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.

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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 [Balasubramanian et al., 2017; Turner et al., 2019]. Based on multi-locus-sequence typing (MLST) analysis, *S. aureus* strains have been assigned to distinct evolutionarily related clonal complexes (CCs) [Feil et al., 2003]. Most strains car-

Fig. 1. Schematic illustration of Sa3int phages. Genetic organization of the representative Φ13 phage (top) of strain 8325. Annotations are based on Iandolo et al. [2002] 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*.

ry up to four prophages in their genome, many of which harbor accessory genes encoding staphylococcal virulence factors [Xia and Wolz, 2014; Ingmer et al., 2019b]. 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 [Kwan et al., 2005; Goerke et al., 2009; Kahánková et al., 2010; Deghorrain and Van Melderren, 2012; Ingmer et al., 2019b; Oliveira et al., 2019]. *S. aureus*-infecting siphoviruses have been classified according to polymorphisms of the integrase gene (*int*) [Goerke et al., 2009; Kahánková et al., 2010; Deghorrain and Van Melderren, 2012]. The *int* type dictates chromosomal integration at cognate *attB* sites and is closely associated with the virulence gene content of the prophage [Goerke et al., 2009]. Sa3int phages were first described as triple-converting phages and are by far the most prevalent *S. aureus* phages [van Wamel et al., 2006; Goerke et al., 2009; Verkaik et al., 2011]. Up to 96% of human nasal isolates were observed to carry Sa3int phages integrated into the *hlb* locus that encodes β-hemolysin (Hlb), also

named β-toxin. These phages carry genes that encode human-specific immune evasion factors [van Wamel et al., 2006] and other potential virulence factors [Ingmer et al., 2019b]. There is now compelling evidence that Sa3int phages play important roles in the adaptation of *S. aureus* to its human host [Sung et al., 2008; Ingmer et al., 2019a; Bouiller et al., 2020; Matuszewska et al., 2020]. 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.

Phage-Encoded Accessory Genes

Van Wamel and co-workers [2006] 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 inhibi-

tory protein of *S. aureus* (CHIPS), staphylokinase (SAK), and staphylococcal enterotoxin A or P (SEA or SEP), respectively. 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 [van Wamel et al., 2006] (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 Ingmer et al. [2019b]). 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 [Sumby and Waldor, 2003; Goerke et al., 2006a]. This is partially due to a multicopy 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 [Rooijakkers et al., 2005a] (for review, see Garcia et al. [2012]). 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 inac-

tive state [Rooijakkers et al., 2009]. SCIN also promotes the formation of convertase dimers [Jongerijs et al., 2010], 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 [Rooijakkers et al., 2005a]. Of note, several SCIN homologues have been identified at other genome locations [Rooijakkers et al., 2009]. Interestingly, EqSCIN was detected in phages of *S. aureus* isolates from horses and shown to specifically inhibit the C3 convertases of horses [de Jong et al., 2018].

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 [Veldkamp et al., 2000], a process that was later shown to be due to the secretion of a 14.1-kDa protein named CHIPS [de Haas et al., 2004]. 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 [Bloes et al., 2015]. 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 [Postma et al., 2004], 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 [de Haas et al., 2004]. 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 *hlyB* with serogroup F bacteriophages was shown to be correlated with SAK activity [Winkler et al., 1965; Coleman et al., 1989]. SAK is a 15-kDa secreted protein that can associate with the surface of *S. aureus* cells [Collen and Lijnen, 1994]. Several functions have been described for SAK (for review, see Bokarewa et al. [2006]). 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 [Lijnen et al., 1991]. The generation of active plasmin is enhanced on the surface of *S. aureus* cells [Mölkänen et al., 2002] and protects against inactivation by plasmin inhibitors that are typically present in human plasma [Silence et al., 1993]. Therefore, plasmin-coated bacteria are prepared to degrade human IgG or C3b [Rooijackers et al., 2005b] 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 [Bokarewa and Tarkowski, 2004]. 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 [Jin et al., 2004].

SAK specifically activates human plasminogen and does not react with murine plasminogen [Kwiecinski et al., 2015; Okada et al., 2000]. To overcome this species specificity, transgenic mice expressing either human or natural mouse plasminogen were compared to analyze the impact of SAK during bacteremia [Kwiecinski et al., 2010]. Surprisingly, the activation of human plasminogen by SAK reduced the severity of systemic staphylococcal infection. The same model was subsequently used to analyze the impact of SAK on skin infection [Kwiecinski et al., 2013]. 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 [Peetermans et al., 2014]. 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 [Kwiecinski et al., 2015]. SAK-dependent activa-

tion 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 [van Wamel et al., 2006], a SprG1/F1 system is located within most IECs [Pichon and Felden, 2005]. Entries for *sprG/F* in the staphylococcal regulatory RNA database (SRD) are listed in Table 1 and Figure 1 [Sassi et al., 2015]. The SprG/F systems were first detected in an sRNA screen and designated small pathogenicity island RNAs (Sprs) G1/F1 to G4/F4 [Pichon and Felden, 2005]. The expression of *sprG1/F1* has been verified in several strains, including N315 and Newman but not for strain NCTC8325 [Pinel-Marie et al., 2014]. 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. 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 [Pinel-Marie et al., 2021]. Two toxic peptides are translated from *sprG*, a major form (SprG1₄₃₉, 44 amino acids) and a shorter form (SprG1₃₁₂, 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 [Pinel-Marie et al., 2014]. Interestingly, SprG1 peptides show activity towards other bacterial species, and the longer peptide SprG1₄₃₉ can also lyse human erythrocytes.

Staphylococcal Enterotoxin A (SEA)

sea was one of the first genes described as being phage encoded in *S. aureus* [Betley and Mekalanos, 1985]. 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

Table 1. Accessory genes of Sa3int phages

	Accession No.	Gene	Location on prophage genome	Distribution in IEC-type	Reference	8325	USA300_FRP3757	Newman	MW2	N315
<i>Immune-evasion cluster (IEC)^a</i>										
Staphylococcal complement inhibitor	YP_500655	<i>scn</i>	3'-end	A,B,C,D,E,F,G	Roijaakers et al., 2005a	SAOUHSC_02167	SAUSA300_1919	NWMN_1876	MW1884	SA1754
Chemotaxis inhibitory protein	YP_500656	<i>chp</i>	3'-end	A,B,C,F	Roijaakers et al., 2006	SAOUHSC_02169	SAUSA300_1920	NWMN_1877	-	SA1755
Staphylokinase	YP_500658	<i>sak</i>	3'-end	A,B,D,E,F,G	Lack, 1948	SAOUHSC_02171	SAUSA300_1922	NWMN_1880	MW1885	SA1758
Staphylococcal enterotoxin A	WP_000750406	<i>sea</i>	3'-end	A,D	Casman et al., 1963	-	-	NWMN_1883	MW1889	-
Staphylococcal enterotoxin P	WP_000034846	<i>sep</i>	3'-end	F,G	Kuroda et al., 2001	-	-	-	-	SA1761
<i>Additional accessory genes</i>										
Srn3840 (SprG1) ^b	KI625227 KI625228	(SprG1 ₃₁₂) (SprG1 ₄₃₉)	3'-end	-	Pinel-Marie et al., 2014	sprG1_sRNA310	sprG1_sRNA310	sprG1_sRNA310	sprG1_sRNA310*	sprG1_sRNA310
Srn3830 (SprF1) ^b	KI625226	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
<i>Avian-adapted genes (Sa3int phage ΦAvβ)</i>										
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].

the massive production of cytokines such as IL-2 and IFN- γ within T cells and TNF- α and IL-1 β 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 diarrhea when consumed orally, which is correlated with their role in staphylococcal food poisoning [Dinges et al., 2000]. 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 [Lina et al., 2004]. 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 (SEI-) toxin” [Lina et al., 2004]. SEA is a 27-kDa protein categorized as a “real” enterotoxin with superantigenic and emetic activity [Bergdoll, 1988; Johnson and Magazine, 1988; Abrahmsén et al., 1995]. 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 [O’Brien et al., 2006].

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 SEI-P based on the recommended nomenclature [Thomas et al., 2007]. Recombinant SEP showed emetic properties in a house musk shrew assay, although at a relatively high dose [Omoe et al., 2005]. SEP/SEI-P is a classical superantigen with high T-cell stimulatory activity [Omoe et al., 2005].

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 Φ 13 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., Φ 12, Φ ETA, Φ PVL, Φ PV83, and Φ SLT) that are assigned to different Sa-int groups. Based on its localization, it was proposed that ORF-C may function as an excisionase [Iandolo et al., 2002], 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 neighboring 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 harbored 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 and 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 [Munson et al., 1998].

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 [Sibbald et al., 2006].

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 Φ UT1) and is also present in different CCs, including LA-MRSA CC398 [Gerlach et al., 2018; Sieber et al., 2020; Xiong et al., 2020]. *TarP* is an alternative glycosyltransferase that alters the glycosylation pattern of *S. aureus* wall teichoic acid (WTA), catalyzing the attachment of GlcNAc in the β -1,3-position of RboP [Gerlach et al., 2018]. 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 does not appear to influence the household transmission of LA-MRSA CC398 [Sieber et al., 2020].

Avian hlb-Converting Prophages

Species-specific Sa3int phages are prevalent in poultry *S. aureus* isolates (designated Φ Av β). These phages lack the typical IEC but rather carry two avian-specific genes at the same location on the 3' end of Φ Av β . 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 -Hemolysin (Hlb)

Hlb was first described in 1935 when Glenn and Stevens [1935] observed a hot-cold hemolysis effect in which *S. aureus* cultivation at 37°C followed by cooling to 4°C resulted in an enhanced hemolysis pattern on blood agar plates. The nucleotide sequence of the gene encoding the 37-kDa protein Hlb was elucidated in 1989 [Projan et al., 1989]. Structural analysis revealed that Hlb belongs to the DNase I folding superfamily, which includes sphingomyelinases [Huseby et al., 2007]. Indeed, Hlb is a phospholipase with specificity towards sphingomyelin to generate ceramide and phosphocholine [Doery et al., 1963]. Hlb exhibits species-dependent hemolytic activity that correlates with the amount of sphingomyelin content in eryth-

rocytes, 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 [Bernheimer et al., 1974]. However, Hlb is able to efficiently target human endothelial cells [Herrera et al., 2017a], human keratinocytes [Katayama et al., 2013] and monocytes [Walev et al., 1996; Huseby et al., 2007]. The Hlb-generated ceramide can act as a second messenger in eukaryotic cells [Li et al., 2019]. 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 [Tajima et al., 2009; Herrera et al., 2017b]. Notably, a second function of Hlb as a biofilm ligase has been described [Huseby et al., 2010]. 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 [Herrera et al., 2016].

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 [Cifrian et al., 1996] and keratitis in rabbits [O'Callaghan et al., 1997]. In a lung infection model, Hlb was shown to promote increased neutrophilic inflammation and the vascular leakage of serum proteins into lung tissue [Hayashida et al., 2009]. 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 hemolytic activity towards erythrocytes, Hlb was also shown to play an important role in skin colonization by damaging keratinocytes [Katayama et al., 2013].

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 [Haag et al., 2019b; Heaton et al., 2020; Matuszewska et al., 2020]. Some *S. aureus* CCs are restricted to a single taxonomic group, suggesting that distinct *S. aureus* populations are solely maintained within a given species [Haag et al., 2019b]. Other CCs are preva-

lent in several species, indicating transmission between humans and animals. Humans are thought to be a major reservoir for *S. aureus* among animal species [Richardson et al., 2018; Matuszewska et al., 2020]. The transmission of *S. aureus* between humans and livestock is of particular concern, as *S. aureus* isolates from farmed animals are often antibiotic resistant [Wulf and Voss, 2008; Richardson et al., 2018]. 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 [McCarthy et al., 2011; Richardson et al., 2018].

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, see 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 [Bouiller et al., 2020]. 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 [Price et al., 2012; Uhlemann et al., 2012]. However, LA-MRSA CC398 may be capable of readapting to the human host through acquisition of an IEC-harboring Sa3int phage [McCarthy et al., 2012; Cuny et al., 2015; Sieber et al., 2020]. The presence of an IEC was shown to be correlated with increased human-to-human transmission and excess disease burden of LA-MRSA [McCarthy et al., 2012; Sieber et al., 2020]. 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, $p = 0.0010$) [Sieber et al., 2020]. 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 [Jung et al., 2017]. 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 *hly* is altered in

CC398 strains. Thus, in these strains, Sa3int often integrates elsewhere in the genome [Kraushaar et al., 2017; Tang et al., 2017; van Alen et al., 2018], 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 harbor an intact *hly* gene and lack the IEC cluster, while truncated *hly* genes and IECs are detected in clade II subtypes [Chen et al., 2018]. These results suggest that a CC9 strain with extraordinarily high virulence potential obtained IEC-carrying Sa3int phages after jumping from pigs to humans [Jin et al., 2020].

In the US, there is a diverse population of LA-MRSA, including organisms of ST5 MRSA lineages. Furthermore, the *hly* 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% [Hau et al., 2015].

Poultry

There is a limited number of *S. aureus* genotypes associated with poultry in different geographic regions [Haag et al., 2019b; Matuszewska et al., 2020]. 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 [Lowder et al., 2009]. 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 (Φ Av β) 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 [Lowder et al., 2009] (Table 1). The Φ Av β 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 Φ Av β occurs between *S. aureus* strains. This finding was reinforced by Price et al. [2012], who tested 34 isolates from domestic turkeys suffering from foot joint infection and observed that all CC398 isolates but one carried Φ Av β in their genomes. Thus in avian isolates, similar to

the human *S. aureus* population, the *hly* 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 harboring an IEC [Walther et al., 2018]. 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 [Jung et al., 2017]. 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 Luk-PQ [de Jong et al., 2018; Mama et al., 2019]. 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 [Spoor et al., 2013; Richardson et al., 2018]. *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 5,500 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. Nine of 23 human isolates and none of 19 bovine or pig isolates contained an IEC-harboring Sa3int phage, indicating that the acquisition of Sa3int phages by these isolates occurred after their transmission to humans [Spoor et al., 2013]. 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 [Kumagai et al., 2007; Resch et al., 2013]. 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 *hly* positive [Moncke et al., 2007].

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 [Viana et al., 2015]. 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 [Mrochen et al., 2018b, 2020]. 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 [Mrochen et al., 2018a]. Similar to mice, free-living and laboratory rats are often colonized with *S. aureus* [Raafat et al., 2020]. 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 harbored 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 2,700 years ago. The development of this monkey-associated clade was accompanied by the loss of Sa3int phage [Senghore et al., 2016], 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 [Coleman et al., 1989] and already indicates that IEC or Hly 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 favorable for the bacteria. A comparison of colonizing and invasive *S. aureus* strain populations revealed that invasive strains are more frequently Hlb positive [Hedström and Malmqvist, 1982; Peacock et al., 2002; Goerke et al., 2009]. Notably, most Sa3int phages remain inducible, leading to the complete restoration of functional Hlb [Goerke et al., 2006b; Salgado-Pabón et al., 2014], and phage induction may be favored 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 [Goerke et al., 2006a; Tang et al., 2017]. 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 [Goerke et al., 2006b] 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 [Treffon et al., 2020], supporting the idea that during CF lung infection, Sa3int-encoded factors are of less importance. Hlb appears also to promote bacteremia. SAK-deficient isolates were shown to be >4 times more likely to cause lethal bacteremia than SAK-positive isolates, suggesting that an intact *hly* gene and/or SAK deficiency may worsen the outcome of patients with *S. aureus* bacteremia [Jin et al., 2003; Kwiecinski et al., 2013]. Boyle-Vavra et al. [2011] compared an isogenic pair of daptomycin-susceptible and daptomycin-resistant MRSA isolates from a patient with recurrent bacteremia. The *hly* 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 *hly* gene was also shown to be associated with catheter-related bacteremia [Pérez-Montarelo et al., 2018], and 45% of isolates from recurrent furunculosis were observed to produce Hlb compared to 19% of those associated with nasal colonization [Hedström and Malmqvist, 1982].

The results of analyses using several animal models support that infection can select for the loss of Sa3int phages. Katayama et al. [2013] 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 mu-

rine skin colonization by *S. aureus*. Hlb-positive MW2 variants also arise in the blood, kidney, and heart vegetation of infected rabbits [Salgado-Pabón et al., 2014]. 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 [Guérillot et al., 2019]. 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 [Burian et al., 2010]. 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 favorable for long-term asymptomatic colonization. However, this hypothesis was challenged by the results of a colonization study [Verkaik et al., 2011] 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 harboring 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 [Goerke et al., 2006b]. Under these conditions, the reconstitution of the phage-interrupted *hly* gene may be advantageous. When these mis-located phages were in-

duced and used to re-infect *S. aureus* in vitro, the phages reintegrated at their dedicated attachment site within *hlyB*. There is also evidence [Goerke et al., 2006b; Deutsch et al., 2018; Tran et al., 2019] that Sa3int phages may perform “active lysogeny,” a process during which a phage is temporally excised from the chromosome without forming intact phage particles [Feiner et al., 2015]. 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 [Goerke et al., 2006a]. 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 *hlyB* gene when needed and may be

achieved via active lysogeny, temporal re-localization 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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