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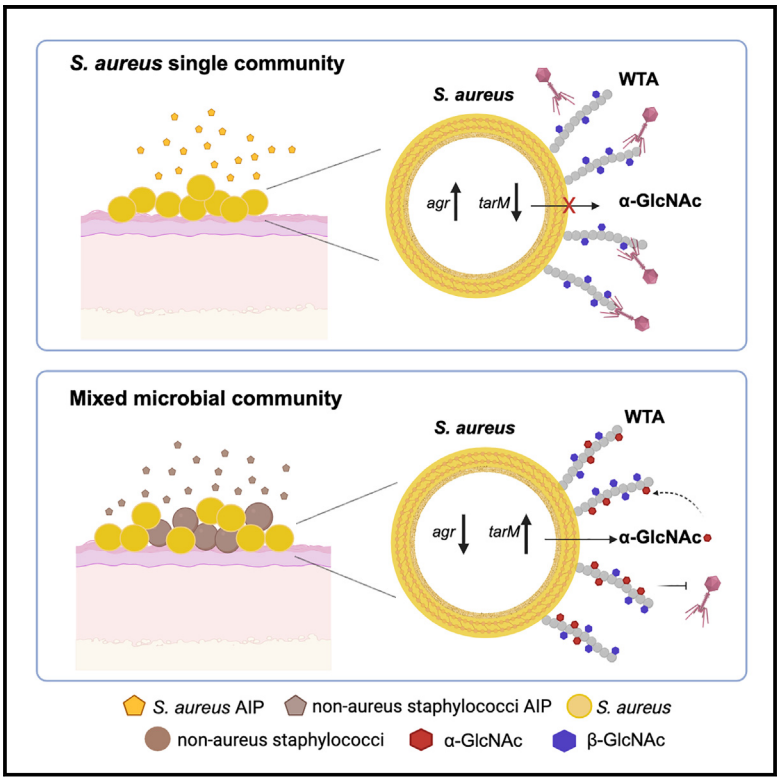
Cross-species communication via *agr* controls phage susceptibility in *Staphylococcus aureus*

Graphical abstract



Highlights

- Lytic phage Stab20 infects *S. aureus* when *agr* quorum sensing is induced
- Quorum-sensing induction reduces *tarM*-encoded α-GlcNAc glycosylation of WTA
- α-GlcNAc glycosylated WTA hinders infection of Stab20 and related phages
- Non-aureus staphylococci protect *S. aureus* from Stab20 killing by *agr* interference

Authors

Jingxian Yang, Janine Zara Bowring, Janes Krusche, ..., Tom Grunert, Andreas Peschel, Hanne Ingmer

Correspondence

hi@sund.ku.dk

In brief

Yang et al. show that α-GlcNAc glycosylation of wall teichoic acids is reduced by *agr* quorum-sensing induction in *S. aureus*. This reduction allows infection by the lytic phage Stab20. In mixed communities with non-aureus staphylococci producing inhibitory quorum-sensing auto-inducing cyclic peptides, *agr* interference protects *S. aureus* from infection.

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Cross-species communication via *agr* controls phage susceptibility in *Staphylococcus aureus*

Jingxian Yang,[1] Janine Zara Bowring,[1] Janes Krusche,[2,3] Esther Lehmann,[1] Benjamin Svejdal Bejder,[4] Stephanie Fulaz Silva,[1] Martin Saxtorph Bojer,[1] Tom Grunert,[5] Andreas Peschel,[2,3] and Hanne Ingmer[1,6,*]

[1]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Copenhagen, Denmark

[2]Department of Infection Biology, Interfaculty Institute for Microbiology and Infection Medicine Tübingen (IMIT), University of Tübingen, 72076 Tübingen, Germany

[3]Cluster of Excellence "Controlling Microbes to Fight Infections (CMFI)," German Center for Infection Research (DZIF), Tübingen, Germany

[4]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark

[5]Functional Microbiology, Institute of Microbiology, Department of Pathobiology, University of Veterinary Medicine, 1210 Vienna, Austria

[6]Lead contact

*Correspondence: hi@sund.ku.dk

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SUMMARY

Bacteria use quorum sensing (QS) to coordinate group behavior in response to cell density, and some bacterial viruses (phages) also respond to QS. In *Staphylococcus aureus,* the *agr*-encoded QS system relies on accumulation of auto-inducing cyclic peptides (AIPs). Other staphylococci also produce AIPs of which many inhibit *S. aureus agr*. We show that *agr* induction reduces expression of *tarM*, encoding a glycosyltransferase responsible for α-*N*-acetylglucosamine modification of the major *S. aureus* phage receptor, the wall teichoic acids. This allows lytic phage Stab20 and related phages to infect and kill *S. aureus*. However, in mixed communities, producers of inhibitory AIPs like *S. haemolyticus*, *S. caprae*, and *S. pseudintermedius* inhibit *S. aureus agr*, thereby impeding phage infection. Our results demonstrate that cross-species interactions dramatically impact phage susceptibility. These interactions likely influence microbial ecology and impact the efficacy of phages in medical and biotechnological applications such as phage therapy.

INTRODUCTION

Bacterial viruses, the bacteriophages (or phages), have received renewed interest as antimicrobial options for treating infections with antibiotic-resistant pathogens. Phages are classified as lytic or temperate with the former infecting and killing bacteria, while the latter also are able to integrate into and replicate with the bacterial genome. In phage therapy, lytic phages are employed to eradicate infections, and historically, there have been examples of life-threatening infections being cured with phages.[1] Yet, phage therapy can fail, for example, due to phage resistance.[2]

One of the pathogens for which phage therapy has been applied is *Staphylococcus aureus*.[3,4] It is an opportunistic pathogen that on the one hand colonizes a third of the human population, asymptomatically, but it also can cause a variety of serious infections.[5] Transition from colonization to infection is in part controlled by the *agr* quorum-sensing (QS) system composed of a two-component response regulator and sensory histidine kinase-sensing, *agr*-encoded, auto-inducing, cyclic peptides (AIPs). In response to cell density and AIP concentration, *agr* mediates the transition from expression of *S. aureus* adhesion and colonization factors at low cell densities to expression of toxins and extracellular enzymes at high cell densities.[6] Curiously, *S. aureus* strains encode one of four *agr* variants (I–IV), where a cognate AIP is required for *agr* induction, while some non-cognate AIPs prevent *agr* induction. AIPs are also produced by a variety of non-aureus staphylococci (NAS) that colonize humans and animals, and a substantial part of these interfere with induction of *S. aureus* QS, including those from *S. epidermidis, S. hominis, S. haemolyticus, S. caprae*, and *S. pseudintermedius*.[7–10] These interactions are likely to be key for both *S. aureus* colonization and infection.[11–14]

The main receptors for *S. aureus* phages are the wall teichoic acid (WTA) glycopolymers that decorate the cell surface by being attached to the cell wall.[15] WTA can be modified by alanylation and glycosylation. While both types of modification are important for interaction with the innate and adaptive immune systems, so far only the latter has been implicated in phage binding.[16] For example, most myophages recognize the WTA backbone regardless of glycosylation,[15,17] whereas α- or β-glycosylated WTA is essential for infection by siphophages and podophages[15,18–20] as well as for a few myophages.[21,22]

WTA synthesis involves initiation (TarO), priming (TarABDF), and polymerization (TarIJLK) of repeating ribitol phosphate (RboP) units, and glycosylation is catalyzed by TarM and TarS

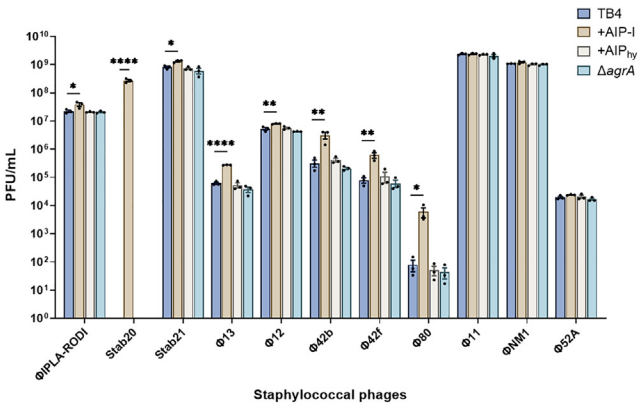

Figure 1. Impact of *agr* on phage infection of *S. aureus*

Overnight cultures of TB4 and TB4Δ*agrA* were diluted to OD_{600} of 0.01 where TB4 was either untreated or supplemented with 0.1 μM AIP-I (inducing) or AIP_{hy} (inhibitory), and all were grown to OD_{600} of 0.35 where cultures were infected with indicated phages. Plaque-forming units (PFUs) were counted by the full plate plaque assay. Data are shown as the mean ± SEM (n = 3). Statistical analysis was done by one-way ANOVA with Dunnett's test for multiple comparison. *p < 0.05, **p < 0.01, ****p < 0.0001.

that add *N*-acetylglucosamine (GlcNAc) to the C4 position of the RboP unit in an α or β configuration, respectively.[23] In addition, some phages encode TarP, which modifies the C3 position with a β-*O* linkage.[24] After synthesis, the WTA is translocated to the cell surface by the ABC transporter, TarGH, where it is further decorated with D-alanine and anchored to the peptidoglycan by the LcpABC family of proteins.[23]

Here, we have examined how *S. aureus agr* affects phage susceptibility, and we find that successful infection by a subset of lytic myophages including Stab20 relies on *agr* induction, resulting from decreased expression of α-glycosylation of the WTA. This demonstrates that *agr* controls phage infections and alters WTA glycosylation patterns. Further, we show that in mixed communities with NAS producing inhibitory AIPs, *S. aureus* is protected from infection by Stab20 and related phages. Thus, our findings indicate that there is a delicate balance between the ubiquitous NAS and *S. aureus* that impacts not only *S. aureus* virulence factor production but also WTA-mediated immune responses, phage susceptibility, and with that entire microbial community. In perspective, our findings may add yet another aspect to how communities can influence susceptibility of bacteria to phages and, thus, may explain why phage therapy occasionally fails.

RESULTS

Induction of *agr* promotes phage infection of *S. aureus*

To explore whether the *S. aureus agr* QS system affects susceptibility to phage infections, we examined a collection of phages including temperate phages belonging to serogroup B (ϕ11, ϕNM1, ϕ80, and ϕ52A), serogroup Fb (ϕ13), and serogroup A (ϕ12, ϕ42b, and ϕ42f) as well as the lytic phages (ϕIPLA-RODI, Stab20, and Stab21) belonging to serogroup D. These phages were used to infect exponentially growing cells of TB4 (a prophage-cured *S. aureus* derivative of strain Newman), an *agrA* deletion mutant, as well as TB4 cells treated with synthetic AIPs that either induce (AIP-I) or inhibit (*S. hyicus*, AIP_{hy}) *S. aureus* QS.[10] In comparison with untreated TB4 cells, we found that addition of AIP-I increased susceptibility to a variety of both lytic and temperate phages, namely ϕIPLA-RODI, Stab20, Stab21, ϕ13, ϕ12, ϕ42b, ϕ42f, and ϕ80, with the greatest effect being on Stab20 infection. For Stab20, we only observed infection when cells were grown in the presence of AIP-I prior to phage infection, whereas no infection was observed for untreated cells, cells lacking *agrA*, or cells treated with the inhibitory AIP_{hy} (Figure 1). The effect of AIP-I on *S. aureus* susceptibility to Stab20 infection was further confirmed in a liquid infection assay where only AIP-I-treated TB4 cells were killed by the phage in contrast to untreated TB4 cells, *agrA* mutant cells, or cells treated with AIP_{hy} (Figure S1). Thus, we decided to focus our studies on Stab20.

Stab20 is a lytic myophage within the genus *Kayvirus* in the subfamily Twortvirinae.[25] It was originally isolated on *S. xylosus* and has a broad host range infecting both methicillin-resistant (MRSA) and susceptible *S. aureus* (MSSA) as well as some strains of *S. epidermidis, S. haemolyticus*, and *S. saprophyticus*.[26] Given that infection of *S. aureus* by Stab20 was stimulated by AIP-I, we examined how susceptibility varied with growth phase. As expected, AIP-I promoted Stab20 infection of TB4 WT cells in both exponential and stationary growth phase, whereas in the absence of exogenous AIP-I, it only infected stationary phase cells. In contrast, phage infection was abolished in stationary phase cells treated with the inhibitory AIP_{hy} and in the Δ*agrA* mutant cells treated with AIP-I (Table S1). These results corroborate that induction of *agr* promotes TB4 susceptibility to Stab20 infection.

To determine whether *agr* impacts Stab20 infection in other *S. aureus* strains than TB4, we grew strains belonging to different clonal complexes and *agr* types in the presence or absence of their cognate AIPs (Table S2) and subsequently infected them with Stab20 in exponential or stationary growth phase (Figure 2).

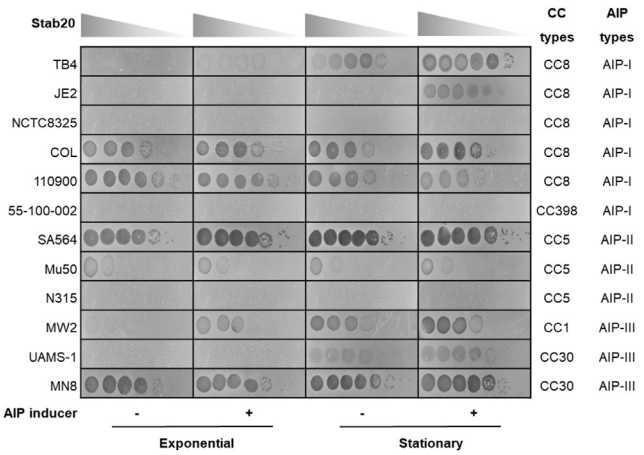

Figure 2. Stab20 infection of *S. aureus* is strain dependent

Stab20 phage lysate dilutions (10^0∼10^{-6}) were spotted onto bacterial lawns of TB4 grown either without or with 0.1 μM of the cognate AIP inducer as indicated and harvested in either exponential (OD_{600} 0.35) or stationary growth phase. Images are representative of three independent experiments.

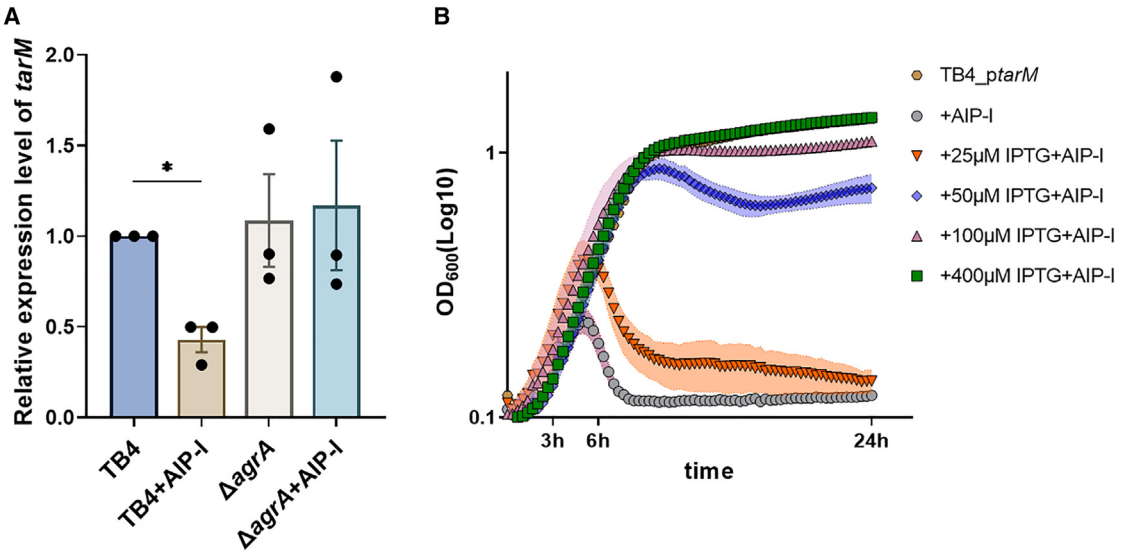

Figure 3. Expression of *tarM* is *agr* regulated and affects Stab20 infectivity

(A) Relative expression of *tarM* was determined by qRT-PCR using *pta* as a reference gene in TB4 or TB4Δ*agrA* mutant cells either untreated or treated with 0.1 μM AIP-I at OD_{600} of 0.35. Data are shown as the mean ± SEM (n = 3), and ΔCt values were analyzed using one-way ANOVA with Dunnett's test multiple for comparison. *$p < 0.05$.

(B) Growth of TB4_p*tarM* treated with or without AIP-I (0.1 μM) upon Stab20 infection. Expression of *tarM* was induced by IPTG at the concentrations indicated in the figure legend. Strains were infected at a multiplicity of infection (MOI) of 0.1. Data are shown as the mean ± SEM (n = 3).

The result shows that several strains were infected independently of growth phase and AIP addition, including COL, 110900, SA564, and MN8, while some were not infected at all (NCTC8325, 55-100-002, and N315). MW2, which belongs to CC1 and encodes an AIP-III, resembled TB4 in being infected upon AIP induction and in stationary growth phase, while JE2 (CC8, AIP-I) was infected only when in stationary phase and exposed to inducing AIPs. UAMS-I (CC30, AIP-III) displayed susceptibility to infection solely in stationary phase, irrespective of addition of inducing AIPs. Thus, susceptibility to infection by Stab20 is strain dependent and is neither associated with the clonal complex nor *agr* type.

Glycosylation of wall teichoic acids affects Stab20 infection

WTA is the primary receptor of staphylococcal phages, and therefore, we speculated that *agr* may affect WTA synthesis or structure. To test this, we infected strain JE2 and its Δ*tarO*, *tarM*, *tarS*, and *tarK* mutant derivatives with Stab20 and examined plaque formation (Figure S2). Here, we observed that deletion of *tarO,* which abolishes WTA entirely, prevented Stab20 infection as has been observed for other phages.[15,18] Further inactivation of *tarK* increased the phage susceptibility of stationary cells. TarK is a bifunctional enzyme that like TarL catalyzes both ribitol phosphate priming and polymerization, and inactivation of *tarK* increases the length of WTA.[27] In contrast, inactivation of *tarS* encoding the β-GlcNAc WTA glycosyltransferase abolished phage infection in stationary phase cells supplemented with AIP-I. The greatest effect, however, was seen for the *tarM* mutant that was infected by Stab20, independent of growth phase and AIP addition. Interestingly, the plaques of *tarM* mutant cells in stationary phase appeared slightly clearer when treated with AIP-I compared to the other examined conditions (Figure S2). Because *tarK* expression is repressed by *agr* induction,[27] this phenomenon may be due to the combined effect of the *tarM* deletion and induction of *agr* reducing *tarK* expression. Collectively, our results suggest that *tarS* is required for phage infection and that expression of both *tarM* and *tarS* may be *agr* dependent.

tarM expression is regulated by *agr* and determines susceptibility to Stab20

To determine whether *tarM* and/or *tarS* are *agr* regulated, we monitored gene expression in TB4 and *agrA* mutant cells either with or without inducing AIP-I. While there were no significant differences in *tarS* expression (Figure S3), we observed that *tarM* transcription decreased significantly ($p < 0.05$) in TB4 cells treated with the inducing AIP-I but not in *agrA* mutant cells (Figure 3A). These results agree with a previous study where a gene (MW0919) was found in MW2 that corresponds to *tarM* and was shown to be downregulated by *agr*.[28]

To monitor the impact of *tarM* expression on Stab20 infectivity, we expressed *tarM* from an IPTG-inducible promoter in pSK9067 (TB4_p*tarM*) and examined infection at various IPTG concentrations (Figure 3B). In the absence of IPTG, Stab20 eliminated AIP-I-treated cells but did not affect untreated cells. However, with increasing concentrations of IPTG and thus increased expression of *tarM,* phage killing was delayed and even prevented at 400 μM IPTG. These results show that *tarM* expression inversely correlates with Stab20 susceptibility and indicate that there may be a threshold concentration of TarM above which phage infection is prevented.

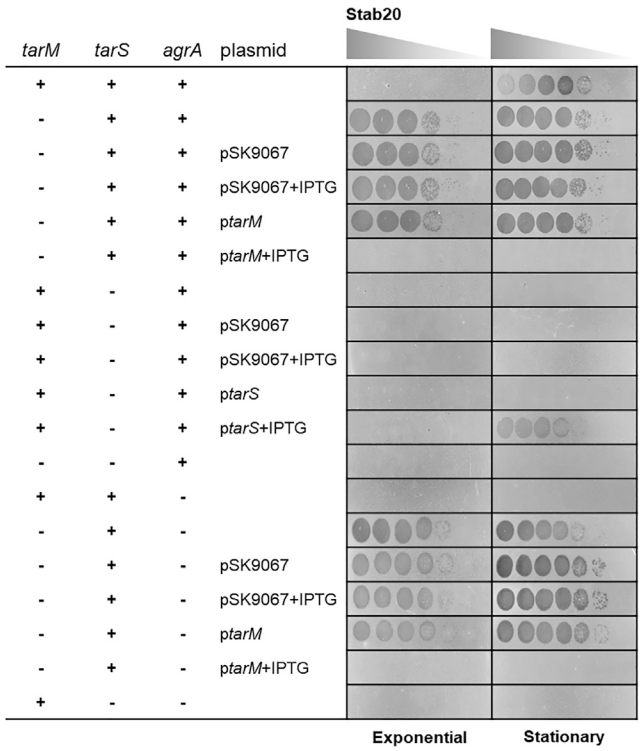

Figure 4. Stab20 infects *tarM* but not *tarS* mutant cells

Phage dilutions (10^0–10^{-6}) were spotted onto bacterial lawns of deletion mutants of *tarM*, *tarS*, and both *tarM* and *tarS* in TB4 or TB4Δ*agrA* as well as their complemented derivatives. IPTG was added at 400 μM. Representative images are shown from three independent experiments.

Lastly, we deleted *tarS* and *tarM* in TB4 and TB4Δ*agrA* mutant cells and examined Stab20 susceptibility. Similar to what we observed in JE2, deletion of *tarM* in TB4 allowed Stab20 to infect independently of growth phase and AIP-I addition, and it even allowed infection of *agrA* mutant cells (Figure 4). Importantly, the phenotype of the *tarM* mutant was complemented by ectopic expression of *tarM*. In contrast, Stab20 was unable to infect *tarS* mutant cells in stationary phase, while infection was restored by plasmid-encoded *tarS*. When deleting both *tarM* and *tarS,* Stab20 infection was prevented entirely (Figure 4). These results are consistent with a model where the TarS-catalyzed β-GlcNAc decoration of WTA is the receptor of Stab20, while the α-GlcNAc decoration catalyzed by TarM interferes with phage infection and is repressed upon *agr* induction.

Induction of *agr* reduces α-GlcNAc modification of WTA

In light of the observation that *agr* promotes phage infection by suppressing *tarM* expression, we monitored anti-α-GlcNAc-WTA F(ab') deposition to TB4 as well as the Δ*tarM* and Δ*tarS* mutant cells by flow cytometry.[29] As predicted, this analysis showed that the Δ*tarM* and the Δ*tarM*Δ*tarS* double mutant had no α-GlcNAc substitutions on their WTA, whereas the Δ*tarS* mutant still had α-GlcNAc substitutions comparable with the TB4 cells. Remarkably, the amount of α-GlcNAc was significantly decreased when cells were treated with inducing AIP-I (Figure 5A).

To understand the strain differences in Stab20 susceptibility, we compared α-GlcNAc glycosylation of TB4 to that of JE2, which requires additional AIP-I for Stab20 to infect in stationary phase (Figure 2). Here, we observed that WTA of JE2 is more heavily α-GlcNAc glycosylated compared to TB4 (Figure 5B). Similar to TB4, we also observed that AIP-I reduced α-GlcNAc glycosylation of JE2 but to a lesser extent than in TB4. When investigating the activity of the *agr* system by monitoring RNAIII expression, we saw that in both TB4 and JE2, AIP-I greatly induced RNAIII levels in exponential phase, but there were no significant differences in stationary phase (Figure S4). Thus, AIP-I addition to exponentially growing cells possibly induces changes in WTA glycosylation that last into stationary phase. In conclusion, strain differences in α-GlcNAc glycosylation levels may explain the observed differences in Stab20 susceptibility. However, other factors are also likely to contribute such as the presence or absence of phage defense systems.

Non-aureus staphylococci inhibit *S. aureus agr* and protect against Stab20 and related phages

In our initial experiments, we had observed that the AIP from *S. hyicus* inhibited *agr* in TB4 to the extent that Stab20 infection was prevented (Figure 1). This led us to examine whether Stab20 infection could also be prevented by spent supernatants from NAS commonly colonizing humans, including *S. epidermidis*, *S. hominis*, and *S. hyicus*.[30] These species have previously been shown to produce AIPs that inhibit *S. aureus agr*.[7] Importantly, we observed that in all cases, spent supernatants from the NAS either eliminated or greatly reduced Stab20 infection of TB4 cells (Figure S5).

Next, we questioned if co-culture of NAS together with *S. aureus* influenced Stab20 infectivity (Figure 6A). To do this analysis, we again chose NAS strains that produce AIPs inhibiting *S. aureus agr*[7,10] but also were not infected by Stab20, namely *S. caprae, S. haemolyticus*, and *S. pseudintermedius* (Figure 6B). Importantly, when co-cultured with TB4, all NAS prevented or strongly inhibited Stab20 infection except for the *agr* deletion mutant of *S. pseudintermedius* ED99 where the inhibition was abolished (Figure 6B). The inhibitory effect of NAS was not due to growth inhibition of TB4, as TB4 formed the greater part of the co-cultures (Table S3). Conversely, when co-cultured with the *tarM* mutant of TB4, none of the NAS strains prevented Stab20 infection (Figure 6B).

Lastly, we set out to determine whether other phages than Stab20 may be affected by *agr* when infecting *S. aureus*. To this end, we searched for phages that may have similar infection patterns as Stab20 by analyzing putative receptor-binding proteins and discovered several myoviruses with similar receptor-binding proteins as those predicted from Stab20, including vB_SauM_EW18, vB_SauM_EW26, vB_SauM_EW29, and vB_SauM_EW72. Like Stab20, these four phages only infected exponential TB4 cells when treated with AIP-I, while they were able to infect TB4 in stationary phase. Furthermore, the phages were unable to infect co-cultures comprising TB4 and *S. pseudintermedius* ED99, and addition of AIP-I to the co-cultures re-established Stab20 infectivity (Figure 7).

These findings show that bacterial communities can affect phage susceptibility of individual bacterial species and that

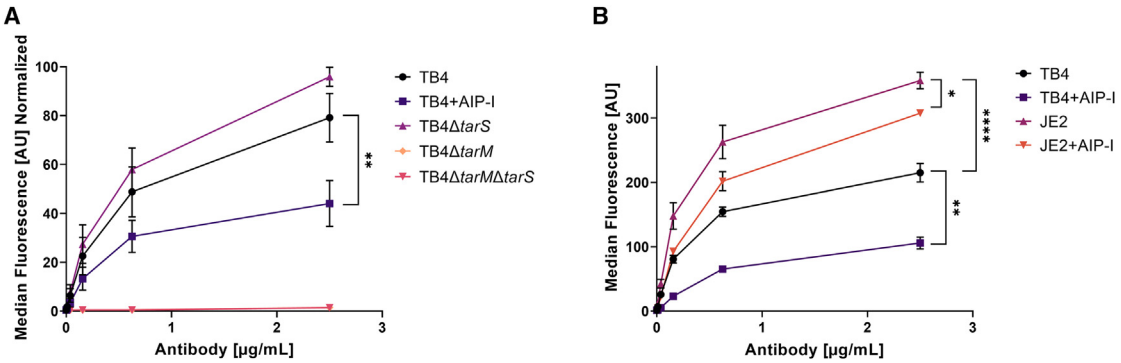

Figure 5. *agr* induction reduces α-GlcNAc substitutions on WTA

(A) TB4, TB4 treated with inducing AIP-I (1 μM), Δ*tarM*, Δ*tarS*, and Δ*tarM*Δ*tarS* at OD_{600} of 0.35 were tested for binding to F(ab') targeting α-GlcNAc-WTA. Data are shown as the mean ± SEM (n = 3). Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test. **p < 0.01.

(B) TB4 and JE2 with or without treatment of AIP-I (1 μM) of stationary growth phase (OD_{600} 4.0) were tested for binding to F(ab') targeting α-GlcNAc-WTA. Data are shown as the mean ± SEM (n = 3). Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test. *p < 0.05, **p < 0.01, ****p < 0.0001.

subtle differences in AIP concentrations and compositions influence whether *S. aureus* is infected by Stab20 and related phages or not.

DISCUSSION

A key finding of this study is that the NAS prevent phage infection of *S. aureus* by producing variant AIPs that inhibit the *S. aureus agr* and reduce α-GlcNAc glycosylation of WTA. Staphylococci are part of the common skin flora of humans and animals with *S. aureus* being the most prominent opportunistic pathogen. On the skin of humans and animals, *S. aureus* exists in tightly interwoven microbial communities where it co-colonizes with NAS such as *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. caprae*, and *S. pseudintermedius*.[31–33] In addition, a wide range of other bacterial species produce molecules that inhibit *S. aureus agr* activation. For example, Lactobacilli and *Bacillus subtilis* produce cyclic peptides different from AIPs,[34,35] while commensal *Corynebacterium* spp. produce yet unidentified compounds that also inhibit *S. aureus agr*.[36] If these bacteria share an ecological niche, intricate interactions are possible via AIP-mediated signaling crosstalk that may impact both *S. aureus* colonization and infection.[8,11–14,37] Our results show that AIP-mediated activation or inhibition of *agr* impacts whether *S. aureus* is infected by Stab20-like lytic phages or not. This in turn influences the composition of the microbial community.

Recently, reports have documented that phages themselves also respond to environmental signals via QS. In *Vibrio cholera* and *Bacillus subtilis,* temperate phages employ QS to make decisions on lysis-lysogeny either by tapping into the host QS systems or by encoding an "arbitrium" sensing system,[38–40] while in *Pseudomonas aeruginosa,* the bacterial QS systems stimulate susceptibility to at least two lytic phages.[41] Bacterial crosstalk was also shown to impact the biology of a temperate *V. cholera* phage where a phage-encoded regulator determines whether the phage lifestyle is lytic or lysogenic.[42] Thus, multi-species bacterial interactions appear to impact phage biology in both Gram-positive and -negative bacteria.

In staphylococci, the structure of WTA is diverse, and the glycoepitopes vary between strains and clones.[43] In *S. aureus*, three forms of WTA glycosylation have been reported,[44] and variation in the corresponding glycosyltransferases determines the host range of staphylococcal phages.[45] The 12 *S. aureus* strains examined in this study all encoded *tarS*, seven harbored additional *tarM*, and three also encoded *tarP* (Table S2). Although Stab20 infection was found to be dependent on *tarS*-mediated β-GlcNAc modification (Figure 4), the *S. aureus* strains differed in their susceptibility as strains NCTC8325, 55-100-002, and N315 had *tarS* but failed to be infected by the phage (Figure 2; Table S2). Intriguingly, the presence of *tarM* appears to be inversely associated with phage susceptibility, as three of five *tarM*-negative strains were susceptible to Stab20, and the two unsusceptible strains carried *tarP* (55-100-002 and N315) (Table S2). This is consistent with earlier studies on podophages and myophages where *S. aureus* strains lacking *tarM* all were susceptible to podophages Φ44AHJD, Φ66, and ΦP68, except those also lacking *tarS*.[18,21] Notably, prophage-encoded *tarP* might impair phage infection in *S. aureus* because *tarP*-carrying 55-100-002 and N315 showed phage resistance even though *tarM* is absent in their genomes (Table S2). Lastly, we observed that the length of WTA may influence phage susceptibility as a *tarK* mutant reported to have increased length of WTA was infected in contrast to the JE2 wild type (Figure S2). As expression of *tarK* is repressed by *agr* induction,[27] this repression may explain the effect of AIP-I addition to stationary phase cells of the *tarM* mutant.

Glycosylation of the WTA is also affected by environmental conditions. For example, under *in vitro* conditions, *S. aureus* strain Newman exclusively produce α-GlcNAc-glycosylated WTA (>90%), whereas under *in vivo* conditions the glycosylation pattern switched to β-GlcNAc.[46] Our finding links induction of *agr* to downregulation of α-GlcNAc modifications on WTA. This strongly suggests that *agr* may be responsible for regulation of growth-phase-dependent shift of WTA glycosylation patterns. In addition to being a phage receptor, WTA is important for *S. aureus* pathogenicity, antibiotic resistance, adhesion, colonization, and immune interaction with the host.[15,16,47–51] Thus, the

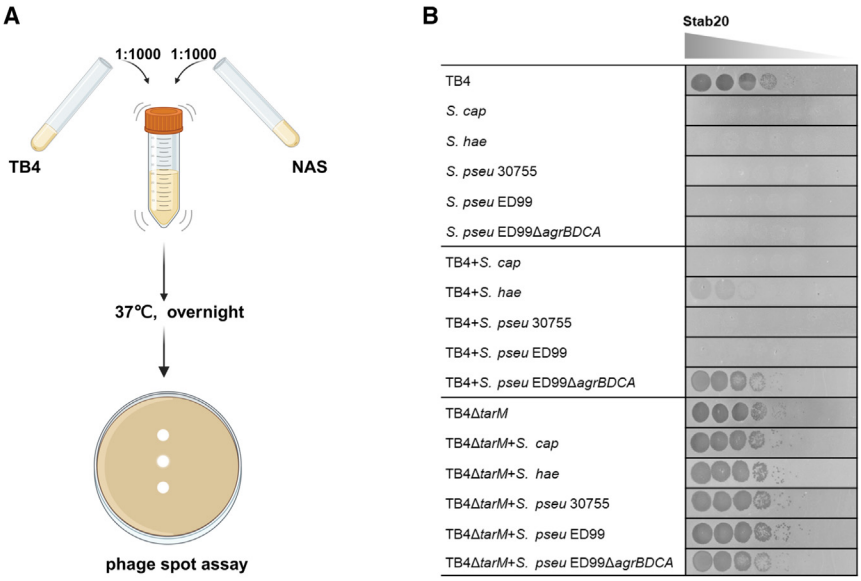

Figure 6. Non-aureus staphylococci inhibit Stab20 infection of *S. aureus*

(A) Cultures of TB4 and indicated NAS were diluted 1:1,000 and either combined ("co-culture") or grown individually overnight to subsequently be applied to form bacterial lawns on which phage lysates were spotted for susceptibility testing.

(B) Susceptibilities of individual and co-cultures of TB4 or TB4Δ*tarM* with the non-aureus staphylococci *S. caprae* (*S. cap*), *S. haemolyticus* (*S. hae*), and *S. pseudintermedius* (*S. pseu*). Representative images are shown from three independent experiments.

agr-mediated control of *tarM* might have broader implications on staphylococcal biology beyond the interaction with phages.

The influence of the microbial community on phage susceptibility has several perspectives. One aspect is that environmental regulation of phage receptors may allow for coexistence of lytic phages and their hosts, where a fraction of the host population expresses the binding epitope, while the remaining do not. Such phenotypic heterogeneity in phage susceptibility has been seen for *Salmonella enterica* with phase variation in expression of the O-antigen phage receptor.[52] Another perspective is phage therapy. While there are numerous reasons for failure of phage therapy, such as *in vivo* instability, delivery challenges, and immunological responses,[53] our results suggests that cross-species repression of *agr* in microbial communities may be an additional factor compromising phage efficacy. Studies of how microbial interactions impact phage therapy is beyond the scope of this study; however, the influence of microbial interactions should be considered when isolating new phages intended for phage therapy or other biotechnological purposes. Our finding that inducing AIPs reverse NAS-mediated repression (Figure 7) suggests that cross-species inhibition of phage receptors may be reversed by communication molecules, which thus could inspire searches for such molecules to be applied as co-factors when phages are used as antimicrobials. Epigenetic regulation of phage receptors is still a large unexplored area, and future studies should be directed at understanding how phages interact with their hosts when present in multi-species and natural environments.

Limitations of the study

This study explored the impact of cross-species interactions by *S. aureus* QS on phage infection, specifically highlighting that *agr* induction enhances the infection of Stab20-like phages. However, the underlying reasons behind the differential infection patterns between Stab20-like phages and other staphylococcal phages remain unresolved. To gain deeper insights into these mechanisms, further bioinformatics analysis of phage binding proteins is deemed imperative. Regarding RNAIII expression in stationary TB4 and JE2 cells, no significant difference was observed between AIP-I-treated and non-treated cells, while α-GlcNAc modifications were less pronounced in AIP-I-treated cells. This suggests the possibility of RNAIII-independent interactions between *agr* and *tarM*, yet the precise mechanism by which *agr* inhibits TarM and α-GlcNAc modifications was not fully clarified in this study. Furthermore, the experimental setting involves the simulation of *S. aureus* within a mixed staphylococci community *in vitro*. While valuable for initial insights, this *in vitro* approach may not fully replicate the complexity of interactions that occur *in vivo*, thus potentially limiting the clinical relevance of the findings. It is worth noting that attempts to use *Galleria mellonella* for phage therapy experiments were unsuccessful. Therefore, considering additional models for validation and extension of the implications of these results is suggested for future studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Bacteria and phages
  - Phage induction and infection
  - Phage susceptibility assay
  - RT-qPCR
  - Gene cloning and mutant construction
  - IgG deposition assay
  - Co-culturing experiments
  - Assessment of NAS supernatants
  - Circumvention of *agr* repression
- QUANTIFICATION AND STATISTICAL ANALYSIS

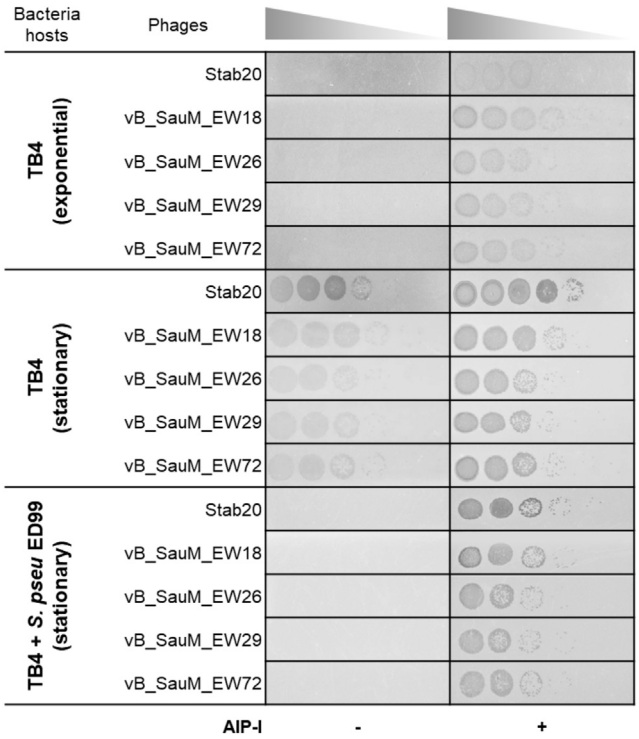

Figure 7. *agr* interference in staphylococci community affects infectivity of Stab20-like phages

Phage Stab20 along with vB_SauM_EW18, vB_SauM_EW26, vB_SauM_EW29, and vB_SauM_EW72 were used to infect bacterial lawns of TB4 either untreated or treated with AIP-I (0.1 μM) during both exponential and stationary growth phases. Similarly, bacterial lawns of co-cultured TB4 and *S. pseudintermedius* ED99 were infected with the same phages in the presence or absence of 0.1 μM AIP-I. Representative images of three independent experiments are shown.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.113154.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.Y., J.Z.B., and H.I.; methodology, J.Y., J.Z.B., B.S.B., M.S.B., A.P., and H.I.; investigation, J.Y., J.Z.B., J.K., E.L., S.F.S., and T.G.; formal analysis, J.Y., J.Z.B., J.K., and H.I.; visualization, J.Y., J.Z.B., and H.I.; writing – original draft, J.Y.; writing – review & editing, J.Y., J.Z.B., and H.I.; supervision, J.Z.B. and H.I., funding acquisition, J.Z.B. and H.I.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-α-GlcNAc WTA IgG	Hendriks et al.[29]	N/A
F(ab')2-Goat anti-Human IgG-FITC	Sigma-Aldrich	Cat#AQ112F; RRID:AB_92761
Bacterial and virus strains		
Bacterial Strains, see Table S4	This paper	N/A
Phage Strains, see Table S4	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Agar No.1	Thermo Fisher Scientific	Cat#LP0011
Ampicillin sodium salt	Sigma-Aldrich	Cat#A0166
Chloramphenicol	Sigma-Aldrich	Cat#C0378
Erythromycin	Sigma-Aldrich	Cat#E5389
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Thermo Fisher Scientific	Cat#R0392
Mitomycin C	Sigma-Aldrich	Cat#M0503
Nutrient broth No.2	Thermo Fisher Scientific	Cat#CM0067
S. aureus AIP-I	Gless et al.[10]	N/A
S. aureus AIP-II	Gless et al.[10]	N/A
S. aureus AIP-III	Gless et al.[10]	N/A
S. hyicus AIP	Gless et al.[10]	N/A
TSA (Tryptone soya agar)	Thermo Fisher Scientific	Cat#CM0131
TSB (Tryptone soya broth)	Thermo Fisher Scientific	Cat#CM0129
X-Gal (5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside)	Thermo Fisher Scientific	Cat#R0404
Critical commercial assays		
PrimeScript™ RT Reagent Kit	Takara	Cat#RR047A
FastStart Essential DNA Green Master	Roche	Cat#06402712001
RNeasy Mini Kit	Qiagen	Cat#74106
Oligonucleotides		
See Table S5	This paper	N/A
Recombinant DNA		
See Table S4	This paper	N/A
Software and algorithms		
Graphpad Prism 9.5.1	GraphPad	https://www.graphpad.com/
Biorender	BioRender	https://www.biorender.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hanne Ingmer (hi@sund.ku.dk).

Materials availability

This study did not generate new unique reagents, and all bacterial and phage strains generated in this study are available from the lead contact upon request.

Data and code availability

- No datasets were generated during this study.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The bacteria strains, mutants and phage strains used in this study are described in Table S4.

METHOD DETAILS

Bacteria and phages

Bacterial strains used in this study are described in Table S4. *Staphylococcus* strains were cultured in Tryptone Soya Broth (TSB) or Tryptone Soya Agar (TSA), and *E. coli* strains were grown in Luria–Bertani (LB) or LB agar (LA). Antibiotics (erythromycin 5 μg/mL or 10 μg/mL; ampicillin 100 μg/mL; chloramphenicol 10 μg/mL) were added as required. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for induction of gene expression and X-Gal (5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside) TSA plates were used for bacterial assessment. AIP-I/II/III and AIP_{hy} were synthesized as described.[10] To induce or inhibit the *agr* system in *S. aureus* strains, overnight cultures were diluted to OD_{600} of 0.01 and grown with the addition of 0.1 μM AIP inducers or AIP_{hy}. 1 μM AIP-I was applied in IgG deposition assay.

Phage induction and infection

Lytic phage propagation was performed as described.[54] Briefly, overnight cultures of recipient strains were grown to OD_{600} of 0.15, collected by centrifugation, and resuspended in 1:1 TSB and phage buffer (PHB; 1 mM $MgSO_4$, 4 mM $CaCl_2$, 50 mM Tris-HCl, 100 mM NaCl, pH 8.0). Suspended cells were infected with a phage stock at an appropriate multiplicity of infection before incubated at 30°C, 80 rpm to a complete lysis. Lysates were filtered and stored at 4°C. Phage induction was performed as described.[54] Briefly, lysogens were grown overnight and were subcultured to OD_{600} of 0.15, followed by addition of 2 μg/mL mitomycin C. Cell cultures were then incubated at 30°C, 80 rpm until completely lysed. Lysates were filtered and stored at 4°C.

Phage susceptibility assay

Phage full plate plaque assay was conducted by diluting overnight cultures of *S. aureus* to OD_{600} of 0.01 with TSB and grown with 0.1μM AIPs (inducing or inhibitory, if needed) to either exponential phase (OD_{600} of 0.35) or overnight. Phage lysates were serially diluted in PHB. 100μL recipient cells and 100 μL phage lysates were mixed and incubated for 10 min at room temperature, followed by the addition of 3 mL phage top agar (PTA; 20 g/L Nutrient Broth No. 2; 3.5 g/L Agar No.1), and were plated out on phage base agar plates (PBA; 20 g/L of Nutrient Broth No. 2; 7 g/L Agar No.1) supplemented with 10mM $CaCl_2$. Plaque-forming units (PFU/mL) was determined after 24h incubation at 37°C. Phage spot assay was performed by spotting 10μL serial diluted phage lysates onto PBA plates overlaying with 3mL PTA carrying 100 μL AIP-treated or untreated recipient cells. Images of phage plaques were obtained by scanning the plates after 24h incubation at 37°C.

Phage liquid infection was assessed using a plate reader (Bioscreen C, Oy Growth Curves Ab Ltd), where cultures grown to OD_{600} of 0.15 (with AIPs or IPTG, if necessary) were transferred to honeycomb Bioscreen plates (95025BIO) in 125 μL aliquots. The same volume of phage lysates in PHB were added to each well and OD_{600} was measured every 20 min for 24 h at 30°C with shaking.

RT-qPCR

For RNA sample preparation, overnight cultures were diluted to OD_{600} of 0.01 in fresh TSB and supplemented by 0.1 μM AIP-I (if necessary). Cell cultures at OD_{600}0.35 or overnight (OD_{600}4.0) were collected for RNA isolation by using the RNeasy Mini Kit (Qiagen). PrimeScript RT Reagent Kit (Takara) was used to generate cDNA and FastStart Essential DNA Green Master (Roche) for qPCR in a Lightcycler 96 (Roche). All RT-qPCR experiments were performed in triplicate with three technical replicates, primers are listed in Table S5. Gene *pta* and *gyrB* were set as control. Data analysis was performed in the LightCycler Application Software, version 1.1 (Roche). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method[55] and ΔCt values were used in statistical analysis.

Gene cloning and mutant construction

Plasmids (Table S4) and primers (Table S5) used for gene cloning are listed. TB4Δ*agrA* was constructed as previously described.[56] The pCasSA-*agrA* plasmid was constructed using an insert containing the *agrA* spacer (tgtctacaaagttgcagcga) followed by the sgRNA scaffold and homologous sequences of 1kb upstream and 1kb downstream of the *agrA* gene. This fragment was synthesized and cloned into the pCasSA vector by Twist Bioscience (San Francisco, CA, USA). TB4Δ*tarM*, TB4Δ*agrA*Δ*tarM*, and TB4Δ*tarM*Δ*tarS* mutants were constructed using the SLiCE cloning method.[57] Briefly, primer pairs MA/MB (or SA/SB) and MC/MD (or SA/SD) were used for amplifying upstream and downstream of *tarM* (or *tarS*), the two fragments were then ligated by an overlap extension PCR using MA/MD (SA/SD) primers. pIMAY-Z_Δ*tarM* or pIMAY-Z_Δ*tarS* was obtained by setting a SLiCE reaction by 1 μL DNA insert, 1 μL linearized pIMAY-Z, 6 μL of H_2O, 1 μL of SLiCE and 1 μL 10× ligation buffer at 37°C for 15 min. The plasmid was modified in *E. coli* IMO8B and was then electroporated into wild type TB4. After incubation at 30°C for 48h on BHI agar containing 10 μg/mL chloramphenicol and 100 μg/mL X-Gal, transformants were selected and underwent steps of plasmid integration at 37°C and plasmid excision at 30°C. Finally, gene deletion was checked using primers MA and MD (or SA and SD).

TB4Δ*tarS* was constructed by transducing pIMAY-Z_Δ*tarS* plasmid to wild type TB4. Briefly, phage lysates of ϕ11Δ*int*-pIMAY-Z_Δ*tarS* was obtained by using ϕ11Δ*int* to infect TB4Δ*agrA*_pIMAY-Z_Δ*tarS* stored at 30°C following the above method of phage liquid

infection. Afterward, overnight cultures of strain TB4 were diluted and grown to OD_{600} of 1.4 before being added with 4.4 mM $CaCl_2$. 1mL of TB4 cells together with 100 μL ϕ11Δ*int*-pIMAY-Z_Δ*tarS* lysates were incubated at 37°C for 20 min, following addition of 3mL TTA (30 g/L TSB; 7.5 g/L Agar No.1). Mixtures were then plated on TSA plates containing 17mM sodium citrate and 10 μg/mL chloramphenicol. Transductants were selected after 24h incubation at 37°C and the subsequent steps were carried out as described in SLiCE cloning method.

Complemented and overexpression mutants were constructed using expression vector pSK9067.[58] The primers MO and SO with SalI and EcoRI restriction sites were used to clone *tarM* and *tarS* from TB4. The digested fragments was then ligated with the linearized pSK9067 harboring a P_{spac} promoter to construct the plasmid p*tarM*/S, which was transformed into *E. coli* IM08B before electroporation into *S. aureus*. *S. aureus* transformants were selected on TSA containing 10 μg/mL erythromycin.

The 8325-4 ϕ11Δ*int* mutant strain was constructed by allelic replacement of the up- and downstream flanking regions, using the primers described in Table S5. Cloning of the flanking regions into the pMAD vector was achieved using the SLiCE method as previously described, before transformation into strain 8325-4 ϕ11.[59] Integration of the pMAD plasmid and crossover events were performed as previously described.[60] Gene deletion was confirmed by the extraction of DNA from potential clones and PCR followed by sequencing using oligonucleotides that annealed outside the recombination flanks.

IgG deposition assay

Anti-α-GlcNAc WTA IgG deposition was measured via binding of a secondary fluorescein 5-isothiocyanate (FITC) labeled Fab-Fragment against human IgG. Briefly, *S. aureus* strains were grown overnight at 37°C with agitation, then were subcultured with the treatment of 1 μM AIP-I (if necessary) and diluted to OD_{600}0.4 in PBS+0.1% BSA, and the pellet was collected and resuspended in PBS+0.1% BSA. 25 μL of anti-α-GlcNAc WTA IgG in PBS+0.1% BSA prepared as described[29] were incubated with 25 μL of *S. aureus* suspensions on ice before washing with PBS+0.1% BSA. Afterward, the cells were incubated with F(ab')2-Goat anti-Human IgG-FITC (Goat anti-Human IgG, F(ab')2, FITC Conjugated Affinity Purified, Sigma; diluted 1:500) on ice, with the final anti-IgG concentration ranging from 0 to 2.5 μg/mL. The cells were washed once with PBS+0.1% BSA and fixed in 1% paraformaldehyde in PBS for 15min, followed by washing with PBS. The final suspensions in PBS were transferred to flow cytometry to measure fluorescence. The normalization was done by setting the highest measured median fluorescence of all strains for each replicate to 100%.

Co-culturing experiments

Bacteria were grown at 37°C, 180 rpm overnight and OD_{600} was measured before standardizing to the same. Overnight bacterial cultures were added to fresh TSB at 1:1000 dilution, followed by incubation at 37°C, 180 rpm overnight. Afterward, 100 μL cultures were collected and subsequently used for susceptibility test against tested phages by spot assay. TSA-X-Gal (100 μg/mL) plates were used to count colonies of *S. pseudintermedius* 30755, *S. pseudintermedius* ED99, *S. pseudintermedius* ED99Δ*agrBDCA* or *S. haemolyticus* in the co-cultures, as they produced β-galactosidase, resulting in blue colonies on the plate. Mannitol salt agar plate was used for *S. caprae* as it failed to produce a halo-like zone, which differed from that observed in TB4.

Assessment of NAS supernatants

The tested NAS strains including *S. epidermidis*, *S. hominis* and *S. hyicus* were grown at 37°C, 180 rpm overnight. Following incubation, the cell cultures were centrifuged at 12,000 × *g* at 4°C for 10 min, and the resulting supernatant was filtered through a 0.22-μm syringe filter (Minisart, Sartorius). To assess the phage susceptibility of TB4 cells treated with the collected supernatant, overnight cultures of TB4 were diluted to OD_{600} of 0.01. Subsequently, a mixture was prepared by combining TB4 cells (60%; v/v) with obtained supernatants (40%; v/v), followed by incubation at 37°C, 180 rpm overnight. Phage spot assay was performed on the resulting overnight cultures the following day.

Circumvention of *agr* repression

To assess the impact of introducing *S. aureus* AIP inducers on the reestablishment of phage susceptibility within staphylococcal communities, standardized overnight cultures of TB4 and *S. pseudintermedius* ED99 were inoculated into fresh TSB at a dilution of 1:1000. Subsequently, the mixture was subjected to the treatment with or without 0.1 μM AIP-I, followed by incubation at 37°C, 180 rpm overnight. Phage susceptibility test was performed the next day by spot assay.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed by using GraphPad Prism (GraphPad Software, version 9.5.1). Statistically significant differences were calculated by using one-way ANOVA method with Dunnett's multiple comparison test. Value of *n* represents the number of biological repeats. Details of statistical analysis are indicated in figure legends.