SpoVG Modulates Cell Aggregation in *Staphylococcus aureus* by Regulating *sasC* Expression and Extracellular DNA Release

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**ABSTRACT**  Biofilm formation is involved in numerous *Staphylococcus aureus* infections such as endocarditis, septic arthritis, osteomyelitis, and infections of indwelling medical devices. In these diseases, *S. aureus* forms biofilms as cell aggregates interspersed in host matrix material. Here, we have observed that the level of cell aggregation was significantly higher in the isogenic spoVG-deletion strain than in the wild-type strain. Reverse transcription-quantitative PCR data indicated that SpoVG could repress the expression of *sasC*, which codes for *S. aureus* surface protein C and is involved in cell aggregation and biofilm accumulation. Electromagnetic mobility shift assay demonstrated that SpoVG could specifically bind to the promoter region of *sasC*, indicating that SpoVG is a negative regulator and directly represses the expression of *sasC*. In addition, deletion of the SasC aggregation domain in the spoVG-deletion strain indicated that high-level expression of *sasC* could be the underlying cause of significantly increased cell aggregation formation. Our previous study showed that SpoVG is involved in oxacillin resistance of methicillin-resistant *S. aureus* by regulating the expression of genes involved in cell wall synthesis and degradation. In this study, we also found that SpoVG was able to negatively modulate the *S. aureus* drug tolerance under conditions of a high concentration of oxacillin treatment. These findings can broaden our understanding of the regulation of biofilm formation and drug tolerance in *S. aureus*.

**IMPORTANCE** This study revealed that SpoVG can modulate cell aggregation by repressing *sasC* expression and extracellular DNA (eDNA) release. Furthermore, we have demonstrated the potential linkage between cell aggregation and antibiotic resistance. Our findings provide novel insights into the regulatory mechanisms of SpoVG involved in cell aggregation and in biofilm development and formation in *Staphylococcus aureus*.

**KEYWORDS**  *Staphylococcus aureus*, SpoVG, transcriptional regulation, cell aggregation

*Staphylococcus aureus* is a major human pathogen and is responsible for a variety of chronic and relapsing infections such as sepsis, osteomyelitis, endocarditis, toxic shock, and infections of implanted devices (1, 2). Bacterial biofilms are the matrix-enclosed structures that comprise bacterial cells, extracellular matrix proteins, carbohydrates, and extracellular DNA (eDNA) and adhere to biological or nonbiological surfaces (3, 4). Biofilm formation is often regarded as a virulence factor and plays a significant role in the chronic infectious process, since bacterial cells in the biofilm can escape host immune attack and resist antibiotic treatment. Biofilm development and formation generally consist of five stages, including attachment, multiplication, exodus, maturation, and dispersal (4). During the attachment stage, planktonic cells adhere to surfaces of biological or nonbiological materials and proliferate into sticky aggregations. However, the successional biofilm growth pattern implies a high variability in mushroom structure development and surface coverage (3). A previous study had
pointed to the biological advantage of cell aggregations over single cells during biofilm formation (5). *S. aureus* cell aggregation is a biological process through which cells bind to matrix proteins and form stable clumps to evade host defenses and to adapt to antibiotic stress. In aggregate communities, *S. aureus* cells adjust the distribution of their adhesins and surface proteins to promote their tolerance of hazardous environments (5, 6). Biofilm development and formation can be modulated by various regulatory factors such as sigma B (7), the Agr system (7), SaeRS (8, 9), SarA (8), and MgrA (10, 11), but the regulatory mechanisms of cell aggregation remain largely unknown.

In *S. aureus*, SpoVG is a global transcriptional regulator and binds to the DNA region that contains a characteristic TAATTT/A motif (12). SpoVG can modulate the production of capsule, extracellular nuclease, protease, and lipase (13–15) and the emergence of methicillin and glycopeptide resistance of methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA) (13, 16).

In this study, we found that cell aggregation levels were significantly increased in the *S. aureus* spoVG-deletion strain compared to the wild-type (WT) strain. In addition, reverse transcription-quantitative PCR (RT-qPCR) data identified a potential target gene, sasC. By introducing the *spoVG sasC* double mutant, we demonstrated that SpoVG could modulate cell aggregation by repressing sasC expression and eDNA release. Our results have further demonstrated that cell aggregation is linked with oxacillin tolerance.

**RESULTS**

**The spoVG-deletion strain exhibits stronger cell aggregation.** During the growth of *S. aureus*, we found a significant difference in bacterial behavior between the WT and *spoVG*-deletion strains. The *spoVG*-deletion strain exhibited cell clumps after being grown for 3, 6, 9, and 12 h in transparent glass tubes compared with the WT strain, and the alteration could be reversed by *spoVG* complementation (Fig. 1A). When grown in flat-bottomed conical flasks, cells of the *spoVG*-deletion strain gathered together and formed a hard-to-disperse net structure (Fig. 1B). Since the sedimentation of the clumps formed in the *spoVG*-deletion strain resulted in a clearing of the supernatant, a time course of the supernatant of the WT and *spoVG*-deletion strains over 20 h was used to quantify cell aggregation. The *spoVG*-deletion strain displayed a supernatant variation significantly different from the results seen with the WT and *spoVG*-complemented strains (Fig. 1C). In addition, fluorescence microscopy was employed to determine the morphological features of the *spoVG*-deletion strain with the fluorescent shuttle plasmid pALC. As a result, fairly apparent cell clusters were formed in the *spoVG*-deletion strain after growth overnight (Fig. 1D). These data indicate that SpoVG plays a significant role in the regulation of cell aggregation.

**Cell aggregation of the spoVG-deletion strain is protease sensitive.** To analyze the components of cell aggregation that formed in the *spoVG*-deletion strain, we added protease K and trypsin into the cell aggregation culture, and phosphate-buffered saline (PBS) was added as a control treatment. Cell clusters were dissolved after digestion with protease K and trypsin (Fig. 2), suggesting that the cell clusters formed in the *spoVG*-deletion strain may involve variations in expression of bacterial surface proteins.

**SpoVG represses sasC expression.** SpoVG is a global transcriptional factor and site-specific DNA-binding protein in *S. aureus* (14–16). To get a deeper understanding of the regulatory role of SpoVG in cell aggregation, we performed RT-qPCR to measure the expression levels of 17 potential target genes at the aggregation-formation stage. These genes included those encoding several members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and the coagulating proteins in *S. aureus*, Coa and vWbp. The results showed that the transcriptional levels of 12 genes were altered in the *spoVG*-deletion strain, including 3 upregulated genes (*ebhB*, *isdA*, and *sasC*) and 9 downregulated genes (*clfB*, *sdrC*, *sraP*, *sasG*, *spa*, *sdrE*, *emp*, *eap*, and *coa*). Among these genes, the mRNA levels of *ebhB* and *sasC* were significantly increased in the *spoVG*-deletion strain compared with those in the WT strain (Fig. 3A).
The *ebh* gene encodes the giant staphylococcal surface protein (GSSP), which is membrane anchored and protrudes from the cell surface in a fiber-like manner and therefore inhibits cell-cell interactions (17, 18). The *isdA* gene, which encodes IsdA, has been reported to inhibit bacterial biofilm formation. The *sasC* gene encodes *S. aureus*...
surface protein SasC, which is involved in cell aggregation and biofilm-accumulation processes (19).

RT-qPCR data indicated that SpoVG may modulate cell aggregation by repressing expression of cell wall proteins, especially that of SasC. Meanwhile, RT-qPCR data showed that the significantly increased level of expression of sasC in the spoVG-deletion strain could be reversed by spoVG complementation (Fig. 3B). To verify the regulatory role of SpoVG in the expression of sasC, we constructed a sasC promoter-lacZ fusion reporter plasmid and determined the levels of β-galactosidase activities in the WT and spoVG-deletion strains. As predicted, the promoter activity of sasC was increased in the spoVG-deletion strain compared with that in the WT strain (Fig. 3C), suggesting that SpoVG is a repressor of sasC.

Electrophoretic mobility shift assay (EMSA) was performed to determine whether SpoVG can specifically bind to the sasC promoter region. A shifted band was visible after incubation of SpoVG with the biotin-labeled DNA probe containing the sasC promoter region (Fig. 3D). This shifted band disappeared when unlabeled sasC promoter DNA was added but did not disappear in the presence of unlabeled hu DNA as the unspecific competitor, demonstrating that SpoVG can bind to the sasC promoter region specifically. Taken together, these results indicated that SpoVG could repress the transcription of sasC by directly binding to its promoter region. Furthermore, RT-qPCR data showed that the significantly increased expression of ebhB in the spoVG-deletion strain could be reversed by the spoVG complementation (Fig. 3E).

The SasC aggregation domain was able to form a superpolymer in vitro. The SasC protein consists of the N-terminal signal peptide, the aggregation domain, 17 DUF1542 domains, and the C-terminal LPXTG cell wall-anchored motif (Fig. 4A). To investigate the character of the SasC aggregation domain, we expressed this region with a His tag. The SDS-PAGE and native-PAGE results showed that the SasC aggregation domain may exist in polymer form (Fig. 4B). Fast protein liquid chromatography (FPLC) coupled with multiangle light scattering (FPLC-MALS) analysis results revealed that the SasC aggregation domain indeed formed a superpolymer in vitro, and the molecular weight was about 1,610 kDa (Fig. 4C), implying that the SasC aggregation domain formed more than 35 polymers.

Increased sasC transcription and eDNA release resulted in cell aggregation in the spoVG-deletion strain. To investigate the function of SasC in cell aggregation in the spoVG-deletion strain, we constructed a SasC aggregation domain deletion in the spoVG-deletion strain (Fig. 5A). Compared with the strong evidence of cell clumps seen in the spoVG-deletion strain after growth for 3, 6, 9, or 12 h in transparent glass tubes, the SasC aggregation domain deletion in the spoVG-deletion strain showed an obvious reduction in the level of cell clumps (Fig. 5B). The optical density at 600 nm (OD$_{600}$) of supernatant of the WT, spoVG-deletion, spoVG-complemented, and spoVG sasC double mutant strains showed that the cell aggregation level was decreased to a large extent in the spoVG sasC double mutant strain compared with the spoVG-deletion strain.
SpoVG Regulates Cell Aggregation in S. aureus

Fluorescence microscopy showed similar results (Fig. 5D). These data suggest that SpoVG may modulate cell aggregation by repressing the expression of sasC.

It has been known that eDNA can act as an adhesive and thus strengthen biofilms, which is important for S. aureus biofilm formation. To investigate whether increased cell
aggregation of the spoVG-deletion strain is eDNA dependent, we determined the amount of eDNA present in the cell aggregation structure. The average amount of eDNA present in cell aggregation of spoVG-deletion strain was ~10-fold that represented in the WT strain, and the amount of eDNA in the spoVG-complemented strain was restored to the original level (Fig. 6A), implying a critical role of eDNA in the development of cell aggregation in the spoVG-deletion strain. Moreover, after DNase I treatment, the aggregation of the spoVG-deletion strain weakened or disappeared (Fig. 6B). These results allow us to conclude that high levels of sasC expression and eDNA release can lead to cell aggregation in the spoVG-deletion strain.

Cell aggregation is associated with oxacillin susceptibility and cell survival. The spoVG-deletion strain exhibited significantly decreased oxacillin resistance compared with the WT strain, and the phenotype could be restored by spoVG complementation (Fig. 7A and B); these results are consistent with our previous study (16). Moreover, the spoVG sasC double mutant strain exhibited increased oxacillin resistance compared with that of the spoVG-deletion strain (Fig. 7A and B).

We also tested cell survival of the WT, spoVG-deletion, spoVG-complemented, sasC mutant, and spoVG sasC double mutant strains in Mueller-Hinton (MH) broth exposed to 6.4 mg/ml of oxacillin (representing approximately 100× MIC for the WT strain) for 24 or 48 h. The spoVG-deletion strain exhibited significantly increased drug tolerance after treatment with a high concentration of oxacillin for 24 h compared with the WT
strain, and the phenotype could be restored by the spoVG complementation (Fig. 7C). Moreover, the spoVG sasC double mutant strain exhibited decreased drug tolerance compared with that of the spoVG-deletion strain (Fig. 7C). Oxacillin treatment for 48 h showed similar results (Fig. 7D). These results indicated that cell aggregation is tightly associated with oxacillin susceptibility and drug tolerance under conditions of treatment with oxacillin at a high concentration.

DISCUSSION

Bacteria grow and proliferate depending on their surroundings. In response to certain circumstances, S. aureus can subsist either as single and independent cells or organized in aggregates such as biofilms. Previous studies have shown that in natural environments and during infections, biofilms are seeded by cell aggregations and individual bacterial cells (5), but an explanation of how aggregation formation is controlled remains elusive. In our experiments, we observed that the spoVG-deletion S. aureus strain displayed a high level of cell aggregation, which disappeared after digestion with protease.

Further, we have demonstrated that SpoVG could modulate cell aggregation by repressing sasC expression in S. aureus. SasC is one of the S. aureus surface adhesins that...
has a typical LPXTG cell wall anchor motif and has been reported to be involved in cell aggregation and biofilm accumulation. Our data revealed that the SasC aggregation domain was able to play a significant role in cell aggregation in the spoVG-deletion strain. Furthermore, our data indicated that SpoVG is involved in transcriptional regulation of ebbB, and the underlying mechanism requires to be further studied.

*S. aureus* cells of biofilms are held together in clusters by the electrostatic net formed by eDNA. However, the details of the mechanism by which eDNA is released from *S. aureus* cells remain unknown. Here, we have revealed a negative regulatory effect of SpoVG on eDNA and further revealed that eDNA plays an important role in the cell aggregation process.

Overall, this report can facilitate deeper understanding of the regulatory mechanisms of SpoVG involved in cell aggregation in *S. aureus*, and the function of SpoVG in biofilm formation needs to be further studied. We have also revealed the potential linkage between cell aggregation and antibiotic resistance, and the exact mechanism needs to be further investigated (Fig. 8).

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* Trans1-T1 and BL21(DE3) were grown in Luria broth (LB) medium (Oxoid) with appropriate antibiotics (150 μg/ml ampicillin sodium salt or 50 μg/ml kanamycin sulfate). *S. aureus* and its derivative strains were grown in tryptic soy broth (TSB) medium (BD) with 15 μg/ml chloramphenicol at 37°C with shaking at 220 rpm (~16 × g). Constructed plasmids were purified from *E. coli* Trans1-T1 and transformed into *S. aureus* strain N315 by electroporation. The media were solidified with 1.5% (wt/vol) agar when needed.

**Construction of the sasC single mutation and spoVG sasC double mutation.** To obtain a single mutant with a mutation of the sasC gene and a spoVG sasC double mutant, the plasmid pBTs and an appropriate protocol was used as described previously (20). Briefly, DNA fragments corresponding to the upstream and downstream regions of sasC aggregation domain were amplified by PCR, using *S. aureus* strain N315 genomic DNA as the template. The PCR products were ligated by overlap PCR to form an up-down fragment, which was purified, digested with KpnI and SalI, and cloned into the temperature-sensitive shuttle plasmid pALC containing a temperature-sensitive *S. aureus* origin of replication, a chloramphenicol resistance cassette, and a suicide gene for plasmid maintenance or selection. The resulting plasmid containing the upstream and downstream fragments in tandem was then amplified in *E. coli* Trans1-T1. The recombinant pBTs was then extracted from *E. coli* and transformed into *S. aureus* strain N315 by electroporation at 2.5 kV for modification and subsequently introduced into *S. aureus* strain N315. The transformants that had allelic replacement of sasC were selected on tryptic soy agar (TSA) containing 100 ng/μl anhydrotetracycline (ATC) and were further verified by PCR and DNA sequencing. The spoVG sasC double mutant was constructed using a similar strategy by introducing the sasC mutant plasmid into the spoVG-deletion strain and further confirmed by PCR and DNA sequencing.

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**FIG 6** eDNA was involved in the process of bacterial cell aggregation. (A) The level of eDNA release of the WT strain and spoVG-deletion strain. DNase I treatment was used as a negative control. (B) The cell clumps processed with DNase I treatment for 0, 2, 4, and 8 h were visualized by fluorescence microscopy. The fluorescent shuttle plasmid pALC was transformed into the WT, spoVG-deletion, and spoVG sasC double mutant strains. (Left) WT strain. (Right) spoVG-deletion strain.
Fluorescence microscopy. To further measure cell aggregation of *S. aureus*, fluorescence microscopy was performed. First, cultures of different strains carrying a pALC fluorescence shuttle plasmid with green fluorescent protein (GFP) were grown in TSB for 12 h at 37°C, and then the green fluorescence of the samples excited by blue light were viewed with a fluorescence microscope.

RNA isolation, cDNA generation, and reverse transcription-quantitative PCR. For total RNA extraction, the overnight cultures of *S. aureus* were diluted 1:100 in TSB with appropriate antibiotics and grown to the early exponential phase (OD$_{600}$ of 0.6), midexponential phase (OD$_{600}$ of 2), and stationary phase (OD$_{600}$ of 6). *S. aureus* cells were collected by centrifugation and processed with 900 μl RNAiso plus (TaKaRa) in combination with 0.1-mm-diameter silica-zirconia beads in a FastPrep-24 automated system (MP Biomedicals Solon). The residual DNA was removed with RNase-free recombinant DNase I (TaKaRa; FIG 7 Cell aggregation is associated with oxacillin susceptibility and cell survival. The oxacillin susceptibility of the WT, spoVG-deletion, spoVG-complemented, and spoVG sasC double mutant strains was determined. (A) Results determined by the oxacillin gradient dilution method. (B) Results determined by the plate count method. The WT, spoVG-deletion, spoVG-complemented, and spoVG sasC double mutant strains were grown to the stationary phase and treated with 100× MIC of oxacillin (6.4 mg/ml), and survival rates were determined following 24 or 48 h of incubation. (C) Results from 24 h of incubation. (D) Results from 48 h of incubation. Values represent results from three biological replicates ± SEM. Statistical values were determined by the use of one-way ANOVA and the F test to compare variances. ***, P < 0.001; ****, P < 0.0001.
For reverse transcription, cDNA was synthesized with a PrimeScript first-strand cDNA synthesis kit (TaKaRa) using random primers. Reverse transcription-quantitative PCR (RT-qPCR) was performed with SYBR Premix Ex Taq (TaKaRa) using a StepOne real-time PCR system (Applied Biosystems) and LC96 real-time PCR system (Roche). The quantity of cDNA was measured by the threshold cycle ($2^{-\Delta\Delta CT}$) method with $hu$ as the reference gene (21) and the corresponding control sample as the run calibrator. The primers used in this study are listed in Table 2. All the RT-qPCR assays were repeated at least three times.

**Construction of the LacZ reporter vector.** To construct reporter plasmid pOSsasC, the DNA fragment containing the $sasC$ promoter region was amplified from $S. aureus$ strain N315 genomic DNA using primers listed in Table 2. The fragment was digested with BamHI and EcoRI and cloned into the shuttle vector pOS1. The reporter plasmid was transformed first into $S. aureus$ RN4220 for modification and then into the WT and spoVG-deletion strains.

**β-Galactosidase activity assay.** Analysis of $β$-galactosidase activity was performed as previously described (22). For $β$-galactosidase assay with o-nitrophenyl-$β$-D-galactopyranoside (ONPG) as the substrate, the WT and spoVG-deletion strains were grown to the stationary phase, centrifuged, and then resuspended in $100 \mu$l of ABT-LSA buffer (60 mM K$_2$HPO$_4$, 40 mM KH$_2$PO$_4$, 100 mM NaCl, 0.1% Triton X-100, 50 μg/ml lysostaphin). The samples were maintained under shaking conditions at 37°C until thoroughly lysed. Then, $100 \mu$l ABT buffer and $50 \mu$l ONPG were added to initiate the reaction. The samples were incubated at 37°C until a yellow color became apparent, and 1 ml Na$_2$CO$_3$ (1 M) was then added to stop the reaction. Sample absorbance was read at 420 nm, and units were calculated using the following formula: $\text{units} = (1,000 \times \text{OD}_{420})/(T \times V \times \text{OD}_{600})$ (where $T$ [measured in minutes] was the
TABLE 1 Strains and plasmids used in this study

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**Note:** lowercase letters indicate restriction sites. Underlined letters indicate complementary sequences used for overlap PCR ligation.

incubation time and V [in milliliters] was the volume of culture used in the assay). The assays were repeated at least three times.

**Electrophoretic mobility shift assay.** The biotin-labeled DNA fragment pΔasC containing sasC promoter region was amplified from *S. aureus* strain N315 genomic DNA using primers listed in Table 2. The amplified biotin-labeled pΔasC fragment was incubated at 25°C for 30 min with various amounts of SpoVG-P and SpoVG in incubation buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0) for electrophoretic mobility shift assay (EMSA). After incubation, the mixtures were electrophoresed in a 4% native polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer and then transferred to a nylon membrane in 0.5× TBE buffer. The band shifts were detected using a chemiluminescent nucleic acid detection module (Thermo Fisher) and were imaged with an ImageQuant LAS 4000 system (GE Healthcare). The unlabelled fragment of promoter was added as the specific competitor (SC). The unlabelled ~100-bp DNA fragment derived from the open reading frame (ORF) of *hu* was added as the nonspecific competitor (NC).

**Purification and detection of eDNA.** Purification and detection of eDNA were performed as previously described (22). Samples of overnight bacterial culture were collected by centrifugation, and the samples were first treated with 5 µg/ml proteinase K at 37°C for 1 h. Following treatment, bacterial samples were centrifuged and the supernatant was filtered using a 0.22-µm pore-size polyether sulfone membrane to remove the bacterial cells. The extracellular DNA (eDNA) was extracted through the use of a phenol-chloroform-isoamyl alcohol DNA extraction method. The aqueous phase was added with sodium acetate at a final concentration of 0.3 M and 0.6 volumes of isopropanol. The eDNA precipitation was washed twice with 75% (vol/vol) ethanol, air-dried, and dissolved in 500 µl nuclease-free water.

**Cloning, expression, and purification of recombinant SasC aggregation domain and FPLC-MALS.** The DNA region encoding the SasC aggregation domain was amplified by PCR using *S. aureus* RN220 genomic DNA as a template. The amplified biotin-labeled pΔasC fragment was incubated at 25°C for 30 min with various amounts of SpoVG-P and SpoVG in incubation buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0) for electrophoretic mobility shift assay (EMSA). After incubation, the mixtures were electrophoresed in a 4% native polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer and then transferred to a nylon membrane in 0.5× TBE buffer. The band shifts were detected using a chemiluminescent nucleic acid detection module (Thermo Fisher) and were imaged with an ImageQuant LAS 4000 system (GE Healthcare). The unlabelled fragment of promoter was added as the specific competitor (SC). The unlabelled ~100-bp DNA fragment derived from the open reading frame (ORF) of *hu* was added as the nonspecific competitor (NC).

**TABLE 2 Primers used in this study**

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</table>

**Note:** lowercase letters indicate restriction sites. Underlined letters indicate complementary sequences used for overlap PCR ligation.
strain N315 genomic DNA as the template. The corresponding PCR product was digested by Ndel and XhoI and was then cloned into the pET-28a (+) vector, generating plasmid pETSaC-A. The resulting plasmid was transformed by DNA sequencing and then transformed into E. coli BL21(DE3), and the transformant was grown in LB medium with 50 μg/ml kanamycin at 37°C to an OD₆₀₀ of 0.4 to 0.6 and then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 37°C for additional 3 h. The cells were then harvested, resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0), and lysed by sonication. The His-tagged SasC aggregation domain protein was purified by the use of nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) and Superdex 200 Increase 10/300 GL column (GE Healthcare). SDS-PAGE and the bichromonic acid (BCA) assay were used to analyze the protein purity and concentration, respectively. The accurate molar mass of protein was measured by FPLC-MALS (Wyatt Technology).

Oxacillin susceptibility assay. The oxacillin susceptibility assay was performed as described by Clinical and Laboratory Standards Institute. Bacterial strains were serially diluted and plated on Mueller-Hinton agar with 2% NaCl containing increasing concentrations of oxacillin. CFU counts were determined after overnight incubation at 37°C.

Drug tolerance assay. The drug tolerance assay was performed as previously described (24). Bacterial strains were grown to the early exponential and stationary phase, and the cultures were serially diluted and plated on agar to determine the initial CFU. For drug tolerance detection, bacterial strains (early exponential and stationary phase) were treated with oxacillin for 24 and 48 h at 100× MIC (6.4 mg/ml). Following treatment, cultures were collected, washed with 0.9% NaCl to remove the oxacillin, and then serially diluted and spot plated to determine the posttreatment CFU. The drug tolerance was determined as follows: posttreatment CFU/initial CFU.

Statistical analyses. All experiments were performed in biological triplicate. Values are from three biological replicates ± SEM (standard errors of the means). Statistical values were determined by the use of Student’s t test (for two groups), analysis of variance (one-way analysis of variance [ANOVA], for more than two groups), and the F test to compare variances, with a P value of <0.05 considered significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

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