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# Characterisation of New Potential Vaccine Candidates against Infections caused by *Staphylococcus aureus*

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*Inaugural-Dissertation zur Erlangung des Doktorgrades  
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# Contents

<b>1 Abbreviations</b>	<b>6</b>
<b>2 Introduction</b>	<b>8</b>
2.1 Clinical relevance and pathogenicity of <i>Staphylococcus aureus</i> . . . . .	8
2.2 Antibiotic resistance of <i>S. aureus</i> . . . . .	10
2.3 Vaccination strategies against <i>S. aureus</i> . . . . .	11
2.3.1 Active immunisation . . . . .	11
2.3.2 Passive immunisation . . . . .	12
2.3.3 Antibacterial function of immunoglobulins . . . . .	13
2.4 Aim of the study . . . . .	14
<b>3 Material and Methods</b>	<b>15</b>
3.1 Material . . . . .	15
3.1.1 Chemicals and enzymes . . . . .	15
3.1.2 Bacteria and culture media . . . . .	15
3.1.3 Buffers and solutions . . . . .	16
3.1.4 Technical equipment . . . . .	19
3.1.5 Consumables . . . . .	20
3.1.6 Kits . . . . .	20
3.1.7 Oligonucleotides . . . . .	21
3.1.8 Antibodies . . . . .	22
3.2 Methods . . . . .	23
3.2.1 Depletion of specific IgGs from IVIG . . . . .	23
3.2.2 <i>S. aureus</i> growth curves . . . . .	23
3.2.3 RNA isolation . . . . .	24
3.2.4 Synthesis and labelling of cDNA . . . . .	24
3.2.5 Hybridisation of microarrays . . . . .	25
3.2.6 Analyses of microarrays . . . . .	25
3.2.7 Quantitative realtime PCR (qPCR) . . . . .	26
3.2.8 Isolation of <i>S. aureus</i> anchorless cell wall (ACW) proteins . . . . .	26

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3.2.9	Subtractive proteome analysis (SUPRA) . . . . .	27
3.2.10	Cloning, expression and purification of vaccine candidates . . . . .	27
3.2.11	Enrichment of specific IgGs from IVIG . . . . .	28
3.2.12	Surface localisation of vaccine candidates . . . . .	29
3.2.13	<i>In vitro</i> opsonophagocytosis of <i>S. aureus</i> by human neutrophils . . . . .	29
3.2.14	<i>In vitro</i> opsonophagocytic killing of <i>S. aureus</i> by human neutrophils . . . . .	30
3.2.15	Immunisation of mice . . . . .	30
3.2.16	Murine model of sepsis . . . . .	30
3.2.17	Statistical analysis . . . . .	31
<b>4</b>	<b>Results</b>	<b>32</b>
4.1	Characterisation of the bacteriostatic effect mediated by <i>S. aureus</i> specific IgGs . . . . .	32
4.1.1	Human serum inhibits <i>in vitro</i> growth of <i>S. aureus</i> . . . . .	32
4.1.2	Intravenous immunoglobulin preparation (IVIG) specifically inhibits <i>in vitro</i> growth of <i>S. aureus</i> . . . . .	32
4.1.3	Bacteriostatic effect on <i>S. aureus</i> is mediated by <i>S. aureus</i> specific IgGs . . . . .	35
4.1.4	Gene expression profiling of <i>S. aureus</i> over the course of bacteriostasis . . . . .	37
4.2	Identification and characterisation of potential vaccine candidates against <i>S. aureus</i> . . . . .	46
4.2.1	Subtractive proteome analysis (SUPRA) of anchorless cell wall (ACW) proteins enables identification of potential vaccine candidates against <i>S. aureus</i> . . . . .	46
4.2.2	BT1, BT2 and BT3 are localised on the surface of <i>S. aureus</i> . . . . .	49
4.2.3	Affinity purified IgGs specific for BT1, BT2 and BT3 trigger opsonophagocytosis of <i>S. aureus</i> by human neutrophils <i>in vitro</i> . . . . .	49
4.2.4	Affinity purified IgGs specific for BT1 and BT2 mediate killing of <i>S. aureus</i> by human neutrophils <i>in vitro</i> . . . . .	51
4.2.5	Immunisation with recombinant BT1 and BT3 protects mice from death upon lethal challenge with <i>S. aureus</i> . . . . .	52
<b>5</b>	<b>Discussion</b>	<b>56</b>
5.1	Characterisation of the bacteriostatic effect mediated by <i>S. aureus</i> specific IgGs . . . . .	56
5.2	Identification and characterisation of potential vaccine candidates against <i>Staphylococcus aureus</i> . . . . .	59
<b>6</b>	<b>Abstract</b>	<b>64</b>

<b>7 Zusammenfassung</b>	<b>66</b>
<b>8 Bibliography</b>	<b>68</b>
<b>9 Supplement</b>	<b>78</b>
<b>10 Danksagung</b>	<b>86</b>
<b>11 Erklärung</b>	<b>87</b>
<b>12 Lebenslauf</b>	<b>88</b>

# 1 Abbreviations

aa-dUTP	5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate
ACW-proteins	Anchorless cell wall proteins
ANOVA	Analysis of variance
APS	Ammoniumpersulfate
ATCC	American type culture collection
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFU	Colony forming unit
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
clfA	Clumping factor A
cna	Collagen adhesion protein
CP	Capsular polysaccharides
dEcIVIG	IVIG depleted of <i>E. coli</i> specific IgGs
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dSaIVIG	IVIG depleted of <i>S. aureus</i> specific IgGs
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eno	enolase
FACS	Fluorescence activated cell sorting
Fig.	Figure
fnbPA	Fibronectin binding protein A
fnbPB	Fibronectin binding protein B
fur	Ferric uptake regulator
GFP	Green fluorescent protein
HBSS	Hank's balanced salt solution
HI-serum	Heat inactivated serum

hp2160	hypothetical protein similar to esterase (spot ID 2160)
IEF	Isoelectric focussing
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPG	Immobilised pH gradient
Isd	Iron surface determinant
IVIG	Intravenous immunoglobulin preparation
IVIG-DS	IVIG dialysed against PBS
kDa	Kilo-Dalton
LB	Luria Bertani medium
LD <sub>50</sub>	Dose that kills 50 % of tested animals
LPS	Lipopolysaccharide
MALDI-TOF	Matrix assisted laser desorption/ionisation time of flight
MOI	Multiplicity of infection
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
MWCO	Molecular weight cutoff
NHS-activated	N-hydroxysuccinimide
OD	Optical density
ON	Over night
ORF	Open reading frame
oxo	3-oxoacyl- (acyl-carrier protein) reductase
PBS	Phosphate buffered saline
pI	Isoelectric point
PMN	Polymorphonuclear neutrophils
PSI	Pounds per square inch
qPCR	Quantitative real time PCR
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SERPA	Serological proteome analysis
SUPRA	Subtractive proteome analysis
Tris	Tris(hydroxymethyl)aminomethan

## 2 Introduction

### 2.1 Clinical relevance and pathogenicity of *Staphylococcus aureus*

*Staphylococcus aureus* is a facultatively anaerobic gram-positive coccus, belonging to the group of Staphylococcaceae. The coagulase positive *S. aureus* can be found as commensal on human skin, with the moist squamous epithelium of the anterior nares as its primary habitat (Roche et al., 2003). Although not showing signs of infection about 20 % of the human population is continuously colonised with *S. aureus* and another 60 % is transiently colonised. Thus, increasing the risk for invasive infections of endogenous origin (von Eiff et al., 2001).

As nosocomially or community acquired opportunistic pathogen, *S. aureus* causes minor infections of the skin, but also serious life-threatening infections like pneumonia, endocarditis, sepsis and toxic shock syndrome (Lowy, 1998) in healthy and immunocompromised patients, representing the main causative agent of nosocomial infections (Laupland et al., 2003).

The genome of *S. aureus* encodes a broad range of virulence factors, to effectively establish an infection. To date more than 40 different virulence factors playing an important role in the pathogenicity of staphylococcal infections are known (Arvidson and Tegmark, 2001). Functions of these virulence factors include adherence to host cell structures, evasion from the host immune system and active damage on host cells.

Microbial adhesion to host tissue is essential during the initial steps in the pathogenesis of most infections. Adhesion is mediated by protein adhesins of the MSCRAMM (microbial surface component recognising adhesive matrix molecules) family, which in most cases are covalently linked to the cell wall peptidoglycan (Foster and Hook, 1998). Common to these MSCRAMMs is an N-terminal signal peptide, enabling translocation of the protein across the membrane, and a C-terminal hydrophobic region, harbouring the conserved LPXTG motive essential for covalent linkage to the bacterial cell wall (Kronvall and Jonsson, 1999; Patti et al., 1994; Schneewind et al., 1995). Most prominent members of this family are the fibronectin binding proteins (FnbpA and B) or clumping factor A (ClfA), a fibrinogen binding protein (Foster and Hook, 1998). So far in total 21 proteins containing the LPXTG motive have been identified in *S. aureus* by genome screenings (Roche et al., 2003).



Besides covalently linked protein adhesins, also anchorless cell wall proteins (ACW proteins) mediating adhesion to extracellular matrix components have been identified. These proteins lack a signal peptide as well as a common sorting signal like LPXTG and are secreted by a so far unknown mechanism. Upon secretion they are suggested to be re-associated to the bacterial cell surface (Chhatwal, 2002). One member of this new group of adhesins is staphylococcal enolase, which apart from its function as glycolytic enzyme mediates binding to laminin and thereby might contribute to tissue invasion and blood dissemination (Carneiro et al., 2004).

Apart from adhesion, strategies for evasion from the host immune system are crucial to further promote effective colonisation. Protein A, a covalently linked surface protein harbouring the LPXTG motive, mediates immune evasion by binding to the FC portion of immunoglobulin G, thereby hampering efficient opsonisation and subsequent elimination by professional phagocytes (Foster, 2005). Additional components interfering with the immune system are staphylokinase, the capsular polysaccharides and so called superantigens. By binding to the T-cell receptor and the MHC class II molecule on antigen-presenting cells, superantigens trigger a T-cell activation that results in the release of pro-inflammatory cytokines (Llewelyn and Cohen, 2002). The excessive uncoordinated release of pro-inflammatory cytokines, in particular TNF  $\alpha$ , is thought to be responsible for many of the clinical features of toxic shock syndrome (Miethke et al., 1992). In addition, *S. aureus* avoids the detrimental effects of oxygen free radicals that are formed during the respiratory burst in professional phagocytes by several mechanisms, including the yellow carotenoid pigment as scavenger or expression of two superoxide dismutases, which remove superoxide radicals (Foster, 2005).

Secreted proteins like the pore-forming  $\alpha$ -toxin, hemolysins and other cytolytic toxins (e.g. leukocydins, Pantone-Valentine-leukocysin) lead to the destruction of host cells (Tomita and Kamio, 1997). Besides this, other toxins cause food poisoning (enterotoxins A-O), the toxic shock syndrome (TSST-1) or the staphylococcal scalded skin syndrome, which is triggered by the exfoliative toxins A and B (Ladhani et al., 1999).

Another important feature for the pathogenesis of staphylococcal infections is for example the availability of iron in the iron-limited host environment. To this end *S. aureus* evolved strategies to sequester iron by synthesis of high affinity iron chelators, siderophores, and corresponding membrane associated ABC-transporter systems or covalently linked surface proteins (iron-regulated surface determinant; Isd A, B, C, and H) for the sequestration of heme complexed iron (Maresso and Schneewind, 2006). The importance of iron homeostasis was confirmed by the reduced virulence of a *S. aureus* mutant lacking the ferric uptake regulator (*fur*) in a murine skin abscess model of infection (Horsburgh et al., 2001a). In fact, *S. aureus* infections always depend on multiple virulence factors and the high redundancy in the function of virulence factors is supported by several *in vivo* studies using mutants lacking for example MSCRAMMs, but not preventing colonisation (Darouiche et al.,

1997; Patel et al., 1987; Peacock et al., 1999).

## 2.2 Antibiotic resistance of *S. aureus*

To date antibiotics are the only effective therapy against *S. aureus* infections. However, the steadily increasing incidence of *S. aureus* strains, resistant to multiple antibiotics aggravates the treatment, thereby leading to increased mortality by *S. aureus* infections.

Today MRSA is the common denotation for those multiple resistant *S. aureus*, although it was initially used for 'Methicillin resistant *S. aureus*' conferred by an additional penicillin binding protein (PBP2a). The *mecA* gene encoding PBP2a is located on a DNA element termed SCC*mec* (staphylococcal cassette chromosome *mec*), site-specifically integrated into the staphylococcal genome (Beck et al., 1986; Berger-Bachi et al., 1986).

For the treatment of infections caused by MRSA the glycopeptide antibiotic Vancomycin still represents the antibiotic of choice. However, due to the selective pressure during treatment, Vancomycin intermediate resistant *S. aureus* (VISA) evolved, first described in 1997 by Hiramatsu et al. (1997). In contrast to other antibiotic resistances this intermediate resistance against Vancomycin is not genetically encoded. In 2002 the first case of a genetically determined Vancomycin resistance (VRSA) was reported (Weigel et al., 2003). In this case the *vanA* gene, conferring resistance to Vancomycin, was horizontally transferred from a Vancomycin resistant *Enterococcus faecalis* during co-infection.

Until the late 1990s the predominant pattern of MRSA infections in the USA remained a nosocomially acquired disease, but then MRSA from community-acquired sources (CA-MRSA) without a history of intravenous drug use began to be described more frequently (Corriere and Decker, 2008). In 2006 the overall prevalence of CA-MRSA in the population was 59 %. Due to an almost three times longer stay for inpatients suffering from *S. aureus* infection causing nearly thrice the cost compared to non-infected inpatients, infections with MRSA also became an economic problem. Moreover, the treatment of infections caused by MRSA, which accounts for almost 50 % of *S. aureus* related infections in several US hospitals, even doubles the cost caused by infections with a methicillin susceptible *S. aureus* (MSSA). The mortality rate associated with MRSA infections is with 20.7 % almost thrice as high than observed for infections caused by susceptible *S. aureus* (6.7 %) (Corriere and Decker, 2008). Next to this also the prevalence of CA-MRSA is steadily increasing. In the United States a single clone of CA-MRSA (USA-300) has become the most prevalent cause of staphylococcal soft tissue infections acquired in the community (King et al., 2006; Tenover et al., 2006) and has entered the inpatient setting, causing also invasive diseases (Davis et al., 2006; Gonzalez et al., 2006; Klevens et al., 2007).

The drastic increase in both nosocomially and community acquired MRSA related infections and thereby increased mortality rates highlight the pressing need for alternative strategies to prevent and treat *S. aureus* infections.

## 2.3 Vaccination strategies against *S. aureus*

### 2.3.1 Active immunisation

Since first vaccination studies employing active immunisation using whole bacteria (Greenberg et al., 1987) or staphylococcal phage lysates (Giese et al., 1996) failed to achieve protective effects, single structures of *S. aureus* like the capsular polysaccharides (CP) or adhesion molecules of the MSCRAMM family got into focus of vaccine research. These attempts achieved at least a partial protection against *S. aureus* infections.

By prevention of phagocytic activity the CPs represent an important factor in the pathogenicity of *S. aureus* (Kampen et al., 2005). With over 80 % serotypes 5 and 8 are the most prevalent of the 11 different serotypes among *S. aureus* isolates (Sompolinsky et al., 1985; Poutrel et al., 1988). Since purified CP5 and CP8 demonstrated only weak immunogenicity in mice, purified CPs were conjugated to a non-toxic variant of Exotoxin A (ETA) of *Pseudomonas aeruginosa* to increase immunogenicity. The conjugate induced high titres of CP-specific antibodies and a T-cell dependent immune response (Fattom et al., 1990). Furthermore, active immunisation using a bivalent CP5-CP8 conjugate vaccine (StaphVax™; Nabi Biopharmaceuticals) led to an increased survival rate in mice (Fattom et al., 2004), but failed to pass the Phase III clinical trial in end stage renal disease patients. A second Phase III trial in haemodialysis patients also failed, hence halting the development of StaphVax (Garcia-Lara and Foster, 2009). Currently a combined vaccine including CP5, CP8, polysaccharide component 336 and two staphylococcal toxins is under investigation (Pentastaph; <http://www.nabi.com>).

The polysaccharide intercellular adhesin of *S. aureus* and *S. epidermidis* is a poly-N-acetylglucosamine (PNAG) that enables adhesion to inert surfaces and living tissues. Immunisation using PNAG achieved moderate protection in rabbit models of endocarditis or catheter-associated bacteraemia and a murine model of kidney infection (Maira-Litran et al., 2004).

Also secreted extracellular molecules like TSST-1 have been tested in immunisation studies. For this purpose either a non-toxic mutant or formaldehyde inactivated TSST-1 was used for vaccination of rabbits and achieved a protection from lethal challenge with *S. aureus* (Hu et al., 2003). However, due to the high variability in the set of toxin genes and their expression between different *S. aureus* strains, toxins alone are most likely no promising vaccine target.

Due to their function and surface localisation cell wall associated proteins represent promising targets. To date several cell wall anchored proteins harbouring the LPXTG motive have been tested in immunisation studies. For example, improved survival of mice was observed upon immunisation with the collagen binding protein (Cna) in a murine model of sepsis (Nilsson et al., 1998). However, as Cna is not present in all *S. aureus* strains, it is not eligible as single component vaccine. In another attempt immunisation with IsdA, IsdB, SdrD

or SdrE significantly reduced the bacterial load in infected kidneys (Stranger-Jones et al., 2006). Moreover, as observed in the same study immunisation with a multicomponent vaccine consisting of IsdA, IsdB, SdrD, and SdrE induced opsonophagocytic antibodies against each component in immunised mice, reduced the load of *S. aureus* in kidneys and improved survival upon lethal challenge with *S. aureus*. The iron surface determinant B (IsdB) is also the basis of Merck's V710 vaccine (Garcia-Lara and Foster, 2009), for which a Phase II clinical trial has just been completed (Feb 2010; <http://clinicaltrials.gov/ct2/show/NCT00572910>). Immunisation with IsdA and Clumping factor B (ClfB), two major determinants in nasal carriage, decreased nasal colonisation in a cotton rat and murine model of nasal carriage, respectively (Clarke et al., 2006; Schaffer et al., 2006). Thereby representing a potential option to restrict the spread of CA-MRSA.

Altogether, much effort has been made to characterise potential vaccine candidates in *in vivo* infection models and some promising results have been achieved. But these results, especially those already tested in clinical trials, strongly suggest that due to the high variability in the spectrum of virulence factors for each strain as well as the redundancy in protein function, a single component vaccine will not be sufficient to confer protection. The results obtained by Stranger-Jones et al. (2006) point towards a polyvalent vaccine as the most promising strategy to combat *S. aureus* infections. Thus, the identification and characterisation of eligible candidates is pivotal for the development of a successful multicomponent vaccine. In this regard ACW proteins of *S. aureus* represent a new interesting class of vaccine candidates, since they exhibit both important enzymatic function and surface localisation.

### 2.3.2 Passive immunisation

Since active immunisation will most likely require a booster immunisation to achieve full protection, it is not representing a treatment option for already infected patients or patients short before surgery. In these cases, passive immunisation might represent an interesting alternative. Especially, as it is also applicable in immunocompromised patients, not able to trigger a sufficient humoral response upon active immunisation.

In the pre-antibiotic era the so-called serum therapy represented the first attempt of passive immunisation to overcome infections (Casadevall and Scharff, 1994). Although effective, it caused severe side effects, due to poor purity of the antibody fractions used. Since then antibody purification methods have vastly improved, thereby circumventing adverse effects due to impurities. For example intravenous immunoglobulin preparations (IVIG) derived from human plasma are routinely used in immunocompromised patients to provide antibodies against various pathogens and thereby support the immune system.

Several attempts have been made to test for protective effects by passive immunisation against different target molecules. For example a hyperimmune polyclonal immunoglobulin

preparation targeting CP5 and CP8 obtained from volunteers immunised with StaphVax™ (Altastaph; Nabi Biopharmaceuticals) failed to prove efficacy in two Phase II clinical trials (Benjamin et al., 2006; Rupp et al., 2007).

Biosynexus has developed a humanised mouse chimeric IgG1 monoclonal antibody with *in vitro* opsonophagocytosis activity to *S. epidermidis* lipoteichoic acid (LTA), a membrane bound glycolipid extending into the cell wall of gram-positive bacteria (Pagibaximab). It conferred around 80 % protection against lethal challenge with *S. aureus* in rodent models (Weisman, 2007), and Phase I/II clinical trials in low birth weight (LBW) neonates demonstrated that it is non-immunogenic, opsonic against coagulase negative *S. aureus* and reduces the incidence of bacteraemia (Weisman et al., 2009).

Another target under investigation is clumping factor A (ClfA), which belongs to the family of MSCRAMMs. ClfA mediates adhesion by binding to host ligands like fibrinogen and was previously shown to be required for virulence in a murine septic arthritis model (Josefsson et al., 2001). Passive immunisation with either rat or rabbit anti-ClfA polyclonal antibodies conferred protection in murine models of sepsis and arthritis. Veronate® (Inhibitex) is a human immunoglobulin preparation from plasma donors with naturally occurring high titres of IgG to ClfA and to the *S. epidermidis* fibrinogen binding protein (SdrG). Although protective in animal models (Vernachio et al., 2006), it failed to meet its primary endpoint, a 50 % reduction in late onset *S. aureus* bacteraemia, in a Phase III clinical trial in LBW neonates (DeJonge et al., 2007).

These results further underline the necessity of the identification of *S. aureus* protein candidates conferring protection by either active or passive immunisation, as components of a polyvalent vaccine against *S. aureus*. The major advantage of passive immunisation is the option to combine antibodies against multiple targets exhibiting different functions, e.g. neutralisation of bacterial toxins and opsonisation for subsequent elimination by professional phagocytes, thereby further increasing the protective potential of a polyvalent vaccine.

### 2.3.3 Antibacterial function of immunoglobulins

The main antibacterial functions of immunoglobulins are 1) opsonisation and in turn Fc  $\gamma$ -receptor mediated phagocytosis by professional phagocytes such as neutrophils or macrophages, 2) the neutralisation of adhesion to host cell tissue by binding to the respective pathogen associated protein, and 3) neutralisation of bacterial toxins. Besides, immune complexes of antibodies and toxins, which are not sufficient to induce Fc  $\gamma$ -receptor dependent uptake by phagocytes, are eliminated by activation of the classical pathway of complement activation. Additionally, antibodies have also been shown to amplify or suppress the inflammatory response depending on their specificity, isotype and concentration (Casadevall and Pirofski, 2003).

Apart from this indirect antimicrobial activities, there are some examples for direct an-

timicrobial actions mediated by antibodies. It was shown that IgM or IgG specific for *Borrelia burgdorferi* surface proteins damage the surface protein coat of the organism, thereby leading to a bactericidal effect in the absence of complement (Connolly and Benach, 2001; Connolly et al., 2004). Furthermore, an antibody specific for *Escherichia coli* LPS was found to be bacteriostatic due to interference with the release of an iron chelator (enterochelin), thus preventing iron uptake (Fitzgerald and Rogers, 1980). Similarly, IgM monoclonal antibodies specific for surface proteins of *Acinetobacter baumannii* have been reported to be bactericidal by inhibition of iron uptake (Goel and Kapil, 2001).

In 1971 it was reported that human serum exhibits a bacteriostatic effect on *S. aureus* (Ehrenkranz et al., 1971). The underlying mechanism is not yet described, but an antibody directly inhibiting the growth *S. aureus* would be of great benefit in the context of passive immunisation.

## 2.4 Aim of the study

Due to the worldwide increasing prevalence of *S. aureus* resistant to multiple antibiotics (MRSA) in both hospital and community as well as the rapid emergence of resistances against new antibiotics, the development of alternative strategies for the treatment and prevention of staphylococcal infections is essential. Because *S. aureus* infections always depend on a multitude of virulence factors and there is a high variability in virulence factor genes among *S. aureus* isolates, single target strategies are less promising. Furthermore, passive immunisation strategies are particularly advantageous, due to their short term applicability and potential composition of immunoglobulins of different isotypes and functionalities.

The aim of this study was to investigate a potential bacteriostatic effect on *S. aureus* conferred by specific IgGs, using an intravenous immunoglobulin (IVIG) preparation as source of naturally occurring, *S. aureus* specific IgGs. Due to the enormous potential of a direct bacteriostatic effect elicited by IgGs for passive immunisation, the mechanism underlying bacteriostasis was analysed by gene expression profiling during inhibition of growth.

Additionally, this study aimed to enlarge the pool of potential candidates for a polyvalent vaccine. Thus, immunogenic anchorless cell wall proteins of *S. aureus* were identified and characterised regarding their potential to confer protection against lethal challenge with *S. aureus* upon active immunisation in a murine model of sepsis.

# 3 Material and Methods

## 3.1 Material

### 3.1.1 Chemicals and enzymes

Chemicals were of research grade and purchased from AppliChem, Merck, Sigma-Aldrich, ROTH or Becton Dickinson GmbH unless stated otherwise. Buffers and solutions were obtained from Bio-Rad or prepared using bi-distilled H<sub>2</sub>O from an EASYpure UV/UF H<sub>2</sub>O purification unit (Werner Reinstwassersysteme), degassed and sterilised by autoclaving or filtration through a 0.2 µm membrane, if necessary. Restriction enzymes were obtained from Fermentas and used in recommended buffers. PFU Polymerase was obtained from Promega and recombinant Lysostaphin from Sigma-Aldrich.

### 3.1.2 Bacteria and culture media

<i>E. Coli</i>	Genotype	Provider
<i>E. coli</i> K12 XL1Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI <sup>q</sup> ZΔM15 Tn10 (Tet, <sup>r</sup> )]	Stratagene
DH5α	F-φ80dlacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 tonA hsdR17(r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) phoA supE44 λ -thi-1 gyrA96 relA1	Invitrogen
BL 21	F- dcm ompT hsdS (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) gal	Stratagene

<i>S. aureus</i>	Resistance	Provider
ATCC 29213	MSSA	American Type Culture Collection
ATCC 29213-GFP	Ampicillin/ Chloramphenicol	Inst. for Med. Microbiology, Immunology and Hygiene, S. Leggio

<b>Media / Antibiotics</b>	<b>Composition</b>
LB -Medium	1 % Tryptone; 0.5 % Yeast extract; 0.5 % NaCl; pH 7.0
LB-Agar	1 % Tryptone; 0.5 % Yeast extract; 0.5 % NaCl, 15 % Agar; pH 7.0
SOC-Medium	2 % Tryptone, 0.5 % Yeast extract, 0.5 % NaCl, 25 mM KCl, 100 mM MgCl <sub>2</sub> , 20 mM Glucose; pH 7.0
Ampicillin	Stock solution: 50 mg/ml in H <sub>2</sub> O Working concentration: 50 µg/ml
Chloramphenicol	Stock solution: 10 mg/ml in MeOH Working concentration: 20 µg/ml
Kanamycin	Stock solution: 50 mg/ml in H <sub>2</sub> O Working concentration: 50 µg/ml

### 3.1.3 Buffers and solutions

<b>Buffers and Solutions</b>	<b>Composition</b>
<b>cDNA labelling</b>	
50 x aa-dNTP Mix	25 mM dATP, 25 mM dGTP, 25 mM dCTP, 15 mM dTTP, 10 mM aminoallyl-dUTP
1M Phosphate buffer	9.5 ml 1M K <sub>2</sub> HPO <sub>4</sub> , 0.5 ml 1M KH <sub>2</sub> PO <sub>4</sub> ; pH 8.5-8.7
Phosphate wash buffer	5 mM Phosphate buffer in 80 % Ethanol; pH 8.0
Phosphate elution buffer	4 mM Phosphate buffer; pH 8.0
Dye coupling buffer	100 mM Na <sub>2</sub> CO <sub>3</sub> ; pH 9.0 (freshly prepared)
Cy-Dyes (GE Healthcare)	content of one vial reconstituted in 73 µl DMSO, stored dessicated at -20 °C
Stop solution (Dye coupling)	100 mM NaOAc; pH 5.2
<b>Microarray hybridisation</b>	
20x SSC	3 M NaCl, 0.3 M Natriumcitrate; pH 7.0



<b>Buffers and Solutions</b>	<b>Composition</b>
10 % SDS	100 g SDS in 1000 ml A. bidest.
Prehybridisation	3.5 x SSC, 0.1 % SDS, 10 mg/ml BSA in A. bidest
Wash A	1 x SSC, 0.05 % SDS
Wash B	0.06 x SSC
<b>Agarose gels</b>	
50 x TAE buffer	40 mM Tris, 20 mM Acetic acid, 2 mM Na <sub>2</sub> EDTA, pH 8.5
10 x Loading buffer	200 mM Tris-Acetate, 5 mM Na <sub>2</sub> EDTA, 0,01 % Bromphenolblue, 50 % Glycerin (v/v), pH 8.0 at 37 °C for 3 h stirring
1 Kb DNA-ladder	100 µl 1 Kb Plus DNA-Ladder (Invitrogen), 400 µl 10 x loading buffer, 500 µl A. bidest
1% Agarosegel	1 g Agarose, 100 ml 1x TAE-Puffer, 1 µl Ethidiumbromid (10 µg/µl)
<b>1D- and 2D-SDS-PAGE</b>	
10 x Laemmli buffer	0.06 M Tris-HCl (pH 6.8), 2 % SDS, 25% Glycerol, 0.2 % Bromphenolblue, 10 % 2β-Mercaptoethanol
Rehydration solution	8 M Urea, 2 M Thiourea, 40 mM DTT, 1 % CHAPS, 0.5 % Pharmalyte
Equilibration solution	50 mM Tris-HCL (pH 8.8), 8 M Urea, 2 M Thiourea, 30 % Glycerol, 2 % SDS, 0.05 % Bromphenolblue
TT buffer	3M Tris-HCl, 0,3 % SDS, 1 mM EDTA; pH 8.5
TE buffer	10 mM Tris/HCl, 1 mM EDTA; pH 8.0
<b>Silver staining</b>	
Fixation solution	50 % EtOH, 10 % Acetic acid
Incubation solution	30 % EtOH, 6.8 % NaOAc, 0.5 % Glutaraldehyde, 0.2 % Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> x 5 H <sub>2</sub> O
Staining solution	0.1 % AgNO <sub>3</sub> , 0.02 % Formaldehyde
Developing solution	2.5 % Na <sub>2</sub> CO <sub>3</sub> , 0.01 % Formaldehyde
Stop solution	5 % Acetic acid

<b>Buffers and Solutions</b>	<b>Composition</b>
<b>Coomassie staining</b>	
Staining solution	2.5 % Serva blue R250, 45 % Ethanol, 15 % Acetic acid
De-colourisation solution	45 % Methanol, 15 % Acetic acid
<b>Immunoblotting</b>	
TBST buffer (10x)	1.5 M NaCl, 0.5 M Tris, 0.5 % Tween 20, pH 7.4
PBST buffer (10x)	1.4 M NaCl, 27 mM KCl, 100 mM NaH <sub>2</sub> PO <sub>4</sub> , 18 mM KH <sub>2</sub> PO <sub>4</sub> , 0.2% Tween 20; pH 7.4
Blocking buffer	5 % skim milk powder, 2 % BSA in 1x TBST
Transfer buffer	25 mM Tris-HCl, 192 mM Glycine, 20 % Methanol
Stripping buffer	62.5 mM Tris-HCl, 2 % SDS, 100 mM 2 $\beta$ -Mercaptoethanol; pH 6.8
<b>Colony Blot</b>	
Denaturing solution	0.5 M NaOH, 1.5 M NaCl
Neutralisation solution	1.5 M NaCl, 0.5 M Tris-HCl; pH 7.4
20 x SSC	3 M NaCl, 0.3 M sodium citrate; pH 7.0
<b>Affinity chromatography</b>	
<b>Recombinant proteins</b>	
10 x PBS	1.4 M NaCl 100 mM Na <sub>2</sub> PO <sub>4</sub> , 27 mM KCl, 18 mM KH <sub>2</sub> PO <sub>4</sub> ; pH 7.4
Resuspension buffer	1 x PBS, 500 mM NaCl, 5 % Glycerol, EDTA-free complete protease inhibitor
Binding buffer	1 x PBS, 500 mM NaCl, 40 mM Imidazole; pH 7.4
Elution buffer	1 x PBS, 500 mM NaCl, 250 mM Imidazole; pH 7.4
Dialysis buffer	1 x PBS, 10 % Glycerol; pH 7.4
<b>Antibody enrichment</b>	
Wash A (NHS column)	0.5 M Ethanolamine, 0.5 M NaCl; pH 8.3
Wash B (NHS column)	0.1 M NaOAc, 0.5 M NaCl; pH 4.0
Storage buffer	0.05 M Na <sub>2</sub> HPO <sub>4</sub> , 0.1 % NaN <sub>3</sub> ; pH 7.0
1 x PBS (binding buffer)	140 mM NaCl, 2.7 mM KCl, 10 mM NaH <sub>2</sub> PO <sub>4</sub> , 1.8 mM KH <sub>2</sub> PO <sub>4</sub> ; pH 7.4

<b>Buffers and Solutions</b>	<b>Composition</b>
Elution buffer	0.1 M Glycine-HCl; pH 2.5
Neutralisation buffer	1 M Tris-HCl; pH 9.0

### 3.1.4 Technical equipment

<b>Device</b>	<b>Specification</b>	<b>Provider</b>
Centrifuge	Megafuge 1.0 R	Thermo Scientific
Colony counter	Countermat FLASH	IUL
Developer	AGFA Curix 60	AGFA
Electrophoresis chamber	Criterion Cell	Bio-Rad
Electrophoresis chamber	Ettan Dalt II System	GE Healthcare
ELISA-Reader	MRX Tc	Dynex Technologies
Flow cytometer	FACScalibur	BD Biosciences
FPLC system	ÄKTA™ Purifier	GE Healthcare
French <sup>®</sup> Press Cell	K20, 20000 PSI	Thermo Scientific
French pressure cell press	French <sup>®</sup> Press	Thermo Scientific
Gel documentation	Gel Doc 2000	Bio-Rad
High-speed centrifuge	RC 5 C plus	Sorvall
IEF	Multiphor II	GE Healthcare
Incubator (bacteria)	Kelvitron t	Thermo Scientific
Incubator (cells)	Hera Cell 240	Thermo Scientific
Lightcycler <sup>®</sup>	Lightcycler <sup>®</sup> LC480	Roche
MALDI-TOF MS	Reflex IV	Bruker Daltonics
Microarray scanner	Axon GenePix 4100	Axon Instruments
Microcentrifuge	Centrifuge 5417 R	Eppendorf
Microscope	Axiovert 25	Zeiss
PCR cycler	Thermocycler T3	Biometra
Power supply	Power Pac 3000	Bio-Rad
Power supply for Multiphor II	EPS-3501xL	GE Healthcare
Roller-mixer	Cat RM5	Zipperer
Spectrophotometer	Genesis 20	Thermo Scientific
Spiral plater	Eddy Jet	IUL
Tank-Blotter	Criterion blotter (9.4x15 cm)	Bio-Rad

Device	Specification	Provider
Tank-Blotter	Trans-Blot cell (16x20 cm)	Bio-Rad
Test-Tube-Rotator	Model 34528	Snjiders
Thermomixer	Comfort	Eppendorf

### 3.1.5 Consumables

Designation	Manufacturer
Bottle-Top-Filter PES, 0.45 µm	Nalgene
Centricon-Plus 70, 30 000 MWCO	Millipore
Corning hybridisation chambers	Corning
Criterion™ Empty Cassettes	Bio-Rad
Amersham Hyperfilm ECL	GE Healthcare
Eddy-Jet tips	IUL
HiPrep™ 26/10 Desalting Column	GE Healthcare
HisTrap™ FF crude Columns	GE Healthcare
HiTrap™ NHS-activated HP Columns	GE Healthcare
IEF-Electrode Strips	GE Healthcare
Immobiline™ Dry Strips (3-10NL/4-7 NL)	GE Healthcare
Lifterslips™ 22 x 22 1 mm	Implen
LightCycler® 480 Multiwell Plate 96, white	Roche
Maxisorp™ flat-bottom 96-well plate	Nunc
Mueller-Hinton Agarplatten	Oxoid
Nitrocellulose	Schleicher & Schuell
Sterifix® Injection Filter, 0.2 µm	Braun

### 3.1.6 Kits

Designation	Manufacturer
DC-Protein Assay	Bio-Rad
Amersham™ ECL™ Detection Reagent	GE Healthcare
Ettan Dalt II Gel and Buffer Kit	GE Healthcare
LightCycler® 480 SYBR Green I Master	Roche

Designation	Manufacturer
QIAprep Spin Miniprep	Qiagen
QIAquick PCR Purification	Qiagen
RNeasy Mini Kit	Qiagen
Superscript <sup>®</sup> III first strand synthesis system	Invitrogen
OptiEIA <sup>™</sup> TMB Substrate Reagent Set	BD Biosciences

### 3.1.7 Oligonucleotides

Desalted oligonucleotides were obtained from Operon and a 100  $\mu$ M stock-solution ( TE; pH 8.0) was prepared and stored at -20 °C. Primers were used in a final concentration of 5 pmol/ $\mu$ l for cloning of the vaccine candidates or 10 pmol/ $\mu$ l for qPCR, respectively.

Gene	Name	Sequence (5' to 3')
BT1	BT1-f	CCA TGG CTA AAT CAG TGG CTA
	BT1-r	AAC CTC GAG CAA CTC TGC GAT TAC
BT2	BT2-f	GTT CCA TGG GTC ATC AAG CAG ATG
	BT2-r	GTG CTC GAG TCT ACT TTG CAA GTA
BT3	BT3-f	CCA TGG GAA CAC CAA TTA TAG C
	BT3-r	GCC CTC GAG TTT TGC ACC TTC TAA
qPCR 16SrRNA	16S-f	TCG GGG GAC AAA GTG ACA GGT
	16S-r	AGA GTG CCC AAC TTA ATG ATG GC
qPCR fur	fur-f	GTT ACC AGA AGT TGA AAA TCG AGT TGA
	fur-r	CTA TCC TTT ACC TTT AGC TTG GCA CG
qPCR fhuC	fhuC-f	ACC TGA AGT AGC AGA TGG CTT AAC TG
	fhuC-r	CCA ATC AAT TTC TTT CTT ATC CTC AGC
qPCR fhuD2	fhuD2-f	AGC AAT TGG ACA AGA TGC AAC AGT
	fhuD2-r	ATG CTT GAT ATA ATA CTT CTC CAC CAC G
qPCR ribA	ribA-f	GAT TGT GGT GCT CAA CTT GAA TCG
	ribA-r	ATT TGT TTA ACA ATC CTA TGC CAC GAC
qPCR catalase	cata-f	ATA TTC TCT GAA ATA GGT AAG CAA ACC G
	cata-r	TTA ACG CAA ATC CTC GAA TGT CAC

Gene	Name	Sequence (5' to 3')
qPCR ferritin	ferri-f	CAG CAC CAA AAA TTG ACT TTT CAA G
	ferri-r	TCT TGA CGA GCG ATT TCA GAT AAG TTA

### 3.1.8 Antibodies

Antibody	Characteristics	Provider
Octagam <sup>®</sup> (IVIG) 1:500	Intravenous immunoglobulin preparation	Octapharma
Venimmun <sup>®</sup> N (IVIG) 1:500	Intravenous immunoglobulin preparation	Aventis Behring
Anti-6-His-tag 1:5000	monoclonal mouse anti 6-His-tag clone 13/45/31	Dianova
Anti-human IgG 1:2500	Polyclonal HRP-goat anti-human IgG	Sigma
Anti-human FC $\gamma$ 1:100	Polyclonal PE-goat anti-human FC $\gamma$ F(ab) <sub>2</sub>	Dianova
Anti-mouse IgG 1:5000	Polyclonal HRP-goat anti-mouse IgG	Sigma

## 3.2 Methods

### 3.2.1 Depletion of specific IgGs from IVIG

*S. aureus* ATCC 29213 or *E. coli* K12 XL1blue from an overnight (ON) culture was inoculated 1:100 in 400 ml LB and cultured until an optical density of 2.0 at 600 nm ( $OD_{600}$ ) was reached. Subsequently bacteria were harvested by centrifugation, washed in phosphate-buffered saline (PBS) (pH 7.3) and resuspended in an IVIG preparation (Octagam<sup>®</sup>, Octapharma) or PBS as control. The suspension was rotated slowly ON at 4 °C, subsequently bacteria were removed by centrifugation, and the supernatant was sterile filtered through a 0.2 µm membrane filter. Protein concentration of IVIG depleted of *S. aureus* (dSaIVIG) or *E. coli* specific IgGs (dEcIVIG) was determined using the Bio-RAD DC assay according to the manufacturer's instructions.

### 3.2.2 *S. aureus* growth curves

*S. aureus* ATCC 29213 from ON culture was inoculated 1:100 into LB broth and cultured until  $OD_{600}$  of 0.3 was reached. Subsequently bacteria were harvested by centrifugation at 4 °C, 3000 g for 15 minutes. After washing once with cold PBS, the number of *S. aureus* colony forming units (CFU) was estimated by OD measurement, based on the observation that an  $OD_{600}$  of 3 equates to  $1 \times 10^9$  CFU/ml. For experimental assays  $1 \times 10^4$  CFU were inoculated in 500 µl LB broth. 13 mg/ml of either IVIG, BSA, dialysed IVIG (IVIG-DS), dSaIVIG or dEcIVIG adjusted in 500 µl PBS or solely PBS were added (final volume = 1 ml). Where indicated 25 or 50 % of the total volume (1 ml) were substituted by human serum, heat-inactivated (HI) human serum, IVIG or PBS (control). At defined time points (every hour up to 5 hours) samples were taken, diluted 1:2 in 0.1 % Triton X 100, sonicated for 5 minutes and 50 µl of suitable dilutions were plated on Mueller Hinton agar using the Eddy jet spiral plater (IUL Instruments). Plates were incubated at 37 °C for 16 hours and CFUs were enumerated using the Counterstat Flash (IUL Instruments). Experiments were performed using triplicates.

To enable expression profiling during the course of growth inhibition, RNA was isolated from *S. aureus* cultured in the presence of IVIG, dSaIVIG or PBS. In a total volume of 10 ml,  $1 \times 10^7$  CFU/ml were co-incubated with either 2.5 mg/ml IVIG, dSaIVIG or PBS substituted with 5 mg/ml Maltose (adjusted to the concentration present in IVIG) in a 1:1 ratio with LB broth. To assess growth of *S. aureus* under these conditions, triplicate samples for each treatment were included in parallel to the samples for RNA isolation. At defined time points (every 30 minutes up to 120 minutes, or immediately before RNA isolation) a sample was taken, diluted 1:2 in 0.1 % Triton X 100, sonicated for 5 minutes and 50 µl of suitable dilutions were spirally plated on Mueller Hinton agar. Plates were incubated at 37 °C for 16 hours and CFUs were enumerated. RNA samples were immediately harvested by centrifugation

and the pellets washed once with ice-cold TE pH 8.0, and immediately subjected to RNA isolation

### 3.2.3 RNA isolation

Subsequent to washing with ice cold TE pH 8.0 bacterial pellets were resuspended in 100  $\mu$ l of 0.5  $\mu$ g/ $\mu$ l Lysostaphin-TE (pH 8.0) and incubated for 20 minutes at RT, followed by Proteinase K treatment for 20 minutes at RT. Subsequently 350  $\mu$ l RLT buffer containing  $\beta$ -Mercaptoethanol was added and samples stored at -80 °C until RNA cleanup using the RNeasy<sup>®</sup> Mini kit (Qiagen) according to the manufacturer's instructions. To degrade genomic DNA an on column DNA digestion was included as recommended by the manufacturer. Concentration and purity of RNA diluted in 10 mM Tris pH 7.5 was determined by spectrophotometry. Only RNA samples with an  $A_{260}/A_{280}$  ratio of 1.9 to 2.1 were used for expression profiling. Until use RNA was stored at -80 °C.

### 3.2.4 Synthesis and labelling of cDNA

For use in quantitative realtime PCR (qPCR) 2  $\mu$ g total RNA was reverse transcribed into cDNA using the Superscript<sup>®</sup> III Kit (Invitrogen) using random hexamers according to the manufacturer's instructions. To account for variability in cDNA synthesis, each target RNA was reverse transcribed in triplicates. Absence of contaminating genomic DNA was confirmed by analysis of samples without reverse transcriptase (-RT samples).

For subsequent microarray hybridisation samples were indirectly labelled by incorporation of amino-allyl(aa)-dUTPs during cDNA synthesis, using a protocol developed by the institute for genomic research (TIGR) with modifications. 5  $\mu$ g total RNA were mixed with 6  $\mu$ g random hexamers adjusted to a volume of 14  $\mu$ l DEPC-H<sub>2</sub>O and incubated for 10 minutes at 70 °C (denaturation), subsequently chilled on ice for one minute and briefly centrifuged. Then 50 x aa-dNTP mix was added to achieve a final concentration of 0.5 mM for dATP, dCTP and dGTP, or 0.3 mM dTTP and 0.2 mM aa-dUTP, respectively in 30  $\mu$ l reaction volume. The other components (10 x RT buffer, 25 mM MgCl<sub>2</sub>, DTT and RNaseOut<sup>®</sup>) provided by the superscriptIII<sup>®</sup> Kit were added accordingly. Upon addition of 2  $\mu$ l reverse transcriptase (superscript<sup>®</sup> III) samples were incubated for 30 minutes at 25 °C, followed by ON incubation at 42 °C. Subsequently samples were incubated for 5 minutes at 85 °C, followed by addition of RNaseH and further incubation for 20 minutes at 37 °C. Prior to labelling cDNA samples were purified using the QiaQuick<sup>®</sup> PCR purification kit (Qiagen) according to the manufacturer's instructions with freshly prepared phosphate wash buffer instead of PE-wash buffer. The cDNA was eluted twice with 30  $\mu$ l Phosphate elution buffer, followed by drying using the speedvac. Cy-3 and Cy-5 dye esters were reconstituted as recommended by the manufacturer (GE-Healthcare) and 4.5  $\mu$ l were added to cDNA, resuspended in 4.5  $\mu$ l freshly prepared 0.1 M Na<sub>2</sub>CO<sub>3</sub> pH 9.0, followed by incubation for 1 hour at RT in the dark.



The Dye-coupling reaction was stopped by addition of 35  $\mu$ l 100 mM NaOAc pH 5.2. Samples intended for co-hybridisation were mixed and subjected to QiaQuick<sup>®</sup> PCR purification according to the manufacturer's instructions. Mixed labelled cDNA was eluted in 30  $\mu$ l H<sub>2</sub>O and immediately used for microarray hybridisation.

### 3.2.5 Hybridisation of microarrays

Microarray slides derived from the Bacterial Microarray Group at Saint Georges (B $\mu$ G @S) were pre-hybridised in a coplin jar at 65 °C in 50 ml of prewarmed pre-hybridisation solution for 20 minutes, followed by a 1 minute wash in H<sub>2</sub>O and a final rinse in isopropyl alcohol. Pre-hybridised microarray slides were immediately dried by centrifugation at 425 g for 5 minutes as recommended by B $\mu$ G @S. Upon elution from QiaQuick columns labelled cDNA was adjusted to a final concentration of 4 x SSC and 0.3 % SDS in a total volume of 45  $\mu$ l by addition of 20 x SSC and 2 % SDS. Samples were incubated for 2 minutes at 95 °C, allowed to chill slightly followed by brief centrifugation. Pre-hybridised microarray slides were placed in Corning hybridisation chambers (Corning) and the LifterSlips<sup>™</sup> (Implen) were carefully aligned, to cover the spotted area, then the sample was applied and moved by capillary action to the end of the LifterSlip<sup>™</sup>. To prevent slides from drying, 15  $\mu$ l H<sub>2</sub>O were applied to each of the two wells in the chamber, then chambers were sealed and submerged in a waterbath at 65 °C for 18 hours. After incubation LifterSlips<sup>™</sup> were removed and slides were washed twice in pre-heated wash solution A at 65 °C for 2 minutes, followed by washing twice in wash solution B under same conditions. Slides were dried by centrifugation and kept dry in the dark until scanning.

Samples derived from co-incubation with IVIG were labelled with Cy5 and were co-hybridised either with Cy3-labelled PBS or dSaIVIG treated samples. Four biological replicates for each comparison per time point (t0, t30 and t60) were analysed. To assess Dye-dependent variability, a Dye-swap experiment was performed.

### 3.2.6 Analyses of microarrays

Microarray slides were scanned using the Axon 4100 Scanner and GenepixPro 6.1 Software (Molecular Devices). The PMT gain for each dye (Cy3/Cy5) was adjusted to fulfil two criteria: 1) max 0.05 % saturated pixels and 2) an overall intensity ratio of both dyes close to 1. Slides were scanned with a pixel size of 10  $\mu$ m and averaging the signals upon scanning each line three times. Subsequent to scanning the grid that links spot-location to gene annotation was aligned carefully with the spotted area and the respective spots. When each spot was correctly aligned, analysis was performed and the raw data were exported to an Acuity (Molecular Devices) database for normalisation and post-analysis. In Acuity all arrays were first normalised using LOWESS normalisation followed by creating data sets for each time point (t0, t30 and t60) and co-hybridisation (IVIG vs. dSaIVIG; IVIG

vs. PBS). Recommended settings for filtering criteria were applied to exclude signals from the dataset, which are not exceeding background or signal to noise ratio levels lower than 3. Genes not showing a fold change of about 1.5 in three of the four arrays in a dataset were excluded from further analysis. The remaining dataset was then statistically analysed using student's t-test including Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) of resulting p-values. Candidate genes identified with a p-value less than 0.05, were distributed into functional groups following gene annotation and blast analysis against all completely sequenced *S. aureus* genomes.

### 3.2.7 Quantitative realtime PCR (qPCR)

The expression changes of selected genes identified by microarray analysis were validated by qPCR using the LightCycler<sup>®</sup> LC480 (Roche). As housekeeping gene 16 S rRNA was used. For qPCR 1 µl of a 1:4 dilution of each target-cDNA was used in a total reaction volume of 20 µl, containing primers, 2 × LightCycler<sup>®</sup> 480 SYBR green I mastermix adjusted with nuclease free water. Primers were used at a final concentration of 10 pmol.

#### qPCR program:

Step of program	Temperature	Duration	
Initial denaturing	95 °C	15 min.	
Cyclic denaturing	95 °C	15 sec.	} 45×
Annealing	55 °C	20 sec.	
Elongation	72 °C	20 sec.	
Melting curve	from 50 °C to 95 °C	1 °C/sec.	

Due to differences in the qPCR efficiencies between target and reference (16 S rRNA) genes the  $\Delta\Delta^{ct}$  method could not be used for analysis of the qPCR data. Therefore, a standard curve was determined for all primer pairs, by measurement of 2x serial dilutions of control (PBS, t0) cDNA and included in each run, to enable quantification. Values were normalised to 16 S rRNA and mean and SD of triplicates was calculated.

### 3.2.8 Isolation of *S. aureus* anchorless cell wall (ACW) proteins

*S. aureus* ATCC 29213 from ON culture was inoculated into LB broth adjusting to a starting OD<sub>600</sub> of 0.05. Bacteria were cultured until early exponential growth phase. ACW proteins were extracted from bacterial pellets using a modified protocol of a previously described method (Antelmann et al., 2002). The pellets were extensively washed, resuspended in 1.5 M LiCl, 25 mM Tris-HCl (pH 7.2) containing complete protease inhibitors, and incubated on ice for 30 min. Subsequent to centrifugation, the supernatants were pooled and precipitated with 10% (w/v) trichloroacetic acid ON at 4 °C. The precipitate was washed thrice with ice-cold ethanol and dried under vacuum. Extracted proteins were dissolved in 8 M

urea for 2-D gel electrophoresis (2-DE). Protein concentration was determined using the Bio-Rad DC assay in a 1:4 dilution according to the manufacturer's instructions.

### 3.2.9 Subtractive proteome analysis (SUPRA)

2-DE was performed according to the method described by Bernardo et al. (2004) using the Multiphor II system according to the manufacturer's instructions. Proteins dissolved in 8 M urea were separated on 18-cm immobilised pH gradient (IPG) strips using non-linear pH ranges of 3 to 10 and 4 to 7 (GE Healthcare). Isoelectric focusing was performed using 500 µg protein for Coomassie blue-stained gels and Western blots and 100 µg for silver-stained gels and Western blots. *S. aureus* ACW-proteins were then separated on 12.5% Tris-glycine-SDS gels (25 cm by 20 cm by 1.0 mm) using the Ettan Dalt II system (GE Healthcare).

For immunoblotting, proteins separated by 2-DE were transferred to nitrocellulose membranes using a Trans-Blot cell (Bio-Rad) according to the manufacturer's instructions. Membranes were probed ON at 4 °C either with IVIG at a dilution of 1:500 or with IVIG depleted of *S. aureus* specific IgGs (dSaIVIG) using an equivalent antibody concentration. Specific detection of immune complexes was performed using anti-human IgG-HRP conjugated secondary antibody at a 1:2,500 dilution. After treatment with dSaIVIG the membrane was stripped at 50 °C using stripping buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol and incubated with IVIG. To assess the reproducibility of spot patterns, three separate experiments were performed. Signals of interest were matched with corresponding spots on the Coomassie stained preparative gel, excised, and digested with trypsin, followed by matrix-assisted laser desorption ionisation - time of flight (MALDI-TOF) mass spectrometry performed as previously described (Bernardo et al., 2004). Probability-based scoring (probability based on implementation of the Mowse algorithm for assessing peptide and protein matches (Pappin et al., 1993)) was performed by using the equation  $-10 \times \log(P)$ , where P is the probability that the observed peptide match is a random event. Scores greater than 56 were considered significant.

### 3.2.10 Cloning, expression and purification of vaccine candidates

The ORFs encoding BT1, BT2 and BT3, lacking potential secretion signalling sequences, were amplified from *S. aureus* ATCC 29213 genomic DNA by PCR using Pfu-Polymerase. The PCR products were purified using the QiaQuick PCR purification kit according to the manufacturer's instructions. PCR product and dephosphorylated pET29b vector were restricted using NcoI and XhoI in 2x Tango buffer for 2 hours at 37 °C, subsequently ligated and transformed into *E. coli* DH5 α and selected on Kanamycin-LB plates. Vector pET29b provides a N-terminal S-tag and a C-terminal His-tag in frame with the inserted gene. Correct constructs, identified by colony PCR and restriction analysis, were sequenced and

transformed into *E. coli* BL 21 for protein expression.

Clones with the highest efficiency in the expression of the respective fusion proteins were identified by colony blot analysis. Briefly, the colonies from transformation plates after ON incubation were transferred to a fresh plate containing 1 mM IPTG by nitrocellulose filters with colonies side up. After incubation of these plates for 4 hours at 37 °C, filters were placed colony side up on Whatman paper soaked with 10 % SDS for 10 minutes, then filters were placed on paper soaked with denaturing solution for 5 minutes. Filters were subsequently incubated twice for 5 minutes on neutralising solution, and finally for 15 minutes on 20 x SSC followed by two 5 minute washes in 1 x TBST. Finally membrane filters were blocked for 30 minutes at RT and subsequently incubated with anti-His primary antibody ON at 4 °C. Prior to and after incubation with HRP-conjugated secondary antibody membranes were washed thrice in 1 x TBST, then treated with ECL reagent. Exposed films were developed, detected colonies aligned with original colonies on transformation plates and selected colonies were used for preparation of glycerol stocks for further use. Expression clones from ON culture in Kanamycin-LB were inoculated 1:100 in 400 ml Kanamycin-LB and cultured until an OD<sub>600</sub> of 0.6. Then IPTG (final concentration 1 mM) was added and incubated for 4 hours. Bacteria were harvested, washed twice in PBS and stored in 1 x PBS buffer containing EDTA-free complete protease inhibitor (Roche), 5 % Glycerol and Lysonase™ at -20 °C until lysis of the cells by French Press. Upon disruption of the cells by French press with a pressure of 20 000 PSI cell debris was eliminated by centrifugation at 17000 g.

Prior to loading onto equilibrated HisTrap™FF crude columns (GE Healthcare), lysates were adjusted to a final concentration of 500 mM NaCl and 40 mM Imidazole pH 7.4. Protein was loaded onto the column by ON recirculation at a flowrate of 1 ml per minute at 4 °C using a peristaltic pump. The loaded column was transferred to the pre-equilibrated FPLC system, washed with binding buffer and fusion protein was eluted with 250 mM imidazole in 1 x PBS containing 500 mM NaCl (elution buffer). To eliminate imidazole, eluates were pooled and dialysed twice ON against 1 x PBS containing 10 % Glycerol. Protein concentration was determined using the Bio-RAD DC assay according to the manufacturer's instructions and sterile filtered purified proteins were stored at -20 °C.

### 3.2.11 Enrichment of specific IgGs from IVIG

For enrichment of *S. aureus* specific IgGs, 5 to 10 mg of purified recombinant protein (BT1, BT2 or BT3) was covalently linked to N-hydroxysuccinimide-activated Sepharose (NHS; HiTrap™ NHS activated HP columns) columns according to manufacturer's instructions (GE Healthcare). Binding of IgGs was performed ON at 4 °C by slow recirculation of IVIG-DS. Specifically bound IgGs were eluted from the column by pH shifting using 0.1 M glycine-HCl (pH 2.7) with the ÄKTApurifier liquid chromatography system. The pH of the eluted fractions

was neutralised by addition of a sufficient amount of 1 M Tris-HCl (pH 9.0). IgG-containing fractions were pooled and, after buffer exchange into PBS (pH 7.3) using the HiPrep™ 26/10 desalting column, concentrated using Centricon Plus-70 kDa cut off centrifugal filter units (Millipore) stored sterile filtered at 4 °C .

### 3.2.12 Surface localisation of vaccine candidates

To assess the surface localisation of the antigens, *S. aureus* strain ATCC 29213 GFP was inoculated into LB broth and grown at 37 °C to an OD<sub>600</sub> of 0.3. Bacteria were harvested, washed and adjusted to  $1 \times 10^8$  CFU/ml in PBS containing 5% bovine serum albumin (BSA) and 1% normal mouse serum (MS) to block non-specific binding to Protein A. IVIG, dSaIVIG, or enriched specific IgGs (anti-BT1, anti-BT2, and anti-BT3) were added in a concentration of 5 µg/ml as primary antibodies, incubated for 30 minutes at RT and washed with PBS-0.5% BSA to remove free antibodies. A 1:100 dilution of a phycoerythrin-conjugated goat anti-human Fc $\gamma$  F(ab)<sub>2</sub> fragment (Dianova) was used as secondary antibody and was incubated for 30 minutes at RT in the dark. After removal of free secondary antibodies, samples were diluted 1:5 in PBS-0.5% BSA and analysed by flow cytometry using the FACSCalibur system and CELLQuestPro software (BD Biosciences).

### 3.2.13 *In vitro* opsonophagocytosis of *S. aureus* by human neutrophils

Human polymorphonuclear cells (PMNs) were isolated by dextran sedimentation and Ficoll-Hypaque gradient centrifugation from 50 ml of heparinised blood from a healthy donor according to standard protocols. Isolated human PMNs were resuspended in HBSS pH 7.3, and number of cells was determined by trypan blue exclusion in a Neubauer chamber. For the opsonophagocytosis assay *S. aureus* ATCC 29213-GFP was inoculated into LB broth and grown at 37 °C to an OD<sub>600</sub> of 0.3. Bacteria were harvested, washed and adjusted to  $1 \times 10^9$  CFU/ml. IVIG, dSaIVIG and specific human IgGs (anti-BT1, anti-BT2, and anti-BT3), enriched from IVIG, were added at a concentration of 100 µg/ml and pre-opsonisation of bacteria was allowed for 20 minutes at 37 °C. Bacterial uptake in the presence of 2.5% human serum or HI-serum was used as control.  $2.5 \times 10^6$  human PMNs and bacteria at a multiplicity of infection (MOI) of 10 were incubated for 5 minutes at 37 °C with slow rotation upon initial synchronisation of infection by centrifugation at 400 g for 2 minutes. Phagocytosis was stopped by centrifugation for 5 minutes at 150 g at 4 °C, and samples were washed three times, resuspended in HBSS and samples were taken for FACS analysis (1:5 dilution in PBS-0.5% BSA (pH 7.3); kept on ice until measurement) using the FACSCalibur and Cellquest Pro Software.

### 3.2.14 *In vitro* opsonophagocytic killing of *S. aureus* by human neutrophils

To assess killing of *S. aureus* by neutrophils, in contrast to samples intended for analysis of phagocytosis extracellular bacteria were not removed by differential centrifugation. PMNs and samples were isolated and adjusted as described in 3.2.13. Immediately after addition of bacteria (t<sub>0</sub>) 50 µl of each sample was taken to assess the initial CFU number and subsequently samples were centrifuged at 400 g for 2 minutes at 25 °C, to synchronise infection, followed by incubation in the rotating wheel at 37 °C for 10 minutes (t<sub>12</sub>). At indicated time points upon incubation at 37 °C in the rotating wheel (t<sub>12</sub>, t<sub>22</sub>, t<sub>32</sub> and t<sub>92</sub>) 50 µl of each sample was taken and diluted 1:50 in PMN lysis buffer (5 mM Na<sub>2</sub>CO<sub>3</sub> pH 11), to enable PMN lysis during incubation for 10 minutes at RT. Subsequently samples were further diluted 1:10 in PMN lysis buffer and plated on Mueller-Hinton agar using the spiral plater. Upon incubation at 37 °C for 16 hours CFU were enumerated using the CounterMat Flash (IUL Instruments) and percent killing was calculated by dividing the number of CFUs at the given time point (t<sub>12</sub>, t<sub>22</sub>, t<sub>32</sub> and t<sub>92</sub>) by the initial CFU number (t<sub>0</sub>) of the respective sample multiplied by 100. *S. aureus* incubated without PMNs were included to assess bacterial growth under experimental conditions. Experiments were performed using triplicates.

### 3.2.15 Immunisation of mice

Female BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratories. Mice were injected intraperitoneally with a 1:1 emulsion (total volume, 200 µl) containing 80 µg recombinant protein (BT1, BT2, or BT3) and complete Freund's adjuvant (Sigma) on day 0, followed by subcutaneous administration of two booster doses using an emulsion containing 40 µg antigen and incomplete Freund's adjuvant (1:1) on days 31 and 59. Mice immunised with BSA as non-specific antigen served as controls. For the bivalent immunisation using recombinant BT1 and BT3 50 or 25 µg of each antigen was used for initial or booster immunisation, respectively, to not exceed the total amount of 40 or 20 µg used for single target immunisation. To determine the specific antibody titre, blood samples were taken on days 14, 45, 66 by retro-orbital bleeding under anaesthesia and analysed by ELISA on respective 0.5 µg/ml antigen and total *E. coli* lysate as control antigen in a 5-fold serial dilution using the OptiEIA™ TMB substrate reagent set (BD Biosciences) according to the manufacturer's instructions. The titre is given as the dilution corresponding to the half maximal antibody response.

### 3.2.16 Murine model of sepsis

One week after the last booster immunisation BALB/c mice (n = 11 to 12) were challenged with LD<sub>50</sub> of *S. aureus* ATCC 29213 by intravenous (i.v.) injection of 3 x 10<sup>7</sup> CFU in 300 µl PBS. Mice were monitored daily for clinical signs of infection and mortality for 14 days.

### **3.2.17 Statistical analysis**

Growth curve results were statistically analysed by one-way ANOVA, followed by Bonferroni post analysis using GraphPad Prism version 5.03 for Windows (GraphPad Software, [www.graphpad.com](http://www.graphpad.com)). Microarray data were analysed by unpaired, two-tailed student's t-test including Benjamini-Hochberg correction to minimise the false discovery rate (Benjamini and Hochberg, 1995) using Acuity (Molecular Devices). Survival curves (Kaplan-Meier) were analysed by the Gehan-Breslow-Wilcoxon test implemented in Graphpad Prism 5.

## 4 Results

### 4.1 Characterisation of the bacteriostatic effect mediated by *S. aureus* specific IgGs

#### 4.1.1 Human serum inhibits *in vitro* growth of *S. aureus*

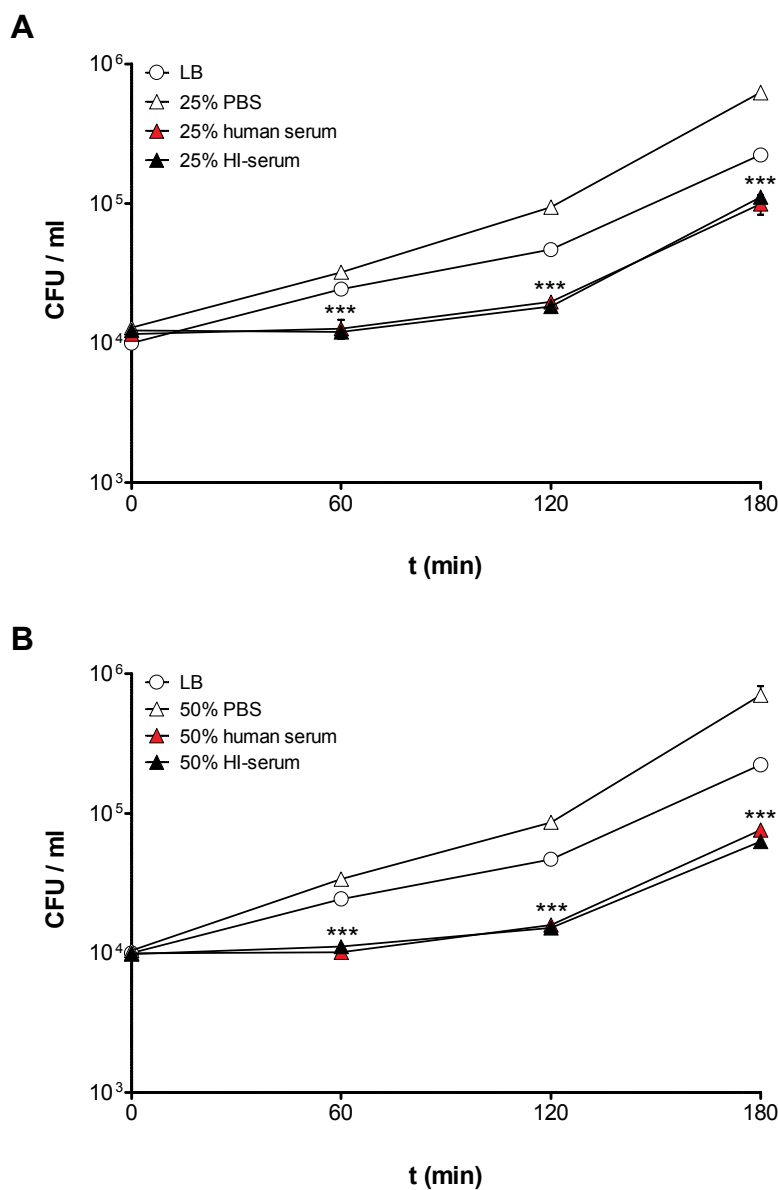
It was observed previously that human serum exhibits a bacteriostatic effect on growth of *S. aureus* and that this bacteriostatic effect most likely is caused by antibodies (Ehrenkranz et al., 1971). Since this effect would be highly beneficial regarding the use of antibodies in passive immunisation, we aimed to confirm this finding. To this end *S. aureus* was co-cultured with 25 or 50% of complete or heat-inactivated (HI) human serum *in vitro*, to measure the potential bacteriostatic effect in a classical colony forming unit (CFU) enumeration assay. As shown in Figure 4.1, growth of *S. aureus* was statistically significantly inhibited in the presence of either 25% (A) or 50% (B) human serum compared to control samples, cultured in the presence of an equal amount of PBS or solely LB. Moreover, no difference in the inhibitory capacity of heat-inactivated human serum (HI-serum) compared to complete human serum was detected, hence indicating that heat-stable components, most presumably IgGs, in the serum cause the bacteriostatic effect.

#### 4.1.2 Intravenous immunoglobulin preparation (IVIG) specifically inhibits *in vitro* growth of *S. aureus*

To determine whether bacteriostasis is mediated by *S. aureus* specific IgGs, an IVIG preparation, previously shown to contain opsonising IgGs specific for *S. aureus* (Glowalla et al., 2009), was tested for effects on bacterial growth. For this purpose *S. aureus* was incubated in the presence of either 25 or 50% IVIG and samples where 25 or 50% of the culture volume was replaced by PBS served as control.

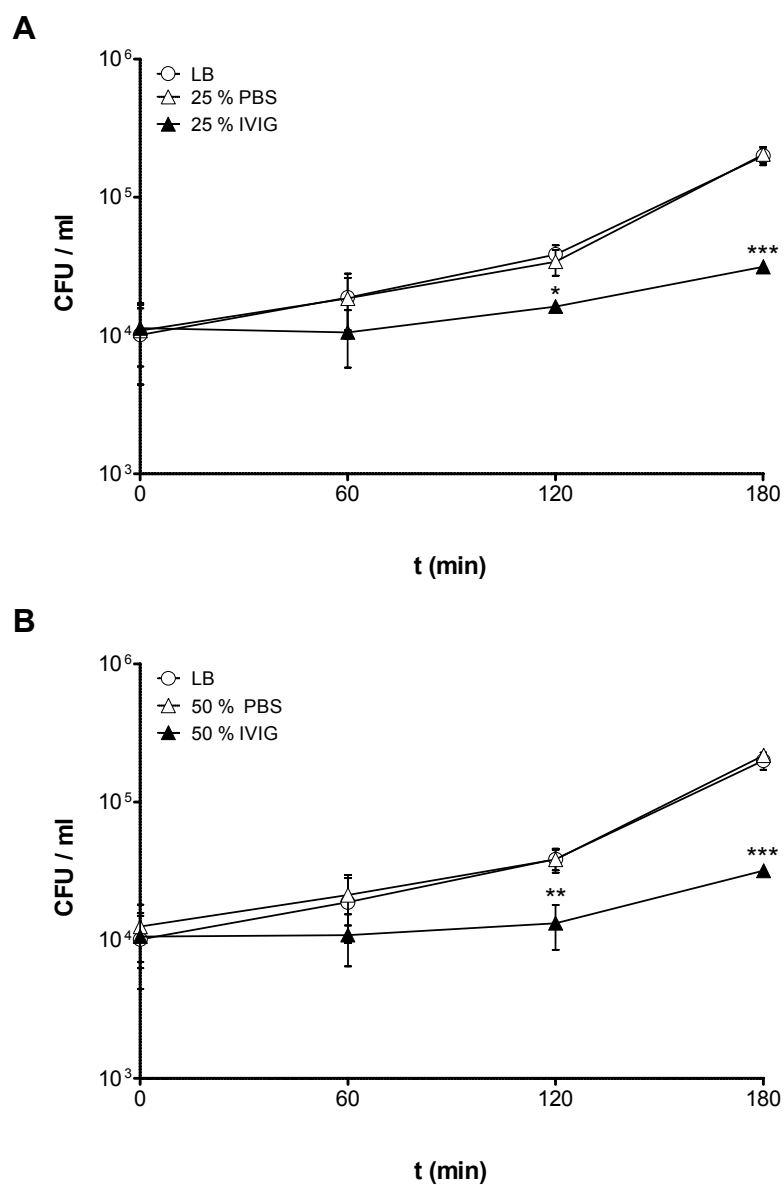
As shown in Figure 4.2, growth of *S. aureus* was statistically significantly inhibited for up to 120 minutes upon addition of IVIG. Although the number of CFUs in IVIG-treated samples slightly increased after 120 minutes, the number of CFUs was significantly lower in comparison to the respective PBS control 180 minutes after addition of IgGs.





**Figure 4.1:**

**Effect of human serum on *in vitro* growth of *S. aureus*.** Growth kinetics of *S. aureus* in LB (open circles) and upon addition of 25% (A) or 50% (B) of human serum (red closed triangles), heat-inactivated human serum (HI-serum; closed triangles) or an equal amount of PBS (open triangles), respectively was assessed using a CFU enumeration assay. At indicated time points 50  $\mu$ l diluted bacterial culture was spirally plated on Mueller-Hinton agar, incubated for up to 16 h at 37 °C and subsequently CFU per ml were counted. Shown are mean and SD of triplicates from a representative experiment. Statistical significance between serum treated compared to the respective PBS control sample was determined by one-way ANOVA followed by Bonferroni post analysis. P-values are indicated by asterisks (\* \* \* =  $p < 0.001$ ).

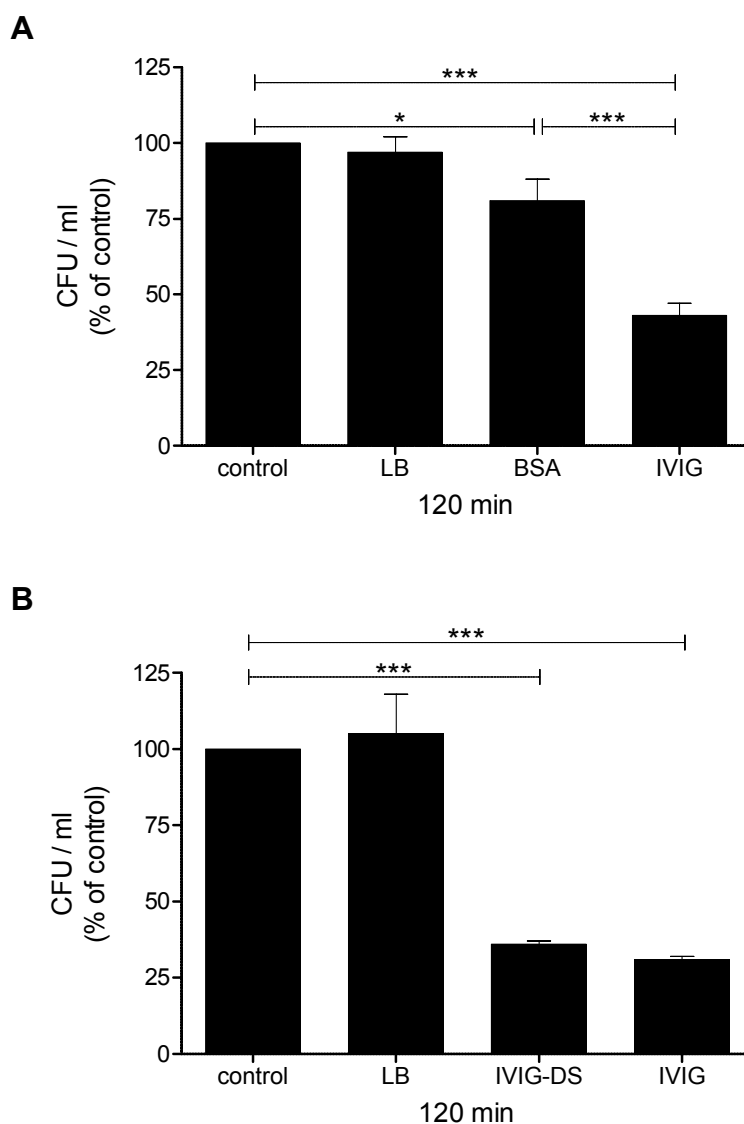


**Figure 4.2:**

**Effect of IVIG on *in vitro* growth of *S. aureus*.** Growth kinetics of *S. aureus* in LB (open circles) and upon addition of 25 % (A) or 50 % (B) IVIG (closed triangles) or an equal amount of PBS (open triangles), respectively was assessed using a CFU enumeration assay as described above. Shown are mean and SD of triplicates from a representative experiment. Statistical significance between IVIG treated compared to respective PBS control sample was determined by one-way ANOVA followed by Bonferroni post analysis. P-values are indicated by asterisks (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ).

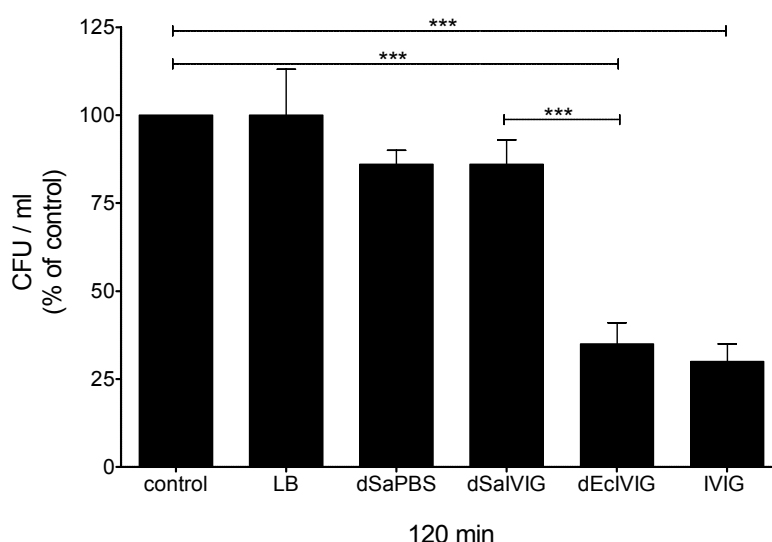
### 4.1.3 Bacteriostatic effect on *S. aureus* is mediated by *S. aureus* specific IgGs

To ensure that the observed inhibitory effect mediated by IVIG is not due to changes in culturing conditions by the addition of protein, the experiments were performed using 13 mg/ml BSA or IVIG adjusted in PBS, respectively. This concentration of IVIG equates to 25% IVIG as used in previously described experiments. In contrast to IVIG treatment the co-cubation of *S. aureus* with BSA led to significant but only minor inhibition of growth (Fig. 4.3 A), thereby indicating that bacteriostasis is a specific effect mediated by IVIG.



**Figure 4.3:**

**Effect of BSA or IVIG-DS on *in vitro* growth of *S. aureus*.** Growth kinetics of *S. aureus* in LB and upon addition of 13 mg/ml BSA (A) or dialysed IVIG (IVIG-DS; B) compared to IVIG in PBS was assessed using a CFU enumeration assay. Samples supplemented with 50% PBS served as control. Shown are mean and SD of triplicates in percent of PBS control 120 minutes after addition of the respective substances. Statistical significance between indicated samples (capped lines) was determined by one-way ANOVA followed by Bonferroni post analysis. P-values are indicated by asterisks (\*\*\*) =  $p < 0.001$ .



**Figure 4.4:**

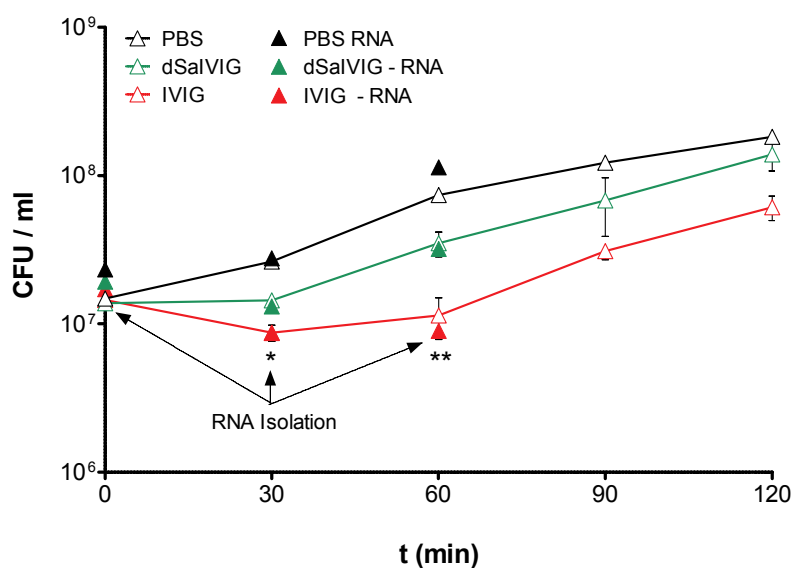
**Effect of IVIG on *in vitro* growth of *S. aureus* upon depletion of *S. aureus* or *E. coli* specific IgGs.** Growth kinetics of *S. aureus* in LB and upon addition of 13 mg/ml IVIG or IVIG upon depletion of *S. aureus* specific IgGs (dSaIVIG) was assessed using a CFU enumeration assay. Samples supplemented with 50% PBS, 50% PBS co-incubated with *S. aureus* (dSaPBS) or 13 mg/ml IVIG depleted of *E. coli* specific IgGs (dEcIVIG) served as control. Shown are mean and SD of triplicates in percent of the given PBS control 120 minutes after addition of the respective substances. Statistical significance between indicated samples (capped lines) was determined by one-way ANOVA followed by Bonferroni post analysis. P-values are indicated by asterisks (\*\*\*) ( $p < 0.001$ ).

Furthermore, when IVIG dialysed against PBS (IVIG-DS) was used, the bacteriostatic effect remained (Fig. 4.3 B), indicating that the observed bacteriostatic effect is solely due to the presence of IgGs and independent of the buffer composition of the original IVIG preparation. To elucidate whether *S. aureus* specific antibodies are responsible for the bacteriostatic effect, IVIG was depleted of *S. aureus* specific IgGs (dSaIVIG) ON at 4 °C and upon sterile filtration used as source of non-specific IgGs in a CFU enumeration assay. To rule out possible side effects caused by the process of depletion, PBS after ON co-incubation with *S. aureus* (dSaPBS) and IVIG depleted of *E. coli* specific IgGs (dEcIVIG) were used as additional controls.

When *S. aureus* was cultured in the presence of dSaIVIG, growth was comparable to bacteria cultured without IgGs (PBS control, LB; Fig. 4.4). In contrast, growth of *S. aureus* cultured in the presence of dEcIVIG was inhibited to a comparable extent as bacteria cultured in the presence of IVIG. Hence, these results imply that *S. aureus* specific IgGs are responsible for the bacteriostatic effect on growth of *S. aureus*.

#### 4.1.4 Gene expression profiling of *S. aureus* over the course of bacteriostasis

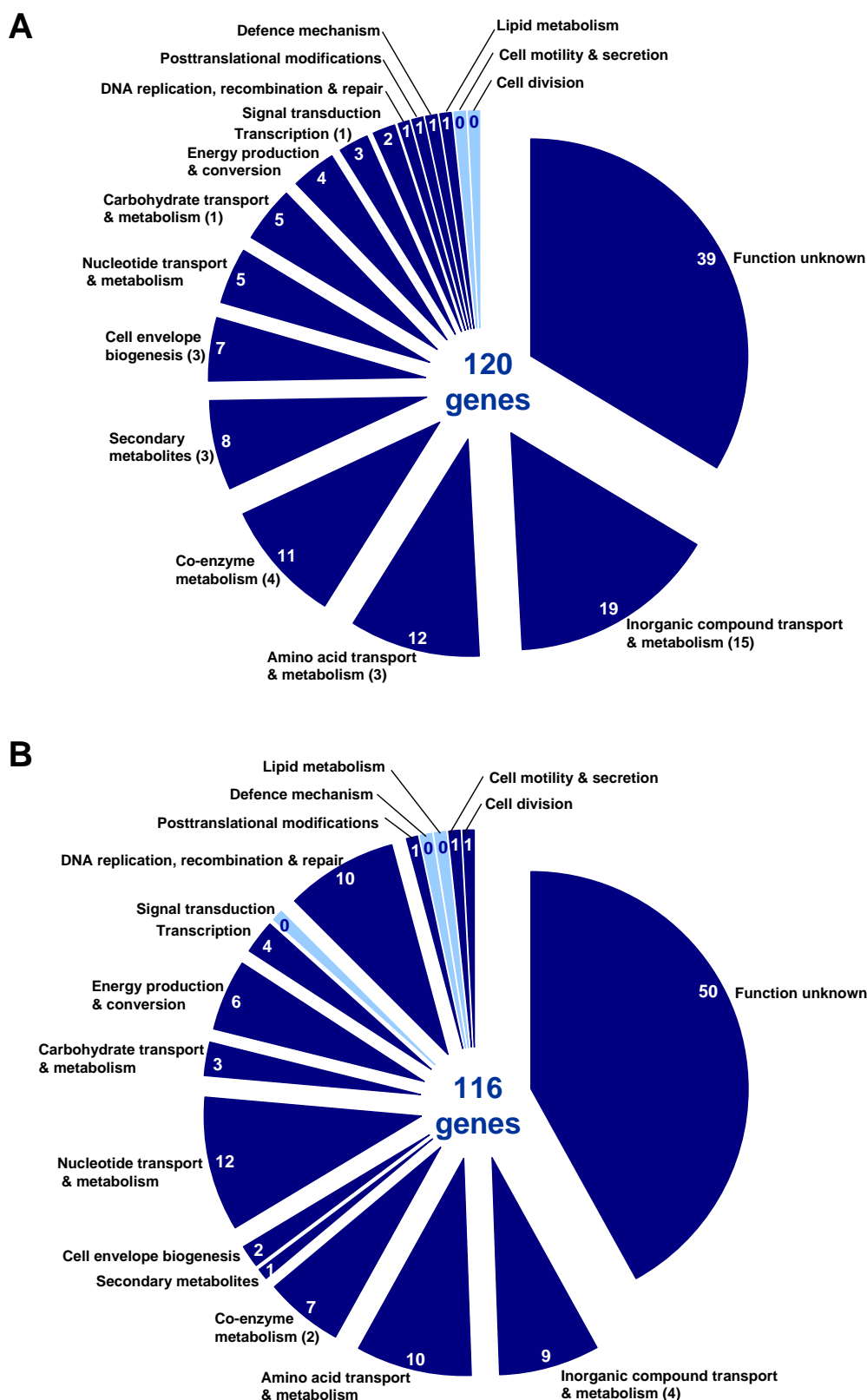
To elucidate the mechanism leading to the observed bacteriostatic effect elicited by specific IgGs on *S. aureus* growth, gene expression profiling was performed. For this purpose the culturing conditions were optimised to the minimal IVIG concentration, sufficient to elicit bacteriostasis and an elevated initial number of *S. aureus*-CFUs ( $1 \times 10^7$  /ml instead of  $1 \times 10^4$  /ml), in order to enable isolation of an adequate amount of RNA for subsequent microarray analyses. Due to these changes in the experimental setup, the duration of the bacteriostatic effect slightly decreased (see Fig. 4.5).



**Figure 4.5:**

**Growth kinetics of *S. aureus* for RNA isolation.** A growth curve of *S. aureus* cultured in the presence of 50% PBS (black open triangles) 2.5 mg/ml IVIG (red open triangles) or dSaIVIG (green open triangles) was assessed by CFU enumeration assay. Shown are mean and SD of triplicates from a representative experiment. Arrows indicate time point of RNA isolation and filled triangles in the respective colour represent CFU/ml in RNA sample immediately prior to RNA isolation. Statistical significance of the difference of IVIG compared to dSaIVIG treated samples was determined by one-way ANOVA followed by Bonferroni post analysis. P-values are indicated by asterisks (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ).

The RNA of IVIG, dSaIVIG and PBS supplemented *S. aureus* cultures was isolated immediately upon addition of IgGs or PBS (t0), during inhibition (t30) and shortly before IVIG treated cultures start to grow (t60). For subsequent hybridisation to the *S. aureus* specific microarray samples were indirectly labelled with Cy5- (IVIG) and Cy3-dyes (dSaIVIG or PBS). The employed microarray comprises all predicted open reading frames present in any of seven fully sequenced *S. aureus*-genomes (Witney et al., 2005), thereby accounting for the high variability in staphylococcal genomes.



**Figure 4.6:**

**Differentially expressed genes in IVIG compared to dSalVIG samples distributed into functional categories.** Differentially expressed genes within IVIG treated samples compared to dSalVIG samples over the whole time course were distributed into functional categories according to gene annotation. All genes showed at least 1.5-fold up- (A) or downregulation (B) in at least three of four arrays with  $p < 0.05$  after Benjamini-Hochberg correction at any of the analysed time points (t0, t30 and t60). Numbers in parentheses indicate number of genes related to iron uptake.

**Table 4.1:**

Differentially expressed genes related to iron uptake and metabolism in IVIG versus dSaIVIG treated samples revealed by microarray analysis

Common <sup>a)</sup>	Predicted function	GI protein	t0		t30		t60	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
<i>Regulation of iron uptake and storage:</i>								
fur	iron uptake regulatory protein	49483748			<b>-1.5</b>	0.019		
<i>Iron storage and prevention of oxidative stress:</i>								
SAR1344	catalase	49483526			<b>-2.8</b>	0.019	<b>-2.0</b>	0.017
SAR1984	ferritin	49484134	<b>-2.6</b>	0.035	<b>-4.7</b>	0.019	<b>-4.1</b>	0.017
SAR2227	non-heme iron-containing ferritin	49484363	<b>-2.9</b>	0.017				
<i>Porphyrin synthesis:</i>								
hemC	porphobilinogen deaminase	49483913					<b>-1.6</b>	0.014
hemD	uroporphyrinogen III synthase	49483912	<b>-1.5</b>	0.012				
<i>Siderophore mediated iron uptake:</i>								
fhuB	ferrichrome transport permease	49482876			<b>2.1</b>	0.026		
fhuC	ferrichrome transport ATP-binding protein	49482875			<b>2.4</b>	0.026	<b>1.9</b>	0.016
fhuD	ferrichrome transport permease	49482877			<b>2.1</b>	0.036	<b>1.8</b>	0.017
fhuD2	ferrichrome-binding lipoprotein precursor	49484499			<b>2.4</b>	0.039		
sirA	lipoprotein	49482358			<b>7.9</b>	0.019	<b>4.4</b>	0.018
sirB	siderophore transport system permease	49482357			<b>5.8</b>	0.019	<b>3.4</b>	0.017
sirC	siderophore transport system permease	49482356					<b>3.5</b>	0.038
sstA	FecCD transport family protein	49482990					<b>3.8</b>	0.034
sstB	FecCD transport family protein	49482991			<b>5.6</b>	0.032	<b>4.1</b>	0.028
sstC	ABC transporter ATP-binding protein	49482992	<b>2.3</b>	0.025	<b>7.8</b>	0.019	<b>4.7</b>	0.017
sstD	lipoprotein	49482993			<b>9.3</b>	0.019	<b>4.6</b>	0.017
SAR2266	FecCD transport family protein	49484395			<b>3.4</b>	0.030	<b>2.3</b>	0.023
SAR2268	transport system binding lipoprotein	49484397			<b>6.0</b>	0.019	<b>3.9</b>	0.019
<i>Associated with siderophore uptake:</i>								
sbnA	pyridoxal-phosphate dependent enzyme	49482359					<b>3.5</b>	0.033
sbnB	put. ornithine cyclodeaminase	49482360					<b>4.7</b>	0.017
sbnC	put. siderophore biosynthesis protein	49482361					<b>3.9</b>	0.012
sbnD	put. transport protein	49482362					<b>3.0</b>	0.012
sbnE	put. siderophore biosynthesis protein	49482363					<b>3.1</b>	0.017
sbnF	put. siderophore biosynthesis protein	49482364					<b>2.6</b>	0.017
sbnG	put. aldolase	49482365					<b>2.8</b>	0.012
sbnH	pyridoxal-dependent decarboxylase	49482366					<b>2.1</b>	0.012
sbnI	hypothetical protein	49482367					<b>2.3</b>	0.012
ribA	riboflavin biosynthesis protein	49484013	<b>4.8</b>	0.024	<b>21.2</b>	0.006	<b>8.8</b>	0.013
ribD	bifunctional riboflavin biosynthesis protein	49484015	<b>5.1</b>	0.017	<b>16.9</b>	0.006	<b>7.9</b>	0.012
ribE	riboflavin synthase alpha chain	49484014	<b>4.7</b>	0.049	<b>15.2</b>	0.019	<b>8.3</b>	0.013
ribH	6,7-dimethyl-8-ribityllumazine synthase	49484012	<b>3.8</b>	0.036	<b>13.0</b>	0.006	<b>6.6</b>	0.013
<i>Uptake of heme or transferrin bound iron:</i>								
isdI	heme-degrading monooxygenase	49482408	<b>1.7</b>	0.049	<b>2.4</b>	0.043	<b>2.4</b>	0.038
isdA	iron-regulated heme-iron binding protein	49483292			<b>3.0</b>	0.030	<b>1.7</b>	0.026
isdC	put. surface anchored protein	49483293			<b>2.7</b>	0.019	<b>2.8</b>	0.020
srtB	sortase B	49483297			<b>5.3</b>	0.037	<b>3.4</b>	0.012

<sup>a)</sup> Prefix SAR, MRSA252 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

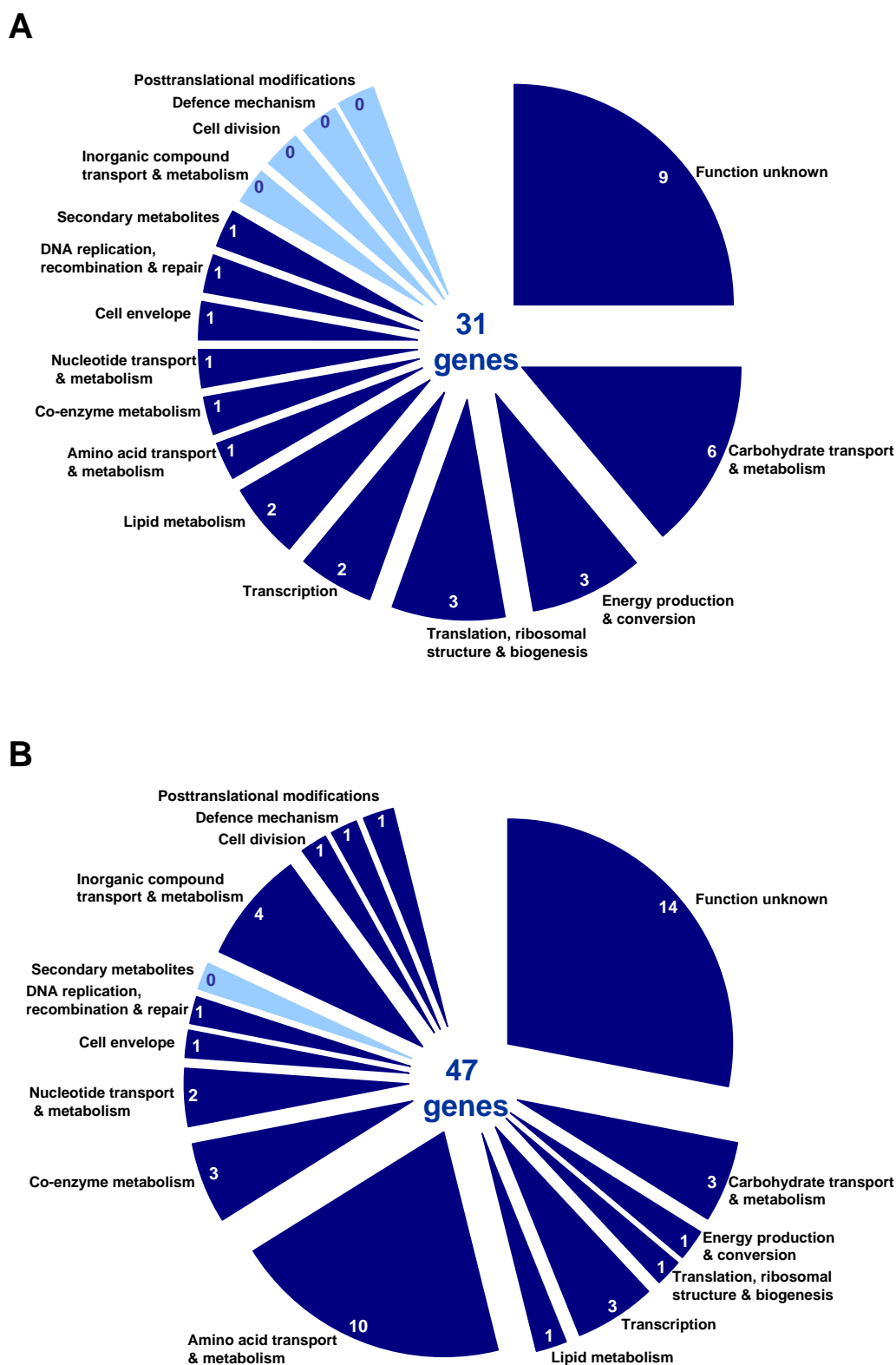
To identify differentially expressed genes in IVIG treated samples at each time point (t0, t30 and t60), microarrays were hybridised in parallel with either IVIG and dSalVIG (source of non-specific IgGs) treated samples (microarray set A), or IVIG and PBS treated samples (microarray set B) for each time point. Only genes showing a minimum fold change of 1.5 in at least three of the four arrays for each time point (biological replicates) with a p-value of less than 0.05 upon Benjamini-Hochberg correction were taken into account. The microarray analysis of IVIG compared to dSalVIG (microarray set A) or PBS (microarray set B) led to the identification of divergent sets of differentially expressed candidate genes.

Concerning the dSalVIG control (microarray set A), Figure 4.6 illustrates the distribution of all genes matching these criteria into functional categories upon co-hybridisation of IVIG with dSalVIG at any of the analysed time points. In total 236 genes were found to be differentially expressed in IVIG compared to dSalVIG samples. As shown in figure 4.6 A, 120 genes were upregulated and the remaining 116 genes were downregulated (Fig. 4.6 B). Since *S. aureus* genomes contain many ORFs coding for so far uncharacterised proteins, it is not surprising that the largest group, with in total 89 genes, represents genes with yet unknown function. Interestingly 19 genes of the in total 28 genes in the second largest group representing genes linked to inorganic compound transport and metabolism are related to iron uptake. In addition, further 17 genes related to iron uptake or metabolism were found in other functional categories (numbers given in parentheses). While the majority of these genes were upregulated, only six genes were downregulated in IVIG treated samples. The ferric uptake regulator *fur* was 1.5-fold downregulated at t30 and the expression of its target genes was altered accordingly (Dale et al., 2004b; Horsburgh et al., 2001b; Speziali et al., 2006). Dependent on the predicted function in iron uptake these genes were arranged and the fold change as well as the respective p-value for each time point are listed in Table 4.1 (for all 236 genes see Table 9.2 in supplement, page 78 ff.).

The in parallel performed microarray analysis of IVIG samples co-hybridised with PBS samples as additional control (microarray set B) resulted in 78 genes matching the filtering criteria for the identification of differentially expressed genes. Only 13 of these genes were also identified as differentially expressed in the other set of microarray analysis (IVIG versus dSalVIG; Table 9.1, page 78). Surprisingly, none of these genes is related to iron uptake or metabolism, hence indicating a strong difference between the two applied controls (dSalVIG and PBS). Furthermore, there was no apparent functional connection between these candidates.

A distribution of all up- or downregulated genes among the 78 candidate genes according to functional categories is depicted in Figure 4.7 A and B, respectively. Again almost one third of the identified genes belong to the group of unknown function. Followed by the two groups of genes related to aminoacid- (in total 11 genes) and carbohydrate transport and metabolism (in total 9 genes). Two or four additional genes related to aminoacid or carbohydrate metabolism, respectively were identified in other functional groups.





**Figure 4.7:**

**Differentially expressed genes in IVIG compared to PBS samples distributed into functional categories.** Differentially expressed genes within IVIG treated samples compared to PBS samples over the whole time course were distributed into functional categories according to gene annotation. All genes showed at least 1.5-fold up- (A) or downregulation (B) in at least three of four arrays with  $p < 0.05$  after Benjamini-Hochberg correction at any of the analysed time points (t0, t30 and t60).

All genes falling into one of these two categories, including potential regulators are listed in Table 4.2 (for all 78 genes see Table 9.3 in supplement, page 84 ff.). Since most of the candidates related to carbohydrate transport and metabolism were not identified as differentially expressed during inhibition (t30), these are rather unlikely involved in bacteriostasis.

**Table 4.2:**

Main clusters of differentially expressed genes in IVIG related to PBS treated samples revealed by microarray analysis

Common <sup>a)</sup>	Predicted function	GI protein	t0		t30		t60	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
<i>Aminoacid transport and metabolism:</i>								
lysP	lysine-specific permease	49483924			<b>-1.6</b>	0.015		
oppB	oligopeptide transport system permease	49483147			<b>-2.1</b>	0.020		
oppC	oligopeptide transport system permease	49483148	<b>-1.8</b>	0.033	<b>-2.1</b>	0.017		
pyrAA	put. carbamoyl-phosphate synthase, pyrimidine-specific, small chain	49483365					<b>-1.9</b>	0.043
rocD	ornithine aminotransferase	49483117			<b>-1.7</b>	0.017		
SAR0243	put. zinc-binding dehydrogenase	49482482					<b>-1.8</b>	0.037
SAR0521	GntR family regulatory protein	49482747	<b>-1.8</b>	0.033	<b>-1.8</b>	0.015	<b>-1.8</b>	0.037
SAR0920	NAD-specific glutamate dehydrogenase	49483118					<b>4.0</b>	0.043
SAR1338	put. homoserine dehydrogenase	49483520			<b>-4.6</b>	0.031		
SAR1702	put. cysteine desulfurase	49483867	<b>-1.6</b>	0.033				
SAR1703	put. oxygenase	49483868					<b>-1.7</b>	0.037
SAR1849	proline dehydrogenase	49484011			<b>-2.6</b>	0.021		
SAR2682	put. aminotransferase	49484800			<b>-2.5</b>	0.017		
<i>Carbohydrate transport and metabolism:</i>								
bglA	6-phospho-beta-glucosidase SAR0264	49482503					<b>3.9</b>	0.043
fdaB	fructose 1,6 bisphosphate aldolase	88196553					<b>2.2</b>	0.037
fruA	PTS transport system, fructose-specific IIBC component	49482956					<b>1.8</b>	0.043
glpD	aerobic glycerol-3-phosphate dehydrogenase	49483464					<b>3.0</b>	0.037
glpF	put. glycerol uptake facilitator protein	49483462					<b>5.3</b>	0.043
SAR0205	put. maltose ABC transporter, ATP-binding protein	49482446			<b>-2.4</b>	0.016		
SAR0660	DhaK subunit	49482878						
SAR2399	put. transcription regulator	49484531					<b>-1.7</b>	0.037
SAR2408	PTS system, arbutin-like IIBC component	49484538			<b>-2.8</b>	0.017		
SAR2409	put. transcription regulator	49484539					<b>-1.8</b>	0.045
SAR2589	put. glucarate transporter	49484713					<b>6.4</b>	0.037
scrR	sucrose operon repressor	49484268					<b>2.1</b>	0.043
uhpT	put. sugar phosphate transport protein	49482454			<b>-2.6</b>	0.029		

<sup>a)</sup> Prefix SAR, MRSA252 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

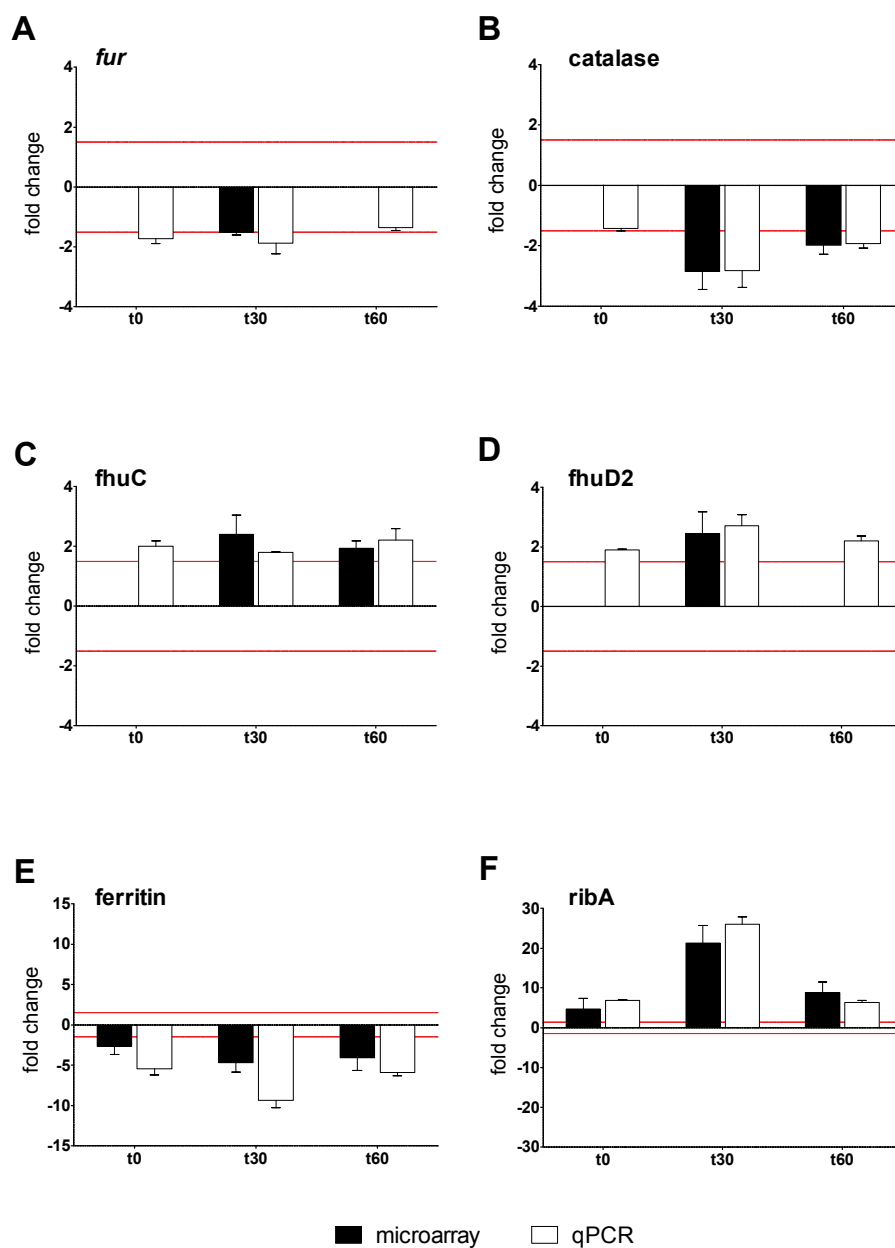
Due to the pivotal role of iron for growth of bacteria, the prominent signature of iron related genes as detected by microarray set A, could explain the observed bacteriostatic effect in IVIG treated samples. Especially, as a direct bacteriostatic effect of immunoglobulins by interference with iron uptake was already shown for other bacterial species (Fitzgerald and

Rogers, 1980; Goel and Kapil, 2001). Thus, quantitative real time PCR (qPCR) for selected iron related genes was applied to validate the microarray results and to analyse, whether the iron signature was just missed by IVIG vs. PBS arrays, due to the stringent filtering criteria.

As shown in Figure 4.8, qPCR results for each analysed gene (*fur*, *catalase*, *fhuC*, *fhuD2*, *ferritin* and *ribA*) show a similar fold change of gene expression as was measured by microarray analysis, hence verifying microarray data. Since qPCR is more sensitive than microarray analyses it is not surprising that in contrast to microarray results three of the tested genes (*fur*, *fhuC* and *fhuD2*) are already more than 1.5-fold up- or downregulated at t0 or t0 and t60 in case of *fhuD2* expression.

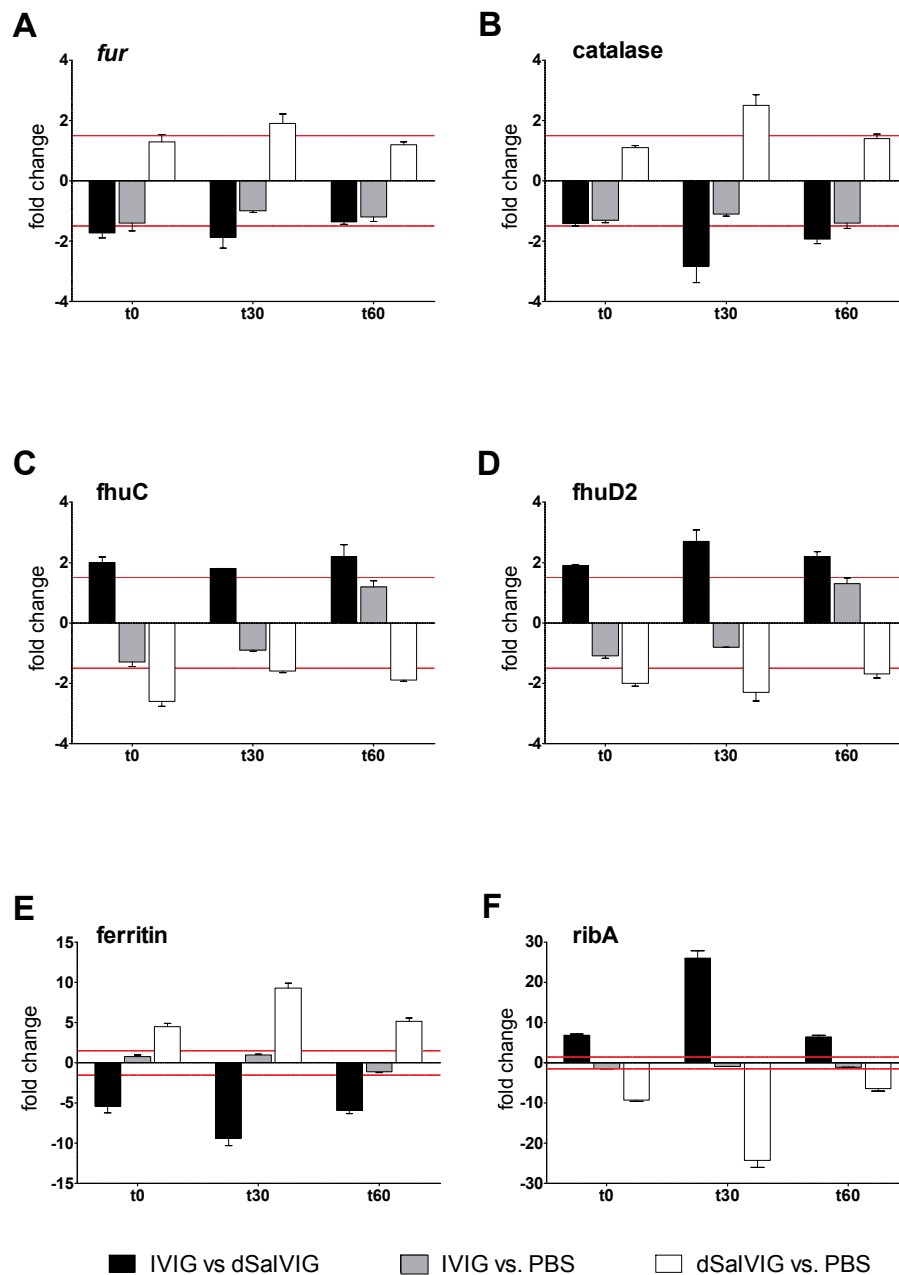
Furthermore, qPCR analysis (Fig. 4.9) confirmed that the iron signature as identified by comparison of IVIG to dSalVIG samples (black bars) is not detectable by analysis of IVIG versus PBS samples (grey bars). Whereas in case of qPCR for *fur* or *catalase* at t0 or t0 and t30, respectively the IVIG vs. PBS samples only marginally failed the fold change filter (1.5- fold), the qPCR results for all other tested genes clearly demonstrated that the iron signature as identified by microarray set A is not present in comparison of IVIG to PBS treated samples (microarray set B). Moreover, qPCR results of dSalVIG related to PBS treated samples (white bars) revealed that the iron signature is most likely derived from dSalVIG, since the obtained results represent the almost exact reciprocal values of IVIG related to dSalVIG (black bars). Hence the iron signature is not representing the mechanism underlying bacteriostasis and, most importantly, the data obtained with dSalVIG are not applicable for further investigation of this mechanism. Since the depletion of *S. aureus* specific IgGs was performed by ON incubation with viable staphylococci at 4 °C, it is likely that next to high affinity iron chelators (siderophores) also other staphylococcal factors affecting microarray analyses are secreted and not eliminated by filtration. For example the most likely pre-existing siderophores in dSalVIG apparently lead to an immediate supply of *S. aureus* with iron at early time points, thus causing the false identification of the iron signature by microarray set A. To overcome this problem in future, a chromatographic purification of IgGs subsequent to depletion of specific IgGs is essential.

Prerequisite for the identification of genes involved in the mechanism underlying the observed bacteriostatic effect mediated by *S. aureus* specific IgGs is a reliable gene expression signature. Consequently, since under the used conditions no distinct signature could be identified, any further characterisation was infeasible. Therefore, the bacteriostatic effect on *S. aureus* can currently not serve as a selection criterion for the search of protective antibodies. Thus, our further search for protective *S. aureus* specific antibodies was based on a subtractive selection procedure recently developed in our laboratory.



**Figure 4.8:**

**Comparison of qPCR results to microarray results.** RNA of IVIG and dSaIVIG samples for each time point (t0, t30 and t60) was reverse transcribed into cDNA in triplicate. The qPCR was performed using primers specific for *fur* (A), *catalase* (B), *fhuC* (C), *fhuD2* (D), *ferritin* (E) and *ribA* (F). Shown is the fold change in expression of the respective gene in IVIG versus dSaIVIG samples (white bars) compared to the respective microarray result (black bars) for each time point. Represented is mean and SD. Red lines indicate a fold change of 1.5.



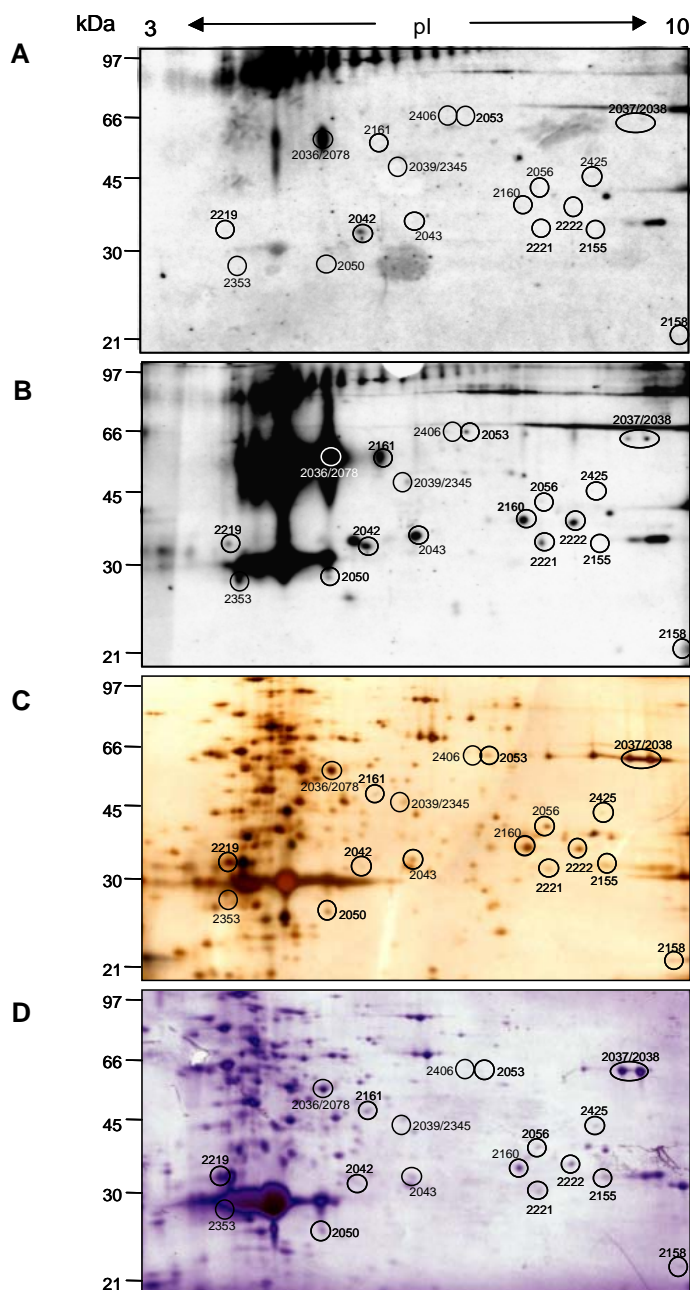
**Figure 4.9:**

**qPCR results of IVIG vs. dSalIVIG and IVIG or dSalIVIG vs PBS.** RNA of IVIG, dSalIVIG and PBS treated samples for each time point (t0, t30 and t60) was reverse transcribed into cDNA in triplicate. The qPCR was performed using primers specific for *fur* (A), *catalase* (B), *fhuC* (C), *fhuD2* (D), *ferritin* (E) and *ribA* (F). Shown is the fold change in expression of the respective gene in IVIG versus dSalIVIG samples (black bars), IVIG vs. PBS (grey bars) and dSalIVIG vs. PBS (white bars) for each time point. Represented is mean and SD. Red lines indicate a fold change of 1.5.

## 4.2 Identification and characterisation of potential vaccine candidates against *S. aureus*

### 4.2.1 Subtractive proteome analysis (SUPRA) of anchorless cell wall (ACW) proteins enables identification of potential vaccine candidates against *S. aureus*

Anchorless cell wall proteins (ACW proteins) from *S. aureus* strain ATCC 29213 were separated by 2-DE, followed by Western blot analysis using IVIG and dSaIVIG to identify antigenic proteins. To obtain high-quality spot resolution and to ensure adequate spot matching and identification, proteins were separated using two different pH ranges, pH 3 to 10 (Fig. 4.10) and pH 4 to 7 (not shown), in a series of three gels in parallel. One gel was used for immunoblotting, probing the same blot twice, first with dSaIVIG (Fig. 4.10 A) and subsequently with IVIG (Fig. 4.10 B). The two remaining gels were stained for spotmatching (silver; C) and excision of protein spots (Coomassie; D). IVIG and dSaIVIG produced reproducibly distinct immunoblot profiles for *S. aureus* ACW proteins in at least three individual experiments. The depletion of *S. aureus* specific, opsonising IgGs (dSaIVIG) resulted in a considerably lower number of protein spots compared to immunoblots probed with IVIG (compare Fig. 4.10 A and B). Proteins recognised by complete IVIG but not dSaIVIG were referred to as potential vaccine candidates, based on the assumption that these are detected by naturally occurring *S. aureus* specific IgGs, hence representing *in vivo* expressed antigens. In contrast, protein spots strongly reacting with both IVIG and dSaIVIG were excluded from further investigation, since these are more likely to be recognised by IgGs that lack specificity for *S. aureus* antigens. Furthermore, protein spots not detected in all three individual experiments were also excluded from subsequent analysis. Protein spots of interest were excised from Coomassie stained gels and used for identification by MALDI - TOF analysis. To account for potential variability in the IgG repertoire, a second IVIG preparation (Venimmun<sup>®</sup> N) was used for Western blot analysis, revealing an almost identical protein pattern as obtained using Octagam<sup>®</sup>. A total of 37 proteins detected by both IVIG preparations were identified using SUPRA (see Table 4.3). Except for four candidate proteins, namely, enterotoxin M and three hypothetical proteins (spot ID 2089, 2222, and 2240), all identified proteins are highly conserved in *S. aureus*, since their sequences were present in all 15 completely sequenced genomes of *S. aureus*, currently available in the GenBank database. These four proteins not conserved among sequenced *S. aureus* strains were excluded from further investigation because ubiquitous occurrence is a major prerequisite for a vaccine candidate.



**Figure 4.10:**

**Subtractive proteome analysis (SUPRA) of *S. aureus* ACW proteins using IVIG and dSaIVIG**  
 ACW proteins isolated from *S. aureus* strain ATCC 29213 were separated according to their isoelectric points on pI 3 to 10 IPG strips. Gels loaded with 100  $\mu$ g protein were used for subtractive immunoblotting. Spots not immunoreactive with dSaIVIG (A) but immunoreactive with IVIG (B) were spots predicted to be promising vaccine candidates (circles). Spots immunoreactive with both IVIG and dSaIVIG were excluded from further investigation. For MALDI-TOF identification corresponding spots were matched with preparative gels loaded with 500  $\mu$ g protein (C and D). SUPRA was performed in collaboration with Eva Glowalla.

**Table 4.3:** Immunogenic anchorless cell wall proteins from *S. aureus* ATCC 29213 identified by SUPRA

	GI protein	Spot ID	Predicted function	pI <sup>a)</sup>	MW kDa) <sup>b)</sup>	MASCOT Total Score <sup>c)</sup>	Sequence Coverage
1	15927930	2160	hypothetical protein, similar to esterase (hp2160) <sup>1,2</sup>	7.2	35.6	92	28
2	15926453	2338	enolase (eno) <sup>1,2</sup>	4.6	47.1	206	47
3	15926814	2357	3-oxoacyl- (acyl-carrier protein) reductase (oxo) <sup>1,2</sup>	5.6	26.2	103	35
4	21204821	2228	alanine dehydrogenase	5.6	40.1	268	55
5	14247692	2053	aldehyde dehydrogenase	6.6	51.9	56	15
6	15924043	2221	autolysin	9.7	102.6	77	11
7	15926504	2077	aminotransferase NifS homologue	5.3	46.4	346	60
8	15927677	2226	ATP synthase beta chain	4.7	51.4	317	55
9	14247509	2237	chorismate mutase homologue	5.8	40.7	112	34
10	15926560	2042	conserved hypothetical protein	5.6	31.8	121	48
11	15926780	2161	conserved hypothetical protein	5.7	34.6	186	45
12	15928275	2342	conserved hypothetical protein	5.3	37.5	188	33
13	14247601	2244	enterotoxin SEM	6.5	27.5	68	28
14	15926324	2247	ferrichrome transport ATP-binding protein	5.6	29.7	99	28
15	6578924	2070	glutamyl-t-RNA-Gln amidotransferase subunit A	5.0	52.3	143	36
16	15926530	2036/2078	hypothetical protein, similar to NADH dehydrogenase	5.4	44.4	79	29
17	15926396	2037/2038	hypothetical protein, similar to anion-binding protein	9.0	74.4	248	38
18	21205078	2222	hypothetical protein	5.8	65.5	63	13
19	15927580	2425	hypothetical protein,	8.6	38.7	78	14
20	15923845	2089	hypothetical protein	5.7	29.4	113	38
21	15928230	2235	hypothetical protein, similar to autolysin precursor	6.0	69.2	72	16
22	15927670	2239	hypothetical protein, similar to SceD precursor	5.5	24.1	70	34
23	15923892	2240	hypothetical protein	6.5	37.1	89	19
24	15928148	2043/2050/2094/ 2096/2099	immunodominant antigen A	6.1	24.2	80	23
25	15926547	2232	NAD-specific glutamate dehydrogenase	5.2	45.9	255	50
26	2239274	2039/2345	peptidoglycan hydrolase	6.1	35.2	63	22
27	15925843	2229/2230	phosphopentomutase	5.0	43.8	229	60
28	14247604	2406	protoporphyrinogen oxygenase	6.3	52.2	126	23
29	15927798	2158	50S ribosomal protein L13	9.3	16.3	228	66
30	15926178	2346	50S ribosomal protein L25	4.4	23.8	104	34
31	15927879	2155	secretory antigen precursor SsaA homologue	9.0	29.4	112	40
32	15926226	2084/2219/2227	translational elongation factor TU	4.7	43.1	98	33
33	15926838	2242	transcription pleiotropic repressor codY	5.9	28.7	178	49
34	15926451	2353	triosephosphate isomerase	4.8	27.4	66	32
35	21205110	2056	truncated beta-hemolysin	8.8	37.5	105	42
36	15926841	2241	uridylyate kinase	6.0	26.3	88	26
37	13701328	2374	Xaa-Pro dipeptidase	5.2	39.6	241	36

<sup>a)</sup> Theoretical pI

<sup>b)</sup> Theoretical molecular weight

<sup>c)</sup> Identification probability of the peptide match

<sup>1</sup> Short name of candidate given in parentheses

<sup>2</sup> Characterisation described in Glowalla et al. 2009



Furthermore, the two most obvious candidates *S. aureus* protein A (Spa, several spots between 45 and 66 kDa) and immunodominant antigen A (IsaA), which was previously reported to be highly immunogenic (Vytvytska et al., 2002) and described as an autolytic enzyme involved in cell wall metabolism (Stapleton et al., 2007) were deferred at first from further analysis, since they are already present at high titres in many individuals and hence unlikely confer protection against *S. aureus* infection.

Three of the identified vaccine candidates, the hypothetical protein 2160 (hp2160), oxo and eno, have already been analysed for efficacy in the prevention of *S. aureus* infections upon immunisation with the respective N-terminal GST-fusion proteins in a murine model of sepsis (Glowalla et al., 2009). Upon active immunisation also GST specific IgGs could be detected in serum of immunised mice by ELISA and complete removal of the GST tag is hampered by the incomplete thrombin cleavage site in pDEST15, the vector used for construction of the N-terminal GST fusion proteins. Therefore, subsequent candidates were expressed as C-terminal His-tag fusion proteins in *E. coli*. Expression and purification conditions of BT1-His, BT2-His and BT3-His were optimised, to achieve the maximum yield of each recombinant protein under native conditions in suitable purity.

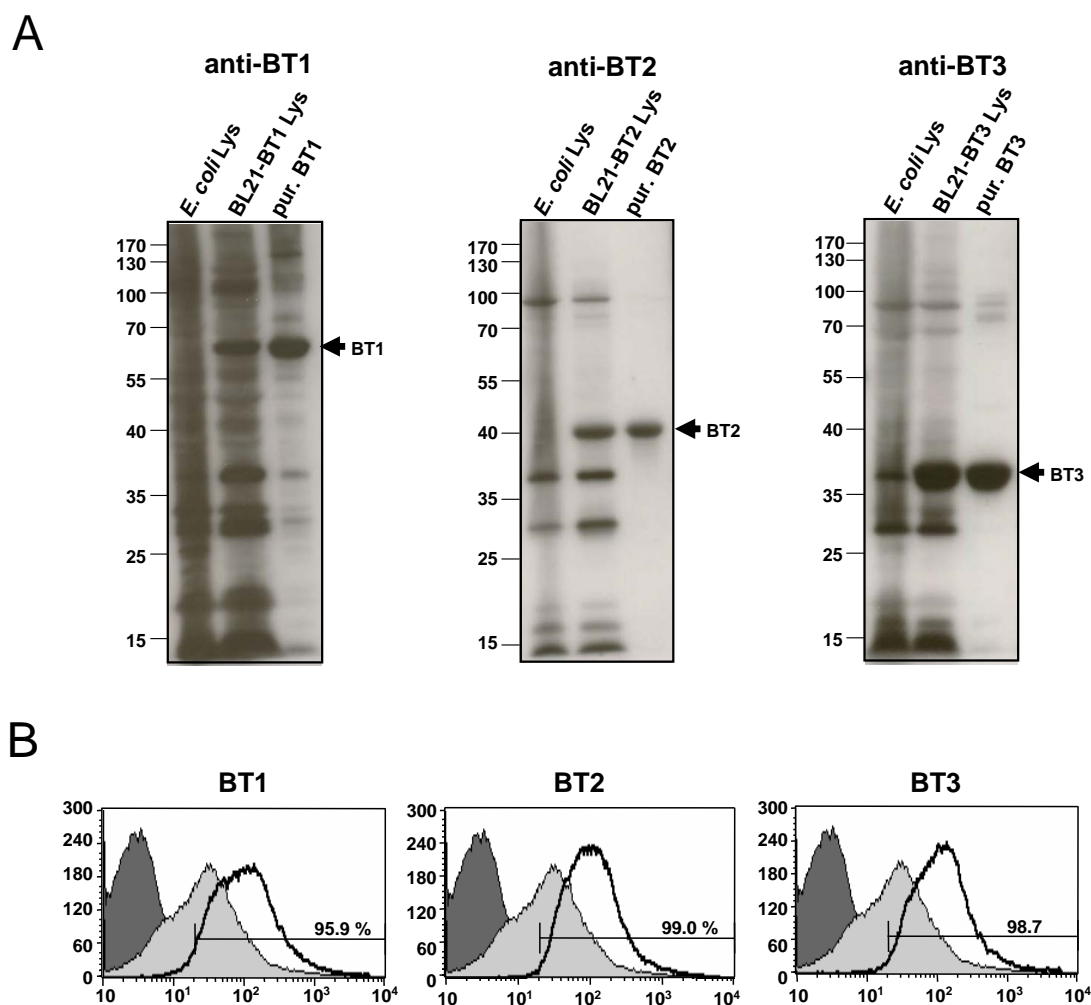
#### **4.2.2 BT1, BT2 and BT3 are localised on the surface of *S. aureus***

As surface localisation of a vaccine candidate is pivotal for opsonisation of the pathogen by IgGs, IgGs specific for BT1, BT2 and BT3 were enriched from IVIG by affinity chromatography using the corresponding purified recombinant protein covalently linked to NHS-activated columns, to enable assessment of surface localisation on viable *S. aureus* and *in vitro* opsonophagocytic assays.

At first, as shown in Figure 4.11 A, purified IgG fractions were analysed for specificity by western blot analysis. Each antibody fraction was able to strongly detect the corresponding recombinant protein and, to a lesser extent, non-specific *E. coli* proteins. Since the major band corresponds to the respective fusion protein the purified IgG fractions were then used as primary antibody, to assess the surface localisation of corresponding *S. aureus*- antigens by flow cytometry. As shown in Figure 4.11 B, all three antibody fractions were able to detect more than 95 % of PE-positive viable *S. aureus*, therefore almost 40 % more PE-positive gated events, than detected in samples where dSaIVIG was used as negative control. Thus, proving surface localisation of the analysed *S. aureus* - antigens BT1, BT2 and BT3.

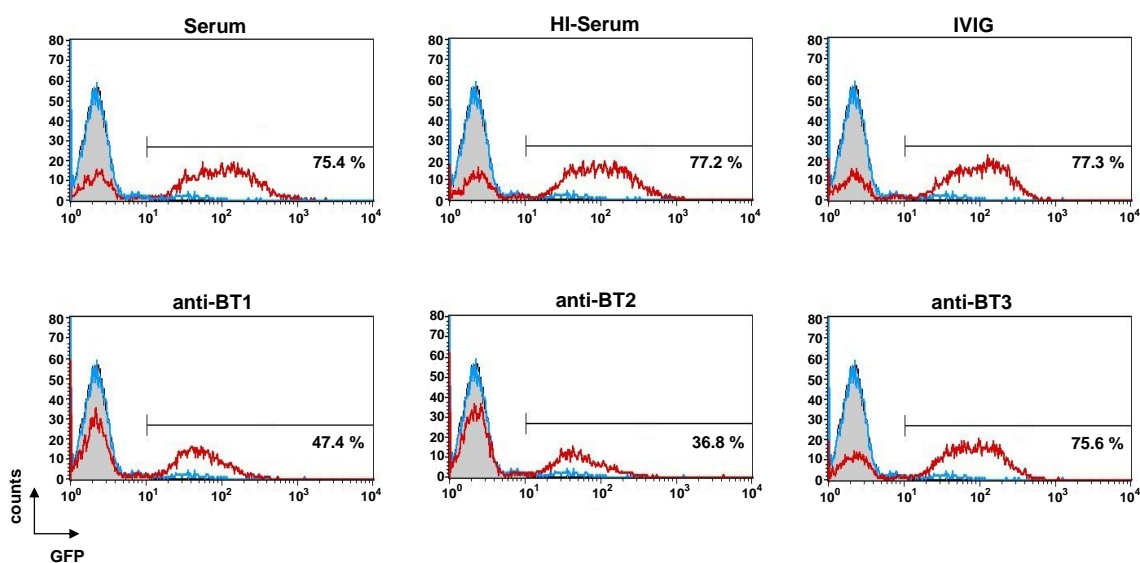
#### **4.2.3 Affinity purified IgGs specific for BT1, BT2 and BT3 trigger opsonophagocytosis of *S. aureus* by human neutrophils *in vitro***

Since IgGs present in IVIG represent naturally occurring antibodies, enriched IgGs for each of the three antigens were tested regarding their potential to promote opsonophagocytosis by human neutrophils.



**Figure 4.11:**

**Specificity of enriched IgGs and surface localisation of vaccine candidates** A) Specificity of affinity enriched IgGs specific for BT1 (left), BT2 (middle) and BT3 (right) was assessed by Western Blot analysis. 10  $\mu$ g of cytoplasmic protein fraction of non-transformed *E. coli* (first lane), *E. coli*-BL21 expressing recombinant BT1, BT2 or BT3 (second lane) or 2.5  $\mu$ g affinity purified BT1, BT2 or BT3 protein (third lane) were separated by SDS PAGE. Immunoblots were probed with affinity purified anti-BT1, anti-BT2 or anti-BT3 (1  $\mu$ g/ml). Arrows indicate the respective recombinant proteins. B) Surface localisation of BT1 (left), BT2 (middle) and BT3 (right) was assessed by incubation of *S. aureus*-ATCC 29213-GFP with 5  $\mu$ g/ml of either dSaIVIG (negative control; lightgrey filled), anti-BT1, anti-BT2 or anti-BT3 for 30 min at RT. After washing, bound IgGs were detected by PE conjugated anti-human FC  $\gamma$  F(ab)<sub>2</sub> fragments and subsequently analysed by flow cytometry. *S. aureus*-ATCC 29213-GFP incubated with secondary antibody only is represented by the dark-grey histogram. Given percentages represent percent of gated events in marked region for each corresponding primary antibody (black line).



**Figure 4.12:**

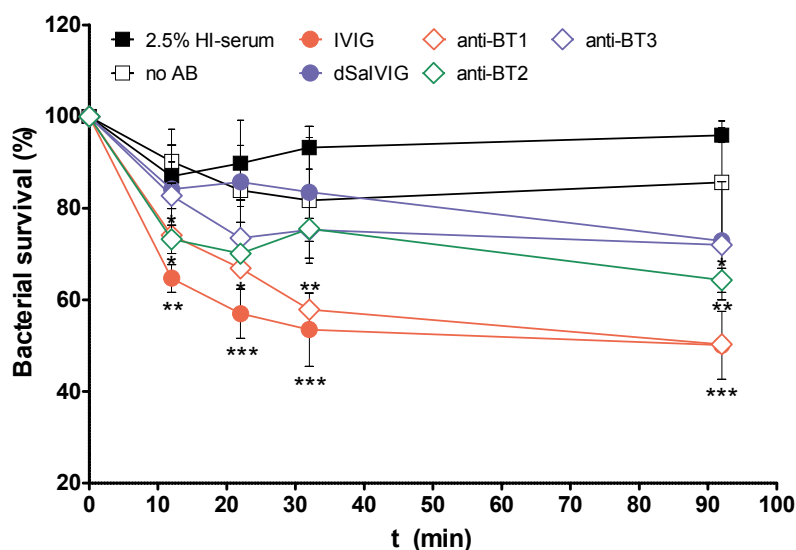
**Opsonophagocytosis of *S. aureus* ATCC29213-GFP by human neutrophils** Human neutrophils, bacteria (MOI 10), and 2.5% human serum, 2.5% HI-serum or 100  $\mu$ g/ml IgGs were incubated for 5 min at 37  $^{\circ}$ C for opsonophagocytosis, followed by differential centrifugation. Human PMNs (lightgrey filled) and bacteria with dSaIVIG (blue line) as opsonin served as negative controls. Red lines represent specific opsonins. Green fluorescence was measured by flow cytometry. Given percentages of GFP-positive human PMNs (marked region) represent the fraction of cells with internalised ATCC 29213-GFP.

As shown in Figure 4.12 human serum, HI-serum and IVIG efficiently triggered opsonophagocytosis, resulting in more than 75% GFP positive PMNs. Whereas the fraction of GFP positive neutrophils was remarkably lower using IgGs specific for BT1 (47.4%) and BT2 (36.8%), IgGs specific for BT3 triggered opsonophagocytosis in an extent comparable to human serum, HI serum and IVIG (75.6%). Since dSaIVIG used as negative control equates to PMNs in the absence of *S. aureus*, IgGs directed against BT1 and BT2 clearly promote phagocytosis.

#### 4.2.4 Affinity purified IgGs specific for BT1 and BT2 mediate killing of *S. aureus* by human neutrophils *in vitro*

Since enriched IgGs specific for each of the three candidates triggered efficient phagocytosis by human neutrophils, they were then tested for their capacity to mediate subsequent elimination of staphylococci.

As shown in Figure 4.13 *S. aureus* pre-opsonised with IVIG are efficiently eliminated by human neutrophils already 12 minutes upon addition of cells, resulting in only 65% surviving bacteria, further decreasing to 50% at t92. Although with a lower effect at early time points, anti-BT1 antibodies mediated a statistically significant killing of staphylococci



**Figure 4.13:**

**Opsonophagocytic killing of *S. aureus* ATCC 29213-GFP by human neutrophils** Human neutrophils, bacteria (MOI 10) without antibodies (black open squares) or pre-opsonised with either 2.5% HI-serum (black closed squares) or 100  $\mu$ g/ml IVIG (red closed circles), dSalVIG (blue closed circles), anti-BT1 (red open diamonds), anti-BT2 (green open diamonds) or anti-BT3 (blue open diamonds) were incubated for 10 min at 37  $^{\circ}$ C, to enable phagocytosis subsequent to synchronisation of infection by a 2 minute centrifugation at 400 g in a pre-warmed centrifuge. To assess elimination of bacteria, samples were removed immediately upon addition of bacteria ( $t_0$ ), upon phagocytosis ( $t_{12}$ ) and at  $t_{22}$ ,  $t_{32}$  and  $t_{92}$ . Upon lysis of PMNs, samples were spread in a 1:500 dilution on Mueller Hinton agar plates. Shown are mean and SD of triplicates in percent of the respective  $t_0$ . Statistical significance in comparison to the sample without antibodies was determined by one-way ANOVA followed by Bonferroni post analysis. P-values are indicated by asterisks (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ).

comparable to IVIG, also resulting in only 50 % surviving bacteria at  $t_{92}$ . The killing effect promoted by anti-BT2 antibodies, though lower than that observed using IVIG or anti-BT1, led to a statistically significant reduction of viable bacteria to 64 % at  $t_{92}$ . In contrast, anti-BT3 antibodies failed to mediate statistical significant killing of bacteria, but results obtained for the early time points demonstrate a killing effect comparable to samples with anti-BT2 as opsonin.

#### 4.2.5 Immunisation with recombinant BT1 and BT3 protects mice from death upon lethal challenge with *S. aureus*

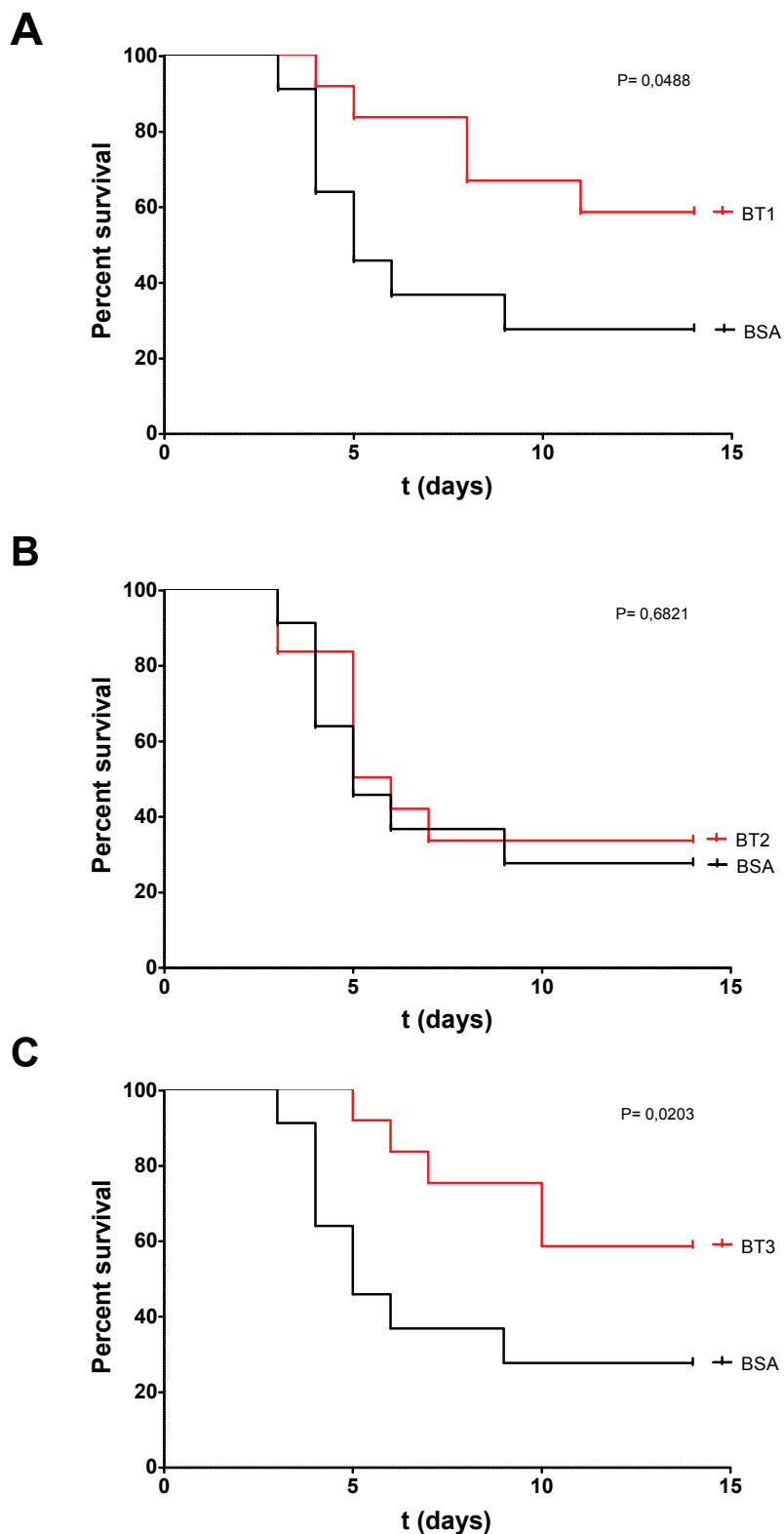
So far no *in vitro* test has the potential to predict, whether a vaccine candidate confers protection or not. Therefore, the purified recombinant proteins BT1, BT2 and BT3 were used for *in vivo* studies in BALB/c mice, to ascertain whether immunisation leads to protection against lethal challenge with *S. aureus*. Immunisation resulted in a high serum titre of IgGs for BT1 (1,199,050) and BT3 (653,048), but only 94,669 for BT2 one week after the second booster dose, prior to infection. To assess the protective potential immunised mice were

challenged intravenously with  $3 \times 10^7$  CFU of *S. aureus* strain ATCC 29213 one week after the second booster immunisation. For two weeks infected mice were monitored daily for clinical signs of infection and mortality. Mice immunised with BSA as non-specific antigen served as control.

As shown in Figure 4.14, 60 % of the mice immunised with either BT1 (A) or BT3 (C) survived, in contrast to only 25 % surviving mice in the group of BSA-immunised mice. In case of immunisation with recombinant BT2 (B) no improved survival compared to infected control mice was observed.

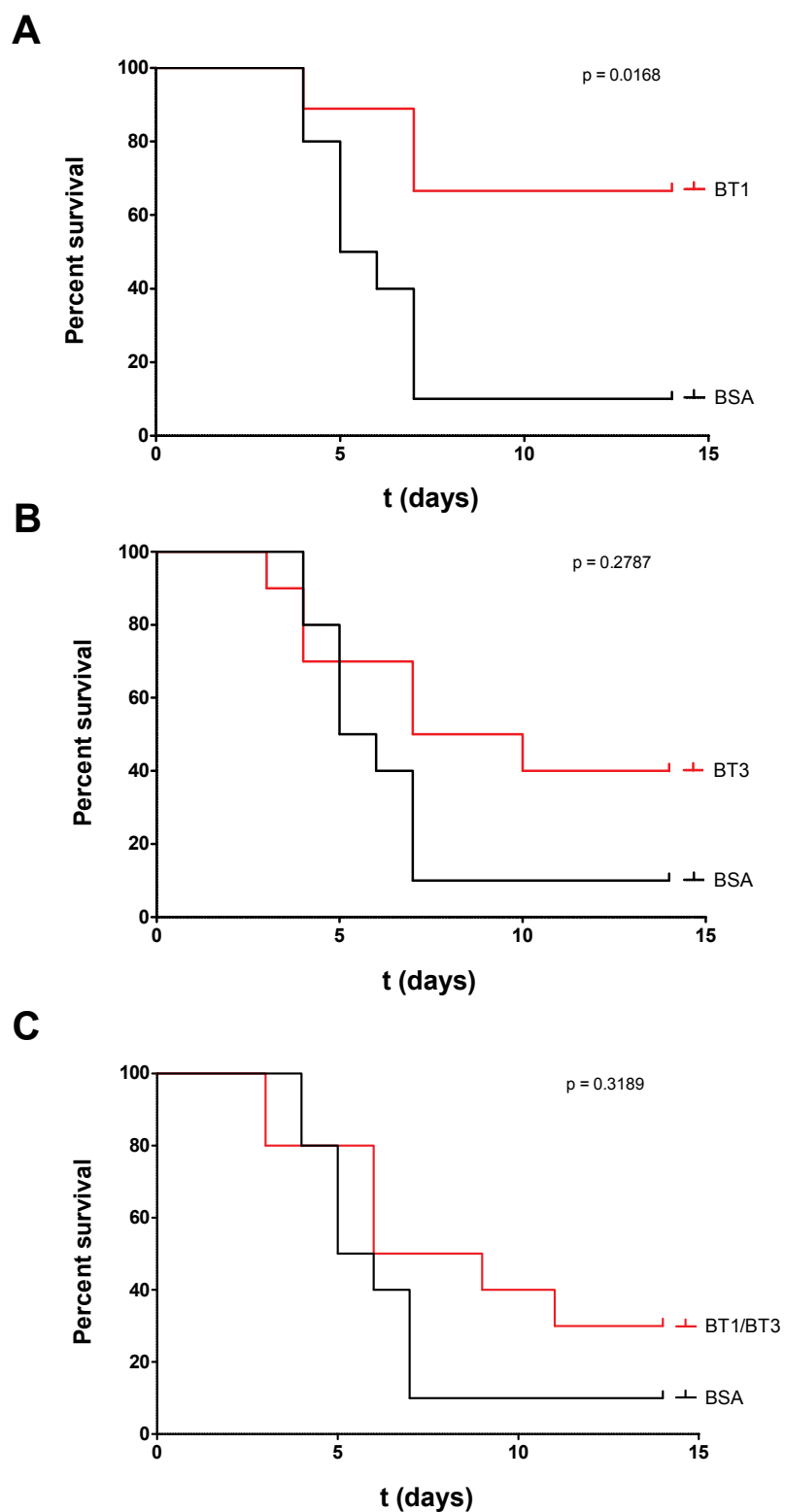
To ascertain, whether immunisation with both candidates could potentiate the observed protective effect, mice were immunised with a mixture of equal amounts of each antigen, resulting in a serum titre comparable to single immunisation or BSA as control. As shown in Figure 4.15, more than 65 % of the mice immunised with BT1 alone (A) survived the challenge with *S. aureus* in contrast to only 10 % in the BSA control group. In mice immunised with BT3 (B) still 30 % more animals survived the challenge compared to BSA immunised mice. In case of immunisation with a mix of recombinant BT1 and BT3 (C) the survival rate further declined to 30 %, thus just 20 % higher than observed for mock immunised mice. The discrepancy between single target immunisation and the bivalent immunisation could be due to the lower amount of antigen used for immunisation, since only half of the amount of each antigen was used for immunisation. Thus, the conditions for polyvalent immunisations have to be further improved.

Altogether, three of the so far characterised proteins, namely hp2160, BT1 and BT3, conferred protection in a murine model of sepsis. The remaining candidates will now be tested consecutively, to enable the formulation of a multivalent mix of vaccine candidates for active immunisation in order to improve the protective effects observed upon monovalent immunisation.



**Figure 4.14:**

**Survival of mice challenged with LD<sub>50</sub> of ATCC29213 upon monovalent immunisation**  
BALB/c mice immunised with recombinant BT1 (A; red line), BT2 (B; red line) and BT3 (C; red line) or BSA as control (black line) were challenged i.v. with  $3 \times 10^7$  CFU of *S. aureus* ATCC 29213 (n = 11 to 12). Survival was monitored for 14 days. Given are exact p-values determined by Gehan-Breslow test



**Figure 4.15:**

**Survival of mice challenged with LD<sub>50</sub> of ATCC29213 upon bivalent immunisation** BALB/c mice immunised with recombinant BT1 (A; red line), BT3 (B; red line) and a mix of BT1 and BT3 (C; red line) or BSA as control (black line) were challenged i.v. with  $3 \times 10^7$  CFU of *S. aureus* ATCC 29213 (n=10). Survival was monitored for 14 days. Given are exact p-values determined by Gehan-Breslow test

# 5 Discussion

*S. aureus* causes apart from minor skin infections severe life-threatening invasive diseases like pneumonia, endocarditis and sepsis in healthy and especially in immunocompromised patients. The rapid emergence of *S. aureus* strains resistant to Methicillin (MRSA) and many other available antibiotics further aggravates the situation, reflected by the drastic increase in both nosocomially and community acquired MRSA related infections and the therewith increased mortality rates (Corriere and Decker, 2008). Thus, highlighting the pressing need for alternative strategies to prevent and treat *S. aureus* infections.

## 5.1 Characterisation of the bacteriostatic effect mediated by *S. aureus* specific IgGs

As passive immunisation is appropriate for short term usage, an antibody-based therapy is highly desirable for the prevention and treatment of *S. aureus* infections. The major risk factor for *S. aureus* infection is for example a post-surgical infection by either an endogenous or nosocomial *S. aureus* strain (Perl et al., 2002; Kluytmans et al., 1997; Wertheim et al., 2005). But, since only few surgeries are scheduled well in advance, a strategy based on active immunisation would most likely not be appropriate. Moreover, passive immunisation is also applicable in immunocompromised patients, not able to trigger a sufficient humoral response.

Furthermore, the combination of antibodies with different functionalities, e.g. neutralisation of toxins or adhesion to host cell tissue, opsonisation and subsequent phagocytosis by professional phagocytes, would further increase the beneficial aspects of passive immunisation. In this respect the observation that most likely the immunoglobulin fraction of human serum caused bacteriostasis of *S. aureus* (Ehrenkranz et al., 1971), could be indicative of a so far uncharacterised direct function mediated by *S. aureus* specific antibodies.

For this reason, after reproducing the results obtained with human serum, we employed an intravenous immunoglobulin (IVIg) preparation as source of *S. aureus* specific IgGs in order to analyse whether these mediate inhibition of staphylococcal growth. In fact IVIg exhibited a bacteriostatic effect on *S. aureus*, and additional control experiments revealed that this inhibition of growth is solely due to IgG. Moreover, IgGs responsible for bacteriostasis



could be depleted by over night co-incubation of IVIG with *S. aureus* (dSaIVIG), thus proving that *S. aureus* specific IgGs mediate the inhibition of growth. Since this is an indication of a so far uncharacterised direct effect of specific IgGs against *S. aureus*, we aimed to analyse the underlying mechanism. For this purpose we employed gene expression profiling, to elucidate the response of *S. aureus* to the action of IgGs during bacteriostasis.

The genetic variation in *S. aureus* is very extensive with around 22 % of the genome being comprised of dispensable genetic material (Fitzgerald et al., 2001), amongst others encoding virulence factors. To account for this, we utilised the seven strain *S. aureus* PCR product microarray provided by the Bacterial Microarray Group at Saint Georges (BµG@S). This microarray is based on the genome of *S. aureus* strain MRSA252 and represents all predicted ORFs present in any of the seven genomes of *S. aureus* strains MRSA252, MSSA476, MW2, N315, Mu50, COL and NCTC8325.

To limit the problems connected with the several steps of microarray analysis and sample variability, each experiment for RNA isolation was performed four times. Only genes showing a minimum fold change of 1.5 in at least three of the four arrays for each time point (biological replicates) with a p-value of less than 0.05 upon Benjamini-Hochberg correction were taken into account. Furthermore, two different controls were applied in parallel sets of microarray experiments; IVIG compared to dSaIVIG as source of non-specific IgGs (microarray set A) or PBS (microarray set B).

Whereas 236 genes matched the filtering criteria for any of the analysed time points (t0, t30 and t60) upon analysis of microarray set A, only 78 genes were found differentially expressed by comparison of IVIG to PBS (microarray set B) indicating a difference between the two applied controls. Moreover, only 13 genes common to both sets of microarrays were identified. Most of these 13 genes are only categorised by function prediction and there is no obvious link between these genes and the observed bacteriostasis. In general the major detriment of a global analysis on *S. aureus* gene expression analysis for the characterisation of an unknown mechanism is the enormous number of so far uncharacterised hypothetical proteins, reflected by the fact that these represent with 86 or 23 differentially expressed genes the largest groups in microarray set A and B, respectively. Thus, almost one third of all identified genes in both sets of arrays were not usable for further characterisation of the mechanism underlying bacteriostasis. Intriguingly, the most prominent signature of genes with altered expression identified by microarray set A was related to iron uptake and metabolism. Since antibodies specific for *E. coli* LPS or surface proteins of *A. baumannii* were found to be bacteriostatic due to interference with iron uptake (Fitzgerald and Rogers, 1980; Goel and Kapil, 2001), this was a very promising finding. However, qPCR on iron relevant genes validated the microarray results and further supported that these did not show a difference in the expression level when IVIG samples were compared to PBS samples. Moreover, qPCR results of dSaIVIG related to PBS treated samples represented the almost exact reciprocal values of IVIG related to dSaIVIG samples, suggesting

that the expression of iron related genes was altered due to dSaIVIG treatment, hence not representing the mechanism underlying bacteriostasis. As iron is essential for basic bacterial physiologic processes such as electron transport or nucleotide synthesis (Marengo and Schneewind, 2006), bacteria evolved several strategies to overcome the iron limitation in the host environment. Next to the sequestration of heme or transferrin bound iron by surface proteins (Mazmanian et al., 2003; Reniere et al., 2007), under iron restricted conditions bacteria secrete high affinity iron chelators (siderophores), subsequently enabling uptake of complexed iron by specialised ABC-transporters (Lindsay and Riley, 1994; Dale et al., 2004a; Park et al., 2005; Speziali et al., 2006; Miethke and Marahiel, 2007). Since dSaIVIG is derived from overnight co-incubation with viable *S. aureus* at 4 °C it is most likely that siderophores are secreted as response to the lack of iron in IVIG, thereby supplying *S. aureus* with pre-existing siderophores and consecutively iron during incubation with dSaIVIG. Thus, the differential expression of genes related to iron uptake is rather due to a higher concentration of iron in dSaIVIG treated samples and hence not the mechanism underlying bacteriostasis.

Another prominent functional group of differentially expressed genes was related to amino-acid transport and metabolism in both sets of arrays, but apart from the putative carbamoyl-phosphate synthetase (pyrAA) distinct candidates were identified depending on the employed control.

Since a reliable signature of genes with altered expression is essential for the analysis of the mechanism underlying the bacteriostatic effect mediated by *S. aureus* specific IgGs, further characterisation based on these results was infeasible. Especially as construction of allelic replacement mutants of *S. aureus* is not trivial and highly laborious the number of investigated genes should be strongly limited.

If IgGs (e. g. purified from IVIG) specific for a single antigen of *S. aureus* also inhibit staphylococcal growth, gene expression profiling could be repeated using these instead of complete IVIG. Hence avoiding the combined action of various IgGs with diverse specificities, potentially affecting *S. aureus*. Nevertheless, the problem of many poorly annotated and characterised genes will persist. Alternatively, since transposon insertion libraries represent *S. aureus* mutants deficient for any of the non-essential genes, IVIG could be used to screen such libraries of *S. aureus* for mutants, whose growth is no longer inhibited by specific IgGs present in IVIG. This could enable the direct identification of genes important for IgG mediated bacteriostasis and might thereby enable the analysis of the underlying mechanism. Potential drawback of this attempt is the identification of genes not directly related to bacteriostasis, but for example metabolic pathways, falsely supporting growth in the presence of otherwise bacteriostatic specific IgGs.

## 5.2 Identification and characterisation of potential vaccine candidates against *Staphylococcus aureus*

The steadily growing interest in a protective vaccination strategy against *S. aureus* is reflected by numerous studies on active and passive immunisation using a variety of *S. aureus* surface components.

Due to their prominent role in immune evasion the capsular polysaccharides (CP) of *S. aureus* were in focus of vaccine research. Of the 11 different serotypes of CPs in *S. aureus* two, namely Cp5 and CP8, are the predominant serotypes (Thakker et al., 1998), covering 75 % of all isolates (Arbeit et al., 1984). Although purified CPs are only poorly immunogenic (Fattom et al., 1990, 1993), conjugation to a non-toxic form of Exotoxin A from *P. aeruginosa* as carrier protein vastly enhanced immunogenicity. Furthermore, active immunisation using a bivalent CP5-CP8 conjugate vaccine (StaphVax<sup>TM</sup>; Nabi Biopharmaceuticals) led to an increased survival rate in mice (Fattom et al., 2004), but failed to pass the Phase III clinical trial in end stage renal disease patients. A second Phase III trial in haemodialysis patients also failed, thereby attenuating the development of StaphVax (Garcia-Lara and Foster, 2009). This failure could be partially due to the fact, that the capsule of *S. aureus* is no key virulence factor as in other pathogenic bacteria like *N. meningitidis* and *S. pneumoniae*, where immunisation with CPs confers more than 90 % protection (Projan et al., 2006). Besides this, the capsule is produced in the post-exponential phase of staphylococcal growth, when, since CP expression is under control of the quorum sensing system *agr* (Luong et al., 2002), already a high density of bacteria is reached at the site of infection. This likely hampers sufficient protection in various groups of chronically sick and immunocompromised patients like end stage renal disease patients (Raskova et al., 1987).

To date several staphylococcal adhesins of the MSCRAMM family have been analysed for their protective potential in immunisation studies. Despite the positive results achieved, no monovalent vaccination strategy proved efficacy in clinical trials, in both settings of passive or active immunisation (Garcia-Lara and Foster, 2009). Hence, it is becoming more and more obvious that a single target strategy could not confer full protection. This is especially true, because the function of staphylococcal virulence factors is highly redundant and their expression is tightly regulated and temporary during the different stages of growth (Bronner et al., 2004; Boisset et al., 2007).

A first attempt testing a multivalent vaccine was conducted by Stranger-Jones et al. (2006). In this study the protective capacity of four different members of the MSCRAMM family containing the LPXTG sorting signal was tested in a murine *in vivo* sepsis model upon monovalent immunisation in comparison to a combined vaccine. In contrast to monovalent immunisation with IsdA, IsdB, SdrD and SdrE the combined vaccine conferred significant protection from lethal challenge using six different clinical isolates of *S. aureus*. These results demonstrate that a highly protective vaccine is feasible, but the challenging task

remains the identification of eligible candidate proteins (Projan et al., 2006). The major prerequisite for a target protein is the expression and accessibility by antibodies under *in vivo* conditions. Additionally, the protein should preferably be essential to circumvent the development of escape mutants. But in terms of multicomponent vaccines this risk is, due to the presence of multiple antigens, already markedly decreased. As gene expression is tightly regulated during the growth of *S. aureus*, it would be beneficial to combine either constantly expressed proteins or components expressed at different growth phases, to cover the entire course of growth stages during disease. For example the members of the MSCRAMM protein family are maximally expressed during early exponential growth and are repressed in response to *agr* activation (Cheung et al., 2004; Yarwood and Schlievert, 2003) at the transition of late exponential to stationary phase, while at the same time toxin and capsule production is activated.

However, most vaccine studies conducted so far concentrate on MSCRAMM proteins (Stranger-Jones et al., 2006; Zhou et al., 2006; Arrecubieta et al., 2008). These are covalently attached to the staphylococcal peptidoglycan upon recognition of the C-terminally located conserved LPXTG motif by sortase A (Navarre and Schneewind, 1999) or sortase B, in case of the iron responsive surface determinants like *IsdA* and *IsdB* (Mazmanian et al., 2002). Due to the conserved LPXTG motif these proteins are readily identified by genome screenings (Roche et al., 2003; Stranger-Jones et al., 2006). Despite the positive results achieved by reverse vaccinology for other pathogens like group B streptococcus (Maione et al., 2005) or serogroup B meningococcus (Giuliani et al., 2006; Pizza et al., 2000), the major drawback of this bioinformatics-based screening for potential vaccine candidates is the dependency on conserved sequence motifs. Thus, missing proteins lacking such motifs or harbouring to date uncharacterised conserved signals, like the group of anchorless cell wall (ACW) proteins described by Chhatwal (2002). ACW proteins are amongst others involved in adhesion to host cell structures in several gram-positive bacteria like *S. pneumoniae*, *S. pyogenes* and group A streptococci. Since they lack common signal sequences, to date it is not known by which mechanism these proteins are secreted (Chhatwal, 2002). Furthermore, these proteins lack the LPXTG motif or cholin-binding-repeats mediating attachment to the bacterial cell wall. In case of  $\alpha$ -enolase from *S. pneumoniae* it was shown that upon secretion the protein re-associates to the bacterial cell wall (Bergmann et al., 2001), where it mediates adhesion by binding to laminin and leads to plasminogen activation. Due to its role in glycolysis enolase also exhibits a pivotal metabolic function, reflected by the fact that the construction of an enolase deficient isogenic mutant of *S. pneumoniae* was infeasible (Bergmann et al., 2001). In group A streptococci, next to enolase also glyceraldehyde-3-phosphate dehydrogenase (GAPDH), another glycolytic enzyme, was localised on the surface, where it mediates adhesion to several mammalian proteins and also acts as ADP-ribosylating enzyme (Pancholi and Fischetti, 1993, 1998). The multifunctional roles of the so far characterised ACW proteins renders them promising vaccine candidates.

Since ACW proteins are not traceable by bioinformatic genome screenings, we applied a proteomic approach on an ACW-protein preparation from *S. aureus* for the identification of potential vaccine candidates. On the basis of serological proteome analysis (SERPA) first used by Vytvytska et al. (2002), we developed a new procedure of subtractive proteome analysis, coined SUPRA. To overcome the limitation in source of specific antibodies accompanying the use of individual patient sera, an IVIG preparation was employed for the detection of immunogenic ACW proteins. The IVIG preparations used, contained *S. aureus* specific IgGs mediating phagocytosis and killing by human neutrophils (Glowalla et al., 2009). Potential vaccine candidates were identified by immunodetection of ACW-proteins using IVIG and IVIG depleted of *S. aureus* specific IgGs (dSaIVIG). Proteins detected by IVIG but not, or to a far lesser extent, by dSaIVIG were considered as potential vaccine candidates, based on the assumption that those are recognised by naturally occurring *S. aureus* specific IgGs, hence representing *in vivo* expressed immunogenic proteins. In total 39 immunogenic proteins matching these criteria were identified. Except for four of these candidates all proteins are present in the 15 currently available completely sequenced genomes of *S. aureus*, hence underscoring their broad distribution among *S. aureus* isolates.

In total, six of these candidates have been characterised regarding their protective potential upon active immunisation in a murine model of sepsis. Whereas immunisation with enolase (Eno) and oxoacyl-reductase (Oxo) led to a reduction in bacterial load in organs after intravenous challenge with *S. aureus*, only immunisation with the hypothetical protein hp2160 conferred significant protection against lethal challenge with *S. aureus* ATCC 29213 and USA 300 (Glowalla et al., 2009). Three other candidates, designated BT1, BT2 and BT3 were in the focus of the current study.

So far next to the pivotal role in the cytoplasm no function on the surface of *S. aureus* has been described for BT1, but flow cytometric analysis confirmed its surface localisation.

For BT2 and BT3 important functions in the cytoplasm and on the surface were proven.

For subsequent *in vitro* and *in vivo* experiments each of the three candidates (BT1, BT2 and BT3) was cloned and expressed as C-terminal His-Tag fusion protein. To ensure correct protein folding, the respective fusion proteins were purified under native conditions. Using IgGs specific for each antigen, enriched from IVIG by affinity chromatography, the surface localisation of BT1, BT2 and BT3 of *S. aureus* strain ATCC 29213 was confirmed by flow cytometry. Subsequently these antibody fractions were employed as opsonin in *in vitro* opsonophagocytosis experiments, revealing that IgGs specific for BT1, BT2 and BT3 promote phagocytosis by human neutrophils. Intriguingly, IgGs specific for BT3 triggered phagocytosis to a comparable extent as the same amount of IVIG. Since all IgG fractions triggered phagocytosis also opsonophagocytic killing was assessed using freshly isolated human neutrophils. Whereas IgGs specific for BT1 mediated elimination of bacteria comparable to IVIG, anti-BT2 IgGs triggered killing of *S. aureus* to a slightly lower extent. Though a killing effect could be observed for anti-BT3 IgGs at early time points, this effect did not

reach statistical significance.

Since the previously obtained results concerning eno, oxo and hp2160 demonstrated that these *in vitro* assays are a prerequisite, but are not sufficient to reliably predict the protective potential *in vivo* (Glowalla et al., 2009), BalbC mice were immunised with purified BT1, BT2 and BT3 in complete or incomplete Freund's adjuvant for initial and the two booster immunisations, respectively. Mice immunised with BSA served as controls. The specific titre for BT1 and BT3 was comparable, whereas immunisation with BT2 achieved only a 6 to 12 times lower serum titre upon second booster immunisation, indicating a lower immunogenicity compared to BT1 and BT3. Accordingly, survival upon lethal challenge with *S. aureus* ATCC 29213 was significantly improved only in mice immunised with BT1 and BT3. Thus representing promising vaccine candidates. In case of BT2 no difference to BSA immunised mice was observed, hence deferred at first from further analysis. Since Stranger-Jones et al. (2006) obtained no significant improvement of survival upon single immunisation with IsdA, IsdB, SdrD and SdrE this exclusion of candidates not conferring significant protection upon monovalent immunisation could be too stringent, as a combination of these four antigens conferred full protection. Especially, as IsdA immunised mice died even earlier than control immunised mice and, as demonstrated by Kim et al. (2010), passive immunisation using polyclonal rabbit antibodies against IsdA and IsdB prolong survival upon lethal challenge. Nevertheless, due to its redundant function, BT2 is a weaker vaccine candidate compared to BT1, BT3 and hp2160.

As proven by the failure or only minor advantages achieved by several monovalent vaccination strategies, a multicomponent vaccine seems to be the only reasonable strategy to prevent and treat *S. aureus* infections. Therefore, we aimed to identify potential components for such a polyvalent vaccine. So far three of the six to date tested candidates identified by SUPRA confer protection in a murine model of sepsis. Two, namely eno and oxo achieved at least a reduction of the bacterial load in organs and their failure in conferring protection against lethal challenge could be due to their pronounced homology to respective murine proteins. To test the potential synergistic effect of BT1 and BT3 upon combined vaccination, immunisation and subsequent lethal challenge of BalbC mice was performed in comparison to single immunisations. Unfortunately, no synergistic effect could be observed under the applied conditions. Whereas BT1 alone still conferred statistically significant protection, no statistically significant improvement in survival of BT3 immunised mice could be observed. Though, in contrast to the BSA control group, 30% more BT3 immunised mice survived the lethal challenge with *S. aureus* ATCC 29213. The mix was comparable to monovalent immunisation using BT3. However, only half the amount of each antigen was used for combined vaccination, to avoid exceeding the total amount of antigen used for single immunisation. Since the optimal conditions for polyvalent vaccinations are not sufficiently studied yet, the optimisation of the amount of antigen used in a combined vaccine might improve the results obtained with the bivalent vaccination using BT1 and

BT3.

Remarkably SUPRA led to the identification of 39 immunogenic proteins within the ACW-proteins of *S. aureus*. The results obtained to date confirmed the efficacy of SUPRA in the identification of potential vaccine candidates. The gradual characterisation of the remaining candidates will further increase the number of protective candidates for a multicomponent vaccine. Combined with other components like IsdA and IsdB as well as the bivalent CP5-CP8 conjugate a highly protective vaccine against *S. aureus* could be feasible. Additionally, the generation of monoclonal antibodies for the most promising candidates like thus far BT1 and BT3 is a definite future objective, to analyse for the protective potential upon passive immunisation.

## 6 Abstract

Due to the rapid emergence of *S. aureus* strains resistant to multiple antibiotics and the therewith increased mortality rates, the development of alternative strategies to prevent and treat *S. aureus* infections is of great clinical and economical importance. Based on the results concerning both monovalent active and passive immunisation, it is getting obvious that only multivalent vaccine strategies might confer full protection from *S. aureus* related infections. Furthermore, due to their short term applicability and potential composition of immunoglobulins of different isotypes and functionalities, strategies based on passive immunisation are particularly advantageous.

Using an intravenous immunoglobulin preparation (IVIG) as source of naturally occurring *S. aureus* specific IgGs, a significant inhibition of staphylococcal growth was observed *in vitro*. Thus, confirming the bacteriostatic effect on *S. aureus* as observed using human serum in the 1970s. Since this inhibitory effect was not observed upon treatment with IVIG depleted of *S. aureus*-specific IgGs (dSaIVIG), bacteriostasis is triggered solely by *S. aureus* specific IgGs. In order to analyse the underlying mechanism, gene expression profiling was conducted, using a *S. aureus*-seven genome PCR-product microarray. Comparison of IVIG to dSaIVIG treated samples led to the identification of 236 differentially expressed genes over the course of bacteriostasis. In contrast, IVIG compared to PBS treated samples as additional control resulted in 78 genes with altered expression. Only 13 genes were identified by both sets of microarrays, indicating a strong difference between the two applied controls. Moreover, the most prominent signature representing genes related to iron uptake and metabolism was only identified by comparison of IVIG to dSaIVIG samples. qPCR on iron related genes not only verified the microarray results, but also indicated that the iron signature was derived from dSaIVIG, thus not representing the mechanism underlying bacteriostasis. Due to the lack of a reliable signature the mechanism underlying bacteriostasis could not be characterised.

Additionally, we aimed to enlarge the repertoire of potential candidates for a polyvalent vaccine. For this purpose a novel subtractive proteomic approach (SUPRA) on anchorless cell wall (ACW) proteins of *S. aureus* was developed. This method is based on immunodetection of *in vivo* expressed, immunogenic proteins separated by 2D gelelectrophoresis with either complete IVIG or dSaIVIG. Proteins immunoreactive with IVIG but not, or to a lesser



extent using dSaIVIG were identified by MALDI-TOF analysis. SUPRA led to the identification of 37 new potential vaccine candidates among ACW proteins. Three of these, BT1, BT2 and BT3 were characterised in this study. The surface localisation of these antigens was confirmed by flow cytometry using specific antibodies enriched from IVIG. Purified IgGs for each antigen mediated opsonophagocytosis and subsequent opsonophagocytic killing by human neutrophils. However, when used for monovalent immunisation of BalbC mice only BT1 and BT3 conferred significant protection against lethal *S. aureus* challenge in a murine model of sepsis. Despite the protective potential upon monovalent immunisation a bivalent vaccination using BT1 and BT3 did not exhibit a synergistic protective effect, most likely due to the reduced amount of antigen used for immunisation.

Among the six so far investigated vaccine candidates identified by SUPRA, three conferred protection against lethal challenge with *S. aureus* (hp2160, BT1 and BT3) and two led to a reduction of bacterial load in organs (eno and oxo). Therefore, SUPRA represents a valuable tool for the identification of promising vaccine candidates for subsequent use in a multicomponent vaccine against *S. aureus*.

## 7 Zusammenfassung

Bedingt durch die rasante Entstehung multiresistenter *S. aureus* (MRSA) Stämmen und der damit einhergehenden gestiegenen Mortalität, ist die Entwicklung alternativer Strategien zur Prävention und Behandlung von *S. aureus* Infektionen von enormer klinischer und ökonomischer Bedeutung. Basierend auf den Ergebnissen bisheriger monovalenter aktiver oder passiver Immunisierungen wird deutlich, dass eine vollständige Protektion vor *S. aureus* Infektionen nur durch eine Kombination von verschiedenen Antigenen erzielt werden kann. Außerdem sind, bedingt durch die kurzfristige Anwendbarkeit und die potentielle Kombination von Immunglobulinen verschiedener Isotypen und Funktionalitäten, Strategien, die auf passiver Immunisierung beruhen, besonders vorteilhaft.

Unter Verwendung einer intravenösen Immunglobulin Präparation (IVIG) als Quelle natürlich vorkommender, *S. aureus* spezifischer IgGs, konnte eine signifikante Inhibition des Wachstums von *S. aureus* beobachtet werden. Somit wurde der in den 1970er Jahren beobachtete bakteriostatische Effekt von humanem Serum auf *S. aureus* bestätigt. Da dieser inhibitorische Effekt nicht beobachtet wurde, wenn *S. aureus* mit IVIG nach Depletion von spezifischen IgGs (dSaIVIG) kultiviert wurde, vermitteln ausschließlich *S. aureus* spezifische IgGs diese Bakteriostase. Um den zugrundeliegenden Mechanismus aufzuklären, wurde das Gen-Expressionsprofil mittels eines *S. aureus* spezifischen PCR-Produkt Mikroarrays ermittelt. Der Vergleich zwischen mit IVIG und dSaIVIG behandelten Proben führte zur Identifikation von 236 differentiell exprimierten Genen im Verlauf des bakteriostatischen Effektes. Im Gegensatz dazu ergab der Vergleich von IVIG zu mit PBS kultivierten Staphylokokken als zusätzliche Kontrolle nur 78 Gene mit veränderter Expression. Nur 13 Gene wurden durch beide Mikroarray Analysen identifiziert. Darüber hinaus wurde die auffälligste Signatur von insgesamt 36 Genen, die in Verbindung mit dem Transport und Metabolismus von Eisen stehen, nur durch den Vergleich von IVIG mit dSaIVIG identifiziert. Mittels qPCR dieser Gene wurden nicht nur die Mikroarray Daten verifiziert, sondern zudem gezeigt, dass die Eisen Signatur durch dSaIVIG eingebracht wurde und daher nicht den Mechanismus darstellt, der der Bakteriostase zugrundeliegt. Da die weitere Analyse nicht zur Identifikation einer zuverlässigen Signatur geführt hat, konnte der zu Bakteriostasis führende Mechanismus nicht aufgeklärt werden.

Außerdem wollten wir das Repertoire geeigneter Antigene für die Verwendung in einer

polyvalenten Vakzine gegen *S. aureus* erweitern. Zu diesem Zweck wurde eine neue subtraktive Proteom Analyse (SUPRA) von Zellwand-assoziierten Proteinen von *S. aureus* entwickelt. Diese Methode basiert auf der Immunodetektion von mittels 2D Gelelektrophorese aufgetrennten, *in vivo* exprimierten immunogenen Proteinen mit IVIG oder dSaIVIG. Proteine die mit IVIG, aber nicht oder deutlich geringer mit dSaIVIG detektiert wurden, wurden mittels MALDI-TOF Analyse identifiziert. SUPRA führte zur Identifikation von 37 neuen potentiellen Vakzinekandidaten in der Gruppe der Zellwand-assoziierten Proteine. Drei dieser Kandidaten, BT1, BT2 und BT3 wurden in dieser Arbeit charakterisiert. Mittels aus IVIG aufgereinigten spezifischen IgGs konnte die Oberflächenlokalisierung der drei Proteine mittels Durchflusszytometrie bestätigt werden. Außerdem vermittelten diese Antikörper Opsonophagozytose und anschließende Elimination durch humane Neutrophile. Nach monovalenter Immunisierung von BalbC Mäusen führten jedoch lediglich BT1 und BT3 zu einer Protektion vor letaler intravenöser Infektion mit *S. aureus*. Eine bivalente Immunisierung mit BT1 und BT3 führte allerdings im Vergleich zur Einzelimmunisierung nicht zu einem synergistischen protektiven Effekt, sehr wahrscheinlich bedingt durch die reduzierte Antigenmenge während der Immunisierung.

Von den bisher insgesamt sechs getesteten, durch SUPRA identifizierten Vakzine Kandidaten, vermittelten drei Protektion vor letaler Infektion mit *S. aureus* (hp2160, BT1 und BT3) und zwei führten zu einer reduzierten Bakterienlast in Organen (eno und oxo). Daher bietet SUPRA eine wertvolle Methode zur Identifikation vielversprechender Kandidaten für die Verwendung in einer polyvalenten Immunisierung.

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## 9 Supplement

**Table 9.1:** Differentially expressed genes in IVIG versus dSalVIG and IVIG versus PBS

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub>		t <sub>30</sub>		t <sub>60</sub>	
			FC 1 <sup>b)</sup>	FC 2 <sup>c)</sup>	FC 1 <sup>b)</sup>	FC 2 <sup>c)</sup>	FC 1 <sup>b)</sup>	FC 2 <sup>c)</sup>
bglA	6-phospho-beta-glucosidase	49482503					7.0	3.9
fabG	3-oxoacyl-[acyl-carrier protein] reductase	49483394					1.9	1.7
menH	put. 2-heptaprenyl-1,4-naphthoquinone methyltransferase	49483658				-1.8	-1.5	
pyrAA	put. carbamoyl-phosphate synthase	49483365	1.7				-1.8	-1.9
SAR0331	put. membrane protein	49482566				-2.1	-1.7	-2.1
SAR0874	conserved hypothetical protein	49483072					2.0	1.7
SAR1599	put. geranyltranstransferase	49483771	-1.5			-2.3		-2.5
SAR1684	conserved hypothetical protein	49483850			1.7		2.5	2.1
SAR1849	proline dehydrogenase	49484011				-2.6	2.6	
SAR1854	hypothetical protein	49484016					-1.9	-1.9
SAR1879	put. lipoprotein	49484040					2.3	3.2
SAR2408	PTS system, arbutin-like IIBC component	49484538	-2.3		-6.5	-2.8		
set15	superantigen-like protein	15923423					-9.3	-2.5

<sup>a)</sup> Prefix SAR, MRSA252 gene ID

<sup>b)</sup> fold change of gene expression in IVIG related to dSalVIG treated sample

<sup>c)</sup> fold change of gene expression in IVIG related to PBS treated sample

**Table 9.2:**

List of the 236 differentially expressed genes in IVIG compared to dSalVIG treated samples

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub>		t <sub>30</sub>		t <sub>60</sub>	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
agrB	put. autoinducer processing protein	49484262					2.4	0.025
arcA	arginine deiminase	49484832	-2.3	0.046			-2.1	0.012
arcB	put. ornithine carbamoyltransferase	49484831	-1.7	0.012			-1.8	0.023
argG	put. argininosuccinate synthase	49483121	-3.1	0.017			-2.1	0.017
argH	put. argininosuccinate lyase	49483120	-2.9	0.015			-1.8	0.012
asp23	alkaline shock protein 23	49484402					3.0	0.028
bglA	6-phospho-beta-glucosidase	49482503					7.0	0.033
citC	isocitrate dehydrogenase	49483936					3.1	0.031
citZ	citrate synthase II	49483937					3.6	0.036
clfB	fibrinogen and keratin-10 binding surface anchored protein	49484827					-1.8	0.017

<sup>a)</sup> Prefix SA, N315 gene ID; SACOL, COL gene ID; SAOUHSC, NCTC8325 gene ID; SAR, MRSA252 gene ID; SAV, Mu50 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

Continued on next page...

## List of the 236 differentially expressed genes in IVIG compared to dSaIVIG treated samples (continued)

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub>		t <sub>30</sub>		t <sub>60</sub>	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
coa	staphylocoagulase precursor [conserved region]	49482463	<b>-1.8</b>	0.036			<b>-8.9</b>	0.014
crtN	squalene synthase	49484762					<b>2.4</b>	0.026
cycA	put. D-serine/D-alanine/glycine transporter	49483938					<b>1.6</b>	0.017
dltA	D-alanine-D-alanyl carrier protein ligase	49483092	<b>1.9</b>	0.012				
dnaA	chromosomal replication initiator protein DnaA	49482254					<b>-1.8</b>	0.020
dnaN	DNA polymerase III, beta chain	49482255	<b>-1.7</b>	0.017			<b>-1.9</b>	0.040
fabG	3-oxoacyl-[acyl-carrier protein] reductase	49483394					<b>1.9</b>	0.017
fda	fructose-bisphosphate aldolase class I	49484802			<b>1.7</b>	0.031		
fhuA	ferrichrome transport ATP-binding protein	49482875			<b>2.3</b>	0.026	<b>1.9</b>	0.016
fhuB	ferrichrome transport permease	49482876			<b>2.1</b>	0.026		
fhuD	ferrichrome transport permease	49482877			<b>2.0</b>	0.036	<b>1.8</b>	0.017
fnb	fibronectin-binding protein precursor						<b>-2.3</b>	0.026
fnbA	fibronectin-binding protein precursor	49484704					<b>-2.8</b>	0.014
fnbB	fibronectin-binding protein precursor	15928081					<b>-2.2</b>	0.018
fur	iron uptake regulatory protein	49483748			<b>-1.5</b>	0.019		
glcB	PTS system, glucose-specific IIBC component	49484739					<b>3.6</b>	0.036
glnA	glutamine synthetase	49483472	<b>2.3</b>	0.015				
gyrB	DNA gyrase subunit B	49482258					<b>-1.8</b>	0.017
hemC	porphobilinogen deaminase	49483913					<b>-1.6</b>	0.014
hemD	uroporphyrinogen III synthase	49483912	<b>-1.5</b>	0.012			<b>-1.7</b>	0.017
icaD	intercellular adhesion protein D	49484862					<b>-1.7</b>	0.019
ilvC	ketol-acid reductoisomerase	49484281			<b>-3.1</b>	0.019		
isdA	iron-regulated heme-iron binding protein	49483292			<b>2.9</b>	0.030	<b>1.6</b>	0.026
isdC	put. surface anchored protein	49483293			<b>2.7</b>	0.019	<b>2.7</b>	0.020
isdI	heme-degrading monooxygenase	49482408	<b>1.7</b>	0.049	<b>2.3</b>	0.043	<b>2.2</b>	0.038
leuD	3-isopropylmalate dehydratase small subunit	49484285			<b>-1.7</b>	0.026	<b>-1.6</b>	0.025
menH	put. 2-heptaprenyl-1,4-naphthoquinone methyl-transferase	49483658					<b>-1.5</b>	0.035
mtIA	PTS system, mannitol-specific IIBC component	49484378			<b>-1.8</b>	0.019		
narG	nitrate reductase alpha chain	49484613	<b>-4.2</b>	0.017				
ndk	put. nucleoside diphosphate kinase	49483656	<b>-1.7</b>	0.025				
nrdD	anaerobic ribonucleoside-triphosphate reductase	49484813	<b>-1.6</b>	0.021				
nuc	thermonuclease precursor	49483047					<b>-2.3</b>	0.017
opuD2	glycine betaine transporter 2	49484405					<b>2.6</b>	0.039
phoB	alkaline phosphatase III precursor (pseudogene)	49487405			<b>-1.6</b>	0.026	<b>-1.6</b>	0.035
proP	put. proline/betaine transporter	49482802	<b>1.8</b>	0.028			<b>1.8</b>	0.014
purA	put. adenylosuccinate synthetase	49482270					<b>-1.7</b>	0.026
purC	put. phosphoribosylaminoimidazole-succinocarboxamide synthase	49483230	<b>-1.7</b>	0.015				
purD	put. phosphoribosylamine-glycine ligase	49483238	<b>-2.2</b>	0.015				
purE	put. phosphoribosylaminoimidazole carboxylase catalytic subunit	49483228	<b>-1.5</b>	0.016	<b>-1.7</b>	0.039		
purH	put. bifunctional purine biosynthesis protein	49483237	<b>-3.0</b>	0.021	<b>-1.7</b>	0.019		
purL	put. phosphoribosylformylglycinamide synthase II	49483233	<b>-3.5</b>	0.022	<b>-1.9</b>	0.024	<b>-1.8</b>	0.018
purM	put. phosphoribosylformylglycinamide cyclo-ligase	49483235	<b>-2.7</b>	0.017	<b>-1.6</b>	0.026		
purQ	put. phosphoribosylformylglycinamide synthase I	49483232	<b>-2.0</b>	0.016			<b>-1.5</b>	0.038

<sup>a)</sup> Prefix SA, N315 gene ID; SACOL, COL gene ID; SAOUHSC, NCTC8325 gene ID; SAR, MRSA252 gene ID; SAV, Mu50 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

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## List of the 236 differentially expressed genes in IVIG compared to dSaIVIG treated samples (continued)

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub>		t <sub>30</sub>		t <sub>60</sub>	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
pyrAA	put. carbamoyl-phosphate synthase, pyrimidine-specific, small chain	49483365	1.7	0.015			-1.8	0.050
pyrAB	put. carbamoyl-phosphate synthase, pyrimidine-specific, large chain	49483366	1.7	0.029			-1.8	0.047
pyrC	put. dihydroorotase	49483364	2.1	0.016			-1.8	0.034
pyrF	put. orotidine 5'-phosphate decarboxylase	49483367	1.6	0.049				
pyrP	put. uracil permease	49483362	2.2	0.016				
recF	DNA replication and repair protein RecF	49482257					-2.3	0.029
ribA	riboflavin biosynthesis protein	49484013	4.3	0.024	20.8	0.006	8.5	0.013
ribD	bifunctional riboflavin biosynthesis protein	49484015	4.7	0.017	16.7	0.006	7.7	0.012
ribE	riboflavin synthase alpha chain	49484014	3.9	0.049	13.6	0.019	8.0	0.013
ribH	6,7-dimethyl-8-ribityllumazine synthase	49484012	3.3	0.036	12.9	0.006	6.4	0.013
rnalll	rnalll regulatory transcript containing the delta-hemolysin structural gene	49484261					2.0	0.033
rpsU	30S ribosomal protein S21	49483823	-1.6	0.031			-1.8	0.016
rsbV	anti-sigma B factor antagonist	49484292					1.9	0.017
SA0191	put. RND family efflux transporter	15925901	-1.5	0.017				
SA0222v	staphylocoagulase precursor [conserved region]						-3.0	0.042
SA0743	similar to staphylocoagulase precursor	15926465					-3.3	0.018
SA1001	formyl peptide receptor-like 1 inhibitory protein	15926739					-1.7	0.017
SA1633	beta-lactamase	15927389					-1.6	0.032
SACOL1381	exonuclease SbcD	57650350					1.7	0.043
SACOL1510	polyprenyl synthetase	57650425					-1.6	0.017
SACOL1511	ubiquinone/menaquinone biosynthesis methyl-transferase	57650426					-1.6	0.019
SACOL1542	MutT/nudix family protein	57650455					-1.7	0.019
SACOL1719	glutamyl-tRNA reductase	57650544	2.0	0.012				
SACOL1975	nicotinate phosphoribosyltransferase	57650665			2.3	0.019	2.2	0.013
SAOUHSC_02559	urease beta subunit	88196204					-3.5	0.034
SAOUHSC_02727	hypothetical protein	88196367					-2.8	0.033
SAOUHSC_03644							1.8	0.012
SAR0012	put. hydrolase	49482265					-1.9	0.029
SAR0110	put. Na <sup>+</sup> /Pi-cotransporter protein	49482350					-2.0	0.020
SAR0148	hypothetical protein	49482389					-1.8	0.028
SAR0172	conserved hypothetical protein	49482413	2.6	0.024				
SAR0174	put. lipoprotein	49482415	1.9	0.046				
SAR0176	conserved hypothetical protein	49482417	2.5	0.025				
SAR0212	put. membrane protein	49482453	-1.8	0.015			-3.8	0.017
SAR0221	hypothetical protein	49482462					-1.9	0.017
SAR0230	put. extracellular solute-binding lipoprotein	49482471	-1.6	0.015				
SAR0231	conserved hypothetical protein	49482472	-1.9	0.015			-1.8	0.017
SAR0233	flavohemoprotein	49482474	-2.1	0.012				
SAR0238	put. PTS multi-domain regulator (pseudogene)				-1.6	0.032		
SAR0240	put. PTS transport system, IIA component	49482479			-2.2	0.019		
SAR0262	GntR family regulatory protein	49482501					2.6	0.018
SAR0263	put. PTS transport system protein	49482502					6.9	0.027
SAR0271	put. transport protein	49482511			2.6	0.036	1.9	0.018
SAR0299	hypothetical protein	49482535					-1.8	0.023

<sup>a)</sup> Prefix SA, N315 gene ID; SACOL, COL gene ID; SAOUHSC, NCTC8325 gene ID; SAR, MRSA252 gene ID; SAV, Mu50 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

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## List of the 236 differentially expressed genes in IVIG compared to dSaIVIG treated samples (continued)

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub>		t <sub>30</sub>		t <sub>60</sub>	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
SAR0301	put. membrane protein	49482536					<b>-1.8</b>	0.023
SAR0320	put. bacterial luciferase family protein	49482555			<b>1.9</b>	0.037		
SAR0321	put. glycine cleavage H-protein	49482556			<b>2.3</b>	0.006		
SAR0324	put. lipolate-protein ligase A	49482559			<b>1.9</b>	0.034		
SAR0331	put. membrane protein	49482566					<b>-1.7</b>	0.038
SAR0332	hypothetical protein	49482567					<b>-2.0</b>	0.033
SAR0390	put. lipoprotein	49482621					<b>2.5</b>	0.019
SAR0405	hypothetical protein	49482638					<b>2.0</b>	0.027
SAR0411	hypothetical protein	49482643					<b>-1.6</b>	0.026
SAR0438	put. lipoprotein	49482665	<b>-1.5</b>	0.035				
SAR0458	sodium:neurotransmitter symporter family protein	49482683					<b>-1.9</b>	0.030
SAR0522	put. pyridoxine biosynthesis protein	49482748			<b>2.4</b>	0.044	<b>2.8</b>	0.042
SAR0523	SNO glutamine amidotransferase family protein	49482749			<b>2.6</b>	0.031	<b>3.3</b>	0.025
SAR0624	put. esterase	49482845					<b>2.2</b>	0.030
SAR0722	put. transposase	49482926			<b>-1.8</b>	0.039		
SAR0735	put. exported protein	49482938					<b>1.5</b>	0.025
SAR0760	put. membrane protein	49482963					<b>-1.9</b>	0.017
SAR0763	put. radical activating enzyme	49482966	<b>3.0</b>	0.012				
SAR0764	put. 6-pyruvoyl tetrahydropterin synthase	49482967	<b>2.7</b>	0.012				
SAR0765	conserved hypothetical protein	49482968	<b>2.4</b>	0.015				
SAR0805	conserved hypothetical protein	49483008			<b>-1.6</b>	0.039	<b>-1.8</b>	0.026
SAR0854	hypothetical protein	49483054					<b>2.0</b>	0.013
SAR0855	hypothetical protein	49483055					<b>1.8</b>	0.018
SAR0861	nitroreductase family protein	49483062			<b>2.1</b>	0.034	<b>1.9</b>	0.025
SAR0874	conserved hypothetical protein	49483072					<b>2.0</b>	0.032
SAR0893	put. membrane protein	49483091	<b>1.7</b>	0.015				
SAR0905	put. transporter protein	49483103					<b>-1.9</b>	0.020
SAR1010	put. membrane protein	49483202					<b>-1.8</b>	0.035
SAR1041	conserved hypothetical protein	49483231	<b>-1.7</b>	0.017				
SAR1049	put. cobalt transport protein	49483239					<b>2.9</b>	0.019
SAR1050	ABC transporter ATP-binding protein	49483240					<b>3.2</b>	0.017
SAR1051	put. membrane protein	49483241					<b>2.8</b>	0.017
SAR1052	hypothetical protein	49483242	<b>-1.7</b>	0.016			<b>-1.6</b>	0.019
SAR1059	put. cytochrome ubiquinol oxidase	49483249	<b>-1.7</b>	0.017			<b>-1.6</b>	0.041
SAR1060	put. membrane protein	49483250	<b>-1.6</b>	0.021			<b>-1.6</b>	0.025
SAR1088	put. pyruvate carboxylase	49483277					<b>-1.5</b>	0.017
SAR1127	put. exported protein	49483317					<b>-4.0</b>	0.019
SAR1210	put. chromosome partition protein	49483397					<b>-1.6</b>	0.026
SAR1253	conserved hypothetical protein	49483440	<b>-1.5</b>	0.035				
SAR1263	hypothetical protein	49483450					<b>2.2</b>	0.017
SAR1281	conserved hypothetical protein	49483469					<b>-2.0</b>	0.035
SAR1285	hypothetical protein	49483473	<b>2.0</b>	0.015				
SAR1344	catalase	49483526			<b>-2.8</b>	0.019	<b>-2.0</b>	0.017
SAR1347	put. GMP reductase	49483529					<b>-2.1</b>	0.017
SAR1398	put. phosphate transport system protein	49483576					<b>-1.8</b>	0.026
SAR1399	ABC transporter ATP-binding protein	49483577			<b>-1.7</b>	0.021		
SAR1400	ABC transporter permease protein	49483578			<b>-1.9</b>	0.033		
SAR1419	put. branched-chain amino acid transporter protein	49483597					<b>-1.8</b>	0.012

<sup>a)</sup> Prefix SA, N315 gene ID; SACOL, COL gene ID; SAOUHSC, NCTC8325 gene ID; SAR, MRSA252 gene ID; SAV, Mu50 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

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## List of the 236 differentially expressed genes in IVIG compared to dSaIVIG treated samples (continued)

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub>		t <sub>30</sub>		t <sub>60</sub>	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
SAR1445	put. membrane protein	49483623	<b>1.5</b>	0.017				
SAR1446	conserved hypothetical protein	49483624					<b>-1.7</b>	0.017
SAR1454	put. membrane protein (pseudogene)						<b>-1.9</b>	0.012
SAR1471	put. membrane protein	49483649	<b>-1.7</b>	0.015				
SAR1472	put. membrane protein	49483650	<b>-2.3</b>	0.016				
SAR1473	conserved hypothetical protein	49483651					<b>-1.8</b>	0.013
SAR1493	put. membrane protein	49483671			<b>2.1</b>	0.019	<b>2.0</b>	0.017
SAR1499	hypothetical phage protein	49483676					<b>-4.0</b>	0.035
SAR1599	put. geranyltransferase	49483771	<b>-1.5</b>	0.041				
SAR1620	put. membrane protein	49483792	<b>-1.8</b>	0.045			<b>-1.9</b>	0.025
SAR1664	conserved hypothetical protein	49483834					<b>-1.8</b>	0.026
SAR1683	put. membrane protein	49483849			<b>1.7</b>	0.019	<b>3.1</b>	0.026
SAR1684	conserved hypothetical protein	49483850			<b>1.7</b>	0.019	<b>2.5</b>	0.034
SAR1687	conserved hypothetical protein	49483853			<b>1.6</b>	0.019	<b>2.2</b>	0.045
SAR1704	hypothetical protein	49483869					<b>2.0</b>	0.035
SAR1705	conserved hypothetical protein	49483870					<b>1.9</b>	0.035
SAR1848	conserved hypothetical protein	49484010			<b>1.8</b>	0.019	<b>1.6</b>	0.012
SAR1849	proline dehydrogenase	49484011					<b>2.6</b>	0.021
SAR1854	hypothetical protein	49484016					<b>-1.9</b>	0.019
SAR1879	put. lipoprotein	49484040					<b>2.3</b>	0.028
SAR1880	put. membrane protein	49484041					<b>2.4</b>	0.038
SAR1919	enterotoxin	49484069					<b>-1.8</b>	0.026
SAR1921	enterotoxin	49484071					<b>-1.7</b>	0.026
SAR1973	put. membrane protein	49484124					<b>2.0</b>	0.022
SAR1984	ferritin	49484134	<b>-2.5</b>	0.035	<b>-4.6</b>	0.019	<b>-3.9</b>	0.017
SAR1985	put. exonuclease	49484135	<b>-2.2</b>	0.030				
SAR2016	put. exported protein	49484165					<b>1.8</b>	0.012
SAR2227	put. non-heme iron-containing ferritin	49484363	<b>-2.8</b>	0.017				
SAR2228	conserved hypothetical protein	49484364	<b>-1.8</b>	0.016				
SAR2232	conserved hypothetical protein	49484368					<b>2.7</b>	0.017
SAR2265	put. membrane protein	49484394	<b>-2.7</b>	0.028			<b>-1.9</b>	0.019
SAR2266	FecCD transport family protein	49484395			<b>3.3</b>	0.030	<b>2.3</b>	0.023
SAR2268	put. transport system binding lipoprotein	49484397			<b>5.7</b>	0.019	<b>3.7</b>	0.019
SAR2269	hypothetical protein	49484398					<b>2.3</b>	0.028
SAR2270	hypothetical protein	49484400					<b>2.4</b>	0.019
SAR2271	put. membrane protein	49484400					<b>2.6</b>	0.017
SAR2274	put. membrane protein	49484403					<b>3.1</b>	0.026
SAR2275	put. membrane protein	49484404	<b>1.6</b>	0.012			<b>3.4</b>	0.028
SAR2296	conserved hypothetical protein	49484426					<b>-2.7</b>	0.012
SAR2297	put. acetolactate synthase	49484427					<b>-2.8</b>	0.013
SAR2338	xanthine/uracil permeases family protein	49484469					<b>-1.8</b>	0.013
SAR2364	FdhD/NarQ family protein	49484495	<b>-1.5</b>	0.034				
SAR2368	put. ferrichrome-binding lipoprotein precursor	49484499			<b>2.4</b>	0.039		
SAR2392	conserved hypothetical protein	49484524			<b>-2.1</b>	0.026		
SAR2393	put. bifunctional protein	49484525			<b>-2.1</b>	0.022		
SAR2403	put. membrane protein	49484535	<b>-2.9</b>	0.012	<b>-3.2</b>	0.034	<b>-2.2</b>	0.017
SAR2408	PTS system, arbutin-like IIBC component	49484538	<b>-2.3</b>	0.021	<b>-6.5</b>	0.036		
SAR2413	put. short chain dehydrogenase	49484543					<b>2.0</b>	0.017

<sup>a)</sup> Prefix SA, N315 gene ID; SACOL, COL gene ID; SAOUHSC, NCTC8325 gene ID; SAR, MRSA252 gene ID; SAV, Mu50 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

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## List of the 236 differentially expressed genes in IVIG compared to dSaIVIG treated samples (continued)

Common <sup>a)</sup>	Predicted function	GI protein	t <sub>0</sub> FC <sup>b)</sup> p-value <sup>c)</sup>	t <sub>30</sub> FC <sup>b)</sup> p-value <sup>c)</sup>	t <sub>60</sub> FC <sup>b)</sup> p-value <sup>c)</sup>
SAR2420	arginase family protein	49484548			1.9 0.017
SAR2429	put. 3-methylpurine glycosylase	49484559		-1.7 0.037	-1.6 0.017
SAR2460	put. acetyltransferase (GNAT) family protein	49484589			2.0 0.026
SAR2469	conserved hypothetical protein	49484598			1.8 0.017
SAR2487	tetrapyrrole (corrin/porphyrin) methylase family protein	49484614	-1.6 0.016		
SAR2558	conserved hypothetical protein	49484685			2.0 0.012
SAR2561	conserved hypothetical protein	49484688			2.2 0.017
SAR2621	put. membrane protein	49484742			-4.8 0.018
SAR2641	put. aminotransferase	49484761			1.5 0.017
SAR2645	put. glycosyl transferase	49484763			3.0 0.017
SAR2646	put. phytoene dehydrogenase related protein	49484764			2.6 0.017
SAR2647	put. membrane protein	49484765			2.0 0.023
SAR2739	conserved hypothetical protein	49484853			1.7 0.017
SAR2773	conserved hypothetical protein	49484886			2.5 0.020
SAR2777	put. DNA-binding protein	49484890			1.8 0.012
SAR2791	put. membrane protein	49484901			-1.6 0.017
sarR	staphylococcal accessory regulator A homologue	49484510			-1.6 0.033
SAV0206v	staphylocoagulase precursor [conserved region]				-5.2 0.019
SAV0902	hypothetical protein	15923892	-1.5 0.019		
sbi	IgG-binding protein	49484634			-3.8 0.017
sbnA	pyridoxal-phosphate dependent enzyme	49482359			3.4 0.033
sbnB	put. ornithine cyclodeaminase	49482360			4.5 0.017
sbnC	put. siderophore biosynthesis protein	49482361			3.9 0.012
sbnD	put. transport protein	49482362			3.0 0.012
sbnE	put. siderophore biosynthesis protein	49482363			3.0 0.017
sbnF	put. siderophore biosynthesis protein	49482364			2.5 0.017
sbnG	put. aldolase	49482365			2.8 0.012
sbnH	pyridoxal-dependent decarboxylase decarboxylase	49482366			2.1 0.012
sbnI	hypothetical protein	49482367			2.3 0.012
set15	superantigen-like protein	15923423			-9.3 0.029
sigB	RNA polymerase sigma-B factor	49484290			1.8 0.017
sirA	lipoprotein	49482358		7.5 0.019	4.1 0.018
sirB	put. siderophore transport system permease	49482357		5.5 0.019	3.3 0.017
sirC	put. siderophore transport system permease	49482356			3.3 0.038
spa	immunoglobulin G binding protein A precursor	49482354			1.6 0.017
srtB	sortase B	49483297		5.1 0.037	3.4 0.012
sstA	FecCD transport family protein	49482990			3.4 0.034
sstB	FecCD transport family protein	49482991		5.0 0.032	3.7 0.028
sstC	ABC transporter ATP-binding protein	49482992	2.3 0.025	7.4 0.019	4.5 0.017
sstD	lipoprotein	49482993		8.9 0.019	4.3 0.017
thiM	put. hydroxyethylthiazole kinase	49484316			3.5 0.032
ureA	urease gamma subunit	49484503			1.6 0.020
yycH	put. exported protein	49482273	-1.7 0.029		

<sup>a)</sup> Prefix SA, N315 gene ID; SACOL, COL gene ID; SAOUHSC, NCTC8325 gene ID; SAR, MRSA252 gene ID; SAV, Mu50 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

**Table 9.3:** List of the 78 differentially expressed genes in IVIG compared to PBS treated samples

Common <sup>a)</sup>	Predicted function	GI protein	$t_0$		$t_{30}$		$t_{60}$	
			FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>	FC <sup>b)</sup>	p-value <sup>c)</sup>
bglA	6-phospho-beta-glucosidase SAR0264	49482503					<b>3.9</b>	0.043
fabG	3-oxoacyl-[acyl-carrier protein] reductase	49483394					<b>1.7</b>	0.045
fdaB	fructose 1,6 bisphosphate aldolase	88196553					<b>2.2</b>	0.037
fruA	PTS transport system, fructose-specific IIBC component	49482956					<b>1.8</b>	0.043
fumC	fumarate hydratase, class-II SAR1942	49484093					<b>2.1</b>	0.044
glpD	aerobic glycerol-3-phosphate dehydrogenase	49483464					<b>3.0</b>	0.037
glpF	put. glycerol uptake facilitator protein	49483462					<b>5.3</b>	0.043
glpK	glycerol kinase	49483463					<b>5.2</b>	0.037
hisS	histidyl-tRNA synthetase	49483876					<b>-1.9</b>	0.043
hup	DNA-binding protein HU	49483660	<b>1.6</b>	0.033	<b>1.7</b>	0.029		
isaA	immunodominant antigen A	49484768			<b>1.8</b>	0.029		
kbl	put. 2-amino-3-ketobutyrate coenzyme A ligase	49482780					<b>3.2</b>	0.043
lysP	lysine-specific permease	49483924			<b>-1.6</b>	0.015		
menH	put. 2-heptaprenyl-1,4-naphthoquinone methyl-transferase	49483658			<b>-1.8</b>	0.020		
mvaS	3-hydroxy-3-methylglutaryl coenzyme A synthase	49484747					<b>1.9</b>	0.037
oppB	put. oligopeptide transport system permease protein	49483147			<b>-2.1</b>	0.020		
oppC	put. oligopeptide transport system permease protein	49483148	<b>-1.8</b>	0.033	<b>-2.1</b>	0.017		
purB	adenylosuccinate lyase	49484149			<b>1.8</b>	0.029		
pyrAA	put. carbamoyl-phosphate synthase, pyrimidine-specific, small chain	49483365					<b>-1.9</b>	0.043
rocD	ornithine aminotransferase	49483117			<b>-1.7</b>	0.017		
rpsG	30S ribosomal protein S7	49482776			<b>1.8</b>	0.016		
SACOL3503					<b>1.7</b>	0.018		
SAR0205	put. maltose ABC transporter, ATP-binding protein	49482446			<b>-2.4</b>	0.016		
SAR0243	put. zinc-binding dehydrogenase	49482482					<b>-1.8</b>	0.037
SAR0312	put. N-acetylneuraminase lyase	49482547			<b>-2.0</b>	0.016		
SAR0331	put. membrane protein	49482566			<b>-2.1</b>	0.018	<b>-2.1</b>	0.043
SAR0367	DNA-binding protein	49482600					<b>2.4</b>	0.037
SAR0490	tetrapyrrole (corrin/porphyrin) methylase family protein	49482716					<b>-1.7</b>	0.037
SAR0521	GntR family regulatory protein/ regulatory protein	49482747	<b>-1.8</b>	0.033	<b>-1.8</b>	0.015	<b>-1.8</b>	0.037
SAR0642	ABC transporter permease protein	49482860					<b>-1.7</b>	0.043
SAR0643	ABC transporter ATP-binding protein	49482861					<b>-1.6</b>	0.037
SAR0786	ribonucleoside-diphosphate reductase beta chain	49482989					<b>-1.7</b>	0.043
SAR0871	ABC transporter permease protein	49483070			<b>-1.7</b>	0.015		
SAR0872	put. lipoprotein /NLPA lipoprotein	49483071	<b>-1.8</b>	0.033				
SAR0874	conserved hypothetical protein / CsbD-like super-family protein	49483072					<b>1.7</b>	0.037
SAR0920	put. NAD-specific glutamate dehydrogenase	49483118					<b>4.0</b>	0.043
SAR0996	conserved hypothetical protein	49483190			<b>-2.9</b>	0.021		
SAR1227	conserved hypothetical protein	49483414	<b>1.6</b>	0.033				
SAR1338	put. homoserine dehydrogenase	49483520			<b>-4.6</b>	0.031		
SAR1345	50S ribosomal protein L33 type 2	49483527			<b>1.6</b>	0.015		
SAR1377	ImpB/MucB/SamB family protein	49483558			<b>-1.8</b>	0.029		

<sup>a)</sup> Prefix SACOL, COL gene ID; SAR, MRSA252 gene ID; SAS, MSSA476 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

Continued on next page...

## List of the 78 differentially expressed genes in IVIG compared to PBS treated samples (continued)

Common	Predicted function	GI protein	$t_0$ FC <sup>b)</sup> p-value <sup>c)</sup>	$t_{30}$ FC <sup>b)</sup> p-value <sup>c)</sup>	$t_{60}$ FC <sup>b)</sup> p-value <sup>c)</sup>
SAR1455	put. exported protein	49483632			<b>-1.7</b> 0.038
SAR1481	hypothetical protein	49483659			<b>-1.8</b> 0.037
SAR1599	put. geranyltranstransferase	49483771		<b>-2.3</b> 0.019	<b>-2.5</b> 0.045
SAR1610	lipoate-protein ligase A protein	49483782			<b>-2.2</b> 0.038
SAR1684	conserved hypothetical protein	49483850			<b>2.1</b> 0.043
SAR1686	put. biotin carboxyl carrier protein of acetyl-CoA carboxylase	49483852		<b>-1.8</b> 0.020	
SAR1693	put. O-methyltransferase	49483859			<b>-1.7</b> 0.037
SAR1702	put. cysteine desulfurase	49483867	<b>-1.6</b> 0.033		
SAR1703	put. oxygenase	49483868			<b>-1.7</b> 0.037
SAR1756	hypothetical protein	49483919			<b>-1.9</b> 0.037
SAR1849	proline dehydrogenase	49484011		<b>-2.6</b> 0.021	
SAR1854	hypothetical protein	49484016			<b>-1.9</b> 0.045
SAR1879	put. lipoprotein	49484040			<b>3.2</b> 0.037
SAR1881	put. lipoprotein	49484042			<b>2.1</b> 0.043
SAR1883	hypothetical protein	49484044			<b>1.9</b> 0.045
SAR1916	enterotoxin	49484066			<b>-1.7</b> 0.039
SAR2052	hypothetical phage protein	49484199		<b>2.0</b> 0.029	
SAR2399	put. transcription regulator	49484531			<b>-1.7</b> 0.037
SAR2408	PTS system, arbutin-like IIBC component	49484538		<b>-2.8</b> 0.017	
SAR2409	put. transcription regulator	49484539			<b>-1.8</b> 0.045
SAR2467	conserved hypothetical protein	49484596			<b>3.1</b> 0.043
SAR2589	put. glucarate transporter	49484713			<b>6.4</b> 0.037
SAR2596	conserved hypothetical protein	49484720			<b>3.5</b> 0.045
SAR2651	put. membrane protein	49484769		<b>-2.1</b> 0.016	
SAR2664	hypothetical protein	49484782			<b>-1.7</b> 0.037
SAR2682	put. aminotransferase	49484800		<b>-2.5</b> 0.017	
SAR2688	hypothetical protein	49484806	<b>-1.9</b> 0.033		
SAR2737	conserved hypothetical protein	15925646			<b>-1.6</b> 0.037
SAR2744	put. capsule synthesis protein	49484858		<b>-1.6</b> 0.015	<b>-1.8</b> 0.037
SAS0036	DNA replication and repair protein RecF	49484948			<b>1.8</b> 0.043
sasF	put. surface anchored protein	49484843		<b>-1.9</b> 0.029	<b>-1.7</b> 0.037
scrR	sucrose operon repressor	49484268			<b>2.1</b> 0.043
sdrE	bone sialoprotein-binding protein [conserved region]	49482792		<b>-1.6</b> 0.015	
set15	exotoxin 15	15926111			<b>-2.5</b> 0.047
tcaR	MarR family regulatory protein	49484572			<b>-1.8</b> 0.047
thl	acetyl-CoA acetyltransferase	49482584			<b>1.6</b> 0.043
uhpT	put. sugar phosphate transport protein	49482454		<b>-2.6</b> 0.029	

<sup>a)</sup> Prefix SACOL, COL gene ID; SAR, MRSA252 gene ID; SAS, MSSA476 gene ID

<sup>b)</sup> fold change of gene expression in IVIG treated samples

<sup>c)</sup> p-value was determined by t-test including Benjamini-Hochberg correction

# 10 Danksagung

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# 11 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschliesslich Tabellen, Karten und Abbildungen -, die anderen Werken entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Martin Krönke und Prof. Dr. Jonathan Howard betreut worden.

Nachfolgend genannte Teilpublikationen liegen vor:

Glowalla, E.; **Tosetti, B.**; Krönke, M. and Krut, O., 2009 *Proteomics-based identification of anchorless cell wall proteins as vaccine candidates against Staphylococcus aureus*, Infect Immun **77** (7) 2719-29

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Köln, 29. Mai 2011

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Bettina Tosetti

# 12 Lebenslauf

## Angaben zur Person

Name	Bettina Tosetti
Geburtsdatum und -ort	21.02.1978, Neuss
Familienstand	ledig
Staatsangehörigkeit	deutsch

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## Schulbildung

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