The Giant Protein Ebh Is a Determinant of *Staphylococcus aureus* Cell Size and Complement Resistance

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Staphylococcus aureus USA300, the clonal type associated with epidemic community-acquired methicillin-resistant *S. aureus* (MRSA) infections, displays the giant protein Ebh on its surface. Mutations that disrupt the *ebh* reading frame increase the volume of staphylococcal cells and alter the cross wall, a membrane-enclosed peptidoglycan synthesis and assembly compartment. *S. aureus ebh* variants display increased sensitivity to oxacillin (methicillin) as well as susceptibility to complement-mediated killing. Mutations in *ebh* are associated with reduced survival of mutant staphylococci in blood and diminished virulence in mice. We propose that Ebh, following its secretion into the cross wall, contributes to the characteristic cell growth and envelope assembly pathways of *S. aureus*, thereby enabling complement resistance and the pathogenesis of staphylococcal infections.

*S*taphylococcus aureus is both a commensal and an invasive pathogen that causes soft tissue infections, sepsis, endocarditis, and pneumonia (1). Owing to the frequent use of antibiotics, staphylococci have evolved resistance to many different drugs (2). These methicillin-resistant *S. aureus* (MRSA) strains are associated with therapeutic failure and increased mortality due to staphylococcal infections (3, 4). In the United States, clones of the USA300 lineage cause epidemics of community-acquired MRSA infections (2). Two drugs, daptomycin and linezolid, were recently licensed for the treatment of these infections (5, 6); however, MRSA strains evolve resistance against new antibiotics (7). Thus, there is a continued need for the identification of new drug targets and therapies that combat MRSA.

S. aureus is a spherical microbe with a thick cell wall envelope that is synthesized at the cross wall, a compartment formed during cell division from the plasma membranes of adjacent daughter cells (8). Upon completion of peptidoglycan synthesis, staphylococci split cross walls down the middle, thereby separating daughter cells that form new cross walls in a plane perpendicular to their previous division plane (9). Cell wall synthesis and cell separation at the cross wall require protein traffic into this compartment (10–13). Several precursor proteins with signal peptides that bear a YSIRK-G/S motif have been shown to be secreted at the cross wall, including sortase-anchored surface proteins (12), Geh (lipase) (14), and LytN, a murein hydrolase responsible for cross wall splitting (10). Although the mechanism of precursor secretion into the cross wall has not been revealed, the purpose of such traffic is either the display of cell wall proteins on the bacterial surface or a contribution of secreted proteins to cross wall synthesis and cell separation (15). Bioinformatic analysis identified Ebh, a giant precursor, as harboring the YSIRK-G/S signal peptide (Fig. 1A) (16, 17). A specific role for Ebh during the cell cycle of *S. aureus* was heretofore not known.

Also described as encoding an extracellular matrix binding protein in *Staphylococcus epidermidis* (18, 19), *ebh* is conserved in *S. aureus*, *S. epidermidis*, *Staphylococcus warneri*, and *Staphylococcus haemolyticus* (20) but is not found in the *Staphylococcus intermedius* group and in *Staphylococcus lugdunensis*, species that do not colonize humans and are infrequently associated with human disease (21, 22). *ebh* appears to be transcribed as a monocistronic, 31,266-nucleotide gene in strain USA300. The predicted 10,421-amino-acid Ebh precursor comprises an N-terminal YSIRK-G/S signal peptide (amino acid [aa] residues 1 to 39), an N-terminal domain annotated as a hyperosmolarity resistance domain (Ebh$_N$; residues 179 to 2530) (23), 7 repeats of the 54-residue FIVAR domain (possibly associated with polysaccharide binding), 50 repeats of the 123-residue FIVAR-GA domain (albumin binding), 7 repeats of DUF1542 (a 72-residue domain of unknown function), a putative SMC domain (structural maintenance of chromosomes; residues 8976 to 9576), a transmembrane domain (residues 10227 to 10247), and, finally, the positively charged cytoplasmic domain (Fig. 1A) (24). Clones of the USA300 lineage, as well as several other clinical isolates, harbor intact *ebh* open reading frames (ORFs) (25). *S. aureus* isolates that have been maintained in research laboratories harbor nucleotide changes truncating the *ebh* ORF at coding sequences for the FIVAR-GA or DUF1542 repeats. For example, in strain Newman, Ebh is only 7,031 residues long (Fig. 1A) (26–28).

Recombinant purified FIVAR domains of Ebh (or its Embp homologue) have been reported to bind fibronectin, heparin, hyaluronate, and plasminogen; nevertheless, the physiological relevance of such associations is not yet appreciated (16, 18, 19). X-ray crystallography revealed FIVAR-GA as double (FIVAR)- and triple (GA)-helical bundle structures with an elongated shape (24, 29). Other work reported that *S. aureus* 8325-4 *ebh* mutants display increased sensitivity to teicoplanin (a glycopeptide antibiotic), osmotic stress, and Triton X-100 (23). To the best of our knowledge, previous studies on *S. aureus ebh* were performed with strains that carry truncating mutations in this gene. Previous work also left unresolved whether the giant Ebh protein contributes to methicillin resistance or to the pathogenesis of staphylococcal infections.
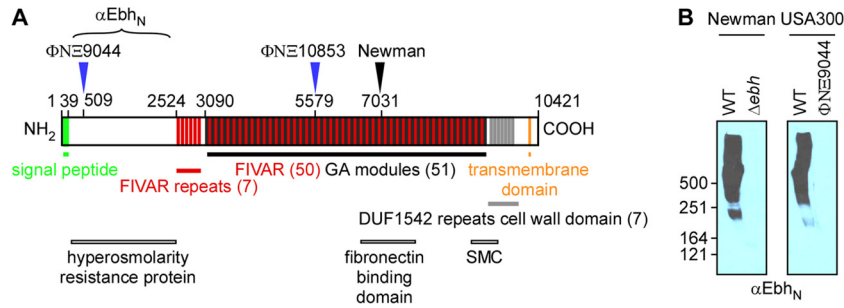
FIG 1 The giant Ebh protein of *Staphylococcus aureus*. (A) Schematic of Ebh in *Staphylococcus aureus* USA300 LAC and its variants. The diagram of Ebh domains includes the N-terminal YSIRK-G/S signal peptide, the hyperosmolarity resistance region, FIVAR and GA domain repeats, the SMC region, DUF1542, and the transmembrane and cytoplasmic domains. Blue arrows denote the positions of transposon insertions ΦNΞ9044 and ΦNΞ10853, which lead to truncated proteins of 509 and 5,579 residues, respectively. Compared to wild-type (WT) strain USA300 LAC, ΦNΞ9044 represents the *ebh* null mutant. *S. aureus* Newman harbors a truncated *ebh* gene with a termination codon after codon 7031 (black arrow). (B) Immunoblot experiments with staphylococcal cell lysates separated by 6% SDS-PAGE to detect Ebh expression in USA300 LAC compared with its ΦNΞ9044 *ebh* variant and in *S. aureus* Newman compared with its Δ*ebh* mutant. Rabbit immune serum against Ebh$_N$ (residues 42 to 2524) was used to detect Ebh. Numbers indicate molecular mass markers (kDa).

MATERIALS AND METHODS

Ethics statement. Experiments with blood from human volunteers involved a protocol that was reviewed, approved, and performed under the regulatory supervision of the University of Chicago's Institutional Review Board (IRB). Written, informed consent was provided by all volunteers. Animal experiments involving *S. aureus* challenge followed protocols that were reviewed, approved, and performed under the regulatory supervision of the University of Chicago's Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC). Animals were handled by the University of Chicago Animal Resource Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care and the Department of Health and Human Services. BALB/c mice and New Zealand White rabbits were purchased from Charles River Laboratories and Harlan Sprague Dawley, respectively. The statistical analysis of mouse survival data during staphylococcal sepsis was analyzed with the two-tailed log rank test. The results of all animal experiments were examined for reproducibility.

Bacterial strains and growth. *S. aureus* strains were cultured on tryptic soy agar (TSA) or in tryptic soy broth (TSB) at 37°C. *Escherichia coli* strains DH5α and BL21(DE3) were cultured on Luria agar or in Luria broth at 37°C. Ampicillin (100 μg/ml) and erythromycin (10 μg/ml) were used for plasmid and transposon mutant selection, respectively.

Transposon mutagenesis. Insertional mutations ΦNΞ9044 and ΦNΞ10853 from the Phoenix library were transduced into *S. aureus* USA300 LAC (30). Each mutant carries the *bursa aurealis* transposon, containing an erythromycin resistance cassette in the *ebh* gene (Fig. 1A); mutations were verified by DNA sequencing (30). Briefly, chromosomal DNA was extracted (Promega Wizard kit), digested with AciI, religated with T4 ligase to form individual episomes, and PCR amplified using Martn-F and Martn-R, primers specific to the *bursa aurealis* transposon (30). PCR products were sequenced to verify the site of transposon insertions in the *ebh* target of USA300 LAC.

Deletion mutagenesis. DNA sequences 1 kb upstream and downstream of *ebh* were PCR amplified using the primers attB1_ebh (GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTAGATCAAGGCTATTAACGC), ebh1_sacII (GGTTCCGCGGGGAGCACCGATTGACATCAC), ebh2_sacII (GGTTCCGCGGCTCCTTATCTTGTTGTTATGTC), and attbB2_ebh (GGGGACCACTTTGTACAAGAAAGCTGGGTGATCAGAATTAGGTGTAACCTC). The fragments were cloned into pKOR1 by use of a BP clonase II kit (Invitrogen) (31). The resulting plasmid, pΔ*ebh*, was electroporated into *S. aureus* Newman (26). Transformants were subjected to a temperature shift, which induced allelic exchange to generate the corresponding Δ*ebh* deletion (31). Mutants were verified by PCR amplification of the gene locus, DNA sequencing, and immunoblot analysis (Fig. 1B).

Localizing Ebh to the staphylococcal cross wall. Plasmid pEbh$_{SP}$-mCherry was created via splicing by overhang extension PCR (SOE PCR). A fusion protein of the signal peptide for Ebh (residues 1 to 126; aa 1 to 42) and mCherry was created by amplification of the mCherry coding sequence with primers mCh_Ebh_f (AACAAATCAACCAGCAAGGGCGAGGAGGATAACATG) and mCh_NheI_rc (GATCGCTAGCTTACTTGTACAGCTCGTCCATGCC). The coding sequence for the Ebh signal peptide was amplified with primers EbhSP_EcoRI_f (GATCGAATTCGTGAATTATCGTGATAAAATTCAAAAG) and EbhSP_mCh_rc (GGTTGATTTGTTTCAGCAGCATGCTCGCCCTTGCT). PCR products were used as templates in the following amplification step, using primers EbhSP_EcoRI_f and mCh_NheI_rc, and the product from this reaction was purified, digested with EcoRI/NheI, and ligated into predigested pMF312 (a derivative plasmid of pOS1 containing the iTet promoter) (32) to generate plasmid pEbh$_{SP}$-mCherry. Transformants were analyzed by DNA sequencing to confirm the correct sequence and subsequently transformed into *S. aureus* RN4220. Plasmid DNA was extracted from transformants and electroporated into *S. aureus* Newman or its Δ*ebh* variant. Expression was determined by fluorescence microscopy. Overnight cultures of *S. aureus* Newman and the Δ*ebh* mutant with empty vector or plasmid pEbh$_{SP}$-mCherry were diluted in fresh culture medium, grown to mid-log phase, and induced with anhydrotetracycline. Cells were fixed, stained with BODIPY-vancomycin to delineate cell walls, and visualized by confocal fluorescence microscopy.

Ebh$_N$ purification and rabbit antiserum. The coding sequence of Ebh$_N$ (codons 42 to 2524) was cloned as a PCR fragment with flanking XhoI and BglII sites into pET15b (33) cut with the same restriction enzymes to generate pT7-Ebh$_N$. PCR amplification used *S. aureus* USA300 LAC template DNA and primers Ebh-XhoI (gaaCTCGAGgctgaaacaaatcaaccagc) and Ebh-BglII (AGTAGATCTTTGTGGGAAATTAACCCAACG). Overnight cultures of *E. coli* BL21(DE3)(pT7-Ebh$_N$) were diluted 1:100 in fresh LB medium, grown at 37°C to an A_{600} of 0.5, and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. Cells were centrifuged at 6,000 × g, suspended in 1× column buffer (0.1 M Tris-HCl [pH 7.5], 0.5 M NaCl), and lysed in a French press at 14,000 lb/in². Lysates were subjected to ultracentrifugation at 40,000 × g for 30 min, and supernatants were subjected to affinity chromatography on Ni-nitrilotriacetic acid (Ni-NTA) columns. Each column was washed with column buffer containing 25 mM imidazole, followed by elution with 500 mM imidazole. The eluate was dialyzed against 1× phosphate-buffered saline (PBS). To remove endotoxin, 1:1,000 Triton X-114 was added, and the solution was chilled for 5 min, incubated at 37°C for 10 min, and centrifuged at 13,000 × g. Supernatant was loaded onto a HiTrap desalting column to remove Triton X-114. A rabbit (6-month-old New Zealand White female) was immunized with 100 μg Ebh$_N$ emulsified in complete

Freund's adjuvant (Difco) for initial immunization or emulsified in incomplete Freund's adjuvant for booster immunizations on days 24 and 48. On day 60, the rabbit was bled. Serum was recovered after centrifugation of coagulated blood at $2,000 \times g$ for 10 min.

Immunoblotting. Overnight cultures of staphylococci in TSB were diluted 1:100 in fresh medium and grown with shaking at 37°C to an A_{600} of 0.4. One milliliter of each culture was lysed by adding 5 μl of lysostaphin from a 2-mg/ml stock for 30 min, followed by the addition of 75 μl of 100% (wt/vol) trichloroacetic acid (TCA) solution. Samples were incubated on ice for 10 min and centrifuged for 10 min at $13,000 \times g$. Protein sediment was washed with 1 ml ice-cold 100% acetone, air dried, and solubilized in 50 μl sample buffer (4% SDS, 50 mM Tris-HCl [pH 8.0], 10% glycerol, and bromophenol blue). Protein samples were separated by 6% SDS-PAGE and subjected to immunoblotting with Ebh_N antiserum and secondary mouse anti-rabbit Alexa Fluor 680 conjugate. Immunoreactive signals were quantified with a Li-Cor Odyssey instrument.

Immunofluorescence microscopy. Overnight cultures of staphylococci in TSB were diluted 1:100 in fresh medium and grown to an A_{600} of 0.4. One milliliter of culture was centrifuged to sediment bacteria. Staphylococci were washed in $1\times$ PBS and fixed (2.5% paraformaldehyde, 0.006% glutaraldehyde in $1\times$ PBS, pH 7.4) for 20 min at room temperature. Bacteria were washed 3 times with PBS and suspended in 100 μl PBS, and a 30-μl droplet was added to a coverslip precoated with poly-L-lysine. For precoating, 60 μl of poly-L-lysine solution (Fisher) was placed on a glass coverslip for 5 min, followed by 3 washes with water. Cells adhering to the coverslip were washed 3 times with 60-μl droplets of PBS and then placed in blocking solution (3% bovine serum albumin [BSA], 1:200 human IgG [Sigma] in PBS) for 30 min. Blocking solution containing the specific rabbit antibody (1:1,000) was added to cells, and coverslips were incubated for 1 h. Coverslips were washed 10 times, and secondary antibody solution was added for 1 h (3% BSA, 1:200 Alexa Fluor 647-conjugated mouse anti-rabbit IgG). Slides were washed again in PBS, and 1:100 BODIPY-vancomycin and 1:1,000 Hoechst dye in PBS were added for 5 min. Coverslips were washed three times, mounted on glass slides with N-propylgallate, and sealed with nail polish. For visualization of mCherry, cells were processed with a similar protocol, but without antibody staining. Slides were stored at 4°C, and images were collected using a Leica SP5 AOBS spectral two-photon confocal microscope.

Transmission electron microscopy. Staphylococcal cells were cultured in TSB to mid-log phase in the presence or absence of 2 μg/ml oxacillin, centrifuged, fixed in 3% paraformaldehyde, embedded in epoxy, and thin sectioned. Sections were stained with uranyl acetate and viewed using a Tecnai F30 (Philips/FEI) transmission electron microscope (field emission gun, 300-kV accelerating voltage, and magnification of $\times 10,000$ to $\times 40,000$) and a high-performance charge-coupled device camera with 4,000-by-4,000 resolution. Images were acquired using Gatan DigitalMicrograph software, contrast adjusted, and processed using Adobe Photoshop.

Oxacillin sensitivity. Overnight cultures of $S.$ $aureus$ were normalized to an A_{600} of 4.0 and diluted 1:1,000 in TSB with or without oxacillin. The absorbance at 600 nm was measured for 18 h in a 96-well plate reader at 37°C with constant shaking.

Staphylococcal survival in blood or plasma. Overnight cultures of $S.$ $aureus$ strains were diluted 1:100 into fresh TSB and grown at 37°C until they reached an A_{600} of 0.4. One milliliter of culture was washed and suspended in 1 ml PBS to obtain 1×10^8 CFU/ml stock. Whole blood from naive 6-week-old BALB/c mice was collected, and lepirudin (Refludan) was added to a final concentration of 50 μg/ml. To obtain fresh plasma, whole blood was centrifuged at $2,000 \times g$ for 3 min to sediment blood cells, and the supernatant was transferred to a fresh tube. Plasma samples were either left untreated or heat inactivated at 60°C for 30 min with occasional mixing. Blood or plasma was aliquoted (450 μl) and mixed with 50 μl bacterial stock to a final concentration of 5×10^6 CFU/ml. Samples were incubated at 37°C with slow rotation. Aliquots (50 μl) were removed at timed intervals, mixed 1:1 with 2% saponin in PBS, and

incubated on ice for 30 min. Five serial 10-fold dilutions were prepared, and 10-μl aliquots were spread on TSA for colony formation and enumeration.

Complement deposition assays. $S.$ $aureus$ (1×10^7 CFU/ml stock) was incubated in PBS with 10% human plasma in a final volume of 1 ml. Reaction mixtures were incubated at 37°C with rotation, and aliquots of 100 μl were removed and quenched 1:10 in ice-cold PBS at 5-min intervals. Samples were washed three times with cold PBS, labeled with fluorescein isothiocyanate (FITC)-conjugated anti-C3 F(ab)$_2$, washed three more times, fixed in 4% formalin, and analyzed by flow cytometry using an LSRII instrument. Aliquots that had been removed at 0, 5, and 15 min were also processed for immunoblotting and immunofluorescence microscopy. For immunoblotting, samples were quenched and then centrifuged at $13,000 \times g$, and the sediment was processed as described above. Briefly, the sediment was suspended in 50 μl sample buffer, boiled for 3 min, and centrifuged at $13,000 \times g$ to remove insoluble material. Solubilized proteins were subjected to immunoblotting with goat anti-human C3 primary antibody and Alexa Fluor 680-conjugated donkey anti-goat antibody. SDS-PAGE gels were imaged using a Li-Cor Odyssey machine. For immunofluorescence microscopy, aliquots were removed at 0 and 5 min, quenched and washed as described above, fixed, and then stained with FITC-conjugated anti-C3 F(ab)$_2$, using the aforementioned protocol for confocal fluorescence microscopy.

Mouse challenge experiments. Overnight cultures of $S.$ $aureus$ were diluted 1:100 in fresh TSB and grown to an A_{600} of 0.4. Bacteria were sedimented by centrifugation at $7,500 \times g$, washed, and suspended in $1\times$ PBS. Six-week-old female BALB/c mice ($n = 10$) were injected in the periorbital venous sinus with 5×10^6 CFU $S.$ $aureus$ USA300 LAC in 100 μl PBS. On day 5 postinfection, mice were euthanized (CO_2 asphyxiation and cervical dislocation), and kidneys were removed during necropsy. All organs were examined for surface lesions, and 8 to 10 right kidneys were analyzed for histopathology by staining thin-sectioned paraffin-embedded tissues with hematoxylin-eosin. These slides were examined by light microscopy for abscess formation. For the lethal challenge model, all experimental conditions remained identical, except that 5×10^7 CFU $S.$ $aureus$ USA300 LAC were injected intravenously and mice were monitored for survival over 14 days postinfection.

RESULTS

***S. aureus ebh* mutants.** Compared with MRSA isolate USA300 LAC and its related clones (25, 34), $S.$ $aureus$ Newman harbors a nucleotide substitution in ebh that truncates the Ebh polypeptide at amino acid 7032 (26). Using pKOR1 allelic replacement technology (31), we deleted the entire ebh open reading frame of $S.$ $aureus$ Newman, thereby generating the Δebh variant. We also transduced two $bursa$ $aurealis$ alleles originally isolated from $S.$ $aureus$ strain Newman (30) into USA300 LAC, thereby terminating the open reading frame of ebh after codon 509 (ΦNΞ9044) or after codon 5579 (ΦNΞ10853) (Fig. 1A). Mutational lesions were verified by DNA sequencing. To analyze the wild-type and mutant strains for the synthesis of ebh gene products, we purified recombinant His-tagged Ebh_N (Ebh residues 42 to 2524) from $E.$ $coli$ lysates by affinity chromatography and raised rabbit immune serum (anti-Ebh_N). Mid-log-phase cultures of $S.$ $aureus$ Newman or USA300 LAC and ebh variants were centrifuged, the peptidoglycan envelope was digested with lysostaphin, and proteins were precipitated with TCA. Proteins were subjected to 6% SDS-PAGE and analyzed by immunoblotting with anti-Ebh_N. Lysates derived from $S.$ $aureus$ Newman and USA300 harbored anti-Ebh_N-immunoreactive species ranging in size from 250 kDa to >500 kDa (Fig. 1B). Neither the Δebh variant of $S.$ $aureus$ Newman nor ΦNΞ9044 generated Ebh_N-immunoreactive species, indicating that ebh expression had been abolished (Fig. 1B). Because of the very large
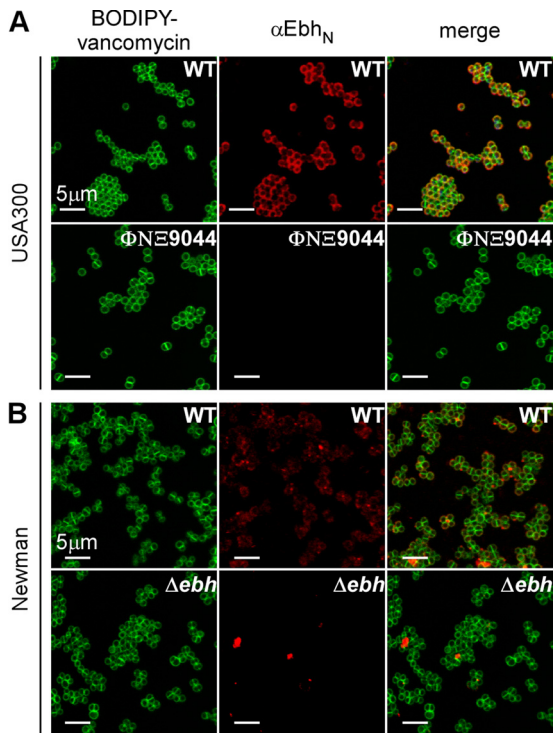
FIG 2 Ebh is displayed on the staphylococcal surface. Mid-log-phase staphylococcal cells were stained with BODIPY-vancomycin (green) to delineate peptidoglycan and with anti-Ebh$_N$ and Alexa Fluor 647 to localize Ebh (red). Immunofluorescence microscopy images were acquired for wild-type strain USA300 LAC and its ΦNΞ9044 *ebh* variant (A) as well as for wild-type strain *S. aureus* Newman and its Δ*ebh* mutant (B). Ebh-specific staining was observed for USA300 LAC and *S. aureus* Newman but not for the ΦNΞ9044 and Δ*ebh* variants.

FIG 3 Ebh$_{SP}$-mCherry is secreted into the cross wall. *S. aureus* Newman and Δ*ebh* cells with or without pEbh$_{SP}$-mCherry were grown to mid-log phase and induced with anhydrotetracycline. Cells were fixed and stained with BODIPY-vancomycin to delineate the cell wall (green). Samples were analyzed via confocal fluorescence microscopy to reveal mCherry staining (red).

size of the *ebh* gene, we were unable to complement the mutant phenotypes with a plasmid-borne copy of the wild-type *ebh* gene.

Ebh is displayed on the surface of *S. aureus*. Immunofluorescence microscopy was used to localize Ebh in bacteria. Staphylococci were grown to mid-log phase, fixed with paraformaldehyde, and stained with either BODIPY-vancomycin (green fluorescence) or anti-Ebh$_N$ and Alexa Fluor 647 conjugate (red fluorescence). BODIPY-vancomycin stains the cell wall envelope of *S. aureus* and is abundantly deposited at the cross wall (11). Anti-Ebh$_N$ antibody staining was found on the surface of *S. aureus* USA300 LAC but not on *S. aureus* ΦNΞ9044 (Fig. 2A). Staphylococci displayed a spherical distribution of anti-Ebh$_N$ staining, similar to that of sortase-anchored surface proteins secreted via YSIRK-G/S-type signal peptides (11). Anti-Ebh$_N$ staining also generated immunofluorescence signals on the surface of *S. aureus* Newman, albeit that the fluorescence signals were less intense and less homogeneous than those for USA300 LAC (Fig. 2B). This phenotype may be due to the truncation of Ebh in *S. aureus* Newman, which eliminates the C-terminal transmembrane domain of the polypeptide (Fig. 1A). It is, however, not clear why truncated Ebh remains associated with the surface of *S. aureus* Newman. We observed occasional aggregates of anti-Ebh$_N$ staining at cell contact sites of *S. aureus* Newman and its Δ*ebh* variant (Fig. 2B); these signals were interpreted as nonimmune binding of antibodies to staphylococcal protein A. Anti-Ebh$_N$ staining on *S. aureus* USA300 a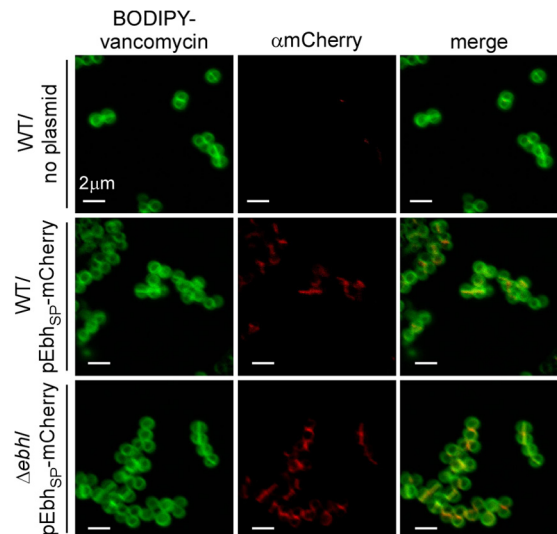nd Newman did not occur at the cross wall, pre-sumably because antibodies cannot access this compartment (Fig. 2A and B).

Ebh$_{SP}$-mCherry is secreted into the cross wall of *S. aureus*. We wondered whether the YSIRK-G/S signal peptide of Ebh is sufficient to direct the secretion of a hybrid mCherry protein into the cross wall, as has been observed for LytN-mCherry and Geh-mCherry (10, 14). A hybrid gene comprising nucleic acid sequences for the tetracycline-inducible promoter and the *ebh* sequence (codons 1 to 62) were fused to the mCherry open reading frame, thereby generating pEbh$_{SP}$-mCherry. This plasmid was transformed into *S. aureus* Newman or its Δ*ebh* variant. Staphylococci were grown to mid-log phase, induced for Ebh$_{SP}$-mCherry expression with anhydrotetracycline (35), fixed, and stained with BODIPY-vancomycin prior to fluorescence microscopy (Fig. 3). As expected, BODIPY-vancomycin (green) stained staphylococcal peptidoglycan, in particular the cross wall (Fig. 3). *S. aureus* Newman (without pEbh$_{SP}$-mCherry) did not generate red fluorescence signals (Fig. 3). Staphylococci that had been transformed with pEbh$_{SP}$-mCherry displayed mCherry signals at the cross wall for both wild-type and Δ*ebh* variant strains (Fig. 3). Cross wall staining of pEbh$_{SP}$-mCherry cells is dependent on the Ebh signal peptide, as staphylococci expressing mCherry alone generated diffuse cytoplasmic staining (10, 14). These data suggest that the YSIRK-G/S signal peptide of Ebh is sufficient to direct reporter proteins to the cross wall, as has been observed for other YSIRK-G/S signal peptides (12, 14). Furthermore, the *ebh* gene is not essential for the secretion of YSIRK-G/S precursors into the cross wall, as Ebh$_{SP}$-mCherry trafficking was observed in both wild-type and Δ*ebh* mutant strains.

***ebh* is a cell size determinant of *S. aureus*.** Staphylococci from mid-log-phase cultures were sedimented by centrifugation, suspended in PBS, and fixed in glutaraldehyde. Samples were embedded in epoxy, thin sectioned, and stained with uranyl acetate prior to viewing by transmission electron microscopy (Fig. 4A to E). To quantify the sizes of individual staphylococci, we measured the
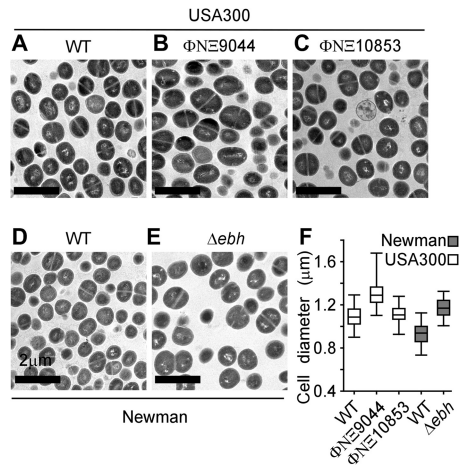
FIG 4 The giant protein Ebh is a cell size determinant of *S. aureus*. (A to E) *S. aureus* USA300 LAC (A) and its *ebh* variants ΦNΞ9044 (B) and ΦNΞ10853 (C), as well as *S. aureus* Newman (D) and its Δ*ebh* mutant (E), were fixed, thin sectioned, and viewed by transmission electron microscopy. (F) The diameters of dividing cells were measured, and the results were plotted in a dot-and-whisker plot. The *ebh* mutant cells were larger than their wild-type parents: for Newman versus its Δ*ebh* variant, $P < 1 \times 10^{-4}$; and for USA300 LAC versus ΦNΞ9044, $P < 1 \times 10^{-4}$. Measurements represent the average diameters for 80 to 100 dividing cells. Statistical significance and *P* values were calculated with the Mann-Whitney U test.

FIG 5 Ebh contributes to *S. aureus* methicillin resistance. Growth of *S. aureus* USA300 LAC and its *ebh* variants ΦNΞ9044 and ΦNΞ10853 was determined for samples diluted from overnight cultures in TSB without (A) or with (B) 2 μg/ml oxacillin. Growth was measured as increased absorbance at 600 nm (A_{600}). *S. aureus* USA300 LAC (C) and its *ebh* variant ΦNΞ9044 (D) were grown to mid-log phase in 2 μg/ml oxacillin, fixed, thin sectioned, and viewed by transmission electron microscopy.

diameters of thin-sectioned cells that had formed a cross wall and displayed two daughter cells with equal volumes, thereby ensuring that spherical cells selected for measurements had been cut across the middle. By calculating the average diameter (± standard error of the mean) for 80 to 100 cells per sample, we noted that *S. aureus* USA300 (diameter of 1.1 ± 0.2 μm) and *S. aureus* Newman (0.9 ± 0.3 μm) differed in size (Fig. 4F). Furthermore, cells of the *ebh* mutant strain ΦNΞ9044 (1.3 ± 0.4 μm) were, on average, 0.2 μm (20%) larger than those of wild-type parent strain USA300 LAC (Fig. 4D). Similar observations were made in determining the average cell diameter for the Δ*ebh* variant of *S. aureus* Newman (1.1 ± 0.3 μm) (Fig. 4F). Note that the ΦNΞ10853 insertion, which truncated *ebh* after codon 5579, did not affect the cell size of USA300 LAC (1.1 ± 0.2 μm) (Fig. 4F). Thus, mutations that abolish *ebh* expression (Δ*ebh* or ΦNΞ9044), but not mutations that truncate *ebh* in the FIVAR-GA module repeats, increase the cell size of staphylococci.

ebh is required for methicillin (oxacillin) resistance. *S. aureus* USA300 LAC, but not *S. aureus* Newman, is resistant to methicillin, a penicillinase-resistant β-lactam compound that is no longer commercially available (34). Over the past decade, oxacillin has been used as a surrogate for methicillin; this β-lactam is also resistant to cleavage by penicillinase. Expression of PBP2a (*mecA*) in *S. aureus* USA300 confers resistance to both methicillin and oxacillin (36). Following the addition of 2 μg ml^{-1} oxacillin to culture media, the growth of wild-type USA300 LAC was initially delayed but quickly resumed, as expression of PBP2a supports resistance to this antibiotic (37) (Fig. 5A and B). In contrast, oxacillin inhibited the growth of the ΦNΞ9044 *ebh* mutant, whereas ΦNΞ10853, encoding a longer Ebh variant, exhibited an intermediate phenotype of oxacillin sensitivity (Fig. 5A and B).

Electron microscopy of USA300 LAC and its *ebh* mutant ΦNΞ9044 grown in the presence of 2 μg ml^{-1} oxacillin revealed
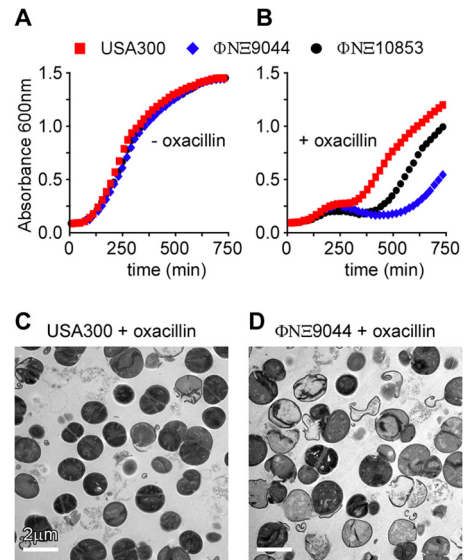
differences in cell wall integrity and cell structure. Wild-type USA300 LAC cells displayed the physiological cell and cell wall envelope architecture (Fig. 5C). In contrast, many cells of the ΦNΞ9044 variant had lysed: empty murein sacculi displayed defects, which occurred as small holes in the peripheral cell wall and as structural deformations of the cross wall (Fig. 5D). These data suggest that Ebh is involved in the physiological assembly, integrity, and separation of the cell wall when staphylococci synthesize peptidoglycan with PBP2a (MecA) in the presence of oxacillin.

ebh is a determinant of S. aureus complement resistance. We wondered whether the structural changes to the envelope of *ebh* mutant staphylococci affect their ability to survive and replicate in host tissues. To begin to address this question, we first inoculated wild-type and *ebh* mutant staphylococci into fresh human blood (38). Studies with blood samples from many different volunteers had shown that the capacity of human blood to kill *S. aureus* USA300 LAC varies by more than 2 orders of magnitude (log CFU) (39). Such phenotypic variation may depend on hereditary factors; however, it is more likely to be influenced by adaptive immune responses that are based on individual encounters with the pathogen. To study the effects of the *ebh* gene, we selected blood samples from human volunteers that showed a moderate level (23.7%) of *S. aureus* USA300 LAC killing over a 30-min incubation period (Fig. 6A). Compared to USA300 LAC, the *ebh* variant ΦNΞ9044, but not ΦNΞ10853, was more susceptible to killing in blood, with 50.3% killing of ΦNΞ9044 (Fig. 6A). A similar phenotype was observed in comparing the survival of *S. aureus* Newman and its Δ*ebh* variant, with 16.8% and 32% killing of staphylococci in human blood, respectively (Fig. 6A). To determine whether *ebh*-mediated survival of staphylococci is specific for human blood, the experiment was performed with blood from naive mice that had been raised under pathogen-free conditions
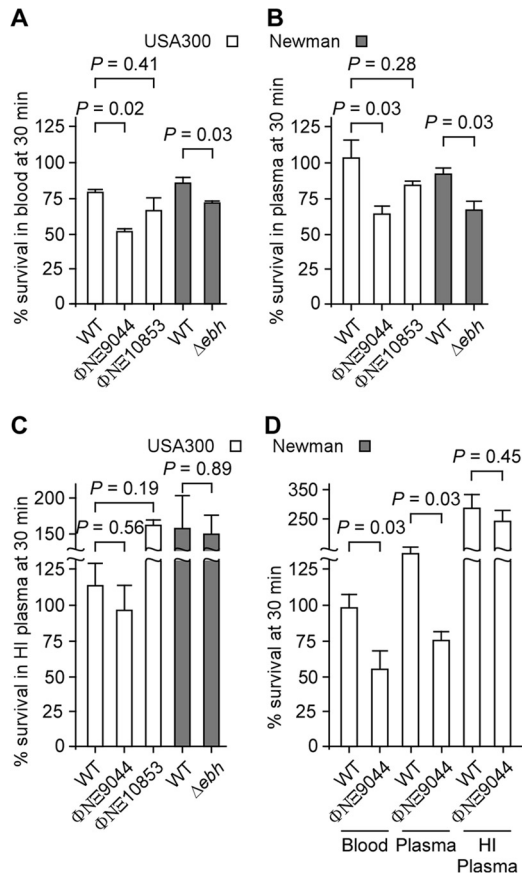
FIG 6 Ebh contributes to *S. aureus* resistance against complement. (A) *S. aureus* USA300 LAC and its *ebh* variants ΦNΞ9044 and ΦNΞ10853, as well as *S. aureus* Newman and its Δ*ebh* mutant, were incubated in human blood for 30 min. Bacterial survival was assessed by enumerating CFU on agar plates before and after incubation in blood and calculating the percent survival. (B) Staphylococci were incubated in human plasma for 30 min, and the percent survival was calculated. (C) Staphylococci were incubated in heat-inactivated (HI) human plasma for 30 min, and the percent survival was calculated. (D) Staphylococci were incubated in mouse blood, mouse plasma, or heat-inactivated mouse plasma for 30 min, and the percent survival was calculated. Data represent the averages for three or four independent trials. Statistical significance and *P* values were calculated with the Mann-Whitney U test.

and lacked antibodies against staphylococci (38). Mouse blood did not affect the survival of USA300 LAC (1.3% killing) or the ΦNΞ10853 variant, whereas 41.8% of ΦNΞ9044 cells were killed during incubation with mouse blood (Fig. 6D). Bacterial killing in mouse blood may be a feature of phagocytic cells that recognize opsonized staphylococci and, following phagocytosis, derive oxygen radicals and lysosomal vesicles to eliminate the pathogen. At least for Gram-negative bacteria, complement deposition in the bacterial envelope triggers the formation of membrane attack complexes (MACs) that kill microbes without assistance from phagocytic cells (40). To distinguish between such possibilities, human blood was centrifuged, causing red blood cells and immune cells to sediment. Plasma samples were removed with the supernatant and used as a source of complement for incubation with staphylococci (Fig. 6B). Incubation in human plasma did not affect the survival of *S. aureus* USA300 LAC, in agreement with the general model that staphylococci cannot be killed by complement. In contrast, 38.9% of the *ebh* mutant ΦNΞ9044 cells were killed in

human plasma (62.7% survival; *P* = 0.0317), whereas 19% of ΦNΞ10853 cells (*P* = 0.285) were killed. To verify whether the observed killing of ΦNΞ9044 in plasma was indeed caused by complement, human plasma was heat treated at 56°C to inactivate C3 convertases; heat treatment indeed abrogated killing of ΦNΞ9044 (Fig. 6C). These data suggest that the deposition of complement in the envelope of the *ebh* mutant may trigger staphylococcal killing in blood.

Complement deposition on the staphylococcal surface. Complement-mediated killing can be initiated via the deposition of antibody or lectin and complement convertase C3b or C5 binding to bacterial surfaces; this triggers a series of proteolytic cascades that result in the formation of a MAC (40). As alluded to above, complement proteins are effective at lysing Gram-negative bacteria, whereas the thick peptidoglycan envelope of Gram-positive pathogens prevents access of MACs to bacterial membranes (41). *S. aureus* isolates are particularly resistant to complement-mediated killing, as these microbes secrete SCIN, Sak, and CHIPS—proteins that neutralize or destroy complement (42–44). Furthermore, *S. aureus* uses cell wall-associated proteins (Eap, Efb, and Sbi) to sequester complement (45–47). To test whether Ebh protects staphylococci from complement deposition, we measured the abundance of C3b in the envelope of wild-type or *ebh* mutant cells by using both flow cytometry and immunofluorescence microscopy. Mid-log-phase wild-type and *ebh* mutant staphylococci were incubated with human plasma, and aliquots were analyzed at 5-min intervals. Samples were stained with anti-human C3b FITC-conjugated antibody and quantified by flow cytometry. We observed increased C3b deposition in the envelope of the *ebh* mutant ΦNΞ9044 compared to wild-type USA300 LAC (Fig. 7A). Wild-type and *ebh* mutant staphylococci were incubated with plasma for various times, washed with PBS, boiled in sample buffer, and subjected to immunoblotting with anti-C3. We observed increased deposition of C3 on *ebh* mutant surfaces (increase in anti-C3 from 0 to 15 min, 0.97% for USA300 LAC versus 69.19% for ΦNΞ9044; *P* = 0.0228), indicating that complement factors adhere to the surfaces of *ebh* mutant cells but not to wild-type staphylococci. We also visualized complement deposition via confocal microscopy (Fig. 7B). Staphylococcal cells were incubated in plasma, and aliquots were removed at timed intervals, stained with C3b FITC-conjugated antibody, and viewed under a confocal microscope. Cells of the *ebh* mutant ΦNΞ9044 were labeled with C3 within 10 s, whereas wild-type staphylococci exhibited very little C3 labeling even after 5 min of incubation. These data suggest that *ebh* expression is required for the assembly of a staphylococcal envelope that is refractory to C3 complement deposition and complement-mediated killing.

Ebh and staphylococcal virulence. To investigate whether *ebh* contributes to staphylococcal virulence, we infected cohorts of BALB/c mice via intravenous injection with either wild-type USA300 LAC or the *ebh* variant ΦNΞ9044 (Ebh$_{1–509}$) or ΦNΞ10853 (Ebh$_{1–5579}$) (Fig. 8A). At a challenge dose of 5 × 10^7 CFU, all BALB/c mice that had been infected with USA300 LAC or ΦNΞ10853 died within 24 to 48 h. In contrast, animals infected with the same dose of the ΦNΞ9044 *ebh* variant displayed a delayed time to death of up to 108 h (for USA300 LAC versus ΦNΞ9044, *P* < 0.005). Following intravenous challenge with 5 × 10^6 CFU, BALB/c mice infected for 5 days with USA300 LAC generated abscesses in kidneys and other internal organs (48). When renal tissues were analyzed for bacterial load, we observed an average of 6.2 log$_{10}$ CFU USA300 LAC g^{-1} tissue
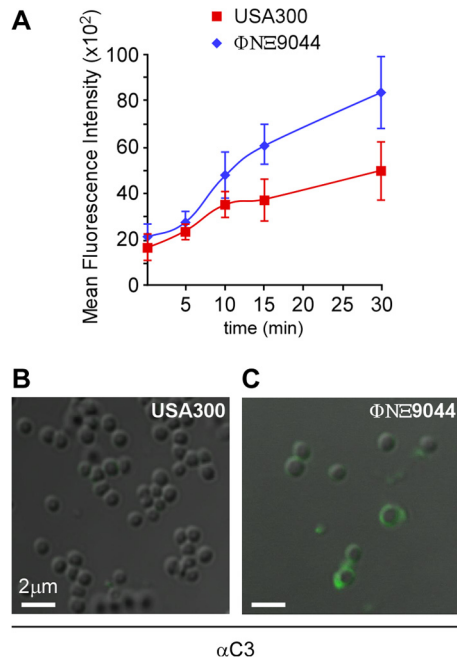
FIG 7 Ebh prevents C3 complement deposition in the envelope of *S. aureus*. (A) MRSA strain USA300 LAC and its *ebh* variant ΦNΞ9044 were incubated with human plasma. At timed intervals (0, 5, 10, 15, 20, 25, and 30 min), aliquots were removed, bacteria stained with FITC-conjugated anti-human C3, and fluorescence quantified via flow cytometry. Samples generated at the 5-min time point for panel A were viewed by confocal microscopy to reveal C3 deposition in the envelopes of *S. aureus* USA300 LAC (B) and ΦNΞ9044 (C).

FIG 8 Ebh contributes to virulence of *S. aureus* in a mouse model. (A) BALB/c mice (*n* = 10) were injected into the periorbital venous plexus with 5×10^7 CFU of *S. aureus* USA300 LAC or its *ebh* variant ΦNΞ9044 or ΦNΞ10853. Animal survival was recorded over 14 days. When analyzed with the log rank test, the *ebh* variant ΦNΞ9044 displayed a significant delay in time to death compared to the wild type (for USA300 LAC versus ΦNΞ9044, $P < 0.05$). (B) BALB/c mice (*n* = 10) were injected into the periorbital venous plexus with 5×10^6 CFU of *S. aureus* Newman or its Δ*ebh* variant and with USA300 LAC or its *ebh* variant ΦNΞ9044. At 5 days postinfection, animals were euthanized and necropsied and their kidneys removed. Renal tissues were homogenized, and staphylococcal loads were determined by plating aliquots on TSA and enumerating CFU. The mean bacterial load was calculated and analyzed for statistical significance by use of the Mann-Whitney U test to compare the wild-type and *ebh* variant strains: for USA300 LAC versus ΦNΞ9044, $P = 0.0103$; and for Newman versus the Δ*ebh* variant, $P = 0.0409$.

(Fig. 8B). In contrast, the ΦNΞ9044 *ebh* variant displayed a 1.4-$\log_{10}$ reduction in CFU g^{-1} tissue (Fig. 8B). A similar defect, namely, a 1.2-$\log_{10}$ reduction in CFU g^{-1} tissue, was observed in comparing wild-type *S. aureus* Newman and its Δ*ebh* variant (Fig. 8B). Thus, mutations that abolish the expression of *ebh* in *S. aureus* Newman or USA300 LAC diminish staphylococcal virulence, likely because *ebh* mutant bacteria are subject to complement-mediated killing in infected host tissues.

DISCUSSION

Enzymes responsible for lipid II synthesis (MurG) and polymerization into peptidoglycan (PBP1, PBP2, PBP2a, and PBP4) are localized to the cross wall (49–53). In the absence of cell division or under conditions of diminished abundance of cell division factors, PBPs cannot localize because the cross wall is not formed (54). Furthermore, depletion of lipid II from staphylococcal cells, as occurs when *S. aureus* is treated with murgocil or vancomycin, diminishes PBP localization to the cross wall (49, 52). PBP2 is the key enzyme of *S. aureus* responsible for the polymerization of glycan strands from lipid II (55). PBP2 cooperates with PBP4 to generate cross-linked peptidoglycan (51, 56). Indeed, most of the Gly$_5$ cross bridges in the cell wall of *S. aureus* are linked to D-Ala of adjacent wall peptides (57, 58). For MRSA strains growing in the presence of β-lactams, PBP2a functions as the sole transpeptidase, replacing PBP1, PBP2, and PBP4, but not PBP2 transglycosylase (51). Sortase A, another enzyme requiring lipid II substrate (59), is also localized to the cross wall during cell division (60).

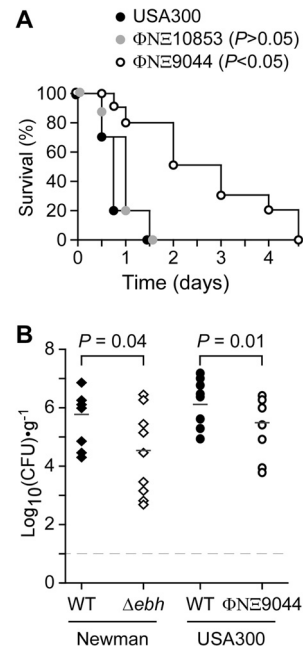Recent work suggests a connection between the assembly of wall teichoic acid (WTA), which does not occur within the cross wall (13), and peptidoglycan synthesis at the cross wall (53). WTA synthesis is initiated by TagO and TagA, which link GlcNAc and ManNAc to bactoprenol (C_{55}-PO_4) (61–63). The product, C_{55}-$(PO_4)_2$-GlcNAc-ManNAc, is extended with glycerol-3-phosphate (GroP) and poly-ribitol-5-phosphate (RboP) (64). Bactoprenol-linked WTA precursors are tethered to the C_6-OH of glycan chains by three enzymes—LcpA, LcpB, and LcpC—that fulfill seemingly redundant functions (65, 66). *S. aureus* mutants lacking *tagO* display aberrant cross wall formation with parallel and incompletely formed structures (62, 67, 68). A similar phenotype is observed with *lcpABC* mutants (65). Finally, *tagO* mutants as well as wild-type staphylococci treated with tunicamycin, an inhibitor of TagO (69, 70), are susceptible to methicillin even when PBP2a is expressed, which implies that PBP2a may not be localized properly to the cross wall when teichoic acid synthesis is blocked (62, 71).

Biogenesis of the cross wall follows a series of temporal and spatially controlled cytokinesis events that are initiated with the polymerization of FtsZ, a tubulin-like GTPase (72). Once tethered to the membrane and associated with other cell division factors, FtsZ constricts as a ring structure, the divisome (72). The purpose of the divisome is the generation of two daughter cells, which is accomplished by separating the membranes of bacteria at a mid-cell position (73). EzrA, a negative regulator of the divisome in

firmicutes (74), contributes to the proper positioning of FtsZ rings and PBPs at the staphylococcal cross wall (75). These observations can be integrated into a simple model. Divisome placement at midcell localizes MurG and lipid II synthesis, triggering accumulation of lipid II at the cell division site and directing PBPs and sortase to the cross wall. Perturbation of WTA synthesis (*tagO* or *lcpABC*) may increase the availability of bactoprenol and disturb the localized accumulation of lipid II at the cross wall, thereby perturbing the localization of PBPs and sortase A. Once the cross wall has formed, i.e., peptidoglycan is synthesized in the extracellular space derived from FtsZ-catalyzed membrane separation, other mechanisms provide for the trafficking of precursors with YSIRK-G/S signal peptides into the cross wall. These proteins are deposited in the cross wall and distributed over the staphylococcal surface (proteins with LPXTG sorting signals) (12). Other YSIRK-G/S precursors, for example, LytN and Ebh, traffic into the cross wall to shape the structure of its peptidoglycan and/or split the cross wall (10).

Experiments described herein reveal that Ebh is a surface protein of *S. aureus* Newman and USA300. The distribution pattern of Ebh is reminiscent of that observed for sortase-anchored surface proteins with YSIRK-G/S signal peptides, for example, protein A (SpA) (11). Unlike sortase A-anchored products, which are linked to peptidoglycan, the giant protein Ebh is thought to span the peptidoglycan layer and the plasma membrane (Fig. 1A). Ebh, similar to sortase A-anchored proteins, is presumably distributed over the staphylococcal surface once the cross wall is split (12). In the absence of *ebh*, mutant staphylococci display a surprising phenotype: the bacterial cell volume is nearly doubled, and cross walls assume irregular shapes and thicknesses. We suspect that the increased sensitivity of *ebh* variants to complement-mediated killing in plasma may be a feature of their cell wall separation defects. Weakened peptidoglycan within cross walls and increased cell size appear to increase the association with complement and may enable the deposition of MACs with membrane-disrupting attributes (76, 77). The aberrantly shaped *ebh* cells are also susceptible to treatment with oxacillin, a β-lactam that impairs the transpeptidation reaction of cell wall synthesis (78). These findings further corroborate our hypothesis that Ebh may enable physiological peptidoglycan synthesis and drug resistance at the cross wall. What might be the molecular mechanisms that lead to the unique phenotypes of *ebh* variants? We presume that Ebh may act as a cytoskeletal element for proper positioning of the cross wall and subsequent cross wall splitting and cell separation. Note that its predicted length (350 nm) would allow Ebh to span the calculated width of the cross wall. If it does so, perhaps staphylococci exploit the shape and repeat structures of Ebh for the proper assembly of peptidoglycan and/or other carbohydrate structures within the cross wall compartment.

Earlier work identified *mecA* (PBP2′ or PBP2a), encoding a transpeptidase that catalyzes the transpeptidation reaction of cell wall synthesis when MRSA cells are grown in the presence of methicillin or oxacillin (79, 80). Methicillin resistance in clinical isolates of *S. aureus* is typically heterogeneous in phenotypic expression, where most cells of a population show low resistance levels and a minor population displays high resistance (81). Reduced methicillin resistance (10- to 50-fold reduction in MIC) in MRSA strains is observed following inactivation of any one of 20 different *fem* genes (factors essential for methicillin resistance) (78). Several of these factors are involved in the synthesis of lipid

II, i.e., C_{55}-$(PO_4)_2$-MurNAc(L-Ala-D-iGln-L-Lys(NH_2-Gly$_5$)-D-Ala-D-Ala)-GlcNAc, for example, by synthesizing the pentaglycine (Gly$_5$) cross bridge (FemAB and FmhB) (82), the di-amino acid L-Lys (FemF/MurE) (83), D-Gln (FemC) (84), or the GlcNAc moiety of lipid II (FemD [GlmM]) (85). Nevertheless, mutations in genes for cell wall- or membrane-associated proteins (FmtA, FmtB, and FmtC [MprF]) can also diminish methicillin resistance, although the underlying mechanisms are not yet understood (86–88). Deletion of the *ebh* gene does not affect the susceptibility of *S. aureus* Newman toward β-lactam antibiotics (MIC of oxacillin is 0.2 μg ml^{-1} for both wild-type and Δ*ebh* strains). However, β-lactam resistance of the MRSA strain USA300 LAC (MIC, 20 μg ml^{-1}) is reduced by 2 orders of magnitude in the USA300 *ebh* mutant (ΦNΞ9044) (MIC, 0.2 μg ml^{-1}).

We observed that the genomes of staphylococcal strains whose DNA sequences were determined in close temporal proximity to their clinical isolation may harbor intact (full-length) *ebh* genes (25). In contrast, strains that had been propagated for years in the laboratory prior to genome sequencing harbored truncated *ebh* variants with 3′ nonsense mutations. Our experiments involved mutants with *bursa aurealis* insertions in the *ebh* gene. Depending on the locations of the mutations, these variants display a gradient of defects, where the most severe phenotypes are associated with 5′ disruptions and nonsense mutations at the 3′ end cause minor phenotypes. From this information, we conclude that expression of full-length *ebh* is not required for staphylococcal growth in the laboratory. Nevertheless, secretion of full-length Ebh into the cross wall facilitates staphylococcal replication in host tissues by providing for envelope stability and protection against host defenses.

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