The Q225P Mutation in SigB Promotes Membrane Vesicle Formation in *Staphylococcus aureus*

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Abstract

Both Gram-positive and Gram-negative bacteria release nano-sized lipid bilayered particles, known as membrane vesicles (MVs), into external environments. Although MVs play a variety of roles in bacterial physiology and pathogenesis, the mechanisms underlying MV formation in Gram-positive microorganisms such as *Staphylococcus aureus* remain obscure. Bacterial MV production can be induced in response to stress conditions, and the alternative sigma factor B (SigB) functions as a central regulator of the stress response in Gram-positive bacteria. In a previous study, we demonstrated that the SigB(Q225P) substitution mutation in *S. aureus* promotes biofilm formation. Here, we report that the SigB(Q225P) mutation also increases MV production in this important pathogen. LacZ reporter assays and electrophoretic mobility shift assays showed that the Q225P substitution reduces SigB binding to the promoter region of the thermonuclease gene (*nuc*), resulting in a significant reduction in Nuc expression. Deletion of *nuc* markedly enhances *S. aureus* MV generation, possibly due to the accumulation of nucleic acids. These results are not only important for understanding MV biogenesis in *S. aureus*, but also useful for the development of a *S. aureus* MV-based platform for MV application.

Introduction

Membrane vesicles (MVs) are bilayered nano-sized lipid particles that are released by bacteria under normal culture conditions. The vesicles play pivotal roles in a variety of functions such as competition, survival, invasion, infection, material exchange, host immune escape, and biofilm formation [1]. MVs were first described in Gram-negative bacteria but were not reported in Gram-positive bacteria until 2009, perhaps because Gram-positive species lack outer membranes and have thick and rigid peptidoglycan layers [2]. The major components of bacterial MVs include proteins, nucleic acids, lipids, and other metabolites [3]. Since

the membrane-anchored lipids and proteins associated with MVs are highly immunogenic, MVs have received increasing attention as promising vaccine carriers and have been exploited to combat several infectious diseases [1, 3–7]. However, MVs are obtained from bacteria at very low yields under ordinary conditions, hindering MV development and application. This shortcoming must be addressed before MV technology can be leveraged for practical large-scale vaccine development.

MV production is controlled by regulatory mechanisms that vary across bacterial species. For example, changes in expression of the outer membrane protease OmpT in enterohemorrhagic *Escherichia coli* dynamically affect MV biogenesis, composition, and size [8]. Similarly, MV formation in *Salmonella enterica* increases upon induction of PagC, specifically within acidified macrophage phagosomes [9]. In the Gram-positive anaerobic pathogen *Clostridium perfringens*, phosphorylation of the sporulation master regulator Spo0A triggers MV production [10]. In contrast, MV biogenesis in group A *streptococcus* (GAS) is negatively regulated by the CovRS two-component system [11]. Taken together, these observations demonstrate that bacterial MV production is complex and is regulated by diverse genetic mechanisms.

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Increased production of bacterial MVs occurs in response to stress conditions such as high temperature [12], oxygen stress [13], antibiotic exposure [14], osmotic pressure [15], and nutrient availability [16]. This adaptive behavior presumably helps bacteria to survive in adverse environments. Sigma factors, which are subunits of RNA polymerases, play a critical role in bacterial stress responses and MV production. For example, the food-borne pathogen *Listeria monocytogenes* secretes approximately 9 times more MVs than does an isogenic sigma factor B (SigB)-deficient mutant [17]. MV protein composition also decreases from 130 proteins to 89 in the wild-type and mutant strains, respectively. Exposure to external stressors increases MV formation in *Pseudomonas aeruginosa*, and is accompanied by elevated levels of AlgU, a member of the sigma E-like family of stress-related sigma factors [18]. Although AlgU is not essential for vesiculation, overexpression of AlgU significantly increases MV production.

Staphylococcus aureus is an important pathogen capable of producing a variety of virulence factors during bacterial growth and invasion. Among them, thermonuclease (Nuc) encoded by the *nuc* gene is one of the most important exoenzymes that could catalyze the hydrolysis of the 5' phosphodiester bond of both DNA and RNA in host cells, thus facilitating bacterial immune escape and pathogenicity [19, 20]. In previous work, we identified a substitution mutation (Q225P) in SigB of *S. aureus* that confers a non-pigmented phenotype and an enhanced ability to form biofilms [21]. We report here that the SigB(Q225P) mutation also promotes the generation of *S. aureus* MVs through the direct downregulation of Nuc. Deletion of the *nuc* gene significantly increases MV production. Our results provide new insights into MV biogenesis in *S. aureus* and establish a foundation for the development of MV-based vaccines.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were grown at 37 °C in brain heart infusion (BHI) medium or on BHI agar. *Escherichia coli* strains were grown in Luria Broth (LB) medium or on LB agar at 37 °C. For plasmid maintenance, cultures were supplemented with 100 µg/ml of kanamycin or 50 µg/ml of ampicillin for *E. coli* and 10 µg/ml of chloramphenicol for *S. aureus*.

Preparation and Quantification of MVs

MVs were isolated from *S. aureus* culture supernatants. Briefly, overnight cultures were diluted 1:100 and inoculated into 100 ml BHI broth, followed by incubation at 37 °C with slow shaking for 6 h to allow cultures to reach an OD_{600} of 2.0. Culture supernatants were collected by centrifugation at $6000 \times g$ at 4 °C for 20 min to remove bacterial cells, and then filtered through 0.22 µm Millex syringe filters (Beyotime) to remove any remaining cell debris. Culture supernatants were then centrifuged at $200{,}000 \times g$ for 3 h at 4 °C, and the MV fractions were resuspended in phosphate-buffered saline (PBS) after two washes in PBS. MVs were stored as aliquots at -80 °C.

For quantitation, MV proteins were separated by 12% SDS-PAGE, and after electrophoresis the gel was stained with Coomassie Brilliant Blue R-250. Gel images were captured and the intensity of protein bands was measured using the image analysis application Quantity One (BioRad). Protein concentration was determined using an enhanced BCA Protein Assay Kit (Beyotime). Lipid

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Reference/source
Strains	Lab collection
S. aureus Newman	Provided by Professor Yu Lu
S. aureus Newman-SigB(Q225P)	[21]
S. aureus Newman-SigB(Q225P)-Δ*nuc*	This work
S. aureus Newman-SigB(Q225P)-Δ*nuc/nuc*	This work
E. coli DH5α	Lab collection
E. coli BL21 (DE3)	Lab collection
Plasmids	
pBT2	Provided by Professor Baolin Sun
pLI50	Provided by Professor Baolin Sun
pOS1	Lab collection
pET30a	Lab collection
pET30a-Nuc	This work

content was determined using the membrane-specific fluorescent lipophilic dye FM4-64 (Invitrogen).

Expression and Purification of Recombinant Nuc Protein and Production of Anti-Nuc Antibodies

Nuc recombinant protein was expressed and purified as described previously with some modifications [22]. Briefly, the *nuc* gene was amplified by PCR from the *S. aureus* Newman strain using specific primers (Supplementary Table S1) and cloned into the *Eco*R I/*Hin*d III sites of the pET30a expression vector using *E. coli* BL21 (DE3) as a host. Cells harboring the recombinant pET30a-Nuc plasmid were grown in fresh LB broth at 37 °C to an OD_{600} of 0.5 and induced with 0.5 mM IPTG at 37 °C for 5 h. Cells were harvested by centrifugation and lysed by sonication in a buffer containing 50 mM Tris-HCl and 200 mM NaCl (pH 8.0). Recombinant Nuc protein was purified from the supernatant using a Ni–NTA affinity column (GE). Protein purity was assessed by SDS-PAGE, and protein concentration was determined using a BCA Protein Assay Kit (Beyotime). Aliquots of the purified protein were stored at −80 °C.

For antibody preparation, mice were immunized by multipoint subcutaneous injection with 50 µg of purified Nuc protein formulated with an equal volume of complete Freund's adjuvant, and boosted at 14 and 28 days after the initial dose. One week after the final immunization, mice were anesthetized and blood was obtained by retro-orbital collection. Blood samples were centrifuged to separate serum. Sera were filtered through a 0.22 µm filter and stored at −80 °C.

SDS-PAGE and Western Blot Analysis

Exponential phase cultures of *S. aureus* (100 µl) were harvested and resuspended in 60 µl PBS, then lysed with 4 µl lysostaphin (1 mg/ml, Sigma) at 37 °C for 30 min. The lysed cells were mixed with 5 × SDS gel loading buffer and immersed in a boiling water bath for 10 min. After centrifugation, supernatants were loaded onto a 12% SDS-polyacrylamide gel and subjected to electrophoresis. Separated proteins in the gel were then electrophoretically transferred to PVDF membranes. The blots were blocked in 5% nonfat skim milk for 1 h at room temperature and then incubated overnight at 4 °C with mouse anti-Nuc or anti-SigB polyclonal antibodies. After washing with PBST (PBS containing 0.05% Tween-20), the membranes were incubated with HRP-conjugated goat anti-mouse secondary antibody (diluted 1:5000 in 5% non-fat skim milk) for 1 h at room temperature. The membranes were washed with PBST once more and developed with a chemiluminescent substrate.

Gene Knockout and Genetic Complementation

An isogenic Δ*nuc* mutant was generated in the *S. aureus* Newman-SigB(Q225P) background by allelic replacement using methods we described previously [23]. Genetic complementation was performed by transforming the Δ*nut* mutant strain with an *E. coli-S. aureus* shuttle plasmid pLI50 construct containing the *nuc* gene and its upstream promoter.

LacZ Reporter Analysis of *nuc* Promoter Activity

nuc promoter activity was measured using β-galactosidase assays as previously described, with some modifications [24]. First, the *nuc* gene promoter was fused to the *lacZ* reporter gene. The resulting pOS1-*nuc* reporter plasmid was transformed into the *S. aureus* RN4220 strain for modification, and then introduced into the wild-type Newman and the SigB(Q225P) mutant strains. Transformants containing the *nuc* promoter-*lacZ* fusion construct were cultured overnight and then diluted 1:100 in 1 ml fresh BHI broth containing 10 µg/ml chloramphenicol. After incubation for 6 h at 37 °C, the bacterial cells were prepared from 100 µl of culture by centrifugation and resuspended in 100 µl of AB buffer (100 mM KH_2PO_4, 100 mM NaCl, pH 7.0). The cell suspension was treated with 2 µl of 1 mg/ml lysostaphin (Sigma) for 30 min at 37 °C, followed by the addition of 900 µl of AB buffer containing 0.1% TritonX-100 (ABT). 50 µl was then mixed with 10 µl of 4-methylumbelliferyl-β-D-galactoside (4 mg/mL, Sigma) and incubated at room temperature for 1 h. Finally, a 20 µl aliquot was diluted in 180 µl of ABT solution, and the fluorescence intensity of the reaction mixture was measured at $Ex/Em = 365/445$ nm/nm.

Electrophoretic Mobility Shift Assays (EMSA)

DNA/protein binding assays were performed as previously described [21]. Briefly, the putative promoter region of the *nuc* gene was amplified by PCR using DNA from the *S. aureus* Newman strain as a substrate. The *saeR* gene promoter was also PCR-amplified and served as a non-specific DNA control. A constant amount of DNA (1 µg) was mixed with increasing amounts of purified SigB and SigB(Q225P) proteins (from 0 to 20 µg) in EMSA/Gel-Shift buffer (Beyotime). After incubation for 30 min at room temperature, mixtures were subjected to electrophoresis through 8% nondenaturing polyacrylamide gels. The gels were then stained with GelRed and imaged under UV light. ImageJ was used to measure the intensity of unbound DNA in the digital images.
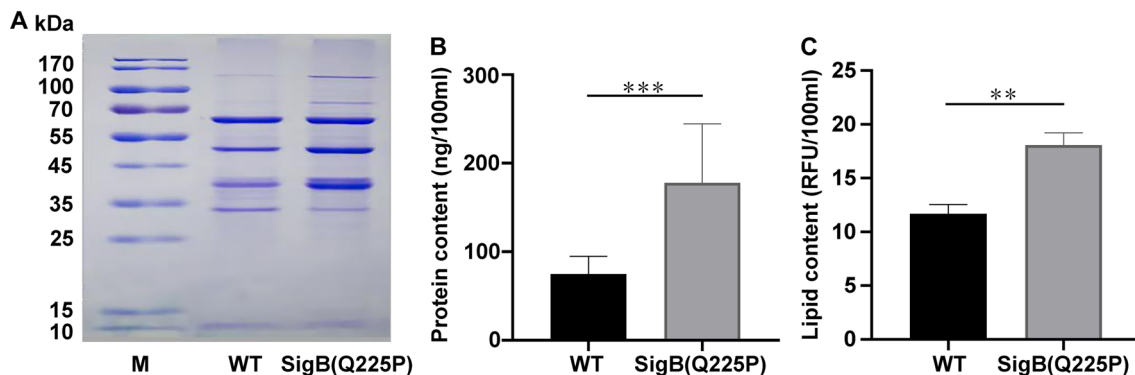
Statistical Analysis

All experiments were conducted in triplicate and repeated at least three times. Statistical analysis was performed using GraphPad Prism 8.0 and SPSS 23.0. An unpaired two-tailed Student's t test was used to assess differences between means. Differences were considered statistically significant at $P < 0.05$.

Results

### The SigB(Q225P) Mutation Promotes MV Production in *S. aureus*

To assess the effect of the SigB(Q225P) mutation on MV production in *S. aureus*, the wild-type Newman and SigB(Q225P) mutant strains were cultured under identical conditions. MVs were then extracted from the culture supernatants and subjected to SDS-PAGE. Protein bands from MVs derived from the SigB(Q225P) mutant were significantly more intense that those from the wild-type strain (Fig. 1A), suggesting that the SigB(Q225P) mutation stimulates MV production. The differences were confirmed by quantitative analysis of the protein and lipid contents in the MVs (Fig. 1B, C). The concentration of proteins in SigB(Q225P) mutant-derived MVs was 177.8 ± 19.2 ng/100 ml, approximately 2.4-fold greater than the concentration in the wild-type strain (74.9 ± 5.8 ng/100 ml). Similarly, the lipid content of MVs from the SigB(Q225P) mutant was 1.5-fold greater than in the wild-type strain (18.1 ± 1.1 vs. 11.7 ± 0.9 RFU/100 ml). Considering that the wild-type Newman and SigB(Q225P) mutant strains have similar growth rates [21], these data indicate that the SigB(Q225P) mutation promotes MV production in *S. aureus*.

Nuc Expression is Reduced in the SigB(Q225P) Mutant

Since SigB mutations affect Nuc expression in *S. aureus* [25, 26], a Western blot analysis was performed to determine Nuc protein levels in the SigB(Q225P) mutant. As shown in Fig. 2B, no significant differences in SigB levels were observed between the Newman and the SigB(Q225P) mutant strains, indicating that the Q225P mutation itself does not alter SigB expression. However, the SigB(Q225P) mutant has lower levels of Nuc protein compared to the wild-type strain (Fig. 2C), suggesting that the SigB(Q225P) mutation in Newman strain decreases Nuc expression.
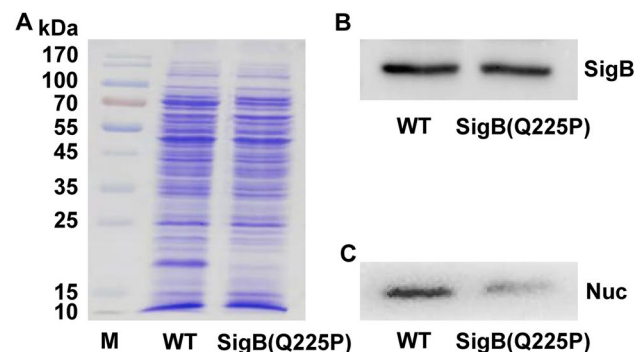
Fig. 2 The SigB(Q225P) mutation decreases Nuc expression. **A** The whole cell bacterial proteins of *S. aureus* strains were separated by 12% SDS-PAGE and loading was adjusted to equalize the intensity of the background protein banding patterns in the two lanes. **B** Western blot showing that the Q225P mutation does not alter SigB expression. **C** Western blot showing that the SigB(Q225P) mutation decreases Nuc expression

Fig. 1 MV production in *S. aureus* Newman and Newman-SigB(Q225P). **A** SDS-PAGE analysis of MVs extracted from an equal volume of bacterial cultures of the wild-type Newman and Newman-SigB(Q225P) strains. **B** Quantitative analysis of protein content of MVs (***$P < 0.001$). **C** Quantitative analysis of lipid content of MVs (**$P < 0.01$)

Deletion of *nuc* Increases MV Production

To assess the role of Nuc in MV production, we constructed a markerless *nuc* deletion mutant (Δ*nuc*) in the Newman-SigB(Q225P) background, and a complemented strain (Δ*nuc/nuc*) using the shuttle plasmid pLI50. MVs were prepared from cultures of both strains and compared by SDS-PAGE. As shown in Fig. 3A, proteins from MVs produced by the Δ*nuc* mutant were more intensely stained than those derived from Newman-SigB(Q225P). Consistent with this result, quantitative analysis of protein and lipid contents showed that Δ*nuc* releases a higher level of MVs compared with the parental Newman-SigB(Q225P) strain (Fig. 3B, C). Functional complementation of *nuc* in the Δ*nuc* mutant restored MV formation at a comparable level to that of Newman-SigB(Q225P). These results demonstrate that Nuc has a functional role in MV production in *S. aureus*.

## The SigB(Q225P) Mutation Directly Affects Nuc Expression

To test whether the SigB(Q225P) mutation is directly responsible for the reduction of Nuc expression, a *nuc* promoter-*lacZ* fusion reporter system was constructed to investigate *nuc* gene regulation in the wild-type Newman and the SigB(Q225P) mutant strains. β-galactosidase assays revealed that *nuc* promoter activity in the SigB(Q225P) mutant decreased by 15% compared with that in the wild-type strain (Fig. 4A). To better examine the mechanism underlying the down-regulation effect, a gel-shift assay (EMSA) was performed using purified SigB and SigB(Q225P) proteins. The results showed that both proteins bind DNA fragments containing the promoter region of the *nuc* gene in a concentration-dependent manner (Fig. 4B, C). However, the wild-type

SigB protein has a higher DNA-binding activity, as shown by its ability to reduce the amount of free DNA probe more rapidly and to lower levels. These results indicate that the Q225P mutation diminishes the ability of SigB to bind to the *muc* gene promoter.

Discussion

Because research on bacterial MVs has primarily focused on Gram-negative bacteria [27, 28], knowledge of the mechanisms of MV biogenesis in Gram-positive strains is limited. Unlike Gram-negative bacteria, Gram-positive strains contain only one inner membrane and a highly cross-linked peptidoglycan layer, suggesting that MV formation in Gram-positive bacteria depends on endolysin-triggered cell lysis [29]. Since Lee and colleagues announced the discovery of MVs produced by the Gram-positive bacterium *S. aureus* [2], several attempts have been made to explore the mechanism of MV biogenesis in this opportunistic pathogen. Kosgodage et al. found that MV production is regulated by peptidylarginine deiminase (PAD)-mediated pathways in *S. aureus*, and that PAD-specific inhibitors reduce the release of MVs [30]. Lee's group reported that staphylococcal α-type phenol-soluble modulins (PSMs) promote *S. aureus* MV production by disrupting the cytoplasmic membrane [31]. The extent of peptidoglycan cross-linking appears to modulate the efficiency of MV biogenesis. Treatment with subinhibitory concentrations of penicillin G or inactivation of the peptidoglycan biosynthesis-associated *pbp4* or *tagO* genes significantly increases MV size and yield [31].

In this study, we demonstrated that the general stress response regulator SigB plays an important role in production of MVs in *S. aureus*. A single amino acid substitution
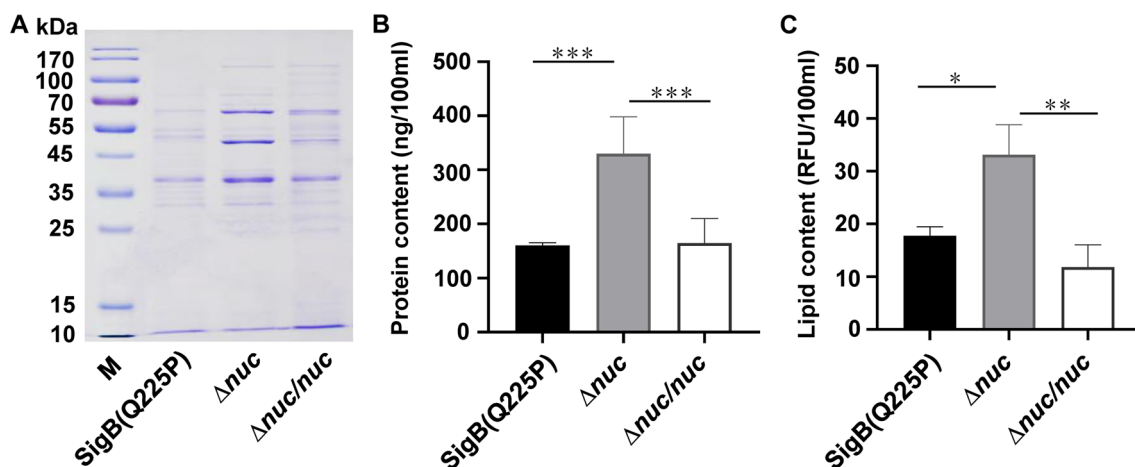
Fig. 3 Inactivation of *nuc* promotes MV formation. **A** SDS-PAGE analysis of MVs extracted from Newman-SigB(Q225P), the Δ*nuc* mutant, and the complemented strain Δ*nuc/nuc*. **B** Quantitative analysis of protein content from MVs derived from three different strains (***$P < 0.001$). **C** Quantitative analysis of lipid content of MVs from three different strains (*$P < 0.05$; **$P < 0.01$)
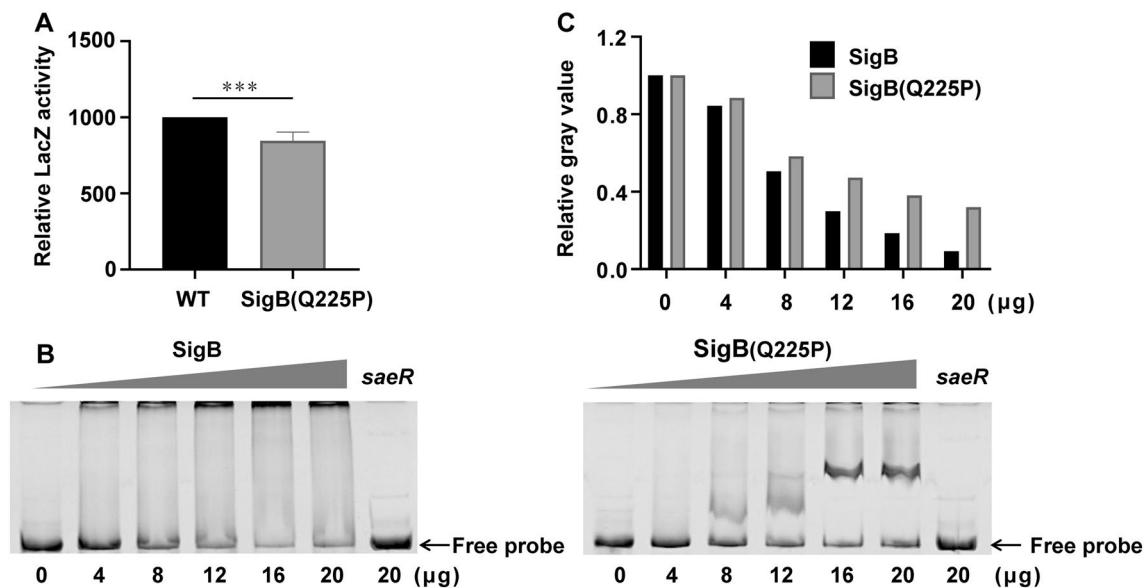
Fig. 4 The SigB(Q225P) mutation directly affects Nuc expression. **A** β-galactosidase assays. The activity of the *nuc* gene promoter was determined in the wild-type Newman and the SigB(Q225P) mutant strains with a *lacZ* reporter gene system. Data are presented as means + standard deviations (SD) (***$P < 0.001$). The error bar is not visible for the WT value at the scale used in this figure. **B** Band shift

assays. A constant amount (1 μg) of DNA probe containing the *nuc* gene promoter was incubated with the increase in concentrations of SigB or SigB(Q225P) proteins. The *saeR* gene promoter was used as a non-specific DNA control. **C** The intensity of the free probe in each lane was quantified using ImageJ. Results shown are representative of three independent experiments

in SigB (Q225P) significantly induces MV production, as measured by SDS-PAGE and analyses of MV protein and lipid content. Given that bacterial species differ in their mechanisms of vesiculogenesis, the widely conserved transcription regulator SigB is a likely common genetic determinant for MV biogenesis in Gram-positive bacteria. The underlying mechanisms may be related to changes in cell membrane fluidity and cell wall integrity [5, 32, 33]. In *L. monocytogenes*, MVs derived from a *sigB* deletion mutant were deformed and less abundant in comparison with those from the wild-type strain [17]. Mitsuwan et al. found that rhodomyrtone reduced SigB activity in *S. aureus* during exponential growth, resulting in significantly lower MV production [34].

Because the Q225P missense mutation occurs in the predicted DNA-binding domain of SigB, it is unsurprising that the DNA-binding properties of the mutant are affected. As expected, results from the β-galactosidase reporter and gel-shift assays demonstrate that the SigB(Q225P) mutation weakens the ability of SigB to bind the *nuc* gene promoter. This also causes a significant reduction in Nuc expression. Nuc is a specific virulence determinant of *S. aureus* that degrades DNA and RNA, and plays an important role in the development of tissue damage and dissemination of bacteria in an infected host [35, 36]. In a previous study, we demonstrated that decreased Nuc expression causes the accumulation of extracellular DNA (eDNA) and promotes biofilm formation [21]. Zhu et al. also reported that deletion of the

SigB-dependent *spoVG* operon reduces nuclease activity in the *S. aureus* Newman strain, leading to high levels of eDNA, cell aggregation, and biofilm formation [26, 37]. It has been suggested that the accumulation of eDNA on the surface of MVs derived from *Helicobacter pylori* may provide a bridging function in MV–MV and MV–cell interactions, thereby promoting MVs aggregation and preventing eDNA degradation by nucleases [38]. Since nucleic acids are one of the major components of bacterial MVs, we hypothesize that down-regulation of Nuc will probably impair the DNA/RNA digestion capability of *S. aureus*, thus causing nucleic acids to accumulate on the MVs surface, which may further facilitate the aggregation of MVs. In addition, the redundant nucleic acids in *S. aureus* cells may also result in stress that ultimately induces MV production. This is consistent with our evidence that deletion of *nuc* significantly increases MV formation in *S. aureus*. Additional experiments will be required to define the precise mechanism of action of Nuc in the production of MVs.

Author Contributions Conceived the study and designed the experiments: ML and RZ; performed the experiments: LQ and YY; analyzed

Data Availability All data generated or analyzed during the study are included in the published paper.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Medical University (No. AMUWEC2019429) and complied with institutional animal welfare and ethical guidelines.

Consent to Participate Not applicable.

Consent for Publication All authors have approved the manuscript and agree with its submission for publication.

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