N-Acetylglucosaminylation of Serine-Aspartate Repeat Proteins Promotes Staphylococcus aureus Bloodstream Infection*

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Lena Thomer1,2, Samuel Becker1,3, Carla Emolo1, Austin Quach4, Hwan Keun Kim5, Sabine Rauch6, Mark Anderson7, James F. LeBlanc7, Olaf Schneewind1,2, Kym F. Faul1 and Dominique Missiakas1,2,3

From the 1Department of Microbiology, The University of Chicago, Chicago, Illinois 60637 and the 2Pasarow Mass Spectrometry Laboratory, Semel Institute of Neuroscience and Human Behavior, and Department of Psychiatry and Biobehavioral Sciences and the 3Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California 90024

Background: Staphylococcus aureus agglutinates in plasma in a manner that requires host fibrinogen and clumping factor A, a bacterial surface protein with serine-aspartate (SD) repeats.

Results: SdgB modifies serine residues in SD repeats with GlcNAc, and this glycosylation contributes to the pathogenesis of sepsis.

Conclusion: Glycosylation of SD repeats aids bacterial escape from host defenses.

Significance: Interference with glycosylation may alter staphylococcal infections.

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1 These authors contributed equally to this work.
2 Members of and supported by the Region V "Great Lakes" Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Consortium (National Institutes of Health Award 1-U54AI-057153).
3 To whom correspondence should be addressed: Dept. of Microbiology, University of Chicago, 920 East 58th St., Chicago, IL 60637. Tel.: 773-834-8161; Fax: 773-834-8150; E-mail: dmissiak@bsd.uchicago.edu.

Staphylococcus aureus secretes products that convert host fibrinogen to fibrin and promote its agglutination with fibrin fibrils, thereby shielding bacteria from immune defenses. The agglutination reaction involves ClfA (clumping factor A), a surface protein with serine-aspartate (SD) repeat that captures fibrin fibrils and fibrinogen. Pathogenic staphylococci express several different SD proteins that are modified by two glycosyltransferases, SdgA and SdgB. Here, we characterized three genes of S. aureus, aggA, aggB (sdgA), and aggC (sdgB), and show that aggA and aggC contribute to staphylococcal agglutination with fibrin fibrils in human plasma. We demonstrate that aggB (sdgA) and aggC (sdgB) are involved in GlcNAc modification of the ClfA SD repeats. However, only sdgB is essential for GlcNAc modification, and an sdgB mutant is defective in the pathogenesis of sepsis in mice. Thus, GlcNAc modification of proteins promotes S. aureus replication in the bloodstream of mammalian hosts.

Staphylococcus aureus is the most frequent cause of human bloodstream infection, sepsis, and endocarditis in the United States (1, 2). This Gram-positive bacterium evolved to clot animal and human plasma, bind fibrinogen, and agglutinate within a meshwork of fibrin fibrils (3). S. aureus agglutination requires two secreted products, Coa (coagulase) and vWbp (von Willebrand factor-binding protein) (4), which each associate with and activate prothrombin to convert fibrinogen to fibrin (5, 6).

ClfA (clumping factor A), a sortase-anchored surface protein (7), tethers bacteria to fibrin fibrils by binding to the C-terminal end of the γ-chain, thereby blocking incorporation of additional fibrin subunits into agglutinated fibrils (4, 8, 9). Agglutination with fibrin fibrils protects staphylococci from phagocytes and promotes the formation of infectious thrombi that contribute to the lethal outcome of staphylococcal sepsis in mice (4, 10). Staphylococcal agglutination is also essential for the pathogenesis of infectious endocarditis and the formation of purulent abscess lesions, which promote bacterial persistence and dissemination in host tissues (11–13).

ClfA is the founding member of the family of serine-aspartate (SD) repeat proteins that are synthesized and secreted as precursors with N-terminal signal peptides (14, 15). Proteins with SD repeats are typically composed of three domains. The N-terminal A region provides for association with specific ligands, predominantly the α-, β-, or γ-chain of fibrinogen (16–18). For S. aureus SdrC, SdrD, and SdrE, but not for ClfA and ClfB, the A domain is followed by B domain repeats with additional ligand binding properties (19). SD repeats tether the N-terminal ligand-binding domains to the C-terminal LPXTG sorting signal, which triggers sortase A-mediated linkage to the cell wall envelope (20–22). SD repeat proteins are also found in other pathogenic staphylococci, for example the opportunistic pathogen Staphylococcus epidermidis (23) and Staphylococcus saprophyticus, a frequent cause of urinary tract infection (24).

Hazenbos et al. (25) isolated a human monoclonal antibody that recognizes proteins with SD repeats from S. aureus and S. epidermidis in a manner requiring post-translational modification by the sdgB-encoded UDP-GlcNAc glycosyltransferase. In vitro experiments with purified glycosyltransferases confirmed that proteins with SD repeats are substrates for SdgB- and SdgA-mediated GlcNAc modification (25). Hazenbos et al. further proposed that glycosylation prevents proteolytic degradation of such proteins by human, but not mouse, neutrophils or cathepsin.
Here, we searched for factors that contribute to *S. aureus* agglutination with fibrin fibrils. We report that mutant alleles of *aggA* and *aggC* display reduced agglutination in human plasma, and *aggC*, but not *aggA*, affects the post-translational modification of ClfA, but not the staphylococcal coagulases Coa and vWbp (12). *aggA* and *aggC* flank the *sdrCDE* locus of *S. aureus* (see Fig. 1A), which prompted further examination of transposon insertions in neighboring genes (*nwmn_0521* through *nwmn_0528*) and identified *aggB* as a second factor affecting ClfA modification. *aggB* and *aggC* encode the previously reported SdgA and SdgB proteins. We demonstrate that the *aggC* (sdgB) gene is required for GlcNAc modification of SD repeats of staphylococcal ClfA. Furthermore, *S. aureus aggC* (sdgB) variants, but not *aggB* (sdgA) variants, are defective in the pathogenesis of bloodstream infections in mice.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Reagents**—The human clinical isolate *S. aureus* Newman (wild-type) (26, 27) was transduced with bacteriophage φ85 lysates derived from variants with inser- tional *bursa aurealis* lesions (28, 29). Mutant alleles from cor- responding chromosomal regions were verified by DNA sequencing of PCR products. *S. aureus* strains were propagated in tryptic soy broth (TSB) or on tryptic soy agar plates at 37 °C with antibiotic selection when necessary. Erythromycin was used at 10 μg/ml to select for *bursa aurealis* insertion vari- ants. For complementation studies, the coding sequences of *aggA*, *aggB* (sdgA), or *aggC* (sdgB) were PCR-amplified from chromosomal DNA of *S. aureus* Newman as a template, cut with XhoI and BamHI, and cloned into the corresponding expression vector pWWW412 (30). PCRs used primer pairs NWMN_0522-XhoI-F (5′-AAAAACTCGAGAAGGATTATATATTATGGCAGTGC-3′) and NWMN_0522-BamHI-R (5′-AAAAAGGTATCTTTTATTTATGTTTATGCAC-3′) for *aggA*, NWMN_0526-Xhol-F (5′-AAAATCTCGAGAATTATTTTGTAGGTAATTAGTTAGG-3′) and NWMN_0526-BamHI-R (5′-AAAAAGGTATCTTTTATTTATGTTTATGCAC-3′) as described above, and identified *aggB* as a second factor affecting ClfA modification. *aggB* and *aggC* encode the previously reported SdgA and SdgB proteins. We demonstrate that the *aggC* (sdgB) gene is required for GlcNAc modification of SD repeats of staphylococcal ClfA.

**Agglutination**—Overnight cultures of *S. aureus* were washed with 1 ml of PBS and suspended to a final concentration of *A*$_{600}$ = 4.0. Bacteria from 1 ml of culture were incubated with 1:500 SYTO 9 (Invitrogen) for 15 min, washed twice with 1 ml of PBS, and suspended in 1 ml of PBS. Bacteria were mixed 1:1 with lepirudin-treated human plasma on glass microscope slides and incubated for 15 min. Samples were viewed and images were captured on an IX81 live cell total internal reflection fluorescence microscope using a 20× objective. For quantification of agglutination, 20 bright-field microscope images were obtained from each strain using a Nikon TE2000-U microscope with 20× objective. The threshold function in ImageJ software was used to convert the image into a dichromatic format in which staphylococci are black and the background is white. The areas of agglutination complexes in each image were measured. The average area of *S. aureus* Newman in saline without plasma was subtracted from all data sets. To statistically compare the wild type and mutant, data were analyzed by two-way analysis of variance (ANOVA) using Prism (GraphPad Software); *p* values <0.05 were deemed significant.

**Immunoblotting**—*S. aureus* Newman and its mutants (*aggA*, *aggB*, or *aggC*) with or without complementing plasmids were grown overnight in TSB. For plasmid stability, chlorampheni- col (10 μg/ml) was added as needed. Overnight cultures were diluted 1:100 into fresh TSB and grown to *A*$_{600}$ = 1.5. One ml of culture was treated with TCA (14%, 0 °C, 30 min) and centrifuged (20,000 × g, 15 min), and the pellet was washed with acetone (1 ml, 0 °C, 15 min). The sediment was suspended in 1 ml of Buffer A (50 mM Tris-HCl (pH 7.5) and 150 mM NaCl with 20 μg of lysozyme and incubated (37 °C, 1.5 h). Solubilized proteins were precipitated with TCA, washed with acetone (as described above), and dried. Proteins were solubilized in 0.5 M Tris-HCl (pH 8.0) and 4% SDS, mixed and heated with sample buffer, and subjected to 8% SDS-PAGE with electro- transfer to PVDF membranes. Membranes were blocked by incubation in bovine serum albumin (3%, 1 h, room tempera- ture). To prevent binding of primary antibodies to protein A, 0.8 mg of human IgG was added to 10 ml of blocking buffer. After incubation (1 h, room temperature), immune serum (pri- mary antibody at 1:5000 dilution) was added, and the mem- branes were incubated for an additional hour. Membranes were washed three times for 10 min with TBS-T (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% and Tween 20) and incubated with Alexa Fluor 680-labeled goat anti-rabbit secondary antibody (1:20,000; LI-COR) for 1 h. Membranes were washed again with TBS-T, and fluorescence at 700 nm was measured with a LI- COR Odyssey infrared scanner.

**Purification of ClfA**—*S. aureus* Newman (wild-type) or its *aggB* (sdgA) and *aggC* (sdgB) mutants harboring plasmid pClfASD5 were grown overnight in TSB with 10 μg/ml-1 chloramphenicol. Cultures were diluted 1:100 into 2 liters of TSB and grown to *A*$_{600}$ = 1.5. Cultures were centrifuged (8000 × g, 15

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$^4$ The abbreviations used are: TSB, tryptic soy broth; TEV, tobacco etch virus; ANOVA, analysis of variance; LC-ESI-MS, combined liquid chromatography-electrospray ionization mass spectrometry with fraction collection; PMN, polymorphonuclear neutrophil.
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min). Culture supernatants were cooled on ice, and ammonium sulfate was added to (w/v) 90%, followed by incubation for 1 h. The precipitate was sedimented by centrifugation (8000 × g, 15 min), suspended in 10 ml of Buffer A, and dialyzed overnight at 4 °C against the same buffer. Dialyzed material was centrifuged, and the supernatants were loaded by gravity flow onto a Streptactin® column (IBA). The column was washed with Buffer A and eluted with 5 mM desthiobiotin (IBA), and the eluate containing ClfASDS proteins was desalted via HiTrap chromatography (GE Healthcare). To cleave ClfASDS, 50 μg of purified protein was treated with 5 μg of TEV protease (Sigma) for 1 h at 30 °C. Samples were analyzed by SDS-PAGE and immunoblotting or subjected to mass spectrometry.

Combined Liquid Chromatography-Electrospray Ionization Mass Spectrometry with Fraction Collection (LC-ESI-MS)—Sample aliquots containing ClfASDS proteins cleaved or not with TEV protease were dried in a vacuum concentrator, dissolved in water (250 μl), and loaded onto a polymeric reverse phase column (Agilent PLRP-S, 300-Å pore size, 5-μm particle size, 2.1 × 150 mm) equilibrated in solvent B (100:0.1 (v/v) water/formic acid) and eluted (100 μl/min) with a linearly increasing concentration of solvent C (100:0.1 (v/v) acetonitrile/formic acid, 0 min/5%, 5 min/5%, 45 min/90%, 50 min/5%, and 60 min/5%). The column effluent was passed through a stream splitter; a proportion (~50%) was directed to a fraction collector (1 min/fraction), and the remainder was directed to an Ionspray™ source connected to a triple quadrupole mass spectrometer (ABI Sciex API III+) scanning in the positive ion MS mode from m/z 500–1850 (step size of 0.3 Da, dwell of 1 ms, 4.82 s/scan, orifice of 90 V). Data were interrogated using Mascot® software (version 3.3.0) for molecular mass calculations from multiply charged ion clusters and BioMultiView® (version 1.3.1) for display of the deconvoluted spectra.

MALDI-MS—Select fractions from the LC-ESI-MS+ experiments were pooled, dried in a vacuum concentrator, and dissolved in water/acetonitrile/formic acid (50:50:0.1), and aliquots (2 μl) were spotted with either α-cyanocinnamic or sinapinic acid matrices (both 5 mg/ml in 40:60:0.1 water/acetonitrile/trifluoroacetic acid) on a stainless steel target, and laser desorption spectra were recorded with a Bruker-Daltonics ultrafleXtreme tandem time-of-flight mass spectrometer (in the MS mode, 10,000 laser shots/spectrum; in the collisionally activated dissociation MS/MS mode, 20,000 laser shots/spectrum with a 12 Da window for precursor ion selection). Data were interrogated with mMass software (version 5.5.0). To compensate for m/z drift during the MS/MS data acquisition process, paired m/z and intensity values from individual spectra were loaded into Microsoft Excel® (version 14.0.7106.5003), and the m/z values were globally shifted (0–10 Da) to align a preselected ion in each spectrum. The shifted spectra were then reloaded into mMass for further interpretation and presentation. Fragmentation predictions from theoretical sequences were obtained using the MS-Product tool within ProteinProspector (version 5.10.13).

Mouse Bloodstream Infection—Overnight cultures of S. aureus Newman (wild-type) and clfA, aggB (sdgA), and aggC (sdgB) mutants were diluted 1:100 into fresh TSB and grown for 2 h at 37 °C. Cultures were centrifuged (4000 × g, 10 min), and the pellets were washed and resuspended in PBS to the desired bacterial concentration. Inocula were quantified by spreading sample aliquots on tryptic soy agar and enumerating cfu. BALB/c mice (groups of 30, n = 30) were anesthetized via intra-peritoneal injection with 65 mg/ml−1 ketamine and 6 mg/ml−1 xylazine per kg of body weight. Mice were infected by injection with 1–2 × 10^8 cfu of S. aureus Newman or mutants into the periorbital venous sinus of the right eye. At 12 h following infection, cohorts of mice (n = 10) were killed by CO₂ inhalation and necropsied. Hearts were removed, and staphylococcal loads were analyzed by homogenizing tissue in PBS containing 0.1% Triton X-100. Serial dilutions of homogenates were spread on tryptic soy agar and incubated for enumeration of cfu. Additional cohorts of animals (n = 10) were killed, and hearts were examined by histopathology. Briefly, hearts were fixed in 10% formalin for 24 h at room temperature, embedded in paraffin, thin-sectioned, stained with hematoxylin/eosin, and inspected by light microscopy to enumerate pathological lesions. The remaining animals (n = 10) were monitored for survival over 10 days. All mouse experiments were performed at least twice and conducted in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at The University of Chicago. The statistical significance of the mouse sepsis model was analyzed by two-tailed log-rank analysis. Bacterial loads in the experimental animal infection model were analyzed by one-way ANOVA to measure statistical significance. Two-way ANOVA was used to analyze the statistical significance of histopathological analysis in the experimental animal infection model. All data were analyzed using Prism. p values <0.05 were deemed significant.

RESULTS

S. aureus agg Mutants Defective in Agglutination—The wild type and insertional mutants were screened with human plasma for factors that contribute to defects in staphylococcal agglutination (4, 29). This search identified several mutants with bursa aurealis insertions in two genes, designated aggA and aggC (Fig. 1A). Unlike clfA or coa/vwb mutants, which are completely defective for agglutination (4), variants with lesions in aggA or aggC displayed reduced staphylococcal agglutination (Fig. 1, B and C). The aggA and aggC genes flank the sdr locus, which is composed of tandem repeats of the genes encoding SD repeat proteins SdrC, SdrD, and SdrE (14). Insertional lesions in these neighboring genes, including sdr genes, did not display a significant defect in agglutination compared with the wild type; however, the aggB variant was examined further because of the homology between the aggB and aggC gene products. Defects associated with clfA, aggA, and aggC were complemented by expressing the wild-type genes on a plasmid (Fig. 1, B and C). The aggB and aggC genes were characterized as UDP-GlcNAc transferases that modify SD repeat proteins and have been designated SdgA and SdgB, respectively (25). SdgA and SdgB are 490 and 496 amino acids long and share 44% identity (E-value of 3e−144) with a conserved GtfA-like domain, named after Streptococcus gordonii GtfA, a protein...
that catalyzes the O-linked glycosylation of the cell surface protein GspB (33).

S. aureus aggB (sdgA) and aggC (sdgB) Mutants Affect Mobility of ClfA and ClfB on SDS-PAGE—To test whether agg mutants affect the production or secretion of known agglutination factors, staphylococcal cultures treated with the murein hydrolase lysostaphin were analyzed by immunoblotting with Coa-, vWbp-, or ClfA-specific antibodies. These experiments revealed that aggA, aggB (sdgA), and aggC (sdgB) mutants produced similar amounts of Coa and vWbp (Fig. 2). Immunoblotting for the sortase A enzyme (SrtA) was used as a loading control (Fig. 2). Although the aggB (sdgA) and aggC (sdgB) mutants harbored similar amounts of ClfA compared with wild-type and aggA mutant staphylococci, their immunoreactive material migrated with increased mobility on SDS-PAGE (Fig. 2). Of note, aggC (sdgB)-mutant ClfA appeared as the fastest migrating species. sdgA-mutant ClfA assumed intermediate mobility, and the mobility of aggA-mutant ClfA was unaffected (Fig. 2). All of these species were specific for ClfA, as the clfA mutant extract did not react with the ClfA-specific antibodies (Fig. 2). Plasmids providing for the expression of wild-type aggB (sdgA) or aggC (sdgB) restored the SDS-PAGE mobility of ClfA in the corresponding mutants to the same level as ClfA in wild-type staphylococci. We wondered whether the SDS-PAGE mobility of other proteins with SD repeats is also affected in the agg mutants. Immunoblotting of staphylococcal cell wall preparations with ClfB-specific antibodies revealed altered mobility of ClfB in aggB (sdgA) or aggC (sdgB) mutant strains (Fig. 2). SdrC, SdrD, and SdrE also displayed differential mobility in these mutants (data not shown). Hereafter, we refer to aggB and aggC as sgdA and sgdB (25).

Purification and Characterization of ClfA<br>

Glycosylation of Staphylococcal SD Repeat Proteins—To analyze the chemical structure of ClfA, we engineered a plasmid-expressed variant, ClfA<sub>SDDS</sub>, with insertion of a TEV protease cleavage site, Strep-tag, five tandem SD repeats, and a C-terminal hexahistidyl tag immediately downstream of the fibrinogen-binding A domain (Fig. 3A). When transformed into wild-type, sdgA mutant, and sgdB mutant staphylococci, pClfA<sub>SDDS</sub> provided for the expression of ClfA<sub>SDDS</sub>, which was partially purified from culture supernatants by ammonium sulfate precipitation and affinity chromatography. When subjected to SDS-PAGE and immunoblotting, ClfA<sub>SDDS</sub> from wild-type, sgdA mutant, and sgdB mutant staphylococci (ClfA<sub>SDDS/WT</sub>, ClfA<sub>SDDS/sdgA</sub>, and ClfA<sub>SDDS/sgdB</sub>) respectively migrated with similar mobility and reacted with antibodies specific for the A domain of ClfA (Fig. 3B, αClfA), hexahistidyl (HIS probe), and Strep-tag (αStrep). These data indicate that Strep-Tactin affinity chromatography of ClfA<sub>SDDS</sub> yielded a partially purified, full-length polypeptide.

These ClfA<sub>SDDS</sub> samples were subjected to LC-ESI-MS<sup>+</sup>. That from wild-type staphylococci (ClfA<sub>SDDS/WT</sub>) generated a series of multiply charged ions that, upon deconvolution, revealed six distinct molecular species with measured masses of 59,857.86, 60,060.48, 60,264.44, 60,465.63, 60,671.95, and 60,875.89 Da (Fig. 3C). The average difference between these species is 203.6 Da, which corresponds to the residue mass of GlcNAc (203.193 Da calculated as the average (chemical) mass for C₉H₁₇NO₉). The sample from sgdB mutant staphylococci (ClfA<sub>SDDS/sgdB</sub>) generated multiply charged ion signals that were deconvoluted into a single species with a measured mass of 59,859.90 Da. This is similar to that of the lightest ClfA<sub>SDDS/WT</sub> species, and both are in reasonable agreement with the predicted mass of unmodified ClfA<sub>SDDS</sub> protein with the 39-amino acid signal sequence.

**FIGURE 1.** Genes that affect <i>S. aureus</i> agglutination in human plasma. A, plan of the organization of <i>sdr</i> and <i>agg</i> genes in strain Newman shown as yellow and gray boxes, respectively. B, agglutination in lepinudin-treated human plasma of SYTO 9-stained <i>S. aureus</i> Newman (WT) or mutants with insertional lesions in <i>clfA</i>, <i>aggA</i>, <i>aggB</i>, and <i>aggC</i> along with complemented strains <i>clfA</i>/pcfIA, <i>aggA</i>/paggA, <i>aggB</i>/paggB, and <i>aggC</i>/paggC. C, quantification of agglutination shown in B. Means ± S.E. were calculated from 12 fields of microscopic view. Statistical significance was assessed in pairwise comparison with WT with Student’s t two-tailed test. * p < 0.01; ** p < 0.0001.
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A

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\begin{array}{c|c|c|c|c}
\hline
& \text{CIAA}_{\text{SDS}} & \text{CIAA}_{\text{SDS}} & \text{CIAA}_{\text{SDS}} & \text{CIAA}_{\text{SDS}} \\
\hline
\text{ss} & 1 & 2 & 3 & 4 \\
\hline
\text{N1} & 1 & 2 & 3 & 4 \\
\hline
\text{N2} & 1 & 2 & 3 & 4 \\
\hline
\text{N3} & 1 & 2 & 3 & 4 \\
\hline
\text{SD5} & 1 & 2 & 3 & 4 \\
\hline
\text{LPXTG} & 1 & 2 & 3 & 4 \\
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FIGURE 4. Structural analysis of CIAA_{SDS} C-terminal fragments. A, purified CIAA_{SDS}WT, CIAA_{SDS}/sdgA, and CIAA_{SDS}/sdgB as shown in Fig. 3 were incubated without (−) or with (+) TEV protease and analyzed by Coomassie Blue-stained SDS-PAGE and immunoblotting with anti-CIAA (α-CIAA), anti-Strep-tag (α-Strep), or anti-hexahistidyl (HIS Probe) antibodies (lanes 1–3). The fifth panel was incubated with succinylated wheat germ agglutinin (sWGA). An aliquot of glycysylated human immunoglobulin (IgG) was loaded in lane 4 as a positive control. The relative intensity was measured for each lane.

B

FIGURE 3. Purification and characterization of CIAA_{SDS}. A, plan of the domain organization of chromosome-encoded CIAA in S. aureus Newman (933 amino acids) and the CIAA_{SDS} construct (600 amino acids) used in this study. The LPXTG cell wall sorting signal sequence (ss), and SD region bearing serine-aspartate repeats are shown. The SD region contains 130 and 5 repeats, respectively in CIAA (left) and the engineered construct (right). A domain with the N1N2N3 regions is responsible for fibrinogen (Fg) binding. Amino acids in green, red, and blue correspond to the TEV recognition motif, Strep-tag, and the hexahistidyl tag used for purification. Numbers indicate the positions of amino acids in the sequence of each protein. B, the CIAA_{SDS}WT, CIAA_{SDS}/sdgA, and CIAA_{SDS}/sdgB products (lanes 1–3) purified from culture supernatants over Strep-Tactin beads were separated by SDS-PAGE and visualized with Coomassie Blue or transferred to PVDF membrane for immunoblotting with antibodies specific for the A domain of CIAA (α-CIAA), hexahistidyl (HIS Probe), and Strep-tag (α-Strep). Numbers to the right indicate the positions of molecular mass markers in kilodaltons. C, tabulation of the measured masses of purified CIAA_{SDS} products obtained by ESI-MS and the corresponding calculated (theoretical) mass of the CIAA_{SDS} protein.

C

D

FIGURE 3. Purification and characterization of CIAA_{SDS}. A, plan of the domain organization of chromosome-encoded CIAA in S. aureus Newman (933 amino acids) and the CIAA_{SDS} construct (600 amino acids) used in this study. The LPXTG cell wall sorting signal, signal sequence (ss), and SD region bearing serine-aspartate repeats are shown. The SD region contains 130 and 5 repeats, respectively in CIAA (left) and the engineered construct (right). A domain with the N1N2N3 regions is responsible for fibrinogen (Fg) binding. Amino acids in green, red, and blue correspond to the TEV recognition motif, Strep-tag, and the hexahistidyl tag used for purification. Numbers indicate the positions of amino acids in the sequence of each protein. B, the CIAA_{SDS}WT, CIAA_{SDS}/sdgA, and CIAA_{SDS}/sdgB products (lanes 1–3) purified from culture supernatants over Strep-Tactin beads were separated by SDS-PAGE and visualized with Coomassie Blue or transferred to PVDF membrane for immunoblotting with antibodies specific for the A domain of CIAA (α-CIAA), hexahistidyl (HIS Probe), and Strep-tag (α-Strep). Numbers to the right indicate the positions of molecular mass markers in kilodaltons. C, tabulation of the measured masses of purified CIAA_{SDS} products obtained by ESI-MS and the corresponding calculated (theoretical) mass of the CIAA_{SDS} protein.

removed following secretion (calculated 59,848.04 Da) (Fig. 3C). The 0.0167% difference between the measured and calculated masses could be due to a single oxidation of one of the four methionine residues in the sequence. Accounting for a single oxidation reduces the difference to 0.009%, which is consistent with the accuracy of molecular mass measurements made by ESI on quadrupole mass spectrometers. The sample from sdgA mutant staphylococci (CIAA_{SDS}/sdgA) generated a series of multiply charged ions that were deconvoluted as three distinct species with measured masses of 59,873.89, 60,072.94, and 60,279.41 Da (Fig. 3C). The average mass difference between these (202.76 Da) is also similar to the chemical mass of GlcNAc (203.193 Da). These data suggest that CIAA_{SDS/WT} is modified with at least five GlcNAc moieties, whereas CIAA_{SDS/sdgB} is not modified, and CIAA_{SDS/sdgA} displays an intermediate level of glycosylation.

Structural Analysis of TEV Fragments of CIAA_{SDS} from Wild-type, sdgA (aggB) Mutant, and sdgB (aggC) Mutant Staphylococci—CIAA_{SDS/WT}, CIAA_{SDS/sdgA}, and CIAA_{SDS/sdgB} were incubated with TEV protease and analyzed by Coomassie Blue-stained SDS-PAGE and immunoblotting (Fig. 4A). TEV protease treatment increased the mobility of CIAA_{SDS/WT} CIAA_{SDS/sdgA}, and CIAA_{SDS/sdgB}. The faster migrating species reacted with anti-CIAA antibody, but not with anti-Strep-tag or anti-hexahistidyl antibody, indicating that the protease had cleaved off the C-terminal SD5 repeat of CIAA_{SDS} (the small size of the C-terminal fragment precluded visualization on SDS-PAGE). The periodic acid—Schiff reaction failed to generate oxidized products with CIAA_{SDS/WT} (data not shown). Nevertheless, succinylated wheat germ agglutinin, a GlcNAc-specific lectin (34), readily bound CIAA_{SDS/WT} prior to TEV protease cleavage, but not after (Fig. 4A, fifth panel). These data suggest that CIAA_{SDS} in wild-type S. aureus is modified with GlcNAc in the C-terminal SD5 repeat in a manner that does not lead to periodate formation.
likely because the hemiacetal of the carbohydrate moiety is tethered to the polypeptide chain. When analyzed by LC-ESI-MS<sup>+</sup>, TEV protease-treated ClfASD<sub>5</sub> samples from wild-type, sdgA mutant, or sdgB mutant staphylococci generated multiply charged ions that, upon deconvolution, yielded masses of 56,083.91, 56,091.42, and 56,083.73 Da, respectively (data not shown). These observations are in agreement with TEV protease cleavage at ClfASD<sub>5</sub> residue 565. These N-terminal fragments encompass 526 amino acids and lack both the signal sequence (first 39 amino acids) and the C-terminal SD5 peptide, and they are not modified with GlcNAc.

LC-ESI-MS<sup>+</sup> of the C-terminal SD5 wild-type sample following TEV protease cleavage yielded another series of ions that, upon deconvolution, showed six different compounds with measured masses of 3794.59, 3997.39, 4200.49, 4404.26, 4607.50, and 4810.99 Da (Fig. 4B). The average mass difference between these species is 203.28 Da. LC-ESI-MS<sup>+</sup> of the TEV protease-treated ClfASD<sub>5sdgA</sub> sample yielded a series of ions that, upon deconvolution, showed peptide species with masses of 3794.96 and 3999.46 Da (difference of 205.5 Da) (Fig. 4C). Finally, LC-ESI-MS<sup>+</sup> of the TEV protease-treated ClfASD<sub>5sdgB</sub> sample gave rise to a single compound with an average mass of 3794.80 Da (Fig. 4D). Together, these data indicate that in wild-type <i>S. aureus</i>, the SD-containing region of ClfASD<sub>5</sub> is modified by GlcNAc in a manner requiring the glycosyltransferase encoded by sdgA and, to a lesser degree, the one encoded by sdgB.

**SD Repeats of ClfA Are Modified with GlcNAc:** To identify the amino acid residues that are modified with GlcNAc, the C-terminal SD peptides of ClfASD<sub>5WT</sub> and ClfASD<sub>5sdgA</sub> were analyzed off-line by MS/MS of the fractions collected during LC-ESI-MS<sup>+</sup>. Collisionally activated dissociation MS/MS experiments during ESI were unsuccessful. However, collisionally activated dissociation MS/MS of the corresponding singly charged precursor ions produced during MALDI (MALDI-MS/ MS) yielded spectra that were interpreted for their y- and b-fragment ions. The non-glycosylated peptide (parent mass of 3793.7 Da) gave a virtually complete y-ion series, partially presented in Table 1 (ions y<sub>2</sub>–y<sub>31</sub>; ions y<sub>20</sub>, y<sub>24</sub>, and y<sub>29</sub> were missing), but with a much less complete b-ion ladder (data not shown). This y-ion ladder confirms the predicted amino acid sequence of the unmodified C-terminal ClfASD<sub>5</sub> fragment (Fig. 5A and Table 1). However, the MALDI-MS/MS spectra of the glycosylated isoforms were less populated with readily assignable fragment ions, and there were no clearly discernable y- or b-ion ladders, precluding unambiguous assignment of the sites of glycosylation. Furthermore, possible heterogeneity of the glycosylation pattern within each isoform would diminish signal intensity for any one fragment ion and would further complicate interpretation of the data. Overlaying the MALDI-MS/MS spectra of the mono-, di-, tri-, tetra-, penta-, and hexaglycosylated isoforms revealed an m/z drift of fragment ions, adding yet another complication to spectral interpretation. This interspectral drift is an unavoidable feature of the instrument with which the data were collected. Nonetheless, all spectra showed a strong signal at m/z 1072, assigned as y<sub>9</sub> (Fig. 5A and Table 1). This signal was then used to align the spectra (Fig. 5B). Comparison of the aligned spectra in a stacked overlay format (Gel View in mMass software) showed diagonal ladders separated by 203 Da originating from y<sub>13</sub> (single-step ladder), y<sub>17</sub> (three-step ladder), y<sub>21</sub> (five-step ladder), y<sub>23</sub> (five-step ladder), and y<sub>25</sub> (six-step ladder) (Fig. 5B). The regular step distances of 203 Da strongly suggest the presence of monoglycosylation at each site because a preponderance of double glycosylation would have resulted in ladders with 406-Da step sizes. Furthermore, ion y<sub>13</sub> with sequence S<sub>27</sub>GS<sub>29</sub> and not y<sub>9</sub> with sequence S<sub>25</sub>D was monoglycosylated (Fig. 5A and Table 1). This signal was then used to align the spectra (Fig. 5A). Comparison of the aligned spectra in a stacked overlay format (Gel View in mMass software) showed diagonal ladders separated by 203 Da originating from y<sub>13</sub> (single-step ladder), y<sub>17</sub> (three-step ladder), y<sub>21</sub> (five-step ladder), y<sub>23</sub> (five-step ladder), and y<sub>25</sub> (six-step ladder) (Fig. 5B). The regular step distances of 203 Da strongly suggest the presence of monoglycosylation at each site because a preponderance of double glycosylation would have resulted in ladders with 406-Da step sizes. Furthermore, ion y<sub>13</sub> with sequence S<sub>27</sub>GS<sub>29</sub> and not y<sub>9</sub> with sequence S<sub>25</sub>D was monoglycosylated (Fig. 5A and Table 1). Additional glycosylation (+ 203 Da) was observed in ions y<sub>17</sub>, y<sub>21</sub>, y<sub>23</sub>, and y<sub>25</sub>, encompassing sequences S<sub>21</sub>D, S<sub>17</sub>D, S<sub>13</sub>D, and S<sub>D</sub>D, respectively. The data also suggest the possibility of some diglycosylation at a single SD site. For example, the ions assigned as y<sub>17</sub> and y<sub>21</sub> in the hexa-GlcNac isoform showed additional rungs that are consistent with diglycosylation at

| TABLE 1 | Summary tabulation of the heaviest y-ion observed in the MALDI-MS/MS spectra of un-, mono-, di-, tri-, tetra-, penta-, and hexaglycosyl precursor ions corresponding to the isoforms of ClfASD<sub>5WT</sub> C-terminal peptide released by TEV protease treatment |
|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Precursor ion | Product ion<sup>a</sup> | m/z<sup>b</sup> | Relative error<sup>c</sup> (ppt)<sup>d</sup> |
| 3793.7 (unglycosylated) | y<sub>9</sub> | 1072.3 | 0.2 |
| 3996.8 (monoglycosylated) | y<sub>9</sub> | 1072.3 | 0.2 |
| 4199.9 (diglycosylated) | y<sub>9</sub> | 1072.3 | 0.2 |
| 4403.0 (triglycosylated) | y<sub>9</sub> | 1072.2 | 0.2 |
| 4606.2 (tetracyglycosylated) | y<sub>9</sub> | 1072.2 | 0.2 |
| 4810.4 (pentaglycosylated) | y<sub>9</sub> | 1072.3 | 0.1 |

<sup>a</sup> The sequence of product ions is shown in Fig. 5A.

<sup>b</sup> The data were obtained from spectra aligned as described under “Experimental Procedures” and shown in Fig. 5B. Assignments for the heaviest glycosylated y-ions are shown.

<sup>c</sup> Relative error (parts per thousand (ppt)) indicates the difference between the observed m/z value for each ion and the calculated value for that ion.

<sup>d</sup> Gel View in mMass software.
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repeats S^{17}D and S^{21}D (Fig. 5B). In support of this assignment is the fact that y17 exhibited a maximum of three glycosylations C-terminal to repeat S^{17}D, whereas the y21 ion contained only one additional SD repeat but harbored two additional glycosylations (Table 1). In addition, close inspection of the molecular ion region showed losses of 203 Da and weak but discernible signals with losses of 406 Da. Although it is impossible to definitively assign the origin of these signals, the 406-Da loss could arise from loss of a di-GlcNAc moiety. In conclusion, these experiments suggest that serine residues within SD repeats of ClfA are modified with O-linked GlcNAc in a manner requiring the SdgB glycosyltransferase. The majority of serine residues appear to be monoglycosylated, although some appear to be diglycosylated, and the interpretation cannot exclude the possibility of even higher order glycosylations at single residues. Because, ClfA_{SD5} is partially glycosylated in the sdgB mutant, it is possible that SdgA may favor additional glycosylation at sites already glycosylated by SdgB.

Glycosylation of SD Repeat Proteins and Pathogenesis of Staphylococcal Infections—We sought to determine whether GlcNAc modification of serine residues in SD repeat proteins contributes to the pathogenesis of S. aureus infections. Equal doses of wild-type Newman or clfA, sdaA, and sdbB mutant S. aureus were injected into the bloodstream of BALB/c mice, and survival was monitored over a period of 10 days. Most animals infected with wild-type S. aureus succumbed to infection within 48 h (Fig. 6A). Mice infected with the clfA mutant displayed a significant delay in time to death as well as survival in some of the infected animals (Fig. 6A). Although all animals infected with the sdbB mutant succumbed to infection, these mice displayed a reproducible delay in time to death (Fig. 6A).

We did not detect a significant delay in time to death between animals that had been infected with wild-type S. aureus or the sdaA variant strain (Fig. 6A).

Heart tissues were examined for bacterial load and the histopathology of staphylococcal lesions. As reported earlier (4), the infectious load of the clfA mutant in heart tissue was reduced by 1.98 log_{10} cfu (Fig. 6B). Histopathology revealed that, compared with wild-type S. aureus, the clfA mutant formed fewer lesions and did not agglutinate in the vasculature, which is characterized by intravascular thrombi of staphylococci and fibrin without immune cell infiltrates (Fig. 6C). The sdbB mutant displayed an intermediate phenotype with reduced bacterial load (Δ0.67 log_{10} cfu g^{-1}) (Fig. 6B) and reduced frequency of infectious lesions in heart tissue (Fig. 6C). Staphylococcal agglutinations without immune cell infiltrate were rarely observed for the sdbB variant. Moreover, mixed histopathological features, including agglutinated bacteria and moderate immune cell infiltrates (staphylococcal agglutinations + polymorphonuclear neutrophils (PMNs)) as well as massive neutrophil inflammation (PMNs), represented the most abundant infectious lesions of sdbB mutant S. aureus (Fig. 6, C and D).

DISCUSSION

Glycosylation of secreted proteins in bacteria has only recently been appreciated (35, 36). In eukaryotic cells, protein oligosaccharyltransferase and differential processing of its N-linked carbohydrate side chains control the folding, traffic-
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plasma and in mouse heart tissues. Of note, the sdgA mutant did not display a significant defect in the mouse bloodstream model, indicating that SdgA-mediated glycosylation of SD proteins is not a contributor to disease in mice. Interestingly, AggA, a predicted NADPH-dependent FMN reductase, contributed to S. aureus agglutination in human plasma but was dispensable during staphylococcal bloodstream infection in mice (data not shown). We presume that GlcNAc modification of ClfA is responsible for the observed SdgB-mediated agglutination and virulence defect, as S. aureus mutants defective in clfB or sdrCDE expression display wild-type agglutination phenotypes (4). It seems unlikely that the sdbB-mediated agglutination defect is due to the proposed mechanism of increased cathepsin B-mediated proteolysis of ClfA (25). First, mouse cathepsin does not cleave non-glycosylated ClfA, yet sdbB mutant staphylococci display virulence defects in mice (25). Second, neutrophil-mediated release of cathepsin does not occur during staphylococcal agglutination in human plasma (4). Future work will need to reveal whether the agglutination phenotype of non-glycosylated ClfA is caused by altered binding activities for fibrinogen and fibrin or whether they impact another, as of yet unknown host ligand.

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