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Temperate Phages of *Staphylococcus aureus*

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ABSTRACT Most *Staphylococcus aureus* isolates carry multiple bacteriophages in their genome, which provide the pathogen with traits important for niche adaptation. Such temperate *S. aureus* phages often encode a variety of accessory factors that influence virulence, immune evasion and host preference of the bacterial lysogen. Moreover, transducing phages are primary vehicles for horizontal gene transfer. Wall teichoic acid (WTA) acts as a common phage receptor for staphylococcal phages and structural variations of WTA govern phage-host specificity thereby shaping gene transfer across clonal lineages and even species. Thus, bacteriophages are central for the success of *S. aureus* as a human pathogen.

INTRODUCTION

The diversity of the *Staphylococcus aureus* species is mainly determined by mobile genetic elements, many of which are prophages or phage-related genomic islands. Strain evolution as a result of short- and long-term adaptation to diverse environments is tightly linked to phages. Many phages carry accessory genes coding for staphylococcal virulence factors, which are important for the success of certain *S. aureus* clonal complexes (CCs). Second, phages support the induction, packaging, and transfer of genomic islands (1, 2). This topic is reviewed elsewhere. Third, phage-mediated transduction is an efficient way to transfer not only extrachromosomal mobile elements, such as plasmids, but also chromosomal markers (albeit with lower efficiency). *S. aureus* is thought not to be naturally competent, so that recombination and horizontal gene transfer are mostly phage mediated and, to a lesser extent, conjugative. Here, we will first give a brief overview of previously used methods to classify *S. aureus* phages. Then we will mainly focus on the impact of temperate phages on the evolution of the bacterial host.

CLASSIFICATION OF *S. AUREUS* PHAGES

All known *S. aureus* phages belong to the order *Caudovirales* (tailed phages) and are composed of an icosahedral capsid filled with double-stranded DNA and a thin filamentous tail. Based on the complete genomes of 27 phages (3), *S. aureus* phages were assigned to three size classes: siphoviruses, with a genome size of 39 to 43 kb, podoviruses, with a smaller genome size of 16 to 18 kb, and myoviruses, with a genome size of 120 to 140 kb. This capsid and genome size-based classification correlates with the tail morphology as observed by electron microscopy: *Podoviridae* have a very short tail, *Siphoviridae*, a long noncontractile tail, and *Myoviridae*, a long contractile, double-sheathed tail (for recent reviews, see 4–6). All of the known temperate staphylococcal phages belong to the *Siphoviridae*, which are the primary focus of this chapter.

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The siphovirus genomes are usually organized into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis (7–9). The evolution of phage lineages was driven by the lateral gene transfer of interchangeable genetic elements (modules), which consist of functionally related genes. A functional module found in one phage can be replaced in another phage by a sequence-unrelated module that fulfils the same or related functions; often, genes within such modules travel together (10). Thus, multiple alignments of *S. aureus* phage genomes reveal a chimeric and mosaic structure resulting from horizontal transfer and recombination (3, 5, 9, 11–13).

Due to this modular structure, phage nomenclature and classification of the *Siphoviridae* are challenging and are a matter of debate. In Table 1 the different designations and properties of a representative set of *S. aureus Siphoviridae* are illustrated. In one approach phages were classified based on protein repertoire relatedness (5, 7). Thereby, *Siphoviridae* from *S. aureus* were clustered into six major clades. Clades 4 to 6 (Sfi21-like phages) share characteristic features with the capsid region of *Escherichia coli* HK97 phage and use the cos-site strategy for DNA packaging. Clades 1 to 3 (Sfi11-like pac-type phages) are related to the *Bacillus subtilis* SPP1 phage (7). The Sfi11/Sfi21-like grouping of *Siphoviridae* is based on genome analysis of lactococcal phages. Gene clusters extending from the DNA packaging genes to the tail genes were found to be represented by two unrelated configurations: one is characteristic of cos-site phages (prototype: phage Sfi21), and the other is characteristic of pac-site phages (prototype: phage Sfi11).

In another approach, *S. aureus Siphoviridae* were classified according to polymorphism of the *int* gene and the modular composition of lysogeny, regulation, replication, structural, and lytic modules (9, 12) (Table 1). The modules can be defined by multiplex PCR or sequence analyses of selected genes within the modules. *S. aureus* prophages were primarily classified on the basis of *int* gene homology (9, 12, 13). By including information on the allelic variation of other modules, a further meaningful subdivision can be achieved. A similar approach to the general classification of bacteriophages has been proposed previously (14, 15). Kahánková *et al.* (9) established a mulitplex PCR assay to distinguish between different types of integrase (10 types), antirepressor (five types), replication proteins (four types), dUTPase (four types), portal protein (eight types), tail appendices (four types), and endolysin (four types). The proposed extended typing scheme covering seven major genomic modules enables not only the differentiation of phages but also the design of a classification system. For the basic description and rapid differentiation of a phage strain, the serological group type, the *int* type, and the endolysin type are the most relevant (located at the left side, the middle, and the right side of the prophage genomes). Using a simplified scheme, e.g., phage φ11 can be classified as Sa5int-Ba-ami1 or phage φ13 as Sa3int-Fb-ami3 (9). Despite the multiple mosaic variants, there are close links between particular modules; e.g., all phages using *polA* for replication are serogroup A.

There are several reasons to classify *S. aureus* prophages primarily based on *int* polymorphism. First, nucleotide sequences are well conserved within *int* groups, making the gene an ideal target for PCR amplification. The *int* grouping has good discriminatory power, reflecting the diversity of the *S. aureus* phage population. Recombination and exchange of certain modules seem to occur more often between phages of the same *Sa-int* group than between phages of different *Sa-int* groups (16). Second, the *int* typing allows for prediction of the chromosomal location of the prophage. Last, the *int* type is closely linked to the virulence gene content of the prophage and can therefore convey information about the pathogenic potential of the bacterial lysogen (12). Most of the *S. aureus* phages can be assigned to one of the major *Sa-int* types 1 to 8.

PHAGE INTEGRATION/EXCISION

All prophages integrate to position the *int* gene in close proximity to the bidirectional origin of chromosome replication. Based on amino acid sequence homology and catalytic residues, most integrases belong to the tyrosine recombinase type family. Only the integrases of Sa7int phages were found to belong to the serine recombinase type family (12). Despite the usually strong association of the *int* type with the integration site, there are also events where a phage may integrate in an illegitimate attachment site. This phenomenon was described to occur for Sa3int phages during chronic lung infections of cystic fibrosis patients (17). Under these conditions, the reconstitution of the phage-interrupted *hlb* gene may be an advantage. When these mislocated phages were induced and used to reinfect *S. aureus in vitro*, the phages reintegrated at their dedicated attachment site within *hlb*. Sa3 phages are only rarely found in livestock-associated *S. aureus* strains of CC398. In these strains the canonical attB site within *hlb* is altered, and therefore Sa3int phages integrate at diverse alternative attB sites (18, 19). The mechanism for excision of *S. aureus Siphoviridae* is much less well understood. Excision followed by replication

TABLE 1 Classification and properties of selected *S. aureus Siphoviridae*[e]

Phage name	Sfi type[a]	Clade[a]	Int[b]	Sero[b]	Holin[c]	Virulence[d]	cos/pac	attB	Phage or attB in 8325[c]
Φ55	Sfi11	II	Sa1	B	438				SAOUHSC_00851:
ΦMu50B	Sfi11		Sa1	B	438		pac	TTCGAAATGGAAGGTAGTA	823130-823148
ΦETA	Sfi11	2	Sa1	B	276	*eta*			
ΦSa1JH1			Sa1	B	276				
ΦETA2			Sa1	B	276	*eta*			
Φ252A			Sa2	A	303				SAOUHSC_01583:
ΦSa2mw	Sfi21		Sa2	A	303	*lukFS-PV*	cos: CGGCGGGGGC	ACCATCACATTATGATGA TATGTTTATTT	Φ12, 1463618-1508581
ΦPVL108	Sfi21	5	Sa2	Fb	303	*lukFS-PV*			
Φ2958PVL	Sfi21	4	Sa2	A	303	*lukFS-PV*			
ΦPVL	Sfi21	5	Sa2	Fb	303	*lukFS-PV*			
ΦSa2USA300	Sfi21		Sa2	A	303	*lukFS-PV*			
ΦSLT	Sfi21	4	Sa2	A	303	*lukFS-PV*			
Φ12	Sfi11	4	Sa2	A	303				
Φ13	Sfi21	5	Sa3	Fb	255a	*sak*, *chp*, *scn*	cos: CGGAGCAGA	TGTATCCAAACTGG	*hlb*: Φ13, 2031923-
ΦN315	Sfi21	6	Sa3	Fa	255a	*sep*, *sak*, *chp*, *scn*			2074632-
ΦSa3mw	Sfi21		Sa3	Fb	255a	*sek*, *seq*, *sea*, *sak*, *scn*			
ΦNM3		6	Sa3	Fa	255a	*sea*, *sak*, *chp*, *scn*			
ΦMu50A	Sfi21	ND	Sa3	Fa	255a	*sea*, *sak*, *scn*			
ΦSa3USA300			Sa3	Fa	255b	*sak*, *chp*, *scn*			
ΦSa3JH9			Sa3	Fa	255b	*sak*, *chp*, *scn*			
Φ42E	Sfi21	IV	Sa3	A	255a				
ΦSa4JH1			Sa4	A	438			CATGTAATTCC	SAOUHSC_00958 *(htrA)*:
ΦSa4ms			Sa4	A	303				933127-933137
Φ11	Sfi11	1	Sa5	B	438		pac	CTTCCCATGG	SAOUHSC_02090: Φ11,
ΦPV83			Sa5	Fb	255a	*lukM*, *lukF-PV*			1923398- 1967013
Φ187	Sfi11	I	Sa5	L	255a				
Φ29	Sfi11	II	Sa5	B	438				
ΦNM1	ND	1	Sa5	B	303				
Φ88	Sfi11	II	Sa5	B	438				
Φ52A	Sfi11	II	Sa6	B	438				SAOUHSC_00300 (*geh*):
Φ80	Sfi11	2	Sa6	B	438				316250-316257
ΦNM4		2	Sa6	B	303			ATCATACAAGGATGGGAT	
ΦSa6JH9			Sa6	B	438				
ΦCOL			Sa6	A	303				
L54a			Sa6	A	?				
Φtp310-2			Sa6	A	303				
Φ53	Sfi11	I	Sa7	B	303				Intergenic, downstream
Φ80alpha	Sfi11	1	Sa7	B	303		pac	AGGTATCTG	SAOUHSC_01079 (*isdB*):
Φ85	Sfi11	I	Sa7	B	303				1042159-1042167
ΦNM2			Sa7	B	438			AGGTATCTG	
Φ6390			Sa7	B	255a	*sak*			
Φ92	Sfi11	II	Sa7	B	438				
φSaov2			Sa7	F					
ΦRF122			Sa8	B	438		pac	CGGAAGGTAAGGGA	SAOUHSC_T00050 (*trnaS*): 1864312-1864325
Φ96	Sfi11	2	Sa9	B	303		pac	ND	tmRNA, 788659
ΦEW	Sfi11	3	Sa11	ND	435		pac	ND	SAOUHSC_00581: 584240

[a]Classification based on reference 5.
[b]Classification based on reference 12.
[c]Location of the native phage or the attB site in the genome of reference strain 8325 (GenBank accession number NC_007795.1).
[d]virulence genes
[e]Abbreviations: ND, Not determined; tmRNA, transfer messenger RNA.

may result not only in bacterial lysis and phage release but also in a process termed "active lysogeny." This term was recently introduced to describe instances where prophages temporally and reversibly excise from the chromosome without lysing the bacterial host (20). This might be seen as a form of bacterial gene regulation that possibly improves bacterial fitness. Evidence for active lysogeny in *S. aureus* comes from a recent sequencing approach to detect extrachromosomal phages in *S. aureus* (21). Furthermore, it could be demonstrated that phages are readily induced under infectious conditions (17, 22–25). Analysis of isolates from cystic fibrosis patients revealed that translocation of the Sa3int phages often leads to a splitting of the bacterial population (17) into Hlb-positive (phage-cured) and phage-positive fractions. Both the phage-encoded virulence factors and Hlb are secreted factors; thus, functional complementation can be assumed.

PHAGE-BACTERIAL RECOGNITION

Adsorption to the bacterial host is the first critical step within the phage life cycle. Interaction of the receptor-binding protein (RBP) of the phage and its receptor initiates the infection cycle and, importantly, determines the host range and specificity (26). Phage receptors have to satisfy the following distinct requirements to be suitable for viral attachment. (i) Accessibility: phage receptors have to be accessible to the phage by random Brownian motion, flow, or diffusion. (ii) Abundance: with attachment being a stochastic process, the abundance of the receptor must be high enough to permit a sufficient probability of RBP-receptor contact. (iii) Constancy: the chemical composition of the receptor must be stable enough to allow evolutionary adaption of the phage to possible changes in the receptor appearance. Wall teichoic acid (WTA) of *S. aureus* fulfills all three requirements imposed on a proper phage receptor and was shown to act as the primary phage receptor. It is a major component of the cell wall and is expressed by all *S. aureus* strains. Most *S. aureus* strains produce WTA comprised of ribitol-phosphate (RboP) repeats. The complex biosynthesis of WTA is reviewed elsewhere (27, 28). WTA is covalently attached to the peptidoglycan of the cell wall (29, 30). Further derivatization of WTA is achieved in two ways. Attachment of *N*-acetyl-glucosamine residues (GlcNAc) occurs at the C4-position of the RboP unit by two glycosyltransferases, TarS and TarM, which attach GlcNAc in the β-position and α-position, respectively (31, 32). Recently, a third prophage-encoded glycosyltransferase, named TarP, was identified (33). Unlike TarS, TarP catalyzes the attachment of GlcNAc in the β-1,3-position.

The second modification of WTA is attachment of D-alanine residues at the C2-position by the Dlt-machinery (34). In contrast, a completely different type of WTA, composed of a glycerol-phosphate (GroP) repeating unit modified with α-*N*-acetyl-galactosamine (GalNAc) residues is found in *S. aureus* strains belonging to CC395 (35). This GroP WTA resembles the WTA structure found in many coagulase-negative staphylococci (36). Early studies identified *B. subtilis* mutants lacking WTA or WTA glycosylation. Those mutants appeared to be resistant against certain *B. subtilis* bacteriophages (37, 38). It was subsequently shown that the sugar moiety of WTA is also important for *S. aureus* phage interaction. Treatment of *S. aureus* with exo-β-acetylglucosaminidase or use of WTA glycosylation mutants leads to a decrease in phage binding (39, 40).

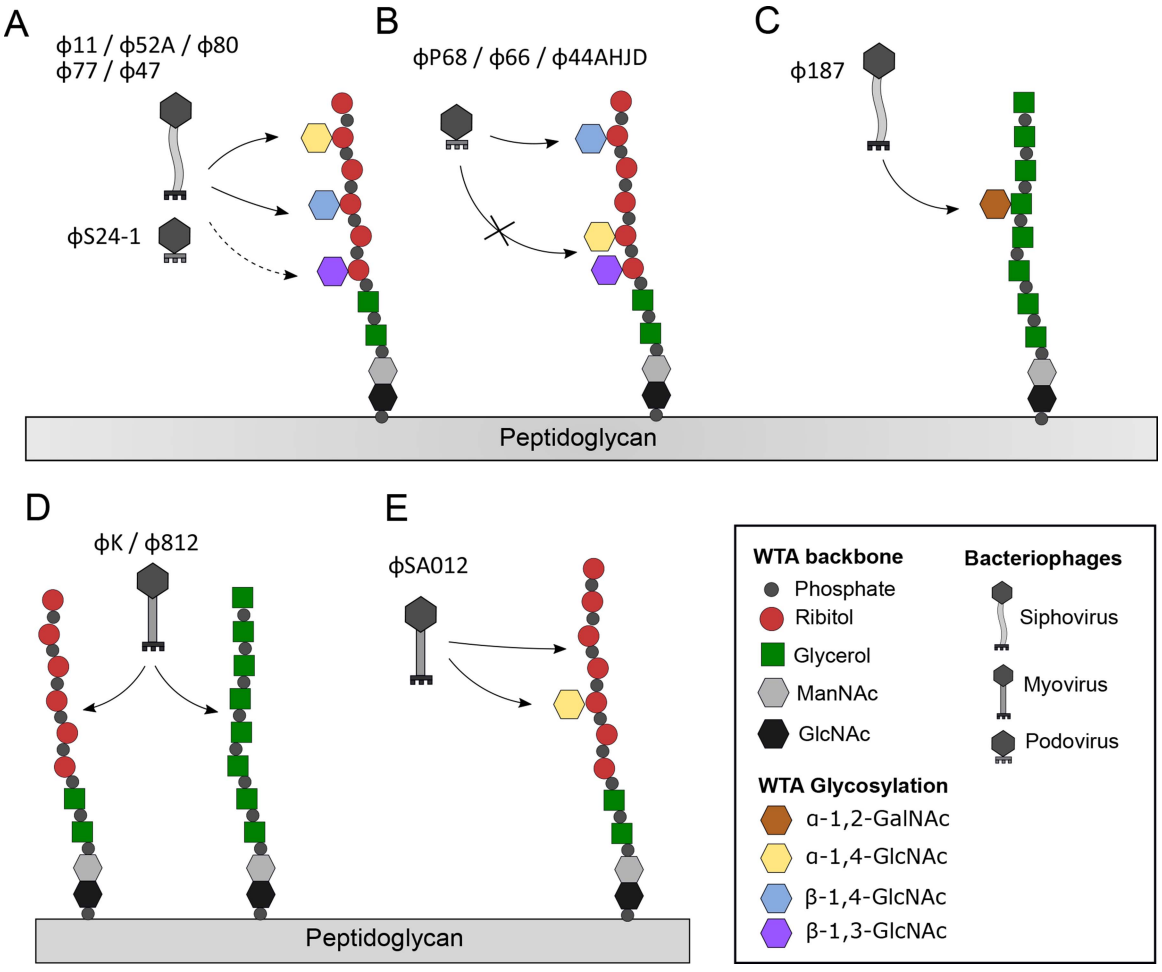
Although a majority of publications describe only carbohydrate-based phage interactions (WTA in combination with peptidoglycan structures), work by Nordström *et al.* (41) suggested that additional proteinaceous factors, prominent among them, surface protein A, interfere with phage adsorption. To our knowledge, this is the only account of surface protein phage interactions reported for *S. aureus*. Interestingly, a secondary protein receptor besides the primary receptor WTA has been described for the Gram-positive model organism *B. subtilis*. The lytic phage Spp1 adsorbs first in a reversible manner to WTA, which accelerates an irreversible interaction with the membrane receptor YueB and injection of viral DNA (42). A similar phenomenon was described for the membrane protein PIP, which serves in lactococcal species as a secondary phage receptor (43). However, PIP homologues in *S. aureus* have not been shown to be necessary for phage adsorption (44). Thus, so far, there is no clear evidence for a secondary proteinaceous receptor in *S. aureus*.

The advent of bacterial genetics allowed further insights into the molecular basis of bacteria-phage interactions. An early study suggested that besides WTA, a different anionic polymer of the *S. aureus* cell envelope, lipoteichoic acid, might serve as a receptor for the tail protein ORF636 of *S. aureus* phage ΦSLT (45). Lipoteichoic acids (LTA) consist of D-alanylated glycerol phosphate repeating units attached to the cell membrane of *S. aureus* (46). However, using an LTA-deficient mutant strain of RN4220, no difference in the infectivity of phages expressing homologues of the tail protein of ΦSLT could be observed (47). In contrast to the LTA-deficient mutant, gene deletion of *tagO*, the first gene of

the WTA biosynthesis pathway, leads to a phage-resistant phenotype (47, 48). By creating a transposon mutant library, Xia *et al.* (32) were able to isolate the RN4220 mutant K6, which is deficient in WTA glycosylation. K6 carries a transposon interrupting the gene coding for the α-glycosyltransferase TarM. K6 additionally carries a premature stop codon in *tarS* coding for the β-glycosyltransferase (31). K6 was resistant to a wide range of siphoviruses (47). Interestingly, as reviewed later, lytic myoviruses (Φ812 and φK) were able to infect K6 despite the lack of WTA glycosylation.

By using defined knockouts of the two identified ribitol-phosphate glycosyltransferases, *tarM* and *tarS*, it could be demonstrated that siphoviruses of serogroup B do not seem to differentiate between α- or β-GlcNAcylation at the ribitol C4 position (31, 44) (Fig. 1A). The receptor promiscuity of siphoviruses might reflect their role as the main vectors of horizontal gene transfer. Of note, Winstel *et al.* (49) demonstrated that *S. aureus* pathogenicity islands (SaPIs) can be transferred between strains expressing α- or β-GlcNAcylated WTA. Notably, β-1,3-GlcNAcylation by TarP seemed to reduce the infection and SaPI-transfer capacity of serogroup B siphoviruses (33) (Fig. 1A). More importantly, the Winstel and coworkers publication established the compatibility of phage and host receptors as a key driver of cross-species horizontal gene transfer (49). Even distantly related bacterial species such as *Listeria monocytogenes*

FIGURE 1 Receptor specificity of *S. aureus* phages. **(A)** Siphoviruses Φ11, Φ80, Φ52A, Φ47, and Φ77 and podovirus SA24-1 recognize α-or β-1,4-GlcNAc-RboP WTA. β-1,3-GlcNAc-WTA is adsorbed to less strongly by Φ80, Φ52A, and Φ11. **(B)** Podoviruses ΦP68, Φ44AHJD, and Φ66 bind to β-1,4-GlcNAc-RboP WTA and are blocked by β-1,3-GlcNAc or α-1,4-GlcNAc modifications. **(C)** Siphovirus Φ187 binds to α-GalNAC-GroP. **(D)** Myovirus ΦK, Φ812, attache to the backbone of GroP and/or RboP. **(E)** ΦSA012 recognizes both the RboP WTA backbone and α-1,4-GlcNAc-RboP by two different RBPs.



can engage in horizontal gene transfer with *S. aureus* (49, 50), as long as they express a compatible phage receptor. In contrast, lack of RboP-GlcNAc WTA excludes bacteria from the exchange of genetic information. For instance, *S. aureus* PS187 (CC395), equipped with GroP-type WTA, is cut off from horizontal gene transfer with RboP-WTA-expressing *S. aureus*, but is able to engage in exchange with coagulase-negative species that share a similar GroP-type WTA (35, 49). Interestingly, ectopic expression of RboP-type biosynthesis genes in coagulase-negative staphylococci with GroP-WTA rendered them susceptible to *S. aureus* phages (49). Hence, it can be speculated that RBP-receptor incompatibility is a major hindrance for horizontal gene transfer between *S. aureus* and coagulase-negative staphylococci. The other two described classes of morphological groups, namely *Myoviridae* and *Podoviridae*, show more diverse receptor specificities, that are discussed later in the article.

Returning to the postulated requirements for a phage receptor (accessibility, abundance, and constancy), one can audit whether they hold true for WTA as the phage receptor of *S. aureus*. WTA is an exposed cell surface molecule attached to the peptidoglycan (51), which would allow easy access to phages. The negative charge of WTA, conferred by phosphate residues, might allow polar or ionic interaction with RBPs. LTA, due to its presence in the more internal cell membrane of *S. aureus*, might therefore be of less utility as a receptor for bacteriophages. Additionally, WTA is a highly abundant glycopolymer that constitutes up to 60% of the cell wall mass in many Gram-positive bacteria. It is a key component of the cell wall, and no natural isolates of *S. aureus* without WTA have been reported so far. The indispensability and abundance of WTA ensure a high probability of phage-receptor contact, which is crucial for the adsorption process. Coagulase-negative bacteria, especially *Staphylococcus epidermidis*, show an increased diversity of WTA glycosylation patterns (36, 52). *S. aureus* appears to encode only one housekeeping glycosyltransferase, TarS. The *tarS* gene is found in almost all *S. aureus* genomes (53). The second glycosyltransferase, TarM, appears to have been acquired very early in *S. aureus* evolution (54). However, certain clonal lineages, such as CC5 and CC398, seem to have deleted *tarM* during their emergence or may have acquired phage-encoded *tarP*. The introduction of more sophisticated WTA glycosylation patterns might lead to less phage interaction and therefore to a decrease in the ability to participate in horizontal gene transfer. The described receptor constancy of WTA together with the widespread absence of clustered regularly interspaced short palindromic repeat (CRISPR)/Cas systems allows rapid acquisition and exchange of genetic elements by phages or phage-like particles (SaPIs), which ensures the role of *S. aureus* as a major human pathogen.

OCCURRENCE AND ROLE OF ACCESSORY PHAGE GENES

Although phages may be regarded as selfish elements, bacteria have learned to use them for their own purposes, and lysogeny can be regarded as a motor for short-term evolution. In many pathogens, phages provide the bacteria with additional genes that enable them to establish a new lifestyle. In *S. aureus*, several such phage-encoded virulence factors have been described, an observation originally described as phage conversion. Genes coding for Panton-Valentine leukocidin (*lukSF*) exfoliative toxin A (*eta*) (55), the cell-wall anchored protein SasX (56), and the immune evasion cluster (IEC) composed of enterotoxin S (*sea*), staphylokinase (*sak*), the chemotaxis inhibitory protein (*chp*), and the staphylococcal complement inhibitor (*scn*) (57) are the best-characterized phage-encoded virulence factors in *S. aureus*. A transposon mutant library was screened for virulence genes, and phage-encoded virulence genes were detected on all four prophages from strain Newman (58). Moreover, small RNAs involved in gene regulation (SprD = teg14) (59) or coding for the type I toxin-antitoxin system (SprF1/SprG1) (60) are encoded on Sa3int phages. With the availability of new phage and *S. aureus* genomes, new putative phage-encoded virulence genes (e.g., those coding for putative Clp protease or phospholipase) are being discovered and are awaiting functional analysis (18, 61–63).

Interestingly, accessory genes are strongly associated with phages of certain *int* groups and are localized at the left or (more frequently) right ends of the phage. There is a link between the encoded virulence factors, the *int* module, and the lytic module (holin and amidase genes), which are localized at the opposite end of the prophages (9). One may assume that it is evolutionarily beneficial to interchange this whole unit. Of note, these modules are in close proximity after phage excision in the circular and/or concatamer form of the phage. The close link of the lytic module and the inserted virulence factors is perhaps favored to optimize the phage control of the expression of the virulence genes (64). For instance, the expression of the virulence genes becomes cotranscribed with the late phage genes upon phage induction (64–66).

Sa1int Phages Carrying *eta*

The exfoliative toxins are virulence factors of *S. aureus* that cause bullous impetigo and its disseminated form, staphylococcal scalded-skin syndrome. The clinical symptoms vary from blisters anywhere on the body to multiple lesions complicated by conjunctivitis and staphylococcal scalded-skin syndrome (67). The exfoliative toxin A gene (*eta*) is carried in the genomes of Sa1int phages (9, 12, 13). An *eta* homolog is also carried by SaPI2 but is not known to be functional (1). However, these phages can be differentiated into at least six types due to variation in different modules (68). These *eta*-phages were associated with outbreaks of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* strains of various CCs in Japan and the Czech Republic (67, 69, 70) and are also present in a subpopulation of CC121 strains associated with superficial infections (25, 71).

Sa2int Phages Carrying *lukSF*

lukSF encodes the bi-component leucotoxin PVL, which targets human phagocytes through interaction with the complement receptors C5aR1 and C5aR2 (72). *lukSF*-encoding phages are strongly associated with skin and soft tissue infection and necrotizing pneumonia, which can also affect young, immunocompetent people (73–76). The majority of *lukSF*-carrying phages are Sa2int phages (66, 77–82). These phages are integrated within a conserved ORF which is surrounded by a cluster of tandemly repeated genes. In CC80 strains, phage induction led to the acquisition of host DNA into the phage genome probably due to a homologous recombination event between direct repeats of the two paralogous genes adjacent to the phage integration site (83). Phage excision was accompanied by an additional chromosomal deletion in this region. PCR-based typing schemes were established and modified to further subtype the *lukSF*-encoding phages (78, 84, 85). These studies confirm the high mosaicism of phage genomes and show that different *S. aureus* lineages have acquired a diverse set of *lukSF* phages. The combination of *lukSF* phages with methicillin resistance is characteristic of community-associated MRSA strains that are spreading in various continents. Strains of the most prominent USA300 lineage (CC8 isolates) are mainly spreading in North America, whereas in Europe CC80 strains are spreading, in Asia, CC59 strains, in the Asia Pacific region, CC30 strains, and in Australia, CC93 strains are more prevalent. The global spread of different *S. aureus* clones containing different *lukSF*-carrying phages supports the idea that PVL provides some fitness advantage to the bacterial host. The most common feature of all these strains is the strong association of *lukSF* phages and superficial, recurrent skin infections (75).

Sa3int Phages Carrying the IEC

Sa3int phages are by far the most prevalent *S. aureus* phages. Up to 96% of human nasal isolates were found to carry Sa3int phages integrated into the *hlb* gene (17, 86). Hlb can modify endothelial cells and platelets by both toxin sphingomyelinase and biofilm ligase activities, thereby increasing infection severity (87). The *hlb*-converting phages carry genes representing the IEC (*sea*, *sak*, *chp*, and *scn*) (57), coding for highly human-specific virulence genes. SEA has been described to modulate the function of chemokine receptors such as CCR1, CCR2, and CCR5 (76). CHIPS blocks two G-protein coupled receptors involved in chemotaxis and phagocyte activation (C5a and the formylated peptide receptor) (88). SAK is a potent plasminogen activator with pleiotropic function including fibrinolysis and antiopsonic activity. The latter occurs through degradation of immunoglobulin G and C3b/C3bi on the surface of staphylococci (89). SCIN is a specific inhibitor of the C3 convertase of the complement system (90). Seven IEC variants were discovered, carrying different combinations *of sea* (or *sep*), *sak*, *chp*, and *scn*. The genes' order was conserved and always in the same 5′ to 3′ orientation. *Scn* was the only gene present in all IECs analyzed, whereas, e.g., *sea* was detectable in only 27% of the IECs (57). Interestingly, genes coding for a type I toxin-antitoxin system (SprF1/SprG1) are also localized within the IEC gene cluster (60).

Sa7int Phages Carrying *sak*

As an exception to the rule, *sak* could be detected not only on Sa3int phages but also occasionally on Sa7int phages. Curiously, such a *sak*-encoding Sa7int phage is found in derivatives of the laboratory strain 8325-4 (17). The widely used phage-cured strain 8325-4 was somehow lysogenized with such a phage and has since been distributed to laboratories worldwide (designated strain RN6390 or ISP479C). A similar phage was detected in most of the prevalent hospital-associated MRSA clones (belonging to the ST5 lineage) isolated in southern Germany (91). These strains were highly successful over the years and were quickly diversifying, as illustrated by differences in pulsed-field gel electrophoresis patterns and antibiotic susceptibility. Notably, these isolates can be clearly discriminated from other CC5 isolates (ST225, a single-locus variant of ST5) circulating in Germany, which are characterized by the high prevalence of a typical Sa1int phage (91, 92).

SasX-Carrying Phage in MRSA

The assumption that mobile genetic elements promote the spread of bacterial clones was emphasized by the emergence of highly epidemic MRSA strains carrying a phage harboring a newly identified cell wall-anchored virulence factor, SasX (56). SasX promotes nasal colonization, bacterial aggregation, and virulence. *sasX*, similar to other phage-carried virulence genes, is located as an accessory gene at the right end of a phiSPβ-like prophage. The genome size of 127 kb is significantly larger than that of a typical *S. aureus* siphovirus and highly similar to a prophage found in *S. epidermidis* strain RP62A (93), indicating that the phage and thus a new virulence trait was acquired from *S. epidermidis*. This phage is spreading between *S. aureus* strains and is also found in MRSA strains of the CC5 lineage (56). It is unclear whether this phage and other genetic elements managed to cross the species barrier and, if so, how.

TarP-Carrying Phages

Recently, the phage-associated WTA-glycosyltransferase TarP was found to glycosylate the WTA in an alternative way (33). The *tarP* gene is located in the lysogenic module of three different *S. aureus* prophages with integrase groups, Sa1int, Sa3int, and Sa9int. The *tarP*-Sa3int phage, ΦN315, additionally encodes the IEC discussed above. *tarP*-encoding phages can be found in clonal lineages CC5 (hospital-associated clones with Sa3int TarP-phages) and CC398 (livestock-associated clones with Sa1int and Sa9int TarP-phages). Modification of WTA by TarP lead to reduced opsonization by IgG in human sera. The immune evasion capabilities of TarP were reflected by lower immunogenicity of TarP-modified WTA in comparison with TarS-modified WTA.

ROLE OF PHAGES IN HUMAN ADAPTATION OF ANIMAL ISOLATES

S. aureus colonizes a variety of animal species and adapts to particular species through changes in the core genome as well as potential phage-encoded virulence genes (94). However, transmission of strains between human and animal reservoirs also occurs. Since the early 2000s MRSA strains with the sequence type ST398 were described as colonizing pigs and also causing infections in humans living in close contact with livestock (95, 96). Strains belonging to the CC398 lineage are commonly found in livestock and are resistant to multiple antibiotics but lack several important virulence factors (97–99). More recently, human to human transmission of CC398 strains was reported. Critical in this jump are Sa3int phages that generally are absent in CC398 strains but are present in most livestock strains infecting humans without livestock contact (61, 100). The importance of Sa3int for virulence of CC398 strains to humans has also been demonstrated more directly, because the presence of Sa3int decreased phagocytosis by human but not by pig polymorphonuclear neutrophils, while β-hemolysin production was abolished due to integration of the phage in the *hlb* gene (101).

Interestingly, the livestock-associated MRSA CC398 strains originated in humans as a methicillin-susceptible *S. aureus* strain, and upon introduction into livestock they lost Sa3int and acquired methicillin resistance and resistance to tetracycline, which is commonly used in livestock production (102, 103). The recent reintroduction of Sa3int into the livestock-associated MRSA strain population appears to be restricted by mutations in the 14-base pair phage attachment site of many CC398 strains (104) that leave the *hlb* reading frame intact but reduce integration of Sa3int (18, 19, 105). In these strains Sa3int integrates elsewhere in the chromosome, and the majority of those integration sites contain a 4-nucleotide sequence (5′-CTGG-3′) that is shared with the bona fide integration site in *hlb* (19). Importantly, the location of the integration appears to influence the stability of the Sa3int prophage in the livestock strains (18, 19), indicating that the success of the livestock MRSA strains in humans may depend on the location of the prophage. Interestingly, Sa3int also can promote animal adaptation, because some of the avian isolates carry a Sa3int-like phage with two putative avian-niche-specific genes (102).

The loss of Sa3int in the original jump of methicillin-susceptible *S. aureus* strains to livestock and the observation that the *hlb* gene of livestock-associated CC398 strains is mutated such that the phage integration site is eliminated but the *hlb* gene is kept intact underscores the role of the phages in lysogenic conversion. It also underscores the probability that Hlb is important for livestock but not for human pathogenesis. Also, the human to bovine jump of CC8 strains has been associated with the loss of Sa3int, similar to the human to livestock jump of CC398 (102, 106).

Phages other than Sa3int have also been associated with the human adaptation of CC398. Several studies document the presence of a phiMR11-like phage in these strains, and further analysis suggested that it is a defective phage that may act as a helper phage which interacts with a coresiding Sa3int phage to promote the expression of phage gene products (61, 107, 108). In another subset of CC398 strains associated with human to hu-

man transmission, Sa7int phages were detected (103). Little is known about this phage, but its association with strains being transmitted between humans suggests that it may be important for human adaptation.

DUAL CONTROL OF PHAGE-ENCODED VIRULENCE GENES: LINK TO HOST REGULATORY SYSTEMS AND PHAGE LIFE CYCLE

Phage-encoded virulence genes are integrated into the regulatory mechanism of the bacterial host and modulated in a manner surprisingly similar to bacterial chromosome-encoded virulence factors. The alternative sigma factor B seems to inhibit the expression of most, if not all, of the currently analyzed phage-encoded virulence factors (109–111). Furthermore, the two-component regulatory system *saeRS* and, to a lesser extent, the quorum-sensing system *agr* (66, 109, 110, 112) are required for the activation of most of the phage-encoded virulence factors, such as *eta*, *pvl*, *scn*, and *chp*. Interestingly, as an exception to this observation, *sak* was not, or was only marginally, influenced by *sae* and/or *agr* (66, 110). Both the *sae* and *agr* regulatory systems are essential for the coordinated expression of many bacterial chromosome-encoded virulence factors, and mutants deficient in these factors are clearly less virulent as shown in different animal models of infection. Thus, the phage-encoded virulence factors are integrated into different regulatory circuits employed by the bacteria. It is likely that the prophages acquired these virulence genes along with their pre-existing chromosomally determined regulatory features. Moreover, the expression of phage-encoded virulence genes is also influenced by subinhibitory concentrations of certain antibiotics. β-Lactam antibiotics, for instance, enhance PVL production presumably via the transcriptional factors SarA and Rot (112).

The expression of these virulence factors is also tightly linked to the phage life cycle. Prophages are induced by environmental conditions that lead to DNA damage, including exposure to reactive oxygen species generated by leukocytes or exposure to exogenous agents such as antibiotics (65, 113, 114). It has been demonstrated that under such phage-inducing conditions, the transcription of the virulence factors that are localized in close proximity to the lysis module of the phage genome is increased (64–66). This phenomenon is partially due to a multicopy effect caused by phage replication. However, it has also been shown that transcription becomes intimately linked to the phage genes through cotranscription with the now derepressed lysis genes (65, 66). In this regard, the use of antibiotics that induce the SOS response, such as quinolones or β-lactam antibiotics, is a special concern. Antibiotic-induced expression of phage-encoded toxin genes is well documented for *E. coli* prophages harboring Shiga-toxin-encoding genes (*stx*). Quinolones enhance *stx* transcription, Stx production, and toxin release from the bacterial cells via phage-mediated lysis and death in mice (115).

MYOIRIDAE AND *PODOVIRIDAE*

Although more is known about the siphoviruses, the phages belonging to the *Myoviridae* and *Podoviridae* are receiving increasing attention because of their therapeutic potential in combating *S. aureus* infections. In this context, their nontemperate and lytic nature does not carry the risk of enabling the spread of virulence or antibiotic-resistance genes upon phage treatment (116).

The receptor interaction of *Myoviridae* and *Podoviridae* demonstrates more diversity in comparison with the discussed *Siphoviridae*. The well-described Myovirus ΦK and Φ812 only require either a GroP or RboP backbone of WTA (Fig. 1D) (35, 47). Structural elucidation of the RBP of Myovirus φ812 demonstrates a sophisticated conformational change of the double-layered baseplate upon binding of the bacterial cell wall (116). Hence, their high receptor promiscuity allows infection of many coagulase-negative staphylococci expressing GroP-WTA (49, 117). Alternatively, Twortlikevirus ΦSA012 appears to distinguish between α- and β-GlcNAcylation (118). At least two RBPs are encoded by ΦSA012: one interacting with the RboP backbone and the other, with α-1,4-GlcNAc residues (Fig. 1E). Interestingly, Podovirus ΦP68, Φ44AHJD, and Φ66, requiring β-1,4-GlcNAcylated WTA, fail to adsorb and infect strains carrying WTA modified with α-GlcNAc or β-1,3-GlcNAc residues in a dominant manner (33, 54). This finding demonstrates that certain WTA glycosylation patterns are able to protect *S. aureus* from viral predation by preventing proper phage adsorption. Furthermore, it shows that the alternative WTA glycosyltransferase, TarM and TarP, play an antiviral role. Obtaining phage resistance by blocking the host receptor is an often-observed feature (119, 120). However, TarM-mediated glycosylation does not block all *S. aureus* podoviruses from infection. Certain podoviruses, such as S24-1, appear to have evolved an RBP that allows adsorption to α- or β-GlcNAcylated WTA (121). Noticeably, this RBP shares key amino acids with the RBP of siphovirus φ11, GP45, also shown to facilitate binding to α- or β-GlcNAcylated WTAs (44).

The application of phages to limit *S. aureus* both *in vitro* and *in vivo* has been tested extensively, and there seems to be some therapeutic potential (122, 123). In the following, we mention just a few examples of how phages may be used to combat *S. aureus*. Additionally, the therapeutic use of phage lytic proteins in *S. aureus* is yielding promising results, showing good efficacy without apparent side effects (124). During dairy production a phage cocktail consisting of *Myoviridae* and *Podoviridae* as well as lytic *Siphoviridae* eradiated a 10^6 CFU/g *S. aureus* population after 14 days in Cheddar cheese curd during ripening at 4°C (125). Also, in biofilms, phages appear to reduce *S. aureus* numbers as exemplified in two studies using members of the *Myoviridae* family (126, 127). However, if present in sublethal doses, the action of the lytic phages may promote DNA release and collectively enhance biofilm formation, suggesting that caution should be used when considering phage therapy for eradication of *S. aureus* biofilms (128). The potency of phage therapy *in vivo* has also been evaluated. In a mouse model of bovine mastitis a phage cocktail significantly reduced infection with a clinical bovine mastitis when applied 4 h postinoculation as demonstrated by improved pathology and decreased bacterial counts. Importantly, phage quantification indicated that the phage cocktail maintained high intramammary phage titers without spreading systemically (129). Future studies are likely to further address the therapeutic potential of targeting *S. aureus* with phages.

PHAGE DYNAMICS: MOVEMENT OF THE PHAGE WITHIN AND BETWEEN STAPHYLOCOCCAL SPECIES

Whereas most *S. aureus* isolates harbor multiple phages, less is known about the prevalence and nature of phages in coagulase-negative staphylococci. Analyses of available phage genome sequences of coagulase-negative staphylococci revealed a modular structure similar to that of *S. aureus* phages. The transfer of phages between different staphylococci is also supported by cluster analyses of phages from different staphylococcal species (130). CRISPR/Cas loci are present in some *S. epidermidis* strains but are lacking in most *S. aureus* isolates. These loci are involved in the recognition and cleavage of foreign DNA. Therefore, it was postulated that the gene flow is uni-directional (131), as indicated by several instances in which genetic material was presumably transferred from *S. epidermidis* to the more pathogenic *S. aureus* species. For example, the staphylococcal cassette chromosome expressing methicillin resistance (SCC*mec*) genomic islands that carry the *mecA* gene conferring resistance to methicillin at least occasionally originate from *S. epidermidis* (132). Genetic exchange might be possible between different staphylococcal species because they live in similar environments, such as on the skin or in the nose. Additionally, phages might persist in a specific environment even though the bacterial host is already eliminated through the action of the immune system or antibiotics. Such phages or transducing particles may then infect coinhabitants, providing them with new properties.

PHAGES MEDIATE HORIZONTAL GENE TRANSFER

Horizontal gene transfer is commonly observed in *S. aureus*, and phages are believed to be major contributors (6, 16). In 1959, transduction was described for a staphylococcal phage (133), and soon thereafter, several phages were shown to be transducing (134), particularly those belonging to serological group B (135), such as Φ11, Φ80a, and Φ80 (136, 137). Transduction is the process by which bacterial DNA, during lytic replication of the phage, is mis-packaged into phage capsids forming transducing particles and upon release can be taken up by bacterial cells. In generalized transduction, the phage machinery recognizes pseudo *pac*-sites that mimic the sequence from where phage DNA packaging is initiated during lytic growth, and thus, in principle, any bacterial DNA can be transferred by this process. Normally, it is thought that the recipient strain should be susceptible to the transducing phage, but this appears not to be the case, because plasmid DNA is effectively transduced into recipient *S. aureus* strains by φ29, φ52A, and φ80α as well as by prophage φ53 in spite of their insensitivity to the lytic action of the transducing phage (138). In *S. aureus*, the transfer of antibiotic resistance genes has mostly been studied, but metabolic traits have also been transduced, as have been various mobile genetic elements (139, 140). The size of the DNA transferred by a transducing particle is limited to that of the *S. aureus* siphophage genome (approximately 43 kbp) (8, 141), and molecules of smaller sizes such as plasmids are transferred as linear multimers (142). The size limitation of the phage capsid prevents transfer of larger chromosomal elements such as the SCC*mec*. The SCC*mec* cassettes vary in size from 20 kbp to 60 kbp, so only the smaller SCC*mec*s are expected to transfer through generalized transduction, as demonstrated for SCC*mec* type IV and SCC*mec* type I (143, 144). Even then, transfer of methicillin resistance is a rare event, and other factors are likely required, such as lysogeny prior to transduction and the presence of a penicillinase in the

recipient strain (145). The need for penicillinase activity was confirmed and explained by the transcriptional regulation of the *mec* gene by the plasmid-borne *blaR1-blaI* regulatory genes (143). Thus, transduction is possibly responsible for the transfer of SCC*mec* elements, although additional factors are required.

In the laboratory, the phages Φ11 and Φ80a in particular have been used extensively for genetic manipulations of *S. aureus* (146). However, outside of the lab a diverse range of transducing phages are being reported (49, 147, 148). One example is Φ187, which only binds and transduces ST395 strains expressing an unusual WTA (Fig. 1C) that, surprisingly, is also present in *S. epidermidis* and *L. monocytogenes*, which are also transducible (49). Another phage, S6, isolated from sewage, proved to be a giant myophage that is able to transduce plasmid and methicillin resistance between *S. aureus* and non-*aureus* staphylococcal species, including *S. epidermidis*, *S. felis*, *S. sciuri*, and *S. pseudintermedius* (148).

Although some phages are able to readily transduce between staphylococcal species, horizontal gene transfer appears generally limited within the species through restriction-modification (R-M) systems. The principle function of these R-M systems is to protect the cell by degrading foreign DNA. If the phage is derived from a host with the same R-M system, the phage DNA becomes methylated at the cognate restriction site and thus is protected. Strains of the major CCs were shown to differ in their R-M specificity genes (149). Thus, mobile genetic elements present in one strain move to a strain of the same lineage at a higher frequency than to strains of other lineages. Consequently, *S. aureus* lineages carry a unique combination of core variable genes, suggesting only vertical transmission of these genes (149). It has also been shown that prophage prevalence is associated with the clonal background of *S. aureus*, indicating that the spread of the phages in the bacterial population is at least partially restricted (12, 13). In certain CCs, some phage groups are completely absent, whereas others are significantly less or significantly more frequent. The most prominent disequilibrium was the finding that CC15 strains do not carry Sa3int phages, although this is the most common phage group found in *S. aureus*, with a prevalence of up to 90% (17, 57, 150). In addition, many isolates from the CC15 complex carried none of the seven prophage groups, suggesting that this lineage is particularly restrictive to the uptake of foreign DNA. From these studies, it is clear that restriction barriers are important in limiting phage transmission and likely also transduction. Other barriers may also be present, such as the CRISPR/Cas system, but their biological importance is unknown because the system has only been reported for a few *S. aureus* strains (151).

Although it is well accepted that transduction is central for horizontal gene transfer in staphylococci, surprisingly little is known of the process under biologically relevant conditions. Recently, we observed that phages spontaneously released from a subpopulation of lysogenic cells can infect, lyse, and efficiently transfer DNA from a phage-susceptible bacterial population back to the intact lysogenic population, which itself survives because of immunity to phage killing (152). The process, which we termed "auto-transduction," is driven by the spontaneous release of phages from the lysogenic population. Upon infection of susceptible cells, phages are formed together with transducing particles containing bacterial DNA and mobile genetic elements such as plasmids or SaPIs. These elements are effectively introduced in the original population, which due to lysogeny resists phage killing (152). Even though the phenomenon of transducing particles entering lysogens was observed in the first transduction experiments (153), we still do not know the extent to which it has biological impact. What, for example, remains to be studied are the conditions that promote the spontaneous release of prophages and those that influence the formation of transducing particles. A recent study showed that the ratio of transducing particles to phages is affected by antibiotics (154), thus stressing the need for *in vivo* studies that assess the potential impact of antimicrobial therapy on transduction.

Recently, another study investigated transduction and found that packaging of bacterial DNA in transducing particles differs depending on whether they arise from an induced, temperate phage or from an infection (155). Interestingly, while infection with a transducing phage leads even to packaging of the bacterial chromosome in transducing particles, the induction of a temperate transducing phage leads to preferential packaging of bacterial DNA downstream of the integration site in a process termed "lateral transduction." By lateral transduction, several hundred kilobases of *S. aureus* DNA is packaged with high frequency, leading to hypermobility of this chromosomal region (155). With these findings in mind, we are beginning to understand how bacteriophages can be the main vehicle of staphylococcal horizontal gene transfer.

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