Funktionelle Charakterisierung neuer Virulenzfaktoren von *Staphylococcus aureus*

(Functional chracterization of new virulence factors of *Staphylococcus aureus*)

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer.nat.)

an der Mathematisch-Naturwissenschaftlichen Fakultät

der

Ernst-Moritz-Arndt-Universität Greifswald

vorgelegt von

Dorothee Grumann

geboren am 03.01.1980

in Fürth

Greifswald, 11.03.2010

Dekan: Prof. Dr. Klaus Fesser

1. Gutachter: Prof. Dr. Barbara M. Bröker

2. Gutachter: Prof. Dr. Alex F. van Belkum

Tag der Promotion: 09. Juli 2010

CONTENTS

PART I INTRODUCTION

Chapter 1 *Staphylococcus aureus* in disease and in health……1

1.1 *S. aureus* – human pathogen and harmless commensal……2

1.2 *S. aureus* comparative genomics……4

1.3 What determines staphylococcal virulence?……6

1.4 Staphylococcal superantigens……7

1.5 Outline of the thesis……14

PART II RESULTS

Chapter 2 Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates.

S. Holtfreter, D. Grumann, M. Schmudde, H. T. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Bröker. 2007. J Clin Microbiol 45:2669-2680……24

Chapter 3 Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages.

C. Goerke, R. Pantucek, S. Holtfreter, B. Schulte, M. Zink, D. Grumann, B. M. Bröker, J. Doskar, and C. Wolz. 2009. J Bacteriol 191:3462-3468……39

Chapter 4 Recurrent furunculosis - associations with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus.*

H. Masiuk, K. Kopron, D. Grumann, C. Goerke, J. Kolata, J. Jursa-Kulesza, S. Giedrys-Kalemba, B. M. Bröker, and S. Holtfreter. 2010. in press……47

Chapter 5 Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*.

N. J. Verkaik, C. P. de Vogel, H. A. Boelens, D. Grumann, T. Hoogenboezem, C. Vink, H. Hooijkaas, T. J. Foster, H. A. Verbrugh, A. van Belkum, and W. J. van Wamel. 2009. J Infect Dis 199:625-632……..69

Chapter 6 Neutralizing antibody response to *Staphylococcus aureus* superantigens in bacteremic patients.

D. Grumann, E. Ruotsalainen, J. Kolata, P. Kuusela, A. Järvinen, V. P. Kontinen, B. M. Bröker, and S. Holtfreter. 2010. submitted……78

Chapter 7 Immune cell activation by enterotoxin gene cluster (*egc*)-encoded and non-*egc* superantigens from *Staphylococcus aureus*.

D. Grumann, S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Völker, and B. M. Bröker. 2008. J Immunol 181:5054-5061……96

PART III SUMMARY

Chapter 8 Summary and discussion……116

8.1 What determines staphylococcal virulence? Molecular-epidemiology of colonizing and invasive *S. aureus* isolates……117

8.2 Anti-staphylococcal antibody response in colonization and bacteremia……122

8.3 Comparison of *egc* and non-*egc* superantigens – What is the reason for the lack of anti-*egc* antibodies?……125

PART IV APPENDIX

Summary……135

Zusammenfassung……138

Abbreviations……142

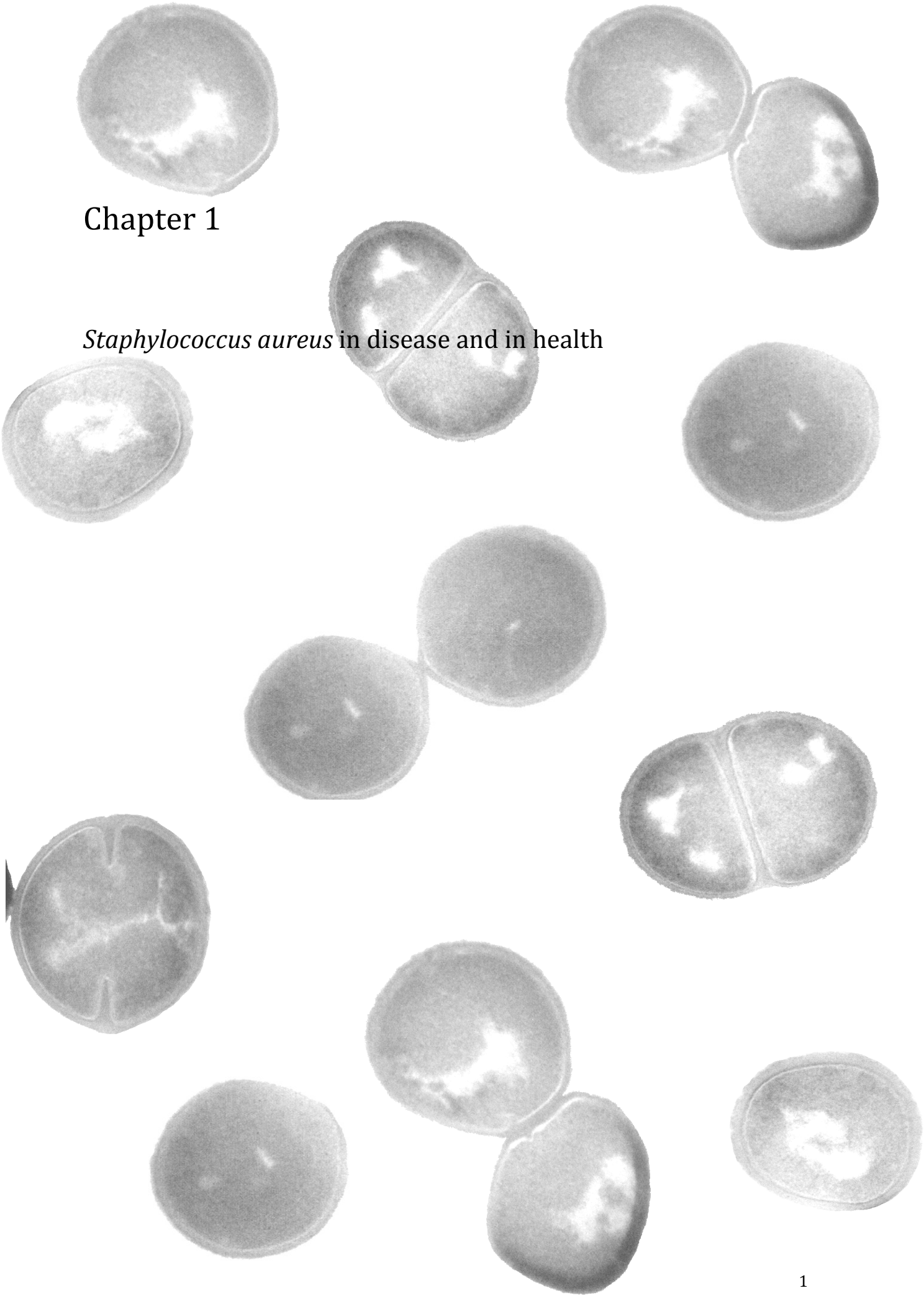
Publications……145

Conferences……..146

Acknowledgments/ Danksagung……148

Eidesstattliche Erklärung……150

Curriculum vitae……151



Chapter 1

Staphylococcus aureus in disease and in health

Staphylococcus aureus in disease and in health

Staphylococcus (S.) aureus is an opportunistic pathogen and a leading cause of bacterial infections worldwide. The spread of antibiotic resistant strains in hospitals as well as in the healthy population is of growing concern. Moreover, up until now, no anti-*S. aureus* vaccine has been approved for medical practice (1).

1.1 *S. AUREUS* – HUMAN PATHOGEN AND HARMLESS COMMENSAL

Clinical impact of *S. aureus*

S. aureus is a major human pathogen capable of causing a wide spectrum of infections, from relatively mild skin infections such as folliculitis and furunculosis to life-threatening diseases, including sepsis, pneumonia, osteomyelitis, and infective endocarditis (2). Infections caused by this pathogen have increased over the past 25 years (2, 3). *S. aureus* (methicillin-sensitive as well as methicillin-resistant *S. aureus;* MSSA and MRSA, respectively) ranks as the most common cause of nosocomial bloodstream infections and leads to increased morbidity, mortality, length of hospital stay, and costs (4).

The treatment of such infections is complicated by the ability of this species to become resistant to antibiotics (5). MRSA strains are widespread in nosocomial environments (hospital-associated MRSA; HA-MRSA), and account for > 60% of *S. aureus* isolates in US intensive care units (6). Of growing concern is the emergence and spread of highly pathogenic MRSA strains in the community outside the hospital setting (community-associated MRSA; CA-MRSA), especially the clone USA300 (7, 8). These strains cause serious infections in otherwise healthy individuals (7, 9). Vancomycin is the drug of choice for therapy of infections due to MRSA, but increase in vancomycin use has led to the emergence of vancomycin intermediate-resistent (VISA) or resistant strains (VRSA) (10). As a consequence of increasing antibiotic resistance non antimicrobial approaches to control *S. aureus* are needed. However, due to the complex interaction between *S. aureus* and the immune system of its host, vaccine development is still a challenging task.

S. aureus nasal carriage patterns

In apparent contrast with its infectious potential, *S. aureus* is also a frequent commensal that colonizes the skin and mucosal surfaces of humans and several animal species (11). The primary ecological niche of *S. aureus* are the anterior nares (11, 12). However, multiple body sites can be colonized including the skin, perineum, pharynx, and less frequently the gastrointestinal tract, vagina, and axillae (13). Longitudinal studies distinguish three nasal carriage patterns in healthy individuals: about 20% (range 12 - 30%) are persistent carriers, approximately 30% (range 16 - 70%) are intermittent carriers, and about 50% (range 16 - 69%) are noncarriers (13). While persistent carriers are usually colonized by a single strain of *S. aureus,* intermittent carriers commonly carry different strains over time (14, 15). Cross-sectional studies yield a prevalence of ~ 35% carriers in the healthy population, which is actually a mix of persistent and intermittent carriers at the time of investigation (16, 17).

It is clinically relevant to distinguish between persistent and intermittent carriers. Persistent carriers have higher *S. aureus* loads, resulting in increased dispersal and a higher risk of infection in comparison to intermittent carriers and noncarriers (13, 18-21). Intermittent carriers and noncarriers show similar infection risks.

Recently van Belkum et al. suggested a reclassification of nasal carriage types (21). An artificial colonization study where volunteers were inoculated with a mixture of *S. aureus* strains showed that intermittent carriers and noncarriers quickly eliminated the inoculated *S. aureus* strains, while persistent carriers preferentially reselected their original resident strain from the inoculum mixture (21, 22). Furthermore, the antibody levels against 17 *S. aureus* antigens were equal in intermittent carriers and noncarriers but higher in persistent carriers. Along with the previously described low risk of infection of intermittent carriers and noncarriers, the authors suggest to distinguish just two types of nasal carriers: persistent carriers and others.

What determines *S. aureus* nasal carriage?

Mechanisms leading to *S. aureus* nasal carriage appear to be multifactorial and are still not fully understood. Bacterial factors (e.g. staphylococcal toxins and cell wall-associated proteins) (2, 23), environmental factors (e.g. hospitalization and crowding) (24, 25) as well as host susceptibility factors (e.g. immune suppression or other serious

underlying diseases) play important roles (11, 24). The results of an artificial colonization study (see above) indicated that host factors are probably the major determinants of the *S. aureus* carrier status and an optimal fit between host and bacteria seems to be essential for long-term colonization (22).

S. aureus nasal carriage is a major risk factor for infection

Carriage of *S. aureus* has been identified as a risk factor for the development of infection in various settings (11, 16, 26-28). Importantly, most of these *S. aureus* infections are caused by the patients´ own flora. Von Eiff et al. demonstrated in a prospective study that nasal strains and subsequent bacteremic strains have the same genotype in more than 80% of the cases (29). Similarly, hemodialysis patients and chronic ambulatory peritoneal dialysis patients have increased infection rates of mostly endogenous origin (16, 30).

Wertheim and coworkers investigated the incidence of bacteremia in carriers as well as noncarriers in a non-surgical patient population (n = 14,008) (31). Nasal carriage increased the risk of nosocomial infection by a factor of three. Surprisingly, the mortality rate from *S. aureus* bacteremia was four times higher in noncarriers than in carriers (8% vs. 32%, $P = 0.006$) (31). An explanation for this observation has not yet been provided, although a role for the immune system has been proposed (32).

Moreover, several studies have demonstrated that eradication of *S. aureus* from nasal and other body sites with the anti-staphylococcal drug mupirocin effectively prevents *S. aureus* infections (15, 33-37).

1.2 *S. AUREUS* COMPARATIVE GENOMICS

Currently, 14 annotated whole genome sequences of *S. aureus* are available. A comparison of these sequences and whole genome microarray analyses revealed that the *S. aureus* genome consists of a core genome (~ 75%), a core variable genome (~ 10%) and mobile genetic elements (MGEs, ~ 15%) (38, 39).

The core genome is highly conserved concerning gene order and gene sequence and comprises house keeping genes, which are essential for growth and survival (5). Allelic variations in the core genome are exploited by sequence-based genotyping methods, like

multilocus sequence typing (MLST) (40) and protein A (*spa*) genotyping (41). MLST determines the phylogenetic relationship of bacterial strains based on the sequence fragments of seven housekeeping genes. In contrast, *spa* genotyping compares the variable repeat region of the protein A gene (*spa*), which differs in sequence and number of *spa* repeats. A *spa* type is assigned to each unique repeat pattern, and closely related *spa* types are referred to as clonal complexes (CCs). Several studies demonstrated that pulsed-field gel electrophoresis (PFGE), MLST and *spa* genotyping provide largely concordant results, however, only sequence-based typing methods can be easily compared between laboratories (41, 42). The genotyping analyses revealed that *S. aureus* has a highly clonal population structure, dominated by ten prevalent clonal lineages (43-45).

The core variable genome includes most surface-associated genes (MSCRAMMs) and regulator genes. Core variable genes are encoded on the bacterial chromosome and are, therefore, typically stable and transferred vertically (39). Lindsay et al. demonstrated that each staphylococcal lineage carries a unique combination of core variable genes (39).

MGEs include bacteriophages, pathogenicity islands, plasmids, transposons, and staphylococcal chromosomal cassettes (SCC) (38, 46). They mainly encode resistance and virulence genes (e.g., Panton-Valentine leukocidin (PVL) genes, superantigen (SAg) genes). MGEs can be distributed either by vertical transmission to daughter cells or by horizontal transfer by bacteriophages or conjugation (38). Microarray analyses revealed substantial variation in the distribution of MGEs within lineages, suggesting frequent horizontal transfer or loss of MGEs (47). However, there is evidence that some MGEs are distributed within certain lineages at higher frequency than between lineages, suggesting the existence of some barriers in horizontal transfer of MGEs (39). The recently described Sau1 type I restriction-modification system, which recognizes and digests foreign DNA may serve as an example (48). The accumulation of such MGEs may result in the emergence of “superbugs” that are increasingly antibiotic resistant and of higher virulence (5).

1.3 WHAT DETERMINES STAPHYLOCOCCAL VIRULENCE?

One of the major questions is, whether all *S. aureus* strains have equal disease-evoking potential or whether invasive disease is associated with particularly virulent genotypes or with particular virulence genes.

Comparison of the staphylococcal core genome by genotyping analyses did not reveal significant differences between colonizing and bacteremia *S. aureus* isolates. Colonizing as well as invasive isolates were found in the same major and minor staphylococcal lineages (39, 44, 45, 49). This suggests that invasion into the bloodstream does not require special bacterial virulence traits but mainly depends on host factors, e.g. barrier breakage, indwelling catheters, or a compromised immune system. This may be different in community-acquired diseases that affect individuals who are not immune compromised, such as furunculosis, or community-acquired pneumonia, and also in chronic infections such as osteomyelitis. For better discrimination, future molecular-epidemiological studies should, therefore, focus on well-defined diseases rather than just comparing colonization with invasion.

Such detailed analyses can provide novel insights into *S. aureus* pathogenicity. By carefully differentiating between different clinical pictures, Melles et al. were able to dissect subclusters of strains with differential degrees of pathogenicity (45). The most prominent example is PFGE type USA300, which emerged as the predominant CA-MRSA genotype and is particularly adept at causing skin and soft-tissue infections (9). This suggests that indeed some clones are more virulent than others (45, 50). The molecular reasons for this are under intensive investigation. Overall, evidence for a decisive role of the core genome in *S. aureus* virulence is limited.

Therefore, it was suggested that virulence determinants encoded on MGEs may determine the virulence of an isolate (5). MGEs carry genes with virulence or resistance functions. However, a clear association between certain virulence genes and disease could only be established for toxin-mediated diseases, such as toxic shock syndrome (TSS; SAgs), staphylococcal scalded skin syndrome (exfoliative toxins), as well as necrotizing pneumonia and deep-seated skin infections (PVL) (51-55). Peacock et al. observed seven determinants (*fnbA, can, sdrE, sej, eta, hlg,* and *ica*) which were significantly more common in invasive isolates (56), but these candidate genes could not be confirmed by Lindsay and coworkers (39). The vast majority of cases of severe

S. aureus diseases cannot be explained by the action of a single virulence determinant and it is likely that a number of factors act in concert during the infective process (39, 52, 54-56). Furthermore, virulence factors have redundant functions (e.g., SAgs) and for individual virulence factors several functions have been described. For example, staphylococcal SAg-like protein 7 (SSL7) blocks IgA-Fc receptor interactions and inhibits complement (57), and SpA impedes phagocytosis by binding the Fc component of IgG and interaction with TNF receptor 1 stimulates a proinflammatory signaling cascade (58).

In conclusion, despite the enormous variation observed between *S. aureus* isolates and the considerable amount of genetic exchange between isolates, so far there is limited evidence that this variation influences pathogenicity (38, 39). Therefore, the key to understand *S. aureus* pathogenesis may lie in the identification of host factors that contribute to colonization, susceptibility to infection and outcome of infection (59, 60).

1.4 STAPHYLOCOCCAL SAGS

The molecular mechanism of *S. aureus* pathogenicity is complex and involves a large number of toxins. Among them are the staphylococcal SAgs, which belong to the most potent T cell mitogens known. Some members stimulate human T cells at femtomolar concentrations. So far, 21 different SAgs have been described: the toxic shock syndrome toxin (TSST-1), the staphylococcal enterotoxins A-E, G-J (SEA-SEE, SEG-SEJ) and the staphylococcal enterotoxin-like toxins K-U (SElK- SElU) (Table I) (52, 61-64). Originally, SAgs were designated as enterotoxins because of their emetic ability when orally ingested. Interestingly, some of the recently identified toxins were reported to lack emetic properties. Therefore, the International Nomenclature Committee for Staphylococcal Superantigens introduced a new nomenclature in 2004 to distinguish those SAgs with proven emetic activity in primates (SEs) from those that remain unconfirmed (SEls) (62).

Table I. Functional properties of staphylococcal SAgs. Adapted from Fraser et al. (65).

SAg	localisation	MW (kDa)	Crystal structure solved	Zinc binding	MHCII binding α/β chain	human TCR Vβ specificity[1]
SEA	bacteriophage (ΦSa3)	27.1	+	+	+ / +	5.2, 5.3, 7.2, 9, 16, 18, 22
SEB	SaPI1	28.4	+	-	+ / -	3, 12, 13.2, 14, 17, 20
SEC	SaPI2, SaPI3	27.6	+	+	+ / -	3, 12, 13.2, 14, 17, 20
SED	plasmid (pIB485)	26.9	+	+	+ / +	1, 5.1, 5.2, 5.3
SEE	bacteriophage?	26.4	-	+	+ / +	5.1, 8, 16, 18, 21.3
SEG	νSaβ	27.0	+	-	+ / -	3, 13.1, 13.2, 14
SEH	SCCmec	25.1	+	+	- / +	6.7, 8, Vα
SEI	νSaβ	24.9	+	+	- / +	1, 5.1, 5.2, 5.3
SEJ	plasmid (pIB485, pF5)	28.5	-	?	?	8, 21.3
SElK	SaPI1	26.0	+	+	- / +	1, 5.1, 5.2, 6.7
SElL	SaPI3	26.0	-	?	?	1, 5.1, 5.2, 5.3, 7.1, 16, 22, 23
SElM	νSaβ	24.8	-	?	?	21.3
SElN	νSaβ	26.1	-	?	?	9
SElO	νSaβ	26.7	-	?	?	7.1
SElP	bacteriophage (ΦSa3)	27.0	-	?	?	5.1, 8, 16, 18, 21.3
SElQ	SaPI1	25.0	-	?	?	6.7, 31.3
SER	plasmid (pIB485, pF5)	27.0	-	?	?	3, 12, 14
SES[2]	plasmid (pF5)	26.2	-	?	?	9, 16
SET[2]	plasmid (pF5)	22.6	-	?	?	?
SElU	νSaβ	27.1	-	?	?	13.2, 14
TSST-1	SaPI2	22.1	+	-	+ / -	2

[1]determined by Thomas et al. (66).

[2]newly described by Ono et al. (64).

The SAgs´ mode of action

Conventional antigens are taken up and processed by antigen-presenting cells (APCs). The resulting antigenic peptides are bound to major histocompatibility complex (MHC) molecules and displayed for T cells on the APC surface. These MHC/ peptide complexes are recognized by T cells via the hypervariable loops of their T cell receptor (TCR) α and β chains.

SAgs can bypass this highly specific interaction between T cells and APCs. They activate a large fraction of T cells by directly cross-linking certain TCR Vβ domains with conserved structures on MHC class II (MHC II) molecules expressed on professional APCs (Figure 1). Both TCR and MHC II are contacted outside their antigen binding sites (67). Every SAg interacts with a defined TCR repertoire determined by the TCR Vβ sequences. As the number of different Vβ elements in humans is restricted to approximately 50 (61), up to 20% of all T cells can be activated by one SAg. In contrast, conventional peptide antigens only stimulate between 0.001% and 0.0001% of naïve T cells, because recognition is dependent on both the variable and junctional segments (D and J) of the TCR α and β chains (67). The Vβ-restricted expansion is the characteristic hallmark of all SAgs (68-70). Interestingly, there is one exception: the SAg SEH also contacts TCR Vα chains (66, 71). In addition to the polyconal T cell activation this cross-link results in a massive systemic release of proinflammatory cytokines, such as IL-2, IFN-γ and TNF-α, which can lead to fever and shock.

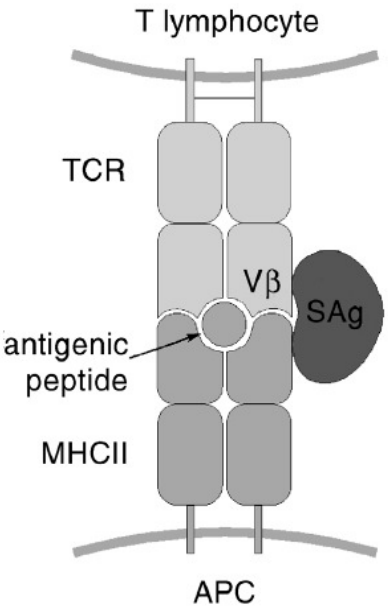


Figure 1. SAg function. From Holtfreter et al. (52). SAgs bypass the conventional antigen recognition by directly cross-linking MHC class II molecules on antigen-presenting cells (APC) with T cell receptors (TCRs) on T lymphocytes.

The T cell proliferation phase is followed by a profound state of unresponsiveness (anergy) in which the T cells fail to proliferate and secrete IL-2 (72) or even undergo cell death by Fas/ Fas ligand-mediated apoptosis (73). It has therefore been proposed that the function of SAgs might be the deletion of T cells that help B cells to mount an effective and highly specific antibody response against the bacteria (67).

Structural similarities of SAgs

So far, the three-dimensional structures of nine staphylococcal SAgs have been solved by x-ray crystallography: SEA, SEB, SEC, SED, SEG, SEH, SEI, SEK and TSST-1 (74-82). Despite very different primary structures (amino acid sequences), all SAgs show remarkable similarities in their secondary and tertiary structure, consisting of two globular protein domains (Figure 2) (61, 65).

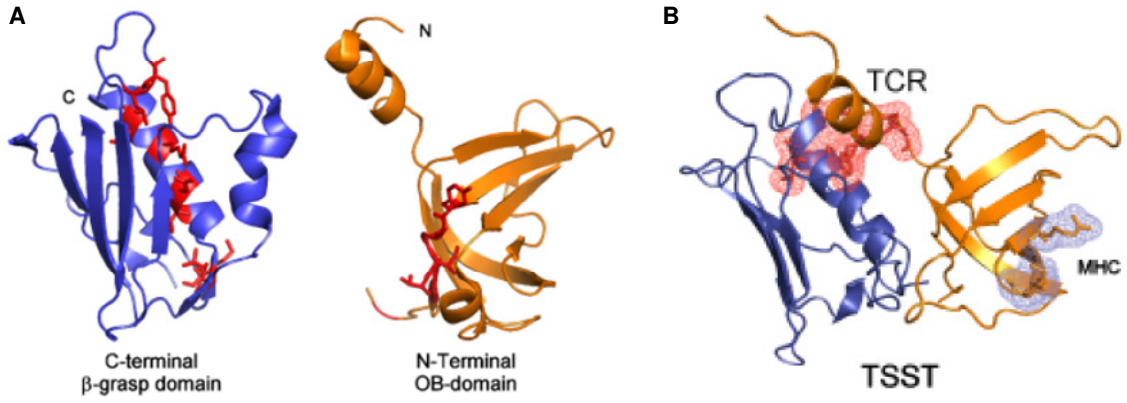


Figure 2. The two domain structure of SAgs. Adapted from Fraser and Proft (65).
A) Staphylococcal SAgs consist of two protein domains. The β-grasp domain (blue) is a twisted β-sheet of four to five antiparallel strands that pack against a highly conserved α-helix. Residues depicted in red are conserved in all SAgs. The OB domain (orange) consists of a five-stranded β-sheets that coils to form a β-barrel and is capped by a small α-helix. B) Structure of TSST-1. The MHC II binding site is in the OB-fold, and the TCR binding residues are located on the face of the C-terminal domain.

The N-terminal domain is an oligosaccharide/ oligonucleotide-binding fold (OB-fold) (65). The OB domain is a common protein domain found in different bacterial toxins such as the AB5 toxin family, including the cholera toxin and pertussis toxin, and in nucleases (65, 83). The larger C-terminal domain is a β-grasp fold consisting of a twisted β-sheet capped by the central α-helix.

Some of the bacterial SAgs are dependent on zinc ions to be functional and to be able to properly bind MHC II. Crystal structures of some of these SAgs in complex with MHC II show that zinc is important for the SAg to interact with MHC II with high affinity (84, 85). In addition, the zinc ion is important for the three-dimensional stability of the SAg itself (86, 87).

Genetic localization of SAgs

Genetic analysis of *S. aureus* clinical isolates, including whole genome sequencing, has shown that ~ 80% of all *S. aureus* clinical isolates harbor SAg genes, on average five to six (88, 89). Furthermore, an extensive heterogeneity of SAg gene patterns between *S. aureus* strains has been observed (88-90). SAgs are encoded on MGEs, such as bacteriophages, plasmids, *S. aureus* pathogenicity islands (SaPI), and genomic islands (table I) (38).

Role of SAgs in staphylococcal virulence

SAgs are the causative agents for several diseases. While their role in staphylococcal food poisoning and TSS is well defined, their contribution in other diseases, like Kawasaki disease and atopic dermatitis, is unclear.

Staphylococcal food poisoning results from ingestion of food contaminated with SAgs. It is characterized by self-limiting nausea, emesis, abdominal pain or cramping, and diarrhea after a short incubation time (91). Due to their impressive stability to denaturing conditions, such as heat and low pH, SAgs are not completely destroyed by mild cooking or digestion of food in the stomach.

TSS is characterized by high fever, rash, desquamation, hypotension and major organ involvement. In TSS, *S. aureus* is predominantly found at mucosal sites (vaginal or nasophryngeal) or in localized abscesses (65). Bergdoll et al. demonstrated that 94% of confirmed menstrual TSS isolates produced high amounts of TSST-1 compared with 4% for non-TSS strains (92). TSST-1 and other SAgs, like SEA, SEB and SEC, have been associated with non-menstrual TSS (93).

The role of SAgs in other forms of sepsis is less well defined. In contrast to gram-negative bacteria, gram-positive bacteria like *S. aureus* do not induce septic shock by

lipopolysaccharid (LPS), but via cell wall components (e.g., peptidoglycans, lipoteichoic acids) or – in case of *S. aureus* and *Streptococcus pyogenes* – by SAgs (94). Peptidoglycans and lipoteichoic acids act as pathogen associated molecular patterns (PAMP), like LPS, and are recognized by pattern recognition receptors (PRR) on macrophages and monocytes. By binding to toll like receptor 2 (TLR2) and CD14 they activate a massive production of proinflammatory mediators, which are responsible for the symptoms of sepsis. In contrast, SAgs activate T cells to secrete high amounts of cytokines. In animal models, SAgs and LPS very effectively synergize in the induction of lethal shock, and these observations motivated the development of the two-hit model of septic shock (95). According to Holtfreter et al. the first hit comprises all PAMPs which activate the innate immune system, and the second hit SAgs or another T cell stimulus (52).

Kawasaki disease is an acute febrile disease in children that resembles TSS. Although the etiological agent is not known, a role for SAgs was suggested (96). Intravenous immunoglobulin therapy is highly effective when given early, suggesting that the agent is a toxin that is neutralized by anti-toxin antibodies contained in pooled human serum.

Recently, a role for SAgs in skin and airway allergies was suggested. For atopic dermatitis a correlation between clinical severity and colonization with SEA- and SEB-producing *S. aureus* as well as with IgE with specificity for SEA and SEB were observed in one study but not confirmed in others (97, 98).

A special case – the *egc* SAgs

The genes of the recently described enterotoxin gene cluster (*egc*) are the most prevalent SAg genes in commensal and invasive *S. aureus* isolates with frequencies ranging between 46 and 66% (47, 99-101). In 2001 Jarraud et al. described the cluster which is located on a staphylococcal genomic island (νSaβ) (88, 102). The *egc* harbors five SAg genes (*seg*, *sei*, *selm*, *seln*, and *selo)* and the pseudogenes *ψ ent1* and *ψ ent2* (88). The SAg gene loci were likely generated through gene duplication and variation from an ancestral gene (88). Letertre et al. have described an additional SAg, designated SElU that is a fusion product of *ψ ent1* and *ψ ent2* (102, 103). In contrast to the non-*egc* SAgs, the *egc* is organized as an operon; its genes are transcribed into a polycistronic mRNA (88). Given that each of the *egc* SAgs showed the strongest homology with one of the

known SAgs encoded outside the *egc* on monocistronic loci (Figure 3), Jarraud et al. suggested the *egc* to be an enterotoxin gene nursery (88).

Although *egc* genes are the most prevalent SAg genes in commensal and invasive *S. aureus* isolates, they appear to cause toxic shock only very rarely (104). In fact, one study suggested that *egc* SAgs are significantly enriched in commensal strains compared to invasive isolates, and their presence is negatively correlated with severity of *S. aureus* sepsis (105, 106). Therefore it was suggested that the *egc* is associated with non-invasiveness and a lower disease-evoking potential.

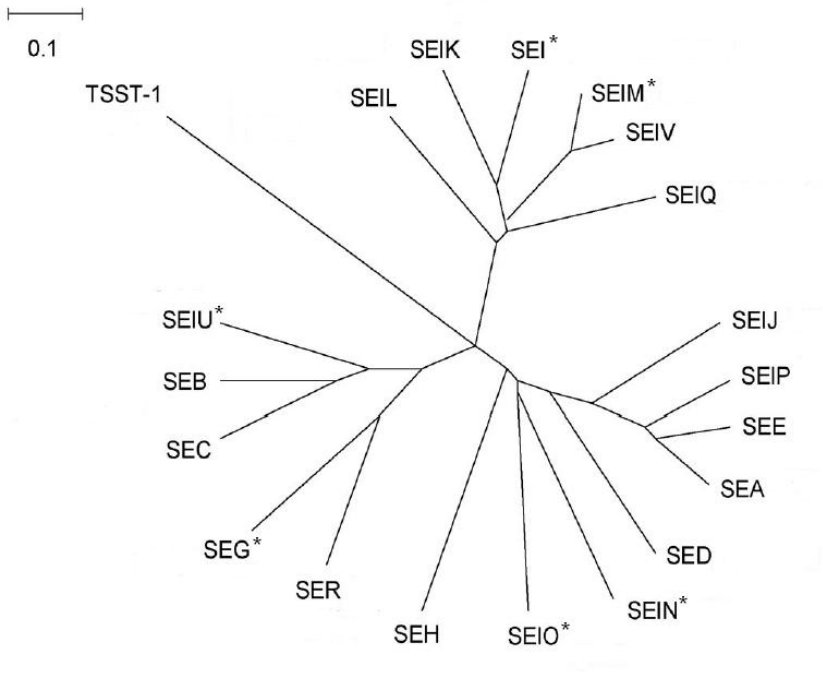


Figure 3. Phylogenetic tree of staphylococcal SAgs. Adapted from Thomas et al. (66). The dendrogram was constructed based on the amino acid sequences using SplitsTree4 software (neighbour joining method). Each *egc* SAg (asterisks) shows strongest homology with one of the non-*egc* SAgs.

Antibody response against staphylococcal SAgs

In addition to their superantigenicity, SAgs, like other proteins, also act as conventional antigens and induce a specific antibody response. Neutralizing serum antibodies against non-*egc* SAgs (e.g., TSST-1, SEA, SEB, and SEC) are common in the healthy population (89, 107-112). Anti-SAg antibodies are clinically important since they have been shown to be protective in patients as well as in animal models (113-116). In *S. aureus* carriers, these antibodies are highly specific for the SAgs of the colonizing strain and very effectively neutralize their mitogenic effects (32). In contrast, neutralizing antibodies against *egc* SAgs are very rare, even among carriers of *egc*-positive *S. aureus* strains (89) (and unpublished observations).

This "*egc* gap" in the antibody response of healthy individuals was unexpected, since *egc* SAgs are by far the most prevalent SAgs in clinical *S. aureus* isolates (47, 99-101). Furthermore, it appears unlikely, that *egc* and non-*egc* SAgs differ systematically in their immunogenicity, because the amino acid sequences of the *egc* SAgs are more closely related to those of individual non-*egc* SAg than to each other (Figure 3) (88, 91). Thus, what could be the reason for the lack of anti-*egc* antibodies?

1.5 OUTLINE OF THE THESIS

To shed light i) on the bacterial virulence determinants and ii) on the role of antibodies in the interaction between *S. aureus* and its host, the aims of the present thesis were

1) to identify virulence determinants by comparing the prevalence of SAg genes and phages among colonizing and invasive *S. aureus* isolates and to correlate it with the clonal background,
2) to determine the presence and the development of anti-SAg antibodies in humans, both in *S. aureus* colonization and bacteremia and
3) to elucidate the reasons for the selective lack of neutralizing serum antibodies specific for a subgroup of SAgs, the *egc*-SAgs.

Chapter 2 to 4 describe studies that aim at identifying staphylococcal virulence determinants in bacteremia and furunculosis. In **Chapter 2**, the highly diverse SAg gene patterns of nasal and blood culture isolates from Western Pomerania were analyzed using multiplex PCR and correlated with the clonal background. **Chapter 3** characterizes the phage profiles of nasal and blood culture isolates from different sources in Germany. In **Chapter 4** virulence genes and phage profiles from nasal and furunculosis isolates from Szczecin, Poland, were determined and correlated with the clonal background.

The following two chapters focus on the role of anti-SAg antibodies in colonization as well as in *S. aureus* bacteremia. **Chapter 5** describes a new and simple method to determine antibody levels using Luminex® technology. The SAg-neutralizing antibody response developing during *S. aureus* bacteremia was characterized in **Chapter 6**. Antibodies against *egc* SAgs were absent at the acute phase of bacteremia and not induced during convalescence.

The lack of neutralizing antibodies against *egc* SAgs, previously observed in healthy individuals (89), and now demonstrated during infection, was surprising, because *egc* SAgs are the most prevalent SAgs in *S. aureus*. Therefore we tested, whether the observed "*egc* gap" in the antibody profiles was due to i) differential immune cell activating properties of *egc* and non-*egc* SAgs or ii) their differential regulation (**Chapter 7**).

REFERENCES

1. Schaffer, A. C., and J. C. Lee. 2008. Vaccination and passive immunisation against *Staphylococcus aureus*. *International journal of antimicrobial agents* 32 Suppl 1:S71-78.
2. Lowy, F. D. 1998. *Staphylococcus aureus* infections. *The New England journal of medicine* 339:520-532.
3. Steinberg, J. P., C. C. Clark, and B. O. Hackman. 1996. Nosocomial and community-acquired *Staphylococcus aureus* bacteremias from 1980 to 1993: impact of intravascular devices and methicillin resistance. *Clin Infect Dis* 23:255-259.
4. van Belkum, A., D. C. Melles, J. Nouwen, W. B. van Leeuwen, W. van Wamel, M. C. Vos, H. F. Wertheim, and H. A. Verbrugh. 2009. Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. *Infect Genet Evol* 9:32-47.
5. Lindsay, J. A., and M. T. Holden. 2004. *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol* 12:378-385.
6. Boucher, H. W., and G. R. Corey. 2008. Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin Infect Dis* 46 Suppl 5:S344-349.
7. Tristan, A., M. Bes, H. Meugnier, G. Lina, B. Bozdogan, P. Courvalin, M. E. Reverdy, M. C. Enright, F. Vandenesch, and J. Etienne. 2007. Global distribution of Panton-Valentine leukocidin--positive methicillin-resistant *Staphylococcus aureus*, 2006. *Emerging infectious diseases* 13:594-600.
8. Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Jama* 298:1763-1771.
9. Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, and D. A. Talan. 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. *The New England journal of medicine* 355:666-674.
10. Perichon, B., and P. Courvalin. 2009. VanA-type vancomycin-resistant *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy* 53:4580-4587.
11. Williams, R. E. 1963. Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. *Bacteriol Rev* 27:56-71.

12. Peacock, S. J., I. de Silva, and F. D. Lowy. 2001. What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol* 9:605-610.

13. Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. *The Lancet infectious diseases* 5:751-762.

14. Noble, W. C., R. E. Williams, M. P. Jevons, and R. A. Shooter. 1964. Some Aspects of Nasal Carriage of Staphylococci. *Journal of clinical pathology* 17:79-83.

15. Kluytmans, J. A., and H. F. Wertheim. 2005. Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection* 33:3-8.

16. Kluytmans, J., A. van Belkum, and H. Verbrugh. 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 10:505-520.

17. Nouwen, J. L., A. van Belkum, and H. A. Verbrugh. 2001. Determinants of *Staphylococcus aureus* nasal carriage. *The Netherlands journal of medicine* 59:126-133.

18. White, F. N., G. Ross, L. Barajas, and E. D. Jacobson. 1966. Hemodynamics of endotoxin shock in the rat and the effects of phenoxybenzamine. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y* 122:1025-1029.

19. Nouwen, J. L., A. Ott, M. F. Kluytmans-Vandenbergh, H. A. Boelens, A. Hofman, A. van Belkum, and H. A. Verbrugh. 2004. Predicting the *Staphylococcus aureus* nasal carrier state: derivation and validation of a "culture rule". *Clin Infect Dis* 39:806-811.

20. Nouwen, J. L., M. W. Fieren, S. Snijders, H. A. Verbrugh, and A. van Belkum. 2005. Persistent (not intermittent) nasal carriage of *Staphylococcus aureus* is the determinant of CPD-related infections. *Kidney international* 67:1084-1092.

21. van Belkum, A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim. 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. *The Journal of infectious diseases* 199:1820-1826.

22. Nouwen, J., H. Boelens, A. van Belkum, and H. Verbrugh. 2004. Human factor in *Staphylococcus aureus* nasal carriage. *Infection and immunity* 72:6685-6688.

23. Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond, and A. Peschel. 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nature medicine* 10:243-245.

24. Bogaert, D., A. van Belkum, M. Sluijter, A. Luijendijk, R. de Groot, H. C. Rumke, H. A. Verbrugh, and P. W. Hermans. 2004. Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet* 363:1871-1872.

25. Peacock, S. J., A. Justice, D. Griffiths, G. D. de Silva, M. N. Kantzanou, D. Crook, K. Sleeman, and N. P. Day. 2003. Determinants of acquisition and carriage of *Staphylococcus aureus* in infancy. *Journal of clinical microbiology* 41:5718-5725.

26. Perl, T. M., J. J. Cullen, R. P. Wenzel, M. B. Zimmerman, M. A. Pfaller, D. Sheppard, J. Twombley, P. P. French, and L. A. Herwaldt. 2002. Intranasal mupirocin to prevent postoperative *Staphylococcus aureus* infections. *The New England journal of medicine* 346:1871-1877.

27. Davis, K. A., J. J. Stewart, H. K. Crouch, C. E. Florez, and D. R. Hospenthal. 2004. Methicillin-resistant *Staphylococcus aureus* (MRSA) nares colonization at hospital admission and its effect on subsequent MRSA infection. *Clin Infect Dis* 39:776-782.

28. Kluytmans, J. A., J. W. Mouton, E. P. Ijzerman, C. M. Vandenbroucke-Grauls, A. W. Maat, J. H. Wagenvoort, and H. A. Verbrugh. 1995. Nasal carriage of *Staphylococcus aureus* as a major risk factor for wound infections after cardiac surgery. *The Journal of infectious diseases* 171:216-219.

29. von Eiff, C., K. Becker, K. Machka, H. Stammer, and G. Peters. 2001. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. *The New England journal of medicine* 344:11-16.

30. Boelaert, J. R. 1994. *Staphylococcus aureus* infection in haemodialysis patients. Mupirocin as a topical strategy against nasal carriage: a review. *Journal of chemotherapy (Florence, Italy)* 6 Suppl 2:19-24.

31. Wertheim, H. F., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh. 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* 364:703-705.

32. Holtfreter, S., K. Roschack, P. Eichler, K. Eske, B. Holtfreter, C. Kohler, S. Engelmann, M. Hecker, A. Greinacher, and B. M. Broker. 2006. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. *The Journal of infectious diseases* 193:1275-1278.

33. Bloom, B. S., A. M. Fendrick, M. E. Chernew, and P. Patel. 1996. Clinical and economic effects of mupirocin calcium on preventing *Staphylococcus aureus* infection in hemodialysis patients: a decision analysis. *Am J Kidney Dis* 27:687-694.

34. Bode, L. G., J. A. Kluytmans, H. F. Wertheim, D. Bogaers, C. M. Vandenbroucke-Grauls, R. Roosendaal, A. Troelstra, A. T. Box, A. Voss, I. van der Tweel, A. van Belkum, H. A. Verbrugh, and M. C. Vos. Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. *The New England journal of medicine* 362:9-17.

35. Boelaert, J. R., Y. A. De Baere, M. A. Geernaert, C. A. Godard, and H. W. Van Landuyt. 1991. The use of nasal mupirocin ointment to prevent *Staphylococcus aureus* bacteraemias in haemodialysis patients: an analysis of cost-effectiveness. *The Journal of hospital infection* 19 Suppl B:41-46.

36. Boelaert, J. R., H. W. Van Landuyt, C. A. Godard, R. F. Daneels, M. L. Schurgers, E. G. Matthys, Y. A. De Baere, D. W. Gheyle, B. Z. Gordts, and L. A. Herwaldt. 1993. Nasal mupirocin ointment decreases the incidence of *Staphylococcus aureus* bacteraemias in haemodialysis patients. *Nephrol Dial Transplant* 8:235-239.

37. Kluytmans, J. A., M. J. Manders, E. van Bommel, and H. Verbrugh. 1996. Elimination of nasal carriage of *Staphylococcus aureus* in hemodialysis patients. *Infect Control Hosp Epidemiol* 17:793-797.

38. Lindsay, J. A., and M. T. Holden. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus. Functional & integrative genomics* 6:186-201.

39. Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds. 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *Journal of bacteriology* 188:669-676.

40. Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* 95:3140-3145.

41. Koreen, L., S. V. Ramaswamy, E. A. Graviss, S. Naidich, J. M. Musser, and B. N. Kreiswirth. 2004. spa typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *Journal of clinical microbiology* 42:792-799.

42. Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W. Friedrich, and W. Witte. 2006. Assignment of Staphylococcus isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. *Journal of clinical microbiology* 44:2533-2540.

43. Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt. 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences of the United States of America* 99:7687-7692.

44. Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day. 2003. How clonal is *Staphylococcus aureus*? *Journal of bacteriology* 185:3307-3316.

45. Melles, D. C., R. F. Gorkink, H. A. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and A. van Belkum. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus. J Clin Invest* 114:1732-1740.

46. Lindsay, J. A. 2010. Genomic variation and evolution of *Staphylococcus aureus. Int J Med Microbiol* 300:98-103.

47. Monecke, S., C. Luedicke, P. Slickers, and R. Ehricht. 2009. Molecular epidemiology of *Staphylococcus aureus* in asymptomatic carriers. *Eur J Clin Microbiol Infect Dis* 28:1159-1165.

48. Waldron, D. E., and J. A. Lindsay. 2006. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *Journal of bacteriology* 188:5578-5585.

49. Wertheim, H. F., W. B. van Leeuwen, S. Snijders, M. C. Vos, A. Voss, C. M. Vandenbroucke-Grauls, J. A. Kluytmans, H. A. Verbrugh, and A. van Belkum. 2005. Associations between *Staphylococcus aureus* Genotype, Infection, and In-Hospital Mortality: A Nested Case-Control Study. *The Journal of infectious diseases* 192:1196-1200.

50. Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet* 367:731-739.

51. Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 13:16-34, table of contents.

52. Holtfreter, S., and B. M. Broker. 2005. Staphylococcal superantigens: do they play a role in sepsis? *Arch Immunol Ther Exp (Warsz)* 53:13-27.

53. Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infection and immunity* 70:631-641.

54. Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne. 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis* 29:1128-1132.

55. Ladhani, S. 2003. Understanding the mechanism of action of the exfoliative toxins of *Staphylococcus aureus*. *FEMS immunology and medical microbiology* 39:181-189.

56. Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day. 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infection and immunity* 70:4987-4996.

57. Langley, R., B. Wines, N. Willoughby, I. Basu, T. Proft, and J. D. Fraser. 2005. The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria. *J Immunol* 174:2926-2933.

58. Gomez, M. I., A. Lee, B. Reddy, A. Muir, G. Soong, A. Pitt, A. Cheung, and A. Prince. 2004. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nature medicine* 10:842-848.

59. Dryla, A., S. Prustomersky, D. Gelbmann, M. Hanner, E. Bettinger, B. Kocsis, T. Kustos, T. Henics, A. Meinke, and E. Nagy. 2005. Comparison of antibody repertoires against *Staphylococcus aureus* in healthy individuals and in acutely infected patients. *Clinical and diagnostic laboratory immunology* 12:387-398.

60. van den Akker, E. L., J. L. Nouwen, D. C. Melles, E. F. van Rossum, J. W. Koper, A. G. Uitterlinden, A. Hofman, H. A. Verbrugh, H. A. Pols, S. W. Lamberts, and A. van Belkum. 2006. *Staphylococcus aureus* nasal carriage is associated with glucocorticoid receptor gene polymorphisms. *The Journal of infectious diseases* 194:814-818.

61. Proft, T., and J. D. Fraser. 2003. Bacterial superantigens. *Clinical and experimental immunology* 133:299-306.

62. Lina, G., G. A. Bohach, S. P. Nair, K. Hiramatsu, E. Jouvin-Marche, and R. Mariuzza. 2004. Standard nomenclature for the superantigens expressed by Staphylococcus. *The Journal of infectious diseases* 189:2334-2336.

63. Schlievert, P. M., and G. A. Bohach. 2007. Staphylococcal and streptococcal superantigens: an update. In *Superantigens: Molecular basis for their role in human diseases*. M. Kotb, and J. Fraser, eds. ASM Press, Washington, DC.

64. Ono, H. K., K. Omoe, K. Imanishi, Y. Iwakabe, D. L. Hu, H. Kato, N. Saito, A. Nakane, T. Uchiyama, and K. Shinagawa. 2008. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. *Infection and immunity* 76:4999-5005.

65. Fraser, J. D., and T. Proft. 2008. The bacterial superantigen and superantigen-like proteins. *Immunological reviews* 225:226-243.

66. Thomas, D., O. Dauwalder, V. Brun, C. Badiou, T. Ferry, J. Etienne, F. Vandenesch, and G. Lina. 2009. *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. *Infection and immunity* 77:2043-2050.

67. Fraser, J., V. Arcus, P. Kong, E. Baker, and T. Proft. 2000. Superantigens - powerful modifiers of the immune system. *Mol Med Today* 6:125-132.

68. White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack. 1989. The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* 56:27-35.

69. Choi, Y. W., A. Herman, D. DiGiusto, T. Wade, P. Marrack, and J. Kappler. 1990. Residues of the variable region of the T-cell-receptor beta-chain that interact with *S. aureus* toxin superantigens. *Nature* 346:471-473.

70. Kappler, J., B. Kotzin, L. Herron, E. W. Gelfand, R. D. Bigler, A. Boylston, S. Carrel, D. N. Posnett, Y. Choi, and P. Marrack. 1989. V beta-specific stimulation of human T cells by staphylococcal toxins. *Science (New York, N.Y* 244:811-813.

71. Petersson, K., H. Pettersson, N. J. Skartved, B. Walse, and G. Forsberg. 2003. Staphylococcal enterotoxin H induces V alpha-specific expansion of T cells. *J Immunol* 170:4148-4154.

72. Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Matis. 1990. In vivo induction of anergy in peripheral V beta 8+ T cells by staphylococcal enterotoxin B. *The Journal of experimental medicine* 172:1091-1100.

73. Alderson, M. R., T. W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. A. Schooley, R. G. Goodwin, C. A. Smith, F. Ramsdell, and D. H. Lynch. 1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *The Journal of experimental medicine* 181:71-77.

74. Schad, E. M., I. Zaitseva, V. N. Zaitsev, M. Dohlsten, T. Kalland, P. M. Schlievert, D. H. Ohlendorf, and L. A. Svensson. 1995. Crystal structure of the superantigen staphylococcal enterotoxin type A. *The EMBO journal* 14:3292-3301.

75. Papageorgiou, A. C., H. S. Tranter, and K. R. Acharya. 1998. Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 A resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors. *Journal of molecular biology* 277:61-79.

76. Papageorgiou, A. C., K. R. Acharya, R. Shapiro, E. F. Passalacqua, R. D. Brehm, and H. S. Tranter. 1995. Crystal structure of the superantigen enterotoxin C2 from *Staphylococcus aureus* reveals a zinc-binding site. *Structure* 3:769-779.

77. Sundstrom, M., L. Abrahmsen, P. Antonsson, K. Mehindate, W. Mourad, and M. Dohlsten. 1996. The crystal structure of staphylococcal enterotoxin type D reveals Zn2+-mediated homodimerization. *The EMBO journal* 15:6832-6840.

78. Fernandez, M. M., S. Bhattacharya, M. C. De Marzi, P. H. Brown, M. Kerzic, P. Schuck, R. A. Mariuzza, and E. L. Malchiodi. 2007. Superantigen natural affinity maturation revealed by the crystal structure of staphylococcal enterotoxin G and its binding to T-cell receptor Vbeta8.2. *Proteins* 68:389-402.

79. Hakansson, M., K. Petersson, H. Nilsson, G. Forsberg, P. Bjork, P. Antonsson, and L. A. Svensson. 2000. The crystal structure of staphylococcal enterotoxin H: implications for binding properties to MHC class II and TcR molecules. *Journal of molecular biology* 302:527-537.

80. Fernandez, M. M., R. Guan, C. P. Swaminathan, E. L. Malchiodi, and R. A. Mariuzza. 2006. Crystal structure of staphylococcal enterotoxin I (SEI) in complex with a human major histocompatibility complex class II molecule. *The Journal of biological chemistry* 281:25356-25364.

81. Gunther, S., A. K. Varma, B. Moza, K. J. Kasper, A. W. Wyatt, P. Zhu, A. K. Rahman, Y. Li, R. A. Mariuzza, J. K. McCormick, and E. J. Sundberg. 2007. A novel loop domain in superantigens extends their T cell receptor recognition site. *Journal of molecular biology* 371:210-221.

82. Acharya, K. R., E. F. Passalacqua, E. Y. Jones, K. Harlos, D. I. Stuart, R. D. Brehm, and H. S. Tranter. 1994. Structural basis of superantigen action inferred from crystal structure of toxic-shock syndrome toxin-1. *Nature* 367:94-97.

83. Mitchell, D. T., D. G. Levitt, P. M. Schlievert, and D. H. Ohlendorf. 2000. Structural evidence for the evolution of pyrogenic toxin superantigens. *Journal of molecular evolution* 51:520-531.

84. Li, Y., H. Li, N. Dimasi, J. K. McCormick, R. Martin, P. Schuck, P. M. Schlievert, and R. A. Mariuzza. 2001. Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. *Immunity* 14:93-104.

85. Petersson, K., M. Hakansson, H. Nilsson, G. Forsberg, L. A. Svensson, A. Liljas, and B. Walse. 2001. Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. *The EMBO journal* 20:3306-3312.

86. Cavallin, A., K. Petersson, and G. Forsberg. 2003. Spectrophotometric methods for the determination of superantigen structure and stability. *Methods in molecular biology (Clifton, N.J* 214:55-63.

87. Cavallin, A., H. Arozenius, K. Kristensson, P. Antonsson, D. E. Otzen, P. Bjork, and G. Forsberg. 2000. The spectral and thermodynamic properties of staphylococcal enterotoxin A, E, and variants suggest that structural modifications are important to control their function. *The Journal of biological chemistry* 275:1665-1672.

88. Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* 166:669-677.

89. Holtfreter, S., K. Bauer, D. Thomas, C. Feig, V. Lorenz, K. Roschack, E. Friebe, K. Selleng, S. Lovenich, T. Greve, A. Greinacher, B. Panzig, S. Engelmann, G. Lina, and B. M. Broker. 2004. *egc*-Encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. *Infection and immunity* 72:4061-4071.

90. Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. Von Eiff. 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *Journal of clinical microbiology* 41:1434-1439.

91. Thomas, D., S. Chou, O. Dauwalder, and G. Lina. 2007. Diversity in *Staphylococcus aureus* enterotoxins. *Chem Immunol Allergy* 93:24-41.

92. Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis. 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome *Staphylococcus aureus* isolates. *Lancet* 1:1017-1021.

93. McCormick, J. K., J. M. Yarwood, and P. M. Schlievert. 2001. Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol* 55:77-104.

94. Blank, C., A. Luz, S. Bendigs, A. Erdmann, H. Wagner, and K. Heeg. 1997. Superantigen and endotoxin synergize in the induction of lethal shock. *Eur J Immunol* 27:825-833.

95. Bannan, J., K. Visvanathan, and J. B. Zabriskie. 1999. Structure and function of streptococcal and staphylococcal superantigens in septic shock. *Infectious disease clinics of North America* 13:387-396, ix.

96. Yarwood, J. M., D. Y. Leung, and P. M. Schlievert. 2000. Evidence for the involvement of bacterial superantigens in psoriasis, atopic dermatitis, and Kawasaki syndrome. *FEMS microbiology letters* 192:1-7.

97. Bunikowski, R., M. Mielke, H. Skarabis, U. Herz, R. L. Bergmann, U. Wahn, and H. Renz. 1999. Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *The Journal of allergy and clinical immunology* 103:119-124.

98. Zollner, T. M., T. A. Wichelhaus, A. Hartung, C. Von Mallinckrodt, T. O. Wagner, V. Brade, and R. Kaufmann. 2000. Colonization with superantigen-producing *Staphylococcus aureus* is associated with increased severity of atopic dermatitis. *Clin Exp Allergy* 30:994-1000.

99. Becker, K., A. W. Friedrich, G. Peters, and C. von Eiff. 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO, and SElN. *Mol Nutr Food Res* 48:488-495.

100. Fueyo, J. M., M. C. Mendoza, M. A. Alvarez, and M. C. Martin. 2005. Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. *FEMS microbiology letters* 243:447-454.

101. Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Broker. 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *Journal of clinical microbiology* 45:2669-2680.

102. Letertre, C., S. Perelle, F. Dilasser, and P. Fach. 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J Appl Microbiol* 95:38-43.

103. Thomas, D. Y., S. Jarraud, B. Lemercier, G. Cozon, K. Echasserieau, J. Etienne, M. L. Gougeon, G. Lina, and F. Vandenesch. 2006. Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. *Infection and immunity* 74:4724-4734.

104. Jarraud, S., G. Cozon, F. Vandenesch, M. Bes, J. Etienne, and G. Lina. 1999. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *Journal of clinical microbiology* 37:2446-2449.

105. Ferry, T., D. Thomas, A. L. Genestier, M. Bes, G. Lina, F. Vandenesch, and J. Etienne. 2005. Comparative prevalence of superantigen genes in *Staphylococcus aureus* isolates causing sepsis with and without septic shock. *Clin Infect Dis* 41:771-777.

106. van Belkum, A., D. C. Melles, S. V. Snijders, W. B. van Leeuwen, H. F. Wertheim, J. L. Nouwen, H. A. Verbrugh, and J. Etienne. 2006. Clonal distribution and differential occurrence of the enterotoxin gene cluster, *egc*, in carriage- versus bacteremia-associated isolates of *Staphylococcus aureus*. *Journal of clinical microbiology* 44:1555-1557.

107. Banks, M. C., N. S. Kamel, J. B. Zabriskie, D. H. Larone, D. Ursea, and D. N. Posnett. 2003. *Staphylococcus aureus* express unique superantigens depending on the tissue source. *The Journal of infectious diseases* 187:77-86.

108. Jozefczyk, Z. 1974. Specific human antibodies to enterotoxins A, B, and C1 of Staphylococcus: their increased synthesis in staphylococcal infection. *The Journal of infectious diseases* 130:1-7.

109. LeClaire, R. D., and S. Bavari. 2001. Human antibodies to bacterial superantigens and their ability to inhibit T-cell activation and lethality. *Antimicrobial agents and chemotherapy* 45:460-463.

110. Notermans, S., W. J. van Leeuwen, J. Dufrenne, and P. D. Tips. 1983. Serum antibodies to enterotoxins produced by *Staphylococcus aureus* with special reference to enterotoxin F and toxic shock syndrome. *Journal of clinical microbiology* 18:1055-1060.

111. Parsonnet, J., R. V. Goering, M. A. Hansmann, M. B. Jones, K. Ohtagaki, C. C. Davis, and K. Totsuka. 2008. Prevalence of toxic shock syndrome toxin 1 (TSST-1)-producing strains of *Staphylococcus aureus* and antibody to TSST-1 among healthy Japanese women. *Journal of clinical microbiology* 46:2731-2738.

112. Schroder, E., G. Kunstmann, H. Hasbach, and G. Pulverer. 1988. Prevalence of serum antibodies to toxic-shock-syndrom-toxin-1 and to staphylococcal enterotoxins A, B and C in West-Germany. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 270:110-114.

113. Bavari, S., B. Dyas, and R. G. Ulrich. 1996. Superantigen vaccines: a comparative study of genetically attenuated receptor-binding mutants of staphylococcal enterotoxin A. *The Journal of infectious diseases* 174:338-345.

114. LeClaire, R. D., R. E. Hunt, and S. Bavari. 2002. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. *Infection and immunity* 70:2278-2281.

115. Nilsson, I. M., M. Verdrengh, R. G. Ulrich, S. Bavari, and A. Tarkowski. 1999. Protection against *Staphylococcus aureus* sepsis by vaccination with recombinant staphylococcal enterotoxin A devoid of superantigenicity. *The Journal of infectious diseases* 180:1370-1373.

116. Ulrich, R. G., M. A. Olson, and S. Bavari. 1998. Development of engineered vaccines effective against structurally related bacterial superantigens. *Vaccine* 16:1857-1864.

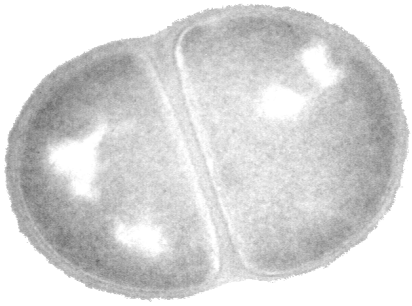
Chapter 2

Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates

S. Holtfreter, D. Grumann, M. Schmudde, H. T. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Bröker

PUBLISHED IN:

J Clin Microbiol 2007; 45:2669-2680



COMMENT:

In this manuscript we reported that two subclusters of the staphylococcal lineage CC25 can be discriminated by *spa* genotyping. *Egc*-positive t078 strains (and relatives) and *egc*-negative t056 strains (and relatives) were grouped into CC25 by BURP-analysis (costs: 5). Meanwhile MLST analyses revealed that they belong to two distinct lineages (http://www.spaserver.ridom.de). The *egc*-positive t078 strains (and relatives) belong to CC25 (MLST-Type ST26) and the *egc*-negative t056 strains (and relatives) to ST101. ST101 strains were significantly overrepresented among nasal isolates in comparison to blood culture isolates (9.3% vs, 2.2%, $P \leq 0.05$) while CC25 strains (*spa* type t078) were found in both cohorts (3.7% vs. 10.2%, not significant). The exfoliative toxin d (*etd*) was not observed in nasal isolates, but in 8 of 9 invasive CC25 (t078) strains.

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2007, p. 2669–2680 Vol. 45, No. 8
0095-1137/07/$08.00+0 doi:10.1128/JCM.00204-07

Clonal Distribution of Superantigen Genes in Clinical *Staphylococcus aureus* Isolates▽†

S. Holtfreter,[1] D. Grumann,[1] M. Schmudde,[1] H. T. T. Nguyen,[1] P. Eichler,[1] B. Strommenger,[4] K. Kopron,[2] J. Kolata,[1] S. Giedrys-Kalemba,[2] I. Steinmetz,[3] W. Witte,[4] and B. M. Bröker[1]*

Institute for Immunology and Transfusion Medicine, University of Greifswald, Greifswald, Germany[1]; Department of Microbiology and Immunology, Pomeranian Medical University, Szczecin, Poland[2]; Friedrich-Loeffler Institute for Medical Microbiology, University of Greifswald, Greifswald, Germany[3]; and National Reference Center for Staphylococci, Wernigerode, Germany[4]

Received 26 January 2007/Returned for modification 30 March 2007/Accepted 17 May 2007

Staphylococcus aureus **is both a successful human commensal and a major pathogen. The elucidation of the molecular determinants of virulence, in particular assessment of the contributions of the genetic background versus those of mobile genetic elements (MGEs), has proved difficult in this variable species. To address this, we simultaneously determined the genetic backgrounds (*spa* typing) and the distributions of all 19 known superantigens and the exfoliative toxins A and D (multiplex PCR) as markers for MGEs. Methicillin- sensitive *S. aureus* strains from Pomerania, 107 nasal and 88 blood culture isolates, were investigated. All superantigen-encoding MGEs were linked more or less tightly to the genetic background. Thus, each *S. aureus* clonal complex was characterized by a typical repertoire of superantigen and exfoliative toxin genes. However, within each *S. aureus* clonal complex and even within the same *spa* type, virulence gene profiles varied remarkably. Therefore, virulence genes of nasal and blood culture isolates were separately compared in each clonal complex. The results indicated a role in infection for the MGE harboring the exfoliative toxin D gene. In contrast, there was no association of superantigen genes with bloodstream invasion. In summary, we show here that the simultaneous assessment of virulence gene profiles and the genetic background increases the discriminatory power of genetic investigations into the mechanisms of *S. aureus* pathogenesis.**

Staphylococcus aureus is a major human pathogen capable of causing a wide range of infections, such as skin and tissue infections, toxin-mediated diseases, pneumonia, and bacteremia. At the same time, *S. aureus* is a persistent colonizer of the human nose in 20% of the population and is intermittently carried by another 30% (61). Colonization with *S. aureus* is a major risk factor for staphylococcal infections (47, 61). In carriers, 80% of nosocomial *S. aureus* bacteremia cases have an endogenous origin, which underlines the importance of host factors (59, 63). On the other hand, there is plenty of evidence that *S. aureus* clones differ in their disease-evoking potentials, but it has been difficult to explain these differences at the molecular level (39, 41, 51).

The species *S. aureus* has a highly clonal population structure with 10 predominant clonal lineages, as demonstrated by various genotyping analyses, such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and *spa* genotyping (9, 11, 41). *spa* genotyping is based on variations of the polymorphic region within the protein A gene (*spa*), which belongs to the staphylococcal core genome (22). It has a high discriminatory power similar to that of PFGE, but results can be more easily compared between laboratories (2, 30, 55). Moreover, *spa* typing, MLST, and PFGE are highly concordant, and *spa*-typing data can be easily mapped onto the MLST *S. aureus* database (http://www.spaserver.ridom.de) (30, 55).

Whole-genome microarrays recently revealed that the *S. aureus* genome consists of a core genome (~75%), a core variable genome (~10%), and mobile genetic elements (MGEs) (~15%) (39). MGEs, such as plasmids, phages, pathogenicity islands, and genomic islands, carry a variety of staphylococcal resistance and virulence genes. MGEs can be distributed by two distinct mechanisms. First, MGEs are passed on to daughter cells by vertical transmission and are, therefore, strongly associated with lineages (38). Secondly, MGEs can be horizontally transferred and thus spread between lineages (38, 40). Sometimes such MGEs are conspicuously absent from certain clonal complexes, presumably due to restrictions on horizontal transmission (31). Consequently, the distribution patterns of MGEs among clonal lineages reflect their mobility (39). For example, several research groups have reported that certain staphylococcal superantigen (SAg) genes are associated with particular clonal lineages (6, 27, 44, 51, 57).

SAgs are secreted toxins that induce a strong activation of large T-cell subpopulations. This can result in toxic shock (25). Eighty percent of all *S. aureus* strains harbor SAg genes, on average five or six, among which the *egc* SAgs are the most prevalent (5, 24, 26, 48). Most of the 19 described *S. aureus* SAgs, SEA to SEE, SEG to SER, SEU, and toxic shock syndrome toxin 1 (TSST-1), are encoded on phages and pathogenicity islands (38). Staphylococcal phage Φ3 carries either *sea* (strain Mu50), *sep* (N315), or *sea-sek-seq* (MW2) (3, 31). A family of related pathogenicity islands carry *seb-sek-seq* (SaPI1 in strain COL), *tst-sec3-sel* (SaPI2 in strains N315 and Mu50), or *sec-sel* (SaPI3 in strain MW2) (3, 17, 31). The enterotoxin gene cluster, *egc*, including *seg-sei-sem-sen-seo* and sometimes

* Corresponding author. Mailing address: Institut für Immunologie, Universität Greifswald, F.-Sauerbruch-Strasse/Diagnostikzentrum, 17487 Greifswald, Germany. Phone: 0 38 34-86 55 95. Fax: 0 38 34-86 54 90. E-mail: broeker@uni-greifswald.de.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 30 May 2007.

TABLE 1. Characteristics of the study population

Characteristic	Value for:			
	Nasal isolates			Blood culture isolates (BK)
	T	SH	SZ	
Study area	Western Pomerania	Western Pomerania	Sczcecin	Western Pomerania
Study population	Healthy blood donors	Healthy blood donors	Healthy blood donors	Hospital patients
No. of subjects	121	114	362	88
Time period	April–October 2002	February 2005–February 2006	March 2006	April 2002–October 2002 (n = 32) February 2005–February 2006 (n = 56)
Mean age (±SD) (yr)	33.8 (±11.4)	33.3 (±11.5)	28.9 (±9.2)	58.3 (±21.3)
Male sex (%)	64.5	62.3	52.8	61.4
No. of *S. aureus* isolates	55	52	108	88
Colonization status (two nose swabs)[a]				
No. of noncarriers (%)	70 (57.8)	63 (55.3)		
No. once positive (%)	29 (24.0)	24 (21.0)		
No. twice positive (%)	22 (18.2)	27 (23.7)		

[a] T and SH studies. Two nose swabs were obtained from healthy blood donors over a course of at least 10 weeks. The blood donors from both studies were on average 56.5% noncarriers, 21.0% persistent carriers (two positive nose swabs), and 22.5% intermittent carriers (one positive nose swab).

seu, is located on the genomic island vSAβ (31, 38). Other SAg genes are found on plasmids (*sed-sej-ser*) or on the antibiotic resistance cassette SCC*mec* (*seh*) (3, 49, 67).

The way in which staphylococcal virulence is determined on a molecular level remains elusive. Regarding the *S. aureus* core genome, nasal and invasive strains probably do not differ fundamentally, because they fall into the same main clusters (11, 41). Similarly, analysis of the core variable genome, which comprises lineage-specific genes for surface proteins and regulatory factors, did not identify factors clearly related to virulence (39). This suggests that staphylococcal virulence might primarily depend on MGE-encoded toxin or resistance genes (27), as has been shown for SCC*mec* and the gene of the Panton-Valentine leukocidin, the PVL locus (10, 41). However, except for some toxins, it has been difficult to assess the contributions of individual virulence determinants to *S. aureus* pathogenicity (32, 36, 39, 51).

Since many MGE-encoded virulence factors are linked to clonal complexes, analyses of their association with invasiveness could be biased by the underlying clonal population structure (51). Consequently, we propose that the simultaneous determination of the genetic background (clonal lineage) and virulence genes will increase the discriminatory power of investigations into the mechanisms of *S. aureus* pathogenesis. However, to date, such studies are rare (7, 27, 50). Because of their extraordinary variability in the species *S. aureus*, we have chosen *S. aureus* SAgs as a model to test this approach.

Here, we show the results of a comprehensive analysis of the diversity of staphylococcal SAgs in correlation with the genetic background in a large collection of *S. aureus* strains, including 107 nasal and 88 blood culture isolates from Western Pomerania in northeastern Germany. Our aims were to investigate to what degree the distribution of the known SAg genes is linked to the underlying clonality of the population and to test whether the analysis of SAg-carrying MGEs within defined clonal complexes would reveal differences between nasal and invasive isolates.

MATERIALS AND METHODS

Study population and bacterial isolates. (i) Nasal-carriage isolates. Two nose swabs were obtained from 121 healthy blood donors at the Institute of Immunology and Transfusion Medicine, University of Greifswald, over a course of at least 10 weeks from April to October 2002 (T strains) (Table 1). In a follow-up study (SH strains), nose swabs were obtained from 114 healthy blood donors between February 2005 and February 2006 (Table 1). A strain shift, as defined by different SAg and accessory gene regulator (*agr*) gene profiles, of the first and second nasal isolates occurred in 5/51 persistent carriers (T009, T098, T166, T169, and SH24). If the SAg and *agr* genes in both isolates were identical, only the first isolate was analyzed further. All participants gave informed consent, and both studies were approved by the Ethics Board of the University of Greifswald.

(ii) Blood culture isolates. Blood culture isolates (BK; n = 88) were obtained by the Friedrich-Loeffler-Institute of Medical Microbiology, University of Greifswald, from May to December 2002 (n = 32) and from January 2005 to February 2006 (n = 56) (Table 1). Most isolates were obtained from patients from different wards of the University Hospital of Greifswald (40 internal medicine, 10 surgery, 9 intensive-care unit, 7 neurology, 3 neurosurgery, 2 neonatology, 2 urology, 1 gynecology, and 4 pediatrics). Six isolates were from a general hospital near Greifswald (Wolgast); four strains were isolated from patients from a neurorehabilitation center in Greifswald. Only one isolate from each patient was included. We observed no spatial or temporal clustering of *S. aureus* genotypes.

(iii) Nasal-carriage isolates from Sczcecin. One hundred eight nasal *S. aureus* isolates (SZ) were obtained from 362 blood donors at the Department of Microbiology and Immunology, Pomeranian Medical University, Sczcecin, Eastern Pomerania, Poland, in March 2006. All participants gave informed consent, and the study was approved by the Ethics Board of the University of Sczcecin.

(iv) Control strains. Control strains for the PCR-based assays included A920210 (*egc*, *eta*, and *agr-4*) (28), CCM5757 (*seb*, *sek*, *seq*, and *agr-1*), Col (*seb*, *sek*, *seq*, *mecA*, and *agr-1*) (3), FRI1151m (*sed*, *sej*, *ser*, and *agr-1*) (27), FRI137 (*sec*, *seh*, *sel*, *egc* plus *seu*, and *agr-2*), FRI913 (*sea*, *sec*, *see*, *sek*, *sel*, *seq*, *tst*, and *agr-1*), N315 (*sep*, *sec*, *sel*, *tst*, *egc*, *mecA*, and *agr-2*) (31), TY114 (*etd* and *agr-3*), and 8325-4 (no SAg genes).

***S. aureus* identification and DNA isolation.** *S. aureus* was identified using standard diagnostic procedures and a gyrase PCR (see below). Total DNA of *S. aureus* was isolated with the Promega Wizard DNA purification kit (Promega, Mannheim, Germany) according to the manufacturer's instructions.

***spa* genotyping.** PCR for amplification of the *S. aureus* protein A (*spa*) repeat region was performed according to the published protocol (2, 22). PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced using two amplification primers from a commercial supplier (SeqLab, Goettingen, Germany). The forward and reverse sequence chromatograms were analyzed with the Ridom StaphType software (Ridom GmbH,

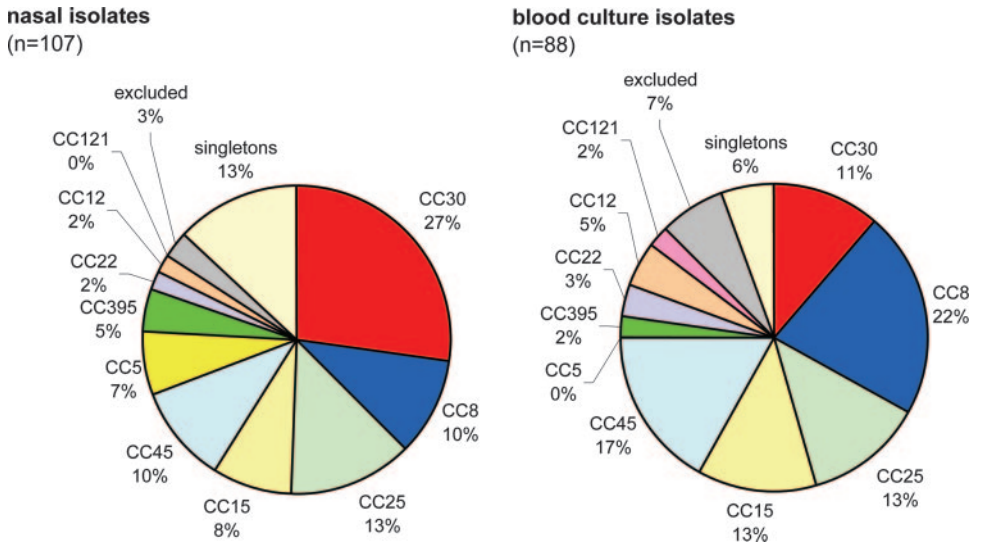


FIG. 1. Distribution of nasal and blood culture isolates within clonal complexes. *spa* types were clustered into 10 CCs by BURP analysis using a cost of 5 as the threshold for clustering. MLST CC nomenclature was deduced from *spa* CCs using the Ridom SpaServer database. CC30 was overrepresented among nasal strains ($P = 0.01$), CC8 was overrepresented among blood culture isolates ($P = 0.05$), and CC5 included only nasal isolates ($P = 0.05$).

Würzburg, Germany). A *spa* type is deduced from the sequence and number of *spa* repeats, which are generated by point mutations and intrachromosomal recombination events. Mutation of a single base pair results in a different *spa* type. With the BURP algorithm (Ridom GmbH), *spa* types were clustered into different groups, the calculated cost between members of a group being ≤5. The calculated cost reflects the evolutionary distance between two isolates. *spa* types shorter than five repeats were excluded from the analysis, because they did not allow the reliable deduction of ancestries.

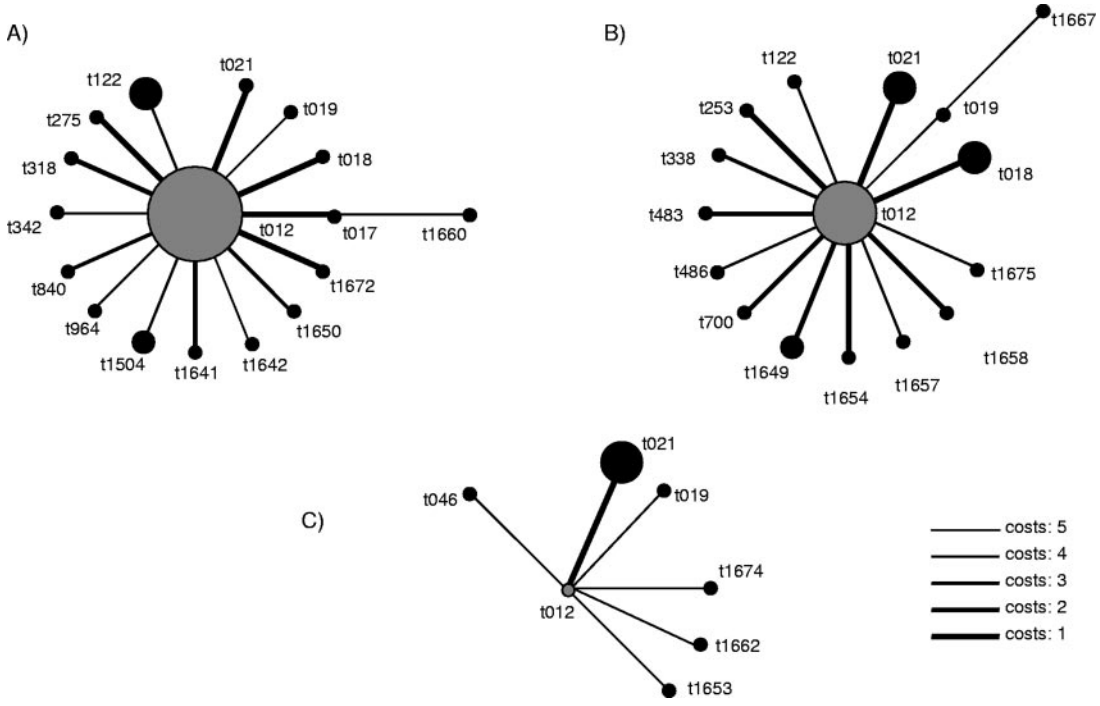


FIG. 2. Clonal relationships of Pomeranian CC30 isolates of different clinical origins. (A) Nasal isolates from Western Pomerania. (B) Nasal isolates from Sczcecin. (C) Blood culture isolates from Western Pomerania. Clusters were created with the BURP algorithm of the Ridom SpaType Software. The size of each *spa* circle reflects the number of isolates belonging to the *spa* type. The thickness of the connecting lines reflects the calculated costs, which express the evolutionary distances. The founder of a cluster, i.e., the *spa* type with the highest number of direct relatives, is shaded in gray. Blood culture isolates from one *spa* type were not outbreak related. *spa* type t037 isolates were excluded from the CC30 cluster because they are known to belong to MLST ST239 (CC8). *spa* t037 isolates have arisen from a single recombination event that involved the exchange of a DNA fragment, including the *spa* t037 gene, between MLST30 and MLST 239 (52, 53).

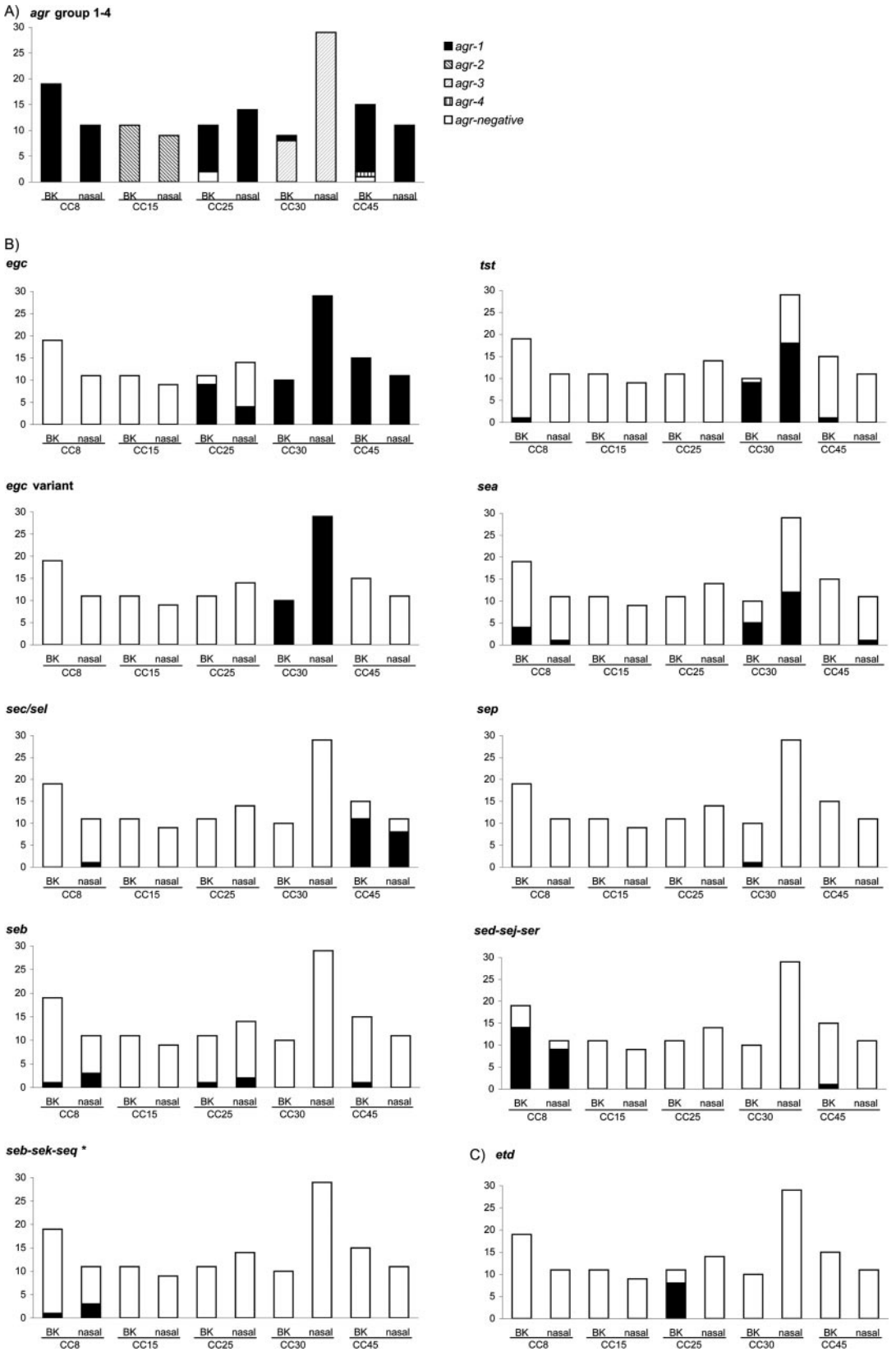


FIG. 3. Distribution of (A) *agr-1* to *-4*, (B) SAg genes or SAg gene combinations, and (C) *etd* within the five major clonal complexes. The overall heights of the bars denote the total number of isolates within the complex. The height of the shaded area represents the number of isolates positive for the respective gene. The minor lineages CC5, -12, -22, -121, and -395 and singletons were excluded from this analysis. An *egc* variant is an *egc* cluster with *seu* and an *sem* allelic variant. *, one strain harbored only *seb-seq* (T198-1; CC8).

MLST genotyping. MLST genotyping was performed on selected *S. aureus* isolates (also see Fig. 4 and 5) according to published protocols (9). Otherwise, MLST clonal complexes (CCs) were deduced from BURP grouping of *spa* types using the Ridom SpaServer database (http://www.spaserver.ridom.de) (55).

SAg multiplex PCRs. 16S rRNA PCR was performed with each DNA preparation to control the DNA quality and absence of PCR inhibitors (24). Gyrase primers allowed the identification of *S. aureus* (37). Methicillin resistance was detected with *mecA*-specific primers (45). All strains were negative for the PVL locus, as tested by PCR for *lukS-lukF* (27). Six sets of multiplex PCRs were established, partially based on published protocols, to amplify (i) *sea*, *seh*, *sec*, and *tst*; (ii) *sed*, *etd*, *eta*, and *sek*; (iii) *see*, *seb*, *sem*, *sel*, and *seo*; (iv) *sen*, *seg*, *seq*, and *sej*; (v) *sei*, *ser*, *seu*, and *sep*; and (vi) *agr-1* to *-4* (see Table S1 in the supplemental material). Primer pairs for detection of *sea* to *see*, *seh*, *sem*, *seo*, *tst*, *eta*, *etd*, the PVL locus, and *agr-1* to *-4* were previously described (27, 28, 35, 66). Primers for *seg*, *sei*, and *sej* were modified from published primer sequences (42), and primers for *sel*, *sek*, *sen*, *sep*, *seq*, *ser*, and *seu* were designed for this study (see Table S1 in the supplemental material).

Single and multiplex PCRs were performed with the GoTaq Flexi DNA polymerase system (Promega). Each reaction mixture (25 μl) contained 5 μl 5× GoTaq reaction buffer, 100 μM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Roche Diagnostics, Mannheim, Germany), 5 mM $MgCl_2$, 150 to 400 nM of each primer, 1.0 U GoTaq Flexi DNA polymerase, and 10 to 20 ng of template DNA. An initial denaturation of DNA at 95°C for 5 min was followed by 30 cycles of amplification (95°C for 30 s, 55°C for 30 s, and 72°C for 60 s), ending with a final extension phase at 72°C for 10 min. All PCR products were resolved by electrophoresis in 1.5% agarose gels (1× Tris-borate-EDTA buffer), stained with ethidium bromide, and visualized under UV light. Positive controls included DNA from SAg gene-positive *S. aureus* reference strains. In addition to standard PCR controls for contamination events, *S. aureus* strain 8325-4 served as a SAg gene-negative control.

***sem* gene sequencing.** The PCR for amplification of the *sem* gene variant was performed with sequencing primers (sems-1 and -2) flanking the *sem* open reading frame using the HotGoldStar Polymerase system (Eurogentec, Seraign, Belgium). Each reaction mixture (50 μl) contained 5 μl 10× HotGoldStar reaction buffer, 200 μM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 1 μM of primers (sems-1 and sems-2), 1.0 U HotGoldstar DNA polymerase, and 10 to 20 ng of template DNA. An initial denaturation of DNA at 95°C for 10 min was followed by 30 cycles of amplification (95°C for 30 s, 56.8°C for 40 s, and 72°C for 60 s), ending with a final extension phase at 72°C for 10 min. Sequencing was performed as described above.

Statistical analysis. Differences between groups were assessed using the chi-square test. *P* values of <0.05 were considered statistically significant. Contingency tables were used to compare the prevalences of a particular SAg gene or *agr* type between clonal complexes.

Nucleotide sequence accession number. The nucleotide sequence of the *sem* gene variant found in CC30 (*sem*_CC30) has been deposited at GenBank (accession number EF551341).

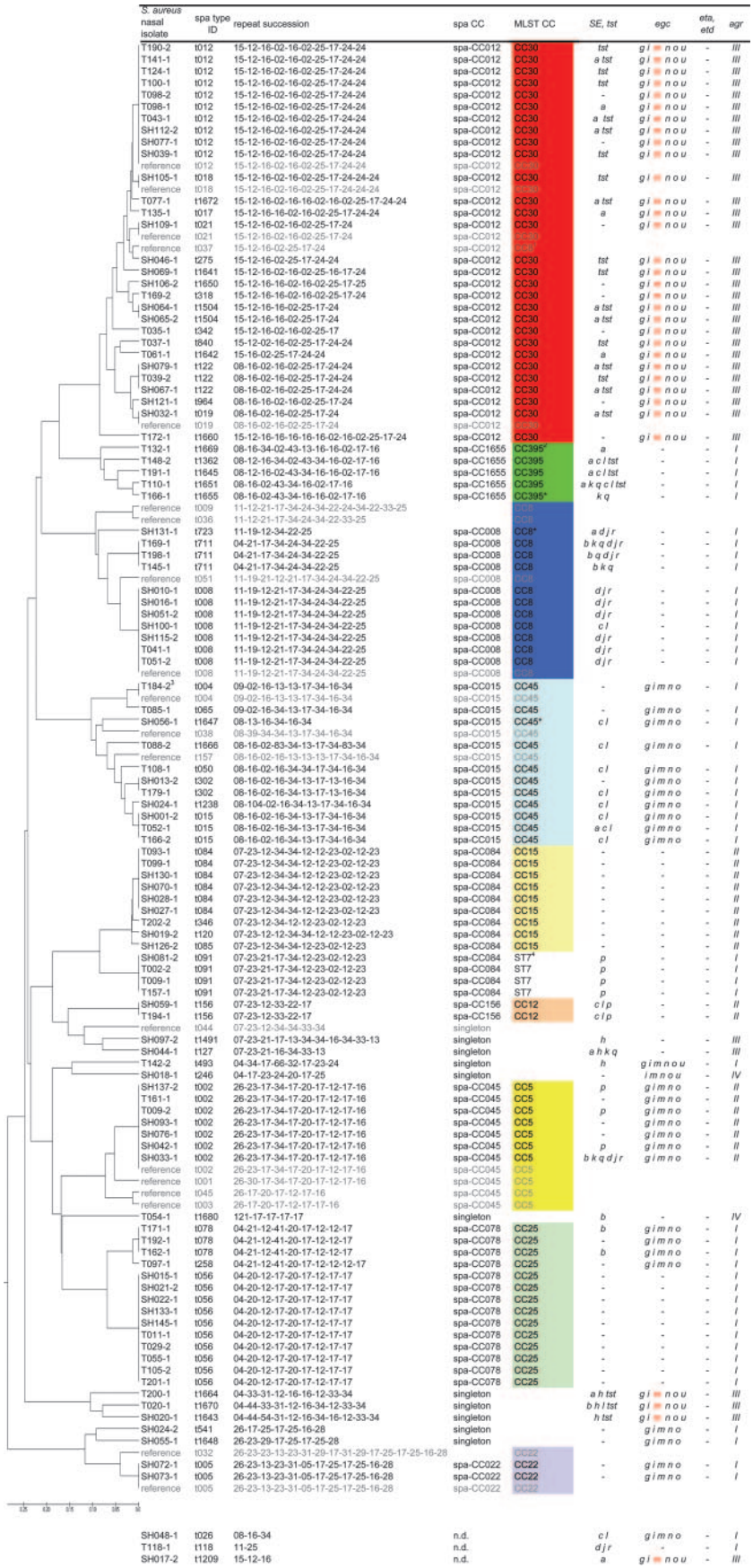
RESULTS

In this study, we investigated the *spa* genotypes, *agr* groups, and SAg gene patterns of nasal and blood culture *S. aureus* isolates from Western Pomerania in northeastern Germany. The demographic data are summarized in Table 1. A total of 107 nasal isolates were obtained from healthy blood donors during two studies on *S. aureus* nasal colonization (T strains and SH strains). Similar colonization patterns were observed in both study groups (Table 1). Only one nasal *S. aureus* strain was methicillin resistant (T184-2). To investigate whether the *spa*-defined core genomes or SAg-carrying MGEs differed between noninvasive and invasive isolates, we additionally screened 88 methicillin-susceptible *S. aureus* (MSSA) blood culture isolates (Table 1). The MSSA isolates were obtained from the University Hospital of Greifswald or other nearby medical facilities over the same time periods as the nasal isolates to avoid the potential confounding effects of differences in geographical locations or time periods. Most isolates were collected in different wards of the University Hospital of Greifswald, most frequently in the internal medicine ward ($n = 40$), surgical ward ($n = 10$), and intensive-care unit ($n = 9$); four strains were isolated from outpatients (for details, see Materials and Methods). The patients were on average 58.3 years old, and 61.4% were male.

Identification of clonal lineages by *spa* typing. *spa* typing of nasal and blood culture isolates from Western Pomerania revealed 93 different s*pa* types, varying in length from 1 (t779) to 13 (t1660 and t379) repeats. Seventy-five *spa* types were present in single isolates, whereas 10 *spa* types were represented by at least five isolates. The largest clone was t008, which comprised 24 isolates (12.3% of all isolates). Moreover, we identified 30 new *spa* types not included in the Ridom SpaServer database. BURP clustering assigned the 93 different *spa* types to five major and five minor CCs (Fig. 1). The major complexes (containing >5% of the isolates) included MLST CC8, CC15, CC25, CC30, and CC45, which together incorporated 73.3% of all isolates. In contrast, the minor complexes CC5, CC12, CC22, CC121, and CC395 accounted for 13.3% of all strains. Singletons that could not be assigned to a major CC by *spa* typing occurred among the nasal ($n = 14$; 13.1%) and blood culture ($n = 5$; 5.7%) strains. Since clustering parameters excluded *spa* types shorter than five *spa* repeats, three nasal and five blood culture isolates were excluded from BURP grouping. Moreover, one isolate (BK067) was nontypeable, because we were not able to amplify the *spa* gene by PCR. Overall, our results confirm the predominance of major clonal lineages as reported previously in other studies (11, 41). However, the frequencies of clonal lineages varied considerably between the different *S. aureus* strain collections (1, 39, 62), suggesting large geographical variations.

Distribution of nasal and blood culture isolates between clonal complexes. As expected, both nasal and blood culture isolates were present in most clonal complexes (11, 41). CC5 ($n = 7$) was exceptional, because it contained only nasal isolates, and all of them belonged to the same *spa* type, t002 (6.5%; $P \leq 0.05$). Moreover, two clonal lineages were clearly represented in different proportions between nasal and invasive *S. aureus* isolates (Fig. 1). CC8 was overrepresented among blood culture isolates compared to nasal isolates (21.6% versus 10.3%; $P \leq 0.05$), while CC30 was underrepresented among blood culture strains (11.4% versus 27.1%; $P \leq 0.01$). In CC30, *spa* type t012 was predominant among nasal strains, whereas t021 was the most frequent *spa* type in blood culture isolates (4/10 versus 1/29; $P \leq 0.01$) (Fig. 2).

To assess the spreading of CC30 within the healthy population, we additionally screened 362 healthy blood donors from Sczcecin, Eastern Pomerania, Poland, in a cross-sectional approach. Similar to the Western Pomeranian population, 26/108 (24.1%) of the nasal isolates from Sczcecin belonged to CC30 (data not shown), which was thus the dominating *S. aureus* lineage in the Pomeranian community. High prevalences of CC30 have also been reported from other geographical areas worldwide (1, 39, 62). *spa* type t012 was the dominant colonizing clone and probably the evolutionary founder of the CC30 cluster in both Western (10/29 CC30 isolates) and Eastern (7/26) Pomerania (Fig. 2). However, there were also remarkable differences in the *spa* type compositions between the two CC30 collections, suggesting a divergent evolution, which was probably enforced by the former East German-Polish border.


S. aureus nasal isolate
spa type ID
repeat succession
spa CC
MLST CC
SE, tst
egc
eta, etd
agr
T190-2 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
T141-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 a tst g i m n o u - III
T124-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
T100-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
T098-2 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 - g i m n o u - III
T098-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 a g i m n o u - III
T043-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 a tst g i m n o u - III
SH112-2 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 a tst g i m n o u - III
SH077-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 - g i m n o u - III
SH039-1 t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
reference t012 15-12-16-02-16-02-25-17-24-24 spa-CC012 CC30
SH105-1 t018 15-12-16-02-16-02-25-17-24-24-24 spa-CC012 CC30 tst g i m n o u - III
reference t018 15-12-16-02-16-02-25-17-24-24-24 spa-CC012 CC30
T077-1 t1672 15-12-16-02-16-16-02-16-02-25-17-24-24 spa-CC012 CC30 a tst g i m n o u - III
T135-1 t017 15-12-16-16-02-16-02-25-17-24-24 spa-CC012 CC30 a g i m n o u - III
SH109-1 t021 15-12-16-02-16-02-25-17-24 spa-CC012 CC30 - g i m n o u - III
reference t021 15-12-16-02-16-02-25-17-24 spa-CC012 CC30
reference t037 15-12-16-02-25-17-24 spa-CC012 CC30
SH046-1 t275 15-12-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
SH069-1 t1641 15-12-16-02-16-02-25-16-17-24 spa-CC012 CC30 tst g i m n o u - III
SH106-2 t1650 15-12-16-16-02-16-02-25-17-25 spa-CC012 CC30 - g i m n o u - III
T169-2 t318 15-12-16-16-02-16-02-25-17-24 spa-CC012 CC30 - g i m n o u - III
SH064-1 t1504 15-12-16-16-02-25-17-24 spa-CC012 CC30 a tst g i m n o u - III
SH065-2 t1504 15-12-16-16-02-25-17-24 spa-CC012 CC30 a tst g i m n o u - III
T035-1 t342 15-12-16-02-16-02-25-17 spa-CC012 CC30 - g i m n o u - III
T037-1 t840 15-12-02-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
T061-1 t1642 15-16-02-25-17-24-24 spa-CC012 CC30 a g i m n o u - III
SH079-1 t122 08-16-02-16-02-25-17-24-24 spa-CC012 CC30 a tst g i m n o u - III
T039-2 t122 08-16-02-16-02-25-17-24-24 spa-CC012 CC30 tst g i m n o u - III
SH067-1 t122 08-16-02-16-02-25-17-24-24 spa-CC012 CC30 a tst g i m n o u - III
SH121-1 t964 08-16-16-02-16-02-25-17-24 spa-CC012 CC30 - g i m n o u - III
SH032-1 t019 08-16-02-16-02-25-17-24 spa-CC012 CC30 a tst g i m n o u - III
reference t019 08-16-02-16-02-25-17-24 spa-CC012 CC30
T172-1 t1660 15-12-16-16-16-16-16-02-16-02-25-17-24 spa-CC012 CC30 - g i m n o u - III
T132-1 t1669 08-16-34-02-43-13-16-16-02-17-16 spa-CC1655 CC395* a - - - I
T148-2 t1362 08-12-16-34-02-43-34-16-02-17-16 spa-CC1655 CC395 a c l tst - - - I
T191-1 t1645 08-12-16-02-43-34-16-16-02-17-16 spa-CC1655 CC395 a c l tst - - - I
T110-1 t1651 08-16-02-43-34-16-02-17-16 spa-CC1655 CC395 a k q c l tst - - - I
T166-1 t1655 08-16-02-43-34-16-16-02-17-16 spa-CC1655 CC395* k q - - - I
reference t009 11-12-21-17-34-24-34-22-24-34-22-33-25 CC8
reference t036 11-12-21-17-34-24-34-22-33-25 CC8
SH131-1 t723 11-19-12-34-22-25 spa-CC008 CC8* a d j r - - - I
T169-1 t711 04-21-17-34-24-34-22-25 spa-CC008 CC8 b k q d j r - - - I
T198-1 t711 04-21-17-34-24-34-22-25 spa-CC008 CC8 b q d j r - - - I
T145-1 t711 04-21-17-34-24-34-22-25 spa-CC008 CC8 b k q - - - I
reference t051 11-19-21-12-21-17-34-24-34-22-25 spa-CC008 CC8
SH010-1 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 d j r - - - I
SH016-1 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 d j r - - - I
SH051-2 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 d j r - - - I
SH100-1 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 c l - - - I
SH115-2 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 d j r - - - I
T041-1 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 d j r - - - I
T051-2 t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8 d j r - - - I
reference t008 11-19-12-21-17-34-24-34-22-25 spa-CC008 CC8
T184-2[3] t004 09-02-16-13-13-17-34-16-34 spa-CC015 CC45 - g i m n o - I
reference t004 09-02-16-13-13-17-34-16-34 spa-CC015 CC45
T085-1 t065 09-02-16-34-13-17-34-16-34 spa-CC015 CC45 - g i m n o - - I
SH056-1 t1647 08-13-16-34-16-34 spa-CC015 CC45* c l g i m n o - I
reference t038 08-39-34-34-13-17-34-16-34 spa-CC015 CC45
T088-2 t1666 08-16-02-83-34-13-17-34-83-34 spa-CC015 CC45 c l g i m n o - I
reference t157 08-16-02-16-13-13-13-17-34-16-34 spa-CC015 CC45
T108-1 t050 08-16-02-16-34-34-17-34-16-34 spa-CC015 CC45 c l g i m n o - I
SH013-2 t302 08-16-02-16-34-13-17-13-16-34 spa-CC015 CC45 - g i m n o - I
T179-1 t302 08-16-02-16-34-13-17-13-16-34 spa-CC015 CC45 c l g i m n o - I
SH024-1 t1238 08-104-02-16-34-13-17-34-16-34 spa-CC015 CC45 c l g i m n o - I
SH001-2 t015 08-16-02-16-34-13-17-34-16-34 spa-CC015 CC45 c l g i m n o - I
T052-1 t015 08-16-02-16-34-13-17-34-16-34 spa-CC015 CC45 a c l g i m n o - I
T166-2 t015 08-16-02-16-34-13-17-34-16-34 spa-CC015 CC45 c l g i m n o - I
T093-1 t084 07-23-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
T099-1 t084 07-23-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH130-1 t084 07-23-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH070-1 t084 07-23-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH028-1 t084 07-23-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH027-1 t084 07-23-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
T202-2 t346 07-23-12-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH019-2 t120 07-23-12-12-34-34-12-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH126-2 t085 07-23-12-34-34-12-23-02-12-23 spa-CC084 CC15 - - - - II
SH081-2 t091 07-23-21-17-34-12-23-02-12-23 spa-CC084 ST7[4] p - - I
T002-2 t091 07-23-21-17-34-12-23-02-12-23 spa-CC084 ST7 p - - I
T009-1 t091 07-23-21-17-34-12-23-02-12-23 spa-CC084 ST7 p - - I
T157-1 t091 07-23-21-17-34-12-23-02-12-23 spa-CC084 ST7 p - - I
SH059-1 t156 07-23-12-33-22-17 spa-CC156 CC12 c l p - - II
T194-1 t156 07-23-12-33-22-17 spa-CC156 CC12 c l p - - II
reference t044 07-23-12-34-34-33-34 singleton
SH097-2 t1491 07-23-21-17-13-34-34-16-34-33-13 singleton h - - III
SH044-1 t127 07-23-21-16-34-33-13 singleton a h k q - - III
T142-2 t493 04-34-17-66-32-17-23-24 singleton h g i m n o u - I
SH018-1 t246 04-17-23-24-20-17-25 singleton - i m n o u - IV
SH137-2 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 p g i m n o - II
T161-1 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 - g i m n o - II
T009-2 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 p g i m n o - II
SH093-1 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 - g i m n o - II
SH076-1 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 - g i m n o - II
SH042-1 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 p g i m n o - II
SH033-1 t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5 b k q d j r g i m n o - II
reference t002 26-23-17-34-17-20-17-12-17-16 spa-CC045 CC5
reference t001 26-30-17-34-17-20-17-12-17-16 spa-CC045 CC5
reference t045 26-17-20-17-12-17-16 spa-CC045 CC5
reference t003 26-17-20-17-12-17-17-16 spa-CC045 CC5
T054-1 t1680 121-17-17-17-17 singleton b - - IV
T171-1 t078 04-21-12-41-20-17-12-12-17 spa-CC078 CC25 b g i m n o - I
T192-1 t078 04-21-12-41-20-17-12-12-17 spa-CC078 CC25 - g i m n o - I
T162-1 t078 04-21-12-41-20-17-12-12-17 spa-CC078 CC25 b g i m n o - I
T097-1 t258 04-21-12-41-20-17-12-12-12-17 spa-CC078 CC25 - g i m n o - I
SH015-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
SH021-2 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
SH022-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
SH133-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
SH145-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
T011-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
T029-2 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
T055-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
T105-2 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
T201-1 t056 04-20-12-17-20-17-12-17-17 spa-CC078 CC25 - - - I
T200-1 t1664 04-33-31-12-16-16-12-33-34 singleton a h tst g i m n o u - III
T020-1 t1670 04-44-33-31-12-16-34-12-33-34 singleton b h l tst g i m n o u - III
SH020-1 t1643 04-44-54-31-12-16-34-16-12-33-34 singleton h tst g i m n o u - III
SH024-2 t541 26-17-25-17-25-16-28 singleton - g i m n o - I
SH055-1 t1648 26-23-29-17-25-17-25-28 singleton - g i m n o - I
reference t032 26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28 CC22
SH072-1 t005 26-23-13-23-31-05-17-25-17-25-16-28 spa-CC022 CC22 - g i m n o - I
SH073-1 t005 26-23-13-23-31-05-17-25-17-25-16-28 spa-CC022 CC22 - g i m n o - I
reference t005 26-23-13-23-31-05-17-25-17-25-16-28 spa-CC022 CC22
SH048-1 t026 08-16-34 n.d. c l g i m n o - I
T118-1 t118 11-25 n.d. d j r - - - I
SH017-2 t1209 15-12-16 n.d. a g i m n o u - III

A subanalysis of strains isolated in 2002 versus 2005–2006 (data not shown) showed that CC395 was not yet detected in 2002 but a total of seven isolates representing different, closely related *spa* types were discovered in 2005–2006. While a sampling bias cannot be excluded, it appears more likely that this *S. aureus* clone was recently introduced into the area, increased in frequency in the population, and rapidly diversified into the observed cluster of closely related *spa* types. Moreover, within the blood culture isolates, we observed an expansion of the lineages CC15, CC45, and CC8, which together accounted for 34.4% of the strains in 2002 compared to 60.7% in 2005–2006. Even though these differences are not significant due to small case numbers, they suggest that the *S. aureus* population structure is highly dynamic.

Distribution of *agr* types. *agr* is a global regulator of virulence gene expression, and four different *agr* subgroups, *agr-1* to *-4*, are known. The *agr* locus belongs to the core variable genome and is strongly linked with clonal lineages (27, 39). In agreement with others (27, 43, 51, 64), we observed that the clonal lineages CC8, CC22, CC25, CC45, and CC395 harbored *agr-1* and all CC5, CC12, and CC15 isolates were characterized by *agr-2* (Fig. 3A, 4, and 5). Moreover, CC30 isolates carried *agr-3*, while *agr-4* occurred only in the CC121 lineage.

We also noted a single *agr-1* isolate within the CC30 cluster (BK085) and one *agr-4* isolate in CC45 (BK004); this had occasionally been observed before (51, 64). Interstrain recombination events could account for these exceptional strains, and this needs further investigation (19, 54, 56). Five blood culture isolates could not be typed with our *agr* multiplex PCR system, presumably due to a deletion of the *agr* locus (18; S. Holtfreter, unpublished data).

Distribution of SAg genes. We then determined the presence of the 19 known SAg genes, as well as of the *eta* and *etd* genes, using a system of five multiplex PCRs. Comprehensive overviews of the *spa*-defined clonal lineages and their respective *agr* types and SAg gene patterns are provided in Fig. 4 for the nasal isolates and Fig. 5 for the blood culture isolates from Western Pomerania.

None of the SAg genes were randomly distributed between the clonal complexes ($P \leq 0.001$; contingency table analysis) but rather were strongly associated with the clonal lineages. This suggests that most MGEs are predominantly transferred vertically while horizontal transmission between different lineages is limited. However, MGEs were also found in strains of divergent clonal lineages that do not share a recent ancestor. Here, the exchange of MGEs appears to be favored between some CCs. The distribution of selected SAg genes, as well as *etd*, within the major CCs is depicted in Fig. 3B and C.

egc SAgs, which cluster on the *S. aureus* genomic island vSAβ, were strictly linked to the clonal background, which is in agreement with previous studies (8, 38, 39). The *egc* cluster was present in all CC5, CC22, and CC45 isolates but completely absent from CC8, CC12, CC15, and CC395. *egc* genes also characterized one subcluster of the CC25 lineage (t078 and relatives), whereas they were missing from the other (t056 and relatives) (Fig. 3B, 4, and 5). Moreover, we observed an *egc* variant that was almost exclusively linked to the CC30 background. This *egc* variant was characterized by an *sem* allelic variant that escaped detection with our standard PCR due to three point mutations within the binding site of the *sem* forward primer (EF551341) and an additional *seu* gene, and it probably corresponds to the reported *egc2* variant (Fig. 3, 4, and 5) (4, 34). Our data clearly show that the *egc*-containing genomic island is transferred only vertically.

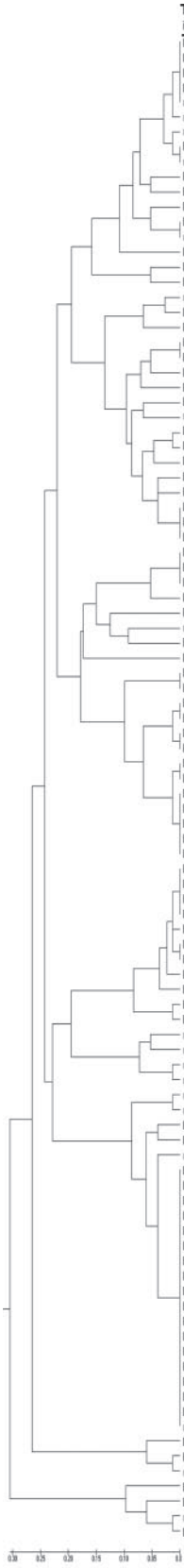
Other SAgs with very strong linkage to certain CCs were *tst*, *sec-sel*, and *sed-sej-ser* (Fig. 3B, 4, and 5). The *tst* gene, which is located on a family of related islands, was strongly linked to the CC30 background, as reported in previous studies (39, 51). *sec-sel* are colocalized on the pathogenicity island SaPI3 and were detected mainly in CC45 isolates. Finally, the plasmid-borne SAg genes *sed-sej-ser* were usually found in CC8. These results suggest that horizontal transfer of the respective islands and plasmids between clonal lineages is rare.

SAg genes with a broader distribution were the phage-borne *sea*, which was occasionally detected in CC8, -30, -45, and -395, and *seb*, on SaPI, which was infrequently found in CC5, -8, -12, -25, and -45 (Fig. 3B, 4, and 5).

Furthermore, we observed some new SAg gene combinations, indicating the existence of yet-undescribed MGE variants. For example, *tst* and *seb* are usually located on two different related SaPIs, either of which integrates into the same genomic locus. The rare observation of *seb* in a *tst*-positive isolate, as detected in this study and by others (33), can be explained by the mosaic structure of MGEs, where short mosaic fragments can spread to other MGEs of the same type by homologous recombination (39). Similarly, *seb-sek-seq* are usually clustered on SaPI1, but we and others found *seb* without *seq-sek* in several strains (16, 48), suggesting a new SaPI variant. This is intriguing and needs more investigation.

***agr* and SAg gene profiles of *S. aureus* clonal complexes.** As a consequence of their linkage to the genetic background, each clonal complex is characterized by typical SAg gene patterns. However, within clonal complexes and even within the same *spa* types, we observed considerable variation in the prevalences of the SAgs that constitute these lineage-specific pat-

FIG. 4. Distribution of SAg genes, *agr* types, and *eta* and *etd* genes within *spa*-defined clonal complexes among nasal isolates ($n = 107$). *spa* types were clustered by BURP analysis into 10 clonal complexes, which are color coded according to the scheme in Fig. 1. For construction of the consensus tree, several reference strains with unknown SAg gene patterns were included in the BURP clustering (shaded in grey). MLST CCs were deduced from BURP grouping of *spa* types (55). MLST CCs labeled with an asterisk were MLST sequenced. SAg genes, *agr* types, and *eta* and *etd* genes were determined by multiplex PCR. All strains tested negative for the PVL locus (not shown). Staphylococcal enterotoxins (SEs) are indicated by single letters (*a = sea*, etc.). Footnotes are as follows. 1, *spa* type t037 isolates were grouped into *spa* CC012 but are known to belong to MLST ST239 (CC8). *spa* t037 isolates have arisen from a single recombination event that involved the exchange of a >200-kb DNA fragment including the *spa* gene between MLST30 and MLST239 (52, 53). 2, *spa* CC1655 isolates were clustered into MLST CC395 after MLST sequencing of two representative strains. 3, T184-2 tested *mecA* positive. 4, *spa* type t091 isolates were grouped into *spa* CC084 but belonged to ST7 (singleton) according to MLST sequencing.



blood culture isolate	spa-type ID	repeat succession	spa-CC	MLST-CC	SEs, *tst* genes	*egc*	*eta*, *etd*	*agr*
BK080	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30	*a tst*	*g i [m] n o u*	-	*III*
BK032	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30	*a tst*	*g i [m] n o u*	-	*III*
BK030	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30	*a tst*	*g i [m] n o u*	-	*III*
BK014	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30	*a tst*	*g i [m] n o u*	-	*III*
reference	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30				
reference	t037	15-12-16-02-25-17-24	spa-CC012	CC30				
reference	t018	15-12-16-02-16-02-25-17-24-24-24	spa-CC012	CC30				
BK002	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	*tst*	*g i [m] n o u*	-	*III*
reference	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30				
BK085	t1674	15-12-16-02-25-34-24-17	spa-CC012	CC30	*p*	*i [m] n o u*	-	*I*
BK087	t1653	15-12-12-16-02-16-02-25-17	spa-CC012	CC30	*a tst*	*g i [m] n o u*	-	*III*
BK028	t046	08-16-02-16-02-25-17-24-24-24	spa-CC012	CC30	*tst*	*g i [m] n o u*	-	*III*
BK089	t019	08-16-02-16-02-25-17-24	spa-CC012	CC30	*tst*	*g i [m] n o u*	-	*III*
reference	t019	08-16-02-16-02-25-17-24	spa-CC012	CC30				
BK018	t1662	15-25-17-24-24	singleton	CC30* [2]	*tst*	*g i [m] n o u*	-	*III*
BK084	t1645	08-12-16-02-43-34-16-16-02-17-16	spa-CC1655	CC395 [3]	*a c l tst e*	-	*etd*	*I*
BK066	t1362	08-12-16-34-02-43-34-16-02-17-16	spa-CC1655	CC395*	*a c l tst*	-	-	*I*
reference	t004	09-02-16-13-13-17-34-16-34	spa-CC583	CC45				
BK037	t061	09-02-16-13-34-17-34-16-34	spa-CC583	CC45	-	*g i m n o*	-	*I*
BK065	t1646	09-20-16-34-13-17-34-16-34	spa-CC583	CC45	-	*g i m n o*	-	*I*
BK051	t230	08-16-02-16-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK052	t230	08-16-02-16-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK004	t1081	08-16-02-43-34-17-34	spa-CC583	CC45	-	*g i m n o*	-	*IV*
BK041	t1663	08-16-20-13-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK098	t465	08-23-16-34-13-17-34-16-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
reference	t038	08-39-34-34-13-17-34-16-34	spa-CC583	CC45				
BK069	t620	08-16-02-16-34-13-17-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK063	t583	08-16-02-16-34-13-16-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK056	t1668	08-16-34-13-34-13-17-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK034	t031	08-16-02-16-34-13-34-16-34	spa-CC583	CC45	*b c l*	*g i m o*	-	-
BK068	t630	08-16-02-16-34-17-34-16-34	spa-CC583	CC45	-	*g i m n o*	-	*I*
BK020	t015	08-16-02-16-34-13-17-34-16-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK021	t015	08-16-02-16-34-13-17-34-16-34	spa-CC583	CC45	*c l*	*g i m n o*	-	*I*
BK055	t015	08-16-02-16-34-13-17-34-16-34	spa-CC583	CC45	*d j r c l tst*	*g i m n o u*	-	*I*
BK058	t156	07-23-12-33-22-17	spa-CC156	CC12*	*c l*	-	-	*II*
BK086	t156	07-23-12-33-22-17	spa-CC156	CC12	*c l p*	-	-	*II*
BK023	t156	07-23-12-33-22-17	spa-CC156	CC12	*c l p*	-	-	*II*
BK050	t160	07-23-21-24-33-22-17	spa-CC156	CC12	*b p*	-	-	*II*
BK019	t1659	07-16-12-23-02-02-34	singleton		*d r tst*	*g i m n o*	-	*II*
reference	t044	07-23-12-34-34-33-34	singleton					
BK026	t127	07-23-21-16-34-33-13	singleton		*h*	-	-	*I*
BK062	t1673	26-22-17-21-34-34-22-34	singleton		-	*g i m n o*	-	*I*
BK091	t091	07-23-21-17-34-12-23-02-12-23	spa-CC084	ST7* [4]	*p*	-	-	*I*
BK099	t091	07-23-21-17-34-12-23-02-12-23	spa-CC084	ST7	*p*	-	-	*I*
BK040	t094	07-23-12-34-34-12-12-23	spa-CC084	CC15*	-	-	-	*II*
BK072	t094	07-23-12-34-34-12-12-23	spa-CC084	CC15	-	-	-	*II*
BK047	t335	07-23-12-34-34-12-23	spa-CC084	CC15	-	-	-	*II*
BK048	t335	07-23-12-34-34-12-23	spa-CC084	CC15	-	-	-	*II*
BK043	t346	07-23-12-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK088	t346	07-23-12-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK017	t084	07-23-12-34-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK027	t084	07-23-12-34-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK031	t084	07-23-12-34-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK044	t084	07-23-12-34-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK081	t084	07-23-12-34-34-12-12-23-02-12-23	spa-CC084	CC15	-	-	-	*II*
BK029	t078	04-21-12-41-20-17-12-12-17	spa-CC078	CC25	-	*g i m o*	*etd*	-
BK075	t078	04-21-12-41-20-17-12-12-17	spa-CC078	CC25	-	*g i m n o*	*etd*	*I*
BK009	t078	04-21-12-41-20-17-12-12-17	spa-CC078	CC25	-	*g i m n o*	*etd*	*I*
BK003	t078	04-21-12-41-20-17-12-12-17	spa-CC078	CC25	-	*g i m n o*	*etd*	*I*
BK001	t1671	04-21-12-41-20-17-12-12-12-12-17	spa-CC078	CC25	*b*	*g i m n o*	*etd*	*I*
BK061	t258	04-21-12-41-20-17-12-12-12-17	spa-CC078	CC25	-	*g i m n o*	*etd*	*I*
BK083	t258	04-21-12-41-20-17-12-12-12-17	spa-CC078	CC25	-	*g i m o u*	-	-
BK057	t1656	04-21-21-12-41-20-17-12-12-12-17	spa-CC078	CC25	-	*g i m n o*	*etd*	*I*
BK025	t1661	04-21-12-41-20-12-12-12-12-17	spa-CC078	CC25	-	*g i m n o*	*etd*	*I*
BK049	t514	04-20-12-17-20-17-12-17-17-17	spa-CC078	CC25	-	-	-	*I*
BK015	t056	04-20-12-17-20-17-12-17-17	spa-CC078	CC25	-	-	-	*I*
reference	t002	26-23-17-34-17-20-17-12-17-16	spa-CC045	CC5				
reference	t001	26-30-17-34-17-20-17-12-17-16	spa-CC045	CC5				
reference	t045	26-17-20-17-12-17-16	spa-CC045	CC5				
reference	t003	26-17-20-17-12-17-17-16	spa-CC045	CC5				
reference	t009	11-12-21-17-34-24-34-22-24-34-22-33-25		CC8				
reference	t036	11-12-21-17-34-24-34-22-33-25		CC8				
BK016	t648	11-21-17-34-24-34-22-25	spa-CC008	CC8	*b k q d j r tst*	-	-	*I*
BK077	t190	11-17-34-24-34-22-25	spa-CC008	CC8	-	-	-	*I*
reference	t051	11-19-21-12-21-17-34-24-34-22-25	spa-CC008	CC8				
BK008	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	-	-	-	*I*
BK010	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*a d j r*	-	-	*I*
BK012	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK022	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK042	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	-	-	-	*I*
BK045	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK053	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*a d j r*	-	-	*I*
BK054	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK059	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK060	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*a d j r*	-	-	*I*
BK064	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	-	-	-	*I*
BK070	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK071	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK092	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
BK096	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*a d j r*	-	-	*I*
BK101	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	-	-	-	*I*
BK035	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8	*d j r*	-	-	*I*
reference	t008	11-19-12-21-17-34-24-34-22-25	spa-CC008	CC8				
BK078	t916	14-44-20-44-13-12-17-17-23-18-17	spa-CC159	CC121	-	*g i m o u*	-	-
BK036	t272	14-44-13-12-17-17-17-23-18-17	spa-CC159	CC121	-	*g i m n o u*	*eta*	*IV*
reference	t159	14-44-13-12-17-17-23-18-17	spa-CC159	CC121				
BK090	t1665	26-23-13-23-31-17-17-25-16-28	spa-CC379	CC22*	-	*g i m n o*	-	*I*
BK005	t1120	26-13-23-31-05-17-25-17-25-16-28	spa-CC379	CC22	-	*g i m n o*	-	*I*
reference	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	CC22				
BK073	t379	26-23-23-13-23-31-29-17-25-17-25-16-28	spa-CC379	CC22	*c l*	*g i m n o*	-	-
BK033	t643	04-21-12-17	n.d.		-	*g i m n o*	*etd*	*I*
BK024	t1644	08-13-16-34	n.d.		*c l*	*g i m n o*	-	*I*
BK007	t779	08	n.d.		*c l*	*g i m n o*	-	*I*
BK011	t1640	08-02-12-23	n.d.		*b*	-	-	*II*
BK006	t1546	26-23-34-34	n.d.		*b*	*g i m n o*	-	*II*
BK067	-	-	no PCR product		*b k q*	-	-	*I*

TABLE 2. SAg gene and *agr* signatures of *S. aureus* clonal complexes[a]

CC	Characteristics	No.	*agr* type	*egc*	Other SAg genes	Reference(s)	*eta*, *etd*
CC5	MSSA	7	*agr-2* (7)	***egc*** (7)	*seb-seq-sek* (1), *sed-sej-ser* (1), *sep* (3)		
	N315 (MRSA)		*agr-2*	***egc***	*tst-sec-sel*	31	
CC8	MSSA	30	*agr-1* (30)		***sed-sej-ser*** (23), *sea* (5), *seb-sek-seq* (3), *seb-seq* (1), *tst* (1), *sec-sel* (1)		
	COL (MRSA); *spa* type t008		*agr-1*		*seb-sek-seq*	17	
	Lyon-MRSA; *spa* type t008	13	*agr-1*		***sea*** (13), *seb* (1), *sed* (3)[b]	12	
CC12	MSSA	6	*agr-2* (6)		***sec-sel*** (5), *sep* (5), *seb* (1)		
CC15	MSSA	20	*agr-2* (20)				
CC22	MSSA	5	*agr-1* (4), *agr*-neg[d] (1)	***egc*** (5)	*sec-sel* (1)		
	EMRSA-15; *spa* type t032		*agr-1*	***egc***	*sec-sel*	29	
CC25	MSSA	25	*agr-1* (23), *agr*-neg (2)	***egc*** (13[c])	*seb* (3), *seu* (1)		*etd* (8[c], all BK)
CC30	MSSA	39	*agr-3* (38), *agr-1* (1)	***egc* variant** (39)	***tst*** (27), ***sea*** (17), *sep* (1)		
	EMRSA-16; *spa* type t018		*agr-3*	***egc* variant**	*tst*, *sea*	12, 23	
CC45	MSSA	26	*agr-1* (24), *agr-4* (1), *agr*-neg (1)	***egc*** (26)	***sec-sel*** (19), *tst* (1), *sea* (1), *seb* (1), *sed-sej-ser* (1), *seu* (1)		
	Berlin MRSA		*agr-1*	***egc***		12	
CC121	MSSA	2	*agr-4* (1), *agr*-neg (1)	***egc*** (2)	*seu* (2)		*eta* (1)
CC395	MSSA	7	*agr-1*		***tst-sec-sel*** (5), ***sea*** (6), *sek-seq* (2), *see* (1)		*etd* (1)

[a] For each staphylococcal clonal cluster, the characteristic *agr* subgroup, SAg genes, and *eta* and *etd* genes are indicated. The number of isolates that tested positive for a virulence gene is given in parentheses. SAg genes that occurred in more than 50% of the isolates of one CC are shown in boldface letters. SAg genes that are clustered on MGEs are linked by hyphens. In addition, the SAg/*agr* signatures of MRSA outbreak clones (Lyon-MRSA, epidemic MRSA-15 [EMRSA-15], ERMSA-16, and Berlin MRSA) and whole-genome sequenced strains (COL and N315) are provided.

[b] *sej* and *ser*, which cluster with *sed* on a plasmid, were not determined in this study (12).

[c] *egc* genes were present in one subcluster of the CC25 lineage (t078 and relatives; $n = 13$), whereas they were missing in the other one (t056 and relatives; $n = 12$). The *etd* gene occurred only in blood culture isolates of CC25 ($n = 8/11$; $P \leq 0.001$).

[d] *agr*-neg, *agr* negative (i.e., a negative result by *agr* PCR).

terns. There were between one (CC15) and eight (CC8) different SAg genotypes within the major clonal lineages. This indicates frequent acquisition and loss of SAg-carrying MGEs within lineages (Fig. 4 and 5).

The characteristic SAg gene profiles and *agr* groups of the clonal lineages are summarized in Table 2. Within CC25, *spa* sequencing discriminated two sublineages, *egc*-positive t078 strains (and relatives) and *egc*-negative t056 isolates (and relatives). The former were more frequent among the invasive strains (9/11 versus 4/14; $P \leq 0.01$). Within this t078 cluster, eight strains additionally harbored the *etd* gene. Importantly, they were found exclusively in the blood culture isolates ($P \leq 0.001$), so that CC25 isolates harboring the pathogenicity island containing *etd* appear to be more virulent than those without it. Others have reported the *etd* locus to be associated with the methicillin-resistant *S. aureus* (MRSA) ST80 lineage, as well as with a lineage carrying *agr-1*, *egc*, and sometimes *seb* (66); the latter likely represents the CC25 t078 subcluster described here.

As shown above, CC30 isolates were more prevalent among nasal strains, while CC8 was significantly overrepresented among blood culture isolates. However, there were no differences in the SAg gene profiles between nasal and invasive isolates, either in CC30 or in CC8 (Fig. 4 and 5). CC15 isolates never harbored SAg genes, presumably due to restrictions on horizontal transmission (60). The SAg gene patterns of the minor lineages CC12, -22, -121, and -395 are based on small case numbers and need to be confirmed. The SAg and *agr* profiles reported for MRSA strains largely correspond to our findings with MSSA isolates, which demonstrates that the SCC*mec* cassettes show a greater horizontal mobility than the MGEs encoding SAgs (Table 2).

FIG. 5. Distribution of SAg genes, *agr* types, and *eta* and *etd* genes within *spa*-defined clonal complexes among blood culture isolates ($n = 87$). For background information, see the legend to Fig. 4. *S. aureus* clinical isolate BK067 was not *spa* typeable and therefore was excluded from this analysis. Footnotes are as follows. 1, *spa* type t037 isolates were grouped into *spa* CC012 but are known to belong to MLST ST239 (CC8) (52, 53). 2, *spa* type t1662 is a singleton according to *spa* typing but was grouped into MLST CC30 after MLST sequencing (ST30). 3, *spa* CC1655 isolates were clustered into MLST CC395 after MLST sequencing of two representative strains. 4, *spa* type t091 isolates were grouped into *spa* CC084 but belonged to ST7 (singleton) according to MLST sequencing.

DISCUSSION

Recent analyses have shown that all *S. aureus* genotypes that efficiently colonize humans have given rise to life-threatening pathogens but that some clonal lineages appear to be more virulent than others (41). Analysis of the core variable genome could not clearly attribute virulence to any of the factors examined (39). In fact, each of the 10 dominant *S. aureus* lineages has a unique combination of core variable genes, such as surface-associated and regulatory genes (27, 39). This suggests that associated resistance and virulence genes carried on MGEs could determine staphylococcal virulence, in which case their horizontal mobility has to be taken into account. Accordingly, we performed a comprehensive survey of the distribution of the 19 staphylococcal SAg genes, the exfoliative toxin genes, and the *agr* types in the known *S. aureus* clonal lineages to answer the following question: are there differences in the core genome and/or the SAg-carrying MGEs between nasal and blood culture isolates?

Our results clearly showed that MGEs carrying SAg genes were strongly associated with the clonal background. Either they did not spread between different genetic lineages at all, such as the *egc*-carrying pathogenicity island vSAβ (8, 38, 39, 57), or the efficiency of such genetic exchange was low, as in the case of *tst*, which is carried by a family of related pathogenicity islands (39, 40), and also in the case of the plasmid-borne SAg genes *sed-sej-ser* (14, 15). The *sea*-carrying phage Φ3 and *seb*, located on SaPI, were distributed more broadly (39, 51). This shows that the genetic distribution of SAg-carrying MGEs occurs mainly by vertical transmission. The degrees of horizontal mobility vary considerably between different MGEs.

Barriers to horizontal transfer could be the incompatibility of related bacteriophages (bacteriophage immunity), SaPIs, and plasmids; varying susceptibilities to transduction or conjugation; or the Sau1 restriction modification system (38, 60). Notably, the lineage CC15 completely lacks SAg genes. It can be assumed that the restriction modification system of this lineage prevents the acquisition of any SAg gene-carrying MGE by horizontal transfer.

Jarraud et al. have reported associations of *agr* types with diseases, in particular with the toxin-mediated toxic shock syndrome and exfoliative diseases (27). Since the *agr* locus belongs to the core variable genes and was strictly associated with clonal lineages, this observation may reflect the links of these clonal lineages and their associated virulence gene patterns with disease. For example, most cases of menstrual toxic shock syndrome are caused by *S. aureus* lineage CC30, which is characterized by *tst* and *agr-3* (6, 13, 27, 46).

As a result of the described restrictions on horizontal gene transfer, each clonal complex was characterized by a typical SAg and exfoliative toxin gene profile and *agr* type, as is described in detail in Results above (Table 2). These typical MGE profiles explain many of the differences in virulence and disease symptoms that are observed between *S. aureus* lineages. However, in cases of tight linkage, the relative contributions of MGEs and the core or core variable genome cannot be resolved using the tools of molecular epidemiology. In other words, a preponderance of virulence-associated genes among invasive *S. aureus* isolates could be caused or, on the contrary, masked by an uneven distribution of clonal complexes between nasal and invasive strains (51). A striking example is the *egc*-carrying genomic island vSAβ, which we found always and exclusively in members of CC5, CC22, CC30, CC45, and a subcluster of CC25. On the CC25 background, *egc* was associated with invasiveness, but on the CC5 background, it characterized nasal isolates. Such associations should therefore be interpreted with caution.

Within each lineage and even within the same *spa* type we observed considerable variation of SAg genes. The transfer of bacteriophages appears to be quite frequent during both colonization and infection (20, 21, 44). The colonizing strain T098 even lost the *sea*-carrying phage between the first and second samplings, since the PCRs for *sea* and the phage-specific integrase gene became negative while the *spa* types, PFGE patterns, and antibiograms remained identical (unpublished observations). This illustrates the high degree of horizontal mobility of phages between strains of similar genetic backgrounds (39, 51). In such cases, the impact of MGEs can be readily assessed by comparing invasive and noninvasive *S. aureus* isolates with similar genetic backgrounds. In our study, the strict association of *etd* with invasiveness on the CC25 genetic background strongly suggests that *etd*—or associated virulence genes on that island—contributes to disease. The exfoliative toxin D induces intradermal blister formation by cleavage of desmoglein 1 and is associated with cutaneous abscesses and furuncles (65). This shows that virulence gene analysis can increase the discriminatory power of other genotyping methods, as has also been suggested by others (27, 51, 58). In contrast, in CC8 and CC30, the SAg gene profiles did not differ between nasal and invasive strains, rendering an important contribution of SAgs to the invasion process unlikely. We conclude that restricting a comparative analysis of virulence factors to those CCs that harbor the respective virulence genes will increase its sensitivity and specificity.

In agreement with our results, similar consensus repertoires of virulence genes (e.g., SAg genes, *agr* groups, and hemolysins) have also been reported for MRSA clones of CC5, -8, and -30 (7). The fact that staphylococcal lineages from different geographical regions show similar MGE profiles suggests that these lineages are evolutionarily old and share a conserved genomic structure. On this conserved genetic background, the *mecA* gene shows highly dynamic behavior, which illustrates the extraordinary selective pressure exerted on the species *S. aureus* by therapeutic intervention. Interestingly, the reported SAg profiles within clonal complexes are less variable in MRSA strains than in our MSSA collection (7). Likely reasons are the relatively recent acquisition of SCC*mec* and the shaping of the population structure of MRSA by local outbreaks in hospitals and communities.

In Pomerania, CC30 was significantly more common among nasal strains than among blood culture isolates. It appears that the local CC30 population is optimized for symptom-free colonization and probably causes systemic infections only under very accommodating conditions. Intriguingly, Wertheim et al. reported that in The Netherlands CC30 isolates tend to be more prevalent among endogenous invasive strains than noninvasive strains (62). Though there are some differences in the ways the Dutch and the Pomeranian strains were collected, this means that the diagnosis "CC30" alone conveys limited information. In support of this, two CC30 MRSA clones, the hos-

pital-acquired MRSA ST36:USA200 and the community-acquired MRSA ST30:USA1100, induce very different disease types, which has been attributed to differences in their virulence gene repertoires (7). A detailed comparison of the Dutch and the Pomeranian CC30 populations will hopefully reveal more factors that predispose to invasiveness.

In addition to virulence gene assessment, the analysis of individual *spa* types or MLST types within CCs can be informative. In CC30, the *spa* type t012 was most prevalent among nasal strains, whereas t021 dominated the blood culture isolates. Similarly, within the CC25 lineage, t078 isolates were overrepresented among invasive strains. However, while high-resolution typing methods based on sequence variations of the core genome may help to identify aggressive *S. aureus* clones, virulence gene typing is much more likely to provide clues to the underlying molecular mechanisms.

In conclusion, we have shown here that *S. aureus* clonal complexes are characterized by consensus repertoires of SAg genes. However, within each lineage, and even within the same *spa* type, there was remarkable variation of SAg gene profiles. For *etd*, our data indicate a role in bloodstream invasion while rendering it unlikely for SAgs. Using SAgs as an example of highly variable virulence genes, we have shown here that the simultaneous assessment of virulence gene profiles and genetic background can provide new insights into *S. aureus* virulence.

REFERENCES

1. **Aires de Sousa, M., T. Conceicao, C. Simas, and H. de Lencastre.** 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. J. Clin. Microbiol. **43:**5150–5157.
2. **Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M. C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X. W. Huijsdens, A. M. Kearns, A. Mellmann, H. Meugnier, J. K. Rasheed, E. Spalburg, B. Strommenger, M. J. Struelens, F. C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte.** 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J. Clin. Microbiol. **44:**619–621.
3. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu.** 2002. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet **359:**1819–1827.
4. **Bania, J., A. Dabrowska, K. Korzekwa, A. Zarczynska, J. Bystron, J. Chrzanowska, and J. Molenda.** 2006. The profiles of enterotoxin genes in *Staphylococcus aureus* from nasal carriers. Lett. Appl. Microbiol. **42:**315–320.
5. **Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. von Eiff.** 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J. Clin. Microbiol. **41:**1434–1439.
6. **Booth, M. C., L. M. Pence, P. Mahasreshti, M. C. Callegan, and M. S. Gilmore.** 2001. Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. Infect. Immun. **69:**345–352.
7. **Diep, B. A., H. A. Carleton, R. F. Chang, G. F. Sensabaugh, and F. Perdreau-Remington.** 2006. Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. **193:**1495–1503.
8. **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. Lancet **367:**731–739.
9. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
10. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. USA **99:**7687–7692.
11. **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. J. Day.** 2003. How clonal is *Staphylococcus aureus*? J. Bacteriol. **185:**3307–3316.
12. **Ferry, T., M. Bes, O. Dauwalder, H. Meugnier, G. Lina, F. Forey, F. Vandenesch, and J. Etienne.** 2006. Toxin gene content of the Lyon methicillin-resistant *Staphylococcus aureus* clone compared with that of other pandemic clones. J. Clin. Microbiol. **44:**2642–2644.
13. **Fitzgerald, J. R., D. E. Sturdevant, S. M. Mackie, S. R. Gill, and J. M. Musser.** 2001. Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. Proc. Natl. Acad. Sci. USA **98:**8821–8826.
14. **Fueyo, J. M., M. C. Mendoza, M. A. Alvarez, and M. C. Martin.** 2005. Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. FEMS Microbiol. Lett. **243:**447–454.
15. **Fueyo, J. M., M. C. Mendoza, and M. C. Martin.** 2005. Enterotoxins and toxic shock syndrome toxin in *Staphylcoccus aureus* recovered from human nasal carriers and manually handled foods: epidemiological and genetic findings. Microb. Infect. **7:**187–194.
16. **Fueyo, J. M., M. C. Mendoza, M. R. Rodicio, J. Muniz, M. A. Alvarez, and M. C. Martin.** 2005. Cytotoxin and pyrogenic toxin superantigen gene profiles of *Staphylococcus aureus* associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. J. Clin. Microbiol. **43:**1278–1284.
17. **Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser.** 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J. Bacteriol. **187:**2426–2438.
18. **Goerke, C., S. Campana, M. G. Bayer, G. Doring, K. Botzenhart, and C. Wolz.** 2000. Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. Infect. Immun. **68:**1304–1311.
19. **Goerke, C., S. Esser, M. Kummel, and C. Wolz.** 2005. *Staphylococcus aureus* strain designation by *agr* and *cap* polymorphism typing and delineation of *agr* diversification by sequence analysis. Int. J. Med. Microbiol. **295:**67–75.
20. **Goerke, C., S. Matias y Papenberg, S. Dasbach, K. Dietz, R. Ziebach, B. C. Kahl, and C. Wolz.** 2004. Increased frequency of genomic alterations in *Staphylococcus aureus* during chronic infection is in part due to phage mobilization. J. Infect. Dis. **189:**724–734.
21. **Goerke, C., C. Wirtz, U. Fluckiger, and C. Wolz.** 2006. Extensive phage dynamics in *Staphylococcus aureus* contributes to adaptation to the human host during infection. Mol. Microbiol. **61:**1673–1685.
22. **Harmsen, D., H. Claus, W. Witte, J. Rothganger, D. Turnwald, and U. Vogel.** 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. **41:**5442–5448.
23. **Holden, M. T. G., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. J. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill.** 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc. Natl. Acad. Sci. USA **101:**9786–9791.
24. **Holtfreter, S., K. Bauer, D. Thomas, C. Feig, V. Lorenz, K. Roschack, E. Friebe, K. Selleng, S. Lovenich, T. Greve, A. Greinacher, B. Panzig, S. Engelmann, G. Lina, and B. M. Broker.** 2004. *egc*-encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. Infect. Immun. **72:**4061–4071.
25. **Holtfreter, S., and B. M. Broeker.** 2005. Staphylococcal superantigens: do they play a role in sepsis? Arch. Immunol. Ther. Exp. **53:**13–27.
26. **Holtfreter, S., K. Roschack, P. Eichler, K. Eske, B. Holtfreter, C. Kohler, S. Engelmann, M. Hecker, A. Greinacher, and B. M. Bröker.** 2006. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain; a potential explanation for their improved survival in severe sepsis. J. Infect. Dis. **193:**1275–1280.
27. **Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch.** 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect. Immun. **70:**631–641.
28. **Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina.** 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol. **166:**669–677.
29. **Johnson, A. P., H. M. Aucken, S. Cavendish, M. Ganner, M. C. Wale, M. Warner, D. M. Livermore, and B. D. Cookson.** 2001. Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the

UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). J. Antimicrob. Chemother. **48:**143–144.
30. **Koreen, L., S. V. Ramaswamy, E. A. Graviss, S. Naidich, J. M. Musser, and B. N. Kreiswirth.** 2004. *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. J. Clin. Microbiol. **42:**792–799.
31. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
32. **Ladhani, S.** 2003. Understanding the mechanism of action of the exfoliative toxins of *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol **39:**181–189.
33. **Layer, F., B. Ghebremedhin, W. Konig, and B. Konig.** 2006. Heterogeneity of methicillin-susceptible *Staphylococcus aureus* strains at a German university hospital implicates the circulating-strain pool as a potential source of emerging methicillin-resistant *S. aureus* clones. J. Clin. Microbiol. **44:**2179–2185.
34. **Letertre, C., S. Perelle, F. Dilasser, and P. Fach.** 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. J. Appl. Microbiol. **95:**38–43.
35. **Lina, G., F. Boutite, A. Tristan, M. Bes, J. Etienne, and F. Vandenesch.** 2003. Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. Appl. Environ. Microbiol. **69:**18–23.
36. **Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne.** 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin. Infect. Dis. **29:**1128–1132.
37. **Lina, G., A. Quaglia, M. E. Reverdy, R. Leclercq, F. Vandenesch, and J. Etienne.** 1999. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrob. Agents Chemother. **43:**1062–1066.
38. **Lindsay, J. A., and M. T. Holden.** 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Funct. Integr Genomics **6:**186–201.
39. **Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds.** 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J. Bacteriol. **188:**669–676.
40. **Lindsay, J. A., A. Ruzin, H. F. Ross, N. Kurepina, and R. P. Novick.** 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. Mol. Microbiol. **29:**527–543.
41. **Melles, D. C., R. F. J. Gorkink, H. A. M. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and A. van Belkum.** 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. J. Clin. Investig. **114:**1732–1740.
42. **Monday, S. R., G. M. Vath, W. A. Ferens, C. Deobald, J. V. Rago, P. J. Gahr, D. D. Monie, J. J. Iandolo, S. K. Chapes, W. C. Davis, D. H. Ohlendorf, P. M. Schlievert, and G. A. Bohach.** 1999. Unique superantigen activity of staphylococcal exfoliative toxins. J. Immunol. **162:**4550–4559.
43. **Monk, A. B., S. Curtis, J. Paul, and M. C. Enright.** 2004. Genetic analysis of *Staphylococcus aureus* from intravenous drug user lesions. J. Med. Microbiol. **53:**223–227.
44. **Moore, P. C. L., and J. A. Lindsay.** 2001. Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: evidence for horizontal transfer of virulence genes. J. Clin. Microbiol. **39:**2760–2767.
45. **Murakami, K., W. Minamide, K. Wada, E. Nakamura, H. Teraoka, and S. Watanabe.** 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. J. Clin. Microbiol. **29:**2240–2244.
46. **Musser, J., P. Schlievert, A. Chow, P. Ewan, B. Kreiswirth, V. Rosdahl, A. Naidu, W. Witte, and R. Selander.** 1990. A single clone of *Staphylococcus aureus* causes the majority of cases of toxic shock syndrome. Proc. Natl. Acad. Sci. USA **87:**225–229.
47. **Nouwen, J., M. Fieren, S. Snijders, H. Verbrugh, and A. van Belkum.** 2005. Persistent (not intermittent) nasal carriage of *Staphylococcus aureus* is the determinant of CPD-related infections. Kidney Int. **67:**1084–1092.
48. **Omoe, K., D. L. Hu, H. Takahashi-Omoe, A. Nakane, and K. Shinagawa.** 2005. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. FEMS Microbiol. Lett. **246:**191–198.
49. **Omoe, K., D.-L. Hu, H. Takahashi-Omoe, A. Nakane, and K. Shinagawa.** 2003. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. Infect. Immun. **71:**6088–6094.
50. **Peacock, S. J., A. Justice, D. Griffiths, G. D. I. de Silva, M. N. Kantzanou, D. Crook, K. Sleeman, and N. P. J. Day.** 2003. Determinants of acquisition and carriage of *Staphylococcus aureus* in infancy. J. Clin. Microbiol. **41:**5718–5725.
51. **Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. J. Day.** 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect. Immun. **70:**4987–4996.
52. **Robinson, D. A., and M. C. Enright.** 2004. Evolution of *Staphylococcus aureus* by large chromosomal replacements. J. Bacteriol. **186:**1060–1064.
53. **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**3926–3934.
54. **Robinson, D. A., A. B. Monk, J. E. Cooper, E. J. Feil, and M. C. Enright.** 2005. Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. J. Bacteriol. **187:**8312–8321.
55. **Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W. Friedrich, and W. Witte.** 2006. Assignment of *Staphylococcus* isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. J. Clin. Microbiol. **44:**2533–2540.
56. **Takahashi, M., M. Takahashi, F. Shinohara, H. Takada, and H. Rikiishi.** 2001. Effects of superantigen and lipopolysaccharide on induction of CD80 through apoptosis of human monocytes. Infect. Immun. **69:**3652–3657.
57. **van Belkum, A., D. C. Melles, S. V. Snijders, W. B. van Leeuwen, H. F. L. Wertheim, J. L. Nouwen, H. A. Verbrugh, and J. Etienne.** 2006. Clonal distribution and differential occurrence of the enterotoxin gene cluster, *egc*, in carriage- versus bacteremia-associated isolates of *Staphylococcus aureus*. J. Clin. Microbiol. **44:**1555–1557.
58. **van Leeuwen, W. B., D. C. Melles, A. Alaidan, M. Al-Ahdal, H. A. Boelens, S. V. Snijders, H. Wertheim, E. van Duijkeren, J. K. Peeters, P. J. van der Spek, R. Gorkink, G. Simons, H. A. Verbrugh, and A. van Belkum.** 2005. Host- and tissue-specific pathogenic traits of *Staphylococcus aureus*. J. Bacteriol. **187:**4584–4591.
59. **von Eiff, C., K. Becker, K. Machka, H. Stammer, and G. Peters.** 2001. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. N. Engl. J. Med. **344:**11–16.
60. **Waldron, D. E., and J. A. Lindsay.** 2006. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. J. Bacteriol. **188:**5578–5585.
61. **Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen.** 2005. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect. Dis. **5:**751–762.
62. **Wertheim, H. F., W. B. van Leeuwen, S. Snijders, M. C. Vos, A. Voss, C. M. Vandenbroucke-Grauls, J. A. Kluytmans, H. A. Verbrugh, and A. van Belkum.** 2005. Associations between *Staphylococcus aureus* genotype, infection, and in-hospital mortality: a nested case-control study. J. Infect. Dis. **192:**1196–1200.
63. **Wertheim, H. F., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh.** 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet **364:**703–705.
64. **Wright, J. S., III, K. E. Traber, R. Corrigan, S. A. Benson, J. M. Musser, and R. P. Novick.** 2005. The *agr* radiation: an early event in the evolution of staphylococci. J. Bacteriol. **187:**5585–5594.
65. **Yamaguchi, T., K. Nishifuji, M. Sasaki, Y. Fudaba, M. Aepfelbacher, T. Takata, M. Ohara, H. Komatsuzawa, M. Amagai, and M. Sugai.** 2002. Identification of the *Staphylococcus aureus etd* pathogenicity island, which encodes a novel exfoliative toxin, ETD, and EDIN-B. Infect. Immun. **70:**5835–5845.
66. **Yamasaki, O., A. Tristan, T. Yamaguchi, M. Sugai, G. Lina, M. Bes, F. Vandenesch, and J. Etienne.** 2006. Distribution of the exfoliative toxin D gene in clinical *Staphylococcus aureus* isolates in France. Clin. Microbiol. Infect. **12:**585–588.
67. **Zhang, S., J. J. Iandolo, and G. C. Stewart.** 1998. The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (Sej). FEMS Microbiol. Lett. **168:**227–233.

SUPPLEMENTAL MATERIAL

Suppl. Table 1: Oligonucleotide primers and control strains used for toxin gene detection.

	Gene	Primers	GenBank accession no.	Sequence (5'-3')	Fragment size (bp)	Tm (°C)	Control strain	Reference
multiplex I	*sea*	Nsea-1	M18970	gaa aaa agt ctg aat tgc agg gaa ca	560	55	FRI913	27
		Nsea-2		caa ata aat cgt aat taa ccg aag gtt c				
	seh	Nseh-1	U11702	caa tca cat cat atg cga aag cag	376	55	FRI137	27
		Nseh-2		cat cta ccc aaa cat tag cac c				
	sec	Nsec-1	X05815	ctt gta tgt atg gag gaa taa caa aac atg	275	55	FRI913	27
		Nsec-2		cat atc ata cca aaa agt att gcc gt				
	tst	Ntst-1	J02615	ttc act att tgt aaa agt gtc aga ccc act	180	55	FRI913	27
		Ntst-2		tac taa tga att ttt tta tcg taa gcc ctt				
multiplex II	*sed*	Nsed-1	M28521	gaa tta agt agt acc gcg cta aat aat atg	492	55	FRI1151m	27
		Nsed-2		gct gta ttt ttc ctc cga gag t				
	etd	Netd-1	AB057421	caa act atc atg tat caa gga tgg	358	55	TY114	67
		Netd-2		cca gaa ttt ccc gac tca g				
	eta	Neta-1	M17347	act gta gga gct agt gca ttt gt	190	55	A920210	27
		Neta-2		tgg ata ctt ttg tct atc ttt ttc atc aac				
	sek	Nsek-1	U93688	atg cca gcg ctc aag gc	134	55	FRI913	this work
		Nsek-2		aga ttc att tga aaa ttg tag ttg att agc t				
		Nsek-3	AAW36439	tgc cag cgc tca agg tg				
multiplex III	*see*	Nsee-1	M21319	caa aga aat gct tta agc aat ctt agg c	482	55	FRI918	27
		Nsee-2		cac ctt acc gcc aaa gct g				
	seb	Nseb-1	M11118	att cta tta agg aca cta agt tag gga	404	55	CCM5757	27
		Nseb-2		atc ccg ttt cat aag gcg agt				
	sem	Nsem-1	AF285760	cta tta atc ttt ggg tta atg gag aac	326	55	FRI137	27
		Nsem-2		ttc agt ttc gac agt ttt gtt gtc at				
	sel	Nsel-1	NC_002745	gcg atg tag gtc cag gaa ac	234	55	FRI137	this work
		Nsel-2		cat ata tag tac gag agt tag aac cat a				
	seo	Nseo-1	AF285760	agt ttg tgt aag aag tca agt gta ga	180	55	FRI137	27
		Nseo-2		atc ttt aaa ttc agc aga tat tcc atc taa c				

multiplex IV	*sen*	Gsen-1	AF285760	cgt ggc aat tag acg agt c	474	55	FRI137	this work
		Gsen-2	NC_002952	gat tga tyt tga tga tta tka g				
	seg	Gseg-1	AF285760	tct cca cct gtt gaa gg	323	55	FRI137	this work
		Gseg-2		aag tga ttg tct att gtc g				
	seq	Gseq-1	AAW36439	acc tga aaa gct tca agg a	204	55	Col	this work
		Gseq-2		cgc caa cgt aat tcc ac				
	sej	Gsej-1	AF053140	tca gaa ctg ttg ttc cgc tag	138	55	FRI1151m	this work
		Gsej-2		gaa ttt tac cay caa agg tac				
multiplex V	*sei*	Gsei-1	AAW36439/	cty gaa ttt tca acm ggt ac	461	55	FRI137	this work
		Gsei-2	AF285760	agg cag tcc atc tcc tg				
	ser	Nser-1	AB075606	agc ggt aat agc aga aaa tg	363	55	FRI1151m	this work
		Nser-2		tct tgt acc gta acc gtt tt				
	seu	Nseu-1	AY205306	aat ggc tct aaa att gat gg	215	55	FRI137	this work
		Nseu-2		att tga ttt cca tca tgc tc				
	sep	Gsep-1	NC_002745	gaa ttg cag gga act gct	182	55	N315	this work
		Gsep-2		ggc ggt gtc ttt tga ac				
multiplex VI	*agr 1 – 4*	pan agr		atg cac atg gtg cac atg c				
	agr-1	agr1	X52543	gtc aca agt act ata agc tgc gat	439	55	Col	35
	agr-2	agr2	AF001782	tat tac taa ttg aaa agt gcc ata gc	572	55	N315	35
	agr-3	agr3	AF001783	gta atg taa tag ctt gta taa taa tac cca g	320	55	TY114	35
	agr-4	agr4	AF288215	cga taa tgc cgt aat acc cg	657	55	A920210	35
single PCR	*16SrRNA*	16SrRNA-		gta ggt ggc aag cgt tat cc	228	58		24
		16SrRNA-		cgc aca tca gcg tca g				
	gyrase	gyr-1		agtaca tcg tcg tat act ata tgg	281	55		37
		gyr-2		atc acg taa cag ttc aag tgt g				
	mecA	mecA-1	NC_002745	aaa atc gat ggt aaa ggt tgg c	533	55	N315	45
		mecA-2		agt tct gca gta ccg gat ttg c				
	pvl	Npvl-1	AB006796	atc att agg taa aat gtc tgg aca tga tcc a	433	55	ATCC49775	27
		Npvl-2		gca tca ast gta ttg gat agc aaa agc				
sequen-cing	*sem*	sems-1		gat agr saw rtt taa wta tag gag aaa ta	780	56.8		this work
		sems-2		tcc ttt wct aag tta tga ttg aa				
	spa	spa-1113f		taa aga cga tcc ttc cgt gag c	variable	60		22
		spa-1514r		cag cag tag tgc cgt ttg ctt				

Chapter 3

Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages

C. Goerke, R. Pantucek, S. Holtfreter, B. Schulte, M. Zink, D. Grumann, B. M. Bröker, J. Doskar, and C. Wolz

PUBLISHED IN:

J Bacteriol 2009; 191:3462-3468

JOURNAL OF BACTERIOLOGY, June 2009, p. 3462–3468 Vol. 191, No. 11
0021-9193/09/$08.00+0 doi:10.1128/JB.01804-08

Diversity of Prophages in Dominant *Staphylococcus aureus* Clonal Lineages▽

Christiane Goerke,[1]* Roman Pantucek,[2] Silva Holtfreter,[3] Berit Schulte,[1] Manuel Zink,[1] Dorothee Grumann,[3] Barbara M. Bröker,[3] Jiri Doskar,[2] and Christiane Wolz[1]

Institut für Med. Mikrobiologie und Hygiene, Universitätsklinikum Tübingen, Tübingen, Germany[1]; Department of Genetics and Molecular Biology, Masaryk University, Brno, Czech Republic[2]; and Institut für Immunologie und Transfusionsmedizin, Universität Greifswald, Greifswald, Germany[3]

Received 23 December 2008/Accepted 23 March 2009

Temperate bacteriophages play an important role in the pathogenicity of *Staphylococcus aureus*, for instance, by mediating the horizontal gene transfer of virulence factors. Here we established a classification scheme for staphylococcal prophages of the major *Siphoviridae* family based on integrase gene polymorphism. Seventy-one published genome sequences of staphylococcal phages were clustered into distinct integrase groups which were related to the chromosomal integration site and to the encoded virulence gene content. Analysis of three marker modules (lysogeny, tail, and lysis) for phage functional units revealed that these phages exhibit different degrees of genome mosaicism. The prevalence of prophages in a representative *S. aureus* strain collection consisting of 386 isolates of diverse origin was determined. By linking the phage content to dominant *S. aureus* clonal complexes we could show that the distribution of bacteriophages varied remarkably between lineages, indicating restriction-based barriers. A comparison of colonizing and invasive *S. aureus* strain populations revealed that *hlb*-converting phages were significantly more frequent in colonizing strains.

Staphylococcus aureus asymptomatically colonizes the anterior nares of humans but also causes a wide spectrum of acute and chronic diseases. Most of the dissimilarity between *S. aureus* strains is due to the presence of mobile genetic elements such as plasmids, bacteriophages, pathogenicity islands, transposons, and insertion sequences (2, 14, 19, 23). Many virulence factors are encoded on such mobile elements (3, 6, 17, 26, 27, 35). In particular, bacteriophages play an important role in the pathogenicity of *S. aureus* either by carrying accessory virulence factors such as Panton-Valentine leukocidin (PVL) (encoded by the *luk*-PV operon), staphylokinase (encoded by *sak*), enterotoxin A (encoded by *sea*), and exfoliative toxin A (encoded by *eta*) or by interrupting chromosomal virulence genes such as those for β-hemolysin (*hlb*) and lipase (*geh*) upon insertion. Additionally, phages are the primary vehicle of lateral gene transfer between *S. aureus* strains, providing the species with the potential for broad genetic variation. We could show that phages increase the genome plasticity of *S. aureus* during infection, facilitating the adaptation of the pathogen to various host conditions (11, 12).

Despite the obvious importance of phages for the biology of *S. aureus*, epidemiological data on the prevalence of phages in this species are limited (28, 33). More than 80 genome sequences of staphylococcal bacteriophages and prophages are available in the public genome databases. Most published *S. aureus* phages belong to the *Siphoviridae* family of temperate, tailed bacterial viruses. Traditionally, *S. aureus* phages were characterized according to their lytic activity, morphology, and serological properties (1, 28). Today, the temperate phages in clinical *S. aureus* isolates can by identified with a multiplex PCR strategy, which is based on sequence differences between viral genes coding for the surface-exposed determinants (28).

In general, the evolution of phage lineages seems to be driven by the lateral gene transfer of interchangeable genetic elements (modules), which consist of functionally related genes. The *Siphoviridae* genomes are usually organized into six functional modules: lysogeny, DNA replication, regulation of transcription, packaging and head, tail, and lysis (4). A functional module found in one phage can be replaced in another phage by a sequence-unrelated module that fulfils the same or related functions. Multiple alignment of *S. aureus* phage genomes also revealed a chimeric and mosaic structure resulting from horizontal transfer and recombination (5, 20). It is an open question whether all phages have access to a common gene pool or whether subpools have developed, which are due to differences in the accessibility of strain variants of the bacterial host species.

It was recently shown that most human *S. aureus* strains belong to one of 10 independent lineages or clonal complexes (CCs) (9, 24). Exchange of DNA is very much lower between different lineages than within the same lineage due to the action of the restriction-modification (R-M) system SauI (34). The prime role of R-M systems in many bacteria is the defense against DNA bacteriophages. These systems usually comprise a DNA methyltransferase and a restriction endonuclease. The former protects self DNA by methylation of specific nucleotides in a certain DNA sequence, whereas the latter cleaves the foreign unmodified DNA at the same sequence motif. If the spread of bacteriophages between different *S. aureus* lineages was controlled by the R-M system or a similar mechanism, an unequal distribution would be expected.

Here we established a classification scheme for staphylococ-

* Corresponding author. Mailing address: Institut für Med. Mikrobiologie und Hygiene, Universitätsklinikum Tübingen, Elfriede-Aulhorn-Str. 6, 72076 Tübingen, Germany. Phone: 49-7071-2985229. Fax: 49-7071-295165. E-mail: christiane.goerke@med.uni-tuebingen.de.

▽ Published ahead of print on 27 March 2009.

cal prophages of the major *Siphoviridae* family which was based on the suggested phage designation of the published *S. aureus* genomes (22, 23). When analyzing a representative *S. aureus* strain collection, we could show that the frequency of certain phage groups varied between *S. aureus* lineages. A comparison of colonizing and invasive *S. aureus* strain populations revealed that *hlb*-converting phages were significantly more frequent in colonizing strains.

MATERIALS AND METHODS

Bacterial isolates. *S. aureus* isolates from different sources were included in this study: nasal carriage isolates and blood culture isolates from the University of Greifswald (15); nasal carriage isolates (12) and clinical isolates from diverse sources from the Department of Medical Microbiology and Hygiene, Tübingen; blood culture isolates from the Kantonsspital Basel; community-associated methicillin-resistant *S. aureus* (MRSA) reference isolates from the Robert Koch Institute, Wernigerode; and reference strains from the Network on Antimicrobial Resistance in *Staphylococcus aureus* strain collection.

Strain typing. *spa* typing was performed as described previously (15) using the Ridom StaphType software (13). Pulsed-field gel electrophoresis (PFGE) typing and Southern blot analysis were performed as described previously (10).

MLST. Multilocus sequence genotyping (MLST) was performed on selected isolates according to published protocols (8). Otherwise, MLST CCs were deduced from BURP grouping of *spa* types or by comparison with the PFGE pattern (31).

Phage integrase multiplex PCRs. Multiplex PCR was performed using the multiplex PCR kit (Qiagen, Hilden, Germany). Each reaction mixture (25 μl) contained 12.5 μl 2× Qiagen multiplex PCR master mix, 200 nM of each primer, and 10 ng of template DNA. An initial denaturation of DNA at 95°C for 15 min was followed by 35 cycles of amplification (95°C for 30 s, 55°C for 60 s, and 72°C for 45 s), ending with a final extension phase at 72°C for 10 min. All PCR products were resolved by electrophoresis in 3% agarose gels, stained with ethidium bromide, and visualized under UV light. The following primers specific for the phage integrase genes were used: for Sa1int, Sa1-F (AAGCTAAGTTC GGGCACA) and Sa1-R (GTAATGTTTGGGAGCCAT) (length, 569 bp); for Sa2int, Sa2-F (TCAAGTAACCCGTCAACTC) and Sa2-R (ATGTCTAAATG TGTGCGTG) (length, 640 bp); for Sa3int, Sa3-F (GAAAAACAAACGGTGC TAT) and Sa3-R (TTATTGACTCTACAGGCTGA) (length, 475 bp); for Sa4int, Sa4-F (ATTGATATTAACGGAACTC and Sa4-R (TAAACTTATATG CGTGTGT) (length, 320 bp); for Sa5int, Sa5-F (AAAGATGCCAAACTA GCTG and Sa5-R (CTTGTGGTTTTGTTCTGG) (length, 375 bp); for Sa6int, Sa6-F (GCCATCAATTCAAGGATAG and Sa6-R (TCTGCAGCTGAGGAC AAT) (length, 167 bp); and for Sa7int, Sa7-F (GTCCGGTAGCTAGAGGTC and Sa7-R (GGCGTATGCTTGACTGTGT) (length, 214 bp). Validation of the multiplex PCR assay was carried out with (i) genome-sequenced *S. aureus* strains, (ii) prophage-less *S. aureus* 8325-4 or *S. aureus* 1039 lysogenized with genome-sequenced phages of the International Typing Set belonging to different *int* gene classes (ϕ55, Sa1int; ϕ47, Sa2int; ϕ42E, Sa3int; ϕ29, Sa5int; ϕ77, Sa6int; or ϕ53, Sa7int), and (iii) triple-lysogenic *S. aureus* NCTC 8325 (harboring prophages ϕ11, ϕ12, and ϕ13) lysogenized with ϕ77 or with ϕ53.

Sequence analysis. Phage sequences were obtained from the NCBI nucleotide database or were assembled from the published *S. aureus* genomes. Open reading frames (ORFs) for the integrase and holin genes were deduced from the whole phage genomes by BLAST analysis. Integrase sequences were aligned by ClustalW. Phages were assigned to serotypes using the primer sequences published by Pantucek et al. to identify the respective genes (28).

For sequencing of the integrase and holin genes of phage ϕ6390, the targets were amplified from strain RN6390 by standard PCR using the primers 6390intseq-for (ATTGGCGAACGAGGTAAC) and 6390intseq-rev (GCCA ATTTTGAGGAGGGAG) for the integrase gene and Holin255-for (ATGA TTAATTGGAAAATTAGAA and Holin255-rev (CTAGTATTTTCTTCTT GGTTCT) for the holin gene. Amplicons were cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) for sequencing. The sequencing was done by 4base lab, Reutlingen, Germany, using the Dynamic sequence kit (Amersham Biosciences, Freiburg, Germany). Sequence data were analyzed using Vector NTI software (InforMax, Frederick, MD).

Statistical analysis. Differences between groups were assessed using the likelihood ratio test with Bonferroni's adjustment.

Nucleotide sequence accession number. The ϕ6390 integrase sequence was deposited in the GenBank and EMBL databases (accession no. FM877489).

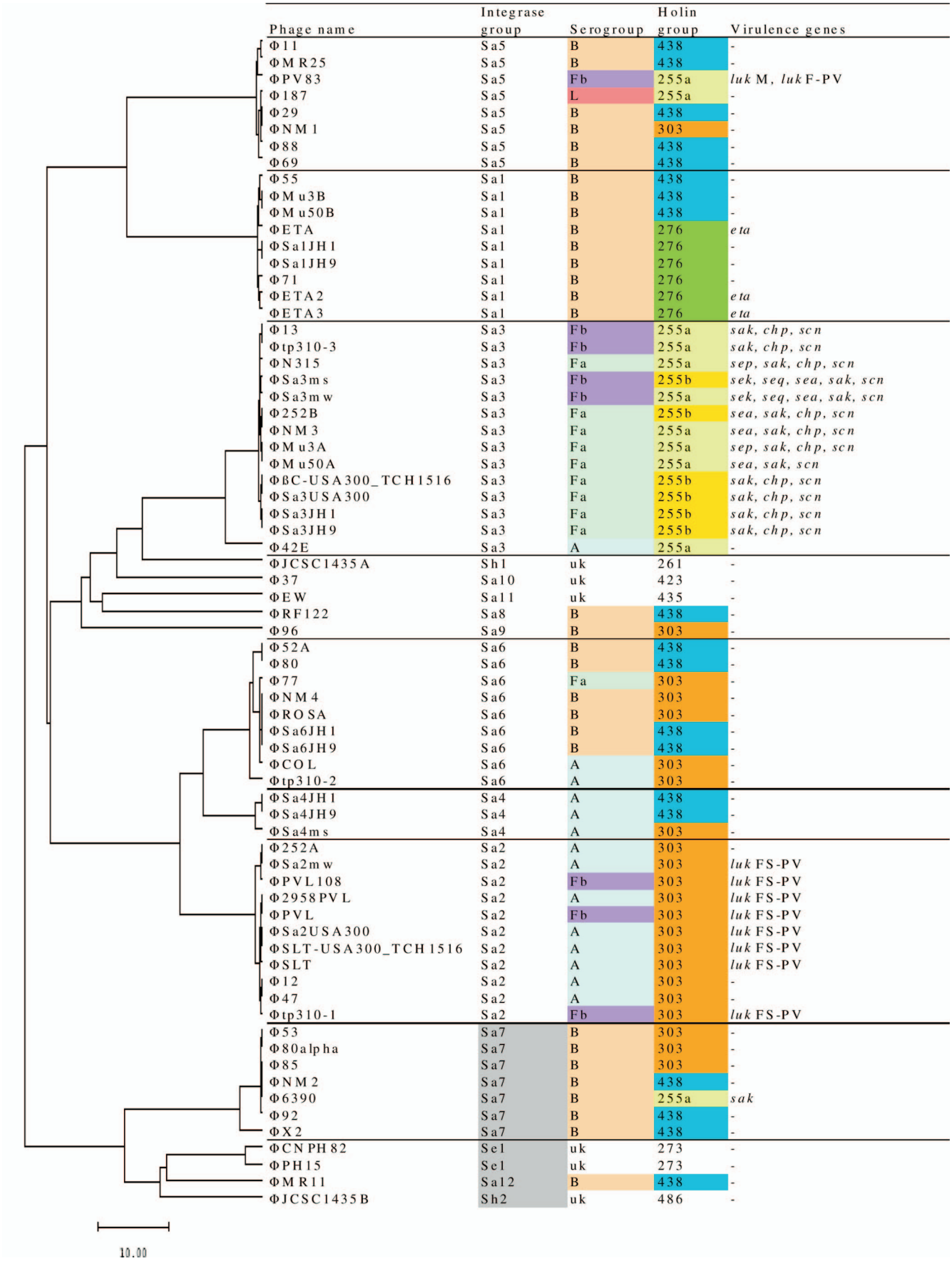
RESULTS

Classification of staphylococcal prophage modules. The identification and description of prophages existing in bacterial strains relies on a clear classification scheme, while the grouping of bacteriophages into distinct phage types is extremely difficult because of high sequence variation even within functionally equivalent genes. Additionally, recombination leads to the emergence of extensive mosaicism in bacteriophage genomes (5, 20). To develop a reliable classification system, we compared all 71 complete bacteriophage genome sequences of the class *Siphoviridae* currently available in the databases; the majority are derived from *S. aureus*, and two each are from *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. The comparison was based on three distinct features present in all phages: (i) differences in the lysogeny module, in particular the integrase, which dictates the insertion site of the phage in the bacterial chromosome; (ii) differences in the phage morphology module, where the serogroup of each phage was determined based on capsid, tail, and tail appendix protein sequences (28), and (iii) differences in the lysis module, where the characteristic holin gene was investigated.

Genes coding for a putative integrase could be identified in all the available staphylococcal phage genomes with the exception of one (ϕ3A). Alignment of the integrase nucleotide sequences clustered the prophages in seven major and eight minor groups (Fig. 1). Within groups, the nucleotide sequence identity was 95% to 100%; between the groups, there was only 38% to 84% identity, which was still compatible with functional analogy. In fact, based on amino acid sequence homology and catalytic residues, most integrases belong to the tyrosine recombinase type family; only Sa7int, Sa12int, Se1int, and Sh2int belong to the serine recombinase type family. Most *S. aureus* prophages clustered in one of the seven major groups (designated Sa1int to Sa7int), and five were singletons (Sa8int to Sa12int). The two known *S. epidermidis* bacteriophages (ϕCNPH82 and ϕPH15) and the two *S. haemolyticus* prophages (ϕJCSC1435A and ϕJCSC1435B) differed strongly from all *S. aureus* phages (Fig. 1).

Next, the serogroup-specifying genes were compared based on capsid, tail, and tail appendix protein sequences (28). The three main serogroups A, B, and F were associated with phage tail appendices. F phages were classified into two subgroups, Fa and Fb, because their DNA-packaging, head, and tail genes belong to different modules. Sequence homology within groups ranged from 85% to 100%, while there was no significant homology between the groups. The majority of *S. aureus* phages could be assigned to one of the four prominent serogroups (A, B, Fa, and Fb). Two *S. aureus* phages (ϕ37 and ϕEW), the two *S. epidermidis* phages, and the two *S. haemolyticus* phages could not be discerned with the applied classification scheme.

Sequence alignment of the holin genes revealed 10 different groups. Sequence relatedness within these groups ranged from 92% to 100% homology. Because sequence homology was closely correlated with gene length, the holin groups were designated by the sequence length polymorphism (number), and additional sequence variations were indicated by letters (255a, 255b, 216, 273, 276, 303, 423, 435, 438, and 486). The majority of the *S. aureus* phages clustered in one of the five



major holin groups. Little sequence homology was observed between the holin genes from the different staphylococcal species.

Mosaicism of staphylococcal phage genomes. When comparing the integrase sequence tree with the results of the other two classification systems for 70 staphylococcal phages (ϕ3A was omitted, because no integrase gene could be detected), different degrees of genome mosaicisms were observed. For the phage groups Sa3int, Sa5int, and Sa6int, a high diversity in the combination of the three marker modules was determined (Fig. 1). The largest group of *S. aureus* phages (14/70) are the Sa3int phages, which differed in serogroup (Fa, Fb, or A), holin genes (255a and 255b are not closely related), and their combination of immune-modulatory virulence factors. Phages of this group typically integrate into the *hlb* gene of *S. aureus*, leading to negative conversion of β-hemolysin production (6). Similarly, the phages of the Sa5int group exhibited a high diversity in their module pattern: three serogroups (B, Fb, and L) and three holin groups (255a, 303, and 438) could be detected. The popular transducing phage ϕ11 of *S. aureus* strain 8325 is placed in this group. Phage ϕPV83 is the only Sa5int member which encodes a known virulence factor (*lukM*). Finally, serogroups A, B, and Fa and two holin genes, 303 and 438, were found in phage group Sa6int. These phages typically integrate into the lipase gene (*geh*) of *S. aureus* (21).

A lower degree of variation regarding the three analyzed modules was found in the *S. aureus* phage groups Sa1int, Sa2int, Sa4int, and Sa7int (Fig. 1). Some Sa1int phages harbor the exfoliative toxin a (*eta*). Sa2int phages integrate into an ORF (SA3121) of unknown function in the *S. aureus* genome, as shown for phage ϕ12 of strain 8325 and the PVL-encoding phages (18). All Sa7int phages contained the serogroup B module and holin gene 303 or 438, with the exception of prophage ϕ6390. This phage encodes holin 255a, which is characteristic for Sa3int phages, the typical *sak*-carrying phages. We recently determined the integration site of ϕ6390 (intergenic region between *rpmF* and *isdB*) in the prototypic *S. aureus* strain RN6390 and showed that it carries the virulence gene *sak* (11).

The remaining *S. aureus* phages harbored unique integrase types but shared serogroups or holin genes with other integrase groups. For instance, the prophage ϕRF122 of the bovine *S. aureus* isolate RF122 (Sa8int) and the Sa9int and Sa12int phages all exhibit the serogroup B module and holin type 438 or 303. None of the *S. aureus* bacteriophage modules could be detected in the phages of the other staphylococcal species.

The results give an overview of phage mosaicism, but they also show strong association between functional units. Especially, the phage-encoded virulence factors were closely linked to the integrase groups.

Identification of *S. aureus* prophages by multiplex PCR. For a prevalence analysis of *S. aureus* prophages in a large strain collection, we focused on the identification of the integrase polymorphism for several reasons. First, nucleotide sequences

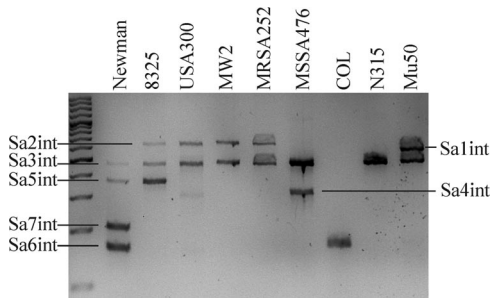


FIG. 2. Multiplex PCR detecting the Sa1int to Sa7int integrase genes in prototypic *S. aureus* strains.

are well conserved within integrase groups making, the gene an ideal target for PCR amplification. Second, the integrase-defined grouping had the best discriminatory power, reflecting the diversity of the *S. aureus* phage population as well as their relatedness. Last, the integrase type is closely linked to the virulence gene content of the prophage and might therefore convey information about the *S. aureus* pathogenic potential (Fig. 1).

We established a multiplex PCR scheme to discriminate between the seven most prominent *S. aureus* integrase families, Sa1int to Sa7int. The method reliably identified the prophage content of the prototypic *S. aureus* strains N315, Mu50, MW2, MRSA252, MSSA476, Newman, and 8325 (Fig. 2). Additionally the method was validated with *S. aureus* strains 8325-4 and 1039 lysogenized with genome-sequenced phages of the International Typing Set. The seven *int* groups were detectable by the multiplex PCR in these isolates (data not shown).

Distribution of phage types in *S. aureus* clonal lineages. To acquire a representative collection of *S. aureus* strains from different clonal lineages, 386 isolates were obtained from the following sources: 161 isolates from nasal colonization of healthy individuals, 115 blood culture isolates, 73 isolates from diverse clinical samples, and 37 reference strains. Both MRSA and methicillin-susceptible *S. aureus* strains were included in this collection. Isolates were typed by either *spa* or PFGE typing and assigned to MLST CCs. After excluding singletons and CCs with fewer than 10 isolates from the strain collection, 291 isolates remained for further analyses. These strains were grouped into seven CCs representing different *agr* types: CC5 (*agr*-2), CC8 (*agr*-1), CC15 (*agr*-2), CC22 (*agr*-1), CC25 (*agr*-1), CC30 (*agr*-3), and CC45 (*agr*-1). No substantial difference in the distribution of CCs was observed in the distinct geographical locations.

Analyzing the prevalence of the seven phage groups in the 291 *S. aureus* isolates revealed that prophages of the groups Sa3int (74%) and Sa2int (33%) (Table 1) were the most frequent ones. Sa7int could be detected in 16%, Sa1int in 9%, Sa6int in 6%, and Sa4int only in 0.2% of the isolates; 13% of the isolates harbored none of the targeted prophages. Next we

FIG. 1. In silico analysis of the integrase groups, serogroups, holin groups, and virulence genes of 70 published staphylococcal bacteriophages of the class *Siphoviridae*. Integrase nucleotide sequences were aligned using the ClustalW algorithm. Identical serogroups and holin groups are color coded. Integrases of the serine recombinase-type family are shaded in gray.

TABLE 1. Distribution of the seven prophage groups Sa1int to Sa7int in common *S. aureus* CCs

CC	% (no.) of isolates	% (no. of isolates)[a] with prophage group:							
		Sa1int	Sa2int	Sa3int	Sa4int	Sa5int	Sa6int	Sa7int	None[d]
15	9 (32)	9 (3)	44 (14)	0 (0)***[b]	0 (0)	3 (1)	19 (6)	6 (2)	31 (10)*[c]
22	4 (13)	8 (1)	23 (3)	85 (11)	0 (0)	0 (0)	0 (0)	0 (0)	15 (2)
25	9 (30)	0 (0)	47 (14)	86 (26)	0 (0)	0 (0)	0 (0)	0 (0)*[b]	10 (3)
30	20 (67)	1 (1)*[b]	58 (39)***[c]	85 (57)	0 (0)	33 (22)***[c]	10 (7)	16 (11)	6 (4)
45	15 (50)	26 (13)**[c]	4 (2)***[b]	72 (36)	0 (0)	18 (9)	2 (1)	14 (7)	24 (12)
5	18 (63)	11 (7)	17 (11)*[b]	89 (56)	2 (1)	5 (3)	5 (3)	38 (24)***[c]	5 (3)
8	11 (36)	6 (2)	39 (14)	83 (30)	0 (0)	3 (1)	0 (0)	11 (4)	11 (4)
All	100 (291)	9 (27)	33 (97)	74 (216)	0.2 (1)	12 (36)	6 (17)	16 (48)	13 (38)

[a] ***, $P < 0.001$; **, $P < 0.005$; *, $P < 0.05$.
[b] Significantly lower than in the whole cohort.
[c] Significantly higher than in the whole cohort.
[d] Absence of any of the seven prophage groups.

asked whether there was a relationship between prophage groups and *S. aureus* clonal lineages. The likelihood ratio test was applied to compare the frequency of phage types in certain CCs with that in the whole cohort. Indeed, the frequency of the different phage types varied remarkably between the tested *S. aureus* CCs. In CC15, the very common Sa3int phages were never detected ($P < 0.001$). This result was verified by Southern analysis using probes specific for the phage-encoded staphylokinase (*sak*) (data not shown). Additionally, in significantly more ($P < 0.05$) CC15 isolates, none of the seven prophage groups could be detected. No CC25 isolate harbored a Sa7int phage ($P < 0.05$). In CC30 isolates, Sa1int phages were significantly less frequent ($P < 0.05$), but the Sa2int and Sa5int phages were significantly more frequent ($P < 0.001$ for both) than in the whole *S. aureus* strain collection. In CC45 the prevalence of Sa1int phages was significantly higher ($P < 0.005$) and that of Sa2int phages significantly lower ($P < 0.001$) than in all isolates. In CC5 also the Sa2int group was less often detected ($P < 0.05$), whereas Sa7int phages were significantly more frequent ($P < 0.001$). The phage prevalences in CC22 and CC8 did not differ from those in the overall cohort.

We next calculated the number of simultaneously occurring prophages per bacterial cell. We could detect none of the seven prophages in 13% (38/291) of the isolates, one prophage in 36% (106/291), two in 38% (110/219), three in 11% (32/219), and four in 2% (4/219). Thus, most isolates contain one or two prophages, but none contain more than four. By linking these results with the genetic background of the isolates, we could show that in CC15, strains with no or only one prophage were strongly overrepresented ($P < 0.0004$), whereas CC30 isolates were more often than average lysogenic for at least two prophages ($P < 0.0001$).

Distribution of phages in invasive versus colonizing isolates. To test whether phage prevalences differ in invasive and colonizing *S. aureus* populations, blood culture isolates were compared to nasal carriage strains. In total 276 isolates were available for analysis, 115 from blood cultures and 161 nasal isolates. When applying the multiplex PCR scheme, we could show that in the colonizing population significantly more isolates harbor Sa3int phages than in the invasive strains ($P < 0.05$) (Table 2). No differences were observed in the prevalence of the other phage types. Most of the isolates were lysogenic for one or more phages: in only 21% of the blood culture and 13% of the nasal isolates could none of the seven phage groups be detected. Additionally, the isolates from both populations also did not differ in the number of prophages per cell (data not shown).

The higher frequency of Sa3int in nasal isolates was not correlated with an overrepresentation of certain CCs (data not shown). In general, no CC was linked to invasive or colonizing strains, and phage distribution was associated only with the genetic background of the strain and not with its origin.

DISCUSSION

Bacteriophages have a tremendous impact on the biology of their bacterial hosts, because they play an important role in bacterial ecology, evolution, and adaptation. For instance, in the human pathogen *S. aureus*, prophages are responsible for the emergence and evolution of new threatening strains such as the community-acquired MRSA strains which carry PVL-encoding prophages. Despite their importance, a comprehensive picture of the distribution of prophages in the *S. aureus* strain populations was lacking. In the present study we could show that prophage prevalence was associated with the clonal background of *S. aureus*, indicating that the spread of the phages in the bacterial population is at least partially restricted. In certain CCs some phage groups were completely absent and others were significantly less or, on the other hand, significantly more frequent. The most prominent disequilib-

TABLE 2. Distribution of the seven prophage groups Sa1int to Sa7int in nasal carriage and blood culture populations

Prophage group	% (no.) of isolates[a] from:		
	Blood culture ($n = 115$)	Nose ($n = 161$)	Total ($n = 276$)
Sa1int	11 (13)	8 (13)	9 (26)
Sa2int	27 (31)	32 (51)	30 (82)
Sa3int	59 (68)	73 (118)*	67 (186)
Sa4int	0 (0)	0 (0)	0 (0)
Sa5int	9 (10)	17 (27)	13 (37)
Sa6int	6 (7)	9 (14)	8 (21)
Sa7int	13 (15)	9 (15)	11 (30)
None	21 (24)	13 (21)	16 (45)

[a] *, $P < 0.05$.

rium was the finding that CC15 strains do not carry Sa3int phages, although this is the most common phage group found in *S. aureus*, with a prevalence of up to 90% (11, 25, 33). In good agreement with this total absence of Sa3int phages from CC15 strains is the earlier observation that this lineage completely lacks staphylococcal superantigen genes (15), some of which (i.e., *sea*, *sep*, *sek*, and *seq*) are carried on Sa3int phages. In addition, many isolates from the CC15 complex carried none of the seven prophage groups, suggesting that this lineage is particularly restrictive to the uptake of foreign DNA. In CC30 an unusually high proportion of Sa2int phages could be detected. In this *S. aureus* lineage an early pandemic clone, which already carried the PVL-encoding phage (members of the Sa2int group), developed into a community-acquired methicillin-resistant clone by acquiring the SCC*mec* type IV cassette (30). CC5 isolates were characterized by the high proportion of Sa7int phages. Interestingly, Sa7int phages of this CC often carry the *sak* gene detected previously on phage ϕ6390 (11) and phages of selected clinical isolates (unpublished data).

The CCs analyzed were shown to differ in their R-M specificity genes (34). The SauI R-M system is a major barrier to horizontal gene transfer in *S. aureus* and seems to delay the evolution of new strains. Mobile genetic elements present in one strain will move to a strain of the same lineage at a higher frequency than to strains of other lineages. As a consequence, *S. aureus* lineages carry a unique combination of core variable genes, suggesting only a vertical transmission of these genes (24). Additional R-M systems were described for *S. aureus*, some of which were shown to be phage encoded, which may also contribute to phage exclusion (7). In addition to the host restriction, the lysogenic immunity of a resident prophage may play a role in prevalence differences. Indeed, Sa1int- and Sa2int-type phages appear to be (in part) mutually exclusive; the simultaneous occurrence of both in a single isolate is uncommon ($P = 0.0095$). In concordance, in CC30, Sa1int phages were rare whereas Sa2int phages were frequent; in CC45, the distribution is vice versa.

When comparing the patterns of phage prevalence in invasive versus colonizing *S. aureus* isolates, no differences were detected with the exception of Sa3int phages, which were significantly more common in colonizing strains. This is in agreement with our own observation that in 96% of nasal isolates Sa3 phages were stably integrated into the *hlb* gene (11). Hlb-converting phages encode the immune-modulatory proteins Sak, Scin, and Chips (6, 33). These may act together to resist the innate immune response encountered during nasal colonization (complement, defensins, and phagocytosis). In contrast, the lack of the Sa3int phages in infecting isolates is correlated to restore Hlb production. This is in concordance with previous findings indicating that Hlb-producing strains are linked to infectious conditions (11, 16, 29). However, when comparing community-acquired invasive isolates with nasal carriage isolates, Lindsay et al. were unable to detect any association between gene and invasive isolates (24). This discrepancy is perhaps due to different criteria for the inclusion of isolates in the invasive group.

In this study we developed a reliable classification scheme for staphylococcal phages of the *Siphoviridae* family, which is the largest, best-described group of temperate *S. aureus* phages. We could show that phages can be clustered into defined groups based on the integrase sequence. This feature fulfils the criteria to be discriminative enough to account for the high diversity of the prophages without being too diverse, thus creating only types represented by single members. Importantly, the integrase identification allows prediction of the chromosomal location of the prophage and gives an indication of the virulence gene content. Analysis of a large *S. aureus* strain collection revealed that most of the isolates contained one to three prophages, which is in line with the phage content of the *S. aureus* strains for which the whole genomes have been sequenced. The most prevalent phages were the Sa3int group, followed by Sa2int. Sa4int was detected only once.

To assess phage diversity, 71 complete staphylococcal bacteriophage genome sequences from the databases were analyzed in three marker regions: lysogeny module, morphogeny module, and lysis module. Various degrees of genome mosaicism could be observed within the different Sa-int groups. The Sa1int and Sa2int groups were characterized by a uniform modular architecture with strong links between the genes for integrase, holin, and encoded virulence factors. Perhaps it is evolutionarily beneficial to interchange this whole unit, which is in proximity in the circular form of the phage. Multiple alignments of several PVL-encoding phages revealed a high degree of mosaic structure of the phage genomes, but the *luk*-PV genes were always located in a 6.4-kb region consisting of the host lysis module, *luk*-PV, *att*P, and the integrase gene (18). We aligned the eight PVL-carrying and the three non-PVL-carrying strains of the Sa2int group to discern the crossover point for integration of the toxin complex. This point appeared to be located at the end of the phage amidase ORF (data not shown). The close organization of the lytic module and the inserted virulence factors is perhaps favored to optimize the phage control of the expression of the pathogenicity genes (32). Interestingly, Sa1int and Sa2int phages, which excluded one another, did not share any modules, suggesting a parallel evolution with no or little contact. It would now be of interest to test whether recombination occurs only within the lineage boundaries or whether phage mosaics are evolutionarily more ancient than the lineage branching. The fact that none of the *S. aureus* phage modules are present in phages from other staphylococcal species argues in favor of the first possibility.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft through grants to C.W. (Wo578/6 and TR34), B.M.B. (TR34 and GRK-840), and D.G. (GRK-840). The contributions of R.P. and J.D. were supported by grant LSHM-CT-2006-019064 from the European Union. Isolates NRS158, NRS161, NRS162, NRS184, NRS187, NRS192, NRS226, NRS229, NRS232, NRS237, and NRS384 were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH contract no. HHSN2722007 00055C.

REFERENCES

1. **Ackermann, H. W., and M. S. DuBow.** 1987. Natural groups of bacteriophages, viruses of prokaryotes, vol. 2. CRC Press, Boca Raton, FL.
2. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu.** 2002. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet **359:**1819–1827.
3. **Betley, M. J., and J. J. Mekalanos.** 1985. Staphylococcal enterotoxin A is encoded by phage. Science **229:**185–187.

4. **Brussow, H., and F. Desiere.** 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. Mol. Microbiol. **39:** 213–222.
5. **Canchaya, C., C. Proux, G. Fournous, A. Bruttin, and H. Brussow.** 2003. Prophage genomics. Microbiol. Mol. Biol. Rev. **67:**238–276.
6. **Coleman, D. C., D. J. Sullivan, R. J. Russell, J. P. Arbuthnott, B. F. Carey, and H. M. Pomeroy.** 1989. *Staphylococcus aureus* bacteriophages mediating the simultaneous lysogenic conversion of beta-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. J. Gen. Microbiol. **135:**1679–1697.
7. **Dempsey, R. M., D. Carroll, H. Kong, L. Higgins, C. T. Keane, and D. C. Coleman.** 2005. Sau42I, a BcgI-like restriction-modification system encoded by the *Staphylococcus aureus* quadruple-converting phage Phi42. Microbiology **151:**1301–1311.
8. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
9. **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day.** 2003. How clonal is *Staphylococcus aureus*? J. Bacteriol. **185:**3307–3316.
10. **Goerke, C., S. Matias y Papenberg, S. Dasbach, K. Dietz, R. Ziebach, B. C. Kahl, and C. Wolz.** 2004. Increased frequency of genomic alterations in *Staphylococcus aureus* during chronic infection is in part due to phage mobilization. J. Infect. Dis. **189:**724–734.
11. **Goerke, C., C. Wirtz, U. Fluckiger, and C. Wolz.** 2006. Extensive phage dynamics in *Staphylococcus aureus* contributes to adaptation to the human host during infection. Mol. Microbiol. **61:**1673–1685.
12. **Goerke, C., M. Gressinger, K. Endler, C. Breitkopf, K. Wardecki, M. Stern, C. Wolz, and B. C. Kahl.** 2007. High phenotypic diversity in infecting but not in colonizing *Staphylococcus aureus* populations. Environ. Microbiol. **9:**3134–3142.
13. **Harmsen, D., H. Claus, W. Witte, J. Rothganger, D. Turnwald, and U. Vogel.** 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. **41:**5442–5448.
14. **Holden, M. T., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill.** 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc. Natl. Acad. Sci. USA **101:**9786–9791.
15. **Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Broker.** 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45:**2669–2680.
16. **Jin, T., M. Bokarewa, L. McIntyre, A. Tarkowski, G. R. Corey, L. B. Reller, and V. G. Fowler, Jr.** 2003. Fatal outcome of bacteraemic patients caused by infection with staphylokinase-deficient *Staphylococcus aureus* strains. J. Med. Microbiol. **52:**919–923.
17. **Kaneko, J., T. Kimura, S. Narita, T. Tomita, and Y. Kamio.** 1998. Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. Gene **215:**57–67.
18. **Kaneko, J., and Y. Kamio.** 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci. Biotechnol. Biochem. **68:**981–1003.
19. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
20. **Kwan, T., J. Liu, M. DuBow, P. Gros, and J. Pelletier.** 2005. The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. Proc. Natl. Acad. Sci. USA **102:**5174–5179.
21. **Lee, C. Y., and S. L. Buranen.** 1989. Extent of the DNA sequence required in integration of staphylococcal bacteriophage L54a. J. Bacteriol. **171:**1652–1657.
22. **Lindsay, J.** 2008. Staphylococcus: molecular genetics. Caister Academics, Norfolk, United Kingdom.
23. **Lindsay, J. A., and M. T. Holden.** 2004. *Staphylococcus aureus*: superbug, super genome? Trends Microbiol. **12:**378–385.
24. **Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds.** 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J. Bacteriol. **188:** 669–676.
25. **Matthews, A. M., and R. Novick.** 2005. Staphylococcal phages, p. 297–318. *In* M. K. Waldor, D. I. Friedman, and S. L. Adhya (ed.), Phages. Their role in bacterial pathogenesis and biotechnology. ASM Press, Washington, DC.
26. **Narita, S., J. Kaneko, J. Chiba, Y. Piemont, S. Jarraud, J. Etienne, and Y. Kamio.** 2001. Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. Gene **268:**195–206.
27. **Novick, R. P.** 2003. Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. Plasmid **49:**93–105.
28. **Pantucek, R., J. Doskar, V. Ruzickova, P. Kasparek, E. Oracova, V. Kvardova, and S. Rosypal.** 2004. Identification of bacteriophage types and their carriage in *Staphylococcus aureus*. Arch. Virol. **149:**1689–1703.
29. **Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day.** 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect. Immun. **70:** 4987–4996.
30. **Robinson, D. A., A. M. Kearns, A. Holmes, D. Morrison, H. Grundmann, G. Edwards, F. G. O'Brien, F. C. Tenover, L. K. McDougal, A. B. Monk, and M. C. Enright.** 2005. Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. Lancet **365:**1256–1258.
31. **Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W. Friedrich, and W. Witte.** 2006. Assignment of *Staphylococcus* isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. J. Clin. Microbiol. **44:**2533–2540.
32. **Sumby, P., and M. K. Waldor.** 2003. Transcription of the toxin genes present within the *Staphylococcal* phage ϕSa3ms is intimately linked with the phage's life cycle. J. Bacteriol. **185:**6841–6851.
33. **van Wamel, W. J., S. H. Rooijakkers, M. Ruyken, K. P. van Kessel, and J. A. van Strijp.** 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. J. Bacteriol. **188:**1310–1315.
34. **Waldron, D. E., and J. A. Lindsay.** 2006. SauI: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. J. Bacteriol. **188:**5578–5585.
35. **Yamaguchi, T., T. Hayashi, H. Takami, K. Nakasone, M. Ohnishi, K. Nakayama, S. Yamada, H. Komatsuzawa, and M. Sugai.** 2000. Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. Mol. Microbiol. **38:**694–705.

Chapter 4

Recurrent furunculosis – associations with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus*

H. Masiuk, K. Kopron, D. Grumann, C. Goerke, J. Kolata, J. Jursa-Kulesza, S. Giedrys-Kalemba, B. M. Bröker, and S. Holtfreter

IN PRESS:

J Clin Microbiol 2010

Recurrent furunculosis - associations with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus*

Helena Masiuk[1], Katarzyna Kopron[1], Dorothee Grumann[2], Christiane Goerke[3], Julia Kolata[2], Joanna Jursa-Kulesza[1], Stefania Giedrys-Kalemba[1], Barbara M. Bröker[2], and Silva Holtfreter[2]

[1] Department of Microbiology and Immunology, Pomeranian Medical University, Szczecin, Poland

[2] Institute for Immunology and Transfusion Medicine, University of Greifswald, Greifswald, Germany

[3] Institute for Medical Microbiology and Hygiene, Universitätsklinikum Tübingen, Tübingen, Germany

ABSTRACT

Staphylococcus aureus is a major cause of skin and soft tissue infections, such as furuncles, carbuncles, and abscesses, but also frequently colonizes the human skin and mucosa without causing clinical symptoms. Panton-Valentine leukocidin (PVL) is a pore-forming toxin that has been associated with soft-tissue infections and necrotizing pneumonia. We have compared genotype, virulence gene repertoire and phage pattern of 74 furunculosis isolates with 108 control strains from healthy nasal carriers.

The large majority of furunculosis strains were methicillin sensitive. CC121 and CC22 accounted for 70% of the furunculosis strains but only for 8% of the nasal isolates. The PVL-enoding genes *luk-PV* were detected in 85% of furunculosis strains, while their prevalence among colonizing *S. aureus* strains was below 1%. *Luk-PV* genes were distributed over several lineages (CC5, 8, 22, 30, 121 and ST59). Even within the same lineages, *luk-PV*-positive phages characterized furunculosis strains, while their *luk-PV*-negative variants were frequent in nasal strains.

The very tight epidemiological linkage between *luk-PV* and furunculosis, which could be separated from the genetic background of the *S. aureus* strain as well as from the gene

make-up of the *luk-PV*-transducing phage, lends support to the notion of an important role for PVL in human furunculosis. These results make a case for the determination of *luk-PV* in recurrent soft tissue infections with methicillin-sensitive as well as -resistant *S. aureus*.

INTRODUCTION

Skin and soft tissue infections (SSTI) are the most frequent disease caused by *S. aureus* outside the hospital setting. SSTI comprise a diverse range of clinical pictures, such as furuncles, carbuncles, subcutaneous abscesses, folliculitis, bullous impetigo and staphylococcal scalded skin syndrome (1). Furunculosis is a very common disease characterized by infection of hair follicles and local accumulation of pus and necrotic tissue. Even mild lesions are painful and unsightly and often heal leaving a scar (2). Antibiotic treatment is frequently not effective and many furunculosis patients suffer from recurrent episodes or develop chronic symptoms over months and years without a period free from outbreaks (2).

Apart from being a major human pathogen, *S. aureus* is also a frequent colonizer of human skin and mucosa (3, 4). The bacteria find their primary ecological niche in the human nose, but are also able to colonize skin, throat, and intestines, sometimes exclusively (5, 6). Around 20% of the healthy population are persistent nasal *S. aureus* carriers (3, 4). Patients suffering from chronic furunculosis are usually *S. aureus* carriers, and skin and nose isolates from a given patient commonly have identical characteristics (7-9).

The species *S. aureus* displays extensive genetic variability. Genotyping analyses, such as multilocus sequence typing (MLST) and protein A (*spa*) sequence typing, demonstrated that the *S. aureus* population structure is highly clonal with 10 major and many minor clonal clusters (CCs) (10-13). Mobile genetic elements comprise 15% of the *S. aureus* genome (14). These plasmids, phages and pathogenicity islands carry a variety of virulence and resistance genes which can strongly enhance virulence (15, 16). For example, staphylococcal prophages, which are classified into the seven types Sa1int to Sa7int, can harbor the genes for the exfoliative toxin A (Sa1int), the pore-forming toxin Panton-Valentine leukocidin (PVL, Sa2int), and superantigens (SAg; Sa3int) (17). Most

mobile genetic elements can readily spread horizontally among *S. aureus* strains of the same clonal cluster while a transfer between clusters is limited (12, 15, 17, 18).

Despite intensive research efforts it still remains elusive how staphylococcal virulence is determined on a molecular level. Numerous studies compared the core genome and virulence gene repertoire of blood culture and colonizing isolates but failed to identify factors clearly related to virulence (10-12). This suggests that invasion into the blood stream does not require special bacterial virulence traits but mainly depends on host factors, e. g. barrier breakage, indwelling catheters, or a compromised immune system. In contrast, the causative virulence factors for a number of toxin-mediated diseases are well known. Toxic shock syndrome and food poisoning are caused by SAgs (19), while staphylococcal scalded skin syndrome and bullous impetigo are associated with exfoliative toxins (20-22).

PVL is a pore-forming toxin, which is composed of two protein components (LukF and LukS), that very efficiently disrupt the cell membrane of neutrophils (23). PVL has been associated with chronic or recurrent skin and soft-tissue infections and with necrotizing pneumonia, which also affect immune competent persons (16, 24-26). One PVL-producing *S. aureus* clone, USA300, a community-acquired methicillin resistant (CA-MRSA) member of the CC8, is epidemic in the US community and causes severe SSTI and necrotizing pneumonia (27).

The aim of this molecular-epidemiological study was to further elucidate the molecular determinants of virulence in chronic furunculosis, in particular to assess the contributions of the bacterial genetic background versus those of virulence factors and phages. By applying *spa* genotyping and PCR-based virulence gene and phage profiling we observed strong associations of PVL and the genetic background with furunculosis.

MATERIALS AND METHODS

Study population and bacterial isolates. *Furunculosis strains: S. aureus* isolates from 74 patients with furunculosis were obtained from a typical mature furuncle (fresh pus) by a physician during the acute phase of skin infection or by a surgeon during abscess incision. In eleven cases nose swabs were taken in parallel. The study was carried out at the Department of Microbiology and Immunology, Pomeranian Medical University, Szczecin, Poland between 2002 and 2008.

Nasal strains: 108 nasal *S. aureus* isolates were obtained from 362 healthy blood donors at the Department of Microbiology and Immunology, Pomeranian Medical University, Szczecin, Poland, in March 2006. Volunteers who reported skin infections during the last 2 years were excluded. All participants gave informed consent, and the study was approved by the Ethics Board of the University of Szczecin. Genotype and virulence genes of a subset of these strains (the 28 CC30 isolates) were previously published by Holtfreter et al. (12).

***S. aureus* identification and DNA isolation.** *S. aureus* was identified using standard diagnostic procedures and a gyrase PCR (12). Total DNA of *S. aureus* was isolated with the Qiagen DNeasy® blood & tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

***spa* genotyping.** PCR for amplification of the *S. aureus* protein A (*spa*) repeat region was performed according to the published protocol (28, 29). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced by a commercial supplier using both amplification primers (Agowa, Berlin, Germany). The forward and reverse sequence chromatograms were analysed with the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). With the BURP algorithm (Ridom GmbH) *spa* types were clustered into different groups, with the following setting: Calculated cost between members of a group less than or equal to five. *Spa* types shorter than five repeats were not clustered, because they do not allow the reliable deduction of ancestries. Since s*pa* typing and multilocus-sequence typing (MLST) are highly concordant (30), *spa* typing data could be easily mapped on MLST types by using the SpaServer database (www.spaserver.ridom.de).

Detection of *S. aureus* virulence factors and phages by PCR. PCR was used to screen for a total of 26 genes. Single PCR was applied for the detection of 16SrRNA, gyrase (*gyr*), methicillin resistance (*mecA*), PVL (*luk-PV*) and exfoliative toxin *etb*. Six sets of multiplex PCRs were applied to amplify I) *sea, seh, sec* and *tst*, II) *sed, etd, eta* and *sek*, III) *see, seb, sem, sel* and *seo*, IV) *sen, seg, seq* and *sej*, V) *sei, ser, seu* and *sep* and VI) *agr* types *1-4* as previously reported (12). Single and multiplex PCRs were performed with the GoTaq® Flexi DNA polymerase system (Promega, Mannheim, Germany) as previously described (12). All PCR products were resolved by electrophoresis in 1.5% agarose gels (1x TBE buffer), stained with Etbr and visualised under UV light. Positive controls

included DNA from SAg gene-positive *S. aureus* reference strains, while *S. aureus* strain 8325-4 served as negative control.

Multiplex PCR for the phage integrase genes *Sa1int -Sa7int* was performed as previously reported (17).

Statistical analysis. Categorial variables were assessed using Person's chi-square test. *P* values of <0.05 were considered statistically significant.

RESULTS

Study cohorts

To identify virulence determinants in *S. aureus* furunculosis, we analysed the genotypes, virulence gene patterns and phage profiles of 74 *S. aureus* isolates from furunculosis patients and 108 nasal isolates from healthy carriers (Table 1).

Table 1: Characteristics of the study cohorts.

	furunculosis	colonization
No. of strains	74	108
age (mean ± SD)	26.6 ± 11.7	29.4 ± 9.4
% male	48.6	88.0
time of sampling	2002 - 2008	march 2006

[1] patient's age unknown for 21/74 samples

[2] patient's gender unknown for 9/74 samples

Spa-defined clonal lineages

To clarify the role of the core genome in furunculosis, we performed *spa* typing on the furunculosis and nasal isolates. This revealed 91 different *spa* types, which were assigned to 10 CCs and 4 sequence types (ST) by BURP clustering. Singletons, i.e. *spa* types which could not be assigned to a CC or ST, occurred among nasal (9/108) and furunculosis strains (1/74). Nine strains were excluded from BURP clustering, because the *spa* repeats were too short, and two strains were *spa*-negative.

As expected, the nasal strains showed a highly diverse population structure (Figure 1). The major lineages (containing more than 5% of the isolates) included CC30 (26%),

CC15 (17%), CC45 (10%) and CC25 (6%), whereas CC5, 8, 12, 121 and ST7, 59 and 109 were detected rarely.

In sharp contrast, 55.4% (41/74; $P<0.001$) of all furunculosis strains belonged to the lineage CC121 (Figure 1). Notably, this lineage was rare among nasal strains (3.7%; 4/108) (Figure 1). *Spa* types were diverse within this lineage: Among the 41 furunculosis-associated CC121 isolates we observed 14 different *spa* types, t159 (13 isolates) and t435 (8 isolates) being the most prevalent. The four commensal CC121 strains all belonged to different *spa* types. Moreover, CC22 was overrepresented among furunculosis strains (14.9% vs. 3.7%, $P < 0.01$). Together, CC121 and CC22 accounted for 70.3% of all furunculosis isolates, and, accordingly, the prevalence of other lineages such as CC15, 25, and 30 was significantly reduced.

Nasal strains were available from 11 of the 74 patients. In all cases, furunculosis and nasal strains were clonally identical (Suppl. table 1). This confirms an earlier study, which reported the same phage type in the nose and the lesion in the majority of furunculosis patients (3).

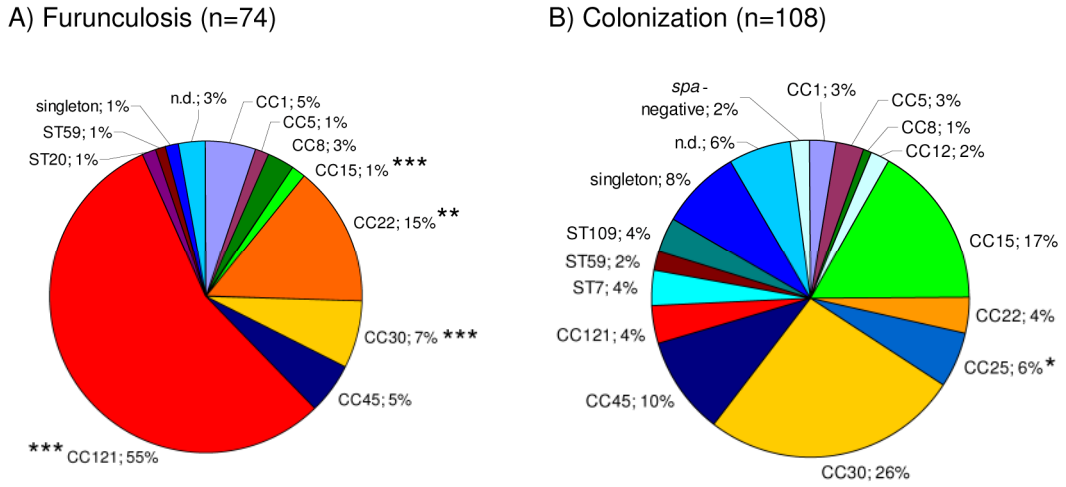


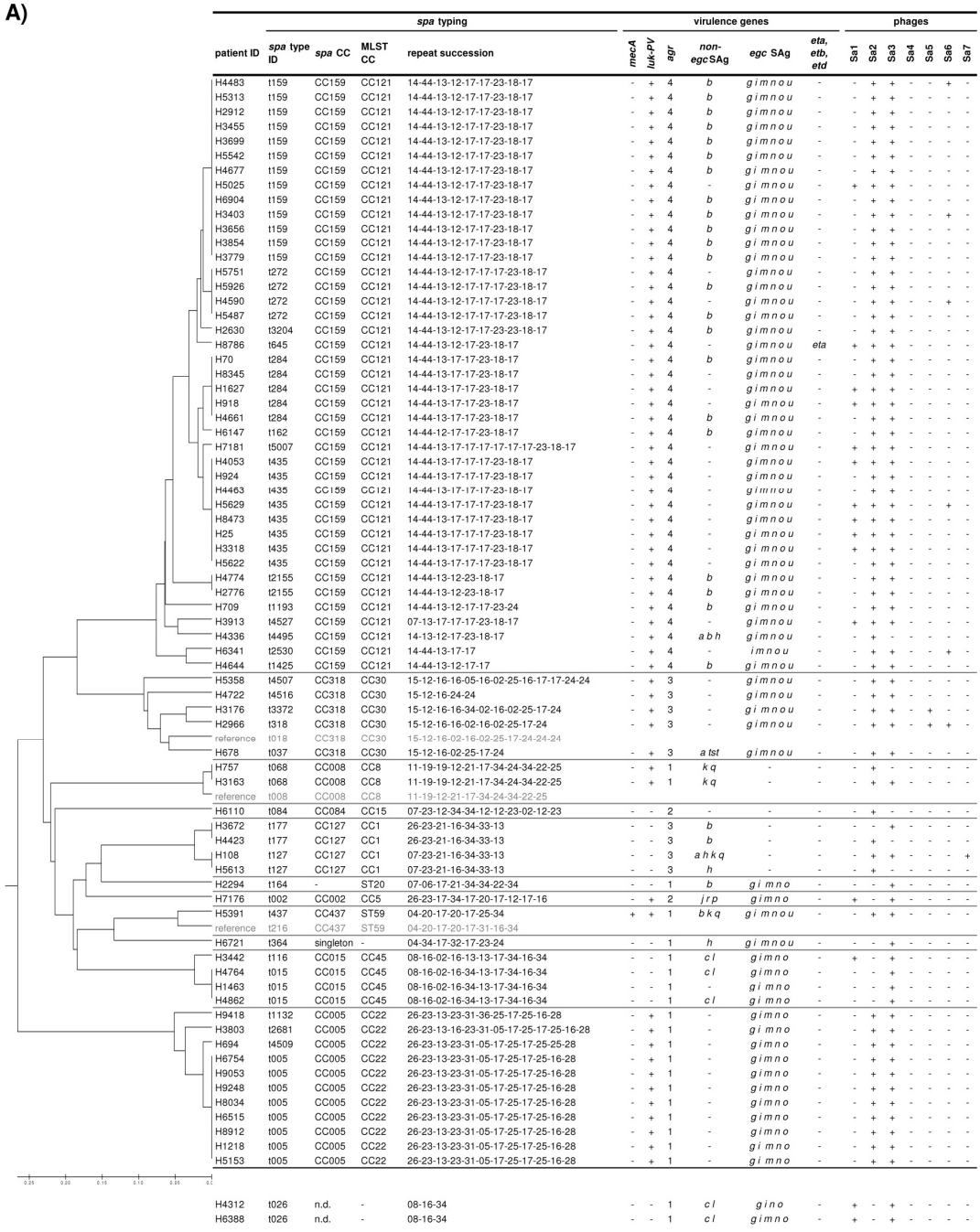
Fig. 1: Prevalence of *spa*-defined CCs among A) furunculosis strains and B) colonizing strains. CC121 and CC22 together accounted for 70.3% of furunculosis strains but only for 8% of the colonizing isolates. *Spa* types were clustered into 10 CCs and 4 STs by BURP analysis. MLST-CC nomenclature was deduced from *spa*-CCs using the Ridom SpaServer database. Chi-Square test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Virulence gene repertoire

To address the contribution of virulence factors to furunculosis, we next determined the genes encoding the methicillin resistance (*mecA*), PVL toxin (*luk-PV*), SAgs (*sea-seu*, *tst*), exfoliative toxins A, B and D (*eta, etb, etd*), and *agr* types 1-4.

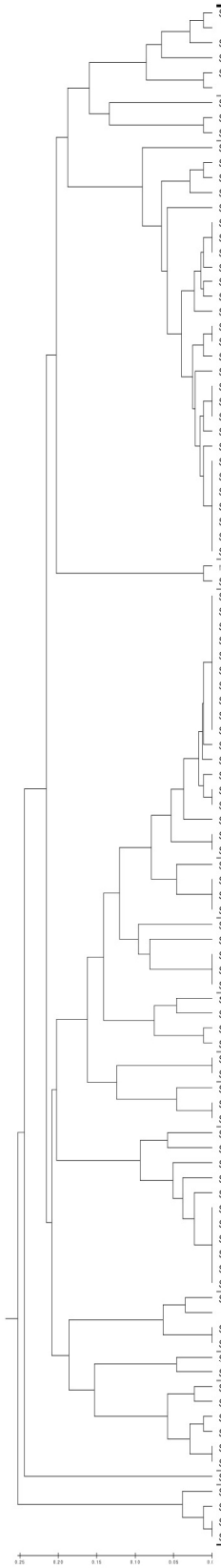
Methicillin resistance: Except for two isolates, all furunculosis and nasal isolates were methicillin-sensitive. Among the nasal strains we detected one MRSA (SZ148) which belonged to CC45, a known MRSA lineage. Moreover, one furunculosis and one nasal isolate (H5391, SZ179) belonged to ST59 and were *mecA*- and *luk-PV*-positive, which is characteristic for CA-MRSA.

A)



	spa typing				virulence genes						phages						
patient ID	*spa* type ID	*spa* CC	MLST CC	repeat succession	*mecA*	*luk-PV*	*agr*	non-*egc* SAg	*egc* SAg	*eta*, *etb*, *etd*	Sa1	Sa2	Sa3	Sa4	Sa5	Sa6	Sa7
H4483	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	+	-
H5313	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H2912	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3455	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3699	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H5542	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H4677	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H5025	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H6904	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3403	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	+	-
H3656	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3854	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3779	t159	CC159	CC121	14-44-13-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H5751	t272	CC159	CC121	14-44-13-12-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	-	+	+	-	-	-	-
H5926	t272	CC159	CC121	14-44-13-12-17-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H4590	t272	CC159	CC121	14-44-13-12-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	-	+	+	-	-	+	-
H5487	t272	CC159	CC121	14-44-13-12-17-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H2630	t3204	CC159	CC121	14-44-13-12-17-17-23-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H8786	t645	CC159	CC121	14-44-13-12-17-23-18-17	-	+	4	-	*g i m n o u*	*eta*	+	+	+	-	-	-	-
H70	t284	CC159	CC121	14-44-13-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H8345	t284	CC159	CC121	14-44-13-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	-	+	+	-	-	-	-
H1627	t284	CC159	CC121	14-44-13-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H918	t284	CC159	CC121	14-44-13-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H4661	t284	CC159	CC121	14-44-13-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H6147	t162	CC159	CC121	14-44-12-17-17-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H7181	t5007	CC159	CC121	14-44-13-17-17-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H4053	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H924	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	-	+	+	-	-	-	-
H4463	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	-	+	+	-	-	-	-
H5629	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	+	-
H8473	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H25	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H3318	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H5622	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	-	+	+	-	-	-	-
H4774	t2155	CC159	CC121	14-44-13-12-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H2776	t2155	CC159	CC121	14-44-13-12-23-18-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H709	t1193	CC159	CC121	14-44-13-12-17-17-23-24	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3913	t4527	CC159	CC121	07-13-17-17-17-23-18-17	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H4336	t4495	CC159	CC121	14-13-12-17-23-18-17	-	+	4	*a b h*	*g i m n o u*	-	-	+	-	-	-	-	-
H6341	t2530	CC159	CC121	14-44-13-17-17	-	+	4	-	*i m n o u*	-	-	+	+	-	-	+	-
H4644	t1425	CC159	CC121	14-44-13-12-17-17	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H5358	t4507	CC318	CC30	15-12-16-16-05-16-02-25-16-17-17-24-24	-	+	3	-	*g i m n o u*	-	-	+	+	-	-	-	-
H4722	t4516	CC318	CC30	15-12-16-24-24	-	+	3	-	*g i m n o u*	-	-	+	+	-	-	-	-
H3176	t3372	CC318	CC30	15-12-16-16-34-02-16-02-25-17-24	-	+	3	-	*g i m n o u*	-	-	+	+	-	+	-	-
H2966	t318	CC318	CC30	15-12-16-16-02-16-02-25-17-24	-	+	3	-	*g i m n o u*	-	-	+	+	-	+	+	-
reference	t018	CC318	CC30	15-12-16-02-16-02-25-17-24-24-24													
H678	t037	CC318	CC30	15-12-16-02-25-17-24	-	+	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	-	-
H757	t068	CC008	CC8	11-19-19-12-21-17-34-24-34-22-25	-	+	1	*k q*	-	-	-	+	-	-	-	-	-
H3163	t068	CC008	CC8	11-19-19-12-21-17-34-24-34-22-25	-	+	1	*k q*	-	-	-	+	+	-	-	-	-
reference	t008	CC008	CC8	11-19-12-21-17-34-24-34-22-25													
H6110	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	-	+	-	-	-	-	-
H3672	t177	CC127	CC1	26-23-21-16-34-33-13	-	-	3	*b*	-	-	-	-	+	-	-	-	-
H4423	t177	CC127	CC1	26-23-21-16-34-33-13	-	-	3	*b*	-	-	-	+	-	-	-	-	-
H108	t127	CC127	CC1	07-23-21-16-34-33-13	-	-	3	*a h k q*	-	-	-	+	+	-	-	-	+
H5613	t127	CC127	CC1	07-23-21-16-34-33-13	-	-	3	*h*	-	-	-	+	-	-	-	-	-
H2294	t164	-	ST20	07-06-17-21-34-34-22-34	-	-	1	*b*	*g i m n o*	-	-	-	+	-	-	-	-
H7176	t002	CC002	CC5	26-23-17-34-17-20-17-12-17-16	-	+	2	*j r p*	*g i m n o*	-	+	-	+	-	-	-	-
H5391	t437	CC437	ST59	04-20-17-20-17-25-34	+	+	1	*b k q*	*g i m n o u*	-	-	+	+	-	-	-	-
reference	t216	CC437	ST59	04-20-17-20-17-31-16-34													
H6721	t364	singleton	-	04-34-17-32-17-23-24	-	-	1	*h*	*g i m n o u*	-	-	-	+	-	-	-	-
H3442	t116	CC015	CC45	08-16-02-16-13-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	+	-	+	-	-	-	-
H4764	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	-
H1463	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
H4862	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	-
H9418	t1132	CC005	CC22	26-23-13-23-31-36-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H3803	t2681	CC005	CC22	26-23-13-16-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H694	t4509	CC005	CC22	26-23-13-23-31-05-17-25-17-25-25-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H6754	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H9053	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H9248	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H8034	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H6515	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H8912	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H1218	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H5153	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H4312	t026	n.d.	-	08-16-34	-	-	1	*c l*	*g i n o*	-	+	-	+	-	-	-	-
H6388	t026	n.d.	-	08-16-34	-	-	1	*c l*	*g i m n o*	-	+	-	+	-	-	-	-

B)



	spa typing				virulence genes						phages						
patient ID	*spa* type	spa-CC	MLST-CC	repeat succession	*mecA*	*luk-PV*	agr	non-*egc*SAg	*egc* SAg	*eta*, *etb*, *etd*	Sa1	Sa2	Sa3	Sa4	Sa5	Sa6	Sa7
SZ235	t272	CC159	CC121	14-44-13-12-17-17-17-23-18-17	-	-	4	-	*g i m n o u*	*eta*	+	-	+	-	-	+	-
reference	t159	CC159	CC121	14-44-13-12-17-17-23-18-17													
SZ034	t435	CC159	CC121	14-44-13-17-17-17-23-18-17	-	-	4	-	*g i m n o u*	-	-	-	-	-	-	+	-
SZ275	t4495	CC159	CC121	14-13-12-17-23-18-17	-	-	4	-	*g i m n o u*	-	-	-	+	-	-	-	-
SZ225	t4499	CC159	CC121	08-17-17-23-18-17	-	-	4	-	*g i m n o u*	*eta, etb*	+	-	+	-	-	+	-
reference	t314	CC159	CC121	08-17-23-18-17													
SZ309	t495	singleton	-	04-34-17-66-66-32-17-23-24	-	-	1	*h*	*g i m n o u*	-	-	-	+	-	+	-	-
SZ178	t1458	singleton	-	121-21-17-17-23-24	-	-	3	*k q tst*	-	-	+	+	+	-	-	-	-
SZ338	t4523	singleton	-	121-21-17-23-24	-	-	3	*k q tst*	-	-	-	+	+	-	-	-	-
SZ095	t1667	CC012	CC30	15-34-02-16-02-25-17-24	-	-	3	-	*g i m n o u*	-	-	-	+	-	+	-	-
SZ076	t122	CC012	CC30	08-16-02-16-02-25-17-24-24	-	-	3	*tst*	*g i m n o u*	-	-	-	+	-	+	-	+
SZ248	t019	CC012	CC30	08-16-02-16-02-25-17-24	-	-	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	-	+
SZ260	t1657	CC012	CC30	15-16-02-16-02-25-25-17-24-24	-	-	3	*tst*	*g i m n o u*	-	-	+	+	-	-	+	-
SZ318	t1675	CC012	CC30	15-21-12-16-02-16-02-25-17-24-24-24	-	-	3	*a*	*g i m n o u*	-	-	-	+	-	-	+	-
SZ150	t018	CC012	CC30	15-12-16-02-16-02-25-17-24-24-24	-	-	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	-	+
SZ215	t018	CC012	CC30	15-12-16-02-16-02-25-17-24-24-24	-	-	3	*a*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ358	t018	CC012	CC30	15-12-16-02-16-02-25-17-24-24-24	-	-	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ348	t1658	CC012	CC30	15-12-16-02-16-02-25-25-17-24-24-24	-	-	3	*k tst*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ228	t486	CC012	CC30	15-12-16-02-16-02-25-17-24-24-24-24-24	-	-	3	*a tst*	*g i m n o u*	-	-	-	-	-	-	-	-
SZ044	t253	CC012	CC30	15-12-16-02-16-02-25-17-24-24-24-24	-	-	3	-	*g i m n o u*	-	-	-	+	-	-	+	-
SZ058	t483	CC012	CC30	15-12-16-16-02-16-02-25-17-24-24-24-24	-	-	3	-	*g i m n o u*	-	-	-	+	-	-	-	-
SZ169	t037	CC012	CC30	15-12-16-02-25-17-24	-	-	3	*a*	*g i m n o u*	-	-	+	+	-	-	+	-
SZ176	t037	CC012	CC30	15-12-16-02-25-17-24	-	-	3	*a*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ219	t338	CC012	CC30	15-21-16-02-25-17-24	-	-	3	*tst*	*g i m n o u*	-	-	+	-	-	+	-	-
SZ288	t1649	CC012	CC30	15-12-16-02-16-02-25-25-17-24	-	-	3	-	*g i m n o u*	-	-	+	+	-	-	-	-
SZ142	t021	CC012	CC30	15-12-16-02-16-02-25-17-24	-	-	3	*a tst*	*g i m n o u*	-	+	-	+	-	-	-	-
SZ281	t021	CC012	CC30	15-12-16-02-16-02-25-17-24	-	-	3	*tst*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ310	t021	CC012	CC30	15-12-16-02-16-02-25-17-24	-	-	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ188	t700	CC012	CC30	15-12-16-02-16-02-25-17-25-17-24	-	-	3	*tst*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ138	t1654	CC012	CC30	15-12-16-02-16-02-25-17-17-24-24	-	-	3	*tst*	*g i m n o u*	-	-	-	-	-	-	+	-
SZ070	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	*tst*	*g i m n o u*	-	-	+	+	-	+	-	-
SZ096	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	*tst*	*g i m n o u*	-	-	-	+	-	+	-	-
SZ100	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	+	-
SZ165	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	*a*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ242	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	*a tst*	*g i m n o u*	-	-	+	+	-	-	-	-
SZ269	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	-	*g i m n o u*	-	-	-	+	-	+	+	-
SZ344	t012	CC012	CC30	15-12-16-02-16-02-25-17-24-24	-	-	3	*tst*	*g i m n o u*	-	-	-	+	-	-	-	-
reference	t008	CC008	CC8	11-19-12-21-17-34-24-34-22-25													
SZ164	t5560	CC008	CC8	11-19-313-21-17-34-24-34-22-25	-	-	1	*a d j r*	-	-	-	+	+	-	-	-	-
SZ040	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	-	-	-	-	-	+	-
SZ087	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	+	-	-	-	-	+	-
SZ092	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	+	-	-	-	-	+	-
SZ113	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	+	-	-	-	-	+	-
SZ114	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	*eta*	+	-	-	-	-	-	-
SZ139	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	-	-	-	-	-	+	-
SZ144	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	*eta*	-	-	+	-	-	-	+
SZ193	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	-	-	-	-	-	+	-
SZ212	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	+	-	-	-	-	-	-
SZ282	t084	CC084	CC15	07-23-12-34-34-12-12-23-02-12-23	-	-	2	-	-	-	-	+	-	-	+	-	-
SZ206	t4513	CC084	CC15	07-23-12-113-34-12-12-23-02-12-23	-	-	2	-	-	-	+	-	-	-	-	-	-
SZ105	t085	CC084	CC15	07-23-12-34-34-12-23-02-12-23	-	-	2	-	-	-	-	+	-	-	-	-	-
SZ049	t360	CC084	CC15	07-23-12-34-12-23-02-12-23	-	-	2	-	-	-	-	+	-	-	+	-	-
SZ279	t346	CC084	CC15	07-23-12-34-12-12-23-02-12-23	-	-	2	-	-	-	-	+	-	-	-	-	-
SZ284	t346	CC084	CC15	07-23-12-34-12-12-23-02-12-23	-	-	2	-	-	-	-	-	-	-	-	-	-
SZ011	t853	CC084	CC15	07-23-34-34-12-12-23-02-12-23	-	-	2	-	-	-	-	-	-	-	-	-	-
SZ056	t2119	CC084	CC15	07-23-12-12-23-02-12-23	-	-	2	-	-	-	+	-	-	-	-	+	-
SZ334	t2119	CC084	CC15	07-23-12-12-23-02-12-23	-	-	2	-	-	-	-	-	-	-	-	+	-
SZ003	t3884	CC091	ST7	14-21-17-34-12-23-02-12-23	-	-	1	*p*	-	-	-	-	+	-	-	-	-
SZ022	t091	CC091	ST7	07-23-21-17-34-12-23-02-12-23	-	-	1	*p*	-	-	-	-	+	-	-	-	-
SZ140	t091	CC091	ST7	07-23-21-17-34-12-23-02-12-23	-	-	1	*p*	-	-	-	-	+	-	-	-	-
SZ236	t091	CC091	ST7	07-23-21-17-34-12-23-02-12-23	-	-	1	*p*	-	-	-	-	+	-	-	-	-
SZ333	t4502	singleton	-	08-16-34-34-34-16	-	-	1	*c tst*	-	-	+	-	-	-	-	-	-
SZ033	t1116	singleton	-	26-23-12-34-34-12-12-23	-	-	2	-	-	-	-	-	-	-	-	+	+
SZ328	t4519	singleton	-	08-34-34-12-23	-	-	2	-	-	-	+	-	-	-	-	+	-
SZ329	t4519	singleton	-	08-34-34-12-23	-	-	2	*l*	-	-	+	-	-	-	-	+	-
SZ350	t4519	singleton	-	08-34-34-12-23	-	-	1	*a c*	-	-	-	+	+	-	-	-	-
SZ342	t209	CC4522	ST109	07-16-12-23-34	-	-	2	-	*g i m n o*	*eta*	+	-	+	-	-	-	-
SZ306	t800	CC4522	ST109	07-16-12-23-02-12-23-02-34	-	-	2	-	*g i m n o*	*eta*	+	-	+	-	-	+	-
SZ110	t4522	CC4522	ST109	07-16-12-23-02-12-23-02-02-02-34	-	-	2	-	*g i m n o*	-	-	-	+	-	-	-	-
SZ149	t4529	CC4522	ST109	07-16-12-23-02-12-23-12-23-02-02-02-34	-	-	2	-	*g i m n o*	-	-	-	+	-	-	-	-
SZ132	t156	CC156	CC12	07-23-12-33-22-17	-	-	2	*c l p*	-	-	+	-	+	-	-	-	+
SZ177	t156	CC156	CC12	07-23-12-33-22-17	-	-	2	*c l p*	-	-	-	-	+	-	-	-	-
SZ324	t114	CC127	CC1	07-16-34-33-13	-	-	3	*a h k q*	-	-	-	+	+	-	-	-	-
SZ024	t127	CC127	CC1	07-23-21-16-34-33-13	-	-	3	*a h k q*	-	-	-	+	+	-	-	-	+
SZ156	t127	CC127	CC1	07-23-21-16-34-33-13	-	-	3	*a h k tst*	-	-	-	+	+	-	-	-	-
SZ072	t397	CC015	CC45	09-02-16-13-17-34-16-13	-	-	1	-	*g i m n o*	-	-	-	+	-	+	-	-
SZ194	t065	CC015	CC45	09-02-16-34-13-17-34-16-34	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
SZ079	t4511	CC015	CC45	08-16-16-02-16-34-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	+	-	+
SZ148	t1078	CC015	CC45	08-16-02-16-34-13-17-34-34	+	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	-
SZ162	t116	CC015	CC45	08-16-02-16-13-13-17-34-16-34	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
SZ012	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	-
SZ013	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	-
SZ064	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	+	-	+	-	-	-	-
SZ067	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	+	-	+	-	-	-	-
SZ075	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*o*	-	-	-	+	-	-	-	-
SZ319	t015	CC015	CC45	08-16-02-16-34-13-17-34-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	+
SZ083	t283	CC053	CC5	26-23-17-13-17-20-17-12-17-12-17-34	-	-	2	*a d j r*	*g i m n o*	-	-	-	+	-	-	+	-
reference	t053	CC053	CC5	26-23-17-34-17-20-17-12-17-34													
SZ002	t002	CC053	CC5	26-23-17-34-17-20-17-12-17-16	-	-	2	*a*	*g i m n o*	-	-	-	+	-	-	-	-
SZ005	t002	CC053	CC5	26-23-17-34-17-20-17-12-17-16	-	-	2	*p*	*g i m n o*	-	-	-	+	-	-	-	-
SZ179	t437	CC216	ST59	04-20-17-20-17-25-34	+	+	1	*a b k q*	-	-	-	+	-	-	-	-	-
SZ199	t216	CC216	ST59	04-20-17-20-17-31-16-34	-	-	1	*b k q*	-	-	-	-	-	-	-	-	-
SZ327	t167	CC078	CC25	04-12-41-20-17-12-12-17	-	-	1	*a h k q*	*g i m n o*	*etd*	-	+	+	-	-	-	-
SZ204	t2909	CC078	CC25	11-12-41-20-17-12-12-12-17	-	-	1	*c l*	*g i m n o*	*etd*	-	-	+	-	-	-	-
SZ163	t1054	CC078	CC25	04-21-12-17-20-17-12-12-12-17	-	-	1	-	*g i m n o*	*etd*	-	+	+	-	-	-	-
SZ172	t349	CC078	CC25	04-21-12-17-20-17-12-12-17	-	-	1	-	*g i m n o*	*etd*	-	-	+	-	-	-	-
SZ089	t078	CC078	CC25	04-21-12-41-20-17-12-12-17	-	-	1	-	*g i m n o*	*etd*	-	+	+	-	-	-	-
SZ186	t078	CC078	CC25	04-21-12-41-20-17-12-12-17	-	-	1	*k q*	*g i m n o*	*etd*	-	+	+	-	-	-	-
SZ097	t1652	singleton	-	04-44-44-33-31-12-16-34-16-12-25-22-34	-	-	3	*h tst*	*g i m n o u*	-	-	-	+	-	+	+	-
SZ107	t223	CC005	CC22	26-23-13-23-05-17-25-17-25-16-28	-	-	1	*tst*	*g i m n o*	-	-	-	+	-	-	-	-
SZ296	t2816	CC005	CC22	26-23-13-23-31-31-05-17-25-17-25-16-28	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
SZ255	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
SZ302	t005	CC005	CC22	26-23-13-23-31-05-17-25-17-25-16-28	-	-	1		*g i m n o*	-	-	-	+	-	-	+	-
SZ167	negative	-	-	-	-	-	1	*a*	-	-	-	-	+	-	-	-	-
SZ268	negative	-	-	-	-	-	2	-	-	-	-	-	-	-	-	+	-
SZ008	t232	n.d.	-	04-17-17	-	-	1	-	-	-	-	-	+	-	-	-	-
SZ082	t386	n.d.	-	07-23-13	-	-	3	*a h k q*	-	-	-	+	+	-	-	-	+
SZ262	t026	n.d.	-	08-16-34	-	-	1	-	*g i m n o*	-	-	-	-	-	-	-	-
SZ272	t026	n.d.	-	08-16-34	-	-	1	*c l*	*g i m n o*	-	-	-	+	-	-	-	-
SZ198	t3625	n.d.	-	08-16-34-25	-	-	1	-	-	-	-	-	+	-	-	-	-
SZ303	t2088	n.d.	-	14-44-17	-	-	4	-	*g i m n o u*	*eta*	+	-	+	-	-	+	-
SZ136	t4494	n.d.	-	26-34	-	-	2	*b*	*g i m n o*	-	-	-	-	-	-	-	-

Fig. 2: Distribution of virulence genes and phages within *spa*-defined CCs among A) furunculosis strains and B) colonizing strains. Luk-PV genes were detected in 85% of furunculosis strains, while their prevalence among nasal strains was below 1%. Furunculosis and colonizing strains did not differ in their SAg gene pattern. For a reliable construction of the consensus tree, some reference *spa* types were included in the BURP clustering (shaded in grey). Virulence genes (SAg genes, *agr*, *eta, etb, etd, luk-PV, mecA)* genes and phage types were determined by PCR. Staphylococcal enterotoxins (SEs) are indicated by single letters (*a _ sea*, etc.).

PVL: The genes encoding the PVL-toxin were a characterizing feature of the furunculosis strains but almost absent from nasal isolates. In total, 85.1 % (64/74) of the furunculosis strains, but only one nasal isolate were *luk-PV*-positive ($P < 0.001$; Figure 2, 3). The phage-encoded *luk-PV* genes were widely distributed among the different lineages. All CC5, CC8, CC22, CC30, CC121 and ST59 isolates were *luk-PV*-positive, whereas strains belonging to CC1, CC15, CC45 and ST20 lacked the *luk-PV* genes (Figure 3, upper panel).
SAg: SAg genes were more or less tightly linked to staphylococcal lineages, which is in agreement with previous studies (Figure 2) (12, 31, 32). For example, *egc* SAgs, which are encoded on the genomic island vSAβ, were strictly linked to CC5, ST20, CC22, CC30, CC45 and CC121. Other SAg with strong CC linkages were *tst* (CC30), *sea* (CC30), *sec* and *sel* (CC45), *sep* (ST7) and *seb* (CC121) (Fig 2). However, within certain CCs and even within the same *spa* type there was remarkable variation of SAg gene patterns. This suggests that horizontal transmission of SAg-encoding mobile genetic elements occurs frequently within lineages, but might be limited between lineages.

To avoid a bias caused by the uneven distribution of CCs among furunculosis and nasal strains, we next compared the SAg gene patterns for each CC separately. The SAg gene *seb* was significantly more frequent among furunculosis-associated than among nasal CC121 strains (23/41 vs. 0/4; $P < 0.05$). Except for *seb*, furunculosis and nasal strains did not differ in their SAg gene patterns. Earlier studies also found no particular association of enterotoxin genes with impetigo or furunculosis (9, 21, 33).

Exfoliative toxins: Exfoliative toxins ETA and ETB but probably not ETD are strongly associated with bullous impetigo and staphylococcal scalded skin syndrome, but absent from furunculosis strains (21, 34). In line with this, *eta*, *etb* and *etd* were rare among our furunculosis and nasal strains. The *etd* gene was strictly linked to CC25 (Figure 2), a lineage which contained only nasal strains. This confirms microarray data by Monecke

et al. who detected the pathogenicity island comprising *edinB* and *etd* exclusively in CC25 strains (13, 31).

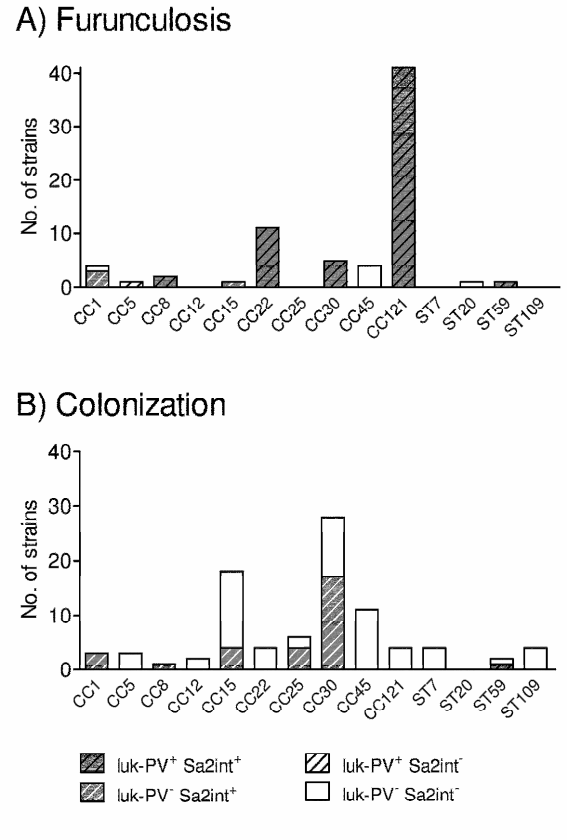


Fig 3: Distribution of *luk-PV* genes and the PVL-encoding phage Sa2int within the *spa*-defined CCs among A) furunculosis and B) colonizing strains. *Luk-PV*-positive phages characterized furunculosis strains, whereas their *luk-PV*-negative counterparts were frequent in colonizing strains. The total number of strains per CC is represented by the overall height of the bar, whereas the number of strains positive for *luk-PV* or Sa2int phage is represented by stripes or grey shading, respectively.

Accessory gene regulator (Agr): Agr is a global regulator of virulence gene expression, and four different *agr* types, *agr*1-4, are known. The *agr* locus belongs to the core variable genome and is thus strictly linked to CCs (15). In agreement with other studies (12, 31, 32), we observed that *agr1* was linked to CC8, CC22, CC45, ST7, and ST59, *agr2* was present in CC5, CC12, CC15 and ST109, *agr3* was associated with CC1 and CC30, and *agr4* was detected in CC121.

Based on our PCR analyses we could define the virulence gene signature of furunculosis CC121 isolates as follows: *mecA-, luk-PV+, egc+, frequently seb+,* and *agr 4*. CC22 furunculosis strains were characterized by *mecA-, luk-PV+, egc+,* and *agr1*.

Phages

Several *S. aureus* virulence factors, including PVL, ETA, and the SAgs SEA, SEP, SEK and SEQ are encoded by staphylococcal phages. To correlate the observed virulence gene profile with the prevalence of phages, we applied a multiplex-PCR for the phage-specific

integrase genes *Sa1int* – *Sa7int* which was recently described by Goerke et al. (17). Almost all strains carried phages (96.7%), usually between one and three. Sa3int phages were by far the most prevalent, followed by Sa2int, Sa1int, Sa6int, Sa5int, and Sa7int (Table 2). The phage profiles of the 108 nasal strains are very similar to the frequencies reported by Goerke and coworkers for nasal isolates from Germany, except for Sa1int and Sa6int which were more abundant in the Polish strain collection (17).

Table 2: Prevalence of phages among furunculosis and colonizing strains.

	No. (%) of positive isolates		
	Furunculosis (n=74)	Colonization (n=108)	*P* value[1]
Sa1int	15 (20.3)	19 (17.6)	
Sa2int	64 (86.5)	36 (33.3)	***
Sa3int	69 (93.2)	80 (74.1)	***
Sa4int	0 (0.0)	0 (0.0)	
Sa5int	2 (2.7)	12 (11.1)	*
Sa6int	6 (8.1)	25 (23.1)	**
Sa7int	1 (1.4)	10 (9.3)	*
No phage	0 (0)	6 (5.5)	*

[1] Chi-Square test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

The prophage prevalence was linked with the *spa*-defined clonal background. For example, Sa2int phages were highly abundant among CC25 and CC30 isolates, but absent from all CC45 and CC5 isolates (Table 2). Similarly, the highly prevalent Sa3int phages were present in all CC5, CC22, CC25, and CC45 strains, but rare among CC15 isolates. This linkage between phage groups and clonal lineages was previously reported by Goerke at al. and indicates that the spread of phages in the *S. aureus* population is at least partially restricted (17).

Phage patterns of furunculosis and nasal strains were remarkably different, which can be partially explained by a bias in CC prevalences. Furunculosis strains carried on average more phages per strain (2.12 vs. 1.69). Sa2int and Sa3int phages were more frequent among furunculosis strains, while phages Sa5int, Sa6int and Sa7int were more often detected among nasal strains (Table 2).

However, the most striking difference concerned Sa2int. In our strain collection we observed *luk-PV*-positive and *luk-PV*-negative variants of this phage group. While *luk-*

PV-positive Sa2int phages were found in 81% of the furunculosis strains but only in one nasal isolate ($P < 0.001$), its *luk-PV*-negative counterpart was present in one third of the nasal strains but only in 5% of the isolates associated with furunculosis ($P < 0.001$; Figure 3). Remarkably, CC30 (and CC8) strains from furunculosis patients harboured *luk-PV*-positive Sa2int phages, whereas commensal strains belonging to the same clonal lineage contained its *luk-PV*-negative variant. All furunculosis-associated isolates from the typical lineages CC121 and CC22 harboured *luk-PV*-positive Sa2int, while the commensal CC121 and CC22 isolates did not contain this phage at all and were therefore *luk-PV*-negative. This very strong association of *luk-PV* genes with furunculosis indicates an important pathogenetic role for the PVL toxin.

DISCUSSION

SSTI are the most common diseases caused by *S. aureus* in the community, and the recent spread of PVL-positive CA-MRSA has spurred scientific and public interest in this neglected disease. In the US, the CA-MRSA clone USA300 causes around 60% of severe SSTI among patients presenting to US American emergency departments (25, 27). However, in Europe the large majority of SSTI is caused by MSSA and PVL-positive CA-MRSA are still uncommon, accounting for fewer than 1% of all MRSA isolates (35). The prevalence was also low in our study: only one CA-MRSA (ST59) was isolated from a furunculosis patient.

A major finding of our study is that only two lineages, CC121 and CC22, accounted for 70% of the furunculosis MSSA strains. Notably, all were *luk-PV*-positive, while their colonizing counterparts never harbored *luk-PV* genes. The pronounced genetic diversity of the CC121 (16 *spa* types) and CC22 (6 *spa* types) isolates from patients and controls cannot be explained by clonal outbreaks but suggests long time endemic persistence and diversification of these lineages. CC121 and CC22 differed in *agr* types but had similar virulence gene signatures: *egc* SAg genes, in CC121 isolates frequently *seb*, but no other SAg genes and no exfoliative toxin genes.

SSTI caused by *luk-PV*-positive CC121 MSSA have been reported from Saxony and Brandenburg in Germany as well as from hospitals in Russia showing that this CC has a world-wide distribution (31, 36-38). Similar strains also caused highly lethal CA-

pneumonia and severe sepsis with progressive metastatic soft tissue infection (38, 39), demonstrating the virulence potential of this lineage.

A different subclass of CC121 isolates harbors *eta* and/or *etb* genes but lacks the *luk-PV* locus. These strains commonly cause staphylococcal scalded skin syndrome and bullous impetigo (40, 41), toxin-mediated staphylococcal diseases, which are associated with *eta* or *etb* in 65-100% of the cases (9, 21, 22, 42, 43). One such CC121 clone, characterized by ST123 and *spa* type t171, is notorious as "epidemic European fusidic acid resistant impetigo clone (EEFIC)" (41, 44-48). This suggests that within a given clonal background, i.e. CC121, the virulence gene repertoire can shape the clinical symptoms.

In spite of the high selection pressure exerted by the frequent antibiotic treatment of patients with severe skin infection, CC121 strains apparently have not acquired the SCCmec cassette (49) (personal communication U. Nübel, Robert Koch Institute, Wernigerode, Germany), suggesting restraints on resistance gene transfer. However, considering the wide distribution of SCCmec among diverse *S. aureus* clonal lineages, this might change in the future and CC121 might emerge as highly virulent CA-MRSA.

In our molecular-epidemiological study, we further consolidated the role of PVL in furunculosis. Generally, the potent PVL toxin is epidemiologically associated with furunculosis, abscesses, and skin lesions, but absent from isolates causing impetigo, blisters or staphylococcal scalded skin syndrome (16, 50). In our cohort 85% of MSSA isolates from patients with furunculosis harbored *luk-PV* genes. Others have reported frequencies between 30 to 97% in SSTI (9, 16, 21, 31, 50). In striking contrast, the *luk-PV* prevalence among colonizing *S. aureus* strains was below 1%, again in agreement with previous studies (12, 13). However, it must be emphasized that not all furunculosis-associated strains harbored the PVL gene, implying that additional factors, either host or pathogen derived, affect the development of furuncles.

For example, the SAg gene *seb* was significantly more frequent among furunculosis-associated than among nasal CC121 strains. However, around half of these CC121 strains were *seb*-negative. Moreover, while PVL was distributed over a broad range of staphylococcal lineages, *seb* was detected only in CC121, CC1 and ST59. This suggests that SEB might contribute to the disease process in CC121 isolates, but its linkage with furunculosis is much weaker than that of PVL.

The present study highlights the genetic diversity of *luk-PV*-positive MSSA strains. *Luk-PV* genes were detected in isolates belonging to CC5, 8, 22, 30, 121 and ST59, confirming and extending findings in Saxony, Germany, that have been reported by Monecke et al. (31). Similarly, the *luk-PV* genes were demonstrated in a range of MRSA isolates worldwide, which belonged to ten different MLST sequence types (ST1, 5, 8, 22, 30, 59/359, 80/583, 88, 93 and 152) (26, 32, 51). This diversity suggests a dominant role for PVL above the clonal background in the pathogenesis of furunculosis. Moreover, it indicates frequent and independent *luk-PV* gene acquisition events by *S. aureus* (31, 32). The high genetic mobility of *luk-PV* can be attributed to its localization on phages of group Sa2int, which transduce PVL genes within the species *S. aureus* (17, 52). However, there appear to be restrictions in the transfer of phages between lineages since we and others (almost) never observed Sa2int phages in CC45 (17, 31). The phage group Sa2int comprises eleven closely related *luk-PV*-positive and *luk-PV*-negative phages (17). In our study, only the *luk-PV*-positive Sa2int phages were closely linked to furunculosis strains, whereas *luk-PV*-negative variants were frequent in colonizing strains. Remarkably, this was also the case when furunculosis-associated and colonizing strains belonged to the same lineage, CC30 and CC8, underlining the very strong association of *luk-PV* with chronic furunculosis.

The importance of PVL as a virulence factor in *S. aureus* infection is currently under debate, because studies addressing its role in mouse models of skin and lung infection produced contradictory results (53-56). This study shows a very tight epidemiological linkage between *luk-PV* and furunculosis, which can be separated from the genetic background of the *S. aureus* strain as well as from the gene make-up of the *luk-PV*-transducing phage. The findings, therefore, lend support to the notion of a causative role for PVL in human furunculosis.

However, this does not explain the strong overrepresentation of CC121 and CC22 among the furunculosis isolates, which indicates that PVL and the core genome act in synergy, as has been proposed by Fan and coworkers (57). Apart from bacterial virulence factors, host disposition, such as immune suppression and gene polymorphisms, exposition and personal hygiene might play an important role (58, 59).

Our results have consequences for the diagnosis and therapy of *S. aureus* infections. *Luk-PV* genes are recognized worldwide as markers for epidemic CA-MRSA (26, 32, 60). This study and work by others from the recent years, have demonstrated that in Europe SSTI

with *luk-PV*-positive MSSA are common (32, 35). As shown in this study, SSTI-associated strains will in most cases be characterized by PVL and/or a permissive genetic background and exhibit particular virulence. Thus, PVL-associated symptoms might be frequently encountered, even in settings, where CA-MRSA is rare. The implementation of a *luk-PV* test in the diagnosis of *S. aureus* infection would therefore provide important information for the treating clinician. Our findings are also relevant for therapy, because we and others have shown that the vast majority of patients with chronic furunculosis (87-100%) are nasal carriers of their infecting *S. aureus* strain (7-9). To prevent repeated re-infection, the elimination of this *S. aureus* strain from the whole body as well as the patient's environment should be the therapeutic aim in chronic furunculosis (36, 61).

ACKNOWLEDGMENTS

This work was supported by grants from the DFG (SFB-TR34, GRK-840) and the BMBF. We thank Ulrich Nübel for helpful comments on the manuscript.

SUPPLEMENTAL MATERIAL

Suppl. table 1: Furunculosis and colonizing isolates from the same patient belonged to on *S. aureus* clone.

		spa typing			virulence genes						phages						
patient ID	**source**	***spa* type ID**	***spa* CC**	**MLST CC**	***mecA***	***pvl***	***agr***	***non-egc* SAg**	***egc* SAg**	***eta, etb, etd***	**Sa1**	**Sa2**	**Sa3**	**Sa4**	**Sa5**	**Sa6**	**Sa7**
H108	pus	t127	CC127	CC1	-	-	3	*a h k q*	-	-	-	+	+	-	-	-	**+**
H108	nose	t127	CC127	CC1	-	-	3	*a h k q*	-	-	-	+	+	-	-	-	**-**
H1463	pus	t015	CC015	CC45	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
H1463	nose	t015	CC015	CC45	-	-	1	-	*g i m n o*	-	-	-	+	-	-	-	-
H1627	pus	t284	CC159	CC121	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H1627	nose	t284	CC159	CC121	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H3176	pus	t3372	CC318	CC30	-	+	3	-	*g i m n o u*	-	-	+	+	-	+	-	-
H3176	nose	t3372	CC318	CC30	-	+	3	-	*g i m n o u*	-	-	+	+	-	+	-	-
H3318	pus	t435	CC159	CC121	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H3318	nose	t435	CC159	CC121	-	+	4	-	*g i m n o u*	-	+	+	+	-	-	-	-
H3699	pus	t159	CC159	CC121	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H3699	nose	t159	CC159	CC121	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H6147	pus	t162	CC159	CC121	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H6147	nose	t162	CC159	CC121	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H694	pus	t4509	CC005	CC22	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H694	nose	t4509	CC005	CC22	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H709	pus	t1193	CC159	CC121	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H709	nose	t1193	CC159	CC121	-	+	4	*b*	*g i m n o u*	-	-	+	+	-	-	-	-
H8034	pus	t005	CC005	CC22	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H8034	nose	t005	CC005	CC22	-	+	1	-	*g i m n o*	-	-	+	+	-	-	-	-
H3163	pus	t068	CC008	CC8	-	+	1	*k q*	-	-	-	+	+	-	-	-	-
H3163	nose	t068	CC008	CC8	-	+	1	*k q*	-	-	-	+	+	-	-	-	-

REFERENCES

1. Iwatsuki, K., O. Yamasaki, S. Morizane, and T. Oono. 2006. Staphylococcal cutaneous infections: invasion, evasion and aggression. *J Dermatol Sci* 42:203-214.

2. RJ Hay, and B. Adriaans. 2004. Bacterial infections. In *Rook's textbook of dermatology.* Burn T, Breathnach S, Cox N, and G. C., eds. Blackwell Publishing Company. 1-85.

3. Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* 5:751-762.

4. van Belkum, A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim. 2009. Reclassification of *Staphylococcus aureus* Nasal Carriage Types. *J Infect Dis* 199:1820-1826.

5. Acton, D. S., M. J. Plat-Sinnige, W. van Wamel, N. de Groot, and A. van Belkum. 2009. Intestinal carriage of *Staphylococcus aureus*: how does its frequency compare with that of nasal carriage and what is its clinical impact? *Eur J Clin Microbiol Infect Dis* 28:115-127.

6. Mertz, D., R. Frei, B. Jaussi, A. Tietz, C. Stebler, U. Fluckiger, and A. F. Widmer. 2007. Throat swabs are necessary to reliably detect carriers of *Staphylococcus aureus*. *Clin Infect Dis* 45:475-477.

7. Toshkova, K., C. Annemuller, O. Akineden, and C. Lammler. 2001. The significance of nasal carriage of *Staphylococcus aureus* as risk factor for human skin infections. *FEMS Microbiol Lett* 202:17-24.

8. Tulloch, L. G. 1954. Nasal carriage in staphylococcal skin infections. *Br Med J* 2:912-913.

9. Durupt, F., L. Mayor, M. Bes, M. E. Reverdy, F. Vandenesch, L. Thomas, and J. Etienne. 2007. Prevalence of *Staphylococcus aureus* toxins and nasal carriage in furuncles and impetigo. *Br J Dermatol* 157:1161-1167.

10. Melles, D. C., R. F. J. Gorkink, H. A. M. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and A. van Belkum. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. *J. Clin. Invest.* 114:1732-1740.

11. Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. J. Day. 2003. How Clonal Is *Staphylococcus aureus*? *J. Bacteriol.* 185:3307-3316.

12. Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Broker. 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *J Clin Microbiol* 45:2669-2680.

13. Monecke, S., C. Luedicke, P. Slickers, and R. Ehricht. 2009. Molecular epidemiology of *Staphylococcus aureus* in asymptomatic carriers. *Eur J Clin Microbiol Infect Dis*.

14. Lindsay, J. A., and M. T. Holden. 2004. *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol* 12:378-385.

15. Lindsay, J. A., and M. T. Holden. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Funct Integr Genomics* 6:186-201.

16. Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne. 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis.* 29:1128-1132.

17. Goerke, C., R. Pantucek, S. Holtfreter, B. Schulte, M. Zink, D. Grumann, B. M. Broker, J. Doskar, and C. Wolz. 2009. Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages. *J Bacteriol* 191:3462-3468.

18. Waldron, D. E., and J. A. Lindsay. 2006. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *J Bacteriol* 188:5578-5585.

19. Fraser, J. D., and T. Proft. 2008. The bacterial superantigen and superantigen-like proteins. *Immunological Reviews* 225:226-243.

20. Amagai, M., N. Matsuyoshi, Z. H. Wang, C. Andl, and J. R. Stanley. 2000. Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nat Med* 6:1275-1277.

21. Gravet, A., P. Couppie, O. Meunier, E. Clyti, B. Moreau, R. Pradinaud, H. Monteil, and G. Prevost. 2001. *Staphylococcus aureus* isolated in cases of impetigo produces both epidermolysin A or B and LukE-LukD in 78% of 131 retrospective and prospective cases. *J Clin Microbiol* 39:4349-4356.

22. Lina, G., Y. Gillet, F. Vandenesch, M. E. Jones, D. Floret, and J. Etienne. 1997. Toxin involvement in staphylococcal scalded skin syndrome. *Clin Infect Dis* 25:1369-1373.

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

24. Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, N. Brousse, D. Floret, and J. Etienne. 2002. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* 359:753-759.

25. Campbell, S. J., H. S. Deshmukh, C. L. Nelson, I. G. Bae, M. E. Stryjewski, J. J. Federspiel, G. T. Tonthat, T. H. Rude, S. L. Barriere, R. Corey, and V. G. Fowler, Jr. 2008. Genotypic characteristics of *Staphylococcus aureus* isolates from a multinational trial of complicated skin and skin structure infections. *J Clin Microbiol* 46:678-684.

26. Vandenesch, F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, and J. Etienne. 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* 9:978-984.

27. Moran, G. J., Krishnadasan, Anusha, Gorwitz, Rachel J., Fosheim, Gregory E., McDougal, Linda K., Carey, Roberta B., Talan, David A., the EMERGEncy ID Net Study Group. 2006. Methicillin-Resistant *S. aureus* Infections among Patients in the Emergency Department. *N Engl J Med* 355:666-674.

28. Harmsen, D., H. Claus, W. Witte, J. Rothganger, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *J Clin Microbiol* 41:5442-5448.

29. Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M. C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X. W. Huijsdens, A. M. Kearns, A. Mellmann, H. Meugnier, J. K. Rasheed, E. Spalburg, B. Strommenger, M. J. Struelens, F. C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *J Clin Microbiol* 44:619-621.

30. Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W. Friedrich, and W. Witte. 2006. Assignment of *Staphylococcus* isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. *J Clin Microbiol* 44:2533-2540.

31. Monecke, S., P. Slickers, M. J. Ellington, A. M. Kearns, and R. Ehricht. 2007. High diversity of Panton-Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus*. *Clin Microbiol Infect* 13:1157-1164.

32. Monecke, S., B. Berger-Bachi, G. Coombs, A. Holmes, I. Kay, A. Kearns, H. J. Linde, F. O'Brien, P. Slickers, and R. Ehricht. 2007. Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. *Clin Microbiol Infect* 13:236-249.

33. Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* Genetic Background, Virulence Factors, *agr* Groups (Alleles), and Human Disease. *Infect. Immun.* 70:631-641.

34. Yamaguchi, T., K. Nishifuji, M. Sasaki, Y. Fudaba, M. Aepfelbacher, T. Takata, M. Ohara, H. Komatsuzawa, M. Amagai, and M. Sugai. 2002. Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun* 70:5835-5845.

35. Robert, J., J. Etienne, and X. Bertrand. 2005. Methicillin-resistant *Staphylococcus aureus* producing Panton-Valentine leukocidin in a retrospective case series from 12 French hospital laboratories, 2000-2003. *Clin Microbiol Infect* 11:585-587.

36. Wiese-Posselt, M., D. Heuck, A. Draeger, M. Mielke, W. Witte, A. Ammon, and O. Hamouda. 2007. Successful termination of a furunculosis outbreak due to lukS-lukF-positive, methicillin-susceptible *Staphylococcus aureus* in a German village by stringent decolonization, 2002-2005. *Clin Infect Dis* 44:e88-95.

37. Vorobieva, V., T. Bazhukova, A. M. Hanssen, D. A. Caugant, N. Semenova, B. C. Haldorsen, G. S. Simonsen, and A. Sundsfjord. 2008. Clinical isolates of *Staphylococcus aureus* from the Arkhangelsk region, Russia: antimicrobial susceptibility, molecular epidemiology, and distribution of Panton-Valentine leukocidin genes. *Apmis* 116:877-887.

38. Baranovich, T., H. Zaraket, Shabana, II, V. Nevzorova, V. Turcutyuicov, and H. Suzuki. 2009. Molecular characterization and susceptibility of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from hospitals and the community in Vladivostok, Russia. *Clin Microbiol Infect*.

39. Schefold, J. C., F. Esposito, C. Storm, D. Heuck, A. Kruger, A. Jorres, W. Witte, and D. Hasper. 2007. Therapy-refractory Panton Valentine Leukocidin-positive community-acquired methicillin-sensitive *Staphylococcus aureus* sepsis with progressive metastatic soft tissue infection: a case report. *J Med Case Reports* 1:165.

40. O'Neill, A. J., A. R. Larsen, R. Skov, A. S. Henriksen, and I. Chopra. 2007. Characterization of the epidemic European fusidic acid-resistant impetigo clone of *Staphylococcus aureus*. *J Clin Microbiol* 45:1505-1510.

41. Larsen, A. R., R. L. Skov, V. Jarlier, and A. S. Henriksen. 2008. Epidemiological differences between the UK and Ireland versus France in *Staphylococcus aureus* isolates resistant to fusidic acid from community-acquired skin and soft tissue infections. *J Antimicrob Chemother* 61:589-594.

42. Yamasaki, O., T. Yamaguchi, M. Sugai, C. Chapuis-Cellier, F. Arnaud, F. Vandenesch, J. Etienne, and G. Lina. 2005. Clinical manifestations of staphylococcal scalded-skin syndrome depend on serotypes of exfoliative toxins. *J Clin Microbiol* 43:1890-1893.

43. Kanzaki, H., M. Ueda, Y. Morishita, H. Akiyama, J. Arata, and S. Kanzaki. 1997. Producibility of exfoliative toxin and staphylococcal coagulase types of *Staphylococcus aureus* strains isolated from skin infections and atopic dermatitis. *Dermatology* 195:6-9.

44. O'Neill, A. J., A. R. Larsen, A. S. Henriksen, and I. Chopra. 2004. A fusidic acid-resistant epidemic strain of *Staphylococcus aureus* carries the *fus*B determinant, whereas *fusA* mutations are prevalent in other resistant isolates. *Antimicrob Agents Chemother* 48:3594-3597.

45. Osterlund, A., T. Eden, B. Olsson-Liljequist, S. Haeggman, and G. Kahlmeter. 2002. Clonal spread among Swedish children of a *Staphylococcus aureus* strain resistant to fusidic acid. *Scand J Infect Dis* 34:729-734.

46. Tveten, Y., A. Jenkins, and B. E. Kristiansen. 2002. A fusidic acid-resistant clone of *Staphylococcus aureus* associated with impetigo bullosa is spreading in Norway. *J Antimicrob Chemother* 50:873-876.

47. Koning, S., A. van Belkum, S. Snijders, W. van Leeuwen, H. Verbrugh, J. Nouwen, M. Op 't Veld, L. W. van Suijlekom-Smit, J. C. van der Wouden, and C. Verduin. 2003. Severity of nonbullous *Staphylococcus aureus* impetigo in children is associated with strains harboring genetic markers for exfoliative toxin B, Panton-Valentine leukocidin, and the multidrug resistance plasmid pSK41. *J Clin Microbiol* 41:3017-3021.

48. Laurent, F., A. Tristan, M. Croze, M. Bes, H. Meugnier, G. Lina, F. Vandenesch, and J. Etienne. 2009. Presence of the epidemic European fusidic acid-resistant impetigo clone (EEFIC) of *Staphylococcus aureus* in France. *J Antimicrob Chemother* 63:420-421; author reply 421.

49. Monecke, S., R. Ehricht, P. Slickers, H. L. Tan, and G. Coombs. 2009. The molecular epidemiology and evolution of the Panton-Valentine leukocidin-positive, methicillin-resistant *Staphylococcus aureus* strain USA300 in Western Australia. *Clin Microbiol Infect*.

50. Holmes, A., M. Ganner, S. McGuane, T. L. Pitt, B. D. Cookson, and A. M. Kearns. 2005. *Staphylococcus aureus* isolates carrying Panton-Valentine leucocidin genes in England and Wales: frequency, characterization, and association with clinical disease. *J Clin Microbiol* 43:2384-2390.

51. Dufour, P., Y. Gillet, M. Bes, G. Lina, F. Vandenesch, D. Floret, J. Etienne, and H. Richet. 2002. Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. *Clin Infect Dis.* 35:819-824.

52. Narita, S., J. Kaneko, J. Chiba, Y. Piemont, S. Jarraud, J. Etienne, and Y. Kamio. 2001. Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. *Gene* 268:195-206.

53. Diep, B. A., A. M. Palazzolo-Ballance, P. Tattevin, L. Basuino, K. R. Braughton, A. R. Whitney, L. Chen, B. N. Kreiswirth, M. Otto, F. R. DeLeo, and H. F. Chambers. 2008. Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *PLoS One* 3:e3198.

54. Bubeck Wardenburg, J., A. M. Palazzolo-Ballance, M. Otto, O. Schneewind, and F. R. DeLeo. 2008. Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis* 198:1166-1170.

55. Brown, E. L., O. Dumitrescu, D. Thomas, C. Badiou, E. M. Koers, P. Choudhury, V. Vazquez, J. Etienne, G. Lina, F. Vandenesch, and M. G. Bowden. 2009. The Panton-Valentine leukocidin vaccine protects mice against lung and skin infections caused by *Staphylococcus aureus* USA300. *Clin Microbiol Infect* 15:156-164.

56. Tseng, C. W., P. Kyme, J. Low, M. A. Rocha, R. Alsabeh, L. G. Miller, M. Otto, M. Arditi, B. A. Diep, V. Nizet, T. M. Doherty, D. O. Beenhouwer, and G. Y. Liu. 2009. *Staphylococcus aureus* Panton-Valentine leukocidin contributes to inflammation and muscle tissue injury. *PLoS One* 4:e6387.

57. Fan, J., M. Shu, G. Zhang, W. Zhou, Y. Jiang, Y. Zhu, G. Chen, S. J. Peacock, C. Wan, W. Pan, and E. J. Feil. 2009. Biogeography and virulence of *Staphylococcus aureus*. *PLoS One* 4:e6216.

58. Emonts, M., A. G. Uitterlinden, J. L. Nouwen, I. Kardys, M. P. Maat, D. C. Melles, J. Witteman, P. T. Jong, H. A. Verbrugh, A. Hofman, P. W. Hermans, and A. Belkum. 2008. Host polymorphisms in interleukin 4, complement factor H, and C-reactive protein associated with nasal carriage of *Staphylococcus aureus* and occurrence of boils. *J Infect Dis* 197:1244-1253.

59. El-Gilany, A. H., and H. Fathy. 2009. Risk factors of recurrent furunculosis. *Dermatol Online J* 15:16.

60. Tristan, A., T. Ferry, G. Durand, O. Dauwalder, M. Bes, G. Lina, F. Vandenesch, and J. Etienne. 2007. Virulence determinants in community and hospital meticillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection* 65:105-109.

61. Zimakoff, J., V. T. Rosdahl, W. Petersen, and J. Scheibel. 1988. Recurrent staphylococcal furunculosis in families. *Scand J Infect Dis* 20:403-405.

Chapter 5

Anti-staphylococcal humoral immune response in persistant nasal carriers and noncarriers of *Staphylococcus aureus*

N. J. Verkaik, C. P. de Vogel, H. A. Boelens, D. Grumann, T. Hoogenboezem, C. Vink, H. Hooijkaas, T. J. Foster, H. A. Verbrugh, A. van Belkum, and W. J. van Wamel

PUBLISHED IN:

J Infect Dis 2009; 199:625-632

Anti-Staphylococcal Humoral Immune Response in Persistent Nasal Carriers and Noncarriers of *Staphylococcus aureus*

Nelianne J. Verkaik,[1] Corné P. de Vogel,[1] Hélène A. Boelens,[1] Dorothee Grumann,[4] Theo Hoogenboezem,[3] Cornelis Vink,[3] Herbert Hooijkaas,[2] Timothy J. Foster,[5] Henri A. Verbrugh,[1] Alex van Belkum,[1] and Willem J. B. van Wamel[1]

Departments of [1]Medical Microbiology and Infectious Diseases and [2]Immunology and [3]Laboratory of Pediatrics, Erasmus Medical Center, Rotterdam, the Netherlands; [4]Department of Immunology, University of Greifswald, Greifswald, Germany; [5]Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin, Ireland

Background. Persistent carriers have a higher risk of *Staphylococcus aureus* infections than noncarriers but a lower risk of bacteremia-related death. Here, the role played by anti-staphylococcal antibodies was studied.

Methods. Serum samples from 15 persistent carriers and 19 noncarriers were analyzed for immunoglobulin (Ig) G, IgA, and IgM binding to 19 *S. aureus* antigens, by means of Luminex technology. Nasal secretions and serum samples obtained after 6 months were also analyzed.

Results. Median serum IgG levels were significantly higher in persistent carriers than in noncarriers for toxic shock syndrome toxin (TSST)–1 (median fluorescence intensity [MFI] value, 11,554 vs. 4291; $P < .001$) and staphylococcal enterotoxin (SE) A (742 vs. 218; $P < .05$); median IgA levels were higher for TSST-1 ($P < .01$), SEA, and clumping factor (Clf) A and B ($P < .05$). The in vitro neutralizing capacity of anti–TSST-1 antibodies was correlated with the MFI value ($R^2 = 0.93$) and was higher in persistent carriers (90.6% vs. 70.6%; $P < .05$). Antibody levels were stable over time and correlated with levels in nasal secretions (for IgG, $R^2 = 0.87$; for IgA, $R^2 = 0.77$).

Conclusions. Antibodies to TSST-1 have a neutralizing capacity, and median levels of antibodies to TSST-1, SEA, ClfA, and ClfB are higher in persistent carriers than in noncarriers. These antibodies might be associated with the differences in the risk and outcome of *S. aureus* infections between nasal carriers and noncarriers.

Staphylococcus aureus is an important pathogen that causes superficial skin infections (furuncles and impetigo) as well as invasive infections that result in abscesses, endocarditis, and bacteremia [1]. Persistent carriers of *S. aureus,* comprising ~20% of the healthy population [2, 3], have an increased risk of developing such infections [4–6], including a 3-fold higher risk of acquiring *S. aureus* bacteremia. Surprisingly, the risk of death in carriers with bacteremia is significantly lower than that in noncarriers with bacteremia [5, 7]. An explanation for this observation has not yet been provided, although a role for the immune system has been proposed. Genotyping has revealed that 80% of strains that cause bacteremia in persistent carriers are endogenous [5, 8]. Because of long-time exposure to their colonizing strain, carriers may have developed antibodies that protect them from bacteremia-related death. Otherwise, noncarriers may harbor antibodies that protect them from nasal colonization [9], and they therefore remain at lower risk of acquiring *S. aureus* bacteremia. Antistaphylolysin titers were found to be higher in carriers than in noncarriers [10], but the 2 groups had similar concentrations of antibodies to teichoic acid [11].

Recently, a higher level of IgG in noncarriers than in carriers was reported for α-hemolysin, major autolysin, iron-responsive surface determinant (Isd) A and H, immunodominant secretory antigen A (IsaA), major histocompatibility complex class II analogue protein w (Map-w), and clumping factor (Clf) B [9, 12]. These

Received 22 July 2008; accepted 1 October 2008; electronically published 22 January 2009.

Potential conflicts of interest: none reported.

Presented in part: International Symposium on Staphylococci and Staphylococcal Infections, Cairns, Australia, 8 September 2008 (abstract 561).

Financial support: Dutch Ministry of Agriculture, Nature and Food Quality (grant V/330224/01/KA).

Reprints or correspondence: Nelianne Verkaik, Dept. of Medical Microbiology and Infectious Diseases, Erasmus MC, 's-Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands (n.j.verkaik@erasmusmc.nl).

The Journal of Infectious Diseases 2009; 199:625–32

0022-1899/2009/19905-0005$15.00
DOI: 10.1086/596743

latter studies focused mainly on antibodies to microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), proteins that are generally considered important for host colonization [13, 14].

At present, little is known about the humoral immune response to staphylococcal enterotoxins (SEs) and immune-modulating proteins in persistent carriers and noncarriers. SEs are superantigens and, therefore, potent proinflammatory agents [15]. They have been implicated in the pathogenesis of toxic shock [15, 16]. The immune modulators staphylococcal complement inhibitor (SCIN) and extracellular fibrinogen-binding protein (Efb) are potent complement inhibitors that lead to diminished phagocytosis and killing by human neutrophils [17, 18]. Chemotaxis inhibitory protein of *S. aureus* (CHIPS) impairs the response of neutrophils and monocytes to formylated peptides and C5a [19]. Consequently, both the SEs and the immune-modulating proteins might play a role in *S. aureus* carriage and disease.

In the present study, we determined levels of antibodies to 9 MSCRAMMs, 7 SEs, and 3 immune-modulating proteins in serum samples and nasal secretions from well-defined persistent carriers and noncarriers and measured the stability of anti-staphylococcal antibody levels over time.

METHODS

Serum samples, nasal secretions, and nasal swab samples. At the beginning of the study, all volunteers completed a questionnaire on age, sex, weight, height, nationality, occupation, smoking and drinking habits, medication (including antibiotic usage), and medical history. Criteria for exclusion were diabetes mellitus, renal insufficiency, chronic obstructive pulmonary disease, heart disease, immunocompromised status, immunosuppressant use, antibiotic use in the last 4 weeks, and skin diseases (such as impetigo and eczema). All 40 participants (median age, 36.9 years; age range, 21–60 years) fulfilled the inclusion criteria and did not suffer from apparent staphylococcal infections during the study period. Venous blood samples and at least 3 consecutive nasal swab samples (at 2-week intervals) were obtained for each of the 40 healthy volunteers. After 6 months, a second blood sample and 2 additional nasal swab samples were collected from 11 of these volunteers. Nasal swab samples were processed as described elsewhere [2]. Subjects were classified as persistent carriers when all nasal swab cultures were positive for *S. aureus,* as intermittent carriers when 1 or 2 nasal swab cultures were positive, and as noncarriers when all nasal swab cultures were negative. Nasal secretions from 13 volunteers were collected at the beginning of the study by vacuum-aided suction without chemical stimulation and processed as described elsewhere [20, 21]. The collected fluid was sonicated in a water bath to disrupt the mucoprotein aggregates and facilitate reproducible handling. The secretions and serum samples were stored at −80°C until use. Human pooled serum (HPS) from 36 healthy donors of unknown *S. aureus* nasal carriage state was used as a standard during Luminex experiments. Volunteers provided written informed consent, and the local medical ethics committee of the Erasmus Medical Center Rotterdam approved the study (MEC-2007-106).

Antigens. The MSCRAMMs ClfA and ClfB, *S. aureus* surface protein (Sas) G, IsdA and IsdH, fibronectin-binding protein A and B, and serine-aspartate dipeptide repeat proteins (Sdr) D and E were expressed with a His tag in *Escherichia coli* XL1-blue strain and purified under denaturing conditions with nickel–nitrilotriacetic acid agarose (Qiagen) recognizing the His tag; quality control was done using SDS-PAGE and mass spectrometry (Ultraflex MALDI-TOF; Bruker Daltonics). Staphylococcal enterotoxin (SE) A was purchased from Sigma. Recombinant proteins SEB, SEI, SEM, SEO, SEQ, and toxic shock syndrome toxin (TSST)–1 were provided by Dr. S. Holtfreter and D. Grumann (University of Greifswald) [16]. Dr. S. Rooijakkers (University Medical Center Utrecht) provided the recombinant proteins CHIPS and SCIN. Prof. J. I. Flock (Karolinska Institutet) supplied the Efb [22–25].

Coupling methods. To quantify antibodies directed against the 19 *S. aureus* proteins simultaneously, the recently introduced microsphere (bead)–based flow cytometry technique (xMAP; Luminex) was applied. The purified proteins were coupled to SeroMAP beads, a carboxylated bead type developed for serological applications. The coupling procedure was performed as described elsewhere [26, 27]. In brief, 25 μg of protein was added to 5.0×10^6 microspheres. This amount of protein was found to be optimal. As an activation buffer, we used 100 mmol/L monobasic sodium phosphate (pH 6.2). To activate the carboxyl groups on the surface of the beads, 10 μL of 50 mg/mL *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used (Pierce Biotechnology). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino)-ethanesulfonic acid (pH 5.0; Sigma-Aldrich). The final concentration of microspheres was adjusted to 4000 beads/μL with blocking-storage buffer (PBS-BN; PBS, 1% bovine serum albumin, and 0.05% sodium azide [pH 7.4]). The microspheres were protected from light and stored at 4°C until use. For control beads, the coupling procedure was done in the absence of *S. aureus* protein. In each experiment, control beads were included to determine nonspecific binding. In case of nonspecific binding, the median fluorescence intensity (MFI) values were subtracted from the antigen-specific results. As a negative control, PBS-BN was included.

Multiplex* S. aureus *antibody assay. The multiplex assay (serum incubated with the different fluorescence-colored antigen-coupled beads mixed in 1 well) was validated by comparing the MFI values for HPS obtained with this multiplex assay with the results for HPS obtained with singleplex assays (serum incubated with each different color of antigen-coupled beads in separate wells). After validation, the different antigen-coupled microspheres were mixed to a working concentration of 4000 beads per color per well. The procedure was the same as de-

scribed elsewhere [26]. Serum samples were diluted 1:100 in PBS-BN for measurement of antigen-specific IgG and IgA and 1:25 for measurement of IgM. Fifty microliters per diluted sample was incubated with the microspheres in a 96-well filter microtiter plate (Millipore) for 35 min at room temperature on a Thermomixer plate shaker (Eppendorf). The plate was washed twice with assay buffer (PBS-BN) that was aspirated by vacuum manifold. The microspheres were resuspended in 50 μL of assay buffer. In separate wells, 50 μL of a 1:200 dilution of R-phycoerythrin (RPE)–conjugated AffiniPure goat anti–human IgG and IgA and 50 μL of a 1:50 dilution of RPE-conjugated donkey anti–human IgM (Jackson Immuno Research) were added. The plate was incubated for 35 min at room temperature on the plate shaker and washed. The microspheres were resuspended in 100 μL of assay buffer. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.2). Tests were performed in triplicate, and the MFI values, reflecting quantitative antibody levels, were averaged. The coefficient of variation (CV) was calculated for each serum sample and averaged per protein and antibody isotype. For nasal secretions, the procedure was identical. Nasal secretions were diluted 1:20, and RPE-conjugated goat anti–human IgG and IgA were diluted 1:50.

TSST-1 neutralization assay. The in vitro TSST-1 neutralization assay was performed as described elsewhere [16, 28]. Initially, the concentration of recombinant TSST-1 that elicited submaximal T cell proliferation was determined (10 pg/mL). Subsequently, 10 pg/mL TSST-1 was incubated with serial dilutions (1:50 to 1:6250) of heat-inactivated serum from the 40 healthy volunteers. At higher serum dilutions, maximal inhibition could no longer be obtained. As a control, TSST-1 was incubated with RPMI 1640 supplemented with 10% fetal bovine serum. After 20 min, 1×10^5 peripheral blood mononuclear cells from healthy blood donors were added to test for TSST-1 neutralizing antibodies. T cell proliferation was determined by the incorporation of [^{3}H]-thymidine after 72 h, quantified by calculating the area under the proliferation curve, and expressed as a percentage of the control without human serum. All measurements were performed in triplicate and repeated in 2 independent experiments.

Statistical analysis. The Mann-Whitney *U* test was used to compare median differences in anti-staphylococcal antibody levels and the median neutralizing capacities of serum from persistent carriers and noncarriers. To compare the antibody levels in the first and second serum samples from an individual, paired *t* tests were used. Correlations between antigen-specific IgG and IgA in serum and nasal secretions were assessed using Pearson's correlation coefficient. Nonlinear regression was used to describe the relation between MFI value and neutralizing capacity. Differences were considered statistically significant when 2-sided *P* values were $<.05$.

RESULTS

Control of the multiplex assay and reproducibility. First, the multiplex assay was validated. The MFI values obtained for HPS with the multiplex assay were between 93% and 116% (median, 100%) of those obtained with the singleplex assays, so it was valid to use the multiplex assay. Serum incubated with control beads (beads without protein coupled on their surface) resulted in median MFI values for IgG, IgA, and IgM of 14 (range, 6–82), 6 (range, 3–22), and 75 (range, 3–957), respectively. This indicates that there was low nonspecific binding (with the exception of IgM in 1 sample). The negative control (PBS-BN) incubated with protein-coupled beads resulted in low MFI values (<10).

Interassay variation was calculated from MFI values obtained from serum samples ($n = 40$) run in 3 separate assays and was averaged per protein and antibody isotype. For IgG, the median CV was 15%, and the range was 5% (CHIPS) to 25% (SEO); for IgA, the median CV was 20%, and the range was 7% (Efb) to 25% (SdrD, SEB, SEI, and SasG); and for IgM, the median CV was 16% and the range was 7% (ClfA) to 43% (SEO; relatively high CV due to MFI values close to 0). Earlier studies found equal CVs for interassay variation [26, 29–31].

Differences in antigen-specific antibodies in serum from persistent carriers and noncarriers. Nineteen volunteers were classified as noncarriers (48%), 6 as intermittent carriers (15%), and 15 as persistent carriers (38%). The MFI valued reflecting serum antibody levels for each person and antibody isotype are shown in figure 1. For most of the antigens there was no apparent quantitative difference in antibody level between persistent carriers and noncarriers. However, the median serum levels of IgG directed against TSST-1 and SEA were significantly higher in persistent carriers than in noncarriers (MFI value, 11,554 vs. 4291 [$P < .001$] and 742 vs. 218 [$P < .05$], respectively). Additionally, the median IgA serum level was significantly higher in persistent carriers than in noncarriers for TSST-1 (973 vs. 155; $P < .01$), SEA (127 vs. 32; $P < .05$), ClfA (1661 vs. 441; $P < .05$), and ClfB (792 vs. 356; $P < .05$). The MFI values reflecting IgG levels were highest for CHIPS, SCIN, and TSST-1; those for IgA were highest for CHIPS, SCIN, and Efb; and those for IgM the were highest for ClfA and SasG.

Stability of anti-staphylococcal antibody levels in serum. To study the stability of the level of *S. aureus* antigen–specific antibodies over time, a second serum sample and 2 more nasal swab samples were collected after 6 months from 11 volunteers. None of these volunteers reported suffering from an apparent *S. aureus* infection between these time points. One of the volunteers (volunteer 2) was classified as an intermittent carrier instead of a noncarrier because of a single positive nasal swab culture after 6 months. For all volunteers, the levels of IgG and IgA to the 19 *S. aureus* proteins did not change significantly during the 6-month period ($P > .05$). Figure 2 shows representative results for the stability of IgG levels for 4 *S. aureus* proteins.

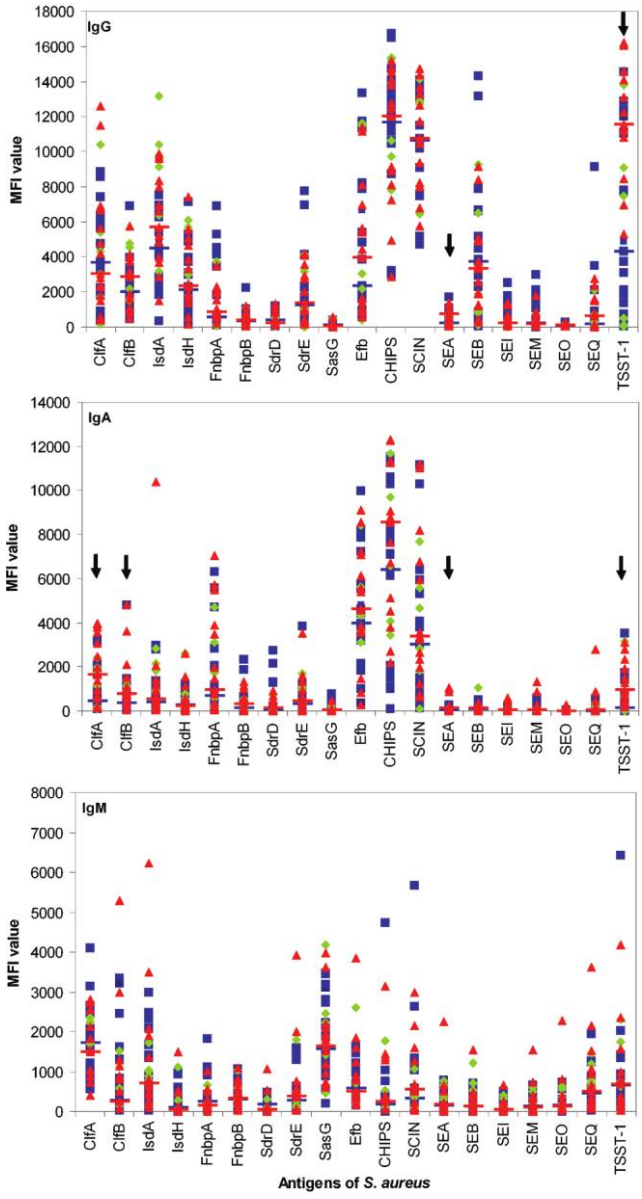


Figure 1. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG, IgA, and IgM for 19 *Staphylococcus aureus* antigens in 40 volunteers. Each symbol represents a single volunteer; red triangles represent persistent carriers, green diamonds represent intermittent carriers, and blue squares represent noncarriers. Horizontal lines indicate median levels of anti-staphylococcal antibodies for persistent carriers and noncarriers, and arrows indicate statistically significant differences in median values (persistent carriers vs. noncarriers, Mann-Whitney *U* test). For IgG, differences were significant for toxic shock syndrome toxin (TSST)–1 (11,554 vs. 4291; $P < .001$) and staphylococcal enterotoxin (SE) A (742 vs. 218; $P < .05$); for IgA, differences were significant for TSST-1 (973 vs. 155; $P < .01$), SEA (127 vs. 32; $P < .05$), and clumping factor (Clf) A and B (for ClfA, 1661 vs. 441; for ClfB, 792 vs. 356; $P < .05$). CHIPS, chemotaxis inhibitory protein of *S. aureus;* Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinants; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate dipeptide repeat protein.

Correlation between anti-staphylococcal antibody levels in serum and nasal secretion. To determine the correlation between anti-staphylococcal antibodies in serum and nasal secretions, these samples were collected simultaneously from 13 volunteers, and the mean IgG and IgA levels (reflected by MFI values) in these samples were calculated for each protein. The correlation coefficient for the comparison between serum and nasal secretions was 0.87 for IgG and correlation 0.77 for IgA (figure 3).

TSST-1 neutralization assay. The neutralizing capacity of TSST-1–specific antibodies in the 40 human samples was determined. The median neutralizing capacity was significantly higher in persistent carriers than in noncarriers (90.6% vs. 70.6%; $P < .05$) (figure 4*A*). The level of IgG binding to TSST-1 is highly related to the neutralizing capacity of the serum samples ($R^2 = 0.93$) (figure 4*B*).

DISCUSSION

We developed an *S. aureus* multiplex immunoassay that enables simultaneous quantification of antibodies to 19 antigens in small serum volumes. This assay is therefore more informative and less time- and serum-consuming than the conventional ELISA technique. The methods was used to determine the levels of antigen-specific IgG, IgA, and IgM in serum samples from persistent carriers, intermittent carriers, and noncarriers of *S. aureus*. An important message of our analyses is that anti-staphylococcal antibody levels showed extensive interindividual variability (figure 1), probably owing to the variable number of previous encounters with different *S. aureus* strains of diverse antigenicity as well as interindividual differences in the ability to mount an antigen-specific humoral immune response. In the group of persistent carriers, differences in carrier strain type (as determined by pulsed-field gel electrophoresis [PFGE]; data not shown) might also contribute to the diversity in antibody levels. Thirteen different PFGE types were found; only 3 of 15 persistent carriers carried the same strain.

The most striking difference between persistent carriers and noncarriers was the median level of IgG to TSST-1 ($P < .001$). An earlier study showed that individuals harboring TSST-1–producing strains had significantly higher levels of serum antibody to TSST-1 than did individuals who carried strains without TSST-1 or who did not carry *S. aureus* at all [32]. In our study, 5 (33%) of the 15 persistent carriers carried a TSST-1–positive strain (as determined by polymerase chain reaction; data not shown), which indicates that current carriage of a TSST-1–positive strain does not fully explain the higher antibody levels in persistent carriers. It is likely that the number of previous encounters with such strains also plays a role. We have shown that the level of anti–TSST-1 IgG is highly correlated with the neutralizing capacity of these antibodies ($R^2 = 0.93$) (figure 4*B*). This implies that these anti–TSST-1 antibodies are functional. It

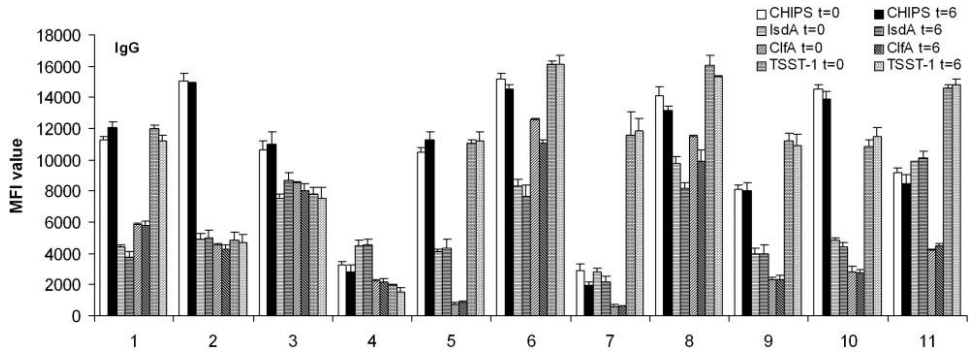


Figure 2. Stability of IgG levels, reflected by median fluorescence intensity (MFI) values for *Staphylococcus aureus* proteins in serum samples from 11 healthy volunteers (1–11) at 0 and 6 months ($t = 0$ and $t = 6$). Error bars represent SEs. CHIPS, chemotaxis inhibitory protein of *S. aureus;* Clf, clumping factor; Isd, iron-responsive surface determinant; TSST, toxic shock syndrome toxin.

is known that humans with high anti–TSST-1 antibody levels do not develop toxic shock syndrome when they become infected with a TSST-1–expressing *S. aureus* strain [33]. As stated elsewhere, it is also known that carriers have a 3-fold higher risk of acquiring *S. aureus* bacteremia than do noncarriers but a significantly lower risk of *S. aureus* bacteremia–related death [5].

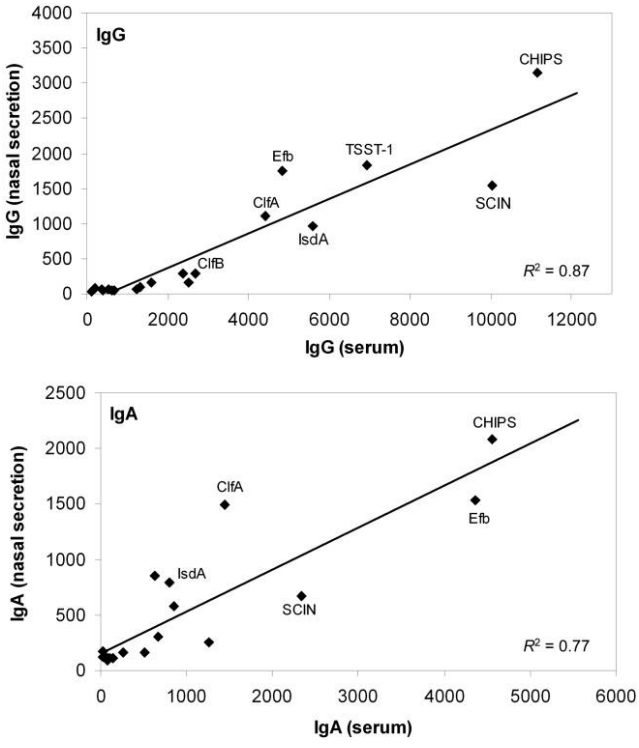


Figure 3. Correlation between IgG and IgA levels in serum and nasal secretions. Mean IgG and IgA levels in serum and nasal secretions, reflected by median fluorescence intensity values, were calculated for each protein. Pearson's correlation coefficient was used. CHIPS, chemotaxis inhibitory protein of *S. aureus;* Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Isd, iron-responsive surface determinant; SCIN, staphylococcal complement inhibitor; TSST, toxic shock syndrome toxin.

Therefore, a possible explanation for this observation is that persistent carriers are protected from toxic shock syndrome because they have a high level of TSST-1–neutralizing antibodies and, consequently, a lower risk of death than noncarriers. These observations should be verified by studying persistent carriers and noncarriers with bacteremia; their anti–TSST-1 antibody levels should be determined and correlated with the outcomes of infection (work in progress).

Other significant differences between persistent carriers and noncarriers were found for IgG directed against SEA ($P < .05$) and IgA directed against TSST-1 ($P < .01$), SEA, ClfA, or ClfB ($P < .05$). These levels were found to be higher in persistent carriers than in noncarriers. Two other studies focusing on anti-staphylococcal antibodies showed higher IgG levels for major autolysin, ClfB, IsdA, IsdH, IsaA, Map-w, and α-hemolysin in noncarriers than in persistent carriers [9, 12]. Although these authors did not measure anti-enterotoxin antibodies or antibodies to immune-modulating proteins, the differences found between persistent carriers and noncarriers differed from our data. One possible explanation for this apparent discrepancy is that the carrier state was less well defined in these studies. Dryla et al. [12] defined persistent carriers and noncarriers as individuals who tested culture positive or negative at least twice, but they did not report whether the carrier state was based on nasal or pharyngeal swab samples (or both) or at what intervals these swab samples were collected. Clarke et al. [9] defined carriers and noncarriers as individuals who were culture positive or negative for *S. aureus* on the basis of just a single nasal swab sample, which cannot reliably distinguish between the different carrier states. Thus, the differences in anti-staphylococcal antibody levels observed in these studies might be explained by the fact that carriage was not defined according to a precise and validated "culture rule," which is based on 2 nasal swab samples and quantitative culture data [2]. In the present study, we used at least 3 nasal swab samples collected at 2-week intervals to define the carrier state.

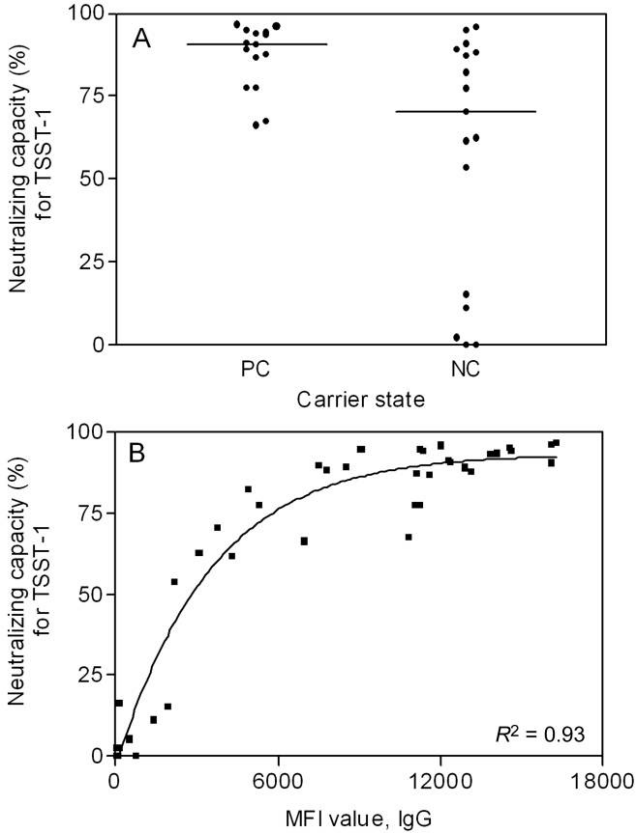


Figure 4. *A,* Higher neutralizing capacity for toxic shock syndrome toxin (TSST)–1 in persistent carriers (PC) than in noncarriers (NC) (median, 90.6% vs. 70.6%; $P < .05$, Mann-Whitney *U* test). Two noncarriers were excluded because of poor technical replicates. *B,* High correlation between the level of IgG antibodies to TSST-1, reflected by median fluorescence intensity (MFI) values, and the neutralizing capacity of the serum samples (nonlinear regression, $R^2 = 0.93$).

The observed IgG and IgA MFI values were highest for CHIPS and SCIN, indicating that these staphylococcal proteins are quite immunogenic. The IgM values were highest for SasG, ClfA, and IsdA. This can be due to a primary immune response to recent exposure to these antigens or to so-called natural IgM antibodies that appear in the absence of stimulation by specific antigens and that are secreted by long-lived, self-renewing B cells belonging to the B1 subset [34]. These natural antibodies are commonly polyspecific and play an important role in the antimicrobial response in humans [35]. Even though the measured IgM antibodies may not be antigen specific, they were directed mostly to MSCRAMMs. This implies that MSCRAMMs are structures that are recognized in an early phase by the immune system and that natural IgM antibodies recognize antigens in a very economic way [34]. Antigen-specific IgG and IgA levels for all volunteers and to all 19 antigens were stable over a period of 6 months. Another study also showed antibody levels to 4 *S. aureus* proteins (IsdH, Map-w, SA0688, and SA2505) remaining stable over time [36]. Stability is the result of humoral memory. Humoral memory is assumed to rely on long-lived plasma cells, which even without antigenic contact will secrete antibodies for many years, and memory B cells, which can be (re)activated by antigen and/or polyclonal stimuli [37].

Components of nasal secretions that complement the innate host defense include IgG and IgA [21, 38]. Therefore, antigen-specific IgG and IgA levels in nasal secretions were determined. IgG and IgA values were highest for CHIPS. Anti-staphylococcal antibody levels in nasal secretions correlated with levels in serum, although for antigen-specific IgA in serum and nasal secretions the correlation was somewhat lower ($R^2 = 0.77$) than that for IgG ($R^2 = 0.87$) (figure 3). There might be an explanation for this observation. In blood, IgA is found predominantly as a monomer, and the ratio of IgA1 to IgA2 is ~4:1. In mucosal secretions, IgA is produced almost exclusively as a dimer, and the ratio of IgA1 to IgA2 is ~3:2 [39, 40]. Therefore, although IgG simply diffuses from the vascular department into the tissues and similarly distributed antigen-specific IgG molecules are measured in blood and nasal secretions, for IgA this is not the case.

In the present study, we focused on nasal carriage. In the absence of nasal carriage, the likelihood of being a throat carrier is 12.6% [41]; a rectal carrier, 3.2% [42, 43]; and an axilla carrier, 2% [44]. In our study, this would mean that only a few of the intermittent and noncarriers would be reclassified into different *S. aureus* carriage types, which would not affect the results significantly. However, it does show the importance of reporting the culture sites when defining the *S. aureus* carriage state.

Although our study was focused exclusively on antibodies directed against *S. aureus* proteins, it should be noted that cell-wall components (such as capsular polysaccharide 5 and 8 [45], peptidoglycan [46] and lipoteichoic acid [47]) are also immunogenic. Therefore, including these cell-wall components in future studies is important; this is the topic of our current methodological investigations.

We have developed a novel high-throughput, low-volume method for detecting levels of antibodies to a wide range of staphylococcal proteins. We showed that anti-staphylococcal antibody levels in serum are highly variable, are stable over time, and correlate well with antibody levels in nasal secretions. Antibodies to TSST-1 have a neutralizing capacity, and median levels of antibodies to TSST-1, SEA, ClfA, and ClfB are higher in persistent carriers than in noncarriers. These antibodies might be associated with the risk of developing *S. aureus* infections and might be responsible for the lower risk of mortality observed in *S. aureus* carriers with bacteremia than in *S. aureus* noncarriers with bacteremia [5].

Acknowledgments

We thank Claudia Brandt-Hagens and Diana Dufour–van den Goorbergh of the Department of Immunology, Erasmus Medical Center, for their help

with the Luminex system. We thank Suzan Rooijakkers and Jan-Ingmar Flock for kindly supplying the *S. aureus* proteins. Special thanks go to Silva Holtfreter for providing the *S. aureus* toxins and for her help with the assay.

References

1. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med **1998**; 339: 520–32.
2. Nouwen JL, Ott A, Kluytmans-Vandenbergh MF, et al. Predicting the *Staphylococcus aureus* nasal carrier state: derivation and validation of a "culture rule." Clin Infect Dis **2004**; 39:806–11.
3. Williams RE. Healthy carriage of *Staphylococcus aureus:* its prevalence and importance. Bacteriol Rev **1963**; 27:56–71.
4. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus:* epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev **1997**; 10:505–20.
5. Wertheim HF, Vos MC, Ott A, et al. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet **2004**; 364:703–5.
6. Toshkova K, Annemuller C, Akineden O, Lammler C. The significance of nasal carriage of *Staphylococcus aureus* as risk factor for human skin infections. FEMS Microbiol Lett **2001**; 202:17–24.
7. Wertheim HF, Melles DC, Vos MC, et al. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis **2005**; 5:751–62.
8. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. N Engl J Med **2001**; 344: 11–6.
9. Clarke SR, Brummell KJ, Horsburgh MJ, et al. Identification of in vivo–expressed antigens of *Staphylococcus aureus* and their use in vaccinations for protection against nasal carriage. J Infect Dis **2006**; 193:1098–108.
10. Bergqvist S. Observations concerning the presence of pyrogenic staphylococci in the nose and their relationship to the antistaphylolysin titre. Acta Med Scand **1950**; 136:343–50.
11. Humphreys DW, Kelly MT, Crowder JG, Martin RR, White A. Immunological studies in nasal carriers of staphylococci. Contrib Microbiol Immunol **1973**; 1:570–80.
12. Dryla A, Prustomersky S, Gelbmann D, et al. Comparison of antibody repertoires against *Staphylococcus aureus* in healthy individuals and in acutely infected patients. Clin Diagn Lab Immunol **2005**; 12:387–98.
13. Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol **1998**; 6:484–8.
14. Roche FM, Meehan M, Foster TJ. The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. Microbiology **2003**; 149:2759–67.
15. Balaban N, Rasooly A. Staphylococcal enterotoxins. Int J Food Microbiol **2000**; 61:1–10.
16. Holtfreter S, Roschack K, Eichler P, et al. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. J Infect Dis **2006**; 193:1275–8.
17. van Wamel WJ, Rooijakkers SH, Ruyken M, van Kessel KP, van Strijp JA. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. J Bacteriol **2006**; 188:1310–5.
18. Jongerius I, Kohl J, Pandey MK, et al. Staphylococcal complement evasion by various convertase-blocking molecules. J Exp Med **2007**; 204: 2461–71.
19. de Haas CJ, Veldkamp KE, Peschel A, et al. Chemotaxis inhibitory protein of *Staphylococcus aureus,* a bacterial antiinflammatory agent. J Exp Med **2004**; 199:687–95.
20. van Belkum A, Emonts M, Wertheim H, et al. The role of human innate immune factors in nasal colonization by *Staphylococcus aureus*. Microbes Infect **2007**; 9:1471–7.
21. Cole AM, Dewan P, Ganz T. Innate antimicrobial activity of nasal secretions. Infect Immun **1999**; 67:3267–75.
22. Rooijakkers SH, Ruyken M, Roos A, et al. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol **2005**; 6:920–7.
23. Rooijakkers SH, van Kessel KP, van Strijp JA. Staphylococcal innate immune evasion. Trends Microbiol **2005**; 13:596–601.
24. Shannon O, Uekotter A, Flock JI. Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* as an antiplatelet agent in vivo. Thromb Haemost **2005**; 93:927–31.
25. Shannon O, Uekotter A, Flock JI. The neutralizing effects of hyperimmune antibodies against extracellular fibrinogen-binding protein, Efb, from *Staphylococcus aureus*. Scand J Immunol **2006**; 63:184–90.
26. Verkaik N, Brouwer E, Hooijkaas H, van Belkum A, van Wamel W. Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins. J Immunol Methods **2008**; 335:121–5.
27. Martins TB, Augustine NH, Hill HR. Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to group A streptococci. J Immunol Methods **2006**; 316:97–106.
28. Holtfreter S, Bauer K, Thomas D, et al. egc-encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. Infect Immun **2004**; 72:4061–71.
29. Lal G, Balmer P, Joseph H, Dawson M, Borrow R. Development and evaluation of a tetraplex flow cytometric assay for quantitation of serum antibodies to *Neisseria meningitidis* serogroups A, C, Y, and W-135. Clin Diagn Lab Immunol **2004**; 11:272–9.
30. Lal G, Balmer P, Stanford E, Martin S, Warrington R, Borrow R. Development and validation of a nonaplex assay for the simultaneous quantitation of antibodies to nine *Streptococcus pneumoniae* serotypes. J Immunol Methods **2005**; 296:135–47.
31. Ray CA, Bowsher RR, Smith WC, et al. Development, validation, and implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum. J Pharm Biomed Anal **2005**; 36:1037–44.
32. Ritz HL, Kirkland JJ, Bond GG, Warner EK, Petty GP. Association of high levels of serum antibody to staphylococcal toxic shock antigen with nasal carriage of toxic shock antigen-producing strains of *Staphylococcus aureus*. Infect Immun **1984**; 43:954–8.
33. Stolz SJ, Davis JP, Vergeront JM, et al. Development of serum antibody to toxic shock toxin among individuals with toxic shock syndrome in Wisconsin. J Infect Dis **1985**; 151:883–9.
34. Vollmers HP, Brandlein S. Natural IgM antibodies: the orphaned molecules in immune surveillance. Adv Drug Deliv Rev **2006**; 58:755–65.
35. Chu Q, Ludtke JJ, Subbotin VM, Blockhin A, Sokoloff AV. The acquisition of narrow binding specificity by polyspecific natural IgM antibodies in a semi-physiological environment. Mol Immunol **2008**; 45:1501–13.
36. Dryla A, Gelbmann D, von Gabain A, Nagy E. Identification of a novel iron regulated staphylococcal surface protein with haptoglobin-haemoglobin binding activity. Mol Microbiol **2003**; 49:37–53.
37. Radbruch A, Muehlinghaus G, Luger EO, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol **2006**; 6:741–50.
38. Kaliner MA. Human nasal respiratory secretions and host defense. Am Rev Respir Dis **1991**; 144:S52–6.
39. Janeway CA Jr. Adaptive immunity to infection. In: Janeway CA Jr, Travers P, Walport M, Shlomchik MJ, eds. Immunology. 6th ed. Vol 1. New York: Garland Science Publishing, **2005**: 409–42.
40. Chuang PD, Morrison SL. Elimination of N-linked glycosylation sites from the human IgA1 constant region: effects on structure, function. J Immunol **1997**; 158:724–32.
41. Mertz D, Frei R, Jaussi B, et al. Throat swabs are necessary to reliably detect carriers of *Staphylococcus aureus*. Clin Infect Dis **2007**; 45:475–7.
42. Wertheim HF, Verveer J, Boelens HA, van Belkum A, Verbrugh HA, Vos MC. Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. Antimicrob Agents Chemother **2005**; 49:1465–7.

43. Acton DS, Tempelmans Plat-Sinnige MJ, van Wamel W, de Groot N, van Belkum A. Intestinal carriage of *Staphylococcus aureus:* how does its frequency compare with that of nasal carriage and what is its clinical impact? Eur J Clin Microbiol Infect Dis **2008**; 8 August (electronically published ahead of print).
44. Dancer SJ, Noble WC. Nasal, axillary, and perineal carriage of *Staphylococcus aureus* among women: identification of strains producing epidermolytic toxin. J Clin Pathol **1991**; 44:681–4.
45. Fattom A, Fuller S, Propst M, et al. Safety and immunogenicity of a booster dose of *Staphylococcus aureus* types 5 and 8 capsular polysaccharide conjugate vaccine (StaphVAX) in hemodialysis patients. Vaccine **2004**; 23:656–63.
46. Verbrugh HA, Peters R, Rozenberg-Arska M, Peterson PK, Verhoef J. Antibodies to cell wall peptidoglycan of *Staphylococcus aureus* in patients with serious staphylococcal infections. J Infect Dis **1981**; 144: 1–9.
47. Wergeland H, Endresen C, Natas OB, Aasjord P, Oeding P. Antibodies to *Staphylococcus aureus* peptidoglycan and lipoteichoic acid in sera from blood donors and patients with staphylococcal infections. Acta Pathol Microbiol Immunol Scand [B] **1984**; 92:265–9.

Chapter 6

Neutralizing antibody response to *Staphylococcus aureus* superantigens in bacteremic patients

D. Grumann, E. Ruotsalainen, J. Kolata, P. Kuusela, A. Järvinen, V. P. Kontinen, B. M. Bröker, and S. Holtfreter

SUBMITTED

Neutralizing antibody response to *Staphylococcus aureus* superantigens in bacteremic patients

Dorothee Grumann[1], Eeva Ruotsalainen[2], Julia Kolata[1], Pentti Kuusela[3,4], Asko Järvinen[2], Vesa P. Kontinen[5], Barbara M. Bröker[1], Silva Holtfreter[1]

[1]Institute of Immunology and Transfusion Medicine, University of Greifswald, Germany

[2]Division of Infectious Diseases, Department of Medicine, Helsinki University Central Hospital, Finland

[3]Division of Clinical Microbiology (HUSLAB), Helsinki University Central Hospital, Finland

[4]Department of Bacteriology and Immunology, the Hartman Institute, University of Helsinki, Finland

[5]National Institute for Health and Welfare, Helsinki, Finland

ABSTRACT

Background: Staphylococcus aureus superantigens (SAgs) are highly potent T cell mitogens. Antibodies against non-enterotoxin gene cluster (*egc)* SAgs are common in healthy adults, whereas neutralizing antibodies against *egc* SAgs are rare. We investigated the anti-SAg antibody response during *S. aureus* bacteremia (SAB).

Methods: This prospective clinical study (www.clinicaltrials.gov, NCT00548002) included 43 injection drug users (IDUs) and 44 group matched nonaddicts with SAB. *Spa* genotypes and SAg gene patterns (multiplex PCR) of the *S. aureus* isolates were determined. The neutralizing capacity of sera obtained at the acute phase and the convalescent phase of SAB was tested against the SAg cocktail of the respective infecting strain and a panel of recombinant SAgs.

Results: The lineages CC59 and CC30 were more prevalent among bacteremia strains from IDUs than from nonaddicts. SAg gene patterns in isolates from IDUs and nonaddicts were similar. At the acute phase of bacteremia, IDUs had more neutralizing antibodies

against non-*egc* SAgs than nonaddicts. Antibody titers frequently increased during infection. Interestingly, antibodies against *egc* SAgs were absent at the acute phase and not induced by SAB.

Conclusions: SAB triggers an antibody response only against non-*egc* SAgs. Preimmunization in IDU patients is probably due to previous exposure to the infecting strain.

INTRODUCTION

Staphylococcus (S.) aureus is a major human pathogen that causes a wide spectrum of infections, such as toxin-mediated diseases, and systemic infections, for instance, bacteremia and endocarditis. At the same time, *S. aureus* is a commensal that colonizes approximately 35% of the healthy population in the nose (1, 2).

Among the numerous toxins of *S. aureus* are the 21 known staphylococcal superantigens (SAgs): the toxic shock syndrome toxin (TSST-1), the staphylococcal enterotoxins (SEA-SEE, SEG-SEJ) and the staphylococcal enterotoxin-like toxins (SElK- SElU) (3-7). They are encoded on mobile genetic elements, like phages and pathogenicity islands (8). SAgs are the causative agents of food poisoning and toxic shock syndrome, but their role in bacteremia is not well defined (5, 9). They can activate a large fraction of T lymphocytes by directly cross-linking certain T cell receptor Vβ domains with conserved structures on major histocompatibility complex class II (MHC II) molecules. This results in a polyclonal T cell activation and massive cytokine release.

The more recently described enterotoxin gene cluster (*egc*) harbors five to six SAg genes (*seg*, *sei*, *selm*, *seln*, *selo* and sometimes *selu*), which cluster on a staphylococcal pathogenicity island (νSaβ) (10, 11). The *egc* genes are the most prevalent SAg genes in commensal and invasive *S. aureus* isolates with frequencies ranging between 52 and 66% (12-14).

We previously reported that SAg genes are not randomly distributed, but rather strongly associated with the clonal lineages (14). Thus, each lineage is characterized by a typical SAg gene profile. However, within each lineage, most SAg genes are mobile (except for *egc* SAgs). Therefore, several SAg genotypes can occur within one clonal complex (CC).

In addition to their superantigenicity, SAgs, like other proteins, also act as conventional antigens and induce a specific antibody response. Antibodies against non-*egc* SAgs (e.g., TSST-1, SEA, SEB, SEC) are common in the healthy population (15-18). In *S. aureus* carriers, these antibodies are highly specific for the SAgs of the colonizing strain and they effectively neutralize their mitogenic effects (19). Surprisingly, neutralizing antibodies against *egc* SAgs are very rare, even among carriers of *egc*-positive *S. aureus* strains (18) (and unpublished observations). This "*egc* gap" in the antibody response of healthy individuals was unexpected because of the high prevalence of *egc* SAg genes in clinical *S. aureus* isolates (12-14). A comparison of recombinant *egc* and non-*egc* SAgs revealed that they do not differ in any of the studied aspects of T cell activation including: gene regulation, cytokine secretion or induction of T cell proliferation (20). Remarkably, *egc* SAgs are secreted by *S. aureus* during logarithmic growth in vitro, whereas non-*egc* superantigens – like most virulence factors – are expressed during stationary growth (20, 21).

The aim of our study was to test whether the differentially regulated *egc* SAgs and non-*egc* SAg elicit an antibody response during systemic infection. In particular, we wanted to investigate the role of *egc* SAgs in *S. aureus* bacteremia (SAB) among nonaddicts previously less exposed to *S. aureus* and among injection drug users (IDUs) with more frequent contact to it.

In a prospective clinical study, we (i) determined the genotype and SAg gene patterns in bacteremia isolates from IDUs and matched nonaddicts and (ii) compared the SAg-neutralizing capacity of sera obtained at the acute phase of bacteremia and in the convalescent phase.

MATERIALS AND METHODS

Patient population. We prospectively collected 430 adult patients with blood culture positive for methicillin-sensitive *S. aureus* (MSSA). Twelve university or central hospitals in Finland participated in this study between January 1999 and May 1999, and between January 2000 and August 2002 (22). In this study, 43 IDUs and 44 group matched nonaddicts as controls were included (Table 1) (23). For each IDU with endocarditis (n=19), we chose a nonaddict with preferably definite endocarditis (n=20; 16 definite

and 4 possible cases). For each IDU without endocarditis (n=24), we chose an age (± 15 years) and sex matched nonaddict, whose randomization time was the nearest possible.

Written informed consent was obtained from all patients or their representatives. The study was approved by the ethics committees of all study sites, and was conducted in accordance with the Declaration of Helsinki.

Table I. Characteristics of IDUs and nonaddicts with methicillin-sensitive *S. aureus* bacteremia (n=87).

Characteristics	Injection drug users (n = 43)	Nonaddicts (n = 44)
Age (years, mean ± SD)	29 ± 8	50 ± 19
Male sex	32 (74.4)	28 (63.6)
Previous *S. aureus* infection[a]	7 (16)	8 (18.2)
Underlying disease		
Liver disease	36 (83.7)	5 (11.4)
HIV positive	6 (13.9)	0 (0.0)
Diabetes	3 (7.0)	12 (27.3)
Coronary artery diseases	0 (0.0)	8 (18.2)
Chronic renal failure	0 (0.0)	6 (13.6)
Malignancy	0 (0.0)	4 (9.1)
McCabe´s classification[b]		
Healthy or nonfatal disease	43 (100)	33 (75.0)
Ultimately or rapidly fatal disease	0 (0)	11 (25.0)
Endocarditis[c]	19 (44.2)	20 (45.5)
Outcome		
Death within 3 months	2 (4.7)	4 (9.1)

NOTE. Data are presented no. (%) of patients, unless otherwise indicated.

[a]superficial *S. aureus* infections or *S. aureus* bacteremia (only IDUs).

[b]Prognosis or severity of underlying diseases classified according to the criteria of McCabe and Jackson.

[c]Classified as definite or possible by using the modified Duke criteria.

***S. aureus* identification and DNA isolation.** Routine bacteriological methods were used to detect *S. aureus* grown in blood (22). Total *S. aureus* DNA was isolated with the Promega Wizard® DNA purification kit (Promega, Mannheim, Germany) according to the manufacturer's instructions.

Serum samples. After the first positive culture for *S. aureus*, serum samples were collected at days 2 to 7 (acute phase) and at days 22 to 28 (convalescent phase) (23). Sera were stored at -20°C for further analysis. Both samples were available from 27 of 43 IDUs and from 37 of 44 nonaddicts. In the other 16 IDUs and 6 nonaddicts, one or both serum samples were missing.

***spa* genotyping.** PCR for amplification of the *S. aureus* protein A (*spa*) repeat region was performed according to the published protocols (24, 25). PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced using both amplification primers by a commercial supplier (MWG Biotech, Ebersberg, Germany). The forward and reverse sequence chromatograms were analyzed with the Ridom StaphType software Version 1.99.11 (Ridom GmbH, Würzburg, Germany). With the BURP algorithm (Ridom GmbH), *spa* types were clustered into different groups (the parameter *calculated cost between members of a group* was set at less than or equal to five). *Spa* types shorter than five repeats were not grouped, because they do not allow the reliable deduction of ancestries. Since *spa* typing and multilocus-sequence typing (MLST) are highly concordant (26), *spa* typing data could be easily mapped on MLST types by using the SpaServer database (www.spaserver.ridom.de).

Virulence gene detection by PCR. PCR was used to screen for a total of 25 virulence genes. Single and multiplex-PCR were applied for the detection of gyrase (*gyr*), methicillin resistance (*mecA*), Panton-Valentine-leukocidin (*pvl*), staphylococcal enterotoxins (*sea*-*seu*), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins (*eta*, *etd*), and agr group 1-4 as previously reported (14).

Neutralization assay. Neutralization assays were performed as described before (18, 19, 27). Initially, the concentrations of bacterial supernatants (post-exponential growth phase) and recombinant SEB, SEC, SElQ, TSST-1 and SEI which elicited submaximal proliferation were determined in T cell proliferation assays (between 1 and 100 pg/ml). They were performed in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Afterwards, the appropriate concentrations of supernatant or recombinant SAgs were incubated in the presence of heat-inactivated patient sera serially diluted in RPMI 1640/10% FBS. For control, supernatants or recombinant SAgs were incubated without human serum. After twenty minutes, 10^5 peripheral blood mononuclear cells (PBMCs) from third-party healthy blood donors were added to measure mitogenic potency and neutralizing serum capacity. T cell proliferation was determined by the incorporation of

[^{3}H]thymidine after 72h, quantified by calculating the area under the proliferation curve (AUC), and expressed as a percentage of the control without human serum. All measurements were performed in triplicates. Two IDU patients (T-29359, T-37698) were excluded, because their invasive *S. aureus* isolate was SAg-negative.

Statistical analysis. Differences in the *spa* genotype and virulence gene pattern between IDUs and nonaddicts were assessed using the Chi-square test. The Mann-Whitney test was used to compare the neutralizing capacity of serum from IDUs and nonaddicts. The neutralizing capacities of the acute and convalescent serum samples from the bacteremic patients were compared with the paired t-test. *P* values ≤ 0.05 were considered statistically significant.

RESULTS

Genotype of SAB isolates from IDUs and nonaddicts

Among the SAB isolates from 43 IDUs and 44 nonaddicts we found 46 different *spa* types, which were assigned to 12 CCs (CC1, 5, 8, 9, 12, 15, 20, 22, 25, 30, 45, and 59). Moreover, we observed six singletons. Eight isolates could not be clustered by BURP analysis, because clustering parameters excluded *spa* types shorter than five repeats, and two strains were non-typable by *spa* PCR.

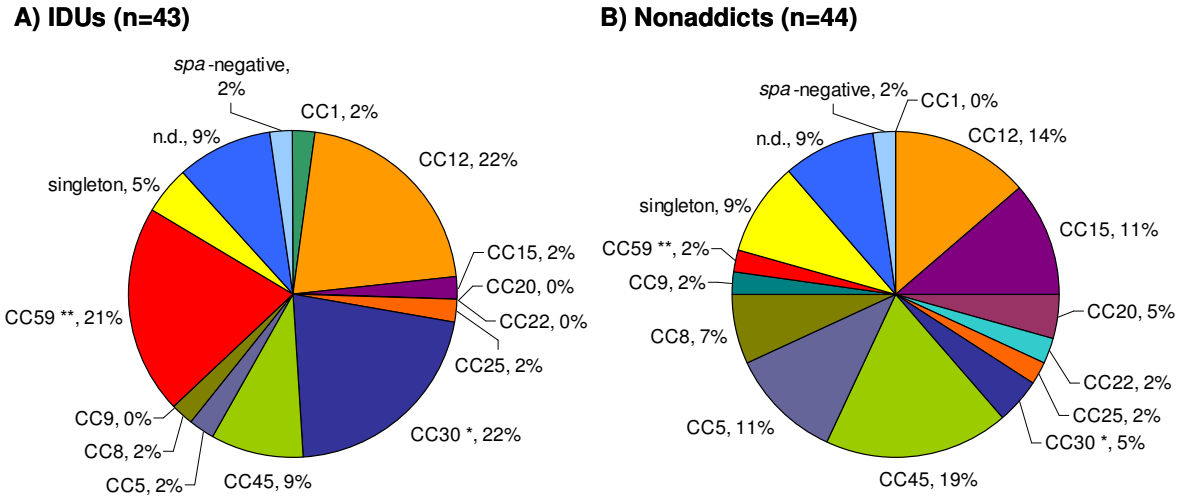
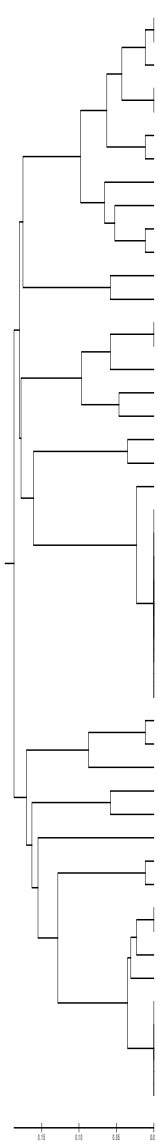


Figure 1. Clonal distribution of SAB isolates from A) IDUs and B) nonaddicts. *Spa* types were clustered into 12 CCs by BURP analysis. MLST-CC nomenclature was deduced from *spa*-CCs using the Ridom SpaServer database. CC59 and CC30 were overrepresented among SAB isolates from IDUs. Chi-Square test. *$P \leq 0.05$, **$P \leq 0.01$.

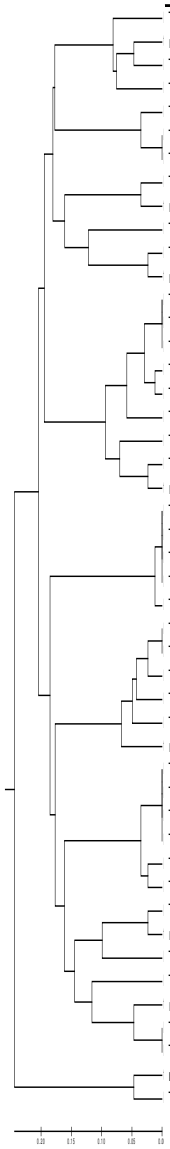
The genetic diversity of SAB strain collections from IDUs and nonaddicts was similar, but CC59 and CC30 were significantly overrepresented among isolates from IDUs in comparison to nonaddicts (CC59: 20.9% vs. 2.3%, $P \leq 0.01$, and CC30: 20.9% vs. 4.5%, $P \leq 0.05$, respectively) (Figure 1). No CC or *spa* type was associated with endocarditis.

A)



	spa typing				virulence genes				
strain ID	*spa* type ID	repeat succession	*spa* CC	deduced MLST CC	non-*egc* SAgs	*egc* SAgs	*eta*, *etd*	*pvl*	*agr*
T-72730[a]	t012	15-12-16-02-16-02-25-17-24-24	CC021	CC30	*a*	*g i m n o u*	-	-	*3*
T-34115[b]	t012	15-12-16-02-16-02-25-17-24-24	CC021	CC30	*tst*	*g i m n o u*	-	-	*3*
T-29165[a,b]	t021	15-12-16-02-16-02-25-17-24	CC021	CC30	*a tst*	*g i m n o u*	-	-	*3*
T-37389[a,b]	t342	15-12-16-02-16-02-25-17	CC021	CC30	*tst*	*g i m n o u*	-	-	*3*
T-34353[a,b]	t342	15-12-16-02-16-02-25-17	CC021	CC30	*tst*	*g i m n o u*	-	-	*3*
T-31257[a,b]	t298	15-12-16-02-17-24	CC021	CC30	*tst*	*g i m n o u*	-	-	*3*
T-36500[a,b]	t096	15-12-16-16-02-17-24	CC021	CC30	*c l tst*	*o*	-	-	*3*
T-32571[a,b]	t2785	35-16-02-25-17-24-24	CC021	CC30	*a tst*	*g i m n o u*	-	-	*3*
T-36895[a,b]	t2786	08-16-02-25-17-24-24-17	CC021	CC30	*a tst*	*g i m n o u*	-	-	*3*
reference	t019	08-16-02-16-02-25-17-24	CC021	CC30					
reference	t138	08-16-02-25-17-24	CC021	CC30					
T-37576[b]	t451	11-12-05-17-34-24-34-22-25	CC451	CC8	*a b k q*	-	-	-	*1*
reference	t008	11-19-12-21-17-34-24-34-22-25	CC451	CC8					
T-71400[a]	t015	08-16-02-16-34-13-17-34-16-34	CC065	CC45	*c l*	*g i m n o*	-	-	*1*
T-36476[a,b]	t015	08-16-02-16-34-13-17-34-16-34	CC065	CC45	*c l*	*g i m n o*	-	-	*1*
T-31859	t2784	08-16-02-16-13-17-34-16-13	CC065	CC45	*c l*	*g i m n o*	-	-	*1*
T-33510[b]	t715	09-02-16-34-13-17-34	CC065	CC45	-	*g i m n o*	-	-	*1*
reference	t065	09-02-16-34-13-17-34-16-34	CC065	CC45					
T-31253[b]	t078	04-21-12-41-20-17-12-12-17	CC078	CC25	*b*	*g i m n o*	*etd*	*+*	*1*
reference	t167	04-12-41-20-17-12-12-17	CC078	CC25					
reference	t216	04-20-17-20-17-31-16-34	CC172	CC59					
T-31648[b]	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-31236[b]	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-29195	t172	04-20-17-45-16-34	CC172	CC59	*b k q*	-	-	-	*1*
T-72830	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-71404	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-31385[a,b]	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-31610[a,b]	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-36780[a,b]	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
T-71405[a]	t172	04-20-17-45-16-34	CC172	CC59	*a b k q*	-	-	-	*1*
reference	t085	07-23-12-34-34-12-23-02-12-23	CC084	CC15					
T-29359	t084	07-23-12-34-34-12-12-23-02-12-23	CC084	CC15	-	-	-	-	*2*
T-37698	t803	07-23-02-12-23	singleton	singleton	-	-	-	-	*2*
reference	t045	26-17-20-17-12-17-16	CC002	CC5					
T-35732[b]	t002	26-23-17-34-17-20-17-12-17-16	CC002	CC5	-	*g i m n o*	-	-	*2*
T-31560	t2730	07-23-178-21-17-34-33-16-16-16-23	singleton	singleton	-	-	-	-	*1*
T-36462[b]	t127	07-23-21-16-34-33-13	CC127	CC1	*h*	-	-	-	*3*
reference	t128	07-23-23-21-16-34-33-13	CC127	CC1					
T-30900[b]	t2133	07-23-21-33-22-17	CC2133	CC12	*b*	-	-	-	*2*
T-29325[b]	t2133	07-23-21-33-22-17	CC2133	CC12	*b p*	-	-	-	*2*
T-36342[b]	t1137	07-23-12-33-22-22-17	CC2133	CC12	*c l p*	-	-	-	*2*
T-33421	t888	07-23-21-24-33-17	CC2133	CC12	*b p*	-	-	-	*2*
T-35093[b]	t160	07-23-21-24-33-22-17	CC2133	CC12	*b p*	-	-	-	*2*
T-34163[a]	t160	07-23-21-24-33-22-17	CC2133	CC12	*b p*	-	-	-	*2*
T-36750[a,b]	t160	07-23-21-24-33-22-17	CC2133	CC12	*b p*	-	-	-	*2*
T-31202[a]	t160	07-23-21-24-33-22-17	CC2133	CC12	-	-	-	-	*2*
T-27360[a]	t160	07-23-21-24-33-22-17	CC2133	CC12	*b p*	-	-	-	*2*
T-31485	t026	08-16-34	n.d.	n.d.	*c l*	*g i m n o*	-	-	*1*
T-31028[a]	t519	04-20-17-25	n.d.	n.d.	-	*i m n o u*	-	-	*4*
T-71385	t519	04-20-17-25	n.d.	n.d.	-	*i m n o u*	-	-	*4*
T-35456	t779	08	n.d.	n.d.	*c l*	*g i m n o*	-	-	*1*
T-27350[b]	no PCR product		-	-	*b k q*	-	-	-	*1*

B)



	spa typing				virulence genes				
strain ID	*spa* type ID	repeat succession	*spa* CC	deduced MLST CC	non-*egc* SAgs	*egc* SAgs	*eta*, *etd*	*pvl*	*agr*
T-72914[b]	t1642	15-16-02-25-17-24-24	CC021/012	CC30	*a q tst*	*g i m n o u*	-	-	*3*
reference	t012	15-12-16-02-16-02-25-17-24-24	CC021/012	CC30					
T-32202[a,b]	t1414	15-12-16-02-16-02-25-24	CC021/012	CC30	*q tst*	*g i m n o u*	-	-	*3*
T-29551[b]	t219	15-12-16-16-02-17-24-24-24	singleton	singleton	*q*	*o*	-	-	*3*
T-71451[a,b]	t024	11-12-21-17-34-24-34-22-25	CC024	CC8	*a*	-	-	-	*1*
T-71452[a,b]	t008	11-19-12-21-17-34-24-34-22-25	CC024	CC8	*a d j r*	-	-	-	*1*
T-31384[a,b]	t008	11-19-12-21-17-34-24-34-22-25	CC024	CC8	*d j r*	-	-	-	*1*
T-29211[a,b]	t078	04-21-12-41-20-17-12-12-17	CC078	CC25	-	*g i m n o*	*etd*	-	*1*
reference	t167	04-12-41-20-17-12-12-17	CC078	CC25					
T-32662[a]	t3302	08-21-17-34-24-33-34	singleton	singleton	-	-	-	-	*1*
T-32183[b]	t172	04-20-17-45-16-34	CC172	CC59	*a b q*	-	*eta*	-	*1*
reference	t216	04-20-17-20-17-31-16-34	CC172	CC59					
T-31149[b]	t015	08-16-02-16-34-13-17-34-16-34	CC116/015	CC45	*c l*	*g i m n o*	-	-	*1*
T-33333[a,b]	t015	08-16-02-16-34-13-17-34-16-34	CC116/015	CC45	*c l*	*g i m n o*	-	-	*1*
T-30803[a,b]	t015	08-16-02-16-34-13-17-34-16-34	CC116/015	CC45	*c l*	*g i m n o*	-	-	*1*
T-76071[a,b]	t073	08-16-02-16-13-17-34-16-34	CC116/015	CC45	*c l*	*g i m n o*	-	-	*1*
T-35124[b]	t116	08-16-02-16-13-13-17-34-16-34	CC116/015	CC45	*c l*	*g i m n o*	-	-	*1*
T-29035[b]	t620	08-16-02-16-34-13-17-34	CC116/015	CC45	*c l*	*g i m n o*	-	-	*1*
T-71442[a,b]	t1122	09-02-16-34-34-17-34-16	CC116/015	CC45	-	*g i m n o*	-	-	*1*
T-30293[b]	t004	09-02-16-13-13-17-34-16-34	CC116/015	CC45	-	*g i m n o*	-	-	*1*
reference	t065	09-02-16-34-13-17-34-16-34	CC116/015	CC45					
T-34788[a]	t084	07-23-12-34-34-12-12-23-02-12-23	CC084	CC15	-	-	-	-	*2*
T-34238	t084	07-23-12-34-34-12-12-23-02-12-23	CC084	CC15	-	-	-	-	*2*
T-31264	t084	07-23-12-34-34-12-12-23-02-12-23	CC084	CC15	-	-	-	-	*2*
T-30869	t084	07-23-12-34-34-12-12-23-02-12-23	CC084	CC15	-	-	-	-	*2*
T-30224[a]	t085	07-23-12-34-34-12-23-02-12-23	CC084	CC15	-	-	-	-	*2*
T-72806[a,b]	t002	26-23-17-34-17-20-17-12-17-16	CC002	CC5	*d j p r*	*g i m n o*	-	-	*2*
T-36108[b]	t002	26-23-17-34-17-20-17-12-17-16	CC002	CC5	*p tst*	*g i m n o*	-	-	*2*
T-30198[a,b]	t242	26-23-17-13-17-20-17-12-17-16	CC002	CC5	*d j r*	*g i m n o*	-	-	*2*
T-24960[b]	t067	26-23-17-34-17-20-17-12-17	CC002	CC5	*p*	*g i m n o*	-	-	*2*
T-72949[a,b]	t539	26-23-17-34-17-12-17-16	CC002	CC5	*p*	*g i m n o*	-	-	*2*
reference	t045	26-17-20-17-12-17-16	CC002	CC5					
T-72942[b]	t160	07-23-21-24-33-22-17	CC2133/888	CC12	*b p*	-	-	-	*2*
T-72805[a,b]	t160	07-23-21-24-33-22-17	CC2133/888	CC12	*p*	-	-	-	*2*
T-32130[a,b]	t160	07-23-21-24-33-22-17	CC2133/888	CC12	*b p*	-	-	-	*2*
T-30221[a,b]	t160	07-23-21-24-33-22-17	CC2133/888	CC12	*b p*	-	-	-	*2*
T-32558[a,b]	t888	07-23-21-24-33-17	CC2133/888	CC12	*b p*	-	-	-	*2*
T-36979[b]	t2133	07-23-21-33-22-17	CC2133/888	CC12	*b p*	-	-	-	*2*
T-71453[b]	t1045	26-23-02-12-23-34-34	CC1045	CC9	-	*g i m n o*	-	-	*2*
reference	t100	26-23-02-12-23-02-34-34	CC1045	CC9					
T-30602[b]	t3303	07-17-23-02-34-34	singleton	singleton	-	*g i m n o*	-	-	*2*
T-33904	t2186	07-23-12-21-17-17-34	singleton	singleton	-	-	-	-	*1*
reference	t195	07-06-17-21-34-34	CC164	CC20					
T-31809[b]	t164	07-06-17-21-34-34-22-34	CC164	CC20	-	*g i m n o*	-	-	*1*
T-31263[b]	t164	07-06-17-21-34-34-22-34	CC164	CC20	*b p*	*g i m n o*	-	-	*1*
reference	t005	26-30-17-34-17-13-17-12-17-16	CC3304	CC22					
T-37613[a,b]	t3304	26-23-20-31-05-17-25-17-25-16-28	CC3304	CC22	-	*g i m n o*	-	-	*1*
T-29575[b]	t026	08-16-34	n.d.	n.d.	*c l*	*g i m n o*	-	-	*1*
T-37342[b]	t026	08-16-34	n.d.	n.d.	*c l*	*g i m n o*	-	-	*1*
T-71932[b]	t026	08-16-34	n.d.	n.d.	*c l*	*g i m n o*	-	-	*1*
T-29349[b]	t3301	09-02-17-34	n.d.	n.d.	-	*g i m n o*	-	+	*1*
T-32666[b]	no PCR product		-	-	-	*g i m n*	-	-	*1*

Figure 2. Distribution of virulence genes within *spa*-defined CCs among SAB isolates from A) IDUs and B) nonaddicts. For construction of the phylogenetic tree, several reference strains were included in the BURP clustering (shaded in grey). Virulence genes (SAg genes, *agr*, *eta*, *etd, mecA* and *pvl genes*) were determined by multiplex PCR. SAB strains from IDUs and nonaddicts did not differ in their virulence gene pattern. Staphylococcal enterotoxins (SEs) are indicated by single letters (*a* = *sea*, etc.). *tst* = toxic shock syndrome toxin, *egc* = enterotoxin gene cluster, *eta*, *etd* = exfoliative toxins a and d, *agr* = accessory gene regulator, *pvl* = Panton-Valentine leukocidin (*lukPV*). [a)] Patients with infective endocarditis. [b)] Serum analyzed in neutralization assays.

Virulence gene repertoire of SAB isolates from IDUs and nonaddicts

To test whether SAB isolates from IDUs and nonaddicts differ in their virulence gene pattern, we next determined the accessory gene regulator (*agr*) type, SAg genes, PVL and exfoliative toxin genes. The *agr* is a global regulator of virulence gene expression and four different *agr* subgroups, *agr 1-4*, are known. In agreement with previous studies (14, 28-31), we observed a strict linkage of *agr* subgroups with the *spa*-derived lineages (Figure 2). PVL and exfoliative toxin genes (*eta* and *etd*) occurred only rarely.

Multiplex-PCR was applied to detect 19 SAg genes. SAg genes were highly prevalent among SAB isolates from both collections (IDUs: 90.7%, nonaddicts: 84.1%) and SAg gene patterns differed remarkably. As previously reported, SAg genes were linked to staphylococcal lineages (14).

The *egc* SAg genes were by far the most prevalent (IDUs: 44.2%, nonaddicts: 61.4%). *Seb* and *sea* were overrepresented among IDU isolates (*seb*: 44.2% vs. 15.9%, $P \leq 0.01$; *sea*: 30.2% vs. 9.1%, $P \leq 0.05$), but this was due to the high prevalence of *sea*/*seb*-positive CC59 and *sea*-positive CC30 isolates among IDU strains. This emphasizes the importance of a simultaneous analysis of virulence genes and genetic background. The comparison of SAg patterns within certain CCs revealed no major differences between isolates from IDUs and nonaddicts. Furthermore, we found no association of SAg genes with endocarditis.

Neutralizing serum antibodies in IDUs and nonaddicts

To test whether *egc* SAgs elicit an antibody response during infection, we analyzed the neutralizing antibody response of SAB patients against (i) the supernatant of their infecting strain and (ii) against representative recombinant SAgs.

At onset of bacteremia, many patients already possessed neutralizing serum antibodies against the SAg cocktail produced by their infecting strain (Figure 3, Figure 4A). While this neutralizing capacity was mostly low in nonaddicts, IDUs already showed high antibody titers at the acute phase. This suggests that they were preimmunized with the SAgs of their infecting strain. In several cases, we observed a rise in antibody concentrations during SAB, again especially among IDUs (Figure 3, Figure 4). In some individuals, titers increased more than 100-fold (T-30900, and T-35093 (figure 3, A2)). However, this was different in patients infected with CC59 strains. These *S. aureus*

isolates harbored a number of non-*egc* SAgs (*sea, seb, sek, selq*) and their supernatants were strongly mitogenic. IDUs infected with these strains had neutralizing antibodies at diagnosis of SAB, but serum concentrations did not further increase thereafter.

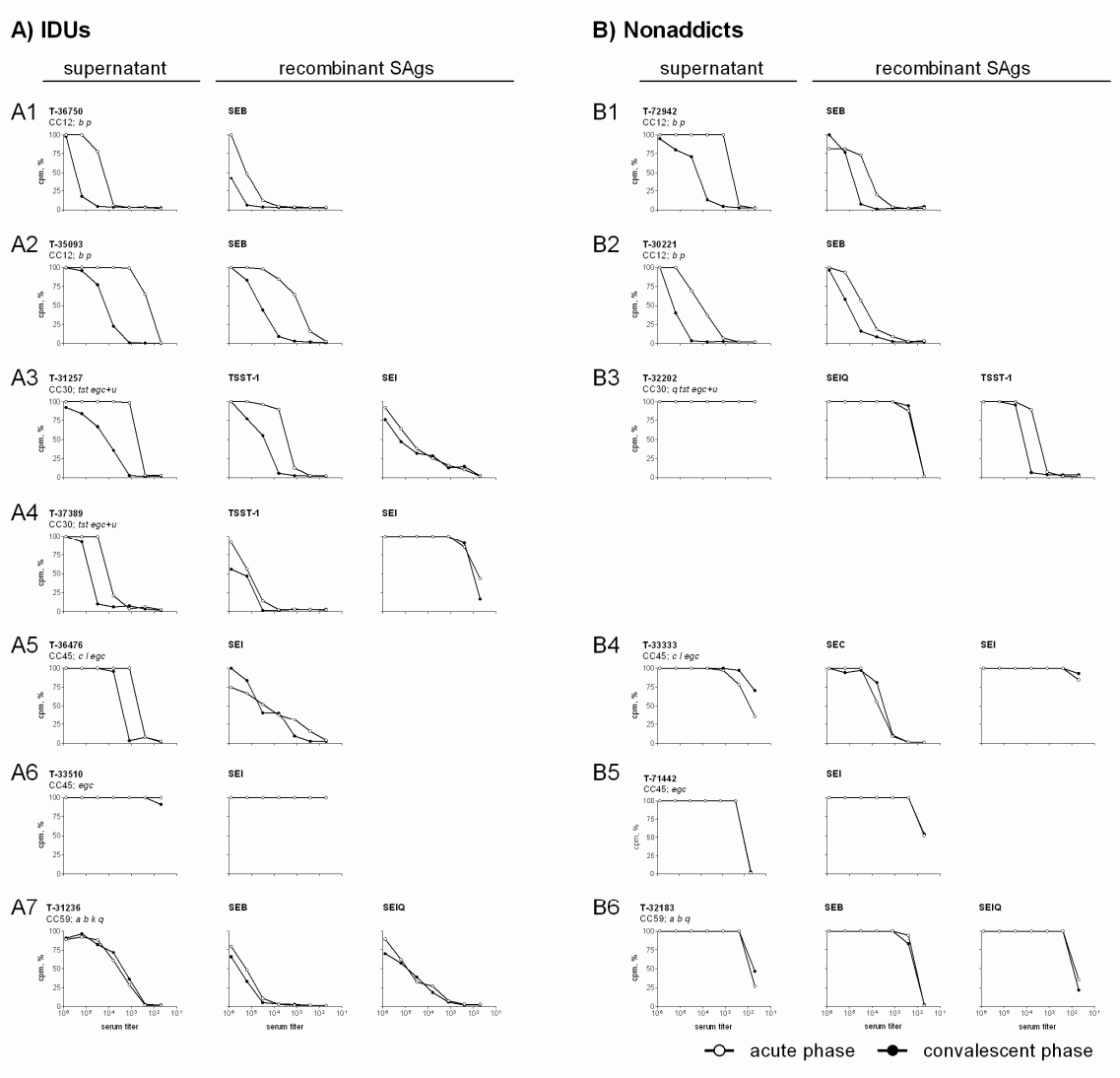


Figure 3. SAg-neutralizing capacity of selected SAB sera from A) IDUs and B) nonaddicts. The neutralizing capacity of SAB sera (acute phase, convalescent phase) against (i) the supernatant from the infecting strain and (ii) representative recombinant SAgs was determined. Graph depicts the SAg-induced proliferation in the presence of serum, expressed as percentage of the control without human serum. In contrast to nonaddicts, most IDUs had neutralizing antibodies already at the acute phase of SAB and antibody titers increased more frequently. In general, neutralizing antibodies against *egc* SAgs were absent and were not induced. Representative data sets are depicted (IDUs: 7/25; nonaddicts: 6/37).

The SAg cocktails in the *S. aureus* culture supernatants maybe close to the clinical situation, but their SAg composition is not known. For molecular definition, we complemented analysis by neutralization assays with recombinant SAgs (TSST-1, SEB,

SEC, SElQ, and SEI), which in most patients confirmed the results obtained with bacterial supernatants. Sera from six patients neutralized recombinant SAgs but not the bacterial supernatants (e.g., Figure 3: B3). The neutralizing effect against individual SAgs was probably obscured by the mitogenic effects of others that were also present in the supernatant.

Notably, neutralizing antibodies against *egc* SAgs were rare exceptions at the acute phase of SAB and such antibodies were not induced during bacteremia. The findings were similar for supernatants from *egc*-positive strains (Figure 3: A6, B5; Figure 4) and for recombinant SEI (Figure 3: A4, A6, B4-B5; Figure 4B). High titer anti-SEI antibodies were present in only two of the 62 tested patient sera (T-31257 and T-36476, Figure 3: A3, A5). Both patients were IDUs and their anti-SEI antibody serum concentrations did not increase during SAB.

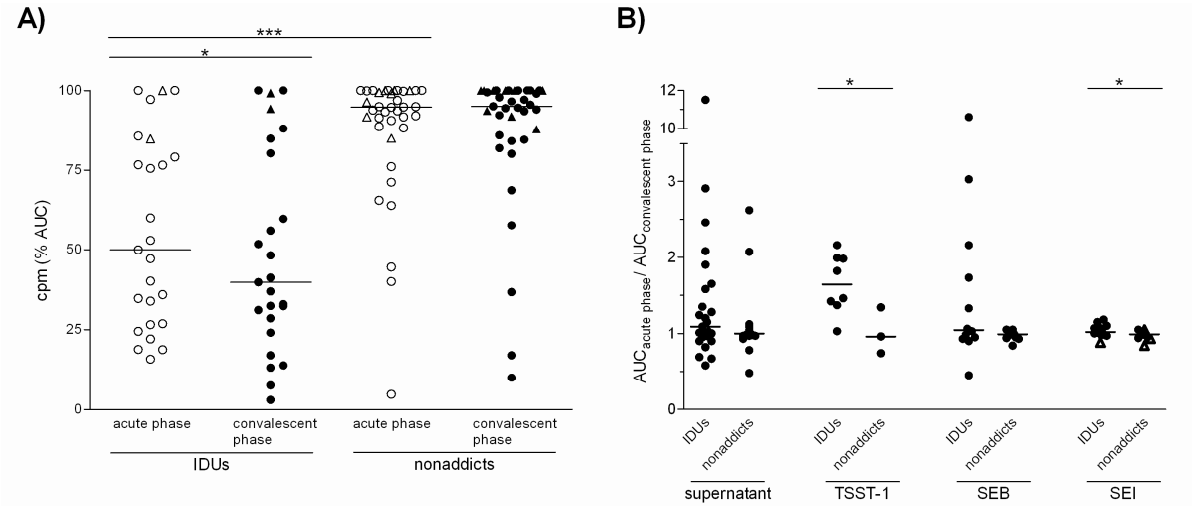


Figure 4. SAg-neutralizing capacity of SAB sera from IDUs and nonaddicts. A) The neutralizing capacity of SAB sera (acute phase, convalescent phase) against the SAg cocktail produced by the infecting strain. SAg-induced proliferation in the presence of serum, quantified by calculating the area under the proliferation curve (AUC) and expressed as percentage of the control without human serum, for all datasets (IDUs: 25; nonaddicts: 37). In contrast to nonaddicts, most IDUs showed an effective antibody response already at the onset of bacteremia. B) Fold-change of the neutralizing capacity of SAB sera (acute phase, convalescent phase) against the *S. aureus* culture supernatants and representative recombinant SAgs. The ratio of the AUC from both serum samples of one patient was calculated. IDUs frequently showed an increase in neutralizing antibody titers against the SAg cocktail and recombinant non-*egc* SAgs. In general, neutralizing capacity against *egc* SAgs (triangle=SAB isolates which were only *egc*-positive) was very low or absent and not triggered during bloodstream invasion. *P* values were calculated using the paired t-test (acute phase vs. convalescent phase) or the Mann-Whitney test (IDUs vs. nonaddicts); median values are indicated. **P* ≤ 0.05, *** *P* ≤ 0.001

DISCUSSION

Neutralizing antibodies against non-*egc* SAgs are common in healthy adults (15-17), whereas neutralizing antibodies against *egc* SAgs are very rare (18). Remarkably, the regulation of SAg release differs fundamentally between both groups of SAgs (20, 21). These findings raised the question, how this differential regulation of *egc* and non-*egc* SAg affects the anti-SAg antibody response during SAB. Comparison of SAB isolates from IDUs and nonaddicts revealed a high prevalence of CC59 and CC30 strains among IDUs, but no differences in SAg gene patterns. We observed a boost of neutralizing antibody titers against non-*egc* SAgs during bloodstream infection. In contrast, *egc* SAgs did not elicit a boost or de novo generation of antibodies.

SAB strains from IDUs and nonaddicts had a highly diverse population structure. In total 12 different staphylococcal lineages and some singletons were observed. The variability of genotypes appeared to be lower among addicts, where the two lineages CC30 and CC59 accounted for over 40% of all strains. While the prevalence of CC30 among IDU isolates was in the same range that was reported for *S. aureus* colonization (16 - 27%) (14, 32-34), CC59 strains are rare in healthy European carriers. Prevalences range from 0% to 4.5% in nasal isolates from the UK, Poland, the Netherlands, and Germany (14, 32-35). However, in IDUs from the UK, methicillin sensitive *S. aureus* strains isolated from abscesses and soft tissue infections frequently belonged to CC59 (30), indicating that this lineage spreads in the internationally connected community of IDUs. We found no correlation of certain clonal lineages or SAg gene patterns with endocarditis.

We previously reported that every *S. aureus* clonal lineage is characterized by a consensus repertoire of *agr* subgroup and virulence genes (14). The SAB isolates in the present study fit well into this picture. In some CCs, the frequencies of the typical SAg genotypes differed between Finland and Germany (14).

Neutralizing antibodies against non-*egc* SAgs are frequent in the healthy population (15-18), and in the present study, this was also found to be the case in SAB patients at the acute phase of bacteremia. However, neutralizing antibody titers were low in most nonaddicts. A possible explanation would be that the majority of them suffered from exogenous infections (19). Unfortunately, the carrier status of our patient cohort is not known. The SAg-neutralizing capacity was much higher in IDUs, suggestive of intensive exposure to *S. aureus,* probably even to the invasive strain. Repeated bacterial

inoculation could be the reason for this, because frequent injections, contaminated drugs, sharing of drug-use equipment and poor hygiene usually characterize the living conditions of IDUs (36, 37). In agreement with this, studies from the seventies showed higher colonization rates and more frequent endogenous infections in IDUs than in the general population (38, 39).

In IDUs but not in nonaddicts, bacteremia increased the neutralizing serum capacity, extending earlier reports about staphylococcal infections (e.g., bacteremia and wound infection) (40, 41). While other groups measured antibody binding, we employed a functional assay, because it has been shown that SAg neutralizing antibodies can be protective in patients as well as in animal models (42-45). The high titers and rapid increases of SAg-neutralizing antibodies are indicative of a vigorous antibody response to many *S. aureus* antigens in IDUs (23). In addition to other factors, such as younger age, lack of pre-existing heart disease, and the predominance of the right-sided heart involvement (46-48), an efficient and protective antibody response might contribute to the more favorable outcome of *S. aureus* endocarditis in IDUs compared to the general population. Only a few nonaddicts responded to bacteremia with an increase of SAg neutralizing antibodies. It appears that bacteremia rarely primes high affinity antibody responses, which would be required for SAg neutralization, but it seems that this immune stimulus is strong enough to boost pre-existing B cell memory.

Overall, the boost of neutralizing antibody titers clearly shows that the immune system is exposed to non-*egc* SAgs during *S. aureus* bloodstream invasion. This remains open for *egc*-encoded SAgs, which did not elicit a boost or de novo generation of specific antibodies, neither against supernatants of *egc*-positive strains nor against recombinant SEI. As we demonstrated previously, the immune cell-activating properties of *egc* and non-*egc* SAgs are very similar (induction of T cell proliferation, cytokine secretion, and gene regulation) and cannot explain the striking differences in the immune response to *egc* and non-*egc* SAgs (20). Because the amino acid sequences of the *egc* SAgs are more closely related to those of individual non-*egc* SAgs than to each other(10, 49), it also appears unlikely, that the two groups of SAgs differ systematically in their immunogenicity. Remarkebly, *egc* SAgs are secreted during logarithmic growth in vitro, whereas non-*egc* SAg – like most virulence factors – are expressed in stationary growth (20, 21). This *egc* gap in the anti-SAg antibody profile, which we observed in healthy adults, *S. aureus* carriers and noncarriers (18), and now also following bloodstream

invasion, shows that differential regulation of antigen expression has a strong impact on the immune response.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (GRK840, "Host-Pathogen Interactions in Generalized Bacterial Infections", SFB-TR34) and the Alfried Krupp Wissenschaftskolleg Greifswald ("A Functional Genomics Approach to Infection Biology"). We thank the FINTROVA and FINLEVO study group investigators for their invaluable participation in patient collection.

REFERENCES

1. van Belkum, A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim. 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. *The Journal of infectious diseases* 199:1820-1826.
2. Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. *The Lancet infectious diseases* 5:751-762.
3. Proft, T., and J. D. Fraser. 2003. Bacterial superantigens. *Clinical and experimental immunology* 133:299-306.
4. Lina, G., G. A. Bohach, S. P. Nair, K. Hiramatsu, E. Jouvin-Marche, and R. Mariuzza. 2004. Standard nomenclature for the superantigens expressed by Staphylococcus. *The Journal of infectious diseases* 189:2334-2336.
5. Holtfreter, S., and B. M. Broker. 2005. Staphylococcal superantigens: do they play a role in sepsis? *Arch Immunol Ther Exp (Warsz)* 53:13-27.
6. Schlievert, P. M., and G. A. Bohach. 2007. Staphylococcal and streptococcal superantigens: an update. In *Superantigens: Molecular basis for their role in human diseases*. M. Kotb, and J. Fraser, eds. ASM Press, Washington, DC.
7. Ono, H. K., K. Omoe, K. Imanishi, Y. Iwakabe, D. L. Hu, H. Kato, N. Saito, A. Nakane, T. Uchiyama, and K. Shinagawa. 2008. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. *Infection and immunity* 76:4999-5005.
8. Lindsay, J. A., and M. T. Holden. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Functional & integrative genomics* 6:186-201.
9. McCormick, J. K., J. M. Yarwood, and P. M. Schlievert. 2001. Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol* 55:77-104.

10. Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* 166:669-677.

11. Letertre, C., S. Perelle, F. Dilasser, and P. Fach. 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J Appl Microbiol* 95:38-43.

12. Becker, K., A. W. Friedrich, G. Peters, and C. von Eiff. 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO, and SElN. *Mol Nutr Food Res* 48:488-495.

13. Fueyo, J. M., M. C. Mendoza, M. A. Alvarez, and M. C. Martin. 2005. Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. *FEMS microbiology letters* 243:447-454.

14. Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Broker. 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *Journal of clinical microbiology* 45:2669-2680.

15. Schroder, E., G. Kunstmann, H. Hasbach, and G. Pulverer. 1988. Prevalence of serum antibodies to toxic-shock-syndrom-toxin-1 and to staphylococcal enterotoxins A, B and C in West-Germany. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 270:110-114.

16. Kunstmann, G., E. Schroder, H. Hasbach, and G. Pulverer. 1989. Immune response to toxic-shock-syndrome toxin-1 (TSST-1) and to staphylococcal enterotoxins A, B and C in *Staphylococcus aureus* infections. *Zentralblb Bakteriol* 271:486-492.

17. Takei, S., Y. K. Arora, and S. M. Walker. 1993. Intravenous immunoglobulin contains specific antibodies inhibitory to activation of T cells by staphylococcal toxin superantigens [see comment]. *J Clin Invest* 91:602-607.

18. Holtfreter, S., K. Bauer, D. Thomas, C. Feig, V. Lorenz, K. Roschack, E. Friebe, K. Selleng, S. Lovenich, T. Greve, A. Greinacher, B. Panzig, S. Engelmann, G. Lina, and B. M. Broker. 2004. *egc*-Encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. *Infection and immunity* 72:4061-4071.

19. Holtfreter, S., K. Roschack, P. Eichler, K. Eske, B. Holtfreter, C. Kohler, S. Engelmann, M. Hecker, A. Greinacher, and B. M. Broker. 2006. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. *The Journal of infectious diseases* 193:1275-1278.

20. Grumann, D., S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Volker, and B. M. Broker. 2008. Immune cell activation by enterotoxin gene cluster (*egc*)-encoded and non-*egc* superantigens from *Staphylococcus aureus*. *J Immunol* 181:5054-5061.

21. Munson, S. H., M. T. Tremaine, M. J. Betley, and R. A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infection and immunity* 66:3337-3348.

22. Ruotsalainen, E., A. Jarvinen, I. Koivula, H. Kauma, E. Rintala, J. Lumio, P. Kotilainen, M. Vaara, J. Nikoskelainen, and V. Valtonen. 2006. Levofloxacin does not decrease mortality in *Staphylococcus aureus* bacteraemia when added to the standard treatment: a prospective and randomized clinical trial of 381 patients. *Journal of internal medicine* 259:179-190.

23. Ruotsalainen, E., M. Karden-Lilja, P. Kuusela, J. Vuopio-Varkila, A. Virolainen-Julkunen, S. Sarna, V. Valtonen, and A. Jarvinen. 2008. Methicillin-sensitive *Staphylococcus aureus* bacteraemia and endocarditis among injection drug users and nonaddicts: host factors, microbiological and serological characteristics. *The Journal of infection* 56:249-256.

24. Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *Journal of clinical microbiology* 41:5442-5448.

25. Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M. C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X. W. Huijsdens, A. M. Kearns, A. Mellmann, H. Meugnier, J. K. Rasheed, E. Spalburg, B. Strommenger, M. J. Struelens, F. C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *Journal of clinical microbiology* 44:619-621.

26. Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W. Friedrich, and W. Witte. 2006. Assignment of Staphylococcus isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. *Journal of clinical microbiology* 44:2533-2540.

27. Verkaik, N. J., C. P. de Vogel, H. A. Boelens, D. Grumann, T. Hoogenboezem, C. Vink, H. Hooijkaas, T. J. Foster, H. A. Verbrugh, A. van Belkum, and W. J. van Wamel. 2009. Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. *The Journal of infectious diseases* 199:625-632.

28. Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day. 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infection and immunity* 70:4987-4996.

29. Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infection and immunity* 70:631-641.

30. Monk, A. B., S. Curtis, J. Paul, and M. C. Enright. 2004. Genetic analysis of *Staphylococcus aureus* from intravenous drug user lesions. *Journal of medical microbiology* 53:223-227.

31. Wright, J. S., 3rd, K. E. Traber, R. Corrigan, S. A. Benson, J. M. Musser, and R. P. Novick. 2005. The *agr* radiation: an early event in the evolution of staphylococci. *Journal of bacteriology* 187:5585-5594.

32. Donker, G. A., R. H. Deurenberg, C. Driessen, S. Sebastian, S. Nys, and E. E. Stobberingh. 2009. The population structure of *Staphylococcus aureus* among general practice patients from The Netherlands. *Clin Microbiol Infect* 15:137-143.

33. Monecke, S., C. Luedicke, P. Slickers, and R. Ehricht. 2009. Molecular epidemiology of *Staphylococcus aureus* in asymptomatic carriers. *Eur J Clin Microbiol Infect Dis* 28:1159-1165.

34. Fowler, V. G., Jr., C. L. Nelson, L. M. McIntyre, B. N. Kreiswirth, A. Monk, G. L. Archer, J. Federspiel, S. Naidich, B. Remortel, T. Rude, P. Brown, L. B. Reller, G. R. Corey, and S. R. Gill. 2007. Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *The Journal of infectious diseases* 196:738-747.

35. Masiuk, H., K. Kopron, D. Grumann, C. Goerke, J. Kolata, J. Jursa-Kulesza, S. Giedrys-Kalemba, B. M. Bröker, and S. Holtfreter. 2010. Recurrent furunculosis - associations with Panton-Valentine

leukocidin and the genetic background of *Staphylococcus aureus*. *Journal of clinical microbiology* accepted.

36. Bassetti, S., and M. Battegay. 2004. *Staphylococcus aureus* infections in injection drug users: risk factors and prevention strategies. *Infection* 32:163-169.

37. Lowy, F. D., and M. Miller. 2002. New methods to investigate infectious disease transmission and pathogenesis--*Staphylococcus aureus* disease in drug users. *The Lancet infectious diseases* 2:605-612.

38. Tuazon, C. U., and J. N. Sheagren. 1974. Increased rate of carriage of *Staphylococcus aureus* among narcotic addicts. *The Journal of infectious diseases* 129:725-727.

39. Tuazon, C. U., and J. N. Sheagren. 1975. Staphlococcal endocarditis in parenteral drug abusers: source of the organism. *Annals of internal medicine* 82:788-790.

40. Kanclerski, K., B. Soderquist, M. Kjellgren, H. Homberg, and R. Mollby. 1996. Serum antibody response to *Staphylococcus aureus* enterotoxins and TSST-1 in patients with septicaemia. *Journal of medical microbiology* 44:171-177.

41. Jozefczyk, Z. 1974. Specific human antibodies to enterotoxins A, B, and C1 of Staphylococcus: their increased synthesis in staphylococcal infection. *The Journal of infectious diseases* 130:1-7.

42. Ulrich, R. G., M. A. Olson, and S. Bavari. 1998. Development of engineered vaccines effective against structurally related bacterial superantigens. *Vaccine* 16:1857-1864.

43. Bavari, S., B. Dyas, and R. G. Ulrich. 1996. Superantigen vaccines: a comparative study of genetically attenuated receptor-binding mutants of staphylococcal enterotoxin A. *The Journal of infectious diseases* 174:338-345.

44. Nilsson, I. M., M. Verdrengh, R. G. Ulrich, S. Bavari, and A. Tarkowski. 1999. Protection against *Staphylococcus aureus* sepsis by vaccination with recombinant staphylococcal enterotoxin A devoid of superantigenicity. *The Journal of infectious diseases* 180:1370-1373.

45. LeClaire, R. D., R. E. Hunt, and S. Bavari. 2002. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. *Infection and immunity* 70:2278-2281.

46. Chambers, H. F., O. M. Korzeniowski, and M. A. Sande. 1983. *Staphylococcus aureus* endocarditis: clinical manifestations in addicts and nonaddicts. *Medicine* 62:170-177.

47. Julander, I. 1985. Unfavourable prognostic factors in *Staphylococcus aureus* septicemia and endocarditis. *Scandinavian journal of infectious diseases* 17:179-187.

48. Fowler, V. G., Jr., J. M. Miro, B. Hoen, C. H. Cabell, E. Abrutyn, E. Rubinstein, G. R. Corey, D. Spelman, S. F. Bradley, B. Barsic, P. A. Pappas, K. J. Anstrom, D. Wray, C. Q. Fortes, I. Anguera, E. Athan, P. Jones, J. T. van der Meer, T. S. Elliott, D. P. Levine, and A. S. Bayer. 2005. *Staphylococcus aureus* endocarditis: a consequence of medical progress. *Jama* 293:3012-3021.

49. Thomas, D., S. Chou, O. Dauwalder, and G. Lina. 2007. Diversity in *Staphylococcus aureus* enterotoxins. *Chem Immunol Allergy* 93:24-41.

Chapter 7

Immune cell activation by enterotoxin gene cluster (*egc*)-encoded and non-*egc* superantigens from *Staphylococcus aureus*

D. Grumann, S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Völker, and B. M. Bröker

PUBLISHED IN:

J Immunol 2008; 181:5054-5061

Immune Cell Activation by Enterotoxin Gene Cluster (*egc*)-Encoded and Non-*egc* Superantigens from *Staphylococcus aureus*[1]

Dorothee Grumann,* Sandra S. Scharf,† Silva Holtfreter,* Christian Kohler,‡ Leif Steil,† Susanne Engelmann,‡ Michael Hecker,‡ Uwe Völker,† and Barbara M. Bröker[2]*

The species *Staphylococcus aureus* harbors 19 superantigen gene loci, six of which are located in the enterotoxin gene cluster (*egc*). Although these *egc* superantigens are far more prevalent in clinical *S. aureus* isolates than non-*egc* superantigens, they are not a prominent cause of toxic shock. Moreover, neutralizing Abs against *egc* superantigens are very rare, even among carriers of *egc*-positive *S. aureus* strains. In search of an explanation, we have tested two non-exclusive hypotheses: 1) *egc* and non-*egc* superantigens have unique intrinsic properties and drive the immune system into different directions and 2) *egc* and non-*egc* superantigens are released by *S. aureus* under different conditions, which shape the immune response. A comparison of three *egc* (SEI, SElM, and SElO) and three non-*egc* superantigens (SEB, SElQ, and toxic shock syndrome toxin-1) revealed that both induced proliferation of human PBMC with comparable potency and elicited similar Th1/Th2-cytokine signatures. This was supported by gene expression analysis of PBMC stimulated with one representative superantigen from each group (SEI and SEB). They induced very similar transcriptional changes, especially of inflammation-associated gene networks, corresponding to a very strong Th1- and Th17-dominated immune response. In contrast, the regulation of superantigen release differed markedly between both superantigen groups. *Egc*-encoded proteins were secreted by *S. aureus* during exponential growth, while non-*egc* superantigens were released in the stationary phase. We conclude that the distinct biological behavior of *egc* and non-*egc* superantigens is not due to their intrinsic properties, which are very similar, but caused by their differential release by *S. aureus*. *The Journal of Immunology,* 2008, 181: 5054–5061.

Staphylococcus aureus is both a successful colonizer and an important pathogen in humans. These bacteria cause a wide spectrum of infectious diseases including several toxin-mediated diseases. Among the numerous toxins of *S. aureus* are the 19 known staphylococcal superantigens (SAgs)[3]: the toxic shock syndrome toxin (TSST-1), the staphylococcal enterotoxins (SEA-SEE, SEG-SEJ), and the staphylococcal enterotoxin-like toxins (SElK-SElR and SElU) (1–4). SAgs are the causative agents of toxic shock syndrome, and might also contribute to septic shock (3, 5). They directly cross-link conserved regions of the variable domains of the TCR β-chain (TCR Vβ) with MHC class II molecules (outside the peptide-binding cleft) on APCs. This results in a strong stimulation of T cells that express the matching TCR Vβ element on their surface. Activated T cells respond with proliferation and massive cytokine release. In this way, SAgs activate up to 20% of all T cells. In contrast, conventional Ags only stimulate around 0.001% of T cells. They require uptake by APCs, processing into peptides, loading onto MHC-II molecules, and presentation on the cell surface, where their specific recognition is mediated by the hypervariable loops of TCR α- and β-chains.

The recently described enterotoxin gene cluster (*egc*) harbors 5 to 6 SAg genes (*seg*, *sei*, *selm*, *seln*, *selo,* and sometimes *selu*), which cluster on a staphylococcal pathogenicity island (νSaβ) (6, 7). The *egc*-genes are the most prevalent SAg genes in commensal and invasive *S. aureus* isolates; frequencies between 52 and 66% have been reported (8–10). However, they appear to cause toxic shock only very rarely (11). In fact, *egc* SAgs are significantly more frequent in commensal strains than in invasive isolates, and their presence is negatively correlated with severity of *S. aureus* sepsis (12, 13). Because of this, it was suggested that SAgs might differ in their pro-inflammatory potential, and Dauwalder et al. (14) reported that in PBMC from healthy donors the non-*egc* SAg SEA induces a stronger Th1-response than the *egc* SAg SEG.

In addition to their superantigenicity, SAgs, like other proteins, also act as conventional Ags and induce a specific Ab response. Abs against non-*egc* SAgs (e.g., TSST-1, SEA, SEB, SEC) are common in the healthy population (15–18). In *S. aureus* carriers, these Abs are highly specific for the SAgs of the colonizing strain and very effectively neutralize their SAg effects (19). In contrast, neutralizing Abs against *egc* SAgs are very rare, even among carriers of *egc*-positive *S. aureus* strains (18) (and unpublished observations). This "*egc*-gap" in the Ab response of healthy individuals was unexpected, because of the high prevalence of *egc* SAg genes in clinical *S. aureus* isolates (8–10).

*Institute for Immunology and Transfusion Medicine, University of Greifswald, Greifswald, Germany; †Institute for Genetics and Functional Genomics, University of Greifswald, Greifswald, Germany; and ‡Institute for Microbiology, University of Greifswald, Greifswald, Germany

Received for publication April 29, 2008. Accepted for publication August 1, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was financially supported by the Deutsche Forschungsgemeinschaft (GRK-840, SFB-TR34).

[2] Address correspondence and reprint requests to Dr. Barbara M. Bröker, Ernst-Moritz-Arndt-Universität Greifswald, Institut für Immunologie und Transfusionsmedizin, Sauerbruchstrasse/ Neubau P, D-17487 Greifswald, Germany. E-mail address: broeker@uni-greifswald.de

[3] Abbreviations used in this paper: SAg, superantigen; TSST-1, toxic shock syndrome toxin-1; *egc*, enterotoxin gene cluster; FDR, false discovery rate; PCA, principal component analysis; SE, staphylococcal enterotoxin; SEl, staphylococcal enterotoxin-like toxin.

Table I. *Cloning primers*

Gene	Primer Sequences (5′→ 3′)[a]
*seb-strep*II	5-ATGGTAGGTCTCAAATGGAGAGTCAACCAGATCCTAAACC
	3-ATGGTAGGTCTCAGCGCTCTTTTTCTTTGTCGTAAGATAAACTTC
*selq-strep*II	5-ATGGTAGGTCTCAAATGGATGTAGGGGTAATCAACCTTAGA
	3-ATGGTAGGTCTCAGCGCTTTCAGTCTTCTCATATGAAATCTCTA
*tst-strep*II	5-ATGGTAGGTCTCAAATGTCTACAAACGATAATATAAAGGATTTG
	3-ATGGTAGGTCTCAGCGCTATTAATTTCTGCTTCTATAGTTTTTATTT
*sei-strep*II	5-ATGGTAGGTCTCAAATGCAAGGTGATATTGGTGTAGGTAAC
	3-ATGGTAGGTCTCAGCGCTGTTACTATCTACATATGATATTTCGAC
*selm-strep*II	5-ATGGTAGGTCTCAAATGGATGTCGGAGTTTTGAATCTTAGG
	3-ATGGTAGGTCTCAGCGCTACTTTCGTCCTTATAAGATATTTCTAC
*selo-strep*II	5-ATGGTAGGTCTCAAATGAATGAAGAAGATCCTAAAATAGAGAG
	3-ATGGTAGGTCTCAGCGCTTGTAAATAAATAAACATCAATATGATAGT

[a] *Bsa*I restriction sites are underlined.

In search of an explanation for these counterintuitive observations, we have tested two non-exclusive hypotheses: 1) *egc* and non-*egc* SAgs have unique intrinsic properties and drive the immune response into different directions and 2) *egc* and non-*egc* SAgs are released by *S. aureus* under different conditions, which shape the immune response to them. To test these hypotheses, we compared the effects of *egc* and non-*egc* SAgs on human blood cells. Their T cell-mitogenic potencies, the elicited cytokine profiles as well as their impact on gene expression were highly similar. Both *egc* and non-*egc* SAgs induced a very strong pro-inflammatory response. In contrast, the regulation of SAg release by *S. aureus* differed markedly between *egc* and non-*egc* SAgs.

Materials and Methods

Bacteria

We used four clinical *S. aureus* isolates from a strain collection from hospitals in Northeast Germany that were investigated previously (18). Their SAg gene repertoire was determined by multiplex-PCR (pSA17: *seb*, *selp;* pSA20: *sec*, *sell*, *selp*; aSA2: *seg, sei, selm, seln, selo, selu*; aSA4: *seg, sei, selm, seln, selo*) as described before (10).

Purification of recombinant SAgs

SEB, SElQ, TSST-1, SEI, SElM, and SElO were produced by recombinant gene technology. The gene sequences were amplified from the sequenced *S. aureus* strains N315 (*tst, sei, selm, selo*) and COL (*seb, selq*) using primers containing *BsaI* restriction sites (underlined) (Table I). PCR products were purified with the Qiagen Quick PCR Purification Kit (Qiagen), digested with *BsaI* and introduced into the *Escherichia coli* plasmid pPR-IBA1 with *Strep*-tag II (IBA). The resulting plasmids were amplified in *E. coli* DH5α and then transfected into *E. coli* BL21 pLysS for overexpression. Recombinant proteins were purified with *Strep*-Tactin Superflow columns according to the manufacturer's instructions (IBA). The purity of the recombinant SAgs was assessed by SDS-PAGE stained with colloidal Coomassie Brilliant Blue. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce). LPS concentrations were determined with a *limulus* amebocyte lysate assay (QCL1000, Lonza). Contaminating LPS was very efficiently removed by two rounds of LPS depletion with the EndoTrap red columns (Profos). In our proliferation and cytokine secretion assays, the final LPS concentrations ranged between 0.4 (0.004 EU/ml) and 31.6 pg/ml (0.316 EU/ml). The final concentrations of LPS in the stimulation experiments for microarray analysis were always below 31.6 fg/ml (3.14×10^{-4} EU/ml).

T cell proliferation assay

T cell proliferation was assessed after 72 h culture by incorporation of [^{3}H]thymidine. Human PBMC (10^5/well) were stimulated with recombinant SAgs titrated over a broad concentration range (0.1 pg/ml to 1 μg/ml) as described previously (18, 19). A control protein (*S. aureus* anti-σ factor RsbW), which was overexpressed and purified in parallel to the recombinant SAgs, did not induce T cell activation. To analyze the growth phase-dependent secretion of SAgs, we cultured two *S. aureus* strains with *egc* SAg genes (aSA2, aSA4) and two strains with non-*egc* SAg genes (pSA17, pSA20) in Luria broth medium. Cultures were sampled at optical densities (OD_{600}) of 0.05, 0.15, 0.4, 1.0, 2.0, 3.0, 4.0, 5.0, and 5.5. Culture supernatants were adjusted by dilution in cell culture medium to OD_{600} 0.05 of the *S. aureus* culture. Their T cell-mitogenic potency was assessed by incubating 10^5 human PBMC/well with 1000-fold dilutions of the adjusted bacterial culture supernatants in 96-well flat-bottom plates in the presence of RPMI 1640 supplemented with 10% FBS.

Cytokine measurement

Human PBMC (10^5/well) were stimulated with recombinant SAgs at concentrations 10-fold above the lowest concentration, which triggered the maximal proliferation (100 pg/ml to 100 ng/ml). Culture supernatants were tested for cytokines after 72 h. The concentrations of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured with the BD Cytometric Bead Assay Human Th1/Th2 Cytokine Kit (BD Biosciences) according to the manufacturer's instructions.

Statistical analysis of T cell proliferation and cytokine data

Differences between *egc* and non-*egc* SAgs were assessed using the Kruskal-Wallis test. For T cell proliferation assays, the SAg concentrations inducing maximal proliferation were compared. The p values below 0.05 were considered statistically significant. Median values are depicted in all images.

RNA isolation and microarray analysis

PBMC from three different blood donors were stimulated with increasing concentrations of the recombinant SAgs SEI and SEB. After 6 h of stimulation, 10^7 PBMC were lysed with TRIzol (Invitrogen) followed by purification of total RNA using an RNeasy Micro Kit (Qiagen) and subsequent precipitation with sodium acetate. RNA concentration and quality were assessed using a Nanodrop ND-1000 (NanoDrop Technologies) and an RNA 6000 Nano LabChip on the Bioanalyzer 2100 (Agilent Technologies). In parallel, the lowest SAg concentrations which elicited maximal responses in 72 h proliferation assays were determined for each case, and RNA samples obtained by stimulation with 10-fold higher (plateau) SAg concentrations (10 to 100 pg/ml) were selected for microarray analysis.

Starting with 2.5 μg of total RNA, labeling was performed using the One-Cycle Target Labeling protocol according to the manufacturer's instructions (Affymetrix). Size distribution of biotin-labeled amplified RNA was analyzed using the Bioanalyzer 2100 and concentration was measured with the Nanodrop ND-1000. Gene expression arrays (GeneChip Human Genome U133 Plus 2.0) were used applying standard protocols (Affymetrix) and arrays were scanned on a GeneChip Scanner 3000 (Affymetrix).

Analysis of array data

The Affymetrix GeneChips used in this study analyze the expression of 54,675 probe sets. Affymetrix array image data were processed with the GeneChip Operation Software (GCOS, Version 1.4, Affymetrix) with the MAS5.0 algorithm and default settings. Expression raw data were transferred to the GeneSpring GX software package version 7.3.1 (Agilent Technologies). Very low raw signal intensities were raised to the threshold level of 10 U to avoid artificially high ratios in the subsequent calculations. Expression values were then subjected to a "per chip" and "per gene" median normalization. Statistical analysis was restricted to probe sets flagged present or marginal by GCOS software in at least two of the three

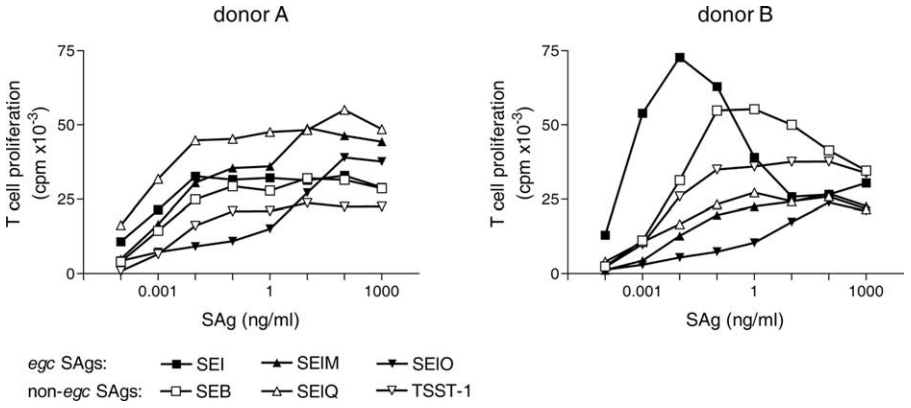


FIGURE 1. Similar T cell-mitogenic potencies of *egc* and non-*egc* SAgs. PBMC from healthy blood donors were stimulated with serial dilutions of recombinant *egc* (SEI, SEIM and SEIO; filled symbols) and non-*egc* SAgs (SEB, SEIQ and TSST-1; empty symbols). Proliferation was assessed after 72 h by [^{3}H]thymidine incorporation. Two representative data sets of nine are depicted.

samples (different blood donors), in untreated controls and/or in samples stimulated with SEI or SEB.

To identify differentially expressed probe sets, intensity ratios between SAg stimulated and untreated cells were calculated for each donor and each SAg. These ratios (PBMC stimulated with SEI vs untreated controls or PBMC stimulated with SEB vs untreated controls) were used for statistical analysis performed with the rank products method (20), which is especially suited for ratio comparison in experiments with small numbers of replicates (21). Data underwent rank product statistics with 1,000 iterations resulting for each probe set in values for the average ratio and two false discovery rates (FDR) (22), one for up-regulation and one for down-regulation. Probe sets with an FDR value less than 0.05 for either up- or down-regulation were considered to represent significant changes in gene transcription.

The signal intensities of all probe sets displaying significant differences in intensity between untreated controls and samples stimulated with SEI or SEB were used for clustering of samples using Pearson correlation; the intensity ratios of the same probe sets were also subjected to a principal component analysis (PCA). Both analyses were performed with GeneSpring GX (Agilent Technologies). The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE11281.

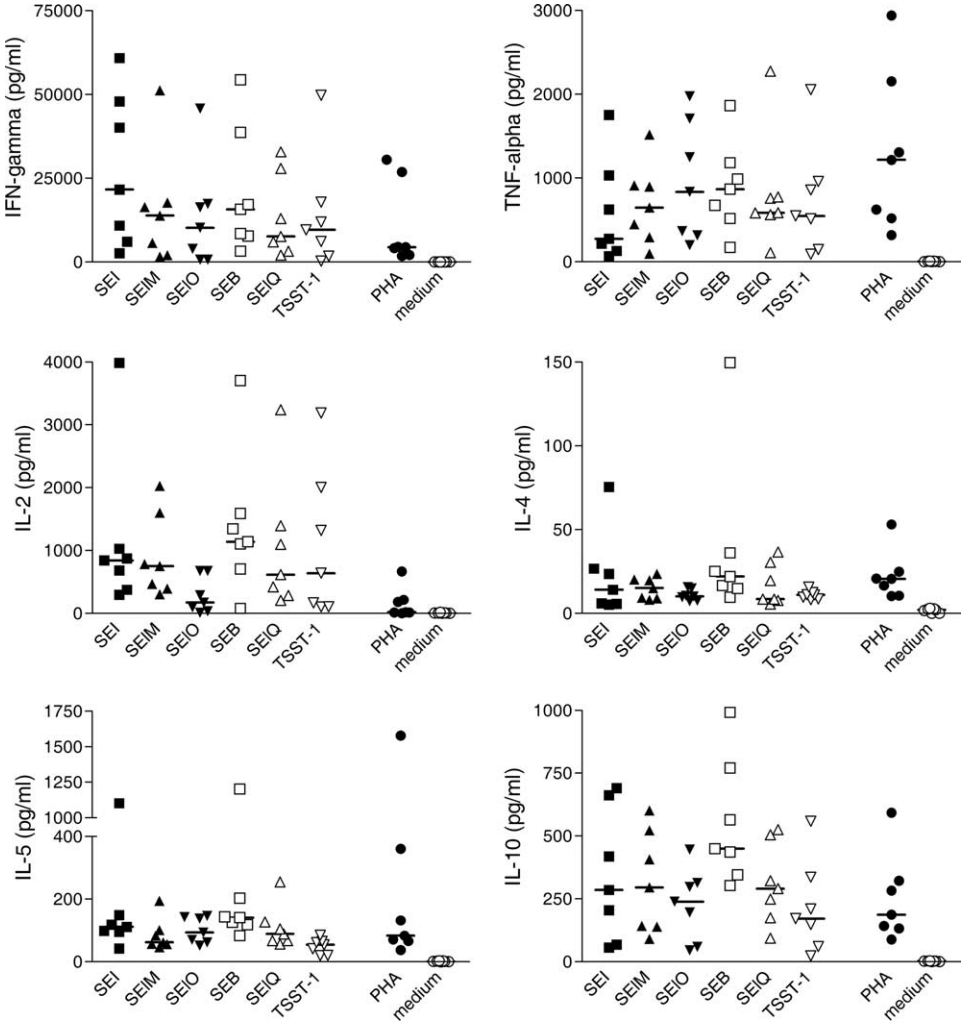


FIGURE 2. Similar cytokine profiles induced by *egc* and non-*egc* SAgs. We compared the release of pro- and anti-inflammatory cytokines by human PBMC after 72 h of stimulation with recombinant *egc* (SEI, SEIM and SEIO; filled symbols) and non-*egc* SAgs (SEB, SEIQ and TSST-1; empty symbols). The T cell mitogen PHA (filled circles) and unstimulated cells (medium; empty circles) served as control. The median values are indicated.

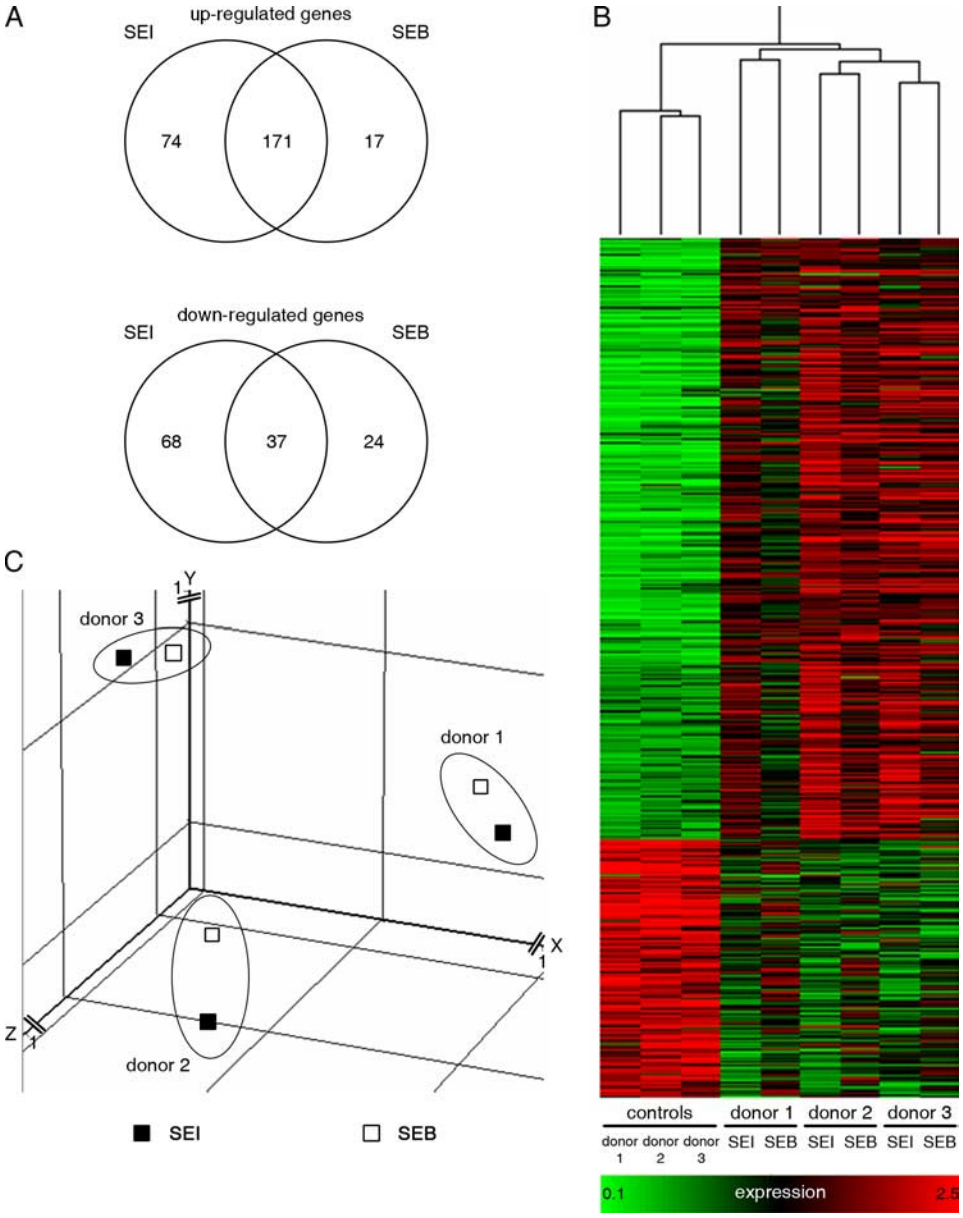


FIGURE 3. Similar gene expression profiles induced by SEI and SEB. Gene expression of human PBMC was analyzed after 6 h of stimulation with SEI and SEB. Probe sets were filtered for an FDR < 0.05 by rank test. *A*, Venn graphs of the up-regulated (*upper panel*) and down-regulated (*lower panel*) genes. Probe sets representing the same gene were combined (see supplemental material Tables I and II). There was a high overlap of genes regulated by SEI and SEB. *B*, Hierarchical clustering (condition tree) of the 511 probe sets with an FDR < 0.05. Expression is shown as deviation from the mean expression value of the probe set over all samples. Samples are grouped based on the similarity of their expression data. *C*, PCA based on the expression values of the 511 probe sets with an FDR < 0.05, normalized to the corresponding control sample (The variances of the axes are: *x*-axis 41.7%, *y*-axis 24.9%, and *z*-axis 20.4%). Interindividual differences outweighed the differences between the stimulating SAgs (*B* and *C*).

Results

Comparison of T cell-mitogenic properties of egc *and non-*egc *SAgs*

To compare the T cell-mitogenic activity of *egc* and non-*egc* SAgs, we stimulated human PBMC from nine healthy blood donors with three *egc* SAgs (SEI, SElM, and SElO) and three non-*egc* SAgs (SEB, SElQ, and TSST-1) and measured their proliferation. All six SAgs induced very strong T cell proliferation in a dose-dependent manner with some interindividual variations in the maximal response to single SAgs (Fig. 1). This likely reflects heterogeneity in the TCR Vβ family composition of the T cell pools from our healthy probands as well as MHC gene polymorphisms, which may affect SAg binding. With the exception of SElO, all SAgs induced strong proliferation already in the pg/ml concentration range. We then determined the SAg concentrations inducing half-maximal proliferation to be ~7 pg/ml for SEB, 1.5 pg/ml for SElQ, 1 pg/ml for TSST-1, 0.5 pg/ml for SEI, 20 pg/ml for SElM, and 2200 pg/ml for SElO (data not shown). Hence, except for SElO ($p = 0.00009$), we found no significant differences between the T cell-mitogenic potencies of *egc* and non-*egc* SAgs.

Comparison of cytokine profiles induced by egc *and non-*egc *SAgs*

Non-*egc* SAgs are notorious for their ability to induce a massive cytokine response in T cells and APCs. Therefore, we next investigated *egc* SAgs for their ability to trigger the release of pro- and anti-inflammatory cytokines by PBMC and compared this with the effects of non-*egc* SAgs. Human PBMC from seven different blood donors were stimulated for 72 h with recombinant SAgs and the cytokine concentrations in the supernatants were determined. We used the SAgs at concentrations just above those which elicited maximal proliferation (plateau concentrations) (Fig. 1). All SAgs induced the secretion of large amounts of pro-inflammatory (IFN-γ, TNF-α, IL-2) and anti-inflammatory (IL-4, IL-5, IL-10) cytokines (Fig. 2). There was considerable interindividual variation in strength of the cytokine response: Some individuals released high amounts of all measured cytokines, while in others the concentrations were uniformly low. Notably, individual recombinant SAgs were as potent as the mitogen PHA.

The dominating cytokine was IFN-γ, the lead cytokine of Th1 cells, with median concentrations between 7570 (SElQ) and 21610

Table II. *Genes regulated >10-fold by SAg treatment*

Gene Symbol[a]	Encoded Protein	NCBI Accession Number	SEI Fold Change[b]	SEB Fold Change[b]
IL2	IL-2	NM_000586	318.4	166.1
CXCL9	CXCL9, Mig	NM_002416	196.5	159.8
UBD	Ubiquitin D	NM_006398	175.7	122.1
IFNG	IFN-γ	M29383	165.8	127.2
CXCL11[c]	CXCL11, I-TAC	AF030514	112.7	90.2
IL22[c]	IL-22	AF279437	109.3	65.0
ANKRD22[c]	Ankyrin repeat domain 22	AI925518	106.2	89.3
IL17A	IL-17A	Z58820	98.5	57.0
IL31RA[c]	IL-31 receptor α-chain	AI123586	74.3	46.7
FAM26F[c]	Family with sequence similarity 26, member F	AV734646	50.6	37.5
CXCL10	CXCL10, IP-10	NM_001565	45.6	47.3
IL3	IL-3	NM_000588	45.6	22.4
SLAMF8[c]	SLAM family member 8	NM_020125	38.9	33.5
LOC729936	Similar to guanylate binding protein 3	BC013288	35.6	38.7
XCL1/XCL2	XCL1, lymphtactin/XCL2, SCM-1β	NM_003175	26.4	17.1
SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	NM_000062	26.0	21.7
SUCNR1	Succinate receptor 1	AF348078	25.2	13.0
IL27	IL-27	NM_145659	24.9	15.4
XCL2	XCL2, SCM-1β	U23772	23.8	16.3
APOL4	Apolipoprotein L, 4	AF305226	22.2	19.2
FCGR1B	FcγRI (CD64), β-chain	L03419	18.6	17.8
FCGR1A	FcγRI (CD64), α-chain	X14355	16.9	15.7
SECTM1	Secreted and transmembrane 1	BF939675	16.8	13.1
CCL8	CCL8, MCP-2	AI984980	15.7	14.5
IL17F	IL-17F	AL034343	15.0	10.9
BATF2	Basic leucine zipper transcription factor, ATF-like 2	AW083820	14.7	15.2
GBP5[c]	Guanylate binding protein 5	BG545653	14.4	16.5
ETV7[c]	Ets variant gene 7 (TEL2 oncogene)	AF218365	14.3	11.9

[a] Genes upregulated >10-fold by SEI and SEB (FDR values ≤ 0.0001). No gene was 10-fold down-regulated.
[b] Mean fold change of three blood donors.
[c] Gene represented by more than one probe set; the probe set with the highest fold change is displayed.

pg/ml (SEI). High levels of TNF-α and IL-2 were also found. Besides these proinflammatory cytokines, SAgs also induced secretion of the Th2-cytokines IL-5 (91.5pg/ml) and IL-4 (11.8pg/ml), though at much lower concentrations. In addition, large amounts of the immunosuppressive cytokine IL-10 were detected, which ranged from median values of 170 (TSST-1) to 450 pg/ml (SEB). As shown in Fig. 2, we observed no significant differences between the cytokine profiles triggered by *egc* and non-*egc* SAgs.

Effects of the SAgs SEI and SEB on PBMC gene expression

For a comprehensive view of the SAg response, we next selected the *egc* SAg SEI and the non-*egc* SAg SEB and used them to stimulate PBMC from three different donors. After 6 h of stimulation, the cells were harvested and their transcription profiles analyzed with Affymetrix expression arrays. The gene expression data showed that 391 genes (511 probe sets, FDR < 0.05) were influenced by stimulation of PMBC with SEI and/or SEB. As shown in Fig. 3*A*, two-thirds of these genes (262 genes) were upregulated and one-third was down-regulated (129 genes). The vast majority of genes was influenced to a similar extent by SEI and SEB (supplemental Tables I and II).[4] In general, the stimulation with SEI was slightly stronger than with SEB, so that more of the observed changes in gene expression reached significance (350 genes vs 249 genes). This fits to the observation that 91% (171 of 188 genes) of all SEB-induced genes were also significantly induced by SEI, while only 17 genes were significantly up-regulated only by SEB (Fig. 3*A*, *upper panel*). Repression of gene expression was generally less impressive, but still more than half of the genes influenced by SEB (61%, 37/61 genes) were also significantly affected by SEI. Notably, no gene was regulated in opposite directions by SEI and SEB (data not shown). The complete list of regulated genes is documented in supplemental Tables I and II.

We then conducted a hierarchical clustering analysis of all probe sets significantly affected by SEI and/or SEB to group samples based on the similarity of their expression data. Fig. 3*B* illustrates the results of this analysis as a condition tree. As expected, unstimulated and SAg-stimulated samples clustered separately. Strikingly, the SAg-exposed samples of the three healthy blood donors clustered separately, but not the two SAgs used for stimulation as one would have expected, if SEI and SEB had different effects on PBMC. Thus, the condition tree clearly shows that interindividual differences between the blood donors outweighed the differences between the stimulating SAgs.

In an independent approach, the data were submitted to a PCA. This PCA was based on the ratios between the probe set signals of stimulated and non-stimulated PBMC, which had been calculated separately for each donor and each SAg. PCA converges the majority of statistical variables of complex data sets to three informative dimensions (principal components), which are visualized in Fig. 3*C*. Similar to the hierarchical clustering, the PCA grouped our samples into three pairs, which comprised the SEI- and SEB-stimulated samples of each donor. Therefore, both analysis tools show that the effects of SEI and SEB on PBMC gene expression were very similar, so that differences between them had less impact than the interindividual variation between the blood donors.

Table II displays those genes, whose transcription was induced >10-fold by SAg treatment. No gene was repressed by a factor of 10 or more. Among the most strongly induced genes were those encoding for IL-2 (SEI: 318.4fold; SEB: 166.1-fold), IFN-γ (SEI: 165.8-fold; SEB: 127.2-fold) and IL-17A (SEI: 98.5-fold; SEB:

[4] The online version of this article contains supplemental material.

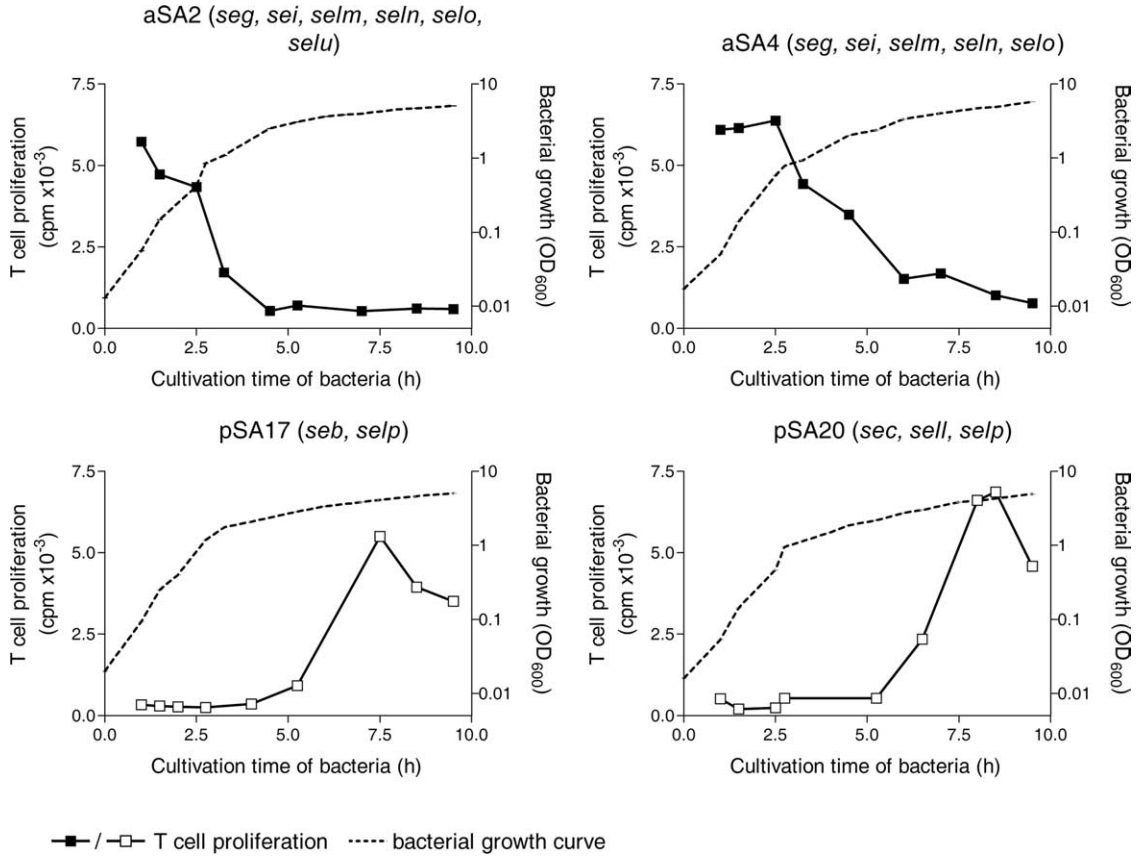


FIGURE 4. Bacterial growth phase-dependent secretion of *egc* and non-*egc* SAgs. Two *S. aureus* strains with *egc* SAg genes (aSA2, aSA4; *upper panel*) and two strains with non-*egc* SAg genes (pSA17, pSA20; *lower panel*) were cultured in Luria broth medium. Cultures were sampled at OD_{600} of 0.05, 0.15, 0.4, 1.0, 2.0, 3.0, 4.0, 5.0, and 5.5. Culture supernatants were normalized to OD_{600} 0.05 by dilution in cell culture medium. Their T cell-mitogenic potency was assessed by incubating human PBMC with 1000-fold dilutions. T cell proliferation was measured after 72 h by [^{3}H]thymidine incorporation. One representative data set of three is depicted.

57.0-fold), indicative of a very strong Th1- and Th17-response. But also the cytokines IL-22, IL-3, and IL-27, the chemokines CXCL9, CXCL10, CXCL11, XCL2, and CCL8 as well as the T cell activation marker CD64 were strongly induced. These findings are in good agreement with the results of our T cell proliferation and cytokine measurements (Figs. 1 and 2). The T cell activation marker genes CD69, CD40L, and CD25; the cell cycle genes cyclin D2 and cyclin-dependent kinase 6 as well as the transcripts for the cytokines IL-4, IL-5, and TNF-α were also significantly induced by both SEI and SEB but $<$10-fold (see supplementary Table I). The only discrepancy concerns IL-10, where protein secretion was detected by cytokine measurements but no induction of gene transcription by SEI or SEB was observed. However, IL-10 is typically produced in the later phase of the immune response, while in this study the gene expression patterns were assessed already after 6 h of stimulation.

Growth phase-dependent secretion of egc *and non-*egc *SAgs*

In search of an explanation for the lack of neutralizing Abs against *egc* SAgs even in carriers of *egc*-positive *S. aureus* strains, we finally focused on the regulation of the SAg release by *S. aureus*. It has been reported that the *egc*-operon is transcribed in the exponential but not in the stationary growth phase, as it is typical for non-*egc* SAgs (6, 23). We cultured two clinical *S. aureus* strains exclusively with *egc* (aSA2: *seg, sei, selm, seln, selo, selu*; aSA4: *seg, sei, selm, seln, selo*) and two strains with only non-*egc* SAgs genes (pSA17: *seb*, *selp*; pSA20: *sec*, *sell*, *selp*) and sampled supernatants at optical densities (OD_{600}) between 0.05 and 5.5. To correct for the higher cell densities and resulting higher concentrations of secreted products at the late growth phase, all samples were adjusted to OD_{600} 0.05 by dilution in cell culture medium (Fig. 4). These normalized bacterial supernatants from different growth phases of the *S. aureus* isolates were then used at 1/1000 dilutions to assess their T cell-mitogenic potency in a proliferation assay. Supernatants from *S. aureus* strains pSA17 and pSA20 showed mitogenic activity only at high OD_{600} ($>OD_{600}$ 3.0), indicating that these non-*egc* SAg proteins are secreted primarily in the late stationary growth phase of *S. aureus*. In contrast, *egc* SAgs were released during exponential growth but secretion stopped at higher bacterial densities. The SAgs were not degraded (data not shown), but on a per cell basis their concentration decreased to submitogenic levels with increasing bacterial densities.

Discussion

Egc SAgs are by far the most prevalent staphylococcal SAgs (18, 24). Despite this, they are not a prominent cause of toxic shock syndrome (4, 11, 25) but their presence appears to be associated mainly with symptom-free carriage and inversely correlated with the severity of *S. aureus* infection (12, 26). Astonishingly, neutralizing anti-*egc* serum Abs are very uncommon in healthy individuals (18). In search of an explanation for these counterintuitive observations, we proposed two hypotheses: 1) *egc* and non-*egc* SAgs have unique intrinsic properties and drive the immune system into different directions and 2) *egc* and non-*egc* SAgs are released by *S. aureus* under different conditions, which shape the immune response to them. Our results lend support only to the latter.

Our purified and rigorously LPS-depleted recombinant *egc* (SEI and SElM) and non-*egc* SAgs (TSST-1, SEB, SElQ) were of similarly high mitogenic potency, inducing half-maximal proliferation at concentrations between ~0.5 and 20 pg/ml. Only the *egc* SAg SEIO was significantly less potent (~2200 pg/ml). These findings corroborate and extend earlier studies where mitogenic concentrations for non-*egc* staphylococcal SAgs were determined to be in the pg/ml or even fg/ml range (27–29). To date, the mitogenic potency of the other *egc* SAgs, SEG, SElN, and SElU, has not been determined in a human T cell proliferation assay. The group of Munson et al. (23) reported that concentrations of 1.84 nM SEG (~40 ng/ml) were necessary to induce maximal proliferation in murine splenocytes, but murine cells are less susceptible to SAg stimulation than human PBMC by several orders of magnitude (25, 30).

If intrinsic properties of *egc* and non-*egc* SAgs were responsible for the different response of the immune system, one might expect them to elicit contrasting cytokine profiles. However, we observed no systematic differences between *egc* and non-*egc* SAgs. Both groups induced the release of high amounts of pro-inflammatory (IFN-γ, TNF-α, IL-2) and lower concentrations of anti-inflammatory (IL-4, IL-5, IL-10) cytokines. IFN-γ was the lead cytokine in our panel, reaching average concentrations of ~17 ng/ml after 72 h. Others have also reported induction of Th1- and Th2-cytokines after stimulation with a number of non-*egc* staphylococcal and streptococcal SAgs (31–33). In contrast, Dauwalder and coworkers (14) recently reported that stimulation of human PBMC with very high concentrations (100 ng/ml) of SEA and the *egc* SAg SEG did not elicit anti-inflammatory cytokine (IL-10) and chemokine (TARC) secretion. Moreover, only SEA but not SEG induced the release of TNF-α and the Th1-chemokine MIP-1α in their system. This suggests that SEG might have unique properties among the *egc* SAgs, but differences in the SAg concentrations and/or SAg production and purification procedures should also be considered as an explanation (34).

These differences prompted us to aim for a comprehensive picture of the response of blood cells to SAg stimulation. We used transcriptional profiling to compare one *egc* SAg (SEI) with one non-*egc* SAg (SEB). Stimulation with SEI and/or SEB changed the transcription of 391 genes, two thirds of which were up-regulated. This is in agreement with Mendis and coworkers (35), who also treated PBMC with SEB. Stimulation with SEI was slightly stronger than with SEB, probably because this SAg activates a larger fraction of T cells. But the hierarchy of induced genes was very similar, and not a single gene was regulated in opposite directions by the two SAgs. In fact, there were interindividual variations of the blood donors in their SAg response, as has also been reported by others (32, 33), and these outweighed the differences between the transcription profiles induced by SEI and SEB. Among the most strongly induced genes were proinflammatory mediators of Th1- as well as cytotoxic T cell-responses: IL-2 and IFN-γ were up-regulated more than 100-fold, supporting our observations on the protein level. Fitting the same response profile, CD40L, lymphotoxin-α, granzymes A and B, TNF-α, IL-12 (p35) and IL-27 were also highly induced. Additionally, we noticed highly increased transcription levels of IL-17A and IL-17F that indicate activation of Th17 cells. We interpret these findings to represent an extremely strong proinflammatory in vitro reaction to both SAgs.

However, in agreement with our cytokine measurements, the Th2-cytokine genes IL-4 and IL-5 were also induced. As expected, the T cell activation marker genes (CD69, CD40L, and CD25) and cell cycle genes (cyclin D2 and cyclin-dependent kinase 6) were up-regulated, and the transcription of chemokines guiding the migration of activated T cells, CXCL9, CXCL11, CXCL10, and CCL8 was also increased (36). In addition, we found enhanced transcription levels of the apoptosis-related genes *trail, fas, casp10, casp7*, which is not surprising, because very strong T cell stimulation may drive activation-induced cell death, and the elimination of SAg-reactive T cell subpopulations is considered to be a hallmark of SAg action (37). In summary, the *egc* SAg SEI and the non-*egc* SAg SEB induced very similar gene expression patterns corresponding to an extremely strong activation of T cells and APCs such as one might expect from SAgs.

Taken together, the immune cell-activating properties of *egc* and non-*egc* SAgs, their superantigenicity, proved very similar in every aspect studied and cannot explain the striking differences in the immune response to *egc* and non-*egc* SAgs, with a high prevalence of neutralizing Abs specific for non-*egc* but not for *egc* SAgs (15–18).

Because the amino acid sequences of the *egc* SAgs are more closely related to those of individual non-*egc* SAgs than to each other (6, 38), it also appears unlikely, that the two groups of SAgs differ systematically in their immunogenicity.

We found only one important aspect, in which *egc* and non-*egc* SAgs were fundamentally different: *S. aureus* strains harboring *egc* SAg genes secreted these toxins during early exponential growth, while strains with non-*egc* SAgs released them mainly in the late stationary phase. Similar observations have been reported on the RNA-level: The polycistronic *egc*-mRNA accumulated maximally during exponential growth (Refs. 6, 23, and unpublished data), while most non-*egc* SAg genes were transcribed in the postexponential growth phase.

Considering the extraordinary stability of SAg proteins, why did the *egc*-related T cell proliferation drop away so rapidly with increasing bacterial density? Presumably transcription of the *egc*-operon was shut down in the late exponential growth phase, so that the concentration of the *egc* SAgs did not further increase with increasing bacterial cell numbers in culture. This would lead to a rapid decline in mitogenic potency of bacterial supernatants on a per cell basis. We have no evidence for proteolytic degradation of the *egc* SAgs, not very surprisingly, because SAg proteins have been shown to be exquisitely stable.

Why the *egc*-operon is selectively transcribed during early exponential growth, is not understood. The regulation of staphylococcal SAgs is complex, and to date, besides the accessory gene regulator, the staphylococcal accessory regulator, the alternative σ factor, and the regulator of toxins also have been shown to play a role (39–43). However, this information has been obtained by investigation of *S. aureus* gene regulation in bacterial cell culture. The challenge will now be to elucidate, which of these processes are effective during the interactions of the microorganism with its host. There is first evidence that the regulatory circuits *S. aureus* employs during infection (and probably also colonization) differ from those characterized in vitro (44, 45). It remains to be seen, how this affects the release of *egc* and non-*egc* SAgs in vivo and the immune response against them.

At this stage, the high prevalence of neutralizing serum Abs against non-*egc* SAgs (18) allows the conclusion that most healthy adults have been exposed to these toxins during their encounters with *S. aureus*. For *egc* SAgs, it remains an open question whether they are 1) expressed in vivo, 2) in which quantities, and 3) under which conditions. In vitro data suggest that *egc* SAgs are generally produced in very small amounts (46). Because *S. aureus* secrets *egc* and non-*egc* SAgs in distinct functional states, they are likely released during different phases of its interaction with the human host. Colonization may not be the same as invasion. Consequently it is possible that the cell populations, which are exposed to *egc*- vs non-*egc* SAgs, differ, and that only in the case of non-*egc* SAgs they are able to orchestrate an efficient adaptive immune response.

In summary, *egc* and non-*egc* SAgs were very similar in all studied aspects of immune cell activation: gene regulation, cytokine secretion and induction of T cell proliferation, but their release from the bacteria was regulated in a fundamentally different manner.

Acknowledgments

We are grateful to Anne-Kathrin Ziebandt and Annette Dreisbach for their help with some of the experiments, and to Robert S. Jack for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Proft, T., and J. D. Fraser. 2003. Bacterial superantigens. *Clin. Exp. Immunol.* 133: 299–306.
2. Lina, G., G. A. Bohach, S. P. Nair, K. Hiramatsu, E. Jouvin-Marche, and R. Mariuzza. 2004. Standard nomenclature for the superantigens expressed by *Staphylococcus*. *J. Infect. Dis.* 189: 2334–2336.
3. Holtfreter, S., and B. M. Broker. 2005. Staphylococcal superantigens: do they play a role in sepsis? *Arch. Immunol. Ther. Exp. (Warsz)* 53: 13–27.
4. Schlievert, P. M., and G. A. Bohach. 2007. Staphylococcal and streptococcal superantigens: an update. In *Superantigens: Molecular Basis for Their Role in Human Diseases.* M. Kotb, and J. Fraser, eds. ASM Press, Washington, DC.
5. McCormick, J. K., J. M. Yarwood, and P. M. Schlievert. 2001. Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol.* 55: 77–104.
6. Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J. Immunol.* 166: 669–677.
7. Letertre, C., S. Perelle, F. Dilasser, and P. Fach. 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J. Appl. Microbiol.* 95: 38–43.
8. Becker, K., A. W. Friedrich, G. Peters, and C. von Eiff. 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO, and SElN. *Mol. Nutr. Food Res.* 48: 488–495.
9. Fueyo, J. M., M. C. Mendoza, M. A. Alvarez, and M. C. Martin. 2005. Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. *FEMS Microb. Lett.* 243: 447–454.
10. Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, et al. 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* 45: 2669–2680.
11. Jarraud, S., G. Cozon, F. Vandenesch, M. Bes, J. Etienne, and G. Lina. 1999. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J. Clin. Microbiol.* 37: 2446–2449.
12. Ferry, T., D. Thomas, A. L. Genestier, M. Bes, G. Lina, F. Vandenesch, and J. Etienne. 2005. Comparative prevalence of superantigen genes in *Staphylococcus aureus* isolates causing sepsis with and without septic shock. *Clin. Infect. Dis.* 41: 771–777.
13. van Belkum, A. 2006. Staphylococcal colonization and infection: homeostasis versus disbalance of human (innate) immunity and bacterial virulence. *Curr. Opin. Infect. Dis.* 19: 339–344.
14. Dauwalder, O., D. Thomas, T. Ferry, A. L. Debard, C. Badiou, F. Vandenesch, J. Etienne, G. Lina, and G. Monneret. 2006. Comparative inflammatory properties of staphylococcal superantigenic enterotoxins SEA and SEG: implications for septic shock. *J. Leukocyte Biol.* 80: 753–758
15. Schroder, E., G. Kunstmann, H. Hasbach, and G. Pulverer. 1988. Prevalence of serum antibodies to toxic-shock-syndrom-toxin-1 and to staphylococcal enterotoxins A, B and C in West Germany. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* 270: 110–114.
16. Kunstmann, G., E. Schroder, H. Hasbach, and G. Pulverer. 1989. Immune response to toxic-shock-syndrome toxin-1 (TSST-1) and to staphylococcal enterotoxins A, B and C in *Staphylococcus aureus* infections. *Zentralbl. Bakteriol.* 271: 486–492.
17. Takei, S., Y. K. Arora, and S. M. Walker. 1993. Intravenous immunoglobulin contains specific antibodies inhibitory to activation of T cells by staphylococcal toxin superantigens (see comment). *J. Clin. Invest.* 91: 602–607.
18. Holtfreter, S., K. Bauer, D. Thomas, C. Feig, V. Lorenz, K. Roschack, E. Friebe, K. Selleng, S. Lovenich, T. Greve, et al. 2004. *egc*-Encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. *Infect. Immun.* 72: 4061–4071.
19. Holtfreter, S., K. Roschack, P. Eichler, K. Eske, B. Holtfreter, C. Kohler, S. Engelmann, M. Hecker, A. Greinacher, and B. M. Broker. 2006. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. *J. Infect. Dis.* 193: 1275–1278.
20. Breitling, R., P. Armengaud, A. Amtmann, and P. Herzyk. 2004. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett.* 573: 83–92.
21. Jeffery, I. B., D. G. Higgins, and A. C. Culhane. 2006. Comparison and evaluation of methods for generating differentially expressed gene lists from microarray data. *BMC Bioinform.* 7: 359.
22. Storey, J. D. 2002. A direct approach to false discovery rates. *J. R. Statist. Soc. B* 64: 479–498.
23. Munson, S. H., M. T. Tremaine, M. J. Betley, and R. A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infect. Immun.* 66: 3337–3348.
24. Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. Von Eiff. 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J. Clin. Microbiol.* 41: 1434–1439.
25. Medina, E. 2007. Novel experimental models for dissecting genetic susceptibility of superantigen-mediated diseases. In *Superantigens: Molecular Basis for Their Role in Human Diseases.* M. Kotb, and J. Fraser, eds. ASM Press, Washington, DC. 183–194.
26. van Belkum, A., D. C. Melles, S. V. Snijders, W. B. van Leeuwen, H. F. Wertheim, J. L. Nouwen, H. A. Verbrugh, and J. Etienne. 2006. Clonal distribution and differential occurrence of the enterotoxin gene cluster, *egc*, in carriage- versus bacteremia-associated isolates of *Staphylococcus aureus*. *J. Clin. Microbiol.* 44: 1555–1557.
27. Orwin, P. M., D. Y. Leung, H. L. Donahue, R. P. Novick, and P. M. Schlievert. 2001. Biochemical and biological properties of staphylococcal enterotoxin K. *Infect. Immun.* 69: 360–366.
28. Orwin, P. M., D. Y. Leung, T. J. Tripp, G. A. Bohach, C. A. Earhart, D. H. Ohlendorf, and P. M. Schlievert. 2002. Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* 41: 14033–14040.
29. Orwin, P. M., J. R. Fitzgerald, D. Y. Leung, J. A. Gutierrez, G. A. Bohach, and P. M. Schlievert. 2003. Characterization of *Staphylococcus aureus* enterotoxin L. *Infect. Immun.* 71: 2916–2919.
30. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med.* 175: 91–98.
31. Rink, L., J. Luhm, M. Koester, and H. Kirchner. 1996. Induction of a cytokine network by superantigens with parallel TH1 and TH2 stimulation. *J. Interferon. Cytokine Res.* 16: 41–47.
32. Norrby-Teglund, A., S. Chatellier, D. E. Low, A. McGeer, K. Green, and M. Kotb. 2000. Host variation in cytokine responses to superantigens determine the severity of invasive group A streptococcal infection. *Eur. J. Immunol.* 30: 3247–3255.
33. Proft, T., B. Schrage, and J. D. Fraser. 2007. The cytokine response to streptococcal superantigens varies between individual toxins and between individuals: implications for the pathogenesis of group A streptococcal diseases. *J. Interferon. Cytokine Res.* 27: 553–557.
34. Brandt, K., J. van der Bosch, R. Fliegert, and S. Gehring. 2002. TSST-1 induces Th1 or Th2 differentiation in naive $CD4^+$ T cells in a dose- and APC-dependent manner. *Scand. J. Immunol.* 56: 572–579.
35. Mendis, C., R. Das, R. Hammamieh, A. Royaee, D. Yang, S. Peel, and M. Jett. 2005. Transcriptional response signature of human lymphoid cells to staphylococcal enterotoxin B. *Genes Immun.* 6: 84–94.
36. Moser, B., and P. Loetscher. 2001. Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2: 123–128.
37. Miethke, T., C. Wahl, K. Heeg, and H. Wagner. 1993. Acquired resistance to superantigen-induced T cell shock: V β selective T cell unresponsiveness unfolds directly from a transient state of hyperreactivity. *J. Immunol.* 150: 3776–3784.
38. Thomas, D., S. Chou, O. Dauwalder, and G. Lina. 2007. Diversity in *Staphylococcus aureus* enterotoxins. *Chem. Immunol. Allergy* 93: 24–41.
39. Novick, R. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48: 1429–1449.
40. Bischoff, M., P. Dunman, J. Kormanec, D. Macapagal, E. Murphy, W. Mounts, B. Berger-Bachi, and S. Projan. 2004. Microarray-based analysis of the *Staphylococcus aureus* σB regulon. *J. Bacteriol.* 186: 4085–4099.
41. Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan. 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J. Bacteriol.* 183: 7341–7353.
42. Said-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan, and B. N. Kreiswirth. 2003. Global regulation of *Staphylococcus aureus* genes by Rot. *J. Bacteriol.* 185: 610–619.
43. Ziebandt, A. K., D. Becher, K. Ohlsen, J. Hacker, M. Hecker, and S. Engelmann. 2004. The influence of *agr* and σ^B in growth phase dependent regulation of virulence factors in *Staphylococcus aureus*. *Proteomics* 4: 3034–3047.
44. Goerke, C., S. Campana, M. G. Bayer, G. Doring, K. Botzenhart, and C. Wolz. 2000. Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. *Infect. Immun.* 68: 1304–1311.
45. Goerke, C., and C. Wolz. 2004. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. *Int. J. Med. Microbiol.* 294: 195–202.
46. Omoe, K., M. Ishikawa, Y. Shimoda, D. L. Hu, S. Ueda, and K. Shinagawa. 2002. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates Harboring seg, seh, or sei genes. *J. Clin. Microbiol.* 40: 857–862.

SUPPLEMENTAL MATERIAL

Supplemental Table I: Genes upregulated after stimulation with SEI and SEB.

				SEI		SEB	
Gene Symbol[a]	**Gene Title**	**NCBI Accession No**	**probe set ID**	**mean fold change**[b]	**FDR**	**mean fold change**[b]	**FDR**
IL2	interleukin 2	NM_000586	207849_at	318.4	< 0.0001	166.1	< 0.0001
CXCL9	chemokine (C-X-C motif) ligand 9	NM_002416	203915_at	196.5	< 0.0001	159.8	< 0.0001
UBD	ubiquitin D	NM_006398	205890_s_at	175.7	< 0.0001	122.1	< 0.0001
IFNG	interferon, gamma	M29383	210354_at	165.8	< 0.0001	127.2	< 0.0001
CXCL11[c]	chemokine (C-X-C motif) ligand 11	AF030514	210163_at	112.7	< 0.0001	90.2	< 0.0001
IL22[c]	interleukin 22	AF279437	222974_at	109.3	< 0.0001	65.0	< 0.0001
ANKRD22[c]	ankyrin repeat domain 22	AI925518	238439_at	106.2	< 0.0001	89.3	< 0.0001
IL17A	interleukin 17A	Z58820	216876_s_at	98.5	< 0.0001	57.0	< 0.0001
IL31RA[c]	interleukin 31 receptor A	AI123586	243541_at	74.3	< 0.0001	46.7	< 0.0001
FAM26F[c]	family with sequence similarity 26, member F	AV734646	229390_at	50.6	< 0.0001	37.5	< 0.0001
CXCL10	chemokine (C-X-C motif) ligand 10	NM_001565	204533_at	45.6	< 0.0001	47.3	< 0.0001
IL3	interleukin 3 (colony-stimulating factor, multiple)	NM_000588	207906_at	45.6	< 0.0001	22.4	< 0.0001
SLAMF8[c]	SLAM family member 8	NM_020125	219386_s_at	38.9	< 0.0001	33.5	< 0.0001
LOC729936	Similar to guanylate binding protein 3	BC013288	1570541_s_at	35.6	< 0.0001	38.7	< 0.0001
XCL1 /// XCL2	chemokine (C motif) ligand 1 /// chemokine (C motif) ligand 2	NM_003175	214567_s_at	26.4	< 0.0001	17.1	< 0.0001
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	NM_000062	200986_at	26.0	< 0.0001	21.7	< 0.0001
SUCNR1	succinate receptor 1	AF348078	223939_at	25.2	< 0.0001	13.0	< 0.0001
IL27	interleukin 27	NM_145659	1552995_at	24.9	< 0.0001	15.4	< 0.0001
XCL2	chemokine (C motif) ligand 2	U23772	206366_x_at	23.8	< 0.0001	16.3	< 0.0001
APOL4	apolipoprotein L, 4	AF305226	1555600_s_at	22.2	< 0.0001	19.2	< 0.0001
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)	L03419	214511_x_at	18.6	< 0.0001	17.8	< 0.0001
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	X14355	216950_s_at	16.9	< 0.0001	15.7	< 0.0001
SECTM1	secreted and transmembrane 1	BF939675	213716_s_at	16.8	< 0.0001	13.1	< 0.0001
CCL8	chemokine (C-C motif) ligand 8	AI984980	214038_at	15.7	< 0.0001	14.5	< 0.0001
ZBED2	zinc finger, BED-type containing 2	NM_024508	219836_at	15.5	< 0.0001	8.0	0.0006
IL17F	interleukin 17F	AL034343	234408_at	15.0	< 0.0001	10.9	< 0.0001
BATF2	basic leucine zipper transcription factor, ATF-like 2	AW083820	228439_at	14.7	< 0.0001	15.2	< 0.0001
GBP5[c]	guanylate binding protein 5	BG545653	229625_at	14.4	< 0.0001	16.5	< 0.0001
ETV7[c]	ets variant gene 7 (TEL2 oncogene)	AF218365	224225_s_at	14.3	< 0.0001	11.9	< 0.0001
---	Transcribed locus, weakly similar to XP_001117086.1 similar to Olfactory receptor 10T2 (Olfactory receptor OR1-3) [Macaca mulatta]	AI694413	235229_at	14.0	< 0.0001	11.0	0.0001
PEG10[c]	paternally expressed 10	AL582836	212094_at	12.7	0.0002	7.4	0.0004
IL21	interleukin 21	NM_021803	221271_at	11.6	< 0.0001	6.4	0.0005
CD40LG	CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)	NM_000074	207892_at	10.2	0.0002	6.2	0.0005
DACT1	dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)	NM_016651	219179_at	10.1	0.0002	6.8	0.0005
XCL1	chemokine (C motif) ligand 1	NM_002995	206365_at	10.1	0.0004	6.9	0.0006
GBP1[c]	guanylate binding protein 1, interferon-inducible, 67kDa	AW014593	231578_at	9.2	0.0003	10.0	< 0.0001
APOL6[c]	apolipoprotein L, 6	AW026509	241869_at	9.0	0.0010	8.2	0.0014
LTA	lymphotoxin alpha (TNF superfamily, member 1)	NM_000595	206975_at	8.9	0.0004	7.7	0.0005
CISH[c]	cytokine inducible SH2-containing protein	D83532	223961_s_at	8.4	0.0005	7.8	0.0004
IL4[c]	interleukin 4	NM_000589	207538_at	8.2	0.0003	4.6	0.0018
NDFIP2[c]	Nedd4 family interacting protein 2	AW290956	224799_at	7.8	0.0005	3.2	0.0162
PDCD1LG2[c]	programmed cell death 1 ligand 2	AF329193	224399_at	7.7	0.0006	5.3	0.0015

KCTD12[c]	potassium channel tetramerisation domain containing 12	AA551075	212188_at	7.6	0.0004	6.8	0.0004
LOC730249	similar to Immune-responsive protein 1	BG236136	240287_at	7.2	0.0006	7.2	0.0004
EGR1[c]	Early growth response 1	AI459194	227404_s_at	7.2	0.0005	6.8	0.0004
MUC1	mucin 1, cell surface associated	AI610869	213693_s_at	7.0	0.0007	5.8	0.0014
GOLM1	golgi membrane protein 1	NM_016548	217771_at	6.7	0.0009	4.7	0.0016
---	CDNA: FLJ20968 fis, clone ADSU00702	AK024621	1561738_at	6.6	0.0008	9.6	0.0001
ENPP3[c]	Ectonucleotide pyrophosphatase/phosphodiesterase 3	AV691872	244044_at	6.6	0.0013	5.0	0.0017
GBP4[c]	guanylate binding protein 4	AW392952	235574_at	6.6	0.0007	5.1	0.0010
CEACAM1[c]	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	X16354	209498_at	6.6	0.0006	5.5	0.0009
CD200R1	CD200 receptor 1	NM_138939	1552875_a_at	6.4	0.0007	3.7	0.0050
PTGER3[c]	prostaglandin E receptor 3 (subtype EP3)	AW242315	213933_at	6.2	0.0008	4.8	0.0016
JAK2[c]	Janus kinase 2 (a protein tyrosine kinase)	BC043187	1562031_at	6.1	0.0013	6.9	0.0005
WARS[c]	tryptophanyl-tRNA synthetase	M61715	200628_s_at	6.0	0.0010	4.9	0.0014
SLAMF1[c]	signaling lymphocytic activation molecule family member 1	NM_003037	206181_at	6.0	0.0013	3.2	0.0128
CD200[c]	CD200 molecule	AF063591	209583_s_at	5.9	0.0012	2.7	0.0461
VAMP5	vesicle-associated membrane protein 5 (myobrevin)	NM_006634	204929_s_at	5.9	0.0013	4.6	0.0018
HAPLN3	hyaluronan and proteoglycan link protein 3	BE348293	227262_at	5.9	0.0013	4.4	0.0025
APOL3	apolipoprotein L, 3	NM_014349	221087_s_at	5.8	0.0013	4.7	0.0017
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	M11734	210229_s_at	5.8	0.0012	4.1	0.0036
NR4A1	nuclear receptor subfamily 4, group A, member 1	NM_002135	202340_x_at	5.6	0.0022	5.8	0.0008
PRRG4[c]	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)	NM_024081	207291_at	5.6	0.0018	5.1	0.0015
APOL1	apolipoprotein L, 1	AF323540	209546_s_at	5.6	0.0015	3.7	0.0064
FCGR2B /// FCGR2C	Fc fragment of IgG, low affinity IIb, receptor (CD32) /// Fc fragment of IgG, low affinity IIc, receptor for (CD32)	M31933	210889_s_at	5.4	0.0037	3.8	0.0146
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7	NM_002562	207091_at	5.3	0.0021	4.5	0.0019
LINCR	likely ortholog of mouse lung-inducible Neutralized-related C3HC4 RING domain protein	AL389981	232593_at	5.3	0.0019	4.6	0.0019
CD40[c]	CD40 molecule, TNF receptor superfamily member 5	NM_001250	205153_s_at	5.3	0.0020	3.8	0.0044
CD69	CD69 molecule	L07555	209795_at	5.2	0.0021	4.4	0.0021
FGD2	FYVE, RhoGEF and PH domain containing 2	AK024456	215602_at	5.2	0.0021	3.9	0.0044
TAGAP[c]	T-cell activation GTPase activating protein	NM_138810	1552541_at	5.1	0.0022	3.6	0.0072
---	Full length insert cDNA clone YT87E05	AF085978	1561654_at	5.1	0.0024	4.5	0.0019
CTLA4[c]	cytotoxic T-lymphocyte-associated protein 4	AI733018	236341_at	5.0	0.0025	3.4	0.0082
CRTAM	cytotoxic and regulatory T cell molecule	NM_019604	206914_at	5.0	0.0035	4.4	0.0029
FRMD4B	FERM domain containing 4B	AU145019	213056_at	4.9	0.0030	4.0	0.0036
KREMEN1[c]	kringle containing transmembrane protein 1	BF221745	227250_at	4.9	0.0031	4.8	0.0014
SAMD4A[c]	sterile alpha motif domain containing 4A	AB028976	212845_at	4.9	0.0028	3.3	0.0141
---	---	BE466400	241237_at	4.8	0.0036	5.1	0.0014
---	---	BF439675	237009_at	4.8	0.0031	3.5	0.0077
KLF4[c]	Kruppel-like factor 4 (gut)	NM_004235	220266_s_at	4.7	0.0051	5.2	0.0013
ARNT2	aryl-hydrocarbon receptor nuclear translocator 2	NM_014862	202986_at	4.7	0.0034	4.0	0.0038
BCL2[c]	B-cell CLL/lymphoma 2	M13994	203684_s_at	4.7	0.0035	2.2	0.1593
CMAH[c]	cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminate monooxygenase)	AW205659	229604_at	4.6	0.0046	3.6	0.0064
---	Clone FLB4630	AF113688	1570621_at	4.6	0.0040	2.1	0.1519

IL19	interleukin 19	NM_013371	220745_at	4.6	0.0072	3.0	0.0237
E2F7	E2F transcription factor 7	AI341146	228033_at	4.6	0.0044	3.9	0.0053
---	Full length insert cDNA clone YX74D05	AI655467	230741_at	4.5	0.0046	3.9	0.0039
LAP3	leucine aminopeptidase 3	NM_015907	217933_s_at	4.5	0.0043	3.9	0.0042
LIF	leukemia inhibitory factor (cholinergic differentiation factor)	NM_002309	205266_at	4.5	0.0044	4.6	0.0018
PDSS1	prenyl (decaprenyl) diphosphate synthase, subunit 1	NM_014317	220865_s_at	4.5	0.0045	2.7	0.0409
JAG1[c]	jagged 1 (Alagille syndrome)	U73936	209099_x_at	4.4	0.0077	3.8	0.0061
---	MRNA; cDNA DKFZp313C1812 (from clone DKFZp313C1812)	AL832657	1562307_at	4.4	0.0045	3.3	0.0111
C11orf75	chromosome 11 open reading frame 75	NM_020179	219806_s_at	4.4	0.0046	3.7	0.0066
C1QB	complement component 1, q subcomponent, B chain	NM_000491	202953_at	4.3	0.0049	4.4	0.0020
TRAFD1	TRAF-type zinc finger domain containing 1	NM_006700	202837_at	4.3	0.0055	2.6	0.0416
SOCS2[c]	suppressor of cytokine signaling 2	AB004903	203372_s_at	4.3	0.0051	3.2	0.0121
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	NM_000593	202307_s_at	4.3	0.0048	3.9	0.0040
TNFSF10[c]	tumor necrosis factor (ligand) superfamily, member 10	U57059	202687_s_at	4.3	0.0055	3.5	0.0093
IL15[c]	interleukin 15	Y09908	217371_s_at	4.2	0.0073	3.4	0.0099
IL9	interleukin 9	NM_000590	208193_at	4.2	0.0088	1.7	0.3179
INHBA[c]	inhibin, beta A	M13436	210511_s_at	4.2	0.0071	3.6	0.0066
BCL2L14*	BCL2-like 14 (apoptosis facilitator)	AK000127	233464_at	4.2	0.0066	3.9	0.0044
---	Transcribed locus	R98767	240593_x_at	4.2	0.0066	3.4	0.0129
TIFA	TRAF-interacting protein with a forkhead-associated domain	AA195074	226117_at	4.2	0.0063	3.7	0.0056
FAM92A1[c]	family with sequence similarity 92, member A1	AW960748	235391_at	4.2	0.0072	4.2	0.0026
---	---	AW392551	238725_at	4.2	0.0068	4.2	0.0030
TNFRSF21[c]	tumor necrosis factor receptor superfamily, member 21	BE568134	214581_x_at	4.1	0.0064	2.7	0.0365
SOCS1[c]	suppressor of cytokine signaling 1	AI056051	209999_x_at	4.1	0.0083	3.1	0.0177
IRF8	interferon regulatory factor 8	AI073984	204057_at	4.1	0.0071	3.7	0.0051
CDK6[c]	cyclin-dependent kinase 6	AW194766	243000_at	4.1	0.0091	2.6	0.0592
IL5	interleukin 5 (colony-stimulating factor, eosinophil)	NM_000879	207952_at	4.0	0.0072	3.9	0.0039
---	Transcribed locus	AW628623	236785_at	4.0	0.0251	3.1	0.0425
IRF4[c]	interferon regulatory factor 4	NM_002460	204562_at	4.0	0.0076	2.9	0.0229
IRF1	interferon regulatory factor 1	NM_002198	202531_at	4.0	0.0086	4.0	0.0038
FAS[c]	Fas (TNF receptor superfamily, member 6)	X83493	215719_x_at	4.0	0.0078	3.0	0.0186
PLAT	plasminogen activator, tissue	NM_000930	201860_s_at	4.0	0.0077	2.5	0.0551
LIMK2[c]	LIM domain kinase 2	NM_005569	202193_at	3.9	0.0085	3.3	0.0106
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	M80536	203716_s_at	3.9	0.0086	2.3	0.0992
PLEKHA7	pleckstrin homology domain containing, family A member 7	AA758861	228450_at	3.9	0.0112	2.9	0.0415
SLC31A2	solute carrier family 31 (copper transporters), member 2	NM_001860	204204_at	3.9	0.0091	3.2	0.0146
HAVCR2	hepatitis A virus cellular receptor 2	BC020843	1555629_at	3.9	0.0140	2.5	0.1158
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	NM_006144	205488_at	3.9	0.0091	1.7	0.3297
LOC284417	hCG1651476	BC020862	1570225_at	3.8	0.0118	2.8	0.0291
---	Homo sapiens, clone IMAGE:5170250, mRNA	BC029255	1559125_at	3.8	0.0090	2.9	0.0265
---	---	N65982	241750_x_at	3.8	0.0091	3.8	0.0054
CD274	CD274 molecule	AF233516	223834_at	3.8	0.0104	3.0	0.0209
C4orf32	chromosome 4 open reading frame 32	AI110850	227856_at	3.8	0.0103	2.8	0.0292
GBP3	guanylate binding protein 3	AL136680	223434_at	3.8	0.0099	3.5	0.0070

C9orf39	chromosome 9 open reading frame 39	AK098502	1559005_s_at	3.7	0.0108	2.4	0.0668
IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	NM_000882	207160_at	3.7	0.0119	2.4	0.0713
RHEBL1[c]	Ras homolog enriched in brain like 1	BC014155	1570253_a_at	3.7	0.0122	3.1	0.0333
EMP1[c]	epithelial membrane protein 1	BC017854	1564796_at	3.7	0.0119	3.0	0.0197
CD86[c]	CD86 molecule	NM_006889	205686_s_at	3.7	0.0117	2.8	0.0290
SNFT	Jun dimerization protein p21SNFT	NM_018664	220358_at	3.7	0.0118	3.2	0.0135
SAMHD1[c]	SAM domain and HD domain 1	AF147427	1559883_s_at	3.7	0.0130	2.8	0.0292
IL15RA	interleukin 15 receptor, alpha	NM_002189	207375_s_at	3.7	0.0118	3.3	0.0104
CCND2	cyclin D2	AW026491	200951_s_at	3.6	0.0119	2.6	0.0476
YES1[c]	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	NM_005433	202932_at	3.6	0.0142	2.6	0.0467
---	Full-length cDNA clone CS0DF024YN14 of Fetal brain of Homo sapiens (human)	AW295340	243222_at	3.6	0.0195	2.3	0.1065
CAMK2D[c]	Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	AA809487	231042_s_at	3.6	0.0206	1.6	0.4270
---	CDNA FLJ12055 fis, clone HEMBB1002049	AU146983	214967_at	3.6	0.0140	2.9	0.0318
ALPK1	alpha-kinase 1	AI760166	227438_at	3.6	0.0124	2.7	0.0375
KCTD14	potassium channel tetramerisation domain containing 14	AI672101	58916_at	3.6	0.0136	2.7	0.0516
C1orf150	chromosome 1 open reading frame 150	NM_145278	1552908_at	3.6	0.0124	2.7	0.0326
CNDP2	CNDP dipeptidase 2 (metallopeptidase M20 family)	NM_018235	217752_s_at	3.6	0.0125	3.1	0.0159
---	---	AI092511	237953_at	3.6	0.0140	2.7	0.0388
TNF	tumor necrosis factor (TNF superfamily, member 2)	NM_000594	207113_s_at	3.5	0.0140	3.0	0.0231
ASCL2	achaete-scute complex homolog 2 (Drosophila)	AI393930	229215_at	3.5	0.0130	2.7	0.0398
LMNB1	lamin B1	NM_005573	203276_at	3.5	0.0138	2.7	0.0368
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	X16323	209960_at	3.5	0.0140	3.2	0.0171
MEFV	Mediterranean fever	NM_000243	208262_x_at	3.5	0.0139	3.1	0.0171
RIPK2[c]	receptor-interacting serine-threonine kinase 2	AF027706	209544_at	3.5	0.0144	3.1	0.0172
RNF125	ring finger protein 125	NM_017831	207735_at	3.4	0.0160	2.1	0.1561
---	Full length insert cDNA clone YR04D03	AF085913	1565915_at	3.4	0.0231	3.1	0.0196
LOC162073	hypothetical protein LOC162073	AI458417	227954_at	3.4	0.0163	2.5	0.0567
---	Transcribed locus	BE674703	241849_at	3.4	0.0163	2.7	0.0366
INDO	indoleamine-pyrrole 2,3 dioxygenase	M34455	210029_at	3.4	0.0200	3.2	0.0145
GBP2	guanylate binding protein 2, interferon-inducible	NM_004120	202748_at	3.4	0.0174	3.3	0.0099
ZNRF1	zinc and ring finger 1	AI144394	225960_at	3.3	0.0195	2.9	0.0267
MYO1B	myosin IB	BF432550	212364_at	3.3	0.0189	2.3	0.0992
IL13	interleukin 13	NM_002188	207844_at	3.3	0.0198	2.3	0.0990
MEOX1	mesenchyme homeobox 1	NM_004527	205619_s_at	3.3	0.0205	2.5	0.0566
EGR3	early growth response 3	NM_004430	206115_at	3.3	0.0195	3.2	0.0138
LIPA	lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	AW961916	236156_at	3.3	0.0197	2.8	0.0290
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	AI128225	213537_at	3.3	0.0190	2.3	0.0861
---	CDNA FLJ11041 fis, clone PLACE1004405	AI343467	227140_at	3.3	0.0239	2.3	0.0885
C2	complement component 2	BC029781	1554533_at	3.3	0.0229	3.2	0.0174
---	Transcribed locus, weakly similar to XP_001117086.1 similar to Olfactory receptor 10T2 (Olfactory receptor OR1-3) [Macaca mulatta]	AI240943	238629_x_at	3.3	0.0249	1.6	0.4790
KIAA0040	KIAA0040	T79953	203143_s_at	3.3	0.0197	2.5	0.0596
---	Transcribed locus, strongly similar to XP_531234.1 hypothetical protein XP_531234 [Pan troglodytes]	R14866	243328_at	3.3	0.0266	2.6	0.0544
ANTXR2	anthrax toxin receptor 2	AU152178	225524_at	3.3	0.0206	2.7	0.0388

C1QC	complement component 1, q subcomponent, C chain	AI184968	225353_s_at	3.3	0.0332	3.1	0.0271
C16orf7	chromosome 16 open reading frame 7	NM_004913	205781_at	3.2	0.0222	3.1	0.0171
AYTL1	acyltransferase like 1	AA789296	239598_s_at	3.2	0.0207	2.5	0.0583
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	J03189	210164_at	3.2	0.0205	2.4	0.0703
CIITA	class II, major histocompatibility complex, transactivator	NM_000246	205101_at	3.2	0.0259	2.7	0.0431
FCER2	Fc fragment of IgE, low affinity II, receptor for (CD23)	NM_002002	206759_at	3.2	0.0308	1.7	0.3358
---	Full-length cDNA clone CS0DD001YA12 of Neuroblastoma Cot 50-normalized of Homo sapiens (human)	BE673665	228573_at	3.2	0.0220	2.6	0.0480
CASP10	caspase 10, apoptosis-related cysteine peptidase	NM_001230	205467_at	3.2	0.0232	2.8	0.0297
PPA1	pyrophosphatase (inorganic) 1	NM_021129	217848_s_at	3.2	0.0219	2.5	0.0626
PDP2	pyruvate dehydrogenase phosphatase isoenzyme 2	AB037769	232861_at	3.2	0.0232	1.1	1.0724
LRRC8C	leucine rich repeat containing 8 family, member C	AL136919	223533_at	3.2	0.0239	2.4	0.0763
C6orf150	chromosome 6 open reading frame 150	AK097148	1559051_s_at	3.2	0.0221	2.5	0.0553
KCNJ15	potassium inwardly-rectifying channel, subfamily J, member 15	U73191	210119_at	3.2	0.0303	2.8	0.0318
NR4A2[c]	nuclear receptor subfamily 4, group A, member 2	S77154	216248_s_at	3.2	0.0232	3.1	0.0171
TLR4[c]	toll-like receptor 4	NM_138557	1552798_a_at	3.2	0.0240	2.4	0.0729
FBXO6	F-box protein 6	AF129536	231769_at	3.2	0.0239	2.5	0.0575
PRPS2	Phosphoribosyl pyrophosphate synthetase 2	AI392908	230352_at	3.2	0.0239	1.7	0.3180
TEP1	telomerase-associated protein 1	NM_007110	205727_at	3.1	0.0358	2.4	0.0887
GIMAP8	GTPase, IMAP family member 8	BE671843	240646_at	3.1	0.0251	1.4	0.4421
BLVRA[c]	biliverdin reductase A	AA740186	203771_s_at	3.1	0.0259	2.2	0.1171
EAF2	ELL associated factor 2	NM_018456	219551_at	3.1	0.0265	2.1	0.1362
---	MRNA; cDNA DKFZp586E151 (from clone DKFZp586E151)	AL050124	234276_at	3.1	0.0304	3.2	0.0160
TRPM6[c]	transient receptor potential cation channel, subfamily M, member 6	AF350881	224412_s_at	3.1	0.0255	2.0	0.2239
RGS16	regulator of G-protein signaling 16	BF304996	209324_s_at	3.1	0.0266	2.4	0.0793
XIRP1	xin actin-binding repeat containing 1	AW755250	235042_at	3.1	0.0300	2.3	0.1543
---	CDNA: FLJ21424 fis, clone COL04157	AK025077	215864_at	3.1	0.0272	1.9	0.2240
KIAA1913	KIAA1913	AA088177	234994_at	3.1	0.0282	2.4	0.0795
LRRK2	leucine-rich repeat kinase 2	AK026776	229584_at	3.1	0.0301	2.9	0.0210
RGS1	regulator of G-protein signaling 1	NM_002922	202988_s_at	3.0	0.0304	2.3	0.0780
MIRH1	microRNA host gene (non-protein coding) 1	AA256157	232291_at	3.0	0.0319	1.9	0.2373
RHOBTB3[c]	Rho-related BTB domain containing 3	NM_014899	202976_s_at	3.0	0.0392	3.4	0.0135
---	Full length insert cDNA clone YA84A05	T56980	1559949_at	3.0	0.0325	2.1	0.1431
SORT1	sortilin 1	BF447105	212807_s_at	3.0	0.0316	2.8	0.0289
DCTN1	dynactin 1 (p150, glued homolog, Drosophila)	NM_021196	204296_at	3.0	0.0318	2.5	0.0559
IL2RA[c]	interleukin 2 receptor, alpha	K03122	211269_s_at	3.0	0.0339	2.3	0.0881
SLAMF7[c]	SLAM family member 7	AJ271869	234306_s_at	3.0	0.0327	2.3	0.0952
RAPGEF2[c]	Rap guanine nucleotide exchange factor (GEF) 2	AL117397	215992_s_at	3.0	0.0324	2.7	0.0391
IL1RL1	interleukin 1 receptor-like 1	NM_003856	207526_s_at	3.0	0.0816	3.1	0.0378
P2RY14	purinergic receptor P2Y, G-protein coupled, 14	NM_014879	206637_at	3.0	0.0318	2.5	0.0594
PPM2C[c]	protein phosphatase 2C, magnesium-dependent, catalytic subunit	BG542521	222572_at	3.0	0.0341	2.6	0.0486
GEM	GTP binding protein overexpressed in skeletal muscle	NM_005261	204472_at	3.0	0.0376	2.0	0.1734
CASP7	caspase 7, apoptosis-related cysteine peptidase	NM_001227	207181_s_at	3.0	0.0333	2.8	0.0289

LASS6	LAG1 homolog, ceramide synthase 6	AI081356	235463_s_at	3.0	0.0323	1.9	0.2424
---	Full-length cDNA clone CS0DK005YO12 of HeLa cells Cot 25-normalized of Homo sapiens (human)	AI870951	228066_at	3.0	0.0392	2.1	0.1482
---	Transcribed locus	AW044663	230127_at	3.0	0.0372	2.7	0.0420
APOL2	apolipoprotein L, 2	BC004395	221653_x_at	2.9	0.0357	2.9	0.0240
OR52K3P	olfactory receptor, family 52, subfamily K, member 3 pseudogene	AF143328	232829_at	2.9	0.0356	2.3	0.0901
---	---	AI608902	227458_at	2.9	0.0380	2.6	0.0392
ARID5B	AT rich interactive domain 5B (MRF1-like)	AA150242	241969_at	2.9	0.0400	1.8	0.2846
RHOH	ras homolog gene family, member H	NM_004310	204951_at	2.9	0.0373	2.5	0.0589
---	Clone FLB4630	AF113688	1570622_at	2.9	0.0356	1.6	0.3347
---	---	BF509371	242907_at	2.9	0.0373	3.0	0.0205
---	Homo sapiens, clone IMAGE:4332461, mRNA	R14890	232397_at	2.9	0.0394	2.9	0.0291
RHOB	ras homolog gene family, member B	BI668074	1553962_s_at	2.9	0.0415	1.6	0.4464
XRN1	5'-3' exoribonuclease 1	AY137776	1555785_a_at	2.9	0.0372	2.2	0.1058
---	Transcribed locus	BF196334	239963_at	2.9	0.0425	1.8	0.2686
C9orf19	chromosome 9 open reading frame 19	AA284532	225604_s_at	2.9	0.0375	2.0	0.1734
---	Full-length cDNA clone CS0DI067YM20 of Placenta Cot 25-normalized of Homo sapiens (human)	AA603344	235964_x_at	2.9	0.0390	2.5	0.0658
---	Homo sapiens, clone IMAGE:5581630, mRNA	BC035773	1569666_s_at	2.9	0.0476	2.4	0.0930
C19orf12	chromosome 19 open reading frame 12	BC004957	223983_s_at	2.9	0.0397	2.3	0.0948
---	MRNA; cDNA DKFZp667K2218 (from clone DKFZp667K2218)	AI125308	1557733_a_at	2.9	0.0408	1.6	0.4463
HSPD1	heat shock 60kDa protein 1 (chaperonin)	BF965447	241716_at	2.9	0.0389	1.8	0.3041
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	AL136139	202149_at	2.9	0.0390	2.2	0.1106
SNX10	sorting nexin 10	NM_013322	218404_at	2.9	0.0393	2.5	0.0566
---	CDNA: FLJ21256 fis, clone COL01402	AK024909	216166_at	2.9	0.0391	2.7	0.0373
---	Transcribed locus	AA579773	243221_at	2.9	0.0437	2.4	0.0741
C21orf71	chromosome 21 open reading frame 71	BU192089	1556414_at	2.8	0.0408	2.2	0.1014
CSF1	colony stimulating factor 1 (macrophage)	M37435	209716_at	2.8	0.0407	1.9	0.2061
RNF24	ring finger protein 24	NM_007219	204669_s_at	2.8	0.0416	2.3	0.0988
TFRC	transferrin receptor (p90, CD71)	N76327	237215_s_at	2.8	0.0461	1.5	0.5255
---	Transcribed locus	AI147211	235971_at	2.8	0.0475	2.8	0.0291
---	Transcribed locus	AA151917	230503_at	2.8	0.0430	3.4	0.0080
---	---	AI377755	236203_at	2.8	0.0681	2.8	0.0366
DOHH	deoxyhypusine hydroxylase/monooxygenase	NM_031304	208141_s_at	2.8	0.0475	2.3	0.0816
STAMBPL1	STAM binding protein-like 1	AI638611	227607_at	2.8	0.0461	2.0	0.1559
STAT2	signal transducer and activator of transcription 2, 113kDa	S81491	217199_s_at	2.7	0.0500	1.9	0.2249
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	AI872403	1568713_a_at	2.7	0.0543	2.6	0.0494
SLC6A12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	U27699	206058_at	2.7	0.0625	2.9	0.0236
TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	NM_003811	206907_at	2.6	0.0704	2.9	0.0240
FHL1	four and a half LIM domains 1	AF220153	214505_s_at	2.5	0.0880	2.9	0.0273
---	---	BC022885	1564656_at	2.4	0.0874	2.8	0.0317
ZC3HAV1	zinc finger CCCH-type, antiviral 1	NM_020119	220104_at	2.3	0.1163	2.6	0.0417
---	Transcribed locus	BF431214	236065_at	2.3	0.1146	2.9	0.0256
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	AB000221	209924_at	2.2	0.1492	2.6	0.0483
ROBO4	roundabout homolog 4, magic roundabout (Drosophila)	NM_019055	220758_s_at	2.1	0.1797	3.3	0.0144
NRP2	neuropilin 2	AF022859	211844_s_at	1.8	0.2115	3.1	0.0171

---	CDNA FLJ13856 fis, clone THYRO1000988	AK023918	215595_x_at	1.6	0.5785	2.7	0.0374
NMNAT3	nicotinamide nucleotide adenylyltransferase 3	AW172570	243738_at	1.5	0.6179	2.6	0.0485
SCUBE3	Signal peptide, CUB domain, EGF-like 3	BE674338	230253_at	1.4	0.7905	3.0	0.0175
---	CDNA FLJ36965 fis, clone BRACE2006075	BU852182	1560755_at	1.3	0.8681	3.1	0.0191
---	---	X76785	234882_at	1.2	1.0279	2.9	0.0269

[a]All upregulated genes with an FDR less than 0.05 with at least one of the stimuli.

[b]Mean fold change of three blood donors.

[c]The probe set with significant changes with both stimuli is shown. In cases where none fulfilled this criterion, the probe set showing the highest fold change value was selected.

Gene expression of human PBMC analyzed after 6 h of stimulation with SEI and SEB. The vast majority of genes was influenced to a similar extend by SEI and SEB.

Supplemental Table II. Genes downregulated after stimulation with SEI and SEB.

Gene Symbol[a]	Gene Title	NCBI Accession No	probe set ID	SEI		SEB	
				mean fold change[b]	FDR	mean fold change[b]	FDR
HEY1[c]	hairy/enhancer-of-split related with YRPW motif 1	NM_012258	218839_at	-6.2	< 0.0001	-3.9	0.0015
ST3GAL6[c]	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	AI989567	213355_at	-6.1	< 0.0001	-4.2	0.0005
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_002993	206336_at	-5.7	< 0.0001	-4.8	0.0010
NT5DC2	5'-nucleotidase domain containing 2	NM_022908	218051_s_at	-5.6	< 0.0001	-2.6	0.0490
MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	BC001875	223723_at	-5.3	< 0.0001	-3.7	0.0036
VCAN[c]	versican	BF590263	204619_s_at	-4.6	0.0001	-5.2	0.0003
---	Transcribed locus	AI494347	240393_at	-4.5	0.0003	-2.6	0.0413
NOG	Noggin	AL575177	231798_at	-4.5	0.0002	-2.6	0.0413
HNMT[c]	histamine N-methyltransferase	BC005907	211732_x_at	-4.2	0.0006	-3.2	0.0093
LOC285181	hypothetical protein LOC285181	AA002166	1561334_at	-4.1	0.0005	-2.9	0.0212
OLIG1	oligodendrocyte transcription factor 1	AL355743	228170_at	-3.9	0.0002	-3.8	0.0021
TFPI	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	AL080215	215447_at	-3.8	0.0011	-3.2	0.0092
C5AR1	complement component 5a receptor 1	NM_001736	220088_at	-3.8	0.0012	-2.7	0.0418
---	---	AA805633	230175_s_at	-3.7	0.0011	-2.8	0.0326
SIGLEC9	sialic acid binding Ig-like lectin 9	AF247180	210569_s_at	-3.7	0.0011	-3.1	0.0136
KCNE3	potassium voltage-gated channel, Isk-related family, member 3	AI692703	227647_at	-3.7	0.0012	-4.2	0.0005
---	MRNA; cDNA DKFZp313E1515 (from clone DKFZp313E1515)	AL833097	1560034_a_at	-3.7	0.0017	-2.3	0.1028
VNN1[c]	vanin 1	BG120535	1558549_s_at	-3.6	0.0008	-3.4	0.0073
STON2	stonin 2	AA632295	227461_at	-3.6	0.0011	-2.4	0.0806
TIMP2[c]	TIMP metallopeptidase inhibitor 2	BE968786	231579_s_at	-3.6	0.0015	-2.5	0.0600
EPHA4[c]	EPH receptor A4	T15545	228948_at	-3.5	0.0015	-2.8	0.0297
---	MRNA full length insert cDNA clone EUROIMAGE 85905	AL080280	1559910_at	-3.5	0.0016	-2.1	0.1945
RNASEH2C	ribonuclease H2, subunit C	AI990526	227543_at	-3.5	0.0024	-1.3	0.8072
CXCL2	chemokine (C-X-C motif) ligand 2	BC005276	1569203_at	-3.3	0.0031	-2.2	0.1344
C4orf18	chromosome 4 open reading frame 18	AF260333	223204_at	-3.3	0.0025	-4.0	0.0016
OLIG2	oligodendrocyte lineage transcription factor 2	AA757419	213825_at	-3.3	0.0017	-2.8	0.0263
---	Transcribed locus	BF724178	229613_at	-3.3	0.0015	-1.5	0.6611
SLC16A6[c]	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	AI873273	230748_at	-3.3	0.0029	-2.5	0.0483
---	Homo sapiens, clone IMAGE:4105785, mRNA	BC016361	1558871_at	-3.2	0.0064	-2.9	0.0293
EREG	epiregulin	BC035806	1569583_at	-3.2	0.0064	-2.4	0.0777
HAS1	hyaluronan synthase 1	NM_001523	207316_at	-3.2	0.0064	-4.7	0.0021
DOCK5[cd]	dedicator of cytokinesis 5	BF447954	230263_s_at	-3.2	0.0052	-2.1	0.1649
FPRL2[c]	formyl peptide receptor-like 2	AW026543	230422_at	-3.1	0.0061	-3.6	0.0025
CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1	NM_000784	203979_at	-3.1	0.0051	-2.6	0.0575
HECTD1	HECT domain containing 1	BC016947	1570251_at	-3.0	0.0084	-2.1	0.1259
FLJ46446	Hypothetical gene supported by AK128305	CA425190	1556402_at	-3.0	0.0060	-2.0	0.1950
---	---	AI694722	242397_at	-3.0	0.0064	-2.9	0.0290
PLK2	polo-like kinase 2 (Drosophila)	NM_006622	201939_at	-3.0	0.0060	-2.4	0.0770
ZFP36L2[c]	zinc finger protein 36, C3H type-like 2	AI356398	201367_s_at	-3.0	0.0096	-3.0	0.0173
---	Transcribed locus	AI401017	244874_at	-3.0	0.0068	-1.7	0.4029

SPON1	spondin 1, extracellular matrix protein	AI885290	213993_at	-3.0	0.0073	-1.4	0.8303
TXLNB	taxilin beta	AL589605	227834_at	-3.0	0.0074	-2.2	0.1358
TNS1[c]	tensin 1	AF116610	218864_at	-3.0	0.0073	-2.7	0.0317
FUCA1	fucosidase, alpha-L- 1, tissue	NM_000147	202838_at	-3.0	0.0109	-2.4	0.0781
CD14	CD14 molecule	NM_000591	201743_at	-3.0	0.0085	-2.6	0.0475
SULT1A3 /// SULT1A4	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3 /// sulfotransferase family, cytosolic, 1A, phenol-preferring, member 4	AI580112	222094_at	-3.0	0.0073	-1.3	0.7235
SLC24A4	solute carrier family 24 (sodium/potassium/calcium exchanger), member 4	W90718	243969_at	-2.9	0.0114	-1.9	0.1951
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	AI368358	240440_at	-2.9	0.0074	-2.9	0.0214
PER1	period homolog 1 (Drosophila)	NM_002616	202861_at	-2.9	0.0061	-2.2	0.1059
C20orf117	chromosome 20 open reading frame 117	NM_015377	207711_at	-2.9	0.0122	-2.9	0.0282
LOC440934[c]	Hypothetical gene supported by BC008048	AW473883	244159_at	-2.9	0.0094	-2.4	0.0848
ETV5[c]	ets variant gene 5 (ets-related molecule)	NM_004454	203349_s_at	-2.9	0.0099	-2.6	0.0607
CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	NM_005211	203104_at	-2.9	0.0104	-2.8	0.0324
IGSF2	immunoglobulin superfamily, member 2	NM_004258	207167_at	-2.9	0.0159	-2.1	0.2000
C5orf29	chromosome 5 open reading frame 29	NM_152687	1552386_at	-2.8	0.0114	-2.3	0.1080
PLD1	phospholipase D1, phosphatidylcholine-specific	AA132961	232530_at	-2.8	0.0098	-1.9	0.2779
---	Transcribed locus	AA573201	240099_at	-2.8	0.0132	-1.8	0.3658
ZBTB1	zinc finger and BTB domain containing 1	NM_014950	205092_x_at	-2.8	0.0103	-1.9	0.2764
TNFRSF8	tumor necrosis factor receptor superfamily, member 8	NM_001243	206729_at	-2.8	0.0052	-2.1	0.1582
PNKD	paroxysmal nonkinesigenic dyskinesia	AB033010	233177_s_at	-2.8	0.0158	-2.8	0.0319
LOC387763	hypothetical LOC387763	AW276078	227099_s_at	-2.7	0.0159	-2.4	0.0750
---	---	AI832594	244605_at	-2.7	0.0256	-1.2	0.9150
---	CDNA clone IMAGE:5787947	BC039553	1559544_s_at	-2.7	0.0251	-1.8	0.3998
TMEM45B	transmembrane protein 45B	AW242836	230323_s_at	-2.7	0.0135	-2.2	0.1381
VAMP1	vesicle-associated membrane protein 1 (synaptobrevin 1)	NM_016830	207100_s_at	-2.7	0.0200	-1.6	0.4263
YPEL2	yippee-like 2 (Drosophila)	AK097253	1556420_s_at	-2.7	0.0156	-1.4	0.6977
PASK	PAS domain containing serine/threonine kinase	U79240	216945_x_at	-2.7	0.0248	-1.9	0.2779
TTLL4	tubulin tyrosine ligase-like family, member 4	NM_014640	203703_s_at	-2.6	0.0218	-2.2	0.1415
---	---	H94882	242936_at	-2.6	0.0269	-2.2	0.1587
DOK3	docking protein 3	BC004564	223553_s_at	-2.6	0.0217	-2.5	0.0520
FCAR	Fc fragment of IgA, receptor for	U56237	211306_s_at	-2.6	0.0209	-2.3	0.0894
RAB7B	RAB7B, member RAS oncogene family	AY094596	1553982_a_at	-2.6	0.0217	-1.7	0.4212
LDLRAP1	low density lipoprotein receptor adaptor protein 1	AL545035	221790_s_at	-2.6	0.0288	-1.5	0.5919
HHEX	hematopoietically expressed homeobox	Z21533	215933_s_at	-2.6	0.0359	-2.1	0.1948
LY9[cd]	lymphocyte antigen 9	AL582804	215967_s_at	-2.6	0.0252	-2.3	0.0971
TFPI2	tissue factor pathway inhibitor 2	AL574096	209277_at	-2.6	0.0330	-1.8	0.2785
NR5A2	nuclear receptor subfamily 5, group A, member 2	AF228413	210174_at	-2.6	0.0381	-2.1	0.1491
ZNF587	zinc finger protein 587	AK055448	1558251_a_at	-2.5	0.0311	-2.0	0.2417
C20orf112	chromosome 20 open reading frame 112	AL034550	230954_at	-2.5	0.0344	-2.7	0.0422
NMT2	N-myristoyltransferase 2	AL134489	215743_at	-2.5	0.0381	-1.9	0.2999

INSR	insulin receptor	AA485908	213792_s_at	-2.5	0.0286	-2.3	0.0982
---	CDNA FLJ12187 fis, clone MAMMA1000831	AA572675	232286_at	-2.5	0.0266	-2.1	0.1412
RGS2	regulator of G-protein signaling 2, 24kDa	NM_002923	202388_at	-2.5	0.0331	-2.2	0.1164
SLC22A15	solute carrier family 22 (organic cation transporter), member 15	AI279062	228497_at	-2.5	0.0381	-1.5	0.6454
CXCR7	chemokine (C-X-C motif) receptor 7	AI817041	212977_at	-2.5	0.0356	-2.5	0.0715
PTGFRN	prostaglandin F2 receptor negative regulator	BF311866	224937_at	-2.5	0.0286	-2.0	0.1772
CXCL5	chemokine (C-X-C motif) ligand 5	BG166705	215101_s_at	-2.5	0.0378	-2.4	0.0911
---	MRNA; cDNA DKFZp666P238 (from clone DKFZp666P238)	AL833038	1563597_at	-2.5	0.0286	-1.4	0.7700
---	Homo sapiens, clone IMAGE:5205388, mRNA	AW303397	229040_at	-2.5	0.0361	-1.5	0.5746
---	Transcribed locus	AI733470	240821_at	-2.5	0.0358	-1.2	0.9783
GLCE	glucuronic acid epimerase	W87398	213552_at	-2.5	0.0392	-1.5	0.5754
---	CDNA clone IMAGE:4544718	BC014231	1570607_at	-2.5	0.0347	-1.5	0.5624
HOM-TES-103	hypothetical protein LOC25900	BC002857	209721_s_at	-2.5	0.0395	-2.3	0.1110
---	Mir-223 transcript variant 1 mRNA, complete sequence	N39230	229934_at	-2.4	0.0390	-1.7	0.4093
TPST1	tyrosylprotein sulfotransferase 1	NM_003596	204140_at	-2.4	0.0420	-3.3	0.0298
CXCL3	chemokine (C-X-C motif) ligand 3	NM_002090	207850_at	-2.4	0.0388	-1.8	0.3705
SPIRE1	spire homolog 1 (Drosophila)	BC016825	1554807_a_at	-2.4	0.0415	-1.4	0.7458
PTCH1	patched homolog 1 (Drosophila)	BG054916	209815_at	-2.4	0.0396	-2.3	0.1031
---	Transcribed locus	AA416756	244677_at	-2.4	0.0425	-1.6	0.4845
CD244	CD244 molecule, natural killer cell receptor 2B4	NM_016382	220307_at	-2.4	0.0424	-1.7	0.3877
FRAT2	frequently rearranged in advanced T-cell lymphomas 2	AB045118	209864_at	-2.4	0.0387	-2.7	0.0312
ARRDC2	arrestin domain containing 2	AK000689	226055_at	-2.4	0.0423	-2.2	0.1087
IRS2	insulin receptor substrate 2	AF073310	209185_s_at	-2.4	0.0491	-2.1	0.1671
---	---	AA808178	235743_at	-2.4	0.0395	-1.5	0.5252
SIGLEC10	sialic acid binding Ig-like lectin 10	AF301007	1552807_a_at	-2.2	0.0705	-2.8	0.0421
LY9[cd]	lymphocyte antigen 9	AF244129	210370_s_at	-2.2	0.0794	-2.9	0.0186
GAS2L3	Growth arrest-specific 2 like 3	AI860012	238756_at	-2.2	0.0799	-2.7	0.0460
KLHL24	kelch-like 24 (Drosophila)	AI961401	242088_at	-2.1	0.0911	-2.7	0.0415
DOCK5[cd]	dedicator of cytokinesis 5	AK024569	222721_at	-2.1	0.1225	-2.6	0.0454
---	---	AW024087	239284_at	-2.0	0.0461	1.1	1.0232
SIGLEC5	sialic acid binding Ig-like lectin 5	NM_003830	220000_at	-2.0	0.1785	-3.4	0.0036
---	Transcribed locus	AW016812	231644_at	-2.0	0.1629	-2.8	0.0303
MEGF9	multiple EGF-like-domains 9	BF110421	212831_at	-1.8	0.2279	-2.7	0.0329
HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	NM_005114	205466_s_at	-1.8	0.2294	-2.7	0.0414
---	CDNA FLJ34016 fis, clone FCBBF2002541	AI935334	235385_at	-1.8	0.2627	-2.6	0.0481
C1orf21	chromosome 1 open reading frame 21	NM_030806	221272_s_at	-1.7	0.2910	-2.9	0.0145
PDLIM7	PDZ and LIM domain 7 (enigma)	BC023629	1569149_at	-1.7	0.2875	-2.7	0.0309
SFRS11	Splicing factor, arginine/serine-rich 11	T90915	236948_x_at	-1.6	0.4361	-2.6	0.0449
DECR2	2,4-dienoyl CoA reductase 2, peroxisomal	NM_020664	219664_s_at	-1.5	0.3851	-3.0	0.0221
ARRB1	arrestin, beta 1	NM_004041	218832_x_at	-1.5	0.4429	-3.4	0.0042
CCPG1	cell cycle progression 1	AK022459	222156_x_at	-1.5	0.4797	-2.6	0.0454
LOC286254	hypothetical protein LOC286254	AI471954	1559765_a_at	-1.5	0.4694	-2.6	0.0485
PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta	AF225513	223551_at	-1.4	0.5512	-2.8	0.0309
---	Transcribed locus	AI935541	236220_at	-1.4	0.4052	-3.0	0.0153
TTC3	tetratricopeptide repeat domain 3	BC026260	1569472_s_at	-1.3	0.7176	-2.6	0.0431
FANK1	fibronectin type III and ankyrin	AU143929	232968_at	-1.3	0.7818	-2.7	0.0419

	repeat domains 1						
SOCS4	suppressor of cytokine signaling 4	NM_080867	1552792_at	-1.2	0.8851	-2.9	0.0227
PDPR	pyruvate dehydrogenase phosphatase regulatory subunit	NM_017990	220236_at	-1.2	0.8907	-2.6	0.0468
ARHGAP24	Rho GTPase activating protein 24	AI743534	223422_s_at	-1.2	0.9224	-3.3	0.0042
MVD	mevalonate (diphospho) decarboxylase	AI189359	203027_s_at	-1.1	0.9687	-2.6	0.0429
BPI	bactericidal/permeability-increasing protein	NM_001725	205557_at	-1.1	0.9984	-2.6	0.0465

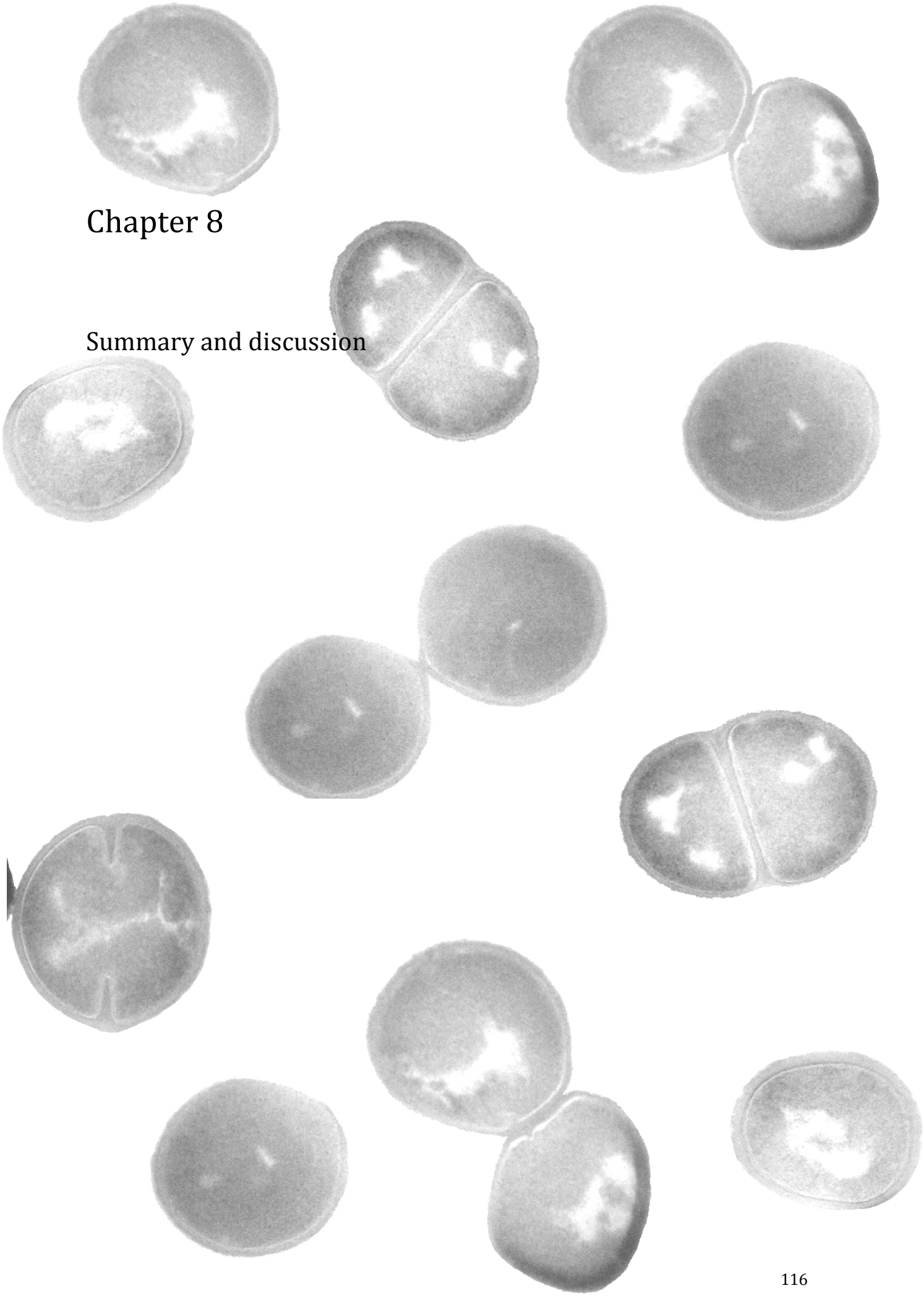
[a]All downregulated genes with an FDR less than 0.05 with at least one of the stimuli.

[b]Mean fold change of three blood donors.

[c]Gene represented by more than one probe set. The probe set with significant changes following both stimuli is shown. If none fulfilled this criterion, the probe set showing the highest fold change was selected.

[d]Gene represented by two probe sets, which are both included in this table.

Gene expression of human PBMC analyzed after 6 h stimulation with SEI and SEB. The vast majority of genes was influenced to a similar extend by SEI and SEB.



Chapter 8

Summary and discussion

Summary and discussion

S. aureus is both a ubiquitous human commensal and a frequent cause of clinically important infections. To date, *S. aureus* is the most common cause of nosocomial infections. The alarming global spread of MRSA has spurred efforts to develop active and passive anti-staphylocoocal vaccines. However, vaccine development is a challenging task, because both the species *S. aureus* and the host response that it induces are highly variable.

Therefore, this thesis was focussed on i) bacterial virulence determinants, in particular SAgs, and ii) on the role of anti-SAg antibodies in the interaction between *S. aureus* and its host. First of all, we analyzed the prevalence of SAg genes and phages among colonizing and invasive *S. aureus* isolates and correlated this with their genetic background. In a second approach the presence and development of anti-SAg antibodies in *S. aureus* colonization and bacteremia were determined. Finally, we performed a comprehensive survey of the intrinsic properties and regulation of *egc* and non-*egc* SAgs, to elucidate the reasons for the selective lack of neutralizing serum antibodies specific for *egc* SAgs.

8.1 WHAT DETERMINES STAPHYLOCOCCAL VIRULENCE? MOLECULAR-EPIDEMIOLOGY OF COLONIZING AND INVASIVE *S. AUREUS* ISOLATES

In the recent years, huge efforts have been made to determine bacterial factors that are important for staphylococcal virulence. Since evidence for a decisive role of the core genome on *S. aureus* virulence is limited (1-3), it was suggested that virulence determinants encoded on MGEs may determine the virulence of an isolate (4).

Each *S. aureus* clonal complex is characterized by a consensus repertoire of superantigen genes and phages

Genotyping analyses, such as MLST and *spa* genotyping, demonstrated that the *S. aureus* population structure is highly clonal with 10 predominant CCs (1, 2, 5, 6). *S. aureus* strains harbor a broad and highly variable repertoire of virulence and resistance genes including the 21 different SAgs. SAgs are encoded on MGEs and can therefore be distributed by two distinct mechanisms: vertical transmission to daughter cells and

horizontal transfer by bacteriophages and conjugation (7, 8). In order to investigate whether SAg genes are randomly distributed among different CCs or rather linked to the clonal background, we determined *spa* genotypes and SAg gene patterns of about 300 *S. aureus* isolates from Western Pomerania **(Chapter 2)**. Additionally, the phage patterns of about 300 *S. aureus* isolates from different sources in Germany were analyzed by multiplex PCR for the seven most prominent *S. aureus* integrase families, Sa1int to Sa7int, and correlated with the clonal lineage **(Chapter 3)**.

Genotype and SAg gene patterns. As expected, we observed 10 predominant staphylococcal lineages (CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45, CC121 and CC395), which have successfully spread globally (Chapter 2) (1, 2, 5, 6). Interestingly, none of the SAg genes were randomly disseminated between the CCs ($P \leq 0.001$; contingency table analysis) but rather strongly associated with the clonal background. For example, the *egc*-encoding genomic island νSAβ was strictly linked to CC5, CC22, and CC45. Other SAgs with strong CC linkages were the SaPI-encoded *tst* and *sec-sel* (CC45), or the plasmid-encoded *sed-sej-ser* (CC8). SAg genes with a broader distribution were the phage-encoded *sea*, and the SaPI-encoded *seb*. These findings of a clonal distribution of SAg genes are in good agreement with other studies on MSSA as well as MRSA isolates (5, 7, 9-14).

To conclude, these results show that most SAg-carrying MGEs are predominantly transferred vertically. However, within a CC and even within the same *spa* type we also observed considerable variation of SAg gene patterns. This suggests that horizontal transfer of SAg-encoding MGEs occurs frequently within lineages, while horizontal transmission between the clonal lineages is limited. This phenomenon can be explained by the action of the lineage-specific restriction-modification systems, like SauI (15).

Phage patterns. The characterization of the prophage content of 291 *S. aureus* isolates revealed a moderate association between prophage prevalence and the clonal background (Chapter 3). Most isolates contained one or two prophages, and Sa3int phages, which encode SAgs like *sea*, *sep*, *sek*, and *seq* (16, 17), were by far the most common with a prevalence of 74%. There was one exception: CC15 strains harbored maximally one prophage ($P \leq 0.0004$) and never carried Sa3int phages. In good agreement, CC15 strain completely lack staphylococcal SAg genes (Chapter 2). In contrast, CC30 isolates were frequently lysogenic for at least two prophages ($P \leq 0.0001$).

As a consequence of the linkage of SAg gene patterns and phages to the CCs, each *S. aureus* lineage is characterized by a typical SAg gene and phage repertoire (Chapter 2, Table 2; Chapter 3, Table 1). Since many MGE-encoded virulence factors are linked to CCs, analyses of their association with invasiveness can be biased by differences in the underlying clonal population structure. Consequently, we highly recommend the simultaneous determination of the genetic background (CC) and virulence genes. Such an approach will increase the discriminatory power of genetic investigations into the mechanisms of *S. aureus* pathogenesis.

SAg genes are not associated with bloodstream invasion

To date it is largely unclear, what determines staphylococcal virulence on a molecular level (2, 7, 14). To investigate, whether SAg genes **(Chapter 2)** or phages **(Chapter 3)** are associated with bloodstream invasion, we compared virulence genes, phage patterns and core genome (*spa* type) of nasal and blood culture isolates from Germany. To avoid a bias due to underlying differences in the clonal composition of the *S. aureus* strain collections, virulence gene and phage patterns were separately compared in each CC. Finally, we characterized blood culture isolates from 43 injection drug users (IDUs) and 44 nonaddicts from Helsinki, Finland **(Chapter 6)**.

Genotype and SAg gene repertoire of nasal and blood culture isolates. *Spa* genotyping showed, as expected, that nasal and blood culture isolates were present in most CCs (Chapter 2) (1, 12). CC5 contained only nasal isolates (6.5%; $P \leq 0.05$). Moreover, CC8 was overrepresented among blood culture isolates compared to nasal isolates (21.6% vs. 10.3%; $P \leq 0.05$), while CC30 was underrepresented among blood culture strains (11.4% vs. 27.1%; $P \leq 0.01$). Intriguingly, in The Netherlands CC30 isolates tend to be more prevalent among endogenous invasive strains that non-invasive strains (3). This suggests, that German and Dutch strains differ in their core variable or variable genome, rendering them more or less invasive, respectively. This requires further investigation. *Spa* genotyping discriminated two subclusters of CC25, t056 and t078. MLST-typing by other investigators later led to the reclassification of one of them – *spa* type t056 - as ST101. The strains of *spa* type t078 (and relatives) still belong to CC25 (MLST-Type ST26). ST101 strains were significantly overrepresented among nasal isolates in comparison to blood culture isolates (9.3% vs. 2.2%, $P \leq 0.05$). CC25 strains of *spa* type t078, were found in both cohorts (3.7% vs. 10.2%, not significant).

Interestingly, *etd* was not seen in the 4 nasal isolates, but in 8 out of 9 invasive CC25 strains ($P \leq 0.001$), which is suggestive of a role for this factor in virulence.

Within staphylococcal lineages there were no differences in the SAg gene profiles between nasal and invasive isolates. Thus, SAgs do not contribute strongly to the invasive potential of blood culture isolates. In contrast, CC25 strains harboring the pathogenicity island that contains *etd* appear to be more virulent than those without it.

Phage patterns of nasal and blood culture isolates. Bacteriophages play an important role in the pathogenicity of *S. aureus* for instance by mediating the horizontal gene transfer of virulence factors. Comparison of phage patterns between nasal and blood culture isolates (Chapter 3) revealed no differences except for Sa3int phages, which were significantly more common in colonizing strains (73% vs. 59%; $P \leq 0.05$). This higher frequency was not biased by an overrepresentation of certain CCs. Sa3int phages integrate into the β hemolysin gene (*hlb*), thereby making it non-functional. They encode immune-modulatory proteins (staphylokinase (Sak), staphylococcal complement inhibitors (SCIN), and chemotaxis inhibitory protein (CHIPS)) (17, 18), which might help to resist the innate immune response during nasal colonization. In contrast, the lack of the Sa3int phages in infecting isolates is correlated with Hlb production. This fits to previous findings indicating that Hlb-producing strains are linked to infections (14, 16).

Genotype and SAg gene repertoire of bacteremia isolates from IDUs and nonaddicts. IDU patients represent a special cohort due to higher colonization rates and more frequent endogenous infections than in the general population (19, 20). The genetic diversity of *S. aureus* bacteremia isolates from IDUs and nonaddicts (Chapter 6) was largely similar, but CC59 and CC30 were significantly overrepresented among isolates from IDUs in comparison to nonaddicts (CC59: 20.9% vs. 2.3%, $P \leq 0.01$; CC30: 20.9% vs. 4.5%, $P \leq 0.05$). While the prevalence of CC30 among IDU isolates is in the same range that was reported for *S. aureus* colonization (16 - 27%), CC59 strains are rare in healthy European carriers (0 - 4.5%) (Chapter 2) (6, 21, 22). However, in the UK Monk et al. frequently observed CC59 isolates in IDUs with abscesses and soft tissue infections (23). This indicates a spread of CC59 strains in the internationally connected community of IDUs. The comparison of SAg gene patterns within individual CCs revealed no major differences between bacteremia isolates from IDUs and nonaddicts.

In summary, the present studies come up with only few virulence gene candidates for bactermia. In the Western Pomerania study the CC25, which carries the MGE harboring *etd* (*spa* type t078), was associated with bacteremia, however such strains were rare among bacteremia isolates from Helsinki, Finland (9% vs. 2.3%). Additionally, a functional *hlb* gene was correlated with bacteremia. In contrast, there was no association of SAg genes with bloodstream invasion. Apart from our own efforts, several other studies compared the core genome and virulence gene repertoire of blood culture and colonizing isolates but failed to identify factors clearly related to virulence (1, 2, 5). This suggests that invasion into the bloodstream does not require special bacterial virulence traits but mainly depends on host factors, e.g. barrier breakage, indwelling catheters, or a compromised immune system.

PVL-positive CC121 isolates are the main cause of furunculosis

Skin and soft tissue infections, like furunculosis, are the most frequent diseases caused by *S. aureus* outside the hospital setting. PVL is a pore-forming toxin and is epidemiologically linked to chronic or recurrent skin and soft-tissue infections. We have used a molecular-epidemiological approach with the aim to dissect linkage of furunculosis with PVL, the PVL-encoding phage and its PVL-negative counterpart, with other virulence genes (SAgs and exfoliative toxins), and with the genetic background of clinical *S. aureus* isolates (almost all MSSA) **(Chapter 4)**. Therefore, we analyzed 74 *S. aureus* isolates from furunculosis patients and 108 nasal *S. aureus* isolates from healthy carriers from Szczecin, Poland. We show that both the genetic background, namely lineages CC121 and CC22 (70% of all furunculosis isolates), and PVL (85%) are tightly linked to the furunculosis-inducing phenotype. The linkage between PVL and furunculosis can be separated from the contribution of the genetic background as well as from the other genes that are located on the PVL-carrying phage. These findings indicate an important role for PVL in human furunculosis. Except for *seb*, which was within CC121 significantly overrepresented among furunculosis isolates in comparison to nasal isolates (56.1% vs. 0%; $P \leq 0.05$), no SAg was associated with furunculosis.

The study demonstrates that comparing clinical isolates from well-defined diseases can indeed provide novel insights into *S. aureus* virulence. Moreover, our results are of clinical importance. PVL is mainly discussed as a virulence factor of CA-MRSA. Most clinicians, however, are not aware that PVL-positive MSSA strains, which are much more

common, at least in Europe, can also cause serious and long-lasting symptoms, which require repeated antibiotic treatment and/or surgical intervention. In our opinion, a test for PVL should be included in the diagnostic work-up of recurrent skin infections. The therapeutic goal should then be the elimination of these *S. aureus* strains of increased virulence from the patient and his environment.

8.2 ANTI-STAPHYLOCOCCAL ANTIBODY RESPONSE IN COLONIZATION AND BACTEREMIA

The fact that a large number of studies, including those described in Chapters 2 and 3, were unable to point to a fundamental difference between *S. aureus* populations isolated from blood culture and the nose, emphasizes the role of host factors, such as immunity. Especially the observation that in bacteremia the mortality of *S. aureus* carriers is significantly lower than that in noncarriers (24), raised the question whether the adaptive immune system establishes immunity to the colonizing strains, which could be of advantage in endogenous infection. In support of this hypothesis, our group recently showed a strain-specific neutralizing antibody response in carriers against the SAg cocktail produced by their colonizing strain (25). It has been shown that such SAg neutralizing antibodies can be protective in patients as well as in animal models (26-29). On the other hand, noncarriers may harbor antibodies that protect them from nasal colonization (30, 31).

Levels of antibody binding to TSST-1 were highly related to neutralizing capacity

There are several methods to measure antigen-specific antibody titers. The most commonly used test is the enzyme-linked immuno-sorbent assay (ELISA), which measures antibody binding to native, immobilized antigens. Verkaik et al. recently established a multiplex immunoassay based on the Luminex® technology that allows the simultaneous quantification of antibodies against 19 *S. aureus* antigens (32) **(Chapter 5)**. Sera from 15 persistent carriers and 19 noncarriers were analyzed for IgG, IgA, and IgM binding to nine microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which mediate *S. aureus* adhesion, three immune-modulatory proteins, which inhibit the innate immune response by interfering with the complement cascade or recruitment of neutrophils, and seven SAgs (SEA, SEB, SEI, SElM, SElO, SElQ, and TSST-1).

In accordance with earlier reports, the healthy individuals harbored a broad range of anti-staphylococcal antibodies with an extensive interindividual variability in the antibody binding pattern (25, 33-35), reflecting the individual history of encounters with *S. aureus*. Median levels of antibodies to TSST-1, SEA, ClfA, and ClfB were higher in persistent carriers than in noncarriers.

Since antibody function is clinically most relevant we performed Luminex® and neutralization assays for TSST-1 in parallel. IgG binding to TSST-1 strongly correlated with the neutralizing capacity of the serum samples (R^2=0.93).

Additionally, the results support earlier observations from neutralization assays, which showed high neutralizing capacity of non-*egc* SAgs, but a lack of neutralizing antibodies against *egc* SAgs in sera of the healthy population (33).

Egc SAgs do not elicit a neutralizing antibody response during bacteremia

It remains largely unkown which conditions (e.g., nasal colonization, minor or major infections) are required to trigger an antibody response against different *S. aureus* antigens. Healthy individuals harbor a broad range of anti-staphylococcal antibodies. In *S. aureus* carriers, these antibodies are specific for their colonizing strain (25, 35). Our group recently demonstrated, that short-term experimental nasal colonization is not sufficient to induce a robust systemic antibody response (34). We therefore assume that most anti-*S. aureus* serum antibody responses are elicited by (minor) infections, as it has been reported for selected antigens (36-38). Therefore, we tested whether *egc* and non-*egc* SAgs elicit a neutralizing antibody response during systemic infection. In particular, we investigated the anti-SAg antibody response in *S. aureus* bacteremia among nonaddicts previously less exposed to *S. aureus* and among IDUs with more frequent contact to it **(Chapter 6)**.

In this clinical trial sera from 25 IDUs and 37 nonaddicst were obtained at the acute phase of bacteremia and three to four weeks thereafter (convalescent phase). Neutralizing capacity of the sera was tested against the SAg cocktail produced by the respective infecting strain as well as a panel of representative recombinant SAgs.

At the acute phase of bacteremia most patients already possessed neutralizing antibodies against non-*egc* SAgs. This finding is in agreement with the frequent observation of neutralizing antibodies against non-*egc* SAgs in the healthy population

(33, 39-41). However, neutralizing antibody titers were low in most nonaddicts. A possible explanation could be that the majority of them suffered from exogenous infections (25). The SAg-neutralizing capacity was higher in IDUs, probably due to intensive exposure to *S. aureus* (42, 43), as higher colonization rates and more frequent endogenous infections are described for IDUs than in the general population (19, 20).

In IDUs but not in nonaddicts, bacteremia increased the neutralizing serum capacity. Similarly, other investigators observed that antibodies against *S. aureus* antigens are generated or boosted during *S. aureus* infections (e.g., bacteremia and wound infection) (31, 38, 44). Only a few nonaddicts responded to bacteremia with an increase of SAg neutralizing antibodies. It seems that bacteremia rarely primes high affinity antibody responses, which would be required for SAg neutralization, but it appears that this immune stimulus is strong enough to boost pre-existing B cell memory.

Notably, neutralizing antibodies against *egc* SAgs were rare at the acute phase of bacteremia and were not induced during infection. The findings were similar for supernatants from *egc*-positive strains and for recombinant SEI and extend earlier findings in the healthy population (33).

In summary, the boost of neutralizing antibody titers clearly shows that the immune system is exposed to non-*egc* SAgs during *S. aureus* bloodstream invasion. This remains unknown for *egc*-encoded SAgs, which did not elicit a boost or de novo generation of specific antibodies, neither in colonization nor in *S. aureus* bacteremia. Furthermore, the strong specific anti-SAg antibody response questions the assumption, that the function of SAgs would be the deletion of T cells that help B cells to mount an effective antibody response (45). According to McLoughlin et al. T cell-derived IFNγ facilitates a CXC chemokine-driven recruitment of neutrophils to the infection side (46). Besides their critical role in *S. aureus* clearance, neutrophils can also contribute to pathogenesis by harboring viable *S. aureus* intracellulary (47). Therefore, SAg-dependent T cell activation might serve *S. aureus* to manipulate the function of granulocytes to its advantage.

8.3 COMPARISON OF *EGC* AND NON-*EGC* SAGS – WHAT IS THE REASON FOR THE LACK OF ANTI-*EGC* ANTIBODIES?

Neutralizing antibodies against *egc* SAgs are very uncommon in both healthy individuals and bacteremia patients. This was surprising because of the high prevalence of *egc* genes in clinical *S. aureus* isolates (Chapter 2) (6, 12, 48). Despite this, *egc* SAgs are not a prominent cause of TSS (49). In search for an explanation, we have tested two non-exclusive hypotheses: i) *egc* and non-*egc* SAgs have unique intrinsic properties and drive the immune system into different directions and ii) *egc* and non-*egc* SAgs are released by *S. aureus* under different conditions, which shape the immune response to them **(Chapter 7)**.

Egc and non-*egc* SAgs have similar T cell activating properties

If intrinsic properties of *egc* and non-*egc* SAgs were responsible for the different antibody response, one might expect them to differ in their T cell-activating properties. Therefore, we compared the effect of three recombinant *egc* SAgs (SEI, SElM, and SElO) and three non-*egc* SAgs (SEB, SElQ, and TSST-1) on human blood cells.

First, we compared the T cell-mitogenic properties of *egc* and non-*egc* SAgs. Therefore, we stimulated human PBMCs from nine healthy blood donors with the three recombinant *egc* and non-*egc* SAgs. All six SAgs induced strong dose-dependent proliferation. In agreement with earlier studies, the mitogenic concentrations for non-*egc* staphylococcal SAgs were in the picogram or even femtogram range (50-52). Hence, except for SElO we found no significant differences between the T cell mitogenic potencies of *egc* and non-*egc* SAgs.

Second, we analyzed the induced cytokine profiles, since non-*egc* SAgs are notorious for their ability to induce a massive cytokine response in T cells and APCs. Human PBMCs from seven blood donors were stimulated with recombinant SAgs in concentrations 10-fold above the lowest concentration, which elicited maximal proliferation (100pg/ml to 100ng/ml). After 72h the cytokine concentrations in the supernatant were determined. All SAgs induced high amounts of pro-inflammatory (IFN-γ, TNF-α, IL-2) and lower concentrations of anti-inflammatory (IL-4, IL-5, IL-10) cytokines. Similarly, others have reported the induction of Th1- and Th2-cytokines after stimulation with a number of non-*egc* staphylococcal and streptococcal SAgs (53-55). Despite considerable

interindividual variations, we observed no significant differences between the cytokine profiles triggered by *egc* and non-*egc* SAgs. In contrast, Dauwalder et al. reported that stimulation of human PBMC with very high concentrations (100ng/ml) of SEA induced a stronger Th1-response than the *egc* SAg SEG.

For a comprehensive view on the SAg response, we finally performed gene expression analysis of PBMC from three blood donors. After 6h of stimulation with one representative SAg of each group (SEI and SEB) transcriptional changes were analyzed with Affymetrix expression arrays. SAgs were used in concentrations 10-fold above the lowest concentration, which elicited maximal proliferation (10pg/ml to 100pg/ml). Stimulation with SEI and/or SEB changed the transcription of 391 genes (511 probe sets, FDR < 0.05), two-thirds of which were up-regulated. This is in agreement with other studies, in which PBMCs were treated with SEA or SEB (56, 57). Importantly, the vast majority of the genes were influenced to a similar extend and no gene was regulated in opposite directions by SEI and SEB. Different analysis tools (hierarchical clustering, and principal component analysis) revealed that the effects of SEI and SEB on PBMC gene expression were very similar. In fact, interindividual differences between the technical replicates which used different blood donors were stronger than the differences between both SAgs. Among the most strongly induced genes were those encoding for the T cell activation markers CD69, CD40L, and CD25 and for the cytokines IL-2, IFN-γ and IL17A. These findings confirmed our results of T cell proliferation and cytokine measurements and indicate a very strong Th1- and Th17 response such as one might expect from SAgs. In contrast, Dauwalder et al. observed a broad transcriptional activation after stimulation of human PBMCs with high concentrations (10ng/ml) of SEA, whereas SEG (10ng/ml) had no detectable effect (56). Either SEG has unique properties among the *egc* SAgs or the different results are due to the different SAg concentrations used and/ or production and purification procedures.

In summary, the immune cell-activating properties of *egc* and non-*egc* SAgs, their superantigenicity, was very similar in every aspect studied and cannot explain the striking differences in the immune response to *egc* and non-*egc* SAgs. Furthermore, a systematically difference in the immunogenicity of both groups of SAgs is unlikely, since the amino acid sequences of the *egc* SAgs are more closely related to those of individual non-*egc* SAgs than to each other (58, 59).

The *egc* gap in the anti-SAg antibody profile is probably due to a differential regulation of SAg gene expression

In search for an explanation for the lack of neutralizing antibodies against *egc* SAgs, we finally focussed on the regulation of the SAg release by *S. aureus*. We cultured two clinical isolates with only *egc* SAg genes and two with only non-*egc* SAgs genes, obtained culture supernatants at different optical densities and used proliferation assays as a read-out system for SAg secretion. Strains encoding non-*egc* SAgs induced proliferation at high optical densities, indicating that these SAgs are secreted in the late stationary growth phase. In contrast, *egc* SAgs were released during exponential growth phase and secretion decreased at higher bacterial densities. Similar observations have been reported on mRNA-level (58, 60, 61).

To date it is unclear, why the *egc*-operon is selectively transcribed during early exponential growth. In vitro data suggest that *egc* SAgs are generally produced in very small amounts (60, 62). The regulation of staphylococcal SAgs is complex, and besides the accessory gene regulator, the staphylococcal accessory regulator, the alternative σ factor, and the regulator of toxins also have been shown to play a role (63-67). Recently, Derzelle et al. analyzed expression of 18 SAgs by quantitative reverse transcription-PCR and observed four distinct expression patterns: i) unchanged mRNA abundance during bacterial growth (*sea, see, sej, sek, seq* and *sep*); ii) decrease in transcript levels (*seg, sei, sem, sen, seo, seu*); iii) drastic inductions of expression at the end of the exponential growth phase (*seb, sec, seh)* or iv) modest post-exponential increase in mRNA level (*sed, ser, sel*) (60). The decrease of *egc* mRNA levels in the late exponential growth phase might be the reason for the rapid drop of T cell proliferation with increasing bacterial densities. Because from this point the concentrations of *egc* SAgs decreases on a per cell basis. However, most research has been performed by investigation of standard laboratory *S. aureus* strains cultivated in rich medium. The challenge will now be to clarify, which of these processes are effective during the interactions of wild type strains with the host. There is evidence that the regulatory circuits *S. aureus* employs during infection (and probably also colonization) differ strongly from those characterized in vitro (68, 69). It remains unknown, how this affects the release of *egc* and non-*egc* SAgs in vivo and the immune response against them.

So far, the high prevalence of neutralizing serum antibodies against non-*egc* SAgs allows the conclusion that most healthy adults have been exposed to these toxins during their

encounters with *S. aureus*. The *egc* gap in the anti-SAg profile, which we observed in healthy adults (Chapter 5) (33), and following bloodstream invasion (Chapter 6) shows that differential regulation of antigen expression has an impact on the immune response. It remains an open question whether *egc* SAgs are i) expressed in vivo, ii) in which quantities, and iii) under which conditions.

In cooperation with Marc Burian, we performed a pilot study to approach this problem. *S. aureus* was isolated from the nose of carriers, and directly ex vivo, RNA was analyzed and the level of transcription of non-*egc* (*sea* and *sec*) SAgs and one *egc* SAg gene was determined by quantitative real-time PCR. In the nose of healthy adults there was strong transcription of the *egc* SAg *selo*, while transcription of *sea* and *sec* was weak (Burian et al., manuscript in preparation). Thus it appears that the functional state of *S. aureus* during nasal colonization – transcription of *egc* SAgs - shares features with exponential growth in vitro, while invasion – expression of non-*egc* SAgs - more closely resembles the post-exponential growth phase in bacterial cell culture.

REFERENCES

1. Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day. 2003. How clonal is *Staphylococcus aureus*? *Journal of bacteriology* 185:3307-3316.

2. Melles, D. C., R. F. Gorkink, H. A. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and A. van Belkum. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. *J Clin Invest* 114:1732-1740.

3. Wertheim, H. F., W. B. van Leeuwen, S. Snijders, M. C. Vos, A. Voss, C. M. Vandenbroucke-Grauls, J. A. Kluytmans, H. A. Verbrugh, and A. van Belkum. 2005. Associations between *Staphylococcus aureus* Genotype, Infection, and In-Hospital Mortality: A Nested Case-Control Study. *The Journal of infectious diseases* 192:1196-1200.

4. Lindsay, J. A., and M. T. Holden. 2004. *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol* 12:378-385.

5. Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds. 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *Journal of bacteriology* 188:669-676.

6. Monecke, S., C. Luedicke, P. Slickers, and R. Ehricht. 2009. Molecular epidemiology of *Staphylococcus aureus* in asymptomatic carriers. *Eur J Clin Microbiol Infect Dis* 28:1159-1165.

7. Lindsay, J. A., and M. T. Holden. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Functional & integrative genomics* 6:186-201.

8. Lindsay, J. A., A. Ruzin, H. F. Ross, N. Kurepina, and R. P. Novick. 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular microbiology* 29:527-543.

9. Monecke, S., B. Berger-Bachi, G. Coombs, A. Holmes, I. Kay, A. Kearns, H. J. Linde, F. O'Brien, P. Slickers, and R. Ehricht. 2007. Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. *Clin Microbiol Infect* 13:236-249.

10. Monecke, S., P. Slickers, M. J. Ellington, A. M. Kearns, and R. Ehricht. 2007. High diversity of Panton-Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus*. *Clin Microbiol Infect* 13:1157-1164.

11. Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet* 367:731-739.

12. Fueyo, J. M., M. C. Mendoza, M. A. Alvarez, and M. C. Martin. 2005. Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. *FEMS microbiology letters* 243:447-454.

13. Fueyo, J. M., M. C. Mendoza, and M. C. Martin. 2005. Enterotoxins and toxic shock syndrome toxin in *Staphylococcus aureus* recovered from human nasal carriers and manually handled foods: epidemiological and genetic findings. *Microbes Infect* 7:187-194.

14. Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day. 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infection and immunity* 70:4987-4996.

15. Waldron, D. E., and J. A. Lindsay. 2006. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *Journal of bacteriology* 188:5578-5585.

16. Goerke, C., C. Wirtz, U. Fluckiger, and C. Wolz. 2006. Extensive phage dynamics in *Staphylococcus aureus* contributes to adaptation to the human host during infection. *Molecular microbiology* 61:1673-1685.

17. van Wamel, W. J., S. H. Rooijakkers, M. Ruyken, K. P. van Kessel, and J. A. van Strijp. 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. *Journal of bacteriology* 188:1310-1315.

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

19. Tuazon, C. U., and J. N. Sheagren. 1974. Increased rate of carriage of *Staphylococcus aureus* among narcotic addicts. *The Journal of infectious diseases* 129:725-727.

20. Tuazon, C. U., and J. N. Sheagren. 1975. Staphlococcal endocarditis in parenteral drug abusers: source of the organism. *Annals of internal medicine* 82:788-790.

21. Donker, G. A., R. H. Deurenberg, C. Driessen, S. Sebastian, S. Nys, and E. E. Stobberingh. 2009. The population structure of *Staphylococcus aureus* among general practice patients from The Netherlands. *Clin Microbiol Infect* 15:137-143.

22. Fowler, V. G., Jr., C. L. Nelson, L. M. McIntyre, B. N. Kreiswirth, A. Monk, G. L. Archer, J. Federspiel, S. Naidich, B. Remortel, T. Rude, P. Brown, L. B. Reller, G. R. Corey, and S. R. Gill. 2007. Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *The Journal of infectious diseases* 196:738-747.

23. Monk, A. B., S. Curtis, J. Paul, and M. C. Enright. 2004. Genetic analysis of *Staphylococcus aureus* from intravenous drug user lesions. *Journal of medical microbiology* 53:223-227.

24. Wertheim, H. F., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh. 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* 364:703-705.

25. Holtfreter, S., K. Roschack, P. Eichler, K. Eske, B. Holtfreter, C. Kohler, S. Engelmann, M. Hecker, A. Greinacher, and B. M. Broker. 2006. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. *The Journal of infectious diseases* 193:1275-1278.

26. Bavari, S., B. Dyas, and R. G. Ulrich. 1996. Superantigen vaccines: a comparative study of genetically attenuated receptor-binding mutants of staphylococcal enterotoxin A. *The Journal of infectious diseases* 174:338-345.

27. LeClaire, R. D., R. E. Hunt, and S. Bavari. 2002. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. *Infection and immunity* 70:2278-2281.

28. Nilsson, I. M., M. Verdrengh, R. G. Ulrich, S. Bavari, and A. Tarkowski. 1999. Protection against *Staphylococcus aureus* sepsis by vaccination with recombinant staphylococcal enterotoxin A devoid of superantigenicity. *The Journal of infectious diseases* 180:1370-1373.

29. Ulrich, R. G., M. A. Olson, and S. Bavari. 1998. Development of engineered vaccines effective against structurally related bacterial superantigens. *Vaccine* 16:1857-1864.

30. Clarke, S. R., K. J. Brummell, M. J. Horsburgh, P. W. McDowell, S. A. Mohamad, M. R. Stapleton, J. Acevedo, R. C. Read, N. P. Day, S. J. Peacock, J. J. Mond, J. F. Kokai-Kun, and S. J. Foster. 2006. Identification of in vivo-expressed antigens of *Staphylococcus aureus* and their use in vaccinations for protection against nasal carriage. *The Journal of infectious diseases* 193:1098-1108.

31. Dryla, A., S. Prustomersky, D. Gelbmann, M. Hanner, E. Bettinger, B. Kocsis, T. Kustos, T. Henics, A. Meinke, and E. Nagy. 2005. Comparison of antibody repertoires against *Staphylococcus aureus* in healthy individuals and in acutely infected patients. *Clinical and diagnostic laboratory immunology* 12:387-398.

32. Verkaik, N., E. Brouwer, H. Hooijkaas, A. van Belkum, and W. van Wamel. 2008. Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins. *Journal of immunological methods* 335:121-125.

33. Holtfreter, S., K. Bauer, D. Thomas, C. Feig, V. Lorenz, K. Roschack, E. Friebe, K. Selleng, S. Lovenich, T. Greve, A. Greinacher, B. Panzig, S. Engelmann, G. Lina, and B. M. Broker. 2004. *egc*-Encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. *Infection and immunity* 72:4061-4071.

34. Holtfreter, S., T. T. Nguyen, H. Wertheim, L. Steil, H. Kusch, Q. P. Truong, S. Engelmann, M. Hecker, U. Volker, A. van Belkum, and B. M. Broker. 2009. Human immune proteome in experimental colonization with *Staphylococcus aureus*. *Clin Vaccine Immunol* 16:1607-1614.

35. Kolata, J., L. G. Bode, S. Holtfreter, L. Steil, H. Kusch, B. Holtfreter, M. Hecker, S. Engelmann, A. van Belkum, U. Volker, and B. M. Broker. Distinctive patterns in the human antibody response to *Staphylococcus aureus* bacteremia revealed by immune secretome analysis. submitted.

36. Burlak, C., C. H. Hammer, M. A. Robinson, A. R. Whitney, M. J. McGavin, B. N. Kreiswirth, and F. R. Deleo. 2007. Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. *Cellular microbiology* 9:1172-1190.

37. Colque-Navarro, P., B. Soderquist, H. Holmberg, L. Blomqvist, P. Olcen, and R. Mollby. 1998. Antibody response in *Staphylococcus aureus* septicaemia--a prospective study. *Journal of medical microbiology* 47:217-225.

38. Kanclerski, K., B. Soderquist, M. Kjellgren, H. Homberg, and R. Mollby. 1996. Serum antibody response to *Staphylococcus aureus* enterotoxins and TSST-1 in patients with septicaemia. *Journal of medical microbiology* 44:171-177.

39. Schroder, E., G. Kunstmann, H. Hasbach, and G. Pulverer. 1988. Prevalence of serum antibodies to toxic-shock-syndrom-toxin-1 and to staphylococcal enterotoxins A, B and C in West-Germany. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 270:110-114.

40. Kunstmann, G., E. Schroder, H. Hasbach, and G. Pulverer. 1989. Immune response to toxic-shock-syndrome toxin-1 (TSST-1) and to staphylococcal enterotoxins A, B and C in *Staphylococcus aureus* infections. *Zentralblb Bakteriol* 271:486-492.

41. Takei, S., Y. K. Arora, and S. M. Walker. 1993. Intravenous immunoglobulin contains specific antibodies inhibitory to activation of T cells by staphylococcal toxin superantigens [see comment]. *J Clin Invest* 91:602-607.

42. Bassetti, S., and M. Battegay. 2004. *Staphylococcus aureus* infections in injection drug users: risk factors and prevention strategies. *Infection* 32:163-169.

43. Lowy, F. D., and M. Miller. 2002. New methods to investigate infectious disease transmission and pathogenesis--*Staphylococcus aureus* disease in drug users. *The Lancet infectious diseases* 2:605-612.

44. Jozefczyk, Z. 1974. Specific human antibodies to enterotoxins A, B, and C1 of Staphylococcus: their increased synthesis in staphylococcal infection. *The Journal of infectious diseases* 130:1-7.

45. Fraser, J., V. Arcus, P. Kong, E. Baker, and T. Proft. 2000. Superantigens - powerful modifiers of the immune system. *Mol Med Today* 6:125-132.

46. McLoughlin, R. M., J. C. Lee, D. L. Kasper, and A. O. Tzianabos. 2008. IFN-gamma regulated chemokine production determines the outcome of *Staphylococcus aureus* infection. *J Immunol* 181:1323-1332.

47. Gresham, H. D., J. H. Lowrance, T. E. Caver, B. S. Wilson, A. L. Cheung, and F. P. Lindberg. 2000. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* 164:3713-3722.

48. Becker, K., A. W. Friedrich, G. Peters, and C. von Eiff. 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO, and SElN. *Mol Nutr Food Res* 48:488-495.

49. Jarraud, S., G. Cozon, F. Vandenesch, M. Bes, J. Etienne, and G. Lina. 1999. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *Journal of clinical microbiology* 37:2446-2449.

50. Orwin, P. M., J. R. Fitzgerald, D. Y. Leung, J. A. Gutierrez, G. A. Bohach, and P. M. Schlievert. 2003. Characterization of *Staphylococcus aureus* enterotoxin L. *Infection and immunity* 71:2916-2919.

51. Orwin, P. M., D. Y. Leung, H. L. Donahue, R. P. Novick, and P. M. Schlievert. 2001. Biochemical and biological properties of Staphylococcal enterotoxin K. *Infection and immunity* 69:360-366.

52. Orwin, P. M., D. Y. Leung, T. J. Tripp, G. A. Bohach, C. A. Earhart, D. H. Ohlendorf, and P. M. Schlievert. 2002. Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* 41:14033-14040.

53. Norrby-Teglund, A., S. Chatellier, D. E. Low, A. McGeer, K. Green, and M. Kotb. 2000. Host variation in cytokine responses to superantigens determine the severity of invasive group A streptococcal infection. *Eur J Immunol* 30:3247-3255.

54. Proft, T., B. Schrage, and J. D. Fraser. 2007. The cytokine response to streptococcal superantigens varies between individual toxins and between individuals: implications for the pathogenesis of group A streptococcal diseases. *J Interferon Cytokine Res* 27:553-557.

55. Rink, L., J. Luhm, M. Koester, and H. Kirchner. 1996. Induction of a cytokine network by superantigens with parallel TH1 and TH2 stimulation. *J Interferon Cytokine Res* 16:41-47.

56. Dauwalder, O., A. Pachot, M. A. Cazalis, M. Paye, C. Faudot, C. Badiou, B. Mougin, F. Vandenesch, J. Etienne, G. Lina, and G. Monneret. 2009. Early kinetics of the transcriptional response of human leukocytes to staphylococcal superantigenic enterotoxins A and G. *Microbial pathogenesis* 47:171-176.

57. Mendis, C., R. Das, R. Hammamieh, A. Royaee, D. Yang, S. Peel, and M. Jett. 2005. Transcriptional response signature of human lymphoid cells to staphylococcal enterotoxin B. *Genes Immun* 6:84-94.

58. Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* 166:669-677.

59. Thomas, D., S. Chou, O. Dauwalder, and G. Lina. 2007. Diversity in *Staphylococcus aureus* enterotoxins. *Chem Immunol Allergy* 93:24-41.

60. Derzelle, S., F. Dilasser, M. Duquenne, and V. Deperrois. 2009. Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. *Food microbiology* 26:896-904.

61. Munson, S. H., M. T. Tremaine, M. J. Betley, and R. A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infection and immunity* 66:3337-3348.

62. Omoe, K., M. Ishikawa, Y. Shimoda, D. L. Hu, S. Ueda, and K. Shinagawa. 2002. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates Harboring *seg*, *seh*, or *sei* genes. *Journal of clinical microbiology* 40:857-862.

63. Bischoff, M., P. Dunman, J. Kormanec, D. Macapagal, E. Murphy, W. Mounts, B. Berger-Bachi, and S. Projan. 2004. Microarray-based analysis of the *Staphylococcus aureus* sigmaB regulon. *Journal of bacteriology* 186:4085-4099.

64. Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan. 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *Journal of bacteriology* 183:7341-7353.

65. Novick, R. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular microbiology* 48:1429-1449.

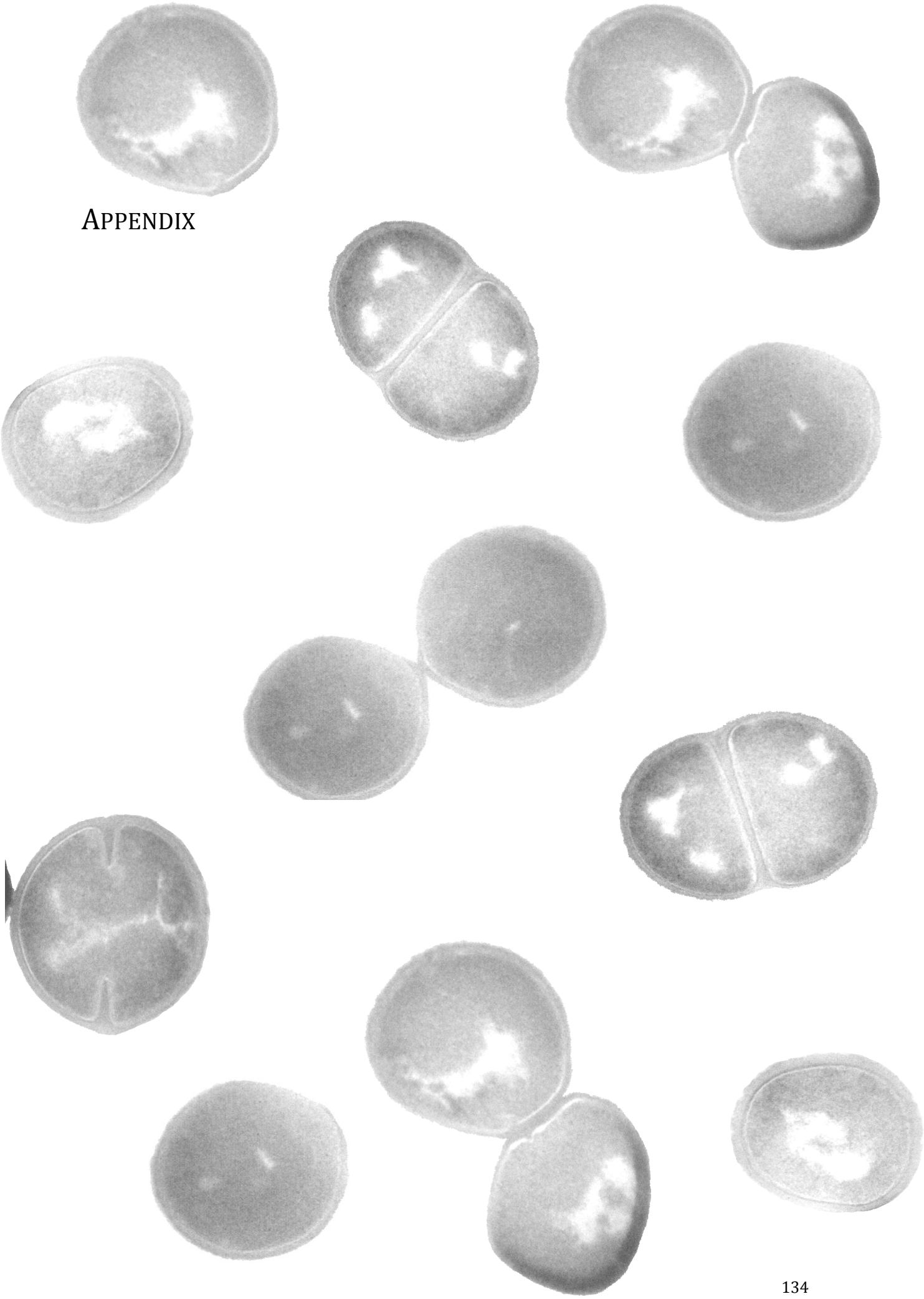
66. Said-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan, and B. N. Kreiswirth. 2003. Global regulation of *Staphylococcus aureus* genes by Rot. *Journal of bacteriology* 185:610-619.

67. Ziebandt, A. K., D. Becher, K. Ohlsen, J. Hacker, M. Hecker, and S. Engelmann. 2004. The influence of agr and sigmaB in growth phase dependent regulation of virulence factors in *Staphylococcus aureus. Proteomics* 4:3034-3047.

68. Goerke, C., S. Campana, M. G. Bayer, G. Doring, K. Botzenhart, and C. Wolz. 2000. Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. *Infection and immunity* 68:1304-1311.

69. Goerke, C., and C. Wolz. 2004. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. *Int J Med Microbiol* 294:195-202.

APPENDIX



SUMMARY

Staphylococcus (S.) aureus is the most common cause of nosocomial infections and the species is becoming increasingly resistant to antibiotics. In contrast, about 35% of the healthy population are colonized with *S. aureus* in the anterior nares and usually do not suffer from serious symptoms. However, nasal colonization is a major risk factor for staphylococcal infection. Paradoxically, in case of bacteremia, which in carriers is usually caused by the colonizing strain, the prognosis of carriers is much better than that of noncarriers.

The genetic make-up of the species *S. aureus* is highly diverse. Mobile genetic elements comprise about 15% of the *S. aureus* genome. They encode many virulence factors like the 21 different known staphylococcal superantigens (SAgs), highly potent activators of T lymphocytes. Besides their well known causative role in food poisoning and toxic shock syndrome, information about SAg involvement in pathogenesis is limited. On the other hand, the human host and its immune response are also highly diverse. The impressive variability of pathogen and host may explain, why up until now efforts to develop an effective anti-*S. aureus* vaccine have not been successful.

This study focuses on SAgs, because they are potent virulence factors that are highly diverse and therefore mirror of the variability of the species *S. aureus*. The goals of this work were

- to search for molecular-epidemiological associations between SAgs and different diseases caused by *S. aureus,*
- to determine the prevalence and the development of anti-SAg antibodies in healthy *S. aureus* carriers and noncarriers as well as in bacteremia patients, and
- to elucidate the reasons for the selective lack of neutralizing serum antibodies specific for a subgroup of SAgs, the *egc* SAgs.

SAgs are encoded on mobile genetic elements, such as bacteriophages, plasmids, and pathogenicity islands, which can be distributed by two distinct mechanisms: vertical transmission to daughter cells and horizontal transfer by bacteriophages and conjugation. We investigated the distribution of SAg genes and/ or bacteriophages by multiplex PCR and correlated this with the clonal background, determined by *spa*

genotyping. Altogether we analyzed more than 700 *S. aureus* isolates from nasal colonization, bacteremia or furunculosis. SAg-encoding mobile genetic elements and bacteriophages were not randomly distributed, but rather strongly associated with the clonal background. As a consequence, each clonal lineage was characterized by a typical SAg gene and phage repertoire. However, within clonal lineages and even within the same *spa* type we observed considerable variation, suggesting that within lineages mobile genetic elements were frequently acquired and lost.

We suggest that the simultaneous assessment of virulence gene profiles and the genetic background strongly increases the discriminatory power of genetic investigations into the mechanisms of *S. aureus* pathogenesis. Indeed, this approach indicated a role for the mobile genetic element encoding exfoliative toxin d, and for the intact β-hemolysin gene in blood stream invasion, while rendering it unlikely for SAgs. Moreover, we observed a strong association between Panton-Valentine leukocidin and furunculosis, which was independent of the clonal background. Except for *seb*, which was significantly more frequent among furunculosis-associated than among nasal CC121 strains, SAg genes were not associated with furunculosis.

While functional neutralization assays closely mimic the protective action of anti-SAg antibodies in vivo, they are labor-intensive and time-consuming. A fast and easy method for the simultaneous quantification of antibody binding to multiple staphylococcal antigens is the Luminex® technology. Using serum samples from 15 persistent carriers and 19 noncarriers we showed a strong correlation between antibody binding and neutralizing capacity against the clinically important SAg TSST-1. This assay confirmed the astonishing lack of antibodies against *egc* SAgs in healthy carriers and noncarriers, which was previously described by Holtfreter and coworkers.

Since colonization is probably not sufficient to induce a robust antibody response as revealed by experimental colonization with *S. aureus*, we propose that (minor) infections are required to induce the high titers of non-*egc* SAg-neutralizing antibodies in healthy adults. To test this, we investigated whether SAgs elicit a neutralizing antibody response during *S. aureus* bacteremia (n = 64). At the acute phase of the disease most patients already had neutralizing antibody against non-*egc* SAgs, and antibody titers frequently increased during infection. Notably, *egc* SAgs did not elicit a boost or de novo generation of specific antibodies.

The "*egc* gap" in the antibody response, which has now been shown in healthy adults, carriers and noncarriers, as well as following systemic infection with *S. aureus* strains that harbor *egc* SAg genes, is astonishing. After all, *egc* SAgs are by far the most prevalent SAgs. In search for an explanation, the intrinsic properties of three recombinant *egc* (SEI, SElM, SElO) and non-*egc* SAgs (SEB, SElQ, TSST-1) were compared in depth. *Egc* and non-*egc* were very similar with regard to induced T cell proliferation, cytokine profiles, and gene expression of human peripheral blood mononuclear cells. Both SAg groups stimulated a strong Th1- and Th17 response. However, there was a striking difference in the regulation of the two groups of SAgs by *S. aureus*: *Egc* SAgs were secreted in exponential growth during bacterial cell culture, while non-*egc* SAgs – like most virulence factors – were secreted in the post-exponential growth phase. We conclude that the differential regulation of *egc* and non-*egc* SAg has an impact on the immune response.

But how are SAgs regulated by *S. aureus* during its interaction with the host? Up until now most research on regulation of virulence factors has been performed in vitro and information about the in vivo behavior of the bacteria is difficult to generate, especially in humans. The immune response can help to shed light on this problem, because it is an exquisitely specific sensor for the exposure to different antigens. The high prevalence of neutralizing serum antibodies against non-*egc* SAgs indicates that most healthy adults have been exposed to these toxins during their encounters with *S. aureus*. In particular invasive episodes trigger such an antibody response as was shown in this work, which provides evidence that non-*egc* SAgs are produced during *S. aureus* bloodstream invasion. For *egc* SAgs this remains an open question. However, initial data indicate that the *egc* SAg genes are primarily transcribed during nasal colonization.

Using SAgs as a model system, this thesis emphasizes the versatility of the species *S. aureus* (and also of individual bacterial strains), in its multifaceted encounters with the human host. Monovalent vaccines are not likely to be protective in these complex interactions. It will be a considerable challenge to determine which of the numerous virulence factors, including the wealth of SAgs, should be included in the antigen cocktail of a carefully composed anti-*S. aureus* vaccine.

ZUSAMMENFASSUNG

Staphylococcus (S.) aureus ist die häufigste Ursache für nosokomiale Infektionen. Die zunehmende Entwicklung von Antibiotikaresistenzen ist deshalb besorgniserregend. Gleichzeitig besiedelt *S. aureus* die Nasenschleimhaut von etwa 35% der gesunden Bevölkerung. Diese sogenannten Carrier haben ein erhöhtes Risiko für *S. aureus*-Infektionen, die meist durch ihren endogenen *S. aureus* Stamm ausgelöst werden. Überraschenderweise haben Carrier im Fall einer Infektion eine deutlich bessere Prognose als Nichtcarrier.

Das Genom der Spezies *S. aureus* ist hoch variabel - etwa 15% besteht aus mobilen genetischen Elementen. Hier werden hauptsächlich Virulenzfaktoren wie die 21 bekannten Superantigene (SAgs) kodiert. SAgs sind sehr potente T-Zell-Mitogene und können *S. aureus* Lebensmittelvergiftungen und das Toxische-Schock-Syndrom auslösen. Über ihre Rolle bei anderen Krankheitsbildern ist wenig bekannt. SAgs werden allerdings nicht immer wirksam, denn neutralisierende Antikörper können SAgs binden und somit vor ihrer toxischen Wirkung schützen. Die hohe Diversität von *S. aureus*-Stämmen und die Komplexität der Immunantwort des Wirts könnten erklären, weshalb bisher alle Anstrengungen, einen effektiven Impfstoff gegen *S. aureus* zu entwickeln, gescheitert sind.

SAgs stehen im Mittelpunkt dieser Arbeit, da diese potenten Virulenzfaktoren mit ihrer Diversität die außergewöhnliche Variablität der Spezies *S. aureus* widerspiegeln. Die Arbeit hatte folgende Ziele:

- Die Suche nach einem molekular-epidemiologischen Zusammenhang zwischen SAgs und verschiedenen durch *S. aureus* verursachten Krankheiten,
- die Bestimmung der Prävalenz und Entwicklung von Antikörpern gegen SAgs bei gesunden *S. aureus* Carriern und Nichtcarriern sowie bei Bakteriämie-Patienten und
- die Aufklärung der Ursache für das selektive Fehlen von neutralisierenden Serumantikörpern gegen eine Untergruppe von SAgs, die *egc*-SAgs.

SAgs sind auf mobilen genetischen Elementen kodiert, auf Bakteriophagen, Plasmiden und Pathogenitätsinseln. Diese Elemente können durch zwei unabhängige Mechanismen

weitergegeben werden, durch vertikalen Transfer auf Tochterzellen oder durch horizontale Übertragung mit Bakteriophagen oder Konjugation. Wir haben die Verteilung von SAg-Genen und/ oder Bakteriophagen mittels Multiplex-PCR analysiert und mit dem genetischen Hintergrund der *S. aureus*-Isolate korreliert, der durch *spa*-Typisierung bestimmt wurde. Insgesamt wurden über 700 *S. aureus*-Isolate aus nasaler Besiedlung, Bakteriämie bzw. Furunkulose untersucht. Die SAg-kodierenden mobilen genetischen Elemente und Bakteriophagen waren nicht zufällig verteilt, sondern stark an den genetischen Hintergrund der Isolate geknüpft: Jede klonale Linie war durch ein typisches SAg-Gen- und Bakteriophagen-Repertoire charakterisiert. Trotzdem wurden erhebliche Unterschiede innerhalb klonaler Linien und auch innerhalb desselben *spa*-Typs beobachtet. Dies lässt vermuten, dass innerhalb einer klonalen Linie mobile genetische Elemente häufig erworben werden oder verloren gehen.

Unsere Daten zeigen, dass die parallele Bestimmung der Virulenzgen- und Bakteriophagen-Profile und des genetischen Hintergrunds die Trennschärfe von genetischen Untersuchungen zu *S. aureus*-Virulenzmechanismen stark erhöht. Der Vergleich von nasalen und Blutkultur-Isolaten mit dem gleichen klonalen Hintergrund zeigte, dass die Exfoliatives Toxin d-kodierende Pathogenitätsinsel und das vollständige β-Hämolysin-Gen, nicht dagegen SAgs, bei Bakteriämie eine Rolle spielen. Außerdem beobachteten wir eine starke Korrelation von PVL mit Furunkulose, die unabhängig vom genetischen Hintergrund der Isolate war. Mit Ausnahme von *seb*, das innerhalb der Linie CC121 bei Furunkulose-Isolaten signifikant häufiger vorkam, waren SAgs nicht mit Furunkulose assoziiert.

Die klinisch relevante protektive Wirkung von anti-SAg Antikörpern kann mit funktionellen Neutralisationsassays gemessen werden, allerdings sind diese Assays zeit- und arbeitsintensiv. Eine elegante Alternative ist die Luminex®-Technologie, bei der zeitgleich die Antikörperbindung an verschiedene *S. aureus*-Antigene quantifiziert wird. Die Analyse von Serumproben von 15 persistierenden Carriern und 19 Nichtcarriern zeigte eine starke Korrelation der im Luminex-System gemessenen Antikörperbindung mit der neutralisierenden Kapazität gegen das SAg TSST-1. Auch mit dieser Technik bestätigte sich, dass Antikörper gegen *egc*-SAgs bei gesunden Carriern und Nichtcarriern selektiv fehlen, wie bereits von Holtfreter et al. berichtet wurde.

Wir gingen davon aus, dass für die Bildung der hohen Antikörpertiter gegen nicht-*egc*-SAgs, wie sie in gesunden Erwachsenen regelmäßig gefunden werden, (subklinische)

Infektionen nötig sind, da experimentelle Besiedlung mit *S. aureus* nicht ausreichte, um eine robuste Antikörperantwort zu generieren. Um dies zu überprüfen, haben wir die Antikörperentwicklung gegen SAgs während *S. aureus*-Bakteriämie untersucht (n = 64). Bereits in der akuten Krankheitsphase hatte ein Großteil der Patienten neutralisierende Antikörper gegen nicht-*egc*-SAgs, die häufig im Verlauf der Infektion weiter anstiegen. Interessanterweise induzierte auch eine systemische Infektion weder Boost noch Neubildung von neutralisierenden Antikörpern gegen *egc*-SAgs.

Die „*egc*-Lücke" in der Antikörperantwort, die jetzt sowohl bei gesunden Carriern und Nichtcarriers als auch nach systemischer *S. aureus*-Infektion gezeigt wurde, war unerwartet, da *egc*-SAgs bei klinischen *S. aureus*-Isolaten die häufigsten SAgs sind. Auf der Suche nach der Ursache haben wir die intrinsischen Eigenschaften von drei rekombinanten *egc*- (SEI, SElM und SElO) und drei nicht-*egc*-SAgs (SEB, SElQ und TSST-1) verglichen. *Egc*- und nicht-*egc*-SAgs verhielten sich in allen untersuchten Aspekten der T-Zellaktivierung sehr ähnlich: Induktion der T-Zell-Proliferation, Zytokinsekretion und Genexpression in humanen Immunzellen. SAgs beider Gruppen induzierten eine starke proinflammatorische Th1- und Th17-Antwort.

Im Gegensatz dazu wurden *egc*- und nicht-*egc*-SAgs unterschiedlich reguliert. *Egc*-SAgs wurden bereits in der exponentiellen Wachstumsphase sezerniert, während nicht-*egc*-SAgs – wie die meisten andern Virulenzfaktoren – erst in der post-exponentiellen Wachstumsphase freigesetzt wurden. Wir folgern daraus, dass die unterschiedliche Regulation von *egc*- und nicht-*egc*-SAgs die adaptive Immunantwort beeinflusst.

Wie werden SAgs bei der Wechselwirkung von *S. aureus* mit seinem Wirt reguliert? Die Forschung der Regulation von *S. aureus*-Virulenzfaktoren beschränkte sich bisher vor allem auf *in vitro*-Bedingungen, da *in vivo*-Untersuchungen besonders beim Menschen sehr schwierig sind. Die hochspezifische adaptive Immunantwort zeigt einen Ausweg: Sie gibt indirekt Auskunft über die *in vivo*-Expression verschiedener *S. aureus*-Antigene. Die hohe Prävalenz von neutralisierenden Serumantikörpern gegen nicht-*egc*-SAgs ist ein Indiz dafür, dass die meisten gesunden Erwachsenen während ihrer Begegnungen mit *S. aureus* mit diesen Toxinen in Kontakt gekommen sind. In dieser Arbeit wurde dokumentiert, dass diese Antikörperantwort bei invasiven Episoden verstärkt wird. Dies zeigt, dass zumindest die nicht-*egc*-SAgs bei einer Bakteriämie gebildet werden. Für *egc*-SAgs lässt sich die Frage bisher nicht beantworten, doch weisen erste Daten darauf hin, dass *egc*-SAgs hauptsächlich während der nasalen Besiedlung transkribiert werden.

Als Modellsystem beleuchten die SAgs in dieser Arbeit die Vielseitigkeit der Spezies *S. aureus* und der Immunreaktion des humanen Wirts. Dass monovalente Impfstoffe gegen *S. aureus* wirksam sein werden, erscheint angesichts der komplexen Wechselwirkungen unwahrscheinlich. Die Herausforderung liegt jetzt darin herauszufinden, welche der zahlreichen Virulenzfaktoren, einschließlich der Fülle von SAgs, zur Antigen-Kombination einer wirksamen *S. aureus*-Vakzine gehören müssen.

ABBREVIATIONS

ACME	arginin catabolic mobile element
AFLP	amplified fragment length polymorphism
agr	accessory gene regulator
APC	antigen presenting cells
AUC	area under the curve
bp	base pairs
CA-MRSA	community-associated MRSA
CC	clonal complex
CHIPS	chemotaxis inhibitory protein of *S. aureus*
chp	CHIPS gene
CI	confidence interval
ClfA, ClfB	clumping factor A, B
CV	coefficient of variation
Eap	extracellular adherence protein
Efb	extracellular fibrinogen binding protein
egc	enterotoxin gene cluster
ELISA	enzyme-linked immunosorbent assay
eta, etb, etd	exfoliative toxin A, B, and D gene
Fc	constant region of Ig
FDR	false discovery rate
FnbpA, FnbpB	fibronectin-binding protein A, B
geh	glycerol ester hydrolase gene
gyr	gyrase
HA-MRSA	hospital-associated MRSA
hlb	beta hemolysin gene
IDU	injection drug user
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IsaA	immunodominant secretory antigen A
IsdA, IsdH	iron-responsive surface determinant A, H

LPS	lipopolysaccharid
LTA	lipoteichoic acid
lukS-lukP,	leukocidin genes S and F, encoding PVL
Luk-PV	operon encoding PVL
Map-w	MHC class II analogue protein w
mecA	methicillin resistance gene
MFI	mean fluorescent intensity
MGE	mobile genetic element
MHC	major histocompatibility complex
MLST	multi locus sequence typing
MRSA	methicillin resistant *S. aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
MSSA	methicillin sensitive *S. aureus*
OB-fold	oligosaccharide/ oligonucleotide-binding fold
OD	optical density
OR	odds ratio
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PHA	phythemagglutinin
PRR	pattern recognition receptor
PVL	Panton-Valentine leukocidin
S. aureus	*Staphylococcus aureus*
SAB	*S. aureus* bacteremia
SAg	superantigens
sak	staphylokinase gene
SaPI	*S. aureus* pathogenicity island
sarA	staphylococcal accessory regulator
SasG	*S. aureus* surface proteinG
SCIN	staphylococcal complement inhibitor

scn	SCIN gene
SdrD, SdrE	serine-aspartate dipeptide repeat protein D, E
SE	staphylococcal enterotoxin
SEl	staphylococcal enterotoxin-like toxin
spa	*S. aureus* protein A gene
SpA	*S. aureus* protein A
SSC*mec*	staphylococcal chromosomal cassette encoding the *mecA* gene
SSL	staphylococcal SAg-like protein
SSSS	staphylococcal scalded skin syndrome
SSTI	skin and soft tissue infections
ST	sequence type
TCR	T cell receptor
TGF	tumorforming growth factor
TLR	toll like receptor
TNFα	tumor necrosis factor alpha
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin 1
VISA	vancomycin intermediate-resistant *S. aureus*
VRSA	vancomycin-resistant *S. aureus*
σ^B	alternative sigma factor
νSa	*S. aureus* genomic island

PUBLICATIONS

Grumann, D., E. Ruotsalainen, J. Kolata, P. Kuusela, A. Järvinen, V. P. Kontinen, B. M. Bröker, and S. Holtfreter. 2010. Neutralizing antibody response to *Staphylococcus aureus* superantigens in bacteremic patients. submitted.

Masiuk, H., K. Kopron, **D. Grumann**, C. Goerke, J. Kolata, J. Jursa-Kulesza, S. Giedrys-Kalemba, B. M. Bröker, and S. Holtfreter. 2010. Recurrent furunculosis - associations with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus. Journal of clinical microbiology.* in press.

Goerke, C., R. Pantucek, S. Holtfreter, B. Schulte, M. Zink, **D. Grumann**, B. M. Bröker, J. Doskar, and C. Wolz. 2009. Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages. *Journal of bacteriology* 191:3462-3468.

Verkaik, N. J., C. P. de Vogel, H. A. Boelens, **D. Grumann**, T. Hoogenboezem, C. Vink, H. Hooijkaas, T. J. Foster, H. A. Verbrugh, A. van Belkum, and W. J. van Wamel. 2009. Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. *The Journal of infectious diseases* 199:625-632.

Grumann, D., S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Volker, and B. M. Bröker. 2008. Immune cell activation by enterotoxin gene cluster (*egc*)-encoded and non-*egc* superantigens from *Staphylococcus aureus. J Immunol* 181:5054-5061.

Holtfreter, S., **D. Grumann**, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Bröker. 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *Journal of clinical microbiology* 45:2669-2680.

CONFERENCES

Burian, M., **D. Grumann**, S. Holtfreter, U. Völker, Ch. Wolz, B. M. Bröker, and C. Goerke. 2010. Expression of *Staphylococcus aureus* superantigens (SAgs) in the authentic human nasal environment. In *3rd Joint conference German Society for Hygiene and Microbiology (DGHM) and Association for General and Applied Microbiology (VAAM)*, Hannover, Germany

Masiuk, H., K. Kopron, **D. Grumann**, C. Goerke, A. Barwich, J. Kolata, R. Bruns, A. Zautner, J. Jursa-Kulesza, M. Nowosiad, C.-F. Classen, I. Steinmetz, S. Giedrys-Kalemba, B. M. Bröker, and S. Holtfreter. 2009. *S. aureus* CC121 is the main cause of chronic furunculosis. In *61th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)*, Goettingen, Germany

Grumann, D., J. Kolata, P. Kuusela, E. Ruotsalainen, A. Järvinen, V. Kontinen, B. M. Bröker, and S. Holtfreter. 2009. Do superantigens play a role in *Staphylococcus aureus* bacteremia? In 61th *Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)*, Goettingen, Germany

Grumann, D., J. Kolata, P. Kuusela, E. Ruotsalainen, A. Järvinen, V. Kontinen, B. M. Bröker, and S. Holtfreter. 2009. Do superantigens play a role in *Staphylococcus aureus* bacteremia? In *2nd European Congress of Immunology*, Berlin, Germany.

Grumann, D., S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Völker, and B. M. Bröker. 2008. Immune cell activation by enterotoxin gene cluster-encoded and non-*egc* superantigens from *Staphylococcus aureus*. In *Pathophysiology of Staphylococci*, Kloster Banz, Germany.

Kolata, J., S. Holtfreter, **D. Grumann**, E. Friebe, K. Rogasch, C. Kohler, S. Thomas-Ecker, S. Engelmann, and B. M. Bröker. 2008. Human T cell response to *Staphylococcus aureus* antigens. In *Pathophysiology of Staphylococci*, Kloster Banz, Germany.

Verkaik, N. J., C. P. de Vogel, H. A. Boelens, **D. Grumann**, H. Hooijkaas, T. J. Foster, H. A. Verbrugh, A. van Belkum, and W. J. B. van Wamel. 2008. Antistaphylococcal humoral immune response in persistent and non-nasal carriers of *Staphylococcus aureus*. In *Pathophysiology of Staphylococci*, Kloster Banz, Germany.

Kolata, J., S. Holtfreter, K. Rogasch, C. Kohler, **D. Grumann**, E. Friebe, S. Thomas-Ecker, M. Hecker, H.-D. Volk, S. Engelmann, and B. M. Bröker. 2008. Human T cell response to *Staphylococcus aureus* antigens. In *60th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)*, Dresden.

Grumann, D., S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Völker, and B. M. Bröker. 2008. T cell activation by enterotoxin gene cluster-encoded and "classical" *Staphylococcus aureus* superantigens. In *13th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI)*, Cairns, Australia.

Grumann, D., S. Holtfreter, C. Kohler, S. Scharf, M. Hecker, U. Völker, S. Engelmann, and B. M. Bröker. 2007. Similar T cell-activating properties of *egc*-encoded and classical *Staphylococcus aureus* superantigens. In *59th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)*, Göttingen.

Holtfreter, S., **D. Grumann**, M. Schmudde, H.T.T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Bröker. 2007. Clonal distribution of superantigen genes in clinical *S. aureus* isolates. In *59th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)*, Göttingen.

Grumann, D., S. Holtfreter, K. Kopron, P. Eichler, M. Schmudde, H. T. T. Nguyen, C. Feig, S. Giedrys-Kalemba, W. Witte, I. Steinmetz, and B. M. Bröker. 2007. Similar T cell-activating properties of *egc*-encoded and classical *Staphylococcus aureus* superantigens. In *3rd Spring School on Immunology*, Ettal, Germany.

Holtfreter, S., **D. Grumann**, K. Kopron, P. Eichler, M. Schmudde, N. T. T. Nguyen, C. Feig, S. Giedrys-Kalemba, W. Witte, I. Steinmetz, and B. M. Broker. 2006. Unusual Superantigen gene pattern in an *S. aureus* lineage, which very efficiently colonizes healthy individuals but is underrepresented among invasive isolates in Pomerania. In *12th International Symposium on Staphylococci & Staphylococcal Infections (ISSSI)*, Maastricht, The Netherlands.

ACKNOWLEDGMENTS/ DANKSAGUNG

Ich bedanke mich ganz herzlich bei allen die zu dieser Arbeit auf verschiedenste Weise beigetragen haben. Beginnen möchte ich mit Barbara M. Bröker für die exzellente wissenschaftliche Betreuung meiner Promotion, die zahlreichen anregenden Diskussionen und das Vertrauen in meine Arbeit.

Dem Graduiertenkolleg GRK840 „Host-Pathogen Interactions in Generalized Bacterial Infections " sowie der Doktorandenschule „A Functional Genomics Approach to Infection Biology" des Alfried Krupp Wissenschaftskolleg möchte ich für die Finanzierung meiner Doktorarbeit danken. Ich danke Monika Müller, Susanne Kühl, Ruth Terodde und Maja Richter für ihr die Organisation und Unterstützung bei wissenschaftlichen Workshops, Seminaren und Kongressreisen – ohne wären die letzten Jahre nicht so turbulent, aber auch weniger spannend und lehrreich gewesen!

Ich danke allen Mitarbeitern unseres Labors für die angenehme Arbeitsatmosphäre. Mein besonderer Dank gilt Silva Holtfreter für die uneingeschränkte Unterstützung in allen „Laborlagen" – sogar aus dem fernen Neuseeland. Ich habe die Zusammenarbeit mit Dir sehr genossen! Julia Kolata danke ich, dass Sie immer ein offenes Ohr für mich hat und mir in der „heißen Phase" des Schreibens den Rücken freigehalten hat. Ich freue mich auf unsere weitere Zusammenarbeit. Außerdem danke ich Erika Friebe, dass sie „den Laden" am laufen hält, und allen Praktikanten die uns in den letzten drei Jahren bei der Charakterisierung unserer *S. aureus*-Stammsammlung tatkräftig unterstützt haben.

Ich danke Sandra Scharf, Leif Steil und Uwe Völker von der Arbeitsgruppe Funktionelle Genomforschung für die Kooperation bei den Transkriptomanalysen. Herzlichen Dank an Sandra und Leif für die Zusammenarbeit bei der Durchführung der Transkriptomanalysen und der Auswertung der entstandenen Datenflut. Mein besonderer Dank geht an Uwe Völker für die anregenden Diskussionen und die Unterstützung beim Schreiben des Manuskripts.

Ich bedanke mich bei Susanne Engelmann, Christian Kohler, Anne Kathrin Ziebandt und Katrin Kusch vom Institut für Mikrobiologie für die fachkundige Unterstützung bei Klonierungsfragen, sowie die Bereitsstellung von Vektoren. Dankeschön auch an Sylvio Redanz für die Bereitstellung der Elmi-Bilder von Staphylokokken.

Pentti Kuusela, Eeva Ruotsalainen, Asko Järvinen und Vesa P. Kontinen danke ich herzlich für die Bereitstellung der *S. aureus*-Isolate und Seren von Bakteriämie-Patienten aus Helsinki. Mein besonderer Dank gilt Eeva Ruotsalainen für die schnelle Bereitstellung der klinischen Daten.

Mein herzlicher Dank geht an Christiane Goerke und Christiane Wolz für die Zusammenarbeit bei der Phagen-Charakterisierung. Sowie an Marc Burian für die Kooperation bei der Aufklärung der Superantigenexpression in vivo.

Nelianne Verkaik, Willem van Wamel und Alex van Belkum danke ich für die Kooperation bei der Analyse der Antikörpertiter in gesunden Probanden. Mein spezieller Dank geht an Nelianne für die freundschaftliche Zusammenarbeit und die aufregenden Telefon-Diskussionen.

Stefania Giedrys-Kalemba, Kasia Kopron und Helena Masiuk danke ich für die Bereitstellung der etwa 200 *S. aureus*-Isolaten aus Stettin. Dankeschön an Kasia und Helena für die nette Zusammenarbeit bei der Charakterisierung dieser Stämme.

Ich danke meinen Eltern, meinem Bruder und meinen Freunden für das geduldige Zuhören, die motivierenden Worte und das Verständis, dass meine Besuche in den letzten Jahren aufgrund des Zeitmangels eher selten waren.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Greifswald, den 11.03.2010 Dorothee Grumann

CURRICULUM VITAE

Persönliche Daten

Name, Vorname	Dorothee Grumann
Geburtstag	03. Januar 1980
Geburtsort	Fürth
Familienstand	ledig

Ausbildung

seit 2006	Promotionsstudentin der Alfried Krupp Doktorandenschule „A Functional Genomics Approach to Infection Biology“ (seit Juni 2009) und des DFG-Graduiertenkollegs 840 „Wechselwirkung zwischen Erreger und Wirt bei generalisierten bakteriellen Infektionen“ (Okt 2006 bis Mai 2009) am Institut für Immunologie und Transfusionsmedizin, Ernst-Moritz-Arndt-Universität Greifswald, Thema der Promotionsarbeit: Funktionelle Charakterisierung neuer Virulenzfaktoren von *Staphylococcus aureus.* Betreuerin: Prof. Dr. med. Barbara M. Bröker
2001 – 2006	Studium der Humanbiologie an der Ernst-Moritz-Arndt-Universität Greifswald Hauptfach: Immunologie Nebenfächer: Virologie, Humangenetik Diplomarbeit am Institut für Immunologie und Transfusionsmedizin, zum Thema „Charakterisierung der Immunantwort bei Carriern auf *Staphylococcus aureus* Virulenzfaktoren“
1999 – 2000	Studium der Oecotrophologie an der Technische Universität München
1999	Abitur, Gymnasium Herzogenaurach