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Surface Glycopolymers Are Crucial for *In Vitro* Anti-Wall Teichoic Acid IgG-Mediated Complement Activation and Opsonophagocytosis of *Staphylococcus aureus*

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The cell envelopes of many Gram-positive bacteria contain wall teichoic acids (WTAs). *Staphylococcus aureus* WTAs are composed of ribitol phosphate (RboP) or glycerol phosphate (GroP) backbones substituted with D-alanine and *N*-acetyl-D-glucosamine (GlcNAc) or *N*-acetyl-D-galactosamine (GalNAc). Two WTA glycosyltransferases, TarM and TarS, are responsible for modifying the RboP WTA with α-GlcNAc and β-GlcNAc, respectively. We recently reported that purified human serum anti-WTA IgG specifically recognizes β-GlcNAc of the staphylococcal RboP WTA and then facilitates complement C3 deposition and opsonophagocytosis of *S. aureus* laboratory strains. This prompted us to examine whether anti-WTA IgG can induce C3 deposition on a diverse set of clinical *S. aureus* isolates. To this end, we compared anti-WTA IgG-mediated C3 deposition and opsonophagocytosis abilities using 13 different staphylococcal strains. Of note, the majority of *S. aureus* strains tested was recognized by anti-WTA IgG, resulting in C3 deposition and opsonophagocytosis. A minority of strains was not recognized by anti-WTA IgG, which correlated with either extensive capsule production or an alteration in the WTA glycosylation pattern. Our results demonstrate that the presence of WTAs with TarS-mediated glycosylation with β-GlcNAc in clinically isolated *S. aureus* strains is an important factor for induction of anti-WTA IgG-mediated C3 deposition and opsonophagocytosis.

Staphylococcus aureus can cause serious infections of the skin, soft tissues, and bloodstream in the community and in hospitalized patients (1). To establish successful infection, *S. aureus* deploys a variety of survival and immune evasion strategies, such as the acquisition of essential nutrients and expression of adhesins, which promote colonization and survival, and the production of virulence factors, such as capsules and toxins, which aid host immune evasion (2, 3). The recent spread of methicillin-resistant *S. aureus* (MRSA) increases the necessity of treating infections better. Unfortunately, many efforts to develop an efficacious vaccine against *S. aureus* have failed (4, 5). The putative reasons for this failure in vaccine clinical trials were assumed to be due to a focus on vaccines with single target antigens stimulating humoral defense rather than vaccines with a combination of target antigens stimulating both humoral and cellular immunity.

S. aureus is a Gram-positive bacterial pathogen that is surrounded by glycopolymers, including wall teichoic acid (WTA), peptidoglycan, lipoteichoic acid, and capsular polysaccharide (CP). These bacterial surface glycopolymers are recognized by serum antibodies and a variety of pattern recognition molecules, including mannose-binding lectin (MBL) (6, 7). Bacterial WTAs are involved in bacterial cell wall maintenance, susceptibility to antimicrobial molecules, biofilm formation, and host interaction (8, 9). Most MRSA strains, such as USA300, COL, and MW2, express poly(ribitol phosphate) (RboP) WTA, which is composed of 10 to 40 RboP repeating units (10). The hydroxyls on the RboP repeats are modified with D-alanine and *N*-acetylglucosamines (GlcNAc) via an α- or β-GlcNAc anomer (11). However, some strains, such as *S. aureus* PS187 (ST395 lineage), have recently been found to produce a unique poly(glycerol phosphate) (GroP) WTA modified with *N*-acetyl-D-galactosamine (GalNAc) (12). Furthermore, the molecular elucidation of WTA biosynthesis pathways in *S. aureus* paved the way for the identification of two WTA glycosyltransferases, TarM and TarS, responsible for modifying RboP with either α-GlcNAc or β-GlcNAc, respectively (13, 14). In addition, analysis of the WTA biosynthesis pathway in the *S. aureus* sequence type 395 (ST395) lineage revealed a novel WTA glycosyltransferase, TagN, which is involved in modification of GroP WTA with α-GalNAc (12, 15). These studies help provide an understanding of how *S. aureus* cells produce variable WTA types and elucidate the functional importance of WTA structure variation during infections.

The human complement system is the first line of host defense responses to invading pathogens (16). Pathogen-specific serum antibodies activate the classical complement pathway (17). Hu-

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man serum MBL binds to a mannose or GlcNAc residue of bacterial surface sugar chains (18) and functions as an opsonin activating the lectin complement pathway (6). The activation of the classical and lectin pathways mediates opsonization by complement fragments, such as C4b and C3b. The opsonized pathogens are engulfed by phagocytes, which are recruited by C3a and C5a anaphylatoxins (17). Therefore, functional determination of the bacterial ligand moiety recognized by serum antibody during opsonophagocytosis is important for understanding the host-microbe interaction and for prevention of *S. aureus* infections.

Recently, we reported that *S. aureus* WTA functions as a ligand of MBL (19). Intriguingly, serum MBL from infants who had not yet fully developed adaptive immunity could bind to *S. aureus* WTA and induce complement C3 deposition. Additionally, the purified anti-WTA IgG from adults' sera strongly induced activation of the classical complement pathway, leading to the opsonophagocytosis of *S. aureus* cells (20). We further determined that anti-WTA IgG and MBL require the GlcNAc residues of *S. aureus* WTAs for complement activation (21). Namely, although anti-WTA IgG-mediated classical and MBL-mediated lectin complement activation and opsonophagocytosis are required for the β-GlcNAc residue of WTA, α-GlcNAc residues of WTA have hardly any and only a low capacity to activate both the classical and lectin complement pathways (21). Also, we have demonstrated *in vivo* the protective efficacy of anti-WTA antibodies against two clinical MRSA strains, COL and MW2 (22).These studies reveal that the sugar moiety of WTA is an important molecular determinant in host immune responses to *S. aureus* infection *in vitro* and *in vivo*.

However, we wondered whether our purified human anti-WTA IgG can recognize other clinically isolated *S. aureus* strains, which may harbor different WTA backbone structures, different glycosylation patterns, or different amounts of CPs on the bacterial surfaces (15, 23). We assumed that the determination of a spectrum of staphylococci recognized by serum anti-WTA IgG may be valuable for designing efficacious passive immunization against infections caused by different staphylococci. Also, to design an efficient WTA vaccine target antigen, it is vital to determine the exact WTA glycosylation pattern and WTA backbone structures of diverse staphylococcal strains.

The staphylococcal CPs play important roles in pathogenesis during *S. aureus* infection by impeding phagocytosis, resulting in bacterial persistence in the bloodstream of infected host organisms (24). Two major staphylococcal CPs, serotype 5 CP (CP5) and serotype 8 CP (CP8), predominate among clinically isolated strains from humans (24). These CPs have been reported to decrease *in vitro* complement-mediated opsonophagocytosis and to increase lethality in a mouse infection model (25). Previously, a careful study was carried out to estimate the ability of the complement C3 component to bind to different *S. aureus* strains by injection of six different encapsulated *S. aureus* strains into intact and C3-depleted mice (26). However, in that study, the molecular reasons why there was no straight relationship between the CP amounts of *S. aureus* strains and the deposited C3 amounts were not clearly answered. Therefore, we supposed that elucidation of the reason why serum antibody-mediated C3 deposition was not induced on some CP-producing strains is important for understanding the molecular interaction between host and microbes.

To examine how the amounts of CP produced, different WTA backbone structures, and the WTA glycosylation pattern affect anti-WTA IgG recognition of 13 different *S. aureus* strains, we analyzed anti-WTA IgG-mediated C3 deposition and opsonophagocytosis, the phage susceptibility, and the genotypes of 13 different staphylococcal strains. Those 13 strains consisted of 6 CP-producing strains, 1 CP-deficient mutated strain, 1 *S. aureus* ST395 clone (PS187), 1 *S. epidermidis* strain, 3 MRSA strains, and 1 laboratory strain (Table 1). Of note, complement C3 deposition on three strains failed, including *S. aureus* Lowenstein and PS187 and *S. epidermidis*, while anti-WTA IgG-mediated opsonophagocytosis of five strains was not induced. Genotyping and phage susceptibility patterns revealed that rare alterations in the WTA backbone structure, glycosylation pattern, or overt CP production can interfere with the binding of anti-WTA IgG to a certain extent. Our combined analytical techniques revealed possible reasons why C3 deposition and opsonophagocytosis were not induced for some of the strains, providing valuable information offering an understanding of the molecular interaction between host and microbes.

TABLE 1 *S. aureus* and *S. epidermidis* strains used in this study

Strain	Relevant characteristics[a]	Reference
S. aureus RN4220	Restriction mutant	39
S. aureus M0107	RN4220 Δ*spa*::Phl	40
S. aureus T790	M0107 Δ*tarM*::Erm	21
S. aureus T803	M0107 Δ*tarS*::Km	21
S. aureus T807	M0107 Δ*tarM*::Erm Δ*tarS*::Km	21
S. aureus USA300	CA-MRSA	14
S. aureus USA300 Δ*tarM*	USA300 Δ*tarM*	41
S. aureus USA300 Δ*tarS*	USA300 Δ*tarS*	14
S. aureus USA300 Δ*tarMS*	USA300 Δ*tarM* Δ*tarS*	41
S. aureus COL	HA-MRSA	42
S. aureus MW2	CA-MRSA	43
S. aureus Becker	CP8	44
S. aureus Reynolds	CP5	44
S. aureus JL022	CP deficient	31
S. aureus M (NCTC 10649)	CP1	45
S. aureus Wright	CP8	46
S. aureus Smith diffuse	CP2	47
S. aureus Lowenstein	CP5	48
S. aureus PS187	ST395 isolate	12
S. epidermidis ATCC 14990 (Fussel)	Coagulase negative	49

[a] CA-MRSA, community-associated MRSA; HA-MRSA, health care-associated MRSA.

MATERIALS AND METHODS

Ethics statement. We obtained approval for this study specifically from the Institutional Review Board of Pusan National University. For the collection of human polymorphonuclear leukocytes (PMNs) from adults, we also obtained written informed content from all healthy participants.

Proteins, sera, bacteria, and reagents. Native human MBL/MBL-associated serine protease (MASP) complex was purified from human serum as described previously (27). *S. aureus*-treated serum was prepared as described previously (20) using the *S. aureus* M0107 strain (Δ*spa*), which is deficient in immunoglobulin-binding protein A. *S. aureus* strains were grown at the appropriate temperature in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing, where appropriate, 100 μg/ml ampicillin, 10 μg/ml erythromycin, 50 μg/ml kanamycin, 12.5 μg/ml chloramphenicol, or 20 μg/ml phleomycin. Encapsulated strains were cultivated in Columbia medium supplemented with 2% NaCl to enhance capsule production. The bacteria were fixed with ethanol in order to (i) inhibit bacterial cell growth, which has been shown in previous studies to be optimal for the *in vitro* interaction of *S. aureus* cells with serum antibodies and human MBL (19–21), and to (ii) exclude the effects

of bacterial secreted protein and cell surface molecules, which might be factors differentially released from various *S. aureus* strains and affect complement activation and opsonophagocytosis.

Purification of WTA from *S. aureus*. *S. aureus* WTA was prepared as described previously (28) with some modifications. In brief, WTA-bound insoluble peptidoglycan was prepared and treated with 5% (wt/vol) trichloroacetic acid for 18 h at room temperature to release the WTA from peptidoglycan. The obtained WTA was further purified by anion-exchange column chromatography as described previously (21).

Purification of anti-WTA IgG. Anti-WTA IgG was purified from commercially available human intravenous IgG (IVIG; Green Cross, South Korea) using a WTA-coated nitrocellulose membrane as described previously (19) with the following modifications. Briefly, 100 μg of peptidoglycan-linked WTA in 200 μl of phosphate-buffered saline (PBS) was prepared from *S. aureus* strain T384 (RN4220 Δ*lgt*::Phl Δ*oatA*::Erm) as described previously (19), spotted onto a nitrocellulose membrane (10 by 90 mm; pore size, 0.45 μm; Whatman), and baked at 100°C for 1 h. The membranes were washed with buffer A (20 mM Tris-HCl, pH 7.4, 250 mM NaCl) and blocked with buffer B (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% bovine serum albumin [BSA]) for 2 h at 4°C. Three sheets of the membrane were incubated with 50 mg of IVIG in 40 ml of buffer C (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% BSA) for 2 h at 4°C. After washing with buffer A, bound IgGs were eluted with 1 ml of 0.1 M glycine (pH 2.8) and immediately neutralized with 1 M KOH to pH 7.5. The glycine in the eluted IgG fraction was removed by passing the fraction through a Vivaspin 20 centrifugal concentrator (Sartorius) three times in the presence of buffer A. To remove the anti-peptidoglycan IgGs, the IgG fraction obtained was incubated at 4°C with *S. aureus* Δ*spa* Δ*tagO* double mutant cells that had been prefixed with formaldehyde. Then, the *S. aureus* double mutant cells were pelleted by centrifugation, and the supernatant was collected, concentrated with the Vivaspin 20 concentrator, and used as a purified anti-WTA IgG fraction.

Flow cytometry analysis of *S. aureus* cells. The amounts of bound IgG on *S. aureus* cells were measured as described previously (21). Briefly, ethanol-fixed *S. aureus* cells (4 μl of a suspension with an A_{600} of 3) were incubated with human anti-WTA IgG (50 ng) in 20 μl of incubation buffer (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10 mM $CaCl_2$, 1% BSA) on ice. The cells were washed with washing buffer (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10 mM $CaCl_2$) and incubated with mouse anti-human IgG monoclonal antibody (MAb; diluted 1:200; Sigma) as the primary antibody, followed by goat $F(ab')_2$ anti-mouse IgG antibodies conjugated with fluorescein 5-isothiocyanate (FITC; diluted 1:200; Beckman Coulter). The washed *S. aureus* cells were sonicated for 15 s to disperse clumped cells before flow cytometry analyses (Accuri C6; Beckman Coulter). To detect C3 deposition, ethanol-fixed cells were incubated with *S. aureus*-treated serum (10%) with or without MBL/MASP (10 ng) or anti-WTA IgG (50 ng) in 20 μl of incubation buffer for 60 min at 37°C. Then, bound C3b was detected using mouse anti-human C3 MAb conjugated with FITC (diluted 1:200; Beckman Coulter).

Isolation of human PMNs and opsonophagocytosis assay. PMNs were isolated from healthy donors using Polymorphprep solution (Nycomed Pharm As, Torshov, Norway) as described previously (20). The PMNs were 99% viable, as shown in a trypan blue dye exclusion test. An opsonophagocytosis assay was performed with minor modifications as described previously (20). *S. aureus* cells grown to a postexponential growth phase in LB or Columbia medium were washed, killed with 70% ethanol, labeled with 0.1 mM FITC (Sigma) in 0.1 M Na_2CO_3 buffer (pH 8.5) for 30 min at room temperature, and resuspended in Hanks' balanced salt solution. FITC-labeled bacteria (in an amount equivalent to 1.5×10^7 CFU) were opsonized with 10% *S. aureus*-treated serum with purified anti-WTA IgG in 20 μl of Hanks' balanced salt solution containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, and 0.4% BSA for 30 min at 37°C with shaking. A PMN suspension (1.5×10^5 cells, 35 μl) was added to 5 μl of opsonized bacteria (corresponding to 3.7×10^6 CFU; multiplicity of infection, ~25) and incubated at 37°C for 60 min with shaking. The phagocytosed FITC-labeled *S. aureus* cells in the PMNs were counted by fluorescent phase-contrast microscopy. More than 100 PMNs were counted. Extracellular FITC-labeled *S. aureus* cells were quenched by 0.2% trypan blue.

Experiments with phages. Phage susceptibility was analyzed using a soft-agar spot assay as described previously (13). Briefly a phage panel including the broad-host-range phages ΦK (13) and Φ812 (29), serogroup B phage Φ11 (13), and serogroup L phage Φ187 (29) was used, and lysates were spotted onto bacterial lawns and analyzed after overnight incubation for macroplaque formation. Phage adsorption was determined as described previously (12), except that the multiplicity of infection was set equal to 0.1 for both phages Φ11 and Φ187. Phages ΦK, Φ812, and Φ11 were propagated on *S. aureus* RN4220 cells. Phage Φ187 was propagated on *S. aureus* PS187 cells as described previously (12).

Molecular genotyping of *S. aureus* WTA glycosyltransferases. The *tarM*, *tarS*, and *tagN* genes were amplified from genomic DNA using the primers (Eurofins Genomics, Germany) listed in Table S1 in the supplemental material. Publicly available reference genomes were analyzed for the presence of *tarM*, *tarS*, or *tagN* using the BLAST program (30).

Sequence analysis of the *tarS* deletion in *S. aureus* Lowenstein. Whole-genome sequencing (WGS) was performed using an Illumina MiSeq system (Illumina, CA). Standard Illumina libraries for two 250-bp runs were made using a Nextera XT kit according to the manufacturer's instructions (Illumina, CA). CLC Bio's Genomic Workbench (version 8.0.1; CLC Bio, Denmark) was used for the *de novo* assembly and alignment of contigs against the *S. aureus* NCTC 8325 reference genome (GenBank accession no. NC_007795).

Processing and statistical analysis. The results from the quantitative analyses are expressed as the mean ± standard deviation (SD) of the data from at least three independent experiments, unless otherwise stated. Other data were representative of those from at least three independent experiments that yielded similar results. The statistical analyses were performed using Student's *t* test. *P* values of less than 0.05 were considered statistically significant and are indicated in the figures. The statistical analyses were performed using SPSS statistical analysis software. Differences between groups were analyzed by an unpaired Student's *t* test. *P* values of less than 0.05 were considered significant and are indicated in the figures.

Accession number. Raw reads of whole-genome sequence data for *S. aureus* Lowenstein were deposited in the NCBI Short Read Archive under the study accession number SRP061258.

RESULTS

WTA β-GlcNAc governs human anti-WTA IgG-mediated C3 deposition against USA300. First, because we used ethanol-fixed bacteria to examine anti-WTA IgG-mediated C3 deposition, it was necessary to address the effects of ethanol treatment on the *S. aureus* strains. For this experiment, we used two different *S. aureus* strains under two different conditions: live and alcohol-fixed *S. aureus* strains RN4220 and USA300. After incubation of these four bacterial samples with 10% Δ*spa* mutant-treated human serum and anti-WTA IgG, C3 deposition capabilities were examined via fluorescence-activated cell sorter analyses. As shown in Fig. S1 in the supplemental material, C3 deposition was satisfactory under all four conditions, while the amount of bound C3 was lower in the live bacteria (groups b and d) than alcohol-fixed bacteria (groups a and c), confirming that ethanol treatment of *S. aureus* cells leads to a clearer C3 deposition capacity and does not inhibit serum-mediated complement activation.

In our previous study, we used cells of the *S. aureus* RN4220 parental strain, a α-GlcNAc-deficient Δ*tarM* mutant, a β-GlcNAc-deficient Δ*tarS* mutant, and a Δ*tarMS* double mutant to examine the relationship between WTA glycosylation and human MBL- or anti-WTA IgG-dependent complement activation (19–21). Since RN4220

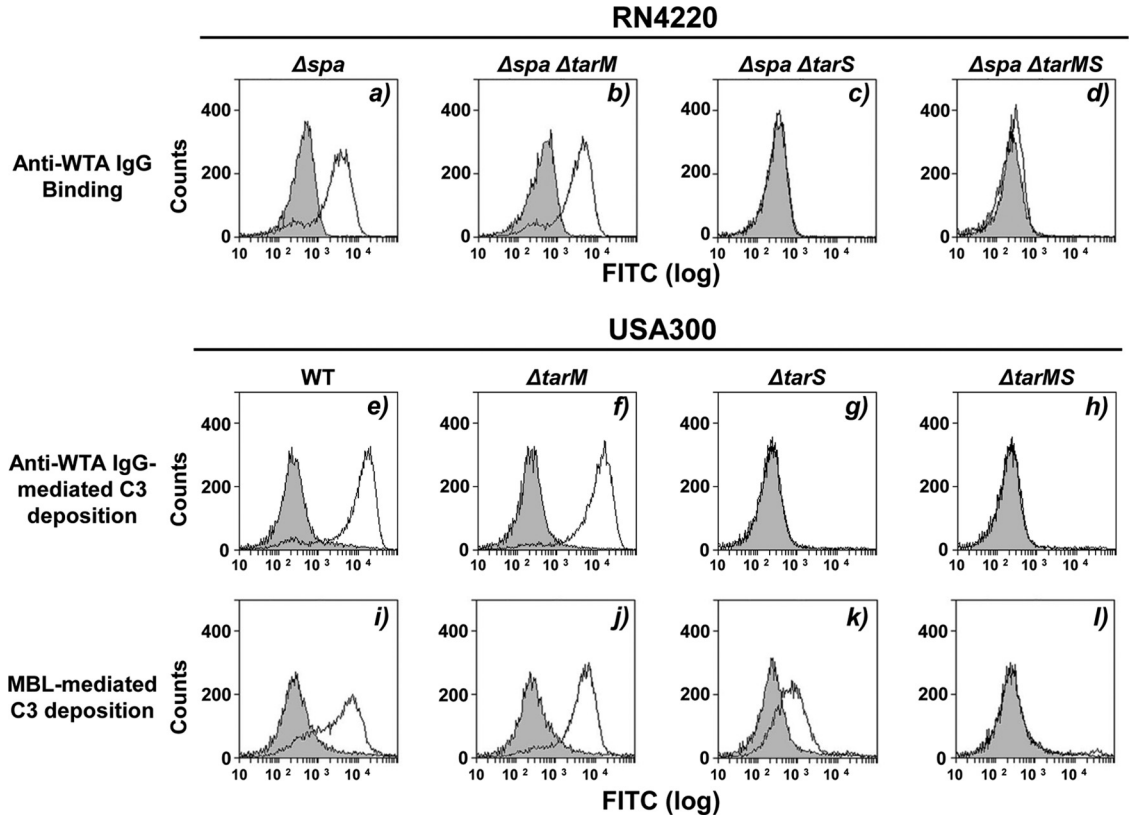

FIG 1 WTA glycosylation-dependent C3 deposition via purified anti-WTA IgG or MBL on *S. aureus* MRSA strain USA300. (a to d) Ethanol-killed *S. aureus* RN4220 mutant cells were incubated without (gray area) or with (area outlined by a black line) anti-WTA IgG (50 ng) in 20 μl of buffer, and bound IgG was detected by flow cytometric analysis. (e to l) Measurement of C3 deposition on USA300 mutant strains incubated in 10% Δ*spa* mutant-treated human serum without (gray area) or with (area outlined by a black line) anti-WTA IgG (50 ng) in 20 μl of buffer. C3 was detected by flow cytometric analysis with specific antibodies. The method used for the preparation of Δ*spa* mutant-treated human serum is described in Materials and Methods. The results are representative of those from three independent experiments.

is a laboratory strain previously mutagenized with a chemical mutagen and we did not examine purified anti-WTA IgG-dependent C3 deposition on clinical strains in our previous study (21), we assumed that C3 deposition and opsonophagocytosis should be retested with clinically important strains, such as the USA300 strain. For this purpose, the binding specificity of purified human serum anti-WTA IgG was addressed (Fig. 1a to d). As described previously, purified anti-WTA IgG bound to RN4220 lacking the IgG-binding protein A (Δ*spa* mutant) and to the Δ*spa* Δ*tarM* double mutant but not to the Δ*spa* Δ*tarS* mutant or the Δ*spa* Δ*tarMS* mutant (Fig. 1a to d), suggesting that the purified anti-WTA IgG has a strong binding specificity for staphylococcal WTA β-GlcNAc residues.

Next, we examined anti-WTA IgG- or human MBL-mediated C3 deposition on the USA300 wild type and corresponding mutants (Fig. 1e to l). Anti-WTA IgG induced C3 deposition on both the USA300 parental and Δ*tarM* strains but not on the Δ*tarS* and Δ*tarMS* mutants (Fig. 1e to h). Human MBL induced C3 deposition on the parental USA300 strain and the Δ*tarM* or Δ*tarS* mutants but not on the Δ*tarMS* double mutant. Of note, β-GlcNAc WTA has a stronger capacity to stimulate MBL-dependent complement activation than α-GlcNAc, as described in our previous study (21). These results suggest that human anti-WTA IgG is specific for β-GlcNAc-modified RboP WTA of the USA300 strain.

C3 deposition and opsonophagocytosis are induced for the majority of *S. aureus* isolates recognized by anti-WTA IgG. We wondered whether purified anti-WTA IgG can also induce complement-mediated C3 deposition on diverse *S. aureus* strains. Four mildly CP-producing strains, *S. aureus* Becker (CP8), Wright (ATCC 49525, CP8), Lowenstein (ATCC 49521, CP5), and Reynolds (CP5), were selected. Also, the two heavily CP-producing strains M (ATCC 49951, CP1) and Smith diffuse (ATCC 13709, CP2) were selected. As a control, a CP-deficient strain (JL022) constructed from the Reynolds strain by allelic replacement mutagenesis of a CP biosynthesis gene was included (31). Also, RN4220 and three clinically isolated MRSA strains (USA300, COL, and MW2), which are known to produce β-GlcNAc- and α-GlcNAc-substituted RboP backbones of WTA (13–15), were included. Finally, two special staphylococcal strains, *S. aureus* ST395 isolate PS187 and *S. epidermidis* strain ATCC 14990, both of which produce distinct WTA backbone structures and glycosylation patterns (12, 32), were included in the analyses.

As shown in Fig. 2a to j, anti-WTA IgG-mediated C3 deposition was induced on the eight strains, supporting the possibility of the presence of exposed WTAs on their bacterial surfaces. However, C3 deposition failed on the three strains *S. aureus* Lowenstein and PS187 and *S. epidermidis* ATCC 14990 (Fig. 2k to m). The failure of C3 deposition on the PS187 and *S. epidermidis* strains was expected due to the previously reported production of GroP WTA types modified with either GalNAc or α-Glc/α-GlcNAc (12, 32), respectively, highlighting the specificity of

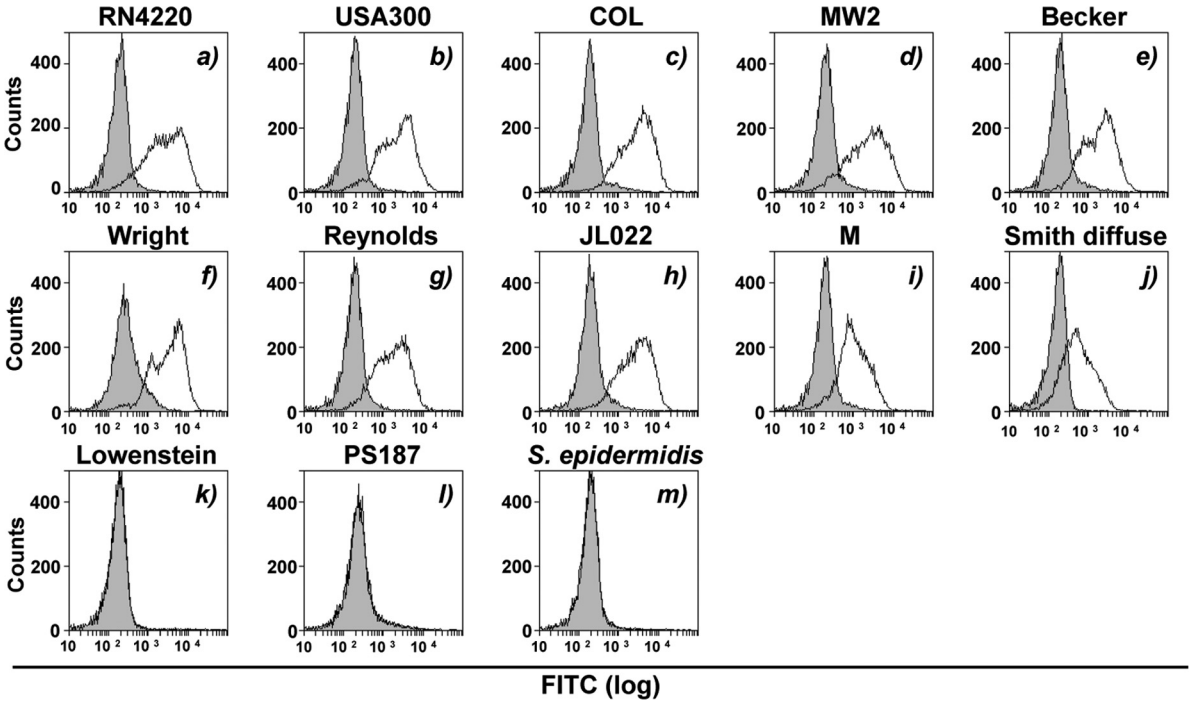

FIG 2 Anti-WTA IgG-mediated C3 deposition on various staphylococcal strains. Cultured bacterial cells were collected by centrifugation, washed with PBS three times, and treated with ethanol for further experiments. C3 deposition was measured in 10% Δ*spa* mutant-treated human serum without (gray area) or with (area outlined by a black line) anti-WTA IgG (50 ng). Bound C3 was detected with specific antibodies by flow cytometric analysis. The results are representative of those from three independent experiments.

our anti-β-GlcNAc antibody for RboP-GlcNAc WTA types. The reason why C3 deposition was not induced on the mildly CP-producing Lowenstein strain is provided below.

To further address the inability of C3 to be deposited on these three strains, we next examined anti-WTA IgG-mediated opsonophagocytosis (Fig. 3). To estimate anti-WTA IgG-mediated opsonophagocytosis, we counted the FITC-labeled bacterial cells engulfed by 100 PMNs under a fluorescence microscope. Anti-

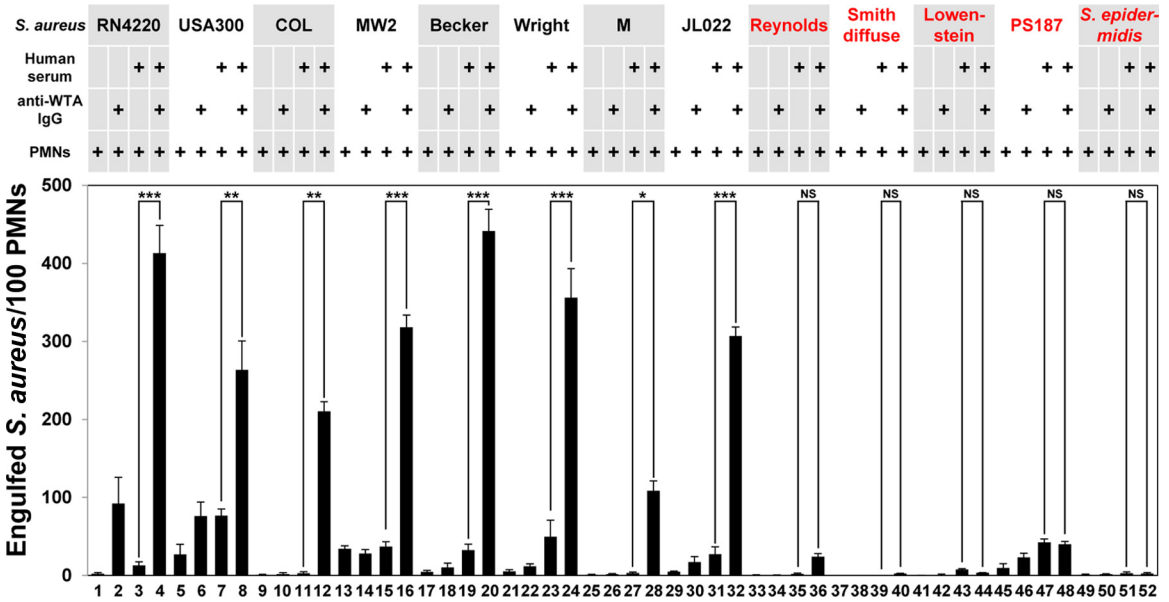

FIG 3 Anti-WTA IgG does not induce opsonophagocytosis of some strains. Ethanol-killed bacterial cells were labeled with FITC (0.1 mM) and opsonized without or with Δ*spa* mutant-treated human serum (10%). Purified anti-WTA IgG (50 ng) was simultaneously added as indicated. Opsonized FITC-labeled bacterial cells were incubated with human PMNs (1×10^5) at a multiplicity of infection of 25 in RPMI 1640 medium at 37°C for 1 h. The number of phagocytosed *S. aureus* cells per 100 PMNs was counted by fluorescent phase-contrast microscopy. Data are presented as the means ± SDs (error bars) of the results from three independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

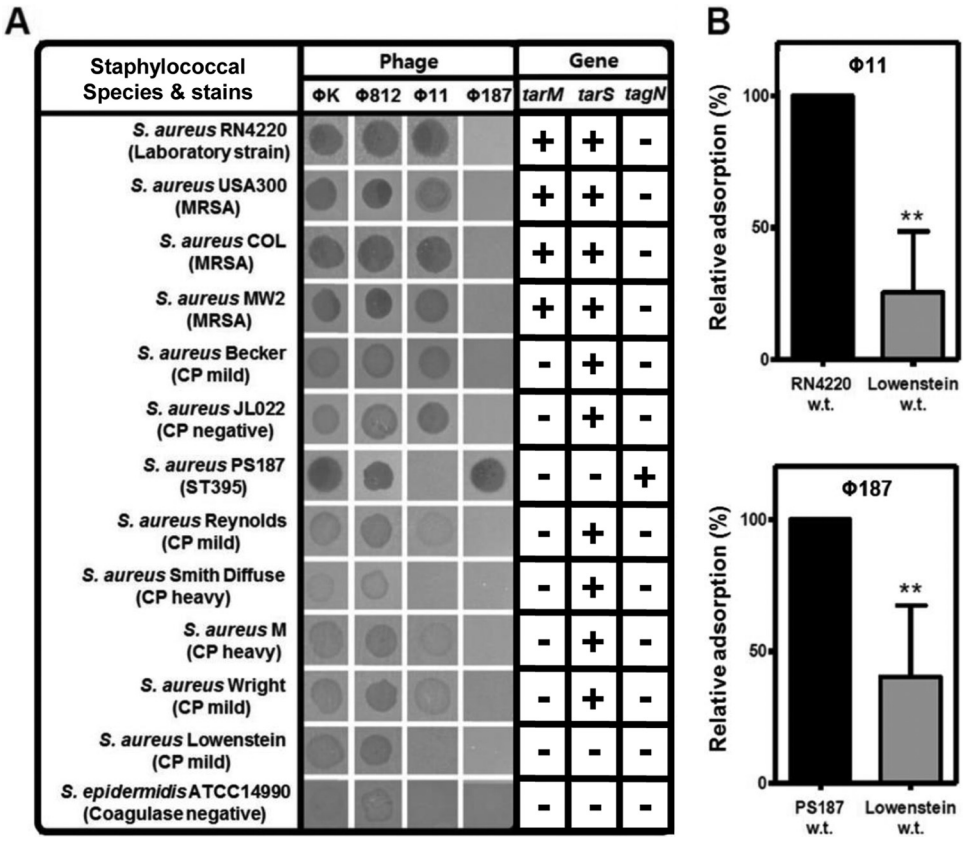

FIG 4 Correlation between phage susceptibility and the presence of WTA glycosyltransferases. The phage susceptibilities of various *S. aureus* wild-type strains and *S. epidermidis* strain ATCC 14990 were determined. Lysates were spotted onto bacterial lawns, and macroplaque formation was analyzed after overnight incubation. The presence (+) or absence (−) of the WTA glycosyltransferase-encoding genes *tarM*, *tarS*, and *tagN* in the corresponding genome is indicated. The *tarM*, *tarS*, and *tarN* genes were amplified from genomic DNA by using the corresponding primers listed in Table S1 in the supplemental material. (B) Rates of phage Φ11 or Φ187 absorption by the *S. aureus* Lowenstein wild-type (w.t.) strain. The results are representative of those from three independent experiments. The rate of phage adsorption relative to the rate of adsorption of phage Φ11 or Φ187 by strain RN4220 or PS187, which was set equal to 100%, is shown, and results are given as means ± SDs ($n = 3$). Statistically significant differences compared with the results for the strains that absorbed the phages were determined by the unpaired two-tailed Student's *t* test. **, $P < 0.001$ to 0.01.

WTA IgG-mediated opsonophagocytosis of RN4220 (Fig. 3, column 4) and three MRSA strains (Fig. 3, columns 8, 12, and 16) was induced. Opsonophagocytosis of mildly CP-producing strains, such as Becker and Wright, and the heavily CP-producing M strain also occurred (Fig. 3, columns 20, 24, and 28), reflecting C3 deposition. However, although C3 deposition was induced on the mildly CP-producing Reynolds and heavily CP-producing Smith diffuse strains (Fig. 2g and j), anti-WTA IgG-mediated opsonophagocytosis of these two strains was not induced in a statistically significant fashion (Fig. 3, columns 36 and 40), indicating that CP production by these two strains may protect these bacteria from opsonophagocytosis. Under the same conditions, the JL022 strain, a CP-deficient mutant constructed from strain Reynolds, was opsonophagocytosed by PMNs (Fig. 3, column 32). As expected, anti-WTA IgG-mediated opsonophagocytosis of three strains, strains Lowenstein and PS187 and the *S. epidermidis* strain, was not induced, as found as described above for C3 deposition (Fig. 3, columns 44, 48, and 52). Taken together, our results suggest that PMN-mediated opsonophagocytosis cannot be directly correlated with CP production on some *S. aureus* strains.

Complement C3 deposition strongly correlates with phage Φ11 susceptibility and glycosylated β-GlcNAc (β-GlcNAcylated) RboP WTA. To further elucidate the relationship between C3 deposition and WTA backbone structures or WTA glycosylation patterns, we used a set of 4 different staphylococcal phages, including ΦK, Φ812, Φ11, and Φ187. Lytic *S. aureus* phages, such as ΦK and Φ812, are known to recognize diverse WTA backbones of either the RboP or GroP type (12, 13, 33, 34), while Φ11 recognizes GlcNAc-modified RboP WTA for efficient absorption and infection (12, 33). In contrast, phage Φ187 is specific for GalNAc-modified GroP WTA (34). As expected, all 13 tested strains were susceptible to ΦK and Φ812, suggesting that WTAs are produced on their surfaces (Fig. 4A). Among them, complement-activating strains, such as RN4220, the three MRSA strains tested, Becker, and CP-negative strain JL022 were susceptible to Φ11, indicating that these strains express GlcNAc-modified RboP WTA on their cell surfaces. However, other CP-producing strains, the Reynolds, M, and Wright strains, retained only weak susceptibility to phage Φ11, supporting the notion that these strains produce GlcNAc-modified RboP WTA but that production of mild CPs may interfere with the absorption of Φ11. Interestingly, Smith diffuse, which was sensitive to C3 deposition, was susceptible to ΦK and Φ812 but resistant to Φ11 and Φ187. Since Smith diffuse bears *tarS* within the WTA *tar* gene cluster, facilitating β-GlcNAc-mod-

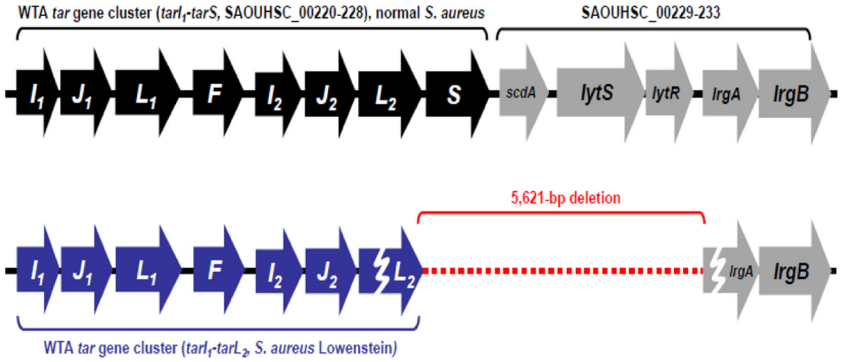

FIG 5 The complement-evading *S. aureus* strain Lowenstein carries a large deletion in the *tarS*-encoding region. Whole-genome sequencing revealed a 5,621-bp deletion in the *tarS*-encoding region which includes the 3′ end of $tarL_2$, *scdA*, *lytS*, *lytR*, and the 5′ end of *lrgA*. The genetic organization of the WTA *tar* gene cluster of *S. aureus* reference strain NCTC 8325 (top) was compared to that of the WTA *tar* gene cluster of strain Lowenstein (bottom). $tarL_2$ and *lrgA* most likely represent pseudogenes in strain Lowenstein (broken arrows). Gene locus numbers are indicated (note that, for some reason, SAOUHSC_002224 is missing in NCTC 8325).

ified RboP WTA biosynthesis, β-GlcNAc-harboring RboP WTA is likely produced (see below; Fig. 4A). The nonsusceptibility of the Smith diffuse strain to phage Φ11 might be caused by heavy CP production. On the other hand, the Lowenstein strain was resistant to C3 deposition, Φ11, and Φ187, suggesting that either (i) CP production may mask WTA glycosylation or (ii) a distinct WTA glycosylation type might be present on this strain (Fig. 4A). When the relative absorption rate of phage Φ11 or Φ187 for *S. aureus* Lowenstein was determined, the relative adsorption rate was decreased about 75% and 60% compared to that for the RN4220 and PS187 strains, respectively (Fig. 4B), supporting the results presented in Fig. 4A. The only Φ187-susceptible strain was PS187, which was in agreement with previous observations (12). Finally, *S. epidermidis* strain ATCC 14990 weakly retained susceptibility to phages ΦK and Φ812 but was resistant to phages Φ11 and Φ187, which is consistent with its WTA structure having a GroP WTA backbone with a α-Glc/α-GlcNAc modification (32).

To explain the different phage susceptibilities of the *S. aureus* strains and their correlation with complement-mediated C3 deposition and opsonophagocytosis, we further checked for the presence or absence of WTA glycosyltransferases encoded by the *tarM*, *tarS*, and *tagN* genes via PCR (Fig. 4A). Notably, all strains on which C3 was deposited, such as RN4220, the three MRSA strains, Becker, M, Wright, and JL022, carried *tarS*, and some also carried *tarM*. However, despite the presence of *tarS*, the heavily CP-producing Smith diffuse strain was resistant to Φ11 and anti-WTA IgG-mediated opsonophagocytosis, suggesting that β-GlcNAc-modified RboP WTA might be masked by surface CPs. Other complement-evading strains were either positive for *tagN* (PS187) or negative for all WTA glycosyltransferases described so far, TagN, TarS, and/or TarM. Moreover, whole-genome sequencing of *S. aureus* strain Lowenstein, on which complement was not deposited and whose genome lacked *tarM* and *tagN*, revealed a unique 5,621-bp deletion encompassing the *tarS*-encoding region (Fig. 4A and 5). The deletion also comprised the 3′ end of $tarL_2$ as well as *scdA*, *lytS*, *lytR*, and the 5′ end of *lrgA*, suggesting that this strain synthesizes nonglycosylated WTA. Accordingly, altered WTA structures strongly correlate with complement evasion (12, 32). Taken together, these results demonstrate that anti-WTA IgG-mediated C3 deposition and opsonophagocytosis strongly correlate with phage Φ11 susceptibility and RboP WTA glycosylated with β-GlcNAc.

DISCUSSION

Our combined analyses of anti-WTA IgG-mediated C3 deposition and phage susceptibility and genotyping of 13 different staphylococcal strains provided us with invaluable information about WTA glycosylation and the WTA backbone structures of these strains (Table 2). Previous studies have shown that injection of *S. aureus* teichoic acids into humans or rabbits results in the induction of circulating antibodies against β-GlcNAc or α-GlcNAc WTA (35, 36). Until recently, the lack of availability of purified homogeneous GlcNAc WTAs from *S. aureus* mutant cells hampered efforts to determine the exact epitope of anti-WTA antibodies. In our previous study (21), the availability of *S. aureus* Δ*tarM*, Δ*tarS*, and Δ*tarMS* mutant cells and WTAs purified from these mutant cells enabled us to determine the exact antigenic determinant of anti-WTA antibodies and MBL. The current study further confirms that human serum anti-WTA IgG recognizes the β-GlcNAc of WTA of clinically isolated staphylococcal strains. Also, we demonstrate that the *S. aureus* β-GlcNAc WTA recognized by serum anti-WTA IgG specifically induces the opsonophagocytosis of diverse *S. aureus* strains harboring the β-GlcNAc residue of WTA. Interestingly, *tarM* was absent from five CP-producing *S. aureus* strains. The absence of *tarM* has also been reported in several health care-associated MRSA strains, such as N315, Mu50, Mu3, and JH1 (15), indicating that the absence of *tarM* is a common feature of certain sequence types. Evolutionarily, it remains unclear if the loss or gain of *tarM* could be an advantage during colonization and infection.

Recent studies suggested that an effective vaccine to prevent *S. aureus* infections must contain multiple antigens that are carefully selected to interrupt *S. aureus* pathogenesis (37). Our study shows that human anti-WTA IgG recognizes the β-GlcNAc of WTA of most of the clinically isolated staphylococcal strains tested. If staphylococcal WTA is proven to be a valuable vaccine target antigen, a mixture of WTA derivatives, such as RboP WTA modified with β-GlcNAc residues and GroP WTA modified with GalNAc residues, should be considered active vaccine candidates that should permit active immunization against infections caused by diverse *S. aureus* strains. Also, because *S. aureus* can persistently colonize the human body, a constant interaction and adaptation between the bacteria and the host immune system will occur. This is supported by the observation that all adults have preexisting serum antibodies capable of binding to *S. aureus* cell surface anti-

TABLE 2 Predicted WTA structures of various staphylococcal strains

Species/strain	WTA type (glycosylation)	Anti-WTA IgG-mediated C3 deposition[b]	Reference or source
S. aureus RN4220	RboP (α- and β-GlcNAc)	○	13
S. aureus USA300	RboP (α- and β-GlcNAc)	○	This study
S. aureus COL	RboP (α- and β-GlcNAc)	○	This study
S. aureus MW2	RboP (α- and β-GlcNAc)	○	14
S. aureus Becker	RboP (β-GlcNAc)	○	This study
S. aureus Reynolds	RboP (β-GlcNAc)	○	This study
S. aureus JL022	RboP (β-GlcNAc)	○	This study
S. aureus M	RboP (β-GlcNAc)	○	This study
S. aureus Wright	RboP (β-GlcNAc)	○	This study
S. aureus Smith diffuse	RboP (β-GlcNAc)	○	This study
S. aureus Lowenstein	RboP (unknown[a])	X	Unknown
S. aureus PS187	GroP (α-GalNAc)	X	12
S. epidermidis ATCC 14990	GroP (α-Glc, α-GlcNAc)	X	32

[a] Most likely produces a nonglycosylated RboP WTA.
[b] ○, deposition; X, no deposition.

gens (38). However, the exact ligand molecule(s) recognized by antibodies preexisting in serum *in vivo* has not yet been determined. Since we demonstrated that the β-GlcNAc WTA recognized by serum IgG specifically induces the opsonophagocytosis of diverse *S. aureus* strains *in vitro*, purified anti-WTA IgG will be a valuable tool for determination of the exact ligand motif of staphylococcal surface molecules which are recognized by serum antibodies *in vivo*. Also, since *S. aureus* is a major pathogen that can be difficult to treat due to drug resistance or its presence at sites of infection that are difficult to reach, the development of a vaccine may help to prevent *S. aureus* infections. Our current work provides some important findings that may help with the identification of a possible vaccine target, and it presents a panel of methods that may be useful for the typing of strains found in the clinic.

Finally, combined technologies, such as analysis of anti-WTA IgG-mediated C3 deposition and opsonophagocytosis, phage susceptibility testing, and genotyping of diverse staphylococcal strains, will be useful tools for determination of the staphylococcal WTA backbone structure and WTA glycosylation patterns in newly emerging drug-resistant *S. aureus* strains.

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