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Staphylococcus aureus SaeR/S-regulated factors overcome human complement–mediated inhibition of aggregation to evade neutrophil killing

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Significance

Staphylococcus aureus is a major causative agent in implant-associated and other biofilm infections. The ability to form aggregates, a "strength in numbers" approach, helps to protect small groups of bacteria from killing by immune cells. We demonstrate that an arm of the innate immune system, serum complement, counteracts this tactic by inhibiting bacterial aggregation, thereby increasing susceptibility to phagocyte killing. Unlike the related opportunistic pathogens *Staphylococcus epidermidis* and *Enterococcus faecalis*, *S. aureus* produces several proteins that combine to inhibit complement. Thus, by blocking complement, these *S. aureus* proteins facilitate bacterial aggregation and subsequent phagocyte evasion. Our study identifies a host defense process and describes protein factors *S. aureus* uses to overcome it.

Staphylococcus aureus **(*S. aureus*) is a frequent culprit in implant-associated infections and employs many virulence factors to escape killing by the host immune system. The specific immune evasion strategies used by small aggregates of *S. aureus* on a surface, precursors to mature biofilm, are still relatively unknown. Time-lapse confocal microscopy was leveraged to quantify interactions between *S. aureus* aggregates and human neutrophils in vitro and identify specific mechanisms of resistance to neutrophil killing. Surface-associated wild-type *S. aureus* rapidly formed small biofilm aggregates when grown in human serum. Conversely, aggregation was inhibited when the SaeR/S two-component gene regulatory system was deleted. Wild-type aggregates began to show individual and population-level resistance to neutrophil killing upon reaching sizes of approximately 50 to 75 μm^2, whereas Δ*sae* clusters failed to reach these sizes and were readily cleared. Aggregation of Δ*sae* strains was impaired by serum complement, and this inhibition required complement proteins C3 and factor B, but not C4 or C5, suggesting that this activity primarily occurs at the level of the alternative pathway. Several complement-inhibiting genes regulated by SaeR/S were identified that collectively facilitate biofilm aggregate formation in human, but not murine serum. Finally, aggregation of two related opportunistic pathogens, *Staphylococcus epidermidis* and *Enterococcus faecalis*, was inhibited by serum. These data demonstrate a function of serum complement, the ability to inhibit bacterial aggregation, that is potently blocked by *S. aureus* through the production of multiple complement-interfering proteins that are regulated by the SaeR/S system.**

biofilm | *Staphylococcus aureus* | complement | neutrophil | microscopy

Chronic biofilm infections are difficult to treat due to their inherent tolerance to both antibiotic therapies and host immune defenses (1, 2). *Staphylococcus aureus* (*S. aureus*) is commonly implicated in biofilm infections associated with orthopedic implants and other indwelling medical devices (3, 4). In addition to readily forming biofilms, *S. aureus* produces many virulence factors that interfere with host immunity (5). It remains poorly understood how small amounts of contaminating bacteria successfully evade host innate immune defenses and subsequently develop into robust biofilm infections. We have previously demonstrated that delayed discovery of contaminating cells on a surface by neutrophils, key early responders to *S. aureus* infections, leads to formation of mechanically resilient and difficult to kill biofilm aggregates (6, 7). *S. aureus* aggregates on a surface that reach an approximate size of ~50 to 75 μm^2 resist killing by human neutrophils and cause neutrophil membrane damage (6). The period in which bacteria are susceptible to clearance is therefore dependent on the initial size of contaminating aggregates, the growth rate of the bacteria in vivo, and the speed of recruitment and successful discovery of bacteria by host immune defenses. An improved understanding of the mechanisms by which *S. aureus* aggregates tolerate neutrophil killing will provide crucial insight into the events that determine whether contaminating bacteria are cleared or persist. In the current study, we sought to identify factors that contribute to *S. aureus* biofilm aggregate tolerance to neutrophil clearance, specifically by investigating genes regulated by the SaeR/S two-component system and the Agr quorum-sensing system.

The SaeR/S system is a major regulator of virulence in *S. aureus* and has shown to be essential for pathogenesis in murine models of skin infection (8, 9), sepsis (10), and bacterial pneumonia (8, 11). The *sae* locus consists of four genes that encode the histidine kinase *saeS*, the response regulator *saeR*, and the two auxiliary membrane proteins *saeP*

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and *saeQ*. SaeS senses external stimuli such as alpha defensin via an extracellular loop (12, 13), leading to SaeR-mediated transcription of genes encoding a variety of toxins and immune evasion factors (9). Thus, the SaeR/S system plays an essential role in the recognition and evasion of host immune defenses. The Agr system is a second important regulator of *S. aureus* virulence that responds to extracellular concentrations of self-made autoinducing peptide to monitor population size. Agr has demonstrated roles in biofilm maturation and detachment (14, 15), neutrophil evasion in the dermis (16), host cell lysis after phagocytosis (17, 18), bacteremia (19), skin infections (20), pneumonia (8), and phagocyte evasion during biofilm infections (21). Together, these two systems regulate or coregulate many important virulence factors such as secreted leukocidins, phenol soluble modulins, hemolysins, proteases, complement inhibitors, and surface proteins (9, 20, 22).

While neutrophils constitute a critical cellular component of early innate immune response against *S. aureus*, the complement system is crucial for early and effective recognition of pathogens and initiation of inflammation. Complement is a tightly regulated proteolytic cascade of serum proteins that can be activated through three separate pathways—the classical pathway, the lectin pathway, and the alternative pathway—that begin with the recognition of foreign surfaces by host proteins and converge at the point of the protein C3. The classical and lectin pathways utilize the classical C3 convertase (C4b2a) to cleave C3 into C3a and C3b, while the alternative pathway produces the alternative C3 convertase (C3bBb). These convertases further complex with C3b to form C5 convertases that cleave C5 to release C5a and C5b. C3a and C5a are powerful anaphylatoxins that help to initiate inflammation by recruiting immune cells and priming neutrophils while microbial surface-deposited C3b serves as an opsonin to assist phagocytes in the recognition and subsequent phagocytosis and killing of foreign microbes (23–25). C5b deposited on the surface of serum-sensitive microbes can lead to lysis by formation of the C5b-9 membrane attack complex. This cascade thus plays a significant role in the effective recognition of microbes, recruitment of immune cells, and subsequent killing by phagocytes.

In this work, we demonstrate that the SaeR/S two-component system is required for the formation of mechanically stable biofilm aggregates in serum and that an isogenic deletion mutant of this system becomes highly susceptible to clearance by human neutrophils in vitro. The defective aggregation phenotype observed in a LACΔ*saePQRS* (hereafter referred to as LACΔ*sae*) mutant was dependent on the alternative complement pathway, as complement inhibition or depletion restored bacterial aggregation. We further found that SaeR/S-regulated genes *ecb*, *efb*, *sbi*, *scn*, *scb*, and *fnbA/fnbB* collectively prevent complement-mediated inhibition of aggregation. Together, these results characterize an additional function of human complement, inhibition of bacterial aggregation, that is strongly impaired by SaeR/S-regulated factors to facilitate the aggregation of *S. aureus* and evasion of human neutrophil killing during early biofilm formation.

Results

***S. aureus* Aggregation Limits Neutrophil Killing on an Abiotic Surface.** We have previously demonstrated that human neutrophils readily clear single cells and very small clusters of methicillin-sensitive *S. aureus* from a surface but struggle to kill larger biofilm aggregates (6, 7). We utilized the same time-lapse confocal microscopy approach to quantify individual neutrophil–bacteria interactions and confirm these results with a clinically relevant methicillin-resistant strain of *S. aureus*, USA300/Los Angeles Clone (LAC). LAC cells expressing green fluorescent protein (GFP) were briefly attached to a glass surface and subsequently incubated in 10% normal human serum (NHS) for 1, 2, or 3 h to allow biofilm aggregates to develop prior to neutrophil addition. Neutrophils were added to wells and *S. aureus*–neutrophil interactions were imaged for 4 h. Bacterial aggregates that were grown for 2 or 3 h demonstrated population-level resistance to neutrophil killing compared to wells containing CFU-matched numbers of nonaggregated cells (Fig. 1 *A* and *B*). Cells that were grown for 1 h demonstrated little additional resistance to neutrophil clearance compared to nonaggregated controls (Fig. 1*C*). After 2 h of growth, many aggregates had formed that were approximately 50 to 60 μm^2, large enough to resist killing by neutrophils (Fig. 1 *D* and *E*). In addition to resisting neutrophil killing, aggregates of this size induced significant neutrophil membrane damage, as determined by neutrophil uptake of propidium iodide (PI) (Fig. 1*F*). Together, these data confirm previous results demonstrated in a laboratory strain of *S. aureus* (6) and more robustly establish that small *S. aureus* aggregates can resist killing by human neutrophils as has been described for mature biofilms (26).

The SaeR/S Gene Regulatory System Is Essential for Biofilm Aggregate Formation and Resistance to Killing by Neutrophils. We sought to explore the mechanisms by which *S. aureus* tolerates neutrophil killing during early biofilm aggregate formation by investigating two major regulators of *S. aureus* virulence: the SaeR/S two-component system and the Agr quorum-sensing system. We attached LACΔ*sae* or LACΔ*agr* cells to a glass surface, incubated in 10% NHS, and observed growth over 4 h by confocal microscopy. While LAC and LACΔ*agr* cells formed dense aggregates (Fig. 2 *A* and *C*), LACΔ*sae* demonstrated a profound aggregation defect (Fig. 2*B* and Movie S1). Cells regularly broke away from aggregates and drifted across the field of view (FOV), demonstrating impaired cell–cell adhesion. To validate the role of the SaeR/S system, we complemented LACΔ*sae* with the *sae* locus via a stable genome integration and observed that aggregation in 10% NHS was restored (Fig. 2*D*). Unlike the wild-type (WT) strain, we observed an increase in the number of LACΔ*sae* aggregates in each FOV as cells broke away from larger aggregates (Fig. 2*E*) and aggregates remained comparatively small (Fig. 2*F*). Conversely, the number of LAC or LACΔ*agr* aggregates remained relatively static (Fig. 2*G*), and these aggregates increased in size (Fig. 2*H*).

We next challenged LACΔ*sae* and LACΔ*agr* cells with human neutrophils to determine whether either system's absence increased susceptibility to clearance. Aggregate tolerance to neutrophil killing in the WT strain began to manifest after approximately 2 h of growth (Fig. 1*B*). Therefore, we incubated bacteria for 2 h prior to the addition of neutrophils. On average, LACΔ*sae* but not LACΔ*agr* aggregates were significantly smaller at the time of discovery by a neutrophil compared to WT (Fig. 3 *A* and *B*). Neutrophil membrane damage was largely prevented in LACΔ*sae* (Fig. 3*C*), while only a moderate but not statistically significant decrease was observed with the LACΔ*agr* strain (Fig. 3*D*). Compared to LAC aggregates, a higher fraction of LACΔ*sae* aggregates were completely cleared by neutrophils (Fig. 3*E*) while LACΔ*agr* aggregates exhibited similar susceptibility to WT (Fig. 3*F*). These data demonstrate that unlike LAC or LACΔ*agr* bacteria, individual LACΔ*sae* aggregates fail to reach the requisite size to be protected from neutrophil killing. At the population level, we saw significantly more clearance of bacteria across an entire FOV with the LACΔ*sae* strain (Fig. 3*G*), but not with the LACΔ*agr* strain (Fig. 3*H*). Thus, SaeR/S but not Agr-regulated factors are essential for the formation of biofilm aggregates in

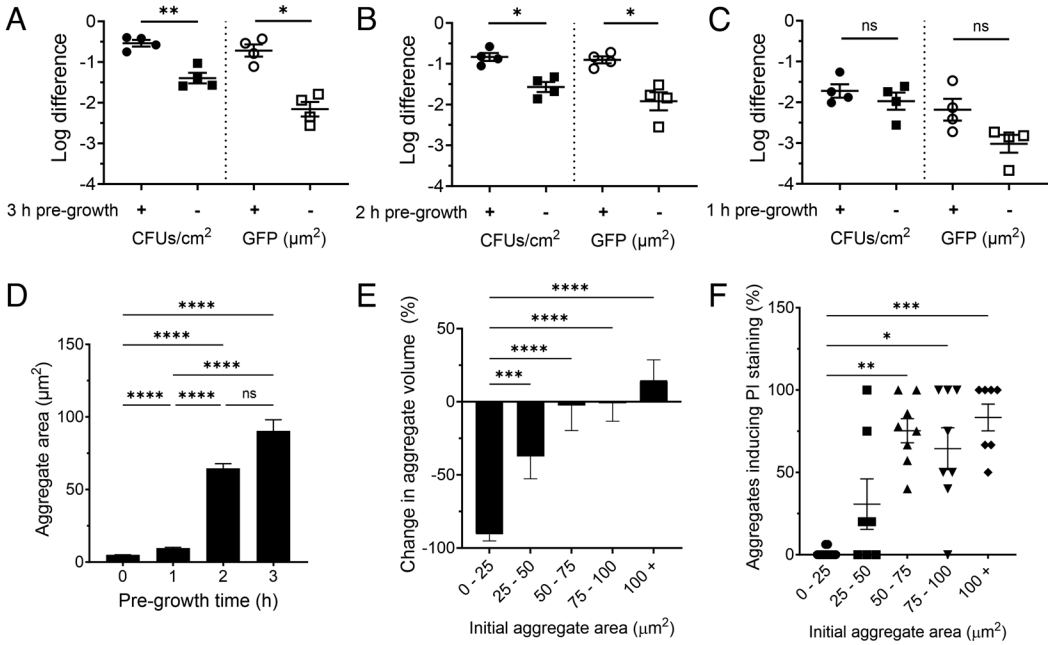

Fig. 1. *S. aureus* (strain LAC) biofilm aggregates rapidly gain resistance to neutrophil killing. Survival of aggregates grown for (*A*) 3 h, (*B*) 2 h, or (*C*) 1 h prior to neutrophil addition (pregrowth +, circles) and CFU-matched nonaggregated controls (pregrowth −, squares), compared to control wells without neutrophils. Log difference calculated as the difference between neutrophil-treated and control wells for either recovered CFUs (closed symbols) or total GFP signal remaining on the surface measured by a stitched tile scan view of the entire well (open symbols) (N = 4 independent experiments each. Paired *t* test $^{*}P < 0.05$, $^{**}P < 0.01$). (*D*) Average bacterial aggregate size at the start of imaging. Bins indicated the amount of time allowed for aggregate growth prior to neutrophil addition (N = 61 to 1,406 aggregates per condition from 12 independent experiments. Kruskal–Wallis test with Dunn's multiple comparisons $^{****}P < 0.0001$). (*E*) Change in aggregate volume following discovery by a neutrophil (N = 15 to 92 aggregates per bin from 12 independent experiments. Kruskal–Wallis test with Dunn's multiple comparisons $^{***}P < 0.001$, $^{****}P < 0.0001$). (*F*) Percentage of aggregates in a FOV that induce PI staining of at least one neutrophil (N = 7 to 12 FOVs per bin. Kruskal–Wallis test with Dunn's multiple comparisons $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Error bars indicate mean ± SEM.

human serum and subsequent resistance to killing by human neutrophils.

Biofilm Aggregation in Serum Is Protein Mediated. SaeP, SaeQ, and SaeS are all membrane-bound proteins and SaeP has been shown to interact with biofilm matrix proteins (27). The aggregation phenotype that we observed did not appear to be due to direct interactions with the SaeP or SaeQ proteins as a LACΔ*saePQ* mutant formed stable aggregates (*SI Appendix*, Fig. S1*A*). To investigate the mechanism of aggregation, we treated WT aggregates with Proteinase K or DNase. Proteinase K treatment led to concentration-dependent fracturing of biofilm aggregates into smaller groups of cells (*SI Appendix*, Fig. S2 *A* and *B*). Cells treated with Proteinase K were released from the static aggregates and drifted across the FOV more frequently than WT (*SI Appendix*, Fig. S2*C*). DNase treatment did not lead to fracturing of aggregates or increased cell release (*SI Appendix*, Fig. S2 *D–F*), suggesting that aggregates at this stage form through a protein-dependent mechanism. These results are consistent with previous reports identifying *S. aureus* proteins that facilitate biofilm formation in vitro in different assays and conditions (28, 29). We utilized this existing literature to identify potential SaeR/S-regulated genes that might be responsible for the poor aggregation phenotype that we observed in the LACΔ*sae* strain. We screened and found no aggregation defect in strains lacking the genes for fibronectin binding proteins (LACΔ*FnbPs*) (30, 31), extracellular adherence protein (LACΔ*eap*) (32, 33), extracellular matrix binding protein (LACΔ*emp*) (32), or protein A (LACΔ*sbi*Δ*spa*) (34) (*SI Appendix*, Fig. S1 *B–E*). Aggregation did not appear to be dependent on coagulation via residual fibrinogen in serum, as a coagulase and von Willebrand factor binding protein knockout did not display an aggregation defect (LACΔ*coa*Δ*vwb*) (35) and no effect was observed in clumping factor A or B deficient strains (LACΔ*clfA*, LACΔ*clfB*) (36) (*SI Appendix*, Fig. S1 *F–H*). Although aureolysin has been demonstrated to be upregulated in a LACΔ*sae* mutant (37), the poor aggregation phenotype is not a product of upregulation of bacterially derived proteases, as a mutant deficient in the major *S. aureus* proteases and SaeR/S still retained the poor aggregation phenotype (*SI Appendix*, Fig. S1*I*). Aggregation was also not dependent on the combined effects of sortase A anchored surface proteins or the production of poly-N-acetylglucosamine (*SI Appendix*, Fig. S1 *J* and *K*). While these experiments only screened a few well-described candidate genes from previous reports, the findings with Proteinase K suggest a separate aggregation mechanism in serum which is protein mediated.

Complement Proteins Prevent Biofilm Aggregation by the SaeR/S Deletion Mutant. We sought to determine whether a host serum protein was mediating WT aggregation in a manner similar to previous reports (36, 38–40), or conversely, inhibiting LACΔ*sae* aggregation. To remove serum proteins, we ethanol-precipitated human serum overnight (39). No difference in WT aggregation was observed between NHS and ethanol-treated serum; however, the LACΔ*sae* strain was able to aggregate in ethanol-treated serum (*SI Appendix*, Fig. S3 *A–D*). This suggested that serum proteins actively inhibited LACΔ*sae* aggregation rather than facilitated WT aggregation. Heat-inactivation of serum complement prior to incubation with bacteria similarly resulted in restoration of aggregation by LACΔ*sae* (Fig. 4 *A* and *B* and Movie S2). To confirm the role of complement rather than other heat-labile proteins, cobra venom factor (CVF), which binds to factor B to form a highly stable C3 and C5 convertase (41), was used to specifically deplete C3 and C5 complement proteins from serum. LACΔ*sae* cells grown in serum treated with CVF for 1 h prior to incubation (CVF-S) showed a restoration of aggregation (Fig. 4*C*).

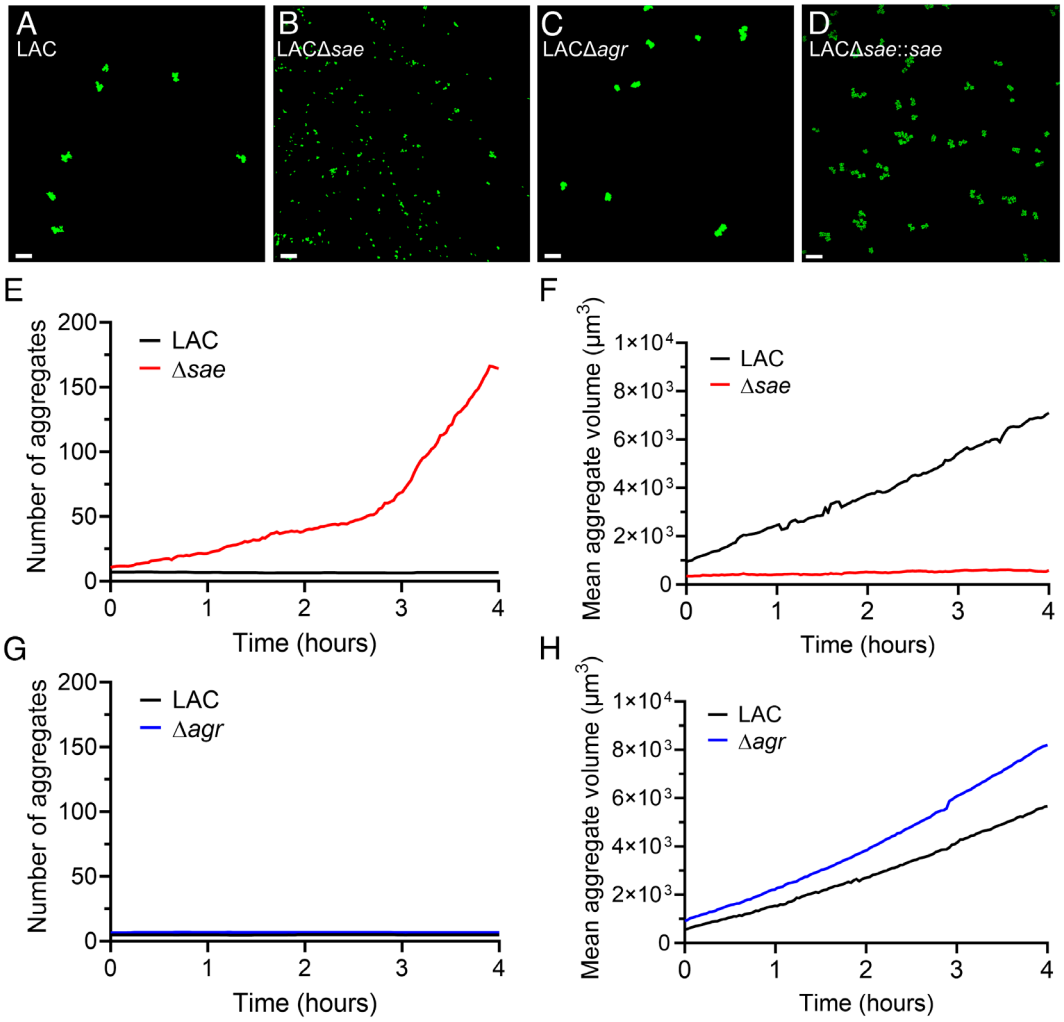

Fig. 2. LACΔ*sae* demonstrates an aggregation defect in human serum. Representative images of (*A*) LAC, (*B*) LACΔ*sae*, (*C*) LACΔ*agr*, and (*D*) LACΔ*sae*::*sae* aggregation in 10% NHS after approximately 4 h of growth. [Scale bar, 50 μm (*A–D*).] (*E-H*) Aggregation phenotype in 10% NHS. Data shown were collected from N = 8 FOVS from four independent experiments per condition.

Growth of WT bacteria in CVF-S did not appear different from growth in NHS. The number of LACΔ*sae* aggregates in each FOV when grown in heat-inactivated serum (HIS) or CVF-S was similar to WT in CVF-S, while LACΔ*sae* aggregates fractured in NHS (Fig. 4*D*). At 4 h, LACΔ*sae* aggregates grown in HIS or CVF-S were significantly larger than those grown in NHS (Fig. 4 *E* and *F*). LACΔ*sae* cell clusters grown in NHS failed to maintain strong cell–cell adhesion and remained small (24.7 ± 48.6 μm^2) whereas WT or LACΔ*sae* cells grown in HIS or CVF-S regularly formed aggregates larger than 750 μm^2 (Fig. 4*F*). Addition of compstatin, a peptide that binds C3 and prevents cleavage by C3 convertases (42), produced a dose-dependent restoration of aggregation by LACΔ*sae* (Fig. 4*G*). Collectively, these findings indicate that complement in serum inhibits aggregation of the LACΔ*sae* mutant.

We next tested whether the ability of serum complement to inhibit aggregation could be observed with additional *S. aureus* strains and other opportunistic pathogens. We confirmed that SaeR/S mutants in two additional MRSA strains, MW2 (USA400) and COL (CC8/ST250/USA500) (*SI Appendix*, Fig. S4 *A–H*) and a methicillin susceptible strain, MnCop (*SI Appendix*, Fig. S4 *I–L*) are unable to form aggregates when grown in NHS. *Staphylococcus epidermidis* (*S. epidermidis*) strain 1457 formed distinct aggregates in HIS but aggregation was completely inhibited in NHS, mimicking the *S. aureus* Δ*sae* phenotype (*SI Appendix*, Fig. S4 *M–P*). *Enterococcus faecalis (E. faecalis*) strain OG1RF formed clusters of long chains in HIS but failed to form significant chains in NHS (*SI Appendix*, Fig. S4 *Q–T*). It is likely that the capacity of human serum to inhibit aggregation of both these species is due to the lack of the mechanism that is present in *S. aureus* to overcome this host defense process.

Together, these data establish that complement proteins downstream of C3 cleavage by C3 convertases can prevent aggregation of LACΔ*sae*. These data further indicate that *S. aureus* overcomes this inhibition of aggregation via SaeR/S-regulated factors, while *S. epidermidis* and *E. faecalis* lack these defenses and demonstrate impaired aggregation in serum.

Inhibition of LACΔsae Aggregation Requires the Alternative Pathway. We next assayed LACΔ*sae* aggregation in human serum that had been depleted of C3 or C5 by immunoaffinity chromatography to identify complement components responsible for inhibition of bacterial aggregation. *S. aureus* requires serum supplementation to grow in HBSS (6) and we observed a severe growth defect in HBSS containing 10% complement-depleted serum, suggesting that key nutrients were removed during the chromatography process. NHS or HIS supplementation in RPMI 1640 medium recapitulated our previously observed aggregation phenotypes (*SI Appendix*, Fig. S3 *E–H*). Due to the slower bacterial growth in RPMI containing 10% complement-depleted serum,

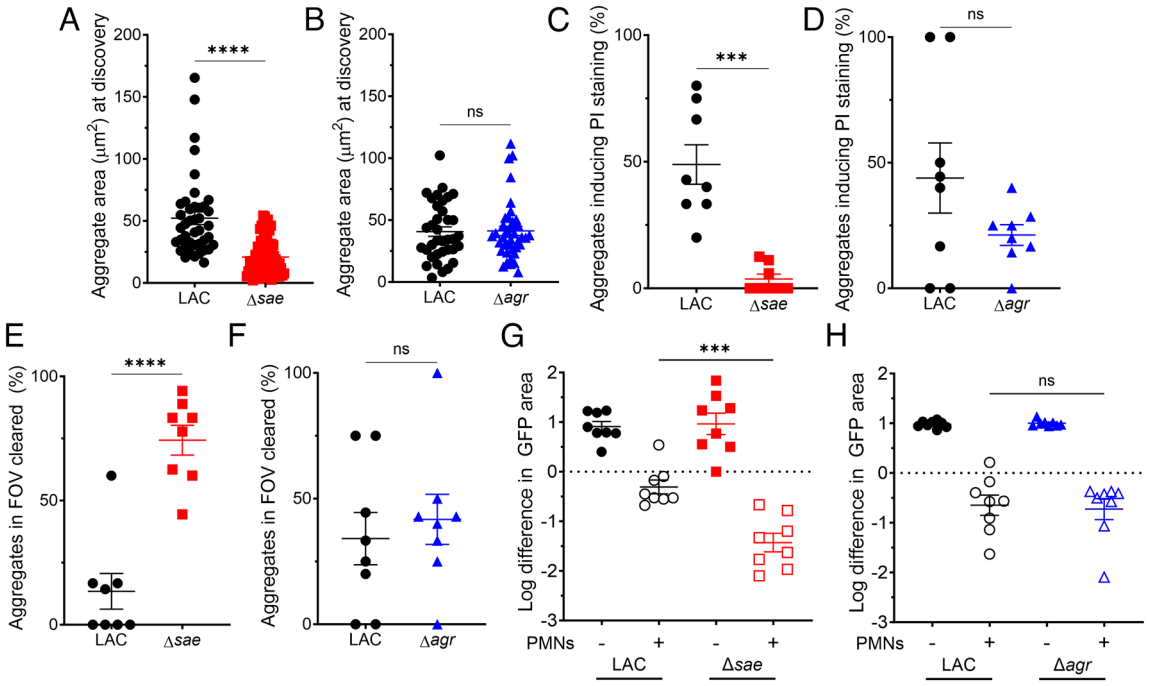

Fig. 3. LACΔ*sae* but not LACΔ*agr* shows increased susceptibility to neutrophil clearance in vitro during early biofilm formation. (*A*) Biofilm aggregate size at the time of initial interaction with a neutrophil (*A*) Δ*sae* [N = 41 (LAC) and 86 (Δ*sae*) aggregates. Mann–Whitney test. ****$P < 0.0001$] and (*B*) Δ*agr* [N = 36 (LAC) and 40 (Δ*agr*) aggregates. Mann–Whitney test]. (*C* and *D*) Percentage of aggregates in a FOV that induce PI staining of at least one interacting neutrophil (Unpaired *t* test with Welch's correction. ***$P < 0.001$). (*E* and *F*) Percentage of aggregates in a FOV that had no detectable GFP signal at 4 h after discovery by a neutrophil (Unpaired *t* test with Welch's correction. ****$P < 0.0001$). (*G* and *H*) Log difference in GFP area within a FOV over 4 h of imaging. Solid symbols indicate control wells without PMNs added. Open symbols indicate wells treated with PMNs (N = 8 FOVs per condition. Unpaired *t* test with Welch's correction ***$P < 0.001$). Error bars indicate mean ± SEM. Data shown were collected from 8 FOVs from four independent experiments each.

we grew cells for longer and quantified aggregate size from 4 to 8 h. Growth of LACΔ*sae* in RPMI containing 10% C3-depleted serum resembled growth in HIS and CVF-S in that aggregates remained cohesive and grew significantly in size over time (Fig. 5 *A*, *E*, and *F* and Movie S3). Conversely, growth of LACΔ*sae* in 10% C5-depleted serum resembled growth in NHS in both the gradual increase in the number of cell clusters over time and the small, relatively constant, aggregate size (Fig. 5 *B*, *E*, and *F* and Movie S4). After 8 h of growth, aggregates in C3-depleted serum were significantly larger than aggregates in C5-depleted serum, and aggregates grown in C5-depleted serum were predominantly less than 100 μm^2 in size (Fig. 5 *G* and *H*). We confirmed that C5 is not required for the inhibition of *S. aureus* aggregation by treating human serum with the C5 inhibitor eculizumab prior to incubation with LACΔ*sae* and found no difference in aggregation (*SI Appendix*, Fig. S5). These data demonstrate that complement C3, but not C5, is required to prevent aggregation of LACΔ*sae*.

To determine whether aggregation inhibition occurred through both the classical/lectin pathway and alternative pathways, we grew LACΔ*sae* cells in C4-depleted (alternative pathway activity only) and factor B (fB)-depleted (classical/lectin pathway activity only) sera. Aggregation remained strongly inhibited in C4-depleted sera while LACΔ*sae* cells aggregated in C3 or fB-depleted serum (Fig. 5 *A*, *C*, and *D* and Movies S5 and S6). C4 depletion did not have a significant effect on aggregation inhibition as aggregates fractured and remained small; however, depletion of C3 or fB prevented aggregation interference (Fig. 5 *I* and *J*). There was a slight decrease in average size between aggregates grown in C3 and fB-depleted sera, but the two shared broadly similar phenotypes (Fig. 5 *A*, *D*, *I*, and *J*). These data demonstrate that neither the classical nor the lectin pathway plays a significant role in aggregation inhibition under the tested conditions; rather this phenomenon occurs primarily via the alternative pathway.

We next queried whether differences in aggregation were due to differential deposition of C3b on the bacterial cell surface. Many complement-interfering proteins produced by *S. aureus* are SaeR/S-regulated and we hypothesized that we would observe significant differences in C3b deposition between WT and LACΔ*sae* (9, 22). Surprisingly, we observed little difference in C3b opsonization of LAC and LACΔ*sae* using either confocal immunofluorescence (*SI Appendix*, Fig. S6*A*) or flow cytometry (*SI Appendix*, Fig. S6*B*). We speculate that given the low density of *S. aureus* cells and the high serum concentration used in this assay, bacterially derived complement-interfering proteins were likely present in insufficient concentrations to fully inhibit complement deposition as has previously been reported (43, 44). Thus, it appears unlikely that the presence of C3b on the surface alone is responsible for the striking difference in aggregation. Rather, disruption of complement-mediated aggregation inhibition may depend more on hampering C3b function or C3bBb(C3b) convertase activity.

***S. aureus* Produces Several Complement-Interfering Proteins That Collectively Are Responsible for Impeding the Ability of Complement to Block Bacterial Aggregation.** We next aimed to identify the SaeR/S-regulated factors that are responsible for the ability of *S. aureus* to overcome complement-mediated inhibition of aggregation in human serum. We sequenced RNA recovered from surface adherent LAC and LACΔ*sae* cells grown in either NHS or HIS to identify differentially expressed genes. Importantly, we detected no differential expression of genes by either LAC or LACΔ*sae* in NHS compared to HIS, demonstrating that the difference in LACΔ*sae* aggregation is not due to altered transcription by *S. aureus* in response to heat-inactivation of the serum (*SI Appendix*, Table S1 and Dataset S1). Comparison of LAC and LACΔ*sae* transcript levels in NHS broadly resembled

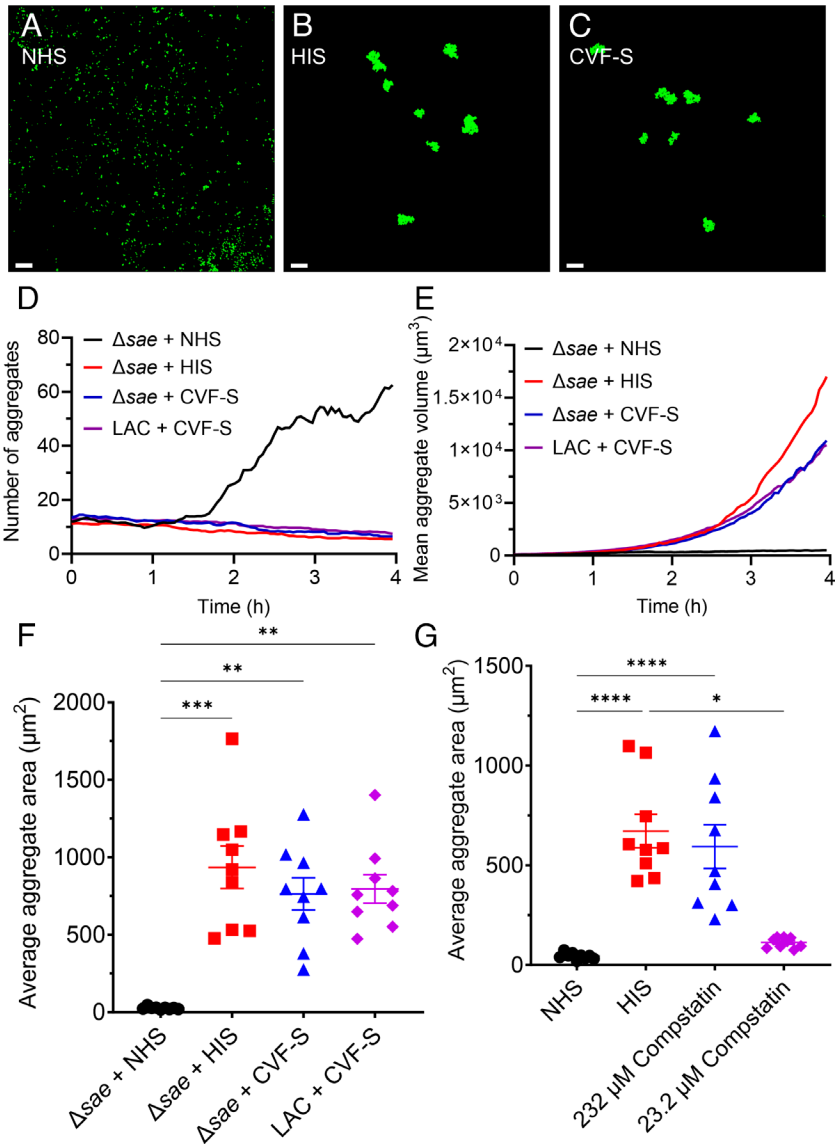

Fig. 4. Complement inhibits aggregation by LACΔ*sae*. Representative images of LACΔ*sae* aggregation in 10% (*A*) NHS, (*B*) HIS, or (*C*) CVF-treated serum (CVF-S) after approximately 4 h of growth. (Scale bar, 50 μm.) (*D*) Average number of bacterial objects detected and (*E*) average aggregate volume in each FOV (N = 6 FOVs per condition). (*F*) CVF or (*G*) compstatin treatment of serum restores Δ*sae* aggregation (N = 9 FOVs per condition. Kruskal–Wallis test with Dunn's multiple comparisons. **P* < 0.05, ***P* < 0.01, ****P* < 0.001). Data shown were collected from N = 3 independent experiments. Error bars indicate mean ± SEM.

previous datasets (9, 22, 37) and demonstrated a massive reduction in expression of known complement-inhibiting proteins (*SI Appendix*, Table S1). Of note, *sbi*, *efb, ecb*, *scn*, *scb*, and *eap*—genes with products known to strongly inhibit complement function—were all downregulated at least 25-fold in LACΔ*sae* (*SI Appendix*, Table S1). These results underscore the idea that SaeR/S is the major regulator of complement-interfering genes in *S. aureus*. To identify the factors that overcome complement-mediated inhibition of aggregation, we created isogenic deletion mutants in the LAC background in several genes known to interfere with the alternative pathway that were also highly downregulated in the LACΔ*sae* strain. Single deletions of *ecb*, *efb*, *sbi*, *scn*, *scb*, and *fnbA/fnbB*, failed to duplicate the LACΔ*sae* aggregation phenotype. As many of these genes have overlapping functions, we then began examining various combinations. Double knockouts of two related inhibitors of the alternative convertase, Δ*scn*Δ*scb* (*SI Appendix*, Fig. S1*L*) and Δ*ecb*Δ*efb* (*SI Appendix*, Fig. S1 *M* and *N*) did not demonstrate obviously impaired aggregation, illustrating the complementary function of some of these proteins. Of the combinations we constructed, only a total knockout of all 7 genes showed clear phenotypic similarity to the Δ*sae* mutant, suggesting that these genes in combination overcome complement-mediated inhibition of aggregation (Fig. 6). The strain LACΔ*ecb*Δ*efb*Δ*sbi*Δ*scn*Δ*scb*Δ*FnbPs*, referred to as ΔC7, showed an inability to form structurally stable biofilm aggregates in 10% NHS but readily aggregated in 10% HIS (Fig. 6 *A*, *B*, *E*, and *F* and Movie S7), similar to the LACΔ*sae* strain. A ΔC5 strain (LACΔ*ecb*Δ*efb*Δ*sbi*Δ*FnbPs*), generated during construction of the ΔC7 strain, showed slight susceptibility to complement-mediated inhibition of aggregation, demonstrating an additive effect by the two Staphylococcal complement inhibitor (SCIN) proteins (Fig. 6 *C*–*F*). The ΔC5 strain also showed more variability in aggregation phenotype between replicates than the ΔC7 strain—in some experiments, only slight inhibition was observed, while in others there was little aggregation (Fig. 6 *E* and *F*). We hypothesize that this variability is due to differences between serum donors. We next verified the role of the 5 secreted genes that were deleted from the ΔC7 strain by generating single gene complementation strains (*SI Appendix*, Fig. S7). We observed differing levels of restoration of aggregation through single gene complementation and substantial

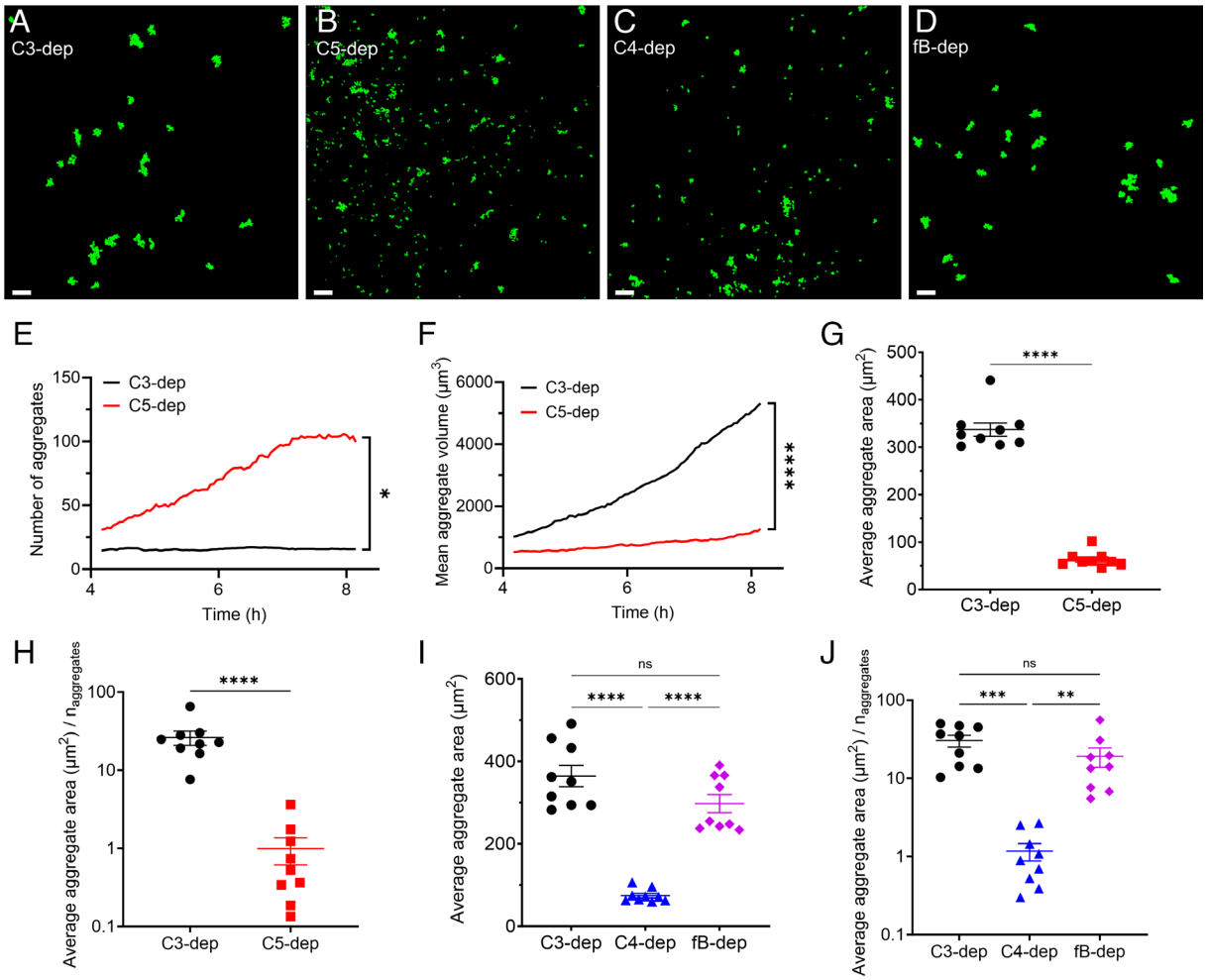

Fig. 5. The alternative complement pathway is required for inhibition of *S. aureus* aggregation. Representative images of LACΔ*sae* aggregation in (*A*) C3-depleted human serum, (*B*) C5-depleted human serum, (*C*) C4-depleted human serum, and (*D*) factor B-depleted human serum after approximately 8 h of growth. (Scale bar, 50 µm.) (*E*) Average number of bacterial objects detected and (*F*) average aggregate volume in each FOV after 8 h (N = 6 FOVs per condition. Unpaired *t* test with Welch's correction $^{*}P < 0.05$, $^{****}P < 0.0001$). (*G*) Average aggregate area and (*H*) aggregation score per representative FOV after 8 h (N = 9 FOVs per condition. Mann–Whitney test. $^{****}P < 0.0001$). (*I*) Average aggregate area per representative FOV after 8 h (N = 9 FOVs per condition. Brown–Forsythe with Dunnett's T3 multiple comparisons test. $^{****}P < 0.0001$). (*J*) Aggregation score after 8 h of growth (N = 9 FOVs collected per condition. Kruskal–Wallis test with Dunn's multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$). Data shown were collected from N = 3 independent experiments. Error bars indicate mean ± SEM.

variability between individual replicates, potentially due to differing serum complement levels between donors. Complementation of *efb* and *scn* had the strongest effect on aggregation, while *sbi* complementation had a moderate effect (*SI Appendix*, Fig. S7 *I–K*). While *ecb* and *scb* complementation failed to produce a strong overall effect, qualitatively we observed that there were slightly more large aggregates than in the control strain (*SI Appendix*, Fig. S7*K*) suggesting a mild effect. Together, these data suggest that the ability of WT *S. aureus* to form aggregates in serum is a result of the combined effects of these proteins. This conclusion is further supported by our observation that the ΔC5 strain, which still retains the *scn* and *scb* genes, produces a phenotype that varies significantly between donors.

To demonstrate that the observed aggregation phenotype is independent from coagulation of fibrinogen, we compared *S. aureus* growth in 10% human serum and human plasma (NHP) (*SI Appendix*, Fig. S8). First, we found that a LACΔ*coa*Δ*vwb* mutant aggregates in serum (*SI Appendix*, Fig. S1*F*), indicating that neither coagulase nor von Willebrand factor binding protein contributes to this phenomenon. Aggregation of the ΔC5 and ΔC7 mutants was restored in 10% NHP. Coagulase (*coa*) and von Willebrand factor binding protein (*vwb*), whose gene expression is regulated by SaeR/S, are known to contribute to coagulation by *S. aureus* (*SI Appendix*, Table S1). Fibrinogen present in NHP failed to restore aggregation in the LACΔ*sae* strain (*SI Appendix*, Fig. S8). Together, these data demonstrate that the SaeR/S regulatory program is essential for aggregation in plasma through the control of genes required for two independent and distinct mechanisms of aggregation—the process of inhibiting complement described herein and that of coagulation.

We additionally assessed whether *S. aureus* can aggregate in mouse serum. Notably, WT LAC failed to form aggregates in 10% fresh mouse serum and was indistinguishable from isogenic Δ*sae*, ΔC5, and ΔC7 strains (*SI Appendix*, Fig. S9). Heat-inactivation of mouse serum improved aggregation of all strains, suggesting that aggregation interference in mouse serum is also dependent on complement. While the anti-complement activities of SCIN and SCIN-B have been demonstrated to be human specific (45), our observation that a LACΔ*scn*Δ*scb* strain can aggregate in human serum suggests that the described phenotypic differences between mouse and human serum are not solely due to a loss of SCIN and SCIN-B activity. Rather, the other genes deleted from ΔC7 may also be poorly adapted to mouse complement, or there may be differences in the potency of complement-mediated aggregation inhibition in mice compared to humans.

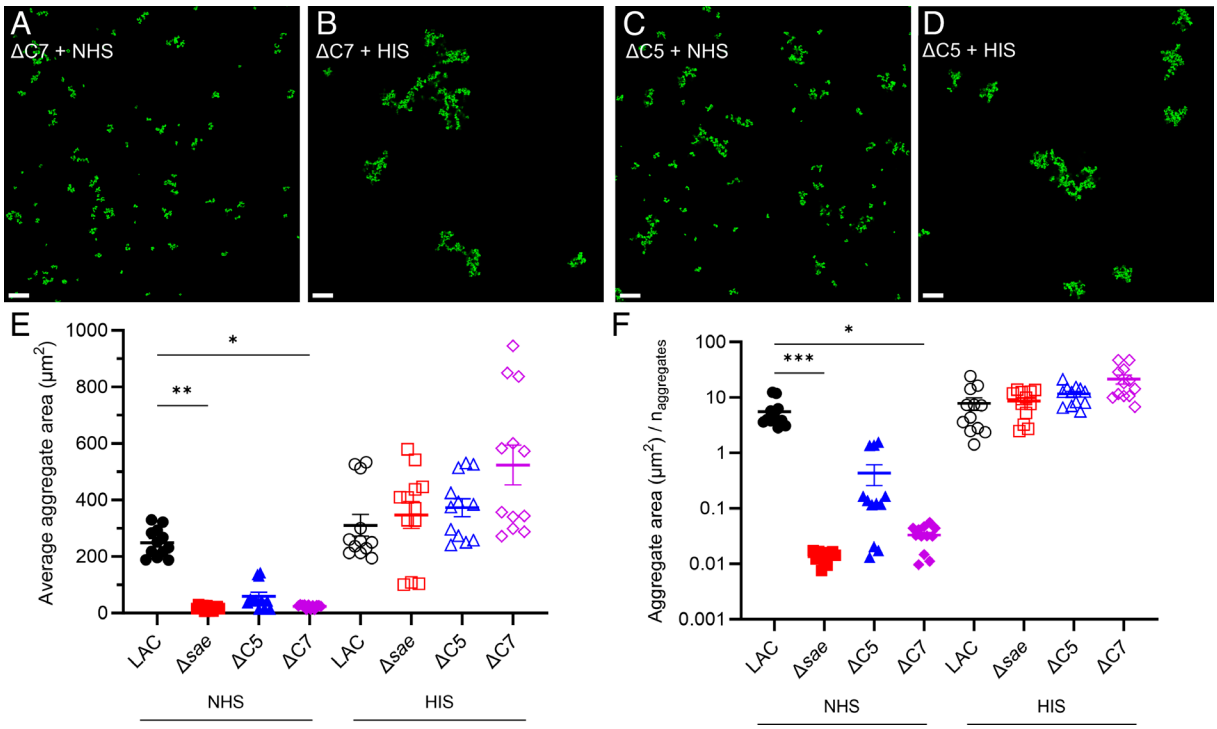

Fig. 6. Multiple SaeR/S-regulated genes contribute to *S. aureus* aggregation in human serum. Representative images of LACΔC7 (LACΔ*ecb*Δ*efb*Δ*sbi*Δ*scn*Δ*scb*Δ*FnbPs*) aggregation in (*A*) NHS and (*B*) HIS after approximately 4 h of growth. Representative images of LACΔC5 (LACΔ*ecb*Δ*efb*Δ*sbi*Δ*FnbPs*) in (*C*) NHS and (*D*) HIS after approximately 4 h of growth. (Scale bar, 20 μm.) (*E*) Average aggregate area per representative FOV collected after approximately 4 h of growth. (*F*) Aggregation score after 4 h of growth. N = 12 FOVs per condition collected from four independent experiments. Kruskal–Wallis test with Dunn's multiple comparisons compared to LAC-NHS condition. $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate mean ± SEM.

Discussion

S. aureus is an important pathogen in implant-associated infections as well as skin infections, pneumonia, infective endocarditis, and sepsis (3, 46, 47). In the context of biofilm infections, there is scant evidence demonstrating how contaminating bacteria evade early host immune defenses, as much of the focus has been on how the pathogen modulates and inhibits immune function later in the infection (26, 48–50). In this work, we demonstrate that *S. aureus* rapidly develops neutrophil-resistant biofilm aggregates in human serum. Importantly, the development of mechanically stable aggregates and resulting recalcitrance to neutrophil killing is dependent on SaeR/S-regulated factors, as aggregate formation by a LACΔ*sae* deletion strain was directly inhibited by serum complement in a C3 and fB-dependent manner. Therefore, we propose a model wherein human complement can prevent bacterial aggregation, but this effect is overcome by the combined effects of several *S. aureus* SaeR/S-regulated virulence factors. While a few studies have established that serum or serum proteases can affect late biofilm formation (51–54), this study demonstrates that complement inhibits bacterial cell–cell adhesion and describes protein factors used by a bacterial pathogen to overcome this effect. The data in this report illustrate that even "small" (50 to 75 μm^2) biofilm aggregates can become recalcitrant to neutrophil killing. Thus, reduction of bacterial aggregation by complement is a potent host defense mechanism as it demonstrably amplifies the ability of phagocytes to effectively clear bacteria. Our observations have implications beyond implant-associated infections, such as in sepsis and infective endocarditis where exposure to complement is expected to play a major role. Importantly, these findings shed light on the earliest stage of *S. aureus* biofilm pathogenesis- the transition from contaminating cells to robust biofilm infection. Previous studies have demonstrated that loss-of-function mutations in the Agr quorum-sensing system during chronic infections result in dense biofilms that provide increased protection from phagocytes and antibiotic therapy (21, 55, 56). In this work, we found no difference in growth or survival against neutrophil challenge between WT and Δ*agr* strains during early biofilm aggregate formation. Our findings therefore support a conceptual model where early biofilm formation is dependent on Sae-mediated immune evasion and long-term resistance depends on acquired Agr mutation.

We showed that *S. aureus* can form aggregates in human serum by the production of several complement inhibitors that affect the alternative pathway. SCIN can inhibit activity for both the classical (C4b2a) and alternative (C3bBb) C3 convertases and interfere with C3b deposition (45, 57, 58). The SCIN homologues SCIN-B and SCIN-C similarly interfere with C3 convertase activity (43). The C3-binding domain of extracellular fibrinogen-binding protein (Efb) can bind surface-bound C3b to block C3b containing convertases from cleaving C3 and C5 (43, 58). Extracellular complement-binding protein (Ecb) can also inhibit C3b containing convertases on the microbe surface (43) and can block C3b recognition by CR1 (59), thereby inhibiting phagocytosis. Additionally, Efb and staphylococcal immunoglobulin-binding protein (Sbi) have been demonstrated to bind host plasminogen, which can be converted to active plasmin by secreted staphylokinase (60), facilitating degradation of C3, C3a, and C3b near the cell surface (61). Sbi can also directly inhibit alternative pathway function through binding and consumption of C3 and by interacting with the alternative pathway regulatory protein Factor H (62–64). The fibronectin binding proteins have been shown to inhibit the alternative pathway through the recruitment of Factor H to the microbe surface through binding with the N2N3 domains (65). We demonstrate that these virulence factors combine to facilitate aggregation through collective inhibition of

complement. Thus, we suggest that the ability of bacteria to aggregate is determined by the overall balance of total serum complement activity versus the capacity of the organism to produce proteins to inhibit complement (*SI Appendix*, Fig. S10). We observed that deletion of individual genes in *S. aureus* was insufficient to prevent aggregation due to the remaining complement inhibitory genes. Conversely, overexpression of some individual genes (*ecb*, *scn*, and *sbi*) in the ΔC7 strain was sufficient to restore some degree of aggregation. The production of several complement-inhibiting genes of similar function ensures the ability of *S. aureus* to aggregate in the presence of serum complement. However, more studies are needed using complemented strains under natural promoters and using strains complemented in more than one gene to determine the exact influence of each of the C7 genes identified in this study.

Based on our observation that complement-mediated aggregation inhibition cannot occur without cleaved C3 or fB, interference with the alternative C3 convertase or C5 convertase is the most likely step at which *S. aureus* can restore aggregation. Efb, Ecb, Sbi, and the SCIN proteins all have demonstrated roles for interfering with the alternative convertases (43, 45, 62) while the FnbPs can further reduce alternative pathway activation by co-opting host regulatory factors (65). Importantly, we found these genes were significantly downregulated in LACΔ*sae* in our RNA sequencing data. Moreover, *sbi* and *scn* were two of the most abundant transcripts detected in WT LAC, suggesting a high level of production. Further work is required to determine the relative contributions of each gene knocked out in the C7 strain (Δ*ecb*Δ*efb*Δ*sbi*Δ*scn*Δ*scb*Δ*FnbPs*) and will likely require many permutations to determine which genes are minimally required to facilitate aggregation and which genes are the most potent inhibitors of complement-mediated aggregation inhibition. *S. aureus* inhibits other aspects of the complement system through virulence factors such as extracellular adherence protein (Eap), serine protease-like protein B (SplB), staphylococcal superantigen-like protein 7 (SSL7), protein A (Spa), clumping factor A (clfA), and surface protein serine-aspartate repeat protein E (SdrE), highlighting broad interference with complement functions as an important strategy for *S. aureus* immune evasion (44, 65–72).

S. aureus produces many surface proteins that can aid in aggregation by binding host proteins found in blood such as collagen, fibronectin, and fibrinogen (73). In attempting to identify the mechanism of aggregation in serum, we were able to rule out a role for the sortase A anchored surface proteins (74). Individual knockouts of SaeR/S-regulated genes encoding proteins with host protein binding function also failed to demonstrate an aggregation defect (*SI Appendix*, Fig. S1). The observation that the genes responsible for coagulation, *coa* and *vwb*, were dispensable for aggregation in serum indicates that coagulation of fibrinogen presents a parallel mechanism to complement inhibition for purposes of aggregation. Future studies will consider the possibility that immune complexes are responsible for *S. aureus* aggregation in serum due to their ability to agglutinate bacteria and the well-established role of complement in their resolubilization (25, 75). A preliminary attempt to investigate this model showed that anti-*S. aureus* IgG induced aggregation in a manner that was disrupted by complement. However, additional experiments are needed to thoroughly explore this hypothesized mechanism and determine whether there are other non-antibody-mediated mechanisms of aggregation in serum that may also be affected by complement.

Aggregation and biofilm formation are general defense strategies that are employed by many bacterial species to impair phagocytosis and increase survival. Complement interference is also a common strategy utilized by many pathogens such *Streptococcus spp.*, *Serratia marcescens*, and *Porphyromonas gingivalis* due to its importance in initiating inflammation and phagocytosis (24, 57, 76). We therefore hypothesize that the ability to resist complement-mediated aggregation inhibition is not specific to *S. aureus* and may be overcome by other bacteria that produce tools to disrupt complement. We have already demonstrated that *S. epidermidis* 1457 and *E. faecalis* OG1RF aggregation is prevented by serum, indicating that they do not possess the defensive means to inhibit complement in the same manner as *S. aureus*. Thus, it will be important to investigate whether other pathogenic organisms can effectively overcome the ability of complement to disrupt bacterial aggregation.

Future work on this subject should assess the suitability of a rabbit or nonhuman primate model for investigating the role of complement-mediated aggregation inhibition in pathogenesis, given the failure of *S. aureus* to aggregate in mouse serum. Furthermore, additional studies will be necessary to describe the specific mechanism by which complement C3 and fB inhibit bacterial aggregation, whether by solubilization of immune complexes, activation of an additional serum protein, or another mechanism. Together, our findings and future investigations could inform the design of therapeutics aimed at disrupting bacterial aggregation, a potent defense mechanism against host innate immune defenses.

Materials and Methods

Bacteria Strains and Preparation. All strains used in this study are listed in *SI Appendix*, Table S2. A detailed description of all cloning procedures can be found in *SI Appendix*. *S. aureus*, *S. epidermidis*, and *E. faecalis* were grown overnight in tryptic soy broth (TSB), or TSB supplemented with 10 μg/mL chloramphenicol or 3 μg/mL tetracycline when required for plasmid maintenance. Overnight cultures were centrifuged, washed in PBS, resuspended in tryptic soy broth, and serially diluted and adjusted by optical density. Bacteria were seeded onto a 4-chambered glass bottom Petri dish (Cellvis, CA, Cat no. D35C4-20-1.5-N) or 8-chambered dish (Cellvis, C8-1.5H-N) to facilitate live-cell imaging. To attach bacteria, 10 μL of diluted cell suspension was added to the surface and incubated for 35 min at 37 °C. Following incubation, unattached bacteria were gently rinsed from the surface with PBS. Unless otherwise noted, each chamber of the Petri dish was filled with 0.5 to 1 mL of 10% fresh human serum or modified serum diluted in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} to condition the surface and opsonize bacteria and incubated at 37 °C for at least 30 min prior to neutrophil addition. *S. epidermidis* was grown in 20% serum diluted in HBSS and *E. faecalis* was grown in 20% serum diluted in RPMI.

Neutrophil Assays. Human neutrophils were isolated from heparinized venous blood obtained from healthy donors following a standard IRB-approved protocol (Protocol numbers JVK040821 (Institutional Review Board, Montana State University) and No. 99-CC-0168 (Institutional Review Board for Human subjects, NIH)). All donors provided written consent to participate in the study. Neutrophils were isolated under endotoxin-free conditions (<25 pg mL^{-1}) and purity (<2% PBMC contamination) and viability (<5% propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry as previously described (10, 77). Neutrophils were kept on ice until stained with LysoBrite™ Red (AAT Bioquest, CA, Cat no. 22645) according to the manufacturer's instructions. In neutrophil challenge experiments, neutrophils were immediately added to the surface following staining and 5 μg/mL propidium iodide was added to each well at this time. To enumerate surviving bacteria, bacteria were removed from the surface with a sterile scraper and pipetting, vortexed in dilution tubes with PBS, and plated on tryptic soy agar in triplicate. Plates without detectable bacteria were counted as 0.5 colonies prior to log transformation.

Proteins and Serum Preparation. Serum was obtained from healthy donors by venous blood draw as described above, in tubes without heparin. HIS was prepared by heating aliquots of human serum to 56 °C for 20 to 30 min. CVF-depleted serum was produced by incubating 10 μg/mL CVF (Complement

Technology, Tyler, TX, Cat no. A150) with serum for 1 h at 37 °C, then diluted to 10% in HBSS. C3, C4, C5, and fB-depleted sera were obtained from Complement Technology (Cat nos. A314, A308, A320, and A335, respectively) and diluted to 10% in RPMI 1640. Compstatin (Selleck Chemicals, Houston, TX, Cat no. S8522) was added to 10% NHS in HBSS to a final concentration of 232.2 or 23.2 μM and mixed just prior to incubation with bacteria. Eculizumab (Thermo Fisher Scientific, Cat no. MA5-41700) was added to NHS at a concentration of 25 μg/mL (78) and incubated at 37 °C for 30 min prior to dilution into HBSS. IgG/IgM/IgA depleted human serum was purchased from Pel-Freez biologicals (Cat no. 34041-1). Anti-*S. aureus* rabbit IgG was purchased from Thermo Fisher Scientific (Cat no. PA1-7246). Ethanol-precipitated serum was prepared by adding 200 proof absolute ethanol to a final concentration of 10% in serum and incubating overnight at 4 °C (39). Serum was centrifuged and the resulting supernatant was diluted 1:10 in HBSS. Proteinase K (Cat no. EO0491) and Dnase I (Cat no. EN0521) were obtained from ThermoFisher Scientific. Mouse sera from adult male and female mice were obtained via cardiac puncture under anesthesia, and sera from two to three same-sex mice were combined for each experiment. Both BALB/c and C57BL/6NCrl mice were used (N = 2 experiments each). Serum was spun down for 10 min at 4,000 × g and diluted to 10% in HBSS. Heat-inactivation was performed as above. Animal work was approved by the Institutional Animal Care and Use Committee of the NIAID (Approval Number: LB1E).

Microscopy. A Leica SP5 inverted confocal laser scanning microscope was utilized for time-lapse imaging. GFP-tagged bacteria and propidium iodide were excited with a 488 nm laser and stained neutrophils were excited with a 561 nm laser. An Okolab (Ambridge, PA) Uno Stage Top Incubator with stand-alone humidity controller or a LiveCell (Pathology Devices, CA) environmental chamber system was utilized to maintain 5% CO_2, 20% O_2, 90% humidity, and 37 °C for sample incubation during imaging. 12 to 20 μm image stacks with 1-μm z-slices were recorded sequentially at 1 to 2 min intervals using a Leica 20×/0.7 NA dry objective lens. Experiments using serum diluted in HBSS were imaged for 4 h while experiments with C3, C4, C5, or fB-depleted serum diluted in RPMI were imaged in two consecutive 4 h segments. Two fields of view were imaged per chamber in each experiment. In neutrophil challenge experiments, each well was subsequently imaged after time-lapse microscopy using a 7 × 7 stitched tile scan with a 10×/0.3 NA dry objective to quantify the total amount of bacteria remaining on the surface. For serum aggregation assays, three representative fields of view were imaged following time-lapse microscopy from each well using a 20× dry objective. Additional imaging was performed using a Leica Stellaris 5 confocal laser scanning microscope with a Leica DMi8 inverted microscope platform, white light laser, and a 20×/0.75 NA dry objective or a Leica Stellaris 8 confocal laser scanning microscope with a 20×/0.75 HC PL APO CS2 dry objective. To assess *E. faecalis* aggregate size, growth medium was removed, and cells were briefly fixed in absolute methanol for 5 min. Cells were washed with PBS, stained with 1 μg/mL DAPI for 5 min at room temperature, washed again, and imaged as above.

Image Analysis. Image analysis was performed using MetaMorph version 7.8.13 (Molecular Devices, San Jose, CA), ImageJ version 1.52n (US NIH, Bethesda, MD), and Imaris version 10.1.0 or earlier (Oxford Instruments, Abingdon, UK). Detailed method descriptions are provided in *SI Appendix*.

C3b Surface Deposition. LAC and LACΔ*sae* aggregates were grown in 10% NHS in HBSS for 4 h prior to blocking and antibody staining. Surface-associated C3b was detected with mouse anti-human C3b antibodies (Invitrogen, Clone 6C9, Cat no. MA1-70053) and Goat anti-mouse secondary antibodies conjugated with Alexa Fluor 633 (Invitrogen, Cat no. A-21052) and imaged by confocal microscopy. C3b deposition on planktonic cells was determined by incubating cells in 10% NHS for 30 min prior to blocking and antibody staining. Antibody binding was quantified using a BD LSRFortessa (Becton, Dickinson and Company, Franklin Lakes, NJ), and data were analyzed using FlowJo v10.8.1.

RNA Extraction and Sequencing. LAC and LACΔ*saePQRS* bacteria were grown in 4-well culture dishes in 10% serum or HIS diluted in Hank's balanced salt solution containing Mg^{2+}, Ca^{2+}, for 4 h postattachment at 37 °C and 5% CO_2. Cells were detached by repeated pipetting and the cell suspension was mixed with RNAprotect bacteria reagent (Qiagen, Hilden, Germany) at 1:2 ratio by volume. Subsequently, samples were centrifuged for 15 min at 4,000 × g and pellets were frozen at −80 °C until RNA isolation. Triplicate samples were prepared on separate days. A detailed description of the RNA extraction, sequencing, and read-mapping can be found in *SI Appendix*.

Statistics. Statistical analysis was performed in GraphPad Prism version 10.0.3 or later. Normality of each dataset was assessed by the D'Agostino–Pearson or Shapiro–Wilk test. The appropriate statistical test was applied based on the assessed normality and is described in each figure legend.

Data, Materials, and Software Availability. All study data are included in the article and *SI Appendix*.

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