Immunantwort gegen Superantigene bei *Staphylococcus aureus* Carriern

(Immune response against superantigens in *Staphylococcus aureus* carriers)

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GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Staphylococcus aureus colonises the anterior nares of circa 35% of the healthy population. However, once the mucosal or skin barrier is broken, e.g. by a lesion of the skin or mucous membranes, catheters insertions and foreign bodies, the bacteria can invade virtually every human tissue and cause a broad spectrum of diseases, ranging from mild skin and wound infections to life-threatening conditions, such as endocarditis, pneumonia and sepsis. To date, *S. aureus* is the most common cause of nosocomial infections and the species is becoming increasingly resistant to antibiotics[1]. In several industrialised nations, including parts of Europe, the USA and Japan, 40-60% of all nosocomial *S. aureus* strains are resistant to methicillin (methicillin-resistant *S. aureus*; MRSA)[2]. Especially the emergence of high-level vancomycin-resistant isolates is alarming and threatens to throw staphylococcal therapy back to the pre-antibiotic age[3]. As a consequence, effective measures to prevent and treat staphylococcal infections are urgently needed.

It is well established that nasal colonisation with *S. aureus* is a major risk factor for staphylococcal infections[4]. Compared to noncarriers, *S. aureus* carriers have a four-fold increased risk of acquiring an *S. aureus* bacteraemia, which is mostly caused by the colonising strain[5]. Intriguingly, once *S. aureus* carriers develop an *S. aureus* bacteraemia, their mortality is lower than in noncarriers[6]. These observations stress the importance of host factors, such as the immune response, for the outcome of *S. aureus*-host interactions. However, despite their high prevalence and the medical need to prevent *S. aureus* infections in the human population, our understanding of the role of the immune system in staphylococcal colonisation and infection is still limited[4]. Therefore, a major task of staphylococcal research is the elucidation of the immunological mechanisms active in *S. aureus* nasal carriage and infection. This knowledge is a prerequisite for the development of new preventive and therapeutic strategies, such as active and passive antistaphylococcal vaccination.

The main aim of this thesis was to investigate adaptive immune responses which *S. aureus* carriers raise against their colonising strain. In our studies we used superantigens (SAgs) as indicator antigens for three reasons. Firstly, SAgs are clinically important virulence factors. They cause the toxic shock syndrome, and are probably also involved in the pathogenesis of staphylococcal sepsis. Secondly, the SAg gene repertoire of clinical *S. aureus* isolates is highly variable, due to their localisation on mobile genetic elements. This enabled us to compare strain-specific immune responses in *S. aureus* carriers and noncarriers. Finally, by exploiting the T cell-mitogenic activity of SAgs, we could easily assess neutralising antibody capacities of different sera in a proliferation assay.

The scope of the present thesis was

i) to analyse the prevalence of SAg genes among colonising and invasive isolates and to correlate it with the clonal background,
ii) to determine the anti-SAg antibody profiles in healthy individuals and
iii) to compare strain-specific antibody responses against staphylococcal SAgs in *S. aureus* carriers and noncarriers.

Chapter 1 provides an overview about *S. aureus* nasal carriage and the associated infection risk, briefly describes *S. aureus* genomics, discusses determinants of staphylococcal virulence and finally summarises the current knowledge about the role of the innate and adaptive immune system in staphylococcal colonisation and infection. **Chapter 2** introduces staphylococcal SAgs, which are in the focus of this thesis. This review gives an overview about the localisation of all 19 SAg genes within the staphylococcal genome and discusses their implications in sepsis.

In **Chapter 3,** the highly diverse SAg gene repertoire of nasal and blood culture isolates from Western Pomerania was analysed using multiplex-PCR and correlated with the clonal background. **Chapter 4** elucidates the heterogeneous activation patterns of different blood cell types induced by various staphylococcal soluble factors. Both studies emphasise the importance of strain-specific analyses of host-pathogen interactions during colonisation and infection.

Once the SAg gene repertoire of clinical strains was determined, we analysed the antibody profiles against staphylococcal SAgs in healthy individuals (**Chapter 5**). Antibodies against SAgs of the enterotoxin gene cluster (*egc*) were detected only rarely, which is 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 T cell-activating properties of *egc* SAgs compared to classical SAgs or ii) their differential regulation (**Chapter 6**).

Finally, to investigate whether colonisation induces an adaptive immune response, we compared antibody profiles of *S. aureus* carriers and noncarriers using SAgs as strain-specific indicator antigens. The results of this study are described in **Chapter 7**.

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Staphylococcal colonisation and infection

DETERMINANTS OF *S. AUREUS* NASAL CARRIAGE

S. aureus nasal carriage patterns

Staphylococcus aureus usually behaves as a commensal micro-organism colonising skin and mucosal surfaces of humans as well as several animal species. The primary ecological niche of *S. aureus* are the anterior nares. However, it also colonises other body sites, including the skin, perineum, pharynx and, less frequently, the gastrointestinal tract, vagina and axillae[1]. Longitudinal studies revealed that about 20% (range 12-30%) of individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers (range 16-70%) and about 50% (range 16-69%) are noncarriers[1]. Cross-sectional studies observed a prevalence of ~35% in the healthy population, which represents a mix of persistent and intermittent carriers at the time of investigation[2,3]. The distinction between persistent and intermittent carriage is clinically relevant. Persistent carriers have higher *S. aureus* loads and, therefore, disperse staphylococci more extensively into the environment. Moreover, persistant carriers have an increased risk of *S. aureus* infection[1,4,5]. In order to prevent such infections, it is important to explore and identify human and bacterial factors that may lead to persistant carriage.

Genotyping data revealed that persistent carriers usually carry one identical strain over time while intermittent carriers are commonly colonised by different strains consecutively[6,7]. The prevailing assumption has been that colonised individuals always carry a single strain. Surprisingly, recent data revealed that approximately 6.6% of *S. aureus* carriers are simultaneously colonised with multiple *S. aureus* clones[8]. Moreover, colonisation rates vary substantially with age. More than 70% of newborns have a positive nasal *S. aureus* culture[9]. Afterwards, infant carriage rates fall from 40-50% during the first 8 weeks to ~20% by 6 months[9]. Furthermore, persistent carriage is more common in children than in adults, and many people shift from persistent carriage to intermittent carriage or noncarriage during adolescence[10]. The reasons for these differences in colonisation patterns remain to be clarified.

S. aureus- host interactions during nasal colonisation

The mechanisms leading to *S. aureus* carriage are multifactorial and still not fully understood. An artificial colonisation experiment using a mixture of different strains showed that the majority of noncarriers and carriers returned to their original carrier state within few weeks. Notably, most persistent carriers tested positive again for their original resident strain[11]. Therefore, host characteristics are probably major determinants of the *S. aureus* carrier status and an optimal fit between host and bacterium seems to be essential[11]. Concordantly, recent whole genome analyses revealed that each staphylococcal lineage harbours a unique pattern of surface molecules (MSCRAMMs), which may interact with the host in unique ways[12]. Thus, it seems likely that different lineages preferentially colonise particular hosts.

The host factors that determine the *S. aureus* carrier status are only poorly characterised. There is evidence that S*. aureus* carriage rates vary between ethnic groups, with higher rates in Caucasians and in men, and depend on age and gender (table 1)[1]. Moreover, certain patient groups, e.g. patients with diabetes mellitus, patients undergoing haemodialysis and patients with HIV, have higher *S. aureus* nasal carriage rates[1]. In contrast, smoking seems to be protective[13]. Besides these clinical and demographic factors, polymorphisms associated with carriage were recently identified in the genes encoding the glucocorticoid receptor[14], TNF-α, C-reactive protein and IL-4[13].

Stable nasal *S. aureus* carriage is preceded by i) exposure to staphylococci, ii) adherence of the bacteria to certain receptors in the nose, iii) evasion of the hosts immune defences and iv) propagation in the nose (table 1)[1]. It is widely known that exposure to staphylococci, especially MRSA, is increased during hospitalisation. Another source of *S. aureus* are heavily colonised household members. Children, for example, often carry the same strain as their mothers[9]. Newborns acquire their strain either from hospital personnel or their mother[9,15,16]. Moreover, skin lesions, minor deformations of the nasal cavity and nose picking were described as risk factors for nasal carriage (table 1)[1,17,18].

Table 1: Mechanisms associated with *S. aureus* nasal carriage. Adapted from Wertheim et al.[1,19].

Mechanism	Host	*S. aureus*
General	Age, sex, ethnicity Antibiotic use Underlying disease (diabetes mellitus, HIV, liver disease, eczema, nasal abnormalities and others) Gene polymorphisms	Virulence Antibiotic resistance
Exposure	(Heavily) colonised partners Hospital environment Nose picking	
Adherence	Available adhesins Keratin type 10 Epithelial membrane Collagen Vitronectin Fibronectin Fibrinogen Laminin Mucins (Extracellular) matrix proteins Surface charge Hydrophobicity	Bacterial interference Clumping factor B (Lipo)teichoic acid wall teichoic acid Capsule Collagen binding protein Vitronectin binding protein Fibronectin binding protein Fibrinogen binding protein Laminin binding protein Capsular polysaccharides MSCRAMMs Suface charge Hydrophobicity
(Evading) immune responses	Mucosal/skin barrier Clearance in mucus by microvilli Antibodies Lysozyme, lactoferrin, antimicrobial peptides Opsonisation Immune status HLA type	Proteases, lipases Host cell internalisation Protein A (binds Fc of IgG) Resistance to antimicrobial peptides Capsule

MSCRAMM = microbial surface components recognising adhesive matrix molecules.

S. aureus can bind to the mucosa and to mucin[1], and several staphylococcal adhesins and their host cell ligands have been described in the last years (table 1). Wall teichoic acid (WTA), a surface-exposed staphylococcal polymer, is essential for nasal colonisation and

mediates adherence to human nasal epithelial cells[20]. A lack of WTA also leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis[21]. Recent studies on patients with chronic rhinosinusitis showed that *S. aureus* can also persist intracellularly in nasal epithelial and glandular cells[22]. Accordingly, Wertheim and coworkers suggested that intermittent carriers are actually "mucosal carriers", whereas in persistent carriers strains use a special niche, such as glands or intracellular residence[1]. Another important determinant of nasal carriage is bacterial interference, i.e. the occupation of the nasal ecological niche by one *S. aureus* strain impedes colonisation with a second strain (see page 9). Moreover, there are hints that colonisation with *S. aureus* is blocked by resident strains of non-*aureus* staphylococci or other bacteria of the normal nasal flora, such as corynebacteria[18].

To colonise the human nares, staphylococci have to evade the host immune response. Nasal secretions, for example, contain immunoglobulins (IgA, IgG), lysozyme, lactoferrin and antimicrobial peptides. *S. aureus* carriers have raised concentrations of alpha-defensins (e.g. human neutrophil peptide 1, 2 and 3) and human beta-defensin 2 in their nasal secretions, indicating a neutrophil- and epithelium-mediated inflammation[23]. However, subsequent studies also showed that staphylococci are resistant against the bactericidal effects of these defensins (see page 11)[24]. These findings suggest that colonisation induces an innate immune response, which is, however, ineffective and insufficient to eradicate the strain. Additionally, there is some evidence that colonisation induces an antibody response (see page 12)[25,26].

CLINICAL IMPACT OF *S. AUREUS* NASAL CARRIAGE

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

S. aureus (MRSA as well as MSSA) is the most common cause of nosocomial infections[27]. Importantly, most of these *S. aureus* infections originate from the patients' own flora. Von Eiff et al. demonstrated in a prospective multicenter study that nasal strains and subsequent bactaeremic strains have the same genotype in more than 80% of the cases[28]. Similarly, hemodialysis patients and patients treated with continuous ambulant peritoneal dialysis have increased infection rates of mostly endogenous origin[2,29].

To date, the pathogenesis of endogenous *S. aureus* infections is not completely resolved. Wertheim et al. suggested that individuals, who carry *S. aureus* in their nose, contaminate their hands, then transfer the micro-organism to other sites on their bodies, thereby causing subsequent infection, especially if the skin, which serves as a first line barrier, is injured[1]. Several studies clearly demonstrate that *S. aureus* nasal carriage is an important risk factor for the development of nosocomial infections in various settings, such as in general hospital populations, surgical patients, patients undergoing hemodialysis or continuous peritoneal dialysis, HIV-infected patients and patients in intensive care units[1,2,28,29].

Wertheim et al. investigated the incidence of *S. aureus* bacteraemia in carriers as well as noncarriers in a nonsurgical patient population (n=14.008)[30]. They observed a threefold increased risk for *S. aureus* nasal carriers to acquire a nosocomial *S. aureus* bacteraemia, compared to noncarriers (95% CI: 2.0-4.7). Again, the bacteraemic strains of the carriers had

the same genotype as their nasal strains in approximately 80% of the cases. Surprisingly, *S. aureus*-related in-hospital mortality was four times higher in noncarriers who developed an *S. aureus* bacteraemia than in carriers (8% vs. 32%, p=0.006)[30]. Thus, carriage triples the risk of staphylococcal bacteraemia, but being a noncarrier is risky, too, since mortality due to staphylococcal bacteraemia is significantly increased. Since this phenomenon could not be explained by differences in the genetic background of endogenous and exogenous strains[31], partial immunity of carriers against their colonising strain may play a pivotal role.

Prevention of infection by eradicating nasal carriage

Since nasal *S. aureus* carriage is one of the most important risk factors for nosocomial and surgical site infections today, it has been reasoned that eradicating the colonising strain should reduce the infection rate. Eradication can be achieved either by local (or systemic) application of antibiotics or by bacterial interference strategies. The antibiotic ointment mupirocin was introduced in the late 1980s and showed high efficacy in the local elimination of *S. aureus.* Mupirocin prophylaxis has been used in several intervention studies. However, the results have been inconclusive in most patient populations. While earlier studies using historical controls have reported substantial reductions of surgical site infections among patients receiving mupirocin[32-35], many recent randomised controlled trials failed to confirm these results (summarised in table 2)[36-38].

Table 2: Randomised controlled mupirocin intervention studies. Adapted from Kluytmans et al.[39].

Author, year of publication	Patient population	Outcome
Perl et al., 2002[37]	surgical	No significant effect on *S. aureus* surgical site infections, but twofold reduction (4.0% mupirocin vs. 7.7% placebo, P=0.02) of nosocomial *S. aureus* infections in carriers. n=3864.
Kalmejer et al., 2002[38]	orthopaedic	No significant effect on *S. aureus* surgical site infection rate (3.8% mupirocin vs. 4.7% placebo). n=614.
Wertheim et al., 2004[36]	nonsurgical, *S. aureus* carriers	No effect on nosocomial *S. aureus* bacteraemia (2,6% mupirocin vs. 2,8% placebo). Development of nosocomial *S. aureus* infection significantly delayed by 19 days. n=1602.
Boelaert et al., 1989[40]	hemodialysis	Significant fourfold reduction in *S. aureus* infection rates (1/104 patient-months (mupirocin) vs. 6/147 patient-months (placebo), P≤0.05). n=34.
Mupirocin study group, 1996[41]	continuous peritoneal dialysis	Signficant threefold reduction in exit-site *S. aureus* infections (14/1390 patient-months (mupirocin) vs. 44/1236 patient-months (placebo) P=0.006). n=267.

Perl et al., Kalmejer et al. and Wertheim et al. conducted randomised placebo-controlled trials to study the efficacy of mupirocin in general surgical, orthopaedic and non-surgical patient populations[36-38]. However, in all three studies mupirocin failed to significantly reduce the *S. aureus* surgical site infection rate, or, in case of nonsurgical patients, the rate of nosocomial *S. aureus* infection. Notably, Perl et al. observed a significant twofold reduction of nosocomial *S. aureus* infections in the subgroup of carriers[37]. Additionally, there is some evidence that decolonisation with mupirocin is effective in patients undergoing hemodialysis and continous peritoneal dialysis[39,40]. Future studies should focus on persistent *S. aureus* carriers

and patients with chronic infections or with a high infection risk, e.g. surgery, since these groups are most likely to benefit from mupirocin treatment.

Another historical strategy to eliminate the colonising strain is bacterial interference, i.e. active colonisation with an *S. aureus* strain with comparably low virulence (*S. aureus* 502A), which then prevents colonisation by other more virulent strains[16,42,43]. The exact mechanisms of bacterial interference have never been elucidated in detail, but competition for binding sites in the nose might be important[16]. Whether interference between different *S. aureus* strains is due to cross-inhibition of the expression of virulence factors by the accessory gene regulator (*agr*), is discussed controversely[8,18,44]. In the 1960s, interference was used very successfully in nurseries during outbreaks of *S. aureus* infections and for treatment of patients with recurrent furunculosis[43,45,46]. However, in rare cases this approach was complicated by serious infections due to *S. aureus* 502A[47], and after one case of a fatal *S. aureus* infection this strategy was not pursued further at that time[48]. Nevertheless, there might be a future for the concept of bacterial interference: A recent study demonstrated that MRSA and MSSA compete for the same ecological niche[49]. Therefore, interference could become a tool in the fight against MRSA infections.

STAPHYLOCOCCAL COMPARATIVE GENOMICS

Over the last five years seven complete *S. aureus* genomes have been published[50-54]. They range in size from 2.592 Mb to 2.748 Mb and are predicted to contain between 2592 and 2748 protein coding sequences[55]. A comparison of the published *S. aureus* genomes and, more recently, 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 (MGE) (~15%)[12].

The core genome is highly conserved regarding gene order as well as gene sequence and comprises house keeping genes, which are essential for growth and survival[12]. Subtle variations within such house keeping genes are exploited by sequence-based genotyping methods, such as multilocus sequence typing (MLST)[56,57] and protein A (*spa*) genotyping[58]. MLST determines the relationship of bacterial isolates on the basis of sequence fragments of seven house keeping genes. In contrast, *spa* genotyping relies on comparisons of the variable repeat region of the protein A (*spa*) gene, which differs in sequence and number of s*pa* repeats. A *spa*-type is assigned to each unique repeat pattern, and closely related *spa* types are referred to as clonal complexes. Several studies clearly demonstrated that pulsed-field gel electrophoresis, MLST and *spa*-typing provide largely concordant results[58]. These genotyping analyses revealed that *S. aureus* has a highly clonal population structure, dominated by approximately 10 highly prevalent clonal lineages[57,59,60].

The core variable genome includes most surface-associated genes (MSCRAMMs) and also some regulatory genes. Core variable genes are encoded on the bacterial chromosome and are, therefore, typically stable and transferred vertically[12]. Recent studies demonstrated that each staphylococcal lineage has a characteristic spectrum of core variable genes[12].

MGEs include bacteriophages, plasmids, pathogenicity islands, chromosomal cassettes, genomic islands and transposons. They encode mainly virulence (e.g. *pvl,* SAg genes) and resistance genes[55,61]. MGEs can be distributed by two distinct mechanisms: firstly, clonal diversification, i.e. vertical transmission to daughter cells, and, secondly, horizontal gene transfer, which is strongly dependent on transduction by phages[55]. Whole genome arrays revealed substantial variation in the carriage of MGEs, suggesting frequent horizontal transfer of these elements between different strains[12,55]. However, several virulence factors are not uniformly distributed among the clonal lineages, indicating limited acquisition and loss of these elements[12]. This exclusion process is possibly controlled by the recently characterised restriction-modification system Sau1[62].

WHAT DETERMINES STAPHYLOCOCCAL VIRULENCE?

One of the major questions in recent staphylococcal research is whether all *S. aureus* strains have equal disease-evoking potential so that host factors are decisive, or whether invasive disease is associated with particularly virulent genotypes or with particular virulence genes.

Comparisons of the staphylococcal core genome by genotyping analyses showed that nasal and invasive isolates fall into the same main clusters[31,57,60]. Therefore, it appears that any *S. aureus* genotype has the potential to cause severe disease, given the appropriate clinical conditions. This suggests that *S. aureus* has primarily evolved for colonisation, and that lineages which are well adapted for colonisation are also good pathogens[57,60]. However, there is some evidence that particular clones may be more or less virulent than others[31,60]. Melles and collegues identified clusters and subclusters in a Dutch strain collection, which were overrepresented among bacteraemia-associated isolates or associated with skin disease (impetigo)[60]. Wertheim et al. found CC45 significantly underrepresented among endogenous invasive strains compared to colonising strains (OR, 0.16; CI: 0.04-0.59)[31]. Remarkably, by MLST analysis they could not identify any *S. aureus* CC that was particularly prone to cause invasive disease[31]. Similarly, analysis of the core variable genome also did not identify any factors clearly related to virulence[12].

Since virulence could not be explained by the underlying clonality, it was suggested that the influx and loss of virulence determinants carried on MGEs could play a large part in determining the virulence of an isolate[55]. MGEs carry hundreds of virulence factors and putative virulence genes, which are involved in adherence to human tissue, evasion of the immune response, toxin secretion and regulation of virulence gene expression[55]. Hence, the increased virulence of particular clones can be due to the acquisition of MGE-carried virulence genes, such as *pvl* and *mecA*[31,60]. However, a clear association between certain virulence genes and disease could only be established for a few toxin-mediated diseases, such as toxic shock syndrome (SAgs) , scalded skin syndrome (exfoliative toxins), as well as necrotising pneumonia and deep-seated skin infections (*pvl*)[63-67]. Importantly, the vast majority of staphylococcal infections cannot be explained by the action of a single virulence determinant, and it is likely that a number of factors act in combination during the infective process[12,65-68].

To conclude, despite intensive research efforts, it is not understood if and how the impressive variation seen in the species *S. aureus* and the considerable genetic exchange between *S. aureus* clones might influence pathogenesis[12,55]. Therefore, the key to understanding the pathogenesis of *S. aureus* disease may lie in the identification of host factors, that contribute to colonisation, susceptibility to infection and outcome of infection, such as the immune status and gene polymorphisms[13,14,25]. This is underlined by the finding that *S. aureus* related mortality is significantly lower in *S. aureus* carriers than in noncarriers, which implies an important role of the adaptive immune response[30]. However, scientists just started to unravel the immune responses elicited by colonisation and infection.

INNATE AND ADAPTIVE IMMUNE RESPONSES AGAINST *S. AUREUS* AND BACTERIAL IMMUNE EVASION MECHANISMS

Innate immunity

To act as a commensal or pathogen, staphylococci have to withstand the attack of the host's immune system. Even though *S. aureus* can invade professional as well as non-professional phagocytes, it is a primarily extracellular pathogen[22,69]. Consequently, host defence relies mainly on innate immune mechanisms and anti-staphylococcal humoral immune responses.

The innate immune system possesses three important effector mechanisms that enable rapid and efficient killing of bacteria: i) antimicrobial peptides, ii) the complement system and iii) phagocytes. Antimicrobial peptides, such as defensins, directly bind to microbial structures and lyse the bacterial membrane[70]. However, sufficient concentrations are probably only reached in the gut and inside the granules of neutrophils. The complement system can be induced by three activation pathways, which all converge in the formation of C3 convertases, that bind to microbial surfaces[71]. C3 convertase formation leads to the release of opsonising complement factors (C3b), chemoattractant molecules (C5a) and, finally, bacterial cell lysis by pore formation. Complement-mediated opsonisation is essential for the elimination of *S. aureus* by the human host[71]. Phagocytes (e.g. neutrophils, monocytes) are able to ingest, kill and digest bacteria within minutes. Neutrophils can recognise staphylococci either directly via toll-like receptor (TLR) 2 and formylated peptide receptor, or indirectly after opsonisation with antibodies or complement[72]. Neutrophil killing is achieved by a vast array of proteases, lipases, antimicrobial peptides, amidases and highly reactive oxygen radicals[71].

Innate immune evasion mechanisms

Gram-positive bacteria, such as staphylococci, are shielded by their thick cell wall against direct attacks of complement and antimicrobial peptides. Therefore, neutrophils are probably the main enemies of *S. aureus* within the human host. However, staphylococci have evolved a multitude of mechanisms to very efficiently evade the host innate immune responses, as outlined in table 2.

Antimicrobial peptides have cationic properties that allow interactions with the bacterial cytoplasmatic membrane, which usually comprises negatively charged phospholipids.

Table 2: Innate immune evasion mechanisms of *S. aureus* (adapted from Rooijakkers et al. [73]).

	Host defence mechanism	Bacterial evasion mechanism
Defensin evasion	α-defensins from neutrophils lyse bacterial cells	**Staphylokinase** inhibits α-defensins
	Cathelicidin LL-37 is bactericidal against staphylococci	**Aureolysin**, a metalloproteinase, inactivates LL-37 by cleavage
	Lysozyme blocks peptidoglycan synthesis	all *S. aureus* strains are completely **lysozyme resistant** by chemical modification of peptidoglycan
	defensins and antimicrobial peptides lyse bacterial cells	*S. aureus* alters the composition and net charge of lipoteichoic acid, wall teichoic acid and phospholipids
Complement evasion	classical pathway	**protein A** blocks complement activation via C1q
	opsonisation	Staphylokinase (**SAK**) converts plasminogen into plasmin, which then cleaves opsonins (IgG, C3b) from the bacterial cell wall
	C3 convertase	extracellular fibrinogen binding molecule (**Efb**) binds C3 (in high concentrations)
		SCIN stabilises and blocks surface-bound C3-convertases
	C5a	Chemotaxis inhibitory protein of *S. aureus* (**CHIPS**) blocks receptors for chemoattractants C5a and formylated peptide
		Staphylococcal superantigen-like protein 7 (**SSL7**) binds C5
Neutrophil evasion	chemotaxis (migration to sites of infection via chemoattractants, e.g. C5a, formylated peptides)	Extracellular adherence protein (**Eap**) blocks extravasation of neutrophils by shielding the endothelial adhesion molecule ICAM-1
		CHIPS blocks the formylated peptide receptor
	phagocytes produce reactive oxygen species	resistance by production of **catalase** and **carotenoid pigments**

Staphylococci, however, circumvent antimicrobial peptide attacks by i) reducing the net negative charge of the bacterial cell envelope via covalent modification of anionic molecules (e.g. lipoteichoic acid, wall teichoic acid and phospholipids)[74,75] ii) expelling antimicrobial peptides through energy-dependent pumps and iii) cleavage with proteases[70].

An elegant example for complement evasion is the staphylococcal complement inhibitor (SCIN)[73]. This 9.8kD polypeptide inhibits the enzymatic activity of C3 convertases, which represent the central step in the classical, alternative and lectin pathway of complement activation, and thereby blocks complement deposition on the bacterial surface[73]. This diminishes phagocytosis and killing by neutrophils. Apart from SCIN, many complement evasion molecules were recently discovered, such as chemotaxis inhibitory protein of *S. aureus* (CHIPS), Staphylococcal superantigen-like protein 7 (SSL-7), staphylokinase (SAK) and extracellular fibrinogen binding molecule (Efb). All these factors interfere with different steps of complement activation, thereby compromising the host's complement attack (reviewed by Rooijakkers et al.[71]). Interestingly, the genes encoding SCIN, CHIPS and SAK as well as staphylococcal enterotoxin A or P form an "innate immune evasion cluster", which is located on a phage and, therefore, can be horizontally transferred between *S. aureus* strains[76].

S. aureus has also developed mechanisms to circumvent destruction by neutrophils. Briefly, staphylococci avoid phagocytosis by producing a polysaccharide capsule and by preventing opsonisation (via protein A, SCIN, SAK), chemotaxis (CHIPS, SSL7) and neutrophil extravasation (Eap) (see table 1). Different *S. aureus* strains display varying abilities to lyse host cells, a feature associated with clear virulence differences in a mouse model of infection[69]. Moreover, the bacteria actively counteract neutrophil-mediated killing by switching on different sets of genes, when being internalised. These include capsule biosynthesis genes, oxidative stress genes and several virulence genes[69].

Antibody responses against staphylococci during colonisation and infection

The current knowledge about the role of adaptive immune responses in protection and disease susceptibility is limited. A number of studies have investigated the humoral immune response to *S. aureus* in infected patients but only few have addressed the response in healthy carriers and noncarriers. Moreover, most serological studies analysed antibody titres against total bacterial lysates or only few selected proteins. To overcome this limitation, future analyses of antibody responses should be based on broad-spectrum and high throughput screening methods, such as protein arrays, bacteriophage expression libraries of *S. aureus*[77] or immunoblots on two-dimensional gels of cellular and secreted staphylococcal antigens.

Evidence for a protective role of antibodies in staphylococcal infections comes from animal experiments as well as from clinical studies. It has been shown that antibodies against certain bacterial components, such as surface adhesins (clumping factor A and B, fibronectin-binding protein and collagen-binding protein)[78-81], surface polysaccharides (types 5 and 8)[82] and poly-N-succinyl beta-1-6 glucosamine are protective[83]. Moreover, intravenous IgG preparations contain antibodies that are effective against *S. aureus*. For example, IgG preparations selected for high anti-clumping factor A antibody concentrations showed neutralising and opsonising activity in in vitro assays and were protective in a rabbit endocarditis model[84]. Furthermore, vaccination trials with type 5 and 8 capsule polysaccharide vaccine decreased the prevalence of bacteraemia in hemodialysis patients in a phase 3 trial[85].

Apart from their function as opsonins, antibodies are important for the neutralisation of secreted toxins, such as SAgs, for three reasons[86,87]. Firstly, anti-SAg antibody titres rise during staphylococcal infections, e.g. bacteraemia and wound infection[88,89]. Secondly, neutralising anti-SAg antibodies protect against SAg-induced toxic shock syndrome in animal models[90-93]. Finally, most TSS patients lack anti-TSST-1 antibodies and there is anecdotal evidence that treatment of TSS patients with immunoglobulin preparations is efficient[87,94,95].

Recently, Dryla et al. compared IgA and IgG antibody titres in healthy persons and patients with invasive disease. They used ELISA methods with a panel of 19 recombinant staphylococcal proteins and observed a pronounced heterogeneity among individuals in both groups[25]. Healthy individuals can have very high levels of circulating anti-staphylococcal antibodies, which probably reflect previous encounters with the micro-organism. Antibodies against the surface proteins clumping factor B, Efb and coagulase were missing or low in infected patients compared to healthy individuals[25].

A promising approach is to screen bacteriophage expression libraries of *S. aureus* with serum samples from infected patients, carriers and noncarriers[77]. Compared to carriers, non-carriers showed higher IgG concentrations against four antigens, including iron-responsive surface determinant (Isd) A and IsdH, and vaccination of cotton rats with IsdA or IsdH protected against nasal colonisation[77]. Moreover, higher IgG concentrations against seven *S. aureus* antigens were found in infected patients compared to healthy controls[77].

In summary, anti-staphylococcal antibodies have been shown to contribute to protection against staphylococcal disease. Therefore, it is reasonable to assume that susceptibility to disease correlates with the concentrations of anti-staphylococcal antibodies. This hypothesis needs to be tested in studies that include persistent and intermittent carriers and noncarriers as well as patients with different staphylococcal diseases. It is likely that antibodies directed against *S. aureus* surface structures as well as antibodies that neutralise secreted virulence factors are particularly important for protection. Since each staphylococcal lineage displays a unique architecture of surface proteins and individual strains differ remarkably in their MGE encoded virulence gene profile, the coordinated investigation of colonising or infecting *S. aureus* clones and the immune responses of their respective hosts promises to be the most informative approach.

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Staphylococcal superantigens: Do they play a role in sepsis?

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SUMMARY

In *Staphylococcus aureus*, 19 different superantigens (SAgs) have been described. Their genes are all located on mobile genetic elements, such as pathogenicity islands, plasmids and phages. SAgs bypass conventional antigen recognition by directly cross-linking major histocompatibility complex class II (MHCII) molecules on antigen-presenting cells with T cell receptors. This leads to massive T cell proliferation and cytokine release, which may end in toxic shock syndrome. The role of SAgs in other forms of sepsis is less well defined. In animal models, SAgs and lipopolysaccharide (LPS) very efficiently synergise in the induction of lethal shock, and on the basis of these observations a two-hit model of sepsis has been proposed: LPS or another monocyte stimulus hits first, then SAg or another T cell stimulus hits. In clinical studies, however, evidence for an involvement of SAgs in sepsis has been difficult to obtain. This may have a number of reasons: differences between humans and rodents in their response to LPS and SAg, heterogeneity of SAg combinations in *S. aureus* clinical isolates, lack of tools to analyse SAg effects in patients, blocking anti-SAg serum antibodies and MHCII polymorphisms.

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INTRODUCTION

Staphylococcus (S.) aureus is a multifacetted bacterium which lives in a state of armed neutrality vis-à-vis mankind. It persists as a commensal bacterium in 10–30% of the human population, but it is also a common cause of food poisoning[1]. Beyond this, *S. aureus* can cause infections of varying severity, ranging from skin abscesses and wound infections to debilitating and even life-threatening diseases such as osteomyelitis, endocarditis, necrotising pneumonia, toxic shock syndrome (TSS) and sepsis[2,3]. The pathogenicity of *S. aureus* is multifactorial. Its ability to cause such a broad range of diseases is due to an abundance of virulence factors which facilitate attachment, colonisation, tissue invasion, toxinosis and immune evasion[2,3]. However, host factors, environmental factors (e.g. intravascular catheters) and bacterial competition also contribute to the pathogenesis of staphylococcal infections[2,3]. Furthermore, several studies have demonstrated that colonisation with *S. aureus* is a significant risk factor for *S. aureus* infections[4-8].

Among the virulence factors of *S. aureus* are the superantigens (SAgs). SAgs are microbial exotoxins which activate large subpopulations of T lymphocytes, causing a massive cytokine release which may end in shock. In animal models it has been clearly demonstrated that SAgs can contribute to the pathogenesis of sepsis and septic shock. However, in clinical studies it has been difficult to show direct evidence for an involvement of SAgs, except for the rare cases of TSS.

The aims of this review are:

- to give an overview of the SAg spectrum encountered in *S. aureus,*
- to summarise recent data about the localisation of the SAg genes on the *S. aureus* genome,
- to outline the evidence for a role of SAgs in sepsis,
- to discuss why it is so difficult to measure SAg effects in clinical situations.

S. AUREUS SUPERANTIGENS

SAgs are microbial toxins which activate large subpopulations of T lymphocytes by bypassing the physiological antigen processing and presentation pathways. Some of them are effective at femtomolar concentrations and belong to the most potent T cell mitogens known, so that the term “superantigen” appears very appropriate[9]. SAgs can be secreted as exotoxins by different strains of *S. aureus*, *Streptococcus pyogenes, Streptococcus equii*, *Streptococcus dysgalactiae*, *Mycoplasma arthritidis* and *Yersenia pseudotuberculosis*, but there are also membrane-bound forms, which are encoded in the genome of mouse mammary tumor viruses[9,10]. Thus, superantigenic toxins, which have a common mechanism of T cell activation, have evolved in parallel in very distant micro-organisms.

This review focuses on the SAgs of *S. aureus*, where 19 different SAgs have been described: the TSS toxin (TSST)-1 and the staphylococcal enterotoxins (SE) A–R and SEU (table 1). Due to the pace of detection of new SAgs, their nomenclature is still subject to change, which may give rise to confusion. In this article, we follow the recommendations of

Table 1: Biochemical and functional properties of staphylococcal superantigens (SAgs).

SAg	Reference strain	Gen bank, accession number	localisation	MW (kDa)	Zinc binding	MHCII α/β chain	emesis	human TCR Vβ specificity	Ref.
TSST-1	N315	NCBI, SA1819	SaPI1	21,9	-	+/-	no	**2.1**, 8	11
SEA	Mu50	NCBI, SAV1948	lysogenic phage (ΦSA3)	27,1	C-term	+/+	+	1.1, 5.3, **6.3**, 6.4, 6.9, **7.3-4**, 9.1, 16, 21.3, 22, 23.1	12
SEB	Col	TIGR, SA0907	SaPI1	28,4	-	+/-	+	1.1, **3.2**, 6.4, 15.1	13
SEC1	-	NCBI, X05815	SaPI1	27,5	cleft	+/-	+	**3**, 3.2, 6.4, 6.9, **12**, **15.1**	
SEC2	-	NCBI, AY450554	SaPI1	27,6	cleft	+/-	+	12, 13.1, 13.2, 14, 15, 17, 20	
SEC3	N315	NCBI, SA1817	SaPI1	27,6	cleft	+/-	+	5.1, **12**	11
SED	pIB485	NCBI, AF053140	plasmid (pIB485)	26,9	C-term, cleft	+/+	+	1.1, **5.3**, 6.9, 7.4, 8.1, **12.1**	14,15
SEE	FRI918	NCBI, M21319	bacteriophage?	26,8	C-term	+/+	+	5.1, 6.3-4, 6.9, **8.1**	16
SEG	N315	NCBI, SA1642	SaPI3	27,0	-	+/-	+	3, 12, 13.1, 13.2, 13.6, **14**, 15	11,17
SEH	MW2	NCBI, MW0051	SCCmec	25,2	C-term	-/+	nd	**Vα10**	12,18
SEI	N315	NCBI, SA1646	SaPI3	24,9	nd	nd/+	+	1.1, **5.1**, 5.2, **5.3**, **6b**, 23.1	11,17
SEJ	pIB485	NCBI, AF053140	plasmid (pIB485, pF5)	28,5	nd	nd/+	nd	nd	14,15,19
SEK	Col	TIGR, SA0886	SaPI1	25,3	C-term?	nd/+	nd	**5.1**, 5.2, 6.7	13,20
SEL	N315	NCBI, SA1816	SaPI1	24,7	nd	nd/+	no	**5.1**, 5.2, 6.7,7, 9, 16, 22	11,21
SEM	N315	NCBI, SA1647	SaPI3	24,8	nd	nd/+	nd	6a, **6b**, 8, 9, 18, **21.3**	11,17
SEN	N315	NCBI, SA1643	SaPI3	26,1	nd	nd	nd	**9**	11,17
SEO	N315	NCBI, SA1648	SaPI3	26,7	nd	nd	nd	5.1, **7**, 9	11,17
SEP	N315	NCBI, SA1761	lysogenic phage (ΦSa3)	26,4	nd	nd	nd	nd	12
SEQ	Col	TIGR, SA0887	SaPI1	26,0	nd	nd	no	2.1, 5.1, **6.7**, 21.3	13,22
SER	Fukuoka 5	NCBI, AB075606	plasmid (pIB485, pF5)	27,0	nd	nd	nd	3, 11, 12, 13.2, **14**	19,23
SEU	MRSA252	NCBI, SAR1918	SaPI3	nd	nd	nd	nd	nd	

Staphylococcal enterotoxins (SEs) and TSST-1 are localised on mobile genetic elements and they activate T cells in a TCRVβ-specific manner (Vβ signature). Usually, one to three TCRVβ families dominate the response (bold), while the involvement of others is less pronounced. Abbrevations: C-term – C-terminal, SE – staphylococcal enterotoxins, TSST-1 – toxic shock syndrome toxin-1, SaPI – staphylococcal pathogenicity island, SCCmec– staphylococcal chromosomal cassette encoding the methicillin resistance gene, MW – molecular weight, MHCII – major histocompatibility complex class II, TCR – T cell receptor, nd – not determined. For reference see also[24-28].

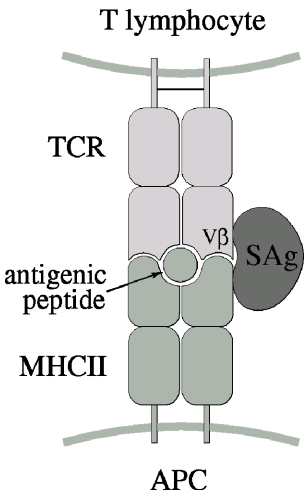

Figure 1: SAg function. SAgs bypass the conventional antigen recognition by directly cross-linking major histocompatibility complex class II (MHCII) molecules on the antigen-presenting cell (APC) with T cell receptors (TCRs) on T lymphocytes.

the International Nomenclature Committee for Staphylococcal Superantigen Nomenclature[29]. The SAgs of *S. aureus* and *Streptococcus pyogenes* form the subgroup of pyrogenic toxin SAgs. Beside acting as SAgs, the members of this group are also pyrogenic and enhance an endotoxin shock in experimental models[30]. In addition, after ingestion the SE can cause staphylococcal food poisoning, a very acute gastroenteritis[26].

Superantigenicity

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 then displayed to T cells on the APC surface. These MHC/peptide complexes are recognised 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 MHCII/peptide complexes by directly cross-linking conserved structures on TCRVβ hains with those on MHCII molecules. Both TCR and MHCII are contacted outside their antigen binding sites (figure 1)[10]. Therefore, while SAg action strictly depends on the presence of MHCII molecules, it is, in contrast to conventional antigen presentation, not restricted by certain MHCII alleles. In spite of this, MHCII alleles may differ greatly in their efficiency of SAg-binding[31,32].

On the T cell side, SAg binding is determined by the Vβ element used in the TCR. There are 47 functional Vβ elements in humans, which have been grouped into 23 TCRVβ families on the basis of sequence similarities[33]. Each SAg can bind to a subset of these Vβ elements, known as its Vβ signature. Usually, one to three TCRVβ families dominate the response, while the involvement of others is less pronounced. For example, TSST-1 binds to all TCRVβ 2-positive T cells, but in some cases activation of TCRVβ 8.1-expressing T cells is also found (table 1).

Consequently, individual SAgs can activate large fractions (5–20%) of the T cell population. In contrast, conventionally processed peptide antigens are recognised by only 1 out of 10^4–10^5 naive T cells. This Vβ -restricted T cell expansion is a characterising feature of all SAgs[30,34]. Interestingly, there is one exception: the SAg SEH contacts TCRVα chains[18].

SAg structure and function

Pyrogenic toxin SAgs are a diverse group of proteins. SEB and SEK, for example, have only 15.5% amino acid sequence homology[28]. However, resolution of their crystal structures has revealed a common three-dimensional structure, consisting of two globular domains, a C-terminal β-grasp motif (A domain) and a smaller N-terminal β-barrel domain (B domain)[35]. The residues determining TCRVβ specificity are located within the C-terminal region of the A domain, which is composed of four β-strands and a flanking α-helix. The B domain has an O/B (oligosaccharide/oligonucleotide-binding) fold, a common feature of different bacterial toxins. Interestingly, different mechanisms of MHCII binding have evolved within the family of pyrogenic toxin SAgs. Some SAgs have a low-affinity MHCII binding site in their B domain, which binds to the invariant α1-domain of MHCII[10]. Others have a second, zinc-dependent binding site in their A domain, which contacts the MHCII β-chain, so that they can cross-link MHCII molecules. Some SAgs can form dimers via their zinc-binding sites, and these dimers then contact two MHCII molecules (see reviews[30,36,37]; table 1).

Effects on target cells

The cross-linking of MHCII and TCR by SAgs induces activation of both APCs and T lymphocytes. TCRVβ-positive T cells proliferate and release large amounts of proinflammatory cytokines (IL-2, IFN-γ and TNF-α). The T cell proliferation phase is followed by a profound state of unresponsiveness or even cell death. Therefore, an expansion as well as a reduction of the proportion of TCRVβ-expressing T cells can be observed following SAg action[34,38].

Monocyte activation requires dimerisation of surface MHCII molecules and/or signaling via CD40[39]. Both can be achieved by SAgs, since they bridge the membranes of APCs and T cells and further induce the expression of CD40-ligand by the T cells. In addition, SAgs with two MHCII-binding sites can induce MHCII cross-linking and thus activate monocytes independently of T cells[40-43]. Activated monocytes secrete TNF-α, IL-1β and IL-6 in response to the SAg stimulus.

The systemic release of proinflammatory cytokines by T cells and monocytes can be detected *in vivo* a few hours after a SAg stimulus. In severe cases this leads to generalised capillary leakage and hypotension. TNF-α and IFN-γ are considered to be the most important mediators of this SAg-induced shock. This TSS-like syndrome is commonly observed after injection of TSST-1 or enterotoxins into rodents[44].

THE LOCALISATION OF SAG GENES IN THE *S. AUREUS* GENOME

While the pathological effects of SAgs have been studied in detail, their physiological functions in bacterial life have remained elusive. Genetic analysis of *S. aureus* clinical isolates, including whole genome sequencing, has shown the following:

- 70–80% of all *S. aureus* clinical isolates harbour SAg genes, 5 on average[17,45,46],
- the heterogeneity of the SAg repertoire between *S. aureus* strains is extensive[17,45-47],
- all staphylococcal SAg genes are localised on mobile genetic elements (table 1).

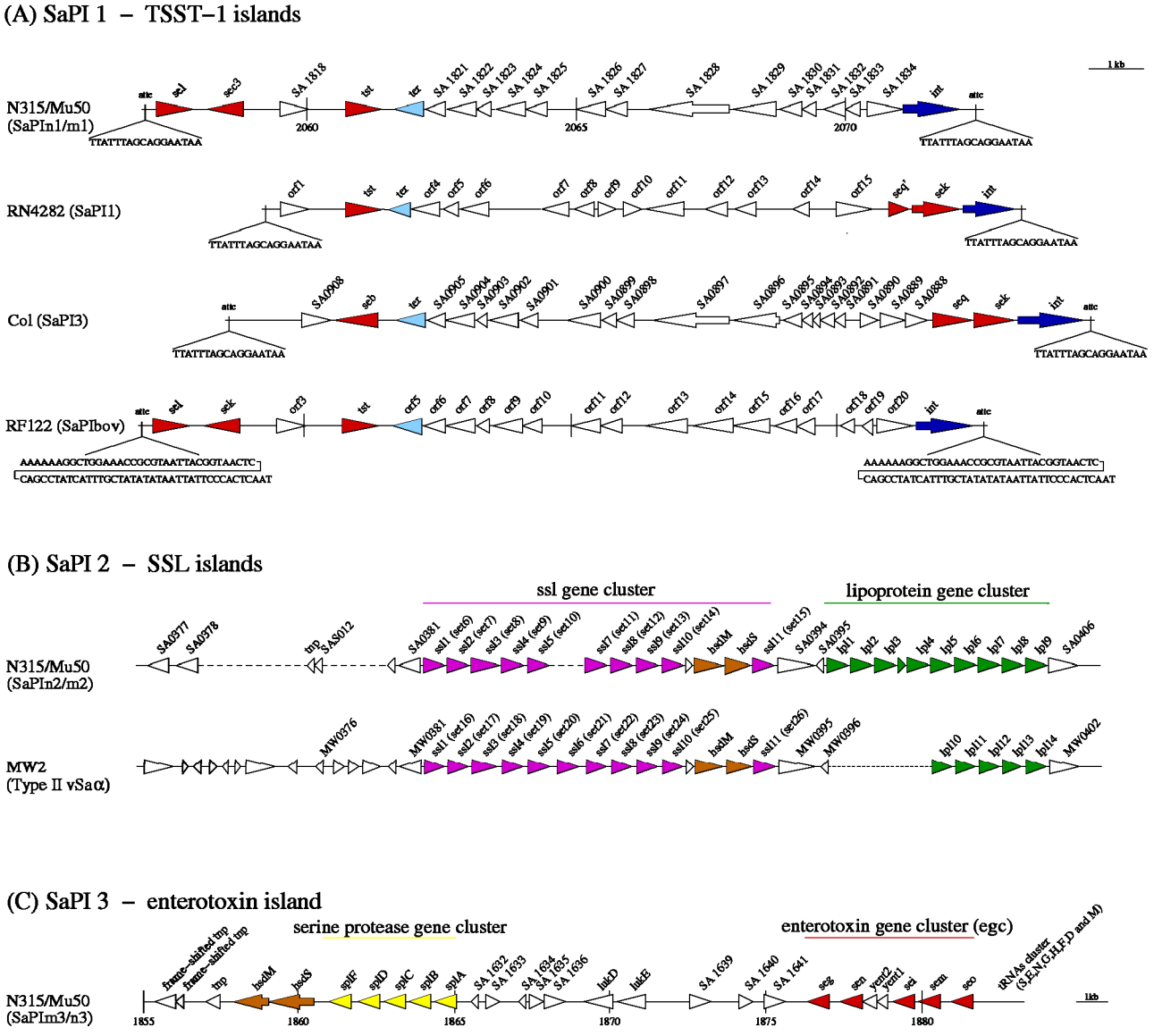

Figure 2: Staphylococcal pathogenicity islands. The staphylococcal pathogenicity islands 1-3 (SaPI1-3) carry superantigen (SAg) genes or staphylococcal superantigen-like (*ssl*) genes[11-13]. The PAI nomenclature is adapted from Kuroda et al.[11], synonyms are shown in brackets. **A)** SaPI1, **B)** SaPI2, **C)** SaPI3. Arrows and arrowheads indicate open reading frames (ORF) and their direction of transcription, while broken lines symbolise missing ORFs. Abbreviations: tnp – transposase gene, int – integrase gene, luk – leucotoxin gene. Color scheme: red – superantigens, magenta – ssl, yellow – serine proteases, green – lipoproteins, blue – integrases, light blue – likely terminase genes, brown – restriction/modification system, white – ORFs with unknown function*.

Whole genome sequencing has revealed that staphylococcal SAg genes are encoded by accessory genetic elements that are either mobile or were formerly mobile, i.e. plasmids, prophages, transposons and pathogenicity islands (table 1)[11,12,48]. The presence of SAg genes on mobile elements along with other virulence factors probably facilitates their horizontal spread between *S. aureus* strains[48,49]. In fact, a comparison of the 5 published *S. aureus* genomes (N315, Mu50, MW2, MRSA252 and MSSA476[11,12,48]) showed marked variation in the distribution and composition of these mobile elements (figure 2). This is reflected by the extensive heterogeneity of the SAg repertoire in *S. aureus* isolates.

Pathogenicity islands*

The pathogenicity islands (PAI) of *S. aureus* are the first clearly defined PAIs in Gram-positive bacteria. PAIs have evolved from former lysogenic bacteriophages and plasmids, and they are defined as large genomic regions (>15 kb) which are commonly present in pathogenic variants, but not in closely related non-pathogenic bacteria. PAIs carry virulence-associated genes, differ in their G+C content from the rest of the chromosomal genome, are flanked by direct repeats and carry mobility genes, including conserved integrases[13,50]. They are widely assumed to be mobile; however, of the staphylococcal PAIs, mobility has only been demonstrated for SaPI1bov[51]. Recently, variants of the staphylococcal PAIs have been discovered which lack virulence genes, so that the more general term “genomic island” is sometimes preferred[12,52].

SaPI1 (TSST-1 island)

The *S. aureus* pathogenicity island (SaPI)1 is the prototypic staphylococcal PAI[51,53]. It is 15.2 kb in length and is flanked by 17-bp direct repeats (att_c). SaPI1 encodes the SAgs TSST-1, SEK (formerly entK) and SEQ (formerly SEI), and it carries a functional integrase gene[53]. Lindsay et al.[51] have demonstrated that SaPI1 can be mobilised by the helper phage Φ80α. During the vegetative growth of Φ80α, the genomic island is excised from its unique chromosomal insertion site att_c, amplified and encapsidated into specialised phage heads. After transduction to a *recA*-deficient *S. aureus* recipient strain, SaPI1 integrates at the att_c site, presumably directed by the self-encoded integrase[53]. In the absence of a helper phage the island is very stable.

Several variants of SaPI1 have been described, which differ in their SAg genes (fig. 2A). SaPIbov from a bovine mastitis isolate contains *tst*, a *sec* variant and *sel*. The SaPI1 homologue of the *S. aureus* reference strain COL, which has been named SaPI3 (not to be confused with the SaPI3 described below), contains *seb* at the same position as *tst* in SaPI1 and, additionally, *sek* and *seq*[20,22]. This explains the phenomenon of toxin gene exclusion: *seb* (on SaPI3) and *tst* (on SaPI1) never coexist in a clinical *S. aureus* isolate[13].

SaPI3 (enterotoxin island)

The SaPI3 is composed of a serin protease gene cluster, a leucocidin gene cluster (*lukD*, *lukE*) and an enterotoxin gene cluster (*egc*) (fig. 2C)[11]. The *egc* contains five SAg genes, *seg*, *sei*, *sem*, *sen* and *seo*, as well as two pseudogenes with sequence homology to enterotoxin genes, ψ*ent1* and ψ*ent2*[11,17]. Recently, a new putative enterotoxin gene locus *seu* has been discovered, which results from a gain of function mutation in the pseudo-genes[54]. Based on phylogenetic analyses, Lina et al.[29] have suggested that the *egc* may be the enterotoxin nursery from which all known *S. aureus* SAg genes have evolved. *egc* SAgs are the most frequent SAgs in *S. aureus*, as 50–60% of clinical isolates contain this gene cluster, but so far they could not be clearly associated with clinical syndromes[17,45,46,55-58]. Transcriptional analysis revealed that the *egc* functions as an operon[17].

* The nomenclature of the staphylococcal pathogenicity islands was recently modified, for comparison refer to table 1 in the appendix (page v).

Prophages

All sequenced *S. aureus* strains (except for COL) have the prophage ΦSa3 integrated into their chromosomes[12,48]. There are several variants of this bacteriophage: The *S. aureus* strain Mu50, for example, carries an *sea* gene on ΦSa3, while in N315 *sep* is present at the same locus[11,12]. MW2 and the closely related strain MSSA476 harbor *sea* and, additionally, genes for *seg2* and *sek2,* the latter two being homologues to *seq* and *sek*, respectively, which are found on SaPI1 in COL[12,48]. ΦSa3 is generally mobile, but some defective variants have been described[59,60]. The gene for SEE, *see*, is probably also located on a phage[16].

Plasmids

The genes for SED, SEJ and the recently discovered SER are carried by plasmids. *sed* and *sej* are co-localised on the penicillinase plasmid pIB485[14,15]. The *ser* gene is either located together with *sed* and *sej* on a pIB485-related plasmid, or only with *sej* on a pF5-related plasmid[19,23].

SaPI2 (SSL island)

On *SaPI2* a large family of putative exotoxins has been identified which share sequence homology with SAgs[11,12]. These proteins were discovered by Williams et al.[61], who described a cluster of five related genes in *S. aureus,* which they called SE-like genes, *set1–set5*. Comparison of *set*-clusters from different strains showed a remarkable heterogeneity in both gene number (7–11 genes) and the encoded peptide sequences[62]. Recently, the International Nomenclature Committee for Staphylococcal Superantigen Nomenclature recommended that the SET family should be renamed staphylococcal superantigen-like (SSL) proteins and that the encoding genes should be designated *ssl1–ssl11*[29] (fig. 2B). Their function is still unknown. Despite their sequence homology to SAgs, SSL-proteins do not have SAg properties[62,63]. Crystal structure analysis revealed a structural similarity with SAgs; however, the residues which are important for MHCII and TCR binding are not conserved[63,64]. Recombinant SSL1 has been shown to induce IL-1β, IL-6 and TNF in peripheral blood mononuclear cells[61]. Moreover, SSL5 and SSL7 have been demonstrated to interact with monocytes and dendritic cells[64]. We propose that SSL proteins play a role in the interaction of *S. aureus* with its host because, firstly, the *ssl* gene cluster is located on a pathogenicity island and, secondly, antibodies against SSL proteins are highly prevalent, implying that the SSL proteins are secreted in the host[11,12,62]. The fact that the cluster is present in every *S. aureus* strain indicates that it is essential[45].

DIFFERENT PATHWAYS INTO SEPTIC SHOCK

Sepsis is defined as a systemic inflammatory response to an infection. The causative agents are micro-organisms or their toxins, which spread from a local infection site and enter the blood stream. Severe sepsis is complicated by organ dysfunction, and the term septic shock refers to the subsequent state of acute circulatory failure[65]. Sepsis and septic shock are the major causes of death in intensive care units[66,67]. Recent USA and European epidemiological studies have reported that severe sepsis accounts for 2–11% of all hospital or intensive care unit admissions and that each year it causes the death of 200,000 patients in the USA

alone[68]. While Gram-negative infections were the predominant cause in the 1960s and 1970s, the incidence of Gram-positive infections increased in the past two decades. Today, Gram-positive organisms account for about half of the cases of severe sepsis[69,70]. Despite intensive research and improvements in supportive care, hospital mortality of severe sepsis and septic shock has remained frighteningly high: 30 and 60%, respectively[71,72].

Gram-negative sepsis

The pathophysiology of Gram-negative sepsis has been thoroughly studied in the last decades and it is now understood in great detail. Lipopolysaccharide (LPS), or endotoxin, which is a major component of the outer cell membrane of Gram-negative bacteria, plays a key role. LPS acts as a pathogen-associated molecular pattern (PAMP) and it very strongly activates monocytes and macrophages, which recognise minute amounts of the endotoxin via specific pattern-recognition receptors. The elucidation of the very complex recognition process constitutes a milestone in immunological research. LPS is bound by the soluble LPS-binding protein (LBP) and transferred to a membrane-bound receptor complex on

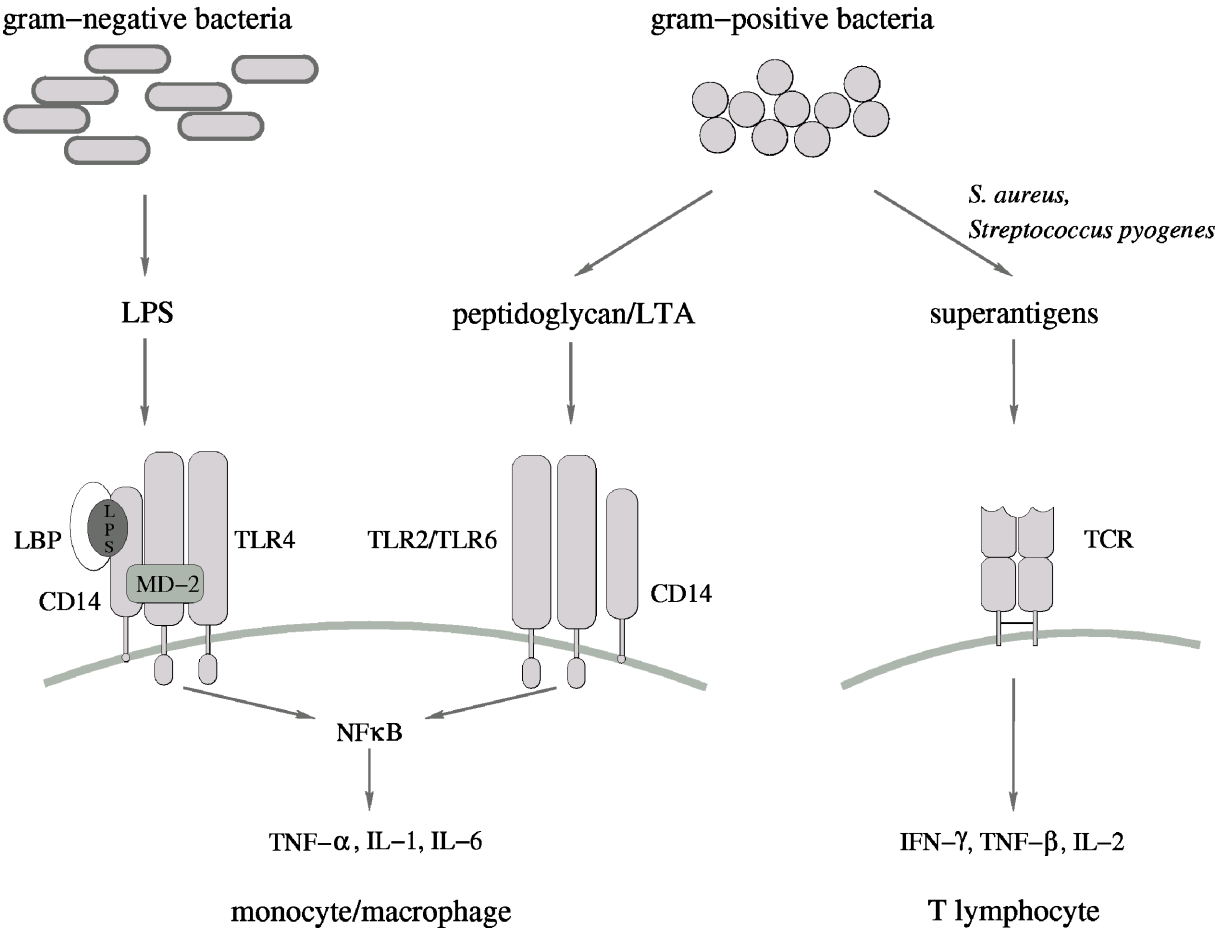

Figure 3: Different pathways into septic shock. Two pathways for triggering lethal shock can be defined: Cell wall components from Gram-negative and Gram-positive bacteria (LPS, peptidoglycan/LTA) interact with pattern-recognition receptors on monocytes, while SAgs activate T lymphocytes. Monocytes and T cells are triggered to release large amounts of proinflammatory cytokines which can eventually induce lethal shock. While TNF-α is the most important cytokine in monocyte-mediated shock, IFN-γ plays a key role in SAg-induced shock. Abbrevations: LPS – lipopolysaccharide, LBP – LPS binding protein, MD-2 – myeloid differential protein 2, TLR – Toll-like receptor, LTA – lipoteichoic acid, TCR – T cell receptor.

monocytes/macrophages, which is composed of CD14, Toll-like receptor (TLR) 4 and myeloid differential protein-2 (MD-2) (Fig. 3)[73-75]. CD14, a GPI-anchored protein, probably helps to load LPS onto the TLR4-MD-2 complexes[76]. The signal is then transduced by TLR4 and results in the activation of the transcription factor NFκB. In response to this signal, monocytes and macrophages synthesise and secrete large amounts of proinflammatory mediators, such as TNF, IL-1, IL-6, chemokines, platelet activating factor, leukotriens, colony-stimulating factors, oxygen radicals and nitric oxide (NO). These mediators contribute to sepsis pathogenesis by inducing vascular leakage, hypotension (especially via NO), haemoconcentration and metabolic acidosis[70,77].

Gram-positive sepsis

S. aureus, coagulase-negative staphylococci and streptococci are the most common causes of Gram-positive sepsis. In contrast to Gram-negative organisms, these bacteria do not contain LPS. However, they produce a variety of extrinsic and intrinsic molecules which can trigger inflammation: cell wall components, such as peptidoglycans and lipoteichoic acid (LTA) on the one hand, and exotoxins, such as SAgs, on the other[78]. Similarly to LPS, LTA and peptidoglycans as well as whole bacteria are recognised by monocytes and macrophages via the pattern-recognition receptors TLR2 and, probably, TLR6[78-81] (fig. 3). This induces the release of proinflammatory cytokines (TNF-α, IL-1, IL-6 and IL-8) and NO. Additionally, peptidoglycan seems to activate coagulation and alternative complement pathways[69,82]. Moreover, LTA and peptidoglycan synergise in the induction of NO formation and the release of TNF and IFN-γ[83] and, importantly, they act synergistically in inducing shock and multiorgan failure in rats[84]. However, in comparison with LPS, their potency is low[80].

While LPS as well as cell wall components of Gram- positive bacteria predominantly activate monocytes, macrophages and other cells of the innate immune system, SAgs primarily target T lymphocytes. As described above, SAgs induce a massive cytokine release from T cells and monocytes, which in rare cases can lead to TSS, which is characterised by fever, hypotension, rash, desquamation and multiorgan failure[85,86].

TSS – SAg-induced shock

As described above, TSS can be considered as a form of Gram-positive sepsis, which is dominated by the pathophysiological effects of staphylococcal or streptococcal SAgs. TSS is a rare disease with an incidence of 1/100,000[87]. It occurs most frequently in young women during their menses due to the usage of highabsorbency tampons, which facilitate the growth of *S aureus* (menstrual TSS), but it can also be a complication of surgery, burns and wound infections (nonmenstrual TSS)[85,87]. The causative agent of menstrual TSS is TSST-1, and a single *S. aureus* clone has been shown to be responsible for the majority of menstrual TSS cases[88]. The strict association of TSST-1 with menstrual TSS is probably due to its unique ability to cross mucosal barriers[89]. Non-menstrual TSS cases represent one third of all TSS cases today[87] and they are associated with TSST-1 or other staphylococcal SAgs[90-92].

Several studies have unequivocally demonstrated SAg effects during clinical streptococcal or staphylococcal TSS. Firstly, the selective activation and expansion (or deletion) of T cells corresponding to the TCRVβ signature of the suspected SAg has been reported in TSS patients[34,38,93]; for example, up to 70% of the peripheral T cells were Vβ2 positive in a case of TSST-1-induced TSS[34]. Secondly, in some cases serum samples from TSS patients are mitogenic for T cells, which indicates the presence of SAgs[9,94]. Additionally, in rare cases SAg could be directly measured in the serum of TSS patients[9,95]. Finally, SAg-neutralising antibodies are beneficial in streptococcal TSS, and treatment with intravenous immunoglobulins reduces mortality[96-99].

THE TWO-HIT MODEL OF SEPTIC SHOCK

Animal models confirm that two pathways into septic shock can be distinguished, one dominated by the innate, the other by the adaptive immune system: On the one hand, SCID-mice, which lack B and T cells, are resistant to SEB-induced shock, but sensitive to LPS. This SAg resistance can be reversed by reconstitution with T cells[44]. On the other hand, SEB can cause lethal shock in endotoxin-resistant C3H/HeJ mice, which lack functional TLR4, despite their deficient macrophage response[100]. Since both pathways lead to the release of large amounts of proinflammatory cytokines and eventually to lethal shock, the question arose whether these pathways may synergise. In most immune responses the innate and the adaptive immune systems cooperate, and the multitude of synergistic and antagonistic interactions at the interfaces of their intricate network are in the focus of intensive research. It appears that an extreme dominance of either the innate or the adaptive response is probably the exception rather than the rule: In an organism which is colonised with huge numbers of different micro-organisms, the isolated exposure to stimuli of the innate immune system, such as LPS (or LTA and peptidoglycans), or to stimuli of the adaptive immune system, such as SAgs, may be a rare event outside of the laboratory. These considerations and the observation that LPS and SAg synergise in rodents have motivated the development of the two-hit model of septic shock by Bannan et al[101]. They suggested that in septic shock the following sequence of events may be typical: A Gram-negative infection causes symptoms of vasodilatation and hypotension (1st hit). Treatment with fluids and antibiotics rescues the patient. However, some days later a Gram-positive insult, typically originating from the skin or the gastrointestinal flora, causes an irreversible shock in the LPS-sensitised patient (2nd hit)[101]. While the sequence of events may differ from the original hypothesis, there is now a large body of experimental data which supports the two-hit model: 1st hit – LPS or another monocyte stimulus; 2nd hit – SAg or another T cell stimulus.

Synergism of SAg and LPS in shock induction

Co-injection of LPS and SAg (SEB) in mice reduces the lethal dose for both shock inducers almost 100-fold and enhances the release of TNF-α, IL-6 and IFN-γ[102]. This synergism is T cell dependent and effectively prevented by cyclosporin A. The key mediator appears to be IFN-γ, since neutralising antibodies to it are inhibitory[102]. The most impressive form of synergism is observed when animals are primed with a sublethal dose of SAg followed by an injection of endotoxin a few hours later[102-106]. The group of Schlievert studied the kinetics of

TSST-1-induced LPS enhancement in a rabbit model. Depending on the doses of TSST-1 and endotoxin given, rabbits showed an up to 50,000-fold enhanced susceptibility to either SAg or endotoxin[105]. Similarly, the injection of a moderate dose of the SAgs SEB or TSST-1, which was not able to trigger a lethal cytokine syndrome, increased the sensitivity to endotoxin in mice[103,104]. A sublethal priming injection of TSST-1 12 hours before LPS-injection reduced the lethal dose of LPS 20-fold and induced a 1000-fold increase in serum TNF-α levels[104]. T cells were essential for this SAg-mediated LPS sensitisation, because T cell-deficient SCID mice neither upregulated TNF-α serum levels nor exhibited enhanced lethality[103,104]. Additionally, the SAg effect was reconstituted by adoptive T cell transfer[104]. Cyclosporin A treatment and anti-IFN-γ antibodies were protective and strongly reduced TNF-α serum levels[104]. Altogether, these data clearly show that the activation of T cells is the basis of the SAg-mediated LPS-priming in mice.

What are the sources of LPS in SAg-primed organisms? The commensal Gram-negative gut flora probably plays an important role. Low levels of circulating endotoxin can be detected even in healthy humans[107], and increased intestinal absorption as well as reduced endotoxin clearance can lead to elevated endotoxin plasma concentrations[108-111]. This has been observed in patients with liver cirrhosis, hemorrhagic shock, cardiac surgery and severe acute pancreatitis[107,112-114]. An elevation of endogenous circulating LPS may even be caused by the SAg itself, since SAg-induced hypotension with splanchnic hypoperfusion and ischaemia injury damages the intestinal barrier function, so that Gram-negative bacteria and/or endotoxin can translocate across the gut wall[109,113].

SAgs (in synergy with LPS) may also be toxic for hepatocytes[115,116], which are crucially involved in endotoxin clearance[108,109]. There is evidence that endogenous endotoxins also contribute to the severity of TSS: In rabbits TSST-1 treatment increased the levels of circulating endotoxin, and death could be prevented by co-administration of the LPS-neutralising drug polymyxin B[111]. Similarly, in a study with 10 human TSS patients the serum concentration of endotoxin was increased in the acute phase and returned to normal values in convalescence[111]. These data show that the intestine can act as an endogenous source for endotoxin. Increases in LPS translocation, especially when combined with reduced endotoxin clearance, may lead to LPS serum concentrations which, in synergy with SAgs, may become life threatening.

Co-infecting Gram-negative bacteria could be an exogenous source of LPS in Gram-positive sepsis. Large proportions of patients with non-Gram-negative sepsis show endotoxaemia, and TSS patients frequently acquire opportunistic infections with Gram-negative bacteria, such as *Haemophilus influenca, Pseudomonas aeroginosa* and *Escherichia coli*[105,117,118]. Polymicrobial infections are reported to account for approximately 10% of bactaeremic episodes[119,120]. However, the incidence of polymicrobial infections may still be underestimated, because they are difficult to detect[70,121].

The SAgs SEA, SEB and TSST-1 strongly enhance the LPS-induced production of cytokines, such as TNF-α, IFN-γ and IL-6[102,106,122]. TNF-α then plays a key role, because in

SAg-primed mice, which lack the p55 TNF receptor, LPS is not lethal[102]. The SAg-induced rise in TNF-α secretion is mainly mediated by the T cell-derived IFN-γ, a strong macrophage activator. Purified IFN-γ enhances TNF-α synthesis by LPS-stimulated monocytes *in vitro* and it induces LPS hypersensitivity in mice, whereas *in vivo* neutralisation of IFN-γ activity prevents it[102,104,123-126]. Bosisio et al.[123] showed that IFN-γ increases the expression of the LPS receptors TLR4 and CD14 by monocytes, which could explain the sensitising effect. Conversely, short-term preincubation with TLR ligands, e.g. LPS, amplifies the IFN-γ signaling via increased phosphorylation of STAT1[127,128]. In the effector phase, IFN-γ augments the toxic TNF-α effects on different tissues[129]. In addition to IFN-γ, secretion of GM-CSF and surface expression of CD40-ligand by SAg-activated T cells may contribute to the overwhelming monocyte activation, which is the hallmark of LPS sensitisation.

DO SAGS AND ENDOTOXIN SYNERGZE IN HUMAN SEPSIS?

Data from animal models have convincingly demonstrated that SAgs and LPS very efficiently synergise in the induction of lethal shock. If SAgs could also sensitise humans to LPS, this might have serious consequences given the high toxicity of endotoxin in man: The lethal LPS dose of 1–2 µg would be reduced to ng or even pg amounts, assuming a similar amplification[130]. However, evidence for the relevance of the two-hit model in human sepsis has been difficult to obtain. This may have a number of reasons:

1. differences between humans and rodents in their response to LPS and SAg,
2. heterogeneity of SAg combinations in *S. aureus* clinical isolates,
3. lack of tools to analyse the SAg effects in patients,
4. blocking anti-SAg serum antibodies and
5. MHCII polymorphisms.

1. Differences between humans and rodents in their response to LPS and SAg

Interactions of SAg and endotoxin are usually assessed in mice and rabbits. In comparison with humans, their susceptibility to the toxicity of LPS and SAgs is low, because small sequence differences in the cellular toxin receptors result in much lower affinities[131-134]. Therefore, in humans even minute concentrations of SAg and/or LPS, which are below the level of detection, might have strong effects.

2. Heterogeneity of SAg combinations in S. aureus clinical isolates

In mouse models of SAg-induced shock, a single purified or recombinant SAg is usually injected into the animal. However, a recent survey revealed that clinical *S. aureus* isolates harbor five SAg genes on average[45]. The effects of complex “SAg cocktails” on the pathogenesis of severe systemic infections are poorly understood.

3. Lack of tools to analyse the SAg effects in patients

By definition, SAgs activate T cells in a TCRVβ-restricted manner. Therefore, SAg effects in patients are usually measured by a shift in the TCRVβ repertoire of the T cell pool. However, the characterisation of the Vβ signatures of individual SAgs is complicated by several factors. Firstly, SAg effects may be variably associated with T cell proliferation or apoptosis.

Secondly, the T cell responses are influenced by MHCII polymorphisms[135]. Finally, while the Vβ signature may predict the shift in the TCRVβ repertoire in *S. aureus* strains with only a single SAg gene, most *S. aureus* strains carry multiple SAg genes, and in these cases their composite Vβ signature cannot be inferred from the determined SAg gene repertoire (Holtfreter, unpublished data). Therefore, the analysis of the T lymphocyte Vβ subset composition does not appear to be suitable for the detection of SAg involvement in most cases of sepsis and related illnesses.

Direct measurements of SAgs in serum have only rarely been successful, since SAgs on their own are effective in femtomolar concentrations, and synergism with LPS may occur at even lower concentrations. However, in a recent study, circulating staphylococcal and streptococcal SAgs were detected in 5/16 sepsis patients and 10/24 patients with septic shock[136]. Additionally, the streptococcal SAg SPEA has been detected in the serum of 4/7 patients with streptococcal TSS or invasive disease immediately after admission[95]. Furthermore, it has been shown that anti-SAg antibody titers rise after staphylococcal infections, such as wound infections or septicaemia[137,138]. However, while serum conversion indicates a systemic release of SAgs during infection, it allows no conclusions about the SAg effects on T cells.

4. Blocking anti-SAg serum antibodies

SAgs are strongly immunogenic and there is a high prevalence of antibodies against SEA, SEB, SEC, SED and TSST-1 in the healthy community[137,139-141]. These antibodies can neutralise SAgs and abolish their proliferative effects on T cells[45]. A notable exception are the SAgs encoded by the *egc* on SaPI3 (Fig. 2C), which are the most prevalent SAgs in *S. aureus*[17]. Surprisingly, neutralising antibodies against the *egc*-encoded SAgs are very rare[45]. Seroconversion against SAgs has been observed in patients with *S. aureus* septicaemia, though minor infections and possibly even *S. aureus* carriage may also induce an anti-SAg antibody response[137,138].

Evidence for a protective role of neutralising anti-SAg antibodies is abundant: While more than 90% of healthy men and women older than 25 years have high anti-TSST-1 antibody titers, these are absent in 90% of patients with menstruation-associated TSS[85,142-144]. A lack of specific antibodies was also found in invasive streptococcal disease[99,145,146]. Intravenous Ig preparations (IVIG), which are prepared from large pools of human plasma, contain considerable amounts of neutralising anti-SAg antibodies, and treatment with IVIG reduced cytokine secretion, bacterial load and mortality in streptococcal TSS[96-98,147-149]. Moreover, there is anecdotal evidence that an IVIG therapy can also decrease mortality in staphylococcal TSS[85]. Therefore, when analysing the role of SAgs in a clinical context, the patient's antibody status has to be taken into account, because it can skew or even abolish the SAg effects on T cells.

5. MHCII polymorphisms

The impact of host genetic factors on the susceptibility to infections as well as on outcome has come into the focus of research, and the influences of age, gender, cytokine genes and,

most importantly, MHC genes have been discussed[150,151]. Host factors which influence the outcome of systemic infections with *Streptococcus pyogenes* have been studied in detail. A single clone of *Streptococcus pyogenes* can cause clinical syndromes of varying severity, ranging from superficial carriage through pharyngitis to toxic shock syndrome[145,152,153]. Recently, MHCII haplotypes were identified to be a key factor in host susceptibility to severe infections with *Streptococcus pyogenes*: Certain MHCII haplotypes are protective, while others increase the risk of disease[154]. Llewelyn et al.[135] have demonstrated that the SPEA binding affinity to different MHC-DQ alleles varies significantly, which results in dramatic differences in T cell proliferation and cytokine production, and which even influences the TCRVβ repertoire of the stimulated T cells. Especially at low SAg concentrations, as might be encountered during sepsis, only strongly binding MHCII alleles mediate TNF-α secretion[135]. However, the possibility that MHC polymorphisms could influence superantigenicity and thus clinical susceptibility to the toxicity of individual SAgs has received little attention until recently. While the findings do not contradict the well-established notion that SAg action strictly depends on the presence of MHCII molecules but is not restricted by certain MHCII alleles (as is the presentation of conventional antigens), they add one more level of complexity to the analysis of SAg effects in patients.

CONCLUSIONS

In experimental settings, shock can be induced by LPS, which activates monocytes via pattern-recognition receptors and also by SAgs, which activate large T cell subpopulations. The two pathways are interconnected and potentiate each other. We suggest that the two-hit model of sepsis should be generalised, because in most cases of systemic bacterial infections both the innate and the adaptive immune system will be stimulated simultaneously or sequentially. The 1st hit would comprise all PAMPs which can activate the cells of the innate immune system after binding to their pattern-recognition receptors. The 2nd hit would include SAgs and other T cell stimuli, for example recall antigens, which elicit a fast and strong memory response. TSS, with its strong bias on T cell stimulation, could be regarded as one extreme form of such a two-hit scenario, while the experimental endotoxin shock would represent the other end of the spectrum.

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Clonal distribution of superantigen genes in clinical *S. aureus* isolates

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ABSTRACT

Staphylococcus aureus is both a successful human commensal and a major pathogen. The elucidation of the molecular determinants of virulence, in particular the 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 have simultaneously determined the genetic background (*spa* typing) and the distribution 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 characterised 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 harbouring the exfoliative toxin D gene. In contrast, there was no association of superantigen genes with blood stream 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.

SUBMITTED

INTRODUCTION

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 bacteraemia. At the same time, *S. aureus* is a persistent coloniser of the human nose in 20% of the population and intermittently carried by another 30%[1]. Colonisation with *S. aureus* is a major risk factor for staphylococcal infections[1,2]. In carriers, 80% of nosocomial *S. aureus* bacteraemia cases have an endogenous origin, which underlines the importance of host factors[3,4]. On the other hand, there is plenty of evidence that *S. aureus* clones differ in their disease-evoking potential, but it has been difficult to explain these differences at the molecular level[5-7].

Whole genome micro-arrays recently revealed that the *S. aureus* genome consists of a core genome (~75%), a core variable genome (~10%) and mobile genetic elements (MGE, ~15%)[6]. The highly conserved core genome comprises house keeping genes located on the bacterial chromosome. Subtle variations of the core genome are exploited by sequence-based genotyping methods, such as multilocus sequence typing (MLST) and protein A (*spa*) genotyping. *Spa* genotyping is based on variations of the polymorphic region within the protein A gene[8]. It has a high discriminatory power similar to pulsed field gel electrophoresis (PFGE) but results can be more easily compared between laboratories[9-11]. Moreover, *spa* typing, MLST and PFGE are highly concordant, and *spa* typing data can be easily mapped onto the MLST *S. aureus* database (www.spaserver.ridom.de)[10,11]. Genotyping analyses revealed a highly clonal population structure with ten predominant clonal lineages in the species *S. aureus* [5,12,13].

The core variable genes are encoded on the bacterial chromosome and mainly codes for surface associated proteins, several toxins as well as regulatory functions such as the accessory gene regulator (*agr*)[6]. They are rather conservative within lineages which results in a unique combination of core variable genes in each of the ten predominant lineages[6,14].

MGEs, such as plasmids, phages, pathogenicity islands and genomic islands, carry a variety of staphylococcal resistance and virulence genes. MGEs can horizontally spread among *S. aureus* of the same lineage and potentially also between lineages[15,16]. Sometimes they are conspicuously absent from certain clonal complexes, presumably due to restrictions on horizontal transmission[17].

It remains elusive how staphylococcal virulence is determined on a molecular level. Regarding the core genome, nasal and invasive strains probably do not differ fundamentally, because they fall into the same main clusters[5,12]. Similarly, analysis of the core variable genome did not identify factors clearly related to virulence. This suggests that staphylococcal virulence might primarily depend on MGE-encoded toxin or resistance genes, such as *mecA* and *pvl* genes[5,14,18]. However, except for some toxins, it has been difficult to assess the contribution of individual virulence determinants to *S. aureus* pathogenicity[6,7,19,20].

Since many MGE-encoded virulence factors are linked to clonal complexes, their association with invasiveness could be influenced by effects of the genetic background[7]. Consequently, we propose that focussing the analysis on those *S. aureus* clonal complexes which harbour certain virulence factors will increase its sensitivity and specificity. This requires the simultaneous determination of the genetic background (clonal lineage) and virulence genes. However, to date, such studies are rare[14,21,22]. Because of their extraordinary variability in the species *S. aureus,* we have chosen the 19 *S. aureus* superantigens, SEA-SEE, SEG-SER, SEU and TSST-1, as a model to test this approach.

SAgs are secreted toxins, which induce a strong activation of large T cell subpopulations. This can result in toxic shock[23]. 80% of all *S. aureus* strains harbour SAg genes, on average 5 to 6, among which the *egc* SAgs are the most prevalent[24-27]. Most *S. aureus* SAg genes are located on phages and pathogenicity islands[15]. Staphylococcal phage Φ3 encodes either *sea* (strain Mu50), *sep* (N315) or *sea-sek-seq* (MW2)[17,28]. A family of related pathogenicity islands carry *seb-sek-seq* (SaPI1, COL), *tst-sec3-sel* (SaPI2; N315 and Mu50) or *sec-sel* (SaPI3; MW2)[*17,28,29]. The enterotoxin gene cluster, *egc*, encoding *seg-sei-sem-sen-seo* and sometimes *seu*, is located on the genomic island vSAβ[15,17]. Other SAg genes are found on plasmids (*sed-sej-ser*) or on SCC*mec* (*seh*)[28,30,31]. Several groups have reported that certain SAg genes are associated with particular clonal lineages[7,14,32-34].

Here we show the results 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 the North-East of Germany. Our aims were to investigate to which 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. *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). All participants gave informed consent, and both studies were approved by the Ethics Board of the University of Greifswald.

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

[*] The nomenclature of the staphylococcal pathogenicity islands was recently modified, for comparison refer to table 1 in the appendix (page v).

neonatology, 2 urology, 1 gynaecology, 4 paediatrics). Six Isolates were from a general hospital near Greifswald (Wolgast); 4 strains were isolated from patients from a neuro-rehabilitation centre, Greifswald. Only one isolate was included from each patient. We observed no spatial or temporal clustering of *S. aureus* genotypes.

Nasal-carriage isolates from Sczcecin: 108 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. The participants had an average age of 33.3 years and 62.3% were male. All participants gave informed consent, and the study was approved by the Ethics Board of the University of Sczcecin.

Control strains for the polymerase chain reaction (PCR)-based assays included A920210 (*egc, eta, agr4*)[35], CCM5757 (*seb, sek, seq, agr1*), Col (*seb, sek, seq, mecA, agr1*)[28], FRI1151m (*sed, sej, ser, agr1*)[14], FRI137 (*sec, seh, sel, egc+seu, agr2*), FRI913 (*sea, sec, see, sek, sel, seq, tst, agr1*), N315 (*sep, sec, sel, tst, egc, mecA, agr2*)[17], TY114 (*etd, agr3*) 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 Wizzard® 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[8,9]. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced using both amplification primers from a commercial supplier (SeqLab, Goettingen, Germany). The forward and reverse sequence chromatograms were analysed with the Ridom StaphType software (Ridom GmbH, 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 less than or equal to five. *Spa* types shorter than five repeats were excluded from the analysis, because they do not allow the reliable deduction of ancestries.

MLST genotyping. MLST genotyping was performed on selected *S. aureus* isolates (indicated with an asterisk in Table 2 and 3) according to published protocols[13]. Otherwise, MLST-CCs were deduced from BURP grouping of *spa* types using the Ridom SpaServer database (www.spaserver.ridom.de)[11].

SAg multiplex PCR Polymerase chain reactions. *16SrRNA* PCR was performed with each DNA-preparation to control DNA quality and absence of PCR inhibitors[24]. G*yrase* primers allowed the identification of *S. aureus*[36]. Methicillin resistance was detected with *mecA*-specific primers[37]. All strains were negative for the Panton-Valentine-leukocidin (*pvl*) genes as tested by PCR for *lukS-lukF*[14]. 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 group I-IV* (suppl. table 1). Primer pairs for detection of *sea-see, seh, sem, seo*, *ser*, *seu*, *tst, eta, etd, pvl* and *agr group I-IV* were previously described[14,35,38-40]. Primers for *seg, sei* and *sej* were modified from published primer sequences [41], and primers for *sel, sek*, *sen, sep,* and *seq* were designed for this study (suppl. table 1).

Single and multiplex PCRs were performed with the GoTaq® Flexi DNA polymerase system (Promega). Each reaction mix (25 µl) contained 5 µl 5x GoTaq® reaction buffer, 100 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Roche Diagnostics, Mannheim, Germany), 5 mM $MgCl_2$, 150-400 nM of each primer, 1.0 U GoTaq® Flexi DNA polymerase and 10-20 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 (1x TBE buffer), stained with Etbr and visualised 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 SAg gene-negative control.

***sem* gene sequencing.** The PCR for amplification of the *sem* gene variant was performed with sequencing primers (sems-1,-2) flanking the *sem* open reading frame using the HotGoldStar Polymerase system (Eurogentec, Seraign, Belgium). Each reaction mix (50 µl) contained 5 µl 10x HotGoldStar reaction buffer, 200 µM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 1 µM of primers (sems-1, sems-2), 1.0 U HotGoldstar DNA polymerase and 10-20 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 prevalence of a particular SAg gene or *agr* type between clonal complexes.

RESULTS

In this study we investigated the *spa* genotypes and SAg gene patterns of nasal and blood culture *S. aureus* isolates from Western Pomerania, in the North-East of Germany. The demographic data are summarised in table 1. A total of 107 nasal isolates were obtained from healthy blood donors during two studies on *S. aureus* nasal colonisation (T strains, SH strains). In both study groups similar colonisation patterns were observed. About 21 % of the volunteers were persistently colonised (two positive nose swabs with a time difference of at least 10 weeks), 22 % intermittently (one positive swab), and 57 % were noncarriers. A strain shift as defined by different SAg and *agr* gene profiles occurred in 5/51 persistent carriers (T009, T098, T166, T169; SH24). If the SAg and *agr* genes in both isolates were identical, only the first isolate was analysed further. 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 non-invasive and invasive isolates, we additionally screened 88 MSSA blood culture isolates (table 1). The MSSA isolates were obtained from the University Hospital of Greifswald or other close-by medical facilities over the same time periods as the nasal isolates to avoid the potential confounding effects of differences in geographical location or time periods.

Table 1: Characteristics of the study population.

	nasal isolates (T)	nasal isolates (SH)	blood culture isolates (BK)
study area	Western Pomerania	Western Pomerania	Western Pomerania
study population	healthy blood donors	healthy blood donors	hospital patients
subjects, no.	121	114	88
time period	apr – oct 2002	feb 2005 – feb 2006	apr 2002 – oct 2002; n=32 feb 2005 – feb 2006; n=56
mean age (±SD), years	33.8 (±11.4)	33.3 (±11.5)	58.3 (±21.3)
male sex, %	64.5	62.3	61.4
S. aureus isolates, no.	55	52	88
colonisation status (2 nose swabs)[1]			
noncarrier, no. (%)	70 (57.9 %)	63 (55.3 %)	-
1x positive, no. (%)	29 (24.0 %)	24 (21.1 %)	-
2x positive, no. (%)	22 (18.2 %)	27 (23.7 %)	-

[1]T, SH study: Two nose swabs were obtained from healthy blood donors over a course of at least ten weeks. Among the blood donors from both studies were on average 57% noncarriers, 21% persistent carriers (2 positive nose swabs) and 22% intermittent carriers (one positive nose swab).

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, t379) repeats. 75 *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 clonal complexes (figure 1). The major complexes (containing >5% of the isolates) included MLST-CC8, 15, 25, 30 and 45, which together incorporated 73.3% of all isolates. In contrast, the minor complexes CC5, 12, 22, 121 and 395 accounted for 13.3% of all strains. The same dominant lineages, except for CC395, were also observed in other studies[5,12]. However, the prevalence of the respective CCs varies considerably between the different *S. aureus* strain collections[6,42,43], suggesting large geographical variations.

Singletons, which could not be assigned to a major CC by *spa* typing, occurred among the nasal (n=14; 13.1%) and blood culture strains (n=5; 5.7%). 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 non-typable, because we were not able to amplify the *spa* gene by PCR. Overall, our data clearly show that the natural MSSA population in the region of Western Pomerania is highly clonal.

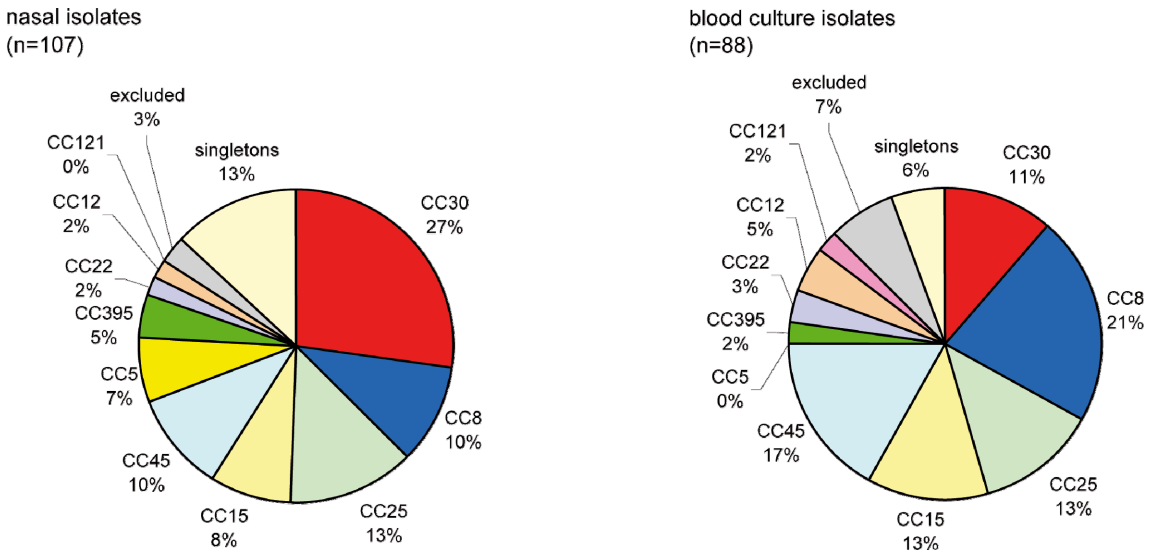


Figure 1: Distribution of nasal and blood culture isolates within clonal complexes. *Spa* types were clustered into 10 clonal complexes by BURP analysis using a cost of 5 as threshold for clustering. MLST-CC nomenclature was deduced from *spa*-CCs using the Ridom SpaServer database. CC30 was overrepresented 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).

Distribution of nasal and blood culture isolates between clonal complexes

As expected, both nasal and blood culture isolates were present in most clonal complexes[5,12]. 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 ≤ 0.05). Moreover, two clonal lineages were clearly represented in different proportions (figure 1) between nasal and invasive *S. aureus* isolates. CC8 was overrepresented among blood culture isolates compared to nasal isolates (21.6% vs. 10.3%, P ≤ 0.05) while, CC30 was underrepresented among blood culture strains (11.4% vs. 27.1 %, P ≤ 0.01). In CC30, *spa* type t012 was dominant among nasal strains, whereas t021 was the most frequent *spa* type in blood culture isolates (4/10 vs. 1/29, P≤0.01, figure 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 (suppl. table 2), which is thus the dominating *S. aureus* lineage in the Pomeranian community. A high prevalence of CC30 has also been reported from other geographical areas worldwide[6,42,43]. *Spa* type t012 was the dominant colonising clone and probably the evolutionary founder of the CC30 cluster in both Western (10/29 CC30 isolates) and Eastern Pomerania (7/26, figure 2). However, there were also remarkable differences in the *spa* type composition between the two CC30 collections suggesting a divergent evolution, which was probably enforced by the former East German-Polish border.

A subanalysis of strains isolated in 2002 versus 2005/06 (data not shown) showed that CC395 was not yet detected in 2002 but a total of 7 isolates representing different, closely related *spa* types were discovered in 2005/06. While a sampling bias cannot be excluded, it

appears more likely that a new *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 2005/06. Even though these differences are not significant due to small case numbers, they suggest that the *S. aureus* population structure is dynamic and that the prevalence of CCs fluctuates over time.

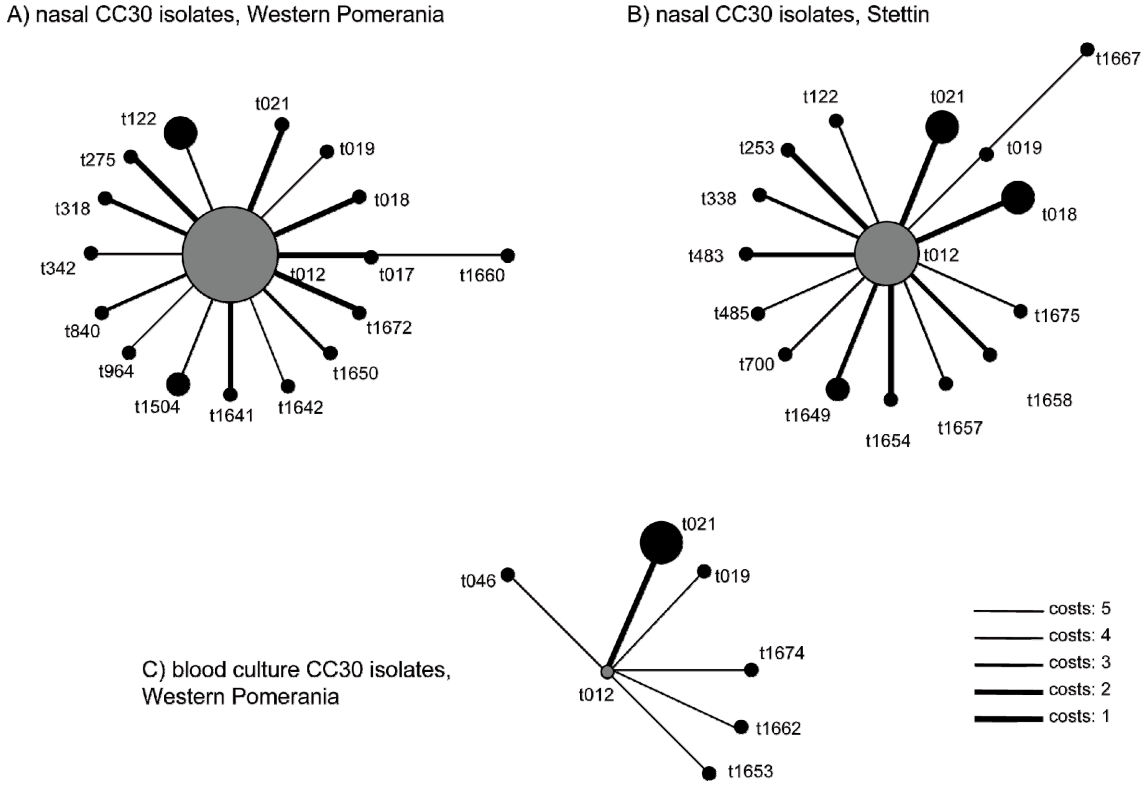


Figure 2: Clonal relationship of Pomeranian CC30 isolates of different clinical origin. A) nasal isolates from Western Pomerania, B) nasal isolates from Sczcecin and C) blood culture isolates from Western Pomerania. Clusters were created with the BURP algorithm of the Ridom SpaType Software. The size of each *spa* circle reflects the number of isolates belonging to this *spa* type. The thickness of the connecting line reflects the calculated costs, which express the evolutionary distance. The founder of a cluster, i.e. the *spa* type with the highest number of direct relatives, is shaded in grey. 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 has arisen from a single recombination event that involved the exchange of a DNA fragment, encoding the *spa* t037 gene, between MLST 30 and MLST 239[44,45].

Distribution of *agr* types

The accessory gene regulator (*agr*) is a global regulator of virulence gene expression, and four different *agr* subgroups, *agr I-IV*, are known. The *agr* locus belongs to the core variable genome and is strongly linked with clonal lineages[6,14]. In our study, the *agr* subgroups were also very tightly, but not absolutely, linked to the underlying clonal background. In agreement with others[7,14,46,47], we found *agr I* to be strongly associated with CC8, 25, 45 and 395, while a*gr II* was present almost exclusively in CC5, 12 and 15 (figure 3A, table 2, 3). Moreover, *agr III* was detected only in CC30 isolates and some singletons, while *agr IV* occurred only in the CC121 lineage.

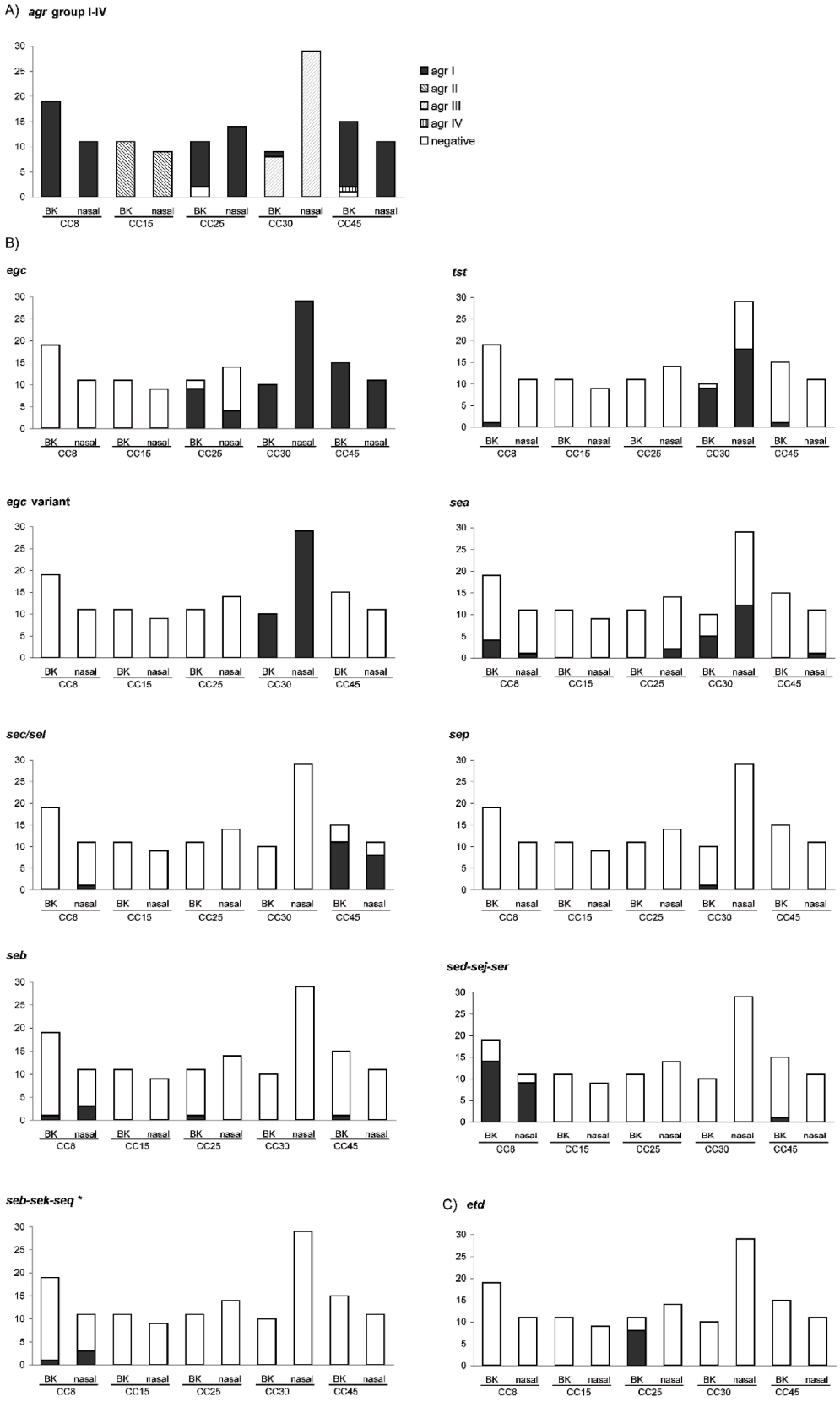


Figure 3: Distribution of A) *agr* subgroups I to IV, B) SAg genes or SAg gene combinations and C) *etd* within the five major clonal complexes. B, C) The overall height of the bars denotes the total number of isolates within the complex. The height of the shaded area represents the number of isolates positive for the respective SAg gene or SAg gene combinations. The minor lineages CC5, 12, 22, 121, 1655 and singletons were excluded from these analyses. *egc* variant = *egc* cluster with *seu* and an *sem* allelic variant. * one strain harboured only *seb-seq* (T198-1, CC8).

We also noted a single *agr I* isolate within the CC30 cluster (BK085) and one *agr IV* isolate among the CC45 isolates (BK004); this was occasionally observed before[7,46]. Interstrain recombination events could account for these exceptional strains, which needs further investigation[48-50]. Five blood culture isolates could not be typed with our *agr* multiplex PCR system. We are currently investigating, whether this phenomenon is due to a deletion of the *agr* locus[51], or to point mutations within the primer binding region.

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. A comprehensive overview on the *spa*-defined clonal lineages, their respective *agr* types and SAg gene patterns is provided in table 2 for the nasal isolates and table 3 for the blood culture isolates from Western Pomerania.

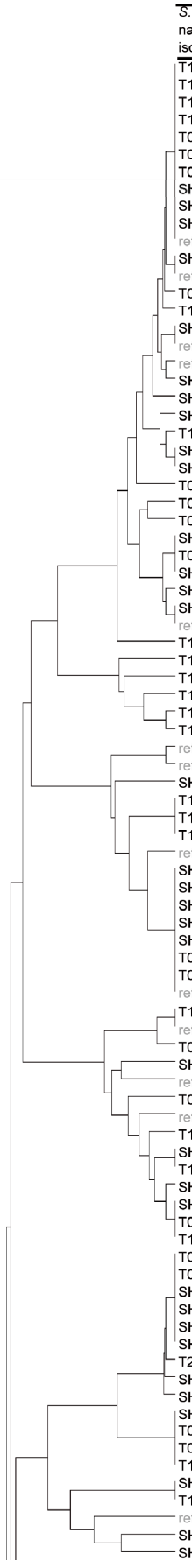
None of the SAg genes or SAg gene combinations was randomly distributed between the clonal complexes ($P \leq 0.001$; contingency table analysis). The association of SAg genes with the lineages suggests that most MGEs are predominantly transferred vertically, while horizontal transmission between different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

SAgs with very strong clonal association were the *egc* SAgs clustering on the *S. aureus* genomic island vSAβ[6,15,52]. The *egc* cluster, as originally described by Jarraud and coworkers,[35] contains *seg-sei-sem-sen-seo* (vSAβ). It was present in all CC5, CC22 and CC45 isolates but completely absent from CC8, CC12, CC15 and CC395. *Egc* genes also characterised one subcluster of the CC25 lineage (t078 and relatives), whereas they were missing from the other (t056 and relatives, fig. 3B, table 2, 3). Moreover, we observed an *egc* variant, which was almost exclusively linked to the CC30 background, where it was present in all isolates. This *egc* variant is characterised by, an *sem* allelic variant that escapes detection with our standard PCR due to three point mutations within the binding site of the *sem* forward primer, and an additional *seu* gene, and probably corresponds to the reported *egc2* variant (fig. 3b, table 2, 3)[53,54]. Our data clearly show that the *egc*-encoding genomic island is very stable.

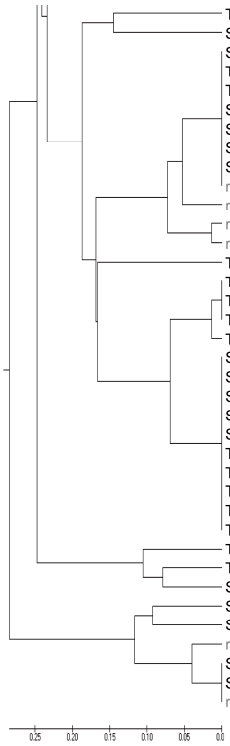
Other SAgs with very strong linkage to certain CCs were *tst, sec-sel* and *sed-sej-ser* (fig. 3B, table 2, 3). The SAg *tst* can be located on a family of related islands which were strongly linked to the CC30 background. *Sec-sel* are co-localised on the pathogenicity island SaPI3 and were detected mainly in CC45 isolates. Finally, the plasmid-encoded SAg genes *sed-sej-ser* were usually found in CC8.

SAg genes with a broader distribution were the phage-encoded *sea*, which was occasionally detected in CC8, 25, 30, 45 and 395, and the SaPI-encoded *seb*, which was infrequently found in CC5, 8, 12, 25 and 45 (figure 3B, table 2, 3).

Table 2: Distribution of SAg genes, *agr* type and *eta* and *etd* genes within *spa*-defined clonal complexes among nasal isolates (n=107).



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	CC8[1]				
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[2]	*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*

Spa-types were clustered into 10 clonal complexes by BURP analysis, which are colour-coded according to figure 1. For construction of the consensus tree, several reference strains with unknown SAg gene pattern were included in the BURP clustering (shaded in grey). Singletons could not be assigned to any clonal complex. MLST-CCs were deduced from BURP grouping of *spa* types[11]. MLST-CCs labelled with an asterisk were MLST-sequenced. SAg genes, *agr* type, *eta* and *etd* genes were determined by multiplex PCR. All strains tested negative for the *pvl* gene (not shown). Staphylococcal enterotoxins (SEs) are indicated by single letters (a = *sea*, …). *tst* = toxic shock syndrome toxin gene, *egc* = enterotoxin gene cluster, *eta, etd* = exfoliative toxin a, d, *agr* = accessory gene regulator.

[1]*Spa* type t037 isolates were grouped into *spa*-CC012, but are known to belong to MLST ST239 (CC8). *Spa* t037 has arisen from a single recombination event that involved the exchange of a >200 kb DNA fragment, encoding the *spa* gene, between MLST30 and MLST239[44,45].

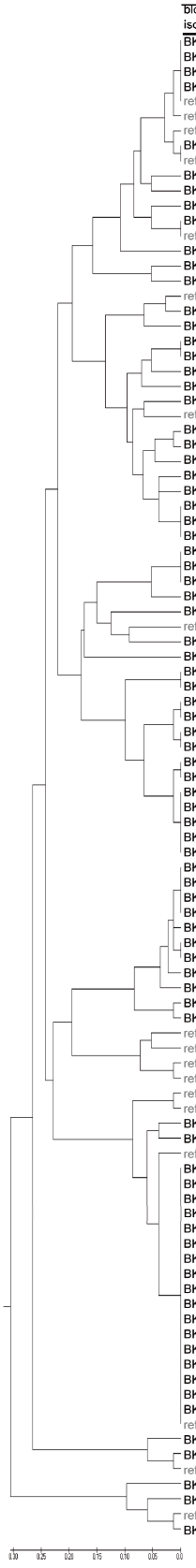
[2]*Spa*-CC1655 isolates were clustered into MLST-CC395 after MLST-sequencing of two representative strains.

[3]T184-2 was tested *mecA* positive.

[4]*Spa* type t091 were grouped into *spa*-CC084, but belonged to ST7 (singleton) according to MLST sequencing.

Furthermore, we observed some new SAg gene combinations indicating the existence of so far 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[55] 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[6]. Similarly, *seb-sek-seq* are usually clustered on SaPI1, but we and others found *seb* without *seq-sek* in several strains[27,56], suggesting a new SaPI variant. This is intriguing and needs more investigation.

Table 3: Distribution of SAg genes, *agr* type and *eta* and *etd* genes within *spa*-defined clonal complexes among blood culture isolates (n=87).



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	CC8[1]				
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	04-21-12-41-20-17-12-12-12-17	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	04-21-21-12-41-20-17-12-12-17	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*

Legend Table 3: For background information please refer to table 2. *S. aureus* clinical isolate BK067 was not *spa*-typable and, therefore, excluded from this analysis.
[1]*Spa* type t037 isolates were grouped into *spa*-CC012, but are known to belong to MLST ST239 (CC8). *Spa* t037 has arisen from a single recombination event that involved the exchange of a >200 kb DNA fragment, encoding the *spa* t037 gene, between MLST30 and MLST 239[44,45].
[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 were grouped into *spa*-CC084, but belonged to ST7 (singleton) according to MLST sequencing.

agr/SAg gene profiles of *S. aureus* clonal complexes

As a consequence of their linkage to the genetic background, individual clonal complexes of *S. aureus* are characterised by different SAg gene patterns. However, within clonal complexes and even within the same *spa* sequence types, we observed considerable variation in the prevalence of the SAgs that constitute these lineage-specific patterns. There were between 1 (CC15) and 8 (CC8) different SAg genotypes within the major clonal lineages. This indicates frequent acquisition and loss of SAg-carrying MGEs within lineages (tables 2 and 3).

The characteristic SAg gene profiles and *agr* groups of the clonal lineages are summarised in table 4. 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 vs. 4/14, $P \leq 0.01$). Within this t078 cluster, eight strains additionally harboured the *etd* gene. Importantly, these were found exclusively in the blood culture isolates ($P \leq 0.001$), so that CC25 isolates harbouring the *etd*-encoding pathogenicity island appear to be more virulent than those without. Others have reported the *etd* locus to be associated with the MRSA ST80 lineage as well as with a lineage carrying *agr I*, *egc* and sometimes *seb*[39]; 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, neither in CC30 nor in CC8 (table 2, 3).

The SAg gene patterns of the minor lineages CC12, 22, 121 and 395 are based on small case numbers and need to be confirmed. One of our two CC121 isolates, both from blood cultures (*agr* IV, *egc* including *seu*), carried *eta,* which is located on a phage. It has been previously reported, that most *eta* producing strains belong to *agr* IV and that these are associated with exfoliative syndromes[14,57,58]. The SAg gene patterns of CC395 isolates were heterogeneous, which suggests a high mobility of MGEs within this rapidly evolving cluster.

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 4).

Table 4: SAg gene and *agr* signatures of *S. aureus* clonal complexes. For each staphylococcal clonal cluster the characteristic *agr* subgroup, SAg genes as well as *eta* and *etd* genes are indicated. The number of isolates tested positive for a virulence gene is provided in brackets. SAg genes that occurred in more than 50% of isolates of one CC are shown in bold letters. SAg genes, which are clustered on MGEs are linked by a hyphen. An addition, the SAg/*agr* signatures of MRSA outbreak clones and whole-genome sequenced strains are provided.

CC	strains	number	*agr* type	*egc*	other SAg genes	*eta, etd*
CC5	MSSA	7	*agr II*	*egc*+	*seb-seq-sek* (1), *sed-sej-ser* (1), *sep* (3)	
	N315 (MRSA)		*agr II*	*egc*+	*tst-sec-sel* [17]	
CC8	MSSA	30	*agr I*	*egc*-	***sed-sej-ser*** (23), *sea* (5), *seb-sek-seq* (3), *seb-seq* (1), *tst* (1), *sec-sel* (1)	*etd* (1)
	COL (MRSA), *spa* type t008		*agr I*	*egc*-	*seb-sek-seq* [29]	
	Lyon-MRSA, *spa* type t008		*agr I*	*egc*-	***sea*** (13/13)*, seb (1/13), sed (3/13)** [59]	
CC12	MSSA	6	*agr II*	*egc*-	***sec-sel*** (5), ***sep*** (5), *seb* (1)	
CC15	MSSA	20	*agr II*	*egc*-	**-**	
CC22	MSSA	5	*agr I* (4), *agr*- (1)	*egc*+	*sec-sel* (1)	
	EMRSA-15, *spa* type t032		*agr I*	*egc*+	*sec-sel* [60]	
CC25	MSSA	25	*agr I* (23), *agr*- (2)	*egc*+ (13 **)	*seb* (3), *seu* (1)	*etd* (8**, all BK)
CC30	MSSA	39	*agr III* (38), *agr I* (1)	*egc-variant*+	***tst*** (27), ***sea*** (17), *sep* (1)	
	EMRSA-16, *spa* type t018		*agr III*	*egc-variant*+	*tst, sea* [59-61]	
CC45	MSSA	26	*agr I* (24), *agr IV* (1), *agr*- (1)	*egc*+	***sec-sel*** (19), *tst* (1), *sea* (1), *seb* (1), *sed-sej-ser* (1), *seu* (1)	
	Berlin MRSA		*agr I*	*egc*+	- [59]	
CC121	MSSA	2	*agr IV* (1), *agr*- (1)	*egc*+	*seu* (2)	*eta* (1)
CC395	MSSA	7	*agr I*	*egc*-	***tst-sec-sel*** (5), ***sea*** (6), *sek-seq* (2), *see* (1)	*etd* (1)

**Sej* and *ser*, which cluster with *sed* on a plasmid, *w*ere not determined in this study[59].

***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, $P \leq 0.001$).

agr = accessory gene regulator, *egc* = enterotoxin gene cluster, *eta, etd* = exfoliative toxin A, D

DISCUSSION

Recent studies showed that all *S. aureus* genotypes that efficiently colonise humans have given rise to life-threatening pathogens, but that some clonal lineages appear to be more virulent than others[5]. Analysis of the core variable genome could not clearly attribute virulence to any of the factors examined[6]. In fact, each of the ten dominant *S. aureus* lineages has a unique combination of core variable genes, such as surface-associated and regulatory genes[6]. This suggests that associated resistance and virulence genes encoded 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* type 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. They either did not spread between different genetic lineages at all, such as *egc*-carrying pathogenicity island vSAβ[6,15,34,52], or the efficiency of such genetic exchange was low, as in the case of *tst*, which is encoded by a family of related pathogenicity islands[6,16], and of the plasmid-encoded SAg genes *sed-sej-ser*[62,63]. The *sea*-carrying phage Φ3 and the SaPI-encoded *seb* were distributed more broadly[6,7]. This shows that the genetic distribution of SAg-carrying MGEs occurs mainly by vertical transmission. The degree of horizontal mobility varies considerably between different MGEs.

Barriers for horizontal transfer could be the incompatibility of related bacteriophages (bacteriophage immunity), SaPIs and plasmids, varying susceptibility to transduction or conjugation, or the Sau1 restriction modification system, as has recently been proposed by Lindsay and co-workers[15,64].

The group of Jarraud have reported associations of *agr* type with diseases, in particular with the toxin-mediated TSS and exfoliative diseases[14]. 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 III*[14,33,65,66].

As a result of the described restrictions of horizontal gene transfer each clonal complex was characterised by a typical SAg and exfoliative toxin gene profile and *agr* type, as is described in detail in the results section (table 2). The typical MGE profile linked to a clonal complex likely contributes significantly to its virulence as well as to the patterns of symptoms elicited by this *S. aureus* lineage. However, in cases of very tight linkeage, as seen for example with the *egc*-encoding MGEs, the relative contributions of MGEs and 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[7]. Though they may play a role, the contribution of MGEs to pathogenicity cannot be separated from the genetic background in these situations. A striking example is the *egc*-encoding genomic island vSAβ which we found always and exclusively in members of CC5, CC30, CC45 and a subcluster of CC25. On the CC25 background, *egc* was associated with invasiveness but on the CC5 background it characterised 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 both during colonisation and infection[32,67,68]. The colonising strain T098 even lost the *sea*-carrying phage between the first and second sampling, since the PCRs for *sea* and the phage-specific integrase became negative, while *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 background[6,7]. In such cases, the impact of MGEs can be readily assessed by comparing invasive and non-invasive *S. aureus* isolates of similar genetic background. 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[69]. This shows that virulence gene analysis can increase the discriminatory power of other genotyping methods, as has also been suggested by others[7,14,70]. In contrast, in the 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.

In agreement with our results, similar consensus repertoires of virulence genes (e.g. SAg genes, *agr* group, hemolysins) have also been reported for MRSA clones of CC5, 8 and 30[22]. The fact that staphylococcal lineages of different geographical regions show similar MGE profiles suggests that these lineages are evolutionary old and share a conserved genomic structure. On this conserved genetic background the *mecA* gene shows a highly dynamic behaviour, 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[22]. Likely reasons are the relatively recent acquisition of SCC*mec* and the shaping of the population structure of MRSA by local outbreaks in hospital and community. The fact that *mecA*-carrying SCC*mec* so far only occurred in five clonal complexes, suggests that, similar to SAg-carrying MGEs, the horizontal transfer of this MGE is restricted[64].

In Pomerania, CC30 was significantly more common among nasal strains than among blood culture isolates. It appears that the local CC30 population is optimised for symptom-free colonisation 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 compared to non-invasive strains[42]. Though there are some differences in the ways the Dutch and the Pomeranian

strains were collected, this means that the diagnosis “CC30” alone is of limited information. In support, two CC30 MRSA clones, the HA-MRSA ST36:USA200 and the CA-MRSA ST30:USA1100, induce very different disease types, which has been attributed to differences in their virulence gene repertoire[22]. 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 for the underlying molecular mechanisms.

To conclude, we have shown here that *S. aureus* clonal complexes are characterised 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 blood stream invasion, while rendering it unlikely for SAgs. Using SAgs as an example for 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.

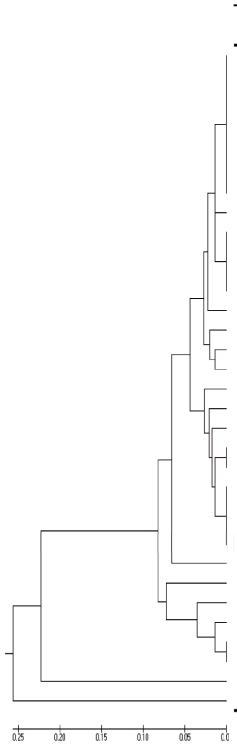
SUPPLEMENTARY TABLES

Suppl. Table 1: Oligonucleotide primers and reference strains used for SAg gene detection.

	Gene	Primers	GenBank accession	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	[14]
		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	[14]
		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	[14]
		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	[14]
		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	[14]
		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	[39]
		Netd-2		cca gaa ttt ccc gac tca g				
	eta	Neta-1	M17347	act gta gga gct agt gca ttt gt	190	55	A920210	[14]
		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	[14]
		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	[14]
		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	[14]
		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	[14]
		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	[40]
		Nser-2		tct tgt acc gta acc gtt tt				
	seu	Nseu-1	AY205306	aat ggc tct aaa att gat gg	215	55	FRI137	[40]
		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				[38]
	agr-1	agr1	X52543	gtc aca agt act ata agc tgc gat	439	55	Col	[38]
	agr-2	agr2	AF001782	tat tac taa ttg aaa agt gcc ata gc	572	55	N315	[38]
	agr-3	agr3	AF001783	gta atg taa tag ctt gta taa taa tac cca g	320	55	TY114	[38]
	agr-4	agr4	AF288215	cga taa tgc cgt aat acc cg	657	55	A920210	[38]
single PCR	*16SrRNA*	16SrRNA-1		gta ggt ggc aag cgt tat cc	228	58		[24]
		16SrRNA-2		cgc aca tca gcg tca g				
	gyrase	gyr-1		agtaca tcg tcg tat act ata tgg	281	55		[36]
		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	[37]
		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	[14]
		Npvl-2		gca tca ast gta ttg gat agc aaa agc				

	Gene	Primers	GenBank accession	Sequence (5'-3')	Fragment size (bp)	Tm (°C)	Control strain	Reference
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		[8]
		spa-1514r		cag cag tag tgc cgt ttg ctt				

Suppl. Table 2: Distribution of SAg genes, *agr* type and *eta* and *etd* genes of CC30 nasal isolates from Sczcecin, Poland. 30 out of 108 nasal isolates from healthy blood donors from Sczcecin, Poland, showed the *agr*/SAg gene profile characteristic for CC30 isolates (*agr III, egc* with *seu* and *sem* variant). *spa* typing demonstrated that 26 of these isolates belong to CC30.



S. aureus nasal isolate	spa type ID	repeat succession	spa-CC	MLST-CC	SEs, *tst* genes	*egc*	*eta, etd*	*agr*
SZ344	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ269	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	-	*g i m n o u*	-	*III*
SZ242	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	*a tst*	*g i m n o u*	-	*III*
SZ165	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	*a*	*g i m n o u*	-	*III*
SZ100	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	*a tst*	*g i m n o u*	-	*III*
SZ096	t012	15-12-16-02-16-02-25-17-24-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ070	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				
SZ138	t1654	15-12-16-02-16-02-25-17-17-24-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ150	t018	15-12-16-02-16-02-25-17-24-24-24	spa-CC012	CC30	*a tst*	*g i m n o u*	-	*III*
SZ215	t018	15-12-16-02-16-02-25-17-24-24-24	spa-CC012	CC30	*a*	*g i m n o u*	-	*III*
SZ358	t018	15-12-16-02-16-02-25-17-24-24-24	spa-CC012	CC30	*a tst*	*g i m n o u*	-	*III*
reference	t018	15-12-16-02-16-02-25-17-24-24-24	spa-CC012	CC30				
SZ348	t1658	15-12-16-02-16-02-25-25-17-24-24-24	spa-CC012	CC30	*k tst*	*g i m n o u*	-	*III*
SZ228	t486	15-12-16-02-16-02-25-17-24-24-24-24-24	spa-CC012	CC30	*a tst*	*g i m n o u*	-	*III*
SZ058	t483	15-12-16-16-02-16-02-25-17-24-24-24-24	spa-CC012	CC30	-	*g i m n o u*	-	*III*
SZ044	t253	15-12-16-02-16-02-25-17-24-24-24-24	spa-CC012	CC30	-	*g i m n o u*	-	*III*
SZ219	t338	15-21-16-02-25-17-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ188	t700	15-12-16-02-16-02-25-17-25-17-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ288	t1649	15-12-16-02-16-02-25-25-17-24	spa-CC012	CC30	-	*g i m n o u*	-	*III*
SZ169	t037	15-12-16-02-25-17-24	spa-CC012	CC8[1]	*a*	*g i m n o u*	-	*III*
SZ176	t037	15-12-16-02-25-17-24	spa-CC012	CC8[1]	*a*	*g i m n o u*	-	*III*
SZ142	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30	*a tst*	*g i m n o u*	-	*III*
SZ281	t021	15-12-16-02-16-02-25-17-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ310	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				
SZ318	t1675	15-21-12-16-02-16-02-25-17-24-24-24	spa-CC012	CC30	*a*	*g i m n o u*	-	*III*
SZ095	t1667	15-34-02-16-02-25-17-24	spa-CC012	CC30	-	*g i m n o u*	-	*III*
SZ260	t1657	15-16-02-16-02-25-25-17-24-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ076	t122	08-16-02-16-02-25-17-24-24	spa-CC012	CC30	*tst*	*g i m n o u*	-	*III*
SZ248	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				
SZ156	t127	07-23-21-16-34-33-13	singleton	-	*a h k tst*	*g i m n o u*	-	*III*
SZ097	t1652	04-44-44-33-31-12-16-34-16-12-25-22-34	singleton	-	*h tst*	*g i m n o u*	-	*III*

[1]*Spa* type t037 isolates were grouped into *spa*-CC012, but are known to belong to MLST ST239 (CC8)[44,45].

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Blood cell activation patterns induced by soluble products of *Staphylococcus aureus*

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ABSTRACT

Coagulopathy is a feature of *S. aureus* sepsis even when bacteraemia is not evident. This suggests that soluble bacterial products are involved in alteration of the clotting system. We tested *S. aureus* culture supernatants from isolates of 20 asymptomatic carriers and of blood cultures of 20 septic patients for induction of T lymphocyte proliferation, monocyte tissue factor (TF) activity, platelet aggregation, and red blood cell lysis. Wild type *S. aureus* and its isogenic *agr*- and *sarA*-deficient mutants were used to evaluate the impact of these global regulators on the procoagulatory capacity of *S. aureus* supernatants. Staphylcococcal supernatants induced T lymphocyte proliferation (28/40) strictly dependent on the presence of superantigen genes; monocyte TF activity (35/40) was caused by heat resistant factors, monocyte lysis was less pronounced if either *agr* or *sarA* were missing; platelet aggregation (17/40) was induced by heat sensitive proteases which were *agr*-regulated. Platelet activation and red blood cell lysis, but not TF expression were inhibited by normal human sera, their IgG fractions, and IVIG. A procoagulatory status of platelets and monocytes and lysis of red blood cells by soluble products released by *S. aureus* may account for the high incidence of coagulation abnormalities in *S. aureus* sepsis even in the absence of high density bacteraemia.

SUBMITTED

INTRODUCTION

The incidence of sepsis due to infection with Gram-positive microorganisms, particularly *S. aureus*, is increasing and this bacterium has been identified in 15-20% of nosocomial haematogenic infections[1,2]. *S. aureus* is a normal human commensal, which is present in the anterior nares of around 30% of the healthy population. These colonising staphylococci may become an endogenous source of bacteraemia and infection[3-6]. *S. aureus* carriers have an increased risk of nosocomial *S. aureus* infection, which in the majority of cases is caused by the colonising strain[5,7,8]. Paradoxically in case septicaemia develops, carriers have a much better outcome than noncarriers[8], which suggests some form of acquired protection.

Inappropriate activation of the coagulation cascade is a major complication of septicaemia, which may lead to disseminated intravascular coagulopathy (DIC)[9,10]. This may be caused in part by direct platelet-*S. aureus* interaction: Bacterial adhesins such as clumping factor or fibronectin-binding protein as well as protein A bind via plasma protein bridges to platelet surface structures and thereby induce platelet activation and aggregation[11-14]. However, even in patients with severe DIC a significant degree of bacteraemia is rarely found[15,16]. This raises the possibility that soluble bacterial products may be responsible for blood cell activation leading to DIC. As carriers of *S. aureus* have a better prognosis than noncarriers when they develop *S. aureus* septicaemia, we also assumed that a specific neutralising immune response to substances released by *S. aureus* might be a plausible explanation.

The initiation of coagulation is primarily mediated by the tissue factor (TF) – factor VIIa pathway[9,10]. Monocytes are able to express TF and generate TF bearing microparticles, which can be integrated in the membrane of platelets[17]. There is growing evidence that soluble products of *S. aureus*, like superantigens (SAgs), or cell wall components, such as peptidoglycans and lipoteichoic acid, can induce TF expression on monocytes and thereby trigger the clotting cascade[18-20]. To address the question, whether *S. aureus*-derived soluble factors can activate blood cell-related clotting, we investigated the procoagulatory capacity of 40 *S. aureus* clinical isolates, among them 20 commensal and 20 invasive strains. To analyse the genetic characteristics of *S. aureus* associated with blood cell activation, we also made use of a wild type *S. aureus* laboratory strain and its isogenic *agr*- and *sarA*-deficient mutants. *Agr* (accessory gene regulator) and *sarA* (staphylococcal accessory regulator) are global regulators of *S. aureus* virulence genes. The *agr* locus comprises a two component system involved mainly in regulation of gene expression in response to cell density (quorum sensing) and in the up-regulation of exoprotein synthesis and the down-regulation of surface-associated proteins[21,22]. The *sarA* locus influences the regulation of both extracellular and cell wall-associated proteins independently from *agr*.

MATERIALS AND METHODS

Bacterial strains. Twenty *S. aureus* isolates from throat swabs of asymptomatic carriers (aSA1-20) and 20 clinical *S. aureus* isolates from blood cultures of patients with septicaemia (pSA1-20) were obtained. They were identified as *S. aureus* by their ability to produce clumping factor and/or protein A (Murex Staphaurex test; Murex Biotech Ltd., Dartford, United

Kingdom). All clinical isolates were methicillin-sensitive. The *S. aureus* laboratory strains RN6390 (RN6390-WT) and its isogenic mutants, the *agr*-deficient strain RN6911 (RN6390Δ*agr*) and the *sarA*-deficient strain ALC136 (RN6390Δ*sarA*), have been described before[22,23]. Their secretomes have been extensively characterised by us (A.K. Ziebandt, S. Engelmann) by two-dimensional (2-D) protein patterns of extracellular protein extracts of *S. aureus* wild-type and its respective regulatory mutants and quantitatively compared using ettan-fluorescence difference gel electrophoresis technique (Amersham Biosciences) (table 1)[24,25]. The strain RN6390WT and its mutants do not harbour any SAg genes.

Bacteria were cultured in Luria broth (LB) medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, 1 mM NaOH) at 37°C up to an optical density (OD) of 3.0 at 540 nm, corresponding to the post-exponential growth phase. The culture supernatants were harvested by centrifugation, filtered (0.22 µm filter), and the cell-free supernatants were used for investigations. For some experiments *S. aureus* culture supernatants were heat treated at 40°C, 56°C, 70°C or 100°C for 1 h, cooled on ice and centrifuged (10,000 x g, 10 min) before use.

Purified bacterial substances. Peptidoglycans from *S. aureus* were obtained from Sigma Aldrich Chemie, Munich, Germany. Recombinant alpha haemolysin (Hla) was kindly provided by Chr. Kohler (Institute of Microbiology and Molecularbiology, University Greifswald).

Protease inhibitor cocktail (EDTA-free) and the single protease inhibitors leupeptin, E-64, pefabloc and phosphoramidon were obtained from Roche Diagnostics GmbH, Penzberg, Germany.

Sera and IgG. Sera were obtained from healthy volunteers. The respective IgG fractions were purified from 1 mL serum by affinity chromatography (protein G column, Pharmacia Biotech, Freiburg, Germany) by standard methods and dialysed over night (membrane cut off 100,000 D, Serva, Heidelberg, Germany) against cell culture medium (RPMI-1640, Gibco BRL, Paisley, UK) for experiments with peripheral blood mononuclear cells (PBMC), or against phosphate buffered saline (PBS, pH 7.2) for platelet experiments. IgG (IVIG; Polyglobin N, Bayer AG, Ludwigshafen, Germany) approved for therapeutic use was used at final concentrations of 0.075-20 mg/mL after ultracentrifugation (100,000 x g, 1 h) to remove immune complexes.

Detection of SAg and haemolysin genes of *S. aureus* strains. The SAg gene pattern (*sea* to *seo* and *tst*) of the clinical *S. aureus* isolates was determined by PCR as described before[26]. In addition, the genes for the recently described SAgs s*er* and *seu* were detected by PCR using the following primer pairs: ser 5': agc ggt aat agc aga aaa tg, ser 3': tct tgt acc gta acc gtt tt, seu 5': aat ggc tct aaa att gat gg, seu 3': att tga ttt cca tca tgc tc. The genes encoding staphylococcal haemolysin variants *hla, hlb, hlc, hlcv* and *hld* were detected by PCR as described previously[27].

Proliferation of T lymphocytes. PBMCs were stimulated for proliferation by incubation with bacterial supernatants, [^{3}H]thymidine incorporation was measured as described previously[26].

Table 1: Secretion products of *S. aureus* RN6390-WT in the post exponential growth phase in comparison to its isogenic mutants RN6390Δ*agr* and RN6390Δ*sarA*. Strains were cultured in Luria broth (LB) to stationary phase, and secreted proteins were analysed by two dimensional gel electrophoresis. Protein expression of wild type and mutant strains was compared using the Delta2D software. For quantification of changes in the level of interesting proteins the Ettan-fluorescence difference gel electrophoresis technique (Amersham Biosciences) was used. The table is a comprehensive overview and comparison of data reported by us before[24,25].

Protein name (*S. aureus* N315)	Gene ID	Function	RN6390-WT : RN6390*Δagr*	RN6390-WT : RN6390*ΔsarA*
Aur	13702595	Aureolysin	↓	↑
Geh[F]	13700235	Glycerolesterhydrolase	↓	↑
GlpQ	13700763	Glycerophosphoryl diester phosphodiesterase	↓	↑
Lip[F]	13702629	Triacylglycerol lipase precursor	↓	↑
Plc	13700011	1-Phosphatidylinositol phosphodiesterase precursor	↓	↑
SspA	13700850	Serine protease SspA, V8 protease	↓	↑
SspB	13700849	Cysteine protease SspB	↓	↑
Hla	13700962	Alpha haemolysin precursor	↓	=
SplA	13701606	Serinprotease SplA	↓	=
SplB	13701605	Serinprotease SplB	↓	=
SplC	13701604	Serinprotease SplC	↓	=
SplF	13701602	Serinprotease SplF	↓	=
Hlb	13701798	Beta haemolysin precursor	↓	↓
LukD	13701612	Leukotoxin LukD	↓	-
SA1812	13701799	hypothetical protein similar to synergohymenotropic toxin precursor	↓	-
SA2097[F]	13702104	Hypothetical protein similar to secretory antigen precursor SsaA	↓	-
Sak	13701739	Staphylokinase	↑	↑
LytM	13700191	Peptidoglycanhydrolase	↑	=
Aly	13702602	Hypothetical protein similar to Autolysin	↑	↓
Geh	13700235	Glycerolesterhydrolase	↑	↓
IsaA	13702519	Immunodominant antigen A	↑	↓
SA0620	13700556	Secretory antigen SsaA homologue	↑	↓
SA2097	13702104	Hypothetical protein similar to secretory antigen precursor SsaA	↑	-
SsaA	13702099	Secretory antigen precursor SsaA homologue	↑	-
IsaA[F]	13702519	Immunodominant antigen A	=	↓
Lip	13702629	Triacylglycerol lipase precursor	=	↓
Nuc	13700682	Nuclease	-	↑
SA0620[F]	13700556	Hypothetical protein similar to secretory antigen precursor SsaA	-	↑
SdrD	13700454	Ser-Asp-rich Fibrinogen-binding protein	-	↑
Stp[F]	13701702	Staphopain, Cysteinprotease	-	↑

↑ protein spots are present in higher amounts in the wild type compared to the corresponding mutant

↓ protein spots are present in lower amounts in the wild type compared to the mutant

= no changes in the amount of the respective protein spot between wild type and mutant were observed

- not determined

[F] fragment of the respective protein

Procoagulatory activity of monocytes[28,29]. Buffy coats of citrated whole blood of normal donors were obtained by centrifugation (4,000 x g, 10 min). PBMCs were isolated by Ficoll density gradient centrifugation (MSL 1077; Biocoll Separating Solution; Biochrom), washed in 5 mM PBS/EDTA and resuspended in endotoxin-free culture medium (RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, Gibco BRL, Paisley, United Kingdom). 2 x 10^5 PBMC per well (96-well flat-bottom plates, 37°C, 5% CO_2) were incubated for 4 h with either lipopolysaccharide (LPS) of *E. coli* serotype 026:B6 (Sigma Aldrich Chemie, Munich, Germany) at 1 µg/mL, with *S. aureus* bacterial culture supernatants, or with *S. aureus* peptidoglycan (390 pg/mL to 1 µg/mL). Supernatants from selected *S. aureus* strains (pSA6, pSA19, RN6390-WT, RN6390Δ*agr* and RN6390Δ*sarA*) were heat treated, coincubated with human serum (1:10), isolated serum IgG or IVIG (0.075 mg/mL to 10 mg/mL). Preincubation of *S. aureus* supernatants with polymyxin B (30 min, 20 µg/mL, Sigma Aldrich Chemie, Munich, Germany) was used to exclude monocyte activation by LPS.

After 4 h of incubation, PBMCs were washed twice with Tris buffered saline (TBS, 0.05 M Tris-HCl, 0.2 M NaCl buffer, pH 8.4) to remove non-adherent cells. The adherent monocytes were incubated with 50 µL/well normal pooled plasma (1:25 in TBS, pool of 30 healthy donors) supplemented with 4 U/mL hirudin and 50 µL/well chromogenic substrate for activated factor X (FXa) (0.5 µM spectrozyme Xa, american diagnostica, Pfungstadt, Germany, 0.05 M $CaCl_2$) (90 min, 37°C) followed by photometric analysis at 405 nm. Each test was performed in duplicate.

Calculations. FXa activity was quantified with a standard curve obtained with purified FXa (0-1 mU/well, Diagnostica Stago, Asnières, France). Experiments were only considered to be valid if the medium control was lower than 0.3 mU FXa/well and the positive control with LPS showed at least a 3 fold increase over the medium control. ODs exceeding the value obtained with 1 mU FXa are reported as 1 mU FXa/well. For each S. aureus supernatant mean values of at least 3 experiments with different donor cells were calculated as percentage of the FXa generation measured after incubation with LPS (=100%) in the same monocyte preparation in the same experiment:

$$\frac{(\text{mU FXa induced by S. aureus supernatant}) - (\text{mU FXa medium control})}{(\text{mU FXa induced by LPS}) - (\text{mU FXa medium control})} \times 100 = \%\ \textit{TF activity}$$

TF activity ≥35% was considered to be positive.

Viability of monocytes, *1. MTT assay[30].* PBMCs (2 x 10^6/well, 24-well-plate) were incubated with S. aureus culture supernatants (pSA19, RN6390-WT, RN6390Δagr and RN6390ΔsarA) in the presence of tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium-bromide] (MTT, Sigma Aldrich Chemie, Munich, Germany) 0.6 mg/mL at a final volume of 500 µL/well (4h, 37°C, 5% CO_2). After removing non-adherent cells by washing with TBS, isopropanol (250 µL, 10 min, room temperature) was added for cell lysis and dissolution of the resulting crystalline formazan and total MTT was quantified photometrically at 550 nm. Cell viability was expressed as percentage of MTT dye measured with the same monocyte preparation after incubation with cell culture medium. *2. Quantification of the CD14 positive*

cell population: PBMCs ($1x10^6$) were incubated in the presence of different concentrations of *S. aureus* culture supernatants (pSA10, pSA16, pSA19, pSA20, aSA17, RN6390-WT, RN6390Δagr and RN6390ΔsarA) or with cell culture medium alone for 4h, at 37°C, in a 5% CO_2 atmosphere. The cells were then harvested, washed once (1,580 x g, 5 min) and resuspended in FACS-Flow medium (Becton Dickinson, Franklin Lakes, USA). Anti-CD14-PE (Diatec, Oslo, Norway) was used to quantify the percentage of CD14-positive monocytes. Mouse IgG_1-PE was used as isotype control (Southern Biotech. Ass., Birmingham, USA).

Platelet aggregation assay. Platelets were obtained by differential centrifugation of ACD-anticoagulated whole blood from healthy donors (180 x g, 20 min), washed, resuspended in Tyrode`s buffer as described[31], adjusted to 300,000-400,000 platelets/µL, incubated for 30 min at 37°C for reversal of minor platelet activation during washing, then incubated with the culture supernatants of *S. aureus* strains (n=40, 1:10 final dilution) or staphylococcal Hla in 96-well microtiter plates. Mixing was achieved with two steel spheres (2 mm diameter, SKF, Schweinfurt, Germany) per well on a magnetic stirrer (1,000 rpm, 45 min, room temperature). The transparency of the suspension was assessed with an indirect light source every 5 min. for 45 min. All experiments were performed in duplicate. Collagen was used as a positive control and buffer as a negative control. Alternatively, platelets (300,000/µL) were washed as described and incubated with selected *S. aureus* supernatants (pSA12, pSA13). Platelet aggregation was measured by aggregometry[32].

Platelet morphology. 10 µL platelet suspension were taken during the test period, added to ice cold EDTA solution (0.5% [w/v], pH 7.2), fixed onto glass slides, stained by May Grünwald Giemsa stain and assessed by light microscopy.

Coincubation with human sera or antibodies. Sera from healthy donors (carrier status unknown) were heat treated (45 min, 56°C) to inactivate thrombin and complement and centrifuged (7,300 x g, 3 min) to remove aggregates. 90 µL serum (undiluted, 1:2 and 1:10), IgG purified from serum of these healthy donors (0.2 to 14 mg/mL) or IVIG solution (0.2, 2, 10, 20 and 40 mg/mL; Polyglobin N, Bayer AG, Germany) were incubated with 90 µL of washed platelet suspension before addition of 20 µL of *S. aureus* supernatants (aSA7, aSA17, pSA9, pSA10, pSA12, pSA14, RN6390-WT and RN6390Δ*sarA*). Buffer in place of serum served as a negative control.

Preincubation with protease inhibitors. *S. aureus* supernatants (aSA7, aSA17, pSA6, pSA9, pSA18, pSA19, RN6390-WT and RN6390Δ*sarA*) were incubated with the protease inhibitors leupeptin (0.1 to 10 µg/mL), E64, pefabloc (0.005 to 2 µg/mL), phosphoramidon (10 to 600 µg/mL) or a cocktail of these protease inhibitors (30 min, 37°C) prior to incubation with washed platelets.

Alpha haemolysin (Hla). Recombinant staphylococcal Hla (0.0065-0.65 µg/mL) was incubated with washed platelets (300,000/µL) on a magnetic stirrer in the presence or absence of sera (1:10, 1:20 and 1:100), IgG fractions (0.1 and 1mg/mL) or IVIG (0.2, 2 and 20 mg/mL). Platelet aggregation was assessed every 5 min as described above.

Peptidoglycan (PG). Washed platelets (300,000/µL) and monocytes were incubated as described above with purified staphylococcal peptidoglycan in a concentration range from 390 pg/mL to 1.0 µg/mL.

Red blood cell lysis. Red blood cells were obtained from citrated whole blood of healthy donors, washed twice with 0.9% NaCl, pH 7.2, resuspended in 0.9% NaCl and incubated with culture supernatants of *S. aureus* clinical isolates (n=40) and the laboratory strains RN6390-WT, RN6390Δ*agr*, RN6390Δ*sarA,* or 0.9% NaCl, (30 min, RT), or with recombinant Hla (0.0065-0.65 µg/mL). After centrifugation (2,300 x g, 5 min), free hemoglobin (Hb) was measured photometrically[33].

Statistical analysis. Fisher's exact test was used to compare activation of monocytes and platelets by *S. aureus* isolates of different origin. The student's t-test was used to compare the lag time until platelet aggregation induced by aSA and pSA supernatants was visible. P-values < 0.05 were considered as statistically significant. The correlation between the induction of T lymphocyte proliferation, platelet activation, monocyte activation and red blood cell lysis by *S. aureus* supernatants were calculated with the Spearman test. Statistical analysis was carried out using the SAS package, Version 9.1 (SAS Institute, Cary, NC).

RESULTS

Influence of *S. aureus* culture supernatants on T lymphocytes, monocytes, platelets and red blood cells

T lymphocytes: In addition to already identified SAg genes[26], we screened all *S. aureus* clinical isolates for the staphylococcal enterotoxin (SE) genes *ser* and *seu* and up-dated some entries (table 2). Thereby, we further confirmed a stringent correlation between the presence of SAg genes and the secretion of T lymphocyte-stimulating factors (table 2).

Monocytes: Of the 40 clinical *S. aureus* strains 35 (87.5%) released factors that induced a procoagulatory surface on monocytes (FXa generation ≥35% of the LPS control). There was no difference between commensal and invasive isolates (p=1.0; table 2). In 34 cases the activity was strongly concentration-dependent with a maximum at the highest concentration tested (1:50 dilution). Commensal and invasive strains were similarly effective: aSA activity ranged from 47.3% to 100% of FXa generation induced by 1 µg/ml LPS, that of pSA from 37.2% to 100% (a representative example is shown in figure 1a). Two *S. aureus* culture supernatants (aSA5, pSA10) showed a bell shaped response curve of FXa generation (figure 1b). The supernatant of aSA17 did not induce TF activity but lysed monocytes at high concentrations (figure 1c). The reduced procoagulatory activity at high concentrations correlated with a decrease of monocyte metabolic activity (reduced MTT conversion, data not shown) and a reduction in the CD14-positive cell population (figure 1b, c), indicating monocyte destruction.

Platelets: Of the 40 clinical *S. aureus* strains 24 (60.0%) released factors that caused platelet aggregation. Representative results of aggregometry and optical evaluation are shown (figure 2 a, b). Commensal and invasive isolates did not show major differences in their

platelet-activating potential or in the average lag time before aggregation occurred (p=0.897; figure 2 c).

Table 2: Supernatants of *S. aureus* isolates from throat swabs of asymptomatic carriers (aSA, n=20) and from blood cultures of septic patients (pSA, n=20) induced divers patterns of T lymphocyte, monocyte, platelet and red blood cell activation. The prevalence of *S. aureus* SAg genes strictly correlated with T lymphocyte proliferation (aSA n=12, pSA n=16) (27), but not with factor Xa generation of monocytes (induced by aSA n=17, pSA n=18), platelet aggregation (aSA n=12, pSA n=12), or red blood cell hemolysis (aSA n=8, pSA n=9). *S. aureus* culture supernatants were prepared as described under methods. *tst*: toxic shock syndrome toxin 1 gene (TSST-1-gene); *egc*: enterotoxin gene cluster; *a*: staphylococcal enterotoxin a (*sea*). +: cell activation occurred; 0: cell activation not detectable.

	Virulence genes			Induction of			
S. aureus supernatant	*staphylococcal enterotoxin* genes, *tst*	*egc* genes	*haemolysin* genes *(hla, hlb, hlc, hlc$_v$, hld)*	T lymphocyte proliferation	FXa generation on monocytes	platelet aggregation	red blood cell haemolysis
aSA11	*d j r*	-	*a, c-v, d*	+	+	+	+
aSA14	*c l*	*g i m n o*	*a, c, d*	+	+	+	+
aSA16		*g i m n o*	*a, c-v, d*	+	+	+	+
aSA19	*c l*	*g i m n o*	*a, c, d*	+	+	+	+
aSA04	-	*g i m n o*	*a, c-v, d*	+	+	+	0
aSA12	*c l*	*g i m n o*	*a, c, d*	+	+	0	+
aSA01	*a tst*	*g i m n o u*	*a, c, d*	+	+	0	0
aSA02	-	*g i m n o u*	*a, c-v, d*	+	+	0	0
aSA03	*a tst*	*g i m n o u*	*a, c, d*	+	+	0	0
aSA06	*a tst*	*g i m n o u*	*a, c, d*	+	+	0	0
aSA05	*d j r*	-	*a, d*	+	0[1]	+	+
aSA18	*d j r p*	*g i m n o*	*a, c-v, d*	+	+	+	0
aSA07	-	-	*a, c-v, d*	0	+	+	+
aSA13	-	-	*a, c-v, d*	0	+	+	0
aSA09	-	-	*a, c-v, d*	0	+	+	0
aSA10	-	-	*a, c-v, d*	0	+	+	0
aSA08	-	-	*a, c-v, d*	0	+	0	0
aSA15	-	-	*a, c-v, d*	0	+	0	0
aSA17	-	-	*a, c-v, d*	0	0[2]	+	+
aSA20	-	-	*a, c-v, d*	0	0	0	0
pSA11	*c l*	*g i m n o*	*a, c, d*	+	+	+	+
pSA12	*b*	*g i m n o*	*a, c-v, d*	+	+	+	+
pSA18	*c l*	*g i m n o*	*a, c, d*	+	+	+	+
pSA19	*c l*	*g i m n o*	*a, c, d*	+	+	+	+
pSA06	-	*g i m n o*	*a, c, c-v, d*	+	+	+	0
pSA10	*a d j r*	*g i m n o*	*a, c-v, d*	+	+[3]	+	0
pSA17	*b p*	-	*a, c-v, d*	+	+	+	0
pSA05	*a*	*g i m n o u*	*a, c, d*	+	+	0	+
pSA13	*b*	-	*a, c-v, d*	+	+	0	+
pSA02		*g i m n o u*	*a, c-v, d*	+	+	0	0
pSA04	*b k q d j r*	-	*a, c-v, d*	+	+	0	0
pSA07	-	*g i m n o u*	*a, c, d*	+	+	0	0
pSA08	-	*g i m n o u*	*a, c, d*	+	+	0	0
pSA20	*c l p*	-	*a, c-v, d*	+	+	0	0
pSA15	*c l*	*g i m n o*	*a, c, d*	+	0	+	+
pSA16	-	*g i m n o u*	*a, c-v, d*	+	0	+	+
pSA14	-	-	*a, c-v, d*	0	+	+	+
pSA01	-	-	*a, c-v, d*	0	+	+	0
pSA09	-	-	*a, c-v, d*	0	+	+	0
pSA03	-	-	*a, c-v, d*	0	+	0	0

[1]aSA5 showed a bell shaped response curve with FXa generation less than 35%.

[2]aSA17 showed cytotoxic effects at high concentrations but did not induced FXa generation in monocytes.

[3]pSA10 showed a bell shaped response curve with FXa generation < 35% at a 1:50 dilution and > 35% at a 1:100 dilution

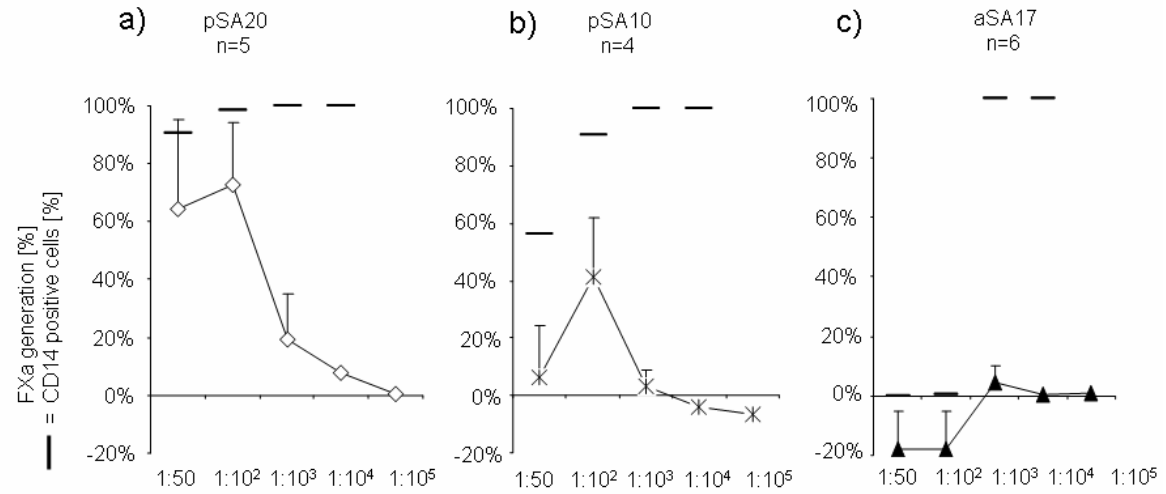


Figure 1: Induction of FXa generation in monocytes by *S. aureus* supernatants. Different patterns of FXa generation by monocytes were observed after incubation with *S. aureus* supernatants (0% FXa = medium control, 100% FXa = activation by LPS [1µg/ml *E. coli*]). **a)** 34 of 40 supernatants induced a procoagulatory surface on monocytes in a dose-dependent manner as shown for pSA20**. b)** A bell shaped response curve was observed after incubation with two supernatants, as shown for pSA10**. c)** aSA17 was one of three supernatants without TF activating capacity. Diminished procoagulatory activity at high concentrations of the *S. aureus* supernatants was correlated with reduced cell vitality (pSA10 at 1:50 dilution) and cell lysis (aSA17 at 1:50 to 1:100 dilution) as shown by reduction or loss of the CD14-positive cell population (—) **(b, c)**.

Red blood cells: Of the 40 clinical *S. aureus* strains tested all had the genes for the *S. aureus* haemolysins *hla-hld* (table 2). However, only 17 (42.5%) of the supernatants lysed red blood cells.

The abilities of *S. aureus* strains to activate T lymphocytes, monocytes, platelets or to lyse red blood cells appeared to be independent features (table 2). There was no correlation in cell activating capacity between the different cell types (correlation coefficients for T lymphocytes vs monocytes: 0.129, p=0.627; T lymphocytes vs platelets: -0.089, p=0.729; T lymphocytes vs red blood cells: 0.232, p=0.179; monocytes vs platelets: -0.107, p=0.631; and for red blood cells vs monocytes -0.136, p=0.144) with one exception: platelet aggregation and red blood cell lysis were weakly correlated (correlation coefficient 0.392; p=0.02).

Characterisation of the activating bacterial components

Heat resistance: Heat treatment up to 100°C for 60 minutes did not influence the monocyte activating capacity of culture supernatants from the strains pSA6 and pSA19, which were used at concentrations inducing maximal TF activity (data not shown). In contrast, treatment at 56°C for 30 minutes completely abolished platelet activation by 6 out of 7 tested supernatants (figure 3a). This shows that monocyte and platelet activation are caused by different factors. The pronounced heat sensitivity of the platelet-activating component(s) suggests that these may be proteins. In one strain (aSA7) partial inhibition was observed. This indicates that more than one factor might be involved in platelet activation.

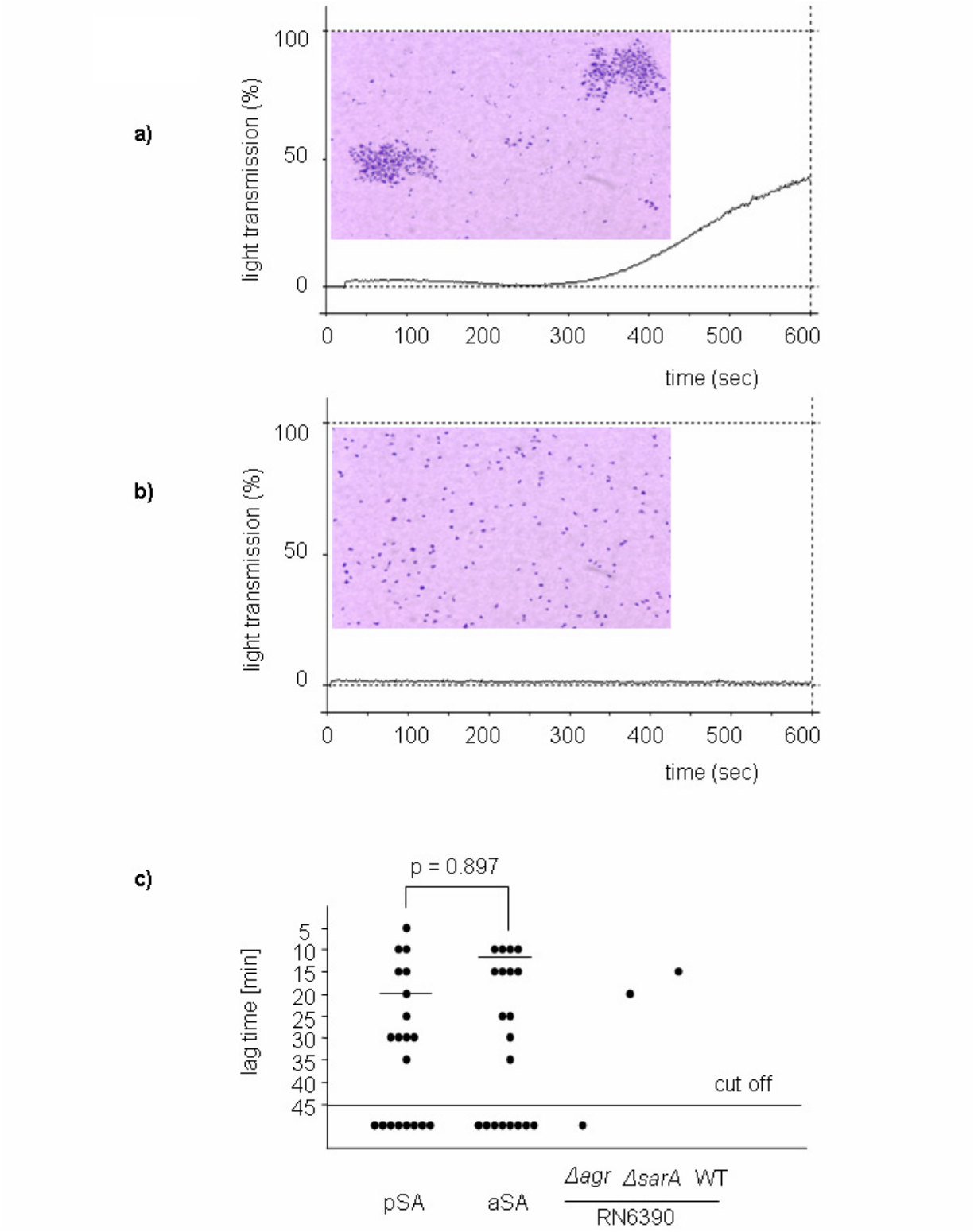


Figure 2: Induction of platelet aggregation by *S. aureus* supernatants. Strong platelet aggregation was observed after incubation of platelets with the culture supernatant of **a)** pSA12, but not **b)** pSA13. Aggregometry curves and platelets fixed onto glass slides and stained by May Grünwald Giemsa stain (inserts) are shown. **c)** The mean lag time of platelet aggregation induced by supernatants of *S. aureus* strains from throat swabs (aSA, n=20) and blood cultures (pSA, n=20) did not differ. In contrast to the lab strain RN6390-WT and its RN6390Δ*sarA* mutant, the RN6390Δ*agr* mutant did not activate platelets.

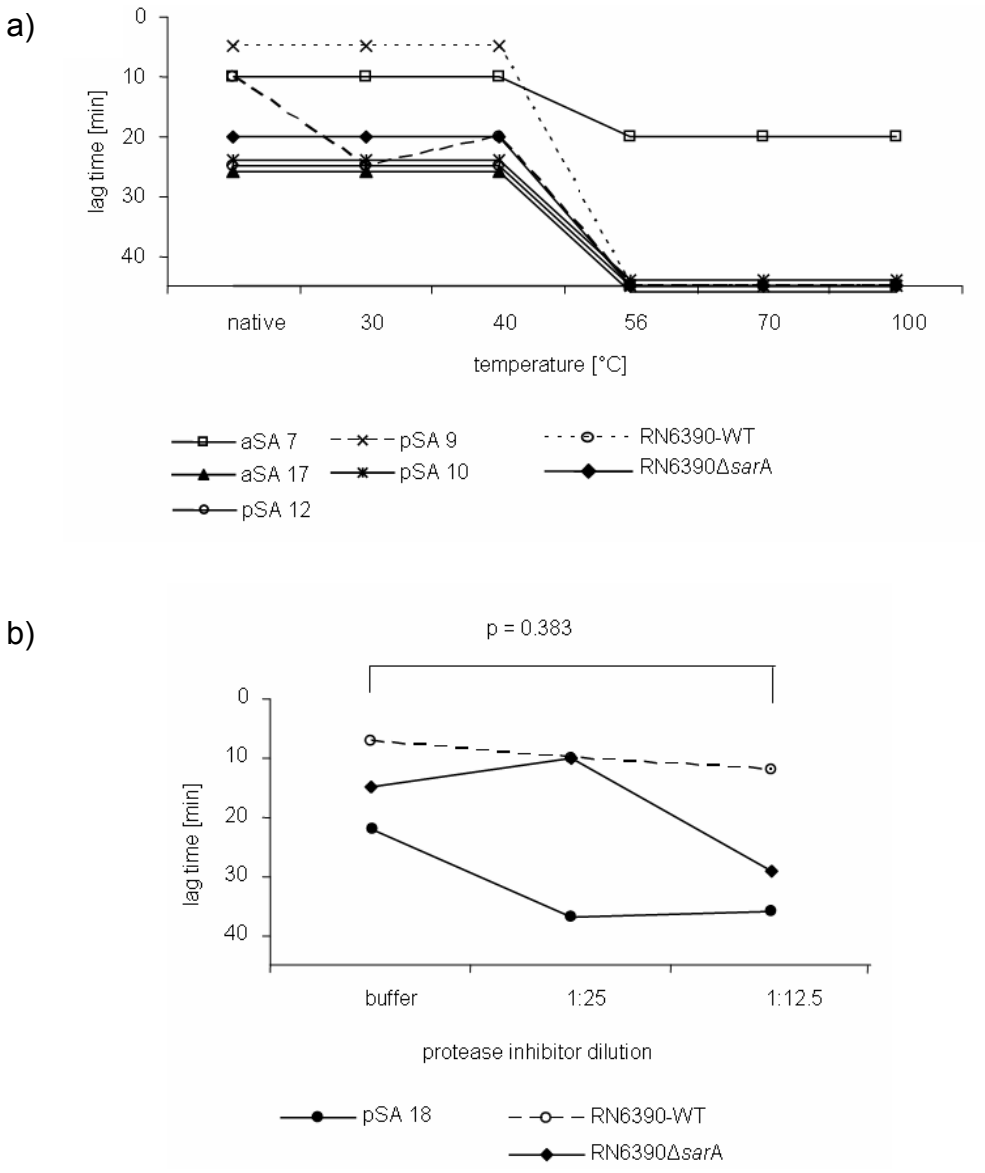


Figure 3: Characterisation of platelet activating bacterial components. a) Platelet aggregation by *S. aureus* supernatants was prevented by heat inactivation in six of seven supernatants tested. In one supernatant heat inactivation only partially inhibited platelet activation. This indicates that different factors might be involved in platelet activation. **b)** Platelet aggregation by *S. aureus* supernatants was partially inhibited by the treatment with a protease inhibitor cocktail indicating that proteases are involved in platelet activation.

Protease inhibitors: Platelet aggregation could be inhibited by pre-incubation of the *S. aureus* culture supernatants with a protease inhibitor cocktail. Figure 3 b) shows the results for the strongly activating supernatant of the lab strain RN6390-WT, its less activating Δ*agr* mutant and of the clinical isolate pSA18, indicating that proteases contribute to platelet activation by *S. aureus* culture supernatants.

Purified bacterial substances: Peptidoglycans are good candidates for the monocyte activeting principle, since they are very heat resistant. While *S. aureus* peptidoglycans induced FXa generation in monocytes in a dose dependent manner (figure 4 a), they did not activate platelets at concentrations up to 1 µg/mL (data not shown).

To test, whether differences in Hla secretion may account for differences in platelet activation and red blood cell lyses, which were observed between the clinical *S. aureus* strains, we incubated platelets and red blood cells with different concentrations of recombinant Hla. This virulence factor induced platelet aggregation at a concentration as low as 32.5 ng/mL. Increasing the concentrations shortened the lag time (data not shown). However, red blood cell lysis was only detectable at Hla concentrations ≥650 ng/ml. This indicates that platelet aggregation is a more sensitive marker for the expression of Hla than haemoglobin release by red blood cells.

Effects of mutations in *agr* and *sarA* on procoagulatory activity

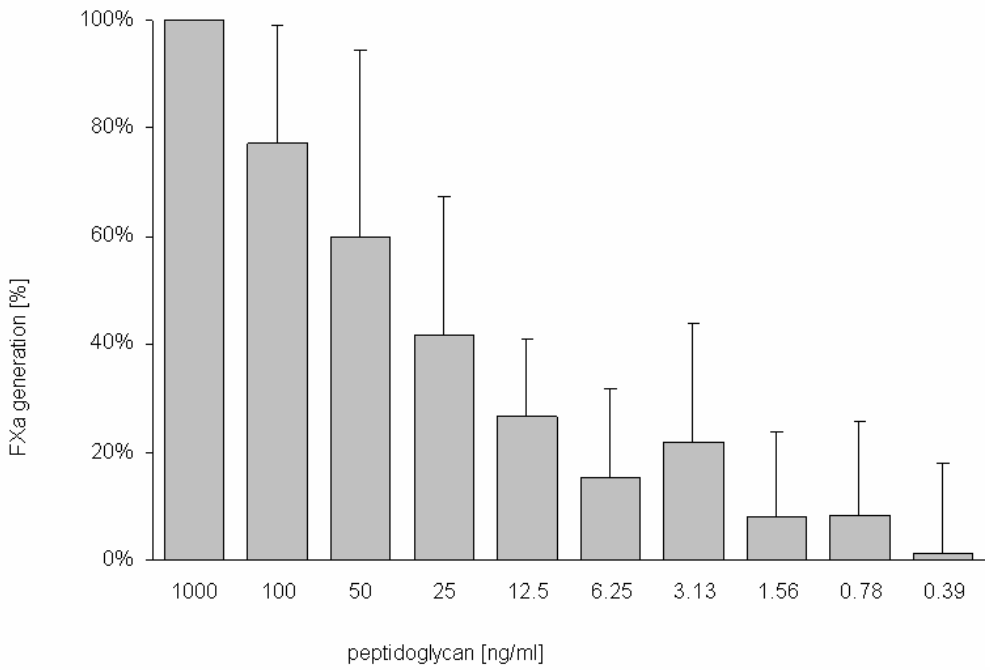
The laboratory strain RN6390-WT lacks SAg genes. Not surprisingly it failed to induce T-cell proliferation, but it still induced monocyte and platelet activation. This shows that activation of the clotting system is independent of *S. aureus* SAgs. Culture supernatants from the laboratory strain RN6390-WT and its isogenic mutants RN6390Δ*agr*, RN6390Δ*sarA* were compared, to assess the role of these global regulators for the release of pro-coagulatory factors by *S. aureus*. In monocytes, supernatants from the wild type strain RN6390-WT induced procoagulant activity in a concentration-dependent manner with a maximum at dilution of 1:12.800 (figure 4b). Supernatants of the RN6390Δ*agr* and RN6390Δ*sarA* mutants also induced procoagulant activity in a concentration-dependent manner. Interestingly, high concentrations of the culture supernatants did not cause FXa generation on monocytes but even lower FXa generation than in the buffer control. This was correlated with disappearance of the CD14 positive cell population as determined by FACS analysis (data not shown) indicating that the release of factors toxic for monocytes is upregulated by both *agr* and *sarA.* Heat treatment of the supernatants from the laboratory strains did not inhibit monocyte activating capacity.

S. aureus RN6390-WT induced platelet aggregation within 5 min and after 10 min platelets were completely lysed. The RN6390Δ*sarA* mutant caused platelet aggregation but no lysis, whereas the supernatant of the RN6390Δ*agr* mutant did neither induce platelet aggregation nor lysis (figure 2c). In a similar way, only RN6390-WT released factors which efficiently lysed red blood cells while supernatants of the *agr*- and *sarA*-mutants did not. Thus lysis of monocytes is enhanced by both global regulators but is not dependent on them. In contrast, for platelet activation gene products regulated by *agr* are of major importance.

Neutralising antibodies

Human sera (n=8), their corresponding IgG fractions and IVIG inhibited *S. aureus*-induced platelet activation in a dose-dependent manner, while the respective IgG-depleted sera had no effect. Examples are given in figure 5 a- c. At a concentration of 5 mg/mL IVIG inhibited the platelet-activating effect of *S. aureus* supernatants, whereas the sera of healthy donors showed marked inter-individual diversity, and different effects on the various *S. aureus* strains. Platelet aggregation in response to Hla was also inhibited by human serum, purified serum IgG and by IVIG in a dose-dependent manner (data not shown). In marked contrast, neither human sera (n=6), their IgG fractions nor IVIG were able to inhibit the induction of a procoagulant surface on monocytes by *S. aureus* supernatants (data not shown).

a)



b)

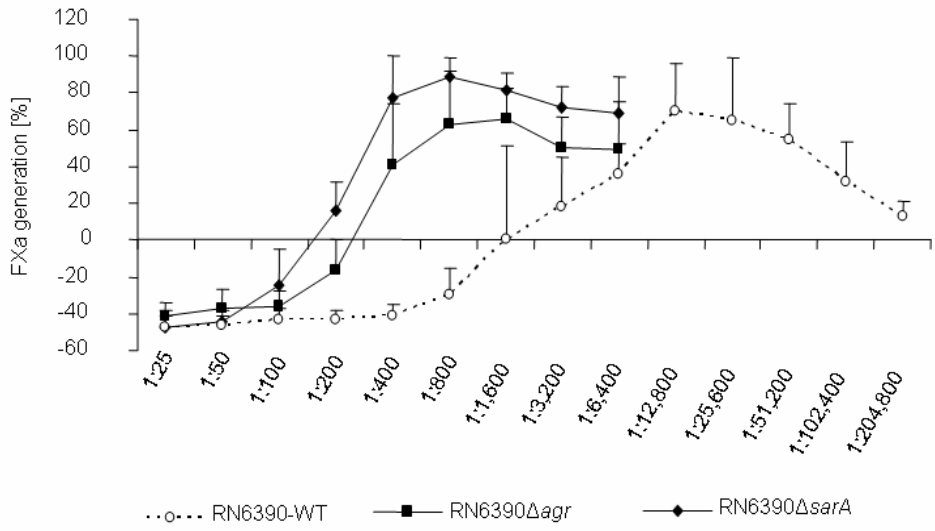


Figure 4: Induction of a procoagulatory surface on monocytes as measured by FXa generation. a) Peptidoglycans of *S. aureus* induced FXa generation on monocytes in a dose dependent manner. **b)** Supernatants of the *S. aureus* lab strain RN6390-WT and its mutants RN6390Δ*agr* and RN6390Δ*sarA* induced FXa generation of monocytes in a dose-dependent manner (0% FXa = medium control, 100% FXa = activation by LPS [1 µg/mL *E. coli*]). All supernatants were cytotoxic at high concentrations. In *agr*- and *sarA*-deficient mutants, cytotoxic factors seemed to be downregulated as compared with RN6390-WT.

DISCUSSION

Here, we report that clinical *S. aureus* isolates release soluble factors, which induce activation of T lymphocytes, platelets, and a procoagulant surface on monocytes, and that several of these factors are inhibited by specific IgG antibodies. Using mutant *S. aureus* strains lacking the regulators *agr* or *sarA*, respectively, we identified *agr* as an important

regulator for *S. aureus* virulence factors inducing platelet activation, while the regulator *sarA* seems to be more important for the expression of virulence factors inducing cell lysis. We further confirmed that SAgs play the decisive role in T lymphocyte proliferation[26], but also show that activation of platelets and monocytes is not dependent on the presence of SAgs.

Our findings may contribute to further understanding of the pathogenesis of severe clotting disorders in *S. aureus* septicaemia. While DIC is a frequent complication in severe *S. aureus* infection, bacteria are rarely detected in large numbers in the circulation of affected patients[15,16]. Therefore, it seems to be unlikely that bacteria-bound platelet-activating proteins, such as clumping factor, fibronectin-binding protein, or protein A, contribute primarily to activation of platelets and monocytes in patients with sepsis-associated DIC[11-14]. Our findings of soluble *S. aureus* products with procoagulatory capacities which activate platelets or monocytes could provide an explanation for the high frequency of coagulation abnormalities in septic patients.

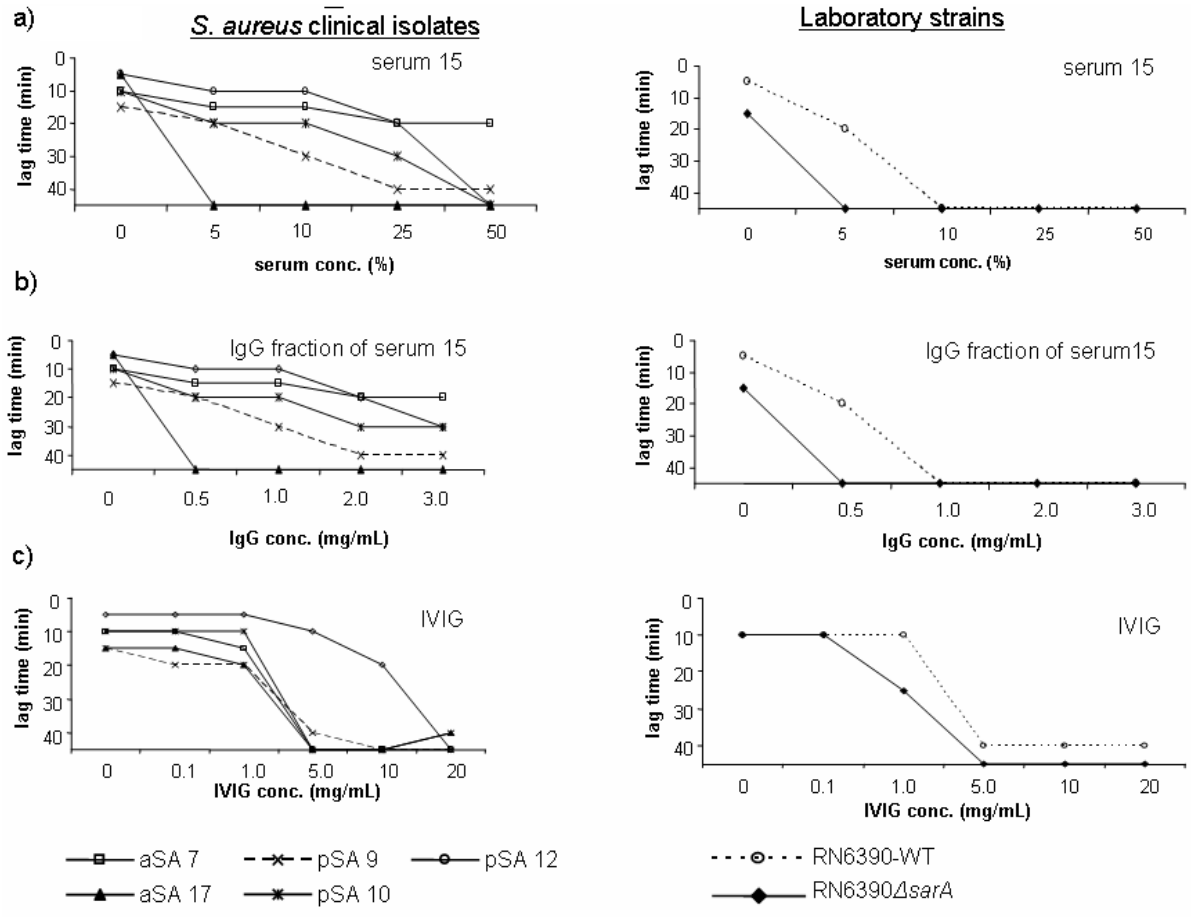


Figure 5: Human IgG antibodies inhibited the induction of platelet aggregation by *S. aureus* supernatants. Platelet aggregation by *S. aureus* supernatants was inhibited by **a)** pre-incubation with human sera and **b)** their respective IgG fractions, as well as by **c)** IVIG in a dose-dependent manner. Column A provides 3 representative examples of clinical isolates and column B the effects of serum, its IgG fraction, and IVIG on the laboratory strains RN6390-WT and its mutant RN6390Δ*sarA*.

Supernatants of *S. aureus* clinical isolates showed a marked heterogeneity in the activation patterns of blood cells, reflecting high variability of different *S. aureus* strains. This may also be reflected in the broad array of clotting disorders, which can be caused by *S. aureus* infection. Importantly, these differences between *S. aureus* strains were not related to whether the isolates were obtained from blood cultures of septic patients, or from nose swabs of healthy individuals. This is in agreement with the observation that in carriers of *S. aureus*, septicaemia is mostly caused by the same strain which colonised the anterior nares[5,6].

It is a not well understood paradox that *S. aureus* carriers, although having a much higher risk for developing *S. aureus* septicaemia, show significantly better outcome than noncarriers when sepsis occurs[8]. A possible explanation can be the tuning of the immune system by the colonising strain, resulting in a strain-specific antibody response directed against those virulence factors, which are produced during colonisation. We have previously demonstrated that neutralising serum antibodies directed against *S. aureus* SAgs (with the exception of *egc*-encoded SAg) are highly prevalent in the healthy population[26,34]. Here we demonstrate that platelet activation by secreted products of *S. aureus* can also be inhibited by IgG antibodies. It is an attractive hypothesis that the improved outcome of carriers with *S. aureus* bacteraemia could, in part, be related to these neutralising antibodies. In contrast, antibodies did not interfere with monocyte activation by soluble *S. aureus* factors. This may be one of the reasons why treatment of septic patients with IVIG was not effective in clinical trials[35-37].

Our study may provide a new explanation for the well known decrease in peripheral monocyte counts in some septic patients. While it was assumed that this is caused primary by monocyte activation or apoptosis, this in vitro study show that at least some *S. aureus* strains release factors which are able to lyse monocytes.

Blood cell activation is mediated by many different soluble products of *S. aureus*, as shown by the fact that heat treatment inhibited platelet, but not monocyte, activation. Purified peptideglycans, which are heat resistant cell wall components of *S. aureus*, induced a tissue-factor dependent procoagulant surface on monocytes in a dose-dependent manner in our experimental setting, which corroborates recent findings of Mattsson et al[19].

In contrast, peptidoglycans did not activate platelets. Platelet activation was dependent on heat sensitive soluble *S. aureus* factors, which were at least partially inhibited by protease inhibitors. Aggregation of platelets was correlated with red blood cell lysis ($p=0.02$) but more supernatants aggregated platelets than were able to lyse red blood cells (60% vs. 42.5%). Using recombinant alpha haemolysin at defined concentrations we provide evidence that platelets are much more sensitive to alpha haemolysin than red blood cells and confirm that alpha haemolysin is one major causative agent for platelet activation by *S. aureus*[38,39]. All *S. aureus* strains assessed (table 2) harbored the genes encoding the haemolysins *hla* - *hld*, but they differed in their capacity to activate platelets and to lyse red blood cells. This strongly suggests that these *S. aureus* strains differentially express their virulence genes resulting in large differences in the amounts of released virulence factors[40]. Interestingly, we found three *S. aureus* strains (aSA12, pSA5, pSA13; table 2) which caused red blood cell lysis but not platelet aggregation. This suggests that, beside alpha haemolysin, *S. aureus*

may release other factors which induce red blood cell lyses but do not cause platelet activation.

To further characterise the soluble *S. aureus* factors causing blood cell activation, we assessed the activation patterns induced by supernatants of the laboratory strains RN6390-WT and its isogenic mutants RN6390Δ*agr* and RN6390Δ*sarA*. Since RN6390-WT and its mutants have no SAg genes, our experiments clearly show that monocyte and platelet activation can occur independently of SAg, although certain SAgs, like TSST-1, enterotoxin A and B, seem also to be able to induce TF expression by monocytes[20].

These mutants also allowed us to assess the impact of the global regulators *agr* and *sarA* on the release of procoagulatory factors. Monocyte- and platelet-activating factors were differentially regulated. Monocyte activation occurred with the supernatants of both, the *agr* and the *sarA* mutant, while platelet aggregation was dependent on *agr* but not on *sarA* and was at least in part inhibited by protease inhibitors. This indicates that the *agr* regulated enzymes, which are expressed by those *S. aureus* strains causing platelet aggregation (table 1 and table 2), are potential candidates for platelet activation by *S. aureus* supernatants.

In conclusion, using a large variety of commensal and invasive *S. aureus* isolates, we demonstrate that most *S. aureus* strains are able to release soluble factors which activate and even lyse blood cells, though at very different concentrations. These soluble factors may induce coagulation even at high dilutions (up to 1:100,000). The capacity to induce T lymphocyte proliferation, FXa generation on monocytes, or platelet aggregation were independent features of *S. aureus* strains, reflecting the large number of virulence factors as well as the diversity of the species *S. aureus*[40,41]. This could account for some of the variability of interactions between *S. aureus* and the host, often resulting in symptom-free carriage but which can also cause severe infection. Well characterised laboratory strains and proteome analysis of *S. aureus* culture supernatants allowed us to further characterise the role of SAgs, to identify haemolysins and several proteases as potentially important candidates for *S. aureus* mediated blood cell activation. Furthermore, host-related properties seem to have an important role. IgG antibodies from many healthy individuals block T lymphocyte and platelet activation by *S. aureus* soluble factors. This may be one explanation for the paradox that in case of sepsis, carriers of *S. aureus*, although at much higher risk to develop septicaemia, have a better prognosis than noncarriers. However, these antibodies did not interfere with the induction of a procoagulant surface on monocytes, which may explain some of the discrepant results obtained with IVIG treatment in patients with *S. aureus* sepsis.

ACKNOWLEDGEMENTS

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Egc-encoded superantigens from *Staphylococcus aureus* are neutralised by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin

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SUMMARY

PCR was employed to determine the presence of all known superantigen (SAg) genes (*sea*, *seq* and *tst*) and of the exotoxin-like gene cluster (*set*) in 40 *Staphylococcus aureus* isolates from blood cultures and throat swabs; 28 isolates harbored superantigen genes, five on average, and this strictly correlated with their ability to stimulate T cell proliferation. In contrast, the *set* gene cluster was detected in every *S. aureus* strain, suggesting a nonredundant function for these genes which is different from T cell activation. No more than 10% of normal human serum samples inhibited the T cell stimulation elicited by *egc*-encoded enterotoxins (staphylococcal enterotoxins G, I, M, N and O), whereas between 32 and 86% neutralised the classical superantigens. Similarly, intravenous human immunoglobulin G preparations inhibited *egc*-encoded superantigens with 10- to 100-fold-reduced potency compared with the classical enterotoxins. Thus, there are surprisingly large gaps in the capacity of human serum samples to neutralise *S. aureus* SAgs.

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INTRODUCTION

Staphylococcus aureus persists as a commensal micro-organism in 10 to 30% of the population, but the organism is also a common cause of food poisoning and infections of different severity such as skin abscesses and wound infections, osteomyelitis, endocarditis, pneumonia, toxic shock syndrome and staphylococcal scarlet fever[1]. *S. aureus* is one of the most frequent causes of hospital-acquired infections, and the emergence and spread of multiresistant strains give rise to concern. The pathogenicity of *S. aureus* is multifactorial, and the versatility of this organism is underscored by recent clinical studies[2-7].

Superantigens (SAgs) activate large subpopulations of T lymphocytes by directly cross-linking certain T cell receptor Vβ domains with conserved structures on major histocompatibility complex class II molecules[8]. They belong to the most potent T cell mitogens known and can induce massive systemic cytokine release, leading to the symptoms of toxic shock syndrome[9]. Among the virulence factors of *S. aureus* are the staphylococcal enterotoxins, the causative agents of food poisoning. They also act as SAgs. Whole-genome sequencing of several *S. aureus* clinical isolates has revealed that all 17 known staphylococcal enterotoxins (staphylococcal enterotoxins (SE) A-SEE and SEG-SEQ) and toxic shock syndrome toxin (TSST) 1 are encoded on mobile genetic elements together with other virulence factors[10-12]. For example, the recently described enterotoxin gene cluster *egc*, which contains the five SAg genes *seg*, *sei*, *sem*, *sen* and *seo*, as well as two pseudogenes is located on the genomic island SaPI3[11*].

Egc is special in that it functions as an operon and its genes are transcribed into a single polycistronic mRNA[13]. In addition, a large cluster of up to 11 genes with sequence homology to SAgs has been discovered on the genomic island SaPI2; they have been termed staphylococcal exotoxin like genes, or *set*[10,11,14,15]. For an overview of the organisation and nomenclature of the *set* gene cluster, see supplemental figure S1 at http://www.medizin.uni-greifswald.de/immun /gk840/holtfreters1.pdf.

It has been known for some time that SAgs and lipopolysaccharides of gram-negative bacteria act synergistically. In mice, lipopolysaccharide and SEA are effective at 100-fold-reduced doses, if both agents are applied simultaneously[16]. In general, SAgs sensitise rodents to the lethal effects of lipopolysaccharide[17]. Such findings form the basis of the two-hit model of sepsis, which suggests that SAgs, besides being the causative agents of gram-positive toxic shock syndrome, may also contribute to septic shock induced by gram-negative or polymicrobial sepsis[18]. Evidence for the two-hit model has been hard to find in humans for a number of reasons. Firstly, most studies have concentrated on a few SAgs, the effects of which may have been masked by the presence of others. Secondly, host factors such as the HLA haplotype modulate the SAg effects[19-21]. Finally, the serum of many healthy individuals contains antibodies, which can neutralise the T cell-stimulatory effects of SAgs; 85 of 100 human serum samples fully inhibited the T cell proliferation induced by all 11 isoforms of the streptococcal SAg SMEZ, and the remaining 15 serum samples at least partially neutralised

* This article uses the old nomenclature for staphylococcal pathogenicity islands; for comparison refer to table 1 in the appendix (page v).

a subset of the variants[22]. In mice, such antibodies have been shown to protect the animals from the toxic effects of SAgs as well as from the lethal consequences of *S. aureus* infection[23-26]. In humans, lack of detectable antibodies to toxic shock syndrome-associated SAgs in the serum was predictive of susceptibility to toxic shock syndrome[27,28], and there is evidence that intravenous immunoglobulin preparations improve the survival of patients with streptococcal toxic shock syndrome[29-31].

The present study addresses whether it is possible, on the basis of our current knowledge about SAgs and exotoxin- like genes, to reliably predict the T cell-stimulating properties of a given *S. aureus* clinical isolate. In addition, in this study, the prevalence of serum factors which can inhibit the T cell stimulation induced by staphylococcal secretion products has been determined.

MATERIALS AND METHODS

Bacterial strains and secretion products. Sequence information was available from the following *S. aureus* reference strains: N315, Mu50 and Col (http: //www.tigr.org/), MW2 (http://www.cib.nig.ac.jp/) and NCTC6571, FRI326 and NCTC8325-4 (http://ncbi.nlm.nih.gov/). The strains FRI722, FRI955, FRI918 and FRI169 were from M. Bentley, University of Wisconsin, Madison[32]. Twenty *S. aureus* clinical isolates from throat swabs of asymptomatic individuals (aSA1 to -20) and 20 clinical isolates from blood cultures (pSA1 to 20) were collected by the Friedrich-Loeffler-Institut für Medizinische Mikrobiologie from hospitals in northeast Germany in 2000. They were identified as *S. aureus* by their ability to produce clumping factor and/or protein A (Murex Staphaurex test; Murex Biotech Ltd., Dartford, United Kingdom). All 40 isolates were methicillin sensitive in an antibiogram. The isolates were cultured in Luria broth (LB) medium (10 g of peptone per liter, 0.5 g of yeast extract per liter, 10 g of NaCl per liter and 1 mM NaOH) at 37°C up to an optical density at 540 nm of 3, corresponding to the postexponential growth phase. After centrifugation, the remaining cell debris was removed from the culture supernatants by filtration through a 0.02 µm filter and the cell-free supernatants were stored at -70°C.

Serum samples, immunoglobulin preparations, blood cells and proliferation assays. For the initial experiment, serum samples were obtained from 100 consecutive blood donors from the Department of Transfusion Medicine. These had an average age of 30.1 years; 49% were female and 51% were male. For all further experiments, serum samples from 23 healthy volunteers (18 female and 5 male), average age 21.8 ± 1.1 years, were used. The design of this study was approved by the Ethics Committee of the Medical Association of the Land Mecklenburg-Vorpommern (Ethikkommission der Ärztekammer Mecklenburg-Vorpommern bei der Ernst-Moritz-Arndt-Universität Greifswald), and informed consent was obtained from all blood donors. Three different preparations of human immunoglobulin for intravenous application were purchased from Novartis Pharma (Nürnberg, Germany; Sandoglobin), Baxter Immuno (Vienna, Austria; Gammagard) and Octapharma Pharmaceuticals (Vienna, Austria; Octagam). Peripheral blood mononuclear cells (PBMC) from healthy blood donors were isolated by density centrifugation over Ficoll. Cells were cultured in 96-well flat-bottomed plates at a density of 10^5/well in RPMI with L-glutamine and penicillin-streptomycin.

Heat-inactivated fetal bovine serum was added to a final concentration of 10%. To test for neutralising serum factors, fetal bovine serum was replaced by individual heat inactivated human serum where indicated. In the initial experiment, the fetal bovine serum was completely replaced by 10% heat-inactivated human serum. Since direct comparisons showed very similar results with only 2% human serum and 8% fetal bovine serum, these conditions were used in all further experiments to save material and thus enable extensive titration experiments with bacterial supernatants and recombinant SAgs. The cells were stimulated for proliferation by incubation with bacterial supernatants, which were titrated over a wide range. The mitogen phytohaemagglutinin (PHA) (Abbot, Wiesbaden, Germany) was used at a final concentration of 0.5 µg/ml. After 72 h of culture in a humidified incubator in the presence of 5% CO_2 at 37°C, [3H]thymidine (Amersham, Freiburg, Germany) was added at 0.5 µCi/well for 16 h. The cells were then harvested and the incorporated radioactivity was determined. All measurements were performed in triplicate, and the standard errors of the mean were below 20% except for values of <1.000 cpm.

Sequence comparisons. The deduced amino acid sequences of the *set* genes were compared with the Blast and Blast2 programs (http://ncbi.nlm.nih.gov/; http://www.tigr.org/).

Detection of enterotoxin and *set* genes. DNA was extracted from staphylococcal cultures and used as the template in PCRs for the detection of sequences corresponding to *sea-seq* and *tst* with the primers shown in table 1[13,33]. For the analysis of the *set* cluster, seven primer pairs were designed to amplify groups of *set* sequences covering the 11 loci as well as their known allelic variants (see supplemental online material, figure S1) as shown in table 1. The nomenclature for the *set* genes in this paper follows that used by Kuroda et al. for the fully sequenced *S. aureus* reference strain N315[11]. The amplifications were performed with *Taq* polymerase in a Biometra thermocycler with the following conditions: initial denaturation at 95°C for 5 min, followed by 30 stringent cycles (1 min of denaturation at 95°C, 1 min of annealing at the temperature indicated in table 1 and 1 min of extension at 72°C) and a final extension step at 72°C for 5 min. The quality of the DNA extracts and the absence of PCR inhibitors were confirmed by amplification of glyceraldehhyde-3-phosphate dehydrogenase or of 16S rRNA. The PCR products were then analyzed by electrophoresis through a 1% agarose gel. At least two independent experiments were performed for each determination.

Production and purification of recombinant enterotoxins. Recombinant enterotoxins were produced as previously described[13]. Briefly, primers were designed for the amplification of full-length *sea* (5' CA*GAATTC*AGCGAGAAAAGCGAAGAAATAAATG and 3' GCCTGCAGTTAACTTGTATATAAATATATATCAATATGAATGTTTTCAG) and *sei* (5' CA*GAATTC*CAAGGTGATATTGGTGTAGGTAACTTAA and 3' GC*CTGCAG*TTAG TTACTATCTACATATGATATTTCGACATCAAG; restriction sites are italic). The 5' primers were chosen within the coding sequence of the genes, omitting the region predicted to encode the signal peptide as determined by hydrophobicity analysis according to Kyte and Doolittle[34], and they contained restriction sites for EcoRI and PstI. After digestion with these enzymes, the PCR products were ligated into the pMAL-c2 expression vector from New England Biolabs (Ozyme), which was restricted with the same enzymes. The resulting plasmids were transfected into *Escherichia coli* TG1. The integrity of the open reading frames was confirmed by DNA sequencing

Table 1: Nucleotide sequences of enterotoxin and exotoxin-like gene-specific primers used in this study, annealing temperatures and anticipated PCR products.

Gene		Oligonucleotide sequence[a]	T_{anneal} °C	Fragment length	Ref.
tst	5 3	GCT TGC GAC AAC TGC TAC AG TGG ATC CGT CAT TCA TTG TTA A	56.2	559	[35]
sea	5 3	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	63.0	520	[35]
seb-sec	5 3	ATG TAA TTT TGA TAT TCG CAG TG TGC AGG CAT CAT ATC ATA CCA	64.0	683	[35]
sec3	5 3	CTT GTA TGT ATG GAG GAA TAA CAA TGC AGG CAT CAT ATC ATA CCA	59.2	283	[35]
sed	5 3	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	61.6	384	[35]
see	5 3	TAC CAA TTA ACT TGT GGA TAG AC CTC TTT GCA CCT TAC CGC	61.6	170	[35]
seg	5 3	CGT CTC CAC CTG TTG AAG G CCA AGT GAT TGT CTA TTG TCG	66.0	327	[35]
seh	5 3	CAA CTG CTG ATT TAG CTC AG GTC GAA TGA GTA ATC TCT AGG	58.0	360	[35]
sei	5 3	CAA CTC GAA TTT TCA ACA GGT AC CAG GCA GTC CAT CTC CTG	67.2	465	[35]
sej	5 3	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	61.6	142	[35]
sek	5 3	ATG GCG GAG TCA CAG CTA CT TGC CGT TAT GTC CAT AAA TGT T	62.0	197	
sel	5 3	CAC CAG AAT CAC ACC GCT TA TCC CCT TAT CAA AAC CGC TAT	63.1	410	
sem	5 3	CTA TTA ATC TTT GGG TTA ATG GAG AAC TTC AGT TTC GAC AGT TTT GTT GTC AT	62.2	325	[13]
sen	5 3	ACG TGG CAA TTA GAC GAG TC GAT TGA TCT TGA TGA TTA TGA G	61.0	475	[13]
seo	5 3	AGT TTG TGT AAG AAG TCA AGT GTA GA ATC TTT AAA TTC AGC AGA TAT TCC ATC TAA C	62.2	179	[13]
sep	5 3	CTG AAT TGC AGG GAA CTG CT ATT GGC GGT GTC TTT TGA AC	64.0	187	
seq	5 3	GAA CCT GAA AAG CTT CAA GGA ATT CGC CAA CGT AAT TCC AC	64.0	209	
set2, set 2b, set2c, set8, set9	5 3	AAG AGC GTA TTA TAC GAA ACC TTT CAA TAA GTT GTT TTC TCA A	46.2	398	
set5, set13	5 3	CTG GTC ACG CGA AAG TAG AA CTT TGT TAT ACC GCC AAC GC	61.0	293	
set7, set2a	5 3	AGC AAC AGG TGT AAA CAC TAC AA TAG AGT ACT TTG CAC CTT CAA ATC	52.1	308	
set 3, set3a, set6, set10	5 3	GAA AGC AAG TTT AGC ATT AGG TCT GTA CTC TTG TGA ATT TTC TA	54.0	239	
set5, set12	5 3	AGC TAA AGC GAT ATT TGT ATT AGG TTC GGC GTT CTT AGA GAC TCA	59.0	396	
set 1a, set1b, set1c, set4, set11, set14	5 3	AAA GCA ACA TTA GCA TTA GG TTC TTT GTT ACA CCA CCA AC	56.0	344	
set3b, set15	5 3	GCT AAA GCA AGT TTA GCA CTA GG TTT ACT GTC TTT AGG TTC TGT CTT A	48.6	543	
16S rRNA	5 3	GTA GGT GGC AAG CGT TAT CC CGC ACA TCA GCG TCA G	58.0	228	[35]
G3PDH	5 3	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA		452	

[a] Nucleotide sequences for the amplification of *sek*, *sel*, *sep* and *seq* as well as the *set*-genes and anticipated sizes of the PCR products were derived from published sequences of reference strains (see materials and methods);

[b] The nomenclature of the *set* genes corresponds to that in the supplemental online material, figure S1.

of the junction between pMAL-c2 and the inserts. The fusion proteins were purified from cell lysates of transformed *E. coli* by affinity chromatography on an amylose column according to the manufacturer`s instructions (New England Biolabs). pMAL-c2 without an insert was also transfected into *E. coli* TG1, and maltose-binding protein (MBP) without enterotoxin was purified in a similar way and used as a control.

RESULTS

Occurrence of SAg genes in clinical isolates of *S. aureus.*

In this study, 40 clinical *S. aureus* strains were analyzed for their ability to secrete T cell-stimulating factors. Supernatants from high-density bacterial cultures were incubated with PBMC from three different donors in the presence of fetal bovine serum. Thymidine incorporation was used as a readout for T cell activation. Both the degree of T cell stimulation and the titers of the supernatants, which caused maximal proliferation, were highly reproducible between the three experiments (data not shown); 16 of 20 *S. aureus* isolates from blood cultures and 12 of 20 commensal *S. aureus* isolates from throat swabs were able to stimulate T cells.

To address the question of whether SAgs and/or staphylococcal enterotoxins could account for the T cell activation, the 40 clinical isolates were analyzed by PCR for the presence of all 17 SAg genes. For the characterisation of the *set* cluster, a PCR system, which allowed the amplification of seven groups of *set* genes covering the whole cluster, was developed (see Materials and Methods). The results of this analysis were compared with the maximal T cell proliferation which could be induced by the secretion products of individual bacterial strains (table 2).

Every strain harboured members of the *set* cluster regardless of whether it was able to stimulate T cells or not. Thus, the proteins encoded by the cluster of *set* genes do not appear to be responsible for the T cell stimulation induced by *S. aureus*. In contrast, the 40 strains were very heterogeneous with regard to the 17 enterotoxin loci. There was an absolute correlation between the presence of members of the enterotoxin gene family in an *S. aureus* isolate and its secretion of T cell-activating substances (table 2), supporting the notion that enterotoxin gene products were the T cell-stimulating agents in the bacterial supernatants.

Neutralising antibodies in normal human serum.

Serum antibodies, which can inhibit the T cell activation by individual SAgs, are frequently present in healthy individuals. However, since the majority of clinical samples of *S. aureus* have multiple SAg genes (table 2), we wished to determine whether the complex mixture of SAgs secreted by clinical *S. aureus* isolates can also be neutralised with similar frequency and efficiency. For an initial screen, T cell activating supernatants from 11 *S. aureus* isolates differing in their SAg gene spectrum were selected. The *S. aureus* reference strain FRI 918, which expresses only a single SAg (staphylococcal enterotoxin E), was also included. Serum samples from 100 healthy adult blood donors were then tested for their ability to inhibit the T cell activation induced by the secretion products of these 12 *S. aureus* strains. The

Table 2: Induction of T cell proliferation by the secretion products of *S. aureus* clinical isolates correlates with the presence of enterotoxin genes but not with that of staphylococcal exotoxin-like genes (*set*).

S.aureus isolates, blood culture	T cell activation, cpm max[a]	enterotoxins, TSST[b]	*egc*-encoded enterotoxins[b]	*set*[c]	*S.aureus* isolates, throat swab	T cell activation, cpm max[a]	enterotoxins, TSST[b]	*egc*-encoded enterotoxins[b]	*set*[c]
pSA4	152914	*b d j k q*[d]	-	+	aSA4	72879	-	*m n o*	+
pSA2	79281	-	*m n o*[d]	+	aSA2	68290	-	*g i m n o*	+
pSA16	65158	-	*g i m n o*	+	aSA12	66989	*c l*	*g i m n o*	+
pSA12	54439	-	*g i m n o*	+	aSA1	56741	*a tst*	*g i n o*	+
pSA10	52038	*a d j l*	*g i m n o*	+	aSA3	48447	*a tst*	*g i n o*	+
pSA19	48624	*c l*	*g i m n o*	+	aSA19	44926	*c l tst*	*g i m n o*	+
pSA17	45499	*b p*	-	+	aSA14	42492	*c l*	*g i m n o*	+
pSA18	44338	*c l*	*g i m n o*	+	aSA16	42241	-	*g i m n o*	+
pSA11	36239	*c l*	*g i m n o*	+	aSA18	41699	*d j p*	*g i m n o*	+
pSA5	33833	*a d*	*n o*	+	aSA6	34671	*a tst*	*g i n o*	+
pSA13	24941	*b*	-	+	aSA11	20062	*d j*	-	+
pSA6	23866	-	*m n o*	+	aSA5	15994	*d j*	-	+
pSA20	18426	*c l p*	-	+	aSA7	1530	-	-	+
pSA15	18156	*c l*	*g i m n o*	+	aSA17	774	-	-	+
pSA8	14375	-	*n o*	+	aSA10	752	-	-	+
pSA7	5751	-	*n o*	+	aSA15	717	-	-	+
pSA1	1609	-	-	+	aSA13	686	-	-	+
pSA3	1495	-	-	+	aSA20	658	-	-	+
pSA14	871	-	-	+	aSA9	467	-	-	+
pSA9	274	-	-	+	aSA8	397	-	-	+

[a] Human PBMC were cultured for 72 h in the presence of 10% FBS and their proliferation was determined by thymidine incorporation. The cells were stimulated with serial dilutions (1/40 – 1/40 000 000) of sterile culture supernatants from 40 *S. aureus* clinical isolates. The titration curves reproducibly peaked at different dilutions; the maximal thymidine incorporation is indicated in the table. Background values in the absence of bacterial supernatant ranged between 175 and 1450 cpm in different experiments. Values greater than 3 times background were considered to be significant T cell activation.

[b] The presence of enterotoxin genes was determined by PCR.

[c] Seven different PCR-reactions were performed to cover all the loci of the *set*-gene cluster. There was substancial heterogeneity in the composition of the *set*-cluster, but this did not correlate with T cell proliferation and is not shown.

[d] Except for TSST-1, the staphylococcal enterotoxins (SE) are indicated by one letter; for example: „a“ means *sea*.

neutralising response was determined as the percent inhibition of thymidine incorporation in the presence of 10% human serum versus 10% fetal bovine serum. To exclude non-specific inhibition, the mitogen PHA was used as a control. Table 3 shows the neutralising activity of 10 representative human serum samples.

None of the 100 human serum samples inhibited the PHA- induced proliferation. On the contrary, the T cells proliferated much more vigorously in the presence of human serum. It is a common observation that human serum under most conditions supports the proliferation of human T cells much better than fetal bovine serum. This was also frequently observed after stimulation with bacterial supernatants, most impressively after stimulation with secretion products of *S. aureus* isolate pSA16 (table 3). Therefore, we considered a reduction in proliferation in the presence of human serum of more than 25% to be significant.

Table 3 shows 10 serum samples with individual patterns of neutralising capacity: serum 54 did not neutralise any of the *S. aureus*-derived mitogenic factors, whereas others inhibited most T cell-stimulating supernatants. Figure 1 summarises the data for all 100 serum samples. There was a correlation between the enterotoxin genes of an isolate and the presence of inhibiting serum factors; the effects of secretion products from *S. aureus* isolates which harbored only members of the *egc* cluster of enterotoxins were inhibited only very rarely.

Table 3: Inhibition of T cell proliferation elicited by culture supernatants of different *S. aureus* strains by 10 individual human sera.

S. aureus isolate	Enterotoxins, TSST	*egc*-encoded enterotoxins	human serum no.									
			55	49	53	39	40	54	65	95	100	88
PHA			-101	-118	-109	-74	-57	-136	-80	-83	-75	-100
FRI 722	*a b q k*		99	83	93	80	99	-10	-234	-230	86	19
pSA5	*a d*	*n o*	79	25	50	32	42	-73	-59	-7	-9	-16
pSA17	*b p*		68	56	84	20	72	1	-20	-3	12	-52
pSA20	*c l p*		99	98	99	98	99	-46	-22	92	-64	98
FRI 955	*d j*		99	53	98	99	98	-51	-19	36	-83	93
FRI 918	*e*		99	26	91	98	97	-135	-48	-85	-55	100
aSA2		*g i m n o*	-81	28	29	3	22	-15	-85	-54	2	-48
pSA12		*g i m n o*	-67	1	16	-39	3	-71	-195	-61	-19	-79
FRI 169	*q k tst*		68	-12	97	89	87	-312	81	-231	93	123
pSA2		*m n o*	-34	29	34	-37	-44	-39	-160	-19	-8	-35
pSA16		*g i m n o*	-187	-50	-49	-119	-101	-98	-246	-45	-99	-68
aSA4		*m n o*	8	100	6	-84	-63	-142	-278	-116	-32	-227

Inhibition: 75-100% 25-74% < 25%

Proliferation in the presence of 10% human serum was compared with that in the presence of 10% FBS (100%), and inhibition is expressed as a percentage of this value. Negative values indicate that there was more vigorous proliferation in the presence of human serum. The bacterial culture supernatants were used at a concentration which, in the presence of FBS, induced a level of proliferation which was just below the plateau.

A more detailed analysis comparing two *egc*-containing *S. aureus* isolates (aSA2 and aSA4) with strains FRI 722 (*sea*, *seb*, *seq* and *sek*) and pSA20 (*sec*, *sel* and *sep*) with 23 new human serum samples confirmed the results obtained in the survey. In this experiment, the bacterial supernatants were titrated over a wide range of concentrations and inhibition was defined as a reduction of the mitogenic potency by a factor of at least 10 in the presence of 2% human serum. For example, with this criterion, the proliferation induced by *S. aureus* FRI722 was inhibited by human serum samples HS4 and HS5 and that induced by *S. aureus* pSA20 was inhibited by HS4 and HS8 (figure 2). As in the initial experiment, however, the addition of human serum frequently enhanced the T cell proliferation. The patterns of inhibition and/or stimulation of T cell activation by individual human serum samples were reproducible in repeat experiments. The results obtained with 23 different serum samples are summarised in table 4. They suggest that the gene products of the *egc* very rarely elicit a strong neutralising antibody response, despite the fact that members of the *egc* cluster were the most frequent SAg genes in our *S. aureus* isolates.

Neutralisation of recombinant SAgs.

To find out whether these differences are an inherent property of the individual SAgs or whether they are caused by other bacterial secretion products contained in the culture, staphylococcal enterotoxin I (rSEI), which is encoded by *egc*, was compared with rSEA. The recombinant SAgs were expressed as fusion products with MBP, which did not induce proliferation by itself (figure 3A). Only 1 of 23 human serum samples reduced the potency of rSEI by a factor of 10, whereas by the same criteria rSEA was inhibited by 16 serum samples. This is in good agreement with the results obtained with the bacterial supernatants (figure 3B).

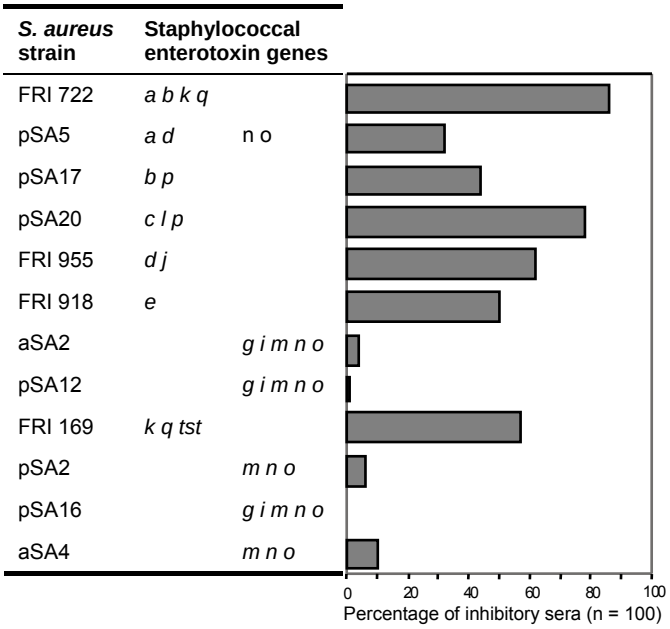

Figure 1: Inhibition of T cell proliferation induced by *S. aureus* secretion products. Twelve *S. aureus* isolates were grown to high density, and their supernatants were titrated and used to stimulate proliferation of human PBMC in the presence of 10% fetal bovine serum. A concentration just off the plateau was then chosen for assessment of the proliferation elicited in the presence of 10% fetal bovine serum compared to 10% human serum, and 100 serum samples from healthy individuals were screened. A reduction of thymidine incorporation of at least 25% was taken as significant inhibition, because cells which were stimulated with the mitogen PHA as a control uniformly proliferated more vigorously in the presence of human serum.

Neutralisation by pooled human immunoglobulin.

A similar difference in neutralising capacity was also observed in three different intravenous immunoglobulin preparations from pooled human serum samples. Inhibition of T cell proliferation induced by FRI722, pSA20, or rSEA was around 100-fold more efficient than that induced by aSA2, aSA4, or rSEI (figure 4). In contrast to human serum, the addition of purified immunoglobulin G did not increase the T cell proliferation, suggesting that this enhancement of proliferation was likely due to human growth factors or hormones rather than to specific interactions of antibodies with SAgs.

DISCUSSION

This survey of all known SAg genes confirms earlier findings that most *S. aureus* clinical isolates harbour subsets of enterotoxin genes. There was extensive variation between individual strains, and generally the isolates harboured multiple enterotoxin genes, usually five. In this investigation, we observed a strict correlation between the presence of enterotoxin genes in an *S. aureus* isolate and its ability to elicit T cell proliferation. Therefore, a genetic analysis of the SAg gene loci could be useful for the prediction of the functional properties of unknown clinical isolates. There are two limitations: Firstly, the presence of a SAg gene does not necessarily mean that the protein is expressed at mitogenic levels. Secondly, while it appears unlikely that many more SAg gene loci still await discovery, allelic variation at the known gene loci could be extensive. Point mutations, however, can abolish primer binding to a functional SAg gene variant (C. Feig, unpublished observation).

In contrast to the enterotoxins, the presence of exotoxin-like genes, which are encoded by the *set* cluster on genomic island SaPI2, did not correlate with T cell stimulation. This is in agreement with the observation that recombinant staphylococcal exotoxin-like proteins 1, 3, 10 and 15 had no superantigenic properties[14,36]. The data reported in this study as well as the published *S. aureus* sequences show that the *set* gene cluster is present in every *S.*

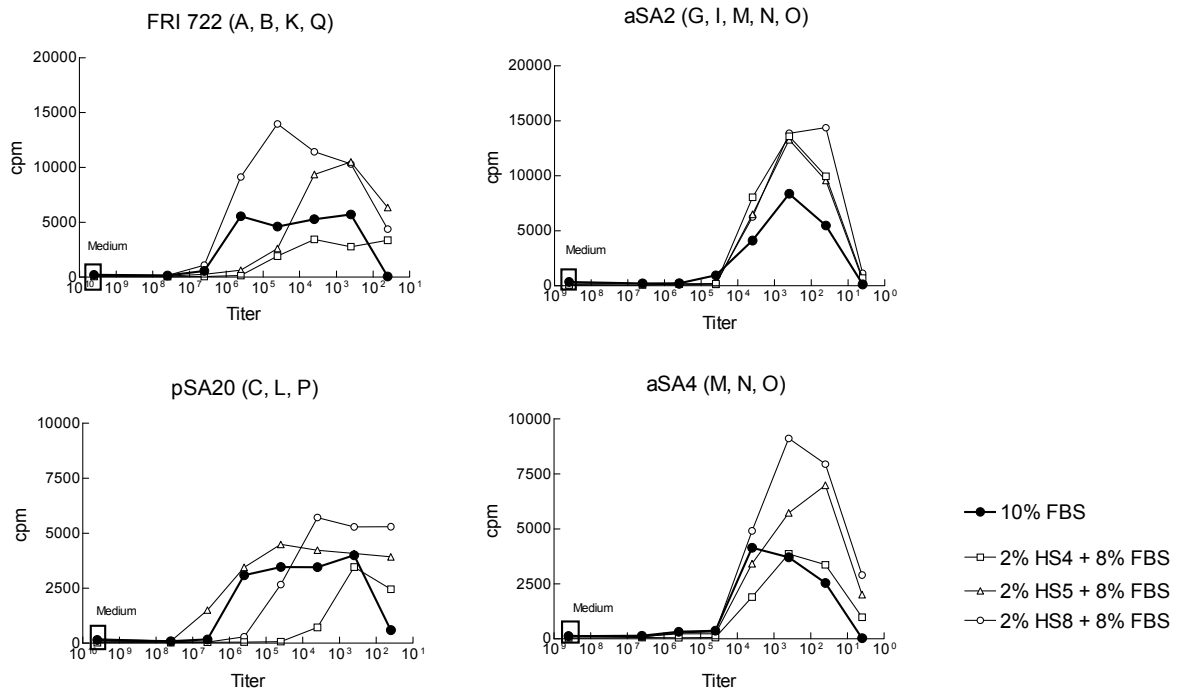

Figure 2: Neutralisation capacity of human serum samples. Four *S. aureus* strains were grown to high density in culture and the supernatants were titrated over a large range and used to stimulate proliferation of human PBMC in the presence of 10% fetal bovine serum (FBS). Proliferation was assessed by thymidine incorporation after 72 h of culture; 23 serum samples from healthy individuals (HS) were tested for their ability to inhibit this proliferation when used at a final concentration of 2% (in the presence of 8% fetal bovine serum). A right shift of the titration curve by at least a factor of 10 in the presence of human serum was considered to indicate significant inhibition. The effect of three typical human serum samples is shown as an example.

aureus strain and that its composition is highly variable[14]. This suggests a nonredundant function of its gene products in *S. aureus*, and the location of the *set* cluster on a genomic island indicates that this function may be relevant for host-pathogen interactions. What that function may be remains to be defined.

Testing of 123 serum samples from healthy blood donors for their ability to inhibit T cell activation by the secretion products of 12 different *S. aureus* strains revealed remarkable heterogeneity and surprisingly large holes in neutralising capacity. None of the tested serum samples neutralised all the *S. aureus* culture supernatants in this investigation, and the secretion products from isolates harboring *egc* but no other enterotoxin genes were neutralised only rarely. This was confirmed by experiments with recombinant SEA and SEI, which excludes that other factors contained in the bacterial supernatants are responsible (table 4). As expected, this difference in neutralising capacity was also mirrored by three different intravenous immunoglobulin preparations, which are prepared from very large pools of human serum (figure 4). The inhibition of *egc* gene products was about 100-fold less efficient than that of other SAgs. These differences in neutralising capacity may result in variations in their therapeutic efficacy in patients infected with different *S. aureus* strains.

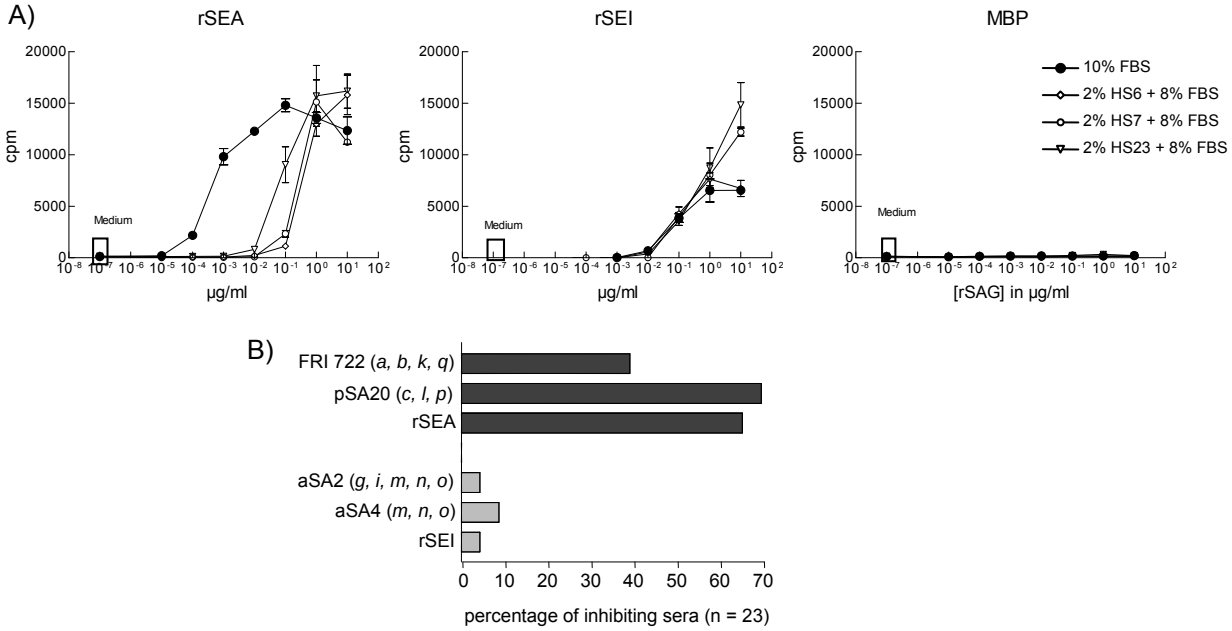

Figure 3: A) Inhibition of T cell proliferation induced by the recombinant SAgs rSEA and rSEI. Fusion proteins of MBP and staphylococcal enterotoxin A or I, respectively, were used to stimulate proliferation of human PBMC in the presence of 10% fetal bovine serum (FBS). PBMC which proliferated in response to MBP alone were excluded from the analysis; 23 serum samples from healthy individuals were then tested for their ability to inhibit this proliferation when used at a final concentration of 2% (in the presence of 8% fetal bovine serum). A right shift of the titration curve by at least a factor of 10 in the presence of human serum was considered to indicate significant inhibition. The effect of three typical human serum samples is shown as an example. **B) Frequency of human serum samples able to inhibit the proliferative effects of *S. aureus* secretion products or of recombinant SAgs**. This figure summarises the data obtained with 23 serum samples which were tested as shown in figure 2 and panel A.

In agreement with our results, Banks and colleagues detected significantly lower serum antibody binding to recombinant SEG and SEI compared with SEA, SEB, or SEC2 in an enzyme-linked immunosorbent assay[4]. Therefore, low concentrations of antibodies specific for *egc*-encoded proteins could at least partially explain the failure of many serum samples to neutralise these SAgs. This is unexpected because *egc* was the most frequent SAg locus in *S. aureus* in the present as well as in earlier studies[13,37]. A survey of around 200 cases of toxic shock syndrome and staphylococcal scarlet fever demonstrated that *egc*-encoded SAgs are able to cause symptomatic staphylococcal toxemias but that these are probably rare[38,39].

Table 4: Sera from 23 healthy volunteers were tested for their abilitiy to inhibit the T cell proliferation elicited by the secretion products of four different *S. aureus* strains[a].

Inhibition factor	No. of sera with neutralising activity against:			
	FRI722	pSA20	sSA2	aSA4
0	14	8	22	21
10	6	2	1	2
100	2	5	0	0
≥ 1000	1	8	0	0

[a] Experiments were conducted as shown in figure 3. PBMC were stimulated with bacterial supernatants over a large range of concentrations in the presence of 10% fetal bovine serum. The inhibition factor was determined from the right shift of the response curve when 2% of the fetal bovine serum was replaced with human serum. Proliferation induced by the mitogen phytohemagglutinin was never inhibited by human serum factors.

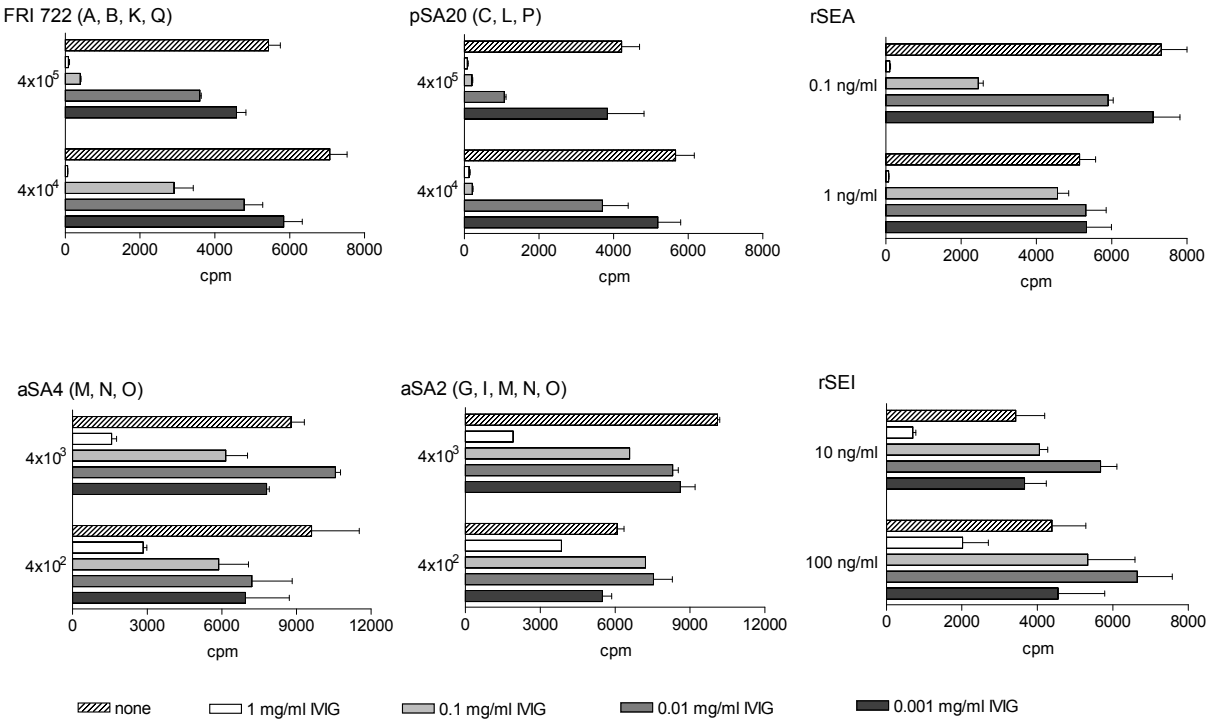

Figure 4: Therapeutic preparations of human immunoglobulin G (IVIG) inhibit *egc*-derived enterotoxins less efficiently than other SAgs. Human PBMC were stimulated with bacterial supernatants or rSEA and rSEI at two different titers or concentrations as indicated on the left of each panel. They were cultured in the presence of 10% fetal bovine serum, and increasing concentrations of intravenous immunoglobulin preparations were added where indicated. In this figure, the effect of a preparation of Sandoglobin (Novartis Pharma) is shown; two other intravenous immunoglobulin preparations (see Materials and Methods) gave very similar results.

Therefore, in spite of the low level of antibody responses against *egc*-encoded SAgs in the population, these do not appear to be a major threat to health.

What might be the molecular reasons for the low efficiency of neutralisation of *egc*-encoded SAgs by serum factors? On the basis of sequence comparisons, the known SAgs of *S. aureus* and *Streptococcus pyogenes* have been grouped into three clusters. Each cluster contains at least one *egc*-encoded SAg, which means that the *egc* genes differ strongly from each other[13]. This makes it unlikely that they share unique structural features which interfere with a strong immune response against them. In addition, our data show that in vitro-generated supernatants of *egc*-harboring *S. aureus* isolates as well as recombinant SEI can efficiently stimulate T cells, further arguing against an inherent lack of immunogenicity. On the other hand, there are indications that *egc*-encoded SAg expression may be regulated differently from classical SAgs. First, with an enzyme-linked immunosorbent assay method, Omoe and colleagues have shown that only a minority of *S. aureus* strains, which harbored *seg* and *sei* genes, secreted the SEG and SEI proteins in detectable amounts in vitro, which was in contrast to the classical SEH[40]. Furthermore *seg* mRNA accumulated in the logarithmic growth phase, in contrast to the other SEs and TSST-1, which are primarily transcribed during postexponential bacterial growth ([41]; G. Lina, unpublished data). SAg protein expression appears to correspond to the mRNA levels (S. Holtfreter, unpublished data).

Unfortunately, little information is available about the regulatory mechanisms which are effecttive at different stages of *S. aureus* interaction with its host in vivo. It is conceivable that not only the amounts but also the spectrum of secreted SAgs differ between *S. aureus* carriage and *S. aureus* infection. Finally, our data underscore the specificity of the SAg- neutralising antibodies. The lack of cross-inhibition has to be taken into account when designing SAg vaccines.

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We have no potentially conflicting commercial interests.

SUPPLEMENTAL ONLINE MATERIAL

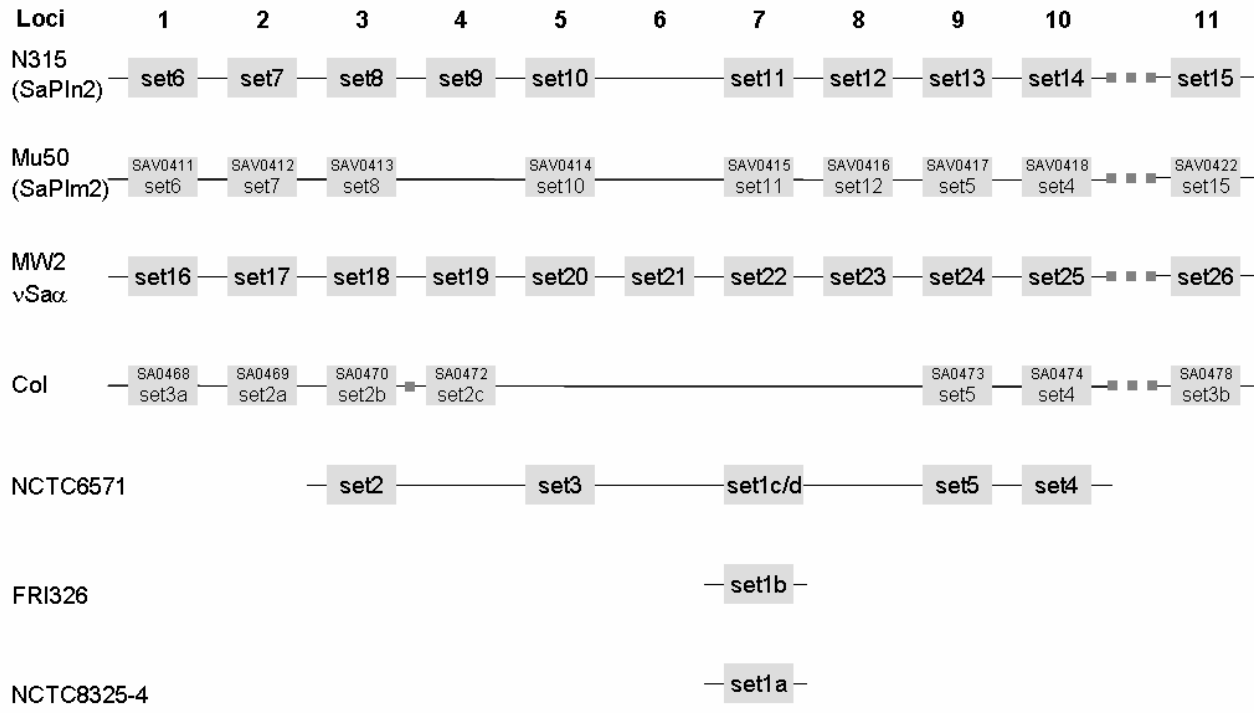

Figure S1: Organisation of the staphylococcus exotoxin-like gene cluster (*set*) on the staphylococcus pathogenicity island 2[†]. The composition of the *set*-cluster is highly variable. There are maximally 11 different loci with 25 – 67% deduced amino acid homology between individual pairs of genes. In addition, there are allelic variants for each of the loci, which are shown in the columns. These variants show a higher degree of homology (75 – 99%). The nomenclature in this paper is according to the completely sequenced *S. aureus* reference strain N315, which is shown at the top.

[†] This paper uses the old nomenclature of staphylococcal pathogenicity islands. For comparison of *set* and *ssl* nomenclature refer to Chapter 2 and table 1 in the appendix (page i).

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Similar T cell-activating properties of *egc*-encoded and classical *Staphylococcus aureus* superantigens

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ABSTRACT

Neutralising serum antibodies against *S. aureus* superantigens are common in the healthy population with one notable exception: Superantigens of the enterotoxin gene cluster (*egc*), by far the most prevalent superantigens in *S. aureus* clinical isolates, only rarely elicit a neutralising antibody response, even in carriers of *egc*-positive *S. aureus* strains. We hypothesised that this "*egc* gap" in the antibody response is due to i) different T cell-activating properties of classical and *egc* superantigens and/or ii) their differential regulation. Three classical (SEB, SEQ and TSST-1) and three *egc* superantigens (SEI, SEM and SEO) were overexpressed in *E. coli*, purified and LPS-depleted, and their T cell-activating properties were studied. The T cell mitogenic potency and cytokine induction (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) did not differ between classical and *egc* superantigens. In contrast, *egc*-encoded proteins were secreted by *S. aureus* during logarithmic growth, while classical superantigens were released in the stationary phase. In conclusion, we propose that, due to their different regulation, classical and *egc* superantigens contact the host's immune system under conditions, which either favour or, in the case of *egc* superantigens, prevent efficient antibody production.

SUBMITTED

INTRODUCTION

Staphylococcus (S.) aureus is both a successful coloniser and an important human pathogen, causing a wide variety of infectious diseases including toxin-mediated diseases. Among the numerous toxins of *S. aureus* are the 19 known staphylococcal superantigens (SAg): the toxic shock syndrome toxin (TSST-1) and the staphylococcal enterotoxins (SEA-SEE, SEG-SER and SEU)[1]. SAgs directly cross-link T cell receptors on T cells with MHC class II molecules on antigen-presenting cells, thereby inducing T cell proliferation and massive cytokine release. SAgs are involved in the pathogenesis of staphylococcal food poisoning, toxic shock syndrome, and probably also septic shock[1,2].

The enterotoxin gene cluster (*egc*) harbours five or six SAg genes (*seg*, *sei*, *sem*, *sen*, *seo* and sometimes *seu*), which are clustered on a staphylococcal pathogenicity island (SaPI2)[3,4]. *Egc* SAgs are by far the most prevalent SAgs in both commensal and invasive *S. aureus* isolates, frequencies between 52 and 66% have been reported[5-8]. However, while antibodies against “classical” SAgs (e.g. TSST-1, SEA, SEB, SEC) are very frequent in the healthy population[9-11], neutralising antibodies against *egc* SAgs are surprisingly rare, even among carriers of *egc*-positive *S. aureus* strains[12] (Holtfreter, unpublished data). To date, the reasons for this “*egc* gap” in the humoral immune response against staphylococcal SAgs are unknown. We hypothesise, that the “*egc* gap” could be due to two non-exclusive mechanisms. Firstly, classical and *egc* SAg may differ in their T cell-activating properties. Secondly, there might be differences in the in vivo expression of classical and *egc* SAgs. Indeed, it was previously reported that the *egc* is transcribed as an operon in the exponential growth phase of *S. aureus*[3,13]. In contrast, most ”classical“ SAg genes are transcribed during post-exponential growth. Low concentrations of *egc* SAgs in vivo or the specific micro-environmental conditions associated with exponential versus post-exponential bacterial growth might prevent an efficient adaptive immune response including the generation of neutralising antibodies.

To test these hypotheses, we firstly produced three classical (SEB, SEQ and TSST-1) and three *egc* SAgs (SEI, SEM and SEO) by recombinant gene technology and assessed their T cell-activating properties by measuring T cell proliferation and cytokine secretion. Secondly, we compared the growth phase-dependent SAg secretion of *S. aureus* clinical isolates harbouring either classical or *egc* SAg genes.

MATERIALS AND METHODS

Production of recombinant SAgs. SEB, SEQ, TSST-1, SEI, SEM and SEO were produced by recombinant gene technology. The respective gene sequence were amplified from the sequenced *S. aureus* strains N315 (*tst, sei, sem, seo*) and COL (*seb, seq*) using the following primers with BsaI restriction sites (underlined) (table 1). PCR products were purified with the Qiagen Quick PCR Purification Kit (Qiagen, Hilden), digested with BsaI and introduced into the *E. coli* plasmid pPR-IBA1 with *Strep*-tag® II (IBA, Goettingen). The

Table 1: Cloning primers.

gene	primer sequences (5'→ 3')
seb-*strep*II	5-ATGGTAGGTCTCAAATGGAGAGTCAACCAGATCCTAAACC 3-ATGGTAGGTCTCAGCGCTCTTTTTCTTTGTCGTAAGATAAACTTC
seq-*strep*II	5-ATGGTAGGTCTCAAATGGATGTAGGGGTAATCAACCTTAGA 3-ATGGTAGGTCTCAGCGCTTTCAGTCTTCTCATATGAAATCTCTA
tst-*strep*II	5-ATGGTAGGTCTCAAATGTCTACAAACGATAATATAAAGGATTTG 3-ATGGTAGGTCTCAGCGCTATTAATTTCTGCTTCTATAGTTTTTATTT
sei-*strep*II	5-ATGGTAGGTCTCAAATGCAAGGTGATATTGGTGTAGGTAAC 3-ATGGTAGGTCTCAGCGCTGTTACTATCTACATATGATATTTCGAC
sem-*strep*II	5-ATGGTAGGTCTCAAATGGATGTCGGAGTTTTGAATCTTAGG 3-ATGGTAGGTCTCAGCGCTACTTTCGTCCTTATAAGATATTTCTAC
seo-*strep*II	5-ATGGTAGGTCTCAAATGAATGAAGAAGATCCTAAAATAGAGAG 3-ATGGTAGGTCTCAGCGCTTGTAAATAAATAAACATCAATATGATAGT

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, Goettingen). The purity of recombinant SAgs was assessed by SDS-PAGE stained with Coomassie Brilliant Blue (figure 1). Proteins concentrations were determined with the Roti®-Quant assay (Roth, Karlsruhe, Germany). LPS concentrations were determined with a limulus amebocyte lysate assay (QCL1000, Cambrex, Walkersville). Contaminating LPS was reduced by two rounds of LPS depletion with the EndoTrap® red columns (Profos, Regensburg) to below 5ng/ml (table 2). Final concentrations of LPS in our assays were always below 30pg/ml (0.3 EU/ml).

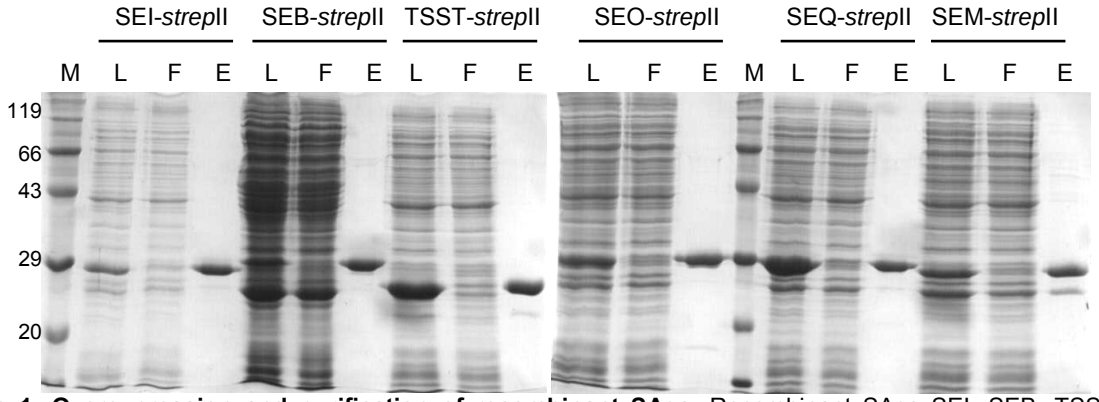

Figure 1: Overexpression and purification of recombinant SAgs. Recombinant SAgs SEI, SEB, TSST-1, SEO, SEQ and SEM were overexpressed in *E. coli* and purified from the bacterial lysates with Strep-Tactin columns. Bacterial lysates (L), flow through fractions (F) and eluted protein samples (E) were analysed by 12% SDS-PAGE.

T cell proliferation assay. T cell proliferation was assessed after 72 h culture by incorporation of [^{3}H]thymidine. To compare the T cell proliferation after stimulation with recombinant SAgs, human peripheral blood mononuclear cells (PBMCs) from two different blood donors were stimulated with recombinant SAgs titrated over a broad range (0.1 pg/ml to 1 µg/ml) as described previously[12,14]. A control protein (*S. aureus* anti-sigma regulatory factor rsbW), which was overexpressed and purified in parallel to the recombinant SAgs, did not induce T cell activation. To analyse the growth phase dependence of SAg secretion, we cultured two strains with classical SAg genes (pSA17: *seb*, *sep;* pSA20: *sec*, *sel*, *sep*) and two strains with *egc* SAg genes (aSA2, aSA4) in LB medium and obtained culture samples at the

Table 2: LPS depletion of purified recombinant SAgs. LPS was depleted with EndoTrap® red columns and quantified with a limulus amebocyte lysate assay.

Protein sample	LPS in ng/ml before LPS depletion	LPS in ng/ml after LPS depletion	LPS depletion rate
SEB-*strep*II	436	1.2	99.7
SEQ-*strep*II	1500	0.63	99.9
TSST-1-*strep*II	130	1.7	98.7
SEI-*strep*II	30	4.8	84.0
SEM-*strep*II	22.9	0.08	99.7
SEO-*strep*II	29.7	0.29	99.0

indicated optical densities. Bacterial culture supernatants were obtained by centrifugation, sterilised by filtration and normalised to OD 0.2 of the bacterial culture. Afterwards supernatants were diluted 1:1000 and incubated with $1x10^5$ PBMC/well in 96-well flat-bottom plates in the presence of RPMI 1640 supplemented with 10% fetal bovine serum.

Cytokine measurement. Human PBMCs were stimulated with recombinant SAgs (100 pg/ml SEB, 100 pg/ml SEQ, 100 pg/ml TSST-1, 10 pg/ml SEI, 100 pg/ml SEM and 100 ng/ml SEO) and 20 µl supernatant were obtained after 24 h and 72 h. 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, Heidelberg) according to the manufacturer's instructions. In principle, six bead populations with distinct fluorescence intensities (FL3 channel) are coated with capture antibodies specific for IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ proteins. These cytokine capture beads were mixed with PE-conjugated detection antibodies (FL2 channel) and then incubated with recombinant cytokine standards or test samples to form sandwich complexes. Samples were measured by flow cytometry and analysed with the BD CBA Analysis Software.

RESULTS

Comparison of T cell-mitogenic properties of classical and *egc* SAgs.

To analyse whether there are substantial differences in the T cell-mitogenic activity of classical and *egc* SAgs we performed T cell proliferation assays with human PBMCs from two healthy blood donors using three classical SAgs (SEB, SEQ, and TSST-1) and three *egc* SAgs (SEI, SEM, and SEO). All six SAgs induced a strong T-cell proliferation in a dose-dependent manner (figure 2). Importantly, except for SEO, all SAgs induced strong proliferation already in the pg/ml range. Moreover, we observed some individual differences in the proliferative response to SAg stimulation. Afterwards, using series of 1:3 dilutions we determined the SAg concentrations inducing half-maximal proliferation to be 7.3 pg/ml for SEB, 1.6 pg/ml for SEQ, 0.8 pg/ml for TSST-1, 0.6 pg/ml for SEI, 18.1 pg/ml for SEM, and 2263 pg/ml for SEO. Hence, except for SEO, we observed no major differences between the T cell-mitogenic potency of classical and *egc* SAgs.

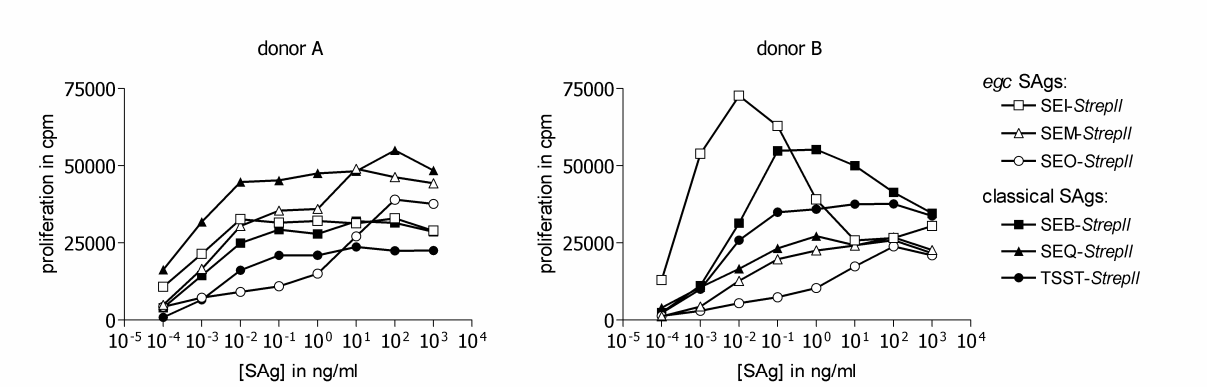

Figure 2: Similar T cell-mitogenic potencies of classical and *egc* SAgs. PBMCs from two healthy blood donors were stimulated with recombinant classical SAgs (SEB, SEQ and TSST-1; filled symbols) and *egc* SAgs (SEI, SEM and SEO; empty symbols). Proliferation was assessed after 72 h by [^{3}H] thymidine incorporation. Results of one out of three independent experiments are depicted.

Comparison of cytokine profiles induced by classical and *egc* SAgs.

SAg stimulation induces a massive cytokine release by both the activated T cells and antigen presenting cells. We compared the release of pro- and anti-inflammatory cytokines by human PBMCs from two different blood donors 24 h and 72 h after stimulation with recombinant classical and *egc* SAgs. The concentrations of recombinant SAgs were previously titrated to elicit strong proliferation just below the maximal response (figure 2), and their effects on cytokine secretion were compared to those of the T cell mitogen phytohemagglutinin (PHA). Unstimulated cells served as control. In general, SAg and PHA stimulation of human PBMCs induced the release of high concentrations of both pro-inflammatory (IFN-γ, TNF-α, IL-2) and anti-inflammatory (IL-4, IL-5, IL-10) cytokines (figure 3). Notably, individual recombinant SAgs were as potent as the mitogen PHA. Moreover, we observed some individual variation in the cytokine response.

The dominating cytokine after SAg stimulation was IFN-γ, which is also the key pro-inflammatory Th1 cytokine. While only small IFN-γ concentrations were present after 24 h, we detected ~50 ng/ml IFN- γ after 72 h SAg stimulation. Moreover, SAg stimulation induced high levels of TNF- α (on average 270 pg/ml, 72 h) and IL-2 (on average 320 pg/ml). Besides these pro-inflammatory cytokines, SAgs also induced the anti-inflammatory cytokines IL-4 and IL-5, though at lower levels. While IL-4 was detected already after 24 h (19 pg/ml), IL-5 was measured only after 72 h (31 pg/ml). Additionally, high levels of IL-10, which can be produced by regulatory T cells and has immunosuppressive functions, were detected after 24 h and 72 h. Importantly, we observed no general differences between the cytokine profiles triggered by classical and *egc* SAgs.

The cytokine pattern after PHA stimulation was similar to that elicited by SAgs. Again, IFN-γ was the dominating cytokine, and high concentrations of TNF-α, IL-2 and IL-10 were also measured. Compared to SAg stimulation, PHA was a more potent inducer of TNF-α and IL-4.

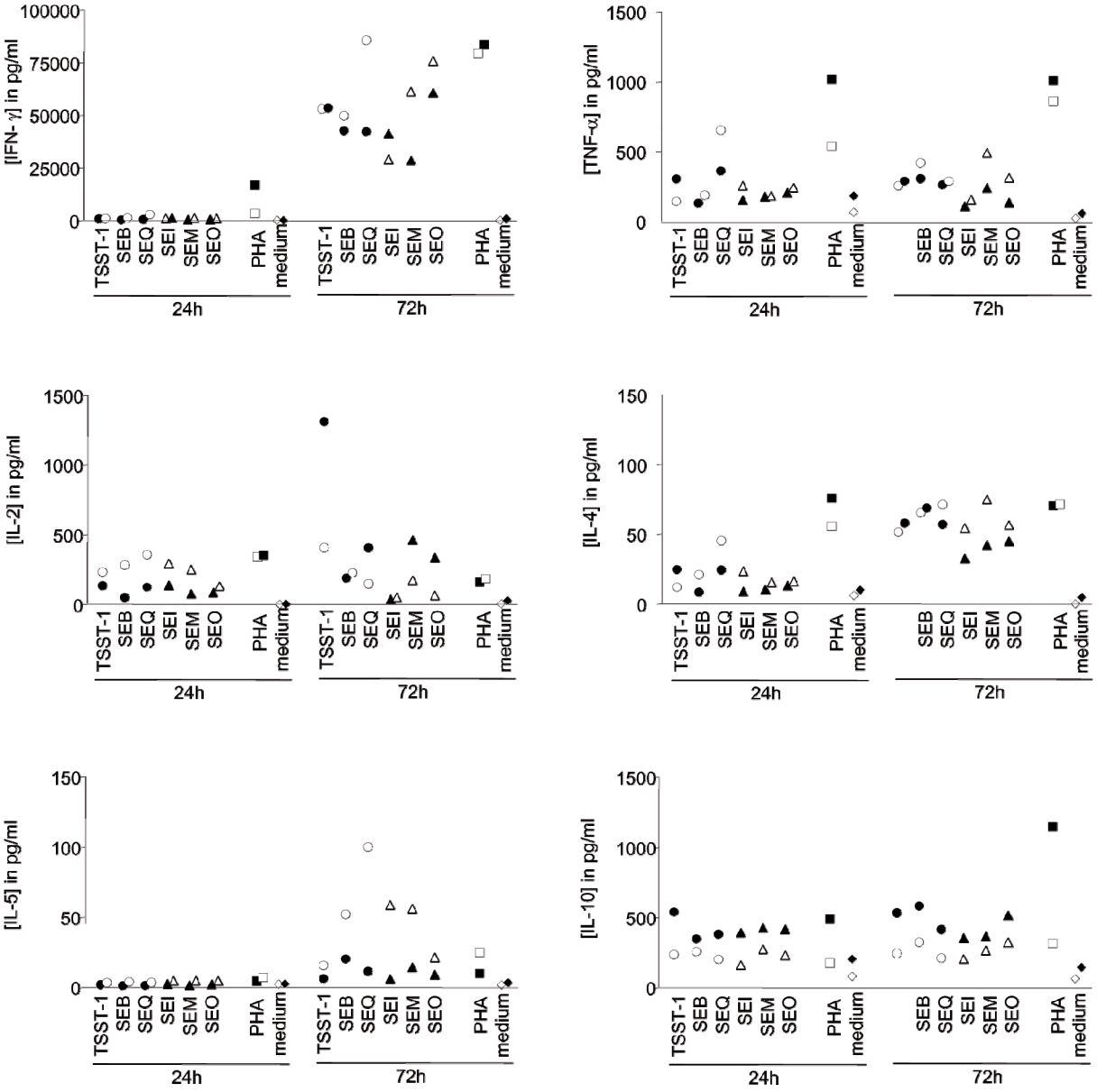

Figure 3: Similar cytokine profiles induced by classical and *egc* SAgs. We compared the release of pro- and anti-inflammatory cytokines by human PBMCs 24 and 72 h after stimulation with recombinant classical (TSST-1, SEB and SEQ, cirlces) and *egc* SAgs (SEI, SEM and SEO; triangles). The T cell mitogen phytohemagglutinin (PHA; squares) and unstimulated cells (medium; diamonds) served as control.

Comparison of the growth phase-dependent secretion of classical and *egc* SAgs.

It was previously reported, that the *egc* operon is transcribed in the logarithmic growth phase, but not in the stationary phase[13]. To assess how this influences SAg secretion in *S. aureus* clinical isolates, we analysed the T cell-mitogenic potency of *S. aureus* culture supernatants in a proliferation assay with human PBMCs. For this purpose, we cultured two strains with classical SAg genes (pSA17: *seb*, *sep*; pSA20: *sec*, *sel*, *sep*) and two strains with *egc* SAg genes only (aSA2, aSA4) in LB medium. Culture samples were obtained at the indicated optical densities.

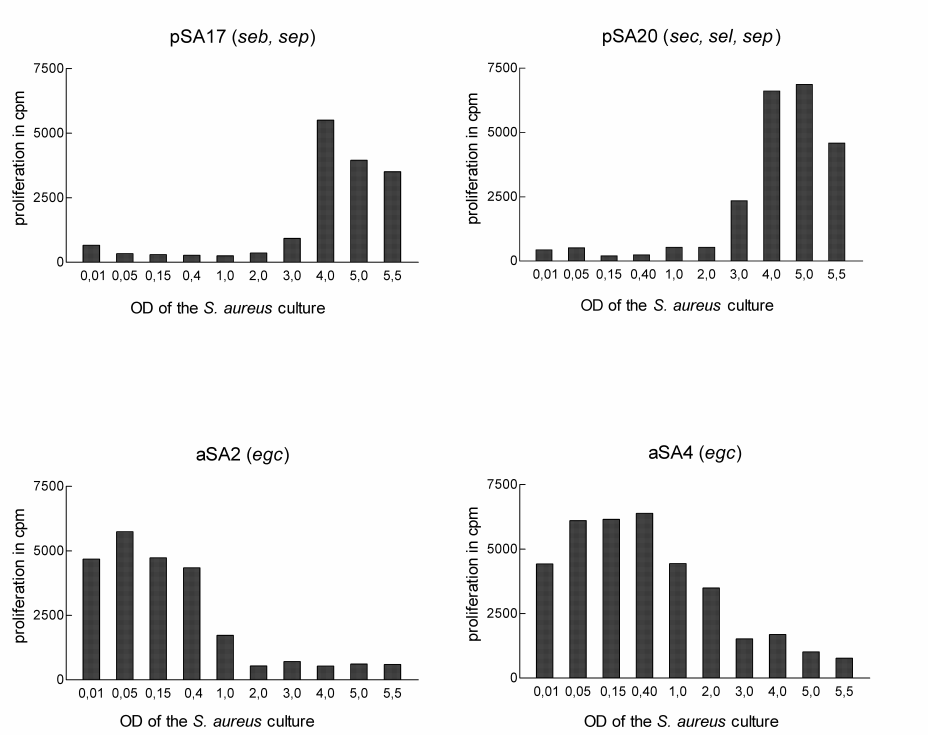

Figure 4: Different growth phase-dependent secretion of classical and *egc* SAgs. Two *S. aureus* strains with classical SAg genes (pSA17, pSA20) and two strains with *egc* SAg genes (aSA2, aSA4) were cultured in LB medium. Culture samples were obtained at the indicated optical densities, filtrated and normalised to OD 0.2. Afterwards, the induction of T cell proliferation in human PBMCs was assessed at a 1:1000 dilution of supernatants.

To correct for the higher cell densities and consequently higher concentrations of secreted products at the late growth phase, all samples were adjusted to OD 0.2 (figure 4). Supernatants of strains pSA17 and pSA20 showed mitogenic activity only at high ODs (> OD 3.0), indicating that these classical SAg proteins are secreted primarily in the late stationary growth phase of *S. aureus*. In contrast, secretion of *egc* SAgs reached a plateau already in the early logarithmic growth phase and quickly declined at higher ODs. Hence, *egc* SAg protein expression corresponded to the reported mRNA regulation[3,13].

DISCUSSION

We and others have previously reported that *egc* SAgs are by far the most prevalent staphylococcal SAgs[12,15]. In spite of this, anti-*egc* serum antibodies are very rare in healthy individuals[12]. To elucidate this phenomenon, we investigated whether this "*egc* gap" in the antibody response is due to i) different T cell-activating properties of classical and *egc* SAgs and/or ii) differential regulation. The comparison of their T cell activating properties revealed no differences between classical and *egc* SAgs. In contrast, we provide evidence that the regulation of *egc* SAg release indeed differs from that of classical SAgs.

To compare the T cell-activating properties of classical (TSST-1, SEB, SEQ) and *egc* SAgs (SEI, SEM, SEI), we performed T cell proliferation assays and analysed the cytokine profile. Proliferation assays revealed that classical and *egc* SAgs had similar T cell-mitogenic potencies. TSST-1, SEB, SEG, SEI and SEM induced half-maximal proliferation at

concentrations between 0.8-18 pg/ml. This very high potency is in agreement with previous studies where mitogenic concentrations for staphylococcal SAgs were determined to be in the pg/ml or even fg/ml range[16-18]. In contrast, the *egc* SAg SEO showed a lower mitogenic potency (~2260 pg/ml). To date, the mitogenic potency of the other *egc* SAgs, SEG, SEN and SEU, has not been determined in a human T cell proliferation assay. Munson et al. reported that 1.84 nM SEG, which corresponds to ~40 ng/ml, induced maximal T cell proliferation in murine splenocytes[13]. However, these data can not be directly compared, because mice are much less susceptible to SAg stimulation than humans.

Besides T cell proliferation, SAg activation induces a massive cytokine release in both T cells and antigen presenting cells. In this study, we compared the capacity of classical and *egc* SAgs to induce cytokine release in human PBMCs. We observed both pro-inflammatory (IFN-γ, TNF-α) and anti-inflammatory (IL-4, IL-5, IL-10) cytokines after SAg stimulation. This is in agreement with data from Rink et al., who also detected a strong Th1 as well as Th2 cytokine induction after stimulation with various staphylococcal and streptococcal SAgs[19]. In our study, IFN-γ was the lead cytokine, reaching concentrations of ~50 ng/ml after 72 h. Moreover, high amounts of TNF-α, IL-2 and IL-10 were detected. Importantly, we observed no striking differences between the cytokine profiles induced by classical or *egc* SAgs. This is in contrast to a recent report from Dauwalder et al., who compared cytokine and chemokine release of human PBMCs after stimulation with 100 ng/ml SEA and SEG[20]. They observed that SEA, but not SEG, induced TNF-α and MIP-1α, a Th1 chemokine. Moreover, in their hands none of the SAgs induced strong anti-inflammatory responses (IL-10, TARC, a Th2 chemokine). This discrepancy with our data may be explained by the use of different read-out systems, different SAg concentrations and different SAg production procedures[21]. Alternatively, the T cell activating properties of SEG could differ from that of the other *egc* members SEI, SEM and SEO, that we have studied.

Using T cell proliferation as read-out for SAg secretion, we demonstrated that strains harbouring *egc* SAg genes secreted those toxins during early logarithmic bacterial growth, while the tested classical SAgs were released mainly in the late stationary phase. This is in agreement with reports that polycistronic *egc* mRNA accumulates maximally during exponential growth[3,13]. In contrast, most classical SAg genes are transcribed in the postexponential growth phase. To date, several global regulators of staphylococcal virulence factors have been characterised, such as the accessory gene regulator (*agr*), the staphylococcal accessory regulator (SarA), the alternative sigma factor (σ^B), and the regulator of toxins (Rot)[22-26]. However, while these regulators have been extensively studied in vitro, their role in *S. aureus* host colonisation and infection remains to be clarified. In fact, recent data suggest that the regulatory circuits *S. aureus* employs during infection (and probably also colonisation) may differ from those characterised in vitro[27,28]. Therefore, while the robust antibody response to classical SAgs in carriers clearly shows that these SAgs are released by the bacteria during colonization[14], it is difficult to estimate at this moment whether *egc* SAg genes (i) are expressed in vivo at all, and, if so, (ii) in which quantities and (iii) under which environmental conditions.

Many studies demonstrated that the *egc* is highly prevalent among *S. aureus* strains in general[3,7,12,15]. Jarraud et al. detected *egc* genes (*seg*, *sei*) in most isolates from various clinical settings, for example colonisation, suppurative infections and toxic shock syndrome[3]. Moreover, a survey of around 200 cases of toxic shock syndrome and staphylococcal scarlet fever demonstrated that *egc* SAgs are able to induce staphylococcal toxaemias, but that these cases are probably rare[29,30]. Van Belkum et al. compared the prevalence of *egc* SAgs in a large collection of commensal and invasive isolates from the Netherlands. *Egc* SAg genes were slightly enriched in strains from healthy carriers (63.7% of commensal isolates vs. 52.9% of invasive isolates, P=0.03)[31]. Similarly, a small-scale analysis of blood culture isolates from patients with sepsis with and without septic shock and patients with suppurative infections suggested that the prevalence of *sea* increased with the severity of infections, whereas the prevalence of the *egc* decreased[32]. The authors concluded that *egc* SAgs have a role in colonisation, so that *egc*-harbouring strains have a slightly lower disease-evoking potential. However, the *egc*-encoding pathogenicity island is likely not mobile and, therefore, strictly linked with the clonal background. Most successful clonal lineages (CC5, 22, 25, 30 and 45) contain the *egc* locus, whereas other clones (CC8, 15) apparently lack the *egc*[31]. Therefore, the contribution of *egc* SAgs in colonisation or invasion can not be separated from the (potential) impact of the genetic background[33].

Therefore, the precise role of *egc* SAgs in host pathogen interaction during staphylococcal colonisation and infection remains to be defined. We have previously reported that *S. aureus* carriers mount an efficient neutralizing antibody response against the classical SAgs of their colonising strain. This antibody response proves that those SAgs are secreted by *S. aureus* during colonisation and/or minor, unnoticed infections. In contrast, carriers of *egc*-positive *S. aureus* strains do not develop high titer anti-*egc* antibodies (Holtfreter, unpublished data). Hence, evidence for the expression of *egc* SAgs during colonization is still lacking.

Furthermore, in vitro data suggest that *egc* SAgs are produced in very small quantities which are often below the detection threshold of an ELISA system (5-10 ng/ml)[13]. With our more sensitive T cell proliferation assay, which has a threshold within the pg/ml range, we could regularly detect the secretion of *egc* SAgs by various clinical isolates (figure 4; unpublished data). Importantly, the growth phase dependency of SAg secretion has to be taken into consideration when comparing the production of *egc* and classical SAgs by clinical isolates. In our experiments, we adjusted the culture samples to the same OD to account for the increasing bacterial density during growth. This approach revealed that equal absolute bacterial cell numbers induce a comparable mitogenic activity, however at different growth phases. In other words, both *egc* and classical SAgs are secreted in comparable amounts on a single bacterial cell level. The low bacterial densities in the logarithmic growth phase explain why total *egc* SAg amounts in culture supernatants are so small, that they may escape detection, unless highly specific methods are applied.

In summary, we have previously reported that although *egc* SAgs are highly prevalent among *S. aureus* isolates, anti-*egc* serum antibodies are rare in healthy individuals. In search for an explanation, here we provide evidence that the protein expression of *egc* SAgs

differs from that of classical SAgs. An assessment of the T cell-mitogenic activity and cytokine induction, however, revealed no differences between classical and *egc* SAgs. Though there is evidence that the *egc* is overrepresented among carriage strains compared to invasive isolates, its possible role in colonization or infection remains to be clarified. It appears unlikely that the *egc* genes, that represent the most prevalent SAg genes in *S. aureus*, should not be expressed in the host at all. Thus, we propose that, due to their different regulation, classical and *egc* SAgs contact the host's immune system under conditions, which either favour or, in the case of *egc* SAgs, prevent efficient antibody production.

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Staphylococcus aureus carriers neutralise superantigens by antibodies specific for their colonising strain: A potential explanation for their improved prognosis in severe sepsis

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ABSTRACT

Staphylococcus aureus is one of the most common causes of hospital-acquired infections. At the same time, 25% of healthy persons are symptom-free *S. aureus* carriers, and they have an increased risk of developing nosocomial *S. aureus* septicemia. Paradoxically, their prognosis is much better than that of noncarriers. We compared the antibody profiles for carriers and noncarriers toward *S. aureus* superantigens. In carriers, we found high titers of neutralising antibodies specific for those superantigens that are expressed by their colonising strain. The results show that carriage status confers strainspecific humoral immunity, which may contribute to protection during *S. aureus* septicemia.

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INTRODUCTION

Staphylococcus aureus is a frequent cause of infection ranging from mild clinical symptoms (e.g., skin infections and food poisoning) to life-threatening conditions (e.g., pneumonia and sepsis). Today, the microorganism is one of the most common causative agents of hospital-acquired infections. At the same time, 20%–30% of healthy persons are colonised with *S. aureus*[1]. When hospitalized, these carriers have an increased risk of developing severe nosocomial *S. aureus* infection and sepsis that is, in ~80% of cases, caused by the colonising strain[2,3]. However, compared with noncarriers who have newly acquired *S. aureus* infections, carriers with *S. aureus* septicemia seem to have a much better outcome. In a prospective trial, Wertheim et al.[3] found mortality due to *S. aureus* bacteremia to be much lower in carriers than in noncarriers (3/40 vs. 13/41; odds ratio, 0.1; P = .013). This indicates that the immune system of *S. aureus* carriers may react to *S. aureus* infections differently than that of noncarriers, which could be due a tuning of the immune response by the colonising *S. aureus* strain[4].

Superantigens (SAgs) can be used to test this hypothesis. First, these secretion products of *S. aureus* are very potent proinflammatory agents, and they have been implicated in the pathogenesis of septic shock[5]. SAgs cross-link major histocompatibility complex class II molecules on antigen-presenting cells with T cell receptors (TCRs) that use certain TCRVβ elements[6]. This leads to very strong T cell proliferation and cytokine secretion, which can result in shock. Moreover, SAgs may induce the upregulation of Toll-like receptor–4 on monocytes and thereby exacerbate their proinflammatory response to endotoxin [7]. Second, the SAg pattern of different *S. aureus* strains varies considerably. To date, 19 SAgs have been described: staphylococcal enterotoxins (SE) A–E , G–R and SEU, as well as toxic shock syndrome toxin (TSST)–1[6]. Approximately 80% of *S. aureus* strains harbor multiple SAg genes on mobile genetic elements, which results in highly heterogeneous SAg expression in clinical isolates[6,8].

Anti-SAg antibodies are highly prevalent in the healthy population, and it has been shown that humans with high anti–TSST-1 antibody titers do not develop toxic shock syndrome when they become infected with a TSST-1–expressing *S. aureus* strain[9]. As a consequence, vaccination strategies have been explored in mice using recombinant SAg toxoids or conserved SAg peptides as antigens[10-12]. The elicited antibody responses conferred protection against toxic shock induced by SAgs, as well as against *S. aureus* sepsis. Some cross-reactivity with heterologous SAgs and some cross-protection has been observed[10,12]. In contrast to these approaches, we used SAgs in the present study as strain-specific indicator antigens to address the question of whether there are differences in the antibody response between *S. aureus* carriers and noncarriers.

MATERIALS AND METHODS

Over the course of at least 3 months, 2 nose swabs and serum samples were obtained from 123 healthy blood donors. All participants gave informed consent, and the study was approved by the ethics board of the University of Greifswald.

S. aureus was identified by morphologic identification of colonies on blood agar plates and a *S. aureus*–specific latex agglutination test (Murex Staphaurex test; Murex Biotech). The SAg gene repertoire of the *S. aureus* isolates was determined by polymerase chain reaction, as described elsewhere[8].

Proliferation assays were performed as described elsewhere[8]. Briefly, peripheral blood mononuclear cells (PBMCs) from healthy blood donors were cultured in 96-well flat-bottom plates at a density of 1 X 10^5 cells/well in the presence of RPMI 1640 supplemented with 10% fetal bovine serum. The cells were stimulated for proliferation by incubation with *S. aureus* supernatants (OD, 3.0) in serial dilutions or by individual SAgs, which were produced by recombinant gene technology (SEA, TSST-1 and SEC3)[13] or purified from bacterial cultures (SEB; Sigma). To test for SAg neutralising serum factors, heat-inactivated human serum was added at a final concentration of 2%. Proliferation was determined by the incorporation of [3H]thymidine, 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 triplicate and in at least 2 independent experiments.

RESULTS AND DISCUSSION

Of 123 healthy blood donors 22 (18%) were twice positive for *S. aureus* (constant carriers), whereas 70 (57%) were twice negative (noncarriers). This is consistent with the results of previous studies[1]. The SAg gene repertoire of the colonising strains was very heterogeneous (table 1). Except for 3 subjects in whom a strain shift occurred, the SAg patterns of the 2 isolates were identical, indicating a clonal relationship, which for 5 samples was confirmed by pulsed-field gel electrophoresis (data not shown).

The prevalence of neutralising antibodies against the complex SAg mixtures, which are found in *S. aureus* supernatants, was analyzed in proliferation assays. We excluded the *S. aureus* isolates harboring no SAg genes, the 3 subjects in whom a strain shift occurred (T9, T166 and T169) and those who only had SAgs encoded by the enterotoxin gene cluster *(egc),* given that we have recently shown that *egc* SAgs only very rarely induce neutralising antibodies[8]. Culture supernatants from the remaining 14 isolates were used to stimulate proliferation in human PBMCs. Taking the AUC as a measurement for inhibition, we compared for each *S. aureus* strain the neutralising capacity of the serum obtained from (1) its carrier, (2) a randomly chosen noncarrier and (3) a carrier whose colonising strain harboured a nonoverlapping spectrum of SAg genes (figure 1A); 13 (93%) of 14 carriers showed a very effective antibody response that almost abolished T cell proliferation induced by the SAgs of their colonising strain (figure 1B). In contrast, when secretion products of *S. aureus* strains expressing different SAgs were assessed, the serum neutralising capacity of *S. aureus* carriers was much lower and did not differ from that of noncarriers (figure 1A and 1*B*). Only approximately one-third of the carriers and noncarriers efficiently neutralised the biological effects of SAgs secreted by unrelated *S. aureus* strains (figure 1B). This likely reflects the individuals' history of immune encounters with different *S. aureus* strains.

Table 1. Superantigen (SAg) gene repertoire of commensal *Staphylococcus aureus* isolates.

S. aureus isolate (nose swab)	SAg genes (SEs or *tst*)	*egc* genes
T9-1	*p*	-
T9-2	*p*	*g i m n o*
T35-1 [b]	-	*g i m n o u*
T35-2	-	*g i m n o u*
T41-1 [a]	*d j r*	-
T41-2	*d j r*	-
T52-1 [a]	*a c l*	*g i m n o*
T52-2	*a c l*	*g i m n o*
T54-1 [a]	*b*	-
T54-2	*b*	-
T55-1 [b]	-	-
T55-2	-	-
T77-1 [a]	*a tst*	*g i m n o u*
T77-2	*a tst*	*g i m n o u*
T85-1 [b]	-	*g i m n o*
T85-2	-	*g i m n o*
T93-1 [b]	-	-
T93-2	-	-
T98-1 [a]	*a*	*g i m n o u*
T98-2	-	*g i m n o u*
T100-1 [a]	*tst*	*g i m n o u*
T100-2	*tst*	*g i m n o u*
T110-1 [a]	*a c k l q tst*	-
T110-2	*a c k l q tst*	-
T118-1 [a]	*d j r*	-
T118-2	*d j r*	-
T135-1 [a]	*a*	*g i m n o u*
T135-2	*a*	*g i m n o u*
T145-1 [a]	*b k q*	-
T145-2	*b k q*	-
T157-1 [a]	*p*	-
T157-2	*p*	-
T161-1 [b]	-	*g i m n o*
T161-2	-	*g i m n o*
T166-1	*k q*	-
T166-2	*c l*	*g i m n o*
T169-1	*b d j k q r*	-
T169-2	-	*g i m n o u*
T179-1 [a]	*c l*	*g i m n o*
T179-2	*c l*	*g i m n o*
T191-1 [a]	*a c l tst*	-
T191-2	*a c l tst*	-
T200-1 [a]	*a h tst*	*g i m n o u*
T200-2	*a h tst*	*g i m n o u*

NOTE. SE, staphylococcal enterotoxin *(a–e, g–r and u); tst,* toxic shock syndrome toxin–1.
[a] These samples were further analyzed in the experiment shown in figure 1*B* and 1*C*.
[b] These samples were further analyzed in the experiment shown in figure 1*C*.

When single recombinant or purified SAgs (SEA, SEB, SEC3 and TSST-1) were tested, serum samples from all carriers very efficiently neutralised those SAgs that were present in their colonising strain (figure 1*C*). Unrelated individual SAgs were generally neutralised more frequently than were the complex SAg mixtures (76%–79%, with 25% proliferation used as a threshold), which are produced by *S. aureus* wild-type strains (figure 1*C*). Neutralisation of the biological SAg effects correlated with antibody binding to purified SAgs as confirmed by ELISA (TSST- 1 and SEB; data not shown). These results are in agreement with those of previous studies, which detected SAg-binding antibodies in 40%–95% of serum samples from healthy donors or patients, irrespective of their carrier status [14-16].

In contrast to our approach, previous studies have mainly focused on the protection conferred by cross-reactive antibodies, which can be induced by vaccination with SAgs or defined conserved SAg peptides[10-12]. In agreement with our data, the antibody titers specific for heterologous SAgs were ~10- fold lower than those directed against the SAgs used for immunization[10]. These differences were strongly correlated with the degree of protection[10].

Our results clearly show that the human immune system reacts specifically against the colonising *S. aureus* strain, leading to almost complete neutralisation of the T cell stimulating effects of its SAgs. Thus, during colonisation, conserved epitopes do not appear to be immunodominant.

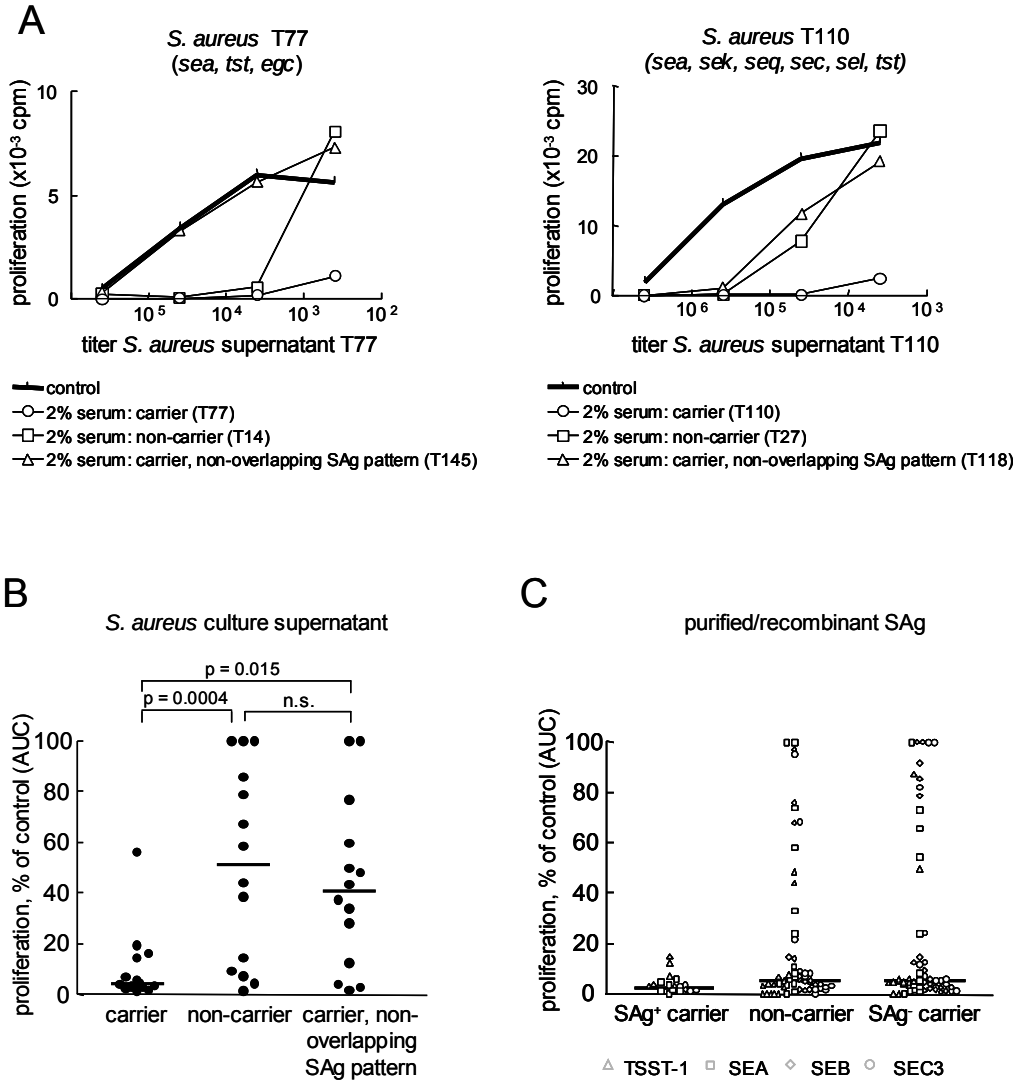

Figure 1. Effective and highly specific antibody responses against superantigens (SAgs) of their colonising strain by *Staphylococcus aureus* carriers. A) Peripheral blood mononuclear cells stimulated with supernatants (OD, 3.0) from colonising *S. aureus* strains in serial dilutions. Serum from (1) the corresponding carrier *(circles),* (2) a randomly chosen noncarrier *(squares) and* (3) a carrier whose strain harbored a nonoverlapping SAg gene spectrum *(triangles)* were analyzed for their neutralising capacity. Two representative data sets out of 14 are shown. **B)** 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 14 data sets. Carriers showed a very effective antibody response that almost abolished the SAg-induced proliferation. Their inhibition of SAgs from unrelated *S. aureus* strains was much less effective and did not differ from noncarriers. *P* values were calculated using the Wilcoxon and Mann-Whitney *U* tests; median values are indicated. **C)** Graph of the results of 4 independent proliferation assays with the recombinant SAgs toxic shock syndrome toxin (TSST)–1 *(triangles),* staphylococcal enterotoxin (SE)–A *(squares),* SEC3 *(diamonds) and* purified SEB *(circles).* We analyzed the neutralising capacity of the same noncarrier serum and all carrier serum samples (as indicated in table 1), except for those in which a strain shift occurred. Again, all carriers showed a very effective antibody response against those SAgs, which are expressed by their colonising strain (SAg^+ carrier). Individual SAgs were neutralised by serum from unrelated carriers (SAg^- carrier) and from noncarriers at a higher frequency than the complex SAg mixtures. NS, not significant.

Because, in rodents, 10-fold differences in SAg neutralising capacity were critical for the outcome of a SAg challenge, our observations could be clinically highly relevant. The specific and effective antibody response in carriers may, in part, explain the paradox observed in a prospective trial. Although *S. aureus* carriers are at higher risk of acquiring *S. aureus* septicemia, in the case of sepsis, their outcome is much better[3]. However, one has to bear in mind that *S. aureus* harbors many more virulence factors besides SAgs. Like SAgs, most of these are encoded on mobile genetic elements, and they are highly variable in clinical isolates. Although we have shown an antibody response against the SAgs of their colonising strain in *S. aureus* carriers that may provide some strain-specific protection, further studies are needed to assess their immune response toward other virulence factors.

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Summary and discussion

S. aureus is a highly versatile pathogen, which is reflected by a whole array of different clinical pictures, ranging from mild skin and wound infections, through toxin-mediated diseases to systemic infections, such as sepsis. Beyond this, *S. aureus* persistently resides in the nasal cavity of ~20% of the population and can be intermittently detected in another 30%. *S. aureus* nasal carriage is a major risk factor for the development of endogenous *S. aureus* infections in various clinical settings, which underlines the importance of host factors[1,2]. However, despite the clinical importance of nasal carriage, our knowledge about adaptive immune responses triggered by colonisation is limited. Especially the potential impact of anti-staphylococcal antibodies on the carriage status as well as the shift from colonisation to infection is only poorly understood. Therefore, this thesis was focussed on the anti-staphylococcal antibody responses of carriers and noncarriers. Staphylococcal SAgs, virulence factors with extraordinary variability in the species *S. aureus,* served as indicator antigens for our studies. First of all, we analysed the prevalence of SAg genes among colonising and invasive isolates and correlated this with their genetic background. Afterwards, we determined the anti-SAg antibody profiles in healthy individuals. Finally, we characterised the antibody responses of *S. aureus* carriers against their colonising strains using SAgs as strain-specific indicator antigens.

Multiplex-PCR analysis reveals a very heterogeneous SAg gene repertoire in *S. aureus* isolates from Pomerania.

Recent genome analyses revealed that staphylococci harbour a broad and highly variable repertoire of virulence genes including 19 different staphylococcal SAgs. However, to date, epidemiological information about the distribution of all 19 SAg gene loci is limited. In order to characterise our *S. aureus* strain collection, we have compared the SAg gene patterns in nasal and blood culture isolates from the region Western Pomerania.

In a first study, we determined the SAg gene patterns of 20 throat isolates and 20 blood culture isolates by conventional PCR (Chapter 5). To accelerate SAg gene analysis, we then established a set of five multiplex PCRs for the detection of all known 19 SAg genes. With this robust and high-throughput technique we characterised more than 300 isolates of different clinical origin, including 107 nasal isolates of *S. aureus* carriers from Western Pomerania, 88 blood culture isolates of hospital patients from Western Pomerania and 109 nasal isolates of *S. aureus* carriers from Szczecin, Poland (Chapter 3).

PCR analyses revealed highly heterogeneous SAg gene patterns in our strain collections, which can be explained by their localisation on mobile genetic elements, namely genomic islands, pathogenicity islands, plasmids and phages. Most isolates (~80%) harboured SAg genes, on average five to six, and *egc* SAgs were by far the most prevalent (~55%). SAgs genes which are co-localised on genomic islands, i.e. *egc* SAgs, pathogenicity islands, i.e. *seb-sek-seq* and *tst-sec-sel*, and plasmids, i.e. *sed-sej-ser*, were usually found in combination (Chapter 2, 3, 5). However, we also observed new SAg gene combinations, which are

probably carried by as yet uncharacterised MGE variants. The heterogeneous SAg gene patterns of nasal isolates make these toxins ideal indicator antigens for studying strain-specific immune responses.

The presence of SAg genes in clinical *S. aureus* isolates predicts their T cell activating properties.

SAgs are extremely potent T cell mitogens. In Chapter 5, we show that it is possible to reliably predict the T cell stimulating properties of a given *S. aureus* isolate by SAg genotyping. In fact, we observed a strict correlation between the presence of SAg genes and the ability to induce T cell proliferation in vitro. Therefore, a genetic analysis of the SAg gene repertoire of a clinical *S. aureus* isolate will allow the prediction of its T cell mitogenic potential. However, the presence of a SAg gene does not necessarily imply that the protein is expressed at mitogenic levels in the host.

Staphylococcal SAg-like (SSL) proteins are no SAgs.

In addition to staphylococcal SAgs, *S. aureus* encodes a large cluster of up to 11 genes with sequence homology to SAgs. They were originally termed staphylococcal exotoxin like genes, or *set*, but were recently renamed staphylococcal SAg-like genes, *ssl* (Chapter 2). The genomic island vSAα (previously termed SaPI2), which encodes the *ssl* cluster, is very stable and likely not transferred horizontally. We and others found an *ssl* cluster in all strains examined to date (Chapter 5)[3]. In contrast to enterotoxins, the presence of *ssl* genes in clinical *S. aureus* isolates (n=40) did not correlate with their T cell mitogenic activity (Chapter 5). Concordantly, recombinant SSL11 had no superantigenic properties (S. Holtfreter, unpublished data). Recent data from other researchers suggest other important functions for SSL proteins in host-pathogen interaction. For example, SSL7 binds the Fc part of IgA, thereby blocking IgA-FcR interactions, and also inhibits complement factor C5[4]. Other SSL proteins target endothelial cells and neutrophils[5,6], and future studies will probably reveal further immunoevasive properties of SSLs.

***Spa* genotyping of nasal and blood culture isolates reveals 10 dominant lineages.**

Genotyping analyses of large *S. aureus* strain collections have recently shown that the *S. aureus* population structure is highly clonal[7,8]. In these studies, nasal and invasive strains fell into the same lineages, demonstrating that strains from all lineages can have a disease-evoking potential. To resolve the population structure of our nasal and blood culture isolates from Western Pomerania, we performed *spa*-genotyping (Chapter 3). *Spa*-genotyping revealed 93 different *spa*-types that clustered into 5 major (CC8, 15, 25, 30, 45) and 5 minor lineages (CC5, 12, 22, 121, 395). The same dominant lineages were observed also in other studies[7,8], except for CC395, suggesting that these CCs are successful lineages that have spread worldwide.

Interestingly, the clonal complex CC8 was overrepresented among blood culture isolates, whereas CC30 was underrepresented. In contrast to our data, CC30 isolates from the

Netherlands tend to be more prevalent in endogenous invasive strains than in non-invasive strains[9]. This suggests, that staphylococcal virulence depends less on the genetic background than on virulence and resistance genes encoded on mobile genetic elements. The differences between the Dutch and Pomeranian CC30 population will be in focus of our future research, which will, hopefully, reveal MGEs that predispose to invasiveness.

SAg-encoding MGEs are not randomly distributed between different clonal lineages, but rather strongly linked to the underlying clonality.

S. aureus MGEs can be distributed by two distinct mechanisms: horizontal transfer by bacteriophages and vertical transmission to daughter cells. Since all SAgs are located on MGEs, we examined whether their distribution was random or rather linked to the *spa*-defined lineages. Interestingly, SAg genes or SAg gene combinations were closely linked to the clonal background (Chapter 3). Each lineage was characterised by defined combinations of *agr* group and SAg genes. However, within a clonal complex and even within the same *spa* type we observed considerable variation, suggesting that SAg gene-carrying MGEs were frequently acquired and lost. Moreover, we found remarkable differences in the horizontal mobility between different MGE types, e.g. genomic islands, plasmids, pathogenicity islands and phages.

In the recent years, huge efforts have been made to determine bacterial factors that are important for staphylococcal virulence, both in the core and the variable genome. Despite the impressive variation seen in the species *S. aureus* and the considerable genetic exchange between *S. aureus* clones[10,11], it is not exactly understood if and how this variation might influence pathogenesis. Here, we demonstrate that SAg-encoding MGEs are linked to clonal lineages. Therefore, their contribution to invasiveness could be masked or shammed by the underlying clonality. Consequently, the power of a comparison of virulence genes between invasive and non-invasive strains will be strongly increased, if the underlying clonality is taken into account. In our study, this approach indicated a role in invasiveness for some MGEs, e.g. the exfoliative toxin D-encoding pathogenicity island, while rendering it unlikely for SAg genes (Chapter 3). To conclude, our data suggest that the simultaneous assessment of virulence gene profiles and the genetic background will increase the discriminatory power of genetic investigations into the mechanisms of *S. aureus* virulence.

Moreover, there is growing evidence that the key to understanding the pathogenesis of *S. aureus* disease may lie in the identification of host factors, e.g. immune status and gene polymorphisms[12-14], that contribute to colonisation, susceptibility to infection and outcome of infection. Especially the finding that *S. aureus* related mortality is significantly lower in *S. aureus* carriers than in noncarriers, implies an important role of the carriers' adaptive immune response[15].

Soluble products of clinical *S. aureus* isolates induce highly diverse blood cell activation patterns.

Disseminated intravascular coagulopathy is a common feature of systemic *S. aureus* infections, even when bacteraemia is not evident. This suggests that soluble *S. aureus* factors may be involved in alterations of the coagulation system. Therefore, we investigated whether clinical *S. aureus* isolates release soluble, procoagulatory factors (Chapter 4). Culture supernatants from 40 clinical *S. aureus* isolates, including 20 throat isolates from healthy persons and 20 blood culture isolates, induced highly diverse blood cell activation patterns, including T cell proliferation, expression of procoagulatory tissue factor on monocytes, platelet aggregation and red blood cell lysis. Apparently, the abilities to activate all these different blood cell types were independent features, reflecting the large number of virulence factors as well as the diversity of the species *S. aureus*. The elucidation of the underlying mechanisms revealed that i) as expected, T cell proliferation was strictly correlated with the presence of SAg genes, ii) tissue factor expression on monocytes was probably triggered by peptidoglycans and iii) platelet activation and red blood cell lysis were likely caused by α-haemolysin, though at different concentrations.

Importantly, most *S. aureus* isolates produced soluble factors, which induced a procoagulatory surface on platelets and/or monocytes. This may explain the high incidence of disseminated intravascular coagulopathy in *S. aureus* sepsis in the absence of bacteraemia. Moreover, this study once more underlines the diversity of clinical *S. aureus* isolates, which, apart from host factors, may explain the broad spectrum of clinical symptoms and the variability of the outcome of encounters between *S. aureus* and its human host.

***Egc* SAgs are neutralised by human sera much less efficiently than classical SAgs.**

At the beginning of the work described in this thesis, it was known that neutralising serum antibodies against SEA, SEB, SEC, SED and TSST-1 are frequently present in healthy individuals. However, the antibody profiles against the other, more recently described SAgs had not been determined so far. Moreover, as described above, the majority of *S. aureus* strains harbour multiple SAg genes. Therefore, we also wanted to determine whether the complex mixtures of SAgs secreted by clinical *S. aureus* isolates can be neutralised with similar frequency and efficiency as single SAgs (Chapter 5).

We screened 123 serum samples from healthy blood donors for their capacity to neutralise the T cell mitogenic factors present in 12 different *S. aureus* supernatants. We observed a marked heterogeneity and surprisingly large “holes” in the neutralising capacity. None of the tested sera neutralised all analysed *S. aureus* supernatants. Interestingly, the secretion products of *egc*-encoding strains as well as recombinant SEI were inhibited only rarely (5-10%), whereas between 32 and 86% of the tested sera neutralised classical SAgs. Similarly, iv IgG preparations inhibited *egc* SAgs with 10-100 fold reduced efficiency compared to classical SAgs.

This "*egc* gap" in the anti-SAg antibody profile is probably not due to different T cell activating properties, but rather to a differential regulation of SAg gene expression.

This "*egc* gap" in the SAg-neutralising antibody profiles of healthy individuals was surprising, since *egc* SAgs are by far the most prevalent SAgs in clinical *S. aureus* isolates (Chapter 3, 5). We hypothesised that the *egc* gap could be due to i) differential T cell activating properties and/or ii) differential regulation of SAg gene expression (Chapter 6). A comparison of T cell proliferation and cytokine profiles after stimulation with three recombinant *egc* SAgs and three classical SAgs revealed no major differences. Using proliferation assays as read-out system for SAg secretion, we demonstrated that strains harbouring *egc* SAg secrete those toxins mainly in the early logarithmic growth phase, while classical SAgs were secreted mainly in the stationary phase. These results complement earlier findings on an early transcription of *egc* genes[16]. Therefore, the differential regulation of *egc* and classical SAgs seems the most plausible explanation for the "*egc* gap". We suggest, that due to their differential regulation, classical and *egc* SAgs come into contact with the host immune system under very different conditions, which could favour or, in the case of *egc* SAgs, prevent efficient antibody production.

Egc-SAgs are highly prevalent among *S. aureus* strains of various clinical origins, but it has been reported in a recent study that *egc* SAgs are slightly enriched among commensal compared to invasive strains (Chapter 3, 6)[17-19]. The authors concluded that *egc* SAgs have a role in colonisation, so that *egc*-harbouring strains have a slightly lower disease evoking potential. However, most successful clonal lineages (CC5, 22, 25, 30 and 45) uniformly contain the *egc* locus, whereas other lineages always lack the *egc* (Chapter 3)[17]. Because of this strict linkage, the role of *egc* SAgs in colonisation or invasion can not be separated from the potential impact of the clonal background by genetic analyses. Therefore, the exact biological function, if any, of *egc* SAgs during colonisation and subsequent infection remains to be clarified.

***S. aureus* carriers generate a strong and highly specific antibody response against the SAgs of their colonising strain.**

S. aureus carriers have an increased risk of developing an *S. aureus* bacteraemia, which is mostly caused by the colonising strain[2]. Intriguingly, there is a considerably higher mortality in noncarriers with invasive *S. aureus* strains, compared to carriers with invasive disease[15]. MLST analyses of exogenous and endogenous strains revealed no single clonal complex that could explain the higher mortality in this patient group[9]. To explain these paradoxical findings, we hypothesise that partial immunity against the colonising strain may contribute to the improved outcome of carriers (Chapter 7).

SAgs are suitable to test this hypothesis for three reasons. Firstly, SAgs are clinically important virulence factors. They cause the toxic shock syndrome, and are probably also involved in the pathogenesis of staphylococcal sepsis (Chapter 2). Secondly, the SAg gene repertoire of clinical *S. aureus* isolates is highly variable, due to their localisation on mobile genetic elements (Chapter 3, 5). This enabled us to study strain-specific immune responses

in *S. aureus* carriers and noncarriers. Finally, by exploiting the T cell-mitogenic activity of SAgs, we could easily assess neutralising antibody specificities of different sera in a neutralisation assay.

Sera from persistent carriers neutralised SAgs of their colonising strain with significantly higher efficiency than sera from noncarriers (Chapter 7). This is an indirect evidence for the production of SAgs during colonisation and/or minor unnoticed infections. Importantly, this antibody response was highly strain-specific, since the antibody response of carriers against other SAgs did not differ from that of noncarriers. In contrast, carriers of *egc*-positive strains did not mount an efficient antibody response, which was expected based on our previously observed "*egc* gap".

In conclusion, colonisation with *S. aureus* confers a strong and strain-specific antibody response against staphylococcal SAgs. We suggest that in carriers neutralising antibodies directed against SAgs and other staphylococcal virulence factors could confer partial protection during systemic infections, and, therefore, explain the improved outcome of carriers with *S. aureus* bacteraemia. In support, mice were protected against a systemic *S. aureus* infection after intranasal vaccination with an SEC toxoid[20]. Furthermore, the high specificity of the antibody response has to be considered for the development of vaccine strategies.

Nevertheless, one has to bear in mind that, besides SAgs, *S. aureus* harbours many more virulence factors with variable prevalence in clinical isolates. Several vaccination studies demonstrated that anti-staphylococcal antibodies can contribute to protection against staphylococcal disease[21-23]. Therefore, it is reasonable to assume that susceptibility to disease correlates with the levels of anti-staphylococcal antibodies. While infected patients would be expected to display certain antibody "gaps" in the acute phase of disease, healthy individuals may possess protective antibodies acquired during previous encounters with *S. aureus* strains.

These hypotheses need to be tested in comprehensive studies including persistent and intermittent carriers and noncarriers as well as patients with different staphylococcal diseases. The most appropriate method for an elucidation of antibody responses towards the multitude of staphylococcal virulence factors are broad-spectrum and high throughput screening techniques, such as protein arrays and two-dimensional western blots. Such investigations will hopefully lead to the identification of antibody specificities that confer protection from colonisation, disease or lethal outcome. This knowledge is a prerequisite for the development of effective preventive and therapeutic vaccination strategies. Treatment of *S. aureus* bacteraemia with pooled immunoglobulins was performed in the past without significant success[24]. Our finding of strain-specific antibody profiles could provide an explanation for this. They further suggest that customised cocktails of monoclonal antibodies could improve the therapeutic efficacy of passive anti-*S. aureus* vaccination.

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SUMMARY

To date, *Staphylococcus aureus* is the most common cause of nosocomial infections and the species is becoming increasingly resistant to antibiotics. Beyond this, *S. aureus* colonises the nasal mucosa of circa 35% of the healthy population, so-called carriers. Importantly, *S. aureus* nasal carriage is a major risk factor for the development of *S. aureus* infections, which are commonly caused by the colonising strain. This underlines the importance of host factors for the outcome of *S. aureus*-host interactions. Despite the clinical importance of nasal carriage, little is known about humoral immune responses triggered by colonisation. Therefore, this thesis was focussed on the anti-staphylococcal antibody responses of *S. aureus* carriers and noncarriers. Staphylococcal superantigens (SAgs) served as indicator antigens for our studies. SAgs are virulence factors with extraordinary variability in the species *S aureus* and act as extremely potent T cell mitogens.

To date, 19 different SAg gene loci are known in the species *S. aureus*, but molecular-epidemiological studies on the distribution of these genes are limited. Therefore, we established five multiplex PCRs for the detection of all known SAgs. With this robust and high-throughput technique we analysed the SAg gene patterns of more than 300 isolates, including 107 nasal isolates of *S. aureus* carriers and 88 blood culture isolates of hospital patients from Western Pomerania. The SAg gene patterns were highly heterogeneous, which can be explained by their localisation on mobile genetic elements (MGE), such as genomic islands, pathogenicity islands, phages and plasmids. Most isolates (~80%) harboured SAg genes, on average five to six, and SAgs of the enterotoxin gene cluster (*egc*) were by far the most prevalent. Additionally, we observed a strict correlation between the presence of SAg genes and the T cell mitogenic potency of clinical isolates.

SAg-encoding MGEs can be distributed by two distinct mechanisms: horizontal transfer by bacteriophages and vertical transmission to daughter cells. To investigate the distribution of SAg genes within the *S. aureus* population, we determined the clonal relationship of our isolates by *spa* genotyping. Interestingly, SAg-gene encoding MGEs were not randomly distributed, but rather closely linked to clonal lineages. Each clonal lineage was characterised by defined combinations of SAg genes. However, within a clonal complex and even within the same *spa* type we observed considerable variation, suggesting that within lineages SAg gene-encoding MGEs were frequently acquired and lost. These data suggest that the simultaneous assessment of virulence gene profiles and the genetic background strongly enhances the discriminatory power of genetic investigations into the mechanisms of *S. aureus* virulence. Indeed, the comparison of virulence genes within each clonal complex indicated a role in invasiveness for some MGEs, e.g. the exfoliative toxin D-encoding pathogenicity island, while rendering it unlikely for SAgs.

It is known that neutralising serum antibodies against the SAgs SEA, SEB, SEC, SED and TSST-1 are frequently present in healthy individuals. However, the neutralising antibody profiles against more recently described SAgs or complex SAg cocktails as secreted by clinical isolates had not been determined so far. Therefore, we screened more than 100 sera for

their SAg neutralising capacity with a neutralisation assay. We observed a marked heterogeneity and surprisingly large “gaps” in the neutralising capacity. Interestingly, the *egc* SAgs were inhibited only rarely (5-10%), whereas between 32 and 86% of the tested sera neutralised “classical” SAgs.

This “*egc* gap” in the SAg-neutralising antibody profiles of healthy individuals was unexpected, since *egc* SAgs are by far the most prevalent SAgs. We could demonstrate that the “*egc* gap” is probably not due to different T cell activating properties of *egc* SAgs compared to classical SAgs, but rather to a differential regulation of SAg gene expression.

S. aureus carriers have an increased risk of developing an *S. aureus* bacteraemia, which is in most cases caused by the colonising strain. Intriguingly, a large prospective clinical trial revealed a considerably higher mortality in noncarriers with invasive *S. aureus* strains compared to carriers with invasive disease. To explain these paradoxical findings, we hypothesised that in carriers partial immunity against the colonising strain may contribute to their improved outcome. SAgs are suitable to test this hypothesis for three reasons. Firstly, SAgs are clinically important virulence factors. They cause the toxic shock syndrome and are probably also involved in the pathogenesis of staphylococcal sepsis. Secondly, the described highly variable SAg gene repertoire of clinical *S. aureus* isolates enabled us to study strain-specific antibody responses in *S. aureus* carriers and noncarriers. Finally, by exploiting the T cell-mitogenic activity of SAgs, neutralising antibody specificities of different sera were easily assessed in a neutralisation assay.

Importantly, sera from persistent carriers neutralised SAgs of their colonising strain with significantly higher efficiency than sera from noncarriers. This antibody response was strain-specific, since the antibody response of carriers against other SAgs did not differ from that of noncarriers. Thus, colonisation with *S. aureus* confers a strong and strain-specific antibody response against staphylococcal SAgs.

We suggest that in carriers neutralising antibodies directed against SAgs and other staphylococcal virulence factors confer partial protection during systemic infections. This could explain the better prognosis of carriers with *S. aureus* bacteraemia compared to noncarriers. Moreover, our data imply that the key to understanding the pathogenesis of *S. aureus* disease may lie in the identification of host factors rather than bacterial factors. Such host factors could be the immune status and gene polymorphisms that contribute to colonisation, susceptibility to infection and outcome of infection. Finally, while the treatment of *S. aureus* bacteraemia with pooled immunoglobulins was performed in the past without significant success, our findings on strain-specific antibody profiles suggest that therapies with customised cocktails of monoclonal antibodies could have a higher efficacy.

ZUSAMMENFASSUNG

Staphylococcus aureus ist der häufigste Erreger nosokomialer Infektionen, und die zunehmende Antibiotikaresistenz dieser Spezies ist besorgniserregend. Zugleich besiedelt *S. aureus* die Nasenschleimhaut bei circa 35% der gesunden Bevölkerung, so genannten Carriern. Die nasale Besiedlung mit *S. aureus* ist ein wichtiger Risikofaktor für die Entstehung von *S. aureus* Infektionen, die zumeist durch den kolonisierenden Stamm verursacht werden. Dies unterstreicht die Bedeutung von Wirtsfaktoren für den Ausgang von *S. aureus*-Wirtsinteraktionen. Trotz der klinischen Bedeutung der nasalen Besiedlung ist über die humorale Immunantwort des Wirtes auf eine solche Besiedlung mit Staphylokokken wenig bekannt. Darum stand die anti-*S. aureus* Antikörperantwort von Carriern und Nichtcarriern im Mittelpunkt dieser Arbeit. Staphylokokken-Superantigene (SAg) dienten in unseren Studien als Indikatorantigene. SAg sind Virulenzfaktoren mit außergewöhnlicher Variabilität in der Spezies *S. aureus* und wirken als hoch potente T-Zellmitogene.

Es sind inzwischen 19 verschiedene SAg-Genloci in der Spezies *S. aureus* bekannt, aber es gibt bisher nur wenige umfassende Untersuchungen zu ihrer Verteilung. Deshalb haben wir zunächst fünf Multiplex-PCRs zum Nachweis aller 19 SAg-Gene etabliert. Mit dieser robusten Hochdurchsatzmethode haben wir das SAg-Genmuster von mehr als 300 Isolaten untersucht, darunter 107 nasale Isolate von *S. aureus* Carriern und 88 Blutkulturisolate von Krankenhauspatienten aus Vorpommern. Die SAg-Genmuster waren sehr heterogen, was sich durch die Lokalisation der SAg-Gene auf mobilen genetischen Elementen (MGE), wie genomischen Inseln, Pathogenitätsinseln, Phagen und Plasmiden, erklären lässt. Die meisten Isolate (80%) besaßen SAg-Gene, im Durchschnitt fünf bis sechs, und die SAg des Enterotoxin-Genclusters (*egc*) waren bei weitem die häufigsten. Außerdem beobachteten wir eine strikte Korrelation zwischen dem Vorhandensein von SAg-Genen und der T-Zellmitogenen Potenz von klinischen Isolaten.

SAg-kodierende MGEs können durch zwei verschiedene Mechanismen verbreitet werden: horizontaler Transfer durch Bakteriophagen und vertikale Übertragung auf Tochterzellen. Um die Verteilung der SAg innerhalb unserer *S. aureus*-Population zu untersuchen, wurde die klonale Verwandtschaft der *S. aureus*-Isolate mittels *spa*-Genotypisierung bestimmt. Interessanterweise waren die SAg-kodierenden MGEs nicht zufällig verteilt, sondern eng an die klonalen Linien gekoppelt. Jede dieser klonalen Linien war durch eine bestimmte Kombination von SAg-Genen charakterisiert. Allerdings beobachteten wir eine beachtliche Variabilität innerhalb der klonalen Komplexe und sogar innerhalb einzelner *spa*-Typen. Dies lässt vermuten, dass MGEs innerhalb von klonalen Komplexen sehr mobil sind, d.h. häufig erworben werden oder verloren gehen. Diese Befunde implizieren, dass die simultane Bestimmung von Virulenzgen-Profilen und dem genetischen Hintergrund die Trennschärfe von genetischen Untersuchungen von *S. aureus* Virulenzmechanismen erheblich erhöht. Durch den Vergleich der Virulenzgene von kommensalen und invasiven Isolaten mit dem gleichen genetischen Hintergrund ließ sich für das exfoliative Toxin D (bzw. die kodierende Pathogenitätsinsel) eine Bedeutung für die Invasivität von *S. aureus* aufzeigen, für SAg dagegen mit großer Wahrscheinlichkeit ausschließen.

Es ist bekannt, dass neutralisierende Serumantikörper gegen die SAg SEA, SEB, SEC, SED und TSST-1 in gesunden Individuen häufig vorkommen. Die neutralisierenden Antikörper

gegen die erst kürzlich beschriebenen SAg oder gegen komplexe SAg-Cocktails, wie sie von klinischen Isolaten sezerniert werden, wurden bisher jedoch noch nicht bestimmt. Darum haben wir über 100 Seren in Neutralisationsassays auf ihre SAg-neutralisierende Kapazität hin untersucht. Wir beobachteten eine erhebliche Heterogenität und erstaunlich große „Lücken" in der anti-SAg-Antikörperantwort. Besonders *egc*-SAg wurden nur selten inhibiert (5-10%), während zwischen 32 und 86% der getesteten Seren „klassische" SAg neutralisierten.

Diese "*egc*-Lücke" in den SAg-neutralisierenden Antikörperprofilen gesunder Individuen war unerwartet, weil die *egc*-SAg mit Abstand am häufigsten vorkommen. Wir konnten zeigen, dass die „*egc*-Lücke" wahrscheinlich nicht auf unterschiedliche T-Zellaktivierung durch *egc*-SAg im Vergleich zu klassischen SAg zurückzuführen ist, sondern auf eine unterschiedliche Regulation der SAg-Genexpression.

S. aureus Carrier haben ein erhöhtes Risiko eine *S. aureus* Bakteriämie zu entwickeln, welche in den meisten Fällen durch den kolonisierenden Stamm verursacht wird. Erstaunlicherweise zeigte eine große prospektive klinische Studie, dass eine Bakteriämie bei Carriern eine deutlich geringere Letalität hat als bei Nichtcarriern. Um dieses Paradoxon zu erklären, haben wir die Hypothese aufgestellt, dass bei Carriern eine partielle Immunität gegen den kolonisierenden Stamm zu ihren verbesserten Überlebensraten beitragen könnte. SAg sind aus drei Gründen geeignet, um diese Hypothese zu testen. Erstens sind SAg klinisch bedeutsame Virulenzfaktoren. Sie verursachen das Toxische Schocksyndrom und sind wahrscheinlich auch an der Pathogenese der Staphylokokken-Sepsis beteiligt. Zweitens ermöglicht die beschriebene hohe Variabilität der SAg-Genausstattung von klinischen *S. aureus*-Isolaten eine stammspezifische Analyse der Antikörperantworten in Carriern und Nichtcarriern. Drittens lassen sich - durch Ausnutzen der T-Zellmitogenen Aktivität der SAg - neutralisierende Antikörperspezifitäten der verschiedenen Seren leicht in einem Neutralisationsassay bestimmen.

Seren von persistierenden Carriern neutralisierten SAg ihres eigenen kolonisierenden Stammes mit signifikant höherer Effizienz als Seren von Nichtcarriern. Diese Antikörperantwort der Carrier war stammspezifisch, da sich die Antikörperantwort der Carrier gegen andere SAg nicht von der der Nichtcarrier unterschied. Somit löst die Besiedlung mit *S. aureus* eine starke, stammspezifische Antikörperantwort gegen Staphylokokken-SAg aus.

Wir vermuten, dass bei Carriern neutralisierende Antikörper, die gegen SAg oder andere Staphylokokken-Virulenzfaktoren gerichtet sind, einen partiellen Schutz bei systemischen Infektionen gewähren. Dies könnte die bessere Prognose von Carriern mit einer *S. aureus*-Bakteriämie erklären. Außerdem implizieren unsere Ergebnisse, dass der Schlüssel zum Verständnis der Pathogenese von *S. aureus* Erkrankungen eher in der Identifizierung von Wirtsfaktoren als von bakteriellen Faktoren liegt. Solche Wirtsfaktoren können beispielsweise der Immunstatus oder Genpolymorphismen sein, die zu Kolonisierung, der Infektionsanfälligkeit oder dem Ausgang einer Erkrankung beitragen. Die Behandlung von *S. aureus* Bakteriämien mit gepoolten Immunglobulinen erbrachte keinen deutlichen Erfolg. Unsere Ergebnisse deuten darauf hin, dass „maßgeschneiderte" Cocktails aus monoklonalen Antikörpern gegen verschiedene *S. aureus* Virulenzfaktoren therapeutisch wirksamer sein könnten.

NOMENCLATURE OF SAG-ENCODING MGES

Table 1: Comparison of the old and new nomenclature of SAg-encoding pathogenicity islands and genomic islands. The old nomenclature was used in Chapter 2 and 5, while the new nomenclature was applied in Chapter 3 and 6. Lindsay et al. suggested the new nomenclature for SAg-encoding MGEs in 2004 (Lindsay, J. A. and M. T. Holden, Trends Microbiol 2004).

old nomenclature	new nomenclature	encoded genes	Reference strain
SaPI1-II	SaPI1	*seb, sek, seq*	Col
SaPI1-I	SaPI2	*sel, sec3, tst*	N315
-	SaPI3	*sec, sel*	MW2
SaPI3	vSAβ	*egc* cluster	N315, Mu50
SaPI2	vSAα	old: *set* cluster new: *ssl* cluster	N315

ABBREVIATIONS

agr	accessory gene regulator
CA-MRSA	community-acquired MRSA
CC	clonal cluster
CHIPS	chemotaxis inhibitory protein of *S. aureus*
CI	confidence interval
Eap	Extracellular adherence protein
Efb	extracellular fibrinogen binding molecule
egc	enterotoxin gene cluster
ELISA	enzyme-linked immunosorbent assay
eta, etd	exfoliative toxin A, D genes
Fc	fragment crystalisable, carboxy-terminal antibody region
FcR	Fc receptor
HA-MRSA	hospital-acquired MRSA
HLA	human lymphocyte antigen (see: MHC)
hla, hlb, hlc, hld	alpha, beta, gamma, delta hemolysin gene
IFN	interferon
Ig	immunoglobulin
IL	interleukine
IVIG	intravenous immunoglobulin
LBP	LPS-binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
lukS-lukF	leucocidine genes S and F, encoding the PVL
mecA	methicillin resistance gene
MGE	mobile genetic element
MHC	major histocompatibility complex
MLST	multilocus sequence typing
MRSA	methicillin resistent *S. aureus*
MSSA	methicillin sensitive *S. aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
NO	nitric oxide
OD	optical density
OR	odds ratio
PAI	pathogenicity island
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis

PHA	phytohemagglutinin
PVL	Panton Valentine leucocidine
RBC	red blood cell
sarA	staphylococcal accessory regulator
S. aureus	*Staphylococcus aureus*
SAg	superantigen
SAK	staphylokinase
SaPI	*S. aureus* pathogenicity island
SAS	*S. aureus* culture supernatants
SCC*mec*	staphylococcal chromosomal cassette encoding the *mecA* gene
SCIN	staphylococcal complement inhibitor
SE	staphylococcal enterotoxin
SET	staphylococcal exotoxin-like protein, new name: SSL
spa	*S. aureus* protein A
SSL	Staphylococcal superantigen-like protein, former name: SET
SSSS	staphylococcal scalded skin syndrome
TCR	T cell receptor
TF	tissue factor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin 1
σ^B	alternative sigma factor
vSA	*S. aureus* genomic island

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Poster Awards

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Ferner erkläre ich, daß ich diese Arbeit selbständig verfaßt und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

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