In vivo and in vitro profiling of global interactions between Staphylococcus aureus and its human host

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Cover
Confocal laser scanning microscopy snapshot of internalized *S. aureus* HG001 by THP-1 human macrophages. GFP-expressing staphylococcal cells are colored green, and actin filaments are colored red. Microscopy image obtained by J.F. da Silva Domingues from the Department of Biomedical Engineering at the University of Groningen.

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Introduction and scope of this thesis

Staphylococcus aureus – harmless commensal or dangerous pathogen?

The ancestors of today's bacteria were single-cell microorganism that were the first forms of life to appear on earth about four billion years ago. Since then, these microorganisms have continuously adapted to very different ecological niches. As a result, bacteria are found in every environment on earth, from cold arctic soils to the warm waters of hot springs and sub-ocean hydrothermal vents. In addition, some bacteria adapted their lifestyles to colonize different hosts, including human beings. Thus, many different bacteria can be found within and on the human body. In most cases, both humans and microbes benefit from this co-evolution as exemplified by a plethora of bacteria in the human gut. However, some apparently commensal bacteria occasionally invade the host, which can lead to life- threatening diseases. One of these opportunistic bacteria is *Staphylococcus aureus* (Figure 1).

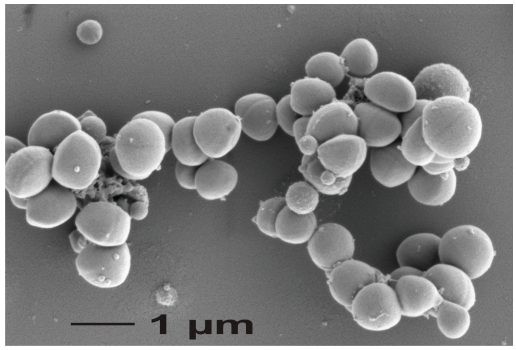

Figure 1. Scanning electron microscopy image of *S. aureus* RN6390

S. aureus is a Gram-positive bacterium frequently found in the nasal cavity of humans and several animal species (120). In humans, the anterior nares are the most frequent carriage site for *S. aureus*. Other known carriage sites include the skin, perineum, pharynx as well as the gastrointestinal tract, vagina and axillae (59, 78). The colonization rate in the healthy human population is established at about 30% (50, 120). However, if the primary barrier function of the skin is disrupted, or if the immune system is compromised, *S. aureus* can become a dangerous pathogen that has the potential to invade almost all tissues and organs causing a broad range of diseases (66). These can vary from mild skin infections, such as impetigo, to life-threatening systemic infections (*e.g*. pneumonia, meningitis, osteomyelitis, and sepsis) (1, 19, 32, 125). Not only the diseases that this pathogen can cause are alarming, but also its high propensity to acquire resistance to antibiotics (65). In the pre-antibiotic era, the mortality of patients with *S. aureus* bacteremia exceeded 80%, and over 70% developed metastatic infections (105). The prognosis of patients with *S. aureus* infections improved

drastically since the introduction of penicillin in the early 1940s. However, already a few years later the first *S. aureus* strains resistant against penicillin emerged in hospitals (90). In the 1960s, a new semi-synthetic antibiotic - methicillin - was introduced for treatment of staphylococcal infections. Also in this case, the first methicillin resistant *S. aureus* strains (MRSA) were already observed within a period of two years (65). The first infections caused by MRSA used to be associated with hospitalized patients. This phenomenon is generally referred to as hospital-acquired MRSA (HA-MRSA). However, in recent years, the spread of so-called community-acquired MRSA (CA-MRSA) strains has laid an additional burden on the healthcare system (77, 95). In contrast to HA-MRSA, the CA-MRSA lineages easily spread within the young and healthy community, and they have now entered into hospitals causing nosocomial infections with even higher mortality rates than the hospital-acquired strains (79, 88).

Till now, the therapy against *S. aureus* infections relies mainly on antibiotics. However, due to the fast development of antibiotic resistance by *S. aureus*, alternative ways to prevent and cure staphylococcal diseases need to be discovered, including immunization strategies. Importantly, there is currently no vaccine or passive immunization therapy available that could stop this successful pathogen from infecting humans. Therefore, various attempts have been made towards the development of anti-staphylococcal vaccines. In this context, efforts have been directed towards the identification of effective targets for vaccination or antibody therapy. These included the polysaccharide capsules of certain strains, or cell surface-exposed proteinaceous factors. Some of these candidate targets were selected for human vaccination studies after positive pre-clinical trials. These include the capsule serotypes 5 and 8, the clumping factor A (ClfA), ClfA in combination with the serine aspartate repeat protein G (SdrG), or the iron-responsive surface determinant B (IsdB). However, none of these have so far passed the stage of clinical trials (7, 22, 39, 94, 119). The probable cause of this is that *S. aureus* possesses a strong ability to escape and to suppress the host immune responses (48, 58, 91). In addition, this pathogen displays an amazing genomic flexibility and heterogeneity in the expression of cell surface-exposed and secreted antigens (4, 37, 73). Other vaccine candidates that gave promising results in animal models, such as the immunodominant staphylococcal antigen A (IsaA) and the iron-responsive surface determinant A (IsdA), still need to be evaluated for their application potential in vaccines (47, 62).

S. aureus– human host interactions

A thorough understanding of the mechanisms by which *S. aureus* can colonize and

survive inside its human host is the key to the development of new anti-staphylococcal therapies and vaccines. *S. aureus* has traditionally been classified as an extracellular pathogen that is combated through innate immune factors, such as antimicrobial peptides and proteins (*e.g.* lysozyme, defensins and complement), or adaptive immune responses (*e.g.* different types of immunoglobulins) (49, 57, 108). However, in the past few years different studies have challenged this concept, showing that *S. aureus* is able to invade and survive in epithelial cells, keratinocytes, endothelial cells, fibroblasts and osteoblasts (6, 41, 76, 108). In addition, *S. aureus* has evolved different mechanisms to invade cell types that are incapable of phagocytosis (103). Via a modified 'zipper-type' mechanism with F-actin rearrangement, *S. aureus* enters the host cells (104), thereby triggering the relapse of *S. aureus* disease (108). *S. aureus* tissue invasion provokes migration of neutrophils and macrophages to the side of infection in order to phagocytose and kill this bacterium (23). The phagocytosis can be promoted either by opsonins, such as complement proteins or specific antibodies, which coat the *S. aureus* cell surface, or by direct binding to surface receptors (93). Once *S. aureus* is phagocytosed it resides in a phagosome, where it is exposed to different antimicrobial agents, such as reactive oxygen species, nitrogen intermediates and degradative enzymes (23). *S. aureus* can avoid such host defenses by (i) interfering with antimicrobial substances (*e.g.* staphylokinase protects against the bactericidal effects of defensins), (ii) resistance to antimicrobial peptides and proteins (*e.g.* lysozyme resistance due to peptidoglycan modification), and (iii) by evading or resisting oxidative and nitrosative stresses (3, 23, 33, 93). The protection against toxic hydrogen peroxide and free radicals relies on protective antioxidants, including catalase, staphyloxanthin and the yellow carotenoid pigment (21, 61, 86). Notably, once it has entered professional phagocytes (Figure 2), *S. aureus* can take advantage of these cells for transport via the bloodstream and dissemination throughout the human body (108). Lastly, *S. aureus* is able to mediate host cell lysis by secreting cytolytic toxins and proteins involved in the manipulation of apoptotic cell death pathways (23, 118). The death of some of the internalized *S. aureus* cells after they have disseminated can lead to the release of surviving bacteria and their toxins in certain tissues, which will then drive local inflammation and infection (108).

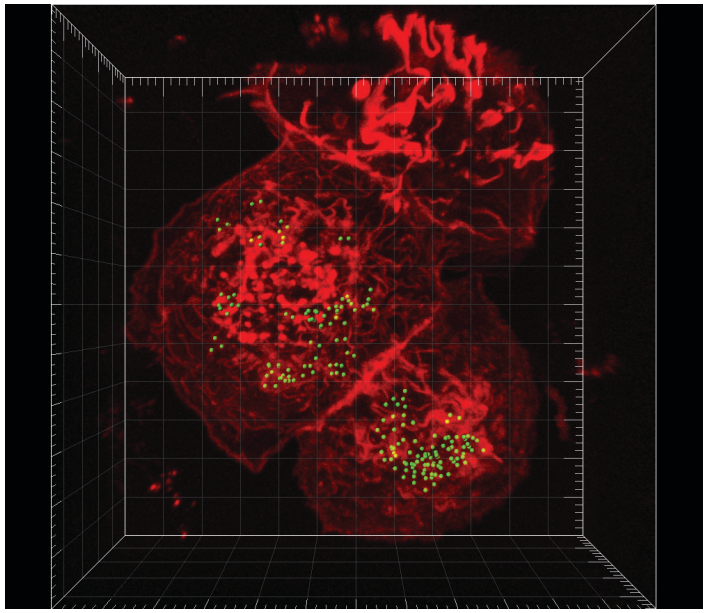

Figure 2. Internalization of *S. aureus* by THP-1 human macrophages. GFP-expressing staphylococcal cells are colored green, and actin filaments are colored red (Image generated in collaboration with J. Domingues and Prof. H.C van der Mei).

The interactions between *S. aureus* and its human host are central in the development of staphylococcal disease. As outlined above, *S. aureus* infections are usually limited by the primary barriers of the skin and mucosa, as well as the innate and adaptive immune responses of healthy individuals. However, the situation is different in patients where these defenses are compromised. Consequently, such patients are often heavily colonized by *S. aureus*. This has been extensively studied in patients with cystic fibrosis or atopic dermatitis (5, 14, 38, 42). In other groups of patients with defective barriers, the interactions with *S. aureus* have been studied to lesser extents. One of these diseases is epidermolysis bulosa (EB). EB refers to a group of inherited disorders caused by mutations in various structural proteins in the skin. Patients with EB develop blisters as a consequence of trivial mechanical trauma (31, 68). Four major EB subtypes can be distinguished, namely EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB), and Kindler syndrome (29). Depending on the type of EB, the symptoms vary in severity from minor blistering of the skin to a lethal form involving other organs (97). The ulceration of the skin in patients with EB leads to the development of wounds that become colonized by different bacteria (9, 75). It has been shown that the most commonly isolated microorganisms from wound cultures of EB patients are *S. aureus*, *Streptococcus* species, and *Pseudomonas aeruginosa* (9).

The presence of bacteria in wounds can be categorized as four different conditions, namely contamination, colonization, critical colonization and infection (Figure 3) (28). All chronic wounds should be considered as contaminated. In this case, mostly non-replicating microorganisms are present within or on the surface of the wound (24). The host defenses are usually able to clear these contaminants and, consequently, they do not interfere with wound healing. Colonization can be defined as the presence of replicating microorganisms adhering to a wound in the absence of tissue damage (24). Critical colonization refers to the transition state between colonization and invasive wound infection. This is represented by conditions where the bacterial bio-burden in the wound reaches levels at which it interferes with healing, but does not produce the classic signs and symptoms of infection (98). Whether the colonizing organism invades the tissue depends on a number of microbe-host interactions, such as the amount of bacteria per gram tissue, virulence and pathogenicity of the respective bacteria and proper innate and adaptive immune responses of the host (121). Wound infection is characterized by the presence of replicating micro-organisms within a wound with subsequent host injury (24). The wound infection symptoms include erythema, warmth, swelling, pain, odor and purulent drainage (60, 89). Notably, wound infections that are not adequately treated can progress into systemic infections and even sepsis (89). The wounds of patients with EB are highly colonized by *S. aureus*, since 95% of the EB patients with chronic wounds and 59% of the patients without chronic wounds tested positive for the presence of *S. aureus* in their wounds (9, 113). This high wound colonization is likely to contribute to the development of chronic wounds (75). In addition, it may predispose EB patients to life-threading infections. This view is critically underscored by the observation that sepsis is one of the leading causes of death amongst infants with EB. Specifically, septicemia was associated with mortality in 14 to 24% of the patients with Herlitz type JEB (JEB-H) (30, 123). JEB-H is caused by null mutations in the genes encoding an adhesion protein in the epidermal basement membrane: laminin-332 (123). In the absence of laminin-332, the skin is prone to generalized painful blistering with persistent erosions and granulation tissue formation (46). Possibly, the high susceptibility of patients with JEB-H to staphylococcal wound infections relates to the high numbers of recurrent erosions found over the diaper area of the skin. These are absent from the wounds of patients with other types of EB. This may explain why patients with such other types of EB seem to be less susceptible for invasive infections by *S. aureus*, despite high colonization rates (124). In the latter case, effective innate and adaptive immune responses may compensate at least partially for the impaired barrier function of the skin (112).

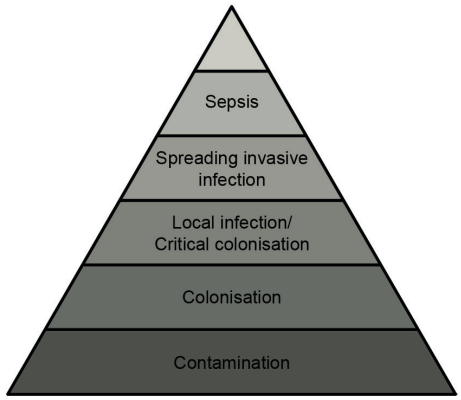

Figure 3. Schematic representation of the presence of bacteria in a wound. The bacterial presence in wounds can be categorized as four different conditions: contamination, colonization, critical colonization and infection. Ultimately, this can lead to invasive diseases, such as sepsis. Adapted from Edwards and Harding (28).

Staphylococcal virulence factors

The pathogenicity of *S. aureus* is caused by a broad range of cell surface-exposed or secreted virulence factors. These include: surface-exposed proteins involved in adherence and colonization of host tissues, surface-exposed factors involved in the inhibition of phagocytic engulfment (capsule and protein A), invasins exported into the host environment to promote the bacterial spread in invaded tissues (*e.g.* leukocidin, kinases, and hyaluronidase), biochemical properties that enhance staphylococcal survival in phagocytes (carotenoid and catalase production), immunological disguises (protein A, Sbi, coagulase, and clotting factor), superantigens (*egc* and non-*egc* SAgs), toxins damaging the membrane of host cells (hemolysins and leukotoxin), and determinants for inherent and acquired resistance to antimicrobial agents (102). It is known that *S. aureus* populations carry a range of unique variants of these virulence factors. The variation in genes coding for surface proteins and immune evasion factors has been shown to be lineage specific (73, 126). In particular, variations were observed for genes encoding surface proteins. These may be completely absent, or they may be truncated, which then affects predicted functional domains (73). The same applies also to genes encoding secreted proteins predicted to interact with the host immune system (73). However, the level of variation in the latter genes is less drastic than observed for genes encoding surface proteins, and their complete absence or truncation is less common (73). Notably, variations in cell surface-exposed and secreted proteins can also relate to different expression levels in different *S. aureus* lineages. The variations in the expression levels of virulence genes may relate to differential activities of specific regulators. One of the key regulators of virulence factor genes is the RNAIII, which is responsible for their

cell density-dependent expression (85). In addition, at least 16 two-component regulatory systems are to different extents involved in staphylococcal virulence (36). Strain-specific differences in gene regulation may results in different exoproteome patterns as has been shown for *S. aureus* cells grown *in vitro* (126). Notably, changes in the expression of virulence factors seem to relate to different host environments (11, 12, 55, 64, 67). In nasally-carried *S. aureus* cells, the genes encoding the most important adhesion molecules (*e.g. clfB*, *isdA*, *fnbA*, *atlA*, *eap*), immune-modulating factors (*sak*, *chp*, *spa*) and cell surface remodeling factors (*sceD*, *oatA*, *atlA*) are highly expressed, while genes encoding major toxins (*hla*, *psm*) are not detectably transcribed (11, 12). In contrast, in cutaneous abscesses, genes encoding toxin components, such as *lukS-PV*, *lukE*, *hlgB* and *hla* are up-regulated, while RNAIII, *bsaB* and *spa* expression seem to be down-regulated (64). In addition, *S. aureus* incubated in human serum or blood up-regulates the genes involved in iron and iron transport-associated molecules (67). Taken together, it can be concluded that *S. aureus* shows major adaptive responses to different host environments by changing the expression of different groups of virulence factors. Consistent with this idea, specific adaptive immune-responses might be raised against different staphylococcal virulence factors, depending on the infection site as well as invading strain.

The immune responses against *S. aureus* and different staphylococcal proteins have been studied extensively both in healthy human individuals as well as patients with different diseases. Thus, it was reported that the levels of antibodies directed against the toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxin A (SEA) and ClfA and ClfB are significantly higher in healthy persistent carriers than in healthy non-carriers (117). Anti-staphylococcal antibody levels were shown to increase strongly during the course of infection in patients with bacteremia (52, 63, 116). This applied in particular to antibodies against IsaA, IsaB, the major cold shock protein (CspA) and the phosphocarrier protein (Hpr) (63). In addition, Kolata *et al.* have shown that during bacteremia caused by exogenous *S. aureus* strains (*i.e.* strains not carried by the patient), there was an increase of immunoglobulins G (IgG) binding to antigens from the invasive strain, but not the colonizing strain (52). These findings indicate that adaptive immune responses are important to combat invasive staphylococcal infections, which would support the concept that active or passive immunization strategies could represent effective antistaphylococcal therapies.

Secretion of virulence factors

To colonize or invade the human host *S. aureus* expresses different virulence factors, which

are first synthesized as precursors with an N-terminal signal peptide to direct their transport from the cytoplasm to an extra-cytoplasmic location, such as the cell wall or extracellular milieu via different transport system (102). The most commonly used pathway for protein transport across the membrane is the general secretory (Sec) pathway. In addition, several other special-purpose pathways for protein transport are known, such as the twin-arginine translocation (Tat) pathway and the pseudopilin-specific (Com) pathway (Figure 4).

The Sec translocation machinery is composed of several subunits. The SecA translocation motor binds pre-proteins and pushes them through the membrane-embedded SecYEG translocation channel via repeated cycles of ATP binding and hydrolysis (27, 87, 102, 122). The structure of the SecA/SecYEG complex from the Gram-negative bacterium *Thermotoga maritime* suggests that one SecA molecule is bound to one set of SecYEG channel proteins. The core of the Sec translocon consists of the SecA, SecY, and SecE proteins, which are essential for growth and viability of bacteria, such as *Escherichia coli*, *Bacillus subtilis* and *S. aureus* (10, 15, 51). In contrast, the channel component SecG is dispensable for growth and cell viability (84, 101, 115). However, unlike the situation in *B. subtilis*, deletion of *secG* from *S. aureus* has a profound impact on the composition of the exoproteome (101). It was shown that various extracellular proteins were present in decreased amounts in the growth medium of *secG* mutant strains, which is consistent with impaired Sec channel function (101). Furthermore, the absence of *secG* caused a serious decrease in the amounts of the cell wall-bound Sbi protein. In addition to the major SecYEG channel proteins, *S. aureus* produces a second set of SecA and SecY proteins, generally referred to as SecA2 and SecY2 (100). Comparison of the amino acid sequences of the SecY1 and SecY2 proteins shows that their similarity is relatively low (about 20% identity) and that the conserved regions are mainly restricted to the membrane spanning domains (101). Interestingly, the deletion of the *secY2* gene had no detectable effect on the exoproteome of *S. aureus*, which suggests that the SecY2 protein has a very specific role in protein export (101). Nevertheless, a *secGsecY2* double mutant strain displayed synthetic growth and secretion defects, indicating that SecY2 may function in synergy with the main Sec channel (101).

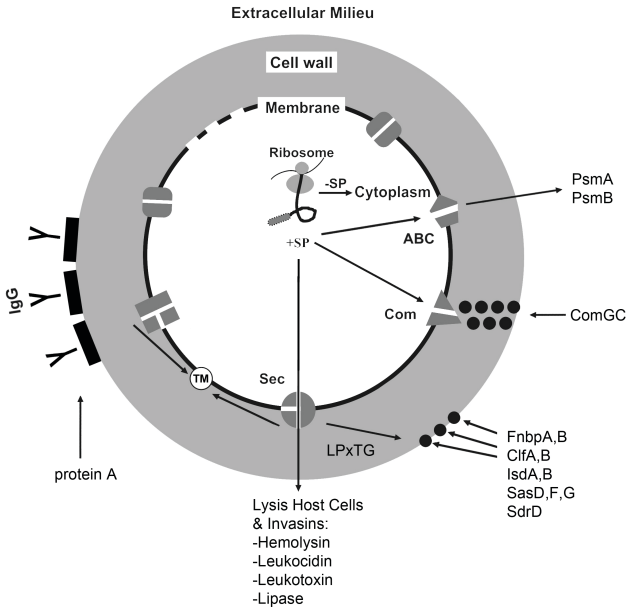

Figure 4. Schematic representation of different protein secretion pathways in *S. aureus*. Proteins synthesized with an appropriate signal peptide are specifically directed to a respective pathway. Furthermore, proteins with a membrane anchor domain (TM) are retained in the cytoplasmic membrane, while proteins with a C-terminal LPxTG motif are covalently bound to the cell wall. Abbreviations: Psm, phenol-soluble modulin; Fnbp, fibronectin- binding protein; Clf, clumping factor; Isd, iron-regulated surface determinant protein; Sas, staphylococcal surface protein; Sdr, serine-aspartate repeat protein. Adapted from Sibbald *et al.* (102).

After membrane translocation of a pre-protein through the Sec channel, the signal peptide is removed by signal peptidase. This is a prerequisite for the release of the translocated protein from the membrane (2, 114). Thus, in order to be retained at the membrane, translocated proteins require specific retention signals (102). Such retention signals may be present in the form of a transmembrane anchor domain or lipid-modification that will, respectively, attach membrane proteins and lipoproteins to the membrane. Other specific amino acid sequence motifs can facilitate non-covalent or covalent cell wall attachment.

The signal for covalent cell wall attachment is the so-called LPxTG motif that is positioned in the C-terminus of the respective exported proteins. The covalent cell wall attachment of LPxTG proteins requires the enzymatic activity of so-called sortases (69). These sortases are membrane-bound transpeptidases, which cleave the LPxTG motif between the Thr and Gly residues and catalyze the formation of an amide bond between the carboxyl group of the Thr residue and the free amino end of a pentaglycine cross bridge in peptidoglycan precursors (35, 56, 99, 107). The sortases are grouped into four classes (A-D) based on phylogenetic criteria (25). *S. aureus* possesses one sortase A (SrtA) enzyme, which recognizes the general LPxTG motif (71, 83). It has been shown that *S. aureus* lacking the *srtA* gene is

impaired in the retention of LPxTG proteins in the cell wall. As a consequence, the absence of SrtA causes a defect in the establishment of acute infections (70). In addition, the *srtA* mutation causes a hyper-spreading phenotype probably because it interferes with cell-cell attachment through surface-exposed LPxTG proteins (111). It has been reported that 19 different *S. aureus* proteins carry the canonical C-terminal LPxTG motif and that two additional proteins carry a C-terminal LPxAG motif (34, 69, 82, 92, 102). These include protein A (Spa), two fibronectin-binding proteins (FnbpA and FnbpB)(40), ClfA, ClfB, three cell wall-anchored proteins with large serine-aspartate repeat domains (SdrC, SdrD and SdrE) (45), a collagen- binding protein (Cna), a plasmin-sensitive protein (Pls) (96), the methicillin resistance determinant B (FmtB) (53), and eleven staphylococcal surface (Sas) proteins.

In addition to SrtA, *S. aureus* contains a second sortase known as SrtB. SrtB is required for the cell wall anchoring of surface proteins that contain an NPQTN motif. So far, the only protein known to be covalently coupled to the cell wall by SrtB is IsdC (72). Another *S. aureus* protein with a motif that resembles NPQTN is the DNA-binding protein II, but this protein is probably not cell wall-bound, because it lacks a signal peptide for export from the cytoplasm (102). It is presently not known how important SrtB is for staphylococcal virulence in general, but *S. aureus* lacking *srtB* was shown to have a small defect in the establishment of arthritis in a mouse model (44).

In recent years, the functions of many secretion machinery components of *S. aureus* have been elucidated, as exemplified by studies on the Sec and Tat pathways (8, 13, 20, 43, 101, 106). Intriguingly however, for several other predicted secretion machinery components, no biological functions were so far described. This applies for example to the pseudopilin export machinery of *S. aureus*. This machinery is very similar to the Com machinery for DNA binding and uptake in *B. subtilis*. Interestingly, the genes coding for most Com proteins are present in the sequenced *S. aureus* strains, suggesting that this bacterium is able to assemble pseudopili of the Com type (102). In *B. subtilis*, the ComGC, ComGD, ComGE, and ComGG proteins form pilin-like structures that are localized to the cytoplasmic membrane and cell wall (16- 18, 109). Assembly of the pseudopilus in *B. subtilis* requires the specific signal peptidase ComC, which processes the N-terminal signal peptides of ComG proteins upon membrane translocation (16, 109, 110). Furthermore, stability of the *B. subtilis* ComGC pseudopilin requires post-translocational disulfide bond formation, which is catalyzed by the thiol-disulfide oxidoreductases BdbC and BdbD (16, 26, 54, 74). In *S. aureus*, the transcription of the respective *com* genes, which are organized in the *comG* and *comE* operons, is directed by the staphylococcal alternative sigma factor σ^H (80). Judged by the presence of these *com* genes and a dedicated transcription factor, it seemed likely that *S. aureus* can employ its Com proteins for DNA uptake. Indeed, this

was recently confirmed by Morikawa *et al.* (81), who showed that *S. aureus* cells producing active σ^H become competent for natural transformation by plasmid or chromosomal DNA. Taken together, the remarkable success of *S. aureus* as an opportunistic pathogen seems to be based on the acquisition of highly effective systems for colonization and subversion of its human host, and for the evasion of innate and adaptive host defenses. On top of that high levels of genomic plasticity and variability in gene expression allow the *S. aureus* 'superbug' to change its appearance, which adds significantly to its capacity to avoid elimination by the human host. Accordingly, novel strategies to combat this pathogen through immunotherapy must be based on an integrated approach that requires the identification of invariant and immune-dominant targets. The identification of such targets requires (i) in-depth analyses on the localization of proteins and other compounds to the staphylococcal cell surface and (ii) a thorough understanding of the human immune responses to these cell surface-exposed compounds.

Scope of this thesis

S. aureus is a commensal bacterium, which colonizes about 30% of healthy human population. However, if the primary barrier function of the skin is disrupted or if the immune system is compromised, *S. aureus* is given the opportunity to invade its human hosts, which can then result in systemic infections. Currently, the treatment of staphylococcal infections relies mainly on the administration of antibiotics. However, due to the high adaptability of this bacterium, many *S. aureus* lineages have become resistant to antibiotics. Therefore, alternative ways to prevent and cure staphylococcal diseases need to be discovered. An attractive but challenging option is the development of active or passive immunization strategies. To be able to develop effective anti-staphylococcal vaccines or therapeutic antibodies, the staphylococcal cell surface as well as exported virulence factors need to be mapped in detail. At the same time the immune responses of the human host to *S. aureus* need to be defined. Ideally, this involves investigations with volunteers, whose immune system has been heavily challenged with different staphylococcal lineages expressing a broad range of virulence factors. The overarching objective of the research presented in this thesis was therefore (i) to identify individuals who are heavily challenged by *S. aureus*, (ii) to map the cell surface-exposed *S. aureus* components that are targeted by the immune responses of these individuals, and (iii) to obtain a better understanding of the mechanisms that govern the expression and export of potential targets for antistaphylococcal therapies. The essential components in the interactions between *S. aureus* and its human host have been introduced in Chapter 1 of this thesis. In addition, **Chapter 1** gives an overview of the different virulence factors produced by *S. aureus* and the mechanisms by which they are exported from the cytoplasm to the cell surface and the extracellular milieu of the bacteria which, in case of an infection, is synonymous with the cells and tissues of the human host.

The studies described in **Chapter 2** reveal that patients with the blistering disease epidermolysis bullosa (EB) are heavily colonized with *S. aureus*. The results of extensive sampling revealed that all EB patients with chronic wounds, and the majority of patients without chronic wounds carry *S. aureus*. Molecular typing of the collected isolates showed that the *S. aureus* population structure in EB patients mirrors the geographical distribution of *S. aureus* types in the Netherlands. Furthermore, typing revealed that self-transmission of *S. aureus* between wounds and the upper respiratory tract of individual EB patients occurs frequently, whereas transmission of *S. aureus* between different EB patients appears to be rare.

Chapter 3 focuses on the colonization of EB patients with *S. aureus* in time and its impact

on adaptive antistaphylococcal immune responses. Analysis of all collected isolates revealed major fluctuations in the *S. aureus* types carried over a period of ~2 years. Overall, the sera of EB patients contained higher antistaphylococcal IgG levels than those of healthy individuals. Specifically, this applied to IgGs against nine important virulence factors, including the superantigens SEM, SEN and SEO. Notably, EB patients carrying different *S. aureus* types contained higher levels of antistaphylococcal antibodies than EB patients colonized by only one type. This suggests that the immune system of EB patients is heavily challenged with *S. aureus* antigens. Notably, some of the IgGs that are present at elevated levels may compensate for the impaired barrier function of the skin of the investigated EB patients.

In **Chapter 4**, the staphylococcal topography of chronic wounds of five EB patients was examined by replica-plating of used bandages. Molecular typing of these isolates revealed that individual chronic wounds contained up to six different *S. aureus* types at one time point of sampling. Some of these isolates were closely related suggesting that they share a recent common ancestry. Importantly, other isolates derived from adjacent wound locations belong to distinct molecular complexes of *S. aureus*. These observations imply that the classical assumption that one individual is predominantly colonized by one type of *S. aureus* does not apply to chronic wounds of patients with EB.

Chapter 5 reports on the use of antibodies from EB patients to identify immunodominant targets in *S. aureus*. Specifically, cell surface-exposed staphylococcal proteins were profiled by tryptic 'surface shaving' approaches and gel-free mass spectrometry (MS). A subset of the identified proteins were screened for interactions with antibodies from EB patients. This led to the identification of immunodominant domains in several surface-exposed proteins, including the covalently cell wall-bound proteins ClfB and IsdB, a 'YkyA-like' cell wall- binding lipoprotein, the membrane proteins EbpS and LtaS, the non-covalently cell wall-bound and secreted proteins Atl, Sbi, IsaA, Emp, and the cytoplasmic proteins Afl1, Eno, and GAPDH. These proteins are thus potentially powerful targets for antistaphylococcal immune therapies.

The studies described in **Chapter 6** report on the application of the surface shaving protocol to profile the binding of human serum proteins to *S. aureus*. As shown by surface shaving with trypsin and subsequent MS analysis of liberated peptides, several components of the complement system, the platelet factor 4 and the isoform 1 of the inter-α-trypsin inhibitor heavy chain H4 are detectable on the staphylococcal cell surface upon incubation in human serum. The surface shaving technique is thus a versatile tool to profile global

interactions between human serum proteins and the *S. aureus* cell surface.

Chapter 7 describes the roles of the non-essential Sec channel components SecG and SecY2 in the biogenesis of the extracellular proteome of *S. aureus*. The results show that SecG is of major importance for protein secretion by *S. aureus*. No secretion defects were detected for strains with a *secY2* single mutation. However, deletion of *secY2* exacerbated the secretion defects of *secG* mutant cells. Furthermore, a *secGsecY2* double mutant strain displayed a synthetic growth defect. This might relate to a slightly elevated expression of *sraP*, encoding the only known substrate for the Sec2 pathway, in cells lacking SecG. Additionally, the results suggest that SecY2 can interact with the Sec1 channel, which would be consistent with the presence of a single set of *secE* and *secG* genes in *S. aureus*.

Studies on the staphylococcal pseudopilin export pathway are documented in **Chapter 8**. The results show that ComGC from *S. aureus* localizes to the cytoplasmic membrane and cell wall, consistent with the formation of pseudopili. Furthermore, the studies show that the thiol-disulphide oxidoreductase DsbA is required for ComGC stability, suggesting that this pseudopilin contains a disulfide bond as is the case for its homologue in *B. subtilis*. The pseudopilin-specific signal peptidase ComC is needed for ComGC processing and optimal cell surface exposure. Notably, chapter 8 represents the first report where biological functions are demonstrated for *S. aureus* DsbA and ComC.

The studies presented in **Chapter 9** were aimed at determining the relevance of sortase activity for *in vitro* staphylococcal growth in human plasma. The results show that, depending on the investigated strain, SrtA can be important for efficient growth in human plasma, whereas SrtB is not required. Transcript profiling of *srtA* mutant cells grown in human plasma revealed a typical phosphate starvation response, even though this 'medium' is known to contain relatively high phosphate concentrations. This suggests that one or more covalently cell wall-bound proteins, such as FnbpA and FnbpB, are involved in phosphate binding or uptake.

Although the sortase-mediated cell wall attachment of proteins has been characterized in great detail, it was thus far not known how sortases shape the actual surface of a staphylococcal cell with respect to proteins that are directly exposed to the extracellular milieu. Therefore, the studies described in **Chapter 10** were aimed at determining the 'surfacomes' of *srtA* or *srtB* mutant cells of *S. aureus*. As shown by 'cell surface shaving' with immobilized trypsin, SrtA has a major impact on the staphylococcal surfacome, whereas SrtB is only of minor importance. Specifically, the results show that the sortase-dependent surfacome consists of

covalently cell wall-bound ‘LPxTG proteins’, non-covalently wall-bound proteins, known secreted proteins and cytoplasmic proteins. Intriguingly, the data suggest that certain LPxTG proteins serve as nodes in the cell wall interactome of *S. aureus*.

Finally, the results presented in this thesis are discussed in **Chapter 11**, where also an outlook for future research is presented.

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2

High genetic diversity of *Staphylococcus aureus* strains colonizing patients with epidermolysis bullosa

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Abstract

Patients with the blistering disease epidermolysis bullosa (EB) frequently suffer from chronic wounds that become colonized by pathogenic bacteria, such as *Staphylococcus aureus*. To determine *S. aureus* colonization rates in EB patients, swabs were collected from the anterior nares, throats and wounds of 52 Dutch EB patients. Swabs were also collected from nares and throats of 13 healthcare workers who occasionally meet the sampled EB patients. All EB patients with chronic wounds and 75% of the patients without chronic wounds were colonized with *S. aureus*. In contrast, 39% of the sampled healthcare workers were colonized with *S. aureus*. Typing revealed a high degree of genetic diversity of 184 collected *S. aureus* isolates. Autoinoculation of *S. aureus* in individual EB patients was shown to occur frequently, whereas transmission of *S. aureus* between EB patients is apparently rare. There was no evidence for *S. aureus* transmission between EB patients and healthcare workers.

Background

Epidermolysis bullosa (EB) is a genetic blistering disease (3, 11). Because the natural barrier function of their skin is breached (2, 13), EB patients are more likely colonized with opportunistic microorganisms. To date, only two studies addressed the microbial colonization of wounds of EB patients, indicating that they are mostly colonized by staphylococci, streptococci and *Pseudomonas aeruginosa* (1). Up to 86% of the EB patients tested positive for *Staphylococcus aureus* (1, 4). *S. aureus* is a commensal bacterium, which colonizes epithelial surfaces, such as the skin and the anterior nares in 25-37% of healthy adults (7, 9, 16). However, under certain circumstances, including a breach of the skin, *S. aureus* can become invasive which may lead to severe infections (8). Importantly, *S. aureus* nasal carriage is an important risk factor for infections (8, 15-17), and the presence of this bacterium in wounds can contribute to the development of chronic wounds (5, 7, 10, 12). Accordingly, one of the leading causes of death amongst infants with EB is sepsis (2).

Question addressed

Despite its clinical relevance, the colonization of EB patients with *S. aureus* has thus far not been investigated in great detail, and it was not known whether colonization of EB patients is biased towards certain *S. aureus* lineages. Therefore, the present studies aimed at defining the *S. aureus* population colonizing patients with EB.

Methods

Patients and bacterial isolates

52 patients with EB were recruited from the Dutch epidermolysis bullosa Registry (DEBR) at the University Medical Center Groningen (UMCG). This patient cohort included 31 male and 21 female individuals, who ranged in age between 7 months and 76 years (mean 25.5 years). Additionally, 13 healthcare workers from the UMCG were included in this study (6 males and 7 females, who ranged in age between 25 and 60 years), who have contacts with individual EB patients about one to four times four year. Samples were taken from 3 distinct wounds, the left and right anterior nares, and the throat of each patient using transswabs (MWE, Corsham, England). As the included healthcare workers lacked wounds, they only donated samples from the left and right anterior nares and throat. The obtained samples were cultivated on blood agar (BA) plates with 5% sheep blood (Mediaproducts BV, Groningen, the Netherlands) and *S. aureus* was identified on the basis of colony morphology, Gram staining, a positive catalase reaction and the Pastorex Staph Plus test from Bio-Rad. All *S. aureus* strains were stored in 8% glycerol at - 80°C. For further analyses, the *S. aureus*

isolates were streaked on BA plates and cultivated overnight at 37°C. Cell lysates were prepared as previously described (14).

Ethical approval

The local medical ethics committee of the University Medical Center Groningen approved of the collection of bacterial samples from patients with EB on the basis of informed consent. The informed consent was obtained from all patients included in the present studies.

Multiple-locus Variable Number of Tandem Repeats Analysis

VNTR PCR reactions were performed as described by Schouls et al. (14). Briefly, repetitive DNA sequences from the genes encoding the V8 serine protease (*sspa*), protein A (*spa*), staphylocoagulase (*coa*) and from 5 non-coding regions were amplified in two multiplex PCR reactions. Diluted PCR products were mixed with the GeneScan 1200 LIZ marker (Applied Biosystems) and after heat denaturation separated on an ABI 3730 DNA sequencer. The number of the repeats of each VNTR was calculated with GeneMArker software (Softgenetics, State Collage,USA). Minimum spanning trees were created according to the procedure described before (14) using Bionumerics software (Applied Maths).

***Spa* typing**

Spa sequence typing was performed according to the RIDOM protocol (http://www. ridom. de) with previously described modifications (14). Shortly, 1µl of staphylococcal lysate was included in a 25 µl PCR HotStart master mix (Qiagen, Hilden, Germany) containing 2.5 pmol of forward (5'-TAAAGACGATCCTTCAGTGAGC-3') and reverse (5'-CAG CAG TAG TGC CGT TTG CTT-3') primers. The resulting PCR product was sequenced and analyzed with Bionumerics software (Applied Maths). The nine novel *spa*-types that were identified have been submitted to the Ridom SpaServer.

Statistical analyses

Statistical analyses were performed with SPSS statistics for Windows. Data were compared using two-tailed independent student *t*-tests, and *P*-values <0.05 were considered significant.

Results

The EB patients were divided into two groups consisting of **EB patients with chronic wounds** (n=20) who had multiple non-healing wounds over periods of more than 3 months, and **EB patients without chronic wounds** (n=32) who had relatively few wounds that healed in shorter periods of time. We collected 178 *S. aureus* isolates from EB patients and 6 from healthcare workers. All patients with chronic wounds were colonized by *S. aureus*

on at least one site, and 75% of the patients without chronic wounds were colonized by this bacterium (Figure 1A). The highest colonization rates were identified in wounds, and the lowest in the throat. There was no specific correlation between patient age and *S. aureus* colonization (not shown). In contrast to the EB patients, 39% of the included healthcare workers carried *S. aureus*, which closely matches the *S. aureus* colonization rates in healthy adults (7, 9, 16).

Typing of all 184 *S. aureus* isolates by Multiple-locus-Variable-Number-of-Tandem-Repeats-Analysis (MLVA) distinguised 67 *S. aureus* types (Table 1). 79 strains were selected for further analysis by *spa*-typing, which identified 51 different types including 9 novel types (Table 1). These analyses revealed that EB patients with chronic wounds were colonized with up to four different *S. aureus* MLVA types, and EB patients without chronic wounds with up to three different MLVA types (Figure 1B-C). To visualize the genetic diversity of the isolated *S. aureus* strains, they were plotted in a minimum spanning tree of ~23,000 *S. aureus* isolates collected in the Netherlands (Figure 2). This showed that the isolated *S. aureus* strains are randomly distributed amongst the different MLVA complexes. In most cases where different MLVA types were detected in one EB patient, they belonged to different MLVA complexes as exemplified for patient 44 (Figure 2).

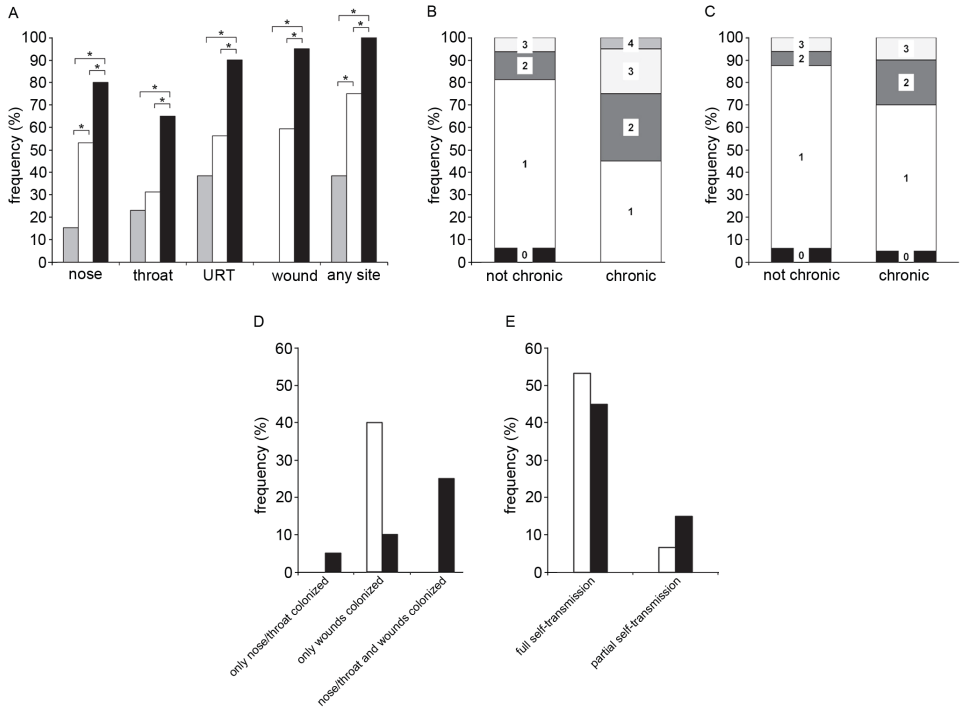

Figure 1. Frequency of *S. aureus* detected in the patients with EB. (**A**) *S. aureus* colonization rates at different body sites of EB patients and healthy healthcare workers. A distinction was made between EB patients with chronic wounds (black bars) and EB patients without chronic wounds (white bars). Grey bars represent the *S. aureus* colonization rates in the sampled healthcare workers. The statistical significance of observed differences was assessed, and differences with *P*-values of = 0.05 are marked with a star (*). URT, upper respiratory tract. (**B**) The frequency of the occurrence of multiple *S. aureus* isolates of different MLVA types within individual patients is represented for the entire sampled EB patient cohort, (**C**) and for the sampled wounds of these patients. Patients with less than 3 wounds were not included in this particular analysis. The numbers of different *S. aureus* MLVA types within individual patients are indicated in the differently marked fields in each bar. (**D**) Absence of autoinoculation is represented by the frequencies of EB patients who were colonized with *S. aureus* only in the nose/throat, or only in their wounds. In certain cases, the nose and/or throat and wounds were colonized by different *S. aureus* MLVA- types. (**E**) *S. aureus* autoinoculation in EB patients with three wounds. In case of 'full autoinoculation', a particular *S. aureus* MLVA type was detectable in all three wounds and in the nose and/ or throat. In case of 'partial autoinoculation', isolates from the nose and/or throat and at least one wound belonged to the same MLVA type. White bars, EB patients without chronic wounds; black bars, EB patients with chronic wounds.

The transmission of *S. aureus* between different EB patients is rare as maximally three patients were colonized with the same MLVA type (Table 1). None of the *S. aureus* MLVA types isolated from healthcare workers was identified amongst the EB patients, indicating the absence of transmission. To determine the autoinoculation rates of *S. aureus* within EB patients, we compared isolates from the wounds of individual patients with isolates from their nose and/or throat. This revealed that extensive autoinoculation of *S. aureus* from the nose/throat to the wounds (or *vice versa*) had occurred (Figure 1D-E). Several

cases of 'partial autoinoculation' were observed (Figure 1E). In these cases, strains isolated from nose or throat and at least one wound isolate belonged to the same MLVA type, while other wound isolates belonged to different MLVA types. In 25% of the patients with chronic wounds all wound isolates belonged to different MLVA types as compared to strains isolated from nose or throat (Figure 1D), suggesting that autoinoculation had not occurred or that it remained undetected.

Conclusion

Here we report an unexpectedly high rate of *S. aureus* colonization among EB patients. Even if only the colonization rates of the upper respiratory tract are compared, the rates determined for EB patients (56-90%) were substantially higher than those measured for healthy individuals (25-37%) (7, 16) or healthcare workers who meet the sampled EB patients (39%). This implies that the wounds of EB patients represent an attractive niche for *S. aureus*. Molecular typing showed that (i) colonization of the EB patients is not limited to specific genetic lineages of *S. aureus*; (ii) individual patients with EB can carry up to four different staphylococcal MLVA types; and (iii) autoinoculation of staphylococci between the upper respiratory tract and wounds of EB patients occurs frequently. The view that colonization of EB patients by *S. aureus* is a random process is supported by *spa*-typing analyses, which show that most of the identified *spa*-types belong to the most predominant *spa*-types in the areas of residence of the respective EB patients (6). Thus, the *S. aureus* population structure in the sampled EB patients mirrors the general *S. aureus* population structure in the Netherlands. An important finding is that the rate of *S. aureus* transmission between different patients in the sampled population is relatively low. The few cases where different EB patients did carry the same *S. aureus* MLVA types concern family members, or individuals who live in an area where the respective *spa*-types are very common also amongst the general population (14).

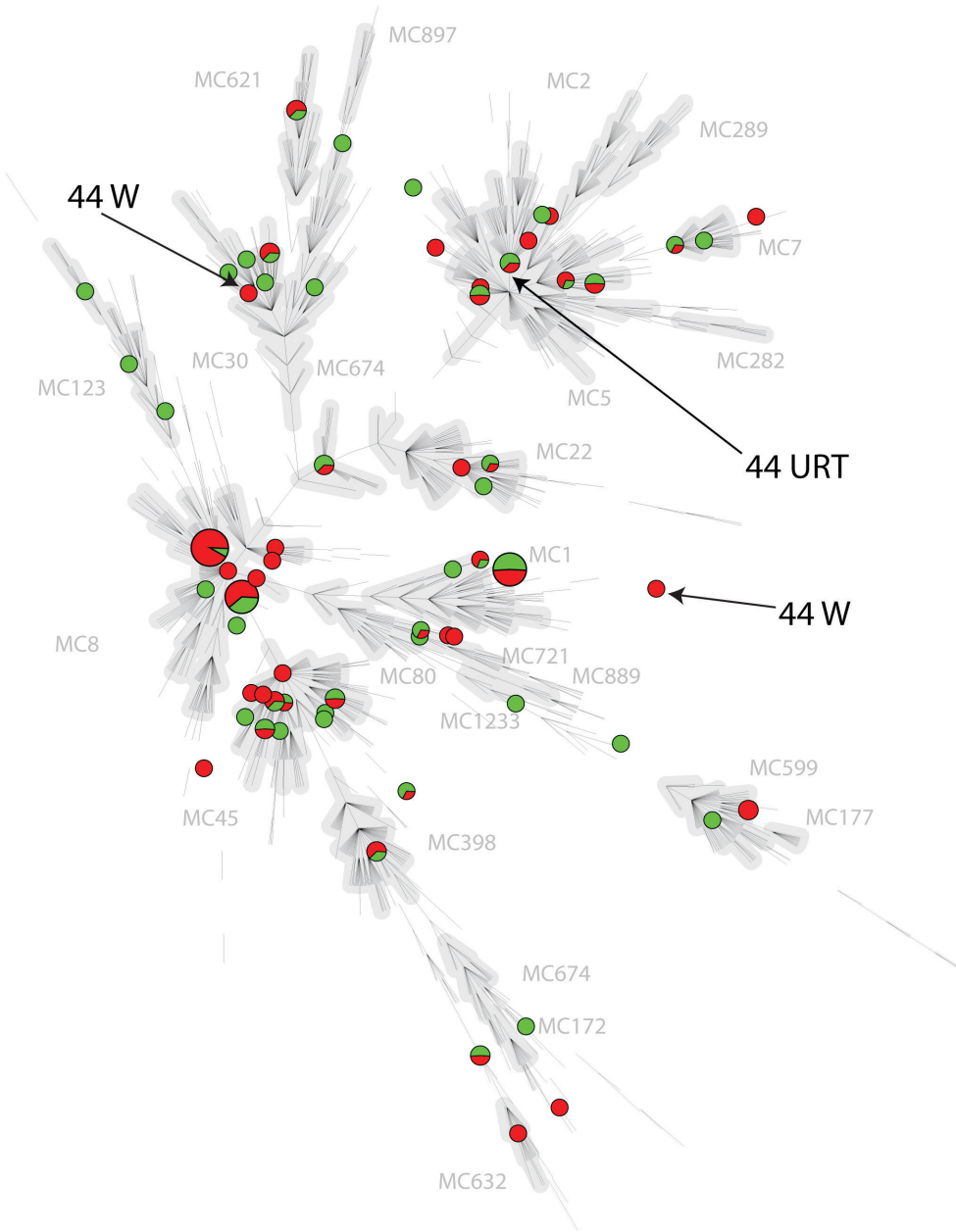

Figure 2. Minimum spanning tree based on MLVA of ~23,000 *S. aureus* isolates of which ~90 % were MRSA. Green circles represent *S. aureus* isolates from the nose and/or throat of EB patients. Red circles represent *S. aureus* isolates from the wounds of EB patients. The size of each circle is indicative for the number of *S. aureus* isolates with this particular MLVA type. Arrows indicate the tree location of *S. aureus* isolates from the upper respiratory tract (URT) or wounds (W) of chronic EB patient 44.

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Table 1. Results of MLVA and *spa*-typing of *S. aureus* strains isolated from 52 patients with EB and the five *S. aureus* carrying healthcare workers.

	Chronic wounds	Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3	
		MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa
Patient 1	Yes	656	t1081	565	nd			656	nd	238	t377	538	t065
Patient 2	Yes	550	t026	550	nd	550	nd	550	nd	550	nd	550	nd
Patient 3	No	2024	t216	2024	nd			2024	nd	2024	nd	2024	nd
Patient 4	Yes	7	t091	7	nd			665	nd	7	nd		
Patient 5	Yes	1965	t179	184	t085	1965	nd	1965	nd	1965	nd	1965	nd
				1965	nd								
Patient 6	Yes	1101	t5515					1162	nd	1162	t008		
Patient 7	Yes	565	t571	656	nd			565	nd	565	nd	565	nd
Patient 8	Yes					332	t723						
Patient 9	Yes	1966	t127	1967	t127	1967	nd	1966	nd	1966	nd		
Patient 10	No	330	t008	1968	t349	1968	nd	330	nd	330	nd	330	nd
Patient 11	No	482	t148	482	nd	482	nd	482	nd	482	nd		
Patient 12	No	538	t065			314	t008	8	t008	8	nd	-	-
Patient 13	No					613	t159			-	-	-	-
Patient 14	Yes							2025	t311	67	t311	67	nd
Patient 15	Yes	330	t008	330	nd	330	nd	330	nd	330	nd	330	nd
Patient 16	No							1969	t084	1969*	nd	1969	nd
								1970	t010				
Patient 17	No									1970*	t010		
Patient 18	Yes	1837	t502	1837	nd	1837	nd	1837	nd	1837	nd	1837	nd
Patient 19	No											-	-
Patient 20	No											-	-
Patient 21	Yes					1971	t4169	1972	t401				
Patient 22	No	321	t1476					321	nd	321	nd	321	nd
Patient 23	No							321	t1476	321	nd	321	nd
Patient 24	No							321	nd	321	t1476	321	nd
Patient 25	No	2026	t1827	2026	nd			2031	t704	-	-	-	-
Patient 26	No	1973	t1252	1973	nd	1973	nd	1973	nd	1973	nd		
Patient 27	No	1973	t1252	1973	t1252	1973	nd	1973	nd	1973	nd		
Patient 28	No							321	t377	321	nd	321	nd
Patient 29	Yes							322	t5511	322	nd	322	nd
Patient 30	Yes	1057	t5508	1057	nd	654	t5513	632	t267	632	nd	632	nd
Patient 31	No	2027	t291	2027	nd			2027	nd	2027	nd	2027	nd
Patient 32	No	534	t026			534	nd	534	nd	534	nd	534	nd
Patient 33	No							45	t050	45	nd	45	nd
Patient 34	Yes	1974	t015	1974	nd	1974	nd	1974	nd	1974	nd	1974	nd
Patient 35	No	490	t5510			490	t5510	490	nd	1975	t5510	1976	t5510
Patient 36	No	1977	t450	1977	nd	1978	t450	1979	t450	1981	t450	-	-
Patient 37	No							-	-	-	-	-	-
Patient 38	No							-	-	-	-	-	-
Patient 39	No							-	-	-	-	-	-
Patient 40	No	1982	t130	510	t065			-	-	-	-	-	-
Patient 41	No	510	t065	510	nd			-	-	-	-	-	-
Patient 42	Yes			46	t1154			46	nd	46	nd		
Patient 43	Yes	1552	t224	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd

	Chronic wounds	Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3	
		MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa
Patient 44	Yes			67	t311	67	n	1983	t098	1983	nd	1316	t012
Patient 45	No							-	-	-	-	-	-
Patient 46	Yes	67	t311			1107	t021			1316	t012	1316	nd
Patient 47	No			770	t018			-	-	-	-	-	-
Patient 48	No			1140	t005							-	-
Patient 49	No												
Patient 50	No	1984	t160			1984	nd	489	t1869	-	-	-	-
Patient 51	Yes	1987	t5512	1987	nd	733	t067	1987	nd	1988	t5514	1989	t5512
Patient 52	No											-	-
HCW 3	N/A					536	t1601	N/A	N/A	N/A	N/A	N/A	N/A
HCW 4	N/A					371	t091	N/A	N/A	N/A	N/A	N/A	N/A
HCW 5	N/A	204	t364	204	nd			N/A	N/A	N/A	N/A	N/A	N/A
HCW 6	N/A					1985	t209	N/A	N/A	N/A	N/A	N/A	N/A
HCW 8	N/A					1986	t159	N/A	N/A	N/A	N/A	N/A	N/A

Abbreviations: nd, not determined; empty cell, negative for *S. aureus*; -, no swab obtained; HCW, healthcare worker; N/A, not applicable. * two isolates with the same type.

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3

High anti-staphylococcal antibody titers in patients with epidermolysis bullosa relate to long-term colonization with alternating types of *Staphylococcus aureus*

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Abstract

Patients with the blistering disease epidermolysis bullosa (EB) develop wounds that are highly susceptible to bacterial colonization. Recently, we reported that over 75% of the EB patients sampled at one particular point of time were colonized with *Staphylococcus aureus*. To determine possible changes in *S. aureus* colonization over time, swabs were collected from the nares, throats and wounds of 61 EB patients at three time points during a period of ~2 years. All *S. aureus* isolates were typed by Multiple-locus Variable Number of Tandem Repeats Analysis and *spa*-typing. This revealed major fluctuations in the *S. aureus* types sampled from individual EB patients. In addition, blood donations were obtained from 13 EB patients to determine their IgG levels against 43 virulence factors or whole cells of *S. aureus*. Overall, the sera of EB patients contained higher anti-staphylococcal IgG levels than those of healthy individuals. Specifically, this applied to IgGs against nine important virulence factors, including the superantigens SEM, SEN and SEO. Notably, EB patients carrying different *S. aureus* types contained higher levels of anti-staphylococcal antibodies than EB patients colonized by only one type. Our findings suggest that the immune system of EB patients is heavily challenged with *S. aureus* antigens.

Introduction

Epidermolysis bullosa (EB) is a genetic blistering disease that renders patients susceptible to colonization by the opportunistic pathogen *Staphylococcus aureus* (1, 3, 10). Recently, we observed that all EB patients with chronic wounds, and 75% of the patients without chronic wounds were colonized with *S. aureus* (15). In contrast, only ~30% of the healthy human population carries this pathogen (22). Persistent *S. aureus* carriers have an increased risk for staphylococcal infections but, compared to non-carriers, their risk of death due to bacteremia is lower (23). This may relate to increased levels of protective anti-staphylococcal antibodies upon long-term exposure to colonizing strains (8). Furthermore, anti-staphylococcal antibody levels were shown to increase strongly during bacteremia (8, 18). Since high exposure to *S. aureus* is a potential health risk for EB patients, our present studies were firstly aimed at defining their *S. aureus* population over time and, secondly, at determining their anti- staphylococcal IgG levels.

Results and Discussion

Based on informed consent, 61 EB patients from the Dutch epidermolysis bullosa Registry were included in our studies. *S. aureus* colonization was determined in three rounds of sampling at half-yearly intervals. In each round, swabs were collected from 3 wounds, the left and right anterior nares, and the throat. 43 EB patients participated in the second sampling round, 40 in the third, and 35 patients participated in all three sampling rounds. Overall, we identified 101 different *S. aureus* types by molecular typing with Multiple- locus Variable Number of Tandem Repeats Analysis (MLVA). Only 18 of these MLVA types were encountered in all rounds (Figure 1A). 118 strains were also *spa*-typed, revealing 48 different *spa*-types (Table 1). Next, we compared the variations in *S. aureus* types isolated from individual EB patients over time. This revealed that the same MLVA type was identified on ~42.5% of all sampled patients with minor variations for different sites of sampling (Figure 1B). Furthermore, 58.3% of the patients with chronic wounds and 43.5% of the patients without chronic wounds carried alternating *S. aureus* MLVA types over time. In 8.7% of the patients without chronic wounds, a different MLVA type was encountered in each sampling round. These findings show that the included EB patients are continuously challenged by different *S. aureus* types and that the carried *S. aureus* population can change rapidly. This seems to challenge the classical dogma that persistent carriers are mainly colonized by one *S. aureus* type (22). However, our studies specifically address a patient group that is highly susceptible to *S. aureus* due to continuous skin defects, which is different from the situation in healthy individuals.

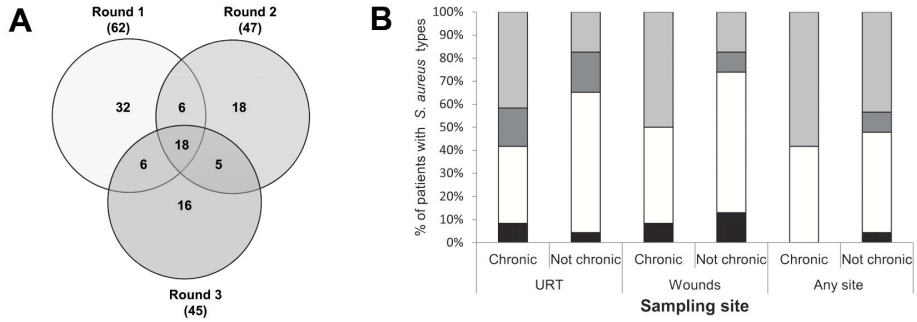

Figure 1. *S. aureus* MLVA types identified in EB patients over a period of ~2 years. (**A**) Summary of the numbers of different MLVA types identified in three rounds of sampling. (**B**) Changes in the *S. aureus* MLVA types isolated from 35 EB patients in three rounds of sampling. The MLVA typing results were analyzed for individual EB patients with chronic wounds (n=12) or without chronic wounds (n= 23). Black bars, percentage of patients not carrying *S. aureus*; white bars, percentage of patients colonized by the same MLVA type in all three sampling rounds; dark gray bars, percentage of patients colonized by different types in all three sampling rounds; light gray bars, percentage of patients with alternating MLVA types. URT, upper respiratory tract.

To assess the anti-staphylococcal IgG levels in EB patients, we first performed whole-cell Enzyme-Linked Immunosorbent Assays (ELISAs) using an *S. aureus* mutant lacking the IgG-binding proteins Spa and Sbi, and IgGs isolated from patients with chronic wounds. Sera from healthy donors were used as controls. This revealed that EB patient sera contained significantly higher anti-staphylococcal IgG levels than the controls (Figure 2). To determine specific responses, the levels of serum IgGs from 13 EB patients against 43 purified *S. aureus* antigens were measured using Luminex technology (Figure 3). As controls, the sera from 14 age-matched healthy individuals were used. For most antigens, the median fluorescence intensities (MFI) were higher in EB patients than in the control group. Especially, the MFI levels for IgGs against the surface proteins IsdA and SasG, the secreted proteins IsaA, SCIN, Nuc and LytM, and the superantigens (SAgs) SEM, SEN and SEO were statistically significantly higher in EB patients. The increased IgG levels against IsaA, SCIN, Nuc, and LytM could be explained by the fact that these proteins are expressed by many *S. aureus* types (24). Also, the *egc* gene cluster-encoded SAgs SEM, SEN and SEO are amongst the most prevalent SAgs of *S. aureus* (52%-66%) (6). Intriguingly, persistent carriers, bacteremia patients and furunculosis patients were found to develop no, or only low levels of antibodies against these SAgs (2, 5, 7). This suggests that EB patients are more significantly challenged by *egc* SAgs than healthy carriers and bacteremia or furunculosis patients.

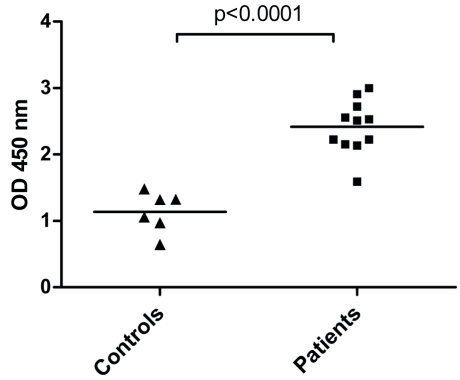

Figure 2. Serum IgG responses of EB patients to *S. aureus* cells. Cells of the *S. aureus* Newman *spa sbi* double mutant were grown till exponential phase, diluted to an OD_{600} of 0.4 and used in whole cell ELISA assays. Human IgG's were applied at 250 ng/ml and the reaction was measured at 450 nm. Squares represent IgGs from patients with EB (n=11), and triangles represent IgGs from healthy control donors (n=6). The difference in the IgG responses of EB patients and the healthy control group to *S. aureus* was statistically significant ($p<0.0001$).

To determine whether carriage of multiple *S. aureus* strains impacts on anti-staphylococcal IgG levels, we compared patients colonized by 1 MLVA type (n= 7) with patients colonized by multiple MLVA types (n=5). Interestingly, the highest MFI levels were observed for IgG's from patients carrying multiple MLVA types. This was particularly evident for IgG's against IsdA, LukD, HlgB, LytM, LukS, LukF and ETA (Figure 3B). Notably, the incidence of LukS/F is very low so, conceivably, the respective Luminex signals represent cross- reactive IgGs against the more common HlgA/B or LukE/D proteins (4). A significant correlation between anti-staphylococcal IgG levels in serum, wound fluid and sterile blister fluid was revealed in samples from one EB patient (Figure 3C). Here, the largest difference concerned 4-fold lowered anti-IsaA levels in wound fluid. This implies that future studies on anti- staphylococcal immune responses in EB patients can be based on non-invasively sampled wound fluid.

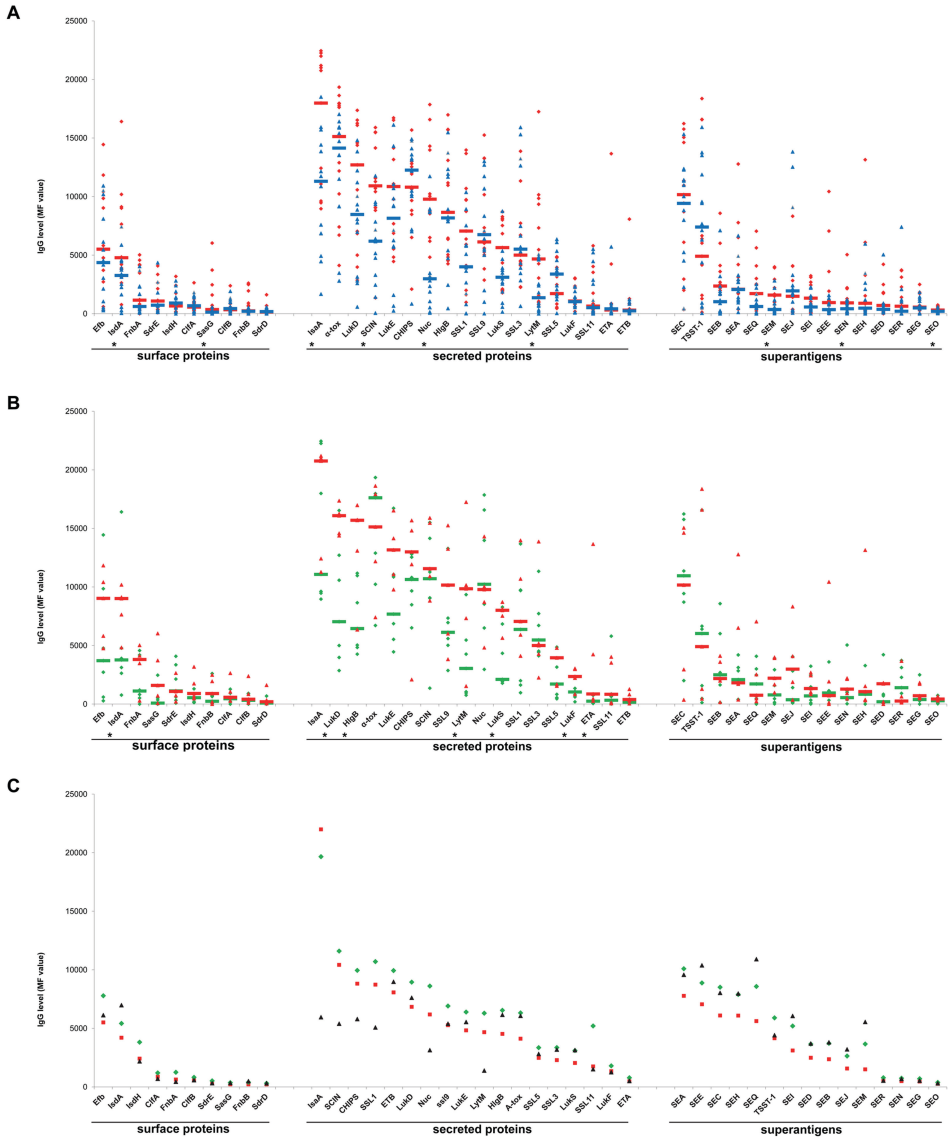

Figure 3. IgG responses of EB patients to staphylococcal antigens. IgG levels were determined by Luminex assays with (**A**) sera of EB patients, (**B**) sera of EB patients colonized by 1 or multiple MLVA types, or (**C**) serum, blister fluid and wound fluid of one EB patient. Median fluorescence intensity (MFI) values reflect the levels of antigen- specific IgGs. (**A**) red diamonds, EB patient samples (n=13); blue triangles, age-matched healthy controls (n=14). (**B**) red triangles, samples from EB patients colonized over time by multiple MLVA types (n=5); green diamonds, samples from EB patients colonized by one MLVA type (n=7). (**C**) Green diamonds, blister fluid; black triangles, wound fluid; red squares, serum. Median levels of IgGs are indicated by horizontal lines. Stars indicate statistically significant differences ($p<0.05$).

In conclusion, EB patients are highly challenged with very diverse *S. aureus* types and carriage of multiple *S. aureus* types seems to elicit the highest humoral responses in these patients. However, we cannot exclude the alternative possibility of increased humoral- and reduced cell-mediated immunity in EB patients, which might impact on *S. aureus* carriage. Notably, EB patients do not frequently suffer from *S. aureus* bacteremia, and none of the patients who donated blood was treated for staphylococcal bacteremia in the 5 years prior blood donation. This suggests that their high anti-staphylococcal antibody titers may be protective against invasive *S. aureus* infections, which would be consistent with the protective effects of IsaA-specific antibodies in mice (9).

Materials and methods

Ethical approval

The authors declare that experiments using human materials were performed with institutional approval upon written informed patient consent, and with adherence to the Helsinki Guidelines. Specifically, the medical ethics committee (METc) of the University Medical Center Groningen (UMCG) approved of the collection of non-invasive samples from patients with EB on the basis of written informed patient consent. Furthermore, the METc of the UMCG approved of the blood donations from patients with EB on the basis of written informed patient consent (approval no. NL27471,042,09). The Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Assen, the Netherlands), approved the protocol for blood donations from healthy volunteers on the basis of written informed patient consent. This protocol is registered by QPS Groningen (code 04132-CS011). The required informed consent was obtained from all EB patients and healthy volunteers included in the present studies.

Patients and bacterial isolates

In total 61 patients with EB from the Dutch epidermolysis bullosa Registry (DEBR) were recruited and sampled as described. Samples were taken from 3 distinct wounds, the left and right anterior nares, and the throat of each patient using transswabs (MWE, Corsham, England). This sampling was performed three times with about half yearly intervals. In the second round of sampling, 43 patients with EB were included and in the third round 40. In total, 35 patients with EB participated in all three sampling rounds. *S. aureus* was isolated from the obtained samples as previously described (15). Cell lysates were prepared as previously described (11).

Bacterial strains and culture conditions for whole-cell ELISA

A *S. aureus* Newman *spa sbi* double mutant strain (12) was grown overnight in RPMI

1640 without phenol red (PAA Laboratories, Austria) under vigorous shaking (250 rpm) at 37°C. The cultures were then diluted in pre-warmed RPMI 1640 medium without phenol red to an OD_{600} of 0.05, and cultivation was continued under the same conditions. Cells in the mid-exponential growth phase were collected by centrifugation, washed in phosphate-buffered saline (PBS) and diluted to an OD_{600} of 0.4. Conditions were optimized using a mix of three mouse anti-peptidoglycan monoclonal antibodies (QED, San Diego, US). These antibodies specifically recognize peptidoglycan of *S. aureus* and *S. epidermidis*.

Multiple-locus Variable Number of Tandem Repeats Analysis and *spa*-typing

Variable Number of Tandem Repeat PCR reactions on *S. aureus* isolates from EB patients were performed as previously described (15). The *spa*-typing was performed according to the RIDOM protocol (http://www.ridom.de) with previously described modifications (15).

Blood plasma, wound fluid and sterile blister fluid

Whole blood samples (12 ml) were donated by 13 patients with EB. The samples were processed for further analyses immediately after collection. In addition, 500 ml samples of whole blood were donated by 6 healthy volunteers from the UMCG and these were also processed immediately after collection. The donated blood was diluted 1:1 in Hanks' Balanced Salt Solution (HBSS, Gibco). Plasma was obtained after separation from blood cells using Ficoll-Paque PLUS (GE Healthcare) according to manufacturer's instructions. The collected human plasma was stored at -30°C. The preparation of control group serum from non-carriers that was used in the Luminex assays was previously described (17, 20, 21). Sterile blister fluid and wound fluid samples were donated by patient EB.60. Wound fluid was collected from a bandage as previously described with some modifications (13). Briefly, a bandage that had covered a wound for 1 day was removed and immediately soaked in sterile HBSS for 1 h at 4°C. The bandage was then placed in a sterile gaze (Medicomp, Germany) that was fixed in the upper part of a 50 ml tube (Corning) using the tube's screw cap. Wound fluid was then separated from the bandage by centrifugation at 3500g for 15 min at 4°C. Blister and wound fluid samples were stored at -30°C.

Purification of IgG from human plasma

IgG's were purified from the plasma of 11 patients with EB and 6 healthy volunteers by fast protein liquid chromatography using Mabselect Protein A columns and the ÄKTA explorer system (GE Healthcare). IgG was eluted in citrate buffer (pH 3.0) and immediately dialyzed against PBS. IgG concentrations were determined using a Nanodrop 2000 (Thermo scientific).

Whole cell ELISA

Microlon High-affinity ELISA microtiter plates (Greiner Bio-one, Frickenhausen, Germany) were coated with 100 µl of *S. aureus spa sbi* double mutant culture and incubated overnight at 4°C. Plates were washed three times with 0.05% Tween-20 (Sigma Aldrich) in PBS (PBS-T20), blocked in 1% (w/v) bovine serum albumin (Sigma Aldrich) in PBS for one hour at 37°C, and washed with PBS three times. Human IgGs of EB patients or healthy volunteers were then applied in the concentration of 250 ng/ml. Next, the plates were incubated for 1 h at 37°C and washed three times. Diluted (1:5000) goat anti-human IgG-Fc-PO (Brunschwig Chemie, Basel, Switzerland) was added to the wells. The plates were incubated for 1 h at 37°C, and washed three times. The reaction was developed by addition of tetramethyl benzidine and stopped by adding 0.2 M sulphuric acid. Readouts were recorded at 450 nm in a Sunrise ELISA reader (Tecan, Männedorf, Switzerland).

Luminex bead-based flow cytometry

The relative amounts of IgGs against 43 *S. aureus* antigens were determined by the bead- based Luminex flow cytometry technique (xMAP®, Luminex Corporation, Austin, Texas, USA) using plasma, sterile blister fluid or wound fluid from patients with EB, and plasma from a healthy non-carrier control group (17, 20, 21). Purification of antigens was described previously (14, 19, 20). The coupling procedure and measurements of antibody levels were performed as described (16, 20). Independent duplicate Luminex assays were performed on different days, and the median florescence intensity (MFI), reflecting the antibody concentrations, was averaged. To determine non-specific binding, control beads without coupled protein were analyzed and the resulting fluorescence was subtracted from the MFI values. Human pooled serum was used as a standard.

Statistical analyses

Statistical analyses were performed with GraphPrism 5. The results of ELISA studies were compared using two-tailed independent student *t*-tests. The differences in the median antibody levels between the patients with EB and healthy individuals, or between EB patients colonized by one or multiple *S. aureus* MLVA types as measured by Luminex assays were assessed using the Mann-Whitney U test. Differences were considered statistically significant when 2-sided *P*-values were <0.05.

Abbreviations

Clf, clumping factor; EfB, extracellular fibrinogen-binding protein; ET, exfoliative toxin; Fnb, fibronectin binding protein; HlgB, gamma-hemolysin B; IsaA, immunodominant antigen A; Isd, iron-responsive surface determinant; Luk, leukocidin; LytM, peptidoglycan hydrolase;

Nuc, endonuclease; SasG, *S. aureus* surface protein G; SCIN, staphylococcal complement inhibitor; CHIPS, chemotaxis inhibitory protein of *S. aureus*; Sdr, serine-aspartate dipeptide repeat protein; SE, staphylococcal enterotoxin; SSL, staphylococcal superantigen-like protein; TSST-1, toxic shock syndrome toxin.

Conflict of Interest

The authors state no conflict of interest. However, it should be noted that G.S.E., H.W., H.P.J.B., and He.G. are employees of IQ Therapeutics.

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Table 1. *S. aureus* MLVA types and *spa*-types collected from individual EB patients. nd not determined; empty cell, negative for *S. aureus*; # two isolates with the same type; -, no swab obtained; * received 5 days oxacilin before swabbing

				ROUND 1										ROUND 2										ROUND 3									
				Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3		Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3		Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3	
№	Blood	EB type	category	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa
1	yes	JEB	chronic wounds	656	t1081	565	nd			656	nd	238	t377	538	t065	372	t091			321	t377	538	t065					322	t652	238	t377			372	nd	238	nd		
2	yes	JEB	chronic wounds	550	t026	550	nd	550	nd	550	nd	550	nd	550	nd													550	t026	550	nd	550	nd	550	nd	550	nd	-	-
3		EBS	not chronic wounds	2024	t216	2024	nd			2024	nd	2024	nd	2024	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4		DEB	chronic wounds	7	t091	7	nd			665	nd	7	nd			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5		EBS	chronic wounds	1965	t179	184 & 1965	t085 & nd	1965	nd	1965	nd	1965	nd	1965	nd	1965	t179	1965	nd	1965 #	t179	1965 #	nd	1965	nd	1965	nd	1965	nd	1965	nd	1965	nd	1965	nd	1965	nd	1965	nd
6		DEB	chronic wounds	1101	t5515					1162	nd	1162	t008			-	-	-	-	-	-	-	-	-	-	-	-	2586	t5153	2586	nd	1162	t008	1162	nd	2586	nd	2586	t5153
7		JEB	chronic wounds	565	t571	656	nd			565	nd	565	nd	565	nd	572	t1451	572	nd	572	nd	565	t571	565	nd	565	nd			565 & 2587	nd & t571	565	nd			565	nd	2587	t571
8		EBA	chronic wounds					332	t723																									-	-	-	-
9	yes	JEB	chronic wounds	1966	t127	1967	t127	1967	nd	1966	nd	1966	nd			1966	t127	1966	nd	1967	nd	1966	nd	1966 #	t127	1966	nd	2588	t015	2588	nd	1967	t127	2588	nd	2588	nd	2588	nd
10		JEB	not chronic wounds	330	t008	1968	t349	1968	nd	330	nd	330	nd	330	nd	330	t008	330	nd	330	nd			330	nd	330	nd	330	nd	330	nd								
11	yes	EBS	not chronic wounds	482	t148	482	nd	482	nd	482	nd	482	nd									482	t148			482	nd			482	nd			482	nd	482	nd		
12			not chronic wounds	538	t065			314	t008	8	t008	8	nd	-	-	538	t065	538	nd			2609	t127					484	nd	538	nd	484	nd								
13		EBS	not chronic wounds					613	t159			-	-	-	-													1165	t385	1970	t010			309	t377				
14	yes	DEB	chronic wounds							2025	t311	67	t311	67	nd	-	-	-	-	-	-	-	-	-	-							67	t311	67	nd	67	nd		
15	yes	JEB	chronic wounds	330	t008	330	nd	330	nd	330	nd	330	nd	330	nd																								
16		EBS	not chronic wounds							1969 & 1970	t084 & t010	1969	nd	1969	nd							2589	t272	2589	nd					1165	t385	1970	t010			-	-	-	-
17		EBS	not chronic wounds									1970	t010			1970	t010					1970	nd																
18		EBS	chronic wounds	1837	t502	1837	nd	1837	nd	1837	nd	1837	nd	1837	nd	-	-	-	-	-	-	-	-	-	-	-	-	2590	t254	2590	nd			2590	nd	2590	nd	2590	nd
19		EBS	not chronic wounds									-	-	-	-																			-	-	-	-		
20		EBS	not chronic wounds									-	-	-	-																	319	t008			-	-	-	-
21		JEB	chronic wounds					1971	t4169	1972	t401					15	t084			1971	t4169	2612	t401	15 #	t084 #	181	t084	181	nd	181	nd	181	nd	181	nd	181	nd		
22		EBS	not chronic wounds	321	t1476					321	nd	321	nd	321	nd	321	t1476	321	nd			321	nd	321	nd	321	nd	321	nd					321	nd	321	nd	321	nd
23		EBS	not chronic wounds							321	t1476	321	nd	321	nd	321	t1476	321	nd			321	nd	321	nd	321	nd	321	nd	321	nd			321	nd	321	nd	321	nd
24		EBS	not chronic wounds							321	nd	321	t1476	321	nd	321	t1476	321	nd			321	nd	321	nd	321	nd												
25		EBS	not chronic wounds	2026	t1827	2026	nd			2031	t704	-	-	-	-	2605	t1827					2591	t704	2591	nd	2591	nd	2031	t704			2591	nd	2591	nd	2591	nd		
26		DEB	not chronic wounds	1973	t1252	1973	nd	1973	nd	1973	nd	1973	nd			1973	t1252	1973	nd			1973	nd	1973	nd	1973 #	nd & t1252	-	-	-	-	-	-	-	-	-	-	-	-
27		DEB	not chronic wounds	1973	t1252	1973	t1252	1973	nd			1973	nd			1973	t1252	1973	t1252			1973	t1252	2592	t1252	1973	nd	-	-	-	-	-	-	-	-	-	-	-	-
28		DEB	not chronic wounds					321	t377	321	nd	321	nd									2593	t177					-	-	-	-	-	-	-	-	-	-	-	-
29		DEB	chronic wounds					322	t5511	322	nd	322	nd									322	t5511	321	t648									321	t648				
30		SFE DS	chronic wounds	1057	t5508	1057	nd	654	t5513	632	t267	632	nd	632	nd	-	-	-	-	-	-	-	-	-	-	-	-	1057	t5508	1057	t5508	2588	t015	2588	nd	2588	nd	2588	nd
31		EBS	not chronic wounds	2027	t291	2027	nd			2027	nd	2027	nd	2027	nd	2027	t291	2027	nd			-	-	-	-	-	-	2027	nd	2610	nd			2027	nd	2610	nd	-	-

				ROUND 1												ROUND 2												ROUND 3											
				Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3		Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3		Left nare		Right nare		Throat		Wound 1		Wound 2		Wound 3	
Nr	Blood	EB type	category	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa	MLVA	spa
32		EBS	not chronic wounds	534	t026			534	nd	534	nd	534	nd	534	nd	534	t026	534	nd	534	nd	534	nd	534	nd	534	nd			534	nd	534	nd	534	nd	534	nd	534	nd
33		EBS	not chronic wounds							45	t050	45	nd	45	nd											-	-	-	-	-	-	-	-	-	-	-	-	-	-
34		DEBt	chronic wounds	1974	t015	1974	nd	1974	nd	1974	nd	1974	nd	1974	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35		EBS	not chronic wounds	490	t5510			490	t5510	490	nd	1975	t5510	1976	t5510											-	-	-	-	-	-	-	-	-	-	-	-	-	-
36		EBS	not chronic wounds	1977	t450	1977	nd	1978	t450	1979	t450	1981	t450	-	-	1981	t450	1981	nd	1981	nd	1981	nd					1981	nd	1981	nd	1981	nd	1981	nd	1981	nd		
37		DEB	not chronic wounds							-	-	-	-	-	-	2573	t837					2573	nd	2573	nd	-	-	2573	nd					2573	nd	2573	nd	-	-
38		DEB	not chronic wounds							-	-	-	-	-	-	2594	t493	2594	t493					-	-	-	-			2573	t837							-	-
39		DEB	not chronic wounds							-	-	-	-	-	-	2594	t493	2594	t493	t837			2573	-	-	-	-									-	-	-	-
40		EBS	not chronic wounds	1982	t130	510	t065			-	-	-	-	-	-	510	t065	510	t065	510	nd	-	-	-	-	-	-	2595	t084	2595	nd	2595	nd	2595	t084	-	-	-	-
41		EBS	not chronic wounds	510	t065	510	nd			-	-	-	-	-	-	510	t065	510	nd					-	-	-	-	510	nd	510	nd			510	nd	-	-	-	-
42	yes	JEB	chronic wounds			46	t1154			46	nd	46	nd					46	t1154															46	nd			-	-
43		JEB	chronic wounds	1552	t224	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	t224	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd	1552	nd
44		DEB	chronic wounds			67	t311	67	n	1983	t098	1983	nd	1316	t012			67	t311			2596	t127	2596	nd	2596	nd	-	-	-	-	-	-	-	-	-	-	-	-
45		DEB	not chronic wounds							-	-	-	-	-	-	2597	t140	2597				2597	nd	2597	nd	-	-							2597	nd	2597	nd	-	-
46		DEB	chronic wounds	67	t311			1107	t021			1316	t012	1316	nd							1316	t012	1316	nd	1316	nd	2607	nd	1376	nd			1376	nd	1376	nd		
47		DEB	not chronic wounds			770	t018			-	-	-	-	-	-	770	t018	770	t018			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48		EBS	not chronic wounds			1140	t005							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49		DEB	not chronic wounds																			-	-	-	-	-	-	1933 #	t701 #	1933	nd			2611 & 1933	t056 & nd	-	-	-	-
50		DEB	not chronic wounds	1984	t160			1984	nd	489	t1869	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			177	t084			489	t1869	489	t1869	489	nd
51	yes	DEB	chronic wounds	1987	t5512	1987	nd	733	t067	1987	nd	1988	t5514	1989	t5512	1988	t5514	1988	nd			1988	nd	733	t067	1988 #	t5514 #	1988	nd	733	t067	1988	nd	1988	nd	1988	nd	733	t067
52		DEB	not chronic wounds											-	-					7	t091					-	-			58	t002			58	t002				
53	yes	JEB	not chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-	371	t091	371	nd			371	nd					-	-	-	-	-	-	-	-	-	-	-	-
54		DEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-	1502	t091	1502	nd			1502	nd	1502	nd	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
55	yes	DEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-					15	t084	15	nd	15	nd	15	nd	-	-	-	-	-	-	-	-	-	-	-	-
56		JEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57		DEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-	138	t127	138	nd			138	nd	138	nd	138	nd	-	-	-	-	-	-	-	-	-	-	-	-
58	yes	DEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-	2598	t002	2599	nd			2598	nd	-	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
59	yes	DEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-							272	t008	272	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60	yes	JEB	chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
61		EBS	not chronic wounds	-	-	-	-	-	-	-	-	-	-	-	-			5	t002			5	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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4

Topography of distinct *Staphylococcus aureus* types in chronic wounds of patients with epidermolysis bullosa

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Abstract

The opportunistic pathogen *Staphylococcus aureus* is known to interfere with wound healing and represents a significant risk factor for wound infections and invasive disease. It is generally assumed that one individual is predominantly colonized by one *S. aureus* type. Nevertheless, patients with the genetic blistering disease epidermolysis bullosa (EB) often carry multiple *S. aureus* types. We therefore investigated whether different *S. aureus* types are present in individual wounds of EB patients and, if so, how they are spatially distributed. The staphylococcal topography in chronic wounds was mapped by replica-plating of used bandages and subsequent typing of *S. aureus* isolates. Individual chronic wounds of five patients contained up to six different *S. aureus* types. Unexpectedly, distinct *S. aureus* types formed micro-colonies that were located in close proximity and sometimes even overlapped. While some adjacent *S. aureus* isolates were closely related, others belonged to distinct molecular complexes. We conclude that the general assumption that one individual is predominantly colonized by one type of *S. aureus* does not apply to chronic wounds of EB patients. We consider this observation important, not only for EB patients, but also for other patients with chronic wounds in view of the potential risk for severe staphylococcal infections.

Introduction

Staphylococcus aureus is a Gram-positive bacterium that colonizes ~30% of the healthy human population (10). However, under certain circumstances, *S. aureus* transforms from harmless commensal into a dangerous pathogen that can cause life-threatening invasive diseases (3). Accordingly, *S. aureus* carriage has been associated with an increased risk of severe infections (21-23). In most carriers, only one type of *S. aureus* is encountered, suggesting an intimate relationship between the carrier and the carried strain (22). However, the presence of multiple *S. aureus* types has also been reported in healthy individuals and in patients with particular diseases, such as cystic fibrosis, atopic dermatitis and the genetic blistering disease epidermolysis bullosa (EB) (7, 11, 15, 19, 20). The presence of multiple *S. aureus* types was shown to be exceptionally high in EB patients with chronic wounds, who were shown to carry up to four *S. aureus* types at one time point of sampling (20). Over longer periods of time, up to six alternating *S. aureus* types were isolated from these patients, and this high exposure to *S. aureus* seems to elicit strong antistaphylococcal immune responses (19).

Notably, in most previous studies, the observed co-existence of multiple *S. aureus* types related to the analysis of swab samples from different body sites where one *S. aureus* colony was analyzed per swab sample (19, 20). In only one study, multiple *S. aureus* colonies derived from individual nasal and perianal swap samples were analyzed, showing that children selected for elective surgery carried up to three *S. aureus* types at one particular body site (15). To date, the co-existence of multiple *S. aureus* types has not been studied quantitatively and, due to the swab sampling methodology, nothing is known about the relative distances at which these co-existing *S. aureus* types are located from each other. Furthermore, little information is available on the genetic relatedness of co-existing *S. aureus* types and hence it is not known whether these are derived from in-patient evolution or the simultaneous acquisition from different sources (8, 13). The present studies were therefore aimed at investigating the co-existence of different *S. aureus* types in relation to their genetic relatedness and spatial distribution *in vivo*. This is only possible by investigating individuals that carry these *S. aureus* types on body sites that are readily accessible for direct sampling without disturbing the microbial topography. As shown here, such requirements are met by the chronic wounds of patients with EB (6), which are extensively colonized with *S. aureus* (4, 16, 20). Briefly, we investigated the spatial distribution of *S. aureus* in the chronic wounds of five EB patients by replica plating of used bandages onto agar plates, and subsequent typing of multiple *S. aureus* colonies. As shown by fluorescence *in situ* hybridization (FISH), the typed colonies on the replica plates represent micro-colonies of *S. aureus* that already existed in the investigated wounds. Up to six co-existing *S. aureus*

types were encountered in individual wounds, often in close proximity. While some adjacent *S. aureus* isolates were closely related, others belonged to distinct molecular complexes.

Results and discussion

S. aureus topography in chronic wounds

To determine the topography of *S. aureus* in chronic wounds of EB patients, used bandages of five patients were replica plated onto cysteine lactose electrolyte-deficient (CLED) agar. These bandages had covered the wounds for ~24 h and they were replica plated immediately upon redressing of the wounds. The plates were then incubated overnight at 37°C. Depending on the patient and the investigated wounds, we observed either confluent growth or separate colonies at different densities. Where possible, regions of the plates with separate colonies (Figure 1) were directly used for further analysis by picking of colonies and subsequent species determination using standard diagnostic methods. In case of patient 62, only overlapping colonies were detectable. Therefore, these colonies were streaked on fresh plates so that individual colonies could be isolated for further analyses. The vast majority of the investigated colonies from all patients included in this study were shown to represent *S. aureus*. Next, we investigated whether such colonies were derived from individual cells or pre-existing micro-colonies in the investigated wounds. To this end, the bandages of one EB patient were pressed onto glass slides and FISH analyses were performed with an *S. aureus*-specific oligonucleotide probe (Figure 2). As a control, FISH was performed with a probe specific for bacteria in general. This confirmed that the vast majority of bacteria present in the investigated wounds were *S. aureus*. Importantly, these *S. aureus* cells were mostly present in micro-colonies, although individual *S. aureus* cells were also detectable. We therefore conclude that the bacterial wound topography as observed through replica plating of used bandages closely reflects the actual bacterial topography in the wounds that were dressed with the respective bandages. In those cases where confluent growth on plates was observed by replica plating, the bacteria in the wounds are apparently present in the form of biofilms (24).

Co-existence of distinct *S. aureus* types in chronic wounds

The presence of different co-existing *S. aureus* types in the investigated wounds was first assessed by Multiple-locus Variable Number of Tandem Repeats Fingerprinting (MLVF), because this technique allows the rapid and high-resolution typing of many *S. aureus* isolates in a short period of time (17). Per replica-plated bandage, we typed 12 to 48 colonies, and for each patient at least one replica-plated bandage was included from which 24 colonies were typed. In total, 368 *S. aureus* colonies were typed (36 to 85 colonies per patient at any particular

time point of sampling), which yielded 27 distinct *S. aureus* MLVF types. Thus, we typed a substantially larger number of colonies per patient than the 20 colonies per patient that were previously recommended for studies with small numbers of carriers (5). As shown in Figure 3A, we detected between two and six different *S. aureus* types in individual chronic wounds of four EB patients at one time point of sampling. By investigating the wound colonization in patient 14 over time, we detected even up to 10 different *S. aureus* types (Figure 3B). In the wounds of patients 1 and 14, dominant MLVF types were identified, which were accompanied by several less abundant MLVF types. On the other hand, less drastic differences were observed in the relative abundance of identified MLVF types in the wounds of patients 62, 63 and 64 (Figure 3A). Intriguingly, when the MLVF results were assessed in relation to the topography of typed *S. aureus* colonies, it became clear that different *S. aureus* types can exist in very close proximity (Figure 1). This conclusion was underscored by the observation that six different types of *S. aureus* were separated from overlapping colonies in the sampled wound of patient 62. To our knowledge this is the first time that the relative numbers of co-existing *S. aureus* types at one particular body site have been quantified, and that their topography has been mapped in detail. Also, the co-existence of different *S. aureus* types in close proximity has not been shown before.

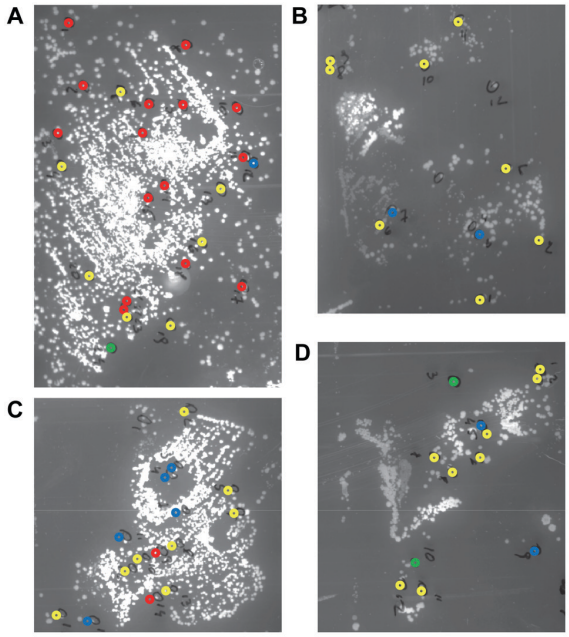

Figure 1. *S. aureus* chronic wound topography. Used bandages from four chronic wounds (**A-D**) of patient 64 were replica-plated onto CLED agar plates. The plates were incubated overnight at 37°C. Individual *S. aureus* colonies were isolated for typing by MLVF. *S. aureus* colonies with different MLVF types are indicated in dfferent color codes: red, type 1; blue, type 2, yellow, type 3; green, type 4.

To approximate the relatedness of the mapped *S. aureus* types, the results of our MLVF analyses were evaluated with GelCompar II (Applied Maths). As shown in the resulting dendrogram, the different *S. aureus* isolates from patient 14 all clustered together due to their similar MLVF patterns (Figure 4). This suggests that these isolates share a common ancestry, and that the dominant MLVF type 1 has been most successful in colonizing the investigated wounds of patient 14. Smaller clusters of two to four related *S. aureus* MLVF types were also identified in the wounds of patients 1, 62, 63 and 64, which suggests that some of the staphylococci in wounds of these patients have recently evolved from a common ancestor. However, individual wounds of the latter patients also contained apparently unrelated MLVF types. Since this suggested that the latter isolates represent very different *S. aureus* types, they were further analyzed in detail by *spa*-typing and Multiple-locus Variable Number of Tandem Repeats Analysis (MLVA). These analyses indeed demonstrated that the wounds of patients 62, 63 and 64 contained *S. aureus* types that belong to distinct molecular complexes (Table 1). Some of these types can actually also be recovered from the nares or throats of the respective patients (data not shown), but these body sites also contain other *S. aureus* types as shown in Figure 4 for patients 1 and 63.

In conclusion, our present study shows that chronic wounds of individual EB patients can be colonized by several distinct *S. aureus* types. As shown here for the first time, these distinct *S. aureus* types can form micro-colonies that are located in close proximity (Figure 1). This implies that these different types are not mutually exclusive. It thus seems that the dogma that one individual is usually colonized by one *S. aureus* type does not apply to the wounds of patients with EB. We consider this finding important, not only for patients with EB, but also for other patients with chronic wounds, because *S. aureus* is known to interfere with wound healing and poses a significant risk factor for infections and invasive disease (12, 14).

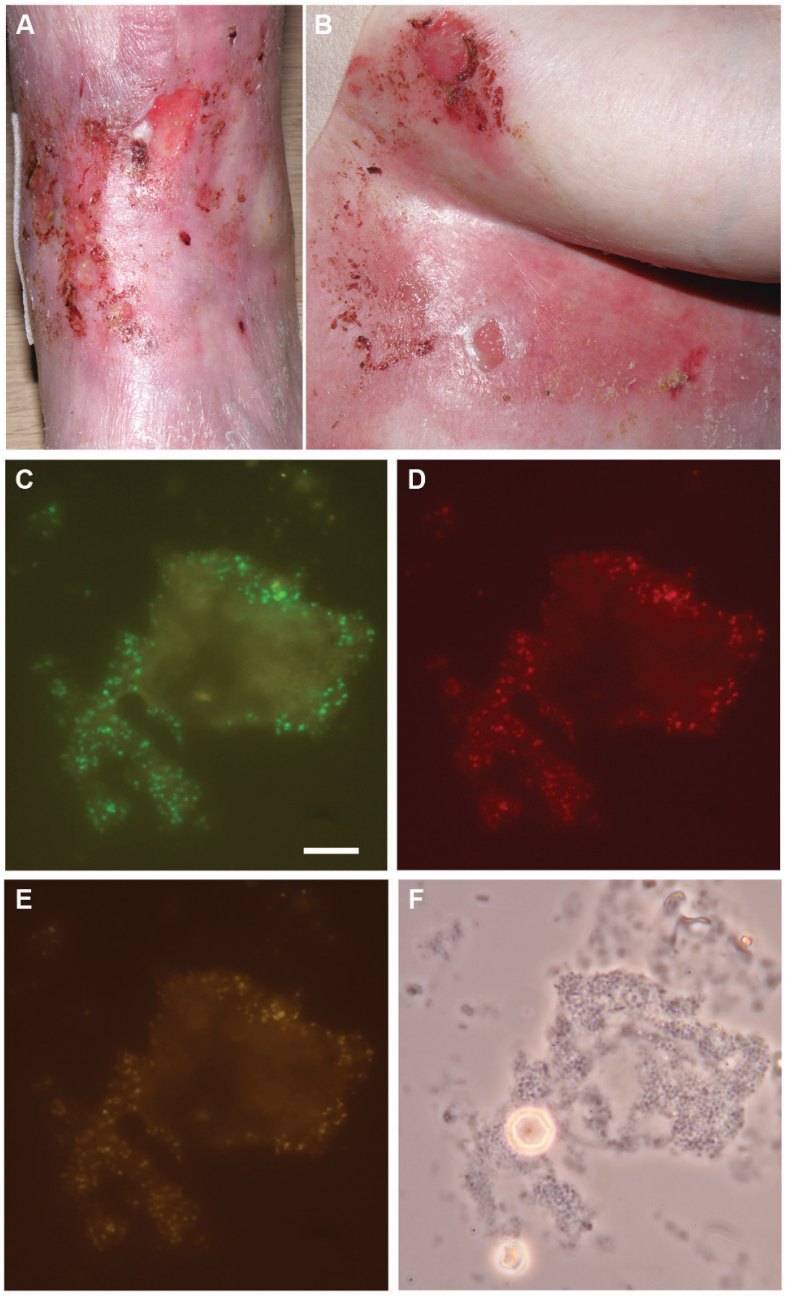

Figure 2. Chronic wounds contain microcolonies of *S. aureus*. To study the micro-topography of *S. aureus* in chronic wounds of patient 14 (**A**, **B**), a glass slide was pressed onto a freshly removed used bandage. The presence of *S. aureus* was visualized by FISH using an *S. aureus*-specific PNA probe. (**C**), FISH with a universal bacterial PNA probe; (**D**), FISH with an *S. aureus*-specific PNA probe; E, overlay of images (**C**-**D**; **F**), phase contrast image. The white bar in panel C indicates 10 μm.

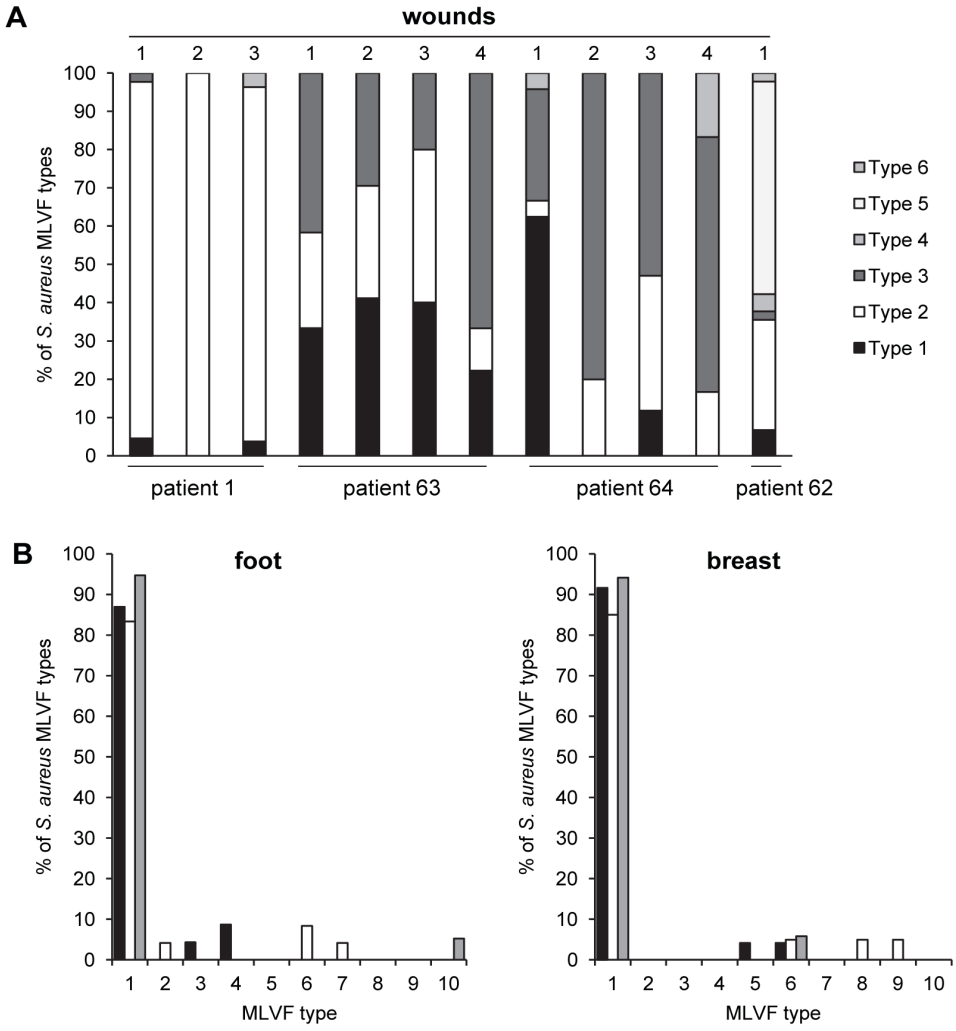

Figure 3. Relative numbers of MLVF types detected in the chronic wounds of five EB patients. (**A**) The percentages of different *S. aureus* MLVF types detected in individual chronic wounds from patients 1, 62, 63 and 64 at one particular time point are indicated by bar diagrams. Different MLVF types are marked in black, white or grey shading. (**B**), percentages of different *S. aureus* MLVF types detected in chronic foot and breast wounds from patient 14. Replica plating of used bandages was performed thrice at two-weekly intervals. Black bars mark the first, white bars the second and grey bars the third time point of sampling.

Figure 4. MLVF dendrogram of different *S. aureus* types isolated from chronic wounds of patients with EB. The dendrogram was generated using the Dice coefficient with Tolerance 0.6 and optimization 0.5. Clusters of isolates were generated with a cut-off value set to 60%. The MLVF types of individual *S. aureus* isolates are indicated in the dendrogram together with the number of the patient from which they were collected.

Materials and methods

Ethical approval

The authors declare that experiments using human materials were performed with institutional approval upon written informed patient consent, and with adherence to the Helsinki Guidelines. Specifically, the medical ethics committee (METc) of the University Medical Center Groningen (UMCG) approved of the collection of non-invasive samples from patients with EB on the basis of written informed patient consent. The required informed consent was obtained from all EB patients included in the present studies.

Patients and bacterial isolates

In total, five patients with EB from the Dutch epidermolysis bullosa Registry (DEBR) were recruited in this study (note that patient numbers used in this paper correspond to van der Kooi-Pol *et al.* (2012a,b). Samples from three patients (62, 63, 64) were taken from three distinct wounds, the left and right anterior nares, and the throat of each patient using transswabs (MWE, Corsham, England). *S. aureus* was isolated from the obtained samples as previously described (20). In addition, the bandages from one to four different wounds were collected from all patients. In case of patient 14, the bandages from two distinct wounds

were collected three times at two-weekly intervals. All collected bandages were immediately replica-plated onto CLED agar (Oxoid) by gently pressing them for 5 to 10 s on the agar surface. To this end, bio assay plates were used (245x245x25 mm; Nunc). After 24 h of incubation at 37°C, 12 to 48 *S. aureus* colonies per wound/bandage were selected for further analysis. The isolation of *S. aureus* was confirmed by standard diagnostic methods.

Molecular characterization of *S. aureus*

The extraction of total DNA from *S. aureus* isolates for PCR amplification was performed as described by Schouls *et al.* (2009). All isolated *S. aureus* colonies were typed by MLVF as previously described (17). Only identical patterns were regarded as the same subtype. Isolate clusters were delineated with a 60% similarity cutoff value. In addition, unique *S. aureus* MLVF types isolated from bandages of EB patients 62, 63 and 64 were characterized by MLVA and *spa*-typing as previously described (20).

Fluorescent *in situ* hybridization

Bandages from EB patient 14 were used for FISH. Slides coated with gelatin-suspension (0.1% gelatin, 0.01% $KCr(SO_4)_2.12H_2O$, Sigma) were pressed onto used bandages (15 s), air-dried, fixed with 96% ethanol (5 min), and stored at -20°C until further processing. FISH was performed as previously described with minor modifications (9, 9). Briefly, cells were fixed with 4% paraformaldehyde for 1 h at 4°C, and permeabilized with 2 U/ml lysostaphin. After dehydration with 96% ethanol, the cells were hybridized for 2 h at 50°C with the STAUR (2) and EUB338 (1) probes (5 ng/µl each) in hybridization buffer, and then washed for 10 minutes in wash buffer (9). Fluorescence microscopy was performed with a Leica DM RXA microscope. The phase contrast and fluorescent images were merged using Adobe Photoshop CS4.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: M.M.v.d.K.P., H.J.M.H. and J.M.v.D.
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Analyzed the data: M.M.v.d.K.-P, A.J.S., and H.J.M.H..
Contributed reagents/materials/analysis tools: J.C.D., and M.F.J.
Wrote the paper: M.M.v.d.K.P. and J.M.v.D.

Table 1. MLVA and *spa*-typing of *S. aureus* isolates from chronic wounds of EB patients.

	MLVF	*spa*	MLVA		VNTR[a] number (24)							
			type	MC[b]	09_01	61_01	61_02	67_01	21_01	24_01	63_01	81_01
Patient 62	Type 1	t254	1298	15	7	5	2	1	1	9	99	4
	Type 2	t254	1298	15	7	5	2	1	1	9	99	4
	Type 3	t6993	3393	none	7	5	2	1	1	8	99	4
	Type 4	t254	1298	15	7	5	2	1	1	9	99	4
	Type 5	t7473	532	45	11	3	3	4	1	12	1	5
	Type 6	t9968	45	45	11	3	3	4	1	11	1	5
Patient 63	Type 1	t002	23	5	14	2	1	3	2	11	7	5
	Type 2	t078	2049	none	12	0	2	2	1	10	6	3
	Type 3	t078	2049	none	12	0	2	2	1	10	6	3
Patient 64	Type 1	t9969	3391	none	14	1	1	1	1	10	7	5
	Type 2	t509	3392	none	14	1	1	1	1	9	6	5
	Type 3	t015	45	45	11	3	3	4	1	11	1	5
	Type 4	t509	3392	none	14	1	1	1	1	9	6	5

[a]VNTR, Variable Number of Tandem Repeats.

[b]MC, molecular complex.

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5

Tryptic striptease of *Staphylococcus aureus* unveils the cell surface localization of immunodominant epitopes

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[#] both authors contributed equally to this work

To be submitted

Summary

The opportunistic pathogen *Staphylococcus aureus* has become a major threat for human health and well-being by developing resistance to antibiotics, and by its fast evolution into new lineages that rapidly spread within the healthy human population. This calls for the development of active or passive immunization strategies to prevent or treat acute phase infections. Since no such anti-staphylococcal immunization approaches are available as yet, the present studies were aimed at identifying new leads for their development. For this purpose, we thoroughly profiled the cell surface-exposed staphylococcal proteome by combining two surface shaving approaches. In parallel, non-covalently cell wall-bound proteins were extracted with KSCN and analyzed by gel-free proteomics, and also the exoproteome was analyzed through gel-free proteomics. Lastly, we screened a selection of the identified cell wall-attached proteins for binding of immunoglobulin G from patients that have been challenged with different types of *S. aureus* over extended periods of time due to chronic wound colonization. The combined results of these analyses highlight particular cell surface-exposed *S. aureus* proteins with highly immunogenic epitopes as potentially powerful targets for the development of protective anti-staphylococcal immunization strategies.

Introduction

The Gram-positive bacterium *Staphylococcus aureus* is an opportunistic pathogen that asymptomatically colonizes approximately 30% of the healthy human population (2, 12, 27, 43, 46, 55). Upon invasive growth, *S. aureus* can cause a wide variety of diseases ranging from relatively mild skin infections to severe sepsis. A major reason for concern is the high propensity of *S. aureus* to acquire resistance to antibiotics (6, 45). This is critically underscored by the rapid development of resistance to the antibiotic methicillin. When methicillin was first introduced into the clinic, only methicillin-sensitive *S. aureus* (MSSA) was encountered. However, within two years, the first methicillin-resistant *S. aureus* (MRSA) isolates were observed (30). While MRSA was initially only a threat for hospitalized, elderly and frail individuals, the last two decades have witnessed the emergence of so-called community-acquired MRSA lineages that rapidly spread within the young and healthy population (31, 36, 44). Importantly, community-acquired MRSA lineages have now also entered nosocomial settings, which gives rise to increased morbidity and mortality rates. It is therefore a major societal challenge to develop novel, effective and long-lasting anti-staphylococcal therapies (5, 36, 38, 41).

In principle, vaccination is a very effective approach for protecting individuals at risk against pathogenic microbes. Unfortunately however, no vaccines against *S. aureus* in general, or MRSA in particular, are currently available (37, 54). While there have indeed been various attempts to develop vaccines for preventing staphylococcal infections, none of the candidate vaccines has successfully passed (pre-) clinical trials. Most likely, this relates to multiple factors, including the ability of *S. aureus* to evade or suppress the human immune system (1, 23, 26, 42, 48) as well as the high genome plasticity and adaptability of this wide-spread pathogen (8, 14, 19, 31, 33, 56). Also, the published attempts to develop anti-staphylococcal vaccines were so far focused on a relatively narrow group of known *S. aureus* antigens, including capsular polysaccharides and a few cell wall-associated or secreted proteins (37, 54). In this context, it is noteworthy that recent proteomics analyses have indicated the presence of at least 449 different proteins in the *S. aureus* cell envelope (8, 9, 13, 17, 47, 53). These proteins may include powerful targets for future anti-staphylococcal immunization strategies, especially if they are exposed to the extracellular environment where they are readily recognizable by the human immune system. However, relatively little is known about the *S. aureus* cell surface-exposed proteome - the ‘surfacome’ - in terms of the particular protein domains that are directly exposed to the extracellular milieu. Even less is known about the presence of possible immunodominant epitopes within such exposed protein domains that could be used for the development of novel immunization approaches.

To pinpoint candidate targets for novel anti-staphylococcal immunization approaches, the present studies were focused on the identification of immunodominant cell surface-exposed protein domains of *S. aureus*. For this purpose, we first performed an in-depth proteomics analysis of the surfacome based on the incubation of *S. aureus* cells with immobilized trypsin that cannot penetrate into the cell wall (*i.e.* 'cell surface shaving' (8)) or with soluble trypsin that can penetrate into the deeper cell wall layers. In parallel, we analyzed proteins released from the cells by spontaneous shedding or by treatment with the chaotropic compound KSCN, and we analyzed the extracellular proteome (*i.e.* the 'exoproteome') of the investigated cells. Lastly, a screen for immunodominant epitopes in the identified cell surface-exposed proteins was performed using peptide arrays and serum immunoglobulin G (IgG) from patients with the genetic blistering disease epidermolysis bullosa (EB). As shown in previous research, these patients are exposed to multiple and alternating types of *S. aureus* over long periods of time due to chronic wound colonization (3, 51, 52). Altogether, the present 'tryptic striptease' of the *S. aureus* cell unveils a set of cell wall-localized and surface-exposed antigens that may serve as targets for novel active or passive immunization approaches to prevent or treat staphylococcal infections.

Methods

Bacterial strains and growth conditions

The *S. aureus* strains Newman, Newman Δ*spa*Δ*sbi* and USA300 were grown overnight in tryptic soy broth (TSB, OXOID) under vigorous shaking at 37°C. The cultures were then diluted into pre-warmed RPMI 1640 medium (PAA) to an OD_{600} of 0.05 and cultivation was continued under the same conditions. Exponentially growing cells were again diluted into fresh and pre-warmed RPMI medium to a final OD_{600} of 0.05 and their cultivation was continued to an OD_{600} of 0.2.

Isolation and processing of sub-proteome fractions

Exoproteome- Cells were separated from the growth medium by centrifugation (15 min, 6750 × g, 4°C). The growth medium fraction thus obtained was filtrated (pore size 0.22 μm), and the exoproteome present in this fraction was precipitated overnight at 4°C with 10% trichloroacetic acid (TCA). Precipitated proteins were pelleted by centrifugation (20 min, 18620 × g, 4°C) and washed with acetone. Protein pellets were dried and resuspended in 50 mM ammonium bicarbonate and overnight digested with trypsin (Promega) at 37°C.

Surfacome shaving with immobilized trypsin- Cells were harvested by centrifugation (10 min, 6080 × g, 4°C) and washed twice with PBS containing 40% sucrose (Acros) and 20 mM sodium azide (Sigma-Aldrich). Immobilized trypsin (Pierce) was activated

with 50 mM ammonium bicarbonate (Sigma-Aldrich), resuspended in 50 µl PBS with 40% sucrose and 20 mM sodium azide, and added to the washed cells. The shaving reaction was conducted for 45 min at 37°C. Released peptides representing the surfacome were isolated, reduced with 10 mM DTT (30 min), alkylated with iodoacetamide (Sigma-Aldrich), and digested with trypsin overnight at 37°C.

Surfacome shaving with soluble trypsin- The protocol for cell shaving with soluble trypsin was essentially the same as the protocol for shaving with immobilized trypsin. In this case immobilized trypsin in 48 µl PBS with 40% sucrose and 20 mM sodium azide was added to the cells.

Spontaneously released proteins- The protocol for analysis of proteins spontaneously released from the cells was essentially the same as the protocol for shaving with immobilized trypsin. In this case, 50 µl PBS with 40% sucrose was added to the cells.

Non-covalently cell wall-bound proteins- Cells were harvested by centrifugation (10 min, 6750 × g, 4°C), washed twice in PBS with 40% sucrose and 20 mM sodium azide, resuspended in 1 M potassium thiocyanate (KSCN), and incubated for 10 min on ice. After centrifugation (10 min, 6750 × g, 4°C), the resulting supernatant was filtrated (pore size 0.22 µm) and proteins in the filtrate were precipitated with TCA. The collected proteins were digested with trypsin overnight at 37°C.

Mass spectrometric analyses

Reduction and alkylation, desalting of the samples, mass spectrometric (MS) analysis and database searches were performed as previously described (8). The strain-specific uniprot databases were used for the *S. aureus* strains Newman and USA300, respectively including concatenated reversed databases with 5250 and 5298 entries. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they exceeded the specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides, respectively. All experiments were conducted in independent triplicates. Peptides were only accepted as identified if they were detected in at least two out of the three replicates per sample set. With these filter parameters no false positive hits were obtained. The identified peptides of strain Newman are listed in Supplemental Table 1 (available on request) and the identified peptides of strain USA300 are listed in Supplemental Table 2 (available on request).

Western Blotting

Protein samples were separated using NuPAGE gels (Invitrogen) and separated proteins

were subsequently transferred to a Protran nitrocellulose membrane (Whatman) by semi- dry blotting (75 min at 1 mA/cm²). Membranes were incubated with specific antibodies against TrxA and bound antibodies were detected as previously described (32).

Immunofluorescence

S. aureus cells were cultured in RPMI as indicated above, harvested by centrifugation (5 min, 18620 × g, 4°C) and washed once in PBS with 20 mM sodium azide. Next, cells were resuspended in 100 µl PBS with 20 mM sodium azide and IsaA-specific (Sakata, Terakubo, & Mukai, 2005) or TrxA-specific antibodies. As a control, cells were incubated with IsaA-specific antibodies that had been pre-incubated with purified IsaA. After 30 min incubation on ice, the cells were collected by centrifugation (5 min, 18620 × g, 4°C) and resuspended in PBS with 20 mM sodium azide containing AlexaFluor® 594 labeled goat-anti-rabbit antibodies (Invitrogen). After 30 min incubation on ice, the cells were washing three times in PBS with 20 mM sodium azide, resuspended in MilliQ water and transferred to Polysine-slides (Thermo Scientific). After drying, Vectashield mounting medium (Vector Laboratories) was applied to the slides to prevent photobleaching. Images were recorded with a Leica DM5500 B fluorescence microscope (Leica Microsystems B.V), and image processing was conducted with the ImageJ 1.43m software.

Pepscan analysis

To map regions of cell surface-exposed *S. aureus* proteins that are recognized by human IgGs, we synthesized libraries of linear 15-mer peptides with an overlap of 11 amino acids on solid support (Pepscan), as previously described (50). For some proteins, libraries of CLIPS™ constrained 15-mers were prepared as previously described (49). The peptide libraries were probed with heat-inactivated human sera, in a dilution of 1:1000, with goat-anti-human-HRP conjugate as a secondary antibody, and developed with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). A charge-coupled device camera was used to register absorbance at 405 nm. For every single Pepscan dataset, the data was normalized to the average signal intensity of the analysis. Furthermore, the signals for every single protein were normalized to the median of the corresponding protein. In addition the standard deviations of the normalized data sets were calculated for each protein. Peptides with a signal exceeding the median plus twice the standard deviation and a normalized signal intensity higher than three were regarded as being immunogenic domains.

Human plasma

Whole blood samples (12 ml) from EB patients were processed immediately after donation by 1:1 dilution in Hanks' Balanced Salt Solution (Gibco). Plasma was obtained after separation

from blood cells using Ficoll-Paque PLUS (GE Healthcare) according to manufacturer's instructions. The collected human plasma was stored at -30°C prior to use. The authors declare that the experiments using human plasma were performed with institutional approval upon the receipt of written informed patient consent, and with adherence to the Helsinki Guidelines.

Results

Complementary protein and peptide identifications in different sub-proteome fractions

To identify protein domains that are exposed on the cell surface of *S. aureus*, a comprehensive gel-free proteomics analysis was performed on the community-acquired MRSA strain USA300 and the MSSA laboratory strain Newman. Specifically, staphylococcal cells were shaved either with trypsin immobilized on agarose beads (Figure 1A, 'shaving 1') or with soluble trypsin ('shaving 2'). Liberated peptides were collected and subsequently identified through MS. In parallel, gel-free proteomics was applied to define spontaneously released proteins ('control'), non-covalently cell wall-bound proteins extracted with KSCN ('wall'), and proteins in the exoproteome of the analysed cells. The rationale of this is approach was that the immobilized trypsin would only access protein domains that stick out from the cell surface, whereas the other approaches would help to distinguish the surface-exposed protein domains from proteins or protein domains that are present in the deeper cell envelope layers or in the exoproteome. Overall, this resulted in the identification of 255 unique proteins from the USA300 strain and 177 from the Newman strain (Figure 1, B and C). The p*I* and Mw values of the identified proteins are summarized in Supplemental Figure 1 (available on request). Furthermore, 1191 unique peptides were identified in all samples derived from strain USA300, and 762 in the samples derived from strain Newman (Figure 1, D and E). Importantly, only two proteins were identified in the control fractions (one protein for each strain), showing that the spontaneous release of proteins from the cells during collection and processing of the different sub-proteome fractions was negligible (Supplemental Tables 1 and 2 available on request). Therefore, the control fraction is not further specified in what follows. It is also noteworthy that shaving with immobilized trypsin identified fewer proteins/peptides than shaving with soluble trypsin, suggesting that the soluble trypsin does indeed reach targets in the deeper layers of the cell wall (Figure 1). The complementarity of the four sub-proteome fractionation approaches is underscored by the relatively small numbers of proteins/peptides that were identified in all samples from each strain (Figure 1). In fact, this underscores the need to combine multiple approaches for a comprehensive description of the cell wall-, cell surface- and extracellular proteomes of *S. aureus*.

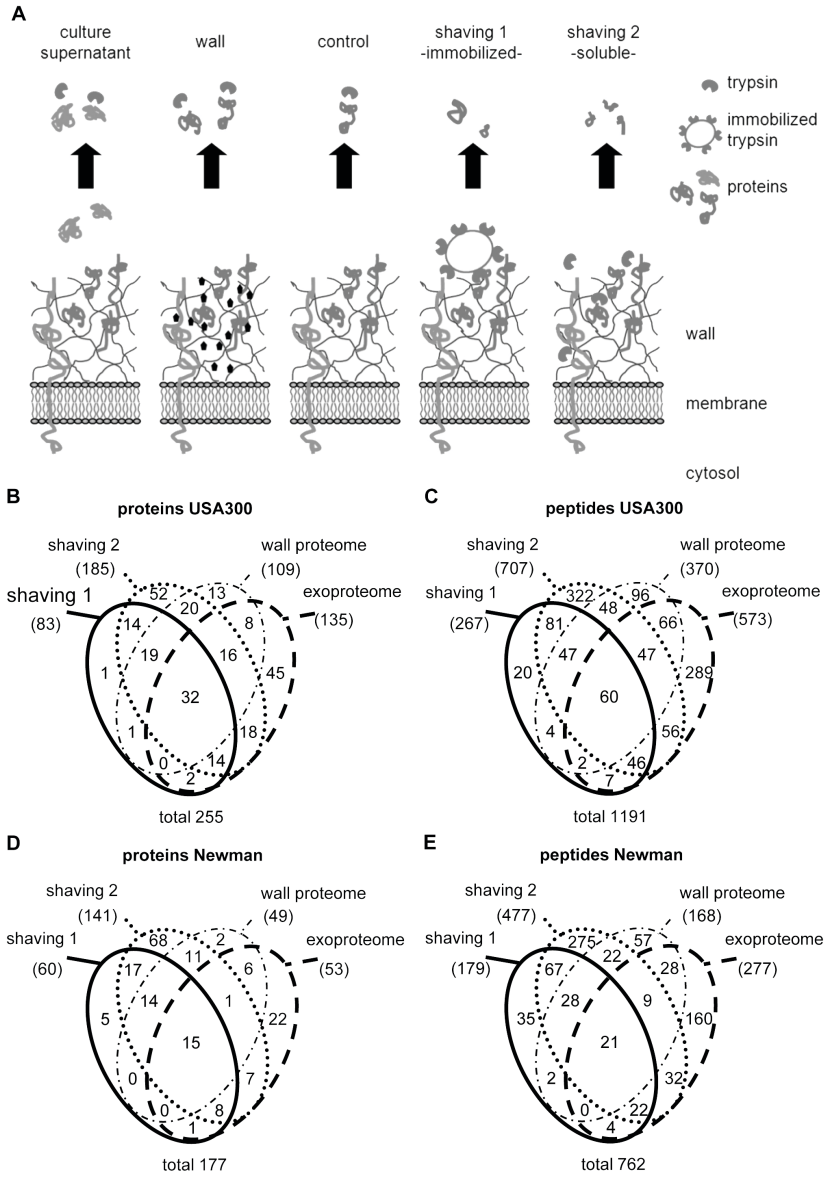

Figure 1. Schematic representation of the analysis of different sub-proteome fractions and proteomics results in numbers. **A**) Cells were harvested at identical optical densities and proteins in the growth medium fraction ('culture supernatant') were precipitated and digested with trypsin. Non-covalently cell wall-attached proteins were extracted with 1 M KSCN and also digested with trypsin ('wall'). Cells were incubated in PBS with 40% sucrose and 20 mM azide alone ('control'), or in the same buffer with either immobilized trypsin ('shaving 1') or soluble trypsin ('shaving 2'). The Venn diagrams summarize the results obtained for strains USA300 (**B**, **C**) and Newman (**D**, **E**) at the protein- (**B**, **D**) and peptide levels (**D**, **E**). 'Shaving 1' and 'shaving 2' respectively mark the results from cell shaving with immobilized or soluble trypsin; 'wall proteome' marks the results from cell wall extraction with KSCN, and 'exoproteome' marks the results from the analysis of culture supernatants.

Strain-specific protein identifications in sub-proteome fractions

A comparison of the total protein identifications revealed that the different analyzed sub-proteome fractions from strain USA300 were more complex than those from strain Newman (Figure 2, Supplemental Figure 2 available on request). Furthermore, a search for potential signal peptides, trans-membrane domains and cell wall-binding domains revealed that most proteins that were exclusively identified in samples from strains USA300 or Newman lack such signals, which suggests a predominant cytoplasmic localization (Figure 2, upper panels). Since about four times more proteins were identified in samples from strain USA300 than in samples from strain Newman, it seems that strain USA300 is more susceptible to lysis. This view is supported by the observation that the bifunctional staphylococcal autolysin Atl- a typical cell wall-bound protein - was identified in the exoproteome of strain USA300, but not in the exproteome of strain Newman. To investigate the presumed lysis of strain USA300, Western blotting experiments were performed in which the localization of the cytoplasmic marker protein TrxA was assessed. As shown in Figure 3A, no TrxA was extracted from the cells with KSCN under conditions that were similar to those applied for cell surface shaving with trypsin. This implies that little if any lysis occurred during the isolation of the different cell-associated sub-proteome fractions. On the other hand, about two-fold more TrxA was detectable in growth medium fractions of strain USA300 than in the equivalent fractions of strain Newman (Figure 3B). This suggests that strain USA300 is more susceptible to cell lysis than strain Newman, and that cell lysis had occurred already during culturing. This is an important observation, because it implies that the cytoplasmic proteins identified on the surface of staphylcoccal cells used for our present sub-proteome analyses had reached the cell surface during culturing. It should be noticed here that, while we think that autolysis during culturing plays an important role in the apparent export of cytoplasmic proteins to the cell surface and growth medium, we cannot exclude the possibility that some of these proteins are actively exported from the cytoplasm via as yet undefined mechanisms.

Proteins common to *S. aureus* strains USA300 and Newman

Despite clear differences, there are also many overlaps in the proteins identified in the respective sub-proteome fractions from *S. aureus* strains Newman and USA300 (Figure 2, Supplemental Table 3 available on request). This is in line with the fact that most of the proteins identified in the present studies are encoded by the genomes of both investigated *S. aureus* strains. Interestingly, seven proteins were identified in all four sub-proteome samples from the USA300 and Newman strains (Supplemental Tables 4 and 5 available on request). These are the IgG-binding protein Spa, the secretory antigen SsaA, the transglycosylase IsaA, the fructose-bisphosphate aldolase class 1, the DNA binding protein HU and the ribosomal proteins L29 and L30. To verify cell surface exposure, an immunofluorescence control

experiment was performed for IsaA, against which a highly specific antibody was available. As shown in Figure 3C, the IsaA antibody bound effectively to the cells of a Δ*spa*Δ*sbi* mutant of strain Newman, which lacks the immunoglobulin-binding proteins Spa and Sbi. Titration of the antibodies with increasing amounts of purified recombinant IsaA confirmed the specificity of the IsaA antibody binding to the cells (Figure 3, D-J). Furthermore, antibodies against the secreted thermonuclease, which was not detected on the cell surface of strains Newman or USA300 did not bind to the cells (Figure 3K), and the same was true for antibodies against the cytoplasmic marker protein TrxA (Figure 3l), which was also not identified as being bound to the cell surface (Figure 3A). Based on these findings, we conclude that the surface shaving approach does indeed yield specific information on the surface exposure of particular *S. aureus* proteins.

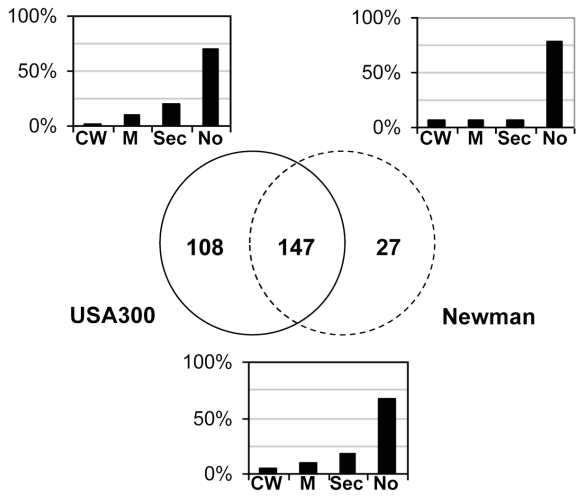

Figure 2. Predicted subcellular localization of the proteins identified for strains USA300 and Newman. The overlapping as well as unique proteins identified in the four sub-proteome fractions from strains USA300 and Newman were analyzed with respect to their predicted subcellular localization as previously described (9). CW, covalently wall-bound proteins; M, transmembrane- and lipoproteins; Sec, secreted proteins; No, proteins with no predicted motif for subcellular localization.

Epitope mapping in surface proteins of *S. aureus*

Altogether, our proteomics analyses led to the identification of 281 unique proteins (Supplemental Table 6, sheet A available on request). Thereof, we selected 54 proteins for further analysis by Pepscan epitope-mapping. The selected proteins include predicted cytosolic, membrane, lipid-modified, cell wall-associated as well as extracellular proteins (Supplemental Table 6, sheet B available on request). For all of these proteins linear 15-mer peptide arrays with 11-mer overlaps were prepared. Additionally, arrays with CLIPS™ constrained 15-mers were prepared for the IsaA, LytM and Nuc proteins. Plasma

donated by seven different EB patients, who have high IgG responses against staphylococcal proteins (51), was then used for the detection of immunogenic domains. In total, we analyzed the interaction of 6821 peptides with IgG's in the plasma of EB patients. This revealed 358 human IgG-binding peptides from 48 different *S. aureus* proteins. Merging of overlapping sequences finally resulted in the delineation of 201 immunodominant domains (Supplemental Table 6, sheet C available on request). The most conserved peptides recognized by IgGs from human serum belong to a Zinc-binding lipoprotein (A6QJP6), the extracellular matrix-binding protein (Emp), and the iron-regulated surface determinant (IsdB) (Supplemental Table 6, sheet D; Supplemental Figure 3; available on request). The relative localization of immunodominant protein regions and peptides identified in the different investigated sub-proteomes is schematically presented in Supplemental Figure 4 (available on request). Notably, the peptides from certain other cell surface-exposed proteins did not bind IgGs from EB patient plasma (*i.e.* FtsL, RS7, the DNA binding protein HU, a putative thioredoxin, two CsbD-like proteins and an uncharacterized), and these proteins can thus be regarded as negative controls for our epitope mapping analysis.

The highest number of IgG-binding peptides was identified for IsdB, and a close analysis of their location within this protein revealed a highly immunodominant domain between amino acids 47 and 129. This domain was also identified by shaving with immobilized trypsin as being cell surface-exposed (Figure 4A, 'shaving 1'). A second immunogenic region was detected in the C-terminal part of IsdB between amino acids 395 and 565, which is also identified by surface shaving. Notably, surface shaving with immobilized trypsin ('shaving 1') identified only peptides from the N- and C-terminal regions in IsdB, whereas shaving with soluble trypsin ('shaving 2') also identified peptides from the central region of IsdB. Peptides from the central region were also identified in extracellular IsdB. Thus, the central region of IsdB is not a substrate for immobilized trypsin, suggesting that it is protected by the cell wall.

A remarkable finding was that especially the surface-exposed N-terminal pro-region of the autolysin Atl was very well recognized by the IgGs from EB patients (Figure 4B). It should be noted that this region is removed from Atl during the processing of the exported pro-Atl into the active amidase and glucosaminidase domains (23). Furthermore, we detected strong immunogenic signals in apparently surface-exposed domains of other proteins, such as the extracellular matrix-binding protein (Emp), coagulase (Coa), fibronectin-binding protein A (FnbpA) and clumping factor B (ClfB) (Supplemental Figure 4 available on request). However, we also observed that for some proteins, such as the chemotaxis inhibitory protein (CHIPS), different protein regions were identified by cell surface shaving and epitope mapping. In the case of CHIPS, we observed an IgG-binding domain in the

N-terminus, next to the signal peptide, whereas proteomics identified most peptides in the C-terminal half of this protein (Supplemental Figure 4 available on request). Interestingly, immunogenic domains positioned next to the signal peptides were observed also for other proteins, such as Atl, Emp, the FPRL1 inhibitory protein (FLIPr), the lipoprotein YkyA, a peptide binding protein (Q2FKI7) and the MHC class II analog protein (Omp7; Supplemental Figure 4 available on request). Lastly, the epitope mapping revealed also numerous IgG-binding domains in typical cytoplasmic proteins that were found to be exposed on the *S. aureus* cell surface. These include the fructose-bisphosphate aldolase Alf1, the enolase Eno, the triosephosphate isomerase Tim, the elongation factors G and Ts, the ribosomal proteins S5, S13 and L25, the phosphoglycerate kinase Pgk and the glyceraldehyde-3-phosphate dehydrogenase GAPDH (Supplemental Figure 4 available on request).

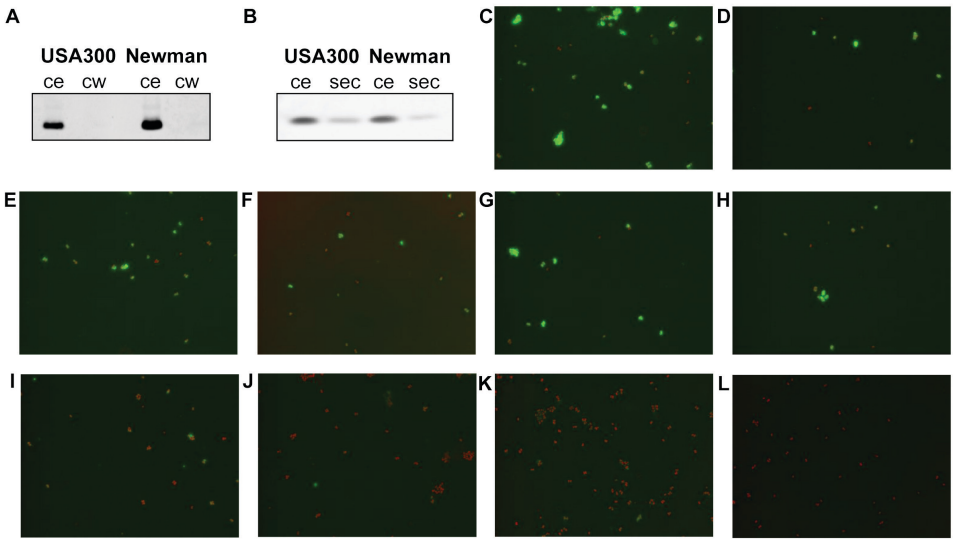

Figure 3. Lysis controls and verification of cell surface localization of IsaA. **A)** Cells were harvested from cultures with an OD_{600} of 0.2, washed, and non-covalently cell wall-bound proteins were extracted with KSCN. The extracted wall proteins (cw) as well as crude cell extracts (ce) were analyzed by Western blotting with specific antibodies against the cytosolic marker protein TrxA. **B)** Cultures were harvested at an OD_{600} of 0.2. Cells were separated from the growth medium by centrifugation, and the presence of TrxA in crude cell extracts (ce) or growth medium fractions (sec) was assessed by Western blotting with specific antibodies. (**C**) Cells of *S. aureus* Newman Δ*spa*Δ*sbi* were harvested at OD_{600} 0.2, incubated with an IsaA-specific antibody plus a secondary antibody labeled with AlexaFluor® 594, and inspected by fluorescence microscopy. As a control for specific antibody binding, the IsaA- specific antibody was pre-incubated with increasing amounts of purified IsaA prior to fluorescence microscopy: (**D**) 10 pg IsaA, (**E**) 100 pg IsaA, (**F**) 1 ng IsaA, (**G**) 10 ng IsaA, (**H**) 100 ng IsaA, (**I**) 1 µg IsaA, (**J**) 10µg IsaA. Antibodies directed against (**K**) the secreted thermonuclease Nuc, or (**L**) the cytosolic marker protein TrxA were applied as negative controls for immuno-fluorescence.

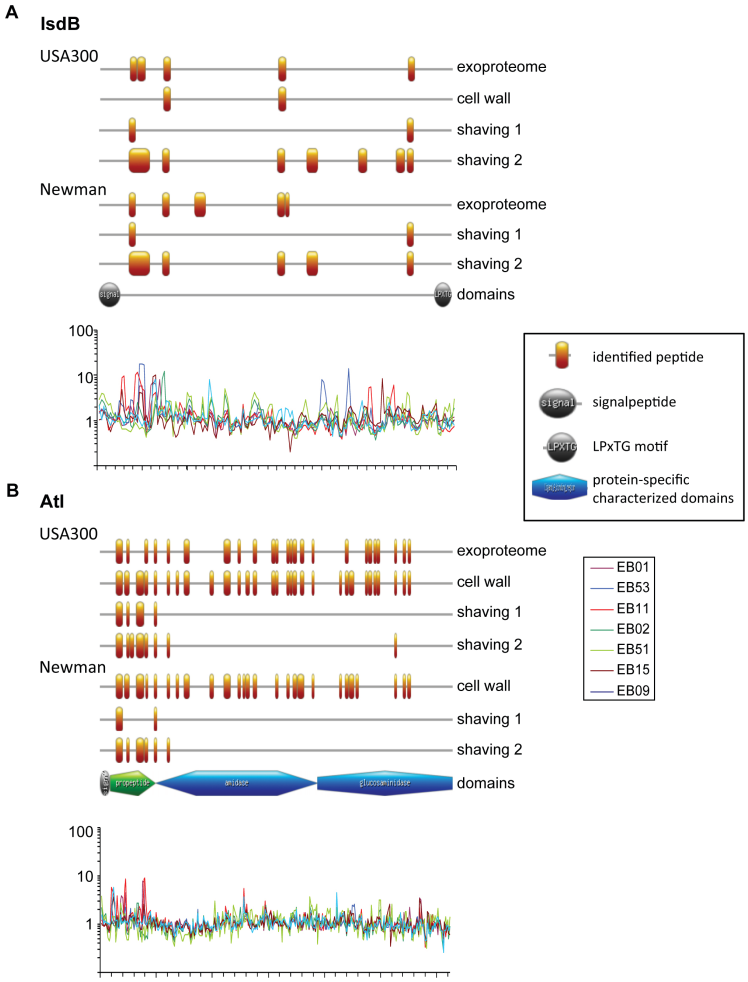

Figure 4. Comparison of proteome and epitope mapping results. Peptides of the IsdB (**A**) and Atl (**B**) proteins as identified by proteomics analyses of four different sub-proteomes of *S. aureus* strains USA300 and Newman are highlighted in the linearly depicted protein sequence. In addition known protein domains are indicated. The graphs display the signals from the epitope mapping normalized to the median signal of the respective protein and serum for the seven different EB patients.

Discussion

The world-wide spread of highly antibiotic-resistant lineages of *S. aureus* calls for the development of novel anti-staphylococcal therapies. Such therapies could very well include active or passive immunization. Unfortunately, attempts to develop anti-staphylococcal immunotherapy have so far remained unsuccessful, which may relate to the selected

antigens. We therefore set out to define cell surface-exposed immunodominant epitopes in two important model strains for *S. aureus*, namely the laboratory MSSA strain Newman and the community-acquired MRSA strain USA300.

Various elegant studies on the staphylococcal exoproteome, wall proteome and surfacome were published in recent years (8, 17, 47, 53, 56). However, the usefulness of these studies for the rational design of novel antistaphylococcal immunotherapy was limited by the fact that they were based on different *S. aureus* isolates grown in different media under different conditions, and that very different technical approaches were applied (9). Therefore, we designed an integrated work flow in which the cell wall proteomes, surfacomes and exoproteomes of two different strains (Newman and USA300) grown under identical conditions were analyzed in parallel following exactly the same protocol. An important outcome was that, despite substantial differences, there was indeed a significant overlap in the identified proteins of both investigated strains. A major pool of differentially identified proteins was formed by typical cytoplasmic proteins that were detected in and on the wall of both strains. While it is currently debated how such proteins leave the cytoplasm to become localized to the cell wall or the extracellular milieu, it is becoming increasingly clear that some of them have so-called 'moonlighting functions' in virulence (18). In line with this notion, our present studies show that the plasma of EB patients suffering from chronic staphylococcal wound colonization does indeed contain IgGs that specifically bind to a range of cytoplasmic proteins and that there is significant overlap between the IgG-binding domains and domains identified in our proteomic analysis (*e.g.* Tim, RS5, Eno, GAPDH). Notably, antibodies directed against Eno have been demonstrated to cause opsonophagocytic killing of *S. aureus* and this protein is a known protective antigen on the cell surface of *Streptococcus suis* (11, 13). Furthermore vaccination of rats with recombinant Eno caused protection against dental caries (7). First results also indicate GAPDH as a candidate for a broad spectrum vaccine to reduce infection in aquacultures (28).

Notably, several surface-exposed proteins that were subjected to epitope mapping revealed clusters of epitopes that were specifically localized to certain domains within these proteins. This is best exemplified by the wall-anchored IsdB protein, the autolysin Atl, the adhesin Emp and the transglycosylase IsaA. The significance of these results is underlined by recent efforts to develop an IsdB-based vaccine, which showed that this protein is highly immunogenic (15, 16), and a study in which the binding site of a human monoclonal antibody was located to the IsdB domain between residues 50 to 285 (10). In the present studies, we identified the IsdB domain between residues 47 and 129 as a hot spot for recognition by IgGs from EB patients. This overlap is striking, especially because of the very different approaches that have been applied. Less effort has as yet been put into the development of

vaccines containing Emp or Atl, but passive immunization with antibodies against Emp did show a reduction of staphylococcal loads in an animal model (4), and passive immunization with a monoclonal antibody against IsaA gave protection against *S. aureus* in a central venous catheter-related infection model and a sepsis survival model (29). Furthermore, Atl was previously identified as a strong antigen through gel-based immunoproteomic approaches on growth medium fractions of *S. aureus* (20, 24). On this basis, it would be interesting to test the application potential of the N-terminal segment of Atl as a target for active or passive immunization.

Interestingly, the IgGs from different EB patients did not always bind to the same *S. aureus* proteins or protein domains. This may have different reasons. Firstly, the Pepscan approach was based mainly on linear peptides and, therefore, certain conformational epitopes of particular proteins recognized by IgGs of the EB patients might be overlooked. A second reason for the observed variability in IgG responses could be that the different patients did not carry the same *S. aureus* types (51). Thus, it is conceivable that different surface-exposed antigens were either produced at low levels or not at all. This is a realistic possibility since an analysis of 58 different *S. aureus* genome sequences showed large variations in the composition and presence of genes for 25 surface-bound and/or immune-evasive proteins (33). Eighteen of these proteins were identified by our proteomic analyses of strains USA300 and Newman (*i.e.* ClfA, ClfB, Coa, Eap, Efb, EbpS, Emp, EsxA, FLIPr, FnbpA, IsdA, IsdB, SasG, Sbi, SCIN, SdrD, Spa, VWbp), and 12 were analyzed by our Pepscan approach showing that they were recognized by antibodies from at least one EB patient (*i.e.* ClfB, Coa, EbpS, Efb, Emp, EsxA, FLIPr, FnbpA, IsdB, SasG, Sbi, SdrD). It is relevant to note that some of the latter proteins, such as Coa, IsdA, IsdB and Spa, have been implicated as potentially effective targets for immunotherapy.

Altogether, our present analyses highlight several immunodominant cell surface-exposed proteins of *S. aureus* and specific sub-domains of these proteins as potential targets for novel active or passive immunization approaches. These include the covalently cell wall-bound proteins ClfB and IsdB, a YkyA-like cell wall-binding lipoprotein, the membrane proteins EbpS and LtaS, the non-covalently cell wall-bound and secreted proteins Atl, Sbi, IsaA, Emp, and the cytoplasmic proteins Afl1, Eno, and GAPDH. Future studies will show whether any of these proteins can indeed serve as effective targets for antistaphylococcal immunotherapy.

Acknowledgements

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6

Surface shaving as a versatile tool to profile global interactions between human serum proteins and the *Staphylococcus aureus* cell surface

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Abstract

The human commensal bacterium *Staphylococcus aureus* is renowned as a causative agent of severe invasive diseases. Upon entering the bloodstream, *S. aureus* can infect almost every tissue and organ system in the human body. To withstand insults from the immune system upon invasion, several immune-evasive mechanisms have evolved in *S. aureus*, such as complement inhibition by secreted proteins and IgG-binding by surface-exposed protein A. While it is generally accepted that *S. aureus* cells bind a range of host factors for various purposes, no global analyses to profile staphylococcal host factor binding have so far been performed. Therefore, we explored the possibility to profile the binding of human serum proteins to *S. aureus* cells by 'surface shaving' with trypsin and subsequent mass spectrometric analysis of liberated peptides. This resulted in the identification of several components of the complement system, the platelet factor 4 and the isoform 1 of the inter-α-trypsin inhibitor heavy chain H4 on the staphylococcal cell surface. We conclude that surface shaving is a versatile tool to profile global interactions between human serum proteins and the *S. aureus* cell surface.

Introduction

Staphylococcus aureus is an important pathogen that can cause a wide range of infections ranging from superficial skin infections to severe invasive diseases (9, 18). The risk of intravascular and systemic infection by *S. aureus* rises significantly when the epithelial barrier is disrupted by surgery, intravascular catheters, implants, mucosal damage or trauma. Especially the surface-exposed and secreted proteins of *S. aureus* have major roles in the host-pathogen interactions that underlie staphylococcal diseases. Interestingly, recent genomic and proteomic analyses have revealed that the extracellular and surface-exposed proteomes of different *S. aureus* strains are highly variable, which seems to relate to a combination of genome plasticity and differential gene expression in different strains (11, 46). This heterogeneity impacts on the potential success of vaccination approaches, especially since the most promising candidate targets for vaccine development are the surface-exposed proteins.

S. aureus produces a variety of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which play critical roles in cell adhesion and invasion. In the past, the interaction between bacterial cell surface-exposed proteins and host proteins has mostly been addressed by targeted analyses in which genes for candidate bacterial interaction partners were either deleted or overexpressed. Subsequently, the particular binding regions in the identified bacterial proteins were mapped. The best characterized MSCRAMMs are the clumping factors A and B (ClfAB) as well as the fibrinogen-binding proteins A and B (FnbpAB) (5, 8, 13, 26,29, 42, 43). However, there are most likely additional MSCRAMMs exposed on the surface of *S. aureus* that can interact with host proteins. Besides the MSCRAMMs *S. aureus* also expresses proteins that interact with the host immune system thereby supporting immune evasion, like the staphylococcal complement inhibitor (SCIN) (34), the chemotaxis inhibitory protein (CHIPS) (33) and the staphylococcal superantigen-like protein 7 (SSL7) (3). While these proteins are secreted into the host environment, the immunoglobulin G (IgG)-binding protein A resides on the cell surface and binds host IgG in order to prevent immune recognition and subsequent destruction of the staphylococcal cell (15).

Recently, we have developed a cell surface shaving approach that allows the identification of exposed domains of cell envelope proteins that can engage in interactions with extracellular biomacromolecules from the human host. In the present studies, we have investigated whether this surface shaving approach can be applied also to identify human serum proteins that bind to the *S. aureus* cell surface. Furthermore, we used two distinct *S. aureus* strains (*i.e.* USA300 and Newman) with highly different surfacome composition (11) to investigate to what extent differences in surfacome composition impact on the binding of serum proteins. Importantly,

the USA300 strain represents the most successful community-associated methicillin-resistant *S. aureus* (MRSA) lineage (21, 27, 30, 31, 39), while the methicillin-sensitive strain Newman was recently shown to be highly virulent in animal experiments (6, 17, 23). The obtained results represent the proof-of-principle that surface shaving can be successfully applied to identify human serum proteins that bind to the *S. aureus* cell surface.

Materials and methods

Bacterial strains and culture

Staphylococcus aureus strains Newman (2) and USA300 FPR3757 (10) were grown overnight in tryptic soy broth (TSB, OXOID) under vigorous shaking (250 rpm) at 37°C. The cultures were then diluted into pre-warmed RPMI 1640 medium (PAA) to an OD_{600} of 0.1 and cultivation was continued under the same conditions. Exponentially growing cells were again diluted into fresh and pre-warmed RPMI medium to a final OD_{600} of 0.05 and the cultivation was continued until an OD_{600} of 0.2 was reached.

Human serum

80 ml of fresh human blood from a healthy volunteer was collected in tubes (BD Vacutainer® SST™ II Advance) and incubated for 30 min at 37°C in a water bath. Subsequently the coagulated blood fraction was pelleted at 3000 rpm at room temperature for 5 min. The serum was transferred to fresh tubes and centrifuged for 10 min at 8000 rpm, 4°C. The resulting supernatant fraction was used for further experiments.

Sample preparation

Three ml of each of the *S. aureus* cultures grown in triplicates were harvested at OD_{600} 0.2 by centrifugation (8000 rpm, 4°C, 10 min) and washed twice with PBS 40% sucrose (Acros), 20 mM sodium azide (Sigma-Aldrich). Thereafter, cells were resuspended in 3 ml of fresh human serum and incubated at room temperature for 15 min. Human serum without bacteria was treated in the same way and used as a control for unspecific binding of serum components to the reaction vessels. Thereafter, the samples were centrifuged to pellet the bacterial cells (8000 rpm, 4°C, 10 min), and the cells were washed twice with shaving buffer (PBS 40% sucrose, 20 mM sodium azide). The cells were then subjected to the shaving procedure (11). To verify the reproducibility of this approach the experiment was repeated three months later with fresh serum from the same donor.

Mass spectrometric analyses

Half of the peptides obtained from shaving were separated by liquid chromatography and measured online by ESI mass spectrometry. LC-MS/MS analyses were performed using

a nanoACQUITY UPLC™ system (Waters) coupled to an LTQ Orbitrap™ mass spectrometer (Thermo Fisher Scientific), creating an electro spray by the application of 1.5 kV between Picotip™ Emitter (SilicaTip™, FS360-20-10 Coating P200P, New Objective) and transfer capillary. Peptides were loaded onto a trap column (nanoAcquity UPLC TM column, Symmetry® C18, 5 µm, 180 µm inner diameter x 20 mm, Waters) and washed 3 min with 99% buffer A (0.1% (v/v) acetic acid) with a flow rate of 10 µl/min. Elution was performed onto an analytical column (nanoAcquity UPLC TM column, BEH130 C18 1.7 µm, 100 µm inner diameter × 100 mm, Waters) by a binary gradient of buffer A and B (100% (v/v) ACN, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 400 nl/min.

For MS/MS analysis a full survey scan was performed in the Orbitrap (m/z 300–2000) with a resolution of 30,000. The full scan was followed by MS/MS experiments of the five most abundant precursor ions acquired in the LTQ via CID. Precursors were dynamically excluded for 30 s, and unassigned charge states as well as singly charged ions were rejected. For protein identification tandem mass spectra were extracted using Sorcerer™v3.5 (Sage-N Research). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (ThermoFinnigan, version v.27, rev. 11), applying the following search parameters: peptide tolerance, 10 ppm; tolerance for fragment ions, 1 amu; b- and y-ion series; an oxidation of methionine (15.99 Da) and alkylation of cysteine (57.02 Da) were considered as variable modification (max. three modifications per peptide). The raw data was searched against strain-specific bacterial databases (both containing a concatenated reversed database, 5250 and 5298 entries) and the human IPI database (v3.75, containing a concatenated reversed database, 179070 entries) assuming the digestion enzyme trypsin. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides. Protein identifications were accepted if present in at least two biological replicates per serum batch. With these filter parameter no false positive hits were obtained.

Data evaluation

Proteins were regarded as significantly identified when detected in at least two of the three replicates of serum samples from two independent blood donations. The normalized spectral abundance factor (NSAF) was calculated and averaged for the different sample sets (47). For statistical analyses the natural logarithm was calculated. To prevent problems during the logarithmic transformation, zero spectral counts were replaced by the value 0.1. The Student *t*-test was applied to the lnNSAF values and *P*-values below 0.05 were regarded as significant.

Purification of human IgG

Plasma IgG was purified after separation of MNCs from human plasma using Ficoll-Paque PLUS (GE Healthcare). IgG was then purified by FPLC using Mabselect Protein A columns and the AKTA explorer system (GE Healthcare). IgG was eluted in citrate buffer (pH 3.0) and immediately dialyzed against PBS. IgG concentrations were subsequently determined with the Biorad DC protein assay.

Evaluation of trypsin digestion

Human IgGs or bovine serum albumin (BSA, Sigma) were diluted to a final concentration of 1 µg/µl by adding either PBS or PBS with 8 M urea (final concentration 7.6 M urea). All samples were then diluted with PBS resulting in a concentration of 3.4 M urea in the urea-treated samples and 30 mM DTT was added to a subset of the samples. After the addition of trypsin, the samples were incubated at 37°C for 45 min. As controls, human IgGs or BSA were incubated without trypsin on ice or at 37°C. Thereafter the proteins were separated by NuPAGE electrophoresis (Invitrogen) and stained with SimplyBlue SafeStain (Invitrogen) according to the manufacturers' instructions.

Evaluation of bacterial cell stability after incubation in human serum

S. aureus cells were incubated in human serum or shaving buffer. Subsequently, the cells were washed twice with shaving buffer and, then, incubated in shaving buffer at 37°C for 45 min. The samples were centrifuged (8000 rpm, 4°C, 10 min) and the resulting supernatant was precipitated overnight with a final concentration of 10% TCA. The precipitates were washed with ice cold acetone and resuspended in sample buffer (LDS, Invitrogen). Cells were resuspended in sample buffer and disrupted using the PreCellys24 bead beater (Bertin Technologies; three cycles of 30 s 6800 rpm and 30 s pauses). Cell debris and beads were pelleted and the supernatant was transferred to a fresh tube and designated crude extract. After separation by NuPAGE the proteins were transferred to a Protran nitrucellulose membrane (Whatman) by semi-dry blotting (75 min at 1 mA/cm²). Membranes were incubated with specific antibodies against TrxA (28). The signals were detected using the Odyssey system (LI-COR Biosciences) after incubation with IRDye 700 goat anti-rabbit antibodies.

Results and Discussion

Application of the surface shaving approach to *S. aureus* cells incubated with human serum resulted in the reproducible identification of 44 human proteins (Table 1, Supplemental Table 1 available on request). Notably, 32 of the identified proteins were also reproducibly detected in reaction vessels incubated with serum without added bacteria, indicating that these

serum proteins have a high affinity for the plastic surface of the reaction vessels. The most abundant of these 32 proteins were the serum albumin precursor and the apolipoproteins A-I, C-III, A-IV and A-II. While we initially anticipated that this approach might be hampered by high-level binding of IgGs to the staphylococcal Protein A, this turned out not to be the case. To test whether this could be due to resistance of Igs to tryptic digestion under the applied conditions, we incubated purified human IgG's with trypsin for 45 min at 37°C. As shown in Figure 1, the purified native IgG's were not degraded by trypsin, even after incubation with 7.6 M urea and 30 mM DTT. In contrast, the BSA was degraded by trypsin in the absence of DTT or urea, and the proteolysis of this control protein was significantly enhanced by the addition of urea and DTT (Figure 1). This finding is consistent with earlier publications showing that 6 M guanidinium-HCl and reduction with DTT are needed to completely unfold and digest IgGs (4, 16). In fact, the observed trypsin resistance of the IgGs is probably the main reason why it turned out possible to identify less abundant serum proteins that are enriched on the *S. aureus* cell surface.

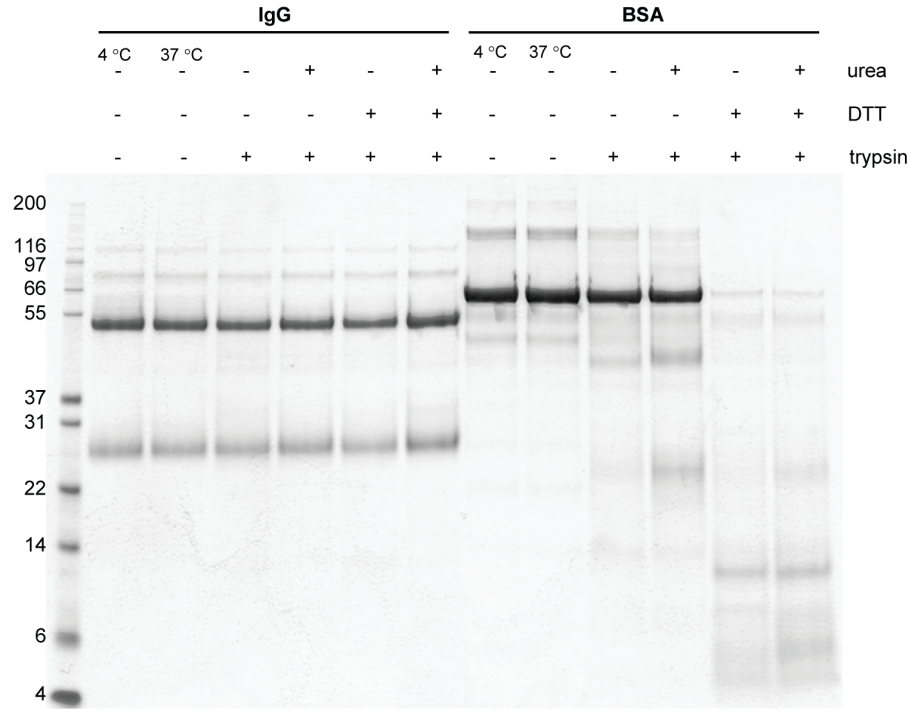

Figure 1. Trypsin resistance of human IgG. 5µg of purified human IgGs were subjected to tryptic digestion at 37°C for 45 min. As a control the Bovine serum albumin (BSA) was used. Prior to the addition of trypsin, the samples were denatured with urea and/or reduced with DTT to disrupt the tertiary structure of the IgGs and BSA. As a negative control, both proteins were incubated on ice as well as at 37°C without the addition of trypsin. Subsequently, the proteins were separated using NuPAGE and visualized with SimplyBlue SafeStain (Invitrogen). Molecular mass markers are indicated on the left.

All obtained MS data were quantified by spectral counting (Supplemental Table 2 available on request). The results showed that nine proteins were specifically enriched on the surfaces of the tested *S. aureus* strains. Five serum proteins were significantly identified in all bacterial samples, while these proteins were barely detectable in the negative controls. Four of these proteins are components of the complement system, namely factor H (fH), the fH-related proteins 1 and 5, and component 7 (C7; Figure 2, A-D). The enrichment of fH on the cell surface of *S. aureus* shown by our shaving experiments is consistent with recent binding studies from Sharp and Cunnion (36). Furthermore these authors demonstrated that binding of fH to the staphylococcal cell surface inhibits the alternative pathway. The complement component C7 is part of the so-called membrane attack complex (MAC). C7 was not very abundantly present in our samples, but it showed a strong enrichment on the staphylococcal cell surface. Interestingly, we identified exclusively the C-terminal domain of this complement component (Supplemental Table 1 available on request). This might indicate that components of the MAC cannot penetrate the cell wall, which could result in MAC resistance as has been described for Gram-positive bacteria in general (25). However, it is also conceivable that the C7 protein is proteolyzed in the serum or on the *S. aureus* cell surface, and that the resulting C-terminal peptide binds to the bacterial cell surface. Our shaving methodology does not distinguish between these two possibilities.

Significant amounts of a fifth protein, the platelet factor 4 (PF4), were detectable in the samples derived from the surface of strain Newman (Figure 2E). In contrast, PF4 was not identified on serum-treated USA300 cells. This indicates a differential binding specificity of the two *S. aureus* strains for this serum protein. Notably, PF4 is a kinocidin. The antimicrobial activity of this small protein was previously shown to be located in the C-terminal part (44). Interestingly, the C-terminal part of PF4 contains numerous positively charged amino acids, which are also involved in binding to polyanions like heparin (45). A recent study demonstrated the binding of PF4 to the cell surface of various bacteria, such as *Escherichia coli*, *S. aureus* and *Streptococcus pneumoniae*. Furthermore, it was shown that the PF4-coating of bacteria results in increased phagocytosis (24). The differential binding of PF4 to *S. aureus* cells as observed for strains Newman and USA300 may thus have implications for their survival upon invasive growth. Potentially, the lack of PF4 binding by *S. aureus* USA300 could represent yet another staphylococcal adaptation to avoid the human host defences.

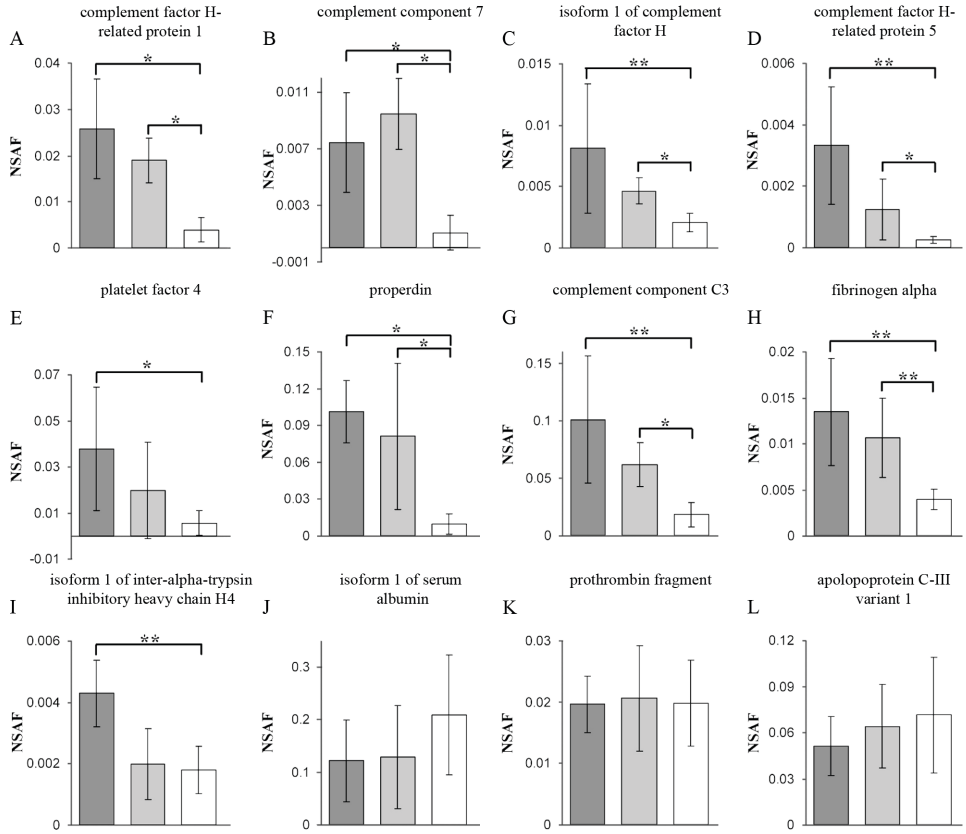

Figure 2. Human serum proteins identified on the cell surface of *S. aureus* strains Newman and USA300. Bacterial cells were grown to early exponential growth phase (n=6), washed twice, incubated with human serum and washed again twice with shaving buffer. As a negative control, we incubated reaction vessels with human serum without adding bacteria. Immobilized trypsin was added to cleave surface-bound proteins. The resulting peptide samples were subjected to mass spectrometric analyses, and the results were evaluated qualitatively as well as quantitatively. Given are the normalized spectral abundance factors (NSAF) for proteins identified in the strains Newman (dark grey shading), USA300 (light grey shading) or control samples (no shading). Results with a P<0.05 are labeled with one star (*). Two stars (**) indicate results with P<0.001. (**A-E**), proteins were not reproducibly detectable in the control samples; (**F-I**), proteins were reproducibly detectable in all three samples; (**J-L**), examples of proteins that show now enrichment on the bacterial cell surface.

Four serum proteins were clearly enriched in the bacterial samples, while residual amounts of these proteins were also detectable in the negative controls (Figure 2, F-I). Properdin was 10-and 8.3-fold enriched on the cell surfaces of the Newman and USA300 strains, respectively. With enrichment factors of 5.6 and 3.4 respectively, the complement component C3 was the second most enriched serum protein on the cell surfaces of these two bacterial strains. Notably, properdin is a positive regulator of complement activation and this protein was shown to bind to the processed complement component C3 (C3b) (19, 35). Both proteins are

well known to interact with the bacterial cell surface, which supports our present study (1, 14, 38). The C3b protein also interacts with complement factor B (fB), which was not identified in the present study. However, a protein with high similarity to fB (*i.e*. the cDNA FLJ55673 protein) was increased about 3-fold on the bacterial cell surfaces with a *P*-value of 0.06, just below our significance criteria. These findings suggest that not only fB, but also the cDNA FLJ55673 protein can bind to the *S. aureus* cell surface. The cDNA FLJ55673 protein might thus be an alternative interaction partner of the C3b protein.

Fibrinogen alpha was identified with enrichment factors of 3.4 and 2.7, respectively, on strains Newman and USA300. This is another clear indication for the relevance of the present approach as this human protein is known to bind to various surface-exposed proteins like clumping factor A (ClfA) and fibronectin-binding protein A (FnbpA) (22, 26, 42). Furthermore, 2.4-fold enrichment of the isoform 1 of the inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) was exclusively shown for strain Newman. In contrast, other constituents of human serum, such as the isoform 1 of serum albumin, prothrombin (fragment) and apolipoprotein C-III variant 1 were not enriched on the bacterial cell surface (Figure 2, J-L), which underscores the specificity of the presented assay. The identification of the ITIH4 on the surface of strain Newman was really unexpected. This protein has not yet been reported to interact with bacteria, but it is a well-known acute-phase serum protein. It was shown that an experimental infection of cattle with a mixture of *Actinomyces pyogenes, Fusobacterium necrophorum* and *Peptostreptococcus indolicus* induced a significant increase of the ITIH4 serum levels (32). Comparable results were obtained when inflammation was induced in mice by cutaneous burn injuries with a superimposed *Pseudomonas aeruginosa* infection (12). In addition, ITIH4 has been reported as a biomarker for several cancer types (41). It should be noted that the purified homologous protein from pigs does not inhibit trypsin, which rules out the possibility that ITIH4 interfered with our surface shaving assay. It will thus be relevant to study the physiological role of ITIH4 in relation to staphylococcal infections. Since we observed an enrichment of the ITIH4 exclusively on the cell surface of strain Newman, it seems likely that this protein interacts with a surface-exposed factor that is absent from the USA300 strain. Consistent with this view, we have previously demonstrated that the surface proteomes of these two strains vary considerably (11), which may be due to a combination of genomic plasticity and differences in gene expression as was also shown for secreted virulence factors (46). The observed surface proteome differences might thus lead to differential binding of host factors. However, it remains to be investigated whether the observed difference for ITIH4 binding relates to the presence or absence of certain cell surface-exposed proteins, or to differences in non-proteinaceous cell surface components. It will furthermore be of interest to investigate how binding of ITIH4 and other serum

proteins is affected by frequently occurring staphylococcal adaptations to the conditions in the host. Prominent examples of such adaptations are the dysfunction of the accessory virulence gene regulator *agr*, and the acquisition or loss of mobile genetic elements, like the staphylococcal cassette chromosome *mec* (7, 37, 49).

Various *S. aureus* proteins were also identified in the present studies (Table 2, Supplemental Table 3; available on request). With one exception, these proteins were also identified in our previous *S. aureus* cell surface shaving analyses (Table 2) (11). Three secreted proteins, namely coagulase, fibronectin binding protein A and the secretory antigen SsaA were identified in the strain Newman samples. The surface-exposed coagulase and fibronectin-binding protein A are both known to interact with human fibrinogen (20, 42). The analyses of serum-treated *S. aureus* strain USA300 resulted in the identification of typical cytosolic proteins, including four ribosomal proteins, the elongation factor TU and the glyceraldehyde-3-phosphate dehydrogenase. This was also the case in our previously published studies in which the cells were not exposed to human serum (11). To check for cell lysis, the stability of the bacterial cells after incubation in human serum was assessed by Western blotting using the cytosolic protein TrxA as a lysis marker (Figure 3). The results show that the bacteria remained stable upon incubation in human serum, indicating that the identified cytoplasmic proteins on the surface of the USA300 strain were already present on this location before the incubation with serum. This is consistent with our previous surface shaving results (11).

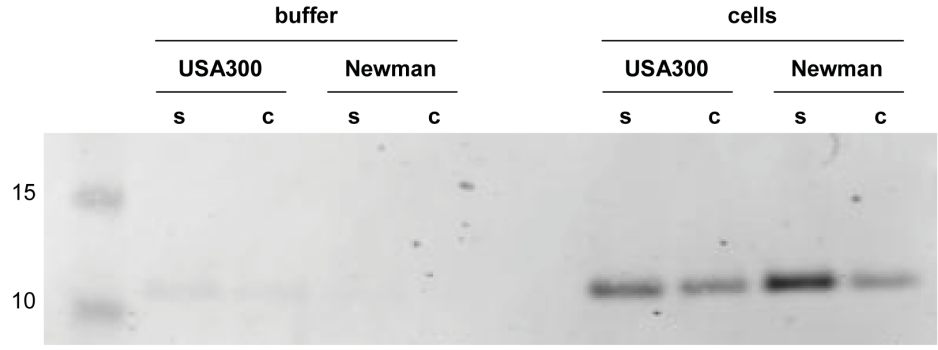

Figure 3. Cell stability after serum treatment. Bacterial cells were grown to the early exponential growth phase, washed twice, and incubated with human serum (s) or shaving buffer (c). After two additional washing steps, the cells were incubated for 45 min in shaving buffer. Cells were pelleted and the resulting supernatant was precipitated with 10% TCA. Crude extract (corresponding to 0.16 OD units) as well as the precipitated proteins from the incubation buffer (corresponding to 0.3 OD units) were separated by NuPAGE and subjected to Western blotting to detect the lysis marker TrxA. Molecular mass markers are indicated on the left.

Concluding remarks

To the best of our knowledge, a global analysis of serum protein binding to microbial cell surfaces has thus far never been performed, neither for *S. aureus* nor any other bacterium. Previous studies describing interactions between bacteria and serum proteins were focused on specific proteins from bacteria and their host, and the experimental set-ups involved pull-down assays and protein-protein interaction studies. Taken together, the present studies provide important proof-of-principle that our surface shaving approach can be applied for the profiling of human serum proteins that bind to the *S. aureus* cell surface. We are convinced that applications for this approach are not limited to studying the adherence of serum proteins to bacterial surfaces, but that it can also be used to investigate bacterial interactions with proteins in other body fluids or samples containing solubilized human cell envelope proteins. The surface shaving technique thus seems to be a versatile generally applicable tool for monitoring bacteria-host interactions. Depending on the specific aims, such applications may require further refinement of the developed protocol, such as the addition of reducing agents, detergents, and/or urea to make tightly folded cell surface-bound human proteins more susceptible to cleavage by trypsin or other proteases. Importantly, surface shaving can even be applied to profile the binding of human proteins to biomaterials as is underscored by our negative controls in which human serum proteins with a high propensity for the binding to naïve polypropylene were profiled. It thus seems that the shaving of surface-attached proteins opens up new avenues for studies on the interactions of bacterial and human proteins with each other, with plastics and other materials that are commonly used in medical implants.

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Conflicts of Interest

The authors have no relevant financial conflict of interest.

Table 1. Human serum proteins binding to the cell surface of *S. aureus* strains Newman and USA300, or the reaction vessel (x marks identified proteins and E marks enriched proteins)

Protein name	IPI number	Newman	USA300	reaction vessel
properdin	IPI00021364.1	E	E	x
complement C3 (Fragment)	IPI00783987.2	E	E	x
fibrinogen alpha	IPI00021885.1, IPI00029717.1	E	E	x
cDNA FLJ55673, highly similar to Complement factor B	IPI00019591.2	x	x	x
isoform 1 of inter-alpha-trypsin inhibitor heavy chain H4	IPI00896419.3	E	x	x
complement C1s subcomponent	IPI00017696.1, IPI00749179.2	x	x	x
immunoglobulin J chain	IPI00178926.2	x	x	x
complement C4	IPI00032258.4, IPI00892604.1, IPI00892547.1, IPI00887154.2, IPI00843913.3, IPI00654875.1, IPI00643525.1, IPI00418163.3	x	x	x
cDNA FLJ55606, highly similar to alpha-2-HS-glycoprotein	IPI00022431.2, IPI00953689.1	x	x	x
neutrophil defensin 1	IPI00005721.1, IPI00021827.3	x	x	x
prothrombin (Fragment)	IPI00019568.1	x	x	x
cDNA FLJ14473 fis, clone MAMMA1001080, highly similar to Homo sapiens SNC73 protein (SNC73) mRNA	IPI00386879.1, IPI00449920.1, IPI00426060.3	x	x	x
immunoglobulin heavy constant mu	IPI00892870.1, IPI00946337.1, IPI00896380.1	x	x	x
alpha-2-macroglobulin	IPI00478003.2	x	x	x
inter-alpha (Globulin) inhibitor H2	IPI00305461.4, IPI00645038.1	x	x	x
apolipoprotein A-	IPI00021841.1	x	x	x
apolipoprotein A-IV	IPI00304273.2	x	x	x
apolipoprotein C-III variant 1	IPI00657670.1	x	x	x
serotransferrin	IPI00022463.1	x	x	x
plasminogen	IPI00019580.1	x	x	x
clusterin	IPI00400826.1, IPI00291262.3, IPI00795633.1, IPI00954954.1	x	x	x
apolipoprotein A-II	IPI00021854.1	x	x	x

Protein name	IPI number	Newman	USA300	reaction vessel
apolipoprotein E	IPI00021842.1	x	x	x
vitronectin	IPI00298971.1	x	x	x
isoform LMW of Kininogen-1	IPI00215894.1	x	x	x
haptoglobin	IPI00641737.1	x	x	x
isoform 1 of serum albumin	IPI00745872.2	x	x	x
putative uncharacterized protein DKFZ-p686I15212	IPI00418153.1, IPI00168728.1	x	x	x
apolipoprotein L1	IPI00186903.4, IPI00914948.1, IPI00514475.5	x	x	x
actin, aortic smooth muscle	IPI00008603.1, IPI00514530.5, IPI00025416.3, IPI00023006.1, IPI00021428.1	x	x	x
plasma kallikrein	IPI00654888.4, IPI00966520.1			x
fibronectin	IPI00339223.2, IPI00022418.2, IPI00339224.2, IPI00339225.2, IPI00339226.2, IPI00339227.5, IPI00339228.2, IPI00339319.2, IPI00414283.7, IPI00479723.5			x
complement factor H-related protein 1	IPI00011264.2	x	x	
platelet basic protein	IPI00022445.1	x	x	
complement component C7	IPI00296608.6	E	E	
isoform 1 of complement factor H	IPI00029739.5	E	E	
platelet factor 4	IPI00022446.1	E		
hemopexin	IPI00022488.1	x		
complement factor H-related protein 5	IPI00843942.1, IPI00006543.2	E	E	
beta-2-glycoprotein 1	IPI00298828.3, IPI00910625.1	x		
heparin cofacto	IPI00292950.4, IPI00879573.1	x		
gelsolin	IPI00026314.1, IPI00647556.2	x		
complement component 6 precursor	IPI00879709.3	x		
apolipoprotein (a)	IPI00029168.1	x		

Table 2. Bacterial proteins identified on the cell surface after incubation with human serum.

protein name	function	previously identified on the staphylococcal cell surface (11)
strain Newman		
A6QDK6	coagulase	yes
A6QJD9	secretory antigen SsaA, function unknown	yes
A6QJY9	fibronectin binding protein	yes
strain USA300		
RS10	30S ribososmal protein S10	yes
RL13	50S ribososmal protein L13	yes
RL11	50S ribososmal protein L11	yes
RS6	30S ribososmal protein S6	yes
Q2FIM1	Glyceraldehyde-3-phosphate dehydrogenase, type I	yes
EFTU	Elongation factor Tu	no

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7

Synthetic effects of *secG* and *secY2* mutations on exoproteome biogenesis in *Staphylococcus aureus*

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Abstract

The Gram-positive pathogen *Staphylococcus aureus* secretes various proteins into its extracellular milieu. Bioinformatics analyses have indicated that most of these proteins are directed to the canonical Sec pathway, which consists of the translocation motor SecA and a membrane-embedded channel composed of the SecY, SecE and SecG proteins. In ddition, *S. aureus* contains an accessory Sec2 pathway involving the SecA2 and SecY2 proteins. Here we have addressed the roles of the non-essential channel components SecG and SecY2 in the biogenesis of the extracellular proteome of *S. aureus*. The results show that SecG is of major importance for protein secretion by *S. aureus*. Specifically, the extracellular accumulation of nine abundant exoproteins and seven cell wall-bound proteins was significantly affected in the *secG* mutant. No secretion defects were detected for strains with a *secY2* single mutation. However, deletion of *secY2* exacerbated the secretion defects of *secG* mutants, affecting the extracellular accumulation of one additional exoprotein and one cell wall protein. Furthermore, the *secG secY2* double mutant displayed a synthetic growth defect. This might relate to a slightly elevated expression of *sraP*, encoding the only known substrate for the Sec2 pathway, in cells lacking SecG. Additionally, the results suggest that SecY2 can interact with the Sec1 channel, which would be consistent with the presence of a single set of *secE* and *secG* genes in *S. aureus*.

Introduction

Staphylococcus aureus is a well-represented component of the human microbiota as nasal carriage of this Gram-positive bacterium has been shown for 30-40% of the population (32). This organism can, however, turn into a dangerous pathogen that is able to infect almost every tissue in the human body. *S. aureus* has become particularly notorious for its high potential to develop resistance against commonly used antibiotics (20,49). Accordingly, the *S. aureus* genome encodes an arsenal of virulence factors that can be expressed when needed at different stages of growth. These include surface proteins and invasins that are necessary for colonization of host tissues, surface-exposed factors for evasion of the immune system, exotoxins for the subversion of protective host barriers, and resistance proteins for protection against antimicrobial agents (37,57).

Most proteinaceous virulence factors of *S. aureus* are synthesized as precursors with an N-terminal signal peptide to direct their transport from the cytoplasm across the membrane to an extracytoplasmic location, such as the cell wall or the extracellular milieu (38,45). As shown for various Gram-positive bacteria, the signal peptides of *S. aureus* are generally longer and more hydrophobic than those of Gram-negative bacteria (38,54). Based on signal peptide predictions using a variety of algorithms, it is believed that most exoproteins of *S. aureus* are exported to extracytoplasmic locations via the general Secretory (Sec) Pathway (38). This seems to involve precursor targeting to the Sec machinery via the Signal Recognition Particle instead of the well-characterized proteobacterial chaperone SecB, which is absent from Gram-positive bacteria (16,19,53). The pre-proteins are then bound by the translocation motor protein SecA (38,45). Through repeated cycles of ATP binding and hydrolysis, SecA pushes the protein in an unfolded state through the membrane-embedded SecYEG translocation channel (12,30,33,52). Upon initiation of the translocation process, the proton-motive force is thought to accelerate pre-protein translocation through the Sec channel (26). Recently, the structure of the SecA/SecYEG complex from the Gram-negative bacterium *Thermotoga maritima* was solved at 4.5 A resolution (58). In this structure, one SecA molecule is bound to one set of SecYEG channel proteins. The core of the Sec translocon consists of the SecA, SecY and Sec proteins, which are essential for growth and viability of bacteria, such as *Escherichia coli* and *Bacillus subtilis* (6,9,22). In contrast, the channel component SecG is dispensable for growth, cell viability and protein translocation (26,48). Nevertheless, SecG does enhance the efficiency of pre-protein translocation through the SecYE channel (26,48). This is of particular relevance at low temperatures and in the absence of a proton-motive force (17). Several studies suggest that *E. coli* SecG undergoes topology inversion during pre-protein translocation (25,27,43). Even so, van der Sluis *et al*. reported that SecG cross-linked to

SecY is fully functional despite its fixed topology (46). During or shortly after membrane translocation of a pre-protein through the Sec channel, the signal peptide is removed by signal peptidase. This is a prerequisite for the release of the translocated protein from the membrane (1,47).

Several pathogens, including *Streptococcus gordonii*, *Streptococcus pneumoniae*, *Bacillus anthracis*, *Bacillus cereus*, and *S. aureus* contain a second set of chromosomal *secA* and *secY* genes named *secA2* and *secY2*, respectively (39). Comparison of the amino acid sequences of the SecY1 and SecY2 proteins shows that their similarity is low (about 20% identity), and that the conserved regions are mainly restricted to the membrane spanning domains. It has been shown for *S. gordonii* that the transport of at least one protein is dependent on the presence of SecA2 and SecY2. This protein, GspB, is a large cell-surface glycoprotein that is involved in platelet binding (4). The protein contains an unusually long N-terminal signal peptide of 90 amino acids, large serine-rich repeats, and a C-terminal LPxTG motif for covalent cell wall binding. The *gspB* gene is located in a gene cluster with the *secA2* and *secY2* genes. Two other genes in this cluster encode for the glycosylation proteins GftA and GftB, which seem to be necessary for stabilization of pre-GspB. Furthermore, the *asp4* and *asp5* genes in the *secA2 secY2* gene cluster show similarity to *secE* and *secG*, and they are important for GspB export by *S. gordonii* (44). Despite this similarity, SecE and SecG cannot complement for the absence of Asp4 and Asp5, respectively. The *secA2*/*secY2* gene cluster is also present in *S. aureus*, but homologues of the *asp4* and *asp5* genes are lacking. This seems to suggest that SecA2 and SecY2 of *S. aureus* share the SecE and SecG proteins with SecA1 and SecY1. The *sraP* gene in the *secA2/secY2* gene cluster of *S. aureus* encodes a protein with similar features as described for GspB. Siboo and colleagues (41) have shown that SraP is glycosylated and capable of binding to platelets. Importantly, the disruption of *sraP* resulted in a decreased ability to initiate infective endocarditis in a rabbit model. Consistent with the findings in *S. gordonii*, SraP export was shown to depend on SecA2/SecY2 (40). However, it has remained unclear whether other *S. aureus* proteins are also translocated across the membrane in a SecA2/ SecY2-dependent manner.

The present studies were aimed at defining the roles of two Sec channel components, SecG and SecY2, in the biogenesis of the *S. aureus* exoproteome. The results show that *secG* and *secY2* are not essential for growth and viability of *S. aureus*. While the absence of SecY2 by itself had no detectable effect, the absence of SecG had a profound impact on the composition of the exoproteome of *S. aureus*. Various extracellular proteins were present in decreased amounts in the growth medium of *secG* mutant strains, which is consistent with impaired Sec channel function. However, a few proteins were present in increased amounts. Furthermore, the absence of *secG* caused a serious decrease in the amounts of

the cell wall-bound Sbi protein. Most notable, a *secG secY2* double mutant strain displayed synthetic growth and secretion defects.

Material & Methods

Bacterial strains and plasmids

All strains used in this study are listed in Table 1. Unless stated otherwise, *E. coli* strains were grown in Luria-Bertani broth (LB). *S. aureus* strains were grown at 37°C in tryptic soy broth (TSB) or B medium (1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose) under vigorous shaking, or on trypic soy agar (TSA) plates or B plates. If appropriate, media for *E. coli* were supplemented with 100 µg/ml ampicillin or 100 µg/ ml erythromycin, and media for *S. aureus* with 5 µg/ml erythromycin, 5 µg/ml tetracyclin or 20 µg/ml kanamycin. To monitor β-galactosidase activity in cells of *E. coli* and *S. aureus*, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to the plates at a final concentration of 80 µg/ml.

Construction of *S. aureus* mutant strains

Mutants of *S. aureus* were constructed using the temperature-sensitive plasmid pMAD (2) and previously described procedures (23). Primers (Table 2) were designed using the genome sequence of *S. aureus* NCTC8325 (http://www.ncbi.nlm.nih.gov/nuccore/NC_007795). All mutant strains were checked by isolation of genomic DNA using the GenElute™ Bacterial Genomic DNA Kit (Sigma) and PCR with specific primers.

To delete the *secG* or *secY2* genes, primer pairs with the designations F1/R1 and F2/R2 were used for PCR amplification of the respective upstream and downstream regions (each ~500 bp), and their fusion with a 21 bp linker. The fused flanking regions were cloned in pMAD, and the resulting plasmids were used to delete the chromosomal *secG* or *secY2* genes of *S. aureus* RN4220. To delete the *secG* or *secY2* genes from the *S. aureus* SH1000 genome, the respective pMAD constructs were transferred from the RN4220 strain to the SH1000 strain by transduction with phage φ85 (29).

To create the *spa sbi* double mutant of *S. aureus* Newman, the *sbi* gene was deleted from a *spa* mutant strain kindly provided by T. Foster (31). For this purpose, the kanamycin resistance marker encoded by pDG783 was introduced between the *sbi* flanking regions via PCR with the primer pairs *sbi*-F1/*sbi*-R1, *sbi*-F2/*sbi*-R2 and *kan*-F1/*kan*-R1. The obtained ~1000 bp fragment was ligated into pMAD, and the resulting plasmid was used to transform competent *S. aureus* Newman *spa* cells. Blue colonies were selected on TSA plates with erythromycin and kanamycin, and the *spa sbi* double mutant was subsequently identified following the previously described protocol (23).

For complementation studies, the *secG* or *secY2* genes were cloned into plasmid pCN51 (11). Expression of genes cloned in this plasmid is directed by a cadmium-inducible promoter. Primer pairs with the F3/R3 designation (Table 2) were used to amplify the *secG* or *secY2* genes. These primers contain an *Eco*RI restriction site at the 5' end and a *Sal*I restriction site at the 3' end of the amplified gene. PCR products were purified using the PCR Purification Kit (Roche), and ligated into the TOPO-vector (Invitrogen). The resulting constructs were then cut with *Eco*RI and *Sal*I, and the *secG* or *secY2* genes (284 and 1233 bp, respectively) were isolated from an agarose gel and ligated into pCN51 cut with *Eco*RI and *Sal*I. This resulted in the *secG*- and *secY2*-pCN51 plasmids. Competent *S. aureus* RN4220 Δ*secG*, Δ*secY2* or Δ*secG* Δ*secY2* cells were transformed with these plasmids by electroporation and colonies were selected on TSA plates containing erythromycin. The plasmids were then transferred to *S. aureus* SH1000 by transduction as described above.

Analytical and preparative two-dimensional (2-D) PAGE

Extracellular proteins from 100 ml culture supernatant were precipitated, washed, dried, and resolved as described previously (56). The protein concentration was determined using Roti®-Nanoquant (Carl Roth GmbH & Co, Karlsruhe, Germany). Preparative 2-D PAGE was performed by using the immobilized pH gradient technique (5,13). The protein samples (350 µg) were separated on immobilized pH gradient strips (Amersham Pharmacia Biotech, Piscataway, NJ) with a linear pH gradient from 3 to 10. The resulting protein gels were stained with colloidal Coomassie Blue G-250G (10) and scanned with the light scanner. Each experiment was performed at least three times.

For identification of proteins by MALDI-TOF MS, Coomassie-stained protein spots were excised from gels using a spot cutter (Proteome Work™) with a picker head of 2 mm and transferred into 96-well microtiter plates. Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI targets were performed automatically in an Ettan Spot Handling Workstation (GE-Healthcare, Little Chalfont, United Kingdom) using a modified standard protocol. MALDI-TOF MS analyses of spotted peptide solutions were carried out on a Proteome-Analyzer 4700/4800 (Applied Biosystems, Foster City, CA) as described previously (13). MALDI-TOF-TOF analysis was performed for the three highest peaks of the TOF spectrum as described previously (13,51). Database searches were performed using the GPS explorer software version 3.6 (build 329) with the organism-specific databases.

By using the MASCOT search engine version 2.1.0.4. (Matrix Science, London, UK) the combined MS and MS/MS peak lists for each protein spot were searched against a database containing protein sequences derived from the genome sequences of *S. aureus* NCTC8325. Search parameters were as described previously (51). For comparison of protein spot volumes, the Delta 2D software package was used (Decodon GmbH Germany). The induction

ratio of mutant to parental strain was calculated for each spot (normalized intensity of a spot on the mutant image/normalized intensity of the corresponding spot on the parental image). The significance of spot volume differences of two-fold or higher was assessed by the Student´s *t* test ($\alpha<0.05$; Delta 2D "statistics" table).

Transcriptional analysis

Total RNA from *S. aureus* RN4220 was isolated using the acid-phenol method (14). Digoxigenin-labeled RNA probes were prepared by *in vitro* transcription with T7 RNA polymerase, using the Dig-RNA labeling mixture (Roche, Indianapolis, IN) and appropriate PCR fragments as templates. The PCR fragments were generated by using the respective oligonucleotides (Table 2) and chromosomal DNA of *S. aureus* RN4220 isolated with the chromosomal DNA isolation kit (Promega, Madison, WI) according to the manufacturer's recommendations. Reverse primers contain the T7 RNA polymerase recognition sequence at the 5' end. Northern blot and slot blot analyses were performed as described previously (50,57). Before hybridization, each RNA blot was stained with methylene blue in order to check the RNA amount blotted onto the membrane. Only blots showing equal amounts of 16S and 23S rRNA for each sample loaded onto the respective gels were used for hybridization experiments. The hybridization signals of the Northern blots were detected with a Lumi-Imager (Roche, Indianapolis, IN) and analyzed with the software package LumiAnalyst (Roche, Indianapolis, IN). Slot blot signal detection was performed with the Intas ChemoCam system and analysed with LabImage1D software (Intas Science Imaging Instruments GmbH, Göttingen, Germany). In slot blot experiments, the induction ratios were calculated by dividing the volumes obtained for the different RNA samples by the volume of the signals of the exponentially grown RN4220 parental strain. An internal RNA standard was spotted onto each membrane to correct for inter-membrane variations.

Cell fractionation, SDS-PAGE, and Western blotting

Overnight cultures were diluted to an OD_{540} of 0.05 and grown in 25 ml TSB under vigorous shaking. For complementation of mutant strains with pCN51-based plasmids, $CdSO_4$ was added after three hours of growth to a final concentration of 0.25 µM. Samples were taken after six hours of growth and separated in growth medium, whole cell and non-covalently cell wall-bound protein fractions. Cells were separated from the growth medium by centrifugation of 1 ml of the culture. The proteins in the growth medium were precipitated with 250 µl 50% trichloroacetic acid (TCA), washed with acetone and dissolved in 100 µl Loading Buffer (Invitrogen). Cells were resuspended in 300 µl Loading Buffer (Invitrogen) and disrupted with glass beads using a Precellys®24 bead-beating homogenizer (Bertin Technologies). From the same culture 20 ml was used for the extraction of non- covalently

bound cell wall proteins using KSCN. Cells were collected by centrifugation, washed with PBS, and incubated for 10 min with 1M KSCN on ice. After centrifugation the non-covalently cell wall bound proteins were precipitated from the supernatant fraction with TCA, washed with acetone and dissolved in 100 µl Loading Buffer (Invitrogen). Upon addition of Reducing Agent (Invitrogen), the samples were incubated at 95ºC. Proteins were separated by SDS-PAGE using precast NuPage gels (Invitrogen) and subsequently blotted onto a nitrocellulose membrane (Protran®, Schleicher & Schuell). The presence of a cytoplasmic marker protein (TrxA), a lipoprotein (DsbA), and several cell wall-associated proteins (Sle1, Aly, ClfA, IsaA) or extracellular proteins (Sle1, Aly, IsaA, SspB) was monitored by immunodetection with specific polyclonal antibodies raised in mice or rabbits. Bound primary antibodies were visualized using fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-mouse/anti-rabbit from LiCor Biosciences). Membranes were scanned for fluorescence at 800 nm using the Odyssey Infrared Imaging System (LiCor Biosciences).

Mouse infection studies

All animal studies were approved by the Animal Care and Experimentation Committee of the district government of Lower Franconia, Germany and conformed to University of Würzburg guidelines. Female Balb/C mice (16-18 g; Charles River, Sulzfeld, Germany) were housed in polypropylene cages and received food and water *ad libitum*. *S. aureus* isolates were cultured for 18 hours in B-medium, washed three times with sterile PBS and suspended in sterile PBS to $1.0x10^8$ CFU/100 µl. As a control, selected dilutions were plated on B agar. Mice were inoculated with 100 µl of *S. aureus* via the tail vein. Control mice were treated with sterile PBS. For each strain, eight mice were used. Three days after challenge, kidneys and livers were aseptically harvested and homogenized in 3 ml of PBS using Dispomix (Bio-Budget Technologies Gmbh, Krefeld, Germany). Serial dilutions of the organ homogenates were cultured on mannitol salt-phenol red agar plates for at least 48 h at 37°C. CFUs were calculated as CFU/organ. The statistical significance of bacterial load was determined using Mann Whitney tests.

Results

The exoproteomes of *secG* and *secY2* mutant *S. aureus* strains

To investigate the roles of SecG and SecY2 in the biogenesis of the *S. aureus* exoproteome, the respective genes were completely deleted from the chromosome of *S. aureus* strain RN4220. This resulted in the single mutant strains Δ*secG* and Δ*secY2*, and the double mutant Δ*secG* Δ*secY2*. Next, cells of these mutants were grown in TSB medium until they reached the stationary phase (Figure 1; not shown for the Δ*secY2* strain).

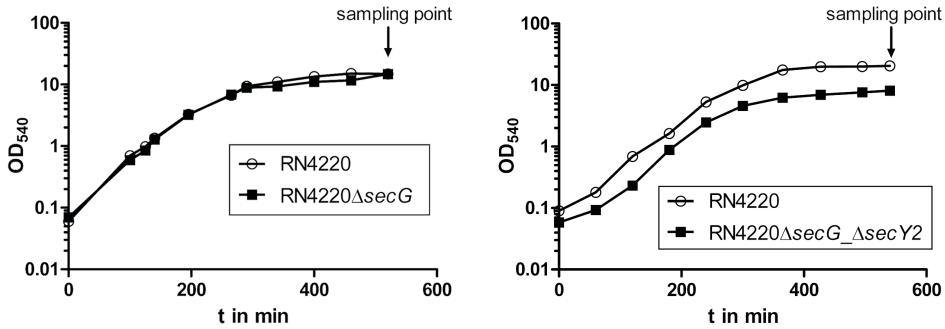

Figure 1 Growth of *S. aureus secG* and *secG secY2* mutants. The *S. aureus* strains RN4220 Δ*secG* (A), Δ*secG* Δ*secY2* (B), and the parental strain RN4220 were grown in 100 ml TSB medium under vigorous shaking at 37°C. Sampling points for the preparation of extracellular proteins for 2-D PAGE analyses in Figure 2 are indicated in the growth curves by arrows.

All three mutants displayed similar exponential growth rates as the parental strain. However, the *secG secY2* double mutant entered the stationary phase at a lower optical density (OD_{540}=8) than the parental strain and the Δ*secG* mutant (OD_{540}=15). Since the amounts of most exoproteins of *S. aureus* increase mainly in the stationary growth phase at high cell densities (37,56), extracellular proteins for 2-D PAGE analyses were collected from the supernatant of cell cultures that had reached stationary phase (Figure 1 and 2). Comparison of the exoproteomes of the *secG* mutant and its parental strain revealed that eleven proteins with Sec-type signal peptides and type I signal peptidase cleavage sites (*i.e.* SAOUHSC-00094, SdrD, Sle1, Geh, Hlb, HlY, HlgB, HlgC, Plc, SAOUHC-02241 and SAOUHSC-02979) were present in significantly decreased amounts when SecG was absent from the cells. This was also true for the secreted moiety of the polytopic membrane protein YfnI, which is processed by signal peptidase I as was previously shown for the YfnI homologue of *B. subtilis* (1). In contrast, the amounts of three other exoproteins (*i.e.* IsaA, Spa and SsaA) were considerably increased due to the *secG* deletion (Figure 2A; Table 3). These effects of the *secG* mutation were fully compensated when *secG* was ectopically expressed from plasmid *secG*-pCN51 (Figure 2C). Northern blot analyses revealed similar transcript levels for *geh*, *hlb* and *spa* in the s*ecG* mutant and the parental strain RN4220. This shows that the changes in the amounts of the respective exoproteins in the *secG* mutant were not caused by a decreased transcription of the corresponding genes (Figure 3). Deletion of the *secY2* gene encoding a channel component of the accessory Sec system in *S. aureus*, did not affect the extracellular protein pattern (data not shown). However, the deletion of both *secG* and *secY2* caused additional changes in the extracellular proteome compared to the *secG* single mutant (Figure 2B). Specifically, one additional exoprotein was identified in decreased amounts (*i.e.* LipA) and one additional exoprotein (*i.e.* LytM) was

identified in increased amounts (Table 3). Furthermore, proteins such as IsaA, Spa, and SsaA were secreted in higher amounts not only by the *secG* mutant, but also by the *secG secY2* double mutant. This effect was significantly exacerbated for IsaA and SsaA in the *secG secY2* double mutant. It is interesting to note that IsaA, LytM, Spa, and SsaA represent cell surface-associated proteins (34,37,42). In contrast, most proteins that were secreted in reduced amounts in the *secG* or *secG secY2* mutants are secretory proteins without retention signals, except for SAOUHSC-00094 (Table 3). Importantly, also the secretion and growth defects of the *secG secY2* mutant strain could be fully reversed by ectopic expression of *secG* from plasmid *secG*-pCN51, and the synthetic effects of the *secG* and *secY2* mutations could be reversed by plasmid *secY2*-pCN51 (data not shown).

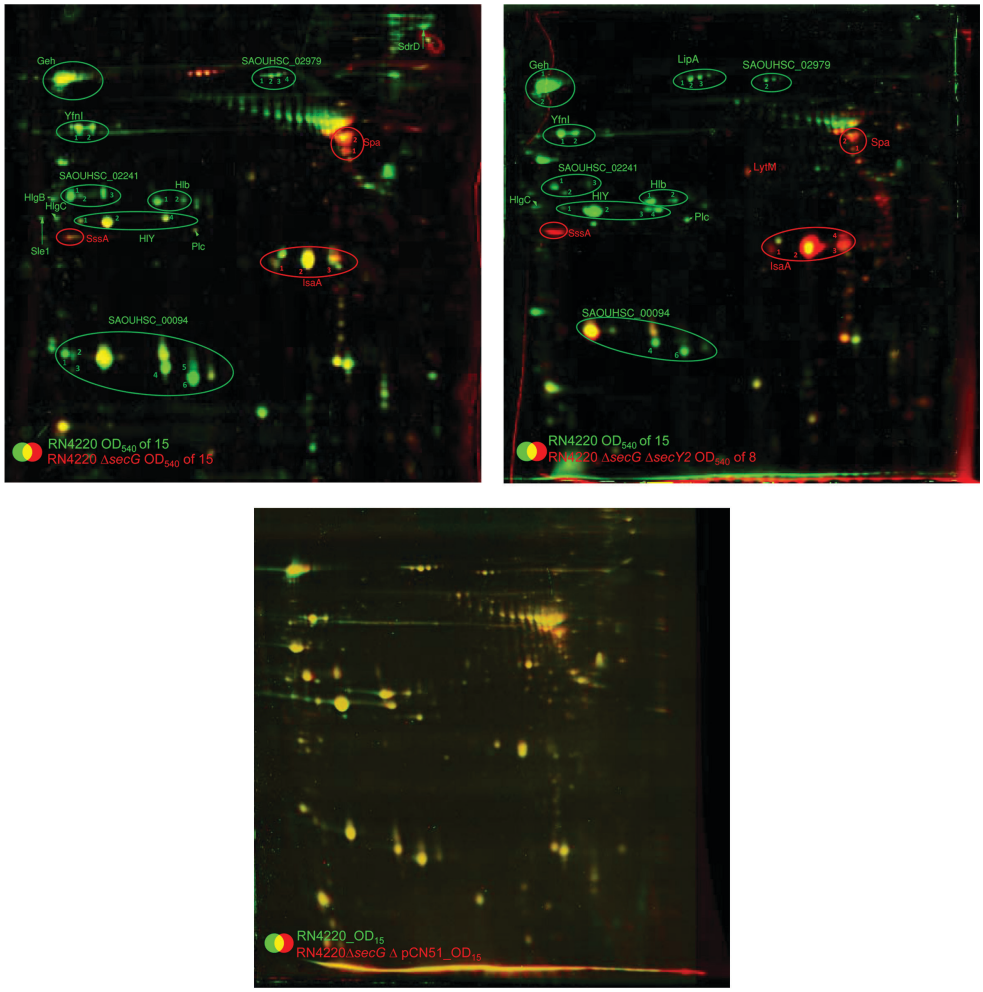

Figure 2. The extracellular proteomes of *S. aureus secG* and *secG secY2* mutants. (**A**) False-colored dual-channel image of 2-D gels of extracellular proteins of *S. aureus* RN4220 (green) and *S. aureus* RN4220 Δ*secG* (red). Proteins (350 µg) isolated from the supernatant of *S. aureus* RN4220 and *S. aureus* RN4220 Δ*secG* grown in TSB medium to an OD_{540} of 15 were separated on 2-D gels by using immobilized pH gradient strips with a linear pH range of 3-10. Proteins were stained with colloidal Coomassie Brilliant Blue. Protein spots present in equal amounts in both strains appear in yellow, protein spots present in higher amounts in the *secG* mutant appear in red, and protein spots present in higher amounts in the parental strain appear in green. Only proteins with a Sec-type signal peptide of which the extracellular amounts were reproducibly affected by the *secG* mutation have been marked. (**B**) False-colored dual-channel image of 2-D gels of extracellular proteins of *S. aureus* RN4220 (green) and *S. aureus* RN4220 Δ*secG* Δ*secY2* (red). For experimental details see (**A**). Protein spots present in equal amounts in both strains appear in yellow, protein spots present in higher amounts in the *secGsecY2* mutant appear in red, and protein spots present in higher amounts in the parental strain appear in green. (**C**) False-colored dual-channel image of 2-D gels of extracellular proteins of *S. aureus* RN4220 (green) and *S. aureus* RN4220 Δ*secG secG*-pCN51 (red). For experimental details see (**A**). All protein spots are yellow, indicating that both strains secreted the respective proteins in equal amounts.

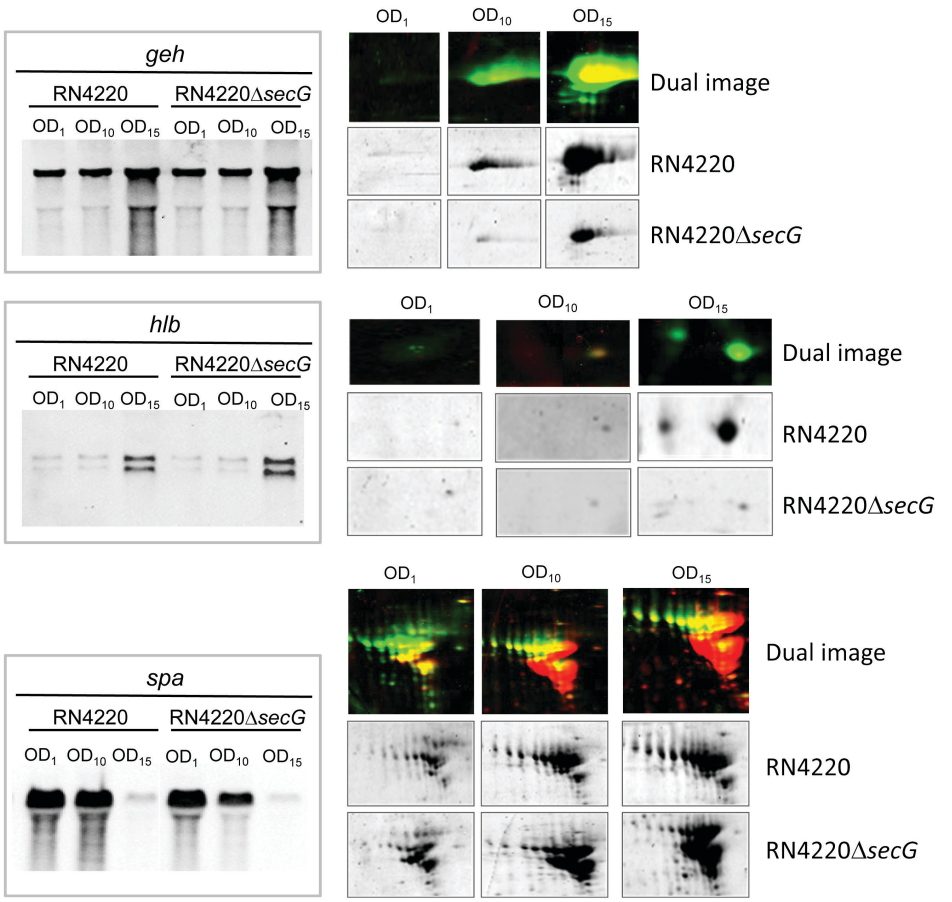

Figure 3. Expression of SecG-dependent exoproteins. RNA and exoproteins were collected from *S. aureus* RN4220 and *S. aureus* RN4220 Δ*secG* grown in TSB medium at 37°C. Samples were collected at three different points during growth (OD_{540} of 1, 10 and 15). In the Northern blotting experiments, membranes were hybridized with digoxigenin-labeled RNA probes specific for *geh*, *hlb* or *spa*. Protein spots from 2-D PAGE analyses of the respective proteins collected at OD_{540} of 1, 10, and 15 are shown for the *secG* mutant and its parental strain both separately and as dual-channel images.

Elevated expression of *sraP* in *secG* mutant cells during the transition phase

To test whether the synthetic effects of the *secG secY2* double mutation might relate to jamming of the SecYE translocation channel by SraP, the only known substrate for the Sec2 pathway, we tried to construct a *secG secY2 sraP* triple mutant. Unfortunately, despite several attempts we did not manage to obtain this triple mutant for reasons that have so far remained obscure. To obtain further insights into the expression of *sraP* under the conditions tested, we performed Northern blotting and slot blot experiments with RNA extracted from the *secG* single mutant, the *secG secY2* double mutant and the parental strain

RN4220. These experiments revealed that *sraP* expression is highest in the transient phase between the exponential and stationary growth phases (Figure 4). Furthermore, the deletion of *secG* reproducibly triggered a two-fold elevated *sraP* transcript level during the transient phase. This moderate but reproducible effect was observed both in the *secG* single mutant and in the *secG secY2* double mutant, which argues to some extent against the possible jamming of SecYE by SraP, at least when SecY2 is still present in the cells.

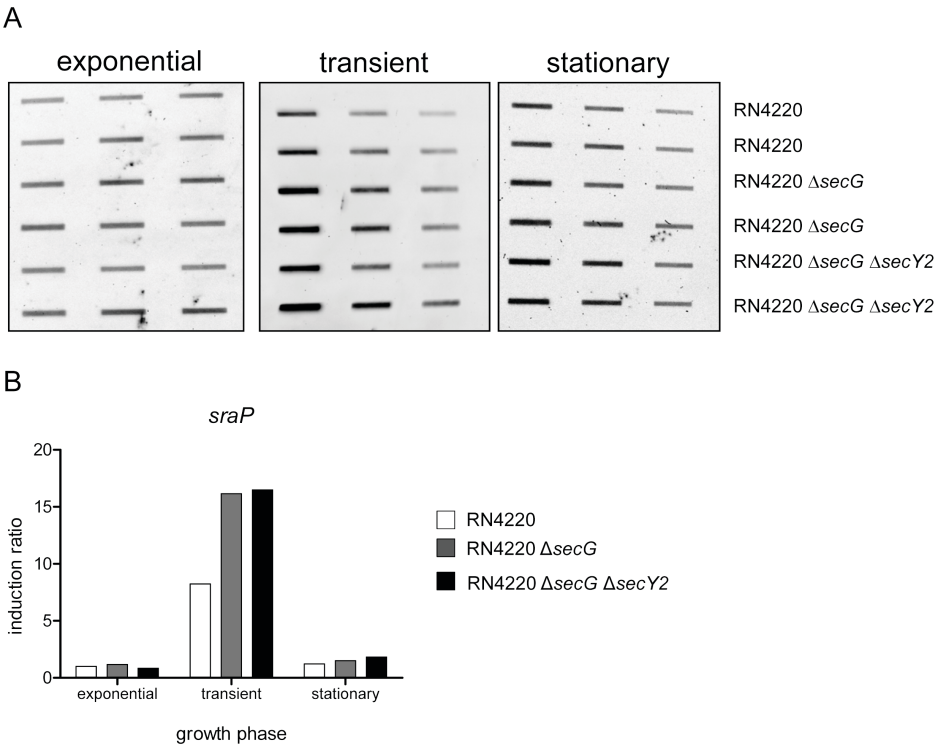

Figure 4. Transcriptional analysis of *sraP*. RNA was prepared from *S. aureus* RN4220, *S. aureus* RN4220 Δ*secG* and *S. aureus* RN4220 Δ*secG* Δ*secY2* cells grown in TSB medium (37°C) at three different stages of growth: exponential phase (OD_{540} 1), transient phase (RN4220 and Δ*secG* at OD_{540} 10; Δ*secG* Δ*secY2* at OD_{540} 6), and stationary phase (RN4220 and Δ*secG* at OD_{540} 15; Δ*secG* Δ*secY2* at OD_{540} 8). (**A**) Serial dilutions of total RNA of the wild type and the mutant strains were blotted and cross-linked onto positively charged nylon membranes. The membrane-bound RNA was hybridized with a digoxigenin labelled RNA probe complementary to *sraP*. (**B**) Quantification of changes in the *sraP* mRNA levels during growth of *S. aureus* RN4220 and its Δ*secG* or Δ*secG* Δ*secY2* mutant derivatives. Induction ratios relate to *sraP* mRNA levels in exponentially growing cells of the RN4220 parental strain as described in the Materials and Methods.

Impaired export of cell wall-bound Sbi in *secG* mutant cells

Western blotting experiments were performed to investigate whether particular protein export defects of the *secG* and *secY2* mutants had remained unnoticed in the proteomic analyses.

These analyses included secreted proteins in the growth medium (Sle1, Aly, IsaA, SspB), non-covalently attached cell wall proteins (Sle1, Aly, IsaA), a covalently attached cell wall protein (ClfA), a lipoprotein (DsbA) and a cytoplasmic marker protein (TrxA) in *S. aureus* strains RN4220 and *S. aureus* SH1000. For most tested proteins no differences were detectable between the *secG* and/or *secY2* mutant strains and their parental strain. However, these analyses showed that a band of ~50 kDa, which was cross-reactive with all tested sera, had disappeared from the fraction of non-covalently bound cell wall proteins of the *secG* mutant. It is known that proteins, such as protein A (36) and Sbi (55) have IgG-binding properties. To investigate whether the missing band would relate to protein A or Sbi, protein fractions from a *spa* mutant, and a *spa sbi* double mutant, were included in the Western blotting analyses. As shown in Figure 5A, the band of ~50 kDa that was missing from the non-covalently bound cell wall proteins in the *secG* mutant was also missing from these proteins in the *spa sbi* double mutant, but not in the *spa* single mutant (only the results for *S. aureus* SH1000 are shown but essentially the same results were obtained for *S. aureus* RN4220). Taken together, these findings show that Sbi is non-covalently bound to the cell wall of *S. aureus* RN4220 and SH1000, and that SecG is required for export of Sbi from the cytoplasm to the cell wall. As was the case for the secreted *S. aureus* proteins detected by proteomics, Sbi export to the cell wall was not affected by the absence of SecY2 (Figure 5B). Finally, it is noteworthy that Sbi is only detectable amongst the non-covalently bound cell wall proteins of *S. aureus* RN4220 and SH1000, whereas it is detectable both in a cell wall-bound and a secreted state in *S. aureus* Newman.

Deletion of *secG* and *secY2* does not affect virulence in a mouse model

To test whether the deletion of *secG* and/or *secY2* would affect the virulence of *S. aureus* SH1000, a mouse infection model was used. The results revealed no significant differences in virulence of the Δ*secG*, Δ*secY2*, or Δ*secG* Δ*secY2* strains as compared to the parental strain SH1000 (Figure 6). This shows that SecG and SecY2 have no important roles in the virulence of strain SH1000 in the context of the used mouse infection model.

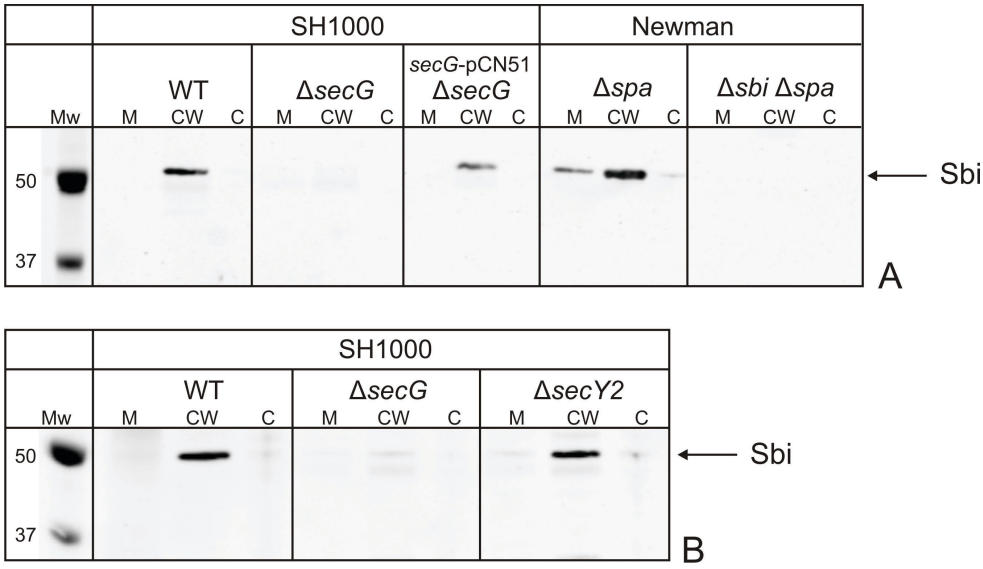

Figure 5. Sbi localization to the cell wall of *S. aureus* depends on SecG. (**A**) *S. aureus* SH1000 (WT), *S. aureus* SH1000 Δ*secG*, and *S. aureus* SH1000 Δ*secG secG*-pCN51 were grown in TSB medium at 37°C till the early stationary phase. Samples of extracellular proteins isolated from the growth medium (M), non-covalently cell wall-bound proteins (CW) and total cells (C) were used for Western blotting and immunodetection with serum of mice immunized with IsaA. As a contol for Sbi production, the strains *S. aureus* Newman Δ*spa* and *S. aureus* Newman Δ*spa* Δ*sbi* were included in the analyses. (**B**) Proteins of *S. aureus* SH1000 (wt), *S. aureus* SH1000 Δ*secG*, and *S. aureus* SH1000 Δ*secY2* were used for Western blotting and immunodetection as in (**A**). The position of Sbi is marked with an arrow.

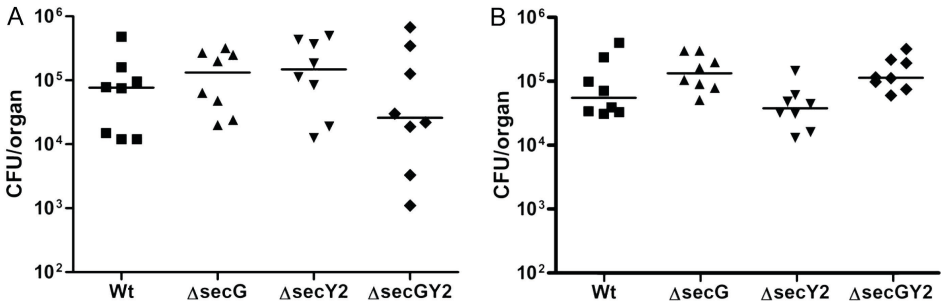

Figure 6. Mouse infection studies with *S. aureus secG* and *secG secY2* mutants. Eight mice were challenged with 1 x 10^8 CFU of *S. aureus* SH1000 Δ*secG*, *S. aureus* SH1000 Δ*secY2*, *S. aureus* SH1000 Δ*secG* Δ*secY2* or the parental strain SH1000 (wt). After 3 days, the bacterial load of the kidneys (**A**) and livers (**B**) was determined as described in the Materials and Methods.

Discussion

The extracellular and surface-associated proteins of bacterial pathogens, such as *S. aureus*, epresent an important reservoir of virulence factors (38,39,57). Accordingly, protein export mechanisms will contribute to the virulence of these organisms. While protein export has been well characterized in model organisms, such as *E. coli* and *B. subtilis*, relatively few functional studies have addressed the protein export pathways of *S. aureus*. Notably, the Sec pathway is generally regarded as the main pathway for protein export but, to date, this has not been verified experimentally in *S. aureus*. Therefore, the present studies were aimed at assessing the role of the Sec pathway in establishing the extracellular proteome of *S. aureus*. We focused attention on the non-essential channel component SecG as this allowed a facile co-assessment of the non-essential accessory Sec channel component SecY2. Our results show that the extracellular accumulation of proteins is affected to different extents by the absence of SecG: some proteins are present in reduced amounts, some are not affected and some are present in elevated amounts. Furthermore, the effects of the absence of SecG are exacerbated by deletion of SecY2, suggesting that SecY2 directly or indirectly influences the functionality of the general Sec pathway. This is all the more remarkable since the absence of SecY2 by itself had no detectable effects on the composition of the extracellular proteome of *S. aureus*.

The observation that the secretion of a wide range of proteins was affected by the absence of SecG is consistent with the fact that all of these proteins contain Sec-type signal peptides. On the other hand, this finding is remarkable since studies in other organisms, such as *E. coli* (26) and *B. subtilis* (48), have shown that deletion of *secG* had fairly moderate effects on protein secretion *in vivo*. In *B. subtilis*, a phenotype of the *secG* mutation was only observed under conditions of high overproduction of secretory proteins (48). Clearly, our present data show that SecG is more important for Sec-dependent protein secretion in *S. aureus* than in *B. subtilis* or *E. coli*. Importantly, the transcription of genes for three proteins (Geh, Hlb and Spa) that were affected in major ways by the absence of SecG was not changed, and all observed effects of the *secG* mutation could be reversed by ectopic expression of *secG*. This suggests that the observed changes in the exoproteome composition of the *S. aureus secG* mutant strain relate to changes in the translocation efficiency of proteins through the Sec channel rather than regulatory responses at the gene expression level. This could be due to altered recognition of the respective signal peptides or mature proteins by the SecG-less Sec channel, or combinations thereof. However, some indirect effects, for example at the level of translation of exported proteins, post-translocational folding, proteolysis or cell wall binding of proteins like IsaA, LytM, Spa and SsaA, can currently not be excluded especially since no proteins were found to accumulate inside

the *secG* mutant cells (data not shown). It remains to be shown why the extracellular accumulation of particular proteins is affected by the absence of SecG, while that of other proteins remains unaffected.

Unexpectedly, our studies revealed that export of the IgG-binding protein Sbi to the cell wall was almost completely blocked in *secG* mutant strains. The reason why this export defect was not detected by 2-D PAGE relates to the fact that Sbi is predominantly cell wall-bound in the tested *S. aureus* strains under the experimental conditions used. It has been proposed previously that Sbi would remain cell wall-attached through a proline-rich wall-binding domain and electrostatic interactions (55). Nevertheless, Burman and colleagues showed that Sbi is extracellular and they suggested that cell surface-bound Sbi might be disadvantageous for the bacterium due to its role in modulating the complement system (8). On the other hand, cell surface localization of Sbi would be appropriate for interference with the adaptive immune system through IgG binding (3). Irrespective of these previously reported findings, our Western blotting analyses show that Sbi is non-covalently bound to the cell wall, not only in *S. aureus* SH1000 and *S. aureus* RN4220, but also in *S. aureus* Newman. However, consistent with the findings of Burman *et al.,* Sbi was also detected in the growth medium of *S. aureus* Newman, which indicates that the location of Sbi in the cell wall or extracellular milieu may differ for different *S. aureus* strains. In case of the Newman strain, the release of Sbi into the growth medium could be due to the fact that this strain produces Sbi and several other cell wall-bound proteins at increased levels compared to the RN4220 and SH1000 strains (35). Conceivably, this increased production of wall-bound proteins might lead to a saturation of available cell wall binding sites for Sbi. Remarkably, the absence of SecG was shown to impact on the relative amounts of various extracellular proteins, while effects of the absence of SecG were detected for only one cell wall-associated protein, namely Sbi. We do not believe that these differences in the numbers of identified proteins relate to the method that was used to monitor effects of the absence of SecG. Especially, the analysis of proteins secreted by *secG* mutant strains via regular 1-D SDS PAGE already revealed major differences in the composition of the exoproteome (not shown). It was for this reason that we initiated our 2-D PAGE analyses to identify the affected proteins. On the other hand, a 1-D SDS PAGE analysis of cell wall-associated proteins did not reveal any major differences, and this was in fact the reason why we investigated potentially wall-associated proteins by Western blotting. Furthermore, we have no evidence from the different studies that we performed that the time point at which the sampling was done during the stationary phase had any major influence on the outcomes of our analyses.

Many of the proteins of which the extracellular amounts are changed due to the absence of SecG are considered to be important virulence factors of *S. aureus*. These proteins are

involved in host colonization (*e.g.* the serine-aspartic acid repeat proteins SdrC and SdrD), invasion of host tissues (*e.g.* hemolysins and leukocidins), cell wall turnover (LytM), and evasion of the immune system (Spa, Sbi). The altered amounts of these proteins suggest that *S. aureus* strains depleted of SecG might perhaps be less virulent. However, in the applied mouse infection model no changes in virulence of the *S. aureus* SH1000 *secG*, *secY2*, or *secG secY2* mutant strains could be detected. This implies that the presence or absence of SecG or SecY2 is not critical for the virulence of *S. aureus* SH1000, at least under the conditions tested in the applied mouse infection model. Clearly, this does not rule out the possibility that such mutants are attenuated in virulence in other infection models that were not tested so far.

Since we were unable to detect secretion defects for *secY2* single mutant strains, our studies confirm that only very few proteins are translocated across the membrane in a SecA2/ SecY2-dependent manner as has previously been suggested by Siboo *et al.* (40). Furthermore, we did not detect differences in the export of glycosylated proteins by the *secY2* mutants (data not shown), which is in line with the suggestion that glycosylated proteins are not strictly dependent on the accessory Sec pathway for export (40). It was therefore quite surprising that the *secY2* mutation exacerbated the secretion defect of the *S. aureus secG* mutant. In fact, the secretion of two additional proteins was found to be affected in the *secG secY2* double mutant. Moreover, a synthetic growth defect was observed for this double mutant. At this stage, it is possible that both the growth defect and the secretion defects are consequences of an impaired Sec channel function. In addition, the exacerbated secretiondefects may relate to SecYE jamming by SraP, which is the only known SecA2/ SecY2 substrate. As shown by Northern blotting analyses, the deletion of *secG* somehow triggers a two-fold elevated *sraP* transcript level during the transition between exponential and post-exponential growth, not only in the *secG* single mutant but also in the *secG secY2* double mutant. This argues to some extent against the jamming of SecYE by SraP, at least when SecY2 is still present in the cells. In the absence of SecG and SecY2, indeed jamming of SecYE by the overexpressed SraP may occur in the transient phase. On the other hand, *sraP* expression seems relatively low in the stationary phase during which we harvested the extracellular proteins for proteomics analyses, which would suggest that any jamming effects of SraP are relatively low in this growth phase. Unfortunately, we have so far not been able to assess the possibility of SecYE jamming by SraP directly, because we were unable to obtain a *secG secY2 sraP* triple mutant. Notably, it is also possible that the exacerbated secretion defects are, to some extent, a secondary consequence of the growth defect of the double mutant. Irrespective of their primary cause, these synthetic effects of the *secG* and *secY2* mutations suggest that the regular Sec channel can somehow

interact with the Sec2 channel. Whether this means that mixed Sec channels with both SecY and SecY2 exist remains to be determined. However, this possibility would be consistent with the observation that *S. aureus* lacks a second set of *secE* and *secG* genes. It would thus be important to focus future research activities in this area on possible interactions between the regular Sec channel components and SecY2.

Acknowledgements

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Table 1. Plasmids and bacterial strains used

Plasmids	**Properties**	**Reference**
TOPO	pCR®-Blunt II-TOPO® vector; Km^R	Invitrogen Life technologies
pCN51	*E. coli* / *S. aureus* shuttle vector that contains a cadmium-inducible promoter	(11)
pMAD	*E. coli* / *S. aureus* shuttle vector that is temperature-sensitive in *S. aureus* and contains the *bga*^{B} gene, Ery^R, Amp^R	(2)
pUC18	Amp^R, ColE1, F80dLacZ, *lac* promoter	(28)
pDG783	1.5-kb kanamycin resistance cassette in pSB118; Amp^R	(15)
secG-pCN51	pCN51 with *S. aureus secG* gene, Amp^R; Ery^R	This work
secY2-pCN51	pCN51 with *S. aureus secY2* gene, Amp^R; Ery^R	This work
Strains	**Genotype**	**Reference**
E. coli		
DH5α	*supE44*; *hsdR17*; *recA1*; *gyrA96*; thi-1; *relA1*	(18)
TOP10	Cloning host for TOPO vector; F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)Φ80*lac*ZΔM15	Invitrogen Life technologies
	Δ*lac*X74 *recA1 ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	
S. aureus RN4220		
Parental strain	Restriction-deficient derivative of NCTC 8325, cured of all known prophages	(24)
Δ*secG*	*secG*	This work
ΔsecY2	*secY2*	This work
Δ*secG* Δ*secY2*	*secG secY2*	This work
S. aureus SH1000		
Parental strain	Derivative of NCTC 8325-4 *rsbU+*, *agr+*	(21)
Δ*secG*	*rsbU+, agr+, secG*	This work
Δ*secY2*	*rsbU+*, *agr+*, *secY2*	This work
Δ*secG* Δ*secY2*	*rsbU+*, *agr+*, *secG secY2*	This work
S. aureus Newman		
Δ*spa*	*spa*	(31)
Δ*spa* Δ*sbi*	*spa sbi*	This work

Table 2. Primers used in this study

Primer	Sequence (5'→3')
secG-F1	TTAAAACAGGACGCTTTATTG
secG-R1	**TTACGTCAGTCAGTCACCATGGCA** AAATTGTCCTCCGTTCCTTAT
secG-F2	**TGCCATGGTGACTGACTGACGTAA** GGTCCGGCGATGTAAATGTCG
secG-R2	GCGTGCATATTCTAAAAAGCC
secY2-F1	TGTCTGGTTCACAAAGCATTT
secY2-R1	**TTACGTCAGTCAGTCACCATGGCA** GTTGCACCTCTTTTATATCAA
*secY2*2-F2	**TGCCATGGTGACTGACTGACGTAA** GGAGGTAATTATGAAATACTT
secY2-R2	GCCTCTCCCTGATCATCAAAA
sbi-F1	TGTGTTCCTTTATTTTCTGCG
sbi-R1	**GAACTCCAATTCACCCATGGCCCCC** CCCCAACTAGCAACTTCGAG
sbi-F2	**CCGCAACTGTCCATACCATGGCCCCC** GGAAATAATCAATCAAAAATATCTTCTC
sbi-R2	CTATTAAACCAACTGCTAAAGTTGC
kan-F1	GGGGGCCATGGGTGAATTGGAGTTCGTCTTG
kan-R1	GGGGGCCATGGTATGGACAGTTGCGGATGTA
secG-F3	GGGGGGTCGACGGGATATACTACTTGTCGTATATA
secG-R3	GGGGGGAATTCCCTTACATACCAAGATAACTTATGCA
secY2-F3	GGGGGGTCGACGTCTTTTTAATGTTTTTGATA
secY2-R3	GGGGGGAATTCCCTTACCAATACTGGTTTAAAAATGG
*spa*_for	ACCTGCTGCAAATGCTGCGC
*spa*_reva	*CTAATACGACTCACTATAGGGAGA* GGTTAGCACTTTGGCTTGGG
*geh*_for	CACATCAAATGCAGTCAGG
*geh*_reva	*CTAATACGACTCACTATAGGGAGA* AATCGACATGATCCCATCC
*hlb*_for	ATCAAACACCTGTACTCGG
*hlb*_reva	*CTAATACGACTCACTATAGGGAGA* CGTAGTAATATGGGAACGC
*sraP*_for	CCATCTAATGTAGCTGGTGG
*sraP*_reva	*CTAATACGACTCACTATAGGGAGA* CACTGATTGTCCAGCATTCG

Overlap in primers are in bold; restriction sites are underlined

[a] Oligonucleotides containing the recognition sequence for T7 polymerase at the 5' end (shown in italic)

Table 3. Extracellular and cell wall proteins with altered secretion patterns in *S. aureus* Δ*secG* and Δ*secG*Δ*secY*2.

Protein[a]	Function	M_r/pI mature	ORFID *S. aureus* NCTC8325	Accession NCBI	Predicted location[b]	Relative level compared to parental strain	
						Δ*secG*/ WT	Δ*secG* Δ*secY2*/ WT
IsaA[1]	immunodominant antigen A	24.2/6.6	SAOUHSC_02887	88196515	cell wall	2.0	4.2
IsaA[2]	immunodominant antigen A	24.2/6.6	SAOUHSC_02887	88196515	cell wall	2.4	8.6
IsaA[3]	immunodominant antigen A	24.2/6.6	SAOUHSC_02887	88196515	cell wall	2.9	4.8
IsaA[4]	immunodominant antigen A	24.2/6.6	SAOUHSC_02887	88196515	cell wall		12.4
LytM	peptidoglycan hydrolase, putative	34.3/6.7	SAOUHSC_00248	88194055	cell wall		4.3
SAOUHSC_00094[1]	hypothetical protein	21.8/9.4	SAOUHSC_00094	88193909	cell wall	0.2	
SAOUHSC_00094[2]	hypothetical protein	21.8/9.4	SAOUHSC_00094	88193909	cell wall	0.3	
SAOUHSC_00094[3]	hypothetical protein	21.8/9.4	SAOUHSC_00094	88193909	cell wall	0.4	
SAOUHSC_00094[4]	hypothetical protein	21.8/9.4	SAOUHSC_00094	88193909	cell wall	0.3	0.3
SAOUHSC_00094[5]	hypothetical protein	21.8/9.4	SAOUHSC_00094	88193909	cell wall	0.5	
SAOUHSC_00094[6]	hypothetical protein	21.8/9.4	SAOUHSC_00094	88193909	cell wall	0.2	0.3
SdrD1	SdrD protein, putative	14.6/3.9	SAOUHSC_00545	88194324	cell wall	0.3	
Sle1 (Aaa)	autolysin precursor, putative	35.8/9.9	SAOUHSC_00427	88194219	cell wall	0.4	
Spa[1]	protein A	55.6/5.4	SAOUHSC_00069	88193885	cell wall	3.7	3.3
Spa[2]	protein A	55.6/5.4	SAOUHSC_00069	88193885	cell wall	5.0	2.9
SsaA	secretory antigen precursor, putative	29.3/9.1	SAOUHSC_02571	88196215	cell wall	5.0	9.9
Geh	lipase precursor	76.4/9.6	SAOUHSC_00300	88194101	extracellular	0.4	0.2
Hlb[1]	truncated β-hemolysin	31.3/8.2	SAOUHSC_02240	88195913	extracellular	0.1	0.2
Hlb[2]	truncated β-hemolysin	31.3/8.2	SAOUHSC_02240	88195913	extracellular	0.4	0.4
HIY[1]	α-hemolysin precursor	35.9/9.1	SAOUHSC_01121	88194865	extracellular	0.3	0.5
HIY[2]	α-hemolysin precursor	35.9/9.1	SAOUHSC_01121	88194865	extracellular	0.4	0.2
HIY[3]	α-hemolysin precursor	35.9/9.1	SAOUHSC_01121	88194865	extracellular		0.2
HIY[4]	α-hemolysin precursor	35.9/9.1	SAOUHSC_01121	88194865	extracellular	0.4	0.2
HlgB	leukocidin F subunit precursor	36.7/9.8	SAOUHSC_02710	88196350	extracellular	0.3	

Protein[a]	Function	M./pI mature	ORFID *S. aureus* NCTC8325	Accession NCBI	Predicted location[b]	Relative level compared to parental strain	
						Δ*secG*/ WT	Δ*secG* Δ*secY2*/ WT
LipA[1]	lipase	76.7/7.7	SAOUHSC_03006	88196625	extracellular		0.5
LipA[2]	lipase	76.7/7.7	SAOUHSC_03006	88196625	extracellular		0.3
LipA[3]	lipase	76.7/7.7	SAOUHSC_03006	88196625	extracellular		0.4
Plc	1-phosphatidylinositol phosphodiesterase precursor, putative	37.1/8.6	SAOUHSC_00051	88193871	extracellular	0.3	0.3
SAOUHSC_02241[1]	hypothetical protein	38.7/9.1	SAOUHSC_02241	88195914	extracellular	0.1	0.2
SAOUHSC_02241[2]	hypothetical protein	38.7/9.1	SAOUHSC_02241	88195914	extracellular	0.3	0.4
SAOUHSC_02241[3]	hypothetical protein	38.7/9.1	SAOUHSC_02241	88195914	extracellular	0.2	0.4
SAOUHSC_02979[1]	hypothetical protein	69.3/6.3	SAOUHSC_02979	88196599	extracellular	0.4	
SAOUHSC_02979[2]	hypothetical protein	69.3/6.3	SAOUHSC_02979	88196599	extracellular	0.3	0.3
SAOUHSC_02979[3]	hypothetical protein	69.3/6.3	SAOUHSC_02979	88196599	extracellular	0.3	
SAOUHSC_02979[4]	hypothetical protein	69.3/6.3	SAOUHSC_02979	88196599	extracellular	0.3	
Yfnl[1]	Polytopic membrane protein, signal peptidase I substrate	74.4/9.5	SAOUHSC_00728	88194493	extracellul	0,4	0,2
Yfnl[2]	Polytopic membrane protein, signal peptidase I substrate	74.4/9.5	SAOUHSC_00728	88194493	extracellul		

[a]Several proteins are detectable as multiple spots. The spot numbers as marked in Figure 2 are indicated in superscript.
[b]Protein localization was predicted as described in Sibbald *et al.* (38); SceD, SsaA and IsaA were shown to be bound ionically to the cell wall by Stapleton *et al.* (42), Sle1 (Aaa) has three LysM domains that can bind to peptidoglycan (7).
[c]The induction ratio of mutant to parental strain was calculated for each spot (normalized intensity of a spot on the mutant image/normalized intensity of the corresponding spot on the parental image). The significance of spot volume differences of two-fold or higher was assessed by the Student´s t test (α<0.05; Delta 2D "statistics" table).

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8

The signal peptidase ComC and the thiol-disulfide oxidoreductase DsbA are required for optimal cell surface display of the pseudopilin ComGC in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is an important Gram-positive bacterial pathogen producing many secreted and cell surface-localized virulence factors. Here we report that the staphylococcal thiol-disulfide oxidoreductase DsbA is essential for stable biogenesis of the ComGC pseudopilin. The signal peptidase ComC is indispensable for ComGC maturation and optimal cell surface exposure.

Introduction

Staphylococcus aureus is a major Gram-positive bacterial pathogen causing a broad range of infections. To subvert its mammalian hosts, *S. aureus* relies on different virulence factors that are localized at the cell surface or secreted into the host milieu (11,20). For export of newly synthesized virulence factors from the cytoplasm, their translocation across the membrane, and post-translocational modifications an intricate secretion machinery has evolved (20). In recent years, the functions of many secretion machinery components of *S. aureus* have been elucidated (3,4,9,13,19,21). Intriguingly however, for several other potential secretion machinery components no biological functions have been described so far. For example, this applies to the extracytoplasmic thiol-disulfide oxidoreductase (TDOR) DsbA, which is known as one of the strongest bacterial TDORs (12,15). Likewise, the role of the pseudopilin export machinery of *S. aureus* has not yet been documented. This machinery is very similar to the Com machinery for DNA binding and uptake in the related Gram-positive bacterium *Bacillus subtilis*. Interestingly, the genes coding for most Com proteins are present in the sequenced *S. aureus* strains (20). The transcription of these genes, which are organized in the *comG* and *comE* operons, is directed by the staphylococcal alternative sigma factor σ^H (17).

The biogenesis of the Com pseudopilin system has been well studied in *B. subtilis*. Among the *B. subtilis* Com proteins with orthologues in *S. aureus* are those encoded by the *comG* operon (1). Specifically, the *B. subtilis* ComGC, ComGD, ComGE, and ComGG proteins form pilin-like structures that are localized to the cytoplasmic membrane and cell wall (6-8,22). Assembly of the pseudopilus in *B. subtilis* requires the specific signal peptidase ComC, which processes the N-terminal signal peptides of ComG proteins upon membrane translocation (6,22,23). Furthermore, stability of the *B. subtilis* ComGC pseudopilin requires post-translocational TDOR-mediated disulfide bond formation (6,10,15,16).

Results and Discussion

ComGC of *S. aureus* is processed by ComC and stabilized by DsbA

To study the processing and stability of *S. aureus* ComGC, the expression of the *com* genes was induced through constitutive expression of *sigH* from plasmid pRIT: *sigH* (17). As shown with specific polyclonal antibodies raised tagainst ComGC, exponentially growing cells of the *S. aureus* strains RN4220, SH1000, RN6911 and Newman produced only the precursor form of this pseudopilin (Figure 1). ComGC production depended strictly on ectopic expression of σ^H (Figure 1). Notably, relatively small amounts of mature ComGC were detectable when the investigated strains were grown to stationary phase (Figure 1A). This inefficient ComGC processing was due to limited ComC signal peptidase activity

as shown by ComC overexpression from plasmid pCN51:*comC*. This resulted in close-to-complete ComGC processing (Figure 1B). Conversely, ComGC processing in the post-exponential growth phase was completely abolished by a *comC* deletion. Consistent with these observations, no *comC* expression was detectable in exponentially growing *S. aureus* cells, and low-level *comC* transcription was detectable in the late stationary growth phase (data not shown). Together, these findings show that ComC is the signal peptidase needed for ComGC processing, and that the investigated strains produce limiting amounts of ComC under the tested conditions.

To investigate whether the stability of *S. aureus* ComGC depends on TDOR activity, the production of this protein was analyzed in strain RN4220 lacking the *dsbA* gene. Western blotting analyses showed that ComGC was barely detectable in cells lacking DsbA. This effect did not relate to possible changes in the σ^H levels, which remained unaltered in the *dsbA* mutant (Figure 2 lower panel). These observations indicate that the intramolecular disulfide bond of *B. subtilis* ComGC is conserved in *S. aureus* ComGC, and that the formation of this disulfide bond between Cys46 and Cys87 of *S. aureus* ComGC is catalyzed by DsbA. Furthermore, this disulfide bond, which is positioned within the predicted extracytoplasmic domain of *S. aureus* ComGC, would be necessary to stabilize ComGC upon export from the cytoplasm. To test this idea, the reducing agent β-mercaptoethanol was added to the growth medium at concentrations up to 2.5 mM, which is the highest concentration of β-mercaptoethanol that can be added to the cells without affecting growth and cell viability (data not shown). Clearly, in the presence of 2.5 mM β-mercaptoethanol, ComGC was barely detectable (Figure 2, upper panels), whereas σ^H production remained unaffected (Figure 2, lower panels). The most simple explanation for these observations is that the TDOR activity of DsbA is required for disulfide bond formation in ComGC and that this disulfide bond is essential for ComGC stability. Nevertheless, it is possible that DsbA is indirectly involved in the stabilization of ComGC. To our knowledge, this is the first report describing a biological function for DsbA in *S. aureus*.

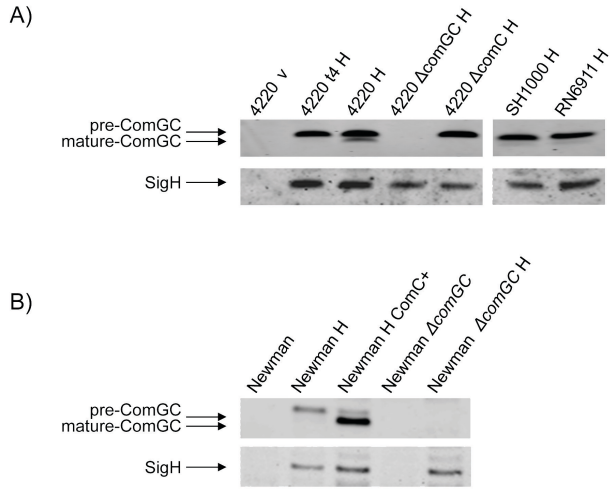

Figure 1. ComGC processing by ComC. *S. aureus* strains were grown overnight at 37°C in Luria-Bertani broth (LB) supplemented with 12.5 µg/ml chloramphenicol to select for pRIT:*sigH* or pRITH5, or 5 µg/ml erythromycin to select for pCN51:*comC*. Samples for Western blotting analyses with polyclonal rabbit antibodies against ComGC or chicken antibodies against SigH were collected after 4 h (t4) or 7 h of growth (**A**), or after 5h of growth (**B**). Cell extracts were prepared as previously described (19). Proteins were separated by SDS-PAGE (NuPage gels, Invitrogen) and blotted onto nitrocellulose membranes (Protran, Schleicher & Schuell). Immunodetection was performed with fluorescent secondary antibodies (IRDye 700 CW goat anti-rabbit, IRDye 800 goat anti-chicken, LiCor) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). The chromosomal *comGC* or *comC* genes were deleted from *S. aureus* strains RN4220 or Newman Δ*spa* Δ*sbi* (19) as previously described (2,14). Primers used for strain and plasmid constructions are listed in Table 1. Lanes relating to strains that carry pRIT:*sigH* for σ^H production are labeled with H; lanes relating to control strains with the empty vector pRIT5H that do not produce σ^H are labeled with v; lanes relating to strains that carry pCN51:*comC* (5) for ComC production are labeled with ComC+.

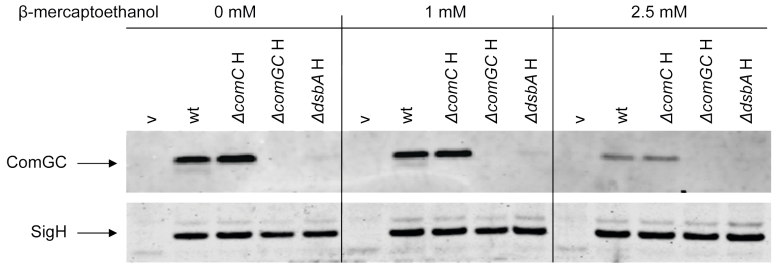

Figure 2. ComGC stabilization by DsbA. *S. aureus* strains were grown for 7h in LB broth as described in Figure 1 in the presence or absence of β-mercaptoethanol (final concentrations 1 mM or 2.5 mM). The preparation of cell extracts, SDS-PAGE and Western blotting with specific antibodies against ComGC or σ^H was performed as described in Figure 1. The chromosomal *dsbA* gene was deleted from *S. aureus* strain RN4220 as previously described (2,14). Primers used for strain construction are listed in Table 1. Lanes relating to strains that carry pRIT:*sigH* for σ^H production are labeled with H; lanes relating to control strains with the empty vector pRIT5H that do not produce σ^H are labeled with v.

ComGC localizes to the membrane, cell wall and cell surface of *S. aureus*

To determine the localization of ComGC, cells of *S. aureus* strain RN4220 or strain RN4220 overproducing ComC were subjected to subcellular fractionation. Cells were first protoplasted. Next, the protoplasts were separated from liberated cell wall proteins (*i.e*. the cellwall fraction) by centrifugation. The collected protoplasts were then disrupted by osmotic shock, and cytosolic proteins were separated from the membranes by ultracentrifugation as previously described (24). Proteins in all collected fractions were separated by SDS-PAGE and the presence of ComGC and thioredoxin A (TrxA) in each fraction was analyzed by Western blotting with specific antibodies (Figure 3A). TrxA was used as a cytoplasmic control protein. This analysis showed that both pre-ComGC and mature ComGC from *S. aureus* localize to the cytoplasmic membrane and cell wall. This dual localization is consistent with the localization of the homologous protein in *B. subtilis*. Furthermore, in *S. aureus* cells overproducing the ComC protein, we observed slightly increased amounts of ComGC in the cell wall fraction as compared to cells of the parental control strain that do not produce ComC under the tested conditions. Next, we investigated by immunofluorescence microscopy whether ComGC is detectable on the cell surface. For this purpose, we employed cells of strain Newman lacking the IgG-binding proteins Spa and Sbi, which displayed negligible background fluorescence (Figure 3B; compare panels for the *spa sbi* mutant and the parental strain Newman). Importantly, σ^H-producing cells showed elevated levels of immune fluorescence and strongly enhanced immune fluorescence was observed when the signal peptidase ComC was overexpressed together with σ^H. These observations are consistent with the view that the signal peptide of ComGC facilitates membrane translocation and exposure of ComGC to the cell wall, irrespective of signal peptide processing by ComC. Enhanced signal peptide processing upon ComC overproduction would then allow more of the translocated mature ComGC to penetrate the cell wall and to become exposed at the cell surface. These findings thus show that ComC-dependent processing of ComGC is of important for optimal cell surface exposure of ComGC. It should be noted that, for unknown reasons, cells overproducing ComC have a larger diameter which seems to relate mostly to a thickened cell wall (Figure 3B).

In summary, we show that biogenesis of the pseudopilin ComGC of *S. aureus* requires the TDOR DsbA for stability and the signal peptidase ComC for precursor maturation and cell surface exposure. This is thus the first report where biological functions are demonstrated for *S. aureus* DsbA and ComC. In *B. subtilis*, the Com system is needed for DNA uptake during genetic competence. Whether this is also true in *S. aureus* remains to be demonstrated, but natural competence has been reported for *S. aureus* (18). Our present findings suggest that expression of *comC* could be a limiting factor in competence development, even if *S. aureus*

cells overproduce σ^H for expression of other *com* genes.

Acknowledgments

We thank Tarek Msadek for helpful discussions and Kazuya Morikawa for providing plasmids pRIT5H and pRIT*sigH*, and anti-σ^H antibodies. This research was supported by CEU project LSHG-CT-2006-037469 and the Top Institute Pharma project T4-213.

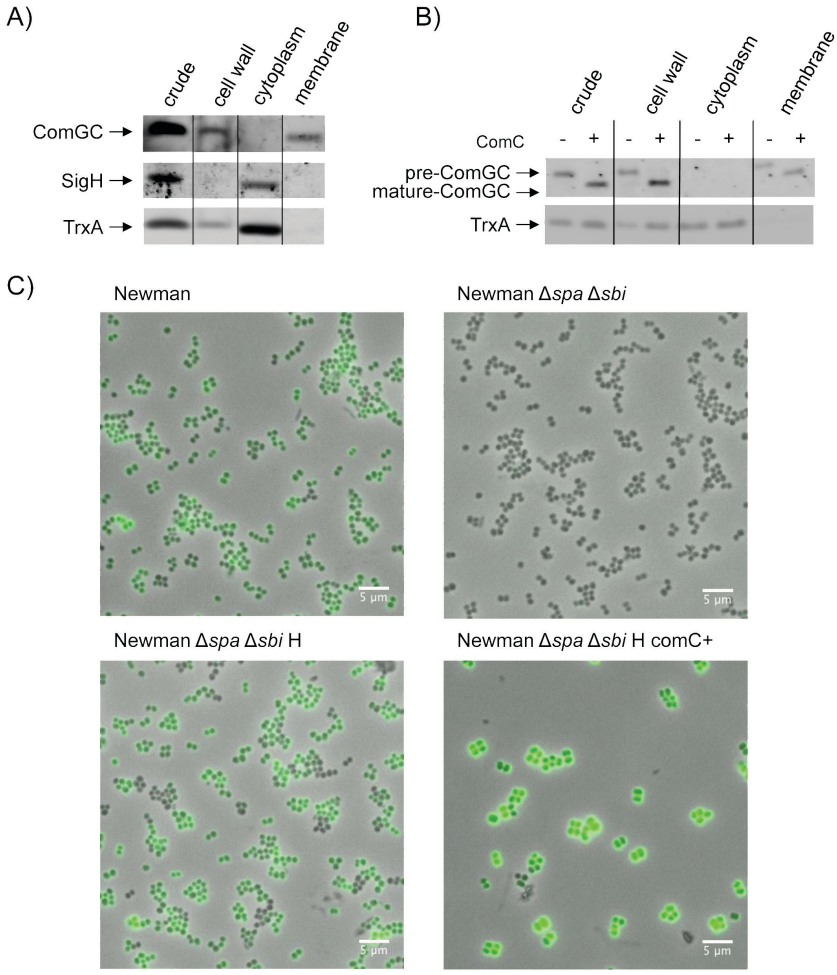

Figure 3. ComGC localizes to the membrane, cell wall and cell surface of *S. aureus*. (**A**), To determine the subcellular localization of ComGC in *S. aureus* RN4220 pRIT: *sigH*, or *S. aureus* RN4220 pRIT:*sigH* containing pCN51:*comC*, the cells were grown in LB broth for 5 h, collected by centrifugation and incubated for 1 h at 37°C in protoplast buffer (50mM Tris-HCl pH 7.6, 0.145 M NaCl, 30% sucrose, 0.01% DNase, and EDTA-free Complete protease inhibitors, Roche). The cell wall fraction (*i.e.* protoplast supernatant) was obtained by centrifugation (20 min, 3000xg, 4°C). Protoplasts were disrupted by osmotic shock in 0.05 M Tris-HCl pH 7.6, 30 min incubation on ice, and vortexing at 5 min intervals. Cytosolic and solubilized membrane proteins were collected as previously described (24). SDS-PAGE and Western blotting with specific antibodies against ComGC or the cytoplasmic control protein TrxA was performed as described in Figure 1. (**B**), Cell surface exposure of ComGC was assessed in *S. aureus* Newman Δ*spa* Δ*sbi* or the parental strain (Newman) by immunofluorescence microcopy. For this purpose, cells were grown for 5 h in LB broth and 1 OD_{600} unit of cells was collected by centrifugation (8000 rpm, 5', 4°C). The cell pellet was resuspended in PBST+2% BSA, and incubated for 10 min on ice. Next, the cells were incubated for 60 min with ComGC-specific polyclonal rabbit antibodies (1:400 in PBST+1%BSA). Unbound antibodies were removed by three washes in PBST, and cell-bound ComGC antibodies were visualized using goat-anti-rabbit alexaFluor488 antibodies (Life technologies) and a Leica DM5500 B microscope. The overlay of phase contrast and fluorescence microscopy images was done with imageJ. The strains containing pRIT:*sigH* for σ^H production are labeled with H; the strain containing pCN51:*comC* for ComC production is labeled with ComC+. The magnification is indicated by scale bars.

Table 1. Primers used in this study. Overlapping parts are shown in bold. Restriction sites used for cloning are underlined and shown in parentheses.

Primer	Sequence (5'→3')
	Construction of *S. aureus comC* mutant
ComC-F1	CGAGATGGTCAAACATTTAAG
ComC-R1	**TCACGTCAGTCAGTCACCATGGCA**ATGACAACCTCCTTATGTAAA
ComC-F2	**TGCCATGGTGACTGACTGACGTGA**AAATTAAAGAAATGGTAA
ComC-R2	AACTGCGATGATTGCATTGGC
	Construction of *S. aureus comGC* mutant
ComGC-F1	GCTCAATAAGATAAACTTTGT
ComGC-R1	**CTACGTCAGTCAGTCACCATGGCA**ATATTAACCTCCATTATTTTA
ComGC-F2	**TGCCATGGTGACTGACTGACG**TAGAAAGCAGTCAGCATTTAC
ComGC-R2	GATTCATCATTGGTATCAATA
	Construction of *S. aureus dsbA* mutant
DsbA-F1	ATTTCTTTGGATATTTATATT
DsbA-R1	**CTACGTCAGTCAGTCACCATGGCA**ATATTAACCTCCATTATTTTA
DsbA-F2	**TGCCATGGTGACTGACTGACG**TAGAAAGCAGTCAGCATTTAC
DsbA-R2	CTTTCGTTATAGTTTTCCCAC
	Construction of pCN51:*comC* for *S. aureus comC* overexpression
ComC-F	CAGCCGGATCCCATAAGGAGGTTGTCATTTGGTAG (*Bam*HI)
ComC-R	CGGAATTCCTTTAATTTTCAAAAATATACGCCTCC (*Eco*RI)

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9

Staphylococcal sortase A mutant cells display a phosphate starvation response in human plasma

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To be submitted

Abstract

Surface proteins of *Staphylococcus aureus* play important roles in the pathogenicity of this bacterium. Several of these proteins are covalently attached to the cell wall by the sortases SrtA and SrtB. The present studies were aimed at determining the relevance of sortase activity for *in vitro* staphylococcal growth in human plasma. The results show that, depending on the investigated strain, SrtA can be important for efficient growth in human plasma, whereas SrtB is not required. Transcript profiling of *srtA* mutant cells grown in human plasma revealed a typical phosphate starvation response, even though this 'medium' is known to contain relatively high phosphate concentrations. This suggests that one or more covalently cell wall-bound proteins are involved in phosphate binding or uptake. Intriguingly, the *fnbpA* gene and, to lesser extent, the *fnbpB* gene were induced in SrtA-deficient cells. Similar to genes encoding the phosphate transporter Pst and the alkaline phosphatase PhoB, the *fnbpA* and *fnbpB* genes are preceded by a typical Pho box, indicating that they are part of the phosphate starvation-inducible pho regulon of *S. aureus*. Since the FnbpA and FnbpB proteins are known SrtA substrates, our observations implicate these two covalently cell wall-bound proteins in the binding and uptake of phosphate by *S. aureus*.

Introduction

Staphylococcus aureus is a Gram-positive commensal bacterium carried by about 20-30% of the human population. However, this commensal is far from harmless, because under certain circumstances it can become invasive causing potentially life-threatening infections, such as sepsis (26). The ability of *S. aureus* to invade and colonize almost all human tissues depends on various cell surface-associated and secreted virulence factors. These virulence factors are first synthesized with an N-terminal signal peptide to direct them into one of the secretion pathways of the staphylococcal cell (44). After their translocation across the membrane, the signal peptide is proteolytically removed, which is necessary for release from the extracytoplasmic side of the membrane (1, 50). The translocated proteins will then pass the cell wall and are released into the extracellular environment, unless they are specifically retained in the cell wall. The retention of proteins in the cell wall requires specific signals that facilitate covalent or non-covalent attachment to particular cell wall components (44).

The signal for covalent attachment of translocated proteins to the cell wall is formed by a so-called LPxTG motif, which is located in the C-terminus of the exported proteins. This LPxTG motif is recognized by membrane-associated transpeptidases known as sortases (27). Upon binding to the LPxTG motif, sortase hydrolyses the bond between the Thr and Gly residues and, then, it covalently attaches the liberated C-terminus of the Thr residue to the free amino group of a pentaglycine cross-bridge in the cell wall peptidoglycan (14, 24, 42, 47). *S. aureus* possesses two sortase enzymes, namely sortase A (SrtA), which recognizes the canonical LPxTG motif, and sortase B (SrtB), which specifically recognizes an NPQTN motif (29, 34). It has been reported that 21 different *S. aureus* proteins carrying an LPxTG motif are attached to the cell wall by SrtA (13, 27, 33, 40, 44). These include protein A (Spa), two fibronectin-binding proteins (FnbpA and FnbpB) (16), two clumping factors (ClfA, ClfB), three cell wall-anchored proteins with large serine-aspartate repeat domains (SdrC, SdrD and SdrE) (22), a collagen-binding protein (Cna), a plasmin-sensitive protein (Pls) (41), the methicillin resistance determinant B (FmtB) (23), and eleven staphylococcal surface (Sas) proteins. The iron-regulated surface determinant protein C (IsdC) is the only protein known to be covalently coupled to the cell wall by SrtB (30).

It has been shown that *S. aureus* lacking the *srtA* gene is impaired in the retention and proper display of LPxTG proteins at the cell surface (43, 49). This explains why SrtA is of major importance for the establishment of systemic and localized staphylococcal infections (28, 52). Specifically, *S. aureus srtA* mutant cells are unable to bind fibrinogen

in solution (52). Furthermore, *S. aureus* cells lacking SrtA were shown to be affected in the binding to cardiac cells, which is probably due to a reduced ability for fibrin binding by otherwise covalently attached cell surface proteins (52). A *srtA* mutant was also found to be impaired in the establishment of arthritis in a mouse model, which seems to be related to defects in the adhesion to collagen and fibrinogen in the target tissue of the joints (21). Unlike the *srtA* mutation, a *srtB* mutation was shown to cause only a minor defect in the establishment of arthritis in a mouse model (20).

Altogether, the impact of *srtA* mutations on protein binding to the staphylococcal cell wall and binding of staphylococci to particular compounds and tissues of the host has been studied in substantial detail. In contrast, nothing was so far known about possible staphylococcal stress responses that are elicited by impaired sortase activity. Therefore, the aim of the present study was to examine how the global transcription of *S. aureus* genes is influenced in the absence of *srtA* or *srtB*. The rationale behind these experiments was that possible stress responses would shed further light on the specific roles of proteins that are covalently bound to the staphylococcal cell wall. To mimic the *in vivo* situation, all analyses were performed with cells grown in human plasma. Interestingly, the present results reveal a phosphate starvation response in *srtA* mutant cells. Our data imply that certain LPxTG proteins, especially FnbpA and FnbpB, are involved in phosphate acquisition.

Materials and methods

Ethical approval

The Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Assen, the Netherlands) approved the protocol for blood donations from healthy volunteers. This protocol is registered by QPS Groningen (code 04132-CS011). The required informed consent was obtained from all healthy volunteers included in the present studies.

Human plasma

Whole blood samples (500 ml) were donated by 3 healthy adult volunteers. The samples were processed for further analyses immediately after collection. The donated blood was diluted 2:1 with Hanks' Balanced Salt Solution (HBSS, Gibco), which is composed of 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$-$6H_2O$, 0.4 mM $MgSO_4$-$7H_2O$, 5.3 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 138 mM NaCl, 0.3 mM Na_2HPO_4 and 5.6 mM D-Glucose (Dextrose). Plasma was obtained after separation from blood cells. The collected human plasma was stored at -30°C. Thawed plasma from the different volunteers was mixed and then heat-inactivated for 30 min at 56°C in a water bath. Subsequently, heat-inactivated plasma was centrifuged for 15 min at 12000 rpm, 4°C and filtered with a 65 µm millipore filter. The resulting fraction

was stored at 4°C and used within 12 h for further experiments.

Bacterial strains and culture

The *S. aureus* strains SH1000 and HG001 and their Δ*srtA* or Δ*srtB* derivatives (49) were grown overnight in tryptic soy broth (TSB, OXOID) under vigorous shaking (250 rpm) at 37°C. The bacterial cultures were diluted to an OD_{600} of 0.05 in pre-warmed TSB and cultivation was continued under the same conditions. Exponentially growing cells (OD_{600} ~0.5) were then diluted into pre-warmed human plasma to a final OD_{600} of 0.05 and the cultivation was continued under the same conditions. Finally, exponentially growing cells were again diluted into fresh and pre-warmed human plasma to a final OD_{600} of 0.05 and the cultivation was continued until four hours after the entry into stationary phase.

Extraction of total RNA

Total RNA was isolated as previously described (35). Briefly, 15 OD units of the parental strain *S. aureus* HG001 and its Δ*srtA* or Δ*srtB* derivatives grown in human plasma were harvested at OD_{600} 0.5, and at four hours after entering into the stationary growth phase (Figure 1B). Samples withdrawn from the different cultures were added to 0.5 volumes of frozen killing buffer (20 mM Tris HCl pH 7.5, 5 mM $MgCl_2$, 20 mM NaN_3) and immediately centrifuged (10 min, 3279 × *g*, 4°C). The collected cells were then immediately frozen in liquid nitrogen and stored at -80°C. Cells were mechanically disrupted in a Mikro-Dismembrator (Santorius) for 2 min at 2600 rpm and resuspended in pre-warmed lysis solution (4 M guanidine thiocyanate, 25 mM sodium acetate [pH 5.2], 0.5% N-laurylsarcosinate 40 [wt/vol]). Total RNA was isolated by acid-phenol extraction and precipitated with isopropanol, washed with 70% ethanol and dissolved in 100 µl of RNase-free water. For transcriptome analyses, 35 µg RNA was DNase-treated using the RNase-Free DNase Set (Qiagen) and purified using the RNA Clean-Up and Concentration Micro Kit (Norgen). The RNA concentration was measured using a NanoDrop spectrophotometer and the RNA quality was assessed with an Agilent 2100 Bioanalyzer as previously described (35).

Microarray experiments

For the microarray experiments, the BaSysBio Sau T1 NimbleGen 385K array was used (12). 20 µg of each RNA sample was labeled and hybridized to an array using the BaSysBio protocol for strand-specific gene expression analysis (35, 38). All tiling array experiments were performed in triplicate using RNA isolated from independent cultures.

An aggregated expression value for each Genbank annotated CDS was computed as the median log2 intensity of probes lying entirely within the corresponding region. Possible cross-

hybridization artefacts were controlled by BLAST-alignment as previously described (12, 51). The aggregated intensity values of the individual samples were normalized by median centering. The statistical analysis of differential mRNA levels between the parental strain and the respective Δ*srtA* or Δ*srtB* mutant strains was performed using the moderated *t*-test (GeneSpring 12, Agilent Technologies). Genes were considered to be differentially expressed at fold changes (FC) of ≥1.8 with a *p*-value <0.05. The microarray data set is available at the NIH Gene Expression Omnibus (GEO) database under record number GSE44761.

Results

Growth of *srtA* or *srtB* mutant *S. aureus* cells in human plasma

Since human plasma is a poorly defined growth medium, we performed extensive growth experiments with different *S. aureus* strains and their *srtA* or *srtB* mutant derivatives. Unexpectedly, the results showed that a *srtA* mutant of strain SH1000 grew at a much lower rate in human plasma than the respective parental strain or a *srtB* mutant (Figure 1A). In contrast, the *srtA* mutant derivative of strain HG001 did not show this growth defect (Figure 1B). These findings indicated that SrtA can be of major importance for staphylococcal growth in human blood, depending on the strain. By contrast, SrtB appeared to be dispensable for growth in human plasma, at least in the case of strains SH1000 and HG001. For an optimal comparison of global transcript profiles of parental and mutant bacterial strains, it is important that these strains grow at comparable rates so that growth-specific effects on gene expression can be excluded. Therefore, all further experiments were performed with strain HG001 and its *srtA* or *srtB* mutant derivatives, which displayed comparable growth in human plasma (Figure 1B).

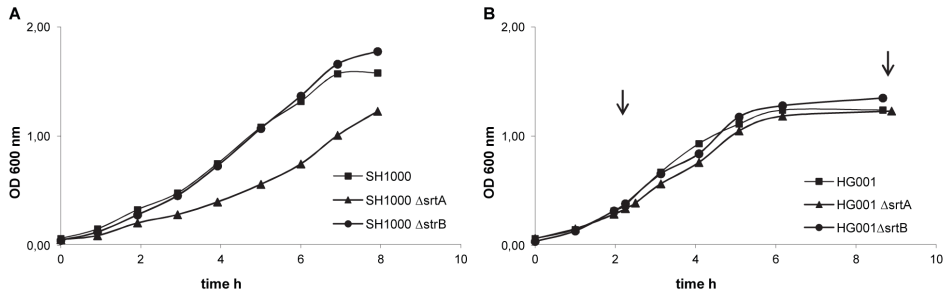


Figure 1. Growth of *S. aureus srtA* and *srtB* mutants in human plasma. *S. aureus* strains were grown in human plasma under vigorous shaking at 37°C strains A) Growth curves of *S. aureus* SH1000 and its Δ*srtA* or Δ*srtB* mutant derivatives. B) Growth curves of *S. aureus* HG001 and its Δ*srtA* or Δ*srtB* mutant derivatives. Time points at which aliquots were withdrawn from the cultures for RNA isolation are indicated by arrows.

Transcriptome of *srtA* or *srtB* mutant strains

To examine whether the absence of sortase genes influences the genome-wide gene expression profile of *S. aureus*, we performed tiling array experiments. For this purpose total RNA was isolated from exponentially growing as well as stationary phase cells of *S. aureus* HG001 and its *srtA* or *srtB* mutant derivatives. As show in Table 1, deletion of the *srtA* gene resulted in the differential expression of 39 genes, from which 31 were up-regulated, and 8 were down-regulated. Functional annotation clustering of up-regulated genes revealed an enrichment of two clusters (17, 18). The first cluster contains eight genes known to be involved in the pathogenesis of *S. aureus*, namely *fnbA*, *hlb*, *psmβ1*, *psmβ2*, *hlgA* and *hlgC*. The second cluster contained five genes (*yjbB*, *pstS*, *pstC*, *pstA*, *pstB*), which encode the phosphate transport system. Notably, the *pstSCAB* operon was up-regulated both in exponentially growing *srtA* mutant cells and *srtA* mutant cells in the stationary phase. This implies that the *srtA* mutant grown in human plasma displays a typical phosphate starvation response. In contrast, deletion of *srtB* did not influence the transcriptome of *S. aureus* cells grown in human plasma with exception of the gene coding for the cold shock protein SAOUHSC_03045 (data not shown).

The *S. aureus* Pho box

To investigate whether the phosphate starvation response in *S. aureus* is similar to that of the closely related Gram-positive bacterium *Bacillus subtilis*, we compared both systems. *B. subtilis* responds to phosphate starvation via the PhoP-PhoR two-component regulatory system (19, 46). In *B. subtilis*, the PhoP response regulator controls the expression of 31 genes in 10 operons, acting as a transcriptional activator or repressor depending on the respective promoter (5). Importantly, the comparison of PhoP from *S. aureus* with PhoP of *B. subtilis* revealed an overall amino acid sequence identity of 68% (Figure 2). A more detailed inspection of both protein sequences showed that all residues required for DNA binding at the so-called Pho box are identical in both proteins (Figure 2, circles) (7). Furthermore, all residues required for transcriptional activation through the interaction with RNA polymerase (Figure 2, diamonds) (7), and all residues involved in PhoP dimer formation (Figure 2, triangles and hexagons) (4, 8) are conserved in both proteins.

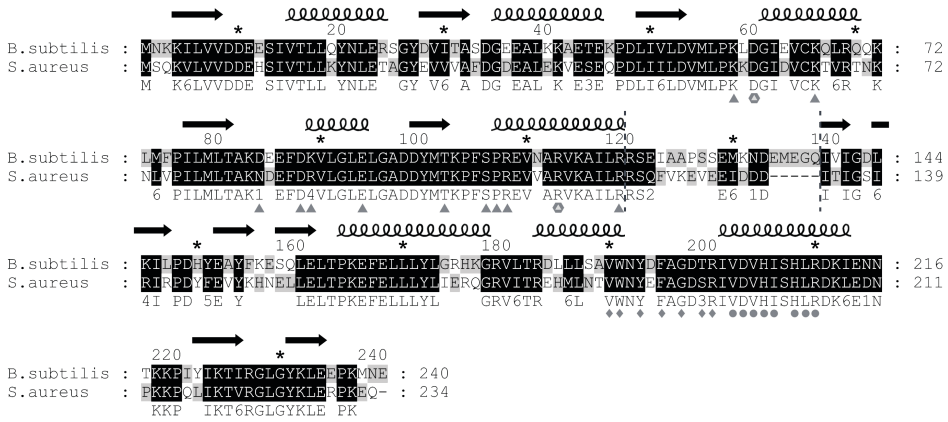
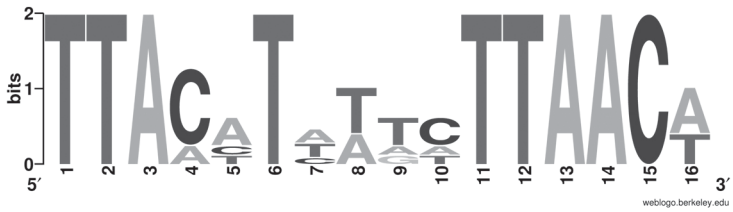


Figure 2. Sequence alignment of PhoP from *S. aureus* and *B. subtilis*. The secondary structure elements of PhoP are indicated by coils for α helices and arrows for β strands. Identical residues are indicated with a black background. Residues positioned at the PhoP dimer interface are indicated by triangles, and two residues involved in PhoP protein-protein interactions via salt a bridge are indicated with hexagons (8). PhoP residues that are essential for transcriptional activation (interaction with RNAP) are indicated by diamonds. PhoP residues involved in DNA binding are indicated by circles (7).

The *B. sublilis* Pho box to which both phosphorylated and non-phosphorylated PhoP dimers bind is composed of two to four 6-bp direct repeats with the consensus sequence TT(A/C/T) A(C/T)A and a spacing of 3 to 7 bp (11). To determine whether this consensus sequence is present in *S. aureus*, the Pho box regions of the *B. subtilis glpQ*, *phoA*, *phoB* and *pstS* genes were used for a motif search in the *S. aureus* genome following the Virtual Footprint software package (32). These Pho box regions were chosen for the motif search, because the genomic organization of the respective genes is conserved in *B. subtilis* and *S. aureus*. This search yielded 87 matches in the *S. aureus* genome (Supplemental Table 1 available on request). Seven of the matching sequences were located upstream of genes that were found to be upregulated in the *srtA* mutant grown in human plasma, namely *yjbB*, *pstS*, *phoB*, *mapW*, *hlb* (*phosholipase C*), *hlgC* and SAOUHSC_02842 (Table 1). The promoter regions of *yjbB*, *pstS*, *phoB*, *hlb* and SAOUHSC_02842 were then used for a gapped alignment (15) and the resulting consensus sequence TT[AT]AC[AT] was defined as a putative Pho box of *S. aureus* (Figure 3). To approximate the potential Pho regulon of *S. aureus*, the upstream sequences of all genes were searched with the Virtual Footprint software for the presence of the putative Pho box consensus TT[AT]AC[AT]. These analyses resulted in 35 hits (Supplemental Table 2 available on request). As expected, *yjbB*, *pstS*, *phoB*, *hlb* and SAOUHSC_02842 were included among these genes. Importantly, several other genes identified by Virtual Footprint encode proteins with an LPxTG motif. These proteins include

FnbpA, FnbpB, SasA, SasF and SasG. Notably, the mRNA levels of *fnbpA* and *fnbpB* were increased in the *srtA* mutant, whereas no changes were observed for the *sasA*, *sasF* and *sasG* mRNA levels (Table 1). In this respect, it is noteworthy that the TT[AT]AC[AT]-like sequences in the promoter regions of *sasF* and *sasG* are located on the opposite strand.

A



B

TTAACA-like sequence

	1	2	3	4	5	6
G						
A			5	10	1	6
T	10	10	5			3
C					9	1
Consensus:	**T**	**T**	**(A/T)**	**A**	**C**	**(A/T)**

Figure 3. The putative Pho box sequence of *S. aureus*. (**A**). The consensus sequence for a Pho box site was generated using the WebLogo tool (http://weblogo.berkeley.edu/) by alignment of the upstream sequences of the 5 potential regulon genes identified *in silico*. (**B**) Determination of a consensus sequence for putative *S. aureus* PhoP binding site repeats. The TTAACA-like sequences in five Pho-regulon promoters were aligned and the nucleotide positions in the TTAACA-like sequence were labeled 1-6. The numbers tabulated represent the number of times that the respective nucleotide appears at the indicated position.

Discussion

Cell wall-associated proteins play important roles in the pathogenicity of *S. aureus*. Several of these proteins are covalently attached to the cell wall by the transpeptidases SrtA or SrtB. In particular, cell wall-attached 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs) promote the adherence of *S. aureus* to human host matrix molecules, such as fibronectin, fibrinogen and collagen, in a the SrtA-dependent manner. The present study, was aimed at assessing the roles of SrtA and SrtB in the *in vitro* growth of *S. aureus* in human plasma, which mimics to some extent the *in vivo* growth condition encountered during bacteremia. As exemplified with the *S. aureus* strain SH1000, the results show that *srtA* can be an important determinant for efficient growth of certain staphylococcal strains in human plasma. Importantly, transcript profiling analyses showed that a *srtA* mutation affected the expression of 39 genes of which eight are potentially phosphate starvation- inducible as judged by the presence of a typical Pho box motif. In contrast, no

phosphate stress response was detectable in *srtB* mutant cells grown in human plasma.
Among the genes affected by the *srtA* mutation, the genes putatively required for phosphate acquisition by *S. aureus* appeared to be enriched. These include the genes for the phosphate-specific transport (Pst) system, which is similar to the Pst system from *B. subtilis*, and the gene for a secreted alkaline phosphatase (PhoB; Table 1). In *B. subtilis* the *pst* operon is a member of the Pho regulon (37), which is controlled by the PhoP-PhoR two-component regulatory system (25). The high similarity between the PhoP regulator components of *S. aureus* and *B. subtilis* suggested that the Pho box motif could be conserved in these two species. Indeed, a Pho box-like motif was identified in the upstream region of the *S. aureus pstSCAB* and *phoB* genes. Together, these findings imply that the *srtA* mutant cells grown in human plasma displayed a phosphate starvation response. Since SrtA is responsible for the proper covalent cell wall attachment of LPxTG proteins, this would suggest that one or more of these proteins are involved in the binding and/or uptake of phosphate. In this respect, it is noteworthy that human serum contains about 1.12 to 1.45 mM phosphate of which ~15% is bound to proteins (3). Thus, after 2:1 dilution of serum with HBSS, the final phosphate concentration in the plasma used for our experiments was about 1 to 1.2 mM. At these concentrations, a phosphate starvation response seems rather unlikely, unless cells are impaired in their ability to bind or take up phosphate. By comparison, a phosphate starvation response in *B. subtilis* is triggered by phosphate levels in the growth medium of about 0.1 mM or lower (31).
Interestingly, our tiling array data show that the gene encoding the LPxTG protein FnbpA, was 2.3 times up-regulated in the *srtA* mutant. In addition, the *fnbpB* gene was also up-regulated, albeit only 1.7-fold, which was just below the somewhat arbitrarily chosen cut-off of 1.8 fold (data not shown). This would suggest that FnbpA and possibly also FnbpB have a role in phosphate acquisition. Both proteins are important in infections caused by *S aureus*, because of their central role in the adhesion to and invasion of host cells. Especially, FnbpA was shown to be a multifunctional adhesin with capacity to bind to fibrinogen, fibronectin, elastin and several other proteins (10, 36, 39, 45). Intriguingly, it has been shown that soluble fibronectin contains covalently bound phosphate (48). Thus, it is conceivable that Fnbp's are somehow involved in the scavenging of phosphate from fibronectin, thereby contributing to the phosphate homeostasis in *S. aureus*. This idea is further supported by the fact that putative Pho boxes are present in the promoter regions of both *fnbA* and *fnbB*. On the other hand, most phosphate in human serum is present in an unbound state, at least according to the literature data (3). This would imply that FnbpA and FnbpB could also be involved in the binding of free phosphate.

Lastly, two small genes, *vraX* and *cwrA*, were most strongly induced in the absence of *srtA*.

The *vraX* gene is known to be regulated by the VraTSR three-component regulatory system, which responds to cell wall and antibiotic stress (6). Likewise, *cwrA* is induced upon cell wall damage (2). This might suggest that the *srtA* mutant cells experience some cell wall stress when grown in human plasma. On the other hand, the *srtA* mutant cells displayed no typical cell wall stress signatures as reported for *vraTSR*, *graRS* or *walKR* mutant strains (2, 9, 12). In fact, apart from the *pstSCAB* and *phoB* genes, none of the other differentially regulated genes in the *srtA* mutant could be directly connected to previously defined stress signatures. Thus, the biological meaning of these gene regulatory responses to the SrtA-deficiency and their connection to particular LPxTG proteins remains to be determined.

In summary, the present studies revealed that *srtA* mutant cells grown in human plasma display a typical phosphate starvation response. Upregulation of *fnbpA* and, to lesser extent, *fnbpB* suggests that the encoded fibronectin-binding proteins, which are known SrtA substrates, may have roles in phosphate acquisition by *S. aureus*. How exactly these proteins could be involved in this process is currently not known, but it is conceivable that the Fnbp's are needed to acquire phosphate that is bound to particular serum proteins, like fibronectin, as well as free phosphate.

Acknowledgements

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Table 1. Differentially regulated genes of *srtA* mutant cells grown in human plasma.

NCTC 8325 ORF[a]	Gene	Description of predicted protein function	FCt_{exp}	FCt_4
00035		hypothetical protein		2.0
00036		hypothetical protein		1.9
00060#	*yjbB*	phosphate:Na+ symporter	2.1	
00192	*coa*	staphylocoagulase	1.8	
00561	*vraX*	hypothetical protein	6.7	
00898	*argH*	argininosuccinate lyase	3.5	
00899	*argG*	argininosuccinate synthase	3.7	
01135	*psmB1*	phenol-soluble modulin beta	2.1	
01136	*psmB2*	phenol-soluble modulin beta	1.8	
01384	*phoU*	PhoU family transcriptional regulator	1.8	
01385	*pstB*	phosphate ABC transporter ATP-binding protein	2.5	1.8
01386	*pstA*	phosphate ABC transporter permease protein	3.4	2.0
01387	*pstC*	phosphate ABC transporter permease protein	4.2	2.4
01388		hypothetical protein	4.2	2.6
01389#	*pstS*	phosphate ABC transporter periplasmic phosphate-binding protein	7.7	2.8
01570		PVL orf 37-like protein		1.8
01571		SLT orf 71-like protein		2.3
01573		conserved hypothetical phage protein		1.8
01574		helix-turn-helix domain protein		2.0
01761		conserved hypothetical protein	2.1	
01990	*glnQ*	amino acid ABC transporter ATP-binding protein	1.9	
01991		putative ABC transporter permease protein	1.8	
02160		truncated-mapW	2.0	
02161#	*mapW*	MHC class II analog protein	2.4	
02163#	*hlb*	phospholipase C	3.7	
02708	*hlgA*	gamma-hemolysin component A	2.1	
02709#	*hlgC*	gamma-hemolysin component C	2.3	
02803	*fnbA*	fibronectin-binding protein A	2.3	
02842#		conserved hypothetical protein	5.1	
02872	*cwrA*	cell wall responsive for antibiotics	6.9	
02958#	*phoB*	alkaline phosphatase III precursor	2.4	
00169		peptide ABC transporter, permease component		-2.0
00170		RGD-containing lipoprotein		-2.0
00171		gamma-glutamyltranspeptidase		-1.8

NCTC 8325 ORF[a]	Gene	Description of predicted protein function	FCt_{exp}	FCt_4
00555		hydrolase haloacid dehalogenase-like	-1.8	
01079	*isdB*	iron-regulated surface determinant protein B	-1.9	
02555		hypothetical protein		-1.8
02697		amino acid ABC transporter, ATP-binding protein		-1.8
02834	*srtA*	sortase A	-135.3	-131.0

[a]Gene names according to the annotated *S. aureus* NCTC 8325 genome sequence SAOUHSC_
[b]FC, fold change; determined as the ratio of the signal values in the HG001 Δ*srtA* strain and the parental strain.
[#]Genes with a Pho box as predicted with the *B. subtilis* Pho box.

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10

Contributions of the sortases A and B to surfacome biogenesis in *Staphylococcus aureus*

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To be submitted

Abstract

Cell surface-exposed proteins of Gram-positive bacterial pathogens have important roles in the binding of ligands and tissues of their human host. Such proteins can be covalently bound to the cell wall through the action of sortases. In *Staphylococcus aureus*, sortase A (SrtA) and sortase B (SrtB) recognize C-terminal 'LPxTG' motifs, which are cleaved by the respective sortase and covalently attached to peptidoglycan. Although this process has been characterized in great detail, it was thus far not known how sortases shape the actual surface of a staphylococcal cell with respect to proteins that are directly exposed to the extracellular milieu. Therefore, the present studies were aimed at determining the 'surfacomes' of *srtA* or *srtB* mutant cells of *S. aureus*. As shown by 'cell surface shaving' with immobilized trypsin and subsequent mass spectrometric analyses (ProteomeXchange identifier PXD000179), SrtA has a major impact on the staphylococcal surfacome, whereas SrtB is only of minor importance. Specifically, our results show that the sortase-dependent surfacome consists of covalently cell wall-bound 'LPxTG proteins', non-covalently wall-bound proteins, known secreted proteins and cytoplasmic proteins. The secreted and cytoplasmic proteins are most likely retained at the surface of SrtA-proficient cells by binding to one or more LPxTG proteins. These LPxTG proteins thus seem to represent crucial nodes in the cell wall interactome of *S. aureus* that bind not only factors of the human host, but also other staphylococcal proteins. This may have important implications for the actual, direct or indirect roles of LPxTG proteins in the virulence of *S. aureus*.

Introduction

Staphylococcus aureus is a Gram-positive bacterium frequently encountered in the nasal cavity of humans (34). However, if the primary barrier function of the skin is disrupted or if the immune system is compromised, *S. aureus* has the potential to invade almost all tissues and organs causing a broad range of diseases (18). These can vary from mild skin infections, such as impetigo, to life-threatening systemic infections (*e.g.* pneumonia, meningitis, osteomyelitis, and sepsis) (1, 3, 7, 36). The pathogenicity of *S. aureus* is caused by a broad range of cell surface-exposed or secreted virulence factors, such as toxins, exoenzymes, adhesins, and immune-modulating proteins (5, 6, 30). The majority of these virulence factors are first synthesized as precursors with an N-terminal signal peptide to direct their export from the cytoplasm via the general secretory (Sec) pathway (30). During or after membrane translocation, the signal peptide is removed by a signal peptidase in order to release the translocated protein from the *trans* side of the membrane (2, 33). Furthermore, if certain retention signals in the form of specific amino acid sequence motifs are present, the translocated proteins can be attached to the cell wall (30). The covalent cell wall attachment of proteins with a so-called LPxTG motif requires the activity of specific transpeptidases known as sortases (19). These enzymes cleave the LPxTG motif between the Thr and Gly residues and catalyze the formation of an amide bond between the carboxyl group of the Thr residue and the free amino end of a pentaglycine cross bridge in peptidoglycan precursors (9, 17, 28, 31). *S. aureus* possesses one sortase A (SrtA) enzyme, which recognizes the canonical LPxTG motif (21, 25). It has been shown that *S. aureus* lacking the *srtA* gene has defects in the retention of 'LPxTG proteins' in the cell wall (29). As a consequence, the absence of SrtA causes a defect in the establishment of acute infections (20). In addition, the mutation of *srtA* was shown to cause a hyper-spreading phenotype, allowing *S. aureus* to migrate over soft agar plates at an increased rate. This probably relates to the fact that surface-exposed LPxTG proteins promote cell-cell attachment, thereby antagonizing spreading (32).

It has been reported that 19 different *S. aureus* proteins carry a canonical C-terminal LPxTG motif and that two additional proteins carry a C-terminal LPxAG motif (8, 19, 24, 26, 30). These include protein A (Spa), two fibronectin-binding proteins (FnbpA and FnbpB)(13), ClfA, ClfB, three cell wall-anchored proteins with large serine-aspartate repeat domains (SdrC, SdrD and SdrE) (15), a collagen-binding protein (Cna), a plasmin-sensitive protein (Pls) (27), the methicillin resistance determinant B (FmtB) (16), and eleven staphylococcal surface (Sas) proteins. In addition to SrtA, *S. aureus* contains a second sortase known as SrtB. SrtB is required for the cell wall anchoring of surface proteins that contain an NPQTN motif. So far the only protein known to be covalently coupled to the cell wall by SrtB is

IsdC (22). It is presently not known how important SrtB is for staphylococcal virulence in general, but *S. aureus* lacking *srtB* was shown to have a small defect in the establishment of arthritis in a mouse model (14).

While the cell wall attachment of proteins by sortases has been well documented, it was to date not known to what extent these enzymes shape the actual surface of a staphylococcal cell in terms of proteins that are exposed to the extracellular milieu. These proteins, collectively termed the surfacome, are of particular importance for staphylococcal pathogenesis as they can potentially bind to cells and tissues of the host. Also, they could be relevant targets for novel antistaphylococcal therapies. Therefore, the present studies were aimed at determining the surfacomes of *srtA* or *srtB* mutant cells of *S. aureus*. Briefly, the results obtained by surface shaving with immobilized trypsin show that the surfacomes of *srtA* or *srtB* mutant cells display significant differences that may either be directly or indirectly caused by the respective sortase-deficiencies.

Materials and Methods

Bacterial strains and culture

The *S. aureus* strain SH1000 (12) and its Δ*srtA* or Δ*srtB* derivatives (29) were grown overnight in tryptic soy broth (TSB, OXOID) under vigorous shaking (250 rpm) at 37°C. The different *S. aureus* cultures were then diluted into pre-warmed RPMI 1640 medium (PAA) to an OD_{600} of ~0.05 (SH1000, Δ*srtB*), or to an OD_{600} of ~0.03 (Δ*srtA*). The cultivation was continued under the same conditions. Exponentially growing cells (OD_{600} of ~0.2) were again diluted into fresh and pre-warmed RPMI medium to a final OD_{600} of ~0.04 (SH1000, Δ*srtB*), or to a final OD_{600} of ~0.02 (Δ*srtA*), and the cultivation was continued until the stationary phase was reached.

Shaving sample preparation

S. aureus cultures were harvested at OD_{600} 0.25 (SH1000, Δ*srtB*) or OD_{600} 0.17 (Δ*srtA*), and at 1 h after transition into the stationary growth phase. Cells were collected by centrifugation (10 min, 6080 × g, 4°C) and washed twice with PBS containing 40% sucrose (Acros), and 20 mM azide (Sigma-Aldrich). Immobilized trypsin (Pierce) was activated with 50 mM ammonium bicarbonate (Sigma-Aldrich) and resuspended in 50 µl PBS plus 40% sucrose and 20 mM azide. The activated immobilized trypsin was then added to the cells. The shaving reaction was conducted for 45 min at 37°C. Released peptides were isolated, reduced with 10 mM DTT (30 min, room temperature), alkylated with iodoacetamide (30 min in the dark at room temperature), and digested with trypsin overnight at 37°C (5). As a control for spontaneous release of proteins from the cells during

the shaving reaction, the cells were incubated with 50 µl PBS containing 40% sucrose, but lacking the immobilized trypsin.

Mass Spectrometry

Reduction and alkylation, desalting of the samples, MS analysis and database searches were performed as previously described (5). The strain-specific uniprot database was used for the NCTC8325 strain (including a concatenated reversed database, 5862 entries). Validation of MS/MS based peptide and protein identifications was performed with Scaffold (version Scaffold_3.6.5, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides. All experiments were conducted in independent triplicates. Peptides were only accepted as being identified if they were detected in at least two out of the three replicates per sample set. With these filter parameters no false positive hits were obtained. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000179. All peptide identifications are listed in Supplemental Table 1 (available on request).

Results and discussion

Impaired growth of *srtA* mutant *S. aureus* cells in RPMI medium

S. aureus SH1000 and its sortase mutant derivatives were grown in RPMI medium, which is also suited for culturing of human blood cells. Accordingly, this medium is believed to be more physiologically relevant than other commercially available growth media that are used to culture *S. aureus* (5). As shown in Figure 1, deletion of *srtA* drastically affected the growth rate of *S. aureus* in RPMI. Furthermore, the optical density reached in the stationary phase was about two-fold lower than that of the parental strain SH1000. Unlike the *srtA* mutation, the *srtB* mutation did not affect the growth *S. aureus* SH1000 in RPMI (Figure 1). This shows that SrtA is important for the growth of *S. aureus* in RPMI medium. Interestingly, our previous studies showed that the *srtA* mutation did not affect growth in TSB medium (29, 32). This indicates that *S. aureus* cells lacking the *srtA* gene have a defect in the cell wall localization of one or more LPxTG proteins that are necessary for the proper growth of *S. aureus* SH1000 in RPMI medium. This prompted us to investigate the surfacome of sortase mutant cells using the previously developed shaving technique that makes use of immobilized trypsin (5). As previously shown (5), this method mainly identifies protein domains exposed on the cell surface, but not those that are buried within the cell wall.

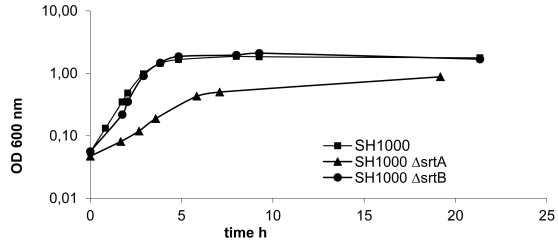

Figure 1. Growth of *S. aureus srtA* and *srtB* mutants. The *S. aureus* strains SH1000 Δ*srtA*, Δ*srtB* and the parental strain were grown in RPMI medium under vigorous shaking at 37°C.

The surfacome of *srtA* or *srtB* mutant cells

To investigate the influence of the *srtA* and *srtB* mutations on the *S. aureus* surfacome, shaving experiments were performed with exponentially growing cells as well as cells that had entered the stationary growth phase. Notably, our previous cell surface shaving studies were focused on early-exponential phase cells, because cell lysis was minimal under these conditions and because the low cell density would more closely reflect the low cell densities encountered during bacteremia. However, it is known that many secreted *S. aureus* proteins are up-regulated at late growth stages, some of which can also be detected in a cell wall-bound state (5, 30). Therefore, we also investigated cells in the stationary phase.

The analysis of exponentially growing cells of the parental strain SH1000, the Δ*srtA* mutant and the Δ*srtB* mutant resulted in the identification of 27, 11 and 17 surface-exposed proteins, respectively (Figure 2A). It should be noted that a few of these proteins (*e.g.* Atl, GAPDH and the putative lipoprotein Q2G2D8) were also detected in control experiments where the spontaneous protein release in the absence of immobilized trypsin was investigated (Supplemental Table 1 available on request). When the cells had reached the stationary growth phase, the numbers of identified surface-exposed proteins of each of these strains were increased to 31, 23 and 19 proteins, respectively (Figure 2B). These findings indicate that not only the numbers of secreted *S. aureus* proteins increase in the stationary phase, but also the numbers of cell surface-exposed proteins. This is most clearly evident for the *srtA* mutant strain.

The comparison of all identified surface-exposed proteins in *srtA* or *srtB* deficient cells, or the parental strain showed that 12 proteins were detectable in all three strains. In contrast, 12 proteins were exclusively detectable in cells of the parental strain, 5 exclusively in *srtA* mutant cells, and 2 exclusively in *srtB* mutant cells (Figure 2C). The highest degree of overlap in identified proteins was observed for the *srtB* mutant cells and cells of the parental strain (Figure 2). Only two proteins, DnaK and the cell wall-bound SsaA2 protein, were detected exclusively on the surface of *srtB* mutant cells. It is presently not clear why these

proteins were detected on the surface of *srtB* mutant cells, but not on the surfaces of the *srtA* mutant cells or cells of the parental strain. Notably, DnaK is a protein that is normally active in the bacterial cytoplasm. However, it belongs to the class of HSP70 heat shock proteins of which human homologues are known to bind to the autolysin Atl at the *S. aureus* cell surface (11, 23). Since Atl was detected in the *srtB* mutant (Table 1), it is conceivable that the detected DnaK was bound to this protein.

Our analyses revealed that the identified surface-exposed proteins of the parental strain included eight LPxTG proteins (Figure 2D, Table 1). Of these eight, only the SasG protein was identified exclusively in the parental strain. Five LPxTG proteins, namely SdrD, ClfA, ClfB, IsdA and IsdB were identified in the parental strain, but not in the *srtA* mutant. This suggests that these proteins require SrtA for proper cell surface exposure, or retention at this location. This idea is consistent with the view that proteins with an LPxTG motif will not be covalently anchored to the cell wall if SrtA is absent, and that such proteins may be secreted into the growth medium or that they are degraded due to an aberrant cell wall localization. Consistent with this view, processed forms of the SdrD and ClfA proteins were previously detected in the cell culture medium of cells lacking SrtA (29). Despite the fact that the lack of detection of SdrD, ClfA, ClfB, IsdA and IsdB at the cell surface of *srtA* mutant cells is consistent with our expectations, a note of caution is in place, because for technical reasons certain proteins may not be identified by MS even though they are present. For example, such proteins may be present in small amounts or they may not have surface-exposed trypsin cleavage sites.

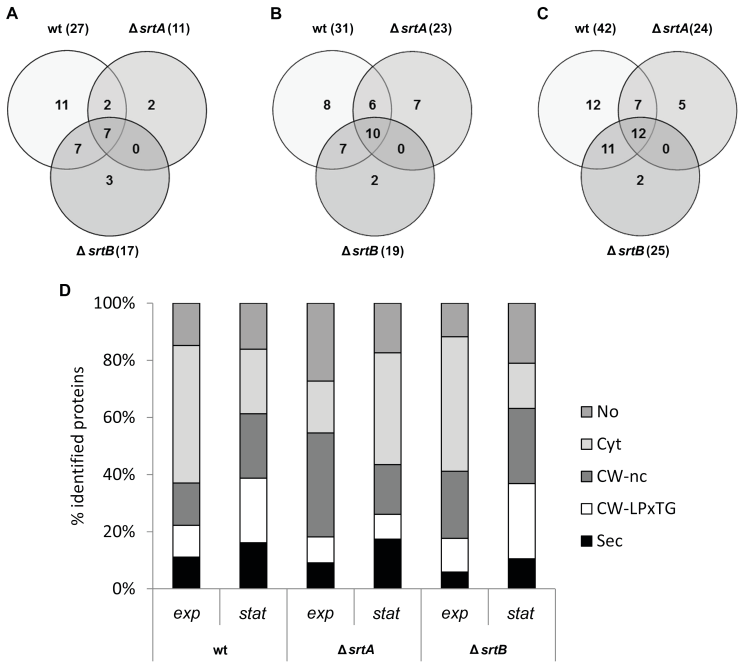

Figure 2. Proteins identified at the surface of *srtA* or *srtB* mutant cells. The numbers of proteins identified by cell surface shaving are indicated in venn diagrams. (**A**) Proteins detected in exponentially growing cells (OD_{600} of ~0.2); (**B**) proteins detected in cells in the postexponential growth phase (1 h after entering into stationary phase); and (**C**) all identified proteins. (**D**) Predicted subcellular localization of proteins identified at the surface of cells in the exponential (exp) or stationary (stat) growth phases. No, proteins lacking known motifs for subcellular localization; Cyt, cytoplasmic proteins; CW-nc, non-covalently cell wall-bound proteins; CW-LPxTG, covalently cell wall-bound proteins; Sec, known secreted proteins.

Intriguingly, the LPxTG proteins Spa and SasF were not only identified at the surface of cells of the parental strain, but also at the surface of *srtA* mutant cells (Table 1). Previous studies have shown that, indeed, some Spa remains bound to the cell wall of *srtA* mutant *S. aureus* cells, albeit in reduced amounts, while this protein is secreted into the growth medium at substantially increased levels (29). Our present findings now show that the wall-bound Spa of SrtA-deficient mutant cells is exposed at the cell surface, but potentially in a different manner than in SrtA-proficient cells. Specifically, our MS analyses identified only three peptides corresponding to the first and second IgG-binding domains as surface exposed in SrtA-proficient cells (Figure 3). In contrast, additional peptides from the second and third IgG-binding domains and the C-terminal LysM domain of Spa were identified as being surface-exposed in SrtA-deficient cells (Figure 3). Possibly, this reflects a mis-localization of Spa in the absence of its covalent binding to the peptidoglycan. The LysM domain, which is still present in the non-covalently cell wall-bound Spa, is most likely responsible for the observed cell wall retention of this protein. However, at least a part of the LysM domain

is cell surface exposed, which may explain why Spa is only loosely bound to SrtA-deficient cells. Like Spa, the LPxTG protein SasF was detected at the surface of *srtA* mutant cells and cells of the parental strain (Figure 3). However, in case of SasF there was no difference in the identified peptides, and this protein was only detectable in the stationary phase.

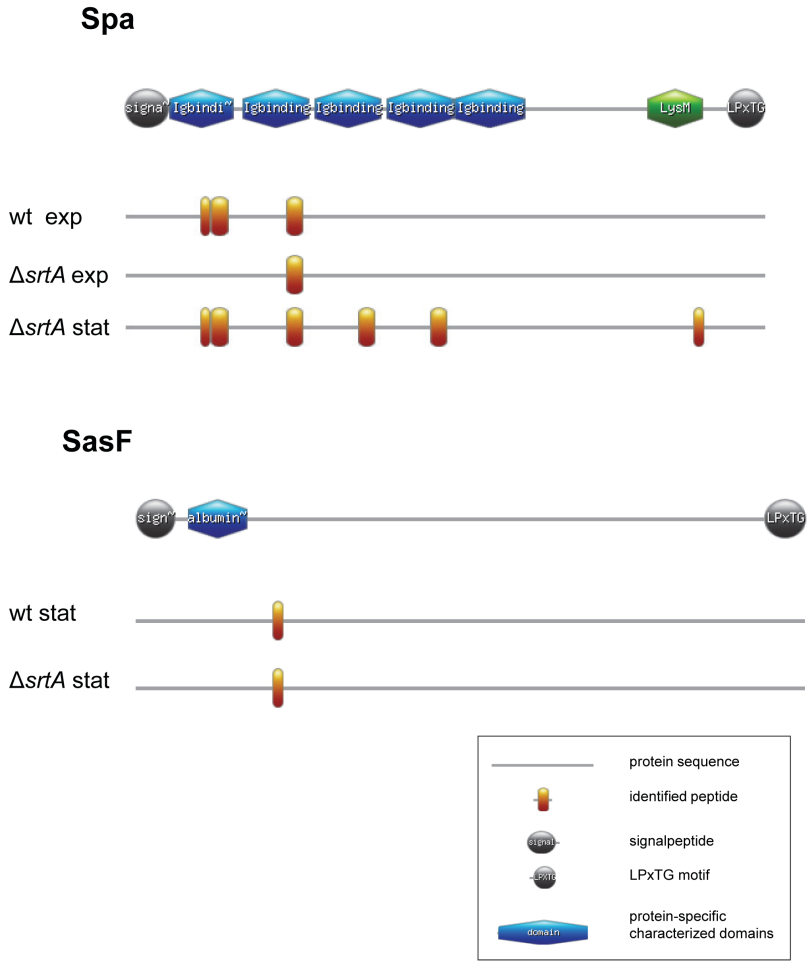

Figure 3. Location of identified peptides in the Spa and SasF proteins. The peptides identified by MS upon surface shaving of *S. aureus* strain SH1000 (wt) and its Δ*srtA* or Δ*srtB* mutant derivatives are highlighted in the linear protein sequences of the IgG-binding protein Spa and the staphylococcal surface protein F (SasF). In addition, known protein domains are indicated as explained in the associated legend.

It is noteworthy that six of the eight LPxTG proteins identified in this study (*i.e.* ClfA, ClfB, IsdB, SasG, SdrD, Spa) contain the so-called YSIRK/GS motif in their signal peptides. It was previously shown that proteins with the YSIRK/GS motif are directed to the cross-wall, which is the peptidoglycan layer that forms during cell division to separate the daughter

cells (4, 35). Our observation that surface shaving with immobilized trypsin preferentially detects LPxTG proteins with the YSIRK/GS motif thus indicates that cross wall proteins are readily detectable with this technique. The fact that the IsdA and SasF proteins lacking the YSIRK/GS motif are also detected suggests that there is no major bias for the identification of cross wall-localized proteins. This further supports the view that our surface shaving approach with immobilized trypsin is broadly applicable for studies on the composition of the *S. aureus* surfacome.

Lastly, several non-LPxTG proteins were identified at the cell surface of the parental SH1000 strain, which were not detected at the surface of the *srtA* mutant cells. These proteins include β-hemolysin, δ-hemolysin, the Efb homologous protein (Ehp) and several ribosomal subunits. During host invasion β-hemolysin and δ-hemolysin are secreted to attack the host cells, while Ehp serves to inactivate complement (10). Our present findings suggest that such non-LPxTG proteins could be retained in the cell wall of SrtA-proficient cells by binding to certain LPxTG proteins that are not effectively localized to the surface of SrtA-deficient cells.

In conclusion, the present cell surface shaving studies with immobilized trypsin provide a first proteomic view of the sortase-dependent surfacome of *S. aureus*. This sub-proteome is shown to consist of covalently cell wall-bound LPxTG proteins, non-covalently wall-bound proteins, known secreted proteins and cytoplasmic proteins. Our findings indicate that the latter secreted and cytoplasmic proteins are retained at the surface of SrtA-proficient cells by binding to one or more LPxTG proteins. If so, these LPxTG proteins represent crucial nodes in the cell wall interactome of *S. aureus*, which may have important implications for their roles in the virulence of this important pathogen.

Acknowledgments:

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Table 1. Suface-exposed proteins of *srtA* or *srtB* mutant *S. aureus* cells.

locus tag[a]	protein name	total pred[b]	exponential growth[c]			stationary phase[c]			total[c]		
			wt	Δ*srtA*	Δ*srtB*	wt	Δ*srtA*	Δ*srtB*	wt	Δ*srtA*	Δ*srtB*
SAOUHSC_00069	Immunoglobulin G-binding protein A	CW-LPxTG		1		1	1		1	1	
SAOUHSC_00130	Heme-degrading monooxygenase IsdI	Cyt				1			1		
SAOUHSC_00348	30S ribosomal protein S6	Cyt	1						1		
SAOUHSC_00356	Putative uncharacterized protein	No	1	1	1	1	1	1	1	1	1
SAOUHSC_00365	Alkyl hydroperoxide reductase subunit C	CW-nc				1			1		
SAOUHSC_00411.4	Phenol-soluble modulin alpha 1 peptide	CW-nc	1	1		1		1	1	1	1
SAOUHSC_00518	50S ribosomal protein L11	Cyt	1						1		
SAOUHSC_00530	Elongation factor Tu	Cyt	1	1	1	1	1	1	1	1	1
SAOUHSC_00545	Serine-aspartate repeat-containing protein D	CW-LPxTG	1		1	1		1	1		1
SAOUHSC_00617	Putative uncharacterized protein	No				1	1	1	1	1	1
SAOUHSC_00634	ABC transporter, substrate-binding protein, putative	CW-nc	1	1	1	1	1	1	1	1	1
SAOUHSC_00795	Glyceraldehyde-3-phosphate dehydrogenase, type I	Cyt	1	1	1	1	1	1	1	1	1
SAOUHSC_00799	Enolase	Cyt					1			1	
SAOUHSC_00812	Clumping factor A	CW-LPxTG	1					1	1		1
SAOUHSC_00834	Thioredoxin, putative	Cyt					1			1	
SAOUHSC_00845	UPF0337 protein	No	1	1		1	1		1	1	
SAOUHSC_00994	Bifunctional autolysin	Sec	1	1	1	1	1	1	1	1	1
SAOUHSC_01024	Putative uncharacterized protein	No					1			1	
SAOUHSC_01079	Iron-regulated surface determinant protein B	CW-LPxTG				1		1	1		1
SAOUHSC_01081	Iron-regulated surface determinant protein A	CW-LPxTG				1		1	1		1
SAOUHSC_01110	Fibrinogen-binding protein-related	Sec	1			1		1	1		1
SAOUHSC_01135	Putative uncharacterized protein- PsmB1	CW-nc	1		1	1	1	1	1	1	1
SAOUHSC_01144	Cell division protein FtsL	Cyt	1			1	1		1	1	
SAOUHSC_01150	Cell division protein FtsZ	Cyt	1			1			1		
SAOUHSC_01177	DNA-directed RNA polymerase subunit omega	Cyt					1			1	
SAOUHSC_01236	Ribosome-recycling factor	Cyt	1				1		1	1	
SAOUHSC_01501	Elastin-binding protein EbpS	CW-nc		1		1	1	1	1	1	1
SAOUHSC_01683	Chaperone protein DnaK	Cyt			1						1
SAOUHSC_01729	Putative uncharacterized protein	No	1				1		1	1	
SAOUHSC_01977	UPF0342 protein	No	1		1				1		1

locus taq[a]	protein name	total pred[b]	exponential growth[c]			stationary phase[c]			total[c]		
			wt	Δ*srtA*	Δ*srtB*	wt	Δ*srtA*	Δ*srtB*	wt	Δ*srtA*	Δ*srtB*
SAOUHSC_02013	Uncharacterized protein	Cyt					1			1	
SAOUHSC_02163	Phospholipase C	Sec				1			1		
SAOUHSC_02260	Delta-hemolysin	Sec	1			1		1	1		1
SAOUHSC_02333	Probable transglycosylase SceD	Sec				1	1		1	1	
SAOUHSC_02441	Alkaline shock protein 23	No	1	1	1	1	1	1	1	1	1
SAOUHSC_02477	30S ribosomal protein S9	Cyt	1		1			1	1		1
SAOUHSC_02493	50S ribosomal protein L30	Cyt	1		1				1		1
SAOUHSC_02494	30S ribosomal protein S5	Cyt	1						1		
SAOUHSC_02496	50S ribosomal protein L6	Cyt	1		1				1		1
SAOUHSC_02511	50S ribosomal protein L4	Cyt	1						1		
SAOUHSC_02571	Staphylococcal secretory antigen SsaA2	CW-nc			1						1
SAOUHSC_02690	Putative uncharacterized protein	CW-nc				1			1		
SAOUHSC_02706	Immunoglobulin-binding protein Sbi	CW-nc				1			1		
SAOUHSC_02798	Surface protein G	CW-LPxTG				1			1		
SAOUHSC_02887	Probable transglycosylase IsaA	Sec	1	1	1	1	1	1	1	1	1
SAOUHSC_02926	Fructose-bisphosphate aldolase class 1	No			1	1	1		1	1	1
SAOUHSC_02963	Clumping factor B	CW-LPxTG	1		1	1		1	1		1
SAOUHSC_02979	N-acetylmuramoyl-L-alanine amidase domain-containing protein	Sec				1			1		
SAOUHSC_02982	Putative uncharacterized protein	CW-LPxTG				1	1		1	1	

[a]Locus tags according to the annotated *S. aureus* NCTC 8325 genome sequence
[b]Abberviations: No, proteins lacking known motifs for subcellular localization; Cyt, cytoplasmic proteins; CW-nc, non-covalently cell wall-bound proteins; CW-LPxTG, covalently cell wall-bound proteins; Sec, known secreted proteins.
[c]The number 1 indicates that peptides belonging to a particular protein were detected in at least two of the three biological replicates.

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11

General summary and discussion

The most diverse organisms on earth are bacteria. They adapted their lifestyles to colonize different ecological niches, including human beings. It is estimated that 500 to 1000 species of bacteria live in the human gut and a roughly similar number of bacteria can be identified on the skin. In most cases, both humans and microbes seem to benefit from this co-existence. However, in some cases the commensal bacteria invade the human host, which can then lead to life-threatening diseases. One of these opportunistic commensal bacteria is *Staphylococcus aureus*. The *S. aureus* colonization rate in the healthy human population is established at about 30%. Especially, if the primary barrier function of the skin is disrupted, or if the immune system is compromised, *S. aureus* has an increased potential to invade almost all tissues and organs causing a broad range of diseases. As introduced in **Chapter 1** of this thesis, the pathogenicity of *S. aureus* is dependent on a broad range of cell surface-exposed, or secreted virulence factors. These include: surface-exposed proteins involved in adherence and colonization of host tissues, invasins exported into the host environment to promote the bacterial spread in invaded tissues, biochemical properties that enhance staphylococcal survival in phagocytes, immunological disguises, superantigens, and toxins damaging the membrane of host cells.

Not only the diseases that this pathogen can cause are alarming, but also its high propensity to acquire resistance to antibiotics. Till now, the therapy against *S. aureus* infections relies mainly on antibiotics. However, due to the fast development of antibiotic resistance by *S. aureus*, alternative ways to prevent and cure staphylococcal diseases need to be discovered, including immunization strategies. Importantly, there is currently no vaccine or passive immunization therapy available that could stop this successful pathogen from infecting humans. Accordingly, novel strategies to combat this pathogen through immunotherapy must be based on an integrated approach that requires the identification of invariant and immune-dominant targets. The identification of such targets requires (i) in-depth analyses on the localization of proteins and other compounds to the staphylococcal cell surface and (ii) a thorough understanding of the human immune responses to these cell surface-exposed compounds. This thesis therefore describes investigation on the interactions between *S. aureus* and its human host *in vivo* and *in vitro*. In particular, studies of immune responses as a result of *S. aureus* colonization in patients with the genetic blistering disease epidermolysis bullosa (EB) revealed several highly immunogenic staphylococcal proteins and their epitopes. In addition, *in vitro* studies have pinpointed various mechanisms that are important for the cell surface exposure of different *S. aureus* proteins that are known to be involved in staphylococcal infections. Taken together, the findings reported in this thesis contribute to a better understanding of the interactions between humans and *S. aureus* during colonization and infection.

EB refers to a group of inherited disorders caused by mutations in various structural proteins in the skin. Patients with EB develop blisters as a consequence of trivial mechanical trauma. The resulting ulceration of the skin in patients with EB leads to the development of wounds that become readily colonized by different bacteria. In **Chapter 2** an unexpectedly high rate of *S. aureus* colonization among EB patients is reported. All EB patients with chronic wounds and 75% of the patients without chronic wounds were shown to be colonized with *S. aureus* on at least one body site. Even when only the colonization rates of the upper respiratory tract were compared, the rates determined for EB patients (56-90%) were substantially higher than those measured for healthy individuals (25-37%) or healthcare workers who occasionally meet the sampled EB patients (39%). This implies that the wounds of EB patients represent an attractive niche for *S. aureus*. Molecular typing showed that (i) colonization of the EB patients is not limited to specific genetic lineages of *S. aureus*; (ii) individual patients with EB were carrying up to four different staphylococcal MLVA types; and (iii) autoinoculation of staphylococci between the upper respiratory tract and wounds of EB patients must occur frequently. The view that colonization of EB patients by *S. aureus* is a random process is supported by *spa*-typing analyses, which showed that most of the identified *spa*-types belong to the most predominant *spa*-types in the areas of residence of the respective EB patients. Thus, the *S. aureus* population structure in the sampled EB patients mirrors the general *S. aureus* population structure in the Netherlands. An important finding was that the rate of *S. aureus* transmission between different patients in the sampled population is relatively low. The few cases where different EB patients did carry the same *S. aureus* MLVA types concerned family members, or individuals who lived in an area where the respective *spa*-types are very common also amongst the general population.

Research described in **Chapter 3** revealed the variation in *S. aureus* types colonizing patients with EB over time, as well as the influence of high-level colonization on anti-staphylococcal antibody titers in these patients. The data show that the same *S. aureus* MLVA type was identified only in ~42.5% of all sampled patients with minor variations for different sites of sampling. Furthermore, 43.5%-58.3% of the patients with EB carried alternating *S. aureus* MLVA types over time. These findings show that the included EB patients are continuously challenged by different *S. aureus* types and that the carried *S. aureus* population can change rapidly. This seems to challenge the classical dogma that persistent carriers are mainly colonized by one *S. aureus* type. Most importantly, it was found that the sera of EB patients contained higher anti-staphylococcal IgG levels than those of healthy individuals. Specifically, this applied to IgGs against nine important virulence factors: the surface proteins IsdA and SasG, the secreted proteins IsaA, SCIN, Nuc and LytM, and the superantigens (SAgs) SEM, SEN and SEO. Notably, EB patients carrying different *S. aureus* types contained higher

levels of anti-staphylococcal antibodies than EB patients colonized by only one type. This was particularly evident for IgGs against IsdA, LukD, HlgB, LytM, LukS, LukF and ETA. Altogether, these observations show that EB patients are highly challenged with very diverse *S. aureus* types, and that carriage of multiple *S. aureus* types apparently elicits the highest humoral responses in these patients.

In most previous studies, the colonization with *S. aureus* was investigated by taking swabs from different body sites, which was followed by the analysis of only one *S. aureus* colony per swab. This is likely to result in an underestimation of the number of colonizing *S. aureus* types, especially in hosts that are more susceptible to *S. aureus* colonization than healthy individuals. Therefore, the studies described in **Chapter 4** were aimed at investigating the co-existence of different *S. aureus* types in wounds of EB patients in relation to their genetic relatedness and spatial distribution *in vivo*. Upon replica-plating of used bandages of five different EB patients, either confluent growth or separate colonies at different densities were observed. Species determination of 12 to 48 colonies per investigated wound revealed that most obtained isolates were *S. aureus*. As showed by fluorescence *in situ* hybridization, *S. aureus* was mostly present in micro-colonies, although individual *S. aureus* cells were also detectable upon replica plating of bandages. It was therefore concluded that the bacterial wound topography as observed through replica plating of used bandages closely reflects the actual bacterial topography in the wounds. Typing of all collected *S. aureus* isolates revealed that individual chronic wounds contained up to six different *S. aureus* types at one time point of sampling and even up to 10 different *S. aureus* types when the wound was investigating over time. Some of these isolates are closely related suggesting that they share a recent common ancestry. Importantly, other isolates derived from adjacent wound locations belong to distinct molecular complexes of *S. aureus*. It can therefore be concluded that the general assumption that one individual is predominantly colonized by one type of *S. aureus* does not apply to chronic wounds of patients with EB.

As shown in **Chapters 2, 3** and **4**, patients with EB are highly colonized by various *S. aureus* strains. This seems to result in increased IgG levels directed against *S. aureus* in general and, consistent with this view, high IgG titers against particular staphylococcal proteins were identified. To date, it is not known whether these antistaphylococcal IgGs are protective against invasive disease caused by *S. aureus*, but this is an interesting possibility that focused attention on the, so far, unsuccessful attempts to develop antistaphylococcal immunotherapy. To design a rational approach for the development of novel antistaphylococcal immunotherapies, the studies described in **Chapter 5** combined an in-depth assessment of the staphylococcal cell wall- and cell surface proteomes with an

array-based screen for epitopes in identified cell wall-bound or surface-exposed proteins of *S. aureus*. For the epitope screen, plasma donated by EB patients was used. The proteomics analyses identified a large set of proteins on the surface of two different *S. aureus* strains, the MSSA strain Newman and the community-acquired MRSA strain USA300. In particular the USA300 strain was found to expose many typically cytoplasmic proteins on its surface. Previous studies by others have also reported on this remarkable phenomenon. Importantly, for several exported cytoplasmic proteins, so-called moonlighting functions in pathogenesis have been documented and, in accordance with the idea that cytoplasmic proteins may be exposed on the staphylococcal cell surface also *in vivo*, it was observed that the plasma of EB patients indeed contained IgGs against cytosolic proteins, such as the fructose-bisphosphate aldolase (Alf1), the enolase (Eno), the triosephosphate isomerase Tim, the elongation factors G and Ts, the ribosomal proteins S5, S13 and L25, the phosphoglycerate kinase Pgk and the glyceraldehyde-3-phosphate dehydrogenase GAPDH. Antibodies directed against Eno have previously been demonstrated to cause opsonophagocytic killing of *S. aureus* and this protein is a known protective antigen on the cell surface of *Streptococcus suis*.

Interestingly, the studies documented in **Chapter 5** also showed that some of the proteins identified on the *S. aureus* cell surface display clusters of epitopes that appear to be exposed into the extracellular host milieu. The best examples for this group of proteins are the cell wall-anchored IsdB protein, the bifunctional autolysin Atl, the adhesin Emp and the transglycosylase IsaA. Notably, the IgGs from different EB patients did not always bind to the same epitopes of particular investigated proteins. This may have several possible reasons. In the first place, with a few exceptions, the epitope mapping was performed with arrays that contained mostly linear peptides. Accordingly, IgGs that recognize only conformational epitopes will be overlooked by this approach. Another possible reason for the high variability in the binding of IgGs from EB patients to the peptide libraries may relate to the fact that different EB patients were colonized by different *S. aureus* types. These different types of *S. aureus* might express different sets of cell surface-exposed antigens and, thus, elicit different immune responses. In this respect, it is important to take into account the previously demonstrated variability of different *S. aureus* lineages with respect to the presence of particular genes for surface-exposed or exported proteins. Nevertheless, the analyses described in **Chapter 5** have altogether highlighted several immunodominant cell surface-exposed proteins of *S. aureus* and specific sub-domains of these proteins as potential targets for novel active or passive immunization approaches. These include the covalently cell wall-bound proteins ClfB and IsdB, a 'YkyA-like' cell wall-binding lipoprotein, the membrane proteins EbpS and LtaS, the non-covalently cell wall-bound and secreted proteins Atl, Sbi, IsaA, Emp, and the cytoplasmic proteins Afl1, Eno, and GAPDH. Future

studies will show whether any of these proteins can indeed serve as effective targets for antistaphylococcal immunotherapy.

Chapter 6 describes the profiling of global interactions between human serum proteins and the *S. aureus* cell surface. The quantification of MS data by spectral counting showed that nine serum proteins were specifically enriched on the cell surfaces of the *S. aureus* strains USA300 and Newman. These include components of the complement system, namely factor H (fH), the fH-related proteins 1 and 5, and component 7. In addition, significant amounts of a fifth protein, the platelet factor 4 (PF4), were detectable in samples derived from the surface of strain Newman, but not USA300. In addition, four serum proteins were clearly enriched on the staphylococcal cell surface, namely properdin, complement component C3, fibrinogen-α, and isoform 1 of the inter-α-trypsin inhibitor heavy chain H4 (ITIH4). It was previously shown that almost all of these proteins are involved in the human host defences against *S. aureus*. One exception is ITIH4, which was enrichment only on the cell surface of strain Newman. This protein has not yet been reported to interact with bacteria, but it is a well-known acute-phase serum protein, as was previously shown in a mouse model for cutaneous burn injuries. In addition to human proteins, various surface-exposed *S. aureus* proteins were also identified. These included Coa, FnbpA, the secretory antigen SsaA, and several typical cytosolic proteins, including four ribosomal proteins, the elongation factor TU and GAPDH. Taken together, the findings reported in **Chapter 6** show that the surface shaving technique is a versatile generally applicable tool for monitoring bacteria-host interactions.

To colonize or invade the human host, *S. aureus* expresses different virulence factors that can, in fact, also represent targets for the human immune defenses as was shown and discussed in **Chapters 3** and **5**. These virulence factors are first synthesized as precursors with an N-terminal signal peptide to direct their transport from the cytoplasm to an extra-cytoplasmic location, such as the cell wall or extracellular milieu via different transport systems. As outlined in the introductory **Chapter 1** of this thesis, the most commonly used pathway for protein transport across the membrane is the general secretory (Sec) pathway. The translocation via this pathways can be divided into three stages: (i) chaperoning of newly synthesized proteins to the membrane-embedded translocon, (ii) membrane translocation, and (iii) post-translocation modification and processing. In the present PhD research, several components that are involved in the latter two stages have been analyzed.

The Sec machinery for protein translocation is composed of several subunits. The SecA

translocation motor binds pre-proteins and pushes them through the membrane-embedded SecYEG translocation channel via repeated cycles of ATP binding and hydrolysis. The core of the Sec translocon consists of the SecA, SecY and SecE proteins, which are essential for growth and viability. In addition to the major SecYEG channel proteins, *S. aureus* produces a second set of SecA and SecY proteins, generally referred to as SecA2 and SecY2. In **Chapter 7**, the analyses of isogenic *secG* and *secY2* mutants of *S. aureus* is described. While the deletion of *secY2* had no detectable effects on protein secretion, the importance of SecG became clearly evident upon analysis of the exoproteome of a *secG* mutant by 2-D PAGE. Specifically, the extracellular accumulation of nine abundant exoproteins and seven cell wall-bound proteins was significantly affected in the *secG* mutant. Among these proteins are some known virulence factors involved in host colonization (*e.g.* the serine-aspartic acid repeat proteins SdrC and SdrD), invasion of host tissues (*e.g.* hemolysins and leukocidins), cell wall turnover (LytM), and evasion of the immune system (Spa). Another interesting finding was that the second IgG-binding protein Sbi was almost completely absent from the cell wall of the *secG* mutant strain. Interestingly, deletion of *secY2* exacerbated the secretion defects of *secG* mutants, affecting the extracellular accumulation of one additional exoprotein and one cell wall protein. Furthermore, the *secG secY2* double mutant displayed a synthetic growth defect. This might relate to a slightly elevated expression of *sraP*, encoding the only known substrate for the Sec2 pathway, in cells lacking SecG. Additionally, the results suggest that SecY2 can interact with the Sec1 channel, which would be consistent with the presence of a single set of *secE* and *secG* genes in *S. aureus*. Notably, infection experiments in a mouse model did not reveal any attenuation of the *secG* mutant strain, suggesting that this component of the secretion machinery is dispensable for host subversion by *S. aureus*. This implies that the presence or absence of SecG or SecY2 is not critical for the virulence of *S. aureus* SH1000, at least under the conditions tested in the applied mouse infection model.

In recent years, the functions of many secretion machinery components of *S. aureus* have been elucidated as exemplified by studies on the Sec pathway. Intriguingly however, for several other predicted secretion machinery components, no biological functions had been described at the start of the present PhD research. This applied for example to the pseudopilin export machinery of *S. aureus*. This machinery is very similar to the Com machinery for DNA binding and uptake in *B. subtilis*. Assembly of the pseudopilus in *B. subtilis* requires the specific signal peptidase ComC, which processes the N-terminal signal peptides of ComG proteins upon membrane translocation. Furthermore, stability of the *B. subtilis* ComGC pseudopilin requires post-translocational disulfide bond formation, which is catalyzed by the thiol-disulfide oxidoreductases (TDOR) BdbC and BdbD. Interestingly, the genes coding

for most Com proteins are present in the sequenced *S. aureus* strains, suggesting that this bacterium is able to assemble pseudopili of the Com type. **Chapter 8** describes studies on the processing and stability of *S. aureus* ComGC. In *S. aureus*, the transcription of most com genes, including *comGC*, is directed by the staphylococcal alternative sigma factor σ^H. Upon constitutive expression of σ^H, exponentially growing cells of *S. aureus* produced only the precursor form of ComGC, while cells in the post-exponential growth phase showed low-level ComC-dependent processing of ComGC. Interestingly, the inefficient ComGC processing was due to limited ComC signal peptidase activity as shown by ComC overexpression. Furthermore, ComGC was barely detectable in cells lacking the TDOR DsbA, and this was also the case when cells were grown in the presence of the reducing agent β-mercaptoethanol. Together, these observations imply that the TDOR activity of DsbA is required for disulfide bond formation in ComGC and that this disulfide bond is essential for ComGC stability. Notably, both pre-ComGC and mature ComGC were found to localize to the cytoplasmic membrane and cell wall of *S. aureus*. However, as shown by immunofluorescence microscopy, the overproduction of ComC resulted in strongly enhanced surface exposure of ComGC. These findings thus show that ComC-dependent processing of ComGC is of importance for the optimal cell surface exposure of ComGC. It is presently not clear, why *S. aureus* cells seem to produce ComC in limiting amounts for ComGC biogenesis, but this observation may explain why *S. aureus* shows only marginal levels of natural competence.

The ability of *S. aureus* to invade and colonize almost all human tissues depends on various cell surface-associated and secreted virulence factors. As described above these virulence factors are first synthesized with an N-terminal signal peptide to direct them into one of the secretion pathways of the staphylococcal cell. The translocated proteins will then pass the cell wall and are released into the extracellular environment, unless they are specifically retained in the cell. The signal for covalent attachment of translocated proteins to the cell wall is formed by a so-called LPxTG motif, which is located in the C-terminus of the exported proteins. This LPxTG motif is recognized by membrane-associated transpeptidases known as sortases. *S. aureus* possesses two sortase enzymes, named sortase A (SrtA) and sortase B (SrtB). SrtA recognizes the canonical LPxTG motif. In contrast, SrtB recognizes a degenerate LPxTG motif, namely NPQTN.

The research described in **Chapter 9** was aimed at assessing the roles of SrtA and SrtB in the *in vitro* growth of *S. aureus* on human plasma, which mimics to some extent the *in vivo* growth condition encountered during bacteremia. As exemplified with the *S. aureus* strain SH1000, the results show that *srtA* can be an important determinant for efficient growth of certain staphylococcal strains in human plasma. Importantly, transcript profiling analyses

showed that a *srtA* mutation altered the expression of 39 genes. In contrast, no stress response was detectable in *srtB* mutant cells grown on human plasma. Among the genes affected by the *srtA* mutation, the genes putatively required for phosphate acquisition by *S. aureus* appeared to be enriched. These include the genes for the phosphate-specific transport (Pst) system, which is similar to the Pst system from *B. subtilis*, and the gene for a secreted alkaline phosphatase (PhoB). In *B. subtilis* the *pst* operon is a member of the Pho regulon, which is controlled by the PhoP-PhoR two-component regulatory system. The high similarity between the PhoP regulator components of *S. aureus* and *B. subtilis* suggested that the Pho box motif could be conserved in these two species. Indeed, a Pho box-like motif was identified in the upstream region of the *S. aureus pstSCAB* and *phoB* genes. Together, these findings imply that the *srtA* mutant cells grown in human plasma displayed a phosphate starvation response. Interestingly, our tiling array data show that the gene encoding the LPxTG protein FnbpA, was 2.3 times up-regulated in the *srtA* mutant. In addition, the *fnbpB* gene was also up-regulated, albeit only 1.7-fold, which was just below the somewhat arbitrarily chosen cut-off of 1.8 fold. This would suggest that FnbpA and possibly also FnbpB have a role in phosphate acquisition. Both proteins are important in infections caused by *S. aureus*, because of their central role in the adhesion to and invasion of host cells. Especially, FnbpA was shown to be a multifunctional adhesin with the capacity to bind to fibrinogen, fibronectin, elastin and several other proteins. Intriguingly, it has been shown that soluble fibronectin contains covalently bound phosphate. Thus, it is conceivable that Fnbp's are somehow involved in the scavenging of phosphate from fibronectin, thereby contributing to the phosphate homeostasis in *S. aureus*. This view is further supported by the fact that putative Pho boxes are present in the promoter regions of both *fnbpA* and *fnbpB*. On the other hand, most phosphate in human serum is present in an unbound state, at least according to the available literature data. This would imply that FnbpA and FnbpB could also be involved in the binding of free phosphate.

Chapter 10 addresses the influence of *srtA* or *srtB* mutations on the *S. aureus* growth in RPMI medium as well as on the surfacome. The growth experiments of *S. aureus* SH1000 and its sortase mutant derivatives showed that only the deletion of *srtA*, but not *srtB* drastically affected the growth rate of *S. aureus*. This shows that SrtA is important for the growth of *S. aureus* in RPMI medium, as was also shown to be the case for growth in human plasma in **Chapter 9**. Importantly, the surfacome analysis of exponentially growing cells of the parental strain SH1000, the Δ*srtA* mutant and the Δ*srtB* mutant resulted in the identification of 27, 11 and 17 surface-exposed proteins, respectively. In stationary growing cells these numbers were increased to 31, 23 and 19 proteins, respectively. These findings indicate that not only the numbers secreted *S. aureus* proteins increase in the stationary phase, but also

the numbers of cell surface-exposed proteins. More importantly, our analyses revealed that the identified surface-exposed proteins of the parental strain included 9 LPxTG proteins. Of these nine, only the SasG protein was identified exclusively in the parental strain. Five LPxTG proteins, namely ClfA, ClfB, IsdA, IsdB and SdrD were identified in the parental strain, but not in the *srtA* mutant. This suggests that these proteins require SrtA for proper cell surface exposure. Intriguingly, two LPxTG proteins, Spa and SasF, were identified at the surface of both *srtA* mutant cells and cells of the parental strain. Previous studies have shown that indeed some Spa remains bound to the wall of *srtA* mutant *S. aureus* cells, albeit in reduced amounts, while this protein is secreted into the growth medium at substantially increased levels. Our findings show that the wall-bound Spa of SrtA-deficient mutant cells is exposed at the cell surface, but potentially in a different manner than in SrtA-proficient cells. Specifically, our MS analyses identified additional peptides from the second and third IgG-binding domains and the C-terminal LysM domain of Spa as being surface-exposed in SrtA-deficient cells. Possibly, this reflects a mis-localization of Spa in the absence of its covalent binding to the peptidoglycan. The LysM domain, which is thus still present in the non-covalently cell wall-bound Spa, is most likely responsible for the observed cell wall retention of this protein.

In conclusion, the research described in this thesis has addressed genome- and proteome-wide 'global' interactions between *S. aureus* and its human host. To this end, different studies were performed *in vivo* and *in vitro*, addressing (i) the colonization of chronic wounds by *S. aureus*, (ii) the IgG responses of colonized patients to particular staphylococcal proteins, (iii) the subcellular localization of these staphylococcal proteins, and (iv) the mechanisms by which these proteins are localized to the staphylococcal cell wall, cell surface and exoproteome. As such, it can be concluded that the main goals of the present thesis research have been achieved. The main challenges for future research now lie in the translation of the present findings into novel strategies for antistaphylococcal therapy. An important question that should be addressed on the short term would be how to apply the knowledge gained on wound colonization by *S. aureus* for the development of new approaches to achieve better wound care and faster wound healing. This would be highly relevant for EB patients who suffer from chronic wounds. An important long-term research goal is the implementation of the identified immunodominant cell surface-exposed targets for the development of novel vaccines or protective antibodies to prevent or combat *S. aureus* infections.

12

Nederlandse samenvatting

De meest diverse groep van levende organismen op aarde wordt gevormd door bacteriën. Bacteriën hebben hun levensstijl aan verschillende ecologische niches aangepast, waaronder de mens. In de humane darm leven bijvoorbeeld 500 tot 1000 verschillende bacteriesoorten en vergelijkbare aantallen kunnen op de menselijke huid geïdentificeerd worden. In de meeste gevallen lijken zowel de bacteriën als de mens te profiteren van deze coëxistentie, maar in sommige gevallen kunnen commensale bacteriën de menselijke gastheer binnendringen, hetgeen tot levensbedreigende ziektes kan leiden. Eén van deze opportunistische bacteriën is *Staphylococcus aureus*. *S. aureus* koloniseert ongeveer 30% van de gezonde humane populatie. Wanneer echter de primaire barrièrefunctie van de huid verstoord is of wanneer het immuunsysteem is aangetast, dan heeft *S. aureus* de mogelijkheid om invasief te groeien en bijna alle weefsels en organen van het lichaam binnen te dringen en een breed scala aan ziektes te veroorzaken. Zoals in **hoofdstuk 1** van dit proefschrift is aangegeven hangt de pathogeniciteit van *S. aureus* af van de aanwezigheid van verschillende virulentiefactoren. Deze omvatten eiwitten aan het celoppervlak van de bacterie die betrokken zijn bij de binding en kolonisatie van gastheerweefsels, geëxporteerde invasines die de bacteriële verspreiding binnen het geïnfecteerde weefsel bevorderen, verschillende moleculen die de kans op overleving van staphylokokken na opname door fagocyterende immuuncellen vergroten, eiwitten die helpen het immuunsysteem van de gastheer te ontwijken en toxines die de membranen van gastheercellen beschadigen.

Niet alleen de ziektes die door *S. aureus* veroorzaakt worden zijn zorgwekkend, maar ook het sterke vermogen van deze bacterie om resistentie tegen antibiotica te ontwikkelen. Tot nu toe zijn de therapieën tegen *S. aureus* infecties voornamelijk gebaseerd op het gebruik van antibiotica. Vanwege de snelle toename van antibioticum-resistente *S. aureus* varianten is er echter een groeiende behoefte aan alternatieve therapieën, waaronder actieve en passieve immunisatie om ziektes veroorzaakt door *S. aureus* te voorkomen of te genezen. Momenteel zijn er echter nog geen vaccins of beschermende antilichamen beschikbaar om deze zo succesvolle ziekteverwekker te stoppen. Daarom moeten nieuwe strategieën om deze ziekteverwekker te bestrijden door middel van immuuntherapie gebaseerd zijn op een geïntegreerde aanpak, die leidt tot de identificatie van invariante immunodominante targets die in alle cellen van *S. aureus* aanwezig zijn. De identificatie van deze targets vereist (i) een gedetailleerde kennis van de lokalisatie van eiwitten en andere moleculen op het celoppervlak van *S. aureus* en (ii) een goed begrip van de menselijke immuunreacties tegen deze componenten. Het onderzoek beschreven in dit proefschrift was daarom gericht op de bestudering van de interacties tussen *S. aureus* en de menselijke gastheer, zoveel *in vivo* als *in vitro*. Het beschreven onderzoek naar de immuunresponsen van patiënten met de erfelijke blaarziekte epidermolysis bullosa (EB) ten gevolge van langdurig *S. aureus*

dragerschap heeft geleid tot de identificatie van meerdere zeer immunogene eiwitten van *S. aureus* en de immunodominante epitopen in deze eiwitten. Daarnaast hebben *in vitro* studies verschillende mechanismen opgehelderd die van belang zijn voor localisatie van *S. aureus* eiwitten op het celoppervlak. Tezamen dragen de bevindingen beschreven in dit proefschrift bij aan een beter begrip van de interacties tussen *S. aureus* en zijn menselijke gastheer tijdens kolonisatie en infectie.

EB is de verzamelnaam voor een groep van erfelijke huidaandoeningen, die veroorzaakt worden door mutaties in verschillende structurele eiwitten in de huid. Als gevolg van kleine mechanische beschadigingen van de huid ontwikkelen patiënten met EB blaren. De resulterende ulceratie van de huid leidt tot de ontwikkeling van wonden die gemakkelijk gekoloniseerd worden door verschillende bacteriën. In **hoofdstuk 2** van dit proefschrift wordt beschreven, dat EB-patiënten een onverwacht hoog percentage van *S. aureus*-dragerschap vertonen. Alle EB-patiënten met chronische wonden en 75% van de patiënten zonder chronische wonden bleken gekoloniseerd te zijn met *S. aureus* op ten minste één plaats op het lichaam. Zelfs wanneer uitsluitend naar de kolonisatie van de bovenste luchtwegen gekeken werd, bleek het *S. aureus* kolonisatiepercentage bij EB-patiënten (56 tot 90%) aanzienlijk hoger te liggen dan bij gezonde personen (25 tot 37%) of zorgverleners die regelmatig contact hebben met EB-patiënten (39%). Dit betekent dat de wonden van EB-patiënten een aantrekkelijke niche voor *S. aureus* vormen. Moleculaire typering van de *S. aureus*-isolaten met behulp van de zogenaamde MLVA methode liet zien (i) dat de kolonisatie van EB-patiënten niet beperkt is tot specifieke *S. aureus* types, (ii) dat individuele patiënten met EB maar liefst vier verschillende *S. aureus* types kunnen dragen op een willekeurig tijdstip en (iii) dat overdracht van stafylokokken tussen de bovenste luchtwegen en wonden van EB-patiënten regelmatig voorkomt. De opvatting, dat kolonisatie van EB-patiënten door *S. aureus* een willekeurig proces is werd verder ondersteund door zogenaamde *spa*-typeringsanalyses, waaruit bleek dat het grootste deel van de bij EB-patiënten gevonden *S. aureus spa*-types behoren tot de meest dominante *spa*-types in het woongebied van de respectievelijke EB-patiënten. Dit betekent dat de *S. aureus* populatiestructuur in de bemonsterde EB-patiënten de algemene *S. aureus* populatiestructuur in Nederland weerspiegelt. Een andere belangrijke bevinding was, dat de mate van *S. aureus* transmissie tussen verschillende EB-patiënten in de bemonsterde populatie relatief laag is. In de weinige gevallen, waarbij verschillende EB-patiënten dezelfde *S. aureus* types bij zich droegen betrof dit gezinsleden of personen die in een gebied leven waar de respectievelijke *S. aureus* types ook frequent bij andere personen voorkomen.

Onderzoek beschreven in **hoofdstuk 3** was gericht op analyse van (i) de *S. aureus* kolonisatie

van EB-patiënten over een lange periode en (ii) de invloed van *S. aureus* kolonisatie op de titers van immuunglobulines G (IgG) tegen *S. aureus* bij deze patiënten. De resultaten laten zien, dat eenzelfde *S. aureus* type alleen aangetoond werd bij ~42,5% van de onderzochte patiënten. Daarentegen bleken de overige onderzochte EB-patiënten over een langere periode gekoloniseerd te worden door afwisselende *S. aureus* types. Deze waarnemingen laten zien, dat EB-patiënten voortdurend geconfronteerd worden met verschillende *S. aureus* types en dat de koloniserende *S. aureus* populatie in de tijd snel kan veranderen. Verder liet het onderzoek zien, dat sera van EB-patiënten hogere niveaus van anti-*S. aureus* IgG's bevatten dan sera van gezonde personen. Dit geldt in het bijzonder voor IgG's tegen negen belangrijke virulentiefactoren: de oppervlakteeiwitten IsdA en SasG, de geëxporteerde eiwitten IsaA, SCIN, Nuc en LytM en de superantigenen SEM, SEN en SEO. EB-patiënten die met verschillende *S. aureus* types gekoloniseerd waren bevatten hogere niveaus van anti-*Staphylococcus* antilichamen dan EB-patiënten gekoloniseerd door slechts één *S. aureus* type. Dit was vooral evident voor IgG's tegen de IsdA, LukD, HlgB, LytM, LukS, LukF en ETA eiwitten van *S. aureus*. Tezamen tonen deze waarnemingen aan, dat het immuunsysteem van EB-patiënten die verschillende *S. aureus* types dragen zeer sterk wordt uitgedaagd door deze bacterie en dat het dragerschap van meerdere *S. aureus* types de hoogste humorale responsen bij deze patiënten opwekt.

In de meeste voorgaande studies werd mogelijk *S. aureus*-dragerschap onderzocht door middel van uitstrijkjes van verschillende plaatsen van het lichaam met behulp van wattenstaafjes. Vervolgens werden deze wattenstaafjes gebruikt om de daarin aanwezige bacteriën te isoleren, waarna slechts één *S. aureus* kolonie per wattenstafje verder werd geanalyseerd. Dit heeft waarschijnlijk geresulteerd in een onderschatting van het aantal koloniserende *S. aureus* soorten, vooral bij personen die verhoogd vatbaar zijn voor *S. aureus*-kolonisatie. Daarom waren de studies beschreven in **hoofdstuk 4** gericht op het onderzoeken van de mogelijke coëxistentie van verschillende *S. aureus* types in individuele wonden van EB-patiënten. Hiertoe werd de 'replica plating' methode gebruikt voor het verzamelen van koloniserende bacteriën in plaats van de eerder gebruikte wattenstaafjes. Na replica plating van gebruikte verbanden van vijf verschillende EB-patiënten werd in sommige gevallen confluënte bacteriegroei en in andere gevallen de vorming van losse kolonies waargenomen. Uit de analyse van 12 tot 48 bacterie-isolaten per wond bleek, dat dit in de meeste gevallen *S. aureus* betrof. Met behulp van fluorescentie *in situ* hybridisatie werd vervolgens zichtbaar gemaakt, dat *S. aureus* meestal aanwezig was in de vorm van microkolonies, hoewel afzonderlijke *S. aureus* cellen ook gedetecteerd werden. Op grond van deze waarnemingen kon geconcludeerd worden, dat de bacteriële wondtopografie, zoals waargenomen door de replica plating van de gebruikte verbanden, de werkelijke bacteriële *S. aureus* topografie in

de wonden van de onderzochte EB-patiënten weerspiegelt. Het typeren van alle verkregen *S. aureus* isolaten liet zien, dat individuele chronische wonden wel zes verschillende *S. aureus* types konden bevatten op één bepaald moment, en maar liefst 10 verschillende *S. aureus* types over langere tijdsintervallen. Sommige van de gevonden *S. aureus* types blijken nauw verwant te zijn wat suggereert, dat ze een recente gemeenschappelijke afstamming hebben. Andere isolaten afkomstig van aangrenzende wondlocaties behoren tot zeer verschillende *S. aureus* types. Er kan daarom geconcludeerd worden, dat de algemene veronderstelling dat één persoon door één type *S. aureus* gekoloniseerd wordt met zekerheid niet geldt voor de chronische wonden van patiënten met EB.

Zoals in de **hoofdstukken 2, 3** en **4** beschreven, is de *S. aureus*-kolonisatie bij patiënten met EB zeer hoog. Hoogst waarschijnlijk leidt dit tot de waargenomen verhoging in de IgG-niveaus tegen verschillende componenten van *S. aureus*. Tot op heden was echter niet bekend of deze anti-*Staphylococcus* IgG's bescherming bieden tegen invasieve ziektes veroorzaakt door *S. aureus*. Dit is echter een interessante mogelijkheid voor verder onderzoek, met name met het oog op de ontwikkeling van een immunotherapie tegen *S. aureus* infecties. De studies beschreven in **hoofdstuk 5** combineren daarom een proteomics-gebaseerde identificatie van de celwand- en celoppervlak-geassocieerde eiwitten van *S. aureus* met een array-gebaseerde screening voor immuno-dominante epitopen in de geïdentificeerde eiwitten. Voor de epitoop-screening werd door EB-patiënten gedoneerd bloedplasma gebruikt. Via proteomics analyses werd een groot aantal eiwitten op het celoppervlak van twee verschillende *S. aureus* stammen, de meticilline-gevoelige (MSSA) stam Newman en de 'community-acquired' meticilline-resistente (MRSA) stam USA300, geïdentificeerd. Op het celoppervlak van de USA300 stam werden naast bekende en voorspelde celwandeiwitten veel typisch cytoplasmatische eiwitten gevonden. Dit opmerkelijke fenomeen werd ook in eerdere studies waargenomen en voor enkele van de geïdentificeerde cytoplasmatische eiwitten wordt een zogenaamde 'moonlighting functie' in pathogenese verondersteld. In overeenstemming met het idee, dat deze cytoplasmatische eiwitten ook *in vivo* op het celoppervlak van *S. aureus* aanwezig zijn, werd gevonden dat het plasma van EB-patiënten inderdaad IgG's tegen cytosolische eiwitten bevatte, waaronder de fructose-bisfosfaataldolase Alf1, de enolase Eno, de triosefosfaatisomerase Tim, de elongatiefactoren G and Ts, de ribosomale eiwitten S5, S13 en L25, de fosfoglyceraatkinase Pgk en de glyceraldehyde-3-fosfaatdehydrogenase GAPDH. Het is eerder aangetoond, dat antilichamen gericht tegen Eno de opsonophagocytische eliminatie van *S. aureus* faciliteren en dat Eno op het celoppervlak van *Streptococcus suis* een beschermende antigeenfunctie heeft.

Een interessante waarneming beschreven in **hoofdstuk 5** is, dat een aantal eiwitten op het

S. aureus celoppervlak clusters van epitopen lijken te bevatten die geëxponeerd zijn in het extracellulaire milieu van de gastheer. De beste voorbeelden van dergelijke eiwitten zijn het celwand-verankerde IsdB eiwit, het bifunctionele autolysine Atl, het adhesine Emp en de transglycosylase IsaA. Opmerkelijk was dat de IgG's van verschillende EB-patiënten niet altijd bleken te binden aan hetzelfde epitoop van de onderzochte eiwitten. Dit kan verschillende oorzaken hebben. Ten eerste werden de meeste epitoopmapping-analyses uitgevoerd met arrays die voornamelijk lineaire peptiden bevatten. Hierdoor kunnen IgG's die uitsluitend conformationele epitopen herkennen over het hoofd gezien worden. Een tweede mogelijke reden voor de hoge variabiliteit in de binding van IgG's van verschillende EB-patiënten aan de peptide arrays is gelegen in het feit, dat verschillende EB-patiënten gekoloniseerd zijn door verschillende *S. aureus* types. De expressie van de verschillende celoppervlakte-eiwitten kan verschillen in verschillende *S. aureus* types en hierdoor kunnen verschillende immuunreacties in verschillende patiënten opwekt worden. Niettemin hebben de analyses beschreven in **hoofdstuk 5** geleid tot de identificatie van verschillende immunodominante celoppervlakte-eiwitten van *S. aureus* en specifieke sub-domeinen van deze eiwitten die als mogelijke targets voor nieuwe actieve of passieve immunisatie kunnen dienen. Hiertoe behoren de covalent gebonden celwandeiwitten ClfB and IsdB, een 'YkyA-achtig' celwand-bindend lipoproteine, de membraaneiwitten EbpS en LtaS, de geëxporteerde en niet-covalent gebonden celwandeiwitten Atl, Sbi, IsaA en Emp en de cytoplasmatische eiwitten Afl1, Eno en GAPDH. Toekomstige studies zullen moeten aantonen of deze eiwitten inderdaad als effectieve targets voor immunotherapieën tegen *S. aureus* ingezet kunnen worden.

Hoofdstuk 6 beschrijft de karakterisering van de globale interacties tussen humane serumeiwitten en het *S. aureus* celoppervlak. Hiertoe werden *S. aureus* cellen geïncubeerd in humaan plasma. De kwantificering van massaspectrometriegegevens toonde aan dat, na incubatie in plasma, negen serumeiwitten specifiek verrijkt aanwezig zijn op het celoppervlak van de *S. aureus* stammen USA300 en Newman. Hiertoe behoren componenten van het complementsysteem, namelijk factor H (fH), de fH-gerelateerde eiwitten 1 en 5 en component 7. Bovendien waren aanzienlijke hoeveelheden van een vijfde eiwit, de platelet factor 4 (PF4), detecteerbaar in monsters afkomstig van het celoppervlak van stam Newman. Daarnaast waren vier serumeiwitten duidelijk verrijkt aanwezig op het *S. aureus* celoppervlak, namelijk properdin, complement component C3, fibrinogeen-α en isovorm 1 van de inter-α-trypsine inhibitor heavy chain H4 (ITIH4). Het was al eerder aangetoond, dat bijna al deze eiwitten betrokken zijn bij humane afweerreacties tegen *S. aureus*. Een uitzondering is ITIH4, waarvan nog niet bekend was dat het interacties aangaat met bacteriën. ITIH4 is echter wel een bekend acute fase serumeiwit, zoals eerder werd aangetoond in

een muismodel voor brandwonden. Naast de humane eiwitten werden ook verschillende *S. aureus* oppervlakte-eiwitten geïdentificeerd, waaronder Coa, FnbpA, het gesectereerde antigeen SsaA en enkele cytosolische eiwitten, waaronder vier ribosomale eiwitten, de elongatiefactor TU en GAPDH. Tezamen laten de bevindingen gerapporteerd in **hoofdstuk 6** zien, dat de oppervlakte shaving-techniek een veelzijdig en algemeen toepasbaar instrument is voor de analyse van bacterie-gastheer interacties.

Om de menselijke gastheer te koloniseren of binnen te dringen, brengt *S. aureus* verschillende virulentiefactoren tot expressie, die ook targets voor de menselijke afweer vertegenwoordigen, zoals werd aangetoond en besproken in de **hoofdstukken 3** en **5**. Deze virulentiefactoren worden eerst gesynthetiseerd als precursors met een N-terminaal signaalpeptide om hun transpoort van het cytoplasma naar een extracytoplasmatische locatie, zoals de celwand of het extracellulaire milieu via verschillende transportsystemen te initiëren. Zoals beschreven in het inleidende **hoofdstuk 1** van dit proefschrift is het algemene secretie (Sec) systeem de meest gebruikte route voor eiwittransport over de cytoplasmamembraan. Het eiwittransport via deze route kan in drie fases verdeeld worden: (i) targeting van nieuw-gesynthetiseerde eiwitten naar de translocatie-machinerie in de cytoplasmamembraan, (ii) membraantranslocatie, en (iii) post-translocationele modificatie en processing. In de context van het onderhavige promotieonderzoek zijn verschillende componenten van de laatste twee stappen geanalyseerd.

Het Sec-systeem bestaat uit verschillende subeenheden. De SecA translocatiemotor bindt precursor-eiwitten en duwt deze door het SecYEG translocatiekanaal in de membraan via herhaalde cycli van ATP-binding en hydrolyse. De kern van het Sec-translocon bestaat uit de SecA, SecY en SecE eiwitten die essentieel zijn voor groei en levensvatbaarheid van bacteriecellen. Naast de belangrijke SecYEG eiwitten produceert *S. aureus* een additionele set SecA en SecY eiwitten, SecA2 en SecY2 genaamd. In **hoofdstuk 7** wordt de analyse van isogene *secG* en *secY2* mutanten van *S. aureus* beschreven. Terwijl de deletie van het *secY2* gen geen detecteerbaar effect had op de eiwitsecretie, bleek de deletie van het *secG* gen de eiwitsecretie in aanzienlijke mate te beïnvloeden. Exoproteoomanalyses lieten zien, dat de extracellulaire accumulatie van negen gesecreteerde eiwitten en zeven celwand-gebonden eiwitten significant beïnvloed was in de *secG* mutant. Tot deze eiwitten behoren een aantal bekende virulentiefactoren betrokken bij de gastheerkolonisatie (de 'serine-aspartic acid repeat proteins' SdrC en SdrD), de invasie van gastheerweefsels (hemolysines en leukocidines), de celwand-turnover (LytM), en het ontwijken van het immuunsysteem (Spa). Een andere opmerkelijke bevinding was, dat het tweede IgG-bindende eiwit van *S. aureus*, Sbi, bijna volledig afwezig was van de celwand van de *secG* mutant. Deletie

van het *secY2* gen versterkte de secretiedefecten van de *secG* mutant, wat resulteerde in de extracellulaire accumulatie van een extra gesecreteerd eiwit en een celwandeiwit. Bovendien vertoonde de *secG secY2* dubbelmutant een synthetisch groeidefect. Dit kan betrekking hebben op de enigszins verhoogde expressie van het *sraP* gen, dat codeert voor het enige tot dusver bekende substraat van het Sec2-systeem. De resultaten suggereren, dat SecY2 een interactie kan aangaan met het reguliere Sec-kanaal. Dit is in overeenstemming met het gegeven, dat er slechts één paar *secE* en *secG* genen in *S. aureus* aanwezig is. Bovendien lieten infectie-experimenten zien, dat een *secG* mutant geen verminderde virulentie heeft in een muismodel. Dit suggereert, dat deze component van het Sec-systeem overbodig is voor gastheer-subversie door *S. aureus*. Dit betekent tevens, dat de aanwezigheid van SecG en/of SecY2 onder de geteste omstandigheden en in het toegepaste muisinfectiemodel niet essentieel is voor de virulentie van *S. aureus*.

In de afgelopen jaren zijn de functies van vele secretiesysteemcomponenten van *S. aureus* opgehelderd, zoals geïllustreerd met de voornoemde studies aan het Sec-systeem. De biologische functies van enkele andere secretiesysteemcomponenten waren echter nog niet bekend bij aanvang van het onderhavige promotieonderzoek. Dit gold bijvoorbeeld voor het pseudopilinesysteem van *S. aureus*. Dit systeem is zeer vergelijkbaar met het Com-systeem voor DNA-binding en opname door *Bacillus subtilis*. Assemblage van Com-pseudipili in *B. subtilis* vereist de activiteit van het specifieke signaalpeptidase ComC, dat de N-terminale signaalpeptides van verschillende ComG-eiwitten tijdens membraantranslocatie afsplitst. Daarnaast is de vorming van disulfidebruggen vereist voor de stabiele productie van het ComGC pseudopiline van *B. subtilis*. De vorming van deze disulfidebruggen die wordt gekatalyseerd door de thiol-disulfide oxidoreductases (TDORs) BdbC en BdbD. Interessant is, dat de genen die coderen voor de meeste Com-eiwitten van *B. subtilis* ook in alle *S. aureus* stammen aanwezig zijn. Dit suggereert dat de bacterie Com-type pseudopili kan vormen. **Hoofdstuk 8** beschrijft daarom onderzoek naar de processing en stabiliteit van *S. aureus* ComGC. In *S. aureus* wordt de transcriptie van de meeste com genen, inclusief *comGC*, aangestuurd door de alternatieve sigma factor σ^H. Bij constitutieve expressie van σ^H produceerden exponentieel groeiende cellen van *S. aureus* alleen de precursorvorm van ComGC. In de post-exponentiële groeifase was daarentegen een laag gehalte matuur ComGC in de *S. aureus* cellen detecteerbaar. Dit was het resultaat van ComC-afhankelijke pre-ComGC processing. Door middel van ComC-overexpressie kon aangetoond worden, dat de inefficiënte processing van ComGC in wild-type cellen het gevolg was van beperkte ComC-expressie. Een opmerkelijke bevinding was, dat ComGC nauwelijks detecteerbaar was in cellen zonder de TDOR DsbA en dit was ook het geval wanneer de cellen gekweekt werden in de aanwezigheid van het reductiemiddel β-mercaptoethanol. Samen laten deze

waarnemingen zien, dat de TDOR-activiteit van DsbA essentieel is voor de vorming van disulfide-bruggen in ComGC en dat de gevormde disulfide-bindingen essentieel zijn voor ComGC-stabiliteit. Opmerkelijk was ook, dat zowel pre-ComGC als matuur ComGC in de cytoplasmamembraan en de celwand van *S. aureus* aantoonbaar waren. Overproductie van ComC resulteerde in een sterk verhoogde oppervlakte-presentatie van ComGC. Deze bevindingen tonen samen aan, dat ComC-afhankelijke maturatie van ComGC van belang is voor een optimale presentatie van ComGC aan het celoppervlak. Het is momenteel niet duidelijk, waarom *S. aureus* cellen de signaalpeptidase ComC in beperkte mate produceren, maar deze waarneming verklaart mogelijk ten dele waarom *S. aureus* slechts marginale niveaus van natuurlijke competentie vertoont.

Het vermogen van *S. aureus* tot invasie en kolonisatie van bijna alle humane weefsels hangt af van verschillende cel-geassocieerde en gesecreteerde virulentiefactoren. Zoals hiervoor beschreven worden deze virulentiefactoren eerst gesynthetiseerd met een N-terminaal signaalpeptide om ze naar één van de secretieroutes van de *Staphylococcus* cel te dirigeren. De getransloceerde eiwitten zullen vervolgens de celwand passeren om uiteindelijk losgelaten te worden in het extracellulaire milieu, tenzij ze specifiek vastgehouden worden in de cel. Het signaal voor covalente binding van getransloceerde eiwitten aan de celwand is het zogenaamde LPxTG motief, dat zich in de C-terminus van sommige geëxporteerde eiwitten bevindt. Dit LPxTG motief wordt herkend door membraan-geassocieerde transpeptidases die bekend staan onder de naam sortases. *S. aureus* bezit twee sortase enzymen die sortase A (SrtA) en sortase B (SrtB) genoemd worden. SrtA herkent het standaard LPxTG motief terwijl SrtB het afwijkende LPxTG motief NPQTN herkent.

Het onderzoek beschreven in **Hoofdstuk 9** was gericht op het bepalen van de rollen van SrtA en SrtB in de *in vitro* groei van *S. aureus* in humaan plasma. Dit weerspiegelt tot op zekere hoogte de *in vivo* groeicondities die de bacteriën tegenkomen bij een bacteriëmie. Zoals aangetoond voor de *S. aureus* stam SH1000 kan SrtA een belangrijke factor zijn voor efficiënte groei van *S. aureus* in humaan plasma. Transcriptie-profiling analyses lieten zien, dat een *srtA* mutatie leidt tot veranderde expressie van 39 genen. Daarentegen werd geen stress-reactie waargenomen in *srtB* mutante cellen die gekweekt waren in humaan plasma. Onder de genen die beïnvloed waren door de *srtA* mutatie waren genen, die nodig zijn voor de fosfaatopname door *S. aureus*. Hiertoe behoren de genen voor het fosfaat-specifieke transportsysteem Pst, dat sterk lijkt op het Pst-systeem van *B. subtilis*, en het gen voor de gesecreteerde alkalische fosfatase PhoB. In *B. subtilis* is het pst operon onderdeel van het Pho-regulon, dat gecontroleerd wordt door het twee-componenten regulatorische systeem PhoP-PhoR. De hoge mate van overeenkomst tussen de PhoP regulatoren van *S. aureus* en

B. subtilis suggereren, dat het Pho-box motief waaraan PhoP bindt geconserveerd is in deze twee bacteriesoorten. Een Pho box-achtig motief kon inderdaad geïdentificeerd worden in het promotergebied van de *S. aureus pstSCAB* en *phoB* genen. Tezamen impliceren deze waarnemingen, dat de *srtA* mutante cellen een fosfaathonger-reactie vertonen wanneer ze gekweekt worden in humaan plasma. In dit opzicht is het interessant, dat onze transcriptie analyses lieten zien dat het gen voor het LPxTG-eiwit FnbpA 2,3 keer verhoogd tot expressie kwam in de *srtA* mutante cellen die in humaan plasma gekweekt waren. Bovendien kwam het *fnbpB* gen onder deze condities ook 1,7 keer verhoogd tot expressie. Dit suggereert dat FnbpA en wellicht ook FnbpB een rol hebben in de fosfaatopname. Beide eiwitten zijn van belang bij *S. aureus* infecties, omdat ze een centrale rol spelen bij de adhesie aan en invasie van gastheercellen. Met name FnbpA blijkt een multifunctioneel adhesine te zijn, dat het vermogen bezit om te binden aan fibrinogeen, fibronectine, elastine en verschillende andere gastheereiwitten. Eerder onderzoek heeft laten zien, dat oplosbaar fibronectine covalent gebonden fosfaat bevat. Het is derhalve denkbaar dat Fnbp's op de een of andere manier betrokken zijn bij het verkrijgen van fibronectine-gebonden fosfaat en alzo een bijdrage leveren aan de fosfaat-balans van de *S. aureus* cellen. Dit idee wordt verder ondersteund door het gegeven, dat potentiele Pho-boxen aanwezig zijn in de promotergebieden van de *fnbpA* en *fnbpB* genen. Hierbij dient opgemerkt te worden, dat het meeste fosfaat in humaan serum in een ongebonden staat aanwezig is. Dit zou kunnen suggereren, dat FnbpA en FnbpB ook betrokken zouden kunnen zijn bij de binding en opname van ongebonden fosfaat.

Hoofdstuk 10 behandelt de invloed van *srtA* en *srtB* mutaties op de groei van *S. aureus* in RPMI medium en de samenstelling van het oppervlakte proteoom van *S. aureus*. Groei-experimenten met *S. aureus* SH1000 en sortase mutanten van deze stam lieten zien, dat een deletie van het *srtA* gen een aanzienlijk groeiremmend effect had, terwijl deletie van het *srtB* gen geen effect had. Hieruit kan geconcludeerd worden, dat SrtA belangrijk is voor de groei van *S. aureus* in RPMI medium, zoals ook het geval was voor groei in humaan plasma (**hoofdstuk 9**). De analyses van het oppervlakteproteoom van exponentieel groeiende cellen van stam SH1000 en de Δ*srtA* of Δ*srtB* mutanten resulteerde in de identificatie van respectievelijk 27, 11 en 17 oppervlakte-geëxponeerde eiwitten. De aantallen geïdentificeerde oppervlakte-eiwitten in de stationaire groeifase waren respectievelijk 31, 23 en 19. Deze waarnemingen geven aan, dat niet alleen het aantal gesecreteerde *S. aureus* eiwitten toeneemt in de stationaire fase, maar ook het aantal celoppervlak-geëxponeerde eiwitten. Onze analyses lieten tevens zien, dat 9 LPxTG eiwitten detecteerbaar waren op het celoppervlak van de wild-type stam. Van deze negen eiwitten werd alleen het SasG eiwit exclusief in de wild-type stam geïdentificeerd. Vijf andere LPxTG eiwitten, te weten ClfA, ClfB, IsdA, IsdB en SdrD werden in de wild-type stam geïdentificeerd, maar niet in

de *srtA* mutant. Dit suggereert dat deze eiwitten afhankelijk zijn van SrtA voor hun correcte presentatie op het celoppervlak. Weer twee andere LPxTG eiwitten, Spa and SasF, werden geïdentificeerd op het oppervlak van zowel *srtA* mutante cellen als ook op het oppervlak van de wild-type stram. Eerdere studies hadden al laten zien, dat Spa nog steeds gebonden bleef aan de celwand van een *S. aureus srtA* mutant, maar wel in verminderde hoeveelheden, terwijl deze mutant aanzienlijk verhoogde hoeveelheden Spa in het groeimedium secreteerde. De waarnemingen beschreven in **hoofdstuk 10** laten zien, dat het celwand-gebonden Spa van SrtA-deficiënte mutante cellen geëxponeerd wordt op het celoppervlak, maar mogelijk wel op een andere manier dan in SrtA-proficiënte cellen. Massa-spectrometrische analyses toonden namelijk aan, dat additionele peptides van het tweede en derde IgG-bindingsdomein en het C-terminale LysM domein van Spa geëxponeerd waren aan het celoppervlak van SrtA-deficiënte cellen. Dit weerspiegelt wellicht een verkeerde localisatie van Spa als dit eiwit niet covalent gebonden wordt aan het peptidoglycaan van de celwand. Het LysM domein, dat nog steeds aanwezig is in het non-covalent celwand-gebonden Spa, is waarschijnlijk verantwoordelijk voor de waargenomen celwand-retentie van dit eiwit in *srtA* mutante cellen.

Samenvattend kan geconcludeerd worden, dat het onderzoek beschreven in dit proefschrift de genoom- en proteoom-wijde 'globale' interacties tussen *S. aureus* en de humane gastheer beschrijft. Hiertoe zijn verschillende *in vivo* en *in vitro* experimenten uitgevoerd, die een dieper inzicht hebben verschaft in: (i) de kolonisatie van chronische wonden van EB-patiënten door *S. aureus*, (ii) de niveaus van IgG's tegen bepaalde *S. aureus* eiwitten in verschillende EB-patiënten, (iii) de subcellulaire localisatie van deze *Staphylococcus* eiwitten en (iv) de mechanismes, waarmee deze eiwitten gelocaliseerd worden in de celwand, op het celoppervlak en in het exoproteoom van *S. aureus*. Alzo zijn de belangrijkste doelstellingen van het onderhavige promotieonderzoek gehaald. Belangrijke uitdagingen voor toekomstig onderzoek liggen in de translatie van de huidige resultaten naar nieuwe strategieën voor de voorkoming en bestrijding van *S. aureus* infecties. Een belangrijke vraag die op de korte termijn beantwoord zou moeten worden is, hoe de verkregen kennis over wondkolonisatie door *S. aureus* vertaald kan worden naar nieuwe benaderingen voor verbeterde wondzorg en snellere wondgenezing. Dit zou zeer relevant zijn voor EB-patiënten die leiden aan chronische wonden. Een belangrijk doel voor de langere termijn is de toepassing van de geïdentificeerde immunodominante celoppervlakeiwitten van *S. aureus* als doelwitten voor de ontwikkeling van nieuwe vaccins of beschermende antilichamen om infecties veroorzaakt door *S. aureus* te voorkomen of te vermijden.

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