An accessory wall teichoic acid glycosyltransferase protects Staphylococcus aureus from the lytic activity of Podoviridae

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Many Staphylococcus aureus have lost a major genetic barrier against phage infection, termed clustered regularly interspaced palindromic repeats (CRISPR/cas). Hence, S. aureus strains frequently exchange genetic material via phage-mediated horizontal gene transfer events, but, in turn, are vulnerable in particular to lytic phages. Here, a novel strategy of S. aureus is described, which protects S. aureus against the lytic activity of Podoviridae, a unique family of staphylococcal lytic phages with short, non-contractile tails. Unlike most staphylococcal phages, Podoviridae require a precise wall teichoic acid (WTA) glycosylation pattern for infection. Notably, TarM-mediated WTA α-O-GlcNAcylation prevents infection of Podoviridae while TarS-mediated WTA β-O-GlcNAcylation is required for S. aureus susceptibility to podoviruses. Tracking the evolution of TarM revealed an ancient origin in other staphylococci and vertical inheritance during S. aureus evolution. However, certain phylogenetic branches have lost tarM during evolution, which rendered them podovirus-susceptible. Accordingly, lack of tarM correlates with podovirus susceptibility and can be converted into a podovirus-resistant phenotype upon ectopic expression of tarM indicating that a “glyco-switch” of WTA O-GlcNAcylation can prevent the infection by certain staphylococcal phages. Since lytic staphylococcal phages are considered as anti-S. aureus agents, these data may help to establish valuable strategies for treatment of infections.

Horizontal gene transfer (HGT) events are prerequisites for bacterial evolution. Bacteria, including many Gram-positive pathogens, employ different mechanisms for the exchange of genetic information. Major mechanisms include bacteriophage- (phage) mediated transduction, conjugation, and transformation\textsuperscript{1,2}. These factors substantially contribute to bacterial evolution but vary in their impact depending on the bacterial species.

During evolution, many bacteria evolved various protective mechanisms that interfere with or impede HGT events. “Clustered regularly interspaced palindromic repeats” (CRISPR/cas) loci, for example, recognize invading DNA and confer bacterial adaptive immunity to phage infection\textsuperscript{1}. Other strategies to avoid HGT include restriction modification (R-M) systems, which most likely evolved in order to avoid
uptake of foreign DNA from sources other than the same or related bacterial species1,4,6. However, in many pathogenic bacteria including the major human pathogen Staphylococcus aureus, particular phage-mediated transduction is probably the most efficient and important mechanism to exchange genetic information8,9. Typically, S. aureus benefits from phage-mediated HGT events, since many staphylococcal phages mobilize resistance plasmids, genomic islands or other genomic loci with determinants of bacterial virulence9,10, thus substantially contributing to the evolution, pathogenicity, and global spread of this pathogen. Hence, protective mechanisms, which interfere with or even completely prevent phage infection and phage-mediated HGT events, can appear disadvantageous and maintain pathogens such as S. aureus in an evolutionary “dead-end.” Such a scenario is probably a reason for the emergence of phylogenetically isolated branches, as reported recently for the unique S. aureus lineage sequence type (ST) 395, which completely changed the phage adsorption receptor properties rendering it resistant from HGT with other S. aureus lineages11,12. However, such dramatic changes in the phage receptor properties are probably very rare among S. aureus clones and do not represent a frequent strategy to prevent phage adsorption or other phage-mediated HGT events.

Apart from ST395 isolates, which synthesize a unique glycerol-phosphate (GroP) WTA substituted with D-alanine and α-O-N-Acetylgalactosamine (GalNAc)13,12, most S. aureus clones synthesize a ribitol-phosphate (RboP) WTA repeating unit substituted with three tailoring modifications, D-alanine, α-O-N-acetylgalactosamine (GlcNAc), and β-O-GlcNAc13,14. The GlcNAc moieties are attached to RboP by two independent enzymes, the α-O-GlcNAc WTA glycosyltransferase TarM15, and the β-O-GlcNAc WTA transferase TarS16. Most S. aureus phages and phage-related S. aureus pathogenicity island (SaPI) particles target these WTA O-GlcNAc moieties for adsorption and subsequent infection15,16-17. Apparently, the stereochmical linkage of WTA glycosylation is dispensable for the phage infection process since strains lacking either one of the two WTA glycosyltransferases are still phage- or SaPI-particle susceptible11,16. In contrast, staphylococcal Myoviridae simply require WTA polymers, regardless of the polyol type or WTA O-GlcNAcylation11,12,17. Nevertheless, since WTA polymers have many other crucial functions in S. aureus pathogenesis and resistance13,14, most staphylococcal phages seem to be well-adapted to a rather conserved and important cell surface molecule, which S. aureus presumably does not mutate frequently. Accordingly, phage infection-preventing mutations in WTA biosynthesis genes have not been described so far. Thus, phage-mediated HGT events among S. aureus clones frequently occur and are rather beneficial for S. aureus evolution and adaptation to changing selection pressures, which is, conversely, also supported by the notion that many S. aureus clones if not all (as suggested by a recent in silico study18) have lost CRISPR/cas loci, which otherwise disable or even completely block HGT. Accordingly, staphylococcal phage protection mechanisms most likely evolved to prevent phage lysis, caused by lytic but not by transducing or beneficial phages.

Here, a novel strategy of S. aureus is described to prevent adsorption and infection of Podoviridae, a specific class of staphylococcal lytic phages with very short, non-contractile tails. This strain-specific barrier, which was lost by various S. aureus lineages during evolution, can completely block the Podoviridae infection process thereby providing new insights into bacterial strategies to counteract phage infections.

Results
Infection of S. aureus by Podoviridae is strain-dependent. Lytic S. aureus phages, for example staphylococcal Myoviridae, usually have a broad host-range and can even infect other staphylococcal species11,19. Accordingly, the broad host-range phages ΦK and Φ812 (Myoviridae) infected and lysed nearly all S. aureus test strains including strains of dominant MRSA lineages, albeit with different potencies (Table 1). However, a collection of another family of lytic staphylococcal phages (Podoviridae; here phages Φ44AHJD, Φ66 and ΦP68) failed to infect certain myovirus-susceptible strains, for instance the two American pandemic CA-MRSA clones USA300 (NRS384) and USA400 (MW2), and the HA-MRSA isolate 605, a member of the predominant Asian ST239 lineage (Table 1). However, a collection of another family of lytic staphylococcal phages (Podoviridae; here phages Φ44AHJD, Φ66 and ΦP68) failed to infect certain myovirus-susceptible strains, for instance the two American pandemic CA-MRSA clones USA300 (NRS384) and USA400 (MW2), and the HA-MRSA isolate 605, a member of the predominant Asian ST239 lineage (Table 1). Even though some test strains were susceptible to Podoviridae, these phages seem to have a narrower host-range than other lytic staphylococcal phages.

Podovirus-susceptible S. aureus strains were found among several clonal lineages suggesting that Podoviridae probably do not require an ST-specific receptor for adsorption and infection, as reported recently for the S. aureus ST395-specific phage Φ18711,12 (Table 1). In line with this notion, the strains PS44A, PS66, and P68 recommended for propagation of different podovirus strains20 were found to belong to different, unrelated STs, when they were multi locus sequence-typed (MLST) (Table 1). Thus, staphylococcal Podoviridae have a specific host-range different from that of other lytic staphylococcal phages such as Myoviridae.

Peptidoglycan-anchored surface proteins are dispensable for host specificity of Podoviridae. The specific host-range of Podoviridae suggests that these phages might fail to infect and lyse certain S. aureus strains due to unique barriers preventing adsorption, infection, or reproduction. Since the commonly used laboratory and podovirus-resistant S. aureus strain RN4220 (see Fig. 1 and Supplementary Fig. S1) lacks R-M systems, prophages, and CRISPR/cas loci previously shown to impede HGT, an intracellular barrier facilitating resistance to Podoviridae seems implausible. More likely, alterations in peptidoglycan modifications, for example specific cell-surface exposed molecules such as peptidoglycan-anchored ‘microbial surface components recognizing adhesive matrix molecules'
(MSCRAMMs), might block adsorption and infection in certain S. aureus. However, S. aureus RN4220 mutants and mutants derived from the clinical CA-MRSA isolate USA300 lacking functional surface proteins (ΔsrtA) were resistant to Podoviridae indicating that factors other than MSCRAMMs interfere with the podovirus infection process (Supplementary Fig. S1).

Thus, S. aureus peptidoglycan-anchored surface proteins do not influence the unusual host-range of staphylococcal Podoviridae.

The S. aureus α-O-GlcNAc WTA glycosyltransferase TarM prevents the lytic activity of Podoviridae. Because all studied staphylococcal phages require WTA polymers or O-GlcNAcylated WTA polymers for adsorption and infection17, adsorption of Podoviridae to their designated cell surface receptors may also be influenced by WTA polymers. Of note, all podovirus-susceptible strains were simultaneously susceptible to the WTA-dependent phages ΦK and Φ812, which excludes that podovirus-susceptible strains fail to produce WTA polymers (Table 1). In line with this assumption, Podoviridae still failed to adsorb to and infect S. aureus RN4220 or USA300 mutants lacking either WTA (ΔtagO) or WTA glycosylation (ΔtarMΔtarS) (Fig. 1a,b).

While well-studied WTA-GlcNAc dependent S. aureus phages such as phage Φ11 do not seem to require a specific stereochemistry of WTA O-GlcNAc for infection14 the tested podoviruses exhibited an unexpected preference for TarS-glycosylated but not TarM-glycosylated WTA. Strikingly, lack of WTA α-O-GlcNAcylation (ΔtarM) resulted in dramatically increased binding capacities of phage ΦP68 and rendered strain RN4220 ΔtarM highly susceptible to podovirus infection (Fig 1a,b). In contrast, lack of tarS did not lead to phage susceptibility of RN4220 (Fig. 1a). Complementation of the WTA-glycosylation deficient ΔtarMΔtarS mutant with one of the two S. aureus WTA glycosyltransferases TarM or TarS demonstrated that, (i) Podoviridae require TarS-mediated WTA β-O-GlcNAcylation, but (ii) are inhibited by TarM-mediated WTA β-O-GlcNAcylation (Fig 1a,b). Similar results were obtained for S. aureus USA300 strongly suggesting that TarM diminishes the adsorption and infection of Podoviridae to S. aureus (Fig. 1a,b). Because TarM is an intracellular protein it appears highly unlikely that it interferes with podovirus binding directly but impedes podovirus binding by α-O-GlcNAcylated WTA.

Thus, the α-O-GlcNAc WTA glycosyltransferase TarM prevents the adsorption and infection by staphylococcal Podoviridae.

Lack of tarM correlates with susceptibility to Podoviridae. In order to confirm the inhibitory effect of TarM on podovirus susceptibility, genomes of S. aureus test strains were screened for the presence of tarM and tarS. A ΔtarM mutant with one of the two S. aureus WTA glycosyltransferases TarM or TarS demonstrated that, (i) Podoviridae require TarS-mediated WTA β-O-GlcNAcylation, but (ii) are inhibited by TarM-mediated WTA β-O-GlcNAcylation (Fig 1a,b). Similar results were obtained for S. aureus USA300 strongly suggesting that TarM diminishes the adsorption and infection of Podoviridae to S. aureus (Fig. 1a,b). Because TarM is an intracellular protein it appears highly unlikely that it interferes with podovirus binding directly but impedes podovirus binding by α-O-GlcNAcylated WTA.

Thus, the α-O-GlcNAc WTA glycosyltransferase TarM prevents the adsorption and infection by staphylococcal Podoviridae.

### Table 1. Lack of tarM in S. aureus correlates with susceptibility to Podoviridae.

| S. aureus strain | Sequence type | tarM | tarS | Phage susceptibility
|------------------|---------------|------|------|----------------------|
|                  |               |      |      | Myoviridae | Podoviridae |
|                  |               |      |      | ΦK | Φ812 | Φ44AHJD | Φ66 | ΦP68 |
| MW2              | 1             | +    | +    | +  | +  | — | — | — |
| Mu50             | 5             | —    | +    | (+) | +  | +  | +  | +  |
| USA300           | 8             | +    | +    | +  | —  | — | — | — |
| NRS184           | 22            | —    | +    | (+) | +  | +  | +  | +  |
| P68              | 25            | —    | +    | (+) | (+) | — | — | — |
| UAMS-1           | 30            | +    | +    | +  | —  | — | — | — |
| PS66             | 39            | +    | +    | +  | +  | +  | +  | +  |
| USA600           | 45            | —    | +    | (+) | —  | — | — | — |
| JH1              | 105           | —    | +    | +  | +  | +  | +  | +  |
| ED133            | 133           | —    | —    | +  | (+) | — | — | — |
| RF122            | 151           | +    | +    | +  | +  | +  | +  | +  |
| 605              | 239           | +    | +    | (+) | (+) | — | — | — |
| Col              | 250           | +    | +    | +  | —  | — | — | — |
| PS187*           | 395           | —    | —    | +  | —  | — | — | — |
| 82086            | 398           | —    | +    | +  | +  | +  | +  | +  |
| PS44A            | 707           | —    | +    | +  | +  | +  | +  | +  |

*PS187 synthesizes a poly-glycerol phosphate WTA type modified with α-O-N-Acetylgalactosamine (mediated by the ST395-specific WTA glycosyltransferase TagN2). Phage susceptibility was analyzed via soft agar overlay method. Phage susceptibility (+) or resistance is indicated (−). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—). Diminished plaque formation (+) or resistance is indicated (—).
or absence of the genes encoding WTA glycosyltransferases TarM and TarS via PCR or BLASTN of available genomes21. Most strains contained TarS except for strains PS187, which produce an entirely different type of WTA 11,12, and ED133, which does not encode any of the so far described WTA glycosyltransferases (Table 1). In contrast, several strains lacked TarM. As proposed, most TarM- plus TarS-encoding S. aureus strains were podovirus-resistant (Table 1). Conversely, S. aureus wild type and strains lacking WTA (ΔtagO), WTA α-O-GlcNAcylation (ΔtarM), WTA β-O-GlcNAcylation (ΔtarS), WTA glycosylation (ΔtarM ΔtarS), and the complemented mutants (ΔtarM ΔtarS pRB474-tarM, ΔtarM ΔtarS pRB474-tarS) are indicated. Values are given as means and standard deviations (SD, n = 3). Statistical significant differences calculated by one-way ANOVA with Bonferroni’s multiple comparison test are indicated: not significant (ns), P > 0.05; *P < 0.05, **P < 0.01.

Figure 1. The α-O-GlcNAc WTA glycosyltransferase TarM protects S. aureus from the lytic activity of Podoviridae. (a) S. aureus RN4220 and USA300 susceptibility to the broad-host-range lytic phage ΦK (Myoviridae), and to the lytic phages Φ44AHJD, Φ66 and ΦP68 (Podoviridae) was analyzed using a soft-agar overlay approach. A representative experiment is shown. (b) Podovirus ΦP68 adsorption rates (%) to S. aureus RN4220 and USA300 variants. S. aureus wild type and strains lacking WTA (ΔtagO), WTA α-O-GlcNAcylation (ΔtarM), WTA β-O-GlcNAcylation (ΔtarS), WTA glycosylation (ΔtarM ΔtarS), and the complemented mutants (ΔtarM ΔtarS pRB474-tarM, ΔtarM ΔtarS pRB474-tarS) are indicated. Values are given as means and standard deviations (SD, n = 3). Statistical significant differences calculated by one-way ANOVA with Bonferroni’s multiple comparison test are indicated: not significant (ns), P > 0.05; *P < 0.05, **P < 0.01.
Figure 2. Point mutations in TarM render \( \Phi 66 \) propagation strain PS66 susceptible to Podoviridae.

(a) A sequence alignment of wild-type TarM and PS66 TarM is shown. Position of mutations (Gln-453 with Lys; Ala-464 with Glu) and the end of the open reading frame (493) are indicated. (b) \( S. aureus \) RN4220 susceptibility to the broad host-range lytic phage \( \Phi K \) (Myoviridae), and to the lytic phages \( \Phi 44 \), \( \Phi 66 \), and \( \Phi P 68 \) (Podoviridae) was analyzed using a soft-agar overlay approach. \( S. aureus \) RN4220 wild type and strains lacking WTA \( \alpha-O\)-GlcNAcylation (\( \Delta tarM \)), and the complemented mutants (\( \Delta tarM \) pRB474-\( tarM \), \( \Delta tarM \) pRB474-\( tarM \) (Q453K; A464E)) are indicated. A representative experiment is shown.

(Supplementary Fig. S4) suggesting that podovirus sensitivity of PS66 is linked to \( tarS \)-mediated \( \beta-O\)-GlcNAcylated WTA and to a strain-specific dysfunction of TarM.

Next, \( tarM \) was expressed in various podovirus-susceptible strains, including the \( \Phi 44 \) and \( \Phi 66 \) propagation strains PS44A and PS66. Even at very high phage titers, expression of \( tarM \) rendered most susceptible strains completely resistant, confirming the importance of \( tarM \) in diminishing infection by staphylococcal Podoviridae (Fig. 3). In addition, the expression of a plasmid-born copy of \( tarM \) in strain PS66 also caused complete resistance to Podoviridae, further suggesting that the \( tarM \) gene of PS66 is most likely non-functional or less active (Fig. 3).

Thus, Podoviridae require \( \beta-O\)-GlcNAcylated WTA but cannot infect \( S. aureus \) with \( \alpha-O\)-GlcNAcylated WTA.

Tracking the evolution of TarM reveals an ancient origin in other staphylococcal species and vertical inheritance during \( S. aureus \) evolution. TarM is encoded outside of the \( S. aureus \) WTA gene clusters but does not appear to be encoded on a mobile genetic element\(^2\). Nevertheless, it is tempting to assume that it has been acquired by \( S. aureus \) at some point in evolution to interfere with podovirus infection.

To track the emergence of TarM in \( S. aureus \), the genome sequences of 98 \( S. aureus \) strains including those of most \( S. aureus \) laboratory test strains used in this study were obtained to infer their genetic relatedness (Fig. 4a,b). Of note, the presence of \( tarM \) in the most deeply branching \( S. aureus \) isolates MSHR1132 and FSA084, which were recently proposed as novel staphylococcal species \( Staphylococcus argenteus \) sp. nov. and \( Staphylococcus schweitzeri \) sp. nov.\(^2\), revealed that the presence of \( tarM \) is probably an ancient genetic trait of \( S. aureus \) (Fig. 4a). Still, homologues of \( tarM \) are also encoded by certain coagulase-negative staphylococci (e.g. specific \( S. epidermidis \) isolates) and even by non-staphylococcal species such as \( Exiguobacterium oxitolerans \) and \( Tetragenococcus halophilus \). Thus, the early evolution of \( tarM \) probably involved an ancient HGT event to the last common ancestor of contemporary \( S. aureus \) clones, further supported by the notion that \( tarM \) is flanked by a gene possibly related to conjugation (SACOL1042) (Fig. 4c). However, at a later stage of \( S. aureus \) evolution, different types of genetic rearrangements occurred in emerging phylogenetic branches such as CC5 or CC398, leading to a deletion of \( tarM \), which rendered these podovirus-susceptible (Fig. 4c).

Discussion

Staphylococcal Podoviridae infect an unusually wide panel of staphylococcal species but remain avirulent for certain \( S. aureus \) lineages probably as a result of the activity of the \( \alpha-O\)-GlcNAc WTA glycosyltransferase TarM. In \( tarM \)-encoding strains, WTA polymers are probably glycosylated preferentially with \( \alpha-O\)-GlcNAc, suggesting that TarM might be more active than TarS. Consequentially, TarS-mediated \( \beta-O\)-GlcNAcylation is probably affected by the activity of TarM, thus preventing the adsorption and infection of Podoviridae. Even though it cannot be excluded that TarM potentially has additional and undiscovered functions, which may interfere with the adsorption or infection process, the drastically increased adsorption of \( \Phi P 68 \) in isogenic \( \Delta tarM \) mutants suggests that \( \alpha-O\)-GlcNAcylated WTA prevents the adsorption of Podoviridae to \( S. aureus \). Nevertheless, one of the designated podovirus propagation strains
PS66) encoded both WTA glycosyltransferases suggesting that certain strains, despite encoding tarM, are potentially podovirus-susceptible. Here, TarM might be non-functional, dis-regulated, or mutated as observed in PS66, and cannot interfere with the activity of TarS. Nevertheless, this TarM-mediated phenomenon limits the host-range of Podoviridae, and thus, their therapeutic potential compared to other lytic staphylococcal phages such as Myoviridae.

Apart from this, it remains intriguing as to why certain strains and lineages have lost tarM during evolution to become podovirus-susceptible. Since both S. aureus and S. aureus-like species such as S. schweitzeri and S. argenteus encode tarM and tarS, and many human-associated S. aureus lineages have lost tarM during evolution, it can be assumed that tarM is probably not essential for continued adaptation to the human host. This is in agreement with the observation that both types of WTA O-GlcNAcylation, can mediate S. aureus binding to nasal epithelial cells and thus nasal colonization 24. Also, human sera contain preferentially serum antibodies directed against TarS-dependent β-O-GlcNAcylated WTA, but not against TarM-mediated α-O-GlcNAcylated WTA25, suggesting that tarM may be down-regulated or less immunogenic than β-O-GlcNAcylated WTA during infections. It can be assumed that some S. aureus lineages did not eliminate tarM because WTA α-O-GlcNAcylation may provide S. aureus with a fitness benefit, whose basis remains to be identified in the future.

However, bearing tarM and TarM-mediated α-O-GlcNAcylated WTA protects S. aureus at least against the lytic activity of staphylococcal Podoviridae via a modification of the designated phage adsorption receptor. Such alterations of cell-surface structures serving as viral receptors are only one of many bacterial strategies to counteract phage infection and have also been described for other bacterial species26–28, but does not seem a general strategy of S. aureus to avoid phage adsorption and infection. Since other lytic staphylococcal phages such as Myoviridae are capable of infecting tarM-encoding S. aureus isolates, prevention of podovirus infection could be the result of a highly specific WTA-dependent mechanism in S. aureus, presumably as the result of adaptation to specific podovirus-rich environmental niches. In addition, altered phage-receptor binding proteins may easily change the host-range of Podoviridae to render tarM-bearing clones susceptible. Whereas bacterial phage resistance mechanisms such as CRISPR interference appear more efficient and widespread in prokaryotes these can also be

**Figure 3.** Ectopic expression of TarM protects podovirus-susceptible S. aureus against Podoviridae. The α-O-GlcNAc WTA glycosyltransferase TarM was ectopically expressed in various tarM-lacking and podovirus-susceptible S. aureus strains, and the phage susceptibility using a phage panel encompassing the lytic phages Φ44AHJD, Φ66 and ΦP68 (Podoviridae) was analyzed using a soft-agar overlay approach. Various dilutions of phage lysates, S. aureus wild type strains (tarS positive, but tarM negative (or encoding a mutated tarM, strain PS66)), and engineered strains expressing tarM (pRB474-tarM), or empty plasmid control (pRB474) are indicated. A representative experiment is shown.
bypassed, for example, by CRISPR-evading phages\textsuperscript{29} suggesting that host-virus interaction is a constantly evolving process.

**Methods**

**Bacterial strains and growth conditions.** All bacterial strains used in this study are listed in Supplementary Table S1. Unless otherwise noted, bacteria were grown in basic medium (BM) (1\% tryptone, 0.5\% yeast extract, 0.5\% NaCl, 0.1\% K\textsubscript{2}HPO\textsubscript{4}, 0.1\% glucose) or lysogeny broth (Becton Dickinson) supplemented with appropriate antibiotics (Chloramphenicol 10\(\mu\)g/ml, Ampicillin 100\(\mu\)g/ml).

**Molecular genetic methods.** *S. aureus* RN4220 and USA300 \(\Delta\)tarM, \(\Delta\)tarS, \(\Delta\)tarM \(\Delta\)tarS, and \(\Delta\)tagO deletion mutants were described elsewhere\textsuperscript{11,16,24}. For the construction of marker-less RN4220 \(\Delta\)srtA mutant, or a PS66 \(\Delta\)tarS mutant, the previously described *E. coli/S. aureus* shuttle vectors pIMAY or pKOR1 were used\textsuperscript{30,31}. The corresponding primers are listed in Supplementary Table S2. Gene disruption by using pKOR1 or pIMAY was performed as described before\textsuperscript{30,31}. Briefly, pKOR1-tarS, or pIMAY-srtA were isolated from an appropriate *E. coli* strain, and transformed into electrocompetent *S. aureus* RN4220 cells (and reisolated and transformed into PS66). Electroporation conditions were described before\textsuperscript{11}. Knock-out plasmids were integrated onto the *S. aureus* genome at the permissive temperatures (37\(^\circ\)C, pIMAY; 43\(^\circ\)C, pKOR1) and in the presence of chloramphenicol (10\(\mu\)g/ml). Counterselection was performed by using anhydrotetracycline (1\(\mu\)g/ml). Resulting colonies were patched onto BM agar plates with and without chloramphenicol (10\(\mu\)g/ml) and screened for plasmid loss. Gene deletion was confirmed via PCR in chloramphenicol-sensitive colonies.

For complementation studies (or tarM expression in tarM-lacking strain backgrounds), the previously described *E. coli/S. aureus* shuttle vector pRB474 was used\textsuperscript{12}. pRB474-tarM (Q453K; A464E) has been described elsewhere (formerly pRB474-H-tarM)\textsuperscript{15}.

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**Figure 4.** Phylogenetic distribution of tarM reveals an ancient origin in other staphylococci and vertical inheritance during *S. aureus* evolution. (a,b) Phylogenetic network representing the inferred relationship of 98 *S. aureus* strains and two closely related species, *S. argenteus* and *S. schweitzeri*. Strains are indicated by their multilocus sequence types (STs). ST\(^{*}\) and ST\(^{**}\) are single-locus variants of ST30 and ST1148, respectively. Strains encoding tarM are indicated in black, while strains lacking tarM are indicated in red, purple, and blue. (c) Genetic organization of the tarM region in *S. aureus*. The intact tarM region is shown in the upper cluster. Gene locus numbers refer to *S. aureus* strain COL (GenBank accession no. CP000046). Lower clusters indicate distinct deletion events involving tarM.
PCR-typing, sequencing, and multiple locus sequence typing (MLST). For verification (and sequencing) of tarM and tarS in S. aureus genomes, PCR analysis using primers listed in Supplementary Table S2 was used. MLST typing of podovirus propagation strains PS44A, PS66 and P68 was performed as described previously using published primers33.

Experiments with phages. All phages used in this study are listed in Supplementary Table S1. Phages were propagated on S. aureus strains P68 or RN4220 ΔtarM (Φ44AHJD, Φ66 and ΦP68), or RN4220 wild type (ΦK, Φ812) as described previously34. Briefly, the cognate S. aureus host strains were grown overnight at 37°C in BM and diluted in phage-containing lysates (approximately 1 × 10^9 plaque forming units (PFU) per millilitre; titrated on cognate host strains) to a final optical density OD 600 nm of 0.4. Subsequently, CaCl_2 was added to a final concentration of 4 mM. The bacteria/phage mixture was incubated for 30 min at 37°C without agitation and afterwards for at least 3 h at 30°C with mild agitation until complete lysis occurred. In order to remove cell debris, the lysate was centrifuged for 10 min (5,000 × g, 4°C). Lysates were filter-sterilized (0.22 μm) and stored at 4°C.

Phage susceptibility was analyzed as described elsewhere17. Briefly, a phage panel encompassing the broad host-range phages ΦK and Φ812 (Myoviridae), and three serogroup G phages Φ44AHJD, Φ66 and ΦP68 (Podoviridae) were used. 6 μl (approximately 1 × 10^9 PFU/ml, or appropriate dilutions) of freshly propagated phage lysates were spotted onto bacterial lawns using the soft-agar overlay method described by Xia et al.17.

Phage adsorption to S. aureus strains was analyzed as described previously17. Briefly, the phage adsorption rate was analyzed using a multiplicity of infection (MOI) of 0.01 for phage ΦP68. Adsorption rate (%) was calculated by determining the number of unbound PFU in the supernatant and subtracting from the total number of input PFU as a ratio to the total number of input PFU.

Phylogenetic analysis. The chromosomes of all S. aureus and S. argenteus and S. schweitzeri labelled as complete were obtained from GenBank (Supplementary Table S3) and aligned against the chromosome of S. aureus CC45 strain CA-347 (GenBank accession ID NC_021554) after identification and deletion of duplicated regions using MUMmer v 3.235. The 98 publicly available genomes were aligned using MUMmer. Based on the identified core of ~1,9 Mb (~67%) among all strains, a total of 312,427 SPNs was identified, from which the phylogenetic relationship was inferred using the NeighbourNets algorithm in SplitsTree v4.13.1.36.

RNA isolation and preparation. RNA was isolated as described previously24. Briefly, BM over-night cultures were diluted in BM. Bacteria were grown at 37°C until lag, log, or stationary growth phases. Subsequently, bacteria were harvested and resolved in 1 ml TRIzol (Invitrogen/Life Technologies, Carlsbad, CA, USA). Total RNA was isolated according to the manufacturer’s instructions and stored at −80°C.

RNA was transcribed into cDNA and qRT-PCR was performed according to the manufactures instructions using the Brilliant II SYBR Green 1-Step Master Mix (Agilent). Relative quantifications were analyzed by using Roche’s LightCyler480II. Transcription levels of target genes analyzed in this study were normalized against the expression of the housekeeping gene gyrB. All primers used for qRT-PCR are listed in Supplementary Table S2.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla USA, Version 5.04). Statistically significant differences were calculated by using appropriate statistical methods as indicated. P values < 0.05 were considered significant.

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Designed and supervised this study: X.L., A.P. and V.W. Performed the experiments: X.L., D.G., X.D., J.L., M.S., P.K., G.X. and V.W. Analyzed the data: X.L., D.G., X.D., J.L., M.S., P.K., G.X., A.P. and V.W. Wrote the manuscript: A.P. and V.W.

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