SpoVG modulates cell aggregation by regulating sasC expression and eDNA release in Staphylococcus aureus

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ABSTRACT

Biofilm formation is involved in numerous *Staphylococcus aureus* infections such as endocarditis, septic arthritis, osteomyelitis, and infections on in-dwelling medical devices. In these diseases, *S. aureus* forms biofilms as cell aggregates interspersed in host matrix material. Here, we have observed that cell aggregation was significantly higher in the isogenic spoVG-deletion strain compared with that of 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 has shown 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 have found that SpoVG can negatively modulate the *S. aureus* drug tolerance under 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 has revealed that SpoVG can modulate cell aggregation by repressing \textit{sasC} expression and 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, biofilm development and formation in \textit{Staphylococcus aureus}.

**Key words:** \textit{Staphylococcus aureus}, SpoVG, transcriptional regulation, cell aggregation

**INTRODUCTION**

\textit{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 non-biological 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 include five stages, attachment, multiplication, exodus, maturation, and dispersal (4). During the attachment stage, planktonic cells adhere to surfaces of biological or non-biological materials and proliferate into sticky aggregations. However, the successional biofilm growth pattern implies a high variability in mushroom.
structure development and surface coverage (5). A previous study has pointed to the advantage of cell aggregations over single cells during biofilm formation (6). *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 to hazardous environments (7, 8). Biofilm development and formation can be modulated by various regulatory factors such as Sigma B (9), Agr system (9), SaeRS (10, 11), SarA (10), and MgrA (12, 13), 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 (14). SpoVG can modulate the production of capsule, extracellular nuclease, protease, lipase (15-17), and emergence of methicillin- and glycopeptide-resistance of methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA) (15, 18).

In this study, we found that cell aggregation was significantly increased in the *S. aureus* spoVG-deletion strain compared with that of the wild-type (WT) strain. In addition, 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 forms 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 grown for 3, 6, 9, 12 hours in transparent glass tubes compared with the WT strain, and the alteration could be reversed by the 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 hours was detected to quantify cell aggregation. The spoVG-deletion strain displayed a significantly different supernatant variation compared with the WT and spoVG-complemented strains (Fig. 1C). In addition, a fluorescence microscopy was employed to determine the morphological feature 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 formed in the spoVG-deletion strain, we added proteinase K and trypsin into the cell aggregation culture, and PBS was added as a control treatment. Cell clusters were dissolved after digested with proteinase K and trypsin (Fig. 2A-B), suggesting that the cell clusters formed in the spoVG-deletion strain may involve the expression variation of bacterial surface proteins.
SpoVG represses *sasC* expression

SpoVG is a global transcriptional factor and site-specific DNA-binding protein in *S. aureus* (16-18). 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 include 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 up-regulated genes *ebhB*, *isdA* and *sasC*, and 9 down-regulated 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 of 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 (19, 20). The *isdA* gene, encodes IsdA, which 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 (21).

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

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 disappeared 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).

SasC aggregation domain can form a superpolymer in vitro

The SasC protein consists of the N-terminal signal peptide, the aggregation domain, 17 unknown function domain DUF1542, and the C-terminal LPXTG cell wall-anchored motif (Fig. 4A). To investigate the character of SasC aggregation domain, we expressed this region with his-tag. The SDS-PAGE and native-PAGE results showed that SasC aggregation domain may exist in polymer form (Fig. 4B). The fast protein liquid chromatography (FPLC) coupled with multi-angle light scattering (FPLC-MALS) analysis result revealed that SasC aggregation domain indeed formed a superpolymer
in vitro and the molecular weight was about 1610 kDa (Fig. 4C), implying that SasC aggregation domain formed higher than 35 polymers.

Increased sasC transcription and eDNA release result in cell aggregation in the spoVG-deletion strain

To investigate the function of SasC in cell aggregation in the spoVG-deletion strain, we constructed the SasC aggregation domain deletion in the spoVG-deletion strain (Fig. 5A). Compared with the strong cell clumps exhibiting in the spoVG-deletion strain after grown for 3, 6, 9, 12 hours in transparent glass tubes, the SasC aggregation domain deletion in the spoVG-deletion strain showed obvious reduction of cell clumps (Fig. 5B). The OD₆₀₀ 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 that of the spoVG-deletion strain (Fig. 5C). And the fluorescence microscopy showed the 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 strengthen biofilm, which is important for S. aureus biofilm formation. To investigate whether increased cell aggregation of the spoVG-deletion strain is eDNA-dependent or not, we detected 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 folds of that presented in the WT strain, and the amount of eDNA in the spoVG-complemented strain was restored (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 spoVG-deletion strain weakened or disappeared (Fig. 6B). These results allow us to conclude that high level of sasC expression and eDNA release may 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 the spoVG complementation (Fig. 7A-B), and these results are consistent with the previous study (18). Moreover, the spoVG sasC double mutant strain exhibited increased oxacillin resistance compared with that of the spoVG-deletion strain (Fig. 7A-B).

We also tested cell survival of the WT, spoVG-deletion, spoVG-complemented, sasC mutant, and spoVG sasC double mutant strains in MH broth exposed to 6.4 mg/ml of oxacillin (representing approximately 100×MIC for the WT strain) for 24 or 48 hours. The spoVG-deletion strain exhibited significantly increased drug tolerance after treated with high concentration of oxacillin for 24 hours 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 hours showed the similar results (Fig. 7D. These results indicated that cell aggregation is tightly associated with oxacillin susceptibility and drug tolerance under the high concentration oxacillin treatment.
DISCUSSION

Bacteria grow and proliferate depending on their surroundings. In response to a certain circumstance, *S. aureus* can subsist either as single and independent cells or organized in aggregates like biofilms.

Previous studies have shown that in natural environments and during infections, biofilms are seeded by cell aggregations and individual bacterial cells (6), but how aggregation formation is controlled remains elusive. In our experiments, we observed that the *spoVG*-deletion *S aureus* strain displayed severe 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 SasC aggregation domain could play a significant role in cell aggregation in the *spoVG*-deletion strain.

Furthermore, our data have indicated that SpoVG is involved in transcriptional regulation of *ebhB*, and the underlying mechanism requires to be further studied.

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

Overall, this study can facilitate us to add a deeper understanding of the regulatory
mechanisms of SpoVG involved in cell aggregation in \textit{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. 8A-B).

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains, plasmids, and growth conditions}

The bacterial strains and plasmids used in this study are listed in Table 1. \textit{Escherichia coli} Trans1-T1 and BL21 (DE3) were grown in Luria broth (LB) medium (Oxoid) with appropriate antibiotics (150 \(\mu\)g/ml ampicillin sodium salt or 50 \(\mu\)g/ml kanamycin sulfate). \textit{S. aureus} and its derivative strains were grown in tryptic soy broth (TSB) medium (BD) with 15 \(\mu\)g/ml chloramphenicol at 37° C with shaking at 220 rpm (~16 g). Constructed plasmids were purified from \textit{E. coli} Trans1-T1 and transformed into \textit{S. aureus} RN4220 as the initial recipient and then \textit{S. aureus} strain N315 by electroporation. The media were solidified with 1.5\% (w/v) agar when needed.

\textbf{Construction of the sasC single mutation and spoVG sasC double mutation}

To obtain a single mutant of the \textit{sasC} gene and \textit{spoVG sasC} double mutation, the plasmid pBTs and a protocol was used as described previously (22). Briefly, DNA fragments corresponding to the upstream and downstream regions of \textit{sasC} aggregation domain were amplified by PCR, using \textit{S. aureus} strain N315 genomic DNA as 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 pBTs 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 RN4220 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 TSB agar containing 100 ng/μl anhydrotetracycline (ATC), and 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.

Fluorescence microscopy

To further measure cell aggregation of S. aureus, a fluorescence microscopy was performed. First, cultures of different strains carrying a pALC fluorescence shuttle plasmid with GFP were grown in TSB for 12 hours at 37°C, then 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 (OD_{600}=0.6), mid-exponential (OD_{600}=2), and stationary (OD_{600}=6) phases. 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, 5U/μl). For reverse transcription, cDNA was synthesized with a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) using random primers. Reverse transcription-quantitative PCR (RT-qPCR) was performed with SYBR Premix Ex Taq (TaKaRa) using the StepOne Real-Time PCR System (Applied Biosystems) and LC96 Real-Time PCR System (Roche). The quantity of cDNA was measured by 2^ΔΔCt method with hu as the reference gene (23), 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 the 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 first transformed into *S. aureus* RN4220 for modification, and then the WT and spoVG-deletion strains.

**β-Galactosidase activity assay**

The β-galactosidase activity analysis was performed as previously described (24). For β-galactosidase assay with o-Nitrophenyl-b-D-galactopyranoside (ONPG) as the substrate, the WT and spoVG-deletion strains were grown to the stationary phase, centrifuged and then resuspended in 100 μl of ABT-LSA buffer (60 mM K2HPO4, 40 mM KH2PO4, 100 mM NaCl, 0.1% Triton X-100, 50 μg/ml lysostaphin). The samples
were treated under shaking conditions at 37°C until thoroughly lysed. Then, 100 μl ABT buffer and 50 μl ONPG were added to initiate the reaction. The samples were incubated at 37°C until a yellow color became apparent, and 1 ml Na₂CO₃ (1M) was added to stop the reaction. Sample absorbance was read at 420 nm and units were calculated as the following formula: units = (1,000×OD₄₂₀)/(T×V×OD₆₀₀). The assays were repeated at least three times. T (measured in minutes) was the incubation time, and V (in milliliters) was the volume of culture used in the assay.

**Electrophoretic mobility shift assay**

The biotin-labeled DNA fragment, psasC containing sasC promoter region was amplified from *S. aureus* strain N315 genomic DNA using primers listed in Table 2. The biotin-labeled psasC was incubated at 25°C for 30 minutes 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×TBE buffer and then transferred to a nylon membrane in 0.5×TBE buffer. The band shifts were detected using the Chemiluminescent Nucleic Acid Detection Module Kit (ThermoFisher), and imaged with the ImageQuant LAS 4000 (GE Healthcare). The unlabeled fragment of promoter was added as the specific competitor (SC). The unlabeled ~100 bp DNA fragment derived from the ORF of *hu* was added as the nonspecific competitor (NC).

**Purification and detection of eDNA**

Purification and detection of eDNA was performed as previously described (25). Overnight culture of bacterial samples were collected by centrifugation and the samples
were first treated with 5 μg/ml protease K at 37°C for 1 h. Following treatment, bacterial samples were centrifuged and the supernatant was filtered using 0.22 μm polyethersulfone membrane to remove the bacterial cells. The extracellular DNA (eDNA) was extracted through phenol-chloroform-isoamyl alcohol DNA extraction method. The aqueous phase was added with sodium acetate at a final concentration of 0.3M and 0.6 times volume 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* strain N315 genomic DNA as template. The corresponding PCR product was digested by NdeI and XhoI, and then ligated into the pET-28a (+) vector, generating the plasmid pETSasC-A. The resulting plasmid was verified by DNA sequencing, 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 OD$_{600}$ of 0.4-0.6, and then induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at 37°C for additional 3 hours. 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 Ni-NTA resin (Qiagen) and Superdex™ 200 increase 10/300 GL column (GE Healthcare). SDS-PAGE and the 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).
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. The colony-forming units (CFU) were determined after overnight incubation at 37°C.

Drug tolerance assay

The drug tolerance assay was performed as previously described (26). 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 the drug tolerance detection, bacterial strains (early exponential and stationary phase) were treated with oxacillin for 24 and 48 hours 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 post-treatment CFU. The drug tolerance was determined as follows: post-treatment CFU/initial CFU.

Statistical analyses

All experiments were performed in biological triplicates. Values are from three biological replicates ± SEM (the standard errors of the means). Statistical values were determined by the Student T test (for two groups), analysis of variance (one way 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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### Table 1 Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype | Reference or source |
|-------------------|-------------------|---------------------|
| **S. aureus**     |                   |                     |
| RN4220            | 8325-4, r'        | NARSA               |
| WT                | N315 HA-MRSA, SCCmec type II | NARSA |
| ΔspoVG           | N315 strain deletion of *spoVG* | (18) |
| ΔsasC            | N315 strain deletion of the aggregation domain of *sasC* | This study |
| ΔspoVGΔsasC     | N315 *spoVG* *sasC* double mutant | This study |
| **E. coli**      |                   |                     |
| Trans1-T1        | Host strain for cloning | TransGen |
| BL21(DE3)        | Express strain    | TransGen            |
| **Plasmid**      |                   |                     |
| pBTs             | Shuttle vector, temp sensitive, amp′ chl′ | (22) |
| pBTsΔsasC        | pBTs derivative, for *rot* deletion, amp′ chl′ | This study |
| pETSasC-A        | pET28a(+) derivative, with SasC aggregation domain, kan′ | This study |
| pETSpoVG         | pET28a(+) derivative, with ORF of *spoVG*, kan′ | (18) |
| pDuet-Stk1-SpoVG | pRSF-Duet derivative, co-express SpoVG and Stk1 kinase domain, kan′ | (27) |
| pOS1             | Shuttle vector, with *lacZ* ORF lacking first 6 amino acids, amp′ chl′ | (28) |
| pOSsasC          | POS1 derivative, harboring the *sasC* promoter and 18 bp of *sasC* coding sequence from strain N315, amp′ chl′ | This study |
| pL1S0            | Shuttle cloning vector, amp′ chl′ | (29) |
| pL1SpoVG         | pL1S0 derivative, harboring ORF of *spoVG* and its promoter, amp′ chl′ | (18) |

a. r−, restriction system negative; kan′, kanamycin resistant; amp′, ampicillin resistant; chl′, chloramphenicol resistant.

b. NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*. 
| Primer | Sequence (5’-3’)a | Application |
|--------|------------------|-------------|
| sasC-up-F-kpnI | GCCggtaccGAGAACAGACACACAGGA | sasC cell aggregation domain deletion |
| sasC-up-R | TGTTACGTACTGGTCGAC | sasC cell aggregation domain deletion |
| sasC-down-F | GTGCACAGTGAGAACATTACAAATCTACGACATTACGT | sasC cell aggregation domain deletion |
| sasC-down-R-salI | GCGgattcGTTGCGTTATCAACATCA | sasC cell aggregation domain deletion |
| psasC-F-EcoRI | GCCgattcTCCGTAATTTAATTCAT | pOSrot |
| psasC-6aa-BamHI | GCCgattcGTTTCTTTAACAAATTCAT | pOSrot |
| probe-psasC-F | TCCGTAATTTAATTTCAT | EMSA |
| probe-psasC-Biotin-R | TTTTCTTAAAATTTCAT | EMSA |
| RT-sasC-f | GCACCAGTGCAATTACAG | RT-qPCR |
| RT-sasC-t | AGACAGCCTTCGTAGG | RT-qPCR |
| sasC-aggre-NdeI-F | GGAATTCCatatgTACACGCGAATTCGATCC | sasC cell aggregation domain expression |
| sasC-aggre-Xhol-R | CCGcgcaggTTATCTATCAACCTCGGCTGTA | sasC cell aggregation domain expression |

a Lowercase letters indicate restriction sites. Underlined letters indicate complementary sequences used for overlap PCR ligation.
Fig 1. The spoVG-deletion strain displayed increased cell aggregation. (A) The WT, spoVG-deletion, and spoVG-complemented strains were grown in TSB medium. After 3h, 6h, 9h, 12h growth, the spoVG-deletion strain exhibited rapid cell clumping, resulting in a clearing of the supernatant. (B) The WT, spoVG-deletion, and spoVG-complemented strains were grown in flat-bottomed conical flasks. The spoVG-deletion strain exhibited cell-net-structure after incubating 12 hours at room temperature. (C) After overnight growth, the WT, spoVG-deletion, and spoVG-complemented strains were adjusted to OD$_{600}$=2 and incubated at room temperature. The OD$_{600}$ of the supernatant was detected within 20 hours. Values are from three biological replicates ± SEM. Statistical values were determined by the Student T test and the F test to compare variances. * P < 0.05, ** P < 0.01. (D) The cell clumps formed in the spoVG-deletion were visualized using fluorescence microscopy. The fluorescent shuttle plasmid pALC was transformed into the WT and the spoVG-deletion strains. The cell aggregations were photographed after growth overnight. Above: the WT strain. Below: the spoVG-deletion strain.
Fig 2. The cell aggregation of the spoVG-deletion strain is protease-sensitive. The cell aggregation of the spoVG-deletion strain was digested with protease K and trypsin, PBS was used as control. (A) Results in small centrifugal tubes. (B) Results in 96-well plate.

Fig 3. SpoVG represses the transcription of sasC. (A) The transcriptional levels of 17 genes encoding extracellular proteins in the WT and spoVG-deletion strains were detected by RT-qPCR (OD₆₀₀=2). (B) The transcriptional levels of sasC in the WT, spoVG-deletion, and spoVG-complemented strains were detected by RT-qPCR (OD₆₀₀=6). (C) The β-galactosidase activity of sasC in the WT and spoVG-deletion strains. Bacterial cells were collected at OD₆₀₀=6, and the β-galactosidase activity was detected with the substrate ONPG. The spoVG-deletion strain carrying pOSrot was used as positive control, and PBS as negative control. (D) EMSA of non-phosphorylated SpoVG (SpoVG) or the hyper-phosphorylated SpoVG (SpoVG-P) with the biotin labeled promoter psasC. The promoter region of sasC was amplified by PCR, and incubated with purified SpoVG (top) and SpoVG-P (bottom), respectively. The unlabeled probe was used as the specific competitor, and the unlabeled partial fragment of hu ORF region as the non-specific competitor. (E) The transcriptional levels of ebhB in the WT, spoVG-deletion, and spoVG-complemented strains were detected by RT-qPCR (OD₆₀₀=6). Values are from three biological replicates ± SEM. Statistical values were determined by the Student T test and the F test to compare variances. * P < 0.05, ** P < 0.01, *** P < 0.001.
Fig 4. The SasC aggregation domain can form a superpolymer \textit{in vitro}. (A) Distribution of SasC domain. SasC consists of the N-terminal signal peptide (represented by blue arrow), the aggregation domain (represented by purple diamond), 17 unknown function domain DUF1542 (represented by orange diamond), and the C-terminal LPXTG cell wall anchored motif. Black line represents the SasC aggregation domain (41.5 kDa).

(B) The results of SDS-PAGE (left) and native PAGE (right) of SasC aggregation domain. (C) The result of FPLC-MALS of SasC aggregation domain. The molecular weight of superpolymer SasC aggregation domain was marked.
Fig 5. Increased sasC transcription in the spoVG-deletion strain lead to cell aggregation. 

(A) Structure of sasC. The deletion region for sasC (SasC aggregation domain) was represented by red dotted box. (B) After overnight growth, the WT, spoVG-deletion, spoVG-complemented, and spoVG sasC double mutant strains were adjusted to OD₆₀₀=2 and incubated at room temperature for 3, 6, 9, 12 hours. (C) After overnight growth, the WT, spoVG-deletion, spoVG-complemented, sasC mutant, and spoVG sasC double mutant strains were adjusted to OD₆₀₀=2 and incubated at room temperature. The OD₆₀₀ of the supernatant was detected within 36 hours. Values are from three biological replicates ± SEM. Statistical values were determined by the Student T test and the F test to compare variances. ** P < 0.01. (D) The cell clumps were visualized using fluorescence microscopy. The fluorescent shuttle plasmid pALC was transformed into the WT, spoVG-deletion, and spoVG sasC double mutant strains. Cell aggregations were photographed after growth overnight. Above: the WT strain. Middle: the spoVG-deletion strain. Below: the spoVG sasC double mutant strain.
Fig 6. The eDNA was involved in the process of bacterial cell aggregation. (A) The level of eDNA release of the WT and spoVG-deletion strain. DNase I treatment was used as negative control. (B) The cell clumps with DNase I treated for 0, 2, 4, 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: the WT strain. Right: the spoVG-deletion strain.
Fig 7. Cell aggregation is associated with oxacillin susceptibility and cell survival. Oxacillin susceptibility of the WT, spoVG-deletion, spoVG-complemented, and spoVG sasC double mutant strains. (A) The results of oxacillin gradient dilution method. (B) The results of 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 was determined following 24 or 48 hours of incubation. (C) The results of 24 hours. (D) The results of 48 hours. Values are from three biological replicates ± SEM. Statistical values were determined by the One-way ANOVA, and the F test to compare variances. *** P < 0.001. **** P < 0.0001.
Fig 8. Proposed schema of the SpoVG regulatory mechanism. SpoVG modulates cell aggregation by repressing sasC expression and eDNA release. (A) The expression of sasC and release of eDNA are repressed by SpoVG in the WT strain. (B) The high expression of sasC and release of eDNA in the spoVG-deletion strain, leading to cell aggregation. Black arrows represent the transcription of sasC, whereas bars represent repression. Orange arrows represent spoVG and blue circles represent sasC. Chains with different domains represent SasC, double helix structures represent eDNA, and orange circle represents S. aureus strains.
