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Impact of Extracellular Nuclease Production on the Biofilm Phenotype of *Staphylococcus aureus* under *In Vitro* and *In Vivo* Conditions

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Recent studies suggest that extracellular DNA promotes biofilm formation in *Staphylococcus aureus* and, conversely, that extracellular nucleases limit the ability to form a biofilm. *S. aureus* produces at least two extracellular nucleases, and in the study described in this report, we examined the impact of each of these nucleases on biofilm formation under both *in vitro* and *in vivo* conditions. Our results demonstrate that both nucleases impact biofilm formation in the clinical isolate UAMS-1. Under certain *in vitro* conditions, this impact is negative, with mutation of either or both of the nuclease genes (*nuc1* and *nuc2*) resulting in an enhanced capacity to form a biofilm. However, this effect was not apparent *in vivo* in a murine model of catheter-associated biofilm formation. Rather, mutation of either or both nuclease genes appeared to limit biofilm formation to a degree that could be correlated with increased susceptibility to daptomycin.

A defining characteristic of many *Staphylococcus aureus* infections is formation of a biofilm. Because this compromises the efficacy of antimicrobial therapy, it is important to understand the mechanistic basis for biofilm formation. One factor recently shown to be relevant in this regard is extracellular DNA (eDNA). Under *in vitro* conditions, the two possible sources of eDNA are the growth medium and the bacteria themselves. In fact, recent data suggest that it is the latter that play a primary role (13). Current models also suggest that the *S. aureus lytSR* two-component regulatory system and CidR collectively control the release of eDNA by influencing expression of the *cid* and *lrg* operons, with the latter two operons serving opposing roles with respect to each other in modulating the production of murein hydrolases and, consequently, cell lysis (18). Specifically, mutation of *cidA* results in reduced production of murein hydrolases, reduced release of eDNA, and a reduced capacity to form a biofilm, while mutation of the *lrgAB* operon has the opposite effects (13, 19). Additional results supporting this model include the fact that extracellular nuclease, whether applied exogenously or produced by *S. aureus*, limits biofilm formation, at least under certain *in vitro* conditions (3, 13, 22).

The production of extracellular nuclease has also been associated with reduced susceptibility to phagocytosis owing to an enhanced capacity to escape from neutrophil extracellular traps (NETs) (4). Thus, from a pathogenesis point of view, the production of staphylococcal extracellular nuclease potentially plays the opposing roles of promoting escape from NETs but limiting the ability to form a biofilm. These opposing roles may be largely hypothetical owing to the ability of *S. aureus* to regulate nuclease production such that it is produced under conditions when avoiding phagocytosis is the primary concern (e.g., in the bloodstream) but repressed during the process of colonization and biofilm formation. However, these two conditions are not mutually exclusive, as evidenced by the observation that neutrophils have been shown to penetrate *S. aureus* biofilms and ingest biofilm-associated staphylococcal cells (7). Thus, it is possible that these potentially opposing roles are clinically and/or therapeutically relevant. Based on this, we examined the impact of mutating each of two genes (SA0746 or *nuc1* and SA1160 or *nuc2*) encoding extracellular nucleases on biofilm formation under *in vitro* and *in vivo* conditions with a specific emphasis on relative antibiotic susceptibility in the context of an established biofilm.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All experiments were done with the clinical osteomyelitis isolate UAMS-1 and its isogenic *nuc1* (SA0746) and/or *nuc2* (SA1160), *nuc1 nuc2*, and *sarA* mutants (3). The *in vitro* assay employed tryptic soy broth (TSB) supplemented with 3.0% NaCl and 0.5% glucose (1). The capacity of each strain to form a biofilm under *in vitro* conditions was assessed using a model of catheter-associated biofilm formation done with and without precoating of the substrate with 20% human plasma (23). Biofilms were allowed to form for 24 h before harvesting of catheters ($n = 6$) and processing to determine viable count as previously described (23). All *in vitro* experiments were done twice, with the results combined for statistical analysis. The relative daptomycin susceptibility of each strain was assessed by Etest (bioMérieux SA, Marcy l'Etoile, France) using Mueller-Hinton agar as the growth medium.

Nuclease assay. Quantitative assays of nuclease activity were done using a fluorescence resonance energy transfer (FRET)-based assay (11). Briefly, supernatants from overnight cultures (16 h) grown in TSB with and without supplementation as described above were standardized, clarified by centrifugation, and filter sterilized. Aliquots of 25 μl were then mixed with an equal volume of FRET substrate diluted to 2 μM in buffer consisting of 20 mM Tris, pH 8.0, and 10 mM $CaCl_2$. The FRET substrate was the same as previously described (11), except that the 5′ label was a 4,4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX) fluorophore. Results were assessed after a 10-min incubation at 30°C using a BioTek Synergy 2 apparatus (BioTek Instruments, Winooski, VT) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Results are reported as fluorescence units.

Assessment of biofilm formation and relative daptomycin susceptibility *in vivo*. Biofilm formation was assessed *in vivo* using a murine model of catheter-associated biofilm formation (24). Briefly, uncoated

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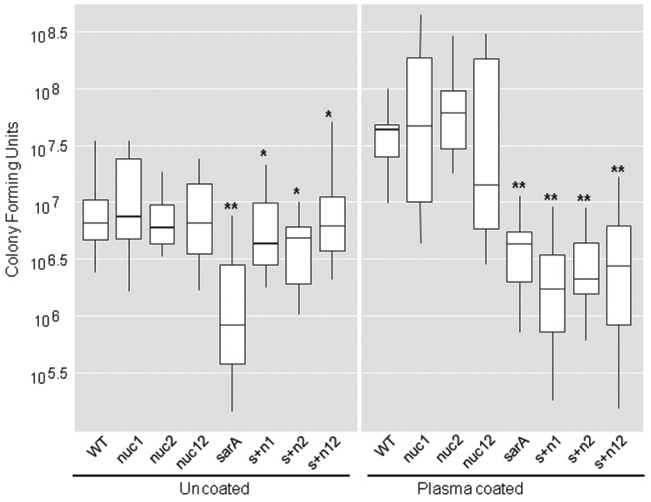

FIG 1 *In vitro* biofilm formation in *sarA* and nuclease mutants with and without plasma coating. Biofilm formation was assessed using an *in vitro* model of catheter-associated biofilm formation with and without first coating the catheters with human plasma (23). Results are shown for UAMS-1 (wild type [WT]), its isogenic derivatives carrying mutations in *nuc1*, *nuc2*, or both (*nuc12*) with and without concomitant mutation of *sarA* (*sarA*). Strain designations: s+n1, *sarA nuc1* double mutant; s+n2, *sarA nuc2* double mutant; s+n12, *sarA nuc1 nuc2* triple mutant. *, statistical significance ($P < 0.05$) by comparison to the results observed with the *sarA* mutant under the same assay condition; **, statistical significance ($P < 0.05$) by comparison to the wild-type strain.

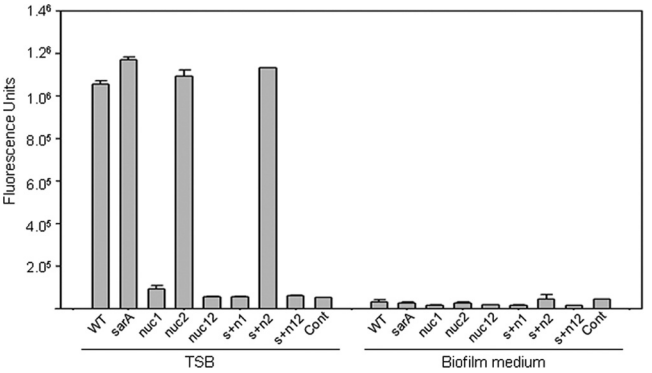

FIG 2 Production of extracellular nuclease. Nuclease production was assessed using a FRET-based assay with supernatants from cultures grown in TSB with (right) and without (left) supplementation of the medium with glucose and salt. Strain designations are the same as described in the Fig. 1 legend.

catheters were implanted into each flank of NIH Swiss mice and inoculated with 10^5 CFU of the test strain in a total volume of 100 μl of phosphate-buffered saline (PBS) by direct injection into the lumen of each catheter. After 24 h, mice were randomly divided into experimental groups ($n = 5$). Because each mouse had two catheters implanted and because previous experiments have confirmed the absence of cross-contamination between catheters in opposite flanks of the same mouse (24), each catheter was treated as an independent data point ($n = 10$). In untreated mice, 100 μl of sterile PBS was injected in the lumen of each catheter at daily intervals. In the treated groups, 100 μl of sterile PBS containing 20× daptomycin (20 μg/ml) was injected into the lumen, also at daily intervals. This concentration corresponds to 20 times (20×) the concentration defined by the Clinical and Laboratory Standards Institute (CLSI) as the breakpoint MIC for *S. aureus* (≤1.0 μg/ml) (23). Treatment was continued for 7 days. All *in vivo* experiments were also done twice, with the results combined for statistical analysis.

Statistical analysis. Bacterial count data from *in vitro* plasma-coated and uncoated experiments were analyzed separately using a three-factor analysis of variance (ANOVA) model. The three factors included in the model were the mutation statuses of *sarA*, *nuc1*, and *nuc2*. For both coated and uncoated analyses, three-way and all two-way interactions were tested. Bacterial count data from *in vivo* harvested catheters were analyzed using a two-factor ANOVA model to assess the impact of *sarA* and *nuc1* mutations for daptomycin-treated and untreated animals separately. Because no viable bacteria were detected on many catheters, *P* values were calculated using permutation tests. All statistical analyses were performed using R (version 2.7; The Foundation for Statistical Computing), with *P* values of ≤0.05 considered significant.

RESULTS

For the *in vitro* experiments, a catheter-associated model of biofilm formation was used with and without precoating of the substrate with 20% human plasma (23). In the clinical osteomyelitis isolate UAMS-1, mutation of *nuc1* and/or *nuc2* was found to have no significant impact on biofilm formation, irrespective of whether catheters were first coated with plasma proteins (Fig. 1). In contrast, mutation of *sarA* was found to limit biofilm formation to a statistically significant degree both with and without plasma coating. When the assay was repeated using *nuc1* and *nuc2* mutants generated in a UAMS-1 *sarA* mutant, which produces extracellular nucleases at elevated levels by comparison to the parent strain (3), mutation of either or both of the nuclease genes was found to enhance biofilm formation to a statistically significant degree, but only when the assay was done without coating with plasma proteins (Fig. 1). The fact that mutation of *nuc2* had an impact on biofilm formation comparable to that observed with mutation of *nuc1* was surprising, in that, as assessed using both DNase agar (3) and a FRET-based assay, mutation of *nuc1* eliminated nuclease production even in a UAMS-1 *sarA* mutant, while mutation of *nuc2* had no discernible effect (Fig. 2).

Importantly, our FRET-based assay also demonstrated that nuclease production is dramatically reduced in our biofilm medium by comparison to TSB (Fig. 2). This suggests that the impact of eliminating nuclease production is likely to be minimized in our *in vitro* catheter assay. This limitation is difficult to overcome *in vitro* because UAMS-1 does not form a robust biofilm unless the medium is supplemented with both NaCl and glucose (1). Both for this reason and, more importantly, to address the discrepancy associated with plasma coating in our *in vitro* assays, we compared UAMS-1 *nuc1*, *sarA*, and *sarA nuc1* mutants *in vivo* using a murine model of catheter-associated biofilm formation (24). Because reduced antibiotic susceptibility is a defining feature of *S. aureus* biofilms (23, 24), we also assessed the relative antibiotic susceptibility of established biofilms using daptomycin as the test antibiotic. Importantly, as assessed *in vitro*, none of the mutants examined in this study exhibited altered susceptibility to daptomycin (Fig. 3). As with our *in vitro* experiments employing plasma-coated catheters, mutation of *nuc1* had little impact on biofilm formation in UAMS-1 (Fig. 4). However, by comparison to UAMS-1, the *nuc1* mutant did exhibit increased susceptibility to daptomycin. We also attempted to complement the *nuc1* mutation in *trans* but were unable to do so because the complementing plasmid was unstable *in vivo* (data not shown). However, we previously confirmed complementation of the *nuc1* mutation under *in vitro* conditions (22), thus confirming that the increased sus-

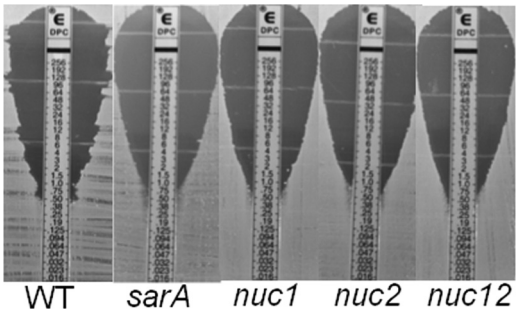

FIG 3 Impact of *sarA* and *nuc* mutations on daptomycin susceptibility *in vitro*. Relative susceptibility to daptomycin was assessed by Etest using Mueller-Hinton agar as the growth medium.

ceptibility of the *nuc1* mutant observed here is unlikely to be due to polar effects unrelated to the production of extracellular nuclease. The increased susceptibility of a UAMS-1 *sarA* mutant was also apparent even by comparison to the isogenic *nuc1* mutant (Fig. 4), which is consistent with our hypothesis that the impact of mutating *sarA* on biofilm formation in *S. aureus* extends beyond its impact on the production of extracellular nucleases (3, 22).

At the same time, our *in vitro* experiments indicated that mutation of *nuc2* also has an impact on the ability of a *sarA* mutant to form a biofilm, at least in the absence of coating with plasma proteins (Fig. 1). Based on this, we also examined the impact of mutating both *nuc1* and *nuc2* (*nuc12*) in UAMS-1 and its *sarA* mutant. In the parent strain, the results were comparable with those observed with the *nuc1* mutant (Fig. 4). However, the UAMS-1 *nuc12* mutant exhibited increased susceptibility to daptomycin to a degree that was comparable to that of the isogenic *sarA* mutant and exceeded that of the *nuc1* mutant (Fig. 5). These

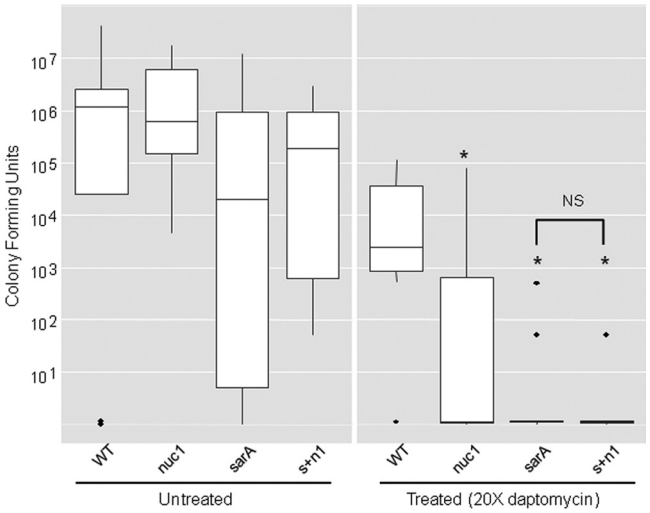

FIG 4 Impact of *sarA* and nuclease production on susceptibility of UAMS-1 to daptomycin *in vivo*. Biofilm formation and relative susceptibility to daptomycin were assessed using an *in vivo* model of catheter-associated biofilm formation (24). Results are shown for UAMS-1 (wild type [WT]) and its isogenic derivatives carrying mutations in *sarA* and/or SA0746 (*nuc1*). Asterisks indicate statistical significance ($P < 0.05$) by comparison to the results observed with the wild-type strain under the same assay condition. The NS above the bracket indicates the lack of statistical significance between the *sarA* and *sarA nuc1* mutants. Solid dots represent outlying observations.

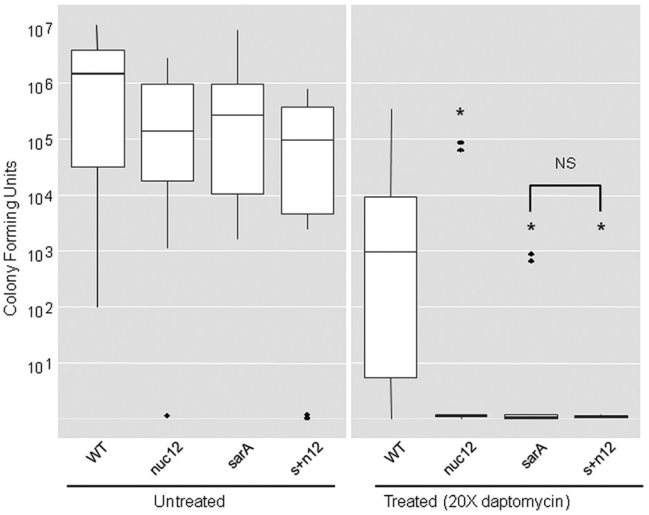

FIG 5 Impact of *sarA* and nuclease production on susceptibility of UAMS-1 to daptomycin *in vivo*. Biofilm formation and relative susceptibility to daptomycin were assessed using an *in vivo* model of catheter-associated biofilm formation (24). Asterisks indicate statistical significance ($P < 0.05$) by comparison to the results observed with the wild-type strain under the same assay condition. The NS above the bracket indicates the lack of statistical significance between the *sarA* and *sarA nuc12* mutants. Solid dots represent outlying observations.

results demonstrate that the extracellular nuclease encoded by *nuc2* is functional under *in vivo* conditions. However, the more important point is that, under *in vivo* conditions, mutation of either nuclease gene was associated with decreased biofilm formation, at least as defined by relative antibiotic susceptibility, while the opposite was true when biofilm formation was assessed *in vitro*.

DISCUSSION

Biofilm formation in *S. aureus* is a complicated process impacted by multiple factors (5, 12, 14, 20). The cumulative data suggest that these factors include eDNA, surface-associated proteins, and the polysaccharide intercellular adhesin (PIA), with the relative impact of each being dependent on both the strain under study and the methods used to assess biofilm formation (1, 3, 10, 15, 21, 22). Much of our work has focused on the staphylococcal accessory gene regulator (*sarA*), mutation of which has been shown to limit biofilm formation in all of the strains that we have examined, other than those known to carry defects that directly impact either these regulatory circuits or the production of specific effector molecules like the fibronectin-binding proteins (1, 3, 22–24). Mutation of *sarA* results in the increased production of extracellular proteases and nucleases (3) and decreased production of PIA (2), any or all of which could contribute to the biofilm-deficient phenotype of *sarA* mutants. Our studies have led us to conclude that it is the increased production of extracellular proteases that plays the primary role in this regard (3, 22), and independent studies from other laboratories have provided strong support for the hypothesis that extracellular proteases are important in *S. aureus* biofilm formation (5, 6, 8, 13). This is consistent with the observation that specific surface-associated proteins known to be sensitive to protease-mediated degradation, including the fibronectin-binding proteins (FnbA and FnbB) and protein A (Spa), have been shown

to promote biofilm formation in *S. aureus* (14, 15). It is also consistent with the observation that biofilm formation was enhanced in our *in vitro* assay when the substrate was coated with plasma proteins. However, most of these studies, including our own, were limited to *in vitro* experiments that may or may not reflect the more therapeutically relevant *in vivo* conditions.

Extracellular nuclease has also been shown to have a negative impact on biofilm formation (9, 13, 17, 19), and this suggests that exogenous nuclease could be used to limit biofilm formation and thereby reduce the therapeutic recalcitrance of biofilm-associated *S. aureus* infections. In contrast, nuclease production has also been shown to promote the ability of *S. aureus* to cause disease by promoting escape from neutrophil extracellular traps (NETs) (4, 16). This brings up the possibility that the therapeutic use of exogenous nucleases could have the adverse effect of promoting the escape of *S. aureus* from phagocytes. In this study, we attempted to address these potentially opposing roles by examining the impact of extracellular nucleases on biofilm formation under both *in vitro* and *in vivo* conditions. Under *in vitro* conditions, limiting nuclease production was shown to enhance biofilm formation, but only in a UAMS-1 *sarA* mutant, which produces increased amounts of extracellular nucleases by comparison to the parent strain (3) and only when the assay was done without coating the substrate with plasma proteins. This is in contrast to reports demonstrating that exonucleases, including those produced by *S. aureus*, limit biofilm formation even in assays done with a plasma-coated substrate (13, 19). It was also demonstrated using the same murine model employed here that a UAMS-1 *cidA* mutant releases reduced amounts of DNA and has a reduced capacity to form a biofilm *in vivo* that is comparable to that observed with the isogenic *sarA* mutant (19). However, while highly suggestive, this does not prove a cause-and-effect relationship between DNA release and biofilm formation.

Thus, to date, the role of *S. aureus* exonucleases in biofilm-associated infection remains unclear. To address this, we extended our *in vitro* studies to include an *in vivo* model of biofilm formation. Unlike previous reports, we also included consideration of relative antibiotic susceptibility *in vivo*, based on the realization that it is the reduced susceptibility of biofilm-associated infections that constitutes the greatest clinical concern. The results of these experiments led us to two conclusions that significantly contrast with those obtained using *in vitro* assays, including our own. The first and arguably most important is that limiting the production of extracellular nucleases in *S. aureus* does not enhance biofilm formation *in vivo* but rather has the opposite effect, particularly when assessed on the basis of relative susceptibility to daptomycin. Indeed, while few studies examining the role of *S. aureus* nucleases in biofilm formation have taken the step of considering antibiotic susceptibility, a recent report did demonstrate that, under *in vitro* conditions, an *S. aureus nuc1 nuc2* mutant was capable of forming a biofilm to an extent that could be associated with dramatically reduced susceptibility to daptomycin (10). Thus, our results demonstrating increased susceptibility in *nuc* mutants provide an additional contrast regarding the role of extracellular nucleases under *in vitro* versus *in vivo* conditions. The second is that, despite the fact that mutation of *nuc2* had no discernible impact on nuclease production *in vitro*, it did further enhance this susceptibility even in a *nuc1* mutant. This is important, in that many previous studies were limited to the analysis of *nuc1* on the basis of the absence of a nuclease-deficient phenotype with a *nuc2* mutant under *in vitro* conditions (3, 13, 22).

It is interesting to note that, when both *nuc1* and *nuc2* were mutated, the degree of increased daptomycin susceptibility was comparable to that of a *sarA* mutant. This was a surprising result, in that *sarA* mutants produce increased amounts of extracellular nuclease (3, 22). If the increased production of extracellular nucleases were responsible for the decreased capacity of a *sarA* mutant to form a biofilm and its increased antibiotic susceptibility (3, 23, 24), then mutation of the *nuc* genes would be expected to result in decreased rather than increased susceptibility. Thus, these results are consistent with our hypothesis that the primary impact of *sarA* on biofilm formation is mediated through factors other than its impact on the production of extracellular nucleases. One possible explanation is that exonucleases do in fact serve a protective role *in vivo* that is unrelated to biofilm formation. Whether or not this is true, the results that we present clearly demonstrate that the production of extracellular nucleases by *S. aureus* does not limit biofilm formation under *in vivo* conditions. This is not to say, however, that nuclease production is not important. For instance, one of the factors contributing to the ability of *S. aureus* to cause disease is its ability to persist in the environment, and that may well be enhanced by both the *cidA*-mediated release of extracellular DNA and reduced DNA degradation. Nor is it to say that exogenous nucleases could not be used to therapeutic advantage, particularly since they could be applied in nonphysiological amounts. At the same time, these results do suggest caution in utilizing such an approach, as it could potentially have an adverse therapeutic effect.

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