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Molecular Basis for Preferential Protective Efficacy of Antibodies Directed to the Poorly Acetylated Form of Staphylococcal Poly-*N*-Acetyl-β-(1-6)-Glucosamine▽

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Poly-*N*-acetyl-glucosamine (PNAG) is a staphylococcal surface polysaccharide influencing biofilm formation that is also under investigation for its vaccine potential. Antibodies that bind to PNAG with either low (<15%) or high (>90%) levels of acetate are superior at opsonic and protective activity compared with antibodies that bind to PNAG with only high levels (>70%) of acetate. PNAG is synthesized by four proteins encoded within the intercellular adhesin (*ica*) locus *icaADBC*. In *Staphylococcus epidermidis*, *icaB* encodes a deacetylase needed for the surface retention of PNAG and optimal biofilm formation. In this study, we confirmed that *icaB* plays a similar role in *Staphylococcus aureus* and found that an *icaB* mutant of *S. aureus* expressed significantly less surface-associated PNAG, was highly susceptible to antibody-independent opsonic killing that could not be enhanced with antibody raised against deacetylated PNAG (dPNAG), and had reduced survival capacity in a murine model of bacteremia. In contrast, an *icaB*-overexpressing strain produced primarily surface-associated PNAG, was more susceptible to opsonophagocytosis with antibody to dPNAG, and had increased survival in a murine bacteremia model. The highly acetylated secreted PNAG was more effective at blocking opsonic killing mediated by a human monoclonal antibody (mAb) to native PNAG than it was at blocking killing mediated by a human mAb to dPNAG, which by itself was a more effective opsonin. Retention of dPNAG on the surface of *S. aureus* is key to increased survival during bacteremia and also provides a molecular mechanism explaining the superior opsonic and protective activity of antibody to dPNAG.

Staphylococcus aureus and coagulase-negative staphylococci are the most frequent causes of nosocomial bloodstream infections (43). A critical virulence determinant in such infections is the production of a high-molecular-weight polymer of β-1-6-linked *N*-acetyl-glucosamine (PNAG) that is involved in adherence to polymeric substrates, bacterial intercellular adhesion, biofilm formation, and protection against antibody-independent opsonic killing (3, 4, 15, 26, 40). Proteins encoded by the *icaADBC* genes of the intercellular adhesin (*ica*) locus synthesize PNAG (6, 10–12, 30, 31). IcaA is a *trans*-membrane glucosyltransferase and can synthesize short PNAG polymers in vitro using UDP-*N*-acetyl-glucosamine as a substrate (10). IcaD increases the biosynthetic efficiency of IcaA (10). IcaC is also a transmembrane protein and appears to be involved in linking short polymers to make longer oligomers of PNAG (10). In its mature form, PNAG, also referred to by some researchers as polysaccharide intercellular adhesin (PIA) (12, 44), is a mixture of polymers such that about 10 to 20% of the amino groups are not acetylated, which could give some of the polymers within the polysaccharide complex a net positive charge (16, 27). It is not known whether PNAG consists of a minority of highly deacetylated molecules mixed in with a majority of highly acetylated molecules or if there is a range of acetyl substituents spread over individual molecules.

In a recent study of PNAG (called PIA) synthesis in *Staphylococcus epidermidis*, it was shown that IcaB exhibits deacetylase activity (40). In that same study, those authors demonstrated that the IcaB-induced partial deacetylation of PNAG is necessary for its association with the bacterial cell surface and that this association is required for biofilm formation and for PNAG-mediated evasion of phagocytosis. In *S. epidermidis*, a loss of the *icaB* gene resulted in the reduced persistence of one strain in a murine model of device-related infection (40). However, while several studies have shown that PNAG produced by *S. epidermidis* is an important virulence factor for coagulase-negative staphylococcus infections involving biofilm formation on implanted biomaterials (25, 37, 38), this has not been found to be the case with *S. aureus* biomaterial implant infections (8, 9, 21). Other studies indicated that for *S. aureus*, PNAG may be more important as a virulence factor for infections involving the systemic spread of the organism, as a recent study showed that the loss of the *ica* locus in *S. aureus* significantly decreased the virulence of three *S. aureus* strains in three murine models: bacteremia, renal infection, and lethal sepsis following peritonitis (22). However, in one *S. aureus* strain, strain Newman, PNAG did not appear to be a virulence factor in a murine model of pneumonia (42). Thus, it appears that there may be somewhat different roles for PNAG in the virulence of *S.*

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epidermidis versus *S. aureus* as well as differences in PNAG-dependent virulence in different anatomic sites of infection. Whether virulence is affected by the loss of IcaB deacetylase activity in *S. aureus* infections where PNAG elaboration is important has not been determined.

In addition, PNAG has been used as a target antigen in a strategy to develop an antistaphylococcal vaccine (16, 27–29). We recently reported that antibodies raised against diphtheria toxoid-conjugated, highly acetylated (>90%) PNAG lacked protective efficacy against PNAG-positive *S. aureus* strains and exhibited significantly lower opsonic killing than antibodies raised against diphtheria toxoid-conjugated chemically deacetylated PNAG (dPNAG) (~15% acetylation) (29). In addition, in contrast to antibodies to highly acetylated PNAG, the antibodies to dPNAG were protective in murine models of bacteremia and lethal peritonitis (29). Confirmatory findings were reported for human antibodies to native PNAG and dPNAG based on the properties of affinity-purified antibodies obtained from the sera of *S. aureus*-infected cystic fibrosis patients (20). Given that Vuong et al. (40) previously demonstrated that IcaB in *S. epidermidis* is a PNAG deacetylase, and that antibodies raised against highly acetylated PNAG are not optimally protective in mice, we hypothesized that the superior opsonic and protective activity of antibodies to the deacetylated form of dPNAG was related to the preferential surface retention of this form of the antigen. Such retention would obviously be essential for effective opsonic killing and protection mediated by antibody specific to this isoform of PNAG. In addition, we evaluated whether secreted, highly acetylated, native PNAG, which can bind antibody raised against dPNAG (29), can act as a decoy antigen, allowing *S. aureus* to evade antibody to PNAG. To test these hypotheses, we constructed in-frame *icaB* deletion mutants and *icaB*-overexpressing strains in *S. aureus* strain 10833, evaluated the surface retention and secretion of PNAG, and correlated these findings with biologic outcomes using biofilm formation in vitro, opsonic killing assays, and in vivo outcomes in a murine model of *S. aureus* bacteremia.

MATERIALS AND METHODS

Staphylococcal strains and media. *S. aureus* strain MN8, obtained from a patient with toxic shock syndrome, was provided by Patrick Schlievert (Minneapolis, MN). Strain MN8m is a spontaneous mutant isolated from a chemostat culture of strain MN8 that overproduces PNAG due to a 5-bp deletion in the middle of the promoter for the *ica* locus (13). *S. aureus* strain NCTC 10833 (ATCC 25904) is a clumping factor-positive variant of a throat swab isolate. Partial deletion of the *ica* locus to produce *S. aureus* strain 10833*ica*::*tet* was performed as described previously by Cramton et al. (6). The strains were grown at 37°C on tryptic soy agar plates containing the appropriate antibiotic. Liquid cultures were grown in either tryptic soy broth (TSB) lacking glucose (17 g/liter peptone from casein, 3 g/liter peptone from soymeal, 5 g/liter NaCl, 2.5 g/liter K_2HPO_4) or TSB plus an additional 1% glucose (TSBG).

Plasmids, primers, and cloning and expression of genes in the *ica* locus. All plasmid purifications were performed with the QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA). All primers were custom synthesized by QIAGEN Operon (Alameda, CA). Restriction enzymes and DNA-modifying enzymes were purchased from Invitrogen. Plasmid pMUC was derived using a previously described vector (13) by ligating the constitutively transcribed *ica* locus from strain MN8m into the shuttle vector pRB473. To create plasmid pMUCΔ*icaB*, a nonpolar, in-frame deletion which removed more than 80% of the *icaB* gene was generated by amplifying the plasmid by PCR with the following primer pair: icaBdelFWD (5′-CCATCCAGTGTGCTTACAGGC-3′) and icaBdelREV (5′-TCCATTAAGAGATGGGACGGATTCC-3′). The ends of the PCR product were phosphorylated using T4 kinase, and the linear DNA was circularized using Ready-2-Go T4 ligase (Amersham). The plasmid was sequenced at the Harvard Medical School Microbiology Core Facility to confirm that the *icaB* deletion did not cause a frameshift mutation and that the rest of the *ica* locus was free from any other mutations. The plasmid was transduced into *S. aureus* 10833*ica*::*tet* to *trans*-complement the chromosomally deleted *ica* locus and produce *S. aureus* strain 10833*ica*::*tet*+pMUCΔ*icaB*.

In order to construct a vector for the constitutive transcription of inserted DNA, the *ica* promoter was amplified from genomic DNA in the *ica*-constitutive strain MN8m by PCR using the primer pair icaprovectorFWD (5′-GGGGGATCCCCCTACTGAAAATTAATCACACTATG-3′) and icaprovectorREV (5′-CCCCCCGGGCAATTTCTTTACCTACCTTTCGTTAG-3′) and cloned into the SmaI and BamHI sites of pRB473 to produce plasmid pKJ3. The *icaB* gene was amplified from MN8 genomic DNA using primers icaBFWD (5′-GTGAAGTATAGAAAATTTATAATTTTAGTGTTGAG-3′) and icaBREV (5′-GGGGAGCTCCTAATCTTTTTCATGGAATCCGTCC-3′), digested with SstI, and cloned into pKJ3 to create p*icaB*. All plasmid constructs were initially transformed into the restriction-deficient *S. aureus* strain RN4220 according to a method described previously by Lee (23). Constructs were transferred to other strains of *S. aureus* by transduction using phage 80 (18).

RT-PCR. *S. aureus* cultures were grown in TSB at 37°C overnight. The following day, 1:10 dilutions of the cultures were made in TSBG and incubated at 37°C for 4 h with shaking. RNA was extracted from 10^9 cells using the RNeasy Protect Miniprep kit (QIAGEN) according to the manufacturer's instructions except that cells were lysed by vortexing in the presence of 4-mm glass beads for 10 min. Turbo DNase was added to digest contaminating DNA that was then inactivated by heating according to the manufacturer's instructions (Ambion). RNA concentrations were determined by the absorbance at 260 nm, and 1 μg of each sample was analyzed using the Superscript II One-Step reverse transcription-PCR (RT-PCR) kit from Invitrogen and primers specific for *icaA* (5′-GTCTATTTACTGGATTGTCGGTC-3′ and 5′-GTCTGACTTCGCTTAATACAGCC-3′), *icaC* (5′-CCTTAGTGTTACAATTTTACATTCG and 5′-CGTTCGTAGTTATAACCCATATATGC-3′), or gyrase B, as a positive control (5′-TTATGGTGCTGGGCAAATACAAG-3′ and 5′-CACCATGTAAACCACCAGATAC-3′). Agarose gels were stained with ethidium bromide and photographed. Each experiment was performed at least three times.

Biofilm assay. Microtiter plate assays for biofilm production were performed essentially as described previously by Christensen et al., with minor modifications (5). Cultures were grown overnight in 2 ml of TSBG, diluted 1:200 in TSBG, and aliquoted into 96-well polystyrene flat-bottom microtiter plates from Corning (Corning, NY). After 24 h at 37°C, the wells were emptied and washed twice with phosphate-buffered saline (PBS). The plates were dried at ambient temperature, stained for 30 s with safranin, washed under gently running tap water, and scanned using a digital scanner. The stained biofilms were resuspended in 100 μl PBS by gentle sonication, transferred into new microtiter wells, diluted 1:4 in PBS, and analyzed by spectrophotometry at an optical density at 450 nm (OD_{450}) using an enzyme-linked immunosorbent assay reader.

Production of human mAbs. Human monoclonal antibodies (mAbs) were produced as previously described (19). Briefly, B cells from a patient recovering from *S. aureus* infection were transformed with Epstein-Barr virus and screened for their ability to bind either acetylated PNAG or dPNAG. Immunoglobulin variable region genes from hybridomas of interest (binding to either PNAG, dPNAG, or both) were cloned into the immunoglobulin G1 (IgG1)-TCAE6 vector and transfected into CHO cells for the production of fully human IgG1 mAbs. The mAbs secreted by the F598 hybridoma (high binding to both acetylated PNAG and dPNAG) and the F628 hybridoma (high binding only to acetylated PNAG) resulting from these manipulations (19) were used in the studies described here.

Immunological detection of PNAG on the cell surface and in culture supernatants. PNAG blots were performed essentially as described previously (7), with minor modifications. Bacteria were grown overnight in 5 ml TSBG. The cultures were diluted in TSB to produce an OD_{600} of ~1.5, and 10 ml was centrifuged to recover the bacterial cells. The culture supernatant was saved, and the cell pellet was washed once with 1 ml PBS and then resuspended in 100 μl 0.5 M EDTA with gentle sonication, followed by boiling for 5 min. The extracts were cleared by centrifugation, and extracts and culture supernatants were treated separately with 200 μg proteinase K and incubated at 60°C for 1 h and then at 85°C for 30 min to inactivate the protease. The cell surface extracts and culture supernatants were immobilized on nitrocellulose using a vacuum manifold. A 1:1 dilution represents one-half of the surface extract from each culture and one-quarter of the culture supernatant. Dilutions (1:10 and 1:100) were made using Tris-buffered saline (TBS). Blots were blocked for 1 h in 1% bovine serum albumin (BSA) in TBS, probed with 1:1,000-diluted goat antiserum specific for PNAG (29), which was affinity-purified as described previously (19),

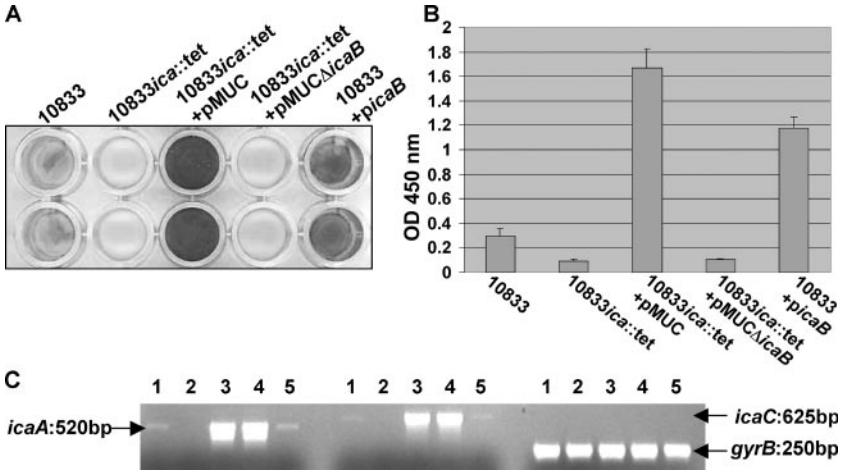

FIG. 1. Role of IcaB in biofilm formation by *S. aureus*. *S. aureus* strain 10833 produces a weak biofilm, which is lost by the deletion of the entire *ica* locus (10833*ica*::*tet*). Biofilm formation is augmented by the presence of the pMUC plasmid (10833*ica*::*tet*+pMUC); however, deletion of *icaB* from pMUC (10833*ica*::*tet*+pMUCΔ*icaB*) results in a phenotype similar to that of the *ica* mutant. Overexpression of *icaB* in the parental strain (10833+p*icaB*) enhances biofilm formation. (A) Safranin-stained biofilms of bacteria grown overnight in TSBG in microtiter wells. (B) Quantitative analysis of the stained biofilms after homogenization by sonication in PBS. Bars reflect the mean OD of eight samples, and error bars indicate the standard deviations. Statistical comparisons (all unpaired *t* tests) were as follows: for 10833 versus 10833*ica*::*tet*, the *P* value was <0.001; for 10833 versus 10833+p*icaB*, the *P* value was 0.001; for 10833*ica*::*tet* versus 10833*ica*::*tet*+pMUC, the *P* value was <0.001; for 10833*ica*::*tet*+pMUC versus 10833*ica*::*tet*+pMUCΔ*icaB*, the *P* value was <0.001. (C) RT-PCR using RNA from strain 10833 (lane 1), strain 10833*ica*::*tet* (lane 2), strain 10833*ica*::*tet*+pMUC (lane 3), strain 10833*ica*::*tet*+pMUCΔ*icaB* (lane 4), and strain 10833+p*icaB* (lane 5). Primers specific for *icaA* transcript produced a 520-bp PCR product (lanes 1 to 5, left), primers specific for *icaC* produced a 625-bp product (lanes 1 to 5, center), and primers specific for the positive control, *gyrB*, yielded a 250-bp product (lanes 1 to 5, right).

diluted in TBS–0.05% Tween 20 containing 1% BSA (24) for 2 h at room temperature, washed, and probed with 1:10,000 swine anti-goat IgG horseradish peroxidase conjugate in TBS–0.05% Tween 20–1% skim milk for 1 h at room temperature. Bands were visualized using the ECL kit (Amersham) and autoradiography.

Immunofluorescence microscopy. Glass-bottom microwell plates (Mattek, MA) were coated with 4% Celltak (BD Biosciences, MA) solution for 30 min and then washed twice with sterile distilled water. Freshly harvested bacterial suspensions were added to the plates, allowed to sit for 20 min at room temperature, and then washed twice with PBS. *S. aureus* 10833*ica*::*tet*+pMUC and *S. aureus* 10833*ica*::*tet*+pMUCΔ*icaB* were fixed in 4% (wt/vol) formaldehyde in PBS for 30 min at 4°C and then washed twice with PBS. Blocking solution containing 1% BSA and 2% normal rabbit serum (to block nonspecific binding to protein A) in PBS was added and incubated for 1 h at room temperature with rocking. After this time, the plates were washed, incubated with a primary antibody solution to either PNAG (human IgG1 mAb F598) or an isotype control (human IgG1 mAb F429 specific for *Pseudomonas aeruginosa* alginate) (34) diluted to 20 μg/ml in blocking buffer, and left for 2 h at room temperature with rocking. After being washed four times with PBS for 5 min each (with rocking), a secondary antibody (anti-human IgG) (Alexa 488; Molecular Probes, Corvallis, OR) diluted 1:5,000 in blocking buffer was added. The plate was left rocking at room temperature for 1 h and then washed again with PBS four times for 5 min each with rocking at room temperature. The plates were examined by phase-contrast and fluorescence microscopy. Images of the same field viewed by the two different microscopic methods were acquired by a camera, and images were processed by computer using the LSM 5 image analysis system.

Opsonophagocytic assays. White blood cells (WBC) were prepared from fresh human blood collected from healthy adult volunteers. Twenty-five milliliters was mixed with an equal volume of dextran-heparin buffer and incubated at 37°C for 1 h. The upper layer containing the leukocytes was collected, the cells were pelleted by centrifugation, and hypotonic lysis of the remaining erythrocytes was accomplished by resuspension of the cell pellet in 1% NH_4Cl and incubation for 10 min at room temperature. WBC were then washed three times and resuspended with RPMI with 15% fetal bovine serum (RPMI-FBS). Using trypan blue staining to differentiate dead from live leukocytes, the final WBC count was adjusted to 2.5×10^7 WBC per ml. The complement source (1 ml of baby rabbit serum diluted 1:10 in RPMI-FBS) was adsorbed at 4°C for 45 min with continual mixing using bacteria resuspended from a pellet containing $\sim 10^9$ CFU of *S. aureus* strain MN8. After adsorption, the complement solution was centrifuged and filter sterilized. The bacterial strains to be evaluated for phagocyte-dependent killing activities of antibody were grown overnight in TSBG, adjusted to an OD_{650} of 0.1 with fresh TSBG, and allowed to grow to an OD_{650} of 0.4. A 1:100 dilution was then made in RPMI-FBS for use in the killing assay. The protocol for the use of human WBC in these assays was approved by the Brigham and Women's Hospital Institutional Review Board.

The opsonophagocytic assay was performed with 100 μl of leukocytes, 100 μl of bacteria, 100 μl of the complement solution, and 100 μl of protein G-purified mAb F598 or mAb F628 at four different concentrations. Controls were made by replacing the antibody with RPMI-FBS. The reaction mixture was incubated on a rotor rack at 37°C for 90 min. The tubes were vortexed for 15 s and diluted in TSB with 0.25% Tween to prevent bacterial aggregation, and samples were plated onto tryptic soy agar plates. The percentage of killing was calculated by determining the ratio of the CFU surviving in the tubes with bacteria, leukocytes, complement, and antibody to the CFU surviving in the tubes with all these components but lacking antibody. For antibody-independent phagocytosis, we calculated the percentage of killing of bacteria by leukocytes and complement only, comparing the surviving counts with those in tubes lacking either leukocytes or complement. The assay was performed with triplicate samples and repeated two to three times.

Inhibition of phagocytosis by competitive assay. PNAG was purified from a culture of *S. aureus* MN8m as previously described (16, 27), and a molecule that was >95% acetylated was obtained. Since mAbs F598 and F628 mediate different levels of opsonic killing per microgram of protein, we standardized the assay by using concentrations of the antibodies that would result in approximately equivalent levels of opsonic killing. PNAG diluted in RPMI-FBS was used in the opsonophagocytic reactions described above at three different concentrations, based on the ratio of IgG used in each assay. Due to the low solubility of PNAG at neutral pH, a maximum concentration of 200 mg PNAG/liter could be used in these assays. Control samples consisted of bacteria, leukocytes, complement, mAb, and PBS without PNAG. The percentage of inhibition of killing was calculated by determining the ratio of the CFU surviving in the tubes with PNAG to the CFU surviving in the tubes without PNAG. The assay was done with triplicate samples and repeated two to three times.

Murine bacteremia model. The animal model used was previously described (29). Briefly, groups of six mice (FVB-NJ; female, 5 to 7 weeks of age) were challenged intravenously (i.v.) with a dose of 1.0×10^7 CFU of *S. aureus* strains in 0.2 ml of PBS. Mice were sacrificed 90 min after bacterial challenge, blood samples were withdrawn, numbers of surviving bacteria were determined by

serial diluting and plating of blood samples, and the results were expressed as CFU/ml of blood. All animal studies were approved by the Harvard Medical Area Institutional Animal Care and Use Committee.

Statistical analysis. Quantitative assays were compared using one-way analysis of variance by applying the Levene's test of homogeneity of variances, the Tukey multiple-comparisons test, and also unpaired-sample *t* tests using SPSS software (Statistical Package for the Social Sciences). All tests were performed with a confidence level of 95%.

RESULTS

IcaB is required for biofilm formation in *S. aureus*. We first analyzed the biofilm-forming capacity of *icaB*-overexpressing and deletion mutant strains of *S. aureus*. *S. aureus* strain 10833 produced a moderate biofilm when grown in TSBG, and the *ica* deletion mutant, which is unable to produce any PNAG, elaborated very little biofilm (Fig. 1). When the *icaB* gene was overexpressed in strain 10833, however, biofilm formation increased approximately fourfold ($P = 0.001$, unpaired *t* test). When strain 10833*ica*::*tet* was complemented with pMUC, which contains the *ica* locus from the PNAG-overproducing strain MN8m, it produced a heavy biofilm. In-frame deletion of the *icaB* gene from pMUC to produce pMUCΔ*icaB* resulted in a strain that was phenotypically similar to *S. aureus* 10833*ica*::*tet* that lacks the *ica* genetic locus in that *S. aureus* 10833*ica*::*tet*(pMUCΔ*icaB*) could not produce a biofilm ($P < 0.001$, unpaired *t* test between strains carrying pMUC and those carrying pMUCΔ*icaB*).

Deletion of *icaB* in pMUC did not induce a frameshift mutation in the *ica* locus; nonetheless, we considered the possibility that the mutation might affect the transcription of the downstream gene *icaC*. To assess the transcription of the *ica* locus in the absence of *icaB*, we performed RT-PCR to measure levels of *icaA* and *icaC* transcripts (*gyrB* transcript levels were measured as a control). As expected from our previous findings that *ica* is constitutively transcribed in pMUC, *icaA* and *icaC* transcript levels in *S. aureus* 10833*ica*::*tet*(pMUC) were much greater than those in *S. aureus* 10833, and the levels of *icaA* and *icaC* RNA in *S. aureus* 10833*ica*::*tet*(pMUCΔ*icaB*) were comparable to those obtained from *S. aureus* strain 10833*ica*::*tet*(pMUC), indicating that the *icaB* deletion mutation does not affect the transcription of the *icaADC* genes in pMUCΔ*icaB* (Fig. 1C). Figure 1C also demonstrates, as expected, that the overexpression of *icaB* in *S. aureus* strain 10833(p*icaB*) does not affect the transcription of the *icaA* and *icaC* genes.

IcaB is required for the association of PNAG with the bacterial cell surface. Immunoblots were used to characterize the levels of PNAG in bacterial cell surface extracts and culture supernatants from the isogenic *S. aureus* 10833 strains. As expected, strain 10833*ica*::*tet* did not produce any detectable PNAG (Fig. 2). Some of the PNAG produced by wild-type *S. aureus* strain 10833 was found in the supernatant, whereas the majority of the polysaccharide was cell surface associated (Fig. 2). Placing the pMUC plasmid into strain 10833*ica*::*tet*, which leads to high levels of *ica* transcripts, augmented PNAG production. In *S. aureus* strain 10833*ica*::*tet*+pMUC, the majority of the PNAG was surface associated. In *S. aureus* strain 10833*ica*::*tet*+pMUCΔ*icaB*, however, a minority of the PNAG was on the surface, and most of it was secreted. In addition, the IcaB-overexpressing strain (10833+p*icaB*) had more PNAG on the surface than the wild-type parental strain, and none was detected in the supernatant.

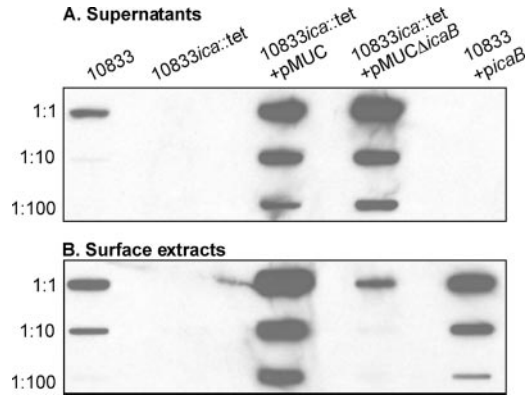

FIG. 2. IcaB is required for the association of PNAG with the bacterial cell surface. *S. aureus* 10833 produces PNAG, the majority of which is surface associated. Strain 10833*ica*::*tet* does not produce detectable PNAG. Complementation of the *ica* deletion with plasmid pMUC (10833*ica*::*tet*+pMUC) resulted in enhanced PNAG production, with the majority present in surface extracts. Deletion of *icaB* from plasmid pMUC (10833*ica*::*tet*+pMUCΔ*icaB*) did not prevent PNAG synthesis but resulted in the secretion of most of the polysaccharide into the culture supernatant. Overexpression of *icaB* in the parental strain (10833+p*icaB*) resulted in an increase in PNAG production, with essentially all of the polysaccharide retained on the cell surface.

We also evaluated the surface association of PNAG by immunofluorescent microscopic analyses. *S. aureus* strains 10833*ica*::*tet*+pMUC and 10833*ica*::*tet*+pMUCΔ*icaB* were stained with dPNAG-specific mAb F598, and immunofluorescence analysis indicated that the mAb bound well to the surface of *S. aureus* 10833*ica*::*tet*+pMUC, but staining of *S. aureus* 10833*ica*::*tet*+pMUCΔ*icaB* was not seen (Fig. 3). At no magnification under which these cells were examined could any fluorescence be detected. No fluorescence more than 10% above the background was observed with either *S. aureus* strain when a human IgG1 isotype control antibody was used in place of mAb F598 (not shown). Together, these results indicate that, similar to *S. epidermidis*, IcaB is required for the optimal association of PNAG with the bacterial cell surface in *S. aureus* as well as the formation of a strong biofilm in tissue culture wells.

Opsonophagocytic assays. We compared phagocyte and complement-dependent killing by human mAb F598, which reacts with both dPNAG and native PNAG antigens, and mAb F628, which reacts optimally with the highly acetylated native PNAG molecule but has little antigen binding activity when acetates are removed (19). In addition, mAb F598 has notably better antigen binding activity to native PNAG than mAb F628, which is indicative of a potentially greater affinity in binding to this antigen (19). Killing of *S. aureus* 10883 and *S. aureus* 10833+(p*icaB*) mediated by mAbs F598 and F628 showed that mAb F598 had higher killing activity than mAb F628 for both strains ($P < 0.001$, unpaired *t* test) (Fig. 4A). Overexpression of IcaB in *S. aureus* 10833+(p*icaB*) enhanced the killing by dPNAG-binding mAb F598 compared to the opsonic killing of wild-type *S. aureus* 10833 ($P < 0.001$, unpaired *t* test) (Fig. 4A). There was no opsonic killing in the

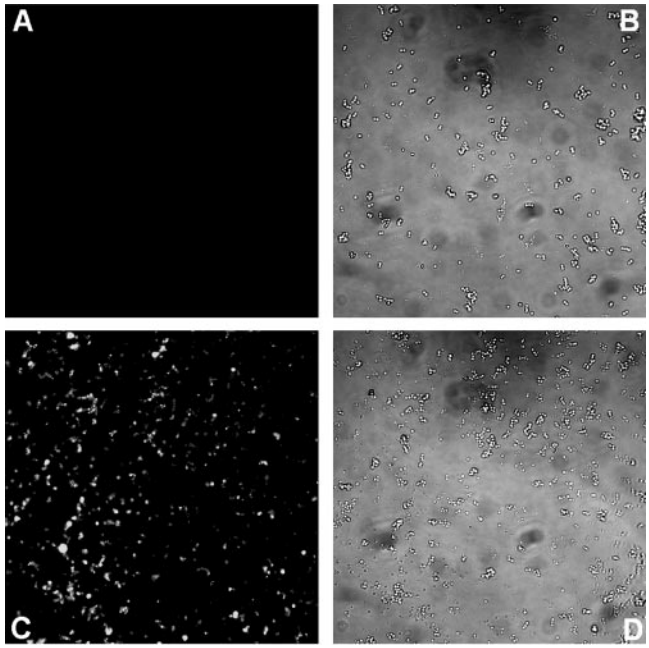

FIG. 3. Evaluation of surface-associated PNAG by *S. aureus* strains by immunofluorescence. (A and B) Fluorescence (A) and phase-contrast (B) microscopy of immunostained *S. aureus* strain 10833*ica*::*tet*+pMUCΔ*icaB*. (C and D) Fluorescence (C) and phase-contrast (D) microscopy of immunostained *S. aureus* 10833*ica*::*tet*+pMUC. PNAG was visualized by reactions with human mAb F598 and secondary anti-human IgG conjugated to Alexa 488.

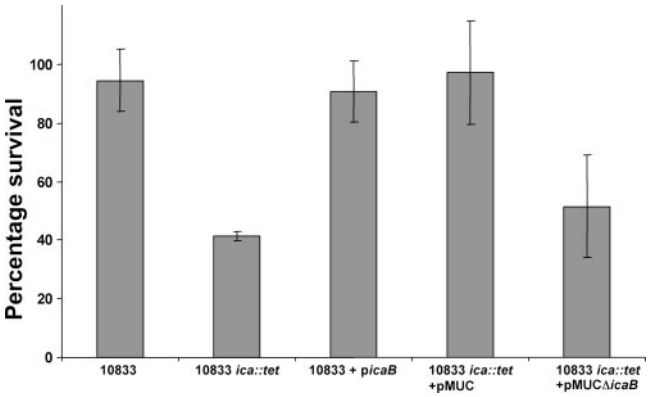

FIG. 5. Antibody-independent phagocytosis of *S. aureus* strains. Survival of an initial inoculum of ~5×10^5 CFU of each strain after incubation at 37°C for 90 min with 2×10^5 PMN and 20% infant rabbit serum as a complement source is shown. Bars represent means, and error bars represent standard deviations. *S. aureus* strain 10833 has a significantly higher survival rate than strain 10833*ica*::*tet* ($P < 0.001$, unpaired *t* test), whereas 10833*ica*::*tet*+pMUC has a higher survival rate than strain 10833*ica*::*tet*+pMUCΔ*icaB* ($P < 0.001$, unpaired *t* test).

absence of mAb, polymorphonuclear leukocyte (PMN), or complement in the reaction tubes, as these values served to calculate the percentage of bacteria killed.

Figure 4B presents the results of the opsonic killing assays using *S. aureus* strains 10833*ica*::*tet*+pMUC and 10833*ica*::*tet*+pMUCΔ*icaB*. The PNAG-overproducing *S. aureus* strain 10833*ica*::*tet*+pMUC was effectively killed by dPNAG-binding mAb F598 but relatively resistant to the opsonic killing effect of native PNAG-binding mAb F628 ($P < 0.001$, unpaired *t* test). Of note, in the absence of mAb, ~60% of *S. aureus* strain 10833*ica*::*tet* and 50% of *S. aureus* strain 10833*ica*::*tet*+pMUCΔ*icaB* were killed by phagocytes and absorbed complement alone, indicating that a loss of the cell surface PNAG resulted in a marked increase in the susceptibility to antibody-independent killing compared to killing in the absence of either phagocytes or complement (Fig. 5). There was no killing of any strain in the absence of PMN or complement (data not shown). When we tested mAbs F598 and F628 for opsonic killing of *S. aureus* strain 10833*ica*::*tet*+pMUCΔ*icaB* (Fig. 4B), there was no enhancement of bacterial killing over that achieved in the presence of only phagocytes and complement ($P > 0.05$, unpaired *t* test), consistent with the low level of PNAG on the surface of this strain.

Inhibition of phagocytosis by competitive assay. To test the hypothesis that non-cell-associated PNAG can act as a decoy

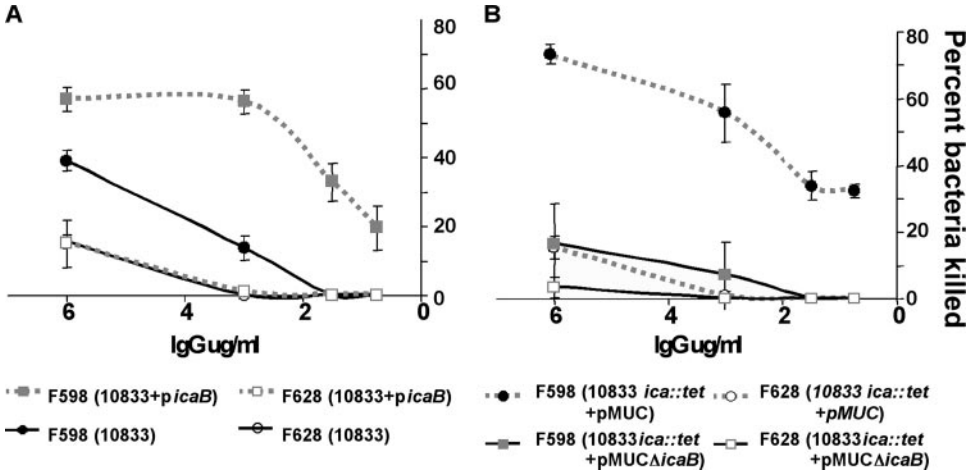

FIG. 4. Opsonophagocytic activity of human IgG1 mAbs F598 (dPNAG and native PNAG binding) and F628 (native PNAG binding only) against *S. aureus* strains. The legend indicates which mAb was used against the four different *S. aureus* strains. Symbols represent means, and error bars represent standard deviations.

TABLE 1. Inhibition of opsonophagocytic activity of human IgG1 mAbs F598 and F628 against *S. aureus* wild-type strain 10833 by highly acetylated PNAG

mAb used	Concn (mg/liter)	PNAG:IgG ratio	% Inhibition
F598	6	1:1	6 ± 7
		4:1	25 ± 12
		10:1	36 ± 5
F628	50	1:1	32 ± 12
		4:1	57 ± 13

to the PNAG-specific opsonic antibody, we added purified, highly acetylated (~95%) PNAG to opsonophagocytic reactions mediated by either mAb F598 or mAb F628 against *S. aureus* strain 10833. Table 1 presents the percentage of inhibition of killing achieved compared to that of control tubes without added PNAG. When we used a 1:1 (wt/wt) ratio of PNAG antigen to mAb, phagocytosis mediated by dPNAG-binding mAb F598 was inhibited only 6%. When we used native PNAG-binding mAb F628 under the same conditions, the inhibition level, 36%, was significantly higher ($P = 0.001$, unpaired *t* test). The greater ability of highly acetylated PNAG to inhibit killing mediated by the mAb to native PNAG compared to the mAb that can also bind to dPNAG was further verified with the other PNAG-to-IgG ratios that we tested (Table 1). We could not test inhibition by higher ratios of PNAG to mAb F628 due to the poor solubility of the antigen at neutral pH at higher concentrations.

Murine bacteremia model. The role of *icaB* in *S. aureus* survival in the blood of mice following i.v. injection was evaluated using two different comparisons of paired strains. The first involved comparing the survival of the IcaB-overexpressing strain with that of the wild-type parental strain, and the second involved comparing the *ica* deletion strain carrying pMUC to restore PNAG production with the same strain carrying pMUCΔ*icaB*. When IcaB was overexpressed in *S. aureus* 10833, significantly more bacteria survived in the blood after 90 min ($P < 0.001$, unpaired *t* test) than in the wild-type parental strain (Fig. 6A). On the other hand, when the *icaB* gene was deleted from the pMUC locus (Fig. 6B), a significantly lower number of bacteria survived than that of bacteria carrying the intact pMUC plasmid ($P < 0.001$, unpaired *t* test). Thus, the overexpression of IcaB enhanced the surface retention of PNAG and enhanced resistance to mouse mediators of bacterial clearance from the blood, whereas the deletion of *icaB* increased the clearance of *S. aureus* from mouse blood.

DISCUSSION

One known strategy that pathogens use to avoid host immune defenses is to elicit high levels of poorly protective antibodies. The protective efficacy of antibodies may be poor due to low antibody affinity (35), the production of an inappropriate antibody isotype (2, 32), or specificity for nonprotective epitopes (39). This last strategy appears applicable to the immune response to staphylococcal PNAG, wherein antibodies that bind best to the immunodominant epitopes on highly acetylated antigen function poorly in opsonic killing and protection assays (29). Overcoming this evasive strategy by immunizing animals with a conjugate vaccine containing poorly acetylated dPNAG elicited antibodies that were superior to those to native PNAG in opsonic killing and protection against systemic *S. aureus* infection (29). However, the molecular basis for the superior efficacy of the dPNAG-specific antibodies was not apparent in the data reported in that prior publication.

The results reported here indicate that in the absence of the IcaB protein, less PNAG is preferentially retained on the *S. aureus* cell surface, as it is for *S. epidermidis* (40), presumably due to a lack of *N*-deacetylase activity in the absence of IcaB. We speculate that in the presence of IcaB deacetylase activity, the PNAG retained on the *S. aureus* cell surface is less able to react with antibody to native PNAG and better able to bind antibody to dPNAG, potentially improving opsonic killing and protection. Increasing the *N*-deacetylase activity by the overexpression of *icaB* increased *S. aureus* susceptibility to killing by a human IgG1 mAb able to bind well to dPNAG but had no effect on killing by a human IgG1 mAb that binds only to native PNAG. Also, increased expression of *icaB* leads to greater survival of this strain in mouse blood, indicating a crucial role for surface-associated PNAG in bacterial resistance to antibody-independent opsonic killing. In contrast, a loss of *icaB* resulted in a strain with decreased surface retention of PNAG, increased susceptibility of the strain to antibody-independent killing, and reduced survival in the blood of infected mice. These findings point to a key role for the IcaB protein in pathogenesis due to its ability to influence the amount of PNAG on the *S. aureus* cell surface.

Vuong et al. (40) initially determined that the *icaB* gene is responsible for the deacetylation of PNAG (referred to as PIA) in *S. epidermidis* and that the deacetylation is necessary for the surface retention of PNAG. The results from our study confirmed that IcaB is needed for the surface retention of PNAG in *S. aureus* strains. Given the high homology of the *icaB* genes in *S. epidermidis* and *S. aureus* and the identical effects that they mediate in regard to PNAG surface retention in both organisms, it is almost certain that IcaB in *S. aureus* also functions as an *N*-deacetylase. Surface retention of PNAG promotes the adherence of staphylococci to various plastics and glass and is necessary for biofilm formation in many, but

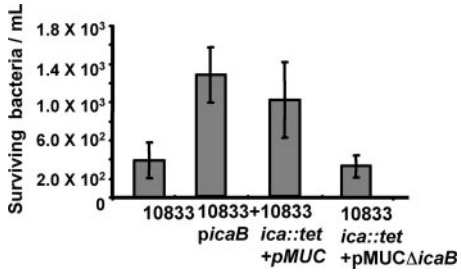

FIG. 6. Murine bacteremia model demonstrates the role for IcaB in enhanced survival of *S. aureus* in vivo. The bars represent the mean numbers of bacteria that were recovered from blood 90 min after i.v. infection, and the error bars indicate standard deviations. Wild-type *S. aureus* 10833 survives less well than *S. aureus* 10833+p*icaB* (IcaB overexpresser) ($P < 0.001$, unpaired *t* test); *S. aureus* 10833*ica*::*tet*+pMUC survives better than *S. aureus* 10833*ica*::*tet*+pMUCΔ*icaB* ($P < 0.001$, unpaired *t* test).

not all (1), strains. Therefore, without *icaB*, PNAG can no longer function in the role of a polysaccharide adhesin, and biofilms cannot form in the absence of surface-retained PNAG. As it is well established that biofilm formation renders bacterial cells more resistant to the immune system (24) and to antibiotic therapy (14) and biofilm formation is an important virulence factor in *S. epidermidis* device-related infections (36, 37), the IcaB protein of staphylococci clearly plays a key role in the pathogenesis of biofilm infections by modulating the attachment of PNAG to the staphylococcal cell surface.

The results reported here, along with those reported previously by Vuong et al. (40), indicate that the PNAG polymer must be anchored to the bacterial cell to form biofilms; PNAG cannot function merely as a sort of intercellular glue binding cells together. Although PNAG was first found to be associated with the production of biofilms in staphylococcal strains, it has now been shown to play a role in biofilm formation by a variety of bacterial species including *Escherichia coli*, *Yersinia pestis*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Actinobacillus pleuropneumoniae*, and *Bordetella pertussis* among others (17, 33, 41). Whether IcaB homologs within the genomes of these other organisms have a similar function in regard to biofilm formation is not known. However, Wang and colleagues showed that a deletion of the *icaB*-homologous gene in *E. coli*, designated *pgaB*, resulted in a loss of biofilm formation for this organism (41).

Our findings also suggest that the secreted form of PNAG may act as a decoy by binding potentially opsonic antibodies away from the cell surface, preventing them from mediating killing. Importantly, the inhibition of binding of a human IgG1 mAb that reacts only with native PNAG compared to the inhibition achieved with a human IgG1 mAb that can also bind to dPNAG suggests that non-cell-associated PNAG may contribute to bacterial resistance to antibody-dependent opsonic killing when antibodies to native PNAG are present.

When the findings reported here are integrated with the findings of other studies that indicate that highly acetylated PNAG may also play a role in virulence by eliciting a less protective antibody response to this antigen (29), it appears that there are multiple properties of this molecule that contribute to the pathogenesis of staphylococcal infections. Effectively overcoming the immunodominance of the poorly protective epitopes associated with a high level of acetylation by use of dPNAG conjugate vaccines was a critical step in furthering the development of PNAG as a vaccine target (29). Confirming the superior opsonic activity of a human mAb capable of binding to dPNAG over that of a human mAb that binds best to native PNAG (19) provided further support for our conclusion that antibody to the surface-retained form of PNAG provides better protection against staphylococcal infection. Studies of antibodies with specificity for either native PNAG or dPNAG isolated by affinity chromatography from the sera of *S. aureus*-infected cystic fibrosis patients (20) also showed superior opsonic killing mediated by the antibodies with specificity to dPNAG. The results from the studies reported here showing the effects of the loss of expression of IcaB and overexpression of IcaB on the ability of *S. aureus* to form biofilms, resist antibody-independent opsonic killing, and survive in mouse blood not only explain, in part, the role of PNAG in these varied aspects of *S. aureus* virulence but also point to a reason why antibody to dPNAG has greater opsonic and protective efficacy. With preferential retention of the deacetylated form of the antigen on the cell surface, there is now a molecular basis for understanding why the antibodies to dPNAG function in a superior fashion compared to those that bind less well to the antigen when acetate groups are reduced. This insight should help guide further vaccine development based on the PNAG molecule, supporting the pursuit of the dPNAG form of the antigen as a vaccine candidate.

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