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Eidesstattliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig verfaßt und keine anderen als die angegebenen Hilfsmittel benutzt habe.

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List of Abbreviations[a]

2-D IB	Two-dimensional immunoblot
Ab	Antibody
Ag	Antigen
APS	Ammonium per sulphate
BCIP	5-brom-4-chlor-3-indoxylphosphate
BSA	Bovine serum albumin
DIGE	Differential in gel electrophoresis
dpi	dots per inch
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix molecules
IAA	Indoleacetic acid
IEF	Isoelectric Focusing
IPG	Immobilized pH gradients
LB	Luria-Bertani (medium)
m/z	mass to charge ratio
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization-Time of Fly
MS	Mass Spectrometry
Mr	Molecular mass
MRSA	Meticillin resistance *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NBT	Nitro blue tetrazolium, chloride
NCBI	National Centre for Biotechnology Information

NCTC	National Collection of Type Culture
OD	Optical density (absorbance)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCA	Principal component analysis
pI	Isoelectric point
PVDF	Polyvinylidene diflouride
rpm	revolutions per minute
RT	Room Temperature
S/N	Signal to noise ratio
SAgs	Superantigens
SDS	Sodium dodecyl sulphate
SERAMs	Secretable expanded repertoire adhesive molecules
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl) amino methane
TSB	Tryptic Soya Broth (medium)
wt	wild type

[a] All the abbreviations of protein names can be found in Table 1.2 and supplemental Table A.1.

Summary

S. aureus is a highly versatile pathogen. On the one hand, *S. aureus* causes a wide range of human diseases ranging from superficial abscesses and wound infections to systemic infections such as osteomyelitis, infective endocarditis, and sepsis. On the other hand, ~35 % of the community are colonized by *S. aureus* and around one fourth of individuals are *S. aureus* persistent nasal carriers. *S. aureus* carriers are usually symptom-free. If hospitalized, they have an increased risk of *S. aureus* infection, which is mostly caused by their colonizing strain. Interestingly, in case of *S. aureus* bacteremia, the *S. aureus*-related mortality is significantly lower in carriers compared to noncarriers. This suggests (at least partial) protection by an adaptive immune response. In support, our group has recently demonstrated that carriers mount a strong and highly specific antibody (Ab) response against the superantigens of their colonizing strain. We propose that the *S. aureus*-specific adaptive immune reaction plays a central role in the development and outcome of an *S. aureus* infection.

However, superantigens, which are known to induce a strong neutralizing Ab response in carriers, are just the tip of the iceberg. *S. aureus* harbors multiple other virulence factors, which contribute to its pathogenicity. Therefore, a comprehensive investigation of anti-*S. aureus* Ab profiles promises important insight into the humoral immune response against *S. aureus*.

In this thesis, an immune proteome approach was applied to the investigation of the anti-*S. aureus* Ab profiles in healthy individuals. The extracellular proteome of a well characterized *S. aureus* laboratory strain, NCTC8325-4 was studied by the combination of two-dimensional gel electrophoresis (2-D PAGE) and mass spectrometry (MALDI-TOF-MS). 2-D immunoblotting was then the method of choice to determine IgG binding to *S. aureus* extracellular proteins.

In the following, the four main work packages and their most important results are briefly described.

1. Characterization of the extracellular proteome of S. aureus NCTC8325-4

Reference maps of the extracellular proteins of *S. aureus* NCTC8325-4 were produced at pH ranges 6-11 and 4-7. In total, 119 (pH 6-11) and 177 (pH 4-7) protein spots were identified, corresponding to 48 and 114 proteins, respectively. Among them were many well-known virulence factors such as alpha-hemolysin (Hla), beta-hemolysin Hlb, gamma-hemolysin subunits (HlgA-C), hyaluronate lyase (HysA) and staphylococcal superantigen-like protein 11 (Ssl11). We also detected various extracellular enzymes,

which can cause tissue degradation and are involved in nutrient acquisition, for example, autolysin (Atl), glycerol ester hydrolase (Geh), lipase (Lip), thermonuclease (Nuc), several serine proteases SplA-F (SplA-F), V8 protease (SspA), cysteine protease (SspB), staphopain thiol proteinase (88195808, SspP). Many of these proteins probably also contribute to the virulence of *S. aureus*.

2. Optimization of a 2-D immunoblot (IB) method for the comprehensive investigation of IgG binding to S. aureus extracellular proteins (strain NCTC8325-4)

The immune proteome of *S. aureus* NCTC8325-4 was revealed by probing 2-D blots of *S. aureus* extracellular proteins at the two pH ranges 6-11 and 4-7 with a pool of sera from 16 volunteers. IgG binding was detected with high sensitivity using a peroxidase-coupled secondary Ab in combination with an ECL-substrate. With application of the software package Delta2D, we could clearly define 66 immune reactive spots on the immunoblots (IBs) of pH range 6-11 and 38 spots on IBs of pH range 4-7. 72 of these 104 immune reactive spots could be identified by matching the IBs with the protein reference maps. These spots represented 36 identified proteins, many of which are known virulence factors, or they are involved in bacterial cell wall biosynthesis and degradation. Generally, the most abundant proteins were also highly immune reactive, but there was no strict correlation between protein abundance and immune reactivity. Some low abundance proteins, especially basic proteins, showed high immune reactivity on 2-D IBs, for example, Atl, 88195808 (SspP) and iron-regulated surface determinant protein A (IsdA). On the other hand, we observed proteins, which were present in large amounts but did not bind IgG such as peptidoglycan hydrolase (LytM) and a hypothetical protein 88193909 (SAOUHSC_00094).

3. Determination of the anti-staphylococcal Ab profiles of S. aureus carriers and noncarriers

Comparing the serum IgG binding patterns of sera from the 16 individual volunteers, we observed pronounced heterogeneity in total IgG binding, spot patterns and spot intensities. Five spots were stronger in carriers than in noncarriers ($P < 0.05$, Mann-Whitney U test). These spots represent IgG binding to SspA, SspB, IsaA, and two hypothetical proteins. A principal component analysis based on differential IgG binding to these spots showed that the carriers were more closely related to each other than the noncarriers, but that they could not be clearly separated from the noncarriers.

4. Does experimental colonization induce changes of the anti-staphylococcal Ab profiles?

Finally, we tested whether symptom-free experimental colonization of the 16 volunteers with *S. aureus* NCTC8325-4 elicited an IgG response. When we compared sera obtained before colonization with those taken 4 weeks after the inoculation with the laboratory *S. aureus* strain, we did not observe major changes in the Ab patterns. We conclude that short- term colonization with a strain of low virulence does not suffice to induce an Ab production, which is comparable to that present already before the colonization. Thus, either long term high density colonization is required, or as we consider most likely, the adaptive immune response is primarily triggered by (minor) *S. aureus* infections.

Taken together, in this work we have separated the soluble proteins from complex extracellular *S. aureus* protein extracts with good reproducibility, large coverage (pH 6-11 and 4-7) and high resolution. With application of an ECL substrate, our 2-D immunoblotting procedure resulted in the highly sensitive detection of IgG binding over a wide range of signal intensities. The most important finding with this technique was the pronounced variability of anti-staphylococcal Ab profiles in healthy adults. This could well explain differences in susceptibility to *S. aureus* infection and its complications. The Ab responses are presumably triggered by long-term colonization or, more likely, by minor infections with *S. aureus*, since experimental nasal colonization of healthy volunteers with a bacterial strain of low virulence did not induce impressive changes in the Ab profiles.

Chapter 1

INTRODUCTION

1.1 *Staphylococcus aureus*-human interaction in colonization and infection

Staphylococcus aureus (*S. aureus*) is a Gram-positive pathogen and can be found as part of the physiological human resident flora. *S. aureus* permanently colonizes the moist squamous epithelium of the anterior nostrils of 20 % of the population, and is transiently associated with another 60 % [1, 2]. However, this member of our benign natural flora can become a formidable intruder when the body defense is compromised, causing diseases ranging in severity from minor superficial skin infections, such as abscesses and impetigo, to life threatening invasive infections, such as septic arthritis, osteomyelitis and endocarditis [4]. In addition, *S. aureus* can also cause toxin-mediated diseases, such as toxic shock syndrome, staphylococcal food poisoning and staphylococcal scalded skin syndrome. Over the last 20 years both community-acquired and hospital-acquired *S. aureus* infections have increased in frequency. To date, *S. aureus* is the most common cause of nosocomial infections [5-7]. Despite antibiotic therapy, these infections can result in severe consequences, stressing the importance of prevention [8-10].

S. aureus produces a large panel of virulence factors and can easily gain resistance to many available antibiotics. In 1961 meticillin resistance was first noted and since the 1970s, endemic strains of meticillin-resistant *S. aureus* (MRSA) carrying multiple resistance determinants became a worldwide nosocomial problem [11-14]. When compared with meticillin-susceptible *S. aureus* (MSSA) bacteremia, bacteremia caused by MRSA resulted in a threefold excess length of hospital stay and a threefold attributable cost [15]. At present, in case of MRSA treatment, the last resort is vancomycin, a drug with rather weak bactericidal potency against prevailing MRSA and strong side effects [16]. However, in 1997 a partially vancomycin-resistant *S. aureus* (VRSA) strain was isolated in Japan and now VRSA strains have been also isolated from USA, France, Korea, South Africa, Brazil and Scotland [11, 12, 17-19]. As infections caused by multidrug-resistant *S. aureus* limit therapeutic options and prolong hospitalization, efforts are being made to develop new treatments and prevention strategies. Until now, the development of effective anti-staphylococcal vaccines is a major task of staphylococcal research. However, in spite of many efforts no vaccine against staphylococcal infection is available so far [20-22].

Factors hindering anti-staphylococcal vaccine development include the multifactorial nature of *S. aureus* pathogenesis. This bacterium has a diverse arsenal of components and products that contribute to the pathogenesis of infection. Furthermore, because approximately one-third of *S. aureus* genome is variable, different *S. aureus* isolates harbor different repertoires of these virulence factors. Therefore, it is not clear to date which factors are good vaccine targets. In order to generate effective vaccines against *S. aureus*, not only more knowledge about the contribution of these factors to the pathogenesis is essential, but also an understanding of their interaction with each other and with host factors [4, 8].

1.1.1 *S. aureus* genetics and virulence factors

S. aureus is a member of the Micrococcaceae family. It was characterized as Gram-positive, cluster-forming coccoid, non-motile and non-spore forming facultative anaerobe. *S. aureus* grows in the form of golden yellow colonies on agar. It can be distinguished from other staphylococci by its ability to ferment mannitol and by the characteristic production of catalase and coagulase [8].

Currently, the genomes from thirteen *S. aureus* strains have been sequenced, including one laboratory strain (NCTC8325), one bovine strain (RF122) and eleven clinical isolates from humans (N315, Mu50, COL, MRSA 252, MSSA476, MW2, USA300, JH9, JH1, Newman, Mu3) (Table 1.1). The laboratory strain NCTC8325 is the parent of non-lysogenic NCTC8325-4 (formerly RN6390, *rsbU* positive) [23-25]. *S. aureus* NCTC8325-4 was derived from *S. aureus* NCTC8325 by curing it from prophages and so far it is the most frequently used strain for molecular, physiological and clinical studies due to its tractable genome and low virulence [25-27].

The *S. aureus* genome has an average size of 2800 Mb and is predicted to contain approximately 2700 genes. Pair-wise comparisons of *S. aureus* genomes revealed co-linearity between them. Approximately 78 % of the genes are conserved among strains and constitute the core genome. The remaining 22 % of the genes comprise an accessory genome with mobile genetic elements (MGE) including genomic islands (e.g. SCC, staphylococcal cassette chromosomes), pathogenicity islands (SaPIs), prophages, integrated plasmids, and transposons [28]. Genes governing virulence and resistance to antibiotics are mainly encoded on MGE and, as the term suggests, they can be transferred horizontally between bacteria [8, 23, 24, 29].

The unique variability and plasticity of the *S. aureus* genome are reflected by the extreme diversity in its proteome, particularly extracellular proteome [30]. Over the years, a

number of *S. aureus* factors has been implicated in pathogenesis, among which, extracellular proteins (cell surface-associated and secreted proteins) are of special interest due to their role as virulence factors, contributing to the establishment or maintenance of an infection.

Table 1.1: Overview of known *Staphylococcus aureus* genomes

***S. aureus* isolate (genome accession number)**	**Year**	**Antibiotic resistance[a]**	**Clinical source**	**References**
N315 (NC_002745)	1982	HA-MRSA	Human	[31]
Mu50 (NC_002758)	1997	HA-MRSA, VISA	Human	[31, 32]
COL (NC_002951)	1963	MRSA	Human	[33, 34]
MRSA252/ USA200 (NC_002952)	1997	HA-MRSA, EMRSA	Human	[35, 36]
MSSA476 (NC_002953)	1998	CA-MSSA	Human	[37]
MW2/USA400 (NC_003923)	1998	CA-MRSA	Human	[38]
NCTC8325 (NC_007795)	1963	MSSA	Human	[39, 40]
USA300/FPR3757/LAC (NC_007793)	2000	CA-MRSA	Human	[41]
JH9 (NC_009487)	2000	HA-MRSA, VISA	Human	[40, 42]
JH1 (NC_009632)	2000	HA-MRSA, VISA	Human	[40, 42]
Newman (NC_009641)	1952	MSSA	Human	[40]
Mu3 (NC_009782)	1996	Hetero-VISA	Human	[43, 44]
RF122 (NC_007622)	n.d.	n.d.	Bovine	[45]

[a] CA-: community-acquired; HA-: hospital-acquired; EMRSA: endemic meticillin resistance *Staphylococcus aureus*; VISA: Vancomycin-intermediate *Staphylococcus aureus*; n.d.: not determined

Cell surface-associated proteins

Many staphylococcal surface proteins have structural features in common including an N-terminal secretory signal sequence, a C-terminal positively charged cytoplasmic anchor with a hydrophobic membrane-spanning domain and a cell wall-anchoring region. Some of these proteins function as adhesins, which have ability to adhere to host components as well as host cells [46]. Adhesins binding to extracellular matrix molecules (ECM) such as matrix and plasma proteins, e.g. fibronectin, fibrinogen, vitronectin, thrombospondin, and collagen, have been designated microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). To date, various MSCRAMMs have been suggested to play an important part in the ability of staphylococci to colonize host tissue [4, 46-50]

MSCRAMMs from *S. aureus* include: (i) clumping factors A and B (Clf A and B) that bind fibrinogen [51-53]; (ii) Collagen binding protein (Cna) [54]; (iii) fibronectin-binding

proteins A and B (FnbpA and B) [55-57]; and (iv) serine-aspartate repeat containing proteins (Sdr family) comprising SdrC, D, E whose ligands are yet to be defined [52] and (v) the bone sialoprotein-binding proteins (Bbp) [58] (Table 1.2). All these MSCRAMMs are structurally related and currently under investigation as vaccine components or as immunotherapy targets (*see* 1.1.3).

Recently described MSCRAMMs include EbpS, an elastin-binding protein whose binding site sequence is strikingly similar to that of Fnbps [59], and Ebh which is an ECM-binding protein homologue. Ebh was found to specifically bind human fibronectin. It is growth phase-regulated and produced during human infection, since serum samples taken from patients with confirmed *S. aureus* infections were found to contain anti-Ebh antibodies [60, 61].

One potential adhesin interacting with ECM proteins is protein A (Spa) which is expressed by almost all *S. aureus* strain. Spa is a cell wall component consisting of a single polypeptide chain (Mr 42,000) with little or no carbohydrate [62]. It has anti-phagotic properties based on the ability to bind the Fcγ of immunoglobulin [63, 64]**.** Spa has four high-affinity ($K_\alpha=10^8$ M^{-1}) binding sites that are capable of interacting with the Fc region of IgGs of several species. However, the affinity of Spa to IgG differs between species and subclasses of IgG. For example, human IgG_1, IgG_2 and IgG_4 bind strongly to Spa, while IgG_3 does not bind [65]. Spa is heat-stable and retains its native conformation even after exposure to denaturing reagents such as 4 M urea, 4 M thiocyanate and 6 M guanidine hydrochloride [66]. As well as being anti-phagocytic, Spa also acts as a B-cell superantigen through interactions with the heavy-chain variable part of Fab fragments (VH3 region, adjacent to the Ag-binding domain) [67-71]. While Spa does not seem to play a significant role in the persistence of nasal colonization by *S. aureus* [72], it could act as a virulence factor in infection as Spa-deficient mutants of *S. aureus* are phagocytosed more efficiently by neutrophils *in vitro* and show decreased virulence in several animal infection models [73, 74] (Table 1.2).

There are two other cell wall-associated protein families including the *S. aureus* surface proteins (Sas) protein and iron-regulated surface determinant (Isd) family. SasG and H are potential vaccine targets because serum Ab levels against them increase during *S. aureus* infection [75, 76]. Isd family proteins, as the name suggests, have important functions in iron acquisition by *S. aureus* but they also have broad–spectrum ligand-binding activity including fibrinogen and fibronectin. In recent studies, vaccination with Isd proteins protected mice against nasal carriage [27] and against lethal challenge with

human clinical *S. aureus* isolates [77]. Additionally, according to Kuklin *et al.*., IsdB vaccination did not only increase survival in a murine *S. aureus* sepsis model but also induced rapid Ab responses in *Rhesus macaques* [78].

Secreted proteins

Anchorless adhesins: Apart from the covalently cell wall-anchored adhesins (MSCRAMMs) mentioned above, there is another group of adhesins that are secreted, the secretable expanded repertoire adhesive molecules (SERAMs). Unlike in other Gram-positive bacteria, SERAMs and MSCRAMMs are not structurally related to each other in *S. aureus* [46] (Table 1.2).

Exotoxins: *S. aureus* produces numerous toxins that are grouped based on their mechanisms of action. Thus far there are, (i) pyrogenic toxin superantigens (SAgs) comprising toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEA-E, SEG-R, SEU-V), (ii) exfoliative toxins (ETA, and ETB), and (iii) cytotoxins including four hemolysins: α, β, γ and δ (Hla, Hlb, Hlg and Hld respectively), and Panton-Valentine leukocidin (PVL). Each of these toxins has potent effects on cells of the immune system [79] (Table 1.2).

Both Hlg and PVL are leukocidins and share a common structure. Together, they constitute the bicomponent β-barrel pore forming toxin (bicomponent β-PFT) group implicating that they are composed of two different proteins. Hlg is formed by association of Hlg2 (HlgA) with either HlgB or HlgC and PVL is formed by combination of LukF-PV (LukD) and LukS-PV (LukE) [80]. The Hlg proteins are derived from the *hlg* locus (*hlg)*, and have been found in 99% of clinical isolates of *S. aureus*. In contrast, LukF-PV and LukS-PV are derived from the PVL locus (*pvl*) which is distinct from the *hlg* locus, and only a small percentage of clinically isolated *S. aureus* strains carries *pvl*.

Table 1.2: Overview of *S. aureus* virulence factors

(adapted from, [4, 31, 46-50, 79])

Abbreviation	Full name protein	Experimentally demonstrated ligands or functions	References
MSCRAMMs (Microbial surface components recognizing adhesive matrix molecules)			
Cna	Collagen binding protein	Collagen	[54]
EbpS	Elastin-binding protein	Elastin	[59]
Ebh-A, -B (GSSP)	ECM-binding protein homologue (giant staphylococcal surface protein)	Fibronectin	[58]
Fnbp-A, -B	Fibronectin-binding protein A	Fibronectin, Fibrinogen, Elastin	[55-57]
	Fibronectin-binding protein B	Fibronectin	[55-57]
Isd family	Iron-regulated surface determinant A, B, etc.	Fibronectin, Fibrinogen, Lactoferrin	[81, 82]
ClfA (SdrA)	Clumping factor A (Serine-aspartate repeat protein A)	Fibrinogen γ chain, Fibrin	[51-53]
ClfB (SdrB)	Clumping factor B (Serine-aspartate repeat protein B)	Fibrinogen α -and β-chain, Type I cytokeratin 10	[52, 83-85]
Sdr family	Bone sialoprotein-binding protein	Bone sialoprotein	[58]
	Serine-aspartate repeat containing proteins: SdrC-E	n.d.	[52]
Sas protein family	*Staphylococcus aureus* surface protein: SasA, SasC-K; Pls (Plasmin-sensitive protein); Asp (Accumulation-associated protein)	*In vitro*: isolated keratinocytes	[75, 76, 86]
Spa	Staphylococcal protein A/ IgG binding protein A	Von Willebrand factor	[87]
SERAMs (Secretable expanded repertoire adhesive molecules)			
Eap (Map, p70)	Extracellular adherence protein (MHC class II-analogous protein)	Fibrinogen α-chain, Fibronectin, Prothrombin, Vitronectin, Collagen	[88-90]
Efb	Extracellular fibrinogen binding protein	Fibrinogen α-chain, C3b	[91, 92]
Emp	Extracellular matrix binding protein	Fibronectin, Fibrinogen, Vitronectin	[93]
vWbp	Von Willebrand factor binding protein	Von Willebrand factor	[94]
Coa*	Coagulase	Fibrinogen, Prothrombin	[95-99]
Exotoxins			
Hla, Hlb, Hlg, Hld	α -hemolysin (α-toxin)	Integrate to cell membrane (pore formation), hemolytic, dermonecrotic and neurotoxic activity	[100, 101]
	β -hemolysin (Sphingomyelinase C)	Integrate to cell membrane, hemolytic activity, phosphorylase *c* activity	[102-107]
	γ -hemolysin	Integrate to cell membrane, hemolytic and inflammatory activity	[108-113]

	δ -hemolysin (δ-toxin, δ-lysin)	Integrate to cell membrane, hemolytic and cytolytic activity	[114-119]
PVL	Panton-Valentine leukocidin	Integrate to cell membrane, hemolytic and inflammatory activity	[108, 109, 111-113]
SEA-E, SEG-R, SEU-V	Staphylococcal enterotoxins	SAgs, cause TSS and SFP, sepsis	[120-124]
TSST-1	Toxic shock syndrome toxin-1	SAg, causes TSS and SFP	[122, 123, 125]
ETA-C*	Exfoliative (epidermolytic) toxins	Lyse proteins (act as esterase)	[126, 127]
Extracellular enzymes			
Aur	Aureolysin (Metalloprotease)	Degrade various staphylococcal surface-associated proteins, human proteins	[128-130]
Atl	Autolysin	Hydrolytically degrade peptidoglycan	[131, 132]
HysA	Hyaluronidase	Degrade hyaluronic of connective tissue	[133]
Nuc	Nuclease (Tnase)	Degrade host nucleic acids	[134]
SAL	Lipase	Hydrolytically degrade lipids	[135]
Sak	Staphylokinase	Convert plasminogen to plasmin	[136, 137]
SspA	V8 protease/ Serine glutamylendopeptidase	Degrade proteins, eg Fnbps, Spa	[126, 138]
SplA-SplF	Serine protease-like proteins	Possibly degrade proteins	[139, 140]
StpA (ScpA), StpB/SspB,	Staphopains (Cystein proteases)	Degrade proteins	[126, 129, 141, 142]
Others			
PBPs	Penicillin-binding proteins	Participate in cell wall synthesis	[97, 143, 144]

*: also plays as an extracellular enzymes: Coa converts fibrinogen to fibrin, ETs act as esterases, ETC is serologically different from ETA, ETB; n.d.: not determined

The bicomponent leukotoxins above are closely related to Hla, also a member of the β-PFT family, which oligomerizes to form a homoheptamer pore on host cell membranes. Hla plays a role in the pathogenesis of staphylococcal disease, as *S. aureus* mutants lacking *hla* display reduced virulence in invasive disease models [73, 145]. Active immunization in mice with a mutant form of Hla (Hla_{H35L}), which cannot form pores, induces Ag-specific IgG responses and confers protection against staphylococcal pneumonia [146]. Moreover, transfer of Hla-specific antibodies protects naive mice against *S. aureus* challenge and prevents the injury of human lung epithelial cells during infection [146].

Other hemolysins, Hlb and Hld, damage membranes of a variety of mammalian cells. However, their roles in disease are not clearly understood [79].

Extracellular enzymes and other bacterial components: Nearly all *S. aureus* strains secrete of extracellular enzymes, which include nuclease (Nuc), several proteases (SspA, SspB), lipases (SAL), hyaluronidase (HysA) and collagenase. The main function of these proteins is probably to degrade and digest organic compounds and macromolecules of the host tissue to provide nutrients and allow tissue invasion by *S. aureus*. However, their role in the pathogenesis of diseases has not yet been well defined [79].

S. aureus proteases include the serine protease SspA (V8 protease, named after the first *S. aureus* strain from which the protein were isolated) that could play a role in controlling microbial adhesion as well as growth and survival of *Staphylococcus aureus* in different infection models [141] . The *sspA* gene forms an operon together with *sspB*, which encodes a 40.6-kDa cysteine protease, and with *sspC*, which encodes a 12.9-kDa cytoplasmic protein. SspA is secreted as an inactive precursor form, which is processed by the metalloprotease aureolysin (Aur) to form the mature serine protease [147]. SspA is required for maturation of the protease SspB, while SspC very specifically binds and inhibits sspB and interfere with its intracellular activity [148]. Thus, the operon encodes the protease SspB as well as a regulatory system for its enzymatic activity.

Aur, an activator of SspA, is a neutral, calcium-stabilized and zinc-dependent metalloprotease. It belongs to the superfamily of M4, or thermolysin-like, endopeptidases. Aur is also expressed as a precursor form [149], but its mechanism of maturation is not known. Most, if not all, staphylococcal strains display a functional gene encoding Aur [149], which is synthesized and secreted throughout the bacterial growth cycle [129]. Regarding presumed targets, Aur as well as SspA cleave staphylococcal surface-associated proteins (i.e. Fbnp, Spa and ClfB), a property that is thought to support

the transition from an adherent to an invasive phenotype [150] and also human proteins (i.e. Ig, antimicrobial peptides) [126, 151]. In addition, the proteases, have been suggested to downregulate the virulence of *S. aureus* in specific niches *in vivo* by degradation of toxins such as Hla [152].

An interesting staphylococcal protein is IsaA, the immunodominant antigen A. It presents not only in the culture supernatant but also in the cell wall fraction. As the name suggests, IsaA is one of the major Ags of *S. aureus*. It has a putative soluble lytic domain suggesting that the protein might participate in the peptidoglycan (PG) turnover [153]. Thus, IsaA is probably a member of the class of lytic transglycolases. This protein group also contains autolysins (Atl) which hydrolyze specific bonds within the bacterial cell wall PG and assist in cell wall expansion, turnover, bacterial growth, and cell separation [131].

1.1.2 Correlation of *S. aureus* nasal carriage and risk of infection

S. aureus nasal carriage

Humans are a natural host of *S. aureus* and the primary ecological niche of *S. aureus* human strains are the anterior nares, i.e. the nostrils. However, *S. aureus* can be isolated from multiple sites of skin and mucosal surfaces of carriers, including throat, axilla and perineum.

S. aureus nasal carriage patterns can be divided into 3 types: persistent, intermittent and non-carriage. The carriage status can be determined by two nasal swabs obtained at a 1-week interval and is defined as persistent-, intermittent-or non-carriage in case of 2, 1, or 0 nasal swab cultures positive for *S. aureus*, respectively. This simple test has a reliability of 93.6% [2].

S. aureus nasal carriage rates range between 20 % and 55 % in cross-sectional studies and patterns differ between individuals. About 10 to 35 % of individuals carry *S. aureus* persistently, while 5 to 50 % never carry *S. aureus* in their nose. Moreover, the carriage patterns also vary with age. Most newborns are exposed to *S. aureus* within the first hours of life and more than 70% have a positive nasal *S. aureus* culture. The rate decreases to 40-50 % during the first 8 weeks of life and to ~ 20 % by 6 months of age. Persistent carriage is then more common in children than in adults, and many people shift from persistent to intermittent carriage during adolescence [154]. The reasons for the intra-individual as well as age-related differences in colonization patterns are not known.

Interestingly, the number of *S. aureus* bacteria in the anterior nostrils is significantly higher in persistent carriers, which may partly explain their increased risk for *S. aureus* infections. Furthermore, persistent carriers are often colonized by a selected single strain of *S. aureus* over long times, while intermittent carriers acquire many different strains over time [1]. Obviously, persistent carriage prevents the acquisition of other strains, and the determinants of persistent and intermittent carriage appear to be different.

Nasal colonization *by S. aureus* is likely facilitated by its surface structures. These range from peptidoglycan molecules and cell wall teichoic acids to a wide variety of proteins belonging to the family of MSCRAMMs such as Fnbps and ClfB [49, 155, 156]. Furthermore, host factors, e.g. gene polymorphisms [1, 157-159], as well as environmental factors are recognized determinants of the *S. aureus* nasal carrier state [160, 161].

To study factors involved in *S. aureus* nasal colonization, different approaches were taken including clinical or microbiological observations [162] and *in vitro* or animal model experiments [163-165]. Mice and cotton rats are the most frequently used animal models for *S. aureus* nasal colonization. The nasal mucosa of cotton rats closely resembles that of humans [165], and the animals maintain *S. aureus* in their nose longer than mice, but genetic investigations in this model are hampered by the lack of inbred and knockout strains. However, neither cotton rats nor mice are natural *S. aureus* carriers. Therefore, even though these animal models may help to define the molecular mechanisms leading to colonization of the nose, human colonization will be most informative. To our knowledge, only one research group in Rotterdam, the Netherlands, has performed such studies in humans under strict health safety precautions and guidance by an ethic committee. In one experiment, the group inoculated volunteers with different *S. aureus* strains which helped to demonstrate the role of human host factors in colonization. Nearly all the volunteers returned to their original carriage state after the experimental inoculation: Noncarriers rapidly rejected the inoculated *S. aureus* strain, and in persistent carriers their original colonizing strains eventually dominated again [166]. Another experiment, showed an important role for ClfB in the persistence of *S. aureus* nasal carriage by parallel inoculation of a wt *S. aureus* strain and its syngeneic ClfB-mutant [155].

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

S. aureus nasal carriage is a major risk factor for the development of *S. aureus* infections in various clinical settings, e.g. in patients after operation (pneumonia and wound infections), in patients on continuous ambulatory peritoneal dialysis and hemodialysis, and in patients infected with human immunodeficiency virus (HIV) [1, 9].

When investigating the incidence of *S. aureus* bacteremia in carriers and noncarriers in a non-surgical patient population, Wertheim *et al.*. documented a threefold increased risk for *S. aureus* nasal carriers to acquire a nosocomial *S. aureus* bacteremia, compared to noncarriers. In approximately 80% of the cases, the bacteremia strains of the carriers had the same genotype as their nasal strains. Surprisingly, however, the *S. aureus*-related in-hospital mortality was 4 times lower in carriers who developed bacteremia than in noncarriers (8 % vs. 32 %, p= 0.006) [167]. For an immunologist, this is paradoxical, because higher infection rates are usually associated with a higher mortality. One explanation could be that carriers mount a (partially) protective immune response against their colonizing strain. This suggestion was supported by a recent study of Holtfreter *et al.*. They showed that carriers have high titers of neutralizing Abs specifically against SAgs expressed by their colonizing strain. The results indicate that the carriage status confers strain-specific humoral immunity, which may contribute to protection during *S. aureus* bacteremia [168]. These findings raised the question whether symptom-free colonization by *S. aureus* is sufficient to elicit this highly effective Ab response or whether this is due to minor *S. aureus* infections as they may commonly occur in carriers.

Because a large proportion of *S. aureus* infections are of endogenous origin, which mean that carriers are usually infected by their own *S. aureus* isolate, it has been intensively investigated, whether nasal *S. aureus* eradication reduce infection rates. There are three treatment strategies which may eliminate *S. aureus* in carriers: locally applied antibiotics or disinfectants, systemic antibiotics and bacterial interference [169] (Table 1.3).

For the two first approaches, several antibiotics and their combinations were studied for their efficiency in decolonizing *S. aureus* from the anterior nares [170-172]. Nowadays, mupirocin calcium ointment (pseudomonic acid) is one of the most frequently used agents. It was introduced in the late 1980s and shows high efficacy in the local elimination of *S. aureus* [173-177]. It has a unique mechanism of action: It binds to isoleucyl transfer RNA synthetase, and thereby inhibits the incorporation of the amino acid into nascent proteins [178]. So far, mupirocin is still effective in *S. aureus* nasal

eradication and has been used in many intervention studies. However, which patient groups profit from this treatment is still controversial. Some studies reported substantial reductions of surgical site infections among patients receiving mupirocin prophylaxis, but other randomized controlled trials failed to confirm it [179-181].

Table 1.3: Potential strategies using in nasal decolonization
(adapted from [169])

Nasal carriage treatment strategy	**Examples**
Systemic antibiotics	Rifampin
	Trimethoprim/sulfamethoxazole
	Ciprofloxacin
	Combinations of systemic antibiotics
	TMP/SMX and rifampin
	Minocycline and rifampin
Systemic and local antibiotics	TMP/SMX and bacitracin
	Rifampin and bacitracin
	Local antimicrobials and antiseptics
	Bacitracin
	Mupirocin
	Povidone-iodine
	Chlorhexidine
Bacterial interference	*S. aureus* 502 A

Bacterial interference is a historical approach to control *S. aureus* nasal carriage and has been used in the 1960s and 1970s. In this strategy, a comparably low virulent *S. aureus* strain or other non-pathogenic bacteria were employed to actively colonize nostrils. These bacteria then prevented colonization by other more virulent strains [171, 182-186]. In the 1960s, interference by a *S. aureus* strain of apparently negligible virulence was reported to be successful in nurseries during outbreaks of *S. aureus* infections and for treatment of patients with recurrent furunculosis [184, 185, 187-189]. This strain, 502A, which was isolated from the nose of a healthy nurse, is penicillin-sensitive and is lysed by phages 6 / 7 / 42E / 44D / 53 / 54 / 75/ 81. However, in rare cases, serious infectious complications due to the 502A strain were observed, and after one case of fatal bacteremia caused by this very strain, bacterial interference was put on hold [190, 191].

1.1.3 Interactions between human immune system and *S. aureus*- Perspective of anti-staphylococcal vaccines

The human immune response against *S. aureus* during colonization and infection

S. aureus is primarily an extracellular pathogen although it can sometimes penetrate host cells and persist intracellularly in a semi-dormant form referred to as small colony variants [192-194]. Therefore, host defense against *S. aureus* depends primarily on innate immune mechanisms and the anti-staphylococcal humoral immune response.

The first stage of staphylococcal or, indeed, microbial infection is colonization: the establishment of the pathogen at the appropriate port of entry. Once the outer physical barriers of the body, the skin or the mucous surfaces, have been breached by *S. aureus*, the organism is confronted with the host immune system, comprising both innate and adaptive responses.

S. aureus infection of the skin stimulates a strong inflammatory response, involving the immigration of neutrophils and macrophages to the site of infection [195]. These cells engulf and dispose of the invading organisms, a process that is greatly enhanced by opsonization by Abs that may be present in the host serum and extracellular fluids, and/ or by complement. This mechanism has an important role in combating staphylococcal infection.

In principal, the innate immune system attacks *S. aureus* with three important effector mechanisms: I) antimicrobial peptides, II) the complement system and III) phagocytes.

Antimicrobial peptides are produced in high amounts by cells such as platelets and neutrophils, and are present on mucosal surfaces, in the airways and on skin. Their antimicrobial activity is due to disruption of the integrity of lipid bilayers. They also function similarly to beta-lactam antibiotics to activate nascent autolytic wall enzymes, leading to bacteriolysis [196].

The complement system consists of proteins and their proteolytic fragments that have many functions in innate and acquired immunity, including opsonization, chemotaxis and direct lysis of the bacteria [197].

Phagocytes include neutrophils and macrophages. These cells have specific receptors for fragments of complement and formylated peptides that enhance the efficiency of phagocytosis. They also express specific receptors for the Fc region of immunoglobulin G (IgG) and bound to the bacterial surface. The engagement of these receptors facilitates uptake and killing of the bacteria which is meant by the term opsonization [4].

B cells, their product Abs, and T cells constitute the adaptive immune system. At the initial stage of the host immune response to infection, the invading microorganism and its products are taken up by macrophages and other Ag-presenting cells and transported to lymph nodes, where Ag-specific B cells are stimulated to differentiate into plasma cells and secrete Abs that neutralize toxins and opsonize the bacteria. In the case of *S. aureus*, the current knowledge about the role of adaptive immunity in protection and disease susceptibility is limited. It is now known that Abs to *S. aureus* Ags are usually present in healthy adults, and there is evidence that titers rise following infection [61, 198]. However, it remains unclear how much Abs can contribute to protection.

Until now, a number of studies have investigated the Ab response to *S. aureus* in infected patients but few have addressed the response in healthy carriers and noncarriers. Furthermore, most serological studies analyzed Ab titers against total bacterial lysates or only few selected proteins. For a more comprehensive analysis of the Ab response, broad-spectrum screening methods such as protein arrays, bacteriophage expression libraries of *S. aureus* or two-dimensional immunoblots (2-D IBs) of staphylococcal Ags could be of value. Moreover, since Ab responses could be highly strain-specific, analyzing matched samples of strain and serum will be the most informative approach.

Some animal model experiments and clinical studies showed that Abs against certain bacterial components, such as surface adhesins (Clfs, Fnbps and Cna) [199-202], surface polysaccharides (type 5 and 8) [203] and poly-N-succinyl β-1-6 glucosamine [204] are protective. In addition, the role of anti-staphylococcal Abs as neutralizing factors for secreted toxins like SAgs was in the focus of many studies. They indicated that anti-SAg Ab titres rise during staphylococcal infections, e.g. bacteremia and wound infection [205, 206] and showed that carriers mount a strong and highly specific Ab response against the SAgs of their colonizing strain [168]. Furthermore, anti-SAg Abs protect against SAg-induced toxic shock syndrome in animal models [207-210]. The protective function of anti-SAg Abs is confirmed by the observation that most TSS patients lack anti-TSST-1 Abs and there is anecdotal evidence that treatment of TSS patients with Ig preparations is efficient [211-213]. These findings are first evidence that anti-staphylococcal Abs contribute to protection against staphylococcal disease. However, in view of the heterogeneity of the species *S. aureus*, the very broad spectrum of symptoms and diseases that can be caused by *S. aureus*, and most importantly, because serious *S. aureus* infections are fatal too frequently, much remains to be done.

S. aureus immune evasion

The human immune system has multiple mechanisms to protect the body from *S. aureus* colonization and infection. However, it is not always effective because *S. aureus* at the same time harbors various factors to evade the immune attack (Table 1.4).

More than half of all *S. aureus* strains secrete the **ch**emotaxis **i**nhibitory **p**rotein of **s**taphylococci CHIPS that can bind avidly to the formyl peptide receptors and the C5a receptor. This binding will prevent the formyl peptide and C5a-mediated chemotaxis of neutrophils to the site of infection. Another protein likely acting in concert with CHIPS to inhibit neutrophil recruitment to the site of infection is Map (Eap). Map binds to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, thereby blocks the binding of its ligand on the surface of neutrophils, the lymphocyte function-associated antigen (LFA-1), and thus prevents leukocyte adhesion, diapedesis and extravasation [214].

Most *S. aureus* strains produce toxins that damage the membranes of host cells. Leukotoxins, cytolytic toxins that damage leukocytes, leukotoxins, kill neutrophils that should engulf and kill the bacteria, thereby contributing to abscess formation. These leukotoxins comprise Hla, Hlg, PVL, as well as LukE/D and Luk M/F-PV-like [215]. The hemolysins Hla, Hlg lyse erythrocytes and leukocytes, whereas PVL is toxic only for leukocytes [215].

Table 1.4: Overview on immune evasion mechanisms by *S. aureus*

Mechanism	Virulence factors	References
Inhibition of neutrophil recruitment	CHIPS, Map	[214]
Killing of leukocytes	Hla, Hlg, PVL	[214]
Resistance to phagocytosis, Inactivation of complement	Spa, Capsular polysaccharides Sak, Efb, ClfA	[214, 216]
Resist to phagocytic killing	Yellow carotenoid pigments, SodA, SodM	[4]
Resistance to antimicrobial peptides	Sak, Aur, modified cell wall and membrane components	[4, 151, 217]
Resistance to lysozymes	Modified cell wall components	[218]
Suppression of the immune system	SAgs, Map, Spa	[69, 214, 219]

The ability of *S. aureus* to avoid opsonins present in normal serum is another important factor in the success of infection. Nearly all *S. aureus* clinical isolates express a thin microcapsular layer that is composed of serotype 5, serotype 8 or serotype 336 capsular

polysaccharides [220]. These capsular polysaccharide can compromise neutrophil access to bound complement and Abs. Moreover, *S. aureus* also avoids opsonophagocytosis by various secreted and cell wall-associated proteins such as Spa, Efb, ClfA, Sak. Spa binds to the Fc region of IgG, thus coats the surface of *S. aureus* with IgG molecules in the incorrect orientation so that they can not be recognized by Fc receptors on phagocytes [64]. Efb catches complement factor C3b and blocks its deposition on the bacterial cell surface [92]. ClfA binds the γ-chain of fibrinogen thus protects *S. aureus* from phagocytosis because bacterial cells become coated with fibrinogen, a host protein, which in turn inhibits deposition of, or access to, opsonins. The extracellular staphylokinase Sak activates cell-bound plasminogen and cleaves IgG and C3b, resulting in reduced phagocytosis by neutrophils [216].

Once *S. aureus* is successfully engulfed by a neutrophil, it can still avoid phagocytic killing, by interfering with phagosome endosome fusion and by resistance against released antimicrobial substances [221]. The latter is partially due to the natural modifications to cell wall components, lipoteichoic acid and membrane phospholipid, for example, D-alanine substitutions of ribitol teichoic acid and lipoteichoic acid and L-lysine additions to phosphatidylglycerol [222]. The modifications not only repel the cationic defensins which are released into the neutrophil phagosome but they also protect *S. aureus* from the positively charged antimicrobial proteins in serum, such as phospholipase A2 and lactoferrin [222]. In addition, *S. aureus* can interfere with the lethal effects of free oxygen radicals released by neutrophils during the oxidative burst. For example, the yellow carotenoid pigment of *S. aureus* scavenges oxygen free radicals [223]. *S. aureus* anti-oxidant enzymes such as superoxide dismutase enzymes (Sod A and Sod M) and catalase (KatA) protect *S. aureus* by removing harmful superoxide radicals [224] [225].

Furthermore, to neutralize antimicrobial peptides, *S. aureus* secretes several proteins that can bind and cleave them. Aur cleaves and inactivates the human defensin peptide cathelicidin LL-37 and contributes significantly to resistance to the peptide *in vitro* [151]. Sak also has potent defensin-peptide-binding activity and contributes to the protection of bacteria [137, 217].

S. aureus can also resist lysozyme, an enzyme which causes bacterial cell lysis by cleaving the glycosidic linkage between N-acetylglucosamine and N-acetyl muramic acid of cell wall PG. This ability was recently attributed to a membrane-bound O-acetyltransferase that modified the C6 hydroxyl group of muramic acid [226].

Besides interfering with innate immunity, *S. aureus* harbors various immune modulatory molecules that suppress or alter adaptive immune functions. For example, Map can bind to the T-cell receptor, which reduces T-cell proliferation, resulting in T-cell suppression as exemplified by a reduced delayed-type hypersensitivity response [219]. Map also can cause a shift from a Th1 response to a Th2 response, which affects cell-mediated immunity [219]. As well as being anti-opsonic, Spa also functions as a B cell SAg because of its ability to bind to Ig heavy chains using the VH3 elements that is adjacent to the antigen-binding domain of IgM molecules exposed on the surface of B lymphocytes. B cells bearing VH3 Ig on their surface are stimulated to proliferate and undergo apoptosis, leading to depletion of a significant proportion of the B cell repertoire [71]. Multiple T cell SAgs such as TSST-1 and various SEs further contribute to the failure of immune system to generate an appropriate Ab response. SAgs bind the exterior surface of the MHC class II protein on the surface of antigen-presenting cells and link it to T-cell receptors on the surface of a T helper cell [227]. Up to 30% of T cells can become activated. This prevents development of a normal immune response because antigen-specific T cells fail to proliferate in response to antigens that are presented in the conventional way on MHC class II [228].

Perspectives of active and passive anti-staphylococcal vaccines

As one of the leading causes of hospital-based blood stream infections, endowed with ever-increasing antimicrobial resistance, *S. aureus* evoked intense efforts to develop new prevention and treatment strategies. These strategies include the development of subunit vaccines (active vaccination) and immunoglobulin preparations (passive vaccination) targeted at virulence factors expressed *in vivo* by staphylococci (Table 1.5). As described above, there is a large number of known and putative staphylococcal virulence factors, but only few have been tested as vaccine candidates (several capsular Ags and cell wall-associated proteins) and only a small fraction of these reached clinical trials.

StaphVAX® (Staphylococcal Polysaccharide Conjugate Vaccine) is a bivalent vaccine containing *S. aureus* capsular polysaccharides 5 and 8 conjugated to a non-toxic recombinant *P. aeruginosa* exotoxin. After phase I, II and the first phase III trial, the results were encouraging. There was induction of both IgM and IgG Abs to capsular polysaccharides after injection [229]. Unfortunately, the last trial involving 3600 hemodialysis patients failed to meet its primary endpoint. The study found no reduction in *S. aureus* types 5 and 8 infections in the StaphVAX group as compared to the placebo

group. Therefore, the development of StaphVAX was put on hold [230]. At the same time, Altastaph, which is a StaphVAX-induced hyperimmune polyclonal Ab preparation, failed to reduce *S. aureus* infection in very low birth weight infants [231]. Consequently, the Altastaph development was also stopped.

Apart from capsular Ags, MSCRAMMs such as ClfA, ClfB, FnbpA, FnbpB and Cna could also be important Ab targets because they mediate the binding of *S. aureus* to human extracellular matrix proteins and to foreign material like vascular catheters.

INH-A21 (Veronate®) is a polyclonal human donor-selected immunoglobulin preparation based on SA-IVIG (*S. aureus*-hyperimmune intravenous immune globulin) that contains high concentrations of Abs to ClfA of *S. aureus* as well as SdrG (Ser-Asp dipeptide repeat G), a fibrinogen-binding MSCRAMM present on the majority of *S. epidermidis* strains [232, 233]. This Ig preparation was protective in a neonatal rat model of *S. epidermidis* infection and successful in the prophylaxis and therapy of rabbits with experimental endocarditis caused by either *S. epidermidis* or MRSA [232]. After clinical phase I and II trials in neonates, the product appeared safe and tolerable [233, 234]. However, in the pivotal phase III study involving 2000 low birth weight infants, INH-A21 showed no reduction in the frequency of *S. aureus* infection. Apparently, the development of this product was put on hold.

Aurexis® (Tefibazumab; MAb T1-2) is a humanized monoclonal Ab with high affinity and specificity for clumping factor A (ClfA). Pre-clinical studies showed that Aurexis® was effective in preventing and treating *S. aureus* infections, including those caused by MRSA. A Phase I study in healthy volunteers and in subjects with end-stage renal disease and a Phase IIa study in 60 patients with complicated *S. aureus* bacteremia are completed. In the subjects studied to date, Aurexis® has proved safe and it was well-tolerated [235-237].

Two other Ab preparations are on the way to clinical testing, Aurograb® and Pagibaximab®. Aurograb® is a human recombinant single chain Ab fragment (scFv) that binds to an ABC transporter, GrfA, an immunodominant surface Ag of *S. aureus*. Aurograb® has an *in vitro* activity against multiple strains of *S. aureus* and acts synergistically with vancomycin against MRSA and VISA [238, 239]. Pagibaximab® (BSYX-A110) is a humanized mouse chimeric monoclonal Ab directed against lipoteichoic acid (LTA), a major cell wall component of Gram-positive bacteria. This product has been reported to provide protection in rodent models of infection with either *S. aureus* or *S. epidermidis*, and it is currently being developed for clinical use [240].

In brief, to date there is no effective vaccine against *S. aureus* in clinical practice. This is due to both the multifactorial character of *S. aureus* virulence and the complexity of the *S. aureus* – host interaction. Therefore, to succeed in the vaccine development, vaccination strategies should not rely on a single virulence factor. A better understanding on the host-pathogen interaction is mandatory. Because of the increasing spread of antibiotic resistance, an active vaccine will be highly beneficial for patients at high risk of staphylococcal infections like dialysis patients, trauma patients, surgical patients, and premature neonates. Patients with manifest infections might benefit from passive vaccination with anti-*S. aureus* Ab preparations.

With this work, I wish to contribute to the huge task. With proteome as well as immune proteome techniques, I hope to reveal new potential molecular targets for vaccine development.

Table 1.5: Active and passive *S. aureus* vaccines. (adapted from [20])

Target	Product	Pharmaceutical company	Patient group	Status	References
Active immunization: subunit vaccine					
S. aureus capsular polysaccharide type 5 and 8	StaphVAX® (*S. aureus* conjugate)	Nabi Biopharmaceuticals	Prevention of *S. aureus* infection	On hold StaphVAX® is currently extended to a multivalent vaccine to also include *S. aureus* 36 and *S. epidermidis* PS-1 and possibly *S. epidermidis* GP-1 and PVL	[230, 241]
Passive immunization					
S. aureus capsular polysaccharide type 5 and 8	Altastaph® (*S. aureus* Ig)	Nabi Biopharmaceuticals	Prevention of *S. aureus* infection in at-risk neonates	On hold Altastaph® must wait for the next generation of StaphVAX®	[231]
S. aureus ClfA, *S. epidermidis* SdrG	INH-A21 (Veronate®)	Inhibitex Inc	Prevention of staphylococcal sepsis in very low birth weight infants	Failed phase III trial	[242, 243]
S. aureus ClfA	Aurexis® Tefibazumab (MAb T1-2)	Inhibitex Inc	Adjunctive therapy of *S. aureus* bacteremia	Completed phase II	[237]
S. aureus ABC transporter GrfA	Aurograb® (anti-MRSA monoclonal Ab)	Neutec Pharma plc	Adjunctive therapy of *S. aureus* bacteremia	Phase III	[239]
Lipoteichoic acid	Pagibaximab (BSYX-A110)	Medimmune	Prevention of *S. aureus* infection	Completed phase IIa	[244]

ABC = adenosine triphosphate-binding cassette

1.2 **Immune proteomics**

In the post-genomic era, an increasing number of studies deals with clinical proteomics. The proteome, all proteins of a cell, is far more complex than the genome [245, 246]. While most cells of an organism contain identical genomes, different cell types as well as functional states are characterized by different proteomes.

Proteome analysis by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a potent method for analyzing complete proteomes of cells, cell lines, organs and tissues. The proteins in a complex sample can be resolved by 2-D PAGE. The first dimension separates proteins according to their isoelectric point (pI), the second by their mass (Mr).

For identification, peptide mass fingerprinting is applied to the resolved proteins (proteomics). In most cases, MALDI-TOF-MS (tandem mass spectrometry), the most popular type among peptide mass fingerprinting techniques, is used in calculating probable amino acid sequences of the peptides. Besides, MALDI-TOF-MS could work as a supporting technique to detect post-translational modifications, which are important features of the proteome. High-resolution 2-D PAGE and unambiguous identification are prerequisites for reliable results [247]

The proteins resolved by 2-D PAGE can be blotted onto membranes and probed for Ab-binding by overlaying the proteins with patient sera (2-D immunoblotting). The sum of all Ags bound by Abs presents the immune proteome of a microorganism (Figure 1.1). When studying bacterial infections, the immune proteome contains candidate antigens for diagnosis or vaccination as well as targets for therapy.

So far, the 2-D PAGE technique in combination with 2-D immunoblotting has been successfully applied to the discovery of Ags from *Helicobacter pylori* [248], *Chlamydia trachomatis* [249, 250], *Borrelia garinii* [251] and *Candida albicans* [252]. In the search of novel *S. aureus* vaccine candidates, science may also benefit from 2-D immunoblotting techniques.

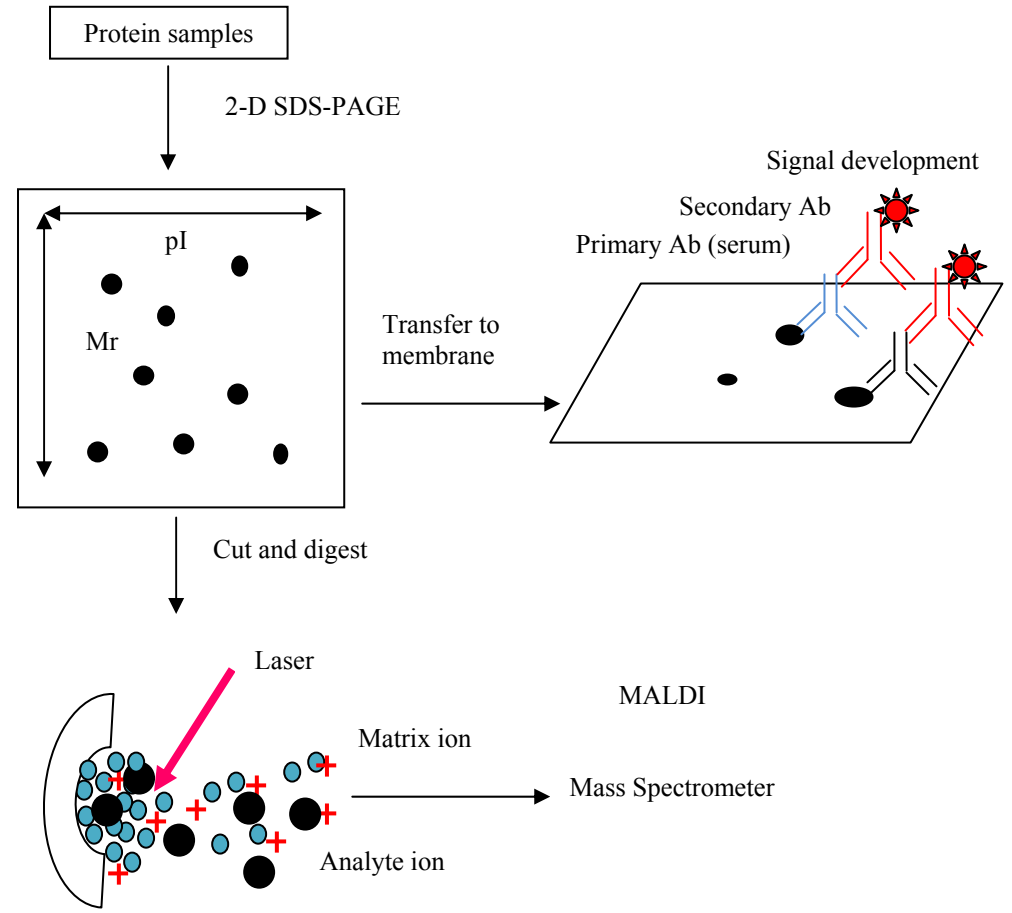


Figure 1.1: Schematic representation of 2-D immunoblotting. Protein samples were resolved on 2-D PAGE. They were transferred to membrane and recognized by specific Abs in serum. The binding was revealed by a signal developing system consisting of secondary Abs, which were linked to an enzyme (horse radish peroxidase) and its substrate. In parallel, different protein spots resolved with 2-D PAGE were identified with mass spectrometry.

: Matrix spot; : analyte; + cation

Aims of the study

Around 25% of the healthy population are persistently colonized by *S. aureus*. These *S. aureus* carriers usually remain symptom-free, but they have an increased risk of developing nosocomial *S. aureus* bacteremia with their own strain. Interestingly, in the case of bacteremia, their prognosis is much better than that of noncarriers. We propose that carriers raise an antibody response against their colonizing strain, which (partially) protects them from the complications of *S. aureus* infection. In order to test this hypothesis, first a comprehensive view of the humoral immune response to *S. aureus* in the healthy population is required.

In this study, I wanted to characterize the anti-staphylococcal antibody profiles in healthy individuals, carriers and noncarriers, and address the question whether symptom-free colonization by *S. aureus* elicits a humoral immune response. Through cooperation with the research group of Alex van Belkum (Erasmus Medical Center, Rotterdam) we had access to sera from 16 healthy individuals, who had been experimentally colonized with a *S. aureus* laboratory strain of low virulence, NCTC8325-4.

The aims of this thesis were:

1. To characterize the extracellular proteome of *S. aureus* NCTC8325-4.
2. To optimize a two-dimensional immunoblot (2-D IB) method for a comprehensive investigation of IgG binding to *S. aureus* extracellular proteins (strain NCTC8325-4)
3. To determine the anti-staphylococcal antibody profiles of carriers and noncarriers
4. To test whether experimental colonization induces changes of these antibody profiles (comparison of sera obtained before and 4 weeks after experimental colonization with *S. aureus* NCTC8325-4)

Chapter 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 *S. aureus* strains

S. aureus NCTC8325-4: provided by H. Wertheim and A. van Belkum, Erasmus university of Rotterdam. This strain was used for experimental nasal colonization of the human volunteers as described below (2.1.2). The 8325–4 strain differs from 8325 by the absence of three prophages [25]. 8325–4 has a defect in the stationary-phase sigma factor (SigB) [253]. This strain is therefore unlikely to colonize and persist as well as 8325 strain and thus facilitated a safe inoculation protocol for humans.

S. aureus NCTC8325-4 *spa* mutant (DU5875, produced by allele replacement): provided by S. Engelmann, Institute of Microbiology, EMAU Greifswald (constructed by T. Foster research group [73]).

2.1.2 Human sera

Experimental colonization: An experimental colonization was performed at the Erasmus university of Rotterdam [155]. Sixteen healthy volunteers (6 males and 10 females, median age 32 years old [range 19-51 years old]) were included in the study. Prior to enrolment, potential participants received a medical check-up and informed consent was obtained. The study protocol was approved by the Medical Ethical Committee of Erasmus MC (MEC 156.137/1996/186). Nasal, pharyngeal and rectal swabs were obtained with sterile cotton-wool swabs. Swabs were inoculated on phenol red-mannitol salt agar (PHMA) and in phenol red-mannitol salt broth (PHMB) as described earlier by Nouwen *et al.*. 2004. On day one, nasal, pharyngeal and rectal cultures were obtained. After one week, cultures of the same anatomical locations were repeated in order to differentiate between persistent and intermittent/noncarriers according to the culture rule described by Nouwen *et al.*. 2004. Decolonization treatment was started for all volunteers. Nasal mupirocin was applied twice daily for five days in combination with daily washing with chlorhexidin containing soap for five days [Hibiscrub®, Regent Medical, United Kingdom]. Five weeks after mupirocin treatment, nostrils, pharynx, and perineum samples were cultured again to define whether decolonization was successful and experimental nasal colonization could be transducted. To perform this colonization, firstly *S. aureus* 8325-4 strain was cultured until log phase in TSB, a medium that is free

from serum components of animal origin. Secondly, 10^7 colony forming units (CFU) were applied to each nostril of volunteers and finally, culture of swabs were taken at day 1, 2, 3, 4, 7, 14, 21 and 28 to observe the maintenance of the strain in volunteers' nostrils. *S. aureus* identification was confirmed by a standard agglutination test (Slide® Staph Plus, bioMérieux, Marcy l'Etoile, France). The result of the colonization experiment was described in Table 2.1.

Volunteer No.	**Carriage status**	**Negative culture since day**
12	PC[a]	4
216	PC	8
601	PC	Remain carrier
603	PC	29
606	PC	3
607	PC	22
165	NC[b]	4
211	NC	4
600	NC	29
604	NC	3
605	NC	3
608	NC	15
609	NC	15
610	NC	28
141	IC[c]	Remain carrier
602	IC	Remain carrier

Table 2.1: Features of volunteers donating sera used in the study

[a] PC: persistent carrier (6 volunteers); [b] NC: Noncarrier (8 volunteers); [c] IC: Intermittent carrier (2 volunteers)

Human sera:

Serum used in the optimization steps, Hubi 23, was obtained from a 23 year-old female carrier and has high Ab titers against SAgs of *S. aureus* (personal communication, S. Holtfreter).

Sera used in the study were obtained from the volunteers in the experimental colonization described above directly before the inoculation (pre-colonization sera) and at day 28 afterwards (post-colonization sera). Serum pool was prepared by mixing 2µl each of 32 sera (from those 16 volunteers, before and after colonization). Sera were aliquoted to avoid repeated freezing and thawing and stored at -80 °C.

2.1.3 Chemicals

Chemical	Mr (Da)	Supplier
2-Propanol (Isopropanol, $CH_3CHOHCH_3$)	60.0	Merck
3-[3-(Cholamidopropyl)dimethylammonio]-1-proanesulfonate (CHAPS, $C_{32}H_{58}N_2O_7S$)	614.9	Sigma
5-Brom-4-chlor-3-indoxylphosphate (BCIP, $C_{40}H_{30}C_{12}N_{10}O_6$)	817.7	Roth
Acetic acid (CH_3COOH)	60.05	Roth
Acetonitrile (ACN)		Merck
Acrylamide 4K solution (40%) Mix 37.5:1		AppliChem
Agarose		Invitrogen
Alkaline phosphatase-conjugated AffiniPure Goat Anti-Human IgG (H+L)		Jackson Immunoresearch
Ammonium bicarbonate (NH_4HCO_3)	79.06	Roth
Ammonium persulphate (APS, $H_8N_2O_8S_2$)	228.2	Amersham Biosciences
Ammonium sulphate ($(NH_4)_2SO_4$)	132.14	Merck
Bovine serum albumin (BSA)		Sigma
Brommophenol Blue (sodium salt, $C_{19}H_9Br_4O_5SNa$)	691.94	Sigma
Coomassie brilliant blue G250 ($C_{47}H_{48}N_3NaO_7S_2$)	854.0	Merck
Cy2, Cy3, Cy5 dyes for DIGE		GE Healthcare
DeStreak™ Rehydration Solution (HED, hydroxyethyl disulfide)		Amersham Biosciences
Dimethylformamide (DMF, $(CH_3)_2NC(O)H$)	73.09	Roth
Disodium hydrogen phosphate (Na_2HPO_4)	141.96	Sigma
Dithiothreitol (DTT, $C_4H_{10}O_2S_2$)	154.24	Amersham Biosciences
DryStrip Cover Fluid (Mineral oil)		Amersham Biosciences
Ethanol (C_2H_5OH)	46.07	Merck
Formaldehyde 37% (HCHO)	30.03	Roth
Glycerol 87% ($C_3H_8O_3$)	92.09	Amersham Biosciences
Glycine ($C_2H_5NO_2$)	75.07	Roth

Hydrochloric acid (HCl)	36.5	Roth
Immobilon Polyvinylidene fluoride (PVDF) transfer membrane		Millipore
Iodoacetamide (IAA, ICH_2COOH)	186.0	Sigma
IPG (immobilized pH gradients) strips 4-7 (7/24 cm), 3-10 (7/24 cm), 6-11 (7/18 cm)		GE Healthcare
LB (Luria-Bertani) Broth base (Lennox L Brothbase)		Invitrogen
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	203.30	Roth
Methanol (CH_3OH)	32.0	Merck
Milk powder (blotting grade)		Roth
Nitro blue tetrazolium, chloride (NBT, $C_8H_6NO_4BrClP$ x C_7H_9N)	433.6	Roth
Pelikan Ink 4001 (Fountain pen Ink)		Pelikan
Peroxidase-conjugated AffiniPure Goat Anti-Human IgG (H+L)		Jackson Immunoresearch
Pharmalyte 3-10, 8-10.5 for IEF		Amersham Biosciences
Phosphoric acid (H_3PO_4)	98.0	Roth
Potassium chloride (KCl)	74.55	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	Roth
Silver nitrate ($AgNO_3$)	169.87	AppliChem
Sodium carbonate (Na_2CO_3)	105.99	Merck
Sodium chloride (NaCl)	58.44	Roth
Sodium dihydrogen phosphate (NaH_2PO_4)	119.98	Sigma
Sodium dodecyl sulphate (SDS, $CH_3(CH_2)_{11}OSO_3Na$)	288.38	Merck
Sodium hydroxide (NaOH)	40.00	Roth
Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$)	248.17	Roth
Tetramethylethylenediamine (TEMED, $C_6H_{16}N_2$)	116.21	Amersham Biosciences
Thiourea (Thiocarbamide, CH_4N_2S)	76.12	Sigma
Trifluoroacetic acid (TFA)		Merck
Tris (hydroxymethyl)aminomethane (TRIS-base, $C_4H_{11}NO_3$)	121.1	Merck
Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl, $C_4H_{11}NO_3$*HCl)	157.6	Merck
Tryptone soya broth (Soybean-Casein digest medium U.S.P)		Oxoid

Tween-20 ($C_{58}H_{114}O_{26}$)	1226	Sigma
Urea ($CO(NH_2)_2$)	60.06	Merck
Water (H_2O, HPLC grade)	18.01	J.T. Baker

2.1.4 Media, buffers and solutions

Medium	**Components**
LB (Luria-Bertani) liquid medium Microbiology Laboratory	2 % (w/v) LB Broth base Dissolved in distilled water Autoclaved at 121°C for 15min
TSB (Trypticase Soy Broth) medium (Soybean Casein Digest Broth) Basal medium	3% (w/v) Tryptone soya broth Dissolved in distilled water Autoclaved with autoclave pot for 15 min Features of the product: pH: 7.3±0.2 at 25 °C 1.7 % Pancreatic digest of casein (Tryptone) 0.3 % Papaic digest of soybean meal (Soya peptone) 0.5 % Sodium chloride (NaCl) 0.25 % Di-basic potassium phosphate (K_2HPO4) 0.25 % Glucose

Buffer/Solution	**Components**
12.5 % SDS separating gel solution	12.5 % (v/v) Acrylamide;375 mM Tris-HCl, pH 8.8 0.1 % (w/v) SDS; 0.05 % (w/v) APS; 0.025 % (v/v) TEMED
Alkaline phosphatase buffer (AP buffer)	100 mM Tris-HCl, pH 9.5 100 mM NaCl; 50 mM $MgCl_2$. $6H_2O$
Alkaline-phosphatase-substrate solution	AP buffer 0.76 mM NBT; 0.2 mM BCIP
Blocking solution	TBS/T 5 % (w/v) non-fat dry milk
Coomassie fixing solution	40 % (v/v) Ethanol 10 % (v/v) Acetic acid
Coomassie staining solution	0.08 % (w/v) Coomassie brilliant blue G-250 8 % (w/v) $(NH_4)_2SO_4$; 0.96 % (v/v) H_3PO_4 85%; 20 % (v/v) CH_3OH
Cup-loading solution (for strip pH 6-11)	10 % (v/v) Isopropanol; 5 % (v/v) Glycerol; 162 mM DTT; 1 % (v/v) Pharmalyte 3-10; 1 % (v/v) Pharmalyte 8.5-10.5 30 µg protein /7 cm strip or 100 µg protein /24 cm strip a trace of bromophenol blue RHB added up to 100 µl/cup

Developing solution	6 % (w/v) Na_2CO_3; 0.0004 % (w/v) $Na_2S_2O_3$. 5 H_2O 0.05 % Formaldehyde
Ink-staining solution	PBS/T 1 % (v/v) Acetic acid; 0.1 % (v/v) Pelikan Ink
PBS buffer	137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 HCl to pH 7.4
PBS/T buffer	PBS buffer 0.1 % (v/v) Tween-20
Peroxidase-substrate solution (enhanced chemiluminescent substrate, ECL)	50 % (v/v) Luminol/ Enhancer Solution 50 % (v/v) Stable Peroxide Solution (provided in the SuperSignal West Femto Maximum Sensitivity Substrate Kit, supplier: Pierce)
Primary-Ab-dilution solution	TBS/T 5 % (w/v) BSA
Rehydration buffer (RHB)	8 M Urea; 2 M Thiourea 33 mM Chaps
Rehydration solution (for strip pH 3-10)	1.2 % (v/v) DeStreak; 2 % (v/v) Pharmalyte 3-10 a trace of bromophenol blue 30 µg protein /7cm strip or 100 µg protein /24 cm strip RHB added up to 125 µl/7cm strip or 450 µl/24 cm strip
Rehydration solution (for strip pH 4-7)	28 mM DTT; 2 % Pharmalyte 3-10 a trace of bromophenol blue 30 µg protein /7 cm strip or 100 µg protein /24 cm strip RHB added up to 125 µl/7 cm strip or 450 µl/24 cm strip
Rehydration solution (for strip pH 6-11)	10 % (v/v) Isopropanol; 5 % (v/v) Glycerol; 162 mM DTT 1 % (v/v) Pharmalyte 3-10; 1 % (v/v) Pharmalyte 8.5-10.5 a trace of bromophenol blue RHB added up to 125 µl/7 cm strip or 450 µl/24 cm strip
SDS Equilibration (SE) solution (solution A and B)	6 M Urea; 375 mM Tris-HCl, pH8.8 20 % (v/v) Glycerol; 4 % (v/v) SDS Solution A: SE solution with 65 mM DTT Solution B: SE solution with 135 mM IAA, a trace of bromophenol blue
SDS running buffer	19 mM Tris-HCl 192 mM Glycine; 3.5 mM SDS
SDS stacking gel solution	5 % (v/v) Acrylamide 125 mM Tris-HCl, pH 6.8 0.1 % (w/v) SDS; 0.05 % (w/v) APS; 0.07 % (v/v) TEMED
Silver fixing solution	50 % (v/v) Ethanol 12 % (v/v) Acetic acid; 0.05 % (v/v) Formaldehyde

Silver staining solution	0.2 % (w/v) $AgNO_3$ 0.075 % (v/v) Formaldehyde
TBS buffer	20 mM Tris-HCl, pH 7.6 137 mM NaCl
TBS/T buffer	TBS buffer 0.1 % (v/v) Tween-20
TE buffer (Tris-EDTA)	10 mM Tris-HCl, pH 7.5; prepared from 1 M stock of Tris-Cl (pH 7.5) 1 mM EDTA; prepared from 500 mM stock of EDTA (pH 8.0).
Transfer buffer	25 mM Tris-HCl, pH 8.5 200 mM Glycine; 20 % (v/v) Methanol

2.1.5 Equipments

Equipment	**Supplier**
4700-or 4800-proteomics-analyzer	Applied Biosystems
7-tesla Finnigan LTQ-FT mass spectrometer	Thermo Electron
Biorad-protein plus Dodeca cell	Bio-rad
Centrifuge 5840R	Eppendorf
Centrifuge /Biofuge fresco	Heraeus
Chemical hook	Wessenmann
Electrophoresis power supply-Power Pac 200, 300, 3000	Biorad
Epson Expression 1680 pro scanner	Epson
Ettan spot handling work station	GE Healthcare
Chemiluminescence scanner (LumiImager)	Boehringer Mannheim
Glas bead ribolyser	
Hera freeze (-80 °C)	Heraeus
IEF device: Multiphor Temperature stabilizer (MultiTemp III), Electrophoresis power supply-EPS 3500, EPS3501 XL	Amersham Biosciences
Laminar box HeraSafe, HS12	Heraeus
Magnetic mixer	IKA-Labortechnik
Microwave	Melissa
Mini-Protean 3 Dodeca Cell	Bio-rad
pH-Meter	Schott Instruments
Plastic foil sealing machine (Folio)	Severin
Refridgerator ultralow (-80 °C)	Sanyo
Schnelltopf (Autoclave pot)	WMF
Semi-dry blotter (MilliBlot Electroblotter II)	Millipore
Shakers (GFL3015)	Großburg wedel
Shakers for bacterial culture	Großburg wedel

(GFL 1086, 1083)	
Shakers for big bacterial culture (OSL 200)	Grant
Sonicator	Bandelin Sonopuls
Sorval RC-5B refrigerated superspeed	DuPont
Spectrophotometer Novaspec II	Pharmacia Biotech
Temperature stabilizer (Thermo Haake K20)	Haake
Tube shaker (Thermomixer comfort)	Eppendorf
UV/visible Spectrometer (Ultrospec 2100 pro)	Amersham Biosciences
Variable speed pump	Bio-rad
Varioklav Dampfsterilisatoren 16204 (Autoclave machine)	H+P Labortechnik
Vortexer GFL	Großburg wedel

2.1.6 Softwares

Software	**Supplier**
Delta2D 3.6	Decodon
Genespring 7.3	Agilent Technologies
GraphPadPrism 5.01	GraphPad Software
ImageQuant 5.2	Molecular Dynamics
LumiAnalyst	Boehringer Mannheim
Microsoft Office 2007	Microsoft
Photoshop 7.0, CS2	Adobe
Swift II Quantitation (accompanied with Ultrospec 2100 pro)	Biochrom Ltd

2.2 Methods

2.2.1 Protein isolation

Bacteria culture

Glycerol stock

S. aureus was streaked onto a LB agar plate and incubated for 24 h at 37 °C. Then, one colony was picked and cultured in LB liquid medium at 120 rpm and 37 °C in a water incubator. At OD540 0.5, the culture was stopped and glycerol stock was made (84 % v/v bacterial culture in sterile glycerol) and aliquots were stored at – 80 °C.

Pre-culture (Overnight or small culture)

Culture from glycerol stock: A glycerol stock was thawed and transferred into TSB medium (5 % v/v glycerol stock in TSB). Next, 1: 400 and 1: 400,000 dilutions of this

culture were produced and all dilutions were incubated overnight at120 rpm and 37 °C in a water incubator.

Culture from blood agar plate: bacteria picked up from blood agar plate were mixed with TSB (1 colony/ 20 ml TSB); then from this, 1:200, 1: $4x10^4$ and 1: $8x10^8$ dilutions were prepared. Finally, all dilutions were incubated overnight at120 rpm and 37 °C.

Main culture

The optical density (OD) of differently diluted pre-cultures was measured. The pre-culture reaching OD540 ~5 was then used to inoculate in a 5l-flask containing 1.5l TSB. The OD540 of the main culture was adjusted to 0.05 at the start. The culture was incubated at 37 °C, 110 rpm. The culture was measured every hour until it came to stationary phase (its OD540 reached 8 to 10).

OD measurement

The optical density (OD) was measured at wavelength of 540nm, OD540, by a spectrophotometer (Novaspec II). Before OD540 reached 0.5, samples of the culture were taken under sterile box to prevent contamination.

Protein extraction

To extract the extracellular or intracellular protein, the bacterial culture was centrifuged for 15 min, 8,000 rpm, at 4 °C.

Extraction of intracellular proteins

The bacterial pellets were resolved in TE buffer and then transferred into tubes and centrifuged for 5 min, 8,000 rpm at 4 °C. After discarding supernatant, pellets were resolved in TE buffer and transferred into micro tubes containing glass beads (diameter: 0.1 mm, Sartorius). A Ribolyser was used in the next step to destroy the cells at 6.5 m/s for 30 sec; afterwards, the tubes were centrifuged for 20 min, 15,000 rpm and supernatant was transferred into new tubes for another centrifugation for 20 min, 15,000 rpm. The final supernatant, which presents the intracellular protein in TE buffer, was transferred into a new tube for protein concentration measurement and storage.

Extraction of extracellular proteins

The supernatant was transferred into centrifuge bottles. To precipitate the proteins the supernatant was incubated over night, at 4 °C after an addition of TCA to a final concentration of 10 % then. Afterwards, it was centrifuged for 1 h, 10,000 rpm, at 4 °C. After discarding the supernatant, protein pellets were resolved in 70 % ethanol, and transferred into tubes to centrifuge for 5 min, 10,000 rpm, at 4 °C. Next, the protein pellet

was washed six to eight times in 70 % ethanol and once with 100 % ethanol and centrifuged for 5 min, 10,000 rpm, at 4 °C. After that, the pellet was air-dried for 2 hours, then resolved in RHB and centrifuged for 10 min at 13,000 rpm, RT to remove insoluble proteins. Finally, the supernatant presenting extracellular protein extract in RHB, was transferred into a new tube for protein concentration measurement and storage.

Protein quantitation: Bradford method

Firstly, a standard curve was generated by using BSA-standard solution (0.1 µg/µl). This solution is diluted to concentrations of 0-12 µg/ml as described in the following table:

Protein amount (µg)	**0 (Ref)**	**1**	**2**	**4**	**6**	**8**	**10**	**12**
BSA solution (µl)	0	10	20	40	60	80	100	120
H2O (µl)	800	790	780	760	740	720	700	680

Secondly, 200µl of Bradford reagent was added into each tube, thoroughly mixed by vortexing and then incubated for 5 min at RT. The absorbance was measured at wavelength 595 nm by using a spectrophotometer (Ultrospec 2100 pro). The standard curve was created by using software Swift II Quantitation Assay.

Finally, for the sample measurement, the extract was diluted 1:10 and measured by performing Bradford assay as triplicates. The protein concentrations of the samples were calculated using the previously generated standard curve in Swift II Quantitation Assay.

2.2.2 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

For the first dimension using IEF, the IPG dry strips were firstly rehydrated for 24 h, at 20 °C to their original thickness of 0.5 mm with an appropriate rehydration solution (see 2.1.5). To perform IEF, strips were placed vertically in a Multiphor II horizontal electrophoresis system. In case of strip pH gradient 6-11, cup-loading solution that contains proteins was cup-loaded at the anode. IEF was run at 20 °C by the different programs set up according to the types of strips (see Table 2.2). After IEF, proteins were separated by their pI on the IPG strips (first dimension).

For the second dimension, IPG strips were sequentially treated with SDS-equilibration solution A containing DTT and B containing IAA for 15 min each in order to allow the separated proteins to fully interact with SDS, reduce disulfide bonds (DTT), prevent reoxidization (IAA) and improve protein transfer from the first to the second dimension (glycerol, urea and thiourea). After equilibration, the strips were placed on top of 12.5 %, SDS-PAGE gels and fixed with 0.5 % agarose solution.

For 7 cm strips, electrophoresis was performed on a Mini Protean II Multi cell, at 20 °C, at a constant of 10 mA per gel for 30 min then 15 mA for 90 min. For 18 or 24 cm strips, electrophoresis was performed in Protean Dodeca cell (Bio-rad), at 20 °C, at a constant of 0.5 W per gel for 1 h then 2 W for 14 hours. After electrophoresis, proteins were separated by their Mr on the gel (second dimension).

To visualize protein spots, gels were stained with silver or Coomassie staining.

To perform immunoblotting, gels were transferred to PVDF membranes.

Table 2.2: IEF programs for different IPG strips

Program for 7 cm IPG strip pH gradient 6-11, 3-10 (ca. 2:30h)

	V			Vh	Time
Step 1	200V	2mA	5W	1	00:01
Step 2	3500V	2mA	5W	2800	01:30
Step 3	3500V	2mA	5W	3700	01:05
Total				6500	02:35

Program for 7 cm IPG strip pH gradient 4-7 (ca. 3h)

	V			Vh	Time
Step 1	200V	2mA	5W	1	00:01
Step 2	3500V	2mA	5W	2800	01:30
Step 3	3500V	2mA	5W	5200	01:30
Total				8000	03:00

Program for 24cm Strip pH 4-7 (ca. 17:50h):

	V			Vh	Time
Step 1	500V	2mA	5W	1	00:01
Step 2	3500V	2mA	5W	3000	01:30
Step 3	3500V	2mA	5W	57000	16:20
Total				60000	17:50

Program for 18 cm Strip pH 6-11 (ca. 8h)

	V			Vh	Time
Step 1	500V	2mA	5W	1	00:01
Step 2	3500V	2mA	5W	3000	01:30
Step 3	3500V	2mA	5W	22000	06:20
Total				25000	07:50

2.2.3 Visualization of proteins on gels

Colloidal Coomassie staining

After electrophoresis, gels were fixed in Coomassie fixing solution for 1 h, and then washed in distilled water for 10 min twice. Subsequently, gels were stained overnight in

Coomassie staining solution. Finally, gels were destained with 20 % methanol and washed in distilled water. Staining and destaining were performed at RT while shaking. Gels were scanned by an Epson Expression 1680 pro scanner with a resolution of 300 dpi.

Silver staining

After electrophoresis, gels were fixed in silver fixing solution for at least 1 h, and then washed in 50 % ethanol twice, 20 min each time. Subsequently, gels were sensitized in 0.02 % $Na_2S_2O_3.5\ H_2O$ for 1 min and then washed 3 times with distilled water, 20 sec each time. After that, gels were incubated in silver staining solution for 20 min. Before being developed in developing solution in 1 to 3 min, gels were washed twice with water, 20 sec each time. The developing reaction was stopped by 1 % glycine for 10 to 30 min. Finally, gels were washed in distilled water. Staining and destaining were performed at RT while shaking. Gels were scanned by Epson Expression 1680 pro scanner with a resolution of 300 dpi.

DIGE staining

Preparation of CyDye working solution: The frozen CyDye was kept for 5 min at RT. After that, a defined volume of DMF was added to the CyDye to give a stock solution of 1 mM, vortexed vigorously for 30 seconds and then centrifuged for 30 seconds at 13000 rpm. The CyDye stock solution was used or stored at -20 °C. It was taken care that the DMF used to reconstitute CyDye was of high quality and has not been open for longer than 3 months because breakdown products of DMF include amines, which will compete with the protein for the CyDye labeling. The CyDye working solution was prepared by mixing one volume of CyDye with 1.5 volumes of DMF (40 % stock CyDye solution (1 mM) and 60 % DMF).

In order to minimize experimental variation between samples, all samples should first be adjusted to the same protein concentration with lysis buffer 1 prior to performing DIGE labeling. About 350 µg of each protein sample was adjusted to a pH of 8.5 with 50 mM NaOH using pH indicator strips. After adjusting pH, the protein concentration of samples was recalculated. One hundred µg protein of each sample were mixed with 800 pmol (2 µl the CyDye working solution) of either Cy3 or Cy5. One hundred µg proteins of each sample were combined and this protein mixture was used as an internal standard that was labeled with Cy2.

The labeling reaction was carried out on ice in the dark for 30 min. The labeling reaction was stopped by adding an equivalent (CyDye working solution) volume of 10 mM lysine and left for 10 min on ice in the dark again. The samples were now ready for performing 2-D PAGE or were stored at -70 °C for up to three months in the dark. Due to the fluorescent properties, Cy2, Cy3 and Cy5 can be adversely affected by exposure to light and thus, it is recommended that the exposure to light sources is kept to a minimum.

After the labeling reaction, labeled proteins were analyzed on mini 2-D gels (BioRad). Images were acquired using a Typhoon 9400 laser scanner (GE Healthcare, Munich, Germany) at excitation/emission wave length of 532/670 nm. To correlate protein abundance with the prevalence of binding serum Abs, IBs were performed with the quantitative gels after scanning. Blots were incubated with serum pool, mixed up of pre- and post-colonization sera from all 16 volunteers. Overlays of the Cy3-stained quantitative gel and the corresponding IBs were generated in Delta2D.

2.2.4 Identification of protein by MALDI-TOF-MS

For protein identification by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS), 350 µg portions of protein extracts were separated on 24 x 20 cm 2-D gels, and the proteins were stained with Coomassie brilliant blue G-250. Stained protein spots were cut from gel by hand or using the spot cutter Proteome Work™ (GE Healthcare, Little Chalfont, United Kingdom) with a picker head of 2 mm and transferred into 96-well microtiter plates loaded with 100 µl J. T Baker water (HPLC grade) per well. Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI targets were performed automatically in an Ettan spot handling workstation (GE-Healthcare) using a modified standard protocol [254].

MALDI-TOF-MS analyzes of spotted peptide solutions were carried out on a Proteome-Analyzer 4700/ 4800 (Applied Biosystems, Foster City, CA). The spectra were recorded in a reflector mode in a mass range from 900 to 3700 Da. Automatic or manual calibration was performed as described previously [254].

After calibration, the peak lists were created using the "peak to mascot" script of the 4700 Explorer software with the following settings: mass range from 900 to 3700 Da; peak density of 15 peaks per range of 200 Da, minimal area of 100 and maximal 60 peaks per protein spot and minimal signal to noise (S/N) ratio of 15. Peptide mixtures that at least twice yielded a Mowse score of at least 64 were regarded as positive identifications. MALDI-TOF-MS analysis was performed for the three highest peaks of the TOF

spectrum. For one main spectrum 25 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point calibration if the mono-isotopic arginine (M+H)+ m/z at 175.119 or lysine (M+H)+ m/z at 147.107 reaches an S/N of at least 5. The peak lists were created using the "peak to mascot" script of the 4700 ExplorerTM software with the following settings: mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; peak density of 15 peaks per 200 Da; minimal area of 100 and maximal 65 peaks per precursor and a minimal S/N ratio of 10.

Database searches were performed using the GPS explorer software version 3.6 (build 329) with the organism specific databases. The combined MS and MS/MS peak lists were searched against a *S. aureus* 8325 protein database obtained from Entrez genome database site (http://www.ncbi.nlm.nih.gov/sites/entrez) using the Mascot search engine version 2.1.0.4 (Matrix Science, London, UK). The accession for the fasta file is NC_007795. Search parameters were as follows: trypsine digestion with one missed cleavage permitted, variable modifications (oxidation of methionine and carbamidomethylation of cysteine), mass tolerance for MS data 50 ppm, and mass tolerance precursor ions 0.6 Da. Search results that yielded a Mowse score of at least 64 ($p < 0.05$) were regarded as positive identifications.

2.2.5 Immunoblotting

To perform immunoblotting, 30 µg portions of protein extracts were separated on 7 x 8.5 cm 2-D gels. The gels were electroblotted onto PVDF membranes, which were already activated in methanol and hydrated in transfer buffer. The blotting were performed by using a semi-dry blotter (MilliBlot Electroblotter II) at 0.08 A per 7 x 8.5 cm gel/membrane for 2 h. Membranes were stained with ink-staining solution for 15 min and scanned using an Epson Expression 1680 pro scanner with a resolution of 300 dpi. After destaining in PBS/T, membranes were blocked in blocking solution for 1 h then washed in TBS/T for 5 times, 5 min each time. Next step was incubation of membranes with human serum (1:10,000 in Primary-Ab-dilution solution) overnight, at 4 °C, followed by a washing step in TBS/T. Specific binding of IgG was visualized by incubation with either alkaline phosphatase-or peroxidase-conjugated goat anti-human IgG (H+L), with dilution 1: 10,000 or 1:50,000, respectively in blocking buffer. The incubation lasted for 1 h at RT, again followed by a washing step in TBS/T and finally, signal development

was performed with alkaline-phosphatase-substrate solution or peroxidase-substrate solution, respectively.

The combination of NBT/BCIP in alkaline-phosphatase-substrate solution is an ideal system for blotting and staining applications with alkaline phosphatase. Together, they yield an intense, black purple precipitate that provides much greater sensitivity than either substrate alone. However, like other chromogenic reactions, sensitivity of this system is limited. In contrast, the peroxidase co-operated with chemiluminescence substrate (SuperSignal West Femto Maximum Sensitivity Substrate) used in this study yields the greatest sensitivity of any available detection method because the enhancers in this substrate greatly intensify the emitted light and extend the signal duration. Using chemiluminescence allows a large linear response range and multiple exposures to be performed to obtain the best image.

For alkaline-phosphatase detection system, membranes were developed in 15 min, washed in water and air-dried before being scanned with Epson Expression 1680 pro scanner. For peroxidase detection system, membranes were developed in 5 min, transferred into new plastic foils and scanned with a chemiluminescence scanner (LumiImager). Blocking, washing and incubating with first and secondary Ab were performed while shaking.

2.2.6 Evaluation of gels and blots

Spot detection and quantitation

All images obtained with the LumiImager scanner (*tiff file) were cropped in ImageQuant, saved in gel file format to retain the high resolution of images (16 bit/pixel) and then imported to the Delta2D program (Delta2D software package version 3.4; Decodon GmbH, Greifswald, Germany). Delta2D automatically adapts the image grey scale to the overall spot intensity on each IB. This strongly affects the IB image viewing, but does not affect the calculated spot intensities. Therefore, in case of presenting image data, gel files were saved as 8-bit tiff file and exported directly from ImageQuant at the same grey scale (300-20,000) to PowerPoint.

The analysis of the 2-D IB images of pH range 6-11 and 4-7 were performed in two separate Delta2D projects. In each project, the images were matched to a master IB by applying the exact warp mode. The master IB used in the analysis was a representative IB developed with the serum pool, which is composed of 32 individual sera. A fused image was obtained by fusing all images in the project with the union fuse option. Spots on the

fused gel image were automatically detected and manually validated by comparing the original gel images with the fused gel image. Subsequently, the spot map and the corresponding labels of the fused gel image were transferred to all gel images included in each project, thus ensuring uniform analysis throughout the study. Spot intensities were calculated based on the area and pixel intensities of spots. The intensity values (spot volumes) were corrected based on the local background. in grey units. The grey unit is defined as one black pixel with no background has absolute quantity 1. As the different sera differed strongly in signal intensity, we did not perform a normalization based on the total signal intensity, but used the raw data instead. In the end, the Delta2D quantitation table was exported to Excel then imported to Genespring or GraphPadPrism to analyze and compare spot number and density.

The spot map of IBs comprised 117 and 119 individual spots for pH range 6-11 and 4-7, respectively.

To reveal the proteins behind the IB spots, the reference map with identified protein spots was matched with the master-IB containing the spot map and corresponding spot labels.

Highly basic proteins (pH> 9.8) were blotted to PVDF membranes with varying efficiency. Therefore, the 29 most basic proteins were excluded from data analysis, including Atl, IsaB, IsdA. However, in case of presenting immune reactive protein spots with master IBs, they were still included.

Statistical analysis

Statistical analysis was performed with GeneSpring (PCA) and GraphPadPrism (Mann-Whitney U test). Means and medians are given with their standard deviations or interquartile range, respectively.

Master IBs:

The spot signal intensity of master IBs was quantified in a different project with 8 replicates for each pH range, 6-11 and 4-7. Significant immune reactive spots were defined by median not less than 0.5 grey unit (cut-off: 0.5 grey unit).

Pre-colonization IBs:

Since pre-colonization IBs of pH range 6-11 contained generally more strong spots than 4-7 images, 6-11 and 4-7 pre-colonization IBs were analyzed separately. Each blot was performed in 2-4 technical replicates. For each spot the mean spot intensities of technical replicates was calculated. As a global measure for serum IgG binding, we calculated the total spot intensity, i.e. the sum of spot intensities for each volunteer.

To investigate the prevalence of strong IB signals among the 16 volunteers, we set a threshold for "strong spots". This threshold was defined as the top 10% of all spots detected in both pH ranges, 6-11 and 4-7 of all IBs (cut-off: 5.6 grey units).

Nonparametric tests (Mann-Whitney U test, GraphPadPrism) were estimated for comparison of mean spot intensities of carriers and noncarriers on single spot level. p-values, two-tailed, below 0.05 were considered statistically significant. Significant spots from this U test (n=8) were subjected to a principal component analysis (PCA), performed in GeneSpring.

Ratios pre- vs. post-colonization:

To reveal colonization induced changes in the IB spot intensities, we determined the ratio of the spot intensity 28 days after colonization and before colonization. The ratios were determined for each technical replicate (2-4) and then the mean ratio was calculated.

We predefined stringent selection criteria in order to minimize the number of false positive results and defined the change in the level of an IB spot as significant only when the following three criteria were fulfilled: i) the mean intensity ratio between post- and pre-colonization had to exceed 5, ii) the intensity ratio had to exceed 2.5 in at least 2 technical replicates and iii) the difference in the spot intensities of post- and pre-colonization sera had to exceed 2x standard deviation of the spot intensity of pre-colonization sera.

Chapter 3
RESULTS

3.1 Optimization of the experimental procedures

3.1.1 Two-dimensional immunoblots (2-D IBs)

In the beginning, experiments were performed to adapt the *S. aureus* culture conditions and the 2-D IB procedures to the purpose of this study, which was to obtain comprehensive information about serum Ab binding to *S. aureus* proteins.

To harvest the extracellular and intracellular proteins of *S. aureus* 8325-4, the strain was first cultured in LB medium. However, it failed to grow to high density (stationary growth at OD540 3) in this medium, resulting in low total protein yields. Therefore, LB was replaced by TSB medium, which is a nutrient-rich medium containing high concentration of free amino acids and peptides [255]. In TSB medium, *S. aureus* grew well and both cellular and extracellular proteins were harvested from bacterial culture at late exponential phase (OD540 3.0) and early stationary growth phase (OD540 9.0) (Figure 3.1).

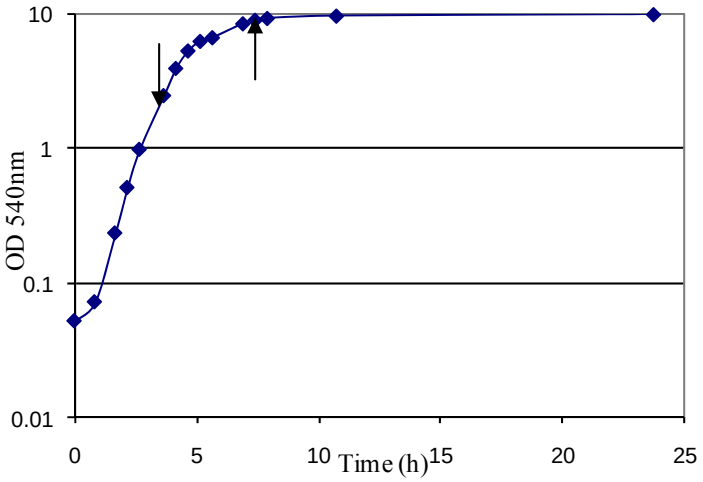


Figure 3.1: Growth curve of *S. aureus* 8325-4 in TSB medium. Bacteria were cultured in TSB. Cells and supernatants were harvested at late exponential (OD540 3.0, downward arrow) and early stationary phase (OD540 9.0, upward arrow).

Both intracellular and extracellular protein extracts from exponential and stationary growth phase were resolved on 2-D mini gels at pH gradient 4-7 and visualized by silver staining (Figure 3.2). There was no major difference between the patterns of cellular proteins harvested at late exponential (Figure 3.2A) and early stationary growth phase (Figure 3.2B). In contrast, the protein pattern of extracellular proteins harvested at the early stationary phase (Figure 3.2D) was much more complex than that from those

obtained during exponential growth (Figure 3.2C). Thus, protein extracts from the early stationary phase were chosen for further experiments.

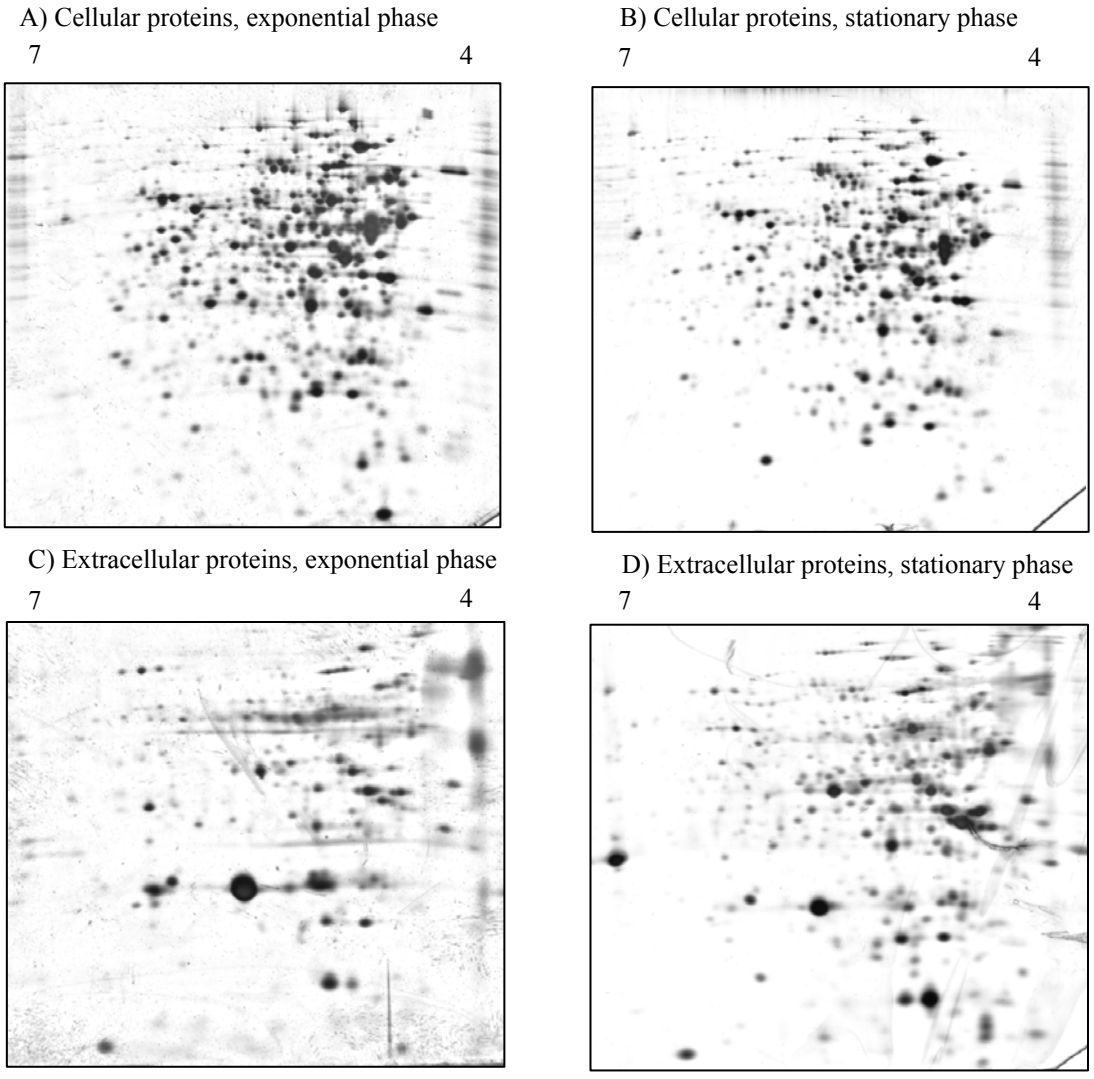


Figure 3.2: Comparison of the cellular and extracellular protein patterns of *S. aureus* 8325-4 harvested at the exponential and stationary growth phase. Cellular (A, B) and extracellular (C, D) protein extracts obtained at the exponential (A, C) and stationary growth phase (B, D) were separated by 2-D PAGE. In the stationary growth phase (D), more different proteins were secreted by *S. aureus* 8325-4 than in the exponential growth phase. Experimental conditions: IPG strips of 7 cm, pH gradient 4-7, silver staining.

To compare Ab binding to cellular and extracellular proteins, the two stationary-phase protein samples were blotted onto PVDF membranes after 2-D resolution (pH range 4-7) and incubated with a human serum. IgG binding was visualized with an anti-human IgG detection system (Figure 3.3). In spite of their higher abundance, there was less Ab binding to cellular proteins than to extracellular proteins. This was expected, because

intracellular proteins of *S. aureus* are usually not exposed to the humoral immune system while extracellular proteins are most likely to contact it in either *S. aureus* epithelial colonization and/ or infection. Furthermore, *S. aureus* releases many virulence factors such as hemolysins, proteases and SAgs into the extracellular space, especially during post-exponential growth. Therefore, to obtain the most informative IBs, we focus our investigation on serum IgG binding to these Ags.

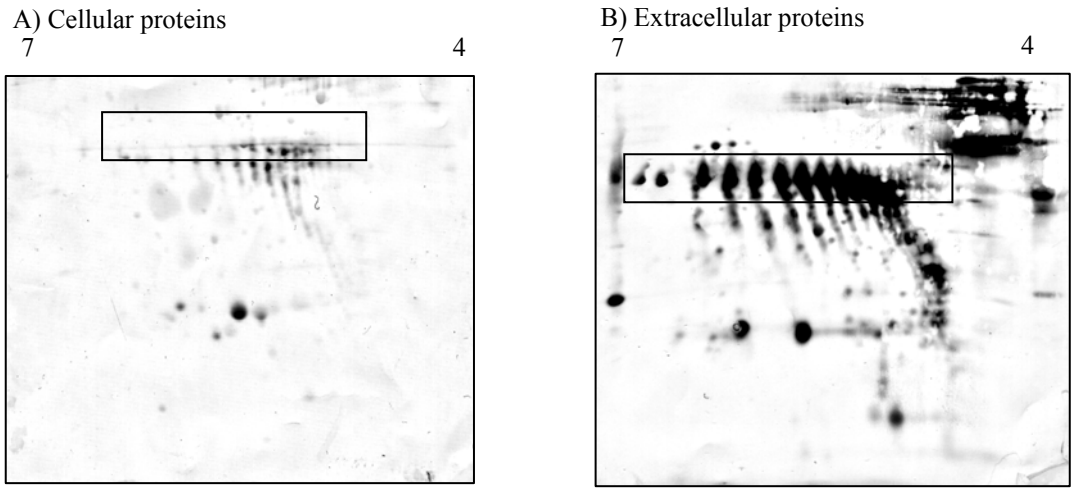


Figure 3.3: Comparison of human Ab binding to cellular and extracellular proteins of *S. aureus* 8325-4. Cellular (A) and extracellular (B) protein extracts were resolved on 2-D gels, blotted onto PVDF membranes and incubated with a human serum. Human IgG binding was then visualized with a specific detection system. There was stronger binding to the extracellular proteins. In both cases, there were strong signals, which co-localized with protein A (boxes). These were caused by protein A binding to the Fc parts of human IgG. Experimental conditions: protein extract of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 4-7, a human serum, Hubi 23, (1: 10,000), detection system alkaline phosphatase-conjugated goat anti-human IgG (H+L) and NBT/ BCIP substrate.

On both blots, strong signals were observed in the low pH range, corresponding to the localization of protein A (Spa) and its degradation products (Figure 3.3). Spa, which is a cell wall-bound protein produced by almost all *S. aureus* strains, has high affinity to the Fc part of human IgG and thus binds serum IgG irrespective of the Ag specificity of the Abs (*see* 1.1.1.1). Moreover, there were unresolved immune reactive proteins with pI above 7 (Figure 3.3). Therefore, gradients covering the alkaline pH range (pH 6-11 and 3-10) were also tested. Silver staining of extracellular protein extracts showed that a combination of pH gradients 6-11 and 4-7 resulted in better spot resolution than a single

pH range 3-10 (Figure 3.4). In addition, many more proteins that are basic were resolved at pH 6-11 (Figure 3.4B) but not in pH 3-10 (Figure 3.4A).

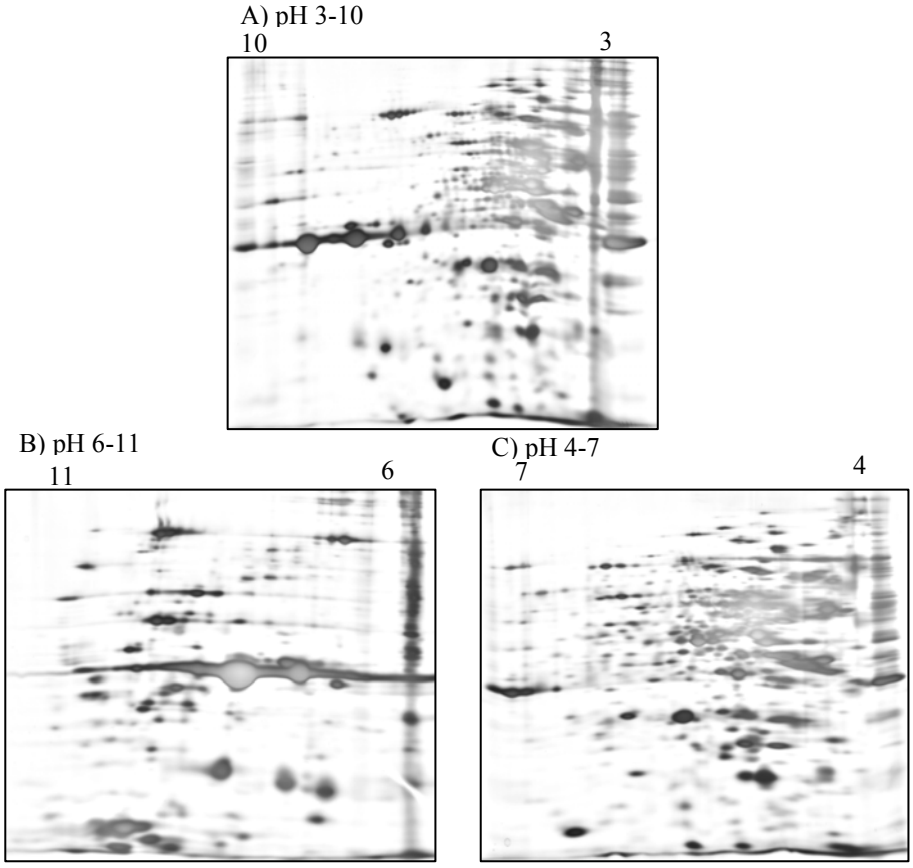


Figure 3.4: Resolution of extracellular protein patterns analyzed in different pH gradients. Proteins were separated at pH gradients 3-10 (A), 6-11 (B) and 4-7 (C). Many proteins were not separated at pH 3-10 while the combination of pH 6-11 and 4-7 provided a good resolution for most proteins. Experimental conditions: extracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, silver staining.

The detection system for human IgG initially used on the IBs was a combination of alkaline phosphatase-conjugated goat anti-human IgG polyclonal Abs with NBT/ BCIP substrate. It was later replaced by peroxidase-conjugated antihuman IgG polyclonal Abs with ECL substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) (see 2.2.5) because of the latter's superior sensitivity. Furthermore, the long-lasting chemiluminescence glow of activated ECL permits serial exposures with increasing scanning time, which guarantees measurements in the linear range of the reaction and thus spot signal quantitation.

Titration of the detection Ab (peroxidase-conjugated goat anti-human IgG) showed the best S/N ratio at a dilution of 1: 50,000, which was consequently used in all subsequent

experiments (Figure 3.5). Since the pH gradient 6-11 largely excluded Spa and its strong non-specific signal, *S. aureus* proteins were resolved in this pH gradient for the initial comparison of Ab binding patterns of different human sera (Figure 3.5). To include the human Ab profile against other acidic and neutral *S. aureus* proteins, extracellular proteins of *spa*-mutant *S. aureus* (DU5875) were applied for 2-D IB at pH range 4-7 (Figure 3.6). This *spa*-mutant *S. aureus* strain derived from strain 8325-4 by *spa* allele replacement. Thus, *S. aureus* 8325-4 Δ*spa* lost its ability to produce Spa, which affects IB at pH 4-7 (Figure 3.3).

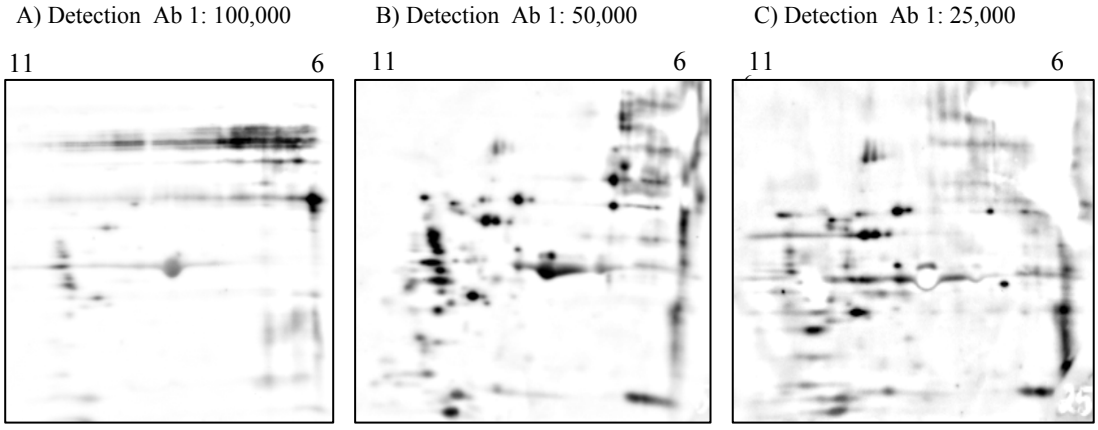


Figure 3.5: Titration of the detection Ab on 2-D IBs of extracellular proteins from *S. aureus* 8325-4. The detection Ab peroxidase-conjugated goat anti-human IgG (H+L) was titrated at dilution of 1: 100,000 (A); 1: 50,000 (B) and 1: 25,000 (C). High concentration of applied Ab leads to strong IB signals, resulting in white spots, technically known as 'ghost hallow', which are due to rapid substrate exhaustion (C). Dilution 1:50,000 that provided best S/N ratio was used in all followed experiments. Experimental conditions: extracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11, human serum Hubi 23 (1:10,000) and ECL substrate.

One frequent problem with 2-D IB is the reproducibility because this technique includes multiple steps, which could be affected by different factors. Therefore, at least two 2-D IB replicates were performed for each serum and in each replicate; the serum pool (see 2.1.2) was applied in parallel to monitor the variation for the experiment. Three representative blots of serum pool for pH gradient 6-11 and 4-7 are shown in Figure 3.6. They indicated that basic and high Mr protein spots provided variable results. This is because these basic spots are not always separated on 2-D PAGE. It is also well known that basic and high Mr proteins are transferred from gel to membrane with varying efficacy. Therefore, highly basic spots were excluded from spot quantitation and data analysis in all subsequent experiments.

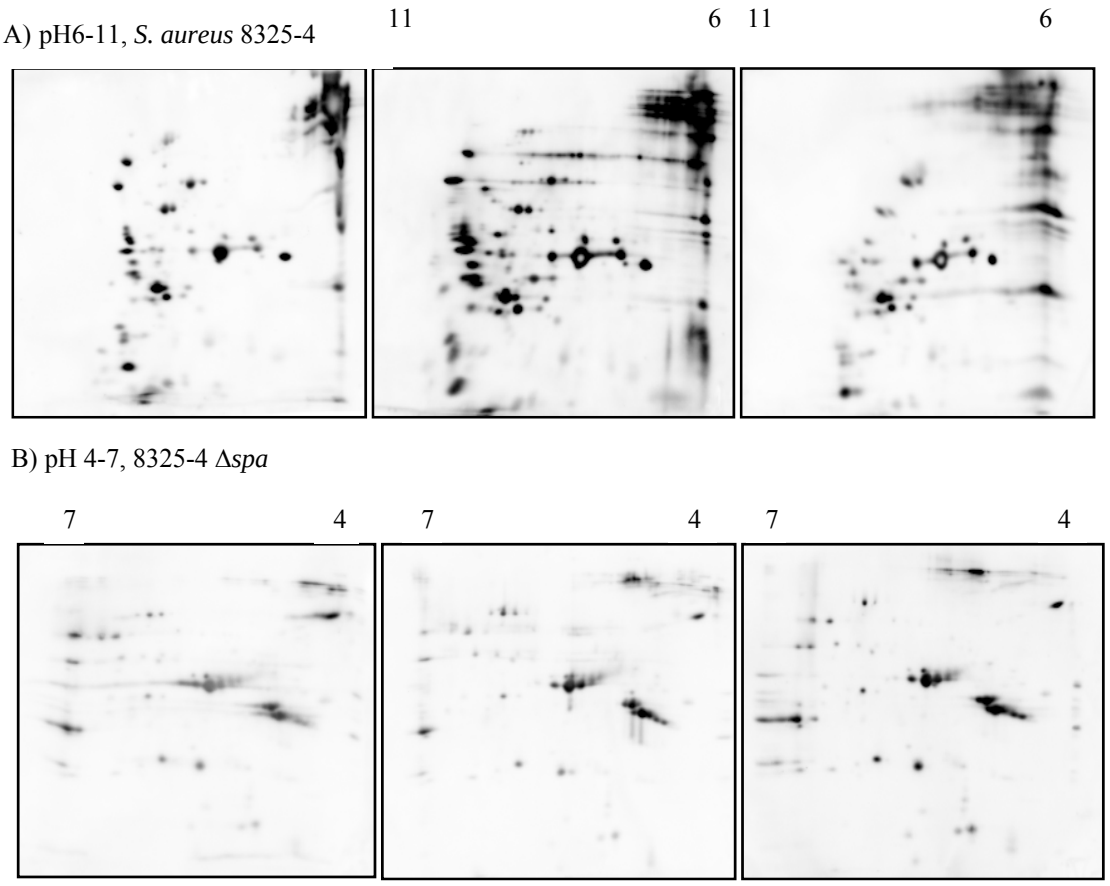


Figure 3.6: Variation among technical replicates. IBs of the serum pool in three different experiments (left to right) at pH range 6-11 (A) and 4-7 (B). Variation was seen with basic and high Mr spots. Experimental conditions: IPG strips of 7 cm, pH gradient 4-7, 6-11, extracellular proteins of *S. aureus* 8325-4 or 8325-4 Δ*spa* at stationary growth phase, serum pool (1:10,000) and ECL substrate. All IB replicates of the serum pool are documented in supplemental figures, Figure A.1.

3.1.2 Optimization of data analysis

To minimize technical variation, six IBs were processed and scanned simultaneously. They were then separately imported into Delta2D program, which permits the handling, matching and quantitation of many images. To test whether the signal intensities obtained with Delta2D were comparable to those from ImageQuant, the intensity of eight well separated spots with signals ranging from weak to very strong, were quantified with both programs. While the arbitrary intensity scales of the two programs were very different, the ratios between signal intensities were well conserved. In other words, Delta2D and ImageQuant gave comparable results (Figure 3.7)

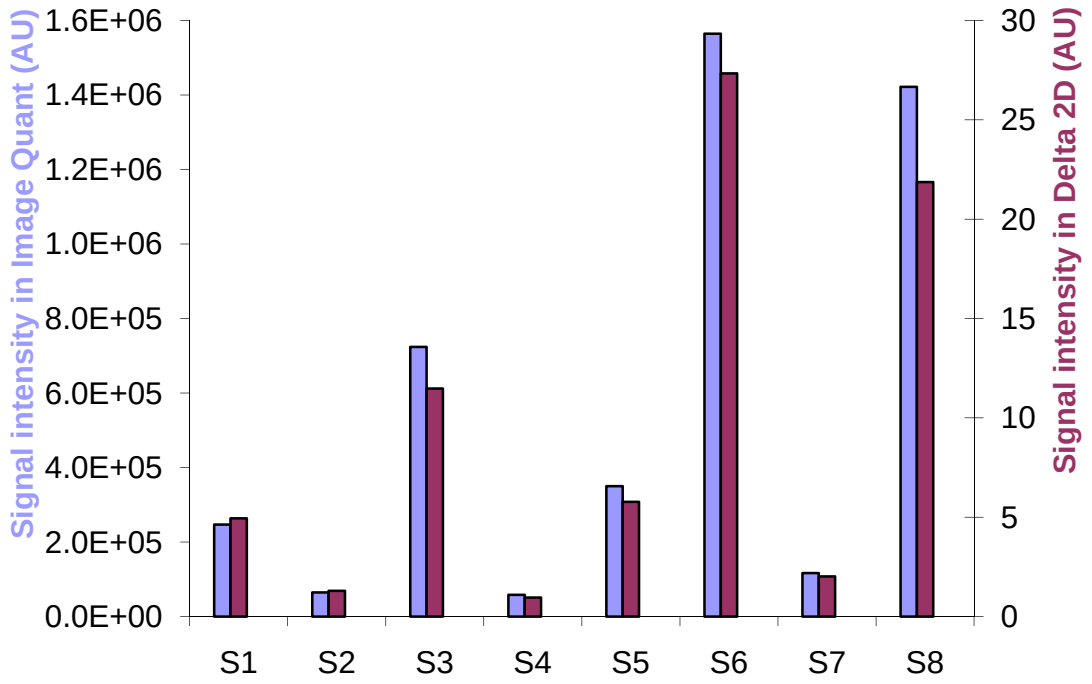


Figure 3.7: Comparison of spot quantitation using two different image-processing programs ImageQuant and Delta2D. Eight well-separated spots (S1-S8) with different intensities were quantified with the two programs. The ratios of the spot intensities obtained by the two programs were very similar. Experimental conditions: extracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11, serum pool, detection system peroxidase-conjugated goat anti-human IgG (H+L) with ECL, exposure time 1 min.

To establish the optimal exposure time, the same IB was serially scanned for time periods between one second and seven minutes after addition of ECL substrate. The signal intensities of eight well-resolved spots, giving both weak and strong signals; were then recorded to determine the linear range of the reaction. The intensities of the strong signal reached a plateau first but were almost linear up to 180 seconds. After 60 seconds, the S/N ratio and the discrimination between weak and strong spots were optimal and all signals were in the linear range (Figure 3.8). Therefore, all subsequent IB images were scanned for 60s.

Most steps of our immunoblotting have been standardized, however, variation between different technical replicates could not completely avoided (Figure 3.9). It was caused by the multifactorial nature of 2-D immunoblotting as well as the high sensitivity detection system used in our study.

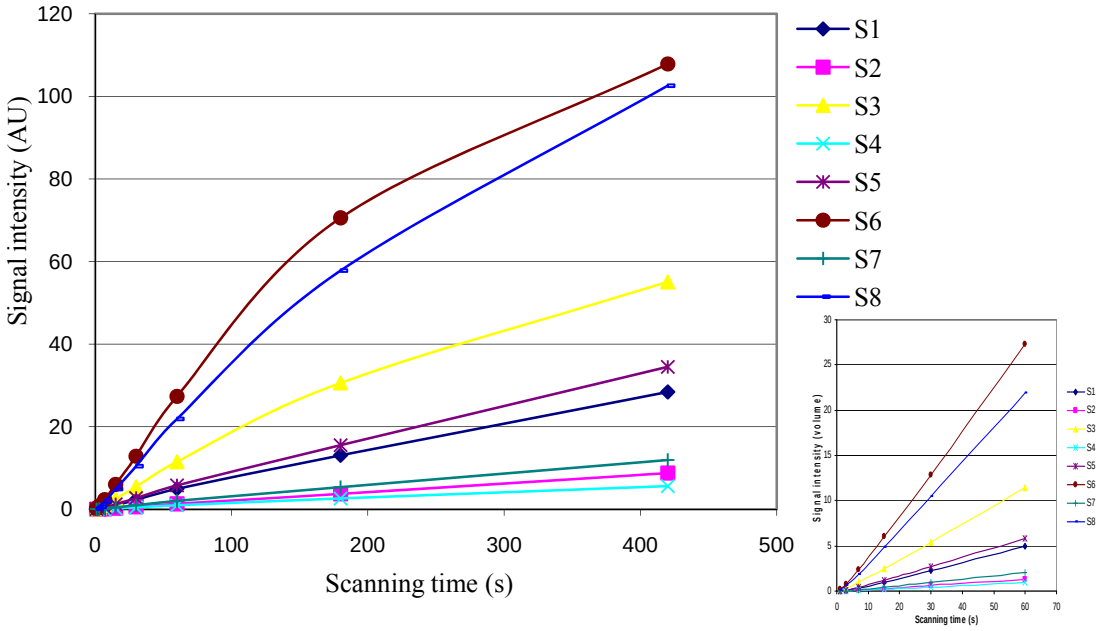


Figure 3.8: Correlation of the signal intensity with exposure time. A 2-D IB was serially scanned with a LumiImager for exposure times between 1 and 420 seconds right after addition of the ECL substrate (left panel). Small panel on the right clarified the cut off between 1 and 60 seconds. Intensity of eight well-separated spots was quantified with the Delta2D to determine the linear range of the reaction. An exposure time of 60 secs proved to be optimal because both weak and strong spots were quantified in the linear range (detail, small panel right graph). Experimental conditions: extracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11, serum pool, detection system peroxidase-conjugated goat anti-human IgG (H+L) and ECL.

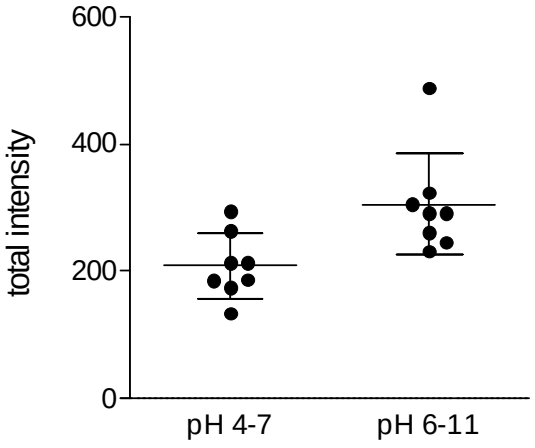


Figure 3.9: Variation in total spot intensity between technical replicates. Over the course of all IB experiments, eight blots were probed in parallel with a pool of all 32 sera applied in this study. As a measure for variation, we determined the total intensity of all identified spots for these eight technical replicates each for pH range 6-11 and 4-7. Human serum IgG reacted more frequently to basic proteins than to neutral or acidic proteins. The standard deviation was around 25 % (6-11: 305.1 ± 80.4 (mean ± SD); 4-7: 208.2 ± 50.9). IB images are documented in supplemental Figure A.1.

3.2 Reference maps of *S. aureus* 8325-4 extracellular proteins

For a comprehensive understanding of the extracellular proteins of *S. aureus* 8325-4, reference maps were produced at pH gradient 6-11 and 4-7. Extracellular proteins were resolved on 20x 24 cm 2-D gels of the pH gradient 6-11 and 4-7. All protein spots visualized by Coomassie staining were excised and digested with trypsin. The digested peptides were analyzed by MALDI-TOF-MS (see 2.2.4). To confirm the identification of protein spots, the MS analysis was repeated at least two times. The extracellular proteomes of 8325-4 at pH ranges 6-11and 4-7 are displayed in Figure 3.10 and 3.11, respectively. The protein annotation on the two reference maps was based on the annotation of *S. aureus* strain NCTC8325. However, due to the incomplete annotation of 8325, some protein names were also adapted from the comprehensive annotations of the *S. aureus* strain N315 and COL. Proteins without assigned name were labeled according to their accession numbers obtained from Entrez genome database site (see 2.2.4; http://www.ncbi.nlm.nih.gov/sites/entrez).

At pH range 6-11 there were 119 defined spots which belong to 48 proteins. 177 spots belonging to 114 proteins were observed at pH range 4-7. Some *S. aureus* proteins appeared in several spots on the reference maps (Table 3.1). This phenomenon is frequently observed on 2-D gels and is caused by protein modification, denaturation and fragmentation [30, 256-258]. This often occurs with high Mr proteins such as autolysin (Atl), aureolysin (Aur), lipase (Lip), glycerol ester hydrolase (Geh), alpha-hemolysin (Hla), V8 protease (SspA), cystein protease (SspB), staphopain thiol protease (88195808 (SspP)) and immunodominant antigen A (IsaA). Protein modifications also lead to the difference between theoretical and observed Mr and pI of identified spots on 2-D gels. This difference is also known for proteins with few or no hydrophobic residues, with modifications and/or with cross-links [259] (Table 3.1).

In few cases, more than one protein could be detected within one spot, which could further resulted in ambiguous immune reactive protein spots on 2-D IB. This uncertain identification is caused by the similarity in pI and Mr of fragments from different proteins. For example, serine protease SplF and SplD were found in one spot. Theoretically, both have pI and Mr value 9.73 and 9.68 kDa, respectively, thus it is not unexpected that they were not separated on 2-D gels (Table 3.1).

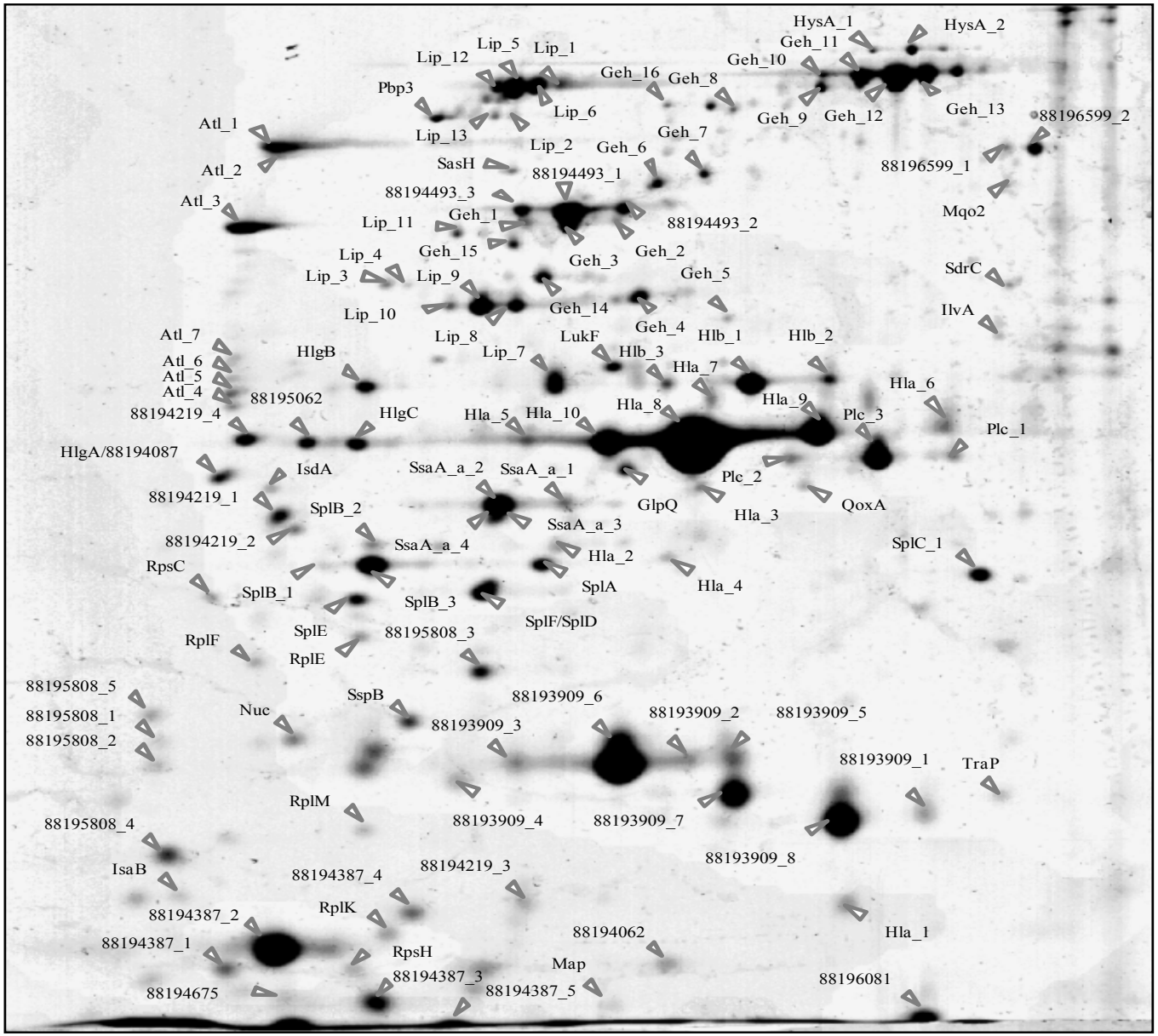


Figure 3.10: Annotated reference map of the extracellular proteome of *S. aureus* 8325-4 at pH 6-11. Protein extracts were from supernatant of bacteria grown to stationary phase in TSB medium. Proteins were obtained by TCA precipitation, separated by 2-D PAGE, stained with Coomassie and identified by MALDI-TOF-MS. A detailed list of identified protein spots is presented in supplemental Table A.2A.

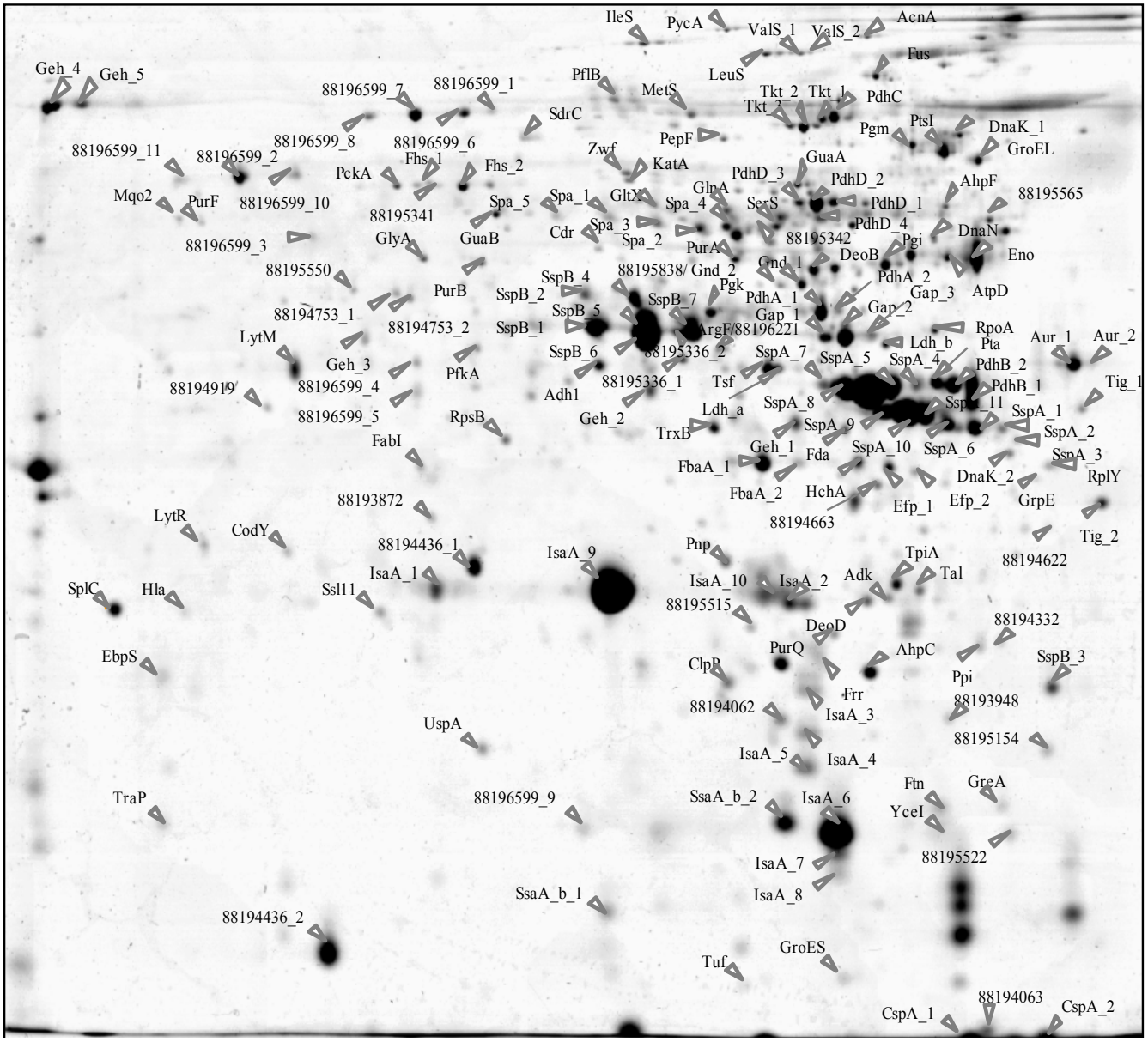


Figure 3.11: Annotated reference map of the extracellular proteome of *S. aureus* 8325-4 at pH 4-7. Protein extracts were from supernatant of bacteria grown to stationary phase in TSB medium. Proteins were obtained by TCA precipitation, separated by 2-D PAGE, stained with Coomassie and identified by MALDI-TOF-MS. A detailed list of identified protein spots is presented in supplemental Table A.2B.

Table 3.1: Multivalent identification results

	Proteins present in more than 1 spot[a]	**Spots contain more than 1 protein**
pH 6-11	15 proteins: 88193909 (8), 88194219 (4), 88194387 (5), 88194493 (3), 88196599 (2), Atl (7), Geh (16), Hla (10), Hlb (3), HysA (2), Lip (13), Plc (3), SplB (3), SsaA_a (4), 88195808 (SspP) (5).	2 spots: HlgA/88194087 SplF/SplD
pH 4-7	24 proteins: 88194436 (2), 88194753 (2), 88195336 (2), 88196599 (11), Aur (2), CspA (2), DnaK (2), Efp (2), FbaA (2), Fhs (2), Gap (3), Geh (5), Gnd (2), IsaA (10), PdhA (2), PdhB (2), PdhD (4), Spa (5), SsaA_b (2), SspA (11), SspB (7), Tkt (3), ValS (2), Tig (2).	2 spots: Gnd/88195838 ArgF/88196221

[a] The number of spots is provided in bracket

On our 2-D gels, we identified several proteins like Atl, Aur, IsaA, peptidoglycan hydrolase (LytM), Spa, secretory antigens SsaA_a and SsaA_b, which were previously described as exponential-phase proteins [257]. However, most of our proteins such as Geh, GlpQ, Hla, beta-hemolysin (Hlb), gamma-hemolysin subunits HlgA, B and C, Lip, phosphatidylinositol phosphodiesterase (Plc), SplA, SplB, SplC, SplE, SplF, SspA, SspB and 88195808 (SspP) belong to the group of post-exponential-and stationary-phase proteins [257].

Among our identified extracellular proteins, there is a big group of extracellular enzymes including proteases, such as Aur, SspA, SspB and 88195808 (SspP), SplA-F, lipases like Lip, Geh, a hyaluronidase HysA and nucleases, such as thermonuclease (Nuc) and DnaK. These enzymes play a role in degradation and/ or modification of proteins and lipids present in the growth environment [260-263]. Besides, we identified proteins engaged in the degradation and turnover of *S. aureus* cell wall like Atl, LytM, IsaA [131, 132].

Even though *S. aureus* 8325-4 is a laboratory strain and has low virulence, we still detected several pore-forming toxins such as Hla, Hlb and all 3 Hlg subunits as well as hyaluronate lyase (HysA), leukocidin subunit F (LukF) and staphylococcal superantigen-like protein 11 (Ssl11).

Several putative stress response proteins were also identified, for example, putative cold shock protein (CspA), chaperone protein HchA, catalase (KatA).

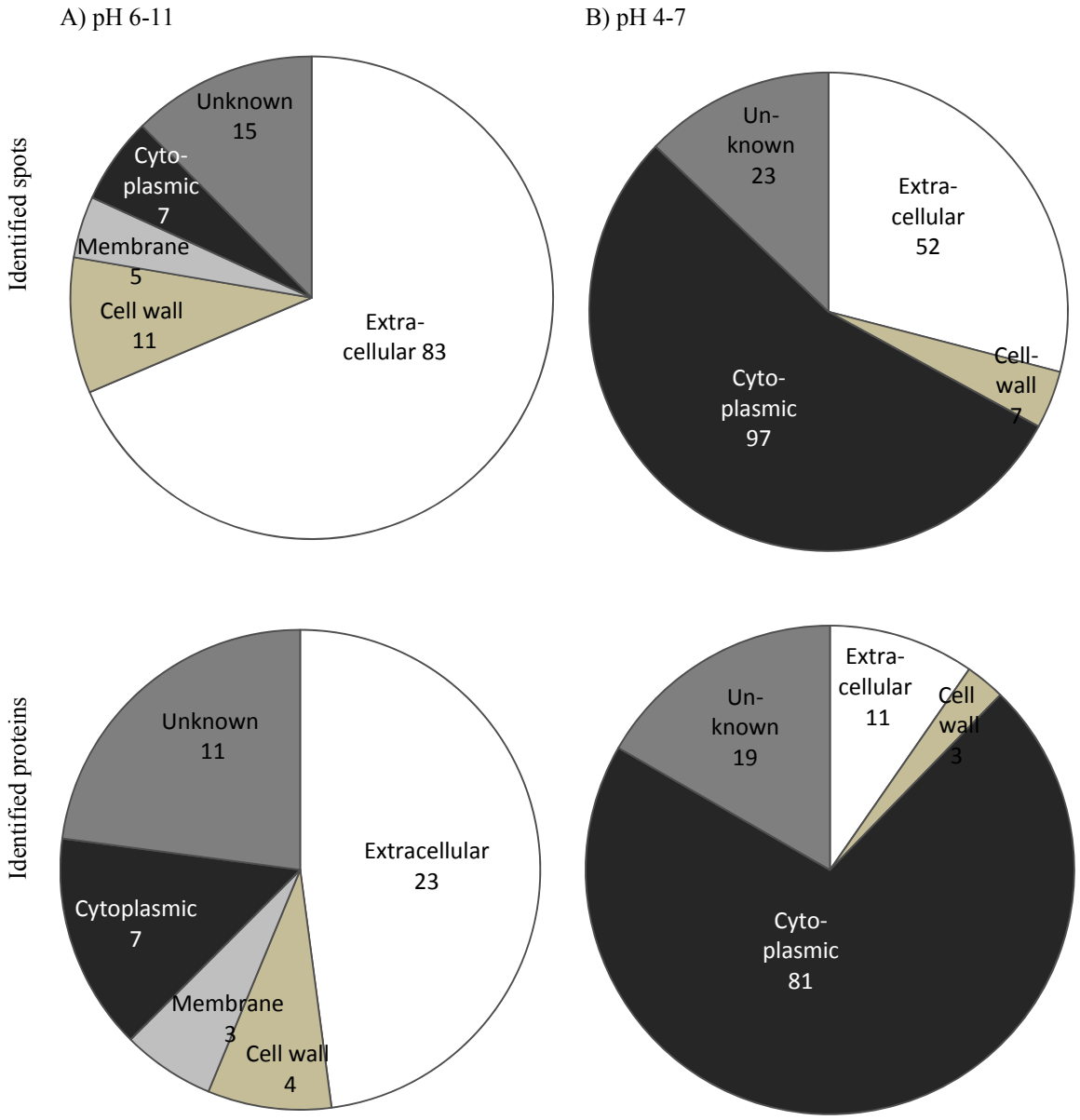


Figure 3.12: Classification of identified proteins at pH gradient 6-11 and 4-7. Identified protein spots (above) and identified proteins (below) were sorted by PSORT, a computer program, for the prediction of the cellular location of proteins based on their aminoacid sequence (http://aureusdb.biologie.uni-greifswald.de/). As some proteins especially the ones predicted to be extracellular were represented by several spots, the upper charts (of identified spots) reflect better protein patterns on 2-D gels. The number of cytoplasmic protein spots is high within pH range 4-7. Nevertheless, they were faint spots on the gels. The basic proteins were mainly extracellular proteins.

In addition to the genuine extracellular proteins, some predicted cytoplasmic proteins like ribosomal proteins (Rps-, Rpl-) or metabolism proteins (aconitate hydratase Acn1, L-lactate dehydrogenase Ldh, enolase Eno) were also identified, especially in pH range 4-7 (Figure 3.12). The appearance of these proteins in the supernatant could not be

completely avoided probably because of bacterial lysis during cell culture [30, 257, 264]. Nevertheless, they are not dominant in the protein extract (Figure 3.10, 3.11).

About 7 % of the identified proteins (10 out of 153) have unknown functions including immunodominant antigen B (IsaB) and several hypothetical proteins. The localization of these proteins was also unable to predict through their sequence, except two proteins were supposed to be intracellular protein. It will be important to determine whether some of these proteins have a role in virulence. All identified proteins of both pH ranges, 6-11 and 4-7 were categorized by function and documented in supplemental Table A.1.

3.3 Comprehensive immune proteome of *S. aureus* 8325-4 extracellular proteins

A major aim of this thesis was to describe the immune proteome of *S. aureus* 8325-4 extracellular proteins. To obtain a broad overview on the Ab profile of healthy individuals, we performed 2-D IB of the extracellular extract with a serum pool. This serum pool contained pre-and post-colonization sera from 16 volunteers including noncarriers (n=8), intermittent carriers (n=2) and persistent carriers (n=6). Figure 3.13 presents 2-D IBs of the serum pool at pH range 6-11 and 4-7.

To identify the immune reactive spots on these 2-D IBs, we produced reference maps of 8325-4 extracellular proteins as described in 3.2. Afterwards, 2-D IBs were compared to these reference maps by using the Delta2D software. Thereby, we were able to annotate the major immune reactive *S. aureus* proteins.

We have detected 117 individual immune reactive spots for IBs of pH range 6-11 and 119 spots for IBs of pH range 4-7. Among these immune reactive spots, 52 (44.4 %) and 43 (36.1 %) could be identified on pH range 6-11 and 4-7, respectively by matching the IB maps with the related reference maps. The IB maps showing the IB spot labels of all detected spots as well as corresponding proteins were provided in supplemental Figure A.4.

We have quantified the intensity of all detected spots on all IB replicates probed with serum pool (pH range, 6-11 and 4-7). Because some of the spots were low spot intensity, we determined a median intensity cut-off of 0.5 (grey units of spot volume) to define significant immune reactive spots. In this way, we obtained 66 immune reactive spots in pH range 6-11 and 38 spots in pH range 4-7 (Supplemental Table A.4). Among these 104 immune reactive spots, 72 (69.2 %) could be identified. These spots correspond to 36 proteins since several proteins were represented by more than one spot (Table 3.2).

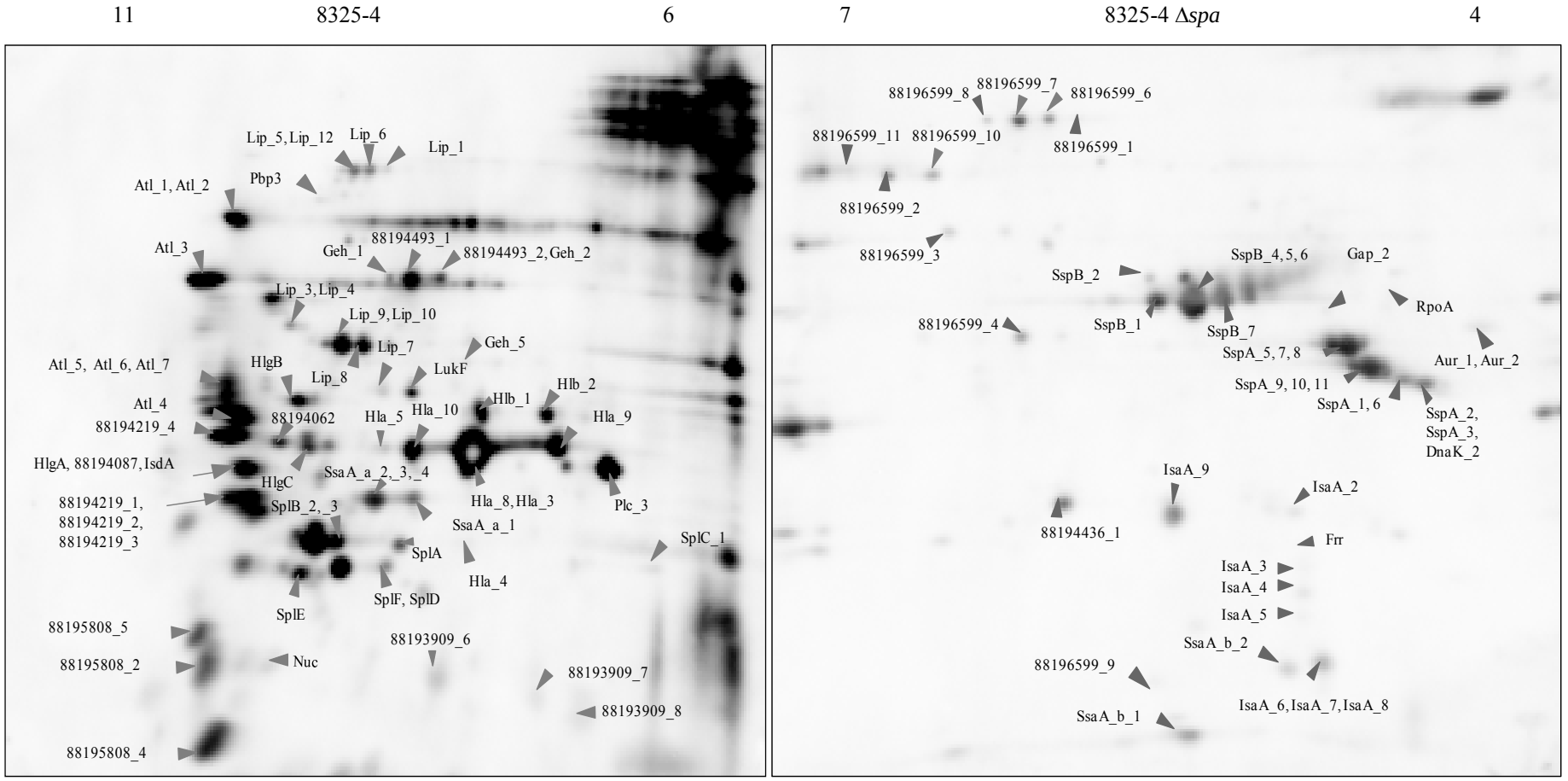


Figure 3.13: Overview of *S. aureus* immune reactive proteins. Two representative 2-D IBs of serum pool for pH ranges 6-11 (A) and 4-7 (B) are depicted. Major immune reactive proteins were indentified by comparison to the reference maps. Human serum IgG reacted to many *S. aureus* extracellular proteins, but more frequently to basic proteins than to neutral or acidic proteins. Experimental conditions: IPG strips of 7 cm, pH gradient 6-11 or 4-7, extracellular proteins of *S. aureus* 8325-4 or 8325-4 Δ*spa*, respectively, at stationary growth phase, serum pool (1:10,000) and ECL substrate.

Apparently, pH 6-11 offered more immune reactive proteins with higher immune reactivity than pH 4-7 (66 to 38 spots). Many basic proteins such as Atl, Hla, Hlg subunits, Lip, SplA, SplB, SplE, iron-regulated surface determinant protein A (IsdA) and several unidentified proteins are highly immune reactive. At pH 4-7, IsaA, Aur, SspA and SspB constitute the most dominant immune reactive proteins (Figure 3.13, Table 3.2).

Interestingly, many identified immune reactive proteins are known virulence factors, for example, Hla, Hlb, Hlg subunits, Aur, Spl proteins or are involved in biosynthesis and degradation of *S. aureus* cell wall like Atl and IsaA (Table 3.2).

Intracellular proteins were usually not immune reactive. However, we also detected a few proteins with moderate IgG binding such as DnaK protein (DnaK), glucose-6-phosphate isomerase (Pgi), phosphoglycerate kinase (Pgk) and 6-phosphogluconate dehydrogenase (Gnd) (Figure 3.13, Table 3.2).

Besides, the correlation between reference and immune proteome maps showed that different spots, which belong to one protein, differed strongly in immune reactivity for example Atl_1 to Atl_5, Hla_5 to Hla_10, Lip_8 to Lip_9, IsaA_1 to IsaA_9, and SsaA_b_1 to SsaA_b_2 (supplemental Table A.4). It is probably due to the variability in the protein quantity, and/ or the presence of different epitopes on these protein fragments (Figure 3.13, supplemental Figure A.4).

Table 3.2: Identified immune reactive proteins of *S. aureus* 8325-4 at pH range 6-11 and 4-7

Protein name	Accession No.	Full name [a]	Locali-zation [b]	pI	Mr	6-11 or 4-7 [c]
Toxins and Hemolysins [d] (6/8)[e]						
Hla	88194865	alpha-hemolysin precursor	ec	9.2	35973	both
Hlb	88195913	truncated beta-hemolysin	ec	8.2	31256	6-11
HlgA	88196348	gamma-hemolysin component A	ec	10.1	34956	6-11
HlgB	88196350	gamma-hemolysin component B	ec	9.8	36711	6-11
HlgC	88196349	gamma-hemolysin component C	ec	9.7	35614	6-11
LukF	88195914	leukocidin/hemolysin toxin family F subunit	ec	9.1	38686	6-11
Virulence/Defense mechanisms (13/18)						
Aur	88196592	aureolysin, putative	ec	4.7	54986	4-7
Geh	88196625	glycerol ester hydrolase (geh)	ec	7.7	76675	both
Lip	88194101	lipase precursor	ec	9.6	76387	6-11
Nuc	88194577	thermonuclease precursor	ec	9.7	25120	6-11
Plc	88193871	1-phosphatidylinositol phosphodiesterase precursor, putative	ec	8.6	37087	6-11
SplA	88195636	serine protease SplA	ec	9.5	25549	6-11
SplB	88195635	serine protease SplB	ec	9.7	26096	6-11
SplD	88195633	serine protease SplD	ec	9.7	25678	6-11
SplE	88195631	serine protease SplE	ec	9.8	25679	6-11
SplF	88195630	serine protease SplF, putative	ec	9.7	25655	6-11
SspA	88194745	glutamyl endopeptidase precursor, putative, V8 protease	ec	4.8	36326	4-7
SspB	88194744	cysteine protease precursor, putative	ec	5.8	44519	both
88195808	88195808	staphopain thiol proteinase (UniProt: SspP)	ec	10.1	44262	6-11
Cell wall biosynthesis and degradation (9/19)						
Atl	88194750	autolysin precursor, putative	ec	10.1	137384	6-11
IsaA	88196515	immunodominant antigen A, putative	ec	6.6	24203	4-7
IsdA	88194829	iron-regulated surface determinant protein A	cw	10.3	38745	6-11
SsaA_a	88196215	secretory antigen precursor, putative	ec	9.1	29327	6-11
SsaA_b	88196220	secretory antigen precursor SsaA, putative	unkn	6.2	17399	4-7
88193909	88193909	hypothetical protein	cw	9.5	21847	6-11
88194219	88194219	autolysin precursor, putative	ec	10.0	35836	6-11
88194436	88194436	secretory antigen SsaA-like protein	ec	6.6	28187	4-7
88196599	88196599	N-acetylmuramoyl-L-alanine amidase	ec	6.3	69253	both
Stress response proteins (1/11)						
DnaK	88195389	DnaK protein, putative	ic	4.4	66361	4-7

Energy metabolism (4/37)						
Gnd	88195316	6-phosphogluconate dehydrogenase, decarboxylating	ic	4.8	51803	4-7
Pgi	88194657	glucose-6-phosphate isomerase	ic	4.5	49822	4-7
Pgk	88194556	phosphoglycerate kinase, putative	ic	5.0	42602	4-7
88194493	88194493	putative sulfatase	ic/ m	9.5	74400	6-11
Amino acid biosynthesis (1/6)						
88195838	88195838	aminotransferase, putative	unkn	4.9	48119	4-7
Transport and binding proteins (1/2)						
88194087	88194087	5'-nucleotidase, lipoprotein e(P4) family	unkn	10.1	33352	6-11
Unknown function (1/10)						
88194062	88194062	staphyloxanthin biosynthesis protein, putative	unkn	6.3	33032	both

[a] Protein names according to the annotated S . aureus strain 8325, Col, N315 and Newman genome sequences based on the aureusDB database (http://aureusdb.biologie.uni-greifswald.de/), the Entrez database (http:// www.ncbi.nlm.nih.gov/sites/entrez) and UniProt database (http://www.uniprot.org).
SsaA were found in the public databases (see footnote a) with two similar but different sequences and are therefore numbered with _a and _b.

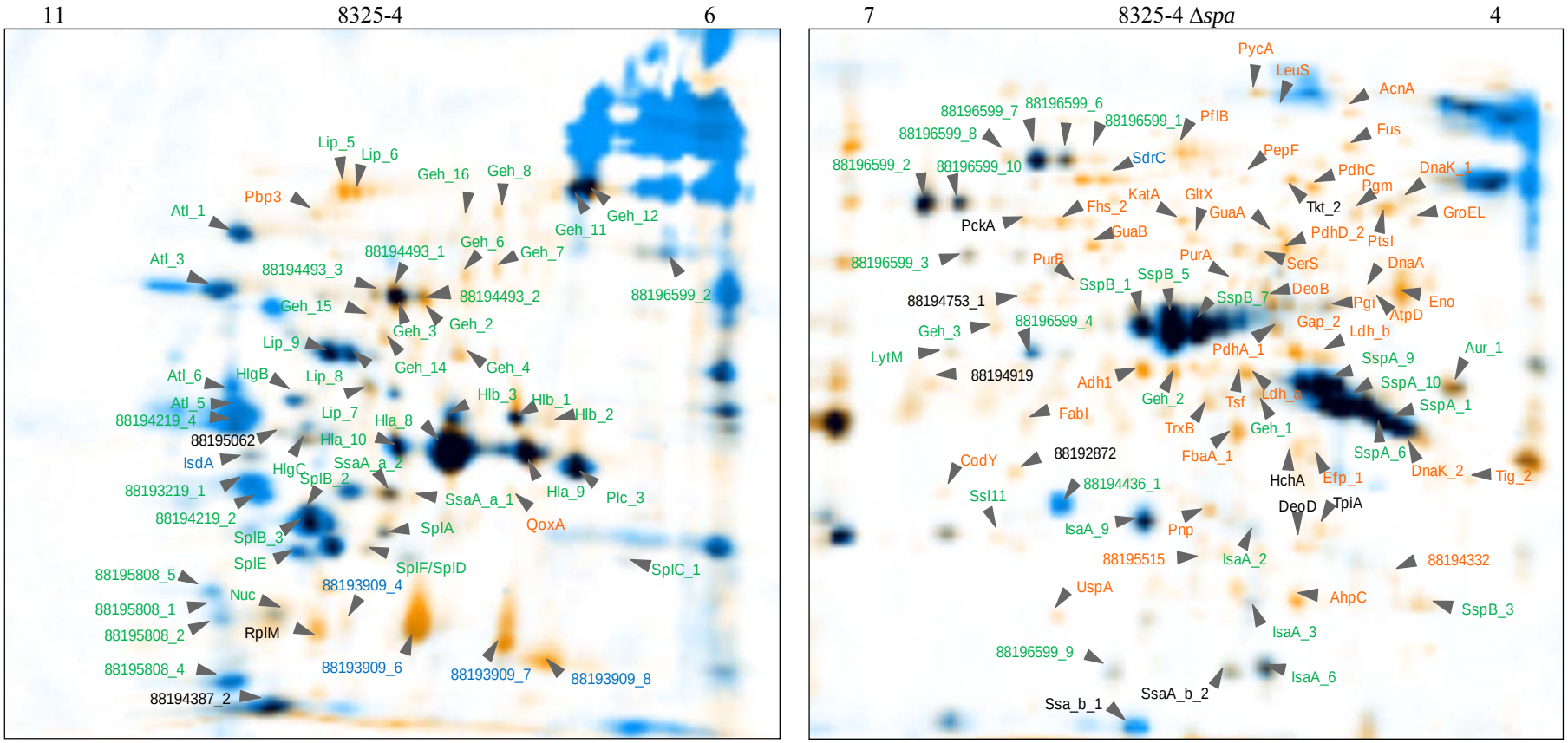
[b] Protein localization was predicted by P-SORT. ic: intracellular, ec: extracellular, m: membrane, cw: cell wall, unkn: unknown.

[c] Protein was detected on 2-D gels of pH range 4-7 or 6-11 or both.

[d] Protein classifications were adapted from Burlak *et al.* [264] and Tigr database (http://www.cmr.tigr.org).

[e] Immune reactive proteins out of total identified proteins on 2-D gels (supplemental Table A.1).

Principally, strong signals observed on 2-D blots are caused by strong Ab binding, however, the abundance of the *S. aureus* proteins also plays a role. To correlate the abundance of proteins to their immune reactivity, overlays of protein-stained gels and 2-D blots of the very same gels were produced. For protein staining on gels, DIGE was used instead of Coomassie blue or silver nitrate because it is a highly sensitive dye and it allows subsequent immunoblotting of the same gel. DIGE stains like Cy3 can detect as little as 125 pg of protein. DIGE staining is suitable for protein quantifications because it provides broad linear range of the signal intensity. In comparison, silver staining detects 1–60 ng of protein with less than a hundred-fold dynamic range (GE Healthcare).



IgG IB with serum pool/Cy3-labeled protein staining;

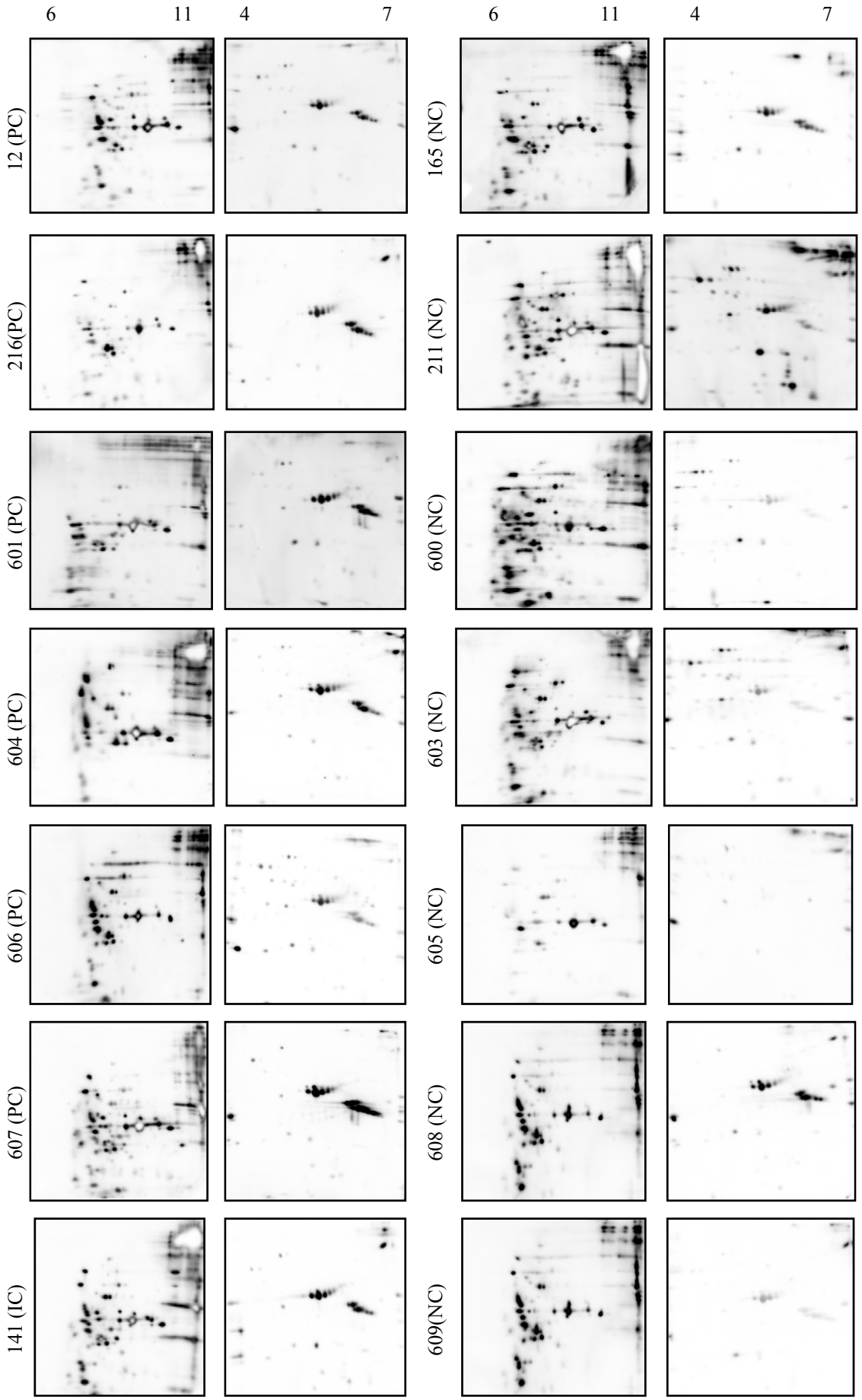
Figure 3.14: Protein abundance versus IgG binding. Overlays of DIGE stained 2-D gels (orange) and their corresponding 2-D IB (blue) were generated in Delta2D. Many *S. aureus* proteins are immune reactive, especially the highly basic proteins which induce very strong Ab binding. Many low abundant acidic spots (pH 4-7) are not recognized by serum IgG. The predicted localization of proteins are color- coded as green (extracellular), blue (cell wall), orange (intracellular) and black (unknown). Experimental conditions: IPG strips of 7 cm, pH gradients 6-11 or 4-7, Cy3-labeled extracellular proteins of *S. aureus* 8325-4 or 8325-4 Δ*spa*, respectively, at stationary growth phase, serum pool (1:10,000) and ECL substrate.

Figure 3.14 shows the overlays of DIGE staining of 2-D gels (orange) and the IB of these gels with serum pool (blue) at pH range 6-11 and 4-7. The overlays revealed proteins which are immune reactive but not so abundant (blue) and vice versa (orange), as well as proteins which are both immune reactive and abundant (both colors give a black overlay). Basic proteins seemed to be highly immune reactive and of low abundance. Consequently, some of them could not be identified (Figure 3.13, supplemental Figure A.4); Many of the low abundance orange spots especially at pH 4-7 represented cytoplasmic proteins (Adh1, Eno, Fus, PycA and TrxB). These might be the result of some bacterial lysis, which cannot be completely avoided. They become visible with the highly sensitive DIGE stain.

3.4 Inter-individual variability of baseline IgG binding to *S. aureus* Ags

Independent from the experimental colonization, we found a marked inter-individual variation in IgG binding, when we probed 2-D blots of the early stationary extracellular proteome of *S. aureus* 8325-4 with the pre-colonization sera from the 16 volunteers. Representative IBs of from all volunteers are depicted in Figure 3.15, which shows serum IgG binding to bacterial proteins resolved in the pH ranges 6-11 (left) and 4-7 (right). Replicates are documented in the supplemental figures, Figure A.2. We observed an impressive heterogeneity in IB patterns, which concerned total IgG binding, spot patterns as well as spot intensities.

The inter-individual variability first concerned total IgG binding. Figure 3.16 shows that the sums of spot intensities differed up to a factor of 20 in the pH range 4-7 and a factor of 3.6 in the pH range 6-11, where more highly immune reactive proteins were present. While serum 605 showed the weakest spot intensities for both pH range 6-11 and 4-7, strong total spot intensities were observed for serum 211. *S. aureus* carriers and noncarriers did not differ significantly in total IgG binding, but numbers are small. Moreover, total IgG binding did not correlate with the duration of experimental colonization (Figure 3.15 and 3.16).


6
11
4
7
12 (PC)
165 (NC)
216(PC)
211 (NC)
601 (PC)
600 (NC)
604 (PC)
603 (NC)
606 (PC)
605 (NC)
607 (PC)
608 (NC)
141 (IC)
609(NC)

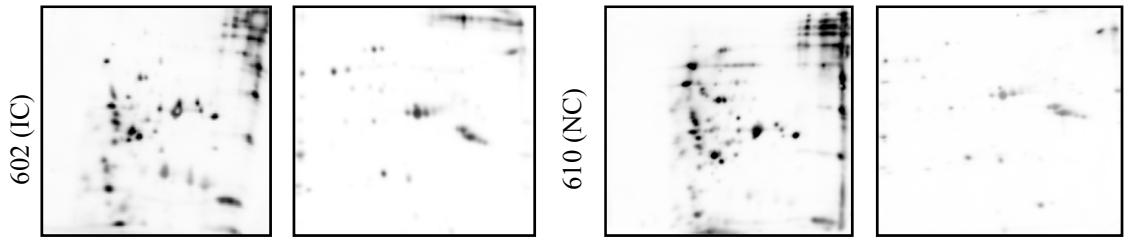


Figure 3.15: High inter-individual variation in serum IgG binding to *S. aureus* extracellular proteins in pH ranges 6-11 and 4-7. IBs were performed with pre-colonization sera from 16 volunteers. Each panel shows one representative blot out of 2-4 technical replicates. Experimental conditions: extracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11 and 4-7, volunteer serum (1:10,000) and ECL substrate. All IB replicates of pre- and post-colonization sera from 16 volunteers are documented in supplemental Figure A.2. PC: persistent carrier; IC: intermittent carrier; NC: noncarrier.

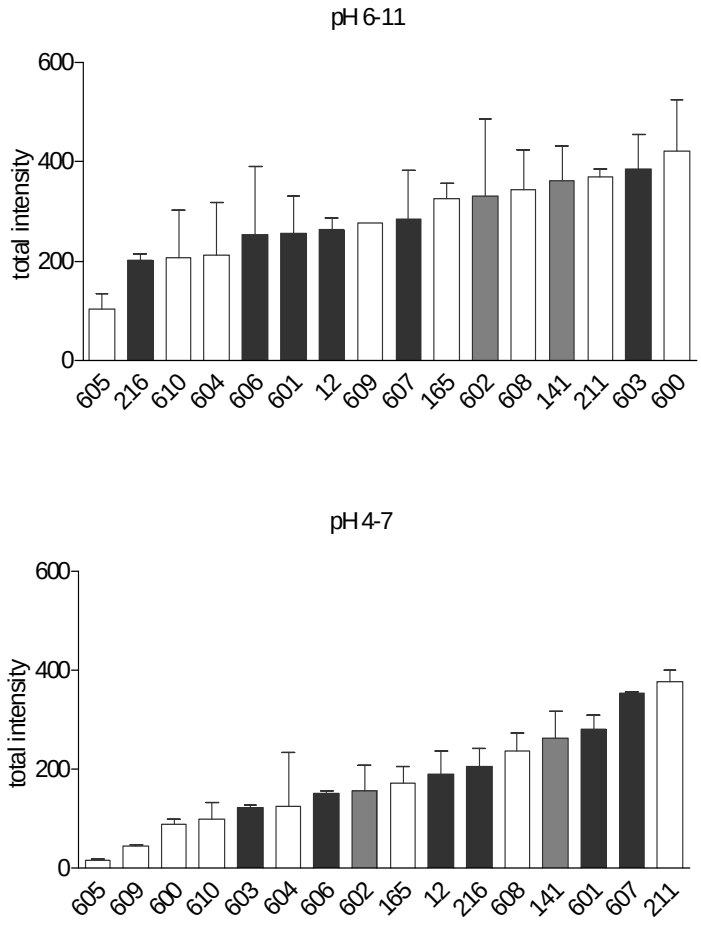


Figure 3.16: Variation of total IgG binding to *S. aureus* 8325-4 extracellular proteins in 16 healthy volunteers (before colonization). Total spot intensity of all analyzed spots was taken as a measure for global serum IgG binding. Human serum IgG reacted more frequently to basic proteins (A) than to neutral or acidic proteins (B). For the 16 volunteers total spot intensity varied by a factor of 20 for IBs with the pH range 4-7. Differences in pH range 6-11 were smaller (factor 3.6). Each value is based on between 2-4 technical replicates. Mean and standard deviation are depicted.

□: Noncarrier; ▒: Intermittent carrier; ■:Persistent carrier

Finally, individuals also differed considerably in spot intensities on single spot level. They differed in both total spot intensities and spot patterns (Figure 3.15). For example, serum 605 recognized only few staphylococcal proteins, especially in the pH range 4-7.

In contrast, serum 211 contains high titer serum IgG against a diverse range of secreted staphylococcal Ags. Additionally, it showed a remarkably strong IgG binding to immunodominant staphylococcal protein A (IsaA).

To reveal the dominant strong spots in Ab profiles of 16 analyzed individuals, we determined the 10 % strongest spots over all 16 volunteers as a measure for strong IgG binding. The 10 % cut-off (5.6 grey units) was determined simultaneously for all analyzed IBs and for both pH ranges. In Table 3.3, we provided an overview of the distribution of the 10 % strongest spots, depicted as grey squares. These spots were ranked by their frequency in the IBs of 16 volunteers (rows). Similarly, volunteers (columns) were ranked by their number of strong spots. A rank product test confirmed that this table contains all spots with a false discovery rate below 0.05 (data not shown). An annotated reference blot with the spot numbers is provided in supplemental Figure A.4.

On the top of Table 3.3 are proteins with IgG binding in many individuals. These spots represent the pattern of immune dominance or the core immune proteome of *S. aureus* 8325-4. Most proteins could be identified by matching the IBs with the protein reference maps of strain 8325-4 (Figure 3.13 and 3.14). Among the 10 % strongest spots present in at least 75% (12/16) of individuals we found many virulence factors such as Hla, SspB, Plc, SplB, SspA, and IsdA. Nevertheless, IgG binding to these immune dominant Ags differed between individuals by a factor of 2.3 (6-11_68, Plc) to 18.9 (4-7_59, SspA). The strongest spot in every individual represents Hla (6-11_59), but Hla was also the most abundant extracellular protein on the 2-D PAGE. The intensity of this spot is likely still underestimated, because in this single case spot quantification was out of the linear range. The substrate was rapidly catabolized by the HRP resulting in spots with a white center (Figure 3.5).

Finally, the lower part of Table 3.4 shows that many immune reactive spots were present in only few individuals. 28 out of the 52 strongest spots appeared in less than half of the volunteers, underlining the impressive inter-individual diversity in the adaptive immune response against *S. aureus* in the healthy population.

The rank of spot intensities was different in different volunteers. These differences in spot intensities are of relevance because the intensities were quantified with good reproducibility. Variation of total spot intensity of technical replicates was around 25 % (Figure 3.9).

Table 3.3: Proteins with strong IgG binding

Spot No.[a]	Protein name[b]	NC[c] 211	IC 141	IC 602	NC 603	PC 601	PC 12	NC 165	NC 608	NC 600	PC 607	PC 216	PC 606	PC 604	NC 609	NC 610	NC 605	volunteers with strong spots[d] No. of volunteers[e]	median	interquartile range
6-11_59	Hla_8, Hla_3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	16	40.9	37.3 - 43.9
6-11_78	SplB_2, SplB_3	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	15	31.1	27.2 - 33.7
4-7_43	SspB_4, SspB_5, SspB_6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	15	28.0	18.7 - 33.5
6-11_60	Hla_9	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	15	20.7	15.4 - 29.0
6-11_68	Plc_3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	15	18.9	17.1 - 26.1
6-11_55	Hla_10	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	14	15.1	12.9 - 22.1
6-11_87	n.i.	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	□	14	13.4	11.7 - 18.2
4-7_40	SspB_7	■	■	■	■	■	■	■	■	□	■	■	■	■	□	■	□	13	18.8	14.7 - 22.1
6-11_85	SplE	■	■	■	■	■	■	■	■	■	■	■	■	□	■	□	■	13	10.5	7.1 - 13.4
4-7_59	SspA_5, SspA_7, SspA_8	■	■	■	□	■	■	■	■	□	■	■	■	■	□	■	□	12	30.6	17.9 - 37.5
4-7_67	SspA_9, SspA_10, SspA_11	■	■	■	□	■	■	■	■	□	■	■	■	■	□	■	□	12	26.5	19.6 - 37.8
6-11_30	Lip_9, Lip_10	■	■	■	■	□	□	■	■	■	□	□	■	■	■	■	■	11	14.2	11.6 - 19.3
4-7_37	Pgk	■	■	■	□	■	■	■	■	□	■	■	■	■	□	■	□	11	9.0	6.7 - 10.7
4-7_49	SspB_1	■	■	□	□	■	■	■	■	□	■	■	■	■	□	■	□	10	8.6	6.7 - 11.8
6-11_31	Lip_8	■	■	■	■	□	■	■	■	■	□	□	■	■	■	■	□	9	9.6	6.9 - 12.4
6-11_57	HlgC	□	■	■	■	■	■	□	□	■	■	■	□	■	■	■	□	9	7.3	6.7 - 9.9
6-11_72	SsaA_a_2, SsaA_a_3, SsaA_a_4	■	■	■	■	■	□	□	□	■	■	□	■	■	□	■	□	8	12.8	7.0 - 15.6
4-7_69	SspA_1, SspA_6	□	■	■	□	■	■	■	■	■	■	□	■	■	□	■	□	8	11.5	8.3 - 13.2
4-7_38	88195838, Gnd_1, Gnd_2	■	■	□	■	□	■	■	■	■	■	■	■	□	■	□	■	8	9.6	8.6 - 10.4
6-11_49	Hlb_1	■	■	■	■	□	■	□	■	■	■	□	■	■	■	□	■	8	9.1	7.4 - 9.6
4-7_71	SspA_2, SspA_3, DnaK_2	□	■	□	□	■	■	■	■	□	■	■	■	■	□	■	□	7	13.8	9.1 - 15.1
6-11_48	HlgB	□	■	■	■	□	■	■	■	■	■	□	■	■	□	■	□	7	13.3	9.8 - 15.4
4-7_8	88196599_2	■	■	■	■	■	□	□	□	■	□	□	■	■	□	■	□	7	7.2	6.5 - 9.3
6-11_18	88194493_1	■	■	□	■	□	■	□	□	■	□	□	■	■	□	■	□	6	13.8	8.4 - 18.6
6-11_8	n.i.	□	■	■	□	□	□	■	■	■	□	□	■	■	■	□	□	6	10.2	8.1 - 11.6
6-11_51	Hlb_2	□	■	■	□	□	■	■	■	□	□	■	□	□	□	□	□	6	9.6	5.9 - 13.7
6-11_25	n.i.	□	□	■	□	■	□	■	■	■	□	□	□	□	■	□	□	6	8.0	6.4 - 9.4
4-7_89	88194436_1	□	□	■	■	■	■	■	□	■	□	□	□	□	□	□	□	6	7.9	6.6 - 9.3
6-11_46	LukF	■	■	□	□	□	■	□	□	■	□	□	□	□	□	□	□	4	8.3	7.1 - 9.5
4-7_1	88196599_7	■	□	■	■	■	□	□	□	□	□	□	□	□	□	□	□	4	7.9	6.6 - 10.5
6-11_98	88193909_6	■	□	■	■	■	□	□	□	□	□	□	□	□	□	□	□	4	7.3	6.6 - 10.4
4-7_86	IsaA_9	■	■	□	■	■	□	□	□	□	□	□	□	□	□	□	□	3	11.0	9.0 - 29.6
6-11_20	88194493_2, Geh_2	■	■	□	■	□	□	□	□	■	□	□	□	□	□	□	□	3	8.0	7.2 - 9.9
6-11_82	SplA	□	■	□	□	□	□	■	□	□	□	□	□	□	□	□	□	2	12.0	10.9 - 13.1
4-7_9	88196599_10	■	□	□	■	□	□	□	□	□	□	□	□	□	□	□	□	2	8.4	7.3 - 9.5
6-11_69	n.i.	□	□	□	□	□	□	■	■	■	□	□	□	□	□	□	□	2	7.2	7.1 - 7.3
4-7_58	88196599_4	■	□	□	■	□	□	□	□	□	□	□	□	□	□	□	□	2	6.6	6.2 - 7.0
4-7_119	IsaA_6, IsaA_7, IsaA_8	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	39.5	n.d.
4-7_117	n.i.	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	21.6	n.d.
4-7_110	SsaA_2	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	10.7	n.d.
6-11_64	Hla_5	□	□	□	□	□	□	□	□	□	□	□	□	□	■	□	□	1	9.2	n.d.
6-11_107	88193909_8	□	□	■	□	□	□	□	□	□	□	□	□	□	□	□	□	1	9.0	n.d.
4-7_107	IsaA_4	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	8.7	n.d.
4-7_91	IsaA_2	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	7.7	n.d.
6-11_2	Lip_5, Lip_12	□	□	□	□	□	□	□	□	□	□	□	□	■	□	□	□	1	7.6	n.d.
6-11_58	88194062	□	□	□	□	□	□	□	□	■	□	□	□	□	□	□	□	1	7.2	n.d.
6-11_81	Hla_4	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	7.1	n.d.
6-11_61	Hla_6	□	□	□	■	□	□	□	□	□	□	□	□	□	□	□	□	1	6.9	n.d.
6-11_12	n.i.	□	□	□	□	□	□	□	□	□	□	□	■	□	□	□	□	1	6.7	n.d.
4-7_118	IsaA_5	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	6.1	n.d.
6-11_22	Geh_1	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	1	6.1	n.d.
6-11_110	88193909_7	□	□	■	□	□	□	□	□	□	□	□	□	□	□	□	□	1	5.9	n.d.
No. of spots		35	28	27	26	24	23	23	22	21	20	18	18	15	13	11	4			

[a] As a measure for immunodominance we identified the 10% strongest spots (grey squares) on all 2-D IBs (cut-off: 5.6 grey units). Table combines the results for pH range 6-11 and 4-7. An annotated reference blot with the spot numbers is provided in supplemental Figure A.4.

[b] IB spots were ranked by their frequency in the 16 volunteers; n.i.: not identified

[c] Carrier status of the 16 volunteers is indicated. Volunteers were ranked by their number of strong spots. NC: noncarrier, IC: intermittent carrier, PC: persistent carrier.

[d] Median and interquartile range (in grey units) are provided for those volunteers, in whom the respective spot was defined as strong (cut-off: 5.6 grey units); n.d.: not determined

[e] Number of volunteers, in whom the respective spot was defined as strong (cut-off: 5.6 grey units).

3.5 Differences between carriers and noncarriers in baseline IgG binding to *S. aureus* Ags

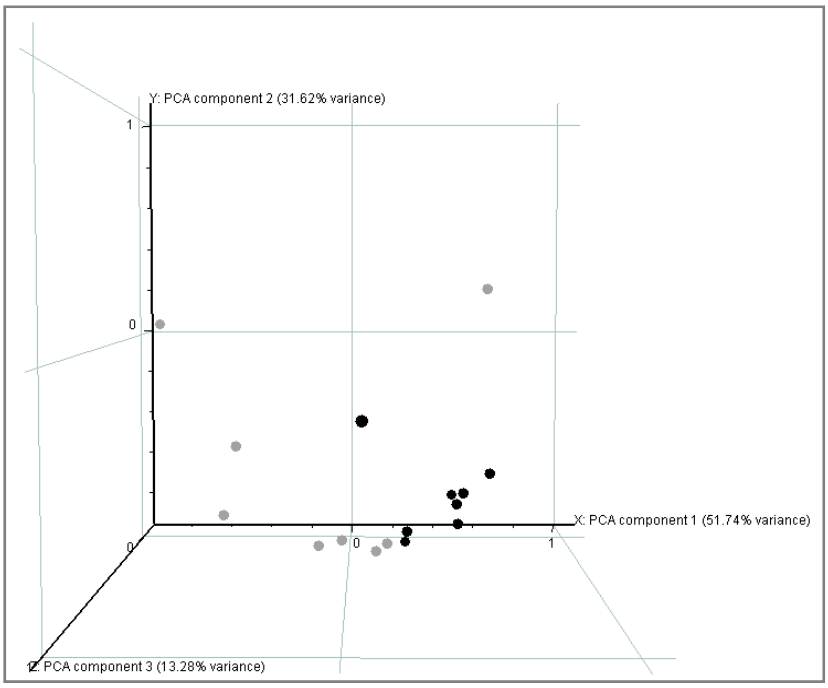
The broad inter-individual variability in baseline IgG binding to *S. aureus* Ags raised the question, whether Ab binding differed between *S. aureus* carriers and noncarriers. We compared mean spot intensities of individual spots for the group of carriers vs noncarriers. In fact, 5 spots were stronger in carriers ($P < 0.05$), but none in noncarriers (Table 3.4). All of them had pI values between 4 and 7, where variability of total spot intensity was more pronounced (Figure 3.15 and 3.16). These spots represent IgG binding to SspA, SspB, IsaA as well as to two unidentified proteins. SspA, SspB, IsaA are represented on 2-D gels by several spots, which all show the same tendency, i.e higher spot intensities in carriers compared to noncarriers.

Table 3.4: Differences in IgG binding of carriers and noncarriers before colonization

		carriers (PC + IC)		noncarriers		
Spot label	protein[a]	median	interquartile range	median	interquartile range	p-value[b]
4-7_101	n.i.	0.7	0.4 - 1.3	0.3	0.2 - 0.4	0.027
4-7_119	IsaA_6, IsaA_7, IsaA_8	1.4	1.1 - 1.9	0.5	0.4 - 0.7	0.027
4-7_49	SspB_1	8.6	6.2 - 12.9	4.3	2.6 - 6.6	0.036
4-7_69	SspA_1, SspA_6	9.6	5.6 - 12.6	2.7	0.8 - 5.5	0.046
4-7_70	n.i.	0.1	0.1 - 0.2	0.1	0.1 - 0.1	0.046

[a] only proteins that differ significantly in IgG binding between carriers and noncarriers are listed; n.i. = not identified
[b] Mann-Whitney U test

Based on differential Ab binding to these spots, a principal component analysis (PCA) was performed. PCA is a statistical tool used to reduce dimensionality of a data set to reveal the characteristics that contribute most to its variance. It keeps lower-order principal components and ignores higher-order ones. In our case, PCA showed that the carriers were more closely related to each other than the noncarriers. However, the carriers could not be clearly separated from the noncarriers (Figure 3.17).



C vs NC, p< 0.05

Figure 3.17: Principal component analysis (PCA) based on differential IgG binding to proteins within pH range 4-7. PCA was based on 10 spots, where IgG binding was stronger in carriers (persistent and intermittent carriers) than noncarriers ($P < 0.05$, Mann-Whitney-U test, see Table 3.3). Carriers (black spots) were more closely related to each other than the noncarriers (grey spots), but they could not be clearly separated from the noncarriers. The variances of the axes are X-axis 41.21 %, Y-axis 23.5 %, and Z-axis 12.52 %.

3.6 Impact of experimental colonization on the Ab response

While there were pronounced inter-individual differences in baseline Ab patterns, experimental colonization induced only moderate changes. Figure 3.18 depicts 2-D IBs of pre- and post-colonization sera from 5 representative volunteers including carriers and noncarriers. No clear difference between before and after colonization was seen in serum IgG binding to basic, neutral as well as acidic proteins. Only 19 spots from both pH ranges, 6-11 and 4-7, gained intensity by a factor of at least 5 (Table 3.5). Such moderate to strong increases in IgG binding were mainly observed among the weak spots (exceptions are 4-7_8 and 4-7_77). These spots are also low abundance proteins, which as well hampered spot identification. Additionally, colonization-induced IgG increases occurred for different spots in different individuals.

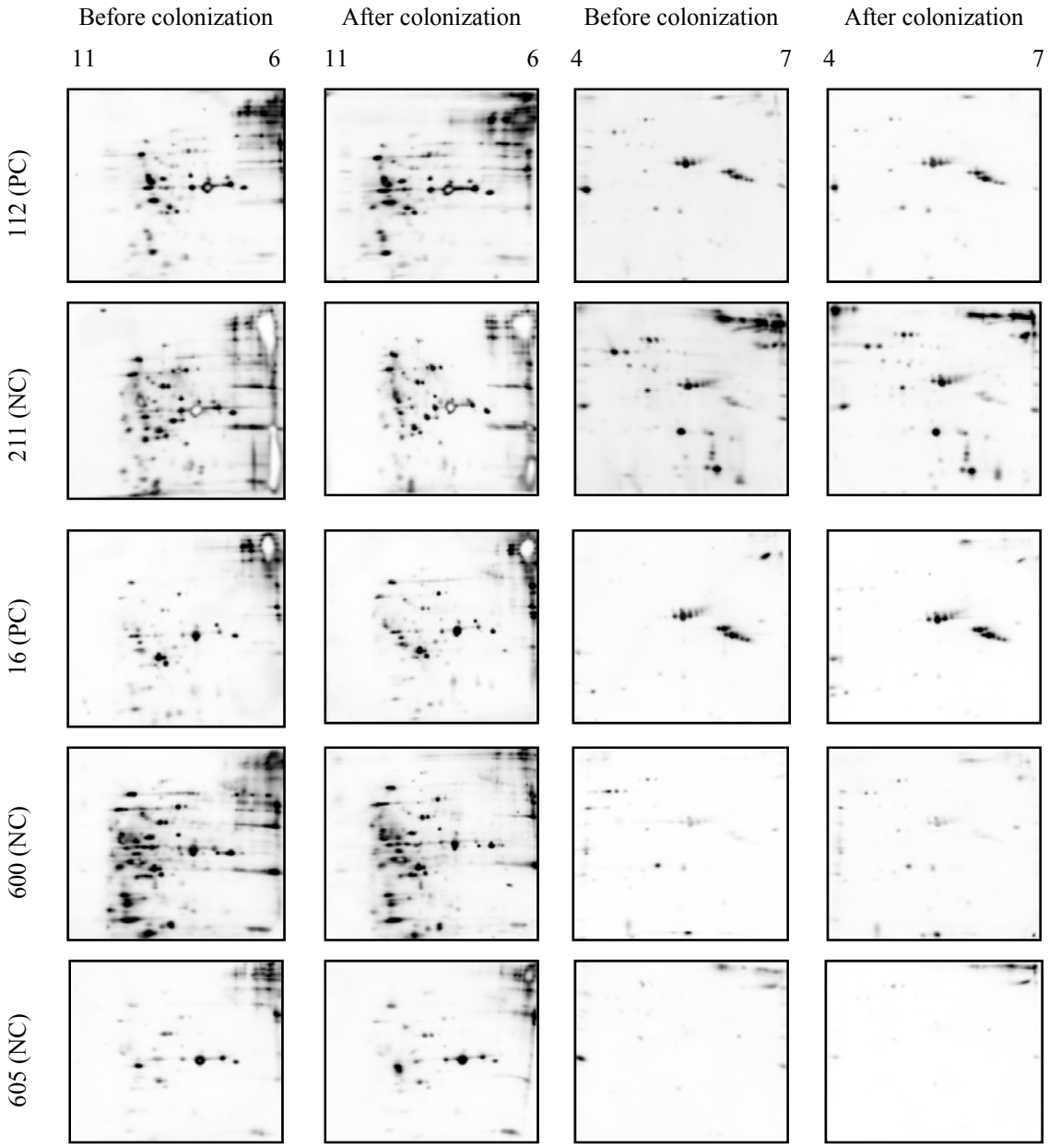


Figure 3.18: Serum IgG binding to *S. aureus* extracellular proteins before and after experimental colonization. Pre- and post-colonization sera from 5 representative volunteers: 12, 211, 216, 600, 605. Each panel shows one representative blot out of 2-4 technical replicates. There is a great variation in anti-staphylococcal IgG amount among volunteer sera but little change between pre- and post-colonization sera. Experimental conditions: extracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11 and 4-7, volunteer serum (1:10,000) and ECL substrate. All IB replicates of pre- and post-colonization sera from 16 volunteers are documented in supplemental figures, Figure A.2. PC: persistent carrier; NC: noncarrier.

Table 3.5: Spots with more than 5-fold increase after experimental colonization

spot label	protein	volunteer	carriage status	median (grey scale)[a]	Min - Max (grey scale)	spot intensity (grey scale)[b]
4-7_34	n.i.	165	NC	11.5	4.2 - 18.7	0.1
4-7_31	n.i.	165	NC	7.7	4.7 - 10.7	0.1
4-7_5	88196599_1	165	NC	6.4	3.4 - 9.3	0.1
6-11_71	n.i.	165	NC	5.9	2.1 - 11.0	0.3
6-11_15	n.i.	604	NC	8.4	7.2 - 9.6	0.1
6-11_42	n.i.	604	NC	7.4	3.2 - 11.6	0.3
4-7_8	88196599_2	608	NC	12.0	8.1 - 15.8	4.0
4-7_9	88196599_10	608	NC	8.6	3.3 - 13.8	2.3
4-7_61	n.i.	608	NC	6.6	2.6 - 10.5	0.1
6-11_21	n.i.	601	PC	7.1	3.7 - 11.6	0.9
6-11_113	n.i.	601	PC	2.7	2.6 - 17.9	0.3
4-7_77	n.i.	603	PC	5.6	5.6 - 5.7	2.6
4-7_115	SsaA_b_1	606	PC	24.8	19.4 - 30.2	0.3
4-7_3	88196599_8	606	PC	9.7	2.5 - 16.9	0.7
4-7_23	PdhD_1, PdhD_2, PdhD_3	606	PC	7.4	5.0 - 9.9	0.1
4-7_5	88196599_1	606	PC	5.9	4.3 - 7.4	0.2
4-7_88	n.i.	606	PC	5.3	4.8 - 5.7	0.1
6-11_6	Pbp3	607	PC	18.6	13.6 - 23.6	0.2
6-11_7	Lip_13	607	PC	15.4	14.9 - 16.0	0.2

[a] Median of the ratios from 2-4 technical replicates

[b] Median spot intensity of technical replicates of IBs developed with post-colonization sera

Chapter 4

DISCUSSION

S. aureus is an opportunistic pathogen and thus, has multiple encounters with a large proportion of the human population without causing serious diseases. Around one fourth of individuals are persistent nasal carriers of *S. aureus*. Nevertheless, information about the Ab responses of distinct individuals, carriers or noncarriers, to *S. aureus* is very limited.

In this study, an immune proteome approach based (combination of 2-D PAGE, MS and 2-D immunoblotting) was applied to identify *S. aureus* immune reactive proteins in the extracellular proteome of *S. aureus* strain 8325-4. Following this, we compared the anti-*S. aureus* Ab profiles from different healthy individuals, and finally, we tested whether there experimental colonization with *S. aureus* alters these Ab profiles.

4.1 The immune proteome approach for the analysis of the humoral immune response against identification of *S. aureus*

Over the years, different approaches including ELISA (Enzyme-linked immunosorbent assay), immunoblotting (Western blot), expression library and protein array have been developed for identifying staphylococcal immune reactive proteins (Table 4.1).

Table 4.1: Different approaches in studying *S. aureus* immune proteome

Approach	Ag nature	Ag recognition	Ag load	Technical complexity	Time required
ELISA	native	Ag-Ab specificity	single	simple	short
Luminex	native	Ag-Ab specificity	multiple	complex	short
1-D IB	denatured	Mr, Ag-Ab specificity	multiple	simple	short
2-D IB	denatured	Mr, pI, Ag-Ab specificity	multiple	medium	medium
Protein microarray	native	Ag-Ab specificity	multiple	complex	short
Expression library	native	Recombinant, Ag-Ab specificity	multiple	complex	long

The most frequently used approach to quantify Ab binding is ELISA, a highly versatile technique. However, this versatile method is limited by the high amount of serum needed

and its single plex approach in case of assessing Ab binding to defined Ags. A promising further development of the ELISA-approach is the Luminex system, which allows multiplexing of up to 50 Ag-specific ELISAs in a single test. Nevertheless, this technique has not yet been widely applied because of costly establishment and requirement of expensive reagents, i.e. unique fluorescence beads (http://www.luminexcorp.com /technology/index.html).

In conventional immunoblotting (1-D IB), Ags were defined as bands according to their Mr [265, 266]. Nevertheless, the 1-D IB is sometimes misleading because one band on 1-D gels often consists of many proteins.

The immune proteome method, 2-D PAGE, 2-D IB and MS, generates a comprehensive picture of immune reactive proteins from a complex mixture with moderate investments of time and effort.

With a sensitivity comparable to that of an ELISA, our 2-D blots required only a minute amount of serum for each blot (1 µl). Additionally, in 2-D IBs, Ab responses against a complex proteome (cellular, extracellular ...) are detected within a single experiment. Moreover, the analysis of Ab responses is not restricted to defined sets of Ags. In fact, we have detected more than 100 different immune reactive protein spots within the *S. aureus* extracellular protein extract. As proteomes of different strains are easily obtained, 2-D immune proteomics also facilitates the investigation of strain-specific immune responses.

The advantages of 2-D IB over expression library in screening immune reactive proteins are its relative economy and simplicity. Expression library requires multiple steps from selecting and cloning genomic fragments to transferring and expressing them *E. coli*. In order to reveal immune reactive proteins, this must be followed by serum screening and clone sequencing [61, 267, 268]. The number of immune reactive proteins identified by expression library-based method is comparable to 2-D IB. Nevertheless, 2-D IB in conjunction with MS is simpler (see 2.2.2-.5) and could identify multiple immune reactive proteins regardless of their genomic localization (either genomic or plasmid-encoded).

2-D IBs have been applied in some recent studies for screening immune reactive proteins of several pathogens including *S. aureus* [264, 269-274]. However, this is the first study to provide IBs of two pH ranges, 6-11 and 4-7, in parallel and with high resolution.

Moreover, the employment of peroxidase/ ECL detection system in our 2-D IB not only provided high sensitivity (~ 10^{-14} g of biotinylated-horseradish peroxidase, http://www.piercenet.com) but also allowed quantitation of signal intensities over a broad

dynamic range. The data were subsequently analyzed with Delta2D software, which allows comparative signal quantitation of multiple IBs. We have selected a linear range of signal intensities for both weak and strong signal IB spots, thus retained the relative signal variation among IB spots. There was only one exception: Ab binding to Hla (6-11_59) was frequently too strong resulting in substrate depletion, which was reflected by the white center within the IB spots (see Figure A.2A).

Limitations of our 2-D IB include the failure to detect low abundant proteins on the reference maps. This hampers identification of some of the immune reactive proteins, because IBs were far more sensitive than protein staining and subsequent mass spectrometry. Additionally, Ab-Ag binding in 2-D IB does not cover conformational epitopes because resolved proteins were generally denatured. Besides, this approach is limited by the number of proteins expressed by *S. aureus* under *in vitro* growth conditions [275]. Finally, since the Ags are present in varying amounts in the protein preparations, Ab binding on the 2-D IB can not be translated into Ab titers (as in ELISAs).

Membrane and cell wall proteins, which are potential immune reactive proteins of high clinical relevance, were not extensively studied since many of them are hydrophobic, basic and hardly soluble. These proteins remain a challenge not only for 2-D PAGE but also for liquid chromatography (LC)-based proteomic approaches. In this case, Luminex would be a suitable choice.

4.2 The extracellular proteome of *S. aureus* 8325-4

In general, *S. aureus* extracellular proteins can be divided into at least two groups based on their growth-phase dependent expression: (i) proteins expressed and secreted by *S. aureus* during exponential and post-exponential growth phases and (ii) proteins only expressed during post-exponential and stationary growth phases [257]. To reveal as many extracellular proteins as possible, we obtained our protein extracts from supernatants of *S. aureus* culture in the post-exponential growth phase, which contained proteins of both groups (see 3.1.1).

A limited number of proteome-based studies have investigated extracellular proteins of *S. aureus*, typically using virulent strains like COL, MW2, LAC, RN6390 and several clinical MRSA isolates [30, 256-258, 264]. Many extracellular proteins were reported to promote pathogenesis, including proteases [260, 262, 263], enterotoxins and exotoxins [79, 276], leukotoxins and hemolysins [80]. Our analysis of *S. aureus* 8325-4, a

laboratory strain with low virulence, revealed proteins playing a role in the growth of *S. aureus* (Aur, Atl, IsdA, Geh, Lip and Nuc) as well as proteins involved in pathogenesis (Hla, Hlb, Hlg subunits, HysA, LukF, Spa, Spl proteins, SspA, SspB and 2 and Ssl11) in the extracellular proteome.

It should be taken into account that proteins, which are important for *S. aureus* growth, could also play a role in its virulence and pathogenicity. For example, Lip and Geh degrade lipids, likely from host cells, which help *S. aureus* to acquire nutrients and probably damage host tissue [277]. IsdA that is required for the hemin uptake of *S. aureus* can also act as a protease inhibitor and protect *S. aureus* against the bactericidal activity of apolactoferrin [278].

The cell wall-attached Spa sequesters host Abs [62, 64, 69]. SspA, an extracellular protease, effectively cleaves Fnbp and Spa [260, 263]. Aur is responsible for cleavage of ClfB [279]. Proteases like Aur and SspA, were suggested to downregulate the virulence of *S. aureus* by degradation of toxins.

The cystein protease SspB (StpB), in contrast, is believed to have an important role in *S. aureus* virulence [150]. SspB directly cleaves kininogen and works in concert with ScpA (StpA) to promote vascular leakage and lower the blood pressure [261, 262]. HysA, which is capable of degrading the acidic mucopolysaccharide hyaluronic acid, a major component of the intercellular ground substance of human and animal connective tissue, was suggested to be an important virulence factor in early stages of subcutaneous infections [280]. Ssl11, one of 14 Ssl proteins, was shown to target myeloid cells by binding sialyllactosamine-containing glycoproteins [281].

We detected all six Spl proteases, SplA-F, which are encoded in one operon [140]. These putative proteases with similarity to SspA and epidermolytic toxins have potential roles in pathogenicity [139].

We observed a high amount of Hla in our extracellular extracts. This well characterized pore-forming toxin, is accused to be one of the main virulence factors in *S. aureus* pneumonia. High level secretion of Hla has been suggested to be an essential determinant of the exceptional virulence of the notorious CA-MRSA strain USA300 [100, 146, 282].

While previous studies of *S. aureus* extracellular proteins mainly focused on acidic and neutral pH [30, 256-258, 264], we included highly basic proteins up to pH 11, which allowed, for example, the detection of the very basic Hlg subunits that had escaped earlier investigations [264].

Some proteins which have been characterized by others in the *S. aureus* extracellular proteome, were missing in our extracts. These were mainly virulence factors such as enterotoxins (SEA, SEB, SEQ, SEK) [121, 256] [283], some staphylococcal enterotoxin-like proteins (Ssl1, Ssl2, Ssl7) [256], coagulase (Coa) [256], staphylokinase (Sak) [30], some leukotoxins (LukD, LukE) [30, 256], immunoglobulin binding protein (Sbi), and a Fnbp homologue (SdrD) [52]. Possibly, they were missed in our identification due to technical reasons (pH range, detection method, detection sensitivity,…), or they are not present at all in our protein extract. It is not surprising as *S. aureus* strains are highly variable in the protein patterns because of the highly variable genome and furthermore, the protein patterns depend much on cultivation conditions and growth phases [30, 256, 257, 264].

4.3 IgG binding to *S. aureus* extracellular proteins

The presence of Abs against a particular Ag in human serum is indicative of its *in vivo* expression during exposure, colonization, or disease and demonstrates that individuals are able to mount an immune response against the respective protein. However, it cannot be excluded that some of these Abs were elicited by other pathogens and cross-react with *S. aureus*. With this caveat in mind, the immune reactive proteins revealed by 2-D IBs can be considered as potential vaccine candidates.

By matching 2-D IBs probed with a human serum pool with the reference maps, 36 significant immune reactive proteins could be identified in the extracellular proteome of *S. aureus* 8325-4. The majority of toxins and known virulence Aur, Geh, Hla, Hlb and Hlg subunits, Lip, Nuc, Spl proteins, SspA, SspB and 88195808 (SspP) were strongly bound by the serum IgG. Proteins, which are responsible for *S. aureus* cell wall synthesis and degradation like Atl, IsaA, IsdA and SsaA also showed high immune reactivity. This indicates that these proteins are expressed *in vivo* and that the adaptive immune system responds to them.

The immune reactive proteins are almost ubiquitous in the species *S. aureus* (Table A.3). However, most of the genes encoding for the identified immune-reactive toxins (5 out of 6) and virulence factors (8 out of 13) appeared in other than 1 homologous copy among different *S. aureus* strains (Table A.3). They, in the other word, belong to variable proteome. Immune reactive proteins of other functional categories (e.g., cell wall biosynthesis and degradation, energy metabolism and regulation), in contrast, belong to the core protein, since they are more conserved and usually present in one gene copy.

Because we tested Ab binding to an *S. aureus* strain, which the probands had not encountered before, our results are biased towards IgG binding to conserved *S. aureus* proteins. In fact, some of these Ags have been recognized previously to be immune reactive (see Table A.6 in the appendix for an overview).

The most prominent among these immune reactive proteins is Hla, a pore-forming toxin that efficiently lyses host cells. Our 2-D IBs and reference maps indicated that Hla was the most abundant extracellular protein on 2-D gels and showed highest immune reactivity. Probably, Hla is regularly secreted *in vivo* and provokes a strong response of the host immune system. Wardenburg and Schneewind recently reported that immunization against Hla protected mice from *S. aureus* pneumonia [146]. In addition, this group also suggested that Hla but not PVL was the crucial pathogenic factor in *S. aureus* pneumonia caused by CA-MRSA [146, 282]. In our study, we found high-titer anti-Hla Abs found in every tested individual. These findings question the effectiveness of an 'monovalent' anti-Hla vaccine in human.

Another major immune reactive protein revealed by our 2-D IBs is IsaA. This protein, which was discovered as being immunodominant in sepsis patients, is currently tested as potential vaccine candidate [284]. IsaA is a putative autolysin since it possesses peptidoglycan hydrolytic activity [153]. Sera from individuals with confirmed *S. aureus* disease contain significantly higher anti-IsaA IgG titers than sera from healthy individuals [198]. Our data further support that IsaA is expressed *in vivo*, and is antigenic.

Our approach showed strong IgG binding to some proteins, which were not known to be immunogenic. Some of them have virulence potential: Hlb, HlgA, HlgC, SspA, SsaA_a and SsA_b.

Several immune reactive proteins are annotated as hypothetical proteins in the *S. aureus* databases: 88193909, 88194219, 88194436, 88196599, 88195838, 88194087 and 88194062. Among them, 88194219, 88194436 and 88196599 are predicted to be extracellular proteins because they contain a signal sequence required for export and/ or a trans-membrane domain (e.g., signal peptide, LPXTG), 88193909 is predicted to be cell wall-attached. Only 88194493 is predicted to be an intracellular protein and the remaining three proteins cannot be classified on the basis of their sequences. The study clearly demonstrates that all these hypothetical proteins are expressed and released by *S. aureus in vivo* and *in vitro*. Their role in the host- pathogen interaction therefore merits further studies.

In summary, we were able to provide an overview of immune-reactive proteins in the *S. aureus* 8325-4 extracellular proteome. While this is probably much less than comprehensive for the many reasons discussed, it contributes to our understanding of the behavior of *S. aureus* during its encounter with the human host. The results further show that the adaptive immune system responds to a very large spectrum of *S. aureus* virulence factors with humoral immunity, which may explain in part, why life-long carriage of *S. aureus* usually does not cause symptoms.

4.4 Factors determining the immune reactivity of *S. aureus* extracellular proteins

To correlate IgG binding to different proteins with their relative abundance on 2-D gels, we produced overlays of Cy3-stained protein gels with the corresponding IBs. Many of the highly abundant proteins, such as Hla, Hlb, SspA, SspB and Lip, were well recognized by the pooled human serum IgG. Interestingly, there were some spots with comparably strong Ab binding. Especially the highly basic proteins, such as Atl, were highly immune reactive. Unfortunately, they were transferred to the PVDF membrane with varying efficacy. This is a well-known problem in 2-D IB. Therefore, we decided to exclude them from the quantitative evaluation.

In general, immune reactivity did not strictly correlate with protein abundance. Many strongly immune reactive proteins such as Atl, IsaA, IsdA appeared as faint spots on 2-D gels. This suggests their expression in colonization and/ or infections as well as their strong interaction with the immune system. It is widely known that the protein expression pattern of a pathogen could be strongly different under *in vivo* and *in vitro* growth conditions [285]. Thus, these proteins of low abundance and high immune reactivity probably are highly expressed *in vivo*, however, this could not always be the case since it may take quite little protein to generate a strong Ab response and vice versa .

On the other hand, we observed proteins, which were present in large amounts but showed weak IgG binding such as peptidoglycan hydrolase (LytM) and the hypothetical protein 88193909. Not only low immunogenicity but also low expression *in vivo* or absence from many *S. aureus* strains are possible explanations for this low immune reactivity.

Additionally, we also observed many faint protein spots, which did not bind IgG on IBs. Many of them represent cytoplasmic proteins. A small amount of cytoplasmic proteins in the bacterial culture supernatant is not unexpected, as cytolysis often occurs during

growth, and thereby intracellular proteins are released into culture medium [30, 256, 286]. These 'contaminating' intracellular proteins became visible with the highly sensitive DIGE-stain. Since IgG did not bind to them, the immune system of carrier is obviously predominantly confronted with those proteins, which are released by the microorganism. Bacterial lysis either does not occur at a large scale *in vivo*, or it happens only inside phagocytes but outside the reach of B cells and Abs.

We have additionally performed IBs of 8325-4 cytoplasmic proteins and probed with the serum pool (Figure A.3). Our result showed the same: low immune reactivity of *S. aureus* cytoplasmic proteins, which is consistent with observation by others [198, 270, 287].

4.5 Anti-*S. aureus* Ab profiles are heterogeneous among individuals

We and others have shown a strong humoral immune response against a broad spectrum of extracellular *S. aureus* proteins in most healthy individuals [27, 61, 168, 198, 267, 287]. How can this be explained?

S. aureus is ubiquitous, and most individuals are exposed to the microorganism during the first hours of life. Later, about 30 % of healthy adults are colonized on their nasal mucosa. Additionally, there is a considerable fluctuation of *S. aureus* nasal carriage, as 50 % of the populations are intermittently colonized. Finally, even in noncarriers, there is probably a high frequency of encounters with *S. aureus*, which may be facilitated by minor lesions [1, 11, 154, 160, 288]. How is the individual history of encounters with *S. aureus* reflected in the individuals anti-*S. aureus* Ab profiles?

When we probed 2-D IBs of the post-exponential extracellular proteome of *S. aureus* 8325-4 with the sera from the 16 healthy volunteers, we found a marked inter-individual variation in IgG binding. This is consistent with previous studies [168, 198]. Dryla and co-workers showed variability of anti-staphylococcal Ab levels among healthy individuals [198]. About 2.5 % to 3.0 % of the total serum IgG reacted with staphylococcal lysate in high-titer sera whereas only 0.1 % of total IgG reacted in the lowest-titer samples [198]. Holtfreter *et al.* reported that neutralizing Ab patterns against SAgs, which represent highly variable virulence factors, were highly diverse among healthy blood donors [168].

Our study extends these findings. We observed heterogeneity among healthy volunteers in the total serum IgG binding to the IBs in the anti-staphylococcal IgG profiles, namely spot patterns and spot signal intensities.

Several factors may be responsible for this diversity.

Firstly, *S. aureus* species is genetically variable. This high genetic diversity of *S. aureus* species is caused by the presence or absence of MGEs, which carry many virulence and resistance genes. This flexibility results in up to 20 % variation in the genome of two *S. aureus* strains [23]. Thus, each encounter with a new *S. aureus* strain will leave a different imprint on the adaptive immune system.

Secondly, *S. aureus* is capable of perfectly adapting to its environment and thus produces different sets of virulence factors and other bacterial components under different conditions, i.e. encounters between *S. aureus* and the human host.

Thirdly yet importantly, humans differ from each other. People are genetically different and thus react differentially to *S. aureus* [289, 290]. Some are likely to become a nasal carrier of *S. aureus* [289], some are not. Some are susceptible to infections with the microorganism [167], others are more resistant.

As a result, every individual has its own history of immune encounters with *S. aureus*, which undoubtedly leaves its imprint on the Ab profiles.

4.6 The core and the variable immune proteome of *S. aureus*

We predicted that the comparison of anti-*S. aureus* Ab patterns will reveal proteins commonly recognized by human sera, i.e. the core immune proteome, and proteins which are recognized only by a small fraction of human sera, i.e. the variable immune proteome. To characterize the core immune proteome, we compared the prevalence of strong spots between the 16 volunteers. Eleven spots were detected in at least 75 % of all volunteers, and by our definition represent the core immune proteome of *S. aureus* 8325-4. Among these immune dominant spots were the conserved virulence factors Hla, Plc, SplB, SplE, SspA and SspB.

In contrast, 28 out of the 52 strongest spots appeared in less than half of the volunteers, underlining the impressive inter-individual diversity in the adaptive immune response against *S. aureus*. But this may only be the tip of an iceberg, because our investigation permits only limited insight into the true extent of diversity. The reason is that we have probed all Ab responses with *S. aureus* 8325-4, the strain which was later used for experimental colonization. This laboratory isolate is not related to those strains, which have triggered the immune response that was present prior to colonization. As a result, it is likely that mainly Abs reacting to conserved immune reactive proteins would show up in our study. Moreover, 8325-4 is a prophage-cured strain, thus provides an extracellular

proteome close to the core proteome of *S. aureus*. Consequently, our results are biased towards conserved *S. aureus* epitopes contained in the core proteome of the species.

To explore the variable immune proteome of *S. aureus*, it will be necessary to test a large number of individuals for their humoral immune response against their own colonizing or infecting strain. We would expect to find many more variable immune reactive spots in this case.

This approach has been applied previously by our group to probe the neutralizing Abs against *S. aureus* SAgs, exotoxins with high genetic variability in *S. aureus*. In fact, the spectrum of neutralizing Abs was of unexpected diversity in healthy blood donors, and in carriers it was highly specific for the SAgs of the colonizing strain [168]. Heterogeneity in anti-*S. aureus* Ab profiles to selected antigens has also been reported by others. Dryla *et al.*. determined serum IgG Abs against whole bacteria as well as selected *S. aureus* antigens by ELISA and also observed broad heterogeneity in Ab titers against their studied Ags [198].

One has to bear in mind that all studies addressing Ab responses against *S. aureus* including ours have been performed with *S. aureus* grown *in vitro* or with recombinant Ags. These, however, may only partially overlap with the Ag spectrum produced by *S. aureus in vivo*. Vytvytska and colleges reported that one *S. aureus* strain grown in different culture media could produce a different set of proteins and consequently, a different set of immune reactive proteins [287]. Until now, information about the behavior of the micro-organism in the different phases of its encounters with the host is scarce. Nevertheless, only those proteins that are expressed *in vivo* can induce an Ab response, in other words, the binding of Abs to a protein indicates its expression *in vivo*. Our data imply that the virulence factors contained in the core immune proteome are commonly produced *in vivo*, because the immune system of most individuals has been exposed to them.

4.7 Differences between carriers and noncarriers in baseline IgG binding to *S. aureus*

Carriers are per definition constantly exposed to *S. aureus* while noncarriers probably have only short-term contacts. Thus, there is possibility that carriers differ from noncarriers in their anti-staphylococcal IgG profile. In our study, among the anti-staphylococcal Ab profiles of 16 healthy volunteers including carriers and noncarriers, there was a large inter-individual variation in total serum IgG binding before colonization

but there were no impressive differences between carriers and noncarriers. This is consistent with a study of the groups of Dryla and Clarke, who also did not find a strong correlation between carriage and Ab levels [198],[27]. In our study, some spots tend to be stronger in carriers than noncarriers: IsaA, SspA, SspB and two hypothetical proteins. Investigation of larger cohorts might uncover more subtle differences between carriers and noncarriers, but at this stage it is clear already that some noncarriers have very strong serum IgG binding to *S. aureus* extracellular proteins. The most likely explanation is that these may have a history of intensive contact with *S. aureus*, such as for example minor infections .

Holtfreter *et al.* previously reported that there was a strong, strain-specific Ab response in carriers against the SAgs of their colonizing strain and only a moderate Ab response in noncarriers [168], but this study, in contrast to all others, focuses on proteins with highly variable expression in the species *S. aureus*.

We did not observe spots that were stronger in noncarriers than in carriers. This is different from the study of Clarke *et al.* who reported that noncarriers (n= 75) had significantly higher IgG titers for four out of 11 tested Ags, Hla, IsdA, IsdH and IsaA, the latter in direct contrast to our results [27]. In the case of Hla, it is possible that differences in IgG binding have gone undetected in our study because the anti-Hla signal was saturated on many 2-D IBs. The group of Clarke proposed that Abs against Hla, IsdA, IsdH and IsaA play a role in protecting noncarriers from colonization. However, in view of the controversial results, this requires further investigation.

Nasal carriage in healthy individuals is likely to be controlled by both local innate and adaptive immune functions. Mucosal Abs i.e. IgA could have an important role and there may be tendency for higher IgG and lower IgA levels in permanent carriers compared to noncarriers [198]. It would therefore be worthwhile to compare IgA responses to *S. aureus* with 2-D IBs. A pilot experiment performed by Silva Holtfreter shows that this will be possible and suggests that IgA binds to a different set of proteins than IgG (data not shown).

Since our study focuses on *S. aureus* soluble proteins, which probably do not mediate adhesion, it may not have addressed the most important determinants of *S. aureus* carriage. However, bacterial cell wall components and membrane proteins cannot be resolved by 2-D PAGE. Coupling them to Luminex-beads would allow the analysis of Ab responses to membrane proteins with possible functions in adhesion and colonization.

4.8 Impact of experimental colonization with *S. aureus* on the serum IgG response

When we compared the Abs profiles against *S. aureus* extracellular proteins of all 16 volunteers before and after the experimental colonization with *S. aureus* 8325-4, we observed almost no difference. There was only a minor increase of IgG binding during colonization occurring in weak spots. There were not conserved between individuals. However, we can not completely exclude that differences in IgG binding to strong spots were underestimated due to signal saturation.

S. aureus nasal colonization occurs on the epithelial surface. Thus, it might well be that the colonizing bacteria are ignored by the systemic adaptive immune system and do not elicit IgG Abs. It is well known that secretory IgA probably plays an essential role in protecting mucosal surfaces from invading bacteria. Thus, we propose that in contrast to serum IgG secretory IgA could play an important role in symptom-free colonization. However, since nasal secretions were not obtained from the 16 volunteers, we could only study serum IgA responses. We compared serum IgA binding in 2-D IBs of four volunteers before and after colonization (data not shown). However, again the IgA binding patterns before and after colonization were very similar.

In conclusion, short-term colonization with a strain of low virulence is not sufficient to induce a serum Ab production, which is comparable to that present already before colonization. Thus, either long-term high density colonization is required, or as we consider most likely, the adaptive immune response is primarily triggered by (minor) *S. aureus* infections. We therefore propose that these Abs contribute to protection against infection with *S. aureus* and its complications.

For further studies, it would be worthwhile to apply the same technique to study Ab responses in colonization against strains other than 8325-4, which shows a reduced fitness in colonizing humans. We would expect a better interpretation of Ab profiles (both IgG and IgA) before and after colonization and probably we could indicate which anti-staphylococcal Ab specificities are induced by colonization and correlated with rejection of the inoculated strain. However, the use of a more potent *S. aureus* in a human experiment may not be ethical and practical.

Chapter 5

CONCLUSIONS AND PERSPECTIVES

In this study, we applied proteome-based approaches to investigate the human humoral immune response to extracellular proteins produced by *S. aureus* strain 8325-4. We have observed strong IgG responses against a large panel of *S. aureus* virulence factors. These responses are common in the healthy population and are likely elicited in the course of minor infections [167]. We hypothesize that these Abs are protective in the case of *S. aureus* infection.

Our results may have implications for vaccine development. Most staphylococcal researchers would agree that an effective vaccine should comprise (1) highly immunogenic Ags, that are (2) conserved throughout the species and (3) are protective in animal models.

But not all highly immunogenic and conserved Ags may be good vaccine candidates. Of course, antigenic proteins that show little sequence variation and are conserved throughout the species *S. aureus* may be expected to induce an Ab response with substantial cross-reaction to the numerous strains of this bacterium. However, our data show that most healthy individuals already possess a strong Ab response against these conserved *S. aureus* proteins, presumably due to frequent exposure. This raises the question, how much a vaccine based on these conserved Ags could add to the high level of protection in the general population. Experimental animals, in contrast, are usually immunologically naïve, when they are used in pre-clinical vaccine efficacy studies. If generated in such settings, the predictive value of humoral protective responses in mice should not be overestimated.

Moreover, the Ab binding in an IB does not necessarily mean that they are protective. In deed, Abs directed at the bacterial surface can opsonize and facilitate the uptake of the pathogen by neutrophils. However, opsonization and uptake does not necessarily lead to the killing of *S. aureus*. Consequently, the presence of Abs directed against the bacterial surface may not provide protection. This notion is supported by the two unsuccessful phase III clinical trials designed to test the efficacy of active immunization against *S. aureus* Ags CP5, CP8 and ClfA [21].

Another function of Abs is the neutralization of toxins produced by the pathogen. There are numerous examples of successful vaccination against bacterial toxins, including

botulinum, diphtheria and tetanus toxins. These toxins are known to be the primary causative agents of the diseases induced by the respective microorganisms. In contrast, *S. aureus* produces many toxins whose relative contributions to human diseases are often not known. Recently, it was reported that immunization against Hla, a ubiquitous cytolytic toxin, protects mice from lethal pneumonia caused by CA-MRSA strains [146]. But we have found high-titer anti-Hla Abs in every human individual. Dryla *et al.* showed that such Abs are functionally active, because hemolytic activity of Hla decreased when incubated with human sera with high titer Abs [198]. This result indicated the presence of functional anti-Hla Abs in healthy individuals. Consequently, a vaccine against a single virulence factor such as Hla is unlikely to be effective in humans. If Abs really protect against *S. aureus* infection and its complications, why is IVIG-treatment (intravenous immunoglobulin) not more effective? Our results might provide an explanation for this. IVIG contains Abs against core *S. aureus* core proteome at high titers, but so does in most cases the patient serum. In contrast, individuals exposed to the variable *S. aureus* virulence factors are rare in the population, and their Abs are diluted in the IVIG pool. Thus, the decisive protecting Abs, which are lacking in the patient, may not be present in the IVIG preparation at sufficient concentrations.

It is well known that previous infections with *S. aureus* do not protect individuals from subsequent infections. This finding was usually explained (1) either by the transient nature of anti-staphylococcal Ab levels or (2) by a negligible role of Abs in anti-staphylococcal defense [291].

Ab stability, however, does not appear to be the problem. The point is rather not likely as Dryla *et al.*. showed that anti-*S. aureus* IgG titers in healthy individuals were stable for at least 12 months [198].

However, to which degree Abs can protect against *S. aureus* infections remains an open question. Wertheim *et al.*. reported that in bacteremic patients, *S aureus* bacteremia-related death was significantly higher in non-carriers than in carriers (13/41 [32%] vs. three/40 [8%], p=0.006) [167]. This result suggested the possibility of Ab protection from lethal infection. This notion is also supported by Holtfreter *et al.* whose study show that carriers harbor high titers of Abs against their own *S. aureus* strains [168].

In summary, we propose that a successful anti-staphylococcal vaccine would have to be a multi-component subunit vaccine, which includes important variable virulence factors. In the case of acute *S. aureus* infections, an ambitious but worthwhile aim would be the

development of an individualized therapy: mAb-cocktails that fill the gaps in the patients' anti-*S. aureus* Ab profiles.

Considering the extreme diversity both of *S. aureus* and its host, the big challenge will now be to find out which of the many Ab specificities observed can significantly contribute to protection. Investigating the changes in human Ab profiles against *S. aureus* during the course of infection may provide leads. Our preliminary data show that the IgG memory response to some *S. aureus* Ags strongly increased during infection. A large scale comparison of patients with different disease outcomes will hopefully reveal the Ab gaps in nonsurvivors and, on the other hand, provide point at protective Abs, which will be present in survivors from the beginning or emerge during reconvalesence.

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Appendix

Table A.1: Annotation of identified proteins of *S. aureus* 8325-4 at pH ranges 6-11 and 4-7.

Protein name	Locus tag (*S. aureus* 8325)	Accession No.	Full name [a]	Localization [b]	Psort score	pI	Mr	4-7 or 6-11 [c]
Toxins and Hemolysins[d] (8)								
Hla	SAOUHSC_01121	88194865	alpha-hemolysin precursor	ec	10.0	9.2	35973	both
Hlb	SAOUHSC_02240	88195913	truncated beta-hemolysin	ec	10.0	8.2	31256	6-11
HlgA	SAOUHSC_02708	88196348	gamma-hemolysin component A	ec	9.7	10.1	34956	6-11
HlgB	SAOUHSC_02710	88196350	gamma-hemolysin component B	ec	9.7	9.8	36711	6-11
HlgC	SAOUHSC_02709	88196349	gamma-hemolysin component C	ec	9.7	9.7	35614	6-11
HysA	SAOUHSC_02463	88196115	hyaluronate lyase	ec	10.0	7.8	91985	6-11
LukF	SAOUHSC_02241	88195914	Leukocidin/hemolysin toxin family F subunit	ec	9.7	9.1	38686	6-11
Ssl11	SAOUHSC_00399	88194194	staphylococcal superantigen-like protein 11	ec	10.0	9.1	25366	4-7
Virulence/Defence mechanisms (18)								
Aur	SAOUHSC_02971	88196592	aureolysin, putative	ec	10.0	4.7	54986	4-7
EbpS	SAOUHSC_01501	88195217	elastin binding protein	cw	9.1	6.3	53221	4-7
Geh	SAOUHSC_03006	88196625	glycerol ester hydrolase	ec	9.7	7.7	76675	both
Lip	SAOUHSC_00300	88194101	lipase precursor	ec	10.0	9.6	76387	6-11
Nuc	SAOUHSC_00818	88194577	thermonuclease precursor	ec	10.0	9.7	25120	6-11
Plc	SAOUHSC_00051	88193871	1-phosphatidylinositol phosphodiesterase precursor, putative	ec	10.0	8.6	37087	6-11
SasH	SAOUHSC_00025	88193846	putative 5-nucleotidase	cw	10.0	10.0	83424	6-11
Spa	SAOUHSC_00069	88193885	protein A	cw	10.0	5.4	56437	4-7
SplA	SAOUHSC_01942	88195636	serine protease SplA	ec	9.7	9.5	25549	6-11
SplB	SAOUHSC_01941	88195635	serine protease SplB	ec	9.7	9.7	26096	6-11
SplC	SAOUHSC_01939	88195634	serine protease SplC	ec	9.7	6.8	26098	both
SplD	SAOUHSC_01938	88195633	serine protease SplD	ec	9.7	9.7	25678	6-11
SplE	SAOUHSC_01936	88195631	serine protease SplE	ec	9.7	9.8	25679	6-11
SplF	SAOUHSC_01935	88195630	serine protease SplF, putative	ec	9.7	9.7	25655	6-11
SspA	SAOUHSC_00988	88194745	glutamyl endopeptidase precursor, putative, V8 protease	ec	10.0	4.8	36326	4-7
SspB	SAOUHSC_00987	88194744	cysteine protease precursor, putative	ec	9.6	5.8	44519	both
Tig	SAOUHSC_01779	88195480	trigger factor	ic	8.9	4.1	48609	4-7
88195808	SAOUHSC_02127	88195808	staphopain thiol proteinase (Uniprot: SspP)	ec	9.7	10.1	44262	6-11

Stress response proteins (11)								
AhpC	SAOUHSC_00365	88194163	alkyl hydroperoxide reductase subunit C	ic	10.0	4.7	20977	4-7
AhpF	SAOUHSC_00364	88194162	alkyl hydroperoxide reductase, subunit F, putative	ic	10.0	4.4	54721	4-7
ClpP	SAOUHSC_00790	88194551	ATP-dependent Clp protease, proteolytic subunit ClpP	ic	9.7	4.9	21514	4-7
CspA	SAOUHSC_01403	88195129	cold shock protein, putative	ic	10.0	4.2	7321	4-7
DnaK	SAOUHSC_01683	88195389	DnaK protein, putative	ic	10.0	4.4	66361	4-7
GroEL	SAOUHSC_02254	88195925	chaperonin GroEL, putative	ic	10.0	4.3	57664	4-7
GroES	SAOUHSC_02255	88195926	chaperonin GroES, putative	ic	10.0	4.6	10416	4-7
HchA	SAOUHSC_00533	88194313	chaperone protein HchA	unkn	2.5	4.7	32177	4-7
KatA	SAOUHSC_01327	88195057	catalase	ic	10.0	5.0	54943	4-7
TrxB	SAOUHSC_00785	88194546	thioredoxin reductase	ic	9.7	5.0	33616	4-7
88195522	SAOUHSC_01822	88195522	thiol peroxidase	unkn	2.5	4.3	18005	4-7
Cell wall biosynthesis and degradation (19)								
Atl	SAOUHSC_00994	88194750	autolysin precursor, putative	ec	10.0	10.1	137384	6-11
Ftn	SAOUHSC_02108	88195790	ferritin, putative	ic	10.0	4.4	19589	4-7
IsaA	SAOUHSC_02887	88196515	immunodominant antigen A, putative	ec	10.0	6.6	24203	4-7
IsdA	SAOUHSC_01081	88194829	iron-regulated surface determinant protein A	cw	10.0	10.3	38745	6-11
LytM	SAOUHSC_00248	88194055	peptidoglycan hydrolase, putative	ec	10.0	6.7	34316	4-7
LytR	SAOUHSC_02583	88196226	transcriptional regulator, putative	unkn	3.3	8.7	33804	4-7
Map	SAOUHSC_02466	88196118	truncated MHC class II analog protein	unkn	3.3	9.3	15447	6-11
Pbp3	SAOUHSC_01652	88195360	penicillin-binding protein 3	ic/ m	9.8	9.7	77238	6-11
SdrC	SAOUHSC_00544	88194324	sdrC protein, putative	cw	10.0	3.9	107795	both
SsaA_a[e]	SAOUHSC_02571	88196215	secretory antigen precursor, putative	ec	9.0	9.1	29327	6-11
SsaA_b	SAOUHSC_02576	88196220	secretory antigen precursor, putative	unkn	3.3	6.2	17399	4-7
88193872	SAOUHSC_00052	88193872	staphylococcal tandem lipoprotein, putative	unkn	3.3	8.7	29787	4-7
88193909	SAOUHSC_00094	88193909	hypothetical protein	cw	9.9	9.5	21847	6-11
88194219	SAOUHSC_00427	88194219	autolysin precursor, putative	ec	10.0	10.0	35836	6-11
88194436	SAOUHSC_00671	88194436	secretory antigen SsaA-like protein	ec	9.7	6.6	28187	4-7
88194675	SAOUHSC_00918	88194675	truncated MHC class II analog protein	unkn	3.3	9.8	15838	6-11
88194919	SAOUHSC_01180	88194919	conserved hypothetical protein	unkn	3.3	8.1	35907	4-7
88195062	SAOUHSC_01332	88195062	hypothetical protein	unkn	2.5	9.8	33590	6-11
88196599	SAOUHSC_02979	88196599	N-acetylmuramoyl-L-alanine amidase	ec	9.7	6.3	69253	both
Regulatory functions (4)								
CodY	SAOUHSC_01228	88194963	transcriptional regulator CodY	ic	8.9	6.1	28755	4-7

TraP	SAOUHSC_01964	88195657	signal transduction protein TraP	ic	8.9	6.6	19548	both
88194753	SAOUHSC_00997	88194753	transcriptional regulator, putative	unkn	3.3	6.4	45685	4-7
88195154	SAOUHSC_01430	88195154	phosphotransferase system enzyme IIA, putative	ic	10.0	4.3	17960	4-7
Energy metabolism (35)								
AcnA	SAOUHSC_01347	88195075	aconitate hydratase 1	ic	10.0	4.6	98969	4-7
Adh1	SAOUHSC_00608	88194378	alcohol dehydrogenase I, putative	ic	10.0	5.2	36047	4-7
AtpD	SAOUHSC_02341	88196007	ATP synthase F1, beta subunit	ic	8.9	4.4	51400	4-7
Cdr	SAOUHSC_00908	88194665	coenzyme A disulfide reductase, putative	ic	10.0	5.2	49290	4-7
Eno	SAOUHSC_00799	88194559	enolase	ic	10.0	4.3	47117	4-7
FabI	SAOUHSC_00947	88194704	enoyl-(acyl-carrier-protein) reductase	ic	8.9	5.7	28022	4-7
FbaA	SAOUHSC_02366	88196029	fructose-bisphosphate aldolase	ic	8.9	4.7	30836	4-7
Fda	SAOUHSC_02926	88196553	fructose-bisphosphate aldolase class-I, putative	unkn	2.5	4.6	33054	4-7
Gap	SAOUHSC_00795	88194555	glyceraldehyde-3-phosphate dehydrogenase, type I	ic	10.0	4.6	36281	4-7
Gnd	SAOUHSC_01605	88195316	6-phosphogluconate dehydrogenase, decarboxylating	ic	8.9	4.8	51803	4-7
Ldh_a[e]	SAOUHSC_00206	88194016	L-lactate dehydrogenase	ic	10.0	4.7	29447	4-7
Ldh_b	SAOUHSC_02922	88196549	L-lactate dehydrogenase	ic	9.7	4.5	34420	4-7
Mqo2	SAOUHSC_02927	88196554	malate:quinone-oxidoreductase	unkn	4.1	6.5	55999	both
PckA	SAOUHSC_01910	88195605	phosphoenolpyruvate carboxykinase (ATP)	unkn	2.5	6.0	59377	4-7
PdhA	SAOUHSC_01040	88194792	pyruvate dehydrogenase complex, E1 component, alpha subunit, putative	ic	8.9	4.6	41383	4-7
PdhB	SAOUHSC_01041	88194793	pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase beta subunit, putative	ic	8.9	4.4	35246	4-7
PdhC	SAOUHSC_01042	88194794	dihydrolipoamide S-acetyltransferase component of pyruvate dehydrogenase complex E2, putative	ic	10.0	4.6	46354	4-7
PdhD	SAOUHSC_01043	88194795	dihydrolipoamide dehydrogenase	ic	10.0	4.7	49481	4-7
PfkA	SAOUHSC_01807	88195507	6-phosphofructokinase, putative	ic	8.9	5.5	33397	4-7
PflB	SAOUHSC_00187	88193997	formate acetyltransferase	ic	10.0	5.1	84862	4-7
Pgi	SAOUHSC_00900	88194657	glucose-6-phosphate isomerase	ic	10.0	4.5	49822	4-7
Pgk	SAOUHSC_00796	88194556	phosphoglycerate kinase, putative	ic	9.9	5.0	42602	4-7
Pgm	SAOUHSC_00798	88194558	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	ic	10.0	4.5	56424	4-7
Pta	SAOUHSC_00574	88194349	phosphate acetyltransferase	ic	8.9	4.5	34952	4-7
PycA	SAOUHSC_01064	88194813	pyruvate carboxylase	ic	8.9	4.9	128547	4-7
QoxA	SAOUHSC_01002	88194758	quinol oxidase AA3, subunit II, putative	ic/ m	10.0	9.0	41777	6-11
Tal	SAOUHSC_01901	88195596	transaldolase	unkn	2.5	4.5	25705	4-7
Tkt	SAOUHSC_01337	88195066	transketolase	unkn	2.5	4.7	68359	4-7
TpiA	SAOUHSC_00797	88194557	triosephosphate isomerase	unkn	2.5	4.5	27292	4-7

Zwf	SAOUHSC_01599	88195310	glucose-6-phosphate 1-dehydrogenase	ic	8.9	5.2	56965	4-7
88194332	SAOUHSC_00553	88194332	hexulose-6-phosphate synthase, putative	ic	8.9	4.3	22436	4-7
88194493	SAOUHSC_00728	88194493	putative sulfatase	ic/ m	10.0	9.5	74400	6-11
88195341	SAOUHSC_01632	88195341	glycine cleavage system P-protein subunit ll, putative	ic	8.9	5.8	54782	4-7
88195342	SAOUHSC_01633	88195342	glycine cleavage system P-protein subunit I, putative	ic	8.9	4.8	49716	4-7
88196221	SAOUHSC_02577	88196221	D-isomer specific 2-hydroxyacid dehydrogenase	ic	10.0	4.9	34674	4-7
Protein synthesis (19)								
Efp	SAOUHSC_01625	88195335	translation elongation factor P	ic	10.0	4.5	20554	4-7
Frr	SAOUHSC_01236	88194969	ribosome recycling factor	ic	10.0	4.8	20353	4-7
Fus	SAOUHSC_00529	88194309	translation elongation factor G	ic	10.0	4.5	76612	4-7
GltX	SAOUHSC_00509	88194290	glutamyl-tRNA synthetase	ic	9.7	5.0	56289	4-7
IleS	SAOUHSC_01159	88194899	isoleucyl-tRNA synthetase	ic	10.0	5.1	104885	4-7
LeuS	SAOUHSC_01875	88195572	leucyl-tRNA synthetase	ic	9.7	4.8	91786	4-7
MetS	SAOUHSC_00461	88194248	methionyl-tRNA synthetase, putative	ic	10.0	4.9	74885	4-7
RplE	SAOUHSC_02500	88196150	50S ribosomal protein L5, putative	ic	8.9	10.0	20267	6-11
RplF	SAOUHSC_02496	88196147	ribosomal protein L6, putative	ic	8.9	10.1	19786	6-11
RplK	SAOUHSC_00518	88194299	ribosomal protein L11	ic	8.9	9.6	14874	6-11
RplM	SAOUHSC_02478	88196130	ribosomal protein L13	unkn	2.5	9.7	16333	6-11
RplY	SAOUHSC_00474	88194261	ribosomal 50S rRNA E-loop binding protein	ic	8.9	4.1	23788	4-7
RpsB	SAOUHSC_01232	88194965	ribosomal protein S2	ic	8.9	4.9	27733	4-7
RpsC	SAOUHSC_02506	88196156	ribosomal protein S3	ic	8.9	10.4	24100	6-11
RpsH	SAOUHSC_02498	88196148	ribosomal protein S8, putative	ic	8.9	9.9	14831	6-11
SerS	SAOUHSC_00009	88193832	seryl-tRNA synthetase	ic	10.0	4.7	48640	4-7
Tsf	SAOUHSC_01234	88194967	translation elongation factor Ts	ic	10.0	4.8	32494	4-7
Tuf	SAOUHSC_00530	88194310	translation elongation factor Tu	ic	10.0	4.5	43104	4-7
ValS	SAOUHSC_01767	88195468	valyl-tRNA synthetase	ic	10.0	4.7	101724	4-7
Protein fate: Degradation of proteins, peptides, and glycopeptides (5)								
GrpE	SAOUHSC_01684	88195390	co-chaperone GrpE	ic	8.9	4.1	24008	4-7
PepF	SAOUHSC_00937	88194694	oligoendopeptidase F	ic	10.0	4.9	69819	4-7
Ppi	SAOUHSC_00891	88194648	peptidyl-prolyl cis-trans isomerase, cyclophilin-type, putative	ic	10.0	4.3	21619	4-7
88195336	SAOUHSC_01626	88195336	proline dipeptidase, putative	ic	8.9	5.1	39338	4-7
88195565	SAOUHSC_01868	88195565	putative peptidase	ic	8.9	4.3	52824	4-7
Amino acid biosynthesis (6)								
ArgF	SAOUHSC_02968	88196589	ornithine carbamoyltransferase	ic	10.0	4.9	37763	4-7

GlnA	SAOUHSC_01287	88195018	glutamine synthetase, type I	ic	10.0	4.8	50841	4-7
GlyA	SAOUHSC_02354	88196017	serine hydroxymethyltransferase, putative	ic	10.0	6.0	45172	4-7
IlvA	SAOUHSC_01451	88195171	threonine dehydratase	ic	8.9	6.5	37306	6-11
88195550	SAOUHSC_01852	88195550	conserved hypothetical protein	ic	8.9	6.0	40618	4-7
88195838	SAOUHSC_02158	88195838	aminotransferase, putative	unkn	2.5	4.9	48119	4-7
Nucleotide biosynthesis and degradation (11)								
Adk	SAOUHSC_02490	88196141	adenylate kinase, putative	ic	10.0	4.5	23974	4-7
DeoB	SAOUHSC_00101	88193914	phosphopentomutase	ic	8.9	4.8	43795	4-7
DeoD	SAOUHSC_02380	88196043	purine nucleoside phosphorylase	unkn	2.5	4.6	25908	4-7
Fhs	SAOUHSC_01845	88195544	formate-tetrahydrofolate ligase, putative	ic	8.9	5.8	59856	4-7
GuaA	SAOUHSC_00375	88194173	GMP synthase, putative	ic	8.9	4.8	58230	4-7
GuaB	SAOUHSC_00374	88194172	inosine-5-monophosphate dehydrogenase	ic	8.9	5.7	52850	4-7
Pnp	SAOUHSC_00097	88193911	purine nucleoside phosphorylase	ic	8.9	4.9	25992	4-7
PurA	SAOUHSC_00019	88193840	adenylosuccinate synthetase	ic	8.9	4.9	47579	4-7
PurB	SAOUHSC_02126	88195807	adenylosuccinate lyase	ic	8.9	5.7	49603	4-7
PurF	SAOUHSC_01014	88194768	amidophosphoribosyltransferase	ic	8.9	6.6	54397	4-7
PurQ	SAOUHSC_01012	88194766	phosphoribosylformylglycinamidine synthase I	ic	8.9	4.7	24527	4-7
Transcription and replication (4)								
DnaN	SAOUHSC_00002	88193825	DNA polymerase III, beta subunit	ic	8.9	4.4	41914	4-7
GreA	SAOUHSC_01714	88195419	transcription elongation factor GreA	ic	8.9	4.2	17743	4-7
RpoA	SAOUHSC_02485	88196136	DNA-directed RNA polymerase alpha chain, putative	ic	10.0	4.4	35012	4-7
88194063	SAOUHSC_00257	88194063	conserved hypothetical protein	unkn	2.5	4.3	11036	4-7
Transport and binding proteins (2)								
PtsI	SAOUHSC_01029	88194782	phosphoenolpyruvate-protein phosphotransferase	ic	10.0	4.4	63219	4-7
88194087	SAOUHSC_00284	88194087	5'-nucleotidase, lipoprotein e(P4) family	unkn	3.3	10.1	33352	6-11
Fatty acid and phospholipid metabolism: Degradation (1)								
GlpQ	SAOUHSC_00897	88194654	glycerophosphoryl diester phosphodiesterase, putative	unkn	4.9	9.1	35311	6-11
Unknown function (10)								
IsaB	SAOUHSC_02972	88196593	immunodominant antigen B, hypothetical	unkn	3.3	10.2	19370	6-11
UspA	SAOUHSC_01819	88195519	conserved hypothetical protein	ic	8.9	5.7	18475	4-7
YceI	SAOUHSC_03022	88196640	conserved hypothetical protein	unkn	2.5	4.4	18655	4-7
88193948	SAOUHSC_00135	88193948	conserved hypothetical protein	ic	8.9	4.4	19257	4-7

88194062	SAOUHSC_00256	88194062	staphyloxanthin biosynthesis protein, putative	unkn	3.3	6.3	33032	both
88194387	SAOUHSC_00617	88194387	hypothetical protein	unkn	3.3	9.6	18594	6-11
88194622	SAOUHSC_00865	88194622	conserved hypothetical protein	unkn	2.5	4.2	27946	4-7
88194663	SAOUHSC_00906	88194663	fumarylacetoacetate (FAA) hydrolase family protein, putative	ic	8.9	4.6	33113	4-7
88195515	SAOUHSC_01815	88195515	metallo-beta-lactamase superfamily protein, putative	ic	8.9	5.0	25251	4-7
88196081	SAOUHSC_02425	88196081	hypothetical protein	unkn	2.5	6.5	10006	6-11

[a] Protein names according to the annotated *S . aureus* strain 8325, Col, N315 and Newman genome sequences based on the aureusDB database (http://aureusdb.biologie.uni-greifswald.de/), the Entrez database (http:// www.ncbi.nlm.nih.gov/sites/entrez) and UniProt database (http://www.uniprot.org).

[b] Protein localization was predicted by P-SORT. ic: intracellular, ec: extracellular, m: membrane, cw: cell wall, unkn: unknown.

[c] Protein was detected on 2-D gels of pH range 4-7 or 6-11 or both.

[d] Protein classifications were adapted from Burlak *et al.* and Tigr database (http://www.cmr.tigr.org).

[e] Ldh and SsaA were found in the public databases (see footnote a) with two similar but different sequences and are therefore numbered with _a and _b.

Table A.2: Identification data of protein spots on 2-D gels resolving *S. aureus* 8325-4 extracellular proteins at pH ranges 6-11 (A) and 4-7 (B).

A) pH 6-11

Accession No.	Protein name[a]	Full name	PC[b]	SC	PS
88193846	SasH	putative 5-nucleotidase	28	0.43	328
88193871_1	Plc_1	1-phosphatidylinositol phosphodiesterase precursor, putative	12	0.38	148
88193871_2	Plc_2	1-phosphatidylinositol phosphodiesterase precursor, putative	12	0.42	100
88193871_3	Plc_3	1-phosphatidylinositol phosphodiesterase precursor, putative	14	0.53	147
88193909_1	88193909_1	hypothetical protein	5	0.62	221
88193909_2	88193909_2	hypothetical protein	6	0.69	223
88193909_3	88193909_3	hypothetical protein	8	0.75	123
88193909_4	88193909_4	hypothetical protein	9	0.75	85
88193909_5	88193909_5	hypothetical protein	7	0.69	99
88193909_6	88193909_6	hypothetical protein	5	0.54	39
88193909_7	88193909_7	hypothetical protein	8	0.75	72
88193909_8	88193909_8	hypothetical protein	5	0.62	42
88194062	88194062	staphyloxanthin biosynthesis protein, putative	4	0.2	69
88194087	88194087	5'-nucleotidase, lipoprotein e(P4) family	9	xxx	139
88194101_1	Lip_1	lipase precursor	23	0.35	483
88194101_2	Lip_2	lipase precursor	19	0.3	298
88194101_3	Lip_3	lipase precursor	18	0.21	272
88194101_4	Lip_4	lipase precursor	13	0.21	151
88194101_5	Lip_5	lipase precursor	25	0.46	361
88194101_6	Lip_6	lipase precursor	24	0.4	282
88194101_7	Lip_7	lipase precursor	13	0.27	68
88194101_8	Lip_8	lipase precursor	19	0.26	262
88194101_9	Lip_9	lipase precursor	20	0.28	395
88194101_10	Lip_10	lipase precursor	11	0.25	53
88194101_11	Lip_11	lipase precursor	21	0.31	218
88194101_12	Lip_12	lipase precursor	21	0.35	281
88194101_13	Lip_13	lipase precursor	18	0.29	168
88194219_1	88194219_1	autolysin precursor, putative	6	0.3	232
88194219_2	88194219_2	autolysin precursor, putative	7	0.24	322
88194219_3	88194219_3	autolysin precursor, putative	3	0.14	89
88194219_4	88194219_4	autolysin precursor, putative	11	0.43	223
88194299	RplK	ribosomal protein L11	9	0.72	305
88194324	SdrC	sdrC protein, putative	14	0.17	396
88194387_1	88194387_1	hypothetical protein	12	0.54	370
88194387_2	88194387_2	hypothetical protein	13	0.62	318
88194387_3	88194387_3	hypothetical protein	10	0.5	255
88194387_4	88194387_4	hypothetical protein	10	0.57	86
88194387_5	88194387_5	hypothetical protein	7	0.47	59
88194493_1	88194493_1	putative sulfatase	25	0.46	338
88194493_2	88194493_2	putative sulfatase	30	0.53	409
88194493_3	88194493_3	putative sulfatase	28	0.5	339
88194577	Nuc	thermonuclease precursor	8	0.35	230
88194654	GlpQ	glycerophosphoryl diester phosphodiesterase, putative	18	0.57	353
88194675	88194675	truncated MHC class II analog protein	9	0.59	353
88194744	SspB	cysteine protease precursor, putative	9	0.2	170
88194750_1	Atl_1	autolysin precursor, putative	27	0.28	270
88194750_2	Atl_2	autolysin precursor, putative	27	0.28	270
88194750_3	Atl_3	autolysin precursor, putative	27	0.25	252
88194750_4	Atl_4	autolysin precursor, putative	24	0.24	171
88194750_5	Atl_5	autolysin precursor, putative	25	0.21	294
88194750_6	Atl_6	autolysin precursor, putative	17	0.16	182
88194750_7	Atl_7	autolysin precursor, putative	23	0.21	280

88194758	QoxA	quinol oxidase AA3, subunit II, putative	8	0.29	205
88194829	IsdA	iron-regulated surface determinant protein A	12	0.32	367
88194865_1	Hla_1	alpha-hemolysin precursor	6	0.2	139
88194865_2	Hla_2	alpha-hemolysin precursor	5	0.24	168
88194865_3	Hla_3	alpha-hemolysin precursor	9	0.32	236
88194865_4	Hla_4	alpha-hemolysin precursor	5	0.24	162
88194865_5	Hla_5	alpha-hemolysin precursor	10	0.37	347
88194865_6	Hla_6	alpha-hemolysin precursor	11	0.44	351
88194865_7	Hla_7	alpha-hemolysin precursor	12	0.48	339
88194865_8	Hla_8	alpha-hemolysin precursor	3	0.19	142
88194865_9	Hla_9	alpha-hemolysin precursor	15	0.59	329
88194865_10	Hla_10	alpha-hemolysin precursor	14	0.58	365
88195062	88195062	hypothetical protein	23	0.65	355
88195171	IlvA	threonine dehydratase	15	0.46	164
88195360	Pbp3	penicillin-binding protein 3	23	0.35	431
88195630	SplF	serine protease SplF, putative	13	0.75	240
88195631	SplE	serine protease SplE	17	0.73	187
88195633	SplD	serine protease SplD	12	xxx	112
88195634_1	SplC_1	serine protease SplC	14	0.55	140
88195635_1	SplB_1	serine protease SplB	11	0.42	452
88195635_2	SplB_2	serine protease SplB	11	0.42	340
88195635_3	SplB_3	serine protease SplB	13	0.44	226
88195636	SplA	serine protease SplA	10	0.46	423
88195657	TraP	signal transduction protein TraP	15	0.89	456
88195808_1	88195808_1	staphopain thiol proteinase (UniProt: sspP)	10	0.29	234
88195808_2	88195808_2	staphopain thiol proteinase (UniProt: sspP)	10	0.34	82
88195808_3	88195808_3	staphopain thiol proteinase (UniProt: sspP)	8	0.18	264
88195808_4	88195808_4	staphopain thiol proteinase (UniProt: sspP)	10	0.29	168
88195808_5	88195808_5	staphopain thiol proteinase (UniProt: sspP)	14	0.38	109
88195913_1	Hlb_1	truncated beta-hemolysin	14	0.62	211
88195913_2	Hlb_2	truncated beta-hemolysin	15	0.7	212
88195913_3	Hlb_3	truncated beta-hemolysin	12	0.48	162
88195914	LukF	Leukocidin/hemolysin toxin family F subunit	16	0.45	316
88196081	88196081	hypothetical protein	7	0.67	81
88196115_1	HysA_1	hyaluronate lyase	18	0.32	184
88196115_2	HysA_2	hyaluronate lyase	18	0.33	104
88196118	Map	truncated MHC class II analog protein	7	0.61	196
88196130	RplM	ribosomal protein L13	11	0.73	226
88196147	RplF	ribosomal protein L6, putative	17	0.75	267
88196148	RpsH	ribosomal protein S8, putative	7	0.59	68
88196150	RplE	50S ribosomal protein L5, putative	13	0.86	293
88196156	RpsC	ribosomal protein S3	11	0.5	354
88196215_1	SsaA_a_1	secretory antigen precursor, putative	8	0.52	455
88196215_2	SsaA_a_2	secretory antigen precursor, putative	8	0.52	516
88196215_3	SsaA_a_3	secretory antigen precursor, putative	7	0.48	335
88196215_4	SsaA_a_4	secretory antigen precursor, putative	11	0.62	411
88196348	HlgA	gamma-hemolysin component A	7	0.4	145
88196349	HlgC	gamma-hemolysin component C	14	0.56	332
88196350	HlgB	gamma-hemolysin component B	12	0.43	297
88196554	Mqo2	malate:quinone-oxidoreductase	18	0.47	276
88196593	IsaB	immunodominant antigen B, hypothetical	12	0.46	191
88196599_1	88196599_1	N-acetylmuramoyl-L-alanine amidase	16	0.36	356
88196599_2	88196599_2	N-acetylmuramoyl-L-alanine amidase	20	0.41	240
88196625_1	Geh_1	glycerol ester hydrolase (geh)	13	xxx	159
88196625_2	Geh_2	glycerol ester hydrolase (geh)	14	0.26	168
88196625_3	Geh_3	glycerol ester hydrolase (geh)	15	0.26	269
88196625_4	Geh_4	glycerol ester hydrolase (geh)	14	0.25	337
88196625_5	Geh_5	glycerol ester hydrolase (geh)	17	0.28	197

88196625_6	Geh_6	glycerol ester hydrolase (geh)	22	0.37	214
88196625_7	Geh_7	glycerol ester hydrolase (geh)	19	0.31	176
88196625_8	Geh_8	glycerol ester hydrolase (geh)	24	0.41	315
88196625_9	Geh_9	glycerol ester hydrolase (geh)	20	0.35	190
88196625_10	Geh_10	glycerol ester hydrolase (geh)	23	0.42	177
88196625_11	Geh_11	glycerol ester hydrolase (geh)	22	0.48	255
88196625_12	Geh_12	glycerol ester hydrolase (geh)	20	0.43	243
88196625_13	Geh_13	glycerol ester hydrolase (geh)	22	0.43	250
88196625_14	Geh_14	glycerol ester hydrolase (geh)	23	0.32	264
88196625_15	Geh_15	glycerol ester hydrolase (geh)	20	0.29	206
88196625_16	Geh_16	glycerol ester hydrolase (geh)	21	0.36	245

B) pH 4-7

Accession No.	**Protein name**	**Full name**	**PC**	**SC**	**PS**
88193825	DnaN	DNA polymerase III, beta subunit	19	0.58	541
88193832	SerS	seryl-tRNA synthetase	16	0.46	239
88193840	PurA	adenylosuccinate synthetase	19	0.51	525
88193872	88193872	staphylococcal tandem lipoprotein, putative	7	0.23	145
88193885_1	Spa_1	protein A	5	0.22	103
88193885_2	Spa_2	protein A	9	0.39	52
88193885_3	Spa_3	protein A	5	0.22	62
88193885_4	Spa_4	protein A	9	xxx	52
88193885_5	Spa_5	protein A	9	0.35	118
88193911	Pnp	purine nucleoside phosphorylase	11	0.47	440
88193914	DeoB	phosphopentomutase	16	0.59	426
88193948	88193948	hypothetical protein	7	0.45	165
88193997	PflB	formate acetyltransferase	29	0.48	393
88194016	Ldh_a	L-lactate dehydrogenase	20	0.59	368
88194055	LytM	peptidoglycan hydrolase, putative	6	0.28	218
88194062	88194062	staphyloxanthin biosynthesis protein, putative	3	0.14	89
88194063	88194063	conserved hypothetical protein/virulence factor EsxA	7	0.48	130
88194162	AhpF	alkyl hydroperoxide reductase, subunit F, putative	14	0.48	111
88194163	AhpC	alkyl hydroperoxide reductase subunit C	8	0.56	337
88194172	GuaB	inosine-5-monophosphate dehydrogenase	20	0.61	256
88194173	GuaA	GMP synthase, putative	21	0.52	296
88194194	Ssl11	staphylococcal superantigen-like protein 11	10	0.52	193
88194248	MetS	methionyl-tRNA synthetase, putative	26	0.44	359
88194261	RplY	ribosomal 50S rRNA E-loop binding protein	7	0.35	247
88194290	GltX	glutamyl-tRNA synthetase	23	0.49	349
88194309	Fus	translation elongation factor G	27	0.53	349
88194310	Tuf	translation elongation factor Tu	6	0.2	72
88194313	HchA	chaperone protein HchA	14	0.49	361
88194324	SdrC	sdrC protein, putative	13	0.17	267
88194332	88194332	hexulose-6-phosphate synthase, putative	6	0.5	148
88194349	Pta	phosphate acetyltransferase	14	0.66	427
88194378	Adh1	alcohol dehydrogenase I, putative	19	0.66	436
88194436_1	88194436_1	secretory antigen SsaA-like protein	6	0.29	135
88194436_2	88194436_2	secretory antigen SsaA-like protein	2	0.14	189
88194546	TrxB	thioredoxin reductase	15	0.62	278
88194551	ClpP	ATP-dependent Clp protease, proteolytic subunit ClpP	8	0.34	102
88194555_1	Gap_1	glyceraldehyde-3-phosphate dehydrogenase, type I	14	0.54	266
88194555_2	Gap_2	glyceraldehyde-3-phosphate dehydrogenase, type I	17	0.62	323
88194555_3	Gap_3	glyceraldehyde-3-phosphate dehydrogenase, type I	11	0.5	178
88194556	Pgk	phosphoglycerate kinase, putative	13	0.5	333
88194557	TpiA	triosephosphate isomerase	11	0.61	274
88194558	Pgm	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	20	0.38	515

88194559	Eno	enolase	22	0.59	474
88194622	88194622	conserved hypothetical protein	10	0.46	326
88194648	Ppi	peptidyl-prolyl cis-trans isomerase, cyclophilin-type, putative	7	0.46	382
88194657	Pgi	glucose-6-phosphate isomerase	20	0.54	544
88194663	88194663	fumarylacetoacetate (FAA) hydrolase family protein, putative	12	0.7	226
88194665	Cdr	coenzyme A disulfide reductase, putative	17	0.5	139
88194694	PepF	oligoendopeptidase F	22	0.34	292
88194704	FabI	enoyl-(acyl-carrier-protein) reductase	17	0.73	413
88194744_1	SspB_1	cysteine protease precursor, putative	16	0.54	389
88194744_2	SspB_2	cysteine protease precursor, putative	13	0.16	271
88194744_3	SspB_3	cysteine protease precursor, putative	5	0.23	171
88194744_4	SspB_4	cysteine protease precursor, putative	15	0.46	167
88194744_5	SspB_5	cysteine protease precursor, putative	16	0.56	171
88194744_6	SspB_6	cysteine protease precursor, putative	17	0.6	213
88194744_7	SspB_7	cysteine protease precursor, putative	16	0.53	243
88194745_1	SspA_1	glutamyl endopeptidase precursor, putative, V8 protease	9	0.3	199
88194745_2	SspA_2	glutamyl endopeptidase precursor, putative, V8 protease	7	0.22	278
88194745_3	SspA_3	glutamyl endopeptidase precursor, putative, V8 protease	5	0.18	159
88194745_4	SspA_4	glutamyl endopeptidase precursor, putative, V8 protease	11	0.31	273
88194745_5	SspA_5	glutamyl endopeptidase precursor, putative, V8 protease	11	0.35	399
88194745_6	SspA_6	glutamyl endopeptidase precursor, putative, V8 protease	10	0.44	307
88194745_7	SspA_7	glutamyl endopeptidase precursor, putative, V8 protease	13	0.56	267
88194745_8	SspA_8	glutamyl endopeptidase precursor, putative, V8 protease	13	0.56	331
88194745_9	SspA_9	glutamyl endopeptidase precursor, putative, V8 protease	11	0.51	189
88194745_10	SspA_10	glutamyl endopeptidase precursor, putative, V8 protease	13	0.52	298
88194745_11	SspA_11	glutamyl endopeptidase precursor, putative, V8 protease	13	0.44	343
88194753_1	88194753_1	transcriptional regulator, putative	10	0.38	295
88194753_2	88194753_2	transcriptional regulator, putative	9	0.32	308
88194766	PurQ	phosphoribosylformylglycinamidine synthase I	15	0.82	277
88194768	PurF	amidophosphoribosyltransferase	13	0.33	297
88194782	PtsI	phosphoenolpyruvate-protein phosphotransferase	16	0.38	512
88194792_1	PdhA_1	pyruvate dehydrogenase complex, E1 component, alpha subunit, putative	12	0.45	382
88194792_2	PdhA_2	pyruvate dehydrogenase complex, E1 component, alpha subunit, putative	7	0.22	64
88194793_1	PdhB_1	pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase beta subunit, putative	16	0.62	338
88194793_2	PdhB_2	pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase beta subunit, putative	9	0.4	262
88194794	PdhC	dihydrolipoamide S-acetyltransferase component of pyruvate dehydrogenase complex E2, putative	15	0.39	354
88194795_1	PdhD_1	dihydrolipoamide dehydrogenase	15	0.42	606
88194795_2	PdhD_2	dihydrolipoamide dehydrogenase	24	0.57	482
88194795_3	PdhD_3	dihydrolipoamide dehydrogenase	24	0.54	657
88194795_4	PdhD_4	dihydrolipoamide dehydrogenase	14	0.4	94
88194813	PycA	pyruvate carboxylase	26	0.32	161
88194865	Hla	alpha-hemolysin precursor	7	0.25	255
88194899	IleS	isoleucyl-tRNA synthetase	22	0.33	301
88194919	88194919	conserved hypothetical protein	8	0.4	268
88194963	CodY	transcriptional regulator CodY	12	0.55	248
88194965	RpsB	ribosomal protein S2	14	0.47	173
88194967	Tsf	translation elongation factor Ts	18	0.7	365
88194969	Frr	ribosome recycling factor	9	0.51	164
88195018	GlnA	glutamine synthetase, type I	19	0.45	332
88195057	KatA	catalase	18	0.43	253
88195066_1	Tkt_1	transketolase	17	0.4	246
88195066_2	Tkt_2	transketolase	22	0.45	229
88195066_3	Tkt_3	transketolase	16	0.39	250
88195075	AcnA	aconitate hydratase 1	35	0.48	552
88195129_1	CspA_1	cold shock protein, putative	3	0.78	131

88195129_2	CspA_2	cold shock protein, putative	3	0.78	137
88195154	88195154	phosphotransferase system enzyme IIA, putative	8	0.65	201
88195217	EbpS	elastin binding protein	6	0.11	153
88195310	Zwf	glucose-6-phosphate 1-dehydrogenase	18	0.63	302
88195316_1	Gnd_1	6-phosphogluconate dehydrogenase, decarboxylating	23	0.56	378
88195316_2	Gnd_2	6-phosphogluconate dehydrogenase, decarboxylating	16	xxx	198
88195335_1	Efp_1	translation elongation factor P	6	0.44	157
88195335_2	Efp_2	translation elongation factor P	5	0.4	124
88195336_1	88195336_1	proline dipeptidase, putative	18	0.58	413
88195336_2	88195336_2	proline dipeptidase, putative	15	0.49	160
88195341	88195341	glycine cleavage system P-protein subunit ll, putative	17	0.43	227
88195342	88195342	glycine cleavage system P-protein subunit I, putative	13	0.4	166
88195389_1	DnaK_1	DNAk protein, putative	16	0.41	465
88195389_2	DnaK_2	DNAk protein, putative	9	0.2	284
88195390	GrpE	co-chaperone GrpE	6	0.37	360
88195419	GreA	transcription elongation factor GreA	6	0.67	118
88195468_1	ValS_1	valyl-tRNA synthetase	21	0.31	160
88195468_2	ValS_2	valyl-tRNA synthetase	18	0.31	356
88195480_1	Tig_1	trigger factor	10	0.26	76
88195480_2	Tig_2	trigger factor	7	0.13	256
88195507	PfkA	6-phosphofructokinase, putative	14	0.47	149
88195515	88195515	metallo-beta-lactamase superfamily protein, putative	13	0.86	299
88195519	UspA	conserved hypothetical protein	14	0.86	265
88195522	88195522	thiol peroxidase (tpx)	10	0.9	390
88195544_1	Fhs_1	formate-tetrahydrofolate ligase, putative	13	0.36	383
88195544_2	Fhs_2	formate-tetrahydrofolate ligase, putative	18	0.43	489
88195550	88195550	conserved hypothetical protein	10	0.32	246
88195565	88195565	putative peptidase	25	0.59	472
88195572	LeuS	leucyl-tRNA synthetase	18	0.31	317
88195596	Tal	transaldolase	22	0.9	258
88195605	PckA	phosphoenolpyruvate carboxykinase (ATP)	25	0.54	265
88195634	SplC	serine protease SplC	15	0.55	254
88195657	TraP	signal transduction protein TraP	15	0.77	339
88195790	Ftn	ferritin, putative	7	0.39	214
88195807	PurB	adenylosuccinate lyase	23	0.55	408
88195838	88195838	aminotransferase, putative	10	0.3	250
88195925	GroEL	chaperonin GroEL, putative	20	0.55	477
88195926	GroES	chaperonin GroES, putative	10	0.8	150
88196007	AtpD	ATP synthase F1, beta subunit	22	0.6	354
88196017	GlyA	serine hydroxymethyltransferase, putative	7	0.23	285
88196029_1	FbaA_1	fructose-bisphosphate aldolase	15	0.73	339
88196029_2	FbaA_2	fructose-bisphosphate aldolase	13	0.63	236
88196043	DeoD	purine nucleoside phosphorylase	12	0.41	407
88196136	RpoA	DNA-directed RNA polymerase alpha chain, putative	14	0.43	207
88196141	Adk	adenylate kinase, putative	13	0.64	131
88196220_1	SsaA_b_1	secretory antigen precursor SsaA, putative	1	0.09	123
88196220_2	SsaA_b_2	secretory antigen precursor SsaA, putative	1	0.09	105
88196221	88196221	D-isomer specific 2-hydroxyacid dehydrogenase	10	xxx	72
88196226	LytR	transcriptional regulator, putative	10	0.36	263
88196515_1	IsaA_1	immunodominant antigen A, putative	2	0.15	82
88196515_2	IsaA_2	immunodominant antigen A, putative	3	0.19	95
88196515_3	IsaA_3	immunodominant antigen A, putative	2	0.15	64
88196515_4	IsaA_4	immunodominant antigen A, putative	2	0.15	67
88196515_5	IsaA_5	immunodominant antigen A, putative	2	0.13	75
88196515_6	IsaA_6	immunodominant antigen A, putative	1	0.09	72
88196515_7	IsaA_7	immunodominant antigen A, putative	1	0.09	82
88196515_8	IsaA_8	immunodominant antigen A, putative	1	0.09	60
88196515_9	IsaA_9	immunodominant antigen A, putative	4	0.23	164

88196515_10	IsaA_10	immunodominant antigen A, putative	2	0.13	85
88196549	Ldh_b	L-lactate dehydrogenase	18	0.66	296
88196553	Fda	fructose-bisphosphate aldolase class-I, putative	17	0.73	414
88196554	Mqo2	malate:quinone-oxidoreductase	11	0.33	244
88196589	ArgF	ornithine carbamoyltransferase	20	0.54	287
88196592_1	Aur_1	aureolysin, putative	17	0.44	584
88196592_2	Aur_2	aureolysin, putative	12	0.35	376
88196599_1	88196599_1	N-acetylmuramoyl-L-alanine amidase	12	0.33	167
88196599_2	88196599_2	N-acetylmuramoyl-L-alanine amidase	13	0.3	559
88196599_3	88196599_3	N-acetylmuramoyl-L-alanine amidase	9	0.21	312
88196599_4	88196599_4	N-acetylmuramoyl-L-alanine amidase	12	0.27	439
88196599_5	88196599_5	N-acetylmuramoyl-L-alanine amidase	4	0.07	106
88196599_6	88196599_6	N-acetylmuramoyl-L-alanine amidase	21	0.4	288
88196599_7	88196599_7	N-acetylmuramoyl-L-alanine amidase	22	0.46	445
88196599_8	88196599_8	N-acetylmuramoyl-L-alanine amidase	25	0.48	373
88196599_9	88196599_9	N-acetylmuramoyl-L-alanine amidase	11	0.18	68
88196599_10	88196599_10	N-acetylmuramoyl-L-alanine amidase	16	0.35	115
88196599_11	88196599_11	N-acetylmuramoyl-L-alanine amidase	16	0.32	129
88196625_1	Geh_1	glycerol ester hydrolase (geh)	8	0.19	128
88196625_2	Geh_2	glycerol ester hydrolase (geh)	6	0.16	131
88196625_3	Geh_3	glycerol ester hydrolase (geh)	6	0.16	117
88196625_4	Geh_4	glycerol ester hydrolase (geh)	24	0.41	311
88196625_5	Geh_5	glycerol ester hydrolase (geh)	24	0.41	311
88196640	YceI	conserved hypothetical protein	9	xxx	187

[a] Protein names according to the annotated *S . aureus* strain 8325, Col, N315 and Newman genome sequences based on the aureusDB database (http://aureusdb.biologie.uni-greifswald.de/), the Entrez database (http:// www.ncbi.nlm.nih.gov/sites/entrez) and UniProt database (http://www.uniprot.org).

Ldh and SsaA were found in the public databases (see footnote a) with two similar but different sequences and are therefore numbered with _a and _b.

Multiple spots belonging to one protein are indicated by a number followed the accession number or the protein name: eg 88193871_1; Plc_1

[b]: The representative Mascot search results displayed for each spot are from one out of 2 or 3 identification replicates; PC: peptide count; SC: sequence coverage, PS: protein score.

Table A.3: Presence of genes encoding proteins homologous to immune reactive proteins in this study

Protein name[a]	Accession No.	Locali-zation [b]	Col[c]	MRSA 252	MSSA 476	Mu50	MW2	N315	8325	Newman	RF122	JH9	USA300
Toxins and Hemolysins[d] (5/6)[e]													
Hla	88194865	ec	1	0	1	1	1	1	1	1	1	1	1
Hlb	88195913	ec	1	0	0	1	1	1	1	1	1	1	1
HlgA	88196348	ec	3	2	3	3	4	3	3	3	4	3	4
HlgB	88196350	ec	2	1	2	2	3	2	2	2	3	2	3
HlgC	88196349	ec	3	2	3	3	4	3	3	3	4	3	4
LukF	88195914	ec	1	1	1	1	1	1	1	1	1	1	1
Virulence/Defence mechanisms (8/13)													
Aur	88196592	ec	1	1	1	1	1	1	1	1	1	1	1
Geh	88196625	ec	1	2	2	2	2	2	2	2	2	2	2
Lip	88194101	ec	1	2	2	2	2	2	2	2	2	2	2
Nuc	88194577	ec	1	1	1	1	1	1	1	1	1	1	1
Plc	88193871	ec	1	1	0	1	1	1	1	1	0	1	1
SplA	88195636	ec	1	0	1	1	1	1	1	1	0	1	1
SplB	88195635	ec	1	0	1	1	1	1	1	1	1	1	1
SplD	88195633	ec	2	0	1	2	1	2	2	2	1	1	2
SplE	88195631	ec	1	1	0	0	0	0	1	1	0	0	1
SplF	88195630	ec	2	0	1	2	1	2	2	2	1	1	2
SspA	88194745	ec	1	1	1	1	1	1	1	1	1	1	1
SspB	88194744	ec	1	1	1	1	1	1	1	1	1	1	1
88195808	88195808	ec	1	1	1	1	1	1	1	1	1	1	1
Cell wall biosynthesis and degradation (0/9)													
Atl	88194750	ec	1	1	1	1	1	1	1	1	1	1	1
IsaA	88196515	ec	1	1	1	1	1	1	1	1	1	1	1
IsdA	88194829	cw	1	1	1	1	1	1	1	1	1	1	1
SsaA_a	88196215	ec	2	2	2	2	2	2	2	2	2	2	2
SsaA_b	88196220	unkn	1	1	1	1	1	1	1	1	1	1	1
88193909	88193909	cw	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
88194219	88194219	ec	1	1	1	1	1	1	1	1	1	1	1
88194436	88194436	ec	1	1	1	1	1	1	1	1	1	1	1
88196599	88196599	ec	1	1	1	1	1	1	1	1	1	1	1
Stress response proteins (0/1)													
DnaK	88195389	ic	1	1	1	1	1	1	1	1	1	1	1
Energy metabolism (0/4)													
Gnd	88195316	ic	1	1	1	1	1	1	1	1	1	1	1
Pgi	88194657	ic	1	1	1	1	1	1	1	1	1	1	1
Pgk	88194556	ic	1	1	1	1	1	1	1	1	1	1	1
88194493	88194493	ic/ m	1	1	1	1	1	1	1	1	1	1	1
Amino acid biosynthesis (0/1)													
88195838	88195838	unkn	1	1	1	1	1	1	1	1	1	1	1
Transport and binding proteins (0/1)													
88194087	88194087	unkn	1	1	1	1	1	1	1	1	1	1	1
Unknown function (1/1)													

88194062	88194062	unkn	1	1	1	1	1	1	1	1	0	1	1

[a] Protein names according to the annotated *S . aureus* strain 8325, Col, N315 and Newman genome sequences based on the aureusDB database (http://aureusdb.biologie.uni-greifswald.de/), the Entrez database (http:// www.ncbi.nlm.nih.gov/sites/entrez) and UniProt database (http://www.uniprot.org).
Ldh and SsaA were found in the public databases (see footnote a) with two similar but different sequences and are therefore numbered with _a and _b.

[b] Protein localization was predicted by P-SORT. ic: intracellular, ec: extracellular, m: membrane, cw: cell wall, unkn: unknown.

[c] Genes were searched for number of copies. In each of 11 sequenced strains, the numbers of gene copies encoding for proteins, which are homologous to the immune reactive proteins identified in our study, are indicated. The analysis was based on the aureusDB database (http://aureusdb.biologie.uni-greifswald.de/).

[d] Protein classifications were adapted from Burlak *et al.* and Tigr database (http://www.cmr.tigr.org).

[e] Number of proteins with variable presence in the 11 sequenced strains/ total number of identified immune reactive proteins.

Table A.4: Intensity of significant immune reactive protein spots detected on 2-D IBs probed with serum pool at pH ranges 6-11 (A) and 4-7 (B).

A)

IB spot label	Protein name[a]	Median[b]	Interquartile range		
6-11_59	Hla_8, Hla_3	**57.30**	47.02	-	60.50
6-11_78	SplB_2, SplB_3	**39.22**	36.75	-	43.26
6-11_17	Atl_3	**25.66**	12.63	-	29.12
6-11_56	88194219_4	**24.26**	13.91	-	27.10
6-11_68	Plc_3	**23.84**	21.39	-	25.28
6-11_73	88194219_1, 88194219_2, 88194219_3	**20.36**	18.43	-	26.22
6-11_50	Atl_4	**20.34**	16.01	-	22.79
6-11_5	Atl_1, Atl_2	**19.22**	8.02	-	31.37
6-11_116	88195808_4	**18.22**	14.88	-	20.36
6-11_60	Hla_9	**16.19**	13.08	-	19.12
6-11_87	n.i.	**13.83**	11.10	-	16.84
6-11_18	88194493_1	**12.04**	11.34	-	13.42
6-11_30	Lip_9, Lip_10	**11.51**	10.62	-	13.51
6-11_55	Hla_10	**11.42**	10.32	-	14.68
6-11_38	Atl_5, Atl_6, Atl_7	**9.89**	6.33	-	12.36
6-11_67	HlgA, 88194087 (1 spot); IsdA	**7.64**	6.46	-	11.29
6-11_31	Lip_8	**6.62**	5.12	-	8.70
6-11_85	SplE	**6.03**	5.15	-	7.91
6-11_72	SsaA_a_2, SsaA_a_3, SsaA_a_4	**5.84**	2.56	-	7.33
6-11_103	88195808_2	**5.77**	5.04	-	6.89
6-11_51	Hlb_2	**5.04**	4.68	-	6.43
6-11_94	88195808_5	**4.50**	2.67	-	6.05
6-11_48	HlgB	**4.36**	3.77	-	6.14
6-11_20	88194493_2, Geh_2	**4.27**	3.77	-	4.84
6-11_57	HlbC	**4.23**	3.57	-	4.63
6-11_2	Lip_5, Lip_12	**4.20**	3.25	-	5.27
6-11_49	Hlb_1	**3.83**	2.63	-	5.78
6-11_98	88193909_6	**3.17**	2.51	-	3.96
6-11_22	Geh_1	**3.15**	1.19	-	4.60
6-11_25	n.i.	**3.03**	1.08	-	7.07
6-11_99	88195808_1	**2.89**	2.48	-	3.68
6-11_76	n.i.	**2.64**	1.45	-	3.17
6-11_58	88194062	**2.45**	1.89	-	3.21
6-11_84	n.i.	**2.40**	1.66	-	6.03
6-11_82	SplA	**2.37**	2.04	-	3.47
6-11_46	LukF	**2.20**	1.36	-	4.29
6-11_27	n.i.	**2.13**	0.18	-	2.63
6-11_8	n.i.	**2.01**	1.19	-	3.51
6-11_52	n.i.	**1.76**	0.33	-	2.14
6-11_101	Nuc	**1.75**	1.62	-	2.08
6-11_3	Lip_6	**1.66**	0.72	-	1.95
6-11_74	SsaA_1	**1.62**	0.89	-	2.59
6-11_86	SplF, SplD	**1.61**	1.13	-	2.20
6-11_21	n.i.	**1.60**	0.58	-	2.00
6-11_65	n.i.	**1.55**	1.12	-	1.71
6-11_107	88193909_8	**1.47**	1.22	-	1.61
6-11_19	n.i.	**1.20**	0.30	-	1.90
6-11_28	Lip_3, Lip_4	**1.19**	0.55	-	1.99
6-11_100	n.i.	**1.09**	0.86	-	1.22

6-11_105	n.i.	**1.08**	0.21	-	1.53
6-11_13	n.i.	**1.06**	0.55	-	1.29
6-11_89	n.i.	**1.05**	0.89	-	1.25
6-11_69	n.i.	**1.00**	0.91	-	1.15
6-11_36	n.i.	**0.98**	0.40	-	1.42
6-11_71	n.i.	**0.97**	0.48	-	1.67
6-11_24	n.i.	**0.86**	0.29	-	1.35
6-11_45	Lip_7	**0.79**	0.63	-	1.07
6-11_110	88193909_7	**0.74**	0.67	-	0.91
6-11_66	n.i.	**0.70**	0.07	-	2.24
6-11_4	Lip_1	**0.59**	0.11	-	0.71
6-11_115	n.i.	**0.57**	0.18	-	0.81
6-11_41	n.i.	**0.52**	0.27	-	0.67
6-11_12	n.i.	**0.52**	0.21	-	3.00
6-11_95	n.i.	**0.51**	0.43	-	0.59
6-11_108	n.i.	**0.51**	0.29	-	0.73
6-11_106	88193909_4	**0.50**	0.42	-	0.59

B)

IB spot label	Protein name	Median	Interquartile range		
4-7_43	SspB_4, SspB_5, SspB_6	**27.7**	26.1	-	30.9
4-7_67	SspA_9, SspA_10, SspA_11	**27.6**	24.6	-	31.8
4-7_59	SspA_5, SspA_7, SspA_8	**24.9**	24.1	-	27.2
4-7_40	SspB_7	**15.8**	12.8	-	18.0
4-7_86	IsaA_9	**8.7**	6.6	-	10.0
4-7_69	SspA_1, SspA_6	**7.9**	6.5	-	10.0
4-7_71	SspA_2, SspA_3, DnaK_2	**7.3**	6.3	-	9.1
4-7_49	SspB_1	**6.8**	5.5	-	8.4
4-7_37	Pgk	**6.6**	5.8	-	8.7
4-7_38	88195838, Gnd_1, Gnd_2	**6.0**	5.8	-	7.5
4-7_89	88194436_1	**5.7**	4.8	-	6.5
4-7_8	88196599_2	**5.7**	3.4	-	7.6
4-7_119	IsaA_6, IsaA_7, IsaA_8	**4.3**	3.3	-	5.1
4-7_1	88196599_7	**3.8**	3.4	-	5.3
4-7_9	88196599_10	**2.8**	1.9	-	3.6
4-7_58	88196599_4	**2.6**	2.2	-	3.6
4-7_114	88194436_2	**2.1**	1.1	-	3.0
4-7_110	SsaA_b_2	**1.7**	1.4	-	2.1
4-7_117	n.i.	**1.6**	0.4	-	2.2
4-7_4	88196599_6	**1.5**	1.2	-	2.5
4-7_25	88196599_3	**1.2**	0.9	-	1.9
4-7_35	n.i.	**1.0**	0.5	-	1.2
4-7_56	Aur_1, Aur_2	**0.9**	0.8	-	1.1
4-7_112	88196599_9	**0.9**	0.4	-	1.0
4-7_101	n.i.	**0.9**	0.6	-	1.2
4-7_33	n.i.	**0.8**	0.7	-	0.9
4-7_46	n.i.	**0.7**	0.5	-	1.1
4-7_107	IsaA_4	**0.7**	0.5	-	0.8
4-7_91	IsaA_2	**0.6**	0.5	-	0.7
4-7_42	SspB_2	**0.6**	0.5	-	0.8
4-7_14	88196599_11	**0.6**	0.1	-	0.8
4-7_115	SsaA_1	**0.6**	0.5	-	0.8
4-7_28	n.i.	**0.5**	0.4	-	0.6
4-7_108	n.i.	**0.5**	0.3	-	0.8
4-7_118	IsaA_5	**0.5**	0.5	-	0.6
4-7_36	Pgi	**0.5**	0.4	-	0.7
4-7_77	n.i.	**0.5**	0.2	-	0.7
4-7_3	88196599_8	**0.5**	0.4	-	0.8

[a] Protein names were referred according to reference maps. IB spots with high signal intensity sometimes cover several protein spots on the reference map, thus, are indicated in several protein names. Some IB spots still could not be identified through matching with the reference maps (n.i.).

[b]: Spot signal intensity of master IBs were quantified in a different project with 8 replicates for each pH range, 6-11 and 4-7. Significant immune reactive spots were defined with median of spot intensity not less than 0.5 grey unit (cut-off: 0.5).

Table A.5: Studies addressing Ab binding to *S. aureus* proteins

Study Year	Origin of proteins	Origin of screening sera	Test for protectivity	Reference
1, 2002	Expression library with genomic fragments from *S. aureus* COL	Pooled human sera[a]; Individual human sera[b]	No	[61]
2, 2002	Total lysate and surface proteins of *S. aureus* COL in 2-D IB resolved at pH range 4-7	Pooled sera Individual human sera	No	[276]
3, 2003	Expression library with genomic fragments from *S. aureus* COL	Pooled sera Individual human sera	No	[261]
4, 2005	A collection of recombinant proteins[g]	Individual human sera[c]	No	[194]
5, 2006	Expression library	Individual human sera[d]	Vaccination of cotton rats with IsdA or IsdH	[27]
6, 2006	Total protein (intracellular, membrane and cell wall proteins) of a biofilm-forming *S.aureus* clinical strain in 2-D IB resolved at pH range 4-7	Rabbit sera[e]	No	[264]
7, 2007	Extracellular proteins of CA-MRSA (MW2, LAC) in 2-D IB resolved at pH 4-9	Mouse sera[f]	No	[258]

[a]: Sera with high anti-staphylococcal Ab titer were pooled separately from patients in the acute phase of an *S. aureus* infection and from healthy individuals

[b, c, d] : Sera from healthy individuals (carriers and noncarriers) and patients in the cute phase of an *S. aureus* infections (b, c) or from patients with in severe *S. aureus* bacteremia (d)

[e, f]: Sera from rabbits/ mice infected with the *S. aureus* clinical strains- MW2 or LAC.

[g]: The collection contained 11 cell wall , 4 membrane, 2 surface and 2 secreted proteins

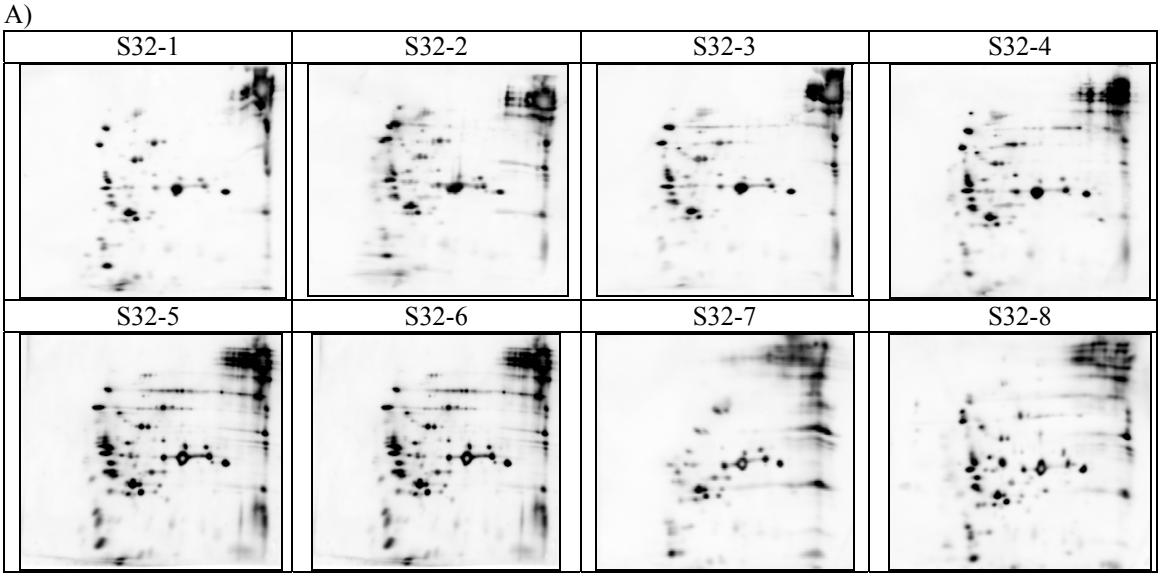
Table A.6: Overview about common immune reactive proteins from *S. aureus* based on published investigations

Immune reactive protein	Full name, putative function	Immune reactivity[a]
Atl	Autolysin	P, H: Study 1 [61]; P, H: Study 3 [261] P, H: Study 5 [27] I: Study 6 [264] NI, IM: Study 7 [258]
Aur	Aureolysin, Zinc metalloproteinase	P, H: Study 1 [61] P > H: Study 3 [261]
Hla	Alpha-hemolysin	P> H: Study 5 [27] I: Study 6 [264]
HlgB	Gamma-hemolysin B	P> H, Study 5 [27]
IsaA	Immunodominant secretory Ag A	P> H: Study 1 [61] P, H: Study 2 [276] P, H: Study 5 [27]
IsdA	Iron-regulated surface determinant A	P> H, cotton rats (protective): Study 5 [27]
Lip	Lipase	P> H: Study 1 [61] H> P: Study 3 [261] I: Study 6 [264] IM: Study 7 [258]
Plc	1-phosphatidylinositol phosphodiesterase	IM: Study 7 [258]
SplA	Serine protease SplA	P> H: Study 5 [27]
SspB	Cystein protease	IM: Study 7 [258]

[a]: The immune reactivity of corresponding proteins were tested with sera from either healthy individual (H)/Patient (P); Non- (NI)/Immune (IM) mice; or I: *S. aureus* infected-rabbit, depending on the respective studies which were listed in Table A.5.

Figure A.1: Variation between IB replicates of serum pool performed at pH range 6-11 and 4-7. Serum from all 16 volunteers before and after colonization was pooled and used to probe *S. aureus* proteins blotted to PVDF membranes. Representative blots from eight independent experiments show good reproducibility except for very basic proteins where blotting efficiency was variable. -Experimental conditions: extracellular proteins of *S. aureus* 8325-4 (A) or *S. aureus* 8325-4 Δ *spa* (B) at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11 (A) or 4-7 (B), serum pool (1:10,000) and ECL substrate.

A)



B)

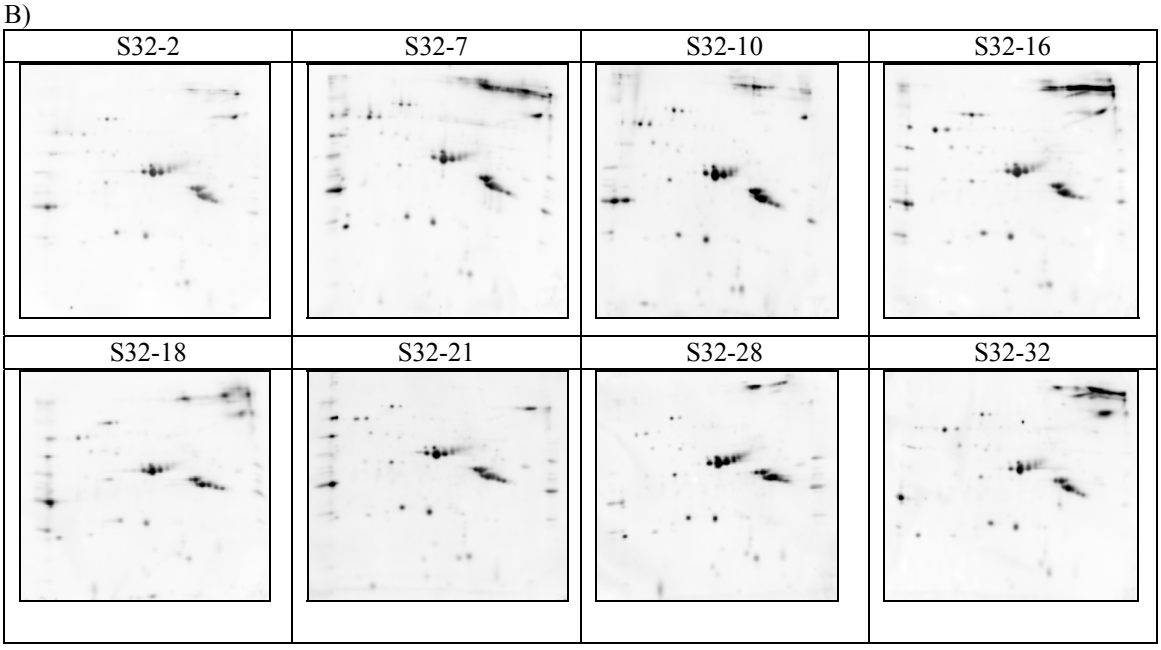
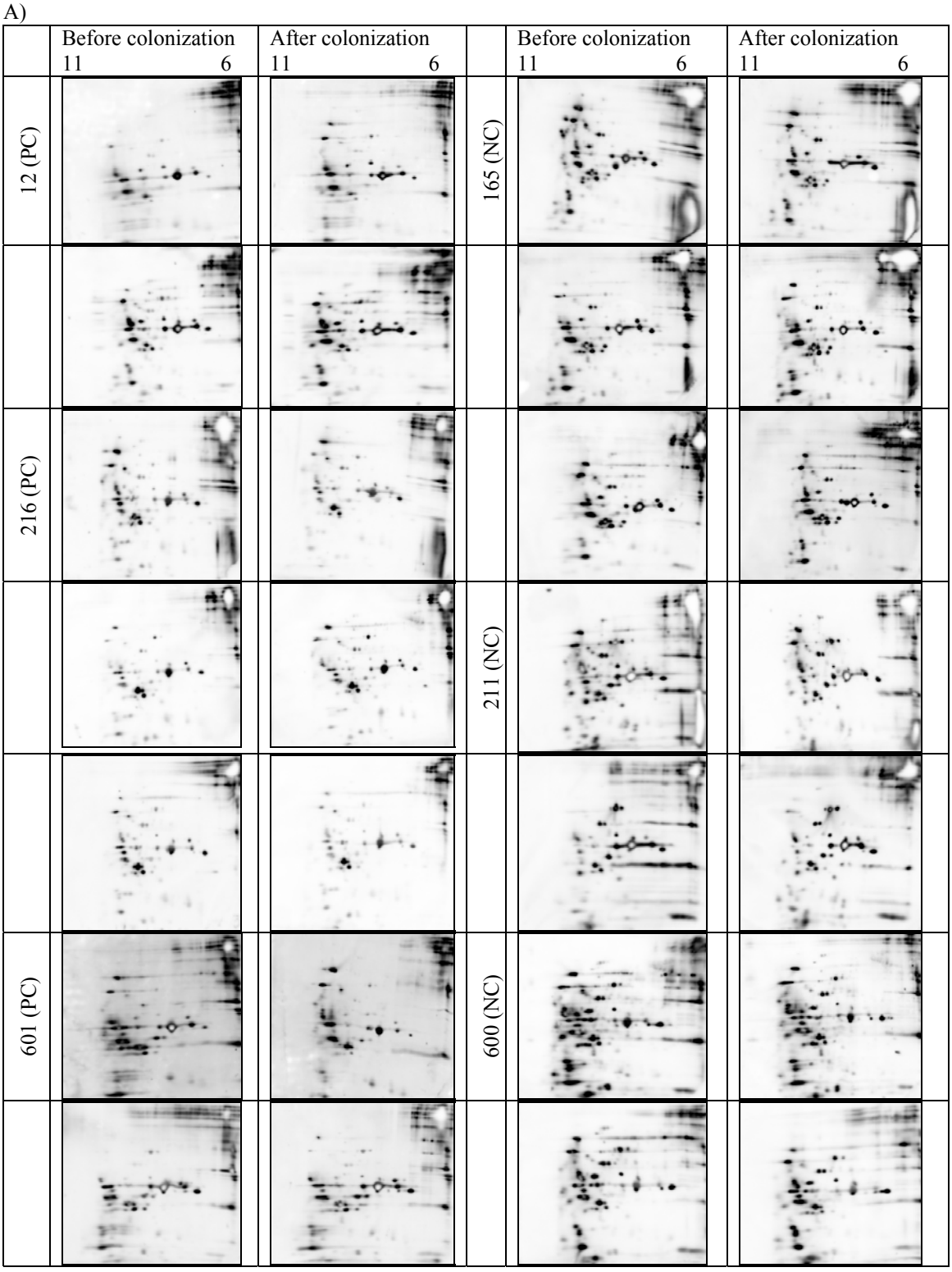
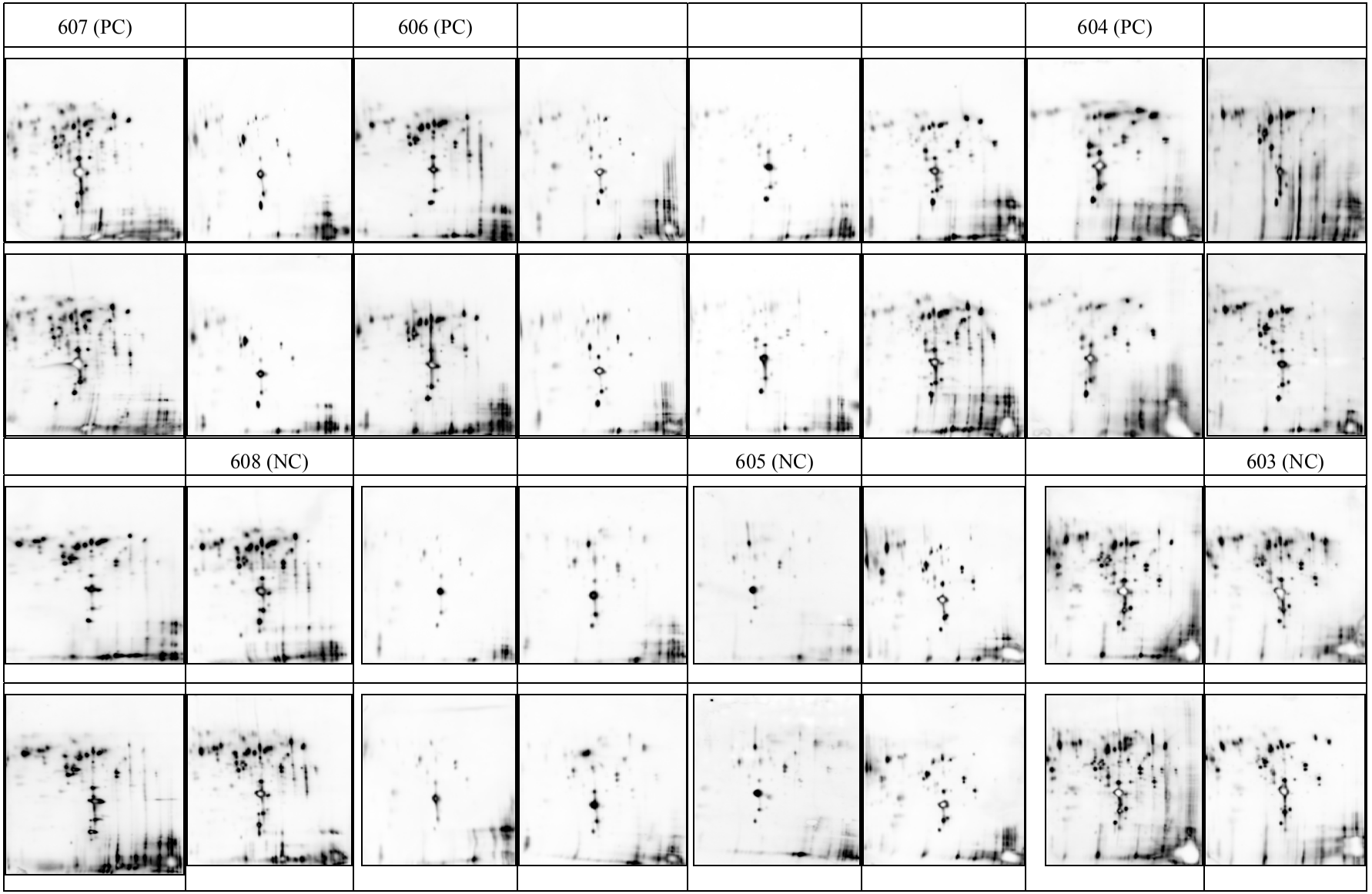
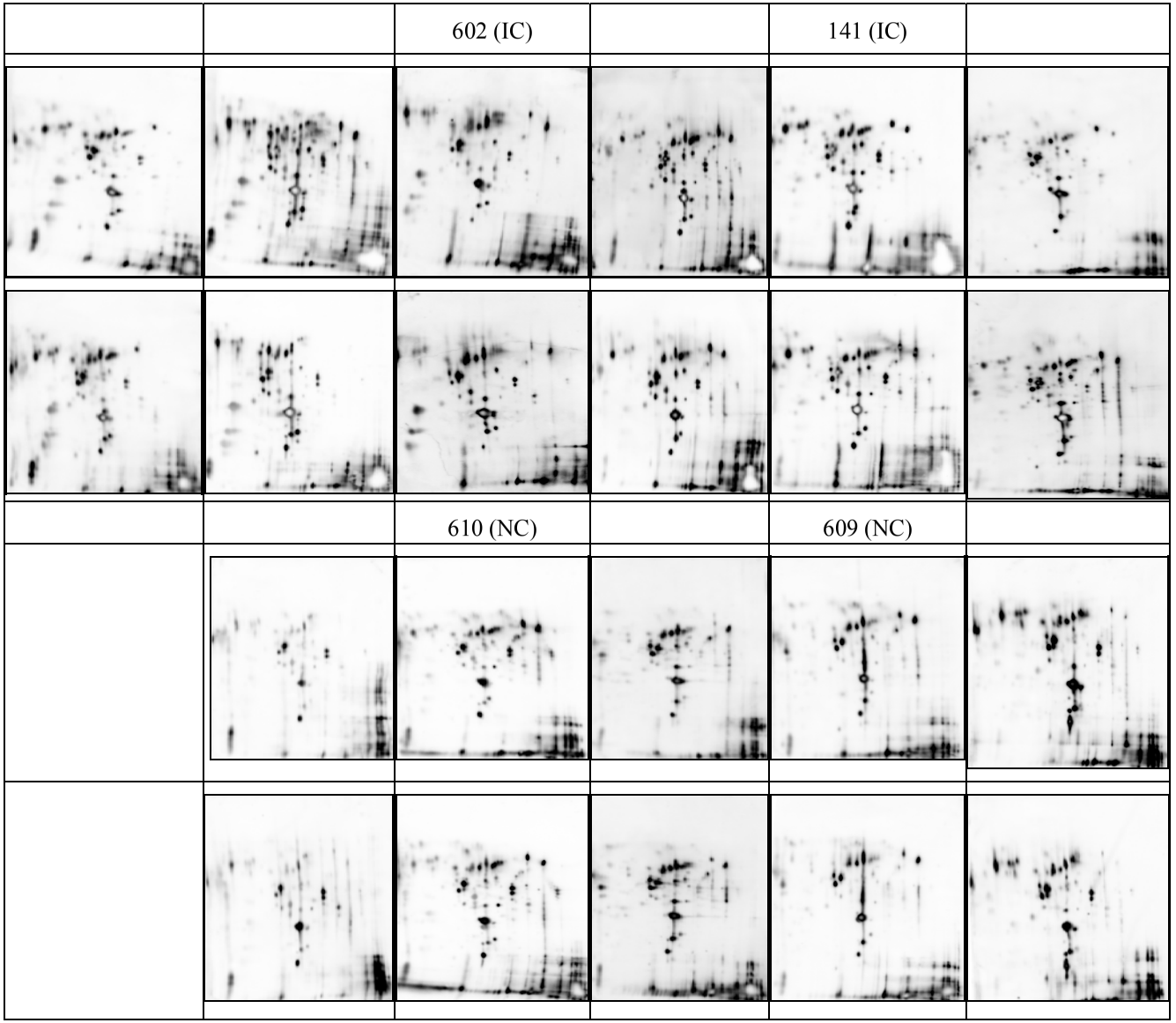


Figure A.2: IB replicates of all pre- and post-colonization sera of the 16 volunteers (pH 6-11 and 4-7). Two or three technical IB replicates were shown for each particular serum. Experimental conditions: extracellular proteins of *S. aureus* 8325-4 or *S. aureus* 8325-4 Δ *spa* at stationary growth phase, IPG strips of 7 cm, pH gradient 6-11 and 4-7, respectively, volunteer serum (1:10,000) and ECL substrate. PC: pesistent carrier; IC: intermittent carrier; NC: noncarrier.

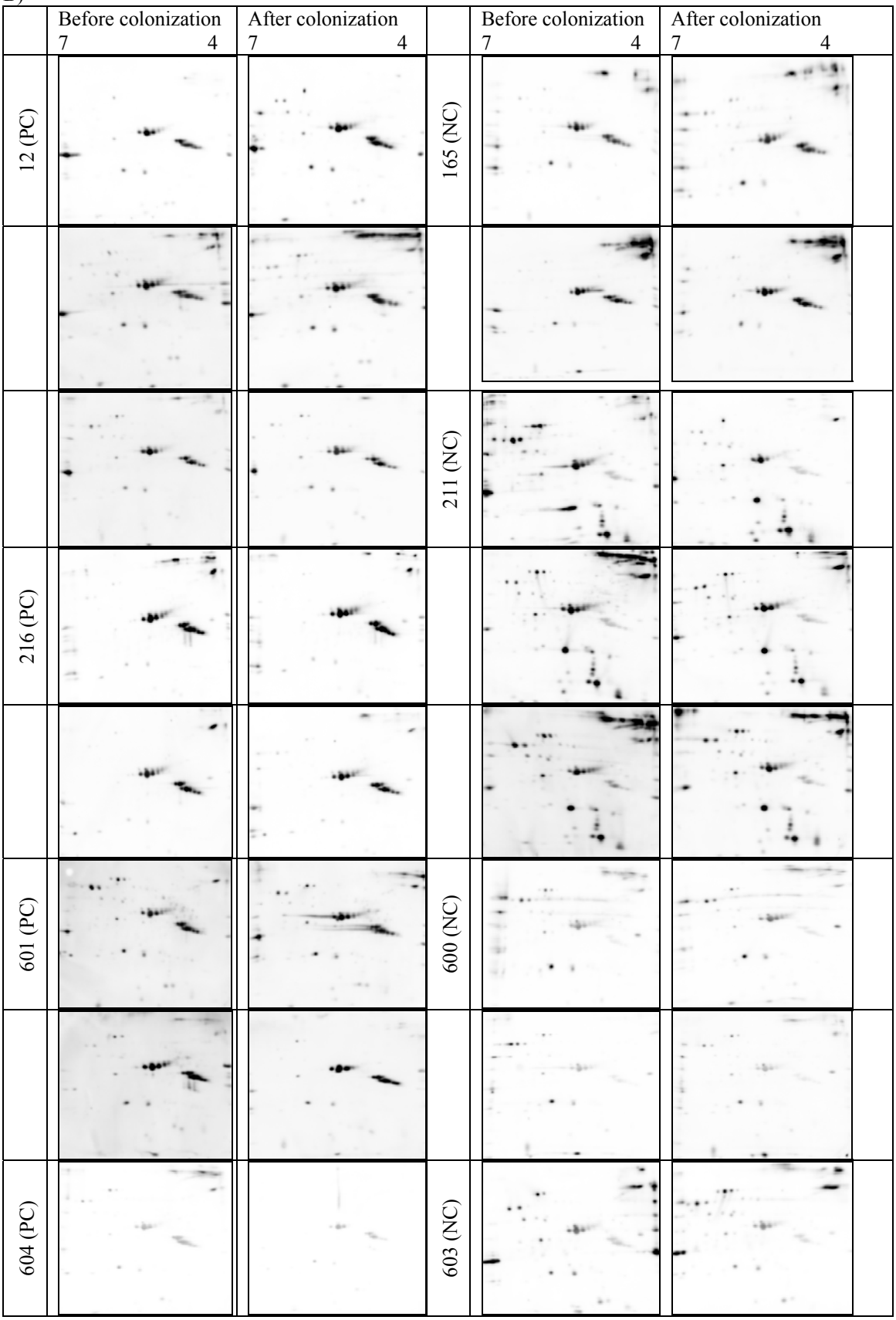
A)




607 (PC)
606 (PC)
604 (PC)
608 (NC)
605 (NC)
603 (NC)


602 (IC)
141 (IC)
610 (NC)
609 (NC)

B)


Before colonization
7
4
After colonization
7
4
Before colonization
7
4
After colonization
7
4
12 (PC)
165 (NC)
211 (NC)
216 (PC)
601 (PC)
600 (NC)
604 (PC)
603 (NC)

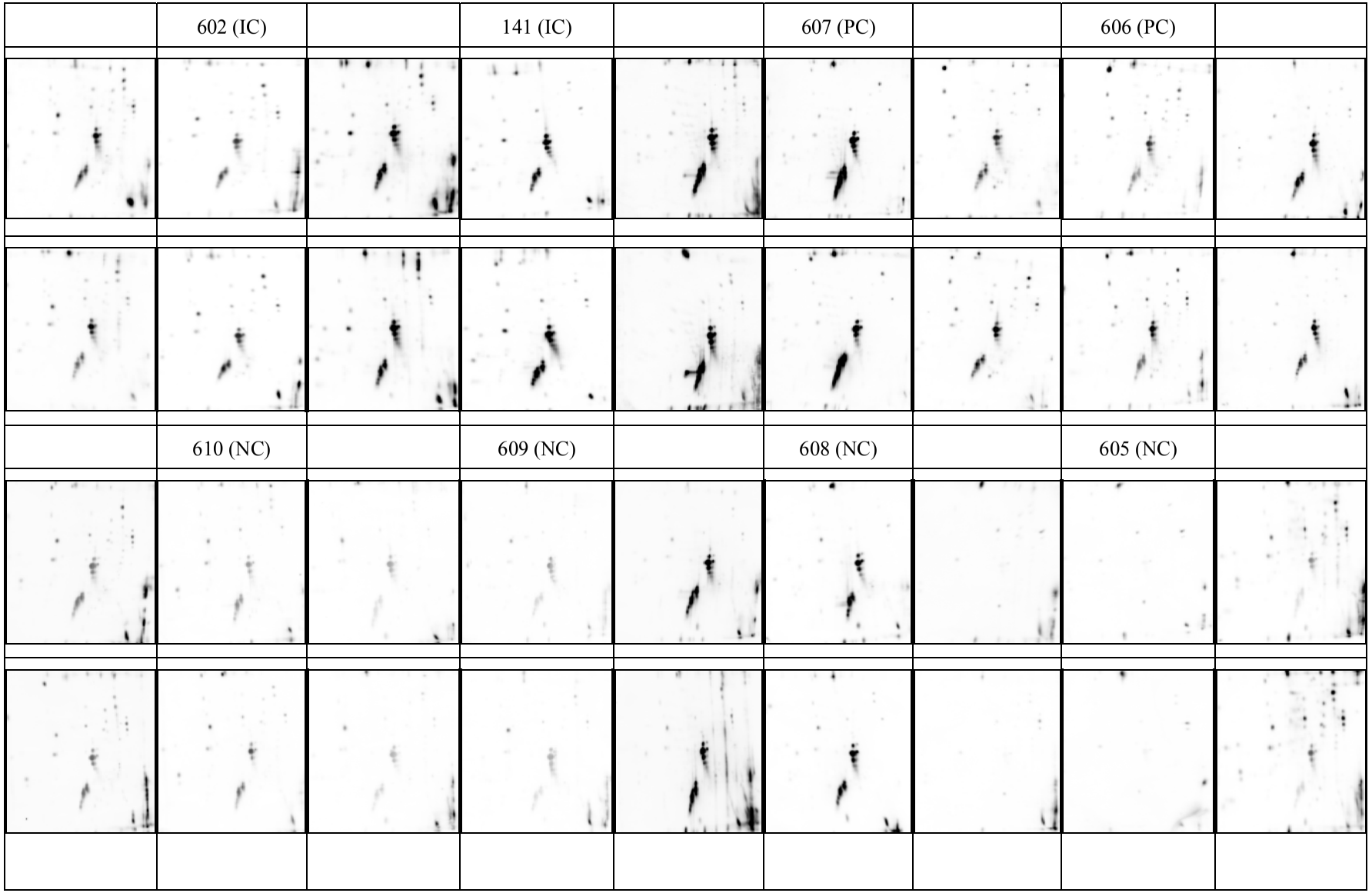

602 (IC)
141 (IC)
607 (PC)
606 (PC)
610 (NC)
609 (NC)
608 (NC)
605 (NC)

Figure A.3: Binding of human IgG to *S. aureus* extracellular and intracellular proteins. The *S.aureus* extracellular protein pattern was less complex than the intracellular one (Coomassie staining). However, there were more immune reactive proteins in the extracellular extract than in the intracellular extract (IB replicate 1 and 2). Experimental conditions: extracellular proteins or intracellular proteins of *S. aureus* 8325-4 at stationary growth phase, IPG strips of 7 cm, pH gradient 4-7, serum pool (1:10,000) and ECL substrate.

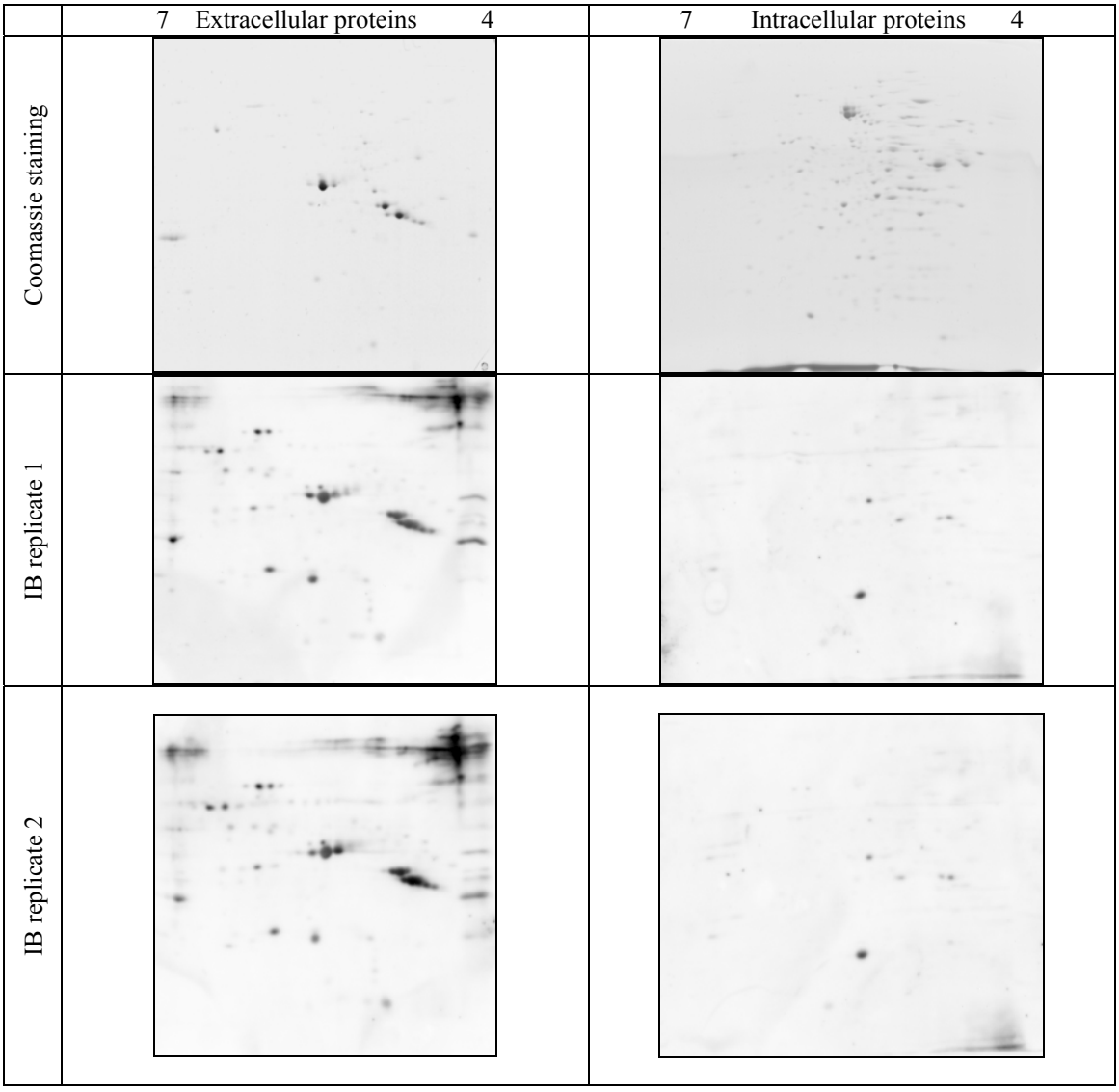
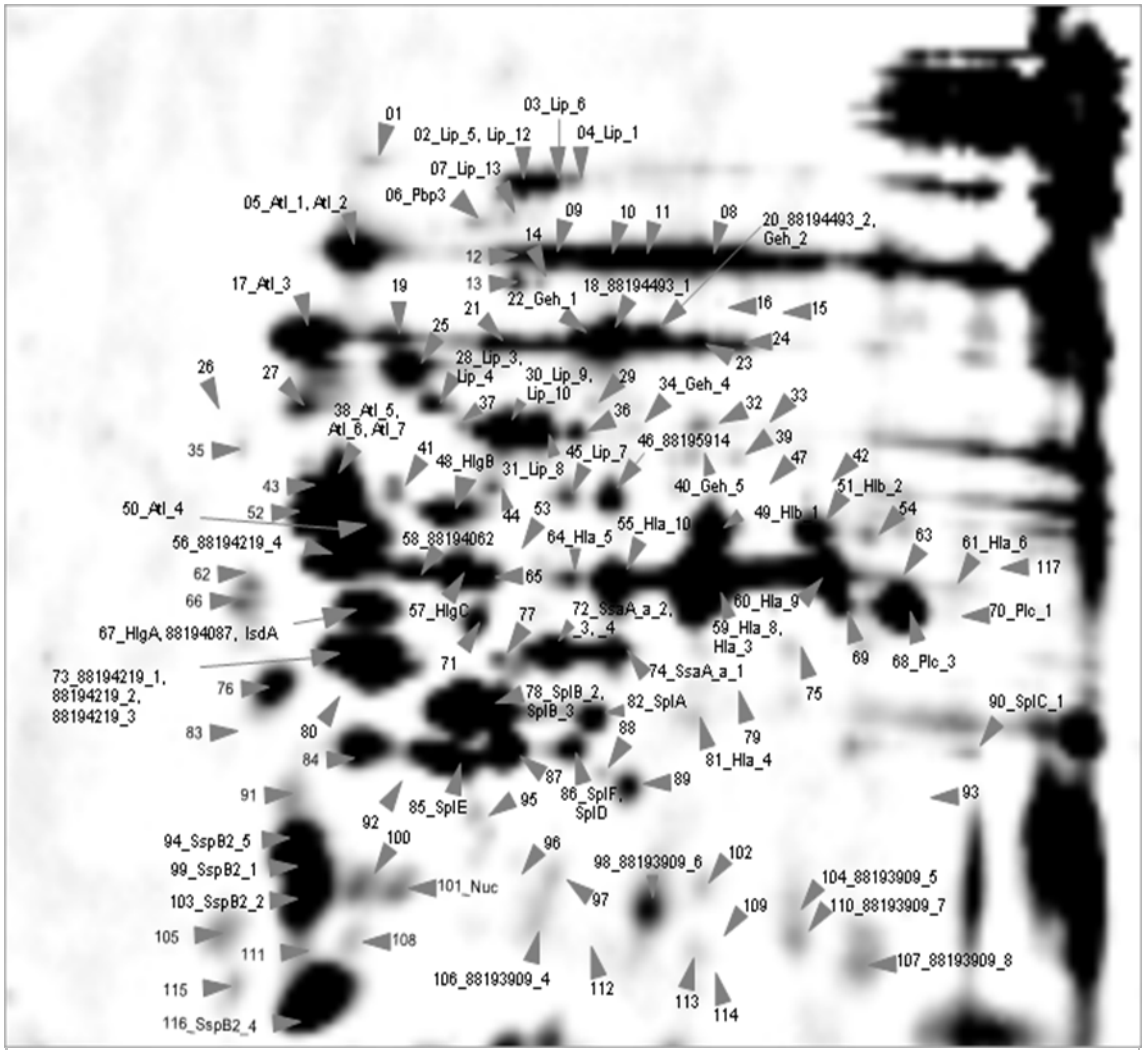
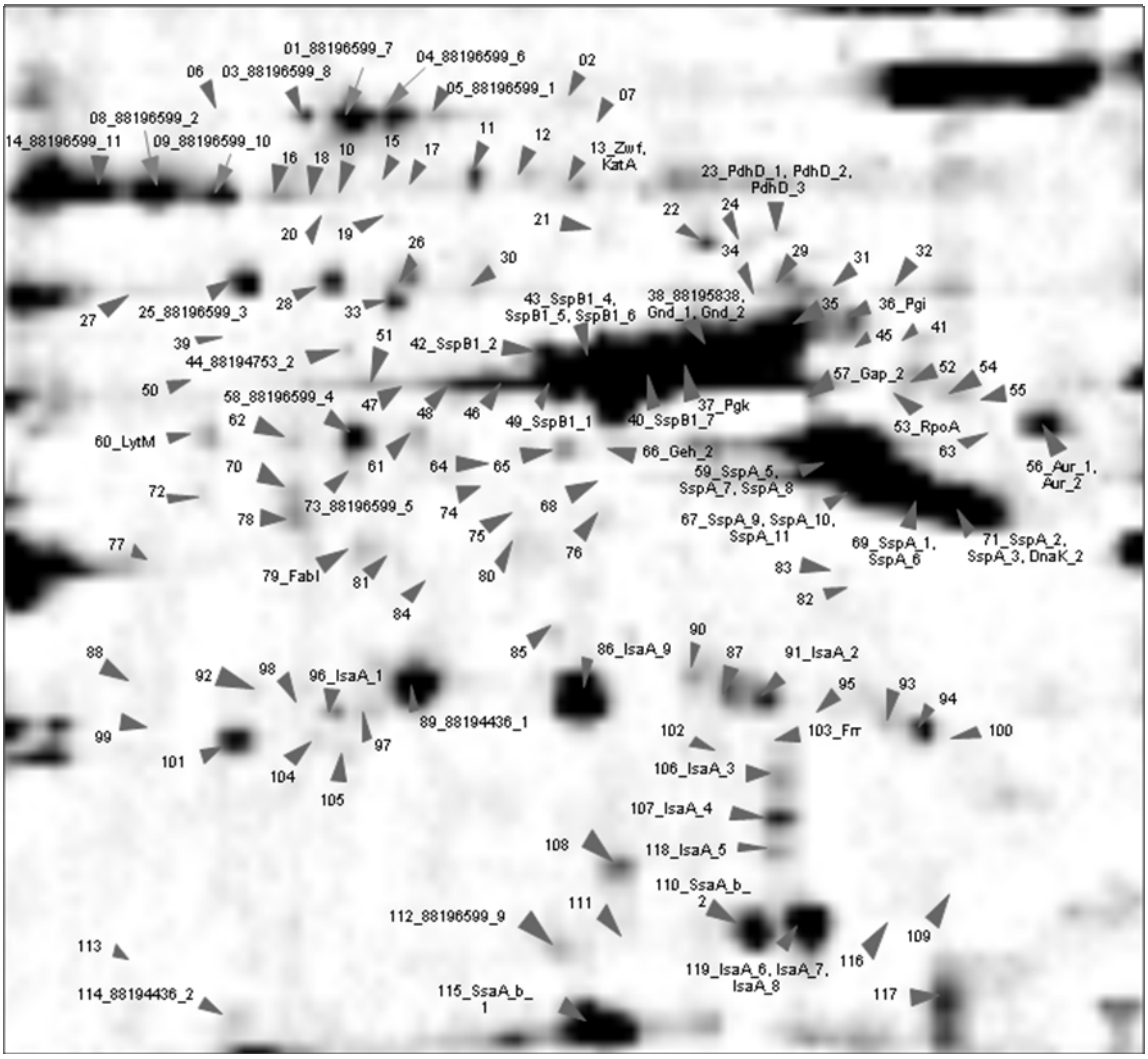


Figure A.4: Spot annotation on the 2-D IBs. One representative IB was used as master image for each pH range 6-11 and 4-7. Gray scale was artificially set at maximum to visualize also the faint IB spots. 117 spots were detected for IBs in the pH range 6-11 and 119 spots in the pH range 4-7. IB spot labels and the corresponding protein names are shown.

A)



B)


01_88196599_7
04_88196599_6
06
03_88196599_8
02
05_88196599_1
07
08_88196599_2
14_88196599_11
09_88196599_10
16
18
10
15
17
11
12
13_Zwf, KatA
23_PdhD_1, PdhD_2, PdhD_3
22
24
21
20
19
26
34
29
31
32
30
27
25_88196599_3
28
33
43_SspB1_4, SspB1_5, SspB1_6
38_88195838, Gnd_1, Gnd_2
35
36_Pgi
45
41
39
44_88194753_2
51
42_SspB1_2
57_Gap_2
52
54
50
58_88196599_4
47
48
46
49_SspB1_1
40_SspB1_7
37_Pgk
55
53_RpoA
62
60_LytM
63
56_Aur_1, Aur_2
70
61
64
65
66_Geh_2
59_SspA_5, SspA_7, SspA_8
72
73_88196599_5
74
68
78
67_SspA_9, SspA_10, SspA_11
75
77
69_SspA_1, SspA_6
71_SspA_2, SspA_3, DnaK_2
79_FabI
81
80
76
83
82
84
90
85
86_IsaA_9
87
91_IsaA_2
88
92
98
96_IsaA_1
95
93
94
89_88194436_1
99
97
102
103_Frr
100
101
104
105
106_IsaA_3
107_IsaA_4
108
118_IsaA_5
110_SsaA_b_2
111
112_88196599_9
109
113
116
119_IsaA_6, IsaA_7, IsaA_8
114_88194436_2
115_SsaA_b_1
117

Curriculum Vitae

Personal Information	
Name	Nguyen Thi Thu Hoai
Birthday	08 July, 1981
Birthplace	Hoa Binh, Vietnam
Nationality	Vietnamese
Family	One daughter, born 02 January, 2008
Address	Institut fuer Immunologie und Transfusionsmedizin Universität Greifswald Sauerbruchstraße, D-17487 Greifswald.
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Education and Training	
1999-2003	Bachelor Course in Biology, Honor program, Hanoi College of Science, Hanoi National University Scholarships: Hanoi College of Science (4 years) Awards: 3rd Prize for student research in life sciences with the topic: 'Study on isozymes from *Aedes aegypti* strains of some residential areas in Vietnam'
2003-2005	2003-2005: Doctoral program at Hanoi College of Science Scholarships: Hanoi College of Science (2 years) 2004-2005: Joint-Training Education Program between Hanoi College of Science and Greifswald University Scholarships: Vietnamese Ministry of Education and Training (1 year), DAAD (2 months)
2005-present	PhD student at Institute of Immunology and Transfusion Medicine, Greifswald University Member of the Graduiertenkolleg GRK840 (http://www.medizin.uni-greifswald.de/immun/gk840) PhD thesis: 'The human antibody response to experimental colonization with *Staphylococcus aureus* NCTC8325-4' Scholarships: Vietnamese Ministry of Education and Training (3 years), DAAD (3 years), DFG Graduiertenkolleg 840 (3 years)

Core Capabilities

Techniques	DNA isolation, PCR, multiplex PCR,
	PBMC isolation and culture, proliferation assay with ^{3}H-incorporation
	Bacterial culture, protein isolation, 1D and 2D-electrophoresis
	Western blot
Softwares	Image Quant, Delta2D, Genespring, GraphPad Prism
	MS office Applications (Word, Excel, Powerpoint), Paint, Photoshop,
	EndNote; Pascal (basic)

Additional Capabilities

Languages	Vietnamese: native; English: fluent (speaking, reading and writing)
	French: basic; German: basic
Training	Scientific Soft Skills Building Program, Forschungsverbund
courses	Mecklenburg-Vorpommern e.V., Greifswald.
	02/2006: Scientific writing
	09/2006: How to create and present a poster
	03/2007: Powerpoint presentation and poster design
	03/2007: Negotiate to close

Research Interests

Infection Immunology

Microbiology

Tumorbiology

Publications

Original publications:

1. Do Ngoc Lien, **Nguyen Thi Thu Hoai**, Vu Duc Huong. Study on isozymes from *Aedes aegypti* strains of some residential areas in Vietnam.Vnu Journal of Science, Nat. Sci & Tech., 2003; 19(4) ; 28-34.

2. Holtfreter S, Grumann D, Schmudde M, **Nguyen HTT**, Eichler P, Strommenger B, Kopron K, Kolata J, Giedrys-Kalemba S, Steinmetz I, Witte W, Bröker BM. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J Clin Microbiol. 2007 Aug;45(8):2669-80.

Submitted manuscripts:

1. **Thi Thu Hoai Nguyen**, Silva Holtfreter, Leif Steil, Quoc Phong Truong, Harald Kusch, Dirk Albrecht, Anne-Kathrin Ziebandt, Susanne Engelmann, Heiman Wertheim, Alex van Belkum, Michael Hecker, Barbara M. Bröker, Uwe Völker. Immune proteome analysis of extracellular *Staphylococcus aureus* proteins. Proteomics.

2. Silva Holtfreter, **Thi Thu Hoai Nguyen**, Heiman Wertheim, Leif Steil, Harald Kusch, Quoc Phong Truong, Susanne Engelmann, Michael Hecker, Uwe Völker, Alex van Belkum, Barbara M. Bröker. Heterogeneity of anti-*Staphylococcus aureus* antibody profiles in healthy humans. PloS.

Posters:

1. Holtfreter, S., Grumann, D., Kopron, K., Eichler, P., Schmudde, M., **Nguyen, H. T. T.,** Feig, C., Giedrys-Kalemba, S., Witte, W., Steinmetz, I., and Broker, B. M. Unusual superantigen gene pattern in an *S. aureus* lineage, which very efficiently colonizes healthy individuals but is underrepresented among invasive isolates in Pomerania., 12th International Symposium on Staphylococci & Staphylococcal Diseases. Maastricht, Austria, 2006.

2. Holtfreter, S., **T. T. H. Nguyen**, H. Wertheim, P. Eichler, A.-K. Ziebandt, T. T. H. Le, K. Roschack, H. Kusch, Q. P. Truong, K. Eske, L. Steil, M. Hecker, S. Engelmann, A. van Belkum, U. Völker, B. M. Bröker. Clinical relevance of serum antibody responses elicited by nasal colonization with *Staphylococcus aureus.* 59. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Göttingen, Germany, 2007.

3. **Thi Thu Hoai Nguyen**, Silva Holtfreter, Thi Thu Hong Le, Anne-Kathrin Ziebandt, Harald Kusch, Quoc Phong Truong, Leif Steil, Michael Hecker, Susanne Engelmann, Alex van Belkum, Uwe Völker, Heiman Wertheim, Barbara M. Bröker. Human antibody response to experimental colonization with *Staphylococcus aureus*. 37th Annual meeting of the German Society for Immunology and 40th years DGfI, Heidelberg, Germany. 2007.

4. Holtfreter, S., **Thi Thu Hoai Nguyen**, Heiman Wertheim, Leif Steil, Harald Kusch, Quoc Phong Truong, Susanne Engelmann, Alex van Belkum, Uwe Völker, and Bröker, B. M. Human IgG response to experimental colonization with *Staphylococcus aureus*. International Conference on Staphylococci and Staphylococcal Infections (ISSSI). Cairns, Australia, 2008.

5. **Thi Thu Hoai Nguyen**, Silva Holtfreter, Thi Thu Hong Le, Anne-Kathrin Ziebandt, Harald Kusch, Quoc Phong Truong, Leif Steil, Michael Hecker, Susanne Engelmann, Heiman Wertheim, Alex van Belkum, Barbara M. Bröker, Uwe Völker. Immune proteome analysis of extracellular *Staphylococcus aureus* proteins. Pathophysiology of

Staphylococci, Kloster Banz, Germany, 2008.

6. Holtfreter, S., **Thi Thu Hoai Nguyen**, Heiman Wertheim, Leif Steil, Harald Kusch, Quoc Phong Truong, Susanne Engelmann, Alex van Belkum, Uwe Völker, and Bröker, B. M. Human IgG response to experimental colonization with *Staphylococcus aureus*. Pathophysiology of Staphylococci, Kloster Banz, Germany, 2008.

7. J. Kolata, S. Holtfreter, D. Grumann, **H. Nguyen**, E. Friebe, K. Rogasch, C. Kohler, S. Thomas-Ecker, S. Engelmann and B. M. Bröker. Human T cell response to *Staphylococcus aureus* antigens. Pathophysiology of Staphylococci, Kloster Banz, Germany, 2008.

Greifswald, December 2008

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