
# Molecular characterization of the staphylococcal two component system *sae* and its role in the regulation of the adhesin Eap under SDS stress stimulation

# Die molekulare charakterisierung des zwei komponenten-systems *sae* in staphylokokken und seiner rolle in der regulation des Eap adhäsins unter SDS vermittelten stress bedingungen

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I hereby confirm that my thesis entitled:

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**To my family: I sustain myself with your warm love. When life turns unbearable…always remember: *Psalm 133***

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# 1. Summary

The *Staphylococcus aureus* two component system (TCS) *sae* governs expression of numerous virulence factors, including Eap (extracellular adherence protein), which in turn among other functions also mediates invasion of host cells. The *sae* TCS is encoded by the *sae*PQRS operon, with *sae*S coding for the sensor histidine kinase (SaeS) and *sae*R encoding the response regulator (SaeR). The *sae*RS system is preceded by two additional open reading frames (ORFs), *sae*P and *sae*Q, which are predicted to encode a lipoprotein (SaeP) and a membrane protein (SaeQ), respectively. Earlier, we have shown that SDS-containing subinhibitory concentrations of biocides (Perform®) and SDS alone activate *sae* transcription and increase cellular invasiveness in *S. aureus* strain Newman. The effect is associated with an amino acid exchange in the N-terminus of SaeS (L18P), specific to strain Newman.

In this work, the role of whether the two additional genes, *sae*PQ coding for the accessory proteins SaeP and SaeQ, respectively, are involved in SDS-mediated *sae*RS was investigated. It could demonstrated that the lack of the SaeP protein resulted in an increased *sae*RS transcription without SDS stress in both $SaeS^{L/P}$ variants, while the SDS effect was less pronounced on *sae* and *eap* expression compared to the Newman wildtype, suggesting that the SaeP protein represses the *sae* system. Also, SDS-mediated inductions of *sae* and *eap* transcription along with enhanced invasion were found to be dependent on presence of the $SaeS^{P}$ variant in Newman wildtype. On the other hand, the study also shows that the *sae*PQ region of the *sae* operon is required for fully functional two-component system *sae*RS under normal growth conditions, but it is not involved in SDS-mediated activation of the *sae*S signaling and *sae*-target class I gene, *eap*.

In the second approach, the study investigates whether SDS-induced *sae* expression and host cell invasion is common among *S. aureus* strains not carrying the (L18P) point mutation. To demonstrate this strain Newman, its isogenic *saeS* mutants, and various *S. aureus* isolates were analysed for *sae*, *eap* expression and cellular invasiveness. Among the strains tested, SDS exposure resulted only in an increase of *sae* transcription, Eap production and cellular invasiveness in strain Newman wild type and MRSA strain ST239-635/93R, the latter without

an increase in Eap. Interestingly, the epidemic community-associated MRSA strain, USA300 LAC showed a biphasic response in *sae* transcription at different growth stages, which, however, was not accompanied by increased invasiveness. All other clinical isolates investigated displayed a decrease of the parameters tested. While in strain Newman the SDS effect was due to the *saeS*$^{P}$ allele, this was not the case in strain ST239-635/93R and the biphasic USA300 strains. Also, increased invasiveness of ST239-635/93R was found to be independent of Eap production. Furthermore, to investigate the global effect of SDS on *sae* target gene expression, strain Newman wild-type and Newman Δ*sae* were treated with SDS and analyzed for their transcription profiles of *sae* target genes using microarray assays. We could show that subinhibitory concentrations of SDS upregulate and downregulate gene expression of several signaling pathways involved in biosynthetic, metabolic pathways as well as virulence, host cell adherence, stress reponse and many hypothetical proteins.

In summary, the study sheds light on the role of the upstream region *sae*PQ in SDS-mediated *sae*RS and *eap* expression during *S. aureus* SDS stress. Most importantly, the study also shows that subinhibitory SDS concentrations have pronounced strain-dependent effects on *sae* transcription and subsequent host cell invasion in *S. aureus*, with the latter likely to be mediated in some strains by other factors than the known invasin Eap and FnBP proteins. Moreover, there seems to exist more than the *sae*S$^{P}$-mediated mechanism for SDS-induced *sae* transcription in clinical *S. aureus* isolates. These results help to further understand and clarify virulence and pathogenesis mechanisms and their regulation in *S. aureus*.

# 1. Zusammenfassung

Das Zwei Komponenten-Systems (TCS) Sae in *S. aureus* reguliert die Expression einer Vielzahl von Virulenzfaktoren, dazu gehört unter anderem das extrazelluläre Adhärenzprotein Eap, welches neben weiteren Funktionen, die Invasion in eukaryotische Wirtszellen vermittelt. Die Gene des *sae* TCS sind in einem Operon organisiert (*saePQRS*), wobei *saeS* für die sensorische Histidinkinase (SaeS) und *saeR* für den „Response Regulator" (SaeR) kodieren. Diesen Genen sind zwei weitere Genabschnitte, *saeP* und *saeQ,* vorangestellt, wobei *saeP* vermutlich für ein Lipoprotein (SaeP) und *saeQ* für ein Membranprotein (RelQ) kodieren. In einer früheren Arbeit konnten wir zeigen, dass SDS-haltige Biozide (Perform©) unter sub-inhibitorischen Konzentrationen, sowie reines SDS, die *sae* Transkription aktiviert und dadurch zu einer erhöhten Invasion des *S. aureus* Stamms Newman in Wirtszellen führt. Dieser Effekt ist assoziiert mit einem spezifischen Aminosäureaustausch im N-terminus von SaeS (L18P) des Stamm Newman.

In dieser Arbeit soll nun die Beteiligung der zwei zusätzlichen Gene, *saeP* und *saeQ*, an der SDS vermittelten transkriptionellen Induktion von *saeR/S* untersucht werden. Es konnte gezeigt werden, dass ohne SaeP, die *saeR/S* Transkription in beiden $Sae^{L/P}$ Varianten erhöht war, wobei eine zusätzliche SDS Behandlung hierfür nicht notwendig war. Im Gegenteil, es zeigte sich, dass der SDS Effekt auf die *sae* und *eap* Expression in der *saeP* Mutante deutlich weniger ausgeprägt ist als im Wildtyp Stamm. Das läßt vermuten, dass das Lipoprotein SaeP repremierend auf das *sae* System einwirkt. Des Weiteren wurde festgestellt, dass die SDS vermittelte transkriptionelle Induktion von *sae* und *eap,* zusammen mit der erhöhten Invasion, abhängig vom vorhanden sein der $SaeS^{P}$ Variante im Newman Wildtyp Stamm ist. Die Arbeit zeigt, dass die *saePQ* Region wichtig ist für die vollständige Funktion des Zwei Komponenten Systems SaeRS unter normalen Wachstumsbedingungen. Jedoch ist diese Region nicht involviert in der Aktivierung von SaeS, mit SDS als Signalgeber, sowie der darauffolgenden Aktivierung des *sae* Zielgens *eap*.

In einem zweiten Ansatz wurde untersucht, ob die SDS induzierte *sae* Expression und Wirtszellinvasion auch häufig in *S. aureus* Stämmen auftritt, welche keine (L18P)

Punktmutation besitzen. Dafür wurde Stamm Newman, die isogene *saeS* Mutante und verschiedene *S. aureus* Klinikisolate auf ihre *sae*, *eap* Expression, sowie zelluläre Invasionsfähigkeit hin analysiert. Von den getesteten Stämmen reagiert nur Wildtyp Stamm Newman und ein MRSA Stamm ST239-635/93R mit gesteigerter *sae* Transkription, Eap Produktion und zellulärer Invasion. Der MRSA Stamm jedoch ohne erhöhte Eap Produktion. Interessanterweise zeigt der „community- associated" MRSA Stamm USA300 LAC eine biphasische *sae* Transkription in verschiedenen Wachstumsphasen, welche jedoch nicht einhergeht mit erhöhter Invasion. Alle anderen Klinikisolate zeigten abnehmende Tendenzen in den getesteten Parametern. Während im Stamm Newman der SDS Effekt auf das $saeS^P$ Allel zurückzuführen ist, gilt dies nicht für den Stamm ST239-635/93R, sowie den biphasischen Stamm USA300. Außerdem konnte gezeigt werden, dass die erhöhte Invasion des Stamms ST239-635/93R unabhängig von seiner Eap Produktion ist. Des Weiteren zeigten wir den globalen Effekt von SDS auf die *sae* Zielgenexpression. Dafür behandelten wir Wildtyp Stamm Newman mit SDS und analysierten die Transkription der *sae* Zielgene mittels Microarray Analyse. Wir konnten zeigen, dass subinhibitorische SDS Konzentrationen, induzierende als auch repremierende Auswirkungen auf die Genexpression haben. Dabei sind Gene betroffen, die involviert sind in verschiedene Signalwege, Biosynthese/Metabolismus als auch in Virulenz, Wirtzelladhärenz und Stressantwort.

Zusammenfassend gibt die Arbeit Aufschluss über die Rolle der „upstream" Region *saePQ* hinsichtlich der SDS-abhängigen *saeRS* und *eap* Expression in *S. aureus*. Am wichtigsten ist hierbei die Erkenntnis, das subinhibitorische SDS Konzentrationen einen deutlichen stammabhängigen Effekt auf die *sae* Transkription und daraus folgernd auf die Wirtszellinvasion von *S. aureus* haben. Letzteres wird vermutlich in manchen Stämmen durch andere Faktoren als die bekannten Invasinproteine Eap und FnBP vermittelt. Außerdem scheint es in den klinischen *S. aureus* Isolaten mehr als nur den $saeS^P$ abhängigen Mechanismus der *sae* Induktion durch SDS zu geben. Diese Ergebnisse helfen uns die Virulenz und pathogenen Mechanismen als auch deren Regulation in *S. aureus* zu verstehen. Die Beobachtungen tragen zu unserem Verständnis bei, wie das *sae* System Signale der Umgebung detektieren kann. Dies ist bis jetzt eine Fragestellung mit vielen Unbekannten.

# 2. Introduction

## 2.1 *Staphylococcus aureus*

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive, facultative anaerobe, cocci bacterium belonging to the *Staphylococcaceae* family and the genus of *Staphylococcus* (120,143). The bacteria often present in grape-like clusters or as single cells under microscope. In routine diagnostics, *S. aureus* has been distinguished in the past from other *Staphylococcus* species by the ability to produce coagulase, which causes clotting (coagulation) of blood (26). Also, *S. aureus* is a catalase positive, non motile and non-sporulating bacterium, which is capable of fermenting glucose (111). The bacterium is approximately 0.5 µm-1 µm in diameter. In the laboratory, *S. aureus* grows rapidly on blood agar under both aerobic and anaerobic conditions. On blood agar, the colonies appear smooth and gold-yellowish due to the ability of the bacterium to produce carotenoids. Depending on the strain, the colonies often produce pronounced β-hemolysis on blood agar. Therefore, *S. aureus* can frequently also be distinguished from other staphylococcal species based on the hemolysis on blood agar in the laboratories. Amongst the currently known 42 staphylococcal species, *S. aureus* signifies the most virulent species in clinical medicine. Of clinical relevance are also coagulase negative staphylococci (CoNS), most frequently *S. epiderdimis*, which can cause diseases associated with indwelling catheters, prosthesis and implants (32,44,48). Other clinically important CNS includes *Staphylococcus saprophyticus*, which commonly causes urinary tract infections in younger women, and *Staphylococcus lugdunensis,* which can cause osteomyelitis and endocarditis (8,21,85,130).

Discovered in 1880 by Sir Alexander Ogston, subsequently named by Rosenbach in 1884, *S. aureus* has remained a dangerous human pathogen with endemic ramifications worldwide (150). The organism is both a commensal and a facultative pathogen. *Staphylococcus aureus* is frequently isolated in both community-associated and nosocomial infections and has the ability to colonize healthy individuals asymptomatically (111,167). The main ecological niche of *S. aureus* is the anterior nares with a carrier prevalence of 20-30% (157). Colonization is one of the main reservoirs for *S. aureus* infections in both community and health-care

settings (165). Other common colonization sites are skin and throat (121). Infections can be introduced by aspiration, indwelling catheters, injuries and surgery, but can also occur by the hematogenous route. Infections caused by *S. aureus* range from mild infections including skin and soft tissue infections to life threatening conditions such as pneumonia, endocarditis, osteomyelitis and sepsis (35,109,111). *Staphylococcus aureus* has globally been also implicated in a number of food poisoning outbreaks and is one of the common causes of gastroenteritis as a result of consumption of contaminated food (13,16). In addition, *S. aureus* can cause exclusively toxin-mediated syndromes such as staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS) (110,128). The success and significance of the pathogen is mainly based on two factors; i) the ability of the bacterium to rapidly develop resistance to multiple drugs used to treat clinical infections and ii) the ability to produce a wealth of virulence factors that promote persistence and infection by *S. aureus* (111)

### 2.1.1 *S. aureus* environmental tolerance and survival

*Staphylococcus aureus* can occupy various niches in the host, which substantially differ with respect to their physico-chemical properties. Thus, *S. aureus* encounters a large range of environmental stress conditions. The adaptability to different environmental conditions also contributes to the versatility of the species. For example, temperature has been shown to affect *S. aureus* strains with 37$^{o}$C (human body temperature) as the optimal growth temperature. *Staphylococcus aureus* strains can grow at ranging from 7$^{o}$C and 47.8$^{o}$C and has a higher tolerance to temperature variance than many other bacteria. Several genes have been shown to be up-regulated by heat-shock including *hla* and *dnaK* genes with cold-shock resulting in the up-regulation of *cspA,* which is responsible for pigment production in *S. aureus* strains (9). Acid stress can also induce changes in the cell membrane composition and gene expression. In one study, *S, aureus sig*B mutants were shown to have reduced tolerance to acid stress (36). Consequently, both temperature and acid stress seem to be important in *S. aureus* related food-borne infections particularly in preserved foods.

Recently, several studies reported that sub-inhibitory concentrations of antibiotics influence gene expression in *S. aureus* strains (53,126,134,155). Ohlsen *et al*., previously

demonstrated that the β-lactam antibiotic nafcillin, which is commonly used for treatment of *S. aureus* infections, can induce α-toxin expression and increase lethality in murine model (126). Another β-lactam antibiotic, cefoxitin has also been shown to induce hemolytic activity *in vitro* primarily through *sae*RS-mediated regulation (104). An increased expression of *fnbA* by subinhibitory concentrations of ciprofloxacin has also been reported (24). In addition, Chen *et al*., reported that sub-inhibitory concentrations of vancomycin induced *sig*B in VRSA strains, consequently enhancing the cytotoxicity to BEAS-2B cells (39,105). Kuroda *et al*., also reported the induction of *sae*RS by glycopeptides in *S. aureus* strains (105). On the other hand, sub-inhibitory concentrations of salicylic acid, the primary metabolite of aspirin produced *in vivo*, induced Eap expression in clinical *S. aureus* isolates and diminished the capsular polysaccharide type 5 expressions (6,7,7).

In the food industry and medical setting, disinfectants are often used to remove *S. aureus* contamination and biofilms. Commonly used surface disinfectants include oxidative (peroxygens) and surface-active ammonium salts compounds, as well as chlorine-releasing compounds and oxygen-based biocides such as Perform®, containing SDS-like detergents to disinfect surfaces and medical devices. It has been previously shown that sub-lethal concentrations of the oxidant, hypochlorite (bleach), were shown to increase cell aggregation and reduce cell size in *S. aureus* (2). Perform® has a broad antibacterial activity which includes *S. aureus* and is used health-care institutions. Our group previously demonstrated that sub-inhibitory concentrations of Perform and SDS increased the expression of Eap in *S. aureus* strain Newman (142). As a result, increased Eap production led to functional consequences, such as enhanced cellular invasion of 293 cells. The data of this study form the basis of this study (discussed later in objectives of the study). The regulation of stress response is coordinated by several global regulators such as two-component signaling system global regulators, *agr*CA, *sae*RS, *arl*SR and *lyt*RS and other systems including *sig*B and *sar*A. Most of these global regulators consist of signal transduction systems known as two component systems (TCS), which enable an organism to elicit an adaptive response to external or internal stimuli primarily through gene regulation [127,128]. Two component systems play important roles in the response and adaptation to environmental changes and stress including temperature, pH, osmolarity, auto-inducing

compounds, oxygen pressure and host cell molecules [131]. Furthermore, TCS are important in the regulation of virulence factor expression and susceptibility to antibacterial agents in many bacterial species. Hence, they could serve as as potential targets for novel antimicrobial agents in bacteria [132].

### 2.1.2 Antibiotic resistance of *S. aureus*

In the 1940s, penicillin was the primary drug of choice for the treatment of *S. aureus* infections (56). Since many strains rapidly became penicillin resistant due to penicillinase production, methicillin was introduced in 1960 as a penicillinase-stable derivative (18). Newer related compounds, such as flucloxacillin are still a mainstay for treatment of *S. aureus* infections. In 2002, it was documented that 90% of *S. aureus* strains were resistant to penicillin (118). Already in 1961, methicillin resistant *S. aureus* (MRSA) strains were identified (18). These strains of MRSA were mainly prevalent in hospitals and did not spread to the community. Later, however, other MRSA strains appeared which were genetically distinguishable from the previously strains. Recently, further MRSA strains emerged which are closely associated with livestock infections (152). Hence, there are several populations of MRSA, health-care-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA) with a related group, livestock-asociated MRSA (LA-MRSA).

Resistance to methicillin is mediated by the *mec*A gene, which is harbored on a mobile genetic element known as the Staphylococcal Cassette Chromosome (SCC*mec*) (92,99). The expression of *mec*A results in an altered penicillin binding protein 2a (PBP 2a), which leads to reduced binding to β-lactam antibiotics (92,99). Five main SCC*mec* types (SCC*mec* I-V) have been described, each with a specific signature of resistance profile and epidemiology (92,99). SCC*mec* type II and III, which are larger and carry more resistant genes have been associated with nosocomial MRSA strains. More importantly, SCC*mec* type IV, which is the smallest cassette of the five SCC*mec* elements is associated with CA-MRSA strains (92,99). Furthermore, most CA-MRSA strains have been shown to express the Panton-Valentine leukocidin, which is associated with skin/soft tissue infections and necrotizing pneumonia (92,99).

Approximately 478 000 cases of health-care associated infections in the USA were attributed to *S. aureus* with a 58% MRSA prevalence. Of these *S. aureus* cases, roughly 2% of these patients die (100). Consequently, the cost of health-care programmes and services implemented for the control and treatment of *S. aureus* were reported to be as high as $9.3 billion in 2001 (122). In Europe Germany, although not mandatory reporting exists, approximately 132 000 cases of MRSA are reported yearly with approximately **€**380 million cost in hospital stays (101). Of these MRSA cases, MRSA accounts for approximately 20% of inpatient culture positive *S. aureus* specimens. Alarming prevalence rates of more than 50% have been reported in countries such as the USA, Japan (2000), Singapore (1997), Colombia (2002) and Egypt (2006) (72) (Fig. 1). These studies included only invasive infections including blood stream infections, skin and soft tissue infections and pneumonia.

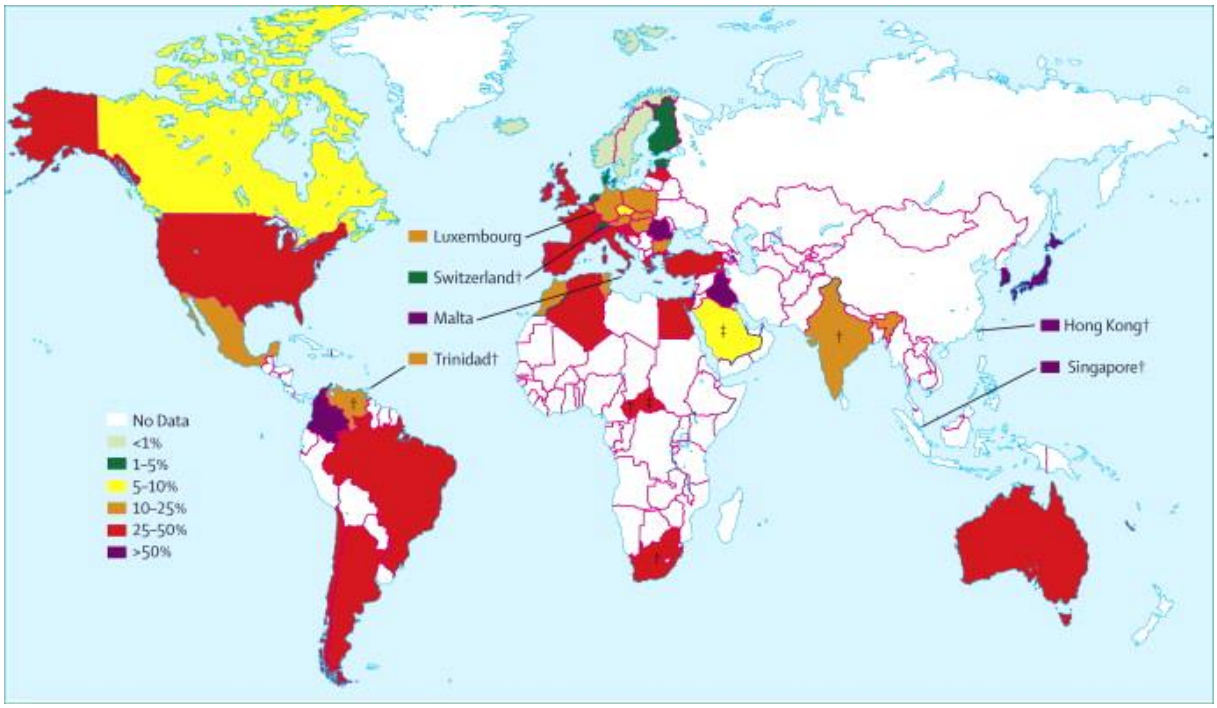



**Figure 1: Global prevalence of MRSA based of peer reviewed studies from 1998-2005.**
MRSA prevalences for the USA, Canada, Latin America, Europe and the Western Pacific accounts for only bloodstream isolates.

Even more alarming is the emergence of vancomycin-resistant *S. aureus* strains (82). Vancomycin still is a major drug for the treatment of multidrug-resistant MRSA infections (83). Presently, *S. aureus* strains that have acquired resistance to all currently used antibiotics including novel alternatives such as daptomycin and linezolid have been reported (79,168).

### 2.1.3 Colonization and disease by *S. aureus*

The main sources of *S. aureus* colonization include direct contact with colonized asymptomatic individuals and those with infected lesions. Colonization is the main reservoir for *S. aureus* transmission in both the hospital and community settings (70). Carrier rate is approximately 20% in persistently colonized individuals and 30% in intermittently colonized (70). Nosocomial transmission is particularly high with poor hand hygiene since hand-to-hand contact is the main mechanism of spread (57,111). Hand hygiene has been shown to significantly decrease *S. aureus* transmission in health-care and community settings (23,67). Susceptibility to colonization and infection is increased in certain groups of individuals such as children, health-care employees, military recruits and prison in-mates (4,5,81). Moreover, *S. aureus* (LA-MRSA) is able to colonize and cause infections in animals such as cattle, poultry and swine (131,152). With animals being an emerging reservoir, MRSA colonization prevalence has been shown to be as high as 45% in individuals with direct contact to livestock (151). High risk groups of LA-MRSA colonization and infection include farmers and veterinarians (58,74). Because an individual can be colonized with either MSSA (17% incidence) and MRSA (8% incidence) strains, eradication of strains can pose a challenge (45). Approximately 6.6% of colonized patients present with multiple *S. aureus* strains (34). To minimize transmission, nasal decolonization of *S. aureus* carriage has been implemented mainly by topical application of decolonizing agent mupirocin (46,52). Between 81% to 100% of patients can be successfully decolonized with mupirocin if there are no adverse factors present, such as ulcera or other chronic wounds which are colonized (46). However, the emergence of *S. aureus* strains resistant to mupirocin has already been reported (86,146). In addition, mupirocin has been shown to alter virulence factor gene expression in some *S. aureus* strains resulting in improved adaptation and increased virulence of the strains (135).

### 2.1.4 Pathogenesis and Virulence factors

*Staphylococcus aureus* possesses a variety of cell-associated and extracellular virulence factors that contributes to the pathogenesis and virulence potential of the strain. These factors include cell associated components, toxins and enzymes which are carefully coordinated at different stages of growth of the bacterium. Furthermore, *S. aureus* strains

can produce host adherence and evasion molecules which are primarily responsible for cellular invasion and evading host immune defenses. In the next section, these *S. aureus* components are briefly discussed.

#### 2.1.4.1 Cell wall of *S. aureus*

The cell wall of *S. aureus* is composed of a thick peptidoglycan layer which contributes to virulence of the bacterium and two polymers known as wall teichoic acid (WTA) and lipoteichoic acids (LTAs) which play a role in adherence to host cells (111). The peptidoglycan links many adhesive proteins and also stimulates cytokine production, which results in activation of the complement system and aggregation of platelets (156,164). Wall teichoic acids contains ribitol and glycerol that are linked by phosphodiester bonds (111). Wall teichoic acids are known to activate neutrophil cytokine generation and are important for nasal colonization and resistance to antimicrobials in *S. aureus* (163,164). On the other hand, LTAs polyanions containing repeating units of ribitol and glycerol phosphate are linked to the lipid membrane of the bacterium (107). *Staphylococcus aureus* forms a (micro-) capsule surrounding the cell wall (111). Ninety percent of *S. aureus* strains are encapsulated with 11 capsular polysaccharides have been reported and approximately 80% of human strains belong to either cap5 of cap8 (10,11). The capsule provides the first interaction with the host molecules and enhances resistance to immune attack; hence the *S. aureus* capsule has been extensively studied for vaccine development of staphylococcal infections (59,137).

#### 2.1.4.2 Cell wall-anchored adhesins

*Staphylococcus aureus* adherence to host cell surface forms the first phase in host cell colonization and invasion (76,148,149). This phase is facilitated by proteins called microbial surface component recognizing adhesive matrix molecules (MSCRAMMS) also known as cell wall-anchored adhesins (111). Classically, MSCRAMMS consist of a consensus motif (LPXTG) for sortase (117). To this end, sortase covalently anchors the adhesins to the penta-glycine side chains of the peptidoglycan (149). These proteins recognize plasma and extracellular matrix (ECM) components such as collagens, fibronectin and fibrinogens (70). Classic *S. aureus* adhesion proteins belonging to MSCRAMMs include protein A (Spa), clumping factor A and B, fibronectin-binding proteins A and B (FnBPs A/B) (149).

#### 2.1.4.2.1 Protein A

Protein A (Spa) binds to IgG and von-Willerbrand-factor, a glycoprotein mediating platelet adhesion at endothelial damage site (78). It also interferes with the opsonization by phagocytes and activates TNF receptor 1 and EGF receptor (EGFR) signaling cascades that can perturb the cytoskeleton (69). Protein A is conserved in all *S. aureus* strains, therefore it can be used as a marker for *S. aureus* diagnostic detection.

#### 2.1.4.2.2 Clumping factor A/B

Clumping factor A is able to bind to human fibrinogen, while ClfB contributes to colonization by binding to cytokeratin-10 in nasal-epithelial cells (166). Furthermore, ClfA has been shown to be responsible for the clumping of fibrinogen found in host cell surfaces (111). Que *et al*., previously showed that the expression of ClfA and FnBPs in a less pathogenic *Lactobacillus lactis* bacterium induced endocarditis in experimental rat model (132).

#### 2.1.4.2.3 Fibronectin binding proteins (A/B)

Fibronectin is a protein found in the extracellular matrix of most mammalian host cells including phagocytes. Several invasins produced by *S. aureus* strains mediate the invasion of bacteria to host cells by binding to fibronectin (75,97,148). Most well studied *S. aureus* invasins include proteins that belong to the MSCRAMMs family such as FnBPA and FnBPB which are encoded by *fnbA* and *fnbB* located on the same operon, respectively. Both FnBPA and FnBPB contain a 500 amino acid N-terminal with a fibronectin binding domain that consists of 4-5 conserved repeats of 50 aa each (71,161). Fibronectin binding proteins are anchored to the bacterial cell wall at the C-terminus which consists of hydrophobic residues (73). *Staphylococcus aureus* FnBPs are mainly expressed in the early exponential growth phase and downregulated as the bacterial load increases (post exponential phase) (98,141). The mechanism of FnBP-dependent cellular invasion has been elucidated (see cellular invasion section 2.1.4.4). FnBPs are virtually conserved in all *S. aureus* strains, however, some strains including 8325-4 and Wood46 are regarded as regulatory mutants, thus FnPBA/B regulation in these strains is impaired rendering them less invasive to host cells (73,148). Most notably, in strain Newman, there is an introduction of a stop codon in the *fnbA* and *fnbB* genes that results in truncated FnBPs in the C-terminal of the protein (73). The truncated domain renders the lack of LPXTG motif for cell wall anchoring, thus FnBPs are secreted and not anchored in strain Newman. As a result, this leads to several impaired

FnBP-dependent functions in the strain Newman (73). The expression of FnBPs is regulated by multifactoral global systems including SarA, SaeRS, and AgrCA.

#### 2.1.4.3 Soluble adhesins

Another group of adhesins are known as secreted expanded repertoire adhesive molecules (SERAMs). This group of proteins contains no LPXTG motif and therefore is not anchored by the sortase to the cell wall but is secreted by *S. aureus* (38). SERAM molecules play an important role in adhesion and invasion into host cells and also contain some immunomodulatory functions (38). These adhesins are further described individually in more detail below.

##### 2.1.4.3.1 Extracellular adherence protein (Eap)

Discovered in 1991, Eap, previously also termed MHC class II-analogous protein (MAP) is another virulence factor that acts as an invasin which is encoded by all *S. aureus* strains, but not other staphylococcal species (28,91). Eap consists of 4 to 6 repeats of 110 amino acids with relatively high sequence identity, however it does not encode the sortase A recognition motif LPXTG (117). Therefore, Eap is an anchorless adhesin, which is secreted and bound non-covalently to the *S. aureus* cell wall via a not yet characterized mechanism. Eap belongs to a class of proteins/adhesins termed SERAMs (secretable expanded repertoire adhesive molecules) (19). It has a broad binding specificity to numerous extracellular matrix including fibrinogen, fibronectin, vitronectin and collagens as well as plasma proteins such as thrombospondin. Expression of Eap is strongly regulated by the SaeRS two component signaling system and to a lesser extent by Agr and SarA global regulatory systems (77). The degree of expression of Eap expression is strain-dependent. For example, in *S. aureus* strain Newman, Eap is highly expressed compared to other investigated strains. Consequently, in strain Newman Eap partially compensates for the loss of functional FnBPs for cellular invasion (73). Maximal expression of Eap occurs during the late-exponential phase of growth and is regulated by *agr*, *sar* and *sae* (54,77). In *S. aureus* strain Newman, Eap is produced in large amounts compared to other described *S. aureus* strains. In this way, because the FnBPs are truncated, strain Newman compensates for the loss of FnBP functionality by Eap overexpression. The elucidated mechanism for this overexpression is dependent on the point mutation in the SaeS protein of the Sae system (73).

#### 2.1.4.3.2 Extracellular matrix protein (Emp)

Another soluble protein that is able to interact with collagen, fibronectin, fibrinogen and vitronectin is Emp (38). Cloned from strain Newman, Emp has a molecular weight of 38, 5 kDa (89). Furthermore, Emp remains conserved in *S. aureus* strains, however lacks in some *S. epidermidis* strains (89). The role of Emp is the pathogenesis of *S. aureus* remains unelucidated, however, under low-iron conditions; Emp regulates iron-mediated biofilm formation (95).

### 2.1.4.4 Cellular invasion

Classically, *S. aureus* adhesins mediate adherence to host cells. Consequently, the second phase involves internalization and invasion into non-professional phagocytes. The internalizationmechanism is mediated by β2-integrins in professional phagocytes. In contrast, cellular invasion in non-professional phagocytes proceeds through a modified zipper type mechanism (Fig. 2). In brief, FnBPs specifically bind to the glycoprotein, fibronectin, which interacts with intergrin $\alpha_5\beta_1$ initiating an actin polymerization-dependent zipper-type mechanism (148).

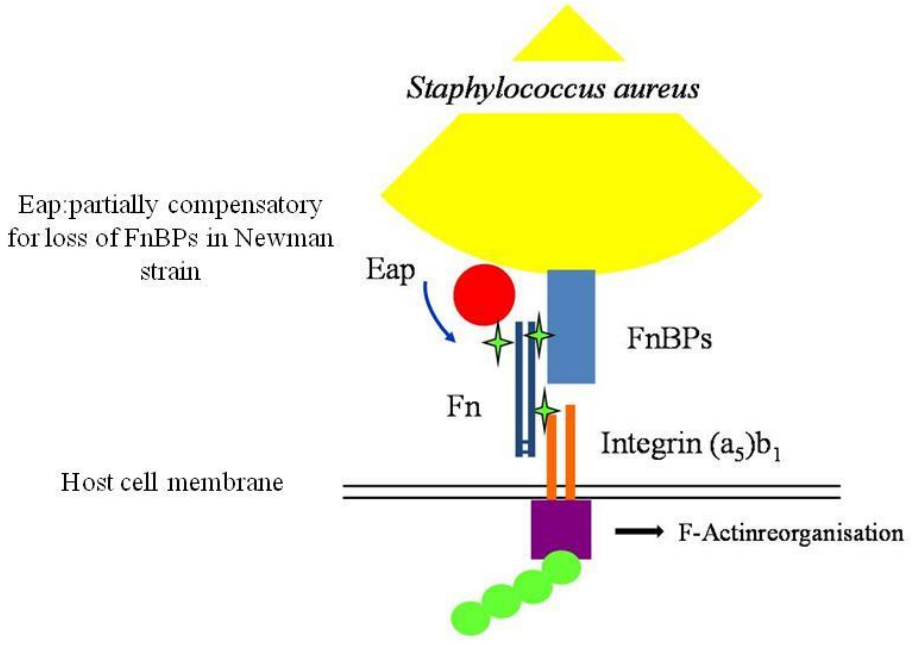



**Figure 2: Model of the host cell invasion by *S. aureus* (149).** In *S. aureus* strain Newman, Eap compensates for the truncated FnBPs, however not all details of the molecular mechanism of Eap in strain Newman have been formally addressed.

Fibronectin consists of three structural modules (type 1, 2 and 3) with the N-terminal of the glycoprotein consisting of five type 1 modules. These five modules are composed of β-strands with a high affinity to FnBP binding motifs (145). The interaction initiates clustering of integrins and activates a signaling pathway, which causes actin cytoskeleton rearrangement and bacterial uptake (148). In strain Newman, internalization by epithelial cells is mediated by Eap due to the lack of cell-wall anchored FnBPs in this strain (76). However, to date the binding receptor has not been identified.

In recent years, there has been increasing evidence of the existence of another non-FnBP and Eap-mediated internalization to non-professional phagocytes by *S. aureus* and *S. epidermidis* strains (84). The novel mechanism involves the staphylococcal autolysin (Atl) and AtlE in *S. aureus* and *S. epidermidis*, respectively. Although, this mechanism has not been fully elucidated it has been proposed to represent a 'backup' mechanism in *S. aureus* strains and possibly the sole mechanism in *S. epidermidis* for internalization. In this study, internalization involved the binding of Atl to the heat shock cognate protein Hsc70 receptor and possible interaction with host cell intergrin $\alpha_5\beta_1$ through fibronectin as a bridging molecule. Furthermore, Atl has been shown to mediate adherence to polystyrene and biofilm formation (25).

#### 2.1.4.5 Immune evasion

*Staphylococcus aureus* produces other proteins that can have an effect on the innate and adaptive immune response. Inhibition of the host defense systems initiate by the excretion of small molecules that inhibit several steps of the immune response. Some of these immune evasion molecules include the bacteriophage IEC components such as CHIPS, SCIN, SAK, Eap, and extracellular fibrinogen binding protein (Efb) (29,76,138,159). Staphylococcal complement inhibitor (SCIN) is a C3 convertase inhibitor with molecular weight of 9.8 kDa that blocks the formation of C3b on the bacterial surface (138). SCIN also prevents phagocytosis following opsonization of *S. aureus* by C3b presentation on its surface (138). Staphylokinase (SAK) is an extracellular protein that stimulates the conversion of human plasminogen to plasmin (29). Jin *et al*. previously reported that patients infected with SAK-negative *S. aureus* isolates were 4-times more likely to develop bacteremia than patients

infected with isolates expressing high levels of SAK. Another protein expressed on the bacteriophage is CHIPS, which is a 14.1 kDa protein found in 60% of *S. aureus* clinical isolates (49). The chemotaxis inhibitory protein (CHIPS) attenuates C5a receptor (C5aR) response as well as the response of formylated peptide receptor of human neutrophils, which leads to neutrophil activation and chemotaxis (49). In addition to its role in host cell adhesion and invasion, Eap has an immune-modulatory effect on host response by binding intercellular adhesion molecule 1 (ICAM-1, CD54), which blocks the adhesion of monocytes and T-cells to activated endothelial cells (37). This binding stimulates the secretion of proinflammatory TNFα and IL-6.

In order to spread to adjoining tissues, *S. aureus* secretes strain specific toxins that aggravate inflammation by forming pores in host cells leading to cell lysis. Common S*. aureus* toxins include α, β, δ and γ-hemolysins and the Panton Valentine leukocidin toxin (PVL). **Alpha-hemolysin** is encoded by the *hla* gene and although all *S. aureus* strains contain the *hla* gene, some strains do not express it (170). Alpha-hemolysin mutant *S. aureus* strains show reduced virulence in animal infections such as endocarditis, mastitis, septic arthritis and pneumonia (20,30). The α-hemolysin is able to bind to specific host cell receptors and stimulate cell signaling cascades including the activation of caspase 8 and 9, which result in caspase 3 activation and leads to DNA degradation and apoptosis (22).

On the other hand, **β-hemolysin** is a neutral sphingomyelinase capable of hydrolyzing sphingomyelin, a plasma membrane lipid (111). β-hemolysin has a molecular mass of 35 kDa and at 37$^{o}$C, the β-hemolysin interacts with red blood cells without lysing them (87). Only when exposed to low temperatures, the β-hemolysin is activated to lyse red blood cells (87). In *S. aureus*, the β-hemolysin encoding gene can be disrupted by a bacteriophage which carries genes encoding immune evasion cluster (IEC) such as staphylokinase (SAK), SEA and SEP, staphylococcal complement inhibitor (SCIN) and the chemotaxis inhibitory protein (CHIPS) (47). The majority of human-derived clinical *S. aureus* strains carry at least 2-4 IEC components. On the other hand, Aarestrup *et al*. reported that β-hemolysin was expressed in 72% of bovine mastitis isolates (1)

**Panton Valentine leukocidin** is another well-characterized pore-forming *S. aureus* toxin. It is a bicomponent cytolysin that is composed of the secreted LukF-PV and LukS-PV proteins. PVL has a high affinity to leukocytes and is known to be associated with certain CA-MRSA strains (15,31), but is also found on MSSA strains. *Staphylococcus aureus* strains expressing PVL have been associated with skin and soft tissue infections and necrotizing pneumonia (50). The role of PVL in the virulence of *S. aureus* is still not been completely understood. Diep *et al*., demonstrated the role of PVL as a cause of necrotizing pneumonia in a rabbit model (51). Also, evidence that the activity of PVL on neutrophils is species specific has been reported which also substantiated that PVL has an important cytotoxic function in human neutrophils. The transcription of PVL was reported to depend on *sar*, *agr* and the *sae* global regulatory systems (33,158). Voyich *et al*., also showed that the deletion of the *sae*RS in strain USA300 LAC (CA-MRSA) resulted in significantly reduced levels of PVL expression. A group of novel secreted cytolytic peptides called phenol soluble modulins have become popular in *S. aureus* research recently (127). **Phenol-soluble modulins** belong to a family of amphipathic, α-helical peptides with surfactant-like properties (42). These peptides are produced by human and animal-related staphylococci (154). *Staphylococcus aureus* produces 7 types of PSMs named PSMα1-α4, PSMβ1 and PSMβ2 and the δ-toxin (160). Interestingly, the δ-toxin is located within the RNAIII molecule encoded by the *agr* global regulatory locus. Therefore, *psm* expression is regulated by the *agr* system (154).

#### 2.1.4.6 Superantigens

*Staphylococcus aureus* secretes superantigenic exotoxins including the most famous superantigen known as the toxic shock syndrome toxin-1 which causes toxic shock syndrome. These toxins are pyrogenic and potent mitogens which are largely associated also with food poisoning. Superantigens trigger T cell activation and proliferation by non-specific interaction with the MHC II, therefore, they do not require antigen processing for immune activation (43). Several *S. aureus* superantigens have been described with Staphylococcal enterotoxin B and C (SEB/C) being well characterized in the literature. For example, SEB has been widely implicated as an aerosolized agent in biological warfare and terrorism (169). Other superantigens particularly SEC has been implicated in promoting infective endocarditis, sepsis and kidney injury (139).

### 2.1.5 Gene regulation of virulence factors in *S. aureus*

The ability to adapt, grow and survive in divergent niches is highly coordinated by several regulatory systems. Stress tolerance and response regulation has been shown extensively to be regulated by the expression of global regulators including Agr, Sae, the SigB and the SarA systems [133]. Global regulatory response results in activation or inactivation of appropriate target genes. In addition, global regulators are activated at different growth phases of the bacterium [138]. For example, the Sae and the Sar systems are reported to be activated at the exponential growth phase and are optimal during the transition from late exponential to stationary phase [139,140]. Better understanding of the mechanisms and functions of virulence factor and resistance gene expressions are needed to further improve the prognosis of patients infected with *S. aureus*. The section below described the general stress response and gene regulation of *S. aureus* to different environmental stress.

#### 2.1.5.1 Sigma factor B

The alternative sigma factor B (SigB) is an important global regulator responsible for response to conditions such as alkaline, heat, oxidative stress and salinity [131,141]. Specifically, SigB positively regulates the expression of α-hemolysin, clumping factor A, coagulase and FnBPA, and negatively regulates serine protease [141]. Deletion of *sig*B in *S. aureus* results in unchanged virulence in a murine abscess model of infection [143]. The *sig*B operon consists of 4 genes including *sig*B, *rsb*U, *rsb*V and *rsb*W [144]. Regulation of expression by the *sig*B system is complex, transcribing multiple transcripts including a bicistronic *sig*B-*rsb*W transcript and a monocistronic *sig*B transcript [144]. Some *S. aureus* strains do not produce functional RsbU protein, which is required for the activation of SigB pathways [145]. However, in these strains, *sig*B activation still occurs independent of RsbU suggesting possible existence of other *sig*B activation mechanisms [145]. In addition, SigB directly affects *sar*A expression by binding to the P3 promoter of *sar*A [146]. Furthermore, SigB impacts on biofilm formation through the positive regulation of the *ica* gene which encodes the factors necessary for polysaccharide intercellular adhesin (PIA) [147]. Furthermore, *sig*B mutant *S. aureus* strains were described as having pigment loss, increased

α-hemolysis activity and reduced peroxide resistance [148]. In strain Newman, *sig*B represses the expression of *sae*RS by an unknown mechanism (own data).

#### 2.1.5.2 Staphylococcal accessory regulator A (SarA)

Similar to other virulence regulators, the *sar*A system is growth-phase dependent system [149]. Initially, *sar*A was thought to negatively regulate the effects of *agr* but later was shown to have additive effects on *agr* system [150]. It functions mainly through the activation of the *agr* promoters and induce regulation of *agr*-dependent genes [151]. In addition, the *sar*A system can activate virulence genes in a *agr*-independent manner [152]. The *sar*A system activates the expression of extracellular and cell wall associated proteins such as FnBPs and represses the expression of collagen adhesion gene in *S. aureus* [153,154]. Mutations in the *sar*A system attenuates virulence in a rabbit model of endocarditis and also results in decreased biofilm formation and increased susceptibility to antistaphylococcal agents [155–157]. Several homologues of SarA have been identified in staphylococci which includes small proteins and large proteins homologues such as SarR, SarT and SarX as well as larger proteins such as Rot, SarS, SarU amongst others [158]. The *sar* locus is located within a 1.2 kbs locus and consists of 3 overlapping transcripts initiated by 3 promoters, *sar*P1, *sar*P2 and *sar*P3 which are active at different growth phases *in vitro* [159].

#### 2.1.5.3 Accessory gene regulator (Agr)

Rescei *et al*., first described the Agr system in 1986 and subsequently by Vandenesch *et al*., in 2001 showing its importance in the regulation of several exoproteins including α-hemolysin, β-hemolysin, TSST-1 and leukocidins during post-exponential phase [98,160–162]. The Agr global regulatory system also represses cell wall-associated proteins activated during the exponential phase including protein A, FnBPs and protein A [162]. The *agr* locus is composed of two promoters, P2 and P3 encoding divergent transcripts RNAII and RNAIII (Fig. 3) [164]. RNAII transcribes 4 *agr* genes called *agr*ABCD with the *agr*A and *agr*C genes encode the two-component system, sensor histidine kinase AgrC and response regulator AgrA that responds to autoinducing peptide (AIP). The *agr*D gene encodes a cell to cell communication system known as quorum sensing in Gram-positive bacteria [165]. The membrane protein AgrB is involved in processing and production as a thiolactone-modified cyclic oligopeptide [164].

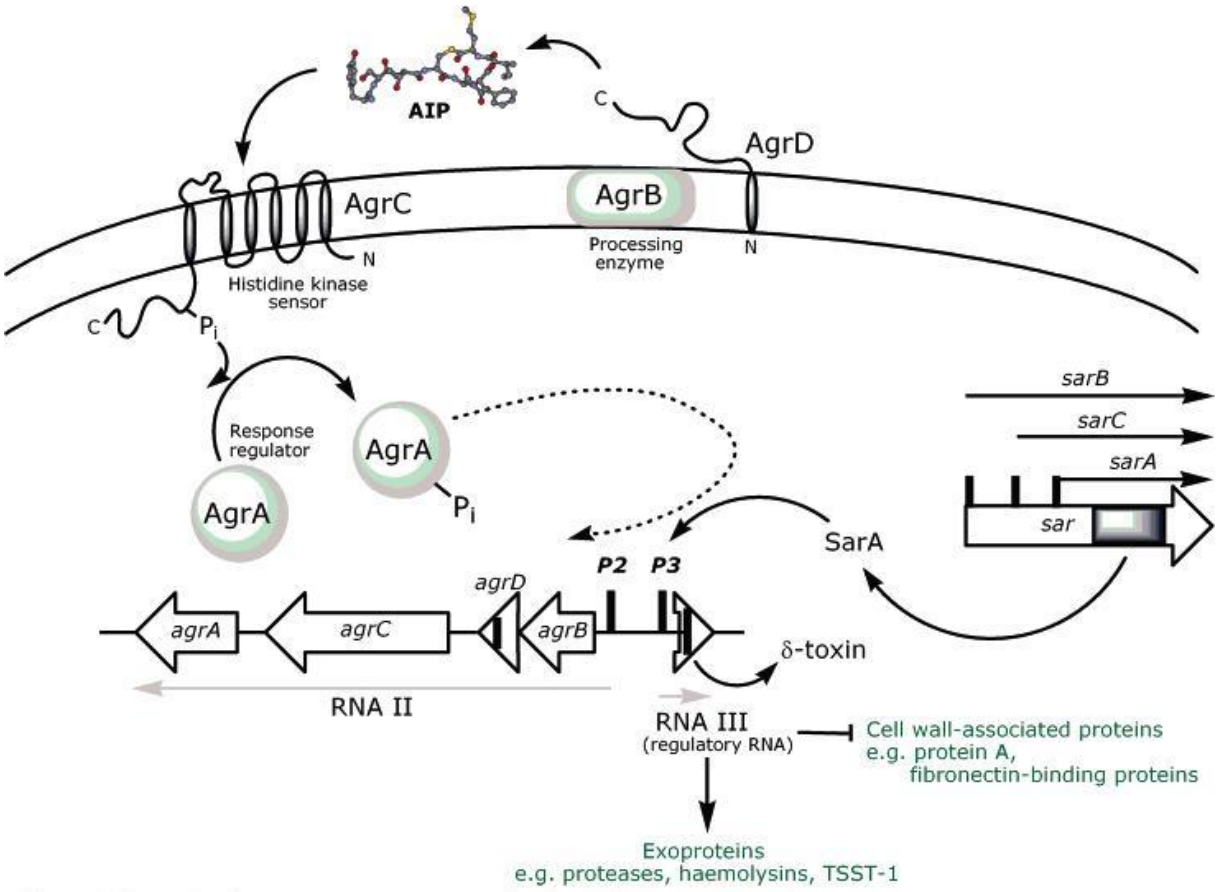



**Figure 3: Schematic representation of the *agr* system of *S. aureus***

RNAII transcribes 4 genes, *agr*BDCA involved in signal transduction and regulation of virulence factors. Two of these genes, *agr*A and *agr*C encode the two-component system, sensor histidine kinase AgrC and response regulator AgrA that responds to autoinducing peptide (AIP). The *agr*D gene encodes a cell-to-cell communication system known as quorum sensing in Gram-positive bacteria.

The activation of the TCS, AgrCA, induces the transcription of 0.5 kb RNAIII transcript leads to the activation of AIP [165]. RNAIII also functions as a regulatory effector molecule for the TCS through translational repression of the virulence gene inhibitor Rot, which represses expression of toxins in *S. aureus* [166]. Moreover, RNAIII encodes δ-toxin which is a 26 aa peptide that inserts into and disrupts cell membranes [167]. In addition, the δ-toxin has been shown to prevent biofilm formation and stimulate oxidative burst of neutrophils [168]. Also, low *agr* activity is required for optimal biofilm development in *S. aureus* [169].

### 2.1.5.4 *Staphylococcus aureus* exoprotein (Sae)

The SaeRS system was first described by Giraudo in 1994 through the characterization of a transposon *Tn551* insertional mutant in *S. aureus* strain RC106 (66). It is a two-component signaling system which regulates the expression of many virulence factors involved in adhesion, immune evasion and toxicity at a transcriptional level (62). Virulence factors such as FnBPs, Eap, α, β and γ-hemolysins are positively regulated by the *sae*RS system while protein A and the *cap* operon is negatively regulated by *sae*RS (63,77,136,153). The deletion of the *sae* locus decreases the virulence in *S. aureus* an murine soft tissue infection model (Fig. 4) (125).


**Figure 4: Virulence of *S. aureus* in murine soft-tissue infection model.**

Comparison of mice injected with USA300 LAC wild type vs Δ*sae*RS. Ulceration produced day 4 after infection (125).

The *S. aureus sae*RS system is reported to be important for the pathogenesis by transcriptionally up-regulating many virulence genes in response to environmental and host-specific signals (104,123,142). Furthermore, other genes and regulators such as *fur, agr* and *sar* are also required for *sae* activation in most *S. aureus* strains (96,123). So far, *sae* is present in all strains analysed. However, the level of transcription of the individual transcripts is strain-dependent (61,136). Recently Mrak *et al*. reported the *sae* system to be implicated in the regulation of protease production and promoting biofilm formation as well (119).

The *sae* operon which codes for the two-component system SaeRS consists of four genes including *saeP*, *saeQ*, *saeR* (0.687 kb) and *saeS* (1.062 kb) with the latter two genes encoding the two component system SaeRS (153). The two component system consists of the SaeS and SaeR which display a strong homology to a membrane-spanning sensor histidine kinase and a cognate cystolic response regulator, respectively (115). The N-terminal of the response regulator, SaeR, contains an aspartate phosphorylation site which is conserved in bacterial response regulators (114). The C-terminus of the sensor histidine kinase, SaeS, contains an auto-phosphorylated histidine residue and two transmembrane domains found in bacterial sensor proteins (116). The function of *sae*P and *sae*Q has not been fully elucidated; however, they have a predicted to express a lipoprotein (SaeP) and a membrane protein (SaeQ), respectively (94). Although, the function of these two proteins has not been fully described, one study has reported that SaePQ is not required in the signaling of theTCS, SaeRS (94). In addition, two promoters, P1 and P3 are located upstream of *saeP* and *saeR* (central to *saeQ*), respectively producing 4 overlapping transcripts which include the primary transcript, T1 (3.0 kb) which initiating from the P1 promoter and terminating downstream of *saeS*, the primary monocistronic transcript, T4 initiated from the P1 promoter terminating upstream of *saeQ*, the primary transcript T3 initiated from the P3 promoter terminates downstream of *saeS* (153). T2 is a product of endonucleolytic cleavage of the primary transcript, T1 by an endoribonuclease RNase Y which has also been reported to have a major role in *S.aureus* gene regulation (113). The T2 transcript also represents the most abundant and stable of the *sae* transcripts (113).

The interaction of the *sae* system with other virulence factors such as *agr* and *sigB* are contradictory, possibly due to strain differences in regulation. However, it has been reported that *sae* is positively regulated by the *agr* and the *sar*A global regulators whilst SigB and Rot represses *sae* expression (61,108,123). In particular, *S. aureus* strain Newman shows an elevated constitutive *sae* expression due to a substitution of an amino acid (L18P) within the N-terminal of the first membrane-spanning domain of the SaeS protein (61,153). The mutation in the sensor histidine kinase results in overproduction of *sae*R target genes such as coagulase, CHIPS and Eap (112,142). However, the mutation of SaeS does not affect the expression of *agr*, *sar*A, *sig*B (68,108). The P1 promoter has a 2-30 times higher activity than

the P3 promoter and is activated by sub-inhibitory concentrations of antibiotics (β-lactam, florfenicol and mupirocin), salicylic acid, $H_2O_2$ and α-defensins (6,27,61,103). On the other hand, environmental stressors such as low pH, high NaCl concentrations repress the *sae* system (124). In one study, the global regulator Fur was shown to be required for the induction of *sae* in low-iron condition (96).

The phosphorylated response regulator, SaeR, recognizes two binding sites on the P1 promoter (125). Two distinct groups of *sae*R target genes have been identified: class I and class II. Class I genes include *coa, fnbA, eap* and the *sae* P1 promoter (SaePQ) that require highly phosphorylated SaeR, whereas class II target genes such as *hla* and *hlb* were activated by low basal phosphorylation activity (94,112). Since the *sae* P1 promoter was reported to elicit 2-30 times phosphorylation activity when compared to the *sae* P3 promoter, class II target genes could be activated by only P3 promoter (112). Hence, the P1 promoter which transcribes *sae*PQ is thought to be dispensable in *sae* signaling. In addition to SaeP and SaeQ are not required in SaeRS signaling, Jeong *et al*., showed that when present, SaeQ stabilizes the sensor histidine kinase, SaeS in strain Newman (94). Also, *Jeong et al*., showed that SaePQ form a protein complex with SaeS and suppress SaeRS mediated signaling by dephosphorylation of the response regulator SaeR (93). Furthermore, Jeong *et al*., could demonstrate by microarray analysis that the deletion of SaePQ results in higher transcription levels of *sae* target genes (93).

## 2.2 Objectives of this study

Previously, we have shown that SDS-containing sub-inhibitory concentrations of biocides (Perform®) and SDS alone activate *sae* transcription and increase cellular invasiveness in *S. aureus* strain Newman through enhanced Eap production (142). The effect is associated with an amino acid exchange in the N-terminus of SaeS (L18P), specific to strain Newman, while most other *S. aureus* strains carry a leucine residue ($SaeS^L$) at this position. The sensor histidine kinase, SaeS, along with its response regulator, SaeR are encoded by the *sae*PQRS operon. The involvement of the upstream region *sae*PQ in the the two component system has not been fully elucidated. Using strain Newman and SDS stress as a model, we examine the possible involvement of the *sae*P and *sae*PQ region in SDS-mediated *sae*/*eap* expression and Eap-dependent host cell invasion by real-time qRT-PCR/Western or Northern blot and flow cytometry analysis, respectively. More importantly, we investigate whether SDS-induced *sae* expression and Eap-dependent host cell invasion is common among *S. aureus* strains without the SaeS point mutation.

In summary, these results may help to further understand the virulence mechanism existing in the laboratory adopted strain Newman and other clinical *S. aureus* strains. Strain Newman has a high virulence potential compared to several laboratory and clinical *S. aureus* strains, as observed by systemic murine challenge (80). This high virulence may, at least partially, be attributed to the SaeS point mutation exisiting in strain Newman. Therefore, investigation of the other *sae* components could help to further clarify *S. aureus* virulence mechanisms and their regulation.

# 3. Materials and Methods

## 3.1 Bacterial strains, Plasmids and Oligonucleotides

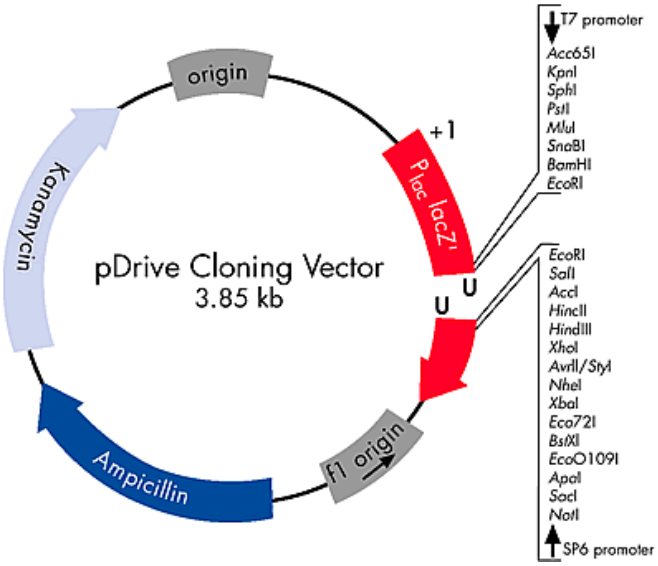
**Table 1: Bacterial strains used in this study**

| Strain or plasmid | Description | Reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| TOP10 | Competent *E. coli* for plasmid transformation | Invitrogen |
| *S. aureus* | | |
| RN4220 | Restriction-deficient *S. aureus* strain, $r^-$ | (102) |
| Newman | Wild type | (55) |
| Newman-29 | Newman, Δ*sae::kan* | (61) |
| Newman-31 | Newman, Δ*saeP::kan* | C. Wolz, Iniversity of Tübingen, Germany |
| NewmanHG | Newman, with $SaeS^L$ from strain RN1 | (112) |
| ISP479C | 8325-4 derivative, with $SaeS^L$ allele | (129) |
| ISP479C -29 | ISP479C, Δ*sae::kan* | (61) |
| ISP479C -31 | ISP479C, Δ*saeP::kan* | C. Wolz, University of Tübingen, Germany |
| 6850 | Wild type | (17) |
| ATCC29213 | MSSA reference strain | DSMZ, Germany |
| LAC | CA-MRSA (USA300) | (50) |
| MW2 | CA-MRSA (USA400) | (14) |
| ST239-635/93 R | MRSA SCC*mec* type III strain | This study |
| ST239-635/93 W | MRSA SCC*mec* type III reference strain | F. Layer, Robert Koch Institute Wernigerode, Germany |
| ST239-635/93 THW89 | MRSA SCC*mec* type III isolated from skin & soft tissue infection | W. Oosthuysen, Tygerberg Hospital, South Africa |
| ST239-635/93 THW99 | MRSA SCC*mec* type III isolated prosthetic device associated infection | W. Oosthuysen, Tygerberg Hospital, South Africa |
| ST239-635/93 465 | Zoonotic isolate | Knut Ohlsen, University of Würzburg, Germany |
| ST239-635/93 966 | Zoonotic isolate | Knut Ohlsen, University of Würzburg, Germany |
| Cowan I | Wild type | ATCC 12598 |
| *S. carnosus* | | |
| TM300 | Wild type | (132) |
| Newman-SDM-SaeP | Newman, Δ*sae::* pCWSAE148 | This study |
| ISP47C-SDM-SaeP | ISP479C, Δ*sae::* pCWSAE149 | This study |

**Table 2: Plasmids used in this study**

| Plasmid | Description | Reference/Source |
|---|---|---|
| pCWSAE33 | pCL84 with *sae*PQRS from Newman | (112) |
| pCWSAE28 | pCL84 with *saePQRS* from ISP479C | (112) |
| pCWSAE42 | pCL84 with saeRS from Newman | Tobias Geiger, University of Tübingen, Germany (unpublished) |
| pCWSAE47 | pCL84 with *sae*RS from ISP479C | (61) |
| pCWSAE148 | Mutation in the Shine-Dalgarno box of SaeP in $saePQRS^{P}$ (based on pCWSAE33) | This work |
| pCWSAE149 | Mutation in the Shine-Dalgarno box of SaeP in $saePQRS^{L}$ (based on pCWSAE28) | This work |
| pMad | Vector for allelic replacement | (12) |

**Map: TA cloning vector:**
http://www.ebiotrade.com/buyf/productsf/qiagen/fig_pDrive_Cloning_Vector.gif




**Map: *sae*PQRS plasmid vector: (106)**

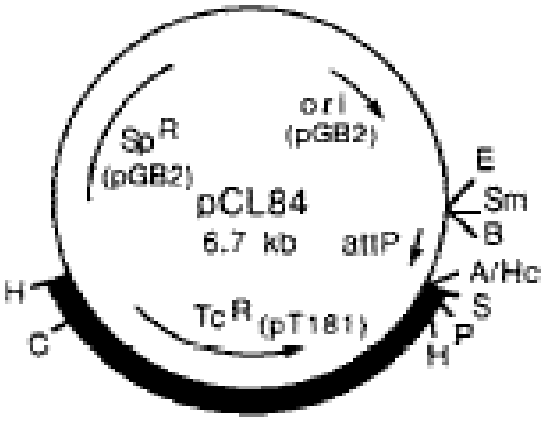



**Figure 5: Vector maps of selected plasmids used in the study.**

**Table 3: Oligonucleotides used in the study**

| Primer | Sequence (5'→ 3') |
|---|---|
| qRT-PCR | |
| sae1F | AAACTTGCTTGATAATGCGCTAAA |
| sae1R | GTTCTGGTATAATGCCAATACCTTCA |
| eap1F | AAGCGTCTGCCGCAGCTA |
| eap1R | TGCATATGGAACATGGACTTTAGAA |
| gyrB 1F | TTAGTGTGGGAAATTGTCGA |
| gyrB1R | CCGCCGAATTTACCACCAGC |
| fnbAF | TGCAATACGACAGATACTT |
| fnbAR | TTGGCCACCTTCATAACCTA |
| *sae* sequencing | |
| Sae1up | TTATTGTGGCAAAAGGTTTA |
| Sae1dw | ATTATTAGGCGGCATACAG |
| DIG labeled probes | |
| sae1980-for | TGGTCACGAAGTCCCTATGC |
| sae2458-rev | TGCTTGCGTAATTTCCGTTAG |
| Map w 98 (eap) | AATAATAATGAAGCGTCTGC |
| Map w 650 (eap) | CGGTAATACCTCTATTTGATT |
| Site directed mutagenesis | |
| SaeP amplification: saeP-up | GACCCCTATTTATTTAAATC |
| saeP-dw | TTATTTTAATTTAGCGCC |
| Shine-Dalgarno mutagenesis: saeSD-up | GAAGAAATTATCAGTTAGCATGAATAC |
| saeSD-dw | GTATTCATGCTAACTGATAATTTCAATTTG |
| | |

## 3.2 Chemicals and disposables

Chemicals used in this study were purchased from different companies including:
Applichem, Amersham Bioscience, Invitrogen, GE Healthcare, New England Biolabs, Thermo Fischer Scientific, Sigma Aldirich, Merck, Riedel-de Haen, , Roche , Roth, Applied Bioscience, Life technologies and Biorad.

Disposables used in this study were purchased mainly from these companies:
Sarstedt, Millipore, A. Hartenstein, BD Bioscience, Schubert and Weiss, Greiner Bio-One, MP Biomedicals and Biorad.

**Table 4: List of equipment used in the study**

| | |
|---|---|
| ABT12-5DM Balance | Kern, Germany |
| Autoclave MM Selectomat 2000 | Holzner, Germany |
| Automatic Micropipette | Gilson, Epperndorf; USA |
| BD FACSCalibur™ Flow cytometer | Becton Dickinson, Germany |
| Biorevo-BZ9000 Fluorescence microscope | Keyence, USA |
| Clean bench | Hera safe, Heraeus |
| $CO_2$ incubator INCOmed | Memmert, Germany |
| Electrophoresis chamber Mini-Protean III | Biorad, Germany |
| Electrophoresis power supply | Biorad, Germany |
| EPI2500 gene pluser (electroporator) | Fischer, Germany |
| Fast-Prep 24 shredder | MP Biomedicals, USA |
| Incubator | Heraeus, Germany |
| L46 Vortexer | Labinco, Netherlands |
| LaminAir HB 2472 | Dietma Müller labotchnik, Germany |
| Microarray system | Agilent Technologies, USA |
| Microtiter plates, Costar 24 Well Culture Cluster | Corning Inc, NY, USA |
| Microwave | Sharp, Japan |
| ND-100 Nanodrop spectrophometer | Thermo Fischer Scientific, Germany |
| Olympus light microscope | Olympus, USA |
| PCR thermocycler | T3, Biometra, Germany |
| pH Electrodes and pH meter | Van-London Phoenix, USA |

| | |
|---|---|
| Phero-Shaker | Biotec-Fischer, Reiskirchen |
| Refrigerator | Liebherr, Germany |
| Rotilabo mini-centrifuge | ROTH, Germany |
| Seesaw shaker | Desaga-Sarstedt Group, Germany |
| Certomat® H Shaking incubator | Sartorius, Germany |
| Speedvac Plus SC110A | Thermo Fischer Scientific, Germany |
| StepOnePlus™ Real-Time PCR System | Applied Biosystems, USA |
| Thermobil heating block | Liebisch, Germany |
| Turboblotter, Northern blot capillary transfer | Whatman® Scheicher & Schuell, |
| U-2000 Spectrophotometer | Hitachi, USA |
| UV camera and UV imager | Herolab, Germany |
| Waterbath | Memmert, Germany |
| Western blot apparatus | Bio-Rad, Germany |

**Table 5: Reaction kits and enzymes**

| Reagent/Enzyme | Manufacturer |
|---|---|
| 5'/3' RACE Kit, 2nd Generation kit | Roche, Germany |
| CSPD chemiluminescence substrate | Roche, Germany |
| Cy3-dCTP and Cy5-dCTP | GE Healthcare, UK |
| CyScribe GFX Purification kit | GE Healthcare,UK |
| DIG Wash and Block Buffer set | Roche, Germany |
| dNTPs | Invitrogen, USA |
| First Strand Buffer | Invitrogen, USA |
| Microarray random primers | Promega, USA |
| PCR DIG probe Synthesis kit | Roche, Germany |
| PHUSION polymerase and buffer | NEB, USA |
| Pierce® ECL Plus Western blotting substrate | ThermoFischer Scientific, USA |
| QAIprep Spin Miniprep kit (plasmid extraction) | Qaigen, Germany |
| Qiagen- PCR cloning kit | Qaigen, Germany |
| Qiagen-RNeasy Mini Kit | Qaigen, Germany |
| QIAquick PCR-purification kit | Qaigen, Germany |
| QIAquick gel-extraction kit | Qaigen, Germany |

| | |
|---|---|
| QuantiTect Reverse Transcription kit | Qaigen, Germany |
| Restriction enzymes and buffers | NEB, USA |
| RNaseH | Invitrogen, USA |
| RNasin® Plus RNase Inhibitor | Promega, USA |
| SuperScript II Reverse Transcriptase | Invitrogen, USA |
| SYBR Green PCR Master Mix | Applied Biosystems, Lifetechnologies, USA |
| T4-DNA Ligase and buffer | NEB, USA |
| Terminator 5'-phosphate-dependent exonuclease | Epicenter Technologies, |
| Tobacco Acid Pyrophosphatase (TAP) | Epicenter Technologies |
| Two-Color RNA Spike-in kit | Agilent Technologies, USA |
| Proteinase K | Qaigen, Germany |
| RNase-free DNase set | Qaigen, Germany |
| HyperLadder I | Bioline, UK |
| Expand High Fidelity system | Roche, Germany |

## 3.3 Buffers, media and solutions

Preparation of all solutions in this study was done using pyrogen free Ampuwa-Water (Fresenius Kabi, Germany).

Dulbecco's PBS (Life Technologies, CA, USA) was used in this study.

### 3.3.1 Media

| | |
|---|---|
| Trypsic soy broth (pH 7,3) | 17 g/L Tryptone |
| | 3,0 g/L Soytone |
| | 2,5 g/L glucose |
| | 5,0 g/L Sodium Chloride |
| | 2,5 g/L Dipotassium Hydrogen Phosphate |
| Mueller Hinton Broth | 17,5 g/L Acid Hydrolysate of Casein |
| | 3,0 g/L Beef extract |
| | 1,5 g Starch |

### 3.3.2 Antibiotics

**Table 6: List of antibiotics used in the study**

| Antibiotic | Stock solution concentration (mg/ml) | Dissolved in | Final concentration (µg/ml) |
|---|---|---|---|
| Ampicillin | 100 | $dH_2O$ | 100 |
| Chloramphenicol | 10 | Ethanol | 10 |
| Erythromycin | 10 | Ethanol | 10 |
| Kanamycin | 10 | Ethanol | 50 |
| Tetracycline | 10 | 70% ethanol | 10 |

## 3.4 Phenotypic methods

### 3.4.1 Bacterial culture maintenance and growth

*Staphylococcus aureus* strains (Tab. 1) were stored as frozen stocks in 70% glycerol at -80$^{o}$C. To grow the cultures, sterile stabs were plated on blood agar plates and incubated overnight at 37$^{o}$C. After confirmation of culture purity, single colonies were inoculated in 20ml TSB (BD, NJ, USA) in 50 ml PPN tubes (Falcon, BD) and incubated at 37$^{o}$C overnight while shaking at 200 rpm. For DNA extraction, *S. aureus* strains were grown in 20 ml MHB instead of TSB.

### 3.4.2 Determination of minimum inhibitory concentration to SDS

The MIC for each *S. aureus* strain to SDS was determined by modified two-fold serial dilution assay according to the CLSI guidelines (162). The micro-dilution assay was performed in 96-well microtitre round bottom plates by adding 100 µl of TSB to all the plate wells. Subsequently, 100 µl of 1% SDS was added to the first well of the row and two-fold serial diluted in all wells of the row except the last well, which was used as a negative control. After the dilution of SDS, 100 µl $OD_{600}$ 0.05 was added to each well. The plate was the incubated at 37$^{o}$C overnight to grow the bacteria. The following day the MICs were determined as wells with no growth (clear TSB) compared to the negative control.

### 3.4.3 Growth curves

After determination of MICs, SDS at 30% of the MIC (0.0046% SDS) was used as the induction concentration of *S. aureus* strains. Hence, growth curves were determined by hourly measuring the OD as an indicator of *S. aureus* growth in TSB with SDS 30% MIC for 8 h. Hourly measurements of optical density were taken with a spectrophotometer at 600 nm. When the OD was above 1.0, bacterial cultures were diluted 1:10 in TSB before measuring.

## 3.5 Molecular biology methods

### 3.5.1 DNA extraction

To isolate genomic DNA, specific *S. aureus* strains were grown overnight in Mueller-Hinton broth. The following day, 2 ml of overnight culture was centrifuged at 13 000 rpm for 5 min. The supernatant was then discarded and the pellet was resuspended in 600 µl TNE buffer. Subsequently, 20 ug/ml was added to the suspension and incubated at 37$^{o}$C for 30 min. Following incubation, the suspension was boiled for 10 min. After lysing the bacterial cells, 10 µl of proteinase K was added and reaction was incubated at 55$^{o}$C for 1 h. Equal volumes of phenol:chloroform:isoamylalcohol (25:24:1) was added. The suspension was centrifuged at 13 000 rpm for 5 min. Subsequently, the upper was transferred to a new tube was centrifuged and was mixed with equal volume of chloroform:isoamylalcohol (24:1). The suspension was centrifuged at 13 000 rpm for 15 min and the supernatant was transferred to a new tube. The suspension was mixed with 0.3 M sodium acetate. Precipitation was performed by adding equal volumes of ice-cold absolute ethanol and incubated overnight at -20. Following precipitation, the DNA pellet was washed twice with 70% ethanol. The DNA pellet was dried by inverting the tubes to dry and resuspended in 100 µl of $dH_2O$.

### 3.5.2 Plasmid extraction

The extraction of plasmid from *E. coli* was performed using a QIAprep Mini kit. In short, single colony of bacteria was inoculated in MHB overnight. The following day, 5 ml of the culture was pelleted at 13 000 rpm for 5 min. Isolation of plasmid was performed following manufacturer's instructions.

### 3.5.3 Agarose gel electrophoresis

To confirm the presence of DNA, agarose gel electrophoresis was used. To prepare the agarose gel, the following components were used in a 500 ml for a 1.5% gel; 4.5 g agarose and 300 ml Tris-acetate-EDTA (TAE) buffer. The mixture was boiled in a microwave for 7 min and then poured to solidify in appropriate gel trays with combs. After cooling at room temperature (~25$^{o}$C), the gels were placed in gel tanks containing 1X TAE buffer. To analyze samples, 10 µl of the sample was mixed with 1 µl of loading buffer and loaded in the gel wells for separation. Electrophoresis of nucleic acid was performed at 120V for 1 h. The gel was then stained in ethidium bromide solution and visualized under UV light.

### 3.5.4 Excision of DNA fragments from agarose gels

To isolate and purify DNA fragments from agarose gels, a QIAquick gel extraction kit (Qaigen, Germany) was used. Briefly, the DNA fragment of interest was excised from the gel using a sterile scapel. The gel piece was placed in a clean 2 ml epperndorf tube. A total of 700 µl of buffer QG was added to the tube and incubated at 50$^{o}$C for 15 min with repeated vortexing until the gel has dissolved. After dissolving the gel piece, 700 µl of isopropanol was added to the tube and mixed thoroughly. The sample was then loaded onto the QIAquick spin columns for binding and elution of pure DNA. Following the loading of the sample, the rest of the procedure was performed according to the manufacturer's protocol and the DNA was eluted in the elution buffer provided with the kit or with $dH_2O$.

### 3.5.5 Polymerase chain reaction

Amplification of target genes was performed by PCR and purified accordingly. Two main PCRs were used in this study including, the standard PCR to amplify target genes using standard Taq DNA polymerase with standard buffer (NEB, Massachusetts, USA) and the amplification of cloned PCR products which require high fidelity proof-reading PHUSION Taq polymerase (NEB, USA).

#### 3.5.5.1 PCR amplification of target genes reaction mix

To amplify target genes using standard Taq DNA polymerase, the following reaction mix was used:

Standard PCR (50 µl):

| | |
|---|---|
| Ampuwa® $H_2O$ | 36.1 µl |
| dNTP (25 mM each) | 0.4 µl |
| 10X ThermoPol-buffer | 10.0 µl |
| Oligonuclotide 5' (10 µM) | 1.0 µl |
| Oligonuclotide 3' (10 µM) | 1.0 µl |
| DNA template (20-50 ng) | 1.0 µl |
| Taq DNA polymerase | 0.5 µl |

Cycling conditions:

| | | | |
|---|---|---|---|
| Initial denaturation: | 95°C | 2 min | |
| Denaturation: | 95°C | 30 s | } 30 cycles |
| Annealing: | (cf. primers, Tab. 3) | 30s | |
| Extension | 72°C | (1 min/1kb) | |
| Final Extension: | 72°C | 10 min | |

High fidelity PCR (50 µl):

| | |
|---|---|
| Ampuwa® $H_2O$ | 34.5 µl |
| dNTP (25 mM each) | 0.5 µl |
| 10X Phusion-buffer | 10.0 µl |
| Oligonuclotide 5' (10 µM) | 1.5 µl |
| Oligonuclotide 3' (10 µM) | 1.5 µl |
| DNA template (20-50 ng) | 1.0 µl |
| PHUSION DNA polymerase | 0.5 µl |

Cycling conditions:

| | | | |
|---|---|---|---|
| Initial denaturation: | 98°C | 2 min | |
| Denaturation: | 98°C | 20 s | 30 cycles |
| Annealing: | (cf. primers, Tab. 3) | 30s | |
| Extension | 72°C | (15 s/1kb) | |
| Final Extension: | 72°C | 10 min | |

### 3.5.6 Extraction and purification of RNA from *S. aureus*

RNA isolation and purification from *S. aureus* was performed with the RNeasy Mini kit (Qiagen, Germany) with minor modifications following manufacturer's instructions. Briefly, 2 ml of *S. aureus* culture was pelleted by centrifugation at 13 000 rpm for 5 min and the supernatant was discarded immediately after. The remaining pellet was washed with 1 ml of PBS and centrifuged at 13 000 rpm for 5 min. After the washing step, the pellet was resuspended in 700 µl RLT buffer. The suspension was transferred to 0.1 mm silica spheres lysing matrix tubes (MP Biomedicals Ohio, USA) and lysed by mechanical disruption in with the Fastprep-24 (MP Biomedicals Ohio, USA) at 6500 rpm for 45 sec. Following disruption of the bacterial cells, the supernatant was transferred to a 2 ml tube and centrifuged at 13 000 rpm for 10 min at 4°C. The supernatant was transferred to a new 1.5 ml tube and equilibrated with 70% ethanol. The mixture was then transferred to an RNeasy column and from this step on the manufacturer's instructions were followed. mRNA was resuspended in RNase free water provided with the RNeasy Mini Kit.

### 3.5.7 cDNA synthesis

Reverse transcription of *S. aureus* mRNA was performed with the Quantitect RT-PCR kit (Qiagen, Germany) following manufacturer's instructions. The cDNA was then used to the expression of target genes by real-time PCR. The following modified manufacturer's protocol was used:

Elimination of genomic DNA

| | |
|---|---|
| mRNA | 1.0 µg |
| gDNA Wipeout buffer | 2.0 µl |

Add $dH_2O$ to final volume of 14 µl and incubated at 42°C for 2 min

Following DNA elimination, the mRNA was placed on ice and reverse transcribed with the following protocol:

RT-PCR

| | |
|---|---|
| Quantiscript Reverse Transcriptase | 1.0 µl |
| 5X Quantiscript RT buffer | 4.0 µl |
| RT Primer mix | 1.0 µl |
| mRNA | 14.0 µl |

The reaction was incubated at 42$^{o}$C for 30 min. Subsequently the RT-PCR was deactivated by incubation at 95$^{o}$C for 3 min. For qRT-PCR analysis, the reaction was diluted 10X in RNase free $H_2O$ provided with the kit.

### 3.5.8 Quantitative RT-PCR

Subsequent to reverse transcription of total mRNA, target gene expression was quantified using real-time PCR analysis using the SYBR Green PCR Master Mix (Applied Biosystems; Warrington, UK) following manufacturer's protocol performed with the 7300 Fast System Software (Applied Biosystems, Warrington, UK). In short, 6 ul of cDNA was added to 14 ul mastermix. The reaction was performed in triplicate in a 96-well plate. Thermal cycling, amplification and detection were performed with the StepOnePlus$^{TM}$ Real-Time PCR system (Applied Biosystems; Warrington, UK) using the following cycling conditions:

**Table 7: Real-time PCR cycling conditions**

| Step | T$^{o}$C | Time | Number of Cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 5 min | 1 |
| **Denaturation** | 95 | 20 s | 35 |
| **Annealing** | 60 | 20 s | 35 |
| **Extension** | 72 | 30 s | 35 |
| **Melting curve** | 95 | 20 s | 1 |
| | 45 | 60 s | 1 |
| | 95 | 10 s | 80 |
| **Cooling** | 40 | 30 s | 1 |

For gene expression, *gyrB* was used as an endogenous control for all target gene quantification. Data analysis was performed with the 7300 Fast System Software. Expression of target genes was calculated by the $\Delta\Delta C_T$ method with the expression of Newman WT strain as a reference and presented as fold change (144).

### 3.5.9 Microarray analysis

For transcriptional profiles of *S. aureus* strains with SDS stress conditions, microarray analysis was performed at the University of Greifswald (Ulrike Mäder, Uwe Völker). Fluorescently labeled cDNA synthesis and purification were performed as previously described with minor modifications (60). Briefly, 10 µg of mRNA was mixed with random primers and Spike-ins (Agilent Technologies, CA, USA). The RNA/primer reaction was incubated at 70$^{o}$C for 10 min and subsequently placed on ice for 5 min. After, 10 µl of 5X First Strand Buffer, 5 µl of 0.1 M DTT, 0.5 µl of dNTPs (10 mM dATP, dGTP, dTTP, 2.5 mM dCTP), 1.25 µl of Cy3-dCTP/Cy5dCTP and 2 ul of Superscript II reverse transcriptase (Invitrogen, USA) were added. The reaction was incubated at 42$^{o}$C for 1 h and then heated at 70$^{o}$C for 10 min and subsequently placed on ice for 5 min. Then, RNA was degraded with 2 units of RNaseH at room temperature (RT) for 30 min. Purification of fluorescently labeled cDNA was performed using the CyScribe GFX Purification kit.The reference pool was Cy3 labeled and the samples were labeled with Cy5. Following cDNA labeling, 200 ng of each Cy3 and Cy5-labeled cDNA were hybridized together to microarray following Agilent's instructions and protocol (Two-Color Microarray-based Gene expression Analysis, version 5.5).

### 3.5.10 Northern blotting

The total RNA from *S. aureus* culture was isolated and purified by the Qaigen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. mRNA was further quantified by the Nanodrop at A260/A280 and the quality was visualized on a 1.5% MOPS/formaldehyde agarose gel by ethidium bromide staining. Northern blot analysis was performed as previously described [27].

Briefly, 2 µg of mRNA was loaded on 1% MOPS/formaldehyde agarose gel and ran for 3 hours at 37 volts for 3 h. After, the presence of RNA was determined by visualization under UV light. The RNA was then transferred and blotted onto a positively charged nylon

membrane by alkaline transfer using the turbo-blotter rapid transfer system (Fig. 6). Subsequently, RNA was cross-linked with the membrane with a UV cross-linker at 1200 kJ for 30 s. The blot was gently washed in 2X SSC for 5 min to remove the high salt content.


Figure 6: Turbo-blotter rapid transfer system(http://www.gelifesciences.co.jp/catalog/1500.asp)

Probes for detection of the target genes were synthesized using the PCR DIG Probe Synthesis Kit (Roche, Switzerland) following the manufacturer's instructions with minor modifications (adjusting the annealing temperature). The specific primers used for the generation of probes are listed in Tab. 3. The probes were diluted in pre-warmed hybridization buffer (1:10000). The blot was warmed in hybridization buffer at 64$^{o}$C for 30 min. Hybridization of the blot was performed at 68$^{o}$C overnight. The following day, the blot was washed twice in 2X SSC/0.1% SDS at RT for 5 min and washed twice once more in 0.2X SSC/0.1% SDS at 68$^{o}$C for 15 min. After stringent blot washes, the blot was further washed and blocked with the DIG Wash and Block Buffer set according to manufacturer's instructions. The labeled probes were detected by anti-digoxigenin antibody conjugated to alkaline phosphatase and detected by CSPD chemiluminescence substrate. Detection was performed on X-ray film (Fujifilm, Tokyo, Japan) with automated development in a Curix 60 (Agfa, Greenville, USA).

### 3.5.11 Tobacco acid pyrophosphatase (TAP) based 5'-RACE (rapid amplification of complementary DNA ends)

To analyze the transcriptional start sites for the *sae* promoters (P1 and P3) in our strains and to differentiate between primary and processed *sae* transcripts, the TAP based 5' -RACE assay was utilized as previously described with minor modifications [30, 31]. First 10 µg RNA-treat with 1 unit of terminator 5'-phosphate-dependent exonuclease (Epicenter Technologies) to eliminate the monophosphate group in a total volume of 20 µl and incubated at 37$^{o}$C for 30 min. Subsequently, 5 units of TAP (Epicenter technologies) and 40 units RNasin (Promega, Madison, USA) were added and incubated at 37$^{o}$C for 60 min. Control RNA was incubated under the same conditions without TAP treatment. The reactions were terminated by phenol extraction and ethanol precipitation and RNA pellet in was resuspended 20 µl RNase free water. Following the TAP treatment, 500 pmol of 5' RNA adapter and 120 units of T4 RNA ligase (NEB, Ipswich, UK) were added and incubated for 1h at 37$^{o}$C. The RNA was reverse transcribed into cDNA Omniscript RT-PCR kit and PCR amplification with Phusion Taq High Fidelity DNA polymerase (NEB, Ipswich, UK) was performed using a second 3' gene specific primer and RNA oligo primer specific for the RNA adapter. The PCR product was sequenced to determine the TSS.

### 3.5.12 Site directed mutagenesis

To introduce specific mutations in the Shine-Dalgarno sequence of *saeP*, plasmid vector, pCL-55 carrying the entire *sae* operon was amplified using primers listed in Tab. 3. Mutation sequences of the primers are underlined. After amplification, 1 µl of *DpnI* (NEB, Ipswich, USA) was added to the reaction to digest methylated DNA. The remaining mixture contained only the mutated plasmids. Plasmids were transformed into *E. coli*. Transduction into a complete *sae* knockout mutant of Newman and ISP479C backgrounds was performed in the group of C. Wolz (University of Tübingen).

### 3.5.13 TA cloning

Cloning of PCR products was performed with the Qiagen PCR cloning kit (Qaigen, Germany). To clone blunt-ended PCR products, the Phusion Taq DNA polymerase (NEB, USA) was used; first the A overhangs were added to 3'- ends of the products to increase ligation efficiency

with the pDrive vector. After purifying the PCR products to remove the Phusion Taq, the following protocol was used to prepare the cloning product:

Adding A overhangs:

| | |
|---|---|
| dATPs (10 mM) | 1.0 ul |
| 10X ThermoPol-buffer | 1.0 µl |
| PCR product | 5 µl (DNA varies according to size, i.e. for 1kb: 0.1-1 µg) |
| Taq DNA polymerase | 0.2 µl |
| Add dH2O to | 50.0 µl and incubate at 72$^{O}$C for 20 min. |

The final product was immediately ligated with the pDrive cloning vector as A overhangs gradually deteriorate with storage. For an efficient ligation mix, the following protocol was followed:

Ligation reaction mix:

| | |
|---|---|
| PCR product (A overhangs) | 4.0 µl |
| pDrive Cloning Vector (50 ng/µl) | 1.0 µl |
| Ligation Master Mix, 2x | 5.0 µl |
| Add $dH_2O$ to | 10.0 µl |

Incubate at 4$^{O}$C for 1 h and store until further use.

### 3.5.14 Transformation of *E. coli*

A transformation procedure was performed using standard heat-shock protocol (140). Briefly One Shot® TOP10 chemically competent cells (Invitrogen, USA) were thawed on ice for 5 min. Subsequently, 5 µl of the ligation mix was added to the cells and the suspension was placed on ice for another 5 min. The cells were heat-shocked in a heating block at 42$^{O}$C for 1 min and immediately placed on ice. After the heat shocking the cells, 200 µl of SOC medium was added to the reaction tube and shaken at 170 rpm for 30 min. The transformation mixture was then plated onto LB agar plates supplemented with X-gal (80µg/ml) and ampicillin (100 mg/ml) and incubated at 37$^{O}$C for 18-2 h for blue/white screening.

## 3.6 Protein biochemistry methods

### 3.6.1 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

For surface protein profile analysis, 5 ml of *S. aureus* culture grown to a desired growth phase was pelleted and washed with 5 ml of PBS. Subsequently, 1 ml of the washed culture ($OD_{600}$ of 1.5) was pelleted and resuspended in 40 µl of laemmli buffer. The suspension was boiled at 100$^{o}$C for 15 min and subsequently centrifuged at 5 000 rpm for 5 min. A total of 8 µl of the supernatant was loaded onto 12% polyacrylamide gel and run at 100 V for 2 hours. Working solutions were as follows:

Polyacrylamide gel composition:

- Solution A:

  Lower-Tris: 50 ml 1M Tris/HCl pH 6.8; 4ml 10% SDS, 46 ml $H_2O$
- Solution B:

  Upper Tris: 75 ml 2 M Tris/HCl pH 8.8; 4ml 10% SDS, 21 ml $H_2O$
- TEMED: N,N,N',N'-tetramethylethane-1,2-diamine
- APS: 16% $(NH_4)_2SO_4$ dissolved in $H_2O$

**Table 8: Separation gel concentrations and volumes**

| **Solution:** | **10%** | **12%** | **15%** |
|---|---|---|---|
| **Solution A** | 2.5 ml | 2.5 ml | 2.5 ml |
| **Gel 30** | 3.33 ml | 4 ml | 5 ml |
| **$H_2O$** | 4.12 ml | 3.45 ml | 2.45 ml |
| **APS** | 50 µl | 50 µl | 50 µl |
| **TEMED** | 5 µl | 15 µl | 15 µl |

**Table 9: Stacking gel concentration**

| Solution | 5% |
|---|---|
| **Solution B** | 125 ml |
| **Gel 30** | 650 µl |
| **$H_2O$** | 3.07 ml |
| **APS** | 25 µl |
| **TEMED** | 15 µl |

### 3.6.2 Silver staining

After electrophoresis, proteins were silver stained as previously described (133). Briefly, the polyacrylamide gel was incubated in a protein fixing solution (50% EtOH, 12% acetic acid, 38% $H_2O$) overnight. The gel was then washed for 20 min in 30% EtOH. Following, the washing step, the gel was incubated in a sensitizing solution (66 µl 37% formaldehyde, 25 µl 43% $Na_2S_2O_3$ in $H_2O$) for 1 min. The gel was then washed three times with $H_2O$ for 1 min. Subsequent to washing, the gel was silver stained (0.2 g $AgNO_3$, 66 µl 37% formaldehyde) for 180 min. Following the silver staining, the gel was washed twice with $H_2O$ for 30 s each. Finally, the gel was incubated in developing solution (50 µl 37% formaldehyde, 1.8 43% $Na_2S_2O_3$, 6 g $Na_2CO_3$, 100 ml $H_2O$) until it was optimally developed and analyzed by GS800 calibrated densitometer. The developed gel reaction was stopped with EDTA solution (13.7 g/l). Identification of proteins was determined previously by liquid chromatography-tandem mass spectrophotometry (142).

### 3.6.3 Western blot

For Western blot analysis, proteins from SDS-PAGE were transferred to a nitrocellulose membrane (Watman, Dassel, Germany) using the Mini Transblot Cell system (Biorad) at 350 mA for 1 h. The membrane was blocked with blocking solution (5% skim milk and 1% Tween) for 1 hour. Subsequent to blocking, nitrocellulose membranes were incubated overnight on a seesaw at $4^oC$ with diluted (1:5000) FnBPA primary antibody. The membrane was then washed three times with washing buffer (25 mM Tris-HCl, 192 mM Glycin, 20% Methanol) for 10 min each. Following the washing step, the membrane was incubated with an anti-mouse immunoglobulin G horseradish peroxidase-conjugated (Jackson Immunoresearch,

Germany) secondary antibody for 1 h on a shaker. The membrane was washed again three times with a washing buffer. Detection was done with an ECL chemiluminescence kit (GE Healthcare, UK) following manufacturer's instructions and exposed to an X-ray film. The X-ray film was developed using an automated developer, Curix 60 (Agfa, Greenville, USA).

### 3.6.4 Storage and cryopreservation of 293 cells

For long-term culture stock storage, human embryonic kidney 293 cells were washed with 5 ml 1x PBS. After the washing step, the cells were detached from the plates with Trypsin/EDTA. The trypsin activity was stopped with DMEM medium, transferred to a 15 ml tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in ice-cold DMSO/HBSS medium (5x the volume of the pellet). The cells were prepared in 1 ml aliquots in sterile cryopreservation tubes. Subsequently, the cells were frozen at -80$^{o}$C overnight. For long-term preservation, the cells were stored in liquid nitrogen.

## 3.7 Cultivation and maintenance of cell lines

### 3.7.1 Cell culture maintenance and seeding

293 cells (DSMZ) were maintained in 75 cm$^2$ tissue culture flasks. The cells were split every 3-4 days. Briefly, cells were grown in DMEM red medium supplemented with 10% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin at 37$^{o}$C and 5$^{o}CO_2$. To split the cells, the old medium was aspirated from the culture flask and the cells were washed with 5 ml 1x PBS. The cells were then detached from the surface of the flask by adding 1 ml Trypsin/EDTA for a few seconds. The cells were aspirated up and down with a 2 ml pipette to break the cell clumps. Subsequently, 9 ml of the DMEM medium was added to stop the trypsinization of the cells. The cells were then diluted resuspended and transferred to a sterile 15 ml falcon tube 1:5 with full medium. The flasks were either incubated at 37$^{o}$C and 5% $CO_2$ or used for seeding of the cells in 24-well plates for invasion assay.

### 3.7.2 Cellular invasion assay

To prepare the cells for bacterial invasion, 293 cells were counted using a Neubauer chamber. Briefly, 10 µl of trypan blue was added with 10 µl of the cell suspension and mixed gently but thoroughly. After mixing the two, 10 µl of the suspension was added to the chamber using a 10 µl pipette. The cells were counted under a light microscope. The cells were counted as the mean count of viable cells per 4 corner squares. The concentration of the cells per ml was calculated as follows:

Number of cells/ml = number of cells x dilution with trypan blue x 1000 (conversion factor of chamber). Cells were then seeded in 24-well plates at a concentration of (3 X $10^5$ cells/well) at 37$^o$C and 5% $CO_2$ for 18 h.

Cellular invasiveness of *S. aureus* strains was determined as previously described with minor modifications [16]. Briefly, 5 ml of *S. aureus* culture with/out SDS as previously described was pelleted and washed with PBS. Subsequent to washing, the bacteria were harvested by centrifugation at 4000 rpm for 5 min at 4$^o$C. The bacteria were labeled with 3 ml of fluorescein isothiocyanate (FITC) in dimethyl sulfoxide for 30 min at 37$^o$C. The bacteria were then washed with 5 ml of PBS and centrifuged for again at 4000 rpm for 5 min. After centrifugation, the pellet was resuspended in 1% human serum albumin-PBS (HSA)-PBS to a final $OD_{540}$ of 1. Preparations of 293 cells (3 X $10^5$ cells/well) were prepared the day before the experiment as previously described. The following day, for bacterial invasion assay, HEK 293 cells were prepared as previously described. Cells were washed with invasion medium (1% HSA; 10 mM HEPES) (Dulbecco's Modified Eagle's Medium; Life technologies, Carlsbad, CA). After washing of the cells, 0.5 ml of the invasion medium was added to the cells. Subsequently, 50 µl FITC-labeled bacteria were added to the HEK 293 cells and were allowed to sediment for 1h at 4$^o$C. Following sedimentation, the culture dishes were incubated at 37$^o$C in for 3 h with 5% $CO_2$. After cellular invasion cells were washed with 1 ml PBS, harvested by trypsinization (trypsin/EDTA, add conc) and treated with monensin (conc final; in EtOH stock) for pH neutralization to eliminate fluorescence quenching. Propidium iodide (conc) (Fluka Biochemicals, Switzerland) was added in order to differentiate between live and dead cells. Cell-associated fluorescence as a marker for cellular invasiveness was measured by using the FACSCalibur (BD; California, USA). Cellular invasiveness of *S. aureus*

strains was determined as percentage relative of invasiveness of the *S. aureus* Cowan I reference strain.

### 3.7.3 Analyis of flow cytometric data

To determine cellular invasiveness, both FITC-labeled bacteria and host cells, which were infected with *S. aureus* were analysed. For all experiments, similar settings were used. To analyse only intact cells, both the cell debris and aggregates were excluded by predefined gates, as follows: Events were measured by forward scatter (FSC) which measures cell size and side scatter (SSC) that measures the granularity of the cells. Using the parameters of the FSC and SSC, the region of interest designated R1 and the corresponding gate were defined as G1 = R1. By applying the Fl-3 channel, region R2 was constructed in order to include propidium negative cells (5 mg/ml PI addition), hence, gate 2 included the R1 and R2 (G2 = R1 + R2). Measurement of host cells infected with FITC-labeled bacteria was performed in the G2 gate and contained cell population, which included R1 and was propidium negative. M1 contained events with a higher Fl-1 signal compared to the negative control (only cells) (Fig. 7).

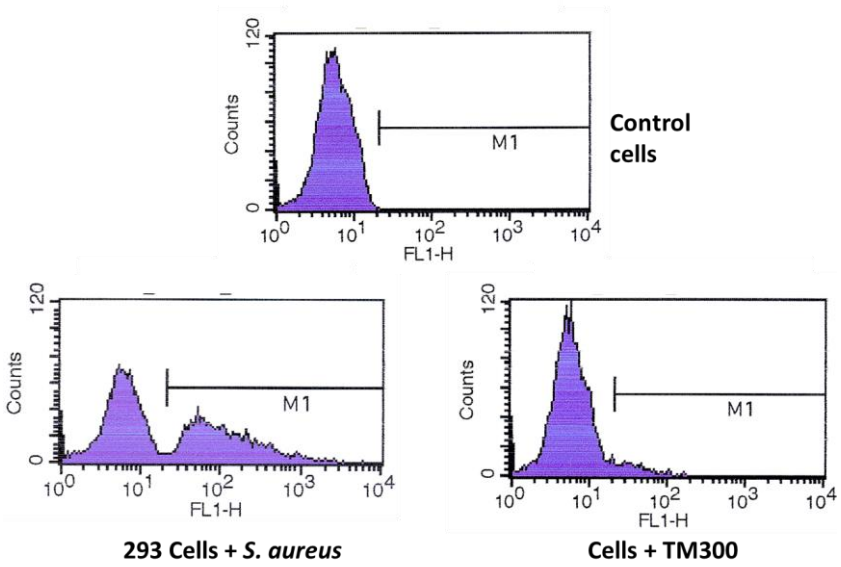



**Figure 7: Flow sytometry histograms of cellular invasion of 293 cells by *S. aureus.***

*S. aureus* strain Newman is compared to non-infected and *S. carnosus* (TM300) infected.

As a parameter for invasiveness, cell-associated Fl-1 height was taken. For this, the non-standard fluorescence units (AFU) were calculated as the product of the percentage of cells in M1 and the average of the Fl-1 height.

Calculation of the AFU was as follows:

$$1\ \text{AFU} = \frac{\text{mean of Fl-1 height for cells x percentage of gated cells in M1}}{\text{mean of Fl-1 height of bacteria x 100}}$$

## 3.8 Computational and statistical analysis

Nucleotide and amino acid comparisons were performed with the Basic alignment search tool for DNA and protein sequences addressed: http://blast.ncbi.nlm.nih.gov/

For multiple alignment of sequence used in this study such as Clustal W sequence alignments and mapping of transcriptional start sites, the BioEdit Sequence Alignment Editor (version 7.0.0) was used.
Prediction of protein domain was done with the PSIPRED Protein Structure Prediction Server software, http://bioinf.cs.ucl.ac.uk/psipred/

Relative gene expression for target genes was performed using the $\Delta\Delta C_T$ method, with the Applied Biosystems 7300 Fast System Software (Applied Biosystems 7300 manual).

i.e. ΔΔCT = ΔCT test sample – ΔCT calibrator sample

Data extraction and processing for microarray analysis was performed using the Feature Extraction software (version 10.5, Agilent Technologies, USA)

Flow cytometric analysis was performed with the BD CellQuest™ Pro version 5.2.

Statistical analyses were performed using a two-tailed, unpaired student's t-test with error bars indicating the standard error of the mean or standard deviation, as indicated. Statistical significance is depicted by asterix compared to untreated control bacteria

# 4. Results

## 4.1 Phenotypic characteristics

Since SDS activation of *sae*RS and *eap* was dependent on the SaeS point mutation harbored by the strain Newman, it was relevant to investigate whether a similar induction could be observed in clinical strains as well. Therefore, several well-characterized strains including the previously discussed ISP479C; the epidemic community-associated MRSA strains USA300-LAC (USA300) and MW2 (USA400); 6850; the MSSA reference strain ATCC29213 and 6 MRSA ST239-635/93 clones were investigated for phenotypic similarities to strain Newman.

### 4.1.1 Determination of the Minimum Inhibitory Concentration

First, the antibacterial activity of SDS by MIC determination of a few selected *S. aureus* strains was performed by the broth microdilution method according to the CLSI guidelines. These strains included Newman WT, Newman Δ*sae*, Newman HG (which represented Newman carries the allele at which the SaeS mutation (L18P) was reverted to (P18L). In addition, the ISP479C, USA300-LAC, 6850, ATCC29213 and 2 ST239-635/93 strains (R and W) were investigated. All selected *S. aureus* strains had MIC values of 0.0156% to SDS.

### 4.1.2 Growth kinetics of *S. aureus* strains in sub-inhibitory concentrations of SDS

To test the effect of sub-inhibitory concentrations of SDS on the growth kinetics of clinical strains, we performed growth curves assays for 8 h. Growth curves were analyzed in strain Newman wild type (WT), Δ*sae*, *sae*S repaired (HG) and selected clinical isolates. A concentration of 30% MIC of SDS (0.0046%) did not significantly affect the growth of *S. aureus* strains (Fig. 8). Concentrations above 0.0046% retarded the growth of *S. aureus* strains, therefore were not investigated further. Interestingly, strain Newman Δ*sae*, ISP479C and the USA300 LAC strains were slightly enhanced by 30% SDS compared to the untreated control. No inhibition on growth was observed in clinical strains.

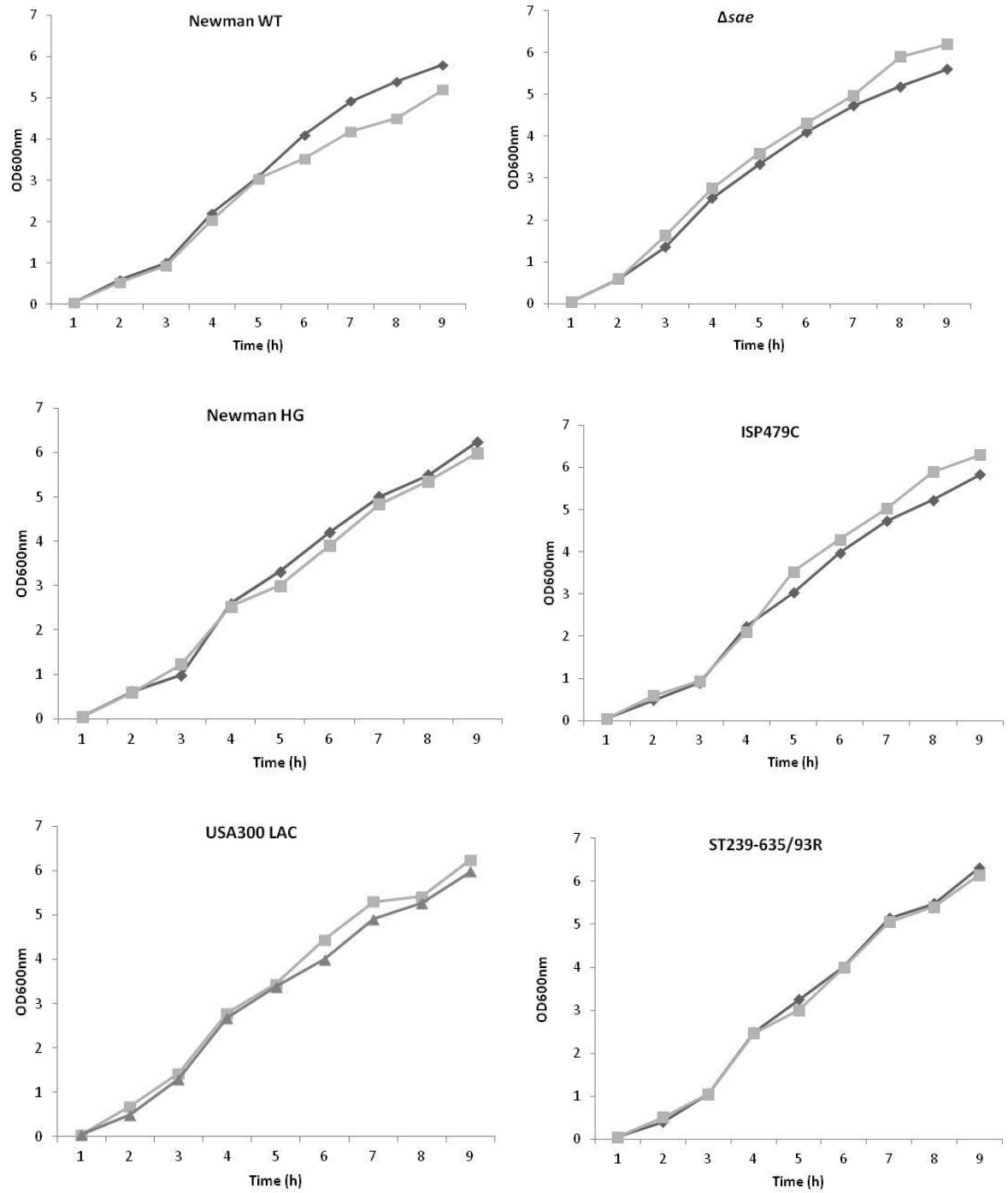



**Figure 8: Growth curves of strain Newman wild-type and other clinical strains in sub-inhibitory concentrations of SDS (30% MIC).**

Strains were grown in TSB supplemented with sub-inhibitory concentrations of SDS (grey line) and without SDS (black line). Growth curves represent one of three independent experiments. (Δ*sae* represents the Newman *sae* mutant strain).

## 4.2 Role of the *sae*RS upstream region *sae*PQ

As the first objective, the *sae*RS upstream region *sae*PQ was investigated for its influence on the two component system *sae*RS. In note, previously, it was shown that the point mutation in the SaeS protein specific for the Newman strain altered the response to SDS stress. The first approach was to generate *sae*P mutants in which chromosomal *sae*P gene was deleted and replaced with kanamycin cassette in both the Newman (carrying the $SaeS^P$ allele) and ISP479C (carrying the $SaeS^L$ allele) backgrounds. The response to SDS of the wild types and their respective mutants were analyzed using different methods. RNA was harvested for *sae*R and SaeR expression by qRT-PCR, which was confirmed by western blot analysis (Fig. 9A and 9B upper panel). The *sae*-dependent invasin, *eap*/Eap was monitored by qRT-PCR (Fig. 9C) and silver stained SDS PAGE, respectively. Furthermore, because Eap is known to mediate cellular invasion of 293 cells, invasion assays were also performed utilizing the wild types and their respective mutants.

### 4.2.1 Effect of the upstream region *sae*P on SDS-mediated *sae*RS activity

SDS stress resulted in a significant activation of *sae*R expression in the Newman strain, but a non-significant decrease in the ISP479C strain (Fig. 9A and 9B). The deletion of *sae*P did not affect the activation or repression of *sae*R in either the Newman or ISP479C strains (Fig. 9A). The expression of SaeR protein was futher confirmed by Western blot analysis.

Next, the study focused on the role of *sae*P on the expression the *sae*-dependent invasion, Eap by silver stained SDS-PAGE analysis (Fig. 9B) and its functionality by cellular invasion assay. As expected, SDS treatment resulted in a significant increase in the expression of *eap* in the Newman WT strain (Fig. 9B lower panel). In the ISP479C, SDS repressed *eap* expression (Fig. 9B lower panel). The deletion of *sae*P did not affect the SDS-induced effect on Eap expression in strain Newman. In the ISP479C Δ*sae*P strain, Eap expression was considerably upregulated by the deletion of *sae*P without SDS stress, however, this was not significant (Fig. 9C).

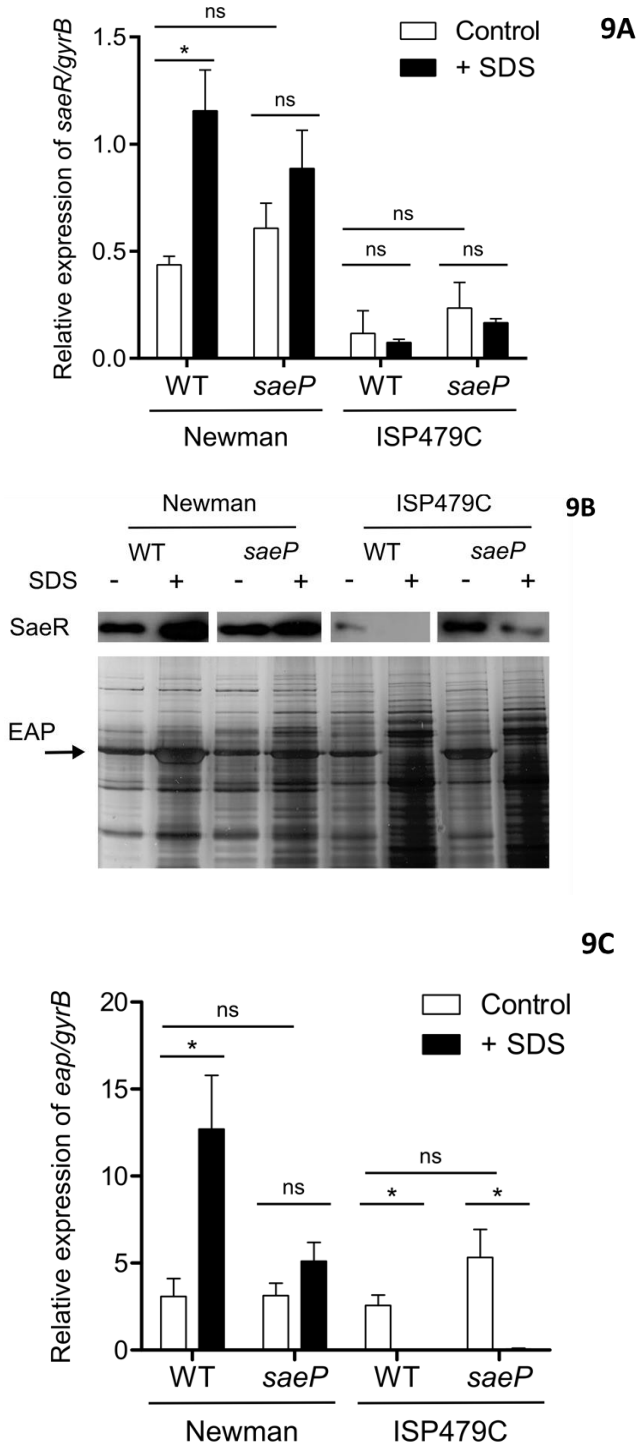



**Figure 9: The upstream region, *sae*P does not affect SDS-mediated *sae*RS activity**

Newman and ISP479C strains vs their respective Δ*sae*P mutants grown in TSB without (-) and with (+) SDS (30% MIC) until late exponential growth phase**.** (**9A)** *sae*R expression was monitored by qRT-PCR. The results represent means ± SEM of at least three independent experiments performed in triplicates. (**9B**) Eap expression was analyzed by SDS PAGE analysis (lower panel) and SaeR by Western blot analysis (upper panel). (9C) Relative *eap* expression in relation to gyrB was assesed by qRT-PCR. Asterix represents the significance of comparisons (***P<0,001; **P=0,001-0,01; *P=0,01-0,05; ns represents P>0,05).

Upon SDS stress of the ISP479C Δ*sae*P strain, Eap production was abolished similar to the ISP479C wild type, which further substantiates that *sae*P is not required for the *sae*-mediated response to SDS. Furthermore, the SDS-activation of *sae* is dependent on the (L18P) mutation located in the *sae*S operon.

### 4.2.2 S*ae*P has an effect on Eap-dependent cellular invasion under SDS stress

To demonstrate the functional requirement of Eap-dependent invasion, cellular invasion assays in Newman wild type compared with Newman Δ*sae*P as well as the ISP479C backgrounds were performed. Untreated Newman Δ*sae*P showed a significantly increased cellular invasiveness compared to Newman wild type: 60.3% vs. 39.8%, respectively (Fig. 4.2). Also consistent with Eap expression, a reduced increase in invasiveness in Newman Δ*sae*P after SDS treatment was observed. The ISP479C Δ*sae*P mutant showed an invasiveness of 62.1% compared to the invasiveness of 41.8% in its parental wild type ISP479C strain. Cellular invasiveness was reduced by SDS stress in both mutant strains. These results indicate that *sae*P/SaeP is not required for *sae*-mediated signaling and Eap-dependent cellular invasion under normal conditions. However under SDS stress, *sae*P/SaeP is partially required for optimal expression of *sae*RS and regulation of *eap* expression and consequently Eap-dependent cellular invasion: Newman Δ*sae*P displays a reduced dynamic range for the SDS response.

**Fig. 10**

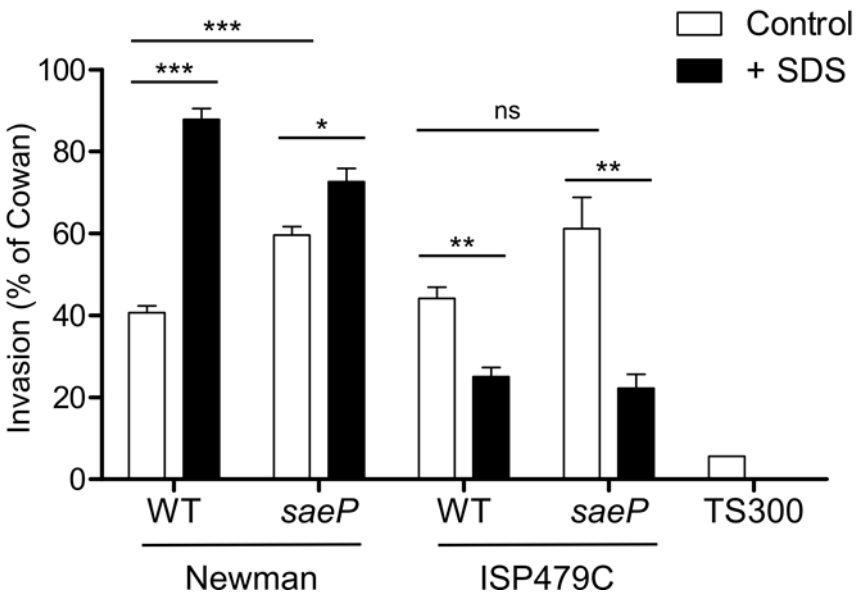



**Figure 10: Cellular invasiveness of 293 cells was measured and expressed as relative invasiveness compared to *S. aureus* Cowan I.**
The strains were grown until late exponential phase (6h) in TSB with or without SDS (30% MIC). Asterix indicate the significance of comparisons (***P<0,001; **P=0,001-0,01; *P=0,01-0,05; ns represents P>0,05).

### 4.2.3 *sae*PQ is not required for *sae*-mediated response to SDS

Since the TSS of the primary transcripts, T1 and T3 were identified which transcribe *sae*PQRS and *sae*RS, respectively, their roles in *sae* signaling under stress conditions were examined. To achieve this, strains already used by Jeong *et al*., and Maineiro *et al*., were utilized ((94,112). To test for their function, a comparison was performed of SDS-induced *sae* and *eap* transcription as well as host cell invasion in strain Newman wild type [SaeS (L18P)], its isogenic variant carrying an SaeS in which the proline ($^{Pro}$) in position 18 was reversed to leucine ($^{Leu}$) designated PQRS$^{Leu}$, as well as a *sae*PQRS deletion mutant complemented with the T3 transcript (only *sae*RS) either with the (L18P) or the (P18L) allele designated RS$^{Pro}$ and RS$^{Leu}$, respectively. As predicted, in the PQRS$^{Pro}$ and PQRS$^{Leu}$ strains and transcripts (T1, T2 and T3) were detectable (Fig.11A). A faint band of the transcript T5 observed in this experiment in the Newman SaeS$^{P}$ variants whereas the RS$^{Pro}$ and RS$^{Leu}$ strains only produced the T3 transcript.

Fig. 11A

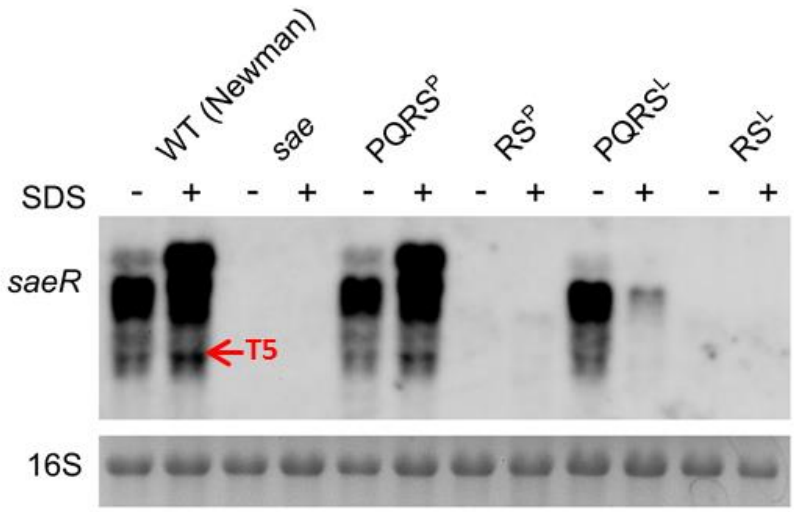



Fig. 11B

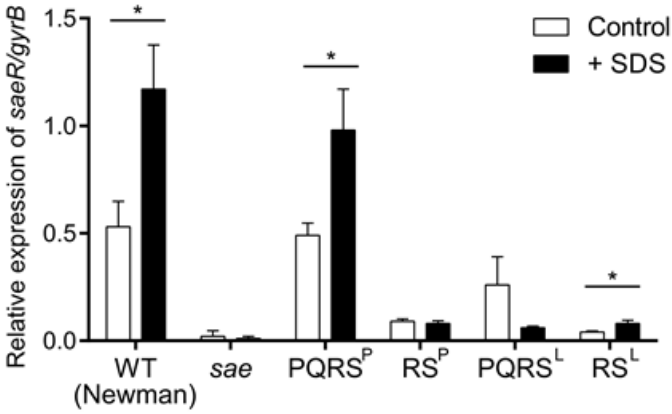



**Figure 11: *sae*PQ is not needed for *sae*-mediated response to SDS.**

Wild type (Newman), *sae*-deleted, and *sae*-deleted strains complemented with *saePQRS*$^{P}$, *saeRS*$^{P}$, *saePQRS*$^{L}$, *and saeRS*$^{L}$ were grown in TSB with or without SDS (30% MIC) until late exponential phase. **(11A)** RNA was hybridized with a digoxigenin-labelled *saeR*-specific probe. 16S rRNA detected in ethidium bromide-stained gels is shown as a loading control. **(11B)** Relative expression of *saeR* in relation to *gyrB* assessed by qRT–PCR. Results represent means ± SEM of at least three independent experiments performed in triplicates.

After SDS treatment, an increase in *sae* transcription was observed in Newman WT and in Δ*sae* complemented with *sae*PQRS$^{Pro}$ but not in the *sae*PQRS$^{L}$ strain. When the mutant was complemented with T3 (only *sae*RS$^{Pro/Leu}$) a weak expression of *sae*RS was observed, which was not significantly altered by SDS exposure. Quantification of *sae* expression by real-time PCR confirmed the effect of SDS on *sae* transcription in these strains by qRT-PCR (Fig. 11B).

In accordance with the Northern blot analyses, SDS treatment resulted in a significant increased *sae* expression (2.5 fold) in Newman WT and in PQRS$^{Pro}$ and in a decreased *sae* expression in the SaeS repaired strain (PQRS$^{Leu}$). In strains possessing either RS$^{P}$ or RS$^{L}$ only, no significant alteration of *sae*R transcription was seen after SDS stress in the Northern blot (Fig. 11A).

Next, the impact of the lack of *saePQ* on the modulation of *eap* expression by SDS was monitored by Northern blot analyses with the use of a specific probe against *eap* (Fig. 12A) and by cellular invasion assays (Fig. 12B). An SDS modulation of *eap* expression was observed in all strains analyzed except for the *sae* deletion mutant. As expected, SDS treatment led to increase or decrease of *eap* expression in the *sae*PQRS$^{P}$ and in the *sae*PQRS$^{L}$ complemented strain, respectively (Fig. 12A). Interestingly, *eap* expression in the strains complemented with *saeRS$^{P}$* or with *saeRS$^{L}$* is still responsive to SDS stress (Fig. 12A), despite very low and SDS independent *sae*RS expression (Fig. 11B).

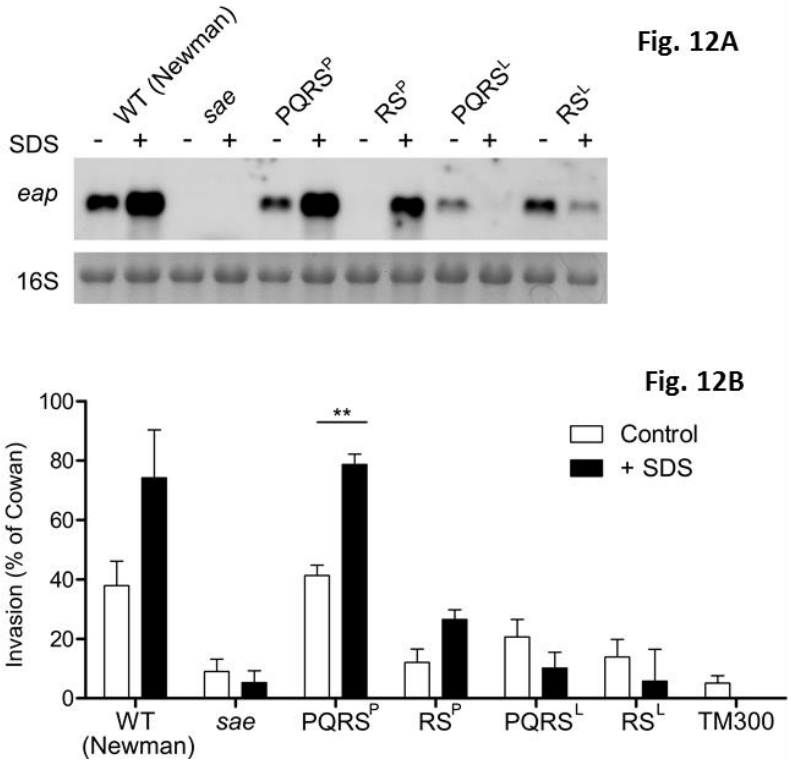



**Figure 12: *sae*P is not needed for *eap* mediated cellular invasion response to SDS.**

**(12A)** RNA was hybridized with a digoxigenin-labeled *eap*-specific probe. **(12B)** Cellular invasiveness was measured in 293 cells and expressed as relative invasiveness compared to *S. aureus* strain Cowan I. Results represent means ± SEM of at least three independent experiments performed in duplicates. Asterisks indicate the significance of comparisons (** P= 0.001 – 0.01; * P= 0.01 – 0.05).

This is in accordance to previous results showing that target gene expression is mostly dependent on SaeS activity with a minor impact of the SaeRS concentration (93,94). These data were corroborated by the cellular invasion assay (Fig. 12B) which strongly correlates with *eap* expression. Interestingly, an increase in a number of *sae*-dependent proteins, including Efb, CHIPs, LukFS and LukE, as well as overall protein production in the $RS^P$ strain treated with SDS was observed suggesting that under SDS stress, *sae*PQ not only represses Eap but other proteins as well (Fig. 13).

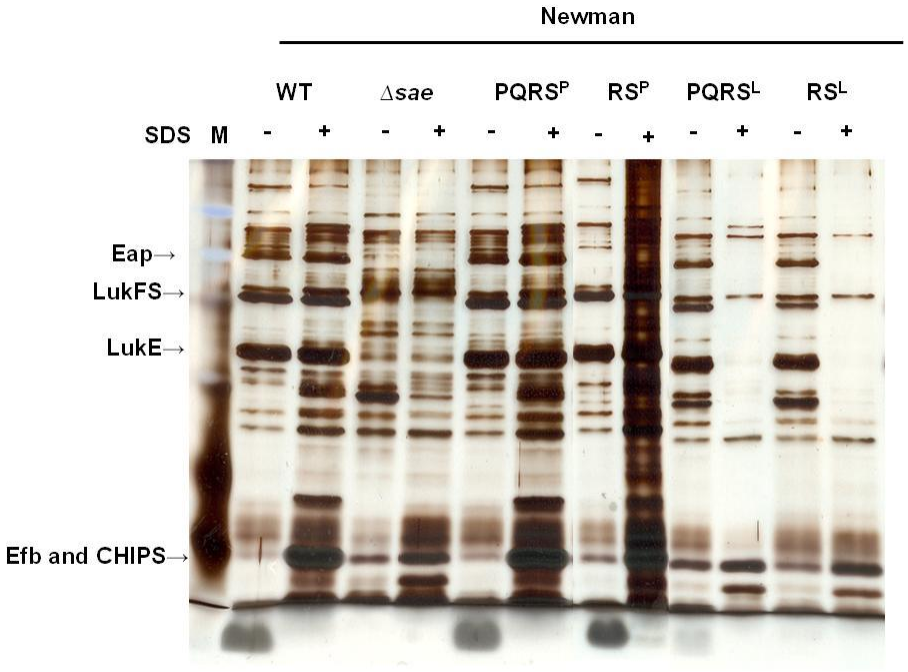



**Figure 13: SDS PAGE showing differential protein expression profiles of strain Newman wild type and its isogenic mutants in response to SDS (30% MIC).**

Strains were grown in TSB with (+) and without (-) and harvested at late exponential phase (6h). Lane M indicates protein marker.

From these results it can be can speculated that SDS might possibly interact with the transmembrane part of SaeS protein either directly or through membrane perturbation. In strains harboring the native $SaeS^L$ allele, SDS presumably leads to conformational changes resulting in a shift from kinase to the phosphatase activity. Lately, phosphatase activity was proposed to be enhanced by interaction with SaePQ [8]. However, SDS seems to control this switch independently of SaePQ. Of note, in strain Newman, harboring the $SaeS^P$ allele, SDS has the opposite effect leading to a further activation of the already hyper-activated SaeS.

This could be due to a further increase in kinase activity or alternatively to an inhibition of the low residual phosphatase activity in this strain. However, more experimental work is needed to substantiate this hypothesis.

## 4.3 Mapping of *sae* transcripts

Although *sae* transcripts and their respective transcriptional sites were previously identified, the experimental Northern blots detecting saeRS transcripts showed additional transcripts which have not been identified (61,94). To do this, the transcriptional start sites (TSS) of *sae* expressed mRNA were first to be confirmed because previous studies suggested that the TSS of *sae* mRNA may be strain dependent (94). Therefore to determine the TSS of *sae* transcripts in the Newman WT strain, two assays including the Tobacco acid pyrophosphatase/5'-RACE PCR assay (TAP) and the 5'/3' RACE Kit**,** 2nd Generation (Roche, Germany) were utilized. The advantage of using the TAP assay as opposed to the 5'/3' RACE Kit is that the TAP assay is able to distinguish between primary and processed transcripts whilst the latter cannot. Using *sae*RS specific primers, the 5'RACE assay revealed 5 transcripts including the previously identified *sae* transcripts (T1, T2 and T3) (Fig. 14). Interestingly, another *sae*RS probe-specific transcript designated T5 was identified by Northern blot analysis. The T5 transcript was also confirmed by the 5'RACE assay (Fig. 14) andsequencing thereafter. NCBI Blast and multiple alignment tools confirmed the existence of the T6 transcript initiating 160-nt downstream of the T3 TSS. Another smaller transcript was also observed. However, analysis of this 5$^{th}$ transcript revealed a non-specific amplification of the 16S rRNA gene, which was later confirmed (personal communication, Christiane Wolz). This may have been due to the high abundance of the rRNA transcript compared to a potential further *sae* transcript rather than non-specific binding of the probes. As shown in the gel electrophoresis (Fig. 14), PCR amplification of the TAP/5'RACE products, could only identify 2 transcripts, T1 and T3 which were also confirmed by sequencing of the excised PCR products. Sequencing of the excised TAP amplification products revealed that these two primary transcripts, T1 and T3, initiate as previously described (94,153), with the latter transcript initiating ±78 nucleotides downstream of previously reported TSS. Indeed, the -35 and -10 promoter sequence analysis could also be

identified. Jeong *et al*., proposed that the TSS identified by Steinhuber *et al*., might be an RNA processing site rather than a real TSS (94,153). These findings suggest that there are several additional endonucleolytic cleavage mechanisms of *sae* transcripts possibly due to different environmental conditions. Hence this also explains the existence of additional transcripts in these experiments. Based on these results, it was concluded that the *sae* operon produces several transcripts including primary transcripts T1, T3 and processed transcripts T2; T5, the latter produced through an unknown processing mechanism.

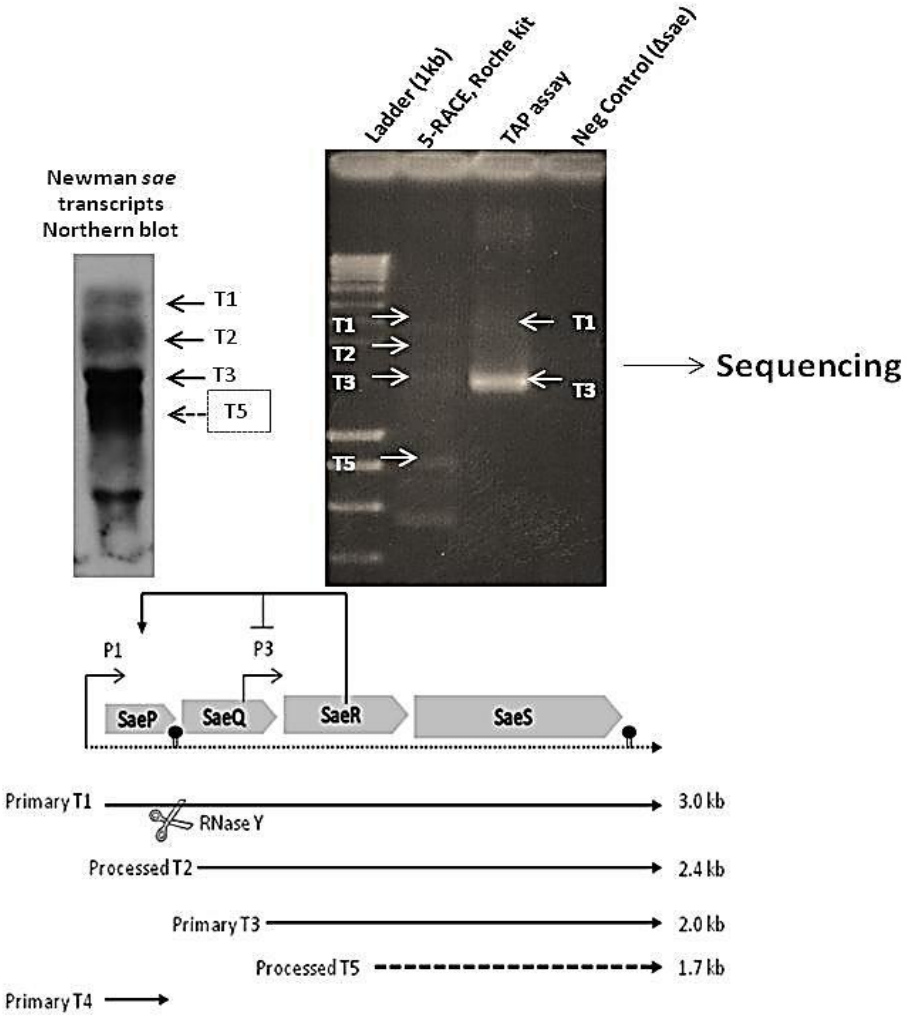



**Figure 14: Identification of *sae* transcripts in strain Newman using 5 RACE'and TAP assay.**

Northern blot analysis (top left) of *sae* transcripts using *sae*RS specific probe detected several transcripts including T1, T2 and T3 as well as and a smaller additional transcript (T5) below the T3 transcript. The processed T5 transcript could also be detected by 5'RACE assay of reverse transcribed cDNA, but not by the TAP-based 5'-RACE which is able to identify primary transcripts. Sequencing and mapping of the T5 transcript indicated the TSS location 160 nt downstream of T3 TSS. Note: the apperance of the non-specific band below the T5 which could not be sequenced using *sae* primers.

## 4.4 *sae*RS and *eap* expression is affected by global regulators *agr*, *sar* and *sig*B

Since previous experiments suggest an additional regulatory mechanism to the *sae*RS signaling and *eap* regulation under SDS stress, the influence of other global regulators, which have been shown to interact with *sae* was then tested. To investigate this, the effect of SDS on *sae*RS and *eap* expression in Δ*agr*, Δ*sar*A and *sig*B in the Newman background strains was analysed by Northern blot (Fig. 15A) and real-time PCR (Fig. 15B). At basal level, the expression of *sae*RS was significantly downregulated in the Δ*agr* (-4.75 fold) and Δ*sar*A (-4.4 fold) strains (Figure 15B).

**Fig. 15A**

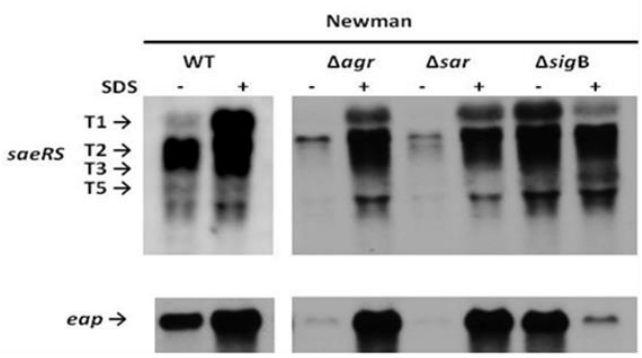



**Fig. 15B**

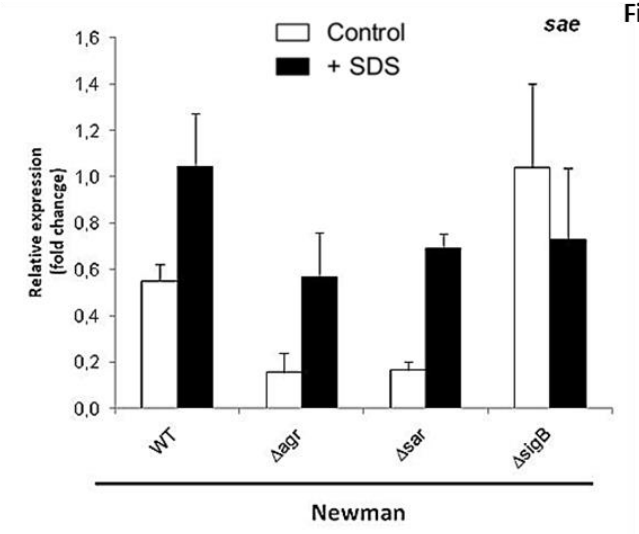


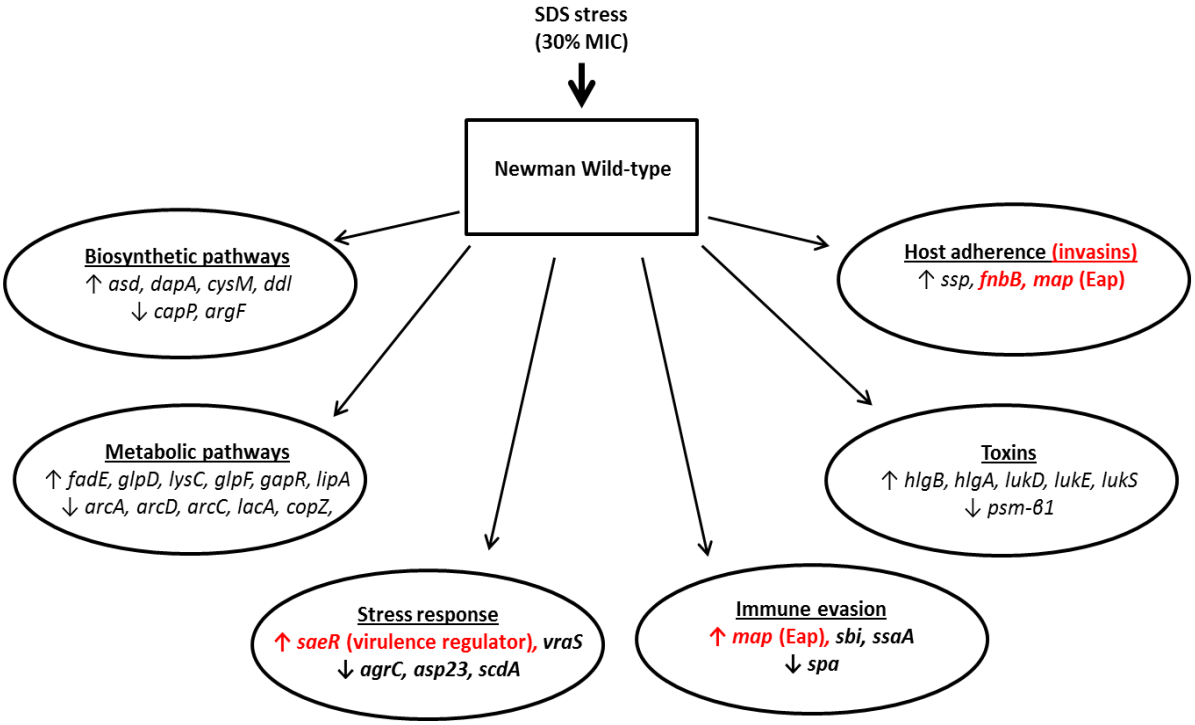

**Figure 15: Global regulators *agr*, *sar*A and *sig*B affect the expression of *sae* and *eap* under SDS stress conditions.**

Northern blots show the expression of *sae*RS and *eap*, the former was quantified by RT-PCR. Results of the RT-PCR are means including ±SEM of at least three independent experiments performed in triplicate, and are expressed as relative fold compared to strain Newman WT.

However, in the Δ*sig*B mutant, *sae*RS was upregulated (1.8 fold) without SDS stress. As expected, the transcription of *eap* in the mutant strains was also severely affected by the expression of *sae*RS. These results corroborate that under these conditions of 6h growth (late exponential phase), *sae*RS transcription is affected by other global regulators, with *agr* and *sar* positively regulating *sae* and *sig*B suppressing the system.

## 4.5 SDS affects the global gene expression of *S. aureus* strain Newman

The data presented above strongly show that several other *sae*-dependent proteins are upregulated by SDS in strain Newman. To investigate this further, transcriptional profiling by microarray analysis was performed by comparing the upregulated and downregulated genes by SDS in strain Newman WT compared with the Newman Δ*sae* strain at late exponential phase of growth. Sixty ORFs were overexpressed (>2.5 fold) in Newman WT upon SDS stress (Supplementary Tab 10.). Several genes in various categories including a number of hypothetical proteins were upregulated by SDS. These categories included genes involved in biosynthetic and metabolic pathways; stress response, host adherence, immune evasion and toxins (Fig. 16A). In the stress response catergory, the response regulator *saeR* was upregulated 3.37 fold, confirming the qRT-PCR results reported in this study. Interestingly, the vancomycin stress response sensor histidine kinase, vraS and the metal stress response regulator, merR were also upregulated 3.59-fold and 8.19-fold, respectively. Host adherence factors including extracellular matrix and plasma binding protein (*ssp*), *fnbB* and *eap* (depicted as *map*) were also upregulated by SDS in the microarray experiments. Twelve toxins which included *sae*-dependent leukocidins; *lukD*, *lukE*, *lukS* and 5 superantigen enterotoxins showed a relatively high upregulation by SDS stress at late exponential phase. Microarray analysis of the downregulated genes in the Newman WT strain showed that 57 genes were downregulated by SDS stress at late exponential phase including 22 genes involved in metabolic pathways. Of interest in the downregulated genes, were the response regulator *agrC* and the *agr*-dependent gene *asp23*, which is involved in the response to alkaline shock (Fig. 16A). In addition, the cytolytic toxin PSM-β1 was also downregulated by SDS stress.



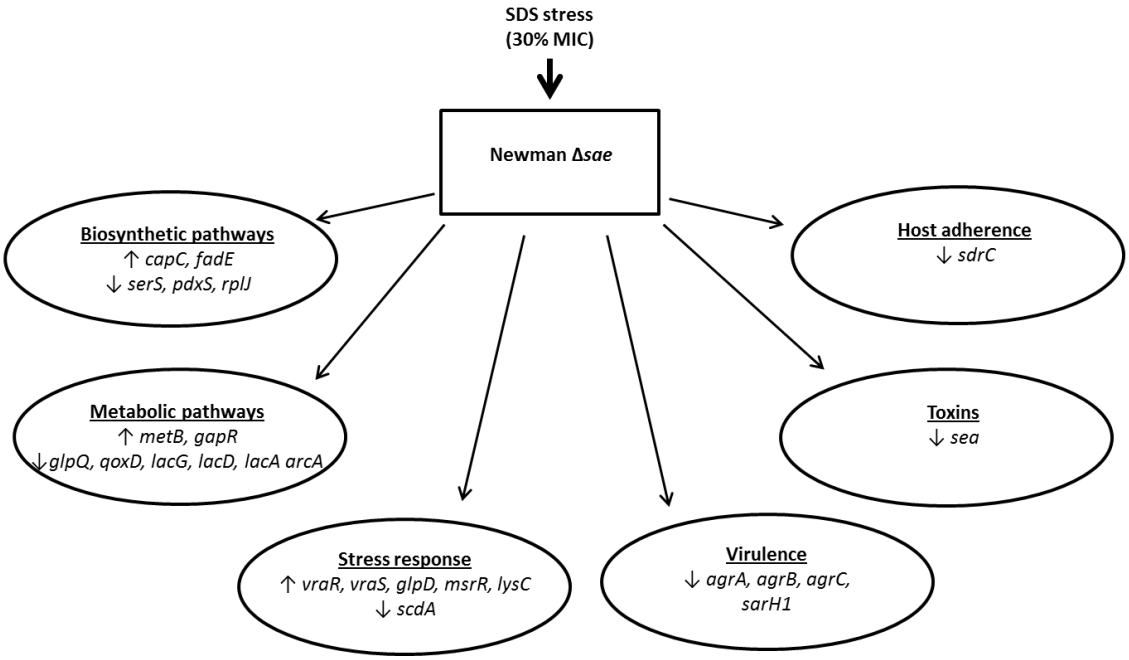




**Figure 16: Summary of genes upregulated and downregulated in strain Newman and its isogenic *sae* mutant in response to SDS stress.**

Strains were grown in TSB with and without SDS (30% MIC) for 6h. Upregulated (↑) and downregulated (↓) genes were identified using atwo-color microarray-based gene expression analysis version 5.5 software. *For full list see supplementary Table 9*.

In the Newman Δ*sae*, SDS upregulated 66 genes as well, including several genes involved in biosynthetic and metabolic pathways; stress response, transport and hypothetical proteins (Supplementary Tab. 12). SDS stress also downregulated gene expression of 50 genes involved in biosynthetic and metabolic pathways, virulence, stress response, host adherence, genes regulating toxins and hypothetical proteins (Supplementary Tab. 13). Interestingly, these downregulated genes included genes involved in global regulation of virulence factors of the *agr* family proteins.

When the gene expression profiles of the Newman WT and Newman Δ*sae* strains were compared, 16 genes could be extrapolated from the two lists, which were upregulated by SDS stress (Supplementary Tab. 14). Equally, 16 genes were also found to be downregulated by SDS conditions (Supplementary Tab. 14). These data suggests that SDS not only affects the expression of *sae*-dependent genes but other genes as well. It should be noted that statistical significance of these data could not be performed due to the high variability between the three samples.

## 4.6 SDS stress affects *S. aureus sae* expression in a strain-dependent manner

So far, the only *S. aureus* strain that resulted in an up-regulation of the *sae* response by SDS was strain Newman. Several clinical strains were monitored for the SDS response by specific *saeR* qRT-PCR and cellular invasion assay (Fig. 17). Strain Newman wild-type, Newman Δ*sae* and Newman HG (where the saeS point mutation was repaired to the wild type (112)) were included in the analyses. With the notable exceptions of isolate ST239-635/93R, treatment with SDS resulted in down-regulation of *sae* expression in all clinical strains (Fig. 17 A). Since an SDS-dependent up-regulation of *sae* expression in the ST239-635/93R strain was identified, several other previously identified clinical ST239-635/93 clones (i.e. ST239-635/93-W; –THW89, -THW-99, -(465) and -(966) and were further tested. A down-regulation in *sae* expression was observed in all of these strains. Thus, the isolate ST239-635/93R seems to be unique with regard to the SDS response although no mutations in *sae*S or *sae*PQR were detectable.

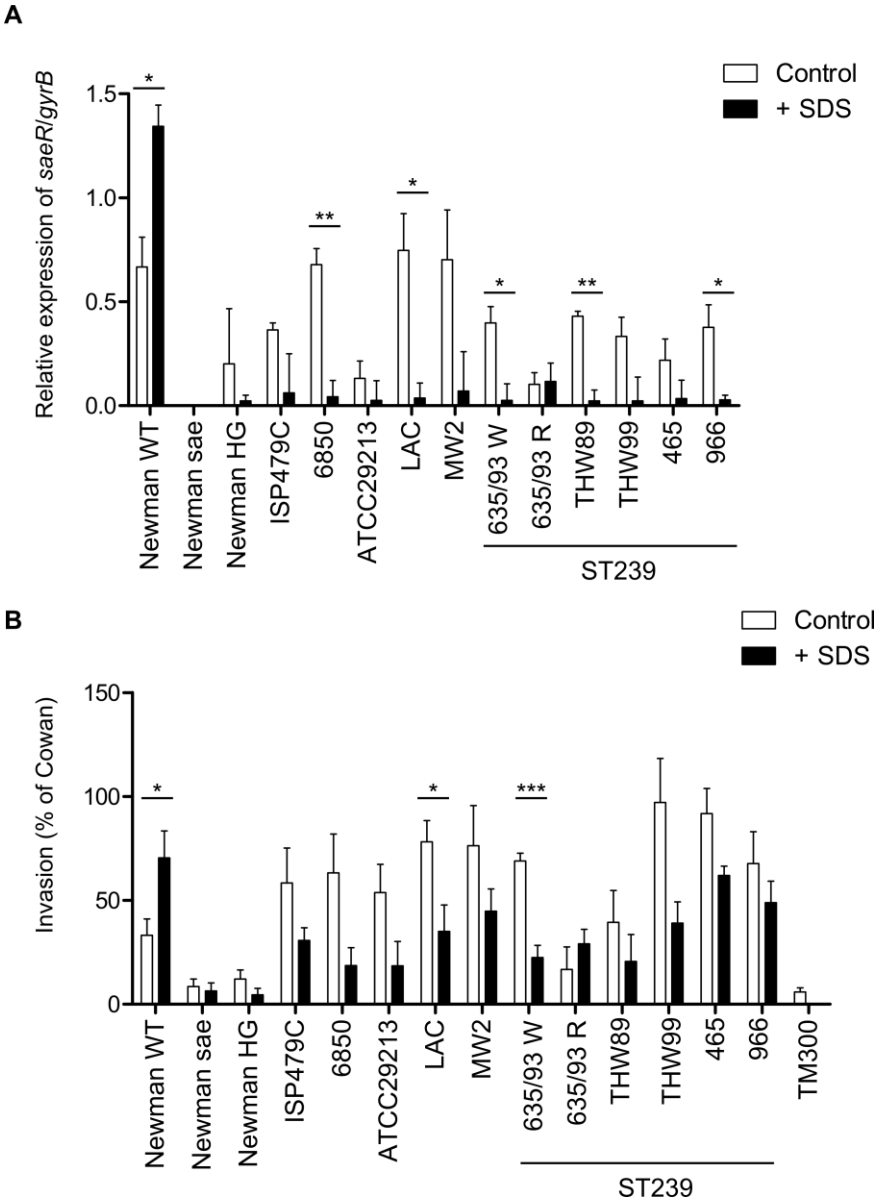



**Figure 17: SDS stress (30% MIC) affects *S. aureus sae* signalling in a strain-dependent manner.**

Relative expression of *saeR* in relation to *gyrB* was assessed by qRT–PCR in various clinical isolates and in control strains at **(A)** late exponential phase of growth. Results represent means ± SEM of at least three independent experiments performed in triplicates. **(B)** Cellular invasiveness into 293 cells was measured and expressed as relative invasiveness compared to *S. aureus* strain Cowan I. Asterix indicate the significance of comparisons (***P<0,001; **P=0,001-0,01; *P=0,01-0,05; ns represents P>0,05).

Since it was shown previously that SDS increases host cell invasiveness of strain Newman through upregulation of *sae*-mediated *eap* expression, the study next focused on the invasiveness of the various strains on human embryonic kidney 293 cells. SDS-treated and untreated *S. aureus* strains were harvested at late exponential phase and subsequently FITC-

labeled for flow cytometric invasion assay analysis as previously described. Consistent with our previous data, SDS treatment of strain Newman increased the cellular invasiveness from 33.2% to 70.5% (Fig. 17B). Upon SDS exposure, a decrease in invasiveness was observed in the Newman *Δsae*PQRS mutant, in strain Newman HG carrying a *sae*$S^L$ allele and in the other clinical isolates, with exception of strain ST-239-635/93R. Preliminary data suggested that the invasion mechanism in these strains is not Eap-mediated, but dependent on FnBPs.

Since, Schäfer *et al*., (142) previously showed that biocide/SDS increases cellular invasiveness of strain Newman in a Eap-dependent way and because one clinical strain was identified, ST239-635/93R, with a similar phenotype to strain Newman i.e. showed increase *sae* expression and cellular invasiveness by SDS stress, the strain was then tested for Eap expression under these conditions. Strains treated or not treated with SDS were harvested at late exponential phase and subsequently investigated for protein expression.

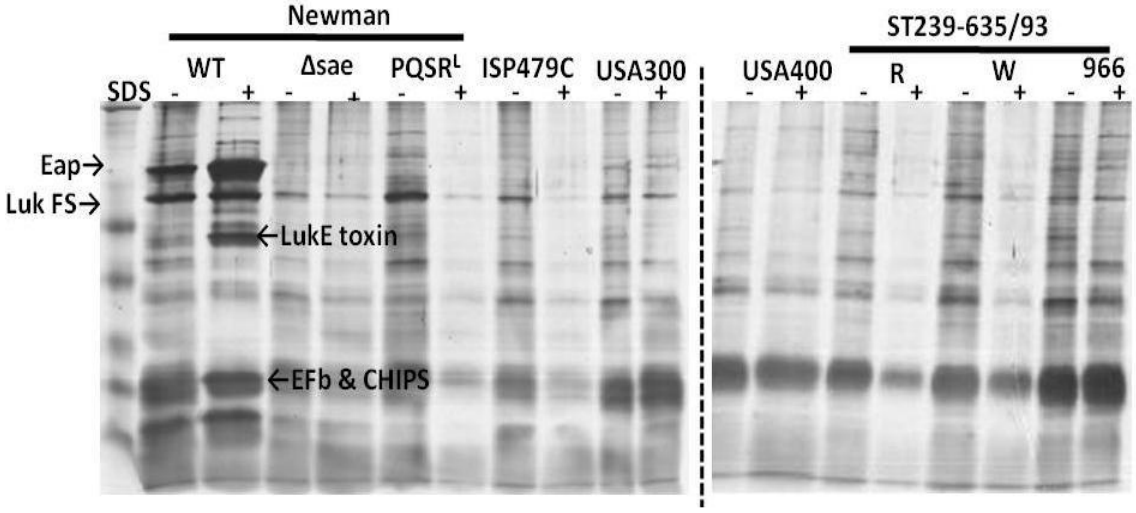



**Figure 18: SDS PAGE showing differential protein expression profiles of Newman WT and its isogenic mutants compared to clinical strains in response to SDS stress.**

Strains were grown in TSB with (+) and without (-) SDS (30% MIC), and harvested at late exponential phase (6h). Eap and other *sae*-dependent proteins are enhanced by SDS treatment in the Newman strain but not in the clinical strains that do not contain mutation in the sensor histidine kinase. Hatched line indicates two different gels.

Consistent with Schäfer *et al*., SDS treatment of strain Newman resulted in increased Eap, Efb CHIPS, and LukFS/E proteins (Fig. 18). No Eap increase was observed in the strain Newman *Δsae*, in the *saeS* repaired and in the other clinical isolates after SDS treatment.

Strain ST-239-635/93R also showed no increase in Eap, therefore the mechanism of enhanced invasiveness by SDS was not attributed to an increase in Eap in this strain. To date, with the exception of strain Newman, cellular invasiveness in *S. aureus* strains is mediated by FnBPs.

# 5. Discussion

Numerous studies have demonstrated the effects of environmental stimuli including pH, temperature, human neutrophils, SDS-based detergents and sub-inhibitory concentrations of antibiotics such as β-lactams, florfenicol, quinolones, clindamycin and vancomycin on *S. aureus* functions (27,104,123,142,142). In addition, several global regulators such as *agr*, *sig*B and *sae* have been recognized as stress regulators responding to some of these stimuli (40,61,104). For example, *sig*B has been described as an essential component of *S. aureus* stress response for coping with alkaline, heat, oxidative and salt stress (147). Perhaps, most importantly, these regulons are also required for the gene regulation of virulence factors. In particular, the *sae*RS two component system directly or indirectly affects the expression of coagulase, chemotaxis inhibitory protein (CHIPS), FnBPs; adhesins Eap and Emp; cytolysins PVL, α- and β-hemolysins (53,64,104). Several of the previously mentioned stimuli have been reported to induce the *sae* system including β-lactams (cefoxitin), florfenicol, mupirocin, salicylic acid, $H_2O_2$, and α-defensins, while low pH conditions, glucose and high salt conditions repress *sae* transcription (6,27,61,103,123). As a result, the *sae* system has been implicated as one of the major regulators of *S. aureus* virulence and pathogenesis (66,125).

In our group, it was previously demonstrated that sub-inhibitory concentrations of the disinfectant Perform (a commonly used oxygen radical-liberating biocide) and a detergent component of it, SDS alone activate *sae* transcription and increase cellular invasiveness in *S. aureus* strain Newman through enhanced Eap production in the Newman strain independent of oxidative stress (142). Interestingly, the SDS effect was associated with an amino acid exchange in the N-terminus of SaeS (L18P), specific to strain Newman, while most other *S. aureus* strains carry a leucine residue at this position (142). Since the signaling of the respective *S. aureus* global regulator, *sae*, consists of the classical *sae*RS two component system, the assumption would be that the response regulator, SaeR encoded by *sae*R is responsible for the transcriptional regulatory activities of the system. The *sae*RS system is preceded by two additional open reading frames (ORFs) in the *sae* operon, *sae*P and *sae*Q, which are predicted to encode a lipoprotein (SaeP) and a membrane protein (SaeQ), respectively (153). These two proteins have been shown to induce phophatase

activity of the sensor histidine kinase (SaeS) (93). However, their role in regulating the TCS *sae*RS under stress conditions have not been entirely elucidated.

## 5.1 Role of *sae*P

Using strain Newman and SDS stress as a model, the first objective was to investigate the role of the *sae*RS upstream components, *sae*P and *sae*PQ, in the signaling of the Sae TCS and the regulation of a *sae*-dependent gene, *eap*, under SDS stress conditions in strain Newman. Secondly, to demonstrate the importance of these genes, isogenic Δ*sae*P mutants in Newman (carrying a proline residue in SaeS) and ISP479C (carrying a leucine residue in the SaeS wild type allele) were analyzed for *sae*R expression (Fig. 9A). The deletion of *sae*P in the Newman Δ*sae*P led to a slightly elevated SaeR protein expression compared to its parental strain suggesting that *sae*P has a regulatory role in *sae*R transcription, however this was not significant. To further determine the role of *sae*P, Newman wild type and its isogenic *saeP* mutant were induced with SDS with a particular focus on expression of *sae*R and a *sae*-dependent gene, *eap*. This was further monitored for its functional role in cellular invasion on 293 cells (Fig. 10). Upregulation of *sae*R and *eap* by SDS was observed in the Δ*sae*P mutant. However, the SDS effect was less pronounced in the deletion mutant compared to wild type. Cellular invasion assays strongly correlated with Eap expression i.e. invasion was also enhanced by SDS stress. However, it was less pronounced in the Δ*sae*P mutant than in the wild type, further emphasizing an independent SDS response by SaeS.

Because the enhanced Eap expression and cellular invasion by SDS was only observed in strain Newman due to the SaeS mutation (L18P), another strain, ISP479C (P18L) was analyzed. In the ISP479C Δ*sae*P mutant, an elevated *sae*R and Eap expression compared to the wild type was observed, however this was not significant (Fig 9A and 9C). In both ISP479C strains, SDS clearly abolished the expression of Eap (Fig. 9C). More interestingly, with SDS treatment an increased overall protein production (except for Eap) was observed by SDS treatment in the ISP479C and in its isogenic Δ*sae*P mutant (Fig. 9B). This is rather in contrast to the previous report by Schäfer *et al*., (142). Perhaps this was due to the difference in culture conditions or harvesting times for protein expression. From these

results it could be concluded that SDS directly interacts with SaeS function. In strains harboring the wild type $SaeS^{L}$ allele, SDS might lead to conformational changes resulting in a shift from kinase to the phosphatase activity. Lately, phosphatase activity was proposed to be enhanced by interaction with SaePQ (93). However, SDS seems to control phosphatase activity independently of SaePQ, possibly by changes in the membrane properties or through other membrane proteins. Of note, in strain Newman harboring the $SaeS^{P}$ allele, SDS has the opposite effect leading to a further activation of the already hyper-activated SaeS. This could be due to a further increase in kinase activity or alternatively to an inhibition of the low residual phosphatase activity in this strain (93,94). In contrast to these conclusions, in our study, the deletion *sae*P alone resulted in significant SaeR expression at least in strain ISP479C. In strain Newman, this repressive effect was not statistically significant (corroborating Jeong's findings) (94). The differences in these results might be explained by the utilization of different *S. aureus* strains in both studies.

## 5.2 Mapping of *sae* transcripts

The P3 promoter is located centrally in the *sae*Q gene (Fig. 14). Because of this, it precluded isolated analysis of this gene without inflicting further major changes to the SaeQ protein and the whole operon. Therefore, the next focus was on the role of both *sae*PQ in the regulation of *sae*RS. It is well known that the *sae* operon consists of two promoters, *sae*P1 and *sae*P3 encoding for *sae*PQRS and *sae*RS, respectively (Fig. 14) (153). To examine the role of *sae*PQ, the transcriptional start sites (TSS) of *sae* transcripts T1, T2 and T3 were first determined. It has recently been suggested that these initiating sites are strain-dependent (94,153). When *sae* transcription was analyzed by Northern blot using a *sae*RS probe, three mRNAs including T1, T2, T3 as expected as well as an additional transcript designated T5 were observed. Usually, a 5'RACE primer extension assay is used to identify transcriptional start sites of mRNA. However, the results from the 5'RACE primer extension assay cannot be conclusive since this assay cannot differentiate between primary and processed transcripts. Because of this, a tobacco acid pyrophosphate (TAP)-mediated 5'RACE method was optimized to distinguish primary mRNA from processed mRNA. It is based on the fact that primary transcripts carry a triphosphate group at the 5'end, which cannot be ligated to the adapter primer except if the triphosphate group is transformed into a monophosphate by

TAP treatment. Thus, by utilizing the 5'RACE primer extension and the TAP-5'RACE assay the existence of this transcript (observed in the Northern blot) was confirmed by sequencing. It is processed 160 nucleotides downstream of T3 transcriptional start site (TSS) It should be cautioned, however, that under different conditions *sae* transcript patterns may vary due to different batches of medium, aeration, growth times and changes in pH. The T5 transcript was less pronounced or apparently lacking in some experiments performed. These findings have also been reported by Adhikari and Novick in earlier studies on *sae*RS system (3). The analyses of the TSS of T1 were identical as previously reported (142). In this study the TSS of T3 was found to be located 78 nucleotides downstream of the previously reported TSS by Steinhuber *et al*., (153). Recently, similar results were reported by Jeong *et al*., thus confirming the correctly identified TSS of T3 (94). The differences might be explained by the utilization of different methods, strain variation or culture conditions as Steinhuber *et al*., had previously utilized CYPG media for growth conditions and ($\gamma^{32}$P) ATP-based method for TSS analysis (153). T2 could not be amplified with the TAP assay suggesting that it is indeed a processed product from endonucleolytic cleavage of T1 by RNase Y as recently demonstrated (113).

## 5.3 Role of *sae*PQ

Based on these findings, a set of experiments was performed to investigate if the entire upstream region, *sae*PQ, is required at all in the SDS-induced transcription of *sae*RS and *eap*. The *sae*P1 which transcribes T1 (*sae*PQRS) has the strongest activity of the two promoters in the Newman strain and like many other global regulatory systems is positively autoregulated (61). Two studies, Mainiero *et al*., and Jeong *et al*., show evidence that the *sae*PQ region is not required for the expression of *sae*-dependent genes (94,112). In these two studies, *sae*-target genes were classified into class I genes which constitutes of those genes that require high levels of hyperactivation by the autoregulatory P1 promoter, such as *fnb*A, and *coa* and class II genes; *hla* and *hlb* which are not dependent on the high phosphorylation activity of the *sae*P1 promoter in strain Newman. Class II genes therefore do not require *sae*PQ to be induced and can be activated at basal level by *sae*R. The evidence in these studies was based alone on the disruption of the P1 promoter did not affect the transcription of Coa and Hla

(94). Hence, in contrast to Mainiero *et al*., and Jeong *et al*., in this study the possible involvement of the *sae*P1-driven *sae*PQ region in SDS-mediated *sae* and *eap* expression under SDS stimulus conditions was investigated. To examine this, mutant strains lacking the *sae*PQ region in the Newman background carrying either the proline or leucine residue were investigated for *sae*RS, *eap* expression and Eap-dependent invasion. SDS-mediated induction of *sae* could only be observed in strains with the *sae*PQ. The deletion of *sae*PQ resulted in abolished *eap* expression in the strain carrying the SaeS (L18P) strain suggesting that *sae*PQ is required for the transcription of class I *sae* target gene, *eap.* One of the unexpected findings was that under SDS stress conditions, SaeRS$^{P}$ resulted in a significant upregulation of *eap* (Fig. 12A) and also increased overall protein expression. Several previously identified *sae*-dependent proteins were upregulated including CHIPS, Eap, LukFS and LukE. It is noted that in contrast to our study, Jeong *et al*., limited their investigation to Coa and Hla (94). Thus, the *sae* regulation of *eap* is possibly different for the class I target gene, *coa*, in their focus. In summary, these results suggested an existence of another regulatory mechanism, which regulates *sae* and *eap* under SDS stress conditions.

## 5.4 Role of other global regulators

Based on these data and strong evidence from previous studies showing that global regulators are strongly linked functionally to each other, the role of other virulence regulatory systems *agr*, *sar* and *sig*B on *sae* and *eap* transcription were therefore investigated (41,65,68). As a result SDS effect on *sae*RS and *eap* transcription in knockout mutants of *agr*, *sar*A and *sig*B in the Newman background was tested. In the *agr* and *sar*A mutant strains, significant downregulation of *sae*RS was observed compared to the wild type suggesting that the global regulators *agr* and *sar* are required for a fully functional *sae* system. These observations confirmed previous studies including Giraudo *et al*. (65)and Harraghy (77) *et al*. that showed that these two regulators are required for *sae* expression (65)[167]. A significantly higher *sae* activity was observed in the *sig*B mutant strain suggesting that *sig*B represses *sae* thus confirming an enhanced invasion potential of this strain as reported previously (142). The most interesting observation was that after SDS treatment, the transcription of *sae*RS was significantly upregulated in the *agr* and *sar* mutant

strains. After SDS treatment in the *sig*B-deficient strain, *sae* expression was reduced implicating that *sig*B is also required for the SDS induction. These results differ to the previous study by Schäfer *et al*. in which SDS treatment in the Δ*sig*B mutant had no effect on the Eap-dependent cellular invasion in strain Newman (142). As a result, transcription of *eap* in these mutant strains was then tested. Indeed, transcription of *eap* was dependent on *sae*RS levels in the *agr* and *sar* mutant strains. Furthermore, *eap* was highly transcribed in the *sig*B mutant compared to the wild type strain. Upon SDS an elevation of *eap* was evident in the Δ*agr* and Δ*sar*A strains. In the *sig*B mutant, *eap* expression was almost abolished by SDS contrary to Schäfer's findings. The reason for these differences could not be elucidated and may be attributed to different growth conditions between the two studies.

## 5.5 Global gene expression to SDS stress

Transcriptional profiles were also determined for gene expression of the Newman WT compared to the Newman Δ*sae* under SDS stress conditions. The global changes in the Newman gene expression shown here provide a comprehensive view of the genes that potentially promote *S. aureus* virulence and adherence to host cells. Because our efforts focused on SDS mediated *sae*-dependent differentially-regulated gene expression, the results are likely to provide new insights into the pathogenesis of *S. aureus* infection and may be used in identification of new *S. aureus* therapeutic targets. To our knowledge *sae*-dependent transcriptional profiles have not yet been elucidated in *S. aureus*.

## 5.6 Strain-dependant response to SDS

This study further shows that the activation of *sae* by SDS is strain-dependent. In most strains analysed, SDS resulted in significant decrease of *sae*RS mRNA which could be correlated with decrease in expression of the target gene *eap* as well as in cellular invasion. Dependent on the strains, the effect on invasion was mediated by down-regulation of *eap* and/or via concomitant downregulation of *fnbA*. Previously, Eap has been shown to be involved in attachment to and invasion of eukaryotic cells (75,90). Harraghy *et al*. previously showed that *sae* is essential for the transcription of *eap* in strain Newman and is markedly repressed in the presence of glucose due to changes in pH (77). Moreover, Eap has been

demonstrated to be an important immune response modulator by inhibiting the delayed-type sensitivity reaction and by interfering with neutrophil recruitment (76). It is also noted that Eap is conserved in *S. aureus* strains and a potent inhibitor of angiogenesis (88).

The upregulation of *sae*RS by SDS stress could be demonstrated in Newman wild type as previously demonstrated (142). In this study, SDS stress resulted in an unusual effect on *sae* and its target gene in one other strain. ST239-635/93R showed increased cellular invasiveness when treated with SDS (Fig. 17B). However, in ST239-635/93R, the increased invasiveness by SDS could not be attributed to an elevation in Eap production (Fig. 18). Preliminary results by qRT-PCR and Western blot analysis showed that FnBPs were decreased after SDS treatment (data not shown). Furthermore, Northern blot analysis indicated that only T3 was transcribed in the strain ST239-635/93R similar to the $RS^L$ complemented mutant in strain Newman. Sequencing of the entire operon showed that there was no mismatch in the *sae* sequence compared to other published database sequences (depicted by strain ISP479C in NCBI BLAST) carrying the $SaeS^L$ allele (Supplementary Fig. 1). These findings indicate that there may be other regulatory mechanisms involved. Interestingly, the expression of *sae* was diminished in strain ST239-635R compared to other related ST239-635/93 strains irrespective of SDS stimulation. Interestingly, Alvarez *et al*., observed an up-regulation of Eap in strain Newman under salicylic acid (SAL) treatment, which also increased cellular internalization of MAC-T cells (6). More so, the SAL effect on Eap expression was demonstrated in *S. aureus* strains other than strain Newman such as Wood46 and the Brazillian clone. Although the point mutation in SaeS (L18P) leads to high virulence factor expression, Steinhuber *et al*. and this study (Fig. 17A) also showed that several clinical isolates express higher level of *sae* than strain Newman (153). However, the effect of *sae* expression has so far been analyzed only by surrogate parameters and not directly correlated with virulence and disease.

In summary, in this study, it was demonstrated that the SaeS polymorphism in strain Newman leads to high expression of Eap on a transcript level, presumbably due to the requirement of high SaeR phosphorylation of SaeR. Two *sae* promoters, P1 and P3 are currently known in *sae* signaling. However only the P1 promoter is strongly autoactivated

(61). Class I target genes for *sae*, including, *eap*, *coa* and *fnbA*, need high phosphorylation for activation. However, for *sae*-dependent expression of these genes, only low *sae* expression is sufficient (94). The low expression of the native P3-derived *sae*RS expression is obviously enough to drive also *eap* expression in the *sae*S$^{L}$. Of note, this expression can still be modulated by SDS exposure. Dependent on the *saeS* allele, SDS stress results in up-regulation (*sae*S$^{P}$) or down-regulation of *eap* (*sae*S$^{L}$). Eap expression was again tightly linked to the invasion capacity of *S. aureus* strain Newman. Taken together, our data suggest that sub-inhibitory concentrations of SDS activate *saeRS* transcription independently of *sae*PQ, thereby leading to alteration of the expression of the *sae* target gene *eap* and consequently host cell invasion. SDS seems to interfere directly with the SaeS kinase/phosphatase activity and this activity is closely linked to the expression of Eap and the capacity of *S. aureus* to invade host cells. Interestingly a single amino acid exchange (Leu to Pro; L18P) in the putative transmembrane domain of SaeS leads to an opposite output of the SDS mediated signal. This suggests that SaeS activity can be directly modulated by structurally non-complex environmental signals, possibly by altering its kinase/phosphatase activity.

*Staphylococcus aureus* remains a frequent cause of nosocomial and community-associated infections. The success of the pathogen in these settings is partially attributed to the ability to adapt to different environments by activation of several two-component systems that control the expression of virulence genes as well as resistance genes. One such two component system is the SaeRS global regulator which is discussed in this study. The SaeRS system is necessary for successful establishment of skin and soft tissue infections as well as survival from phagocytosis by human neutrophils. Collectively, our findings provide insight into SaeRS gene regulation in *S. aureus* when under SDS stress conditions and highlight the importance of investigation downstream events of gene-regulatory systems upon their stimulation i.e. SaePQ auxiliary system. Furthermore, an improved understanding of the molecular mechanisms used by *S. aureus* to evade the host immune system (eg. secretion of *sae*-dependent protein, Eap) will enable the development of novel treatments for *S. aureus* infections. Using micro-array analysis, our study further demonstrates many more genes which can be potential vaccine antigens and targets for therapeutics designed to control *S.*

*aureus* infections. In this regard, further investigation includes the interaction of SaeRS with other global regulators and other mechanisms involved in cellular invasion such as autotlysin (Atl). With such complex mechanisms in place by this organism, this study contributes to a further understanding of *S. aureus* and its interaction with different environments and the importance of strain variation within the species.

# 6. Supplementary

```
>  emb|AJ556795.1|  Staphylococcus aureus regulatory operon sae (saeR and
saeS genes),
ORF4 & ORF3, strain ISP479C
Length=3515

 Score = 1836 bits (955),  Expect = 0.0
 Identities = 957/958 (99%), Gaps = 0/958 (0%)
 Strand=Plus/Minus

Query  91    GCTTGTAATTATTGTCGTTAAGGTCAAAGTTAGTGTCATATGGCCGTTAAACCACATTAA
150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2372  GCTTGTAATTATTGTCGTTAAGGTCAAAGTTAGTGTCATATGGCCGTTAAACCACATTAA
2313

Query  151   AATATATGCAATTGCTAAAATAGTTGAAGTTAATGGTATACTCGATACGACGCCAATAAT
210
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  2312  AATATATGCAATTGCTAAAATAGTTGAAGTTAATAGTATACTCGATACGACGCCAATAAT
2253

Query  211   GATTTGACTTCTAATTGATAACACCATTATCGGCTCCTTTCAAATTTATATCCTAATCCC
270
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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2193

Query  271   CATACAGTTGTGATGGTATATGTTGTAAAGCTCTCTTTTTCTAATTTTTCTCTAATACGG
330
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2192  CATACAGTTGTGATGGTATATGTTGTAAAGCTCTCTTTTTCTAATTTTTCTCTAATACGG
2133

Query  331   TGTATATGGACATTCACGGTATTAGCATCTTCGTAATAGTCATATCCCCAAACTTTTTCA
390
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Sbjct  2132  TGTATATGGACATTCACGGTATTAGCATCTTCGTAATAGTCATATCCCCAAACTTTTTCA
2073

Query  391   AGTAATTCTGATTTAGAAATAACTTCATTTTCTCTAGAAGCTAAATACCACAATAACTCA
450
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Sbjct  2072  AGTAATTCTGATTTAGAAATAACTTCATTTTCTCTAGAAGCTAAATACCACAATAACTCA
2013

Query  451   AATTCCTTAATACGCATAGGGACTTCGTGACCATTTACAGTCACAACTTTACTTAAGTTA
510
```

**Figure 19: *sae*S blast sequence alignment of strain Newman with *sae*S of strain ISP479C.**

Sequencing was performed using *sae* specific primers (Tab. 3) through recommended instructions by Eurofins MWG Operon, Ebersburg, Germany. *sae*S point mutation, T53C (reverse complement) resulting in substitution of leucine to proline in strain Newman.

**Table 10: Genes upregulated by SDS stress in strain Newman WT**

| Biological function | Gene | Synonym | Product | Annotated predicted function | Mean fold change | SEM | p value |
|---|---|---|---|---|---|---|---|
| **Biosythetic pathways** | - | NWMN_0171 | acetyl-CoA/acetoacetyl-CoA transferase | biosynthetic pathways | 12,42 | 5,30 | p < 0.01 |
| | asd | NWMN_1305 | aspartate semialdehyde dehydrogenase | 'de novo' L-methionine biosynthetic process | 10,15 | 5,52 | p < 0.05 |
| | cysM | NWMN_0424 | cysteine synthase | cysteine biosynthetic process from serine | 5,17 | 2,44 | p < 0.05 |
| | ddl | NWMN_1987 | D-alanyl-alanine synthetase A | peptidoglycan biosynthetic process | 4,76 | 1,82 | p < 0.01 |
| | dapA | NWMN_1306 | dihydrodipicolinate synthase | diaminopimelate biosynthetic process | 7,55 | 4,21 | p < 0.05 |
| **Metabolic pathways** | | | | | | | |
| | - | NWMN_0923 | acetyltransferase, GNAT family protein | Metabolic pathways Transferase | 4,38 | 1,22 | p < 0.05 |
| | - | NWMN_2271 | acetyltransferase, GNAT family protein | Metabolic pathways Transferase | 5,81 | 3,01 | p < 0.01 |
| | fadE | NWMN_0170 | acyl-CoA synthetase FadE-like protein | Metabolic pathways lipid metabolim | 10,17 | 4,95 | p < 0.05 |
| | glpD | NWMN_1209 | aerobic glycerol-3-phosphate dehydrogenase | glycerol catabolic process | 6,85 | 0,91 | p < 0.01 |
| | - | NWMN_2104 | aldo/keto reductase family protein | oxidoreductase activity | 2,72 | 0,10 | p < 0.01 |
| | lysC | NWMN_1304 | aspartate kinase | Metabolic pathway | 19,46 | 15,1 | p < 0.05 |
| | - | NWMN_1461 | ATP-dependent RNA helicase DEAD/DEAH box | ATP binding | 3,42 | 0,76 | p < 0.05 |
| | glpF | NWMN_1207 | glycerol uptake facilitator protein | Transport | 8,23 | 2,11 | p < 0.01 |
| | gapR | NWMN_0740 | glycolytic operon regulator | carbohydrate binding | 8,59 | 4,34 | p < 0.01 |
| | lipA | NWMN_0624 | lipase/esterase LipA | hydrolase activity | 10,81 | 8,63 | p < 0.01 |
| | - | NWMN_0870 | oligoendopeptidase F | proteolysis | 9,19 | 4,79 | p < 0.01 |
| | - | NWMN_2225 | phosphosugar-binding transcriptional regulator | carbohydrate metabolic process | 2,62 | 0,08 | p < 0.01 |
| **Stress response** | saeR | NWMN_0675 | DNA-binding response regulator SaeR | Stress response (SDS-based detergents, antibiotics) | 3,37 | 1,47 | p < 0.01 |
| | vraS | NWMN_1823 | sensor histidine kinase VraS | Stress response (Vancomycin stress response) | 3,59 | 0,41 | p < 0.05 |
| | - | NWMN_2105 | transcriptional regulator MerR family | Stress response (Metal stress response) | 8,19 | 0,84 | p < 0.01 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | protein | | | | |
| | | | | | | | |
| **Host adherence** | ssp | NWMN_0758 | extracellular matrix and plasma binding protein | Host adherence | 28,49 | 11,3 | p < 0.01 |
| | fnbB | NWMN_2397 | fibronectin binding protein B precursor | Host adherence | 6,77 | 3,28 | p < 0.05 |
| | map | NWMN_1872 | MHC class II analog protein (Eap) | Immune evasion and Host adherence | 31,09 | 14,3 | p < 0.01 |
| | | | | | | | |
| **Immune evasion** | - | NWMN_1067 | formyl peptide receptor-like 1 inhibitory protein | Immune evasion | 9,57 | 9,03 | p < 0.05 |
| | sbi | NWMN_2317 | immunoglobulin G-binding protein Sbi | Immune evasion | 3,47 | 0,61 | p < 0.01 |
| | map | NWMN_1872 | MHC class II analog protein (Eap) | Immune evasion and Host adherence | 31,09 | 14,4 | p < 0.01 |
| | - | NWMN_2203 | secretory antigen precursor SsaA | Immune evasion | 15,24 | 7,87 | p < 0.01 |
| | | | | | | | |
| **Toxins** | hlgB | NWMN_2320 | gamma hemolysin, component B | Cell lysis | 6,94 | 5,11 | p < 0.05 |
| | hlgA | NWMN_2318 | gamma-hemolysin component A | Cell lysis | 12,73 | 8,09 | p < 0.01 |
| | hlgC | NWMN_2319 | gamma-hemolysin component C | Cell lysis | 4,51 | 1,33 | p < 0.05 |
| | glpK | NWMN_1208 | glycerol kinase | glycerol catabolic process | 6,30 | 2,73 | p < 0.01 |
| | lukD | NWMN_1718 | leukocidin LukD precursor | leukocidins | 14,37 | 5,22 | p < 0.05 |
| | lukE | NWMN_1719 | leukocidin LukE precursor | leukocidins | 23,81 | 8,49 | p < 0.05 |
| | lukS | NWMN_1928 | leukocidin/hemolysin toxin subunit S | leukocidins | 15,86 | 10,7 | p < 0.01 |
| | set2nm | NWMN_0389 | superantigen-like protein | Staphylococcal enterotoxin-like toxin | 4,53 | 1,48 | p < 0.01 |
| | set3nm | NWMN_0390 | superantigen-like protein | Staphylococcal enterotoxin-like toxin | 12,47 | 10,8 | p < 0.01 |
| | set8nm | NWMN_0395 | superantigen-like protein | Staphylococcal enterotoxin-like toxin | 7,98 | 2,61 | p < 0.01 |
| | set9nm | NWMN_0396 | superantigen-like protein | Staphylococcal enterotoxin-like toxin | 18,90 | 12,54 | p < 0.01 |
| | - | NWMN_1076 | superantigen-like protein | Staphylococcal enterotoxin-like toxin | 36,53 | 9,92 | p < 0.01 |
| | | | | | | | |
| **Others** | topA | NWMN_1160 | DNA topoisomerase I | DNA topological change | 3,42 | 0,72 | p < 0.01 |
| | - | NWMN_0362 | hypothetical protein | Unknown | 11,20 | 9,47 | p < 0.05 |
| | - | NWMN_0434 | hypothetical protein | Unknown | 5,11 | 2,49 | p < 0.01 |
| | - | NWMN_0644 | hypothetical protein | Unknown | 4,31 | 2,31 | p < 0.05 |
| | - | NWMN_0764 | hypothetical protein | Unknown | 6,11 | 2,52 | p < 0.01 |
| | - | NWMN_0847 | hypothetical protein | Unknown | 3,07 | 0,47 | p < 0.01 |
| | - | NWMN_0925 | hypothetical protein | Unknown | 6,02 | 1,56 | p < 0.01 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | - | NWMN_0958 | hypothetical protein | Unknown | 4,97 | 3,95 | p < 0.05 |
| | - | NWMN_0115 | hypothetical protein | Unknown | 3,27 | 0,08 | p < 0.01 |
| | - | NWMN_0116 | hypothetical protein | Unknown | 6,47 | 2,55 | p < 0.05 |
| | - | NWMN_0118 | hypothetical protein | Unknown | 3,50 | 0,27 | p < 0.01 |
| | - | NWMN_0150 | hypothetical protein | Unknown | 9,28 | 5,63 | p < 0.05 |
| | - | NWMN_1069 | hypothetical protein | Unknown | 8,27 | 2,15 | p < 0.01 |
| | - | NWMN_1070 | hypothetical protein | Unknown | 8,33 | 1,31 | p < 0.01 |
| | - | NWMN_1825 | hypothetical protein | Unknown | 6,19 | 2,58 | p < 0.05 |
| | - | NWMN_1913 | hypothetical protein | Unknown | 4,78 | 2,60 | p < 0.05 |
| | - | NWMN_2332 | hypothetical protein | Unknown | 4,78 | 1,23 | p < 0.01 |
| | - | NWMN_2389 | hypothetical protein | Unknown | 5,78 | 4,03 | p < 0.01 |
| | - | NWMN_2400 | hypothetical protein | Unknown | 3,62 | 0,45 | p < 0.01 |
| | - | NWMN_2545 | hypothetical protein | Unknown | 3,90 | 1,03 | p < 0.05 |

**Table 11: Genes downregulated by SDS stress in strain Newman WT**

| Biological function | Gene | Synonym | Product | Annotated predicted function | Mean fold change | SEM | p value |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| **Biosynthesis process** | - | NWMN_0124 | 4'-phosphopantetheinyl transferase superfamily protein | Transferase | 0,23 | 0,07 | p < 0.01 |
| | - | NWMN_1933 | acetyltransferase, GNAT family protein | Transferase | 0,25 | 0,15 | p < 0.01 |
| | capP | NWMN_0110 | capsular polysaccharide biosynthesis protein CapP | lipopolysaccharide biosynthetic process | 0,26 | 0,13 | p < 0.05 |
| | - | NWMN_0906 | glycosyl transferase, group 1 family protein | Transferase | 0,09 | 0,07 | p < 0.01 |
| | - | NWMN_1852 | nitric oxide synthase oxygenase | Nitric oxide biosynthetic process | 0,33 | 0,09 | p < 0.01 |
| | argF | NWMN_1078 | ornithine carbamoyltransferase | Arginine biosynthesis, | 0,15 | 0,10 | p < 0.01 |
| | | | | | | | |
| **Metabolic pathways** | - | NWMN_0249 | 5'-nucleotidase, lipoprotein e(P4) family protein | acid phosphatase activity | 0,37 | 0,05 | p < 0.05 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | - | NWMN_0659 | anion transporter family protein | Sodium ion transport | 0,16 | 0,05 | p < 0.01 |
| | arcA | NWMN_2534 | arginine deiminase | arginine catabolic process to ornithine | 0,24 | 0,12 | p < 0.01 |
| | arcD | NWMN_2532 | arginine/ornithine antiporter | amino acid transmembrane transporter activity | 0,23 | 0,14 | p < 0.05 |
| | - | NWMN_0130 | branched-chain amino acid transport system II carrier protein | branched-chain amino acid transmembrane transporter activity | 0,30 | 0,05 | p < 0.01 |
| | arcC | NWMN_1079 | carbamate kinase | purine metabolism, glutamate metabolism, arginine and proline metabolism | 0,09 | 0,06 | p < 0.01 |
| | - | NWMN_2457 | cation-transporting ATPase E1-E2 family protein | Involved in copper export | 0,33 | 0,06 | p < 0.01 |
| | uvrA | NWMN_0727 | excinuclease ABC subunit A | transport activity | 0,32 | 0,07 | p < 0.01 |
| | fda | NWMN_2503 | fructose-1,6-bisphosphate aldolase | Glycolysis | 0,26 | 0,08 | p < 0.01 |
| | lacA | NWMN_2099 | galactose-6-phosphate isomerase subunit LacA | lactose metabolism | 0,05 | 0,02 | p < 0.01 |
| | gapB | NWMN_1580 | glyceraldehyde 3-phosphate dehydrogenase 2 | Oxidoreductase | 0,16 | 0,10 | p < 0.05 |
| | copZ | NWMN_2458 | heavy metal-binding protein | copper transport activity | 0,29 | 0,08 | p < 0.01 |
| | ipdC | NWMN_0132 | indole-3-pyruvate decarboxylase | Catalytic activity, magnesium ion binding | 0,18 | 0,15 | p < 0.05 |
| | bsaG | NWMN_1709 | lantibiotic ABC transporter protein | Transport activity | 0,28 | 0,07 | p < 0.05 |
| | arcB | NWMN_2533 | ornithine carbamoyltransferase | Arginine metabolism | 0,15 | 0,15 | p < 0.01 |
| | ulaA | NWMN_0322 | PTS system ascorbate-specific transporter subunit IIC | phosphoenolpyruvate-dependent sugar phosphotransferase system | 0,07 | 0,04 | p < 0.01 |
| | lacF | NWMN_2095 | PTS system, lactose-specific IIA component | sugar:hydrogen symporter activity | 0,17 | 0,07 | p < 0.01 |
| | pdp | NWMN_2040 | pyrimidine-nucleoside phosphorylase | Glycosyltransferase | 0,32 | 0,05 | p < 0.01 |
| | qoxB | NWMN_0929 | quinol oxidase polypeptide I QoxB | Copper ion binding, cytochrome-c oxidase activity, heme binding | 0,33 | 0,03 | p < 0.05 |
| | qoxC | NWMN_0928 | quinol oxidase polypeptide III | cytochrome-c oxidase activity | 0,27 | 0,09 | p < 0.05 |
| | sdhB | NWMN_1062 | succinate dehydrogenase iron-sulfur subunit | tricarboxylic acid cycle, electron carrier activity | 0,27 | 0,06 | p < 0.01 |
| | lacD | NWMN_2096 | tagatose 1,6-diphosphate aldolase | Lactose metabolism, tagatose-6-phosphate kinase activity | 0,16 | 0,11 | p < 0.01 |
| | - | NWMN_0137 | transcriptional regulator | carbohydrate metabolic process | 0,20 | 0,08 | p < 0.01 |
| | | | | | | | |
| **Stress** | agrC | NWMN_1945 | accessory gene regulator protein C | AgrC response regulator | 0,26 | 0,03 | p < 0.01 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **response** | | | | | | | |
| | asp23 | NWMN_2086 | alkaline shock protein 23 | alkaline shock | 0,31 | 0,05 | p < 0.05 |
| | scdA | NWMN_0193 | cell wall biosynthesis protein ScdA | Response to oxidative stress | 0,13 | 0,07 | p < 0.05 |
| | - | NWMN_2291 | transcriptional regulator DegU family protein | NreB/NreC involved in the control of dissimilatory nitrate/nitrite reduction in response to oxygen | 0,26 | 0,04 | p < 0.05 |
| | | | | | | | |
| **Toxins** | - | NWMN_1084 | PSM-β1 | Cytolytic toxin | 0,13 | 0,08 | p < 0.05 |
| | | | | | | | |
| **Immune evasion** | spa | NWMN_0055 | immunoglobulin G binding protein A precursor (protein A) | Immune evasion, antigen, antiphagocytic, virulence | 0,28 | 0,12 | p < 0.01 |
| | | | | | | | |
| **Others** | isaB | NWMN_2537 | immunodominant antigen B | Antigen | 0,20 | 0,11 | p < 0.01 |
| | - | NWMN_2303 | formate/nitrite transporter family protein | DNA damage, repair | 0,25 | 0,03 | p < 0.01 |
| | - | NWMN_0041 | hypothetical protein | Unknown | 0,14 | 0,12 | p < 0.01 |
| | - | NWMN_0047 | hypothetical protein | Unknown | 0,15 | 0,05 | p < 0.01 |
| | - | NWMN_0048 | hypothetical protein | Unknown | 0,18 | 0,12 | p < 0.05 |
| | - | NWMN_0078 | hypothetical protein | Unknown | 0,12 | 0,02 | p < 0.01 |
| | - | NWMN_0256 | hypothetical protein | Unknown | 0,21 | 0,11 | p < 0.01 |
| | - | NWMN_0323 | hypothetical protein | Unknown | 0,06 | 0,03 | p < 0.01 |
| | - | NWMN_0324 | hypothetical protein | Unknown | 0,07 | 0,04 | p < 0.01 |
| | - | NWMN_0325 | hypothetical protein | Unknown | 0,09 | 0,06 | p < 0.01 |
| | - | NWMN_0601 | hypothetical protein | Unknown | 0,20 | 0,14 | p < 0.05 |
| | - | NWMN_0602 | hypothetical protein | Unknown | 0,21 | 0,15 | p < 0.05 |
| | - | NWMN_0660 | hypothetical protein | Unknown | 0,34 | 0,04 | p < 0.01 |
| | - | NWMN_0752 | hypothetical protein | Unknown | 0,19 | 0,13 | p < 0.05 |
| | - | NWMN_0905 | hypothetical protein | Unknown | 0,08 | 0,07 | p < 0.01 |
| | - | NWMN_1689 | hypothetical protein | Unknown | 0,22 | 0,11 | p < 0.05 |
| | - | NWMN_1934 | hypothetical protein | Unknown | 0,22 | 0,15 | p < 0.05 |
| | - | NWMN_2304 | hypothetical protein | Unknown | 0,23 | 0,06 | p < 0.05 |
| | - | NWMN_2484 | hypothetical protein | Unknown | 0,32 | 0,02 | p < 0.05 |
| | - | NWMN_2485 | hypothetical protein | Unknown | 0,28 | 0,10 | p < 0.01 |
| | - | NWMN_2607 | hypothetical protein | Unknown | 0,29 | 0,04 | p < 0.01 |
| | - | NWMN_2608 | hypothetical protein | Unknown | 0,27 | 0,02 | p < 0.01 |

**Table 12: Genes upregulated by SDS stress in the Newman Δ*sae***

| Biological function | Gene | Synonym | Product | Annotated predicted function | Mean fold change | SEM | p value |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| **Biosynthesis pathways** | capC | NWMN_0097 | capsular polysaccharide synthesis enzyme CapC | Peptidyl-tyrosine dephosphorylation | 3,51 | 0,90 | p < 0.05 |
| | - | NWMN_0145 | peptide ABC transporter permease | Peptidoglycan catabolic process | 9,01 | 5,29 | p < 0.01 |
| | - | NWMN_0150 | hypothetical protein | lysine biosynthetic process via diaminopimelate, aspartate kinase activity | 8,74 | 8,92 | p < 0.05 |
| | fadE | NWMN_0170 | acyl-CoA synthetase FadE-like protein | Oxidoreductase, lysine biosynthetic process via diaminopimelate | 4,08 | 1,15 | p < 0.05 |
| | - | NWMN_0171 | acetyl-CoA/acetoacetyl-CoA transferase | Amine and polyamine biosynthesis; betaine biosynthesis via choline pathway | 12,60 | 15,49 | p < 0.05 |
| | | | | | | | |
| **Metabolic pathways** | metB | NWMN_0425 | cystathionine gamma-synthase | Catalytic activity | 14,18 | 3,67 | p < 0.05 |
| | - | NWMN_0513 | chaperone protein HchA | Transferase, ketone body catabolic process | 12,64 | 6,63 | p < 0.05 |
| | - | NWMN_0542 | hypothetical protein | Catalytic activity | 10,37 | 1,72 | p < 0.01 |
| | gapR | NWMN_0740 | glycolytic operon regulator | Chaperone lyase | 5,42 | 2,38 | p < 0.05 |
| | - | NWMN_0834 | hypothetical protein | Carbohydrate binding | 5,00 | 0,68 | p < 0.05 |
| | - | NWMN_0873 | hypothetical protein | N-acetyltransferase activity | 3,90 | 0,53 | p < 0.01 |
| | - | NWMN_0896 | hypothetical protein | Tranferase | 9,40 | 3,67 | p < 0.01 |
| | - | NWMN_0923 | acetyltransferase, GNAT family protein | Oxidoreductase, glycerol-3-phosphate dehydrogenase activity | 12,36 | 7,01 | p < 0.01 |
| | - | NWMN_0958 | hypothetical protein | - | 4,68 | 1,49 | p < 0.01 |
| | - | NWMN_0995 | phage anti-repressor protein | Protein peptidyl-prolyl isomerization | 4,80 | 2,23 | p < 0.05 |
| | - | NWMN_0996 | hypothetical protein | Hydrolase, proteolysis activity | 5,07 | 2,11 | p < 0.01 |
| | - | NWMN_1028 | phage tape measure protein | Nitrogen metabolism; urea degradation; CO(2) and NH(3) from urea | 3,59 | 1,74 | p < 0.05 |
| | - | NWMN_1039 | phage amidase | Carbohydrate binding, carbohydrate metabolic process | 3,29 | 0,06 | p < 0.05 |
| | glpF | NWMN_1207 | glycerol uptake facilitator | Transferase | 4,59 | 0,55 | p < 0.01 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | protein | | | | |
| **Stress response** | glpD | NWMN_1209 | aerobic glycerol-3-phosphate dehydrogenase | Mercury and metal stress regulator sequence-specific DNA binding transcription factor activity | 4,59 | 0,73 | p < 0.01 |
| | msrR | NWMN_1274 | peptide methionine sulfoxide reductase regulator | Response regulator | 2,85 | 0,22 | p < 0.05 |
| | lysC | NWMN_1304 | aspartate kinase | Sensor histidine kinase | 5,99 | 0,90 | p < 0.01 |
| **Transport** | dapB | NWMN_1307 | dihydrodipicolinate reductase | Transport activity | 5,95 | 1,31 | p < 0.01 |
| | - | NWMN_1404 | hypothetical protein | Phosphoenolpyruvate-dependent sugar phosphotransferase system | 3,72 | 0,60 | p < 0.05 |
| **Transcription regulation** | - | NWMN_0037 | hypothetical protein | DNA binding capacity | 5,13 | 2,33 | p < 0.05 |
| **Others** | - | NWMN_1552 | hypothetical protein | | 8,40 | 3,20 | p < 0.05 |
| | - | NWMN_1553 | hypothetical protein | | 46,14 | 26,91 | p < 0.01 |
| | - | NWMN_1621 | hypothetical protein | | 9,61 | 7,68 | p < 0.05 |
| | prsA | NWMN_1733 | peptidyl-prolyl cis/trans-isomerase | | 3,50 | 0,77 | p < 0.01 |
| | - | NWMN_1778 | hypothetical protein | | 9,56 | 6,83 | p < 0.01 |
| | - | NWMN_1779 | hypothetical protein | | 3,59 | 1,12 | p < 0.05 |
| | - | NWMN_1781 | hypothetical protein | DNA binding | 7,25 | 2,05 | p < 0.01 |
| | - | NWMN_1784 | hypothetical protein | | 5,95 | 1,42 | p < 0.05 |
| | - | NWMN_1785 | hypothetical protein | | 4,98 | 2,82 | p < 0.05 |
| | - | NWMN_1786 | hypothetical protein | | 3,56 | 1,12 | p < 0.05 |
| | - | NWMN_1787 | hypothetical protein | | 9,32 | 4,62 | p < 0.01 |
| | - | NWMN_1788 | hypothetical protein | | 11,57 | 1,89 | p < 0.01 |
| | - | NWMN_1789 | phage head protein | | 5,00 | 1,50 | p < 0.05 |
| | - | NWMN_1790 | hypothetical protein | | 7,12 | 0,71 | p < 0.01 |
| | - | NWMN_1791 | phage head morphogenesis protein | | 10,93 | 7,64 | p < 0.01 |
| | - | NWMN_1792 | phage portal protein | | 6,59 | 2,44 | p < 0.05 |

| | | | | | |
|---|---|---|---|---|---|
| - | NWMN_1793 | phage terminase large subunit | 6,31 | 3,25 | p < 0.01 |
| - | NWMN_1794 | phage terminase small subunit | 8,10 | 4,97 | p < 0.01 |
| vraR | NWMN_1822 | DNA-binding response regulator VraR | 7,19 | 3,40 | p < 0.01 |
| vraS | NWMN_1823 | sensor histidine kinase VraS | 9,59 | 5,99 | p < 0.01 |
| - | NWMN_1824 | hypothetical protein | 10,55 | 7,17 | p < 0.05 |
| - | NWMN_1825 | hypothetical protein | 9,91 | 6,64 | p < 0.05 |
| - | NWMN_1834 | hypothetical protein | 5,41 | 2,10 | p < 0.05 |
| - | NWMN_1905 | hypothetical protein | 7,75 | 3,66 | p < 0.01 |
| - | NWMN_1918 | phage anti repressor | 6,15 | 2,84 | p < 0.01 |
| - | NWMN_1929 | succinyl-diaminopimelate desuccinylase | 7,57 | 3,14 | p < 0.01 |
| mtlF | NWMN_2057 | PTS system, mannitol-specific IIBC component | 7,07 | 2,27 | p < 0.01 |
| - | NWMN_2105 | transcriptional regulator MerR family protein | 6,88 | 1,53 | p < 0.01 |
| ureB | NWMN_2189 | urease subunit beta | 12,03 | 4,65 | p < 0.01 |
| - | NWMN_2225 | phosphosugar-binding transcriptional regulator | 4,10 | 0,36 | p < 0.05 |
| - | NWMN_2262 | hypothetical protein | 3,57 | 0,98 | p < 0.05 |
| - | NWMN_2271 | acetyltransferase, GNAT family protein | 4,50 | 0,85 | p < 0.01 |
| - | NWMN_2332 | hypothetical protein | 251,97 | 222,16 | p < 0.01 |
| - | NWMN_2370 | hypothetical protein | 9,92 | 5,44 | p < 0.01 |
| - | NWMN_2456 | hypothetical protein | 3,17 | 0,05 | p < 0.05 |
| - | NWMN_2510 | glycine betaine aldehyde dehydrogenase | 29,38 | 10,30 | p < 0.01 |
| - | NWMN_2545 | hypothetical protein | 6,05 | 2,30 | p < 0.05 |
| - | NWMN_2583 | hypothetical protein | 7,85 | 5,54 | p < 0.01 |
| - | NWMN_2584 | hypothetical protein | 18,76 | 12,90 | p < 0.01 |

**Table 13: Genes downregulated by SDS stress in strain Newman Δ*sae***

| Biological function | Gene | Synonym | Product | Annotated predicted function | Mean fold change | SEM | p value |
|---|---|---|---|---|---|---|---|
| **Biosynthesis pathway** | serS | NWMN_0008 | seryl-tRNA synthetase | Catalyzes the attachment of serine to tRNA(Ser), Aminoacyl-tRNA biosynthesis; selenocysteinyl-tRNA(Sec) biosynthesis | 0,04 | 0,02 | p < 0.01 |
| | pdxS | NWMN_0481 | pyridoxal biosynthesis lyase PdxS | Involved in the production of pyridoxal phosphate, probably by incorporating ammonia into the pyridine ring | 0,22 | 0,13 | p < 0.05 |
| | rplJ | NWMN_0501 | 50S ribosomal protein L10 | Ribosome biogenesis | 0,18 | 0,05 | p < 0.01 |
| | - | NWMN_0906 | glycosyl transferase, group 1 family protein | Transferase | 0,12 | 0,06 | p < 0.01 |
| | | | | | | | |
| **Metabolic pathway** | glpQ | NWMN_0830 | glycerophosphoryl diester phosphodiesterase | Lipid and glycerol metabolism, glycerophosphodiester phosphodiesterase activity | 0,16 | 0,07 | p < 0.01 |
| | qoxD | NWMN_0927 | quinol oxidase polypeptide IV | Oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor | 0,18 | 0,08 | p < 0.01 |
| | qoxC | NWMN_0928 | quinol oxidase polypeptide III | Cytochrome-c oxidase activity | 0,19 | 0,06 | p < 0.01 |
| | qoxB | NWMN_0929 | quinol oxidase polypeptide I QoxB | Aerobic respiration, Cytochrome-c oxidase activity | 0,23 | 0,12 | p < 0.01 |
| | ilvA | NWMN_1348 | threonine dehydratase | Threonine catabolic process | 0,16 | 0,09 | p < 0.01 |
| | lacG | NWMN_2093 | 6-phospho-beta-galactosidase | Lactose catabolic process via tagatose-6-phosphate | 0,12 | 0,09 | p < 0.01 |
| | lacD | NWMN_2096 | tagatose 1,6-diphosphate aldolase | Lactose catabolic process via tagatose-6-phosphate | 0,09 | 0,05 | p < 0.01 |
| | lacA | NWMN_2099 | galactose-6-phosphate isomerase subunit LacA | Lactose and galactose catabolic process | 0,06 | 0,02 | p < 0.01 |
| | - | NWMN_2530 | transcriptional regulator Crp/Fnr family protein | Positively regulates the expression of the arcABDCR operon under anaerobic conditions, thus playing an essential role in arginine catabolism | 0,17 | 0,10 | p < 0.05 |
| | arcC | NWMN_2531 | carbamate kinase | Arginine metabolic process | 0,19 | 0,10 | p < 0.05 |
| | arcD | NWMN_2532 | arginine/ornithine antiporter | Arginine catabolic process | 0,19 | 0,14 | p < 0.05 |
| | arcB | NWMN_2533 | ornithine carbamoyltransferase | Transferase, arginine catabolic process to ornithine | 0,17 | 0,11 | p < 0.05 |
| | arcA | NWMN_2534 | arginine deiminase | Hydrolase, arginine catabolic process to ornithine | 0,19 | 0,11 | p < 0.01 |
| | | | | | | | |
| **Virulence** | sarH1 | NWMN_0056 | accessory regulator A-like protein | Transcriptional regulator that controls expression of some virulence factors in a cell density- | 0,12 | 0,05 | p < 0.05 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | dependent manner | | | |
| | agrB | NWMN_1943 | accessory gene regulator protein B | Essential for the production of a quorum sensing system signal molecule, the autoinducing peptide (AIP) | 0,19 | 0,02 | p < 0.01 |
| | agrC | NWMN_1945 | accessory gene regulator protein C | Receptor histidine protein kinase | 0,19 | 0,11 | p < 0.01 |
| | agrA | NWMN_1946 | staphylococcal accessory gene regulator A | Response regulator, virulence gene activation | 0,13 | 0,01 | p < 0.05 |
| **Transport** | ulaA | NWMN_0322 | PTS system ascorbate-specific transporter subunit IIC | Phosphoenolpyruvate-dependent sugar phosphotransferase system | 0,13 | 0,15 | p < 0.05 |
| | lacE | NWMN_2094 | PTS system, lactose-specific IIBC component | Sugar transport phosphoenolpyruvate-dependent sugar phosphotransferase system | 0,10 | 0,04 | p < 0.01 |
| | lacF | NWMN_2095 | PTS system, lactose-specific IIA component | Sugar transport, hydrogen symporter activity | 0,10 | 0,05 | p < 0.01 |
| | - | NWMN_2303 | formate/nitrite transporter family protein | | 0,18 | 0,08 | p < 0.05 |
| | - | NWMN_2458 | heavy metal-binding protein | Copper ion binding | 0,24 | 0,08 | p < 0.01 |
| **Cell adhesion** | sdrC | NWMN_0523 | Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrC | Cell surface-associated protein which possibly mediates interactions of S.aureus with components of the extracellular matrix of higher eukaryotes | 0,19 | 0,04 | p < 0.05 |
| **Stress response** | scdA | NWMN_0193 | Iron-sulfur cluster repair proteinScdA | Response to oxidative stress | 0,18 | 0,08 | p < 0.01 |
| **Toxins** | sea | NWMN_1883 | enterotoxin type A precursor | Intoxication staphylococcal food poisoning syndrome | 0,22 | 0,06 | p < 0.01 |
| **Transcriptional regulator** | - | NWMN_0636 | AraC family transcription regulator | sequence-specific DNA binding transcription factor activity | 0,23 | 0,08 | p < 0.01 |
| **Others** | - | NWMN_1352 | hypothetical protein | Unknown | 0,23 | 0,12 | p < 0.05 |
| | - | NWMN_1397 | hypothetical protein | Unknown | 0,31 | 0,11 | p < 0.05 |
| | - | NWMN_1690 | hypothetical protein | Unknown | 0,16 | 0,04 | p < 0.01 |
| | - | NWMN_1691 | hypothetical protein | Unknown | 0,11 | 0,06 | p < 0.05 |
| | - | NWMN_1922 | phage exonuclease | Unknown | 0,27 | 0,10 | p < 0.01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| - | NWMN_0022 | hypothetical protein | Unknown | 0,23 | 0,06 | p < 0.05 |
| - | NWMN_0138 | hypothetical protein | Unknown | 0,25 | 0,10 | p < 0.05 |
| - | NWMN_0219 | hypothetical protein | Unknown | 0,21 | 0,15 | p < 0.05 |
| - | NWMN_0323 | hypothetical protein | Unknown | 0,10 | 0,12 | p < 0.01 |
| - | NWMN_0324 | hypothetical protein | Unknown | 0,09 | 0,10 | p < 0.01 |
| - | NWMN_0325 | hypothetical protein | Unknown | 0,09 | 0,10 | p < 0.01 |
| - | NWMN_0614 | hypothetical protein | Unknown | 0,33 | 0,10 | p < 0.05 |
| - | NWMN_1923 | hypothetical protein | Unknown | 0,14 | 0,06 | p < 0.05 |
| - | NWMN_1924 | hypothetical protein | Unknown | 0,15 | 0,05 | p < 0.01 |
| - | NWMN_2113 | hypothetical protein | Unknown | 0,34 | 0,05 | p < 0.05 |
| - | NWMN_2115 | hypothetical protein | Unknown | 0,26 | 0,08 | p < 0.05 |
| - | NWMN_2199 | secretory antigen precursor SsaA | Surface antigen | 0,28 | 0,05 | p < 0.05 |
| - | NWMN_2442 | hypothetical protein | Unknown | 0,25 | 0,14 | p < 0.05 |

**Table 14: Non *sae*-dependent genes upregulated by SDS stress in strain Newman WT**

| Biological function | Gene | Synonym | Product | Mean fold change | SEM | p value |
|---|---|---|---|---|---|---|
| **Biosynthesis pathway** | - | NWMN_0906 | glycosyl transferase, group 1 family protein | 0,12 | 0,06 | p < 0.01 |
| **Metabolic pathway** | ulaA | NWMN_0322 | PTS system ascorbate-specific transporter subunit IIC | 0,13 | 0,15 | p < 0.01 |
| | qoxC | NWMN_0928 | quinol oxidase polypeptide III | 0,19 | 0,06 | p < 0.05 |
| | qoxB | NWMN_0929 | quinol oxidase polypeptide I QoxB | 0,23 | 0,12 | p < 0.05 |
| | lacF | NWMN_2095 | PTS system, lactose-specific IIA component | 0,10 | 0,05 | p < 0.01 |
| | lacD | NWMN_2096 | tagatose 1,6-diphosphate aldolase | 0,09 | 0,05 | p < 0.01 |
| | lacA | NWMN_2099 | galactose-6-phosphate isomerase subunit LacA | 0,06 | 0,02 | p < 0.01 |
| | copZ | NWMN_2458 | heavy metal-binding protein | 0,24 | 0,08 | p < 0.01 |
| | arcD | NWMN_2532 | arginine/ornithine antiporter | 0,19 | 0,14 | p < 0.05 |
| | arcB | NWMN_2533 | ornithine carbamoyltransferase | 0,17 | 0,11 | p < 0.01 |
| | arcA | NWMN_2534 | arginine deiminase | 0,19 | 0,11 | p < 0.01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Stress response** | scdA | NWMN_0193 | cell wall biosynthesis protein ScdA | 0,18 | 0,08 | p < 0.05 |
| | agrC | NWMN_1945 | accessory gene regulator protein C | 0,19 | 0,11 | p < 0.01 |
| | | | | | | |
| **Others** | - | NWMN_0323 | hypothetical protein | 0,10 | 0,12 | p < 0.01 |
| | - | NWMN_0324 | hypothetical protein | 0,09 | 0,10 | p < 0.01 |
| | . | NWMN_0325 | hypothetical protein | 0,09 | 0,10 | p < 0.01 |

**Table 15: Non *sae*-dependent genes downregulated by SDS stress in strain Newman WT**

| Biological function | Gene | Synonym | Product | Mean fold change | SEM | p value |
|---|---|---|---|---|---|---|
| **Biosythetic pathway** | - | NWMN_2510 | glycine betaine aldehyde dehydrogenase | 6,71 | 3,07 | p < 0.05 |
| | - | NWMN_0171 | acetyl-CoA/acetoacetyl-CoA transferase | 12,42 | 5,30 | p < 0.01 |
| | | | | | | |
| **Metabolic pathway** | gapR | NWMN_0740 | glycolytic operon regulator | 8,59 | 4,34 | p < 0.01 |
| | - | NWMN_2225 | phosphosugar-binding transcriptional regulator | 2,62 | 0,08 | p < 0.01 |
| | glpD | NWMN_1209 | aerobic glycerol-3-phosphate dehydrogenase | 6,85 | 0,91 | p < 0.01 |
| | lysC | NWMN_1304 | aspartate kinase | 19,46 | 15,15 | p < 0.05 |
| | fadE | NWMN_0170 | acyl-CoA synthetase FadE-like protein | 10,17 | 4,95 | p < 0.05 |
| | - | NWMN_0923 | acetyltransferase, GNAT family protein | 4,38 | 1,22 | p < 0.05 |
| | - | NWMN_2271 | acetyltransferase, GNAT family protein | 5,81 | 3,01 | p < 0.01 |
| | | | | | | |
| **Stress response** | - | NWMN_2105 | transcriptional regulator MerR family protein | 8,19 | 0,84 | p < 0.01 |
| | vraS | NWMN_1823 | sensor histidine kinase VraS | 3,59 | 0,41 | p < 0.05 |
| | | | | | | |
| **Transport** | glpF | NWMN_1207 | glycerol uptake facilitator protein | 8,23 | 2,11 | p < 0.01 |
| | | | | | | |
| **Others** | - | NWMN_0958 | hypothetical protein | 4,97 | 3,95 | p < 0.05 |
| | - | NWMN_0150 | hypothetical protein | 9,28 | 5,63 | p < 0.05 |
| | - | NWMN_1825 | hypothetical protein | 6,19 | 2,58 | p < 0.05 |
| | - | NWMN_2332 | hypothetical protein | 4,78 | 1,23 | p < 0.01 |

| | | | | | |
|---|---|---|---|---|---|
| - | NWMN_2545 | hypothetical protein | 3,90 | 1,03 | $p < 0.05$ |

# 7. Abbreviations

| | |
|---|---|
| **Δ** | deletion (delta) |
| **aa** | amino acid |
| **APS** | ammonium peroxide sulphate |
| **ATP** | adenosine 5‘-triphosphate |
| **bp** | base pair |
| **BSA** | bovine serum albumin |
| **cDNA** | complementary DNA |
| **cfu** | colony forming units |
| **$CO_2$** | carbon dioxide |
| **$dH_2O$** | distilled water |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | deoxyribonucleic acid |
| **Dnase** | Deoxyribonuclease |
| **dNTP** | deoxynucleotide triphosphate |
| **E. coli** | Escherichia coli |
| **EDTA** | ethylene diamine tetraacetic acid |
| **Ery** | Erythromycin |
| **EtOH** | Ethanol |
| **FACS** | flourescence activated cell sorting |
| **g** | Gramm |
| **h** | Hour |
| **HEPES** | *N*-(2-hydroxyethyl)-piperazine-*N‘*-2-ethasulfonic acid |
| **HSA** | human serum album |
| **kanA** | Kanamycin |
| **kb** | kilo base pair |
| **kDa** | kilo-dalton |
| **l** | Liter |
| **M** | Molar |
| **mg** | Milligram |
| **min** | Minute |
| **mRNA** | messenger RNA |
| **MRSA** | methicillin resistant *Staphylococcus aureus* |
| **MSSA** | methicillin susceptible Staphylococcus aureus |
| **MW** | molecular weight |
| **NEB** | New England Biolabs |
| **nm** | Nanometer |
| **nm** | Nanometer |
| **O/N** | Overnight |
| **°C** | degree celcius |
| **OD** | optical density |
| **PAGE** | polyacrylamide gel electrophoresis |
| **PBS** | phosphate buffered saline |
| **PCR** | polymerase chain reaction |
| **pH** | hydrogen potential |
| **RNA** | ribonucleic acid |
| **RNase H** | Ribonuclease H |
| **rpm** | revolutions per minute |

| | |
|---|---|
| **RT** | room temperature |
| **RT-PCR** | reverse transcriptase polymerase chain reaction |
| **s** | Second |
| **SDS** | sodium dodecyl sulphate |
| ***Taq*** | thermus aquaticus |
| **TEMED** | N, N, *N'*, *N'*-tetramethylethylendiamide |
| **Tet** | Tetracycline |
| **Tris** | tris-(hydroxymethyl)-aminomethane |
| **U** | Unit |
| **UV** | Ultraviolet |
| **µg** | Microgram |
| **µl** | Microliter |

# 8. Publications and Conference contributions

**Parts of this work are submitted for publication in peer reviewed journals and have been presented at international meetings as oral or poster presentations (indicated by asterix*)**

**Publications:**

*__Phuti E Makgotlho__, Gabriella Marincola, Daniel Schäfer, Tobias Geiger, Elizabeth Wasserman, Christiane Wolz, Wilma Ziebuhr W, Bhanu Sinha. SDS interferes with SaeS signaling independently of SaePQ. PLos One, 8: e71644.

Britta Ballhausen, Philipp Jung, André Kriegeskorte, **Phuti Edward Makgotlho**, Ulla Ruffing, Lutz von Müller, Robin Köck, Georg Peters, Matthias Herrmann, Wilma Ziebuhr, Karsten Becker, Markus Bischoff. LA-MRSA CC398 differ from classical community acquired-MRSA and hospital acquired-MRSA lineages: functional analysis of infection and colonization processes.Accepted for publication in *International Journal of Medical Microbiology*.

Heidi Orth, Zubeida Salaam-Dreyer, **Edward Makgotlho**, Wilhelm Oosthuysen, Bhanu Sinha, Elizabeth Wasserman. Characterization of *Staphylococcus aureus* bacteraemia at Tygerberg hospital. (2013). *South African Journal of Epidemiology and Infection*. 28 (1)

**Phuti E. Makgotlho**, Marleen M. Kock, Anwar Hoosen, Ruth Lekalakala, Shaheed Omar, Michael Dove, Marthie M. Ehlers. Molecular identification and genotyping of MRSA isolates from Steve Biko academic hospital. (2009). *FEMS Immunology and Microbiology*. 57, 104-115

Ehlers, MM, Veldsman, C, **Makgotlho PE**, Dove MG, Hoosens, AA and Kock, MM Detection of $bla_{SHV}$, $bla_{TEM}$ and $bla_{CTX-M}$ antibiotic resistance genes in randomly selected bacterial pathogens from the Steve Biko Academic Hospital. (2009). *FEMS Immunology and Medical Microbiology* 56, 191-196

**Conferences:**

***Phuti E Makgotlho**. Strongly elevated *sae* transcription in response to sub-inhibitory SDS treatment in clinical MRSA isolate ST239-635/93 (EMRSA SCC*mec* III). Oral presentations at the 2nd international IRTG Meeting, Kloster Banz, Germany (14-15 May 2010); Host-pathogen interactions in bacterial infections Greifswald, Germany (01-03 June 2010) and at the Symposium der Fachgruppe Mikrobielle Pathogenität Bad Urach, Germany (21-23 June 2010)

***Phuti E Makgotlho**. Effect of defined stress stimuli on the *S. aureus* Sae system. Oral presentation at the 3rd international IRTG Meeting, Stellenbosch (24-27 February 2011)

***Phuti E Makgotlho**. Identification of novel *sae* transcripts and their functional role in *S. aureus* under SDS stress condition. Poster presentation at the IRTG 1522 International Symposium HIV and associated infectious diseases", Würzburg, Germany

***Phuti E Makgotlho**, Daniel Schäfer, Heidi Linß, Elizabeth Wasserman, Knut Ohlsen, Christiane Wolz, Bhanu Sinha. Strongly elevated *sae* transcription in response to sub-inhibitory SDS treatment in clinical MRSA isolate ST239-635/93 (EMRSA SCC*mec* III). Poster presentation at ISSSI September 2010, (Bath, UK)

Heidi Orth, Zubeida Salaam Dreyer, **Edward Makgotlho**, Bhanu Sinha, Elizabeth Wasserman. Genotypic characterization of *Staphylococcus aureus* isolates causing bacteraemia at Tygerberg Hospital, Western Cape Province, South Africa. Poster presentation (P824) at ECCMID 7-10 May 2011, Milan, Italy

W.F. Oosthuysen, **P.E. Makgotlho**, H. Orth, C. Lombard, B. Sinha and E. Wasserman. Molecular characterization and *in vitro* analysis of selected *S. aureus* clinical isolates from Tygerberg Hospital, South Africa. Poster presentation at "Verbraucherschutz in DART, Forshungserkenntnisse und-perspektiven zu Antibiotikaresistenzen 22-23 May 2012, Berlin

# 9. Curriculum vitae

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**EDUCATION:**

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| April 2009-(Current): | University of Wuerzburg (Germany)<br>PhD (Microbiology)<br>Certificate in Appropriate Laboratory Technologies endorsed by the Medical Mission Institute, Wuerzburg |
| January 2006-March 2009 | University of Pretoria<br>MSc (Medical Microbiology)<br>Good Clinical Practice Certificate (AGCP accreditation)<br>Research methodology course (School of Public Health) |
| January 2005-December 2005 | University of the Western Cape<br>BSc Honours (Medical Bioscience) |
| January 2002-December 2004 | University of the Western Cape<br>BSc (Medical Bioscience) |

**PROFESSIONAL EXPERIENCE:**

| | |
|---|---|
| April 2012-Current: | University of Würzburg, Germany<br>Institute of Hygiene and Microbiology<br>Research PhD student scientist<br>*In vitro* characterization of Zoonotic *S. aureus* isolates (Federal Ministry of Education and Research project) |
| January 2006-Dec 2008 | University of Pretoria<br>Intern scientist, involved in assistance of supervision and training of medical microbiology undergraduate and postgraduate students |
| January 2005-Dec 2005 | University of Western Cape<br>Student assistant, support in organizing of medical microbiology practicals for undergraduate students in the department |

**RESEARCH EXPERIENCE:**

| | |
|---|---|
| April 2009-March 2012: | University of Würzburg (Germany)<br>Institute of Hygiene and Microbiology<br>PhD thesis: Molecular characterization of the staphylococcal two component system *sae* and its role in the regulation of the adhesin Eap under SDS stress stimulation |
| January 2006-March 2009 | University of Pretoria<br>Department of Medical Microbiology<br>MSc thesis: Molecular characterization of MRSA strains from Steve Biko Academic Hospital |
| January 2003-December 2005 | University of the Western Cape<br>Department of Medical Biosciences<br>BSc Hons dissertation: Inhibition of *C. albicans* by *E. Coli* through quorum sensing |

**AWARDS AND HONORS:**

| | |
|---|---|
| April 2009-March 2012 | International Research Training Group 1522 DFG Scholarship for doctoral studies in HIV/AIDS and associated Infectious Diseases in South Africa |
| November 2010 | Best publication by a young researcher, runner-up 2009. University of Pretoria Faculty of Health Sciences (non-clinical) |
| January 2008-December 2008 | University of Pretoria, NRF freestanding Masters Scholarship |
| January 2007-December 2007 | University of Pretoria, Medical Research Council Local Masters Scholarship |

**PROFESSIONAL MEMBERSHIPS:**

| | |
|---|---|
| April 2012-December 2012 | MedVet Staph project: Principal Investigator, Wilma Ziebuhr |
| April 2009-December 2012 | Associate member: SFB/Transregio34: Pathophysiology of Staphylococci, Germany |

| | |
|---|---|
| April 2009-current | Graduate School of Life Sciences, University of Würzburg, Germany |
| April 2009-2012 | International Research Training Group 1522 "HIV/Aids and associated infectious diseases in Southern Africa" (http://www.gk-1522.uni-wuerzburg.de/home/) |
| March 2006-2014 | Health Professions Council of South Africa; intern medical scientist, MW S 0006297 |

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