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Temporal and Stochastic Control of *Staphylococcus aureus* Biofilm Development

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ABSTRACT **Biofilm communities contain distinct microniches that result in metabolic heterogeneity and variability in gene expression. Previously, these niches were visualized within *Staphylococcus aureus* biofilms by observing differential expression of the *cid* and *lrg* operons during tower formation. In the present study, we examined early biofilm development and identified two new stages (designated "multiplication" and "exodus") that were associated with changes in matrix composition and a distinct reorganization of the cells as the biofilm matured. The initial attachment and multiplication stages were shown to be protease sensitive but independent of most cell surface-associated proteins. Interestingly, after 6 h of growth, an exodus of the biofilm population that followed the transition of the biofilm to DNase I sensitivity was demonstrated. Furthermore, disruption of the gene encoding staphylococcal nuclease (*nuc*) abrogated this exodus event, causing hyperproliferation of the biofilm and disrupting normal tower development. Immediately prior to the exodus event, *S. aureus* cells carrying a *nuc*::*gfp* promoter fusion demonstrated Sae-dependent expression but only in an apparently random subpopulation of cells. In contrast to the existing model for tower development in *S. aureus*, the results of this study suggest the presence of a Sae-controlled nuclease-mediated exodus of biofilm cells that is required for the development of tower structures. Furthermore, these studies indicate that the differential expression of *nuc* during biofilm development is subject to stochastic regulatory mechanisms that are independent of the formation of metabolic microniches.**

IMPORTANCE **In this study, we provide a novel view of four early stages of biofilm formation by the human pathogen *Staphylococcus aureus*. We identified an initial nucleoprotein matrix during biofilm development that is DNase I insensitive until a critical point when a nuclease-mediated exodus of the population is induced prior to tower formation. Unlike the previously described dispersal of cells that occurs after tower development, we found that the mechanism controlling this exodus event is dependent on the Sae regulatory system and independent of Agr. In addition, we revealed that the gene encoding the secreted staphylococcal nuclease was expressed in only a subpopulation of cells, consistent with a model in which biofilms exhibit multicellular characteristics, including the presence of specialized cells and a division of labor that imparts functional consequences to the remainder of the population.**

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Biofilms are multicellular communities of bacteria aggregated by a self-produced extracellular matrix (ECM) comprising proteins, carbohydrates, and extracellular DNA (eDNA) (1). In pathogenic bacteria, such as *Staphylococcus aureus*, the formation of biofilms within host tissues and on implanted medical devices leads to chronic infections due to their recalcitrance to antimicrobial therapies and host immune responses (2). Indeed, *S. aureus* is a leading cause of a variety of diseases ranging from skin and soft tissue infections to more serious illnesses, including endocarditis, necrotizing pneumonia, and osteomyelitis (3–5), and its ability to form biofilms is an important determinant of virulence in many of these infections (6).

S. aureus biofilm development has previously been described to occur in three successive steps: (i) attachment, (ii) accumulation/maturation, and (iii) detachment/dispersal (1). The initial attachment step has been shown to involve different surface factors, including teichoic acids (7), potentially through surface charge interactions and several different surface-associated proteins that allow the cells to adhere to either polymeric surfaces or host matrix components (8, 9). As the biofilm matures, synthesis of the ECM components allows the cells to mature into three-dimensional structures (10, 11). Production of the self-produced proteases (12), phenol-soluble modulins (PSMs) (13), and nucleases (11, 14) mediates ECM disruption and the switch from the biofilm lifestyle to planktonic growth. Indeed, adding exogenous enzymes or peptides targeting various ECM components, including DNase I (eDNA), proteinase K (proteins), synthetic PSMs (proteins), and dispersin B (polysaccharide intercellular adhesion [PIA]) have been shown to cause biofilm disassembly (11, 13, 15, 16).

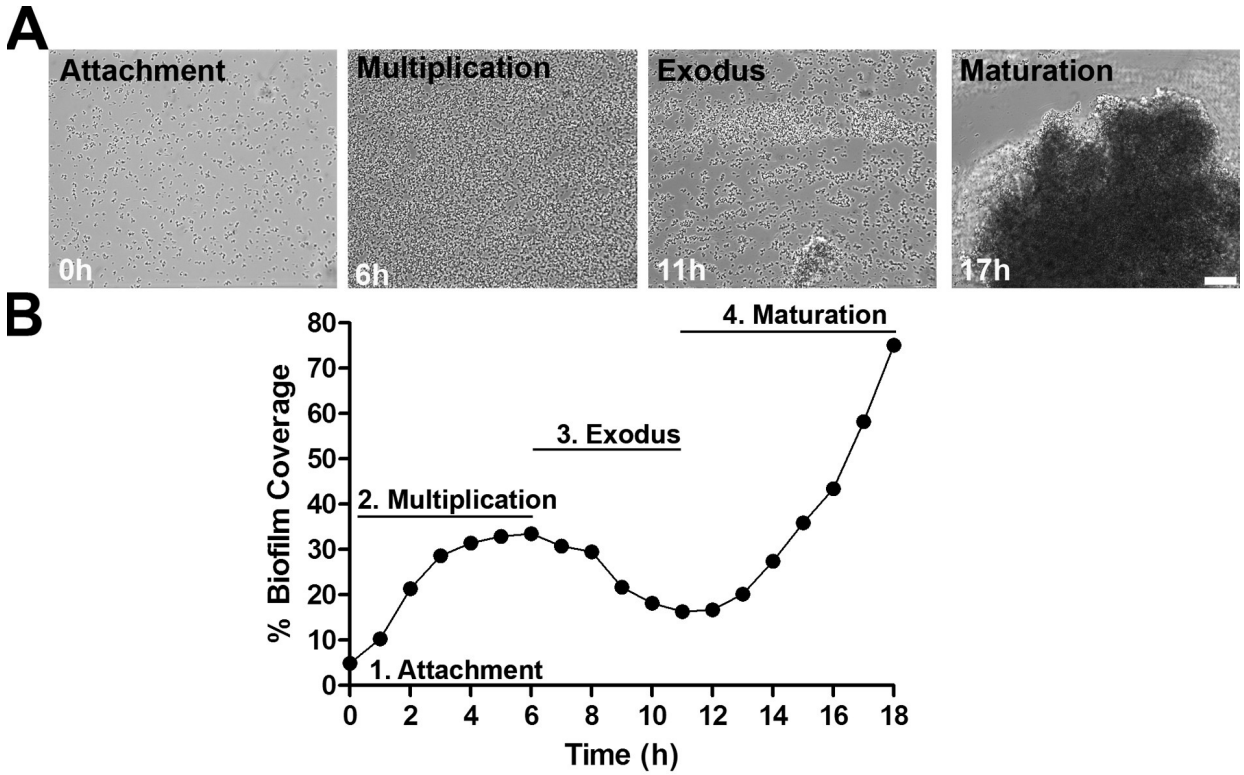

FIG 1 Early stages of *Staphylococcus aureus* biofilm development. *S. aureus* UAMS-1 (wild-type) biofilms were grown in a BioFlux microfluidics system, and bright-field images were captured throughout an 18-h time course experiment. (A) Representative images at the indicated time points of a typical UAMS-1 *S. aureus* biofilm depicting four stages of development: attachment (stage 1), multiplication (stage 2), exodus (stage 3), and biofilm maturation (stage 4). Attachment of cells to the glass bottom plate is quickly followed by the multiplication of the cell population into a confluent "lawn." An exodus event after multiplication is followed by robust tower formation. Scale bar, 50 μm. (B) Quantification of typical *S. aureus* biofilm development presented as a percentage of biofilm coverage plotted versus time. Labels indicate the duration during which each biofilm stage is occurring. See Movie S1 in the supplemental material for a video depiction of the developmental stages of *S. aureus* biofilm formation.

Recently, our laboratory utilized BioFlux technology to monitor the differential control of gene expression during *S. aureus* biofilm development. While most microtiter plate and flow cell systems involve restricted acquisition of microscopic images during the development of a limited number of biofilms, this microfluidics system allows for real-time serial image acquisition of up to 24 simultaneous biofilms under biologically relevant flow conditions, thus, providing much greater resolution of the different stages of biofilm development that may otherwise be overlooked in typical biofilm assays (10, 17). Using this system, the existence of tower structures exhibiting distinct patterns of gene expression and, presumably, distinct physiological characteristics was revealed (10). Specifically, expression of the cell death-associated *cid* and *lrg* operons was primarily contained within two different tower structures: (i) large towers displaying constitutive *lrg* expression and hypoxia-induced *cid* expression within the interior and (ii) small towers exhibiting constitutive *cid* expression. While the large towers demonstrated prominent staining with propidium iodide (PI), indicative of eDNA and/or dead cells, the smaller towers exhibited undetectable PI staining, illustrating the fundamental differences between these tower types.

In the present study, we examined the early stages of biofilm development and assessed the matrix composition as the biofilm matured. Unexpectedly, we identified a distinct transition in matrix composition immediately prior to a previously unrecognized exodus of a subpopulation of the biofilm, which was initiated prior to the development of tower structures. In addition, we demonstrated that exodus was dependent on the coordinated, stochastic expression of the gene encoding the secreted staphylococcal nuclease. Together, these findings suggest the existence of a complex regulatory strategy that controls matrix composition during the early stages of biofilm development and provide novel insight into a nuclease-mediated mechanism involved in the exodus of biofilm cells and subsequent tower formation.

RESULTS

Defining the early stages of *S. aureus* biofilm development. In our previous investigation of *S. aureus* UAMS-1 biofilm development using a BioFlux microfluidics system, the formation of tower structures exhibiting differential gene expression in response to different physiologic signals was demonstrated (10). Closer inspection of the events preceding tower formation (Fig. 1A; see Movie S1 in the supplemental material) revealed that primary attachment of the *S. aureus* cells is followed by rapid multiplication into a confluent "lawn." At about the 6-h time point, an apparent exodus in a subpopulation of the biofilm was observed, followed by distinct foci of robust biofilm growth, resulting in tower formation.

To further study these early events in biofilm development, we established a method to quantify the amount of biofilm coverage

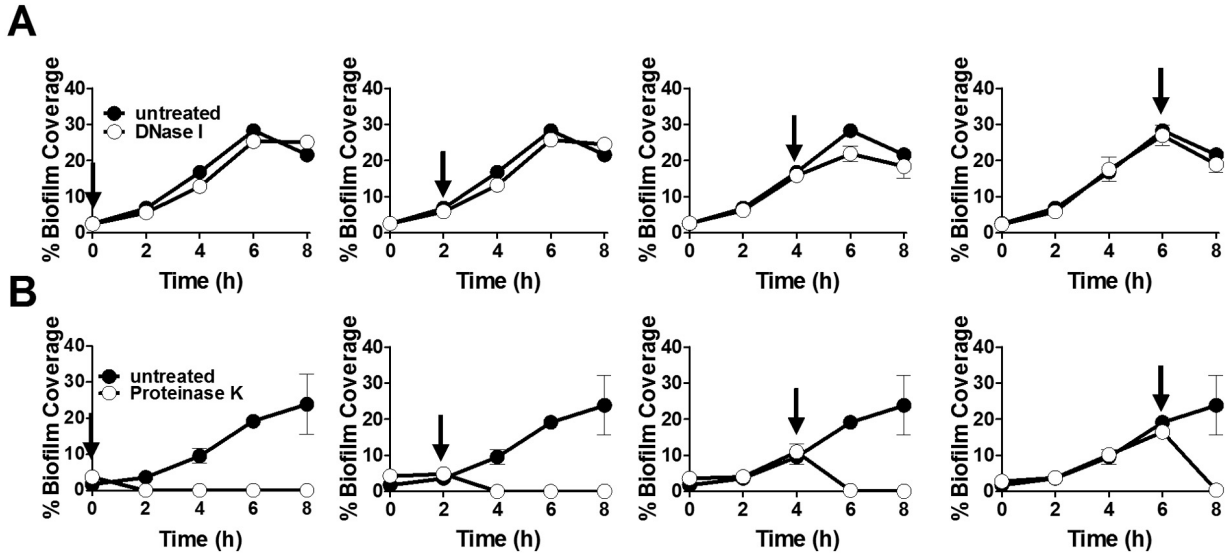

FIG 2 Effects of exogenous proteinase K and DNase I on biofilm attachment and multiplication. *S. aureus* wild-type (UAMS-1) biofilms were grown in the BioFlux system with (open circles) or without (closed circles) exogenously added (A) DNase I (0.5 U ml^{-1}) or (B) proteinase K (100 μg ml^{-1}) at 0, 2, 4, and 6 h after the initiation of the experiment. Arrows in graphs indicate time points at which either proteinase K or DNase I was added to developing biofilm. Each graph shows the mean percentage of biofilm coverage in 2-h intervals. The data represent the means from two independent experiments, each containing at least two technical replicates. Error bars show the standard errors of the means (SEM) from the two independent experiments.

occurring over time (Fig. 1B). To accomplish this, we set a threshold for dark objects (biofilm cells) within each bright-field image and measured the percentage of the area that these objects covered. The data were then plotted as percentage of biofilm coverage versus time. As seen in Fig. 1B, after the initial attachment of cells (0 h), there was a gradual increase in the biofilm coverage observed until about 6 h, at which time the population began to contract until about 11 h. It was during this exodus stage (at approximately 8 h) that we observed the initial signs of tower development, which proceeded until the termination of the experiment at 18 h. To delineate between previously used terminologies of biofilm formation, the terms "multiplication" and "exodus" were chosen to describe these previously uncharacterized stages of biofilm development (Fig. 1).

Protein-dependent attachment and multiplication. To characterize the early stages of biofilm development, the contributions of different ECM components to *S. aureus* biofilm attachment and multiplication were examined using the BioFlux system. Unlike other *S. aureus* strains that produce biofilms with a PIA-based matrix (18, 19), biofilms produced by both *S. aureus* UAMS-1 and USA300 LAC strains have been reported to be PIA independent (20, 21). In agreement with this, we observed no difference in early biofilm formation with UAMS-1 or JE2 (a USA300 LAC derivative) mutants in which the genes encoding the PIA biosynthesis machinery have been disrupted (data not shown).

In previous studies, we reported that eDNA is an important matrix component in *S. aureus* biofilm development and that modulation of the eDNA matrix has a dramatic effect on biofilm maturation (11, 14, 22, 23), including during the initial stages of development in a static assay (22). To gain a better understanding of the contribution of eDNA during early biofilm development under the flow cell conditions used in the present study, we added exogenous DNase I (0.5 U ml^{-1}) at various time points (2-h intervals) during the biofilm attachment and multiplication phases. Similar to a recent study demonstrating DNase I insensitivity during early biofilm development (24), the addition of DNase I had no effect on the biofilm through 8 h of growth (Fig. 2A), suggesting that the initial attachment and multiplication stages lack eDNA under these conditions or that the eDNA present in the matrix during this time is insensitive to DNase I treatment.

S. aureus produces a number of surface-associated and secreted proteins important for adherence. Considering the findings that the attachment and multiplication stages are DNase I insensitive (Fig. 2A), we hypothesized that proteins may play a critical role in these early biofilm formation events. In agreement with this, the staphopain proteases have recently been shown to modulate *S. aureus* biofilm integrity (12). To test the role of proteins, we performed a similar experiment to that described above, except with proteinase K (100 μg ml^{-1}) added at 2-h intervals. As shown in Fig. 2B, the addition of proteinase K detached the entire biofilm at every time point tested. Identical results were observed when the same experiment was performed using strain JE2, demonstrating that this protease sensitivity is consistent among different *S. aureus* lineages (data not shown). Taken together with the DNase I data, these results indicate that the attachment and multiplication stages are dependent on protein components produced by the bacteria.

In an attempt to identify specific proteins important in the attachment and multiplication stages, we utilized the BioFlux system to screen the Nebraska Transposon Mutant Library (NTML) (25) for mutants that are defective in the production of MSCRAMM proteins, including fibronectin-binding proteins A and B (FnbA and FnbB) (26), Empbp (27), clumping factors A and B (ClfA and ClfB) (28, 29), protein A (30), elastin-binding protein (EbpS) (31), Sas family proteins (32, 33), and serine-aspartate repeat (Sdr) family proteins (34, 35). In addition, we examined an *srtA* mutant that is defective in the processing of several LPXTG motif-containing MSCRAMM proteins into the

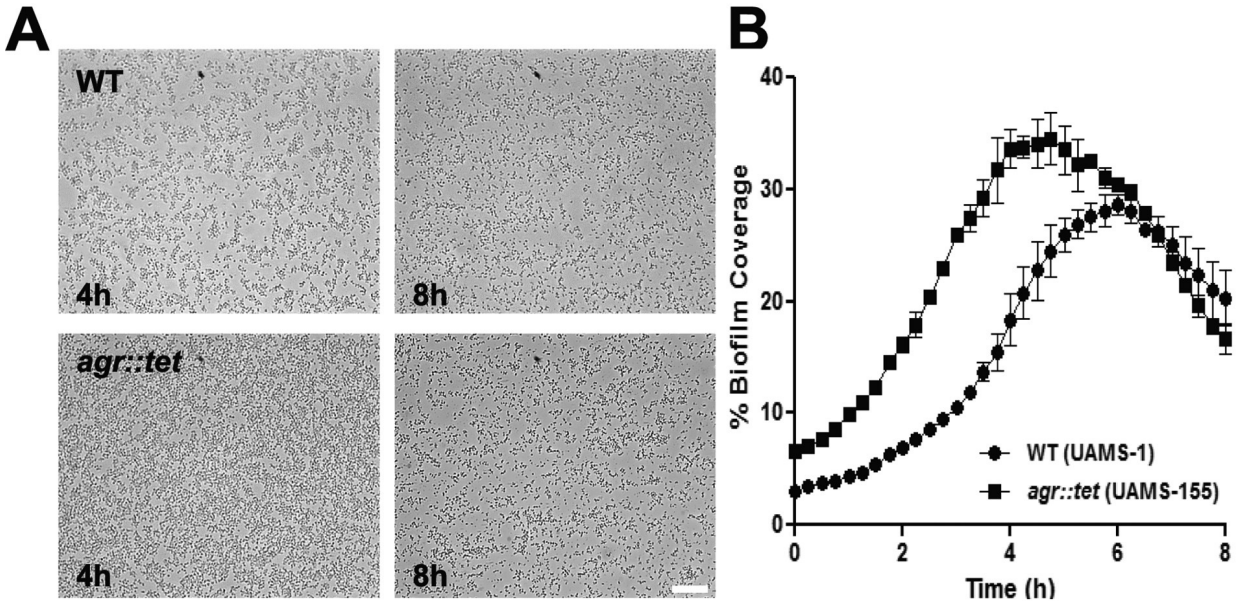

FIG 3 Effect of *agr* quorum sensing on early biofilm development. The *S. aureus agr* mutant strain, UAMS-155 (*agr*::*tet*), was inoculated in parallel with UAMS-1 (wild type) in the BioFlux system and allowed to form a biofilm for 18 h (A) Images selected at 4 h and 8 h are representative of wild-type (WT) and *agr*::*tet* biofilms from multiple experiments. Scale bar, 50 μm. (B) The graph depicts the percentage of biofilm coverage in 15-min intervals of wild-type (WT) and *agr*::*tet* mutant biofilms over 8 h of growth. The data represent the means from two independent experiments, each containing three technical replicates. Error bars show the SEM from the two independent experiments.

cell wall (36). However, none of these mutants exhibited observable changes in biofilm development when grown in the BioFlux system (see Table S1 in the supplemental material). We also selected mutants defective in the production of secreted proteins, such as the as α- and β-hemolysins, which have been shown previously to play a role in biofilm development (37, 38) (see Table S1). Again, no noticeable differences in biofilm formation were observed. Finally, we also tested an *atlA* mutant, in which the primary autolysin is disrupted, for early biofilm defects. Consistent with previous findings (39), we saw limited cell attachment and no biofilm multiplication (see Table S1) using this strain, suggesting its role in these early stages of biofilm formation. Overall, these results support a role for Atl in biofilm attachment and/or multiplication but fail to identify a role for other cell surface and secreted proteins in these processes, although the requirement for a combination of proteins cannot be ruled out.

Exodus is mediated by staphylococcal nuclease. Quorum sensing is the coordinated expression of genes in response to cell density. In *S. aureus*, it is accomplished through the Agr system, which contributes to biofilm dispersal after tower development through activation of proteases and PSMs (13, 16). Thus, we hypothesized that this exodus stage is also controlled by the Agr quorum-sensing system. To test this, we obtained an *agr*::*tet* mutant (UAMS-155) derivative of UAMS-1 and observed its ability to form a biofilm as described above. Interestingly, although the *agr*::*tet* mutant exhibited increased initial attachment and biofilm multiplication, exodus of a subpopulation was still clearly evident (Fig. 3), indicating that this event is largely independent of the Agr system. These results were not specific to this strain as an *agrA*::ΦNΣ transposon mutant of JE2 displayed a similar pattern of exodus during this time period (see Fig. S1 in the supplemental material). Since previous studies have demonstrated that the Agr P3 promoter is activated in a subpopulation of cells in *S. aureus* biofilms (13, 40), we also tested a green fluorescent protein (GFP) reporter that was driven by the Agr-dependent P3 promoter. Consistent with the lack of involvement of *agr* in this exodus event, no P3 promoter activity was detected in the UAMS-1 strain until 13 h of biofilm growth, where expression was primarily limited to the towers (data not shown) as previously observed (40). Collectively, these findings indicate that the exodus of the biofilm population observed in these assays is independent of the Agr quorum-sensing system and that this event is distinct from the previously described Agr-dependent dispersal of cells that occurs after tower formation.

S. aureus synthesizes a myriad of extracellular proteins, the stability and processing of which are modulated by 10 secreted proteases (41). Our observation that proteinase K disrupted biofilms suggests that *S. aureus*-secreted proteases may play a role in the biofilm exodus event. Indeed, recent studies have demonstrated that the staphopain proteases can modulate biofilm integrity (12). However, a USA300 LAC derivative deficient in all 10 secreted proteases (AH1919) (42) showed no defects in exodus of the biofilm subpopulation compared to its parental strain (AH1263) (see Fig. S2 in the supplemental material), suggesting that this event is independent of these proteases. In this regard, several reports have demonstrated that deletion of the secreted nuclease (Nuc) in *S. aureus* causes an increase in biofilm formation as a result of decreased eDNA degradation in the biofilm ECM (11, 14, 43). Based on this, we hypothesized that exodus of the biofilm population is mediated by the function of staphylococcal nuclease in the degradation of eDNA. To test this, we cultured biofilms produced by the wild-type (UAMS-1) and Δ*nuc* mutant (UAMS-1471) strains harboring a "leaky" anhydrotetracycline (aTet)-inducible expression vector (pRMC2-*nuc*) driving low-level *nuc* transcription and then quantified the coverage of the developing biofilm as described above. In support of a role for

nuclease in the exodus event, the Δ*nuc* mutant containing the empty vector (pRMC2) failed to initiate exodus, which resulted in considerably thicker biofilms compared to those of the wild type harboring either pRMC2 or pRMC2-*nuc* (Fig. 4A; see Movie S2 in the supplemental material). Quantification of the biofilm over the first 8 h showed considerably more biofilm present past 6 h of growth in the Δ*nuc* mutant containing pRMC2 and a reversion to wild-type levels of exodus when grown with pRMC2-*nuc* (Fig. 4B). To determine if this phenomenon was conserved in another *S. aureus* strain, we also grew wild-type JE2 and its *nuc* mutant derivative (*nuc*::ΦNΣ) biofilms in parallel. Like the UAMS-1 strain, the *nuc*::ΦNΣ mutant demonstrated biofilm growth that lacked the exodus event, accumulating to a higher cell density over time compared to the wild-type JE2 strain (see Fig. S1 in the supplemental material). Together, these data demonstrate that the *S. aureus*-secreted nuclease plays a major role in the exodus of the biofilm population prior to tower formation and suggest a change in the matrix to nuclease sensitivity immediately prior to the exodus event.

To determine if the exodus defect in the Δ*nuc* mutant could be restored by the addition of exogenous nuclease, we grew wild-type, Δ*nuc* mutant, and *nuc* complement biofilms with or without medium supplemented with DNase I (0.5 U ml^{-1}) starting at the 0-h time point. As shown in Fig. 5, the addition of DNase I had little effect on biofilm multiplication or exodus in the wild-type strain. In contrast, the presence of DNase I caused exodus of the Δ*nuc* mutant biofilm, but not until approximately 6 h of biofilm development, when the exodus event is normally observed (Fig. 5; see Movie S3 in the supplemental material). Similar results were observed using commercially available staphylococcal nuclease (0.5 U ml^{-1}) with wild-type and Δ*nuc* mutant biofilms (data not shown). To assess whether nuclease insensitivity prior to 6 h was a result of the absence of eDNA in the matrix during this time, we isolated eDNA at 4 h from wild-type (UAMS-1) and Δ*nuc* mutant (UAMS-1471) biofilms grown in the absence or presence of active DNase I (0.5 U ml^{-1}). Although eDNA is clearly detectable during the multiplication stage, both wild-type and Δ*nuc* mutant biofilms cultured with and without DNase I showed no significant differences in eDNA levels (data not shown), suggesting that it is protected from the activity of this nuclease.

Biofilm exodus is preceded by nuclease expression. The data generated so far demonstrate that biofilm exodus is reproducibly initiated in a nuclease-dependent manner at approximately 6 h after the initiation of biofilm development. Based on these results, we hypothesized that *nuc* expression would precede biofilm exodus at approximately 6 h. To test this, the UAMS-1 and JE2 strains containing a previously constructed *nuc*::*gfp* promoter fusion plasmid (pCM20) were studied to determine the temporal expression of *nuc* during biofilm development. As anticipated, *nuc* expression was induced just prior to the exodus event at 6 h, albeit in only a subpopulation (<1%) of the cells (Fig. 6A; see Fig. S3 and Movie S4 in the supplemental material). To quantify the induction of *nuc* expression, we set a threshold that would enumerate all of the light objects (fluorescent cells) in each image and plotted this as the percentage of fluorescence coverage over time. As seen in Fig. 6B and Movie S4, *nuc* expression was initially observed at 3 h and maximally expressed near 5 h of biofilm growth, preceding biofilm exodus. These results indicate that both temporal and stochastic regulatory mechanisms control *nuc* promoter activity during biofilm development.

Previous data have shown that the Sae two-component regulatory system regulates nuclease expression (44). Hence, to gain insight into the regulation of the exodus event, we tested wild-type AH1263 (USA300 LAC derivative) and its *saeQRS*::*spc* mutant (AH1558) for biofilm development and *nuc* expression. Consistent with its role as a positive regulator of secreted nuclease, the *saeQRS*::*spc* mutant biofilm developed in a way that was similar to that of a *nuc* mutant lacking biofilm exodus and accumulating to a high cell density (Fig. 7; see Fig. S4 and Movie S5 in the supplemental material). However, the *saeQRS*::*spc* mutant also exhibited an apparent decrease in the rate of biofilm multiplication, suggesting a role for this regulator in the production of some factor(s) important in this process. Additionally, the *saeQRS*::*spc* mutant demonstrated much reduced *nuc* expression compared to wild-type AH1263 (Fig. 7; see Fig. S4 and Movie S5), indicating that the temporal and/or stochastic control of *nuc* expression requires the Sae regulatory system.

DISCUSSION

Our current understanding of *S. aureus* biofilm development is based on the characterization of three basic steps: (i) attachment, (ii) maturation, and (iii) dispersal (1). The complexity of these processes was first highlighted in a study by Yarwood et al. (40), who used time-lapse video microscopy to visualize waves of growth and detachment that appeared to coincide with *agr* expression. More recent studies have revealed that detachment is largely dependent on expression of surfactant-like molecules known as "phenol-soluble modulins" (PSMs) (13). In the present study, we applied BioFlux microfluidics technology to provide enhanced resolution of the events occurring during the early stages of *S. aureus* biofilm development. In doing so, we have identified two additional developmental stages referred to as "multiplication" and "exodus" (Fig. 1; see Movie S1 in the supplemental material), which are distinct from the *agr*-mediated dispersal events that occur after tower formation. In addition, these studies provide greater insight into tower development associated with biofilm maturation, as well as the complex regulatory strategies that precede this process.

Attachment. The observation that the initial attachment of cells to the substrate could be inhibited by the addition of proteinase K indicates that this process is mediated by protein components associated with the cells (Fig. 2B). Given that the Agr system is known to regulate expression of secreted and cell wall-associated proteins in *S. aureus* (45), we tested this strain for its ability to attach in our biofilm assay. As shown in Fig. 3B, we observed an increase in cell attachment in an *agr* mutant derivative of the UAMS-1 strain. This observation is consistent with a previous report showing that *agr*-defective strains exhibit increased adherence to polystyrene in static biofilm assays (46). Within this study, it was demonstrated that increased attachment of the *agr* mutant strains was the result of decreased production of the PSM, delta-toxin, which these authors speculated may act as a strong surfactant preventing hydrophobic interactions between the cell surface and the polystyrene substrate.

In fact, *S. aureus* produces numerous surface proteins, including the Sas family of proteins, fibronectin-binding proteins, clumping factors, elastin-binding proteins, protein A, and AtlA, which have all been shown to be important for attachment and biofilm maturation (8, 47). To determine the potential role of these proteins in our system, we identified NTML mutant strains,

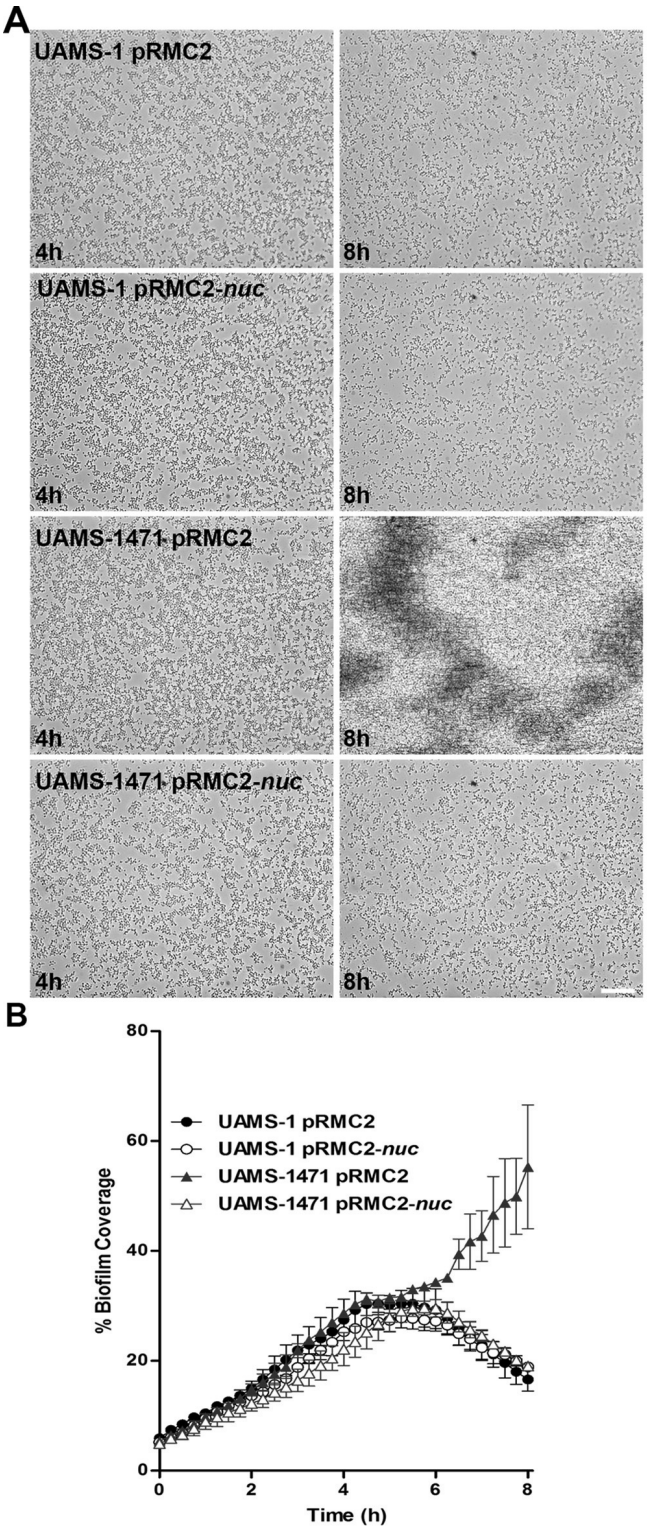

FIG 4 Exodus requires staphylococcal nuclease. Biofilms of the *S. aureus* wild-type (UAMS-1) and Δ*nuc* mutant (UAMS-1471) containing pRMC2 or pRMC2-*nuc* were grown in the BioFlux system. (A) Selected bright-field images at 4 h and 8 h are representative of bioflims of the wild-type (UAMS-1) or Δ*nuc* mutant (UAMS-1471) containing pRMC2 or pRMC2-*nuc* from multiple experiments. Scale bar, 50 μm. (B) The graph shows the mean percentage of biofilm coverage in 15-min intervals of biofilms of the wild-type (UAMS-1) and Δ*nuc* mutant (UAMS-1471) containing pRMC2 or pRMC2-*nuc* over 8 h of growth. The data represent the means from two independent experiments, *(Continued)*

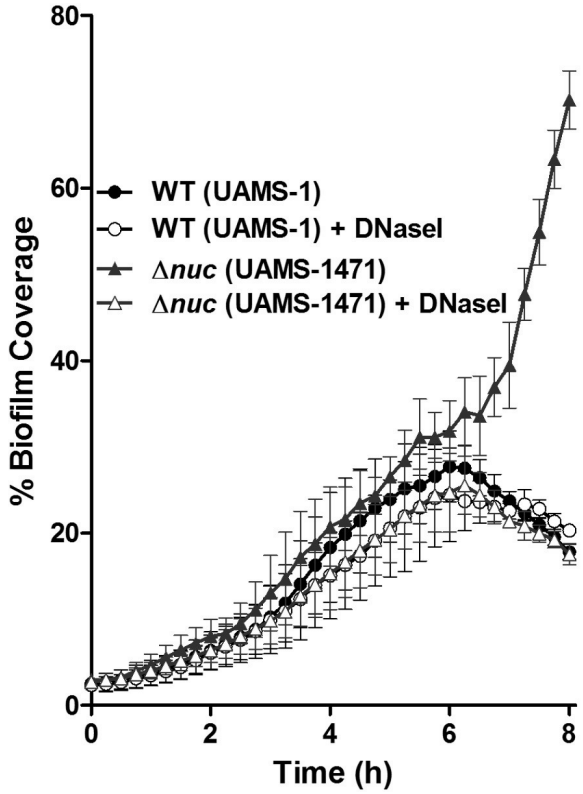

FIG 5 Functional complementation of the *nuc* mutant biofilm phenotype by addition of DNase I. *S. aureus* wild-type (UAMS-1) and Δ*nuc* mutant (UAMS-1471) cells were grown in the BioFlux with or without DNase I (0.5 U ml^{-1}). The graph shows the mean percentage of biofilm coverage in 15-min intervals of wild-type (WT) and Δ*nuc* biofilms grown in the presence or absence of DNase I. The data represent the means from two independent experiments, each containing three technical replicates. Error bars show the SEM from the two independent experiments. For a video compilation of the Δ*nuc* mutant biofilm grown in the presence of DNase I, see Movie S3 in the supplemental material.

as well as others, that contain defects in the synthesis of cell surface-associated molecules (i.e., LPXTG motif proteins, intercellular adhesion, and capsule proteins) and screened them for initial attachment (see Table S1 in the supplemental material). This analysis revealed that only the *atlA*::ΦNΣ mutant exhibited a defect in the initial attachment of cells (see Table S1). These data are in agreement with a recent study that demonstrates that the *S. aureus* biofilm matrix relies less on cell surface-associated proteins, including protein A and the fibronectin-binding proteins, and more so on cytoplasmic proteins released during the stationary phase of growth (48). In addition, while AtlA has been reported to serve as an adhesin (47), results have also indicated that the enzymatic activity of this protein is required for biofilm formation, suggesting the involvement of autolysis and the subsequent release of genomic DNA (39). Arguing against this possibility is the observation that the addition of DNase I to the inoculum

Figure Legend Continued

each containing at least three technical replicates. Error bars show the SEM from the two independent experiments. For a video compilation of the Δ*nuc* mutant containing pRMC2 over 10 h of biofilm growth, see Movie S2 in the supplemental material.

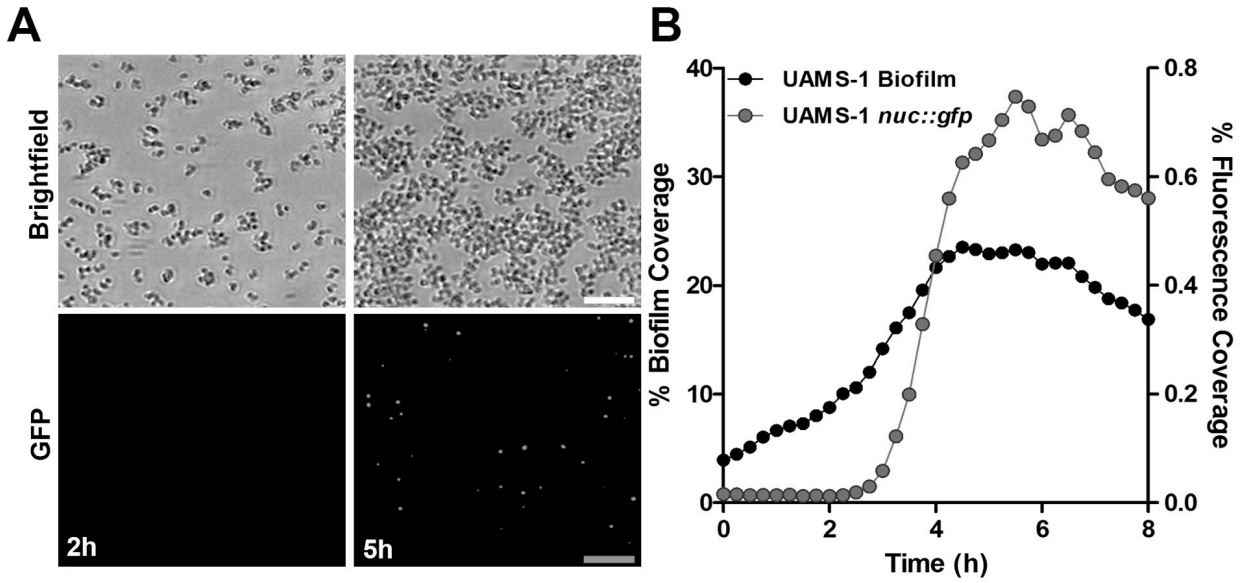

FIG 6 Expression of *nuc* precedes exodus of a biofilm subpopulation. *S. aureus* wild-type (UAMS-1) cells containing the *nuc*::*gfp* reporter plasmid (pCM20) were grown in the BioFlux system. Bright-field and epifluorescence microscopic images were acquired in 5-min intervals at ×200 magnification. (A) Bright-field and epifluorescence (GFP) images at 2 h and 5 h are representative of multiple experiments. Scale bar, 20 μm. (B) The plot depicts biofilm growth as the mean percentage of biofilm coverage and the *nuc*-expressing cells as the mean fluorescence coverage in 15-min intervals over 8 h of growth. The data represent the means from two independent experiments, each containing at least two technical replicates. Error bars were omitted for clarity. For a video compilation observing *nuc* expression, see Movie S4 in the supplemental material.

(data not shown) or at the 0-h time (Fig. 2A) had little effect on the attachment of cells. Complicating the interpretation of these results further is the propensity of the *atlA* mutant to form large clusters of cells, which could have detrimental effects on cell attachment. Furthermore, the fact that many of the mutants tested did not show a defect in biofilm development is not completely unexpected since most of the surface proteins are important for binding host matrix components (i.e., MSCRAMMs), which are absent in our biofilm assays. Overall, the finding that attachment was affected by the *agr* and *atlA* mutations, but none of the other cell surface protein mutations, is consistent with the hypothesis that AtlA and delta-toxin are required for this process (46).

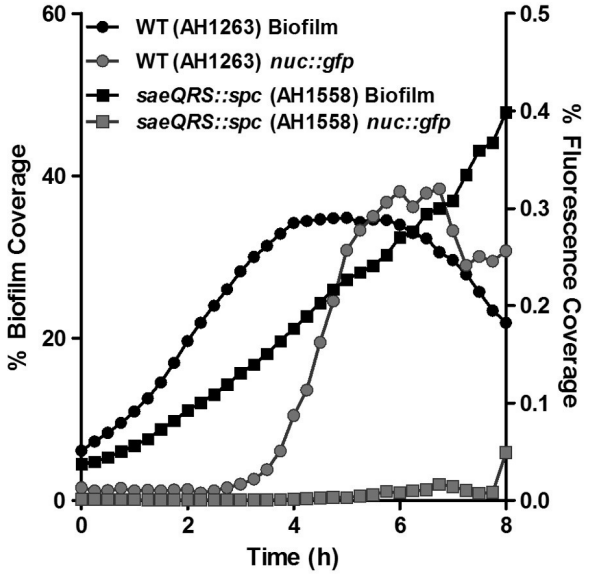

FIG 7 Nuclease-mediated exodus is regulated by Sae. *S. aureus* wild-type (AH1263) and *saeQRS*::*spc* mutant (AH1558) strains carrying the *nuc*::*gfp* reporter plasmid (pCM20) were grown in the BioFlux system. The plot depicts biofilm growth as the mean percentage of biofilm coverage and the *nuc*-expressing cells as the mean fluorescence coverage in 15-min intervals over 8 h of growth. The data represent the means from two independent experiments, each containing at least two technical replicates. Error bars were omitted for clarity. For representative images, see Fig. S4 and for a video compilation of a *saePQRS*::*spc* mutant biofilm see Movie S5 in the supplemental material.

Multiplication. Similar to cell attachment, the multiplication stage was also found to be sensitive to protease treatment (Fig. 2B); however, screening of the NTML for proteins involved in this stage failed to identify a protein important in this process (see Table S1 in the supplemental material). The present studies demonstrated that DNase I had little effect on the biofilm during the multiplication stage (Fig. 2A and 5). This is in agreement with a recent study demonstrating DNase I insensitivity during early *S. aureus* biofilm development (24). Although these results appear to conflict with our previous findings (11, 22), it is important to note that the biofilm growth conditions used here were distinctly different from the static assay conditions used previously. In addition, the BioFlux assay affords greatly increased resolution of the early events in biofilm maturation through real-time microscopic imaging of the cells, thus enabling the visualization of developmental events that were previously undetectable in the static assays. In addition, isolation of eDNA from the biofilms at 4 h treated with or without active DNase I showed no significant changes in eDNA levels (data not shown). Based on these data, we hypothesize that there is a functional shift in the biofilm matrix prior to the exodus event from a protein-based matrix to one that is dependent on both eDNA and protein, most likely the result of eDNA and protein interactions occurring as the biofilm matures.

Indeed, precedence for this includes the demonstration that *S. aureus* beta-toxin, normally known for its role as a hemolysin, can bind eDNA and covalently cross-link to itself, forming an insoluble nucleoprotein matrix within a biofilm (37). However, UAMS-1 does not produce beta-toxin due to an insertion of the bacteriophage in the *hlb* gene (37). Like beta-toxin, the immunodominant surface antigen B (IsaB) has also been shown to bind DNA, yet an *isaB* mutant previously exhibited no defect in biofilm formation (49).

In contrast to these extracellular DNA-binding proteins, recent reports have demonstrated that cytoplasmic nucleoid-associated proteins (NAPs), normally known for intracellular chromosomal structuring, have emerged as possible biofilm scaffolds in different bacteria. Specifically, biofilm produced by *Burkholderia cenocepacia*, nontypeable *Haemophilus influenzae*, and *Escherichia coli* have all shown a requirement for integration host factor (IHF) and/or histone-like protein (HU). In fact, treatment of established biofilms of these species, as well as *S. aureus* and *Staphylococcus epidermidis*, with antisera specific for these proteins resulted in a considerable decrease in the total amount of biofilm generated (50, 51). The precedence for NAPs in bacterial biofilms and the plethora of NAPs identified in *S. aureus* (52) suggest that these proteins may be important contributors to biofilm development in *S. aureus*. Studies to identify specific eDNA-binding proteins important in biofilm integrity are currently in progress in our laboratory.

Exodus. The observation that Agr P3 promoter activity was not observed until well after tower development, in combination with the absence of an effect of an *agr* mutation on the exodus event (Fig. 3), suggests that the Agr quorum-sensing circuit and the PSMs are not required for this event. Instead, given the role of eDNA in biofilm development, we hypothesized that staphylococcal nuclease may be required. Consistent with this hypothesis was the observation that the biofilm became DNase I sensitive after 6 h of development (Fig. 5), and the Δ*nuc* mutant failed to initiate exodus at this time point (Fig. 4). Given the precise timing of the exodus event, we also examined *nuc* expression and, remarkably observed expression limited to subpopulation of cells preceding the exodus event (Fig. 6; see also Fig. S3 and Movie S4 in the supplemental material), suggesting that these specialized cells have an impact on the remainder of the biofilm population, much like the specialization that is seen in *Bacillus subtilis* biofilm formation (53). In addition, these data also suggest a model in which early Nuc-mediated exodus allows for tower formation and eventual late Agr-mediated dispersal for further dissemination of *S. aureus* cells.

The observation that *nuc* exhibited temporal and stochastic patterns of expression during biofilm development indicates it is subject to complex regulatory control. While previous reports have suggested that nuclease is regulated by the Agr quorum-sensing circuit (54–56), new evidence demonstrates *nuc* expression is more directly controlled by the Sae regulatory system (57–59). Indeed, recent promoter mapping, Nuc activity measurements, and immunoblot studies have confirmed the Sae-dependent regulation of nuclease expression (44). In support of these findings, an *agr* mutant exhibited both temporal and stochastic regulatory control of *nuc* expression during biofilm development similar to that of the wild-type strain (data not shown). Additionally, an *saeQRS*::*spc* mutant failed to initiate exodus of the biofilm population and exhibited much reduced *nuc* expression compared to its parental strain, AH1263 (Fig. 7; see Fig. S4 and Movie S5 in the supplemental material). The *saePQRS* operon encodes two auxiliary proteins, SaePQ, and a two-component system, SaeRS, that globally regulate multiple *S. aureus*-secreted proteases (60) and virulence factors, such as alpha-toxin, beta-toxin (*hlb*), coagulase (*coa*), fibronectin-binding proteins (*fnbA* and *fnbB*), and extracellular adherence protein (*eap*) (61, 62). Whether or not the *nuc*-expressing cells also specifically express these other virulence factors remains to be tested.

Biofilm maturation. Based on the results of this study, it is apparent that the early exodus event during *S. aureus* biofilm development is essential for the formation of distinct tower structures, which based on our previous studies (10) have variable physiology and metabolism, as seen by the presence or absence of eDNA and dead cells as well as differential gene (*cid* and *lrg*) expression. However, two major questions remain to be answered. First, how do these eDNA-containing towers remain intact if nuclease is active? A recent report testing DNase I efficacy in UAMS-1 *S. aureus* biofilms demonstrated carbohydrates and eDNA staining within tower structures, and the towers appear to be DNase I insensitive (24). The authors suggest that carbohydrates may be interacting with eDNA and protecting it from DNase I degradation; however, further investigation must be conducted to confirm these findings.

Second, what is the function of towers in *S. aureus* biofilm? Some evidence suggests that tower structures are important for the pathogenesis of *S. aureus* biofilms formed on native heart valves during infective endocarditis, where they detach and travel to secondary sites of infection (63–65). In other organisms, the development of specialized structures is important for survival and/or resistance to environmental stresses. For example, the cystic fibrosis pathogen *Pseudomonas aeruginosa* forms robust tower-like structures that have been shown to be important in resisting microbial biocides, such as sodium dodecyl sulfate (SDS) detergent and tobramycin (66, 67), as well as mediating biofilm dispersal (68). Additionally, the predator bacterium *Myxococcus xanthus* demonstrates complex multicellularity and intercellular signaling through coordinated gene expression to form raised aggregates of cells called "fruiting bodies" (69–71). Indeed, much like the reduction of the cell population during the exodus phase that precedes tower formation in *S. aureus*, *M. xanthus* demonstrates a reduction of the cell population preceding fruiting body development (72, 73). Based on these similarities, it is likely that tower structures produced by *S. aureus* are also important in survival during environmental stress.

Finally, the results of our studies suggest an alternative to the model proposed by Otto (1) describing how *S. aureus* biofilms develop their characteristic structure. In this model, it is envisioned that the PSMs act upon a preexisting thick mat of cells, causing cell detachment and leaving behind structures of various forms (e.g., towers and channels). However, the structures present in the biofilms produced in our biofilm system are clearly not generated in this way. Rather, they are formed after the mass exodus of the bulk of the early biofilm and appear to arise as a result of the rapid growth of only a few remaining cells. In the absence of staphylococcal nuclease, these tower structures are not observed, either because they are overwhelmed by the presence of an unusually robust accumulation of biomass or because a key developmental switch fails to trigger. Continued studies are required to provide a greater understanding of these fascinating developmen-

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

Strain or plasmid	Description	Reference
Strains		
RN4220	Highly transformable strain, restriction deficient	74
UAMS-1	Clinical isolate	75
UAMS-1471	UAMS-1 Δ*nuc*	76
UAMS-155	UAMS-1 *agr*::*tet*	77
USA300 LAC JE2	USA300 LAC derivative	25
JE2 *agrA*::ΦNΣ	*bursa aurealis agrA* mutation in JE2	25
JE2 *nuc*::ΦNΣ	*bursa aurealis nuc* mutation in JE2	25
AH1263	USA300 CA-MRSA Erm^s (LAC*)	78
AH1558	AH1263 *saeQRS*::*spc*	44
AH1919	AH1263 Δ*aur* Δ*sspAB* Δ*scpA spl*::*erm* (protease knockout)	42
Plasmids		
pCM20	*nuc* promoter::sGFP	14
pRMC2	Anhydrotetracycline-inducible plasmid	76
pRMC2-*nuc*	Anhydrotetracycline-inducible plasmid containing *nuc*	14

tal processes, as well as the functions of the structures that are formed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Staphylococcus aureus* strains used in this study are described in Table 1. All *S. aureus* strains were grown in tryptic soy broth (TSB) (EMD Biosciences, Gibbstown, NJ) or on TSB containing 1.5% agar. All experiments were started from fresh overnight TSB cultures grown at 37°C with shaking at 250 rpm. When needed, chloramphenicol (5 μg ml^{-1}), erythromycin (5 μg ml^{-1}), tetracycline (5 μg ml^{-1}), and spectinomycin (1,000 μg ml^{-1}) were added to the growth medium.

Movement of plasmids into the UAMS-1 and JE2 *S. aureus* strains. The plasmids pCM20, pRMC2, and pRMC2-*nuc* were purified using the Wizard Plus SV Minipreps DNA purification system (Promega Corporation) from the *S. aureus* strains AH1263 and SH1000 containing pCM20, pRMC2, and pRMC2-*nuc*, respectively. The plasmids were then electroporated into the highly transformable, restriction-deficient *S. aureus* strain RN4220. Transduction of the plasmids into the UAMS-1 and JE2 strains was performed using φ11 phage propagated on the plasmid-containing RN4220 strain.

BioFlux1000 biofilm assays. *S. aureus* biofilm development was assessed using a BioFlux1000 microfluidic system (Fluxion Biosciences, Inc., San Francisco, CA) as described previously (10). Using BioFlux1000 48-well plates, the biofilm growth channels were primed by adding 200 μl of TSB to the output wells and initiating a reverse flow for 5 min at 5.0 dynes/cm^2. To seed the channels with bacteria, excess TSB in the output wells was replaced with 200 μl of fresh overnight-grown *S. aureus* cells diluted to an optical density at 600 nm (OD_{600}) of 0.8 and pumped into the channels at 2.0 dynes/cm^2 for 5 s. Cells were then allowed to attach to the surface of the plate for 1 h at 37°C. The remaining inoculum was aspirated from the output well, and 1.3 ml of 50% TSB was added to the input wells and pumped at 0.6 dyne/cm^2 for 18 h. Bright-field and epifluorescence images were acquired in 5-min intervals for a total of 217 time points. All epifluorescence images observing GFP expression were acquired using a fluorescein isothiocyanate (FITC) filter kept at constant acquisition settings (gain, 20; exposure, 500 ms). To ensure nuclease was actively being made using the aTet plasmid system (pRMC2 and pRMC2-*nuc*) under noninducing conditions, wild-type and Δ*nuc* mutant strains containing pRMC2 or pRMC2-*nuc* supernatants from effluents of 3-h biofilms grown in the BioFlux without aTet were incubated with *S. aureus* genomic DNA overnight and separated in an agarose gel (data not shown).

To determine the effects of proteinase K (Invitrogen, Inc.) or DNase I (Fermentas, Inc.) on biofilm development, channel priming and cell seeding were performed as described above, and 1 ml of 50% TSB or 1 ml of 50% TSB supplemented with either 100 μg ml^{-1} of proteinase K or 0.5 U ml^{-1} of DNase I was pumped at 0.6 dyne/cm^2 for 8 h. Where indicated, flow was stopped intermittently, and 1 ml of 50% TSB was replaced with 1 ml of 50% TSB supplemented with proteinase K or DNase I. After the flow was restarted, bright-field images were acquired in 2-h intervals.

Quantification of acquired BioFlux biofilm images. Using BioFlux Montage software (Fluxion Biosciences, Inc.), representative bright-field and epifluorescence images were selected and adjusted to similar brightness and calibrated to 0.323445 μm/pixel. For bright-field images, a threshold was set using the Threshold tool and Slider tool to include all dark objects (biofilm cells) within each image. The total percentage of area of coverage of the dark objects was designated the percentage of biofilm coverage and plotted over time. For epifluorescence images, a threshold was set similar to that described above to cover all light objects (fluorescent cells) in each image. The total percentage of the area of coverage was designated the percentage of fluorescence coverage and plotted over time. All time points were plotted in either 1-h or 15-min intervals using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA).

Isolation of eDNA from BioFlux biofilms. *S. aureus* wild-type (UAMS-1) and Δ*nuc* mutant (UAMS-1471) biofilms were grown with or without active DNase I (heat inactivated for 10 min at 95°C) for 4 h in the BioFlux system in four identical channels as described above. In the output wells, 200 μl of 50 mM TES buffer (Tris-HCl [pH 8.0], 10 mM EDTA, 500 mM NaCl) containing 10 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} proteinase K were added to inhibit cell growth in the effluent. After 4 h of biofilm growth, excess medium and effluent were removed from the input and output wells, and the output wells were wiped clean with sterile cotton-tipped applicators. To extract biofilms from channels, 400 μl of 50 mM TES buffer containing 100 μg ml^{-1} proteinase K was added to the output wells and then pumped into the input wells for 10 min at 5.0 dynes/cm^2 and 10 min at 20.0 dynes/cm^2. After ensuring the biofilms had been completely removed from the channels, 350 μl of the flowthrough from the four replicate channels was pooled into prechilled tubes and centrifuged for 5 min at 14,000 rpm, and 1 ml of the supernatant was transferred to new tubes. Excess biofilm supernatants were discarded, and pellets were resuspended in 1 ml of water and kept on ice until the OD_{600} was determined. The eDNA from supernatants was extracted once with 1 ml of phenol-chloroform-isoamyl alcohol (25:24:1) and once with 900 μl of chloroform-isoamyl alcohol (24:1). To precipitate the eDNA, 500 μl of the aqueous phase was mixed with 50 μl of 3 M potassium acetate (pH 5.0) and 1.5 ml of ice-cold 100% ethanol and then stored at −20°C overnight. The following day, the precipitated DNA was collected by centrifugation (15,000 × *g*) for 20 min at 4°C, washed with ice-cold 75% (vol/vol) ethanol, air dried at room temperature, and dissolved in 200 μl of Tris-EDTA (TE) buffer. To quantify the amount of eDNA present, quantitative reverse transcription-PCR (qRT-PCR) was performed on each sample with

LightCycler DNA master SYBR green I (Roche) using gyrase primer sets as previously described (22), and the eDNA concentrations (ng μl^{-1}) were normalized to the total OD_{600} in 1 ml of water.

Statistical analysis. Differences in eDNA present within the biofilms produced by different strains and under different experimental conditions were analyzed by performing a one-way analysis of variance (ANOVA) test with a Tukey's posttest using GraphPad Prism (GraphPad Software, La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01341-14/-/DCSupplemental.

Figure S1, PDF file, 0.6 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 0.3 MB.
Movie S1, MP4 file, 0.3 MB.
Movie S2, MP4 file, 0.3 MB.
Movie S3, MP4 file, 0.3 MB.
Movie S4, MP4 file, 0.3 MB.
Movie S5, MP4 file, 0.3 MB.
Table S1, PDF file, 0.1 MB.

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