Interaction of Sa3int Prophages with their Host Bacterium *Staphylococcus aureus*

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Summary

Prophages are highly abundant within *S. aureus* isolates and represent a major part of the staphylococcal accessory genome with some prophages providing *S. aureus* with additional virulence traits. Staphylococcal prophages are categorized according to homology of their respective integrase gene (*int*). Those of the Sa3int category are of particular importance, especially in the context of host jump and adaption to the human host. Sa3int prophages reside in most human colonizing *S. aureus* isolates whereas in animal isolates they are mostly absent. In addition, Sa3int prophages carry several human specific virulence genes on their genomes, thereby aiding survival of their host bacterium in the human host.

The major aims of this thesis were to 1) identify differences in the phage life cycle in different host strain backgrounds and to 2) decipher molecular mechanisms involved in the interaction between the host bacterium and the phage.

In this work, I show that the biology of Sa3int prophages (Φ13 and ΦN315) is dependent on the staphylococcal host strain background. This indicates that so far, ill-defined bacterial factors interfere with phage biology. In detail, spontaneous transfer within co-culture, lysogenization and induction of Sa3int prophages is distinctive within different *S. aureus* isolates. This is caused by differences in phage replication, which resulted from differences in transcription of lytic phage genes, in particular of replication- and morphogenesis-associated genes. Prediction of transcriptional start sites (TSSs) on the Sa3int prophage Φ13 revealed strain-specific differences, which I hypothesize to be a consequence of variations in posttranscriptional processing of mRNAs. Furthermore, this implies that phage gene regulation is more complex than initially thought and putatively involves regulatory sRNAs. To answer the question whether presence of Sa3int prophages influences host gene expression in a strain-specific manner, I performed differential gene expression analysis. Several genes that show altered transcription in presence of prophage Φ13 were identified, for instance several proteases (*sspA*, *sspB* and *aur*). That indicates that modulation of the expression of host virulence genes might influence bacterial colonization. Using a customized bioinformatical analysis workflow, we successfully performed the first differential gene expression analysis for the direct comparison of two *S. aureus* isolates that belong to different clonal lineages. Application of this workflow led to the identification of UvrA, playing a previously unknown role in phage replication. The thesis in hand gives general and comprehensive insights in strain specific differences in key stages of the Sa3int phage life cycle and the interaction with its host *Staphylococcus aureus*.

Zusammenfassung

Prophagen sind sehr häufig in *S. aureus* Isolaten zu finden und stellen einen großen Teil des akzessorischen Genoms dar. Zudem sind sie in der Lage zusätzliche Virulenzeigenschaften auf *S. aureus* zu übertragen. Prophagen von Staphylokokken werden entsprechend der Ähnlichkeit ihrer Integrase (*int*) in unterschiedliche Kategorien eingeteilt. Prophagen der Kategorie Sa3int spielen vor allem in Bezug auf Besiedlung und Anpassung an den humanen Wirt eine besondere Rolle. Sie kommen in den meisten von Menschen isolierten *S. aureus* Stämmen vor, sind jedoch kaum in Tierisolaten zu finden. Zudem tragen Sa3int Prophagen humanspezifische Virulenzgene, die zur Anpassung von *S. aureus* an den menschlichen Wirt beitragen und dessen Virulenz erhöhen.

Die Ziele dieser Arbeit war es zum einen, wirtsspezifische Unterschiede in der Phagenbiologie aufzuklären und im zweiten Schritt die zugrundeliegenden molekularen Mechanismen, welche die Wechselwirkung zwischen Bakterien und Phagen regulieren, zu entschlüsseln.

In dieser Arbeit konnte ich zeigen, dass die Biologie von Sa3int Modellphagen (Φ13 und ΦN315), speziell in Bezug auf den Stammhintergrund, starke Unterschiede ausweist. Dies deutet darauf hin, dass bislang unzureichend untersuchte bakterielle Faktoren, die Phagenbiologie mit beeinflussen. Im Einzelnen wurden Unterschiede in der spontanen Übertragung von Sa3int Phagen in Ko-Kulturen, in der Lysogenisierung und in der Induktion zwischen den verschiedenen Wirtsstämmen als Zielgröße herangezogen. Die Abweichungen waren auf Unterschiede in der Phagenreplikation zurückzuführen. Die wiederrum durch Unterschiede in der Transkription von lytischen Phagengenen, genauer von im Replikations- und Morphogenesemodul kodierten Genen, begründet waren.

Die Vorhersage von Transkriptionsstartstellen (TSSs) auf Sa3int Prophage Φ13 zeigte ebenfalls stammspezifische Unterschiede. Meiner Meinung nach sind diese eine Konsequenz von Unterschieden in der posttranskriptionellen Prozessierung der mRNAs. Dies deutet darauf hin, dass Genregulation auf Prophagen deutlich komplexer ist, als ursprünglich angenommen wurde und möglicherweise regulatorische sRNAs involviert. Des Weiteren habe ich untersucht, ob die Präsenz von Sa3int Prophagen die Genexpression des Wirtsbakteriums beeinflusst. Differenziellen Genexpressionsanalysen identifizierten mehrere Gene, die in Präsenz des Prophagen ein geändertes Expressionsmuster zeigten. Als Beispiel können Proteasen (wie *sspA*, *sspB* und *aur*) genannt werden. Dies deutet auf eine Anpassung der Expression von Virulenzgenen des Wirtes hin, die möglicherweise Einfluss auf den Kolonisationsprozess hat. Wir haben einen, bioinformatischen Arbeitsablauf etabliert welcher erfolgreich für die erste differentielle Genexpressionsanalyse und somit den direkten

Vergleich zweier *S. aureus* Stämme aus unterschiedlichen klonalen Komplexen angewandt wurde. Mit Hilfe dieses bioinformatischen Ansatzes fanden wir heraus, dass UvrA einen bislang unbekannten Einfluss auf die Phagenreplikation hat. Die vorgelegte Arbeit gibt einen umfangreichen Einblick in die stammspezifischen Unterschiede auf verschiedenen Ebenen des Lebenszyklus von Sa3int Phagen und dessen Interaktion mit dem Wirtsbakterium *Staphylococcus aureus*.

List of publications

Part I

Accepted publications

The role of *hlb*-converting bacteriophages in *Staphylococcus aureus* host adaption

Carina Rohmer and Christiane Wolz

Review article

Microbial Physiology (2021); 31:109-122. doi: 10.1159/000516645

Part II

Manuscript I ready for submission

Influence of *Staphylococcus aureus* strain background on Sa3int phage life cycle

Carina Rohmer[1], Ronja Dobritz[1], Esther Lehmann[1], Shilpa E. George[1,2], Dilek Tuncbilek-Dere[3], Jeffrey J. Power[4], Natalya Korn[1], David Gerlach[1,5], Kay Nieselt[3], Taeok Bae[6], Christiane Wolz[1,4]

Part III

Manuscript II ready for submission

Mutual interaction between staphylococcal factors and Sa3int prophages

Carina Rohmer[1], Jeffrey J. Power[2], Esther Lehmann[1], Shilpa E. George[1,3], Ronja Dobritz[1] , Natalya Korn[1], Christiane Wolz[1,2]

Personal contributions to publications and manuscripts

Accepted publication:

The role of *hlb*-converting bacteriophages in *Staphylococcus aureus* host adaption
Carina Rohmer and Christiane Wolz

For this review article, I was mainly involved in conception of the article and contributed by doing literature research and writing main parts of the manuscript (Abstract, Introduction, Main text, Conclusion) under the supervision of Christiane Wolz. In cooperation with Christiane Wolz, I was involved in manuscript editing and Figure design.

Manuscripts ready for submission

Influence of *Staphylococcus aureus* strain background on Sa3int phage life cycle
Carina Rohmer[1], Ronja Dobritz[1], Esther Lehmann[1], Shilpa E. George[1,2], Dilek Tuncbilek-Dere[3], Jeffrey J. Power[4], Natalya Korn[1], David Gerlach[1,5], Kay Nieselt[3], Taeok Bae[6], Christiane Wolz[1,4]

For this research article, I made major contributions to the conception of this manuscript and experimental design under the supervision of Christiane Wolz. I performed all biological assays which are not stated below as being performed by co-workers. Biological assays concerning phage transfer assays (Figure 1), induction assays (Figure 4 and 5) and Northern blot (Figure 5) were in part performed by Ronja Dobritz. Adsorption assay (Figure 3) was performed by Esther Lehmann. Conception and design of mutants was done by me, production of lysogens and mutants was performed by Natalya Korn and me. Construction and production of ΦN315-tet was performed by David Gerlach. USA300c and Newman-c were kindly provided by T. Bae. Adaption of reference sequences used for RNAseq and differential gene expression analysis was done by Shilpa E. George and me. I performed differential gene expression analysis with help by Jeffrey J. Power. Prediction of transcriptional start sites was performed by Dilek Tuncbilek-Dere and Kay Nieselt. I was mainly involved in data analysis and interpretation of all biological experiments, differential gene expression analysis and interpretation of TSS prediction as well as figure design and writing of the manuscript under the supervision of Christiane Wolz.

Mutual interaction between staphylococcal factors and Sa3int prophages

Carina Rohmer[1], Jeffrey J. Power[2], Esther Lehmann[1], Shilpa E. George[1,3], Ronja Dobritz[1], Natalya Korn[1], Christiane Wolz[1,2]

For this research article, I made major contributions to the conception of this manuscript and experimental design under the supervision of Christiane Wolz. I performed all biological assays which are not stated below as being performed by co-workers. Conception of mutants was done by me, mutant construction was performed by Natalya Korn. Induction assay of mutant strains was in part performed by Ronja Dobritz. Gene alignment serving as basis for differential gene expression analysis was done by Esther Lehmann and me. Adaption of reference sequences for differential gene expression analysis was done by Shilpa E. George and me. Differential gene expression analysis concerning prophage influence onto host bacterium and influence of mitomycin C addition was performed by me. Establishment of DeSeq2 workflow was done by Jeffrey J. Power. Analysis and data evaluation of obtained differential gene expression analysis using DeSeq2 was done by Jeffrey J. Power and me. I was mainly involved in data analysis and data interpretation as well as figure design and writing of the manuscript under the supervision of Christiane Wolz.

General introduction

Staphylococcus aureus

Definition, epidemiology and lineages

The gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is an important human pathogen that persistently colonizes the nasal cavity of 20-30% of the human population. In addition, another 30% of the population are intermittent carriers and therefore not constantly colonized by *S. aureus* (VandenBergh et al., 1999; Wertheim et al., 2005). Colonization is dependent on several factors like human genetic factors, host immunity, bacterial composition and also environmental factors (Mulcahy & McLoughlin, 2016). In general, carriage of *S. aureus* in the nasal cavity represents a major risk factor for infection (van Belkum et al., 2009; Wertheim et al., 2005). These vary from skin infections to invasive infections like bacteremia, endocarditis, sepsis and toxic shock syndrome (Lowy, 1998). Often, they are hard to treat due to increasing resistance of *Staphylococcus au*reus towards several antibiotics. Resulting from antibiotic pressure, resistance against methicillin or vancomycin developed in staphylococcal isolates (MRSA or VISA, respectively) - for review, see (Appelbaum, 2006). Methicillin-resistance is associated with carriage of the *mecA* gene, a penicillin binding protein encoded on a staphylococcal cassette chromosome (SCCmec) of which different types are described (Ito et al., 2014). Further, antibiotic-resistant *S. aureus* isolates are often classified in an epidemiological context. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) occur in individuals within communities whereas healthcare-associated MRSA strains are predominantly isolated from hospital-acquired infections but also linked to healthcare and nursing homes (Millar et al., 2007). Further, livestock-associated *S. aureus* isolates (LA-MRSA) are associated with pig-farmers or other persons with close animal contact, revealing high risk for human and animals concerning the zoonotic potential of cross-species infection. It has been shown that *S. aureus* LA-MRSA is actively transmitted between human and animals as well as between humans (Armand-Lefevre et al., 2005; Voss et al., 2005). Epidemiology is monitored by typing methods based on sequence comparison of seven specific house-keeping genes of the staphylococcal core genome, with multi-locus sequence typing (MLST) being widely accepted (Enright et al., 2000; Saunders & Holmes, 2007). Based on the similarity of these house-keeping genes, isolates are categorized into different sequence-types (ST) which are basis for categorization into clonal complexes (CC) (Feil et al., 2003). For instance, CC1 or CC8 are predominantly composed of human isolates (Feil et al., 2003) whereas CC398 or CC97 are predominantly associated with pigs or cattle isolates, respectively (Price et al., 2012; Spoor et al., 2013). So far no

association between specific clonal complexes and human invasive *S. aureus* isolates has been identified (Lilje et al., 2017; Lindsay et al., 2006).

Wall-teichoic acids (WTA)

Adhesion to epithelial surfaces is a key step in colonization by *S. aureus*. This process is mediated by a large repertoire of adhesion molecules, including the cell wall glycopolymer wall teichoic acid (WTA), a major component of the gram-positive cell envelope. WTA of *S. aureus* is composed of a poly-ribitol-phosphate (RboP) backbone, which is attached to peptidoglycan by phosphodiester bonds (Koç et al., 2015). Secondary modifications consist of D-alanine and *N*-acetylglucosamine (GlcNAc) residues, the latter of which are attached by glycosyltransferases TarM, TarS and TarP (Gerlach et al., 2018; Weidenmaier et al., 2004; Winstel et al., 2014). Besides its role in initial stages of nasal colonization, WTA plays a crucial role in the establishment of endovascular infections and in abscess formation (Weidenmaier & Lee, 2017). The function of this glycopolymer-adhesin as a virulence factor is further underlined by being the major phage receptor of *S. aureus* (Koç et al., 2016; Xia et al., 2011). WTA is therefore essential for recognition and binding by staphylococcal phages.

Mobile genetic elements (MGEs) and restriction modification (R-M) systems

The *S. aureus* genome is comprised of the core genome and in addition, approximately 15% of DNA assigned to MGEs, that represent a major part of the *S. aureus* accessory genome (Lindsay & Holden, 2004). The ability of *S. aureus* to cause severe infection is associated with a vast number of virulence genes encoded on both the accessory as well as the core genome (Gill et al., 2011). MGEs comprise of transposons, plasmids, staphylococcal cassette chromosomes (SCCs), *S. aureus* pathogenicity islands (SaPIs) and prophages. SCCs and plasmids are predominantly associated with resistance genes whereas SaPIs and prophages are associated with carriage of virulence genes (Lindsay & Holden, 2004; Lindsay & Holden, 2006). MGEs can be exchanged between different *S. aureus* strains in a process known as horizontal gene transfer (HGT). This is mainly mediated by bacteriophages which, by chance, pack random pieces of host DNA instead of own phage DNA. These phages are also able to bind to WTA present on other bacterial cells, resulting in ejection and transfer of carried nucleic acid. Transfer of genetic material by bacteriophages is termed transduction. However, uptake of foreign DNA is controlled by restriction-modification (R-M) systems. R-M systems restrict transfer of foreign DNA and protect the integrity of the host genome (Murray, 2000). Type-I R-M systems (Sau1), which are present in all *S. aureus* isolates, consist of three genes (*sau1hdsR*, *sau1hdsM* and *sau1hdsS*) that are responsible for restriction, modification and specificity, respectively. Variations in Sau1-system limit the exchange of

DNA between different *S*. *aureus* lineages and thereby contribute to *S*. *aureus* clonal evolution (Waldron & Lindsay, 2006). Other R-M systems of *S*. *aureus* provide additional genetic barriers, such as SauUSI, the type-IV R-M system that recognizes specific cytosine methylations and thereby limits direct transformation of *E*. *coli* DNA to *S*. *aureus* (Xu et al., 2011). Thus, type-I and type-IV R-M systems represent barriers for transfer of DNA not only between bacterial species but also between different lineages of *S*. *aureus* (Jones et al., 2015). Regardless of the presence of R-M systems, HGT remains the major contributor to genome plasticity and the spread of virulence and resistance genes, especially within the same clonal lineage (McCarthy & Lindsay, 2012; McCarthy et al., 2014).

Staphylococcal regulatory network

The *S*. *aureus* genome encodes for a huge repertoire of virulence genes associated with immune evasion, host invasion and pathogenicity (Otto, 2010, 2014; Wang et al., 2019; Zecconi & Scali, 2013). The environmental conditions that *S*. *aureus* faces during the progression of an infection are changing fast, and therefore require strict regulation of virulence factors. Multiple regulators control expression of these genes, thereby creating a complex and intertwined regulatory network. Agr (accessory gene regulator) is a two-component system regulating, among many other processes, the expression of WTA, cell-wall associated proteins and polysaccharides like the polysaccharide capsule, as well as several toxins (alpha- and beta-hemolysin, leukocidins) and proteases (Arvidson & Tegmark, 2001; Bronner et al., 2004; Wanner et al., 2017). SarA (staphylococcal accessory regulator) is a DNA-binding protein and founding member of the SarA protein family that contains ten further SarA-homologues (e.g. SarS, Rot, MgrA etc.) (Cheung et al., 2008b). As a global transcriptional regulator, SarA is known to influence expression of many genes by binding to their promotors but also regulates its own expression (Cheung et al., 2008a). Three distinct promotors regulate *sarA* expression. Promotor 1 (P1) and P2 are controlled by the transcriptional sigma-factor σ^A, whereas P3 is controlled by the alternative transcriptional sigma-factor σ^B in response to metabolic stress (Manna et al., 1998). The alternative sigma-factor σ^B itself is involved in general stress responses and is regulated by anti-sigma factors RsbW and RsbV and phosphatase RsbU (Senn et al., 2005).

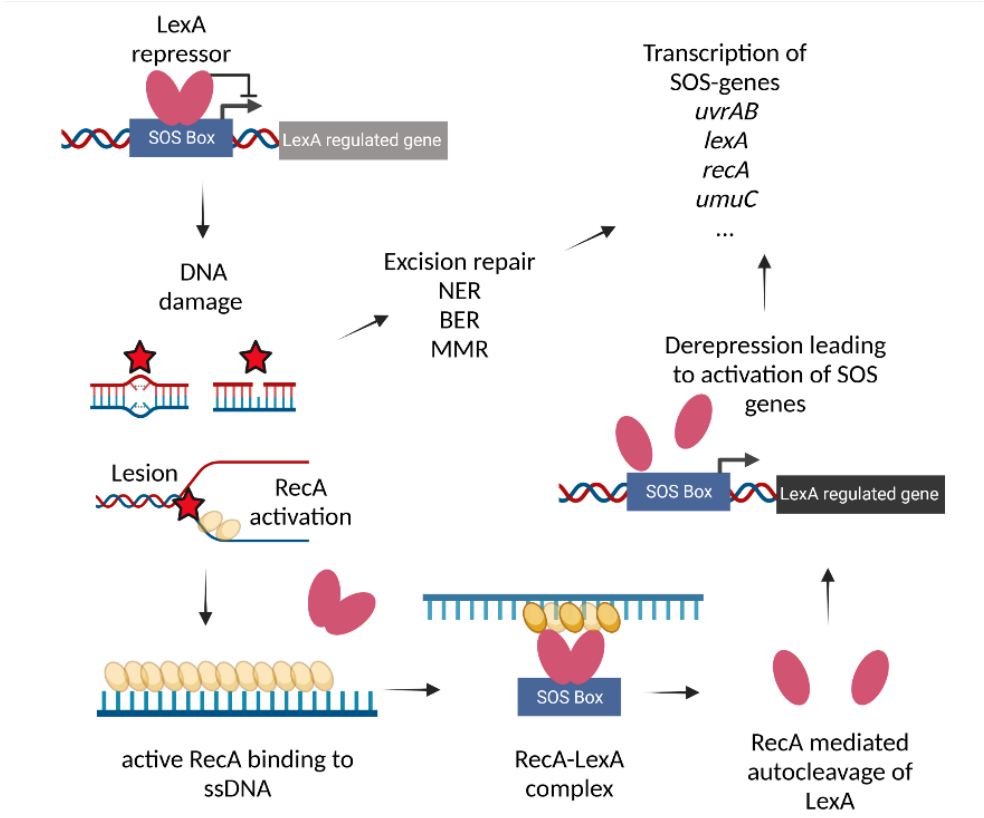
Rapid response to environmental changes on the transcriptional level not only involves sigma-factors or small RNAs, but also regulators reacting specifically to different kinds of cell stress. Presence or absence of oxygen and nutrients requires fast adaption of metabolism to ensure survival in the changing environment, such as different infection sites. In *S*. *aureus*, the carbon catabolite protein A (CcpA) regulates transcription of several genes in response to changes in glucose or fructose concentrations (Seidl et al., 2009), and is furthermore involved in arginine biosynthesis (Nuxoll et al., 2012). Repressor Rex enables adaption of *S.*

aureus to anaerobic conditions by sensing changing NAD+/NADH ratios. Inactivation of Rex results in de-repression of rex-regulated genes (Pagels et al., 2010). Anaerobic conditions are further known to induce genes that are involved in nitrite reduction, regulated by the oxygen-sensing nitrogen regulon (nreABC) (Schlag et al., 2008). ArcR is a regulator controlling expression of genes that enable usage of arginine as energy source under anaerobic conditions (Makhlin et al., 2007). In addition to carbon and oxygen, also iron is necessary for bacterial survival and is mostly available in complexed forms. Fur (ferric uptake repressor) is a repressor that is active in presence of intracellular iron, causing repression of iron-regulated genes by binding to their promotor region. Upon iron starvation, Fur is released allowing transcription of formerly repressed genes (Haley & Skaar, 2012; Maresso & Schneewind, 2006). In *S*. *aureus*, two further Fur-homologues, PerR, Zur and MntR are described (Tuomanen et al., 2001).

The SaeRS (*S*. *aureus* exoprotein expression) two-component system mainly regulates genes coding for secreted virulence factors (like hemolysins and leukocidins) involved in killing of neutrophils (Voyich et al., 2009). SaeRS therefore plays an important role during host infection were opsonophagocytic killing by leukocytes like neutrophils is the primary mode of defense against staphylococcal infections. However, *S*. *aureus* is able to survive phagocytosis by neutrophils, although the intracellular milieu (phagolysosome) contains antimicrobial agents, reactive oxygen species (ROS) and other enzymes to digest bacteria (Bongers et al., 2019). In *S*. *aureus*, DNA-damage by ROS is counteracted by yet another important regulatory system for bacterial survival – the SOS response.

SOS-response in Staphylococcus aureus

The SOS response pathway is induced by DNA damage, for instance by antibiotics, exposure to UV-light or reactive oxygen species. DNA-damaging antibiotics do so either directly by interacting with the nucleic acids, or indirectly through induction of ROS production. Direct interaction is described for ciprofloxacin, a fluoroquinolone antibiotic that induces double-stranded DNA breaks and delays of replication forks (Phillips et al., 1987), or mitomycin C, which inhibits DNA synthesis by forming covalent DNA cross-links (Suresh Kumar et al., 1997). DNA-damage is recognized by proteins of the SOS-response, a global regulatory network of which LexA and RecA are the major components (Cirz et al., 2007; Maslowska et al., 2019).



Schematic illustration of SOS-response
LexA-repressor protein blocks transcription of SOS genes by binding to their promotor regions. When DNA damage occurs, lesions (displays as stars) are recognized by RecA which binds to so single-stranded DNA forming a complex. Bound RecA promotes LexA autocleavage resulting in derepression of LexA-regulated genes followed by transcription of SOS genes. In parallel, other processed like mismatch repair (MMR), base exision repair (BER) and nucleotide exchange repair (NER) are induced.

LexA acts as repressor of the SOS-response by binding to specific promotor sequences (SOS-boxes) blocking transcription of SOS-related genes (Walker, 1984). When DNA-damage is sensed within the cell, LexA is cleaved by itself, leading to derepression of SOS genes. Beside LexA, RecA is constitutively present within bacterial cells and is involved in DNA repair. RecA forms complexes with single-stranded DNA that results from DNA damage. Thereby, it catalyzes strand exchange (homologous recombination) and promotes LexA-cleavage due to co-protease properties (Michel, 2005). Furthermore, RecA is described to cleave repressor molecules of bacteriophages in a similar way as LexA-cleavage, leading to derepression of lytic genes and initiate prophage induction (Roberts & Roberts, 1975). The SOS response subsequently induces multiple pathways involved in single strand DNA damage repair, like mismatch repair (MMR), base excision repair (BER) and nucleotide exchange repair (NER) (Ha & Edwards, 2021).

Nucleotide exchange reaction (NER)

The nucleotide excision repair (NER) pathway is one out of three known pathways involved in single strand damage repair. This pathway exists in both prokaryotes and eukaryotes, although they differ in the number of involved polypeptides (three in prokaryotes versus 19 in eukaryotes) (Friedberg et al., 2005). In prokaryotes, the NER system is best described for *E. coli* and *B. subtilis* (Houten, 1990; Lenhart et al., 2012). The activity of this pathway is mediated by three proteins (UvrA, UvrB and UvrC) that perform the first steps of DNA damage repair. The NER system recognizes helix-distorting lesions like thymine dimers or DNA crosslinks by UvrA and the $UvrA_2B$ complex. This triggers UvrA dissociation and recruitment of UvrC to the site of DNA damage. UvrC subsequently catalyzes removal of 10 to 15 nucleotides (Lenhart et al., 2012). Incisions made by these UvrABC excinucleases are further repaired by proteins like helicase, polymerase and ligases (Sancar, 1996). UvrD is also part of NER in *E. coli*, functioning as a helicase and causing the release of both the excised nucleotide-fragment and UvrC (Sancar, 2020).

In *E. coli* and *B. subtilis*, UvrA, UvrB and UvrC are regulated by LexA. In contrast, in *S. aureus uvrC* is encoded outside of operon and not SOS-response regulated (Cirz et al., 2007; Lenhart et al., 2012). In addition, UvrD is not present in *B. subtilis* or *S. aureus*, and is functionally replaced by the helicase PcrA.

Bacteriophages

General introduction

Bacteriophages in general are viruses which are only able to infect bacterial cells. Their discovery dates back to 1915 and 1917 when Frederick Twort and Felix d´Herelle independently discovered bacteriophages (Herelle, 1917; Twort, 1915) The total population of bacteriophages in the biosphere is estimated with 10^{31} particles (Comeau et al., 2008). Lytic bacteriophages use bacterial host cells only for propagation, resulting in death of the host cell due to lysis and release of progeny virions. However, many bacteriophages are able to establish a stable relationship with their host bacteria due to stable integration into the genome or by extra-chromosomal circulation as a plasmid (lysogenic conversion). In this state, the bacteriophage is residing as a prophage within its host bacterium, now termed a lysogen (Casjens, 2003). Prophages are distributed widely and so far, research indicates that they reside in most bacterial genomes. Prophages play an important role in bacterial virulence by carrying virulence genes. This has been described for cholera toxin CTX encoded on phage CTXΦ of *Vibrio cholerae* (Waldor & Mekalanos, 1996), neurotoxin C encoded by prophages Ceβ and CEγ of *Clostridium botulinum* (Eklund et al., 1971) and

diphteria toxin encoded by β-phage of *Corynebacterium diphteriae* (Freeman, 1951). By contributing to virulence, phages contribute to survival of their host and additionally protect it from infection by other phages (a phenomenon known as superinfection exclusion). Therefore, lysogenic conversion can be mutual advantageous. However, the prophage also exploits its host for DNA replication, maintaining the ability to cause cell death and release of progeny virions due to lysis (Canchaya et al., 2003).

Staphylococcal prophages

For *S*. *aureus*, multitudes of prophages are known and different *S*. *aureus* isolates usually carry one up to four prophages in their genomes. All known staphylococcal phages belong to the order of *Caudovirales* which are defined to contain double-stranded DNA as nucleic acid and are composed of head and tail morphology (Ackermann, 1999). Furthermore, prophages of *S*. *aureus* are assigned to the family of *Siphoviridae* whereas strictly lytic staphylococcal phages can also belong to the families of *Myoviridae* or *Podoviridae* (Deghorain & Van Melderen, 2012).

In 2019, Oliveira and colleagues investigated 205 staphylococcal phage genomes in a comparative genomic analysis. Staphylococcal phages possess a mosaic structure due to exchange, deletion or acquisition of open reading frames (ORF) or whole ORF modules with other phages. Furthermore, they also correlated the presence of prophages with the presence of virulence genes (Oliveira et al., 2019). Association of virulence genes and prophages as a consequence of lysogenic conversion was already described for staphylokinase, staphylococcal enterotoxin A and Panton-Valentine-Leukocidin (PVL) (Coleman et al., 1989; Kaneko et al., 1997). A more detailed association of virulence genes and specific prophage-categories was described by (Goerke et al., 2009). They showed for several virulence genes to be associated with specific integrase-types of staphylococcal prophages, introducing a new classification system. Prophages of the Sa3int type carry several virulence genes encoded on the so called immune evasion cluster (IEC) at the 3´-end of the prophage genome. These virulence genes are highly human specific (van Wamel et al., 2006). Sa3int prophages majorly influence the niche adaption of *S*: *aureus* to the human host by playing an important role in immune evasion. Their presence or absence in animal strains is further associated with host jump of *S*. *aureus*.

Detailed description of Sa3int prophages and their specific impact on *S*. *aureus* was reviewed by Rohmer and Wolz and is part of an article collection on “bacterial survival strategies”.This review article (Rohmer & Wolz, 2021) was accepted in 2021 and is included in this thesis. It serves as part of the introduction (see the following section: accepted manuscript).

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Part I: Accepted publication

The following accepted publication serves as part of the introduction

Review Article

The role of *hlb*-converting bacteriophages in *Staphylococcus aureus* host adaption

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Abstract

As an opportunistic pathogen of humans and animals, *Staphylococcus aureus* asymptomatically colonizes the nasal cavity but is also a leading cause of life-threatening acute and chronic infections. The evolution of *S. aureus* resulting from short- and long-term adaptation to diverse hosts is tightly associated with mobile genetic elements. *S. aureus* strains can carry up to four temperate phages, many of which possess accessory genes encoding staphylococcal virulence factors. More than 90% of human nasal isolates of *S. aureus* have been shown to carry Sa3int phages, whereas invasive *S. aureus* isolates tend to lose these phages. Sa3int phages integrate as prophages into the bacterial *hlb* gene, disrupting the expression of the sphingomyelinase Hlb, an important virulence factor under specific infection conditions. Virulence factors encoded by genes carried by Sa3int phages include staphylokinase, enterotoxins, chemotaxis-inhibitory protein, and staphylococcal complement inhibitor, all of which are highly human-specific and probably essential for bacterial survival in the human host. The transmission of *S. aureus* from humans to animals is strongly correlated with the loss of Sa3int phages, whereas phages are regained once a strain is transmitted from animals to humans. Thus, both the insertion and excision of prophages may confer a fitness advantage to this bacterium. There is also growing evidence that Sa3int phages may perform “active lysogeny”, a process during which prophages are temporally excised from the chromosome without forming intact phage particles. The molecular mechanisms controlling the peculiar life cycle of Sa3int phages remain largely unclear. Nevertheless, their regulation is likely fine-tuned to ensure bacterial survival within different hosts.

Introduction

Staphylococcus aureus is a major opportunistic pathogen of humans and animals that asymptomatically colonizes the nasal mucosa but is also a leading cause of life-threatening acute and chronic infections [1, 2]. Based on multi-locus-sequence typing (MLST) analysis, *S. aureus* strains have been assigned to distinct evolutionarily related clonal complexes (CCs) [3]. Most strains carry up to four prophages in their genome, many of which harbour accessory genes encoding staphylococcal virulence factors [4, 5]. The extra genes present in prophage genomes have no obvious phage function but may act as fitness factors for lysogenic bacteria. All known temperate staphylococcal phages belong to the family *Siphoviridae*. The genomes of siphoviruses are typically organized into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis. The evolution of phage lineages is driven by the lateral gene transfer of interchangeable genetic elements (modules), which consist of functionally related genes [4, 6-10].

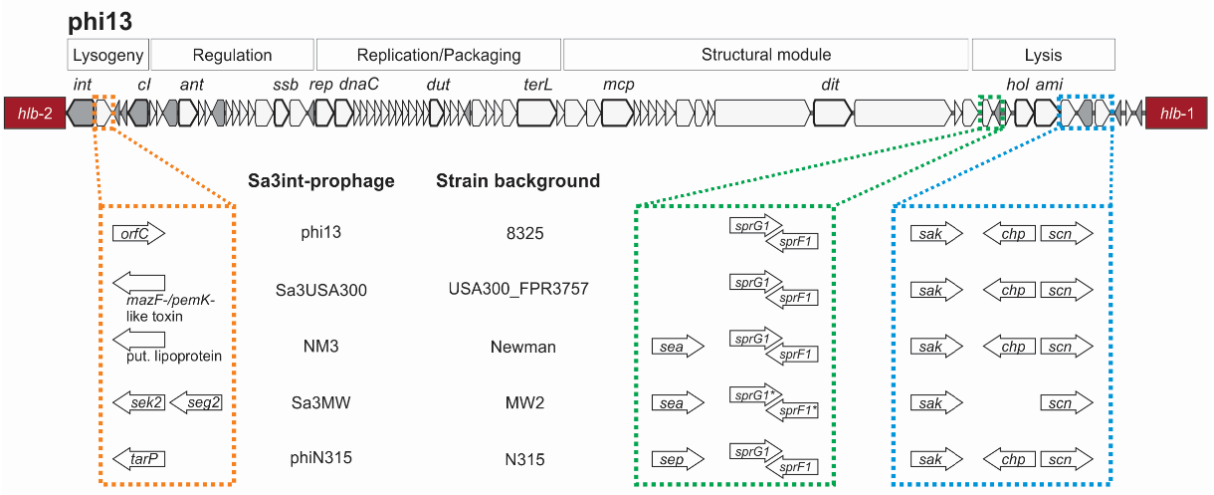

Fig. 1: Schematic illustration of Sa3int phages. Genetic organisation of the representative Φ13 phage (top) of strain 8325. Annotations are based on [11] and manually curated. Zoomed-in view (bottom) on phage-encoded accessory genes as located in the prototypic *S. aureus* strains 8325, USA300, Newman, MW2 and N315. Orange box marks accessory genes located in proximity to *int*. Green and blue boxes represent genes of the immune evasion cluster (IEC) (van Wamel et al., 2006) and the recently discovered toxin-antitoxin system sprG1/F1.

S. aureus-infecting siphoviruses have been classified according to polymorphisms of the integrase gene (*int*) [7-9]. The *int* type dictates chromosomal integration at cognate attB sites and is closely associated with the virulence gene content of the prophage [8]. Sa3int phages were first described as triple-converting phages and are by far the most prevalent *S. aureus* phages [8, 12, 13]. Up to 96% of human nasal isolates were observed to carry Sa3int phages integrated into the *hlb* locus that encodes ß-haemolysin (Hlb), also named ß-toxin. These phages carry genes that encode human-specific immune evasion factors [12] and other potential virulence factors [4]. There is now compelling evidence that Sa3int phages play important roles in the adaptation of *S. aureus* to its human host [14-17]. The transmission of *S. aureus* from humans to livestock is strongly correlated with a loss of Sa3int phages, whereas these phages are regained once the strain is transmitted from animals to humans. The loss of Sa3int phages is associated with restoration of the *hlb* gene and subsequent Hlb synthesis, which is important for specific infections. In this review, we first summarize recent insights into the function of phage-encoded accessory factors and phage-inactivated Hlb. We will then compile epidemiological data supporting the predominant role of Sa3int life cycle switches in bacterial survival and adaptation under different infectious conditions.

Main text

Phage-encoded accessory genes

Van Wamel and co-workers first described the so-called immune evasion cluster (IEC) comprising the genes *scn*, *chp*, *sak*, and *sea/sep*, which encode staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylokinase (SAK) and staphylococcal enterotoxin A or P (SEA or SEP), respectively [12]. Seven IEC variants have been identified that carry different combinations of scn, *chp*, *sak*, or *sea* (or *sep*), always in the same 5`-to-3` orientation near the lysis module at the 3´ end of Sa3int phages. *Scn* is present in every IEC type, whereas *sak*, *chp* and *sea/sep* are only observed in some IEC combinations [12] (shown in Fig. 1). The functions of SCIN, CHIPS, SAK and SEA are highly human specific, supporting the hypothesis that this cluster has evolved to support bacterial adaptation to the human host. In addition, sequencing of diverse *S. aureus* isolates and functional analyses has resulted in the identification of several additional accessory genes carried by Sa3int phages, some of which are located at the opposite 5´ end of the phage in proximity to *int* (shown in Fig. 1).

Phage-encoded virulence genes are integrated into the regulatory system of the bacterial host and modulated in a manner surprisingly similar to bacterial chromosome-encoded virulence factors (for Review see [4]). The alternative sigma factor B, the two-component regulatory system, *saeRS*, and to a lesser extent the quorum-sensing system, *agr*, are involved in regulation of *eta, pvl, scn,* or *chp*. However, the expression of these virulence factors is also tightly linked to the phage life cycle. Under phage-inducing conditions, the transcription of the virulence factors is increased [18, 19]. This is partially due to a multi-copy effect caused by phage replication, but transcription is also increased through co-transcription with the de-repressed lysis genes.

In the following section, we will summarize the major properties of Sa3int-encoded accessory genes, following their order in the phage genome (shown in Fig. 1). Notably, many more factors are present in Sa3int genomes that await further analysis.

Staphylococcal complement inhibitor (SCIN): SCIN was first described as a 9.8 kDa secreted phage-encoded protein categorized as a new class of convertase inhibitors [20] [for review see [21]]. SCIN is able to block classical and alternative pathways of complement activation. These pathways intersect at the conversion of complement component C3 into its bioactive fragments C3a and C3b. C3b bound to the bacterial surface can form a complex with circulating complement factor B. SCIN interacts with the C3bBb complex and impairs downstream complement function by trapping the convertase in a stable but inactive state [22]. SCIN also promotes the formation of convertase dimers [23], which is important for *S. aureus* immune evasion by modulating complement recognition by phagocytic receptors. SCIN is highly specific for human complement and does not block complement activation in

the sera of other animals [20]. Of note, several SCIN homologues have been identified at other genome locations [22]. Interestingly, EqSCIN was detected in phages of *S. aureus* isolates from horses and shown to specifically inhibit the C3 convertases of horses [24].

Chemotaxis inhibitory protein of S. aureus (CHIPS): *S. aureus* supernatants can cause the downregulation of specific receptors on immune cells that are involved in chemotaxis [25], a process that was later shown to be due to the secretion of a 14.1 kDa protein named CHIPS [26]. Chemotaxis is a general mechanism by which cells move towards chemoattractants and is used to recruit immune cells to the site of bacterial infection. Chemoattractants effectively bind to highly specific receptors such as formylated peptide receptors (FPRs) or C5aR expressed on immune cells. FPRs react to formylated methionine or other bacteria-derived chemotactic peptides [27]. C5aR is expressed on several types of white blood cells and recognizes the chemoattractant C5a. Remarkably, CHIPS acts as a potent inhibitor of this chemotactic response by specifically binding to FPR and C5aR [28], and it is often used as a tool to specifically inhibit these pathways. A study evaluating the binding of FITC-labelled CHIPS to isolated neutrophils of different animal species revealed a low level of CHIPS binding to neutrophils of other tested species compared to human neutrophils [26]. Thus, CHIPS appears to play an important role in the ability of *S. aureus* to circumvent its recognition by human immune cells by inhibiting the chemoattractant-mediated recruitment of neutrophils to the site of bacterial infection.

Staphylokinase (SAK): Early on, the lysogenization of *hlb* with serogroup F bacteriophages was shown to be correlated with SAK activity [29, 30]. SAK is a 15 kDa secreted protein that can associate with the surface of *S. aureus* cells [31]. Several functions have been described for SAK [for review see [32]]. The best known property of SAK is its function as plasminogen activator. Plasminogen is the inactive precursor of plasmin, a broad-spectrum serine protease that degrades fibrin and non-collagenous proteins of extracellular matrices. SAK is able to convert plasminogen into the proteolytic, active form plasmin by promoting the formation of a stoichiometric complex [33]. The generation of active plasmin is enhanced on the surface of *S. aureus* cells [34] and protects against inactivation by plasmin inhibitors that are typically present in human plasma [35]. Therefore, plasmin-coated bacteria are prepared to degrade human IgG or C3b [36] or extracellular matrices. The second function ascribed to SAK is its ability to bind α-defensins, human antimicrobial peptides that can protect the host from bacterial invasion [37]. The interaction of SAK with α-defensins results in inhibition of their bactericidal activity. The binding site for α-defensins is different from that responsible for plasminogen binding [38].

SAK specifically activates human plasminogen and does not react with murine plasminogen [39, 40]. To overcome this species specificity, transgenic mice expressing either human or natural mouse plasminogen were compared to analyse the impact of SAK during

bacteraemia [41]. Surprisingly, the activation of human plasminogen by SAK reduced the severity of systemic staphylococcal infection. The same model was subsequently used to analyse the impact of SAK on skin infection [42]. Although SAK had no impact on skin infections in immunocompetent mice, in neutropenic mice, SAK promoted the establishment of skin infections in humanized plasminogen-expressing mice and increased bacterial penetration through skin barriers by activating plasminogen. However, the interaction between SAK and plasminogen did not promote systemic dissemination but instead induced the opening and draining of abscesses and decreased disease severity. In a similar humanized infection model involving adenoviral expression of human plasminogen, SAK-mediated plasmin activity increased the local invasiveness of *S. aureus*, leading to larger lesions with skin disruption as well as decreased bacterial clearance by the host [43]. However, SAK-induced proteolysis appears to be confined to the immediate surroundings of the site of infection, where high concentrations of fibrin and bacteria prevent inactivation but are rapidly neutralized further away from the abscess site. SAK was also shown to attenuate biofilm-associated catheter infections in a mouse model [39]. SAK-dependent activation of plasmin-dependent proteolysis and fibrinolysis results in the breakdown of biofilm architecture and bacterial detachment. Thus, these animal models support the idea that bacteria are covered with SAK-plasmin complexes *in vivo*. Although proteolytic activity of plasmin appears to contribute to local dissemination, SAK does not promote systemic infections and even seems to protect against severe bacteraemia.

Toxin-antitoxin system (SprG1/F1): Although not included in the first description of an IEC [12], a SprG1/F1 system is located within most IECs [44]. Entries for *sprG/F* in the staphylococcal regulatory RNA database (SRD) are listed in Table 1 and Fig. 1 [45]. The SprG/F systems were first detected in an sRNA screen and designated small pathogenicity island RNAs (Sprs) G1/F1 to G4/F4 [44]. The expression of *sprG1/F1* has been verified in several strains, including N315 and Newman but not for strain NCTC8325 [46]. However, an alignment of *sprG1/F1* sequences showed high conservation of these systems among *S. aureus* genomes, including 8325 and MW2, with only minor sequence variations detected. T The antitoxin *sprf1* overlaps with *sprg1* in the antisense direction and does not encode any peptide but rather functions as a non-coding cis-antisense RNA that regulates *sprG1*. *sprF1* mRNA was also shown to reduce protein synthesis under hyper-osmotic stress by binding to ribosomes. This leads to translation attenuation and enhanced persister cell formation [47]. Two toxic peptides are translated from *sprG*, a major form ($SprG1_{439}$, 44 amino acids) and a shorter form ($SprG1_{312}$, internal start codon, 31 amino acids). SprG1 peptides act as secreted pore-forming toxins that can accumulate at the bacterial membrane. The overexpression of *sprG1* results in growth inhibition followed by cell death [46]. Interestingly, SprG1 peptides

show activity towards other bacterial species, and the longer peptide $SprG1_{439}$ can also lyse human erythrocytes.

Staphylococcal enterotoxin A (SEA): *sea* was one of the first genes described as being phage encoded in *S. aureus* [48]. Staphylococcal enterotoxins are pyrogenic toxin superantigens, a group of proteins that also includes exoproteins from *Streptococcus pyogenes*. Superantigens interact with MHC II molecules present on T cells as well as with variable parts of T cell receptors. This interaction leads to the massive production of cytokines such as IL-2 and IFN-γ within T-cells and TNF-α and IL-1β within macrophages, which is likely responsible for the typical clinical outcome of toxic shock syndrome (TSS) with high fever. Staphylococcal enterotoxins are typical superantigens that can stimulate T cells. The designation “enterotoxin” originates from their emetic properties, causing vomiting and diarrhoea when consumed orally, which is correlated with their role in staphylococcal food poisoning [49]. However, as several enterotoxins have been discovered that lack these emetic properties, a new standardized nomenclature was proposed in 2004 by the International Nomenclature Committee for Staphylococcal Superantigens for newly discovered enterotoxins based on the emetic properties of enterotoxins [50]. In this proposal, enterotoxins are only designated as enterotoxins when emetic properties are demonstrated within a primate animal model. Otherwise, if this property is lacking or experiments are not performed, the discovered toxin should be named “staphylococcal enterotoxin-like (SEl-) toxin [50]. SEA is a 27 kDa protein categorized as a “real” enterotoxin with superantigenic and emetic activity [51-53]. Nontoxic concentrations of SEA induce interleukin-8 production by nasal epithelial cells, indicating that SEA can induce an inflammatory response at the site of colonization [54].

Staphylococcal enterotoxin (-like) P (SEP): In some strains (e.g., strain N315, shown in Fig. 1), *sep* is located at the same position as *sea* in other Sa3int phages. SEP and SEA show 78% identity at the amino acid level, and SEP is also designated SEl-P based on the recommended nomenclature [55]. Recombinant SEP showed emetic properties in a house musk shrew assay, although at a relatively high dose [56]. SEP/SEI-P is a classical superantigen with high T-cell stimulatory activity [56].

Accessory genes located in proximity to int: In many Sa3int phages, different ORFs with unknown function are located in the lysogenic module between *int* and the gene coding for the phage repressor protein. It is assumed these genes do not have a function for the maintenance of the prophage state itself but rather give advantage to their host bacterium. Some of these genes are divergently transcribed from the lysogenic transcriptional unit, indicating their autonomous regulation. For instance, ORF-C in Φ13 from strain 8325 encodes a 204 residue membrane protein that is divergently transcribed from the lysogenic module (shown in Fig. 1). ORF-C homologues are present in several other staphylococcal

phages (e.g., Φ12, ΦETA, ΦPVL, ΦPV83 and ΦSLT) that are assigned to different Sa-int groups. Based on its localization, it was proposed that ORF-C may function as an excisionase [11], although a deletion mutant for the encoding gene is not impaired in excision (unpublished data). Thus, the function of this conserved protein remains to be elucidated.

MazF/PemK-like toxin: SAUSA300_1971 is located next to *int* in the Sa3int phage of strain USA300. The predicted protein (237 amino acids) was annotated as a PemK-like/MazF-like toxin of the type II toxin-antitoxin system. The same gene is located at the same position in the Sa3int phages of *S. aureus* strain JH9 and JH1 (SaurJH9_2059 and SaurJH1_2096, respectively) and in Sa6int phage of strain Col (SACOL0319). MazF/PemK-like toxins belong to the protein family PF02452. This family comprises the toxin molecule of typical bacterial toxin-antitoxin systems that includes different toxins, such as MazF or PemK. Toxicity is typically restricted through a neighbouring antitoxin. Interestingly, a putative antitoxin is not detectable next to the phage-encoded MazF homologue. The function of this putative toxin remains to be investigated.

Sek2 (MW1938) and seg2 (MW1937): Two ORFs next to *int* of Sa3int phage of strain MW2 were annotated as *sek2* and *seg2*. The predicted proteins SEK2 and SEG2 show > 95% identity to staphylococcal enterotoxin K (SACOL0886) and enterotoxin G (SACOL0887) of *S. aureus* strain COL, respectively. The *sek* and *seg* genes are harboured on a pathogenicity island in strains USA300 or COL that contains additional enterotoxins. The *sek2/seg2* gene cluster is also present in the Sa3int phage of strain MSSA476 (Sumby & Waldor, 2003). The expression of both enterotoxin variants is increased after phage induction with mitomycin C and is likely co-transcribed with the gene coding for the repressor cI, which is located upstream of both toxins. No data regarding the emetic properties of the enterotoxin SEK/SEK2 are available. However, for SEG, emetic properties were shown in a primate animal model when administered at 80 mg/kg animal weight [57].

NWMN_1924 (hypothetical protein): In strain Newman, an ORF (154 residue protein) is located next to *int* that exhibits 100% identity to SAR2104 of strain MRSA252 and is described as a putative lipoprotein [58].

Glycosyltransferase TarP: *TarP* was first described in Sa3int phages of strain N315 and other clinical CC5 strains . However, *tarP* is also carried by other prophages (Sa1int, Sa3int, Sa7int, Sa9int, and ΦUT1) and is also present in different CCs, including LA-MRSA CC398 [59-61]. TarP is an alternative glycosyltransferase that alters the glycosylation pattern of *S. aureus* wall teichoic acid (WTA), catalysing the attachment of GlcNAc in the β-1,3-position of RboP [60]. WTA alteration by TarP not only influences the ability of several bacteriophages to recognize *S. aureus* but also subverts antibody-mediated immune recognition. However, TarP-mediated protection against anti-WTA antibodies do not appear to influence the household transmission of LA-MRSA CC398 [61].

Avian hlb-converting prophages: Species-specific Sa3int phages are prevalent in poultry *S. aureus* isolates (designated ΦAvβ). These phages lack the typical IEC but rather carry two avian-specific genes at the same location on the 3´ end of ΦAvβ. The first gene (SAAV_2008) encodes a novel ornithine cyclodeaminase sharing 38% identity to an enzyme from *Bacillus cereus* at the protein level. The second gene (SAAV_2009) is of unknown function but is annotated as an avian-specific protease that contains a CAAX domain with 27% identity to a membrane-bound protease produced by *Lactobacillus plantarum*.

β-Haemolysin (Hlb)

Hlb was first described in 1935 when Glenny and Stevens observed a hot-cold haemolysis effect in which *S. aureus* cultivation at 37°C followed by cooling to 4°C resulted in an enhanced haemolysis pattern on blood agar plates [62]. The nucleotide sequence of the gene encoding the 37 kDa protein Hlb was elucidated in 1989 [63]. Structural analysis revealed that Hlb belongs to the DNase I folding superfamily, which includes sphingomyelinases [64]. Indeed, Hlb is a phospholipase with specificity towards sphingomyelin to generate ceramide and phosphocholine [65]. Hlb exhibits species-dependent haemolytic activity that correlates with the amount of sphingomyelin content in erythrocytes, where sheep, cow, and goat erythrocytes are highly sensitive to the toxin, rabbit and human erythrocytes exhibit intermediate sensitivity, and murine and canine erythrocytes are resistant [66]. However, Hlb is able to efficiently target human endothelial cells [67], human keratinocytes [68] and monocytes [64, 69]. The Hlb-generated ceramide can act as a second messenger in eukaryotic cells [70]. Furthermore, the sphingomyelinase/ceramide ratio regulates the internalization of bacteria into the host cell, the subsequent cytokine release, the inflammatory response, and the initiation of host cell apoptosis. Thus, ceramide generation is likely the cause of the Hlb-dependent inhibition of the chemoattractant IL-8 observed in endothelial cells [71, 72]. Notably, a second function of Hlb as a biofilm ligase has been described [73]. Independent of its sphingomyelinase activity, Hlb forms covalent cross-links to itself in the presence of DNA, producing an insoluble nucleoprotein matrix that stimulates biofilm formation. Hlb mutants lacking either of these activities were shown to have a decreased ability to induce the formation of vegetation during infective endocarditis [74].

The role of Hlb as an important virulence factor has been demonstrated in several animal models. First, Hlb was shown to worsen infections of bovine mammary glands [75] and keratitis in rabbits [76]. In a lung infection model, Hlb was shown to promote increased neutrophilic inflammation and the vascular leakage of serum proteins into lung tissue [77]. Neutrophil-mediated lung injury was observed to be associated with Hlb-stimulated ectodomain shedding of syndecan-1, a major heparin sulfate proteoglycan present in

epithelial cells. In addition to its well-known haemolytic activity towards erythrocytes, Hlb was also shown to play an important role in skin colonization by damaging keratinocytes [68].

Table 1. Accessory genes of Sa3int phages

Immune-evasion cluster (IEC)[a]	Accession No.	*Gene*	Location on prophage genome	Distribution in IEC-type	reference	8325	USA300_FRP3757	Newman	MW2	N315
Staphylococcal complement inhibitor	YP_500655	*scn*	3´-end	A,B,C,D,E,F,G	Roijaakers et al., 2005	SAOUHSC_02167	SAUSA300_1919	NWMN_1876	MW1884	SA1754
Chemotaxis inhibitory protein	YP_500656	*chp*	3´-end	A,B,C,F	Roijaakers et al., 2006	SAOUHSC_02169	SAUSA300_1920	NWMN_1877	-	SA1755
Staphylokinase	YP_500658	*sak*	3´-end	A,B,D,E,F,G	Lack 1948	SAOUHSC_02171	SAUSA300_1922	NWMN_1880	MW1885	SA1758
Staphylococcal enterotoxin A	WP_000750406	*sea*	3´-end	A,D	Casman et al., 1963	-	-	NWMN_1883	MW1889	-
Staphylococcal enterotoxin P	WP_000034846	*sep*	3´-end	F,G	Kuroda et al., 2001	-	-	-	-	SA1761
Additional accessory genes										
Srn3840 (SprG1)[b]	KJ625227 KJ625228	($SprG1_{312}$) ($SprG1_{439}$)	3´-end	-	Pinel-Marie et al.,2014	sprG1_sRNA310	sprG1_sRNA310	sprG1_sRNA310	sprG1_sRNA310*	sprG1_sRNA310
Srn3830 (SprF1)[b]	KJ625226	SprF1	3´-end	-	Pinel-Marie et al.,2014	srn_3830	srn_3830	srn_3830	srn_3830*	srn_3830
SAOUHSC_02238	YP_500722	OrfC	5´-end	-	Carroll et al., 1995	SAOUHSC_02238	-	-	-	-
SAUSA300_1971	YP_494622	mazF/pemK-like	5´-end	-	Diep et al., 2006	-	SAUSA300_1971	-	-	-
NWMN_1924	BAF68196	lipoprotein	5´-end	-	Baba et al., 2008	-	-	NWMN_1924	-	-
MW1938 MW1937	BAB95803 BAB95802	Sek2 Seg2	5´-end	-	Baba et al., 2002	-	-	-	MW1938 MW1937	-
SA1808	NP_835519	TarP	5´-end	-	Gerlach et al., 2018	-	-	-	-	SA1808
Avian-adapted genes (Sa3int phage ΦAvβ)										
SAAV_2008	ACY11858	Putative ornithine cyclodeaminase	3´-end (ΦAvβ)	-	Lowder et al., 2009	-	-	-	-	-
SAAV_2009	ACY11859	Putative CAAX-protease	3´-end (ΦAvβ)	-	Lowder et al., 2009	-	-	-	-	-

a) Categorization of IEC-types according to van Wamel et al., 2006
b) Accession No. based on staphylococcal regulatory RNA database (SRD) (Sassi et al., 2015)

Sa3int phages and human-to-animal jumps

S. aureus has been detected in a taxonomically diverse range of animals, including mammals, reptiles, crustaceans and birds [16, 78, 79]. Some *S. aureus* CCs are restricted to a single taxonomic group, suggesting that distinct *S. aureus* populations are solely maintained within a given species [78]. Other CCs are prevalent in several species, indicating transmission between humans and animals. Humans are thought to be a major reservoir for *S. aureus* among animal species [16, 80]. The transmission of *S. aureus* between humans and livestock is of particular concern, as *S. aureus* isolates from farmed animals are often antibiotic resistant [80, 81]. Epidemiological studies have shown that *S. aureus* has jumped between species many times, resulting in the dynamic gain and loss of host-specific adaptive genes that are typically located on mobile genetic elements [80, 82].

Sa3int phages are of particular interest. They are often lost upon transfer from humans to different animals. In several instances, the animal-adapted strain was back-transmitted to humans, and these livestock-originating strains often reacquire Sa3int phages, emphasizing their important role in human colonization (for detail show below). The loss of Sa3int in many animal-derived strains and the observation that Hlb is always functional after phage excision indicate that Hlb plays an important role in animal pathogenesis/colonization.

Pigs: Since the early 2000s, MRSA strains with the sequence type ST398 have been reported to colonize pigs and have since spread worldwide, with these strains also causing infections in humans living in close contact with livestock [15]. These livestock-associated MRSA (LA-MRSA) CC398 isolates are descendants of a human MSSA strain that gained methicillin and tetracycline resistance but lost the Sa3int phage [83, 84]. However, LA-MRSA CC398 may be capable of readapting to the human host through acquisition of an IEC-harbouring Sa3int phage [61, 85, 86]. The presence of an IEC was shown to be correlated with increased human-to-human transmission and excess disease burden of LA-MRSA [61, 85]. In addition, the proportion of secondary cases was observed to be significantly higher in IEC-positive household contacts (11/17) than in IEC-negative households (16/74) (PR 2.99, p = 0.0010) [61]. The importance of Sa3int phages in *S. aureus* virulence towards humans has also been more directly demonstrated, as the presence of Sa3int decreased phagocytosis by human but not pig polymorphonuclear neutrophils [87]. The strong selection for Sa3int phages during human colonization is even more astonishing in light of the finding that the common attB integration site located within *hlb* is altered in CC398 strains. Thus, in these strains, Sa3int often integrates elsewhere in the genome [88-90], and the location of the integration appears to influence the stability of the Sa3int prophage in livestock strains.

In Asia, the prevalent LA-MRSA CC9 strains can also cause severe human diseases. These strains can be grouped into two major clades, where clade I subtypes harbour an intact *hlb*

gene and lack the IEC cluster, while truncated *hlb* genes and IECs are detected in clade II subtypes [91]. These results suggest that a CC9 strain with extraordinarily high virulence potential obtained IEC-carrying Sa3int-phages after jumping from pigs to humans [92].

In the US, there is a diverse population of LA-MRSA, including organisms of ST5 MRSA lineages. Furthermore, the *hlb* gene is intact in these livestock ST5 strains, indicating the absence of Sa3int phages. In contrast, the prevalence of Sa3int phages in MRSA ST5 strains from humans with no exposure to swine was determined to be 90.4% [93].

Poultry: There is a limited number of *S. aureus* genotypes associated with poultry in different geographic regions [16, 78]. The majority of these isolates are belonging to a single CC lineage (CC5) that is also one of the most successful human-associated lineages. All poultry isolates are closely related and originate from a single human-to-poultry host jump that occurred approximately 40 years ago in or near Poland [94]. The poultry ST5 clade has undergone genetic diversification from its human progenitor strain via the acquisition of novel mobile genetic elements from an avian-specific accessory gene pool and by the inactivation of several proteins important for human disease pathogenesis. In particular, a novel Sa3int phage (ΦAvß) lacking the IEC was acquired by these isolates. Instead of an IEC this phage contains genes encoding a novel ornithine cyclodeaminase and a putative novel protease that is likely involved in adaption to birds [94] (Table 1). The ΦAvß phage has been detected in all 13 avian strains of the CC5 poultry clade as well as in other avian-specific lineages (CC385, ST1345 and ST1), suggesting that frequent horizontal gene transfer of ΦAvß occurs between *S. aureus* strains. This finding was reinforced by Price et al., who tested 34 isolates from domestic turkeys suffering from foot joint infection and observed that all CC398 isolates but one carried ΦAvß in their genomes [83]. Thus in avian isolates, similar to the human *S. aureus* population, the *hlb* gene becomes inactivated by phage conversion.

Horses: In addition to pigs, CC398 strains are also often found in horses. Interestingly, horse *S. aureus* isolates are positive for Sa3int phages harbouring an IEC [95]. This may potentially benefit these isolates since IEC carriage in MRSA-ST398 appears to promote bacterial survival in the presence of human and equine polymorphic neutrophils [87]. Several other equine lineages (e.g., CC9 or CC1) have acquired a Sa6int phage encoding a novel equine-specific allele SCIN (eqSCIN) as well as an equine-specific form of the bi-component leukocidin LukPQ [24, 96] . In these lineages, the Sa3int phage is typically absent.

Cattle: Cows have been shown to be the most frequent recipient of *S. aureus* but also appear to be the primary animal reservoir for reinfection of humans and the emergence of animal-derived human epidemic clones [80, 97]. *S. aureus* strains belonging to MLST CC97 are a leading cause of bovine mastitis in Europe, Asia, and North and South America and represent a major economic burden on the global dairy industry. The initial human-to-bovid switch was estimated to have taken place approximately 5500 BP, coinciding with the

expansion of cattle domestication throughout the Old World. However, CC97 has also been shown to be an emerging cause of human infections since approximately 40 years. These data indicate that CC97 isolates circulating among human populations are the result of livestock-to-human host jumps occurring on at least 2 independent occasions. The bovine-human host jump of CC97 clade A was estimated to have occurred between 1894 and 1977. 9 of 23 human isolates and none of 19 bovine or pig isolates contained an IEC-harbouring Sa3int phage, indicating that the acquisition of Sa3int phages by these isolates occurred after their transmission to humans [97]. CC8 strains isolated from bovines suffering from subclinical mastitis emerged following the human-to-bovine jump, which is associated with the loss of Sa3int phages [98, 99]. An analysis of *S. aureus* isolates from cattle in Germany showed that the majority of isolates belonged to the closely related CC8, CC25, and CC97 (34.4% combined) or were related to the sequenced bovine strain RF122. Interestingly, 82% of these isolates were also *hlb* positive [100].

Rabbits: CC121 is a globally distributed, highly virulent CC in humans but has also been associated with disease in farmed rabbits. The origin of the rabbit CC121 lineage was traced back to a human-to-rabbit host jump that occurred approximately 40 years ago [101]. Comparative analysis of the accessory genomes of human ST121 strains showed that all except one contained a Sa3int phage, whereas all ST121 strains from rabbits were Sa3int phage negative.

Wild rodents and mice from animal facilities: Wild rodents are frequently colonized by different mouse-adapted *S. aureus* lineages (e.g., CC49 and CC88. CC130. CC1956) lacking human-specific virulence factors, such as superantigens and the IEC [102, 103] . Interestingly, laboratory mice also carry a large variety of *S. aureus* CCs, most of which likely originate from the human population but also lack Sa3int phages. While CC88 has spread across several continents for three decades, other CCs are sporadically introduced into animal facilities with limited expansion [104]. Similar to mice, free-living and laboratory rats are often colonized with *S. aureus* [105]. Free-living rats were shown to be predominantly colonized with CC130 and CC49, while captive rats from pig farms were mainly colonized with livestock strain CC398. In addition, laboratory rats were most frequently colonized with CC15 and CC8 strains of human origin. Only 2.7% of free-living rats and none of the captive wild rats were observed to carry IEC-encoded genes. However, 59% of the laboratory rats harboured IEC genes, supporting a recent human-to-rat jump.

Monkeys: There are multiple anthroponotic transmissions of *S. aureus* from humans to green monkeys, and the emergence of a monkey-associated clade of *S. aureus* occurred approximately 2700 years ago. The development of this monkey-associated clade was accompanied by the loss of Sa3int phage [106], indicating that the specificity of the IEC excludes non-human primates.

Role of phage conversion in human infection/colonization

The term phage conversion was introduced with the first description of a Sa3int phage [30] and already indicates that IEC or Hlb may fulfil distinct functions under different conditions. However, the relevance of switching between phage integration and excision with respect to the outcome of infections or colonization remains unclear. The human specificity of the IEC hampers analysis in appropriate animal models. Nevertheless, based on observational studies in humans, it appears that Hlb reconstitution during some infectious conditions is favourable for the bacteria. A comparison of colonizing and invasive *S. aureus* strain populations revealed that invasive strains are more frequently Hlb-positive [8, 107, 108]. Notably, most Sa3int phages remain inducible, leading to the complete restoration of functional Hlb [109, 110], and phage induction may be favoured under infectious conditions. Reactive oxygen species generated during infection or other DNA-damaging factors (e.g., quinolone antibiotics) are well known for their phage-inducing capabilities [19, 88]. An analysis of follow-up isolates from cystic fibrosis (CF) patients revealed that Sa3int phage translocation often leads to a splitting of the bacterial population [109] into Hlb-positive (phage-cured) and phage-positive fractions. Sa3int phage-negative CC398 strains were also shown to persistently colonize CF patients without acquiring Sa3int phages during long-term colonization [111], supporting the idea that during CF lung infection, Sa3int-encoded factors are of less importance. Hlb appears also to promote bacteraemia. SAK-deficient isolates were shown to be > 4 times more likely to cause lethal bacteraemia than SAK-positive isolates, suggesting that an intact *hlb* gene and/or SAK deficiency may worsen the outcome of patients with *S. aureus* bacteraemia [42, 112]. Boyle-Vavra et al. compared an isogenic pair of daptomycin-susceptible and daptomycin-resistant MRSA isolates from a patient with recurrent bacteraemia [113]. The *hlb* gene was interrupted by a prophage in the daptomycin-susceptible strain, but this phage was missing in the daptomycin-resistant follow-up isolate. An undisrupted *hlb* gene was also shown to be associated with catheter-related bacteraemia [114], and 45% of isolates from recurrent furunculosis were observed to produce Hlb compared to 19% of those associated with nasal colonization [108].

The results of analyses using several animal models support that infection can select for the loss of Sa3int phages. Katayama et al. observed the loss of the IEC-encoding prophage in *S. aureus* MW2 during adaptation to murine skin. Interestingly, the strain started to produce Hlb, which promoted a >50-fold increase in murine skin colonization by *S. aureus* [68]. Hlb-positive MW2 variants also arise in the blood, kidney and heart vegetation of infected rabbits [110]. Infection-associated *S. aureus* isolates frequently present as small colony variants (SCVs) that are often unstable and show attenuated virulence, although this phenotype is reversible. These SCVs are often associated with Sa3int prophage activation, which results in the production of circular excised forms (25-fold higher compared to wild-type and normal

colony variants) but cannot replicate [115] . It was assumed that phage excision leads to a higher copy number of IEC-encoded genes, resulting in overall higher expression.

To date, under which conditions and why the expression of phage-encoded IEC genes would be advantageous for bacteria remains largely unclear. The epidemiological data point to some advantage of the IEC in establishing or maintaining nose colonization. Indeed, *sak* and *chp* are highly expressed during *S. aureus* colonization, as revealed by gene expression analyses performed on nose swabs from persistent *S. aureus* carriers [116]. One can speculate that SAK could provide some advantages based on its antiphagocytotic properties. The inactivation of defensins present in the nasal cavity via SAK may also provide an additional bacterial survival advantage. CHIPS prevents chemotaxis and thus immune activation, which may be favourable for long-term asymptomatic colonization. However, this hypothesis was challenged by the results of a colonization study [13] in which volunteers were artificially colonized with *S. aureus* strain NCTC 8325-4 with or without the Sa3int phage phi13. Intranasal survival was monitored for 28 days after inoculation, and surprisingly, the strain harbouring phi13 was eliminated faster than the phage-free strain. Thus, this Sa3int phage is not essential during the first stages of *S. aureus* nasal colonization.

Molecular switch mechanisms

The maintenance and mobilization/loss of phages are likely controlled by distinct molecular mechanisms. Despite the typically strong association of the *int* type with the location of the cognate attB site, there are also events during which a phage may integrate at an illegitimate attachment site. This phenomenon was shown to occur for Sa3int phages during chronic lung infections of CF patients [109]. Under these conditions, the reconstitution of the phage-interrupted *hlb* gene may be advantageous. When these mis-located phages were induced and used to re-infect *S. aureus in vitro*, the phages reintegrated at their dedicated attachment site within *hlb*. There is also evidence [109, 117, 118] that Sa3int phages may perform “active lysogeny”, a process during which a phage is temporally excised from the chromosome without forming intact phage particles [119]. Through this process, bacteria can simultaneously activate phage virulence genes as well as the gene that is typically inactivated by phage integration. This phenomenon may be seen as a form of bacterial gene regulation that possibly improves bacterial fitness. Furthermore, phages are likely induced under various infectious conditions, which enhances the transcription of phage accessory genes such as SAK [19]. However, molecular analyses to elucidate such switching mechanisms are currently lacking for *S. aureus* phages. While the presence of a cI-like repressor can often be predicted from the genome sequence, the frequency and function of other regulatory factors involved in the lysogenic-lysis switch needs to be experimentally

characterized. Remarkably, few studies have been dedicated to elucidating the regulatory systems of *S. aureus* phages. Consequently, the roles of most of the gene products impacting the phage life cycle remain elusive.

Conclusion and outlook

Epidemiological data strongly indicate that Sa3int phages have coevolved with the *S. aureus* host to facilitate the adaptation of this bacterial species to the human host. The phages remain highly mobile to relieve expression of the interrupted *hlb* gene when needed and may be achieved via active lysogeny, temporal re-localisation of the phage or phage curing in a distinct fraction of the bacterial population. For *S. aureus* phages, *in vivo* analyses of such switching mechanisms are rare, and the underlying mechanism controlling the phage life cycle has yet to be elucidated. Moreover, the genetic make-up of the host strains is likely to determine the rate of phage mobilization during infection, a feature that might determine the speed at which specific strains can achieve host adaptation. With the exception of RecA, staphylococcal factors controlling the phage life cycle remain to be discovered.

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Conflict of interest

The authors have no conflicts of interest to declare

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Aims of this thesis

Sa3int prophages highly impact their host bacterium, *S*. *aureus*, by providing additional virulence factors via lysogenic conversion. In particular, these factors are highly human-specific and are beneficial for *S*. *aureus* in regard to increased pathogenicity and enhanced survival during infection. During the infection process or after transmission to an animal host, these prophages are mobilized or lost. We hypothesize that switches from lysogenic to lytic life cycle are controlled by molecular factors of the host bacterium and the phage alike. We postulate several bacterial factors to be highly strain-specific, as the different *S*. *aureus* strains are often highly niche adapted and differentially interact with Sa3int prophages.
As the genetic make-up of the host strains may determine the rate of phage mobilization during infection, a feature which might determine the speed at which certain strains can achieve host adaption. I aimed to decipher the molecular mechanisms involved in the strain-specific lysogenic-lytic switch of Sa3int prophages and to further elucidate phage-bacterial interference.

We aim to

- Characterize the Sa3int phage life cycle in different host backgrounds.
- Define the transcriptional units and gene regulatory modules in the prototypic phage Φ13.
- Elucidate whether phage lysogeny impacts bacterial gene expression.
- Define bacterial factors which influence the phage life cycle.

Part II: Manuscript I (ready for submission)

Unpublished manuscript. This manuscript is ready for submission and might differ from the published version

Influence of *Staphylococcus aureus* strain background on Sa3int phage life cycle

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Abstract

Sa3int prophages in *Staphylococcus aureus* play a major role in adaption to the human host by providing human-specific virulence genes which are encoded on the prophage genome. These gene products support escape of *S. aureus* from the human immune system and are influenced by staphylococcal regulators. Additionally, transcription of prophage encoded virulence genes are known to be dependent on phage copy numbers resulting from replication process, which causes elevated gene expression due to multi-copy effect. In this study, we investigated the biology of Sa3int prophages Φ13 and ΦN315 in different *S. aureus* isolates which revealed strain-specific differences in prophage behavior. Differences in phage transfer frequency in co-culture resulted in high and low phage-transferring *S. aureus* isolates associated with varying lysogenization capacity. It was found that these differences are mainly due to differences in phage replication process. Transcriptional analysis using tagRNA-seq approach revealed differentially expressed genes that particularly affect genes in morphogenesis and replication modules, indicating influence by yet unknown host factors. Additionally, transcriptomic architecture of prophage Φ13 was deciphered by prediction of transcriptional start sites (TSSs), indicating strain-specific differences in mRNA processing and unravel putative targets which can be influenced by different staphylococcal factors. Taken together, this study provides deep insight into the complex interaction of Sa3int prophages and their host bacterium and will help to unravel yet unknown mechanisms by which *S. aureus* interferes with phage biology.

Introduction

Staphylococcus aureus is a major human pathogen that also colonizes and causes infections in different animal species (Balasubramanian et al., 2017; Haag et al., 2019; Matuszewska et al., 2020; Sakr et al., 2018; Turner et al., 2019). Transmission of *S. aureus* between humans and livestock is of particular concern as *S. aureus* isolates from farmed animals are often antibiotic resistant (Richardson et al., 2018). Adaptation to the different mammalian hosts is in large part driven by acquisition/loss of mobile genetic elements. *S. aureus* has jumped between species many times, resulting in the dynamic gain and loss of host-specific adaptive genes many of which are prophage encoded (McCarthy et al., 2012). Most prominent is the repeated loss of Sa3int phages upon transfer from humans to different animals. In several instances, the animal-adapted strain was back-transmitted to humans, and these livestock-originating strains often reacquire Sa3int phages, emphasizing their important role in human colonization (Bouiller et al., 2020; Ingmer et al., 2019; Matuszewska et al., 2020; Rohmer & Wolz, 2021; Sung et al., 2008). Up to 96% of human nasal isolates were observed to carry Sa3int phages integrated into the *hlb* locus that encodes β-hemolysin (Hlb). The association of Sa3int prophages with human *S*. *aureus* isolates is mainly due to highly human-specific immune evasion factors encoded in the so called immune evasion cluster (IEC) at the 3´-end of Sa3int prophage genomes (Goerke et al., 2009; van Wamel et al., 2006). Further, several other (putative) virulence factors are encoded close to the integrase gene of these phages (Rohmer & Wolz, 2021). The absence of Sa3int phages in many animal-derived strains and the active, precise excision of prophage under certain conditions in humans turns *hlb*-gene functional, like described by (Goerke et al., 2004), further indicate selection pressure towards an intact *hlb*-gene under specific conditions.

Temperate staphylococcal phages belong to the family of *Siphoviridae*. The genomes of siphoviruses are typically organized into functional modules: lysogeny, DNA replication, morphogenesis, and lysis. The evolution of phage lineages is driven by the lateral gene transfer of interchangeable genetic elements (or modules), which consist of functionally related genes (Deghorain & Van Melderen, 2012; Goerke et al., 2009; Kahánková et al., 2010; Kwan et al., 2005; Oliveira et al., 2019). *S*. *aureus* infecting siphoviruses have been classified according to polymorphisms of the integrase gene (*int*). The integrase dictates chromosomal integration at cognate *attB* sites on the bacterial genome and is further associated with the virulence gene content of the prophage (Goerke et al., 2009). However, due to the mosaic nature of *S. aureus* siphovirus genomes, the distinct phage modules often show high homology between different staphylococcal phages and can be exchanged between members (Oliveira et al., 2019).

The molecular interactions between the *S. aureus* host and its temperate phages are largely unknown. The high number of phage genes encoding hypothetical proteins highlights how little is known about temperate phages and their influence on bacterial life style. Previous analysis of Sa2int prophages revealed that inducibility of the very same phage can be significantly different compared between different *S*. *aureus* isolates (Wirtz et al., 2009).
In this study we analyzed whether strain specific features of diverse *S*. *aureus* isolates also affect the phage life cycle of Sa3int phages. Therefore, we constructed or used several Sa3int mutant phages with focus on two prototypic Sa3int phages, namely Φ13 and ΦN315. Φ13 is derived from *S. aureus* reference strain 8325 of clonal complex (CC) 8 which is widely used for genetic analysis of *S*. *aureus*. ΦN315 is derived from the methicillin resistant strain N315 (CC5) encoding for *tarP*. TarP was described to alter WTA glycosylation of *S*. *aureus* which in turn is known as phage receptor. Modified phages were integrated into different phage-cured *S. aureus* strains (8325-4, SH1000, USA300c, Newman-c and MW2c). Usage of these highly relevant *S*. *aureus* isolates allowed us a comprehensive insight into strain-specific influences of the bacterial host on prophage behavior. Further, we elucidate the transcriptomic architecture of Φ13 using tagRNA-seq approach. We show that different host strain background severely impact mobilization of Sa3int prophages in regard to phage transfer, lysogenization capability and induction. Further, transcriptomic analysis revealed significant differences in gene expression levels of prophage Φ13 which are dependent on the host strain background.

Results

Sa3int phage transfer during co-cultures is determined by the bacterial host strain

To facilitate the analysis of the phage life cycle of Φ13, kanamycin resistance cassette *aph*A3 was introduced at the 3´-end of the phage in exchange for virulence genes *scn* and *chp* (Tang et al., 2017). For comparison of phage transfer in different bacterial strains, Φ13-kana single-lysogenic isolates were generated in different phage free host strains: 8325-4, SH1000, MW2c, Newman-c, USA300c (supplemental table 1). Phage transfer was monitored after 4 hours of co-culture of the Φ13-kana single-lysogens with an isogenic streptomycin (strep)-resistant phage free recipient (Figure 1A). A high transfer rate was observed for strain MW2c and strain Newman-c as enumerated by streptomycin/kanamycin double resistance.
Newly generated lysogens were negative for β-hemolysin on blood agar plates and phage integration into the *hlb* gene was verified by PCR using integration specific oligonucleotides (hlb675 and Sa3intfor). Phage transfer using 8325-4 or USA300c strain pairs revealed significant lower phage transfer rates compared to Newman-c or MW2. During the analyses we observed that the strain 8325-4 tented to aggregate during the incubation period. A *rsbU*

repaired derivative of 8325-4 (strain SH1000) was described to form less aggregates (Horsburgh et al., 2002). To rule out any artefacts due to clumping we also generated a SH1000-Φ13-kana lysogen. This strain indeed did not aggregate but still shows a significantly lower phage transfer rate compared to Newman-c or MW2c. In summary, we could confirm that the host background significantly influences phage life cycle. We could discriminate between high (Newman-c and MW2-c) and low (8325-4, SH1000 and USA300) phage transfer strains.

To analyze whether the strain background similarly influences the transfer rate of other Sa3int phages we included ΦN315-tet derived from strain N315 into the analysis. This phage was labelled with a tetracycline (tet) resistance cassette. The experimental setup for ΦN315-tet followed the same procedures as for Φ13-kana and was mobilized and used for production of single-lysogens of four former phage cured isolates (SH1000, USA300c, Newman-c and MW2c). Phage transfer assays revealed transfer rate of ΦN315-tet to be lower compared to Φ13-kana (Figure 1B). However, again Newman-c and MW2c exhibited higher phage transfer compared to the low transfer strains SH1000 and USA300.

Strain dependent Sa3int phage transfer is determined by the recipient strain

S. *aureus* isolates 8325, SH1000, USA300 and Newman are assigned to CC8 with no obvious restriction barrier (Moller et al., 2019). MW2 belongs to CC1 and gene transfer between CC8 and CC1 strains is restricted due to different restriction-modification systems (Lindsay, 2014). Accordingly, phage transfer between CC8 strains an MW2 was found to be severely impaired (Figure 1C). We next analyzed whether heterogenic transfer between low (SH1000) and high (Newman-c) transfer strains of the same clonal complex is determined by the donor or recipient strain. Strain Newman used as recipient showed high phage acquisition when incubated with either SH1000-Φ13-kana or Newman-c-Φ13-kana as donor (Figure 1D). Strain SH1000 used as recipient, low phage acquisition is detected even when incubated with high transfer donor strain Newman-cΦ13-kana. Thus, the strain dependent phage transfer rate is determined by the recipient.

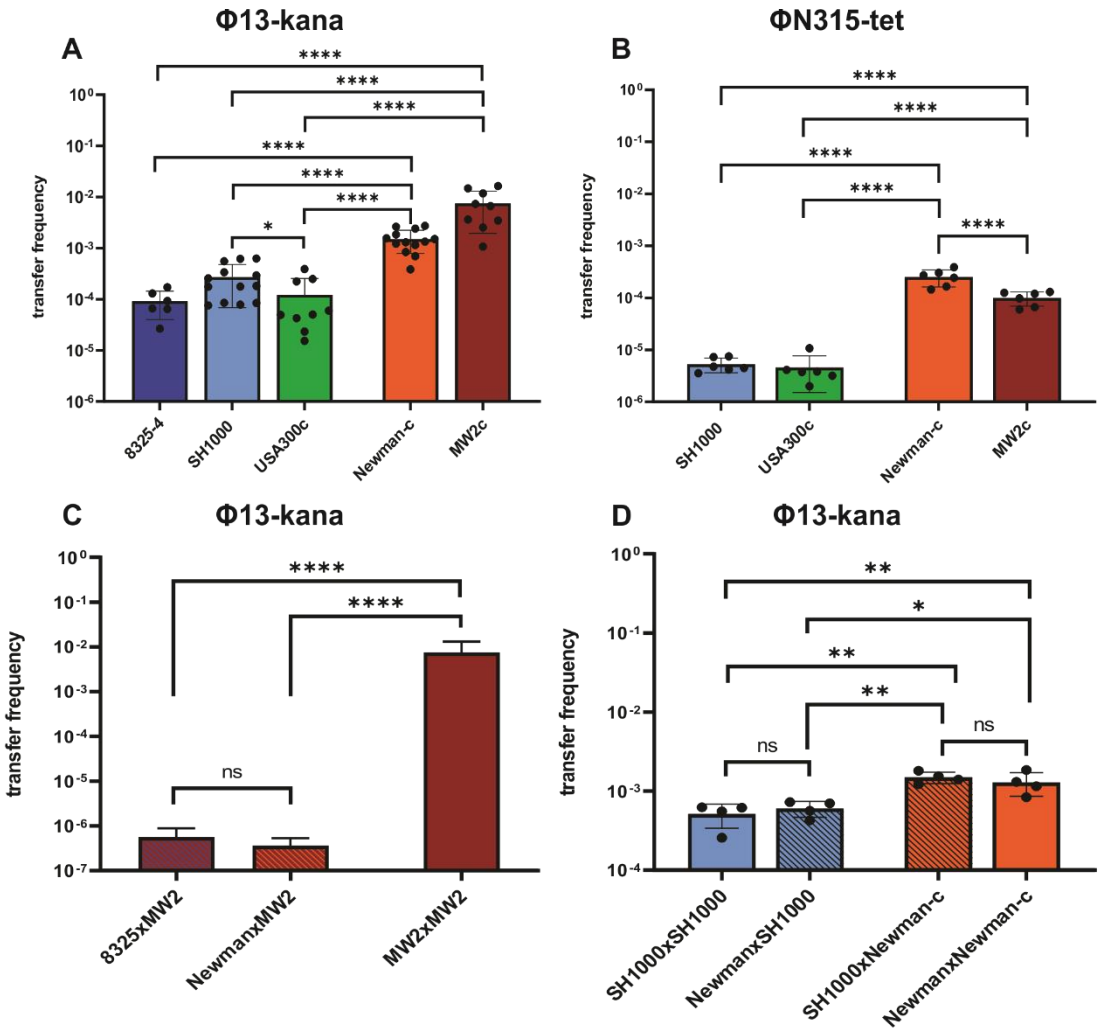

Fig. 1: Phage transfer frequency of **A)** Φ13-kana and **B)** ΦN315-tet in homogenic transfer assay, **C)** in transfer between CC8 donor and CC1 recipient strain and **D)** heterogenic transfer within CC8. Donor-lysogens were mixed with isogenic, phage-cured, streptomycin-resistant recipient derivates of 8325-4 (dark blue), SH1000 (light blue), USA300c (green), Newman-c (orange), MW2c (dark red) with MOI of 1 in tryptic soy broth and co-cultured for 4 hours. Phage transfer frequency was determined by calculating the ratio of CFU of double-resistant colonies (kanamycin/streptomycin for Φ13-kana or tetracycline/streptomycin for ΦN315Tet, respectively) divided by CFU on streptomycin (representing recipient). Values are independent biological replicates referring to mean±SD. Statistical analysis was performed on log-transformed data using one-way ANOVA and back-transformed for visualization of transfer rate.

Strain-dependent lysogenization of Sa3int prophages

We next analyzed whether the different bacterial recipients differ in their impact on phage integration and/or replication. Φ13-kana was induced from Newman and phage titer was measured via plaque assay to adjust the lysate for experimental procedures. Phage lysate was used to lysogenize CC8 isolates (SH1000, USA300c, Newman-c). MW2 was excluded from this assay due to restriction barrier, neither allowing proper titer determination via plaque assay nor usage of Φ13-kana derived from Newman background. Φ13-kana was incubated with recipient strains for 4 h and lysogens selected on agar plates supplemented with antibiotics. Significantly more Φ13-kana lysogens were recovered for high transfer

strains Newman-c compared to the low transfer strains USA300c and SH1000 (Figure 2A). Higher lysogenization frequency of strain Newman-c compared to SH1000 and USA300c was also observed for ΦN315-tet (Figure 2B).

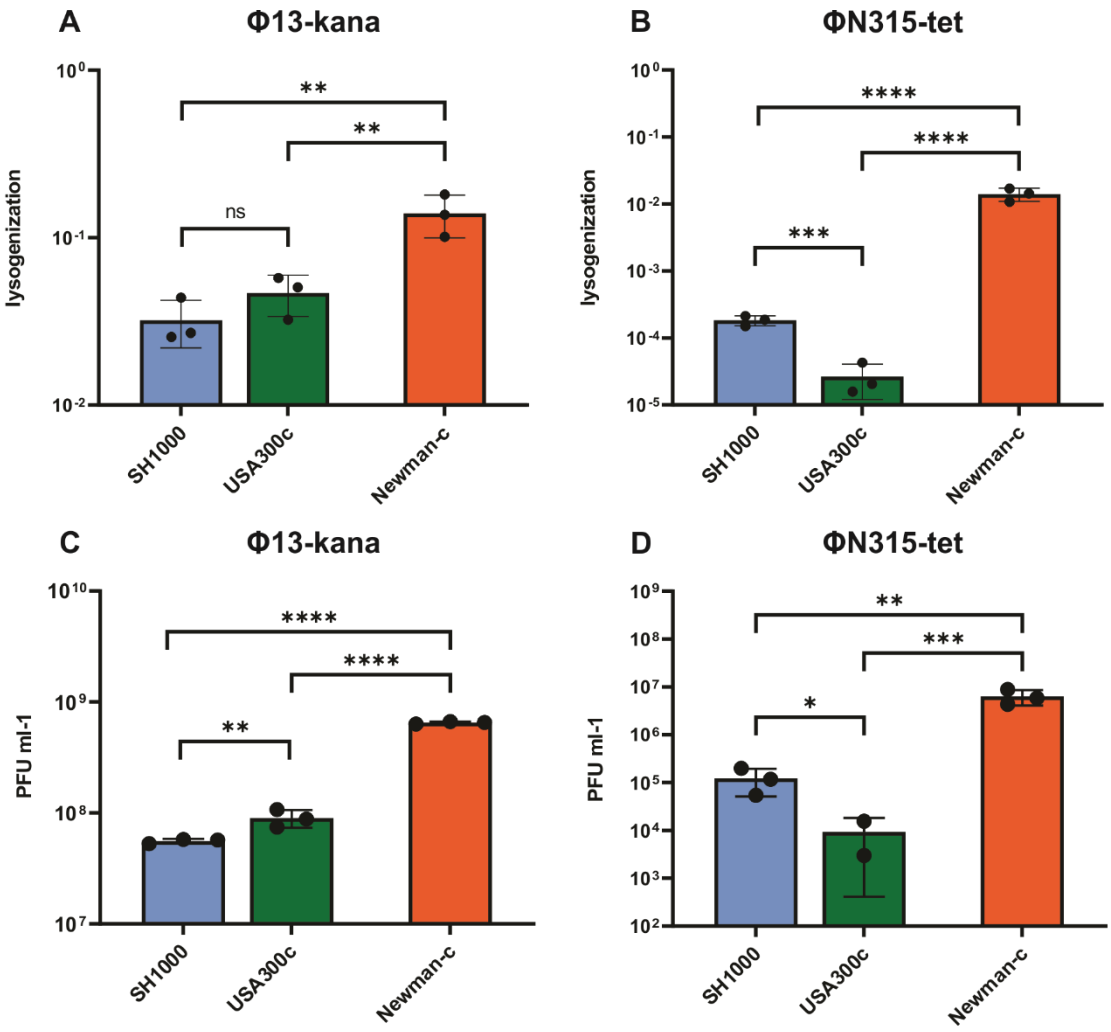

Fig. 2: Lysogenization of phage-cured SH1000 (blue), USA300c (green), Newman-c (orange) recipient with **A)** Φ13-kana and **B)** ΦN315-tet. 10^6 phage particles were mixed with 10^8 phage-cured recipient bacteria to an MOI of 0,01 in tryptic soy broth and co-cultured for 4 hours. Lysogenization rate was determined by CFU grown on TSA-plates containing kanamycin 50 µg ml-1 (representing lysogenized colonies) divided by total CFU on blood-agar plates. Corresponding detection of free phages via plaque-assay of **C)** Φ13-kana and **D)** Φ13N315-tet was determined by agar-overlay method of lysate collected from samples after 4 hours of incubation on the indicator strain, respectively. Values are three independent biological replicates referring to mean±SD. Statistical analysis was performed using one-way ANOVA of log-transformed data. Data are back-transformed for visualization of lysogenization frequency (**A+B**) and phage titer (**C+D**)

Supernatants of 4 h cultures were used to determine free phage titers. Interestingly, more free phage particles of both Φ13-kana and ΦN315-tet were observed for strain Newman-c compared to USA300c and SH1000 (Figure 2C and 2D). Of note, total CFU on nonselective media revealed no significant difference compared to no-phage control cultures (data not shown).

Thus, high transfer strain Newman also has a higher lysogenization capacity. This might in part be due to enhanced production of free phages, resulting from enhanced prophage replication. Higher abundance of free phage particles could, in turn, increase the chance of successful phage adsorption and infection of the host bacterium, followed by lysogenization.

Strain-dependent lysogenization is not mediated by differences in phage adsorption

Wall-teichoic acids (WTA) present at the bacterial surface serve as receptor for staphylococcal phages (Koç et al., 2016; Xia et al., 2011). Importantly, *S. aureus* strains differ in their WTA content (Wanner et al., 2017), which could therefore mediate the observed lysogenization phenotype. To exclude that differences observed in lysogenization and transfer frequency between *S. aureus* isolates result from varying amounts of WTA present at the bacterial surface, adsorption assays were performed (adapted from (Xia et al., 2011)).

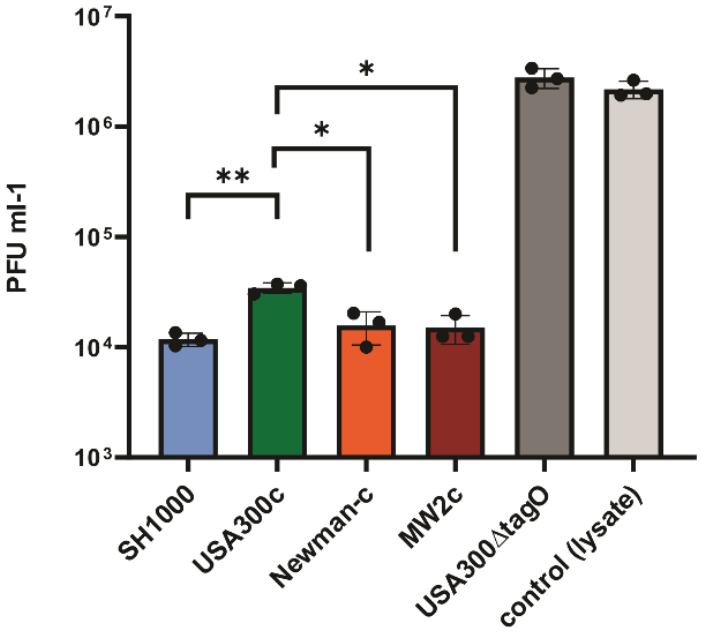

Fig. 3: Adsorption assay of Φ13-kana on phage cured SH1000 (blue), USA300c (green), Newman-c (orange) and MW2c (dark red). WTA-deficient USA300 Δ*tagO* (dark grey) and lysate control adjusted to 10^6 PFU ml-1 (light grey) represent negative control.
10^6 phage particles were incubated with 10^8 phage-cured recipient bacteria for 10 minutes followed by centrifugation to separate bacteria with bound phages from unbound, free phage particles. Titer of free phage were determined by plaque assay (determination of PFU ml-1). Values are three independent biological replicates with mean±SD. Statistical analysis was performed using one-way ANOVA.

We could verify that WTA represents phage receptor for Φ13 since adsorption was not detectable in USA300 Δ*tagO*, a WTA deficient strain (Figure 3). Only minor differences in phage adsorption were detected between isolates with USA300c showing slightly reduced phage adsorption compared to others. However, differences in phage adsorption do not correspond to the observed strain-specific differences in lysogenization capacity. Thus, processes following the initial phage infection are responsible for strain specific differences in phage lysogenization and transfer.

Induction of Sa3int prophages is strain-dependent

Differences in free phage particles measured in lysogenization assays among *S. aureus* isolates indicate that more phage particles are produced in Newman (and MW2) strain background compared to 8325, SH1000 and USA300 which might result from variations in induction and replication. To test this hypothesis, single-lysogenic isolates were analyzed for spontaneous induction in late exponential growth phase. Furthermore, we used mitomycin C, an antibiotic commonly used for prophage induction, at subinhibitory concentrations. We used two methods for phage quantification. First, free phage particles in filtered culture supernatants were quantified by plaque assay. However, MW2 (CC1) could not be used in this assay therefore the indicator strain belongs to CC8 which prevents plaque formation as a result from restriction barrier. Second, a qPCR approach targeting the attachment site (*attP*) was used for absolute quantification (Anderson et al., 2011). We opted to target *attP* for this, since it is present only when phage nucleic acid is either circularized, in concatemer formation or within assembled phage particles, as schematically illustrated in Figure 4A. Importantly, this approach was also applicable to CC1 strain MW2.

Under non-inducing conditions, quantification of free phage particles showed no differences between the isolates tested (Figure 4B). However, quantification of produced phage genomes via qPCR revealed slight differences with higher amount of *attP* detected in Newman and MW2. Addition of mitomycin C revealed increased numbers for free phage particles and *attP* sites compared to untreated condition, as expected (Figure 4B). Furthermore, higher numbers of free phage particles (PFU ml-1) in Newman and phage genomes (*attP* ml-1) in Newman and MW2 could be detected. Discrepancies in absolute values between PFU and *attP* measurement can be explained due to higher sensitivity of the qPCR measurement. Additionally, as not all phage genomes produced are finally packed and released as infectious particles, as they are detected in the qPCR quantification only. In conclusion, observations of increased phage genomes (and released phage particles) in Newman and MW2 background indicate that prophage replication is influenced by yet unknown staphylococcal factors resulting from strain-specific differences.

Phage gene expression is dependent on the host strain background

Most genes encoded on phage Φ13 are of unknown function. Recently, the regulatory switch region of Φ13 was investigated (Kristensen et al., 2021). The region is composed of cI repressor gene (*cI*) and a divergently transcribed *mor* (modulator of repression) gene. We monitored transcription of cI repressor gene (cI-repressor) involved in maintenance of lysogenic state as well as transcription of *mor*, an early lytic gene.

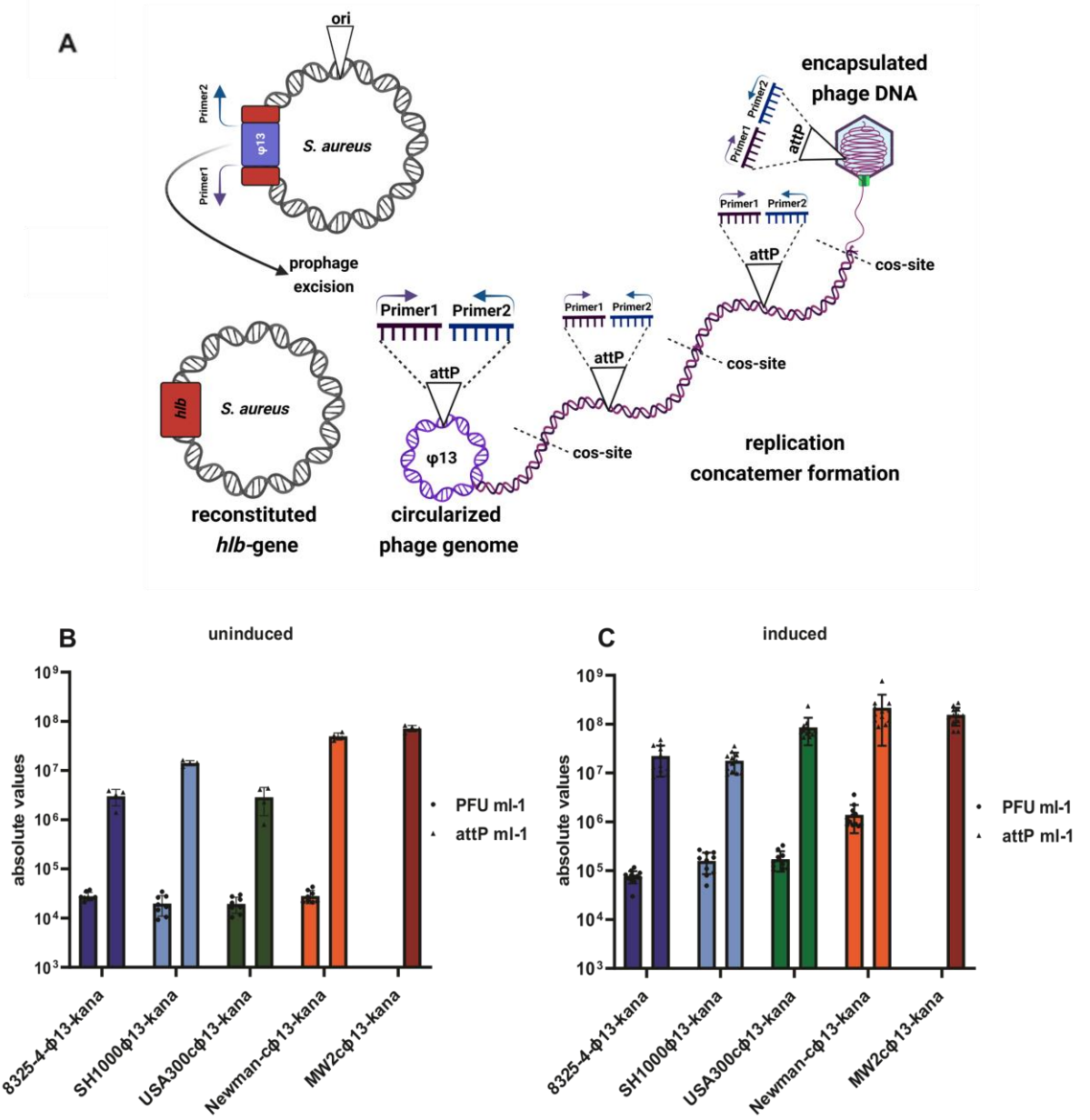

Fig. 4: (A) Schematic illustration of prophage excision, reconstruction of *attP* and replication. Prophage genome (blue) is integrated into and thereby disrupting *hlb*-gene (red) in the prophage state. Excision of prophage genome due to mitomycin C treatment leads to reconstitution of the *hlb*-gene and the attachment site of phage (*attP*) due to circularization by connecting left and right prophage genome ends (below). *attP* is amplified via PCR with primers circlefor and circlerev. Replication following rolling circle principle forms multiple interlinked phage genomes (concatemer) which are cleaved from each other at cos-sites resulting in encapsulation of one phage genome per phage capsid. Quantification off phage particles and genomes under **B)** uninduced and **C)** induced (addition of mitomycin C 300 ng ml-1) condition in phage lysate. Lysate was obtained from 8325 (dark blue) SH1000 (light blue), USA300 (green), Newman (orange) and MW2 (red) single-lysogens carrying Φ13-kana. Quantification of free phage particles (circles) was performed via plaque assay (PFU ml-1), quantification of produced phage genomes (triangles) was performed via absolute quantification using qPCR approach (attP ml-1). Values are three independent biological replicates with mean±SD.

Further, transcription of *orfC*, a gene encoded between integrase (*int*) and cI-repressor as well as expression of bacterial *recA* for control of SOS response was evaluated. Northern blot analysis of Φ13 (Figure 5C) revealed increased expression of *recA* upon mitomycin C treatment but no differences between the strains, indicating that MW2 is as sensitive in SOS response as 8324. Analysis of OrfC and cI-repressor expression revealed transcription of both genes under uninduced conditions. Addition of mitomycin C led to increased expression of both genes which is more pronounced in the MW2 background. Transcription of lytic genes (*mor*) was is only detectable after mitomycin induction. One major transcript was detectable representing co-expression of *mor* with downstream lytic genes. Moreover, the presence of multiple bands indicate processing of the initial major transcript. The expression of these lytic genes is elevated in MW2 background. This is likely due to a multi-copy effect resulting from phage replication after induction. These observations are consistent with biological data obtained from quantification of phage genomes produced which revealed higher copy number in MW2 background (Figure 4B and C).

Since the observed multi-copy effect, due to phage replication, might hide other strain-specific differences in prophage gene expression, we constructed a replication-deficient mutant (Φ13-kanaΔrep) in different *S*. *aureus* single-lysogens. No phage particles were detectable in plaque assay after mitomycin C treatment (data not shown). To test whether the mutant phage was still able to excise, quantification of *attP* via qPCR was performed to detect excision and circularization. Calculation of excised prophage genome per bacterial cell was performed by absolute quantification of *attP* in relation to *recA* (reference for bacterial genome copy numbers). Mitomycin C treatment revealed Φ13-kanaΔrep is still able to excise but replication is abolished (*attP* / *recA* <1; Figure 5B) compared to wildtype Φ13 (Figure 5A). This further indicates that phage induction/excision is not significantly different between strains but that the replication process resulting in a higher phage genome copy number is more pronounced in MW2. Northern blot analysis of the same set of genes in Φ13-kanaΔrep revealed expression of bacterial *recA* to be unaffected, as expected (Figure 5D). Overall, we detected a clear reduction of phage gene transcripts for *orfC* and *mor*, confirming that the multi-copy effect detected for Φ13 wildtype phage is abolished in the replication-deficient mutant. For cI-repressor expression, the decrease in transcript level is not that pronounced compared to wild-type Φ13, indicating that the genes necessary for lysogenic functions are not affected by replication deficiency. Since the multi-copy effect was abolished in the Φ13-kanaΔrep mutant, the observed differences in phage gene expression can be attributed to strain background.

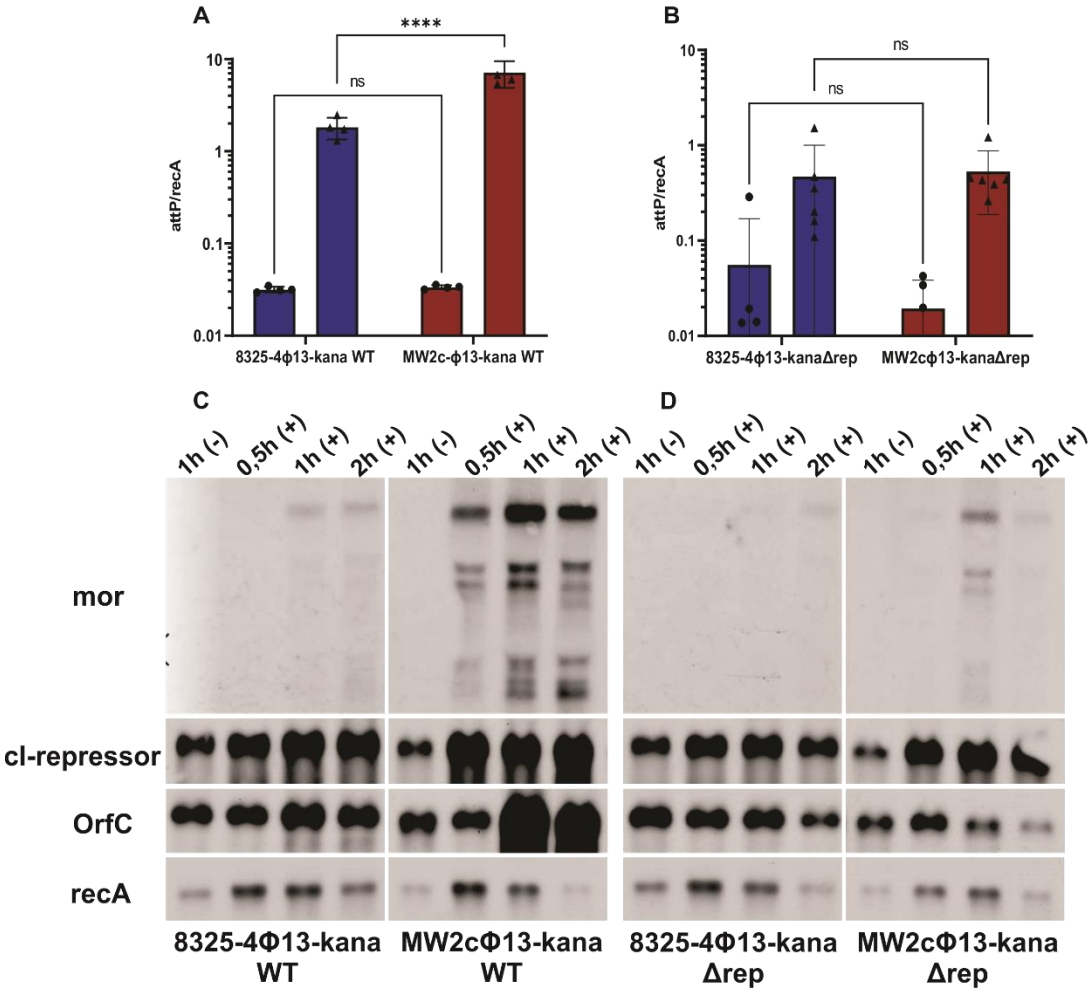

Fig. 5: Absolute quantification of phage genomes per bacterial cell (attP/recA) via qPCR approach for Φ13-kana WT prophage **A)** and Φ13-kanaΔrep **B)** under non-induced (circles) and mitomycin C treated (triangles) conditions in 8325 (blue) or MW2 (red) background. Uninduced conditions correspond to 1h (-), induced conditions correspond to 1h (+) of Northern blot analysis of transcripts from isolated RNA of 8325 and MW2 lysogens carrying Φ13-kana WT **C)** and Φ13-kanaΔrep **D)** as prophage. Visualization of transcripts via hybridization using DIG-labelled DNA-probes specific for recA, OrfC, cI-like repressor and mor under uninduced (1h(-)) or induced conditions at 0,5h, 1h and 2h.

tagRNA-seq approach unravels transcriptional organization of prophage Φ13

Due to these initial observations, we aimed to unravel the transcriptional organization of Φ13 using an optimized method termed tagRNA-seq (Innocenti et al., 2015). tagRNA-seq combines common RNA sequencing for differential gene expression analysis with differential tagging of transcriptional (TSS) and processed start sites (PSS) which was performed on 8325 and MW2 single-lysogens carrying replication deficient phage mutant Φ13-kanaΔrep. Isolates were checked under non-induced and mitomycin C treated conditions in late exponential growth phase, as performed for northern blot analysis (1h(-) and1h (+)) and qPCR (Figure 5B and 5D), respectively. Of 3 datasets revealed from tagRNA-seq approach, TSS enriched fastq data obtained from tagRNA-seq approach were analyzed using

TSSpredator which were adapted to be used on tagRNA-seq data (Tuncbilek-Dere, 2021). Complete TSS prediction datasets are listed in “Additional data files” at the end of this thesis. TSSs predicted for prophage regions were extracted (supplemental table 3) and further analyzed.

In total, 18 TSSs were predicted in both *S*. *aureus* backgrounds under both conditions (Figure 6, grey arrows). They are located in three separate phage modules: 1) in the lytic-lysogenic switch region (cI-repressor and *mor*, position 5 and 6), 2) at positions 10/12/21 for genes in the lysogenic direction but located in the lytic module, and 3) at immune evasion cluster (IEC)-encoded genes like *sak* (position 36) and the SprG/F TA-system (30 and 33). However, position 15 and 16 are not assigned to a gene in the lysogenic direction, indicating the presence of an unknown coding region. For genes in lysogenic direction but encoded in lytic module (SAOUHSC_02232, SAOUHSC_02226 and SAOUHSC_02218), transcripts have already been detected via northern blot and are conform with annotated gene length (data not shown). This indicates that several genes encoded in the same direction of transcription as cI-repressor and integrase might be involved in lysogenic phage life cycle, like described for cII and cIII in model phage λ (Casjens & Hendrix, 2015). 14 TSSs were only enriched in the 8325 background (blue arrows), with five of them only detectable in the uninduced (2, 22, 37, 53, 55) state and eight only under induced conditions (7, 14,18, 20, 26, 38, 39, 45). Only one predicted TSS was detected under both conditions (position 43). In the MW2 background, 14 TSSs were predicted and enriched as well. However, 12 of them were only enriched under induced conditions (8,9,11,13,24, 25,27,28, 32, 34, 35, 51), one only under uninduced conditions (31), and one under both conditions (3). 11 enriched TSSs were predicted for both strains, of which four only under uninduced (29,40,41,52) and five only under induced conditions (4, 17, 23, 48, 49). Position 29 and 40 have a special role since both were predicted under uninduced conditions in both strains, whereas under induced conditions, these TSSs were only enriched in the 8325 background. Interestingly, several predicted TSSs were positioned very closely to each other, but in opposite directions (e.g. 8/9 or 18/20 and 19/21). These locations could indicate putative small RNAs that might function as cis-acting RNAs. However, this requires further investigation. Furthermore, MW2 revealed several predicted TSSs within the morphogenesis module (24, 25, 27, 28), whereas for 8325 only one such position was predicted (26). These observations are important for the next chapter with regards to differential expression analysis comparing Φ13 gene expression in MW2 versus 8325 host strain background.

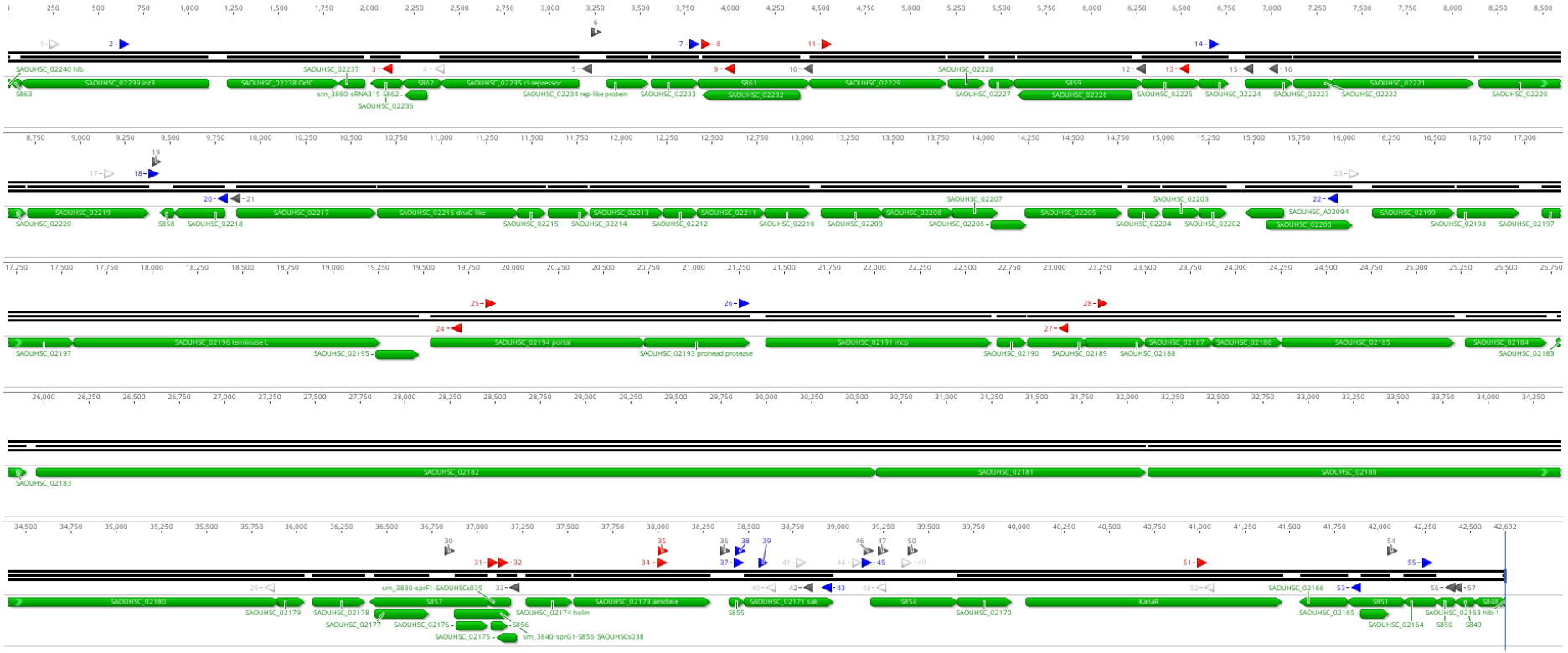

Figure 6: Visualization of predicted and enriched TSS on extracted Φ13 prophage region produced and annotated in Geneious ®. Black line displays phage genomic sequence, green annotations below represent prophage gene annotations from position 1 / 42692 corresponding to base position on prophage genome. Extracted region (wrapped view) spans from SAOUHSC_02239 int (position 1 upper left) to SAOUHSC_02164 (position 42647), right side below with intervals of 250 bp. Drawn, numbered arrows represent predicted an enriched TSS corresponding to table 1. Grey arrows represent TSS predicted in both strain background under both conditions, blue arrows are only enriched in 8325 background and red arrows are only enriched in MW2 background. White arrows are partly detected on both strain background but only in one condition.

Table 1 : Summary of 57 enriched TSS predicted using TSSpredator from extracted prophage genomic region and color code corresponds to Figure 6. Each row represents a predicted and enriched TSS. Grey row filling indicates TSS predicted in both strain background under both conditions. Predicted TSSs enriched only in 8325 background are marked in blue and TSS predicted only in MW2 marked in red. White background resembles TSS predicted in both strains but only under one condition. Asterisk (lines 41 and 29) mark TSS predicted under uninduced condition for both strains but under induced condition only in 8325.

TSS Position on prophage	Condition			Strain			Direction (reference genome)	TSS starting position (prophage genome)	Sequence
	Uninduced	induced	both	8325	MW2	both			
1	✓	×				✓	Sense	232	TGAGTGTGTCTTAATGCGTGCGATGTAATAATTGGTATATTATTGACTCTA
2	✓	×		✓	×		sense	619	ATTTTTCTTGTTCTGACTTCGTAGGTTTTCCGGCTTTATAATTAACTTTA
3			✓	×	✓		Antisense	2,075	AACCATTCACTTATGAGTTACCTAAAGATTTATCGTCACATAATGCGCGTA
4	×	✓				✓	Antisense	2,363	TAATGGGCAAAGTTGTATTGTGATTAATAACGTATATTTAGCGCTTTAATA
5			✓			✓	Antisense	3,181	TTATGAACTTTTTTGTTGAATTTTTGTTCAACAAGCTTTATTATGAAGTTA
6			✓			✓	sense	3,233	TCATGAATTTTTGTATTGACTTGATTCAAAACAAGGTGTAAAGTATAGTTA
7	×	✓		✓	×		Sense	3,776	TGAAAAAGTTAGACGAAAAAATCAACGCCACTCCAATTAAAGAGTGACGGA
8	×	✓		×	✓		Sense	3,841	AATGTTTAAGGTTTTAAATGATATAAAAACTTCTTTAAAAAACCATCCTTG
9	×	✓		×	✓		Sense	3,969	AAAGTCACTACCAACAAAGGAATCTATATAAAGCCTGTTATCATAGAATCG
10			✓			✓	Antisense	4,408	GTTTTTAATGCTTGCATATTGTTTATGCTCCTTTCGTGTATAATGTTGTTA
11	×	✓		×	✓		Sense	4,508	GAAATCGCAATAAGTGCAAGAGAGTTATATAAAGCTTTGGAAGTTAAAAAG
12			✓			✓	Antisense	6,249	CGTTTAAAATATGTGGCATTTCTATCTTTCCTTTCGTGTATAATGTTGTTA
13	×	✓		×	✓		Antisense	6,491	TCTTGCGTTAAATCTCCAGCGATTAAAATTCTCATCTGGGTAATGCACAAT
14	×	✓		✓	×		Sense	6,654	AATTGTACTTATGCCGTTTCTATACTTCACTACTGCATGGTCGATTGCGGG
15			✓			✓	Antisense	6,844	CATTAATTACTTTTTGACATACATCGCCAATTTTGTAGTACATTGTTGACA
16			✓			✓	Antisense	6,983	AATATGTCTAATGCTGTGTATAGATCATTCTCATCTGTTATATTTATACCG
17	×	✓				✓	Sense	9,135	CCGCAACTGTGTAATTTGTGGAAAGCCTCACGCAGACCTAGCGCATTATGA
18	×	✓		✓	×		Sense	9,380	GAATAGACTAAGAATAATAAAAATAGCACTCCTAATCGTCATCTTGGCGGA
19			✓			✓	Sense	9,398	AAAAATAGCACTCCTAATCGTCATCTTGGCGGAAGAGATTAGAAGCGCTAA
20	×	✓		✓	×		Antisense	9,765	GGGGTGAATAAAATATGGAAAAACCTTATATGTTAACATATGATTTAAACT
21			✓			✓	Ântisense	9,835	AAACTCTAAATGTTGCCATTTCGTTATCTCCTTTCTGGTATAATTTTGTTA
22	✓	×		✓	×		Antisense	15,911	TGCAAGTTATAAAGTATATTTACTATATCTTTTACTCTAGAATAAAAATTG
23	×	✓				✓	Sense	16,029	AGTTCGGACTAATTTTGATGCTACATATTGTTTTTTATTATAATTGCTGTG
24	×	✓		×	✓		Antisense	19,657	TCACTATAATTAATTTGGCCGTTCACTGTCACCCTAATTGGCATGCGCGCC
25	×	✓		×	✓		Sense	19,846	GAATTTAACGTTCAGAAAGACATCCGAAATAGAATTGAAATCAGACGCAAG
26	×	✓		✓	×		Sense	21,251	TAAAAAAGAATTAGAAATAAGACTAAAGAAACACTCTATATTAAATAATAT
27	×	✓		×	✓		Antisense	23,021	AAGCAATACGGTTTTCAAAATAGTAACTTGCTAAAGGGTATATAGCACGAG
28	×	✓		×	✓		Sense	23,071	GAAATCCGATGAAACCGAAGACGAAAATATTATACTCTTGTTTCGCTTGCA
29	✓*	✓	✓*	✓	✓*		Antisense	23,288	TTTCTTGATTCTTAGTTGTTGTTCTTTGATAACAGGGATAAGATGAATCCA
30			✓			✓	Sense	35,873	CTTCGGTACTGACTTTTTATTTATTGTTGTAATTATGGTAATATGCAGAAG
31	✓	×		×	✓		Sense	36,871	TCGCTAACTTTGGCTGGTTTCGATGGTTAAATGGTTATTAATTTAATCTTT
32	×	✓		×	✓		Sense	37,110	AATAGCCACCGTCTTTTTAACGGGCTCATTAGGGTAACATGTTTGCGCATG
33			✓			✓	Antisense	37,168	TAATAAGCCGTCTATTTGATATTTATATTATGGTGTGTTAATTTATATATA
34	×	✓		×	✓		Sense	37,231	AAGCAGTCGGTAAATCTGCAAGTAAAATAACAGTTGGAAGTAAAGCGCCTT
35	×	✓		×	✓		Sense	38,047	AGTCGGTAAATCTGCAAGTAAAATAACAGTTGGAAGTAAAGCGCCTTATAA
36			✓			✓	Sense	38,051	CTTTTAATATTTTATTGATTTTTAATATTTTTTCGATATAAAATGAAGTTG
37	✓	×		✓	×		Sense	38,396	TGTTAGCTATAAAAAGAGATAAATAAAAACAAATATATTATATTTGGAGGA
38	×	✓		✓	×		Sense	38,473	ATAAAAAGAGATAAATAAAAACAAATATATTATATTTGGAGGAAGCGCCAT
39	×	✓		✓	×		Sense	38,481	TGCATCAAGTTCATTCGACAAAGGAAAATATAAAAAAGGCGATGACGCGAG
40	✓	×				✓	Antisense	38,607	AGTCACATTTACCATCAAATACGGGCCTGTTGGTTCAAAATAACTCGCGTC
41	✓*	✓	✓*	✓*	✓	✓*	Sense	38,650	TGCGACAGCATATAAAGAGTTTAGAGTAGTTGAATTAGATCCAAGCGCAAA
42			✓			✓	Antisense	38,817	CTTCTTTTTTCTTATTCTTATCATAATAAGTGACTTCGATCTTTGCGCTTG
43			✓	✓	×		Antisense	38,858	TATAATAACCTTTGTAATTAAGTTGAATCCAGGGTTTTTAATATGCTCTGA
44	✓	×				✓	Sense	38,959	CTAATCAGATATTAGGTGACTTATGGGGAGAAATCAGTTAGGATGAAAAAG
45	×	✓		✓	×		Sense	39,127	ATAATCCTTTTTTAGGCAGGTACTTCGGTACTTGCCTATTTTTTTATGTTA
46			✓			✓	Sense	39,181	TTAGGCAGGTACTTCGGTACTTGCCTATTTTTTTATGTTATAATCTTTCTA
47			✓			✓	Sense	39,192	TTGTATGTTATAGCTAGCTTTCGGGCTAGTTTTTTGTTATGATGTGTTACA
48	×	✓				✓	Antisense	39,270	ATCATAACAAAAAACTAGCCCGAAAGCTAGCTATAACATACAATCTAAAAA
49	×	✓				✓	Sense	39,262	TACTACTCCCTCGTAGTATATATGACTTTAGCATTCCCGTATAATAGTTTAC
50			✓			✓	Sense	39,406	CATTCCCGTATAATAGTTTACGGGGTGCTTTTTATGTTATAATTAACTGTA
51	×	✓		×	✓		Sense	39,436	GTGGATTGCGAAAACTGGGAAGAAGACACTCCATTTAAAGATCCGCGCGAG
52	✓	×				✓	Antisense	41,038	AGTTCCTCTTCGGGCTTTTCCGTCTTTAAAAAATCATACAGCTCGCGCGGA
53	✓	×		✓	×		Antisense	41,079	TATTTTTTATTTTCTACACTTCTGTGTTTTACTTTTGTTAAAATATATAGG
54			✓			✓	Sense	41,891	AAATGAGTTAATGAGTTGACTATAACTAATAAGATATTAATTATATTTGTA
55	✓	×		✓	×		Sense	42,092	TCTATTATTAATTTCTTGACTTCTTTTTAAAGATTTATTACTTTTACATTC
56			✓			✓	Antisense	42,412	TATAATAATATTGCTTGACATATCAAACTAGATAGTACTATTTTGAATATA
57			✓			✓	Antisense	42,450	TATAATAAATTAGAGTAGACAACTCAGAATTCCAATTTTATAATAATATTG

Differential gene expression analysis of prophage Φ13

Data files obtained from tagRNA-seq approach were used for differential gene expression analysis. Complete dataset of differential gene expression analysis is listed in “Additional data files” at the end of this thesis and in Supplemental Material of Manuscript II. Differential gene expression data of prophage regions were extracted (Supplemental Table 4) and further analyzed, allowing direct comparison of prophage gene expression levels (Supplemental Table 4). Significance was set to FDR-value <0,05 and log2 fold-change (log2-FC) <-1 or >+1. Datasets obtained from the 8325 background were set as control.

Differential gene expression analysis of Φ13 revealed only nine genes to be significantly overexpressed under uninduced conditions (Figure 7). Of these, eight are significantly higher expressed in the 8325 background (since 8325 is set as control for expression analysis, negative log2-FC values imply higher expression in 8325). This included *OrfC*, corroborating the northern blot analysis (Figure 5D). Furthermore, SAOUHSC_02232 and SAOUHSC_02218 were significantly higher expressed in the 8325 background. These genes are transcribed in the lysogenic transcription direction but are localized within the lytic phage region, with TSSs predicted for these genes (Figure 6, Table 1 and Supplemental Table 3). Additionally, IEC genes were higher expressed in 8325 compared to MW2, specifically *sak* (coding for staphylokinase) and SAOUHSC_02175 (coding for sprF1, belonging to TA-system sprF1G1). For *sak* it was already described that expression is dependent on the host strain background, with expression being lower in MW2 compared to RN6390 (another derivate of 8325) (Wirtz et al., 2009). SAOUHSC_02175 and SprF1 annotation largely overlap and therefore presumably represent the same gene. SAOUHSC_02175 stems from the original annotation (reference sequence obtained from NCBI database), whereas the SprF1 annotation was manually inserted, based on a publication on the SprFG TA-system in which SprF1 is defined as a cis-acting antisense RNA regulating the SprG1 RNA and peptide (Pinel-Marie et al., 2014). SAOUHSC_02191, coding for major capsid protein (mcp), was the only gene showing significant higher expression in MW2. This was unexpected, since all other genes of the lytic module did not show significantly different expression. Interestingly, TSS prediction revealed several enriched TSSs within the morphogenesis module for capsid production. However, TSS 25, enriched only in MW2, is located upstream within SAOUHSC_02194, coding for portal protein. In contrast, TSS 26 was enriched in 8325 background only, but located in close proximity upstream of the mcp-coding gene. Regardless, these findings indicate the presence of a promotor that results in active and strain-specific transcription of this particular gene.

Differential gene expression analysis of Φ13-kanaΔrep in mitomycin C treated (induced) condition revealed 40 prophage-encoded genes to be significantly regulated, of which 35 were higher expressed in the MW2 background (Figure 8). All of these were located in the regulation / replication and morphogenesis modules. The lack of significantly differentially

expressed genes in the replication/packaging module spanning several genes might result from deletion of the replication factor. However, these findings indicate that expression of lytic genes of prophage Φ13 are more pronounced in the MW2 background.

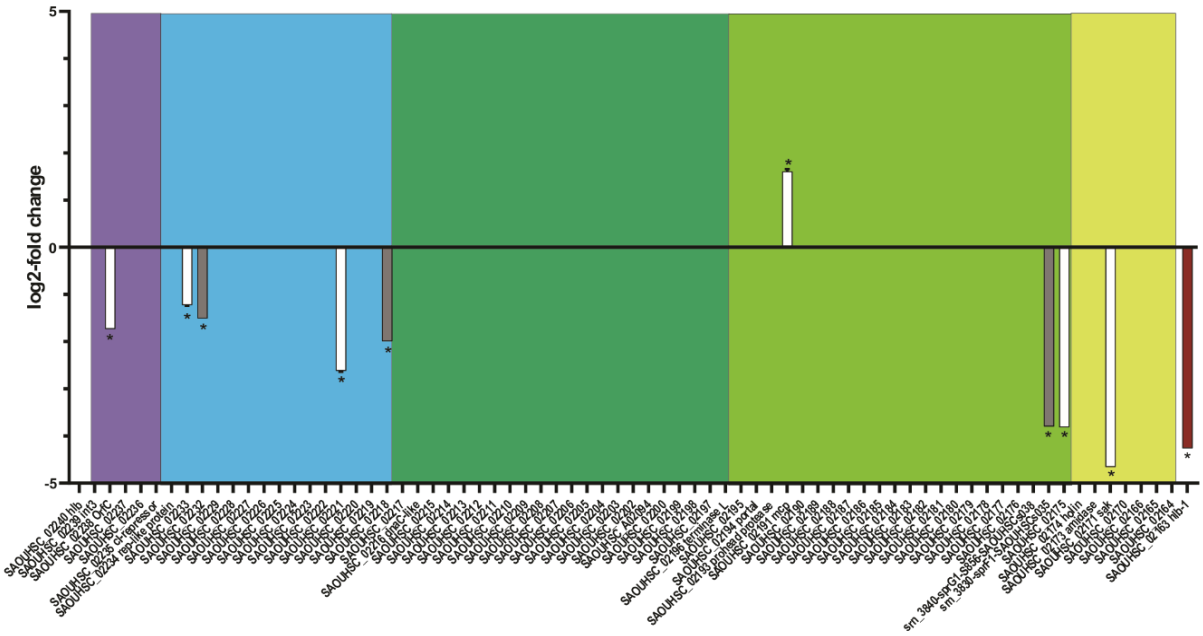

Figure 7: Differential gene expression of prophage Φ13-kanaΔrep by comparison of MW2- versus 8325-background under uninduced conditions. Prophage genes are visualized on the x-axis while log2-fold change is depicted on the y-axis. Only significant genes are visualized (FDR-value <0,05; log2fold change >+1 and <-1) and marked with asterisks. White bars represent genes encoded in lytic direction, grey bars represent genes in lysogenic direction. Since 8325 is set as control for expression analysis, negative values represent higher expression in 8325 background, positive values represent higher expression in MW2 background. Statistics were performed using Wald-test (CLC genomics workbench). Genetic modules are colored in violet (lysogeny), blue (regulation), dark green (replication), light green (morphogenesis) and yellow (lysis / IEC).

This results in a higher genome copy number, which is followed by enhanced expression of the lytic module, in turn leading to higher phage production. In 8325, higher expression under induced conditions was only detected for *sak*, *sprF1*, SAOUHSC_02218 and *orfC*, all of which were already detected to be higher expressed under uninduced conditions. Furthermore, expression of *orfC* is consistent with the northern blot analysis (Figure 5D).

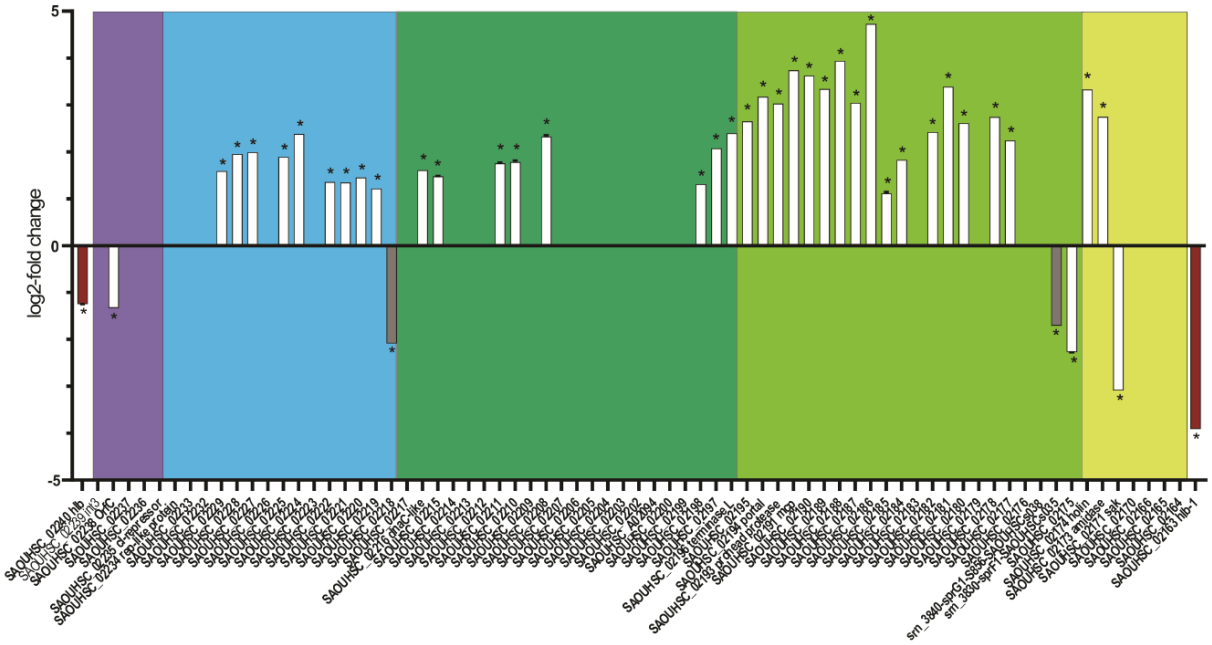

Figure 8: Differential gene expression of prophage Φ13-kanaΔrep by comparison of MW2- versus 8325-background under induced conditions. Prophage genes are visualized on the x-axis while log2-fold change is depicted on the y-axis. Only significant genes are visualized (FDR-value <0,05; log2fold change >+1 and <-1) and marked with asterisks. Therefore 8325 is set as control for expression analysis, negative values represent higher expression in 8325 background, positive values represent higher expression in MW2 background. Statistics were performed using Wald-test (CLC genomics workbench) White bars represent genes encoded in lytic direction, grey bars represent genes in lysogenic direction. Genetic modules are colored in violet (lysogeny), blue (regulation), dark green (replication), light green (morphogenesis) and yellow (lysis / IEC).

Next, we analyzed differential gene expression between induced and uninduced conditions separately for 8325 (Figure 9A) and MW2 (Figure 9B). The majority of prophage genes in 8325 (40 out of 73) and MW2 (56 out of 73) were significantly upregulated after mitomycin C treatment, which was expected. Although we used a replication-deficient phage mutant in this study, expression data revealed a strong upregulation of nearly all phage genes in response to mitomycin C, which was even pronounced in the MW2 background. Since northern blot analysis revealed no strain-specific differences in *recA* transcription, we speculate that yet unknown staphylococcal factors affect phage replication efficiency.

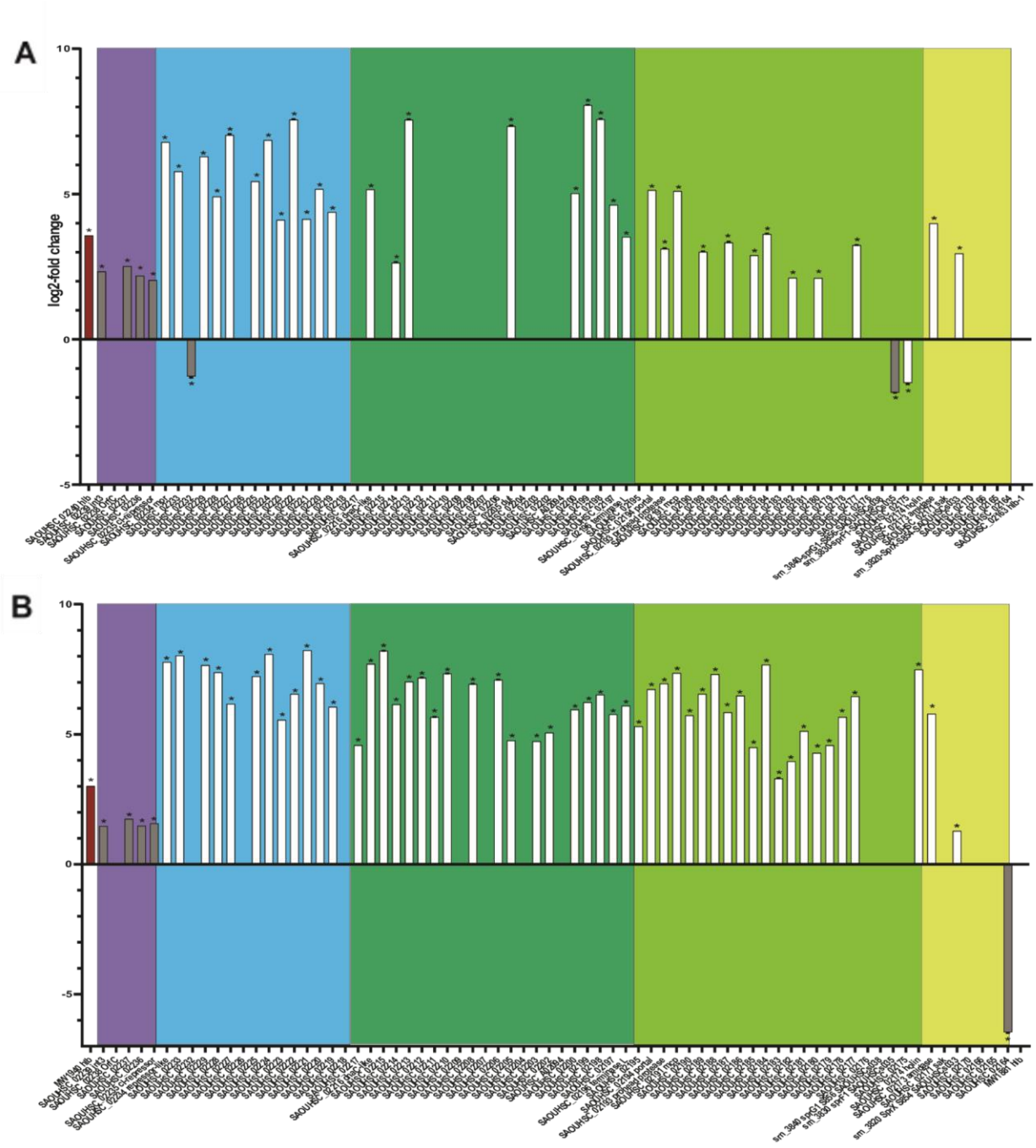

Figure 9: Differential gene expression analysis of prophage Φ13-kanaΔrep in A) 8325 and B) MW2 background by comparison of induced (mitomycin C treated) versus control condition. Prophage genes are visualized on the x-axis while log2-fold change is depicted on the y-axis. Only significant genes are visualized (FDR-value <0,05; log2fold change >+1 and <-1) and marked with asterisks. Therefore uninduced condition is set al control, negative values represent decreased gene expression upon mitomycin C treatment and positive values represent increased gene expression upon mitomycin C treatment. Statistics were performed using Wald-test (CLC genomics workbench); White bars represent genes encoded in lytic direction, grey bars represent genes in lysogenic direction. Genetic modules are colored in violet (lysogeny), blue (regulation), dark green (replication), light green (morphogenesis) and yellow (lysis / IEC).

Discussion

Sa3int prophages are known to play an important role in adaption to the human host, whereas infection of other mammals is associated with loss of Sa3int phages (Rohmer & Wolz, 2021). Sa3int phages encode human-specific virulence genes on an immune evasion cluster (IEC) like *sak*, *chps* and *scn* (Rooijakkers et al., 2006; Rooijakkers et al., 2005; van Wamel et al., 2006). Their expression is dependent on staphylococcal regulators (Rooijakkers et al., 2006) but also on phage replication (Sumby & Waldor, 2003). Distribution of Sa3int prophages within a bacterial population is of high importance, as evidenced by active phage-mediated horizontal gene transfer of these virulence genes. This depends on transfer, lysogenization and induction of phages, which are regulated by multiple factors.

In this study, we shed light on these processes by investigating two Sa3int prophages in regard to their biological behavior (spontaneous phage transfer, lysogenization and induction) as well as on the genetic level investigating gene expression levels in different *S. aureus* isolates. Transfer frequency of Sa3int prophages was strain-dependent and was mainly reliant on the recipient strain. Furthermore, lysogenization frequency showed also strain-dependent behavior, indicating influence of staphylococcal factors in the establishment of the lysogenic life cycle. Strain-specific transfer and lysogenization give rise to the hypothesis that Sa3int phages are able to sense yet unidentified signals that allows for detection of bacterial density and presence of lysogens. Lysogens are resistant against infection of the same phage as a consequence of prophage carriage, which is termed superinfection immunity or superinfection exclusion (Zinder, 1958). Such phage-associated communication systems are described, e.g. for Vibrio phage to sense quorum-sensing (QS) autoinducers produced by the host (Silpe & Bassler, 2019) mediating lysis-lysogeny decision. Further, SPbeta phages of *Bacillus* ssp. use an “arbitrium” communication system to measure small peptides produced by prophages, which allows measurement of lysogen content within bacterial population to decide for lytic-lysogenic switch (Erez et al., 2017). Therefore, it is plausible that Sa3int prophages of *S. aureus* might also use a yet unknown way of “sensing” lysogen content within the population, influencing the pace of establishing a lysogenic life cycle. Furthermore, TSS prediction and RNAseq analysis revealed positions that could encode putative sRNAs expressed on the prophage genome. It is possible that these sRNAs either act within a yet unknown communication system or specifically cis-regulate gene expression. Several prophages were described to encode sRNAs regulating gene expression of own phage-encoded genes like described for Salmonella phage Gifsy-1, or to regulate specific host genes like described for EHEC (Hershko-Shalev et al., 2016; Sudo et al., 2014). In this sense, it remains to be investigated if sRNAs are transcribed from putative locations assumed by TSS prediction. Prediction of TSSs revealed several within the lytic modules, which outlines several starting points for regulated transcription. This might be analogous to *E. coli* phage λ, in which protein Q acts as transcriptional antiterminator,

expressed from gene *Q* that is encoded at the end of the early-genes operon. Acting as transcriptional antiterminator, Q thereby allows expression of the late operon (Roberts et al., 1998). It might be possible that Sa3int prophages also encode genes acting like *Q* in order to delay expression of late prophage genes.

Differences in produced progeny virions were strain-dependent due to differences in lytic gene expression. Higher abundance of lytic transcripts encoding for structural proteins and more abundant phage genomes produced, leads to the conclusion that more phage particles can be packed and released. Alternatively, it is possible that lysis of the host cells is dependent on the strain influenced by cell wall composition. Nevertheless, replication and gene expression were elevated in the MW2 strain background, indicating specific regulation by yet unknown host factors. We could exclude differences in the SOS-response due to similar *recA* expression levels which might have led to increased lytic switch behavior in the MW2 background. This is further strengthened by the fact that we do not detect significant differences in the differential expression of the lytic switch region (*cI*-repressor and *mor*). Therefore, the switch to the lytic cycle is most likely not influenced by host factors. So far, external influence on phage replication was described for lactococcal phage P008, which showed elevated burst size and adsorption rates, but this was dependent on temperature and pH(Müller-Merbach et al., 2007).

It was described that phage abundance is strongly influenced by the extent of host machinery usage by temperate phages, which rely on DNA signatures on the phage genomes to specifically influence replication (Blaisdell et al., 1996). These also include methylation, which is known to be diverse within *S*. *aureus* due to different restriction-modification systems. However, if methylation would have influence on phage replication efficacy, this would only explain differences between clonal complexes as seen for 8325 (CC8) and MW2 (CC1). Since we also detect highly increased phage replication in Newman (CC8), it is rather unlikely that replication efficacy of Sa3int prophages is dependent on DNA modifications. For T4 phage of *E*. *coli*, four promotors have been identified which are responsible for expression of late lytic genes but are not utilized directly by host RNA polymerase (Kassavetis et al., 1983). Utilization involves prophage-encoded σ-factors, activator proteins and co-factors to be bound (Kolesky et al., 1999), representing a tightly regulated activation of late gene transcription of T4 phage. Prediction of TSSs indicates that promotors might be present within the replication and morphogenesis module in Φ13, which could thereby specifically influence the expression of replication- and structural genes in response to yet unknown staphylococcal factors. Further studies investigating putative promotor activity under diverse conditions and in different strains will be performed using fluorescence-labelled promotor-fusion assays of designated regions.

Concluding remarks:

In conclusion, we found strain-specific differences in transfer and lysogenization rate for phages of the Sa3int group in diverse *Staphylococcus aureus* isolates, which is associated with phage production. These differences result from differential replication efficacy, which in turn was dependent on differential gene expression of phage lytic genes in relation to the host strain background. Prediction of transcriptional start sides shed light on the transcriptomic organization of phage Φ13 and revealed several regions that are potentially directly influenced by yet unknown staphylococcal factors. This study highlights the importance of deep investigation of regulatory elements present on this phage group, since they strongly influence gene expression of several modules and might represent locations specifically influenced by host factors. This will contribute to better understanding of Sa3int prophage distribution within bacterial populations. It should further be considered that spontaneous phage release, which is also described in this study, resembles a reservoir of available virulence genes for *S. aureus*, thereby potentially enhancing its pathogenicity

Methods

Strains and growth conditions

Staphylococcus aureus derivates and phages used in this work are listed in supplemental table 1. Unless otherwise stated, single-lysogens of *Staphylococcus aureus* carrying Φ13-kana (Tang et al., 2017) or ΦN315-tet (this study) are used. *S. aureus* was grown in Tryptic Soy Broth (Oxoid) at 37°C, 180 rpm. Precultures and Tryptic Soy Agar plates were supplemented with appropriate antibiotics: kanamycin (kana, 50 µg ml-1), tetracycline (tet, 3 µg ml-1), streptomycin (strep, 500 µg/ml), chloramphenicol (chloro, 10 µg ml-1),.

Strain construction

Oligonucleotides used in this study are listed in Supplemental table 2

Generation of phage cured USA300 (USA300c)

Native prophages (Sa2int and Sa3int) of *S. aureus* strain USA300 were deleted using plasmid pKOR1 as it was described for *S. aureus* Newman (Bae et al., 2006). In brief: a 2 kb DNA-fragment containing the respective *attB* sequences were PCR-amplified with following primers (for Sa2int prophage: primer627 and primer628 ; for Sa3int prophage primer434 and primer435). PCR fragment was inserted into pKOR1 and mutagenesis performed as described.

Construction of streptomycin-resistant strains:

S. *aureus* isolates were grown in tryptic soy broth to OD_{600} = 0,7 and supplemented with streptomycin (500 µg ml-1). After 4 hours of growth, serial dilution was produced ins PBS and 100 µl plated on tryptic soy agar plates supplemented with streptomycin (500 µg ml-1). Resistant colonies were selected and growth was compared to parental strain. Only streptomycin-resistant clones unimpaired in growth were used in this study.

Generation of single-lysogens

Usage of single-lysogens was indispensable for all experiments performed in this study to avoid interference with other prophages. Phage lysates of Φ13-kana or ΦN315-tet were obtained after mitomycin C (500 ng ml-1) induction in liquid culture (OD600 = 0,7). After 2h 37°C additional mitomycin C (500 ng ml-1) was added and supernatant collected after further incubation for 1h (37°C). Supernatant was filtered (0,45 µm pore size (Merck) and phage titer enumerated by plaque assay if possible. Single lysogens were obtained by incubation of 10^6 phages with phage-cured *S*. *aureus* strains (10 ml) from exponential growth phase (OD_{600} = 0,7) for 4 h, 37°C. Selection of lysogens was performed on TSA-plates containing kanamycin (50 µg ml-1) or tetracycline (3 µg ml-1) ,respectively. Single-lysogens were subcultivated four times and phage integration at the cognate *attB* site within *hlb*-gene verified by phenotypical loss of β-hemolysin production and PCR using oligonucleotides hlb257 and Sa3intrev.

Construction of phage ΦN315-tet

Phage ΦN315 was labelled with a tetracycline resistance cassette as follows: tetK resistance cassette was amplified from plasmid pT181 using primer pair Tet2-F BamHI and Tet2-R BamHI. ΦN315 IEC specific overhangs were amplified using primer pair IEC::tet A+B and C +D. The 3 resulting fragments were fused using overlap extension PCR, ligated into plasmid pBASE6 (Geiger et al., 2012) and cloned into *E*. *coli* DC10B. The vector was subsequently transferred into *S*. *aureus* N315 using electroporation and mutagenesis performed as described (Geiger et al., 2012). The mutation was verified by PCR and resulted in replacement of phage encoded *chp* and *scn* with the tet cassette, as performed for Φ13-kana

.

Construction of phage Φ13-kanaΔrep

Left and right flanking regions of prophage encoded replication factor (SAOUHSC_02217) were amplified via PCR using oligonucleotides PiMAYrepdelrev/ repdelrev and repdelfor/PiMAYrepdelfor (supplemental table 2) and cloned into shuttle vector piMAY (Monk et al., 2012) by Gibson assembly in *E*. *coli* DC10B. Plasmid was transferred from *E*. *coli* DC10B into 8325-4Φ13-kana and MW2cΦ13-kana via electroporation and mutagenesis

performed as described (Monk et al., 2012). Gene deletion was verified by sequencing of PCR amplicons spanning the mutation site. Further, lysogens were checked for phenotype (β-hemolysin negative) and prophage induction using mitomycin C followed by plaque-assay.

Phage transfer assay

Cultures of donor- (single lysogens) and recipient (phage-cured, streptomycin resistant) strains were grown to exponential phase (OD_{600} = 0,7), mixed at a ratio of 1:1, and co-cultivated for 4 h, 37°C, 180 rpm. Single and mixed cultures were diluted in PBS and CFU determined on sheep blood-containing agar plates (Oxoid) and TSA agar plates supplemented with respective antibiotics (kanamycin 50 µg ml-1, tetracycline 3 µg ml-1, streptomycin 500 µg ml-1) or double antibiotics (kanamycin 50 µg ml-1+streptomycin 500 µg ml-1 or tetracycline 3 µg ml-1+streptomycin 500 µg ml-1), respectively. Phage transfer frequency was determined by CFU grown on double antibiotic containing plates divided by CFU grown on TSA plates containing streptomycin 500 µg ml -1.

Lysogenization assay

Phage-cured derivates of *S. aureus* isolates were grown to exponential growth phase (OD_{600} = 0,7) and infected to an MOI of 0,01 with $3x10^6$ phage particles followed by incubation for 4 h, 37°C, 180 rpm. Cultures were diluted in PBS and CFU determined on agar plates and TSA agar plates supplemented with either kanamycin (50 µg ml-1) or tetracycline (3 µg ml-1) for selection of lysogens. Single colonies were picked and subcultivated on sheep blood containing agar plates to verify loss of β-hemolysin activity. Lysogenization frequency was determined by CFU counted on agar plates supplemented with antibiotics divided by total CFU count.

PFU enumeration via plaque-assay

Phage titer was determined by softagar overlay method as described (Kropinski et al., 2009) using LS1 (CC8) as indicator strain. Briefly, indicator strain was grown to OD_{600} = 0,7 in tryptic soy broth. Softagar (0,5%) was melted and cooled to 55°C. 100 µl bacterial culture was mixed with 3 ml liquid softagar and poured on tryptic soy agar-plate. After solidification, 10 µl of sterile-filtered phage lysate and dilutions thereof are dropped on the bacterial law and incubated at 37°C to enumerate plaque forming units (PFU) the next day.

Phage adsorption assay

Phage adsorption assay was performed according to (Xia et al., 2011) with slight modifications. In brief, 100 µl ($3x10^6$ phages) were incubated with $3x10^8$ bacteria in 1 ml tryptic soy broth for 10 minutes at room temperature under non-shaking conditions. Bacteria

were pelleted (5000 x g, 5 min), supernatant filtered (0,45 µm pore size, Labsolute) and used for PFU enumeration.

Spontaneous phage induction and induction using mitomycin C

Single-lysogens were grown to exponential growth phase (OD_{600} = 0,7) and split into 10 ml aliquots. Aliquots were further incubated with and without subinhibitory concentrations of mitomycin C (300 ng ml-1, Sigma) for 1 h. Supernatant was filtered using membrane filters (0,45 µm pore size, Merck) and used for PFU enumeration or stored at -20°C for qPCR. For absolute quantification of free phage DNA 100 µl of phage lysate was incubated with Proteinase K (AppliChem) for 1 hour at 55°C followed by heat inactivation at 95°C , 10 min. Phage DNA was quantified by qPCR using SYBR Green qPCR Kit (QIAGEN) and primers circlefor and circlerev spanning the reconstituted *attP* site of the phage. For quantification of excised phages within bacteria, *attP* and the chromosomal *recA* (using primers recAF1 and recA661) was quantified within bacterial pellets. For sample preparation, bacteria were mechanically lysed using zirconia/silica beads in high speed homogenizer (6500 rpm, Fastprep). Lysed pellet were boiled for 10 min and stored at -20°C until use. 1µl of a 1:100 dilution (RNase-free water, Ambion) was used for qPCR.

Northern blot analysis and preparation of RNA-probes

RNA isolation and northern blot analysis were performed as previously described (Goerke 2000). Bacteria were grown to OD_{600} = 0.7 followed by 1h, 37° with or without mitomycin C (300 ng ml-1) addition. In brief: bacterial pellet was resuspended in TRIzol (Thermo Fisher Scientific) and mechanically destroyed using zirconia/silica beads in a high speed homogenizer. RNA was isolated as recommended by Thermo Fisher Scientific. Transcripts on northern blots were visualized in hybridization using digoxigenin-labelled DNA probes.
RNA aliquots were subjected to tagRNAseq (Vertis Biotechnologie AG). Library preparation on rRNA depleted RNA samples was performed as follows: first Illumina TruSeq sequencing adapter (CTGAAGCT) was ligated to RNAs containing a 5´monophosphate end (resulting from processing events and thereby represent PSS) followed by treatment with TEX (Terminator Exonuclease, Lucigen) to remove unligated 5´P-ends. Next, RNA 5´Polyphosphatase (5´PP, Lucigen) was used to convert triphosphate groups at 5´-RNA ends to monophosphate 5´-RNA ends. Formed monophosphate ends were then tagged by ligation of a second Illumina TruSeq sequencing adapter (TAATGCGC) (representing TSS). After fragmentation, oligonucleotide adapter was ligated to 3´ end of RNA fragments and cDNA synthesis performed using M-MLV reverse transcriptase. cDNA was PCR amplified within 16 cycles using high fidelity DNA polymerase. cDNA was purified using Agencourt AMPure XP Kit (Beckman Coulter Genomics). Last, cDNA pool was single-read sequenced on an Illumina NextSeq 500 system using 75 bp read length. Output raw data sets include

unassigned read-files used for differential expression analysis, read-files assigned to either transcriptional start site (TSS) or processed start site (PSS) used for evaluation of transcriptional start sites.

Differential expression analysis of phage-encoded genes using tagRNAseq

Reference genome of *Staphylococcus aureus* 8325 was downloaded from NCBI (NC_009975) and deletion of native phages was performed using Geneious®, followed by addition of Φ13-kana genomic sequence at cognate *attB* site within the *hlb*-gene and SNP-correction based on (Berscheid et al., 2012). Raw data files including raw reads were trimmed using CLC genomics workbench (QIAGEN). Trimmed reads of were mapped against the respective reference genome and data files were obtained from CLC genomics workbench containing RPKM-values for each annotated gene, which are normalized considering gene length. RPKM-values were used for differential expression analysis using Wald test for statistical analysis (CLC genomics workbench, QIAGEN). Significance was set to FDR-value of <0,05 and log2-FC <-1 or >+1. From resulting datasets, genes encoded on prophage were extracted and further analyzed (Supplemental Table 4).

Determination of Transcriptional start sites

To prepare the raw read data for TSS identification, reads were preprocessed, mapped and a coverage per base was computed. For this, the RNA-Seq analysis pipeline READemption version 0.5.0 (Förstner et al., 2014) was used, in particular the subcommands align and coverage. All read samples were mapped to the respective reference sequence with the subcommand align, which integrates the mapper segemehl version 0.3.4 (Hoffmann et al., 2009). For the mapping, the following parameters were used: (--adapter AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC, --processes 4, --segemehl_accuracy 95, --segemehl_evalue 5.0, --poly_a_clipping, --min_phred_score 20 --fastq, --progress).The subcommand coverage calculates one position based coverage files, also called wiggle files, resulting in three file sets: the unnormalized raw wiggle files, files normalized by the total number of mapped reads (TNOAR) and multiplied by one million (mil_normalized), and files normalized by the total number of mapped read and multiplied by the lowest number of mapped reads taking all libraries in consideration (min_normalized). The min_normalized wiggle files were used for TSS calling. The TSS identification using the normalized wiggle files of the tagRNA-seq reads was conducted with TSSpredator 1.1 (Bischler et al., 2015; Dugar et al., 2013). For all of the TSSpredator runs, the following parameters were used: stepHeight: 0.3, step height reduction 0.2, step factor: 2.0, step factor reduction: 0.5, enrichment factor: 2.0, procesing site factor: 1.5, step length: 0.0, base height: 0.0, normalization percentile: 0.9, enrichment normalization percentile: 0.5, cluster method:

HIGHEST, TSS clustering distance: 3, allowed cross-genome shift (allowed cross-condition shift): 1, allowed cross-replicate shift: 1, matching replicates: 2, UTR length: 300, antisense UTR length: 100. TSSpredator expects two types of reads, one from the so-called enriched library and one from the so-called normal or unenriched library. For the tagRNA-Seq data, we use the TSS labelled reads as the enriched libraries, and the PSS labelled reads as the normal, control libraries. The experimental setup of this study used three strains and compared two conditions. Therefore TSSpredator was run both with the strain-setup and condition-setup, to analyze this data. For the cross-condition analysis each strain was considered separately. For the cross-strain analysis TSSpredator expects wiggle files normalized across all input libraries as input, therefore all wiggle files of all strains were normalized manually for the cross-genome analysis. For this, the lowest number of aligned reads over all replicates regarding both conditions was calculated and then each library was multiplied by this minimum. From each TSSpredator run the resulting MasterTables for each condition were combined manually and phage region was extracted (suppl. Table X). For the cross-condition analysis each strain was considered separately, resulting in three independent TSSpredator runs and MasterTables. The MasterTable contains all predicted TSS classified into five TSS classes. Depending on the relative occurrence to an annotated gene defined by the given UTR length, the identified TSS are classified in primary, secondary, internal, antisense and orphan TSS. A detailed description of TSSpredator parameters, TSS classes and output files can be taken from the UserManual available at: http://it.inf.uni-tuebingen.de/?page_id=190

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Conflict of interest

The authors have no conflicts of interest to declare

Supplemental material

Supplemental Table S1: Strains used in this study

Bacterial strain	Clonal complex (CC)	Property	Origin
8325-4	CC8	Phage-cured	Dorte Frees, University of Copenhagen, Denmark
8325-4-Strep	CC8	Phage-cured, resistant against streptomycin	This study
8325-4Φ13-kana	CC8	Single-lysogen	This study
8325-4ΦN315-tet	CC8	Single-lysogen	This study
8325-4Φ13-kanaΔrep	CC8	Single-lysogen carrying replication-deficient mutant	This study
SH1000	CC8	Phage cured	Susanne Engelmann, TU Braunschweig, Germany
SH1000-Strep	CC8	Phage-cured, resistant against Streptomycin	This study
SH1000Φ13-kana	CC8	Single-lysogen	This study
SH1000ΦN315-tet	CC8	Single-lysogen	This study
USA300c	CC8	Phage-cured	This study
USA300c-Strep	CC8	Phage-cured, resistant against Streptomycin	This study
USA300cΦ13-kana	CC8	Single-Lysogen	This study
USA300cΦN315-tet	CC8	Single-lysogen	This study
Newman-c	CC8	Phage-cured	T. Bae et al., 2006
Newman-c-Strep	CC8	Phage-cured, resistant against Streptomycin	This study
Newman-cΦ13-kana	CC8	Single-Lysogen	This study
Newman-cΦN315-tet	CC8	Single-Lysogen	This study
MW2c	CC1	Phage-cured	Tang et al., 2017
MW2c-Strep	CC1	Phage-cured, resistant against Streptomycin	This study
MW2cpΦ13-kana	CC1	Single-Lysogen	This study
MW2cΦN315-tet	CC1	Single-lysogen	This study
MW2cΦ13-kanaΔrep	CC1	Single-lysogen carrying replication-deficient mutant	This study
USA300_Δ*tagO*	CC8	WTA-deficient isolate used as control for adsorption-assay	Wanner et al., 2008
E. coli DC10B	-	Used for cloning procedures	Monk et al.,2012

Name	Sequence
circlefor	TTTTATTTTATATGGGGTATTATTGA
circlrev	GTGTATTCTCATTTGTTAGAAGAAAA
hlb675	GCTATCATTATCGAATCCAC
IEC::tet_A	GACGAATTCGTGAAAAGGGTTGTTTATGGGGC
IEC::tet_B	CTTATATTTTGTTCTAGGATCCCTGTGAATAGTCATAGGCGTCCATACATAATC
IEC::tet_C	GAGTTTTTAGAACAAGGATCCGGTAAAGAAAGTGTTAGGTTACTAGGCCACTTAAC
IEC::tet_D	CTCGAGCTCCCCTGGATTCAACTTAATTACAAAGG
phi13cIDIGfor	TCATACTTCGGATTTAGAGATACC
phi13cIDIGrev	CGAAACCTTATCAAAAGAAACTAGG
phi13croDIGfor	CGGTAAAGTTGGTTGGAA
phi13croDIGrev	ATTGGAGTGGCGTTGATT
phi13sieDIGrev	GAAATCGCTACCAGCTGA
Phi13sieDIGfor	CGCTTCTTCTTACAGGAGTT
Primer434	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGTTCACAGTGATTGTGTATGG
Primer435	GGGGACCACTTTGTACAAGAAAGCTGGGTGCCTGCTACATAGAATGTAGTAGG
Primer627	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATTACATCATCAACTGTATTGTC
Primer628	GGGGACCACTTTGTACAAGAAAGCTGGGTGATGCGTTGAGTAAACTGATTAC
recAF1	GCTCAAGCATTAGGCGTAGAT
recA661	ATTTTAATGCACGTCCACCTGG
sa3intfor	GAAAAACAAACGGTGCTAT
Tet2-F BamHI	TTCACAGGGATCCTAGAACAAAATATAAG
Tet2-R BamHI	TCTTTACCGGATCCTTGTTCTAAAAACTC

Supplemental Table S2: Oligonucleotides used in this study

Supplemental Table 3: TSS prediction on extracted prophage genomic region. Table includes information on predicted TSSs obtained from TSSpredator (https://uni-tuebingen.de/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/informatik/lehrstuehle/integrative-transkriptomik/software/tsspredator/). Detailed description of included information are given in the user guide for TSSpredator V1.1 (https://itnc.informatik.uni-tuebingen.de/index.php/s/en3s7fegaCzWQQy). In brief: information contained in Mastertable are given for each predicted TSS (listed in lines). SuperGenome position (**Column A**) and SuperStrand (**Column B**). MapCount displays the number of strains in which TSS was detected (**Column C**). Detected (**Column F**) and Enriched (**Column G**) contain a "1" if TSS was detected/enriched. Classification as primary, secondary, internal, antisense (**Columns S, T, U, V**) contain a 1 if detected TSSs can be assigned with respect to the gene stated in "locusTag" (**Column N**). Sequence -50 nt upstream + TSS (51nt) contains the base of the TSS and the 50 nucleotides upstream of the TSS (**Column AB**). This table is included on external data carrier (CD attached in this thesis).

Supplemental table 4: Differential gene expression analysis of extracted prophage genomic region. Table contains combined data obtained from CLC genomics workbench (QIAGEN) including expression analysis for prophage genomic region for comparison of MW2 versus 8325 gene locus (**Column A**). Expression values of uninduced condition of MW2vs8325 comparison is marked in yellow (**Column D-F**), expression values for induced condition MW2vs8325 comparison are marked in green (**Column G-I**). Expression values for comparison of condition (induced versus control) are marked in dark blue for 8325 (**column J-L**) and dark red for MW2 (**Column M-O**). RPKM values are listed in Column P-AA and marked in light blue for 8325 and red for MW2 for both conditions. This table is included on external data carrier (CD attached in this thesis).

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Part III: Manuscript II (ready for submission)

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Mutual interaction between Staphylococcal factors and Sa3int prophages

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Abtract

Staphylococcus aureus (*S*. *aureus*) is a known pathogen able to infect humans and animals. Human *S*. *aureus* isolates are often associated with carriage of Sa3int prophages combined with loss of beta-hemolysin production due to phage disruption, whereas animal isolates are positive for beta-hemolysin associated with absence of Sa3int prophages. Sa3int prophages are known to contribute to staphylococcal fitness and virulence in human host by providing human-specific virulence genes encoded on the prophage genome. Strain-specific differences in regard to phage transfer, lysogenization and induction are attributable to yet unknown staphylococcal factors specifically influencing prophage gene expression. In this work we used tagRNA-sequencing approach to specifically search for these unknown host factors. For this purpose, we established a workflow revealing the first direct comparison for differential gene expression analysis on two distinct *S*. *aureus* isolates. Additionally, we investigated influence of Sa3int prophage onto host gene expression. Several genes were identified to show different expression pattern due to prophage presence / absence. Further, global gene expression patterns were investigated in two *S*. *aureus* isolates upon mitomycin C treatment. This provides new insights into the tightly linked host-phage interaction network.

Introduction

Staphylococcus aureus (*S*. *aureus*), as a successful human pathogen, is able to cause a wide variety of diseases, ranging from skin infection to endocarditis, sepsis and toxic shock syndrome. The ability to cause such a wide variety of diseases is mainly due to virulence genes of which some are encoded on prophages integrated in the host genome. Many prophage-encoded virulence genes are associated with specific types of prophages categorized by means of integrase (int)-types (Goerke et al., 2009). Prophages of the Sa3int type are of particular interest as they encode human-specific virulence factors on a region designated as an immune evasion cluster (IEC) and other (putative) virulence genes in proximity to integrase (Rohmer & Wolz, 2021; van Wamel et al., 2006). Adaption to the human host is further reinforced by the association of Sa3int prophages with human *S*. *aureus* isolates and the marked absence of these phages in animal isolates (Matuszewska et al., 2020; Sung et al., 2008). Additional studies revealed that also host jump between animals and humans is associated with the gain or loss of Sa3int prophages (McCarthy et al., 2011; Richardson et al., 2018). Lysogenic conversion of bacteria by toxin-carrying bacteriophages during niche adaption and colonization not only occurs in *S*. *aureus*, but is also suggested for *Streptococcus pyogenes* (Broudy & Fischetti, 2003) and *Corynebacterium diphteriae* (Pappenheimer & Murphy, 1983). Virulence genes encoded on Sa3int prophages have shown to be dependent on prophage life cycle. An example of this is the expression of *sak*, which is highly elevated during prophage induction (Goerke et al., 2006). However, *sak*, as well as other IEC-encoded genes *chp* and *scn*, are also regulated by several staphylococcal regulators such as *agr*, the two-component system *sae* and the alternative sigma-factor σ^B (Rooijakkers et al., 2006). It is currently not well understood how expression of genes encoded on Sa3int prophages is influenced by the host bacterium and how Sa3int prophages might influence *S*. *aureus* gene expression vice-versa.

Due to the carriage of human-specific virulence genes, and their gain or loss during host-jump, Sa3int prophages are expected to play an important role in colonization and persistence of *S*. *aureus* within the human host. As an opportunistic pathogen, *S*. *aureus* is able to adapt rapidly to changing environments, and thus, finely adjust its regulatory network to given conditions. These changes are often related to RNA processing, since post-transcriptional modifications are important to transcript stability and functionality. To date, deep insight into these processes is still missing. Several RNA-sequencing approaches to investigate transcriptional start sites (TSS) have been developed. These methods rely on the presence of a triphosphate group at the 5´-end of mRNA from transcriptional start sites (TSS) and a monophosphate-group at the 5´-end from cleavage sites, known as processed start sites (PSS). Different methods for TSS detection are available, such as differential RNAseq

(dRNA-seq) used in *Heliobacter pylori* or SMRT cappable-seq used in *E*. *coli* (Sharma et al., 2010; Yan et al., 2018). Labelling distinct mRNA-ends with two unequal RNA-oligonucleotides to distinguish TSS and PSS transcripts represent a more specific method (Fouquier d'Hérouel et al., 2011). Coupling of differential labelling with traditional RNAseq analysis was termed tagRNA-sequencing, which has allowed the first insights into transcriptomic architecture in *Enterococcus faecalis* (Innocenti et al., 2015).

Former work revealed strain-specific differences in phage transfer, lysogenization frequency and induction for prophage Φ13 which was the result of differences in replication efficacy influenced by yet unknown staphylococcal factors (Rohmer et al., manuscript I). However, in this study we only focused on the phage itself. In the current study, we set out to identify *S*.. *aureus* factors that are able to influence prophage behavior. Additionally, we investigated influence of prophage presence onto host gene expression. Furthermore, we used a tagRNA-sequencing approach to investigate differential gene expression within two different *S*. *aureus* derivates to unravel strain-specific differences which influence phage replication. Finally, we detected influence of the NER excision repair system on phage replication which was in part independent from the LexA-regulated SOS-response.

Results and discussion

tagRNA-sequencing datasets used in this study

The tagRNA-seq transcriptomic analysis performed in this study consists of three separate parts. First, we investigated the influence of prophage Φ13 on its initial host isolate, *S*. *aureus* 8325. For this purpose, transcriptomic data sets derived from single-lysogenic 8325 carrying Φ13 mutant phage were compared to phage-cured derivate 8325-4, separately in two different conditions (uninduced and mitomycin C treated). Second, the differential gene expression of 8325 and MW2 isolates in both conditions were calculated to resolve the influence of mitomycin C treatment. Last, we adapted a workflow for direct comparison of gene expression levels between MW2 and 8325, to unravel the initial question which strain-specific factors influence the biology of Sa3int prophages (Manuscript I). Table S1 contains a summary of raw data files produced by the tagRNA-seq approach, grouped into separate datafiles (TSS, PSS and unassigned) with respective read counts. Data files termed unassigned are prepared and used for differential gene expression analysis using CLC genomics workbench (QIAGEN).

Impact of Φ13 integration on bacterial gene expression

We have previously described the influence of unknown staphylococcal factors on prophage replication (Rohmer et al., manuscript 1). However, we also hypothesized the prophage Φ13

to have an impact on bacterial host gene expression, based on publications for other prophages. As an example, direct interaction is known for several prophages e.g. in *E. coli* for downregulation of gluconeogenesis genes by prophage-encoded cI-repressor (Chen et al., 2005) or indirect interaction of prophage and host due to phage regulatory switches, like shown in *Listeria monocytogenes* (Feiner et al., 2015). Therefore it was crucial to investigate putative influence of Φ13 onto its host gene expression. Subsequently, we performed tagRNA-seq differential expression analysis on late exponential phase single-lysogen (8325-4-Φ13-kanaΔrep) with and without mitomycin C addition and used phage-cured 8325-4 as the reference, also separated for treated or untreated conditions. Differential gene expression analysis was performed with significance set to P_{adj} <0.05 and log2-FC (abs. > +1 and < -1) setting phage-cured 8325-4 as reference. Significant genes were assigned to different functional categories adapted from (Overbeek et al., 2005) and to regulons adapted from AureoWiki (Fuchs et al., 2018) and (Horvatek et al., 2021), and visualized for untreated (Fig.1B,C) and mitomycin C treated conditions (Fig. 1E,F). Differential expression analysis of uninduced lysogen versus phage-cured 8325 revealed 212 bacterial genes (excluding phage genes and t/sRNAs) to be significantly differentially expressed in the lysogen (Supplemental Table S2 grey columns and Figure 1A). Of these, 119 were significantly upregulated and 93 significantly downregulated in presence of the prophage. Interestingly, several significant genes are associated to regulons of Fur (ferric uptake repressor) and NrdR (repressor for ribonucleotide reductases) with all being upregulated in the presence of prophage. Upon mitomycin treatment, only 44 genes were differentially expressed in the lysogen compared to phage-cured 8325-4 (excluding s/tRNAs and prophage encoded genes), of which 32 genes were upregulated and 12 downregulated (Supplemental Table 2 red columns and Figure 1D). Here again, genes assigned to NrdR-regulon were solely upregulated. Upregulation of *nrd*-genes lead to ribonucleotide reductase activity involved in synthesis of precursors for DNA synthesis (Herrick & Sclavi, 2007). Therefore it is expected that synthesis of precursors are redirected to support phage propagation, which was already hypothesized by other groups (Howard-Varona et al., 2020).

Comparison of both differential gene expression datasets from uninduced and induced condition revealed that a total of 28 genes detected in both analyses and had a similar differential expression pattern (Table 1). This indicates that their expression might be modulated via phage encoded factors and/or disruption of the *hlb*-gene. These genes are scattered along the chromosome and are part of different regulons. Interestingly, one of the differentially expressed genes, SAOUHSC_03046 is predicted as a putative transcriptional regulator of the Xre-family (Ibarra et al., 2013). However, its localization on the genome is not associated with phage-like elements and furthermore, the function of this putative regulator is not known. The strongest impact of Φ13 on gene expression was on the *hlb*-gene (log2-FC -

7,83), which can be explained by the gene interruption due to the integrated prophage resulting in abolished expression.

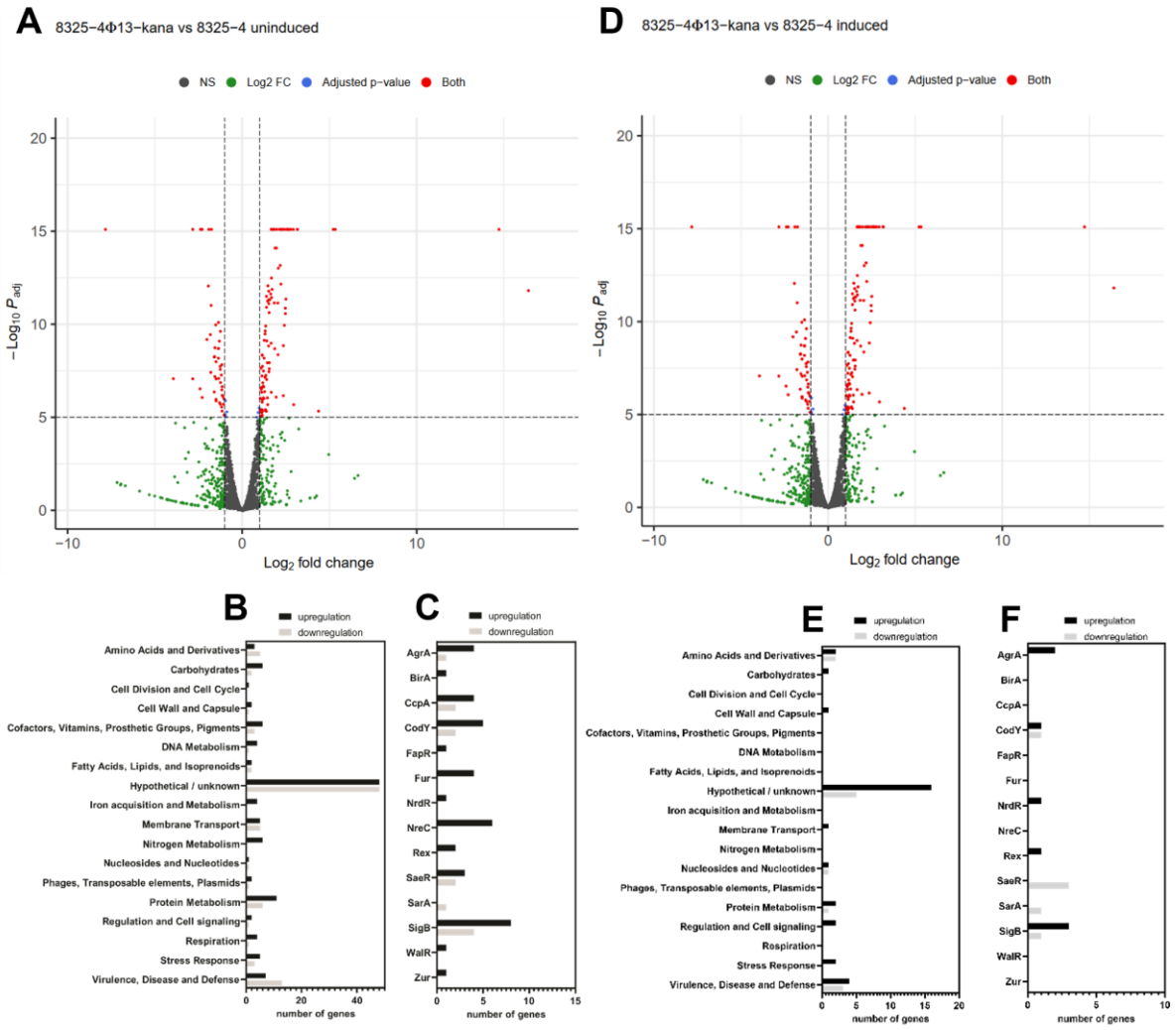

Figure 1: Volcano plots (**A and D**) representing total gene count with corresponding P_{adj} (y-axis) and log2-FC (x-axis) of comparison 8325-4Φ13-kanaΔrep versus 8325-4 in uninduced (**A**) and induced (**D**) condition. Significance was set to P_{adj} (y-axis) <0.05 and log2-FC (x-axis) to <-1 or >+1 with red dots representing significant genes. Blue, green and grey dots are excluded from analysis. **B and E** show association of significant genes to different functional categories under non-induced (**B**) and induced (**E**) conditions, respectively. **C and F** represent association to regulons under non-induced (**C**) and induced (**F**) conditions, respectively. All datasets are included in supplemental table S2.

Of particular interest is the downregulated expression of genes coding for extracellular proteases, in particular *sspA*, *sspB*, and *aur* which were all found to show decreased expression in the lysogen. Additionally, SAOUHSC_01025 and *scdA* were upregulated in presence of Φ13. SAOUHSC_01025 is auxiliary factor A (*auxA*) encoding for a transmembrane protein influencing antibiotic-resistance in *S. aureus* (Mikkelsen et al., 2021). *ScdA*, a gene coding for di-iron-protein ScdA is described to be involved in protecting *S. aureus* from damage caused by nitric oxide and hydrogen oxide exposure (Overton et al.,

2008). Particularly, the upregulation of *scdA* expression, as direct consequence of prophage carriage, could resemble some way for protection for Φ13 because reactive oxygen species are known to the induce lytic phage life cycle upon SOS-response induction. Interception of these molecules by ScdA could be an additional step to maintain the prophage state.

Altogether, we show that the Sa3int phage Φ13 influences the expression of a limited number of bacterial genes that were previously unknown. So far, several studies have been made to investigate how lytic bacteriophages interact with their host at the transcriptional level, e.g. in *Pseudomonas aeruginosa* (Blasdel et al., 2018; De Smet et al., 2016; Wicke et al., 2021). However, direct influence of inherent prophages on their hosts has rarely been studied, one of few examples of this being ΦCD38-2 in *Clostridium difficile* (Sekulovic et al., 2015). In this study, the authors detected 39 genes to be differentially expressed due to prophage carriage, with most prominent influence of the phage on cell surface protein CwpV and several genes involved in glucose, fructose and sorbitol uptake/metabolism. In *S. aureus*, the presence of prophage Φ11 and Φ80α induced enhanced biofilm formation and staphyloxanthin production in lysogens compared to phage-cured background, in addition to several transcriptional changes in the regulatory network (Fernández et al., 2018). Of note, they detected downregulation of *sspA* in Φ11-lysogens, as we also did for Φ13. These observations imply that phage carriage can result in gene regulatory changes and that different phages modulate host gene expression differentially. Our study is the first attempt to investigate the direct influence of a prophage belonging to the Sa3int group on *S. aureus* at the transcriptional level, excluding interference with other prophages. We show that presence of Φ13 significantly downregulates gene expression of proteases that are known to be important for skin colonization and adherence (Lindsay & Foster, 1999; McGavin et al., 1997). We therefore speculate that Φ13 facilitates bacterial adaptation to the human host not only via encoded immune-modulatory factors but also repression of extracellular proteases, which in turn might strengthen bacterial adhesion. However, further investigations regarding the hypothesis of direct influence of Sa3int prophages on proteases SspA, SspB and aur are necessary. Different from the replication-deficient phage used in this study, we expect that the effects seen here will be even more pronounced in the native phage. This includes naturally occurring multi-copy effects that might severely increase differential gene expression values of specific candidate genes identified in this work.

Table 1: Genes differentially expressed due to prophage carriage. Table contains differential gene expression of 28 genes significant for 8325-4Φ13-kanaΔrep versus 8325-4 phage-cured under both uninduced and induced conditions. Data include locus tag, gene name, log2-FC and corresponding Padj-values dependent on the condition, assignment to TheSEED category and described function (obtained from entries of AureoWiki, Fuchs et al., 2018)

8325 locus tag	Gene name	Log2-FC uninduced	Padj uninduced	Log2-FC induced	Padj induced	TheSEED	Function
SAOUHSC_00097 deoD1	deoD1	-2.78	1.89E-05	-1.60	1.95E-04	Nucleosides and Nucleotides	purine nucleoside phosphorylase
SAOUHSC_00202	SAOUHSC_00202	1.68	3.29E-13	1.38	6.08E-06	Hypothetical Protein	hypothetical protein
SAOUHSC_00204 hmp	hmp	1.21	2.10E-07	1.11	2.56E-02	Stress Response	globin domain-containing protein
SAOUHSC_00229 scdA	scdA	2.78	0.00E+00	2.22	7.66E-09	Nitrogen Metabolism	Iron-sulfur cluster repair protein
SAOUHSC_00350 rpsR	rpsR	2.61	0.00E+00	1.89	4.44E-02	Protein Metabolism	30S ribosomal protein S18
SAOUHSC_00408	SAOUHSC_00408	1.72	1.34E-12	1.15	1.26E-03	Hypothetical Protein	hypothetical protein
SAOUHSC_00545 sdrD	sdrD	1.10	8.68E-05	1.30	1.99E-02	Virulence, Disease and Defense	fibrinogen-binding protein SdrD
SAOUHSC_00806	SAOUHSC_00806	1.53	4.80E-12	1.10	4.53E-03	Hypothetical Protein	hypothetical protein
SAOUHSC_00987 sspB	sspB	-1.94	8.85E-13	-1.79	8.82E-06	Hypothetical Protein	cysteine protease
SAOUHSC_00988 sspA	sspA	-1.37	2.02E-09	-1.54	2.13E-04	Protein Metabolism	V8 protease
SAOUHSC_01025	SAOUHSC_01025	2.30	0.00E+00	1.39	2.89E-02	Hypothetical Protein	auxiliary factor A
SAOUHSC_01044	SAOUHSC_01044	2.56	0.00E+00	1.47	1.10E-03	Hypothetical Protein	hypothetical protein
SAOUHSC_01114 efb	efb	-1.19	8.53E-04	-1.24	3.21E-03	Virulence, Disease and Defense	fibrinogen-binding protein
SAOUHSC_01119	SAOUHSC_01119	1.06	1.34E-02	2.04	4.00E-06	Hypothetical Protein	hypothetical protein
SAOUHSC_01128 argF	argF	2.29	0.00E+00	3.23	0.00E+00	Amino Acids and Derivatives	ornithine carbamoyltransferase
SAOUHSC_01129 arcC1	arcC1	1.78	3.08E-04	1.61	1.03E-02	Amino Acids and Derivatives	carbamate kinase
SAOUHSC_01136 psmβ2	psmB2	1.04	2.34E-05	1.60	2.62E-05	Virulence, Disease and Defense	anitbacterial protein
SAOUHSC_01729	SAOUHSC_01729	1.96	8.00E-15	1.04	2.55E-02	Hypothetical Protein	hypothetical protein
SAOUHSC_01755 rpmA	rpmA	1.66	0.00E+00	1.44	1.10E-03	Protein Metabolism	50S ribosomal protein L27
SAOUHSC_01783	SAOUHSC_01783	2.25	1.29E-04	2.21	4.24E-02	Hypothetical Protein	hypothetical protein
SAOUHSC_01935 splF	splF	-1.35	2.00E-09	-1.33	7.14E-05	Hypothetical Protein	serine protease
SAOUHSC_02240 hlb	hlb-2	-7.83	0.00E+00	-3.93	0.00E+00	Virulence, Disease and Defense	hemolysin
SAOUHSC_02332	SAOUHSC_02332	3.17	0.00E+00	1.92	2.14E-04	Hypothetical Protein	hypothetical protein
SAOUHSC_02941 nrdG	nrdG	1.16	4.61E-09	1.26	4.72E-04	Nucleosides and Nucleotides	anaerobic ribonucleotide reductase
SAOUHSC_02971 aur	aur	-1.80	1.12E-05	-1.75	5.80E-04	Virulence, Disease and Defense	zinc metalloproteinase aureolysin
SAOUHSC_03046	SAOUHSC_03046	2.93	0.00E+00	2.10	1.34E-05	Regulation and Cell signaling	helix-turn-helix domain-containing protein
SAOUHSC_03047	SAOUHSC_03047	2.18	6.87E-14	1.93	7.68E-08	Hypothetical Protein	hypothetical protein
SAOUHSC_03048	SAOUHSC_03048	2.37	1.41E-09	2.12	1.91E-07	Hypothetical Protein	hypothetical protein

Mitomycin C treatment reveals upregulation of the LexA regulon

Previously, we showed (Rohmer et al., Manuscript I) that the host background also impacts gene expression of prophage Φ13. Φ13 replication was significantly lower when 8325- was compared to MW2-lysogens upon mitomycin C treatment. However, we also wanted to investigate the global effect of mitomycin C treatment on the bacterial gene expression pattern. For this purpose, we performed differential gene expression analysis for each strain background (8325- and MW2-lysogens and 8325-4) with (and without) regard to mitomycin C treatment (complete datasets are combined in Supplemental Table S3). For analysis, uninduced condition was set as reference. For all separate analyses performed for mitomycin C treatment effects, genes associated with DNA metabolism appeared significantly upregulated and additionally all belong to the LexA-regulon. These data are consistent with data obtained from Cirz et al. who described the SOS-regulon of *S. aureus* in response to ciprofloxacin treatment (Cirz et al., 2007). Ciprofloxacin inhibits bacterial replication due to DNA gyrase stalling, which activates the bacterial SOS-response. The SOS-response is also initiated by mitomycin C treatment due to DNA cross-linking (Bass et al., 2013). Of particular interest, *uvrA* and *uvrB* expression is LexA-regulated whereas *uvrC* is not (Cirz et al., 2007). This is constitent with our data, where *uvrC* is also not detected among significant genes. *uvrA* and *uvrB* were already described in *Listeria monocytogenes* to be induced by mitomycin C, in parallel with *pcrA* (van der Veen et al., 2010). However, for these datasets, we could not directly compare 8325 and MW2 gene expressions because the genome content varied. We therefore developed a new workflow for direct comparison of transcriptomic datasets from two different *S. aureus* strain backgrounds.

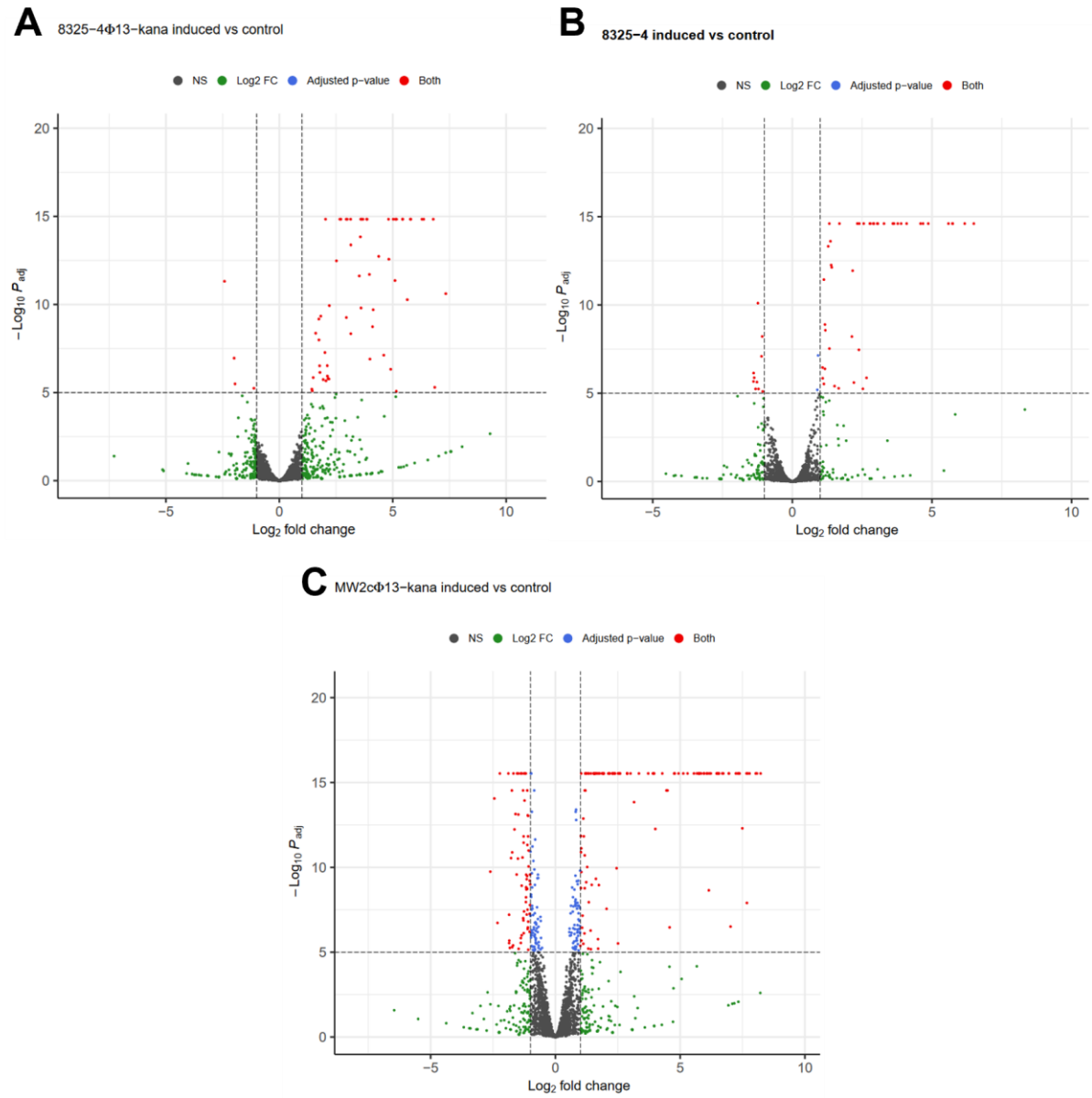

Figure 2: Volcano plots comparing 8325-4Φ13-kanaΔrep (**A**), 8325-4 (**B**) and MW2cΦ13-kanaΔrep (**C**) in induced versus control condition. Red dots represent genes with significant P_{adj} and log2-FC values. Complete datasets are listed and combined in **Supplemental Table S3)**

Differential gene expression analysis of common genes of MW2 versus 8325

References genomes of 8325 (NC_009975) and MW2 (NC_003923) were obtained from the NCBI database and adapted to reflect the single-lysogenic strains used in this study. Gene content of both reference genomes was compared using the AureoWiki database. Genes only present on one of the genomes were excluded from further analysis (Supplemental Table S4, excluded genes). The most obvious differences in gene content are in the accessory gene repertoire, with MW2 containing a type IV SCCmec (starting from MW0025) whereas 8325 does not contain any SCCmec. MW2-encoded genes MW0368 to MW0401 belong to type II *v*Saα genomic island containing several characteristic virulence genes (lpl). Further, among several other genes, *splD* and *splE* are encoded in 8325 but not in MW2. These data are consistent with data published by (Baba et al., 2002). In sum, 227 genes are only present on one genome. All of these genes could represent a putative influencing factor onto phage replication we aim to identify, but could not be investigated using this analysis.

A total of 2693 annotated genes were homologous between the two reference genomes and considered for further analysis. At this stage, CLC genomics workbench reached its technical limitation and we decided to switch to usage of DeSeq2 to compare transcriptional differences. DeSeq2 is able to utilize the prepared tables containing homologous genes with respective unique gene reads for direct comparison. To control for potential differences in the analysis algorithms, DeSeq2 analysis was performed on datasets obtained from condition analysis (previous section) within the same host strain background. DeSeq2 took unique gene reads obtained from initial RNAseq analysis performed on CLC workbench (QIAGEN) as input and the resulting expression values were compared with RPKM values also obtained from the initial CLC genomics workbench expression analysis, as described. Expression values (log2-FC) obtained from the DeSeq2 analysis (Figure 3A and 3B, y-axis) were compared to expression values obtained from RNAseq analysis performed on CLC genomics workbench (Figure 3A and 3B, x-axis) and show only very little variation. The lack of variation showed the two used analysis methods were similar enough to be compared and that we further use DeSeq2 for comparison of homologous genes in MW2 versus 8325.

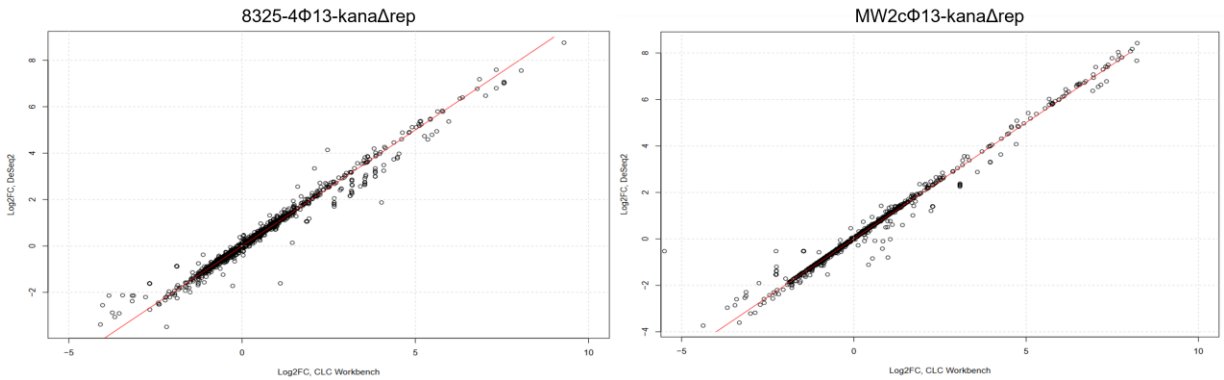

Figure 3: Correlation plot of differential gene expression value (log2-FC) of induced versus control for 8325-4Φ13-kanaΔrep **(left)** and MW2cΦ13-kanaΔrep **(right)** with CLC genomics workbench ® values (plotted on x-axis) and DeSeq2 values (plotted on y-axis).

Differential gene expression analysis of MW2c-Φ13-kanaΔrep versus 8325-4 Φ13-kanaΔrep using DeSeq2

The initial aim of this study was to search for host factors that are responsible for different behavior of prophage Φ13 within two *S. aureus* host strain backgrounds, 8325 and MW2 (see **Rohmer et al., manuscript I**). We therefore established an analysis pipeline using DeSeq2 on a set of genes present on both reference genomes. To our opinion, this was the most reasonable way for detection of host factors influencing prophage gene expression. Therefore strain-specific differences were not only detected for 8325 and MW2 but also for USA300 and Newman (**Rohmer et al., manuscript I**), influencing factors are expected to be present on all strains tested and present among homologous genes. To our knowledge this is the first time that a direct comparison for transcriptional analysis is performed on two *S. aureus* isolates belonging to distinct clonal complexes. Detailed information on the analysis pipeline is described in Methods. In brief: unique gene reads were obtained from RNAseq-analysis using CLC genomics workbench (QIAGEN). Genes were manually curated to obtain only homologous gene pairs (see Supplemental Table S5; as stated above, heterologous genes were excluded from this analysis and are listed in Supplemental Table S4). Differential expression analysis of homologous genes was performed using DeSeq2 (v1.32.0, Bioconductor v.3.13) in R (v4.1.1) for uninduced and mitomycin C treated conditions.

Function and regulons

Differential gene expression analysis of MW2 versus 8325 was performed for uninduced and mitomycin C treated conditions. With 8325 as the baseline, significant upregulation meant higher and downregulation lower expression levels in MW2 compared to expression levels in 8325. In the uninduced condition, 807 out of 2696 homologous genes were significantly up- or downregulated, with P_{adj}-value <0,05 ; log2-FC >+1 and <-1 (Figure 4A and Supplemental Table S5). Excluding s/r/tRNAs and prophage-encoded genes, 758 genes remained for

further analysis. Of these, 398 are up- and 360 are downregulated. With focus on regulons, beside sigma-factor σ^B, the regulators CcpA and CodY are outstanding with a total of 31 and 35 genes differentially regulated, respectively. For CcpA, four showed higher expression in MW2 background whereas 28 showed higher expression in 8325-background. CcpA functions as a regulator for genes assigned to various SEED-categories, like amino acid or carbohydrate metabolism, whereas CodY is associated with regulation of virulence-associated genes. Genes of the *sbn*-gene cluster are downregulated (*sbnABCDGHI*), indicating higher expression in 8325 background. This gene cluster is required for staphyloferrin B synthesis and regulated by Fur (Cheung et al., 2009). Fur-regulated genes were already detected in the first analysis and are probably influenced by prophage Φ13. Because the *sbn*-gene cluster expression is elevated in 8325 background, it is likely that a prophage-encoded gene product which appeared upregulated in differential expression analysis of Φ13 (Rohmer et al., Manuscript I) might regulate this gene cluster. Also putative sRNAs present on Φ13 cannot be excluded to have an influence. Effects of Sa3int prophages on iron-acquisition would be an interesting study objective since this might correlate with adaption to specific niches during *S. aureus* infection process.

Furthermore, eight genes associated with nitrogen metabolism and regulated by NreC are significantly downregulated (higher expression in 8325). Last, most genes which are significantly differentially expressed are regulated by σ^B as a primary regulator (in total 133 of which 124 are upregulated in MW2). This is consistent with our expectation that σ^B-regulated genes to be most pronounced in this comparison because 8325 is deficient in σ^B, due to mutation in the *rsbU* gene whereby RsbU itself regulates sigma-factor σ^B. Only ten primarily σ^B-regulated genes show increased expressed in 8325 background, hypothesizing further regulators other than σ^B influence these genes.

Using Horvatek et al., 2021 as a template, we detailed the influence of sigma-factor σ^B and found 679 of the 758 genes were influenced at least to some extent by σ^B (supplemental table S5). Excluding phage-encoded genes, 71 genes remain which are not associated to σ^B regulation, and are discussed below. In the induced condition, a total of 674 genes were significant. Excluding t/s/rRNAs and prophage genes, 606 genes remained for further analysis, of which 302 were up- and 304 downregulated (Fig 4B and Supplemental Table S5). Significant genes were assigned to different regulons, as described previously. 127 genes were significantly differentially expressed and associated to the σ^B-regulon, of which 120 are up- and seven are downregulated.

Because we could exclude influence of σ^B in Rohmer et al., manuscript I (σ^B-repaired SH1000 shows the same behavior of Φ13 as σ^B-deficient 8325), we focused on genes not associated to the σ^B regulon. Based on data available from AureoWiki and Horvatek et al.,

2021, 71 genes of uninduced and 61 genes of induced comparison are not assigned to the σ^B regulon (Supplemental Table S5), of which 39 were detected in both conditions.

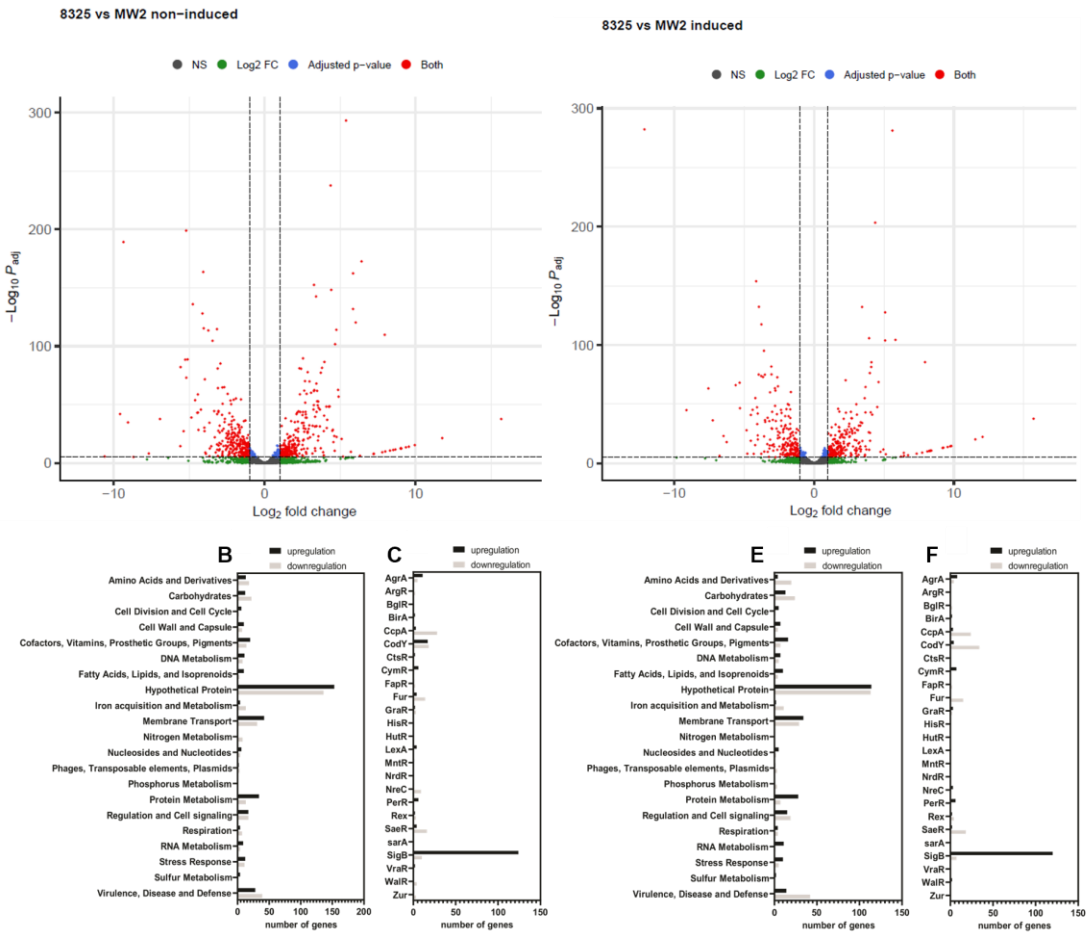

Figure 4: Volcano plots (**A and D**) representing total gene count with corresponding P_{adj} (y-axis) and log2-FC (x-axis) of comparison MW2cΦ13-kanaΔrep versus 8325-4Φ13-kanaΔrep in uninduced and induced condition. Significance was set to P_{adj} (y-axis) <0.05 and log2-FC (x-axis) to <-1 or >+1 with 8325 set as reference. Red dots represent significant genes. Blue, green and grey dots are excluded from analysis. **B and E** show association of significant genes to different functional categories under non-induced (**B**) and induced (**E**) conditions, respectively. **C and F** represent association of significant genes to regulons under non-induced (**C**) and induced (**F**) conditions, respectively. All datasets are included in Supplemental Table S5.

The majority of the significantly differentially expressed genes are associated to protein metabolism (14 in uninduced and 17 in induced conditions) and code for ribosomal proteins. This could already be influenced by phage replication, as higher amount of ribosomal proteins present in MW2 could enable faster and more efficient phage replication. Interestingly, two genes assigned to DNA metabolism appear in both induced and uninduced conditions, *recJ* and *uvrB*. RecJ is a single-stranded DNA-specific exonuclease whereas UvrB is a subunit of exinuclease complex UvrAB, regulated by LexA. Its appearance, especially under non-induced conditions, suggests UvrB to have a role independent of the bacterial SOS-response. *uvrB*-gene is naturally truncated in 8325 background by two ORFs, of which one is annotated as SAOUHSCH_00778 (AureoWiki database) that is 100%

identical to an IS5-transposase of *E. coli* K12. IS5-transposase was detected in bacteriophage λ interrupting the *cI*-repressor gene (Blattner et al., 1974) and to interrupt the *bgl* operon in *E. coli* responsible for β-glucoside utilization (Schnetz & Rak, 1992). IS5 is suggested to act as switching mechanism with transpositional regulating expression of the *bgl*-operon. Active transposition is therefore also expected for IS5 integrated in *uvrB* in 8325 background and should be further investigated, but this was not part of this work. Due to *uvrB* truncation by IS5 transposase, we decided to produce a transposon-mutant of *uvrA*. Therefore UvrAB is known to be active in complexed form, we expect a phenotype similar to an *uvrB* mutant with putative direct influences by IS5 left unaffected. *uvrA::erm* transposon mutants were produced using transposon transduction from NARSA transposon library resulting in *uvrA::erm* transposon mutants in SH1000 and Newman-c single-lysogenic strains carrying Φ13 wildtype phage (SH1000Φ13-kana *uvrA*::erm and Newman-c Φ13-kana *uvrA*::erm, respectively). As described in (Rohmer et al., Manuscript I), a high number of free phages were obtained from *S. aureus* Newman whereas moderate levels of free phages resulted from the SH1000 background. Indeed the *uvrA::erm* -transposon mutant heavily impaired phage replication, as shown in Figure 5.

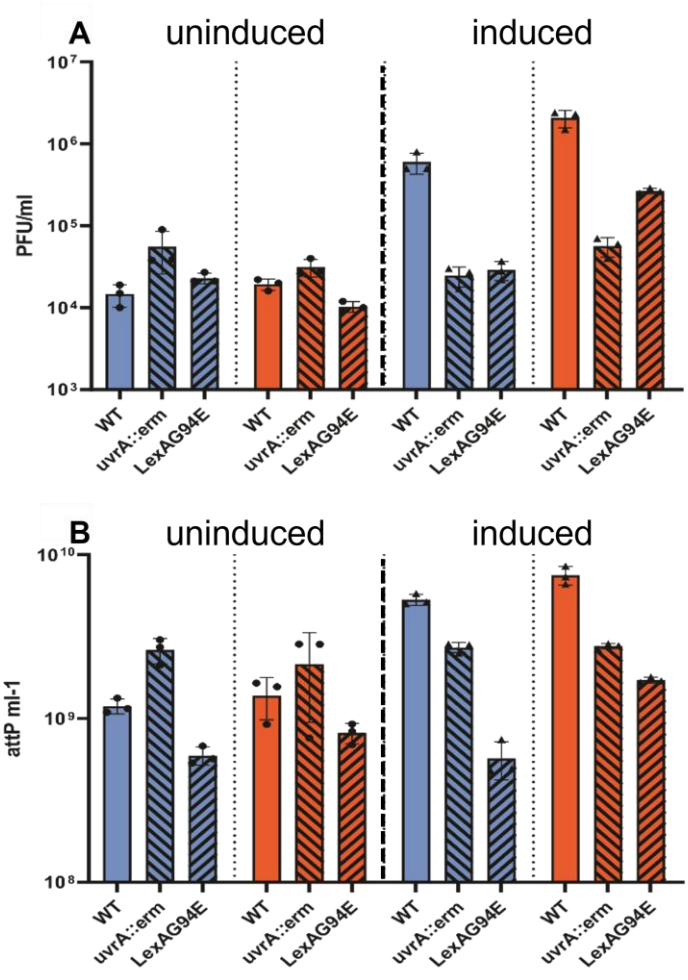

Figure 5: Effect of *uvrA::erm* transposon mutant and *LexAG94E* -mutants on Φ13-kana phage particle production (**A**) and genome replication (**B**) in SH1000 (blue) and Newman (orange) strain backgrounds under uninduced (circles) and mitomycin C treated conditions (triangles). Infectious phage particles were determined via plaque-assay (**A**) and phage genome production was determined via absolute quantification using qPCR approach on collected lysate (**B)**.

However, it was surprising that SH1000Φ13-kana *uvrA*::erm and Newman-cΦ13-kana *uvrA*::erm mutants showed strongly impaired production and release of free phage particles after mitomycin C induction (Figure 5A, right panels) and genome production was highly reduced (Figure 5B right panels). No previously detected strain-specific differences in phage count were detected anymore. Because we hypothesized that LexA, a known *uvrA*-regulator, is responsible for these findings, *lexA*-mutants were produced by exchanging the sequence of one amino acid in the catalytic site (G94E), as described by (Maiques et al., 2006). Loss of the cleavage site rendered LexA inactive and it is therefore not able to regulate the SOS-response or NER-associated genes *uvrAB*. However, the *lexAG94E* mutant still produced slightly more free phage particles than the *uvrA*::erm mutant in the Newman strain background (Figure 5A), which was not detected in the SH1000 strain background (Figure 5A). Nevertheless, detected phage particles after induction decreased compared to SH1000Φ13-kana wildtype. Interestingly, quantification of phage genomes showed a reduction compared to the *uvrA::erm* mutant (Figure 5B). This implies two different functions of *LexA* and *UvrA* on phage biology, with LexA most probably acting on phage induction due to repressor cleavage at start of phage replication, whereas UvrAB probably acts at a different site, potentially affecting gene expression in the morphogenesis module, which will have to be confirmed in future investigations.

In summary, phage replication was not completely abolished in both mutant strains with infectious particles produced by spontaneous induction (Figure 5A).l*exAG94E* was already described to reduce but not completely abolish SaPI induction, suggesting already that a LexA-independent pathway exists for replication and induction of mobile genetic elements (Kelley, 2006). Ampicillin treatment on Φ11 (Sa5int) resulted in the same titer obtained as for untreated RN451 in the *lexAG94E* mutant, although ampicillin is known to induce Φ11 resulting in enhanced phage particle production (Maiques et al., 2006). These findings suggest a LexA-independent pathway for ampicillin-mediated SOS induction of Φ11. Friedberg and colleagues already suggested a putative function of UvrB involved in DNA-replication, under certain circumstances, see (Friedberg et al., 2005) and references herein. Additionally, they stated that TFIIH, the eukaryotic homologue to UvrB, is a critical transcription initiation factor and involved in semiconservative DNA replication and recombination in eukaryotes.

Conclusion

Sa3int prophages are described to be associated with human *S. aureus* isolates, carrying virulence genes beneficial for staphylococcal infection of human host by providing new traits to escape the human immune system. This study further revealed the impact of Sa3int

prophage Φ13 onto its host, *S*. *aureus*. Downregulation of specific proteases like SspA, SspB and aureolysin can be associated to adaption during human host colonization, a consequence of enhanced adhesion due to reduced protease presence. Direct comparison of MW2 versus 8325 backgrounds needed establishment of a workflow which used homologous genes present on both reference genomes for analysis for differential gene expression analysis. Heterologous genes only present on one genome might also influence prophage induction and replication (in particular mobile genetic elements like SaPIs and SCCmec cassette in MW2 background), but these experiments were outside the scope of this project. Direct comparison of homologous genes showed large numbers of differentially regulated genes of which most were a result of σ^B deficiency. Additionally, we identified that the NER system is associated with phage replication in a LexA-regulation independent manner. The exact mechanism behind this remains to be elucidated.

Methods

Bacterial strains and growth conditions

Staphylococcus aureus derivates used in this work are listed in Supplemental Table 6. Single-lysogens of *Staphylococcus aureus* carrying Φ13-kana wildtype (Tang et al., 2017) or replication-deficient Φ13-kanaΔrep mutant (Rohmer et al., manuscript I) were used for all experiments. *S*. *aureus* was grown in Tryptic Soy Broth (Oxoid) at 37°C, 180 rpm. Precultures and Tryptic Soy Agar plates were supplemented with appropriate antibiotics: kanamycin (kana, 50 µg ml-1), chloramphenicol (chloro, 10 µg ml-1), erythromycin (10 µg ml-1).

Strain construction

Oligonucleotides used in this study are listed in Supplemental Table 7

Transposon donor lysate was prepared by infecting USA300 NE145 (Nebraska Transposon mutant library, kindly provided by F. Götz) with phage Φ11, followed by lysate collection and sterile filtration to obtain pure Φ11 lysate. Due to occasionally wrong packaging, some Φ11 viral particles contain transposon instead of own phage DNA which can be transmitted to target isolates. Target *S*. *aureus* isolates SH1000Φ13-kana and Newman-cΦ13-kana were incubated with Φ11 lysate and subsequently plated on tryptic soy agar plates supplemented with erythromycin (10 µg ml-1) and left for incubation for 2-3 days. Erythromycin-resistant colonies grown on plates were checked for transposon integration at the correct insertion site via PCR amplification using primers uvrANE145outsidefor and uvrANE145outsiderev spanning the insertion flanking regions. Transposon integration was verified by uvrANE145outsidefor and TnBuster or TNUpstream, respectively.

lexAG94E mutant in SH1000Φ13-kana and Newman-cΦ13-kana strain background was produced using lexApMAD plasmid which was provided by J.R. Penadés (Maiques et al., 2006). Plasmid was introduced into stated isolates and mutagenesis performed as described (Arnaud et al., 2004).

tagRNA-seq approach

Detailed description of RNA extraction procedure and sample preparation for tagRNA-sequencing approach was performed as stated in Manuscript I and previously described by (Wirtz et al., 2009). Purified RNA was prepared by Vertis Biotechnologie (Straubing, Germany) as described in Manuscript I. Output datafiles obtained in fastq format were further analyzed. In sum, 18 biological samples resulted in 72 datasets comprising raw data file (unsorted), TSS-enriched, PSS-enriched and unnassigned fastQ-files. Supplemental Table 1 includes a summary of distribution from all counts separated into sorted reads with their respective distribution against each unsorted file. Datasets termed unassigned were used for differential expression analysis

Reference genomes:

Reference genome sequence of *S. aureus* 8325 (NC_009975) and MW2 (NC_003923) were adapted as described in Manuscript I. In brief: native phages genomic sequences were removed from original genome sequence files using Geneious ® followed by insertion of Φ13-kana prophage sequence at appropriate *attB* site in *hlb*-gene. SNP correction was performed in accordance to (Berscheid et al., 2012), sRNAs were annotated in accordance to (Carroll et al., 2016). Adapted reference genomes were used for differential gene expression using CLC genomics workbench (QIAGEN), for production of supergenome for TSS prediction and for establishment of supplemental table S5 used for differential gene expression analysis using DeSeq2.

Differential expression analysis due to condition (same reference strain)

Raw data files of reads (unassigned) were trimmed using CLC genomics workbench (QIAGEN). Trimmed reads of were mapped against the respective reference genome and datasets obtained from CLC genomics workbench contained unique gene reads and RPKM-values (normalized considering gene length). RPKM-values were used for differential expression on the same reference genome using Wald test for statistical analysis. Significance was set to FDR-value of <0,05 and log2-FC <-1 or >+1. From resulting datasets, genes encoded on prophage were extracted and further analyzed (Supplemental Table S3).

Cross-comparison differential gene expression analysis

Homologous genes in both reference genomes were identified using the AureoWiki database (Fuchs et al., 2018) and sequence alignments of the two genomes (obtained from NCBI) using Geneious 10.0.9. Genes were considered homologous if the alignment had fewer than 20 SNPs and the gene was found in a respectively similar genomic position. Unique gene reads for homologous genes were obtained from CLC genomics workbench ® (QIAGEN) output data files and used as input in DeSeq2 (v1.32.0, Bioconductor v.3.13, R v4.1.1) to calculate differential expression levels for all strain and treatment conditions. Genes with an FDR value <0,05 and log2-FC <-1 or >+1. Results from differential gene expression are listed in Supplemental Table 5.

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Supplemental Material: Part of the Supplemental material is included on external storage (CD) attached to this thesis

Supplemental Table S1: List of raw data files obtained from tagRNA-seq approach with given read counts of the original fastq-file and the respective sorted read-files assigned to PSS, TSS and unassigned data-files (Column 1). For each data file, number of reads (Column 2) with given percentage of reads (Column C) compared to original file.

Supplemental Table S2: Complete dataset of differential gene expression analysis for 8325-4Φ13-kanaΔrep versus 8325-4 (phage cured). Sheet 1 contains expression values for all genes (locustag, **Column A**) annotated on 8325 reference genome with the respective gene name if known (**Column B**). Additional information for TheSEED category and known Regulators are listed in **Column C,E,F,G** for each gene, respectively. Differential expression values obtained from analysis using CLC genomics workbench (QIAGEN) are given in **Column J-O** for uninduced (marked in grey) and induced marked in light blue (**Column P-U**). RPKM values obtained from RNAseq analysis performed in CLC genomics workbench are

listed in columns V-AG with lysogen marked in green and phage-cured marked in red. Sheet 2 only contains significant gene expression values for uninduced condition, Sheet 3 contains significant gene expression values for induced condition.

Supplemental Table S3: Complete datasets of differential gene expression analysis of 8325-4Φ13-kana (Sheet 1), 8325-4 phage-cured (Sheet 2) and MW2cΦ13-kana (Sheet 3) for induced versus control condition obtained from CLC genomics workbench (QIAGEN). RPKM values obtained from RNAseq analysis (CLC genomics workbench) are marked in blue for uninduced and red for induced condition, respectively.

Supplemental Table S4: genes excluded from DeSeq2 analysis (non-homologous genes) are listed in this table with genes only present on 8325 marked in blue and genes only present on MW2 marked in red (**Column A,** locus tag). Unique gene reads obtained from initial RNAseq analysis (CLC genomics workbench, QIAGEN) used for differential gene expression analysis in DeSeq2 are listed for each replicate and marked in blue for 8325 and red for MW2.

Supplemental Table S5: Mastertable including all homologous genes used for DeSeq2 differential expression analysis for comparison of MW2 versus 8325 in uninduced and induced condition. Table contains gene names listed for 8325 (marked in blue) and MW2 (marked in red). Unique gene reads obtained from initial RNAseq analysis (CLC genomics workbench, QIAGEN) were marked in blue for 8325 and in red for MW2, respectively. Sheet 1 contains all genes with differential gene expression performed for uninduced condition, Sheet 2 contains all genes with different gene expression performed for induced condition.

Supplemtenal Table S6: strains used in this study

Bacterial strain	Clonal complex (CC)	Property	Origin
8325-4	CC8	Phage-cured	Rohmer et al., Manuscript I
8325-4Φ13-kanaΔrep	CC8	Single-lysogen, replication-deficient mutant	Rohmer et al., Manuscript I
SH1000Φ13-kana *uvrA*::erm	CC8	Single-lysogen, transposon mutant	This study
SH1000Φ13-kana *lexAG94E*	CC8	Single lysogen, non-cleavable LexA	This study
Newman-cΦ13-kana	CC8	Single-lysogen	Rohmer et al., Manuscript I
Newman-cΦ13-kana *uvrA*::erm	CC8	Single-lysogen, transposon mutant	This study
Newman-cΦ13-kana *lexAG94E*	CC8	Single-lysogen, non-cleavable LexA	This study
MW2cΦ13-kanaΔrep	CC1	Single-lysogen carrying replication-deficient mutant	Rohmer et al., Manuscript I
USA300_FPR3757 (NE145)	CC8	Nebraska Transposon Library NE145	F. Götz (Tübingen)

Supplemental Table S7: Oligonucleotides used in this study

Name	Sequence
circlefor	TTTTATTTTATATGGGGTATTATTGA
circlrev	GTGTATTCTCATTTGTTAGAAGAAAA
lexADIGfor	AGAATTAACAAAACGACAAAGCGA
lexADIGrev	CCAATTACTTTCCCAATTACAGCAAC
uvrAne145outsidefor	AAGAACGTCAAAAGACAATC
uvrAne145outsiderev	CCATCGATTCTTAAACGTAC
TnBuster	GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC
TnUpstream	CTCGATTCTATTAACAAGGG

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General Discussion

Prophages are tightly linked to evolution of bacteria. Investigation of publicly available microbial genomes revealed that about 40 - 50 % carry at least one prophage (Roux et al., 2015; Touchon et al., 2016). Phage genomes often carry virulence genes that are acquired by bacteria upon lysogenic conversion. Thereby, prophages provide their host bacteria accessory genes, leading to enhanced bacterial survival and host competitiveness. In turn, enhanced survival also ensures the survival of the prophage itself. This is a common strategy as reviewed by (Brüssow H., 2004; Canchaya et al., 2004; Casjens, 2003; Correa et al., 2021; Davies et al., 2016; Howard-Varona et al., 2017). The importance of staphylococcal prophages of the Sa3int group for its host, *Staphylococcus aureus*, is highlighted in Part I (Rohmer & Wolz, 2021) of this thesis. The prevalence of Sa3int prophages within human *S. aureus* isolates indicate their relevance for niche adaption within the human host. This is mainly linked to virulence genes encoded on the immune evasion cluster (IEC) on the phage genome, coding for staphylokinase, chemotaxis inhibitory protein and staphylococcal complement inhibitor. Although these prophage-encoded virulence factors do not have a known function for the phage life cycle itself, they provide *Staphylococcus aureus* with improved survival during infection of the human host, allowing it to hide and escape from the human immune system. This is further underlined by several studies investigating the transmission of *S. aureus* between human and different animal hosts, which is associated with gain and loss of Sa3int prophages (Price et al., 2012; Spoor et al., 2013). In particular, LA-MRSA isolates of CC398 lost Sa3int phages due to host switch from human to animal (Price et al., 2012). The increased household transmission of LA-MRSA CC398, which circulates in low number within the Danish human population, was shown to be associated with acquisition of Sa3int prophages (and their virulence genes encoded on the IEC) (Sieber et al., 2020). Furthermore, in some poultry and horse *S. aureus* isolates, Sa3int prophages were detected but have adapted to the respective host by carrying avian- (e.g. ornithine cyclcedeaminase) or equine-specific (eqSCIN) instead of human-specific virulence genes (de Jong et al., 2018; Lowder et al., 2009).

When a prophage is induced, replication starts and viral genomes are multiplied. Thereby, the abundance of prophage-encoded virulence genes is also increased due to the multi-copy effect. Higher gene copy number paired with elevated gene expression results in an immense increase in abundance of prophage-encoded virulence factors, for example for Shiga-toxin (Stx1) production in EHEC (Wagner & Waldor, 2002) or cholera toxin (CT) production in *Vibrio cholerae* (Lazar & Waldor, 1998). In *S. aureus*, this was also described for staphylococcal enterotoxin A and staphylokinase encoded on Sa3int prophages (Goerke

et al., 2006a; Goerke et al., 2006b; Sumby & Waldor, 2003) as well as for PVL encoded on Sa2int prophages (Wirtz et al., 2009). Further, prophage-encoded virulence genes on the IEC are described to be directly regulated by staphylococcal regulators encoded on the core genome. *Sak*, *scn* and *chp* are described to be regulated by the agr-system, as well as by sarA and the sae two-component system (Rooijakkers et al., 2006). However, it was still unknown if specific staphylococcal factors also influence the expression of other genes encoded on prophages of *S*. *aureus*, especially of Sa3int members.

This knowledge gap was investigated in part II of this thesis (Manuscript I) with regard to Sa3int prophage behavior within different host strain backgrounds. We detected strain-specific differences in phage transfer, lysogenization frequency and progeny production, indicating a drive of the Sa3int prophage to spread throughout the bacterial population. *In vitro* studies already showed spontaneous transfer of Sa3int prophages in co-culture as well as phage mobilization and transfer trigger by biocides (Tang et al., 2017) or antibiotics (Goerke et al., 2006a). These observations were further confirmed by transfer assays performed in Rohmer et al., Manuscript I, revealing high phage transfer frequency in specific isolates. Phage transfer was shown to be dependent on the recipient strain, indicating that Sa3int prophages might be able to sense the abundance of phages or lysogens within a bacterial population. This suggests that a putative sensing mechanism would trigger spontaneous phage release, resulting in phage transfer and distribution followed by acquisition of phages in phage-free bacterial cells to establish a certain amount of phage-carrying cells within the population. Such sensing mechanisms are described for several other phages, for instance for Vibrio phage VP882, which are able to actively sense the quorum-sensing (QS) autoinducer produced by its host, *Vibrio cholerae* (Silpe & Bassler, 2019). Sensing of this autoinducer starts a pathway leading to depression of lytic genes and production of progeny virions. With this, Vibrio phage VP882 is able to sense bacterial density which allows lysogeny-lysis decision-making. Alternatively, *Clostridium difficile* prophage PhiCDMH1 carries a QS-cassette encoding three genes homologous to *agrD*, *agrC* and *agrB* on its genome (Hargreaves et al., 2014).

Similar to *S*. *aureus*, the agr-system of *C*. *difficile* is regulating fitness, indicating that the prophage-encoded agr-homologues either sense signal-peptides that influence lysis-lysogeny decision, or directly influence the host bacterium to alter regulatory mechanisms. As a last example, SP-beta phages infecting *Bacillus* ssp. use a communication system termed “arbitrium”, which relies on production of small peptides (six amino acids in length) during infection of host cells (Erez et al., 2017). SPbeta phages sense the concentration of these small peptides and establish a lysogenic life cycle if the concentration reaches a certain threshold. Staphylococcal Sa3int prophages might also be able to sense signals allowing decision-making for the lysogenic or lytic cycle. This could be sensed either directly

or indirectly, based on altered bacterial metabolism as a result of changing environmental conditions or cell density. This is supported by the transcriptomic analysis, which revealed phage-encoded genes of the replication and morphogenesis modules to be differentially regulated dependent on the host strain, resulting in elevated phage particle production. Since we identified several transcriptional start sites on the Φ13 genome that were only enriched under induced conditions, we assume there is a direct influence of specific host factors on phage gene expression.

In bacteria, the SOS-response is regulated by LexA-repressor that blocks transcription of SOS-genes by binding to specific promotor sequences (Walker, 1984). In *S*. *aureus*, *uvrA*, a gene involved in NER-system, is also part of the LexA-regulon (Cirz et al., 2007). The SOS-response pathway is induced by DNA-damage, for instance by antibiotics like mitomycin C or ciprofloxacin, exposure to UV-irradiation or reactive oxygen species. When DNA-damage is sensed, the LexA-repressor is cleaved. Cleavage is by the co-protease RecA. Loss of LexA repression results in transcription of SOS-genes and DNA repair mechanisms. In parallel, phage repressors (e.g. cI-repressor) are also cleaved, resulting in derepression of lytic genes and start of phage replication (Eguchi et al., 1988). By this mechanism, phages directly sense if their host bacterium is facing stressful conditions. Not only prophages being mobile genetic elements (MGEs), but also *S*. *aureus* pathogenicity islands (SaPIs) are described to be regulated by LexA (Úbeda et al., 2007). It is noteworthy that SaPIs exploit prophages like Φ11, Φ147 or 80α to produce capsids which are smaller in size to ensure that SaPI genome gets encapsulated and transferred (Ruzin et al., 2001; Ubeda et al., 2005). Thus, LexA is highly involved in induction and movement of MGEs. In our study, we confirmed LexA to influence replication of prophage Φ13. LexAG94E mutants produced in the SH1000Φ13-kana and the Newman-cΦ13-kana strain background, harboring an inactive LexA due to a non-functional cleavage site, showed decreased but not completely abolished phage replication and release. However, we still had strain-specific differences with Newman producing more infective phage particles and genomes compared to the SH1000 background. We also confirmed the influence of UvrA on the phage life cycle using *uvrA::erm* mutants. Deficiency in UvrA activity led to severely decreased replication efficacy of phage Φ13 compared to the wildtype strain background. This phenotype is at least in part independent on LexA-regulation, therefore no strain-specific differences in the amount of released infective phage particles or produced genomes could be detected, between SH1000Φ13-kana *uvrA::erm* and Newman-c13-kana *uvrA::erm*. However, it remains to be elucidated which specific genes of Sa3int prophages are influenced by UvrAB. For bacteriophages M13 and X174, rolling-circle replication is initiated by a prophage-encoded replication protein that introduces nicks in the DNA molecule (Petit et al., 1998). This is influenced by the *E*. *coli* host helicase Rep, a single-stranded DNA-dependent ATPase,

through interaction with the prophage-encoded replication-protein (Yarranton & Gefter, 1979). In *S*. *aureus*, *pcrA* (plasmid copy number reduction) shows 40% sequence similarity to *E*. *coli* Rep. Additionally, UvrD is described to affect replication of plasmid pT181 (Iordanescu, 1993, 1995) and to be essential for cell viability (Petit & Ehrlich, 2002). It is therefore likely that the UvrAB complex fulfills a similar role as pcrA.

Transcriptomic analysis until today rarely used to investigate bacteriophage gene expression and mostly limited to lytic phages during the infection process (Ceyssens et al., 2014; Chevallereau et al., 2016; Wicke et al., 2021). Further, the RNAseq approaches facilitate the detection of small regulatory RNAs (sRNAs). sRNAs play an important role in regulation of cellular processes in bacteria (Beisel & Storz, 2010). For *S*. *aureus*, several studies addressed the identification of sRNAs (Broach et al., 2016; Carroll et al., 2016). Importantly, phage gene expression is also known to rely on regulation by sRNAs. Prophage encoded sRNAs regulate prophage encoded genes as well as host encoded genes in several bacterial species (Altuvia et al., 2018). Also for *Listeria* (*L*.) *monocytogenes* A118-like phages, that show some similarity to Sa3int prophages, sRNAs are described (Mraheil et al., 2011). This further underlines the necessity to investigate putative encoded sRNAs on Sa3int prophages and their impact on prophage-encoded genes as well as on *S*. *aureus* gene regulatory network. In this work, fundamental data and specific workflows were obtained to follow up on this topic.

Additionally, *L*. *monocytogenes* A118-like phages are generally employed as a model for Sa3int prophages with regard to a regulatory switch termed “active lysogeny”. *L*. *monocytogenes* A118-like prophages can precisely excise from the *comK*-gene in which they integrate. This results in reconstitution and expression of *comK*, a mechanism similar to Sa3int prophage excision leading to reconstitution of the *hlb*-gene. Furthermore, this mechanism is reversible in this case, as reintegration of prophages lead to arrest of *comK* expression. Prophage excision followed by *comK* expression was induced when *L*. monocytogenes resided within macrophages facing intracellular conditions. Of note, although the prophage is excised, no phage particles are produced. Active ComK activates transcription of the Com system (competence) leading to assembly of a pseudopilus and a membrane translocation channel. This was required for efficient phagosomal escape (Argov et al., 2017; Argov et al., 2019; Rabinovich et al., 2012). Beside reversible active lysogeny, non-reversible active lysogeny is described for several bacteria, meaning that after excision, no reintegration of mobile elements is detected and the excised fragments are lost (Feiner et al., 2015). In *B*. *subtilis*, skin (sigK-intervening DNA element) excision results in reconstitution of sigma-factor σ^K that regulates several genes involved in late stages of mother cell differentiation (Kimura et al., 2010). In Cyanobacteria like *Anabaena* spp., nitrogen-fixing cells (heterocysts) carry three genes involved in nitrogen fixation each of which is interrupted

by phage-like elements. In late stages of heterocyst differentiation, these elements are excised resulting in functional, expressed genes (Golden et al., 1985). For Sa3int prophages in *S*. *aureus*, a regulatory switch was already described under specific stages of infection. Excision followed by temporary circularization or integration at alternative positions on the staphylococcal genome takes place allowing active production of β-hemolysin (Goerke et al., 2006b; Salgado-Pabón et al., 2014; Tran et al., 2019). Temporary *hlb*-expression is assumed to play a yet unknown role in human infection processes and is assumed to be tightly linked to the regulatory network of *S*. *aureus* (Tran et al., 2019). During the colonization process, precise Sa3int excision followed by *hlb*-expression revealed highly elevated colonization efficiency compared to *hlb*-negative isolates (Katayama et al., 2013). The hypothesis for a regulatory switch mechanism of Sa3int prophages is further underlined with results from this study (Rohmer et al., Manuscript I) with multiple TSSs predicted on the Sa3int prophage indicating differences in RNA processing. Furthermore, predicted promotor regions might be bound by repressors under specific conditions, such as described for A118-like phages in *L*. *monocytogenes* that abolish phage replication but still allow excision and circularization (Argov et al., 2019). In sum, this study provides important insights into evidence on this complex interconnected regulatory network of Sa3int prophages and their host bacterium, *Staphylococcus aureus*.

Conclusion

The work presented in this thesis provides new insight into the life style of highly important Sa3int prophages of *S*. *aureus*. These prophages are not only important for virulence of their host by encoding highly human specific virulence genes but also due to their dynamic behavior in regard to transfer, lysogenization and induction. This is tightly linked to the host regulatory system, but features important strain-specific differences. In regard to active lysogeny phages are highly important for *S*. *aureus* survival within different human and animal niches and majorly contributing to host adaption and survival. TSS prediction of prophages and host bacteria represent an initial step in the elucidation of the intricate regulatory mechanisms that are key to phage dynamics.

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Additional data files

Additional data files are stored on CD attached to this thesis and includes a folder termed "Supplemental-Material_Dissertation_Rohmer_Carina"

This folder contains 3 subfolders:

Supplemental Material_Manuscript I: suppl. Table S3 and S4

Supplemental Material Manuscript II: suppl. Table S1 to S5

Supplemental Material – Thesis:

- Original tables obtained from Bioconductor (correspond to Manuscript II) including : **ind_8325vsMW2** (induced) and **non_8325vsMW2** (uninduced)
- RNAseq-datatables-RPKM-unique gene reads-CLC (correspond to Manuscript I and Manuscript II). Contains 18 single files obtained from RNAseq analysis performed on each sample obtained from CLC genomics workbench
- TSS-predator-Mastertables (correspond to Manuscript I). obtained from TSSpredator and contains tsv files of combined table (**MasterTable_combinedRightReps**) and single output tables (**MasterTable_MW2_RightReps**, **MastertableNC_007795_fully** and **MasterTableNC_007795phagecured_RightReps**).

Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel:

“Interaction of Sa3int prophages with their host bacterium *Staphylococcus aureus”*

selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe. Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen beachtet wurden. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe. Meine eigenen Beiträge zu Gemeinschaftsarbeiten habe ich in der „declaration of author contribution“ dargestellt

Tübingen, den 21.12.2021

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