by Melles et al. (20). The method used for 2D clustering of the AFLP data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic means (UPGMA). The similarity metric used was Tanimoto, which defines similarity for binary data (0 and 1) based on the number of positive attributes that two records have in common. The resulting dendrogram was ordered by mean value.

Principal component analysis (PCA) is a standard multivariate method used to reduce the dimensional space of the data to its principal components (PCs). PCA aims to reduce a large number of variables to a smaller set that explain most of the variation in the data. It is, basically, a rotation of axes after centering data to the means of the variables, the rotated axes being the PCs, which are linear combinations of the original variables. The PC computation is displayed as a 3D scatter plot in which the position along the axes shows the PCA score of the strain. The distribution of the strains in the four phylogenetic branches was defined on the basis of PCA. Hierarchical and PCA cluster analysis was performed using Spotfire DecisionSite 7.2 software (http://www.spotfire.com).

To compare the distribution of strain categories in different genetic clusters, chi-squared analysis was used. Logistic regression analysis was used to adjust for possible confounding factors. A two-sided *P*-value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Previously, we determined the overall population structure of *S. aureus* isolated from healthy nasal carriers in the Rotterdam area using ht-AFLP. This revealed four major genetic lineages (I, II, III and IV) of *S. aureus* (20, 24). This earlier study also revealed that the AFLP-clustering matched strongly with the major clonal complexes as defined by MLST (20). Moreover, computer algorithms used to solve the *S. aureus* population structure based on MLST data (eBURST) generated clustering identical to the AFLP Spotfire analyses. In the present study,

using the subset of 400 *S. aureus* carriage strains obtained from children, a total of 147 different genetic markers per strain were generated, covering 58,800 AFLP fragments (Fig. 1). The AFLP data revealed two distinct, homogeneous clusters (II and III) and several other smaller subclusters within the two main ht-AFLP clusters I and IV (Fig. 1, Fig. 2a). MLST analysis of the *S. aureus* isolates identified different clonal complexes in AFLP group I (including CC5, CC7, CC8, CC15, CC20 and CC25), indicating its heterogeneity. In contrast, AFLP-clusters II and III harbour single clonal complexes, CC30 and CC45, respectively. The fact that these two clonal complexes account for 47.3% of all carriage isolates in our study population suggests that they have evolved to be very successful in colonizing humans (20). Clusters IVa and IVb are associated with CC22 and CC121, respectively (see Fig. 1).

We studied potential determinants of carriage of different *S. aureus* genotypes. Table 1 summarizes the main characteristics of the study population. Age, gender, zip code, active smoking and co-colonization with N. menigitidis or *S. pneumoniae* were not significantly associated with carriage of a particular *S. aureus* genotype as defined by AFLP. Although age is an important determinant of *S. aureus* colonization in children, with highest incidences at the age of 11 years (9), we found no association between age distribution and the various genotypes of *S. aureus*. This suggests that unknown host or environmental factors are more

Table 1. Demographic and microbiological characteristics of the study population.

	Study-population (*n* = 400)
Age (years)	
Mean	9.93
Range	1 – 18
Age – no. (%)	
1 – 6 years	113 (28.3)
7 – 12 years	175 (43.8)
13 – 18 years	112 (28.0)
Gender (male) – no. (%)	234 (58.5)
Zip-code (area) – no. (%)	
North	69 (17.3)
Center	257 (64.3)
South	54 (13.5)
Suburbs	20 (5.0)
Co-colonization – no. (%)	
N. meningitidis	7 (1.8)
S. pneumoniae	74 (18.5)
Vaccine-type	24 (32.4)
Non-vaccine type	42 (56.8)
Non-typable	8 (10.8)
Active smoking – no. (%)	22 (5.5)
Passive smoking – no. (%)	186 (46.5)

likely to be prime determinants of the parabolic age-versus-carriage distribution in children (9). Seventy-four (18.5%) of the 400 children with *S. aureus* carriage were co-colonized with *S. pneumoniae*. Twenty-four (32.4%) of the 74 pneumococcal isolates expressed capsular serotypes included in PCV7 (vaccine-type pneumococci) (Table 1). We found no association between co-colonization with vaccine- or non-vaccine-type pneumococci and the different genotypes of *S. aureus* (Table 2, Fig. 2a). In addition, when the RFEL types obtained for the pneumococci were included in the comparison, no additional overlaps in co-colonizing types were observed (Fig. 2b). Notably, genetic heterogeneity among the pneumococci was much higher (showing no genetic clusters; Fig. 2b) (21), than in *S. aureus* (showing four major genetic clusters; Fig. 2a).

Analysis of the zip code categories versus genotypes of *S. aureus* again revealed no significant association ($P = 0.268$; Table 2). This suggested that AFLP clusters were evenly distributed geographically; this contrasts with *S. aureus* infection in specific disease categories, where local spread of certain clones is well established (25).

However, passive smoking was significantly associated with carriage of *S. aureus* AFLP-cluster IV ($P = 0.003$; see also footnote to Table 2). An earlier study had already revealed that

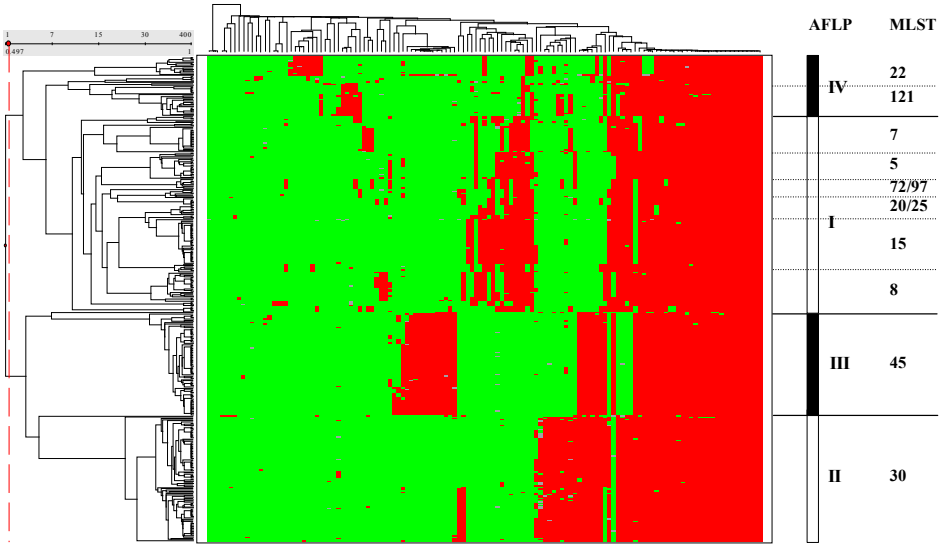

Figure 1. Two-dimensional hierarchical clustering of the 400 *S. aureus* strains.

The green/red figure represents 58,800 binary outcomes, generated by ht-AFLP with 147 marker fragments. Green represents marker absence and red represents marker presence. The dendrogram on the *y*-axis represents the phylogenetic clustering of the 400 strains. The dendrogram on the *x*-axis shows the clustering of the 147 AFLP-markers, many of which segregate in specific groups. In conjunction with principle component analysis, 4 major clusters (I, II, III, and IV) could be identified, represented by the black-and-white bar on the right of the figure. Several subclusters could be identified, which are indicated by dotted lines. MLST data are plotted next to this bar. The numbers indicate clonal complexes determined by MLST analysis. (For color see the online version of this thesis.)

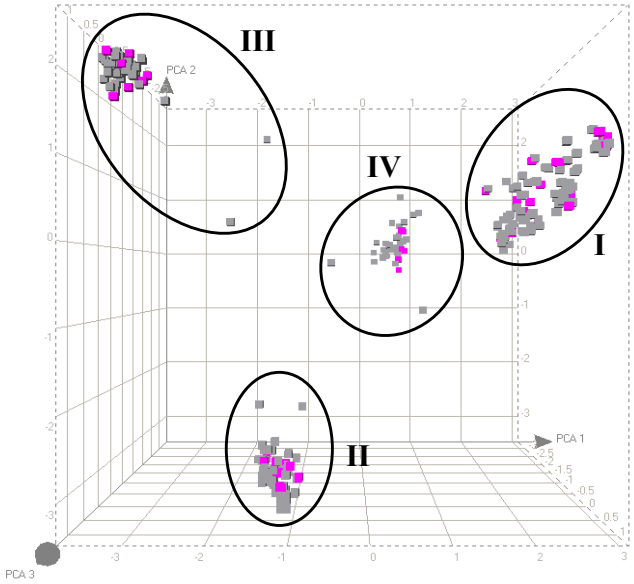

Figure 2a. Principle component analysis of the AFLP data of 400 *S. aureus* isolates.
Each cube in the figure represents one strain. The *S. aureus* strains that are marked purple were isolated from children with co-colonization of *S. pneumoniae*, and the strains marked grey were isolated from children without *S. pneumoniae* co-colonization. (For color see the online version of this thesis.)

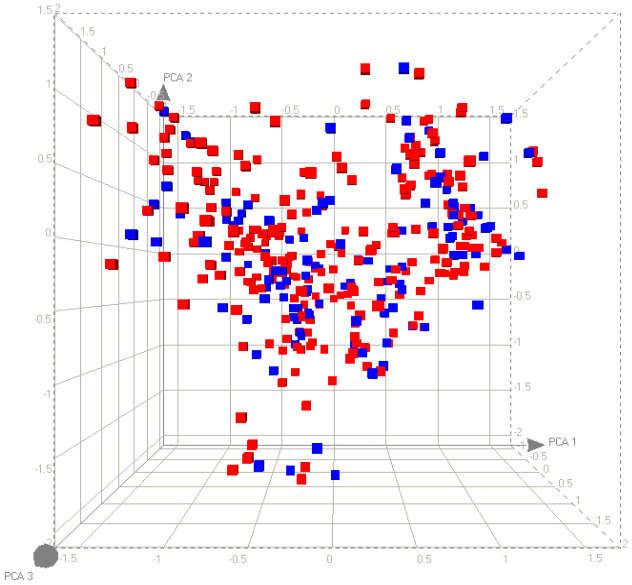

Figure 2b. Principle component analysis of the RFEL data of 578 *S. pneumoniae* isolates.
Each cube in the figure represents one strain. The *S. pneumoniae* strains that are marked blue were isolated from children with co-colonization of *S. aureus*, and the strains marked red were isolated from children without *S. aureus* co-colonization. (For color see the online version of this thesis.)

Table 2. Determinants of nasal carriage for each genetic lineage of *S. aureus*.

	AFLP cluster				
	I	II	III	IV	Chi-Square
	(*n* = 162)	(*n* = 103)	(*n* = 86)	(*n* = 49)	*P*-value (2-sided)
Age (%)					0.722
1 – 6 years	32.1	26.2	25.6	24.5	
7 – 12 years	39.5	48.5	43.0	49.0	
13 – 18 years	28.4	25.2	31.4	26.5	
Gender (male) (%)	63.0	49.5	57.0	65.3	0.278
Zip-code (%)					0.268
Center	66.0	64.1	62.8	61.2	
North	14.8	19.4	18.6	18.4	
South	13.6	15.5	14.0	8.2	
Suburbs	5.6	1.0	4.7	12.2	
Co-colonization (%)					
S. pneumoniae	19.8	18.4	17.4	16.3	0.943 [a]
Vaccine-type	6.8	4.9	7.0	4.1	0.839 [a]
Non-vaccine type	9.9	12.6	9.3	10.2	0.907 [a]
Non-typepable	3.1	1.0	1.2	2.0	0.606 [a]
N. meningitidis	1.2	1.9	2.3	1.8	0.927
Active-smoking (%)	6.2	3.9	5.8	6.1	0.870
Passive-smoking (%)	40.7	44.7	47.7	67.3	0.012 [b]

[a] compared to number (percentage) of children with cultures negative for pneumococci

[b] Chi-square (2-sided): AFLP cluster IV vs. I+II+III; $P = 0.003$

passive smoking is associated with an increased risk of *S. aureus* colonization, with active smoking being protective against colonization (9). This suggests that passive smoking excites colonization opportunities for specific genotypes of *S. aureus* that have been shown to be possibly hypervirulent before (AFLP cluster IV) (20). We additionally performed logistic regression analysis with AFLP cluster IV as outcome, and active smoking, passive smoking, age, gender, cocolonization and zip code as possible confounders in the model. This analysis revealed that passive smoking was independently associated with carriage of AFLP cluster IV (odds ratio 2.8; confidence interval 1.5–5.5; $P = 0.002$). Additional statistical analysis of the different subclusters from major AFLP clusters I and IV (dotted lines in Fig. 1) did not reveal novel associations with carriage of specific *S. aureus* genotypes (data not shown).

In our search for factors co-determining success of colonization with certain *S. aureus* genotypes, we found no evidence for involvement of age, gender, zip code, active smoking and co-colonization with *N. menigitidis* and vaccine or non-vaccine serotypes of *S. pneumoniae*. This suggests that with respect to *S. aureus*, bacterial inter-species competition in the nasopharynx probably depends on host characteristics or currently unspecified microbial features (e.g. receptor and ligands), rather than AFLP-defined overall *S. aureus* genotypes. Further studies involving molecular typing or gene expression testing at a more detailed

level may still generate data that could help identify strain- rather than clone-specific factors involved in bacterial interference. Maturation of the nasopharyngeal niche, including its diverse innate immunity factors during ageing, might be an important driving force, but this hypothesis should be substantiated by further investigations. Recently, it was suggested that hydrogen peroxide production by *S. pneumoniae* is important in the bacterial interference process, a lead that warrants further investigation (26). In conclusion, neither of the staphylococcal clones identified by AFLP has a better competitive edge over the pneumococcus. Thus, success of *S. aureus* clones is not explained on the basis of improved competition with co-colonizing pneumococci. Furthermore, we found an association between passive smoking and carriage of a specific *S. aureus* cluster in children.

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AFLP is a registered trademark of Keygene NV and the AFLPH technology is covered by patents (US 6 045 994A, EP0534858B1) and patent applications owned by Keygene NV.

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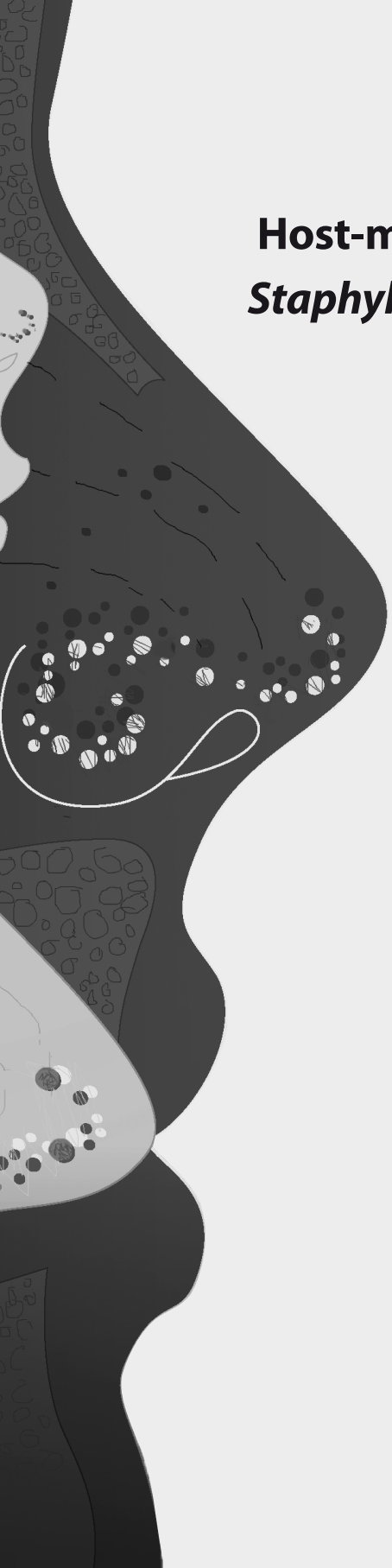

Chapter 8

Host-microbe interplay in persistent *Staphylococcus aureus* nasal carriage in HIV patients

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ABSTRACT

It has been shown that persistent *Staphylococcus aureus* nasal carriage results in increased bacterial dispersal and a higher risk of infection compared to non-or-intermittent *S. aureus* carriage. Although many studies investigated *S. aureus* nasal carriage in HIV patients, none compared persistent carriage to non-persistent carriage nor were studies performed in the HAART era. We investigated the host-microbe interplay of persistent *Staphylococcus aureus* nasal carriage in HIV-infected patients by studying host determinants of persistent carriage as well as the genetic structure of *S. aureus* strains isolated. We compared this genetic structure with the previously determined population structure of *S. aureus* isolates obtained from healthy individuals. Between February 2004 and June 2005 all HIV patients visiting the outpatient department of Erasmus MC (Rotterdam, The Netherlands) were asked to participate in this study. Participants were interviewed and screened for persistent *S. aureus* carriage using two semi-quantitative nasal swab cultures. For 443 patients two cultures were available, 131 (29.6%) were persistent carriers, which is significantly higher as compared to healthy individuals from the same geographic region (17.6%; $P < 0.0001$). Male sex (odds ratio [OR], 2.22; 95% confidence interval [CI], 1.32-3.73), current smoking (OR, 0.58; 95% CI, 0.38-0.90), *Pneumocystis jiroveci*-pneumonia (PCP) prophylaxis (OR, 0.39; 95% CI, 0.16-0.97) and antiretroviral therapy (OR, 0.61; 95% CI, 0.38-0.98) were independent determinants of persistent carriage. Only two strains were *mecA* positive (1.2%) and no PVL positive strains were detected. The population structure of *S. aureus* strains isolated from HIV patients appeared to be strongly overlapping with that of *S. aureus* isolates from healthy individuals.

INTRODUCTION

Staphylococcus aureus is a major human pathogen capable of causing a wide range of infections. Although colonization of multiple body sites occurs, the anterior nares are the most frequent carriage site for *S. aureus* and nasal carriage appears to play a key role in the epidemiology and pathogenesis of infection (1, 2). Moreover, the elimination of *S. aureus* from the nares results in the subsequent disappearance of *S. aureus* from other areas of the body (3-5). Several risk-factors have been identified for *S. aureus* nasal carriage, including diabetes mellitus, hemodialysis, continuous ambulatory peritoneal dialysis (CAPD), and intravenous drug abuse (6). Regular and long-term puncture of the skin by needles and/or intravascular catheters seems to be the common factor in these groups, although additional physiological features may be important as well (6). Another important risk-group are patients infected with the human immunodeficiency virus (HIV), who have an apparently increased risk of nasal carriage (7-11) and subsequent *S. aureus* infections (12-14). In HIV-positive patients, increased rates of *S. aureus* bacteremia and deep soft tissue infections have been observed. Nguyen et al. (13) showed that nasal carriage, presence of a vascular catheter, low CD4 count, and neutropenia were significantly associated with *S. aureus* infection in this patient group. Notably, the carriage rates in HIV-positive patients may even be higher than those actually observed, because of the widespread use of antibiotic prophylaxis and therapy in this particular group of patients (6). Studies excluding intravenous drug addicts still report clearly higher carriage rates in HIV patients (6). The reason for the higher carriage rates in HIV-positive patients is unclear, but the obvious immunological defects could be an explanation. With the introduction of highly active anti-retroviral therapy (HAART) in 1996, morbidity and mortality from HIV infection have dropped dramatically. Hence, HIV infection has become a chronic rather than a lethal disease, at least in the Western world. The effect of HAART on *S. aureus* carriage has not yet been addressed in detail.

Several longitudinal studies demonstrated that three basic nasal carriage patterns can be distinguished among individuals: persistent carriers, intermittent carriers and noncarriers (1, 15, 16). About 20% (range: 12-30%) of the individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers (range: 16-70%) , and about 50% (range: 16-69%) non-carriers (1, 6, 16-18). Persistent carriage seems to have a protective effect on the acquisition of other strains and the load of *S. aureus* is highest in persistent carriers (19). This results in increased bacterial dispersal and a higher risk of infection (17, 20, 21).

The objectives of the present study were to investigate specific determinants of **persistent** *S. aureus* nasal carriage in HIV-infected patients, as well as to compare the genetic structure of *S. aureus* strains isolated from HIV patients with the previously determined population structure of *S. aureus* isolates obtained from healthy individuals (22). Furtermore, we determined the prevalence of methicillin-resistance (MRSA) and Panton-Valentine Leukocidin (PVL) positive *S. aureus* strains within this population of HIV-patients in The Netherlands.

MATERIALS AND METHODS

Study population

Between February 2004 and June 2005 all HIV patients visiting the outpatient department of the Erasmus University Medical Center (Rotterdam, The Netherlands), were asked to participate in this study. Participants were interviewed and screened for *S. aureus* carriage using two quantitative nasal swab cultures by a trained research assistant (taken at a 1- to 2- week interval). For each (quantitative) nasal culture a single swab was used to sample both anterior nares. According to an earlier validated culture rule, two carriage patterns were distinguished: non-or-intermittent versus persistent carriage. This "culture rule" combines qualitative and quantitative results of 2 nasal swab cultures, which accurately predicts the persistent *S. aureus* carriage state with a reliability of 93.6% (17).

Table 1. Main characteristics of the study population (*n* = 507)

	***n* (%)**
Sex (male)	359 (70.8)
Hospitalized in the past year	104 (20.5)
Institutionalized	24 (4.7)
Current active smoking	222 (43.7)
Drug addicts	92 (18.1)
Skin lesions	
Eczema	64 (12.6)
Furunculosis	26 (5.1)
Diabetes mellitus (diagnosis)	22 (4.3)
Antiretroviral therapy (*n* = 506)	382 (75.3)
PCP prophylaxis (*n* = 505)	53 (10.5)
Co-infection hepatitis B and/ or C (*n* = 506)	64 (12.6)
	Mean (range)
Age (years)	41.7 (19 – 78)
Glucose (mmol/l; *n* = 472)	4.8 (2.1 – 15.2)
CD4 count (x 106/l)	410 (10 – 1680)
Viral load (copies/ml); *n* = 503)	<50 [a] (<50 – >100.000)
***Staphylococcus aureus* culture data**	***n* (%)**
2 cultures negative	239 (47.1)
2 cultures positive	142 (28.0)
1 culture negative/ 1 positive	62 (12.2)
1 culture available (and negative)	64 (12.6)
persistent SA carriage according to 'Culture Rule'	**131 (25.8)**
Non-/ intermittent SA carriage according to 'Culture Rule'	**376 (74.2)**

[a] median viral load

Assessment of determinants

Risk factors for persistent nasal carriage of *S. aureus* were evaluated by obtaining personal and clinical data (personal interviews), review of patients' medical files, clinical examination of the patients and assessment of laboratory parameters (see Table 1). We assessed the following basic characteristics, including risk-factors that have been associated with a higher *S. aureus* carriage rate: sex, age, hospitalized in the past year, institutionalized, active smoking, skin lesions, diabetes mellitus, and blood glucose level. Furthermore, we obtained data of HIV-related risk-factors: most recent CD4 cell count, most recent viral load, current use of antiretroviral therapy, PCP-prophylaxis, current or former intravenous drug use, and co-infection with hepatitis B and/or C.

Cultures, DNA isolation and detection of *mecA* and PVL-genes

Nasal swab cultures were obtained according to a standard operating procedure, as described elsewhere (15). Swabs were cultured quantitatively on phenol-red mannitol salt agar (PHMA) and in phenol red mannitol salt broth (PHMB) to distinguish the two carriage patterns (17). For DNA isolation, bacteria were grown overnight at 37°C on Columbia III agar (Becton Dickinson, Etten-leur, The Netherlands) supplemented with 5% sheep blood. Three to five colonies were suspended in TEG buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose) containing lysostaphin (50 µg/ml) and incubated at 37°C for 1 h. DNA was extracted with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) using the MagNA Pure LC Instrument (Roche Diagnostics, Lelystad, The Netherlands) and stored at –20°C. We determined the presence of the *mecA*- and PVL genes by PCR in a subset of the *S. aureus* isolates (n = 170) (23, 24). These strains were collected during the first year of this study (2004). In this period we included 170 patients with at least one positive culture for *S. aureus*. If both cultures were positive, we selected the culture with the highest quantity of *S. aureus*.

Amplified fragment length polymorphism (AFLP)

AFLP analysis was performed for strains derived from 93 out of the 131 persistent carriers as described by Melles et al. (22). These 93 strains were collected from the persistent carriers included in the first year of this study (2004). Using the predictive software package Recomb (25), optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *Mbo*I and *Csp*6I and the linker oligonucleotide pair for *Mbo*I (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGCAGTCTAC-3') and for *Csp*6I (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3') were ligated. Subsequently, a non-selective pre-amplification was performed using the *Mbo*I primer (5'-GTAGACTGCGTACCGATC-3' and *Csp*6I primer: 5'-GACGATGAGTCCTGACTAC-3'). In the final amplification, a ^{33}P-labeled *Mbo*I primer containing one selective nucleotide (either +C or +G) and a *Csp*6I primer containing two selective nucleotides (+TA) were used. Amplified material was analyzed using standard polyacrylamide slabgels and autoradiography. Marker

fragments were scored and a binary table scoring marker fragment absence (0) or presence (1) was constructed.

AFLP database of *S. aureus* isolates from healthy individuals

We compared the genetic structure of *S. aureus* isolated from HIV patients with the (previously determined) natural population structure of *S. aureus* obtained by nasal culture from healthy individuals, described by Melles et al. (22). This study includes high-throughput (ht)-AFLP fingerprints of 829 non-clinical *S. aureus* carriage isolates. These strains were collected during two different carriage surveys among children and the elderly. In total, 3,198 children from the city of Rotterdam, aged 1 to 19 years that participated in a national Meningococcal Vaccination Campaign (in 2002), were enrolled. *S. aureus* was isolated from 1,116 of these children (26). All isolates were stored at -80 ˚C in broth containing glycerol. A random sample of 400 *S. aureus* carriage isolates was drawn for this cohort (22). The second collection originated from a community-based prospective study of elderly in Rotterdam (27). Nasal swabs were obtained from 3,851 persons over 55 years of age, between April 1st 1997 and December 31st 1999. *S. aureus* was isolated from 1,043 elderly (28). All isolates were stored at -80 ˚C in glycerol containing broth. A random sample of 429 carriage isolates was drawn (22). The genotyping data and demographic characteristics for the 829 Dutch strains have been described previously (22). AFLP patterns for the current set of HIV patient-derived isolates were added and included in this database.

AFLP data analysis

Analysis of the AFLP data was performed as described previously by Melles et al. (22). The method used for two-dimensional clustering of the AFLP data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic mean (UPGMA). The similarity metric used was Tanimoto (Spotfire DecisionSite 7.2; Spotfire), which defines similarity for binary data (0 and 1) based on the number of positive attributes that two records have in common. The resulting dendrogram (Figure 1) was ordered by average value.

Principle component analysis (PCA) is a standard multivariate method to reduce the dimensional space of the AFLP data to its principle components (PCs). PCA aims to reduce a large number of variables that explain most of the variation in the data. It is basically a rotation of axes after centering data to the means of the variables, the rotated axes being the PCs, which are linear combinations of the original variables. The PC computation is displayed as a 3-dimensional scatter plot in which the position along the axes shows the PCA score of the strain. PCA was used to identify subgroups of AFLP clusters as hidden by a 2-dimensional representation of hierarchical clustering. Hierarchical cluster analysis and PCA were performed using Spotfire DecisionSite 7.2 software (www.spotfire.com).

MLST

Multilocus sequence typing (MLST) was carried out for 45 *S. aureus* strains using DNA arrays (29). The selected strains were equally distributed across a AFLP dendrogram by selecting approximately 1 out of 20 carriage strains isolated from healthy individuals, going from top to bottom through the AFLP dendrogram (22).

Statistical analysis

Potential determinants of persistent *S. aureus* nasal carriage were first tested univariately. Chi-square test or Fisher's exact test (two-tailed) was used to compare categorical variables, and the unpaired Student's *t* test was used for continuous variables with a normal distribution, and the Mann-Whitney U test for continuous variables without a normal distribution. Variables with a *P*-value of less than 0.20 in the univariate analysis, were further analyzed multivariately using logistic regression analysis. Sex and age were entered in all regression models to correct for potential confounding. Results are reported as odds ratios with 95 percent confidence intervals. Two-sided *P*-values of less than 0.05 were considered to indicate statistical significance. All statistical calculations were performed with the SPSS software package (version 12.0).

RESULTS

S. aureus carriage patterns in the HIV cohort

Between February 2004 and June 2005, 542 patients were screened for persistent *S. aureus* nasal carriage. Two quantitative nasal swab cultures were available in 443 HIV-patients, with mean time between both cultures of 16.7 days (range 3-66 days). According to the culture rule (17), 131 (29.6%) of these 443 patients were classified as persistent carrier. One quantitative nasal swab culture was available in 99 HIV patients. Of these patients (with only one culture available), 64 patients could still be classified as non- or intermittent carrier, because their single nasal culture was negative for *S. aureus* and they were, therefore, very unlikely to be persistent carrier. Patients with only one nasal culture available and positive for *S. aureus* were excluded (n = 35), because it was not possible to classify them in one of the two groups. Therefore, 507 HIV-infected persons (359 men [70.8%]), with a mean age of 41.7 years (range 19 to 78), were included in this study (for other baseline characteristics see Table 1). Herewith, the total number of persistent carriers was 131, whereas 376 non- or intermittent carriers were identified. Data obtained for these HIV patients were used to search for potential determinants of persistent carriage. Additional baseline characteristics; 300 (78.7%) of the 381 patients on HAART (with a known viral load), had an undetectable viral load, compared to 9 (7.4%) of the 121 patients not on HAART. In this study cohort, the

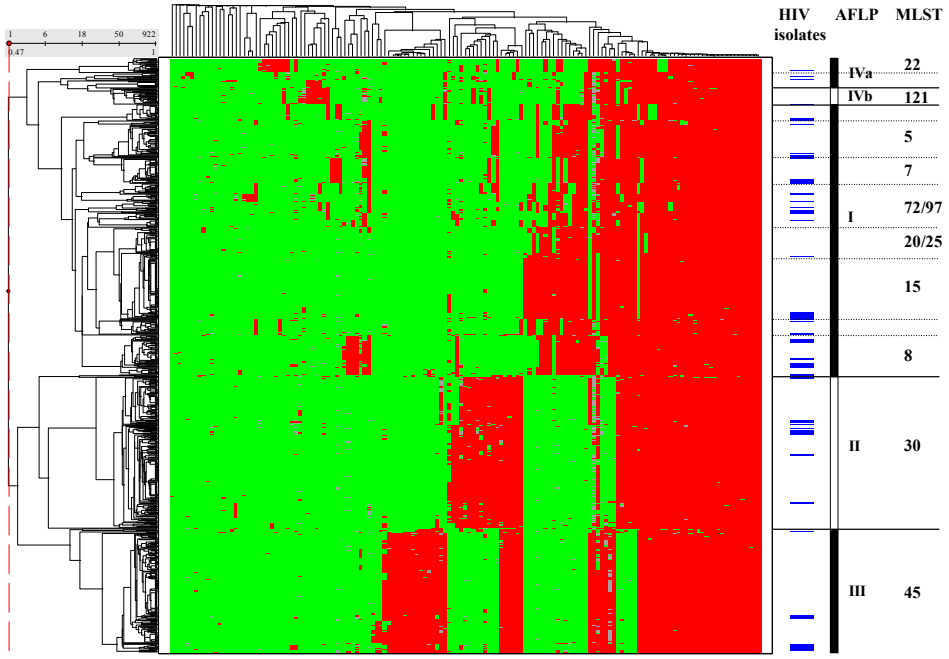

Figure 1. Two-dimensional hierarchical clustering of the 922 *S. aureus* strains.
The green/red figure represents 135,534 binary outcomes, generated by ht-AFLP with 147 marker fragments. Marker-absence corresponds with green and marker-presence with red (grey represents ambiguous positions [i.e. weak bands] which are scored as marker absence in the mathematical analyses). The dendrogram on the *y*-axis represents the phylogenetic clustering of the 922 strains. The dendrogram on the *x*-axis shows the clustering of the 147 AFLP-markers, many of which segregate in specific groups. The blue striped-bar on the right represents the distribution of the carriage strains isolated from HIV patients. In conjunction with principle component analysis 3 major (I, II, III) and 2 (IVa, IVb) minor branches could be identified, represented by the black-white bar on the right of the figure. MLST data is plotted next to this bar (see also reference 22).

use of antiretroviral therapy was very significantly associated with viral load ($P < 0.0001$), but not associated with CD4 counts ($P = 0.799$).

Determinants of persistent nasal carriage of *S. aureus* in HIV patients

Potential risk-factors were first analyzed univariately, which revealed that male sex ($P = 0.007$) was positively associated with persistent carriage and antiretroviral therapy ($P = 0.025$), and *Pneumocystis jiroveci*-pneumonia (PCP) prophylaxis ($P = 0.008$) were negatively associated with persistent *S. aureus* carriage (Table 2). Multivariate logistic regression analysis showed that male sex (odds ratio [OR], 2.22; 95% confidence interval [CI], 1.32 to 3.73; $P = 0.003$), the use of antiretroviral therapy (OR, 0.61; 95% CI, 0.38 to 0.98; $P = 0.042$), PCP prophylaxis (OR, 0.39; 95% CI, 0.16 to 0.97; $P = 0.039$), and also current smoking (OR, 0.58; 95% CI, 0.38 to 0.90; $P = 0.015$) were independent determinants of persistent carriage in this cohort. CD4 cell counts and viral load were not associated with persistent carriage ($P = 0.629$, and

P = 0.267, respectively; see table 2). The viral load was undetectable (<50 copies/ml) in 310 (61.6%) of the 503 HIV patients (with a known viral load). An un-or detectable viral load (as categorical variable) was also not associated with persistent *S. aureus* carriage (*P* = 0.531; Table 2). Furthermore, age, diabetes mellitus (and blood glucose level), co-infections with hepatitis B and/ or C, drug-abuse, skin lesions and history of hospital admission were no determinants of persistent *S. aureus* carriage in this study-cohort. Similar results were obtained when the logistic regression analysis was restricted to the group of 443 patients with both cultures available.

Table 2. Determinants of persistent nasal carriage of *S. aureus* in HIV patients (*n* = 507)

	Persistent carrier (*n* = 131)	**Non-/ intermittent carrier (*n* = 376)**	**OR (95% CI)**	***P* value**	**Adjusted OR (95% CI)**	***P* value**
	***n* (%)**	***n* (%)**				
Sex (male)	105 (80.2)	254 (67.6)	1.9 (1.2-3.1)	**0.007**	2.22 (1.32-3.73)	**0.003**
Hospitalized (past year)	22 (16.8)	82 (21.8)	0.7 (0.4-1.2)	0.259	NI [a]	
Institutionalized	2 (1.5)	22 (5.9)	0.2 (0.1-1.1)	0.054	0.35 (0.08-1.57)	0.170
Current active smoking	49 (37.4)	173 (46.0)	0.7 (0.5-1.1)	0.102	0.58 (0.38-0.90)	**0.015**
Drug addicts	21 (16.0)	71 (18.9)	0.8 (0.5-1.4)	0.512	NI	
Skin lesions						
Eczema	17 (13.0)	47 (12.5)	1.0 (0.6-1.9)	0.879	NI	
Furunculosis	8 (6.1)	18 (4.8)	1.3 (0.5-3.1)	0.645	NI	
Diabetes mellitus	5 (3.8)	17 (4.5)	0.8 (0.3-2.3)	1.000	NI	
Antiretroviral therapy [b]	89 (67.9)	293 (78.1)	0.6 (0.4-0.9)	**0.025**	0.61 (0.38-0.98)	**0.039**
PCP prophylaxis [c]	6 (4.6)	47 (12.6)	0.3 (0.1-0.8)	**0.008**	0.39 (0.16-0.97)	**0.042**
Co-infection hepatitis B and/ or C [b]	16 (12.2)	48 (12.8)	0.9 (0.5-1.7)	1.000	NI	
Undetectable viral load [d]	77 (59.2)	233 (62.5)	0.9 (0.6-1.3)	0.531	NI	
	Mean	**Mean**	**95% CI of difference**	***P* value**	**Adjusted OR (95% CI)**	***P* value**
Age (years)	41.9	41.7	-2.2-1.9	0.847	1.0 (0.98-1.02)	0.855
Glucose (mmol/l) [e]	4.6	4.7	-0.2-0.5	0.395	NI	
CD4 count (x 106/l)	419	407	-59.3-35.9	0.629	NI	
	Median	**Median**				
Viral load (copies/ml) [d]	<50 [f]	<50 [g]		0.267	NI	

[a] NI = not included

[b] *n* = 506

[c] PCP = *Pneumocystis jiroveci*-pneumonia; *n* = 505

[d] *n* = 503

[e] *n* = 472

[f] viral load (copies/ml) 25th – 75th percentiles for persistent carriers: <50 – 10.500

[g] viral load (copies/ml) 25th – 75th percentiles for non/intermittent carriers: <50 – 1.670

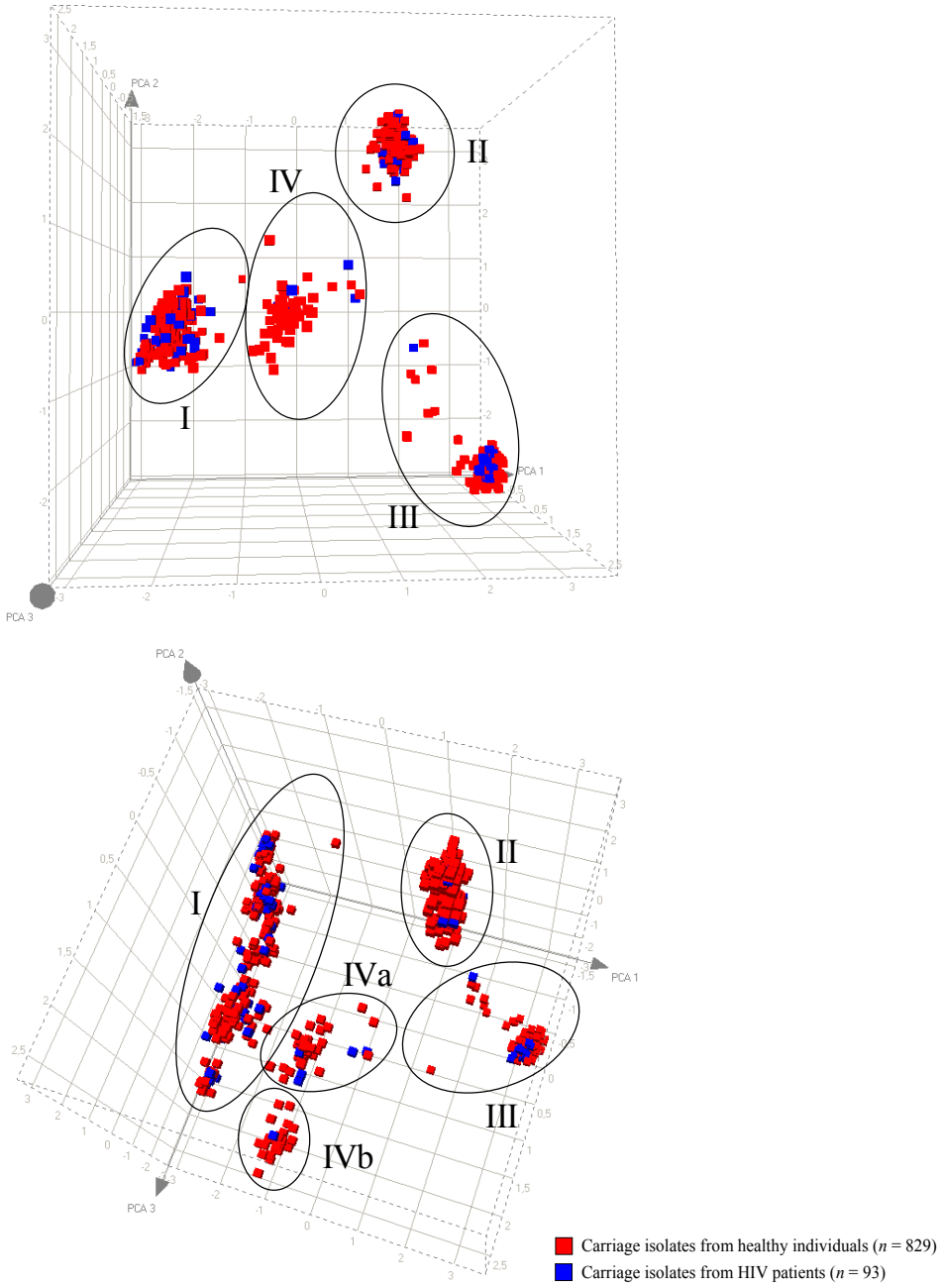

Figure 2. Principle component analysis of the 922 *S. aureus* strains.
The different cubes (plotted in 3-dimensional space), colored according to the source, represent every strain in the study. Each axis represents the score calculated for that strain on each principle component. The circles indicate the different genetic AFLP-clusters.

Table 3. Distribution of *S. aureus* strains in the five genetic AFLP clusters

	AFLP-cluster [n (%)]					
	I	II	III	IVa	IVb	
Carriage isolates						
Healthy individuals (*n* = 829)	367 (44) [a]	216 (26)	176 (21)	46 (6)	24 (3)	
HIV patients (*n* = 93)	52 (56) [a]	18 (19)	17 (18)	5 (5)	1 (1)	

[a] slight overrepresentation of carriage isolates in the HIV cohort (Fisher's exact test; $P = 0.04$)

Population structure of *S. aureus* in HIV patients and *mecA* and PVL status

The population structure of *S. aureus* strains isolated from HIV patients appears to be strongly overlapping with the natural population structure of *S. aureus* isolates from healthy individuals from the same geographic region (Table 3, Figure 1 and 2). The distribution of the strains in the five phylogenetic branches was defined on the basis of principle component analysis of the AFLP data (see Figure 2). The same genetic lineages of *S. aureus* were identified in both study cohorts. However, major cluster I strains were slightly overrepresented in the HIV cohort ($P = 0.04$), compared to strains isolated from healthy individuals (see Table 3). MLST analysis of the *S. aureus* isolates identified different clonal complexes in AFLP-group I (including CC5, CC7, CC8, CC15), indicating its heterogeneity (Figure 1). In contrast, AFLP-clusters II and III harbor single clonal complexes, CC30 and CC45, respectively. Cluster IVa and IVb are associated with CC22 and CC121, respectively (Figure 1). Only two strains were *mec*A positive (1.2%) and no PVL positive strains were detected.

DISCUSSION

S. aureus nasal carriage and subsequent infections are supposedly more common in patients infected with the human immune deficiency virus (7-14). Also (multi-drug) resistant *S. aureus* strains are more common in HIV-patients. It is assumed that most of the *S. aureus* infections derive from nasal carriage, with the nose acting as the primary ecological reservoir of *S. aureus*. Furthermore, it has been shown that persistent carriers stand a higher chance of acquiring staphylococcal infections compared to non-or-intermittent *S. aureus* carriers (21).

Many studies investigated *S. aureus* nasal carriage in HIV patients but none compared persistent carriage to non-persistent carriage nor were studies performed in the HAART era.

In our HIV cohort, we identify 131 persistent carriers of *S. aureus* (29.6%), which is significantly different from healthy individuals in the same geographic region (17.6%; $P < 0.0001$) (28). This confirms earlier studies, showing higher carriage rates (intermittent + persistent) in HIV-positive patients (7-11). Although this study was performed in the HAART era (75% of the patients was using HAART), we still show a significant higher carriage rate compared to healthy individuals. A recently published longitudinal study of 282 community-based drug users showed that both incidence and persistence of *S. aureus* carriage were increased among HIV-seropositive individuals, which is in agreement with our findings (30). Our study confirms that male sex is independently associated with a higher risk of persistent *S. aureus* carriage and active smoking and PCP are independently associated with a lower risk of persistent *S. aureus* nasal carriage in HIV-patients. The PCP prophylaxis used in this cohort was mainly trimethoprim-sulfamethoxazole (TMP-SMX) (approximately 95%), which is usually active against *S. aureus*. Earlier studies already showed that male sex and active smoking are independent determinants of *S. aureus* carriage, but the etiological basis of these observations are still unclear (28, 31). Furthermore, we show that the use of antiretroviral therapy is negatively associated with persistent *S. aureus* carriage. This may indicate an indirect relation between persistent nasal carriage and the immune system or a direct effect of antiretroviral therapy. The CD4 cell level was not associated with persistent carriage in this study. However, it should be noted that the mean CD4 count was high and those with low counts are more likely to receive PCP prophylaxis. Therefore, a true association between CD4 count and persistent carriage would be difficult to find. No PVL positive strains were found to be circulating and the prevalence of MRSA was low (1.2%) in this Dutch HIV cohort, as expected in Netherlands (32, 33).

The population structure of *S. aureus* strains, isolated from HIV patients appears to be strongly overlapping with the natural population structure of *S. aureus* isolates from healthy individuals (22). This suggests that no specific *S. aureus* clones, which could be responsible for the higher carriage rates, are circulating among HIV-patients in the Rotterdam area (The Netherlands). The proportional distribution of the major phylogenetic branches defined by AFLP (clusters I, II, III and IV) within the overall population of *S. aureus*, isolated from humans (including healthy individuals and HIV patients), appears to be fairly stable over time and apparently comparable for different groups (in our geographical region) (22). Notably, AFLP cluster II and III, identical to MLST clonal complex 30 and 45, respectively, account for 46.3% of all carriage isolates in our study population (47.3% of the carriage isolates from healthy individuals and 37.6% of the carriage isolates from HIV patients), suggesting that these two clonal complexes have evolved to be very successful in colonizing humans (22). An earlier study revealed that the AFLP-clustering matches strongly with the major clonal complexes as defined by MLST. Moreover, computer algorithms used to solve the *S. aureus*

population structure based on MLST data (eBURST) generate clustering which is identical to the AFLP Spotfire® and OmniViz® analyses (22).

In conclusion, HIV patients have an increased risk of persistent *S. aureus* nasal carriage compared to healthy individuals. On the other hand, demographic determinants associated with *S. aureus* carriage in this group are not widely differing from those observed in healthy volunteers. However, the most frequent chemotherapies used in HIV patients, PCP prophylaxis and antiretroviral therapy, do protect against *S. aureus* carriage. Whether these therapies also protect against *S. aureus* disease should be investigated further.

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Chapter 9

Panton-Valentine leukocidin genes in *Staphylococcus aureus*

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ABSTRACT

Panton-Valentine leucocidin (PVL) is associated with community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections. However, the prevalence of PVL among nasal carriage isolates of *S. aureus* from healthy individuals is largely unknown. We investigated the prevalence of PVL-carrying *S. aureus* strains, obtained from both nasal carriers in the community as well as patients with invasive infections. We found a low prevalence of 0.6% in 829 carriage isolates of methicillin-susceptible *S. aureus* (MSSA). Furthermore, we conclude that PVL has entered into distinct staphylococcal lineages, with different gene prevalences per genetic group of *S. aureus*. We corroborate that PVL is associated with skin and soft-tissue infections, but also document that PVL is not associated with bacteremia. It appears that the involvement of PVL in the pathogenesis of CA-MRSA infections is not a new staphylococcal feature, since *S. aureus* isolates harboring PVL are encountered throughout several genetic backgrounds of the *S. aureus* population.

The pathogenicity of *Staphylococcus aureus* depends on various bacterial surface components and extracellular proteins. However, the precise role of single virulence determinants in relation to infection is hard to establish. The frequent recovery of staphylococcal isolates that produce leukocidal toxins from patients with deep skin and soft tissue infections, particularly furunculosis, cutaneous abscesses, and severe necrotising pneumonia, suggest that the Panton-Valentine leucocidin (PVL) is one such a virulence factor that has a major role in pathogenicity (1-5).

In 1932, Panton and Valentine described PVL as a virulence factor belonging to the family of synergohymenotropic toxins (6). These toxins form pores in the membrane of host defense cells by synergistic action of two secretory proteins, designated LukS-PV and LukF-PV, which are encoded by two cotranscribed genes of a prophage integrated in the *S. aureus* chromosome (7, 8). PVL is mostly associated with community acquired methicillin-resistant *S. aureus* (CA-MRSA) infections and distinguishable from nosocomial MRSA by non-multidrug resistance and carriage of the type IV staphylococcal chromosome cassette element (SCC*mec* type IV) (3, 9-13).

Despite the presumed importance of PVL as a virulence factor, few data are available on its prevalence among *S. aureus* isolates from the nares of healthy persons compared with

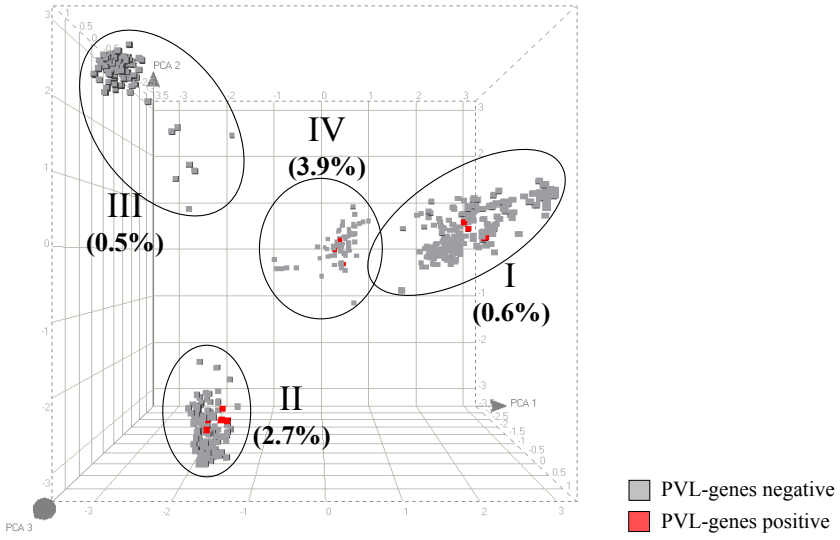

Figure 1. Principal component analysis of the AFLP data of 1,033 *S. aureus* strains.
The different cubes (plotted in 3-dimensional space) represent every strain in the study. Each axis represents the score calculated for that strain on each principle component. The 4 circles indicate the different genetic AFLP-clusters. Cluster IV could be subdivided in two minor clusters (see also reference 14). The percentages (below each cluster) indicate the relative number of PVL-positive strains in each major AFLP cluster. The single PVL-positive strain in cluster III is not visible behind the grey cubes. (For color see the online version of this thesis.)

Table 1. PVL distribution among carriage and invasive isolates per genetic cluster of *S. aureus*

	AFLP-cluster					
	I	**II**	**III**	**IVa**	**IVb**	**Total**
	(*n* = 462)	**(*n* = 261)**	**(*n* = 208)**	**(*n* = 62)**	**(*n* = 40)**	**(*n* = 1033)**
	No. (%) of PVL positive isolates					
Carriage-isolates (*n* = 829)	1	1	1	0	2	5 (0.6) [a]
Bacteremia-isolates (*n* = 146)	1	1	0	0	1	3 (2.1) [b]
Soft-tissue infection (*n* = 18)	1	5	0	1	0	7 (38.9) [c]
Impetigo-isolates (*n* = 40)	0	0	0	0	0	0 (0.0)
Total (*n* = 1033)	3 (0.6) [d]	7 (2.7)	1 (0.5) [e]	1 (1.6)	3 (7.5) [f]	15 (1.5)
MLST data of PVL-positive isolates	CC 1 (*n* = 2) CC 8 (*n* = 1)	CC 30 (*n* = 7)	CC 45 (*n* = 1)	CC 22 (*n* = 1)	CC 121 (*n* = 3)	

[a] versus [c] Fisher's exact test (two-sided); $P < 0.0001$

[b] versus [c] Fisher's exact test (two-sided); $P < 0.0001$

[d] versus [f] Fisher's exact test (two-sided); $P = 0.0079$

[e] versus [f] Fisher's exact test (two-sided); $P = 0.0140$

strains isolated from infections. This lack of data led us to investigate the frequency of PVL gene-positive *S. aureus* strains, obtained from the nares of healthy carriers in the community. For this purpose a single polymerase chain reaction method was used to detect both *lukS-PV* and *lukF-PV* genes (2).

In a previous study, the population structure of *S. aureus*, isolated from the nares of healthy persons in the Rotterdam area, The Netherlands, was elucidated (14). Strains were obtained from healthy children (<19 years) and elderly persons (>55 years). Furthermore, invasive strains (blood-culture, skin and soft-tissue infections and impetigo isolates) were included in this study (see Table 1). All carriage and clinical isolates (*n* = 1,033) were *mecA* negative. We used the same strain collection to study the PVL prevalence in carriage and invasive isolates of *S. aureus* from a single geographic region.

Five PVL positive *S. aureus* strains (0.6%) were found in the carriage group (*n* = 829) and 3 (2.1%) of 146 blood-culture isolates carried the PVL gene (see Table 1). This finding is in agreement with previous reported low PVL-prevalences by Von Eiff et al. (1.4% in 210 carriage-isolates and 0.9% in 219 blood-culture isolates) and Prevost et al. (0% in 31 carriage isolates and 1.4% in 69 blood-culture isolates) (15, 16). However, a higher prevalence of PVL (38.9%) was found in *S. aureus* strains causing abscesses and arthritis (Fisher's exact test; $P < 0.0001$) (14). This finding is also in agreement with the proposed involvement of PVL in severe and invasive (soft tissue) staphylococcal infections (1, 2, 4). No significant differences were found in the presence of PVL when carriage isolates were compared with

invasive blood-culture isolates. PVL was found in each major genomic amplified fragment length polymorphism (AFLP) cluster, indicating that PVL has been introduced in distinct phylogenetic subpopulations of *S. aureus* (Figure 1). Multilocus sequence typing (MLST) analysis of a subset of the strain collection showed that the 15 PVL-positive strains were within clonal complex (CC) 30 ($n = 7$), CC 121 ($n = 3$), CC 1 ($n = 2$), CC 8 ($n = 1$), CC 22 ($n = 1$), and CC 45 ($n = 1$) (see Table 1) (14). Although PVL was found among several staphylococcal genotypes, it was slightly overrepresented in AFLP-cluster IVb (CC 121) compared with major cluster I and III. Whether the prevalence of PVL in carriage- and blood-culture isolates is higher and differs among distinct genetic clusters of *S. aureus* in countries with endemic (CA-)MRSA has to be investigated further.

In conclusion, we have shown that the PVL-encoding phage has entered distinct staphylococcal lineages, although its prevalence differs per clonal group. PVL is associated with skin and soft-tissue infections, but not with bacteremia, which suggests that PVL is not likely to be involved in the pathogenesis of bacteremia. Infections caused by PVL-positive *S. aureus* strains have been documented since the 1930s. Expansion and increased incidence of such infections, however, are more recent, and further epidemiologic studies for tracking this phenomenon are still warranted.

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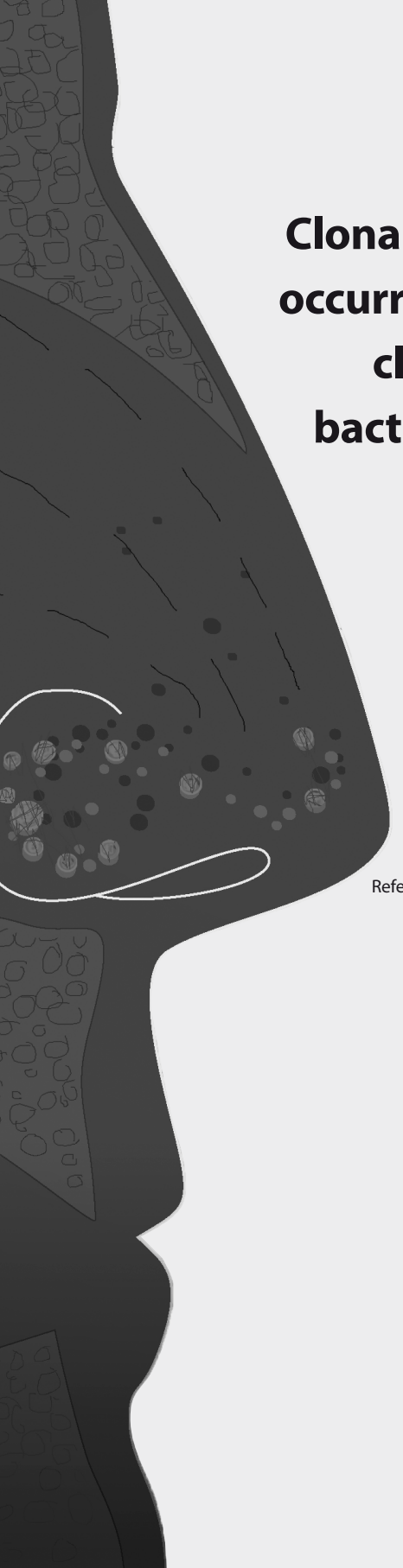

Chapter 10

Clonal distribution and differential occurrence of the enterotoxin gene cluster, *egc*, in carriage- versus bacteremia-associated isolates of *Staphylococcus aureus*

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ABSTRACT

The *Staphylococcus aureus* enterotoxin gene cluster, *egc*, was detected in isolates from healthy individuals and in those from patients with bacteremia. The *egc* genes co-occur and are slightly enriched in strains from healthy carriers (present in 63.7% of carriage-associated isolates versus 52.9% of invasive isolates; $P = 0.03$). Multilocus sequence typing revealed that successful staphylococcal clones usually harbour the *egc* locus.

Staphylococcal enterotoxins are responsible for food-associated outbreaks of diarrhoea among humans. The toxins generate visible pathological lesions in the stomach and the upper part of the small intestine (1). Many of the enterotoxins display superantigen characteristics and are obvious targets for anti-staphylococcal therapies. Genes encoding several of the enterotoxins are physically clustered in the *Staphylococcus aureus* genome (2, 3). The locus encoding the enterotoxins SEG, SEI, SEM, SEN and SEO is currently known as *egc* (enterotoxin gene cluster) (2). Although this cluster is highly prevalent among *S. aureus* strains in general, antibodies are rarely raised against *egc* enterotoxins which is a unique feature of this group of enterotoxins (4). It is intriguing that the prevalence of *egc* genes in isolates of *S. aureus* is negatively correlated with the severity of infection (5). For SEA the situation is precisely opposite: the toxin gene is significantly more often present in the invasive isolates (5, 6). The conclusion could be that one or more of the *egc*-encoded enterotoxins provide protection against severe sepsis. Additional research into this phenomenon is clearly warranted.

We here investigate whether strains isolated during Dutch *S. aureus* screening studies of carriage per sé and *S. aureus* strains causing bacteremia (7, 8) can be differentiated on the basis of the absence or presence of the *egc* locus.

Three hundred and ninety one strains of *S. aureus* were included in the present study (see Table 1). Most of the carriage isolates (n = 118) derived from the study by Wertheim *et al.* (7) in which patients were sampled at time of hospitalisation (MUP isolates). Another set of carriage strains (n = 86) derived from a group of elderly persons (over 50 years of age) in the open community (ERGO isolates) (8). As case strains, bacteremia-associated *S. aureus* strains (n = 180) from both these populations were included (7, 8). In addition, the group

Table 1. Presence of *egc* locus in carriage versus invasive isolates (n = 391)

	No. (%) of isolates		
	Negative for SEM	**Positive for SEM**	**Total**
Carriage-isolates			
MUP	45 (38.1)	73 (61.9)	118 (100)
ERGO	29 (33.7)	57 (66.3)	86 (100)
Total	**74 (36.3)**	**130 (63.7)** [a]	**204 (100)**
Invasive-isolates			
MUP	47 (50.0) [b]	47 (50.0) [c]	94 (100)
ERGO [d]	41 (44.1)	52 (55.9)	93 (100)
Total	**88 (47.1)**	**99 (52.9)** [a]	**187 (100)**

[a] proportionally more SEM positive isolates in the carriage group as compared to the invasive group (Fisher's exact test, two-sided; P = 0.0316)

[b] Forty-three blood culture isolates and 4 pus-isolates

[c] Forty-four blood culture isolates and 3 pus-isolates

[d] All 93 blood-culture isolates

Table 2. Association of PCR results for SEM with those for other enterotoxins of *egc* (validated by 212 isolates)

	No. (%) of isolates	
Enterotoxin	**Positive**	**Negative**
SEM	120	92
SEI	120 (100)	92 (100)
SEO	119 (99.2)	86 (93.5)
SEN	119 (99.2)	90 (97.8)
SEG	117 (97.5)	92 (100)
GIMNO	116 (96.7)	85 (92.4)

of invasive MUP isolates included strains isolated from pus (n = 7), and the nasal *S. aureus* carriage state for the bacteremic patients was also determined (9).

Strains were grown on blood agar plates (Becton-Dickinson, Le Pont de Claix, France) and DNA was extracted using the bacterial DNA kit III and a Magnapure system (Roche Molecular Systems, Lelystad, The Netherlands). The SEM gene was amplified from 10 ng DNA using SEM specific primers (40 cycles at 1 min 94°C, 2 min at 55°C and 3 min at 72°C) (5). PCR mixtures contained Supertaq DNA polymerase (Sphaero Q, Leiden, The Netherlands). PCR products were analyzed by agarose gel electrophoresis. The PCR products were of the expected length. PCRs specific for SEG, SEO, SEI, and SEN were performed for the MUP strains (n = 212) (5), as was multi locus sequence typing (MLST) (8, 10, 11). Statistical analyses involved Fisher's exact test (two-sided), with a P value of < 0.05 considered significant.

Table 2 shows that a positive score in the SEM PCR is strongly associated with positive scores for the SEI, SEO, SEN and SEG enterotoxin genes. Overall, when positive in the SEM PCR, 96.7% (116/120) of these strains were positive for all of the toxins associated with *egc*. When negative in the SEM PCR, 85/92 (92.4%) of these strains were negative for all of the other toxin genes. When carriage strains were compared to all invasive isolates (blood- and pus-derived) it appeared that the carriage isolates harboured the *egc* locus significantly more often (130/204 versus 99/187; P = 0.0316; see Table 1). Although a clear biological rationale is currently lacking, this observation suggests that the presence of *egc* is associated with non-invasiveness and a lower disease invoking potential, as suggested earlier (5). When the pus isolates were excluded from the analysis and the bacteremia-associated strains were compared separately to the carriage strains, the significance was maintained. However, the possible protective effect of *egc* does not involve changes in mortality once bacteremia has developed (49.4% [39/79] *egc* positive in bacteremia with no *S. aureus* related mortality versus 53.3% [8/15] with *S. aureus* related mortality; P = 1.0). Again, a biological explanation is lacking and it has to be emphasized that numbers of cases are low.

Clonality is an important feature of the enterotoxin gene content of strains. Some clones seem to be missing the *egc* element (clonal complexes [CCs] 1, 7, 8, and 15; see Table 3).

Table 3. Presence of the *egc* locus in different genetic clusters of *S. aureus* (*n* = 212)

CC or characterization by multilocus sequence typing	No. (%) of isolates		
	Negative for SEM	Positive for SEM	Total
1	8 (88.9)	1 (11.1)	9 (100)
5	0 (0.0)	12 (100)	12 (100)
7	12 (92.3)	1 (7.7)	13 (100)
8	19 (100)	0 (0.0)	19 (100)
9	1 (33.3)	2 (66.7)	3 (100)
12	1 (100)	0 (0.0)	1 (100)
15	32 (100)	0 (0.0)	32 (100)
22	0 (0.0)	2 (100)	2 (100)
25	0 (0.0)	10 (100)	10 (100)
30	4 (7.5)	49 (92.5)	52 (100)
45	1 (2.7)	36 (97.3)	37 (100)
Singleton	12 (63.2)	7 (36.8)	19 (100)
New sequence type	2 (100)	0 (0.0)	2 (100)
Total	92 (43.4)	120 (56.6)	212 (100)

Strains from CCs 5, 22, 25, 30 and 45 are positive in at least 92.5% of cases. Apparently, the *egc* element is associated with specific staphylococcal lineages, some of which are successful in the Rotterdam region (8).

It is interesting that the *egc* enterotoxins may be associated with staphylococcal toxic shock syndrome and scarlet fever (12) and that some of these toxins are enriched in genital isolates (13). Furthermore, Becker et al. showed that *seg* and *sei* genes were found in strict combination in 53.0% of the invasive- and 57.1% of the colonizing strains of *S. aureus,* whereas the *sed* and *sej* genes were found significantly more often in blood isolates ($P = 0.037$) (14). Ferry et al. showed that the presence of the *egc* locus was lower in *S. aureus* strains isolated from patients with septic shock than in those isolated from septic patients without shock, in strains isolated from patients with suppurative disease, and in carriage strains (5). The levels of antibodies against SEG and SEI are elevated in women; this may reflect mucosal adaptation of strains and a higher contact frequency for women (15). However, some of these studies were performed with limited numbers of strains and sometimes controversial data are published. The association of SEG, SEH, SEI, SEO and SEM with food poisoning, for instance, is still ill-defined (16, 17).

We here describe the presence of *egc* in a large collection of carriage and invasive isolates of *S. aureus* deriving from the same geographical region. *egc* is [1] slightly enriched among carriage strains, [2] not associated with mortality in patients suffering from staphylococcal bacteremia and [3] coupled to certain clonal lineages. There are surprising gaps in the capacities of different human sera to neutralise these superantigens (4). Furthermore, *egc* toxins seems to be produced in lesser quantities and have a lower immunogenic response than other enterotoxins (5), which may also explain why strains producing these *egc* entero-

toxins are tolerated in the nose. The function of the enterotoxins and their immune tolerance need to be studied in detail. Also, the precise enterotoxin gene content of the *egc* locus is still unknown. For instance, the SEU toxin gene was detected only recently (18). The host range of staphylococci with different toxin gene repertoires needs additional investigation as well (19). We conclude that the *egc* locus may enhance the "carriage potential" of an *S. aureus* strain, which might be explained by the fact that most of the successful clones do uniformly contain the *egc* locus.

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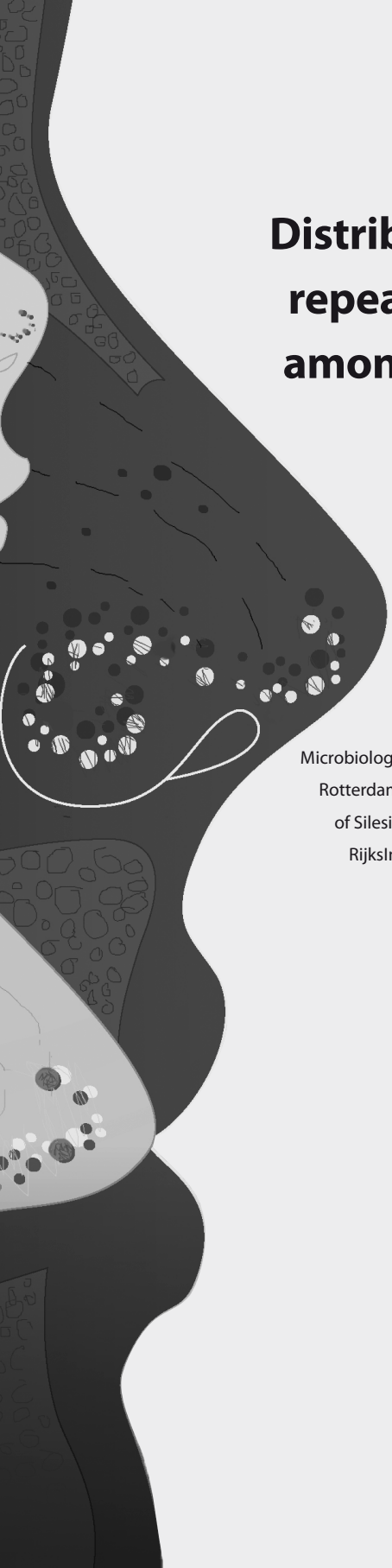

Chapter 11

Distribution of the serine-aspartate repeat protein-encoding *sdr* genes among nasal-carriage and invasive *Staphylococcus aureus* strains

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ABSTRACT

The *sdr* locus was found in all 497 investigated *Staphylococcus aureus* strains, although in 29 strains it contained only the *sdrC* gene (*sdrD* negative, *sdrE* negative). The *sdrC*-positive, *sdrD*-negative, *sdrE*-negative gene profile was exclusive to methicillin-sensitive *S. aureus* (MSSA) strains (Fisher's exact test; $P = 0.0005$) and was not found in the strains collected from bone infections ($P = 0.0019$). We also found a strong association between the presence of the *sdrD* gene and methicillin-resistant *S. aureus* strains ($P < 0.0001$). Our findings suggest that MSSA strains with the newly uncovered *sdrC*-positive, *sdrD*-negative, *sdrE*-negative gene profile have a substantially decreased potential to establish bone infection.

Staphylococcus aureus is an extremely versatile and frequent pathogen of humans both in the community and in hospitals. Infections caused by this organism involve bacterial adhesion to the host extracellular matrix. *S. aureus* adhesins are mostly cell wall-anchored proteins and are grouped into a single family named microbial surface components recognizing adhesive matrix molecules (MSCRAMM).

The Sdr proteins are members of the MSCRAMM family that are encoded by the tandemly arrayed *sdrC*, *sdrD*, and *sdrE* genes, of approximately 2.8, 3.9, and 3.5 kbp, respectively, located in the *sdr* locus (1). The Sdr proteins are members of a family of surface proteins which are characterized by the presence of an R region containing various numbers of the Ser-Asp dipeptides encoded by DNA repeats in the 3' region of the *sdr* genes. The Sdr proteins have a comparable structural organization. A signal peptide is followed by an A domain which is similar in size among the different members of the Sdr family. However, they are not closely related, with only 20 to 30% identical amino acid residues. This suggests that different Sdr proteins have different roles in *S. aureus* pathogenicity. However, a ligand was defined only for Bbp (bone sialo-binding protein), which is an allelic variant of SdrE (2). The Sdr proteins have two, three, or five additional 110- to 113-residue sequences (B motifs) that are tandemly repeated in SdrC, SdrE, and SdrD, respectively. The B repeats bind Ca^{2+} with high affinity, and their structure unfolds when calcium ions are removed. The function of the B domains remains unknown. The B motifs are followed by segments composed of the SD repeats (R region). The C-terminal end (region M) of the Sdr proteins is involved in anchoring the proteins to the bacterial cell wall.

At least two *sdr* genes are present in all tested *S. aureus* strains (1) and always include *sdrC* (3). Therefore, the lack of *sdr* genes must be explained by the absence of *sdrD* or *sdrE*. Peacock and colleagues (3) demonstrated a strong correlation between *S. aureus* invasiveness and the presence of one of the allelic variants of the *sdrE* gene. Moreover, Trad et al. (4) reported a significantly higher prevalence of the *sdrD* gene in *S. aureus* strains responsible for bone infections.

S. aureus multiple-locus variable-number tandem-repeat analysis (MLVA) (5, 6) utilizes polymorphism of seven individual genes (*sspA*, *spa*, *sdrC*, *sdrD*, *sdrE*, *clfA*, and *clfB*). During MLVA characterization of *S. aureus* strains, we found several strains possessing only five bands instead of six or seven in a pattern. After analysis using simplex PCRs with primer pairs designed for the MLVA method, we determined that the subset of *S. aureus* strains had only a single gene in the *sdr* locus. Similarities in the DNA sequence flanking the SD repeats of the *sdr* genes allowed for the selection of a single pair of primers for amplification of all three individual genes in the *sdr* locus. It was not possible to determine which of the *sdr* genes is present in the *sdr* locus. Therefore, we designed a novel triplex PCR procedure to examine the distribution of the *sdr* genes among nasal-carriage and invasive *S. aureus* strains as well as methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) (Table 1).

Table 1. Primers used in this study [a]

Gene	Primer	Nucleotide sequence	Cycling conditions [b]
sdrC	sdrCF	5′-AAAAGGCATGATACCAAATCGA	Den, 94°C/30 s; Ann, 53°C/30 s;
	sdrCR	5′-AATTCTCCATTCGTATGTTCTG	Elon, 72°C/15 s
sdrD	sdrDF	5′-AGTGGGAACAGCATCAATTTTA	
	sdrDR	5′-GTGGTAGATTGTACACTTTCTT	
sdrE	sdrEF	5′-AGAAAGTATACTGTAGGAACTG	
	sdrER	5′-GATGGTTTTGTAGTTACATCGT	
Azoreductase	azoF	5′-GTGAATAATAG(C/T)CCATATGAAC	Den, 94°C/30 s; Ann, 50°C/30 s;
Putative glycosyltransferase	glyR	5'-CTACACCTGTAATTTTACTTTC	Elon, 72°C/1 min

[a] The primers were designed based on *S. aureus* genomes available from the Institute for Genomic Research (http://www.tigr.org) (COL strain), the University of Oklahoma (http://www.genome.ou.edu) (strain 8325), the Sanger Institute (http://www.sanger.ac.uk) (strains MRSA252 and MSSA476), the National Institute of Technology and Evaluation (http://www.bio.nite.go.jp) (strain MW2), and Juntendo University (http://www.juntendo.ac.jp) (strains Mu50 and N315).

[b] Den, denaturation temperature and time; Ann, annealing temperature and time; Elon, elongation temperature and time.

A total of 497 strains (Table 2) were obtained during this study between 1990 and 2005, and these were selected from previously characterized collections (7-9). Only one isolate per person was tested. In addition, none of these strains were part of an outbreak, and none were determined to be from patients or personnel with obvious cross-infection. All strains in the collections were characterized by at least one genome typing method, including pulsed-field gel electrophoresis, multilocus sequence typing, and MLVA. The strains showed a large degree of diversity, and not a single clonal type was overrepresented (data not shown).

DNA was extracted with the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) or the MagnaPure LC DNA system (DNA isolation kit III; Roche, Almere, The Netherlands) as previously described (6, 10). The triplex PCR products specific in size for *sdrC* (144 bp), for *sdrD* (272 bp), and for *sdrE* (433 bp) were analyzed by electrophoresis in 2% agarose SeaKem LE gels (FMC). The two-tailed Fisher exact test was used to analyze the distribution of the *sdr* genes among *S. aureus* strains originating from different hosts. *P* values less than 0.05 were considered a statistically significant difference.

The *sdrC* gene was present in all investigated strains (n = 497), which was concordant with a previous report (3). However, in 29 MSSA strains (of the total 382 MSSA strains), only the *sdrC* gene (*sdrD* negative, *sdrE* negative) was found in the *sdr* locus. The result was confirmed by PCR amplification of a sequence covering the *sdr* locus, using primer pair azoF and glyR (Table 1), targeting the sequences in flanking genes encoding azoreductase (assigned as SACOL0607 in the COL genome) and putative glycosyltransferase (SACOL0611). The amplicon sizes around 3.5 kbp confirmed the presence of only a single *sdr* gene in the *sdr*

Table 2. Distribution of *sdrD* and *sdrE* among *S. aureus* strain collections used in this study

Source of isolation	No. of strains	No. of strains carrying						Geographic origin(s)	Reference(s)
		sdrD	*sdrE*	*sdrD* and *sdrE*	*sdrD* but not *sdrE*	*sdrE* but not *sdrD*	Neither *sdrD* nor *sdrE*		
Community-acquired strains from nasal swabs of asymptomatic carriers	116 MSSA	53	99	51	2	48	15	United Kingdom	7
	1 MRSA	1	1	1	0	0	0		
Community-acquired strains from nasal swabs of asymptomatic carriers	111 MSSA	43	104	40	3	64	4	Holland	9
Hospital-acquired strains collected from blood infections	52 MSSA	23	44	21	2	23	6	Holland	9
Hospital-acquired strains derived from a variety of non-invasive human infections	12 MSSA	3	12	3	0	9	0	Bulgaria, Czech, Republic, Croatia, England, Germany, Lithuania, Poland, Russia, Slovenia, Turkey	8 and this study
	85 MRSA	85	79	79	6	0	0		
Community-acquired strains from nasal swabs of asymptomatic carriers	31 MSSA	15	27	15	0	12	4	Poland	This study
Community-acquired strains collected from osteomyelitis	60 MSSA	46	58	44	2	14	0	Poland	This study
	29 MRSA	28	21	20	8	1	0		
Summary	382 MSSA	183	344	174	9	170	29		
	115 MRSA	114	101	100	14	1	0		
Total	497 MSSA and MRSA	297	445	274	23	171	29		

Table 3. Distribution of *sdrC*-positive, *sdrD*-negative, *sdrE*-negative gene profile among sequence types defined by multilocus sequence typing

ST (allelic profile)	No. of strains	Source of isolation
1 (1,1,1,1,1,1,1)	3	Nasal carriage
12 (1,3,1,8,11,15,11)	2	Nasal carriage (*n* = 1)
		Blood infection (*n* = 1)
25 (4,1,4,1,5,5,4)	2	Nasal carriage
30 (2,2,2,2,6,3,2)	6	Nasal carriage (*n* = 2)
		Blood infection (*n* = 4)
34 (8,2,2,2,6,3,2)	3	Nasal carriage
39 (2,2,2,2,2,2,2)	2	Nasal carriage (*n* = 1)
		Blood infection (*n* = 1)
45 (10,14,8,6,10,3,2)	2	Nasal carriage (*n* = 1)
		Blood infection (*n* = 1)
47 (10,11,8,6,10,3,2)	3	Nasal carriage
182 (18,18,6,2,13,15,18)	2	Nasal carriage
New-1 (3,1,31,1,29,5,3)	1	Nasal carriage
New-2 (3,51,1,1,4,4,3)	3	Nasal carriage

locus (data not shown). Two or three *sdr* genes were always detected in all MRSA strains (*n* = 115). A significant association between the *sdrC*-positive, *sdrD*-negative, *sdrE*-negative gene profile and MSSA strains was found (29/353 versus 0/115; Fisher's exact test; $P = 0.0005$). The strains with only the *sdrC* gene in the *sdr* locus represented different sequence types (STs) defined by multilocus sequence typing (Table 3). In the same STs we found strains with different combinations of the genes in the *sdr* locus, suggesting a high degree of variability. In contrast, the *sdrD* and *sdrE* genes were heterogeneously distributed. Among the tested strains, *sdrD* was significantly associated with MRSA strains (183/199 versus 114/1; Fisher's exact test; $P < 0.0001$), whereas the *sdrE* distribution did not differ between the MSSA and MRSA strains (344/38 versus 101/14; Fisher's exact test; $P = 0.4898$).

We checked the distribution of the *sdr* genes between the strains collected from nose swabs of asymptomatic carriers (*n* = 259) and invasive infections, osteomyelitis (*n* = 89), and blood infections (*n* = 52). The strains possessing only the *sdrC* gene in the *sdr* locus were not found in the strains collected from bone infections (23/236 versus 0/89; Fisher's exact test; $P = 0.0019$). This indicates strongly that the carriage strains with only the *sdrC* gene have abolished or substantially decreased the potential to establish bone infection. In contrast to osteomyelitis, the *sdrC*-positive, *sdrD*-negative, *sdrE*-negative gene profile was found in the strains isolated from blood infections (23/236 versus 6/46; Fisher's exact test; $P = 0.6001$). There was no significant difference between the strains associated with nasal colonization and those associated with both types of infections ($P > 0.05$). However, the *sdrD* gene was significantly associated with osteomyelitis (112/147 versus 74/15; Fisher's exact test; $P < 0.0001$) but not with blood infections (112/147 versus 23/29; Fisher's exact test; $P = 1.0$). There was no significant cor-

relation of *sdrE* with blood infections (231/28 versus 44/8; Fisher's exact test; $P = 0.3457$) and osteomyelitis (231/28 versus 79/10; Fisher's exact test; $P = 1.0$).

In previous investigations a contribution to the pathogenic process of the allelic variants (*sdrE*/*bbp*) of the *sdrE* gene was explored. Peacock and colleagues (3) have shown a possibility that one (*sdrE*) of the allelic variants of the *sdrE* gene is associated with invasive disease while another (*bbp*) is evenly distributed among isolates recovered from healthy individuals and from patients with invasive *S. aureus* disease. Tristan and colleagues (11) have investigated only one of the allelic variants of the *sdrE* gene, and they have revealed that the distribution of *bbp* is significantly associated with osteomyelitis/arthritis. There was no difference in the incidence of *bbp* when carriage-associated strains were compared to invasive isolates. During our investigations we designed the primers which had sequences corresponding to regions of both allelic variants of the *sdrE* gene. Therefore, our results strengthen the observation of Peacock et al. that the allelic variants at a given locus may have different contributions to the pathogenic process. Our data are also concordant with the studies by Trad et al. (4) in which a significantly higher prevalence of only the *sdrD* gene and not the *sdrE* gene in bone infections has been found. The sequence alignments of the *sdrC* and *sdrD* genes (sequences obtained from the *S. aureus* genomes for which websites are given in Table 1) show that their polymorphism level is comparable to that of the *sdrE* gene (data not shown). It is very important to address future studies to determine sequence variability of the *sdr* genes and on the basis of their polymorphism to investigate the pathogenic potential of allelic variants of the *sdrC*, *sdrD*, and *sdrE* genes.

It is not clear why *sdrC* alone seems to be limited to MSSA strains only. This might be a reflection of the fact that MRSA primarily consists of a limited number of highly successful pandemic clones. Katayama and colleagues (12) have demonstrated that genetic background profoundly influences the stability of *mecA* in *S. aureus*. We can only hypothesize now that the same genetic mechanisms could play a role in acquisition and stability of SCC*mec* and the *sdr* genes as well as other genetic elements.

Most infections caused by *S. aureus* result from the combined action of a variety of factors. However, the contribution of particular virulence factors to *S. aureus* pathogenicity in humans is poorly understood. The results obtained in experimental models suggest that *S. aureus* strains producing receptors for bone sialoprotein, collagen, and fibronectin are associated with osteomyelitis and arthritis (2, 13, 14). However, our results show that strains lacking the *sdrD* and *sdrE* genes have decreased potential to infect bones. Studies in which strains with knockouts of *sdrD* and/or *sdrE* are used in experimental models should be performed to cast light on the role of these genes in osteomyelitis. Furthermore, studies to investigate the differential distribution of genes encoding virulence factors in a larger number of *sdrD*- and/or *sdrE*-positive strains and strains lacking both *sdrD* and *sdrE* are needed. The understanding of specific pathogenetic mechanisms may have an important prophylactic and therapeutic impact. The results obtained during this study suggest that both *sdrD* and

sdrE may play comparable and important roles in bone infections. Strains lacking the *sdrD* gene had also potential to give rise to osteomyelitis, but then they always possessed the *sdrE* gene. These findings show that most strains are capable of bone infections. For adequate and cost-effective infection prevention, it is important to distinguish nasal colonizers which may be more aggressive from those with abolished or substantially decreased potential to establish an invasive infection.

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Chapter 12

Capsular and surface polysaccharide serotyping of Dutch *Staphylococcus aureus* strains from carriage and infection

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ABSTRACT

International epidemiological studies have shown that clinical isolates of *Staphylococcus aureus* are usually capsulated with either type 5 or 8 capsular polysaccharides. Since all non-capsulated strains were found to be cross-reactive with polysaccharide 336 (336PS) antibodies, the non-capsulated strains were denoted as type 336PS. We determined the capsular types of 162 Dutch methicillin-susceptible *S. aureus* strains derived from individuals living in the Rotterdam area. The serotype distribution was 28.4% serotype 5, 53.7% type 8, and 17.9% type 336PS. Serotyping was in agreement with genotyping by amplified fragment length polymorphism (AFLP) and multi locus sequence typing (MLST). Among 49 nasal carriage isolates from healthy children 24.5% belonged to serotype 5, 67.3% were type 8 and 8.2% were type 336PS. For 28 adult patients on chronic ambulatory peritoneal dialysis (CAPD) the serotype incidences among carriage isolates obtained from the nose, catheter exit-site, and abdominal skin were 45.1%, 41.2% and 13.7%, respectively. Among *S. aureus* strains deriving from blood cultures, the serotype incidences were 17.7% serotype 5, 53.2% type 8 and 29.0% type 336PS. Apparently, type 336PS strains are more prevalent ($P = 0.017$) among bacteremia isolates as compared to the nasal carriage isolates obtained from healthy children and CAPD patients. In conclusion, all Dutch *S. aureus* isolates belonged to types 5, 8 or 336PS, which is in agreement with data from other countries. Thus, addition of the 336 polysaccharide conjugate to a type 5- and type 8- capsular polysaccharide protein conjugate vaccine would significantly extend the vaccine coverage.

INTRODUCTION

Throughout the world and for many years, *Staphylococcus aureus* has been a leading and persistent pathogen. It plays a central role in hospital-acquired infections and it has shown a high adaptability and developed resistance mechanisms to almost all antibiotics that were introduced over the last few decades. Recently, highly pathogenic, methicillin resistant strains have emerged in the community outside the hospital setting causing serious infections in otherwise healthy individuals (1-3). For years investigators were convinced that vancomycin and other newly discovered antibiotics that were introduced to the marketplace would take care of *S. aureus* infections indefinitely. However, the emergence of the vancomycin intermediate strains (VISA) followed by the isolation of a truly vancomycin resistant *S. aureus*, that probably acquired the *vanA* gene from *Enterococcus*, has become a reality (4, 5) . The emergence and rapid dissemination of community acquired methicillin resistant *S. aureus* (CA-MRSA) strains and the devastating outcomes of these infections underline the need for alternatives to antibiotic treatment. A new approach to combat the highly versatile and adaptable *S. aureus* should address its virulence factors that are associated with disease. Immunotherapeutic strategies for prevention and treatment of *S. aureus* infections need to be designed and to that aim a multivalent vaccine, which targets several virulence factors of *S. aureus*, is under development (Taylor and Fattom, unpublished data).

The discovery of capsular polysaccharides in 1931 by Gilbert (6), the development of an antibody-based capsular polysaccharide serotyping scheme by Karakawa and Vann (7, 8), and the confirmation by Sompolinski et al. (9) revealed that although 13 putative capsular types exists only two capsular serotypes, type 5 and type 8, are clinically relevant and are associated with human disease. None of the other capsules were found in human clinical isolates. Subsequently this was confirmed by several other investigators where roughly 80-90% of *S. aureus* human isolates including MRSA isolates were capsular types 5 and 8 (10-14). These unique capsular polysaccharides (CPs) (8), type 5 and type 8 (CP5 and CP8), had brought about the opportunity to develop vaccines and immunotherapy against these pathogens. Bacterial CPs are considered virulence factors and efficacious vaccine candidates that were shown to elicit protective antibodies. *S. aureus* CPs were shown to be anti-phagocytic (8), and type 5 capsule-specific antibodies were shown to mediate a type-specific opsonophagocytosis, which substantiates the only known mechanism for clearing of *S. aureus* infections (15). Moreover, evaluation of CP5-conjugate-based vaccines and antibodies in animal models resembling different types of *S. aureus* infections in man, revealed that CP-based vaccines and antibodies were able to prevent the infections in challenged animals (16, 17). Similar protection outcomes were observed against type 8 challenge strains when animals were actively immunized with a CP8-conjugate vaccine or passively administered CP8 specific antibodies generated in humans or animals by vaccination with CP8 conjugate vaccine (manuscript under preparation). StaphVAX (Nabi Biopharmaceuticals), a bivalent

CP5 and CP8 conjugate vaccine, was associated with a significant reduction of bacteremia in hemodialyis patients between 3 to 40 weeks post-vaccination in a phase 3 clinical trial (18). However, a second phase III trial failed to confirm efficacy. A subsequent investigation revealed subtle and significant changes in elements of the manufacturing process for the lot used in the confirmatory Phase III study. These changes resulted in differences in the CP structure itself that are believed to be important for producing high-quality and high-affinity antibodies.

Although capsular types 5 and 8 comprise roughly 80-90% of all strains in the United States, the remaining 10-20% of *S. aureus* clinical isolates that are non-reactive to type 5 and type 8 antibodies have lost their ability to produce capsule (19), and were historically denoted as non-typeable (9, 10, 12, 13). Subsequently, these non-typeable strains were found to possess a cell wall surface polysaccharide, denoted as 336 polysaccharide (336PS), which was shown to be anti-phagocytic and enhance virulence through increased complement resistance as compared to classical Wood teichoic acid strains (unpublished data, Nabi Biopharmaceuticals). This polysaccharide is a chemical variation of the prototype cell wall teichoic acid and although the composition of this polysaccharide was identical to the prototype teichoic acid, preliminary analysis, primarily NMR spectra, revealed a unique and different structure for the 336PS. Thus, non-typeable strains that are cross-reactive to 336 antibodies were denoted as the 336 "serotype" (type 336PS) (14, 20, 21). Similar to CP antibodies, 336-conjugate derived antibodies from animals as well as humans were found to mediate *in vitro* opsonophagocytosis and to confer protection against 336PS type strains in animal models (manuscript under preparation). While the prevalence of the capsular and surface polysaccharides among *S. aureus* isolates is important for designing an efficacious *S. aureus* vaccine, establishing the serology of *S. aureus* infections from different geographic locations and monitoring the distribution of infectious isolates for serotype shifts is of great importance.

To study the potential relevance of a putative trivalent *S. aureus* polysaccharide-conjugate vaccine, we aimed to determine the surface and/or capsular polysaccharide serotype of 162 well-characterized *S. aureus* strains from the Erasmus University Medical Center in Rotterdam, The Netherlands. In addition, we determined if there is an association between capsular type and strain genotype. This collection of isolates, which is representative of Dutch methicillin-susceptible *S. aureus* (MSSA), includes isolates obtained from nasal swabs of healthy children (1-18 years) (22, 23), nasal carriage, skin-, as well as catheter exit site isolates from patients undergoing continuous ambulatory peritoneal dialysis (CAPD) (24), and recent blood-culture isolates.

MATERIALS AND METHODS

Rabbit antisera

Type 5 capsular polysaccharide (CP5), type 8 capsular polysacharide (CP8) and 336 polysaccharide (336PS) antisera were generated by immunizing New Zealand. White rabbits intramuscularly three times two weeks apart with Freunds adjuvant and 50 μg of CP5, CP8, or 336PS conjugated to *Pseudomonas aeruginosa* exoprotein A (rEPA), or diphtheria toxoid (DT). Animals were bled on day 42 to collect serum. Rabbits were boosted monthly there after for up to 6 months and production bleeds were obtained weekly for three weeks after each boost.

S. aureus isolates

A collection of 162 methicillin-susceptible *S. aureus* (MSSA) strains was assembled. Forty-nine isolates were obtained from nasal swabs of healthy children (1-18 years) (22, 23), who were taking part in a massive meningococcal vaccination program at the AHOY Center in Rotterdam 2002 (AHOY Study). Forty-five (92%) of these isolates have been typed before by amplified fragment length polymorphism (AFLP) analysis, pulsed field gel electrophoresis (PFGE) and multiple locus sequence typing (MLST) (23, 25). MLST analysis was carried out by using DNA arrays as described earlier (26). This has generated a framework in which AFLP groupings (clusters 1, 2, 3, 4, and 5) and MLST defined clonal clusters (CCs) can be visualized. In addition, 51 isolates from 28 different patients undergoing continuous ambulatory peritoneal dialysis (CAPD) were included. These patients were screened for *S. aureus* carriage starting in January 1998 (24). After an initial screening period nasal, catheter exit site, and contra-lateral abdominal skin swabs (i.e. opposite to the catheter exit site) were taken every 12-weeks during follow up visits. Patients were followed-up for approximately 4 years or until the date ending their CAPD treatment. The isolates we used were cultured from nasal, catheter exit-site and abdominal screening swabs collected from October 1998 through February 2002. Single isolates per sampling site were included from individual patients except for those patients (n = 3) in whom we identified different CP or PS types (per sampling site). Sixty-two recent blood isolates from the Erasmus University Medical Center in Rotterdam (The Netherlands) were included as well. These isolates were cultured between March 2004 and August 2005.

Preparation of bacterial isolates

All isolates were first streaked on both sheep blood agar and Columbia / Colistin-nalidixic acid (CNA) plates, and were incubated at 37°C overnight in order to produce pure, isolated, single *S. aureus* colonies. For each isolate a single colony was picked from the CNA plate and grown for 24 hrs at 37°C on new Columbia agar plates containing 2% $MgCl_2$ and 0.5% $CaCl_2$, a growth medium that facilitates maximum capsulation for serotyping.

Serotyping

Serotyping of the isolates was performed by slide agglutination using antibodies specific to CP5, CP8, or 336PS. For each isolate, several colonies were re-suspended in 0.9% saline to approximately 0.5 OD at 600 nm, and tested for slide agglutination. Briefly, 10 µL of each antiserum and 10 µL of bacterial suspension were spotted onto a glass slide and rocked gently to mix. Positive agglutination was observed by visible clumps present within 5-10 seconds. Saline (0.9%) was used as a negative control. Confirmation of all serotypes was carried out by immuno-diffusion of crude *S. aureus* isolate digests. Crude digests were prepared by re-suspending one loopful of cells in 500 µl of 50 mM Tris-HCl, pH 7.0 containing 2 mM $MgCl_2$. Twenty µL of lysostaphin (3-4 mg/mL), and 2 µL of 10 mg/ml solutions of RNase and DNase were added to the cell suspension and incubated at 37°C while mixing for 3 hours. The digests were then microfuged to remove the cell debris. The digest supernatants were then analyzed by immuno-diffusion using 1% agarose gels containing 1x PBS buffer on GelBond film. All cell digests were used neat, except when set-up against anti-336-rEPA, where they were diluted (1:10). To each central well, a 10 µL of antibodies specific either to CP8, CP8 or 336PS was loaded and a 10 µL of the cell lysates were placed in the surrounding wells and incubated overnight at 4°C. The homologous purified polysaccharide was used as a positive control.

Statistical analysis

To compare the distribution of serotypes across the different strain categories, Fisher's exact test was used. A two-sided *P*-value of less than 0.05 was considered significant. Because multiple isolates were included per patient for the CAPD group (i.e. one per sampling site), we only included the nasal carriage isolates of the CAPD collection in the statistical analysis, when comparing the distribution of the serotypes across the different strain categories.

RESULTS AND DISCUSSION

All 162 *S. aureus* isolates were serotyped by slide agglutination and immunodiffusion using either CP5, CP8-, or 336PS-conjugate polyclonal antibodies. Two serotyping methods, slide agglutination and immunodiffusion, were carried out in order to confirm the serotype for strains that were weakly reactive with type 5 or type 8 antibodies in the slide agglutination test. Serotyping results from slide agglutination and immunodiffusion were in agreement in that all type 5 strains were reactive to type 5 antibodies in slide agglutination and immunodiffusion, while all type 8 isolates were reactive to type 8 antibodies. All 336PS type isolates were non-reactive to type 5 and type 8- antibodies using both tests and were positive with 336-antibodies. Interestingly, the majority of type 5 and type 8 isolates, 44/46 (95.7%) and 82/87 (94.3%) respectively, were also reactive to 336 antibodies using both assays.

Table 1. Summary of serotyping results

Isolate collection	No. (%) of isolates			
	Total	Type 5	Type 8	Type 336PS
Healthy children (n = 49) (AHOY)- nasal colonization	49 (100)	12 (24.5)	33 (67.3)	4 (8.2)
CAPD-patients (n = 28) [a]	51 (100)	23 (45.1)	21 (41.2)	7 (13.7)
Nasal colonization	29 (100)	11 (37.9)	13 (44.8)	5 (17.2)
Catheter exit-site	12 (100)	6 (50.0)	4 (33.3)	2 (16.6)
Abdominal skin swab	10 (100)	6 (60.0)	4 (40.0)	0 (0.0)
Blood-culture (n = 62)	62 (100)	11 (17.7)	33 (53.2)	18 (29.0) [b]
Total	162 (100)	46 (28.4)	87 (53.7)	29 (17.9)

[a] The nasal isolates (n = 29), the catheter exit-site isolates (n = 12), and the abdominal screening isolates (n = 10) of the CAPD patients, were obtained from 27, 9, and 10 different CAPD patients, respectively.

[b] proportionally more type 336PS strains in the blood-culture collection compared to the nasal carriage isolates obtained from the healthy children and CAPD patients (P = 0.017).

Among the 162 MSSA isolates 28.4% were serotype 5, 53.7% were type 8 and 17.9% were type 336PS (Table 1). Among the 49 nasal colonization isolates from healthy children (AHOY-study) 24.5% were type 5, 67.3% were type 8 and 8.2% were type 336PS (Table 1). Serotype 8 appears to be the most prevalent serotype in this study, which is even more evident among carriage isolates from healthy children and which is corroborated by other studies. An Indian study investigated 221 nasal carriage isolates, which revealed a serotype distribution of 22.2% for type 5 and 52.9% for type 8 (27). The prevalence of serotype 8 strains was even higher (64%) among invasive isolates in that study. Verdier et al. (21) showed a serotype distribution among clinical isolates (n = 195) of 42% for type 5 and 45% for type 8. However, they found a predominance of serotype 5 strains (64%) among the MRSA isolates (n = 69) and a predominance of serotype 8 (60%) for the MSSA isolates (n = 126). Roghmann et al. (14) also found that MRSA isolates were significantly more likely to be serotype 5 than methicillin-susceptible isolates (66% vs. 39%, $P < 0.001$). In the current study serotype 5 strains were also predominant among CAPD isolates (see below).

The genotype of 45 nasal carriage isolates was determined before by PFGE, MLST and AFLP (23). Serotyping results demonstrate that there is an association between capsular serotype and genotype as defined by AFLP, MLST and PFGE (Table 2 and Figure 1). *S. aureus* isolates can be grouped into 5 major genetic clusters based on AFLP (23). Interestingly, our serotyping results demonstrate that the major AFLP cluster 1, which is the most genetically diverse, is associated with both type 5 and type 8 isolates. Note that 2 of these isolates are type 336PS and seem to have lost their ability to produce capsule (Table 2). The genetically homogeneous major AFLP clusters 2 and 3 are both associated with capsular type 8. AFLP clusters 4 and 5 are largely associated with capsule type 5 and type 8, respectively. Figure 1 gives a survey of all typing data obtained and shows that in the heterogeneous AFLP cluster

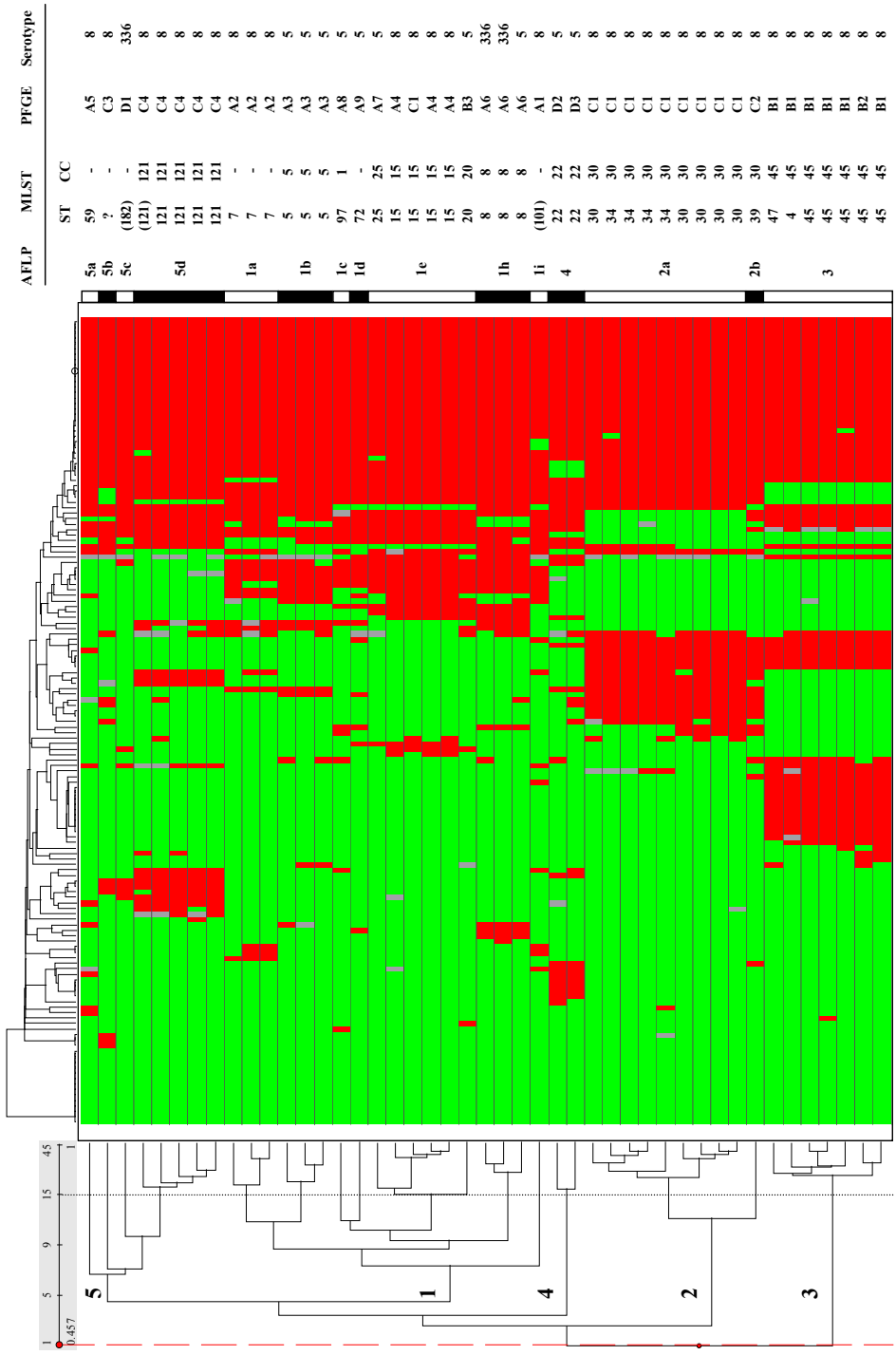
AFLP
MLST
PFGE
Serotype
ST
CC
5a 59 - A5 8
5b ? - C3 8
5c (182) - D1 336
(121) 121 C4 8
5d 121 121 C4 8
121 121 C4 8
121 121 C4 8
121 121 C4 8
1a 7 - A2 8
7 - A2 8
7 - A2 8
1b 5 5 A3 5
5 5 A3 5
1c 5 5 A3 5
1c 97 1 A8 5
1d 72 - A9 5
25 25 A7 5
1e 15 15 A4 8
15 15 C1 8
15 15 A4 8
15 15 A4 8
20 20 B3 5
1h 8 8 A6 336
8 8 A6 336
1i 8 8 A6 5
(101) - A1 8
4 22 22 D2 5
22 22 D3 5
2a 30 30 C1 8
34 30 C1 8
34 30 C1 8
34 30 C1 8
34 30 C1 8
30 30 C1 8
30 30 C1 8
30 30 C1 8
2b 30 30 C1 8
39 30 C2 8
47 45 B1 8
3 4 45 B1 8
45 45 B1 8
45 45 B1 8
45 45 B2 8
45 45 B1 8
1 5 9 15 45
0.457
5
1
4
2
3

Table 2. Serotyping results of the AHOY nasal colonization isolates as sorted by genotype

AFLP cluster	AFLP subcluster	MLST (CC)	PFGE type(s)	No. of isolates			
				Type 5	Type 8	Type 336PS	Total
1	1a	7	A2		3		3
	1b	5	A3	3			3
	1e	15	A4 (C1)		4		4
		20	B3	1			1
		25	A7	1			1
	1h	8	A6	1		2	3
2	2a	30	C1		9		9
3	3	45	B1 (B2)		7		7
4	4	22	D2/D3	2			2
5	5d	121	C4		5		5
Total				**8**	**28**	**2**	**38**

Only AFLP sub-clusters with more than 1 isolate are shown (n = 38)

I, a subdivision can be made on the basis of the PFGE and MLST data. Note that such PFGE and MLST subclusters are essentially homogeneous with respect to their serotype (e.g. PFGE type A2 is serotype 8, whereas PFGE type A3 is serotype 5).

The nasal carriage rate is estimated to be 50% among CAPD patients (28). Persistent nasal carriage of *S. aureus* was shown to be a major risk factor among CAPD-patients for all-cause CAPD-related infections (exit site infections and peritonitis). In a study conducted by Nouwen and colleagues (24), 77 of 78 (98.7%) *S. aureus* infections from persistent carriers were of endogenous origin with colonizing and infecting strains sharing the same genotype. We serotyped 51 *S. aureus* isolates, which were cultured from nasal, catheter exit-site and abdominal screening swabs of CAPD patients. Among the 29 nasal colonization isolates 37.9% were type 5 and 44.8% were type 8, whereas 17.2% were type 336PS. Among 22 CAPD-related isolates 12/22 or 54.5% were type 5 and 8/22 or 36.4% were type 8 (Table 1).

The majority of blood isolates (71.0%) were capsular types 5 and 8. Among the 62 blood-culture isolates 17.7% were type 5, 53.2% were type 8 and 29.0% were 336PS (Table 1). It is

Figure 1. Two-dimensional hierarchical clustering of the AFLP data of 45 *S. aureus* strains.

The green/red figure represents 6,615 binary outcomes, generated by ht-AFLP and based on 147 marker fragments. Green represents marker absence and red represents marker presence (grey represents ambiguous positions [i.e. weak bands] which are scored as marker absence in the mathematical analyses). The dendrogram on the *y*-axis represents the genetic clustering of 45 *S. aureus* strains. The dendrogram on the *x*-axis shows the clustering of the 147 AFLP-markers, many of which segregate in specific groups. Five AFLP major clusters and 15 AFLP subclusters could be identified (as defined by the dotted bar indicating an arbitrarily chosen cut-off), represented by the black-white bar on the right. MLST data, PFGE data, and the serotype data are plotted on the right side of this bar. For additional information on the AFLP, MLST and PFGE data of the 45 *S. aureus* strains, see reference 23. With respect to the codes in the column describing the MLST data: ? = unknown ST; (..) = 6 of the 7 loci are similar to the particular ST listed. (For color see the online version of this thesis.)

notable that proportionally fewer (but not significant) type 5 strains were identified among the bacteremia isolates as compared to nasal carriage isolates included in this study (11/62 versus 23/78, $P = 0.12$). Furthermore, type 336PS strains were clearly overrepresented in the blood-culture collection compared to the nasal carriage isolates obtained from the healthy children and CAPD patients (18/62 versus 9/78, $P = 0.017$). An earlier study also concluded that type 5 strains were less prevalent among invasive-isolates compared to carriage isolates ($P = 0.0015$) (27). However, a recent study from Baltimore, Maryland showed that 42% of a total of 234 blood-culture isolates were capsular type 8, whereas 50% were type 5 (14). The prevalence of methicillin-resistance (MRSA) in that study was 40%. While capsular serotype is highly associated with genotypes, the local dominance of specific genotypes of methicillin-susceptible or resistant *S. aureus* in healthcare centres would be reflecting the serotype prevalence.

In conclusion, serotyping of diverse MSSA strains from The Netherlands shows that the majority of strains are capsular types 5 and 8 and the remaining were non-capsulated denoted as type 336PS. The prevalence of capsular types 5 and 8 varied between (clinical) collections. A clear association of capsular type with genotype was observed. Thus a vaccine containing *S. aureus* CP5-, CP8- and 336PS- conjugates would target nearly 100% of all Dutch *S. aureus* isolates.

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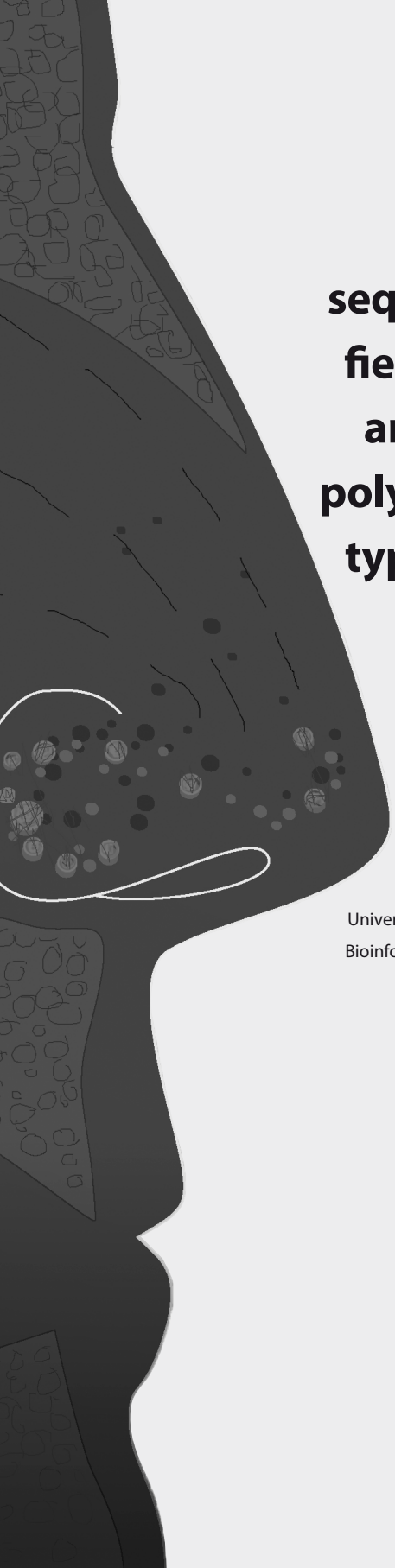
Part III

Technical aspects of defining genomic variability in *Staphylococcus aureus*



Chapter 13

Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*

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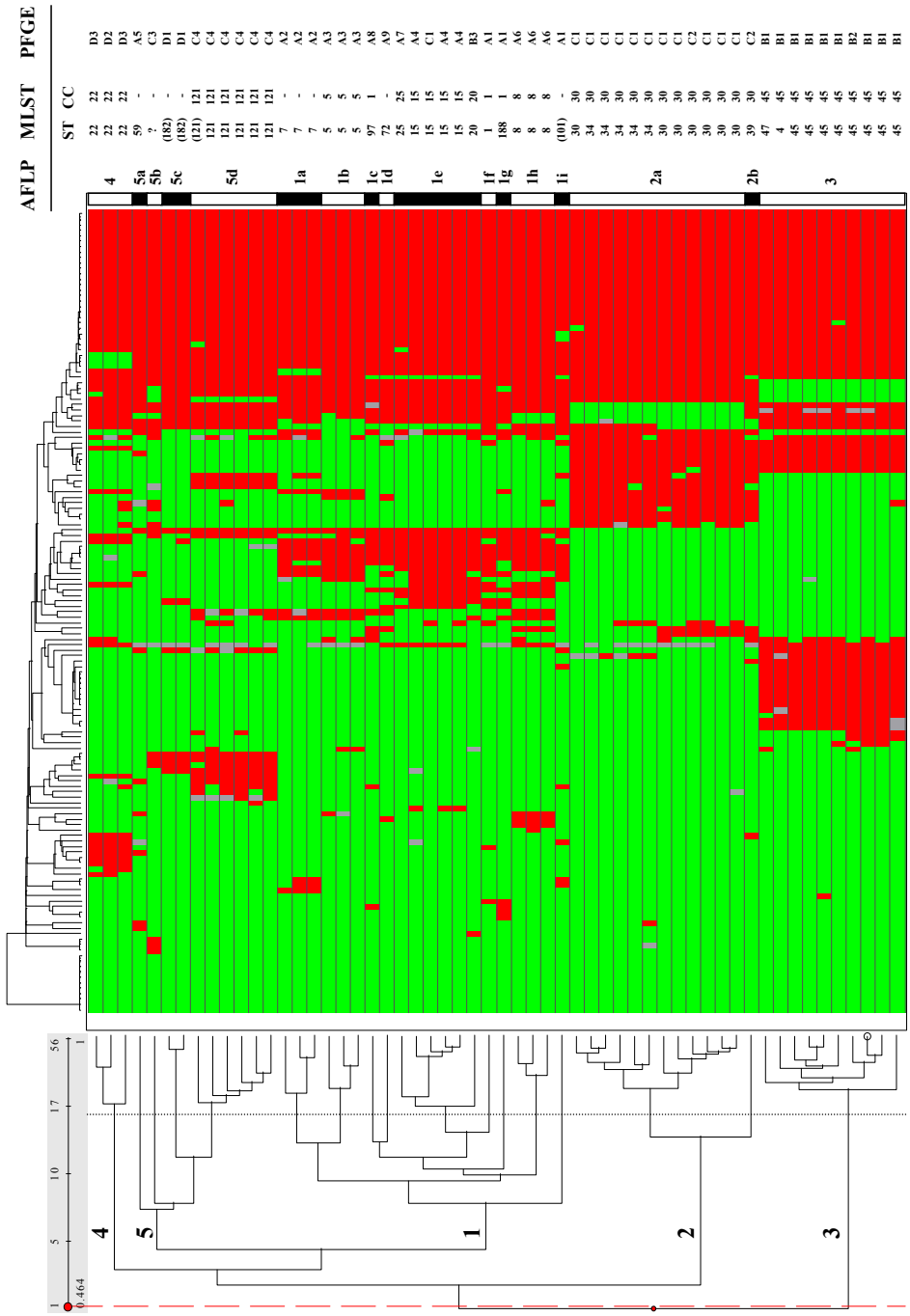
ABSTRACT

We compared multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for typing of *Staphylococcus aureus* and show that the methods yield similar results, although with differences in resolving power and reproducibility. Epidemiological conditions should determine which is the optimal typing method to be used.

Many different pheno- and genotyping methods have been used to distinguish *Staphylococcus aureus* strains. Such methods include antibiotic susceptibility testing, bacteriophage typing, multilocus enzyme electrophoresis (MLEE), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and multilocus sequence typing (MLST) (1-4). All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility. Furthermore, next to the choice of the typing method, knowledge of the overall genetic structure of the natural bacterial population is important for accurate interpretation of the typing results (5). Various typing studies performed on large numbers of *S. aureus* strains have revealed that *S. aureus* is essentially clonal, in contrast to other pathogenic species such as for instance *Streptococcus pneumoniae* (2-6).

It depends on the epidemiological or population genetics' question asked, which method is the most appropriate to use for *S. aureus* characterization. For monitoring short-term, local outbreaks molecular typing methods based on hyper-variable loci are most appropriate (e.g. sequence-based *Spa* or coagulase gene sequencing) (7, 8). However, epidemiologically related strains might still appear to be different using these high-powered procedures, because of frequent sequence changes that do occur in real-time. Typing methods based on sequencing of more stable housekeeping genes (e.g. MLST) have been proposed for long-term global studies and for the assessment of evolutionary relationships among *S. aureus* strains (9). However, the slow accumulation of genetic variation within these housekeeping loci might lead to a lack of discriminatory power between epidemiologically unrelated strains (9). Consequently, a typing approach that targets a combination of highly variable as well as more conserved DNA regions should be used "to provide high-resolution local epidemiological data within the context of a comprehensive population framework" (9).

The aim of the current study was to compare three well-known and frequently used staphylococcal typing methods, MLST, AFLP, and PFGE. All three methods have been used to map outbreaks and to study the natural population structure of methicillin-susceptible and -resistant *S. aureus* strains (MSSA/MRSA). MLST characterizes bacterial isolates on the basis of sequence polymorphism within internal fragments of seven housekeeping genes, representing the stable "core" of the staphylococcal genome. Each gene fragment is translated into a distinct allele, and each isolate is classified as a sequence type (ST) by the combination of alleles of the seven housekeeping loci (3). Conversely, amplified fragment length polymorphism (AFLP®) (10), a "whole genome" typing method, also documents the contribution of "accessory genetic elements" next to genome-core polymorphisms. AFLP scans for polymorphism in actual restriction sites and the nucleotides bordering these sites. As such it documents nucleotide sequence variation, insertions and deletions across entire genomes (10). PFGE, yet another whole genome typing method, detects genetic variation between strains using rare-cutting restriction endonucleases followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used technique for outbreak investigations. However, the stability of PFGE may


AFLP
MLST
PFGE
ST CC
4
5a
5b
5c
5d
1a
1b
1c
1d
1e
1f
1g
1h
1i
2a
2b
3
0.464
4
5
1
2
3

be insufficient for reliable application in long term epidemiological studies (7). Despite the efforts to standardize protocols and interpretation criteria of PFGE data, comparison of interlaboratory results usually remains difficult.

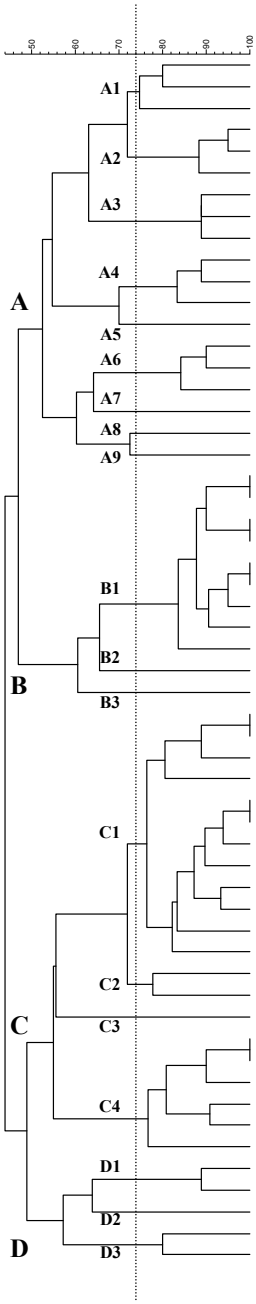
In the current study 45 *S. aureus* carriage- and 11 invasive (bacteremia [n = 9] and soft-tissue infections [n = 2]) isolates, obtained from children below 18 years of age, were included. This collection harbours a representative cross-section of the natural population structure of *S. aureus*. MLST was performed for these 56 *S. aureus* strains using DNA arrays (11). AFLP and PFGE were performed as described (2, 4).

Cluster analysis of the AFLP data was performed using Spotfire DecisionSite 7.2 software (4). Relatedness among the PFGE profiles was evaluated with Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium). A band-based dendrogram was produced by using the Dice coefficient and an unweighted pair group method using arithmetic averages (UPGMA). Band tolerance was set at 1.0%

The results generated by the three typing methods are summarized in Figures 1 and 2. MLST analysis revealed 23 different sequence types. Forty-seven (83.9%) of the 56 strains could be subdivided in 10 clonal complexes defined by MLST. Clonal complexes (CC) 30 and 45 account for almost half (41.1%) of all strains included in this analysis, which is in agreement with the European natural population structure of *S. aureus* in the community (4). AFLP distinguished all strains, showing its high discriminatory power. The AFLP analysis identified 5 *S. aureus* lineages, which is in agreement with earlier data (4). The major AFLP clusters (especially the heterogeneous AFLP clusters 1 and 5) could be subdivided in 17 subclusters as defined by the dotted bar in Figure 1 (an arbitrarily chosen cut-off). AFLP clusters 2, 3, and 4 correspond with MLST clonal complex 30, 45, and 22, respectively. Apparently, these clusters are genetically very homogeneous, but significant genetic heterogeneity could still be visualized by AFLP (Figure 1). PFGE analysis identified 4 major lineages, which were essentially similar to the major lineages as defined by AFLP. These 4 PFGE lineages could be subdivided in 19 subclusters (using the arbitrarily chosen cut-off of 74% as shown in Figure 2), which also strongly match with MLST clonal complexes and the different subclusters defined by AFLP. Obviously, there are discrepancies between the results of the three typing methods, albeit in small numbers. In comparison with MLST, 1 of the 4 ST15 strains is

Figure 1. Two-dimensional hierarchical clustering of the 56 *S. aureus* strains.

The green/red figure represents 8,232 binary outcomes, generated by ht-AFLP and based on 147 marker fragments. Green colored squares represent marker absence and red ones represent marker presence (grey squares represent ambiguous positions (i.e. weak bands) which are scored as marker absence in the mathematical analyses). The dendrogram on the *y*-axis represents the genetic clustering of the 56 strains. The dendrogram on the *x*-axis shows the clustering of the 147 AFLP-markers, many of which segregate in specific groups. Five AFLP major clusters and 17 AFLP subclusters could be identified, represented by the black-white bar on the right. MLST data and PFGE data are plotted on the right side of this bar. With respect to the codes in the column describing the MLST data: ? = unknown ST; (..) = 6 of the 7 loci are similar to the particular ST listed. (For color see the online version of this thesis.)



MLST		AFLP
ST	CC	
(101)	-	1i
1	1	1f
188	1	1g
7	-	1a
7	-	1a
7	-	1a
5	5	1b
5	5	1b
5	5	1b
15	15	1e
15	15	1e
15	15	1e
59	-	5a
8	8	1h
8	8	1h
8	8	1h
25	25	1e
97	1	1c
72	-	1d
4	45	3
45	45	3
45	45	3
45	45	3
47	45	3
45	45	3
45	45	3
45	45	3
45	45	3
45	45	3
20	20	1e
34	30	2a
34	30	2a
15	15	1e
34	30	2a
30	30	2a
30	30	2a
34	30	2a
34	30	2a
30	30	2a
30	30	2a
30	30	2a
30	30	2a
39	30	2b
30	30	2a
?	-	5b
121	121	5d
121	121	5d
(121)	121	5d
121	121	5d
121	121	5d
121	121	5d
(182)	-	5c
(182)	-	5c
22	22	4
22	22	4
22	22	4

Figure 2 Dendrogram of the PFGE data of the 56 *S. aureus* strains

The *S. aureus* strains are divided in 4 major clusters and 19 subclusters as defined by the vertical dotted line. The MLST data and the AFLP data are plotted next to the dendrogram.

clustered separately by PFGE, but not by AFLP (Figure 2), and 1 of the 3 CC1 strains is clustered separately by both AFLP and PFGE. It is obvious that AFLP and PFGE have a higher discriminatory power than MLST. Furthermore, the hierarchical order (going from top to bottom) of the different sub-clusters is different in the PFGE dendrogram, compared to the AFLP dendrogram, but this is due to different topologies of essentially similar dendrograms. Clearly, all methods used show a high degree of concordance in their results.

PFGE and AFLP are both image-based DNA fingerprinting techniques, which compare DNA fragment patterns generated by restriction or restriction in combination with amplification, respectively. PFGE, which does not include a selective amplification step, is less reproducible and, therefore, less useful for long-term epidemiological surveillance or for the study of the evolution and phylogenetic relationships among *S. aureus* strains. Although AFLP is more reproducible than PFGE and probably more suited for inter-laboratory data exchange, it is still less reproducible than DNA sequence-based MLST. However, it should be emphasized that MLST does not have the discriminatory power to study epidemic spread of certain *S. aureus* strains within a limited time frame.

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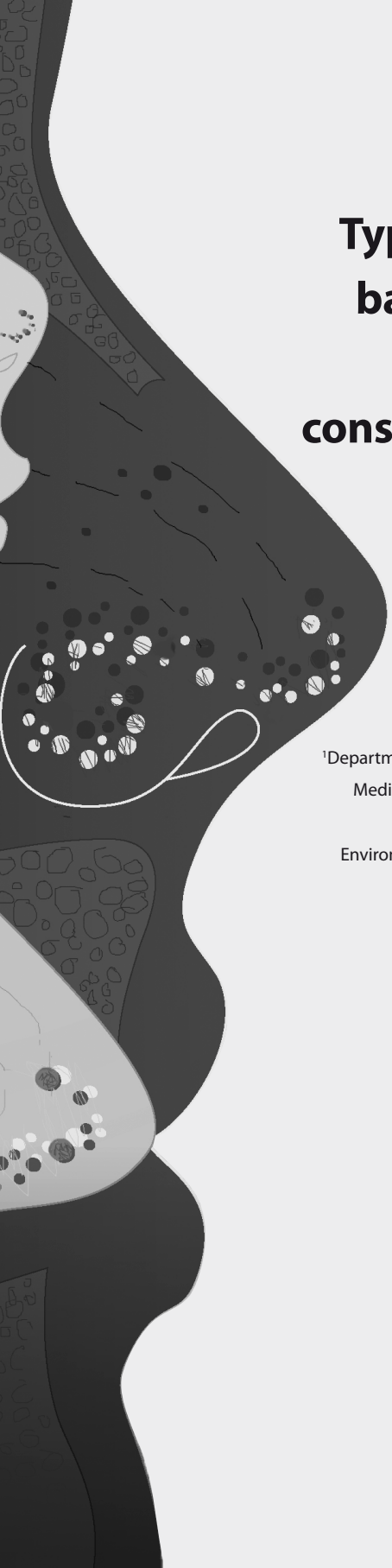

Chapter 14

Typing of *Staphylococcus aureus* based on differentially variable target sequences displays conservation of strain relatedness

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Submitted

ABSTRACT

It is generally assumed that, in bacteria, genetic variation in functionally constrained genomic regions accumulates at a lower rate than in regions of hypermutability such as DNA repeat loci. We compared whole genome polymorphism (using high-throughput amplified fragment length polymorphism) as well as short sequence repeat length variation (using multi-locus variable number of tandem repeat analysis) for 994 *Staphylococcus aureus* strains isolated from both healthy carriers and invasive infections. We here demonstrate that there is a difference in the rates of cross-genome mutation versus regional repeat variability in the clonal bacterial pathogen *S. aureus*. However, despite these differences in the observed degree of genetic variability, a conservation of type assignments as based upon these two inherently different typing techniques was observed.

INTRODUCTION

Staphylococcus aureus is a relatively clonal microorganism that lives in intimate contact with human beings (1-3). Although multiple body sites can be colonized in humans, the anterior nares are the most frequent carriage site for *S. aureus* and nasal carriage appears to play a key role in the epidemiology and pathogenesis of infection (3). It is assumed that *S. aureus'* conserved genome composition provides the organism with the potential to interact intimately with its human host, whereas the accessory genome, including "core-variable genes", harbors the specific colonization and virulence genes rendering it infectious (4, 5). Interestingly, a recent study using microarrays could not identify specific genes, alone or in combination, that were associated with invasive disease. It was suggested that *S. aureus* gene combinations necessary for invasive disease may also be necessary for nasal colonization and that the ability to cause invasive disease is mostly dependent on host factors (6).

A variety of molecular strategies has been used to distinguish and catalogue core genome versus core-variable and mobile elements in *S. aureus* (6). Clonality has been defined on the basis of, among others, multi-locus sequence typing (MLST) and high-throughput amplified fragment length polymorphism (ht-AFLP) studies whereas core-variable and mobile elements have been identified by for instance DNA array hybridization studies (2, 6, 7).

Data obtained by different genome screening methods, focusing on constraint versus hypermutabily regions, for a large collection of bacterial isolates have rarely been linked in order to compare the assignment of genetic types. We here provide an example of such an analysis. We have compared whole genome polymorphism (as defined by ht-AFLP) and short sequence repeat length variation (using multi-locus variable number of tandem repeat analysis [MLVA]) for approximately 1,000 *S. aureus* strains isolated from both healthy carriers and invasive infections. Ht-AFLP is a whole genome typing method that scans for polymorphism in restriction sites and the nucleotides bordering these sites. As such it documents nucleotide sequence variation, insertions and deletions across entire genomes. Recently, MLVA has been introduced as a typing method for a large number of bacterial pathogens (8-10). In MLVA, the variability in the numbers of short tandem repeated sequences is utilized to create DNA fingerprints for epidemiological studies (9). We here visualize the genetic variability of *S. aureus* based on ht-AFLP versus MLVA and discuss the differences and overlaps in the inter-strains relatedness.

MATERIALS AND METHODS

Bacterial isolates and DNA isolation

Carriage and clinical isolates of methicillin-susceptible *S. aureus* (n = 994) have been described before (7). Strains analyzed in the present study included carriage (n = 805), bac-

teremic (*n* = 132), skin-and soft-tissue infection (*n* = 17), and impetigo-derived isolates (*n* = 40) from children and elderly individuals. These strains were isolated at different time points (between 1997 and 2002) from persons living in the greater Rotterdam area in The Netherlands. DNA was isolated from all isolates using culture on blood agar and automated DNA extraction using the Roche MagnaPure and the Bacterial DNA III isolation kit (Roche, Almere, The Netherlands).

Amplified fragment length polymorphism (AFLP) and multiple-locus variable number of tandem repeat analysis (MLVA)

All strains were genetically typed using a high-throughput AFLP (ht-AFLP) approach as described previously (7). Optimal enzyme and primer combinations were selected using the predictive software package Recomb (Keygene NV, Wageningen, The Netherlands). Bacterial DNA was digested with the enzymes *Mbo*I and *Csp*6I and the linker oligonucleotide pairs for *Mbo*I (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGCAGTCTAC-3') and for *Csp*6I (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3') were ligated. Subsequently, a non-selective pre-amplification was performed using the *Mbo*I primer (5'-GTAGACTGCGTACCGATC-3') and *Csp*6I primer (5'-GACGATGAGTCCTGACTAC-3'). In the final amplification, a ^{33}P-labeled *Mbo*I primer containing one selective nucleotide (either +C or +G) and a *Csp*6I primer containing two selective nucleotides (+TA) were used. Amplified material was analyzed using polyacrylamide slabgels and autoradiography. Marker fragments (147 AFLP makers per isolate) were scored and a binary table, scoring marker fragment absence (0) or presence (1), was compiled (7, 11).

In addition, multiple-locus variable number of tandem repeat analysis (MLVA) was performed for all isolates according to (12) and modified by (13). In short, repetitive DNA from the V8 serine protease (*sspA*), protein A (*spa*), Ser-Asp-rich fibrinogen-binding protein (*sdrCDE*), clumping factor B (*clfB*), clumping factor A (*clfA*), fibronectin-binding protein (*fnBP*), collagen adhesion A (*cna*), methicillin-resistant surface protein (*pls*), and cell wall surface-anchored protein (*sas*) genes were amplified in a single multiplex PCR, also including the non-repeat containing methicillin resistance (*mecA*) gene. Amplified material obtained by PCR on purified genomic DNA (1 ng) was subjected to capillary electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA, USA) providing 90 second tracing files containing fluorescence values for amplicons ranging from 50 base pairs to 11 kbp. The BioAnalyzer reagent kit contains upper and lower molecular weight markers used to normalize all profiles.

MLST

Multi-locus sequence typing (MLST) was carried out for a selection of 53 (5.3%) of the 994 *S. aureus* strains using DNA arrays (14). These strains were equally distributed across a AFLP

dendrogram by selecting approximately 1 out of 20 strains going from top to bottom through the AFLP dendrogram (7).

Data analysis

For the comparative analyses of both the AFLP and the MLVA data sets, minimum spanning trees (MSTs) were calculated using the Bionumerics software package (Applied-Maths, Sint-Martens-Latem, Belgium). The binary AFLP data were clustered using a categorical coefficient. Complexes were created if the maximum neighbor distance was 2 changes (except for Figure 1a: 16 changes). The MLVA profiles obtained by capillary electrophoresis were normalized based on the lower and upper band present in all samples and band positions were automatically assigned by the Bionumerics software and manually adjusted where needed. Band based Dice clustering was performed using a 0.5% band position tolerance and 1% optimization. The distance matrix that was thus obtained was used to construct a MST using a 6% similarity bin size to designate MLVA profiles as being identical. For the construction of MLVA complexes a maximum neighbor distance of 1 change was used.

RESULTS AND DISCUSSION

Figure 1 shows the type assignments for all of the strains based on separate analyses of the ht-AFLP and MLVA datasets. The ht-AFLP data of the 994 *S. aureus* isolates are visualized using two minimum spanning trees (MSTs) with different cut-off values (Figure 1a and b with a maximum neighbor distance of 16 and 2, respectively). It should be emphasized that the (dotted) lines between the clusters do not indicate evolutionary relationship between these different clusters. As described earlier, the genuine population structure of *S. aureus* can be subdivided in three major AFLP clusters (denoted **1**, **2** and **3**) and two minor AFLP (**4** and **5**) clusters (Figure 1a) (7, 15). In addition, two very small outlier clusters are visible (brown complex [n = 3] and dark blue complex [n = 2]). The AFLP MST in Figure 1a essentially confirms the clonal structure of *S. aureus*, which is in contrast with those of other pathogenic species such as *Streptococcus pneumoniae* (11, 16).

Figure 1b shows that the 3 major and 2 minor AFLP clusters could be subdivided in several sub-clusters by applying more stringent segregation parameters during MST construction. The largest cluster (blue) is a genetically heterogeneous cluster in contrast with the two other major clusters (red [**2**] and green [**3**]). This is in agreement with the previously reported data on the natural population structure of *S. aureus* (7). Major AFLP clusters **2** and **3** are genetically homogeneous lineages and correspond with MLST clonal complexes 30 and 45, respectively. In the analysis where we applied a maximum neighbor distance of 2 changes for the formation of AFLP complexes or clusters (Figure 1b, 2a, and 3a), 22 different sub-clusters with more than 5 *S. aureus* isolates were identified, and 17 of these sub-clusters or complexes

Figure 1a

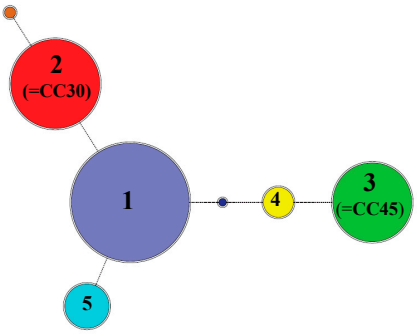

Figure 1b

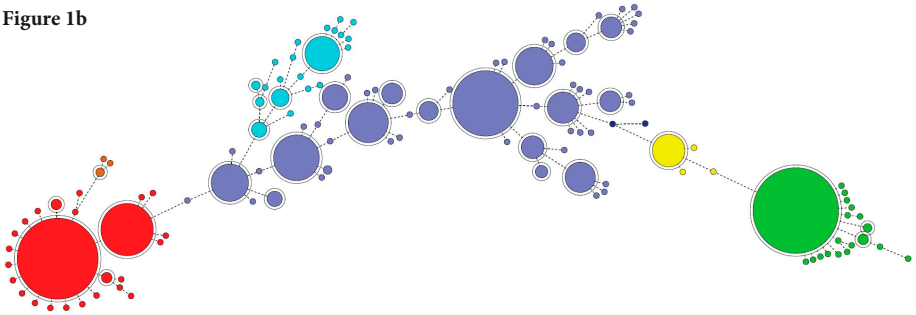

Figure 1c

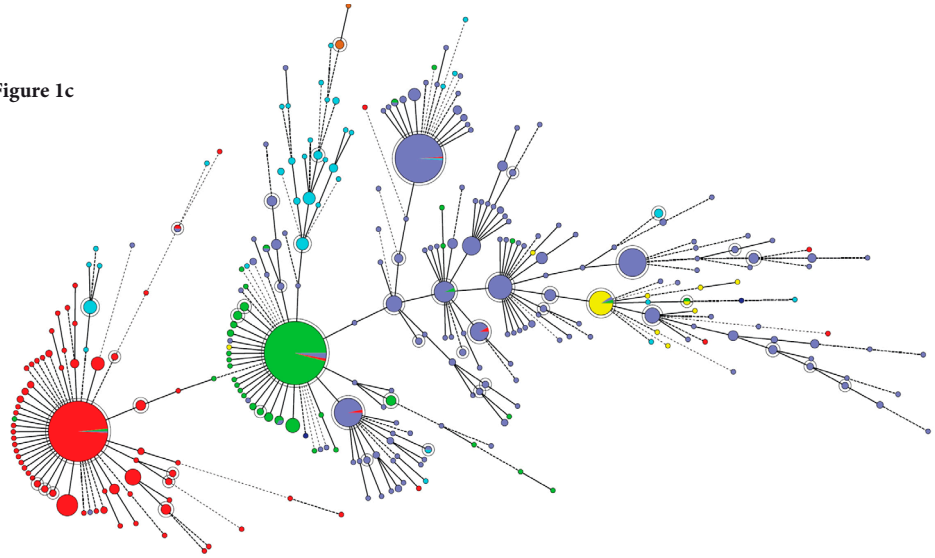

Figure 1a, b and c.

Minimum spanning trees based on the:

a) AFLP data (n = 994); complexes were created if the maximum neighbour distance was 16 changes;

b) AFLP data (n = 994); complexes were created if the maximum neighbour distance was 2 changes.

c) MLVA data (n = 994); complexes were created using a cut-off value of 94%.

carried more than 10 isolates. Several of these clusters coincided with MLST sequence types (ST) (Figure 2a). Earlier studies already showed that AFLP clustering matches strongly with the major clonal complexes as defined by MLST (7, 15).

When the same set of strains was analyzed by MLVA (Figure 1c) the layout of the MST clearly revealed enhanced variability: a large number of minor genetic variants are visualized when a cut off similarity value of 94% was used to identify types. However, the majority of the clonal complexes defined in Figure 1b again co-segregate on the basis of MLVA. For example, the red, green, and yellow complexes (cluster **2**, **3** and **4**, respectively) that are identified as closely related by AFLP also remain distinct groups after MLVA based MST

Figure 2a

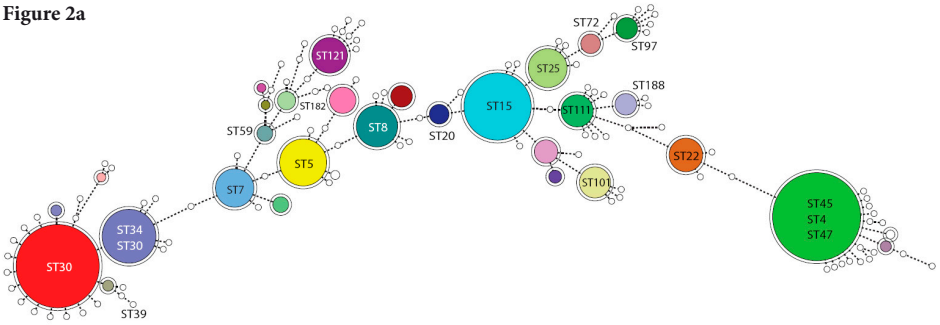

Figure 2b

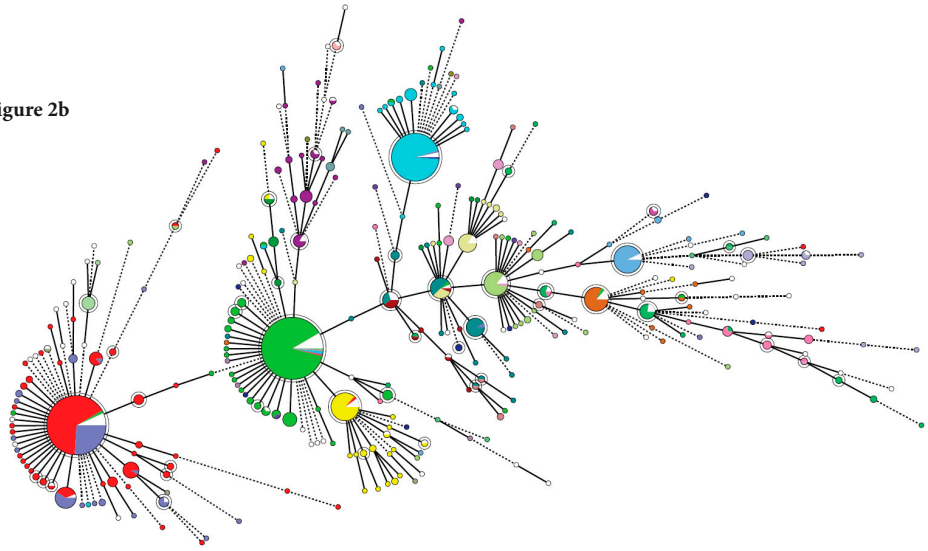

Figure 2a and b.

Minimum spanning trees based on the:

a) AFLP data (n = 994); MLST data of 53 *S. aureus* isolates are included in the figure. Each complex in this figure was assigned a different color.

b) MLVA data (n = 994); the colors, which are used in this figure, are based on the different *S. aureus* AFLP complexes which are shown in Figure 2a.

Figure 3a

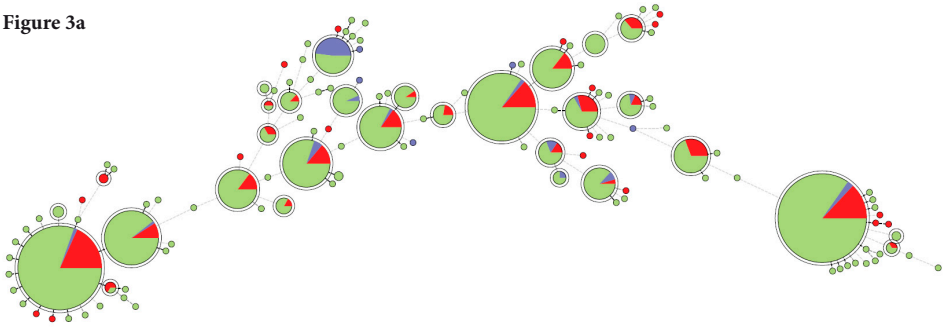

Figure 3b

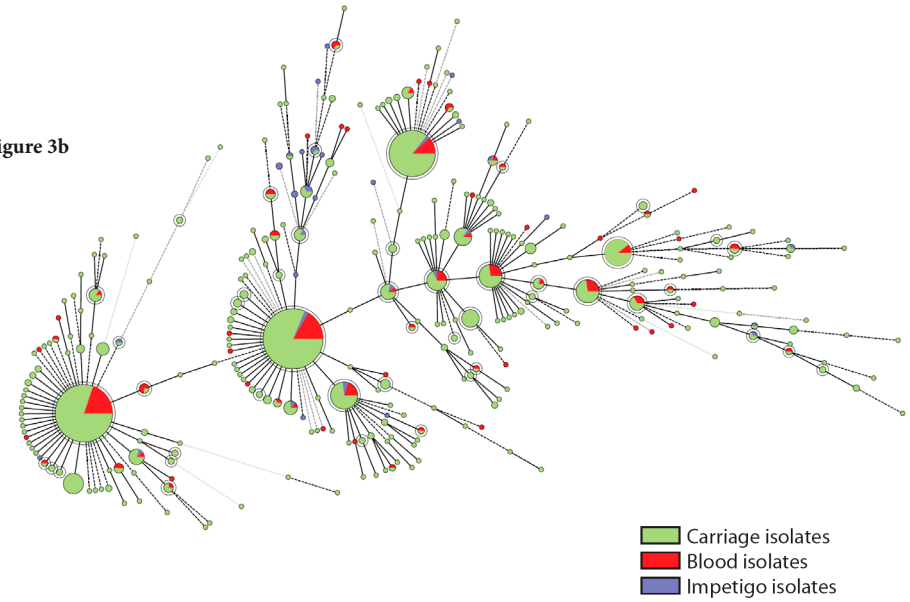

Figure 3a and b.
Minimum spanning trees based on the:
a) AFLP data (n = 994)
b) MLVA data (n = 994)
The colors, which are used in this figure, are based on the source (carriage or invasive) of the *S. aureus* isolates. Light green: carriage isolates (n = 805); red: "blood isolates" (n = 149) including a few skin- and soft-tissue infection isolates (n = 17); blue: impetigo-isolates (n = 40).

construction. In short, despite the apparently increased complexity of the MLVA MST, there still is significant similarity with the topology of the AFLP MST.

Finally, Figure 3 displays the association between the AFLP and MLVA MSTs and the clinical features of the strains. The conclusion is that both invasive and colonizing isolates are present in all *S. aureus* clusters, whether these are defined by AFLP (Figure 3a) or MLVA (Figure 3b). In case of the impetigo isolates a certain degree of clonal expansion could be

hypothesized on the basis of the presence of several strains in a single AFLP cluster. Furthermore, some of the AFLP clusters (Figure 3a) show a slight overrepresentation of bacteremia isolates compared to the average number of such isolates in the other clusters, which corroborates earlier data (7). However, it should be noted that studies using MLST data could not identify hypervirulent clones of *S. aureus* (2, 17). In addition, a recent study using microarrays was unable to identify any association between lineage or gene and invasive isolates (6).

It is obvious that populations of *S. aureus* show genetic diversification. It is thought that the generation of such variation in hypermutable loci such as short sequence repeats proceeds with enhanced speed (18). We here show that there indeed is a difference in the rates or speed of whole genome versus repeat variation in *S. aureus* with repeat-derived type assignments showing enhanced diversification (see Figure 2a versus 2b). However, a clear topological overlap in the MSTs calculated on the basis of complex repeat patterns versus those calculated on the basis of whole genome polymorphism can still be observed. This strongly suggests that even hypervariation of repeat loci remains within the framework set out by overall genome variation. This is in agreement with earlier studies showing that typing techniques using highly variable genes (e.g. adhesion genes) are at least as informative for phylogenetic reconstruction as the more slowly evolving housekeeping genes (e.g. MLST) (19-22).

We here demonstrate that for a clonal bacterial pathogen such as *S. aureus*, analysis of DNA targets with a distinct degree of genetic variability results in overlapping type assignments. This strengthens such models of inter-strain relatedness and suggests that, despite the enhanced variability of repeats, clusters of strains remains traceable. In other words, measuring repeat polymorphisms in clonal microorganims provides a solid basis for genetic type assignment useful for application in epidemiological tracing.

ACKNOWLEDGEMENTS

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AFLP is a registered trademark of Keygene N.V. and the AFLP® technology is covered by patents (US006045994A, EP0534858B1) and patent applications owned by Keygene N.V. The REcomb software is covered by patent applications (WO 00/44937) of Keygene N.V.

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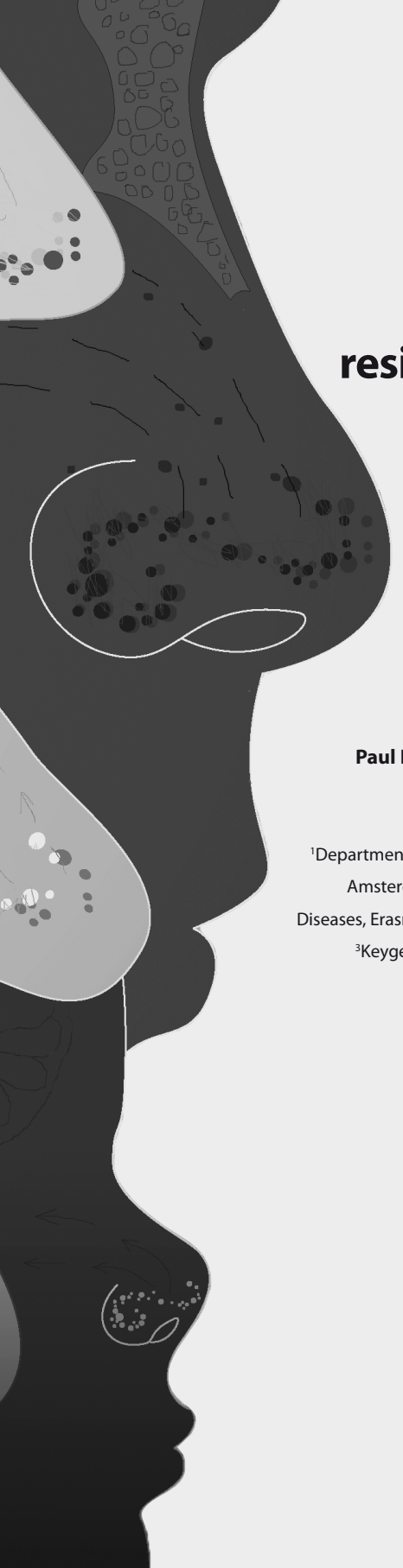

Chapter 15

High density whole genome fingerprinting of methicillin-resistant and -susceptible strains of *Staphylococcus aureus* in search of phenotype-specific molecular determinants

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ABSTRACT

AFLP is a selective restriction fragment amplification method generating DNA fingerprints for microbial isolates. We present high-throughput AFLP (ht-AFLP) to characterize molecular markers associated with bacterial phenotypes. Methicillin-resistant and -susceptible isolates of *Staphylococcus aureus* have been used for this model study in conjunction with the available *S. aureus* genome sequences. This facilitates the calculation of theoretical AFLP fingerprints, comparison of these fingerprints with genuine experimental fingerprints, and the subsequent identification of polymorphic AFLP markers without sequence analysis. Analysis of 46 MRSA and 46 MSSA strains by 39 different AFLP reactions generated more than 2,500 fragments per strain and an overall number of 6,180 scorable markers within all strains. We successfully identify MRSA specific markers and elaborate on the general applicability of the ht-AFLP approach. This method can be applied to any microbial species for which at least one full-genome sequence is available.

INTRODUCTION

One of the most prominent gram positive human pathogens is *Staphylococcus aureus*. This pathogen is involved in a multitude of human infections including bacteremia, septicaemia, skin infections, endocarditis and pneumonia (1, 2). Although most of the infections are rather mild the pathogen can cause serious infections in immuno-compromised patients and other groups of susceptible individuals including neonates or the elderly (3). In general, *S. aureus* infections can be treated with a variety of suitable antibiotics unless the staphylococcal cassette chomosome mec (SCC*mec*) is present resulting in methicillin resistance. This resistance is encoded by the *mecA* gene producing a methicillin–insensitive penicillin binding protein. Until now several types of SCC*mec* elements are characterized (SCC*mec* Type I, II, III, IV, and V) including variants within these types differing in size and composition (4-10). Most of the MRSA strains are multi-resistant due to the presence of the mec-regulon and are only susceptible to a very limited number of antibiotics. It appears that some MRSA strains behave more epidemic than others (EMRSA), hence their apparent hypervirulence (5, 11, 12).

During recent years many researchers have used different typing techniques to possibly identify MRSA strains from EMRSA and methicillin-susceptible *S. aureus* (MSSA) strains (13-18). Among these techniques are many different PCR-mediated approaches but for various microorganisms DNA macro-restriction analysis by pulsed field gel electrophoresis (PFGE) is the current gold standard typing procedure (12). PFGE is an excellent method for the identification of relatedness between isolates derived from a hospital setting. PFGE data frequently identify well-defined clusters of outbreak strains but the identification of supra-clusters (i.e. dissemination between institutions or nation wide) is relatively cumbersome due to the lack of technological reproducibility and standardization (19). Nevertheless there are a few successful applications available (http://www.cdc.gov/pulsenet/index.htm and HARMONY: Microbial Typing).

The identification of major staphylococcal lineages requires alternative approaches. A technique well suited for more long term global epidemiology is multi locus sequence typing (MLST) (15, 20). This recently described and powerful method can indeed identify the long term evolutionary pathways and provide ancestral information on strains from diverse origins (5, 6, 14).

Another typing technique, known as Amplified Fragment Length Polymorphism or AFLP (21, 22), combines the power of PCR with that of restriction fragment analysis. The method can be used for bacterial species identification and strain differentiation but, more importantly, it can generate DNA fingerprints of great complexity with relative ease (23-25). Due to the fact that both the restriction enzymes employed and the PCR primers applied can be varied, it is also suited for high throughput application (26).

For *S. aureus*, several complete genomes are available varying from hospital acquired MRSA (MRSA 252, N315, COL) including a vancomycin intermediate-resistant isolate (MU50) as well as community acquired MRSA (MW2, USA300), community acquired MSSA (MSSA 476) and the bovine strain RF122 (7, 11, 27, 28). This enables a combination of the AFLP typing procedure and theoretical genome comparison. On the basis of the known position of the restriction sites and their neighbouring nucleotide sequence elements in the genome sequences, immediate linkage between the presence or absence of a fragment in an experimental fingerprint with genomic alteration in a predefined region in the staphylococcal chromosome can be made. This will simplify the identification of the possible AFLP marker fragments since their precise genomic location can be deduced from the *in silico* AFLP fingerprint and fragment identification by sequencing may be no longer a prerequisite. In addition, new epidemic isolates can be rapidly characterized and compared to known whole genome sequences for relatedness.

In the present study we introduce ht-AFLP for the assessment of genome polymorphism among several MSSA and MRSA isolates, including epidemic MRSA (EMRSA) strains. We validate the *in silico* analysis as compared to radioactive AFLP fingerprinting and AFLP analysis using an automated DNA sequencer. Differences between strains are defined by accumulation of 39 different AFLP fingerprints and possible MSSA, MRSA- or EMRSA specific markers are identified by sequencing and comparison to the theoretical AFLP fingerprints.

METHODS

Bacterial strains

A total of 92 strains were collected including international hospital acquired MRSA strains (n = 30) (12), local hospital acquired MRSA strains (n = 14) and MSSA strains (n = 46), including ATCC 12228 (Table 1). As internal quality controls, the MRSA genome strains Mu50 and N315 were included (Table 1).

Culture conditions and DNA isolation

All strains were cultured on 5% sheep blood agar during 24hr at 37°C. DNA isolation was carried out with the MagNAPure DNA isolation robot using the bacterial genomic DNA isolation kit III (Roche Diagnostics, Almere, The Netherlands). Briefly, 200µl of a bacterial suspension was lysed in a guanidine lysis buffer. Subsequently, the DNA was bound to silica-coated magnetic particles followed by a washing procedure for removal of residual debris. Finally, the DNA was eluted from the particles at 65°C in a total volume of 100µl elution buffer. DNA concentration was estimated by agarose gel-electrophoresis.

Table 1. Collection of MRSA and MSSA strains analyzed with htAFLP

	MRSA	Strains				MSSA	Strains	
	No.	Isolate	Origin	Sensitive		No.	Origin	Resistant
1	306	Clin. isolate	Sputum	G/F/M/R/T/V	1	85	Sputum	None
2	307	Clin. isolate	Pus	F/M/V	2	87	ATCC 12228	None
3	344	Clin. isolate	Sputum	F/M/V	3	90	Pus	None
4	345	Clin. isolate	Sputum	G/F/M/R/T/V	4	203	capd	P
5	506	Clin. isolate	Nose	F/M/V	5	204	bc	F
6	507	Clin. isolate	Pus	F/M/V	6	209	bc	P
7	508	Clin. isolate	Nose	E/M/V	7	211	capd	P
8	619	Clin. isolate	Sputum	F/R/T/V	8	214	bc	P
9	634	Clin. isolate	Sputum	T/G/F/R/V	9	215	cv	P
10	636	Clin. isolate	Sputum	F/M/V	10	299	Sputum	P
11	639	Clin. isolate	Pus	F/M/V	11	304	Sputum	P
12	646	Clin. isolate	Throat	F/V	12	385	Sputum	P
13	660	Clin. isolate	Sputum	F/M/V	13	446	Sputum	P
14	797	Clin. isolate	Nose	F/R/V	14	449	Sputum	P
15	992	162	A, Fr	G/F/M/R/T/V	15	450	Nose	P
16	995	97121	B, Fr	E/G/F/M/R/T/V	16	451	Pus	P
17	996	BM 10828	C, Fr	M/V	17	453	Sinus	P
18	1000	54518	E7, Finl	F/M/V	18	454	ctip	P
19	1001	61974	E1, Finl	F/MR/T/V	19	455	Sputum	P
20	1002	62176	E10, Finl	F/M/V	20	456	Sputum	P
21	1006	75916	E5, Finl	F/R/T/V	21	457	Sputum	P/F
22	1010	98541	E24, Finl	F/M/R/V	22	620	Pus	P
23	1011	E MRSA 1	NCTC 11939, UK	C/F/M/R/V	23	621	Sputum	P
24	1012	E MRSA 3	M 307, UK	C/F/M/R/V	24	622	Pus	P
25	1013	E MRSA 15	90/10685, UK	G/F/M/R/T/V	25	623	Sputum	None
26	1015	E MRSA 16	96/14719, UK	G/F/M/R/T/V	26	624	Pus	None
27	1017	5	E1, Sp	M/V	27	625	Sputum	P
28	1018	3680	GR1, Gr	M/V	28	626	bc	P
29	1021	97 S96	E1, Bel	F/M/V	29	811	Sputum	None
30	1023	97 S99	E2, Bel	G/F/M/R/T/V	30	812	Sputum	P
31	1025	97 S101	E3, Bel	E/F/G/M/V	31	814	bc	P
32	1026	134/93	N German 1, Ger	M/V	32	815	bc	P
33	1027	100/93	Hannover III, Ger	F/M/R/V	33	816	Sputum	P
34	1029	825/96	Berlin IV, Ger	G/F/M/R/T/V	34	1045	ATCC 12228	None
35	1032	1155-2/98	S. German 1a, Ger	F/M/R/T/V	35	1102	Pus	P
36	1034	N8-890/99	VI, Swe	E/G/M/R/C/V	36	1103	Sputum	P
37	1035	N8-3756/90	I, Swe	F/M/R/V	37	1107	Pus	P
38	1036	CC4G 38266	II, Swe	G/F/M/R/T/V	38	1108	Pus	None
39	1038	AK 541	IV, Swe	F/M/R/V	39	1109	Pus	P
40	1039	ON 408/99	VII, Swe	F/M/V	40	1110	Pus	P
41	1040	AO 9973-97	III, Swe	G/F/M/R/T/V	41	1298	Pus	P
42	1041	NCTC 8325	Swe	E/G/F/M/R/C/T/V	42	1300	Pus	P
43	1313	48018	Sputum	G/F/M/R/T/V	43	1301	Pus	P
44	1315	6727,1	Sputum	F/M/R/T/V	44	1303	Pus	P
45	1580	MU50		F/M/V	45	1304	Pus	P
46	1581	N315		G/F/M/R/C/T/V	46	1305	Pus	None

Strain numbers 15-42 belong to the Harmony collection (12). MRSA strains MU50 and N315 are indicated. For the MRSA strains the sensitivity to antibiotics is shown whereas for the MSSA strains the antibiotic resistance is shown.

E: erythromycin; G: gentamicin; F: fusidic acid; M: mupirocin; R: rifampin; C: ciprofloxacin; T: tetracycline; V: vancomycin. P: penicillin (only MSSA).

In silico AFLP

For *in silico* ht-AFLP the total genome of the strains Mu50 and N315 were downloaded from the NCBI site (www.ncbi.nlm.nhi.gov). This genome was "theoretically digested" with the restriction enzymes *Csp*6I and *Mbo*I and the resulting AFLP fingerprint was presented by the software package RECOMB (Keygene, Wageningen, The Netherlands). The resulting fragments that are between 50 and 500 basepairs in length were highlighted and compared to the actual results of these strains when analyzed with an automated DNA sequencer (MegaBACE, Amersham, Roosendaal, The Netherlands) and by radioactive gel analysis.

Ht-AFLP procedure

The principle of ht-AFLP is identical to that of standard AFLP (22, 26). Two frequently cutting restriction enzymes were selected using the software package RECOMB again. The preference for certain restriction enzymes was determined by their capability to efficiently digest genomic DNA into fragments with an average length of approximately 250 basepairs. An overall number of 3,500 fragments was generated for each *S. aureus* genome. Final choices were the restriction enzymes *Mbo*I and *Csp*6I, both recognizing a four nucleotide site (5'-GATC-3' and 5'-GTAC-3', respectively). After digestion (1 hr, 37°C) and inactivation of the restriction enzymes the restriction fragments were ligated to two double stranded oligonucleotide adaptors consisting of the following two partially complementary primer pairs: 5'-CTCGTAGACTGCGTACC-3' with 3'-CATCTGACGCATGGCTAG-5' and 5'-GACGATGAGTCCTGAG-3' with 3'-TACTCAGGACTCAT-5'. A non-selective pre-amplification was carried out with the following primers MboI (5'-GTAGACTGCGTACCGATC-3') and *Csp*6I (5'-GACGATGAGTCCTGAGTAC-3'). Subsequently, a selective amplification was performed with a γ-^{33}P-endlabeled and/or fluorescently endlabelled (FAM) *Mbo*I primer containing one selective nucleotide *Mbo*I (5'-AGACTGCGTACC*GATC*+N-3') and a *Csp*6I primer containing two selective nucleotides *Csp*6I (5'-GATGAGTCCTGA*GTAC*+NN-3'). This resulted in 4^1 MboI primers and 4^2 Csp6I primers leading to 64 possible different primer combinations.

Ht-AFLP marker identification

AFLP fragments, not present in all strains were identified as putative polymorphic markers. Fragments possibly pinpointing consistent differences between MRSA and MSSA were visually selected. Fragments that were present in the highest number of strains of either MRSA or MSSA origin ($n = 17$ and $n = 16$, respectively) were isolated from the radioactive slab gels and re-amplified with the original primers. In addition, some polymorphic fragments present in both MRSA as well as MSSA strains were selected ($n = 30$). Subsequently, the DNA sequence of these fragments was determined by Dye-terminator sequence reactions followed by product separation using an automated sequencer according to the manufacturer's instructions (MegaBACE, Amersham) (26, 29). Compiled and quality controlled DNA sequences were

analyzed with BLAST searches to identify possible specific DNA fragments or genes (30). In addition, the whole genome sequences of the staphylococcus strains of N315, MU50, MW2, MRSA252, COL, MSSA 476 and RF122 were analyzed for the presence and location of these unique MRSA and MSSA ht-AFLP fragments on these genomes as well as some ht-AFLP fragments identified in both MRSA and MSSA strains.

Based on the marker score data table three similarity matrices, consisting of similarity indices for all combinations of lines, were obtained using NTSYSpc software. The similarity matrices were calculated using the Simple Matching (SM = m/n), Jaccard (J = a/n-d) and the Dice (Dice = $2a/2a+b+c$) coefficients respectively. a ,b ,c ,d ,m ,n , and u are defined as follows for a two-way frequency table for all pairs of two objects i and j.

	j		
		+	-
i	+	*a*	*b*
	-	*c*	*d*

$m = a + d$ (number of matched); $u = b + c$ (number of unmatched); $n = u + m$ (total sample size)

To visualize the relationship between the lines, a dendrogram was generated by the Sequential Agglomerative Hierarchical Nested (SAHN) clustering method following Unweighted Pair Group Method Arithmetic average (UPGMA) for all three matrices.

To evaluate to what extent the dendrograms are a good representation of the similarity matrices, the cophenetic value matrix was calculated for each dendrogram separately. This cophenetic correlation is an indication for the degree to which the dendrograms represent the similarity matrices. The highest cophenetic correlation value is 1. The highest value of the corresponding cophenetic correlation coefficient for the dendrograms is 0.99. This implies that the dendrograms very well represent the similarities between the lines.

RESULTS

Validation of ht-AFLP

The ht-AFLP technique was initially validated using the strains Mu50 and N315. The genome sequences of these strains were digested *in silico* and an AFLP pattern with fragments ranging from 50-500 basepairs was calculated with the software package Quantar (Keygene, Wageningen, The Netherlands). Experimental ht-AFLP was carried out in the laboratory with the same strains and enzymes. The radioactive/fluorescently labeled AFLP fragments were analyzed on a poly acrylamide slab gel and an automatic sequencer, respectively. The results show that all three patterns were nearly identical (Figure 1). Based on the results presented in Figure 1 a total of 67 fragments is visualized. Although differences are visible with the two real AFLP patterns approximately 65/67 (97%) identity is observed. The differ-

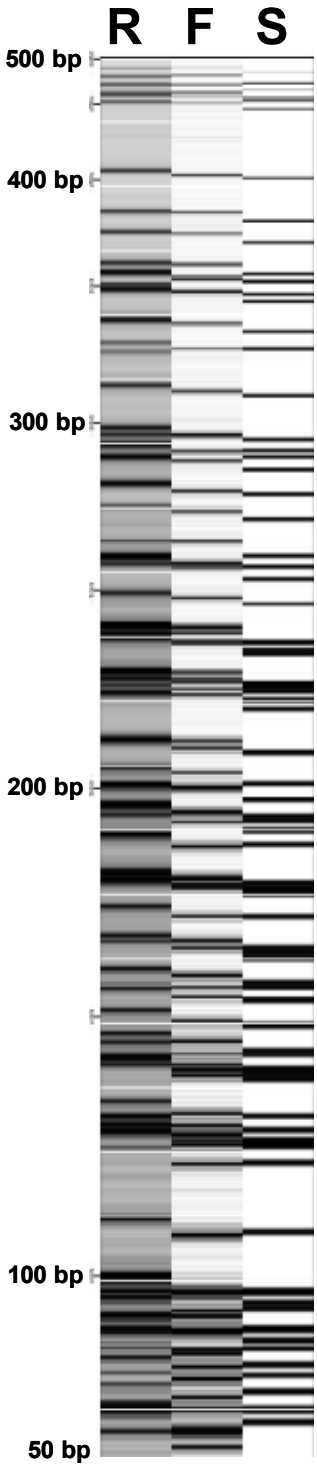


Figure 1. Comparison of *in silico* predicted ht-AFLP pattern (S) of strain N315 with radioactive ht-AFLP analysis on a PAA gel (R) and fluorescent ht-AFLP on an automated DNA sequencer (F). The molecular weights of the fragments are between 50 bp and 500 bp.

ences are most likely due to electrophoretic aberrations such as compression or otherwise due to problems with normalization between the platforms, rather than differences between the AFLP fragments. In addition, patterns for the reference strains Mu50 and N315 could be adequately reproduced from experiment to experiment (data not shown). In general, the *in silico* ht-AFLP results are highly similar to both the radioactive as well as the fluorescent ht-AFLP carried out on the same strains. Furthermore, the resolution of the slab gel systems seems to be significantly lower than that of the automated sequencer.

Ht-AFLP cluster analysis

A total of 46 MRSA (including Mu50 and N315) and 46 MSSA strains were subjected to ht-AFLP using the restriction enzyme combination *Mbo*I/*Csp*6I and 39 different primer combinations. This resulted in more than 2,500 markers per strain, amounting in 6,180 different scorable markers overall. Figure 2 shows a dendrogram, based on all of the different AFLP analyses for all strains. As presented in Figure 2, MRSA and MSSA strains do not segregate

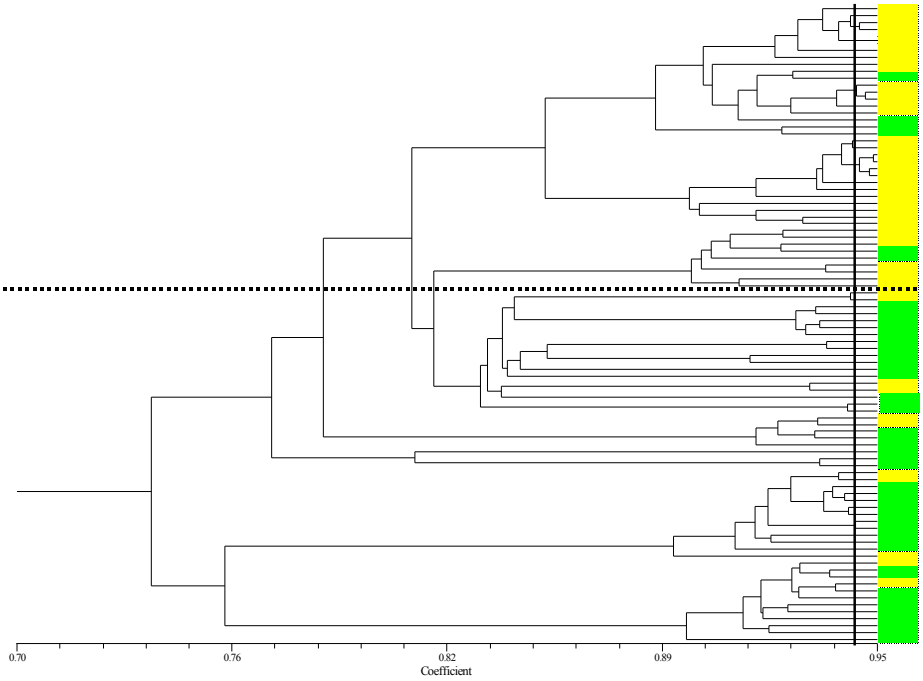
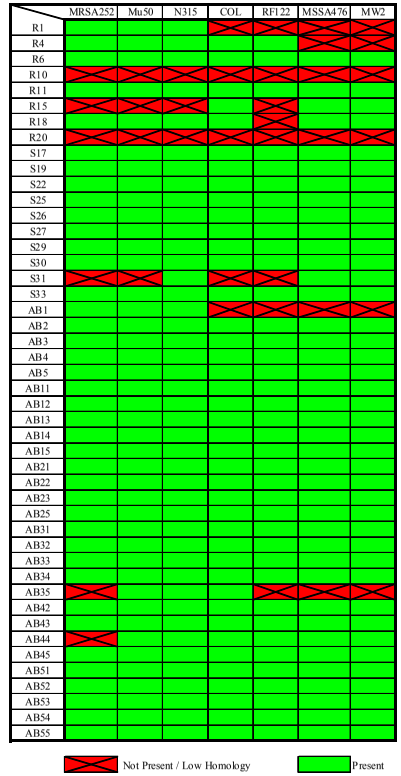


Figure 2. Tree plot of all MRSA and MSSA strains based on ht-AFLP.

The dendrogram is generated by the Sequential Agglomerative Hierarchical Nested (SAHN) clustering method following Unweighted Pair Group Method Arithmetic Average (UPGMA) for all three matrices. A maximum cophenetic correlation coefficient of 0.99 was calculated which indicates a high reliability of the similarity matrices. The SAHN correlation is indicated on the *x*-axis. The vertical line indicates the cut off value for identical strains whereas the horizontal dotted line divides the analysis in a more variant MSSA lower part and less variable MRSA upper part (dark-color = MSSA; light-color = MRSA).

Fragment	MRSA/MSSA	Homology	Genomes
R1	20/0	*Staphylococcus* /*Enterococcus* /*Bacillus* plasmid recombination enzyme	MRSA252, Mu50, N315, Type II SCCmec, Plasmids (*S.aureus* , *S.saprophyticus* , *E.faecalus* , *B.subtilis*)
R4	15/0	alkaline phosphatase synthesis sensor protein	MRSA252, Mu50, N315, COL, RF122.
R6	6/0	*S.aureus* ABC transporter ATP-binding protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
R10	8/0	*Staphylococcus epidermidis* RP62A, complete genome: conserved hypothetical protein	8 MRSA's and 0 MSSA's contained this band, but the only homology found was with a *S.epidermidis* sequenced genome.
R11	8/0	*Staphylococcus* putative membrane protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
R15	5/0	(IS200) Transposase *S.aureus* and others.	COL, MSSA476, MW2. Note: Also homology found with Mu50 and N315 but on the same level as several non *aureus Staphylococcal* , *Streptococcal* and *Bacillus* genomes, among others.
R18	10/0	*Staphylococcus* cassette chromosome recombinase A type 1 (ccrA1) gene	Most: COL, lesser: MSS476, MRSA252, MW2, Mu50, N315, *S.epidermidis* . Also: SCCmec type II, III and IV.
R20	11/0	*Staphylococcus aureus* DNA, type III *staphylococcal* cassette chromosome Mec	SCCmec type III, no homology with the 7 *S.aureus* whole genome sequences.
S17	0/8	*S.aureus* ferrichrome ABC transporter	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, and to a lesser extend: *S.epidermidis*
S19	0/7	*S.aureus* /*S.haemolyticus* nitrite reductase large subunit	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S21	0/7	*S.aureus* Putative N-acetylmannosamine-6-phosphate 2-epimerase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S22	0/7	*S.aureus* haloacid dehalogenase-like hydrolase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S25	0/5	*S.aureus* /*S.haemolyticus* primosomal protein n'	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S26	0/5	*S.aureus* 3-hydroxy-3-methylglutaryl CoA synthase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S27	0/5	*S.aureus* probable capsular biosynthesis protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S29	0/5	*S.aureus* hypothetical protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
S30	0/5	*Staplylococcus* /*Bacillus* 23s-rRNA	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *Bacillus species* .
S31	0/5	*S.aureus* exotoxin and cassette chromosome recombinase B	N315, MW2, MSSA476
S33	0/5	*S.aureus* putative cell division protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB1	20/1	Diverse Plasmids, Plasmid recombination enzyme	MRSA252, Mu50, N315, plasmid pUB110 (and others), SCCmec type II
AB2	45/45	*S.aureus* transcription regulator protein kdpE (sceMEC) / conserved hypothetical protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB3	45/45	*Staphylococcus* putative phosphate acetyltransferase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB4	10/12	*S.aureus* 3-phosphoshikimate 1-carboxyvinyltransferas	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB5	45/45	*Staphylococcus* FtsK/SpoIIIE family protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB11	43/42	*Staphylococcus* /*Streptococcus* formate--tetrahydrofolate ligase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.haemolyticus*
AB12	45/45	*Staphylococcus* 50S ribosomal protein L25	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *S.haemolyticus*
AB13	45/42	*S.aureus* putative transglycosylase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB14	41/38	*Staphylococcus* putative iron-sulphur protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *S.haemolyticus*
AB15	43/36	*S.aureus* histidinol dehydrogenase and ATP phosphoribosyltransferase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB21	44/44	*Staphylococcus* putative methicillin resistance expression factor (FemB)	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *S.haemolyticus*
AB22	42/33	*Staphylococcus* and *Bacillus* putative peptidase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *S.haemolyticus*
AB23	45/44	*S.aureus* putative acyl-CoA dehydrogenase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB25	40/17	*S.aureus* o-succinylbenzoic acid synthetase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB31	45/45	*S.aureus* putative membrane protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB32	45/43	*S.aureus* haloacid dehalogenase-like hydrolase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB33	45/45	*Staphylococcus* 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis*
AB34	42/35	*Staphylococcus* phosphoglycerate kinase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *S.haemolyticus*
AB35	34/6	*S.aureus* transposase for IS1181	Mu50, N315, COL
AB42	45/45	*S.aureus* conserved hypothetical protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB43	41/35	*S.aureus* similar to ABC transporter permease, similar to ABC transporter (ATP-binding pr	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB44	38/12	*S.aureus* leukotoxin F-subunit, gamma-hemolysin component B, leukotoxin, LukD and Pan	Mu50, N315, COL, RF122, MSSA476, MW2, to a lesser extend: MRSA 252
AB45	45/45	*S.aureus* pur operon repressor homologue and translation initiation inhibitor homologue	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus* , *S.haemolyticus*
AB51	45/45	*S.aureus* putative glutamate racemase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB52	45/45	*S.aureus* DNA polymerase III alpha chain PolC-type and nitric-oxide reductase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2
AB53	42/40	*Staphylococcus* glycerol kinase	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.saprophyticus*
AB54	45/43	*Staphylococcus* putative potassium-transporting ATPase B chain	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.haemolyticus*
AB55	45/44	*S.aureus* tetrapyrrole (corrin/porphyrin) methylase family protein	MRSA252, Mu50, N315, COL, RF122, MSSA476, MW2, *S.epidermidis* , *S.saprophyticus*



completely. A vertical line indicates a level of identical strains based on the ATCC 12228 strain (MSSA 87 and MSSA 1045). A few identical MRSA strains can be recognized but no identical MSSA strains were observed. In general, there is no clear indication of distinct MRSA or MSSA clusters. In addition, the correlation coëfficient on the X-axis is lower between the MSSA strains compared to the MRSA strains, which indicates that there is more variation between the different MSSA strains than between the MRSA strains. The two genomic strains N315 and MU50 are in the upper area (indicated by the dotted line) of the dendrogram where the genetic differences between strains seem somewhat lesser according to the dendrogram. Next to these two strains, 33 MRSA and only 6 MSSA are present whereas in the lower part of the dendrogram only 11 MRSA and 40 MSSA strains are present (Fisher's exact test [2-sided P-value] $P < 0.0001$). The dendrogram shows the total analysis of all the different AFLP markers but no single marker resulted a better separation between the MRSA and MSSA strains.

MRSA-MSSA marker identification

Out of all ht-AFLP fragments several potential markers were further analyzed. A total of 66 fragments were successfully picked out of the radioactive slab gel, re-amplified and subsequently sequenced. The sequence results of three fragments were not reliable. The resulting 63 sequences were BLASTed against the Genbank database and against the genome sequences of N315, MU50, MRSA252, MW2, COL, MSSA476 and RF122. The analyzed markers were divided into three categories: R) marker fragments only present in MRSA strains and NOT on MSSA strains ($n = 17$); S) marker fragments only present in MSSA strains but NOT in MRSA strains ($n = 16$); and AB) marker fragments present in both MRSA as well as MSSA strains ($n = 30$). Table 2 shows all details of the fragments. Blast search of the sequences identified 75% of these fragments with known *S. aureus* sequences (47 out of 63 fragments). Thirteen sequences other than *S. aureus* were found and three searches did not have any match. Most of the *S. aureus* fragments were also identified in all genome strains except for two fragments (R10 and R20) which were not present in any of the genome sequences and nine fragments were present in some genome sequences.

Homology to the known genome sequences was found for 6/17 R fragments; 11/16 S fragments and 28/30 AB fragments. None of the markers or marker combinations yielded a 100% separation between MRSA and MSSA. Within the 17 R fragments only two fragments (R6, R11) (Table 2) showed homology with all *S. aureus* genome sequences, including

Table 2. Identification of ht-AFLP markers after comparison with BLAST search.

A total of 47 fragments with staphylococcal sequence homology are presented. For each fragment the number of MRSA versus MSSA strains harboring this fragment is presented as well as the corresponding gene function if known. In addition, the presence of these sequences on the known genome sequences is given. The table is accompanied with a schematic overview showing the relation between the different fragments and their identification on the staphylococcal genome sequences.

MSSA476. The fragment R10 was only identified in eight other MRSA strains whereas R20 showed homology with a *S. aureus* SCC mec type II sequence not present in any of the whole genome sequences. The fragment R15 (transposase) was not found in MRSA252 and RF122 whereas R18 was not present in RF122. One fragment (R1) encodes a plasmid recombination enzyme (pre) and is only present in N315, Mu50 and MRSA252.

From the 16 S fragments 10 fragments showed homology with all *S. aureus* genomes. One fragment (S31) showed homology to an exotoxin not recognized in Mu50 and MRSA 476.

The 30 AB fragments were present in most *S. aureus* genomes although a few differences were notified. One fragment (AB1) showed to be identical to one of the 17 R marker fragments (R1: plasmid recombination enzyme), present in hospital acquired MRSA strains whereas one marker (AB35) encodes a IS1181 transposase only identified in N315 and Mu50 (8 and 10 locations resp., see Figure 3). Finally, fragment AB44 showed homology with the LukD and hlgB genes and also for MW2 with LukF. This latter is in concordance with the fact that the MW2 contains the Panton-Valentine leukocidin genes.

Location on the *S. aureus* genomes

The fragments (sequences) which showed homology with the known S. aureus genomes (8R, 11S, 28AB) were positioned on the seven *S. aureus* genome sequences in Figure 3. To accomplish this, the seven genomes were positioned on top of each other. As depicted in this figure 38/45 fragments are located on (nearly) identical positions in all genomes. As mentioned earlier the fragments R10 and R20 were not present on the currently known genome sequences. In addition, 5 fragments (AB43, AB54,AB2, R3, S29) are found at two positions in all genomes but both positions were identical among the genomes.

Remarkable is that fragment AB35 (transposase) was only found in multicopies in the genomes of N315, Mu50 and COL whereas fragment AB44 (leukotoxin gene) is found either in multicopy or as single genes in all genomes. Finally, fragment R15 seems to be located differently only for RF122.

DISCUSSION

Molecular epidemiology is based on the detection of differences between micro-organisms at the DNA level. Several molecular methods are available to accurately determine such DNA differences (31). The most accurate technique is unequivocally whole genome sequencing (27, 28). For obvious reasons this is not feasible for larger collections of strains. All other methods, however, analyze a small part of the total genome only and may therefore not detect minor but significant differences between strains. On the other hand, more and more complete genome sequences are determined and these can serve as a platform for large scale comparisons with additional strains. This, however, also requires a typing technique that

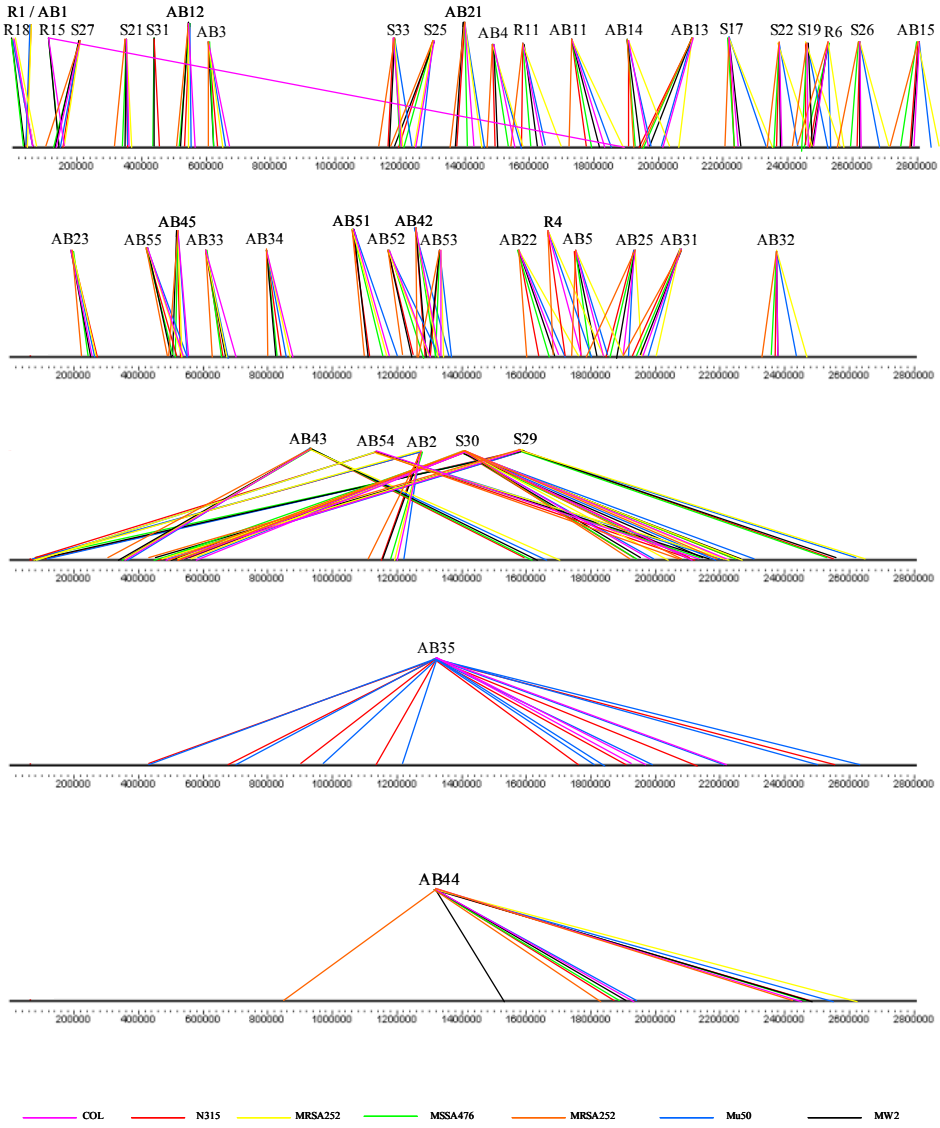


Figure 3. Positioning of AFLP markers on 5 superposed staphylococcal genome sequences.
Fragments corresponding with the different genome sequences are indicated by different colors. The upper two lines show 38 fragments present on one location on each genome sequence. The third line shows 5 fragments present on two different locations on the genomes. The fourth and fifth lines show each fragment present on some of the genomes on several different locations. R = MRSA marker; S = MSSA marker; AB = both MRSA and MSSA. (For color see the online version of this thesis.)

covers a substantial part of the total genome. We here show that ht-AFLP is an obvious candidate technology in this respect. It combines species specific stratification with sub-specific, and even strain-specific information delivery (32). The aim of the current analysis was to

cover a significant fraction of the staphylococcal genome by ht-AFLP thereby enabling the identification of phenotype-specific molecular markers on a collection of strains.

In silico ht-AFLP

This approach was validated by preliminary studies on the sequences available for the two genome strains Mu50 and N315. Before starting the experimental phase, an *in silico* ht-AFLP was carried out which generated an optimal restriction enzyme combination. Subsequently, AFLP was carried out in practice for the genome strains. The results show that the same AFLP patterns were obtained with *in silico*, slab gel and fluorescent AFLP analyses. This is in accordance with earlier, exploratory *in silico* AFLP analyses carried out for *E. coli* (33). The current ht-AFLP method, however, analyses more fragments per gel and provides significantly more genetic information. Ht-AFLP fragments identical to known fragments from complete genome sequences can be linked directly to a genomic localization whereas unknown markers are recognized and further analyzed by sequencing. Subsequently, after the sequence data of these unknown markers are known the position in the total genome can be determined.

Discrimination of MRSA and MSSA

Ht-AFLP analysis of a collection of 92 *S. aureus* strains resulted in a dendrogram (Figure 2) where no absolute clustering of either MRSA or MSSA strains with ht-AFLP is recognized. Earlier PGFE analysis of the Harmony collection showed a similar lack of clustering (12). However, the genetic diversity of the MSSA strains is larger than that among the MRSA strains. This is reflected by the non-symmetrical distribution of the MSSA and MRSA strains in the AFLP dendrogram ($P < 0.0001$). This finding is in agreement with the earlier reports which show that methicillin-resistance has developed in a limited number of phylogenetic clusters of *S. aureus*.

AFLP marker identification

Many adequate laboratory tests for the detection of methicillin-resistance in *S. aureus* have been described (34-38). However, additional targets for interrogating unknown features of methicillin resistance are still scientifically and diagnostically challenging. A total of 63 AFLP markers were analyzed in detail. Although specific markers were identified for both MRSA as well as MSSA strains, none of these markers seem to encode for a unique or unknown gene. The best candidate seems fragment R1 (= AB1) since this fragment is only identified in three HA-MRSA strains and seems related to the SCC*mec* type II. This fragment, however, is only present in 20 out of 46 MRSA strains. All other fragments are present in a smaller fraction of the MRSA strains or also in MSSA strains. Markers from the MRSA strains identified genes encoding enzymes, transporter proteins and hypothetical proteins. Remarkable was the identification of the R1 fragment as an MSSA AFLP marker encod-

ing the Type II resistance island. This is in agreement with suggestions by Holden et al. (7) showing that part of the SCC*mec* is still present in MSSA476. As suggested this SCC*mec* genecluster does not contain the *mec* gene. Fragments that did not show any reliable homology to staphylococcal sequences may code for yet unknown genes but neither of these fragments is unique to MRSA or MSSA strains and as such will not be useful as an strong marker for MRSA. In general, all other makers were identified on the genome sequences of MW2, Mu50, N315, COL, MSSA476, MRSA252 and RF122 possibly encoding "normal" genes. The fact that these genes are found can be explained by point mutations introducing or deleting an *Mbo*I or *Csp*6I restriction site. This has been described before for *M. tuberculosis* (26) and for *S. aureus* (16): several markers derived from normal housekeeping genes. The ht-AFLP method can be considered as universally applicable and suited to compare large numbers of strains. We believe that ht-AFLP has built a bridge between whole genome sequencing and other typing techniques and has proven to give reliable results.

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AFLP is a registered trademark of Keygene N.V. and the AFLP® technology is covered by patents (US006045994A, EP0534858B1) and patent applications owned by Keygene N.V. The REcomb software is covered by patent applications (WO 00/44937) of Keygene N.V.

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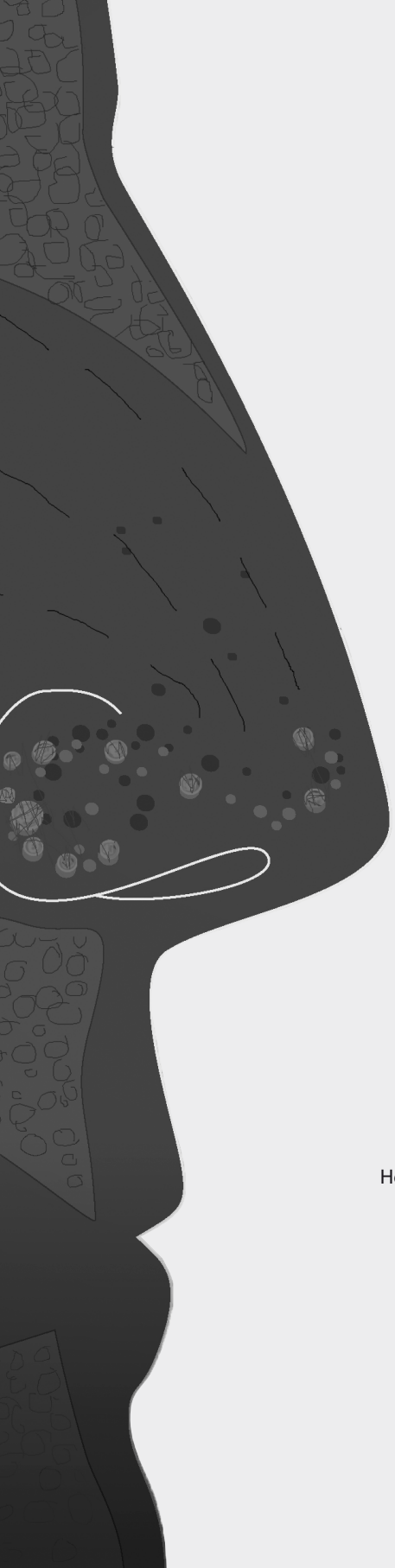
Discussion



Chapter 16

General discussion

Damian C. Melles

In part adapted from:

Heiman F.L. Wertheim, **Damian C. Melles**, Margreet C. Vos, Willem van Leeuwen, Alex van Belkum, Henri A. Verbrugh and Jan L. Nouwen.

The role of nasal carriage in *Staphylococcus aureus* infections.

Lancet Infectious Diseases 2005; 5 (12): 751-762

&

Alex van Belkum and **Damian C. Melles**.

Not all *Staphylococcus aureus* strains are equally pathogenic.

Discovery Medicine 2005; 5 (26), 148-152

GENERAL

The bacterial species *Staphylococcus aureus* is a leading and persistent pathogen throughout the world. It plays a central role in hospital-acquired infections and the frequent and increasing resistance of this pathogen to various antibiotics complicates treatment (1). Recently, the emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) causing serious infections in otherwise healthy individuals poses an additional public health threat (2-4). Effective measures to prevent *S. aureus* infections are therefore urgently needed.

The anterior nares (of the nose) are the primary ecological reservoir of *S. aureus* in humans (5), and it has been determined that most infections result from endogenous nasal carriage (6-10). Longitudinal studies show that about 20% of individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers, and about 50% noncarriers (11-14). As there is considerable evidence that carriage is an important risk factor for invasive infection with *S. aureus*, it is surprising that so little is known about the host factors as well as bacterial factors that promote colonization (15). Recent studies substantiate that the mechanisms leading to *S. aureus* nasal carriage are multifactorial and that an optimal fit between host and bacteria seems to be essential. The specificity of the host-pathogen association has been indicated by artificial inoculation studies where volunteers received mixtures of *S. aureus* strains in their nose. In the majority of the persistent carriers the strain that was previously encountered was re-selected from the inoculum mixture (16). Apparently, carriers become "accustomed" to their resident strain.

Age, sex, fasting glucose levels, diabetes mellitus and active smoking were recently demonstrated to be independent determinants of *S. aureus* nasal carriage (17, 18). In addition, the first human DNA polymorphisms have been identified, which are associated with *S. aureus* nasal carriage (19). It was concluded that polymorphisms in the glucocorticoid receptor gene, influencing glucocorticoid sensitivity, are significantly associated with persistent nasal carriage of *S. aureus*.

An increased understanding of how *S. aureus* colonizes the nares could indicate or improve methods for controlling nasal and skin carriage and subsequent infection (15). From the bacterial perspective, many features have been implicated in the host pathogen interaction. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), e.g. clumping factor B and *S. aureus* surface protein SasG, were demonstrated to be adhesins involved in nasal colonization (20, 21). In addition, a recent study of mutant strains defective in wall teichoic acid (WTA) in a rat model of nasal colonization also implicated WTA in colonization (22). However, all of the current studies have been performed *in vitro*, *ex vivo* or in artificial animal models, and so far, there is no direct evidence for the involvement of a single common bacterial or human genetic or phenotypic characteristic leading to successful colonization. An important question, still unanswered in staphylococcal biology, is whether the molecular determinants for infection and colonization are shared or separate. Simultane-

ously, there is controversy over whether all strains of *S. aureus* have equal disease-invoking potential or whether invasive disease is associated with particularly virulent genotypes. Obviously, to assess differences in the virulence potential of various strains of *S. aureus*, insight into the natural (non-clinical) population structure is essential.

In summary, elucidation of the mechanisms behind *S. aureus* nasal carriage and subsequent infection will facilitate new preventive strategies. In the current thesis we for the first time describe the detailed population structure of naturally occurring methicillin-susceptible *S. aureus* (MSSA) isolated from the nose of healthy persons living in the community. We provide evidence that essentially any *S. aureus* genotype carried by humans can be a life-threatening human pathogen but that certain clones may be more virulent than others. In addition, we studied several determinants of nasal carriage and infection with *S. aureus* in different human populations and animals. We, for instance, investigated several staphylococcal toxins and their relation with *S. aureus* colonization and infection. Finally, we used several *S. aureus* typing techniques and applied them to define the molecular evolution of this important human pathogen. In this chapter we will discuss the overall results described in this thesis and some directions for future research will be given.

POPULATION DYNAMICS OF *STAPHYLOCOCCUS AUREUS*

Genetic variation among bacteria can be extensive. Different species can be distinguished on the basis of sequence polymorphism in important genes such as those encoding the ribosomal RNA molecules, which are major structurally and functionally conserved components of the translation machinery. In addition, extensive genomic variation within a bacterial species can be documented as well, for instance by comparing isolates derived from different humans. Sometimes variation is so abundant that a microorganism is called panmictic: frequent, nearly free recombination takes place between members of the species and a multitude of so-called genotypes can be defined on the basis of DNA characteristics. Other bacterial species are less promiscuous and clear lineages can be identified (subgroups of strains that share certain genomic features). Homogeneous subgroups are defined as "clones". Genetic variation can be used to track microorganisms as they migrate through the human population. A DNA fingerprint can be used to identify inter strain relatedness and, hence, the putative origin of a certain bacterial strain. In addition, genetic typing can reveal how a population of bacterial isolates is structured (23). The important issue of relative or differential pathogenicity (disease-invoking potential) or colonization capacity of certain groups of microbial isolates or clones can be investigated by microbial genotyping.

Several typing techniques have been used to describe the natural population structure of *S. aureus*, including multilocus enzyme electrophoresis (MLEE) (24), pulsed-field gel electrophoresis (PFGE) (25), *spa* typing (26-30), and most often multilocus sequence typing

(MLST) (31, 32). These studies have revealed that *S. aureus* is highly clonal, in contrast to other pathogenic species such as *Streptococcus pneumoniae* (33). MLST characterizes bacterial isolates on the basis of the sequence of internal fragments of seven housekeeping genes that represent the stable "core" of the bacterial genome. For each gene fragment, the different sequences are translated into distinct alleles, and each isolate is defined by the combination of the seven alleles, named the allelic profile or sequence type (ST) (31). These MLST studies have placed most *S. aureus* isolates (colonizing as well as invasive isolates of MSSA and MRSA) in five major universally occurring clusters, clonal complex (CC) 8, CC30, CC5, CC22, and CC45 (32, 34, 35). CCs are defined as clusters of closely related STs, where single differences in the allelic profile are tolerated (36).

However, most studies describing the population structure of *S. aureus* were biased by inclusion of mostly clinical isolates and collections of nosocomial MRSA (25, 37). In **Chapter 3** we for the first time describe in detail the population structure of naturally occurring methicillin-susceptible *S. aureus* (MSSA) isolated from the nose of persons living in the community. We aimed to perform a high-density search for *S. aureus* genetic polymorphism associated with different levels of disease-invoking and colonizer potential. In order to do so we established a large collection of strains derived from healthy nasal carriers, both children (< 18 years) and elderly adults (> 55 years). In addition, strains derived from clinically relevant infections were included. This involved isolates from children suffering from impetigo, from patients with skin- and soft tissue infections and from patients with bacteremia. The number of *S. aureus* isolates exceeded 1,000 and all of these strains were investigated genetically with two so-called amplified fragment length polymorphism (AFLP®) reactions. AFLP is a DNA technology that generates isolate-specific DNA fingerprints. Using selective amplification of distinct subsets of genomic restriction fragments, multiple fingerprints can be generated by separate PCRs for each individual strain (high-throughput AFLP). In our case two reactions were performed for 1,056 strains, which generated 147 different marker fragments per strain and an overall number of 155,232 genetic markers. Cluster analysis of all of the fingerprints revealed the existence of three major (I, II, III) and two minor (IVa and IVb) genetic clusters of *S. aureus*. Major AFLP cluster I could be subdivided in 10 different sub-clusters, which matched with different MLST sequence types, indicating its heterogeneity. In contrast, major AFLP clusters II and III, identical to MLST CC30 and CC45, respectively, account for almost half (47%) of all carriage isolates, suggesting that these two clonal complexes have evolved to be very successful in colonizing human beings (Figure 1).

We identified the same major clusters as the MLST studies (Oxford database, UK; http://www.mlst.net). Apparently, these clonal clusters have spread successfully worldwide. In addition, our study also indicates that genetic change in *S. aureus* is mainly due to point mutations rather than large scale deletions or insertions. Recombination seems to be of a lesser importance as well. Ultimate proof for this hypothesis should be provided by detailed physical mapping and large-scale sequencing studies, however. Apparently, *S. aureus* is relatively clonal,

with five successful groups that can be conveniently defined by AFLP. The question is whether either one of these groups is more virulent or has a higher colonizer capacity than the others.

Feil et al. (32) found no significant differences in the distribution of genotypes between strains isolated from carriers and those from patients with invasive disease. There was, therefore, no evidence for the existence of hyper-virulent *S. aureus* clones. By contrast, subclusters of strains with differential degrees of pathogenicity were observed in our study (**Chapter 3**), in which we identified subclusters with an overrepresentation of bacteremia isolates. Furthermore, expansion of multidrug-resistant clones or clones associated with skin disease (impetigo) was observed as well. Another study found that patients infected with *S. aureus* strains belonging to a CC (in general) suffered from a higher mortality than those infected with strains not belonging to a CC, which indicates the co-evolution of *S. aureus* virulence and spread in humans (9). This study also concluded that (major) CC45 was significantly under-represented among invasive strains, which corroborated our findings (see **Chapter 3**). Furthermore, Peacock and colleagues (38) provided evidence of considerable horizontal transfer of virulence-associated genes in a clonal background.

These findings suggest that clinical impact could possibly be predicted by overall genotype grouping, but the impact of individual genes should certainly not be underestimated. Genes giving rise to resistance to antimicrobial agents are important in this respect, since the possibility to overcome therapy gives a microorganism a selective advantage in the hospital setting where antibiotics are used in large amounts. Our study also revealed that methicillin-resistant strains of *S. aureus* (MRSA) occur in all of the major clusters, suggesting that acquisition of the *mecA* regulon encoding this resistance trait has occurred across distinct phylogenetic subpopulations of *S. aureus*, which has been described before (37, 39).

Strains from impetigo patients were enriched for the occurrence of the gene encoding exfoliative toxin B (40). This gene encodes a proteinase that essentially degrades the protein desmoglein, an anchor-like protein responsible for skin integrity. The involvement of such a gene in an exfoliating disease such as impetigo is comprehensible. Furthermore, strains enriched for the gene encoding the Panton Valentin leukocidin (PVL), an important staphylococcal toxin, were primarily involved in deep skin and soft tissue infections (see also **Chapter 9**). However, a recent study by Lindsay et al. (41) compared 61 community-acquired *S. aureus* isolates to 100 nasal carriage isolates and suggested that the *S. aureus* gene combinations necessary for invasive disease may also be necessary for nasal colonization and that community-acquired invasive disease is strongly dependent on host factors.

In summary, in **Chapter 3** we have largely solved the population structure of *S. aureus* of non-clinical origin. We provide evidence that essentially any *S. aureus* genotype carried by humans can transform into a life-threatening human pathogen but that certain clones are more virulent than others. It remains to be seen whether the possibility of identifying the more pathogenic clones of *S. aureus* in the laboratory can be translated into a reliable diagnostic tool with clinical relevance in the future.

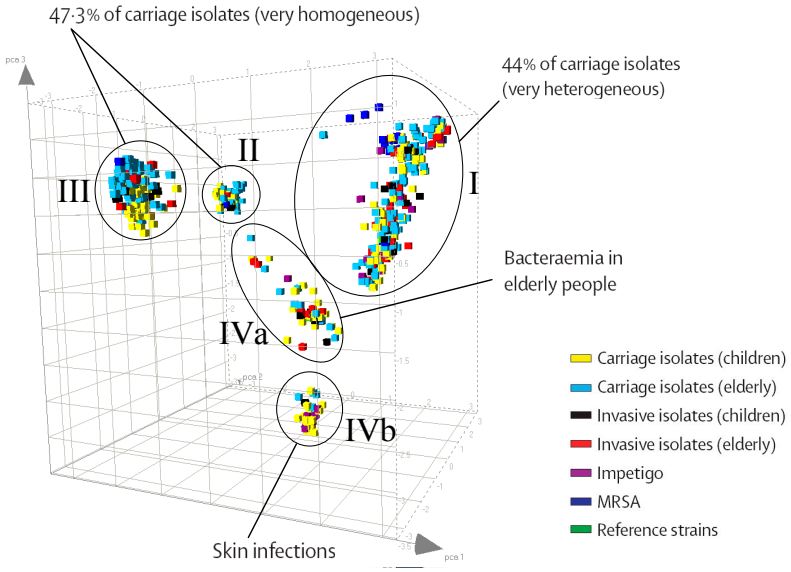


Figure 1. Principal component analysis of 1,056 *S. aureus* strains reveals genetic clusters of hypervirulent clones (15, 104).

The different cubes, plotted here in a three-dimensional space and colored according to their source, represent each *S. aureus* strain analyzed in the study described in **Chapter 3** (104). The 5 circles indicate the 3 major (I, II, and III) and 2 minor (IVa and IVb) different phylogenetic clusters identified by AFLP. Although strains from each of the genetic clusters are essentially able to cause invasive disease, some clusters contain proportionally more invasive isolates. (For color see the online version of this thesis.)

In **Chapter 4** we applied comparative genomics to assess genetic differences between *S. aureus* strains derived from infected animals versus those strains from colonized or infected humans. We aimed to identify genetic markers of *S. aureus* associated with host specificity. A total of 77 veterinary isolates were genetically characterized by high-throughput AFLP (ht-AFLP). Bacterial genotypes of these 77 strains were introduced in the AFLP database with 1,056 human-associated isolates (see **Chapter 3**). The data gathered for the veterinary strains integrated well into the AFLP database, which suggests that several of the traits leading to infections in animals or humans are shared. *S. aureus* strains isolated from animals in close contact with humans (e.g. pet animals) were predominantly classified in one of the five main clusters (genetically heterogeneous AFLP cluster I). In addition, mastitis-associated strains from diverse host species (sheep, goat, and cow) genetically clustered and formed a subgroup in AFLP cluster IVa, signifying a certain degree of tissue specificity for these strains.

Cross-infection with *S. aureus* between humans and domestic animals in the household setting has been described previously (42-45). Cats were involved in an outbreak of an epidemic methicillin-resistant *S. aureus* in a geriatric ward (42). Recently, the first documented case of direct transmission of MRSA between cows and humans have been reported (46). In **Chapter 4**, we support these findings on the basis of an integrated comparison between

human and animal isolates and show that many of these veterinary isolates from various animal hosts fall within the same genomic classes as the human isolates. Additionally, in this chapter we analyzed 10 different virulence genes and determined their distribution in these various *S. aureus* populations isolated from several animal species and humans. The gene encoding the toxic shock syndrome protein (*tst*) was more often encountered among veterinary strains and even more in the mastitis-related strains compared to human isolate results. The gene encoding the collagen binding protein (*cna*) was rarely detected among invasive human strains. We conclude that the presence of (combinations of) virulence factors plays an important role in host- or even tissue-specificity in *S. aureus* infections.

In **Chapter 5** we have addressed the question, whether the distribution of *S. aureus* genotypes from various geographical locations differs significantly. We show that the same major AFLP clusters (I, II, III, and IV) are identified in both Dutch (n = 829) and American (n = 391) community-based nasal isolates of *S. aureus*. Notably, AFLP cluster II and III, which represent MLST CC30 and CC45, respectively, account for 46.4% of all carriage MSSA isolates in the current study population (47.3% of the Dutch carriage isolates and 44.0% of the American MSSA carriage isolates), suggesting that these two clonal complexes have evolved to be extremely successful in colonizing humans (corroborating earlier findings; see **Chapter 3**). As these two clonal types (CC30 and 45) as found in The Netherlands have a seemingly different potential to cause invasive infection (9), it would be worthwhile to seek confirmation of this observation in the American setting. In **Chapter 5** we conclude that the population structures of nasal *S. aureus* isolates from humans are congruent on both sides of the Atlantic. Apparently, both MRSA and MSSA show similar epidemic behavior with some clones being notably more successful colonizers of the human vestibulum nasi.

As already mentioned in the Introduction (**Chapter 1**), a hot topic currently in the Netherlands, as well as other parts of Europe, is the "pig-farming" associated MRSA. The massive colonization of Dutch pigs with a single sequence type of MRSA (ST398) came as a sudden and unpleasant surprise, especially in a country with one of the lowest MRSA prevalences in the world (47-50). Molecular searches for their origin were immediately initiated (47, 51). Unfortunately, molecular typing of the ST398 MRSA was initially compromised, because standard MRSA typing by PFGE failed. The DNA of these strains proved resistant to *Sma*I digestion, leading to uninterpretable results in standard PFGE analysis (52). Whether or not the ST398 sequence type is prevalent among Dutch MSSA strains from an urban region was not clear. In **Chapter 6** we show that ST398, using ht-AFLP analysis, is very rare among Dutch MSSA strains that are colonizing healthy individuals (only two out of 829 strains isolated from nasal swabs of healthy individuals [0.2%]). However, a relatively high number of MSSA isolates genetically homologous to the ST398 MRSA was derived from bacteremic patients (3 out of 146 [2.1%; P = 0.026]). These 3 bacteremia isolates were epidemiologi-

cally non-related, isolated from different patients in different medical departments over an extended period of time. In **Chapter 6** we have established the position of the ST398 MRSA in the overall phylogenetic tree for *S. aureus*. ST398 MRSA is clonal and segregates from the general *S. aureus* population. Our findings pose a warning to public health surveillance: if the ST398 MSSA virulence towards humans would be maintained within the ST398 MRSA lineage from pigs, care should be taken not to introduce this strain into humans. We consider it to be likely that ST398 MRSA from pigs is capable of causing serious infection in humans even though its primary host seems to be porcine.

DETERMINANTS OF NASAL CARRIAGE AND INFECTION WITH *STAPHYLOCOCCUS AUREUS*

Next to elucidating the natural population structure of *S. aureus*, we also focused on several determinants of nasal carriage (e.g. "bacterial interference") or invasive infection with *S. aureus* in different population groups, including a cohort of HIV patients. In addition, we studied several staphylococcal proteins and toxins and their association with colonization or infection with this pathogen. Finally, we studied the potential relevance of a putative trivalent *S. aureus* polysaccharide-conjugate vaccine, which might have an important role in controlling this organism in the future (15).

In **Chapter 7** we studied the putative bacterial interference between *S. aureus* and *Streptococcus pneumoniae* in the nasopharynx of children. Bacterial interference has been postulated to be a major determinant of the *S. aureus* carrier state, or rather, noncarrier state. When an ecological niche is already occupied by certain bacteria, other bacteria do not seem to have the means to replace this resident bacterial population (53). The resident flora must be reduced or eliminated before other bacteria can successfully "interfere" with the resident bacterial population (54). Bacterial interference by active colonization using a nonpathogenic *S. aureus* strain (502A) was successful in nurseries during outbreaks of *S. aureus* infections in the 1960s and for treatment of patients with recurrent furunculosis (54, 55). The early practice of artificial inoculation with *S. aureus* 502A was abandoned after alleged complications (56) and the advent of newer antistaphylococcal antibiotics in the early 1970s.

Two recent large-scale population studies revealed an inverse relationship between nasopharyngeal *S. aureus* colonization and the presence of vaccine-type strains of *S. pneumoniae*, suggesting a natural competition between these bacterial species in the nasopharyngeal niche (17, 57). Another study confirmed the inverse relationship between the nasopharyngeal carriage of *S. pneumoniae* and *S. aureus* in HIV-negative children (58). However, they did not find a negative association between the two pathogens in HIV-positive children. Interestingly, these data are in agreement with the results that were recently obtained in a

cohort of HIV-infected adults (59). Bogaert et al. hypothesized that CD4 cells might play an important role in the pathway of interaction between *S. pneumoniae* and *S. aureus* (59).

What could be the clinical impact of the above mentioned bacterial interference? A trial with the 7-valent pneumococcal-conjugate vaccine (PCV7) in children with recurrent acute otitis media (AOM) showed a shift in pneumococcal colonization towards non-vaccine serotypes and an increase in *S. aureus*-related AOM after vaccination (60). A similar trend was recently observed for bacteremia in young children (61). Hence, bacterial interference might have important clinical implications for the widespread use of PCV7 in young children.

In **Chapter 7** we aimed to determine whether the capacity of *S. aureus* to compete with *S. pneumoniae* is dependent on bacterial genotype, the main question being whether certain successful staphylococcal clones are better equipped to compete with pneumococci. In addition we studied demographic and bacteriological determinants of carriage of specific genotypes of *S. aureus* in a cohort of 400 children. We found that age, gender, zip code, active smoking and co-colonization with *N. meningitidis* or *S. pneumoniae*, both vaccine- and non-vaccine types, were not associated with colonization by specific *S. aureus* genotypes (defined by ht-AFLP). Based on the whole-genome typing data obtained, there was no obvious correlation between staphylococcal and pneumococcal genotypes during co-colonization. Further studies involving molecular typing or gene expression testing at a more detailed level should demonstrate whether differences in bacterial interference are due to more subtle genetic changes, rather than clone-specific factors. Recently, it was suggested that hydrogen peroxide production by *S. pneumoniae* is important in the bacterial interference process, a lead that warrants further investigation (62). Surprisingly, in our study passive smoking showed a significant association with carriage of a specific *S. aureus* cluster. The mechanism behind this finding is unclear and a false-positive (chance-) finding should not be ruled out.

Several subgroups of patients have been associated with higher *S. aureus* carriage rates compared to healthy individuals, including diabetic patients, patients on hemodialysis or continuous ambulatory peritoneal dialysis (CAPD), and intravenous drug abusers (13). Regular and long-term puncture of the skin by needles and/or intravascular catheters seems to be the common factor in these groups (13). Another important risk-group are patients infected with the human immunodeficiency virus (HIV), who have an apparently increased risk of nasal carriage (63-67) and subsequent *S. aureus* infections (7, 68, 69). In HIV-positive patients, increased rates of *S. aureus* bacteremia and deep soft tissue infections have been observed. Nguyen et al. (7) showed that nasal carriage, presence of a vascular catheter, low CD4 count, and neutropenia were significantly associated with *S. aureus* infection in this patient group. It has been suggested that recurrent *S. aureus* infection in patients with HIV infection has been attributed to phagocytic dysfunction or impaired intracellular killing by monocytes and granulocytes (70-72). The reason for the higher carriage rates in HIV-positive patients is unclear, but the obvious immunological defects could be an explanation.

It has been shown that persistent carriers stand a higher chance of acquiring staphylococcal infections compared to non-or-intermittent *S. aureus* carriers (73). Although many studies investigated *S. aureus* nasal carriage in HIV patients, none compared persistent carriage to non-persistent carriage nor were studies performed in the HAART era (since 1996). This led us to investigate specific determinants of **persistent** *S. aureus* nasal carriage in the largest study executed among HIV positive patients ever performed (n = 507) (**Chapter 8**). We used the "culture rule" (14) to differentiate between persistent and non-or-intermittent carriers. Furthermore, we compared the genetic structure of the *S. aureus* strains isolated from HIV patients with the previously determined population structure of *S. aureus* isolates obtained from healthy individuals (see **Chapter 3**).

In our HIV cohort, as described in **Chapter 8**, we identified 29.6% persistent carriers of *S. aureus*, which is significantly different from healthy individuals in the same geographic region (17.6%) (18). This confirms earlier studies, showing higher carriage rates (intermittent + persistent) in HIV-positive patients (63-67). Although 75% of the HIV patients were using HAART and the majority of the patients had an undetectable viral load, the HIV-infected individuals still show a significantly higher carriage rate compared to healthy individuals. A recently published longitudinal study of 282 community-based drug users showed that both incidence and persistence of *S. aureus* carriage were increased among HIV-seropositive individuals, which is in agreement with our findings (74).

In addition, our study revealed that male sex is independently associated with a higher risk of persistent *S. aureus* carriage. In contrast, active smoking and *Pneumocystis jiroveci*-pneumonia (PCP) prophylaxis were independently associated with a lower risk of persistent *S. aureus* nasal carriage in our HIV cohort. The finding that PCP prophylaxis is associated with a lower risk is not surprising. Trimethoprim-sulfamethoxazole, which was mainly used as prophylaxis, is an antibiotic that is usually effective against *S. aureus*. Earlier studies already showed that male sex (+) and active smoking (-) are independent determinants of *S. aureus* carriage, but the etiological basis of these observations are still unclear (18, 75). Furthermore, we showed that the use of antiretroviral therapy is negatively associated with persistent *S. aureus* carriage. This may indicate an indirect relation between persistent nasal carriage and the immune system or a direct effect of antiretroviral therapy. The CD4 cell level was not associated with persistent carriage in this study. However, it should be noted that the mean CD4 count was high and patients with low counts are more likely to receive PCP prophylaxis. Therefore, a true association between CD4 count and persistent carriage would be difficult to find. It was already mentioned earlier (see above), that CD4 cells might play an important role in the pathway of interaction between *S. pneumoniae* and *S. aureus* in the nasopharynx, though the exact mechanism is still unknown (59).

Finally, the population structure of *S. aureus* strains, isolated from HIV patients appears to be strongly overlapping with the natural population structure of *S. aureus* isolates from healthy individuals. This suggests that no specific *S. aureus* clones, which could be respon-

sible for the higher carriage rates, are circulating among HIV-patients in the Rotterdam area (The Netherlands).

It is well known that *S. aureus* strains can express a wide variety of potential virulence factors, including surface proteins that promote adherence to damaged tissue and that bind proteins in blood to help evade antibody-mediated immune responses (76). Depending on the strain, *S. aureus* is capable of secreting a number of membrane-damaging toxins and superantigen toxins that can cause tissue damage and the symptoms of septic shock, respectively (15). In addition, recent studies also reveal that *S. aureus* has multiple mechanisms for evading both innate immunity mediated by polymorphonuclear leukocytes (77, 78) and

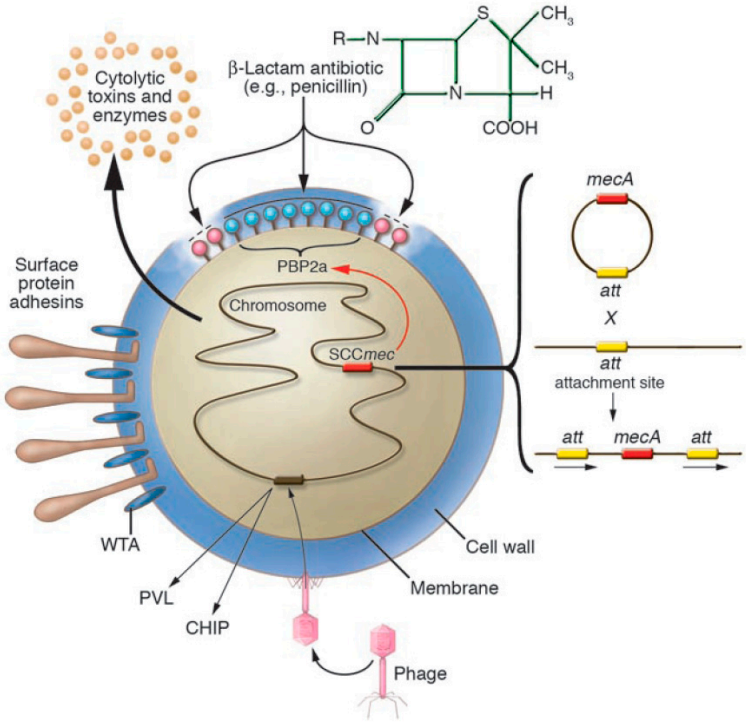


Figure 2. Schematic diagram illustrating how *S. aureus* acquires resistance to methicillin and its ability to express different virulence factors (adapted from reference 15).

The bacterium expresses surface protein adhesins and WTA and also secretes many toxins and enzymes by activation of chromosomal genes. Adhesins and WTA have been implicated in nasal and skin colonization. Resistance to methicillin is acquired by insertion of a horizontally transferred DNA element called SCC*mec*. Five different SCC*mec* elements can integrate at the same site in the chromosome by a Campbell-type mechanism involving site-specific recombination. The *mecA* gene encodes a novel β-lactam-insensitive penicillin binding protein, PBP2a, which continues to synthesize new cell wall peptidoglycan even when the normal penicillin binding proteins are inhibited. Some virulence factors such as PVL and the chemotaxis inhibitory protein, CHIP, are encoded by genes located on lysogenic bacteriophages.

induced immunity mediated by both B and T cells (15, 79). Some virulence factors are expressed by genes that are located on mobile genetic elements, so-called pathogenicity islands (e.g. toxic shock syndrome toxin-1 and some enterotoxins; (80) or lysogenic bacteriophages (e.g. PVL) (80, 81) and factors associated with suppressing innate immunity such as the chemotaxis inhibitory protein (CHIPS) and staphylokinase (77), which are integrated in the bacterial chromosome (Figure 2).

Although several bacterial surface components and extracellular proteins have been associated with the pathogenesis of *S. aureus*, the precise role of single virulence determinants in relation to colonization and/or infection is hard to establish. In the **Chapters 9, 10 and 11** we studied different staphylococcal virulence factors (toxins and adhesins) and their relation with *S. aureus* colonization and infection. As already mentioned, recent studies show that the exotoxin Panton-Valentine leukocidin (PVL) plays a major role in pathogenesis of *S. aureus*. In 1932, Panton and Valentine described this virulence factor belonging to the family of synergohymenotropic toxins (82). This toxin has been associated with skin infections (furuncles and abscesses), community-acquired/associated MRSA (CA-MRSA) infections, and necrotizing pneumonia. The PVL genes code for the production of cytotoxins that cause tissue necrosis and leukocyte destruction by forming pores in cellular membranes. Pore formation requires the presence of the 2 components of the toxin, LukS-PV and LukF-PV.

Despite the presumed importance of PVL as a virulence factor in *S. aureus*, few data are available on its prevalence among *S. aureus* isolates from the nares of healthy persons compared with strains isolated from infections. This lack of data led us to investigate the frequency of PVL gene-positive *S. aureus* strains obtained from the nares of healthy carriers in the Dutch community (**Chapter 9)**. For this study we used the well-characterized *S. aureus* strain collection including 829 carriage isolates and 204 clinical isolates, which has been described in **Chapter 3**. We found a very low PVL-positive prevalence among carriage isolates (0.6%) and blood-culture isolates (2.1%), which is in agreement with several other studies (83, 84). In contrast, we found a significantly higher percentage in *S. aureus* strains causing abscesses and arthritis (38.9%), which is in agreement with the proposed involvement of PVL in severe and invasive soft tissue infections (85-87). PVL was found in each major genomic amplified fragment length polymorphism (AFLP) cluster, indicating that PVL has been introduced in distinct phylogenetic subpopulations of *S. aureus*. Recently, there are some reports that PVL prevalence is rising among carriage strains isolated from healthy persons in the community. This might be associated with the emergence of CA-MRSA in several countries, but further epidemiologic studies for tracking this phenomenon are still warranted.

In addition, we determined the prevalence of PVL-positive and methicillin-resistant positive *S. aureus* strains within the population of HIV-patients in The Netherlands (see also **Chapter 8**). The use of antimicrobials as both prophylactic and therapeutic agents for opportunistic infections in HIV-infected individuals may increase the likelihood of nasal carriage of antimicrobial-resistant *S. aureus* (74, 88). No PVL positive strains were found

to be circulating and the prevalence of MRSA was low (1.2%) in this Dutch HIV cohort, as expected due to the low prevalence of MRSA in Netherlands (49).

Although PVL has been linked by epidemiological studies to (the current outbreak of) community-associated MRSA (CA-MRSA) disease and necrotizing pneumonia, the role that PVL plays in the pathogenesis of these infections has not been tested directly. Recently, some contradictory reports were published on this subject. Voyich et al. (89) used mouse infection models to compare the virulence of PVL-positive with that of PVL-negative CA-MRSA. Unexpectedly, strains lacking PVL were as virulent in mouse sepsis and abscess models as those containing the leukotoxin. Moreover, lysis of human neutrophils and pathogen survival after phagocytosis were similar between wild-type and mutant strains. They concluded that PVL is not the major virulence determinant of CA-MRSA strains, although the toxin may be a highly linked epidemiological marker for CA-MRSA strains. A lot of criticism was raised on this study after its publication. One of the comments, among others, was that mouse leukocytes are not lysed by Panton-Valentine leukocidin (PVL) in contrast to rabbit and human cells. However, this statement was again countered by the original authors. Interestingly, another recent study investigated the role of PVL in pulmonary disease (90). They tested the pathogenicity of clinical isolates, isogenic PVL-negative and PVL-positive *S. aureus* strains, as well as purified PVL, in a mouse acute pneumonia model. It was concluded that PVL producing *S. aureus* is sufficient to cause pneumonia and that the expression of this leukotoxin induces global changes in transcriptional levels of genes encoding secreted and cell wall-anchored staphylococcal proteins, including the lung inflammatory factor staphylococcal protein A (Spa). Another study showed that LukS-PV, 1 of the 2 components of PVL, was detected in lung sections of patients with necrotizing pneumonia together with DNA fragmentation, suggesting that PVL induces apoptosis in vivo and thereby is directly involved in the pathophysiology of necrotizing pneumonia (91). It is obvious that more insight is still required to be able to fully understand the role that PVL plays in the pathogenesis in *S. aureus* infections.

Another set of important virulence factors are the staphylococcal enterotoxins (SEs), which can be responsible for food-associated outbreaks of diarrhea among humans (92). Many of the enterotoxins display superantigen characteristics and are obvious targets for antistaphylococcal therapies. It has been shown that genes encoding several of the enterotoxins are physically clustered in the *S. aureus* genome (93). The locus encoding the enterotoxins SEG, SEI, SEM, SEN, and SEO is currently known as *egc* (enterotoxin gene cluster). Although this cluster is highly prevalent among *S. aureus* strains in general, antibodies are rarely raised against egc enterotoxins, which is a unique feature of this group of enterotoxins (94). It is intriguing that the prevalence of *egc* genes in isolates of *S. aureus* seems to be negatively correlated with the severity of infection (95). For SEA, the situation is precisely opposite: the toxin gene is significantly more often present among invasive isolates (38, 95). The conclu-

sion could be that one or more of the egc-encoded enterotoxins provide protection against severe sepsis. In **Chapter 10** we studied the prevalence of the *egc* locus in *S. aureus* nasal carriage isolates (*n* = 204) compared to bacteremia isolates (*n* = 187). Most of the carriage and clinical isolates included in this study derived from the study by Wertheim et al. (96), in which patients were sampled at the time of hospitalization and monitored for development of bacteremia. The other carriage and invasive isolates that were included derived from the study described in **Chapter 3**. In **Chapter 10** we concluded that *egc* is (a) slightly, but still significantly enriched among carriage strains, (b) not associated with mortality in patients suffering from staphylococcal bacteremia, and (c) coupled to certain clonal lineages. Although a clear biological rationale is currently lacking, our study suggests that the presence of *egc* is associated with non-invasiveness and a lower disease-invoking potential, as suggested earlier (95). In addition, we showed that clonality is an important feature of the enterotoxin gene content of strains. Most successful *S. aureus* lineages (MLST CC 5, 20, 22, 30, and 45) harbor the *egc* cluster, which is again in agreement with the suggestion that *egc* enhances the carriage potential of an *S. aureus* strain. Another, recently published study also concluded that superantigen-encoding mobile genetic elements in *S. aureus* are linked more or less tightly to the genetic background (97). They showed that each *S. aureus* clonal complex was characterized by a typical repertoire of superantigen and exfoliative toxin genes, although within each clonal complex the virulence gene profiles varied remarkably. They did not find an association of superantigen genes with bloodstream invasion.

In **Chapter 11** we studied another set of important proteins of *S. aureus* and their relation with invasiveness in humans. It is obvious that infections caused by *S. aureus* involve bacterial adhesion to the host extra-cellular matrix. *S. aureus* adhesins are mostly cell wall-anchored proteins and are grouped into a single family named microbial surface components recognizing adhesive matrix molecules (MSCRAMM). In **Chapter 11** we have focused on the serine-aspartate repeat (Sdr) proteins, which are members of the MSCRAMM family that are encoded by the tandemly arrayed *sdrC*, *sdrD*, and *sdrE* genes. Next to PVL and *egc* (**Chapters 9 and 10**), we have studied the distribution of the serine-aspartate repeat protein-encoding (*sdr*) genes among nasal carriage isolates (*n* = 259) and strains isolated from osteomyelitis (*n* = 89) and bloodstream infections (*n* = 52). The *sdr* locus was encountered in all *S. aureus* strains, although in 29 strains it contained only the *sdrC* gene (*sdrD* negative, *sdrE* negative). The *sdrC*-positive, *sdrD*-negative, *sdrE*-negative gene profile was exclusive to methicillin-sensitive *S. aureus* (MSSA) strains and was not found in the strains collected from bone infections. The finding that this profile is limited to MSSA isolates, might be a reflection of the fact that MRSA primarily consists of a limited number of highly successful pandemic clones. We found a strong significant association between the presence of the *sdrD* gene and methicillin-resistant *S. aureus* strains. In addition, the *sdrD* gene was significantly associated with osteomyelitis but not with blood infections. Trad et al. also showed an asso-

ciation with *sdrD* and bone infections (98). There was no significant correlation of *sdrE* with blood infections and osteomyelitis. Studies in which strains with knockouts of *sdrD* and/or *sdrE* are used in experimental models should be performed to cast light on the role of these genes in osteomyelitis.

As mentioned before, *S. aureus* is a highly versatile and adaptable microorganism, which developed resistance mechanisms to almost all antibiotics that were introduced over the last decades. The emergence and rapid dissemination of community acquired methicillin resistant *S. aureus* (CA-MRSA) strains and the devastating outcomes of these infections underline the need for alternatives to antibiotic treatment. Immunotherapeutic strategies might play an important role in controlling *S. aureus* infections in the future. In **Chapter 12** we studied the potential relevance of a putative (trivalent) *S. aureus* polysaccharide-conjugate vaccine. International epidemiological studies have shown that clinical isolates of *S. aureus* are usually capsulated with either type 5 or 8 capsular polysaccharides (CP5 and CP8). The remaining isolates are non-capsulated and were found to be cross-reactive with polysaccharide 336 (336PS) antibodies and therefore denoted as type 336PS (99, 100). These unique (capsular) polysaccharides had brought about the opportunity to develop vaccines and immunotherapy against *S. aureus*. Several vaccines are at different development stages and the use of one, StaphVAX (Nabi Biopharmaceuticals), a bivalent CP5 and CP8 conjugate vaccine, was associated with a significant reduction of bacteremia in hemodialyis patients between 3 to 40 weeks post-vaccination in a phase 3 clinical trial (101). However, at 54 weeks no significant reduction was recorded and a second phase III trial failed to confirm efficacy, hence, optimization of vaccine type or usage is still warranted.

In **Chapter 12** we aimed to determine the surface and/or capsular polysaccharide serotype of 162 well-characterized MSSA strains from both carriage and clinically relevant infection. In addition, we determined if there was an association between capsular type and strain genotype. In this study we included isolates obtained from nasal swabs of healthy Dutch children (1-18 years; $n = 49$) (see also **Chapter 3**), nasal carriage as well as catheter exit site isolates from patients undergoing continuous ambulatory peritoneal dialysis (CAPD; $n = 51$) (73), and recent blood-culture isolates ($n = 62$).

Our study revealed that the overall serotype distribution was 28.4% serotype 5, 53.7% type 8, and 17.9% type 336PS. Serotype 8 was the most prevalent serotype in this study, which is corroborated by other studies (100, 102). We showed that type 5 strains are less (but not significant) and type 336PS strains are significantly more prevalent among bacteremia isolates as compared to the nasal carriage isolates obtained from the healthy children and CAPD patients. An earlier study also concluded that type 5 strains were less prevalent among invasive-isolates compared to carriage isolates (102). Serotyping was in agreement with genotyping by amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST). So, a clear association of capsular type with genotype was observed.

We concluded that serotyping of diverse MSSA strains from The Netherlands shows that the majority of strains are capsular types 5 and 8 and the remaining were non-capsulated, denoted as type 336PS. Thus a vaccine containing *S. aureus* CP5-, CP8- and 336- polysaccharide conjugates would target 100% of all Dutch *S. aureus* isolates.

A recently published study by Von Eiff et al. (103) also studied the potential applicability of a putative trivalent *S. aureus* polysaccharide conjugate vaccine in Germany. They tested 714 German MSSA and MRSA strains for their capsular and surface polysaccharide serotype. They also concluded that the addition of the newly described type 336PS to a capsular polysaccharide-protein conjugate vaccine could extend the coverage substantially and would include virtually all MSSA and MRSA strains currently circulating in Germany.

TECHNICAL ASPECTS OF DEFINING GENOMIC VARIABILITY IN *STAPHYLOCOCCUS AUREUS*

In the final part of this thesis we have focused on the technical aspects of molecular typing of *S. aureus*. Next to studying the (natural) population structure of *S. aureus*, molecular typing is also an important tool for understanding the changing epidemiology of MSSA and MRSA infections and for evaluating the efficacy of MRSA outbreak intervention and prevention strategies. Many different pheno- and genotyping methods have been used to distinguish *S. aureus* strains. Such methods include antibiotic susceptibility testing, bacteriophage typing, multilocus enzyme electrophoresis (MLEE), pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and multilocus sequence typing (MLST) (31, 104-106). All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility. Several factors determine which method is the most appropriate to use for *S. aureus* type characterization. For monitoring short-term, local outbreaks, molecular typing methods based on hyper-variable loci are most appropriate (e.g. sequence-based *spa* or coagulase gene analysis) (26, 107). However, epidemiologically related strains might still appear to be different using these high-powered procedures, because of frequent sequence changes that do occur in real-time. Typing methods based on sequencing of more stable housekeeping genes (e.g. MLST) have been proposed for long-term global studies and for the assessment of evolutionary relationships among *S. aureus* strains. However, the slow accumulation of genetic variation within these housekeeping loci might lead to a lack of discriminatory power between epidemiologically unrelated strains (108). Consequently, a typing approach that targets a combination of highly variable as well as more conserved DNA regions should be used "to provide high-resolution local epidemiological data within the context of a comprehensive population framework" (108). In **Chapter 13** we compared three well-known and frequently used staphylococcal typing methods, MLST, PFGE, and AFLP. For this study we used a strain collection (n = 56),

covering a representative cross-section of the natural population structure of *S. aureus* (see **Chapter 3**).

PFGE detects genetic variation between strains using rare-cutting restriction endonucleases, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used technique for outbreak investigations. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies (26). Despite the efforts to standardize protocols and interpretation criteria of PFGE data, comparison of interlaboratory results usually remains difficult. The typing methods MLST and AFLP have been discussed earlier in this chapter (see also **Chapter 3**). All three methods have been used to map outbreaks and to study the natural population structure of both MSSA- and MRSA-isolates. **Chapter 13** reveals that these typing methods show a high degree of concordance in their results, although with differences in resolving power and reproducibility. PFGE and AFLP are both image-based DNA fingerprinting techniques, which compare DNA fragment patterns generated by restriction or restriction in combination with amplification, respectively. PFGE, which does not include a selective amplification step, is less reproducible and, therefore, less useful for long-term epidemiological surveillance or for the study of the evolution and phylogenetic relationships among *S. aureus* strains. Although AFLP is more reproducible than PFGE and probably more suited for inter-laboratory data exchange, it is still less reproducible than DNA sequence-based MLST. However, it should be emphasized that MLST does not have the discriminatory power to study local epidemic spread of certain *S. aureus* strains within a limited time frame. We concluded that epidemiological conditions should determine which is the optimal method to be used for molecular typing of *S. aureus*.

It is well known that bacterial populations diversify through a variety of genetic mechanisms and it is generally assumed that genetic variation in functionally constrained genomic regions accumulates at a lower rate than in regions of hypermutability such as DNA repeat loci. Data obtained by different genome screening methods, focusing on constraint versus hypermutabily regions, for a large collection of bacterial isolates have rarely been linked in order to compare the assignment of genetic types. In **Chapter 14** we provide an example of such an analysis. We compared whole genome polymorphism (as defined by ht-AFLP) and short sequence repeat length variation (using multi-locus variable number of tandem repeat analysis [MLVA]) obtained for 994 *S. aureus* strains from both carriage and clinically relevant infection. MLVA has been introduced as a typing method for a large number of bacterial pathogens (109-111). In MLVA, the variability in the numbers of short tandem repeated sequences is utilized to create DNA fingerprints for epidemiological studies (110). For this study we used the same strains collection (and AFLP data) as described in **Chapter 3**. We show that there is indeed a difference in the rates of cross-genome mutation versus regional repeat variability in the clonal bacterial pathogen *S. aureus*. However, despite these differ-

ences in the observed degree of genetic variability, a conservation of type assignments as based upon these two inherently different typing techniques was observed. This strengthens the population structure model and suggests that, despite the enhanced variability of repeats, clusters of strains remains traceable. In other words, measuring repeat polymorphisms in clonal microorganims provides a solid basis for genetic type assignment useful for application in epidemiological tracing.

The most accurate molecular typing technique is unequivocally whole genome sequencing (112-114). For obvious reasons this is not feasible for larger collections of strains. All other methods, however, analyze only a small part of the total genome and may therefore not detect minor but biologically significant differences between strains. On the other hand, increasing numbers of complete genome sequences are determined and these can serve as a platform for large scale comparisons with additional strains. This, however, also requires a typing technique that covers a substantial part of the total genome. In **Chapter 15** we show that ht-AFLP is an obvious candidate technology in this respect. AFLP combines the power of PCR with that of restriction fragment analysis. The method can be used for bacterial species identification and strain differentiation (115-117) but, more importantly, it can generate DNA fingerprints of great complexity with relative ease. Due to the fact that both the restriction enzymes employed and the PCR primers applied can be varied, it is also suited for high-throughput application (118). For *S. aureus*, several complete genomes are available varying from hospital acquired MRSA (MRSA 252, N315, COL) including a vancomycin intermediate-resistant isolate (MU50) as well as community-acquired MRSA (MW2, USA300), community-acquired MSSA (MSSA 476) and the bovine strain RF122 (112, 113, 119, 120). This enables a combination of the AFLP typing procedure and theoretical genome comparison. On the basis of the known position of the restriction sites and their neighbouring nucleotide sequence elements in the genome sequences, immediate linkage between the presence or absence of a fragment in an experimental fingerprint with genomic alteration in a predefined region in the staphylococcal chromosome can be made. This will simplify the identification of the possible AFLP marker fragments since their precise genomic location can be deduced from the *in silico* AFLP fingerprint. In addition, new epidemic isolates (e.g. the "pig-farming" associated MRSA; see **Chapter 6**) can be rapidly characterized and compared to known whole genome sequences for genetic relatedness.

In **Chapter 15** we used ht-AFLP to characterize molecular markers associated with bacterial phenotypes. Methicillin-resistant and -susceptible isolates of *S. aureus* have been used for this model study in conjunction with the above mentioned, available *S. aureus* genome sequences. The aim of the analysis was to cover a significant fraction of the staphylococcal genome by ht-AFLP thereby enabling the identification of phenotype-specific molecular markers on a collection of strains.

During recent years many researchers have used different typing techniques to possibly identify ordinary MRSA strains from epidemic (E)MRSA and MSSA strains (32, 36, 104, 121-124). We analyzed MRSA (n = 46) and MSSA (n = 46) strains by 39 different AFLP reactions, which generated more than 2,500 fragments per strain and an overall number of 6,180 scorable markers among all strains. We successfully identify MRSA specific markers and elaborate on the general applicability of the ht-AFLP approach. This method can be applied to any microbial species for which at least one full-genome sequence is available. We believe that ht-AFLP has built a bridge between whole genome sequencing and other typing techniques and has proven to give reliable results.

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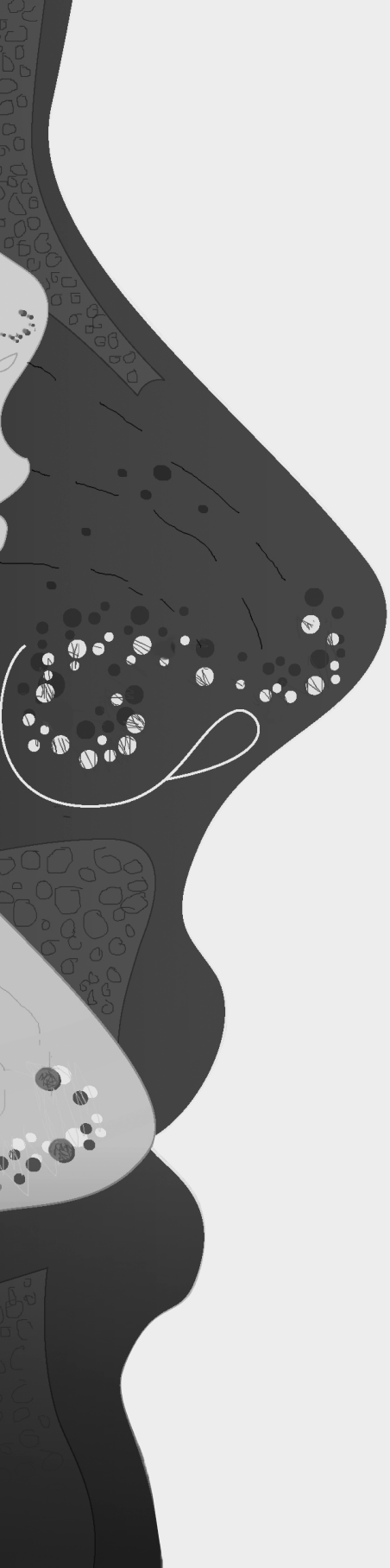

Chapter 17

Summary

Damian C. Melles

Staphylococcus aureus is both a human commensal and a frequent cause of clinically important infections, including relatively mild skin infections as well as life-threatening infections, such as sepsis, osteomyelitis, and deep abscesses. The numbers of both community-acquired and hospital-acquired staphylococcal infections have significantly increased over the past 25 years (1-3). *S. aureus* is the second most important cause of nosocomial blood stream infections leading to increased morbidity, mortality, hospital stay, and costs of medical care (4-7). The anterior nares (of the nose) form the primary ecological reservoir of *S. aureus* in humans (8), and it has been determined that most infections are endogenous, resulting from nasal carriage (9-13). As there is considerable evidence that carriage is an important risk factor for invasive infection with *S. aureus*, it is surprising that relatively little is known about the host factors as well as bacterial factors that promote colonization (14).

S. aureus has shown a high degree of adaptability and it developed or acquired resistance mechanisms to almost all antibiotics that were introduced over the last few decades. Recently, the emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) causing serious infections in otherwise healthy individuals poses a public health threat (15-17). It is obvious that effective measures to prevent *S. aureus* infections are urgently needed. To be able to develop preventive strategies we must elucidate the mechanisms behind *S. aureus* nasal carriage and identify the driving factors of subsequent infection. The main goal of the research described in the present thesis was to determine the population structure and dynamics of nasal carriage versus invasive strains of *S. aureus* as guided by whole genome polymorphism analysis and specific searches for the differential presence of genes deemed important in colonization and/or infection.

Population dynamics of *Staphylococcus aureus*

In **Part I** of the current thesis we have described the detailed population structure of naturally occurring methicillin-susceptible *S. aureus* (MSSA) isolated from the nose of persons living in the community (**Chapter 3**). We suggest that essentially any *S. aureus* genotype that is carried by humans can transform into a life-threatening human pathogen, but strains from some clonal lineages are more virulent than others. Veterinary isolates from various animal host species fell within the same genomic classes as the human *S. aureus* isolates, which suggests that several traits facilitating infection in animals and humans are shared (**Chapter 4**). On the other hand, mastitis-associated strains from diverse host species (sheep, goat, and cattle) clustered in a specific subgroup on the basis of genetic fingerprints, signifying tissue specificity in *S. aureus* pathogenesis. In **Chapter 5** we concluded that the population structures of nasal *S. aureus* isolates from humans are congruent between The Netherlands and the USA. The same successful clones are present although their relative frequency may vary with geographic origin. Apparently, both MRSA and MSSA show similar epidemic behavior with some clones being more successful than others. In **Chapter 6** we positioned the recently discovered "pig-farming" associated MRSA in the overall phylogenetic tree for *S. aureus*

and show that this clone is capable of causing serious infection in humans even though its primary host is most likely porcine.

Determinants of nasal carriage and infection with *Staphylococcus aureus*

In **Part II** we focused on several determinants of nasal carriage or invasive infection with *S. aureus* in different population groups. In addition, we studied several staphylococcal proteins and toxins and their association with colonization or infection with this pathogen. In **Chapter 7** we studied the bacterial interference between *S. aureus* and *Streptococcus pneumoniae* in the nasopharynx of children. We concluded that age, gender, zip code, active smoking and co-colonization with *N. meningitidis* or *S. pneumoniae*, both vaccine- and non-vaccine types, were not associated with colonization by specific *S. aureus* genotypes. Based on our whole-genome typing data obtained, there was no obvious correlation between staphylococcal and pneumococcal genotypes during co-colonization. In **Chapter 8** we confirmed that HIV infected individuals have an increased risk of persistent *S. aureus* nasal carriage compared to healthy individuals and that male gender is independently associated with a higher risk of persistent *S. aureus* carriage. In contrast, active smoking and *Pneumocystis jiroveci*-pneumonia (PCP) prophylaxis were independently associated with a lower risk of persistent *S. aureus* nasal carriage in our HIV cohort. In addition, the population structure of *S. aureus* strains, isolated from HIV patients appears to be strongly overlapping with the natural population structure of *S. aureus* isolates from healthy individuals.

In **Chapter 9** we defined the prevalence of the Panton-Valentine leukocidin (PVL) in carriage as well as clinical isolates of *S. aureus*. We found a very low PVL prevalence among carriage- and blood-culture isolates. However, we found a significantly higher percentage PVL-positive *S. aureus* strains causing abscesses and arthritis, which is in agreement with the proposed involvement of PVL in severe and invasive soft tissue infections. In **Chapter 10** we concluded that the enterotoxin gene cluster (*egc*) is slightly, but still significantly enriched among carriage *S. aureus* strains, not associated with mortality in patients suffering from staphylococcal bacteremia, and coupled to certain important bacterial clonal lineages. Although a clear biological rationale is currently lacking, our study suggests that the presence of *egc* is associated with non-invasiveness and a lower disease-invoking potential, as suggested earlier (18). In addition, we showed that most successful *S. aureus* lineages harbor the *egc* cluster, which is again in agreement with the suggestion that *egc* enhances the carriage or dissemination potential of an *S. aureus* strain. In **Chapter 11** we have focused on the Sdr proteins, which are members of the MSCRAMM family that are encoded by the tandemly arrayed *sdrC*, *sdrD*, and *sdrE* genes. The *sdr* locus was found in all *S. aureus* strains investigated. We found a strongly significant association between the presence of the *sdrD* gene and MRSA strains. In addition, the *sdrD* gene was significantly associated with osteomyelitis but not with blood infections.

In **Chapter 12** we elucidated the potential relevance of a putative (trivalent) *S. aureus* polysaccharide-conjugate vaccine. We determined the surface and/or capsular polysaccha-

ride serotype of 162 well-characterized MSSA strains from both carriage and clinically relevant infection. In addition, we determined if there is an association between capsular type and strain genotype. All Dutch *S. aureus* isolates belonged to capsular types 5, 8 or type 336PS, which is in agreement with data from other countries. Thus a vaccine containing *S. aureus* CP5-, CP8- and 336- polysaccharide conjugates would target 100% of Dutch *S. aureus* isolates.

Technical aspects of defining genomic variability in *Staphylococcus aureus*

In the final part, **Part III**, of this thesis we have studied the genomic variability of *S. aureus*, using several molecular typing methods. In **Chapter 13** we compared three well-known and frequently used staphylococcal typing methods, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), PFGE, and amplified fragment length polymorphism (AFLP). We show that the methods yield similar results, although with differences in resolving power and reproducibility. We concluded that epidemiological parameters should determine which would be the optimal typing method to be used. In **Chapter 14** we determined AFLP-defined whole genome polymorphism as well as length variation in short sequence repeat loci for 994 *S. aureus* strains from both carriage and clinically relevant infection. We corroborate that there is a difference in the rates of cross-genome mutation versus regional repeat variability in the clonal bacterial pathogen *S. aureus*. However, despite these differences in the observed degree of genetic variability, a conservation of type assignments as based upon these two inherently different typing techniques was observed. In **Chapter 15**, finally, we used ht-AFLP to characterize molecular markers associated with MSSA and MRSA phenotypes in conjunction with the available *S. aureus* genome sequences. We successfully identify MRSA specific markers and show that this method can be applied to any microbial species for which at least one full-genome sequence is available. We believe that ht-AFLP has built a bridge between whole genome sequencing and other typing techniques and has proven to give reliable results.

In conclusion, we have solved the population structure of *S. aureus* from a non-clinical origin. We provide evidence that essentially any *S. aureus* genotype carried by humans can transform into a life-threatening human pathogen but that certain clones are more virulent than others. It remains to be seen whether a laboratory-based identification of more pathogenic clones of *S. aureus* can be translated into a reliable, clinical relevant diagnostic tool in the future. We studied the genomic variability of *S. aureus* and discussed the applicability, reliability and clinical relevance of several molecular typing methods for this pathogen. Finally, we have identified several important determinants of carriage and infection with *S. aureus* in both humans and animals. These studies will hopefully contribute and lead to the development of new and more effective measures to prevent *S. aureus* infections in the future.

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Chapter 18

Nederlandse samenvatting

Damian C. Melles

De bacterie *Staphylococcus aureus* is zowel een commensaal als een belangrijke opportunistische verwekker van infecties bij mens en dier. De infecties variëren van milde huidinfecties, zoals furunculosis (steenpuisten) en impetigo (krentenbaard) tot zeer ernstige infecties, zoals sepsis en endocarditis (infecties van de bloedbaan en hartkleppen), osteomyelitis (botinfecties), pneumonie (longontsteking) en diepe abcessen. Vaak ontstaan deze infecties bij mensen met een verminderde afweer, die opgenomen liggen in ziekenhuizen (bijvoorbeeld na een operatie). In de afgelopen 25 jaar is het aantal infecties veroorzaakt door *S. aureus* sterk toegenomen, zowel in ziekenhuizen als in de open bevolking (1-3). *S. aureus* is de op één na meest voorkomende verwekker van bloedbaaninfecties wat leidt tot verhoogde morbiditeit en mortaliteit, verlengde opnameduur en toename van de kosten in de gezondheidszorg (4-7). Daarnaast wordt de behandeling van *S. aureus* gecompliceerd door de toenemende resistentie tegen de verschillende beschikbare antibiotica. Hoewel het percentage meticilline-resistentie onder de klinische *S. aureus* isolaten (ook wel MRSA of "ziekenhuisbacterie" genoemd) in Nederland en sommige Scandinavische landen (nog) erg laag is (< 1%), loopt dit percentage in veel andere landen op tot boven de 50% (8). De lage MRSA prevalentie in Nederland is te danken aan zowel het restrictieve antibiotica beleid als het nationale "Search and Destroy" beleid, opgesteld door de Werkgroep Infectie Preventie (WIP) (9). Recentelijk (augustus 2007) heeft het ministerie van Volksgezondheid, Welzijn en Sport op advies van de Gezondheidsraad ook geconcludeerd dat het Nederlandse MRSA-beleid zinvol is. "Loslaten van het Nederlandse MRSA-beleid zal waarschijnlijk leiden tot een hogere prevalentie van MRSA en daardoor meer ziekte en mogelijk sterfte. Volgens de Gezondheidsraadcommissie wegen de kosten van het MSRA beleid op tegen de kosten van het loslaten van dit beleid. De commissie beveelt dan ook aan om het strikte Nederlandse MRSA-beleid te handhaven."

Hoewel tot enkele jaren geleden MRSA voornamelijk een probleem was in de ziekenhuizen, tonen nieuwe studies aan dat MRSA zich nu ook verspreid in de open bevolking (bijv. in de Verenigde Staten) (10-12). Deze plotselinge opkomst van "community-acquired MRSA (CA-MRSA)", die ernstige infecties kunnen veroorzaken bij gezonde kinderen en volwassenen, leidt tot veel onrust en soms zelfs paniek. In het afgelopen jaar zijn er in de Verenigde Staten regelmatig high schools gesloten in verband met een epidemie van CA-MRSA infecties bij gezonde kinderen. Al deze ontwikkelingen geven aan dat effectieve maatregelen om *S. aureus* infecties te voorkomen dringend nodig zijn.

Hoewel *S. aureus* ernstige infecties kan geven, leeft deze bacterie ook als commensaal op de huid en slijmvliezen van veel gezonde mensen (2, 13). De binnenkant van de punt van de neus ("the anterior nares") is daarbij de belangrijkste niche (lokalisatie) van dragerschap bij de mens (14). Longitudinale studies hebben aangetoond dat ongeveer 20% van de mensen continu nasaal drager is van deze bacterie, 30% soms en 50% nooit drager is van *S. aureus* (15-18). Het mechanisme achter *S. aureus* dragerschap en de reden waarom de één continu drager is en de ander nooit is tot op dit moment nog onduidelijk. Maar waarom is dra-

gerschap van *S. aureus* relevant? Verschillende studies hebben aangetoond dat de meeste infecties ontstaan vanuit "endogeen" dragerschap: de gastheer wordt ziek van de *S. aureus* stam waar men zelf drager van is (19-23). Het eradiceren (verwijderen) van *S. aureus* uit de neus bij dragers blijkt ook infecties te voorkomen in bepaalde patiëntengroepen, zoals hemodialyse patiënten en algemene chirurgische patiënten (23, 24). Hoewel *S. aureus* dragerschap dus een zeer belangrijke risicofactor blijkt te zijn voor het ontstaan van infectie, is het opmerkelijk dat er zo weinig bekend is over de predisponerende factoren voor en de mechanismen achter *S. aureus* dragerschap (25). Om nieuwe preventieve maatregelen te ontwikkelen om *S. aureus* infecties in de toekomst te kunnen voorkomen, is meer inzicht nodig in zowel de gastheerfactoren, de bacteriële factoren, als de omgevingsfactoren die de dragerschapstatus van de mens beïnvloeden. Een belangrijk onderdeel van de zoektocht naar relevante bacteriële factoren van zowel dragerschap als infectie in de mens is het bestuderen van de populatiestructuur van *S. aureus*. Hiermee kan zowel de genetische achtergrond als de genetische verschillen tussen dragerschapsstammen en klinische stammen van *S. aureus* worden bestudeerd.

Het doel van het onderzoek beschreven in dit proefschrift was het verkrijgen van een beter inzicht in zowel de natuurlijke populatie structuur van *S. aureus* als de relatie tussen dragerschap en infectie met deze klinisch relevante bacterie. De hoofdstukken in dit proefschrift zijn verdeeld in drie delen met elk een specifiek thema.

Populatiestructuur van *Staphylococcus aureus*

In **Deel I** hebben we ons geconcentreerd op de populatiestructuur van *S. aureus*. Moleculaire typering van een klonaal micro-organisme (zoals *S. aureus*), maakt het mogelijk om verschillende genotypes aan te tonen en te onderscheiden en de genetische populatiestructuur in kaart te brengen. Dit geeft onder andere de mogelijkheid het dragerschaps-vermogen ("carriage potential") van verschillende *S. aureus* genotypen te kunnen bestuderen. Een aanvullende belangrijke en relevante vraag is of elke *S. aureus* bacterie stam in staat is om ernstige infecties te veroorzaken bij de mens (26, 27). Om verschil aan te kunnen tonen in ziektemakend vermogen tussen verschillende *S. aureus* stammen of genotypen, is het echter noodzakelijk om eerst inzicht te krijgen in de natuurlijke populatiestructuur van *S. aureus*. In **Hoofdstuk 3** is voor het eerst deze natuurlijke populatiestructuur van meticilline-gevoelige *S. aureus* (MSSA) in kaart gebracht. Daarvoor zijn meer dan 2000 *S. aureus* stammen geisoleerd uit de neus van gezonde kinderen en volwassenen buiten het ziekenhuis (een unieke stammen collectie in de wereld) (28, 29). Deze stammen zijn genetisch getypeerd met behulp van "high-throughput amplified fragment length polymorphism (ht-AFLP) analyse". Deze typeringsmethode documenteert zowel nucleotide sequentie variatie, inserties (invoeging of tussenvoeging van een extra stukje DNA) als deleties (het verliezen van een stukje DNA) over het hele *S. aureus* genoom. Om eventueel virulente (meer ziektemakend) *S. aureus* ge-

notypen aan te kunnen tonen hebben we tevens klinische isolaten (bloedkweek stammen en stammen geassocieerd met huid- en weke-delen infecties) meegenomen in de analyse. De conclusie van deze studie was dat in essentie elke *S. aureus* stam, die als commensaal leeft op de mens, kan transformeren tot een levensbedreigend pathogeen, hoewel sommige klonen meer virulent blijken dan anderen.

Om te beoordelen of dezelfde *S. aureus* genotypen infecties veroorzaken in mens en dier, hebben we in **Hoofdstuk 4** klinische isolaten van verschillende dieren (waaronder honden, katten, varkens, schapen, etc.) vergeleken met klinische en dragerschaps isolaten van mensen. Het blijkt dat veel veterinaire isolaten genetisch samenvallen met de humane isolaten, wat aangeeft dat dezelfde stammen zowel dieren als mensen kunnen infecteren. Echter, we vonden ook een apart genetisch cluster van dierenstammen, geassocieerd met mastitis (uierontsteking) bij schapen, geiten en koeien. Dit toont mogelijk toch een bepaalde mate van ziekte- of weefsel-specificiteit aan in de pathogenese van *S. aureus*. Tevens hebben we in deze studie gekeken naar de selectieve aanwezigheid van 10 virulentie genen en concludeerden dat er voor een aantal van deze genen (bijv. het toxic shock syndroom toxine) wel een duidelijk verschil in frequentie van aanwezigheid blijkt te zijn tussen stammen geïsoleerd van infecties bij dieren en bij mensen. We concludeerden dat de aanwezigheid van virulentie factoren een belangrijke rol speelt in de gastheer- en weefsel-specificiteit van *S. aureus* infecties.

Een andere relevante vraag met betrekking tot de populatiestructuur van *S. aureus*, is de vraag of dezelfde genotypen gevonden worden in de verschillende landen of werelddelen. In **Hoofdstuk 5** hebben we de natuurlijke populatiestructuur van neusdragerschaps-stammen van gezonde mensen uit Nederland met die van mensen uit de Verenigde Staten vergeleken. Deze studie liet zien dat dezelfde succesvolle *S. aureus* klonen gevonden worden aan beide kanten van de Atlantische Oceaan. De twee meest succesvolle *S. aureus* klonen in Nederland (in dit proefschrift "AFLP cluster II en III" genaamd) bleken ook de meest voorkomende dragerschapsstammen in de Verenigde Staten.

Het onderzoek beschreven in **Hoofdstuk 6** heeft betrekking op een zeer actueel onderwerp: de zogeheten "varkensgerelateerde MRSA". Recentelijk is gebleken dat varkens (en daarmee ook veel varkensboeren) in Nederland en in andere delen van Europa massaal gekoloniseerd zijn met MRSA (30-32). Aangezien in Nederland de MRSA prevalentie onder mensen zeer laag is, kwam dit als een onverwachte en onplezierige verrassing. Om de verspreiding van deze MRSA in de samenleving en in de ziekenhuizen te voorkomen, heeft de WIP ook de MRSA richtlijnen recentelijk aangepast. Indien varkenshouders, personeel van slachthuizen of dierenartsen in een ziekenhuis worden opgenomen, moeten zij in isolatie verpleegd worden, totdat MRSA dragerschap is uitgesloten. In **Hoofdstuk 6** bestudeerden wij de genetische structuur van deze varkensgerelateerde MRSA. We bevestigden dat het een zeer klonale groep *S. aureus* stammen betreft, die genetisch duidelijk afwijken van de verschillende *S. aureus* genotypen, die we hebben gevonden bij mensen (**Hoofdstuk 3**). Daarnaast toonden we aan dat de meticilline-gevoelige variant van de varkens MRSA relatief

vaak geïsoleerd wordt uit bloedkweken van zieke patiënten in vergelijking met dragerschaps stammen van gezonde mensen. We concludeerden dat de varkensgerelateerde MRSA zeker in staat is om ernstige infecties te veroorzaken in mensen, hoewel de primaire gastheer waarschijnlijk toch het varken is.

Determinanten van neusdragerschap en infectie met *Staphylococcus aureus*

Naast het in kaart brengen van de natuurlijke populatiestructuur van *S. aureus* bestudeerden we in **Deel 2** determinanten van dragerschap en infectie met *S. aureus* in verschillende studiepopulaties. Tevens bestudeerden we een aantal eiwitten en toxinen van *S. aureus* en hun relatie met kolonisatie en/of infectie in de mens. In **Hoofdstuk 7** hebben we ons toegespitst op het fenomeen van "bacteriële interferentie". Wanneer een niche (zoals de neus) "bezet" is door een bepaalde groep bacteriën, blijkt het vaak moeilijk voor andere bacteriën om deze lokale bacteriële populatie te verdrijven en te vervangen (33). Verschillende studies hebben aangetoond dat er een omgekeerde (inverse) relatie bestaat tussen dragerschap van *S. aureus* en *Streptococcus pneumoniae* (pneumokok) in de nasopharynx (neusholte), wat een soort natuurlijke competitie suggereert tussen deze twee bacteriële species (28, 34). Wat kan deze competitie voor klinische consequenties hebben? Recente studies hebben laten zien dat na het toedienen van het pneumokokken-conjugaat vaccin (sinds april 2006 ook in het Rijksvaccinatieprogramma in Nederland) er een toename zichtbaar is van het aantal *S. aureus* infecties bij kinderen (35, 36). Door het toedienen van het vaccin wordt pneumokokken dragerschap mogelijk vervangen door *S. aureus* dragerschap, wat in het vervolg weer aanleiding kan geven tot *S. aureus* infecties. In **Hoofdstuk 7** hebben we onderzocht of de bacteriële interferentie tussen *S. aureus* en *S. pneumoniae* afhankelijk is van het bacteriële genotype. Daarnaast hebben we de determinanten van dragerschap van verschillende *S. aureus* genotypen in 400 kinderen in kaart gebracht. We concludeerden dat noch leeftijd, geslacht, postcode gebied, actief roken of co-kolonisatie met *Neisseria meningitidis* en *S. pneumoniae* geassocieerd is met kolonisatie van specifieke *S. aureus* genotypen. Op basis van onze genotyperingsdata vonden wij geen duidelijke relatie tussen stafylokokken en pneumokokken genotypen tijdens co-kolonisatie.

Studies hebben aangetoond dat verschillende subgroepen van patiënten vaker drager zijn van (en daarmee ook vaker infecties oplopen met) *S. aureus* in vergelijking met gezonde mensen, waaronder diabeten, hemodialyse patiënten en HIV patiënten (17). In **Hoofdstuk 8** bestudeerden we determinanten van persisterend (continu) *S. aureus* dragerschap in een groot cohort van HIV-geïnfecteerden. We bevestigden dat HIV patiënten significant vaker persisterend drager zijn van *S. aureus* dan gezonde mensen. Daarnaast toonden wij aan dat het mannelijke geslacht onafhankelijk geassocieerd is met een hogere kans op persisterend *S. aureus* dragerschap. Actief roken en *Pneumocystis jiroveci*-pneumonia (PCP) profylaxis zijn juist geassocieerd met een lagere kans op persisterend dragerschap. Vervolgens hebben we gekeken naar de populatiestructuur van de *S. aureus* stammen geïsoleerd van de HIV patiën-

ten en deze blijkt niet af te wijken van de populatie structuur van *S. aureus* geïsoleerd van gezonde mensen. Specifieke *S. aureus* klonen zijn dus niet verantwoordelijk voor het hogere percentage dragerschap onder HIV patiënten.

Het is bekend dat *S. aureus* een verscheidenheid aan potentiële virulentie factoren kan produceren, zoals bepaalde oppervlakte eiwitten die adherentie (vasthechting) aan beschadigd weefsel bevorderen en kunnen binden aan eiwitten in bloed om te ontkomen aan de antilichaam-gemedieerde immuun response (37). Daarnaast kan *S. aureus* verschillende toxinen produceren, die weefselschade kunnen veroorzaken en aanleiding kunnen geven tot een septische shock (25). In de **Hoofdstukken 9**, **10** en **11** bestudeerden we enkele van deze potentiële virulentie factoren en hun relatie met dragerschap en infectie met *S. aureus*. Recentelijk zijn er veel studies verschenen over het *S. aureus* toxine: "Panton-Valentine leukocidin (PVL)" (38-42). Dit toxine wordt geassocieerd met huidinfecties, CA-MRSA infecties en zeer ernstige longontstekingen. Hoewel PVL dus geassocieerd lijkt met verschillende relevante infecties, is er weinig bekend over de prevalentie (aanwezigheid) van de PVL genen in *S. aureus* geïsoleerd uit de neus van mensen in de open bevolking. In **Hoofdstuk 9** toonden wij aan dat de PVL prevalentie onder dragerschaps-stammen en bloedkweek isolaten erg laag is. De PVL prevalentie onder *S. aureus* stammen, geïsoleerd uit abcessen en gewrichtinfecties, was echter significant hoger. Dit is in overeenstemming met de veronderstelling dat het PVL toxine geassocieerd is met huid- en weke-delen infecties. Tevens toonden wij aan dat de PVL genen aanwezig zijn in verschillende *S. aureus* klonen en zich dus niet beperken tot een enkele kloon of genotype.

Een andere groep van belangrijke *S. aureus* virulentie factoren zijn de enterotoxinen, die geassocieerd zijn met voedsel-gerelateerde uitbraken van diarree onder mensen (43). In **Hoofdstuk 10** bestudeerden we een bijzondere groep van deze enterotoxinen, die (indien aanwezig) geclusterd liggen op het *S. aureus* genoom: het "*egc* locus" (44). In deze studie concludeerden we dat dit *egc* locus vaker aanwezig blijkt zijn in dragerschapsstammen dan in stammen geïsoleerd uit bloedkweken. Daarnaast bleken de meeste succesvolle *S. aureus* klonen dit gen locus te bevatten. Deze bevindingen suggereren dat de aanwezigheid van het *egc* locus mogelijk geassocieerd is met non-invasiviteit (een lager ziektemakend vermogen), wat tevens in overeenstemming is met andere studies (45).

Naast PVL en *egc* keken we in **Hoofdstuk 11** naar de aanwezigheid van specifieke genen op het *S. aureus* genoom, die coderen voor bepaalde oppervlakte eiwitten, en hun relatie met ziektemakend vermogen in mensen. In deze studie gaat het om de zogeheten: "serine-aspartate repeat (Sdr) proteins", die een onderdeel vormen van de familie van de "microbial surface components recognizing adhesive matrix molecules (MSCRAMM)" (46). Het *sdr* locus bestaat uit verschillende genen (*sdrC*, *sdrD* en *sdrE*), die coderen voor verschillende eiwitten die echter ook afzonderlijk voor kunnen komen op het *S. aureus* genoom. In **Hoofdstuk 11** lieten wij zien dat alle *S. aureus* stammen een bepaalde vorm van het *sdr* locus

bezitten. Het *sdrC* gen blijkt namelijk universeel aanwezig te zijn op het genoom van alle onderzochte *S. aureus* stammen. Uit deze studie konden we verder concluderen dat de aanwezigheid van het *sdrD* gen sterk geassocieerd was met MRSA stammen. Daarnaast was er een sterke relatie tussen de aanwezigheid van het *sdrD* gen en *S. aureus* stammen die osteomyelitis veroorzaakt hadden. Deze associatie werd niet gevonden voor stammen geïsoleerd uit bloedbaan infecties.

Tot slot eindigden we **Deel II** van dit proefschrift met een studie, waarin we de relevantie van een potentieel *S. aureus* polysaccharide-conjugaat vaccin bestudeerden. Immunotherapeutische strategieën zouden immers een belangrijke rol kunnen spelen in het controleren van *S. aureus* (en CA-MRSA) infecties in de toekomst. Hiervoor onderzochten we in **Hoofdstuk 12** de verschillende serotypen (kapseltypen) van zowel dragerschaps- als invasieve *S. aureus* stammen geïsoleerd in Nederland. Uit dit onderzoek blijkt dat de meeste *S. aureus* stammen behoren tot de serotypen 5 en 8, waarmee we data uit eerdere studies bevestigen (47, 48). De overige stammen zijn kapselloos, maar kruisreageren wel allemaal met antilichamen tegen het polysaccharide "336" en worden daarom type 336PS genoemd. In deze studie blijken type 336PS stammen oververtegenwoordigd te zijn onder de bloedkweek stammen in vergelijking met *S. aureus* stammen van gezonde neusdragers. De serotypering blijkt bovendien in overeenstemming met AFLP genotypering: de stammen uit één *S. aureus* cluster hebben allemaal hetzelfde serotype. Met behulp van deze studie konden we concluderen dat een vaccin, dat zowel *S. aureus* kapsel polysaccharide type 5 en 8 en daarnaast ook 336PS polysaccharide bevat, 100% van de Nederlandse *S. aureus* stammen zal dekken.

Technische aspecten van moleculaire typering van *Staphyloccocus aureus*

In het laatste gedeelte van dit proefschrift (**Deel III**) hebben wij ons gefocust op de technische aspecten van moleculaire typering van *S. aureus*. Naast het bestuderen van de (natuurlijke) populatie structuur van *S. aureus*, is moleculaire typering ook een zeer belangrijk instrument voor het in kaart brengen van de epidemiologische veranderingen van MSSA en MRSA infecties, zowel regionaal, nationaal als internationaal (49). Daarnaast is het van belang voor het evalueren van de effectiviteit van ons nationale Search & Destroy beleid ten aanzien van MRSA. Er zijn in het verleden al veel verschillende fenotypische en genotypische methoden gebruikt om *S. aureus* stammen van elkaar te onderscheiden (50-53). In **Hoofdstuk 13** vergeleken we drie bekende en veel gebruikte *S. aureus* typeringsmethoden met elkaar: "multilocus sequence typing (MLST), pulsed field gel electrophoresis (PFGE), en amplified fragment length polymorphism (AFLP)". Deze methoden zijn vaak gebruikt voor het in kaart brengen van epidemieën en het bestuderen van de populatiestructuur van *S. aureus*. In deze studie toonden wij aan dat deze drie typeringsmethoden in essentie vergelijkbare resultaten genereren, hoewel met duidelijke verschillen in onderscheidend vermogen en reproduceerbaarheid. Wij concludeerden dat de epidemiologische vraagstelling zal

moeten bepalen welke typeringsmethode het meest geschikt is om adequate antwoorden te genereren.

Het is bekend dat bacteriële populaties veranderen door een verscheidenheid aan genetische mechanismen. Daarbij wordt aangenomen dat genetische variaties in geconserveerde gebieden van het genoom minder snel accumuleren dan in hypermutabele (snel veranderende) gebieden van het genoom zoals repetitief DNA ("DNA repeat loci", gebieden met zich meermaals herhalende, vaak relatief korte DNA sequenties). In **Hoofdstuk 14** bestudeerden we twee typeringstechnieken, die zich richten op verschillende delen van het genoom en we vergeleken de populatiestructuren van *S. aureus* gegenereerd door deze beide methoden. Enerzijds gebruikten we een typeringsmethode die genetische polymorfismen scoort over het gehele *S. aureus* genoom (high-throughput-AFLP). Anderzijds gebruikten we een methode die specifiek veranderingen in de hypermutabele DNA repeat sequenties in kaart brengt ("multilocus variable number of tandem repeat analysis [MLVA])". Uit deze studie konden we concluderen dat ondanks het gebruik van uiteenlopende typeringstechnieken er een opvallende overlap bestaat in de gegenereerde populatiestructuren. Deze bevinding versterkt het populatiestructuur model voor *S. aureus* gepresenteerd in **Hoofdstuk 3**.

De meest accurate moleculaire typeringstechniek is "whole genome sequencing" (het in kaart brengen van het gehele genoom door middel van sequencen) (54-57). Dit is (met de huidige technologie) nog niet haalbaar voor grote groepen isolaten in verband met de hoge kosten en de arbeidsintensiteit. Alle andere typeringstechnieken analyseren slechts een relatief klein gedeelte van het genoom, waardoor kleine maar mogelijk biologisch significante verschillen tussen stammen onopgemerkt kunnen blijven. Op dit moment zijn de complete genomen bekend van 13 verschillende *S. aureus* stammen (zie: http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). In **Hoofdstuk 15** toonden we aan dat met behulp van ht-AFLP het relatief eenvoudig is om voor een grote groep stammen (met in deze studie als voorbeeld de *S. aureus* bacterie) een groot deel van het genoom te screenen op relevante polymorfismen. Daarnaast kunnen de gegeneerde data met specifieke software direct vergeleken worden met de op dat moment beschikbare complete genoomsequenties van *S. aureus* door middel van de zogeheten "theoretische ht-AFLP analyse" van deze bekende genomen. Met behulp van deze techniek is het dus mogelijk om snel een epidemische stam (bijvoorbeeld de varkens-gerelateerde MRSA) te vergelijken met de bekende complete genomen om genetische verwantschap te kunnen analyseren. In **Hoofdstuk 15** concludeerden wij dat ht-AFLP een "brug" heeft gebouwd tussen "whole genome typing" en andere moleculaire typeringstechnieken.

Concluderend, in dit proefschrift hebben we de natuurlijke populatiestructuur van *S. aureus* in kaart gebracht. We hebben aangetoond dat in essentie elke *S. aureus* stam kan transformeren tot een levensbedreigend pathogeen, hoewel sommige genotypen meer virulent blijken dan anderen. We vergeleken verschillende typeringstechnieken van *S. aureus* en bespraken

de relevantie van deze technieken voor de praktijk. Daarnaast hebben we verschillende belangrijke determinanten van dragerschap en infectie met *S. aureus* in mens en dier bestudeerd en aangetoond. De studies naar deze determinanten zullen hopelijk in de toekomst leiden tot nieuwe en meer effectieve maatregelen om *S. aureus* infecties te voorkomen.

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Curriculum Vitae

Damian Christian Melles werd geboren op 6 september 1977 te Utrecht. In 1995 behaalde hij zijn VWO diploma aan het Baarnsch Lyceum te Baarn. In datzelfde jaar begon hij aan de studie Geneeskunde aan de Medische Faculteit van de Erasmus Universiteit te Rotterdam. Zijn afstudeeronderzoek: "Follow-up bij kinderen met chronische darmontsteking (IBD)" deed hij in 1999 voor de afdeling gastro-enterologie van het Sophia Kinderziekenhuis (Erasmus MC) te Rotterdam. In 1999 behaalde hij zijn doctoraalexamen Geneeskunde. Van januari tot juni 2000 deed hij een onderzoeksstage bij het "Intermountain Health Care Institute for Health Care Delivery Research" in Salt Lake City (Verenigde Staten). Na het afleggen van het Artsexamen begon hij in juli 2002 aan zijn promotieonderzoek aan de afdeling Medische Microbiologie en Infectieziekten (onder begeleiding van Prof. dr. A. van Belkum). Sinds mei 2004 is hij op dezelfde afdeling tevens werkzaam als arts-assistent in opleiding tot arts-microbioloog (opleider Prof. dr. H.A. Verbrugh). Deze opleiding hoopt hij in mei 2009 succesvol af te ronden.

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