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Phages of *Staphylococcus aureus* and their impact on host evolution

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ABSTRACT

Most of the dissimilarity between *Staphylococcus aureus* strains is due to the presence of mobile genetic elements such as bacteriophages or pathogenicity islands. These elements provide the bacteria with additional genes that enable them to establish a new lifestyle that is often accompanied by a shift to increased pathogenicity or a jump to a new host. *S. aureus* phages may carry genes coding for diverse virulence factors such as Panton-Valentine leukocidin, staphylokinase, enterotoxins, chemotaxis-inhibitory proteins, or exfoliative toxins. Phages also mediate the transfer of pathogenicity islands in a highly coordinated manner and are the primary vehicle for the horizontal transfer of chromosomal and extra-chromosomal genes. Here, we summarise recent advances regarding phage classification, genome organisation and function of *S. aureus* phages with a particular emphasis on their role in the evolution of the bacterial host.

1. Introduction

The majority of both colonising and infectious *Staphylococcus aureus* isolates can be placed into a limited number of mostly pandemic clonal complexes (Lindsay, 2010; Lindsay and Holden, 2004; Melles et al., 2004). In general, *S. aureus* is regarded as a highly clonal species with a conserved core genome (Feil et al., 2003) that has evolved mainly through mutation, as illustrated by single-nucleotide polymorphisms detected by multilocus sequence typing (MLST) of selected housekeeping genes or through whole-genome sequencing. Thus, the diversity of the *S. aureus* species is mainly determined by the presence of mobile genetic elements, many of which are prophages or phage-related genomic islands. Both the horizontal transfer of most of the mobile elements and strain evolution are tightly linked to phages. First, phages can be mobilised and transferred to recipient strains. It is known that many accessory genes carried by phage genomes encode for staphylococcal virulence factors, which are important for the success of certain *S. aureus* strains. Second, phages support the induction, packaging and transfer of genomic islands. This interesting topic was recently reviewed (Christie and Dokland, 2012; Novick et al., 2010) and will not be the focus of the present review. Third, phage transduction is an efficient means to transfer not only extra-chromosomal mobile elements, such as plasmids, but also chromosomal markers (albeit with lower efficiency). In general, for *S. aureus,* it is believed that phages are the primary tool for diversification because the species is thought not to be naturally competent. However, this long-standing notion was recently challenged by the finding that at least under certain circumstances, an alternative sigma factor H can be expressed in subpopulations of bacteria in which a competence apparatus becomes activated to mediate the uptake of naked DNA (Morikawa et al., 2012). Interestingly, sigma factor H has also been shown to interact with the conserved promoter region of phage integrase (*int*) genes, which seems to result in the stabilisation of the lysogenic state (Tao et al., 2010). Thus, sigma factor H may serve as a regulatory tool involved in modulating horizontal evolution (at least under some thus-far-poorly-defined conditions), a topic that clearly needs further evaluation.

Here, we will first give a brief overview of previously used methods to classify *S. aureus* phages as well as basic insights into the genome structure of selected phages. Then, we will mainly focus on the impact of phages on the evolution of the bacterial host.

2. Serogroups and morphology of phages infecting *S. aureus*

All known *S. aureus* phages belong to the order Caudovirales (tailed phages), which are composed of an icosahedral capsid filled with double-stranded DNA and a thin filamentous tail. Based on the tail morphology, they can be further classified into three major families: Podoviridae, which have a very short tail; Siphoviridae, which have a long non-contractile tail; and Myoviridae, which have a long, contractile, double-sheathed tail, as shown in Fig. 1.

In early studies, *S. aureus* phages were compared, grouped and classified according to their reaction to polyclonal antiserum, which can neutralise phage infection. Based on the phage neutralisation tests, 39 phages were classified into six serogroups (Rountree, 1949). With more sera and more staphylococcal phage isolates, a total of 11 serogroups (A–H and J–L) were defined (Rippon, 1952, 1956). Group E, J, and K phages were found to be

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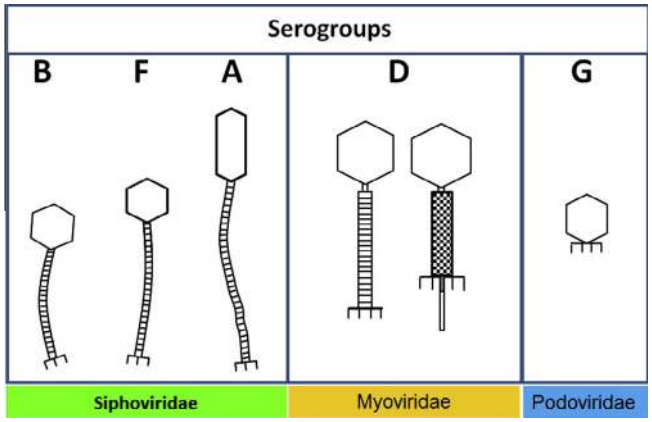

Fig. 1. Schematic representation of major groups of *S. aureus* phage. Modified according to (Brandis and Lenz, 1984).

specific for coagulase-negative staphylococci and avirulent to *S. aureus*. Most of the temperate phages infecting *S. aureus* could be assigned to serogroups A, B and F.

With the development of electron microscopy, morphology-based classification of *S. aureus* phages became feasible. Ackermann and Brandis et al. proposed a similar classification system including three major morphological groups, A–C or 1–3, which correspond to the phage families Myoviridae, Siphoviridae and Podoviridae, respectively (Ackermann, 1975; Brandis and Lenz, 1984). According to the proposal by Brandis and Lenz, all phages in morphological group 2 (Siphoviridae) have non-contractile tails. Based on a tail length longer or shorter than 200 nm and the shape of the head, staphylococcus phages in this group can be further assigned to subgroups 2.1, 2.2.1, and 2.2.2, which roughly correspond to serogroups B, A and F, respectively. While phages from serogroups B and F have isometric capsids, phages from serogroup A have distinct prolate heads (Fig. 1). Most serogroup B phages have tails shorter than 200 nm, while phages in serogroups A and F have tails longer than 200 nm. Phages in serogroups D and G seem unable to lysogenise host cells and can be assigned to Myoviridae or Podoviridae, respectively.

3. Genome organisation of *S. aureus* phages

The identification and differentiation of *S. aureus* phages has been continuously improved through the development of molecular biology techniques. Since the release of the full sequence of phi-PVL (Kaneko et al., 1998) there have been many genomes of staphylococcal phages and prophages fully sequenced. In a representative study (Kwan et al., 2005) based on the complete genomes of 27 phages, *S. aureus* phages are organised into three size classes: staphylococcal siphoviruses, with a genome size of 39–43 kb; podoviruses, with a smaller genome size of 16–18 KD; and myoviruses, with a genome size of 120–140 kb (for recent reviews, see (Deghorain and Van Melderen, 2012; Lobocka et al., 2012)).

The genome maps of four representative siphoviruses that belong to different serogroups (A, B and F) are shown in Fig. 2. The siphovirus genomes are usually organised into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis. Nucleotide sequence analysis revealed that phage genomes of different serogroups share the most homology at the replication module. Two serogroup B phages, ϕ11 and ϕ80α, are among the best studied *S. aureus* phages – partially because both phages have very high transducing efficiency and were thus often used experimentally to transfer gene mutations between *S. aureus* strains. Although there are 50–70 ORFs encoded by each siphovirus genome, for the majority of the ORFs, we have only putative functional annotation. In the following part, only experimentally characterised staphylococcal phage genes will be reviewed. For an updated, expert annotated genome maps of representative phage gnomes please refer to Fig. 2.

3.1. Lysogeny module

As shown in Fig. 2, the integrase and regulator proteins, CI and Cro, respectively, are encoded in the lysogeny modules. The switch between lysogenic and lytic growth is most likely controlled by a molecular circuitry similar to that of the λ phage: The phage will remain in the lysogenic state if CI predominates but will be transformed into the lytic cycle if Cro predominates. In vitro analysis demonstrated that the ϕ11 CI-operator complex resembles those of lambdoid phages at the structural level. The mode of action of the ϕ11 CI, however, may be distinct from that of the repressor proteins of λ and related phages (Ganguly et al., 2009).

3.2. Module for DNA replication

Downstream of the lysogeny module, a dozen of the ORFs in the ϕ11 and ϕ80α genomes may be involved in the redirection of host DNA metabolism for phage DNA replication, as various DNA-binding motifs and putative nucleases could be identified in this region using *in silico* approaches. However, experimental proof is still lacking for most of the ORFs, with a few exceptions. In a recent study, three genes in this region were found to be involved in the mobilisation of *S. aureus* pathogenicity islands (SaPIs). SaPIs are a family of 14–27 kb genetic elements that usually stably reside in the *S. aureus* genome, similarly to prophages, and contain phage-like repressor, integrase and terminase genes but do not contain genes encoding for phage structural proteins. SaPIs also carry a variety of accessory genes including superantigen toxins, antibiotic resistance factors and other virulence factors. The excision and replication of SaPIs needs a helper phage. Both ϕ11 and ϕ80α are used as model helper phages to study SaPI mobilisation. Following the induction of a resident helper phage or superinfection by a helper phage, the SaPI genome is excised, replicated and packed into the structural proteins of the helper phage to form infectious SaPI particles whose capsid is usually 1/3 of the size of its helper phage (Christie and Dokland, 2012; Novick et al., 2010). The depression of different SaPIs requires different proteins from the helper phage. For example, SaPI1, SaPIbov1 and SaPIbov2 are depressed by Sri (ORF22), Dut (ORF32) and ORF15 of ϕ80, respectively (Tormo-Mas et al., 2010). Interestingly Sri was previously identified in ϕ77 as a DnaI-binding protein that inhibits host DNA replication (Liu et al., 2004), while *dut* codes for a dUTPase (Tormo-Mas et al., 2010).

Phage proteins that redirect bacterial metabolic pathways to the phage reproduction cycle have also been identified from the staphylococcal myovirus phages G1 and Twort. ORF67 from phage G1 interacts with the *S. aureus* RNA polymerase σ subunit and blocks cell growth by inhibiting transcription (Dehbi et al., 2009; Osmundson et al., 2012), whereas ORF240 from phage G1 binds tightly to the DNA sliding clamp and prevents both its loading onto DNA and its interaction with DNA polymerase C, leading to DNA replication arrest and cell death (Belley et al., 2006).

3.3. DNA Packaging and morphogenesis modules

In ϕ11 and ϕ80α, the packaging and head modules are localised between the DNA replication and tail module. Recently, the functions of several genes in this region were studied in detail. The RinA protein exerts a regulatory function – it binds to the operator situated upstream of the *terS* gene and activates the transcription of the late operon covering the morphogenesis and lysis modules (Ferrer et al., 2011). The transcription activator activity of both RinA and RinB were previously demonstrated to modulate the

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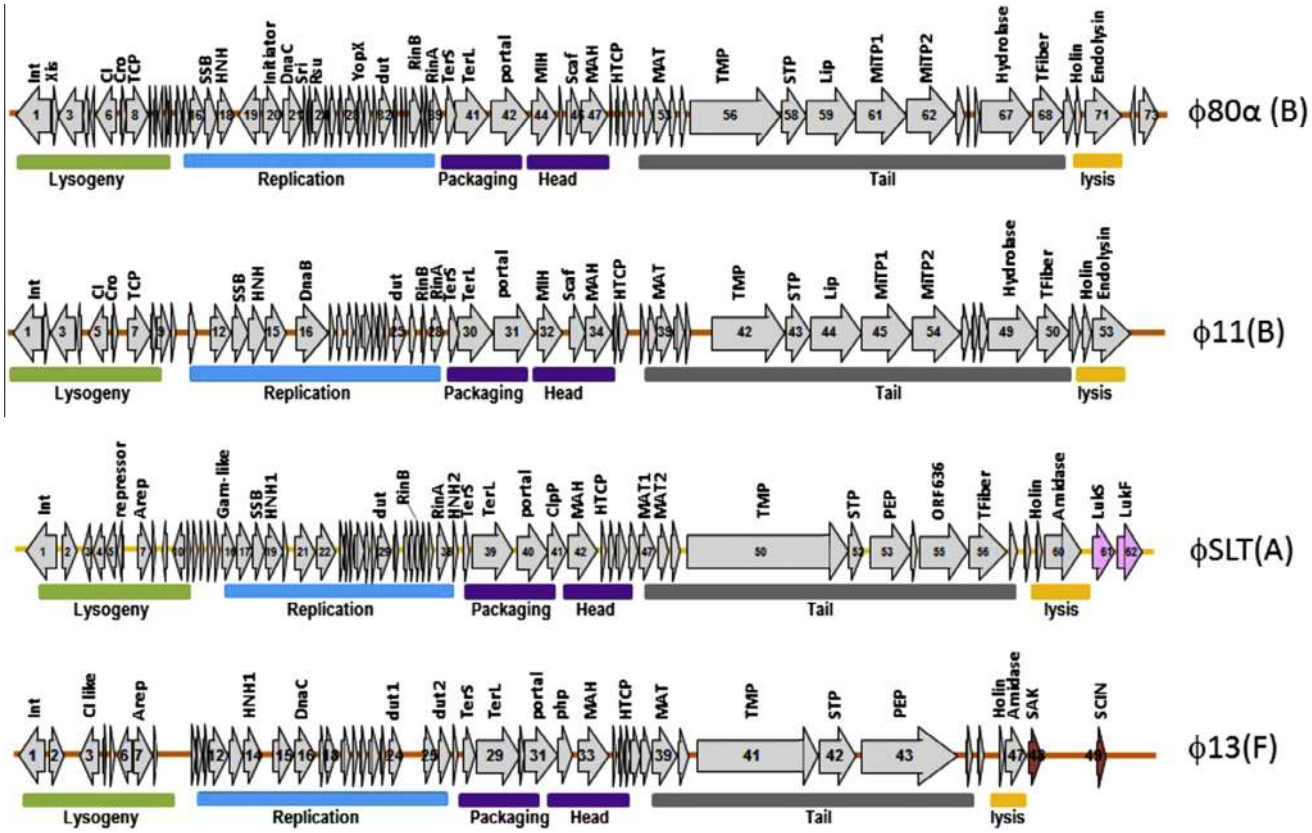

Fig. 2. The genome map of representative *S. auerus* phages. ϕ11 (Genbank entry NC_004615.1), ϕ80α (NC_009526.1), ϕSLT (NC_002661), ϕ13 (NC_004617) were drawn to scale. The putative functions of the ORF were given above the ORF. Int (integrase), Xis (excisioniase), CI (CI like protein), Cro (Cro like protein), SSB (single strand DNA binding protein), HNH (HNH nuclease), TerL (terminase, large unit), TerS (terminase small unit), MIH (minor head protein), MAH (Major head protein), Scaff (scaffold protein), HTCP (head tail connector protein), TMP (tape measure protein), STP (siphon tail protein), Lip (Lipase), MiTP (minor tail protein), MaTP (major tail protein), Tfiber (tail fibre protein), PEP (phage endopeptidase), Arep (antirepressor).

expression of ϕ11 *int* (Ye and Lee, 1993). In another study, it was found that co-expression of ϕ80α capsid protein and scaffolding proteins in *S. aureus* but not in *Escherichia coli* leads to the formation of procapsid-related structures. Moreover, ϕ80α capsid and scaffolding proteins undergo normal N-terminal processing, suggesting that a host protease was involved in the assembly of the capsid (Spilman et al., 2012).

The virion proteins of the helper phages ϕ11 and ϕ80α are highly homologous (Tallent et al., 2007; Tormo et al., 2008). The major head protein and major tail protein are the most abundant proteins in the mature virions. Surprisingly, protein GP8 (ORF8 localised in the lysogeny module of ϕ80α) was found to be associated with CsCl-purified 80α procapsid fractions (Poliakov et al., 2008), suggesting a dual role of GP8 in regulation and virion structure, in a manner similar to protein Psu of phage P4 (Dokland et al., 1993; Pani et al., 2006).

A siphovirus tail is usually composed of major and minor tail proteins, tape measurement proteins, baseplate proteins, cell wall hydrolyses, and tail fibre proteins. Interestingly, the tail protein (ORF636) from ϕSLT interacts with cell membrane-anchored lipoteichoic acid (Kaneko et al., 2009), and HydH5 protein from phage phiPLA88 was shown to bind to and lyse *S. aureus* cells (Rodriguez et al., 2011). Homologues of these two proteins were identified in the tail module of ϕ80α, with ORF62 being 60% identical to ORF636 and ORF67 being 80% identical to HydH5.

3.4. Lysis module

The two-component lysis system consisting of a holin and an endolysin was characterised in podovirus ϕ68 (Takac et al., 2005) and myovirus phage Twort (Loessner et al., 1998). While the gene *lys*16 of ϕ68 encodes the cell wall-degrading enzyme, the gene *hol*15 was found embedded in the −1 reading frame at the 3′ end of *lys*16 and encodes for a class I holin (Takac et al., 2005). The substrate specificities of several endolysins were studied in detail (Navarre et al., 1999). Notably, the ϕ11 endolysin encoded by ORF53 has D-alanyl-glycyl endopeptidase activity in addition to N-acetylmuramyl-L-alanyl amidase activity.

4. Phage classification based on integrase homology

Traditionally, *S. aureus* phages were characterised according to their lytic activity, morphology and serological properties. The evolution of phage lineages seems to be driven by the lateral gene transfer of interchangeable genetic elements (modules), which consist of functionally related genes. The Siphoviridae genomes are usually organised into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis (Fig. 2) (Brussow and Desiere, 2001; Iandolo et al., 2002; Kahankova et al., 2010). 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 (Hatfull and Hendrix, 2011). Thus, multiple alignments of *S. aureus* phage genomes reveal a chimeric and mosaic structure resulting from horizontal transfer and recombination (Canchaya et al., 2003; Goerke et al., 2009; Kwan et al., 2005; McCarthy et al., 2012b).

Due to this modular structure, phage nomenclature and species definition is challenging, and comparative genomics-based approaches are useful (Nelson, 2004). Multiplex PCR strategies have

been developed to classify the *S. aureus* Siphoviridae according to their modules (lysogeny, regulation, replication, structural and lytic modules) (Goerke et al., 2009; Kahankova et al., 2010) (Fig. 3). It seems reasonable to classify the *S. aureus* prophages primarily on the basis of *int* gene homology (Goerke et al., 2009; Kahankova et al., 2010; McCarthy et al., 2012b). First, nucleotide sequences are well conserved within integrase groups, making the gene an ideal target for PCR amplification. The integrase-defined grouping has a good discriminatory power, reflecting the diversity of the *S. aureus* phage population. Second, the integrase identification allows for prediction of the chromosomal location of the prophage. Last, the integrase type is closely linked to the virulence gene content of the prophage and might therefore convey information about the pathogenic potential of *S. aureus* (Goerke et al., 2009). Most of the *S. aureus* phages can be assigned to one of the eight major Sa-int families (Fig 3). Based on amino acid sequence homology and catalytic residues, most integrases belong to the tyrosine recombinase type family. Only the integrases of Sa7int phages

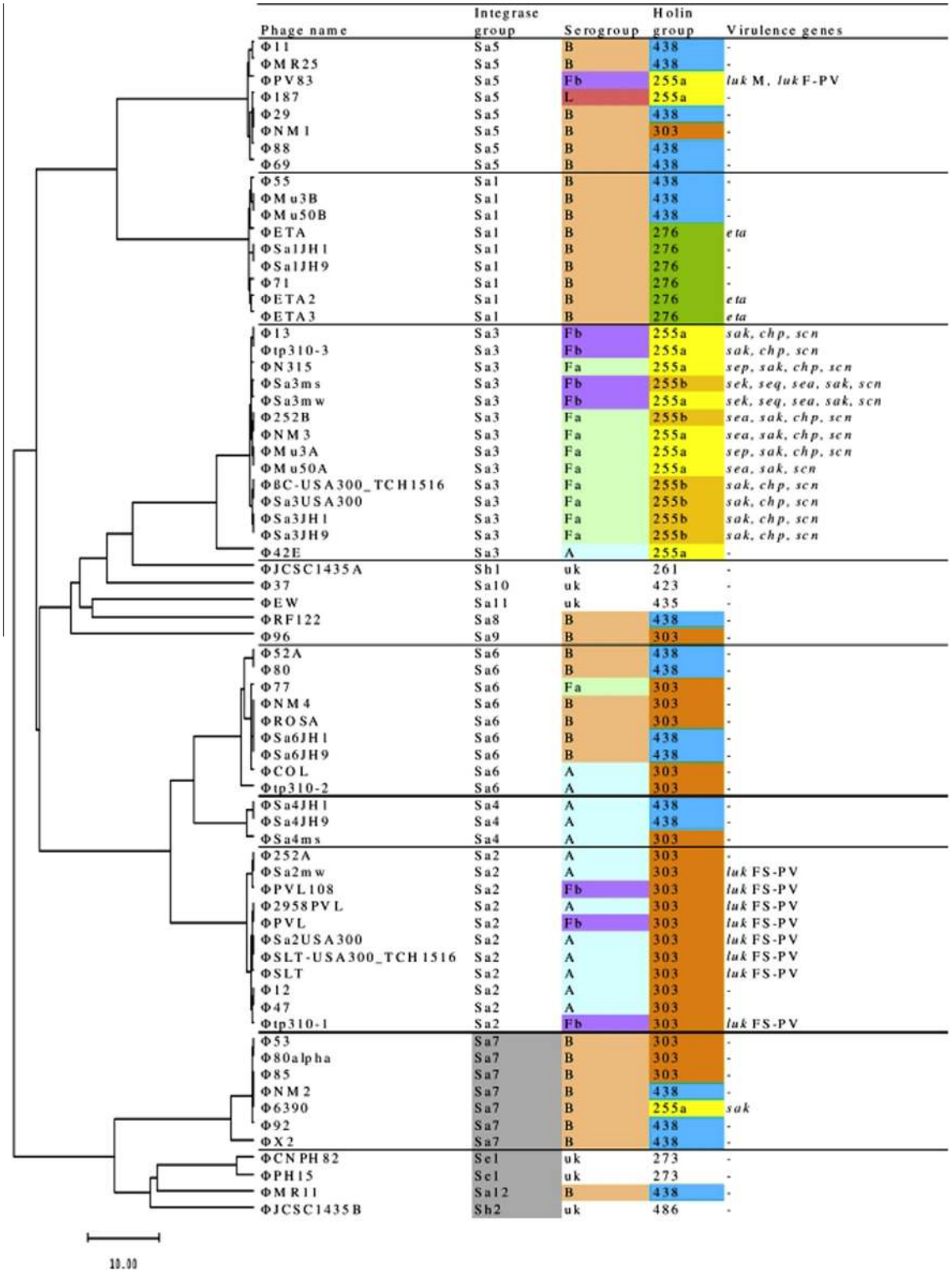

Fig. 3. Classification of *S. aureus* phages based on integrase serogroup and holin group reproduced from (Goerke et al., 2009).

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were found to belong to the serine recombinase type family (Goerke et al., 2009). Recently, one unusual phage with an *int* gene (not related to any of the main Sa-int groups of *S. aureus* but highly similar to Se-*int* from a ϕSPβ-like prophage of *Staphylococcus epidermidis*) was shown in emerging, highly epidemic MRSA strains (sequence type ST239) from Asia (Holden et al., 2010; Li et al., 2012). Additionally, the insertion site of this phage within SAR2132 (coding for a putative membrane protein) has thus far not been described for any *S. aureus* phage.

Despite the usually strong association of the integrase 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 (Goerke et al., 2006b). Under these conditions, the reconstitution of the phage-interrupted *hlb* gene may be of advantage. When these mislocated phages were induced and used to re-infect *S. aureus* in vitro, the phages reintegrated at their dedicated attachment site.

In general, prophages are vertically transferred with the bacterial host genome, and the integration site is tightly linked with the virulence repertoire conferred by the phage. Recombination and exchange of certain modules seem to occur more often within phages of the same Sa-int family than between different bacteriophage *int*-families (McCarthy et al., 2012b).

5. Phage-bacterial recognition

Phage adsorption to the *S. aureus* cell is the first critical step in phage replication. In spite of the importance of this event, the interaction partners are poorly characterised. Gram-positive cell envelopes feature a unique anionic glycopolymer, the peptidoglycan-anchored wall teichoic acid (WTA). WTA is one of the most abundant molecules of the outmost layer and functions as a phage receptor. The two major types of WTAs are either composed of repetitive 1,3-glycerol-phosphate (GroP) or 1,5-ribitol-phosphate (RboP), which are modified with sugar residues and alanyl groups (Weidenmaier and Peschel, 2008). Recent studies on the *Bacillus* siphovirus Spp1 revealed that adsorption of the phage to the host cell initially depends on reversible binding to GroP-type WTA, which accelerates the later irreversible binding to the membrane receptor YueB (Baptista et al., 2008). The tail spike protein GP21 of phage SPP1 binds to YueB and triggers the release of phage DNA (Vinga et al., 2012).

Most *S. aureus* strains express polyribitol phosphate WTA substituted with N-acetylglucosamine (GlcNAc) and D-alanine (Xia et al., 2010). Using isogenic mutants with altered WTA structures, we recently demonstrated that WTAs, but not LTAs, are required for siphovirus and myovirus infection of *S. aureus*. While the siphoviruses need the GlcNAc on WTA for adsorption, the myoviruses seem to adsorb to the backbone of WTA (Xia et al., 2011). Chen and Novick reported on the transduction of SaPIs from *S. aureus* to *Listeria monocytogenes* (Chen and Novick, 2009). Interestingly, some *L. monocytogenes* serotypes were known to produce WTA similar to that of *S. aureus* (Uchikawa et al., 1986), which may well serve as the adsorption receptor of the infectious SaPI particles and enable the intergeneric transfer of toxin genes.

6. Accessory phage genes as major determinants for *S. aureus* evolution

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. Positive lysogenic conversion of different virulence factors was described for the genes coding for Panton-Valentine leukocidin (*lukSF)* (Kaneko et al., 1998), exfoliative toxin A (*eta*) (Yamaguchi et al., 2001), the cell-wall anchored protein SasX (Li et al., 2012) and the immune evasion cluster (IEC) composed of enterotoxin S (*sea*), staphylokinase (*sak*), the chemotaxis inhibitory protein (*chp*) and the staphylococcal complement inhibitor (*scn*) (van Wamel et al., 2006). The disruption of chromosomal factors through phage integration was termed negative conversion, as exemplified by the integration of the Sa3int and Sa6int phages into the β-haemolysin (*hlb*) or lipase (*geh*) genes, respectively (Carroll et al., 1993; Lee and Iandolo, 1986). In most cases, phage conversion could be linked to specific disease outcomes.

6.1. Distribution and localisation of phage-encoded accessory factors

Interestingly, accessory genes are strongly associated with phages of certain *int* groups and are localised at the left or (more frequently) rightward ends of the phage (Fig 2). For instance, multiple alignments of several *lukSF* phages revealed a high mosaic structure of the phage genomes, but the *luk*SF genes were always located in a 6.4 kb region consisting of the host lysis module, *luk*SF, *att*P, and the *int* gene (mostly Sa2int) (Kahankova et al., 2010; Kaneko et al., 1998). The *eta* and *ear* genes are found at the end of Sa1int phages, and the immune evasion gene cluster (IEC)-containing genes *sea*, *sak*, *scn*, and *chp* are found at the end of Sa3int phages. There is a link between the encoded virulence factors and not only the *int* module but also the lytic module (holin and amidase genes), which is localised at the opposite end of the phages. One may assume that it is evolutionarily beneficial to interchange this whole unit, which is in proximity in the circular form of the phage. The close organisation of the lytic module and the inserted virulence factors is perhaps favoured to optimise the phage control of the expression of the pathogenicity genes (Sumby and Waldor, 2003). For instance, the expression of the virulence genes becomes co-transcribed with the late phage genes upon phage induction (Goerke et al., 2006a; Sumby and Waldor, 2003; Wirtz et al., 2009).

6.2. LukSF-carrying phages

LukSF-carrying phages are strongly associated with skin and soft tissue infection and necrotising pneumonia, which can affect young, immunocompetent persons (Shallcross et al., 2013; Vandenesch et al., 2003; Zanger et al., 2012). The phages may differ in the composition of their internal modules, and they typically integrate into one of two lineage-specific insertion sites (Boakes et al., 2011; Chen et al., 2012; Kaneko et al., 1998; Ma et al., 2008; Narita et al., 2001; Otter et al., 2010; Wirtz et al., 2009).

The combination of *lukSF*-phages with methicillin resistance is characteristic of community-associated MRSA strains that are spreading in different continents. Strains of the most prominent USA300 lineage (CC8 isolates) are mainly spreading in North America, whereas in Europe and Asia, CC80 strains and CC30 or CC59 strains are more prevalent, respectively. However, the most common feature of all these strains is the strong association of *lukSF* phages and superficial, recurrent skin infections (Shallcross et al., 2013). This trait might also be the reason for the success of these strains, as they are presumably more transmissible compared to nasal isolates. This hypothesis is supported by recent data indicating that skin and soft tissue infections serve as a source for bloodstream infections (Tattevin et al., 2012).

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6.3. Sa3int phages carrying the IEC (immune evasion cluster)

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 (Goerke et al., 2006b; Verkaik et al., 2011). The encoded immune modulatory proteins (Sea, Sak, Scin and Chips) may act together to resist the innate immune response encountered during nasal colonisation. The virulence factors of the immune evasion cluster are highly human specific, which is in good accordance with the observation that these phages are less prevalent in animal isolates (Verkaik et al., 2011) and are lost when *S. aureus* changes from human to animal hosts (McCarthy et al., 2012a; Price et al., 2012; Resch et al., 2013). Nevertheless, it could be shown in a mouse abscess model that curing of a Sa3int-phage (ΦNM3) in strain Newman resulted in a significant reduction in the ability of the strain to replicate in the liver (Bae et al., 2006).

Notably, most of the Sa3int phages remain inducible, leading to the complete restoration of functional Hlb. Hlb-positive strains are more frequently found among animal isolates (Verkaik et al., 2011) but are also more frequent in human strain collections composed of infectious isolates (Goerke et al., 2009). Furthermore, it could be demonstrated that Sa3int phages are readily induced under infectious conditions (Boyle-Vavra et al., 2011; Goerke et al., 2004; Goerke et al., 2006b; Jin et al., 2003; Peacock et al., 2002). Analysis of follow-up isolates from cystic fibrosis patients revealed that translocation of the Sa3int phages often leads to a splitting of the bacterial population (Goerke et al., 2006b) 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.

6.4. Sak containing Sa7int phages in ST5 MRSA strains

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 (Goerke et al., 2006b). The widely used phage-cured strain 8325-4 was somehow lysogenised with such a phage and has since then been distributed to different laboratories worldwide (designated as strain RN6390 or ISP479C). On the other hand, a very similar phage may have originated in the Tübingen region of Germany (Schulte et al., 2013). Sak-Sa7int phages are present in most of the prevalent, hospital-associated MRSA clones (belonging to the ST5 lineage) isolated in southern Germany. These strains were highly successful over years and are quickly diversifying as illustrated by differences in PFGE patterns and antibiotic susceptibility. Notably, this strain can be clearly discriminated from other CC5 isolates (ST225, a single-locus variant of ST5) circulating in Germany, which are characterised by the high prevalence of a typical Sa1int phage (Nubel et al., 2010; Schulte et al., 2013). The reason that the related lineages ST5 and ST225 differ with respect to local spreading has not yet been elucidated. In general, the reason for the high success of CC5 isolates as hospital-associated lineages is unclear, especially because data from whole-genome sequencing did not reveal any obvious mutations within the core genome that might be associated with this trait (Nubel et al., 2008). One may speculate that the accessory elements may drive the spreading of these strains because the most obvious difference is the phage content.

6.5. ETA-containing phages

The exfoliative toxins (ET) are virulence factors of *S. aureus* that causes bullous impetigo and its disseminated form, staphylococcal scalded-skin syndrome (SSSS). The ETA gene (*eta*) is carried in the genomes of Sa1int phages (Goerke et al., 2009; Kahankova et al., 2010; McCarthy et al., 2012b). However, these phages can be differentiated into at least six different types due to variation in different modules (Holochova et al., 2010). These *eta*-phages were associated with outbreaks of MRSA and MSSA strains of different CCs in Japan and the Czech Republic (Ruzickova et al., 2012; Shi et al., 2011; Yamaguchi et al., 2002) and are also present in a subpopulation of CC121 strains associated with superficial infections (Kurt et al., 2013). The clinical symptoms described for infections with strains harbouring ETA-phages varied from blisters anywhere on the body to multiple lesions complicated by conjunctivitis and SSSS (Ruzickova et al., 2012).

6.6. SasX in MRSA

The assumption that mobile genetic elements promote the spreading of bacterial clones was recently emphasised by the emergence of highly epidemic MRSA strains carrying a phage harbouring a new cell wall-anchored virulence factor, SasX (Li et al., 2012). SasX promotes nasal colonisation, bacterial aggregation and virulence. Again, *sasX*, similarly to other phage-carried virulence factors, is located as an accessory gene at the right end of a phiSPß-like prophage with a genome size of 127 kb, which is significantly larger than that of a typical siphovirus of *S. aureus* and highly similar to a prophage found in *S. epidermidis* strain RP62A (Holden et al., 2010), indicating that the phage and thus a new virulence trait was acquired from *S. epidermidis*. This phage is now spreading between *S. aureus* strains and is also found in MRSA strains of the CC5 lineage (Li et al., 2012). It remains to be elucidated if and how this phage and other genetic elements managed to cross the species barrier.

6.7. Phage-encoded virulence genes in animal isolates

There are now growing genome data available for *S. aureus* isolates from non-human mammals. These strains are adapted to particular species through changes in the core genome as well as new potential phage-encoded virulence genes whose functions are so far not clear (Guinane et al., 2011). Recently, it was shown that livestock-associated *S. aureus* strain isolates originated in humans (Price et al., 2012; Resch et al., 2013). Interestingly, some of the avian isolates carried a Sa3int-like phage with two putative avian-niche specific genes (Price et al., 2012).

7. 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 analysed phage-encoded virulence factors (Bronner et al., 2000; Kato et al., 2011; Rooijakkers et al., 2006). Furthermore, the two-component regulatory system, *saeRS*, and to a lesser extent the quorum-sensing system, *agr* (Dumitrescu et al., 2011; Kato et al., 2011; Rooijakkers et al., 2006; Wirtz et al., 2009), 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* (Rooijakkers et al., 2006; Wirtz et al., 2009). 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 clearly integrated into different regulatory circuits employed by the bacteria to survive within the hostile environment during infection.

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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 (Wagner and Waldor, 2002; Goerke et al., 2006a; Maiques et al., 2006). In addition, a soluble phage-inducing factor produced by human pharyngeal epithelial cells has been described (Broudy et al., 2002). It has been demonstrated that under such phage-inducing conditions, the transcription of the virulence factors that are localised in close proximity to the lysis module of the phage genome is increased (Goerke et al., 2006a; Sumby and Waldor, 2003; Wirtz et al., 2009). This phenomenon is partially due to a multi-copy effect caused by phage replication. However, it has also been shown that transcription becomes intimately linked to the phage genes through co-transcription with the now de-repressed lysis genes (Goerke et al., 2006a; Wirtz et al., 2009). In this regard, the use of antibiotics that induce the SOS response, such as quinolones or β-lactam antibiotics, is of special concern.

8. Phage dynamics: movement of the phage within and between staphylococcal species

Whereas most *S. aureus* isolates harbour multiple phages, less is known about the prevalence and nature of phages in coagulase-negative staphylococci. Analyses of the few available phage genome sequences for coagulase-negative staphylococci revealed a modular structure similar to that of *S. aureus* phages and indicated possible exchange of modules. The transfer of phages between different staphylococci is also supported by cluster analyses of phages from different staphylococcal species (Deghorain et al., 2012). Of note, CRISPR (clustered, regularly interspaced, short palindromic repeat) loci are present in some *S. epidermidis* strains but are lacking in *S. aureus*. These loci are involved in the recognition and cleavage of foreign DNA. Therefore, it was postulated that the gene flow is uni-directional (Otto, 2013), 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 SCCmec genomic islands that carry the *mecA* gene conferring resistance to methicillin at least occasionally originate from *S. epidermidis* (Wielders et al., 2001). 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 although the bacterial host is already eliminated through the action of the immune system or antibiotics. Such phages or transducing particles may then infect co-inhabitants, providing them with new properties.

Nevertheless, gene transfer between *S. aureus* strains is certainly much higher than between different staphylococcal species mainly because of phage receptor specificity and the restriction barrier. However, horizontal gene transfer is 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 has become 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 (Waldron and Lindsay, 2006). Thus, mobile genetic elements present in one strain will 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 (Waldron and Lindsay, 2006). Additional R-M systems described for *S. aureus* were shown to be phage-encoded, which may also contribute to phage exclusion (Dempsey et al., 2005). 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 (Goerke et al., 2009; McCarthy et al., 2012b). In certain CCs, some phage groups were completely absent, whereas others were 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% (Goerke et al., 2006b; Matthews and Novick, 2005; van Wamel et al., 2006). 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.

9. Conclusion and open questions

Most *S. aureus* strains carry several phages, some of which encode virulence genes. The phages are integrated at distinct chromosomal locations that are determined by the cognate *int* gene carried by the phage. The mobilisation of these phages is crucial for the short-term evolution of the bacterial species and for the emergence of new virulent *S. aureus* lineages. This is best exemplified by the multiple emergences and spreading of several distinct *S. aureus* strains carrying different *pvl*-containing phages. The circumstances leading to phage mobilisation and transfer in the in vivo situation (e.g., during certain infection or during colonisation) are only partially understood. There is some evidence that infectious conditions favour phage mobilisation (Goerke et al., 2004) and possibly transduction, and thereby contribute to accelerated evolution of the bacterial species. Restriction barriers and phage exclusion through receptor modifications limit the transfer between species and between strains of different CCs. These effects most likely play major roles in the species diversification of staphylococci, for which phage transduction is thought to be the primary mechanism of horizontal gene transfer. Thus, deeper insights into phage biology will be beneficial for the understanding of bacterial evolution. With new powerful molecular methods and sequencing technologies, old questions can be re-addressed. For instance, the molecular basis underlying the tight phage-bacterial interaction can be unravelled. However, it is already emerging that phages are far more versatile than previously thought based on the analysis of some model phages derived from *E. coli*. New mechanisms for integration, excision, and phage replication can be expected. Additionally, the mutual interaction between phage- and bacterial-encoded factors is only partially understood but is most likely quite common. Importantly, methods to analyse and quantify the three-partner interaction between phage, bacteria and the mammalian host should be established. By such means, the emergence of new virulent or resistant strains might be rendered more predictable.

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