International Journal of Medical Microbiology 309 (2019) 39–53

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Transcriptional regulation of virulence factors Spa and ClfB by the SpoVG-Rot cascade in *Staphylococcus aureus*

Qing Zhu, Wen Wen, Wanying Wang, Baolin Sun*

Division of Molecular Medicine, Hefei National Laboratory for Physical Sciences at Microscale, the CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences, University of Science and Technology of China, China

ARTICLE INFO

Keywords:
Staphylococcus aureus
SpoVG
Transcriptional regulation
Rot
spa
clfB

ABSTRACT

Staphylococcus aureus can produce numerous surface proteins involved in the adhesion and internalization of host cells, immune evasion, and inflammation initiation. Among these surface proteins, the microbial surface components recognizing adhesive matrix molecules contain many crucial cell wall-anchored virulence factors. The Sar-family regulatory protein Rot has been reported to regulate many important extracellular virulence factors at the transcriptional level, including Spa and clumping factor B. SpoVG, a global regulator in *S. aureus*, is known to control the expression of numerous genes. Here, we demonstrate that SpoVG can positively regulate the transcription of *rot* by directly binding to its promoter. SpoVG can also positively regulate the transcription of *spa* and *clfB* through direct-binding to their promoters and in a Rot-mediated manner. Furthermore, SpoVG can positively modulate the human fibrinogen-binding ability of *S. aureus*. In addition, phosphorylation of SpoVG by the serine/threonine kinase, Stk1, can positively regulate its binding to the promoters of *rot*, *spa*, and *clfB*. The human cell infection assay showed that the adhesion and internalization abilities were reduced in the *spoVG* mutant strain in comparison to those in the wild-type strain. Collectively, our data reveal a SpoVG-Rot regulatory cascade and novel molecular mechanisms in the virulence control in *S. aureus*.

1. Introduction

Staphylococcus aureus is a community- and hospital-acquired bacterial pathogen that can cause both local and systemic infections in humans, such as sepsis, endocarditis, and pneumonia (Archer and Climo, 2001; Lowy, 1998). The abilities of *S. aureus* to colonize a wide range of organs and to cause acute and chronic diseases are mainly due to the large arsenal of virulence factors such as toxins, proteases, nucleases, lipases, and adhesive proteins (Bunikowski et al., 2000; Dinges et al., 2000; Foster, 2005, 2009; Foster and Hook, 1998). The production of virulence factors in *S. aureus* is controlled by a network of interacting regulators, mainly including two-component systems, sigma factors, regulatory proteins, and regulatory RNAs (Arvidson and Tegmark, 2001; Bronesky et al., 2016; Bronner et al., 2004; Cheung et al., 2004).

The SarA family, a group of proteins homologous to the virulence global regulator SarA, is one of the foremost families of global regulators in *S. aureus* (Cheung et al., 2008; Cheung and Zhang, 2002). The 133 residue-long regulator Rot (Manna and Ray, 2007), first identified as a repressor of *hla* in *S. aureus* (McNamara et al., 2000), is a single-domain protein of the SarA protein family (Cheung et al., 2008). It has been recognized that Rot can positively regulate *spa*, *clfB*, *sdrC*, and *thrB*, and negatively regulate *hlb*, *splABCDEF*, *sspABC*, *geh*, *hlgB*, and *hlgC* (Said-Salim et al., 2003). The transcription of *rot* is repressed by SarA through its binding to the *rot* promoter (Manna and Ray, 2007). The translation of Rot is negatively regulated by the accessory gene regulator (Agr) system through the binding of RNAIII to *rot* mRNA, which can prevent the binding of the 30S small ribosomal subunit, and subsequently facilitate the degradation of the RNA-RNA complex (Boisset et al., 2007), a regulatory mechanism that was also observed for *spa* (Huntzinger et al., 2005), *coa* (Chevalier et al., 2010), and *sbi* (Chabelskaya et al., 2014).

SpoVG was originally identified as a factor involved in the sporulation in *Bacillus subtilis* (Frisby and Zuber, 1991; Matsuno and Sonenshein, 1999; Rosenbluh et al., 1981; Segall and Losick, 1977). In *B. subtilis*, SpoVG affects the physiological processes of sporulation (Rosenbluh et al., 1981), asymmetric cell division (Matsuno and Sonenshein, 1999), and hemolysin production (Pan et al., 2014). In the non-sporulating bacterium *S. aureus*, SpoVG influences the production of capsule, extracellular nuclease, protease, and lipase by regulating the transcription of *cap*, *nuc1*, *splE*, and *lip* (Meier et al., 2007; Schulthess et al., 2011; Schulthess et al., 2009). Moreover, SpoVG is involved in

* Corresponding author.
E-mail address: sunb@ustc.edu.cn (B. Sun).

https://doi.org/10.1016/j.ijmm.2018.10.006
Received 27 April 2018; Received in revised form 25 September 2018; Accepted 18 October 2018

methicillin and glycopeptide resistance of methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA) (Liu et al., 2016; Schulthess et al., 2009) by regulating the expression of cell wall synthesis gene *femA* and cell wall degradation genes *lytN* and *lytS* (Liu et al., 2016). SpoVG has been reported to preferentially bind to the DNA region that contains a characteristic TAATTT/A motif (Jutras et al., 2013) in *S. aureus*. The DNA-binding property of SpoVG can be enhanced through phosphorylation mediated by staphylococcal Ser/Thr protein kinase Stk1 (Bischoff et al., 2016). The expression of *spoVG* locus, which is located in the *yabJ-spoVG* operon in *S. aureus*, is positively modulated by the alternative sigma B factor (σB) (Bischoff et al., 2004; Meier et al., 2007; Schulthess et al., 2009). In addition, the *spoVG* locus can be negatively regulated by the small RNA SprX at the translational level through the interaction between SprX and *yabJ-spoVG* mRNA (Eyraud et al., 2014). Although numerous reports are available that demonstrated a DNA-binding activity of SpoVG, a recent study by Burke and Portnoy (2016) indicated the SpoVG homolog of *Listeria monocytogenes* to have an RNA-binding activity (Burke and Portnoy, 2016).

In this study, we found that the transcript level of *rot* was significantly decreased in the *S. aureus spoVG* mutant strain compared with that of the wild-type (WT) strain, and that SpoVG was able to bind to the *rot* promoter specifically. By introducing the *spoVG rot* double mutant, we demonstrated that SpoVG played multiple regulatory roles in the transcription of *spa* and *clfB* through direct-binding to the respective promoters and in a Rot-mediated manner. Our results further suggested that Stk1-mdieated phosphorylation of SpoVG could promote its binding to the promoters of *rot*, *spa*, and *clfB*. Moreover, the *spoVG* mutant strain displayed significantly decreased fibrinogen-binding and host cell infection abilities compared with the WT strain.

2. Materials and methods

2.1. 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. Constructed plasmids were purified from *E. coli* Trans1-T1 and transformed into *S. aureus* RN4220 as the initial recipient and then *S. aureus* strain N315 by electroporation. The media were solidified with 1.5% (w/v) agar when required.

2.2. Construction of the rot single mutant and spoVG rot double mutant strains

To introduce a single mutant of the *rot* gene, the plasmid pBTs was used as described previously (Hu et al., 2015). Briefly, DNA fragments corresponding to the upstream and downstream regions of *rot* were amplified by PCR, using *S. aureus* 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* N315. The transformants that had allelic replacement of *rot* were selected on TSB agar containing 200 ng/μl anhydrotetracycline (ATC), and further verified by PCR and sequencing. The *spoVG rot* double mutant was constructed using a similar strategy by introducing the *spoVG* mutant plasmid into the *rot* mutant strain, and further confirmed by PCR and sequencing.

2.3. Complementations of the spoVG, rot, and double mutant strains

For *spoVG* complementation, both the plasmid pRMC*spoVG* that allows the expression of *spoVG* under the control of an ATC inducible promoter and *spoVG* chromosomal complementation were employed. To construct pRMC*spoVG*, the fragment encompassing the open reading frame (ORF) of *spoVG* was amplified from *S. aureus* N315 genomic DNA. The fragment was digested with KpnI and EcoRI, and then cloned into the shuttle plasmid pRMC2 with an ATC inducible promoter. The recombinant plasmid was transformed by electroporation into *S. aureus* RN4220 for modification and subsequently into *S. aureus* N315 to derive the complemented strains. The WT and Δ*spoVG* strains (Liu et al., 2016) were transformed with the plasmid pRMC2 as the control strains, resulting in the WT + pRMC2 and Δ*spoVG* + pRMC2 strains, respectively. To construct the *spoVG* chromosomal complementation, the DNA fragment covering the ORF of *spoVG* and the flank upstream and downstream regions was amplified by PCR, using N315 genomic DNA as template. The PCR products were then digested with KpnI and SalI, and then ligated into the pBTS vector, generating the plasmid pBTS-up-*spoVG*-down. This plasmid was electroporated into *S. aureus* RN4220, then transformed into the *spoVG* mutant and *spoVG rot* double mutant strains. The transformants with homologous recombination were selected on TSB agar containing 200 ng/μl ATC, and further verified by PCR and sequencing.

For *rot* complementation, the plasmid pLI50 was used to construct pLI*rot* complemented plasmid. First, the fragment encompassing the ORF of *rot* and its native promoter was amplified from N315 genomic DNA. The fragment was digested with EcoRI and BamHI and cloned into the shuttle plasmid pLI50 to derive the plasmid pLI*rot*. The recombinant plasmid was transformed into *S. aureus* RN4220 by electroporation and then into the N315 *rot* mutant and double mutant strains to derive the complemented strains. Similarly, the WT, *rot* mutant, and *spoVG rot* double mutant strains were transformed with the plasmid pLI50 as the control strains.

2.4. RNA isolation, cDNA generation, and quantitative reverse transcription-PCR

For total RNA isolation, 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 recombination DNase I (TaKaRa, 5 U/μl). For reverse transcription, cDNA was synthesized with a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) using random primers. Quantitative reverse transcription-PCR (qRT-PCR) 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 $2^{-\Delta\Delta Ct}$ method with *hu* as the reference gene (Valihrach and Demnerova, 2012), and the corresponding control sample as the run calibrator. The primers used in this study are listed in Table 2. All the qRT-PCR assays were repeated at least three times.

2.5. Western blot analysis

To detect the production of Spa, the strains were grown in TSB medium with appropriate antibiotics as required at 37 °C with shaking for 4 and 8 h. The production of Spa in the supernatants of the *S. aureus* cultures was determined by western blot analysis. After standardization

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype[a]	Reference or source[b]
Strains		
S. aureus		
RN4220	8325-4, r^-, initial recipient for modification of plasmids	NARSA
N315	HA-MRSA, SCC*mec* type II	NARSA
SH1000	8325-4, rsbU repaired	Horsburgh et al. (2002)
MW2	CA-MRSA, SCCmec type IV	NARSA
Δ*spoVG*	N315 strain deletion of *spoVG*	Liu et al. (2016)
Δ*rot*	N315 strain deletion of *rot*	This study
ΔΔ	N315 *spoVG rot* double mutant	This study
C-*spoVG*	*spoVG* chromosomal complementation of Δ*spoVG*	This study
ΔΔ C-*spoVG*	*spoVG* chromosomal complementation of ΔΔ	This study
E. coli		
Trans1-T1	Host strain for cloning	TransGen
BL21(DE3)	Express strain, λ prophage DE3, lac UV5 promoter	TransGen
Plasmids		
pBTs	Shuttle vector, temp sensitive, Amp^r Chl^r	Hu et al. (2015)
pBTsΔ*rot*	pBTs derivative, for *rot* deletion, Amp^r Chl^r	This study
pBTsΔ*spoVG*	pBTs derivative, for *spoVG* deletion, Amp^r Chl^r	Liu et al. (2016)
pBTs-up-*spoVG*-down	pBTs derivative, for *spoVG* chromosomal complementation, Amp^r Chl^r	This study
pET28a(+)	Expression vector with a hexa-histidine tag, Kan^r	Novagen
pETSpoVG	pET28a(+) derivative, with ORF of *spoVG*, Kan^r	Liu et al. (2016)
pRSF-Duet	pET vector derivative, designed for the co-expression of two proteins under T7 lac promoter induction, Kan^r	Novagen
pDuet-Stk1	pRSF-Duet derivative, with Stk1 kinase domain, Kan^r	This study
pDuet-Stk1-SpoVG	pRSF-Duet derivative, co-express SpoVG and Stk1 kinase domain, Kan^r	This study
pOS1	Shuttle vector, with *lacZ* ORF lacking first 6 amino acids, Amp^r Chl^r	Schneewind et al. (1993)
pOS*rot*	POS1 derivative, harboring 540-bp region of *rot* promoter and 18 bp of *rot* coding sequence from strain N315, Amp^r Chl^r	This study
pLI50	Shuttle vector, Ap^r Cm^r	Lee et al. (1991)
pLI*rot*	pLI50 derivative, harboring ORF of *rot* and its promoter, Amp^r Chl^r	This study
pRMC2	Shuttle vector, anhydrotetracycline inducible, Amp^r, Cm^r	Corrigan and Foster (2009)
pRMC*spoVG*	pRMC2 derivative, harboring ORF of *spoVG*, Amp^r, Cm^r	This study

[a] r^-, restriction system negative; Kan^r, kanamycin resistant; Amp^r, ampicillin resistant; Chl^r, chloramphenicol resistant.
[b] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

Table 2
Primers used in this study.

Primer	Sequence (5′–3′)[a]	Application
rot-up-F-KpnI	GCGggtaccCCAATGCCAACGCTATTAATG	*rot* deletion
rot-up-R	CATGCTAAACATCTCCCAATTA	*rot* deletion
rot-down-F	TAATTGGGAGATGTTTAGCATGTGCAAGTAGAGCAACAGCAATG	*rot* deletion
rot-down-R-SalI	GCGgtcgacGGTGATGGTCCAATAGATATCAT	*rot* deletion
C-*rot*-f-EcoRI	GCGgaattcTTTAATTTGTTAAATTTAAG	*rot* complementation
C-*rot*-r-BamHI	GCGggatccTAATCATGCTCCATTCATTT	*rot* complementation
C-*spoVG*-F-KpnI	GCGggtaccAATTGCCAGTATTTACATGG	*spoVG* chromosomal complementation
C-*spoVG*-R-SalI	GCGgtcgacTATTCACCTTGCGCATTATC	*spoVG* chromosomal complementation
pRMC2-*spoVG*-F-KpnI	GCGggtaccTTAATATGTTTAATCAAGC	*spoVG* complementation
pRMC2-*spoVG*-R-EcoRI	GCGgaattcATAAAATTGATTAAGCTT	*spoVG* complementation
p*rot*-F-EcoRI	GCGgaattcGTTGAAGATGACGAAATT	pOS*rot*
p*rot*-6aa-R-BamHI	GCGggatccGGACAAGTATTAACAAACAT	pOS*rot*
probe-p*rot*-F	AAGATGAGACAGTAGATGCT	EMSA
probe-p*rot*-Biotin-R	CTTGTATGTGCTAACTTATGC	EMSA
probe-p*spa*-F	GTGTGCTGTATTCTAAAGTG	EMSA
probe-p*spa*-Biotin-R	AATGTTTTTCTTTTTCAA	EMSA
probe-p*clfB*-F	AATAGGAAGAAAACAAATTTTACG	EMSA
probe-p*clfB*-Biotin-R	TACTTATTCTGCTTATTCGACAA	EMSA
RT-*rot*-f	TCCTGTTGACGATGAAAGAA	qRT-PCR
RT-*rot*-r	CATTGCTGTTGCTCTACTTG	qRT-PCR
RT-*spa*-f	AAGACGGCAACGGAGTACATGTCG	qRT-PCR
RT-*spa*-r	CAAGTTCTTGACCAGGTTTGATC	qRT-PCR
RT-*clfB*-f	TTTGGGATAGGCAATCATCA	qRT-PCR
RT-*clfB*-r	TCATTTGTTGAAGCTGGCTC	qRT-PCR
RT-*hu*-f	AAAAAGAAGCTGGTTCAGCAGTAG	qRT-PCR
RT-*hu*-r	TTTACGTGCAGCACGTTCAC	qRT-PCR
E-*spoVG*-F-BamHI	CGggatccGATGAAAGTGACAGATGTA	SpoVG-P expression
E-*spoVG*-R-SalI	GCGgtcgacTTAAGCTTCTTCTGAATC	SpoVG-P expression
E-*stkI*-K-F-NdeI	GGAATTCcatatgATAGGTAAAATAATAA	Stk1 expression
E-*stkI*-K-R-XhoI	CCGctcgagTAAAACACTACTCAAATC	Stk1 expression

[a] Lowercase letters indicate restriction sites. Underlined letters indicate complementary sequences used for overlap PCR ligation.

by total protein concentration determined by SDS-PAGE and the bicinchoninic acid (BCA) assay, equivalent volumes (20 µl each) of supernatants were separated on SDS-12% polyacrylamide gels and electrophoretically blotted to nitrocellulose membrane (Millpore). After blocking with 5% (w/v) nonfat milk in TBST buffer at room temperature for 1 h, the membrane was incubated with a rabbit anti-Spa IgG antibody (Sigma–Aldrich) at a 1/2500 dilution. Bound antibody was detected with the goat anti-rabbit conjugated to horseradish peroxidase (ThermoFisher) at a 1/5000 dilution and visualized with the ImageQuant LAS 4000 (GE Healthcare).

2.6. Cloning, expression, and purification of SpoVG derivatives

The coexpression vector pRSF-Duet was used to express the phosphorylated SpoVG (SpoVG-P). First, the DNA region encoding the Stk1 kinase domain was amplified by PCR using *S. aureus* N315 genomic DNA as template and primers (Table 2) containing BamHI and SalI restriction sites. The corresponding PCR product was digested by appropriate enzymes, and then ligated into the pRSF-Duet vector, generating the plasmid pDuet-Stk1. The *spoVG* gene was then cloned into the pDuet-Stk1 vector using a similar strategy, generating the plasmid pDuet-Stk1-SpoVG. All constructs were verified by DNA sequencing. The resulting plasmid pDuet-Stk1-SpoVG was then transformed into *E. coli* BL21 (DE3) competent cells. Strains carrying pETSpoVG (Liu et al., 2016) and pDuet-Stk1-SpoVG were 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 (IPTG) at 37 °C for additional 5 hours. The cells were then harvested, resuspended in lysis buffer (50 mM Tris–HCl, 300 mM NaCl, pH 8.0), and lysed by sonication. His-tagged SpoVG and SpoVG-P fusion proteins were purified by Ni-NTA resin (Qiagen), eluted with elution buffer (200 mM imidazole, 50 mM Tris–HCl, 300 mM NaCl, pH 8.0), passed through an ultrafiltration system to remove imidazole, and then stored in 10% glycerol at −80 °C until use. Protein purity and concentration were determined by SDS-PAGE and the BCA assay.

2.7. Electrophoretic mobility shift assay

Three biotin-labeled DNA fragments, p*rot*, p*spa*, and p*clfB* containing *rot*, *spa*, and *clfB* promoter regions respectively, were amplified from *S. aureus* N315 genomic DNA using primers listed in Table 2. The biotin-labeled p*rot*, p*spa*, and p*clfB* were 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 fragments of each promoter were added as the specific competitors. The unlabeled ~100 bp DNA fragment derived from the ORF of *hu* was added as the non-specific competitor.

2.8. Mass spectrometry analysis

The His-tagged SpoVG-P protein purified from the *E. coli* BL21 (DE3) cells carrying pDuet-Stk1-SpoVG was first run on SDS-PAGE and visualized by staining with coomassie blue, and the gel band was excised and in-gel digested with trypsin, and the tryptic peptides were subjected to LC–MS/MS analysis with a LTQ mass spectrometer (ProteomeX-LTQ, ThermoFisher), with the His-tagged SpoVG as control. Sequence and peptide fingerprint data were analyzed to determine the SpoVG phosphorylation sites.

2.9. Fibrinogen-binding assay

Binding of cells to fibrinogen immobilized on plates was performed using a modified method as described previously (Ní Eidhin et al., 1998). Fibrinogen was diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to give a range of concentrations from 0.1 to 10 µg/ml, and 100 µl was used to coat 96 well flat bottomed ELISA plates (CorStar) at 4 °C overnight. Control wells containing carbonate buffer only were treated as described above. After washing with 150 mM NaCl containing 0.05% Tween 20, the plates were blocked with 1% BSA and 0.05% Tween in PBS buffer at 37 °C for 1 hour, and incubated with 100 µl cell suspension at 37 °C for 2 hours. The adherent cells were fixed by 100 µl of 25% aqueous formaldehyde at room temperature for 30 minutes and stained by crystal violet for 15 minutes. The bound cells were measured by the respective absorbance (Abs) at 560 nm using the ELX800 microwell plate reader (BioTek). The assays were repeated three times.

2.10. Construction of the LacZ reporter vector

To construct the reporter plasmid pOS*rot*, the DNA fragment containing the *rot* promoter region was amplified from *S. aureus* N315 genomic DNA using primers listed in Table 2. The fragment was digested with BamHI and EcoRI and cloned into the upstream region of the reporter gene *lacZ* of the shuttle vector pOS1. The reporter plasmid was first transformed into *S. aureus* RN4220, and then the WT and *spoVG* mutant strains.

2.11. β-Galactosidase activity assay

For β-galactosidase assay with ONPG (o-Nitrophenyl-b-D-galactopyranoside) as the substrate, overnight cultures of the WT and *spoVG* mutant strains were diluted 1:100 in TSB with chloromycetin (15 µg/ml) and grown for 4 and 8 hours. First, bacterial cells were centrifuged and then resuspended in 100 µl of ABT-LSA buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl, 0.1% Triton X-100, 50 µg/ml lysostaphin). Cells 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 Na_2CO_3 (1 M) was added to stop the reaction. Sample absorbance was read at 420 nm and units were calculated as the following formula: $\text{units} = (1000 \times OD_{420})/(T \times V \times OD_{600})$. 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.

2.12. S. aureus adhesion and internalization assay with HEK293 T and THP-1 cells

For HEK293 T adhesion assay, cells were first cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% FBS (ThermoFisher) and penicillin/streptomycin (penicillin, 100 U/ml; streptomycin, 0.1 mg/ml), and grown at 37 °C and 5% CO_2. Cells were digested by the addition of 0.25% trypsin and incubated at 37 °C for 1 min, and then washed gently with PBS. After washing, cells were resuspended with fresh DMEM and then transferred into 24 well plates (CorStar) with about 10^6 cells per well. After attachment to the flat bottom of the plate for 3 h, cells were washed with PBS to remove the unattached cells, and then incubated with 10^7 bacterial cells of the WT, *spoVG* mutant, and complemented strains per well at 37 °C and 5% CO_2 for 90 min. After incubation, the HEK293 T cells were washed with PBS to remove the bacterial planktonic cells, and lysed with cell lysis buffer (10 mM EDTA, 0.25% Triton-X100 in PBS). The surviving bacterial cells were determined by colony-forming unit (CFU) assay. Data were standardized and normalized by the corresponding survival of the WT strain. For cell internalization assay, HEK293 T cells and bacterial cells

were treated in the similar ways. First, 10^6 HEK293 T cells per well were incubated with 10^7 bacterial cells of the WT, *spoVG* mutant, and complemented strains at 37 °C and 5% CO_2 for 90 min, and the extracellular bacterial cells were killed by 100 μg/ml gentamicin at 37 °C and 5% CO_2 for 2 h. After the treatment of gentamicin, the HEK293 T cells were washed with PBS to remove the bacterial planktonic cells, and lysed by cell lysis buffer (10 mM EDTA, 0.25% Triton-X100 in PBS). The surviving intracellular bacterial cells were determined by CFU assay. The data were standardized and normalized by the corresponding survival of the WT strain. The bacterial cells in the HEK293 T cell adhesion and internalization assay process were collected, then total RNA was extracted, and qRT-PCR was performed. The assays were repeated at least three times.

The infection assay with THP-1 was very similar with HEK293 T except the medium and the treatment of the cells. THP-1 cells were grown in RPMI 1640 medium (Cellgro) supplemented with 10% FBS (ThermoFisher), 2 mM glutamine, 10 mM Hepes, and 1 mM sodium pyruvate, and then induced with 6 ng/μl phorbol myristate acetate (PMA) for 3 h to differentiate into macrophages. The cells were collected, centrifuged at 1000 rpm for 5 min, resuspended with fresh RPMI 1640, and then transferred to 24 well plates for 36-h attachment to the flat bottom of the plate. Incubation with bacterial cells of the WT, *spoVG* mutant, and complemented strains and treatment procedures with gentamicin were the same as in the adhesion and internalization assays of HEK293 T cells. Then the cells were washed with PBS to remove the bacterial planktonic cells, and lysed with cell lysis buffer (10 mM EDTA, 0.25% Triton-X100 in PBS). The surviving bacterial cells were determined by CFU assay and the data were standardized and normalized by the corresponding survival of the WT strain. Meanwhile, the bacterial cells in the cell adhesion and internalization assay were collected and total RNA were extracted, followed by qRT-PCR. The assays were repeated at least three times.

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's *t* test (for two groups), 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. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3. Results

3.1. SpoVG positively regulates the transcription of rot

SpoVG is considered to be a site-specific DNA-binding protein that plays regulatory roles in the capsule synthesis (Meier et al., 2007), the expression of virulence factors (Schulthess et al., 2011), and antibiotic resistance (Liu et al., 2016) in *S. aureus*. To get a deeper understanding of the regulatory functions of SpoVG, we performed qRT-PCR to identify the potential target genes. The results showed that the transcript levels of 6 genes encoding the SarA family proteins were altered in the *spoVG* mutant strain, including 5 up-regulated genes *mgrA*, *rot*, *sarT*, *sarU*, and *sarX*, and 1 down-regulated gene *sarV*. Among these SarA protein family encoding genes, the transcript level of *rot* was significantly decreased in the *spoVG* mutant for more than 100-fold compared with that of the WT strain (Fig. 1A), suggesting that SpoVG may play a critical role in the regulation of the transcription of *rot* in *S. aureus* strain N315.

To verify the results, we measured the growth of the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains, and found no significant difference (Fig. 1B). We then examined the transcript levels of *rot* in different growth phases. Our qRT-PCR results showed that the transcript levels of *rot* were significantly decreased in the *spoVG* mutant strain in early exponential, mid-exponential, and stationary phases compared with those of the WT strain, and that the *spoVG* chromosomal complementation exhibited an efficient restoration effect (Fig. 1C). In addition, we also constructed a complemented plasmid pRMC*spoVG* that allows *spoVG* expression under the control of an ATC inducible promoter. The qRT-PCR results showed that the significantly decreased expression of *rot* in the *spoVG* mutant strain could also be reversed by the plasmid complementation strategy (Fig. 1D).

To verify the regulatory role of SpoVG, we constructed a *rot* promoter-*lacZ* fusion reporter plasmid, and detected the β-galactosidase activities in the WT and *spoVG* mutant strains. As shown in Fig. 1E, the promoter activity of *rot* decreased in the *spoVG* mutant strain compared with that in the WT strain, suggesting that SpoVG is a positive regulator of *rot*. Strain N315, a methicillin-resistant *S. aureus* strain isolated in 1982 from the pharyngeal smear of a Japanese patient (Kuroda et al., 2001), does not express RNAIII, the *agr* regulatory RNA molecule (Pichon and Felden, 2005; Somerville et al., 2003). To investigate whether SpoVG can affect the transcription of *rot* in *agr*-positive *S. aureus* strains, we constructed the *spoVG* mutant in the *agr*-positive *S. aureus* strains SH1000 and MW2 and performed qRT-PCR. Similar with strain N315, no significant difference was observed in the growth of the WT and *spoVG* mutant strains (Fig. 1F and G). The qRT-PCR results showed that the transcript levels of *rot* in the *spoVG* mutant strains were significantly decreased compared with those of the WT strains (Fig. 1H), which were coincident with the result from *agr*-negative strain N315. These data further revealed that SpoVG can positively regulate the transcription of *rot* in both *agr*-positive and *agr*-negative strains.

3.2. SpoVG positively regulates the transcription of spa

Since *spa* has been proposed to be regulated by Rot (Said-Salim et al., 2003), it is reasonable to assume that SpoVG may have an effect on the expression of *spa*. The qRT-PCR results showed that the transcript levels of *spa* decreased significantly in the *spoVG* mutant strain compared with those in the WT strain, and these alterations could be reversed in the complemented strains (Fig. 2A and B). Moreover, the transcript levels of *spa* in the *rot* mutant strain were much lower than those in the WT strain (Fig. 2C), which agreed with the previous report (Said-Salim et al., 2003). To investigate the role that SpoVG played in the regulation of *spa* and to determine if the transcriptional change of *spa* was associated with Rot, we constructed the *spoVG rot* double mutant strain. The transcript levels of *spa* in the *spoVG rot* double mutant strain were significantly decreased in the growth phases of $OD_{600} = 0.6$ and $OD_{600} = 2$ compared with those of the *rot* single mutant (Fig. 2D). Furthermore, the *rot* complementation can restore the decreased transcription of *spa* in the *spoVG rot* double mutant in different growth phases. At the meantime, the *spoVG* complementation also exhibited a compensatory role in the growth phase of $OD_{600} = 0.6$ and $OD_{600} = 2$ (Fig. 2E). In addition, in the *agr*-positive *S. aureus* strains SH1000 and MW2, the transcript levels of *spa* decreased significantly in the *spoVG* mutant strain compared with those in the WT strain (Fig. 2F), indicating that SpoVG can positively regulate the transcription of *spa* in both *agr*-positive and *agr*-negative strains. Taken together, these results revealed that both SpoVG and Rot can regulate the expression of *spa*, and Rot plays a crucial role in the regulation of *spa* by SpoVG.

To further demonstrate that SpoVG can modulate the expression of *spa*, western blot analysis was performed with cultural supernatants. The production level of Spa in the *spoVG* mutant strain was much lower than the WT and complemented strains in different growth phases (Fig. 3A and B). Similarly, the production level of Spa in the *rot* mutant strain was much lower than those in the WT and *rot* complemented strains (Fig. 3C). The *spoVG rot* double mutant strain with *rot* complementation displayed a distinctly higher production level of Spa than that in the *spoVG rot* double mutant, while the *spoVG* single complementation did not (Fig. 3D). These data emphasized the critical role of Rot in the regulation of Spa by SpoVG.

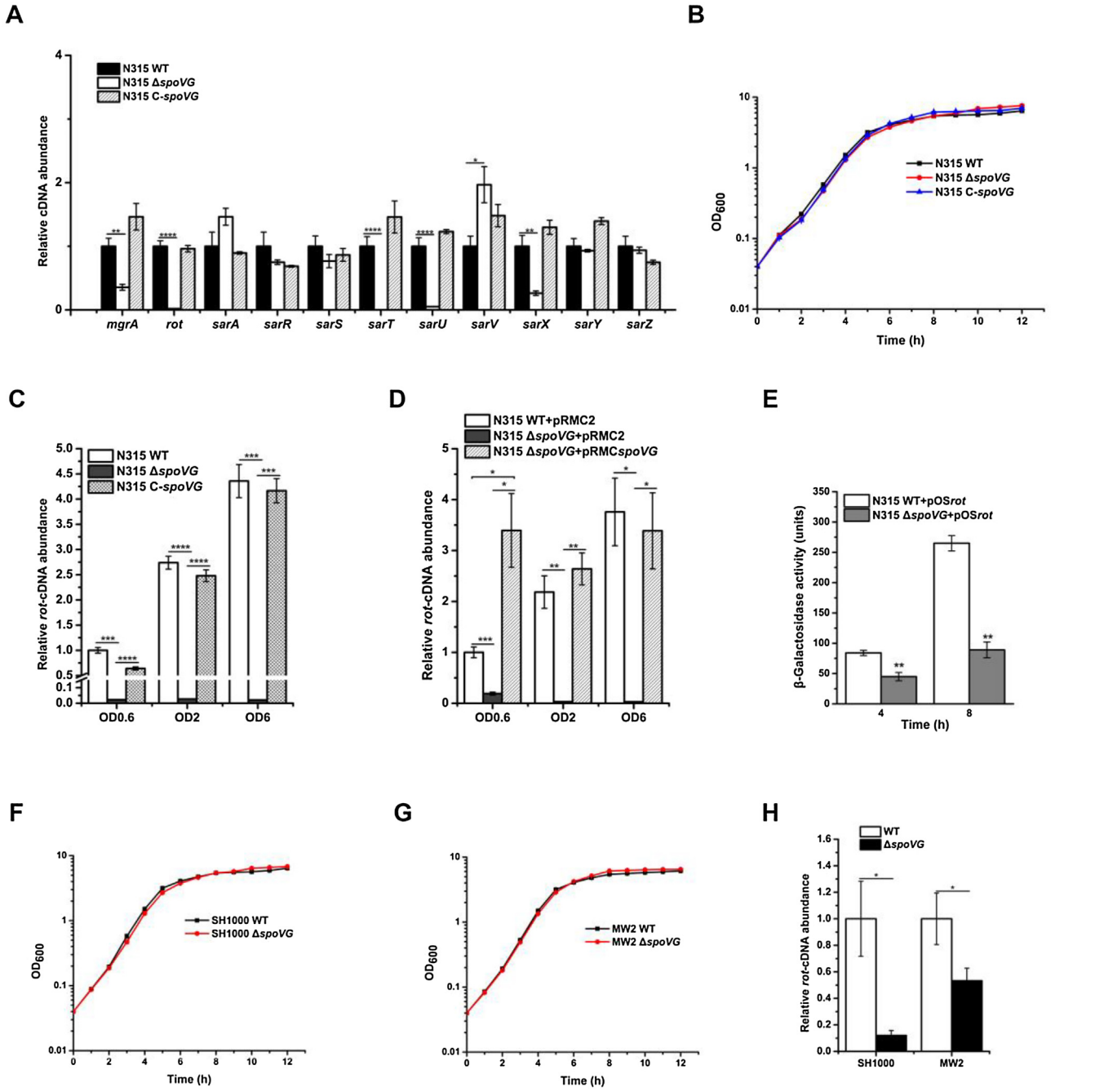

Fig. 1. Positive regulation of *rot* by SpoVG. (A) The transcript levels of 11 genes encoding SarA family proteins in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains were detected by qRT-PCR. (B) Growth of the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains. (C) The transcript levels of *rot* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains. (D) The transcript levels of *rot* in the WT, *spoVG* mutant, and *spoVG* complemented strains carrying pRMC*spoVG*. (E) The β-galactosidase activity driven by the promoter of *rot* in the WT and *spoVG* mutant strains. Bacterial cells were collected at 4 and 8 h, and the β-galactosidase activity was detected with the substrate ONPG. (F) Growth of the SH1000 WT and *spoVG* mutant strains. (G) Growth of the MW2 WT and *spoVG* mutant strains. (H) The transcript levels of *rot* in the WT, *spoVG* mutant strains of SH1000 and MW2 (OD_{600} = 2). Values are from three biological replicates ± SEM. Statistical values were determined by the Student's *t* test, one-way ANOVA, and the *F* test to compare variances. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.3. SpoVG positively regulates the transcription of *clfB*

The qRT-PCR results showed that the transcript levels of *clfB* decreased significantly in the *spoVG* mutant strain compared with those in the WT strain, and the change could be reversed by chromosomal complementation and by introducing pRMC*spoVG* into the *spoVG* mutant strain (Fig. 4A and B). In the *agr*-positive *S. aureus* NCTC 8325, Rot is the activator of *clfB*, and *agr*/RNAIII regulates the expression of *clfB* via Rot (Xue et al., 2012). In the *agr*-negative *S. aureus* N315, the transcript levels of *clfB* in the *rot* mutant strain were lower than those in the WT strain in different growth phases (Fig. 4C). These data were consistent with the previous conclusion (Xue et al., 2012), and revealed that Rot can function as the activator of *clfB* in both *agr*-positive and *agr*-negative strains. Since SpoVG can promote the transcription of *rot*, we expected that SpoVG may affect the transcription of *clfB* through Rot. However, according to our qRT-PCR results, the transcript levels of

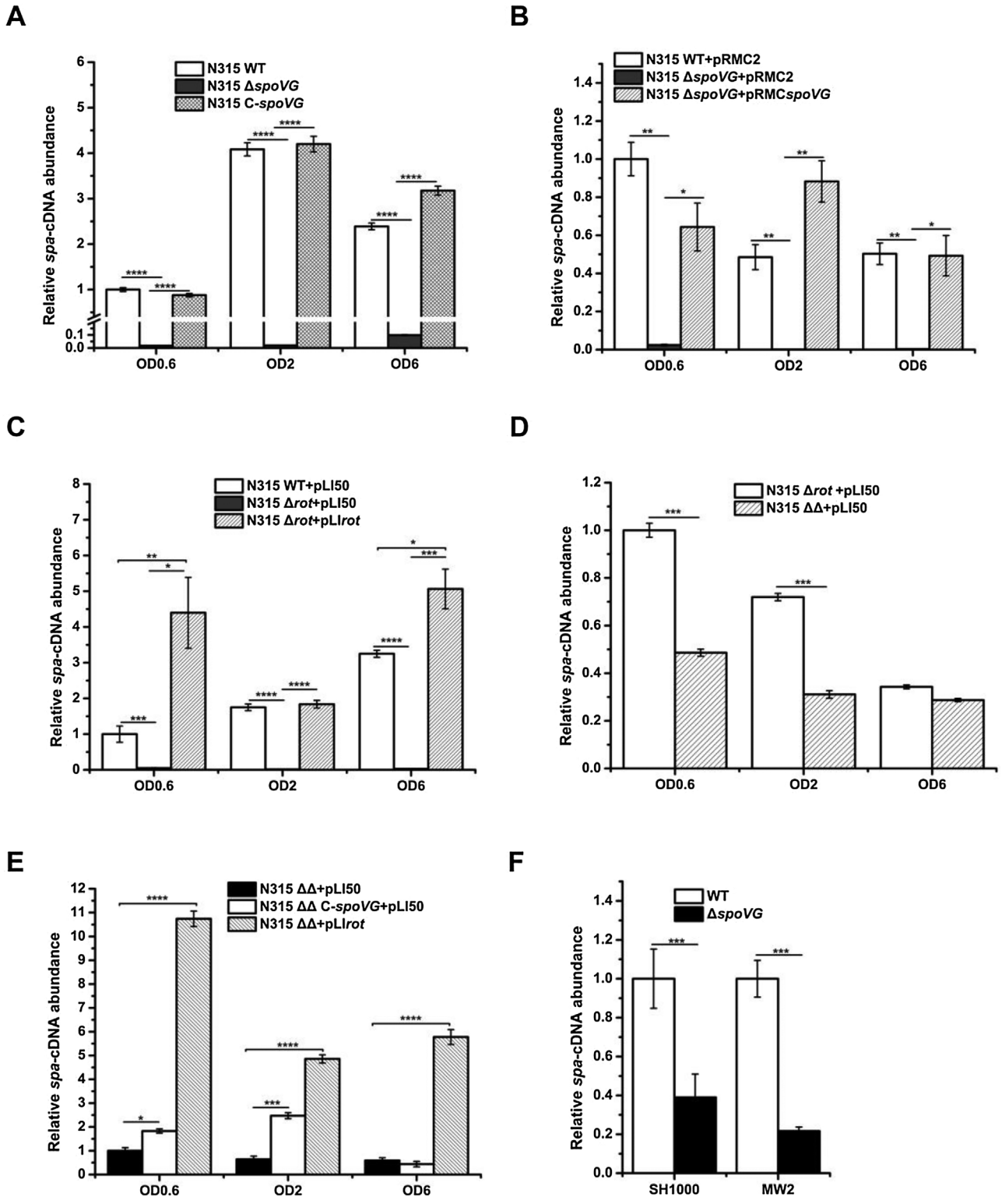

Fig. 2. SpoVG positively regulates the transcription of *spa*. The transcript levels of *spa* were measured by qRT-PCR in different growth phases. (A) The transcript levels of *spa* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains. (B) The transcript levels of *spa* in the WT, *spoVG* mutant, and *spoVG* complemented strains carrying pRMC*spoVG*. (C) The transcript levels of *spa* in the WT, *rot* mutant, and *rot* complemented strains. (D) The transcript levels of *spa* in the *rot* mutant and *spoVG rot* double mutant strains. (E) The transcript levels of *spa* in the *spoVG rot* double mutant strains, and double mutant with *spoVG* chromosomal complemented or *rot* complemented strains. (F) The transcript levels of *spa* in the WT and *spoVG* mutant strains of SH1000 and MW2 (OD_{600} = 2). Values are from three biological replicates ± SEM. Statistical values were determined by the Student's *t* test, one-way ANOVA, and the *F* test to compare variances. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

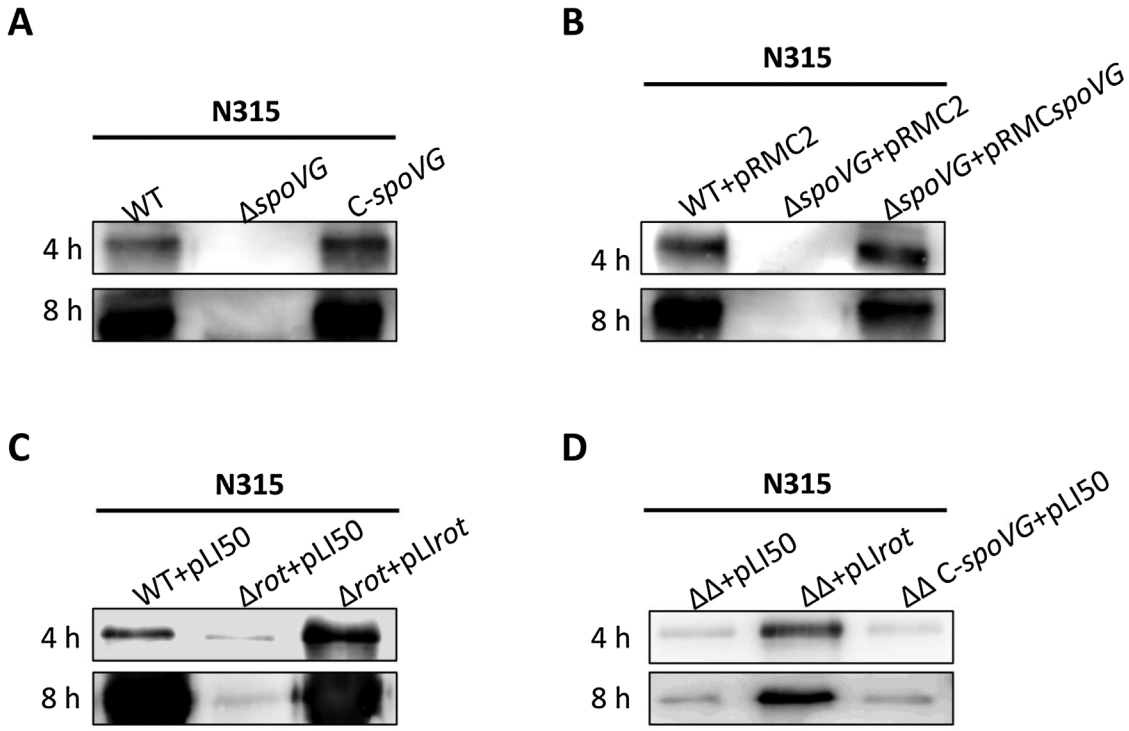

Fig. 3. Western blot analysis of Spa. Strains were grown in TSB medium for 4 or 8 h. (A) Western blot analysis of Spa production in the supernatants of the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains. (B) Western blot analysis of Spa production in the supernatants of the WT, *spoVG* mutant, and *spoVG* complemented strains carrying pRMC*spoVG*. (C) Western blot analysis of Spa production in the supernatants of the WT, *rot* mutant, and *rot* complemented strains. (D) Western blot analysis of Spa production in the supernatants of the *spoVG rot* double mutant strains, double mutant strains with single *rot* complementation and *spoVG* chromosomal complementation.

clfB in the *spoVG rot* double mutant strain were significantly decreased in the growth phases of OD_{600} = 0.6 and OD_{600} = 2 compared with those of the *rot* single mutant strain (Fig. 4D), suggesting that the modulation of *clfB* by SpoVG is not only through Rot. In addition, the *spoVG rot* double mutant strain with the *spoVG* chromosomal complementation showed higher *clfB* expression levels than those of the double mutant strain in the growth phases of OD_{600} = 0.6, OD_{600} = 2, and OD_{600} = 6 (Fig. 4E). These results support the assumption that SpoVG can regulate *clfB* beyond the effect of Rot. In the *agr*-positive *S. aureus* strains SH1000 and MW2, the transcript levels of *clfB* decreased significantly in the *spoVG* mutant strain compared with those in the WT strain (Fig. 4F), suggesting that SpoVG can positively regulate the transcription of *clfB* in both *agr*-positive and *agr*-negative strains.

3.4. Both SpoVG and Rot can positively affect the bacterial fibrinogen-binding ability

Fibrinogen is an important factor of the host extracellular matrix components and usually acts as a mediator of the host cell during *S. aureus* infection (Ko and Flick, 2016). Previous studies have indicated that Rot has a regulatory effect on the fibrinogen-binding ability by controlling the expression of *clfB* (Said-Salim et al., 2003; Xue et al., 2012). To determine whether SpoVG can affect the bacterial fibrinogen-binding ability, we performed the fibrinogen-binding assays. As shown in Fig. 5A, the *spoVG* mutant strain exhibited weaker fibrinogen-binding ability compared with that of the WT strain, and the phenotype could be restored by chromosomal complementation. Moreover, the fibrinogen-binding ability of the *rot* mutant strain was also weaker than that of the WT strain (Fig. 5B). In addition, the *spoVG rot* double mutant strain displayed weaker fibrinogen-binding ability than that of the *rot* single mutant strain (Fig. 5C), indicating that SpoVG affects the ability of bacterial binding to human fibrinogen not only through Rot. Furthermore, the *spoVG rot* double mutant strain with *spoVG* chromosomal complementation and *rot* complementation exhibited higher fibrinogen-binding ability compared with the double mutant strain (Fig. 5D). These data indicated that both SpoVG and Rot can positively modulate the bacterial fibrinogen-binding ability.

3.5. SpoVG can specifically bind to the promoter region of rot and clfB

SpoVG is a transcriptional factor in *S. aureus* with the DNA-binding ability and can control the transcription of *cap*, *lytS*, *lytN*, and *femA* by directly binding to their promoters (Liu et al., 2016). Therefore, EMSA was performed to determine whether SpoVG can bind to the *rot* promoter region. A shifted band was visible after incubation of SpoVG with the biotin-labeled DNA probe containing the *rot* promoter region (Fig. 6A). This shifted band disappeared when an approximate 200-fold excess of unlabeled *rot* promoter DNA was added, but did not disappeared in the presence of 300-fold excess of unlabeled *hu* DNA. This result demonstrated that SpoVG was able to bind to the *rot* promoter region specifically. Taken together, these results indicated that SpoVG could positively regulate the transcription of *rot* by directly binding to the promoter region of *rot*. We further performed EMSA to determine whether SpoVG can bind to the *spa* promoter region and the result showed no legible binding when adding up to 4 nmol SpoVG (Fig. 6B). SpoVG could retard the mobility of the *clfB* promoter region in a dose-dependent manner, and the shifted band of SpoVG-p*clfB* disappeared when an approximate 200-fold excess of unlabeled *clfB* promoter DNA was added, but did not disappear in the presence of 300-fold excess of unlabeled *hu* DNA (Fig. 6C). These results suggest that the transcription of *clfB* is both directly and indirectly regulated by SpoVG in a multi-faceted manner. On one hand, SpoVG can promote the expression of *clfB* through directly binding to its promoter; on the other hand, SpoVG can also influence the expression of *clfB* indirectly through the modulation of Rot.

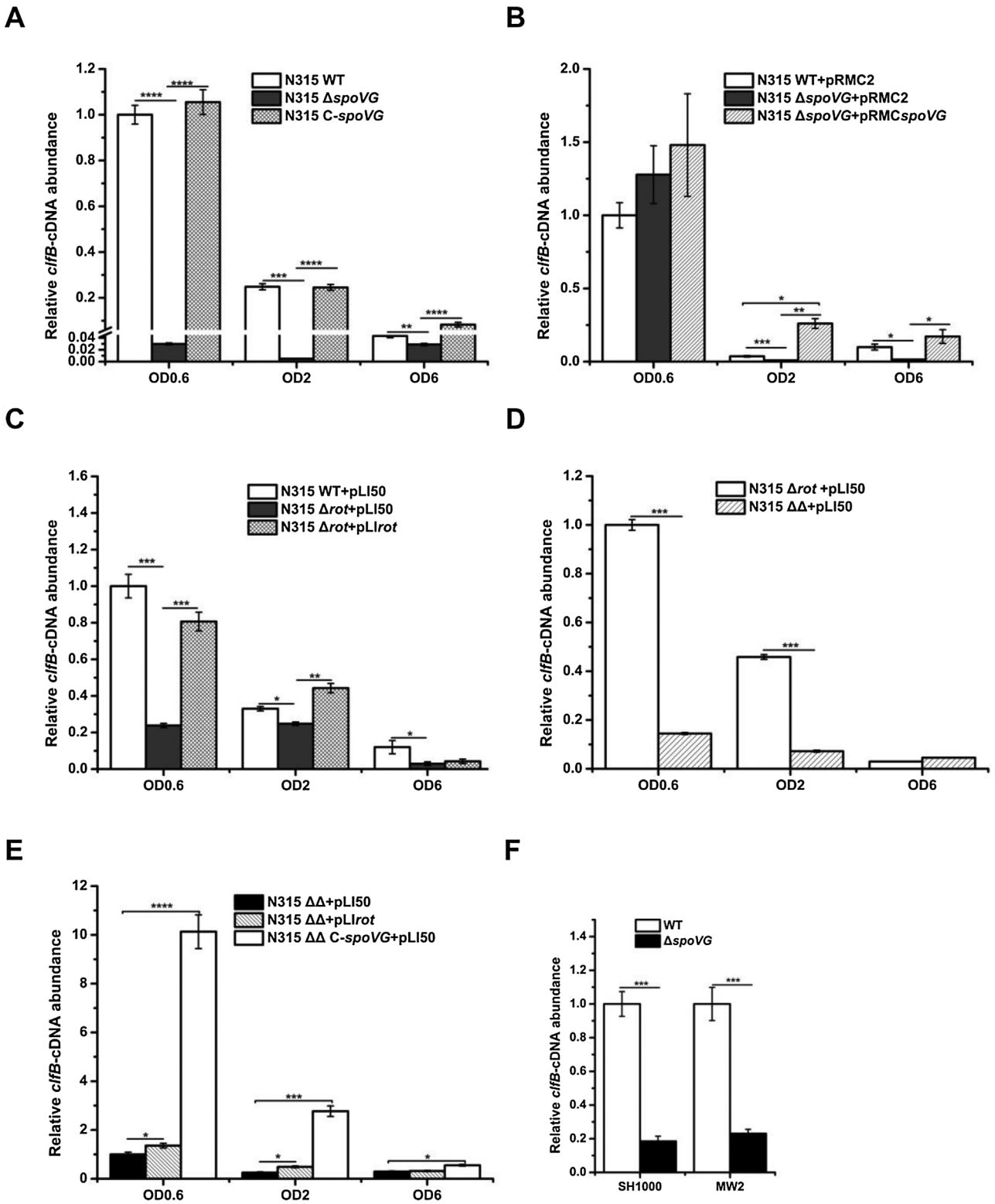

Fig. 4. SpoVG positively regulates the transcription of *clfB*. The transcript levels of *clfB* were measured by qRT-PCR in different growth phases. (A) The transcript levels of *clfB* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains. (B) The transcript levels of *clfB* in the WT, *spoVG* mutant, and *spoVG* complemented strains carrying pRMC*spoVG*. (C) The transcript levels of *clfB* in the WT, *rot* mutant, and *rot* complemented strains. (D) The transcript levels of *clfB* in the *rot* mutant and *spoVG rot* double mutant strains. (E) The transcript levels of *clfB* in the *spoVG rot* double mutant strains, and double mutant strains with *rot* complementation or *spoVG* chromosomal complementation. (F) The transcript levels of *clfB* in the WT and *spoVG* mutant strains of SH1000 and MW2 (OD_{600} = 2). Values are from three biological replicates ± SEM. Statistical values were determined by the Student's *t* test, one-way ANOVA, and the *F* test to compare variances. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

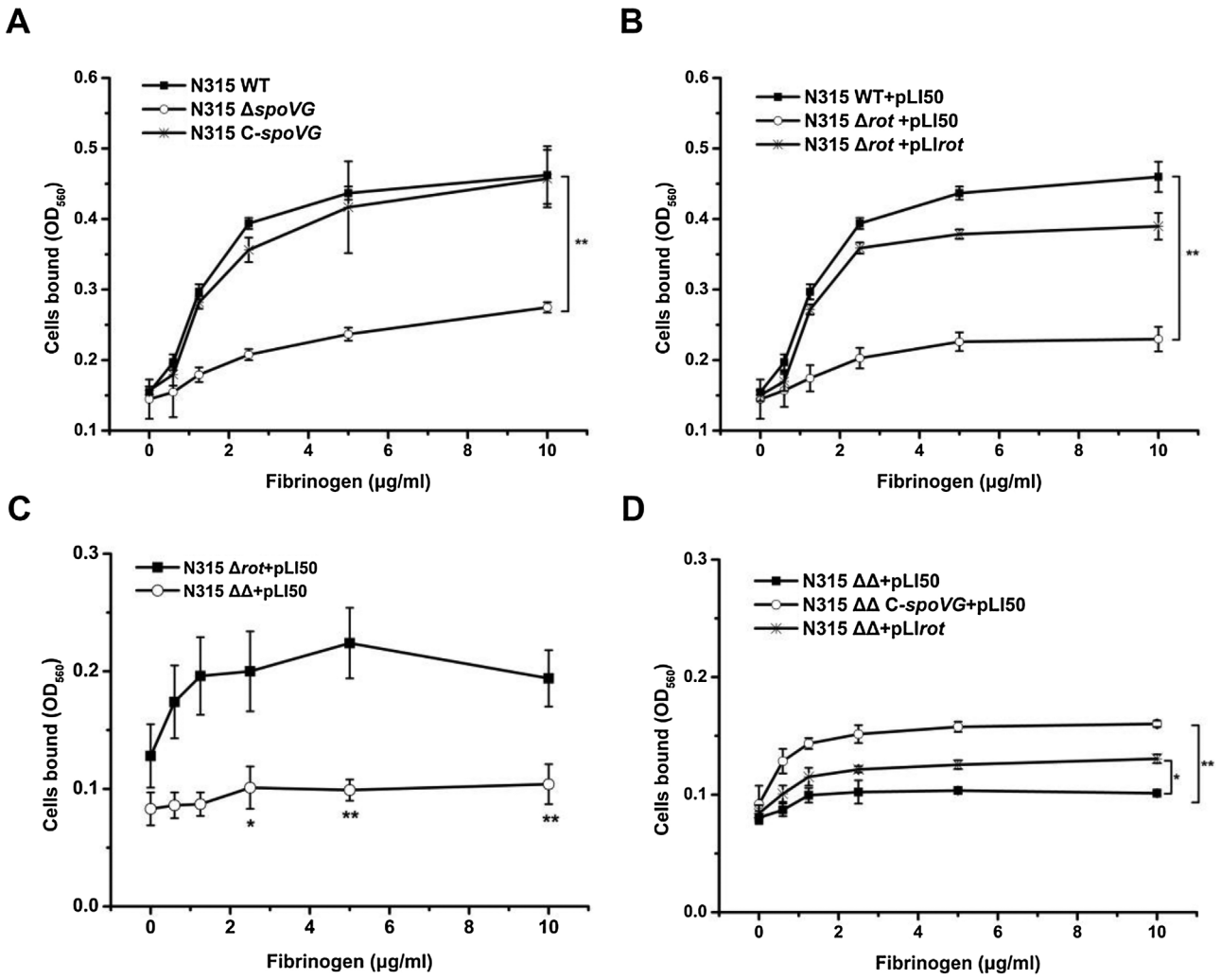

Fig. 5. Both SpoVG and Rot positively affect the bacterial fibrinogen-binding ability. (A) The binding abilities of the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains to human fibrinogen immobilized on plates were measured. (B) The fibrinogen-binding abilities of the WT, *rot* mutant, and *rot* complemented strains. (C) The fibrinogen-binding abilities of the *rot* mutant and *spoVG rot* double mutant strains. (D) The fibrinogen-binding abilities of the *spoVG rot* double mutant strain, and double mutant strain with *rot* complementation or *spoVG* chromosomal complementation. Values are from three biological replicates ± SEM. Statistical values were determined by the Student's *t* test and the *F* test to compare variances. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.6. Phosphorylation of SpoVG positively regulates its DNA-binding property

To further investigate whether phosphorylated SpoVG can interact differently from nonphosphorylated SpoVG with DNA, comparative EMSA was performed between promoter regions of p*rot*, p*spa*, p*clfB* and either the non-phosphorylated SpoVG form (SpoVG) or the hyper-phosphorylated SpoVG form (SpoVG-P). SpoVG-P, phosphorylated by the protein kinase Stk1 during coexpression, was analyzed by mass spectrometry, and the result of phosphorylated residues was listed in Table 3. Concerning p*rot*, a shift could be obtained by adding 1 nmol SpoVG (Fig. 6A), while 0.3 nmol SpoVG-P could give rise to the shift (Fig. 6D). For p*spa*, 1 nmol SpoVG-P was required for a shift (Fig. 6E), whereas no shift band was observed when up to 4 nmol SpoVG was added (Fig. 6B), suggesting a weaker SpoVG-p*spa* interaction in vitro. For p*clfB*, 1 nmol SpoVG could retard the mobility of the *clfB* promoter region (Fig. 6C), while only 0.3 nmol SpoVG-P was needed to obtain the shift (Fig. 6F). These data suggested that the phosphorylation of SpoVG can positively regulate its DNA-binding property in vitro and in vivo, which is coincident with a previous finding (Bischoff et al., 2016).

3.7. SpoVG is involved in the cell adhesion and internalization of S. aureus

S. aureus can express a broad range of surface proteins covalently attached to peptidoglycan, which are known as cell wall-anchored proteins. These cell wall-anchored proteins are important virulence factors that play crucial roles in the survival of *S. aureus* in the commensal state with the host cell. Since SpoVG can positively regulate the virulence regulator Rot and cell wall-anchored proteins Spa and ClfB, it may affect the virulence of *S. aureus*. To investigate this hypothesis, we performed human cell infection assays with HEK293 T and THP-1 cells, and determined the adhesion and internalization ability of *S. aureus* by detecting the survival of *S. aureus*. The *spoVG* mutant strain exhibited significantly reduced adhesion abilities with HEK293 T and THP-1 cells compared with the WT strain, and these alterations could be reversed by the *spoVG* chromosomal complementation (Fig. 7A), indicating that SpoVG is involved in the regulation of cell adhesion ability of *S. aureus*. Furthermore, the internalization abilities of the *spoVG* mutant strain with HEK293 T and THP-1 cells were also reduced significantly compared with those of the WT strain, and the alterations could be reversed by the *spoVG* chromosomal complementation (Fig. 7B), indicating that SpoVG can positively affect the cell internalization ability of *S. aureus*. In addition, we also collected the bacterial cells in the cell adhesion and

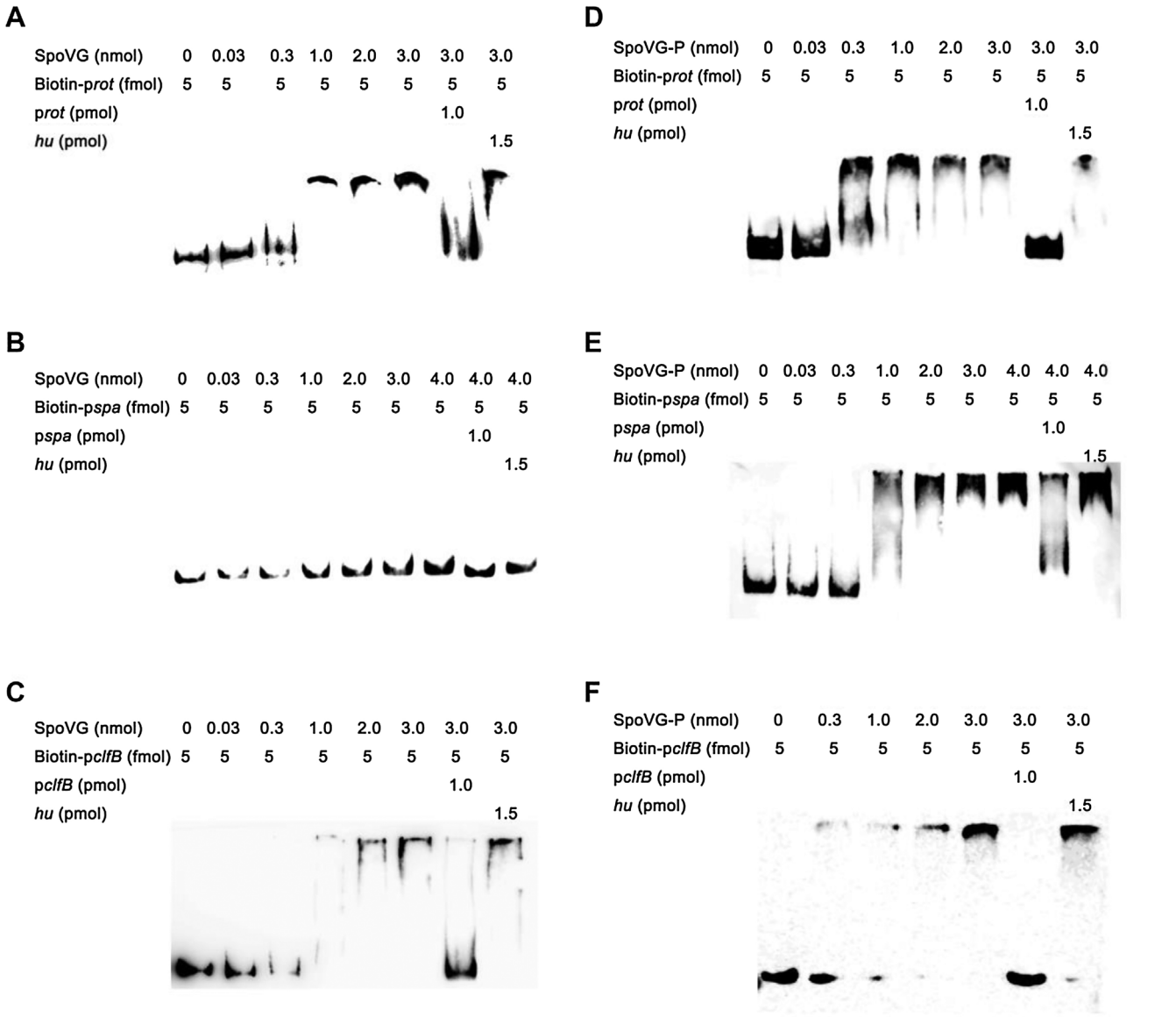

Fig. 6. The DNA-binding activity of the SpoVG derivatives. EMSA of non-phosphorylated SpoVG (SpoVG) or the hyper-phosphorylated SpoVG (SpoVG-P) with the biotin labeled promoters p*rot*, p*spa*, and p*clfB*. The promoter regions of *rot*, *spa*, and *clfB* were amplified by PCR, and incubated with purified SpoVG (A, B, C) and SpoVG-P (D, E, F), respectively. The unlabeled probes were used as the specific competitors, and the unlabeled partial fragment of *hu* ORF region was used as the non-specific competitor.

internalization processes, extracted total RNA, and performed qRT-PCR. The transcript levels of *rot*, *spa*, and *clfB* were decreased significantly in the *spoVG* mutant strains after adhesion and internalization of *S. aureus* with HEK293 T and THP-1 cells, and these changes could be reversed by *spoVG* chromosomal complementation (Fig. 7C–F).

4. Discussion

The coordinated expression of virulence factors in *S. aureus* is mainly controlled by multiple regulatory elements such as two-component systems and transcriptional factors. At least 16 two-component systems have been identified so far in *S. aureus*. They can participate in multiple cellular processes such as sensing and responding to various environmental stimuli, and the transcription regulation of numerous genes and operons (Bronesky et al., 2016; Ibarra et al., 2013; Stock et al., 2000). SpoVG, a highly conserved eubacteria protein, is known to play key roles in multiple cellular processes in *S. aureus*, which mainly depends on SpoVG-DNA interactions (Liu et al., 2016; Meier et al., 2007; Schulthess et al., 2009, 2011, 2012). These reports support a potential role of SpoVG as a crucial regulator. Rot, one of the single domain SarA homologs, plays an important role in the regulation of virulence gene expression in *S. aureus*. A previous report has indicated

Table 3
Phosphorylated residues of SpoVG-P.

Gene name	UniProtKB/Swiss-Prot	Description	Phosphorylated residue[a]
spoVG, SA0456	Q7A7B5.2	Putative septation protein SpoVG [*Staphylococcus aureus* subsp. aureus N315]	T4, T13, S22, S41, T83

[a] T, Thr, threonine; S, Ser, serine.

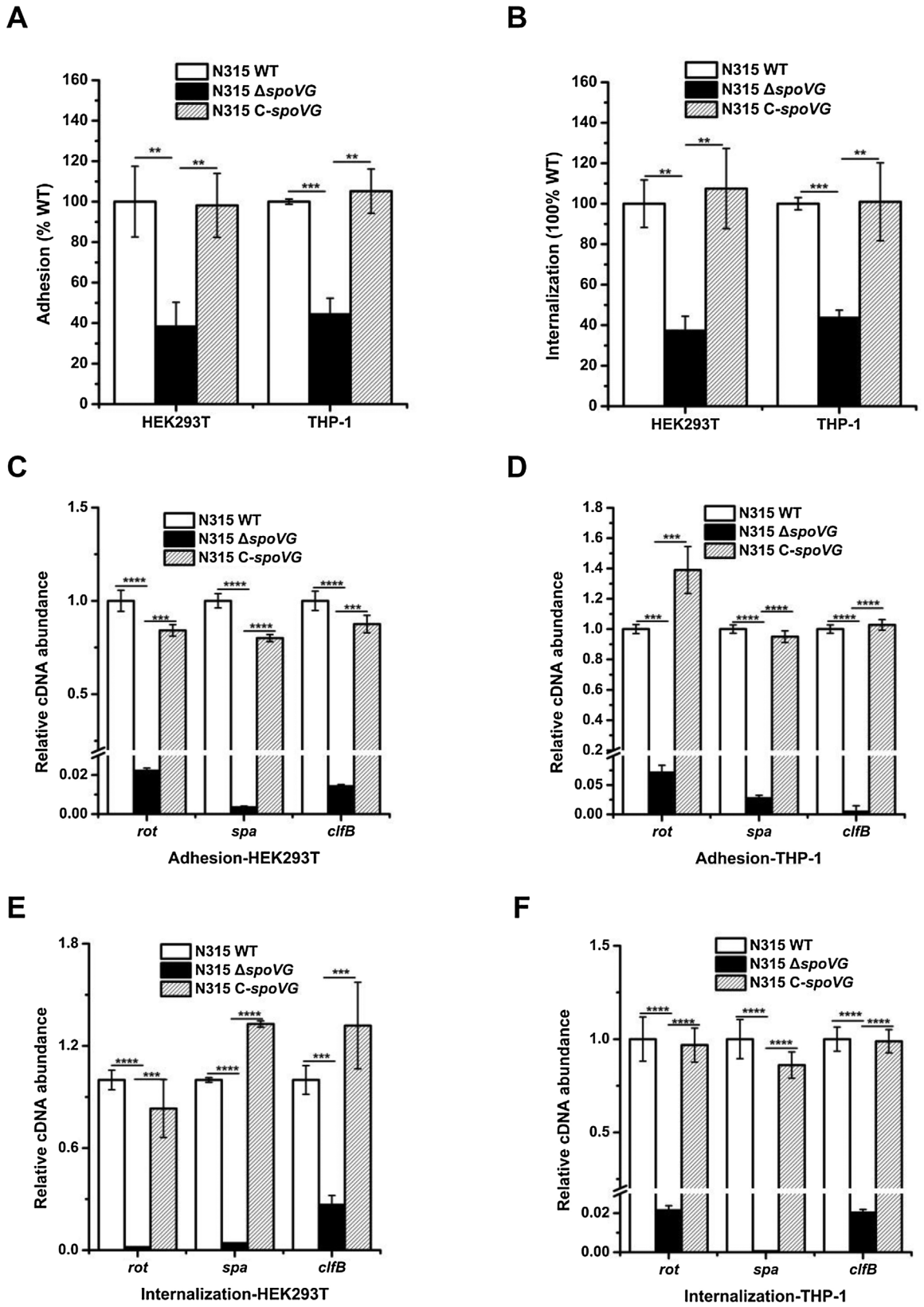

Fig. 7. SpoVG is involved in the cell adhesion and internalization of *S. aureus*. Adhesion and internalization were determined and normalized against the WT strain. (A) Adherence of the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains to HEK293 T and THP-1 cells. (B) Internalization of the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains in HEK293 T and THP-1 cells. (C) The transcript levels of *rot*, *spa*, and *clfB* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains in adhesion with HEK293 T cells. (D) The transcript levels of *rot*, *spa*, and *clfB* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains in adhesion with THP-1 cells. (E) The transcript levels of *rot*, *spa*, and *clfB* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains in internalization with HEK293 T cells. (F) The transcript levels of *rot*, *spa*, and *clfB* in the WT, *spoVG* mutant, and *spoVG* chromosomal complemented strains in internalization with THP-1 cells. Values are from three biological replicates ± SEM. Statistical values were determined by the one-way ANOVA, and the *F* test to compare variances. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

that Rot is involved in the regulation of cell surface adhesins, superantigen-like proteins, and secreted toxins and enzymes (Said-Salim et al., 2003). Rot is thought to function downstream of the Agr system, considering the translation inhibition of Rot by RNAIII (Boisset et al., 2007). The transcription of *rot* is positively modulated by SigB and negatively regulated by SarA (Hsieh et al., 2008; Manna and Ray, 2007). Nevertheless, no other regulators have been reported that could directly regulate the expression of *rot* in *S. aureus*.

Our previous work has demonstrated that SpoVG is involved in oxacillin resistance of MRSA by regulating the expression of genes involved in cell wall synthesis and degradation (Liu et al., 2016). However, the detailed regulatory pathways mediated by SpoVG still remain unclear. In this study, we have investigated the expression of genes encoding SarA family proteins. Our data indicated that the transcript level of *rot* was significantly decreased in the *spoVG* mutant strain compared with that of the WT strain. In addition, we have found that SpoVG can positively influence the promoter activity of *rot* through the *rot* promoter-*lacZ* fusion reporter strategy, and that SpoVG can bind to the promoter region of *rot* specifically. All these data have demonstrated that SpoVG can regulate the expression of *rot* in *S. aureus* strain N315 by directly binding to the *rot* promoter.

Previous reports have found that Rot can positively regulate the virulence factor Spa and surface-located fibrinogen-binding protein ClfB in *S. aureus* (Gao and Stewart, 2004; Said-Salim et al., 2003; Xue et al., 2012). Since SpoVG can positively regulate the expression of *rot*, it is reasonable to assume that SpoVG may have an effect on the expression of *spa* and *clfB*. We found that both SpoVG and Rot could positively regulate the expression of *spa* and *clfB*. Moreover, we have demonstrated that either SpoVG or its phosphorylated derivative can bind to the promoter regions of *spa* and *clfB* specifically. These results have demonstrated that SpoVG and Rot can constitute a regulatory cascade in which Rot acts downstream of SpoVG, and SpoVG can positively regulate the transcription of *spa* and *clfB* through direct-binding to their promoters and in a Rot-mediated manner. Previous full genome DNA microarray analysis indicated that 19 operons were downregulated and 8 were upregulated in the isogenic *yabJ-spoVG* mutant of *S. aureus* Newman (Schulthess et al., 2011). However, neither genes *rot*, *spa*, and *clfB*, nor genes *femA*, *lytN*, and *lytS* that were reported previously to be regulated by SpoVG (Liu et al., 2016) have been identified in the *S. aureus* Newman microarray data, perhaps in part because of the strain variability.

Human fibrinogen can serve as an early barrier of defense against infection of pathogens (Ko and Flick, 2016). *S. aureus* has evolved and maintained a series of variant extracellular proteins that can bind to human fibrinogen, including *S. aureus* microbial surface components recognizing adhesive matrix molecules, ClfA and ClfB (Hartford et al., 2001; Ní Eidhin et al., 1998; Walsh et al., 2008), the fibronectin-binding proteins FnbpA and FnbpB (Stemberk et al., 2014; Wann et al., 2000), the bone sialoprotein-binding protein Bbp (Vazquez et al., 2011), the *S. aureus* surface protein SdrE (Tung et al., 2000), the coagulase (Ko et al., 2016), the extracellular fibrinogen-binding protein Efb (Lee et al., 2004), the extracellular matrix-binding protein Emp, and the extracellular adherence protein Eap (Hussain et al., 2001; Jonsson et al., 1995). Our results revealed that both SpoVG and Rot can positively regulate the expression of *clfB*. It has been reported that ClfB can bind to the α-chain of fibrinogen (Ní Eidhin et al., 1998), with a core-binding motif as GSSGXGXXG in fibrinogen (Xiang et al., 2012). Here we have shown that both SpoVG and Rot can positively affect the bacterial fibrinogen-binding ability, indicating that the SpoVG-Rot regulatory cascade could affect the fibrinogen-binding ability by regulating the *clfB* expression.

S. aureus can express numerous cell wall-anchored proteins with multiple functions, including adhesion and internalization of host cells and tissues, biofilm formation, and evasion of host immune responses. Previous reports have found that Spa is involved in opsonophagocytosis and inflammation (Cedergren et al., 1993; Foster et al., 2014; Gomez et al., 2004), and ClfB is involved in adhesion to desquamated epithelial cells and nasal colonization (Mulcahy et al., 2012; Ní Eidhin et al., 1998). It is believed that the adhesion of *S. aureus* to the host cell surface is a prerequisite for infection (Lowy, 1998; Reichert and Stern, 1984), and the ability of internalization within the host cell can protect *S. aureus* from host defense (Alexander and Hudson, 2001). The human cell infection assays showed that SpoVG could positively affect the cell adhesion and internalization abilities of *S. aureus*, which may depend on the coordinated transcriptional regulation mediated by SpoVG. A previous study has revealed that no detectable alteration of transcriptome was observed in *S. aureus* during the extracellular and adhesion process (Garzoni et al., 2007). In this study, the expression alteration of *spa* and *clfB* in the adhesion process was observed, which implied that virulence genes such as *spa* and *clfB* may be involved in the adhesion process. Several virulence factors of *S. aureus* have been proved to be involved in bacterial invasion into human cells, including Fnbps (Sinha et al., 1999; Sinha and Herrmann, 2005), Eap (Haggar et al., 2003), autolysin Atl (Hirschhausen et al., 2010), and iron-regulated surface determinant B (IsdB) (Zapotoczna et al., 2013). In this study, SpoVG was found to influence the internalization of *S. aureus* into HEK293 T and THP-1. By detecting the expression of genes in the internalization process, we found that the transcription of virulence genes *spa* and *clfB* was significantly decreased in the *spoVG* mutant strain compared with that in the WT strain. ClfB was shown previously not to be involved in the internalization of *S. aureus* by primary oral keratinocytes (Kintarak et al., 2004), and whether Spa can mediate the internalization still remains uncertain. The surface molecules that directly participate in the internalization process mediated by SpoVG need to be further studied.

The staphylococcal Ser/Thr protein kinase, Stk1, has been considered to be involved in multiple cellular pathways, including antibiotic resistance and virulence regulation (Burnside et al., 2010; Debarbouille et al., 2009; Tamber et al., 2010). Moreover, Stk1 can phosphorylate several regulatory factors, such as SarA (Didier et al., 2010), VraR (Canova et al., 2014), CcpA (Leiba et al., 2012), LuxS (Cluzel et al., 2010), and MgrA (Truong-Bolduc and Hooper, 2010). Phosphorylation of these proteins can confer specific properties such as DNA binding and enzymatic activity. In addition, one report has indicated that the DNA-binding property of SpoVG can be enhanced through phosphorylation mediated by Stk1 (Bischoff et al., 2016). Our results are consistent with these findings and further demonstrated that the phosphorylation of SpoVG can positively regulate its DNA-binding property.

The increasing associations between virulence gene expression and pathogenesis of *S. aureus* have emphasized the importance of understanding the regulators and regulatory pathways. So far, multiple strategies such as RNA sequencing and peptide pull-down assay have been used to discover novel regulators or regulatory networks in *S. aureus*. The study of novel regulatory cascades can facilitate us to reach a deeper understanding of the regulatory patterns of gene expression involved in virulence and pathogenicity of *S. aureus*. Our findings have shown that SpoVG and Rot can form a regulatory cascade in which Rot acts downstream of SpoVG and can be positively regulated by SpoVG. The SpoVG-Rot regulatory cascade can positively control the expression of virulence genes *spa* and *clfB* via direct and indirect pathways (Fig. 8). We further have identified new functions of the SpoVG-Rot regulatory cascade in *S. aureus*, which links the expression of virulence genes with human fibrinogen-binding and cell adhesion and internalization abilities together.

5. Conclusions

This study has revealed that a novel SpoVG-Rot regulatory cascade can positively regulate the transcription of *spa* and *clfB*, and SpoVG is involved in the control of human fibrinogen-binding and cell adhesion and internalization abilities of *S. aureus*.

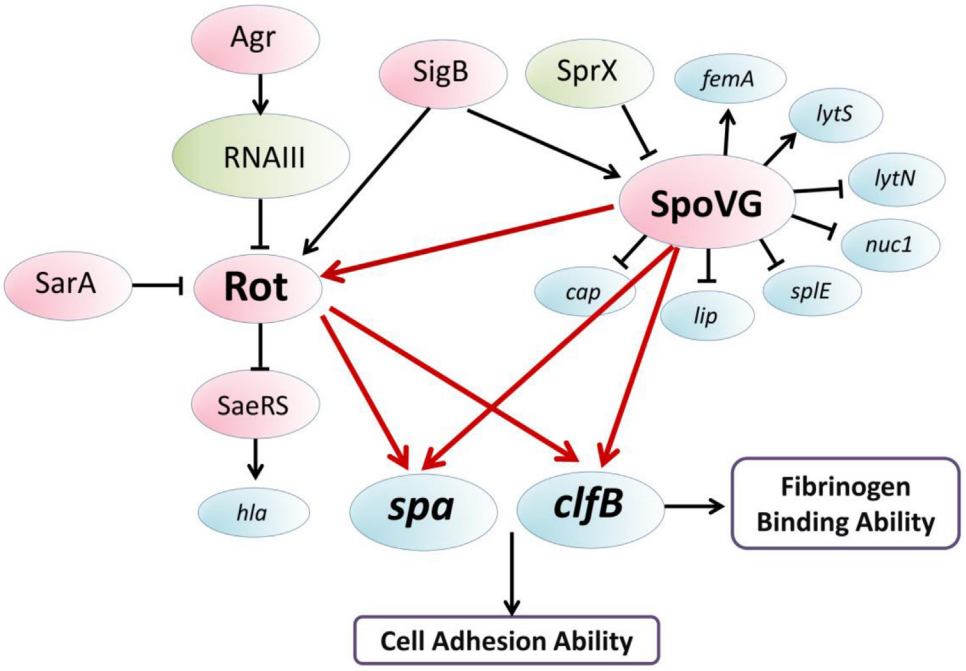

Fig. 8. Regulatory circuits involved in SpoVG and virulence gene expression. The SpoVG-Rot regulatory cascade is represented by red line, the transcriptional regulatory proteins are in pink, the regulatory RNAs are in green, and the target genes are in blue. Arrows represent activation, whereas bars represent repression. All the regulatory circuits represented here are derived from the literatures and this study. SpoVG positively regulates the transcription of *rot* by binding to the promoter region of *rot*. As a global regulator, SpoVG positively regulates the expression of *spa* and *clfB* through direct-binding to their promoters and in a Rot-mediated manner.

Acknowledgments

We thank Shijie Zhang and Xiaoyu Liu for technical assistance. This study was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB29020000).

References

Alexander, E.H., Hudson, M.C., 2001. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. Appl. Microbiol. Biotechnol. 56, 361–366.
Archer, G.L., Climo, M.W., 2001. *Staphylococcus aureus* bacteremia – consider the source. N. Engl. J. Med. 344, 55–56.
Arvidson, S., Tegmark, K., 2001. Regulation of virulence determinants in *Staphylococcus aureus*. Int. J. Med. Microbiol. 291, 159–170.
Bischoff, M., Brelle, S., Minatelli, S., Molle, V., 2016. Stk1-mediated phosphorylation stimulates the DNA-binding properties of the *Staphylococcus aureus* SpoVG transcriptional factor. Biochem. Biophys. Res. Commun. 473, 1223–1228.
Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B., Projan, S., 2004. Microarray-based analysis of the *Staphylococcus aureus* sigma(B) regulon. J. Bacteriol. 186, 4085–4099.
Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A., others, 2007. Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev. 21, 1353–1366.
Bronesky, D., Wu, Z.F., Marzi, S., Walter, P., Geissmann, T., Moreau, K., Vandenesch, F., Caldelari, I., Romby, P., 2016. Staphylococcus aureus RNAIII and its Regulon link quorum sensing, stress responses. Metabolic adaptation, and regulation of virulence gene expression. Annu. Rev. Microbiol. 70, 299–316.
Bronner, S., Monteil, H., Prevost, G., 2004. Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiol. Rev. 28, 183–200.
Bunikowski, R., Mielke, M.E.A., Skarabis, H., Worm, M., Anagnostopoulos, I., Kolde, G., Wahn, U., Renz, H., 2000. Evidence for a disease-promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis. J. Allergy Clin. Immunol. 105, 814–819.
Burke, T.P., Portnoy, D.A., 2016. SpoVG is a conserved RNA-binding protein that regulates listeria monocytogenes lysozyme resistance, virulence, and swarming motility. mBio 7, e00240–16.
Burnside, K., Lembo, A., de Los Reyes, M., Iliuk, A., Binhtran, N.T., Connelly, J.E., Lin, W.J., Schmidt, B.Z., Richardson, A.R., Fang, F.C., Tao, W.A., Rajagopal, L., 2010. Regulation of hemolysin expression and virulence of *Staphylococcus aureus* by a serine/threonine kinase and phosphatase. PLoS One 5, e11071.
Canova, M.J., Baronian, G., Brelle, S., Cohen-Gonsaud, M., Bischoff, M., Molle, V., 2014. A novel mode of regulation of the *Staphylococcus aureus* vancomycin-resistance-associated response regulator VraR mediated by Stk1 protein phosphorylation. Biochem. Biophys. Res. Commun. 447, 165–171.
Cedergren, L., Andersson, R., Jansson, B., Uhlen, M., Nilsson, B., 1993. Mutational analysis of the interaction between Staphylococcal protein-a and human Igg(1). Protein Eng. 6, 441–448.
Chabelskaya, S., Bordeau, V., Felden, B., 2014. Dual RNA regulatory control of a *Staphylococcus aureus* virulence factor. Nucleic Acids Res. 42, 4847–4858.
Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H., Xiong, Y.-Q., 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. 40, 1–9.
Cheung, A.L., Nishina, K.A., Trotonda, M.P., Tamber, S., 2008. The SarA protein family of *Staphylococcus aureus*. Int. J. Biochem. Cell Biol. 40, 355–361.
Cheung, A.L., Zhang, G.Y., 2002. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. Front. Biosci. 7, D1825–D1842.
Chevalier, C., Boisset, S., Romilly, C., Masquida, B., Fechter, P., Geissmann, T., Vandenesch, F., Romby, P., 2010. *Staphylococcus aureus* RNAIII binds to two distant regions of coa mRNA to arrest translation and promote mRNA degradation. PLoS Pathog. 6, e1000809.
Cluzel, M.E., Zanella-Cleon, I., Cozzone, A.J., Futterer, K., Duclos, B., Molle, V., 2010. The *Staphylococcus aureus* autoinducer-2 synthase LuxS is regulated by Ser/Thr phosphorylation. J. Bacteriol. 192, 6295–6301.
Corrigan, R.M., Foster, T.J., 2009. An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. Plasmid 61, 126–129.
Debarbouille, M., Dramsi, S., Dussurget, O., Nahori, M.A., Vaganay, E., Jouvion, G., Cozzone, A., Msadek, T., Duclos, B., 2009. Characterization of a serine/threonine kinase involved in virulence of *Staphylococcus aureus*. J. Bacteriol. 191, 4070–4081.
Didier, J.P., Cozzone, A.J., Duclos, B., 2010. Phosphorylation of the virulence regulator SarA modulates its ability to bind DNA in *Staphylococcus aureus*. FEMS Microbiol. Lett. 306, 30–36.
Dinges, M.M., Orwin, P.M., Schlievert, P.M., 2000. Exotoxins of *Staphylococcus aureus*. Clin. Microbiol. Rev. 13, 16.
Eyraud, A., Tattevin, P., Chabelskaya, S., Felden, B., 2014. A small RNA controls a protein regulator involved in antibiotic resistance in *Staphylococcus aureus*. Nucleic Acids Res. 42, 4892–4905.
Foster, T.J., 2005. Immune evasion by Staphylococci. Nat. Rev. Microbiol. 3, 948–958.
Foster, T.J., 2009. Colonization and infection of the human host by staphylococci: adhesion, survival and immune evasion. Vet. Dermatol. 20, 456–470.
Foster, T.J., Geoghegan, J.A., Ganesh, V.K., Hook, M., 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat. Rev. Microbiol. 12, 49–62.
Foster, T.J., Hook, M., 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol. 6, 484–488.
Frisby, D., Zuber, P., 1991. Analysis of the upstream activating sequence and site of carbon and nitrogen source repression in the promoter of an early-induced sporulation gene of *Bacillus subtilis*. J. Bacteriol. 173, 7557–7564.
Gao, J.X., Stewart, G.C., 2004. Regulatory elements of the *Staphylococcus aureus* protein A (Spa) promoter. J. Bacteriol. 186, 3738–3748.
Garzoni, C., Francois, P., Huyghe, A., Couzinet, S., Tapparel, C., Charbonnier, Y., Renzoni, A., Lucchini, S., Lew, D.P., Vaudaux, P., Kelley, W.L., Schrenzel, J., 2007. A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. BMC Genom. 8, 171.
Gomez, M.I., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A., Prince, A., 2004. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. Nat. Med. 10, 842–848.
Haggar, A., Hussain, M., Lonnies, H., Herrmann, M., Norrby-Teglund, A., Flock, J.I., 2003. Extracellular adherence protein from *Staphylococcus aureus* enhances internalization

into eukaryotic cells. Infect. Immun. 71, 2310–2317.
Hartford, O.M., Wann, E.R., Hook, M., Foster, T.J., 2001. Identification of residues in the *Staphylococcus aureus* fibrinogen-binding MSCRAMM clumping factor A (ClfA) that are important for ligand binding. J. Biol. Chem. 276, 2466–2473.
Hirschhausen, N., Schlesier, T., Schmidt, M.A., Gotz, F., Peters, G., Heilmann, C., 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. Cell. Microbiol. 12, 1746–1764.
Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K., Foster, S.J., 2002. sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. J. Bacteriol. 184, 5457–5467.
Hsieh, H.-Y., Tseng, C.W., Stewart, G.C., 2008. Regulation of Rot expression in *Staphylococcus aureus*. J. Bacteriol. 190, 546–554.
Hu, J., Zhang, X., Liu, X., Chen, C., Sun, B., 2015. Mechanism of reduced vancomycin susceptibility conferred by walK mutation in community-acquired methicillin-resistant *Staphylococcus aureus* strain MW2. Antimicrob. Agents Chemother. 59, 1352–1355.
Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C., et al., 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate spa gene expression. EMBO J. 24, 824–835.
Hussain, M., Becker, K., von Eiff, C., Schrenzel, J., Peters, G., Herrmann, M., 2001. Identification and characterization of a novel 38.5-kilodalton cell surface protein of *Staphylococcus aureus* with extended-spectrum binding activity for extracellular matrix and plasma proteins. J. Bacteriol. 183, 6778–6786.
Ibarra, J.A., Perez-Rueda, E., Carroll, R.K., Shaw, L.N., 2013. Global analysis of transcriptional regulators in *Staphylococcus aureus*. BMC Genom. 14, 126.
Jonsson, K., Mcdevitt, D., Mcgavin, M.H., Patti, J.M., Hook, M., 1995. *Staphylococcus aureus* expresses a major histocompatibility complex class-Ii analog. J. Biol. Chem. 270, 21457–21460.
Jutras, B.L., Chenail, A.M., Rowland, C.L., Carroll, D., Miller, M.C., Bykowski, T., Stevenson, B., 2013. Eubacterial SpoVG homologs constitute a new family of site-specific DNA-binding proteins. PLoS One 8, e66683.
Kintarak, S., Whawell, S.A., Speight, P.M., Packer, S., Nair, S.P., 2004. Internalization of *Staphylococcus aureus* by human keratinocytes. Infect. Immun. 72, 5668–5675.
Ko, Y.P., Flick, M.J., 2016. Fibrinogen is at the interface of host defense and pathogen virulence in *Staphylococcus aureus* infection. Semin. Thromb. Hemost. 42, 408–421.
Ko, Y.P., Kang, M.S., Ganesh, V.K., Ravirajan, D., Li, B., Hook, M., 2016. Coagulase and Efb of *Staphylococcus aureus* have a common fibrinogen binding motif. mBio 7, e01885–15.
Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L.Z., Oguchi, A., Aoki, K., Nagai, Y., Lian, J.Q., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., Hiramatsu, K., 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet 357, 1225–1240.
Lee, C.Y., Buranen, S.L., Ye, Z.H., 1991. Construction of single-copy integration vectors for *Staphylococcus aureus*. Gene 103, 101–105.
Lee, L.Y.L., Liang, X.W., Hook, M., Brown, E.L., 2004. Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb). J. Biol. Chem. 279, 50710–50716.
Leiba, J., Hartmann, T., Cluzel, M.E., Cohen-Gonsaud, M., Delolme, F., Bischoff, M., Molle, V., 2012. A novel mode of regulation of the *Staphylococcus aureus* catabolite control protein A (CcpA) mediated by Stk1 protein phosphorylation. J. Biol. Chem. 287, 43607–43619.
Liu, X., Zhang, S., Sun, B., 2016. SpoVG regulates cell wall metabolism and oxacillin resistance in methicillin-resistant *Staphylococcus aureus* strain N315. Antimicrob. Agents Chemother. 60, 3455–3461.
Lowy, F.D., 1998. Medical progress – *Staphylococcus aureus* infections. N. Engl. J. Med. 339, 520–532.
Manna, A.C., Ray, B., 2007. Regulation and characterization of rot transcription in *Staphylococcus aureus*. Microbiology (Reading, England) 153, 1538–1545.
Matsuno, K., Sonenshein, A.L., 1999. Role of SpoVG in asymmetric septation in *Bacillus subtilis*. J. Bacteriol. 181, 3392–3401.
McNamara, P.J., Milligan-Monroe, K.C., Khalili, S., Proctor, R.A., 2000. Identification, cloning, and initial characterization of rot, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. 182, 3197–3203.
Meier, S., Goerke, C., Woiz, C., Seidl, K., Homerova, D., Schulthess, B., Kormanec, J., Berger-Baechi, B., Bischoff, M., 2007. sigma(B) and the sigma(B)-dependent arlRS and yabJ-spoVG loci affect capsule formation in *Staphylococcus aureus*. Infect. Immun. 75, 4562–4571.
Mulcahy, M.E., Geoghegan, J.A., Monk, I.R., O'Keeffe, K.M., Walsh, E.J., Foster, T.J., McLoughlin, R.M., 2012. Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. PLoS Pathog. 8, e1003092.
Ní Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Höök, M., Foster, T.J., 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. Mol. Microbiol. 30, 245–257.
Pan, X., Chen, X., Su, X., Feng, Y., Tao, Y., Dong, Z., 2014. Involvement of SpoVG in hemolysis caused by *Bacillus subtilis*. Biochem. Biophys. Res. Commun. 443, 899–904.
Pichon, C., Felden, B., 2005. Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. Proc. Natl. Acad. Sci. U.S.A. 102, 14249–14254.
Reichert, R., Stern, G., 1984. Quantitative adherence of bacteria to human corneal epithelial-cells. Arch. Ophthalmol.-Chic 102, 1394–1395.
Rosenbluh, A., Banner, C.D., Losick, R., Fitz-James, P.C., 1981. Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. J. Bacteriol. 148, 341–351.
Said-Salim, B., Dunman, P.M., McAleese, F.M., Macapagal, D., Murphy, E., McNamara, P.J., Arvidson, S., Foster, T.J., Projan, S.J., Kreiswirth, B.N., 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J. Bacteriol. 185, 610–619.
Schneewind, O., Mihaylova-Petkov, D., Model, P., 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. EMBO J. 12, 4803–4811.
Schulthess, B., Bloes, D.A., Berger-Baechi, B., 2012. Opposing roles of sigma(B) and sigma (B)-controlled SpoVG in the global regulation of esxA in *Staphylococcus aureus*. BMC Microbiol. 12, 17.
Schulthess, B., Bloes, D.A., Francois, P., Girard, M., Schrenzel, J., Bischoff, M., Berger-Bachi, B., 2011. The sigma(B)-dependent yabJ-spoVG operon is involved in the regulation of extracellular nuclease, lipase, and protease expression in *Staphylococcus aureus*. J. Bacteriol. 193, 4954–4962.
Schulthess, B., Meier, S., Homerova, D., Goerke, C., Wolz, C., Kormanec, J., Berger-Bachi, B., Bischoff, M., 2009. Functional characterization of the sigma(B)-dependent yabJ-spoVG Operon in *Staphylococcus aureus*: role in methicillin and glycopeptide resistance. Antimicrob. Agents Chemother. 53, 1832–1839.
Segall, J., Losick, R., 1977. Cloned *Bacillus subtilis* DNA containing a gene that is activated early during sporulation. Cell 11, 751–761.
Sinha, B., Francois, P.P., Nusse, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., Krause, K.H., 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha(5)beta(1). Cell. Microbiol. 1, 101–117.
Sinha, B., Herrmann, M., 2005. Mechanism and consequences of invasion of endothelial cells by *Staphylococcus aureus*. Thromb. Haemost. 94, 266–277.
Somerville, G.A., Sayd-Salim, B., Wickman, J.M., Raffel, S.J., Kreiswirth, B.N., Musser, J.M., 2003. Correlation of acetate catabolism and growth yield in *Staphylococcus aureus*: implications for host–pathogen interactions. Infect. Immun. 71, 4724–4732.
Stemberk, V., Jones, R.P.O., Moroz, O., Atkin, K.E., Edwards, A.M., Turkenburg, J.P., Leech, A.P., Massey, R.C., Potts, J.R., 2014. Evidence for steric regulation of fibrinogen binding to *Staphylococcus aureus* fibronectin-binding protein A (FnBPA). J. Biol. Chem. 289, 12842–12851.
Stock, A.M., Robinson, V.L., Goudreau, P.N., 2000. Two-component signal transduction. Annu. Rev. Biochem 69, 183–215.
Tamber, S., Schwartzman, J., Cheung, A.L., 2010. Role of PknB kinase in antibiotic resistance and virulence in community-acquired methicillin-resistant *Staphylococcus aureus* strain USA300. Infect. Immun. 78, 3637–3646.
Truong-Bolduc, Q.C., Hooper, D.C., 2010. Phosphorylation of MgrA and its effect on expression of the NorA and NorB efflux pumps of *Staphylococcus aureus*. J. Bacteriol. 192, 2525–2534.
Tung, H.S., Guss, B., Hellman, U., Persson, L., Rubin, K., Ryden, C., 2000. A bone sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family. Biochem. J. 345, 611–619.
Valihrach, L., Demnerova, K., 2012. Impact of normalization method on experimental outcome using RT-qPCR in *Staphylococcus aureus*. J. Microbiol. Methods 90, 214–216.
Vazquez, V., Liang, X.W., Horndahl, J.K., Ganesh, V.K., Smeds, E., Foster, T.J., Hook, M., 2011. Fibrinogen is a ligand for the *Staphylococcus aureus* microbial surface components recognizing adhesive matrix molecules (MSCRAMM) bone sialoprotein-binding protein (Bbp). J. Biol. Chem. 286, 29797–29805.
Walsh, E.J., Miajlovic, H., Gorkun, O.V., Foster, T.J., 2008. Identification of the *Staphylococcus aureus* MSCRAMM clumping factor B (ClfB) binding site in the alphaC-domain of human fibrinogen. Microbiology (Reading, England) 154, 550–558.
Wann, E.R., Gurusiddappa, S., Hook, M., 2000. The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. J. Biol. Chem. 275, 13863–13871.
Xiang, H., Feng, Y., Wang, J., Liu, B., Chen, Y., Liu, L., Deng, X., Yang, M., 2012. Crystal structures reveal the multi-ligand binding mechanism of *Staphylococcus aureus* ClfB. PLoS Pathog. 8, e1002751.
Xue, T., You, Y., Shang, F., Sun, B., 2012. Rot and Agr system modulate fibrinogen-binding ability mainly by regulating clfB expression in *Staphylococcus aureus* NCTC8325. Med. Microbiol. Immunol. (Berl.) 201, 81–92.
Zapotoczna, M., Jevnikar, Z., Miajlovic, H., Kos, J., Foster, T.J., 2013. Iron-regulated surface determinant B (IsdB) promotes *Staphylococcus aureus* adherence to and internalization by non-phagocytic human cells. Cell. Microbiol. 15, 1026–1041.