

Wall Teichoic Acid Glycosylation Governs *Staphylococcus aureus* Nasal Colonization

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ABSTRACT Nasal colonization by the human pathogen *Staphylococcus aureus* is a major risk factor for hospital- and community-acquired infections. A key factor required for nasal colonization is a cell surface-exposed zwitterionic glycopolymer, termed wall teichoic acid (WTA). However, the precise mechanisms that govern WTA-mediated nasal colonization have remained elusive. Here, we report that WTA GlcNAcylation is a pivotal requirement for WTA-dependent attachment of community-acquired methicillin-resistant *S. aureus* (MRSA) and emerging livestock-associated MRSA to human nasal epithelial cells, even under conditions simulating the nutrient composition and dynamic flow of nasal secretions. Depending on the *S. aureus* strain, WTA O-GlcNAcylation occurs in either α or β configuration, which have similar capacities to mediate attachment to human nasal epithelial cells, suggesting that many *S. aureus* strains maintain redundant pathways to ensure appropriate WTA glycosylation. Strikingly, a lack of WTA glycosylation significantly abrogated the ability of MRSA to colonize cotton rat nares *in vivo*. These results indicate that WTA glycosylation modulates *S. aureus* nasal colonization and may help to develop new strategies for eradicating *S. aureus* nasal colonization in the future.

IMPORTANCE Nasal colonization by the major human pathogen *Staphylococcus aureus* is a risk factor for severe endogenous infections and contributes to the spread of this microbe in hospitals and the community. Here, we show that wall teichoic acid (WTA) O-GlcNAcylation is a key factor required for *S. aureus* nasal colonization. These data provide a mechanistic explanation for the capacity of WTA to modulate *S. aureus* nasal colonization and may stimulate research activities to establish valuable strategies to eradicate *S. aureus* nasal colonization in high-risk hospitalized patients and in the general community.

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Staphylococcus aureus is one of the most important bacterial pathogens in hospital- and community-acquired (CA) infections. *S. aureus* colonizes the anterior nares of approximately 20 to 30% of the healthy population asymptotically. Nasal colonization by *S. aureus* has been found to be a major risk factor for severe endogenous infections and contributes to the transmission and spread of *S. aureus* in hospitals (1, 2).

S. aureus nasal colonization is a complex and multifactorial process which is understood only incompletely. Both microbial and human host factors appear to mediate and modulate *S. aureus* nasal colonization. Host factors shaping nasal colonization include, for example, the presence of hemoglobin in nasal secretions (3), altered expression of antimicrobial peptides in human skin (4, 5), nutrient limitation (6), and polymorphisms in immunomodulatory mediators or receptors (7, 8). Equally importantly, *S. aureus* nasal colonization and stable persistence require bacterial factors which govern efficient attachment to squamous or ciliated epithelial cells located in different areas of the nasal cavity (9, 10). In particular, various *S. aureus* proteins belonging to the group of “microbial surface components recognizing adhesive matrix mol-

ecules” (MSCRAMMs) have been shown to mediate interaction with components of human desquamated nasal epithelial cells (11–13), above all, with cytokeratin-10 and loricrin-binding clumping factor B (ClfB) (14, 15) and iron-regulated surface determinant A (IsdA) (16). *isdA* and *clfB* are upregulated during nasal colonization in humans and *in vivo* models of experimental colonization compared to *in vitro* growth in broth cultures (17–19). *S. aureus* mutants lacking ClfB cannot persist in the human nose over a longer time period, underscoring the key role of ClfB in *S. aureus* nasal long-term colonization (20). However, during early-stage colonization, *clfB* and *isdA* are usually expressed only at low levels. In contrast, genes required for the biosynthesis of another cell wall-anchored macromolecule, designated wall teichoic acid (WTA), are upregulated during this period (17, 18). Typically, *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA) and highly virulent clones such as American pandemic clone USA300, synthesize WTA polymers composed of 11 to 40 ribitol-phosphate (RboP) repeating units modified with three tailoring modifications, D-alanine, α -O-N-acetylglucosamine (GlcNAc), and β -O-GlcNAc (10, 21). Interestingly, *S. aureus* mutants lacking

TABLE 1 Distribution of WTA glycosyltransferases TarM and TarS in nasal *S. aureus* isolates

Parameter	Value for WTA glycosyltransferase-encoding gene(s):		
	<i>tarM</i>	<i>tarS</i>	<i>tarM</i> and <i>tarS</i>
No. of gene-positive nasal isolates ^a	25	70	25
Rate of gene-positive nasal isolates (%) ^b	35.7	100	35.7

^a A total of 70 *S. aureus* isolates obtained from healthy carriers or hospital patients was tested. *spa* typing revealed 49 different *spa* types associated with at least 15 different sequence types (see also Table S1 in the supplemental material).

^b Values (%) were calculated by dividing the number of positive isolates by the total number of tested isolates.

WTA lost their capability to colonize cotton rat nares, while mutants that lost the ability to modify teichoic acids by D-alanylation (WTA and lipoteichoic acid) exhibited reduced colonization. Both types of mutants feature strongly reduced binding capacities to living human primary nasal epithelial cells (22, 23). A recently discovered type F scavenger receptor (SREC-I), present on human nasal epithelial cells, governs *S. aureus* nasal colonization via charge-dependent interaction with WTA polymers, indicating that the D-alanine modification of WTA contributes to *S. aureus* nasal colonization (24). However, the role of the WTA amino-sugar modification with GlcNAc in nasal colonization, which occurs in two different stereochemical configurations (α - and β -O linkages), remains elusive.

Here, we analyzed the role of *S. aureus* WTA O-GlcNAcylation during nasal colonization and reveal molecular determinants that contribute to WTA-mediated *S. aureus* nasal colonization *in vitro* and *in vivo*.

RESULTS AND DISCUSSION

***S. aureus* nasal isolates encode WTA glycosyltransferases.** Two recently discovered enzymes, the α -O-GlcNAc WTA glycosyltransferase TarM and the β -O-GlcNAc glycosyltransferase TarS, can glycosylate *S. aureus* WTA (25, 26). With only very few exceptions, *tarS* is present in all sequenced *S. aureus* genomes. In contrast, *tarM* is absent in certain clonal complexes (CC), for example, in the emerging CC398, which includes livestock-associated MRSA clones (LA-MRSA CC398). This raises the question of whether TarM-dependent WTA α -O-GlcNAcylation may play a role in *S. aureus* host tropism (21).

Here, 70 isolates from a collection of genetically diverse nasal *S. aureus* isolates (49 different *spa* types associated with at least 15 different sequence types) obtained from healthy volunteers or hospital patients were typed by PCR for the presence or absence of *S. aureus* WTA-glycosyltransferase-encoding genes. All tested nasal isolates bore *tarS*, while only a minority (35.7%) of these strains bore both *tarM* and *tarS*, indicating that, in principle, nasal *S. aureus* strains should be capable of glycosylating WTA (Table 1).

Impact of nasal growth conditions on *S. aureus* WTA glycosylation. In order to analyze if such nasal strains indeed have glycosylated WTA, their capacity to bind WTA GlcNAc-dependent bacteriophages (phages) such as Φ 11 (27) was investigated upon growth in a recently developed synthetic nasal medium (SNM3, containing 0.2 mM bipyridine) providing

suitable conditions for simulating the nasal habitat (6). All tested SNM3-grown *S. aureus* strains were susceptible to phage Φ 11, and, interestingly, the phage adsorption rate was increased compared even to that seen with bacteria cultivated in standard laboratory media (Fig. 1A and B). Moreover, even though *tarM* was downregulated in SNM3, the gene for the TarS β -O-GlcNAc WTA glycosyltransferase was upregulated in community-associated MRSA (CA-MRSA; *tarM*- and *tarS*-positive) strain USA300 during the logarithmic- and stationary-growth phases in SNM3 compared to the level seen in bacteria grown in rich B-medium (BM) (Fig. 1C). Thus, WTA polymers are most probably glycosylated under nasal growth conditions but may differ with respect to the type of GlcNAc linkage.

WTA glycosylation affects interaction of MRSA with epithelial cells. Next, the impact of WTA glycosylation on nasal colonization and binding to human epithelial cells was clarified by using various *S. aureus* isolates, including *tarM*- and *tarS*-containing CA-MRSA strain USA300 and the RN4220 laboratory strain as well as LA-MRSA CC398 strain 82086 naturally lacking *tarM* plus corresponding mutants completely lacking WTA glycosylation. Importantly, a lack of WTA glycosylation did not alter expression levels of MSCRAMMs such as ClfB, SdrC, SdrD, IsdA, and SasG, which are known to interact with components of nasal squames (see Fig. S1A in the supplemental material). Accordingly, the ability of *S. aureus* variants lacking WTA glycosylation to interact with extracellular matrix proteins such as cytokeratin-10, fibronectin, and fibrinogen was equal to that of the wild-type strain, indicating that matrix-protein binding MSCRAMMs are fully functional in the mutants (see Fig. S1B to D).

However, the absence of WTA glycosylation caused a strong reduction in the binding capacity of *S. aureus* to living human airway epithelial cells (A549) and to primary epithelial cells derived from cotton rat (CRNEC) or human nares (HNEpC) (Fig. 2; see also Fig. S2 in the supplemental material). Complementation by either the TarM α -O-GlcNAc WTA glycosyltransferase or the TarS β -O-GlcNAc WTA glycosyltransferase restored the binding capacity of WTA glycosylation-deficient mutants to levels comparable to those seen with the corresponding parental strains, suggesting that WTA glycosylation is required for efficient interaction with nasal epithelial cells but that the stereochemistry of WTA glycosylation is of minor importance for this interaction. Indeed, the binding capacity of *S. aureus* USA300 mutants, which still glycosylate WTA but lack one of the two glycosyltransferases (TarM or TarS), to A549 cells or to primary nasal epithelial cells was not affected (see Fig. S3). Similar effects were observed when adherence to primary nasal epithelial cells was monitored under mild shear forces mimicking the dynamic movement of nasal mucus, thereby emphasizing the importance of WTA-GlcNAcylation for interaction with epithelial cells (Fig. 3). However, *S. aureus* mutants completely lacking WTA polymers (Δ *tagO*) were also strongly abrogated in binding to epithelial cells compared to the parental strain, which is in agreement with previous reports (22, 23) (Fig. 2 and 3B and C). The more pronounced effect in the Δ *tagO* mutant than in the glycosylation-deficient mutants may result from the absence of zwitterionic WTA repeating units and their GlcNAcylation, which both appear to be important for interaction with epithelial cell receptors. Together, these findings indicate that backbone glycosylation is an important component

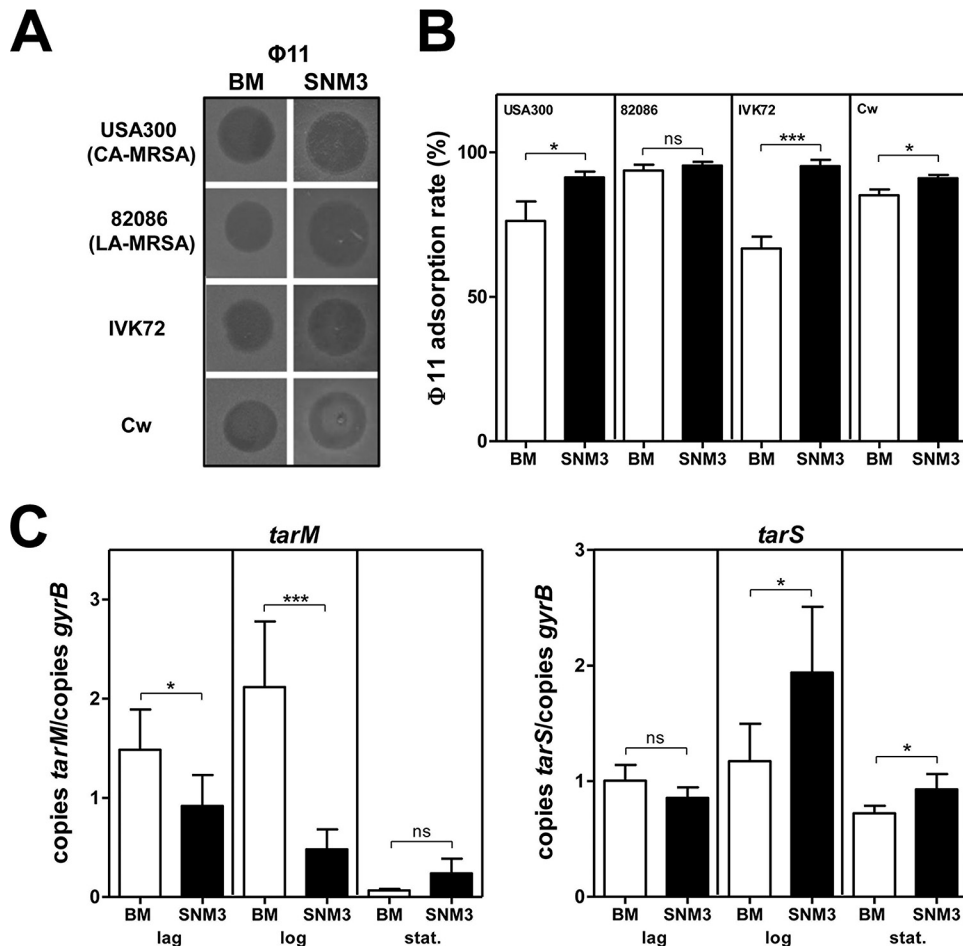


FIG 1 Impact of nasal growth conditions on WTA composition and WTA glycosylation. (A and B) *S. aureus* susceptibility to the WTA GlcNAc-dependent bacteriophage $\Phi 11$ (A) along with $\Phi 11$ adsorption levels (B). (C) qRT-PCR analysis of WTA glycosyltransferase gene expression levels in *S. aureus* USA300. mRNA was isolated from lag-, log-, or stationary (stat.)-phase-grown bacteria. For bacteriophage susceptibility and adsorption analysis or analysis of gene expression levels, strains were grown under rich conditions (B-medium [BM]; white columns) or nasal growth conditions (SNM3 [containing 0.2 mM bipyridine]; black columns). Values are given as means and standard deviations (SD; $n = 3$ to 6). Statistically significant differences calculated using an unpaired two-tailed Student's *t* test are indicated as follows: ns (not significant), $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of WTA polymers required for efficient interaction with human epithelial cells.

WTA glycosylation affects *S. aureus* nasal colonization *in vivo*. Finally, the impact of WTA glycosylation on *S. aureus* nasal colonization was analyzed by using a cotton rat *in vivo* colonization model (22, 28). Cotton rat nares share histological properties with human nares and permit stable long-term nasal colonization with *S. aureus* (29). Moreover, cotton rats are susceptible to various human respiratory bacterial pathogens, thereby representing an excellent *in vivo* nasal colonization model (30). Notably, lack of WTA glycosylation significantly abrogated *S. aureus* nasal colonization in the cotton rat model 3 days after inoculation (Fig. 4). Since absence of WTA glycosylation did not affect *S. aureus* susceptibility to killing by antimicrobial peptides such as the cathelicidin LL-37 (see Fig. S4 in the supplemental material) or growth in various laboratory media, including the SNM3 synthetic nasal medium (see Fig. S5), the reduced nasal colonization capacity is very likely due to diminished interaction with nasal epithelial cells. Thus, WTA glycosylation affects *S. aureus* nasal colonization *in vivo*.

In summary, WTA glycosylation represents a key requirement for efficient *S. aureus* nasal colonization that most probably operates by modulating interaction with nasal epithelial cells. Since the majority of tested nasal *S. aureus* isolates encoded only *tarS* and not *tarM*, it is tempting to speculate that β -O-GlcNAcylated WTA might be more critical than α -O-GlcNAcylated WTA for *S. aureus* during nasal colonization. Nevertheless, WTA interaction with epithelial cells does not seem to depend on a specific stereochemistry of GlcNAc. The different types of WTA glycosylation might be more relevant for shaping other host-pathogen interaction processes such as activation of the human complement system via WTA β -O-GlcNAc-specific antibodies or mannose-binding lectin (31). However, WTA-deficient mutants exhibited even more diminished binding capacities to nasal epithelial cells than WTA glycosylation-deficient mutants, suggesting that the zwitterionic WTA repeating units and their GlcNAcylation are probably of the same importance for interaction with host cell receptors. While it has been demonstrated before that the zwitterionic properties of WTA polymers depending on free aminogroups of the D-alanine substituents govern interaction with the hu-

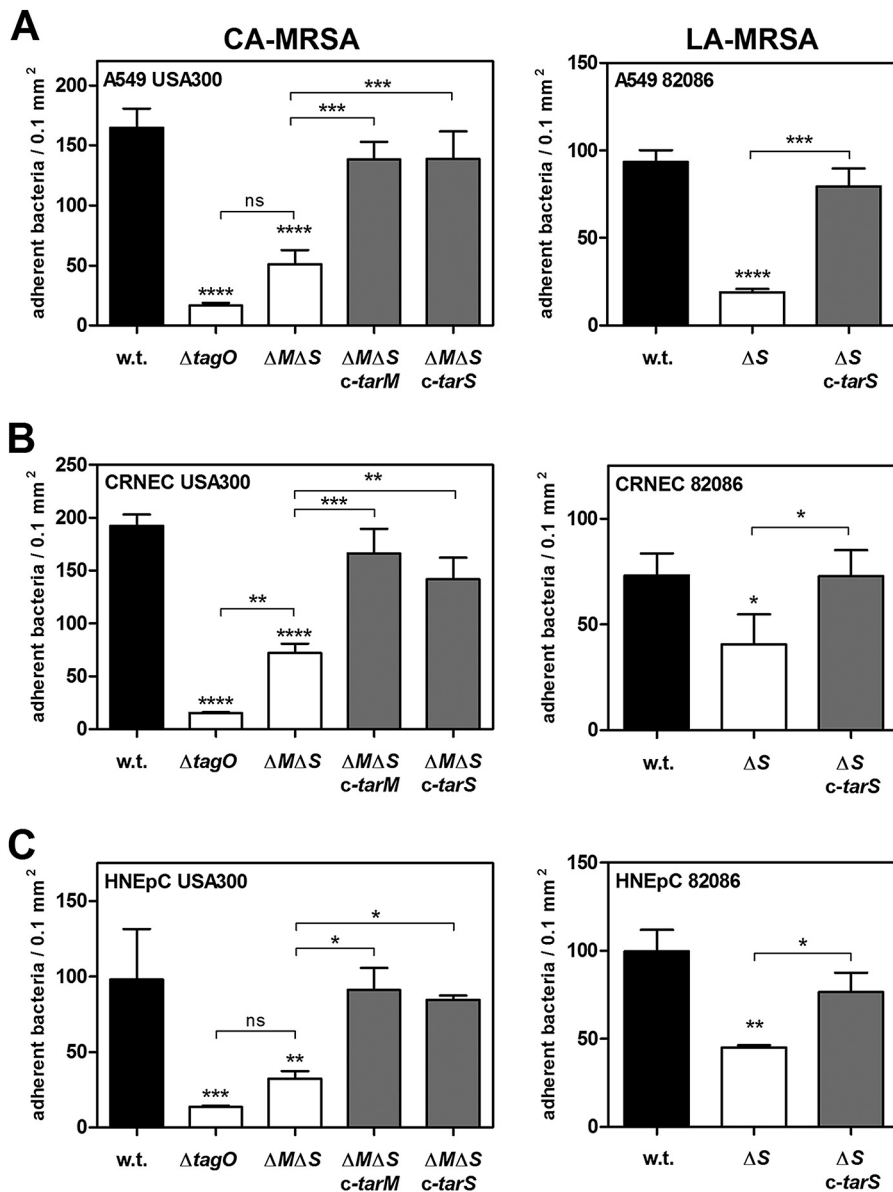


FIG 2 WTA glycosylation affects interaction of MRSA with epithelial cells. Data represent binding capacities of *S. aureus* variants to A549 lung epithelial cells (MOI30) (A), cotton rat nasal epithelial primary cells (CRNEC) (MOI20) (B), or human nasal epithelial primary cells (HNEpC) (MOI30) (C) under static conditions. The binding capacities of various strains encompassing the CA-MRSA USA300 strain and a LA-MRSA CC398 strain (82086) to confluent epithelial cell monolayers were analyzed. The *S. aureus* wild-type (w.t.) strain and strains lacking WTA ($\Delta tagO$) or WTA glycosylation ($\Delta tarM \Delta tarS$ [$\Delta M\Delta S$] or $\Delta tarS$ [ΔS], LA-MRSA CC398 strain 82086) and the complemented mutants ($\Delta M\Delta S c-tarM$, $\Delta M\Delta S c-tarS$, or $\Delta S c-tarS$, LA-MRSA CC398 strain 82086) are indicated. Values are given as means and standard deviations (SD; $n = 3$ to 4). Statistically significant differences calculated using one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison test are indicated as follows: ns (not significant), $P > 0.05$; *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

man scavenger receptor SREC-I in a charge-dependent manner (24), the identity of the host cell receptor responsible for interaction with WTA glycosyl residues remains unclear. Since SREC-I does not exhibit an obvious carbohydrate recognition domain, other human scavenger receptors have to be considered in the future for WTA glycosylation-dependent binding to epithelial cells. Consequently, antibody-based targeting of these receptors or their bacterial ligands, potentially in combination with nasal elimination of *S. aureus* by the antibiotic mupirocin (32), might become a suitable strategy for eradicating *S. aureus* nasal coloni-

zation in high-risk hospitalized patients and in the general community.

MATERIALS AND METHODS

Bacterial strains and growth media. All bacterial strains listed in Table S1 in the supplemental material were grown in lysogeny broth (Becton, Dickinson), B-medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4 , 0.1% glucose), or Mueller-Hinton broth (MHB) or in a recently described synthetic nasal medium (SNM3, containing 0.2 mM bipyridine) (6) at permissive temperatures and were supplemented with the

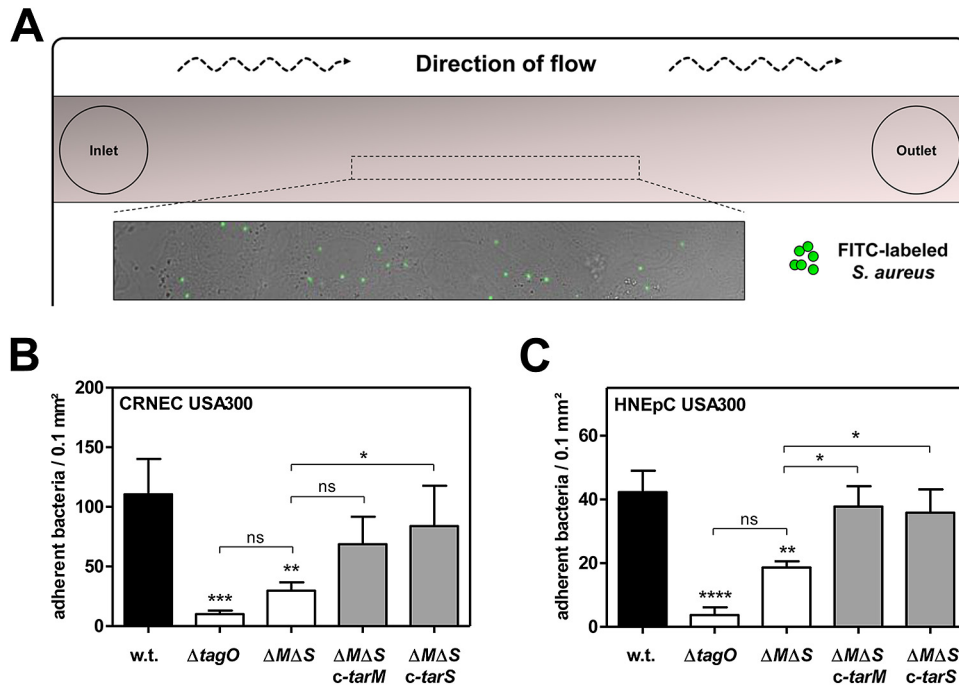


FIG 3 WTA glycosylation mediates adherence to primary epithelial cells under dynamic flow conditions. (A) Principle of flow-chamber-based attachment assays mimicking dynamic nasal conditions. (B and C) The binding capacities of *S. aureus* variants to monolayers of confluent cotton rat nasal epithelial primary cells (CRNEC) (MOI60) (B) or human nasal epithelial primary cells (HNEpC) (MOI60) (C) under conditions of mild shear stress were analyzed. The *S. aureus* USA300 wild-type (w.t.) strain, strains lacking WTA ($\Delta tagO$) or WTA glycosylation ($\Delta tarM \Delta tarS$ [$\Delta M\Delta S$]), and the complemented mutants ($\Delta M\Delta S$ c-*tarM* and $\Delta M\Delta S$ c-*tarS*) are indicated. Values are given as means and standard deviations (SD; $n = 3$ to 4). Statistically significant differences calculated using one-way ANOVA with Bonferroni's multiple-comparison test are indicated as follows: ns (not significant), $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

appropriate antibiotics (chloramphenicol [10 $\mu\text{g/ml}$], streptomycin [250 to 500 $\mu\text{g/ml}$], and ampicillin [100 $\mu\text{g/ml}$]). Growth characteristics were monitored at 37°C.

Molecular genetic methods. For deletion of *tarM*, *tarS*, *tarM* and *tarS*, or *tagO* in *S. aureus* USA300 or in a LA-MRSA CC398 strain (82086), a

previously described *Escherichia coli-S. aureus* shuttle vector, pKOR1, was used (33). For deletion of *srtA*, an *E. coli-S. aureus* shuttle vector, pIMAY, was used (34). Gene disruption via pKOR1 or pIMAY was performed via allelic replacement as described before (33, 34). Briefly, knockout plasmids were isolated from appropriate *E. coli* strains, transformed into electrocompetent *S. aureus* RN4220 cells, reisolated, and subsequently electroporated in a target strain (USA300 or 82086). Electroporation conditions were described elsewhere (35). Knockout plasmids were integrated onto the genome of target strains at permissive temperatures (for pKOR1, 43°C; for pIMAY, 37°C) and in the presence of chloramphenicol (10 $\mu\text{g/ml}$). Following plasmid integration, a counterselection step was performed at 30°C using anhydrotetracycline (1 $\mu\text{g/ml}$). Resulting colonies were patched onto BM agar plates with or without chloramphenicol (10 $\mu\text{g/ml}$) and screened for plasmid loss. Chloramphenicol-sensitive colonies were screened via PCR to confirm gene deletion. The *S. aureus* RN4220 variants were described elsewhere (35). For complementation studies, the previously described pRB474 *E. coli-S. aureus* shuttle vector was used (36).

The presence or absence of *S. aureus* WTA glycosyltransferase-encoding genes *tarM* and *tarS* in nasal *S. aureus* isolates was verified via PCR using the gene-specific primers listed in Table S2 in the supplemental material. *spa* typing was performed as described previously (37). Briefly, the *spa* repeat region was amplified using the published primers listed in Table S2, sequenced, and analyzed using Ridom StaphType 1.5.21 software (37).

Cell culture. Primary human nasal epithelial cells (HNEpC) were purchased from PromoCell (PromoCell, Heidelberg, Germany) and grown in airway epithelial growth medium (PromoCell) according to the manufacturer's instructions. Primary cotton rat nasal epithelial cells (CRNEC) were isolated from cotton rat nares and cultivated in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) (Gibco-BRL) supplemented with 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 10% heat-inactivated fetal bovine serum, and 2 mM glutamine as previously described (24). A549

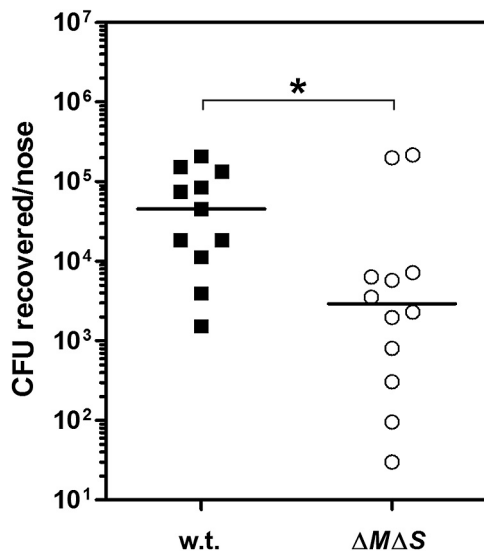


FIG 4 WTA glycosylation affects *S. aureus* nasal colonization *in vivo*. Cotton rats were intranasally challenged with the *S. aureus* USA300 wild-type (w.t.) strain and the USA300 $\Delta tarM \Delta tarS$ ($\Delta M\Delta S$) mutant lacking WTA glycosylation. Colonization status was analyzed 3 days after bacterial instillation. Statistically significant differences calculated using a D'Agostino and Pearson omnibus normality test and a subsequent Mann-Whitney test are indicated as follows: *, $P < 0.05$.

lung epithelial cells (American Type Culture Collection [ATCC CCL-185]) were purchased from the ATCC and grown in DMEM-F12 medium (Gibco-BRL) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 10% heat-inactivated fetal bovine serum, and 2 mM glutamine. Epithelial cells were grown at 37°C under 5% CO₂.

Interaction of *S. aureus* with epithelial cells. Adherence of *S. aureus* to epithelial cells was analyzed as described elsewhere (22). Briefly, approximately 5×10^4 A549, 2.5×10^4 CRNEC, or 2.5×10^4 HNEpC cells were seeded to 24-well culture plates and grown at 37°C under 5% CO₂ in appropriate cell culture media. Bacterial overnight cultures grown in B-medium at 37°C were diluted in MHB and grown at 37°C to the mid-logarithmic-growth phase, washed three times with phosphate-buffered saline (PBS), and labeled with fluorescein isothiocyanate (FITC; 0.1 mg/ml) for 1 h at 37°C. FITC-labeled bacteria were washed three times with PBS and resuspended in RPMI medium (Sigma, St. Louis, MO, USA). Bacterial cell numbers were adjusted using a Neubauer chamber. Confluent epithelial cell monolayers were washed twice with RPMI medium and inoculated with FITC-labeled bacteria for 1 h at 37°C under 5% CO₂. Parameters of multiplicities of infection (MOI) were chosen in a strain-dependent manner and are individually given in the corresponding figure legends. Subsequently, epithelial cell monolayers were washed twice with RPMI and subsequently with PBS. Cells were fixed at room temperature for 10 min using 3.5% paraformaldehyde (PFA)-PBS. PFA was removed, and wells were coated with 1 ml PBS. No morphological changes were observed after this procedure. Adherent bacteria per 0.1 mm² were counted (in at least 6 random fields of 0.1 mm²) using a fluorescence microscope.

Adherence under shear stress conditions was monitored in chamber slides (μ -Slide VI0.4; ibidi, Martinsried, Germany) as described before (24) with minor modifications. Briefly, approximately 2×10^4 HNEpC or 2×10^4 CRNEC cells were seeded to chamber slides and cultivated as described before. Confluent epithelial cell monolayers were washed twice with RPMI medium. Subsequently, peristaltic pumps (Amersham) were used for infection (20 min) with FITC-labeled bacteria at a flow rate of 1 ml/h, mimicking mild shear stress conditions at 0.3 dynes/cm². MOI parameters are given in the corresponding figure legends. Cell monolayers were washed in two consecutive steps using RPMI medium and PBS and fixed as described before. Adherent bacteria per 0.1 mm² were counted (in at least 8 random fields of 0.1 mm²) using a fluorescence microscope.

Adherence to extracellular matrix proteins. Adherence to extracellular matrix proteins was analyzed as described elsewhere (23) with minor modifications. Briefly, 96-well microtiter plates were coated with keratin (50 μ g/well), fibrinogen (2.5 μ g/well), or fibronectin (15 μ g/well) (all obtained from Sigma, St. Louis, MO, USA) mixed in sodium carbonate buffer (50 mM, pH 9.6) overnight at 4°C. Next, wells were blocked by using 100 μ l 2% bovine serum albumin (BSA)-PBS for 2 h at room temperature. Wells were washed with 200 μ l PBS and incubated with 100 μ l bacteria (5×10^9 CFU/ml) for 1.5 h at 37°C. Wells were washed three times with 200 μ l PBS. Bacteria were stained using 100 μ l crystal violet (2 mg/ml in PBS) for 2 min at room temperature. Subsequently, wells were washed five times with 400 μ l PBS. A 100 μ l volume of (70%) ethanol was added to each well, and the absorbance at 600 nm (A_{600}) was determined.

Experiments with phages. Phage Φ 11 susceptibility of *S. aureus* was analyzed as described before (35). When bacteria were grown under nasal growth conditions using SNM3 (containing 0.2 mM bipyridine), phage susceptibility was analyzed on solid SNM3 agar overlaid with SNM3 soft agar (0.4% agar). Phage Φ 11 adsorption to various *S. aureus* isolates grown in rich medium (B-medium) or in SNM3 (containing 0.2 mM bipyridine) was analyzed as described previously (35). Briefly, phage Φ 11 was propagated on the *S. aureus* RN4220 wild-type strain and used for adsorption experiments. The MOI was set to 0.1, and percent phage adsorption to test strains was calculated by determining the number of PFU of unbound phages in supernatants and subtracting that value from the total number of input PFU as a ratio to the total number of input PFU.

RNA isolation and preparation. B-medium overnight cultures were diluted in fresh media (B-medium or SNM3 [containing 0.2 mM bipyri-

dine]) and grown at 37°C. Bacteria were harvested in the lag-, log-, or stationary-growth phase and subsequently resolved in 1 ml TRIzol (Invitrogen/Life Technologies, Karlsruhe, Germany). Bacteria were mechanically disrupted with 0.5 ml zirconia-silica beads (Carl-Roth, Karlsruhe, Germany) (0.1 mm in diameter) using a FastPrep 24 homogenizer (MP Biomedicals) (2 cycles of 20 s at a speed of 6.5 m/s). Samples were stored at -80°C or subsequently used for further preparation. Chloroform (200 μ l) was added, and samples were thoroughly mixed for 60 s and incubated for 3 min at room temperature. Samples were centrifuged ($12,000 \times g$, 15 min, 4°C), and the supernatant was mixed with 500 μ l isopropanol. Samples were incubated for 10 min at room temperature and centrifuged ($12,000 \times g$, 30 min, 4°C). The pellet was washed with 500 μ l (70%) ethanol, and the sample was centrifuged at $7,500 \times g$ for 5 min (4°C). The pellet was air dried and dissolved in 50 μ l nuclease-free water. After incubation at 55°C for 10 min, the sample was mixed well for 4 min and 5 μ g RNA was digested with DNase I (Roche) and stored at -80°C .

qRT-PCR. The primers used for quantitative real-time PCR (qRT-PCR) are listed in Table S2 in the supplemental material. RNA was transcribed into cDNA, and qRT-PCR was performed according to the instructions of the manufacturer using Brilliant II SYBR green 1-Step master mix (Agilent). Relative quantifications were analyzed using Roche's LightCycler 480 II system. Transcription levels of target genes analyzed in this study were normalized against the expression of the *gyrB* housekeeping gene (38).

***S. aureus* killing by antimicrobial peptides.** Killing by antimicrobial peptides was analyzed as described before (22) with minor modifications. Briefly, *S. aureus* overnight cultures were diluted in MHB and grown to the mid-logarithmic-growth phase. Cells were washed 3 times in potassium phosphate buffer (PPB) (pH 7.5) containing 0.005% human serum albumin (HSA) (PPB-HSA). Approximately 2.0×10^5 bacteria (in PPB-HSA) were treated with 10 μ g/ml LL-37 (Sigma, St. Louis, MO, USA). Killing was analyzed over a time period of 180 min. Each reaction was stopped by addition of 250 μ l ice-cold PPB-HSA. Serial dilutions were plated on appropriate agar plates to analyze killing by determination of CFU, and the results are given as a ratio to the total number of input CFU.

Cotton rat nasal colonization model. For analysis of *S. aureus* nasal colonization *in vivo*, a cotton rat model was used (22). Cotton rats continuously received drinking water containing 2.5 mg/ml streptomycin to reduce natural nasal flora 3 days before the experiment. The cotton rats were anesthetized and instilled intranasally with 10 μ l of 1×10^7 CFU of the *S. aureus* USA300 wild type or USA300 Δ tarM Δ tarS. Three days after bacterial instillation, animals were euthanized and nares were removed surgically. Nares were subjected to vortex mixing for 30 s in 1 ml PBS containing 0.5% Tween 20. Serial dilutions were plated on appropriate agar plates to calculate nasal colonization data.

Ethics statement. All animal experiments were performed in strict accordance with the German regulations of the Gesellschaft für Versuchstierkunde/Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the Regierungspräsidium Tübingen, Tübingen, Germany (permit no. T1/10).

Statistical analysis. Statistical analysis was performed by using Graph-Pad Prism (GraphPad Software, Inc., La Jolla, USA; version 5.04). Statistically significant differences were calculated by using appropriate statistical methods as indicated. *P* values of ≤ 0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00632-15/-/DCSupplemental>.

Figure S1, TIF file, 0.3 MB.

Figure S2, TIF file, 0.2 MB.

Figure S3, TIF file, 0.2 MB.

Figure S4, TIF file, 0.1 MB.

Figure S5, TIF file, 0.5 MB.

Table S1, DOCX file, 0.04 MB.

Table S2, DOCX file, 0.02 MB.

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