Thermonucleases Contribute to Staphylococcus aureus Biofilm Formation in Implant-Associated Infections–A Redundant and Complementary Story

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Biofilms formed by Staphylococcus aureus are one of the predominant causes of implant-associated infections (IAIs). Previous studies have found that S. aureus nucleases nuc1 and nuc2 modulate biofilm formation. In this study, we found low nuc1/nuc2 expression and high biofilm-forming ability among IAI isolates. Furthermore, in a mouse model of exogenous IAIs, Δnuc1/2 exhibited higher bacterial load on the surface of the implant than that exhibited by the other groups (WT, Δnuc1, and Δnuc2). Survival analysis of the hematogenous IAI mouse model indicated that nuc1 is a virulence factor related to mortality. We then detected the influence of nuc1 and nuc2 on biofilm formation and immune evasion in vitro. Observation of in vitro biofilm structures with scanning electron microscopy and evaluation of bacterial aggregation with flow cytometry revealed that both nuc1 and nuc2 are involved in biofilm structuring and bacterial aggregation. Unlike nuc1, which is reported to participate in immune evasion, nuc2 cannot degrade neutrophil extracellular traps. Moreover, we found that nuc1/nuc2 transcription is negatively correlated during S. aureus growth, and a possible complementary relationship has been proposed. In conclusion, nuc1/nuc2 are complementary genes involved in biofilm formation in exogenous IAIs. However, nuc2 contributes less to virulence and is not involved in immune evasion.

Keywords: Staphylococcus aureus, biofilm, thermonuclease, implant associated infections, periprosthetic joint infection

INTRODUCTION

Orthopedic implants are mainly used for bone fixation and joint replacement. Owing to locally compromised host defense, implanted foreign structures are highly susceptible to microbial colonization (Zimmerli and Moser, 2012; Zimmerli, 2014). As a devastating complication after arthroplasty or internal fixation, implant-associated infections (IAIs) frequently lead to the failure
of the prosthetic device or requirement of implant replacement and are associated with substantial patient morbidity (Kapadia et al., 2016; Depypere et al., 2020). Orthopedic IAIs are often caused by *Staphylococcus aureus*, although many other pathogens can lead to such infections (Arciola et al., 2005; Pulido et al., 2008). IAIs can be classified as exogenous or hematogenous (Zimmerli, 2014; Wang et al., 2017; Arciola et al., 2018). Exogenous infections, which are the most common type, occur as a consequence of direct seeding from external contaminants or contiguous spread during the perioperative period. Hematogenous infections involve bacterial seeding on implants through the bloodstream. Although hematogenous infections occur less frequently, they represent up to 20% of prosthetic joint infections (PJs) (Sendi et al., 2011; Konigsberg et al., 2014; Tande et al., 2016).

In contrast to other infections such as bacteremia and skin abscess, microbes in IAIs generally form biofilms, which are aggregated structured bacterial communities encased in an extracellular matrix. Biofilms are responsible for the recalcitrance of implant infection to therapy and serve as a source of bacterial dissemination (Arciola et al., 2018). Biofilms are characterized by the production of extracellular polymeric substances (EPSs), which commonly comprise lipids, extracellular proteins, extracellular DNA (eDNA), and exopolysaccharides (Hobley et al., 2015; Schilcher and Horswill, 2020). EPSs typically account for 90% or more of the biofilm dry weight and perform various functions for the inhabitants, such as providing structural rigidity or protecting them from external environmental stress (Flemming and Wingender, 2010; Flemming, 2016). Researchers found that methicillin-sensitive *S. aureus* (MSSA) strains commonly produce polysaccharide intercellular adhesin (PIA)-dependent biofilms. In contrast, the release of eDNA and cell surface expression of a number of sortase-anchored proteins have been implicated in the biofilm phenotype of methicillin-resistant *S. aureus* (MRSA) (Mccarthy et al., 2015).

Extracellular DNA has been recognized as a component of the EPS matrix for a long time. However, its role in the EPS was underestimated until the discovery that it is an essential component in *Pseudomonas aeruginosa* biofilms (Whitchurch et al., 2002). Further investigation revealed that eDNA stabilizes the biofilm matrix and promotes antimicrobial resistance (Hall-Stoodley et al., 2012). In addition, two clinical studies have recently reported a relationship between the presence of eDNA in the biofilm and the outcome of orthopedic IAIs (Zatorska et al., 2017, 2018).

Extracellular DNA is released through bacterial autolysis and digested by nucleases (Okshevsky et al., 2015). According to previous reports, *S. aureus* secretes thermonuclease enzymes to regulate biofilm formation by modulating eDNA (Kiedrowski et al., 2011; Tang et al., 2011). To our knowledge, the chromosome of *S. aureus* encodes two thermonucleases, *nuc1* and *nuc2* (Tang et al., 2008; Hu et al., 2012). *nuc1*, also called micrococcal nuclease, was the first documented thermonuclease, and it is a secreted virulence factor controlled by the SaerS two-component system (Olson et al., 2013). *nuc2* is a cell surface-binding protein with functional nuclease activity (Kiedrowski et al., 2014). Previous studies have reported that *S. aureus* secretes *nuc1* to degrade neutrophil extracellular traps (NETs) and kill phagocytes (Berends et al., 2010; Thammovongs et al., 2013; Sultan et al., 2019). Interestingly, the two abovementioned phenotypes (biofilm formation and immune evasion) seem incompatible because *nuc1* upregulation contributes to immune evasion, whereas *nuc1* downregulation leads to biofilm formation. The mechanism by which nucleases regulate the survival of *S. aureus* in the IAI microenvironment remains unknown. In addition, the contribution of *nuc2* to *S. aureus* pathogenesis in biofilm-related infections and whether *nuc2* contributes to immune evasion are particularly unclear because this nuclease was more recently discovered than *nuc1* and has received limited attention.

In this study, we evaluated the activity of thermonucleases in IAI isolates. We also examined the impact of *nuc1* and *nuc2* on biofilm formation and immune evasion under in vitro and in vivo conditions. Finally, we discussed the relationship between the two nucleases and their function in *S. aureus* survival and adaptation in the IAI microenvironment.

## RESULTS

### Low Thermonuclease Expression and High Biofilm-Forming Ability in IAI Strains

We analyzed the transcription levels of *S. aureus* thermonucleases among 28 clinical isolates using quantitative PCR (qPCR; Figures 1A,B). Significantly lower transcription levels of both *nuc1* (*p < 0.01*) and *nuc2* (*p < 0.05*) were observed in IAI strains (*n = 14*) than in non-IAI strains (*n = 14*). *nuc1* and *nuc2* transcription levels in the IAI group were 2.57- and 2.47-fold lower than those in the non-IAI groups, respectively. Since gene expression is highly dependent on the involved environment, we included human synovial fluid to mimic the environment encountered by the bacteria in the host, and the results were similar to isolates grown in TSB (Supplementary Figure 1).

To determine whether there were differences in nuclease enzyme activity between the two groups, thermonuclease activity was measured directly using toluidine blue DNA agar, and the zones of clearing were measured. The majority of strains from the IAI group had smaller zones of clearing than those of the strains from the non-IAI group (*p < 0.01*), which indicated lower thermonuclease activity. Representative images for each group are shown in Figure 1C.

Previous reports demonstrated that *S. aureus* nuclease (*nuc1*) could affect biofilm formation by modulating eDNA (Kiedrowski et al., 2011). Here, we noted an increased biofilm eDNA in the IAI group (Supplementary Figure 2A). By relating the eDNA amount to *nuc1* and *nuc2* expression levels, we found a moderate correlation between *nuc1* and eDNA (Pearson *R = −0.4592*; Supplementary Figure 2B), but no significant correlation was found between *nuc2* and eDNA (Pearson *R = −0.2983*, *p > 0.05*). Then, we wonder if the IAI isolates with low thermonuclease activity also have higher biofilm-forming ability. Static microtiter biofilm assay found that IAI isolates had higher biofilm-forming ability (Figure 1D). Considering that...
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The expression levels of thermonucleases and biofilm-forming ability in clinical isolates. Expression of nuc1 (A) and nuc2 (B) in IAI and non-IAI isolates \((n = 14/\text{group})\) determined by qPCR. (C) Nuclease activity in toluidine blue DNA agar represented in the diameter of red zones. Representative images are also presented. Red zones indicate nuclease activity. (D) Biofilm formation of IAI (\(n = 14\)) and non-IAI isolates (\(n = 14\)) on different materials including polypropylene 96-well plates (non-IAIs = 1.334 ± 0.103), titanium disk (non-IAIs = 1.664 ± 0.229), and UHMWPE (non-IAI = 1.407 ± 0.284). Biofilm biomass was stained with crystal violet. Statistical significance was calculated using two-tail Student’s \(t\)-test in panels (A–C); the multiple \(t\)-test (Bonferroni–Dunn’s test) was used in panel (D). *\(p < 0.05\); **\(p < 0.01\) vs. non-IAI strains.

**FIGURE 1** | The expression levels of thermonucleases and biofilm-forming ability in clinical isolates. Expression of nuc1 (A) and nuc2 (B) in IAI and non-IAI isolates \((n = 14/\text{group})\) determined by qPCR. (C) Nuclease activity in toluidine blue DNA agar represented in the diameter of red zones. Representative images are also presented. Red zones indicate nuclease activity. (D) Biofilm formation of IAI (\(n = 14\)) and non-IAI isolates (\(n = 14\)) on different materials including polypropylene 96-well plates (non-IAIs = 1.334 ± 0.103), titanium disk (non-IAIs = 1.664 ± 0.229), and UHMWPE (non-IAI = 1.407 ± 0.284). Biofilm biomass was stained with crystal violet. Statistical significance was calculated using two-tail Student’s \(t\)-test in panels (A–C); the multiple \(t\)-test (Bonferroni–Dunn’s test) was used in panel (D). *\(p < 0.05\); **\(p < 0.01\) vs. non-IAI strains.

### Construction and Characterization of nuc1/nuc2 Mutant Strains

To study the pathogenesis of *S. aureus* nuc1 and nuc2 in IAI, we constructed nuc1 and/or nuc2 mutants using the clinical IAI isolate ST1792, which we termed Δnuc1, Δnuc2, and Δnuc1/2. After in-frame mutation, the strain genotypes were validated by Sanger sequencing (Supplementary Figure 3A). Interestingly, the colony formed by Δnuc1/2 was much stickier (Supplementary Figure 3B) than that formed by wild type (WT), Δnuc1, and Δnuc2. The same phenomenon was also observed in the USA300 nuc1/nuc2 isogenic mutant (BD1281).

Nuclease activity was then compared among the four strains (Δnuc1, Δnuc2, Δnuc1/2, and WT) using toluidine blue DNA agar (Figure 2A). No observable difference was found between Δnuc2 and WT with a wide area of the clearing zone. In contrast, no detectable nuclease activity was...
observed for Δnuc1 and Δnuc1/2. We also quantified the biofilm-forming capacity of these strains. Following crystal violet staining, we observed that the biomass of Δnuc1 and Δnuc1/2 increased significantly (Figures 2B–D, Δnuc1: p < 0.01, Δnuc1/2: p < 0.001) in various materials, including titanium, UHMWPE, and polypropylene 96-well plates. However, the biomass of the Δnuc2 biofilm varied with the materials. For example, when grown in UHMWPE, Δnuc2 bacteria developed a more robust biofilm than developed by the WT bacteria. However, biofilms grown on titanium disks and 96-well plates were comparable to the WT biofilms. In addition to quantifying biofilm biomass, the number of culturable cells was also assessed. The results showed that Δnuc1/2 biofilms contained more bacterial cells than the other genotypes (Supplementary Figure 4).

**Δnuc1/2 Has Higher Biofilm-Forming Capacity in the Exogenous IAI Mouse Model**

By inoculating bacteria around the implant locally, we constructed an exogenous IAI mouse model. All mice were euthanized 7 days after infection. No significant differences were found among the groups (WT, Δnuc1, Δnuc2, and Δnuc1/2) when evaluating the bacterial load in the peri-implant tissues (Figures 3A,B). However, when quantifying adherent bacteria on the implant, a higher bacterial load was exhibited by Δnuc1/2 than by the WT (Figures 3C,D). Interestingly, the adherent bacterial load showed no statistical difference among the Δnuc1, Δnuc2, and WT groups.

To investigate the effect of nucleases on environmental adaptations in vivo, a competitive assay was conducted. Bacteria with different genotypes and fluorescent labels were mixed and inoculated in vivo. The implants were harvested on day 7 and observed under a fluorescence microscope. Groups infected with a mixture of Δnuc1 and Δnuc2 presented overlapping red and green fluorescence, and no difference was detected between them (Figure 4). The Pearson correlation test showed a strong correlation (R = 0.83) between Δnuc1 (green) and Δnuc2 (red) signals (Supplementary Figure 5A). However, in groups infected with a mixture of WT and Δnuc1/2, a difference in bacterial distribution (Figure 4B) and a low Pearson correlation (Supplementary Figure 5B) were observed. Specifically, Δnuc1/2 strains labeled with mCherry exhibited broad and even distribution, whereas WT strain labeled with superfolder GFP (sfGFP) was distributed in clusters with less area covered.
FIGURE 3 | Bacterial burden in an exogenous IAI mouse model. CFU for peri-implant tissue and biofilms on implant was determined 7 days after infection. (A) Bacterial count for peri-implant tissues and representative photos (B). (C) Bacterial count for biofilms on implant and representative photos (D). Statistical significance was calculated using ANOVA with Dunnett multiple column comparisons. n = 10/group. *p < 0.05 vs. WT.

FIGURE 4 | Implant from a competition infection mouse model observed with a fluorescent microscope. Implants were harvested on the seventh day since infection and observed with a fluorescence microscope. (A) Implant from mice infected with a ∼1:1 mixture of Δnuc1 (green) and Δnuc2 (red). (B) Implant from mice infected with a ∼1:1 mixture of WT (green) and Δnuc1/2 (red). Scale bar = 100 µm, n = 3/group.

Δnuc1/2 Affects Bacterial Aggregation and Biofilm Structure in vitro

Next, we examined biofilm structure, in vitro, using scanning electronic microscopy (SEM). The biofilm structure of Δnuc1/2 was different from that of the other three genotypes (Figure 5A). Δnuc1/2 bacteria developed “valley and mountain-like” structures, whereas the other bacterial strains did not. However, this difference was only observed at ×50 magnification. When the biofilm was observed at ×2,000 magnification, no difference was detected (Supplementary Figure 6). In order to observe the extracellular matrix, we used a confocal microscope. We noticed that the biofilms formed by Δnuc1/2 were thicker and had higher PI signals, which represent both eDNA and dead cells.

We also measured the percentage of bacterial aggregation using flow cytometry (Figures 5B,C). Δnuc1/2 was more likely to aggregate between bacterial cells (p < 0.01). No statistical difference was observed among the remaining groups (Δnuc1, Δnuc2, and WT).

nuc2 Is Not a Virulence Factor Like nuc1 in a Hematogenous Mice Model

The work mentioned above was based on an IAI mouse model induced by surgical site contamination. However, hematogenous...
infections represent up to 20% of IAIs (Wang et al., 2017). Therefore, we investigated the pathogenesis of Δnuc1 and/or Δnuc2 strains in a hematogenous IAI mouse model. Based on our observations, the group infected with WT and its isogenic nuc2 mutant had significantly higher mortality rates ($p < 0.05$, Figure 6A).
FIGURE 6 | nuc1 and nuc2 involvement in immune evasion. (A) Survival curve for a hematogenous IAI mouse model infected with ST1792 WT, Δnuc2 (n = 8 each), and Δnuc1 and Δnuc1/2 (n = 7 each). (B) Representative fluorescent images for NET degradation assay. S. aureus were incubated with PMA-stimulated neutrophils (n = 3/group). A green signal represents NETs and cell nucleus, scale bar = 100 µm. (C) THP-1 macrophages were incubated with DNA and S. aureus. Cell viability was determined by trypan blue staining (n = 3/group). The survival curve was analyzed with a log-rank (Mantel–Cox) test. ANOVA with Dunnett multiple column comparisons was used in panel (C). **p < 0.01; ***p < 0.001 vs. WT. The four groups are presented in Supplementary Figure 8. Interestingly, most of the death events occurred within 7 days of infection. However, no difference was detected in the bacterial load in peri-implant tissue and on the implant among the four groups (Supplementary Figure 9).

According to previous reports, nuc1 is involved in immune evasion (Tang et al., 2011; Thammavongsa et al., 2013). Hence, we speculated whether nuc2 had the same function. In the NET degradation assay, in which WT and Δnuc1/2 were considered positive and negative controls, respectively, we did not detect any difference when comparing Δnuc1 with Δnuc1/2 (Figure 6B). A previous study reported that nuc1 could lead to immune cell death (Thammavongsa et al., 2013). In line with this result, the trypan blue staining assay (Figure 6C) showed increased THP-1 cell viability in the Δnuc1 and Δnuc1/2 groups when compared with the WT. However, there was no difference in cell viability between the WT and Δnuc2 groups or between the Δnuc1 and Δnuc1/2 groups.

Staphylococcus aureus Sequentially Expresses nuc1 and nuc2 for Environmental Adaptation

Our study indicates that nuc1 and nuc2 are both essential for biofilm formation. However, the redundancy of thermonucleases in the S. aureus chromosome prompted us to investigate the underlying mechanism. By analyzing the public microarray dataset GSE25454, we found that nuc1 and nuc2 were negatively correlated (Figure 7A, $R = -0.59$, $p < 0.001$). Our qPCR results confirmed this phenomenon: during S. aureus growth in tryptic soy broth (TSB), nuc2 was upregulated in 2–4 h and then decreased. In contrast, nuc1 transcription peaked in the post-exponential stage (Figure 7B). Also, the correlation between nuc1 and nuc2 in our study was similar to what we found in the dataset GSE25454 (Figure 7C, $R = -0.8520$, $p < 0.001$). To exclude the possible regulation between the two genes, we also investigated nuc1/nuc2 transcription in Δnuc2/Δnuc1 (Figure 7D), and the data obtained showed no regulation between nuc1 and nuc2. Such a negative correlation led to our hypothesis that nuc1 and nuc2 are complementary. Therefore, it is possible that nuc2 functions in the early growth phase and that nuc1 plays its role during the later phase.

Staphylococcus aureus Modulates Nuclease Transcription When Exposed to Antibiotics

In the first part of our study, we found low expression of nucleases in IAI isolates. Considering patients with IAIIs generally require long-term antibiotic administration, we further speculated whether antibiotics would affect nuc1/2 expression. We then exposed MRSA (USA300) and MSSA (ST1792) to several of the most commonly used antibiotics at sub-minimum inhibitory concentration (MIC) levels. First, we determined the
MIC and sub-MIC of both strains, and the results are listed in Table 1. Following sub-MIC exposure, qPCR was used to measure nuc1 and nuc2 transcriptions (Figure 8).

We found that MSSA and MRSA had different responses to antibiotics. Fewer differences in expression patterns were seen for nuc1 in the two strains with only a major shift seen for vancomycin (decreased in MRSA but increased in MSSA). In both ST1792 and USA300, nuc1 was upregulated when exposed to ciprofloxacin and downregulated after exposure to ceftriaxone and daptomycin. However, for nuc2 expression, an increase was observed in both MRSA and MSSA strains after linezolid. Ciprofloxacin, ceftriaxone, and daptomycin increased nuc2 expression only in the MRSA strain. Finally, nuc2 expression increased in response to vancomycin for the MSSA strain, which is similar to nuc1.

**DISCUSSION**

To our knowledge, this is the first study reporting that S. aureus IAI isolates have low nuclease (nuc1 and nuc2) expression levels, which may be relevant for the high biofilm-forming capacity of IAI isolates. By constructing nuclease mutant strains, we found that ∆nuc1/2 exhibited higher biofilm-forming capacity
in an exogenous IAI mouse model. However, a previous study reported that nuc1 and nuc2 had no significant impact on biofilm formation using a murine model of catheter-associated biofilm formation (Beenken et al., 2012). Although the reason underlying this discrepancy with our results is unclear, we noticed that the strain used is different, which could explain, in part, the discrepancies observed.

According to previous reports, nuc1 regulates biofilm formation by modulating eDNA in the biofilm matrix (Kiedrowski et al., 2011; Tang et al., 2011). However, the impact of nuc2 on biofilms has received limited attention. In this study, we noticed that Δnuc1/2 strains formed sticky colonies and distinct biofilm morphology. One possible explanation for this phenomenon is that the mutation of both nuc1 and nuc2 leads to the accumulation of eDNA, which in turn may increase colony and biofilm viscosity. This hypothesis is partially corroborated by a previous study (Kaito et al., 2011), where it was reported that the presence of eDNA increases extracellular matrix viscosity. Also, biofilms observed with confocal microscopy showed that Δnuc1/2 formed thicker biofilms with higher PI signal. According to a previous report, eDNA degradation is involved in the “exodus” and “dispersal” steps during biofilm maturation (Moormeier et al., 2014). Their study prompted us to speculate that a high eDNA content in the biofilm matrix may make both live and dead bacteria unable to egress from the biofilm and get trapped, thus resulting in a
thicker biofilm with a higher PI signal. Nevertheless, we cannot exclude other possible mechanisms accounting for the observed phenotypes. Since previous studies have not determined if nuc1 and nuc2 have an influence on other biofilm-related genes, the detected phenomenon could also be caused by the regulation between nucleases and other genes.

By analyzing nuc1 and nuc2 transcription levels at different time points, we found that nuc1 and nuc2 transcription levels were temporally regulated during S. aureus growth, and this was also reported in a previous study (Hu et al., 2012). It seems nuc2 was expressed when cell density was low, and nuc1 was prone to be expressed at high cell density. Such temporal gene regulation was most likely dependent on a quorum-sensing system. However, agr, the most well-studied quorum sensing, is not directly associated with nuc1 or nuc2 regulation (Olson et al., 2013; Kiedrowski et al., 2014). Hence, other quorum-sensing systems (e.g., LuxS) and stimuli rather than population density might be involved in the temporal regulation of thermonucleases. Meanwhile, it should be noted that biofilms are not synchronized in terms of growth phase and that it would lead to special difference in gene expression. Previous studies found that nuc1 and nuc2 are highly expressed in the peripheral colony, highlighting the need to study in more detail the spatial regulation of nuc1 and nuc2 in biofilms (Kaito et al., 2011).

The redundancy of nuc1 and nuc2 does not mean equal contribution to S. aureus virulence. For instance, nuc1 in hematogenous IAI strains contributed to the mortality rate of infection while nuc2 did not. Interestingly, we observed that most deaths, in the hematogenous IAI model, occurred during the first 7 days of infection. Based on the knowledge that adaptive immunity takes 4–7 days to mount a response (Iwasaki and Medzhitov, 2010), we speculated that nuc1 may be relevant for innate immune evasion instead of adaptive immune evasion. This was consistent with published researches which demonstrated that S. aureus escapes innate immune defense through NET degradation and phagocyte apoptosis (Berends et al., 2010; Thammavongsa et al., 2013; Winstel et al., 2018). However, concerning nuc2, no observable innate evasion function was detected in our study. It may be due to its reported low enzymic activity (Kiedrowski et al., 2014), and thus, NETs could not be sufficiently degraded with nuc2.

Considering patients with IAIs require long-term administration of antibiotics, our study also examined the impact of antibiotics on nuc1 expression. Although MSSA and MRSA had different responses to antibiotics, ceftriaxone and daptomycin reduced nuc1 levels in both MSSA and MRSA. Because MRSA strains are resistant to ceftriaxone, which seems to downregulate nuc1 expression, the use of this antibiotic could lead to increased biofilm formation. As such, caution should be taken when adopting ceftriaxone for IAI treatment before the antibiotic sensitivity testing is clear.

Our study has some limitations. First, the higher biofilm-forming capacity of IAI groups cannot be fully explained by the low expression of thermonucleases, as other factors such as sarA, clfA/B, srtA, and agr loci also contribute to biofilm formation regulation (Paharik and Horswill, 2016; Otto, 2018). Second, the regulation mechanism of nuc2 transcription in S. aureus remains unknown. Finally, in our hematogenous IAI mouse model, we did not check the bacterial load in other systemic organs, which could help us understand the capacity of these strains to disperse to distant sites. Also, the bacterial load in the hematogenous IAI mouse model showed no significant differences even in the Δnuc1/2 group. Although the mechanism underlying the discrepancy observed between the two mouse models remains unclear, we speculated that the virulence adopted by S. aureus to colonize bone implants and develop biofilms might vary depending on how they invade the human body.

In summary, we identified temporal regulation for nuc1 and nuc2. The pathogenesis of both nucleases was explored using two types of infection models. Low expression of both nuc1 and nuc2 is essential in S. aureus IAIIs caused by surgical site contamination. However, in hematogenous IAIIs, upregulation of nuc1, rather than nuc2, contributes to S. aureus pathogenesis. Our study may provide new insights into the prevention and treatment of IAIs.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Staphylococcus aureus strains used in this study were either strains that were maintained in our laboratory or clinically isolated. To construct a fluorescence-labeled strain, pRN11 and pCM29 plasmids (Pang et al., 2010; de Jong et al., 2017) expressing mCherry and sfGFP, respectively, were introduced into S. aureus competent cells RN4220 via electroporation using a MicroPulser (Bio-Rad, United States). After adding 0.5 µg of plasmid into 50 µl of RN4220 competent cells, the default Staph program was performed (2-mm gap, 1.8 kV, 2.5 ms). Then, the cells were immediately resuspended in 1 ml of TSB (Haibo, Qingdao, China) and cultured on a shaking incubator at 200 rpm for 1 h at 37°C. A total of 100 µl of the recovery culture was grown overnight at 37°C on a TSB-chloramphenicol (10 µg/ml) agar plate. The next morning, a single chloramphenicol-resistant colony harboring pRN11 or pCM29 plasmids was selected and grown overnight in 4 ml of TSB with chloramphenicol (10 µg/ml). Next, according to a previously described bacteriophage transformation method (Olson, 2014), the plasmid was transformed into S. aureus ST1792, which was isolated from an infected prosthesis, with bacteriophage 11.

Thermonuclease Activity Detection

Bacterial cultures were grown in TSB on a shaking incubator at 37°C and 200 rpm for 6 h and then heat-inactivated at 100°C for 10 min. Toluidine blue DNase agar was used to detect thermonuclease activity according to the manufacturer’s instructions (Haibo, Qingdao, China). A total of 80 µl of inactivated culture was added into a 5-mm-diameter hole in the agar plate made with a sterile pellet tip. The plate was incubated for 6 h at 37°C, and the diameter of the clearing zone was measured.

Construction of Thermonuclease Mutants

In-frame deletion of nuc1 and nuc2 genes in clinical isolate ST1792 was performed by allelic replacement using the plasmid.
pKOR1 as previously described elsewhere (Bae and Schneewind, 2006). The primers used are listed in Supplementary Table 1. Briefly, after amplifying the upstream and downstream regions of the target gene, we used SOE-PCR to ligate the upstream and downstream fragments. The PCR product was cloned into pKOR1, and the resulting recombinant plasmids pKOR1-nuc1 and pKOR1-nuc2 were further transformed into *S. aureus* competent cells RN4220 via electroporation and maintained using chloramphenicol (10 µg/ml). Next, the plasmid was transformed into *S. aureus* ST1792 using bacteriophage 11. *S. aureus* ST1792 containing the plasmid constructed was used for construction of mutants by allele replacement with temperature sensitivity as described previously (Bae and Schneewind, 2006). Candidate mutant strains were validated by Sanger sequencing.

**In vitro Static Biofilm Assays**

All bacterial strains involved in this experiment were cultured overnight at 37°C in TSB supplemented with 0.25% glucose (TSBG). The overnight culture was serially diluted to a concentration of ~1 x 10⁶ CFU/ml, and then, 100 µl of the culture was inoculated into a 96-well plate with a flat bottom (BIOFIL, Guangzhou, China). UHMWPE disks (5-mm diameter) were sterilized and placed on a 96-well plate with a round bottom (BIOFIL) and then inoculated with 100 µl of the bacterial culture followed by incubation. Titanium disks (10-mm diameter) were sterilized and placed on a 24-well plate with a flat bottom (BIOFIL) and then inoculated with 1 ml of bacterial culture followed by incubation. After incubation at 37°C for 24 h, the culture medium was aspirated from each well, and wells were washed three times with either 200 µl of PBS in case of the 96-well plate or 1 ml of PBS in case of the 24-well plate. After fixation with methanol, the plate was air-dried, and the biofilm was stained with 200 µl of crystal violet. The crystal violet bound at the bottom of the well was dissolved in 200 µl of 33% acetic acid, and 100 µl aliquots from each well were transferred into a new 96-well plate with a flat bottom. Optical absorbance was measured at 590 nm using a microplate reader (BioTek Instruments, Inc., United States) to quantify the biofilm biomass.

**Biofilm eDNA Content Measurement**

*Staphylococcus aureus* biofilms were grown as described above in a six-well plate (1.5 ml TSBG per well). After gently removing the supernatant, biofilm cells were resuspended with 1 ml of PBS and then filtered using a 0.2-µm filter. To measure eDNA content, 100 µl of filtered resuspension was mixed with 100 µl of 2 µM SYTOX Green (Invitrogen, United States). Fluorescence was measured by using a plate reader (BIO-TEK, ELX 800, United States) with excitation and emission wavelengths of 485 and 520 nm.

**Staphylococcus aureus** RNA Isolation and Quantitative PCR

To investigate the transcription levels of thermonucleases between an IAI strain and a non-IAI strain, *S. aureus* was cultured in TSB (or TSB supplied with 20% human synovial fluid) in a shaking incubator at 37°C and 200 rpm for 6 h. To investigate thermonuclease expression at different time points, ST1792 was cultured in TSB (37°C/200 rpm) and collected at several time points (1, 2, 4, 6, and 8 h). To determine nuc1/2 expression in ST1792 and its isogenic mutants (Δnuc1 and Δnuc2), bacteria were cultured in TSB and incubated at 37°C and 200 rpm for 6 h. To explore nuc1/nuc2 transcription changes after exposure to antibiotic, ST1792 and USA300 were cultured in TSB supplied with sub-MIC antibiotics for 6 h (37°C/200 rpm). The abovementioned bacteria were harvested and transferred to a tissue lyser (Scientz™, Ningbo, China), and the cell wall was physically disrupted for 30 s at a frequency of 50 Hz. RNA was isolated using the EZ-press RNA Purification Kit (EZBioscience, United States) according to the manufacturer's instructions. The quality of the RNA was measured using a Nanodrop device (Thermo Fisher Scientific, United States), and RNA samples with absorbance ratios of 260 nm/280 nm and 260 nm/230 nm higher than 2.0 were selected for reverse transcription. After adding DNase to remove gDNA, 1 µg fresh RNA was immediately reverse-transcribed into cDNA using an RT-PCR kit (EZBioscience, United States). cDNA was diluted with ddH₂O (1:5 dilution) and subsequently used as the DNA template for qPCR, performed with the kit 2 × SYBR Green qPCR Master Mix (EZBioscience). DNA amplification was performed by thermal cycling: initial denaturation at 95°C for 5 min, followed by 40 amplification cycles at 95°C for 10 s and at 60°C for 30 s using a Roche LightCycler 480 (Roche, Switzerland). The primers used in this study and other related information such as product size, primer efficiency, and cycling parameters are provided in Supplementary Table 1. Relative gene expression levels were quantified using the 2⁻ΔΔCT method, with the expression levels of gyrB as the internal reference.

**Biofilm Structure Observation Using SEM and Confocal Microscopy**

Overnight-grown ST1792 biofilms were formed on a titanium disk as described above. After gently being washed with PBS, the samples were fixed with 2.5% glutaraldehyde at 4°C for 4 h, dehydrated using a graded ethanol series (50, 70, 80, 90, 95, and 100% v/v) for 10 min, freeze-dried, coated with platinum, and visualized using SEM (Magellan 400, FEI, United States).

For confocal microscopy observation, biofilms grown on titanium disk were stained with a Live/Dead BacLight bacterial viability kit (Invitrogen, United States) according to the instructions of the manufacturer. Stained biofilms were observed immediately using a confocal microscope (Leica TCS SP8, Germany). The optimal exposure time and laser intensity for both channels (excitation: 488 and 555 nm) were manually set to ensure no overexposure among groups. Then, all of the images were acquired with the same setting for comparability among groups. Raw data were imported into Imaris 9.0.1 for biovolume and biofilm thickness calculation.

**Detection of Bacterial Clumps Through Flow Cytometry**

sfGFP-labeled ST1792 and its isogenic mutants were incubated in TSB at 37°C and 200 rpm overnight in the presence of
chloramphenicol. Then, overnight bacterial cultures were used for flow cytometry (Beckman Coulter CytoFLEX, United States). The sample flow rate was 10 µl/min, and 20,000 events were recorded for further analysis. First, the GFP+ cell population, comprising the bacteria, was selected. Then, single/clumping populations were labeled using the correlation between FITC-H and FITC-A.

**Neutrophil Extracellular Traps Degradation Assay**

Neutrophils were isolated from the blood of healthy donors. The anti-coagulated whole blood (5 ml) was carefully layered over 5.0 ml of Polymorphprep™ (Alere Technologies, Norway) in a 15-ml centrifuge tube. Tubes were centrifuged at 500×g for 30 min at 18–22°C. PMNs were gently separated, and 3 ml of RPMI medium was added to restore normal osmolality. Samples were centrifuged at 400×g for 10 min to collect cells. Finally, the cells were resuspended in the RPMI medium containing 10% fetal bovine serum (FBS). For NET induction, phorbol-12-myristate-13-acetate (PMA, Sigma, United States) was added to the culture medium to reach a final concentration of 90 nM, and samples were incubated at 37°C with 5% CO₂ for 4 h. Then, heat-inactivated bacterial culture (ST1792 and its isogenic mutants) was added (MOI = 100) and incubated for 2 h to degrade the NETs. After fixation with 4% paraformaldehyde, NETs were stained with SYTOX Green (Invitrogen, United States) and visualized using light microscopy.

**Cellular Cytotoxicity Assays Using Trypan Blue Staining**

Human monocytes THP-1 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). A total of 1 × 10⁵ THP-1 cells were cultured, overnight, in an RPMI medium containing 10% FBS and penicillin/streptomycin together with heat-inactivated WT, Δnuc1, Δnuc2, or Δnuc1/2 bacterial suspensions (MOI = 10) and DNA (final concentration of 100 ng/µl). Subsequently, cells were stained with trypan blue and visualized using light microscopy.

**Bacterial MIC Assay**

We adopted a macrolution method to determine the MIC of different antibiotics for MRSA and MSSA, and 1 ml of twofold serial dilutions of antibiotics dissolved in TSB was added to 1 ml of TSB, which contained nearly 10⁶ CFU/ml, in separate tubes. After overnight incubation at 37°C, the turbidity of the test tubes was visually inspected, as turbid test tubes were indicative of bacterial growth, whereas tubes that remained clear indicated no growth. The MIC of the antibiotics tested was considered to be the lowest concentration that inhibited growth. Gentamycin, linezolid, daptomycin, ciprofloxacin, and ceftriaxone were purchased from Aladdin (Shanghai, China).

**GEO Microarray Data Analysis**

Public microarray dataset GSE25454 including 74 samples was selected. First, we downloaded the raw data (.CEL files) from the GEO database (GSE25454). Then, the following process was performed in R (4.0.1). We used the readAffy function (limma package; Ritchie et al., 2015) to import the .CEL files and performed background correction with the gcma function (GCRMA package; Lim et al., 2007). Note that the resulting expression data were represented as intensity and transformed into log-2 scale according to descriptions in the GCRMA package manual. After that, we used the “normalizeWithinArray” function (from the limma package; Ritchie et al., 2015) to normalize the data among samples to remove batch effects. Finally, we extracted the nuc1 and nuc2 expression values for each sample at various time points and performed a Pearson correlation test. The scatter plot was presented with the ggscatter function (ggpubr package).

**Implant-Associated Infections Mouse Model**

**Mice Model of Exogenous IAIs**

Twenty BALB/c mice (6 weeks old) were randomly divided into four groups (WT, Δnuc1, Δnuc2, and Δnuc1/2). Mice were intraperitoneally anesthetized with 1% pentobarbitalum natrium (provided by the animal center), and both knees were shaved and disinfected. Then, the distal femur was exposed through a medial parapatellar incision, and a narrow channel was created at the femoral end using a 25G needle. Subsequently, the prepared sterile titanium wires (0.5-mm diameter) were inserted in a retrograde direction into the intramedullary canal. The overlying subcutaneous tissue and skin were closed using absorbable subcuticular sutures. Finally, 25 µl of the corresponding bacterial inoculum (ST1792 and its isogenic mutants, ∼1 × 10⁷/ml) was injected intraarticularly into the knee joint space. All mice were anesthetized and euthanized by cervical dislocation 7 days after the infection. Peri-implant tissues were harvested and homogenized in 1 ml of sterile saline before CFU counting. The biofilm on the titanium wires together with 1 ml of sterile saline was subjected to sonication (30 kHz, 10 min) in an ultrasound bath (CQ-200B-DST, Yuejin, China), and the resulting sonicated fluid was used for further bacterial load quantification.

**Competition Infection Model**

To observe the in vivo biofilm structure and explore the adaptability of different S. aureus genotypes, a titanium disk was implanted subcutaneously into the dorsal area of the mice, and ∼1 × 10⁶ CFU of the strain mixture was inoculated around the implant. We labeled Δnuc1/WT with sfGFP and Δnuc1/2/Δnuc1/2 with mCherry. Mice were infected with either a WT/Δnuc1/2 mixture or a Δnuc1/Δnuc2 mixture. All mice were euthanized by cervical dislocation and anesthetized 7 days after the infection. The biofilm on the titanium disks was observed using a fluorescence microscope (Leica DMI8, Germany).

**Mouse Model of Hematogenous IAIs**

Thirty-two adult BALB/c mice were randomly divided into four groups (WT, Δnuc1, Δnuc2, and Δnuc1/2). After the implantation surgery performed as described previously, mice were infected via tail vein injection (ST1792 and its isogenic mutants, 1 × 10⁷ CFU/100 µl). Survival was recorded daily.
mice were anesthetized and euthanized by cervical dislocation 28 days after the infection. Peri-implant tissues and biofilm cells on the implant were prepared as described above. Bacterial load enumeration was performed according to the protocol listed in Section “Bacterial Load Enumeration.”

**Bacterial Load Enumeration**

The bacterial suspension or sonicated fluid was serially diluted tenfold. A total of 100 µl of the diluted suspension was spread on sheep blood agar plates. After incubation at 37°C overnight, CFU counts were performed according to the National Standard of China GB/T 4789.2 protocol. The resulting bacterial load for peri-implant tissues was normalized to tissue weight, and all bacterial loads were presented on a log$_{10}$ scale.

**Statistical Analysis**

The GEO microarray was analyzed with R 4.0.1. The fluorescence distribution pattern in Figure 4 was quantified with a Pearson correlation test using Coloc 2, a program in ImageJ (1.53c, Fiji). The remaining data analysis was performed using GraphPad Prism 8.3.0. Statistical significance was indicated as a two-sided $p < 0.05$. Results are represented as mean ± SD unless stated otherwise.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

Neutrophils were isolated from the blood of healthy donors. Human synovial fluid was collected from osteoarthritis patients before they received intra-articular injection of hyaluronic acid. The procedures were approved by the Ethics Committee of the Shanghai Sixth Peoples Hospital. The handling of mice and related procedures in this study were approved by the Animal Care and Experiment Committee of the Medical College of Shanghai Jiao Tong University affiliated Sixth People’s Hospital.

**AUTHOR CONTRIBUTIONS**

JY contributed to the concept of the study and wrote the manuscript. JD and FZ contributed to the qPCR experiment. YM and JY performed *S. aureus* mutant construction. JT and MH collected clinical strains and performed antibiotic susceptibility testing. JY, FJ, and FZ performed the in vivo experiment. QW and PH contributed to data analysis and data interpretation. HS contributed to the study design, manuscript editing and revision. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.687888/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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