Human Antibody Responses against Virulence Factors of *Staphylococcus aureus* during Infection

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Human Antibody Responses against Virulence Factors of *Staphylococcus aureus* during Infection

De humane antilichaam responsen tegen virulentie factoren van *Staphylococcus aureus* tijdens infectie

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The journey not the arrival matters.

T.S. Eliot

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Chapter 1

General introduction,
aim and outline of this thesis

GENERAL INTRODUCTION

Staphylococcus aureus was first described in 1881 by Scottish surgeon Alex Ogston, who observed round bacterial cells (Greek: kókkos) in grape-like clusters (Greek: staphyle) within pus from abscesses [1]. Four years later, German surgeon Friederich Rosenbach was the first to isolate and culture these bacteria, describing colonies with a yellow/golden colour (Latin: aureus). At that time, a causal link between *S. aureus* and skin infections, abscess formation and blood poisoning (bacteraemia) was readily established. These infections were widespread in the pre-antibiotic era; for instance, it is estimated that about half of all casualties in the trenches during World War I (1914-1918) were due to septic wound infections with *S. aureus* [2].

At present, *S. aureus* is still a leading cause of a wide range of community-acquired infections, including those of the skin and soft tissue [3], heart valves (endocarditis) [4], bone (osteomyelitis) [5, 6] and lungs (pneumonia) [7]. These infections are often associated with significant morbidity and, in the case of a serious infection such as bacteremia, mortality (20 to 30% mortality rate) [8, 9]. In addition, *S. aureus* is the most common cause of health-care associated surgical site infections [10, 11]. These infections lead to increased length of stay within the hospital and increased hospital costs, adding an estimated $5-$10 billion yearly to U.S. health costs, for example [12].

Between 12 to 30% of individuals within the general population is persistently colonized by *S. aureus*, predominantly within the mucosa of the nose [13]. Another 30% (range 16-70%) is intermittent carrier. Although colonization by *S. aureus* is asymptomatic in healthy individuals and does not warrant treatment, persistent carriers have a three to six time higher risk for experiencing health-care-associated *S. aureus* infections [14, 15], mostly caused by the endogenous, colonizing strain [16, 17]. Furthermore, within certain patient groups affected by chronic disease, such as young cystic fibrosis patients, *S. aureus* colonization is associated with unfavourable clinical outcomes [18].

S. aureus virulence factors

S. aureus are gram-positive cocci that can produce many surface-associated and excreted virulence factors which enable the potential of this species to cause the above mentioned infections. These proteins can generally be divided into several groups based on their function. For instance, *S. aureus* expresses multiple surface-anchored proteins, such as Fibronectin binding proteins A and B (FnBPA and B), which can bind to specific host molecules and thereby facilitate interaction with host cells and the extracellular matrix [19-21]. This allows effective colonization of host tissue and indwelling foreign materials coated with host molecules, such as catheters and prosthetic joints [22].

Another group of virulence factors comprises the many toxins secreted by *S. aureus*. Some of these can efficiently lyse host cells, such as the pore-forming alpha-toxin [23]

and the bi-component leukocidins and gamma-hemolysin [24, 25]. These toxins defend *S. aureus* against neutrophils [26] and disrupt host tissue, clinically manifesting itself as necrosis, scalding or abscess formation of the skin or soft tissues [27, 28]. Many toxins, including alpha toxin [29], the enterotoxins [30] and toxic shock syndrome toxin [31], also interfere with the host innate and adaptive immune response. These latter toxins act as superantigens, directly activating T-helper cells which leads to massive cytokine release, ultimately causing clinical syndromes such as food poisoning and toxic shock syndrome.

In addition to toxins, several factors secreted by *S. aureus* interfere with specific parts of the host immune response. For example, the recruitment and phagocytic activity of neutrophils is inhibited by a range of secreted proteins, including the Staphylococcal Superprotein Like proteins (SSLs) [32], the formyl-peptide receptor-like 1 inhibitor (FLIPr) [33] and the Chemotaxis Inhibitory protein of S. aureus (CHIPS) [34].

Many of the surface-associated and secreted virulence factors mentioned above are also involved in biofilm formation of *S. aureus* [35-38], a phenotypic trait which importance for *S. aureus* virulence is increasingly recognized. Biofilms are complex communities of bacteria enclosed in a polymer matrix [39], believed to provide shelter for the bacteria residing within against antibiotics and the host immune system [40-42]. Formation of biofilms are specifically associated with chronic infections such as osteomyelitis and prosthetic joint infections [40, 43].

Treatment of *S. aureus* infections

Apart from local treatment with topical antibiotics (mupirocin, fusidic acid) and surgical interventions, including the drainage of abscesses and removal of infected prostheses, intravenous beta-lactam antibiotics are the first-choice agents for treating systemic *S. aureus* infections. During World War II penicillin was introduced on a large scale to treat septic wound infections among allied soldiers, an intervention which had such an impact on military campaigns that the production process was designated as a military secret. Soon afterwards though, penicillinase-producing *S. aureus* became ubiquitous and nowadays the penicillinase-resistant flucloxacillin is the treatment of first choice within the Netherlands. Unfortunately, additional resistance towards all beta-lactam antibiotics, including the penicillin-related indicator agent methicillin, is now endemic in most countries worldwide [44]. Vancomycin, the alternative therapeutic agent used for treating Methicillin-resistant *S. aureus* (MRSA), is clinically inferior compared to beta-lactam antibiotics and, although still rare, the emergence of vancomycin-resistance among MRSA has become a concern [45].

Apart from antimicrobial resistance, serious *S. aureus* infections can be difficult to treat due to their notorious propensity to cause secondary infections, to relapse or to establish chronic infection. This can have devastating consequences, as illustrated by

osteomyelitis, requiring surgery and prolonged antibiotic treatment with often still uncertain outcome [46].

In this context alternative treatment strategies to treat or prevent infections with *S. aureus* are receiving great scientific and clinical interest. Some of these alternatives, such as antimicrobial peptides and si-RNA interfering with bacterial gene expression, appear promising yet are still far away from clinical use [47, 48]. Other treatment strategies which rely on preventive use of current (local) antibiotics to decolonize carriers of *S. aureus* have been shown to significantly reduce health-care associated infections [49], although decolonization might fail and concerns about practicalities and induction of antibiotic resistance remain [50]. In this light, the development of an anti-*S. aureus* vaccine remains an attractive treatment strategy.

The human immune response against *S. aureus*

The host immune response towards a pathogen generally consist of an innate (non-specific) and adaptive (specific) part, the latter including the antibody-mediated (humoral) immune response. In the case of *S. aureus* infections it is now increasingly recognized that cellular immunity, including the actions of neutrophils and T-helper cells, plays a central part in both innate and adaptive immunity of the host [51, 52]. This is supported by the increased incidence of infections with *S. aureus* observed in patients with defects in cellular immunity, such as neutropenia [53, 54]. Based on *in vitro* and *in vivo* animal models, it appears that especially the actions of T-helper 17 cells are important for the host defence against *S. aureus* (reviewed in [51, 52]). These cells recruit and activate neutrophils at the site of infection and promote the production of antimicrobial peptides within skin surfaces.

In addition to cellular immunity, specific antibodies against virulence factors of *S. aureus* aid in the host defence. Ever since the first observation of anti-hemolysin properties of human serum in 1901 [55], these antibodies have received great scientific interest. Neutralizing antibodies can interfere with the actions of e.g. bacterial toxins and surface-anchored proteins. Furthermore, binding of antibodies to the bacterial cell surface can trigger complement activation and opsonophagocytosis of the bacteria [56]. Currently, the presence of antibodies against many virulence factors of *S. aureus*, ranging from alpha toxin to the fibronectin-binding proteins, has been quantified in humans. In general, specific anti-staphylococcal antibodies are detectable at an early phase of life, firstly being of maternal origin and then being replaced by the infants own antibody production around the first half year [57]. Antibodies against *S. aureus* are ubiquitously detectable in human blood, ranging from blood obtained from healthy non-carriers to patients at the start of a clinically apparent infection [58-60]. This suggests that everyone is frequently exposed to *S. aureus* and that these (often brief but repetitive) exposures induce the production of specific antibodies. Furthermore, persistent carriers of *S. aureus* develop an

antibody repertoire that is specifically directed against their colonizing strain [58]. From this immunological baseline, each person will develop a highly individualized antibody response against the infecting strain of *S. aureus* after the onset of infection [59-61].

In light of the increasingly complicated treatment of *S. aureus* infections, extensive attempts have been made to exploit the humoral immune response for vaccination. Monovalent vaccines targeting a wide range of virulence factors have repeatedly been shown to protect or reduce severity of infections in various animal models [62, 63] and the levels of specific antibodies in human patients are associated with clinical outcome [59, 60, 64, 65]. Subsequently, multiple clinical studies have evaluated the efficacy of vaccines to prevent or treat infections in patients (Table 1). However, despite that all these vaccines induced the production of opsonophagocytic antibodies in patients, so far none was shown to have any clinical benefit [for recent reviews see [52, 63, 66-68]. Currently, data from several clinical studies, evaluating the efficacy of multivalent vaccines targeting different *S. aureus* virulence factors, are still pending (Table 1).

The reason why so far all attempts to develop a clinically effective vaccine in humans failed, despite the plethora of promising data from animal models, remains a matter of debate. The active manipulation of the host immune response by *S. aureus*, the masking of immunogenic epitopes within virulence factors for the host immune system or the lack of expression of a virulence factor, targeted by a vaccine, might all contribute to this. In light of the latter possibility, it is currently unclear whether specific virulence factors, previously evaluated as vaccine targets, are actually expressed by *S. aureus* and present during (specific stages of) infection in patients. This is a prerequisite for an effective vaccine. Furthermore, if present, the immunogenic potential of many virulence factors to ubiquitously induce a protective antibody response in humans remains to be confirmed, both in terms of quantity (does the amount of antibodies increase after the onset of infection?) and quality (are they neutralizing?). Finally, currently little is known about the capacity of human antibodies to interfere with the function of most *S. aureus* virulence factors. To fill in these gaps in our knowledge, at least in part, the studies presented in this thesis were started. In line with the current awareness that multivalent vaccines targeting different virulence factors are likely to have a higher chance of success than monovalent vaccines, these studies were performed using a wide range of functionally diverse, well-characterized virulence factors of *S. aureus*.

Aim of this thesis

The general aim of this thesis is twofold. Firstly, this thesis aims to provide further insights into the presence of a wide range of well-characterized virulence factors of *S. aureus* during growth of clinically isolated strains in *in vitro* and *ex vivo* models, mimicking the *in vivo* situation during infection in humans.

Table 1. Overview of the most important anti- *S. aureus* vaccines developed by pharmaceutical companies.

Vaccine name[1]	Company	Targeted virulence factors	Study phase	Major results	Remarks
Veronate®	Inhibitex	ClfA and SdrG	III	No reduction in late-onset sepsis vs placebo	Pooled human IgG; passive immunization
Aurexis®	Inhibitex	ClfA	II	No reduction in relapse of bacteraemia or death	Humanized monoclonal antibody; passive immunization
StaphVAX®	GSK	Capsular polysaccharide (CP) 5 and 8	III	No reduction in sepsis	Active immunization
PentaStaph®	GSK	CP 5, 8, polysaccharide type 336, alpha toxin and Pantom-Valentine leukocidin	II	No longer listed in 2015 pipeline report	Active immunization
AltaStaph®	GSK	Capsular polysaccharide (CP) 5 and 8	II	No reduction in sepsis or death	IgG pooled from volunteers immunized with StaphVAX®
Aurograb®	Novartis	ABC transporter with unknown function	II	No difference in treatment outcome for deep-seated infections when combined with vancomycin	Vaccine combined with vancomycin treatment
Pagibaximab®	Biosynexus	Lipoteichoic acid	III	No reduction in sepsis	Chimeric monoclonal antibody; passive immunization
V710®	Merck	IsdB	III	No reduction in post-operative wound infections	Active immunization
PF 6290510	Pfizer	ClfA, MntC, CP 5 and 8	II	Results pending (study involving post-operative infections)	Active immunization
NDV3	Novadigm	*Candida albicans* agglutinin-like sequence 3 (Als3)	II	Results pending (study involving safety and immunogenicity)	Recombinant protein of *Candida albicans* surface protein with cross-reactivity against *S. aureus*
4C-Staph	Novartis	FhiD2, EsxA and B, alpha toxin, Sur-2	Pre-clinical	_	Active immunization
MEDI4893	Medimmune	Alpha toxin	II	Results pending (study involving ventilation-associated pneumonia)	Human monoclonal IgG; passive immunization
AR 301	Aridis	Alpha toxin	II	Results pending (study involving ventilation-associated pneumonia)	Human monoclonal IgG; passive immunization

[1] Both previously (upper half) and currently (lower half) evaluated vaccines are shown (partially based on [52, 69, 70]).

Secondly, this thesis aims to further characterize the human antibody response induced by the same *S. aureus* virulence factors during different infections in patients.

Ultimately, these aims help in identifying the virulence factors of *S. aureus* that are important for bacterial pathogenesis in humans and could be potential vaccine targets.

Methods: detecting proteins, antibodies and antibody functionality using the Luminex assay

The Luminex assay, which is a flow cytometry technique based on polystyrene beads (xMAP®, Luminex corporation), is used in all studies in this thesis for different measurements. In brief, Luminex beads can be covalently coupled to recombinant proteins (such as the virulence factors of interest). The beads are color-coded with a mixture of 2 fluorescent dyes (red and infra-red), allowing the inclusion of up to 100 different proteins in one single measurement. Coupling of proteins is performed by activating carboxylated beads with *N*-hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) according to a previously optimized protocol [71]. Protein-coupled beads can then be incubated with human serum, washed, and bound antibodies can be quantified using a fluorescently labeled, secondary anti-human antibody as previously described [61, 71]. This method is used in **Chapters 2**, **3** and **5**.

In **chapters 3** and **4** the alternative Competitive Luminex Assay (CLA) is described. In this assay, which was recently validated [72], serum is first incubated with living bacteria instead of protein-coupled beads. In this way, specific antibodies will bind proteins that are expressed by the bacteria and as such will be absorbed from the serum. After removal, the amount of specific antibody remaining in the serum is measured using the standard protocol as mentioned above, and compared to the original antibody levels in control serum that was not incubated with bacteria. Antibody absorption is expressed as a percentage decrease in antibody levels, which in a semi-quantitative manner reflects the presence of bacterial proteins.

In **chapter 6**, a novel functional Luminex-based assay is described wherein specific binding between FnBPA, coupled to beads, and fluorescently labelled fibrinogen is demonstrated.

Outline of this thesis

In **Chapter 2**, we expand on previous studies by characterizing the human antibody response against 56 virulence factors of *S. aureus* in 21 patients suffering from bacteraemia. IgG and IgA levels are prospectively measured following infection and patient IgG levels are compared with those of age-matched, healthy controls. In addition, all infecting strains are genetically characterized and the expression of 3,626 genes is examined more closely for two strains during growth in human blood, using a micro-array setup.

In contrast to bacteraemia, less is known about the human antibody response against *S. aureus* during a chronic, biofilm-associated infection such as osteomyelitis. Therefore, in **Chapter 3** IgG levels against 50 virulence factors of *S. aureus* are prospectively measured in serum of 10 patients suffering from osteomyelitis. Furthermore, the presence of the same 50 virulence factors is established in biofilms of the infecting strains grown *in vitro* on polystyrene (PS) and human bone using CLA. *In vivo* and *in vitro* data are compared and the relationship between protein presence and antibody responses are discussed.

The presence of 52 *S. aureus* virulence factors during biofilm formation by 5 (methicillin resistant) strains is further established in **Chapter 4** using the CLA and *in vitro* biofilm models on PS and on a previously described human skin model (Leiden Epidermal Models) [73]. The presence of virulence factors are compared between different strains and biofilm models, respectively. Presence of virulence factors is additionally confirmed by RT-PCR and mass-spectrometry and, in the case of the ubiquitously expressed alpha toxin with GFP-reporter technology.

In chapter 5, *in vivo* serologic data are further expanded and associated with clinical outcome in Cystic Fibrosis (CF) patients, another patient group suffering from chronic *S. aureus* infection. As part of this observational prospective longitudinal study, the association between IgG levels against 44 *S. aureus* virulence factors and diverse clinical parameters, including lung function, is assessed. In addition, these IgG levels are compared between 182 patients and 53 healthy nasal carriers of *S. aureus*.

Finally, in **chapter 6** the human antibody response against one specific virulence factor, FnBPA, is studied in more detail. Amongst the 21 infecting strains from the same bacteremia patients who are described in chapter 2, amino acid sequence diversity in the FnBPA protein is established. Subsequently, the influence of this diversity on the human antibody response is characterized. Finally, the capacity of antibodies, obtained from both patients and non-infected controls, to interfere with binding between FnBPA and its substrate fibrinogen is determined using a novel Luminex-based assay.

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Chapter 2

Characterization of the humoral immune response during *Staphylococcus aureus* bacteremia and global gene expression by *Staphylococcus aureus* in human blood

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ABSTRACT

Attempts to develop an efficient anti-staphylococcal vaccine in humans have so far been unsuccessful. Therefore, more knowledge of the proteins that are expressed by *Staphylococcus aureus* in human blood and induce an immune response in *S. aureus*-infected patients is required. In this study we further characterize the serial levels of IgG and IgA antibodies against 56 staphylococcal proteins in multiple serum samples of 21 patients with a *S. aureus* bacteremia (median of 10 serum smaples per patient), compare peak IgG levels between patients and 30 non-infected controls, and analyze the expression of 3626 genes by two genetically distinct isolates in human blood. The serum antibody levels were measured using a bead-based flow cytometry technique (xMAP®, Luminex corporation). Gene expression levels were analyzed using a microarray (BμG@s microarray).

The initial levels and time taken to reach peak IgG and IgA antibody levels were heterogeneous in bacteremia patients. Proteins SA0688 and PrsA were associated with the highest median initial-to-peak antibody fold-increase for both IgG (5.05 and 2.92-fold) and IgA (2.07 and 2.72-fold for SA0688 and PrsA, respectively). Peak IgG levels against 27 proteins, including the protein SA0688, were significantly elevated in bacteremia patients versus controls ($P \leq 0.05$). Expression of diverse genes, including *SA0688* and *PrsA,* was ubiquitously high in both isolates at all time points during incubation in blood. However, only a limited number of genes were specifically up- or downregulated in both isolates when cultured in blood, compared to the start of incubation in blood or during incubation in BHI broth.

In conclusion, most staphylococcal proteins tested in this study, including many known virulence factors, do not induce uniform increases in the antibody levels in bacteremia patients. In addition, the expression of these proteins by *S. aureus* is not significantly altered by incubation in human blood over time or compared to standard growth medium. One immunogenic and ubiquitously expressed protein is the putative iron-regulated ABC transporter SA0688.

INTRODUCTION

Staphylococcus aureus is one of the most common causes of bloodstream infections [1, 2] and *S. aureus* bloodstream infections are associated with serious complications such as infective endocarditis and prosthetic device infection [3-5]. The mortality rate of *S. aureus* bacteremia is approximately 20-30% [6-8]. Unfortunately, due to the increasing antibiotic resistance of clinical *S. aureus* isolates [9, 10] and the simultaneous decrease in the number of newly approved antimicrobial agents [11, 12], the treatment of *S. aureus* bacteremia is becoming increasingly difficult. Therefore, alternative strategies to prevent or treat *S. aureus* bacteremia are much needed.

One potential strategy is the development of a vaccine. However, despite the promising results of anti-staphylococcal vaccines in animal models, efforts to develop an efficient vaccine against *S. aureus* in humans have so far failed [13-15]. Classically, vaccine development has focused on stimulating the humoral immune response during *S. aureus* infection, as this response is considered to play an important role in clearing infections [16]. Although recent work questions the effectiveness of the humoral immune response in clearing infections [15] and suggests a more important role for the Th17 cell-mediated immune response [14, 17], knowledge of which proteins are expressed by bacteria and are immunogenic in infected patients remains essential for new immunotherapies. However, to date the number of reports exploring the immunogenicity of *S. aureus* proteins, especially in humans, is limited and all of these studies have investigated relatively small numbers of bacterial proteins (reviewed in [16]. In short, two of the most recent studies found detectable yet heterogeneous antibody levels in single serum samples of both infected patients and healthy controls against 19 [18] or 8 [19] recombinant *S. aureus* proteins. Another study analyzed the immunogenicity of whole-cell wall protein preparations using 2-dimensional gel electrophoresis (2-DE) immunoblotting of pooled sera from both infected patients and controls [20]. Fifteen immunogenic surface proteins were identified, including SdrE and SA0688, for which significantly increased IgG levels had previously been demonstrated in infected patients, compared to non-infected controls [18]. In the most comprehensive study to date, the antibody levels against 19 staphylococcal proteins were serially measured in multiple serum samples from bacteremia patients [21], and heterogeneity in the antibody levels between different patients was again observed. IsdA was associated with increased antibody levels in the majority of patients and was therefore suggested as a potential vaccine component. However, several proteins analyzed in earlier studies including wall teichoic acid, peptidoglycan, SA0688, alpha toxin and other proteins which are hypothesized to be important virulence factors were not analyzed in this study.

To further characterize the humoral immune response during *S. aureus* bacteremia, we analyzed the levels of IgG and IgA antibodies against 56 staphylococcal proteins

in serial serum samples from 22 bacteremia patients. This is the largest collection of known staphylococcal proteins analyzed to date, including the non-protein proteins wall-teichoic acid and peptidoglycan. In addition, we compared the IgG levels against all 56 proteins in bacteremia patients and non-infected controls. Finally, to gain further insight into the bacterial proteins which are expressed in human blood and could be involved in the pathogenesis of bacteremia, we studied the expression changes of 3626 *S. aureus* genes during the incubation of two genetically distinct strains in human blood using microarray analyses. Based on these investigations and previous data, we discuss the potential of specific staphylococcal proteins as components of human vaccines.

MATERIALS AND METHODS

Ethics statement

All patient serum samples used in this study were obtained from coded left-over material from routine diagnostic blood samples. In concordance with the guidelines of the Erasmus University Medical Hospital and the Dutch federation of Biomedical Scientific Societies (Federatie van Medische Wetenschappelijke Verenigingen), all patients were informed of the possibility that left-over material from diagnostic samples could be used for scientific research and all patients were offered the opportunity to give written refusal to this. Serum samples used in this study were only obtained from patients who did not object to the use of left-over material for scientific research and gave verbal consent for this. This procedure was approved and the acquisition of additional written consent was waived specifically for this retrospective study by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2007-106, addendum 2). All collected serum samples were coded and only qualified physicians of the department of Medical Microbiology and Infectious Diseases had access to the original patient data.

Patients, controls and definitions

Twenty-one adult patients, admitted to the Erasmus Medical Center between March 2007 and March 2011 were followed from the time of diagnosis of *S. aureus* bacteremia until discharge from the hospital or, if applicable, during outpatient appointments after discharge. Bacteremia was defined as the isolation of *S. aureus* from at least one blood culture set. A median number of 10 (interquartile range, 12) serum samples were collected per patient over a median period of 34.5 (interquartile range, 35.8) days. The median age of the bacteremia patients included in the study was 65.5 years (interquartile range, 14 years), of whom 73% were male.

All patients were treated with antibiotics according to hospital guidelines under the supervision of a consultant of the Department of Medical Microbiology and Infectious

Diseases. During admission to the hospital, 4 of the 22 patients died; however, none of these deaths could be directly attributed to staphylococcal bacteremia.

Single serum samples were collected from 30 non-infected patients, admitted to the Erasmus Medical Center between July 2011 and February 2012 for reasons other than any infectious disease. In addition, control patients did not suffer from any clinically apparent infection in at least the past 6 months. The median age of the non-infected control patients was 62 years (interquartile range, 11.5 years), of whom 80% were male. *S. aureus* nasal carrier status was not tested for either the bacteremia patients or the control group. However, previous results [22] and additional data (not shown) suggest that there is no overall significant difference in the IgG levels of persistent carriers and non-carriers for all of the proteins tested in this study, except for TSST-1 and SasG.

S aureus strains, detection of virulence genes and genotyping

S. aureus isolates from bacteremia patients were identified on the basis of colony and cellular morphology and Slidex Staph Plus agglutination testing (bioMérieux, Marcy l'Etoile, France). The identification of all staphylococcal isolates was confirmed by Staphylococcus protein A (*spa*)-PCR [23]. The obtained PCR fragments were sequenced; these sequences formed the basis of *spa*-typing. All of the isolates were methicillin-sensitive, as determined by the cefoxitin disk diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) criteria [24]. Antimicrobial susceptibility to additional antibiotics was determined using the VITEK® 2 system with card AST-P549 (bioMérieux).

For each of the 22 bacteremia patients, the first *S. aureus* isolate obtained from a blood culture was screened using PCR for the presence of the 54 genes encoding the proteins to which the antibody responses were measured. PCR was not performed for peptidoglycan and wall teichoic acid biosynthesis genes, as these were assumed to be obligatorily present in all isolates. Primers were both newly designed (Table 1) or described previously [21, 25]. In addition, pulsed-field gel electrophoresis (PFGE) was performed on *Sma1*-digested chromosomal DNA from all 22 isolates, as described previously [26]. Relatedness among the PFGE profiles was evaluated using Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium).

Bacterial proteins

All *S. aureus* proteins used for Luminex experiments were 6x His-tagged proteins with the exception of the synthetic phenol-soluble modulin α 1-4 peptides and the sugars petidoglycan and wall-teichoic acid. The following proteins were coupled to xMAP® beads (Luminex Corporation, Austin, TX, USA): protein secretion system ESX-1-associated factors EsxA and B; Nuclease (Nuc); peptidoglycan hydrolase LytM; immunodominant protein A (IsaA); glucosaminidase; lipase; peptidoglycan (PG); wall teichoic acid (WTA); foldase-protein PrsA; clumping factor A and B (ClfA and ClfB); SD-repeat containing pro-

teins D and E (SdrD and SdrE); iron-responsive surface determinants A and H (IsdA and IsdH); fibronectin-binding proteins A and B (FnbpA and FnbpB); extracellular fibrinogen-binding protein (Efb); *S. aureus* surface protein G (SasG); staphylococcal complement inhibitor (SCIN); chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS); formyl peptide receptor-like inhibitory protein (FLIPr); phenol-soluble modulin α 1-4 peptides (PSM alpha 1-4), alpha toxin; gamma-hemolysin B (HlgB); leukocidins D, E, F and S (LukD, LukE, LukF and LukS); staphylococcal enterotoxins A-E, G-J, M-O, Q, R (SEA–SEE, SEG-SEJ, SEM-SEO, SEQ, SER); exfoliative toxins A and B (ETA and ETB); toxic shock syndrome toxin 1 (TSST-1); staphylococcal superprotein-like proteins 1, 3, 5, 9, 10 and 11 (SSL1, SSL3, SSL5, SSL9, SSL10 and SSL11) and hypothetical proteins SA0104, SA0486 and SA0688.

Table 1. Newly designed primers used in this study.

Gene	Forward primer	Reverse primer
Hla	CGGGATCCGCAGATTCTGATATTAATATT	AACTGCAGTTAATTTGTCATTTCTTCTT
EsxA	CTTACGGGCAAGGTTCAGAC	CTTGTTCTTGAACGGCATCA
EsxB	GGGTGGATATAAAGGTATTAAAGCA	ATGGGTTCACCCTATCAAGC
ETA	ACTGTAGGAGCTAGTGCATTTGT	TGGATACTTTTGTCTATCTTTTTCATCAAC
ETB	ACAAGCAAAAGAATACAGCG	GTTTTTGGCTGCTTCTCTTG
FlipR	TCGCTGCAGGTCTTTTAACTC	GCTTTCTTCACATCACCTTGG
HlgB	GTCAGAGAGTCCATAATGCATTTAA	CACCAAATGTATAGCCTAAAGTG
IsaA	ACCTGAAGCACCTGATGGGT	TACGCAGCAGGTACAGGACA
Lipase	CAATAGGCGTGGTGTCAGTG	AATCGCCAACTTGTGGTTTC
LukDE	TGAAAAAGGTTCAAAGTTGATACGAG	TGTATTCGATAGCAAAAGCAGTGCA
LukF	ATCATTAGGTAAAATGTCTGGACATGATCCA	GCATCAA(GC)TGTATTGGATAGCAAAAGC
LukS	GCAGACGCGTCAACACAA	TTTTACATTTTCCCTATCTTTTT
LytM	CATGCGAAAGACGCAAGCTG	AGGCGCTGTTGAATTACCCG
Nuc	TTATTAAGTGCTGGCATATGTATG	TTTTCTAATATTAAATACACTTAC
PrsA	AAGCAATACGGCGGTAAAGA	GTGCGCCACCTTGTTTAAGT
SSL1	TTCAATTTTTGCATTTTGAGGTT	TTCTTCATCTGAAGCGAAAGC
SSL3	TCGAGTATGACTTCAATTTGTGC	GAACCACATCAACACAACTTCC
SSL5	GATGACAGCAATTGCGAAAG	ATAGCCGCCATCTTTCATTG
SSL9	ATCGGCCAATGCAGAAGTAG	CCACCGACCGAGTATTTGTC
SSL10	CAGCATTAGCAAAAGCGACA	GCTTTCTATGACTTCCCCCATA
SSL11	GCACTAGGGATTTTAACAACAGG	CCATGCGATGAGGCTGTAAT
SEC	CTTGTATGTATGGAGGAATAACAA	TGCAGGCATCATATCATACCA
SED	GTGGTGAAATAGATAGGACTGC	ATATGAAGGTGCTCTGTGG
SEE	TACCAATTAACTTGTGGATAGAC	CTCTTTGCACCTTACCGC
SEG	CGTCTCCACCTGTTGAAGG	CCAAGTGATTGTCTATTGTCG
SEH	CAACTGCTGATTTAGCTCAG	GTCGAATGAGTAATCTCTAGG
SEN	CGTGGCAATTAGACGAGTC	GATTGATTTGATGATTATAG

The following purified non-staphylococcal proteins were also coupled to xMAP beads as negative controls: *Moraxella catarrhalis* ubiquitous surface protein 1 (UspA1); *Streptococcus pneumoniae* pneumococcal surface adhesin A (PsaA) and human metapneumovirus surface protein (hMPV).

SasG, SdrD, SdrE, ClfB, IsdA, IsdH, FnbpA and FnbpB were expressed and purified as described previously [27]. The constructs were kindly provided by T. Foster (Trinity College, Dublin, Ireland). Alpha toxin, HlgB, LukD, LukE, LukF, LukS, SEA and SEC were prepared as described previously [28]. All other proteins were kindly provided by other research groups, as indicated in the acknowledgments.

The purity of all proteins was confirmed using SDS-page. The proteins were coupled to xMAP® beads as described previously [29, 30] with some modifications for PG, WTA and PSM alpha 1-4. For PG and WTA, the beads were incubated with the cross-linkers adipic acid dihydrazide (ADH; 35 mg/ml) and EDC (200 mg/ml) before incubation with the proteins according to the standard protocol. For PSM alpha 1-4 peptides, the activated beads were firstly coupled to 25 µg streptavidin per reaction according to the standard protocol, and then subsequently coupled to biotin-labeled PSM alpha 1-4 peptides for one hour.

Measurement of anti-staphylococcal antibodies

The levels of IgG and IgA antibodies against 56 staphylococcal proteins in the serum samples of bacteremia patients were measured using a bead-based flow cytometry technique (xMAP®; Luminex Corporation), as previously described [21, 29, 30]. In addition to staphylococcal proteins, the IgG levels against the non-staphylococcal proteins UspA1 (*Moraxella cattharalis*), PsaA (*Streptococcus pneumoniae*) and hMPV (human metapneumovirus) were also determined. Serum samples were diluted 1:100 in PBS and secondary phycoerythrin (PE)-labeled goat anti-human antibodies against either total IgG or IgA were diluted 1:200. All measurements were performed in duplicate and the median fluorescence intensities (MFIs), a semi-quantitative measure of antibody levels, were averaged. Duplicate measurements for which the coefficient of variation was larger than 25% were excluded from further analysis. All measurements were corrected for non-specific background signal by subtracting the MFIs of control beads not coupled to any protein.

For the determination of immunological cross-reactivity, 1:200 diluted serum from one bacteremia patient with high MFIs for all leukocidins was pre-incubated with recombinant proteins serially diluted in PBS for 35 minutes on a thermomixer plate shaker. After incubation the serum was spun down twice for 10 minutes at 3400 RPM and non-bound specific antibodies remaining in the supernatant were measured following the standard protocol.

Microarray experiments

Two *S. aureus* isolates from different bacteremia patients were used for the microarray experiments. Overnight cultures were diluted 100 times in fresh prewarmed brain-heart infusion (BHI) broth and grown at 37°C in 5% CO_2 until an OD_{590} of 0.5 was reached. A volume of 30 ml of the culture was pelleted, and then resuspended in 5 ml of freshly isolated heparinized human blood or BHI broth, and incubated with gentle rotation at 37°C in 5% CO_2. All experiments were independently repeated twice with blood from two healthy volunteers (both nasal *S. aureus* carriers). At time point 0 minutes for BHI and time point 0, 30, 60 and 90 minutes for blood, 10 ml RNA protect (Qiagen, Germantown, MD, USA) was added to the samples and incubated for 5 minutes at roomtemperature. The cultures were then pelleted, cold water was added and subsequently 10 x concentrated PBS was added. After centrifugation the pellets were lysed using 1 ml RLT buffer (Qiagen) and 10 µl β-mercaptoethanol, and finally the bacterial pellets were resuspended in 1 ml RNA Pro solution (Qbiogene Inc., City, CA, USA).

S. aureus RNA was isolated using the FastRNA® Pro Blue Kit according to the manufacturer's instructions (Qbiogene Inc.) using the Fastprep FP120 instrument (Qbiogene; two cycles of 45 seconds at a speed setting of 6.0). After isolation, the RNA was treated with 6 U TURBO DNase (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and then the RNA was further purified using the RNAeasy kit (Qiagen) following the manufacturer's protocol.

Chromosomal DNA was isolated from overnight cultures grown in BHI broth. Bacteria were lysed using FastProtein™ Blue Matrix and the FastPrep® instrument (Qbiogene; two cycles of 45 seconds at a speed setting of 6.0). DNA was then purified using the QIAamp DNA Mini Kit (Qiagen) and treated with 10 µl RNase (Promega, Madison, WI, USA).

Hybridization probes were generated from 5 µg total RNA or 1 µg DNA according to the protocol of the Bacterial Microarray Group (BµG@s; St. George's Hospital Medical School, London, UK). RNA or DNA was mixed with 3 µg random primers (Invitrogen, Breda, The Netherlands), heat denatured and snap cooled on ice. The RNA was reverse transcribed to cDNA to incorporate the Cy5 dCTP (GE Healthcare, Diegem, Belgium) fluorescent analog, and DNA was labeled with Cy3 dCTP (GE Healthcare). Labeled RNA and DNA samples were pooled, and hybridized overnight to an *S. aureus* microarray with PCR amplicons printed on Ultragaps (Corning, NY, USA) glass slides (BµG@S) [31]. The array design is available in BµG@Sbase (Accession No. A-BUGS-17; http://bugs.sgul.ac.uk/A-BUGS-17) and also ArrayExpress (Accession No. A-BUGS-17).

The microarray slides were scanned using the ScanArray Express HT scanner (Perkin Elmer, Groningen, The Netherlands) following the manufacturer's instructions. The spots were quantified using Imagene 6.0 software (BioDiscovery, Marina Del Ray, CA, USA). The fully annotated microarray data have been deposited in BµG@Sbase (accession number E-BUGS-137; http://bugs.sgul.ac.uk/E-BUGS-137) (http://bugs.sgul.ac.uk/E-

BUGS-137%29) and also ArrayExpress (accession number E-BUGS-137). GeneSpring GX version 7.3 Software (Agilent Technologies, Santa Clara, CA, USA) was used for normalization and further data analysis. Expression levels were quantified as the log ratio of the signal derived from RNA isolated from blood divided by the signal derived from DNA isolated from broth. Expression levels were averaged for the duplicate experiments from each blood donor, and then the average expression levels from both donors were averaged.

Statistical analysis

Fold-increases in antibody levels were calculated as the ratio of the peak antibody level divided by the initial antibody level (as measured in the first serum sample). If the antibody level only declined after the initial measurement, than the ratio of the lowest antibody level divided by the initial antibody level was calculated. Both fold-increases and decreases were pooled to determine the median fold-change in antibody levels per protein.

Evaluation of histogram plots and the Kolmogorov-Smirnov test revealed a non-normal distribution of the IgG levels for most proteins. The non-parametric Mann-Whitney U test was used to compare the antibody levels of bacteremia patients and controls. Spearman's correlation coefficient was used for correlation analysis of the microarray data. *P*-values ≤ 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA) or Graphpad Prism version 5 (Graphpad Inc. La Jolla, CA, USA).

RESULTS

Genetic typing and presence of virulence genes in clinical *S. aureus* isolates

PFGE analysis was performed on the first available *S. aureus* isolates from all 22 bacteremia patients. The dendogram in Figure 1 illustrates the overall lack of relatedness between the isolates from different patients, with the exception of the isolates from patients 4, 5 and 14. However, there was no epidemiological relationship between these or any of the patients included in this study. To further characterize the genetic background of the clinical isolates, all strains were *spa*-typed. A broad range of *spa*-types linked to different clonal clusters were observed, including two unknown new *spa*-types. All of the isolates were methicillin-sensitive.

For 54 of the 56 proteins analyzed in this study, the presence of the corresponding genes was determined in all clinical isolates using PCR. In addition to the biosynthesis genes for peptidoglycan and wall teichoic acid which are obligatorily present in each isolate, 11 genes were found to be ubiquitously present in all isolates: alpha toxin,

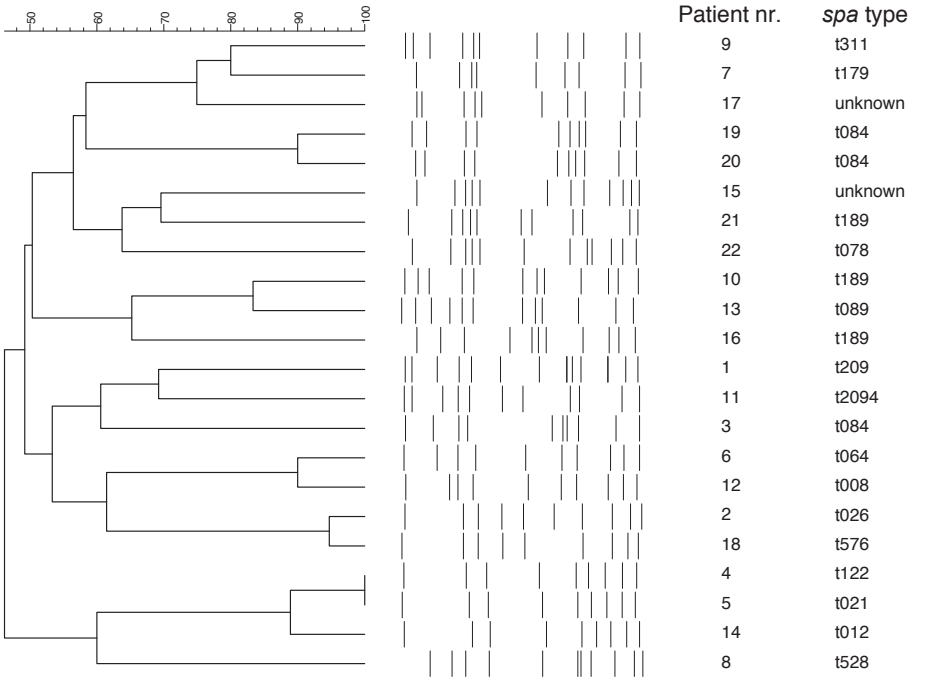

Figure 1. Dendogram of clinical isolates.
Pulsed-field gel electrophoresis data and *spa*-types of *S. aureus* isolates obtained from blood cultures of 22 bacteremia patients are shown.

clumping factor A and B, glucosaminidase, *IsaA*, *IsdA*, lipase, *LytM*, nuclease, *PrsA* and *SA0688*. Five genes were present in only one isolate: exfoliative toxin A, leukocidins F and S, and staphylococcal enterotoxins C and Q. Exfoliative toxin B and enterotoxins E and H were not present in any of the isolates. A summary of the number of isolates containing each gene is presented in Table 2.

Anti-staphylococcal antibodies in bacteremia patients

To study the humoral immune response against a wide array of staphylococcal proteins in bacteremia patients, the total IgG and IgA levels against 56 proteins were measured in serial serum samples from 22 bacteremia patients. The IgG and IgA levels against EsxA, PSM alpha 1-4 peptides, SA0104, SEI and SEJ, and additionally IgA levels against EsxB and SEO were excluded from further analysis, due to the very low signal intensities with coefficients of variation larger than 25% between duplicate experiments.

In general, IgG levels directed against all proteins were already detectable at the time of diagnosis and showed a transient increase in the majority of bacteremia patients. The increases in IgA levels were generally many-fold lower than the increases in IgG levels. The levels of protein-specific IgG, and to a lesser extent IgA, varied extensively

in the first serum sample obtained from each patient (median of 1 day after diagnosis; range, 0-21 days) (Figure 2). The time taken to reach the peak antibody levels varied widely between patients and proteins, and ranged from 7 to 86 days after diagnosis. The course of antibody levels after reaching the peak height was generally characterized by a decrease back towards the initial level and remaining at this level for up to 97 days after diagnosis (Figure 2).

Table 2. Overview of gene presence and associated IgG responses for bacterial proteins

Protein	No. of patients with gene pos isolates (%)	No. of patients with increase in IgG level (%)	Median fold increase from initial to peak level (range)	Significant difference[1]	p value[2]
Alpha toxin	22/22 (100%)	20/22 (91%)	1.3 (0.9-3.47)	ND	ND
CHIPS	14/22 (64%)	16/18 (89%)	1.2 (0.4-2.95)	no	0.389
ClfA	22/22 (100%)	18/20 (90%)	1.6 (0.02-6.09)	yes	0.011
ClfB	22/22 (100%)	14/20 (70%)	1.1 (0.01-5.42)	yes	0.036
Efb	21/22 (96%)	11/15 (73%)	1.5 (0.51-7.96)	no	0.430
EsxA	22/22 (100%)	CV>25%	CV>25%	ND	ND
EsxB	14/22 (64%)	15/17 (88%)	1.4 (0.22-2.91)	yes	0.022
ETA	1/22 (5%)	15/22 (68%)	1.3 (0.29-23.78)	no	0.337
ETB	0/22 (0%)	15/21 (71%)	1.31 (0.29-23.78)	no	0.624
FlipR	16/22 (73%)	18/21 (86%)	1.31 (0.43-8.02)	yes	0.003
FnbpA	20/22 (91%)	14/15 (93%)	1.41 (0.04-5.15)	no	0.274
FnbpB	7/22 (32%)	9/10 (90%)	1.31 (0.75-3.68)	yes	0.040
Glucosaminidase	22/22 (100%)	21/21 (100%)	1.38 (1.02-25.75)	yes	<0.0001
HlgB	17/22 (77%)	22/22 (100%)	1.26 (1.02-5.14)	yes	0.001
IsaA	22/22 (100%)	20/21 (95%)	1.09 (0.9-13.2)	yes	0.001
IsdA	22/22 (100%)	22/22 (100%)	1.68 (1.07-40.45)	yes	0.005
IsdH	21/22 (95%)	16/18 (89%)	1.94 (0.62-6.6)	yes	0.024
Lipase	22/22 (100%)	21/22 (95%)	1.35 (0.85-20.99)	yes	0.002
LukD	16/22 (72%)	22/22 (100%)	1.3 (0.64-4.63)	yes	0.004
LukE	16/22 (72%)	21/22 (95%)	1.3 (0.64-4.63)	yes	0.002
LukF	1/22 (5%)	21/22 (95%)	1.52 (0.95-3.92)	yes	<0.0001
LukS	1/22 (5%)	22/22 (100%)	1.34 (1.02-4.99)	yes	0.002
LytM	22/22 (100%)	19/22 (86%)	1.35 (0.25-32.08)	no	0.641
Nuc	22/22 (100%)	21/21 (100%)	1.57 (1.0-9.48)	yes	0.020
Peptidoglycan	ND	19/21 (90%)	1.26 (0.3-4.53)	yes	0.005
PrsA	22/22 (100%)	13/13 (100%)	2.92 (1.32-34.21)	no	0.096
PSMa peptides 1-4	22/22 (100%)	CV>25%	CV>25%	ND	ND
SA0104	16/22 (73%)	CV>25%	CV>25%	ND	ND

Table 2. Overview of gene presence and associated IgG responses for bacterial proteins (continued)

Protein	No. of patients with gene pos isolates (%)	No. of patients with increase in IgG level (%)	Median fold increase from initial to peak level (range)	Significant difference[1]	p value[2]
SA0486	17/22 (77%)	7/9 (78%)	1.37 (0.46-3.43)	yes	0.030
SA0688	22/22 (100%)	19/20 (95%)	5.05 (0.74-56.96)	yes	<0.0001
SasG	12/22 (55%)	7/10 (70%)	1.17 (0.35-21.64)	no	0.331
SCIN	21/22 (95%)	20/21 (95%)	1.37 (0.92-16.58)	yes	0.0004
SdrD	18/22 (82%)	12/13 (92%)	1.32 (0.01-5.17)	yes	0.047
SdrE	15/22 (68%)	16/18 (89%)	1.69 (0.7-14.59)	no	0.572
SEA	3/22 (14%)	17/22 (77%)	1.21 (0.3-52.14)	no	0.343
SEB	5/22 (23%)	6/8 (75%)	1.13 (0.28-5.21)	no	0.218
SEC	1/22 (5%)	19/22 (86%)	1.14 (0.68-19.37)	no	0.430
SED	2/22 (9%)	19/22 (86%)	1.49 (0.56-8.54)	no	0.089
SEE	0/22 (0%)	16/21 (76%)	1.45 (0.51-7.29)	no	0.222
SEG	11/22 (50%)	16/22 (72%)	1.13 (0.11-5.07)	no	0.157
SEH	0/22 (0%)	14/22 (63%)	1.14 (0.08-3.8)	no	0.240
SEI	11/22 (50%)	CV>25%	CV>25%	ND	ND
SEJ	2/22 (9%)	CV>25%	CV>25%	ND	ND
SEM	8/22 (36%)	9/13 (69%)	1.34 (0.12-7.79)	no	0.075
SEN	10/22 (45%)	18/22 (82%)	1.41 (0.62-4.72)	no	0.060
SEO	11/22 (50%)	11/15 (73%)	1.08 (0.098-2.03)	no	0.084
SEQ	1/22 (5%)	6/11 (55%)	1.06 (0.03-1.42)	no	0.287
SER	2/22 (10%)	15/21 (71%)	1.48 (0.46-10.09)	no	0.309
SSL1	19/22 (86%)	18/22 (82%)	1.46 (0.7-11.21)	yes	0.001
SSL3	20/22 (91%)	21/22 (95%)	1.29 (0.9-3.87)	yes	0.003
SSL5	21/22 (95%)	20/22 (91%)	1.83 (0.65-8.43)	yes	<0.0001
SSL9	6/22 (27%)	18/22 (82%)	1.35 (0.77-10.15)	yes	0.003
SSL10	14/22 (64%)	19/20 (95%)	1.37 (0.84-4.83)	yes	0.030
SSL11	8/22 (36%)	20/22 (91%)	1.58 (0.67-8.67)	yes	0.01
TSST1	3/22 (14%)	17/22 (77%)	1.26 (0.27-10.69)	no	0.279
Wall teichoic acid	ND	18/20 (90%)	1.16 (0.29-6.26)	no	0.420
UspA1	ND	11/14 (79%)	1.21 (0.72-2.07)	ND	ND
PsaA	ND	10/12 (83%)	1.32 (0.7-2.07)	ND	ND
hMPV	ND	12/13 (92%)	1.11 (0.89-1.79)	ND	ND

[1]Comparison of mean IgG levels between 22 patients and 30 healthy controls, where significant differences are indicated by 'yes'.

[2]Comparison *P*-values ≤ 0.05 were considered statistically significant

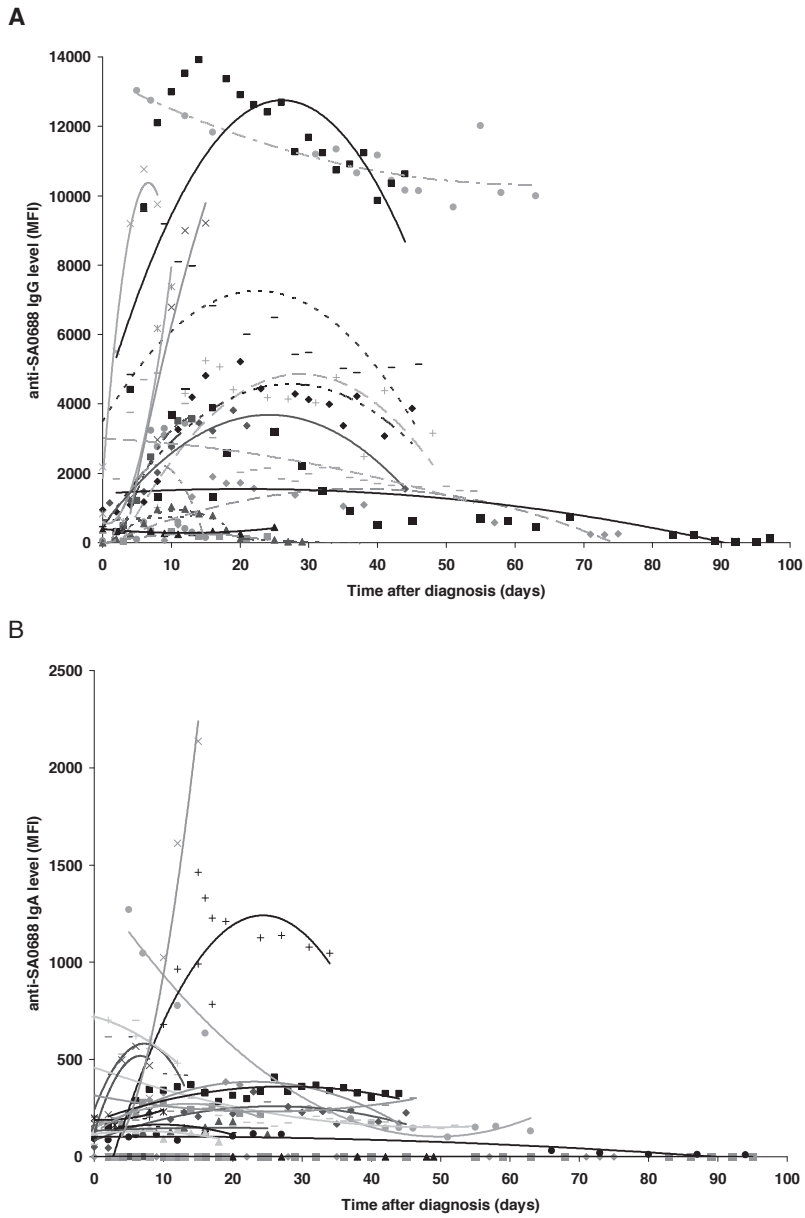

Figure 2. Antibody courses against the protein SA0688 in bacteremia patients.
A: Courses of anti-SA0688 IgG levels in 22 bacteremia patients. Each data point represents the average of duplicate Luminex measurements. Each patient is represented by a serial set of the same symbols and a polynomial trend lines is drawn through this set.
B: Course of anti-SA0688 IgA levels in the same 22 patients. Note that the MFI values on the Y-axis are a factor of 10 lower than in Figure 2A.

For 15 proteins, an increase in IgG levels was observed at some time point after the onset of bacteremia in 95 to 100% of all patients: glucosaminidase, HlgB, IsaA, IsdA, lipase, leukocidins D, E, S and F, nuclease, PrsA, SA0688, SCIN and SSL3 and 10 (Table 2). In contrast to these 15 proteins, only PrsA and Efb were associated with increased IgA levels in 95 to 100% of all patients (Table S1). However, due to the lower signal intensities and coefficients of variation larger than 25% between duplicate experiments, PrsA and Efb-associated IgA levels in the majority of patients were excluded from further analysis. No other proteins were associated with increased IgA and/or IgG levels in at least 95% of all patients.

When the increases in the IgG levels against each bacterial protein in individual patients were combined, the highest median fold increase from the initial serum sample to the peak IgG level was observed for SA0688 (5.05-fold increase; range, 0.74-56.96) followed by PrsA (2.92-fold increase; range, 1.32-34.21; Figure 3, Table 2). PrsA was also associated with the highest median fold increase in IgA levels (2.72-fold increase, range, 1.02-20.83), followed by SasG (2.51-fold increase; range, 0.63-13.02) and SA0688 (2.07-fold increase; range, 0.67-11.31) (Table S2). All other proteins showed median fold increases close to the overall median increase of 1.33 for IgG and 1.42 for IgA.

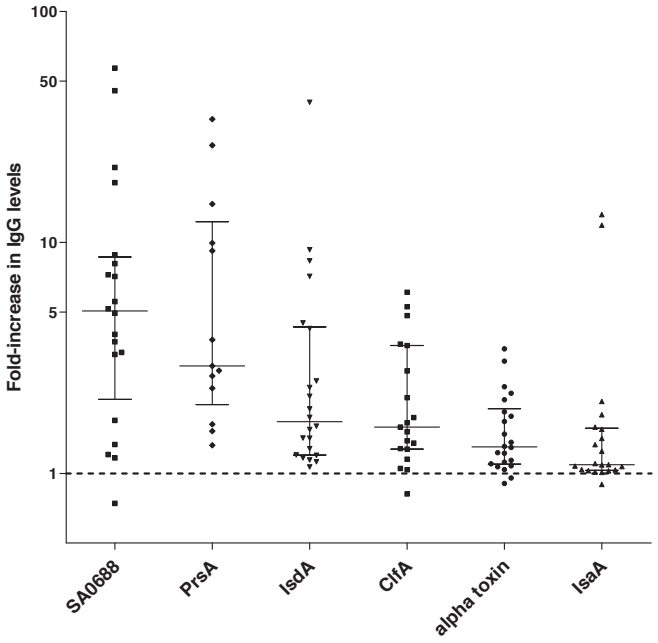

Figure 3. Initial-to-peak fold-increases in IgG levels for 6 bacterial proteins in bacteremia patients.
Each data point represents a single patient; the median fold-increase in IgG levels and interquartile range are indicated by lines. Patients for whom the duplicate Luminex measurements had a CV > 25% were excluded from the analysis. Note the log10 scale of the y-axis.

The median fold increases in the IgG levels for the non-staphylococcal control proteins UspA1, PsaA and hMPV were 1.21 (range 0.72-2.07), 1.32 (range 0.7-2.07) and 1.11 (range 0.89-1.79), respectively.

For 306 (35%) of the 882 observed increases in IgG levels, the corresponding gene was not present in the *S. aureus* isolate from the same patient as determined by PCR. Of these 306 'false-positive' increases, 270 (88%) were observed for known excreted proteins, mainly exfoliative toxins, enterotoxins and hemolysins. When all of the initial-to-peak fold-increases were classified as occurring in either the presence or absence of the corresponding genes in corresponding isolates, an overall median fold increase of 1.37 (range, 0.85-18.07) was observed in the presence of corresponding genes and 1.32 (range, 0.83-2.44) in the absence of corresponding genes.

Comparison of anti-staphylococcal antibodies in patients and controls

To investigate the significance of the increased IgG levels observed in bacteremia patients, the peak IgG levels of all 22 bacteremia patients were compared to the IgG levels of 30 non-infected, age-matched control patients. The IgG levels directed against 27 proteins were significantly higher in bacteremia patients than the non-infected controls (Table 2). The proteins associated with the most significant elevations in IgG levels in bacteremia patients compared to controls were SA0688 (Figure 4) and glucosaminidase ($P < 0.0001$), the immune modulators SSL5 ($P < 0.0001$), SSL1 ($P = 0.0007$) and SCIN ($P = 0.0004$), and the toxins gamma-hemolysin B ($P = 0.0007$) and leukocidin F ($P < 0.0001$).

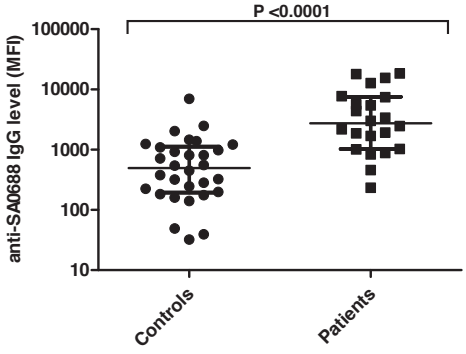

Figure 4. Comparison of anti-SA0688 IgG levels in bacteremia patients and non-infected controls.
Peak IgG levels of 22 bacteremia patients were compared to IgG levels of 30 non-infected controls. The median value and interquartile range are indicated by lines. Note the log10 scale of the y-axis.

In vitro expression of bacterial proteins in human blood

To gain further insight into which bacterial proteins are expressed in human blood and could be involved in the pathogenesis of bacteremia, microarray experiments were performed using the genetically distinct isolates from patients 1 and 4 (Figure 1) to measure the global changes in *S. aureus* mRNA expression during culture in human

blood. The mRNA expression levels of 3626 *S. aureus* genes were measured during log-phase growth in BHI broth and also after 0, 30, 60 and 90 minutes culture in human blood. Compared to the transcriptomes at the start of incubation in blood (0 minutes), only 86 out of the 3626 tested genes showed a two-fold or higher increase in mRNA expression in both isolates at all time points (30, 60 and 90 minutes) when incubated in blood (Table S2). A majority of these upregulated genes have an unknown/unclassified function, are involved in carbon metabolism or are excreted lipoproteins (Figure 5). The only known virulence factors for which mRNA expression was upregulated after culture in blood were the IgG-binding protein *sbi* and the gamma-hemolysin A and B precursors. Thirty genes showed a two-fold or more reduction in mRNA expression in both isolates at all time points, compared to the transcriptomes of both isolates at the start of incubation in blood (Table S2). These downregulated genes are also mainly involved in cellular metabolism or have an unknown function (Figure 5). Comparison of the transcriptomes at each individual time point (30, 60 and 90 minutes) with the transcriptomes of both isolates at the start of incubation in blood (0 minutes), revealed that a total of 360, 420 and 641 genes, respectively, were up- or downregulated two-fold or more. The functional distribution of the differentially expressed genes at each time point was similar to the functional distribution of the differentially expressed genes at all time points combined. In addition to the earlier mentioned upregulation of hemolysin precursors, a more than two-fold upregulation of *IsdA, -B, -C and –F, FnbpA* and *B* and *ClfA* was noted after 90 minutes incubation in blood; whereas only *IsdA, -B, -C, -D* and *FnbpA* were upregulated two-fold or higher after 60 minutes and only *IsdC* was upregulated at least two-fold after 30 minutes, compared to the transcriptomes of both isolates at the start of incubation in blood.

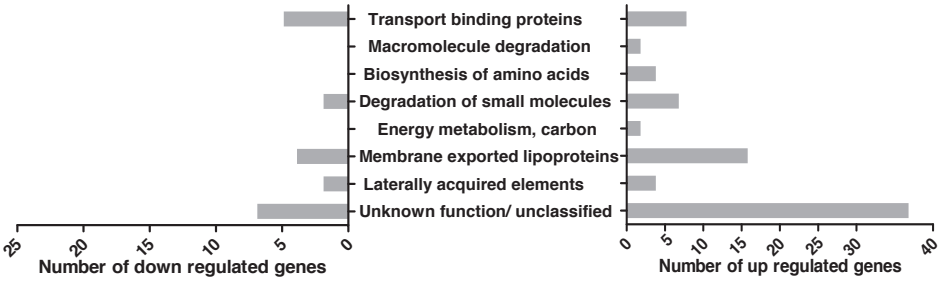

Figure 5. Functional distribution of genes with altered mRNA expression in human blood.
The functional classes are shown for which the largest number of genes showed an at least twofold increased or decreased mRNA expression at all time points in blood (30, 60 and 90 minutes) in both strains compared to trancriptomes at the start of incubation in blood (0 minutes).

Compared to log-phase growth in BHI broth, only 7 of the 3626 analyzed genes showed a two-fold or higher increase in mRNA expression in both strains at all time points (30, 60 and 90 minutes) when cultured in blood: dihydrolipoamide succinyltransferase, the sugar phospate antiporter *uhpT*, the murein hydrolase regulatory gene *IrgA* and the transcripts encoding the putative proteins SA0806, 0211, 0622 and 0761.

Of the 56 bacterial proteins for which the antibody responses were characterized in bacteremia patients, microarray data for 35 genes was available for at least two time points per isolate. In general, the mRNA expression levels of these 35 proteins, quantified as the RNA:DNA log ratios, correlated significantly between both isolates at all time points ($P \leq 0.001$), indicating similar expression levels for these specific 35 genes in both strains. The mRNA expression levels of four genes were consistently high in both isolates during log-phase growth in BHI broth and during all measured time points (0, 30, 60 and 90 minutes) of culture in blood: *SA0688, IsaA, EsxA* and *SCIN* (Table S3). In addition, the mRNA expression level of *PrsA* was high at all time points, except for the 90 minutes time point in one isolate. Compared to the expression level during log-phase growth in BHI broth, none of these 35 genes displayed a two-fold or higher mRNA expression level at any time point when cultured in blood.

DISCUSSION

In this study we investigated the humoral immune response against 56 staphylococcal proteins in bacteremia patients. Firstly, we further demonstrate considerable variation in the IgA and IgG levels of all patients at the time of diagnosis; the time taken to reach peak antibody levels for each protein in each patient was also heterogeneous. Even after extensive visual comparison of specific antibody responses to diverse proteins we could not observe any distinct patterns or profiles between patients. Both patients and proteins were rather all associated with unique combinations of extensively varying antibody responses. These heterogeneous, highly individual antibody responses are in line with previous data [18, 19, 21, 32] and will likely be the result of an individually unique interplay between patients and genetically diverse *S. aureus* strains. Indeed, parallel to the diversity of patient antibody responses we further confirmed the presence of a large genetic diversity amongst infecting strains (figure 1).

The increases observed in the IgG levels of bacteremia patients were generally many-fold higher than the increases in the IgA levels. This may be explained either by hypothesizing that IgA production is not induced by hematogenic bacterial challenge to the same extent as IgG production, or that IgA levels may not alter considerably in blood but may increase more locally on mucosal surfaces. In any case, the relatively low

IgA responses prompted us to focus attention on the more dynamic IgG responses in bacteremia patients.

The IgG levels against fifteen bacterial proteins, including well-described virulence factors such as IsdA and gamma-hemolysin B, were found to increase in at least 95% of the bacteremia patients. Additionally, the peak IgG levels against these 15 proteins were significantly higher in bacteremia patients than age-matched, non-infected patients. The putative ABC transporter SA0688 and the membrane-associated foldase PrsA were associated with the highest median fold increase in IgG levels (5.05 and 2.92-fold, respectively). Although other proteins were also associated with significantly increased IgG levels in individual patients, these data indicate that SA0688 and PrsA appear to be among the most broadly expressed and immunologically recognized proteins. This observation is in line with previous studies which demonstrated the immunogenicity of SA0688 in human serum [18, 20]. In addition, the protein SA0688 showed promising results as part of a multivalent vaccine in an animal model of osteomyelitis [25]. Unfortunately, at current nothing is known about the exact function of SA0688 and how antibodies could interfere in staphylococcal infection through this protein. Moreover, in general other proteins than SA0688 or PrsA which were not associated with significantly increased antibody levels in this study may also provide interesting targets for a vaccine, although we can only speculate about these proteins based on our data and previous studies.

In addition to the question which proteins should ideally be selected for a vaccine component, we can only speculate about whether or not the associated antibody responses will be protective against infection. We observed clearly detectable, pre-existent IgG levels against all proteins in patients at the time of diagnosis, which is in line with previous observations of stable, pre-existent IgG levels in both bacteremia patients and healthy controls [21, 27, 32]. These observations suggest that all individuals have an immunological memory specifically against *S. aureus*, possibly due to earlier, subclinical infections. It remains a question whether a further increase in these pre-existent IgG levels will have an additional protective effect against infection, even though this increase is significant for diverse proteins such as SA0688 and PrsA compared to non-infected controls. In any case, the significant increases in IgG levels for diverse proteins suggests that the corresponding proteins are being expressed *in vivo* in patients, which will be a pre-requisite for any potential vaccine target.

To gain further insight into which bacterial proteins are expressed in human blood, the global changes in the mRNA expression levels of two genetically distinct *S. aureus* isolates during incubation in human blood were investigated. In general, of the 3626 genes investigated, we could only associate limited numbers of genes with significantly altered mRNA expression levels specifically during incubation in blood, compared to the transcriptomes of each isolate at the start of incubation in blood or BHI broth. As noted

for 35 of the 56 proteins investigated in this study, most of the corresponding genes had a relatively constant RNA:DNA log ratio at all time points during culture in blood (Table S3). Most notably, the proteins *SA0688, IsaA, EsxA, SCIN* and, with the exception of one measurement, *PrsA* were highly expressed in both isolates in BHI broth and blood over time. This stable expression of genes by genetically distinct isolates in human blood or tissue would be a first prerequisite for any protein to be a potential vaccine component.

Most of the genes which were up- or downregulated in *S. aureus* specifically during incubation in blood belong to functional classes involved in cellular metabolism or have an unknown function. Exceptions to this were the IgG-binding protein *sbi* and gamma-hemolysin component A precursor, which were upregulated in both isolates at all time points (30, 60 and 90 minutes) in blood compared to the start (0 minutes). In addition, other genes were upregulated at specific time points, mainly surface proteins such as *FnbpA, ClfA* and the diverse iron-regulated surface determinant (*Isd*) proteins. These findings are in agreement with a previous study which reported that a limited number of *S. aureus* genes encoding known virulence factors were specifically upregulated in blood [33]. In this study, mRNA expression of the gamma-hemolysin subunits were found to be most significantly upregulated during incubation in blood.

Although our study demonstrates the *in vitro* expression and *in vivo* immunogenicity of several proteins, there are several limitations in regard to the used techniques. Firstly, in regard to the bead-based flow cytometry assay, we observed significant increases in the antibody levels against leukocidins S and F, for which corresponding genes were present in only one isolate. The increases in specific IgG for these two leukocidin components may be the result of immunological cross-reactivity, where antibodies specific to one toxin component may cross-react with structurally similar components [34-36]. We confirmed the presence of immunological cross-reactivity between the leukocidins F and D and gamma-hemolysin B in our assay (Figure S1). No cross-reactivity was observed between the enterotoxins in our assay (data not shown). Secondly, we used recombinant staphylococcal proteins in our assay, which may lack certain naturally-occurring antibody-binding epitopes or may not have been optimally coupled to our assay beads, thereby possibly missing increases in the levels of specific antibodies. This could provide an alternative explanation for the low signal intensities observed for EsxA, PSM alpha 1-4 peptides, SA0104, SEI and SEJ.

In regard to the analysis of the serially measured IgG levels, we observed a median fold-increase of 1.32 in IgG levels while the corresponding genes were not present in the isolate of the same patient. This median 'false-positive' increase of 1.32 was comparable to that of all PCR confirmed positive increases (1.37) and the overall median fold-increase of 1.33. Furthermore, these increases were also comparable to those of the non-Staphylococcal control proteins UspA1, PsaA and hMPV (1.21, 1.32 and 1.11 fold-increase, respectively). This apparent background signal could on one hand be explained

by a broad, non-specific rise in antibody levels during infection or, alternatively, by the phenomenon that maximum values always tend to be higher than single measurements over an extended time period. Indeed, although IgG levels tend to remain constant over time in both healthy persons [27] and up to three years after infection (unpublished data), small variations in measured IgG levels are consequently observed. This could explain the observed non-specific rise in antibody levels.

In regard to the micro array data, one limitation is that we only investigated protein expression in two out of 21 strains. Investigating protein expression in more genetically diverse strains would allow for more robust conclusions about the expression of specific proteins, however due to practical reasons we were unable to do this. Secondly, as with other *in vitro* models the question remains how well our blood infection model reflects the *in vivo* situation during a bacteremia. Especially the high dose of infecting bacteria is likely to not reflect the real *in vivo* situation and this could influence bacterial mRNA expression, however the adaptation to blood should still be associated with global changes in mRNA expression. Finally, considering the expression of the proteins for which we characterized antibody responses, it should be noted that any direct correlation of *in vitro* bacterial gene expression with the *in vivo* immune response in patients should be interpreted with caution. The mode and phase of bacterial growth *in vivo* may be different and more diverse than the pattern of growth *in vitro*. In addition, the expression of a bacterial protein does not necessarily induce an antibody response *in vivo*, either due to immune modulation by the bacterium or the complex regulatory immune processes within the host. Nonetheless, data on both bacterial gene expression *in vitro* and the *in vivo* immune response can yield valuable insight into the pathogenesis of infection and complement each other for the identification of potential vaccine targets.

To summarize, our study suggests that most of the staphylococcal proteins tested, including many known virulence factors, do not lead to uniform increases in the antibody levels in bacteremia patients. In addition, the expression of these proteins by *S. aureus* is not significantly altered by incubation in human blood over time or compared to standard growth medium. One immunogenic protein is the putative iron-regulated ABC transporter SA0688, which induced a significant antibody response in all bacteremia patients and was stably expressed by genetically distinct isolates under different culture conditions. The ubiquitous expression of this protein will be a prerequisite for any potential vaccine target and our data, together with the previous literature, suggest that SA0688 could be a potential vaccine target to interfere with the growth or virulence of all *S. aureus* cells.

ACKNOWLEDGEMENTS

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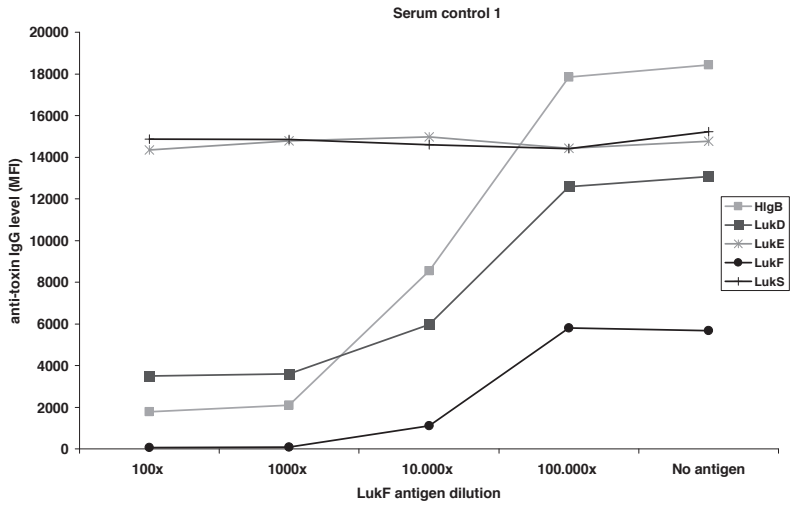
We would also like to thank X. Huijsdens, M. Heck and G. Pluister from the National Institute for Public Health and the Environment (RIVM) for their help with the *spa*-typing of several of our clinical isolates.

SUPPLEMENTAL DATA

A



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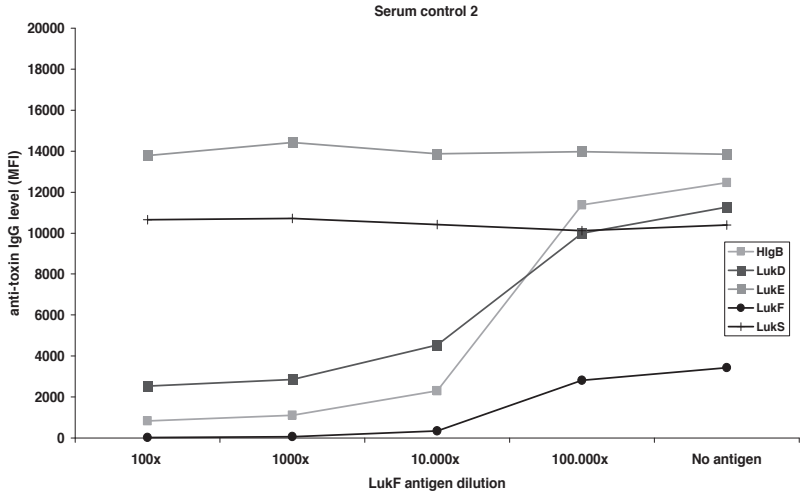

Figure S1. Cross-reactivity between leukocidins F and D and hemolysin gamma-B in human serum.
A: Serial dilutions of recombinant leukocidin F (LukF) were pre-incubated with the serum from a non-infected control with high IgG levels against LukF. After incubation, the remaining IgG levels specific against Leukocidins D, E, F and S and Hemolysin gamma-B were measured. Note the loss in IgG levels specific for LukD and HlgB at lower dilutions of LukF, suggesting immunological cross-talk between these toxin components.
B: The same experiment as for Figure S1A, now with serum from a different non-infected control.

Table S1. Overview of gene presence and associated IgA responses induced by bacterial proteins.

Antigen	No. of patients with gene positive isolates (%)[1]	No of patients with increase in IgA level (%)[2]	Median fold increase from initial to peak level (range
Alpha toxin	22/22 (100%)	17/22 (77%)	1.45 (0.59-4.34)
CHIPS	14/22 (64%)	17/21 (81%)	1.13 (0.52-4.67)
ClfA	22/22 (100%)	19/22 (86%)	1.57 (0.37-19.18)
ClfB	22/22 (100%)	16/20 (80%)	1.31 (0.22-17.9)
Efb	21/22 (96%)	7/7 (100%)	1.29 (1.08-8.32)
EsxA	22/22 (100%)	CV>25%	ND
EsxB	14/22 (64%)	CV>25%	ND
ETA	1/22 (5%)	12/16 (75%)	1.35 (0.41-5.33)
ETB	0/22 (0%)	18/22 (82%)	1.39 (0.2-17.25)
FlipR	16/22 (73%)	13/15 (87%)	1.55 (0.5-78.8)
FnbpA	20/22 (91%)	13/16 (81%)	1.36 (0.25-19.11)
FnbpB	7/22 (32%)	12/16 (75%)	1.42 (0.16-6.24)
Glucosaminidase	22/22 (100%)	18/20 (90%)	1.69 (0.63-5.48)
HlgB	17/22 (77%)	16/22 (73%)	1.25 (0.46-4.63)
IsaA	22/22 (100%)	19/21 (91%)	1.43 (0.57-36.52)
IsdA	22/22 (100%)	19/22 (86%)	1.89 (0.61-10.1)
IsdH	21/22 (95%)	18/20 (90%)	1.98 (0.24-25.4)
Lipase	22/22 (100%)	17/21 (81%)	1.56 (0.27-17.79)
LukD	16/22 (72%)	18/22 (82%)	1.34 (0.67-10.32)
LukE	16/22 (72%)	19/22 (86%)	1.36 (0.76-16.65)
LukF	1/22 (5%)	18/22 (82%)	1.32 (0.15-5.16)
LukS	1/22 (5%)	17/21 (81%)	1.33 (0.63-36.67)
LytM	22/22 (100%)	15/21 (71%)	1.36 (0.17-19.11)
Nuc	22/22 (100%)	14/16 (88%)	1.57 (0.11-16.63)
Peptidoglycan	ND	6/8 (75%)	1.29 (0.66-3.61)
PrsA	22/22 (100%)	6/6 (100%)	2.715 (1.02-20.83)
PSMa peptides 1-4	ND	CV>25%	ND
SA0104	16/22 (73%)	CV>25%	ND
SA0486	17/22 (77%)	11/13 (85%)	1.4 (0.4-23.38)
SA0688	22/22 (100%)	13/14 (93%)	2.07 (0.67-11.31)
SasG	12/22 (55%)	14/15 (93%)	2.51 (0.63-13.02)
SCIN	21/22 (95%)	20/22 (91%)	1.42 (0.85-16.78)
SdrD	18/22 (82%)	16/17 (94%)	1.85 (0.14-7.07)
SdrE	15/22 (68%)	14/15 (93%)	1.46 (0.51-7.03)
SEA	3/22 (14%)	19/21 (91%)	1.58 (0.46-8.69)
SEB	5/22 (23%)	13/17 (77%)	1.32 (0.19-11.95)
SEC	1/22 (5%)	18/22 (82%)	1.28 (0.53-13.82)
SED	2/22 (9%)	18/20 (90%)	1.44 (0.45-3.87)

Table S1. Overview of gene presence and associated IgA responses induced by bacterial proteins. (continued)

Antigen	No. of patients with gene positive isolates (%)[1]	No of patients with increase in IgA level (%)[2]	Median fold increase from initial to peak level (range
SEE	0/22 (0%)	12/16 (75%)	1.31 (0.16-24.28)
SEG	11/22 (50%)	18/21 (86%)	1.4 (0.12-3.34)
SEH	0/22 (0%)	15/18 (83%)	1.58 (0.54-10.04)
SEI	11/22 (50%)	CV>25%	ND
SEJ	2/22 (9%)	CV>25%	ND
SEM	8/22 (36%)	16/18 (89%)	1.45 (0.24-7.96)
SEN	10/22 (45%)	16/19 (84%)	1.49 (0.23-10.09)
SEO	11/22 (50%)	CV>25%	ND
SEQ	1/22 (5%)	8/12 (67%)	1.32 (0.43-10.75)
SER	2/22 (10%)	14/18 (78%)	1.41 (0.41-8.32)
SSL1	19/22 (86%)	17/20 (85%)	1.44 (0.63-5.38)
SSL3	20/22 (91%)	16/22 (73%)	1.39 (0.28-4.96)
SSL5	21/22 (95%)	18/22 (82%)	1.44 (0.59-21.96)
SSL9	6/22 (27%)	17/21 (81%)	1.51 (0.21-10.57)
SSL10	14/22 (64%)	18/22 (82%)	1.33 (0.53-6.29)
SSL11	8/22 (36%)	15/20 (75%)	1.53 (0.31-29.5)
TSST1	3/22 (14%)	17/22 (77%)	1.5 (0.58-8.97)
Wall teichoic acid	ND	18/22 (82%)	1.17 (4.67-0.52)

[1]Presence of genes in 22 isolates

[2]Initial-to-peak fold-increases in IgA levels in 22 bacteremia patients.

CV>25%: Patients for whom the duplicate measurements of the IgA levels had a CV larger than 25% were excluded from the analysis. IgA levels for the antigens EsxA, EsxB, PSM alpha 1-4 peptides, SA0104, SEI, SEJ and SEO were completely excluded because of very low signal intensities with coefficients of variation larger than 25% for a majority of patients.

ND: not determined.

Table S2. List of genes with altered mRNA expression in human blood.

At least twofold increase in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
SAR1524	6,726 (5,471 to 7,471)	0,01 (0,01 to 0,0917)*	5,525	36,02	0,86
SACOL0045	1,699 (0,27 to 4,822)	0,0674	2,298	0,398	200.012
SA0743	4,05 (0,57 to 9,145)	0,428 (0,253 to 0,602)	12,26 (1,808 to 83,14)	6,865	0,896
SA1635	0,509 (0,192 to 2,403)	0,0365 (0,01 to 0,133)	0,655	0,278 (0,157 to 0,559)	0,706 (0,12 to 2,03)
SAR1962	0,634 (0,356 to 1,13)	0,118	1,147 (1,092 to 1,205)	0,998	0,491 (0,406 to 0,594)
hlgA	0,59 (0,178 to 56,8)	0,273 (0,01 to 5,849)	2,437 (0,272 to 12,41)	4,118 (1,388 to 12,33)	3,923 (1,463 to 10,68)
hlgB	0,396 (0,102 to 15,73)	0,166 (0,0105 to 2,615)	1,165 (0,158 to 7,127)	1,893 (0,601 to 6,751)	2,824 (0,99 to 10,25)
SAOUHSC_02294	2,406 (1,416 to 3,905)	1,096	7,05 (2,847 to 17,72)	89,51	3,34 (1,398 to 7,979)
SAR1523	1,637 (0,563 to 11,93)	1,144 (0,377 to 2,406)	7,089 (6,25 to 8,041)	5,18 (3,348 to 8,014)	0,332
uhpT	0,39 (0,156 to 0,793)	0,362 (0,0172 to 2,759)	2,186 (0,318 to 6,009)	4,085 (1,244 to 93,81)	2,366 (1,186 to 5,309)
hlgC	0,379 (0,138 to 14,55)	0,179 (0,01 to 2,539)	1,064 (0,179 to 3,859)	1,81 (0,541 to 6,552)	2,379 (0,765 to 9,058)
SAR0295	0,842 (0,712 to 0,969)	0,0735 (0,01 to 0,722)	0,403 (0,234 to 0,508)	1,198 (0,139 to 19,2)	0,489 (0,454 to 0,524)
SAOUHSC_00745	1,318 (0,414 to 4,042)	0,134 (0,01 to 0,64)	0,714 (0,331 to 1,451)	0,627 (0,282 to 0,798)	2,882 (0,224 to 23,51)
SAR1126	0,837 (0,429 to 1,86)	0,343 (0,01 to 1,605)	1,776 (0,919 to 9,079)	1,433 (1,429 to 1,436)	0,822 (0,73 to 0,926)
SACOL0642	0,539 (0,201 to 1,237)	0,0816 (0,0384 to 0,166)	0,411 (0,394 to 0,428)	0,446 (0,329 to 0,801)	0,921 (0,443 to 1,398)
MW0372	1,018 (0,243 to 29,77)	0,129 (0,01 to 0,62)	0,637 (0,282 to 3,156)	0,499 (0,352 to 0,87)	0,438 (0,01 to 1,001)
8325B-0490	0,433 (0,148 to 1,12)	0,101 (0,017 to 0,924)	0,484 (0,236 to 1,016)	0,439 (0,29 to 0,84)	0,861 (0,485 to 1,237)
SAR2634	1,432 (0,566 to 7,618)	0,787 (0,0275 to 3,978)	3,244 (0,716 to 9,414)	3,267 (1,39 to 5,291)	5,176 (1,62 to 23,56)
MW0393v2	1,551 (0,394 to 103,4)	0,176 (0,01 to 0,797)	0,725 (0,272 to 2,018)	0,653 (0,359 to 1,133)	0,603 (0,468 to 0,82)
MW0369	0,767 (0,183 to 1,913)	0,213 (0,01 to 0,783)	0,776 (0,47 to 0,918)	0,933 (0,67 to 1,448)	1,685 (1,579 to 1,791)
gap2	0,924 (0,476 to 1,934)	1,157 (0,216 to 9,865)	4,188 (0,62 to 11,79)	4,418 (0,984 to 10,7)	121,4 (1,15 to 203.411)
SAR0222	0,693 (0,196 to 56,44)	0,24 (0,0139 to 1,378)	0,856 (0,393 to 3,059)	0,852 (0,203 to 2,582)	0,971 (0,409 to 2,974)

Table S2. List of genes with altered mRNA expression in human blood. (continued)

At least twofold increase in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
repA	1,481 (0,21 to 4,744)	0,16	0,562 (0,377 to 0,908)	0,434 (0,142 to 1,329)	0,769 (0,0802 to 2,562)
SAR2512	0,258 (0,0592 to 17,01)	0,0959 (0,01 to 0,773)	0,33 (0,0972 to 1,289)	0,243 (0,01 to 0,993)	0,524 (0,143 to 1,11)
SA2011	3,869 (0,422 to 36,76)	0,938	3,174 (2,264 to 4,448)	9,72	0,381
SAR1338	1,233 (0,416 to 52,45)	0,414 (0,01 to 1,229)	1,306 (0,525 to 2,676)	1,603 (0,968 to 2,9)	2,1 (0,798 to 5,335)
SAR0210	0,51 (0,259 to 0,878)	0,378 (0,0818 to 0,815)	1,167 (0,193 to 3,149)	1,779 (0,788 to 3,542)	1,537 (0,852 to 4,786)
SAR0169	0,742 (0,24 to 1,544)	0,877 (0,154 to 5,945)	2,659 (0,215 to 7,705)	2,388 (0,9 to 6,896)	2,357 (0,993 to 5,555)
SAR1526	12,15 (2,651 to 53,26)	0,569 (0,467 to 0,671)	1,614 (1,316 to 1,794)	13,78 (3,05 to 62,29)	1,797
SA0357	0,294 (0,107 to 0,753)	0,129 (0,01 to 0,37)	0,363 (0,199 to 0,472)	0,363 (0,119 to 0,667)	0,313 (0,201 to 0,746)
SAR0311	0,162 (0,108 to 0,245)	0,179 (0,0321 to 0,668)	0,498 (0,121 to 6,489)	0,57 (0,328 to 1,596)	0,657 (0,459 to 1,1)
SAR1849	1,27 (0,455 to 2,613)	0,98 (0,01 to 2,974)	2,706 (0,649 to 8,772)	3,273 (1,605 to 8,594)	2,803 (0,958 to 7,525)
lrgB	0,764 (0,0584 to 4,818)	1,276 (0,538 to 4,067)	3,454 (0,646 to 18,86)	4,489 (1,48 to 8,296)	4,906 (3,815 to 7,804)
SAR0761	1,448 (0,504 to 5,323)	2,165 (0,772 to 8,913)	5,776 (1,083 to 12,05)	8,367 (2,418 to 29,99)	7,599 (3,119 to 32,95)
SAR0918	1,135 (0,433 to 3,061)	0,524 (0,01 to 1,668)	1,383 (0,692 to 4,595)	1,281 (0,878 to 1,778)	1,323 (0,7 to 2,364)
SAR0211	0,403 (0,167 to 0,693)	0,382 (0,145 to 0,869)	0,981 (0,229 to 3,496)	1,77 (0,859 to 2,818)	1,465 (0,791 to 2,769)
SAR0208	0,411 (0,235 to 0,722)	0,361 (0,175 to 0,597)	0,922 (0,141 to 2,544)	1,482 (0,748 to 3,076)	1,129 (0,525 to 2,148)
oppD	0,353 (0,136 to 0,583)	0,253 (0,0445 to 0,653)	0,64 (0,151 to 1,934)	0,752 (0,338 to 2,597)	0,951 (0,535 to 1,877)
SAR2668	0,649 (0,281 to 5,646)	0,317 (0,0268 to 0,754)	0,799 (0,354 to 1,489)	1,222 (0,844 to 2,522)	1,356 (0,891 to 3,066)
SAR0719	0,547 (0,2 to 10,93)	0,242 (0,01 to 0,662)	0,608 (0,302 to 1,571)	0,517 (0,27 to 0,924)	0,731 (0,42 to 1,824)
hutI	0,64 (0,226 to 40,76)	0,152 (0,01 to 0,461)	0,378 (0,144 to 0,919)	0,626 (0,313 to 1,047)	0,725 (0,462 to 0,975)
lrgA	0,86 (0,244 to 3,329)	1,309 (0,348 to 3,674)	3,207 (0,35 to 14,07)	4,861 (1,275 to 11,51)	3,413 (1,389 to 7,175)
SAR0414a	1,113 (0,405 to 3,114)	0,219 (0,0655 to 0,728)	0,533 (0,451 to 0,581)	0,587 (0,109 to 1,064)	0,634 (0,453 to 0,746)
SAR0996	1,523 (0,291 to 4,635)	2,564 (0,646 to 6,639)	6,165 (0,869 to 19,6)	10,9 (1,792 to 75,96)	7,25 (2,794 to 19,12)

Table S2. List of genes with altered mRNA expression in human blood. (continued)

At least twofold increase in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
SAR1143	0,802 (0,27 to 49,25)	0,305 (0,01 to 2,07)	0,732 (0,168 to 2,308)	1,346 (0,55 to 4,128)	2,437 (1,119 to 5,195)
SAR2414	0,815 (0,244 to 58,86)	0,168 (0,01 to 0,499)	0,4 (0,223 to 0,833)	0,353 (0,21 to 1,094)	0,43 (0,166 to 0,761)
COLB3543	0,859 (0,188 to 90,35)	0,113 (0,01 to 0,298)	0,268 (0,109 to 1,316)	0,357 (0,131 to 0,808)	0,585 (0,432 to 0,856)
SAR0206	0,459 (0,265 to 0,905)	0,374 (0,184 to 0,785)	0,883 (0,142 to 4,506)	0,846 (0,495 to 1,238)	0,805 (0,453 to 1,613)
fadA	0,444 (0,125 to 30,61)	0,108 (0,01 to 0,433)	0,253 (0,0975 to 0,739)	0,292 (0,137 to 0,5)	0,426 (0,117 to 0,606)
SAP008	1,226 (0,425 to 40)	0,313 (0,01 to 1,217)	0,734 (0,504 to 1,128)	1,007 (0,528 to 2,324)	0,744 (0,587 to 1,014)
hlb	0,506 (0,233 to 0,83)	0,495 (0,178 to 3,262)	1,161 (0,364 to 3,347)	1,699 (0,722 to 3,853)	1,941 (1,058 to 3,578)
SAR0308	0,195 (0,0911 to 0,502)	0,158 (0,0384 to 1,41)	0,369 (0,13 to 1,987)	0,487 (0,197 to 2,126)	1,134 (0,363 to 3,512)
rocD	1,109 (0,517 to 2,064)	1,141 (0,589 to 2,577)	2,65 (0,822 to 8,286)	2,619 (1,38 to 5,197)	2,884 (1,104 to 7,396)
fadX	0,295 (0,0959 to 14,02)	0,096 (0,01 to 0,372)	0,223 (0,0709 to 0,705)	0,264 (0,0803 to 1,035)	0,453 (0,241 to 0,737)
hutU	0,43 (0,155 to 9,417)	0,133 (0,01 to 0,501)	0,303 (0,0898 to 0,873)	0,461 (0,257 to 0,734)	0,692 (0,351 to 1,413)
SAOUHSC_02990	1,945 (0,107 to 28.150)	0,0655 (0,01 to 0,255)	0,149 (0,0791 to 0,195)	0,287 (0,121 to 0,506)	0,208 (0,13 to 0,377)
SAR2641	2,105 (0,242 to 60.139)	0,235 (0,01 to 0,702)	0,532 (0,239 to 1,033)	0,622 (0,361 to 1,075)	0,713 (0,467 to 1,433)
SAR2616	0,747 (0,158 to 1,37)	0,448 (0,01 to 1,129)	1,011 (0,653 to 1,372)	1,192 (0,721 to 2,025)	1,541 (1,227 to 2,314)
SAR0119	0,188 (0,0847 to 0,556)	0,162 (0,01 to 1,429)	0,366 (0,127 to 0,755)	0,479 (0,213 to 1,468)	1,372 (0,622 to 3,992)
SAR1987	1,274 (0,662 to 1,879)	0,41	0,921 (0,561 to 1,557)	0,831 (0,759 to 0,902)	0,924 (0,875 to 0,975)
fadD	0,488 (0,103 to 37,96)	0,0799 (0,01 to 0,389)	0,18 (0,0534 to 0,517)	0,234 (0,114 to 0,645)	0,394 (0,272 to 0,497)
SAR0217	5,957 (0,01 to 28,42)	6,679 (0,231 to 28,36)	14,83 (6,321 to 26,72)	24,25 (14,73 to 55,8)	20,73 (10,55 to 53,49)
asd	0,406 (0,0974 to 8,158)	0,164 (0,01 to 0,872)	0,365 (0,153 to 0,738)	0,455 (0,125 to 3,409)	0,514 (0,212 to 0,908)
SAR2513	0,429 (0,0925 to 136,1)	0,112 (0,01 to 0,595)	0,248 (0,049 to 0,975)	0,287 (0,155 to 0,651)	0,422 (0,193 to 1,197)
pyrC	1,288 (0,285 to 3,188)	1,222 (0,52 to 2,741)	2,703 (0,995 to 7,421)	2,83 (1,056 to 6,317)	2,894 (2,517 to 3,867)
thrC	0,982 (0,453 to 22,15)	0,366 (0,01 to 1,075)	0,808 (0,283 to 1,432)	0,966 (0,495 to 1,842)	1,241 (0,679 to 2,936)

Table S2. List of genes with altered mRNA expression in human blood. (continued)

At least twofold increase in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
SAR2580v	1,085 (0,18 to 3,81)	0,551 (0,01 to 3,511)	1,214 (0,389 to 2,381)	1,447 (0,325 to 6,238)	1,577 (0,452 to 4,953)
SAR0660	0,77 (0,347 to 1,615)	0,832 (0,412 to 1,806)	1,822 (0,601 to 2,98)	1,831 (0,91 to 3,766)	1,71 (1,162 to 2,541)
SAR0312	0,372 (0,149 to 7,184)	0,172 (0,0156 to 0,727)	0,376 (0,175 to 1,263)	0,57 (0,287 to 0,846)	0,728 (0,458 to 1,57)
fmtB	0,439 (0,0946 to 22,45)	0,152 (0,01 to 0,76)	0,333 (0,134 to 0,667)	0,445 (0,135 to 1,265)	0,632 (0,449 to 0,995)
SAR0129	1,306 (0,393 to 3,207)	1,902 (0,343 to 5,725)	4,135 (0,615 to 7,559)	6,704 (2,696 to 32,19)	5,406 (2,419 to 8,16)
SAR2104	0,658 (0,317 to 3,064)	0,475 (0,106 to 0,772)	1,03 (0,495 to 3,354)	1,536 (0,748 to 3,379)	1,051 (0,828 to 1,402)
SACOL0323	0,917 (0,144 to 13,06)	0,162 (0,01 to 0,388)	0,351 (0,147 to 0,811)	0,342 (0,156 to 0,518)	0,494 (0,459 to 0,579)
bioA	0,246 (0,0708 to 8,204)	0,0711 (0,01 to 0,233)	0,152 (0,0518 to 0,708)	0,151 (0,01 to 0,337)	0,249 (0,0679 to 0,465)
COLB0874	0,779 (0,15 to 34,05)	0,107 (0,01 to 0,274)	0,227 (0,121 to 0,389)	0,361 (0,01 to 0,753)	0,431 (0,207 to 0,992)
SAR0209	0,591 (0,364 to 1,164)	0,619 (0,182 to 1,616)	1,319 (0,195 to 5,91)	1,883 (0,671 to 3,155)	1,453 (0,605 to 2,572)
thrB	1,181 (0,418 to 70,58)	0,394 (0,01 to 1,066)	0,835 (0,421 to 1,436)	0,827 (0,49 to 1,563)	0,978 (0,657 to 1,432)
sbi	2,559 (0,804 to 159,6)	2,029 (0,963 to 6,119)	4,296 (2,15 to 7,031)	6,016 (3,985 to 14,87)	4,922 (2,199 to 14,45)
SAR0316	0,427 (0,134 to 5,212)	0,124 (0,01 to 0,39)	0,262 (0,105 to 0,764)	0,268 (0,107 to 0,69)	0,423 (0,01 to 0,872)
SAR0200	0,229 (0,0932 to 0,457)	0,124 (0,01 to 0,617)	0,261 (0,161 to 0,496)	0,401 (0,133 to 1,094)	1,151 (0,968 to 1,334)
sirA	0,664 (0,249 to 2,662)	1,189 (0,297 to 4,779)	2,489 (0,371 to 5,396)	3,179 (0,823 to 8,7)	5,136 (1,91 to 11,77)
SA1789	0,668 (0,134 to 118,8)	0,0624 (0,01 to 0,109)	0,13 (0,0735 to 0,21)	0,236 (0,01 to 9,812)	0,481 (0,423 to 0,654)
SAR0207	0,393 (0,2 to 0,649)	0,355 (0,128 to 0,81)	0,738 (0,157 to 2,501)	1,193 (0,558 to 2,001)	1,001 (0,547 to 1,81)
isdC	0,26 (0,0921 to 0,53)	0,34 (0,0888 to 3,518)	0,691 (0,174 to 2,296)	1,041 (0,428 to 3,383)	2,798 (1,519 to 6,09)
oppF	0,244 (0,108 to 0,517)	0,274 (0,0997 to 0,753)	0,556 (0,199 to 1,454)	0,739 (0,365 to 1,953)	0,944 (0,483 to 2,111)
SAR0760	1,425 (0,536 to 3,851)	1,531 (0,751 to 3,011)	3,08 (1,527 to 5,218)	3,132 (1,634 to 7,887)	3,118 (1,105 to 11,93)

Table S2. List of genes with altered mRNA expression in human blood. (continued)

At least twofold increase in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
At least twofold decrease in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
fadB	0,378 (0,127 to 3,842)	0,0954 (0,01 to 0,545)	0,192 (0,0307 to 0,64)	0,449 (0,206 to 2,577)	0,44 (0,192 to 0,946)
sdrC	2,594 (0,246 to 8,085)	8,388 (6,535 to 10,89)	4,128 (2,567 to 6,087)	3,211 (2,223 to 5,509)	2,001 (1,699 to 2,431)
SAR0972	2,598 (2,567 to 2,629)	3,57	1,7 (0,98 to 3,086)	1,087 (1,047 to 1,126)	1,023
SAR2127	0,86 (0,405 to 1,744)	1,75 (0,421 to 300,1)	0,777 (0,516 to 1,492)	0,874 (0,456 to 2,121)	0,741 (0,201 to 1,249)
cspB	2,904 (0,98 to 5,615)	4,732 (1,9 to 10,09)	2,034 (1,057 to 6,669)	2,071 (1,326 to 2,674)	1,914 (0,858 to 2,694)
uvrC	2,283 (1,939 to 2,626)	1,768 (1,526 to 2,011)	0,722 (0,612 to 0,879)	0,862 (0,558 to 1,662)	0,87 (0,574 to 1,461)
SAR0458	0,841 (0,433 to 2,085)	1,426 (0,919 to 2,157)	0,555 (0,265 to 1,095)	0,522 (0,234 to 1,096)	0,395 (0,257 to 0,767)
SAR1008	4,736 (1,673 to 12,24)	2,597 (2,525 to 2,668)	0,997 (0,83 to 1,209)	0,964 (0,569 to 1,928)	0,724 (0,658 to 0,824)
COLB2006	1,715 (0,127 to 41,13)	3,363 (0,949 to 76,22)	1,267 (0,798 to 2,629)	1,312 (0,893 to 1,672)	0,746 (0,517 to 1,141)
SAOUHSC_02223	1,519 (1,047 to 2,93)	11 (0,993 to 121,7)	4,127 (2,411 to 7,065)	1,976	0,772
COLB3502	6,534 (1,966 to 156,4)	15,69 (1,477 to 84.610)	5,881 (2,704 to 12,83)	41,2 (3,236 to 20.024)	2,737 (1,498 to 3,513)
SAR0905	2,497 (0,662 to 16,49)	2,301 (0,648 to 9,762)	0,83 (0,497 to 2,823)	0,775 (0,353 to 2,209)	0,642 (0,351 to 1,226)
SAR1238	10,03 (0,492 to 273,3)	2,647 (1,732 to 3,983)	0,93 (0,81 to 1,086)	0,956 (0,69 to 1,324)	1,309 (1,186 to 1,604)
SAR0460	1,539 (0,918 to 2,864)	1,448 (1,374 to 1,521)	0,485 (0,278 to 0,818)	0,69 (0,428 to 1,241)	0,585 (0,324 to 1,087)
dltD	5,316 (1,539 to 14,61)	5,071 (1,075 to 22,33)	1,655 (0,875 to 4,597)	1,334 (0,761 to 2,447)	1,294 (0,906 to 2,187)
SAR2049	0,93 (0,495 to 1,767)	0,91	0,247 (0,123 to 0,654)	0,327 (0,256 to 0,397)	0,41 (0,248 to 0,764)
fhuA	0,874 (0,416 to 2,522)	4,626 (0,338 to 229.580)	1,227 (0,649 to 2,116)	0,999 (0,62 to 1,476)	1,212 (0,824 to 1,817)
tsf	7,023 (1,672 to 24,29)	33,13 (4,231 to 95.058)	8,635 (5,317 to 13,46)	8,334 (3,364 to 18,18)	8,638 (3,369 to 21,99)
rpsD	5,996 (2,868 to 17,56)	31,47 (5,429 to 188.403)	7,828 (3,856 to 18,27)	6,648 (2,417 to 21,32)	8,495 (5,273 to 15,47)

Table S2. List of genes with altered mRNA expression in human blood. (continued)

At least twofold increase in mRNA expression[1]		Culture in blood			
Gene	Log phase growth in BHI broth	0 minutes	30 minutes	60 minutes	90 minutes
SAB0725	3,577 (0,712 to 24,23)	50,08 (3,009 to 667.968)	12,23 (4,834 to 51,48)	19,6 (4,994 to 56,22)	103,5 (2,664 to 223.501)
SAR1059	2,32 (1,246 to 6,465)	8,598 (0,683 to 96.257)	1,994 (0,927 to 3,852)	1,698 (0,712 to 3,53)	1,796 (1,266 to 2,253)
atpD	10,6 (3,214 to 17,96)	33,66 (3,131 to 83.754)	7,792 (0,948 to 20,46)	6,696 (1,927 to 25,79)	7,25 (0,959 to 26,59)
rpmD	36,53 (12,99 to 145,9)	131,4 (23,33 to 417.399)	28,73 (13,48 to 77)	30,75 (8,603 to 120,2)	26,67 (7,914 to 71,95)
SAOUHSC_A01455	1,28 (0,887 to 2,292)	19,64 (1,053 to 113.727)	3,386 (1,3 to 8,417)	3,282 (1,628 to 7,026)	0,7
SAR2024	2,704 (0,331 to 30,26)	4,297 (1,149 to 16,07)	0,664 (0,543 to 0,874)	0,925 (0,861 to 0,989)	0,73
SAR0297	0,579 (0,369 to 0,793)	7,384 (0,408 to 242.415)	0,601 (0,15 to 2,22)	0,26 (0,211 to 0,399)	0,352 (0,307 to 0,473)
tnpA2	0,396 (0,306 to 0,517)	7,307 (0,231 to 832.972)	0,401 (0,199 to 1,244)	0,422 (0,0905 to 1,685)	0,358 (0,262 to 0,525)
SAR0473	2,355 (1,298 to 5,564)	34,05 (2,402 to 88.369)	1,809 (1,139 to 3,005)	5,623 (3,873 to 7,839)	2,257 (1,037 to 3,231)
SAR1894	1,78 (0,177 to 24,72)	10,06 (0,243 to 117.324)	0,313 (0,217 to 0,41)	0,294 (0,118 to 0,517)	0,403 (0,114 to 0,686)
dat	5,294 (1,049 to 119)	3.147 (1,751 to 164.598)	2,628 (2,137 to 3,352)	3,725 (2,861 to 4,296)	3,106 (2,138 to 4,598)

[1] Genes are listed for which mRNA expression is respectively at least twofold increased or decreased in both isolates during all time points (30, 60 and 90 minutes) of culture in blood compared to the transcriptomes at the start of culture in blood (0 minutes). mRNA expression is quantified as the average RNA:DNA log ratio of duplo experiments in separate blood samples of two blood donors. Ranges of RNA:DNA log ratios between duplo experiments in separate blood samples are shown, unless only a single measurement from one blood sample was available.

Table S3. mRNA expression levels of 35 genes in two isolates during log-phase growth in BHI broth and human blood.

	Isolate 1				
Gene	Log phase growth in BHI broth[1]	Growth in blood 0min	30 min	60 min	90 min
Luk F	0,41 (0,269 to 0,593)	0,489 (0,276 to 0,75)	0,529 (0,333 to 0,808)	1,414 (0,815 to 2,38)	1,573 (0,811 to 2,186)
SEC	0,551 (0,328 to 1,053)	0,311 (0,174 to 0,444)	0,381 (0,207 to 0,639)	0,433 (0,176 to 0,937)	0,495 (0,374 to 0,686)
SEE	6,292		4,52		
FnbpA	0,659 (0,376 to 1,338)	0,344 (0,01 to 1,424)	1,247 (0,721 to 2,25)	0,985 (0,544 to 1,486)	0,949 (0,828 to 1,035)
FnbpB	0,749 (0,495 to 1,192)	0,815 (0,615 to 1,14)	1,202 (0,437 to 2,115)	0,777 (0,379 to 1,282)	
SEG	0,31 (0,227 to 0,375)	0,329 (0,233 to 0,388)	0,396 (0,278 to 0,594)	0,291 (0,15 to 0,634)	0,299 (0,0967 to 0,584)
SEH	6,238 (1,15 to 33,84)	2,496	1,439 (1,374 to 1,504)		
SdrD	6,652 (4,326 to 8,085)	8,388 (6,535 to 10,89)	4,595 (2,567 to 6,087)	3,211 (2,223 to 5,509)	2,001 (1,699 to 2,431)
SEI	0,302 (0,125 to 0,45)	0,269 (0,189 to 0,423)	0,302 (0,207 to 0,352)	0,251 (0,12 to 0,435)	0,238 (0,0992 to 0,543)
SEM	0,818 (0,339 to 3,195)	0,671 (0,589 to 0,713)	0,662 (0,479 to 0,833)	0,889 (0,688 to 1,85)	1,884 (0,734 to 3,034)
SEN	0,48 (0,208 to 0,941)	0,436 (0,333 to 0,49)	0,368 (0,295 to 0,467)	0,45 (0,298 to 0,624)	1,533 (1,508 to 1,558)
SdrE	1,586 (1,504 to 1,672)	13,46	3,868 (2,514 to 5,221)		
ETA	4,123 (0,831 to 38,44)		0,804	83,84	
ClfA	1,104 (0,652 to 1,442)	1,452 (0,753 to 2,283)	2,013 (1,215 to 4,546)	3,438 (2,8 to 4,156)	2,226 (2,135 to 2,317)
HlgB	0,218 (0,151 to 0,308)	0,0707 (0,0105 to 0,187)	1,303 (0,442 to 3,442)	2,581 (1,274 to 6,751)	2,019 (1,644 to 2,903)
SCIN	3,055 (2,461 to 4,675)	3,409 (2,048 to 5,067)	6,59 (4,503 to 9,112)	10,09 (4,311 to 23,9)	7,915 (5,992 to 9,978)
ClfB	1,141 (0,861 to 1,613)	1,427 (1,036 to 1,92)	1,274 (0,631 to 2,162)	1,285 (0,857 to 2,241)	1,381 (0,991 to 1,836)
CHIPS	2,79 (0,768 to 7,76)	0,908	0,5 (0,483 to 0,518)		
SasG	7,411 (1,627 to 35,52)		2,987		
IsdA	0,684 (0,172 to 3,296)	0,475	0,734 (0,5 to 1,107)	2,687 (1,582 to 4,119)	
Efb	1,907 (1,038 to 3,237)	1,799 (0,738 to 3,319)	5,355 (2,068 to 14,6)	5,27 (3,921 to 7,864)	2,947
Alpha toxin	1,685 (0,377 to 6,354)		0,624 (0,577 to 0,671)		

Table S3. mRNA expression levels of 35 genes in two isolates during log-phase growth in BHI broth and human blood. (continued)

Gene	Log phase growth in BHI broth[1]	Growth in blood 0min	30 min	60 min	90 min
Luk S	1,806 (1,372 to 2,398)	0,48 (0,278 to 0,948)	0,984 (0,692 to 1,399)	0,715	
SEO	0,404 (0,316 to 0,601)	0,45 (0,161 to 0,784)	0,191 (0,0273 to 0,681)	0,229 (0,121 to 0,269)	1,067 (0,799 to 1,335)
SSL 1	0,233 (0,161 to 0,404)	0,185 (0,102 to 0,355)	0,282 (0,213 to 0,419)	0,413 (0,292 to 0,535)	0,446 (0,318 to 0,709)
SSL 11	2,372 (1,583 to 3,161)		0,889		
FlipR	0,627 (0,453 to 0,74)	0,689 (0,616 to 0,762)	1,118 (0,832 to 1,405)	2,097 (1,902 to 2,412)	
PrsA	5,467 (4,4 to 9,422)	8,276 (7,211 to 10,6)	5,117 (1,756 to 11,65)	6,005 (4,759 to 8,495)	5,275 (3,661 to 7,939)
EsxB	0,309 (0,256 to 0,335)	0,303 (0,161 to 0,953)	0,293 (0,203 to 0,388)	0,384 (0,179 to 0,737)	0,334 (0,0997 to 0,594)
EsxA	3,464 (1,14 to 6,933)	4,048 (1,793 to 8,737)	4,764 (2,811 to 9,135)	10,74 (4,461 to 24,58)	6,148 (2,764 to 14,98)
IsaA	6,698 (4,752 to 11,17)	10,43 (7,928 to 19,71)	7,089 (4,009 to 10,3)	7,179 (2,428 to 12,68)	3,928 (3,263 to 4,593)
SA0486	0,324 (0,0804 to 0,787)	0,354 (0,167 to 1,821)	0,488 (0,276 to 0,812)	0,475 (0,286 to 0,675)	0,395 (0,0533 to 0,547)
SA0688	6,522 (3,927 to 11,53)	18,15 (11,86 to 43,04)	14,24 (8,091 to 21,98)	12,02 (8,611 to 18,75)	9,868 (6,401 to 17,13)
lytM	0,629 (0,363 to 1,171)	0,369 (0,0457 to 1,536)	1,874 (1,343 to 2,408)	0,708 (0,425 to 1,603)	0,736 (0,57 to 0,998)
Nuc	0,552 (0,466 to 0,686)	0,269 (0,01 to 1,057)	0,607 (0,28 to 1,033)	0,654 (0,467 to 1,223)	0,574 (0,234 to 0,758)
	Isolate 2				
	Log phase growth in BHI broth	Growth in blood 0min	30 min	60 min	90 min
Luk F	0,572 (0,235 to 2,123)	0,945 (0,553 to 1,659)	1,314 (0,104 to 2,95)	1,236 (0,656 to 2,192)	1,983 (0,852 to 4,115)
SEC	0,349 (0,184 to 1,31)	0,443 (0,212 to 1,027)	0,282 (0,108 to 0,742)	0,267 (0,175 to 0,39)	0,265 (0,0609 to 0,717)
SEE	0,847 (0,479 to 1,214)		2,705		
FnbpA	4,355 (2,51 to 17,82)	2,112 (1,208 to 4,127)	2,086 (1,116 to 3,542)	3,743 (1,44 to 7,45)	3,228 (2,164 to 5,152)
FnbpB	0,801 (0,46 to 1,506)		4,868 (3,074 to 7,709)		0,299
SEG	0,383 (0,163 to 1,264)	0,445 (0,211 to 1,391)	0,309 (0,104 to 0,47)	0,306 (0,155 to 0,555)	0,299 (0,074 to 0,603)
SEH	1,877 (1,767 to 1,987)	3,005	3,539 (1,726 to 6,261)	3,39 (2,823 to 3,808)	2,065 (1,405 to 3,017)

Table S3. mRNA expression levels of 35 genes in two isolates during log-phase growth in BHI broth and human blood. (continued)

Gene	Log phase growth in BHI broth[1]	Growth in blood 0min	30 min	60 min	90 min
SdrD	0,739 (0,246 to 1,434)		2,687		
SEI	0,289 (0,18 to 0,89)	0,392 (0,212 to 0,587)	0,337 (0,263 to 0,503)	0,243 (0,133 to 0,639)	0,285 (0,0681 to 0,76)
SEM	0,544 (0,307 to 1,4)	0,624 (0,431 to 1,17)	0,489 (0,408 to 0,625)	0,374 (0,221 to 0,693)	0,126 (0,0974 to 0,154)
SEN	0,298 (0,205 to 0,523)	0,452 (0,36 to 0,825)	0,592 (0,478 to 0,91)	0,411 (0,302 to 0,52)	0,192
SdrE	1,492 (0,204 to 7,522)	4,945			0,669
ETA	1,667 (0,948 to 2,932)		1,366		0,583
ClfA	1,389 (0,604 to 8,518)	0,819 (0,479 to 1,227)	1,02 (0,624 to 2,407)	1,388 (0,743 to 2,596)	2,333 (1,436 to 3,458)
HlgB	0,72 (0,102 to 15,73)	0,392 (0,131 to 2,615)	1,041 (0,158 to 7,127)	1,389 (0,601 to 2,211)	3,533 (0,99 to 10,25)
SCIN	4,496 (2,576 to 8,511)	7,389 (3,011 to 13,79)	5,935 (2,879 to 11,53)	4,952 (2,075 to 10,8)	9,611 (5,103 to 14,12)
ClfB	2,656 (1,459 to 8,001)	2,074 (1,031 to 6,653)	2,021 (1,078 to 3,827)	3,307 (1,445 to 5,849)	2,477 (1,487 to 4,681)
CHIPS	2,229 (0,839 to 6,71)	0,382	1,166 (0,393 to 2,115)	0,421 (0,358 to 0,483)	1,284
SasG	2,153 (0,312 to 14,97)				0,251 (0,24 to 0,263)
IsdA	0,662 (0,299 to 1,663)	1,08 (0,265 to 5,326)	1,379 (0,3 to 3,318)	1,704 (0,546 to 4,601)	3,238 (1,098 to 7,014)
Efb	2,512 (0,967 to 6,788)	2,701 (0,804 to 5,888)	2,694 (0,709 to 6,002)	2,888 (1,403 to 4,067)	4,691 (2,055 to 7,58)
Alpha toxin	3,034 (1,148 to 19,17)	1,137 (0,794 to 1,481)	0,847 (0,442 to 1,639)	0,954 (0,596 to 1,876)	0,671 (0,43 to 0,944)
Luk S	0,526 (0,332 to 0,72)	0,524			
SEO	0,397 (0,124 to 1,253)	0,506 (0,115 to 1,413)	0,547 (0,285 to 1,046)	0,366 (0,254 to 0,57)	0,293 (0,01 to 0,823)
SSL 1	1,319 (0,551 to 2,498)				0,461 (0,436 to 0,486)
SSL 11	1,343 (0,681 to 2,79)	2,387 (2,204 to 2,571)	1,782 (1,583 to 2,1)	2,887 (1,341 to 4,742)	2,642 (0,186 to 22,58)
FlipR	0,678 (0,398 to 2,052)	2,678 (0,98 to 7,59)	1,85 (0,45 to 5,153)	1,442 (0,897 to 2,323)	2,453 (2,406 to 2,5)
PrsA	1,887 (1,051 to 5,443)	3,718 (2,512 to 6,346)	2,927 (1,561 to 4,533)	2,53 (1,519 to 4,665)	1,856 (0,921 to 3,807)
EsxB	0,686 (0,386 to 0,987)		3,38		1,469
EsxA	2,374 (1,955 to 3,879)	3,057 (1,241 to 6,928)	3,099 (1,551 to 4,395)	3,99 (2,311 to 8,532)	5,093 (3,429 to 8,175)

Table S3. mRNA expression levels of 35 genes in two isolates during log-phase growth in BHI broth and human blood. (continued)

Gene	Log phase growth in BHI broth[1]	Growth in blood 0min	30 min	60 min	90 min
IsaA	8,747 (4,953 to 17,52)	11,68 (6,206 to 34,71)	7,667 (3,484 to 12,99)	8,101 (3,39 to 27,13)	8,322 (5,35 to 15,06)
SA0486	0,226 (0,0902 to 0,677)	0,268 (0,128 to 0,477)	0,304 (0,139 to 0,535)	0,287 (0,17 to 0,507)	0,368 (0,148 to 0,627)
SA0688	14,75 (0,215 to 253,6)	7,648 (2,758 to 14,92)	5,64 (0,972 to 9,216)	7,11 (4,429 to 15,28)	7,364 (5,102 to 12,75)
lytM	1,397 (0,443 to 3,126)	1,17 (0,243 to 2,867)	1,413 (0,962 to 2,004)	1,493 (0,543 to 2,519)	1,875 (1,453 to 2,531)
Nuc	3,39 (1,028 to 58,47)	0,98 (0,6 to 1,826)	0,834 (0,507 to 1,074)	0,889 (0,623 to 1,445)	0,81 (0,639 to 1,25)

[1] Average RNA:DNA log ratios of duplicate experiments in two separate blood samples are given; a RNA:DNA ratio larger than 2 indicates high expression and a RNA:DNA ratio smaller than 0.5 indicates low expression. Range of RNA:DNA log ratios between duplo experiments in seperate blood samples are shown, unless only a single measurement from one blood sample was available.

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Chapter 3

Combining *in vitro* protein detection and *in vivo* antibody detection identifies potential vaccine targets against *Staphylococcus aureus* during osteomyelitis

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ABSTRACT

Currently little is known about the *in vivo* human immune response against *Staphylococcus aureus* during a biofilm-associated infection, such as osteomyelitis, and how this relates to protein production in biofilms *in vitro*. Therefore, we characterized IgG responses in 10 patients with chronic osteomyelitis against 50 proteins of *S. aureus*, analysed the presence of these proteins in biofilms of the infecting isolates on polystyrene (PS) and human bone *in vitro*, and explored the relation between *in vivo* and *in vitro* data.
IgG levels against 15 different proteins were significantly increased in patients compared to healthy controls. Using a novel competitive Luminex based assay, eight of these proteins [(alpha toxin, Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FlipR), glucosaminidase, iron-responsive surface determinant A and H, the putative ABC-transporter SACOL0688, staphylococcal complement inhibitor (SCIN) and Serine-aspartate repeat-containing protein E (SdrE)] were also detected in a majority of the infecting isolates during biofilm formation *in vitro*. However, 4 other proteins were detected in only a minority of isolates *in vitro* while, vice versa, 7 proteins were detected in multiple isolates *in vitro* but not associated with significantly increased IgG levels in patients. Detection of proteins was largely confirmed using a transcriptomic approach.

Our data provide further insights into potential therapeutic targets, such as for vaccination, to reduce *S. aureus* virulence and biofilm formation. At the same time, our data suggests that either *in vitro* or immunological *in vivo* data alone should be interpreted cautiously and that combined studies are necessary to identify potential targets.

INTRODUCTION

Staphylococcus aureus is the most common causative organism of osteomyelitis [1-3], which is defined as an infection of the bone and is associated with significant morbidity [1, 4, 5]. Treatment is often difficult and requires surgery in addition to antibiotics [5]. Osteomyelitis is associated with the formation of bacterial biofilms [3, 6], which are defined as complex communities of bacteria enclosed in a polymer matrix that differ significantly in their gene expression and protein production compared to free-living bacteria [7]. Biofilm formation is believed to increase resistance against antibiotics and the host immune system, which further complicates the treatment of such infections [3, 8, 9].

Together with the increasing incidence of resistant *S. aureus* isolates [10, 11], the difficult treatment of osteomyelitis underscores the need for alternative treatment strategies. One such strategy is the development of a vaccine [12]. However, so far no clinically successful vaccine against *S. aureus* has been developed, despite the promising results of vaccines targeting diverse virulence factors of this pathogen in animal models [13-15], including an animal model for osteomyelitis [16]. Possibly, so far clinically evaluated vaccines might have failed because these were based on single antigens, while the awareness is currently increasing that multiple virulence factors of *S. aureus* should be targeted to undermine bacterial virulence [12, 13].

Although diverse virulence factors of *S. aureus* have been associated with biofilm formation [17-21], so far only a limited number of studies has focussed on the presence of multiple virulence factors simultaneously in biofilms [22, 23]. In addition, most of these studies used *in vitro* and/or animal models, while it remains unclear whether proteins expressed in these models are also involved in infection in humans. Finally, the immunogenic potential of proteins that are specifically expressed during a biofilm-associated infection remains unknown, as studies examining the immune response against *S. aureus* in patients have so far mainly focused on acute infections, such as bacteremia [24-29].

The aims of the present study were to characterize IgG responses in 10 patients with chronic osteomyelitis against 50 proteins of *S. aureus*, to analyse the presence of these proteins in biofilms of *S. aureus* isolates from the same patients on polystyrene (PS) and human bone *in vitro*, and to explore the relation between *in vivo* and *in vitro* data. All 50 proteins are functionally well characterized virulence factors which have been a major focus of many immunoproteomic studies, including vaccination trails, in both animal models [13-15] and humans [25, 29-31]. This study provides further insights into the presence and immunogenicity of these proteins during a biofilm-associated chronic infection in humans.

MATERIALS AND METHODS

Human serum and tissue

All patient serum used in this study was obtained from coded left-over material from routine diagnostic blood samples as described previously [29]. This procedure was approved and the acquisition of additional written consent was waived specifically for this study by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2007-106). Human bone was derived from surplus tissue obtained during routine orthopaedic surgical procedures and all tissue was directly anonymized upon arrival at the laboratory. This procedure was approved and the acquisition of additional written consent was waived by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2004-322). None of the authors were involved in the direct collection of either blood or tissue from patients. Only qualified physicians (MdR and MS) had access to potentially identifying patient information.

Patients

Ten patients diagnosed with chronic osteomyelitis at the Erasmus Medical Center between march 2007 and march 2011 were included in this study (all male, median age 62 years, interquartile range (IQR) 49-69 years). Chronic osteomyelitis was defined as a history of at least one year of clinical and radiological signs indicative of osteomyelitis combined with the isolation of *S. aureus* from at least one deep bone culture. Isolates from 9 patients could be retrieved for this study. The cause of osteomyelitis varied, being iatrogenic following surgery (4 patients), fracture or other trauma (3 patients), diabetic ulcer (2 patients), or hematologic metastasis from another focus (1 patient). Infections were localized in the femur (3 patients), small bones of the foot (3 patients), tibia (2 patients), ulna and sternum (each 1 patient). Nasal carrier status for *S. aureus* was not tested.

A median number of 4 serum samples were collected per patient (IQR 2-5) over a median period of 26 days following the most recent positive bone culture (IQR 9.5-85.5 days). Peak antibody levels of each patient were compared with those measured in single serum samples of 10 previously described bacteremia patients (80% male, median age 64 years, IQR 45- 84) and 20 previously described, age-matched controls (all male, median age 62 years, IQR 57 - 67) who had no record of a clinically apparent infection in at least six months [29]. *S. aureus* nasal carrier status was not tested in patients nor controls.

Bacterial strains and genotyping

S. aureus isolates obtained from deep bone cultures were identified on the basis of colony and microscopic morphology and Slidex Staph Plus agglutination testing (bioMérieux, Marcy l'Etoile, France). Identification was confirmed by *spa*-PCR and all isolates were *spa*-typed [32]. All isolates were methicillin sensitive as determined by cefoxitin disk

diffusion according to the CLSI criteria [33]. Antimicrobial susceptibility to additional antibiotics was determined using the VITEK® 2 system with card AST-P549 (bioMérieux)

Isolates were further typed using pulsed-field gel electrophoresis (PFGE) with *Sma1*-digested chromosomal DNA as described previously [34]. Relatedness among the PFGE profiles was evaluated using Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium). Finally, all isolates were screened with PCR for the presence of 50 genes using previously described primers [29].

Preparation of human serum and measurement of antibodies

Serum samples were collected in BD Vacutainer® SST II Advance plastic serum tubes, which were centrifuged for 3 minutes at 1680g and stored at 4 ^{0}C. For long-term use serum was aliquoted in 1.5 ml Eppendorf® tubes and stored at -80 ^{0}C.

IgG levels against 50 recombinant *S. aureus* proteins were measured in serum samples using a bead-based flow cytometry technique (xMAP®; Luminex® Corporation, Austin, TX, USA), as previously described [29]. All proteins are summarized in Table S1. Proteins were coupled to xMAP® carboxylated beads (Luminex Corporation) as described previously [29, 35]. All measurements were performed in duplicate and the median fluorescence intensities (MFIs), a semi-quantitative measure of antibody levels, were averaged. Duplicate measurements for which the coefficient of variation was larger than 25% were excluded from further analysis. All measurements were corrected for non-specific background signal by subtracting the MFIs of control beads not coupled to any protein.

Preparation of human bone

Fresh human bone was obtained in the operating room from patients receiving a total hip prosthesis, for purposes other than this study, in the Erasmus Medical Center from the period of January 2012 until February 2013. All operations were performed by the same orthopaedic surgeon (dr. P.K. Bos). After removing the femoral head, a small portion of the surplus tissue was cut using a saw and directly transported to the lab in sterile saline. Spongious bone was harvested from the tissue and cut into small pieces fitting in a 96 wells plate. The samples were rinsed repeatedly with saline until all blood was visibly removed.

Biofilm formation on polystyrene and human bone

A routine biofilm model was used as described before [20, 36-38]. Briefly, overnight cultures of *S. aureus* grown on sheep blood agar were suspended in Iscove's Modified Dulbecco's Medium (IMDM, Life technologies, Carlsbad, CA, USA) without phenol red until an OD660 of 2.0 was reached. One µl of this bacterial suspension was added to 199 µl of IMDM in sterile, 96-wells polystyrene plates (Greiner Bio-one GmbH, Kremsmuenster, Austria). Duplicate wells not inoculated with bacteria served as sterile controls. Plates

were then incubated at 37 ^{0}C and gentle shaking at 200rpm for various intervals. Biofilm mass was measured by staining with 1% crystal violet. OD was measured at 490 nm.

Alternatively, standard-sized pieces of freshly isolated human bone were washed in sterile water and then added to 199 µl of IMDM in the same 96-wells plates in which biofilms on PS were grown. One µl of the same bacterial suspension as described above was added before incubation. Duplicate wells with bone tissue were not inoculated to serve as sterile controls.

Multiplex bead assay for assessment of protein levels in biofilms

A previously described multiplex competition Luminex® assay (CLA) was used to indirectly detect the presence of 50 IgG-accessible proteins in biofilms [39, 40]. In brief, biofilms grown on PS and bone were washed once with ice-cold PBS supplemented with 0.5% (wt/v) sodium azide (Sigma-Aldrich) at 1, 8, 24 or 48 hours to remove non-adherent bacteria. Bone tissue with attached biofilms was transferred to a clean well. Biofilms were then incubated for 35 min at 8°C and continuous shaking (500 rpm) with 200 µl of a 1:200 dilution of polyclonal human IgG (PHG), isolated using the HiTrap™ Protein G HP column according to the manufacturer's guidelines (GE Healthcare Bio-sciences, Piscataway, New Jersey, USA), from pooled serum of 40 healthy volunteers [41].

After incubation, the remaining non-bound IgG antibody levels in recovered PHG samples were measured using the multiplex bead-based flow cytometry technique (xMAP®, Luminex corporation), with recombinant proteins covalently coupled to the beads, as described above. As negative controls PHG samples incubated with empty PS wells or sterile bone pieces were included in all experiments.

Next, the percentage decrease in the levels of specific IgG antibodies for each protein was calculated in relation to the negative control. The percentage decrease can be considered as a semi-quantitative measure of the protein-specific antibody absorption from PHG by the biofilm, thus indirectly reflecting the presence of the particular *S. aureus* protein in the biofilm [39, 40]. The average percentage decrease plus two times the standard deviation, obtained at 24 and 48 hours biofilm growth, for two non-*S. aureus* control proteins [*Streptococcus pneumoniae* putative proteinase maturation protein A (PpmA) and human metapneumovirus surface protein (hMPV)] and all *S. aureus* proteins of which genes were not present in an isolate, were chosen as cut-off value (35% at 24 hrs biofilm growth and 42% at 48 hrs, respectively).

Reverse transcriptase PCR

Biofilms were grown on PS for 8 and 24 hrs in 96-well plates in 200 µl of IMDM. Biofilms harvested from 8 wells were washed in PBS, resuspended, pooled and centrifuged at 4000 rpm for 10 min at 4°C. Pellets were resuspended in 200 µl of RNA protect™ Bacterial reagents (Qiagen), stabilized for 5 min and then centrifuged for 10 min at 4°C. The pellet

was dissolved in 1 ml of RNA-pro solution (Fast RNA Pro Blue kit, MP Biomedicals) and stored at -20°C until use. RNA was isolated using the Fast RNA Pro Blue kit according to the manufacturer's protocol. Each 10 µg of isolated RNA was treated twice with 2 U TURBO DNase (Ambion, Life Technologies). The reaction was stopped by adding 0.2 volumes of DNase inactivation reagent (Ambion) and incubation for 2 min at ambient temperature. RNA containing supernatants were collected by centrifugation (1.5 min at 9000 g at ambient temperature) and each 2 µg DNase-treated RNA was treated with 2 U DNase I (Fermentas, Fisher Scientific). One µg of prepared RNA was transcribed into cDNA using 200 U RevertAid H Minus Reverse transcriptase (Fermentas), 4 µl of 5x reaction buffer (Fermentas), 20 U of RiboLock RNase inhibitor (Fermentas) and 2 µl of 10 mM dNTP mix (Fermentas) in a final volume of 20 µl of DEPC-treated water. This was incubated for 60 min at 42°C and then terminated by heating at 70°C for 5 min. For each RNA sample a negative control without reverse transcriptase was processed similarly. The presence of cDNA in all samples was examined using PCR as described previously [29].

Cryo scanning electron microscopy

The clinical isolate from one osteomyelitis patients was allowed to form biofilms on human bone for 24 hours as described above. Next, *S. aureus* in bone was fixed for 15 minutes with 1% (v/v) glutaraldehyde (Sigma) in phosphate buffered saline (PBS) at room temperature. Samples were washed twice with PBS to remove excess fixative and were subsequently serially dehydrated by consecutive incubations in 1 ml of 25% (v/v) and 50% (v/v) ethanol-PBS, 75% (v/v) and 90% (v/v) ethanol-H_2O, and 100% ethanol (2x), followed by 50% ethanol-hexamethyldisilazane (HMDS) and 100% HMDS (Sigma) and air-dried overnight at room temperature. After overnight evaporation of HMDS, bone samples were mounted on 12 mm specimen stubs (12 mm, Agar Scientific) and coated with gold to 1 nm using a Quorum Q150R sputter coater at 20 mA prior to examination with a Phenom PRO Table-top scanning electron microscope (PhenomWorld).

Statistics

Mean IgG levels between patient groups and controls were compared using one-way ANOVA. All data were logarithmically transformed to obtain equal variances between groups, checked with Levene's test for equality of variances. For proteins that were associated with a significant difference in the ANOVA analysis, additional least significant difference (LSD) post-hoc tests were performed for further group comparisons.

Correlation between biofilm mass and the percentage decrease in specific IgG levels, as obtained with CLA, was determined by calculating the non-parametric Spearman's rank correlation coefficient (r_s).

In all cases *p*-values ≤ 0.05 were considered as statistically significant. IBM® SPSS® Statistics version 21 (IBM corporation, Armonk, NY, USA) was used for statistical analysis. Graphics were made using Graphpad Prism version 5 (Graphpad Inc. La Jolla, CA, USA).

RESULTS

Genetic typing of clinical *S. aureus* isolates from osteomyelitis patients

Ten patients that were diagnosed with chronic osteomyelitis caused by *S. aureus*, confirmed by deep bone culture, were included in this study. *S. aureus* isolates could be retrieved from bone cultures of 9 patients and these were genotyped using *spa* typing and PFGE analysis. Eight out of 9 isolates contained different *spa* types and PFGE analysis revealed an overall lack of relatedness between isolates (Figure 1).

In addition to genetic typing, the presence of genes encoding 50 virulence factors of *S. aureus* were examined in the 9 clinical isolates plus the extensively studied reference strain NCTC 8325 using PCR (Table 1, Table S1). Notably, the genes *eta* and *etb*, *lukS* and

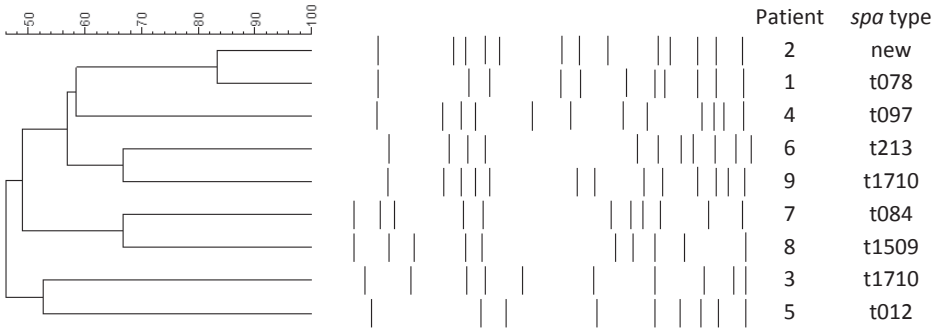

Figure 1. Genotypes of 9 clinical isolates from bone cultures.
Pulsed-field gel electrophoresis data and *spa* types are shown. An isolate of the tenth patient described in this study could not be retrieved.

lukF, *ssl9*, *Tst* and the genes encoding 13 enterotoxins were only detected in a minority of the 10 isolates. The other 31 genes were present in at least half or more of all isolates.

Biofilm formation by clinical *S. aureus* isolates on polystyrene and human bone

Next, the ability of the 9 clinical isolates and the reference strain NCTC 8325 to form biofilms on polystyrene (PS) and freshly isolated human bone were examined. Using crystal violet staining we confirmed that all isolates were able to stably form biofilms on PS, although the amount of biofilm mass varied (Figure 2A). Furthermore, using

Table 1. Presence of genes, mRNA and protein during biofilm formation by 10 *S. aureus* isolates.

Protein[1]	Gene	Functional class	Strains with gene present[2]	Biofilms on polystyrene		Biofilms on polystyrene			Biofilms on human bone		
				mRNA present[2]		Significant reduction in specific IgG[2]			Significant reduction in specific IgG[2]		
				8 hrs	24 hrs	8 hrs	24 hrs	48 hrs	8 hrs	24 hrs	48 hrs
Alpha toxin	*hla*	toxin	10	10	10	5	7	9	6	6	8
CHIPS	*chps*	immmune modulator	7	4	4	3	5	7	6	5	6
ClfA	*clfA*	surface protein	10	4	4	3	6	10	7	9	10
ClfB	*clfB*	surface protein	10	10	10	2	7	9	5	8	8
FlipR	*Flr*	Immune modulator	9	7	5	0	4	5	3	5	5
FnbA	*fnbA*	surface protein	10	10	10	-	2	5	1	5	6
Glucosaminidase	*Atl*	housekeeping	10	10	10	10	10	10	10	10	10
IsaA	*isaA*	housekeeping	10	10	10	10	10	10	10	10	10
IsdA	*isdA*	surface protein	9	9	9	9	9	9	4	8	9
Nuc	*nuc*	housekeeping/ toxin	8	5	1	7	7	8	7	8	8
SACOL0688	*MntC*	housekeeping	10	10	10	9	10	10	10	10	10
SCIN	*scn*	immmune modulator	10	9	8	9	9	9	9	9	9
IsdH	*isdH*	surface protein	10	10	10	3	3	6	2	2	1
Lipase	*lip*	housekeeping/ toxin	10	10	10	1	6	8	1	2	1
SasG	*sasG*	surface protein	7	6	6	1	1	1	7	4	2
SdrD	*sdrD*	surface protein	7	5	5	1	1	3	2	6	6
SdrE	*sdrE*	surface protein	7	7	6	1	1	3	5	5	5

[1]Only proteins are shown that were found in at least half of 9 clinical isolates and strain NCTC8325 with both transcriptomic analysis and CLA at minimally one time point on minimally one surface. From top to bottom protein groups are shown that were detected in a majority of isolates on both PS and bone, mostly on PS, or mostly on human bone, respectively.

[2]Number of isolates in which respectively the gene, mRNA or protein was detected are shown. - indicates that the gene, mRNA or protein was not detected in any isolate.

scanning electron microscopy, layers of tightly adherent bacteria resembling biofilms on human bone after 24 hours incubation were visualized for one clinical isolate (Figure 3A-C). These results indicate the development of mature biofilms on both surfaces.

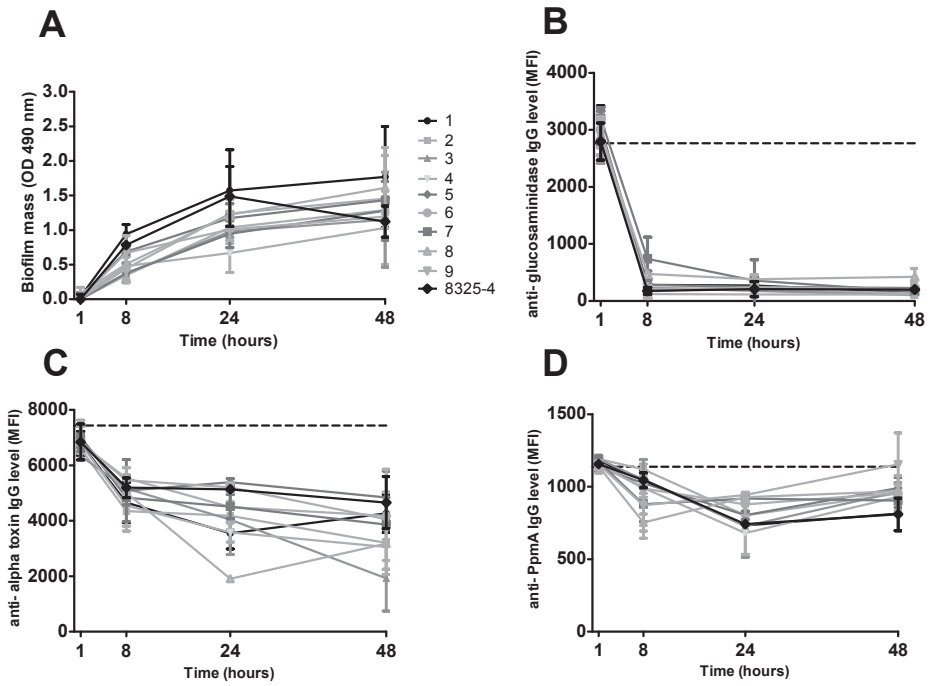

Figure 2. Detection of *S. aureus* proteins during biofilm formation on PS.
A: Biofilm mass formed on PS by 9 clinical isolates and reference strain NCTC 8325. The levels of remaining non-bound IgG directed against specific proteins in PHG after incubation with the biofilms [expressed as mean fluorescence intensity (MFI)], indirectly and inversely reflecting protein presence, are shown for **B:** glucosaminidase, **C:** alpha toxin and **D:** the non-*S. aureus* control protein PpmA. Please note the difference in range of MFI on the y-axis between Figures **B**-**D**. Dashed horizontal lines indicate the average MFI of sterile controls. Symbols and error bars indicate mean and SD of two separate experiments.

Characterization of IgG antibody response against *S. aureus* proteins in osteomyelitis patients

Total IgG levels directed against 50 proteins of *S. aureus* were prospectively measured during variable intervals within the study period (median of 26 days, interquartile range 9.5 – 85.5 days) in serum samples of the 10 patients (median of 4 samples per patient, interquartile range 2 - 5). Measurements for efb, EsxB, PrsA and SA0486 were excluded from further analysis due to low signal intensities with coefficients of variation larger than 25% between duplicate experiments.

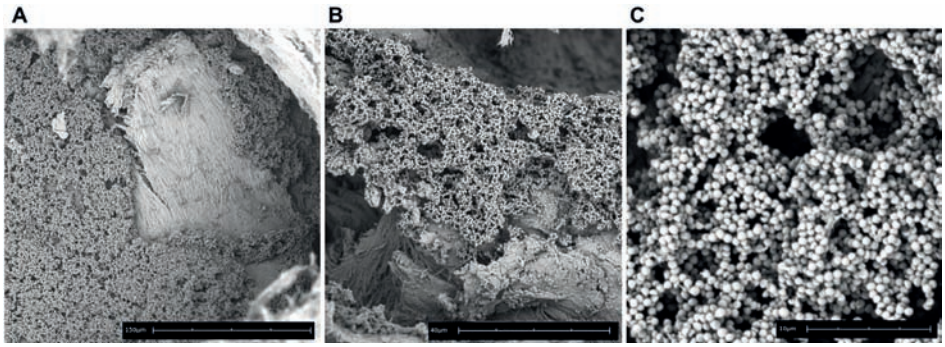

Figure 3. Biofilm formation of *S. aureus* on human bone.
Cryo scanning electron microscopy of one *S. aureus* isolate after 24 hours of biofilm formation on human bone. Please note the different magnifications indicated by the scale bars, respectively being **A:** 150 µm, **B:** 40 µm and **C:** 10 µm.

In line with previous data [24, 26, 28] the height of all protein-specific IgG levels was heterogeneous between patients (Table 2, Table S2), without any clear pattern in the course over time (Figure S1). IgG levels remained detectable up to 250 days within the study period. Comparison of IgG levels between the osteomyelitis patients, 10 patients suffering from a *S. aureus* bacteremia and 20 age-matched non-infected controls revealed that mean IgG levels differed significantly for 15 out of 46 proteins between groups (ANOVA $p < 0.05$, Table 2). Mean IgG levels against 10 proteins [(Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FLIPr), glucosaminidase, gamma-hemolysin B, iron-responsive surface determinant A (IsdA), leukocidins D and F, SACOL0699, staphylococcal complement inhibitor (SCIN), and Staphylococcal superantigen-like proteins 3 and 5 (SSL 3 and 5)] were significantly higher in both osteomyelitis and bacteremia patients compared to controls, IgG levels against 4 proteins (alpha-toxin, exfoliative toxin A (ETA**),** Serine-aspartate repeat-containing protein E (SdrE) and enterotoxin M) were significantly higher only in osteomyelitis patients and IgG levels against 1 protein (IsdH) were only higher in bacteremia patients (Table 2). None of the mean protein-specific IgG levels differed significantly between osteomyelitis and bacteremia patients. Notably, IgG levels against all proteins were readily detectable in the control group and IgG levels against the remaining proteins that are not mentioned above did not differ significantly between patients and controls (Table S2, Fig. S2).

Detection of proteins of *S. aureus* isolates during biofilm formation on PS

We used a competitive Luminex-based assay (CLA) [39, 40] to establish the presence of the same 50 proteins during biofilm formation by 9 of the infecting isolates described above. In line with previous results [39], biofilm mass-dependent absorption of specific IgG for several *S aureus* proteins such as glucosaminidase (Figure 2B) was detected, while no such reduction was seen for IgG specific against non *S. aureus* control proteins such as the

Table 2. Proteins of *S. aureus* associated with significantly increased IgG levels in patients.

Protein[1]	Gene	Function class	Mean IgG level control patients (± SD; N=20)	Mean IgG level bacteremia patients (± SD; N=10) [2]	Mean IgG level osteomyelitis patients (± SD; N=10) [2]	P value ANOVA[3]	P value Post-hoc analysis[4]
FlipR	*flr*	immune modulator	1864 (±1569)	4490 (±3485)	3656 (±2143)	0,007	0,019
Glucosaminidase	*Atl*	housekeeping	5273 (±2827)	86780 (±3612)	8377 (±3882)	0,005	0,019
HlgB	*hlgB*	toxin	6328 (±4290)	10838 (±2924)	10917 (±3627)	0,002	0,007
IsdA	*isdA*	surface protein	3722 (±4532)	6534 (±3993)	5016 (±2756)	0,006	0,023
LukD	*lukD*	toxin	6311 (±3988)	9512 (±3514)	9369 (±3581)	0,024	0,044
LukF	*lukF*	toxin	1105 (±880)	1851 (±772)	2025 (±917)	0,001	0,005
SACOL0688	*MntC*	housekeeping	839 (±650)	3849 (±4290)	2572 (±2432)	0,001	0,005
SCIN	*scn*	immune modulator	3665 (±3322)	7939 (±3805)	7545 (±3782)	<0,000	0,002
SSL3	*ssl3*	immune modulator	4679 (±3068)	8186 (±4746)	6955 (±3673)	0,011	0,042
SSL5	*ssl5*	immune modulator	1929 (±1307)	4675 (±3315)	3932 (±2827)	0,001	0,014
Alpha toxin	*hla*	toxin	8895 (± 4419)	11610,9 (± 5117)	14884,3 (±3749)	0,037	0,011
ETA	*eta*	toxin	893 (±1425)	1238 (±1946)	2874 (±4178)	0,026	0,007
SdrE	*sdrE*	surface protein	293 (±298)	399 (±385)	651 (±441)	0,026	0,007
SEM	*sem*	toxin	526 (±542)	1155 (±1595)	1322 (±966)	0,028	0,011
IsdH	*isdH*	surface protein	825 (±867)	2579 (±3130)	2732 (±4407)	0,034	0,011

[1]Only proteins are shown that were associated with significantly increased IgG levels in at least one patient group. From top to bottom protein groups are shown that were associated with significantly increased IgG levels in both bacteraemia and osteomyelitis patients, only in osteomyelitis patients and only in bacteraemia patients compared to controls, respectively.

[2]Only the peak IgG levels of patients were included for comparison.

[3]P value of ANOVA test comparing all three patient groups. P values < 0.05 were considered as significant.

[4]Groups were additionally compared using least significant difference (LSD) post-hoc tests. The smallest *p*-values, related to the significantly differing groups, are shown.

putative protease maturation protein A (PpmA) of *Streptococcus pneumoniae* (Figure 2D). Similar to the differences in biofilm mass (Figure 2A), the reduction in IgG levels against most proteins, such as alpha toxin, was heterogeneous between isolates (Figure 2C). The average amount of formed biofilm mass and the average percentage reduction in IgG levels correlated significantly for all proteins (e.g. alpha toxin: r_s -0.77, $p < 0.0001$). Based on the percentage decrease in IgG levels against the non-*S. aureus* control proteins and against all *S. aureus* proteins of which the gene was not found in an isolate, cut-off values for protein detection of at least 35% decrease in specific IgG at 24 hrs and 42% at 48 hrs biofilm formation were calculated. CLA measurements for four proteins (ESX-1-associated factor B (EsxB), extracellular fibrinogen-binding protein (efb), foldase-protein PrsA and the putative protein SA0486) were excluded from further analysis due to low MFI's with standard deviations larger than 25% between repeated CLA measurements.

For the 31 genes that were found in at least half of the 10 isolates, 14 proteins were detected in the majority of gene-positive isolates at minimally one time point (8, 24 and/or 48 hours) during biofilm formation on PS (Table 1): the surface proteins clumping factor A and B (ClfA and B), fibronectin-binding protein A (FnbpA), IsdA and H, the housekeeping proteins glucosaminidase, immunodominant antigen A (IsaA), lipase, nuclease and the putative ABC transporter SACOL0688, the immune-modulators FlipR, SCIN and chemotaxis inhibitory protein of *S. aureus* (CHIPS), and alpha toxin.

For the genes that were found in a minority of isolates, the proteins exfoliative toxin A (ETA) and the staphylococcal enterotoxins A, B, D, Q and R were detected in one to maximally three gene-positive isolates during biofilm formation on PS (Table S1). Using the above mentioned cut-off values, no proteins were detected when the corresponding gene was absent in an isolate.

The lack of detection of several secreted proteins, despite the presence of corresponding genes in isolates (Table S1), prompted us to repeat CLA experiments using the medium covering biofilms instead of biofilms self. Results obtained with medium showed similar reduction in IgG levels against all proteins, including secreted proteins such as alpha toxin, compared to results obtained with biofilms (Figure S3). This suggests that the lack of protein detection is not due to false-negative signals. CLA data were further confirmed by the detection of specific mRNA at 8 and/ or 24 hours of biofilm formation for all detected proteins (Table 1, Table S1).

Detection of proteins of *S. aureus* isolates during biofilm formation on human bone

In an attempt to examine the expression of *S. aureus* virulence factors in an environment that more closely resembles the *in vivo* conditions during osteomyelitis, we repeated the above mentioned experiments with biofilms grown on human bone. Generally, the same patterns of reduction in specific IgG levels were observed as for biofilms on PS,

including for glucosaminidase, alpha toxin and the PpmA control protein (Figure 4A-C). Compared to biofilm formation on PS, similar percentages of non-specific reduction in IgG levels were found, prompting us to use the same cut-off values.

For the 31 genes that were found in half or more of the 10 isolates, 15 proteins were detected in the majority of gene-positive isolates at minimally one time point (8, 24 and/or 48 hours) during biofilm formation on human bone (Table 1). These were largely the same proteins that were also detected during biofilm formation on PS, with the exception of IsdH and lipase, which were detected in only a minority of isolates at all time points. On the other hand, *S. aureus* surface protein G (SasG) and SD-repeat containing proteins D and E (SdrD and E) were detected in most isolates during biofilm formation on bone but not on PS at several time points (Table 1).

Notably, for the genes that were found in a minority of isolates, all Staphylococcal enterotoxins were detected in 1 to 4 gene-positive isolates during biofilm formation on bone, which were more isolates compared to the results obtained with biofilms on PS (Table S1).

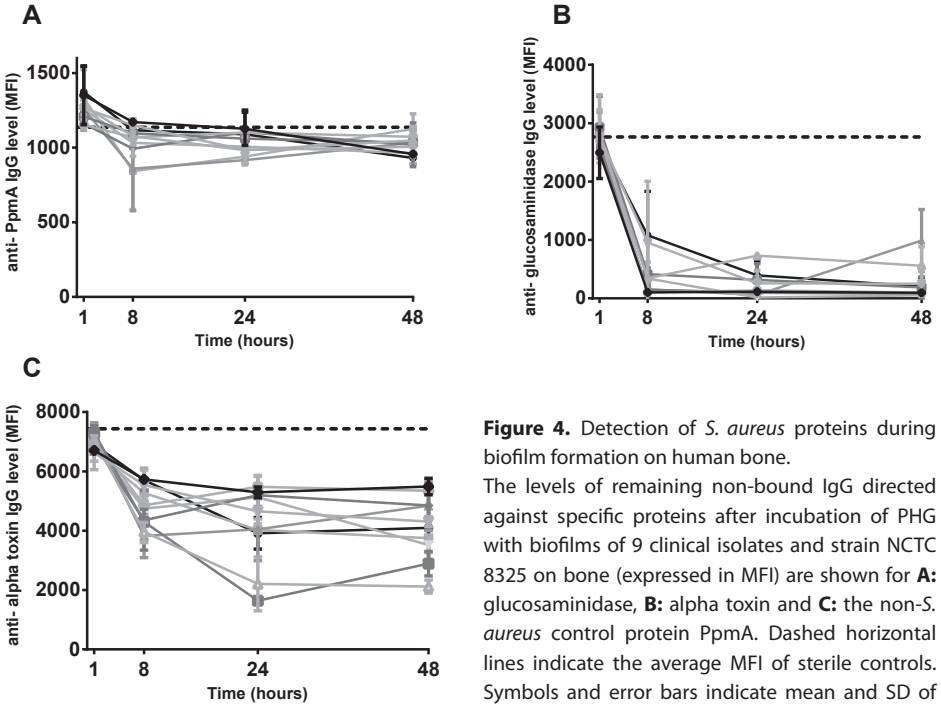

Figure 4. Detection of *S. aureus* proteins during biofilm formation on human bone.
The levels of remaining non-bound IgG directed against specific proteins after incubation of PHG with biofilms of 9 clinical isolates and strain NCTC 8325 on bone (expressed in MFI) are shown for **A:** glucosaminidase, **B:** alpha toxin and **C:** the non-*S. aureus* control protein PpmA. Dashed horizontal lines indicate the average MFI of sterile controls. Symbols and error bars indicate mean and SD of two separate experiments.

Comparison of *in vivo* antibody responses with *in vitro* protein detection

For the 31 genes that were found in at least half of the 10 isolates, 8 *S. aureus* proteins were associated with significantly increased IgG levels in patients and detectable in the

majority of the gene-positive isolates during biofilm formation on PS and/or bone: alpha toxin, FlipR, glucosaminidase, IsdA and H, SACOL0688, SCIN and SdrE. Amongst the less common genes, enterotoxin M was associated with significantly increased IgG levels in patients and it was detectable in multiple isolates specifically during biofilm formation on bone.

In contrast to the above mentioned results, the toxins hemolysin gamma-B and leucocidin D and the immune modulators SSL3 and 5 were associated with significantly increased IgG levels in patients but detected in only a minority of gene-positive isolates during biofilm formation *in vitro*. Vice versa, the surface proteins ClfA and B and the housekeeping proteins IsaA, lipase and nuclease were detected in a majority of strains during biofilm formation *in vitro* but were not associated with significantly increased IgG levels in patients. The latter was also true for the less common enterotoxins B and Q, for which genes were present in 3 and 2 isolates, respectively.

DISCUSSION

In this study we characterized the IgG response against 50 functionally diverse proteins of *S. aureus* in patients with chronic osteomyelitis. IgG levels against 14 diverse virulence factors, such as alpha-toxin, the surface protein IsdA and the housekeeping protein glucosaminidase, were significantly increased in osteomyelitis patients compared to healthy controls. Interestingly, a comparison with peak IgG levels of patients suffering from a *S. aureus* bacteremia revealed little difference, suggesting that immunological exposure to specific virulence factors is similar during both infections. Indeed, bacteria residing within a biofilm can dislodge and potentially cause a bacteremia [42]. This implicates that it might be difficult to find serological markers that can specifically discriminate between a *S. aureus* osteomyelitis and other sorts of infection. Moreover, in the context of identifying potential targets for e.g. vaccination, this implicates that results of immunological *in vivo* studies should be interpreted cautiously when searching for targets that are specifically involved in biofilm formation.

To gain further insight into the involvement of the 50 proteins mentioned above in biofilm formation, we screened for the presence of these proteins during biofilm formation of 9 clinical isolates and reference strain NCTC-8325 on polystyrene (PS) and freshly isolated human bone, using a novel competitive Luminex-based assay (CLA). In general we observed a clear variation in the reduction of protein-specific IgG levels between isolates, which can largely be explained by differences in the amount of biofilm mass formed by each isolate. This variability in biofilm mass is in line with previous results, which might be related to the different genetic backgrounds of the isolates [43].

For the 31 proteins of which genes were present in at least half of the 9 clinical isolates and NCTC 8325, 12 proteins were detected in at least half of all isolates at minimally one time point (8, 24 and/or 48 hours) during biofilm formation on both PS and bone. Several of these proteins, especially surface proteins such as ClfB and FnbpA, already have a known role in biofilm formation [44-46]. In addition, several detected proteins, including IsaA, SACOL0688 and glucosaminidase, have been successfully evaluated as vaccine targets in animal infection models, some specifically in an osteomyelitis model [16, 47]. Our results further indicate the potential use of these proteins as vaccine targets by demonstrating their actual production during biofilm formation by genetically diverse clinical isolates *ex vivo* and, based on antibody responses, also during osteomyelitis in human patients *in vivo* .

Although the CLA results between biofilm formation on PS and bone generally agreed well, detection of a few proteins differed. Lipase and IsdH were mostly detected on PS while the biofilm-associated surface proteins SasG, SdrD and SdrE were mostly detected on bone. In addition, also the genetically less common enterotoxins were detected relatively more often on bone. The presence of SdrE and D specifically during biofilm formation on bone might be explained by bacterial attachment to the calcium-rich extracellular matrix, as these proteins are structurally dependable on calcium [48]. Although we cannot currently explain the other observed differences, we have demonstrated before using CLA that different circumstances can impact protein detection *in vitro* and *ex vivo* [39, 40]. In this context, production of some of the above mentioned proteins, including diverse enterotoxins, is regulated by the accessory gene regulatory (Agr) quorum-sensing system [49, 50]. Possibly, this system is activated differently upon interaction of *S. aureus* with either PS or bone. Lastly, next to culture circumstances also the timing of measurement can be influential, as demonstrated by the detection of SasG mostly during early biofilm formation (8 hrs), which is in line with its established role during the early accumulation phase [51]. Together these findings implicate that results from *in vitro* models should be interpreted cautiously and both timing and circumstances during the measurement should be taken into account.

Combination of *in vitro* and *in vivo* results suggests that 8 proteins, of which most are already discussed above, are both immunogenic in patients and are detectable in the majority of clinical isolates during biofilm formation. At the same time some toxins and immune modulators were associated with significantly increased IgG levels in patients but not detected during biofilm formation *in vitro*. Possibly, our *in vitro* biofilm model might not adequately reflect the *in vivo* situation in patients, or the measured IgG levels might be mounted against proteins produced by planktonic growing bacteria within the patient. Moreover, IgG levels against the leukocidins might be false-positive due to the presence of cross-reactive antibodies, which has been established before in our assay [29]. In contrast to the above mentioned proteins, other proteins such as the ubiq-

uitously present clumping factors and IsaA, were detected *in vitro* but not associated with significantly increased IgG levels in patients. This might well be explained by the high IgG levels that we found in both healthy controls and in patients, thereby eliminating any statistically significant difference between groups. Indeed, antibody titers to diverse *S. aureus* proteins appear to be ubiquitously present in the general population [24, 28] and this appears to be mostly independent of nasal carrier ship for *S. aureus* [41]. cautiously.

A limitation of the current Luminex assay is that its sensitivity is potentially influenced by amino acid sequence diversity, which is described for multiple proteins described in this study [52-55]. However, in general sequence diversity has been described within certain limits and these differences do not significantly impact our assay sensitivity, based on cross-reactivity within our assay as shown earlier for the leukocidin components and hemolysin gamma-B [29] and also the more variable, different isotype forms of the FnBPA A domain [52] (data in submission). In addition, we only compare average IgG levels for each protein separately, further levelling out potential variation in assay sensitivity due to protein diversity. Taken together we are confident that the current assay allows us to screen for the presence of specific antibodies and proteins [29, 40].

The 50 proteins included in the current assay were chosen based on their established roles in *S. aureus* pathogenesis and the corresponding host immune response. However, there are many more e.g. cytosol-based proteins of *S. aureus* that remain less-characterized in this context, yet could also be potentially interesting therapeutic targets. We believe that these proteins will be an interesting addition to future immunoproteomic studies, although this is not the focus of the current study.

We conclude that functionally diverse virulence factors of *S. aureus* are present during biofilm formation by genetically diverse isolates on PS and human bone *in vitro*, and that some of these proteins are immunogenic *in vivo*. These observations merit more mechanistic studies to elucidate the function of specific proteins and the regulation of their expression during *S. aureus* biofilm formation. However, the present data further suggests that multiple proteins, such as the ubiquitously present and immunogenic IsdA or SA0688, could be potential targets for novel agents such as a multi-valent vaccine to prevent or treat biofilm-associated infections in patients. Combined studies using both *in vitro* models and immunological assays in patients *in vivo* can help in identifying novel therapeutic targets.

ACKNOWLEDGEMENTS

We would like to thank dr. P. K. Bos for helping us in obtaining human bone tissue for this study.

SUPPLEMENTAL DATA

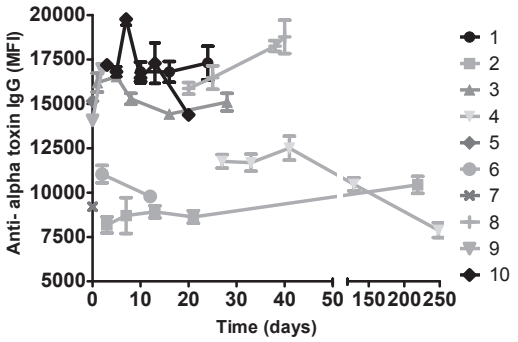

Figure S1. Height and course of anti-alpha toxin IgG levels in 10 patients diagnosed with chronic osteomyelitis.
Time point 0 was arbitrarily defined as the date of the first deep bone culture during the study period. Each data point represents the average of duplicate measurements expressed as the mean fluorescence intensity (MFI).

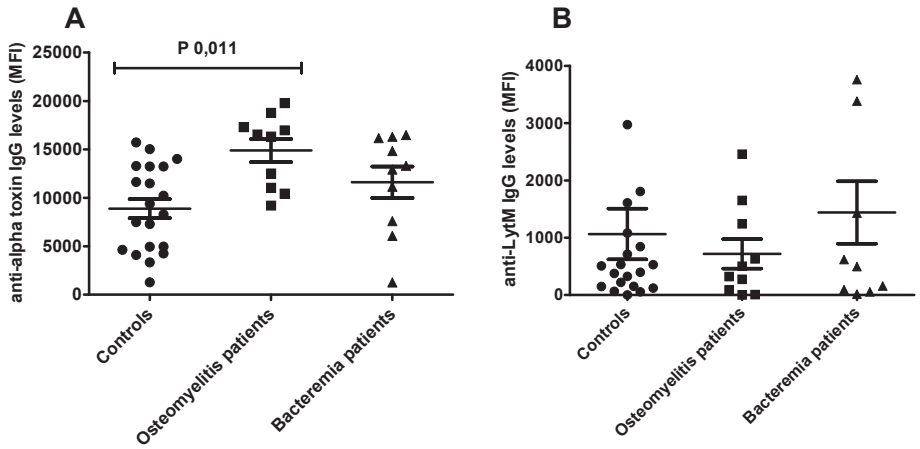

Figure S2. Comparison of IgG levels between patients and non-infected controls.
Peak IgG levels of 10 patients suffering from either chronic osteomyelitis or bacteremia and 20 non-infected age-matched controls are compared for **A:** alpha toxin and **B:** LytM. Average IgG levels against alpha toxin differed significantly between controls and osteomyelitis patients (P value indicated). IgG levels against LytM did not significantly differ between groups. The median value and interquartile range are represented by lines.

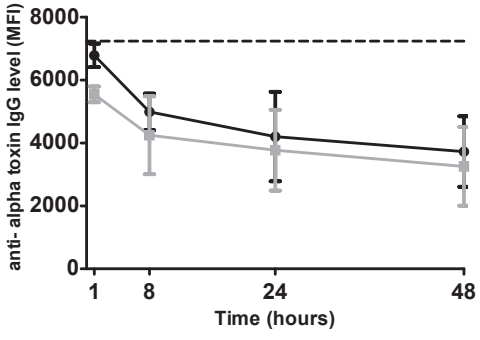

Figure S3. Detection of alpha toxin in 24hrs-biofilms of 10 strains and surrounding medium. The mean remaining non-bound IgG against alpha toxin was separately measured after incubation of PHG with biofilms of 10 strains on PS (dark circles) and after incubation with the IMDM culture medium covering the biofilms (light squares). Error bars indicate standard deviation and the dashed horizontal line indicates the average MFI of sterile controls. Similar results were obtained for the other secreted antigens (data not shown).

Table S1. Detection of genes, mRNA and proteins during biofilm formation of 10 *S. aureus* strains on PS and human bone.

Protein	Gene	Strains with gene present[1]	Biofilms on polystyrene		Biofilms on polystyrene			Biofilms on human bone		
			Strains with mRNA present[1]		Strains with significant reduction in specific IgG[1]			Strains with significant reduction in specific IgG[1]		
			8 hrs	24 hrs	8 hrs	24 hrs	48 hrs	8 hrs	24 hrs	48 hrs
Alpha toxin	*hla*	10	10	10	5	7	9	6	6	8
CHIPS	*chps*	7	4	4	3	5	7	6	5	6
ClfA	*clfA*	10	4	4	3	6	10	7	9	10
ClfB	*clfB*	10	10	10	2	7	9	5	8	8
Efb	*efb*	10	10	10	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
EsxB	*esxB*	10	5	5	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
ETA	*eta*	1	-	-	-	-	1	-	1	1
ETB	*etb*	-	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
FlipR	*flr*	9	7	5	0	4	5	3	5	5
FnbA	*fnbA*	10	10	10	-	2	5	1	5	6
FnbB	*fnbB*	5	5	5	1	3	2	-	2	1
Glucosaminidase	*Atl*	10	10	10	10	10	10	10	10	10
HlgB	*hlgB*	9	9	9	-	-	-	-	1	-
IsaA	*isaA*	10	10	10	10	10	10	10	10	10
IsdA	*isdA*	9	9	9	9	9	9	4	8	9
IsdH	*isdH*	10	10	10	3	3	6	2	2	1
Lipase	*lip*	10	10	10	1	6	8	1	2	1
LukD	*lukD*	8	8	8	0	2	4	1	3	4
LukE	*lukE*	8	8	8	0	2	3	0	1	1
LukF	*lukF*	-	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
LukS	*lukS*	-	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*

Table S1. Detection of genes, mRNA and proteins during biofilm formation of 10 *S. aureus* strains on PS and human bone. (continued)

Protein	Gene	Strains with gene present[1]	Biofilms on polystyrene		Biofilms on polystyrene			Biofilms on human bone		
			Strains with mRNA present[1]		Strains with significant reduction in specific IgG[1]			Strains with significant reduction in specific IgG[1]		
LytM	*lytM*	10	10	10	1	1	4	1	4	2
Nuc	*nuc*	8	5	1	7	7	8	7	8	8
PrsA	*prsA*	10	10	10	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
SACOL0486	*sacol0486*	6	5	5	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
SACOL0688	*MntC*	10	10	10	9	10	10	10	10	10
SasG	*sasG*	7	6	6	1	1	1	7	4	2
SCIN	*scn*	10	9	8	9	9	9	9	9	9
SdrD	*sdrD*	7	5	5	1	1	3	2	6	6
SdrE	*sdrE*	7	7	6	1	1	3	5	5	5
SEA	*sea*	2	2	2	0	0	2	2	2	2
SEB	*seb*	3	3	3	1	2	3	3	3	3
SEC	*sec*	-	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
SED	*sed*	1	1	1	1	1	1	1	1	1
SEE	*see*	-	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
SEG	*seg*	3	3	3	-	-	-	1	2	1
SEH	*seh*	1	1	1	-	-	-	1	1	1
SEI	*sei*	4	3	3	-	-	-	3	3	3
SEM	*sem*	4	4	3	-	-	-	4	2	3
SEN	*sen*	4	4	4	-	-	-	1	1	1
SEO	*seo*	4	4	4	-	-	-	-	1	-
SEQ	*seq*	2	2	2	1	2	2	2	2	2
SER	*ser*	1	1	1	0	1	1	1	1	1

Table S1. Detection of genes, mRNA and proteins during biofilm formation of 10 *S. aureus* strains on PS and human bone. (continued)

Protein	Gene	Strains with gene present[1]	Biofilms on polystyrene		Biofilms on polystyrene			Biofilms on human bone		
			Strains with mRNA present[1]		Strains with significant reduction in specific IgG[1]			Strains with significant reduction in specific IgG[1]		
SSL1	*ssl1*	10	9	8	-	-	1	-	1	1
SSL3	*ssl3*	10	10	9	-	-	1	-	1	1
SSL5	*ssl5*	6	5	6	-	-	-	-	1	-
SSL9	*ssl9*	2	-	-	-	-	-	-	-	-
SSL10	*ssl10*	5	1	2	-	-	-	-	1	1
SSL11	*ssl11*	5	1	2	1	1	2	-	-	-
TSST1	*Tst*	-	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*

[1]Number of isolates in which respectively the gene, mRNA or protein was detected are shown. - indicates that the gene, mRNA or protein was not detected in any isolate.
ND: not determined due to low signal intensities with coefficients of variation larger than 25% between duplicate experiments.

Table S2. *S. aureus* protein-specific IgG levels in controls and patient groups.

Antigen	Functional class	Mean IgG level control patients (± SD; N=20)	Mean IgG level bacteremia patients (± SD; N=10)[1]	Mean IgG level osteomyelitis patients (± SD; N=10)[1]	P value ANOVA[2]	P value Post-hoc analysis[3]
Alpha toxin	toxin	8895 (± 4419)	11610,9 (± 5117)	14884,3 (±3749)	0,037	0,011 (osteomyelitis)
CHIPS	immmune modulator	6578 (±3181)	7788 (±2854)	8670 (±3003)	0,105	
ClfA	surface protein	2375 (±2580)	2935 (±2639)	2601 (±1758)	0,753	
ClfB	surface protein	1736 (±1706)	2244 (±1893)	2775 (±1751)	0,321	
Efb	immmune modulator	ND	ND	ND	ND	
EsxB	housekeeping	ND	ND	ND	ND	
ETA	toxin	893 (±1425)	1238 (±1946)	2874 (±4178)	0,026	0,007 (osteomyelitis)
ETB	toxin	1194 (±1579)	575 (±787)	1210 (±2107)	0,401	
FlipR	immmune modulator	1864 (±1569)	4490 (±3485)	3656 (±2143)	0,007	0,019 (osteomyelitis + bacteremia)
FnbA	surface protein	332 (±358)	596 (±577)	686 (±1248)	0,079	
FnbB	surface protein	599 (±625)	587 (±840)	439 (±275)	0,656	
Glucosaminidase	housekeeping	5273 (±2827)	86780 (±3612)	8377 (±3882)	0,005	0,019 (osteomyelitis + bacteremia)
HlgB	toxin	6328 (±4290)	10838 (±2924)	10917 (±3627)	0,002	0,007 (osteomyelitis + bacteremia)
IsaA	housekeeping	6102 (±3676)	9373 (±4930)	7860 (±5526)	0,240	
IsdA	surface protein	3722 (±4532)	6534 (±3993)	5016 (±2756)	0,006	0,023 (osteomyelitis + bacteremia)
IsdH	surface protein	825 (±867)	2579 (±3130)	2732 (±4407)	0,034	0,011 (bacteremia)
Lipase	housekeeping/ toxin	4072 (±2550)	6557 (±4197)	7155 (±4412)	0,251	
LukD	toxin	6311 (±3988)	9512 (±3514)	9369 (±3581)	0,024	0,044 (osteomyelitis + bacteremia)
LukE	toxin	8859 (±4327)	11743 (±3621)	11510 (±3494)	0,087	
LukF	toxin	1105 (±880)	1851 (±772)	2025 (±917)	0,001	0,005 (osteomyelitis + bacteremia)
LukS	toxin	8382 (±4659)	10988 (±4031)	10518 (±3915)	0,127	

Table S2. *S. aureus* protein-specific IgG levels in controls and patient groups. (continued)

Antigen	Functional class	Mean IgG level control patients (± SD; N=20)	Mean IgG level bacteremia patients (± SD; N=10)[1]	Mean IgG level osteomyelitis patients (± SD; N=10)[1]	P value ANOVA[2]	P value Post-hoc analysis[3]
LytM	housekeeping	950 (±1706)	1171 (±1500)	900 (±1295)	0,971	
Nuc	housekeeping/ toxin	2412 (±2697)	4430 (±3612)	3215 (±1882)	0,132	
PrsA	housekeeping	ND	ND	ND	ND	
SACOL0486	housekeeping	ND	ND	ND	ND	
SACOL0688	housekeeping	839 (±650)	3849 (±4290)	2572 (±2432)	0,001	0,005 (osteomyelitis + bacteremia)
SasG	surface protein	391 (±673)	270 (±293)	299 (±269)	0,786	
SCIN	immmune modulator	3665 (±3322)	7939 (±3805)	7545 (±3782)	<0,000	0,002 (osteomyelitis + bacteremia)
SdrD	surface protein	668 (±480)	1306 (±1620)	992 (±1158)	0,437	
SdrE	surface protein	293 (±298)	399 (±385)	651 (±441)	0,026	0,007 (osteomyelitis)
SEA	toxin	3219 (±3300)	3199 (±3135)	3669 (±2842)	0,571	
SEB	toxin	2996 (±3016)	3969 (±3906)	4613 (±3714)	0,244	
SEC	toxin	9164 (±4334)	8098 (±4407)	9157 (±3739)	0,633	
SED	toxin	866 (±909)	1322 (±1338)	1794 (±2249)	0,231	
SEE	toxin	1344 (±2024)	1190 (±1493)	1266 (±1428)	0,749	
SEG	toxin	745 (±966)	907 (±1913)	958 (±1136)	0,651	
SEH	toxin	2044 (±1920)	1584 (±1667)	3079 (±2799)	0,219	
SEI	toxin	664 (±539)	1268 (±1661)	2009 (±1561)	0,157	
SEM	toxin	526 (±542)	1155 (±1595)	1322 (±966)	0,028	0,011 (osteomyelitis)
SEN	toxin	677 (±910)	665 (±995)	703 (±1141)	0,967	
SEO	toxin	120 (±65)	223 (±317)	361 (±416)	0,060	
SEQ	toxin	1120 (±1599)	1900 (±3039)	3274 (±3907)	0,372	

Table S2. *S. aureus* protein-specific IgG levels in controls and patient groups. (continued)

Antigen	Functional class	Mean IgG level control patients (± SD; N=20)	Mean IgG level bacteremia patients (± SD; N=10)[1]	Mean IgG level osteomyelitis patients (± SD; N=10)[1]	P value ANOVA[2]	P value Post-hoc analysis[3]
SER	toxin	1265 (±1985)	834 (±1187)	1501 (±3436)	0,869	
SSL1	immmune modulator	2794 (±2281)	4848 (±3456)	4887 (±3477)	0,117	
SSL3	immmune modulator	4679 (±3068)	8186 (±4746)	6955 (±3673)	0,011	0,042 (osteomyelitis + bacteremia)
SSL5	immmune modulator	1929 (±1307)	4675 (±3315)	3932 (±2827)	0,001	0,014 (osteomyelitis + bacteremia)
SSL9	immmune modulator	6835 (±3575)	9240 (±3735)	8892 (±4048)	0,115	
SSL10	immmune modulator	3051 (±4038)	5032 (±3893)	5425 (±4038)	0,175	
SSL11	immmune modulator	1087 (±1540)	1332 (±1297)	1793 (±2149)	0,425	
TSST1	toxin	8217 (±6646)	7865 (±3942)	9110 (±4057)	0,545	

[1]Only the peak IgG levels of patients were included for comparison.

[2]P value of ANOVA test comparing all three patient groups. P values < 0.05 were considered as significant.

[3]P value of post-hoc tests with LSD are shown (patient group(s) with significantly higher mean IgG level(s) compared to controls are indicated).

ND: not determined due to low signal intensities with coefficients of variation larger than 25% between duplicate experiments.

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Chapter 4

Detection of alpha-toxin and other virulence factors in biofilms of *Staphylococcus aureus* on polystyrene and a human epidermal model

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ABSTRACT

Background & aim: The ability of *Staphylococcus aureus* to successfully colonize (a)biotic surfaces may be explained by biofilm formation and the actions of virulence factors. The aim of the present study was to establish the presence of 52 proteins, including virulence factors such as alpha-toxin, during biofilm formation of five different (methicillin resistant) *S. aureus* strains on Leiden human epidermal models (LEMs) and polystyrene surfaces (PS) using a competitive Luminex-based assay.

Results: All five *S. aureus* strains formed biofilms on PS, whereas only three out of five strains formed biofilms on LEMs. Out of the 52 tested proteins, six functionally diverse proteins (ClfB, glucosaminidase, IsdA, IsaA, SACOL0688 and nuclease) were detected in biofilms of all strains on both PS and LEMs. At the same time, four toxins (alpha-toxin, gamma-hemolysin B and leukocidins D and E), two immune modulators (formyl peptide receptor-like inhibitory protein and Staphylococcal superantigen-like protein 1), and two other proteins (lipase and LytM) were detectable in biofilms by all five *S. aureus* strains on LEMs, but not on PS. In contrast, fibronectin-binding protein B (FnbpB) was detectable in biofilms by all *S. aureus* biofilms on PS, but not on LEMs. These data were largely confirmed by the results from proteomic and transcriptomic analyses and in case of alpha-toxin additionally by GFP-reporter technology.

Conclusion: Functionally diverse virulence factors of (methicillin-resistant) *S. aureus* are present during biofilm formation on LEMs and PS. These results could aid in identifying novel targets for future treatment strategies against biofilm-associated infections.

INTRODUCTION

Staphylococcus aureus is the causative agent of a variety of infections with generally significant morbidity and mortality. The incidence of both hospital and community acquired infections caused by methicillin-resistant *S. aureus* (MRSA) has increased significantly in the last decades [1–3]. Unfortunately, the treatment of such infections is becoming increasingly complex as current antibiotics may be less effective due to resistance development and biofilm formation [4]. As the number of newly approved antimicrobial agents continues to decrease [5,6], alternative strategies for prevention and/or treatment of bacterial colonization and infection, such as a vaccines [7] and antimicrobial peptides [8], are urgently needed. To date no clinically successful vaccine against *S. aureus* has been developed, despite the promising results of vaccines targeting diverse virulence factors of this pathogen in animal models [9,10]. Currently, the awareness that multiple virulence factors of *S. aureus* should be targeted for any vaccine or other strategy to be successful is increasing [9]. Moreover, some relation between the expression of antibodies against *S. aureus* virulence factors and protection from infection has been made [11].

The capacity of *S. aureus* to cause infections is attributed to its vast array of virulence factors which include adhesive surface proteins, secreted immune modulators, enzymes and toxins [7]. Moreover, many infections such as those of (wounded) skin, mucosae and artificial surfaces [12] are believed to involve biofilm formation by *S. aureus*. Biofilms are defined as complex communities of bacteria encased in an extracellular polymeric matrix and biofilm formation is believed to contribute to bacterial virulence, reduced susceptibility to antibiotics [13–15] and reduced clearance by the immune system. Despite the plethora of studies examining the involvement of biofilm formation [16](1, 2) and/or single virulence factors [17,18] in e.g. skin infections, so far only a few studies has focussed on the involvement of multiple virulence factors in association with biofilm formation by *S. aureus* during infection [19,20].

Biofilm formation by *S. aureus* on polystyrene (PS) has been extensively characterized before [21,22]. However, biofilm formation on human biotic surfaces is much less characterized and the associated pathogen-host interactions are unclear. Earlier we reported that Leiden epidermal models (LEMs) mimic the human skin in many ways, including epidermal morphology and barrier properties [23]. In addition, full thickness human skin equivalents have been used to study skin colonization by (methicillin resistant) *S. aureus* [24,25].

The aim of the current study was to establish the presence of 52 proteins, including virulence factors such as alpha-toxin, during biofilm formation by five different (methicillin-resistant) *S. aureus* strains on LEMs and PS. Using the novel competitive Luminex-based assay (CLA; [26] we detected six proteins (ClfB, glucosaminidase, IsdA,

IsaA, SACOL0688 and nuclease) in biofilms of all biofilm-forming strains on the two surfaces. At the same time, surface- and strain-dependent differences were found for the presence of a wide range of other proteins, such as immune modulators and toxins like alpha-toxin.

MATERIALS AND METHODS

Ethics statement

Human serum was obtained from healthy volunteers who gave written consent for use of serum solely for research purposes within the department of Medical Microbiology and Infectious Diseases at the Erasmus MC Rotterdam. Serum was coded, pooled and has been used for this and earlier studies [27,28]. The original list with documented volunteer names was only accessible to qualified physicians within the department, amongst the current authors only including PMdR. This sampling procedure was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam (MEC-2007-106, addendum 2) [28]. All primary human skin cells from healthy donors used by the Department of Dermatology are isolated from surplus tissue collected according to article 467 of the Dutch Law on Medical Treatment Agreement and the Code for proper Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies [29]. According to article 467, coded anonymous surplus tissue can be used if no objection is made by the patient. All patients were informed of the possibility that surplus tissue could be used for scientific research and all patients were offered the opportunity to give written refusal to this. Only tissue from patients who did not opt out was used. None of the authors were involved in the tissue sampling and only birth date, gender and skin type of the subjects were documented. These data were only accessible to EMH and PHN. Because this procedure, as published previously [8,24-25], is in accordance with national law and additional approval of an ethics committee regarding scientific use of surplus tissue is not required, we did not seek specific approval by our ethics committee. The Declaration of Helsinki principles were followed when working with human tissue.

Staphylococcus aureus strains

The following *S. aureus* strains were used in this study: methicillin-resistant strains LUH14616 (sequence type 247), a kind gift of dr. S. Croes [30]; LUH15051 (ST 239) obtained from dr. M.E.O.C. Heck, (Laboratory of Infectious Diseases and Screening, RIVM, Bilthoven, The Netherlands); USA300 strain Sac042w (ST 8) described earlier [31]; a strain derived from an impetigo patient LUH15091 (ST121) within the Erasmus Medical Center and NCTC 8325-4 (ST 8). All strains were typed using multi locus sequence typing (MLST) [27,32]. Before usage the strains were grown on sheep blood agar plates (Biomerieux).

Biofilm formation on polystyrene plates

A routine biofilm model was used as described before [21,22]. In short, overnight plate cultures of *S. aureus* were re-suspended in Iscove's Modified Dulbecco's Medium (IMDM, Life technologies, Carlsbad, CA, USA) without phenol red until an optical density (OD, 660nm) of 2 was reached. IMDM medium was chosen because of its significant impact on detectable levels of bacterial proteins, e.g. IsdA, ClfB and Efb, expressed by *S. aureus* biofilms on PS [26]. One µl of this bacterial suspension was added to 199 µl of TSB supplemented with 0.5% (wt/v) glucose and 3% (wt/v) NaCl or IMDM without any supplement in sterile 96-wells PS plates (Greiner Bio-one). Plates were then incubated at 37°C with gentle shaking at 200 rpm for various intervals. Biofilm mass was measured by staining with 1% crystal violet. OD was measured at 490 nm.

Leiden epidermal models

The epidermis and dermis of pieces of fresh plastic surgery surplus skin were enzymatically and mechanically separated, and each layer was subsequently digested to obtain single-cell suspensions [33]. The keratinocytes were cultured in keratinocyte medium, i.e. 3 parts DMEM (Gibco/Invitrogen) and 1 part Ham's F12 medium supplemented with 5% (v/v) fetal bovine serum (FBS; HyClone/Greiner), 0.5 µm hydrocortisone, 1 µm isoproterenol, 0.1 µm insulin (all from Sigma–Aldrich, Zwijndrecht, The Netherlands), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Invitrogen). Leiden epidermal models (LEMs; Fig 1A) were made with these primary human keratinocytes as described before [34]. Briefly, one day before generation of the models, medium of the keratinocyte cultures was switched to Dermalife (Lifeline Cell Technology) supplemented with penicillin (10,000 U/ml) and streptomycin (10 mg/ml). The next day 2×10^5 keratinocytes were seeded onto a filter insert (0.4 µM Costar inserts; Corning) in 12-well plates in this Dermalife medium. Three days thereafter the apical medium was removed, leaving the keratinocytes air-exposed. The medium under the filter insert was switched to CnT-02-3D medium (CellnTech) mixed with keratinocyte medium supplemented with 2.4×10^{-2} µM bovine serum albumin and lipids/antibiotics as described above. One day before bacterial inoculation of the LEMs, the medium was switched to this medium without antibiotics. All experiments were performed on 10-day air-exposed cultures.

Colonization of Leiden epidermal model

LEMs were exposed for 1 h at 37°C in 7.3% CO_2 to 300 µl of a log-phase bacterial suspension containing $3.3x10^5$ CFU/ml. Next, the non-adherent bacteria were removed by aspiration and at different intervals thereafter, the viable detachable bacteria were collected in 1 ml of PBS, and serially diluted, and 50 µl of these samples were plated onto diagnostic sensitivity test (DST) agar plates (Oxoid) to determine the number of CFU. To assess the number of adherent bacteria, a model was cut in two equally sized pieces.

One piece was used for histology and the other was homogenized in PBS using a glass Potter-Elvehjem tissue homogenizer, and the homogenates were subsequently serially diluted and plated as described above. The lower limits of detection for detachable and adherent bacteria were 20 and 40 CFU/LEM, respectively.

Histology

One biopsy of each model was fixed in 4% (v/v) formaldehyde, dehydrated, and embedded in paraffin. Next, paraffin blocks were cut into 5-µm sections, deparaffinized, rehydrated, and then stained with hematoxylin and eosin (H&E) staining.

Cryo scanning electron microscopy

For the morphological study of (methicillin resistant) *S. aureus* biofilms on LEM or PS by cryo-scanning electron microscopy (SEM), specimens were quickly frozen in liquid nitrogen slush and transferred directly to the cryo-transfer attachment (Gatan Alto2500). Samples were sublimated at -90 in high vacuum for 5 min and subsequently sputter-coated with a layer of 20 nm gold/paladium and examined in a JEOL JSM6700F scanning electron microscope.

Multiplex bead assay for assessment of the presence of proteins during *S. aureus* biofilm formation

A multiplex competitive Luminex assay [26] (CLA) with minor modifications was used to indirectly detect the presence of 52 IgG-accessible proteins in bacterial cultures (all bacterial proteins are listed in Table S1). In brief, log-phase cultures of *S. aureus* were diluted 1:200 and incubated in PS wells for 1, 8, 24 and 48 hrs. After washing with ice-cold PBS supplemented with 0.5% (wt/v) sodium azide (Sigma-Aldrich), adherent bacteria residing in biofilms on PS or LEMs were incubated at 8°C and continuous shaking (500 rpm) with 200 µl of a 1:200 dilution of polyclonal human IgG (PHG), isolated using the HiTrap™ Protein G HP column according to the manufacturer's guidelines (GE Healthcare Bio-sciences, Piscataway, New Jersey, USA), from pooled serum of 40 healthy volunteers (19 non-nasal carriers, 6 intermittent and 15 persistent nasal carriers of *S. aureus* as determined earlier [35]. After 35 min incubation the PHG samples were recovered from biofilms. The remaining non-bound IgG antibody levels in these samples, specifically directed against 52 proteins of *S. aureus*, were measured using a multiplex bead-based flow cytometry technique (xMAP®, Luminex corporation) wherein recombinant proteins were covalently coupled to the beads as described previously [27,28,36]. As negative controls PHG samples incubated with empty PS wells or sterile LEMs were included in all experiments.

Next, the percentage decrease in the levels of specific IgG antibodies for each protein was calculated in relation to the negative control. The percentage decrease can be

considered a semi-quantitative measure of the protein-specific antibody absorption from PHG by the biofilm, thus indirectly reflecting the presence of the particular *S. aureus* protein by the biofilm [26]. The average percentage decrease plus two times the standard deviation, obtained at 8, 24 and 48 hours biofilm growth, for the three non-*S. aureus* control proteins and all *S. aureus* proteins of which genes were not present in LUH14616 were chosen as cut-off value (35% at 8 and 24 hrs biofilm growth and 40% at 48 hrs, respectively).

In case of bacteria adherent to LEMs, the same protocol was followed with the single modification that PHG samples were directly incubated on top of the LEM. To determine the presence of *S. aureus* proteins in culture supernatants, growth medium that covered biofilms grown on PS was analyzed using the same protocol, with the modification that medium was removed at designated time points and incubated with PHG samples in sterile wells.

Reverse transcriptase PCR

Early biofilms (8 and 16 hrs) were grown in 96-well plates (Cellstar culture plates, Greiner Bio-One) in 200 µl of IMDM (Gibco). Biofilms were resuspended, pooled and centrifuged at 4000 rpm for 10 min at 4°C. Pellets were resuspended in 200 µl of RNA protect™ Bacterial reagents (Qiagen), stabilized for 5 min and then centrifuged for 10 min at 4°C. The pellet was dissolved in 1 ml of RNA-pro solution (Fast RNA Pro Blue kit, MP Biomedicals) and stored at -20°C until use. RNA was isolated using the Fast RNA Pro Blue kit according to the manufacturer's protocol. Each 10 µg of isolated RNA was treated twice with 2 U TURBO DNase (Ambion, Life Technologies). The reaction was stopped by adding 0.2 volumes of DNase inactivation reagent (Ambion) and incubation for 2 min at ambient temperature. RNA containing supernatants were collected by centrifugation (1.5 min at 9000 g at ambient temperature) and each 2 µg DNase-treated RNA was treated with 2 U DNase I (Fermentas, Fisher Scientific). One µg of prepared RNA was transcribed into cDNA using 200 U RevertAid H Minus Reverse transcriptase (Fermentas), 4 µl of 5x reaction buffer (Fermentas), 20 U of RiboLock RNase inhibitor (Fermentas) and 2 µl of 10 mM dNTP mix (Fermentas) in a final volume of 20 µl of DEPC-treated water. This was incubated for 60 min at 42°C and then terminated by heating at 70°C for 5 min. For each RNA sample a negative control without reverse transcriptase was processed similarly. The presence of cDNA in all samples was examined using PCR as described previously [27].

Proteomics

A total of 48 biofilms of strain LUH14616 were grown for 8 hours in PS wells as described above, resuspended in 200 µl of PBS per well, pooled and spun down at 4,000 rpm for 5 min. The resulting pellet was resuspended in 50 µl of PBS, mixed with 50 µl of Laemni

buffer and heated for 5 min at 95°C. Fifty µl of this suspension containing denatured proteins were run on a 15% SDS gel (Biorad) and gel lanes were cut into ~1 mm slices. Lanes were subjected to in-gel reduction with dithiothreitol, alkylation with chloroacetamide and digestion with trypsin (Promega, Leiden, The Netherlands). Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo), operating in positive mode and equipped with a nanospray source. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode by CID. Peak lists were automatically created from raw data files using the Proteome Discoverer (version 1.3; Thermo). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the Uniprot database (release 2013_06.fasta, taxonomy: *S. aureus,* strains USA300, Newman, NCTC 8325-4 and COL). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamido-methylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 65. Individual peptide MS/MS spectra with Mascot scores below 25 were checked manually and either interpreted as valid identifications or discarded.

Construction of the *hla* promotor upstream of GFP_{uvr}

S. aureus strains LUH14616 and Sac042w containing a vector with an *hla* promotor upstream of GFP_{uvr} were prepared as described earlier [36] with some modifications. First, the promotor of *hla* was amplified using primers hlapr1 (cggaattcgatatttctatgtaatggca) and hlapr2 (gctctagacttctatttttttgaacgat) and as a template DNA from *S. aureus* strain Newman. Next, the amplification product was ligated into the EcoRI XbaII site of pALC1484 (a kind gift from dr A.L. Cheung, Dartmouth College, New Hampshire, US) and cloned into *E. coli* DH10beta. From positive colonies on LB agar supplemented with 50 µg of ampicillin/ml, recombinant plasmids were isolated, checked by PCR and sequencing, and then electroporated into *S. aureus* RN4220. Finally, from positive colonies on BHI agar supplemented with 10 µg of chloramphenicol/ml, plasmids were isolated and electroporated into *S. aureus* LUH14616 and Sac042w. As positive and negative control we electroporated respectively pWVW 163, a plasmid containing a phage promotor yielding a strong, constant expression of GFP, and pALC1484, an empty vector [37], into the same *S. aureus* strains as described above.

Data analysis

All data were analysed using Microsoft Excel version 2010 and graphics were made using Graphpad Prism version 5 (Graphpad Inc. La Jolla, CA, USA).

RESULTS

Biofilm formation by MRSA strain LUH14616 on LEMs and PS

Firstly, the ability of the clinical isolate MRSA LUH14616 to form biofilms on both the human skin model (Leiden Epidermal Model: LEM, schematically represented in Figure 1A), and polystyrene (PS) was examined. This MRSA strain was able to adhere to and stably colonize both surfaces, as reflected by an increase in bacterial counts on LEM (Figure 1B) and an increase in crystal violet staining on PS (Figure 1E) within the first 24 hrs after inoculation. Interestingly, haematoxylin-eosin staining of the colonized LEMs showed that the bacteria adhered to the *stratum corneum* and formed small colonies after 16 hrs, but did not invade the epidermis (Figure 1C). To further examine biofilm formation by MRSA strain LUH14616 on these surfaces, bacterial colonization on LEM (Figure 1D) and PS (Figure 1F) was visualized with scanning electron microscopy. Results revealed a tightly adherent layer of bacteria on both LEMs and PS after 24-48 hrs, indicating the development of a mature biofilm on both surfaces. Biofilm-associated bacteria on LEM appeared to be completely encased in an extracellular matrix (Figure 1D), while bacteria on PS appeared to be incompletely encased (Figure 1F).

Detection of toxins, immune modulators and other proteins of MRSA strain LUH14616 during biofilm formation on PS

We used a competitive Luminex-based assay (CLA) to establish the presence of 52 bacterial proteins during biofilm formation by MRSA strain LUH14616 on polystyrene (PS).

In line with previous results [26], biofilm mass-dependent absorption of specific IgG for several *S. aureus* proteins, such as IsdA (Figure 2A), FnbpB (Figure 2C) and glucosaminidase by biofilms was detected, while no such reduction was seen for the levels of IgG antibodies directed against control proteins, e.g. the protein derived from human metapneumovirus (hMPV) (Figure 2B). Based on the percentage decrease in the levels of IgG directed against the three non-*S. aureus* control proteins and against the 28 *S. aureus* proteins of which genes were not found in LUH14616 using PCR, cut-off values of at least 35% decrease in specific IgG at 24 hrs biofilm growth and 40% at 48 hrs were calculated. CLA measurements for five proteins [ESX-1-associated factors EsxA and EsxB, iron surface determinants H (IsdH), Staphylococcal enterotoxin J (SEJ) and foldase-protein PrsA] were excluded from further analysis due to low MFI's with standard deviations larger than 25% between repeated CLA measurements.

Using the above mentioned cut-off values, we detected 8 proteins in 24 hrs and 48 hrs-old *S. aureus* biofilms: the surface proteins fibronectin-binding protein B (FnbpB), ClfB, glucosaminidase, iron-responsive surface determinant A (IsdA), immunodominant antigen A (IsaA), SACOL0688, nuclease, and the immune modulator Efb (Table 1). In addition, a significant decrease in the levels of IgG specifically directed against chemo-

taxis inhibitory protein of *S. aureus* (CHIPS) with 48 hrs-old biofilms, but not 24 hrs-old biofilms, was observed. No significant decrease in the levels of specific IgG for 15 other proteins, despite the presence of corresponding genes in LUH14616 such as for alpha toxin, was observed (Figure 2D).

Additional experiments showed that the secreted proteins alpha-toxin, HlgB, FLIPr and SSL1 could neither be detected in the growth medium covering biofilms, excluding

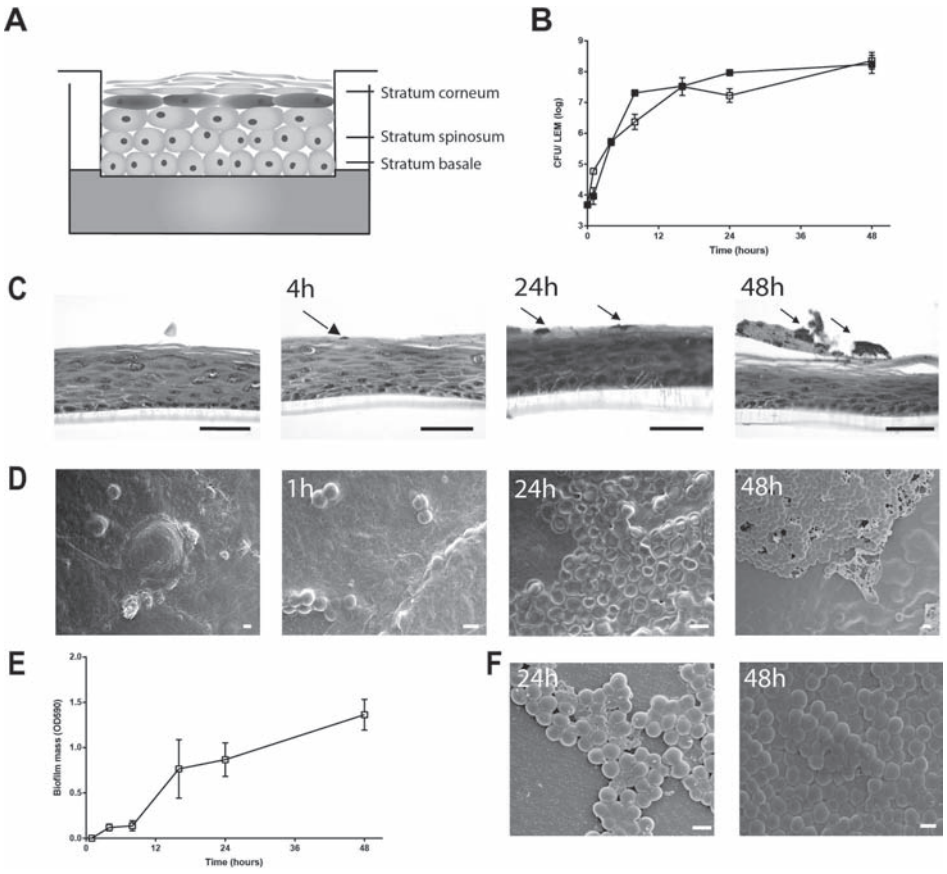

Figure 1. Biofilm formation by *S. aureus* LUH14616 on LEMs and PS surfaces.
A: Schematic representation of LEM. **B**: Bacterial counts were performed on LEM exposed to LUH14616 for various intervals. Adherent bacteria are represented by open symbols and non-adherent/loosely adherent bacteria by closed symbols. Results are displayed as the mean and SD of four experiments.
C: Haematoxilin and eosine staining of LEMs at various intervals after inoculation with LUH14616. Arrows indicate microcolonies, scale bars = 50 µm. **D**: Cryo scanning electron microscopy of LEMs colonized with LUH14616 for various intervals. Photographs are representative for three different keratinocyte donors. **E**: Biofilm formation by LUH14616 on PS in IMDM medium. Results are the mean and SEM of three experiments. **F**: Cryo scanning electron microscopy of *S. aureus* LUH14616 biofilms formed on PS at 24 and 48 hrs after adherence to wells. Scale bars =1 µm.

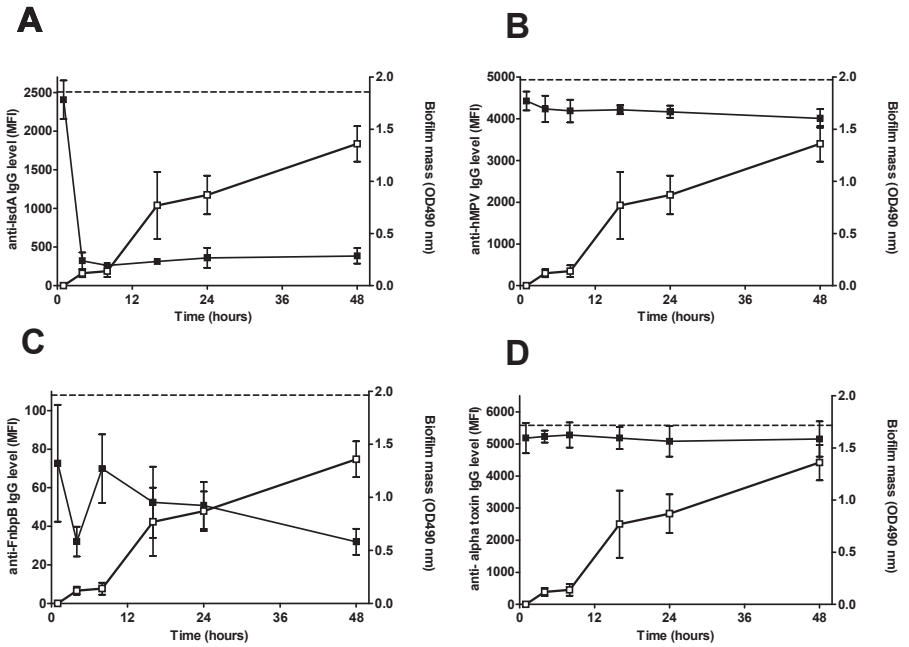

Figure 2. Detection of *S. aureus* proteins during biofilm formation of LUH14616 on PS.
Closed symbols indicate the mean fluorescence intensity (MFI, left Y-axis), reflecting the level of remaining non-bound IgG directed against specific proteins after incubation of PHG with the biofilms, while open symbols indicate biofilm mass (OD490 nm, right Y-axis). Both are plotted against the time of biofilm growth (hrs).
Results are shown for **A:** IsdA, **B:** control protein of human metapneumovirus (hMPV), **C:** FnbpB and **D**: alpha toxin. Dashed horizontal lines indicate the average MFI of sterile controls. Symbols and error bars indicate mean and SD of four experiments.

the possibility of false-negative signals for these secreted proteins (Figure S1). CLA data were further validated by confirming the presence of 7 out of the 8 detected proteins in early (8 hrs) biofilms using mass-spectrometry, while mRNA was detected for 5 of these proteins in early biofilms.

Detection of toxins, immune modulators and other proteins of MRSA strain LUH14616 during biofilm formation on LEMs

Next we screened for the presence of the same 52 proteins in biofilms of LUH14616 grown on LEM. Similar to biofilms grown on PS we observed time dependent absorption of antibodies against diverse antigens such as IsdA (Figure3A) and glucosaminidase, whereas no such reduction was observed for antibodies directed against the non-*S. aureus* control proteins (Figure3B) and the proteins of which the gene was not found in LUH14616. This prompted us to use the same cut-off values.

Table 1. Detection of mRNA and proteins during biofilm formation of LUH14616 on LEMs and PS.

			Biofilms on polystyrene		Biofilms on polystyrene			Biofilms on LEM	
			mRNA present[3]	Protein detectable[4]	Significant reduction in specific IgG[5]			Significant reduction in specific IgG[5]	
Protein[1]	Gene[2]	Functional class	8 hrs	8 hrs	8 hrs	24 hrs	48 hrs	24 hrs	48 hrs
CHIPS	*chp*	immmune modulator	Yes	Yes	-	-	+	-	+
ClfB	*clfB*	surface protein	Yes	Yes	+	+	+	+	+
Glucosaminidase	*Atl*	housekeeping	No	Yes	+	+	+	+	+
IsaA	*isaA*	housekeeping	Yes	Yes	+	+	+	+	+
IsdA	*isdA*	surface protein	Yes	Yes	+	+	+	+	+
Nuc	*nuc*	housekeeping/ toxin	No	Yes	+	+	+	+	+
SACOL0688	*MntC*	housekeeping	Yes	Yes	+	+	+	+	+
Efb	*efb*	immmune modulator	No	Yes	+	+	+	-	-
FnBPB	*fnbB*	surface protein	Yes	No	+	+	+	-	-
Alpha toxin	*hla*	toxin	Yes	No	-	-	-	+	+
FlipR	*flr*	immmune modulator	Yes	No	-	-	-	+	+
HlgB	*hlgB*	toxin	Yes	No	-	-	-	+	+
Lipase	*lip*	housekeeping/ toxin	Yes	No	-	-	-	+	+
LukD	*lukD*	toxin	Yes	No	-	-	-	+	+
LukE	*lukE*	toxin	Yes	No	-	-	-	+	+
LytM	*lytM*	housekeeping	Yes	No	-	-	-	+	+
SSL1	*ssl1*	immmune modulator	Yes	No	-	-	-	-	+
FnBPA	*fnbA*	surface protein	No	Yes	-	-	-	-	-

Table 1. Detection of mRNA and proteins during biofilm formation of LUH14616 on LEMs and PS. (continued)

			Biofilms on polystyrene		Biofilms on polystyrene			Biofilms on LEM	
			mRNA present[3]	Protein detectable[4]	Significant reduction in specific IgG[5]			Significant reduction in specific IgG[5]	
SCIN	*scn*	immmune modulator	Yes	No	-	-	-	-	-
SdrD	*sdrD*	surface protein	Yes	Yes	-	-	-	-	-
SEA	*sea*	toxin	Yes	No	-	-	-	-	-
SSL3	*ssl3*	immmune modulator	Yes	No	-	-	-	-	-
SSL5	*ssl5*	immmune modulator	Yes	No	-	-	-	-	-
SSL10	*ssl10*	immmune modulator	Yes	No	-	-	-	-	-

[1]Only proteins are shown for which corresponding genes were detected in LUH14616 and for which standard deviation between 4 CLA experiments did not exceed 25%. From top to bottom protein groups are shown that were detected on both LEMs and PS, only on PS, only on LEMs or on neither surface, respectively.

[2]Additional ORF IDs for all genes, based on sequences of *S. aureus* strain 8325-4 (SAOUHSC) or Newman (NWMN), are available online (http://www.uniprot.org/).

[3]Presence of mRNA was established using RT-PCR in early 8 hrs biofilms on PS.

[4]Presence of proteins was established using mass spectrometry in early 8 hrs biofilms on PS

[5]Significant reduction in the levels of IgG specific for each protein, indicative of the presence of the protein during biofilm formation, was defined as a reduction in IgG (compared to sterile controls) of at least 35% at 8 and 24 hrs biofilm growth and 40% at 48 hrs.

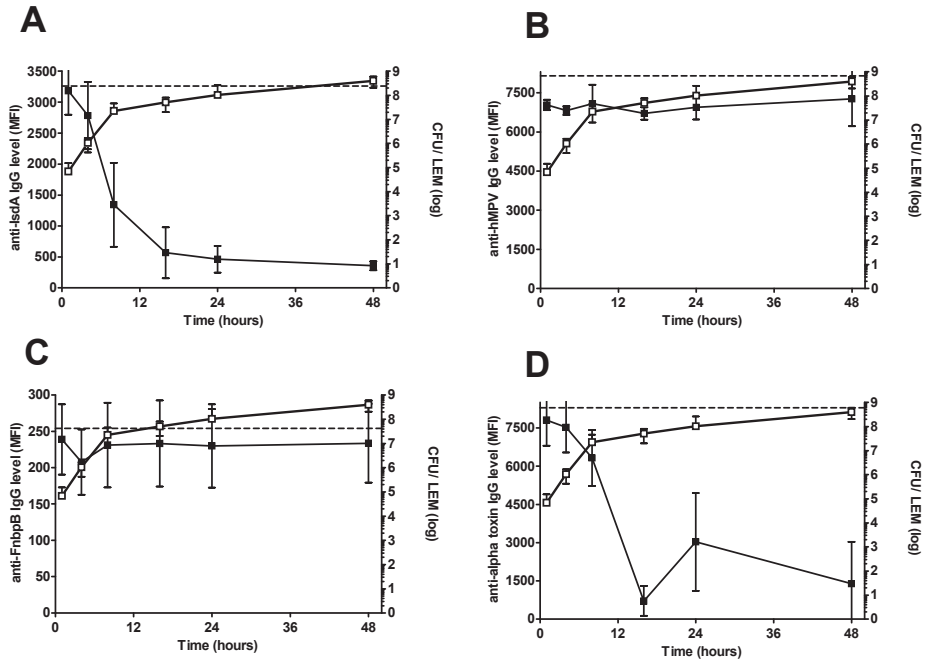

Figure 3. Detection of *S. aureus* proteins during biofilm formation of LUH14616 on LEMs. Closed symbols indicate the mean fluorescence intensity (MFI, left Y-axis), reflecting the level of remaining non-bound IgG directed against specific proteins after incubation of PHG with the bacterial biofilms, while open symbols indicate biofilm mass (OD490 nm, right Y-axis). Both are plotted against the time of biofilm growth (hrs). Results are shown for **A**: IsdA, **B**: control protein of human metapneumovirus (hMPV), **C:** FnbpB and **D**: alpha toxin. Dashed horizontal lines indicate the average MFI of sterile controls. Symbols and error bars indicate mean and SD of four experiments, respectively.

Thirteen proteins were detected in 24 and 48 hrs-old biofilms on LEMs (Table 1): the surface proteins clumping factor B (ClfB), glucosaminidase, IsdA, IsaA, glycyl-glysine endopeptidase (LytM), and SACOL0688; the toxins alpha-toxin (Figure3D), gamma-hemolysin B (HlgB), leukocidins (Luk) D and E, lipase and nuclease; and the immune modulator formyl peptide receptor-like inhibitory protein (FLIPr). In addition, CHIPS and staphylococcal superantigen-like protein 1 (SSL 1) were detected in 48 hrs biofilms, but not 24 hrs biofilms. In contrast to biofilms on PS, no significant reduction was observed for antibodies against FnbpB at any time point (Figure3C).

Detection of proteins during biofilm formation on LEMs and PS by different *S. aureus* strains

To determine whether the results obtained for MRSA LUH14616 are representative for other *S. aureus* strains, experiments with 24 hrs-old biofilms of an additional set of four, genetically diverse *S. aureus* strains were performed: i.e. LUH15051, LUH15091, the USA300 strain Sac042w, and NCTC 8325-4. Results revealed considerable variance in

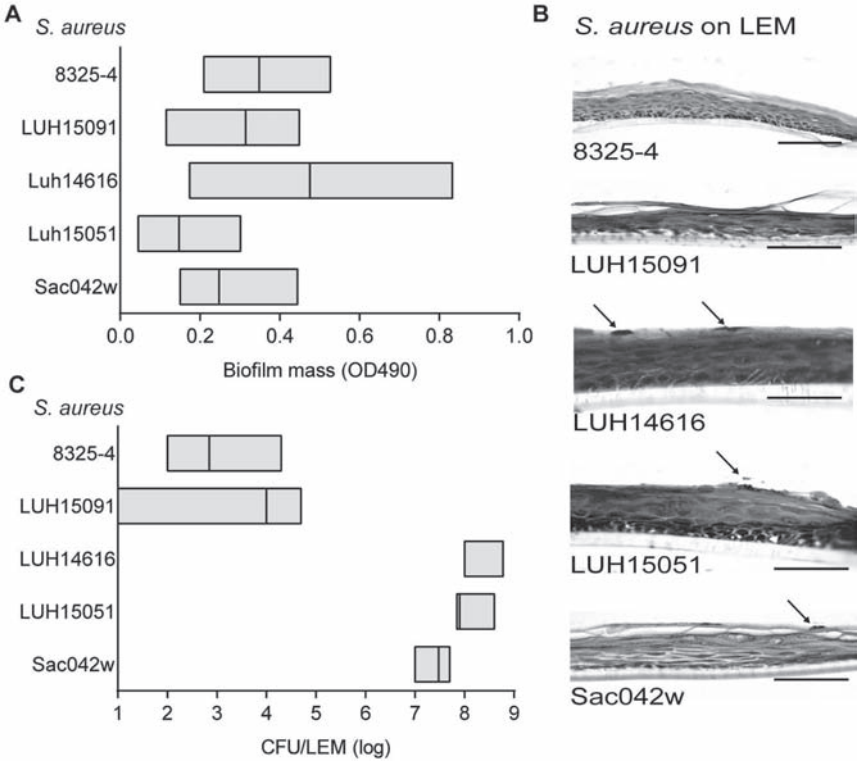

Figure 4. Biofilm formation by 5 different *S. aureus* strains on LEMs and PS surfaces.
A: Biofilm formation after 24 hrs on PS was measured by crystal violet staining. **B**: Hematoxylin and eosine staining of LEMs 24 hrs after exposure to *S. aureus* 8325-4, LUH15091, LUH15051, LUH14616 or Sac042w, arrows indicate *S. aureus*. Photographs are representative for three different experiments. Scale bars = 50 µm. **C**: The number of viable bacteria present on epidermal models after 24 hrs inoculation was determined microbiologically (CFU/LEM). Results are boxplots showing the median and range. Results are means and SEM of three to five experiments.

biofilm mass formed on the PS plates and LEMs among the different strains (Figures 4A-C). Interestingly, strains 8325-4 and LUH15091 formed a significant biofilm on PS, but not on LEMs. The latter two strains were therefore excluded from further analyses. The same cut-off values were used as for LUH14616.

In agreement with the results for LUH14616, the proteins ClfB, IsdA, IsaA, SACOL0688 and glucosaminidase were detected in 24 hrs-old biofilms of LUH15051 and Sac042w on both PS and LEMs (Table S1). In addition, the toxins HlgB, LukD and E and the immune modulator SSL1 were detected in biofilms of both strains only on LEMs, while FnBPB was detected only on PS. In contrast to results obtained with LUH14616, we additionally detected the proteins CHIPS, efb, lipase and lytM (in biofilms of both LUH15051 and Sac042w) and alpha-toxin and FLipR (Sac042w only) on both surfaces. Finally, we detected SEA in biofilms of LUH15051 and Sac042w on respectively LEMS and PS, while SdrD was detected for both strains on LEMS.

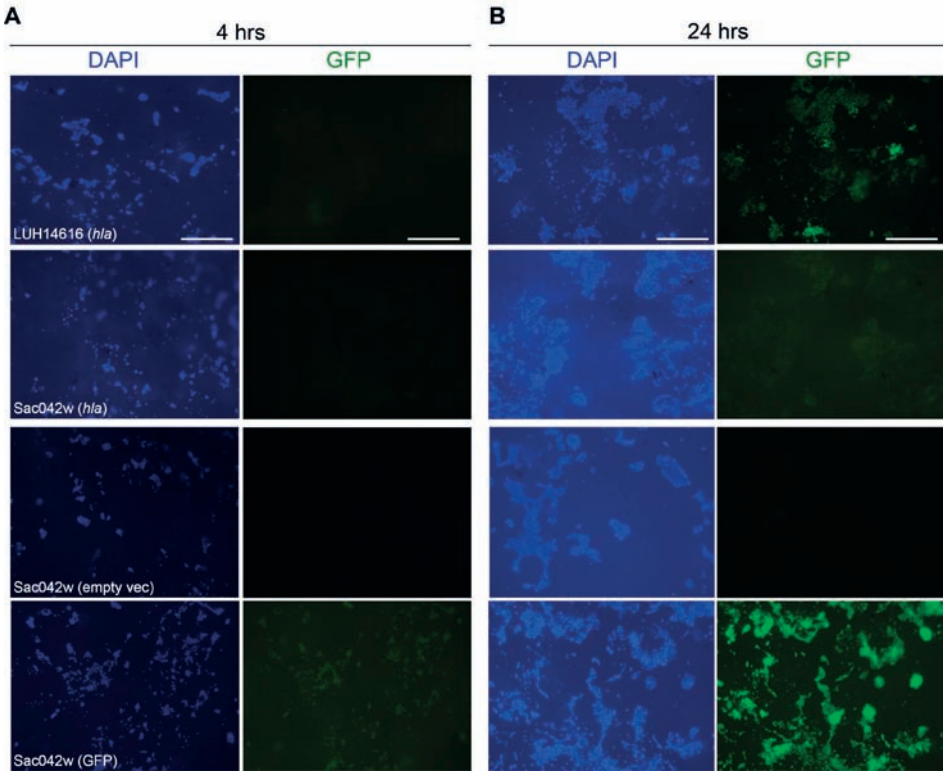

Figure 5. Expression of *hla* by *S. aureus* LUH14616 and Sac042w during biofilm formation on LEMs. LUH14616 and Sac042w containing *hla*-GFP (*hla)*, empty vector (empty vec) or a construct yielding constant GFP expression (GFP), **A**: at 4 hrs and **B**: at 24 hrs after bacterial colonization of LEMs. LEMs were incubated for 4 or 24 hrs with the different bacterial strains, subsequently fixed in 1% paraformaldehyde, and stained with DAPI. *hla* expressing bacteria are presented in green, DAPI staining is presented in blue. Scale bars = 50 µm.

Alpha-toxin expression by MRSA strains LUH14616 and Sac042w during biofilm formation on LEMs and PS

The differential detection of alpha-toxin, an important virulence factor during skin infections caused by *S. aureus*, in biofilms of different strains on LEMs and PS was further investigated using GFP-reporter technology. Visualization of alpha-toxin produced by *S. aureus* was performed by using strains LUH14616 and Sac042w transformed with a vector containing the promoter for *hla*, coupled to GFP. Using fluorescence microscopy, small microcolonies of these bacteria were observed that did not express *hla* after 4 hrs of colonization of epidermal models (Figure 5A), whereas at 24 hrs of colonization LUH14616 highly expressed *hla*, as indicated by the green fluorescent signal (Figure 5B). In contrast to results seen at the protein level, *hla* expression by LUH14616 was also visualized after 24 hrs of colonization of polystyrene (Figure S2), suggesting that the

gene is transcribed but not translated and/or that protein is rapidly degraded. Similar results were obtained for Sac042w, although this was less pronounced than for LUH14616 (Figure 5B, Figure S2).

DISCUSSION

In this study we established the presence of 52 proteins in biofilms of five genetically different *S. aureus* strains on two different types of surfaces, i.e. Leiden epidermal modes (LEMs) and polystyrene (PS). We detected six functionally diverse proteins in biofilms of three different strains on both surfaces. Several of these proteins, including ClfB, glucosaminidase and SA0688, have been previously associated with biofilm formation [38-41], although so far not on a human biotic surface. In this context, surface- and strain-dependent differences in the presence of a wide range of proteins, including alpha-toxin, were found. The detection of multiple toxins (HlgB, LukD/E and alpha toxin) in biofilms of multiple strains on LEMs, but not PS, indicates surface specific protein expression. This implicates that currently used biofilm models, such as those on PS, might not adequately reflect biofilm formation on a more complex surface, such as the human skin. However, we should realize that the biofilms on LEMs and PS were formed under different conditions, i.e. submerged in culture medium for biofilms on PS and on an air-exposed, dry surface in case of biofilm formation on LEMs.

Biofilm formation by strain LUH14616 on LEMs and PS was confirmed using EM. Interestingly, bacteria in a biofilm on LEM but not on PS were completely encased by an extracellular matrix, indicating a phenotypic difference in bacterial biofilm formation on the two models. However, in the current study we did not characterize the material encasing bacteria in more detail, e.g. using immunoelectron microscopy [42].

The detection of *S. aureus* toxins, most notably alpha-toxin, in biofilms on LEMs is in agreement with their well-established roles in the pathogenesis of skin infections [18,43]. The cytolytic pore-forming alpha-toxin [44] lyses human cells including skin tissue, interferes with the innate and adaptive immune responses in a murine skin infection model [45], and is essential for biofilm development on mucosal surfaces [46]. Interestingly, in human skin, the filaggrin protein may inhibit alpha-toxin's cytotoxicity by its ability to regulate the secretion of sphingomyelinase [47]. In line with this, >90% of the atopic dermatitis (AD) patients, who often have reduced filaggrin expression, are colonized by *S. aureus* [48], whereas about 25% of the normal population is persistently colonized by this bacterium [49]. Moreover, *S. aureus* strains isolated from AD patients displayed a higher alpha-toxin production than strains from healthy controls, while the amount of alpha-toxin produced was correlated with disease severity [50].

The other toxins detected in biofilms in this study, including HlgB and the leukocidins D/ E, have also been associated with *S. aureus* skin colonization and infection. This is supported by data from both murine models [51,52] and clinical-epidemiological studies [53,54]. Other data also supports the presence of other, non-toxin proteins detected in this study. For instance, the detected lipase might support the persistence of *S. aureus* in the fatty secretions of mammalian skin [55,56]. A recent study demonstrated that lipases are essential for *S. aureus* biofilm formation [55].

The PHG used in this study to establish bacterial protein presence consisted of a previously described pool of serum from both nasal and non-nasal carriers of *S. aureus* [35]. Specific IgG against all tested proteins was detected in PHG and these IgG levels were generally higher than in serum from individual patients suffering from a *S. aureus* bacteremia [27] (unpublished data). Combined with the high sensitivity of the Luminex assay [57, 58] we think that it is unlikely that the current CLA would not detect antibody absorption by IgG-accessible proteins. However, future studies using other antibody sources (e.g. specific monoclonal antibodies) might further increase the sensitivity of this assay.

A limitation of the PHG used in this study is the aspecific decrease in IgG that was observed against leukocidins S and F, while genes for these proteins were not present in strain LUH14616 and proteomics data could not confirm the presence of HlgB or Luk D/E in 8-hrs biofilms on PS. The known immunological cross-reactivity between Luk D/E, S/F and HlgB [59,60] may explain these conflicting findings. In addition, an incomplete protein library used during mass-spec analysis might explain why CLA results for a particular protein could not be confirmed. Additional mass-spectrometry should be performed on mature biofilms on LEMs to confirm or exclude the presence of these proteins.

Expression on LEMs of *hla*, the gene encoding alpha toxin, was confirmed for two strains using GFP reporter technology. The low levels of *hla* expression by the USA300-derived strain Sac042w may be explained by strain-specific traits [61], possibly caused by mutations in upstream regulators such as *sarA* [62]. Interestingly, for strain LUH14616 *hla* expression was also observed on PS, while CLA nor mass-spectrometry detected alpha toxin at the protein level on this surface. This suggests that *hla* is transcribed but not translated and/or that the protein is rapidly degraded by the bacteria on PS, which has been observed previously for other *S. aureus* strains during planktonic growth [63-65].

Regulation of *hla* and other genes for *S. aureus* virulence factors is influenced by many factors, including the accessory gene regulatory locus (Agr), RNAIII [66], downstream transcription factors Rot [67,68], SarA and -S and Sae [69]. In this connection, we noted that several proteins detected during biofilm formation on LEMs, including alpha-toxin, LytM, SSLs and Spa are (indirectly) regulated by RNAIII [66,70]. Moreover, RNAIII can also directly interfere with mRNA of LytM leading to its down-regulation [71]. Therefore, it may well be that the quorum sensing system of AgrA/RNAIII of *S. aureus* is activated differently upon interaction of *S. aureus* with either LEMs or PS, leading to up- or down-

regulation of specific genes depending on the surface. However, further studies including quantitative mass-spectrometry and transcriptomic analysis are necessary to clarify the role of the diverse regulatory systems [72-74] involved in the expression of *hla* and other genes during biofilm formation on LEMs.

In the context of new anti-infective therapies, such as vaccines, our data indicate that diverse proteins of *S. aureus* in biofilms are accessible to human IgG. Although biofilm-associated bacteria are thought to be more resistant to antimicrobials and effectors of the human immune system [13–15], our data suggests that, in addition to animal models [40], also vaccine-boosted human antibodies can target biofilms. Further insights into the functionality of antibodies, specifically in regard to (the inhibition of) biofilm growth, are required.

Alternatively, it may be interesting to choose an anti-virulence based therapy, for example by targeting interfering RNAs, such as RNAIII that affect the expression of many virulence factors [75]. For example, the RnpA- inhibitor RNPA1000, was shown to have *in vitro* antimicrobial effect against *S. aureus* (and other gram positive pathogens). Moreover, this enzyme dose-dependently protected against the pathogenesis of *S. aureus* in a mouse infection model [76]. Based on our data such an anti-virulence therapy may be effective against biofilms on skin of e.g. AD patients colonized by *S. aureus*, but not against biofilms on abiotic surfaces, such as that of a colonized catheter.

We conclude that functionally diverse virulence factors of (methicillin-resistant) *S. aureus* are present during biofilm formation on PS and LEMs. We specifically confirmed the presence of alpha-toxin during biofilm formation of MRSA strains LUH14616 and Sac042w on LEMs. In addition, the presence of several toxins, including alpha-toxin, immune modulators and other proteins appear to differ depending on the studied strain and surface. These observations merit more mechanistic studies to elucidate the function of specific proteins and the regulation of their expression within *S. aureus* biofilms. However, the present data further suggests that specific proteins, such as the ubiquitously present IsdA or SA0688, could be potential targets for novel agents to prevent biofilm formation and/or to reduce biofilm formation not only in animal models but also on human biotic surfaces.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL DATA

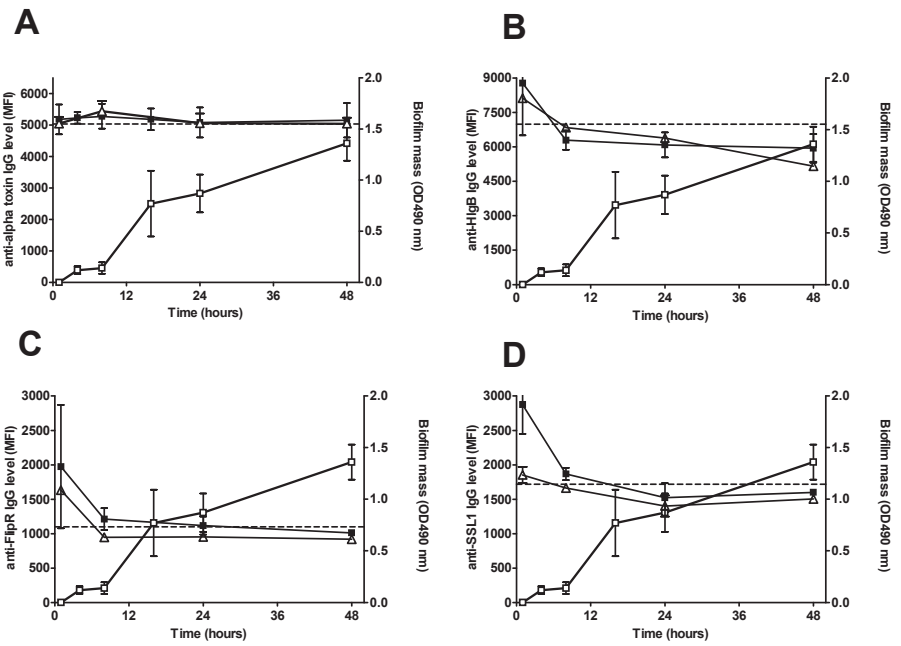

Figure S1. Detection of *S. aureus* toxins and immune-modulators in 24 hrs biofilms of LUH14616 and surrounding medium.

Results are shown for **A**: alpha-toxin, **B**: HlgB, **C:** FlipR, and **D**: SSL1. Remaining non-bound IgG specific against the different proteins was separately measured after incubation of PHG with biofilms on PS and after incubation with the IMDM culture medium covering the biofilms. Closed squares indicate IgG measurements from the biofilm samples and open triangles indicate measurements from medium samples. Biofilm mass on PS is indicated by open squares, which are plotted on the right Y-axis. Dashed horizontal lines indicate average MFI of sterile controls. Results are presented as the mean of 2-4 experiments.

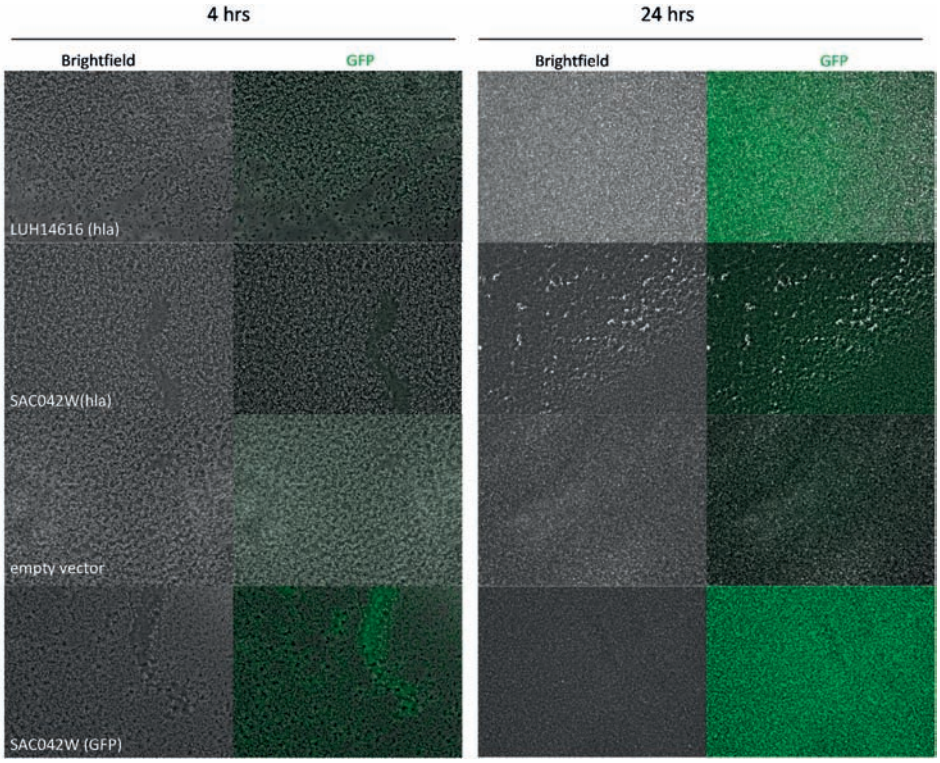

Figure S2. Expression of *hla* by *S. aureus* LUH14616 and Sac042w during biofilm formation on PS. LUH14616 and Sac042w containing *hla*-GFP (*hla)*, an empty vector or a construct yielding constant GFP expression (GFP), at 4 hrs and 24 hrs after bacterial colonization of PS. *hla* expressing bacteria are presented in green.

Table S1. Detection of mRNA and proteins in biofilms of three *S. aureus* strains on LEMs and PS.

Protein	Functional class	Sterile control 24 hrs		Luh15051 24 hrs biofilms			Saco042 (USA300) 24 hrs biofilms		
		Non-bound IgG in MFI ± SD		Non-bound IgG in MFI ± SD, (% reduction)[1]			Non-bound IgG in MFI ± SD, (% reduction)[1]		
		Polystyrene	LEMs[2]	Polystyrene	LEMs[2]	Gene present[3]	Polystyrene	LEMs[2]	Gene present[3]
Alpha toxin	toxin	5033 ± 768	4957 ± 77	4128 ± 89 (18)	185 ± 27 (96)*	yes	2705 ± 323 (46)*	151 ± 68 (97)*	yes
CHIPS	immmune modulator	6917 ± 1277	6508 ± 87	2817 ± 554 (59)*	1274 ± 988 (80)*	yes	746 ± 15 (89)*	1279 ± 675 (80)*	yes
ClfA	surface protein	768 ± 209	767 ± 28	423 ± 74 (45)*	105 ± 9 (86)*	yes	273 ± 2 (64)*	172 ± 124 (78)*	yes
ClfB	surface protein	485 ± 237	356 ± 128	249 ± 73 (49)	41 ± 10 (88)*	yes	179 ± 12 (63)*	92 ± 43 (74)*	yes
Efb	immmune modulator	2706 ± 802	2541 ± 176	485 ± 68 (82)*	502 ± 96 (80)*	yes	967 ± 502 (64)*	1087 ± 883 (57)*	yes
EsxA	housekeeping	ND[4]				yes			yes
EsxB	housekeeping	ND[4]				no			yes
ETA	toxin	192 ± 23	196 ± 18	184 ± 6 (4)	81 ± 18 (59)*	no	179 ± 21 (7)	135 ± 9 (31)	no
ETB	toxin	50 ± 12	49 ± 4	55 ± 2 (0)	45 ± 7 (8)	no	103 ± 46 (0)	47 ± 8 (4)	no
FlipR	immmune modulator	1211 ± 294	1088 ± 317	890 ± 25 (26)	90 ± 51 (92)*	yes	632 ± 81 (46)*	584 ± 231 (47)*	yes
FnbA	surface protein	461 ± 128	399 ± 17	265 ± 51 (43)*	146 ± 22 (63)*	yes	282 ± 19 (39)*	227 ± 107 (43)*	yes
FnbB	surface protein	82 ± 36	59 ± 12	36 ± 5 (56)*	36 ± 5 (39)	yes	52 ± 10 (37)*	47 ± 17 (20)	yes
Glucosaminidase	housekeeping	2088 ± 736	1699 ± 563	1200 ± 39 (43)*	40 ± 8 (98)*	yes	241 ± 26 (88)*	81 ± 7 (95)*	yes
HlgB	toxin	5145 ± 2612	5800 ± 102	3682 ± 1062 (28)	316 ± 263 (95)*	yes	3135 ± 223 (39)*	663 ± 444 (89)*	yes
IsaA	housekeeping	3028 ± 1178	2516 ± 773	370 ± 50 (88)*	92 ± 8 (96)*	yes	469 ± 123 (84)*	385 ± 89 (85)*	yes
IsdA	surface protein	1526 ± 704	1628 ± 60	83 ± 17 (95)*	154 ± 54 (90)*	yes	151 ± 85 (90)*	179 ± 74 (89)*	yes
IsdH	surface protein	ND[4]				yes			yes
Lipase	housekeeping/ toxin	1598 ± 423	1534 ± 49	662 ± 317 (59)*	70 ± 33 (95)*	yes	250 ± 128 (84)*	41 ± 25 (97)*	yes
LukD	toxin	4950 ± 1134	4580 ± 8	3857 ± 74 (22)	1020 ± 498 (78)*	yes	3136 ± 237 (37)*	1601 ± 1125 (65)*	yes
LukE	toxin	4607 ± 1297	4434 ± 87	3588 ± 53 (22)	1208 ± 318 (73)*	yes	3164 ± 190 (31)	1807 ± 952 (59)*	yes
LukF	toxin	672 ± 219	524 ± 204	478 ± 11 (29)	87 ± 9 (83)*	yes	408 ± 78 (39)*	92 ± 18 (82)*	yes

Table S1. Detection of mRNA and proteins in biofilms of three *S. aureus* strains on LEMs and PS. (continued)

Protein	Functional class	Sterile control 24 hrs		Luh15051 24 hrs biofilms			Saco042 (USA300) 24 hrs biofilms		
		Non-bound IgG in MFI ± SD		Non-bound IgG in MFI ± SD, (% reduction)[1]			Non-bound IgG in MFI ± SD, (% reduction)[1]		
		Polystyrene	LEMs[2]	Polystyrene	LEMs[2]	Gene present[3]	Polystyrene	LEMs[2]	Gene present[3]
LukS	toxin	2699 ± 864	2644 ± 127	1878 ± 56 (30)	326 ± 218 (88)*	yes	1352 ± 92 (50)*	206 ± 174 (92)*	yes
LytM	housekeeping	325 ± 73	312 ± 14	199 ± 23 (39)*	53 ± 2 (83)*	yes	197 ± 37 (39)*	82 ± 62 (74)*	yes
Nuc	housekeeping/ toxin	872 ± 198	704 ± 254	208 ± 55 (76)*	90 ± 12 (87)*	yes	299 ± 214 (66)*	156 ± 31 (78)*	yes
PrsA	housekeeping	ND[4]				yes			yes
SACOL0486	housekeeping	234 ± 203	514 ± 12	71 ± 1 (70)*	231 ± 126 (55)*	no	288 ± 276 (0)	139 ± 92 (73)*	no
SACOL0688	housekeeping	297 ± 72	277 ± 91	141 ± 42 (53)*	35 ± 6 (87)*	yes	112 ± 49 (62)*	67 ± 33 (75)*	yes
SasG	surface protein	173 ± 49	144 ± 18	100 ± 15 (42)*	52 ± 7 (64)*	yes	106 ± 37 (39)*	53 ± 20 (63)*	yes
SCIN	immmune modulator	3178 ± 411	4013 ± 196	298 ± 81 (91)*	223 ± 63 (94)*	yes	365 ± 69 (89)*	1804 ± 1139 (55)*	yes
SdrD	surface protein	85 ± 38	83 ± 4	56 ± 2 (34)	53 ± 5 (36)	yes	51 ± 2 (40)*	64 ± 22 (23)	yes
SdrE	surface protein	299 ± 75	299 ± 50	247 ± 21 (17)	95 ± 26 (69)*	yes	317 ± 108 (0)	135 ± 2 (55)*	yes
SEA	toxin	666 ± 221	585 ± 49	435 ± 20 (35)*	290 ± 13 (50)*	no	370 ± 43 (45)*	376 ± 120 (36)	no
SEB	toxin	818 ± 222	751 ± 50	587 ± 30 (28)	378 ± 31 (50)*	no	533 ± 73 (35)*	514 ± 174 (32)	no
SEC	toxin	4476 ± 1094	4241 ± 175	3260 ± 104 (27)	2176 ± 128 (49)	no	2677 ± 268 (40)*	3011 ± 960 (29)	no
SED	toxin	157 ± 45	147 ± 7	114 ± 1 (18)	65 ± 2 (65)*	no	94 ± 8 (40)*	86 ± 48 (42)*	no
SEE	toxin	79 ± 21	75 ± 8	57 ± 7 (28)	40 ± 1 (47)*	no	59 ± 13 (25)	53 ± 16 (29)	no
SEG	toxin	87 ± 15	85 ± 3	98 ± 14 (0)	91 ± 6 (0)	no	209 ± 81 (0)	88 ± 22 (0)	no
SEH	toxin	289 ± 69	300 ± 25	237 ± 23 (18)	169 ± 11 (44)*	no	261 ± 100 (10)	213 ± 84 (29)	no
SEI	toxin	108 ± 38	97 ± 5	101 ± 18 (6)	81 ± 20 (17)	no	165 ± 125 (0)	71 ± 5 (27)	no
SEJ	toxin	ND[4]				no			no
SEM	toxin	107 ± 43	95 ± 7	68 ± 11 (37)*	45 ± 1 (47)*	no	60 ± 20 (44)*	48 ± 18 (50)*	no
SEN	toxin	73 ± 5	91 ± 5	75 ± 8 (0)	58 ± 7 (36)	no	165 ± 158 (0)	109 ± 22 (0)	no

Table S1. Detection of mRNA and proteins in biofilms of three *S. aureus* strains on LEMs and PS. (continued)

Protein	Functional class	Sterile control 24 hrs		Luh15051 24 hrs biofilms			Saco042 (USA300) 24 hrs biofilms		
		Non-bound IgG in MFI ± SD		Non-bound IgG in MFI ± SD, (% reduction)[1]			Non-bound IgG in MFI ± SD, (% reduction)[1]		
		Polystyrene	LEMs[2]	Polystyrene	LEMs[2]	Gene present[3]	Polystyrene	LEMs[2]	Gene present[3]
SEO	toxin	47 ± 14	46 ± 6	36 ± 2 (23)	24 ± 1 (48)*	no	34 ± 9 (28)	32 ± 8 (30)	no
SEQ	toxin	98 ± 31	94 ± 9	66 ± 5 (33)	39 ± 1 (59)*	no	45 ± 3 (54)*	40 ± 16 (58)*	yes
SER	toxin	94 ± 10	118 ± 19	90 ± 2 (4)	49 ± 15 (59)*	no	92 ± 9 (2)	85 ± 14 (28)	no
SSL1	immmune modulator	1754 ± 474	1719 ± 48	1261 ± 113 (28)	274 ± 36 (84)*	no	1071 ± 61 (39)*	938 ± 620 (35)	yes
SSL3	immmune modulator	2902 ± 816	2782 ± 71	2102 ± 79 (28)	1559 ± 68 (44)*	no	1795 ± 223 (39)*	1999 ± 485 (29)	yes
SSL5	immmune modulator	1081 ± 317	1007 ± 70	756 ± 41 (30)	614 ± 34 (39)	no	786 ± 208 (28)	763 ± 147 (24)	yes
SSL9	immmune modulator	2700 744	2379 ± 109	2027 ± 60 (25)	1331 ± 14 (44)*	no	2058 ± 540 (24)	1874 ± 435 (21)	no
SSL10	immmune modulator	2434 ± 825	2224 ± 299	1815 ± 3 (25)	868 ± 260 (61)*	yes	1573 ± 266 (35)*	1611 ± 185 (28)	yes
SSL11	immmune modulator	335 ± 96	350 ± 41	258 ± 3 (23)	105 ± 39 (70)*	no	228 ± 40 (32)	246 ± 48 (30)	no
TSST1	toxin	4208 ± 1087	3999 ± 168	3104 ± 86 (26)	2270 ± 177 (43)*	no	2600 ± 278 (38)*	2944 ± 694 (26)	no

[1]Percentage decrease of specific IgG for each protein was calculated in relation to the negative control and can be considered as a semi-quantitative measure of protein-specific antibody absorption, indirectly reflecting the presence of the protein in the biofilm.

[2]Leiden human Epidermal Models

[3]Presence of genes was established using PCR.

[4]Not Determined, data were excluded due to low MFI's with standard deviations larger than 25% between repeated CLA measurements.

*Reductions in specific IgG surpassing the cut-off values (35% antibody absorption at 24 hrs biofilm growth and 40% at 48 hrs), indicative of protein presence.

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Chapter 5

Factors associated with worse lung function in cystic fibrosis patients with persistent *Staphylococcus aureus*

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ABSTRACT

Background: *Staphylococcus aureus* is an important pathogen in cystic fibrosis (CF). However, it is not clear which factors are associated with worse lung function in patients with persistent *S. aureus* airway cultures. Our main hypothesis was that patients with high *S. aureus* density in their respiratory specimens would more likely experience worsening of their lung disease than patients with low bacterial loads.

Methods: Therefore, we conducted an observational prospective longitudinal multicenter study and assessed the association between lung function and *S. aureus* bacterial density in respiratory samples, co-infection with other CF-pathogens, nasal *S. aureus* carriage, clinical status, antibiotic therapy, IL-6- and IgG-levels against *S. aureus* virulence factors.

Results: 195 patients from 17 centers were followed; each patient had an average of 7 visits. Data were analyzed using descriptive statistics and generalized linear mixed models. Our main hypothesis was only supported for patients providing throat specimens indicating that patients with higher density experienced a steeper lung function decline ($p<0.001$). Patients with exacerbations ($n = 60$), *S. aureus* small-colony variants (SCVs, $n = 84$) and co-infection with *Stenotrophomonas maltophilia* ($n = 44$) had worse lung function ($p = 0.0068$; $p = 0.0011$; $p = 0.0103$). Patients with SCVs were older ($p = 0.0066$) and more often treated with trimethoprim/sulfamethoxazole ($p = 0.0078$). IL-6 levels positively correlated with decreased lung function ($p<0.001$), *S. aureus* density in sputa ($p = 0.0016$), SCVs ($p = 0.0209$), exacerbations ($p = 0.0041$) and co-infections with *S. maltophilia* ($p = 0.0195$) or *A. fumigatus* ($p = 0.0496$).

Conclusions: In CF-patients with chronic *S. aureus* cultures, independent risk factors for worse lung function are high bacterial density in throat cultures, exacerbations, elevated IL-6 levels, presence of *S. aureus* SCVs and co-infection with *S. maltophilia*.

INTRODUCTION

Cystic fibrosis (CF) patients suffer from recurrent bacterial airway infections, which often lead to respiratory insufficiency and reduced life expectancy [1]. *Staphylococcus aureus* is one of the most frequently isolated pathogens from the airways of these patients [2–4]. Recent studies have shown the importance of *S. aureus* in young children with CF by way of eliciting an increased inflammatory response measured in broncho-alveolar lavages [5–9]. Thirty percent of the general population are persistent nasal carriers of *S. aureus* and this percentage is even higher in CF patients [10–12]. Nasal carriers have been shown to have a higher risk for *S. aureus* infections, but a lower rate of lethal infections [13]. It is often difficult to decide whether culturing *S. aureus* in respiratory specimens from CF patients is merely caused by colonization or whether this is also associated with worsening lung disease requiring antibiotic treatment. To support clinical decision, interleukin-6 levels in blood, which have been shown to increase in CF patients during airway inflammation [14], may serve as a potential marker for severity of inflammation.

In the present study, our goal was to determine risk factors associated with worse lung function in CF patients older than six years, who had been persistently colonized by *S. aureus*. We thus conducted a prospective observational longitudinal multi-center study (ClinicalTrials.gov: NCT00669760). We hypothesized that patients with high bacterial loads of *S. aureus* in airway specimens were more likely to experience worse lung function compared to patients with lower bacterial loads. Secondary objectives were the association between lung function and *S. aureus* nasal carriage in CF patients, the role of blood IL-6 levels and IgG levels against 44 *S. aureus* virulence factors, presence of small colony variants (SCVs) of *S. aureus* and co-infection with other CF-related pathogens such as *Stenotrophomonas maltophilia*, methicillin-resistant *S. aureus* (MRSA), *Aspergillus fumigatus*, *Achromobacter xylosoxidans* and *Mycobacterium abscessus* on lung function.

MATERIALS AND METHODS

Study design

We conducted an observational prospective multicenter study to evaluate risk factors for worse lung function in patients with persistent *S. aureus* airway cultures older than 6 years. The treating physicians recruited CF patients for the study by using the following definitions for persistence from the medical records: 1. Patients presented with at least 2 positive cultures 6 months apart within one year before recruitment, or 2. half of the respiratory samples the year before recruitment were *S. aureus* positive. The first patient was recruited in June 2008, the last patient finished the observation period in February 2010. At each visit throat or sputum cultures were obtained and sent to the central study

laboratory located in Münster, Germany. On the basis of a study investigating chronic bronchitis patients [15], we hypothesized that CF-patients with high loads (in sputa ≥1x10^{6}CFU/ml; in throat cultures semi-quantitative score ≥3) of *S. aureus* in their airway specimens are more likely to experience worsening of lung disease due to *S. aureus* than patients with low bacterial loads. To rule out an impact of *P. aeruginosa* or *Burkholderia cepacia* complex (BCC) on lung function, patients with cultures of these two pathogens were excluded. We defined exacerbation by evaluating clinical symptoms established by Fuchs et al. for the assessment of exacerbation [16]. If a patient met at least four out of 11 evaluated symptoms, the clinical status was determined as exacerbation.

Secondary objectives included the association between nasal *S. aureus* carriage, the level of IL-6, the presence of *S. aureus* small colony variants (SCVs) and the co-culture of other important CF pathogens such as *S. maltophilia*, *A. xylosoxidans*, *A. fumigatus* and *M. abscessu*s and the risk for exacerbation or worse lung function.

Inclusion criteria

CF Individuals older than six years were included with persistent *S. aureus* airway cultures one year before recruitment.

Enrolment and follow-up

Patients identified as persistently colonized by *S. aureus* as defined by our inclusion criteria were enrolled in this study by their attending CF specialists. If *P. aeruginosa* or BCC were cultured for more than 6 months during the study period, patients were excluded from further analysis. Patients were followed for a period of 21 months with regular visits (usually every 3 months). Sputum samples were spontaneously produced. There were no requirements for induced sputum cultures. Blood samples were taken from patients once yearly and/or at exacerbations. An ethical statement was obtained at the main study center with the central laboratory in Münster, Germany (2007-496-f-S). Other ethical statements have been obtained from the Universitätsklinikum Tübingen 450/2008B02, Martin-Luther-Universität Halle Wittenberg hm/bu, Ärztekammer Hamburg MC039/09 and the Universitätsklinikum Dresden. All other ethic committees at the Ruhr Universität Bochum, Universitätsklinik Essen, Medizinische Hochschule Hannover, Universitätsklinikum Jena, Charité Universitätsmedizin Berlin, Campus Virchow Klinikum, Kinderkrankenhaus Osnabrück, Universitätsklinik Innsbruck, Universitätsklinik Düsseldorf, Park Schönefeld Klinik Kassel and the Universitätsklinikum Leipzig waived their approval. Written informed consent was obtained from all patients and parents, if patients were younger than 18 years. Clinical trial registered with www.clinicaltrials.gov (NCT00669760).

Investigated Fuchs criteria and clinical report forms (CRFs) [16]

At every visit patients performed lung function tests assessed as forced expiratory volume in 1 second (FEV_1). Other recorded clinical parameters included the presence of fatigue, malaise or lethargy, sinus discharge, cough and hemoptysis as well as sputum volume or color. These data were documented by the treating physicians in CRFs, together with *CFTR* genotype, pancreas sufficiency/insufficiency, physical and radiographic findings on examination of the chest, body mass index and antibiotic therapy. The CRFs were sent together with the respiratory specimens to the central laboratory in Münster, Germany.

Microbiology

All airway and blood samples obtained from patients were sent to the central study laboratory (Medical Microbiology, Münster, Germany) within 24 hours and were cultured according to standard procedures for CF airway cultures [17,18]. All samples were streaked on Columbia sheep blood agar (Becton Dickinson, Heidelberg, Germany), MacConkey agar for Gram-negative bacteria (Becton Dickinson, Heidelberg, Germany), SAID chromogenic agar for *S. aureus*, (bioMerieux, Nürtingen, Germany), all incubated for 48h at 37°C; on chocolate agar (Mast Diagnostica, Reinfeld, Germany) for *Haemophilus influenzae*, incubated for 48h at 37°C with 5% CO_2; on BCSA agar (bioMerieux, Nürtingen, Germany) for *Burkholderia cepacia complex*, incubated at 30°C for 10 days, and on Kimmig agar for fungi. In case of mucoid consistency, sputa were incubated with sputasol (dithiotreitol, Oxoid, Wesel, Germany) for 30 min at 37°C and homogenized by vortexing and vigorous pipetting before further processing. Quantitative cultures of sputa were performed by serial dilution of 500µl of sputum in 4.5ml 0.85% NaCl according to standard procedures. Semi-quantitative analysis was performed for throat and nasal cultures and for sputa of less than 500µl sputum. A score of 1 was assigned to single *S. aureus* colonies on the primary agar plate, a score of 2 to medium density and a score of 3 to highly dense numbers of *S. aureus* colonies on the primary agar plate, respectively. SCVs were identified by sub-culturing isolates of interest from Columbia blood and SAID agar on Columbia blood agar (incubated at 37°C) and on Schaedler agar (incubated at 37°C with 5% CO_2) for 24h [19]. All isolates, which displayed small colony size on Columbia blood and normal size on Schaedler agar were identified as SCVs, which was further confirmed by a positive catalase and positive Pasteurex Staph Plus test (BIO-RAD, München, Germany). In case of negative results, isolates were identified by 16S-RNA sequencing.

Measurements of anti-staphylococcal antibodies

The levels of IgG antibodies against 44 staphylococcal antigens in serum samples of CF patients and 53 healthy nasal carriers, who consisted of volunteers of the Institute of Medical Microbiology and medical students, were measured using a bead-based flow

cytometry technique (xMAP®; Luminex Corporation) as previously described [20,21]. All examined antigens are given in Table S3.

IL-6 measurements

The assay was characterized by intra-assay precisions of 4.2%, 1.6% and 2.0% at low, medium and high analyte levels, respectively. The minimum detectable dose of IL-6 was less than 0.7 pg/mL.The cut-off value for IL-6 (12 pg/mL) corresponds to the highest IL-6 level observed in a control population of 40 apparently healthy individuals and is very close to the cut-off value of 15 pg/mL, which has been suggested in previously published studies [23, 24].

Statistical analysis

Patient data were collected longitudinally. The baseline patient characteristics were analyzed by standard descriptive statistics. Categorical variables were described by absolute and relative frequencies. Continuous variables were described by median and range. The following variables were computed:

Lung function (FEV_1% predicted) was determined on the basis of Quanjer et al. [22]. SCV status (never/ever) of the patients was positive (ever), if SCVs were detected in specimens at any visit. Carrier status was deemed positive, if in at least at 50% of visits *S. aureus* nasal carriage was detected. Exacerbation status (never/ever) was calculated using the symptoms defined by Fuchs [16]. A visit with Fuchs score in excess of 4 was defined as an exacerbation visit. Patients exhibiting at least one exacerbation visit were assigned to the exacerbation ever group. *S. aureus* density [sputa: high ($<10^6$CFU/ml and low $<10^6$CFU/ml according to Mensa and Trilla for patients with chronic bronchitis;[15] nasal and throat swabs: high >/ = +++; low + or ++)] was determined based on quantitative and semi-quantitative data per visit. Patients' co-infection status (never/ever) with MRSA, *S. maltophilia* and *A. fumigatus* was determined by compiling information from all visits.

Outcome parameters of interest were bacterial density (high/low) in different specimens, lung function (FEV_1% predicted) and interleukin-6 (IL-6) levels.

Cross-sectional analyses, e.g. at baseline, were performed using Mann-Whitney-U tests from continuous variables and Chi-Squared tests, or Fisher's exact tests for categorical variables. Longitudinal analyses were performed to assess the effect of different explanatory variables on the outcome parameters. To model patient progress over time we used generalized linear mixed models (GLMM). Bacterial density was modelled by a binary distribution. FEV_1% proved to be normally distributed. IL-6 levels were skewed to the right and a log-normal distribution was used to fit the data. The canonical link function was used. For all models, a random effect for the individual progress of each patient over the study time (days from first visit) was included using sp(pow) as covari-

ance structure. All models were age and gender adjusted, if not otherwise specified. Studentized residual plots were used to examine model requirements.

To analyze the functional dependency of FEV_1% on IL-6 levels a polynomial model was used: $FEV1 = \beta 1 \times [IL6]^{\beta 2}$. The model coefficients were fitted using only baseline measurement using procnlin in SAS.

Each variable for the specific IgG measurements was tested for normal distribution using histogram plots, Q-Q plots and the Kolmogorov-Smirnov test. For the comparison of the IgG levels between patients and healthy controls the multiple testing problem was controlled by Bonferroni correction. Age and gender adjusted GLMM were used to analyse specific IgG-responses according to carrier status (yes/no), density (high/low) in sputa, SCV (ever/never) including a random effect for repeated measurement. q-values were Bonferroni–Holm adjusted p-values to control the FDR on the multiple significance level of 5%. IgG-levels were modelled as continuous factors. Odds ratios (OR) were interpreted as a factor, by which the risk for carriage/high-density/SCV was changed per 100 units in antigen levels. For an association of IL-6 and FEV_1% predicted with IgG-levels, estimates were interpreted as mean change in IL-6 per 1 unit in IgG-levels.

The local significance level for all performed tests was $\alpha = 0.05$. 95%-confidence intervals are given, where appropriate. Statistical tests are performed exploratory and can be interpreted as hypothesis generating. Statistical analysis was performed using SPSS (v. 22, IBM) and SAS (v. 9.4, SAS Institute, Cary, NC).

RESULTS

Demographics and statistics

A total of 195 patients were recruited from 16 German centers and one CF center in Austria, which take care of 1980 CF patients. Thirteen of 195 patients r were excluded due to the chronic culture (> 6 months) of *P. aeruginosa* (n = 12) or *BCC* (n = 1) during the study period. Cystic fibrosis transmembrane regulator (*CFTR*) genotypes were reported for 173 of 195 patients, see Table 1. Patients with non-p.Phe508del *CFTR* genotypes (n = 48; 27.7%) were overrepresented compared to the German CF patient population (Table 1, p<0.0001). The median age of patients was 16 years (range 5.8 y– 42 y). A preponderance of male to female subjects compared to the gender ratio of the German CF registry was reported (Table 1, p<0.001).

The mean number of all visits was seven (range 1–18). Clinical data of patients at baseline are shown and compared with national data concerning German CF patients in Table 1. Lung function decline was -1.2717 FEV_1% predicted per years of age calculated for the study group at the first visit (CI -1.6676/-0.7235; p<0.0001).

Table 1. Characteristics of study patients compared to the German CF population.

Characteristics	study patients[1]	German CF-patients	p-value
number of patients	195	4456	
Age (years)	16 (5.8 - 42)	20 (6-70)	<0.001
Male	120 (61%)	2121 (48%)	<0.001
Ex. panc. suff.[2]	22 (11%)	1426 (32%)	<0.001
FEV_1 (% predicted)	84.1 (13-121)	72.0 (10-145)	<0.001
BMI-Quantils	25% (0.07%-100%)	27% (0.05%-100%)	0.007
Genotype[3]	173	3832	
F508del homo[4]	85 (49%)	1863 (49%)	<0.001
F508del hetero[§§5]	40 (23%)	1429 (37%)	
others	48 (28%)	540 (14%)	

[1]Age, FEV1, BMI and exacerbation numbers are reported as median (range), respectively. Exocrine pancreatic sufficiency and genotype are reported as absolute frequencies (relative frequencies). We did not exclude one patient, who was 5.8 years at the first visit, but was 6.1 years at the second visit with seven reported visits throughout the study period, [2]exocrine pancreatic sufficiency, [3]*Cftr* genotype of patients available, [4]F508del homozygous, [5]F508del heterozygous

Age-bacterial density association

For 98 patients at least one sputum (n = 446, range 1–14) was available for analysis, while for 97 patients only throat cultures (n = 663, range 1–11) were available. The age of patients at the time of the first positive *S. aureus* sputum culture was significantly associated with *S. aureus* density in sputa, indicating that older patients had significantly higher bacterial loads in sputa compared to younger patients (p = 0.018). Patients with low bacterial density in sputa were on average 19.2 years of age, while patients with high bacterial loads were 22.2 years of age, respectively. There was no association between age and *S. aureus* density for patients with only throat (p = 0.7016) or nasal cultures (p = 0.411).

Bacterial density and lung function

Bacterial density according to specimens is shown in Table 2. No significant association, adjusted for age and sex, was reported between *S. aureus* density and FEV_1% predicted in neither patients with sputum (p = 0.5151) nor throat cultures (p = 0.185). There was also no association of FEV_1% predicted with BMI-quantiles for both groups (p = 0.4352; p = 0.1007) neither at baseline nor during the study period.

However, bacterial density in throat specimens was significantly associated with annual lung function decline. Patients with high density lost 1.428% FEV_1 per year compared to patients with low density, Figure 1 (0.8626%FEV_1; p<0.001 of the interaction of age and density). We could not observe similar associations with respect to bacterial density in sputum cultures and lung function decline (data not shown).

Table 2. Positive airway specimens according to *S. aureus* density

Specimens	Low density <1x10^{6}CFU/ml score 1, 2[1]	High density ≥1x10^{6}CFU/ml score ≥3[2]	Sum
Nasal swabs	517 (68.7)	236 (31.3)	753
Nasal lavage	26 (76.5)	8 (23.5)	34
Throat swabs	545 (69.7)	237 (30.3)	782
Sputa	166 (42.1)	228 (57.9)	394
All	1254 (63.9)	709 (36.1)	1963

[1]low bacterial density of sputum (<1x10^{6}CFU/ml) and throat cultures (score 1 and 2)
[2]high bacterial density of sputum (≥1x10^6 CFU/ml) and throat cultures (score ≥3)

The Figs 1–5 show observed FEV_1% predicted measurements over study time (days from first visit). Lines represent the LOESS-fit including 95% confidence intervals of the predicted values of the generalized linear mixed model.

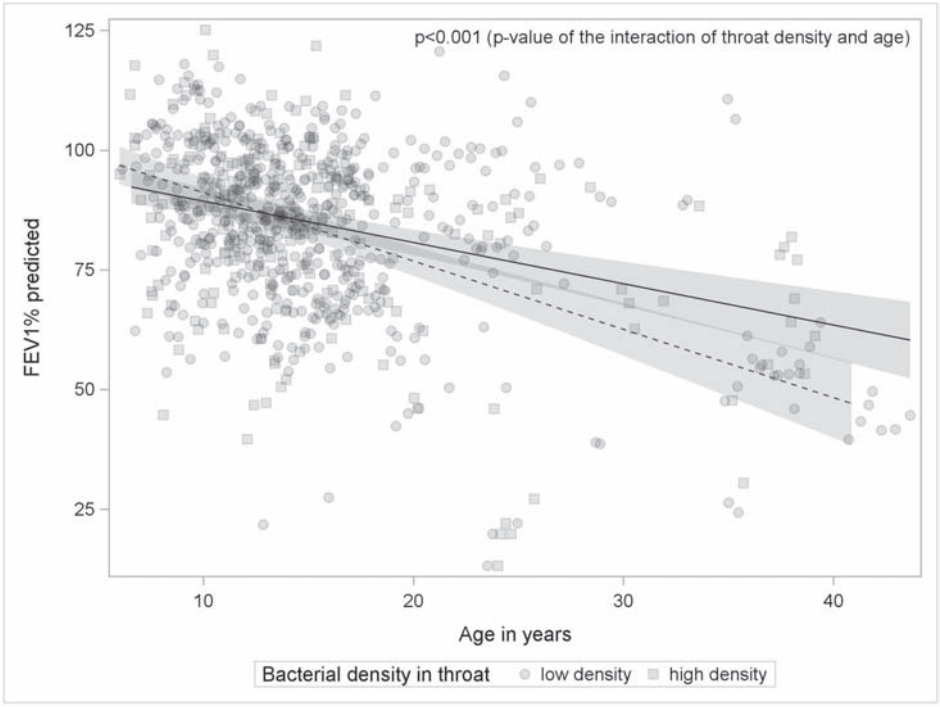

Figure 1. Patients with throat cultures with high bacterial density experience a more rapid lung function decline.
Patients with high bacterial density in throat specimens are indicated by red squares (fitted model prediction represented by dashed line), patients with low bacterial density in throat specimens by blue circles (solid line).

Patients with exacerbations have worse lung function

Sixty of 195 patients (31%; 55% male) experienced exacerbations with a mean number of 0.384 exacerbations per patient (range 0, 4). The age distribution of patients with and without exacerbation did not differ significantly (assessed at baseline, p = 0.5182, S1 Table). Using a longitudinal multivariable model adjusted for age and sex our data showed that patients with exacerbations have worse lung function of 5.40% FEV_1% predicted (95%CI 1.50 to 9.30 FEV_1%) less compared to patients without exacerbations (p = 0.0068, Figure 2). Patients with exacerbations have a lung function decline per year of age of 1.22% FEV_1% predicted (95%CI 0.94 to 1.50 FEV_1%) compared to 0.88% FEV_1% (95%CI 0.64 to 1.12 FEV_1%) predicted in patients without exacerbations (p = 0.0018). Patients with exacerbations received more antibiotics than patients without exacerbations (p = 0.037, OR = 1.175). For patients with exacerbations antibiotic treatment was reported at five visits (mean 4.3), while for patients without exacerbations at three visits (mean 3.43, p = 0.021), respectively.

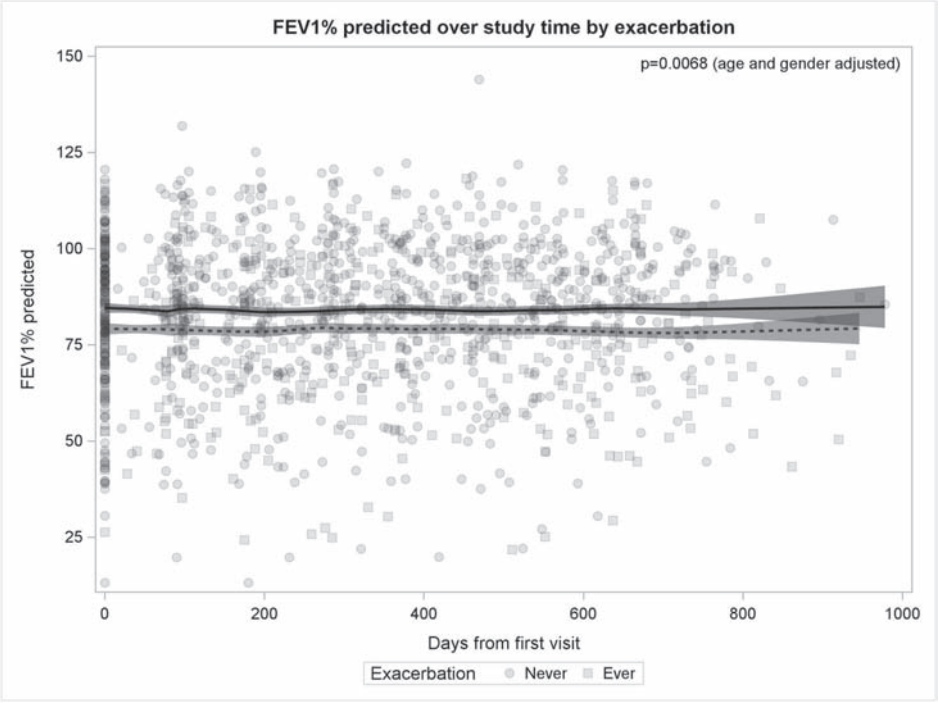

Figure 2. Patients with exacerbations have worse lung function.
Patients with exacerbation are indicated by red squares (fitted model prediction represented by dashed line), patients without exacerbation by blue circles (solid line).

Patients with *S. aureus* nasal carriage

Since persistent nasal *S. aureus* carriers differ from non-carriers in terms of severe *S. aureus* infections [10], we determined the nasal carriage status of our study patients to analyze its impact on CF lung disease. CF patients with persistent nasal carriage (n = 122, 62.6%) were more likely male patients compared to patients without persistent nasal carriage (p = 0.00075, S1 Table) and had significantly more nasal cultures with high bacterial loads (p = 0.0255). *S. aureus* nasal carriers had better lung function throughout the study compared to non-carriers with a difference of 5.758 FEV_1% predicted (95%CI 1.84 to 9.68; p = 0.0042; Figure 3). It is noteworthy that female nasal carriers experienced better lung function throughout the study period compared to male carriers (p = 0.0064, age-adjusted).

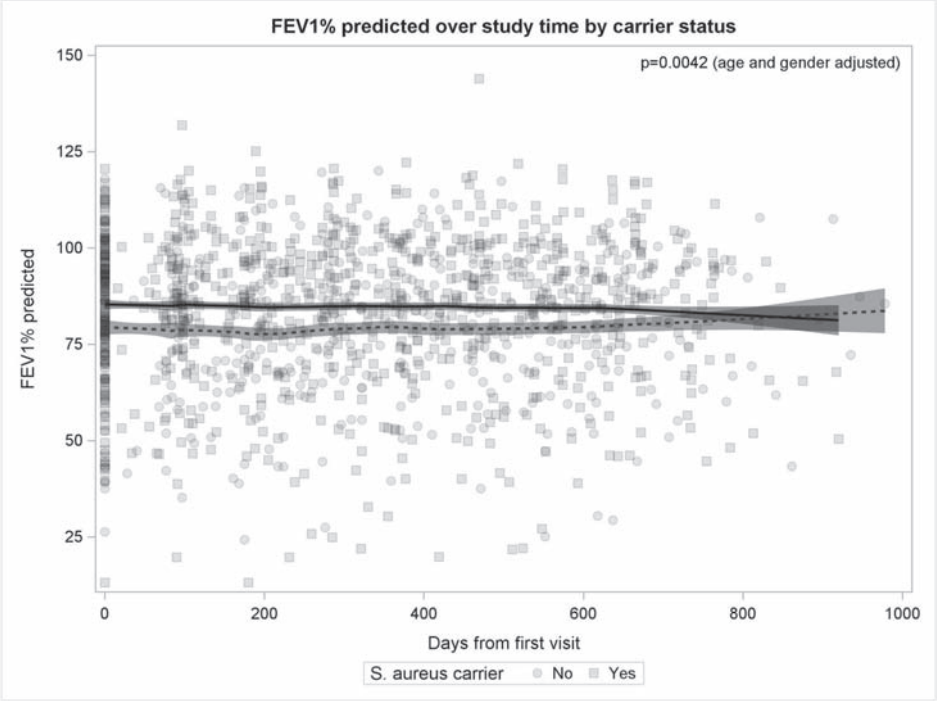

Figure 3. Patients with *S. aureus* nasal carriage have better lung function.
S. aureus nasal carriers are indicated by blue squares (fitted model prediction represented by solid line), non-nasal carriers by red circles (dashed line).

Patients with *S. aureus* SCVs

To determine the impact of *S. aureus* SCVs on lung disease data were analyzed in relation to the culture of SCVs in airway specimens, which were cultured from 84 patients (43%) at least once. Patients with SCVs were significantly older (p = 0.0066), had lower FEV_1% predicted at baseline (p = 0.0133) and during the study period (p = 0.0337, age and gender adjusted; lower lung function of 4.099 FEV_1% predicted (95%CI 0.32 to 7.88

FEV_1%) compared to patients without SCVs (Figure 4). Within a multivariable model also including antibiotic treatment, the association of SCVs with worse lung function was still significant (p = 0.0433, lower lung function in patients with SCVs of 3.89 FEV_1% predicted, 95%CI 0.12 to 7.66 FEV_1%). In addition, patients with SCVs were more likely treated with trimethoprim/sulfamethoxazole (TMP/SMX) compared to patients without SCVs (p = 0.0078).

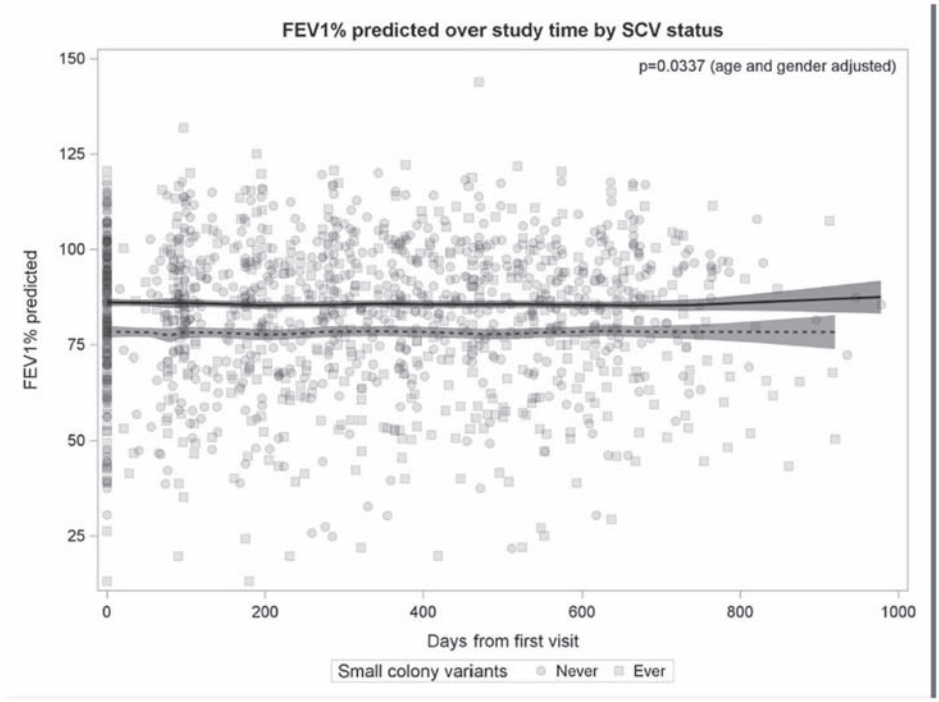

Figure 4. Patients with SCVs have worse lung function.
Patients with SCVs are indicated by red squares (fitted model prediction represented by dashed line), patients without SCVs by blue circles (solid line).

Patients with co-infecting pathogens

To assess the effect of co-infecting pathogens on lung function, we analyzed data of patients according to co-infection by important CF pathogens. Patients with *S. maltophilia* (n = 44) were more likely female (p = 0.0078), less likely nasal *S. aureus* carriers (p = 0.0003), more likely co-infected with SCVs (p<0.0001), experienced more exacerbations (p = 0.0165) and worse lung function of -3.72% FEV_1% predicted throughout the study period (Figure 5, p = 0.0053, 95%CI -6.33 to -1.10). Patients with *A. fumigatus* (n = 60) were older (p<0.0001) and more likely co-infected by SCVs (p = 0.0001).

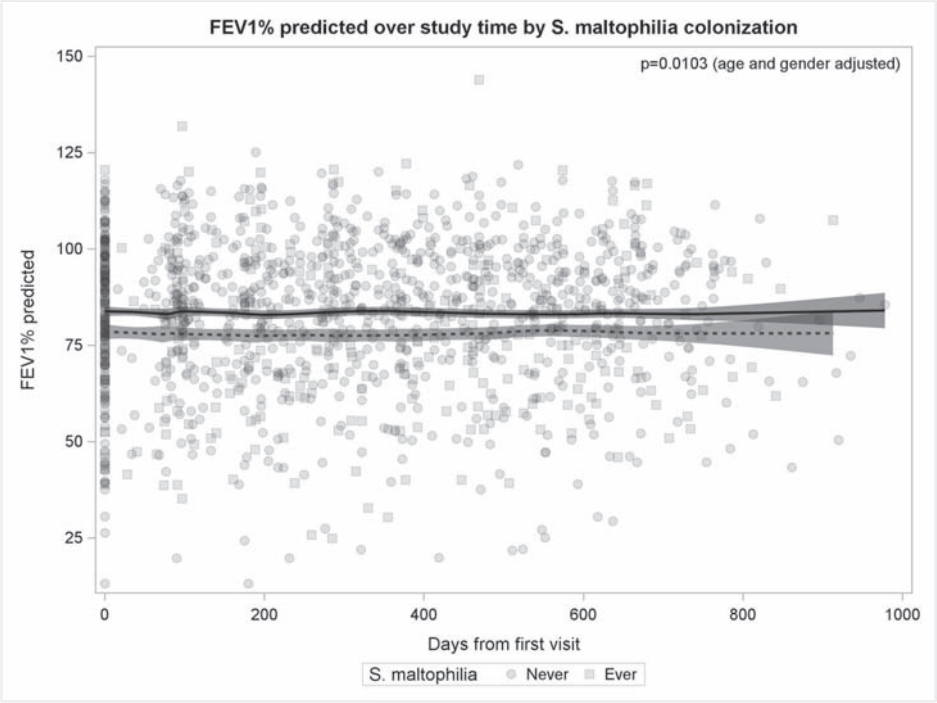

Figure 5. Patients with culture of *S. maltophilia* have worse lung function.
Patients with *S. maltophilia* are indicated by red squares (fitted model prediction represented by dashed line), patients without *S. maltophilia* by blue circles (solid line).
There was a strong association between FEV_1% predicted and bacterial density and cumulative clinical symptoms according to Fuchs criteria [16] for patients who expectorated sputa (95 patients, max. 9 visits; p = 0.0661, S1 Fig), but not for patients with throat cultures (167 patients, max. 9 visits).

IL-6 levels

Since interleukin-6 levels have been shown to be helpful as a potential marker for severity of inflammation [14], we determined IL-6 in sera of the study patients. An inverse association between IL-6 levels and FEV_1% predicted with worse lung function being associated with higher IL-6 at baseline ($p<0.001$, Figure 6). IL-6 levels were also associated with *S. aureus* density in sputa (p = 0.0016; difference of 0.6031 pg/ml in patients with high versus low *S. aureus* density), but not with bacterial density in throat or nasal cultures. Moreover, a significant increase in IL-6 levels in patients with exacerbations (p = 0.0411; difference of 0.2104 pg/ml), SCVs (p = 0.0209; difference 0.2287 pg/ml), *S. maltophilia* (p = 0.0195; difference 0.2622 pg/ml) or with *A. fumigatus* (p = 0.0496; 0.2256 pg/ml) was documented. In addition, significantly more CF patients with high bacterial loads displayed elevated IL-6 levels above the upper normal limit of 12 pg/ml [14,23,24] compared to patients with sputa containing low bacterial loads (p = 0.0142).

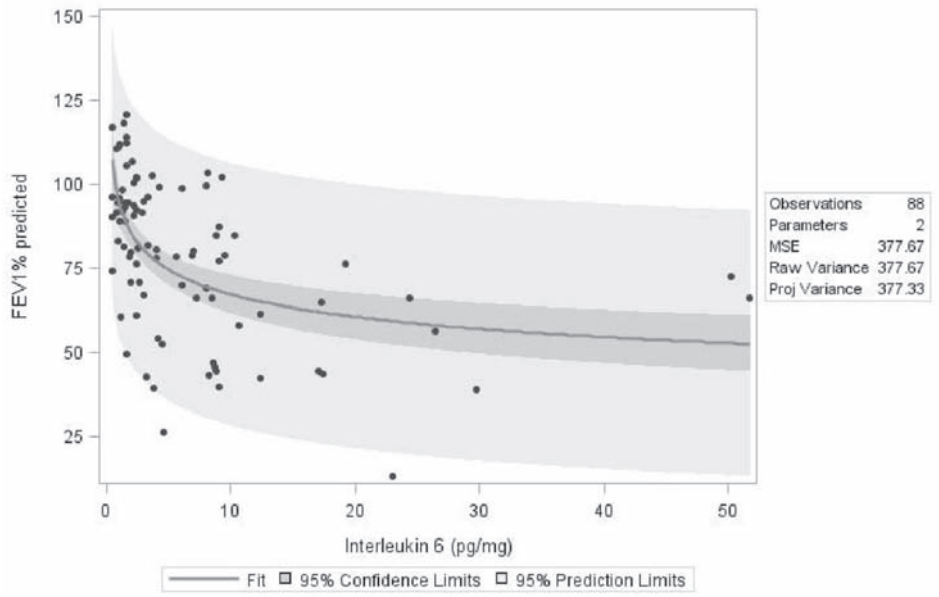

Figure 6. Higher IL-6 is associated with lower FEV1% predicted.
Scatterplot of baseline measurements (n = 88) of all patients with blood samples provided at the first visit. The fit line shows the results of a non-linear regression model. Dark shaded areas show 95% confidence band, lighter shaded area shows 95% prediction band.

Humoral response towards *S. aureus*

Recently, the height of *S. aureus* toxin-specific IgG-levels has been associated with the presence of *S. aureus* in airway specimens of CF patients, the severity of exacerbations and with FEV_1% predicted [25]. To expand on these results, we measured IgG-levels against 44 different virulence factors of *S. aureus* (Supplemental Materials) in single serum samples of 182 CF patients and included the results in our analysis. Furthermore, IgG-levels of patients were compared to those of 53 healthy adult volunteers with persistent nasal *S. aureus* carriage. IgG-levels against 15 out of 44 analysed antigens were significantly higher in patients compared to controls ($p<0.001$, Table 3). It is noteworthy that IgG-levels against 22 virulence factors were significantly and positively associated with IL-6 levels in all CF patients and, in line with previous data, IgG-levels against 18 virulence factors were significantly and inversely associated with FEV_1% predicted (S2 Table). A significant positive association between specific IgG-levels and nasal carriage, presence of SCVs and exacerbations for respectively 14, 16 and 7 antigens (S2 Table) was reported. Finally, no significant differences were found in IgG-levels of patients expectorating sputum with high and low bacterial loads (S2 Table).

Table 3. Significant specific IgG levels against *S. aureus* antigens in CF patients compared to healthy controls.

Antigen	Mean IgG level patients (± SE)[1]	Mean IgG level controls (± SE)[1]	p value[2,5]	Correlation coefficient of IgG and IL-6 levels[3]	p value[4,‖]
Efb	4605 (±210)	2089 (±208)	< 0.0001	0.010	0.840
Glucosaminidase	14992 (±250)	11567 (±571)	< 0.0001	0.147	0.002
HlgB	13621 (±174)	9878 (±407)	< 0.0001	0.061	0.197
IsaA	13288 (±413)	10479 (±678)	0.0009	0.207	< 0.0001
IsdA	10701 (±250)	7638 (±429)	< 0.0001	0.304	< 0.0001
LukD	14176 (±248)	11485 (±464)	< 0.0001	0.14	0.003
LukE	14272 (±238)	11720 (±474)	< 0.0001	0.169	< 0.0001
LukF	4079 (±121)	2718 (±243)	< 0.0001	0.169	< 0.0001
LukS	14097 (±239)	7134 (±524)	< 0.0001	0.206	< 0.0001
LytM	6576 (±308)	3555 (±380)	0.0001	0.27	< 0.0001
Nuc	14405 (±271)	4752 (±493)	< 0.0001	0.0837	0.0795
SA0688	3063 (±259)	760 (±116)	< 0.0001	0.355	< 0.0001
SSL5	2589 (±128)	1616 (±111)	< 0.0001	-0.020	0.669
SSL9	8910 (±194)	7568 (±306)	0.0009	0.079	0.098
Wall teichoic acid	2491 (±124)	319 (±117)	< 0.0001	0.109	0.025

[1]Average IgG levels are given in mean fluorescence activity (MFI).
[2]p-values of difference between 182 patients and 53 healthy controls (Mann-Whitney U test)
[3]Spearman's rank correlation coefficients were calculated for IgG- and IL-6 levels in all patients.
[4]p-values of correlation between IgG and IL-6
[5]adjusted p-values (Bonferroni correction)

DISCUSSION

Using a generalized linear mixed model our data only seem to support our main hypothesis stating that *S. aureus* density has an impact on lung function for patients who provided throat swabs since we could show that patients with higher bacterial density in throat cultures experienced a steeper lung function decline compared to patients with low bacterial density in these airway specimens (Figure 1). However, a closer look at our data showed that this effect was mostly carried by older patients, who provided throat cultures. The effect was lost, if only the group of patients younger than 20 years was analyzed, which is why these results should be interpreted carefully. Yet a trend of worse lung function of patients with high *S. aureus* density in sputa compared to patients with low *S. aureus* density was observed during the study period if cumulative clinical symptoms according to Fuchs were included in the linear mixed model analysis ($p = 0.0661$, S1 Fig).

With our inclusion and exclusion criteria a subgroup of patients was selected that differed from the German CF population by a preponderance of male patients (61.5%) and

overrepresentation of non-p.Phe508del *CFTR* genotypes (27.7%). The fact that our study patients experienced a mean lung function decline of -1.2717 FEV_1% predicted per years of age are consistent with German registry data that carriers of non-F508del genotypes become later in life colonized with *P. aeruginosa* than F508del compound heterozygotes and homozygotes and experience a milder course of the disease[26]. Nevertheless, our study patients still fit into the general CF-population with a lung function decline of 1 to 3 FEV_1% points per year as previously described [27,28] and do therefore not differ in terms of severity of the clinical disease.

Recently, in a retrospective single center study Ahlgren et al. compared CF patients, who were colonized with *S. aureus* or *P. aeruginosa* only, or neither of these pathogens [29]. In their study, which also included more male patients (n = 55%), 24% of 84 patients were colonized by *S. aureus* [29]. In conjunction with our data this may suggest that male patients are more likely colonized by *S. aureus* only and are more likely resistant to colonization or infection caused by *P. aeruginosa*.

Several studies have shown that persistent nasal *S. aureus* carriers are at increased risk for severe *S. aureus* infections, but have a survival advantage during sepsis [13]. We thus determined the impact of nasal *S. aureus* carriage in CF patients on disease progression. Nasal carriage was higher than in other studies assessing nasal carriage as a risk factor for *S. aureus* infection [30,31] both in healthy persons [32] and in CF patients [11], but comparable to the study of Goerke et al. [11,12]. This is most likely attributable to the pre-selection on the basis of our inclusion criteria with the requirement of *S. aureus* persistence in patients' airways.

During the study period the number of patients experiencing at least one pulmonary exacerbation was somewhat lower in our study compared to the study by Sanders et al. (31% versus 40%), who determined the association of frequency of exacerbations and subsequent lung function decline in more than 8.000 CF patients retrospectively [33]. This observation is most likely due to the fact that we excluded patients with chronic *P. aeruginosa* infection who have been shown to experience a more severe course of disease [34].

Our data thus clearly show that the culture of SCVs was independent of *P. aeruginosa* co-infection, but associated with advanced age, lower lung function and treatment with TMP/SMX at both base line and during the study period. Recently, Wolter et al. showed that the recovery of SCVs in airway specimens is independently associated with worse lung function in children [35]. Further studies also indicate that the detection of *S. aureus* SCVs was associated with persistence of *S. aureus* in airway specimens [19], advanced age [36], worse lung function [35–37] and co-infection with *P. aeruginosa* [35,36]. However, in our study we ruled out any impact of *P. aeruginosa* by excluding patients with persistent *P. aeruginosa* infection. In future analyses, it will be investigated

if special geno- or phenotypes of *S. aureus* have an impact on the clinical status of the patients.

In our study, patients co-infected by *S. maltophilia* differed from patients without *S. maltophilia*. They were most likely female, not *S. aureus* nasal carriers, co-infected with *S. aureus* SCVs and experienced more exacerbations. While Goss et al. [38] did not find any impact of *S. maltophilia* on lung function, Waters et al. have recently shown that *S. maltophilia* represented an independent risk factor for pulmonary exacerbations [39], when patients were persistently colonized with *S. maltophilia*. Although we did not distinguish between *S. maltophilia* cultured once or persistently, co-infection by *S. maltophilia* clearly had a negative impact on the clinical status of our patients.

Also, patients co-infected by *A. fumigatus* significantly differed from patients without *A. fumigatus* with respect to age and prevalence of SCVs. Surprisingly, patients with *A. fumigatus* exhibited a better lung function (data not shown) compared to patients without the fungus. This is in contrast to the results reported by Amin et al. [40] showing worse lung function for 37 out of 230 CF patients with persistent *A. fumigatus* infection. This discrepancy may be due to the fact that we did not distinguish between intermittent and chronic infection. It may also be explained by a sampling effect as we primarily cultured *A. fumigatus* from a small number of mostly *A. fumigatus*-positive older patients.

Recently, Horsley et al. documented IL-6 as a key biomarker of inflammation in CF patients with exacerbations [14]. We found that IL-6 levels were significantly correlated with *S. aureus* density in sputa, and with the presence of exacerbations, SCVs, *S. maltophilia* or *A. fumigatus*. Thus, these results suggest that IL-6 is a highly sensitive marker for lung disease. IL-6 may potentially be used to identify patients, which might benefit from initiation of antibiotic therapy.

CF patients mounted higher IgG-levels against numerous virulence factors of *S. aureus* compared to healthy controls. Such an immune response against *S. aureus* is comparable to those of patients that exhibited *S. aureus* bacteremia or osteomyelitis [20]. The fact that the adaptive immune system produces specific antibodies against *S. aureus* underlines that persistently colonized CF patients are immunologically challenged by *S. aureus* although these specific antibodies cannot resolve *S. aureus* persistence. While no significant association between bacterial density in airway specimens of CF patients and IgG-levels was reported, we found a significant association of many specific IgG-values with IL-6-levels, worse lung function, nasal carriage and the culture of SCVs. The latter confirms and expands on another recent study that demonstrated a significant inverse association between *S. aureus* toxin-specific IgG-levels and lung function in CF patients [25]. In contrast to our results, this previous study also showed a significant association between the culture of *S. aureus* in airway specimens and IgG-levels, albeit bacterial density was not quantified as it was done in our study.

There are several limitations to our study. First, our inclusion and exclusion criteria selected a sub-group of CF patients, which do not reflect the entire CF population. However, lung function data of our patients were comparable to other studies [27,41]. Our data should thus provide meaningful results for the general CF population. Second, our selected patient group did experience somewhat less exacerbations compared to groups with *P. aeruginosa* infection, which makes it more difficult to detect significant clinical differences between groups using exacerbation as an outcome measure. Third, the technique of obtaining throat swabs in the different centres by different nurses or physicians could have influenced the microbiological results of the throat swab cultures. Fourth, it seems that our study was underpowered to show significant results for the association between *S. aureus* density and worse lung function of patients, who were able to expectorate sputum. Since *S. aureus* is the most prevalent CF-related pathogen in children, we included patients in our study who were not able to expectorate sputum. We hypothesized that the determination of bacterial densities would be of equal value for throat swabs and sputa. Our patient number calculation was based on this hypothesis. However, there was no association between bacterial density and lung function for patients with throat swabs, but a trend for patients with high bacterial density in sputa if cumulative clinical symptoms for exacerbation were included in the analysis ($P = 0.0661$, S1 Fig).

To conclude, our results suggest that the presence of exacerbations, non-nasal *S. aureus* carriage, female gender, the presence of *S. aureus* SCVs, co-infection with *S. maltophilia* or specific anti-staphylococcal IgG- and IL-6 levels are independent risk factors for worse lung function in CF patients with persistent *S. aureus* infection. Our findings may help to identify *S. aureus* patients at risk for more severe airway infections resulting in worse lung function and will be of value for designing future prospective studies to guide antibiotic therapy in patients chronically infected with *S. aureus*.

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SUPPLEMENTAL DATA

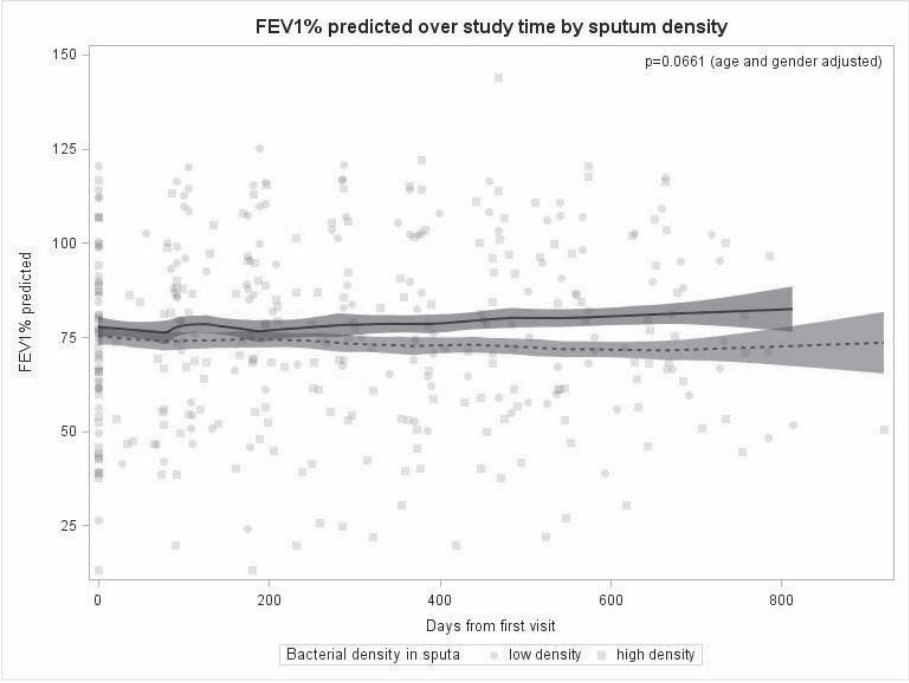

Figure S1. FEV1% predicted over study time by sputum density.
The figure shows observed FEV1% predicted measurements over study time (days from first visit). Lines represent the LOESS-fit including 95% confidence intervals of the predicted values of the generalized linear mixed model. Patients with high bacterial density in sputum are indicated by red squares, patients with low bacterial density in sputum are indicated by blue circles. The fitted model prediction is coloured accordingly.

Table S1. Clinical characteristics of patients at baseline: *S. aureus* nasal carriage, incidence of exacerbations and SCVs.

Characteristics	all patients (range)	nasal carriers (%)	non-carriers (%)	p-value	With exacerbation (%)	without exacerbation (%)	p-value	with SCVs (%)	without SCVs (%)	p-value
# of patients	195	122 (65%)†	66 (35%)		60 (31%)	135 (69%)		84 (43%)	111 (57%)	
Age (years)	16	14.083	13.33	0.45‡	14.95	13.65	0.5182‡	15.41	13.03	0.0066‡
	(6-42)	(6.02-41.58)	(5.82-40.7)		(7.23-36.37)	(5.82-41.7)		(5.82-41.58)	(6.02-40.53)	
Male	120 (61.54%)	86 (70.5%)	30 (45.5%^)	0.00075§	33 (55%)	87 (64.44%)	0.2109§	48 (57.14%)	72 (64.86%)	0.2724§
Ex. panc.suff.	22 (11%)	17 (8.7%)	5 (2.5%)	0.201§	3 (5.08%)	19 (14.18%)	0.067§	9 (10.8%)	13 (12.04%)	0.7978§
FEV_1	84.07	86.96	80.92	0.096‡	81.64	86.27	0.1941‡	79.48	88.56	0.0133‡
(% predicted)	(13.18-120.57)	(13.18-120.57)	(26.35-118.05)		(26.35-116.82)	(13.18-120.57)		(13.18-120.57)	(30.56-115.19)	
BMI-Quantils	25%	28%	23%	ns‡	26.77%	24.93%	0.7740‡	23%	27%	0.3166‡
(0.068%-100%)	(1%-100%)	(0.068%-81%)	(1.7%-97.29%)		(0.068%-99.79%)	(0.068%-92%)		(4%-100%)		
Genotype	173									
F508del homo	85 (49%)	52 (48.1%)	28 (48.3%)	0.993§	27 (54%)	58 (47.15%)	0.1764§	35 (49.3%)	49 (49%)	0.079§
F508del hetero	40 (23.2%)	25 (23.1%)	13 (22.4%)		14 (28%)	26 (21.14%)		11 (15.5%)	28 (28%)	
others	48 (27.7%)	31 (28.7%)	17 (29.3%)		9 (18%)	39 (31.71%)		25 (35.2%)	23 (23%)	

Table S1. Clinical characteristics of patients at baseline: incidence of *Stenotrophomonas* and *Aspergillus*.

Characteristics	With S. maltophilia	Without S. maltophilia	p-value	With A. fumigatus	Without A. fumigatus	p-value
# of patients	44	151		60 (31%)	135 (69%)	
Age (years)	15.78	13.51	0.0847‡	18.10	12.85	<0.0001‡
	(7.33-36.37)	(5.82-41.58)		(8.99-41.58)	(5.82-40.70)	
Male	20 (45.45%)	100 (66.23%)	0.0127§	34 (56.67%)	86 (63.7%)	0.3512§
Ex. panc.suff.	2 (4.55%)	20 (13.61%)	0.0986§	7 (11.86%)	15 (11.36)	0.9202§
FEV_1	80.05	85.89	0.2071‡	80.41	85.60	0.2698‡
(% predicted)	(38.96-120.57)	(13.18-118.05)		(37.56-120.57)	(13.18-118.05)	
BMI-Quantils	23%	25%	0.8861‡	23%	27%	0.2332‡
(5%-92%)	(0.068%-100%)	(1%-97%)		(0.068%-100%)		
Genotype	171			171		
F508del homo	20 (52.63%)	64 (48.12%)	0.8861§	26 (49.06%)	58 (49.15%)	0.9988§
F508del hetero	8 (21.05%)	31 (23.31%)		12 (22.64%)	27 (22.88%)	
others	10 (26.32%)	38 (28.57%)		15 (28.3%)	33 (27.97%)	

Table S2. Staphylococcal specific IgG-levels according to the different patient groups.

Antigen	Mean IgG level patients (± SE)[1]	Mean IgG level controls (± SE)[1]	p value[2,3]	Mean IgG level high bacterial density (± SE)[4]	Mean IgG level low bacterial density (± SE)[5]	p value[2, 3]
Alpha toxin	14405 (±271)	13902 (±511)	0.2653	14430 (±345)	14493 (±282)	0.8085
CHIPS	11492 (±220)	11019 (±349)	0.104	11546 (±319)	11617 (±254)	0.6691
ClfA	5168 (±237)	4302 (±436)	0.081	5617 (±322)	5222 (±262)	0.3415
ClfB	4587 (±222)	4092 (±338)	0.3809	5221 (±321)	4562 (±214)	0.2075
Efb	4605 (±210)	2089 (±207)	< 0.0001	4610 (±290)	4698 (±236)	0.6336
ETA	3999 (±447)	2043 (±424)	0.1180	3868 (±532)	3884 (±421)	0.7338
ETB	613 (±113)	320 (±94)	0.0032	532 (±135)	341 (±48)	0.7725
FlipR	5708 (±251)	4834 (±400)	0.0784	6203 (±333)	5487 (±277)	0.0931
FnbpA	2922 (±201)	2534 (±300)	0.6167	3116 (±252)	2979 (±194)	0.1881
FnbpB	967 (±79)	1327 (±215)	0.1306	1286 (±144)	1036 (±71)	0.2802
Glucosaminidase	14992 (±250)	11567 (±571)	< 0.0001	15507 (±308)	15174 (±256)	0.2996
HlgB	13621 (±174)	9878 (±407)	< 0.0001	13705 (±235)	13624 (±206)	0.7169
IsaA	13288 (±413)	10479 (±678)	0.0009	13963 (±595)	12654 (±466)	0.0524
IsdA	10701 (±250)	7638 (±428)	< 0.0001	11133 (±283)	10871 (±283)	0.0732
IsdH	3220 (±238)	3643 (±380)	0.0910	3281 (±296)	2816 (±207)	0.0981
Lipase	9979 (±317)	8237 (±629)	0.0104	10318 (±439)	9266 (±361)	0.0761
LukD	14176 (±248)	11485 (±463)	< 0.0001	14598 (±314)	13916 (±292)	0.0789
LukE	14272 (±238)	11720 (±473)	< 0.0001	14752 (±305)	13998 (±287)	0.0819
LukF	4079 (±133)	2718 (±243)	< 0.0001	4526 (±200)	4144 (±147)	0.0892
LukS	14097 (±133)	7134 (±524)	< 0.0001	11068 (±396)	10586 (±295)	0.1873
LytM	6576 (±367)	3555 (±380)	0.0001	7059 (±492)	6248 (±382)	0.0542
Nuc	14405 (±271)	4752 (±492)	< 0.0001	10852 (±503)	9881 (±394)	0.0707
SA0486	446 (±26)	341 (±42)	0.0397	450 (±43)	488 (±41)	0.5357
SA0688	3063 (±259)	759 (±116)	< 0.0001	3449 (±346)	3177 (±220)	0.1731
SasG	569 (±70)	636 (±132)	0.1847	623 (±95)	388 (±50)	0.1852
SCIN	10872 (±247)	9657 (±388)	0.0043	11226 (±321)	10470 (±277)	0.0655
SdrD	1084 (±80)	694 (±76)	0.0507	1108 (±105)	937 (±65)	0.2933
SdrE	3139 (±201)	1992 (±221)	0.0253	3513 (±275)	3351 (±199)	0.2916
SEA	3835 (±320)	3613 (±425)	0.2544	4398 (±437)	3539 (±321)	0.1111
SEC	7390 (±441)	8714 (±809)	0.1350	6901 (±581)	6878 (±473)	0.9446
SED	1106 (±118)	1292 (±268)	0.0498	1150 (±153)	1115 (±135)	0.1178
SEE	1333 (±183)	879 (±136)	0.8113	1416 (±214)	1039 (±142)	0.1334
SEG	2047 (±184)	1225 (±270)	0.0544	1941 (±190)	1790 (±190)	0.4405
SEH	2290 (±291)	2174 (±359)	0.0113	2128 (±382)	1950 (±281)	0.9851
SEO	368 (±40)	400 (±51)	0.0266	476 (±79)	394 (±48)	0.5521
SER	1548 (±243)	2001 (±580)	0.3882	1755 (±377)	1436 (±264)	0.2225

Estimated effect on IL-6 levels[6]	p value[3]	Estimated effect on FEV1%[7]	p value[3]	OR nasal carrier status[9]	p value[3]	OR SCV presence[9]	p value[3]	OR exacerbation[10]	p value[3]
-1.33E-6	0,9226	-0,00013	0,6506	1,010	0,0008	0,996	0,2112	0,995	0,0993
0,000019	0,2173	-0,00073	0,0185	1,003	0,4282	1,000	0,9999	1,002	0,4990
-0,00002	0,1179	0,000514	0,1104	1,009	0,0174	1,011	0,0011	1,001	0,6727
3.208E-6	0,8616	0,002079	<.0001	1,005	0,2808	0,995	0,2333	0,995	0,2456
0,000034	0,0650	-0,00071	0,0531	0,995	0,2490	1,001	0,7260	1,000	0,9118
0,000026	0,0173	-0,00061	0,0033	1,004	0,0741	1,000	0,9101	1,001	0,7047
0,000161	0,0014	-0,00700	<.0001	1,032	0,0743	1,014	0,2320	1,002	0,8706
0,000020	0,2025	-0,00040	0,1852	1,009	0,0149	0,999	0,8526	0,998	0,5126
0,000031	0,1775	0,000222	0,6310	1,010	0,0723	1,010	0,0723	1,014	0,0060
0,000053	0,3988	-0,00199	0,1150	0,991	0,5160	1,010	0,4724	0,964	0,0216
0,000061	<.0001	-0,00090	0,0014	1,003	0,3082	1,010	0,0047	1,004	0,2303
0,000063	0,0027	-0,00109	0,0088	1,003	0,4992	1,010	0,0290	1,003	0,5584
0,000032	0,0003	-0,00064	0,0002	1,004	0,0300	1,001	0,6861	1,002	0,2518
0,000085	<.0001	-0,00152	<.0001	1,010	0,0028	1,009	0,0083	1,000	0,9086
0,000063	0,0010	-0,00039	0,3074	0,998	0,7124	1,003	0,4299	1,002	0,6094
0,000032	0,0055	-0,00025	0,2749	1,002	0,5068	1,003	0,2340	1,003	0,2450
0,000052	0,0003	-0,00128	<.0001	1,003	0,4125	1,008	0,0195	1,001	0,7464
0,000067	<.0001	-0,00136	<.0001	1,000	0,9943	1,012	0,0012	1,007	0,0323
0,000100	0,0002	-0,00305	<.0001	0,998	0,7741	1,003	0,5710	1,013	0,0254
0,000064	<.0001	-0,00169	<.0001	0,995	0,0700	1,009	0,0015	1,004	0,1382
0,000054	<.0001	-0,00069	0,0014	0,999	0,7773	1,007	0,0030	1,005	0,0361
0,000022	0,0242	-0,00021	0,2689	1,007	0,0011	1,004	0,0409	0,997	0,1049
4.081E-6	0,9519	0,000285	0,8300	0,971	0,1795	0,995	0,7294	1,004	0,7859
0,000113	<.0001	-0,00217	<.0001	1,000	0,9754	1,008	0,0314	1,007	0,0570
0,000016	0,7673	0,001484	0,1590	1,002	0,8445	1,049	0,0157	1,006	0,5492
0,000039	0,0060	-0,00063	0,0241	1,009	0,0032	1,006	0,0684	0,995	0,1129
0,000138	0,0160	-0,00145	0,1950	0,973	0,0260	1,029	0,0232	0,985	0,2246
0,000068	0,0010	-0,00179	<.0001	1,003	0,4736	1,013	0,0059	1,000	0,9768
-0,00001	0,3454	-0,00007	0,7599	1,005	0,1021	1,004	0,0935	0,997	0,2508
-9.9E-6	0,2951	0,000269	0,1366	1,000	0,9743	1,000	0,9411	0,999	0,6049
0,000018	0,6231	0,001490	0,0377	1,010	0,2704	0,998	0,7832	0,999	0,8861
-6.43E-7	0,9820	-0,00057	0,2779	1,014	0,0608	1,015	0,0193	0,997	0,6557
0,000091	0,0005	-0,00108	0,0264	1,029	0,0003	1,005	0,3829	0,998	0,7464
-0,00001	0,3834	-0,00047	0,0986	0,991	0,0039	1,009	0,0071	1,007	0,0249
0,000185	0,0452	0,001048	0,5874	1,094	0,0135	0,991	0,6676	0,944	0,0436
0,000024	0,2410	0,000166	0,6522	1,007	0,1954	0,999	0,7642	1,007	0,0955

Table S2. Staphylococcal specific IgG-levels according to the different patient groups. (continued)

Antigen	Mean IgG level patients (± SE)[1]	Mean IgG level controls (± SE)[1]	p value[2,3]	Mean IgG level high bacterial density (± SE)[4]	Mean IgG level low bacterial density (± SE)[5]	p value[2, 3]
SSL1	6856 (±410)	5684 (±683)	0.2735	7623 (±542)	6359 (±424)	0.0571
SSL3	5561 (±216)	5123 (±313)	0.4604	5737 (±285)	5621 (±245)	0.5586
SSL5	2589 (±128)	1616 (±111)	< 0.0001	2639 (±170)	2575 (±139)	0.8226
SSL9	8910 (±194)	7568 (±305)	0.0009	9114 (±252)	8917 (±235)	0.5563
SSL10	8754 (±306)	9870 (±552)	0.0869	9107 (±420)	8520 (±336)	0.2516
SSL11	3127 (±255)	2698 (±347)	0.8779	3310 (±334)	2969 (±267)	0.0839
TSST1	7981 (±487)	9076 (±646)	0.499	7585 (±634)	7602 (±545)	0.6607
Wall teichoic acid	2491 (±124)	319 (±16)	< 0.0001	2182 (±119)	2038 (±119)	0.2623

[1] Mean fluorescence intensity (MFI) of IgG levels are shown. MFI values between 182 patients and 53 healthy controls are compared.

[2]p values of difference between patient and controls groups (Mann-Whitney U test)

[3]adjusted p-values (Bonferroni correction)

[4]Mean IgG levels of 228 cases expectorating sputum with high bacterial density

[5]Mean IgG levels of 166 cases expectorating sputum with low bacterial density

[6]IgG levels are modelled as continuous factors. Estimated effects are interpreted as mean change in IL6 per 1 unit of IgG levels. Significant effects are marked bold.

[7]IgG levels are modelled as continuous factors. Estimated effects are therefore interpreted as mean change in FEV1% predicted per 1 unit of IgG levels. Significant effects are marked bold.

[8]Odds ratios (OR) are interpreted as factor by which the risk to be a nasal carrier is changed per 100 units of IgG levels. Significant OR are marked bold.

[9]Odds ratios (OR) are interpreted as factor by which the risk to ever be SCV positive is changed per 100 units of IgG levels. Significant OR are marked bold.

[10]Odds ratios (OR) are interpreted as factor by which the risk to ever experience an exacerbation is changed per 100 units of IgG levels. Significant OR are marked bold.

Estimated effect on IL-6 levels[6]	p value[3]	Estimated effect on FEV1%[7]	p value[3]	OR nasal carrier status[9]	p value[3]	OR SCV presence[9]	p value[3]	OR exacerbation[10]	p value[3]
0,000021	0,0277	-0,00029	0,1199	1,009	0,0002	1,001	0,7920	1,002	0,2828
3.401E-6	0,8398	-0,00051	0,1235	0,996	0,2676	0,995	0,1844	0,988	0,0017
0,000023	0,4368	-0,00015	0,7957	1,013	0,0480	0,981	0,0052	0,997	0,5820
0,000034	0,0620	0,000681	0,0570	1,002	0,5567	1,000	0,9684	0,997	0,3912
0,000020	0,1071	0,000239	0,3165	0,999	0,6832	0,996	0,1613	1,000	0,9705
3.79E-6	0,8488	-0,00154	0,0001	1,006	0,2167	0,994	0,1617	0,992	0,0788
-7.19E-6	0,3762	0,000148	0,3554	1,004	0,0283	0,999	0,4654	0,998	0,1558
0,000070	0,0147	-0,00228	<.0001	1,008	0,2084	1,006	0,3328	1,012	0,0588

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Chapter 6

Human IgG cannot inhibit fibrinogen binding by the genetically diverse A domain of *Staphylococcus aureus* fibronectin-binding protein A

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ABSTRACT

The Fibronectin binding protein A (FnBPA) is a cell-surface associated protein of *Staphylococcus aureus* which mediates adherence to the host extracellular matrix and is important for bacterial virulence. Previously, substantial sequence diversity was found among strains in the fibrinogen-binding A domain of this protein and 7 different isotypes were described. In this study we identify five different FnbpA A domain isotypes in 22 clinical *S. aureus* isolates, obtained from the same number of patients suffering from bacteraemia. Using a bead-based Luminex technique, we establish the presence of total IgG directed against the 7 previously described FnbpA isotypes in all patients at the onset and during the subsequent time course of bacteremia (median of 10 serum samples per patient over a median of 35 days). A significant increase in IgG against the FnbpA A domain, including the isotype carried by the infecting strain, is observed in only three out of 22 patients (14%) after the onset of bacteremia. Using a Luminex-based FnBPA-fibrinogen binding assay we find that pre-incubation of recombinant FnBPA isotypes with IgG from diverse patients does not interfere with binding to fibrinogen. This observation is confirmed using an alternative Luminex-based assay and ELISA.

Importance: Despite the many *in vitro* and murine *in vivo* studies examining the role of FnBPA, the actual presence of this virulence factor during infection in humans is less established. Furthermore, it is currently unknown to what extent sequence variation in virulence factors such as FnBPA affects the human antibody response and the ability of antibodies to interfere with FnBPA function. In this study we establish the ubiquitous presence of human IgG against FnBPA, indicating the importance of this virulence factor during *S. aureus* pathogenesis. Despite this ubiquitous presence of IgG, we find that most patients do not normally mount a significantly increased antibody response against FnBPA after the onset of bacteraemia, indicating lack of expression and/ or immune evasion by FnBPA during this infection. Finally, human antibodies do not interfere with FnBPA-fibrinogen binding. These observations should be taken into account during the development of novel vaccination approaches.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) can both colonize human squamous epithelial surfaces [1, 2] and cause infections that are associated with significant morbidity and mortality, including bacteraemia [3-5]. The success of *S. aureus* to colonize and infect its human host is attributed to many virulence factors, including surface proteins such as the fibronectin-binding protein A (FnBPA) [6]. This protein is encoded by the *fnbA* gene [7, 8] and is expressed by most strains of *S. aureus* [9]. FnBPA has been shown to contribute to disease in diverse animal models of infection diseases [10-12] and it has several functions. FnBPA promotes binding to fibrinogen, elastin and fibronectin [13-15] and it mediates both adhesion to and invasion of endothelial and epithelial cells [16, 17]. The protein also promotes biofilm formation [18, 19]. These latter functions of FnBPA are considered to help *S. aureus* in evading antibiotics and the host immune response [16, 17, 20].

The many functions of FnBPA have made it a potentially interesting target for vaccination approaches against *S. aureus*. However, although FnBPA has been successfully used as a vaccine target in animal models [17, 21, 22], so far this has not led to a valid vaccine in humans. Several factors might contribute to this failure, such as the (lack of) presence of FnBPA during invasive infection, which, despite the plethora of studies examining the role of FnBPA *in vitro* and in animal models *in vivo*, has been less established *in vivo* in humans. Another factor might involve the substantial sequence diversity found in the fibrinogen binding A domain of FnBPA [23], with seven different protein variants (isotypes) sharing only 66 to 76% amino acid identity identified so far [24, 25]. Although all these isotypes bind fibrinogen with similar affinity, murine monoclonal antibodies raised against different isotypes exhibit only limited cross reactivity [25]. The effect of this sequence diversity on the human antibody response against FnBPA remains unclear and has not been taken into account in previous vaccination studies, including both animal models and clinical trials, nor in in our previous studies characterizing patient antibody responses [26-28]. Yet if such sequence diversity in FnBPA among clinical strains of *S. aureus* is present and would affect the human antibody response, this should be taken into account during the development of future vaccines.

It is currently also unknown whether natural antibodies against FnBPA, produced in patients after the onset of infection, can interfere with the binding between FnBPA and fibrinogen. Interestingly, it was previously shown that IgG obtained from patients with *S. aureus* infections did not interfere with the binding between fibronectin and recombinant FnBPA fragments or whole *S. aureus* cells, respectively [29]. On the other hand, rabbit monoclonal antibodies against *S. aureus* FnBP fragments did inhibit fibronectin binding [30], and rabbit polyclonal antibodies raised against clumping factor B, another surface protein of *S. aureus* mediating bacterial adherence, also blocked binding to

fibrinogen [31]. Thus, the ability of naturally occurring antibodies to block the binding between *S. aureus* and constituents of the extracellular matrix remains uncertain.

The aims of this study were to further characterize sequence diversity in the A domain of FnBPA among 22 clinical strains isolated from the blood of the same number of patients suffering from bacteraemia, to characterize the antibody responses against different isotypes in each patient, and to ascertain the ability of human antibodies to interfere with binding between FnBPA and fibrinogen.

RESULTS

Variation of the FnBPA A domain in clinical *S. aureus* isolates

We determined the prevalence of the seven known isotypes (I-VII) of the FnBPA A domain [24, 25] among twenty-two *S. aureus* strains isolated from the blood of patients suffering from bacteremia. The FnBPA A domain of each strain was sequenced and genomic sequences were compared together with those of previously described reference strains for each isotype.

Five isotypes were found among the twenty-two isolates: five strains encoded isotype I (92.7 to 100% amino acid (AA) identity to reference strain 382), five strains isotype II (96 to 100% AA identity to reference strain 3011), five strains isotype III (97.1 to 97.4% AA identity to reference strain 182) and six strains encoded isotype IV (91.9 to 97.1% AA identity to reference strain P1). One strain encoded isotype V (97.5% AA identity to reference strain 3110). The genetic relationships of the *fnbA* A domain of all strains, including the reference strains, is summarized in figure 1. Strains with the same *fnbA* isotype also shared similar *spa* types, while the single strain encoding isotype V had *spa* type t8930.

Since the isotype of the FnBPA A domain is primarily determined by the highly divergent N2N3 subdomains [25], recombinant proteins of these subdomains were expressed for each isotype. These proteins were covalently coupled to color-coded beads in a Luminex set-up, allowing the simultaneous quantification of different antibodies directly in serum samples to characterize patient antibody responses against these variable subdomains.

Characterization of IgG response against FnBPA isotypes in bacteremia patients

Total IgG levels against all seven FnBPA A domain isotypes were prospectively measured in serum samples from patients after the onset of bacteremia (median of 10 serum samples per patient over a median of 35 days). Although patients were infected with a strain carrying one particular isotype, we observed that total IgG from all patients detectably bound all seven isotypes (Figure 2). However, the height of IgG levels, especially

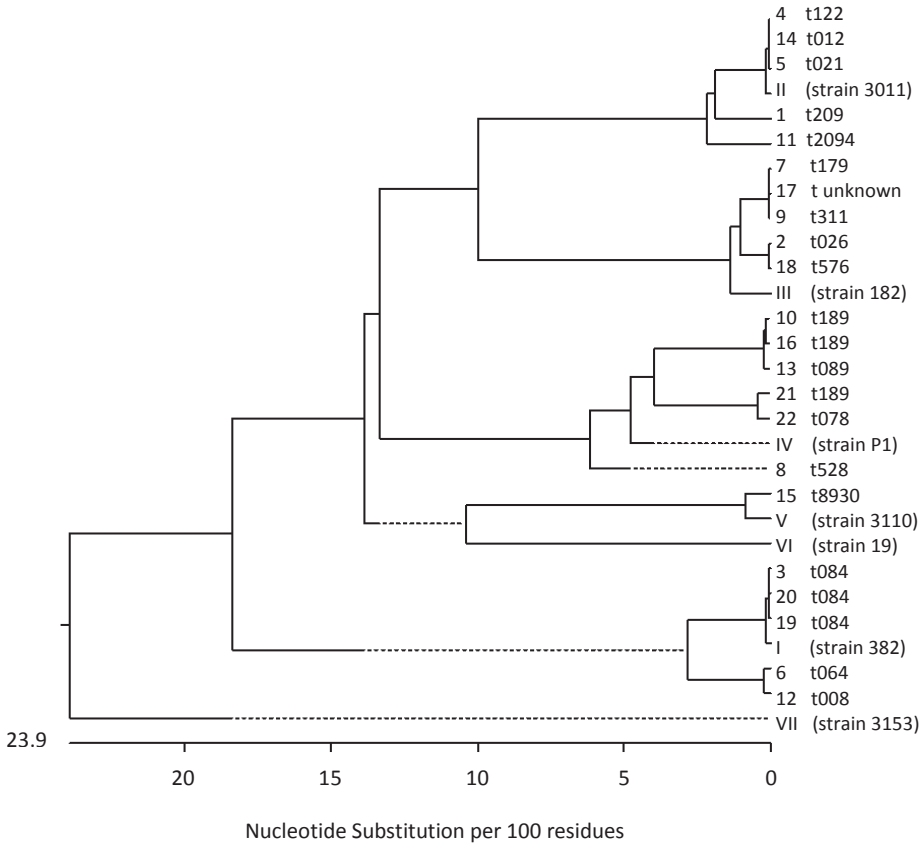

Figure 1. Genetic relationships of *fnbA* A domain between strains.
fnbA gene segments encoding the entire FnBPA A domain were sequenced from 22 clinical isolates. Numbers indicate patient number, followed by the Spa type of each strain. Sequences were aligned together with those of reference strains for the *fnbA* isotypes I to VII.

against the more common isotypes I to IV and isotype V, varied considerably between patients (Figure 3). This is in line with the heterogeneity observed previously in patient antibody responses after the onset of infection by our and other groups [26, 27, 32, 33].

In 18 out of 22 patients, the IgG levels directed against all isotypes did not significantly change after the onset of bacteremia (Figure 2A, Table S1) and we could not identify a specific increase in IgG against the isotype carried by the infecting strain. IgG levels against FnBPA did increase in the remaining four patients after the onset of bacteremia (patients 4, 15, 17 and 20; Table S1), with a significant 10-fold or higher increase observed in three patients (patients 4, 15 and 17). Interestingly, in two out of these latter patients (patients 4 and 15), predominantly IgG levels against the isotype of the infecting strain increased (figure 2B). In the third patient (patient 17), a concomitant, comparable increase in IgG levels against multiple FnBPA isotypes was observed (Table S1).

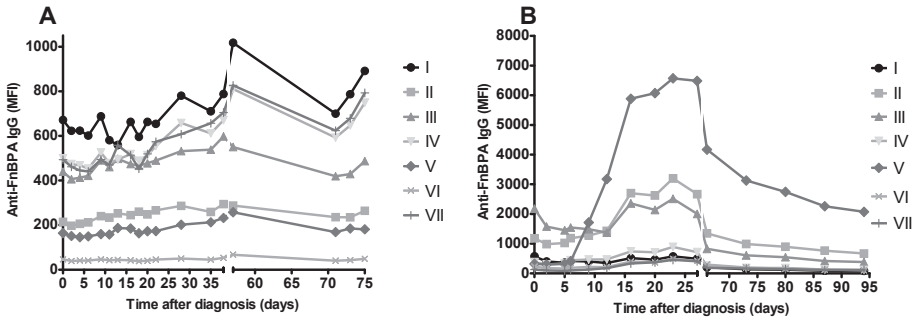

Figure 2. Course of IgG levels against FnBPA isotypes following bacteremia.
A: representative course of IgG levels against 7 FnBPA isotypes (I to VII) following the onset of bacteremia (day 0, defined as the day of the first blood culture positive for *S. aureus*) in patient 1, infected with a strain carrying isotype II. **B:** same plot for patient 15, infected with a strain carrying isotype V. Data points represent the mean of two separate measurements per serum sample.

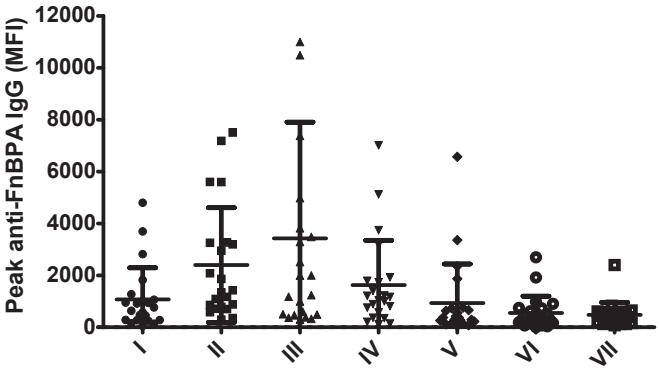

Figure 3. Variation in peak IgG levels against FnBPA isotypes after the onset of bacteremia.
Scatter plot showing the distribution of peak IgG levels against all 7 FnbPA isotypes (I to VII) in 22 patients suffering from bacteremia. Each point represents one patient and horizontal lines represent the median and interquartile ranges, respectively.

FnBPA isotypes bind human fibrinogen

To ascertain the ability of human antibodies, purified from the patients described above, to interfere with binding between FnBPA and fibrinogen, we developed a Luminex-based FnBPA-fibrinogen binding assay. After covalently coupling recombinant FnBPA isotype proteins to beads, we confirmed that all seven isotypes were able to bind R-PE labeled human fibrinogen in a dose-dependent and saturable manner (Supplementary figure 1A). This binding was further confirmed by a competition assay using R-PE labeled and unlabeled fibrinogen, showing that the amount of bound PE-labeled fibrinogen decreased at increasing concentrations of unlabeled fibrinogen (Supplementary figure 1B). Specific binding of fibrinogen by the FnBPA isotypes was confirmed by the lack of

fibrinogen binding observed for the SD-repeat containing protein E (SdrE) and iron-responsive surface determinant A (IsdA), which have low to no affinity for fibrinogen, respectively [34].

Human IgG does not inhibit binding between FnBPA isotypes and fibrinogen

Next, we investigated if purified IgG, isolated from bacteremia patients, could interfere with the binding between the FnBPA isotypes and fibrinogen. We confirmed that total IgG purified from five different patients, each infected with a strain carrying a different FnBPA isotype, and from polyclonal human IgG (PHG) obtained from non-infected volunteers, bound FnBPA isotypes I to V in a specific and dose-dependent manner (supplementary figure 2A and B). Human IgG did not noteworthy bind to fibrinogen (data not shown).

After pre-incubation of FnBPA isotypes I to V with the IgG from either PHG or patients, resulting FnBPA-bound IgG was allowed to bind PE-labeled fibrinogen. Based on results as shown in Supplementary figure 1A we chose a standard concentration of 1 µg PE-labeled fibrinogen per reaction. Pre-incubation of FnBPA isotypes I to V with IgG from neither PHG nor patients interfered with the binding of this protein to PE-labeled fibrinogen, regardless of the IgG dilution (Figure 4A and B). The amount of fibrinogen bound by the isotypes after pre-incubation with IgG dilutions did not significantly differ compared to controls without pre-incubation with IgG (one-way ANOVA, significance level of $P \leq 0.05$). Varying the starting concentration of fibrinogen did not affect these results, nor did simultaneous incubation of fibrinogen, IgG and FnBPA isotypes (data not shown).

To rule out the possibility that the coupling of FnBPA isotypes to the xMAP® beads could somehow affect protein structure and thereby alter binding sites for IgG, which could alternatively explain the lack of interference by IgG as described above, we performed two alternative assays. In the first Luminex-based assay, fibrinogen coupled to beads was incubated with PE-labeled, free recombinant isotype II and III. We confirmed a dose-dependent and saturable binding between the free FnBPA isotypes and immobilized fibrinogen (data not shown). Subsequently, we pre-incubated the free isotype II and III with IgG from PHG or from one of the patients, followed by incubation with the immobilized fibrinogen. In line with the results described above, fibrinogen binding by both isotypes was not inhibited by any dilution of IgG (figure 5A). The amount of bound PE-labeled FnBPA after pre-incubation with IgG did not significantly differ compared to controls without pre-incubation with IgG (one-way ANOVA, significance level of $P \leq 0.05$).

The results described above were further confirmed using ELISA, wherein isotype I and V were immobilized onto 96-wells plates and incubated with fibrinogen. Again, pre-incubation of both isotypes with a concentration range of IgG from neither PHG nor

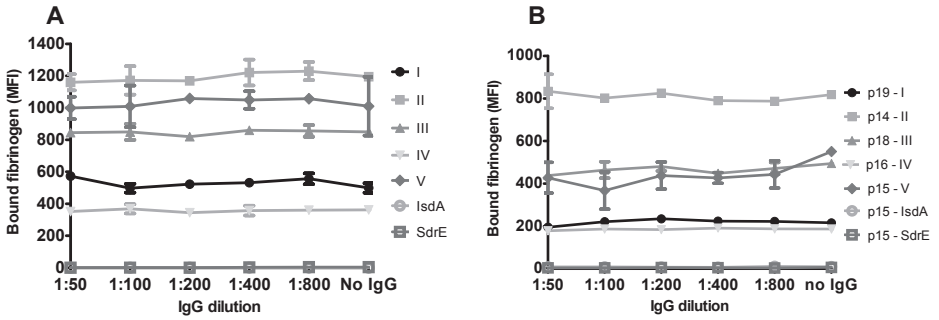

Figure 4. Pre-incubation of FnBPA isotypes with human IgG does not prevent binding of fibrinogen. **A:** The most common isotypes I to V, coupled to beads, were pre-incubated in this Luminex setup with a dilution range of IgG purified from Polyclonal Human IgG (PHG). Binding of a fixed concentration of PE-labeled fibrinogen was then measured. **B:** Results of the same experiment using dilution ranges of IgG purified from different patients. Each isotype was pre-incubated with IgG from a patient that was infected with a strain carrying that isotype (e.g., patient 19 was infected with a strain carrying isotype I).

patients affected the amount of bound fibrinogen (Figure 5B). As a control, we incubated fibrinogen with an increasing concentration of recombinant Clumping Factor A (ClfA) of *S. aureus*, which also binds to the same site on the fibrinogen r-chain as FnBPA, and measured the binding of fibrinogen to immobilized FnbpA. As expected, ClfA inhibited fibrinogen binding to FnbpA (Figure 5C).

DISCUSSION

We found that most patients suffering from serious invasive *S. aureus* infections do not respond to the microbial challenge by increasing production of pre-existing IgG antibody titers directed against the A domain of the most common FnBPA isotypes, including the isotype carried by the infecting strain. Only 3 out of 22 patients (14%) mounted a significantly increased antibody response against FnBPA after the onset of bacteraemia, which was predominantly directed against the isotype of the infecting strain in two patients. The immunoglobulins obtained from these and other patients, as well as from healthy volunteers, were not able to interfere with the binding between FNBPA and human fibrinogen *in vitro*.

In line with previous results we found that *fnbpa* isotypes I to IV were the most common among clinical isolates [25]. Total IgG from patients could detectably bind these isotypes and was ubiquitously present at the onset of bacteremia. Firstly, this ubiquitous presence of IgG against FnBPA suggests an important role of this virulence factor in the pathogenesis of *S. aureus*, presumably during colonization of the host and the

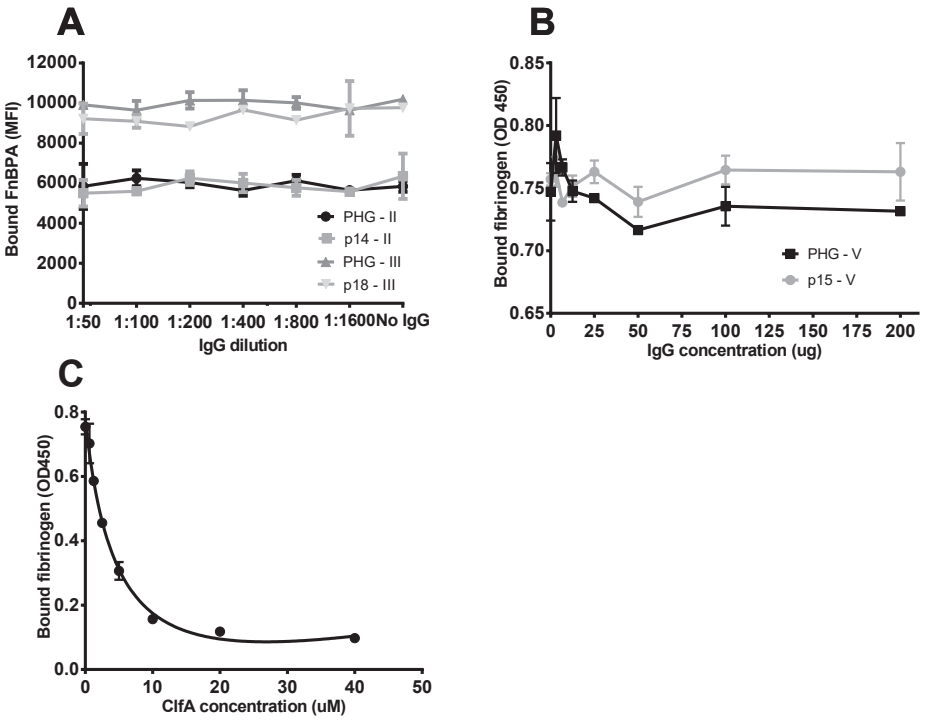

Figure 5. Confirmation of the inability of human IgG to prevent binding between FnBPA isotypes and fibrinogen.

A: PE-labeled, free FnBPA isotypes II and III were pre-incubated with a dilution range of total IgG from either PHG or from patients 14 and 18 (who were infected with strains carrying isotype II and III, respectively). Protein-antibody complexes were then allowed to bind fibrinogen coupled to Luminex beads. **B:** Using an alternative ELISA, pre-incubation of fibrinogen with IgG from patient 15 (infected with a strain carrying isotype V) did not clearly affect subsequent binding to coated FnBPA isotype V. Similar results were obtained for isotype I (not shown). **C:** Using the same ELISA, pre-incubation with ClfA, instead of IgG, did inversely decrease the amount of remaining fibrinogen bounded to FnBPA isotype V.

All data points represent the mean of two separate measurements and are expressed as Mean Fluoresence Itensity (MFI) for **A**; or as Optical Density at 450 nM for **B** and **C**.

early stages of infection. This is in line with the importance attributed to FnBPA during bacterial attachment, colonization and infection in *in vitro* and *in vivo* animal models as described by other authors [6, 11, 12].

Secondly, our results shed new light on the human antibody response towards FnBPA. Pre-existing IgG levels that were detected at the onset of bacteremia against all isotypes -including that of the infecting strain- did not notably change during the course of infection in 18 out of 22 patients. This is in line with our previous results obtained with the same sera, showing that most virulence factors of *S. aureus* do not induce uniform increases in antibody titers after the onset of bacteremia [26]. Notably, these patients

were not immunosuppressed at the time of infection and some virulence factors did induce a significant antibody response. Why other virulence factors, including FnBPA, do not uniformly induce a significant antibody response upon infection is currently unknown. Possibly, active manipulation of the host adaptive immune response by *S. aureus* plays a role [35, 36], or perhaps immunogenic epitopes of the FnBPA A domain are normally not exposed to the host adaptive immune system during infection due to the many interactions of this protein with host extracellular proteins [37]. Finally, it could be that FnBPA is only expressed infrequently on the surface of *S. aureus* during the course of bacteremia, although *fnbpa* mRNA was detectable during *in vitro* bacterial growth in human blood in our previous study [26]. Interestingly, of the three patients that did mount a significant antibody response against FnBPA, one patient was infected with a strain carrying the rare isotype V. Although speculative, perhaps this isotype is associated with less effective immune manipulation, more immunogenic exposure towards the host humeral immune system and/ or higher expression during bacteremia.

We currently do not know to what extent circulating antibodies differ in specificity for the different isotypes. On one hand, as opposed to the limited cross reactivity observed previously for murine monoclonal antibodies raised against different isotypes [25], natural human, polyclonal antibodies might possess cross-reactivity for different isotypes. This would be in line with our previous findings, wherein the same patient IgG was found to cross-react between the structurally similar leukocidin and gamma hemolysin toxin components [26]. Cross-reactivity could explain the simultaneous rise in IgG against multiple isotypes that was observed in two patients. On the other hand, the observation of *de novo* antibody production specifically against the isotype carried by the infecting strain in one patient suggest that specific, non-cross reacting antibodies may be present as well.

The functionality of antibodies against FnBPA remains another subject of debate. Although data from antibodies raised in animal models suggest that they do block binding between *S. aureus* surface proteins and fibronectin [30, 31, 38], other studies, including those with human antibodies, do not show any interference with binding to fibronectin [29, 39]. Our results are in line with the latter, demonstrating no apparent effect at any dilution of human IgG, purified from multiple patients –even from those with a significant antibody response against the isotype carried by the infecting strain, on binding between fibrinogen and FnBPA. A molecular explanation for this observation is currently lacking. Possibly, FnBPA binds with higher affinity to fibrinogen than to antibodies, or the functional binding domain remains immunologically hidden until the ligand is bound [29]. Alternatively, since FnBPA directly complexes with its ligand *in vivo*, ligand binding might induce a conformational change leading to the exposition of other, non-functional binding sites for antibodies. Presence of these Ligand Induced Binding Sites (LIBS) antibodies against FnBPA has been demonstrated in patients suffer-

ing from *S. aureus* endocarditis [40]. Finally, it has even been suggested that antibody binding might stabilize the interaction between FnBPA and fibrinogen [29], thereby actually enhancing binding, as observed for the binding of the FnbA of *Streptococcus dysgalactiae* to fibronectin when pre-incubated with mouse antibodies [39]. Antibodies against FnBPA could have other functions than interfering with ligand binding. For instance, antibodies can assist in the opsonization of *S. aureus* [41] or in augmenting microbicidal killing by phagocytes [42].

We confirmed the inability of IgG to block FnBPA-fibrinogen binding with three alternative assays, to rule out that the coupling of either protein to xMAP® beads could somehow affect correct antibody binding, for instance due to conformational changes. Of note is that the measured mean fluorescence intensities (MFIs) in the Luminex setups were much higher when free PE-labeled FnBPA isotypes were used instead of PE-labeled fibrinogen. Possibly, the large size of fibrinogen [43] would allow only a few PE-labeled molecules to bind to FnBPA-coated beads, while the other way around relatively many, smaller sized, PE-labeled FnBPA proteins could bind to fibrinogen coated beads. Alternatively, the coupling efficacy of both proteins to beads might differ. We currently do not know what the efficacy of the coupling reactions are, however, even if coupling efficiencies would differ the interpretation of our inhibition experiments remains unchanged.

With regard to the attractiveness of FnBPA as a vaccine target our results can be interpreted twofold. On the one hand, we and others found that FnBPA-specific IgG is already circulating prior to the onset of clinical infection, probably resulting from multiple prior exposures to *S. aureus*, yet this immune state does not confer protection from clinical disease [23, 26, 44, 45]. Furthermore, the inability of human IgG to block the binding between FnBPA and fibrinogen would argue against the use of FnBPA as a vaccine target. On the other hand, future vaccination strategies that are based on recombinant protein variants of FnBPA might induce novel, more protective antibodies against functionally important epitopes that are not normally targeted by the humoral immune response. Furthermore, *in vitro* production of antibodies against these functionally important epitopes might also be beneficial in the context of passive immunization. This rationale is supported by previous work demonstrating that serum from unvaccinated human subjects was unable to block the interaction between Clumping factor A (ClfA) and fibrinogen, while antibodies elicited by a recombinant ClfA-containing vaccine could block this binding [46]. Moreover, the level of functional antibody titres, able to block ClfA-fibrinogen binding *in vitro*, could directly be correlated to protection against *S. aureus* infection in an animal model *in vivo* [47]. Further studies into the specific interactions between the FnBPA A domain and antibodies, and their functional consequences, are needed to further elucidate the potential of this protein as a vaccine target in humans.

METHODS

Ethics statement

All patient serum samples used in this study were obtained from coded left-over material from routine diagnostic blood samples. In concordance with the guidelines of the Erasmus University Medical Hospital and the Dutch federation of Biomedical Scientific Societies (Federatie van Medische Wetenschappelijke Verenigingen), all Erasmus MC patients are routinely informed of the possibility that left-over material from diagnostic samples can be used, albeit anonymously, for scientific research; all patients are routinely offered the opportunity to opt out in writing. Serum samples used in this study were only obtained from patients who did not object to the use of left-over material for scientific research and, in addition, gave verbal consent. This procedure was approved and the acquisition of additional written consent was waived specifically for this retrospective study by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2007-106, addendum 2). All serum samples were coded and only qualified physicians of the department of Medical Microbiology and Infectious Diseases had access to the original patient data.

Bacterial strains and patients

Serum samples and *S. aureus* isolates were obtained from 22 patients diagnosed with bacteremia, as described previously [26]. In brief, bacteremia was defined as the isolation of *S. aureus* from at least one blood culture set. Starting from the first positive blood culture, a median number of 10 (interquartile range 5-17) serum samples were collected per patient over a median period of 34.5 (interquartile range 15-50) days.

Sequencing of *fnbA* A domains

DNA was isolated from strains using the QIAamp® DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The A domain of *fnbA* genes was amplified by PCR using previously described flanking primers [25] minus the restriction sites. Products resulting from amplifications were sequenced using a 3100 ABI Prism genetic analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Based on sequences of the flanking regions, individual primer sets were designed for each strain (sequences available upon request) and these were used to amplify and sequence the remaining part of each A domain. Overlapping sequences were merged for each strain and aligned with publicly available reference sequences of all FnBPA isotypes, which have been described previously [25]. The complete sequences of all strains are available upon request. Primer design and alignments were performed using Primer-BLAST® (available at http://www.ncbi.nlm.nih.gov) and MegAlign software (DNAStar Inc., Madison, USA), respectively.

Expression of recombinant FnBPA isotype proteins

pQE30 constructs expressing His-tagged N2N3 A domains of isotypes I to VII were kindly provided by T. Foster [25]. Each construct was verified by sequencing using pQE30-specific primers (sequence available upon request). Constructs were transformed into *E. coli* Topp 10, expressed by induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified under denaturing conditions with Ni-NTA agarose (Qiagen, Valencia, CA) according to instructions of the manufacturer's 'the QIAexpressionist™' handbook. Resulting proteins were quality controlled by SDS-PAGE and dialysed against PBS for 24 hours at 4 ^{0}C.

Measurement of antibodies

IgG levels against all recombinant N2N3 A domains of FnBPA isotypes in the serum samples of bacteremia patients were measured using a bead-based flow cytometry technique (xMAP®; Luminex Corporation, Austin, TX, USA), as previously described [26]. Briefly, recombinant proteins constituting the N2N3 A domains of FnbpA isotypes were covalently coupled to carboxylated xMAP® beads, which were activated with *N*-hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) according to a previously optimized protocol [48]. Protein-coated beads were then incubated with human serum which was diluted 1:100 in PBS. After repeated washing with PBS, bound antibodies were quantified using a 1:200 dilution of secondary phycoerythrin (PE)-labeled goat anti-human IgG antibodies. All measurements were performed in duplicate and the median fluorescence intensities (MFIs), a semi-quantitative measure of antibody levels, were averaged. Eighty-nine out of 1827 duplicate measurements (0,05%) had coefficients of variation larger than 25% and were excluded from further analysis. All measurements were corrected for non-specific background signal by subtracting the MFIs of control beads not coupled to any protein. The course of IgG levels over time were assessed graphically and initial-to-peak increases in IgG levels were calculated for all isotypes in each patient. A more than 4-fold initial-to-peak increase in IgG following within two weeks after the onset of bacteremia was considered proof of a significant immune response.

Luminex-based FnBPA-fibrinogen binding assay

Recombinant FnBPA isotypes and the non-fibrinogen binding surface proteins SdrE and IsdA were coupled to xMAP® beads as described above. Human fibrinogen (Calbiochem, Merck, Darmstadt, Germany) was labeled with phycoerythrin (PE) using the Lightning-Link R-PE conjugation kit (Innova Biosciences, Cambridge, UK) according to the manufacturers protocol, starting with 15mg fibrinogen per 10 ml of 50mM MES buffer. Protein-coated xMAP® beads were incubated with varying concentrations of PE-labeled fibrinogen and, after washing with PBS-1% BSA, median fluorescence intensities (MFIs)

were determined as a measure of bound fibrinogen using the same bead-based flow cytometry technique as described above. To study the effect of potentially interfering antibodies, total IgG was purified from serum of 5 different bacteremia patients and from pooled serum obtained from healthy volunteers (Polyclonal Human IgG, PHG) using a HiTrap ProteinG HP 1 ml column (GE Healthcare, Fairfield, Connecticut, USA) according to the manufacturer's protocol. Purified IgG was dialysed against PBS for 24 hours at 4 ^{0}C and resulting antibody concentrations were 4,7 mg/ml for PHG and a median of 5,1 mg/ml (IQR 4,4-8,8 mg/ml) for patient sera. Bead-bound isotypes were pre-incubated with a dilution range of the purified IgG, washed with PBS-1% BSA, and then incubated with a standard concentration of 1 µg PE-labeled fibrinogen per reaction.

For the alternative Luminex-based binding assay, fibrinogen was coupled to beads and recombinant proteins of FnBPA isotype II and III were PE-labeled as described above. Free FnBPA isotypes were pre-incubated with purified IgG and then incubated with the fibrinogen-coated beads according to the protocol described above. All binding assays were repeated separately twice and figures present the mean of these measurements.

ELISA-based FnBPA-fibrinogen binding assay

An enzyme-linked immunosorbent assay (*ELISA*) was performed to further confirm FnBPA-fibrinogen binding as previously described [49]. In brief, 96-well immulon 4HBX microtiter plates (Thermo Scientific, Waltham, Massachusetts, USA) were coated overnight at 4 °C and gentle rotation with 0.5 µg per well of FnBPA isotypes, diluted in PBS. After blocking the wells with 2% BSA in PBS, plates were washed once with TBS and coated isotypes were pre-incubated with a concentration range of purified IgG, diluted in TBS, for 1 hour at room temperature. Subsequently, after 3 additional wash steps with TBS + 0,1% Tween, plates were incubated for another hour with a fixed concentration of 40 nM fibrinogen (diluted in TBS). After another 3 wash steps, the bound fibrinogen was detected through incubation with horseradish peroxidase (HRP)-conjugated anti-fibrinogen antibodies (1:10.000 dilution) (Thermo Scientific) for 1 hour and quantified after adding the substrate *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich). The resulting absorbance at OD 450 nm was measured in an ELISA microplate reader (Thermomax).

In the case of the ClfA competition assay, 40 nM fibrinogen was pre-incubated for 1 hour with a twofold concentration range of recombinant ClfA (40 to 0,625 µM), and then incubated with FnBPA isotype-coated plates as described above.

Statistical analysis

Fold-increases in antibody levels were calculated as the ratio of the peak antibody level divided by the initial antibody level (as measured in the first serum sample). One-way ANOVA with the duplicate measurements of bound PE-labeled fibrinogen (or FnBPA

isotypes) as dependent variables and the IgG dilutions as factor were used to determine the significance of any differences in bound protein between different IgG dilutions. *P*-values ≤ 0.05 were considered statistically significant. IBM® SPSS® Statistics version 21 (IBM corporation, Armonk, NY, USA) was used for statistical analysis. Graphics were made using Graphpad Prism version 5 (Graphpad Inc. La Jolla, CA, USA).

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SUPPLEMENTAL DATA

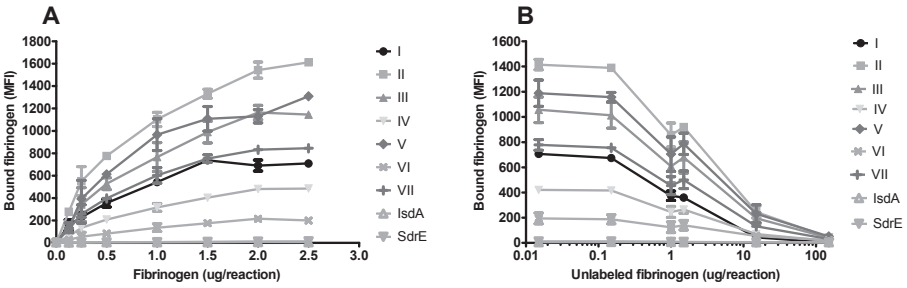

Figure S1. Validation of fibrinogen binding by FnBPA isotypes.
A: increasing concentrations of PE-labeled fibrinogen bind to all FnBPA isotypes but not to the control proteins (IsdA, SdrE and ClfB) in a dose-dependent and saturable manner. **B:** adding increasing concentrations of unlabeled fibrinogen to a fixed amount of 1,5 µg of PE-labeled fibrinogen shows competition for the binding to all FnBPA isotypes. Data points represent the mean of two separate measurements.

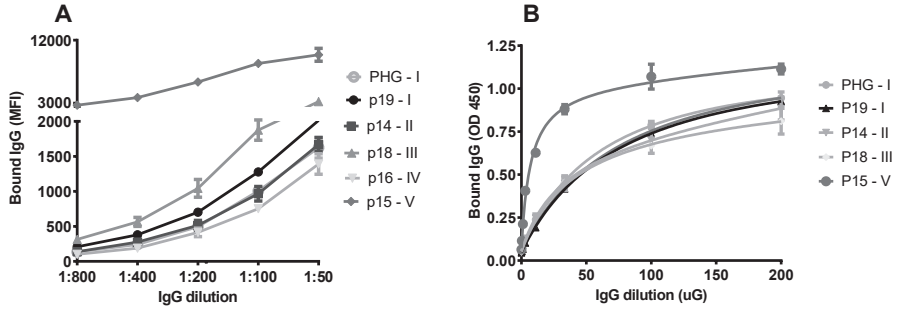

Figure S2. Specific and dose-dependent binding of human IgG to FnBPA isotypes I to V.
A: Beads coated with recombinant FnBPA isotypes I to V were incubated in a Luminex setup with a dilution range of total IgG purified from human pooled serum (PHG) or patients (p14 to p19: for each FnBPA isotype, serum was chosen from a patient that was infected with a strain carrying that isotype). **B:** FnBPA isotypes coated onto wells (0.5 µg/well) were incubated in an alternative ELISA setup with a dilution range of the same IgG, purified from PHG or patients. A plateau in antibody binding at higher concentrations can be observed, indicating that only FnBPA-specific IgG within the polyclonal antibodies bind.

Table S1. Initial- to peak fold-increases in specific IgG levels for FnBPA isotypes in bacteremia patients.

Patient	Isotype infecting strain	Fold-increase in IgG specific for isotype[1]						
		Isotype I	Isotype II	Isotype III	Isotype IV	Isotype V	Isotype VI	Isotype VII
1	II	1,7	1,4	1,4	1,6	1,6	1,4	1,5
2	III	2,8	2,7	3,7	2,7	1,0	1,0	1,0
3	I	1,5	3,8	1,4	1,4	1,5	1,5	1,3
4	II	***7,3***	***23,1***	3,8	1,9	2,3	2,1	2,0
5	II	1,1	1,0	1,0	1,1	1,0	1,0	1,0
6	I	1,7	2,4	2,3	2,7	2,9	2,9	3,1
7	III	1,8	1,6	1,7	1,5	1,6	1,7	1,6
8	IV	1,0	1,1	1,1	1,0	1,0	1,1	1,1
9	III	2,0	1,7	1,3	2,2	1,9	1,7	2,6
10	IV	1,0	1,1	1,0	2,9	1,4	1,4	2,9
11	II	2,0	2,7	2,2	3,1	3,3	2,1	2,0
12	I	1,4	1,0	1,1	1,0	1,2	1,1	1,4
13	IV	2,8	1,2	1,1	2,5	1,1	2,2	1,5
14	II	1,0	1,0	1,1	1,1	1,0	1,0	1,4
15	V	1,0	2,7	1,2	2,4	***20,6***	2,2	3,7
16	IV	1,0	1,0	1,2	1,0	1,0	1,0	1,0
17	III	***7,6***	***4,3***	***9,3***	***10,8***	***7,1***	3,6	1,6
18	III	1,5	2,1	1,7	1,5	2,0	2,0	2,6
19	I	1,2	1,5	1,1	1,5	1,3	1,7	1,5
20	I	2,5	1,4	***4,5***	2,6	2,2	1,8	3,0
21	IV	1,0	1,2	1,3	1,0	1,3	1,0	1,2
22	IV	1,0	1,0	1,2	1,0	1,6	1,0	1,0

[1] A fold-increase of 1,0 means that the IgG level did not change during the course of infection, compared to that of initial measurement at the onset of bacteremia; whereas a fold-increase of e.g. 23.1 fold means that the peak IgG level in that patient after onset of infection was 23.1 times higher compared to the initial measurement. The FnBPA isotype of the infecting *S. aureus* strain, as determined with sequencing, is indicated in the second column. Significant initial-to-peak increases in IgG of more than four-fold are indicated in italic bold.

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Chapter 7

Summary and discussion

SUMMARY

Staphylococcus aureus is an infamous bacterial pathogen which can produce a wide array of virulence factors, enabling it to cause many different infections with significant morbidity and mortality in humans. The ability of *S. aureus* to form biofilm and its rising resistance against antibiotics further complicate treatment of many infections [1, 2]. Therefore, alternative treatment strategies, such as vaccination, are receiving great scientific and clinical interest. However, despite the many different virulence factors that have been targeted by vaccines and their promising effects in animal models, so far all clinical trials failed to demonstrate any favourable effect in humans [3]. Thus, there remains a need for more insights into the presence of *S. aureus* virulence factors and their ability to induce an antibody response during infection in humans.

The general aim of this thesis was twofold; to provide further insights into the presence of a wide range of well-characterized virulence factors of *S. aureus* during growth in *in vitro* and *ex vivo* infection models, mimicking the *in vivo* situation during different infection in humans, and to further characterize the human antibody response during these different infections. The high-throughput, bead-based Luminex assay was used together with confirmation by additional techniques such as RT-PCR and mass-spectrometry throughout this thesis to achieve these aims. Data concerning *in vitro* presence of -and *in vivo* antibody responses against *S. aureus* virulence factors are compared and, together, can help in identifying potential targets for novel vaccination strategies as discussed below.

Main findings

Using a Luminex assay, **Chapter 2** expanded on previous data [4-6] by further characterizing the human antibody response against an expanded set of 56 *S. aureus* virulence factors in 21 patients suffering from bacteraemia. In general, prospective measurement of patient IgG levels following infection further confirmed the large inter-individual heterogeneity found in antibody responses. Specific IgG levels against 15 proteins increased at some time point after the onset of bacteremia in 95 to 100% of patients, of which the highest initial-to-peak fold-increase was observed for the putative ABC membrane transporter SACOL0688. Compared to 30 age-matched, healthy controls, IgG levels against 27 proteins, including those mentioned above, were significantly higher in bacteremia patients (Table 1). These results suggest that these specific proteins are present and induce a significant antibody response *in vivo*. To gain further insight into protein expression during growth in human blood, gene expression in human blood *ex vivo* was determined using micro-array with two genetically different strains isolated from different bacteremia patients. Out of the 56 virulence factors mentioned above, mRNA expression levels of *SA0688, IsaA, EsxA* and *SCIN* genes were consistently high in

both isolates during growth in blood. However, compared to the transcriptomes at the start of incubation, only 86 out of the 3626 tested genes were consistently upregulated in both strains, of which the gamma-hemolysin A and B precursors were the only known virulence factors. In addition, expression of all known virulence factors did not differ during growth in blood or artificial BHI broth, suggesting that their expression is not specifically associated with blood-stream infections.

In contrast to the acute infection caused by bacteremia, little was known about the human antibody response during a more chronic, biofilm-associated infection such as osteomyelitis. This issue was addressed in **chapter 3**, wherein IgG levels against 50 *S. aureus* virulence factors were prospectively measured in 10 patients suffering from osteomyelitis. Again, pronounced inter-individual heterogeneity in IgG levels was found. IgG levels against 14 proteins were significantly higher in osteomyelitis patients compared to 20 age-matched healthy controls (Table 1). Interestingly, none of these IgG levels differed significantly compared to those in the 20 bacteremia patients described above. When *S. aureus* strains isolated from the bone of 9 patients were allowed to form biofilms on polystyrene (PS), 14 different proteins could be detected during biofilm formation by the majority of strains using a competitive Luminex assay (CLA) (Table 1). Twelve of these proteins were also detected in the majority of strains during biofilm formation *ex vivo* on human bone. Comparison between *in vitro* protein detection and the *in vivo* antibody responses revealed that eight proteins were both detectable in the majority of biofilms *in vitro* and induced an antibody response *in vivo*. However, 4 other proteins induced an antibody response *in vivo* but were detected in only a minority of biofilms *in vitro* while, vice versa, 7 proteins were detected in most biofilms *in vitro* but not associated with a significant antibody response *in vivo* (Table 1).

The presence of 52 *S. aureus* virulence factors was further established in **Chapter 4** during biofilm growth of 5 (methicillin resistant) strains on PS and an *ex vivo* model for human skin, the Leiden Epidermal Model (LEM) [7]. Six functionally diverse proteins were consistently detected in biofilms of all strains on both surfaces (Table 1). At the same time, in contrast to the largely similar protein detection in biofilms on PS and human bone as described in **Chapter 3**, eight other proteins were detectable in biofilms of most strains on LEMS but not on PS. These proteins mostly comprised diverse immune modulators and toxins, including the well-characterized alpha toxin. The reverse was true for fibronectin binding protein B, which was detectable on PS but not on LEM. Interestingly, further experiments showed that, although alpha toxin could not be detected at the protein level in biofilms on PS, mRNA and a GFP-construct for this toxin were detectable in biofilms on PS, suggesting that the *hla* gene is expressed but not translated and/or that the protein is rapidly degraded. Lastly, another noteworthy observation was the variable ability of all strains to form biofilms on both surfaces, with two strains (including

Table 1. Summary of Luminex results from **Chapters 2**, **3**, **4** and **5** for the most frequently present virulence factors in studied *S. aureus* strains.

Protein[1]	Function	Presence *in vitro/ ex vivo*				Significant antibody response *in vivo*			Association with clinical outcome
		Consistent expression in blood[2]	Biofilm polystyrene[3]	Biofilm bone[3]	Biofilm skin[4]	Bacteremia[5]	Osteomyelitis[6]	Cystic fibrosis[7]	Cystic fibrosis[8]
Alpha toxin	toxin		+	+	+	ND	+	-	-
CHIPS	immmune modulator		+	+		-	-	-	+
ClfA	surface protein		+	+		+	-	-	-
ClfB	surface protein		+	+	+	+	-	-	+
FlipR	Immune modulator		+	+		+	+	-	-
FnbpA	surface protein		+	+		-	-	-	+
Glucosaminidase	housekeeping		+	+	+	+	+	+	+
HlgB	toxin				+	+	+	+	+
IsaA	housekeeping	+	+	+	+	+	-	+	+
IsdA	surface protein		+	+	+	+	+	+	+
IsdH	surface protein		+			+	-	-	-
Lipase	housekeeping/ toxin		+			+	-	-	-
LukD	toxin					+	+	+	+
LytM	housekeeping					-	-	+	+
Nuclease	housekeeping/ toxin		+	+	+	+	-	+	-
SACOL0688	housekeeping	+	+	+	+	+	+	+	+
SCIN	immmune modulator	+	+	+		+	+	-	-
SdrE	surface protein			+		-	+	-	+
SSL1	immmune modulator					+	-	-	-
SSL3	immmune modulator					-	+	-	+
SSL5	immmune modulator					+	+	+	-
SSL9	immmune modulator					+	-	+	-

[1]only proteins are shown for which genes were consistently present in more than half of all strains in each study, [2]only proteins with significantly increased mRNA expression, detected with micro-array in 2 strains at 0, 30, 60 and 90 minutes growth in blood, are indicated with '+', [3]only proteins that were detectable with CLA in the majority of 9 strains are indicated with '+', [4]only proteins that were detectable with CLA in all 5 strains are indicated with '+', [5]significantly increased IgG levels in 22 patients compared to 30 controls, [6]significantly increased IgG levels in 10 patients compared to 20 controls, [7]significantly increased IgG levels in 182 patients compared to 53 controls, [8] An association with clinical outcome is defined as the presence of either a significant estimated effect of IgG levels on lung function (FEV1%) or a significant Odds ratio for experiencing an exacerbation in 182 patients, ND: not determined

the extensively used laboratory strain NCTC 8325-4) being able to form biofilms on PS but not on LEM.

The human antibody response against 44 *S. aureus* virulence factors was further characterized in Cystic Fibrosis (CF) patients suffering from chronic pulmonary infection caused by *S. aureus* in **Chapter 5**. IgG levels against 15 proteins, being mostly the same proteins that were also found to induce an antibody response in **Chapters 2** and **3**, were significantly higher in 182 patients compared to 53 healthy nasal carriers of *S. aureus* (Table 1). These results thus further suggests the presence of a particular set of *S. aureus* virulence factors during infection *in vivo*. Importantly, this study also demonstrated a significant association between specific IgG levels and several clinical parameters, as was recently already described for IgG levels against alpha toxin and several leucocidin components in CF patients [8]. IgG levels against 18 proteins were significantly and inversely associated with lung function (FEV 1% predicted) and IgG levels against 8 more proteins were significantly associated with the risk (Odds ratio, OR) to experience a pulmonary exacerbation (OR < 1 and > 1 for 3 and 5 proteins, respectively) (Table 1). Finally, IgG levels against respectively 22, 14 and 16 proteins were significantly associated with serum IL-6 levels in CF patients, *S. aureus* nasal carrier status and presence of small colony variants in airway cultures of patients.

The human antibody response against one specific virulence factor, Fibronectin Binding Protein A (FnbpA), is characterized in more detail in the final **Chapter 6**. Furthermore, in addition to the more descriptive data of previous chapters, a first attempt is made to explore the functionality of anti-FnbpA antibodies. In line with previous data [9] a marked sequence diversity is found in the fibrinogen-binding A domain of the *fnbpa* gene amongst 22 strains isolated from the same bacteraemia patients as described in **Chapter 2**. Despite this diversity, IgG from all 22 patients detectably bound the 4 most common FnbpA isotypes and a significant antibody response against the isotype of the infecting strain was observed in only 2 patients, of which one case involved the rare isotype V. These results suggest, in contrast to the limited cross-reactivity observed for monoclonal anti-FnbpA antibodies in animal models [9], the presence of either cross-reactivity of polyclonal human IgG or the presence of different antibodies due to previous exposure to the most common isotypes. Finally, using a novel, Luminex-based FnbpA-fibrinogen binding assay, this study shows that pre-incubation of diverse FnbpA isotypes with human IgG from neither patients nor healthy controls can interfere with the binding of FnbpA to human fibrinogen. These results are confirmed with two independent Luminex- and one ELISA-based assays and are in line with earlier data describing the binding between FnbpA and fibronectin [10, 11]. Although the exact molecular explanation is still unclear, these results might suggest that human antibodies induced by FnbpA *in vivo* normally bind non-functional binding sites of the protein. This could implicate that novel vaccine approaches that involve (the induction of) antibodies

directed against functional epitopes of FnBPA that are not normally targeted by the immune system might more successfully confer protection against infection, as discussed later in this chapter.

Main conclusions

1. A specific set of functionally diverse virulence factors of *S. aureus*, which are summarized in Table 1, are ubiquitously present during bacterial growth in different infection models *in vitro/ ex vivo*, and induce significant antibody responses during different infections *in vivo* (bacteremia, osteomyelitis and chronic pulmonary infection in CF patients). This is established for multiple collections of genetically different *S. aureus* strains.
2. Discrepancies exist for other virulence factors between *in vitro* protein detection and the *in vivo* detected antibody response, suggesting that interpretation of both data combined is most informative in determining the role of a virulence factor during pathogenesis and its potential use as a vaccine target.
3. Both healthy controls without any clinically apparent infection and patients at the start of an infection possess readily detectable, often significant IgG levels against *S. aureus* virulence factors.
4. There exists extensive inter-individual heterogeneity in the titers of IgG antibodies directed against virulence factors of *S. aureus*.
5. In CF patients suffering from chronic pulmonary infection with *S. aureus*, the height of IgG levels against diverse virulence factors of *S. aureus* is significantly associated with clinical parameters such as lung function and risk of experiencing an exacerbation.
6. Although some virulence factors are ubiquitously detectable during biofilm formation *in vitro*, many other virulence factors are more variably detectable amongst genetically different strains, even when corresponding genes are ubiquitously present.
7. Several factors can influence the detection of specific virulence factors during biofilm formation *in vitro*, including the used environment (polystyrene, bone or artificial skin) and the time of measurement.
8. Although some virulence factors, such as alpha toxin, might be expressed at the transcriptomic level during biofilm formation, this does not always lead to detection at the proteomic level. This can be explained by rapid degradation of either transcripts and/or the protein.
9. By far most of the *S. aureus* virulence factors that were specifically upregulated during bacterial growth in human blood *ex vivo* have an unknown function. Compared to bacterial growth in artificial broth, no known virulence factor could be identified that are specifically expressed in blood.

11. Human antibodies against many *S. aureus* virulence factors can be absorbed by biofilms, suggesting that these virulence factors are immunologically accessible within a biofilm environment.
12. Despite the ubiquitous presence of human IgG against the A domain of FnBPA, only a minority of patients mount a significant antibody response against this virulence factor after the onset of bacteremia, indicating lack of expression and/ or immune evasion by FnBPA during this infection.
13. Although human antibodies can bind to the genetically variable A domain of FnbpA, these antibodies are unable to block binding between FnbpA and its substrate fibrinogen.

DISCUSSION

Strengths and limitations of Luminex assay

Most results described in this thesis have been obtained using a Luminex based setup, which continues to be validated for immunological and molecular assays in both research and diagnostic settings [12-14]. An advantage of this multiplex format is its ability for rapid, high-throughput screening of multiple targets simultaneously using small sample volumes. In addition, the Luminex assay has a wider dynamic (up to mean fluorescence intensity [MFI] of 1 x 10^4 arbitrary units) and extended linear range compared to ELISA [15, 16]. Finally, the covalent coupling of proteins to Luminex beads allows for the production of a single batch of protein-coupled beads that can be used throughout an entire study for several months, minimizing intra-assay variation.

However, analogous to ELISA, there are also some general limitations in regard to the Luminex assay. First and foremost, the sensitivity of these assays depends on the number, availability and quality of the recombinant proteins that are coupled to beads or surfaces, respectively. Only antibodies against coupled proteins (or protein domains) will be detected and, although polyclonal human IgG bound diverse FnbpA isotypes in our assay (**Chapter 6**), we cannot completely rule out that sequence diversity of proteins might influence assay sensitivity. Furthermore, just as for ELISA the exact amount of coupled protein remains unknown due to an unknown coupling efficacy. Finally, when determining whether a protein is present or immunogenic during infection, some results should be interpreted cautiously due to immunological cross-reactivity. This was observed in our Luminex assay between gamma-hemolysin and the leucocidin S and F components (**Chapter 2**).

The above mentioned limitations also apply to the Competitive Luminex Assay. In addition, results that are obtained with the CLA can be somewhat difficult to interpret for non-experts, hampering accessibility of the data. Finally, in general the (semi-) quantita-

tive measurement of protein presence and antibody responses described in this thesis only provides descriptive data and does not allow any conclusions about the function or importance of detected proteins and antibodies. Thus, altogether, the Luminex assays used in this thesis should be considered as robust screening tools for the presence of antibodies and (indirectly) proteins, yet they do not provide precise, quantitative nor mechanistic data.

Given these limitations, future use of the Luminex assays should mainly be limited to studies that wish to preliminary screen large number of bacterial strains or serum samples for the presence of proteins or antibodies, respectively. The latter could involve further characterization of antibody responses or, as briefly discussed below, T-cell responses against specific virulence factors. In addition, virulence factors that are identified as potential vaccine targets should be subject to more mechanistic and functional studies, for instance involving transcriptomic approaches to further unravel bacterial regulatory systems or functional antibody binding and neutralization assays, as partly described in **Chapter 6**.

Implications for vaccine development

In the context of vaccination, it seems reasonable to assume that the target for an effective vaccine should at least be present at some time in the majority of clinical strains during colonization or infection. Following this logic, several virulence factors of *S. aureus* could be potential vaccine targets according to data presented in this thesis. Several of the virulence factors that are summarized in Table 1, such as SA0688, IsdA and IsaA, appear to be ubiquitously present in genetically diverse strains under different conditions *in vitro* and induce a significant antibody response in patients *in vivo*, which implies their presence during infection. These virulence factors have indeed been successfully used as vaccine targets in animal models of diverse *S. aureus* infections [17-19]. Yet it appears that merely the presence of virulence factors, although it might be a prerequisite, is not enough for an effective vaccine in humans, nor is the successful evaluation of a vaccine target in animal models. This is for example demonstrated by the results obtained with a vaccine targeting the highly conserved iron surface determinant B (IsdB), which is closely related to the IsdA described in this thesis. Although IsdB induced protection against *S. aureus* infection in animal models and was immunogenic in human patients, a large phase II clinical study failed to show any reduction in the rate of postoperative *S. aureus* infection compared to the placebo, and was actually associated with higher mortality in the vaccinated patients who developed *S. aureus* infections [20].

The failure so far to develop a clinically effective vaccine against *S. aureus* in humans has raised questions about its feasibility [for recent reviews see [3, 21-24]. Besides the discouraging history, there are several other issues that complicate this matter. Firstly, it is currently unclear how important the humoral branch of the human immune response

actually is for controlling infections with *S. aureus*. As shown by data in **Chapters 2**, **3**, **5** and **6** and previous studies [4, 5], antibodies against many virulence factors of *S. aureus* are readily detectable in both healthy controls and patients at the start of a clinically apparent infection, yet these antibodies do not prevent colonization or infection. Furthermore, based on the results presented in **Chapter 6**, it appears that at least some human antibodies are unable to interfere with the actual function of *S. aureus* virulence factors. As already described in **Chapter 1**, it is now increasingly recognized that the cellular rather than humoral immunity, particularly the actions of T helper 17 cells, play a central part in the host defence [3, 25].

A second issue complicating vaccine development is the extensive diversity in the presence and sequences of many genes encoding virulence factors amongst genetically different *S. aureus* strains [26-29]. This means that *S. aureus* strains with a genetically different variant or complete absence of the virulence factor, being targeted by a vaccine, will be unaffected. This scenario becomes even more likely in the light of variable expression levels amongst *S. aureus* strains, as has been established for e.g. alpha toxin [30]. In addition, in case of alpha toxin, *S. aureus* can also alter protein expression post-translationally (**Chapter 5**).

A third issue complicating vaccine development involves the extensive immune evasion abilities of *S. aureus* [31, 32]. As described in **Chapter 1**, many virulence factors secreted by *S. aureus* can inhibit or divert both innate and adaptive immune responses of the host. Furthermore, *S. aureus* can also retreat into either extra- or intracellular persistence [33, 34] and into biofilms [1], which can further dampen the effect of a vaccine-induced antibody response. These immune evasive capabilities of *S. aureus* might reflect its long co-evolution as part of the normal human flora, which contrasts to other, more transiently present species such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. This might partially explain why vaccination against the latter bacterial species but not against *S. aureus* are effective [3].

Based on the above mentioned discussion, it can be concluded that the presence and immunogenicity, established for several virulence factors in this thesis, on its own will likely be insufficient to warrant an effective vaccine in humans. Nevertheless, these virulence factors might still be potential targets in the development of novel vaccination approaches. In contrast to the so far unsuccessfully evaluated monovalent vaccines, such as the IsdB based vaccine, current awareness is rising that a multivalent vaccine is likely to have a higher chance of success [23, 35]. Although whole cell vaccines with inactivated *S. aureus* appear to only moderately induce an antibody response with limited clinical effect in both veterinary and human medicine [36], these vaccines are based on poorly characterized cocktails of bacterial proteins. In contrast, multivalent vaccines consisting of novel recombinant forms of well-characterized virulence factors of *S. aureus* might just induce novel, functional antibodies that are protective against infection.

Whereas naturally occurring antibodies might not functionally interfere with *S. aureus* virulence factors (**Chapter 6**) or protect against infection, this might be achieved by antibodies raised against specific protein epitopes that are not normally targeted by the immune system. This rationale is supported by previous work demonstrating that serum from unvaccinated human subjects was unable to block binding between Clumping factor A (ClfA) and fibrinogen, while antibodies elicited by a recombinant ClfA-containing vaccine could block this binding [37]. Moreover, the level of functional antibody titres, able to block ClfA-fibrinogen binding *in vitro*, could directly be correlated to protection against *S. aureus* infection in an animal model *in vivo* [38].

In addition to the above mentioned, well-characterized virulence factors of *S. aureus*, it should be noted that other virulence factors might also prove to be interesting vaccine targets. These could be currently less-characterized proteins, such as cytosol-based proteins involved in bacterial metabolism. Antibody responses against these proteins are comparably rare and weak [39], yet vaccine-induced antibodies might interfere with bacterial viability. Another group of novel, potential vaccine targets might include specific biofilm-associated virulence factors [40]. This rationale is generally supported by results from **Chapters 3** and **4**, which demonstrate specific antibody binding by biofilms, suggesting that antibodies can bind and therefore target biofilm-associated virulence factors. Indeed, previous work demonstrated that a pentavalent vaccine, combining virulence factors that are mainly associated with either planktonic growth or biofilm formation, effectively cleared infection in a murine tibial osteomyelitis model [41].

Another study from the same group also showed that infection in a rabbit osteomyelitis model was significantly better cleared when vaccination was combined with antibiotic treatment, compared to either vaccination or antibiotic treatment alone [17]. These results suggest the potential use of combining existing and novel treatment options, such as vaccination and currently used antibiotics, to augment each other.

Finally, it should be noted that both this thesis and most research efforts so far have focussed on characterizing antibody responses, yet specific T cell responses against *S. aureus* are only now beginning to be defined [for recent review see [25]. In light of the earlier mentioned, increasingly recognized importance of T-cell mediated immunity against *S. aureus*, the key to an effective vaccine might actually involve induction of specific T-cell mediated immunity, rather than humoral immunity. Interestingly, all patients who were vaccinated with the failed IsdB-based vaccine and subsequently died from post-operative *S. aureus* infection, were shown to have undetectable IL-2 and mostly undetectable IL-17 levels at the time of vaccination and during the course of infection [42]. Based on these findings it can be speculated that the quality of pre-existing T-cell mediated immunity is important for protection against *S. aureus* infection. Further research is needed to firstly determine whether vaccination can influence T-cell mediated

immunity, and secondly whether this can favourably alter the course of infection with *S. aureus*.

When considering novel vaccination approaches, one final question remains as to which (patient) populations might best benefit. Based on the earlier discussed issues complicating vaccine development, it seems unlikely that a universal vaccine that can prevent all *S. aureus* infections will ever be achieved [21]. However, temporal reduction of the risk and/ or severity of infection are more realistic and also valuable endpoints. This could for example involve the active or passive immunization of patients prior to elective surgery to reduce post-operative wound infections, or immunization of patients suffering from chronic infection to reduce morbidity and treatment duration. In addition, although patients suffering from acute infection might not directly benefit from active immunization, severity of especially toxin-mediated infections might be limited by passive immunization, e.g. by infusing antibodies against alpha toxin [43, 44] and Toxic Shock Syndrome Toxin [45]. As discussed earlier all these approaches might be synergistically combined with currently used antibiotic treatments.

In conclusion, several virulence factors of *S. aureus* that are ubiquitously present *in vitro* and induce a significant antibody response *in vivo* are identified in this thesis, and although these observations alone will likely not warrant an effective vaccine in humans, these virulence factors might be useful targets in novel vaccination approaches which are currently being explored. Ever since its first description by Alex Ogston in 1881, the question remains whether effective immunization against *S. aureus* is possible.

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Chapter 8

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Staphylococcus aureus is een grampositieve bacterie welke een breed scala aan infectieziekten in de mens kan veroorzaken, variërend van krentenbaard tot een levensbedreigende bloedvergiftiging (bacteriemie). Bij de totstandkoming van deze infecties spelen verschillende bacteriële virulentie factoren, zoals toxines en adhesie eiwitten, een belangrijke rol. Daarnaast is *S. aureus* in staat om biofilms te vormen, een clustering van bacteriën omgeven door een laag slijm en eiwitten, waarin zij verminderd gevoelig zijn voor antibiotica en de afweer respons van de gastheer. Tenslotte wordt de behandeling van bovenstaande infecties toenemend bemoeilijkt door de wereldwijde opkomst van antibioticaresistentie en het tegelijkertijd ontbreken van alternatieve behandelmogelijkheden, zoals vaccinatie. Maar hoewel er dringend behoefte is aan een effectief vaccin tegen *S. aureus*, ontbreken nog veel van de hiervoor benodigde inzichten in de pathogenese van *S. aureus* en de menselijke antilichaam respons tegen *S. aureus*. Daarom had het onderzoek beschreven in dit proefschrift tot doel om verder inzicht in deze onderwerpen te verkrijgen, wat uiteindelijk kan bijdragen aan de ontwikkeling van nieuwe vaccinatie mogelijkheden.

In **hoofdstuk 2** wordt de humane antilichaam respons tegen 56 virulentie factoren van *S. aureus* in 21 patiënten met een bacteriemie beschreven. Hierbij wordt gebruik gemaakt van een eerder ontwikkelde flowcytometrie (Luminex) techniek die antilichamen tegen meerdere bacteriële eiwitten tegelijk kan meten. Het blijkt dat IgG antilichamen tegen 15 virulentie factoren in sera van een meerderheid van de patiënten stijgen, en dat deze titers gemiddeld significant hoger zijn vergeleken met die van gezonde controles. De meest prominente antilichaam respons is gericht tegen een nog relatief onbekend celmembraan-gebonden transporteiwit van *S. aureus* (SACOL0688) welke eerder geassocieerd is met biofilm vorming. Dit eiwit blijkt samen met diverse andere virulentie factoren ook continu tot expressie te komen in twee klinische isolaten tijdens *in vitro* incubatie in humaan bloed. Verdere vergelijking van eiwitexpressie tussen incubatie in bloed en een kunstmatig groeimedium toont aan dat expressie voor geen enkele virulentiefactor conditie specifiek is.

De humane antilichaam respons tegen een set van 50 virulentie factoren van *S. aureus* wordt verder bestudeerd in **hoofdstuk 3,** deze keer in 10 patiënten met een infectie van het bot (osteomyelitis). Het blijkt dat IgG antilichaam titers tegen 15 virulentie factoren, grotendeels dezelfde als beschreven in **hoofdstuk 2**, significant verhoogd zijn in patiënten vergeleken met gezonde controles. Aangezien osteomyelitis geassocieerd is met biofilm vorming wordt de expressie van alle virulentiefactoren vervolgens *in vitro* gemeten tijdens biofilm vorming op zowel polystyreen als humaan bot door 10 *S. aureus* isolaten, inclusief 9 isolaten uit geïnfecteerd bot van bovenstaande patiënten. Hierbij wordt een nieuw ontwikkelde, op de Luminex gebaseerde techniek gebruikt, waarbij

met behulp van antilichamen indirect bacteriële eiwitten worden gedetecteerd. Acht van de 15 boven genoemde virulentiefactoren, inclusief SACOL0688, worden door alle 10 isolaten consistent in biofilms tot expressie gebracht. Tegelijkertijd blijkt dat er voor andere virulentiefactoren discrepanties zijn tussen de gemeten antilichaam respons *in vivo* en eiwitexpressie *in vitro*. Het waarnemen van een verhoogde antilichaam productie *in vivo* gaat dus niet altijd gepaard met detecteerbare eiwitexpressie *in vitro* en vice versa.

Met behulp van dezelfde techniek als in **hoofdstuk 3** wordt in **hoofdstuk 4** de *in vitro* expressie van 52 virulentiefactoren verder onderzocht in biofilms van 5 (methicilline resistente) stammen op polystyreen en een menselijk huidmodel. Voor diverse virulentiefactoren zoals reeds beschreven in **hoofdstuk 2 en 3** wordt opnieuw consistente expressie in alle stammen waargenomen. Tegelijkertijd blijkt er voor enkele virulentiefactoren ook verschil te zijn tussen de expressie op respectievelijk polystyreen en het huidmodel. Alpha toxine blijkt bijvoorbeeld alleen detecteerbaar te zijn in biofilms op het huidmodel, hoewel transcriptie van het *hla* gen op beide oppervlaktes aantoonbaar is. Deze resultaten wijzen op de complexiteit van translatie en post-translationele modificatie van bacteriële eiwitten, welke *S. areus* in reactie op verschillende omstandigheden lijkt aan te passen.

In **hoofdstuk 5** wordt een link gelegd tussen de antilichaam respons tegen 44 virulentiefactoren van *S. aureus* en diverse klinische parameters in jonge patiënten met taaislijmziekte (Cystic Fibrosis, CF). Vergeleken met een groep van 53 gezonde controles blijken IgG titers tegen 15 virulentiefactoren, hoofdzakelijk dezelfde zoals reeds beschreven in **hoofdstuk 2 en 3**, significant verhoogd in CF patiënten waarbij de luchtwegen persisterend gekoloniseerd zijn met *S. aureus*. Verder blijkt dat de hoogte van IgG titers tegen diverse virulentiefactoren significant geassocieerd is met de longfunctie van patiënten en/of kans op het krijgen van een exacerbatie van luchtwegklachten.

In **hoofdstuk 6** wordt de humane antilichaam respons tegen het eiwitdomein van één specifieke virulentiefactor van *S. aureus*, het Fibronectine Bindend Proteïne A (FnBPA), zowel kwantitatief als kwalitatief gekarakteriseerd. Er blijkt aanzienlijke variatie in de sequentie van dit gen te zijn tussen de 22 klinische stammen geïsoleerd uit de 22 bacteriemie patiënten beschreven in **hoofdstuk 2**. Ondanks deze genetische variatie blijkt dat alle patiënten IgG antilichamen bezitten die de 4 meest voorkomende varianten kunnen binden. Echter, slechts in 3 van de 22 patiënten wordt een stijging van IgG antilichamen waargenomen tijdens het doormaken van een bacteriemie. Dit suggereert dat, hoewel alle patiënten al vóór infectie zijn blootgesteld aan het FnBPA eiwit, dit eiwit tijdens een bacteriemie óf niet tot expressie komt óf afgeschermd wordt van de menselijke antilichaamrespons. Met behulp van een nieuwe, op Luminex gebaseerde techniek wordt verder aangetoond dat antilichamen uit zowel patiënten als gezonde vrijwilligers niet in staat zijn om de binding tussen het FnBPA eiwit en diens ligand fibrinogeen te verstoren.

Op basis van alle resultaten uit **hoofdstukken 2 – 5** blijkt dat een specifieke set van virulentiefactoren van *S. aureus* consistent tot expressie komt en leidt tot een antilichaam respons in de mens, zoals SACOL0688 (samengevat in Tabel 1 in **hoofdstuk 7**). Echter, alleen het aantonen van eiwitexpressie en een antilichaam respons is onvoldoende bewijs voor de geschiktheid van deze virulentiefactoren voor inclusie in een effectief vaccin. Ook duiden de discrepante resultaten tussen *in vitro* eiwitexpressie en *in vivo* antilichaam responsen uit **hoofdstuk 3** erop dat interpretatie van verschillende data nodig is om hierover te kunnen oordelen. Tenslotte is het voor de ontwikkeling van een effectief vaccin nodig om meer inzicht te krijgen in de functie van specifieke antilichamen, welke in het geval van FnBPA bijvoorbeeld de binding van dit eiwit aan fibrinogeen niet kunnen blokkeren (**hoofdstuk 6**). Tezamen kunnen deze nieuwe inzichten in eiwitexpressie, antilichaam respons én functie uiteindelijk leiden tot de ontwikkeling van nieuwe, effectievere vaccinatie strategieën. Hierbij valt bijvoorbeeld te denken aan vaccinatie met een recombinant eiwit dat wel functioneel blokkerende antilichamen induceert, of vaccinatie met biofilm-gerelateerde virulentiefactoren ter bestrijding van biofilm-gerelateerde infecties met *S. aureus*.

Appendices

Curriculum vitae

List of publications

PhD portfolio

Dankwoord

CURRICULUM VITAE

Paul Martijn den Reijer was born on May 18, 1983 in Rotterdam. He completed his secondary education at the Libanon Lyceum in Rotterdam in September 2001 and subsequently studied Biology at Leiden University. During this study he developed a special interest in (Medical) cell- and microbiology and he completed several research internships within these fields, including a research project on the relationship between genetic polymorphisms and cellular aging, under supervision of Prof. dr. R. Westendorp at the department of Geriatrics, Leiden University Medical Center. After obtaining his Master 's degree in Medical Biology *cum laude* in September 2006, he studied Medicine at the same university and performed several clinical- and research internships abroad in respectively Reykjavik, Atlanta and Singapore. After receiving his Master's degree in Medicine in September 2010, he started his PhD project on human antibody responses against virulence factors of *S. aureus* at the department of Medical Microbiology & Infectious Diseases, Erasmus Medical Center Rotterdam, under supervision of Prof. dr. H. Verbrugh and Dr. W. van Wamel. In September 2012 he concurrently started his clinical residency in Medical Microbiology under supervision of Prof. dr. H. Verbrugh, Dr. A. Vonk and Dr. J. Ossewaarde at the departments of Medical Microbiology in the Erasmus Medical Center and Maasstad hospital in Rotterdam, respectively. He will finish both his PhD project and clinical residency in September 2017.

LIST OF PUBLICATIONS

1. **den Reijer PM**, Sandker M, Snijders SV, Tavakol M, Hendrickx AP, van Wamel WJ. 2017. Combining in vitro protein detection and in vivo antibody detection identifies potential vaccine targets against Staphylococcus aureus during osteomyelitis. Med Microbiol Immunol 206:11-22.
2. Junge S, Gorlich D, **den Reijer M**, Wiedemann B, Tummler B, Ellemunter H, Dubbers A, Kuster P, Ballmann M, Koerner-Rettberg C, Grosse-Onnebrink J, Heuer E, Sextro W, Mainz JG, Hammermann J, Riethmuller J, Graepler-Mainka U, Staab D, Wollschlager B, Szczepanski R, Schuster A, Tegtmeyer FK, Sutharsan S, Wald A, Nofer JR, van Wamel W, Becker K, Peters G, Kahl BC. 2016. Factors Associated with Worse Lung Function in Cystic Fibrosis Patients with Persistent Staphylococcus aureus. PLoS One 11:e0166220.
3. **den Reijer PM**, van Burgh S, Burggraaf A, Ossewaarde JM, van der Zee A. 2016. The Widespread Presence of a Multidrug-Resistant Escherichia coli ST131 Clade among Community-Associated and Hospitalized Patients. PLoS One 11:e0150420.
4. **den Reijer PM**, Haisma EM, Lemmens-den Toom NA, Willemse J, Koning RI, Demmers JA, Dekkers DH, Rijkers E, El Ghalbzouri A, Nibbering PH, van Wamel W. 2016. Detection of Alpha-Toxin and Other Virulence Factors in Biofilms of Staphylococcus aureus on Polystyrene and a Human Epidermal Model. PLoS One 11:e0145722.
5. **den Reijer PM**, Lemmens-den Toom N, Kant S, Snijders SV, Boelens H, Tavakol M, Verkaik NJ, van Belkum A, Verbrugh HA, van Wamel WJ. 2013. Characterization of the humoral immune response during Staphylococcus aureus bacteremia and global gene expression by Staphylococcus aureus in human blood. PLoS One 8:e53391.
6. **den Reijer PM**, Sallee D, 3rd, van der Velden P, Zaaijer ER, Parks WJ, Ramamurthy S, Robbie TQ, Donati G, Lamphier C, Beekman RP, Brummer ME. 2010. Hemodynamic predictors of aortic dilatation in bicuspid aortic valve by velocity-encoded cardiovascular magnetic resonance. J Cardiovasc Magn Reson 12:4.
7. **den Reijer PM**, Maier AB, Westendorp RG, van Heemst D. 2008. Influence of the TP53 codon 72 polymorphism on the cellular responses to X-irradiation in fibroblasts from nonagenarians. Mech Ageing Dev 129:175-182.
8. **den Reijer PM**, Knuistingh Neven A, Schimsheimer R.J. 2008. Is valeriaan effectief tegen slapeloosheid? Modern Medicine 32:4.
9. van Heemst D, **den Reijer PM**, Westendorp RG. 2007. Ageing or cancer: a review on the role of caretakers and gatekeepers. Eur J Cancer 43:2144-2152.

PHD PORTFOLIO

Name candidate:	Paul Martijn den Reijer
Erasmus MC department:	Medical Micorbiology & Infectious Diseases
PhD period:	2010-2017
Research school:	Molecular Medicine (MolMed) postgraduate school
Promotor:	Prof. dr. H.A. Verbrugh
Copromotor:	Dr. W.J.B. van Wamel

PhD training

Courses

Basic introduction course on SPSS, Molmed postgraduate school	2011
Virology, Molmed postgraduate school	2012
Dialoog training, Erasmus MC zorgacademie	2012
Phenotypische interpretatie van de gevoeligheidsbepaling, Molmed postgraduate school	2013
Communication, Desiderius school	2013
Samenwerking, Desiderius school	2014
Medische ethiek, Desiderius school	2014
Medische Parasitologische diagnostiek, Leids Universitair Medisch Centrum	2015
Klinische mycologie, Radboud Universitair Medisch Centrum Nijmegen	2015
Openbare Gezondheidszorg, Rijksinstituur voor Volksgezondheid en Milieu	2016
Ziekenhuismanagement, Academie voor Medisch Specialisten	2016
Gezondheidsrecht, Desiderius school	2016
Infectiepreventie, Amphia Academy Infectious Diseases Foundation	2017

Conferences and seminars

Scientific spring meeting NVMM	2011
Scientific spring meeting NVMM (poster presentation)	2012
15th International Symposium on Staphylococci and Staphylococcal Infections Lyon, France (poster presentation)	2012
Research day, Erasmus MC (poster presentation)	2012
NVAMM symposium	2013
MolMed research day	2013
Scientific spring meeting NVMM (poster presentation)	2013
Infectieziekten symposium, AMC Amsterdam	2013
Scientific spring meeting NVMM (poster presentation)	2014
NVP najaarssymposium	2014
NVP voorjaarssymposium (oral presentation)	2015
NVAMM symposium	2015
Golden Oldies Farewell symposium Henri Verbrugh	2015
Scientific spring meeting NVMM, Papendal (poster presentation)	2015

Infectieziekten symposium, AMC Amsterdam	2015
NVAMM symposium	2016
Scientific spring meeting NVMM	2016
Infectieziekten symposium, AMC Amsterdam	2016
NVAMM symposium	2017
Scientific spring meeting NVMM	2017

Teaching activities

Supervision of bachelor students

Supervision of a third year bachelor student 'Biologie en Medisch laboratoriumonderzoek' from the Hogeschool Rotterdam	2012-2013

Supervision of medical students

Supervision of second year medical students during both practical and case sessions of the course 'Microbiology & Infectious diseases'	2011-2017

DANKWOORD

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