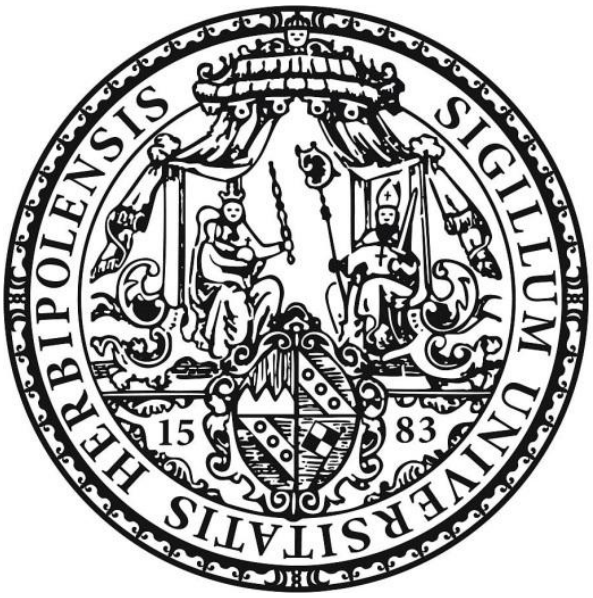

The role of the non-ribosomal peptide synthetase AusAB and its product phevalin in intracellular virulence of *Staphylococcus aureus*

Die Rolle der nicht-ribosomalen Peptidsynthetase AusAB und ihres Produktes Phevalin in der intrazellulären Virulenz von *Staphylococcus aureus*

Doctoral thesis for a doctoral degree
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1. Summary

1.1 Abstract

Staphylococcus aureus is a prevalent commensal bacterium which represents one of the leading causes in health care-associated bacterial infections worldwide and can cause a variety of different diseases ranging from simple abscesses to severe and life threatening infections including pneumonia, osteomyelitis and sepsis.

In recent times multi-resistant strains have emerged, causing severe problems in nosocomial as well as community-acquired (CA) infection settings, especially in the United States (USA). Therefore *S. aureus* has been termed as a superbug by the WHO, underlining the severe health risk originating from it. Today, infections in the USA are dominated by *S. aureus* genotypes which are classified as USA300 and USA400, respectively. Strains of genotype USA300 are responsible for about 70% of the CA infections.

The molecular mechanisms which render *S. aureus* such an effective pathogen are still not understood in its entirety. For decades *S. aureus* was thought to be a strictly extracellular pathogen relying on pore-forming toxins like α-hemolysin to damage human cells and tissue. Only recently it has been shown that *S. aureus* can enter non-professional phagocytes, using adhesins like the fibronectin-binding proteins which mediate an endocytotic uptake into the host cells. The bacteria are consequently localized to endosomes, where the degradation of enclosed bacterial cells through phagosome maturation would eventually occur.

S. aureus can avoid degradation, and translocate to the cellular cytoplasm, where it can replicate. The ability to cause this so-called phagosomal escape has mainly been attributed to a family of amphiphilic peptides called phenol soluble modulins (PSMs), but as studies have shown, they are not sufficient.

In this work I used a transposon mutant library in combination with automated fluorescence microscopy to screen for genes involved in the phagosomal escape process and intracellular survival of *S. aureus*. I thereby identified a number of genes, including a non-ribosomal peptide synthetase (NRPS). The NRPS, encoded by the genes *aus*A and *aus*B, produces two types of small peptides, phevalin and tyrvalin. Mutations in the *aus*AB genes lead to a drastic decrease in phagosomal escape rates in epithelial cells, which were readily restored by genetic complementation in trans as well as by supplementation of synthetic phevalin. In leukocytes, phevalin interferes with calcium fluxes and activation of neutrophils and promotes cytotoxicity of intracellular bacteria in both, macrophages and neutrophils. Further *aus*AB is involved in survival and virulence of the bacterium during mouse lung pneumoniae.

The here presented data demonstrates the contribution of the bacterial cyclic dipeptide phevalin to *S. aureus* virulence and suggests, that phevalin directly acts on a host cell target to promote cytotoxicity of intracellular bacteria.

1.2 Zusammenfassung

Staphylococcus aureus ist ein weit verbreitetes kommensales Bakterium, welches zugleich einer der häufigsten Verursacher von Krankenhausinfektionen ist, und eine Reihe verschiedener Krankheiten, angefangen bei simplen Abszessen, bis hin zu schweren Erkrankungen wie Lungenentzündung, Osteomylitis und Sepsis verursachen kann.
Das Risiko durch nosokomiale sowie epidemische *S. aureus* Infektionen ist in den vergangenen Jahren weiter gestiegen. Dazu beigetragen hat das Auftreten multiresistenter und hoch cytotoxischer Stämme, vor allem in den USA. Als Konsequenz hat die WHO *S. aureus* inzwischen als „Superbug" tituliert und als globales Gesundheitsrisiko eingestuft.

Bei CA-Infektionen dominieren die Isolate der Klassifizierung USA300 und USA400, wobei den Erstgenannten bis zu 70% aller in den USA registrierten CA-MRSA Infektionen der letzten Jahre zugesprochen werden.

Lange Zeit wurde angenommen, dass *S. aureus* strikt extrazellulär im Infektionsbereich vorliegt und die cytotoxische Wirkung von z.B. α-Toxin für Wirtszelltod und Gewebeschädigungen verantwortlich ist. Erst vor kurzem wurde festgestellt, dass *S. aureus* auch durch fakultativ phagozytotische Zellen, wie Epithel- oder Endothelzellen, mittels zahlreicher Adhäsine aufgenommen wird. Die Aufnahme in die Zelle erfolgt zunächst in ein Phagoendosom, in dem die Pathogene durch antimikrobielle Mechanismen abgebaut würden.

Um dies zu verhindern, verfügt *S. aureus* über Virulenzfaktoren, welche die endosomale Membran schädigen. Die Bakterien gelangen so in das Zellzytoplasma, wo sie sich vervielfältigen können, bevor die Wirtszelle schließlich getötet wird. Eine wichtige Funktion in diesem Vorgang konnte bereits in mehreren Studien den Phenol löslichen Modulinen (PSM) zugesprochen werden, Arbeiten unserer Gruppe deuten jedoch darauf hin, dass diese nicht alleine für den phagosomalen Ausbruch von *S. aureus* verantwortlich sind.

In dieser Arbeit verwendete ich eine Transposon Mutantenbibliothek des *S. aureus* Stammes JE2 (USA300) in Verbindung mit automatisierter Fluoreszenzmikroskopie, um Gene zu identifizieren, die den phagosomalen Ausbruch von *S. aureus* beeinflussen. Unter den Mutanten, welche eine Minderung der Ausbruchsraten zeigten, fanden sich auch Mutanten in beiden Genen eines Operons, welches für die nicht-ribosomale Peptidsynthetase AusA/B codiert, die die beiden Dipeptide Phevalin und Tyrvalin produziert. Verminderte Ausbruchsraten konnten sowohl durch genetische Komplementation als auch mittels des Zusatzes synthetischen Phevalins wiederhergestellt werden.

In Leukozyten verhindert Phevalin effizienten Calcium-Flux und die Aktivierung von Neutrophilen. Zudem fördert Phevalin die Cytotoxizität intrazellulärer Bakterien sowohl in Makrophagen, als auch Neutrophilen. Darüber hinaus konnten wir zeigen, dass die NRPS AusAB

und ihre Produkte eine Rolle beim Überleben der Bakterien während einer Infektion im Tiermodell einnehmen.

Die hier präsentierten Daten hinsichtlich des Einflusses von Phevalin auf Virulenz und der Interaktion zwischen Wirt und Pathogen lassen den Schluss zu, dass Phevalin direkt auf einen Wirtszellfaktor wirkt, um die Cytotoxicität intrazellulärer Bakterien zu stärken.

2. Introduction

2.1 *Staphylococcus aureus*

2.1.1 General information

Staphylococcus aureus is a gram-positive, facultative anaerobic coccoid bacterium with a size of 0.8 – 1.2 µm, a genome of approx. 2.8 MB and a GC-content of 33 mol%, that belongs taxonomically to the phylum of Firmicutes and the class of Bacilli (Kuroda *et al.*, 2001). The catalase positive and nitrate reducing Staphylococceae were first identified in findings of Sir Alexander Ogston, isolating the bacteria from the pus of abscesses in humans and were later described by the German researcher Friedrich Julius Rosenbach (1884) who initially classified them in their own genus *Staphylococcus* (Ogston, 1882; Rosenbach, 1884). While Zopf (1885) placed the Staphylococci in the genus of *Micrococcus* this was reversed by Flügge in 1886 (Flügge, 1886; Götz *et al.*, 2006). Only later the three genera *Staphylococcus, Micrococcus* and *Planococcus* were combined in the family Micrococcaceae (Götz *et al.*, 2006). In 2010 the Staphylococci have taxonomically been described as part of the family Staphylococcaceae which, among others, includes *Macrococcus* (Euzeby, 2010). While growing on solid agar plates, *S. aureus,* shows light yellow to orange colored colonies, caused by the anti-oxidant carotenoid staphyloxanthin (Clauditz *et al.*, 2006).

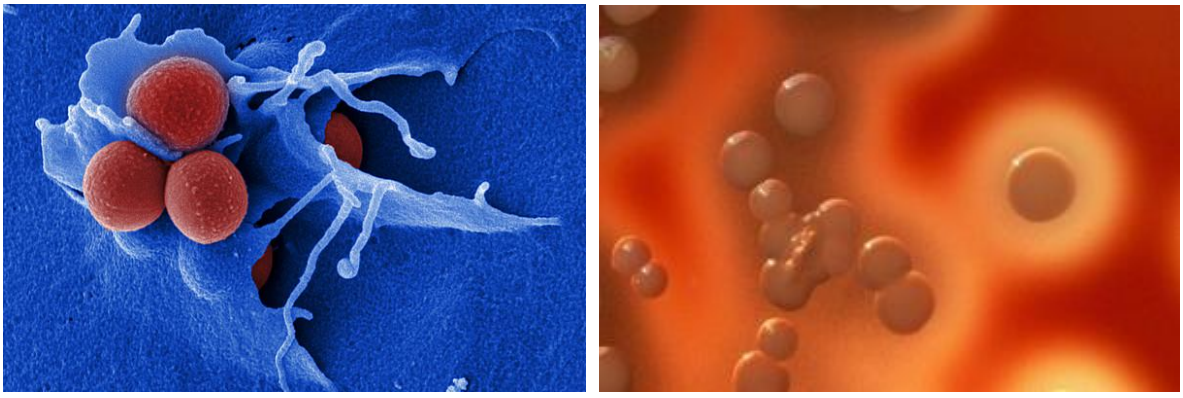

Figure 2.1: Microscopic and macroscopic images of *S. aureus*

Electron-microscopic depiction of *S. aureus* cells on eukaryotic cell membrane and of *S. aureus* colonies on blood agar. ([1]helmholtz-hzi.de; [2]bacteriainphotos.com)

As is the case in other pathogenic microbial organisms, the use of antibiotics in the treatment of bacterial infections in humans has led to the emergence of antibiotic resistance also in *S. aureus*. While infections with *S. aureus* were already untreatable with the first antibiotic in use,

Penicillin, shortly after the introduction of the β-lactam in 1945, the occurrence of resistance against the β-lactam derivative methicillin dates to the early 1960s (Jevons, 1961; Sutherland and Rolinson, 1964) with methicillin being introduced into the clinic only in 1959. Methicillin resistance in *S. aureus* strains is largely dependent on the acquisition of the Staphylococcal Cassette Chromosome (SCC) mec, a large transmissible element carrying the methicillin resistance gene *mec*A, that was likely present in *S. aureus* even before the isolation of the first MRSA strains due to horizontal gene transfer (IWC-SCC, 2009). MecA confers Methicillin and Penicillin resistance by encoding for a low affinity Penicillin-binding protein PBP2a (Ubukata *et al.*, 1990). The emergence of strains with an intermediate susceptibility or resistance to Vancomycin (VISA and VRSA, respectively) currently worsens the problem of antibiotic resistance in clinically relevant strains further (Appelbaum, 2007).

2.1.2 Prevalence of *S. aureus*

2.1.2.1 Colonization

S. aureus strains can be found as both, pathogen and commensal in the human body, colonizing skin, skin glands, gastrointestinal tracts and mucous membranes, especially the epithelium of the anterior nasal vestibule (Cole *et al.*, 2001; den Heijer *et al.*, 2013). Estimates of how widespread *S. aureus* is as a permanent commensal organism with healthy individuals range from 20-30%. Meanwhile up to 60% of the population are intermittent carriers, harboring a strain of *S. aureus* for only a period of time (Kluytmans *et al.*, 1997). Genetic and other host determinants of *S. aureus* carriage are not fully understood, with different studies suggesting a role of factors like age, gender, smoking habits, diet, drug addiction and pre-existing diseases, particularly skin diseases as well as rheumatoid arthritis (Choi *et al.*, 2006; Laudien *et al.*, 2010; Johannessen *et al.*, 2012; Olsen *et al.*, 2012).

Further, up to two percent of the general population is estimated to be permanently carrying MRSA strains, with people working and/or living in an environment associated with livestock showing prevalence rates exceeding 25%. Strains of the clonal complex CC398 make up for the vast majority of the isolates in this setting (Casey *et al.*, 2013; Bosch and Schouls, 2015).

2.1.2.2 Epidemiology

Today, the prevalence of MRSA has developed from mostly nosocomial infections to community-acquired and livestock-associated infections, which are frequently associated with enhanced virulence and transmissibility (Klevens *et al.*, 2006; Mediavilla *et al.*, 2012). In 2005, an

estimated 95,000 severe cases of MRSA bacteremia occurred in the US alone and in 2008 more than 60% of all *S. aureus* isolates from intensive care units (ICUs) in the US were MRSA (Klevens *et al.*, 2007; Boucher and Corey, 2008). However, according to the Center for Disease Control in Atlanta (CDC) cases of hospital-acquired MRSA with a severe course of infection declined by 54% from 2007 to 2011, while cases of CA-MRSA have only dropped by 5%, indicating that measures taken in hospital hygienic standards and patient screening for MRSA carriage lead to less infections, while CA-acquired *S. aureus* remain problematic (Dantes *et al.*, 2013).

While increased sensibility in handling hospitalized MRSA cases as well as raised awareness regarding constantly colonized hospital personnel as a potential risk factor may have lowered HA-MRSA cases, still, patients carrying an MRSA strain commensally have a 13 times higher risk of infection during a stay in a hospital, than patients with MSSA isolates (Cosgrove *et al.*, 2005). *S. aureus* carriers harbor an increased risk of infection with their own strain up to three months after hospitalization especially when the immune system is compromised (Huang and Platt, 2003). While this risk can be decreased by a pre-surgical eradication of the bacteria by use of antibiotics, *S. aureus* remains a leading cause of hospital-acquired infections today (van Rijen *et al.*, 2008). The severity of an infection with *S. aureus* may vary a lot and can lead to superficial soft tissue and skin infections as well as severe infections such as sepsis, necrotizing pneumonia and osteomyelitis (Lowy, 1998).

2.1.2.3 *S. aureus* strains of sequence type 8: The success of USA300

Among the strains associated with CA-acquired *S. aureus*, strains of the sequence type 8 play a major role. In an attempt to establish a national database in the United States for methicillin-resistant *S. aureus,* different strains of sequence type (ST) 8 were classified according to their pulse-field gel electrophoresis (PFGE) patterns and have been designated “USA100” through “USA 800”(McDougal *et al.*, 2003; CDC, 2003). USA300, undoubtedly the most frequent strain identified in CA-associated infections today and during the last 15 years, differs in roughly 20 genes from USA500, the strain which originated evolutionary within the clonal complex 8 by acquisition of a SCCmec type IV cassette (Tenover *et al.*, 2006; Li *et al.*, 2009a). Initially only resistant to semi-synthetic penicillins and macrolides, USA300 over time has acquired further mobile genetic elements and thereby gained more resistances (Han *et al.*, 2007). Resistance to clyndamycin has been acquired with *erm*A and *erm*C, while acquisition of *tet*K and *tet*M led to tetracycline resistance (Tenover and Goering, 2009).

Epidemic USA300 MRSA strains have become the predominant isolate in severe community-acquired *S. aureus* bloodstream infections in the US and have been associated with an increase in skin and soft tissue infections (Tenover and Goering, 2009; Diekema *et al.*, 2014).

First reported by the CDC during an outbreak of MRSA in Colorado among football players in 2000 (CDC, 2003), strains of the USA300 genotype today are well known to be resident in Europe and all over the world with cases reported in France, Switzerland, Colombia, Japan and Germany (Witte *et al.*, 2008; Higuchi *et al.*, 2010; Seidl *et al.*, 2014; van der Mee-Marquet *et al.*, 2015; Bartoloni *et al.*, 2015).

2.2 Virulence factors of *S. aureus*

2.2.1 Toxins

During an infection of the human body, *S. aureus* faces a number of different host immune defense mechanisms ranging from antimicrobial peptides, blood coagulation, the complement system and opsonizing antibodies to phagocytes such as macrophages and PMN, fully equipped to trap and kill invading bacteria. In order to successfully establish infections in the human body *S. aureus* possesses a large arsenal of virulence factors, including a wide range of toxins. Said toxins possess many distinct functions and grant *S. aureus* to attack and defend itself in a number of different ways.

2.2.1.1 Alpha-hemolysin (α-toxin)

Alpha-hemolysin (α-toxin) of *Staphylococcus aureus* is a small beta-barrel structured cytotoxin that functions as a prime example for pore-forming toxins involved in staphylococcal virulence. Research on the secreted and water-soluble toxin has been performed for decades and prompted the understanding that α-toxin plays a crucial part in toxin mediated hemolysis of erythrocytes by *S. aureus* and lysis of other eukaryotic cell types, including keratinocytes and monocytes (Bhakdi *et al.*, 1988; Suttorp *et al.*, 1988; Bhakdi *et al.*, 1989; Walev *et al.*, 1993).

The lytic effect of the 319 amino acid protein is based on the receptor mediated formation of a ring-like, heptameric and water-filled channel with a central pore of approximately 26 Ångström (Å), allowing the efflux of water, ions, as well as low molecular weight molecules of up to 4 kDa from the cell (Bhakdi and Tranum-Jensen, 1991; Song *et al.*, 1996).

The impact of Hla on the development of disease caused by *S. aureus* infection can be regarded as fundamental. *hla*-deficient mutant strains show attenuation in a wide variety of murine disease models, including pneumonia, skin infection, sepsis, endocarditis, infections of the central nervous system and the cornea (Menzies and Kernodle, 1996; Bayer *et al.*, 1997;

O'Callaghan *et al.*, 1997; Kielian *et al.*, 2001; Bubeck Wardenburg *et al.*, 2007b; Kennedy *et al.*, 2010).

The mechanism of action of Hla is dependent on host cell factors functioning as receptors for the secreted toxin monomers. While initially clustered phosphocholine groups and more specifically sphingomyelin were thought to be the defining factor in Hla binding, oligomerization and consequent lysis of the cellular membrane, more recent research identified the metalloprotease A Disintegrin And Metalloprotease (ADAM10) as a high affinity proteinaceous receptor (Valeva *et al.*, 2006; Wilke and Bubeck Wardenburg, 2010). Conditional knockouts in ADAM10 lead to strongly attenuated virulence, explained by the observation, that binding of Hla to epithelial cells prompts an upregulation of the metalloprotease function of ADAM10, causing an increased cleavage of its principal substrate E-cadherin, and consequent damage to adherent junctions as well as the epithelial tissue barrier function (Maretzky *et al.*, 2005; Inoshima *et al.*, 2011; Inoshima *et al.*, 2012).

2.2.1.2 Beta-hemolysin (β-toxin)

Staphylococcus aureus β-toxin is a neutral sphingomyelinase (SMase) initially identified as such in 1963 by (Doery *et al.*, 1963), but probably better known for its hemolytic properties, which coined the toxin the "hot-cold" hemolysin, as beta-toxin binds to red blood cells at 37°C but only lyses them with a consequent exposure to a 4°C cold shock.

The exotoxin possesses a molecular mass of 35 kDa and a complex structure which is closely related to those of SMases in *Staphylococcus schleiferi* and *Bacillus cereus* (Huseby *et al.*, 2007). Its prevalence in human nasal or septicemia isolates is rather low with 11% and 13% respectively, which is caused by a prophage insertion in the genetic locus of beta-toxin in many strains commonly associated with humans (Aarestrup *et al.*, 1999; Diep *et al.*, 2006b). Still, different *hlb*-negative phenotypes have been described, depending on the exact insertion position of the phage (Coleman *et al.*, 1991). The phage most often identified to insert in the beta-toxin genomic region is phage ϕSa3mw, which carries the innate immune-evasion cluster (IEC). The IEC transcribes among others for the immune modulatory gene CHIPS, the staphylokinase (SAK) and the staphylococcal complement inhibitor (SCIN) (van Wamel *et al.*, 2006). IEC genes are important factors in the colonization of the host by *S. aureus* as they function in the evasion of the host immune system. During the course of an infection however, the ϕSa3 prophage has been described to excise from its position in the beta-toxin locus and restore beta-hemolysin production, as was shown in patients suffering from cystic fibrosis (CF) and bacteremia (Goerke *et al.*, 2004; Goerke *et al.*, 2006b; Goerke *et al.*, 2007).

The switch from β-toxin suppression to production, which is thought to be caused by host immune system pressure or antibiotic treatment, may represent an adaptation of the bacterium during differing stages of an infection (Goerke *et al.*, 2006a). While IEC-derived factors might be involved in the protection of small initial populations of *S. aureus* in the early stages of an infection, beta toxin, which shows cytotoxicity against different types of leukocytes and is, via its DNA-binding properties, involved in biofilm formation, could be beneficial as the infection has progressed to levels where pure evasion is no longer an option and the hemolytic properties of beta-toxin weigh in more (Marrack and Kappler, 1990; Walev *et al.*, 1996; Marshall *et al.*, 2000; Huseby *et al.*, 2007; Huseby *et al.*, 2010). As the exact mechanism of the involvement of beta-toxin in various chronic and acute diseases in both, animals and humans, has yet to be shown, other studies suggest functions of the toxin also in colonization of skin and mucosal surfaces, thereby indicating a wide spectrum of β-toxin involvement in *S. aureus* pathogenicity (Hedstrom and Malmqvist, 1982; O'Callaghan *et al.*, 1997; Aarestrup *et al.*, 1999; Diep *et al.*, 2006a; Katayama *et al.*, 2013; Salgado-Pabon *et al.*, 2014).

2.2.1.3 Leukocidins

Leukocidins are bi-component pore-forming toxins that are employed by *S. aureus* in the defense against host leukocytes. A total of six leukocidins have been described, three of them being conserved among all known *S. aureus* strains (HlgAB, HlgCB and LukDE) and 5 being produced by most of the highly cytolytic and clinically relevant strains (HlgAB, HlgCB, LukDE, Panton-Valentine leukocidin (PVL) and LukAB/GH) while family protein number six, LukMF, is exclusively known in zoonotic strains (Alonzo and Torres, 2014).

To reach functionality, all leukocidins require dimerization of two water soluble monomers. The monomers are characterized as S- (slow) and F-subunits (fast) respectively (Finck-Barbancon *et al.*, 1991). In case of γ-hemolysin, both possible monomer combinations (HlgAB and HlgBC) share HlgB as the F-subunit, while HlgA and HlgC constitute separate S-subunits (Kamio *et al.*, 1993). S- and F-subunit of each leukocidin are transcribed in one operon from the same promoter, *hlg*AB being the exception, where the *hlg*A gene lies upstream of the *hlg*B/*hlg*C operon (Alonzo and Torres, 2014). Dimerization occurs almost exclusively at host cell receptors. The complete range of receptors for the leukocidins is poorly understood. Research points towards a set of proteinaceious receptors like the G-protein coupled chemokine receptors CXCR1, CXCR2 for both, LukED and HlgAB. LukED further also targets CCR5 while HlgAB uses CCR2. The human PMN and monocyte associated chemokine receptors C5aR and C5L2R are receptors of LukSF-PV and HlgCB while the Mac-1/CR3 human integrin CD11b is the target of LukAB (GH) (Alonzo *et al.*, 2013; Reyes-Robles *et al.*, 2013; Spaan *et al.*, 2013; DuMont and

Torres, 2014; Spaan *et al.*, 2014) At the host cell receptor the S-subunits of most leukocidins will bind first which in return recruits the F-subunit (Colin *et al.*, 1994). The exception to the rule is HlgAB, where the F-subunit (HlgB) will bind first to the receptor and the S-subunit only later (Kaneko *et al.*, 1997).

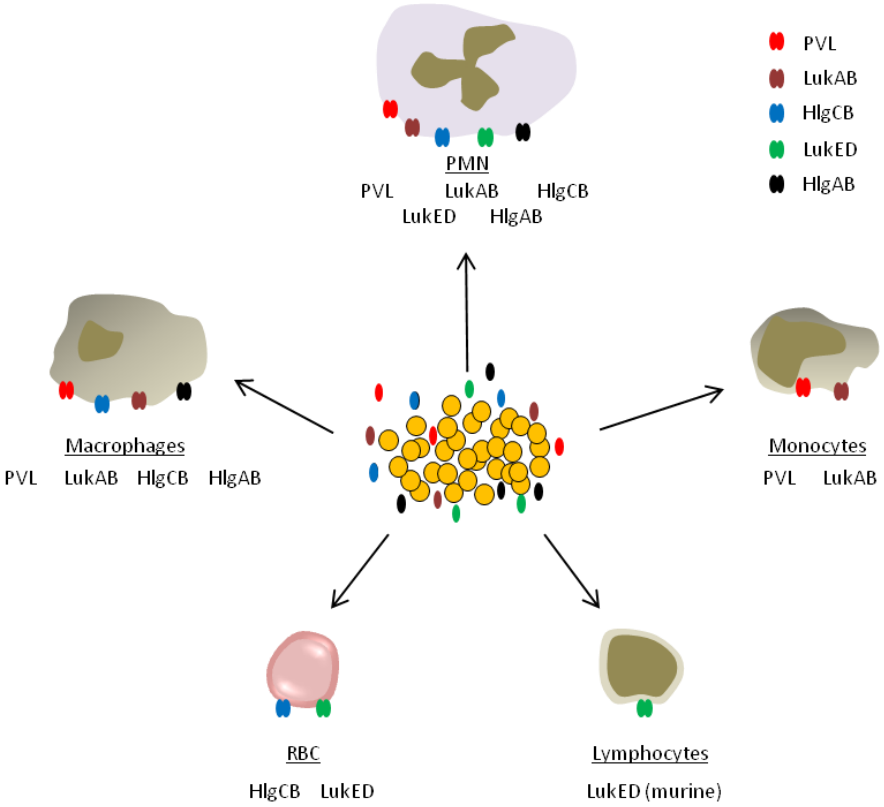

Figure 2.2: Host cell specificity of leukocidins

The schematic depicts the bi-component leukocidins PVL (red), LukAB (brown), HlgCB (blue), LukED (green) and HlgAB (black) as well as their currently known cellular targets. Staphylococci (yellow) release monomeric components of each leukocidins, which will constitute to dimers on the surface of their respective target cells, resulting in lysis of the host cells. Modified from (Alonzo and Torres, 2013).

Upon dimerization the leukocidin dimers assemble into octameric prepores of four alternating S- and F-subunits (Kaneko and Kamio, 2004; Aman *et al.*, 2010; Yamashita *et al.*, 2011). Completion of the prepore octamer on the host cell surface triggers structural changes in the stem domains of the subunits. These domains will unfold, forming a β-barrel pore that disrupts the membrane barrier (Alonzo and Torres, 2014). Pore formation will trigger uncontrolled ion fluxes across the cell membrane, but also the extrusion of cytoplasmic components (Alonzo and Torres, 2014).

The pathogenicity of the leukocidins is defined through their species and cell specificity. This results from the specific interaction of each of the leukocidins with only a single –or very

few- receptor (Alonzo and Torres, 2014). Human polymorphonuclear cells (PMN) are recognized and killed effectively by all leukocidins (Figure 2.2) (Prevost *et al.*, 1995; Gravet *et al.*, 1998; Gauduchon *et al.*, 2001; Morinaga *et al.*, 2003; Loffler *et al.*, 2010; Dumont *et al.*, 2011). The immune system has learned to recognize low concentrations of leukocidins and to boost inflammation reactions as a consequence (Alonzo and Torres, 2014). PVL will cause PMN to secrete pro-inflammatory IL-8 and gamma-hemolysin can cause activation of caspase-1 which in return increases IL-1ß, IL-18 and IL-33 levels (Konig *et al.*, 1995; Staali *et al.*, 1998; Bergsbaken *et al.*, 2009; Munoz-Planillo *et al.*, 2009).

Through their effective killing of host immune cells, the leukotoxins are thought to be crucial virulence factors for *S. aureus* and promote both, bacterial survival through immune evasion, as well as dissemination and infection (Yoong and Torres, 2013; Alonzo and Torres, 2014).

2.2.1.4 Enterotoxins

Probably one of the most devastating toxins in the arsenal of *S. aureus* is the toxic shock syndrome toxin 1 (TSST1), which is directed against T-cells of the host immune system. TSST1 acts as a superantigen and promotes the fast expansion of T-cell populations and the non-specific release of cytokines like IL-1, IL-2 and TNF-α by such cells (Otto, 2014). Strong, undirected release of pro-inflammatory cytokines prevents a focused response of the adaptive immune system and in the worst case, can give rise to a cytokine storm and a toxic shock in the patient (Choi *et al.*, 1990; Ferry *et al.*, 2008; Fraser and Proft, 2008). TSST1 belongs to a family of about 20 secreted enterotoxins, all 20 – 30 kD in mass. They interfere with intestinal functions by cytokine release and subsequent T cell activation and proliferation to cause emesis and diarrhea (Lin *et al.*, 2010; Hennekinne *et al.*, 2012). But, with the exception of SelX, all enterotoxins are present only in a minority of strains (Wilson *et al.*, 2011).

Superantigen-like proteins (SSL) are a family of secreted proteins with an immense bandwith of functions in immune evasion (Williams *et al.*, 2000; Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003). With most of them possessing a human host specific mode of action, they function in the interference of chemokine mediated PMN activation as well as adhesion and rolling inhibition (Bestebroer *et al.*, 2007; Chung *et al.*, 2007; Bestebroer *et al.*, 2009; Thammavongsa *et al.*, 2015). In order to do this, SSL associate with a wider range of glycoproteins on leukocytes, for example TLR2 receptors, which mediate the recognition of staphylococcal lipoproteins and peptidogylcan (Yokoyama *et al.*, 2012; Thammavongsa *et al.*, 2015).

2.2.2 Secreted proteases and nucleases

S. aureus secretes a total of four major proteases. Each of them possesses significant properties, for *S. aureus* pathogenesis.

Aureolysin, a zinc dependent metalloprotease cleaves C3 and C5, both important proteins of the complement system (Laarman *et al.*, 2011; Jusko *et al.*, 2014). Cleavage of C5 to C5a can lead to rapid C5a receptor internalization in PMN, which will limit chemotaxis and ROS production, while C3 cleavage into active C3a and C3b fragments prompts further degradation of C3a by aureolysin itself and of C3b by staphopain B (SspB). The simultaneous loss of both complement proteins limits opsonization of bacteria significantly (Laarman *et al.*, 2011; Jusko *et al.*, 2014). The defense against the complement complex is also the domain of the two cysteine proteases staphopain A (ScpA) and staphopain B (SspB). Both cleave C5 and therefore limit PMN reaction to bacterial infection (Jusko *et al.*, 2014). Further, staphopain A also cleaves the chemokine receptor CXCR2, which results in reduction of PMN chemotaxis, while staphopain B induces a form of atypical cell death in PMN via CD11b cleavage (Smagur *et al.*, 2009b; Laarman *et al.*, 2012). Lastly, the serine protease V8 (SspA), degrades human immunoglobulins and can act on kininogen, thereby influencing the outcome of septic *S. aureus* infections (Molla *et al.*, 1989; Prokesova *et al.*, 1992).

Besides proteases, *S. aureus* also possesses two nucleases, of which Nuc is secreted whereas Nuc2 occurs attached to the bacterial surface (Olson *et al.*, 2013; Kiedrowski *et al.*, 2014). Nuc has been shown to be crucial in the defense against extracellular traps generated by PMN and in the modulation of biofilm formation (Berends *et al.*, 2010; Kiedrowski *et al.*, 2011). A strong phenotype for Nuc2 has yet to be determined (Kiedrowski *et al.*, 2014).

2.2.3 Complement inhibition factors

The complement system of the innate immune system either opsonizes bacteria for subsequent uptake by phagocytes or forms the so-called membrane attack complex (MAC), a bactericidal protein pore complex directed against gram-negative bacteria(Gros *et al.*, 2008; Lambris *et al.*, 2008; Thammavongsa *et al.*, 2015).

While the production of a bacterial capsule often effectively interferes with the complement system, *S. aureus* strains (including strains of genotype USA300) often do not produce a capsule (Thammavongsa *et al.*, 2015). Instead, several virulence factors are involved in the process of complement inhibition. The Staphylococcal Complement Inhibitor (SCIN) acts on the C3 convertase, thereby preventing the generation of the cleavage products C3a and C3b, as well as the cleaving of C5 altogether (Rooijakkers *et al.*, 2005a). The complement factors I (fI)

and H (fH) bind and degrade the membrane binding C3b component (Laarman *et al.*, 2011). The extracellular fibrinogen-binding protein (Efb) and the extracellular complement-binding protein (Ecb) both bind the C3d cleavage product which functions in the activation of both, adaptive and innate immune response (Jongerius *et al.*, 2007).

The staphylococcal protein A (*spA*) binds to immunoglobulins of the classes IgA, IgD, IgG 1-4, IgM and IgE with high affinity and can either inhibit phagocytosis by binding IgG Fcγ domains or it exhibits B cell superantigen activity when interacting with Fab domains of the immunoglobulins (Forsgren and Sjoquist, 1966; Forsgren and Nordstrom, 1974; Goodyear and Silverman, 2003). Accordingly, protein A has been shown to also promote survival of *S. aureus* in human blood (Falugi *et al.*, 2013).

Sbi, the staphylococcal binder of immunoglobulin is a secreted protein which possesses two Ig binding domains and can block both, the classical complement pathway via C1q, as well as the alternative C3/fH pathway (Zhang *et al.*, 1998; Haupt *et al.*, 2008).

CHIPS, FLIPr and FLIPrL form a group of secreted proteins acting in the interference with receptor mediated PMN chemotaxis (Chavakis *et al.*, 2002; Prat *et al.*, 2006; Prat *et al.*, 2009; McCarthy and Lindsay, 2013). Found in most human *S. aureus* isolates, CHIPS binds to the human FPRI and C5aR receptors, the former acting in PMN activation and the latter in complement (de Haas *et al.*, 2004; Postma *et al.*, 2004). FPRI is also the target of both, FLIPr and FLIPrL while FLIPrL also inhibits the FPRII receptor (Prat *et al.*, 2006; Prat *et al.*, 2009). In the case, that immunoglobulins or defensins reach the cell wall of *S. aureus* despite all other counter measures, the metalloprotease staphylokinase forms a new line of defense. The protein can cleave IgG, defensins, fibrin and C3b from the bacterial cell surface to block complement (Jin *et al.*, 2004; Rooijakkers *et al.*, 2005b).

Missing from this list of *S. aureus* toxins is the probably most prominent group of toxins in staphylococcal literature and research. Hemolysins are able to cause the destruction of red blood cells, which coined their name. But the toxicity goes much further. All of them play vital functions in the ability of pathogenic *S. aureus* strains to cause disease.

2.2.4 Phenol-soluble modulins

Phenol-soluble modulins (PSM) constitute a family of exotoxins, identified only recently by the group of Klebanoff, which found them fractionated in the phenol phase of a hot phenol extraction from *Staphylococcus epidermidis* (Mehlin *et al.*, 1999). PSM-like peptides with virulence associated functions have been described before in other *Staphylococcus* species like S*. lugdunensis* and *S. haemolyticus* (Watson *et al.*, 1988; Donvito *et al.*, 1997). Until today PSM

nomenclature is far from consistent across staphylococcal species, as PSMs in *S. epidermidis* obtained their names according to their order of identification whereas *S. aureus* PSM were clustered regarding chemical and sequence properties, reflecting their genomic localization in two operons and inside the regulatory RNAIII (Janzon and Arvidson, 1990; Wang *et al.*, 2007).

S. aureus produces a set of at least eight small amphiphatic PSM, four of them (PSMα 1-4) being transcribed within one operon, while PSMβ 1-2 are encoded by a separate locus and δ within the regulatory RNAIII (Janzon and Arvidson, 1990; Novick *et al.*, 1993; Wang *et al.*, 2007; Verdon *et al.*, 2009; Peschel and Otto, 2013). The last PSM, PSM-mec represents the only family member not transcribed in the core genome of *S. aureus* but in staphylococcal cassette chromosome mec (SCCmec) and is therefore only present in the limited number of strains harboring the SCCmec element (Queck *et al.*, 2009; Cheung *et al.*, 2014). *S. aureus* PSM differ in length, with PSMα 1-4, δ-toxin and PSM-mec being 20-25 AA in size whereas PSMβ 1-2 are 44 AA long. Another differentiating characteristic is net charge, with PSMα 1-4 and PSM-mec being positively, PSMβ 1-2 negatively and δ-toxin neutrally charged (Peschel and Otto, 2013).

Lacking a signal peptide, all PSM are secreted by means of a recently identified specific ABC transporter (Wang *et al.*, 2007). The so-called phenol-soluble modulin transporter (Pmt) is a four-component system, consisting of two ATP-ases and two membrane-associated proteins, with secretion of all PSMs highly dependent on its functionality (Chatterjee *et al.*, 2013). Lacking a functional Pmt, PSMs will accumulate in the cytosol of the bacterium, leading, at least in the case of the highly cytolytic PSMα and δ-toxin, to abnormal cell division, damage to the cytoplasmic membrane and subsequent severe growth defects (Chatterjee *et al.*, 2013). As PSMs can make up for 60% of all secreted proteins in *S. aureus*, a functional PSM export is essential for bacterial survival under *agr*-activating conditions (Cheung *et al.*, 2014). Besides its function in the protection from self-produced highly cytotoxic PSM species, the Pmt has been shown to also confer resistance to non-self PSM (Chatterjee *et al.*, 2013).

The ability of PSMs to interfere with the integrity of membranes is a major factor in *S. aureus* virulence and pathogenesis, as it has been shown that the lysis of a number of different eukaryotic cell types like osteoblasts, monocytes, erythrocytes and PMNs is mediated by cytolytic members of the PSM family, foremost PSMα (Wang *et al.*, 2007; Cheung *et al.*, 2010; Cheung *et al.*, 2012; Cassat *et al.*, 2013). Our understanding of PSM action on membranes is based on research that has been performed on δ-toxin (Pokorny *et al.*, 2002). A similar mode of action for the other PSMs appears likely as a receptor independent effect was advocated (Kretschmer *et al.*, 2010).

While PSMs enable *S. aureus* to kill PMN recruited to the site of infection, they also function as a pathogen-associated molecular pattern (PAMP), leading at very low concentrations to activation, chemo-attraction and interleukin-8 (IL-8) signaling in PMN. This is mediated via

the N-formyl-peptide receptor 2 (FPR2), a paralog of FPR1, present on different types of leukocytes, which has, as of yet, rather been implicated in the recognition of host inflammation markers and not of bacterial peptides (Ye *et al.*, 2009).

In general, PSM expression is regarded as an important marker to determine the virulence potential of a strain. *Staphylococcus epidermidis*, a human commensal staphylococcal species shows much less cytotoxicity compared with *S. aureus.* Accordingly, PSM expression patterns differ considerably between the two species. While *S. aureus* produces the highly cytolytic PSMα peptides in high amounts, they are virtually absent in *S. epidermidis* and also the expression of the strongly cytolytic PSMδ appears low (Cheung *et al.*, 2010). In contrast, highly epidemic and cytotoxic community-acquired MRSA strains as LAC (USA300) and MW2 (USA400) stand out amongst patient isolates regarding their high production rates of strongly cytolytic α-type PSMs (Wang *et al.*, 2007; Otto, 2010; Li *et al.*, 2010; Kobayashi *et al.*, 2011).

Their strong cytolytic properties and their abundance, especially in epidemic and highly virulent strains, render PSMs one of the determining factors in many different types of infections. In skin and soft tissue infections, where *S. aureus* is the responsible pathogen in about 90% of all clinical cases, mutants unable to produce PSM showed significant attenuation in their ability to form abscesses in murine infection models (Wang *et al.*, 2007; DeLeo *et al.*, 2010). But also other onsets of disease depend on PSMs. δ-toxin is involved in the occurrence of atopic dematidis. Mast cells, a type of myeloid stem cell derived granulocyte undergoes degranulation by contact with δ-toxin, thereby contributing to the disease (Nakamura *et al.*, 2013). In osteomyelitis, toxicity of *S. aureus* towards osteoblasts is highly dependent on PSM prodcution and also biofilm-associated infections, where staphylococci are the most commonly found pathogen, depend on PSM production (Otto, 2008; Wang *et al.*, 2011; Cassat *et al.*, 2013; Rasigade *et al.*, 2013). The structuring of biofilms, as well as the promotion of colony formation and the spreading of *S. aureus* on wet surfaces through PSMs illustrate, that this class of toxins does not only influence the ability of *S. aureus* to kill host cells, but also exhibits importance in the adhesion of *S. aureus* to surfaces in a way to colonize and persist (Wang *et al.*, 2007; Omae *et al.*, 2012; Peschel and Otto, 2013; Tsompanidou *et al.*, 2013).

2.2.5 Adhesion to non-organic and organic surfaces

The success of *S. aureus* as a nosocomial pathogen is based to a large degree on the ability to adhere effectively to non-organic surfaces like catheters and prosthetic devices and to the formation of biofilms. Bacterial biofilms are extracellular structures composed of water, DNA, proteins, microbial cells, polysaccharides and nutrients (Tong *et al.*, 2015), which allow for colonization. In infection settings, biofilms function in bacterial defense as they confer high resistance to antimicrobials and the host immune defense (Costerton *et al.*, 1999; Stewart and William Costerton, 2001; Arciola *et al.*, 2012). The adherence and colonization of *S. aureus* to non-organic surfaces like artificial polymeric implants, poses a great threat in hospitals, especially for the health of patients who have just undergone surgery and whose immune response is weakened. Biomedical polymers situated in the human body e.g. heart catheters or hip replacements, are usually covered by platelets, which, in return, give *S. aureus* the opportunity to bind to potential ligands as fibronectin, fibrinogen, collagen or laminin and thereby indirecty, to the polymeric devices (Herrmann *et al.*, 1993; Wang *et al.*, 1993; Patti *et al.*, 1994).

At the same time, adherence to organic surfaces represents an essential capacity for *S. aureus* in both, colonization and infection of its host. *S. aureus* has been shown to adhere to the blood vessel wall before exiting the blood stream again despite the sheer force of the flowing blood. It does so using the von-Willebrand factor protein (vWFp) (Claes *et al.*, 2014). This enables the bacteria to establish secondary sites of infection by spreading through the bloodstream. To facilitate adherence in both settings, *S. aureus* uses a number of adhesins which belong to one of two classes. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) or secreted expanded repertoire adhesive molecules (SERAM) are bacterial surface associated and secreted proteins recognizing a macromolecular ligand within the extracellular matrix of the host cell (Patti *et al.*, 1994; Chavakis *et al.*, 2005). In *S. aureus* the fibronectin-binding proteins take the most prominent role as MSCRAMMS. The two existing homologs, FnBPA and FnBPB show considerable organisational and sequence similarity, both of them anchored via an N-terminal LPXTG motif to the cell wall of *S. aureus* (Keane *et al.*, 2007; Stemberk *et al.*, 2014). While studies show an important role of both proteins in establishing infection and septicemia, they also point out, that FnBPA has a leading role in terms of virulence (Shinji *et al.*, 2011). This seems to be explained by the fact, that while FnBPA is absolutely essential for adhesion and the following internalization into non-professional phagocytes, FnBPB is not. Besides being instrumental in adhesion to host cells, the FnBPs also take part in the formation of biofilms. Mediated by the N-terminal A domains of the FnBPs cell to cell contacts between bacterial cells during biofilm formation are formed (Herman-Bausier *et al.*, 2015).

Other proteinaceous factors involved in bacterial adhesion to the extracellular matrix of human cells include the clumping factor ClfA, which is known to interact with soluble or immobilized fibrinogen and the major integrin αVβ3, which has been shown to be involved in binding of platelets and to cause endocarditis in rats (Siboo *et al.*, 2001; George *et al.*, 2006; Shinji *et al.*, 2011; McDonnell *et al.*, 2016), the immunoglobulin binding staphylococcal protein A (*sp*A), which is also functioning in inhibition of opsonophagocytosis by binding to the vWF (Dossett *et al.*, 1969; Hartleib *et al.*, 2000; Kobayashi and DeLeo, 2013), the extracellular adherence protein (*eap*), which also functions in internalization of bacteria in cells and binds ICAM1 to hinder leukocyte migration, as well as the major autolysin *atl*A, which binds to the heat shock cognate protein Hsc70 and also functions in invasion into host cells, cell separation, cell wall remodeling and the excretion of cytoplasmic proteins (Gilpin *et al.*, 1974; Foster, 1995; Heilmann *et al.*, 1997; Lee *et al.*, 2002; Biswas *et al.*, 2006; Sobke *et al.*, 2006; Athanasopoulos *et al.*, 2006; Chavakis *et al.*, 2007; Vollmer *et al.*, 2008; Hirschhausen *et al.*, 2010; Albrecht *et al.*, 2012; Bur *et al.*, 2013)

2.2.6 Small molecule products of *S. aureus* and their association with virulence

Small molecules are a diverse class of small molecular weight (less than 900 daltons) organic compounds, produced either from gene encoded sequences, enzymatically through a series of proteins or via non-ribosomal peptide synthetase machineries (Felnagle *et al.*, 2008). Small molecules thereby take significant roles in several fields of *S. aureus* physiology and virulence.

Lending *S. aureus* its orange color might be the most obvious attribute of the triterpenoid carotenoid staphyloxanthin, but its actual range of functions goes far beyond this. Produced by the *crt*OPMN-operon encoded enzymes, staphyloxanthin production protects *S. aureus* against photosensitization and oxidative stress (Liu *et al.*, 2005; Clauditz *et al.*, 2006; Kossakowska-Zwierucho *et al.*, 2016). Virulence in an abscess model has been shown to be improved with staphyloxanthin production as well, as the carotenoid protects the bacteria against killing by PMN (Liu *et al.*, 2005).

In infection, the acquisition of iron is an essential task for almost every pathogen known (Weinberg, 1978; Weinberg, 2009). Most of the iron is bound inside cells, mostly in a complex with heme,while extracellular iron exists bound to either transferrin in the blood or lactoferrin in the lymph (Hammer and Skaar, 2011). While heme-bound iron can be taken up by *S. aureus* after lysis of red blood cells (RBC) via Isd proteins, a special class of small molecules, called siderophores competes with host factors for iron binding (Torres *et al.*, 2006). Controlled by the

Fur transcriptional regulator, two enzymatic production chains produce the siderophores staphyloferrin A (*sfn*ABCD) and staphyloferrin B (*sbn*ABCDEFGHI) (Friedman *et al.*, 2006). *S. aureus* requires at least the production of one of these factors for growth in iron-depleted environments (Beasley *et al.*, 2009).

Small molecules in bacteria are often produced by non-ribosomal peptide synthetases (Felnagle *et al.*, 2008). In these cases, proteins with alternating enzymatic domain structures produce molecules via amino acid chain elongation independent of ribosomes (Fischbach and Walsh, 2006). In *S. aureus* one such non-ribosomal peptide synthetase, named AusAB (synonym: PznAB) has been identified (Wyatt *et al.*, 2010; Zimmermann and Fischbach, 2010). While initially connected to virulence, the function of the NRPS and its dipeptide products phevalin, tyrvalin and leuvalin remains debated and elusive (Sun *et al.*, 2010a; Wyatt *et al.*, 2010; Secor *et al.*, 2012).

Among the small molecules regularly transcribed from an ORF, the auto inducing peptide takes undoubtedly the most prominent role. Encoded on the gene *agr*D, the precursor AIP undergoes further processing before being secreted from the cell (Ji *et al.*, 1997; Zhang and Ji, 2004; Zhang *et al.*, 2004). There, AIP functions as part of a quorum sensing system, which ultimately controls a majority of virulence associated *S. aureus* factors, the *agr* system.

2.2.7 Regulation of virulence in *S. aureus*

Virulence factors in *S. aureus* are regulated by an intricate network of global regulators, quorum sensing and two-components systems.

2.2.7.1 The *agr* quorum sensing system

The accessory gene regulator (*agr*) system is a quorum sensing system involved in the cell density-dependent expression of virulence factors in *S. aureus* (Dunman *et al.*, 2001). Agr comprises the key element in the regulation of genes involved in *S. aureus* virulence, governing the expression of the so-called virulon (Novick and Geisinger, 2008). *agr* mutants showed attenuation in several animal disease models, including skin abscesses, endocarditis and septic arthritis (Bunce *et al.*, 1992; Abdelnour *et al.*, 1993; Cheung *et al.*, 1994).

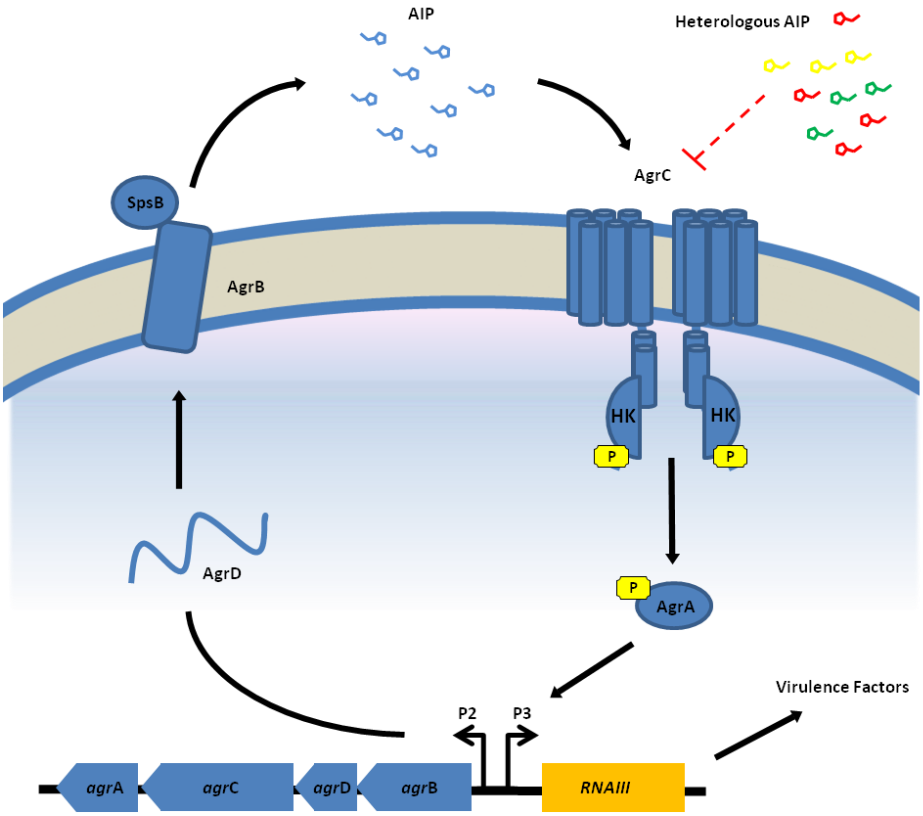

Figure 2.3: The *agr* quorum sensing circuit.

The P2 promoter drives the transcription of the *agr*BDCA operon which encodes all four components of the quorum sensing system. The AIP precursor peptide (AgrD) is directed to the AgrB transmembrane endopeptidase by its N- and C-terminal signal and recognition sequences which are subsequently cleaved by AgrB and the signal peptidase SpsB. Thereby the active AIP is formed which is secreted and at high cell densities and thus high AIP concentrations may be recognized by the transmembrane receptor domain of the homodimeric histidine kinase (HK) AgrC. The response regulator AgrA is phosphorylated by AgrC. High phosphorylation rates drive transcription of the promoter P3 resulting in production of RNAIII. AIP heterologs, produced by different *S. aureus* strains will inhibit AgrC activation. Modified from (Novick and Geisinger, 2008).

The diffusion-sensing or quorum-sensing abilities of *agr* are based on the secretion of a so-called auto-inducing peptide (AIP), which can be sensed by a two-component system. The regulatory circuit thereby is encoded by a four gene operon, *agr*BDCA (Fig. 2.3). The *agr*D mRNA translates into an AIP precursor propeptide, which undergoes consequent processing of its N-terminal amphipatic leader peptide, targeting it to the cell membrane, as well as the C-terminal recognition sequence, which interacts with AgrB (Ji *et al.*, 1997; Zhang and Ji, 2004; Zhang *et al.*, 2004). AgrB functions as a transmembrane endopeptidase which cleaves the C-terminal sequence of the peptide precursor, catalyzing the reversible cyclization of AgrD into a thiolactone intermediate, which is consequently secreted into the extracytoplasmic compartment and released by the cleavage of the N-terminal leader peptide by the signal

peptidase SpsB (Kavanaugh *et al.*, 2007; Wang *et al.*, 2015). Depending on the *S. aureus* strain, this results in the formation of a peptide consisting of seven to nine amino acid residues and a thiolactone ring, which has been found to be essential for the activity of AIP, but also limits the half-life of AIP under physiological conditions (Ji *et al.*, 1997; Mayville *et al.*, 1999; P *et al.*, 2001).

The secreted AIP is recognized by the homodimeric receptor histidine kinase AgrC, consisting of an N-terminal sensor domain which spans the membrane six times, and a highly conserved C-terminal cytoplasmic histidine kinase domain (Lina *et al.*, 1998). AgrC has been shown to possess a low autokinase activity, leading to a baseline of phosphorylation of the response regulator (RR) AgrA. Due to differential affinity of the phosphorylated AgrA to regulatory elements in the P2 and P3 promoter regions, this leads to a baseline transcription of the P2 operon (*agr*BDCA) but not P3. This autokinase activity is enough to complete the autoactivation circuit of the Agr system, without spurring P3-dependent transcription of RNAIII and subsequent activation of the virulon (Novick *et al.*, 1995; Reyes *et al.*, 2011; Wang *et al.*, 2014). Since AgrC shows no detectable phosphatase activity, this leaves the dephosphorylation of AgrA and the consequent deactivation of the *agr*-controlled virulon, to a large degree to the RRs autocatalytic capabilities to dephosphorylate (Wang *et al.*, 2014).

With increasing concentrations of AIP in the environment the AgrC dimers undergo autophosphorylation, which leads to an increased rate of phosphoryl groups transfer to AgrA (Johnson *et al.*, 2015). Transcription of the P2 promoter increases exponentially due to more frequent phosphorylation of AgrA, while at the same time the activation of the P3 promoter is initiated. In staphylococcal evolution four heterologous groups of AIP have emerged, with only one of them being produced by any given strain (Ji *et al.*, 1997; Dufour *et al.*, 2002; Johnson *et al.*, 2015). While the general structure of AIP, including the size of the thiolactone ring, remains largely unchanged between different AIP, even a single amino acid exchange can lead to a different AIP group type (Geisinger *et al.*, 2008; Johnson *et al.*, 2015). A heterologous pairing of AIP and AgrC receptor results in inhibition of the Agr response and a regulatory interference between strains that is independent of growth (Ji *et al.*, 1997).

The promoter P3 governs transcription of the 514 nt long regulatory RNA, RNAIII, which acts as one of the most prominent initiators of *S. aureus* virulon expression (Novick *et al.*, 1993; Dunman *et al.*, 2001). The stable RNA possesses a conserved complex secondary structure and acts at a translational level via an antisense base pairing mechanism on its target mRNAs, binding directly to sequence regions containing either the Shine Dalgarno sequence or the AUG start codon (Benito *et al.*, 2000; Novick, 2003; Huntzinger *et al.*, 2005). Known targets of RNAIII include genes involved in virulence, but most of the effects of RNAIII are facilitated by its binding to the mRNA of intermediary global regulators like the *sar*A family paralog *rot,* the two-component system *sae*RS as well as *mgr*A, which in turn control the expression of hundreds of

genes including such with relevance in virulence, antibiotic resistance and autolysis (Giraudo *et al.*, 2003; Geisinger *et al.*, 2006; Boisset *et al.*, 2007; Gupta *et al.*, 2015). Proteins, which translation is up-regulated under the immediate influence of RNAIII, include the prominent exotoxins α- and β-hemolysin.

While the RR AgrA is primarily thought to control the transcription of the P2 and P3 promoter region, a number of genes could be identified which are regulated by Agr independently of RNAIII (Queck *et al.*, 2008). While most of those genes were found to be negatively regulated, only very few show an upregulation, of which the PSMα and the PSMβ operons were, by far, regulated strongest.

2.2.7.2 SaeRS two-component system

Besides the *agr* quorum sensing system one other transcriptional regulator stands out as a dominant force. The two-component system (TCS) SaeRS (Giraudo *et al.*, 1999). SaeS reacts to stimuli like low pH, sub-inhibitory concentrations of antibiotics and H_2O_2 (Novick and Jiang, 2003; Kuroda *et al.*, 2007; Geiger *et al.*, 2008). Following autophosphorylation, SaeR becomes phosphorylated by the kinase activity of SaeS and binds to specific target sequences in the genome, including a region in the P3 promoter region of *sae*RS, thereby generating a positive feedback loop (Geiger *et al.*, 2008; Sun *et al.*, 2010b; Jeong *et al.*, 2011). The complete *sae* operon, under control of a second promoter (P1), harbors two more genes, the putative lipoprotein SaeP and the transmembrane protein SaeQ and is also activated by phosphorylated SaeR (Juncker *et al.*, 2003; Jeong *et al.*, 2012). Both genes, SaeP and SaeQ engage in a protein complex with SaeS, thereby activating the phosphatase activity of the protein. This in return facilitates the dephosphorylation of the response regulator, thereby reducing transcription of the SaeR targets (Jeong *et al.*, 2012). The P1 promoter, controlling the expression of *sae*P and *sae*Q is induced much slower than the P3 promoter, allowing the expression of *sae* targets for a certain amount of time before the negative feedback loop involving SaeP/Q takes effect and again limits virulence gene expression (Jeong *et al.*, 2012).

Up-regulation of certain genes by the *sae*RS TCS has important functions in terms of general virulence and specifically the defense of the bacterium against the immune cells of the host. Internalization of *S. aureus* by e.g. by human PMN triggers a strong increase of *sae*RS transcription while such a reaction is not equally imminent for the *agr* system (Voyich *et al.*, 2005). A loss of *sae* function leads to attenuated virulence in a series of murine mouse models, including necrotizing pneumonia, skin infections, osteomyelitis and sepsis (Benton *et al.*, 2004; Voyich *et al.*, 2009; Montgomery *et al.*, 2010; Nygaard *et al.*, 2010; Cassat *et al.*, 2013; Beenken *et al.*, 2014).

Depending on the conditions of the experiment and the strain used, SaeRS has been shown to influence up to 8% of all *S. aureus* genes, either direct by binding of the response regulator SaeR to the promoter or indirectly via the influence of *sae* on other regulatory genes (Rogasch *et al.*, 2006; Voyich *et al.*, 2009; Nygaard *et al.*, 2010). SaeR targets include several toxin genes as well as genes encoding immune-modulatory factors. The most prominent examples are genes for α-hemolysin (*hla*), all three components of γ-hemolysin (*hlg*A, *hlg*B, *hlg*C), immunoglobulin binding protein (*sbi*), chemotaxis inhibiting protein CHIPS (*chs*) and several members of the pore forming toxin family of leukocidins, *luk*D, *luk*E, *luk*F and *luk*S, but also the fibronectin-binding proteins (*fnb*A and *fnb*B) which function in adherence and invasion (Nilsson *et al.*, 1999; Ahmed *et al.*, 2001; Postma *et al.*, 2004; Bubeck Wardenburg *et al.*, 2007a; Smith *et al.*, 2011; Reyes-Robles *et al.*, 2013).

2.2.7.3 MarR-type transcriptional regulators: the SarA protein family

The SarA family of proteins is a collection of DNA binding proteins, of which SarA takes a prototypic role and to which all other members are homologues to (Cheung and Zhang, 2002). SarA is a 124 AA protein with a winged helix structure, that has been shown to positively influence expression levels of fibronectin binding proteins as well as toxins but to repress protein A and proteases (Cheung *et al.*, 2004; Zielinska *et al.*, 2012). It does so either by direct binding to promoters of target genes, the stabilization of mRNAs or via the regulation of other global regulators, such as *agr*, *rot* and *sar*V (Cheung *et al.*, 2004; Roberts *et al.*, 2006; Cheung *et al.*, 2008; Priest *et al.*, 2012).

Besides SarA, 10 other family members are known. SarR, SarS, SarT, SarU, SarX, SarZ, SarV, SarY, Rot and MgrA. Regulatory functions inside the protein family are common, as e.g. SarT and SarA regulate *sar*S and MgrA activates *sar*X but represses *sar*V (Cheung *et al.*, 2008). Also other important regulators are affected by SarA protein family members. While SarA, SarU, SarR and MgrA positively regulate *agr*, Rot and SarX repress it (Truong-Bolduc *et al.*, 2003; Said-Salim *et al.*, 2003; Cheung *et al.*, 2004; Manna and Cheung, 2006).

2.2.7.4 CodY

Adjusting to dynamic environmental changes is of prime importance to any bacterium. Escpecially the deprivation of available nutrients represents a situation in which rapid changes in gene expression are necessary. CodY takes an important role in the adaptation to nutrient

starvation in *S. aureus*. When binding to GTP or the branched-chain amino acids leucine, isoleucine and valine, CodY becomes active and limits the expression of more than 200 genes (Pohl *et al.*, 2009; Majerczyk *et al.*, 2010). With nutrient levels being high, CodY represses the expression of a large portion of proteins involved in metabolism and virulence, while it allows MSCRAMM proteins and several enterotoxins to be expressed (Pohl *et al.*, 2009; Majerczyk *et al.*, 2010; Waters *et al.*, 2016).

With an increasing limitation of nutrients, different classes of CodY targets become derepressed. First nutrient uptake genes and genes involved in fatty metabolism are activated. Only with nutrient levels decreasing further, virulence factors, capsule proteins and iron acquisition clusters are expressed, while at the same time MSCRAMM proteins are shut off (Waters *et al.*, 2016). The cascade of changing repression and derepression has been hypothesized to benefit dissemination of the bacteria while limiting factors important for persistence.

2.2.7.5 Alternative sigma factor B (σB)

While prolonged intracellularity is a secondary effect of a mutated *rsp*, the modulation of gene expression in response to internalization is a substantial aspect of an active alternative sigma factor B in *S. aureus*. σB is a subunit of the RNA polymerase holoenzyme which is involved in the regulation of expression of a diverse set of virulence factors, cellular pigmentation, biofilm formation as well as antibiotic resistance (Bischoff *et al.*, 2004; Pane-Farre *et al.*, 2009; Pfortner *et al.*, 2014). σB is tightly controlled by a secondary network of antagonists and anti-antagonists, including the kinase RsbW, the RsbW antagonist RsbV and the phosphatase RsbU (Benson and Haldenwang, 1993; Yang *et al.*, 1996). An active RsbU dephosphorylates RsbV, which in return binds RsbW. As RsbW is occupied in a complex with RsbV, σB gets released and can bind the RNA polymerase core enzyme (Alper *et al.*, 1996) (Fig. 2.4).

Activation of σB has been shown to occur through alkaline shock, spiking temperatures, during entry into stationary growth phase as well as by subinhibitory concentrations of the antibiotics gentamicin, vancomycin and ampicillin (Kullik and Giachino, 1997; Pané-Farré *et al.*, 2006; Pane-Farre *et al.*, 2009; Mitchell *et al.*, 2010; Chen *et al.*, 2011). Further, σB activation has been reported in some, yet not all, cases of internalization of *S. aureus* into host cells (Garzoni *et al.*, 2007; Pfortner *et al.*, 2014). The induction and retention of persistent intracellular forms of *S. aureus*, called small colony variants (SCV), has been shown to coincide with high σB expression levels, and the replication of such SCV appears to dependent on a functional σB (Senn *et al.*, 2005; Moisan *et al.*, 2006; Mitchell *et al.*, 2010; Mitchell *et al.*, 2013).

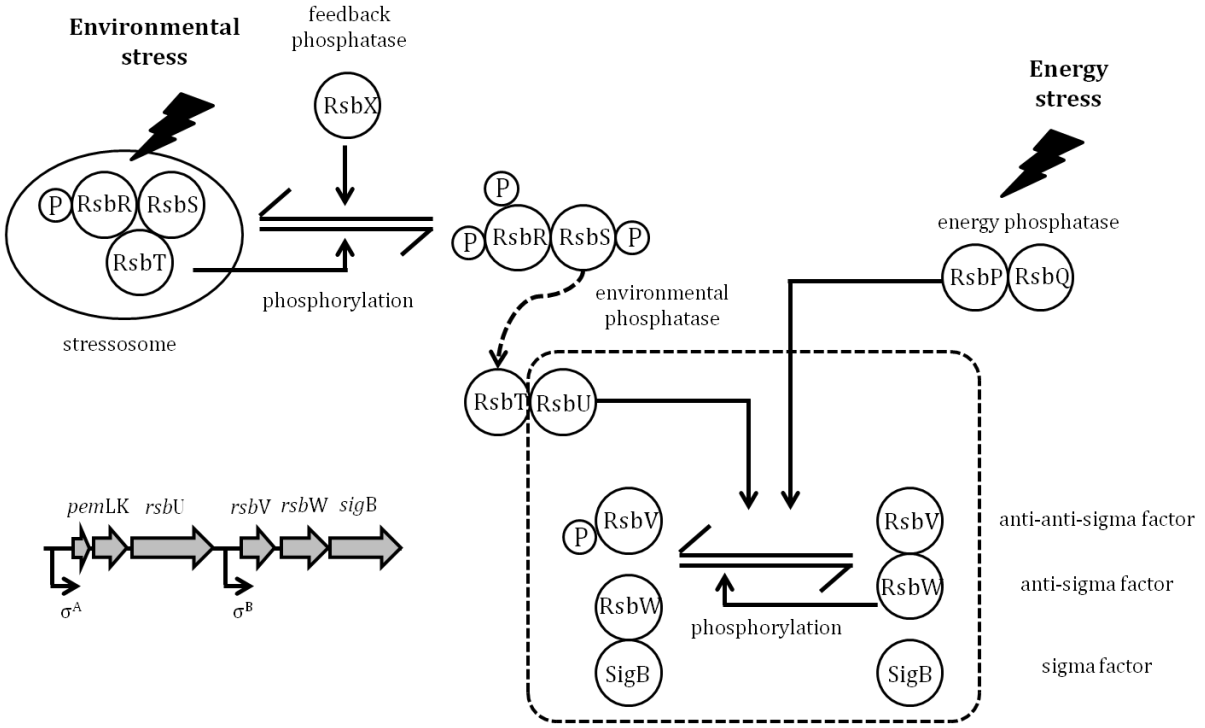

Figure 2.4: The *sig*B regulatory network

Model of SigB activation in *S. aureus*. The two phosphatases RsbU or RsbP react to stress and activate the dephosphorylation of the anti-anti-sigma factor RsbV. Dephosphorylated RsbV displaces the anti-sigma factor RsbW from the complex with SigB. Free SigB will bind to core RNA polymerases and initiates transcription at SigB-dependent promoters. On the bottom left, open reading frames (arrows) and transcription start sites in *S. aureus* are indicated. Modified from (Pane-Farre *et al.*, 2009).

Development of SCV is typical for chronic infections, during which σB is able to silence the cytotoxic capacities of *agr* and *sar*A controlled regulons (Tuchscherr *et al.*, 2015). The connection of σB to chronic diseases is also evident from different studies in mice. While in models of chronic disease, such as septic arthritis, σB expression was shown to be essential, and a σB mutant did not show reduced virulence in acute forms of disease like skin abscesses, wound infection or hematogenous pyelonephritis (Nicholas *et al.*, 1999; Horsburgh *et al.*, 2002; Jonsson *et al.*, 2004).

2.3 Intracellular persistence and virulence

2.3.1 Invasion in non-professional phagocytes

Adhesion to eukaryotic cells by MSCRAMM or SERAM proteins to ligands on host cells enables *S. aureus* to enter non-phagocytic cells. It has been observed, that *S. aureus* enters epithelial and endothelial cells, keratinocytes, fibroblasts and osteoblasts (Jevon *et al.*, 1999; Lammers *et al.*,

1999; Peacock *et al.*, 1999; Dziewanowska *et al.*, 2000; Fowler *et al.*, 2000; Ahmed *et al.*, 2001; Kintarak *et al.*, 2004; Sinha and Fraunholz, 2010; Edwards *et al.*, 2011). I will focus in this part on the FnBPs, the most important type of adhesins, who possess up to 11 repeats of fibronectin-binding motifs showing high affinity towards type 1 structural modules of fibronectin. Type 3 modules of fibronectin in return are recognized and bound by the hosts abundant α5β1 integrin (Foster *et al.*, 2014). The binding of each FnBP to several fibronectin molecules leads to an accumulation of the receptor molecules at the site of adherence (Schwarz-Linek *et al.*, 2003; Bingham *et al.*, 2008). The clustering of integrins then triggers signaling pathways leading to cytoskeleton rearrangement, including the formation of actin-rich membrane protrusions and fibrillar adhesions, the activation of tyrosin kinases and consequently the uptake of the bacteria in a zipper-type like fashion into the host cell (Dziewanowska *et al.*, 1999; Sinha *et al.*, 2000; Agerer *et al.*, 2003; Agerer *et al.*, 2005; Schroder *et al.*, 2006; Carabeo, 2011). However, *S. aureus* can also interact with another molecule on the host cell surface, HSP60 (Dziewanowska *et al.*, 2000).

2.3.2 Small colony variants (SCV)

As nosocomial infections are a severe health risk for patients worldwide, a number of clinics have resorted to the possibility of eradicating the *S. aureus* isolates, commensally carried by patients prior to invasive surgery, as an effective preventive method against post-surgical infections and for the treatment of immune compromised patients of intensive care units in general (van Rijen *et al.*, 2008; Hetem *et al.*, 2016; Septimus and Schweizer, 2016). While this method has proven itself in reducing surgical site infections, decolonization is only in the minority of patients long lasting. Studies have shown that after one year, more than half of all treated patients were colonized with *S. aureus* again, many of them with their own previous isolate (Doebbeling *et al.*, 1993; Fernandez *et al.*, 1995).

This very common reoccurrence of self-isolates points directly towards reservoirs in the human body where *S. aureus* can survive topical disinfection or antibiotic treatment and reemerge after a certain time to colonize again, or in more severe cases, to cause chronic courses of disease, as it is well documented in e.g. cystic fibrosis patients, where *S. aureus* strains persist in the lungs of patients for years despite regular antibiotic treatment (Kahl *et al.*, 2003; Stone and Saiman, 2007).

Prolonged persistence of bacteria inside eukaryotic host cells requires the bacteria to stay undetected by the immune system. *S. aureus* can transition into a reversible state of minimal metabolic activity called small colony variant (SCV) (Proctor *et al.*, 1995). In this physiological state, the virulence regulatory quorum sensing system *agr* is down-regulated and

with it the expression of many secreted virulence factors, such as α-hemolysin (Kohler *et al.*, 2003; Moisan *et al.*, 2006; von Eiff *et al.*, 2006; Kriegeskorte *et al.*, 2011). At the same time adhesins and biofilm formation are up-regulated, both potentially important factors in the evasion of pathogen clearance by the host (Moisan *et al.*, 2006; von Eiff *et al.*, 2006; Mitchell *et al.*, 2010; Singh *et al.*, 2010). All these changes in gene expression in SCV strains favor intracellular survival and persistence as the bacteria are highly invasive and at the same time chemokine release by infected host cells is reduced, which keeps the immune response of the host at a minimum (Tuchscherr *et al.*, 2010). Also, neither antimicrobial peptides nor antibiotics, target SCV strains as effectively as their wild type *S. aureus* counterparts (Koo *et al.*, 1996; Samuelsen *et al.*, 2005; Kahl, 2014).

Initially described for *Salmonella typhi*, small colony variants have been found for many bacterial species (Raven, 1934; Swingle, 1935; Colwell, 1946; Jensen, 1957; Bulger, 1967; Bryan and Kwan, 1981; Baddour *et al.*, 1990). Characterized by their slow growth, lacking pigmentation and reduced hemolysis, the emergence of SCV in *S. aureus* has been found to be caused by a number of circumstances, including exposure to antimicrobial agents as quinolones and triclosan, cold stress or intracellularity in eukaryotic cells (Vesga *et al.*, 1996; Mitsuyama *et al.*, 1997; Bayston *et al.*, 2007; Duval *et al.*, 2010). The mechanism of SCV formation is not entirely understood. It has been observed that the exposure to stresses mentioned above will cause mutations in different genomic regions. Besides mutations in the naphtoate synthase protein *men*B (menadione auxotrophy), *hem*A and *hem*H (hermin auxotrophy), the hydrolase *rsh* (stringent response) and *csp*B (cold shock response), mutations in the thymidylate synthase *thy*A, causing a thymidine auxotrophy, are known SCV phenotype inducing mutations (Schaaff *et al.*, 2003; Chatterjee *et al.*, 2008; Besier *et al.*, 2008; Lannergard *et al.*, 2008; Duval *et al.*, 2010; Gao *et al.*, 2010).

SCV phenotypes are recovered from a multitude of different types of infections like osteomyelitis, respiratory infections, hip abscesses, endocarditis or also bovine mastitis (Proctor *et al.*, 1995; Kahl *et al.*, 1998; Seifert *et al.*, 1999; Maduka-Ezeh *et al.*, 2012; Alkasir *et al.*, 2013). SCV formation of *S. aureus* enables the bacteria to persist for extended periods of time in the host for months or even years, which ultimately may serve as a reservoir for the pathogen thereby contributing to chronic forms of *S. aureus* infection.

2.3.3 Intracellular cycling of *S. aureus*

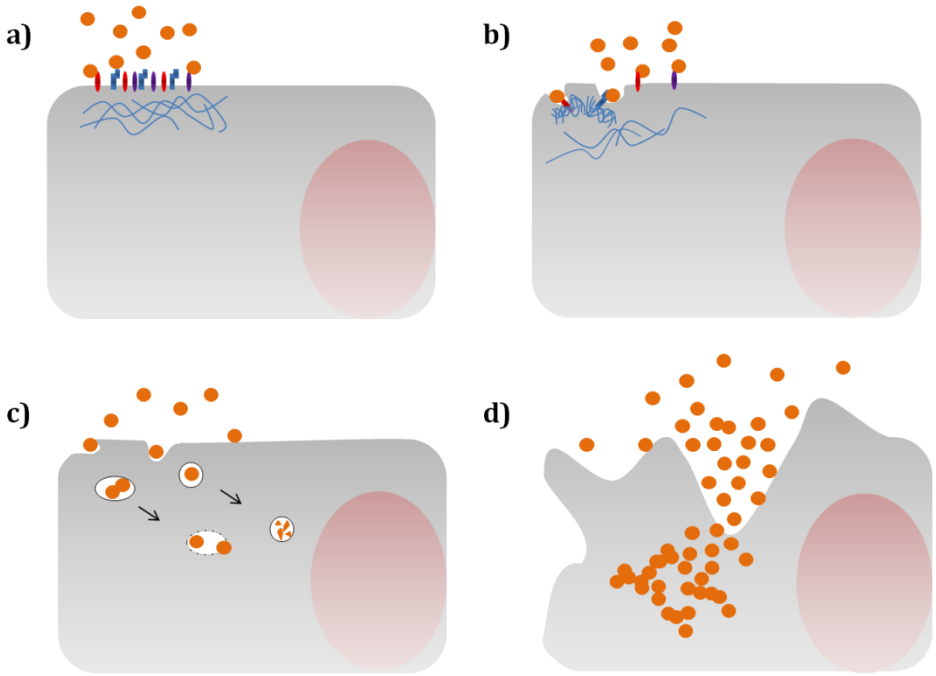

Figure 2.5 Schematic cycle of *S. aureus* infection

Schematic cycle of *S. aureus* infection of a non-professional phagocyte depicted as stages of a) adherence, b) invasion, c) phagosomal escape, d) intracellular replication and consequent apoptosis. a) *S. aureus* adheres to host cells using a set of cell wall anchored proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). b) Uptake into the cell is facilitated through a zipper-type mechanism following cytoskeleton rearrangement, resulting in the engulfment of the bacteria in an endosomal vesicle. c) Bacteria employ membrane targeting toxins in an effort to disrupt the membrane surrounding them, d) thereby reaching the cell cytoplasm and averting degradation through lysosomal acidification. Intracellular replication occurs in the cytoplasm preceding the death of the host cell, causing release of the bacteria and completing the intracellular cycle of *S. aureus*.

Tissue persistence of *S. aureus* may occur also by alternative mechanisms. Phagocytes such as macrophages readily engulf and kill *S. aureus*. Recently it was shown, however, that *S. aureus* can escape degradation by macrophages by lysing the phagocytes, resulting in release of the bacteria, which in turn may be engulfed by other macrophages thereby reinitiating a cycle of intracellularity and release. (Kubica *et al.*, 2008; Jubrail *et al.*, 2016). Bacterial survival in macrophages can be attributed to a failure of acidification of *S. aureus* containing vacuoles (Jubrail *et al.*, 2016). Inside these vacuoles, *S. aureus* can reside for prolonged time and replicate (Flannagan *et al.*, 2015b). Macrophages failing to control an infection with *S. aureus* nevertheless maintain their cellular integrity as bacteria reside inside phagosomal membranes for days before killing the host cells in a manner exhibiting hallmarks of both, apoptosis and necrosis (Flannagan *et al.*, 2015b). Persistence in primary human macrophages and other blood stream

leukocytes, cell types known for their motility within the host body could therefore also serve *S. aureus* as vesicles for dissemination in the host (Kubica *et al.*, 2008; Thwaites and Gant, 2011).

2.3.4 *S. aureus* induced host cell death

Different types of cell death are distinguishable. The most common form of programmed cell death being observed is apoptosis, a caspase-dependent process of cellular disintegration naturally involved in cell aging as well as tissue development and maintenance (Elmore, 2007; Taylor *et al.*, 2008). Caspases, a family of cystein proteases specifically cleaves C-termini of aspartat residues of target substrates including actin, myosin, spectrins, filamin, tubulins and keratins, all of which have functions in cytoskeleton arrangement and integrity (Ku *et al.*, 1997; Stennicke and Salvesen, 1998; Nicholson, 1999; Gerner *et al.*, 2000; Thiede *et al.*, 2005; Adrain *et al.*, 2006). The known caspases can be differentiated into groups according to function. While the caspases 1, 4, 5 and 12 act in controlling the inflammation, caspases 2, 8, 9 and 10 are initiator caspases, instrumental in the control of executioner caspases 3, 6, 7 (McIlwain *et al.*, 2013).

Functional and structural disintegration of the cytoskeleton will lead to rounding of the cells, formation of plasma membrane blebs and fragmentation of the nucleus, all key characteristic of apoptotic cells (Taylor *et al.*, 2008). Later apoptotic bodies are formed by many cell types, which triggers phagocytosis by professional phagocytes, mainly macrophages (Elmore, 2007).

Apoptosis is also the most prevalent version of host cell death observed upon contact with virulent *S. aureus* strains (Menzies and Kourteva, 1998; Bayles *et al.*, 1998; Haslinger *et al.*, 2003; Genestier *et al.*, 2005; Fraunholz and Sinha, 2012). Induction of apoptosis occurs dependent on gene regulation by the global regulator *agr* and the σB, while an involvement of *sar*A is not yet resolved (Wesson *et al.*, 1998; Qazi *et al.*, 2001; Haslinger-Loffler *et al.*, 2005; Jarry and Cheung, 2006; Jarry *et al.*, 2008; Kubica *et al.*, 2008). Among the *agr* controlled bacterial factors, α-toxin has been shown to activate caspases and induce apoptotic pathways independent of CD95/Fas/APO-1 death receptor signaling at very low doses (Bantel *et al.*, 2001; Haslinger *et al.*, 2003; Essmann *et al.*, 2003). Higher doses lead to necrotic forms of cell death in the same studies. As necrosis is accompanied by the release of inflammatory cytokines, the induction of apoptosis is beneficial for bacterial survival. Pyroptosis is a further form of controlled cellular destruction heavily tied to various stimuli accompanied by microbial infection (Bergsbaken *et al.*, 2009). In caspase-1 dependent pyroptosis, the protease causes plasma membrane rupture and the release of pro-inflammatory intracellular contents similar to necrosis (Brennan and Cookson, 2000; Fink and Cookson, 2006; Bergsbaken *et al.*, 2009). But

while necrosis is an energy independent and uncontrolled process, pyroptosis occurs specifically in order to alert the immune system upon recognition of a threat (Bergsbaken *et al.*, 2009). Pyroptosis is largely restricted to leukocytes, where caspase 1 activation has been shown to be beneficial in the clearance of internalized pathogens (Amer and Swanson, 2005; Gurcel *et al.*, 2006).

The significance of the induction of controlled cell death by intracellular bacteria in general was shown for many different kinds of host cells and in particular in the case of primary endothelial cells, where only invasive *S. aureus* strains induced apoptosis (Nuzzo *et al.*, 2000; Tucker *et al.*, 2000; Haslinger-Loffler *et al.*, 2005; Jarry and Cheung, 2006; Chatterjee *et al.*, 2008; Kubica *et al.*, 2008; Lam *et al.*, 2010; Fraunholz and Sinha, 2012). Cell death by intracellular *S. aureus* is thought to be preceded by the phagosomal escape of the internalized bacteria (Grosz *et al.*, 2014). While replication inside intact phagosomes and autophagosomes has been described, efficient progeny of *S. aureus* in many cases has been shown to be dependent on prior disruption of the phagosomal membrane and the transition to the host cell cytoplasm (Schnaith *et al.*, 2007; Kubica *et al.*, 2008; Grosz *et al.*, 2014; Flannagan *et al.*, 2015b).

2.4 *S. aureus* modulation of phagosome maturation and integrity

Invasion of bacteria into non-professional phagocytes and phagocytosis of pathogens by professional phagocytes leads to an uptake of the bacteria into endosomal vesicles that are formed via invagination of the plasma membrane. These vesicles will undergo a series of fusion events (Fairn and Grinstein, 2012). During this so-called maturation phagosomes will fuse in an orchestrated fashion with early endosomes, late endosomes and finally lysosomes (Desjardins *et al.*, 1994). During this succession, highly reactive substances such as reactive oxygen species (ROS), reactive nitrogen species (NOS), antimicrobial peptides and hydrolases are acquired by the continuously acidifying phagosome, leading to an eventual degradation of the phagosomal content. The transformation of the phagosome to a phagolysosome is marked by changes in membrane-associated proteins, such as Rab-family GTPases and lysosomal-associated membrane proteins (LAMP) which associate with and dissociate from the vesicle and thus can be used to visualize maturation (Eskelinen *et al.*, 2003; Rink *et al.*, 2005; Smith *et al.*, 2007; Chen *et al.*, 2015).

Membraneous compartments can also be formed through a second mechanism called autophagy, which are involved in cell maintenance and immune response. Autophagy is defined by the formation of double membrane structures in the cell cytoplasm called phagophores,

which recruit microtubule-associated protein light chain 3 (LC3; also known as ubiquitin-like protein Atg8) to their membrane (Kabeya *et al.*, 2000). Autophagosomes facilitate the degradation of either bulk cytoplasmic material, dysfunctional or surplus organelles, invading microorganisms or foreign proteins. In these functions, autophagosome formation is a direct factor in the health of cell and the whole organism, as autophagy is linked to diseases like cancer, Parkinson's, Alzheimer's as well as muscular disorders (Shintani and Klionsky, 2004). Autophagosome formation is structurally linked to endoplasmic reticulum exit sites (ERES) and Atg9 vesicles originating from the Golgi apparatus (Mari *et al.*, 2010; Yamamoto *et al.*, 2012; Graef *et al.*, 2013; Sanchez-Wandelmer *et al.*, 2015) The phagophore will grow by acquiring membrane lipids from either the plasma membrane, the ER, the Golgi complex or mitochondria (Hayashi-Nishino *et al.*, 2009; Hailey *et al.*, 2010; Ravikumar *et al.*, 2010; Takahashi *et al.*, 2011)

Staphylococcus aureus belongs to a group of intracellular microorganisms, also including *Brucella abortus, Leishmania mexicana, Coxiella burnetii* and *Chlamdia trachomatis*, which can use the autophagosome to replicate (Schaible *et al.*, 1999; Celli *et al.*, 2003; Al-Younes *et al.*, 2004; Gutierrez *et al.*, 2005; Schnaith *et al.*, 2007; Mestre *et al.*, 2010; Mestre and Colombo, 2012). In general, the contribution of autophagy in the course of *S. aureus* infection remains elusive and debated as to whether autophagosomes provide a niche for *S. aureus* to multiply efficiently, or if they represent a vital mechanism in the degradation of intracellular *S. aureus* (Mauthe *et al.*, 2012; Maurer *et al.*, 2015).

To prevent their own degradation, bacteria as *Mycobacterium tuberculosis* or *Legionella pneumonia* developed strategies to prevent the Rab-GTPase-dependent maturation of the phagosome. Other bacteria disrupt the integrity of the surrounding membrane and reach the pH-neutral cytoplasm of the host cell (Meresse *et al.*, 1999; Vieira *et al.*, 2002). For *S. aureus*, the translocation from the phagosome to the cytoplasm has been shown (Bayles *et al.*, 1998; Kahl *et al.*, 2000; Qazi *et al.*, 2001; Shompole *et al.*, 2003; Jarry and Cheung, 2006; Grosz *et al.*, 2014). This process is dependent on an active *agr* quorum sensing system, as *agr* negative mutants lose the ability to disrupt the phagosomal membrane (Qazi *et al.*, 2001; Shompole *et al.*, 2003; Schnaith *et al.*, 2007; Jarry *et al.*, 2008).

Among the virulence factors controlled by *agr*, an involvement of α-toxin (*hla*) in phagosomal escape of *S. aureus* could only be demonstrated in the rather specific cystic fibrosis disease background, where a deletion of *hla* lead to attenuation of escape rates. However, in other cell backgrounds *hla* expression could not be linked to phagosomal escape (Jarry *et al.*, 2008; Giese *et al.*, 2009; Grosz *et al.*, 2014). Other virulence factors work synergistically. Only when both, β-toxin and the PSM δ-toxin are overexpressed in an escape-negative *S. aureus* laboratory strain, the bacteria regain the ability to translocate to the host cytoplasm (Giese *et al.*, 2011).

The strongest effect on phagosomal escape by any virulence factor of *S. aureus* yet was documented for PSMα. Expression of the α-class PSM was shown to be absolute essential for phagosomal escape in non-professional phagocytes (Grosz *et al.*, 2014). Findings, that PMN are killed by *S. aureus* in a PSM dependent manner, specifically after phagocytosis occured, and that the binding of PSM to serum lipoproteins inhibits their cytolytic activity further point to a primarily intracellular action of PSMs (Geiger *et al.*, 2012; Surewaard *et al.*, 2012; Surewaard *et al.*, 2013). After internalization by host cells, PSM production increased strongly and did only decrease again after translocation of the bacterium to the cytoplasm of the host cell. Translocation in general as well as intracellular replication is impeded when the bacteria are not able to produce PSMα (Grosz *et al.*, 2014). Yet, these studies have also shown, that PSMα is not sufficient to mediate phagosomal escape of *S. aureus* since an escape-deficient strain overexpressing PSMα1-4 did not escape the host cell phagosome (Grosz *et al.*, 2014). Other, not yet identified, virulence factors of *S. aureus* must be involved in the facilitation of efficient escape.

2.5 Aims of this work

Recent research has unveiled an intracellular strategy of *S. aureus* to survive in the human host and to establish infections. In the host, *S. aureus* is readily internalized by phagocytes and non-professional phagocytic cells such as epithelial and endothelial cells. *S. aureus* thereby avoids disinfection within phagolysosomes by translocating to host cell cytoplasm. The mechanisms behind the escape of *S. aureus* from the phagosomal compartment remain largely unknown.

In order to identify new factors involved in *S. aureus* virulence the aims of this study were to identify and characterize mutants deficient in phagosomal escape and thereby impeded in their intracellular survival.

To achieve this goal, transposon mutant libraries in a highly cytolytic USA300type CA-MRSA strain should be generated and employed to assess the escape efficiency of single gene mutants in comparison to the wild type. The mutants identified in the screen then were to be tested for their cytotoxicity as well as their virulence potential in different in vitro and in vivo infection settings.

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Bacterial strains

Table 3.1: Bacterial strains used in this study

Name	Properties	Source
E. coli DH5α	DH10b dcm$^-$	Douglas Hanahan
E. coli DC10b	PFGE Type USA300, lacks pUSA03 Erm^S	
S. aureus LAC*	PFGE Type USA300, lacking plasmids pUSA01-03	(Fey *et al.*, 2013)
S. aureus JE2	JE2 *aus*A::bursa, Erm^R	(Fey *et al.*, 2013)
S. aureus JE2 NE119	JE2 *aus*B::bursa, Erm^R	(Fey *et al.*, 2013)
S. aureus JE2 NE964	JE2 NE119 containing p2085_*aus*B, Erm^R, Cm^R	This study
S. aureus JE2 NE964+0182	Cm$^+$	(Joo *et al.*, 2011)
S. aureus JE2 Δabd	MSSA, cytolytic isolate from septic arthritis	(Vann and Proctor, 1987)
S. aureus 6850	Laboratory strain accepting foreign DNA	(Kreiswirth *et al.*, 1983)
S. aureus RN4220	Patient isolate	Riedel, Greifswald
P. aeruginosa	-	
B. subtilis		

A full list of Nebraska library mutants tested is given as table 7.1.

3.1.2 Cell lines

Table 3.2: Cell lines used in this study

Cell Line	Media	Source
HeLa	RPMI, 10% FCS	ATCC CCL-227
HeLa 229	RPMI, 10% FCS	ATCC CCL-2.1
HeLa 229 YFP-cwt	RPMI, 10% FCS	(Grosz *et al.*, 2014)
HeLa Mito-GFP	RPMI, 10% FCS	Suvagata Roy Chowdhury, unpublished
HEK 293T	RPMI, 10% FCS	ATCC CRL-3216
IB3 YFP-cwt	DMEM, 10% FCS	This study
S9 YFP-cwt	DMEM, 10% FCS	This study

3.1.3 Plasmids

Table 3.3: Plasmids used in this study

Name	Properties	Source
pKOR1	Allelic replacement vector, inducible expression of *sec*Y antisense RNA for counterseletion of plasmic backbone	(Bae and Schneewind, 2006)
pBTn	bursa-delivery plasmid; temperature-sensitive Origin of Replication (permissive at 30°C), xylose-inducible expression of Tnp, Erm^R	(Li *et al.*, 2009b)
p2085	shuttle vector for anhydrous tetracyclin-inducible expression of GFP in *S. aureus*, Amp^R, Cm^R	(Giese *et al.*, 2009)
p2085_SarAP1_mRFPmars	Amp^R, Cm^R	(Paprotka *et al.*, 2010)
p2085_SarAP1_*aus*B_mRFPmars	-	This study
pLVTHM	2nd generation lentiviral expressing shRNA from H1 promoter and GFP. pLVTHM was a gift from Didier Trono (Addgene plasmid # 12247)	(Wiznerowicz and Trono, 2003)

psPAX	2nd generation lentiviral packaging vector. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260)	Didier Trono, unpublished
pVSV-G	2nd generation Envelope plasmid. pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259).	Didier Trono, unpublished
pcDNA 6.2 – YFP-TOPO	-	Invitrogen

3.1.4 Oligonucleotides

Table 3.4: qRT Oligonucleotides

Name	5'-3'	Annealing Temp (°C)
0181_for_RT	GTGTCCATTTTCGATGATAGTC	59.9
0181_rev_RT	TGTCAGAGTTATTCTCTAACCG	57.6
0182_for_RT	AACCGCGTACAGTCAACTATAG	59.8
0182_rev_RT	ATAAGCTCACATAGATGGGC	58.2
SecTrans_for_RT	AGTTTTACAAAATTGATTGGTG	57.2
SecTrans_rev_RT	GGTGCCTGTCCTAAGAAAC	58.7
RT-gyrB-F	CGACTTTGATCTAGCGAAAG	59.2
RT-gyrB-R	ATAGCCTGCTTCAATTAACG	58.5

Table 3.5: Oligonucleotides used in cloning

Name	5'-3'	Annealing Temp (°C)
0182_for	CATCCTAGGAGGAAAGTTATGACAGTATTTGTAATGC	71.1
0182_rev	GACCCTAGGTTAACTACTCAATAACTGAAATACAGACAC	69.2
pkor_0181_for_1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGTAATTATATGTTATTGATTTCG	80.5
pkor_0181_for_2	CCGCGGAAAAAGTCCTTCTTTCATATCATAATAC	72.4
pkor_0181_rev_1	CCGCGGGTAGGGAAAGTTATGACAGTATTTGTAATG	75.6
pkor_0181_rev_2	GGGGACCACTTTGTACAAGAAAGCTGGGTGAATTAGTGCGACAACATT TTGTCTATC	84.6
pkor_0182_for_1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTGCTTATCTGATTGAAGTACTAC	81.6
pkor_0182_for_2	CCGCGGAACTTTCCCTACTTACTTATTGAATATTG	72.7
pkor_0182_rev_1	CCGCGGTATGACGTCTGACAGTATCATTGC	77.2

pkor_0182_rev_2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAAAACATTTATTTACCGTTCATCTC	81.9
6850_0181_upstream_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAACTATATGTTAATGATTTCAG	81.2
6850_0182_downstream_for	CCGCGGACATGGCGTCTAACAGTTTTATTTG	77.3
6850_0182_downstream_rev	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCGTCACCTTTTCACCTGTC	86.3

Table 3.6: Oligonucleotides used for detection of Tn-Insertion sites

Name	5'-3'	Annealing Temp (°C)
erm-3.1	TAGGTATACTACTGACAGCTTC	52.1
erm-3.2	ATTCTATGAGTCGCTTTTGTA	55.7
arb1	GGCCACGCGTCGACTAGTCANNNNNNNNNNGATAT	77.6
arb3	GGCCACGCGTCGACTAGTCA	70.9
NE964_c_for	GAAAGTTATGACAGTATTTGTAATGC	58.7
NE964_c_rev	GATACTGTCAGACGTCATATTAACTACTC	60.3
NE119_c_for	CCGAAGCATCCTCAAATTATTAATGG	67.5
NE119_c_rev	CGATATCACCACTTCTATACATCAGCTG	66.3

Table 3.7: Oligonucleotides used in transposon mutagenesis library generation

Name	5'-3'	Annealing Temp (°C)
MultiPlex-Y-Adapt_f	ACACTCTTTCCCTACACGACGCTCTTCCGATC*T	64.5
MultiPlex-Y-Adapt_r	[Phos]GATCGGAAGAGCACACGTCT	77.3
Himar_TnSeq_Read1_v2	ACCGAGATCTACGGACTTATCAGCCAACCTGT	77.7
MP-TnSeq_Index1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT	91.4
MP-TnSeq_Index3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT	90.2
himar-5-PCR	CCATAACTTTAGGGTTAACCATACGC	65.0
himar-3-PCR	CAGCTTCCAAGGAGCTAAAGAGGTCC	70.5
IS-Himar-forward	AATGATACGGCGACCACCGAGATCT	72.8
IS-BC-Reverse	CAAGCAGAAGACGGCATACGAGAT	69.2

3.1.5 Enzymes

Table 3.8: Enzymes used in this study

Name	Supplier
Taq DNA Polymerase	GenAxxon Bioscience
Phusion DNA Polymerase	ThermoFischer
T4 DNA Ligase	Fermentas
FastAP Alkaline phosphatase	Fermentas
Restriction endonucleases	Fermentas
Proteinase K	Roth
RNase A	Fermentas

3.1.6 Buffers and Media

Table 3.9: Buffers for molecular biology and microbiological methods

Buffer	Ingredients
Electroporation Buffer	0.5 M Sucrose, 10% (v/v) Glycerol
Buffer A for RNA isolation	10% Glucose, 12.5 mM Tris-HCl, pH 7.6, 10 mM EDTA Ad DECP-treated H_2O
S. aureus lysis Buffer	200 µg/ml Lysostaphin, 20 mM Tris-HCl, 2 mM EDTA, 1.2% ((v/v) Triton, 20 µg/ml RNAse A
TAE Buffer	242 g Tris base, 57.1 g Acetic acid, 100 ml EDTA (0.5 M pH 8) Ad 1 l H_2O
Opsonization Buffer	HBSS++ with 10% (v/v) pooled Human Serum
TRITC/FITC staining Buffer	10 ml 5.3% (w/v) $NaHCO_3$ 5.6 ml 4.2% (w/v) $NaCO_3$
Permeabilization Buffer	0.2% (v/v) TritonX-100 in 1x PBS
4% PFA	4% (w/v) PFA in 1x PBS, adjust pH to 7.4
Oligonucleotide annealing buffer	100 mM Tris-HCl pH 7.5, 10 mM EDTA, 500 mM NaCl
6x DNA loading dye	1.2 ml Glycerol, 1.2 ml 0,5 mM Na_2EDTA, 300 µl 20% SDS, Bromophenol blue Ad 10 ml H_2O
TE Buffer	1 mM EDTA, 10 mM Tris-HCl (pH8.0)
Tris-Sucrose Buffer	10 mM Tris-HCl, 25% Sucrose, 2.5 mM EDTA

Table 3.10: Buffers for cell culture methods

Media	Ingredients
HBSS++	5 ml 10x Hanks Balanced Salt Solution (HBSS), 20 mM HEPES 1.26 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% (v/v) Human Serum Albumin Ad 50 ml ddH_2O
Opsonization buffer	HBSS++, 10% (v/v) pooled human serum
Full medium	RPMI/DMEM-medium, 10% (v/v) heat-inactivated FCS, 1U Penicillin, 1U Streptomycin, 1 mM Sodium Pyruvate
Infection medium	RPMI/DMEM-medium, 10% (v/v) heat-inactivated FCS, 1 mM Sodium Pyruvate
SILAC-Medium	RPMI 1640, 10% (v/v) dialyzed FCS, 1U Penicillin, 1U Streptomycin, 0.35 mM L-Leucin, 0.195 mM L-Lysin, 0.13 mM L-Arginine
Glycolysis Stress Test Assay Medium	XF Base Medium (Minimal DMEM medium), 1 mM Pyruvate, 2 mM Glutamine, 10 mM Glucose Adjust pH to 7.4 +/- 0.05
PMN Chemotaxis Test Medium	HBSS, 0.05% (v/v) Human Serum Albumin
Calcium Flux Test Medium	RPMI, 0.05% (v/v) Human Serum Albumin
Annexin Labeling Solution	10 mM HEPES pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$
LB Medium	10 g NaCl, 10 g Trypton, 5 g Yeast Extract Ad 1 L H_2O
LB Agar	10 g NaCl, 10 g Trypton, 5 g Yeast Extract, 15 g Agar Ad 1 L H_2O
TSB Medium	30g TSB Ad 1 L H_2O
TSB Agar	30g TSB, 15g Agar Ad 1 L H_2O
TSB Medium +Glucose	27.5 g TSB Ad 1 l H_2O After autoclaving: +5 ml 50% (v/v) Glucose
50% Glucose Solution	50% (w/v) Glucose, Ad 500 ml H_2O
Live Cell imaging medium	RPMI infection medium without phenol red, 20 mM HEPES
B2 Medium	10 g Casein hydrolysate, 25 g Yeast extract, 5 g Glucose, 25 g NaCl, 1g K_2HPO_4 adjust pH to 7.5 Ad 1 L H_2O
SOC Medium	2% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose

3.1.7 Kits

Table 3.11: Kits used in this study

Name	Supplier
TurboDNA Free Kit	Ambion (Life Technologies)
QIAamp DNA Mini Kit	Qiagen
PureLink Quick Plasmid Miniprep Kit	Invitrogen
Lipofectamine 3000	Invitrogen
Cytotoxicity Detection Kit (LDH)	Roche
RevertAid RT Kit	Thermo Scientific
Invisorb Fragment CleanUp	Stratec
Agencourt® AMPure XP PCR Purification beads	Beckman Coulter
NEBNext® End Repair Module	NEB
NEBNext® dA Tailing Module	NEB

3.1.8 Chemicals

Table 3.12: Chemicals used in this study

Name	Supplier
1 kb DNA Ladder	Fermentas
Acetic Acid	Roth
Aceton	Roth
Agar	BD
Agarose	Serva
Ampicillin	Roth
Annexin V	Invitrogen
Antimycin A	Sigma
Aureusimine B	Santa Cruz
Bacto yeast extract	BD
BCECF	Molecular Probes

Bromophenolblue	Roth
BSA	Roth
Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma
Chloramphenicol	Roth
Chloroform	Applichem
DEPC	Roth
Dimethylsulfoxide (DMSO)	Roth
dNTPs	Gennaxxon
EDTA	SERVA
Erythromycin	Roth
Ethanol (technical)	Sigma
Ethanol (preparative)	Roth
Fetal Bovine Serum, dialyzed (FBS)	Sigma
Fetal Calve Serum (FCS)	PAA
Ficoll-Paque™ Plus	GE Healthcare
Fluo-3 AM	Molecular Probes
fMLF	Sigma
GeneRuler 1 kb/100 bp	Fermentas
Geniticin (G418)	Invitrogen
Gentamicin	Gibco
Glucose	Sigma
Glutamine	Sigma
Glycerol	Roth
Gly-Phe β-naphthylamide	Santa Cruz
HCl	Roth
HD Green Plus	Intas
Hepes	Life Technologies
Histopaque – 1119	Sigma
Human Serum Albumin	Sigma
Isopropanol	Roth
Kanamycin	Roth
L-Arginine monochloride	Sigma
L-Leucine	Sigma

L-Lysin monochloride	Sigma
Lysostaphin	AMBI
Monensin	Sigma
Mowiol 4-88	Calbiochem
NaCl	Roth
Nitrocefin	Sigma
Oligomycin	Millipore
Paraformaldehyd	Roth
PEI	Polyscience
Phosphat-buffered Saline (PBS)	Invitrogen
Phosphat-buffered Saline (PBS) Powder	AppliChem
Phytohemagglutinin (PHA)	Sigma
PMA	Sigma
Propidium Iodide	Invitrogen
PVA	Sigma
Pyruvat	Sigma
Rotenon	Sigma
SDS	Roth
Sheep Blood defibrinated	Fiebig Nährstofftechnik
Sodium perchlorate	Sigma
D-Sucrose	Roth
TMRM	Sigma
Tri Reagent	Ambion
Tris	Sigma
Triton X-100	Roth
Tetracycline	Applichem
Trypan Blue	Sigma
Tryptic Soy Broth	Fluka
Tryptic Soy Broth without Dextrose	Fluka
Tryptone	Oxoid
Tween-20	Sigma

3.1.9 Technical Equipment

Benchtop centrifuge Micro200 (Hettich), Cell incubator Heracell 2400 (Thermo Scientific), Plate centrifuge Megafuge 1.0 R (Heraeus), Cooling centrifuge CT15RE (himac), Thermo mixer comfort (Eppendorf), Plate scanner Infinite m200 (Tecan), Confocal fluorescence microscope TCS SP5 (Leica), Refrigerated centrifuge CT15RE (himac), Electroporator Micropulser (BIORAD), pH meter (Hartenstein), Photometer Ultraspec 3100 pro (Amersham Biosciences), Automated Fluorescence microscope Operetta (Perkin Elmer), Nanodrop 1000 Spectrophotometer (Peqlab), StepOne Plus RT-PCR system (Applied Biosciences), Accuri C6 Flow Cytometer (BD), FACSAria III Flow cytometer (BD), FACSCalibur Flow cytometer (BD), pH meter pH720 WTW series (inoLab), Transilluminator DH 40/50 (Biostep), Vortex REAX 2000 (Heidolph), Seahorse Extracellular Flux Analyzer XF^{e}96 (Seahorse Bioscience), FLUOstar Optima Plate Reader (BMG Labtech), Diagenode Bioruptor (Diagenode SA, Belgium), Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Milford, USA), Waters Acquity ultra-high-performance liquid chromatography system (Milford, USA)

3.1.10 Software

Windows 7 (Microsoft), Windows Office 2010 (Microsoft), ImageJ (Schneider *et al.*, 2012), Operetta software Harmony (Perkin Elmer), Artemis (Wellcome Center), ApE plasmid editor (biologylabs.utah.edu), Codon Code Aligner (CodonCode Corp), InkScape (InksScape Community), Corel Draw X5 (Corel), EndNote X6 (Thomson Reuters), NanoDrop ND-1000, SnapGene Viewer (GSL Biotech LLC), CFlow Plus (Accuri C6), Wave (Extracellular Flux Analyzer Software, Agilent), Step OneRT-PCR Software version 2.3 (applied biosystems), ProGenesis (UPLC-MS/Non-linear dynamics)), Masslynx (MS/Waters), Quanlynx (MS/Waters), Chem3D (Perkin-Elmer)

3.2 Methods

3.2.1 Bacterial culture methods

Cultivation of bacteria

E.coli and *S. aureus* strains were cultured overnight at 37°C on respective LB or TSB agar plates or liquid medium at 37°C and 200 rpm. Strains carrying plasmids with temperature sensitive origins of replication were grown at 30°C. If required, the growth medium was supplemented with selective antibiotics.

Bacteria stocks

In order to generate bacterial culture stocks, liquid cultures were grown at 37°C overnight in respective medium. The following day, bacteria were mixed with 50% sterile glycerol to a final concentration of 12.5% (v/v) and stored at -80°C.

Growth curves

To determine differences in bacterial growth, growth curves were recorded using an Infinite m200 plate reader (Tecan). Bacteria were cultured overnight at 37°C and 200 rpm. OD_{600} of the overnight culture (ONC) was determined and bacteria were diluted to an OD_{600} of 0.1in medium before loading 400 µl per sample on a 48-well-plate (Corning).

To warrant statistical significance, three biological replicates were tested using five technical replicates each. Sterile medium was used for reference. The 48-well-plate was placed in a preheated plate reader and OD_{600} was measured every 600 sec for 50 kinetic cycles of orbital shaking with an amplitude of 3 mm accumulating to a total of 8 h. Data was expressed as OD_{600} ± SD.

For application of test substances, the incubation was paused when the indicated OD_{600} of the sample was reached, and wells were treated with substances under investigation of solvent controls. Measurements commenced normally after the spike-in. Data was expressed as OD_{600} ± SD.

Hemolysis assay

To assess the hemolytic activity of *S. aureus* bacterial overnight cultures were grown at 37°C and 200 rpm to stationary growth phase. Cultures were diluted 1:100 in fresh TSB medium and 100 µL of the dilution were spotted on Columbia agar plates supplemented with 5% defibrinated sheep blood. Inoculated agar plates were incubated overnight at 37°C. Bacterial colonies and

hemolytic zones were photographed using an Olympus SZ261 binocular at a magnification of 5x. Diameters were scored using Fiji.

3.2.2 Genetic manipulation of bacteria

Transformation of competent *E.coli*

Chemically-competent *E.coli* were thawed on ice, transferred to a 15 ml Falcon tube and DNA was added. Bacteria were kept on ice for 30 min. Samples were heat shocked for 90 sec at 42°C and chilled on ice for 2 min. 1 ml of LB medium was subsequently added and the bacteria were incubated for 1 h at 37°C and 200 rpm shaking. After incubation the bacteria were plated on selective LB agar plates.

Production of electro competent *S. aureus*

100 ml of fresh TSB medium (flask to medium ratio 1:10) were inoculated to an OD_{600} of 0.05 from a TSB overnight culture and incubated at 37°C and 200 rpm. When OD_{600} 0.5 - 0.6 was reached, bacteria were chilled on ice for 15 min and were pelleted at 4000 g at 4°C for 10 min. Supernatant was discarded and bacteria were resuspended in 100 ml ice-cold 0.5 M sucrose.

Centrifugation was repeated and the pellet was washed with decreasing volumes of 0.5 M sucrose (50 ml, 25 ml, and 12.5 ml). After a final centrifugation, bacteria were taken up in 800 µl of sterile 20% (v/v) glycerol and 50 µl aliquots were frozen at -80°C.

Electroporation of *S. aureus*

To transform *S. aureus* with plasmid DNA, electro competent bacteria, electroporation buffer and DNA were thawed at room temperature for 15 min. 50 µl of bacteria were added to 120 µl of EP and 5-10 µg of plasmid DNA and incubated at room temperature for additional 20 min.

Electroporation was performed in 2 mm cuvettes (VWR) at 1.8 kV for 2.5 ms. Bacteria were taken up in 1 ml of B2 medium and shaken at 37°C and 200 rpm for 2 h in 15 ml conical polypropylene tubes (Greiner). After incubation, bacteria were pelleted by centrifugation and resuspended in a volume of 100-200 µl. For overnight incubation at 37°C, bacteria were plated on TSA plates with selective antibiotics.

Isolation of genomic *S. aureus* DNA

For isolation of genomic DNA from *S. aureus* QIAamp DNA Mini Kit (Qiagen) was used. 1.5 ml of an overnight culture were pelleted in a table centrifuge at 14,000 rpm for 1 min. Supernatant was discarded and bacteria were taken up in 180 µl of lysis buffer. After incubation at 37°C for at

least 30 min, 200 µl Buffer AL and 4 µl of 20 µg/ml Proteinase K were added. The sample was mixed and incubated for 30 min at 56°C.

200 µl ethanol (96-100%) were added and the sample was mixed by pulse-vortexing for 15 sec. All following centrifugation and washing steps were performed according to `DNA Purification from Tissues Manual´ provided with the kit. To elute DNA, 50 µl of preheated ddH_2O was added to the spin column and incubated for 5 min. After final centrifugation, DNA yield was quantified using gel electrophoresis as well as spectrophotometry.

Plasmid extraction

For isolation of plasmids from bacteria, 5 ml of bacterial culture were grown overnight and 1.5 - 3 ml of the ONC were pelleted by centrifugation using a tabletop centrifuge at maximum speed.

Plasmids from *E. coli* were extracted using the Plasmid Extraction Mini Kit (Invitrogen) following the provided manual. Isolation of plasmids from *S. aureus* cultures required the addition of 20 µg/ml Lysostaphin to the bacteria culture after resuspension in resuspension buffer and a subsequent incubation of at least 30 min at 37°C.

Polymerase Chain Reaction

PCR reactions were routinely performed in 200 µl reaction tubes and 50 µl final volume. A standard PCR reaction mix consisted of 10 – 50 ng template DNA, 1x polymerase buffer, 0.2 pmol each of forward and reverse primer and 1U of thermostable polymerase. In applications depending on sequence quality Phusion polymerase (Thermo) with 3'-5' exonuclease activity was used to warrant low error rates during amplification. In all other applications Taq polymerase (Genaxxon) was used. After an 95°C (98°C for Phusion) initiation step for 5 min, up to 32 cycles of 30 sec at 95°C, 30 sec at 50-65°C (depending on primer melting temperature) and a product length dependent 72°C elongation step (30 sec/kb for Phusion; 1 min/kb for Taq) followed. PCR products were subsequently analyzed on 0.8 - 2% TAE agarose gels supplemented with 0,005% HD Green Plus and visualized under UV-light. If necessary, PCR products were purified using a PCR purification kit according to manufacturer's protocol.

Restriction and ligation

Restriction enzymes and T4 DNA ligase were purchased from Fermentas and used according to the manufacturers protocols provided. Vector/Insert ratios for ligation were determined using the formula:

((ng of vector x kb size of insert) / kb size of vector) x (molar ratio of Insert / Vector)

3.2.3 *S. aureus* knock-out strategies

Generation of a transposon insertion mutant library ana analysis by NGS

The generation of a transposon insertion mutant library was performed as described before (Li *et al.*, 2009b). In short, *S. aureus* LAC* containing the temperature sensitive pBTn plasmid was grown in TSB medium (without dextrose) supplemented with 0.5% xylose, chloramphenicol and erythromycin at 30°C for 24 h. During this growth phase transposition of the mobile element into the *S. aureus* genome occurred. From this culture fresh TSB (without dextrose) containing 0.5% xylose and erythromycin were inoculated 1:100. The culture was incubated at 42°C for 24 h and the whole process of re-inoculating fresh cultures and overnight incubation was repeated twice more, once without antibiotics. Bacteria were consequently plated on TSB agar plates containing erythromycin, grown overnight at 37°C and pooled the following day by scraping.

To isolate chromosomal DNA in order to sequence for transposon insertion sites, bacteria were pelleted by centrifugation and resuspended in 200 µl of Tris-Sucrose buffer with 200 µg/ml RNaseA and 100 µg/ml Lysostaphin. The mixture was incubated at 37°C for 30 min before adding 325 µl of TE buffer, 225 µl of 10% SDS and 20 µl Proteinase K (20 mg/ml). After 30 min of incubation at 55°C, 150 µl of 5 M sodium perchlorate was added, followed by 0.5 volumes of chloroform-isoamyl alcohol (24:1). This was followed by 1 hour of incubation at room temperature under vigorous shaking before the aqueous phase was collected and 1 volume of ice cold isopropanol was added in order to precipitate the DNA. The sample was therefore incubated on ice for 30 min and centrifuged at 14,000 rpm for 10 min. The DNA pellet was then washed once using 1 ml of 70% ethanol, air dried and dissolved in 200 µl of nuclease-free water.

Fragmentation of the collected DNA was performed on a Bioruptor sonifier (Diagenode). DNA was first diluted in 1 ml and fragmented for 10 cycles of 30 sec ON and 30 sec OFF at a high power setting (pos H) at 4°C. The ends of 5 µg of the fragmented DNA were repaired using NEBNext repair module and subsequently purified using Agencourt AMPure XP magnetic beads according to manufacturer's protocol. After purification, DNA was taken up in 42 µl of nuclease-free water. DNA fragment size selection (200 - 300 bp) was performed using Agencourt AMPure XP magnetic beads as described (Gilbert *et al.*, 2010). A-tailing of the fragments was done using NEBNext dA tailing module (NEB) and the DNA was again purified using the Agencourt AMPure XP magnetic beads. DNA was dissolved in 30 µl of nuclease-free water.

Multiplex adaptors were generated from oligonucleotides MultiPlex-Y-Adapt_f and MultiPlex-Y-Adapt_r (Table 3.7) by mixing equimolar concentrations and heating them to 94°C for 5 min in 1x Oligo annealing buffer before gradually cooling the mixture to room temperature. The adaptors were ligated to the DNA fragments at 16°C over-night and purified using Agencourt® AMPure XP magnetic beads followed by elution in sterile nuclease-free water.

Subsequent PCR cycles were used to enrich for DNA fragments containing the transposon insertion site while simultaneously Illumina barcodes were introduced by a two-step PCR procedure. During the first 10 cycles of linear PCR using the TNSeq-HimarPCR oligonucleotide I enriched for transposon ends. Subsequently a barcoded MP-TnSeq_Index oligonucleotide was added, and the reaction was continued for 10 additional cycles. To achieve an equimolar ratio, 3´ and 5´ reactions were mixed after purification by AMPure beads. The successful incorporation of the sequences was tested using PCR with IS-Himar-Forward and IS-BC-Reverse primer.

Using the transposon-specific Himar1-Seq primer, the generated libraries were sequenced using an Illumina® Hi-Se2500 platform (single reads). The Illumina adapter sequences were removed via cutadapt version 1.2.1 and were consequently checked for the mosaic end sequence pattern `CAACCTGT` (Martin and Wang, 2011). To be of relevance for further analysis, reads must have had a minimum length of 16 nucleotides and contain the sequence pattern with maximally one mismatch or gap. Reads matching these terms were mapped on the Staphylococcus aureus USA300 LAC genome (Genbank accession NC007793) using Bowtie 2 version 2.1.0 (Langmead and Salzberg, 2012).

In order to identify transposon insertion sites (TIS), alignment start positions of mapped reads were extracted and each alignment start position annotated as TIS. I adjusted the genomic position strand-specifically to account for the 1 bp shift of the reads mapping on the plus or minus strand.

Complete gene knock-out using the pKor1 plasmid

In order to obtain targeted complete gene knock-out mutants for *S. aureus* genes I used the plasmid pKOR1 following published procedures (Bae and Schneewind, 2006). For generation of the knock-out construct, approximately 1 kb regions upstream and downstream of the targeted gene were amplified by PCR using specific primers derived from published genome sequences. Both PCR products were joined by ligation at a SacII restriction site which was added during PCR. Similarly the PCR added *att*B recombination sites at the distal PCR product termini which allowed for BP recombination of the knock-out construct into the vector pKOR1 (Hartley *et al.*, 2000).

The recombinant plasmid was transformed into chemically competent *E.coli*. After first transferring the plasmid through the *S. aureus* strain RN4220, *S. aureus* strain USA300 JE2 was transformed. The consequent allelic replacement was performed as previously described (Bae and Schneewind, 2006).

3.2.4 Cell culture techniques

Passaging

Cells were grown in appropriate medium supplemented with 10% (v/v) fetal calve serum, 1 mM sodium pyruvate, 100 µg/ml Streptomycin and 100 U/ml Penicillin. Cultivation was done at 37°C and 5% CO_2 in T75 cell culture flasks. Passaging of cells was performed depending on confluency every 3-4 days. Cells were washed with PBS twice before adding 1 ml of a 1:10 dilution of Trypsin. Cells were incubated for 5 min at 37°C until adherence to the cell culture flask was dissolved. Cells were taken up in 10 ml of growth medium and diluted 1:20.

Cryo preservation

Cells were grown to a confluency of 75 - 85% in a cell culture flask. Following trypsinization, cells were taken up in fresh full medium and centrifuged at 200 g for 10 min. Cells were resuspended in stocking medium and aliquoted in cryo-tubes with 1 ml of cell suspension each. Stocks were then transferred to -80°C in isopropanol containers for slow cooling. For long time storage, cells were transferred to liquid nitrogen storage after several days at -80°C.

Isolation of primary human neutrophils

PMN were isolated as described before (Hattar *et al.*, 2001). Heparin anticoagulated blood was taken from healthy donors and centrifuged over a Ficoll-Paque gradient for 30 min at 300 g. Erythrocytes were sedimented in saline with 1% Polyvinylalkohol. Remaining erythrocytes were eliminated via hypotonic lysis for 30 sec. PMN were pelleted by centrifugation at 300 g for 5 min and resuspended in 1x Hanks balanced salt solution.

Alternatively, PMN were isolated using a Ficoll/Histopaque gradient centrifugation. After an initial centrifugation for 20 min at 380 g and 22°C without brakes the PMN layer was taken out carefully, the cells were washed once with RPMI medium supplemented with 0.05% HSA and centrifuged for 10 min at 250 g. Residual Erythrocytes were eliminated by water lysis for 30 sec and cells were centrifuged again. PMN were resuspended in RPMI/HSA (0.05%) and counted using a 1:10 dilution in trypan blue.

Isolation and differentiation of PBMCs

Peripheral Blood Mononuclear cells were isolated from healthy donors in accordance to PMN isolation protocol. Following centrifugation over a Ficoll-Paque gradient the buffy appearing cell layer was taken out carefully and diluted with 1x DPBS +1 mM EDTA. Cells were centrifuged for 10 min at 300 g without brakes and washed twice with DPBS +1 mM EDTA. Complete PBMC

population from app. 20 ml of donor blood was taken up in 30 ml of RPMI full media supplemented with 250 ng/ml PHA and seeded in a total of 30 wells in 24 well plates.

After 24 h to 36 h of incubation wells were washed with PBS once and medium was exchanged for medium supplemented with 50 ng/ml of human Macrophage colony stimulating factor (M-CSF). Following 3-4 days of incubation medium was exchanged again for fresh medium with 100 ng/ml M-CSF without any washing steps. On the seventh day post isolation infection experiments were performed.

Preparation of pooled human serum

Blood was taken from healthy donors in non-heparin treated monovettes. Following incubation at room temperature for 30 min samples were centrifuged at 2000 g for 10 min at 20°C. Human serum was taken off, aliquoted and freezed at -20°C.

3.2.5 Cell infection protocols

Infection of adherent epithelial cells with *Staphylococcus* strains

To infect adherent cells with *S. aureus*, cells were grown and passaged as described above. Cells were seeded one day before infection with 100,000 cells per well in 12 well plates and 40,000 cells per well in 24 well plates in growth medium with antibiotics. Medium was exchanged before infection for medium without antibiotics. Overnight cultures of bacteria strains were prepared as described above. From the overnight culture 10 ml of fresh TSB were inoculated to an OD_{600} 0.4 in a 100 ml Erlenmeyer. Bacteria were harvested at 14,000 rpm after 1 h growth.

Bacterial pellets were washed once with PBS and consequently resuspended in infection medium. Bacterial cell densities were calculated using a Thoma hemocytometer. To synchronize the infection well plates were centrifuged for 10 min at 200 g. Plates were then incubated at 37°C and 5% CO_2 until the desired timepoint post infection.

Invasion assays under gentamicin protection

To determine *S. aureus* invasion phenotypes two different methods were conducted. In a gentamicin protection assay, medium was exchanged 1 h post infection for infection medium supplemented with 100 µg/ml gentamicin and 20 µg/ml Lysostaphin, selectively eradicating remaining extracellular bacteria. 90 min post infection cells were washed twice with PBS, lysed using H_2O pH11 for 5 min at room temperature. Lysates were diluted and plated on TSB Agar plates. All experiments were conducted in duplicates.

In addition internalized bacteria numbers were determined by fluorescence microscopy. Cells were seeded in black µ-clear 24 well plates (ibidi #82406; Martinsried, Germany) with 35,000 cells per well. Bacteria were stained using Tetramethylrhodamine (TRITC, mixed isomers; MoBiTec) prior to infection. TRITC was diluted to a concentration of 50 µg/ml in a $NaHCO_3/Na_2CO_3$ buffer. Bacterial pellets were taken up in 250 µl of the staining solution each and incubated with light protection at 37°C and 650 rpm for 30 min. After the staining step, bacteria were washed once with PBS and resuspended in RPMI infection medium for counting. Infection protocol, including gentamicin protection was retained as described above. 90 min post infection cells were fixed with 4% (w/v) paraformaldehyde and kept light protected until analysis on an Operetta automated microscopy system (Perkin-Elmer). Using a 20x PLAN long working distance (WD) objective with a numerical aperture (NA) of 0.45, 10 non-overlapping images (1360 x 1024 px; 675.3928 µm x 508.5311 µm) were acquired. Consequent analysis of the pictures was performed using the microscope embedded Harmony software. Therefore cell cytoplasms were detected using the constitutively expressed YFP signal and the filter set "SpBlue1/YFP" (excitation: 490-510 nm, emission 520-560 nm; 0.75 sec exposure). Internalized TRITC-labeled bacteria were detected and counted via the "Find spots" analysis building block (Method "A", relative spot intensity 0.1, splitting coefficient 1) and the "StdOrange1/Cy3 filter set (excitation: 520-550 nm, emission 560-630 nm; 0.5 sec exposure). All experiments were conducted in duplicates.

Phagosomal escape assay in adherent cells

To detect phagosomal escape, cells stably expressing the YFP-cwt recruitment reporter molecule were seeded in 24 well µ-clear plates (ibidi). Infection and gentamicin protection steps were performed as described above. 90 min post infection medium was exchanged again for infection medium supplemented with 100 µg/ml gentamicin only. Either 3 hpi/6 hpi/24 hpi the cells were washed once with PBS and fixed using 4% (w/v) paraformaldehyde.

Analysis was done as described before using the constitutive YFP fluorescence of the cell and the TRITC fluorescence of stained bacteria. YFP spots were detected using a threshold larger than 100 using the "Corrected Spot Intensity" setting. Differential experimental settings required constant review and variation of threshold settings. Numbers of determined YFP spots were set in correlation to numbers of detected intracellular bacteria to yield phagosomal escape rates.

When paraformaldehyde (PA) fixed bacteria were to be used in infection, bacteria were washed with PBS following staining. Bacteria were resuspended in 4% PA and tumbled overnight at 4°C. Before infection, bacteria were washed once with PBS, remaining PA was quenched using 50 mM of Ammonium chloride for 10 min at RT and sample was washed again

before resuspension in infection medium for counting. All experiments were conducted in duplicates.

Detection of cytotoxicity in infected adherent cells

In order to detect cytotoxicity of internalized bacteria on adherent epithelial cells, 150,000 cells were seeded the day before the experiment in 12 well plates and incubated overnight at 37°C and 5% CO_2.

Bacterial overnight cultures, infection procedures and Lysostaphin/gentamicin protection were performed as described before. At different timepoints, cell supernatants were collected in reaction tubes, cells were washed once with PBS and consequently trypsinized. Trypsinization was stopped by re-addition of supernatants and complete samples were collected. Following centrifugation for 5 min at 500 g, cell pellets were resuspended in labeling solution (Table 3.10) supplemented with 1% (v/v) Annexin V and 1% (v/v) Propidium Iodide (PI) and kept in the dark at room temperature for 15 min. Following incubation, samples were diluted 1:5 using labeling solution and kept on ice.

Analysis was performed using an Accuri C6 flow cytometer. Uninfected cells were used as reference samples. Cell samples were analyzed in terms of cell size, Annexin as well as PI staining in duplicates.

PMN CFU and LDH assays

To evaluate bacterial survival in, as well as bacterial killing of PMN, CFU and LDH assays were performed. Primary human PMN were isolated as described above. Isolated PMN numbers were determined using a Neubauer hemocytometer. PMNs were pelleted at 500 g for 5 min and resuspended in neutrophil infection medium (HBSS++). PMN, were infected at a concentration of 1×10^6 per ml in a reaction tube.

To synchronize the infection, bacteria, tested compounds and DMSO were pipetted in the opened cap of the tube. The reaction tubes were closed and tumbled at 37°C for 10 min to allow phagocytosis of the bacteria. Cells were centrifuged at 500 g for 5 min and supernatant was discarded. PMNs were taken up in fresh HBSS++ and kept tumbling at 37°C.

At each time point, the PMN lysis positive control was separated, 5 µl of lysis buffer (Roche cytotoxicity detection kit) were added and the sample was incubated for 15 min at RT. All other samples were centrifuged for 5 min at 500 g. To measure release of Lactate dehydrogenase (LDH), 100 µl of the supernatant of each sample were taken off and tested in a 96-well plate by adding 100 µl of reaction solution each. The plate was incubated for 10 min at

RT with light protection and 50 µl of stop solution was added to each well. Duplicate samples were then analyzed using an Infinite m200 plate reader at 490 nm.

To check for bacteria CFUs, residual neutrophil infection medium was discarded and cells were incubated in H_2O pH11 for 5 min. Cell lysates were diluted in PBS and plated on agar plates supplemented with appropriate antibiotics.

Detection of phagosomal escape in PMNs using pH-Assay

PMN isolation and bacterial culturing were performed as described. Bacteria were resuspended in 500 µl of FITC staining solution (10 µg/ml FITC in $NaHCO_3/Na_2CO_3$ buffer solution) and kept shaking for 30 min at 37°C and 650 rpm. Bacteria were washed once and counted for infection.

PMN infection was performed as described above. At each time point, PMN were taken up in 1 ml of PBS and subsequently divided in two fractions. One of the fractions was subjected to Monensin treatment (50 µM), the second fraction functioned as a control (50 µM EtOH). Monensin functions as an ionophore that forms complexes with monovalent cations and transport them across lipid membranes of cells (Mollenhauer *et al.*, 1990). Samples were incubated at 37°C for 10 min and then kept on ice. Samples were measured using a C6 Flow cytometer (Accuri).

PBMC CFU and LDH assays

Following differentiation, PBMCs are washed once with PBS and medium was exchanged for RPMI infection medium. Bacterial overnight cultures were treated as described above. To determine bacterial survival in competition with macrophages, cells were infected with an MOI of 5. Plates were centrifuged for 10 min at 1500 g to synchronize the infection. At each time point cells were washed once with PBS and lysed using 1 ml of water pH11 for 5 min at RT. Complete cell lysate was collected and serial dilutions were prepared in PBS. 25 µl of each dilution were plated on TSA plates supplemented with respective antibiotics.

To determine macrophage cell death caused by internalized bacteria, cells were infected at an MOI of 20. Following 30 min of incubation to allow for phagocytosis, medium was exchanged for fresh medium supplemented with 20 µg/ml Lysostaphin to eradicate all residual extracellular bacteria. 30 min later medium was exchanged again for RPMI infection medium without antibiotics. Cells were incubated another 60 min before 100 µl of sample supernatant was transferred to a 96 well plate to determine levels of released LDH using Roche cytotoxicity detection kit. As positive control, complete lysis of cells was achieved in one well by using lysis buffer according to manufacturer protocol. All measurements were performed in duplicates.

3.2.6 PMN tests

Chemotaxis assays

Isolated PMNs were diluted to 3 x 10^6 cells/ml HBSS/HSA (0.05%). 3 µl of BCECF-AM (2 mM) were added to 600 µl cell solution and kept in the dark at RT for 20 min. After incubation, 1 ml of HBSS/HSA (0.05%) was added and the cells were centrifuged for 10 min at 250 g at 4°C. Supernatant was discarded and cells were resuspended in 600 µl of fresh HBSS/HSA (0.05%).

Transwell filters were humidified for 10 min at RT with 100 µl HBSS/HSA (0.05%). Medium was carefully removed and the upper compartment of the transwell plate was filled with 100 µl of BCECF labeled PMN. Lower compartment was filled with 600 µl of HBSS/HSA (0.05%) supplemented with different concentrations of stimulus substance. As 100% migration control I used a positive control with 500 µl HBSS/HSA (0.05%) + 100 µl fluorescent PMN. As a negative control no stimulus was added to the lower compartment, while 1 nM fMLF was added as positive control. The plates were then analyzed in a FLUOstar Optima Plate Reader (BMG Labtech) at chosen time points.

PMN Ca^{2+} flux

Intracellular Ca^{2+} Flux was measured using the calcium sensitive fluorescent dye Fluo3-AM and flow cytometry as described before (Kretschmer *et al.*, 2010). PMN were isolated as described above. 5 x 10^6 cells were incubated with 2 µM of Fluo-3 AM for 20 min at RT in the dark. Cells were washed twice with 5 ml RPMI/HSA (0.05%) and centrifuged for 10 min at 1200 rpm and 4°C. Cells were then diluted to 1 x 10^6 cells per ml in RPMI/HSA (0.05%).

FITC fluorescence of 250 µl dyed PMN was measured to establish the base fluorescence of cells. 2000 gated events were measured with each sample at a flow rate of app. 250 cells/sec. Stimulation of PMN was done by addition of synthetic chemo attractants or bacterial supernatants, used at indicated concentrations in 200 µl of fresh medium. Samples were vortexed and exactly 15 sec after addition of the stimulus, the second measurement was done. Calcium flux is expressed in relative fluorescence units corrected for buffer controls.

To check for inhibition of calcium flux, cells were incubated with 5 µM or 10 µM of phevalin for 30 min at RT before measurements were started.

3.2.7 Detection of AusAB gene expression and aureusimine production

Preparation of pellets for RNA isolation

RNA was isolated from bacterial pellets taken at different growth stages. 100 ml of fresh TSB were inoculated from an overnight culture to an OD_{600} of 0.1 and kept shaking at 200 rpm and 37°C until an OD_{600} 0.6. 1.5 ml of the culture were transferred into a reaction tube and centrifuged at 14,000 rpm for 30 sec. Supernatant was decanted and the pellet shock frozen immediately. The sample was then stored at -80°C. After 8h of growth again samples were taken.

RNA isolation

Pellets were thawn on ice and resuspended in 60 µl of 0.5 M EDTA. 400 µl of buffer A was added and the complete sample was transferred on a Fast-Prep 24 lysis matrix tube (MPbio). Samples were then lysed using a Fastprep-24 sample preparation system at 6 m/sec for 45 sec. Matrix tubes were centrifuged for 10 min at 4°C and 17,900 g.

Supernatant was transferred to a fresh reaction tube and 1 ml Tri reagent solution was added. The mixture was inverted several times and kept at RT for 5 min. 100 µl of 100% chloroform were added to each sample, the mixture again inverted several times and kept at RT for 3 min followed by centrifugation as before. Supernatant was transferred and 200 µl of 100% chloroform were added. Sample was mixed, kept at RT for 5 min and centrifuged for 5 min at 4°C and 17,900 g. The supernatant was again transferred and 500 µl of 2-propanol were added. Sample was mixed and kept at RT for 15 min followed by centrifugation for 15 min at 4°C and 17,900 g. Pellet was washed once with 75% EtOH and centrifuged for 5 min. Supernatant was discarded and the pellets were dried under a sterile bench. Dried pellets were resuspended in 50 µl DNAse-free water. Samples were stored at -80°C.

Preparation of cDNA

RNA samples were thawn on ice and incubated for 5 min at 55°C. RNA concentration was determined using spectrophotometry. 10 µg of RNA were used with TurboDNA Free Kit (Ambion) according to manufacturer's protocol.

Samples were measured via spectrophotometry again and 1 µg of Template RNA was processed with the Revert Aid First strand cDNA Synthesis Kit (Thermo Scientific) using a Random Hexamer primer. The Sample was diluted using 80 µl of DNase free water. For use in qRT-PCRs the sample was later again diluted 1:5 in DNase free water.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed in 96 well PCR microplates (Axygen Scientific). Sample measurements were performed as triplicates. In each experiment the DNA gyrase subunit B (*gyr*B) functioned as the endogenous control. Per well 5 µl of the diluted cDNA was mixed with 15 µl of reaction master mix consisting of 10 µl Sybr Green, 1.8 µl of each primer (900 nM end concentration) and 1.4 µl ddH_2O. Plates were centrifuged and PCR reaction was performed on a RT-PCR system (StepOne Plus).

Detection of phevalin in bacteria culture supernatants using UPLC

Bacteria culture samples were prepared as described before (Secor *et al.*, 2012). 25 ml of TSB overnight culture were normalized for OD_{600} for each strain and centrifuged at 4000 rpm for 10 min at 4°C. Following sterile filtration of the supernatant, 2 ml filtrate were mixed with an equal volume of 100% chloroform by vortexing in glass tubes. Samples were centrifuged at 3000 g for 10 min at 4°C and the organic phase was transferred into a fresh glass tube. Using pressurized air the sample was dried and the pellet resuspended in 100 µl of 20% DMSO.

Samples were analyzed using LC-MS/MS as described before (Seltmann *et al.*, 2010). In short, a Waters Acquity ultra-high-performance liquid chromatography system was coupled to an electrospray interface (ESI) equipped Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Milford, MA, USA).

Aureusimine samples were separated using reverse-phase chromatography on Acquity BEH C18 columns (50 x 2.1 mm, 1.7 µm particle size with a 5 x 2.1 mm guard column; Waters; Milford, MA, USA). The solvent system consisted of water and two additional solvents (solvent A: 0.1% formic acid; solvent B: acetonitrile). The used injection volume was 5 µl. A gradient elution starting from 1% to 100% solvent B was performed at a flow rate of 0.25 mL min–1 within 5 min and a column temperature of 40°C.

Aureusimines were detected by multiple reaction monitoring (MRM). In MRM mode ions are being isolated according to their mass/charge rate in the first quadrupol of the tandem-MS. Instrument parameters for ionization and collision induced dissociation (CID) were optimized by flow injection of phevalin. To ionize samples, the electrospray source was operated in the positive electrospray mode (ESI+) at a capillary voltage of 2.75 kV and a temperature of 120°C. For desolvation, nitrogen was used in combination with a cone voltage of 40 V. Cone gas flow was adjusted with flow rates of 800 L h–1 at 400°C and 10 L h–1, respectively. Using argon as collision gas, fragmentation was performed at a pressure of approximately 3 x 10–3 bar and collision energy (CE) of 22 eV. For each compound, three specific fragments were monitored (phevalin: m/z 229 > 214, m/z 229 > 159, m/z 229 > 81; tyrvalin: m/z 245 > 230, m/z 245 > 175, m/z 245 > 81) with a dwell time of 25 ms per MRM transition.

Detection of phevalin in cells during infection or after phevalin treatment

Cells were incubated in presence of 20 µM phevalin or infected with bacteria as described before. At each time point, cells were detached using Trypsin. 1.5 ml of H_2O was added and the sample transferred to a glass reaction tube. An equal amount of 100% chloroform was added and the samples were subjected to the previously described extraction and analysis protocol.

3.2.8 Detection of the mitochondria phenotype of phevalin

Live cell imaging

Hela cells were seeded in 35 mm µ-dishes (ibidi) two days prior to treatment resulting in a very light confluency of about 30-40% on the day of the experiment. Cells were washed once using pre-warmed PBS and then subjected to phevalin treatment (10 µM) in RPMI medium for 1 h at 37°C and 5% CO_2. Cells were washed once with ice cold PBS and incubated in fresh RPMI medium (+20 mM HEPES) for 10 min at 37°C. Cells were then imaged using a confocal TCS SP5 microscope (Leica) in a 37°C preheated imaging chamber with a 63x oil immersion UV objective (numerical aperture: 1.4).

To score individual fragments per cell, lengths and fragment numbers were measured using a modified version of the object count plugin from FIJI. Continuous networks and individual mitochondria fragments were identified by conversion of detected GFP fluorescence to binary signals and consequent numerical categorization. Equal fluorescence thresholds were retained for every image.

Measurement of mitochondrial respiration using Seahorse XF Analyzer

Hela cells were seeded in a 96-well plate the day before the experiment with 2 x 10^4 cells per well in 80 µl medium (RPMI). Glycolysis stress test assay medium was prepared fresh as described in table 3.10 and the compounds used in the test were diluted in the medium to the following final concentrations:

FCCP	-	100 µM
Oligomycin	-	100 µM
Rotenone	-	50 µM
Antimycin A	-	50 µM

The pH of each compound solution was adjusted to pH7.4 +/- 0.05. Phevalin final concentration was adjusted in glycolysis stress test assay medium without adjustment of pH. The Assay plate was washed twice with medium and mounted with regular medium, medium containing

phevalin or DMSO control. A cartridge plate is loaded with FCCP, Rotenon + Antimycin A or Oligomycin per port with a volume of 25 µl each.

After the measurement finished, measurement plate and replica plate are stained with crystal violet to normalize the results according to cell numbers. Plates are washed once with PBS and fixed using 75% EtOH at RT for 10 min. EtOH is discarded and the plate air dried. 0.1% crystal violet solution is added and the plates are incubated for 30 min at RT tumbling. Plates are washed three times with water and dried overnight. After destaining the plates with 10% (v/v) acetic acid for 5 min tumbling at RT the plates are analyzed in a plate reader at 550/560 nm.

3.2.10 Differences in the phosphoproteome of phevalin-treated cells

Proteomic and phosphoproteomic profiling was performed as described before and exact methodological procedures can be reviewed in the referenced papers (Hochgrafe *et al.*, 2010; Richter *et al.*, 2015).

3.2.11 Mouse infection models

3.2.11.1 Pulmonary lung infection

S. aureus strains dedicated for use in infection were plated on TSA plates supplemented with appropriate concentrations of antibiotics from frozen bacteria stocks and grown overnight at 37°C, shaking at 180 rpm. From the overnight culture, 50 ml of fresh TSB medium were inoculated at an OD_{600} of 0.05 and incubated for three and a half hours at 37°C, shaking again at 180 rpm. Bacteria were collected via centrifugation at 4°C and were resuspended in 20 ml of fresh TSB supplemented with 15% (v/v) glycerol. 2 ml aliquots of the bacterial suspension were stored at -80° until further use. CFUs of the stocks were quantified by plating before titration experiments were performed in order to determine lethal and sub-lethal doses of infection in mice.

When used in infections, strains were revived from glycerol stocks by transferring them to 50 ml of pre-warmed TSB medium. After incubation at 37°C for 30 min, bacteria were pelleted and washed twice with 1x PBS before being resuspended in 1 ml of PBS. After the OD_{600} of the solution was determined, reference growth curves are used to assess bacterial numbers of every strain. Bacterial dilutions were prepared to contain the intended CFU in 20 µl of PBS. Numbers were also confirmed on TSB agar plates.

Table 3.13: Score sheet depicting the determination of severity of disease in infected mice

Observation	Score points
I Body weight	
- No change	0
- Loss of body weight in % = score points; e.g. loss of body weight 8% = 8 points	1-20
- Loss of body weight ≥ 20%	20
II General conditions	
Fur	
- Shining	0
- Matte	2
- Ruffled	4
Eyes	
- Clear and clean	0
- Unclean and sticky, closed or semi-closed	3
Posture	
- Normal	0
- Hunched	10
- Massively hunched	20
Clinical complications	
- Tension, paralysis, tremor	20
- Breath noises	20
- Animal feels cold to the touch	20
III Motility	
- Spontaneous (normal behavior, social contacts)	0
- Spontaneous but reduced	1
- Moderately reduced activity	2
- Motility only after stimulation	5
- Isolation, lethargy, coordination disorders	10
- Self-mutilation, aggression	20
IV Respiration	
- Breathing normal	0
- Breathing slightly changed	1
- Accelerated breathing + 30% (tachypnoea)	10
- Strongly accelerated breathing + 50%	20

Rating	Measures	Sum score pts
Severity level 0	no burden on animals, animals healthy	0-3
Severity level 1	low burden on animals, low sickness, animals are observed	4-9
Severity level 2	moderate burden on animals, moderate sickness, animals are carefully observed	10-15
Severity level 3	moderate to severe burden on animals, moderate to severe sickness, animals are carefully observed, abort of experiment if necessary	16-20
Severity level 4	severe burden on animals, animals moribund, implementation of humane endpoint, abort of experiment	>20

6 week old female Balb/c mice, purchased from Janvier Labs (Saint-Berthevin, France), were kept on a normal diet and in individually ventilated cages in groups of five. At 8 weeks, groups of ten mice were infected intranasally using 20 µl of a bacterial suspension with 2 x 10^8 CFU. After infection, weight loss as well as signs of disease was monitored every 12 h in order to assess a disease activity index for every mouse.

48 hours post infection, the lungs of infected mice were removed and completely homogenized in 1x PBS (GentleMACS, M-tubes, Miltenyi Biotec). Lung lysates were then plated on TSA agar plates to determine CFU loads. Plates were incubated at 37°C for 24 h. The following day, bacterial colonies were counted and bacterial load calculated. Statistical analysis was performed using one-way ANOVA.

3.2.11.2 Muscle abscess model

S. aureus strains used in infection of 6 week old female Balb/c mice, purchased from Janvier Labs, (Saint-Berthevin, France), were revived as described in 3.2.11.1. Mice were kept in individually ventilated cages on a normal diet in six groups of 5. At an age of 8 weeks, groups of 10 mice were infected in their left upper thigh muscle using 50 µl of bacterial suspension (2 x 10^8 cfu per dose) (Hertlein *et al.*, 2011).

48 hours post infection, the infected thigh muscles were removed and completely and homogenized in 1x PBS (GentleMACS, M-tubes, Miltenyi Biotec). The lysates were then serially diluted, the dilutions plated on TSA agar plates and cultivated overnight. The following day, bacterial colonies were counted and bacterial load calculated. Statistical analysis was performed using one-way ANOVA.

4. Results

4.1 Generation of a transposon insertion mutant library in *S. aureus* LAC

In order to screen for single gene mutants lacking an escape phenotype in an unbiased approach we generated a high-density transposon mutant library which, in combination with Next Generation Sequencing, would be used in a set of different experiments to screen for mutants lacking the ability to escape from the host cell phagosome. To generate transposon insertion mutants we used pBTn, a plasmid based on the temperature sensitive *E.coli*/*Staphylococcus* shuttle plasmid pBT2 (Bruckner, 1997). The pBT2 backbone was modified to carry an erythromycin resistance cassette with the *Himar*1 transposase gene, a protein belonging to the class II transposon type which has been shown in different studies to introduce single, random insertions in the bacterial genome and which originates from the hornfly *Haematobia irritans* (Lampe *et al.*, 1998; Rubin *et al.*, 1999; Li *et al.*, 2009b).

Using this plasmid we generated transposon insertion mutants in the highly pathogenic and epidemic MRSA strain LAC*, a variant of the prevalent USA300-type MRSA strain LAC which lacks the pUSA03 plasmid. pUSA03 encodes for constitutive resistance against macrolides, lincosamides, streptogramin B and mupirocin via the genes *erm*C and *ile*S. After the transposon insertion mutagenesis was performed, two pooled libraries of bacteria were collected. The first library (439.D) was collected directly from liquid broth culture and next subjected to DNA isolation. A second library (439.E) was plated on TSA. Bacteria were recovered from the plates by scraping them off the plate surfaces and consequently subjected to DNA isolation. This approach has the benefit of largely eliminating overrepresentation of TIS from DNA of growth inhibited bacteria retained in liquid culture and later amplified during the library preparation.

We then used next-generation sequencing in order to identify the transposon insertion sites (TIS) within the pooled libraries. The analysis of the libraries showed, that we obtained a total of 3123 and 1471 unique insertion sites for libraries 439.D and 439.E respectively, making these libraries less dense than our previously described mutant library in the highly cytotoxic *S. aureus* strain 6850 and also compared to the 18,797 insertion sites obtained in a comparable library generation experiment in the plasmid-free USA300 JE2 (Fey *et al.*, 2013; Fraunholz *et al.*, 2013; Das *et al.*, 2016).

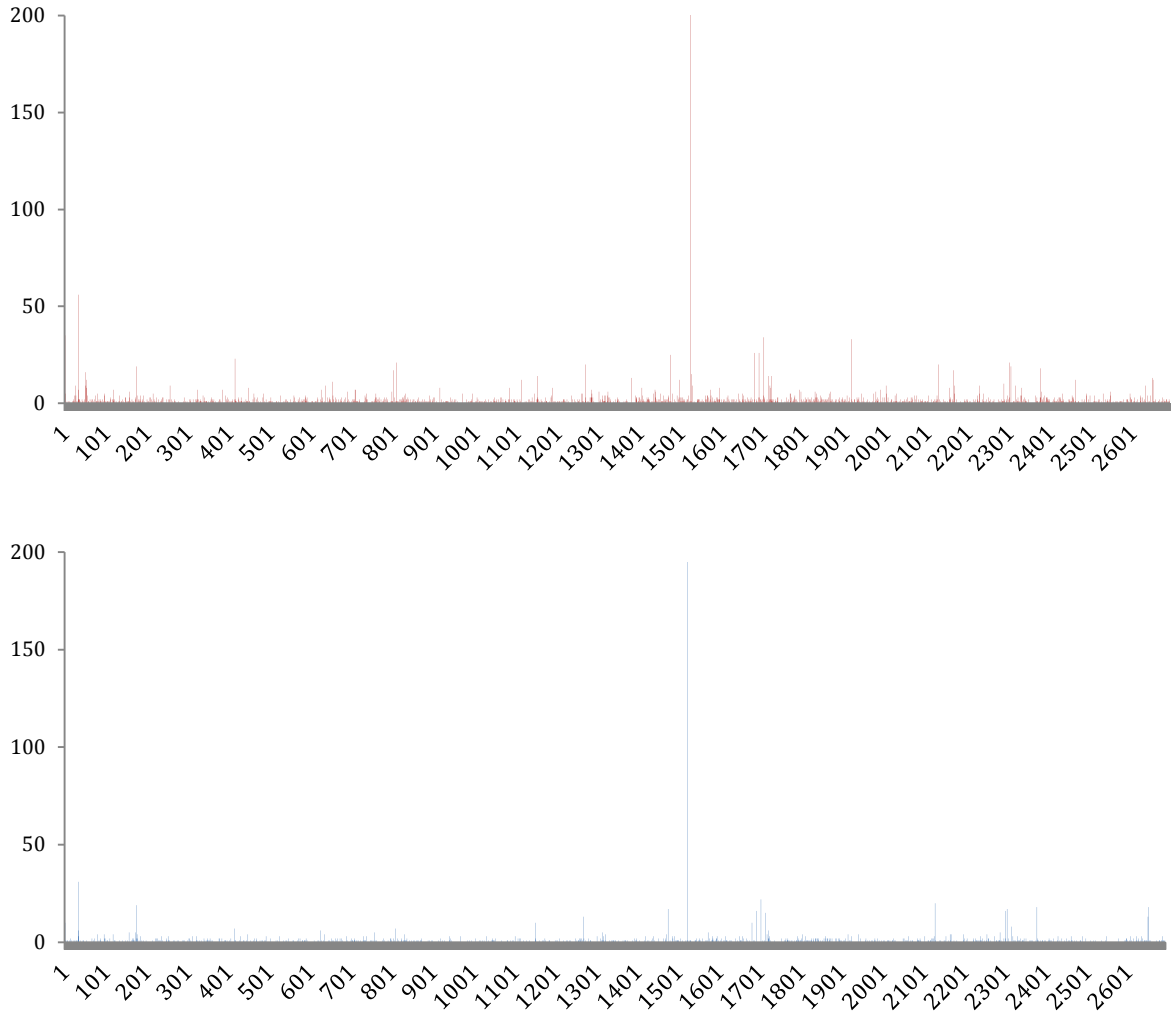

Figure 4.1: Distribution of TIS across the *S. aureus* genome in transposon libraries 439.D (upper graph) and 439.E (lower graph) obtained from the USA300-type strain *S. aureus* LAC*.

S. aureus gene locus numbers are displayed on the x-axis, numbers of individual TIS per gene on the y-axis.

The visualization of TIS across the LAC* genome illustrates a relative even distribution of TIS in the libraries D and E. Only one gene stands out in a way, that it accumulates a large number of TIS. The gene SAUSA300_2326 encodes for the AraC-type transcriptional regulator Rsp, which has recently been shown to be involved in staphylococcal virulence and biofilm formation (Lei *et al.*, 2011; Li *et al.*, 2015; Das *et al.*, 2016). The annotation of TIS revealed, that in a total of 1541 (library D) and 2015 (library E) genes, no TIS was detected. By checking the initial set of sequence reads obtained from Illumina sequencing it became evident, that many of the TIS amplified during the library generation process are actually situated on one of the plasmids of strain LAC.

4.2 Screening for single gene mutant strains deficient in phagosomal escape using automated microscopy

Due to the low coverage of transposon insertion sites obtained for *S. aureus* LAC* (see chapter 4.1) a different approach was chosen for the identification of single gene mutations affecting the capability of *S. aureus* to escape from the endophagosomes.

In order to effectively test specific mutant strains in their ability to escape from the cell phagosome, I chose to use the Nebraska transposon mutant library, a collection of 1952 mutants with unique transposon insertion mutations, covering the majority of non-essential *S. aureus* genes (Fey *et al.*, 2013). This library is made available to the scientific community by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). Transposon insertion mutants were generated in the *S. aureus* JE2, a strain based on USA300 LAC, but missing all three of the strains residing plasmids pUSA01, pUSA02, and pUSA03 (Voyich *et al.*, 2005). All mutants were stocked as frozen samples on 96 well culture plates and stored at -80°C.

In order to screen the available mutants within the NARSA library, I implemented a method that enabled us to screen for escape phenotypes in 12 strains at once by automated fluorescence microscopy making use of the fluorescent recruitment marker system available in our lab (Giese *et al.*, 2009; Giese *et al.*, 2011). In collaboration with the "Functional Genomics" Core Unit situated at the Biozentrum. I adapted the confocal microscopy-based approach to 24 well imaging plates (ibidi), which were subsequently scanned with the Operetta automated microscopy system (Perkin Elmer) of the core unit. Quantification of results was achieved using the embedded Harmony software (Fig. 4.2 and 4.3). Thereby I was able to screen dozens of mutants in a fraction of the time necessary to acquire the same set of information using confocal microscopy.

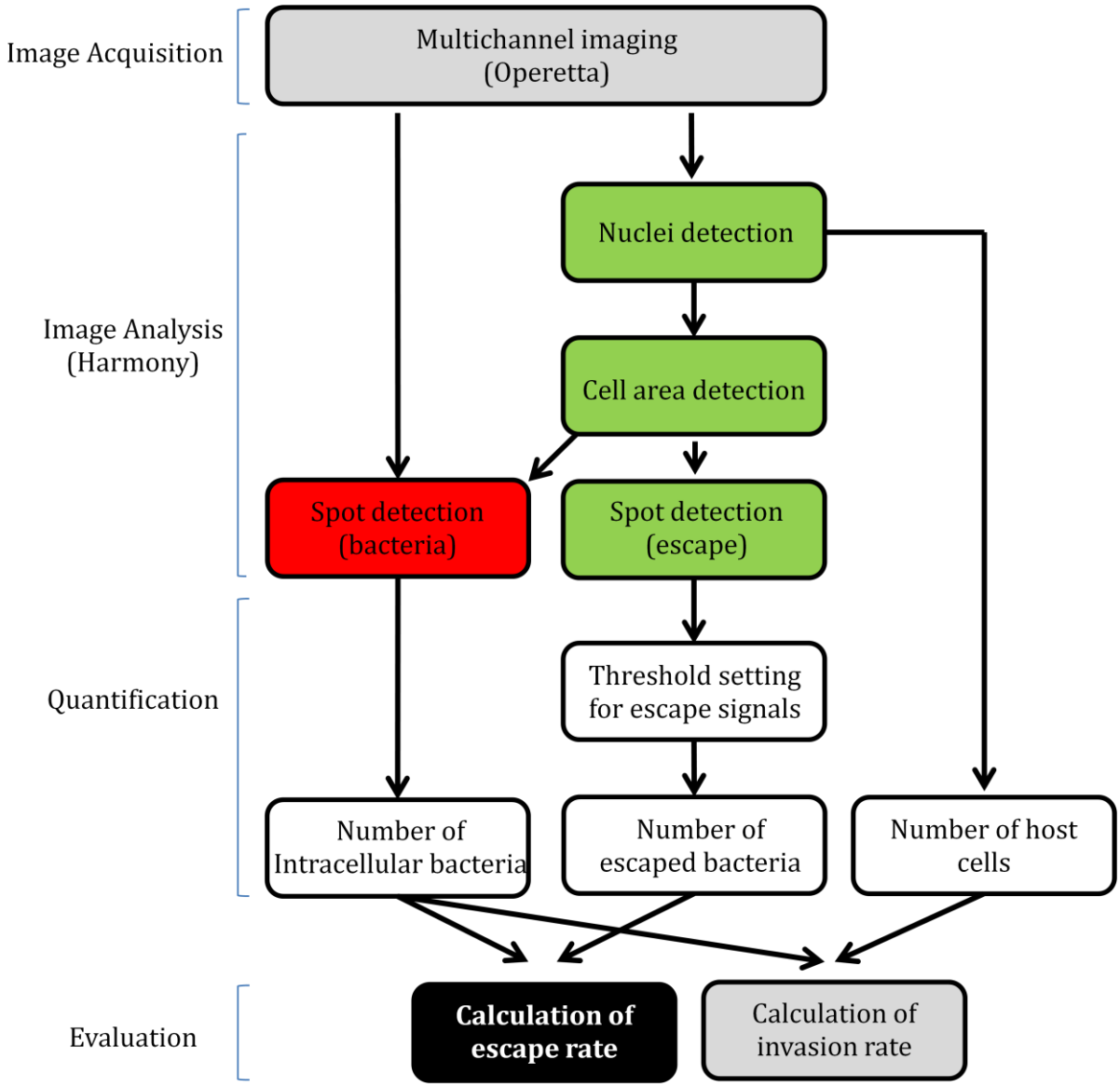

Figure 4.2: Flow chart of the image analysis algorithm used for quantification of phagosomal escape rates.
The flow chart depicts the successive steps of image analysis and quantification performed using the Operetta microscope and the Harmony analysis software. Following multichannel imaging, cell nuclei, cell area and escape spots were detected using YFP fluorescence while bacteria detection was done using TRITC fluorescence. Following the threshold setting for the escape signals, the number of bacteria, the number of escape signals and the number of host cells were used to calculate escape and invasion rates.

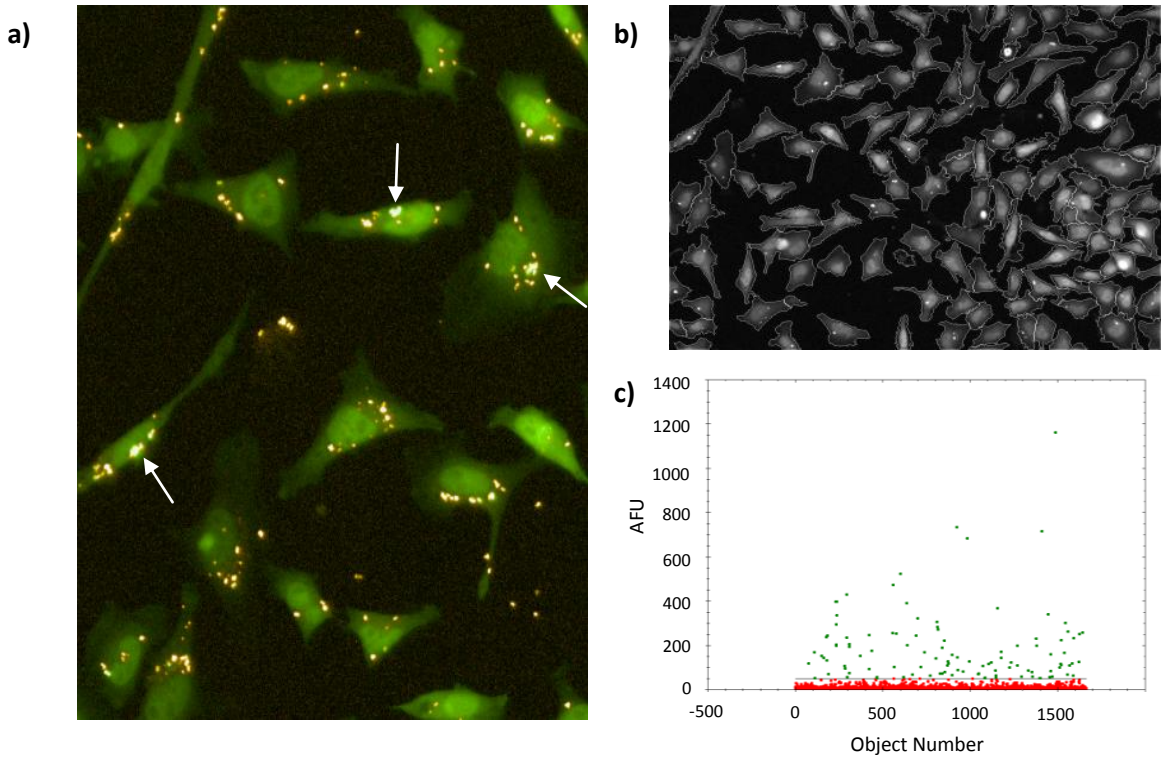

Figure 4.3: Excerpt of the image analysis process using the Harmony software.
Microscopic fluorescence images are acquired using the Perkin Elmer Operetta system. **a)** Pictures are acquired with a 20x PLAN long working disctance objective. To image TRITC (stained bacteria) the "StdOrange1/Cy3" filter set (excitation 520-550 nm, emission 560-630 nm; 0.5 sec exposure time) was used, while YFP fluorescence was detected with the "SpBlue/YFP" filter set (excitation 490-510nm, emission 520-560 nm; 0.75 sec exposure). White arrows point out sites of accumulating YFP fluorescence marker indicating bacteria escaped from the phagosome. **b)** Cell area is determined using the Harmony image analysis building blocks "Find Nuclei" (Method B: Common threshold 0.2; Area > 30 µm²) and "Find Cytoplasm" (Method B: Common threshold 0.45; Individual threshold 0.16)) by detecting the constitutively expressed and evenly distributed YFP fluorescence signal. Internalized *S. aureus* are detected using the "Find Spots" (Method B: Detection sensitivity 0.5; Splitting coefficient 0.5) building block and the TRITC signal of the stained bacteria. Escape signals are counted using the "Find Spots" (Method B: Detection sensitivity 0.5; Splitting coefficient 0.5) analysis building block. **c)** Cutoffs are set in regard of the detected fluorescence intensity. The rate of escaped intracellular bacteria can then be calculated from both, TRITC and selected YFP spot signals.

Although this new methodology allowed me to screen a large number of mutant strains, other constraints still made a selection of mutants to investigate necessary. The selection of strains was done in a rationale driven manner but included also randomly chosen mutants. A complete table of all the mutant strains tested is available as "Table 7.1 Nebraska insertion mutant strains included in screen and their observed relative escape rates".

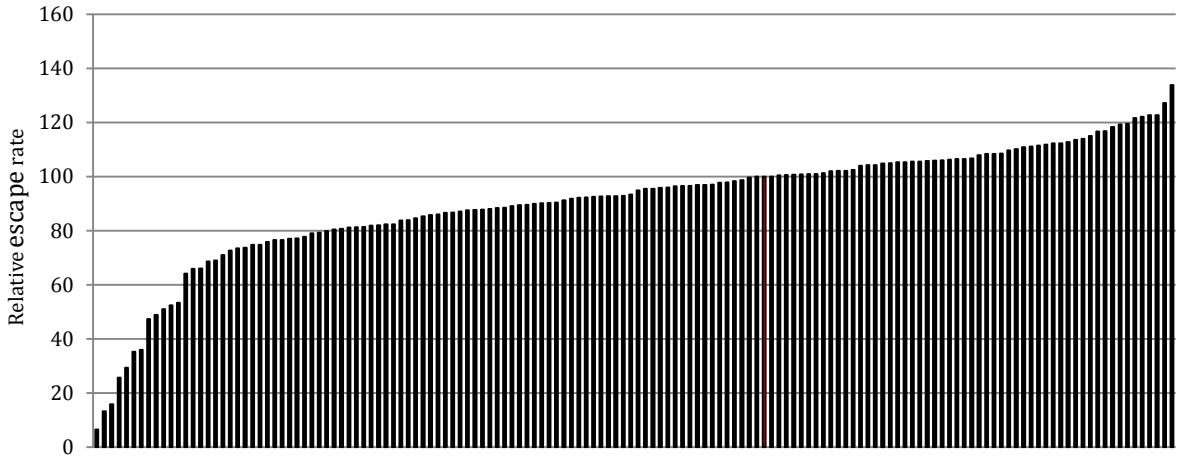

Figure 4.4: Phagosomal escape efficiency of all single gene mutant strains tested.
The phagosomal escape rates of all, Nebraska mutant library derived, single gene mutant strains tested in this study are displayed as relative values. The escape efficiency of the USA300 JE2 wild type (red bar) has been set as 100% while the escape rates of the test strains were normalized to the wild type for each individual experiment before determining a mean value. Number of replicates, mean relative escape values and standard deviations for individual mutants can be drawn from table 7.1.

Phagosomal escape rates were determined by infecting cells expressing the YFP-cwt marker as described in 3.2.5 in 24 well ibidi µ-treat microplate well dishes and fixing the samples 3 hours post infection before analyzing them using fluorescence microscopy. Analysis of detected escape rates of more than 140 tested single gene mutants (Table 7.1) revealed that most mutants tested did not differ in their escape rates with more than 20% in any direction. These were rendered of no interest in our screen and were rather attributed to general variance than actual phenotypic changes.

Phagosomal escape rates of mutants in toxins and regulators

Since virulence of *S. aureus* is governed by global regulators and the expression of toxins, I tested mutants in known genes of both classes.

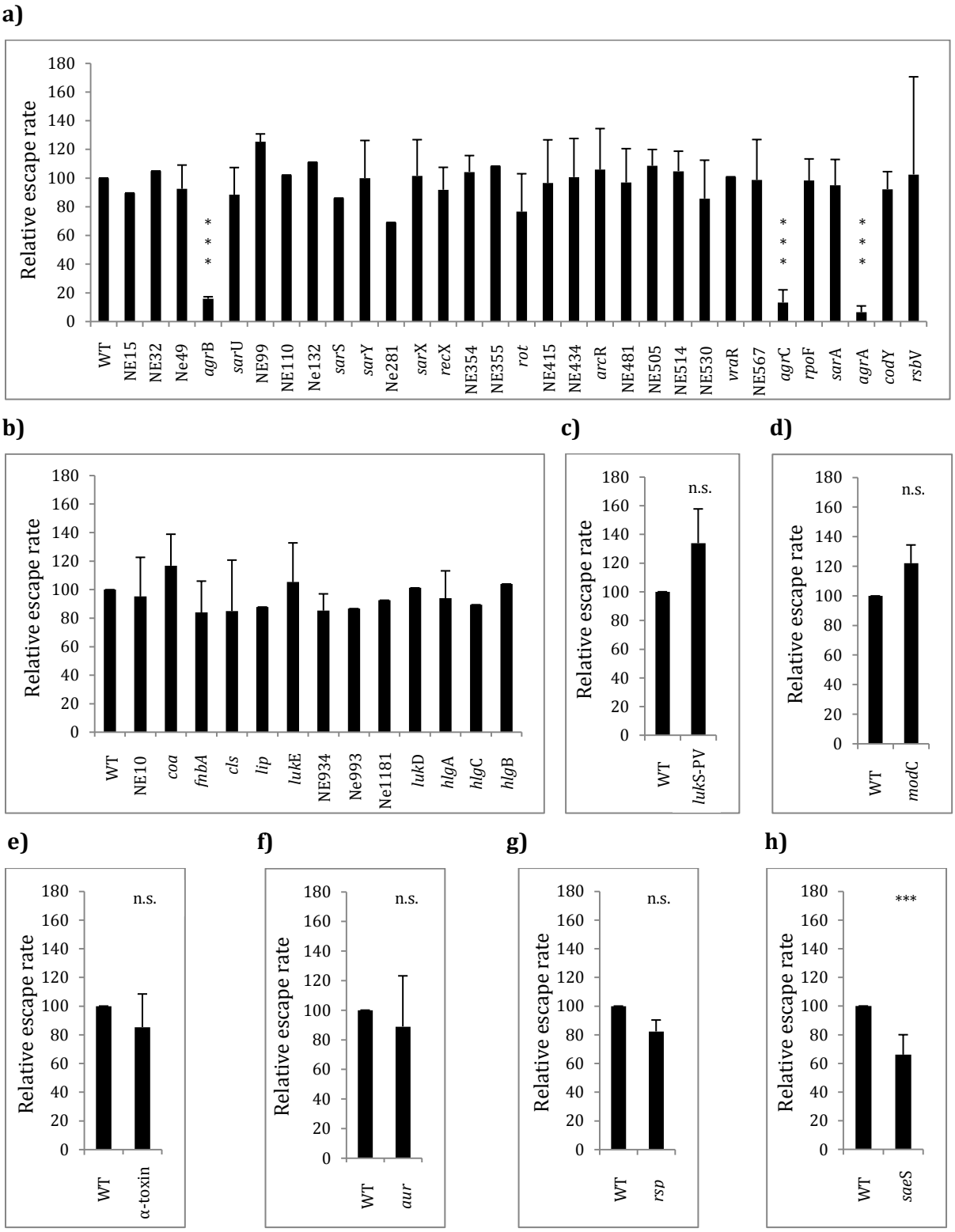

Figure 4.5: Phagosomal escape rates of selected special interest mutant strains.

Phagosomal escape rates of selected mutant strains are depicted relative to the *S. aureus* JE2 wild type strain. The escape efficiency of the wild type has been set at 100%. Graphs show escape efficiencies of all tested known and putative transcriptional regulators (a), known and putative virulence genes of *S. aureus* (b), the leukocidins *luk*S PV (c), the ATP-binding protein *mod*C, the hemolysin *hla* (e), the staphylococcal aureulysin (f), the transcriptional regulator *rsp* (g) and the sensor histidine kinase *sae*S (h). Number of replicates, mean relative escape values and standard deviations for individual mutants can be drawn from table 7.1.

Figure 4.5 and table 4.1 show a large set of gene mutants tested in our escape screen. Most of the genes were revealed to have no impact on phagosomal escape in the described experimental setup. This includes very prominent and otherwise virulence associated toxins like α-hemolysin (NE1354: 85.3 ± 23.1%) (Fig. 4.5 e), β-hemolysin (NE1875: 118.3 ± 38.1%), the γ-hemolysin components A, B and C (*hlg*A, NE1399: 94.0 ± 19.2%; *hlg*B, NE1682: 104%; *hlg*C, NE1449: 89.4%) and the staphylococcal aureulysin (*aur*, NE163: 88.9 ± 34.3%) (Fig. 4.5 f).
A mutation in *pmt*C, one of two ATPbinding proteins of the phenol soluble modulin transporter Pmt shows diminished escape rates (29.3 ± 18.3%; p= .0026). Only two genes showed a considerable up-regulation of phagosomal escape after their respective knock-out, even though in both cases the increase is not yet significant and would require further evaluation. The strongest increase can be observed for the leukocidin toxin family protein *luk*S, the S-subunit of the Panton-Valentine leukocidin (NE1848: 133.8 ± 23.9%) (Fig. 4.5 c). Meanwhile mutants in the monomers of the dimeric leukicidin LukDE (*luk*D, NE1240: 101.2%; *luk*E, NE558: 105.3 ± 27.5%) (all Fig. 4.5 b) showed no effect, while a *luk*A mutant exhibited decreased escape rates (35.9 ± 13.7%; p= .000086). The only other mutation leading to increased escape rates was in the molybdenum ABC transporter ATP binding protein (*mod*C, NE512: 122.0 ± 12.4%) (Fig. 4.5 d).

Transcriptional regulators

This group includes a large number of known and putative transcriptional regulators of which only few showed an effect on phagosomal escape of *S. aureus* JE2. Mutants in three of the four genes of the *agr* quorum sensing system operon (*agr*A, NE1532: 6.4 ± 4.4%; *agr*B, NE95: 15.8 ± 1.4%; *agr*C, NE873: 13.2 ± 8.8%) (Fig. 4.5 a) as well as the sensor histidine kinase of the *sae*RS two-component system (*sae*S, NE1296: 66.0 ± 9.9%) (Fig. 4.5 h), are all well known to be crucially involved in staphylococcal virulence and mutants in the genes lack phagosomal escape (Novick, 2003; Liang *et al.*, 2006). By contrast effects on phagosomal escape rates were neither detectable for tested putative and uncharacterized transcriptional regulators nor for a series of *sar* accessory regulator protein family members, which are known to contribute to virulence gene regulation (*sar*A, NE1193: 94.9 ± 18.0%; *sar*T, NE514: 104.8 ± 13.9%; *sar*U, NE96: 88.4 ± 18.9%; *sar*X, NE296: 101.6 ± 25.2%; *sar*S, NE165: 86.0%; *sar*Y, NE210: 99.9 ± 26.3% and *rot*, NE386: 76.5 ± 26.4%). Also no influence on escape rates was found for the *agr* antagonist *cod*Y (NE1555: 92.2 ± 12.2%) (all Fig. 4.5 a), the repressor of surface proteins (*rsp*, NE1304: 82.3 ± 7.9%) (Fig. 4.5 g) and the alternative sigma factor σB (*rpo*F, NE1109: 98.3 ± 15.0%), even though mutants in two genes of the *sig*B regulatory operon, *rsb*W (25.6 ± 9.2%; p= .00015) and *rsb*U (53.3 ± 24.8%; p= .031) do show a phenotype.

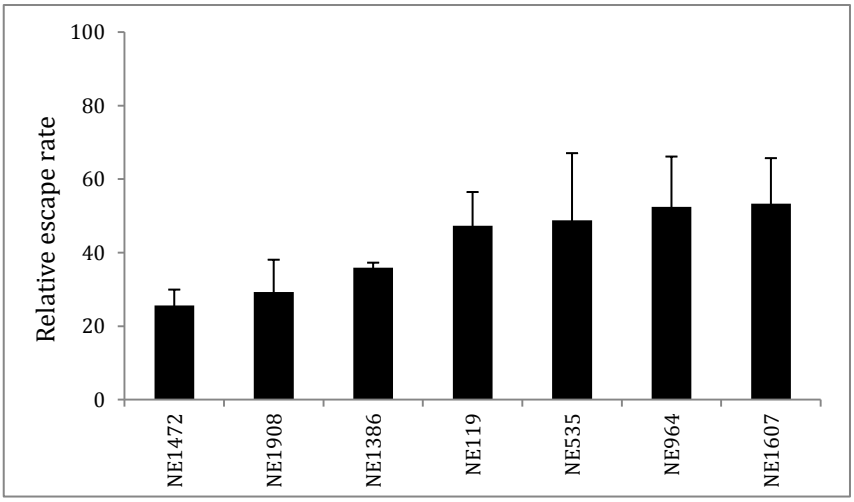

Figure 4.6: Phagosomal escape rates of mutant strains showing strongly reduced escape.
Relative phagosomal escape rates of mutant strains exhibiting significant reduction of their respective escape rates. HeLa cells were infected and escape rates were detected and quantified 3 hours post infection. Escape efficiency of the JE2 wild type has been set at 100%. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

Table 4.1: Annotation, replicate number, mean escape rate and standard deviation of Nebraska library mutant strains strongly attenuated in phagosomal escape.

Library label	Gene Description	Identifier	n	% escape	Std Dev
NE1472	*rsb*W, anti-sigma-B factor, serine-protein kinase	SAUSA300_2023	3	25.6	9.2
NE1908	*pmt*C, ABC transporter ATP-binding protein	SAUSA300_1911	3	29.3	1.,3
NE1386	*luk*A, Leukocidin/Hemolysin toxin family protein	SAUSA300_1974	4	35.9	13.7
NE119	*aus*A, non-ribosomal peptide synthetase	SAUSA300_0181	4	47.3	12.4
NE535	*sps*A, signal peptidase IA	SAUSA300_0867	3	48.8	11.5
NE964	*aus*B, 4'-phosphopantetheinyl transferase superfamily protein	SAUSA300_2640	4	52.4	11.7
NE1607	*rsb*U, sigmaB regulation protein	SAUSA300_2025	3	53.3	24.8

Those genes identified with an reduction of their escape efficiency of more than 40% and a p-value under .05, not yet known to be involved in *S. aureus* virulence, included the signal peptidase IA (*sps*A: 48.8 ± 11.5%; p= .0015) and both genes of the *aus*AB operon (*aus*A: 47.3 ± 12.4%; p= .00015, *aus*B: 52.4 ± 11.7%; p= .00018) (Wyatt *et al.*, 2010; Zimmermann and Fischbach, 2010). As no function of AusAB could be identified yet, despite the fact, that it is the only NRPS identified in *S. aureus* I decided that a deeper investigation of mode of action and phenotypic range of the NRPS would be the most promising venture.

4.3 The non-ribosomal peptide synthetase *aus*AB and its influence on phagosomal escape

The *aus*AB operon in the sequence type USA300 is encoded for by the two genes SAUSA300_0181 and SAUSA300_0182. Upstream of the synthetase the gene SAUSA300_0180 synthesizes a putative multidrug resistance protein, while downstream of the phosphopantetheinyl-transferase the ORF for a hypothetical protein lies in trans. For both genes no immediate connection with the non-ribosomal peptide synthetase or its function is apparent or documented.

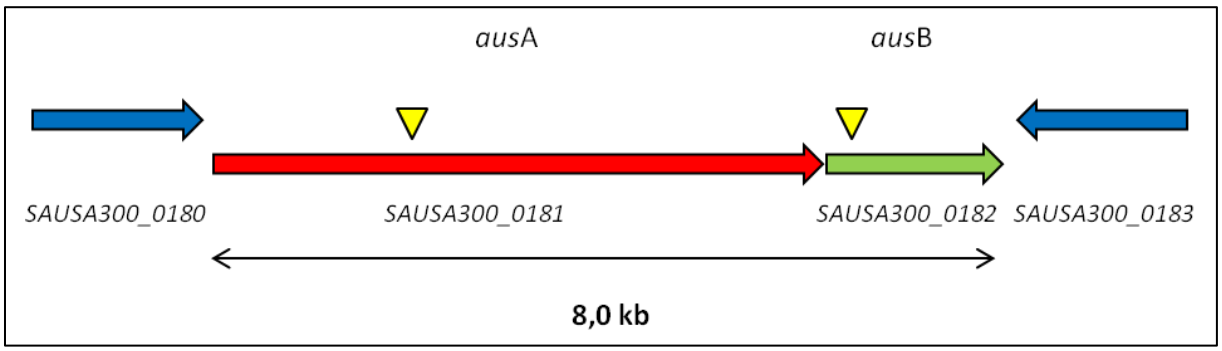

Figure 4.7: Schematic of gene arrangement of the *aus*AB operon in the genome of *S. aureus* strain JE2.
Schematic depiction of the *aus*AB operon, consisting of the 7176 bases long *aus*A ORF and the 645 bases long *aus*B ORF. The lipopreotein *lmr*P, upstream of *aus*A, has a length of 1251 base pairs while the hypothetical protein downstream of *aus*B possesses a length of 495 base pairs. For both *aus*AB adjoining genes, no function is known. Yellow triangles indicate the location of the transposon insertion in the Nebraska library strains NE119 (*aus*A) and NE964 (*aus*B)

The non-ribosomal peptide synthetase *aus*AB was first identified in 2010 by two groups in short succession as such (Wyatt *et al.*, 2010; Zimmermann and Fischbach, 2010). The synthetase *aus*A is composed as a peptide assembly line, in its principles very similar to many others in bacterial organisms (Fischbach and Walsh, 2006). It consists of domains responsible for condensation (C) reactions, facilitating C-N bonds e.g. between the initially bound amino acid and AA forming the consequent peptide chain, adenylation domains (A), which produce adenosine monophosphate esters of the amino acids involved in peptide production in dependency of adenosine triphosphate (ATP), thiolation domains (T), by phosphopantetheinyl-transferase post-translationally modified sites of attachment of the growing peptide chain and a reductase domain (RE), releasing the complete peptide in the form of an aldehyde (Wyatt *et al.*, 2010; Zimmermann and Fischbach, 2010).

In order to reach a functional status, the thiolation domains have to be modified post-translationally by the acyl carrier protein synthase (ACPS) domain of the AusB protein, which catalyzes the priming of both T-sites by attaching the 4'-phospho-pantetheine sidechain of acyl-CoA molecules (Fischbach and Walsh, 2006; Wyatt *et al.*, 2010).

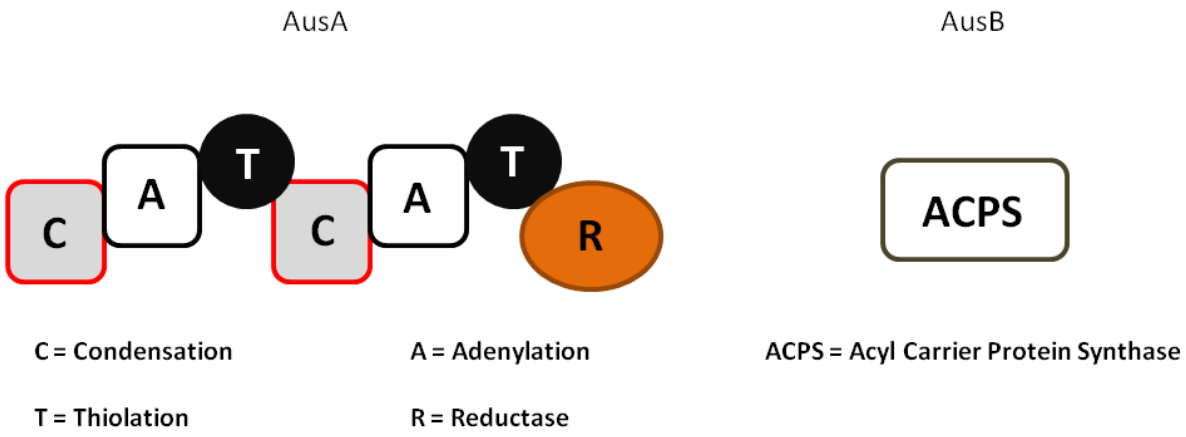

Figure 4.8: Depiction of the domain structures of the NRPS AusA and the 4-phosphopantetheinyl-transferase AusB.

The domain collocation of the proteins AusA and AusB is shown schematically. AusA is composed of two repeats of consecutive condensation, adenylation and thiolation domains, while a reductase domain at the C-terminus represents the functional as well as structural end of the synthetase. AusB possesses only one identified acyl carrier protein sytnhase domain. (Modified from (Zimmermann and Fischbach, 2010))

A set of dipeptides could be identified as the products of the NRPS AusAB. The fact that three peptides are produced in total can be explained because of the looser substrate specificity of the second A-domain, using either tyrosine, phenylalanine or leucine, while the first A-domain strictly processes valine. The results thereof are three cyclized pyrazinones-like products, tyrvalin, phevalin and leuvalin (Wyatt *et al.*, 2010; Zimmermann and Fischbach, 2010).

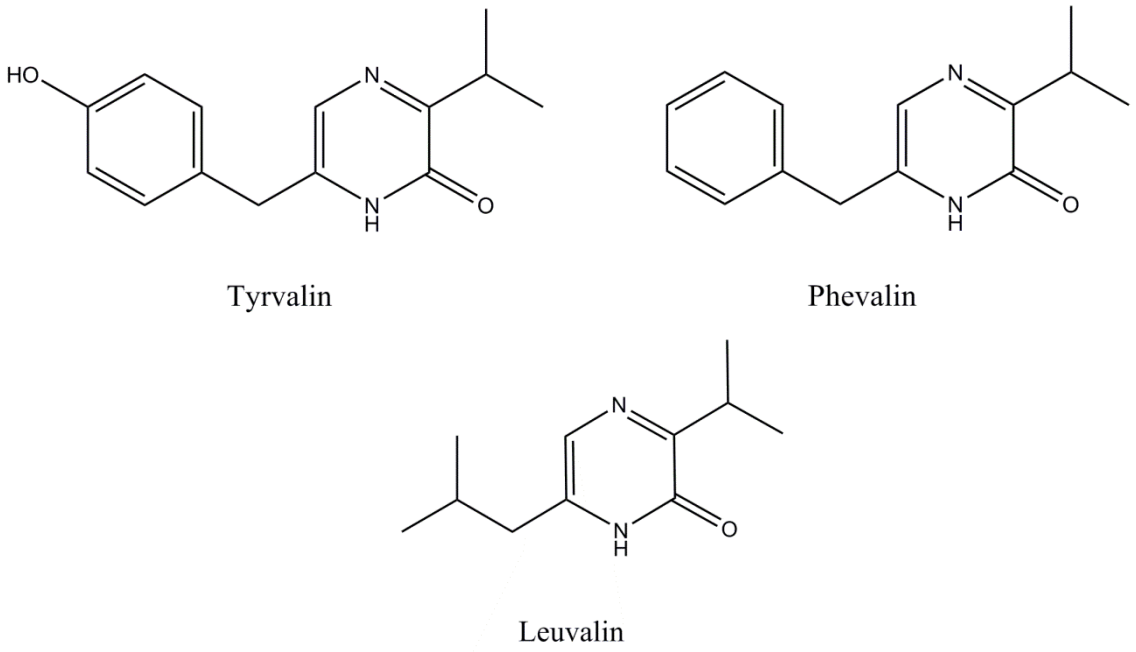

Figure 4.9: Chemical structures of the AusAB produced pyrazinones.

Chemical structures of all three AusAB produced cyclized pyrazinones. Structural specificities of valin, the amino acid first bound by the NRPS can still be found despite the veiling effect of the intramolecular cyclization reaction of the peptide aldehyde intermediate, released by the reductase domain. The two majority products, phevalin and tyrvalin both carry one benzene and one phenol group respectively. (Modified from (Zimmermann and Fischbach, 2010))

All of them undergo spontaneous cyclization via a condensation reaction between the N-terminal amino group and the C-terminal aldehyde generated in the two-electron reductive release reaction by the R domain. A final oxidation then yields the products depicted in Figure 4.9 (Zimmermann and Fischbach, 2010). The abundance of the three peptides was analyzed by mass spectrometry, which revealed that tyrvalin and phevalin are produced in about equal quantities as a main product, whereas leuvalin is only a minor by-product (Zimmermann and Fischbach, 2010).

When relating the USA300 NRPS sequence to other strains it becomes evident, that the NRPS AusAB is highly conserved among *S. aureus*. Using a Blast search I found, that among 50 sequenced genomes of *S. aureus*, identity with the LAC *aus*A was at least at 97.2%, while this value was even better in the case of *aus*B at 98.6%. Also, the NRPS has orthologs among other *Staphylococcus* species, e.g. *Staphylococcus argenteus* (71.2% identity), *S. capitis* (53.8% identity), *S. lugdunensis* (53.2% identity), *S. xylosus* (45.4% identity) and *S. epidermidis* (52.9% identity)

4.3.1 Kinetics of escape in AusAB mutants

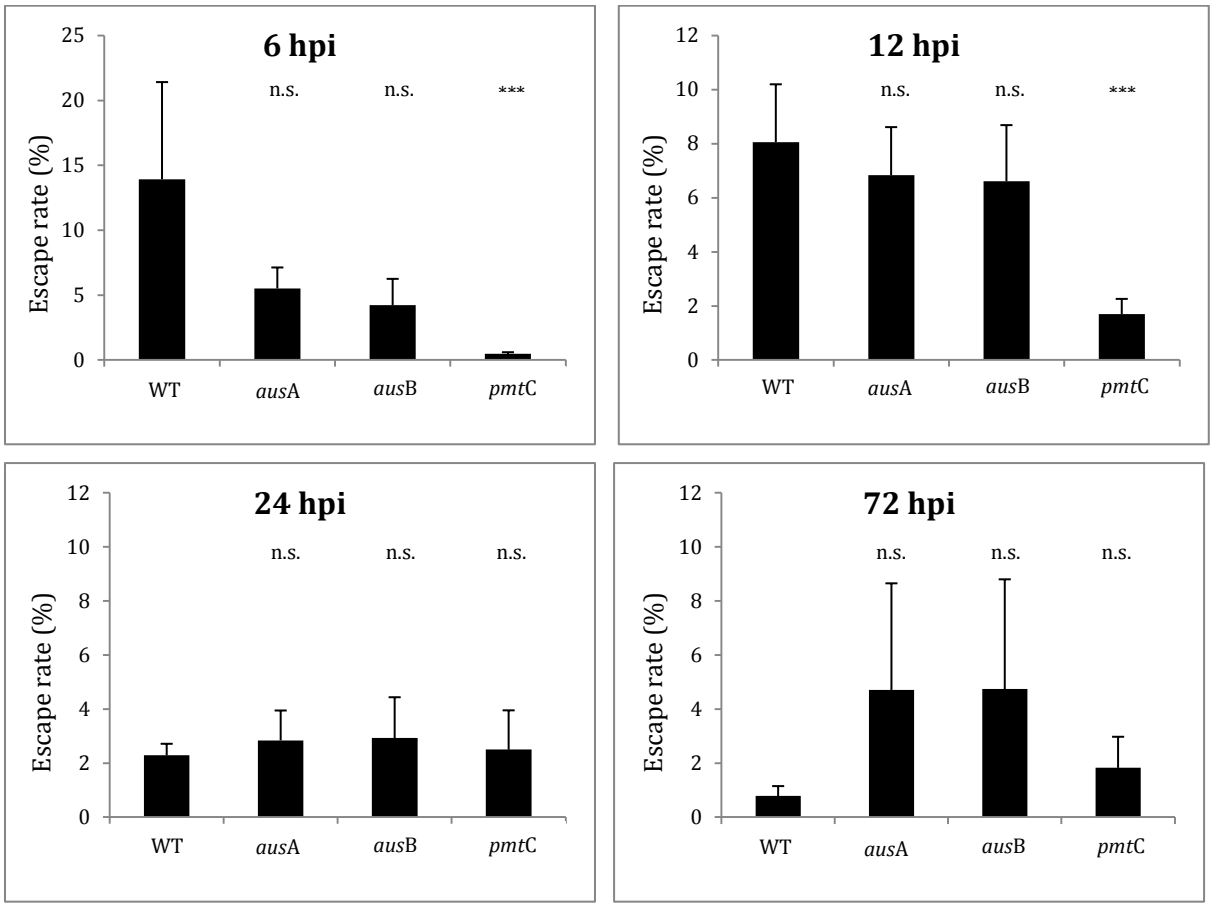

Figure 4.10: Delay of phagosomal escape in AusAB mutants independent of cell type.
IB3 cells expressing the YFP-cwt escape detection marker were infected at an MOI of 10 with wild type or *aus*A, *aus*B and *pmt*C mutant bacteria. Cells were fixed at the indicated time post infection and images were recorded on an Operetta microscopy system with 20x magnification. YFP spot intensity threshold was set at 200. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

I checked for differences in the phagosomal escape kinetics for the *aus*A and *aus*B mutant when compared to both, the JE2 wild type as well as a mutant in the PSM transporter subunit, *pmt*C. In order to exclude that mutations in the *aus*AB operon were delaying the escape phenomenon I infected a lung epithelial cell line with cystic fibrosis genetic markers with wild type bacteria as well as *aus*A, *aus*B and *pmt*C mutants and fixed the cells after 6 h, 12 h, 24 h and 72 h, and analyzed the samples as outlined previously. While the *aus*A and *aus*B mutant escape rates show considerably less escape than the wild type at 6 h post infection, this difference disappears at the 12 h time point (Fig. 4.10). The reduction of the difference in phagosomal escape is due to a lower escape rate in the wild type (13.9 ± 7.5% at 6 hpi vs. 8.1 ± 2.1 at 12 hpi) but also an increase for the *aus*A mutant (5.5 ± 1.6% at 6 hpi vs. 6.8 ± 1.8% at 12 hpi) as well as the *aus*B mutant (4.2 ± 2.0 at 6 hpi vs. 6.6 ± 2.1% at 12 hpi). The *pmt*C mutant does not escape at either

time point (0.5 ± 0.1% at 6 hpi vs. 1.6 ± 0.6% at 12 hpi). At the 24 h and 72 h time point, differences between strains are diminished.

Since *S. aureus* readily kills host cells upon translocation to the cytoplasm (Grosz *et al.*, 2014) I used the Perkin Elmer Harmony Software to enumerate changes in host cell numbers during the course of infection (Fig. 4.11).

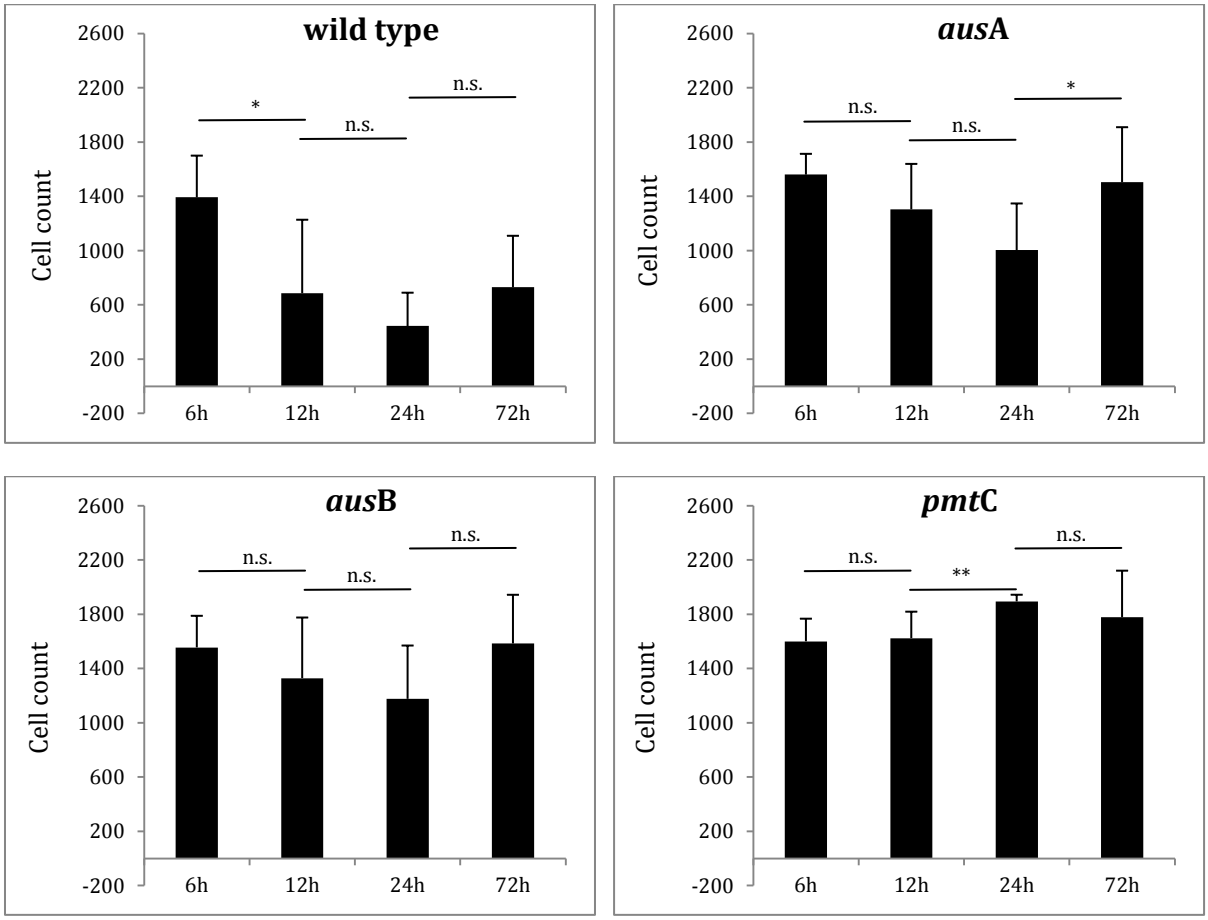

Figure 4.11: Cell counts of AusAB mutant infected cells are reduced to lesser extents than counts of wild type infected cells.

Pictures of infected IB3 YFP-cwt cells were analyzed for the number of detectable adherent cells left in the 10 fields per well imaged using the 20x magnification on the Operetta microscope. For cell detection, the YFP fluorescence constitutively expressed by the IB3 YFP-cwt cell line was employed. Bar graphs show the mean of three independent experiments ± SD. Statistical analysis was performed by t-test.

These data demonstrate that cells infected with the JE2 wild type are lost much faster and in higher numbers from the well plate and therefore also from the analysis than the cells infected with any of the mutant strains. At the 6 h time point cell numbers throughout samples are relative similar, but this changes drastically 12 h post infection, when in the wild type sample only about half of the cell number at 6h is retained (686 ± 541 at 12 hpi vs. 1392 ± 307 at 6 hpi).

This reduction is much smaller for *aus*A (1304 ± 335 at 12 hpi vs. 1560 ± 153 at 6 hpi) and *aus*B (1328 ± 447 at 12 hpi vs. 1554 ± 233 at 6 hpi). No change at all in the cell number is detectable in the *pmt*C sample (1623 ± 195 at 12 hpi vs. 1599 ± 167 at 6 hpi). This lack of cell death in the *pmt*C sample is consistent over the complete time course. The cell count rather increases at the 24 h time point (1893 ± 49) and the host cell number is stable through 72 hpi (1777 ± 343).

In all 3 other samples the cell numbers decrease from 12 hpi to 24 hpi, with the reduction being strongest in the wild type infected cells again (444 ± 246 at 24 hpi vs. 686 ± 541 at 12 hpi). Cell numbers in the *aus*A (1003 ± 345 at 24 hpi vs. 1304 ± 335 at 12 hpi) and *aus*B (1176 ± 393 at 24 hpi vs. 1328 ± 447 at 12 hpi) sample fall as well but only moderately. At the last time point, 72 hours post infection, cell numbers increase in the three samples, wild type, *aus*A and *aus*B.

Following the identification of the two genes of the *aus*AB operon as a putative factor in the facilitation of phagosomal escape of the *S. aureus* strain JE2, we planned to generate a complete gene knock-out mutant (Lambalot *et al.*, 1996). To generate such a mutant we chose the plasmid-based system pKor1, which can be used for seamless and markerless deletion of genes through allelic replacement (Bae and Schneewind, 2006). Attempts of allelic replacement and gene knockout were performed in the strains JE2 as well as the highly cytotoxic strain 6850 (Fraunholz *et al.*, 2013). Both, the deletion of the complete *aus*AB operon, as well as the sole deletion of the *aus*B gene repeatedly failed so that this strategy was abandoned. Also, attempts to transduce the transposon insertion from the Nebraska library mutants into other genetic backgrounds did not succeed as only mixed populations of bacteria were recovered that harbored the transduced genetic element but still expressed the wild type allele. Attempts to scavenge these populations from wild type bacteria through serial plating on TSA plates supplemented with antibiotics failed.

Therefore I decided to use the NE964 mutant (*aus*B::bursa; (Fey *et al.*, 2013)) and transformed this strain with a plasmid constitutively expressing the ORF SAUSA300_0182, encoding the 4'-phosphopantetheinyl transferase superfamily protein AusB, under the control of the SarAP1 promoter. Subsequently I verified the functional complementation of the AusAB non-ribosomal peptide synthetase in the resulting strain NE964 pSarAP1-*aus*B.

4.3.2 UPLC analysis of mutant strain supernatants

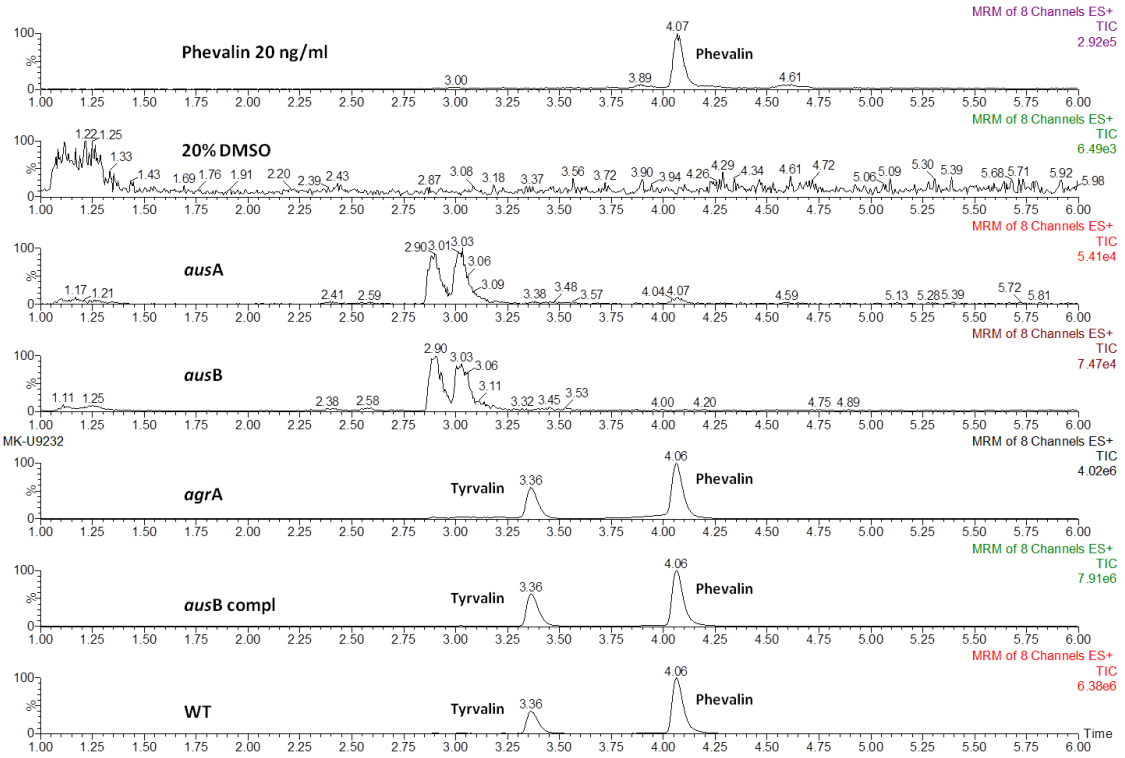

Figure 4.12: Tn-insertion mutants lack aureusimine production, which is restored in the complemented strain.

Conditioned supernatant samples of *S. aureus* JE2 strains as well as a phevalin and a 20% DMSO control were analyzed on a Waters Mass spectrometer using a C18 column. The Figure shows the retention time period (x-axis) of 1 min to 6 min. On the y-axis, the relative intensity is depicted. Exact numbers for the relative intensity in each measurement are shown on the right. Detection occurred via multiple reaction monitoring (MRM) and three specific fragments were monitored for each compound (phevalin: m/z 229 > 214, m/z 229 > 159, m/z 229 > 81; tyrvalin: m/z 245 >359 230, m/z 245 > 175, m/z 245 > 81)

Using an Ultra Performance Liquid Chromatography (UPLC) coupled with Mass spectrometry I investigated the presence of phevalin and tyrvalin in extracts of culture supernatants in collaboration with the chair of pharmaceutical biology. As references for an easier detection of phevalin and tyrvalin we used the molecular masses already published by Zimmerman and Fischbach and synthetical phevalin, commercially available from Santa Cruz Biotech (Heidelberg, Deutschland).

The analysis of strain supernatants was performed as described (Secor *et al.*, 2012). Collected supernatants of the *S. aureus* JE2 wild type strain, the insertional mutants of *aus*A (NE119) and *aus*B (NE964), the *aus*B complemented NE964 strain as well as an *agr*A mutant strain (NE1532). Production and secretion of the two aureusimines, phevalin and tyrvalin was

detected by UPLC in the JE2 wild type, in the *agr*A mutant as well as the complemented *aus*B strain (Fig. 4.12). By contrast, tyrvalin and phevalin were completely absent from the supernatants of both mutants, NE119 and NE964 respectively. The *aus*B complemented strain appeared to produce higher amounts of both dipeptides than the wild type, probably due to higher rates of activation of the *aus*A synthetase by constitutively expressed *aus*B. The *agr*A mutant shows lesser quantities of the aureusimines.

4.3.3 Growth phase dependency of aureusimine production

In ordert to investigate growth phase dependent expression of *aus*AB, I investigated expression oft he genes by quantitative Real time PCR (RT-PCR)

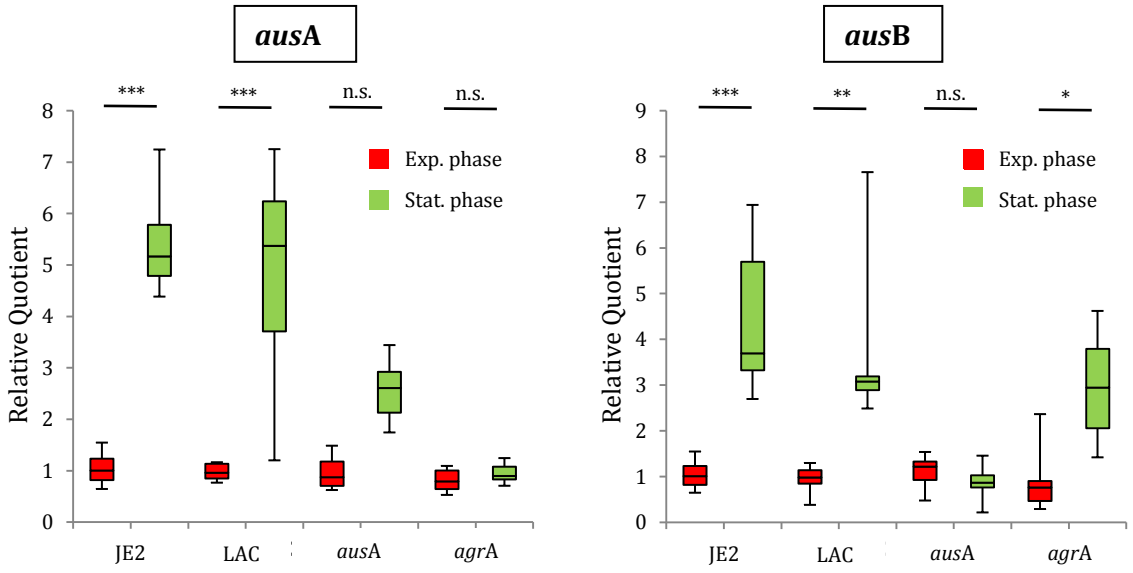

Figure 4.13: Quantitative RT-PCR demonstrates growth phase dependent transcription of *aus*A and *aus*B in different strains.

To test for the abundances of both genes, *aus*A and *aus*B, RNA was prepared from exponential (OD_{600} = 0.6) and stationary growth phase (8h after inoculation) from *S. aureus* wild type strains JE2 and LAC, an *aus*A mutant (NE119) as well as an *agr*A mutant (NE1532). The box-and-whisker-blots illustrate RQ values derived from 3 independent biological replicates. Statistical analysis was performed by t-test. *p<0.05; **p<0.01; ***p<0.001. n.s. not significant

The data obtained from the RT-PCR experiments show a strong dependence of *aus*A and *aus*B transcription on the growth phase of the bacteria (Fig. 4.13). Transcription of *aus*A and *aus*B increased in both wild type strains in stationary growth phase, when compared to exponential phase (5-6 fold and 3-4 fold, respectively). This increase is diminished in case of the *aus*A transposon insertion mutant. *aus*A transcript levels still increase to some degree (Median= 0.87

in exponential phase and 2.61 at stationary phase), probably owed to the location of the transposon insertion site in relation to the primers used in the PCR reaction, an increase in *aus*B levels is missing completely. In the *agr*A mutant, the increase in *aus*A transcripts observed in the wild type is completely absent, thereby indicating dependence of NRPS expression on a functioning and active *agr* system. Interestingely, this is not the case for *aus*B. The relative increase in stationary phase is similar to what we observe in the wild type (Median= 0.76 in exponential phase and 2.95 in stationary phase).

4.3.4 Aureusimines are produced by intracellular *S. aureus*

I next investigated if the aureusimines are produced after *S. aureus* has been internalized in host cells.

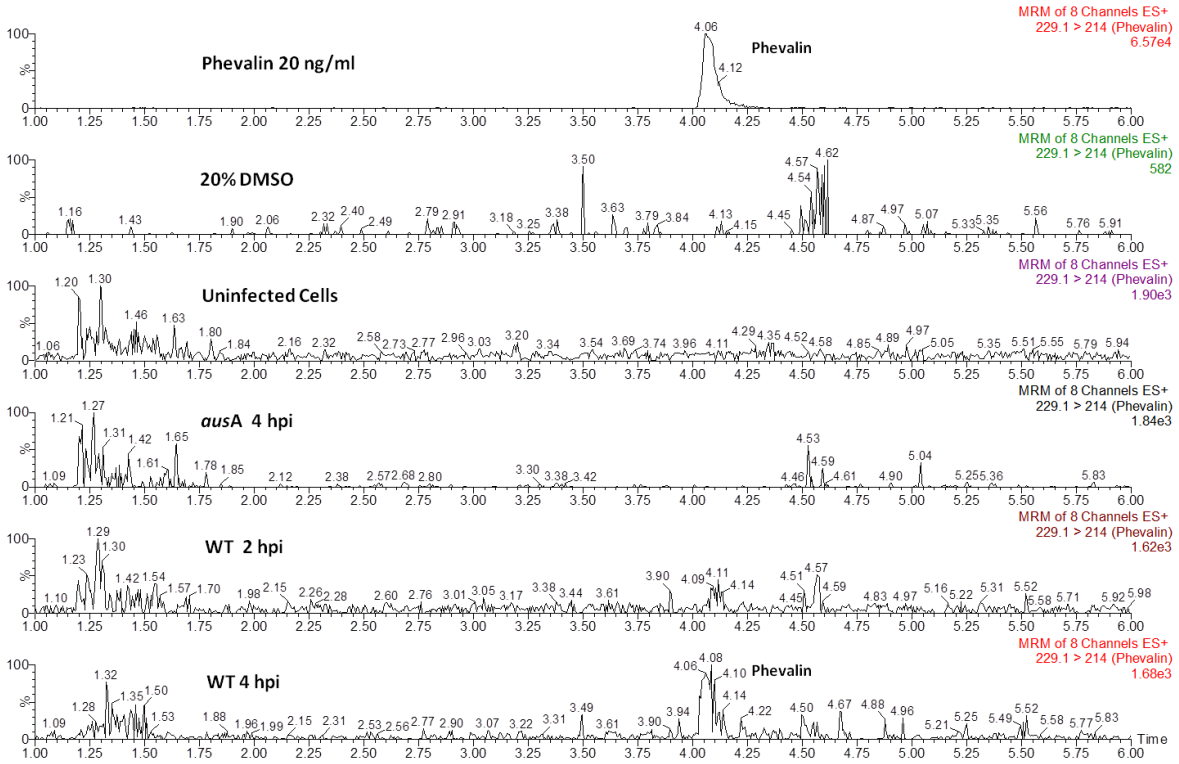

Figure 4.14: Aureusimines are produced by intracellular *S. aureus* and are detectable 4 hours post infection.
HeLa cells were left uninfected, mock treated (DMSO) or were infected with an MOI of 10 of S. *aureus* JE2 wild type or the respective *aus*A mutant and samples were extracted after 2 or 4 hours post infection. Samples were analyzed by UPLC-MS using a C18 column with subsequent detection of small molecules by a Waters Mass spectrometer. Histograms depict the retention time from 1 min to 6 min (x-axis) and the relative intensity (y-axis). Exact numbers for the relative intensity in each measurement are shown on the right. To increase the sensitivity of phevalin detection, only the phevalin specific transition is displayed instead of all MRM.

To evaluate this, cells were seeded in micro titer well plates and infected with either wild type *S. aureus* JE2, the insertional *aus*A mutant, were mocked treated with the solvent DMSO or were left uninfected. As shown in figure 4.14, phevalin was detectable in the cells infected with wild type bacteria but not in the extracts from host cells infected with the *aus*A strain. Also, an increase in the detected phevalin amounts is evident when comparing the 2 and 4 hpi time points of host cell that were infected with the wild type.

4.3.5 Genetic complementation of phevalin synthesis restores phagosomal escape of an *aus*B mutant

In the previous experiments I established that insertional mutants of *aus*A and *aus*B were unable to produce the aureusimines phevalin and tyrvalin and that complementation using a plasmid carrying the *aus*B gene (*aus*B compl.) restored production of the aureusimines in bacterial culture supernatants as well as in infected host cells. I therefore examined if phagosomal escape of *S. aureus* in the cell lines HeLa and S9 was dependent on the production of aureusimines.

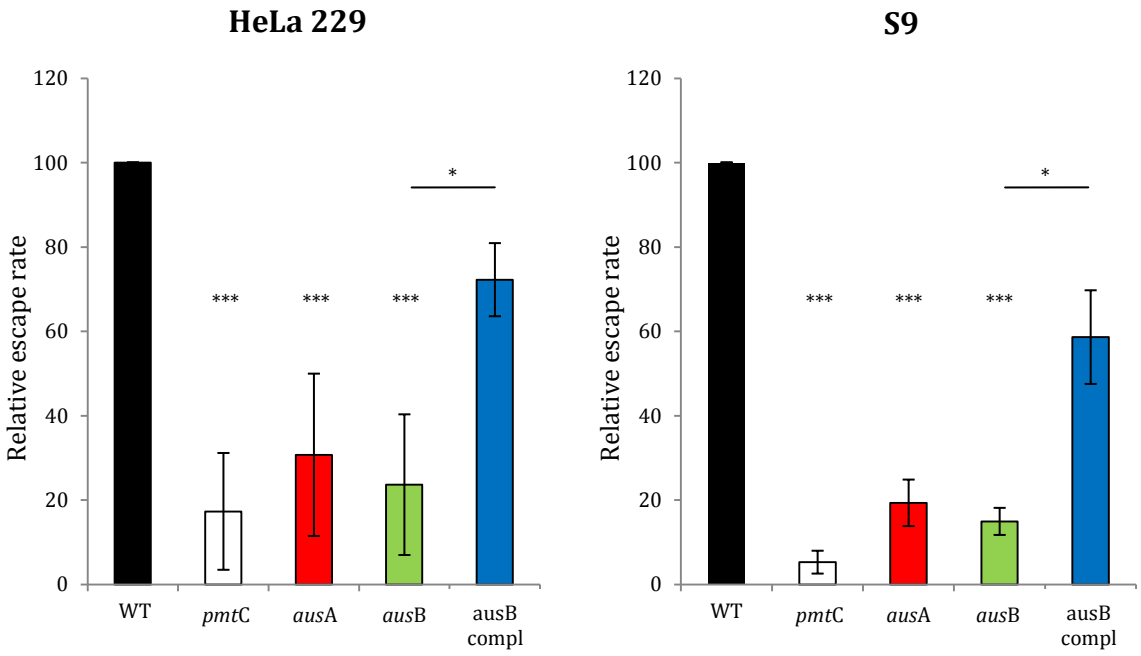

Figure 4.15: Genetical complementation of phagosomal escape.

Hela and S9 cells were infected with either wild type, *aus*A, *aus*B or *pmt*C mutant bacteria as well as the complemented *aus*B mutant strain at an MOI of 10. 4 hpi cells were fixed and images were taken on an Operetta microscope using 20x magnification. YFP spot intensity threshold was set at 100. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

Cells were infected using the previously established protocol (chapter 3.2.5),were fixed four hours post-infection and analyzed by Operetta imaging (chapter 4.3). Insertional inactivation of *aus*A and *aus*B resulted in a strong decrease in phagosomal escape in HeLa (30.7 ± 19.2% and 23.6 ± 16.7%, respectively) and S9 cells (19.3 ± 5.5% and 14.9 ± 3.2%, respectively) when compared to the wild type (set to 100%) thereby corroborating the results obtained during the screen (Fig. 4.15). Relative escape rates for the *pmt*C mutant lie even beneath these values at 17.3 ± 13.8% in HeLa and 5.3 ± 2.7% in S9 (p= .0005 and .0000004, respectively).

Episomal complementation of *aus*B expression led to a restoration of escape rates, since 72.2 ± 8.7% of intracellular *S. aureus* escaped in HeLa (p= .011) and 58.6 ± 11.1% in S9 upper airway epithelial cells (p= .0028).

4.4 Mutants in *aus*A and *aus*B do not affect growth in broth or hemolysis but do affect intracellular cytotoxicity

4.4.1 Mutants in *aus*A and *aus*B do not show a growth defect

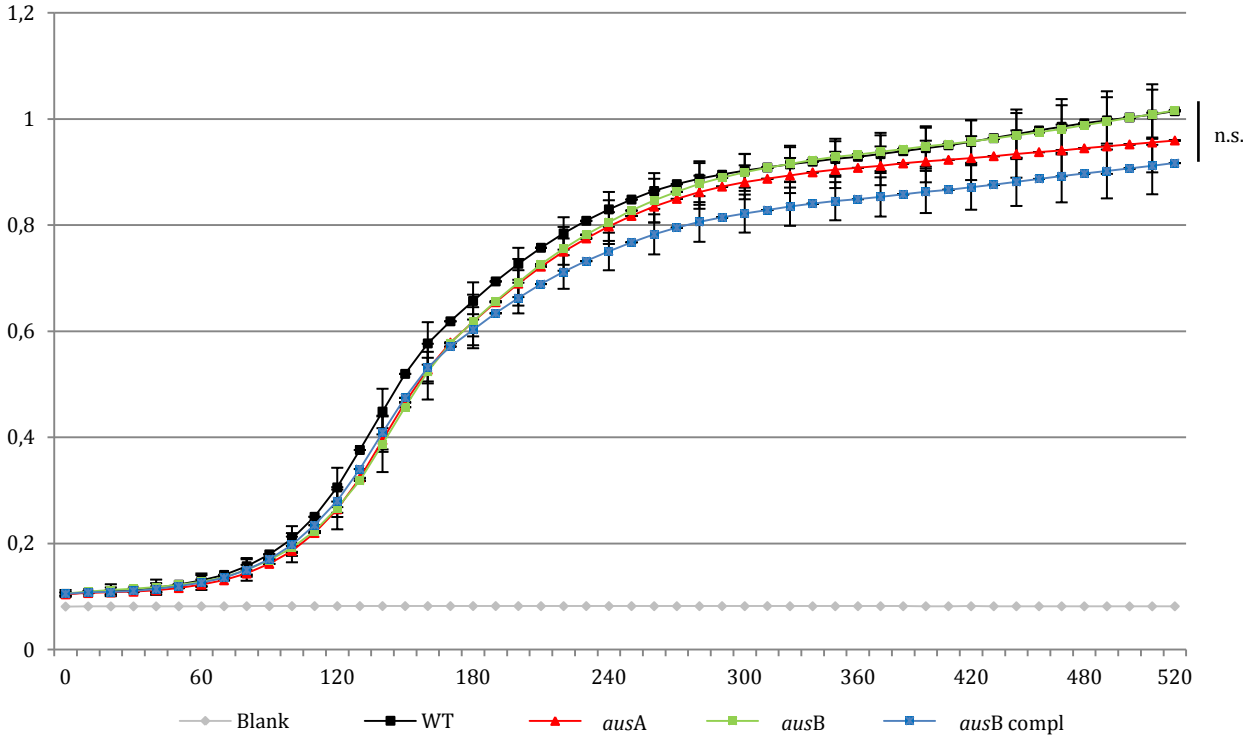

Figure 4.16: Growth curves in TSB medium of bacterial strains shows no significant differences.
S. aureus strains were incubated in TSB medium in microtiter plates inoculated from an overnight culture. Cultures were shaken in a microtiter plate reader and OD_{600} was measured every 10 min for about 9 hours. Each data point represents the mean of three individual experiments. Statistical analysis was performed using t-test.

In order to eliminate a growth defect in the *aus*A and *aus*B mutants as a reason for the attenuated phagosomal escape and virulence, I performed growth curves in TSB medium. The comparison between the JE2 wild type and the mutant strains shows no difference in their ability to grow in tryptic soy broth (Fig. 4.16). While the complemented strain (*aus*B compl) shows a slight reduction in its growth rate over the complete course of the 9 h observation period, this difference is not statistical significant

4.4.2 No differences in hemolysis between AusAB mutants and wild type

Since a deficiency in the production of certain toxins, like PSMs, α-toxin or β-toxin can interfere with the ability of the bacteria to escape from the phagosome (Jarry *et al.*, 2008; Giese *et al.*, 2011; Grosz *et al.*, 2014). I tested bacterial colonies for hemolysis on blood agar plates.

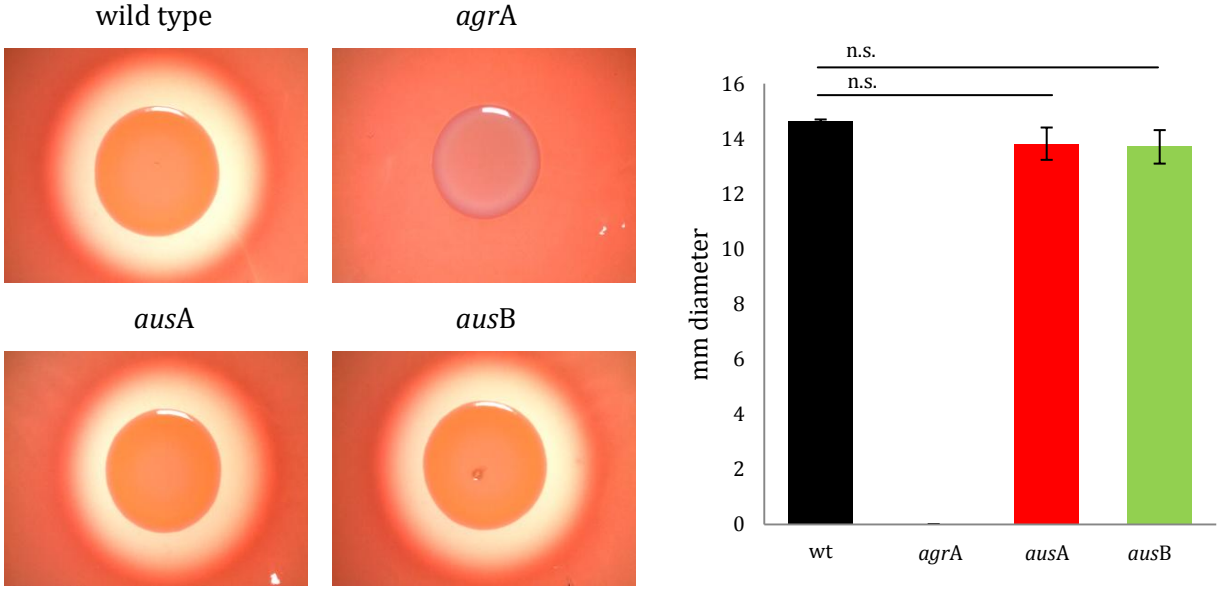

Figure 4.17: α-toxin-based hemolysis is unaffected by mutations in *aus*A and *aus*B.
Qualitative and quantitative analysis of hemolytic patterns of wild type and mutant strains on blood agar plates were established by spotting diluted overnight cultures on Columbia blood agar plates. After overnight incubation, pictures were taken at a 5x magnification. Pictures represent hemolytic patterns around bacterial colonies in 1 out of 2 biological replicates. Graph depicting the mean diameter of the hemolytic area represents data from 2 biological replicates.

Hemolysis of mutant strains in comparison to the JE2 wild type was identical after 24 h at 37°C (Fig. 4.17). Differences in the diameter of hemolysis caused by a colony on Columbia blood agar are minute with wild type at a mean of 14.65 ± 0.05 mm, *aus*A at 13.82 ± 0.59 mm and *aus*B at

13.71 ± 0.61 mm. A mutant in the important virulence regulator *agr*A served as a negative control. No hemolysis could be observed in this mutant.

4.4.3 Invasion into host cells not affected by *aus*AB mutation

Phagosomal escape of bacteria is preceded by the internalization of bacteria into the host cell. In case of professional phagocytes this process is initiated by the host cell and less dependent on bacterial factors. When assessing the internalization into non-professional phagocytes, the expression of certain virulence factors is required for efficient invasion. To exclude an invasion phenotype to be the reason behind less phagosomal escape detectable in cells infected with *aus*A and *aus*B mutants we performed an invasion assay in microtiter plates.

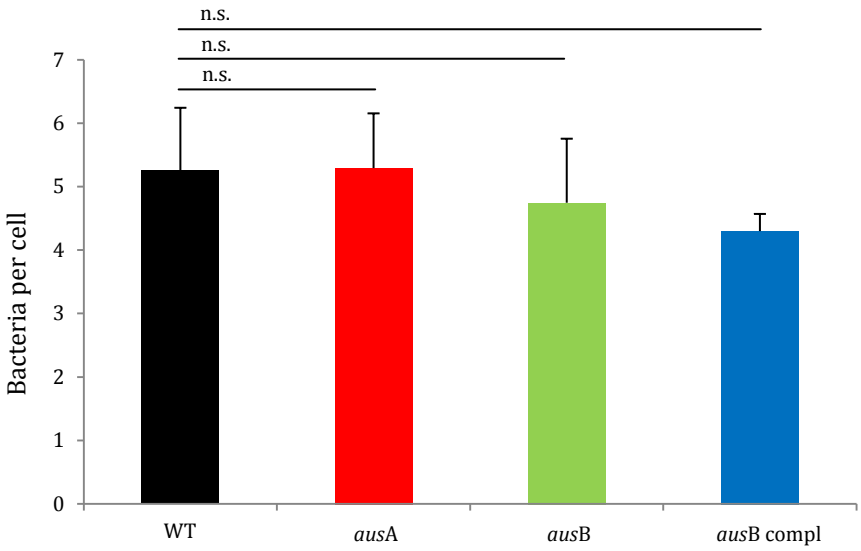

Figure 4.18: Invasion in non-professional phagocytes not dependent on aureusimine production.
HeLa YFP-cwt cells were infected at an MOI of 10 with either wild type or mutant bacteria strains. 1 ½ hours post infection, cells were fixed and an analysis for cell numbers as well as a count of internalized bacteria was performed in compliance with methods used before. No statistically significant changes between strains was detected. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

To detect and quantify invasion, I infected HeLa YFP-cwt cells with TRITC stained bacteria and removed extracellular bacteria by Lysostaphin digestion after co-incubation of 1 hour (chapter 3.2.5). Invasion was measured microscopically. Host cell numbers were enumerated using their constitutive YFP expression whereas fluorescent bacteria were counted in the TRITC channel (Fig. 4.18). While the effective MOI of the wild type strain is at 5.24 ± 0.98, the *aus*A mutant shows a mean MOI of 5.29 ± 0.86 bacteria/host cell. The *aus*B mutant has a slightly lower effective MOI of 4.74 ± 1.00 while the *aus*B complemented strain actually shows the lowest MOI

with 4.29 ± 0.27 bacteria/host cell. Since there are not significant differences between invasiveness of the tested strains, an effect of the non-ribosomal peptide synthetase on bacterial invasion was excluded.

4.4.3 AusAB mutants are attenuated in intracellular cytotoxicity

Internalized bacteria have been shown to cause the death of the host cell (Haslinger-Loffler *et al.*, 2005; Kubica *et al.*, 2008). To evaluate, if the attenuation in phagosomal escape presented by the *aus*AB mutants also influences the ability of the bacteria to kill the host cell from within, we next conducted a cell death experiment using HeLa cells.

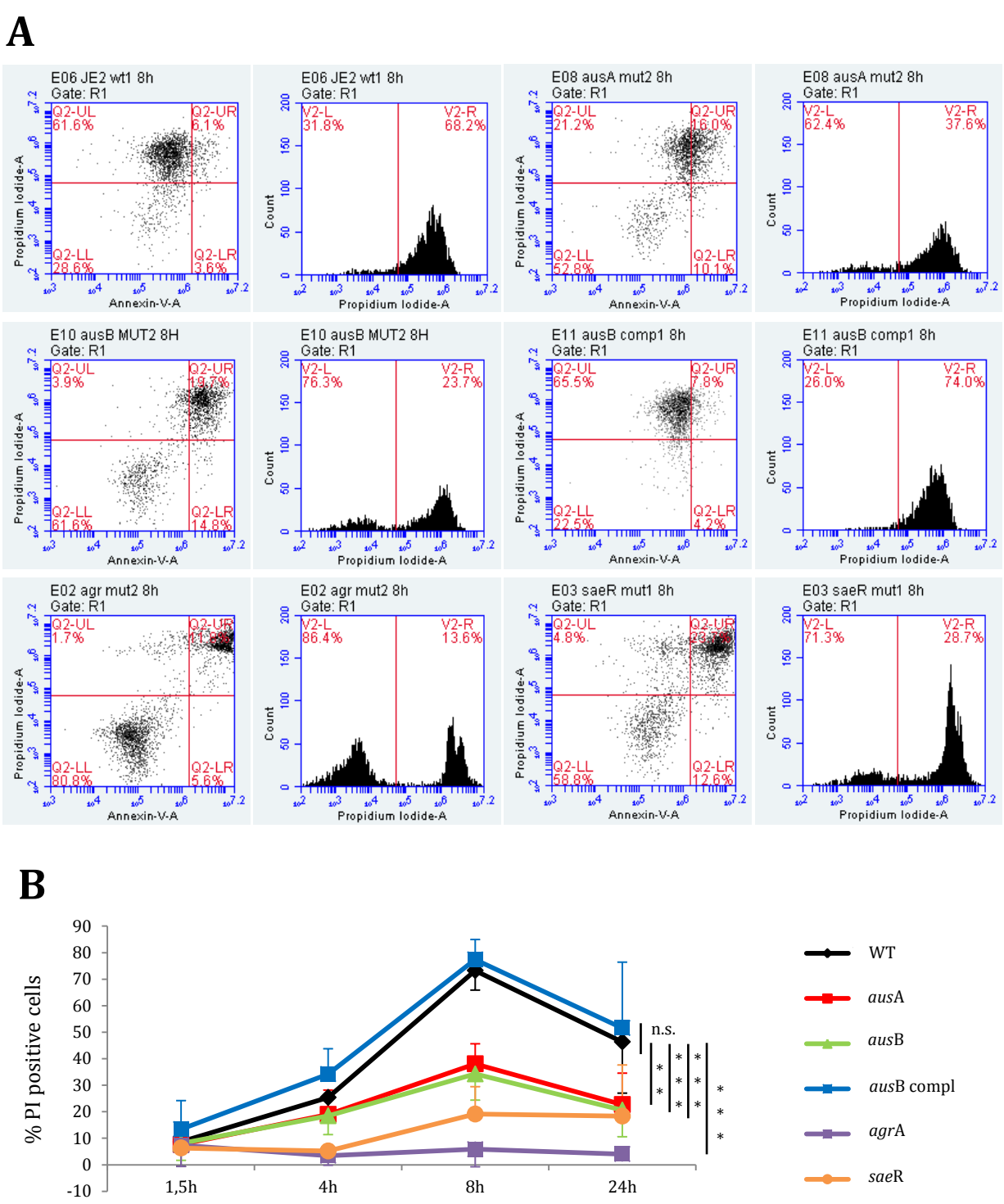

Figure 4.19: Host cell cytotoxicity attenuated in *aus* mutants.

HeLa cells were infected with JE2 wild type as well as a series of mutant strains at an MOI of 10. At 4 different times post infection, cells were trypsinized, subjected to Annexin V and propidium iodide (PI) staining and then analyzed using fluorescence flow cytometry (A). Dot blots and histograms above are representive examples of the cytometric analysis, illustrating differential numbers of PI positive cells in the infected cell samples. Graphs in (B) below summarize a set of three individual biological replicates in terms of percentage of PI positive cells detected at each of the time points. Statistical analysis was performed by linear modeling and ANOVA followed by Tukey's post-hoc analysis.

The analysis of the host cell death kinetics following infection with wild type or mutant strains using Annexin V/PI staining and flow cytometry revealed a strong decrease in the *aus*A and *aus*B mutant to cause cell death (Fig. 4.19).

In the wild type, percentage of PI positive cells increased from 1.5 h (8.67 ± 9.29%) through 4 h (25.38 ± 5.93%) to 8 h (73.25 ± 7.42%) before falling again at the 24 h time point (46.37 ± 19.39%). The complemented *aus*B mutant exhibits a very similar trend with slightly higher values of percent PI positive cells at all timepoints (13.4 ± 10.76% at 1.5 h; 34.18 ± 9.53% at 4 h; 77.41 ± 7.5% at 8 h; 51.58 ± 24.8% at 24 h). Both mutants deficient in the aureusimine production are strongly attenuated in their cytotoxicity in HeLa and show high similarities in their kinetics. Where % PI pos. increases with the *aus*A strain from 7.63 ± 5.36% at 1.5 h to 18.98 ± 9.15% at 4 h and further to 38.07 ± 7.53% at 8 h before declining to 22.7 ± 11.82% at 24 h, *aus*B shows 8.03 ± 6.41% PI pos. cells at 1.5 h, 18.47 ± 7.12% at 4 h, 34.25 ± 9.9% at 8 h and 20.63 ± 10.11% at 24 h. In general, cell death remained at baseline levels 1.5 hours post infection for all strains. From this timepoint, the kinetics developed completely different in the tested strains.

Both mutants still caused more cells to become PI positive than mutants in the two prominent virulence regulators *sae*R and *agr*A. While cytotoxicity in the *sae*R mutant appears to be delayed and heavily attenuated with 6.33 ± 8.54% PI pos cells at 1.5 h, 5.18 ± 2.21% at 4 h, 19.12 ± 10.3% at 8 h and 18.35 ± 19.26% at 24 h, an *agr*A mutation leads to a complete abolishment of all cytolytic properties, where only a mere baseline level of PI positive cells can be detected (7.4 ± 8.01% at 1.5 h; 3.42 ± 3.64% at 4 h; 5.85 ± 6.64% at 8 h and 4.05 ± 2.5% at 24 h).

Looking at the results of the test, we opted for a linear model in terms of statistical analysis, as a linear increase of cell death over time appeared evident (Pearson correlation coefficient R-square = 0.64). TukeyHSD tests were performed for individual comparisons of different time points post infection and individual strains.

4.4.4 Cell death of host cells by *S. aureus* supernatant not influenced by phevalin

The cytotoxicity detected in the experiment above stems with high probability from internalized bacteria only, as the use of gentamicin in the cell culture medium during the infection eliminated viable extracellular bacteria. Since we could not completely rule out effects from released and secreted toxins I next tested possible effects of aureusimine deficiency on toxin secretion and extracellular cytotoxicity of *S. aureus*.

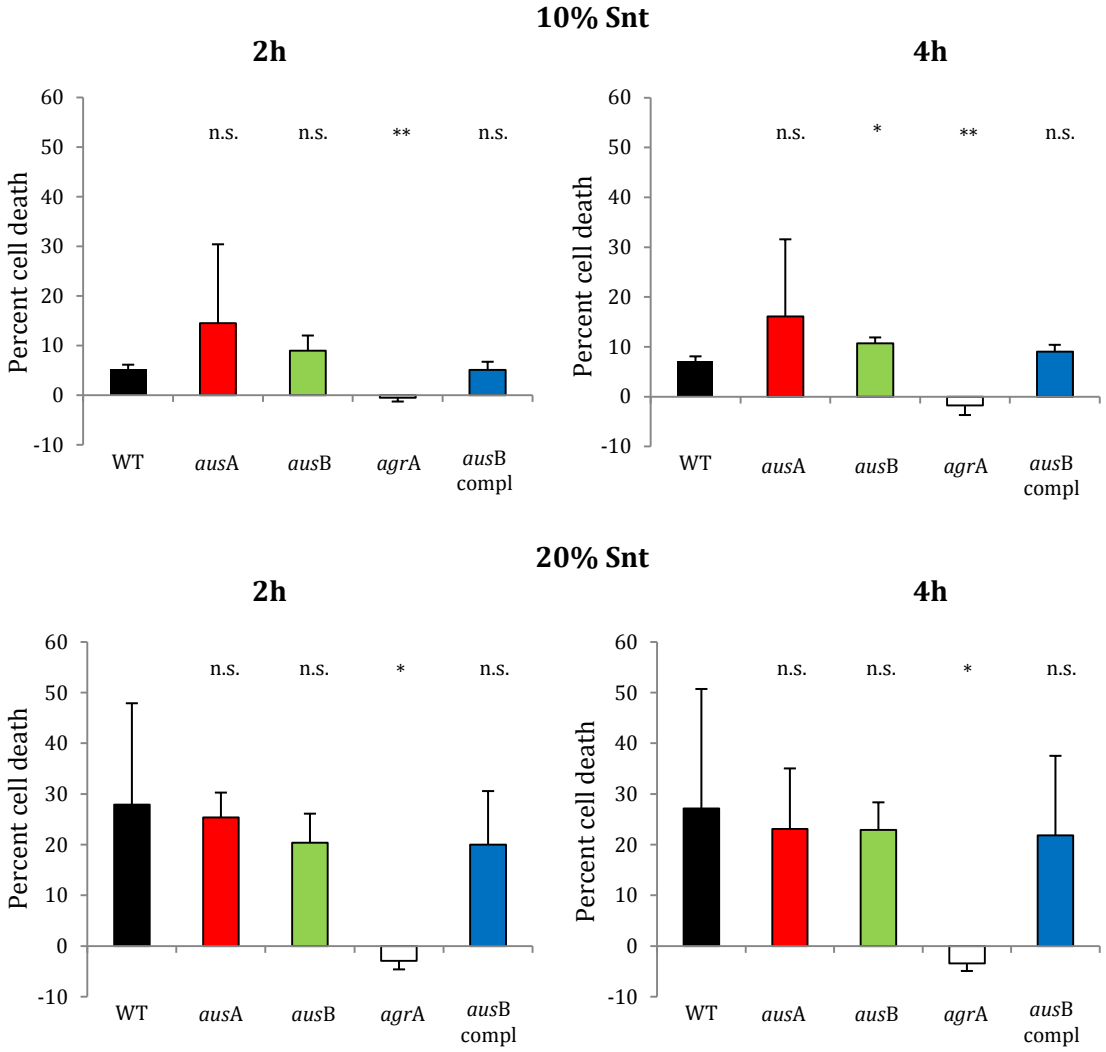

Figure 4.20: NRPS does not influence cytotoxicity of bacterial supernatants.

Host cell death caused by incubation with stationary phase bacterial culture supernatant was observed by measuring released LDH levels in cell culture medium after incubation of HeLa cells with bacterial culture supernatant collected after 16 h shaking at 37°C. LDH measurements were performed either 2 h (left panel) or 4 h (right panel) after addition of either 10% (upper panels) or 20% (lower panels) of bacterial supernatant. Bar graphs show the mean of at least three independent experiments ± SD. Statistical analysis was performed by t-test.

Supernatants were added to cell culture medium in final concentrations of 10% and 20%, respectively. After 2 and 4 hours post-infection host cell death was established by LDH assays (Fig. 4.20). Whereas the culture supernatant of the *agr*A mutant was not cytotoxic, all other tested supernatants demonstrated cytotoxicity against HeLa in the range of the wild type regardless of the investigated time point or supernatant concentration used for intoxication of HeLa. Only at 4 h and with 10% bacterial supernatant, the *aus*B mutant supernatant does cause significantly more cell death than the wild type (WT: 7.1 ± 1.0%; *aus*B: 10.7% ± 1.1% ; p= .016). Therefore these data clearly show that the absence of a functional NRPS/aureusimine production does not decrease cytotoxicity of *S. aureus* supernatants.

4.5 Addition of synthetic phevalin can restore and enhance phagosomal escape of *S. aureus*

As experimental data within the present study demonstrated an involvement of the non-ribosomal peptide synthetase AusAB in the intracellular virulence of *S. aureus*, I next questioned i) if one or more of the AusAB products directly influence phagosomal escape of *S. aureus* and ii) if complementation of the escape phenotype could be achieved by these compounds alone. As leuvalin is only a minor by-product of AusAB (Zimmermann and Fischbach, 2010) I focused on phevalin and tyrvalin.

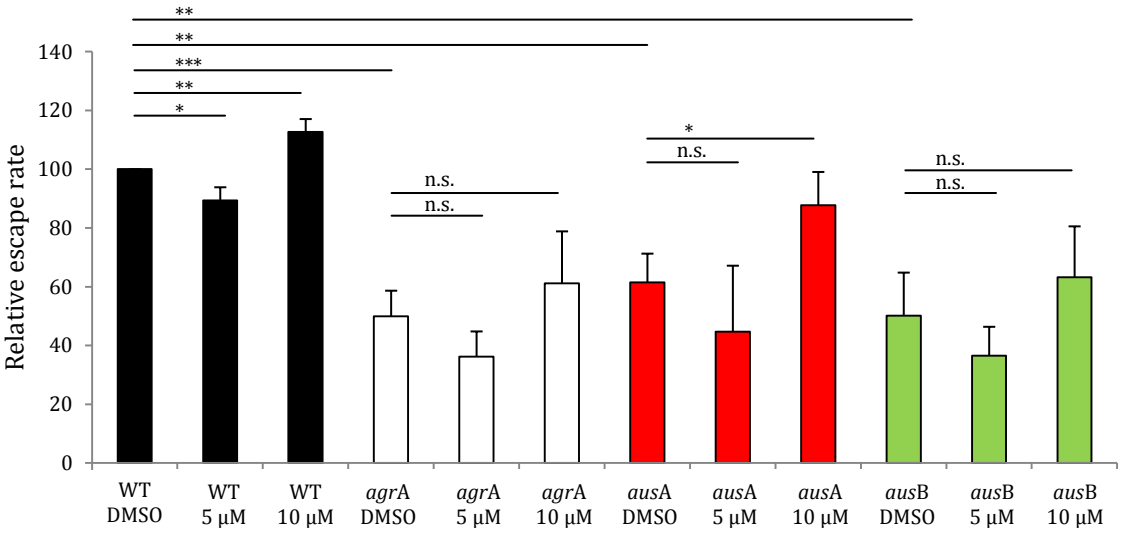

Figure 4.21: Synthetic phevalin influences phagosomal escape of *S. aureus* in a concentration dependent manner.

HeLa cells were infected with bacteria strains as described before. Prior to infection cells were incubated for 1 h with different concentrations of phevalin supplemented to the cell culture medium. Phevalin supplementation was maintained with every medium change until 4 h post infection, when cells were fixed and stored for microscopy. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

I first tested if complementation could be achieved by supplementing synthetic phevalin (CAS 170713-71-0, Aureusimine B, Santa Cruz Biotechnology, Inc.) in concentrations of 5 µM and 10 µM to the infection medium. The results in Figure 4.21 show, that the phagosomal escape phenotype could be restored to a large degree in the *aus*A mutant by addition of 10 µM phevalin (61.4 ± 9.9% in *aus*A DMSO increase to 87.7 ± 11.4% in *aus*A 10 µM phevalin; p= .039). While the effect of phevalin was largest with the *aus*A mutant, an increase could also be observed in every other strain tested with phevalin supplemented at 10 µM (100 ± 0.0% vs. 107.9 ± 4.4% in WT, 49.9 ± 8.7% vs. 61.2 ± 17.7% in *agr*A and 50.1 ± 14.7% vs. 63.3 ± 17.4% in *aus*B). In contrast, when only 5 µM of phevalin were added to the infection medium, relative phagosomal escape

rates rather decreased when compared to the control WT. The degree of reduction of phagosomal escape after treatment with 5 µM phevalin was observed in every strain tested (100 ± 0.0% vs. 89.3 ± 4.5% in WT, 49.9 ± 8.7% vs. 36.2 ± 8.6% in *agr*A, 61.4 ± 9.9% vs. 44.7 ± 22.4% in *aus*A and 50.1 ± 14.7% vs. 36.6% in *aus*B).

I repeated the experiments with tyrvalin, which was kindly provided by Michael Fischbach (UCSF).

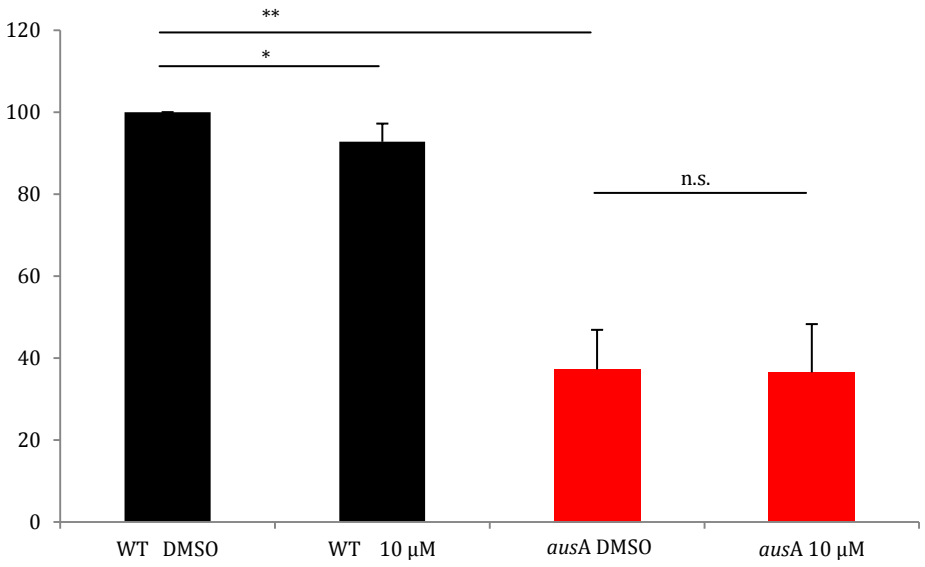

Figure 4.22: Tyrvalin does not complement phagosomal escape.
HeLa cells were infected with *S. aureus* and tyrvalin was supplemented in the infection medium at the time of the infection and throughout the entire experiment at different concentrations to evaluate the concentration necessary to accomplish a restored escape phenotype. Bar graphs with error bars show the mean of three or four independent experiments ± SD, while bar graphs without error bars indicate n=1 with this specific concentration. Statistical analysis was performed by t-test.

While several different concentrations were tested, none provided a significant increase of the escape rate when compared to the DMSO control (Fig. 4.22) in the *aus*A mutant strain tested. As a positive effect on phagosomal escape was only visible when using phevalin (aureusimine B), all following experiments focused on this dipeptide aureusimine B.

4.5.1 Phevalin associates with host cells when added extracellularly

Since phevalin did not alter cytotoxicity of bacterial supernatants but was able to enhance phagosomal escape of intracellular *S. aureus*, I established in cooperation with Markus Krischke

(chair of pharmaceutical biology) by UPLC-MS, if phevalin would enter the host cell after extracellular addition.

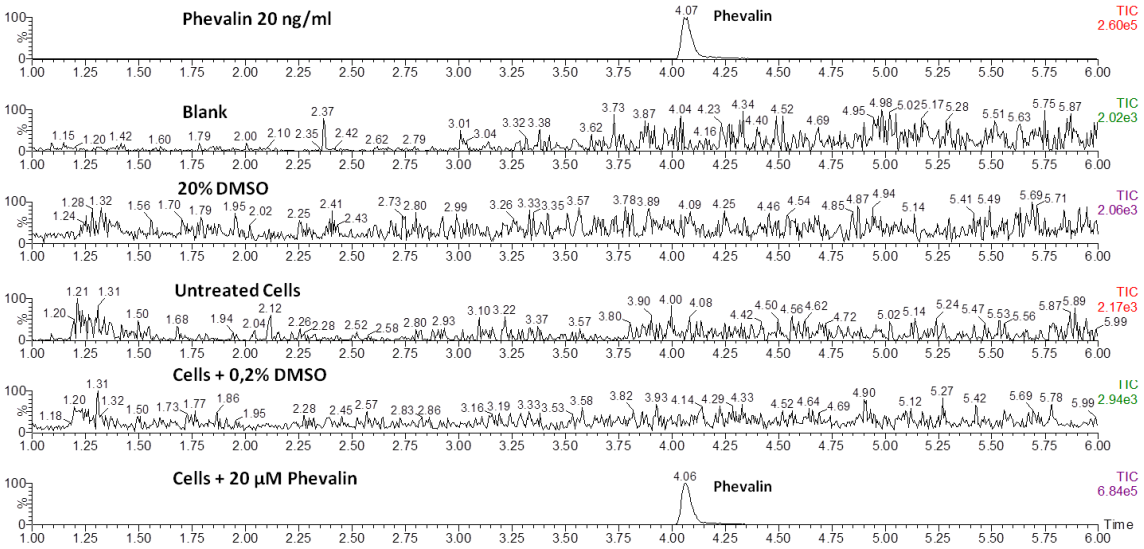

Figure 4.23: Phevalin associates with host cells.

HeLa cells were either left untreated, were treated with either DMSO as solvent control or with 20 µM phevalin for 1 h. Samples were analyzed by UPLC-MS using a C18 column with subsequent detection of small molecules by a Waters Mass spectrometer. Histograms depict the retention time from 1 min to 6 min (x-axis) and the relative intensity (y-axis). Exact numbers for the relative intensity in each measurement are shown on the right. Detection occurred via multiple reaction monitoring (MRM) and three specific fragments were monitored for phevalin (m/z 229 > 214, m/z 229 > 159, m/z 229 > 81)

These data show, that phevalin was readily detectable in extracted host cells after thorough washes, thereby indicating that extracellularly added phevalin can associate with or enter host cells.

4.5.2 Phevalin does not promote the phagosomal release of fixation-killed bacteria

Since several substances have been shown to decrease stability of (phago-) lysosomes (Gonzalez *et al.*, 2012), I questioned, if phevalin alone was sufficient to weaken or disrupt phagosomes in host cells. I therefore fixed *S. aureus* with PFA and infected HeLa with this fixation-killed bacteria. Samples were treated either with DMSO solvent control or several concentrations of phevalin. 3 h post infection, samples were fixed and analyzed.

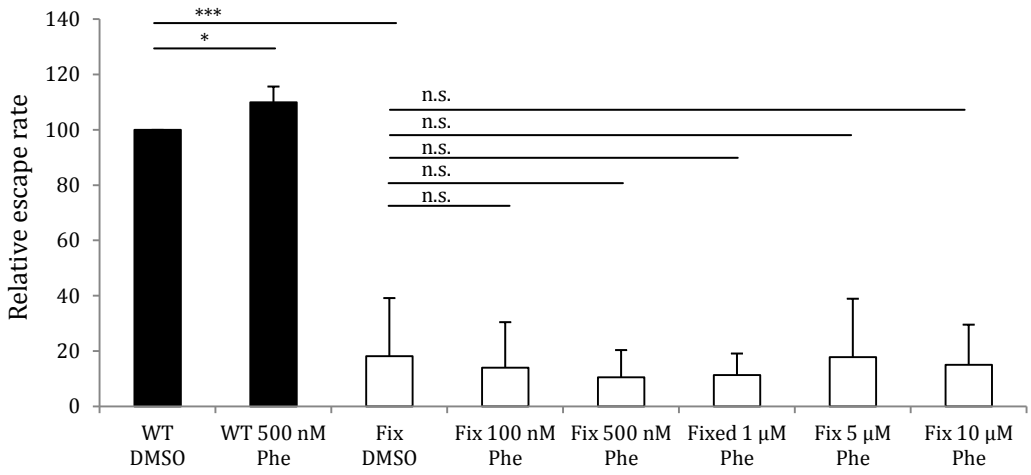

Figure 4.24: Phevalin does not promote the phagosomal release of fixation-killed bacteria.
S. aureus JE2 wild type bacteria were fixed in 4% paraformaldehyde over night before infecting cells with fixed or live bacteria the following day as described before. Phevalin supplementation was maintained at different concentrations from the time of infection throughout the complete infection. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

Although a number of different concentrations of phevalin were tested, no significant disruption of phagosomes was detected in host cells infected with paraformaldehyde-fixed bacteria (Fig. 4.24). Again only when samples with live wild type bacteria were supplemented with phevalin, an increase in phagosomal escape was observed and statistically relevant (109.9 ± 5.8% with 500 nM phevalin vs. 100% in DMSO control; p= .041). These data illustrated that live bacteria were required to mediate phagosomal escape and that the increase in phevalin-treated cells did not result from a general effect on phagosomal membrane integrity.

4.5.3 Phevalin does not act as a classical calpain inhibitor

Phevalin, was first described in *Streptomyces sp*. (Alvarez *et al.*, 1995). In an experiment using calpain inhibitors I therefore investigated, whether phevalin is involved in the process of phagosomal escape by modulating calpain activity.

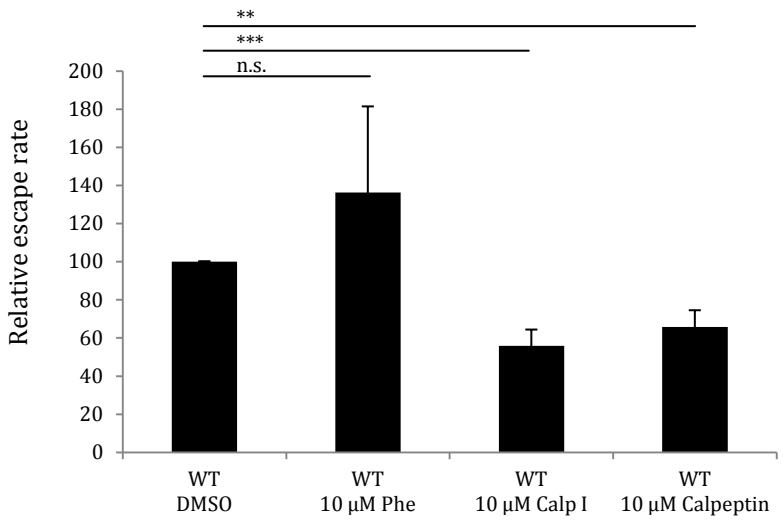

Figure 4.25: Calpains are involved in *S. aureus* phagosomal escape but phevalin modulates escape rates not by calpain inhibition.

HeLa cells were infected with *S. aureus* as described. 1 h prior and throughout the infection, cell infection medium was supplemented with 10 µM of phevalin or either of the two calpain inhibitors Calpain I inhibitor (Calp I) or Calpeptin. 4 h post infection, samples were fixed using 4% paraformaldehyde and stored until microscopy. Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

Supplementing the cell infection medium with the two calpain inhibitors Calpain I inhibitor (Calp I) and Calpeptin significantly decreased phagosomal escape rates in comparison to DMSO-treated controls (CalpI: 55.9 ± 8.5% p= .0008; Calpeptin: 65.7 ± 8.8%, p= .002) This is in stark contrast to the effect we see when using phevalin as a supplement, in this as well as previous experiments (chapter 4.5, figure 4.21).

4.5.4 Phevalin does not exhibit antimicrobial properties

NRPS produced peptides often have antibiotic properties (Felnagle *et al.*, 2008). One of the most recent examples of this product family is lugdunin, which is produced by *S. lugdunensis*. We therefore wanted to test, if phevalin also exhibits antibiotic properties.

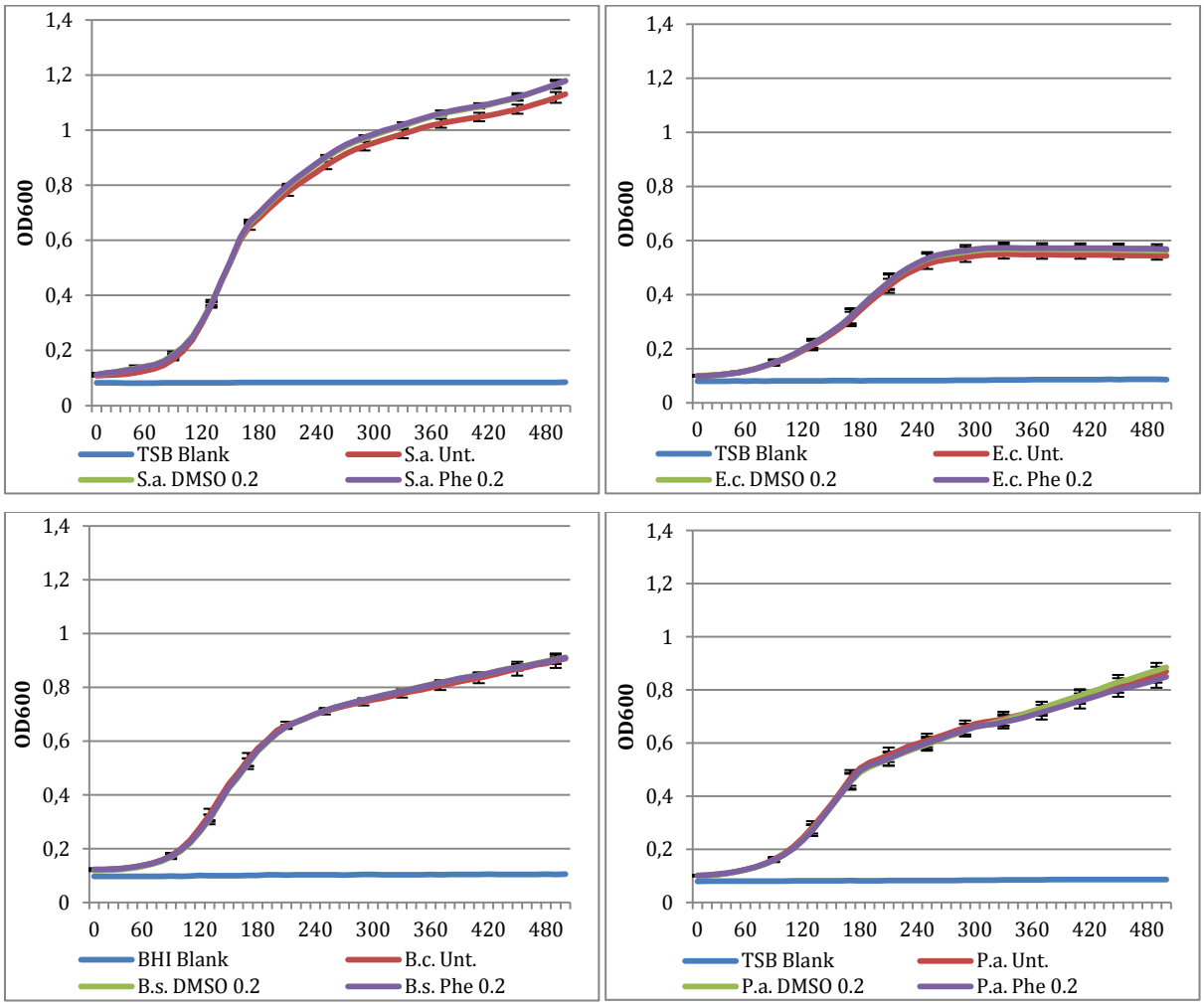

Figure 4.26: Phevalin has no antibiotic activity.

Bacteria strains were grown in microtiter well plates in appropriate medium (TSB for *S. aureus, P aeruginosa* and *E. coli*; BHI *B. subtilis*) at 37°C and 200 rpm shaking in an Infinite m200 plate scanner (Tecan). OD_{600} was measured every 10 min for a total of 520 min. 10 µM of phevalin or a control volume of DMSO were supplemented to the medium as the growing culture reached an OD_{600} of 0.2. Each data point represents the mean of three individual experiments. Representation of error bars was avoided in regards of improved visuality. Statistical analysis was performed using t-test.

To establish potential effects of phevalin on the growth of both, gram-positive as well as gram-negative bacteria, *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* were grown in microtiter plates in appropriate media with or without supplementation of phevalin at 37°C and 200 rpm and growth was recorded by measuring OD_{600} every 10 min in a Tecan Infinite Pro plate reader (Fig. 4.26). However, none of the tested bacteria showed growth impairment following the addition of phevalin, thereby excluding an antibiotic effect for the strains tested.

4.6 Aureusimine expression promotes survival and cytotoxicity in leukocytes

4.6.1 CFU in PMN are not influenced by aureusimine production

As I was able to show the non-ribosomal peptide synthetase AusAB and one of its product, phevalin, to be involved in phagosomal escape of the USA300 type *S. aureus* strain JE2 as well as host cell death following internalization of bacteria, we next wanted to investigate, if cell of the human immune system such as professional phagocytes, were also affected by phevalin in a manner which would benefit the survival of *S. aureus* inside the human host.

Polymorphonuclear neutrophils (PMN) represent the most abundant type of leukocyte in the mammalian body. As PMN comprise the first line of defense against invading pathogens, we initially focused on this cell type. We infected PMN with wild type *S. aureus*, the NRPS mutants within *aus*A and *aus*B as well as the complemented *aus*B mutant in order to assess staphylococcal virulence when faced with the human immune system with and without functional aureusimine synthesis.

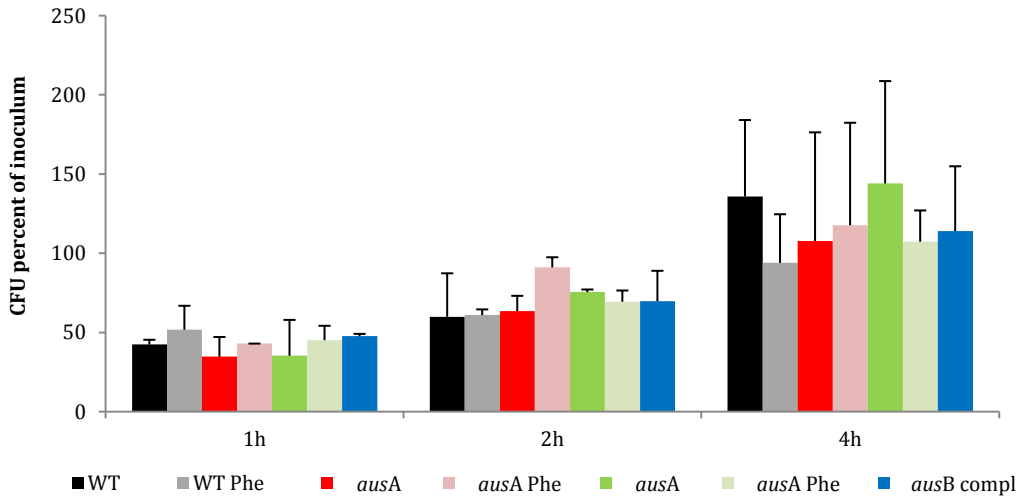

Figure 4.27: *S. aureus* replicates inside PMN independent of aureusimine production.
Primary human PMN were isolated from healthy donors. Cells were infected in PMN infection medium for 10 min and were incubated until the indicated time points. Bacterial CFU were determined on TSA plates following overnight incubation at 37C. Phevalin was added wherever indicated (Phe) at the time of infection at a concentration of 10 µM and was maintained throughout the complete experiment. Recovered CFU at each time point were normalized to bacteria used in inoculums (15 min time point). Bar graphs show the mean of three or four independent experiments ± SD. Statistical analysis was performed by t-test.

The analysis of the CFUs obtained from infected PMN at different time points shows no significant changes in bacterial numbers between the strains used for infection (Fig. 4.27). In all

strains, the recovered CFU drop 1 hour post infection when compared to the original inoculums (10 min time point) only to increase again at 2 hpi and at 4 hpi, where the wild type reaches 135.8 ± 48.3%, the *aus*A mutant 107.7 ± 68.6%, the *aus*B mutant 144.0 ± 64.6% and the *aus*B compl strain 114.0 ± 40.9%.

Supplementation of the samples with 10 µM synthetic phevalin appears to have not much influence on CFU development. While at 1 hpi, the detected CFU are slightly higher in the phevalin sample compared to the DMSO control (51.8 ± 15.1% for WT Phe vs. 42.4 ± 3.0% for WT DMSO; 43.0 ± 6.4% for *aus*A Phe vs. 34.7 ± 12.5% for *aus*A DMSO; 45.2 ± 9.1% for *aus*B Phe vs. 35.4 ± 22.6% for *aus*B DMSO), this effect is lost at 2 hpi and even seems reversed at 4 hpi, when two out of three phevalin-supplemented samples contain less CFU than the respective DMSO control samples (93.9 ± 30.7% for WT Phe vs. 135.8 ± 48.3% for WT DMSO; 117.7 ± 78.1% for *aus*A Phe vs. 107.8 ± 68.6% for *aus*A DMSO; 107.2 ± 19.8% for *aus*B Phe vs. 144.0 ± 64.7% for *aus*B DMSO).

4.6.2 Intracellular cytotoxicity in PMN is decreased in AusAB mutants

While PMN are capable of clearing pathogens effectively from the site of infection, cytotoxic *S. aureus* strains are able to kill PMN thereby interfering with the effectivity of neutrophil activity.

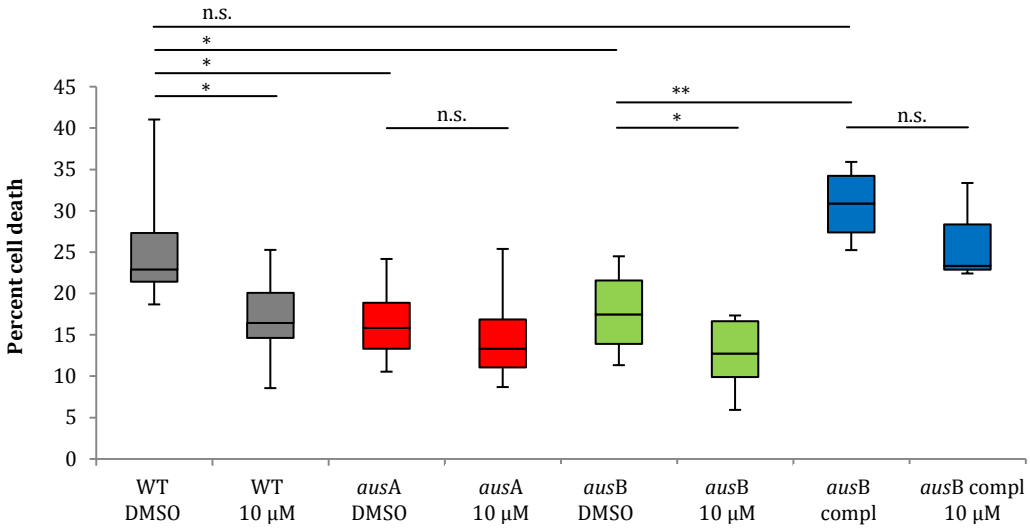

Figure 4.28: PMN cell death is reduced in *aus*AB mutants.

Primary human PMN from healthy donors were infected as before either with the addition of 10 µM phevalin or DMSO treated. Tissue culture supernatants were collected from wells 4 hours post infection to measure LDH levels released during the infection. The box-and-whisker-blots illustrate data points derived from multiple experiments (WT: n=8, *aus*A, *aus*B and *aus*B compl: n=4). 100% lysis was achieved by lysis buffer. Statistical analysis was performed by t-test. *p<0.05; **p<0.01; ***p<0.001.

The mean cell death determined for PMN infected with wild type *S. aureus* JE2 4 hours post infection is at 25.6 ± 7.3%, (median 22.9%). The complemented *aus*B mutant strain surpasses this considerably, while not statistically significant. Four hours after infection, the *aus*B compl strain caused on average 30.7 ± 4.9% cell death. Both NRPS mutants show significantly less cytotoxicity. While the *aus*A mutant kills 16.6 ± 4.9% (median: 15.8%) of host cells, the *aus*B mutant shows 17.7 ± 5.1% (median: 17.5%) cell death. Both are significantly lower than either WT (p= .011 for *aus*A and p= .025 for *aus*B) or complemented *aus*B strain (p= .00085 for *aus*A and p= .0018 for *aus*B).

We further added 10 µM phevalin to infection samples in order to test restoration of host cell death caused by AusAB mutant strains. Interestingely, supplementation with phevalin caused a significant reduction of the detected PMN cell death in the wild type and *aus*B strain, while this reduction was less distinct in the *aus*A and the complemented *aus*B strain (Fig. 4.28). In the WT samples, cell death decreased to a mean of 16.9 ± 5.1% (p= .015 compared to WT), with the *aus*B strain to 12.5 ± 4.3% (p= .045 compared to *aus*B). The decrease in cell death was found to be time independent, as it was also observed at 1 hpi and 2 hpi after phevalin supplementation (data not shown).

4.6.3 Phevalin reduces calcium fluxes in response to FPR-receptor stimuli in PMN

Since the data above suggested that phevalin had a positive effect on PMN survival during an infection with *S. aureus*, I hypothesized, that phevalin would not function in a cytotoxic manner against PMN, but would rather interfere with PMN function on a different level. In collaboration with Dorothee Kretschmer (University of Tübingen) I therefore assessed potential alterations of PMN chemotaxis in response to phevalin (Fig. 4.31).

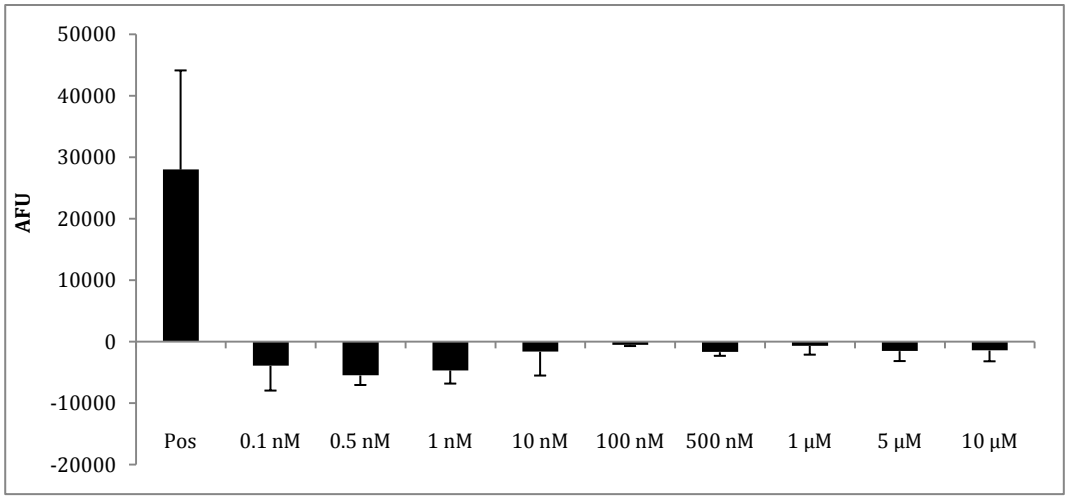

Figure 4.29: Phevalin exhibits no chemotaxis activating properties in PMN

Primary human PMN were isolated from healthy donors and were fluorescence labeled and then incubated in transwell migration assay plates with increasing concentrations of phevalin in the bottom wells. Plates were analyzed in a plate reader for FITC fluorescence. Bar graphs show the mean of three or two independent experiments ± SD. Statistical analysis was performed by t-test.

Primary human PMN were isolated from healthy donors, were fluorescence-labeled and then incubated in transwell migration assay plates with increasing concentrations of phevalin in the bottom wells. Plates were analyzed in a plate reader for FITC fluorescence and normalized to a negative control (no phevalin). Fig. 4.29 shows that none of the tested concentrations of phevalin resulted in PMN chemotaxis in the form of increased directed movement. In fact, with all concentrations a decrease in directed movement and therefore a decrease in FITC fluorescence in the migration well was detected.

As phevalin seemingly decreased movement of the leukocytes, we assessed calcium fluxes in PMN, as to evaluate if phevalin interfered with PMN activation.

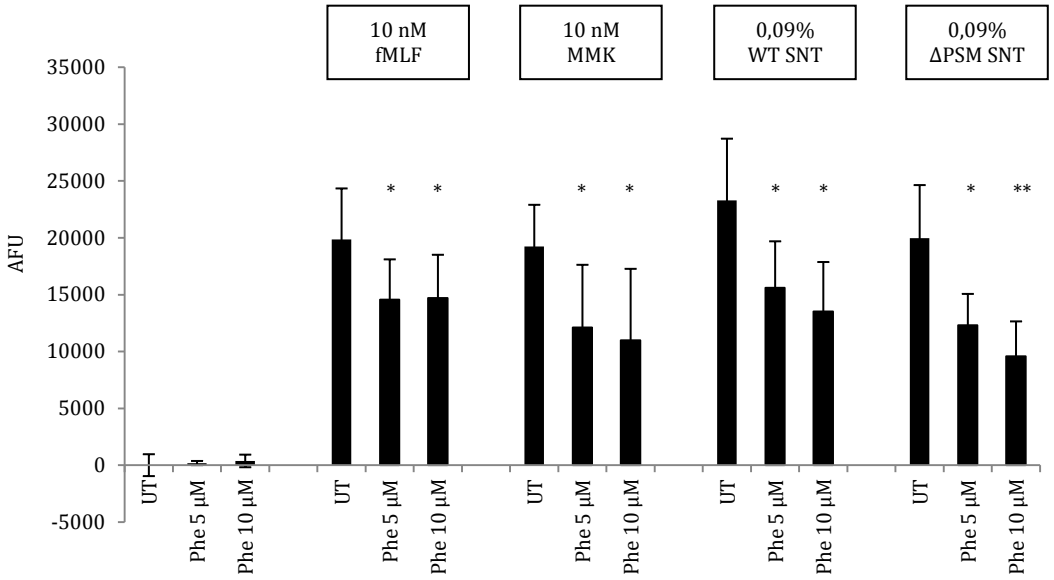

Figure 4.30: Phevalin supplementation interferes with PMN activation.

Primary human PMN were isolated from healthy donors and were incubated with concentrations of 5 µM and 10 µM of phevalin for 30 min and consequently stained using the calcium sensitive dye Fluo3-AM. Fluorescence intensities were measured using a FACSCalibur Flow cytometer (BD). Bar graphs show the mean of three or three independent experiments ± SD. Statistical analysis was performed by t-test.

hPMN were isolated from healthy donors, incubated with 5 µM and 10 µM phevalin for 30 min and were subsequently stained with the calcium-sensitive dye Fluo3-AM. Fluorescence intensities were measured by flow cytometry. When comparing DMSO-treated control to phevalin-treated cells, PMN calcium fluxes were diminished in a concentration-dependent manner (Fig. 4.30). The decrease was independent of the stimuli used for activation. Using the FPRI receptor stimulating (fMLP), we measured a reduction to 73.3 ± 17.8% AFU with 5 µM phevalin and 74.0 ± 19.1% AFU with 10 µM phevalin. An even stronger reduction could be seen with the FPRII stimulant MMK, where the reduction at 5 µM phevalin was to 63.0 ± 28.5% AFU and to 57.1 ± 32.6% AFU with 10 µM phevalin. We also tested stationary phase supernatant of *S. aureus* LAC WT and an isogenic LAC ΔPSM mutant. With wild type supernatant, we saw a reduction to 67.0 ± 17.4% AFU for 5 µM phevalin and to 58.1 ± 18.6% AFU for 10 µM phevalin. For the PSM mutant reductions were about equal to the wild type with 61.8 ± 13.7% AFU for 5 µM phevalin and 48.0 ± 15.3% AFU for 10 µM phevalin.

4.6.4 Survival and cytotoxicity of aureusimine mutants are diminished in macrophages

Second to PMN, macrophages respresent the most abundant cell type of the innate immune system in mammals. Macrophages effectively phagocytose and destroy invading bacteria, but *S. aureus* has been shown to interfere with macrophage action.

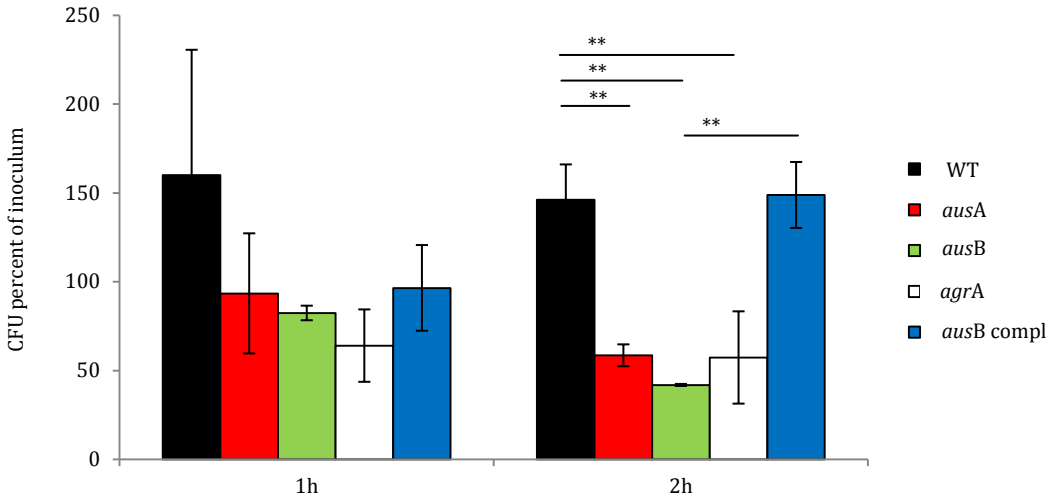

Figure 4.31: Aureusimine mutants show decreased survival in macrophages.
Human PBMC were isolated and differentiated as described in 3.2.4. Macrophages were then infected using an MOI of 5. At indicated time points, cells were lysed and CFU were plated on TSA plates supplemented with appropriate antibiotics and counted after 24 h incubation. Recovered CFU at each time point were normalized to bacteria used in inoculums (15 min time point). Bar graphs show the mean of three or three independent experiments ± SD. Statistical analysis was performed by t-test.

We therefore investigated, if failure to produce aureusimines would influence survival of *S. aureus* in macrophages following phagocytosis. Human primary monocytes (PBMC) were isolated from healthy donors and were differentiated into macrophages (chapter 3.2.4.) Macrophages were then infected with an MOI=5 of *S. aureus* and at several time points post-infection, the phagocytes were lysed and recovered bacterial CFU were plated on TSA plates. Contrasting the results obtained for PMN, aureusimine production proved to be an important factor for *S. aureus* survival (Fig. 4.31). CFU recovered 15 min post infection comprised the effective MOI and was set to 100% for normalization.

WT CFU experience a strong increase at the 1h time point to 160.0 ± 70.5%, which is not followed by another increase at 2 h (146.2 ± 19.8%). The increase at this point becomes statistically significant compared to both aureusimine mutants (p= .002% for *aus*A; p= .006% for *aus*B) and an *agr*A mutant (p= .009). Differences between *aus*B compl CFU and mutant strains

also meet significance 2 hpi (148.8 ± 18.6%; p= .001 for *aus*A; p= .004 for *aus*B and *p*= .007 for *agr*A), while an initial increase at 1 hpi is not detected. Instead of increasing over time, CFU for all three tested mutant strains decrease from 15 min to 1 h and again from 1 h to 2 h. CFU 2 hpi reach 58.5 ± 6.2% for the *aus*A mutant, 41.8 ± 0.6% for the *aus*B mutant and 57.3 ± 26.0% for the *agr*A mutant.

As we were able to detect differences in bacterial survival, we next planned to assess killing of macrophages by internalized bacteria.

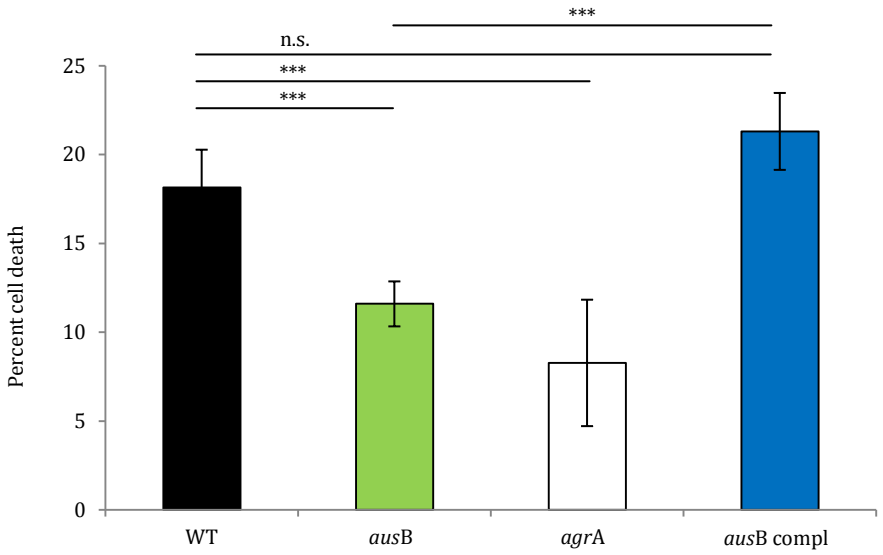

Figure 4.32: An *aus*B mutant causes less cell death in macrophages.

Human PBMC were isolated and differentiated as described before. Cells were infected with selected strains at a MOI of 20. LDH release was measured by aspirating cell culture infection medium at each time point.The shown data is derived from the 2 hpi time point. Bar graphs show the mean of four independent experiments ± SD. Statistical analysis was performed by t-test.

Two hours post infection with an MOI of 20, wild type *S. aureus* JE2 as well as the complemented *aus*B mutant (*aus*B compl.) were strongly cytotoxic as established with LDH assays (18.2 ± 2.1% and 20.3 ± 2.2% cell death, respectively), whereas the *aus*B mutant showed significantly reduced cell death (11.6 ± 1.3% ; *aus*B vs WT: p= .002; *aus*B vs. *aus*B compl.: p= .0002), which reached cell death levels of the *agr*A mutant (8.3 ± 3.6%; (*p*= .003 vs WT and *p*= .0008 vs *aus*B compl., respectively) (Fig. 4.32).

4.7 The role of *aus*AB in *in vivo* mouse infections

4.7.1 Mouse pulmonary lung infection model

Since the data showed dependence of *S. aureus* virulence on aureusimine production in professional as well as non-professional phagocytes in cell culture experiments, we tested, if aureusimines play pivotal roles in animal models of infection. As the previous results indicated phenotypes of the *aus* mutants in macrophages and PMN, we chose a lung infection model, where *S. aureus* encounters alveolar macrophages and infiltrating PMN.

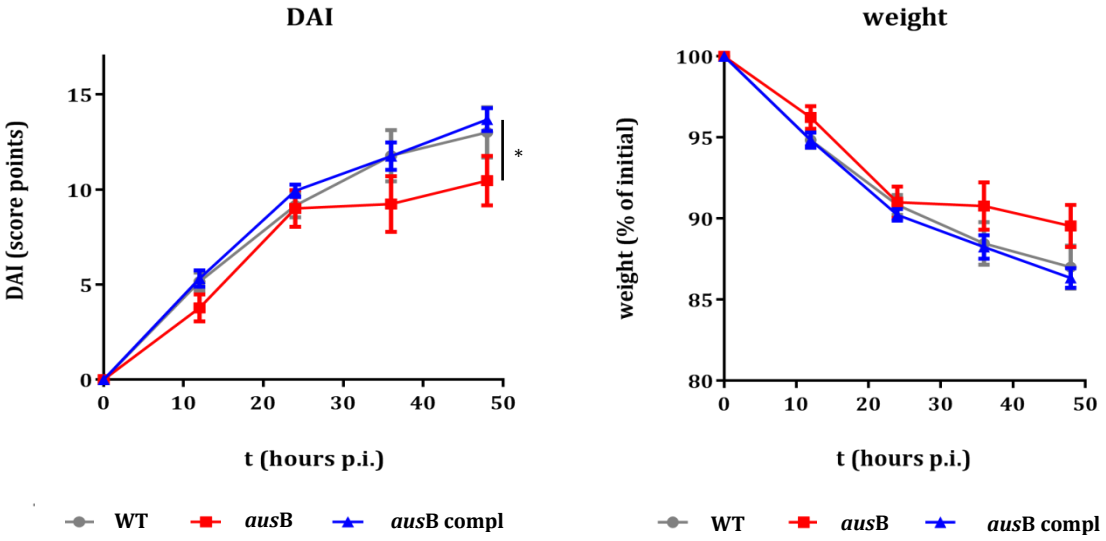

Figure 4.33: An *aus*B mutant shows attenuated virulence in a pneumonia lung infection model.
6 week old female Balb/c mice were infected intranasally with a sublethal bacterial load of 2 x 10^8 CFU using different strains (n=10). Animal weight and other signs of animal health were monitored every 12 hours over a period of 48 hours. Animal disease activity index (DAI) was scored according to ratings displayed in table 3.13.

Six week-old female Balb/c mice (n=10) were infected intranasally with a sublethal bacterial load of 2 x 10^8 CFU using different strains. Animal weight and other signs of animal health were monitored every 12 hours (Fig. 4.33). Disease severity (disease activity index, DAI) of *S. aureus*-infected mice and animal weight were recorded over a 48 h time period after infection with either wild type, complemented *aus*B strain or *aus*B mutant. Differences between the strains are minimal until 24 hpi, whereas the *aus*B mutant shows attenuated virulence at later time points which is indicated by a stabilization of animal weight until the 48 hpi end point and accordingly, a less steep increase in DAI. 48 hours post infection, mice infected with wild type bacteria or complemented *aus*B mutant reach a mean DAI of 13 and 13.7 respectively, and mean relative

weights of 87% and 86.3% of their initial weights. By contrast, at 48 hpi, DAI was decreased for the *aus*B mutant (DAI: 10.5; relative weight: 89.5%).

The data suggested collinearity between disease severity and time for the DAI of mice infected with wild type bacteria. This was confirmed by a linear model in R (r^2 =0.7016; F(4.60)=35.27, p= .864e-15) and significance was reached when DAI scores were analyzed as a function of infection group and time (F(6.188)=61.14, p< 2.2e-16). Mice infected with *aus*B mutants thus show significantly different development of DAI when compared to mice infected with wild type bacteria (p= .0213). No statistically significant difference was determined between wild type and complemented *aus*B mutant (*p*= .89)

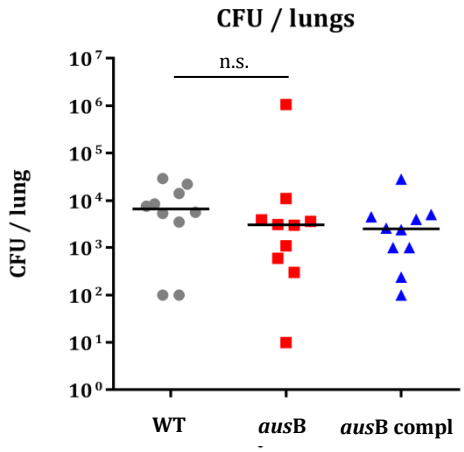

Figure 4.34: CFU recovered from lungs of infected mice 48 hours post infection do not differ significantly.
Lungs of infected mice were recovered 48 hours post infection. The removed tissue was then homogenized in 1x PBS and serially diluted before being plated on TSA plates (n=10). 24 h later, CFU were counted and the bacterial load per lung was determined. For statistical analysis, one-way ANOVA was used.

We further determined the bacterial load in the lung of infected mice as an indicator for bacterial survival in the host. Lungs of infected mice were recovered 48 hours post infection. The removed tissue was then homogenized in 1x PBS and serially diluted before being plated on TSA plates (n=10). 24 h later, CFU were counted and the bacterial load per lung was determined (Fig. 4.34). We found, that differences in recovered CFU are rather small. 48 hours post infection, a median of 6600 wild type CFU, 3500 *aus*B mutant CFU and 2500 *aus*B compl CFU could be found in the mouse lungs per g. Due to very high variations in CFU, expressed in the high standard

deviations of ± 9470 for wild type, 334282 for *aus*B and 8309 for *aus*B compl, no statistical significant differences could be found.

4.7.2 Mouse muscle abscess infection model

After observing differences in disease severity in a pneumonia lung infection model, we chose a second infection model, a muscle abscess model. Mice were injected in their left upper thigh muscle using a injection volume of 50 µl containing 2 x 10^8 bacteria. Infected muscle tissue of mice (n=10) was recovered 48 hours post infection, homogenized in 1x PBS, serially diluted and plated on TSA plates. 24 h later, CFU were counted and the bacterial load per sample was determined.

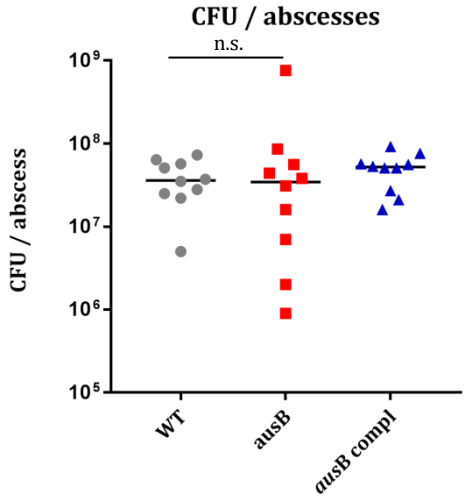

Figure 4.35: CFU recovered from removed muscle tissue of infected mice 48 hours post infection do not differ significantly

Infected muscle tissue of mice was recovered 48 hours post infection. The isolated tissue was then homogenized in 1x PBS and serially diluted before being plated on TSA plates (n=10). 24 h later, CFU were counted and the bacterial load per sample was determined. For statistical analysis, one-way ANOVA was used.

CFU results did not differ considerably between tested strains. Only the *aus*B compl strain showed increased CFU at a mean of 5.2 x 10^7 bacteria per recovered abscess. The numbers of recovered bacteria from wild type strain and *aus*B mutant are very similar at 3.6 x 10^7 and 3.4 x 10^7 CFU respectively. If corrected for outliers, the median of the *aus*B mutant still only decreases to 3.1 x 10^7 cfu and is therefore not significantly different to the wild type.

4.8 Towards identification of the host target of *S. aureus*-produced phevalin

In our experiments, we obtained data suggesting, that phevalin targets a host cell component independent of FPR, possibly on the level of host cell signaling (chapter 4.6.3). This influence also appears to be independent of calpains (chapter 4.5.3). In different studies, phevalin has been shown to upregulate the transcriptional regulator p63 in keratinocytes (Secor *et al.*, 2012). In collaboration with Dr. Falko Hochgräfe (University of Greifswald), we therefore established a proteome/phosphoproteome profile in order to identify signaling components and altered phosphosites upon phevalin treatment of HeLa cells.

We therefore next assessed changes in protein abundance in HeLa cells following phevalin treatment as well as changes in protein phosphorylation status. Phevalin was added to HeLa cells at a concentration of 10 μM and samples were harvested after 30 min and 3 h of incubation. Proteins found with confidence to be regulated in total protein abundance or by (de-) phosphorylation are shown in tables 7.2 to 7.6. In total, 36 proteins were found to be down-regulated in their abundance upon phevalin treatment (Table 7.2 and 7.3), whereas 19 were found to be up-regulated (Table 7.4 and 7.5). Also 86 proteins showed alterations of their phosphorylation (Table 7.6). To determine functional networks among the identified proteins, we used the protein network analysis tool STRING (www.string-db.org).

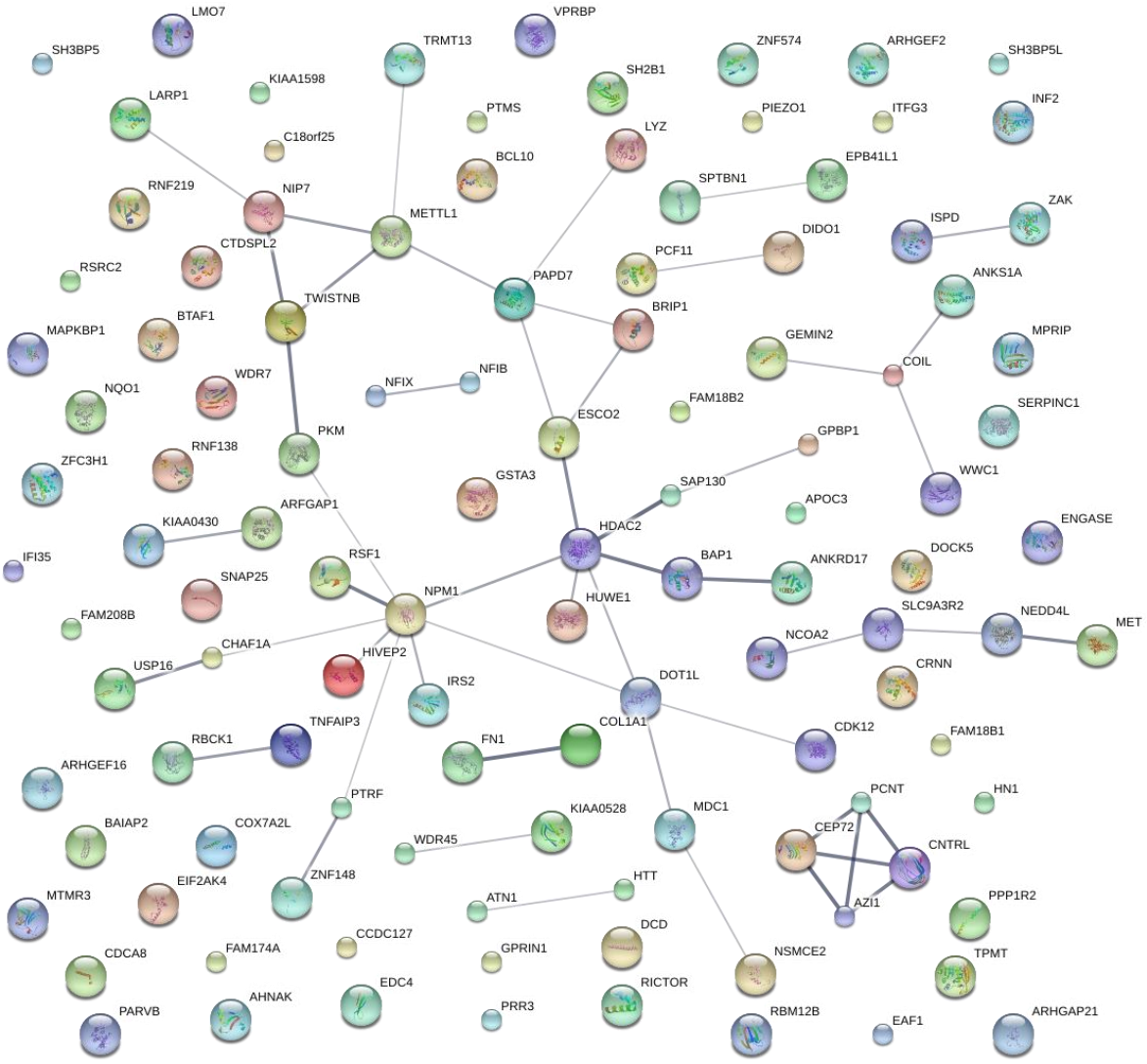

Figure 4.36: String DB analysis of proteins found down-regulated in total abundance and regulated via phosphorylation after phevalin treatment.

The 36 proteins found to be negatively regulated strongest during incubation with 10 µM of phevalin at 30 min or 3 h, as well as all proteins found regulated by phosphorylation or dephosphorylation under the influence of phevalin for 30 min or 3 h were all entered into the "multiple protein" search of STRING DB. Interactions between individual proteins are visualized via direct connections between nodes. The analysis of all 114 entered proteins revealed a strongly branched out network. A medium confidence of 0.4 was chosen as a minimal interaction score, while no shell interactors were permitted.

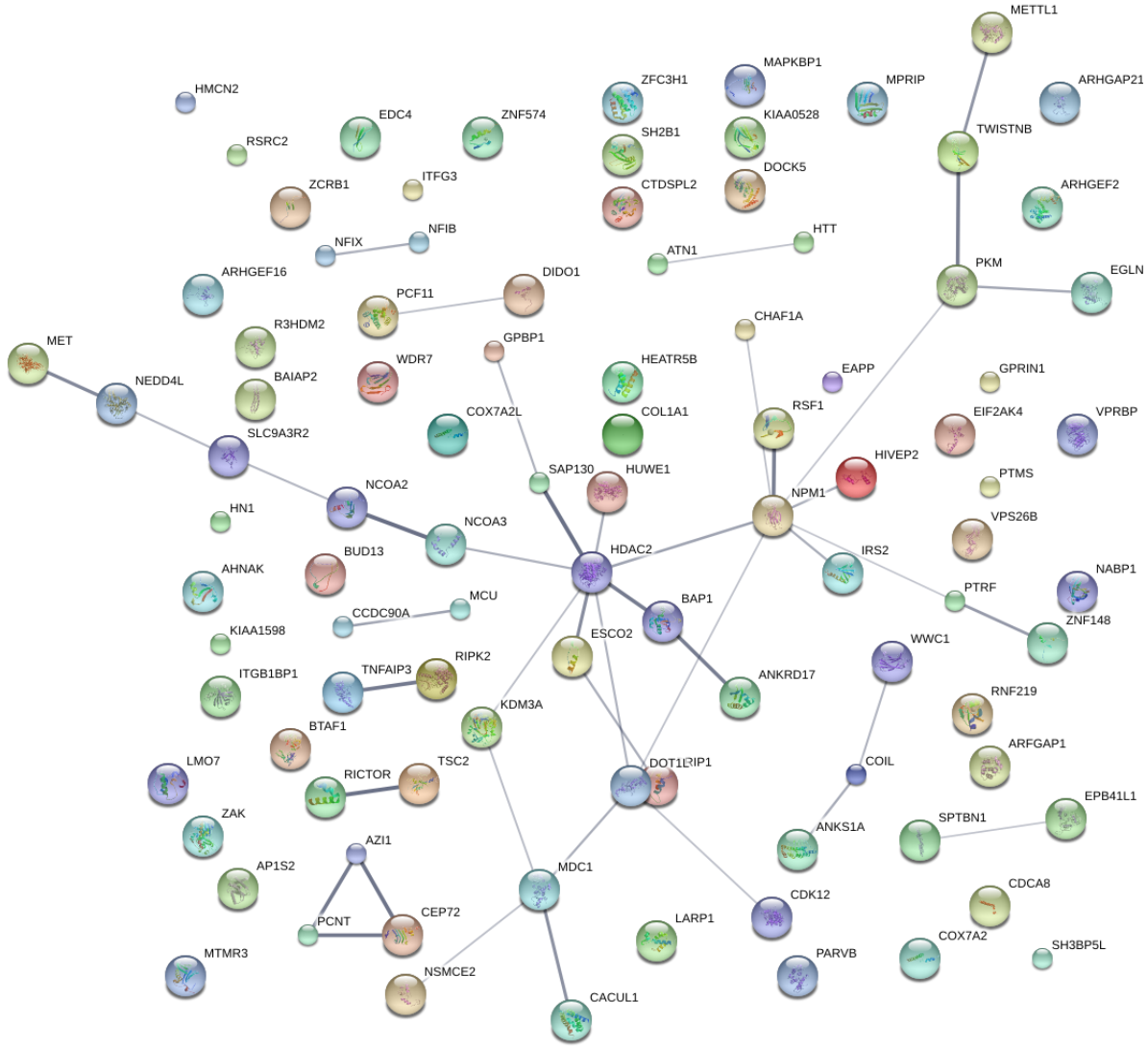

Figure 4.37: String DB analysis of proteins found up-regulated in total abundance and regulated via phosphorylation after phevalin treatment.

The 19 proteins found to be positively regulated strongest during incubation with 10 µM of phevalin at 30 min or 3 h, as well as all proteins found regulated by phosphorylation or dephosphorylation under the influence of phevalin for 30 min or 3 h were all entered into the "multiple protein" search of STRING DB. Interactions between individual proteins are visualized via direct connections between nodes. The analysis of all 98 entered proteins revealed a strongly branched out network. A medium confidence of 0.4 was chosen as a minimal interaction score, while no shell interactors were permitted.

When the proteins found to be regulated negatively in their abundance were analyzed in combination with all proteins found with differential phosphorylation status, multiple interactions between proteins and functional networks were found (Fig. 4.36). The network of 114 proteins contained 52 "edges", which significantly more interactions than statistically expected (35; p= .00367).

Although no functional enrichments in biological processes were detected by STRING, a number of proteins share functional similarities. Two of the genes downregulated strongest at both investigated time points are antimicrobial peptides. Dermicidin (DCD) is predominantly produced in sweat glands and is believed to interact with bacterial membrane phospholipids (Schittek *et al.*, 2001; Li *et al.*, 2009b) and Lysozyme (LYZ), a peptidoglycan hydrolase abundant in tears, saliva and other secretions (Fleming, 1922; Gill *et al.*, 2011). Also fibronectin (FN1) is reduced at the 30 min time point. The glycoprotein functions in host defense, coagulation and wound healing besides being involved in *S. aureus* adhesion and invasion. Other possible functional clusters include DNA double strand break repair (RNF138, PAPD7 and KIAA0430), NFκB regulation (BCL10, NQ01 and RBCK1) and vesicular transport (WDR45, INF2, CNRTL, TVP23C).

Interestingly, a peptide for isoform 2 of mitochondrial fission factor (MFF) shows a reduction of 44% after 3 hours of incubation (Otera et al., 2010). Since in the phosphoproteome data and among the positively regulated proteins more mitochondria associated proteins (COX7A2L, MCU, MCUR1) are found to be regulated, mitochondria morphology and physiology could be effected by phevalin.

For the positively regulated and (de-) phosphorylated proteins, when analyzed with STRING, also more interactions were detected (n=43) than would have been statistically expected (n=29, p=.00763) (Fig. 4.37). Several biological processes were found enriched in this dataset. Nucleic acid metabolic processes ((n=37 genes; 0.0154 false discovery rate (FDR)), organic cyclic compound metabolic processes (n=39; FDR= 0.0283) and positive regulation of cellular metabolic processes (n=26; FDR= 0.0411) are all identified as functional clusters.

STRING finds more interactions than statistically expected (34 vs. 21) when using a medium confidence score of 0.400. Among those genes, one group stands out. All of the proteins LMO7, RICTOR, ARHGEF2, DOCK5, ARHGEF16, ARHGAP21 and MPRIP are involved in the regulation of Rho GTPases or act as co-factors. RhoGTPases are a family of small GTPases whose members are involved in cell migration, cell division, membrane transport and vesicular transport. Possible other genes of interest which are related to this group are the AP152 protein, which is part of the clathrin Assembly Protein Complex, coats vesicles of the Golgi complex and is important in the recognition of sorting signals, as well as VPS2B, the vacuolar protein sorting homolog.

4.9 Mitochondria elongation caused by phevalin treatment

Among the proteins we demonstrated to be regulated following treatment of HeLa cells with phevalin, the occurrence of several mitochondria associated proteins raised our interest. After 3 hours of incubation, MFF levels were detected at only 56% in the phevalin treated sample compared to DMSO control. MFF is a mitochondria outer membrane associated protein, which can bind Dynamin-like protein 1 (Drp1) and recruits it to the mitochondria (Gandre-Babbe and van der Bliek, 2008; Otera *et al.*, 2010). There, Drp1 forms oligomeric rings around the mitochondria and effects mitochondria fission. Lower MFF levels would limit the recruitment of Drp1 and should therefore decrease the number of fission events. To test, if changes in mitochondria morphology occur upon phevalin treatment, we used HeLa229 cells expressing a soluble GFP targeted to the mitochondria using the matrix targeting presequence. Following 2 hours of incubation, we imaged live cells by confocal microscopy.

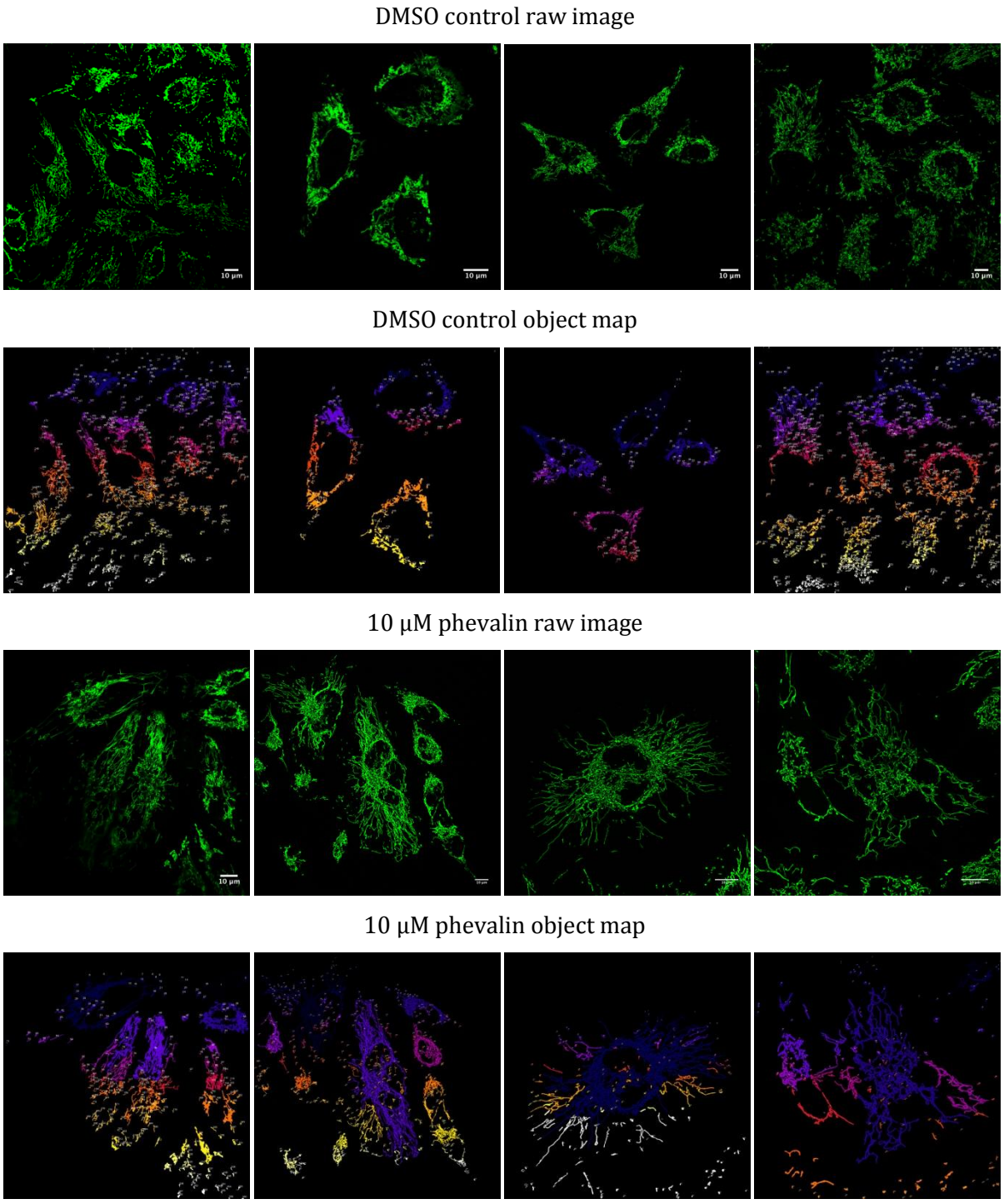

Figure 4.38: Confocal images and object maps of mitochondria morphology in HeLa229 cells demonstrate elongation of mitochondria by phevalin

HeLa229 cells were incubated with phevalin or DMSO for 2 h. Images were taken using a 63x oil immersion objective on a TCS SP5 microscope (Leica). Enumeration of mitochondria was performed via the object count plugin of the image analysis software FIJI. Per experiment 10 pictures, showing a total of 30-40 cells were taken. 3 biological replicates were performed.

Upon the treatment of HeLa229 cells with phevalin, mitochondria morphology changes visibly. Mitochondria appear elongated and more branched when compared to mitochondria in mock-treated/DMSO-treated cells (Fig. 4.38). Object maps of individual cells reinforce this observation and suggest fewer individual mitochondria per cell. To verify these observations, we used the object count plugin of the FIJI image analysis software and assessed both, fragment length and fragment number per individual cell.

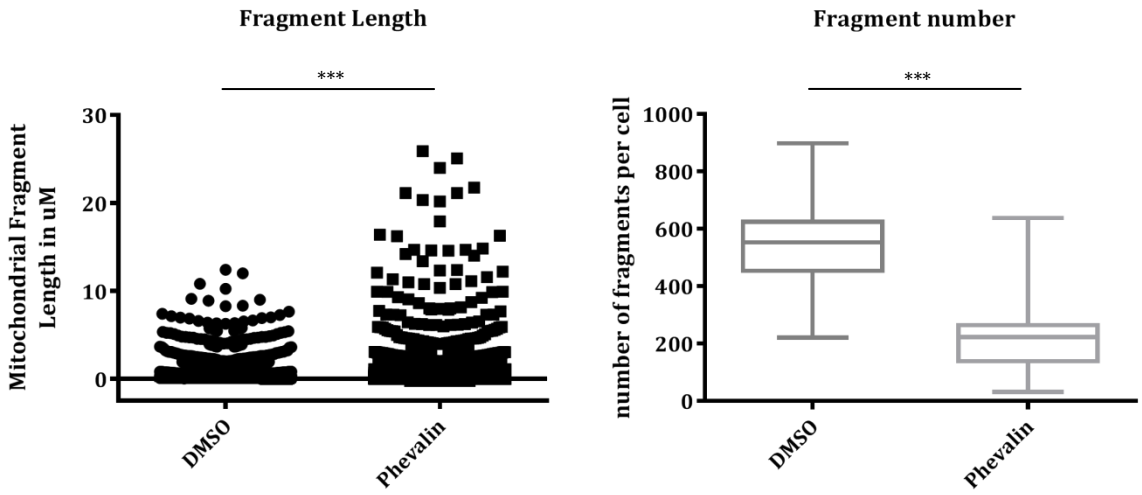

Figure 4.39: Mitochondria length and numbers are changed in phevalin treated cells

Quantification of mitochondria fragment length and count per cell was accomplished with a modified version of the "object count" plugin of the image analysis software FIJI. GFP fluorescence signals of mitochondria were translated to binary signals to enumerate continuous networks and individual fragments. Average lengths of mitochondria fragments were determined in microns. For both conditions, a total of 47 individual cells and 12718 mitochondria fragments were analyzed.

Phevalin treated cells show mitochondria fragments with considerably larger length than control cells (Fig. 4.39). We recorded higher maximal lengths at up to 25.9 µm in phevalin treated cells compared to 12.4 µm in control cells, as well as higher numbers of fragments with a length larger than 1 µm (598 in phevalin treated cells vs. 279 in control cells). Minimal length recorded for mitochondria fragments in the cells imaged differed by one degree of magnitude (0.00068 µm in control cells vs 0.0068 µm in phevalin treated cells). Median length of control mitochondria was 0.00735 µm and 0.0739 µm in treated cells. Using the Mann-Whitney test, we determined significant difference between both samples ($p < .0001$).

Significant differences could also be seen when using the Mann-Whitney test for the analysis of fragment per cell numbers. For the two columns with median fragment numbers of 552 in control cells and 223 fragments in treated cells, a p-value of $< .0001$ could be calculated.

4.9.1 Mitochondrial ATP production is increased in phevalin treated cells

Mitochondria play an essential part in the energy metabolism of the eukarotic cell. The complexes of the electron transport chain, located in the inner membrane of the mitochondrion produce the ATP needed by the cell to maintain its vital functions. When differences in the mitochondrial morphology appear, they will also affect the production of ATP. In elongated mitochondria an increase in cristae densitiy and a larger degree of ATPase oligomerization have been observed (Gomes *et al.*, 2011). To examine, if the morphological changes of mitochondria following phevalin treatment are accompanied by changes in the ATP production of the cell, we used a Seahorse Extracellular Flux (XF) analyzer. The Seahorse XF measures oxygen consumption rates (OCR) as well as extracellular acidification (ECAR) to assess mitochondrial respiration and glycolysis. HeLa cells incubated in medium supplemented with phevalin or mock-treated medium were treated successively with Oligomycin, FCCP and Antimycin A.

The kinetics plot (Figure 4.40) of the oxygen consumption rate per minute shows, how the oxygen consumption rate (OCR) differs from control cells due to treatment with different concentrations of phevalin. OCR functions as an indicator of electron transport chain activity and ATP synthesis. The curve reaches higher consumption rates for 5 µM and 10 µM phevalin. With addition of 20 µM phevalin, the consumption rate drops in comparison to the other concentrations used, but not to control levels. OCR levels allow conclusions of key mitochondria physiology aspects as basal respiratory level, spare respiratory capacity, proton leak and ATP production. The results show, that basal respiration, spare respiratory capacity as well as ATP production all increase in HeLa upon phevalin pre-treatment, while proton leakage remains at control levels. Since this prototype experiment was only conducted once, additional biological replicates will allow more conclusive interpretation of physiological changes of host cell mitochondria upon phevalin addition (see also 5.9/Outlook).

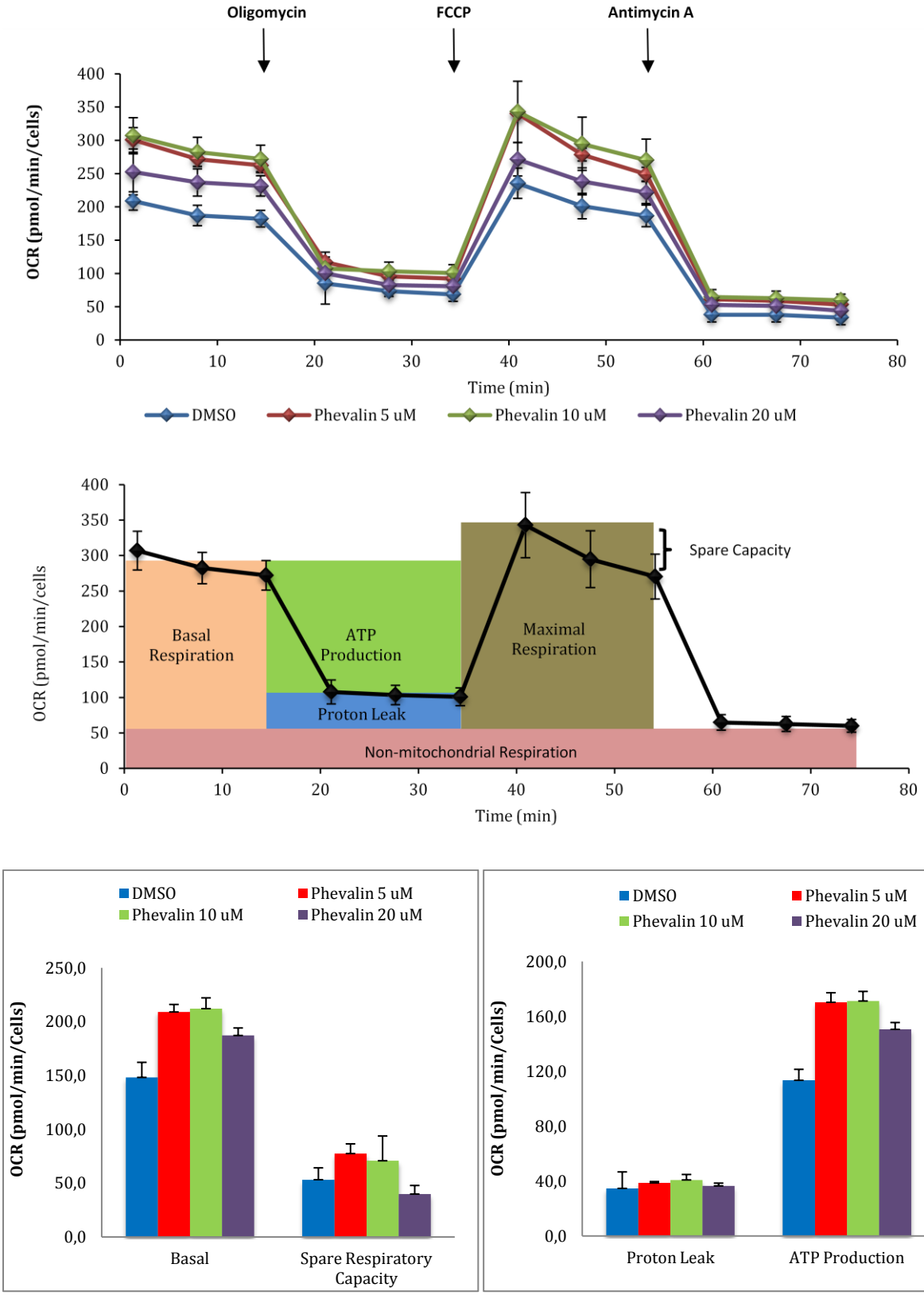

Figure 4.40: Respiratory capacity and ATP production increase in phevalin treated cells

To analyze the effect of phevalin on different parameter of mitochondrial respiration, cells were first treated with different concentrations of phevalin and then subjected to the electron transport chain manipulating drugs Oligomycin, CCCP and Antimycin A. Following measurements, results were normalized for cell numbers per well by crystal violet staining. Y-axis shows oxygen consumption rate per minute by cells. Experiment was performed once.

5. Discussion

5.1 *S. aureus* transposon mutant screen reveals several gene products to be involved in phagosomal escape

S. aureus escapes from phago-endosomes of epithelial cells using a hitherto only incompletely understood set of virulence factors. Whereas the requirement of *agr* and the *agr*-dependently produced phenol-soluble modulins have been shown to be important for escape, PSMs are not sufficient. In order to identify additional bacterial factors, I sought to use genome-wide transposon mutant libraries in a FACS-based escape screen (chapter 3.2.5). Since *S. aureus* of the genotype USA300 have been shown to escape efficiently from endosomes of 293T and HeLa cells (Grosz *et al.*, 2014) my initial experimental approach focused on the generation of a transposon insertion mutant library within *S. aureus* LAC, a highly virulent USA300-type MRSA strain. In recent years research groups have published studies using transposon mutant library pool screens that contained a large number of unique transposon insertion sites (TIS) and by interrogating distribution of TIS before and after a selective procedure with next-generation sequencing (NGS) techniques addressed questions regarding *S. aureus* virulence as well as general fitness and the identification of essential genes (Bae *et al.*, 2004; Li *et al.*, 2009b; Fey *et al.*, 2013; Santiago *et al.*, 2015). In the present study, attempts to generate a high-density transposon library in *S. aureus* LAC were based on using the pBTn plasmid (Li *et al.*, 2009b).

After having followed procedures of transposon mutagenesis that was successfully used in the *S. aureus* strain 6850 (Das *et al.*, 2016) my attempts of implementing this method in the strain USA300 LAC* did not yield the desired high-density transposon mutant libraries. Both generated libraries harbored only 3123 (library D) and 1471 (library E) individual TIS respectively (chapter 4.1). This was far less than the numbers of TIS reported in other studies and less than what could already be achieved in our lab in the strain 6850 (Bae *et al.*, 2004; Li *et al.*, 2009b; Fey *et al.*, 2013; Santiago *et al.*, 2015; Das *et al.*, 2016). Numbers of genes completely without detected TIS (1541 in library D and 2015 in library E) far surpassed the postulated 550 essential genes in *S. aureus* for which a lack of TIS could be readily explained (Fey *et al.*, 2013). The strain LAC*, while missing one of the three plasmids of the parental epidemic isolate LAC, still retains the plasmids pUSA01 and pUSA02. pUSA01 is a cryptic plasmid of unknown function while pUSA02 harbors a resistance cassette against tetracycline (Diep *et al.*, 2006b). Our analysis of the sequence data showed us that many of the reads originated from transposons situated in one of the two plasmids instead of the core genome. Due to the low TIS density of the libraries D

and E, both would not be sufficient for genome-wide identification of virulence factors in future experiments.

I therefore decided to use the established and sequence characterized mutant library made within *S. aureus* JE2 (Fey *et al.*, 2013), a descendant strain of *S. aureus* LAC which has been cured of its plasmids and for which each of the transposon insertions is known. The assessment of phagosomal escape rates by different invasive *Staphylococcus aureus* strains in our lab has been addressed using two different methods in the past. Changes in the pH of the surrounding areas of internalized bacteria can be readily determined using flow cytometry (Lam *et al.*, 2010; Grosz *et al.*, 2014) and can be used to judge if the bacteria did escape from the acidified phagolysosome to the neutral cytoplasm. The second method is based on a host cell line expressing a fluorescent recruitment marker which readily binds to escaped *S. aureus* but does stay evenly distributed in the cell without the occurrence of an escape event (Giese *et al.*, 2009; Giese *et al.*, 2011; Grosz *et al.*, 2014). Such samples can be imaged by fluorescence microscopy and analyzed subsequently by ImageJ.

As it was my goal to test single gene mutants on a medium to large scale, both of these methods in their current state were no optimal solutions. The generation of gene knockouts using established methods like homologous recombination was not feasible in order to screen a large number of mutant strains either. Other methods like the generation of knock-outs using the dCAS-9 system (Mojica *et al.*, 2000; Doudna and Charpentier, 2014) are not yet applicable in *S. aureus* due to low transfection efficiencies. In order to increase the number of strains to be tested I decided to collaborate with the core unit “Functional genomics” in using its high-throughput microscopy and image analysis technology. Compared to other rechniques, such as electron microscopy (Bayles *et al.*, 1998; Qazi *et al.*, 2001; Jarry and Cheung, 2006) as well as the initial technology using the recruitment marker (Giese *et al.*, 2011; Grosz *et al.*, 2014) the new setup enabled us to increase the speed of the analysis considerably by parallezation of much more samples than before. Hence in the present work I could screen 140 different mutants in global regulators such as *agr*, *sae* and *rot*, toxins such as α-toxin and leukocidins, as well as additional miscellaneous protein coding genes for their contribution to phagosomal escape.

The results obtained during the performed screen point to a high reliability of this assay in identifying genes important for phagosomal escape. Most of the mutant strains tested retained phagosomal escape rates with less than 20% difference to the JE2 wild type, which was arbitrarily chosen, showing that false positive hits due to variability of the obtained results are rather unlikely. Genes whose disruption did not convey an escape phenotype include α-hemolysin (*hla*) and the recently identified virulence regulator *rsp,* the function of which had been shown to be required for α-toxin production (Das *et al.*, 2016). Thus my data support that α-toxin is not important for phagosomal escape of *S. aureus* in non-professional phagocytes

(Jarry *et al.*, 2008; Giese *et al.*, 2009; Lam *et al.*, 2010), although α-toxin activity in a cystic fibrosis cell line has been documented (Jarry and Cheung, 2006). Further, genes already known to be involved in phagosomal escape of *S. aureus* like the *agr* quorum sensing system (Qazi *et al.*, 2001; Shompole *et al.*, 2003; Giese *et al.*, 2011; Grosz *et al.*, 2014) and subunits of the PSM transporter protein complex Pmt (Chatterjee *et al.*, 2013) showed strongly reduced phagosomal escape. Noticeably, none of the mutants tested showed a complete lack of detectable YFP signals. The automatic detection of YFP fluorescence by microscopy tends to produce a number of false positive escape signals in each sample. Non-bacteria related accumulations of YFP fluorescence in the cell will be counted as escape. False positive signals could be decreased by changes in the detection algorithm. I tried adjusting the thresholds but found, that a threshold of 100 gives the most consistent results despite the always obtaining a low number of false positives. A higher threshold would also only eliminate a percentage of false signals while reducing recognition of actual escape signals.

Interestingly, only one gene tested in the assay can be marked with some confidence as a factor positively influencing the phagosomal escape of *S. aureus* (p= .070). A *luk*S-PV mutant showed reliably more escape than the wild type (Figure 4.5 c). LukS-PV is one part of the dimeric pore forming leukotoxin LukSF-PV, also called the Panton-Valentine leukocidin (Valentine and Butler, 1940). The role of LukSF-PV in *S. aureus* virulence is disputed to some degree and especially in neucrotizing pneumoniae, as many studies showing effects of LukSF-PV expression base their observation on murine animal models (Voyich *et al.*, 2006; Brown *et al.*, 2009; Yoong and Pier, 2010). Mouse leukocytes however are not lysed by PVL nearly as effectively as human leukocytes are, which might make murine infection models unreliable, even though immune-activating properties of PVL in mice are still present (Loffler *et al.*, 2010; Yoong and Pier, 2012; Alonzo and Torres, 2014). Still, the work performed in other labs, showing that a lack of PVL in mice enhances virulence in a pneumonia model, the same model we chose to investigate the escape factor AusAB, points to the possibility that a lack of PVL can increase phagosomal escape rates in *S. aureus* (Bubeck Wardenburg *et al.*, 2007a). Different leukotoxins have been shown to inhibit each other, for example, LukED is the inhibition target of PVL (Yoong and Torres, 2015). Therefore it is possible that LukS may enhance escape by inhibiting LukA, which I have shown to contribute to escape (Chapter 4.2/Table 4.1/Fig. 4.6). The determination of the exact mechanism by which leukocidins modulate phagosomal escape will require additional work. As of yet, leukocidins were not known to be involved in the phagosomal escape of *S. aureus*.

As the alternative sigma factor B (σB) is one of the crucial transcriptional regulators in the adaptation of *S. aureus* to intracellularity, the fact that *rsb*U and *rsb*W both appear to influence phagosomal escape is reasonable. Both genes take part in the complex control of σB

activity, though in opposite capacities. While *rsb*W functions as an anti-sigma factor, blocking σB, *rsb*U indirectly mediates the release of *rsb*W from σB and therefore functions as an anti-anti-sigma factor (Fig. 2.4) (Pane-Farre *et al.*, 2009). An active σB represses the *agr* quorum sensing system and therefore toxin expression (Bischoff *et al.*, 2001). If σB regulation is imparted and σB activity is changed from the unstressed state, *agr* controlled virulence factors as the PSMs will also be affected and therefore the capability of *S. aureus* to escape the phagosome. Further a mutation in σB did not decrease escape rates (NE1109: 98.3% ± 15.0%). Lesser amounts of σB results in less *agr* control and retained escape rates. Yet, this does not explain why the *rsb*U mutant would cause less escape, since decreased amounts of active RsbU should result in less active σB. The *rsb*U ORF does lie upstream of *rsb*W so that a Tn insertion in *rsb*U could affect *rsb*W transcription as well, but the promoter for *rsb*W has been reported to be situated downstream of *rsb*U (see Fig. 2.4) (Pane-Farre *et al.*, 2009). An explanation for the role of *rsb*U would therefore require additional experiments.

Another gene, the disruption of which did decrease escape rates, was *sps*A, a gene encoding one of two signal peptidases present in *S. aureus* (Cregg *et al.*, 1996). SpsA is highly conserved among *S. aureus* strains but is of unknown function, as it is missing catalytic serine and lysine residues and no peptidase activity was detected (Cregg *et al.*, 1996; Sibbald *et al.*, 2006; Kavanaugh *et al.*, 2007). The *sps*A ORF lies upstream of the *sps*B, which has been shown to mediate the release of the processed AIP from AgrB and of other secreted peptides (Kavanaugh *et al.*, 2007). A lack of AIP release inhibits the *agr* quorum sensing system and therefore toxin expression (Novick *et al.*, 1993). While *sps*B is an essential gene in *S. aureus*, this has not been shown for *sps*A (Cregg *et al.*, 1996). A future project might elucidate the function of *sps*A and its contribution to phagosomal escape of *S. aureus*.

Additionally, I identified two subunits of the nonribosomal peptide synthetase (NRPS) AusAB with effects on phagosomal escape of *S. aureus*. Mutants in both genes in the *aus*AB operon showed significant reductions in phagosomal escape rates (Table 4.1/Fig. 4.6). Although the proteins are highly conserved among *S. aureus* strains and other *Staphylococcus* species like *S. argenteus, S. capitis, S. lugdunensis*, *S. xylosus* and *S. epidermidis* (Zimmermann and Fischbach, 2010), no specific virulence phenotype had been attributed to the NRPS. AusAB expression is increased upon *agr* activation, which might point towards an involvement in virulence (Dunman *et al.*, 2001). Further, the NRPS products phevalin and tyrvalin are present in higher amounts when *S. aureus* grows in biofilms (Secor *et al.*, 2012). An earlier assumed direct involvement of AusAB in *S. aureus* virulence factor transcription and disease had to be ameliorated due to an overlooked secondary mutation in the transcriptional regulator *sae*R (Sun *et al.*, 2010a; Wyatt *et al.*, 2010).

In the following investigations, I demonstrated that the NRPS has a virulence phenotype, modulating escape, causing cell death in macrophages and neutrophils and decreasing neutrophil migration while it does not affect bacterial growth or supernatant cytotoxicity.

5.2 The non-ribosomal peptide synthetase AusAB is involved in phagosomal escape of *S. aureus*

After selecting the AusAB mutants for a following characterization, our first intent was to depict the phagosomal escape phenotype of the mutants in more detail. Our initial screen only presented us a screenshot of escape efficiency in HeLa cells three hours after infection (Table 7.1/Fig. 4.4). We therefore infected a different type of cell, a lung epithelial cell line with *S. aureus* wild type and mutant strains and evaluated the escape rates at four different time points. Using this experimental setup, we were able to determine, that escape in AusAB mutants is rather delayed and reduced than completely abolished. The escape rates of the AusAB mutants resemble neither the JE2 wild type, where escape peaks at 6 hpi and then declines constantly, nor a mutant deficient in PSM secretion, which does hardly escape from phagosomes. Escape in AusAB mutants appears delayed and reaches its peak at 12 hpi. But even here the maximal escape rates of the wild type are not matched. The fact, that phagosomal escape appears to be not completely abolished puts the AusAB mutation in contrast to mutants deficient in PSM production (Grosz *et al.*, 2014). This means, that AusAB expression is not essential for the capability of *S. aureus* to escape, but enhances it. In which way this is, warrants additional experiments. As a transcriptional regulation in *S. aureus* by the NRPS products phevalin and tyrvalin is reported to be minor (Wyatt *et al.*, 2010), a target on host cell is more likely. Phevalin has been shown to influence gene regulation in keratinocytes before (Secor *et al.*, 2012).

AusAB expression must yield a big advantage for *S. aureus*. Production of the protein complex during *agr* activation should represent a tremendous physiological burden on the bacterial cell, especially during a state in which the bacterium increases toxin production. NRPS products usually do bring great benefits to their producers. Many of them are antibiotics or toxins, directed against potential adversaries of the producer in their natural habitat, while others function as siderophores and pigments (Johnson *et al.*, 1945; Totter and Moseley, 1953; Duy *et al.*, 2000; Reverchon *et al.*, 2002). The three dipeptides produced by AusAB belong to a family of pyrazinones and structurally resemble monoketopiperazines. The two amino acids building the dipeptide undergo spontaneous cyclization to form a pyrazinone ring (Zimmermann and Fischbach, 2010).

An involvement of genes upstream and downstream of the *aus*AB operon in synthetase function was ruled out, due to reconstitution of pyrazinone production by expression of AusAB in *E.coli* alone (Wyatt *et al.*, 2012). I therefore limited the ensuing experiments to mutants in the genes *aus*A and *aus*B. By UPLC I showed that aureusimines were detectable in bacterial supernatants following overnight incubation. Aureusimines are therefore secreted, and most likely can be released from the cell without the activity of a dedicated transporter as the aureusimine products possess very low molecular weights. Phevalin and tyrvalin represent the two main products of AusAB with quantities of 1.0 – 1.3 mg L^{-1} tyrvalin, 0.5 – 1.0 mg L^{-1} phevalin and 0.3 mg L^{-1} leuvalin reported in bacteria culture medium of overnight cultures (Zimmermann and Fischbach, 2010; Wyatt *et al.*, 2012). Genetic complementation of aureusimine production in an *aus*B mutant was achieved using an *E. coli*/*S. aureus* shuttle plasmid p2085 encoding the *aus*B ORF under the control of a constitutively active SarAP1 promoter (Liese *et al.*, 2013). Complementation via plasmid lead to aureusimine levels about equal to the wild type, showing that functionality of the synthetase is not dependent on co-translation of the ORFs *aus*A and *aus*B.

Aureusimine production further was detected following internalization of bacteria in HeLa cells. Via UPLC-MS we detected more phevalin 4 hpi in infected cell samples than after 2 hours. As AusAB expression is at least partially under *agr* control (Dunman *et al.*, 2001) and the *agr* quorum sensing system has been shown to be active in internalized bacteria (Qazi *et al.*, 2001) and crucial for efficient escape (Grosz *et al.*, 2014), aureusimine production is certainly switched on after internalization of the bacteria and prior to phagosomal escape. The extent of AusAB control by the *agr* system becomes more differentiated when examining transcriptional changes by quantitative real time-PCR (Fig. 4.13). *aus*A mRNA levels do not increase in an *agr*A mutant during stationary phase, while *aus*B mRNA still increases to levels close to that observed in the wild type. This suggests a differential transcription regulation of *aus*A and *aus*B, thereby contradicting the predicted operon structure. However, when assessing transcription start sites in *S. aureus* we found a second transcription start site (TSS) in the *aus*AB operon situated within the N-terminus of the *aus*B ORF (Fig. 5.1) (Das *et al.*, 2016). This second TSS might explain the independence of *aus*B transcription from *agr* control.

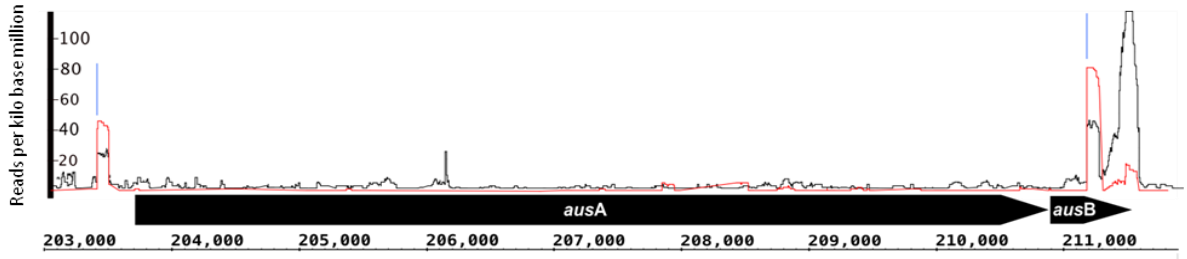

Figure 5.1: RNAseq data of *aus*AB operon shows an additional distinct transcription start site (TSS) in the *aus*B gene

Landscape of transcription start sites at the *aus*AB locus of *S. aureus* LAC. TSS of the wild type *S. aureus* LAC were enriched by treatment with Terminator-5′ phosphate-dependent Exonuclease (WT TEX+) as described before (Das *et al.*, 2016). The black line of the graph shows the normal transcriptome, while the red line represents the TEX treated sample. Large increases of reads per kilo base million for the red line indicate positions of TSS.

In our escape experiments in HeLa and S9 lung epithelial cells, which included an *aus*B mutant strain genetically complemented with the plasmid p2085 SarAP1 *aus*B (*aus*B compl; Fig. 4.15). I did not observe full complementation. The reason for this is probably a concentration dependent efficiency of the aureusimines. Although we detected a functional aureusimine production by UPLC, quantification of the substances was not possible. Thus we cannot exclude differences in the amounts of aureusimine production between the complemented mutant and the wild type four hours after infection.

A better understanding for the unresolved questions in aureusimine expression might arise from the quantification of mRNA levels of bacteria during infection of host cells. Escape positive and escape negative strains can be compared and a kinetic of *aus*AB mRNA expression at different time points can be determined.

5.3 Aureusimine deficiency does not affect staphylococcal growth or invasion into epithelial cells but does reduce host cell death

A lack of aureusimine production did neither inhibit bacterial growth (Fig. 4.16), nor did it influence hemolysis (Fig. 4.17), general toxin production (Fig. 4.20) or invasion into HeLa cells (Fig. 4.18). All of these experiments further underline that a transcription regulation by aureusimines in *S. aureus* is minimal at best. Genes shown previously to have altered expression patterns by a deficiency in aureusimine production include superantigen-like proteins, the γ-hemolysin monomer *hlg*A, the triacyglycerol lipase *lip*, nitrogen regulation genes, a nitrate reductase complex and the transcriptional regulator *sar*Z (Wyatt *et al.*, 2010). None of these

proteins have reported functions in phagosomal escape but are involved in immune evasion and biofilm formation (Fraser and Proft, 2008; Hu *et al.*, 2012). *sar*Z is a *sar*A family protein found to be involved in the regulation of the major transcriptional regulators *sar*A and *agr* (Tamber and Cheung, 2009). While a *sar*Z downregulation demonstrated a negative impact on *hla* production in a published study (Kaito *et al.*, 2006), this was not evident in my experiments (Fig. 4.20). Possible explanations for the difference in the observed effects in the present study/my data and the results of Kaito *et al*. may either originate from different strain backgrounds used (RN4220 in Kaito *et al*.) or remaining *sar*Z levels in the mutant suffice for full hemolysis and supernatant cytotoxicity. How the aureusimine based transcriptional regulation functions is unknown.

Phagosomal escape is thought to be a direct prerequisite for host cell cytotoxicity caused by internalized bacteria (Grosz *et al.*, 2014). In the present study, escape-deficient AusAB mutants also exhibited reduced intracellular cytotoxicity since significantly fewer host cells were killed by the mutants when compared to the wild type or complemented *aus*B mutant (Fig. 4.19) The dynamics of cell death is not entirely different from the wild type, as e.g. observed in a mutant in the transcriptional regulator *rsp* where cell death is only delayed (Das *et al.*, 2016), but the percentage of detected apoptotic cells is reduced at every timepoint. The ability of internalized bacteria to cause host cell death therefore directly correlates with the phagosomal escape phenotype. Further, the effect of aureusimine production on host cell death occurs upon internalization of bacteria only. Extracellular bacteria mainly kill host cells by α-toxin (Bhakdi *et al.*, 1988; Bantel *et al.*, 2001; Essmann *et al.*, 2003; Berube and Bubeck Wardenburg, 2013).

5.4 Phevalin but not tyrvalin acts in phagosomal escape of *S. aureus*

The production of more than one major product is an unusual characteristic of AusAB, as a single major product and possible additional minor products are the norm for NRPS (Fischbach and Walsh, 2006; Zimmermann and Fischbach, 2010). The simultaneous production of both, phevalin and tyrvalin (leuvalin as the minor product left aside) can be attributed to a relaxed substrate specificity of the second adenylation domain of the NRPS (Wyatt *et al.*, 2010; Zimmermann and Fischbach, 2010).

However I found that phevalin but not tyrvalin, used in µM concentrations, can complement the phagosomal escape phenotype of an aureusimine deficient mutant (Fig. 4.21/4.22). Phenotypic complementation using phevalin was concentration dependent and strongest when 10 µM of synthetic phevalin was used in the infection sample. Interestingely, lower as well as higher concentrations lead to decreasing escape rates again. If the experimental

setup was changed, the effective phevalin concentration needed for complementation was also subject to change. While in chapter 4.5, 10 µM phevalin was required to recover the phenotype, 500 nM were used to see a similar effect in chapter 4.5.2. All of these experiments point towards a strict concentration dependency of the phevalin effect. Actual concentrations of phevalin produced by live bacteria in the host cell could not be determined using current methods, as very local phevalin concentrations surrounding or inside the phagosome are most probably responsible for escape. Slight variations in the experimental design could therefore already change the necessary concentration of phevalin used for extracellular addition just as much as batch differences of the synthesized compound. A possible role of tyrvalin in phagosomal escape therefore cannot be excluded completely, as the concentration dependency might apply to tyrvalin as well. A set of different concentrations will have to be tested in escape experiments in order to find a possible effective concentration.

Another possible explanaition for differences in phevalin and tyrvalin activity would be differential membrane permeability. While phevalin is able to can enter host cells and reach its intracellular target, tyrvalin may not be able to do so. Using UPLC we were able to show phevalin entering and/or associating with host cells after incubation in phevalin spiked medium (Fig. 4.23). For lack of purified tyrvalin, which only recently became commercially available, a comparable experiment using tyrvalin is still pending. Based on the structure of both peptides, tyrvalin and phevalin share a light cationic characteristic. Cationic peptides are known to pass cell membranes without using a dedicated transport mechanism but rather by diffusion (Henriques *et al.*, 2006). Phevalin and tyrvalin also fulfill all criteria set for new drugs to be approved for oral ingestion with a logP value of 3.16 for phevalin and 2.68 for tyrvalin (<5/molinspiration.com) and a molecular weight of 228.29 g/mol for phevalin and 244.29 for tyrvalin (between 160 and 480) (Lipinski *et al.*, 2001). Therefore phevalin and tyrvalin should be equally capable of entering host cells upon contact.

Our experiments further illustrate, that phevalin does not permeabilize/weaken phagosomal membranes.. Using paraformaldhyde-fixed bacteria for infection none of the tested phevalin concentrations generated any effect on phagosomal escape rates illustrating that the phagosomes were retaining fixation-killed bacteria irrespective of phevalin treatment. A disruptive function on membranes solely procured by phevalin can therefore be excluded. Live bacteria produce additional factors to enable the bacteria to escape. PSMα peptides are well known to promote phagosomal escape and are therefore one probable factor missing from inactivated bacteria (Grosz *et al.*, 2014). Future studies will have to show if synergies of *aus*AB and PSM exist.

Phevalin was first described by Alvarez et al. (1995) as a natural product of *Streptomyces* sp. In a casein hydrolysis assay, phevalin inhibited calpain activity with an IC_{50} of 1.3 µM

(Alvarez *et al.*, 1995). Calpain inhibition could not be confirmed in later publications but as calpains were shown to modulate phagosomal integrity, we tested phevalin in relation to commercial calpain inhibitors for escape effects (Zeng *et al.*, 2005; Villalpando Rodriguez and Torriglia, 2013). In contrast to phevalin treatment, supplementing the infection medium with calpain inhibitors lead to reduced phagosomal escape rates (Fig. 4.25). Calpains are known to increase plasma membrane permeability and promote the degradation of lysosomal membranes by targeting LAMP2 (Liu *et al.*, 2004; Villalpando Rodriguez and Torriglia, 2013). Its effect on escape therefore excludes calpain inhibition as the relevant task of phevalin in phagosomal escape. This however does not rule a negative effect of phevalin on calpains out completely. As I showed, that lower concentrations than 10 µM of phevalin could negatively affect phagosomal escape rates, the link between calpains and phevalin could be highly concentration dependent. Meanwhile this data points towards a significant influence of calpain activity on phagosomal escape of *S. aureus*. The disruption of the lysosomal membrane appears to be caused not exclusively by bacterial toxins, but is also promoted by host cell proteases. In which manner calpain activation is stimulated by internalized bacteria is not known but calpain1 has been shown to be activated by α-toxin in keratinocytes (Soong *et al.*, 2012).

5.5 Phevalin production promotes *S. aureus* intracellular survival and cytotoxicity in leukocytes

The ability of *S. aureus* to evade degradation by immune cells of the innate immune system in an infection is paramount for survival and persistence inside the host. While PMN pose as the first line of defense, engaging in the phagocytosis of invading bacteria at the site of infection, macrophages will take up everything recognized as a threat to the host such as bacteria, bacteria-containing PMN or apoptotic bodies of destroyed cells (Amulic *et al.*, 2012; Flannagan *et al.*, 2015a). *S. aureus* has developed mechanisms to avoid degradation by macrophages or PMN, to kill the leukocytes or to evade recognition by the immune system altogether (Smagur *et al.*, 2009a; McGuinness *et al.*, 2016). Phagosomal escape is a factor in leukocytes as well, but *S. aureus* recently has been shown to survive and replicate in intact mature phagolysosomes of macrophages (Flannagan *et al.*, 2015b).

Phagosomal escape experiments were conducted in PMN using a method based on the detection of the pH surrounding internalized bacteria and flow cytometry (Lam *et al.*, 2010; Grosz *et al.*, 2014).

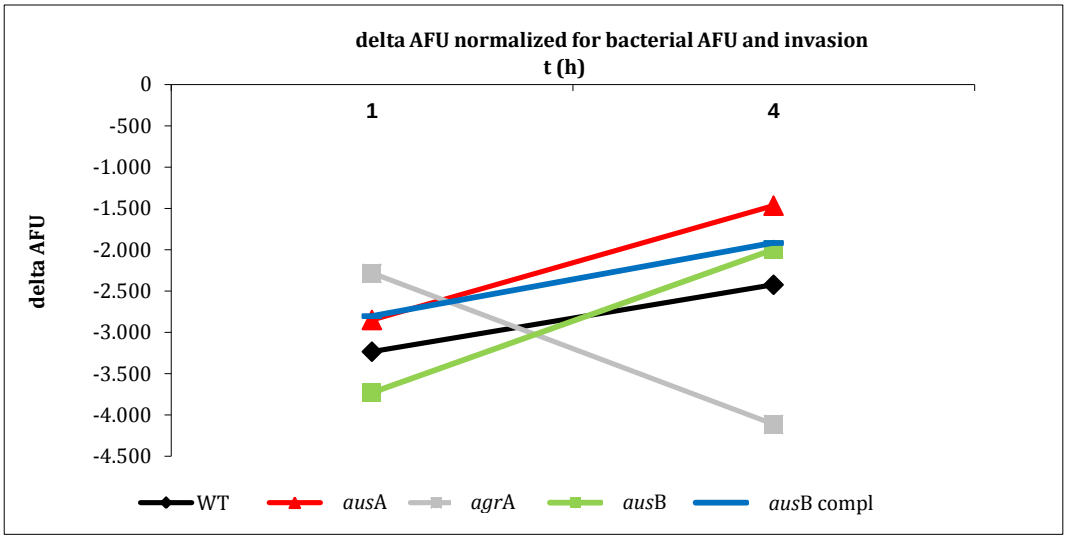

Figure 5.2: Detection of phagosomal escape in PMN using fluorescence flow cytometry

PMN are isolated and infected as before using bacteria stained with FITC as described before (Grosz *et al.*, 2014). 1 and 4 hpi, cells were washed and half of each sample was treated with monensin before FITC fluorescence (arbitrary fluorescence units, AFU) was measured using an Accuri C6 flow cytometer. Results are depicted as differences in monensin-treated and untreated samples (ΔAFU for each time point and strain) (n = 1). An increase in ΔAFU between 1 and 4 hpi indicates translocation of the bacteria from an acidified to a more neutral microenvironment and thereby phagosomal escape of *S. aureus*.

The method proved unreliable when applied to PMN, probably due to the production of highly reactive species in PMN via myeloperoxidase, damaging the pH sensitive FITC dye used to stain bacteria (Bos *et al.*, 1978). The data we were able to collect indicates no differences in phagosomal escape in PMN between wild type and mutant strains (Fig. 5.2). This result is further substantiated by cfu of intracellular bacteria recovered from PMN. I detected no significant differences in intracellular survival between the investigated wild type and mutant strains (Fig. 4.27). MRSA strains such as LAC can survive degradation by PMN and macrophages and will kill the host cell eventually (Flannagan *et al.*, 2015b; Zurek *et al.*, 2015; Jubrail *et al.*, 2016; Yajjala *et al.*, 2016; McGuinness *et al.*, 2016). While neither phagosomal escape nor cfu are affected by aureusimine production in PMN, host cell death was found to be altered by the presence of phevalin (Fig. 4.28). Wild type bacteria and the *aus*B complemented strain killed PMN 4 hours post infection at significantly higher rates than aureusimine mutants. Functional escape and replication inside PMN would suggest no differences between aureusimine mutants and wild type bacteria in host cell death as well. The reduction in cell death upon internalization of *aus*A and *aus*B mutants therefore points to a role of aureusimines in post-escape events in PMN. Defunct aureusimine production delays or inhibits the killing of PMN by intracellular bacteria. Delayed killing amid effective escape and replication is known to mutations in the *rsp* transcriptional regulator of *S. aureus* when internalized. But in the reported case, increasing cfu

inside the cell cytoplasm were detected (Das *et al.*, 2016). In AusAB mutants, no such aggregation was visible. Additional tests will be necessary to assess how an *aus*AB mutation confers reduced cytotoxicity in PMN.

Interestingely, extracellular addition of phevalin to infected or uninfected PMN showed significantly decreased cell death rates after four hours of infection when compared to untreated control cells. These effects were independent from the bacterial strain used (Fig. 4.28). The effect is counter-intuitive as extracellular addition of phevalin to infection samples rather promoted virulence of internalized *S. aureus* strains in other cells. This might be explained by concentration dependencies at certain host cell sites to procure specific effects. A concentration dependency was already visible in the complementation of phagosomal escape in HeLa cells using the extracellular addition of phevalin. This relates to the published inhibitory function of phevalin on calpains (Fig. 4.25) (Alvarez *et al.*, 1995). Calpains are not only known for influencing membrane integrity but claim a multitude of other functions in the cell. The induction of apoptosis is one of these functions (Harwood *et al.*, 2005). An inhibition of calpains could limit the probability of neutrophils to undergo apoptosis, the mode of cell death most commonly caused by *S. aureus* (Bayles *et al.*, 1998; Haslinger-Loffler *et al.*, 2005; Kubica *et al.*, 2008; Fraunholz and Sinha, 2012). Activation of calpains in the initiation of apoptosis is dependent on an increased calcium flux in the cell (Harwood *et al.*, 2005). This coincides with our results, showing that the intracellular calcium flux in neutrophils following FPRI- and FPRII-receptor dependent activation is decreased (Fig. 4.30). From these results I postulate, that the decrease in PMN activation upon phevalin treatment as evidenced by the measurement of PMN calcium fluxes is independent of FPR and the effect on intracellular calcium must be caused by a different, phevalin-sensitive factor in the signaling pathway causing calcium release from endoplasmic calcium storages (Fig. 5.3). This decrease in calcium fluxes would then also inhibit calpain activation and limit apoptosis of PMN.

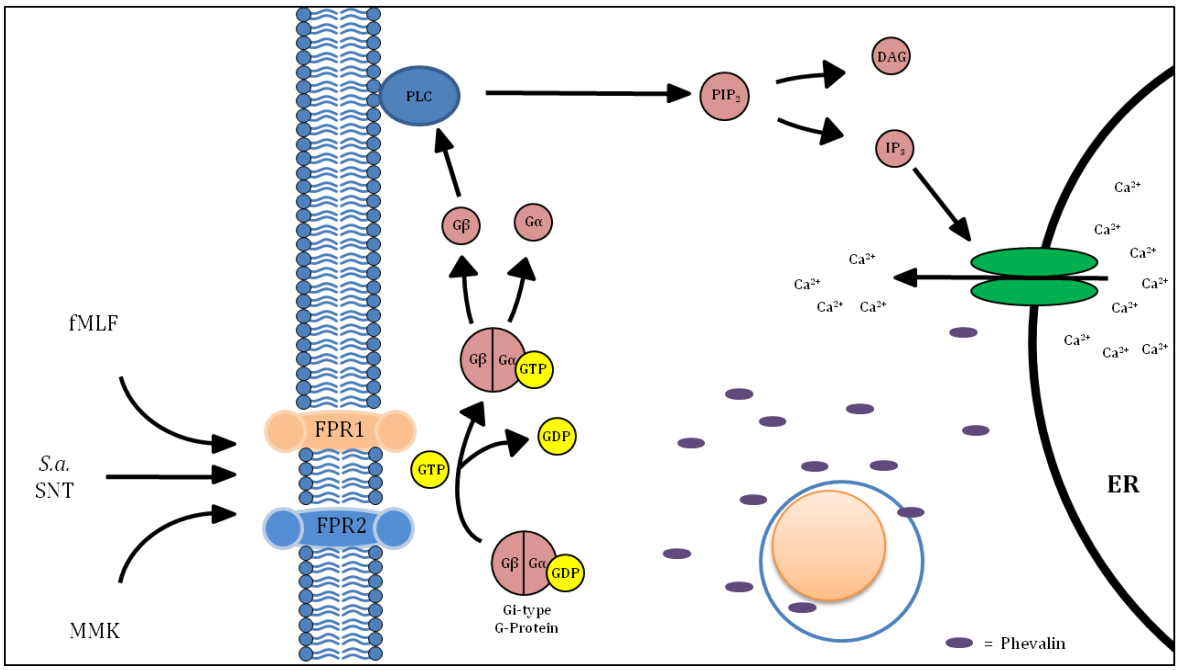

Figure 5.3: Phevalin interferes with an unknown factor of the G-protein coupled signaling pathway governing the calcium release from endoplasmic calcium stores.

In our experiments we used fMLF (FPR1), MMK (FPR2) as well *S. aureus* supernatant as stimuli of the human FPR receptors, to measure the extent of calcium release from endoplasmic stores into the cytoplasm. We showed calcium fluxes to be reduced, independent of the stimuli used, when HeLa cells were first treated with different concentrations of phevalin (Fig. 4.30). Phevalin could affect any step in the G-protein coupled signaling pathway. Stimulation of the FPR receptors results in the replacement of GDP for GTP at the Gi-type G-protein. The G-protein will then dissociate into Gα and Gβ subunits. Gβ subunits are able to activate membrane bound phospholipase C (PLC). PLC in return can cleave phosphatidylinositol 4,5-biphosphate (PIP_2) to form the second messenger molecules phosphatidylinositol 1,4,5-triphosphate (IP_3) and diacyl-glycerol (DAG). IP3 will then mobilize endoplasmic calcium stores. Modified after (Murdoch and Finn, 2000).

By contrast, the situation in macrophages is similar to the results obtained from epithelial cells. Through higher YFP-cwt fluorescence in genetically engineered THP-1 cells and their more spherical structure measurement of phagosomal escape rates was not as reproducible as in epithelial cells. However, cfu collected from macrophages infected with wild type and mutant strains showed a strong deficiency of AusAB mutants to survive and replicate in macrophages as effectively as wild type bacteria or the *aus*B complemented strain. This may be caused by reduced phagosomal escape rates in the mutant when compared to wild type. However it is currently disputed, if the bacteria escape from macrophage phagosomes. *S. aureus* has been shown to not need phagosomal escape to replicate inside macrophages but is rather even dependent on functional phagolysosome acidification for intracellular survival (Flannagan *et al.*, 2015b; Tranchemontagne *et al.*, 2015). Macrophages are known to fail in the control of a *S. aureus* infection (Jubrail *et al.*, 2016). In my experiments, cfu of *S. aureus* wild type and *aus*B

compl are not negatively impacted over the course of 2 hours (Fig. 4.31), whereas in *aus*AB mutants less cfu are recovered 1 and 2 hpi similar to an *agr* mutant.

Differences between wild type and an *aus*B mutant strain also occur in macrophage killing by internalized bacteria. While the *aus*B compl strain kills macrophages even slightly stronger than wild type, an *aus*B mutant causes significantly less macrophage cell death (Fig. 4.32). How exactly aureusimine production influences bacterial survival in macrophages and promote cell death in PMN and macrophages is currently unclear. Since aureusimine production was shown to positively influence the expression of several staphlococcal superantigen-like proteins (Ssl) (Wyatt *et al.*, 2010), of which Ssl3 binds to the TLR2 receptor, thereby decreasing cytokine expression by macrophages (Yokoyama *et al.*, 2012), this process may assist in immune evasion of *S. aureus*. In contradiction to this, the phevalin effect on neutrophil calcium fluxes and activation occurred completely independent of bacteria and therefore independent of a possible transcriptional regulatory effect of phevalin in bacteria. This suggests a direct effect on a host cell target.

5.6 The *S. aureus* NRPS influences infection outcome in a murine lung infection model

The results of the present study demonstrated that survival of *S. aureus* defective in aureusimine production is impaired in professional as well as non-professional phagocytes. At the same time internalized bacteria are less cytotoxic in all tested cell types. To understand if these effects influence disease outcome and bacterial survival in an in vivo setting, I chose a murine lung infection model as well as a murine muscle abscess model. Previous research showed no influence of aureusimines on cfu recovered from mice in a retro-orbital injection model (Sun *et al.*, 2010a). However, the strain used in this study, Newman, does only express truncated fibronectin-binding proteins (FnBPs), rendering the main bacterial factor mediating invasion into host cells in *S. aureus* non-functional (Baba *et al.*, 2008). While Newman still invades cells via the extracellular adherence protein (Eap), internalization is heavily reduced compared to strains with regular FnBPs (Harraghy *et al.*, 2003). As I present a positive influence of phevalin production particularly on *S. aureus* virulence in an intracellular setting, Newman is a suboptimal strain to conduct experiments on the effect on virulence of aureusimines.

LAC, the strain used in this study meanwhile effectively invades bacteria and expresses high levels of intracellular cytotoxicity (Diep *et al.*, 2006b; Date *et al.*, 2014). In lung infection, the host immune system reacts with a massive influx of leukocytes like PMN and pulmonary macrophages. As I observed phenotypes of aureusimine mutants in both cell types, I

hypothesized that this particular model would give us the best indication, if aureusimine production benefits the survival and virulence of *S. aureus* in vivo. In this model we recorded the disease activity (DAI), a measure for the degree of disease severity in mice, which does not require lethal doses of bacterial infection inoculums.

The DAI obtained from the lung infection model revealed a similar course of disease in all mice in the first 24 hours, regardless of the bacterial strain used for infection (Fig. 4.33). Only after 24 hours, mice infected with the *aus*B mutant strain did not show further deterioration of health parameters while the DAI of mice infected with wild type bacteria and an *aus*B compl strain increased further, suggesting that the murine immune system failed to control the infection. Interestingly, cfu isolated from dissected lungs were at comparable levels for all three strains. However, small differences in cfu recovered from infected mice do not necessarily mirror the capacity of the strain to cause disease. USA300 strains with mutations in the two virulence regulators *agr* and *sae*RS are recovered with similar cfu as the wild type in a mouse model of infection, despite significant differences in mouse survival rates (Date *et al.*, 2014). The ability to cause and sustain a serious infection of a strain can therefore often not effectively be judged only by cfu collected from tissue after 48 h or 72 h. The similar DAI scores for all infected mice in the initial phase of infection can be explained due to the fact that many of the symptoms scored in this test originate from the reaction of the murine immune system to bacteria at the site of infection. Massive inflammation due to high bacteria inocula will lead to mice sick appearance (fur, behavior, etc.) and weight loss of the animals. Only when the immune system controls the infection caused by the *aus*B mutant, inflammation is reduced and the mice start to recover. When animals are infected with wild type or *aus*B complemented bacteria the infection can not be controlled by the immune system and the health of the animals further deteriorates.

The in vivo experiments mentioned above show, that a deficiency to produce aureusimines influences the ability of *S. aureus* strains to cause disease in a murine lung infection model. As the in vitro experiments link aureusimine phenotypes particularly with intracellular bacteria, these results directly connect in vivo cytotoxicity to intracellular survival and virulence. In the muscle abscess model, cfu numbers recovered from infected muscle tissue were analyzed. Similar to the lung infection model, differences in cfu between strains were only minor. DAI scores were not collected during the course of this experiment. As the muscle tissue infection stayed locally limited over the complete experiment, even mice infected with wild type bacteria do not suffer from continous loss of weight and do not show sick appearances similar to the lung infection model. As cfu numbers alone do not always display the virulence potential of strains, a definite statement on the importance of aureusimine production for *S. aureus* to cause and sustain an abscess infection can not be issued at this point. It would be necessary to repeat the muscle abscess model for an extended infection period to collect additional data on the

subject. If the *aus*B mutant would prove to not be impaired in its capability to sustain an infection in the abscess model, the differences to the lung infection model could be explained by the fact, that macrophages play such a big role in the lung tissue. 95% of the complete cell burden in broncho-alveolar lavage is made up of alveolar macrophages (Gordon and Read, 2002). Our data show, that the phenotype of aureusimine production deficient mutants is very distinct in macrophages but not as strong in other types of leukocytes like neutrophils.

Phevalin has an effect in *S. aureus*-mediated disease. While this involvement was first described to be primarily caused on the level of transcriptional regulation (Wyatt *et al.*, 2010), this was later revised in large parts (Sun *et al.*, 2010a). The here presented data connects phevalin to intracellular virulence of *S. aureus* and further points to a possible host cell target of phevalin. Although identified in a screen for calpain inhibitors (Alvarez *et al.*, 1995), phagosomal escape is rather being blocked by traditional calpain inhibitors, whereas phevalin enhances this immune evasive strategy of *S. aureus*. Further, phevalin treatment of keratinocytes can alter gene expression (Secor *et al.*, 2012) and influences calcium fluxes in PMN. Thus I questioned which molecular targets exist on the host cell side.

5.7 Host cell proteome changes after phevalin treatment indicate direct effect on host cell gene expression

The experiments presented in this study point at a possible host cell target of phevalin. Phevalin treatment of keratinocytes can alter gene expression and influences calcium fluxes in PMN (Secor *et al.*, 2012). Because of these results, I opted to assess changes in the metabolism and (phospho-) proteome of host cells under phevalin influence. By using synthetic phevalin only, I was able to investigate the immediate effect of the pyrazinones, avoiding deleterious effects of cytolytic toxins like α-toxin and PAMPs, which would cloud changes caused by phevalin (Haugwitz *et al.*, 2006; Kloft *et al.*, 2010).

The proteome as well as the phosphoproteome data saw a number of genes regulated upon phevalin treatment (Table 7.2 – 7.6). Among these, the genes down-regulated strongest included the two antimicrobial peptides (AMP) dermicidin (DCD) and lysozyme (LYZ). For *S. aureus*, the ability to decrease the levels of peptides in the cell designated to destroy bacteria will certainly benefit its survival. Dermicidin is an AMP with a negative net charge, which acts as a main effector of the innate host defense against bacterial pathogens (Rieg *et al.*, 2005). Even though dermicidin is primarily known to be produced in sweat glands and then distributed on the skin (Schittek *et al.*, 2001), our data indicates its presence in HeLa cells as well. Meanwhile

lysozyme is a cationic AMP more widely produced in mammalian body fluids and tissues. It is constitutively produced by phagocytes such as PMN and macrophages (Gordon *et al.*, 1974). Lysozyme facilitates the hydrolysation of 1,4-beta linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan of bacteria. Lysozyme production is well known to be enhanced in cancer cells (Serra *et al.*, 2002). Reductions in lysozyme production in HeLa cells could benefit the survival of internalized bacteria, as AMPs are an integral part of the lysosomal content (Alonso *et al.*, 2007; Hole *et al.*, 2012). If and how a reduction of both AMPs directly benefits *S. aureus* virulence will have to be determined in future studies.

Not in the list of genes downregulated with confidence, but still of interest, as lesser amounts were detected, is the mitochondrial fission factor (Mff). Mff is a tail-anchored membrane protein (Gandre-Babbe and van der Bliek, 2008), which mobilizes Drp1 molecules from the cytosol of the cell to the mitochondria to form a protein complex (Otera *et al.*, 2010). As Drp1 accumulates, it forms rings around the mitochondria which will induce mitochondria fission by constriction. Reduced levels of Mff will result in less fission events which should induce morphological changes in the mitochondrial network. Mff is also not the only gene directly connected to mitochondria which appears affected by phevalin. The cytochrome C oxidase subunit 7A2 (COX7A2) is upregulated. The cytochrome C oxidase is part of the mitochondrial respiratory chain and reduces oxygen to water by transferring electrons from cytochrom C. This reaction is used to transport protons across the mitochondrial inner membrane, generating a proton gradient used to facilitate ATP production. Higher amounts of cytochrome C oxidase could therefore also mean higher ATP production in phevalin treated cells. In our following experiments using mitochondria, both, increased ATP production and elongated mitochondria could be shown to be caused by phevalin treatment (Fig. 4.39 and 4.40). These results represent a first validation of the proteome data.

The strongest regulation in the phosphoproteome is seen in the zinc finger protein 148 (Remington *et al.*, 1997), where 3 positions were found with strong differences in their phosphorylation status. ZNF148, which is also known under the name ZBP-89, is a universally expressed DNA binding protein which has been shown to regulate cell growth arrest (Bai and Merchant, 2001), apoptosis induction (Bai *et al.*, 2004; Chen *et al.*, 2009) and stress induced hematopoiesis (Li *et al.*, 2014). In both aspects, growth and apoptosis control, ZNF148 interacts with the tumor suppressor gene p53 (Bai and Merchant, 2001; Bai *et al.*, 2004; Okada *et al.*, 2006). p53 belongs to the same protein family as p63, which has been shown to be positively regulated in keratinocytes treated with phevalin (Secor *et al.*, 2012). Higher amounts of ZNF148 in the cell stabilize p53 and thereby induce cell cycle arrest (Bai and Merchant, 2001). *S. aureus* has been shown to benefit from cell cycle delay considerably and to promote it upon contact with host cells (Alekseeva *et al.*, 2013). The here described phosphorylation sites of ZNF148 are

uncharacterized as of yet. Additional experiments will be necessary reveal a possible link between ZNF148 phosphorylation, p53 stabilization, cell cycle arrest and a benefit for intracellular survival of *S. aureus*.

How exactly phevalin procures the changes in gene regulation found in this study we can currently only speculate on. Transcriptional regulators as direct binding targets are one possibility. Also, other proteins might be affected in their function by phevalin first, leading to an imbalance in the cell which in return would trigger transcriptional changes and post-translational modifications.

Apart from the individual assessment of genes found in our proteome and phosphoproteome experiment, we also analyzed proteins found to be regulated in both lists combined by STRING analysis (Fig. 4.36). No functional enrichments were detected in the first set of genes including genes down-regulated in abundance. In contrast, the analysis of genes up-regulated in combination with those observed with changed phosphorylation status revealed a number of significantly enriched functional complexes (Fig. 4.37). The complexes included 'nucleic acid metabolic processes', 'organic cyclic metabolic processes' and a 'positive regulation of cellular metabolic processes' complex. The up-regulation of host genes involved in cell metabolism could indicate a negative effect of phevalin on the nutritional state of the cell.

In preliminary analyses of host cell metabolome during phevalin treatment most of the metabolites found to be positively or negatively regulated were identified in hydrophilic interaction chromatography, a type of chromatography used to analyze polar and hydrophilic compounds. Fewer differences were found in fatty acid fractions of metabolites and only few in an analysis specific for lipids. Polar metabolites include sugars, salts as well as a number of amino acids and intermediary compounds ususally originating from the metabolism. From the information I obtained, an effect of phevalin on the metabolome of HeLa cells is very likely. An unequivocal identification of the metabolites found by mass spectrometry will require comparison with compound libraries and will be part of a future study.

5.8 Mitochondrial elongation by phevalin treatment

As a group of proteins, including the mitochondrial fission factor isoform 2 (Mff2), was found to be, regulated in the proteome data I decided to assess mitochondrial morphology upon phevalin treatment, using HeLa cells expressing a GFP variant which is directed to the mitochondrial matrix (S. Roy Chowdhury, personal communication). Mitochondrial network formation strongly changed upon phevalin influence. Less mitochondria were present per imaged cell while single mitochondrial fragments were significantly elongated (Fig. 4.39). Mff is thought to form a transient and dynamic interaction wit Drp1 to recruit it to the mitochondria (Otera *et al.*, 2010).

If Mff levels are decreased, Drp1 will remain cytosolic and can not form scission ring complexes, ultimately leading to mitochondrial elongation. The elongation of the mitochondria observed in our experiments is therefore in accordance with reduction in Mff protein we observed in the proteome data.

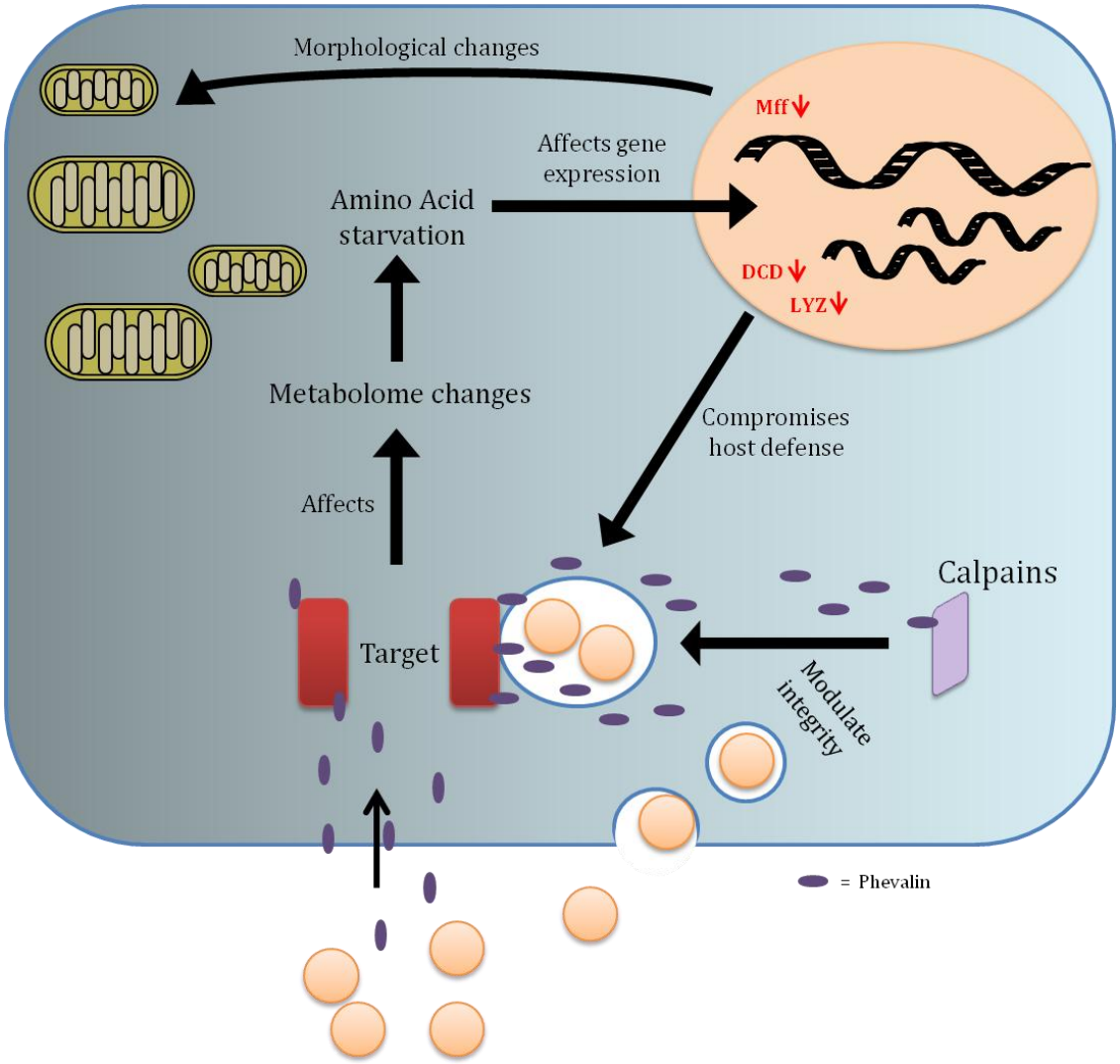

Figure 5.4: Phevalin acts positively on *S. aureus* intracellular survival by inducing changes in the cell metabolome.

The schema shows a model for the effect of phevalin on the host cell during *S. aureus* infection based on my observations in this study. Before and, in increased amounts, after internalization, *S. aureus* produces phevalin. Phevalin enters the cytoplasm of the host cell by a not yet identified mechanism and interacts with its cytoplasmic or possibly membrane bound targets. Whether a direct interaction with calpains, which have been shown to modulate lysosomal integrity, occurs as well is unknown. The interactions of phevalin with host proteins result in changes in the nutritional state of the cell. The cell reacts to the metabolomic imbalance by a differential gene expression, resulting in reduced amounts of the mitochondrial fission factor (Mff), visible through changes in the mitochondrial network, and of the two antimicrobial peptides dermicidin (DCD) and lysozyme (LYZ). The phevalin-induced changes compromise the ability of the cell to counter the bacterial infection and promote intracellular survival of *S. aureus*.

Meanwhile the analysis of extracellular flux showed, that the respiratory capacity of the mitochondria as well as ATP production is elevated in cells treated with phevalin (Fig. 4.40). Increased levels of ATP production are a defining characteristic of mitochondria elongated in response to autophagy (Gomes *et al.*, 2011). Mitochondria elongation protects the mitochondria from degradation due to macroautophagy, the process supplying the cell with amino acids and tricarboxylic acid (TCA) cycle metabolites and ATP in times of starvation and limited nutrient availability (Cecconi and Levine, 2008). Elongated mitochondria possess more cristae, which increases the numbers of dimeric ATP synthases and their activity (Strauss *et al.*, 2008; Gomes *et al.*, 2011). Mitochondria elongation has not been found to be a reaction of the cell to all kinds of starvation, but amino acid and glutamine starvation in particular (Rambold *et al.*, 2011). Amino acid starvation will also increase respiration, protein synthesis and amino acid catabolism in mitochondria, all in line with the phenotype of elongation (Johnson *et al.*, 2014).

Metabolic changes in the cell will have an influence on protein expression. Nutrient limitation will induce autophagy, accompanied by shifts in protein abundances (Mathew *et al.*, 2014; Amaya *et al.*, 2015; Harper and Bennett, 2016). In our experiments, we could see changes in both, protein abundance as well as phosphorylation status. Nutrient starvation has also been shown to promote calpain function through the inactivation of calpastatin, an inhibitor of calpains (Salem *et al.*, 2007). Calpain activity in return is necessary for functioning macroautophagy (Demarchi *et al.*, 2006). Altogether, the mitochondria phenotype observed here only occurs when the cell enters a state in which the nutrient availability, especially of amino acids is limited. Since in our experiments only phevalin was used for cell treatment, the NRPS product itself must affect the cell in a way to limit free amino acids and cause a starvation signal. Phevalin itself is a cyclic dipeptide. Enzymes of the amino acid anabolic pathways could bind phevalin according to their substrate specificities but, because of the cyclized structure of phevalin, the enzymes may not be able to catalyze their normal reaction, eventually causing a disruption of the metabolism and an imbalance in the cell. The cell then would react to this by inducing autophagic pathways. This includes an activation of calpains, which will prompt destabilization of lysosomal membranes and the induction of apoptosis in general (Liu *et al.*, 2004; Villalpando Rodriguez and Torriglia, 2013).

Thus, the mitochondrial phenotype we found is merely a symptom of the influence of phevalin on the nutritional status of the cell. Caused by its cyclic dipeptide structure, phevalin can probably interact with cellular proteins and block essential metabolic pathways.

5.9 Conclusions and outlook

Long thought to be strictly extracellular, the human pathogen *S. aureus* has recently been shown to enter host cells. Inside the cell, the bacteria evade destruction by escaping from the host phagosome in a phenol-soluble modulin (PSM)-dependent manner. Since PSM alone were not sufficient to mediate phagosomal escape, other factors involved in the process were hypothesized.

In the highly virulent methicillin-resistant *S. aureus* strain JE2 I identified a non-ribosomal peptide synthetase AusAB and its product phevalin, as well as several other gene products (*sps*A, *luk*A) as novel bacterial factors that modulate phagosomal escape of the pathogen and verified previously identified escape strategies of S. aureus. I found that the non-ribosomally synthesized cyclic dipeptide phevalin promotes the virulence of internalized bacteria independent of the host cell type and contributes significantly to the severity of disease in a murine infection model. The underlying mechanisms of the phevalin-associated effects have yet to be identified but my experiments suggest a direct host cell target of phevalin. Changes in calcium fluxes, mitochondria morphology, cell metabolism and proteome following treatment of cells with synthetic phevalin provide evidence that *S. aureus* uses phevalin for the modulation of host cells.

The identification of the host cell target will represent the primary task of future experiments. Additional replicates of host cell metabolom changes after phevalin treatment should be performed as well as an identification of found metabolites using substrate libraries. Further, the proteins found to be regulated in the proteome experiment require validation. Changes in protein abundance and phosphorylation status in response to phevalin could be assessed by Western Blots. Identifying direct binding of phevalin to host proteins would ultimately give us the best indication as to how phevalin modulates the cell. One possible method to identify binding targets would be click chemistry. Once a binding target is identified, the signaling pathways leading to the apparent significant changes in the host cell proteome and possibly metabolome need to be investigated in order to completely comprehend the impact, phevalin has on host cells.

Further aspects of this study warrant additional work. In an earlier study Grosz *et al*. showed, that overexpression of PSM in an *agr*-negative strain does not restore the ability to escape in this strain. The experiment should be repeated in a strain overexpressing a combination of PSM and virulence factors found to benefit escape in this study (*aus*AB, *sps*A and *luk*A) in order to find the factor cooperating with PSM in phagosomal escape. In general, the assessment of exact effects of mutations in the genes spsA and lukA, both identified to influence phagosomal escape, should be done. Our escape experiments should also be repeated in primary

epithelial cells, since primary cells provide a more relevant setting and relate more to the in vivo situation.

A potential role of tyrvalin in staphylococcal virulence needs to be addressed by additional experiments. A wider range of concentrations of tyrvalin should be used in escape experiments and experiments with a documented phevalin phenotype like calcium signaling in PMN or the imaging of mitochondria morphology should be repeated using tyrvalin to evaluate the use of the second major dipeptide produced by *S. aureus*.

As we found indications of a yet unknown involvement of host cell calpains in phagosomal escape, the exact effect of changes in calpain activity on escape of *S. aureus* should be investigated in more detail. Experiments using knock-out or knock-down cell lines could be used to first identify the specific calpain causative for the observed phenotype.

In my study I observed a clear phenotype of an *aus*B mutant in the murine lung infection model but histological examinations of infected mouse lungs have to be performed in order to estimate differences in the extent of leukocyte influx into lungs of mice that were infected with either phevalin-proficient or –deficient bacteria. These differences would be of special interest as phevalin showed an influence on chemotaxis in this cell type. Also, additional murine infection settings could lead to a more broadened view on the virulence of *S. aureus* in vivo.

Last but not least, the development of a functional “FACS-able” marker for phagosomal escape of *S. aureus* should be pursued further. The use of such a system would allow for an unbiased and truly genome-wide screening approach for factors involved in phagosomal escape and would represent a powerful tool to assess the yet not fully understood mechanisms of intracellular virulence of *S. aureus*.

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7 Appendix

7.1 Abbrevations

µ	Micro
α	Alpha
AA	amino acid
Agr	accessory gene regulator
amp	ampicillin
β	beta
bp	base pairs
BSA	bovine serum albumin
CA-MRSA	Community-acquired MRSA
cDNA	complementary DNA
CF	Cystic Fibrosis
CFU	colony Forming Units
cm	chloramphenicol
C-terminal	carboxyl-terminal
cwt	cell wall targeting domain
Δ/δ	delta
ddH_2O	double-distilled water
DMEM	Dulbecco`s modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
E. coli	*Escherichia coli*
ECL	enhanced chemoluminescence
e.g.	exempli gratia, for example
erm	erythromycin
EtOH	Ethanol
EDTA	ethylenediaminetetetraacetic acid
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fig.	figure
Fn	fibronectin
FnBP	fibronectin binding protein
fwd /for	forward
g	gramm
GFP	green fluorescent protein
h	hours
ha-MRSA	hospital-acquired MRSA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hla	alpha toxin
Hlb	beta toxin
hpi	hours post infection
HPLC	high performance liquid chromatography
HSA	human serum albumin

ICU	intensive care unit
inf.	infected
kb	kilo base
kDa	kilo Dalton
KO	knock-out
L	litre
LB	lysogeny broth
LukAB	Leukocidin A/B
M	Molar
m	milli
min	Minute
MOI	multiplicity of infection
MRSA	methicillin resistant *S. aureus*
mRNA	Messenger RNA
ms	mouse
nM	nanomolar
n.i.	not infected
N-terminal	Amino-terminal
OD_{490}	optical density measured at a wavelength of 490nm
OD_{600}	optical density measured at a wavelength of 600nm
ONC	overnight culture
P	p-value
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	Paraformaldehyde
p.i.	post infection
PMN	polymorphonuclear leukocytes
Pmt	phenol-soluble modulin transporter
PSM	phenol-soluble modulin
qRT-PCR	quantitative real-time PCR
rev	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temerature
RQ	relative expression levels
sec	seconds
S. aureus	*Staphylococcus aureus*
SCV	small colony variants
S. epidermidis	*Staphylococcus epidermidis*
SOC	super optimal broth with catabolite repression
TBS	Tris buffered saline
TMRM	Tetramethylrhodamine, methyl ester, perchlorate
TRITC	Tetramethylrhodamine
TSB	tryptic soy broth
U	enzyme unit
V	volt
v/v	volume per volume
w/v	weight per volume
wt	wild-type
YFP	yellow fluorescent protein

7.2 List of figures

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7.4 Supplementary information

Table 7.1: Nebraska insertion mutant strains tested for phagosomal escape and their respective relative escape rates

Library label	Gene Description	Identifier	n	% escape[1]	Std Dev
NE4	ABC transporter ATP-binding protein	SAUSA300_0309	1	90,2	-
NE10	putative hemolysin III	SAUSA300_2129	2	95,2	27,4
NE12	drug resistance transporter, EmrB/QacA subfamily	SAUSA300_2126	1	84,6	-
NE15	transcriptional regulator, TetR family	SAUSA300_2509	1	89,6	-
NE17	putative drug transporter	SAUSA300_1705	1	90,4	-
NE26	*coa*; staphylocoagulase precursor	SAUSA300_0224	2	116,8	22,1
NE30	hydrolase, haloacid dehalogenase-like family	SAUSA300_0600	1	81,4	-
NE32	transcriptional regulator, LysR family domain protein	SAUSA300_0095	1	104,9	-
NE49	DNA-binding response regulator, AraC family	SAUSA300_0217	2	92,4	16,6
NE67	isaB; immunodominant antigen B	SAUSA300_2573	1	106,0	-
NE75	Cap1B; capsular polysaccharide biosynthesis protein Cap1B	SAUSA300_2597	1	107,8	-
NE91	*kdp*A; K+-transporting ATPase, A subunit	SAUSA300_2034	1	113,6	-
NE95	*agr*B; accessory gene regulator protein B	SAUSA300_1989	3	15,8	1,4
NE96	*sar*U; staphylococcal accessory regulator U	SAUSA300_2438	3	88,4	18,9
NE99	transcriptional regulator, Fur family	SAUSA300_1448	2	125,4	5,4
NE100	FtsK/SpoIIIE family protein	SAUSA300_1482	1	105,2	-
NE104	putative lipase/esterase	SAUSA300_0641	3	78.2	29.1
NE110	transcriptional regulator, GntRfamily/aminotransferase, class I	SAUSA300_0110	1	102,1	-
NE111	putative surface protein	SAUSA300_0883	1	110,8	-
NE116	putative sensor histidine kinase	SAUSA300_1219	1	116,6	-
NE119	non-ribosomal peptide synthetase	SAUSA300_0181	4	47,3	12,4
NE129	DNA internalization-related competence proteinComEC/Rec2	SAUSA300_1547	1	100,5	-
NE132	putative transcriptional regulator	SAUSA300_2640	1	110,0	-
NE138	putative membrane protein	SAUSA300_0275	1	97,0	-
NE147	sensor histidine kinase	SAUSA300_0254	1	111,7	-
NE163	*aur*; zinc metalloproteinase aureolysin	SAUSA300_2572	3	88,9	34,3
NE165	staphylococcal accessory regulator	SAUSA300_0114	1	86,0	-
NE179	multidrug resistance protein	SAUSA300_2360	1	92,8	-
NE185	siderophore biosynthesis protein, IucA/IucC family	SAUSA300_0122	1	81,2	-
NE186	*fnb*A; fibronectin binding protein A	SAUSA300_2441	2	84,1	21,9
NE210	staphylococcal accessory regulator	SAUSA300_2247	2	99,9	26,3
NE212	putative lysophospholipase	SAUSA300_1710	1	96,5	-
NE214	siderophore biosynthesis protein, IucC family"	SAUSA300_0123	1	89,0	-
NE217	*pkn*B; protein kinase	SAUSA300_1113	1	80,6	-

[1] Normalised to the phagosomal escape rate of the USA300 JE2 wild type detected in the specific experiment. If n >1 then a mean value of all single normalized escape rates is shown.

NE218	sensor histidine kinase family protein	SAUSA300_0218	2	100,0	12,3
NE251	polysaccharide biosynthesis protein	SAUSA300_0482	1	83,8	-
NE258	*cls*; cardiolipin synthetase	SAUSA300_2044	3	85.0	35.7
NE262	DNA-binding response regulator, LuxR family	SAUSA300_1220	2	81,9	9,0
NE274	conserved hypothetical protein	SAUSA300_1867	1	82,3	-
NE286	immunoglobulin G binding protein A precursor	SAUSA300_0113	2	127,1	37,6
NE287	conserved hypothetical protein	SAUSA300_1744	2	82,0	23,9
NE290	transferrin receptor	SAUSA300_0721	2	87,6	0,9
NE294	signal transduction protein TRAP	SAUSA300_1784	3	79,1	14,1
NE296	staphylococcal accessory protein X	SAUSA300_0654	2	101,6	25,2
NE324	regulatory protein RecX	SAUSA300_1854	2	91,9	15,5
NE331	exonuclease RexA	SAUSA300_0870	2	71,0	16,1
NE332	iron transport associated domain protein	SAUSA300_1029	2	102,1	8,6
NE334	Hyaluronate lyase precursor	SAUSA300_2161	1	73,7	-
NE338	*lip*, triacylglycerol lipase precursor	SAUSA300_2603	1	87,8	-
NE354	transcriptional regulator, GntR family protein	SAUSA300_0503	2	104,2	11,4
NE355	transcriptional regulator, TetR family	SAUSA300_2515	1	108,3	-
NE362	putative teichoic acid biosynthesis protein B	SAUSA300_0247	2	100,9	11,3
NE367	arginine repressor	SAUSA300_2571	2	95,4	0,9
NE369	*gua*C, guanosine monophosphate reductase	SAUSA300_1235	1	105,5	-
NE374	*feo*B, ferrous iron transport protein B	SAUSA300_2487	2	119,2	22,7
NE383	Putative membrane protein	SAUSA300_0279	2	105,5	15,7
NE386	*rot*, staphylococcal accessory regulator Rot	SAUSA300_1708	2	76,5	26,4
NE391	*clf*B, clumping factor B	SAUSA300_2565	1	106,5	-
NE392	peptide ABC transporter, ATP-binding protein	SAUSA300_0200	2	90,1	10,0
NE399	preprotein translocase, SecY protein	SAUSA300_2588	3	100,8	27,3
NE400	iron compound ABC transporter, iron compound-binding protein	SAUSA300_2136	3	79,8	26,0
NE406	*fhu*A, errichrome transport ATP-binding protein fhuA	SAUSA300_0633	1	112,3	-
NE410	*ure*C, urease, alpha subunit	SAUSA300_2240	2	96,4	16,2
NE415	putative transcriptional regulator	SAUSA300_2259	4	96,5	30,1
NE420	*pbp*3, penicillin-binding protein 3	SAUSA300_1512	1	105,8	-
NE426	*tat*C, Sec-independent protein translocase TatC	SAUSA300_0347	1	87,6	-
NE431	iron-dependent repressor	SAUSA300_0621	4	92,7	24,6
NE432	*sdr*C protein	SAUSA300_0546	2	104,2	28,3
NE434	phosphosugar-binding transcriptional regulator	SAUSA300_2271	2	100,6	26,9
NE444	aminopeptidase PepS	SAUSA300_1860	2	106,5	5,2
NE447	putative iron-sulfur cluster-binding protein	SAUSA300_1806	2	112,8	19,1
NE453	*sbi*, IgG-binding protein SBI	SAUSA300_2364	1	119,4	-
NE454	*arc*R, transcriptional regulator, Crp/Fnr family	SAUSA300_2566	2	105,9	28,6
NE457	Peptide ABC transporter, permease protein	SAUSA300_0202	2	122,7	0,3
NE462	putative membrane protein	SAUSA300_0917	2	111,5	23,4
NE481	DNA-binding response regulator	SAUSA300_0645	2	96,9	23,6
NE483	*pep*T, peptidase T	SAUSA300_0727	2	102,0	28,5
NE505	transcriptional regulator, LysR family domain protein	SAUSA300_0093	2	108,5	11,4
NE512	*mod*C, molybdenum ABC transporter, ATP-binding protein ModC	SAUSA300_2228	2	122,0	12,4
NE513	putative helicase	SAUSA300_2431	2	88,0	0,2
NE514	*sar*T, staphylococcal accessory regulator T	SAUSA300_2437	4	104,8	13,9
NE519	peptidase, M20/M25/M40 family	SAUSA300_1460	2	95,5	11,6
NE528	putative permease	SAUSA300_0091	2	97,7	11,8
NE530	transcriptional regulator, AraC family	SAUSA300_2248	2	85,7	26,7
NE535	*sps*A, signal peptidase IA	SAUSA300_0867	3	48,8	11,5
NE539	*fad*E, acyl-CoA synthetase FadE	SAUSA300_0228	3	92,2	18,2
NE541	peptide ABC transporter, permease protein	SAUSA300_0201	1	106,7	-
NE551	putative fibronectin/fibrinogen binding protein	SAUSA300_1101	1	108,4	-
NE554	*vra*R, DNA-binding response regulator	SAUSA300_1865	1	100,8	-
NE557	iron transport associated domain protein	SAUSA300_1030	1	97,8	-
NE558	*luk*E, leukotoxin LukE	SAUSA300_1769	3	105,3	27,5

NE567	transcriptional regulator, MarR family	SAUSA300_2331	2	98,6	28,2
NE571	putative transposase	SAUSA300_2263	1	87,0	-
NE578	iron compound ABC transporter, permease protein	SAUSA300_0599	1	77,2	-
NE584	*nar*K, nitrite extrusion protein	SAUSA300_2333	2	122,7	46,6
NE588	*srr*B, staphylococcal respiratory response protein, srrB	SAUSA300_1441	2	88,4	42,5
NE600	putative peptidase	SAUSA300_2087	2	96,9	10,0
NE610	*lys*P, lysine-specific permease	SAUSA300_1628	2	89,9	5,2
NE618	*pho*R, sensory box histidine kinase PhoR	SAUSA300_1638	2	92,7	17,7
NE636	exfoliative toxin A	SAUSA300_1065	1	99,6	-
NE637	*cbf*1, cmp-binding-factor 1	SAUSA300_1791	2	115,0	8,7
NE643	staphylococcal tandem lipoprotein	SAUSA300_0102	2	110,1	15,1
NE647	conserved hypothetical protein	SAUSA300_1485	1	95,8	-
NE648	*glv*C, PTS system, arbutin-like IIBC component	SAUSA300_2270	2	73,5	2,8
NE672	homoserine kinase	SAUSA300_1228	1	113,9	-
NE679	*pbp*4, penicillin-binding protein 4	SAUSA300_0629	1	100,4	-
NE773	drug transporter	SAUSA300_2451	1	79,3	-
NE873	*agr*C, accessory gene regulator protein C	SAUSA300_1991	3	13,2	8,8
NE934	cysteine protease precursor	SAUSA300_0950	2	85,3	11,7
NE964	4'-phosphopantetheinyl transferase superfamily protein	SAUSA300_0182	4	52,4	11,7
NE993	Putative hemolysin III	SAUSA300_2129	1	86,7	-
NE1065	osmoprotectant ABC transporter permease	SAUSA300_0707	1	76,5	-
NE1077	*rbs*K, Ribokinase	SAUSA300_0262	3	74,7	18,1
NE1109	*rpo*F, RNA polymerase sigma factor SigB	SAUSA300_2022	3	98,3	15,0
NE1181	putative membrane protein	SAUSA300_1912	1	92,6	-
NE1188	putative membrane protein	SAUSA300_1912	3	101,9	61,5
NE1193	*sar*A, accessory regulator A	SAUSA300_0605	5	94,9	18,0
NE1224	Fe/Mn family superoxide dismutase	SAUSA300_0135	1	91,2	-
NE1234	*lct*P, L-lactate permease	SAUSA300_0112	3	86.6	30.9
NE1240	*luk*D, leukotoxin LukD	SAUSA300_1768	1	101,2	-
NE1296	*sae*S, sensor histidine kinase SaeS	SAUSA300_0690	5	66,0	9,9
NE1304	*rsp*, transcription regulatory protein	SAUSA300_2326	5	82,3	7,9
NE1354	Alpha-hemolysin precursor	SAUSA300_1058	3	85,3	23,1
NE1363	*srt*B, sortase B	SAUSA300_1034	1	109,6	-
NE1366	catalase	SAUSA300_1232	2	99,9	27,1
NE1386	*luk*A, Leukocidin/Hemolysin toxin family protein	SAUSA300_1974	4	35,9	13,7
NE1399	*hlg*A, gamma-hemolysin component A	SAUSA300_2365	2	94,0	19,2
NE1449	*hlg*C, gamma-hemolysin component C	SAUSA300_2366	1	89,4	-
NE1472	*rsb*W, anti-sigma-B factor, serine-protein kinase	SAUSA300_2023	3	25,6	9,2
NE1532	*agr*A, accessory gene regulator protein A	SAUSA300_1992	5	6,4	4,4
NE1555	*cod*Y, transcriptional repressor CodY	SAUSA300_1148	4	92,2	12,2
NE1607	*rsb*U, sigma-B regulation protein	SAUSA300_2025	3	53,3	24,8
NE1663	diacylglycerol glucosyltransferase	SAUSA300_0918	4	93,3	12,3
NE1682	*hlg*B, gamma-hemolysin component B	SAUSA300_2367	1	104,0	-
NE1714	GTP pyrophosphokinase	SAUSA300_1590	3	74,7	6,5
NE1756	sensor histidine kinase	SAUSA300_0646	1	86,5	-
NE1775	triacylglycerol lipase	SAUSA300_0320	1	95,9	-
NE1787	*srt*A, sortase	SAUSA300_2467	1	112,3	-
NE1848	*luk*S-PV, Panton-Valentine leukocidin, LukS-PV	SAUSA300_1382	3	133,8	23,9
NE1868	*mec*A, penicillin-binding protein 2'	SAUSA300_0032	1	106,2	-
NE1872	*rsb*V, anti-sigma-B factor, antagonist	SAUSA300_2024	2	102,5	68,1
NE1875	truncated beta-hemolysin	SAUSA300_1918	2	118,3	38,1
NE1908	ABC transporter ATP-binding protein	SAUSA300_1911	3	29,3	18,3

Table 7.2: Host cell genes downregulated after 30min of phevalin treatment

Gene name	Protein name	Norm. ratio 1 -3			Mean
Proline-rich protein 3	PRR3	NaN	0,04	0,04	**0,0**
Dermcidin;Survival-promoting peptide;DCD-1	DCD	0,05	0,04	NaN	**0,0**
Apolipoprotein C-III	APOC3	0,01	0,08	NaN	**0,0**
Cornulin	CRNN	NaN	0,04	0,05	**0,0**
Coiled-coil domain-containing protein 127	CCDC127	0,04	0,06	0,08	**0,1**
Lysozyme C	LYZ	0,07	0,07	NaN	**0,1**
Centriolin	CNTRL	0,07	0,10	NaN	**0,1**
ELL-associated factor 1	EAF1	0,08	NaN	0,33	**0,2**
Golgi apparatus membrane protein TVP23 homolog C;Golgi apparatus membrane protein TVP23 homolog B	TVP23C;TVP23B	NaN	0,32	0,30	**0,3**
Fibronectin;Anastellin;Ugl-Y1;Ugl-Y2;Ugl-Y3	FN1	0,28	0,44	0,26	**0,3**
Ubiquitin carboxyl-terminal hydrolase 16	USP16	0,39	NaN	0,39	**0,4**
Gem-associated protein 2	GEMIN2	0,35	0,47	NaN	**0,4**
SH3 domain-binding protein 5	SH3BP5	0,59	0,24	NaN	**0,4**
RanBP-type and C3HC4-type zinc finger-containing protein 1	RBCK1	0,73	0,37	0,46	**0,5**
RNA-binding protein 12B	RBM12B	0,70	0,35	0,66	**0,6**
WD repeat domain phosphoinositide-interacting protein 4	WDR45	0,53	0,65	0,62	**0,6**
Meiosis arrest female protein 1	KIAA0430	NaN	0,55	0,66	**0,6**
tRNA:m(4)X modification enzyme TRM13 homolog	TRMT13	0,47	0,75	0,61	**0,6**
B-cell lymphoma/leukemia 10	BCL10	0,62	NaN	0,66	**0,6**
Protein phosphatase inhibitor 2;Protein phosphatase inhibitor 2-like protein 3	PPP1R2;PPP1R2P3	0,63	0,69	0,61	**0,6**
Inverted formin-2	INF2	0,70	0,64	0,64	**0,7**

Table 7.3: Host cell genes downregulated after 3h of phevalin treatment

Gene name	Protein name	Norm. ratio 1 -3			Mean
Dermcidin;Survival-promoting peptide;DCD-1	DCD	0,03	0,06	0,07	**0,1**
Centriolin	CNTRL	0,09	0,05	0,04	**0,1**
Apolipoprotein C-III	APOC3	0,17	0,04	NaN	**0,1**
Coiled-coil domain-containing protein 127	CCDC127	0,09	NaN	0,13	**0,1**

Antithrombin-III	SERPINC1	0,18	0,18	NaN	**0,2**
60S ribosome subunit biogenesis protein NIP7 homolog	NIP7	0,20	0,29	0,14	**0,2**
Glutathione S-transferase A3	GSTA3	0,37	0,16	NaN	**0,3**
Membrane protein FAM174A	FAM174A	NaN	0,51	0,08	**0,3**
Protein FAM208B	FAM208B	0,32	0,43	0,32	**0,4**
Isoprenoid synthase domain-containing protein	ISPD	NaN	0,47	0,30	**0,4**
Synaptosomal-associated protein 25	SNAP25	NaN	0,57	0,25	**0,4**
Uncharacterized protein C18orf25	C18orf25	NaN	0,27	0,59	**0,4**
Interferon-induced 35 kDa protein	IFI35	NaN	0,35	0,56	**0,5**
Protein phosphatase inhibitor 2;Protein phosphatase inhibitor 2-like protein 3	PPP1R2;PPP1R2P3	0,44	0,49	0,46	**0,5**
NAD(P)H dehydrogenase [quinone] 1	NQO1	0,34	0,76	0,38	**0,5**
Gem-associated protein 2	GEMIN2	0,43	NaN	0,63	**0,5**
Non-canonical poly(A) RNA polymerase PAPD7	PAPD7	0,69	0,35	0,63	**0,6**
Thiopurine S-methyltransferase	TPMT	0,39	0,64	0,67	**0,6**
Cytosolic endo-beta-N-acetylglucosaminidase	ENGASE	NaN	0,62	0,52	**0,6**
Piezo-type mechanosensitive ion channel component 1	PIEZO1	0,62	0,66	0,48	**0,6**
E3 ubiquitin-protein ligase RNF138	RNF138	0,65	0,80	0,60	**0,7**

Table 7.4: Host cell genes upregulated after 30min of phevalin treatment

Gene name	Protein name	Norm. ratio 1 -3			Mean
Hemicentin-2	HMCN2	NaN	35,94	14,45	**25,2**
HEAT repeat-containing protein 5B	HEATR5B	5,66	NaN	4,82	**5,2**
E2F-associated phosphoprotein	EAPP	NaN	6,27	3,70	**5,0**
AP-1 complex subunit sigma-2	AP1S2	NaN	5,97	3,30	**4,6**
CDK2-associated and cullin domain-containing protein 1	CACUL1	2,64	1,82	NaN	**2,2**
Tuberin	TSC2	3,19	1,86	1,44	**2,2**
Cytochrome c oxidase subunit 7A2, mitochondrial	COX7A2	2,69	1,50	1,71	**2,0**
Nuclear receptor coactivator 3	NCOA3	1,33	2,34	1,96	**1,9**
Integrin beta-1-binding protein 1	ITGB1BP1	1,97	NaN	1,54	**1,8**
R3H domain-containing protein 2	R3HDM2	1,89	NaN	1,53	**1,7**
Receptor-interacting serine/threonine-protein kinase 2	RIPK2	1,68	1,86	1,51	**1,7**
Zinc finger CCHC-type and RNA-binding motif-containing protein 1	ZCRB1	1,30	2,19	1,56	**1,7**

Table 7.5: Host cell genes upregulated after 3h of phevalin treatment

Gene name	Protein name	Norm. ratio 1 -3			Mean
BUD13 homolog	BUD13	7,65	3,28	NaN	**5,5**
Lysine-specific demethylase 3A	KDM3A	6,35	1,43	1,92	**3,2**
Egl nine homolog 1	EGLN1	2,16	NaN	4,19	**3,2**
HEAT repeat-containing protein 5B	HEATR5B	NaN	2,56	3,68	**3,1**
Mitochondrial calcium uniporter regulator 1	MCUR1	1,57	1,62	2,99	**2,1**
Vacuolar protein sorting-associated protein 26B	VPS26B	1,78	NaN	1,63	**1,7**
Calcium uniporter protein, mitochondrial	MCU	1,56	NaN	1,81	**1,7**
SOSS complex subunit B2	NABP1	NaN	1,51	1,53	**1,5**

Table 7.6: Protein phosphorylation sites regulated in HeLa cells after 30 min or 3 h incubation with phevalin

Gene name	Protein name	Amino Acid	Position	Mean Reg 30 min	Mean Reg 3 h
Zinc finger protein 148	ZNF148	S	306	**70,97**	**106,40**
Zinc finger protein 148	ZNF148	S	311	**70,97**	**106,40**
Zinc finger protein 148	ZNF148	T	305	**22,64**	**106,40**
LIM domain only protein 7	LMO7	S	417	**11,62**	**5,83**
E3 ubiquitin-protein ligase HUWE1	HUWE1	S	3539	**8,24**	**6,68**
Histone deacetylase complex subunit SAP130	SAP130	S	896	**7,88**	**-**
Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2	T	360	**7,81**	**2,58**
Pre-mRNA cleavage complex 2 protein Pcf11	PCF11	S	368	**7,06**	**-**
Myotubularin-related protein 3	MTMR3	S	633	**5,66**	**2,20**
Pericentrin	PCNT	S	3242	**5,57**	**-**
Shootin-1	KIAA1598	S	494	**5,47**	**-**
SH2B adapter protein 1	SH2B1	S	125	**4,39**	**3,34**
SH2B adapter protein 1	SH2B1	S	126	**4,39**	**2,45**
Borealin	CDCA8	S	219	**4,33**	**2,46**
Band 4.1-like protein 1	EPB41L1	S	578	**3,68**	**1,37**
Histone deacetylase 2	HDAC2	S	392	**3,64**	**0,95**

Pre-mRNA cleavage complex 2 protein Pcf11	PCF11	S	370	**2,93**	**2,87**
Na(+)/H(+) exchange regulatory cofactor NHE-RF2	SLC9A3R2	S	20	**2,91**	**4,09**
Pre-mRNA cleavage complex 2 protein Pcf11	PCF11	S	372	**2,71**	**2,87**
Rapamycin-insensitive companion of mTOR	RICTOR	S	21	**2,42**	**2,19**
Mitogen-activated protein kinase-binding protein 1	MAPKBP1	S	764	**2,40**	**1,88**
Histone-lysine N-methyltransferase, H3 lysine-79 specific	DOT1L	S	1032	**2,35**	**0,79**
Remodeling and spacing factor 1	RSF1	S	573	**2,32**	**12,72**
Protein ITFG3	ITFG3	S	21	**2,16**	**1,01**
Fanconi anemia group J protein	BRIP1	S	128	**2,07**	**1,44**
Chromatin assembly factor 1 subunit A	CHAF1A	S	141	**1,99**	**1,08**
G protein-regulated inducer of neurite outgrowth 1	GPRIN1	S	452	**1,98**	**-**
Insulin receptor substrate 2	IRS2	S	577	**1,98**	**-**
SH3 domain-binding protein 5-like	SH3BP5L	S	362	**1,90**	**1,47**
tRNA (guanine-N(7)-)-methyltransferase	METTL1	S	27	**1,83**	**1,98**
Polymerase I and transcript release factor	PTRF	T	302	**1,79**	**0,68**
UPF0562 protein C7orf55;Putative RNA-binding protein Luc7-like 2	C7orf55;LUC7L2	S	419	**1,77**	**0,89**
Beta-parvin	PARVB	S	7	**1,76**	**0,83**
SH3 domain-binding protein 5-like	SH3BP5L	S	358	**1,65**	**1,76**
E3 ubiquitin-protein ligase NEDD4-like	NEDD4L	S	305	**1,61**	**-**
Ankyrin repeat and SAM domain-containing protein 1A	ANKS1A	S	663	**1,56**	**-**
Remodeling and spacing factor 1	RSF1	S	366	**1,51**	**1,50**
CTD small phosphatase-like protein 2	CTDSPL2	S	104	**1,47**	**2,64**
Nuclear factor 1 B-type	NFIB	S	312	**1,36**	**0,43**
C2 domain-containing protein 5	C2CD5	S	659	**1,29**	**1,33**
Coilin	COIL	T	122	**1,29**	**1,40**
Centrosomal protein of 131 kDa	CEP131	T	93	**1,22**	**0,45**
Mitogen-activated protein kinase kinase kinase MLT	ZAK	S	593	**1,11**	**1,68**
Ankyrin repeat domain-containing protein 17	ANKRD17	S	1939	**1,04**	**0,54**
E3 SUMO-protein ligase NSE2	NSMCE2	S	116	**1,01**	**0,44**
La-related protein 1	LARP1	T	858	**0,99**	**3,91**
Centrosomal protein of 72 kDa	CEP72	S	237	**0,91**	**0,42**
Nucleophosmin	NPM1	T	219	**0,89**	**0,44**

Neuroblast differentiation-associated protein AHNAK	AHNAK	S	5863	**0,86**	**0,54**
Pyruvate kinase PKM	PKM	S	97	**0,81**	**3,46**
Nuclear factor 1 X-type	NFIX	S	293	**0,81**	**0,33**
Hematological and neurological expressed 1 protein;Hematological and neurological expressed 1 protein, N-terminally processed	HN1	S	80	**0,78**	**0,43**
Atrophin-1	ATN1	S	34	**0,72**	**0,49**
Zinc finger C3H1 domain-containing protein	ZFC3H1	S	655	**0,72**	**0,55**
Collagen alpha-1(I) chain	COL1A1	S	1125	**0,67**	**0,59**
Band 4.1-like protein 1	EPB41L1	S	541	**0,67**	**0,46**
Vasculin	GPBP1	S	314	**0,67**	**0,84**
Arginine/serine-rich coiled-coil protein 2	RSRC2	S	17	**0,64**	**0,47**
Mediator of DNA damage checkpoint protein 1	MDC1	T	869	**0,64**	**0,47**
Hepatocyte growth factor receptor	MET	Y	1235	**0,63**	**1,14**
Arginine/serine-rich coiled-coil protein 2	RSRC2	T	16	**0,61**	**0,38**
Protein VPRBP	VPRBP	S	894	**0,60**	
Zinc finger protein 574	ZNF574	T	306	**0,60**	**0,78**
Rho guanine nucleotide exchange factor 2	ARHGEF2	S	668	**0,58**	**0,70**
Spectrin beta chain, non-erythrocytic 1	SPTBN1	S	14	**0,54**	
ADP-ribosylation factor GTPase-activating protein 1	ARFGAP1	S	230	**0,53**	**0,42**
Dedicator of cytokinesis protein 5	DOCK5	S	1824	**0,53**	**0,55**
Rho guanine nucleotide exchange factor 16	ARHGEF16	S	174	**0,49**	**0,53**
WD repeat-containing protein 7	WDR7	S	935	**0,47**	**0,22**
Rho GTPase-activating protein 21	ARHGAP21	S	1095	**0,47**	**-**
RING finger protein 219	RNF219	S	210	**0,45**	**-**
Myosin phosphatase Rho-interacting protein	MPRIP	S	977	**0,42**	**0,93**
Huntingtin	HTT	S	419	**0,41**	**1,00**
N-acetyltransferase ESCO2	ESCO2	S	244	**0,40**	**-**
Eukaryotic translation initiation factor 2-alpha kinase 4	EIF2AK4	T	667	**0,32**	**0,26**
DNA-directed RNA polymerase I subunit RPA43	TWISTNB	T	322	**0,26**	**0,72**
Transcription factor HIVEP2	HIVEP2	S	2300	**0,22**	**0,18**
Cyclin-dependent kinase 12	CDK12	S	274	**0,20**	**0,76**
Cyclin-dependent kinase 12	CDK12	S	276	**0,20**	**0,76**

Nuclear receptor coactivator 2	NCOA2	S	493	**0,19**	**-**
Parathymosin	PTMS	S	2	**0,18**	**0,09**
Parathymosin	PTMS	S	5	**0,18**	**0,09**
Cytochrome c oxidase subunit 7A-related protein, mitochondrial	COX7A2L	Y	2	**0,08**	**0,08**
Remodeling and spacing factor 1	RSF1	S	1251	**0,08**	**0,13**
TATA-binding protein-associated factor 172	BTAF1	S	91	**-**	**0,09**
Tumor necrosis factor alpha-induced protein 3;A20p50;A20p37	TNFAIP3	S	641	**-**	**0,59**
Transcription factor HIVEP2	HIVEP2	S	2301	**-**	**0,14**
Enhancer of mRNA-decapping protein 4	EDC4	S	780	**-**	**2,87**
Protein KIBRA	WWC1	S	931	**-**	**0,51**
Ubiquitin carboxyl-terminal hydrolase BAP1	BAP1	S	325	**-**	**0,24**
Death-inducer obliterator 1	DIDO1	S	1312	**-**	**0,45**

7.5 Publications and poster presentations

Publications

Blättner S., Das S., Paprotka K., Eilers U., Krischke M., Kretschmer D., Rennele CW., Dittrich M., Müller T., Schuelein-Voelk C., Hertlein T., Mueller MJ., Huettel B., Reinhardt R., Ohlsen K., Rudel T., Fraunholz MJ., (2016) Staphylococcus aureus Exploits a Non-ribosomal Cyclic Dipeptide to Modulate Survival within Epithelial Cells and Phagocytes. PLoS Pathog 12(9): e1005857. doi: 10.1371/journal.ppat.1005857

Das S, Lindemann C, Young BC, Muller J, Österreich B, Ternette N, Winkler AC, Paprotka K, Reinhardt R, Förstner KU, Allen E, Flaxman A, Yamaguchi Y, Rollier CS, van Diemen P, **Blättner S**, Remmele CW, Selle M, Dittrich M, Müller T, Vogel J, Ohlsen K, Crook DW, Massey R, Wilson DJ, Rudel T, Wyllie DH, Fraunholz MJ., (2016) Natural mutations in a Staphylococcus aureus virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation. Proc Natl Acad Sci U S A. 31;113(22):E3101-10. doi: 10.1073/pnas.1520255113.

Poster presentations

Blättner S., Das S., Eilers U., Schuelein-Voelk C., Rudel T., Fraunholz MJ., (2014) Identification of factors mediating phagolysosomal escape of Staphylococcus aureus, 3rd Mol Micro Meeting Wuerzburg

Blättner S., Eilers U., Schuelein-Voelk C., Eilers M., Fraunholz MJ., (2014) Novel Staphylococcus aureus phagosomal escape factors identified by automated microscopy, 4. Gemeinsame Konferenz von DGHM und VAAM

Blättner S., Das S., Eilers U., Krischke M., Kretschmer D., Schuelein-Voelk C., Eilers M., Rudel T., Fraunholz MJ., (2015) A non-ribosomal peptide synthase is important for intracellular survival of Staphylococcus aureus, EMBL Symposium: New Approaches and Concepts in Microbiology

7.6 Danksagung

Meinen herzlichen Dank möchte ich ausdrücken an…

…Dr. Martin Fraunholz, dafür, dass er beständig an mich und an meine Arbeit geglaubt hat, dass er mir immer mit seinem Rat zur Seite stand und dafür, dass er mir dabei geholfen hat diese Thesis in ihrer jetzigen Form zu vollenden.

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…meinen ganz besonderen Schatz, Natalie, die mir immer ein Halt war, auch in unruhigeren Zeiten, in denen ich gern an mir und dem Weg, den ich beschritten hatte, zweifelte. Du bist meine Sonne, die mir Kraft gibt und du bist mein Stern, der mir den Weg zeigt, wenn ich einmal nicht weiter weiß. Ich liebe dich.

7.7 Affidavit

I hereby confirm that my thesis entitled "The role of the non-ribosomal peptide synthetase and its product phevalin in intracellular virulence of *Staphylococcus aureus*" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Hiermit erkläre ich an Eides statt, die Dissertation „The role of the non-ribosomal peptide synthetase and its product phevalin in intracellular virulence of *Staphylococcus aureus*" eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

7.8 Curriculum vitae

Berufliche Erfahrungen

[redacted]

10/2012 – 03/2016 Wissenschaftlicher Mitarbeiter, Lehrstuhl für Mikrobiologie, Universität Würzburg

- Pathogen-Wirt Interaktionen in vitro / in vivo
- Analyse diverser Hochdurchsatz-Datensätze in den Bereichen Metabolomics, Proteomics und Phosphoproteomics
- Analyse von mikroskopischen Hochdurchsatz-Experimenten
- Zellkultur und lentivirale Manipulation humaner Zellen

01/2012 – 05/2012 Hilfswissenschaftler, Lehrstuhl für Mikrobiologie, Technische Universität Kaiserslautern

- Genetische Manipulation gram-positiver Bakterien
- Evaluierung bakterieller Antibiotika-Resistenzspektren

Akademische Ausbildung

10/2012 - 12/2016 Promotionsstudent, Lehrstuhl für Mikrobiologie, Universität Würzburg

- Betreuung und Einarbeitung von Studenten auf Master- sowie Bachelor-Niveau
- Erarbeitung und Standardisierung molekular- und mikrobiologischer Experimente

[redacted]

[redacted]

Kenntnisse

Sprachen	• Englisch – Fließend • Französisch - Grundkenntnisse
EDV	• MS Office • Statistica
Weiterbildungen	• Quality Management and Audit in Biotech Industries • Good Clinical Practice (GCP) • Neuere Methoden der Zellanalytik • Patent Law in the Life Sciences • Good Scientific Practice • GLP - GMP

Publikationen

Das S, Lindemann C, Young BC, Muller J, Österreich B, Ternette N, Winkler AC, Paprotka K, Reinhardt R, Förstner KU, Allen E, Flaxman A, Yamaguchi Y, Rollier CS, van Diemen P, **Blättner S**, Remmele CW, Selle M, Dittrich M, Müller T, Vogel J, Ohlsen K, Crook DW, Massey R, Wilson DJ, Rudel T, Wyllie DH, Fraunholz MJ., Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation., Proc Natl Acad Sci U S A. 2016 May 31;113(22):E3101-10.

Blättner S, Das S, Paprotka K, Eilers U, KrischkeM, Kretschmer D, Remmele C, Dittrich M, Müller T, Schühlein-Völk C, Hertlein T, Müller MJ, Hüttel B, Reinhardt R, Ohlsen K, Rudel T, Fraunholz MJ., *Staphylococcus aureus* exploits a non-ribosomal cyclic dipeptide to modulate survival within epithelial cells and phagocytes., Plos Pathog 12(9): e1005857. doi: 10.1371/journal.ppat.1005857

Würzburg, den 01.12.2016