Supplementary Information for

Chemical genetics unveils WTA/PBP2a-PBP2/VraRS as a regulatory axis for virulence gene expression in CA-MRSA

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Supplementary Information Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S2. Unless otherwise noted, *S. aureus* strains were grown in tryptic soy broth (TSB, Difco) at 37°C with shaking (250 rpm), or on tryptic soy agar (TSA, Difco) at 37°C. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with shaking (250 rpm) or on LB agar plates at 37°C. For plasmid maintenance, antibiotics were used at the following concentrations where appropriate: for *S. aureus*, erythromycin (Sangon

Biotech) at 10 μg/ml for RN4220 and 80 μg/ml for USA300 LAC and its derivatives, chloramphenicol (Sangon Biotech) at 15 μg/ml; for *E. coli*, carbenicillin (Sangon Biotech) at 150 μg/ml. For other reagents, tunicamycin and vancomycin (Dalian Meilun Biotech Co., Ltd.), targocil (Shanghai TopScience Co, Ltd.), oxacillin (Shanghai Aladdin Biochemical Technology Co.,Ltd.), imipenem, cefuroxime, ceftizoxime, cefaclor, cefoxitin, and epicatechin gallate from MedChemExpress, cefotaxime, isopropyl β-D-1-thiogalactopyranoside (IPTG), and AIP from Sangon Biotech, anhydrotetracycline (aTc) from APExBIO, Tarocin A1 from Merck, moenomycin complex from GlpBio were used in this study.

Construction of *psmα-lacZ* and *vraX-lacZ* transcriptional fusions

The plasmid pCL-*lacZ* carrying a promoterless *lacZ* reporter gene was used to construct promoter-*lacZ* reporter fusion as previously described (1, 2). For the construction of *psmα-lacZ*, *psmα* promoter region (-523 to -1 of the start codon) was amplified from *S. aureus* USA300 LAC genomic DNA by PCR using the primers *psmα*-pro-F (with *Eco*RI site) and *psmα*-pro-R (with *Kpn*I site) (Table S3). To generate the *vraX-lacZ*, the promoter region of *vraX* (-565 to -3 of the start codon) was amplified from *S. aureus* USA300 LAC genomic DNA by PCR using the primers *vraX*-pro-F (with *Eco*RI site) and *vraX*-pro-R (with *Kpn*I site) (Table S3). The promoter fragments and the plasmid pCL-*LacZ* were digested with *Eco*RI and *Kpn*I, then ligated into pCL-*LacZ* and introduced to *E. coli* DH5α. The construct was confirmed by DNA sequencing, electroporated into RN4220, then transduced into USA300 LAC or its derivatives using bacteriophage Ø85.

Construction of gene deletion mutants

For the construction of the *tarO* null mutant (Δ*tarO*), the upstream fragment (~ 1.1 kb) of the intended deletion was amplified from *S. aureus* USA300 LAC genomic DNA using primers *tarO*-up-F and *tarO*-up-R (Table S3), and the downstream fragment (~ 1.0 kb) of the intended deletion was amplified with primers *tarO*-down-F and *tarO*-down-R (Table S3). The upstream fragment and the downstream fragment were ligated using primers *tarO*-up-F and *tarO*-down-R via overlapping PCR and the product was used for recombination with plasmid pKOR1 (3), yielding pKOR1::*tarO*. The resultant plasmid was introduced to *E. coli* DH5α and sequenced to ensure that no unwanted mutations resulted. Then, the resulting plasmid was transferred by electroporation to *S. aureus* RN4220 and subsequently into *S. aureus* USA300 LAC. The allelic replacement was performed as described previously (1, 2), and the deletion of target gene was confirmed by PCR.

A similar strategy was used to construct Δ*vraR* and Δ*mecA* mutants. For the construction of Δ*vraR*, the upstream fragment (~ 1.0 kb) of the intended deletion was amplified from *S. aureus* USA300 LAC genomic DNA using primers *vraR*-up-F and *vraR*-up-R (Table S3), and the downstream fragment (~ 1.0 kb) of the intended deletion was amplified with primers *vraR*-down-F and *vraR*-down-R (Table S3). The upstream fragment and the downstream fragment were ligated using primers *vraR*-up-F and *vraR*-down-R via overlapping PCR, and the overlapping PCR product was cloned into plasmid pKOR1, yielding pKOR1::*vraR*. For the construction of Δ*mecA*, the upstream

fragment (~ 1.4 kb) of the intended deletion was amplified from *S. aureus* USA300 LAC genomic DNA using primers *mecA*-up-F and *mecA*-up-R (Table S3), and the downstream fragment (~ 1.4 kb) of the intended deletion was amplified with primers *mecA*-down-F and *mecA*-down-R (Table S3). The upstream fragment and the downstream fragment were ligated using primers *mecA*-up-F and *mecA*-down-R via overlapping PCR, and the overlapping PCR product was cloned into plasmid pKOR1, yielding pKOR1::*mecA*. For the construction of Δ*tarO*Δ*vraR*, pKOR1::*tarO* was transferred by electroporation to Δ*vraR*, and the allelic replacement was performed as described above.

Construction of plasmids for gene complementation and gene overexpression

For cloning genes with higher efficiency, we incorporated two restriction enzyme sites (i.e., *Asc*I and *Psp*OMI) into pYJ335 plasmid using site-directed mutagenesis method as previously described (4) with primers pYJ335-PCR-F and pYJ335-PCR-R. The resulting plasmid, pYJ335-1, which contains restriction sites for *Eco*RV, *Asc*I, and *Psp*OMI, enables the cloning of DNA fragments with compatible cohesive or blunt ends. The modified plasmid pYJ335-1 was sequenced to ensure that no unwanted mutations resulted. To construct plasmid p-*tarO*, a ~ 1.1 kb DNA fragment containing *tarO* was amplified from *S. aureus* USA300 LAC genomic DNA with primers *tarO*-F and *tarO*-R (Table S3). The *tarO* PCR fragment was digested with *Psp*OMI, and the plamid pYJ335-1 was digested with *Eco*RV and *Psp*OMI, then ligated, where the *tarO* gene was downstream of the tetracycline-inducible xyl/tetO promoter of pYJ335-1, yielding

plasmid p-*tarO*. Construction of p-*tarO* derivative p-*tarO*^{G152A} was performed as previously described (5) with the QuikChange site-directed mutagenesis kit (StrataGene, catalog no. 200518) and the use of primer pair G152A-F/G152A-R (Table S3).

To construct plasmid p-*vraR*, a ~ 0.7 kb DNA fragment containing *vraR* was amplified from *S. aureus* USA300 LAC genomic DNA with primers *vraR*-F and *vraR*-R (Table S3). The *vraR* PCR fragment was digested with *Psp*OMI, and the plamid pYJ335-1 was digested with *Eco*RV and *Psp*OMI, then ligated, where the *vraR* gene was downstream of the tetracycline-inducible xyl/tetO promoter of pYJ335-1, yielding plasmid p-*vraR*. To generate plasmid p-*sgtB*, a ~ 0.85 kb DNA fragment containing *sgtB* was amplified from *S. aureus* JE2 genomic DNA with primers *sgtB*-F and *sgtB*-R (Table S3). The plasmid pYJ335 was digested with *Eco*RV and ligated with the *sgtB* PCR fragment, where *sgtB* gene was in downstream of the tetracycline-inducible xyl/tetO promoter of pYJ335, yielding plasmid p-*sgtB*. To generate plasmid p-*mecA*, a ~ 2.1 kb DNA fragment containing *mecA* was amplified from *S. aureus* USA300 LAC genomic DNA with primers *mecA*-F and *mecA*-R (Table S3). The plasmid pYJ335 was digested with *Eco*RV and ligated with the *mecA* PCR fragment, where *mecA* gene was in downstream of the tetracycline-inducible xyl/tetO promoter of pYJ335, yielding plasmid p-*mecA*. To construct plasmid p-*murJ*, a ~ 1.7 kb DNA fragment containing *murJ* was amplified from *S. aureus* USA300 LAC genomic DNA with primers *murJ*-F and *murJ*-R (Table S3). Then *murJ* PCR fragment was ligated with the plasmid pYJ335 by homologous recombination, where the *murJ* gene was downstream of the

tetracycline-inducible xyl/tetO promoter of pYJ335, yielding plasmid p-*murJ*.

The resulting plasmid was introduced to *E. coli* DH5α and sequenced to ensure that no unwanted mutations resulted. Then the plasmid was transferred by electroporation to *S. aureus* RN4220 and subsequently into *S. aureus* USA300 LAC and its derivatives.

Construction of *pbp2*-depleted mutant USA300-1

To construct pMutin-HA-*pbp2'* plasmid for the genetic depletion of *pbp2* in USA300 LAC, a DNA fragment covering the ribosome binding site region and the first 630 bp (-20 to + 630 of the start codon) of *pbp2* was amplified from USA300 LAC genomic DNA with primers P*spac*-*pbp2*-F and P*spac*-*pbp2*-R (Table S3). The PCR product and the plasmid pMutin-HA (6) were digested with *Hin*dIII and *Kpn*I and then ligated. The recombination plasmid was introduced to *E. coli* DH5α and sequenced to ensure that no unwanted mutations resulted. The resulting plasmid, pMutin-HA-*pbp2'*, was electroporated into RN4220 and clones were selected on TSA plate supplemented with 10 μg/ml erythromycin and 1 mM IPTG. Integration of pMutin-HA-*pbp2'* into the chromosome at the *pbp2* locus of RN4220 was confirmed by PCR. The integrated plasmids were then transduced with Φ85 into USA300 LAC and clones were selected on a TSA plate supplemented with 80 μg/ml erythromycin and 1 mM IPTG, yielding USA300-1 strain.

Construction of *gfp*-*pbp2* fusion

To construct the *gfp-pbp2* fusion, codon-optimized *gfp* gene (7) was synthesized by Generay Biotech and then amplified with primers *gfp*-F and *gfp*-R (Table S3). The *pbp2* gene was amplified from *S. aureus* USA300 LAC genomic DNA with primers *pbp2*-F and *pbp2*-R (Table S3), then the *gfp* fragment and the *pbp2* fragment were ligated using overlapping PCR with primers *gfp*-F and *pbp2*-R. The resulting *gfp-pbp2* fragment was digested with *Psp*OMI and the plasmid pYJ335-1 was digested with *Eco*RV and *Psp*OMI, then ligated, where the *gfp-pbp2* gene was in downstream of the tetracycline-inducible xyl/tetO promoter of pYJ335-1, yielding plasmid p-*gfp-pbp2*. The resultant plasmid was introduced to *E. coli* DH5α and sequenced to ensure that no unwanted mutations resulted. Then, the resulting plasmid was transferred by electroporation to *S. aureus* RN4220, and subsequently into *S. aureus* USA300 LAC and Δ*tarO*, resulting USA300/p-*gfp-pbp2* and Δ*tarO*/p-*gfp-pbp2* strains, respectively.

WTA extraction and the polyacrylamide gel electrophoresis (PAGE) analysis

Overnight cultures of *S. aureus* strains were diluted into 10 ml fresh TSB which supplemented with 80 μg/ml erythromycin or tunicamycin (where appropriate) in a 50-ml tube with initial $OD_{600} \approx 0.1$ and were cultured for 3 h at 37°C with shaking (250 rpm), then WTAs were extracted as previously described (8). The WTA extractions were subjected to 20% (wt/vol) PAGE in Tris-Glycine running buffer (0.1 M Tris base, 0.1 M Glycine) at 135 V for 120 min and then WTA bands were visualized using the alcian blue-silver staining protocol (9).

Bacterial growth curve measurement

Overnight cultures of the *S. aureus* strains were diluted into 10 ml fresh TSB supplemented with 80 μg/ml erythromycin or the indicated compound in a 50-ml tube with initial OD_{600} ≈ 0.1, and the diluted cultures were grown for 24 h at 37°C with shaking (250 rpm). The growth of bacteria (three biological replicates for each group) was monitored using a nanodrop to measure absorption at 600 nm at the indicated time points.

RNA-seq and data analysis

Overnight cultures of WT *S. aureus* USA300 LAC strain and Δ*tarO* mutant were diluted into 10 ml fresh TSB in a 50-ml tube with initial OD_{600} ≈ 0.1 and were cultured at 37°C with shaking (250 rpm) for 1.5 h (OD_{600} ≈ 0.6) or for 3 h (OD_{600} ≈ 3). The bacteria were harvested by centrifugation, and total RNA was immediately extracted using a Qiagen RNeasy kit (Cat. No. 74104) following the manufacturer's instructions. After rRNA was depleted using the Ribo-off rRNA Depletion kit (bacteria) (Vazyme, Cat#:NR407-2), mRNA was used to generate the cDNA library according to the VAHTS™ Stranded mRNA-seq Library Prep Kit for Illumina protocol (Vazyme, Cat#:NR601-01) and the quality of cDNA library was analyzed to satisfy the sequencing requirements. Then sequencing was performed using the HiSeq X Ten system (Illumina) completed by Sangon Biotech (Shanghai) Co., Ltd.

Bacterial RNA-seq reads were mapped to the *S. aureus* USA300 LAC genome (GenBank: CP055225.1) using Bowtie 2 (version 2.3.2) (10) with two mismatches

allowed. Only uniquely mapped reads were kept for subsequent analyses (11). Differentially expressed genes (DEGs) were determined using DESeq2 Package (12). The false discovery rate (FDR) was used to identify the q value threshold in multiple tests. Fold change ≥ 2 and q-value ≤ 0.05 were used as a threshold to determine significant DEGs. RNA-Seq experiments were performed with three biological replicates for each strain, and the RNA-Seq data have been submitted to the NCBI Sequence Read Archive (SRA) (https://ncbi.nlm.nih.gov/sra/) under BioProject accession number PRJNA746457 with the following Biosample accessions: SAMN20207198 to SAMN20207221.

Quantitative reverse transcriptase PCR (qRT-PCR) analysis

For qRT-PCR analysis, overnight cultures of *S. aureus* strains were diluted into 10 ml fresh TSB in a 50-ml tube (OD_{600} ≈ 0.1). The cultures were incubated at 37°C with shaking (250 rpm) for 1.5 h or 3 h, as indicated. RNA samples were extracted using a Qiagen RNeasy kit following the manufacturer's instructions, and the total RNA sample (2 μg) was treated with gDNA digester (YEASEN, Lot: 11123ES60) and reversely transcribed to synthesize cDNA using the Hifair® II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (YEASEN, Lot: 11123ES60) with random primers according to the manufacture's recommendation. qRT-PCR was carried out in the Bio-Rad 96 well Real-Time PCR System with 20 μl reaction volume, 2 μL of each cDNA dilution was assayed with the Hieff® qPCR SYBR Green Master Mix (YEASEN, Lot:H7901050) and 300 nM primers following the manufacturer's instructions. Melting

curve analysis was performed for verification of product homogeneity. The gene-specific primer pairs used for qRT-PCR for *spa*, *psmα3*, *psmβ1*, RNAIII, *vraX*, *pbp2*, *saeR*, *saeS*, and 16S rRNA are *spa*-RT-F/*spa*-RT-R, *psmα3*-RT-F/*psmα3*-RT-R, *psmβ1*-RT-F/*psmβ1*-RT-R, RNAIII-RT-F/RNAIII-RT-R, *vraX*-RT-F/*vraX*-RT-R, *pbp2*-RT-F/*pbp2*-RT-R, *saeR*-RT-F/*saeR*-RT-R, *saeS*-RT-F/*saeS*-RT-R and 16S-RT-F/16S-RT-R, respectively (Table S3). The amplicon of 16S rRNA was used as an internal control, and the cDNA sample was diluted when analyzing its expression because of high abundance. Relative expression levels of interest genes were calculated by the relative quantification method ($2^{-\Delta\Delta Ct}$ method) (13) and reported as fold-change.

Western blot analysis

For western blot analysis of SpA, overnight cultures of *S. aureus* strains were diluted into 10 ml fresh TSB in a 50 ml tube with initial $OD_{600} \approx 0.1$. The diluted cultures were incubated for 3 h at 37°C with shaking (250 rpm), and the bacteria were harvested by centrifugation, washed once with 1 X TE buffer (10 mM Tris, 1 mM EDTA, pH = 8.0), and resuspended with 1 X TE buffer at a final OD_{600} of 10. Then, 100 µl *S. aureus* suspensions were lysed by lysostaphin (50 µg/ml), and each 100 µl lysed sample was mixed with 25 µl of 5 × SDS loading buffer [250 mM Tris-HCl (pH = 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol and 100 mM DTT] and heated at 100°C for 10 min. Each 10 µl prepared sample was subject to 10% (wt/vol) SDS-PAGE in Tris-Glycine running buffer at 135 V for 90 min, and the proteins on the gel were transferred onto PVDF (Bio-Rad) membranes. The membranes were blocked with 5% (wt/vol) skim milk

for 120 min and then incubated with the primary antibody at 4°C for overnight, washed four times for 80 min with TBST buffer [10 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 0.05% Tween 20], followed by incubating with the secondary antibody for 120 min, and washed four times for 80 min with TBST. The resulting chemiluminescent light was detected by a Tanon-5200 multi (Tanon Science & Technology Co., Ltd.). Primary antibodies anti-SpA (Abcam, 1:5000 dilution) and anti-SrtA (Abcam, 1:2500 dilution) and secondary anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Cwbio, 1:5000) were used. Biostep™ Prestained Protein Marker (Tanon) was used as a molecular weight reference.

Hemolytic activity assay

Overnight cultures of the *S. aureus* strains were diluted into 10 ml fresh TSB in a 50-ml tube with initial $OD_{600} \approx 0.1$, and the diluted cultures were cultured for 3 h at 37°C with shaking (250 rpm) and harvested by centrifugation. Sterilized toothpicks were used to stab the bacteria onto 5% (vol/vol) sheep blood agar plates (Kemajia Microbe Technology Co., Ltd.). Zones of clearance surrounding the bacterial colonies indicated hemolysis and were determined at 30 °C for 24 h and then for 24 h at 4°C after inoculation.

Minimum inhibitory concentration (MIC) assays

Minimum inhibitory concentration (MIC) assays were performed as follows. Briefly, the *S. aureus* strains were cultured in 10 ml fresh TSB in a 50 ml tube. The exponential

phase bacterial suspension was diluted with Mueller-Hinton II broth (cation-adjusted, BD 212322) to approximately 5 x 10^5 CFU/ml. Each 100 µl bacterial dilution was distributed in 96-well plates, and antibiotics were serially diluted twofold. The 96-well plates were incubated at 37°C for 24 h. MIC values were defined as the lowest compound concentration to inhibit bacterial growth completely.

Spot dilution assay

To analyze the resistance of *S. aureus* to moenomycin, a spot dilution test was performed as described previously (14) with some modifications. Briefly, overnight cultures of *S. aureus* strains were diluted into 10 ml fresh TSB in a 50 ml tube with initial OD_{600} ≈ 0.1, and grown at 37°C with shaking (250 rpm) for 3 h. Serial 10-fold dilutions of bacterial suspensions were prepared, and 10 µl of each dilution was spotted onto TSA agar plates containing with or without moenomycin (40 ng/ml). The plates were incubated at 30°C for 24 h and photographed.

Construction of promoter-*lux* fusions

To construct the promoter-*lux* fusions, coding sequences of *luxABCDE* operon were amplified from plasmid pAUL-A Tn4001 of *S. aureus* Xen 36 (15) by PCR using the primers *luxABCDE*-F (with *Eco*RI-*Xho*I-*Pml*I site) and *luxABCDE*-R (with *Kpn*I site) (Table S3), *luxABCDE* product was digested with *Eco*RI and *Kpn*I and then ligated with the pCL-*lacZ* plasmid, generating pCL-*lux*.

For the construction of *psmα*-*lux*, *psmα* promoter region (-523 to -13 of the start

codon) was amplified from *S. aureus* USA300 LAC genomic DNA by PCR using the primers *psmα*-pro-F (with *Eco*RI site) and *psmα*-pro-*lux*-R (with *Xho*I site) (Table S3). To generate the *vraX*-*lux*, the promoter region of *vraX* (-565 to -15 of the start codon) was amplified from *S. aureus* USA300 LAC genomic DNA by PCR using the primers *vraX*-pro-F (with *Eco*RI site) and *vraX*-pro-*lux*-R (with *Xho*I site) (Table S3). The promoter fragments were digested with *Eco*RI and *Xho*I, ligated into pCL-*lux* that was also digested with *Eco*RI and *Xho*I, and then introduced to *E. coli* DH5α. The construct was confirmed by DNA sequencing, electroporated into RN4220, then transduced into USA300 LAC or its derivatives using bacteriophage Ø85.

Monitoring promoter activity by *lux*-based reporters

The *lux*-based reporter assay was performed as previously described with some modification (4). Overnight cultures of the *S. aureus* promoter-*lux* reporter strains were diluted to an OD_{600} ≈ 0.2 in fresh TSB supplemented with or without test compounds, as indicated. Then, 100 µl aliquot of the sample was distributed to a 96-well black-wall clear-bottom plate (Costar, Corning Incorporated), and 50 µl saxoline was added to each well to prevent evaporation. Promoter activities were measured using a Synergy 2 Multi-Mode Microplate Reader (Biotek) at different time points of bacterial growth. For the maintenance of plasmid pYJ335 or its derivatives, 120 µg/ml erythromycin was used, where appropriate.

Expression and purification of His_6-VraR

293 Protein expression and purification were performed as previously described with some
294 modification (4). For the heterogeneous expression of N-terminal 6His-tagged VraR
295 (i.e., His_6-vraR), a ~ 0.65 kb DNA fragment containing *vraR* was amplified from *S. aureus*
296 USA300 LAC genomic DNA with primers His-*vraR*-F and His-*vraR*-R (Table S3). The *vraR*
297 PCR fragment was digested with *Bam*HI and *Xho*I and then ligated into pET28a, which
298 was also digested with *Bam*HI and *Xho*I, generating pET28a-His_6-*vraR*. The construct
299 was introduced to *E. coli* DH5α and sequenced to ensure that no unwanted mutations
300 resulted. Then, the resulting plasmid was transformed into *E. coli* BL21 (DE3).
301 For purification of His_6-VraR, 10 ml overnight LB cultures of *E. coli* BL21 (DE3)
302 carrying the plasmid pET28a-His_6-*vraR* were diluted 1:100 into 1 L fresh LB medium
303 supplemented with 50 μg/ml kanamycin and grown at 37°C with shaking (220 rpm) to
304 an OD_{600} of 0.6. Protein was induced by the addition of 0.5 mM IPTG (Sangon) for 16 h
305 at 16°C. Cells were harvested by centrifugation and resuspended in 50 ml of buffer A
306 (20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), and 0.5 M NaCl). Then, cells were
307 lysed at 4°C by sonication and centrifuged at 12,000 rpm for 25 min at 4°C to remove
308 insoluble material and the membrane fraction. The supernatant was loaded onto a
309 HisTrap HP column (GE Healthcare Life Sciences China), equilibrated with buffer A and
310 eluted with a 0–100% gradient of buffer B (50 mM Tris (pH 8.0), 300 mM NaCl, 1 mM
311 DTT, and 250 mM imidazole). Then the collected fractions containing His_6-VraR were
312 loaded onto the HiTrap Desalting 5 x 5 ml (Sephadex G-25 S) (GE Healthcare Life
313 Sciences China) and eluted with buffer A to remove the imidazole. The purified His_6-
314 VraR protein was verified by 10% (w/v) SDS-PAGE followed by Coomassie blue staining,

and protein concentration of His_6-VraR was determined using a NanoDrop 2000 (Thermofisher China) by A_{280nm}.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed as previously described (5). Briefly, 2 ng/µl DNA fragments and various concentrations of His_6-VraR were mixed with binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 0.05% Nonidet® P-40, 2.5% Glycerol, and 50 mM acetyl phosphate]. The reactions in 20 µl volume were incubated at room temperature for 15 min. Following incubation, the mixtures were separated by electrophoresis in a 6% native polyacrylamide gel in 0.5 x Tris-borate-EDTA (TBE) buffer at 90 V for 110 min at 4°C. Then, the gel was stained with GelRed nucleic acid staining solution for 10 min and the DNA bands were visualized by a Tanon-5200 multi (Tanon Science & Technology Co., Ltd.).

DNA probes (i.e., *spa*-p, *vraX*-p, *vraRS*-p, and *coa*-p) were PCR-amplified from *S. aureus* USA300 LAC genomic DNA using the primers listed in Table S3. For *spa*-p, a 414 bp DNA fragment covering the promoter region of *spa* (-414 to -1 of the start codon) was amplified with primer pair EMSA-*spa*-F/EMSA-*spa*-R. For *vraX*-p, a 350 bp DNA fragment covering the promoter region of *vraX* (-350 to -1 of the start codon) was amplified with primer pair EMSA-*vraX*-F/EMSA-*vraX*-R. For *vraRS*-p, a 147 bp DNA fragment covering the promoter region of *vraRS* (-252 to -106 of the start codon of the vraU in the *vraUTRS* operon) (16) was amplified with primer pair EMSA-*vraRS*-F/EMSA-*vraRS*-R. For *coa*-p, a 228 bp DNA fragment covering the promoter region of *coa* (-199

to +29 of the start codon) was amplified from *S. aureus* USA300 LAC genomic DNA with primers EMSA-*coa*-F/EMSA-*coa*-R. All PCR products were purified with a purification kit (Omega Bio-tek).

Dye primer-based DNase I footprinting assay

The DNase I footprinting assay was performed as previously described (14). Briefly, for the DNase I footprinting assay of *spa* promoter, a 414-bp 6-carboxyfluorescein (FAM)-labeled DNA fragments (-414 to -1 of the start codon) was amplified from *S. aureus* USA300 LAC genomic DNA with primers EMSA-*spa*-F/FAM-*spa*-R. PCR products were purified with a purification kit (Omega Bio-tek). Then, 50 µl reaction mixture containing 300 ng *spa* promoter DNA, 6 µM His_6-VraR, and binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 0.05% Nonidet® P-40, 2.5% Glycerol, and 50 mM acetyl phosphate] was incubated at room temperature for 15 min. Subsequently, 0.05 unit of DNase I was added into the reaction mixture for another 3 min incubation and then stopped the DNase I digestion by adding 90 µl quenching solution (200 mM NaCl, 30 mM EDTA, and 1% SDS). The mixture was extracted with phenol–chloroform-isoamyl alcohol (25:24:1), and the digested DNA fragments were isolated by ethanol precipitation, then dried under vacuum and resuspended in RNase-free water. Next, 5 µl of digested DNA was mixed with 4.9 µl of HiDi formamide and 0.1 µl of GeneScan-500 LIZ size standards (Applied Biosystems). A 3730xl DNA analyser was used to detect the sample, and the results were analysed with GeneMapper software (Applied Biosystems). Thermo Sequenase Dye Primer Manual Cycle

Sequencing Kit (ThermoFisher) was used to determine the sequences of the His_6-VraR protection region more precisely after the capillary electrophoresis results of the reactions were aligned, and the corresponding label-free promoter DNA fragment was used as a template for DNA sequencing. Then, electropherograms were analysed with GeneMarker v1.91 (Applied Biosystems).

For the DNase I footprinting assay of *vraX* promoter, a 350-bp FAM -labeled *vraX* promoter DNA (-350 to -1 of the start codon) was amplified from *S. aureus* USA300 LAC genomic DNA with primers EMSA-*vraX*-F/FAM-*vraX*-R, and the following procedures were similar to those of the *spa* promoter DNase I footprinting assay.

Galleria mellonella larvae infection model

G. mellonella larvae infection assay was performed as previously described (17). Overnight cultures of the *S. aureus* strains were diluted into 10 ml fresh TSB in a 50 ml tube with initial $OD_{600} \approx 0.1$, and the diluted cultures were grown for 3 h at 37°C with shaking (250 rpm), and harvested by centrifugation at 7000 rpm, 4°C for 5 min. The bacterial cells were washed twice and resuspended with pre-cooled (4°C) PBS. Each about 300 mg *G. mellonella* larvae (Tianjin Kaideruixin INTL Trade Co., Ltd.) was injected with sterile PBS or 5×10^6 CFU *S. aureus* in a 10-μl volume, then cultivated at 37 °C and observed per 6 h or 12 h for 102 h to analyze the mortality data of *G. mellonella* larvae.

To test the effect of cefuroxime on *G. mellonella* larvae infection model, each *G. mellonella* larvae was injected with the indicated concentrations of cefuroxime in a 10-

µl volume, and 1 h later, *G. mellonella* larvae was infected by 5×10^6 CFU *S. aureus* USA300 LAC in a 10-µl volume, then cultivated at 37°C and observed per 6 h or 12 h for 120 h to analyze the mortality data. For this experiment, the time of inoculation was designated 0 h.

Mouse infection models

Mouse infection experiments were performed in strict accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (11-14-1988). The protocols of animal study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Public Health Clinical Center (permit 2013P201) and were performed in accordance with the relevant guidelines and regulations. The laboratory animal usage license number is SYXK-HU-2010-0098, certified by Shanghai Committee of Science and Technology.

Overnight cultures of WT USA300 LAC strain and Δ*tarO* mutant were diluted into 100 ml fresh TSB in a 250 ml flask with initial $OD_{600} \approx 0.1$, the diluted cultures were grown for 3 h at 37°C with shaking (250 rpm), and harvested by centrifugation at 7,000 rpm, 4°C for 5 min. The bacteria were washed twice and resuspended with pre-cooled PBS. 6-8 weeks old female BALB/c mice from Shanghai Jiesijie Laboratory Animal Co., Ltd. were intravenously administered with 100 µl bacterial suspension via retroorbital injection. For the abscess formation model, mice were challenged with either 5×10^6 or 2×10^7 CFU of *S. aureus* USA300 LAC or its variant Δ*tarO*. *S. aureus*-infected mice

were humanely euthanized 5 days after infection and kidneys, hearts, and livers were aseptically removed. Organs were homogenized in PBS plus 0.1% Triton X-100 and the homogenates of each organ were serial diluted and spotted onto TSA plates, then cultivated at 30 °C for 24 h and enumerated the CFU. Statistical significance was determined by the Mann-Whitney test (two-tailed). For the lethal challenge model, mice were infected with 1×10^8 CFU of *S. aureus* USA300 LAC and its variant Δ*tarO* by retroorbital injection, and moribund mice were humanely euthanized to obtain survival data; mice were observed for ten days, and the log-rank test was used to analyze mortality data.

Statistical analysis

Statistical analyses and the number of independent biological experiments are presented in the figure legends. The log-rank test was used for survival analysis. Unpaired two-tailed Student's *t* tests, Mann-Whitney *U* test or one-way ANOVA followed by the Tukey's multiple comparisons test were used to compare two datasets.

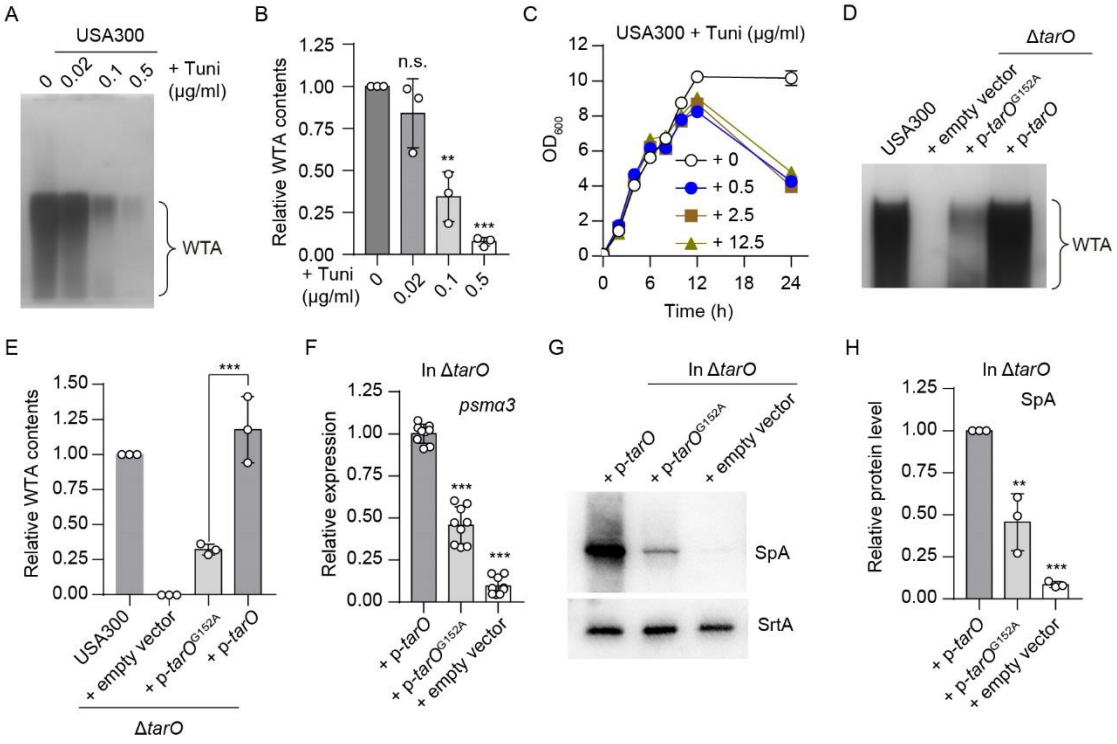


Figure S1. WTA contents positively associate with the expression levels of *psmα* and SpA in USA300 LAC. (A and B) Representative images of WTA PAGE analysis (A) and quantitative analysis (B) of WTAs from WT USA300 LAC strain treated with indicated concentrations of tunicamycin at 37°C for 3 h. The results are reported as fold changes with tunicamycin-untreated control sample set to 1. Data represent mean ± SD from n = *3* independent experiments (n.s., not significant; **P < 0.01; ***P < 0.001 versus USA300 untreated control; one-way ANOVA followed by the Tukey's multiple comparisons test). (C) Effect of tunicamycin on the growth of USA300 LAC in TSB medium. Data represent mean ± SD from n = *3* biological replicates. (D and E) Representative images of WTA PAGE analysis (D) and quantitative analysis (E) of *S. aureus* strains grown in TSB medium for 3 h. The results are reported as fold changes with WT USA300 sample set to 1. Data represent mean ± SD from n = *3* independent experiments (***P < 0.001; one-way ANOVA followed

by the Tukey's multiple comparisons test). (F) qRT-PCR analysis of *psmα3* transcripts in *S. aureus* strains grown in TSB medium for 3 h, presented as relative expression compared with the control sample (Δ*tarO* carrying p-*tarO)* set to 1. Data represent mean ± SD from three technical replicates of three independent experiments (*n* = 9) (***$P < 0.001$ versus Δ*tarO* carrying p-*tarO*, by unpaired two-tailed Student's *t*-test). (G and H) Representative images (G) and quantitative analysis (H) of Western blotting for SpA in *S. aureus* strains grown in TSB medium for 3 h. Sortase A (SrtA) is a loading control. Western blot band intensity of SpA was normalized to the intensities obtained with SrtA, and the results are reported as fold changes with control sample (Δ*tarO* carrying p-*tarO plasmid*) set to 1. Data represent mean ± SD from n = *3* independent experiments (**$P < 0.01$; ***$P < 0.001$ versus Δ*tarO* carrying p-*tarO*; one-way ANOVA followed by the Tukey's multiple comparisons test). In (D) to (H), USA300 LAC harbors an empty pYJ335-1 vector as control, Δ*tarO* carries either an empty pYJ335-1 vector or a p-*tarO* plasmid without (+ p-*tarO*) or with G152A missense mutation in *tarO* (+ p-$tarO^{G152A}$).

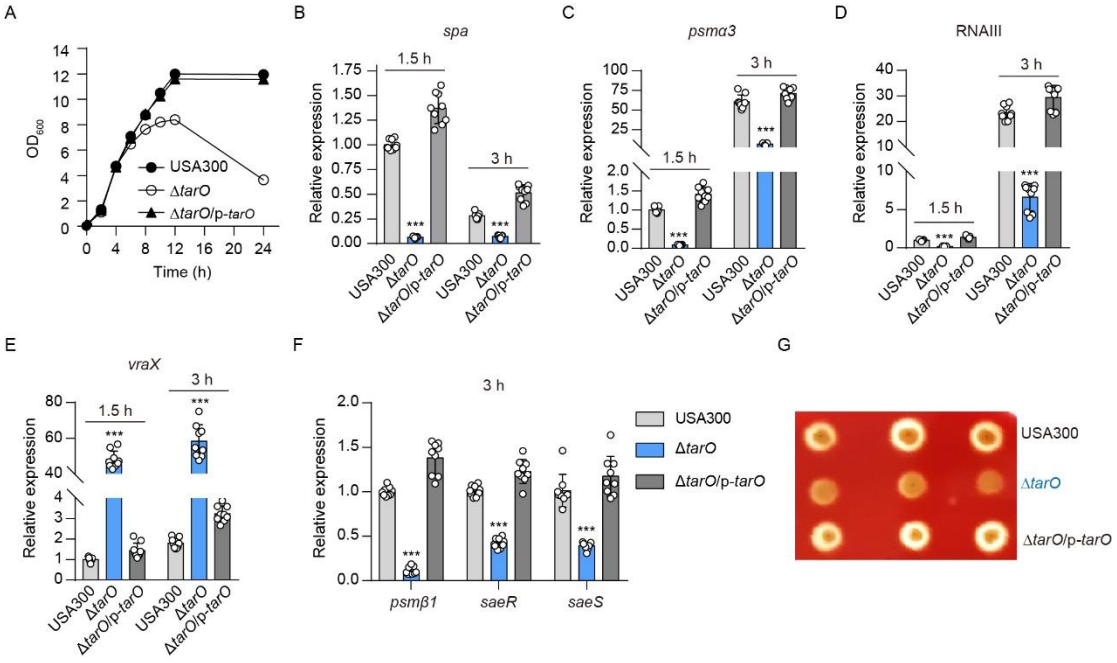


Figure S2. Effect of *tarO* deletion on bacterial growth, virulence gene expression, and the hemolytic activity of USA300 LAC. (A) Growth curve of *S. aureus* strains in TSB medium. Data represent mean ± SD from n = *3* biological replicates. (B to E) qRT-PCR analysis of *spa*, *psmα3*, RNAIII, and *vraX* in *S. aureus* strains grown in TSB medium for 1.5 h and 3 h, presented as relative expression compared with the control sample (WT USA300 at 1.5-h time point) set to 1. Data represent mean ± SD from three technical replicates of three independent experiments ($n = 9$) (***$P < 0.001$; unpaired two-tailed Student's *t*-test). (F) qRT-PCR analysis of *psmβ1*, *saeR*, and *saeS* in *S. aureus* strains grown in TSB medium for 3 h, presented as relative expression compared with the control sample (WT USA300) set to 1. Data represent mean ± SD from three technical replicates of three independent experiments ($n = 9$) (***$P < 0.001$; unpaired two-tailed Student's *t*-test). (G) Hemolytic activity of *S. aureus* strains on the sheep blood agar plate. Bacterial cells were inoculated onto 5% (vol/vol) sheep blood agar plates using sterilized toothpicks. Zones of clearance

surrounding the bacterial colonies indicated hemolysis and were photographed after incubation at 30°C for 24 h and then at 4°C for 24 h. In all panels, USA300 and Δ*tarO* strains harbor an empty pYJ335-1 vector as control, Δ*tarO*/p-*tarO* denotes the complemented strain of Δ*tarO* (Table S2).

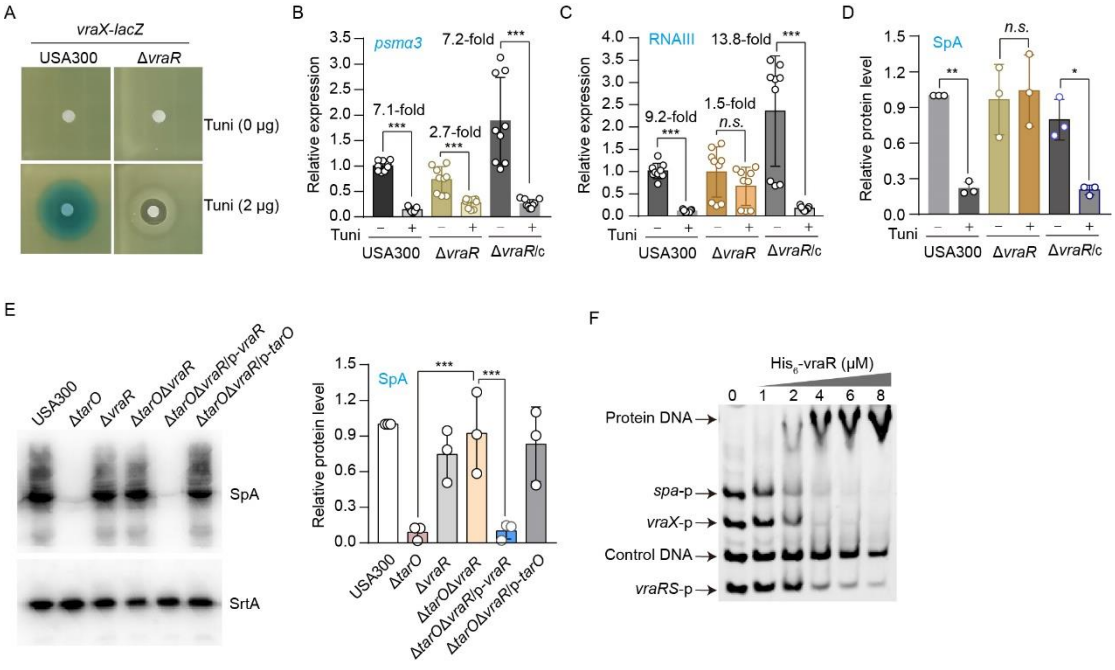
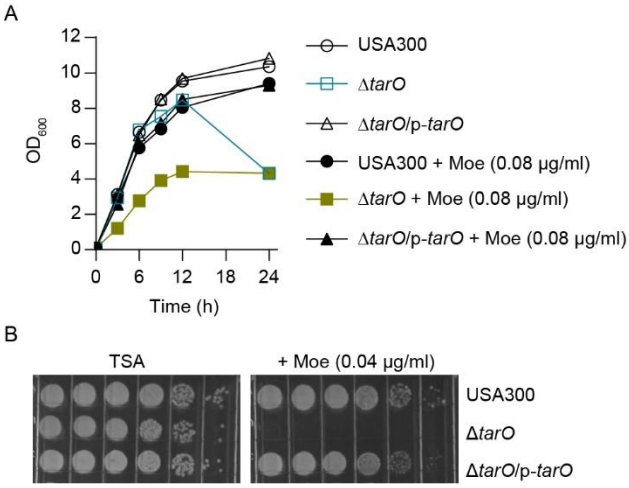


Figure S3. VraR mediates TarO inhibition-induced changes in gene expression. (A) Disk diffusion assay for induction of *vraX-lacZ* by tunicamycin. Disk containing either tunicamycin (Tuni, in a 10-μl volume) or vehicle control (0 μg) was placed on the surfaces of TSA plates lawn with indicated reporter strain. β-galactosidase acting on the substrate X-gal in the medium produced blue rings, and plates were incubated at 37°C for 24 h for colour development. (B and C) qRT-PCR analysis of *psmα3* (B) and RNAIII (C) transcripts in *S. aureus* strains grown in TSB medium supplemented with (+) or without (−) tunicamycin (0.5 μg/ml) for 3 h, presented as relative expression

compared with the tunicamycin-untreated WT USA300, which was set to 1. Data represent mean ± SD from three technical replicates of three independent experiments (n = 9) (***P < 0.001; *n.s.*, not significant; unpaired two-tailed Student's *t*-test). (D) Quantitative analysis of western blotting for SpA in *S. aureus* strains grown in TSB medium supplemented with (+) or without (-) tunicamycin (0.5 μg/ml) for 3 h. Sortase A (SrtA) is a loading control. Results are reported as fold changes with control sample (tunicamycin-untreated USA300, set to 1). Data represent mean ± SD from *3* independent experiments (*P < 0.05; **P < 0.01; *n.s.*, not significant; one-way ANOVA followed by the Tukey's multiple comparisons test). (E) Representative images (*left panel*) and quantitative analysis (*right panel*) of western blotting for SpA in *S. aureus* strains grown in TSB medium supplemented with (+) or without (-) tunicamycin (0.5 μg/ml) for 3 h. Sortase A (SrtA) is a loading control. Results are reported as fold changes with control sample (tunicamycin-untreated USA300, set to 1). Data represent mean ± SD from *3* independent experiments (*P < 0.05; **P < 0.01; *n.s.*, not significant; one-way ANOVA followed by the Tukey's multiple comparisons test). (F) EMSAs showing the DNA-binding ability of purified His_6-vraR to the promoter DNA fragments of *spa* (*spa*-p), *vraX* (*vraX*-p), and *vraRS* (*vraRS*-p). Control DNA, a 228-bp DNA fragment (*coa*-p) covering the promoter region of *coa* gene. In (B to E), USA300, Δ*vraR*, or Δ*tarO*Δ*vraR* strain harbors an empty pYJ335-1 vector as control; In (B to D), Δ*vraR*/c denotes the complemented strain of Δ*vraR* (Δ*vraR*/p-*vraR*).



505

506 **Figure S4. Effect of *tarO* deletion on the susceptibility of USA300 LAC to**

507 **moenomycin.** (A) Growth curve of *S. aureus* strains in a 50-ml tube containing TSB

508 medium supplemented with or without moenomycin. The cultures were shaken (250

509 rpm) at 37°C and the optical density at 600 nm was examined. Data represent mean

510 ± SD from n = *3* biological replicates. USA300 and Δ*tarO* strains harbor an empty

511 pYJ335-1 vector as control, Δ*tarO*/p-*tarO* denotes the complemented strain of Δ*tarO*

512 (Table S2). (A) The *tarO* null mutant strain shows decreased resistance to

513 moenomycin compared with either WT USA300 LAC strain (harboring pYJ335-1) or

514 the complemented strain of Δ*tarO* (i.e., Δ*tarO*/p-*tarO*). For this assay, the diluted

515 overnight cultures were grown in TSB medium at 37°C for 3 h, then serial 10-fold

516 dilutions of bacterial suspension were prepared and spotted (10 µl per spot) onto

517 TSA agar plates supplemented with or without moenomycin, and the plates were

518 cultured at 30 °C for 24 h. Images are representative of two independent

519 experiments.

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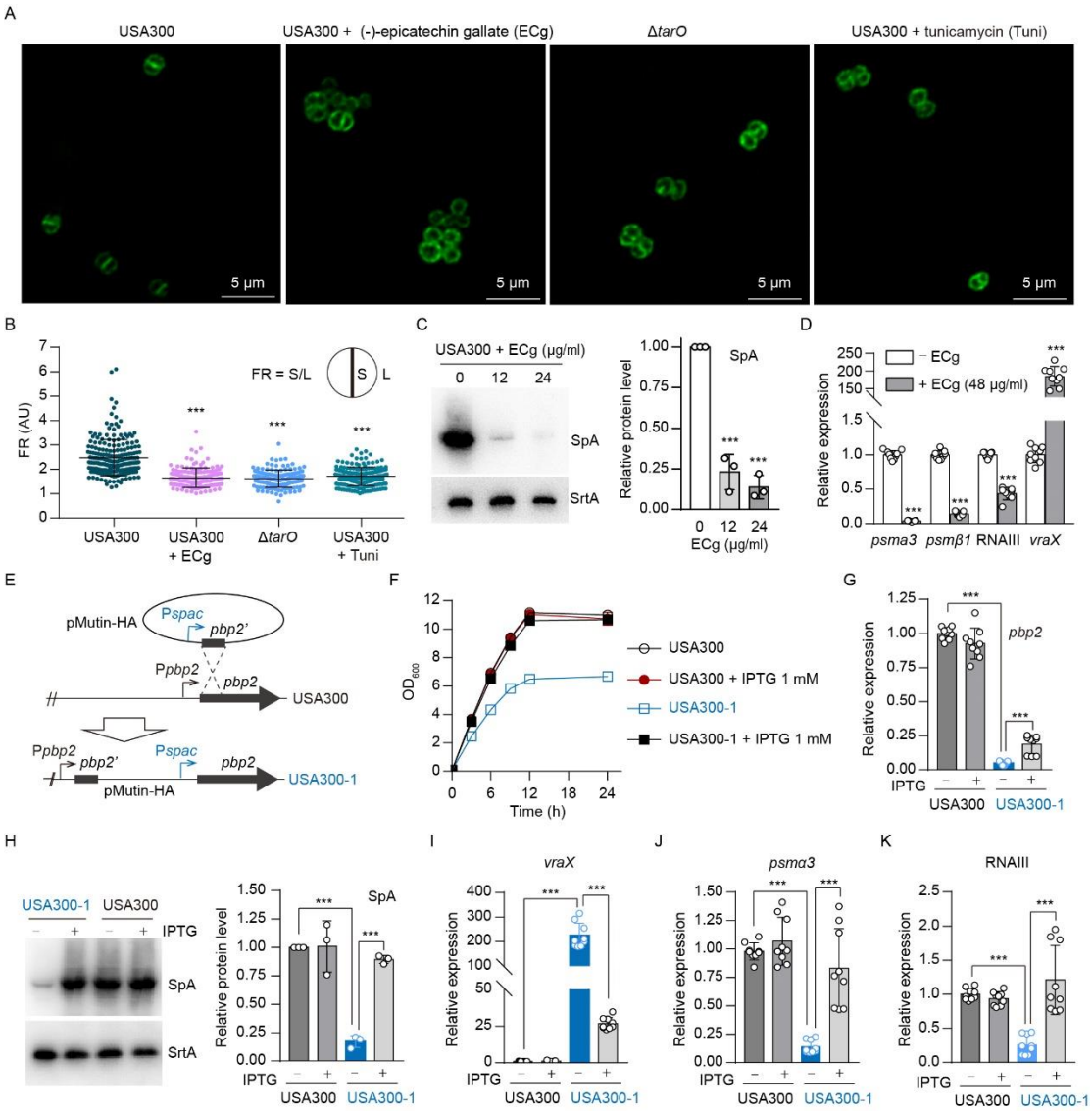

Figure S5. Role of PBP2 in the expression of virulence genes in USA300. (A) Effect of ECg treatment, *tarO* deletion, or tunicamycin treatment on the septal localization of GFP-PBP2. The scale bar is 5 μm. (B) Quantitative analysis of GFP-PBP2 fluorescence at septum (S) versus lateral (L) membrane. Data represent mean ± SD from $n > 130$ cells (***$P < 0.001$ by unpaired two-tailed Student's *t*-test). (C) Representative images (*left panel*) and quantitative analysis (*right panel*) of Western blotting for SpA in WT USA300 strain grown in TSB medium supplemented with indicated concentrations of ECg for 3 h. SrtA is loading controls. Data represent mean ± SD

from n = *3* independent experiments (***P < 0.001 versus WT USA300 control; one-way ANOVA followed by the Tukey's multiple comparisons test). (D) qRT-PCR analysis showing the effect of ECg treatment on expression of *psmα3*, *psmβ1*, RNAIII, and *vraX*. Data represent mean ± SD from three technical replicates of three independent experiments (*n* = 9) (****P* < 0.001, unpaired two-tailed Student's *t*-test). (E) Schematic of construction of *pbp2*-depleted mutant USA300-1. (F) Growth curves of USA300 and USA300-1 cultured in TSB medium supplemented with or without IPTG. (G) qRT-PCR analysis of *pbp2* transcripts in *S. aureus* strains grown in TSB medium supplemented with (+) or without (-) IPTG (1 mM) for 3 h. Data represent mean ± SD from three technical replicates of three independent experiments (*n* = 9) (****P* < 0.001 by unpaired two-tailed Student's *t*-test). (H) Representative images (*left panel*) and quantitative analysis (*right panel*) of Western blotting for SpA in *S. aureus* grown in TSB medium supplemented with (+) or without (-) IPTG (1 mM) for 3 h. SrtA is loading controls. Data represent mean ± SD from n = *3* independent experiments (***P < 0.001; one-way ANOVA followed by the Tukey's multiple comparisons test). (I-K) qRT-PCR analysis of *vraX*, *psmα3*, and RNAIII transcripts in *S. aureus* strains grown in TSB medium supplemented with (+) or without (-) IPTG (1 mM) for 3 h, presented as relative expression compared with the IPTG-untreated controls (set to 1). Data represent mean ± SD from three technical replicates of three independent experiments (*n* = 9) (****P* < 0.001 by unpaired two-tailed Student's *t*-test).

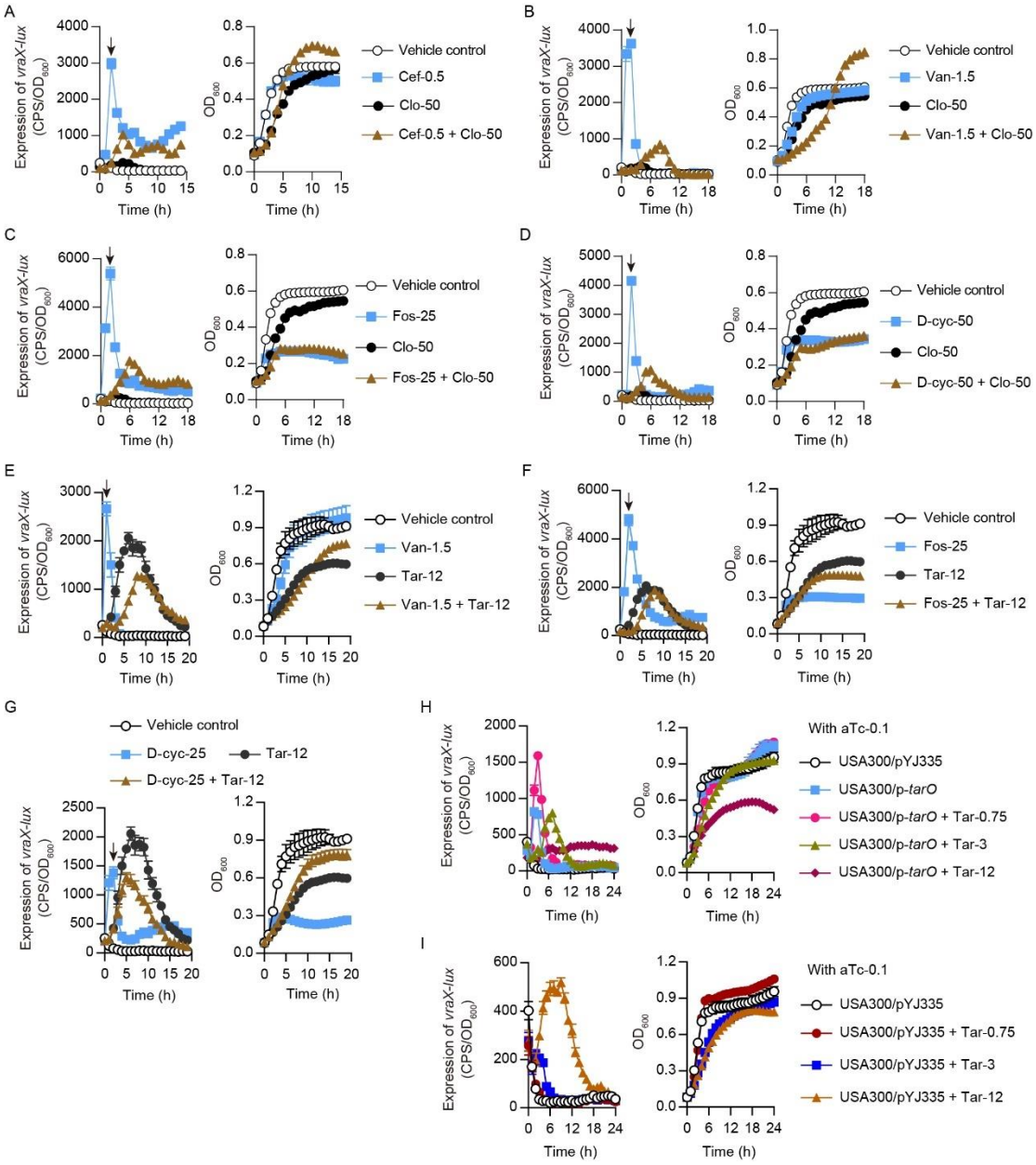

Figure S6. Effect of clomiphene, targocil and *tarO* overexpression on the expression of *vraX-lux*. (A to D) Effect of clomiphene treatment on the *vraX-lux* induction by cefuroxime (A), vancomycin (B), fosfomycin (C), and D-cycloserine (D). Cef-0.5, cefuroxime at a final concentration of 0.5 μg/ml; Van-1.5, vancomycin at a final concentration of 1.5 μg/ml; Fos-25, fosfomycin at a final concentration of 25 μg/ml; D-cyc-50, D-cycloserine at a final concentration of 50 μg/ml; clomiphene-50, clomiphene at a final concentration of 50 μg/ml. Data from *n* = 4 biological replicates

are reported as the mean ± SEM. (E to G) Effect of targocil treatment on the *vraX-lux* induction by vancomycin (E), fosfomycin (F), and D-cycloserine (G). Van-1.5, vancomycin at a final concentration of 1.5 µg/ml; Fos-25, fosfomycin at a final concentration of 25 µg/ml; D-cyc-25, D-cycloserine at a final concentration of 25 µg/ml; Tar-12, targocil at a final concentration of 12 µg/ml. Data from *n* = 4 biological replicates are reported as the mean ± SD. (H and I) Effect of *tarO* overexpression on the expression of *vraX-lux*. The expression of *vraX-lux* in USA300 LAC derivatives grown in TSB medium supplemented with or without targocil in the presence of anhydrotetracycline (aTc-0.1, anhydrotetracycline at a final concentration of 0.1 µg/ml) that induces the tetracycline-inducible *xyl*/*tetO* promoter in pYJ335 plasmid. The effect of targocil on the expression of *vraX-lux* in USA300/pYJ335 strain is also shown (I). Tar-0.75, Tar-3 and Tar-12 denote targocil at a final concentration of 0.75, 3, and 12 µg/ml, respectively. Data from *n* = 4 biological replicates are reported as the mean ± SEM.

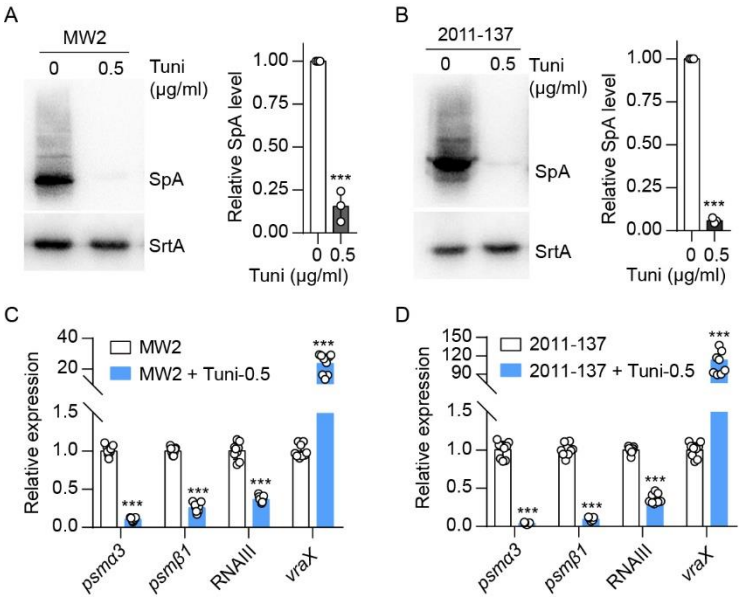

Figure S7. Effect of tunicamycin treatment on virulence gene expression in CA-MRSA isolates USA400 MW2 and 2011-137. (A and B) Representative images (*left panel*) and quantitative analysis (*right panel*) of Western blotting for SpA in USA400 MW2 (A) and 2011-137 (B) strains grown in TSB medium supplemented with (+) or without (-) tunicamycin (Tuni). Sortase A (SrtA) is a loading control. Western blot band intensity of SpA was normalized to the intensities obtained with SrtA, and the results are reported as fold changes with tunicamycin-untreated control sample was set to 1. Data represent mean ± SD from n = *3* independent experiments (***P < 0.001 by unpaired two-tailed Student's *t*-test). (C and D) qRT-PCR analysis of *psmα3*, *psmβ1*, RNAIII, and *vraX* transcripts in USA400 MW2 (C) and 2011-137 (D) strains grown in TSB medium supplemented with (+) or without (-) tunicamycin (0.5 μg/ml), presented as relative expression compared with the untreated controls (set to 1). Data represent mean ± SD from three technical replicates of three independent experiments (*n* = 9) (****P* < 0.001 by unpaired two-tailed Student's *t*-test).

Table S1. Effect of tunicamycin on the antibiotic resistance of CA-MRSA strains

Antibiotics	USA300 (-/+)[a]	FC[b]	USA400 (-/+)[a]	FC[b]	2011-137 (-/+)[a]	FC[b]
Cefotaxime	64/4	16	32/4	8	32/4	8
Cefuroxime	512/4	128	32/2	16	8/1	8
Ceftizoxime	>512/16	>32	512/8	64	128/4	32
Moenomycin	0.25/0.0625	4	0.125/0.03125	4	0.25/0.0625	4

[a]Number indicates the minimum inhibitory concentrations (MIC) (μg/ml), " -" and "+" respectively indicates in the absence and presence of tunicamycin (0.5 μg/ml). [b]FC, MIC fold-change in the absence of tunicamycin relative to those in the presence of tunicamycin. Cefotaxime, cefuroxime, and ceftizoxime bind preferentially to PBP2; moenomycin is a GTase inhibitor.

Table S2. Plasmids and strains used in this study.

Strains or plasmids	Relevant genotype or characteristic	Source
Plasmids		
pCL-*lacZ*	*E. coli-S. aureus* shuttle cloning vector, single-copy integration vector in *S. aureus*	(18)
pCL-*lux*	pCL-*lacZ* derivative carrying *luxABCDE*	this study
pCL-*psmα-lacZ*	pCL-*lacZ* derivative carrying *psmα* promoter	this study
pCL-*psmα-lux*	pCL-*lux* derivative carrying *psmα* promoter	this study
pCL-*vraX-lacZ*	pCL-*lacZ* derivative carrying *vraX* promoter	this study
pCL-*vraX-lux*	pCL-*lux* derivative carrying *vraX* promoter	this study
pYJ335	*E. coli-S. aureus* shuttle vector, Cm^r, Erm^r	(19)
pYJ335-1	*A* modified pYJ335 plasmid containing *Asc*I and *Psp*OMI restriction enzyme sites followed by the blunt-end *Eco*RV site of pYJ335	this study
p-*tarO*	pYJ335-1 derivative carrying *tarO* in the downstream of the xyl/tetO promoter	this study
p-*tarO*^{G152A}	p-*tarO* derivative carrying *tarO* which has alanine substitution mutant at the site of glycine residue 152	this study
p-*vraR*	pYJ335-1 derivative carrying *vraR* in the downstream of the xyl/tetO promoter	this study
p-*sgtB*	pYJ335 derivative carrying *sgtB* in the downstream of the xyl/tetO promoter	this study
p-*murJ*	pYJ335 derivative carrying *murJ* in the downstream of the xyl/tetO promoter	this study
p-*mecA*	pYJ335 derivative carrying *mecA* in the downstream of the xyl/tetO promoter	this study
pET28a	T7 *lac* promoter-operator, N-terminal His tag, Kan^r	Novagen
pET28a-His_6-*vraR*	pET28a derivative carrying *vraR*	this study
pKOR1	Gene replacement vector for *S. aureus* genes, Amp^r, Cm^r	(3)
pKOR1::*tarO*	pKOR1 derivative, for deletion of *tarO*	this study
pKOR1::*vraR*	pKOR1 derivative, for deletion of *vraR*	this study
pKOR1::*mecA*	pKOR1 derivative, for deletion of *mecA*	this study

pMutin-HA	A suicide vector for introducing mutation in *S. aureus* via a single recombination event	(20)
pMutin-HA-*pbp2'*	pMutin-HA derivative carrying a DNA fragment covering the ribosome binding site region and the first 630 bp (-20 to + 630 of the start codon) of *pbp2*	this study
p-*gfp-pbp2*	pYJ335-1 derivative carrying *gfp-pbp2* fusion in the downstream of the xyl/tetO promoter	this study
S. aureus		
RN4220	Derivative of 8325-4 that accepts plasmids	(21)
USA300 LAC	A CA-MRSA USA300 isolate, sequence type 8 (ST8)	(22)
USA400 MW2	A CA-MRSA USA300 isolate; ST1	(22)
2011-137	A clinic isolate of CA-MRSA lineage ST59	(23)
JE2	*S. aureus* LAC cured of all 3 native plasmids; Erm^{s}	(24)
JE2:: *vraX-lacZ*	JE2 strain carrying an integration vector pCL-*vraX-lux*	this study
USA300::*psmα-lacZ*	USA300 LAC carrying an integration vector pCL-*psmα-lacZ*	this study
USA300::*psmα-lux*	USA300 LAC carrying an integration vector pCL-*psmα-lux*	this study
USA300::*vraX-lacZ*	USA300 LAC carrying an integration vector pCL-*vraX-lacZ*	this study
USA300::*vraX-lux*	USA300 LAC carrying an integration vector pCL-*vraX-lux*	this study
RN4220::*vraX-lux*	RN4220 carrying an integration vector pCL-*vraX-lux*	this study
Δ*tarO*	*tarO* deletion mutant of USA300 LAC	this study
USA300/pYJ335-1	USA300 LAC carrying plasmid pYJ335-1	this study
Δ*tarO*/pYJ335-1	Δ*tarO* carrying plasmid pYJ335-1	this study
Δ*tarO*/p-*tarO*	Δ*tarO* carrying plasmid p-*tarO*	this study
Δ*tarO*/p-$tarO^{G152A}$	Δ*tarO* carrying plasmid p-$tarO^{G152A}$	this study
Δ*mecA*	*mecA* deletion mutant of USA300 LAC	this study
Δ*mecA*::*vraX-lux*	Δ*mecA* carrying an integration vector pCL-*vraX-lux*	this study
Δ*vraR*	*vraR* deletion mutant of USA300 LAC	this study
Δ*vraR*/pYJ335-1	Δ*vraR* carrying plasmid pYJ335-1	this study
Δ*vraR*::*psmα-lux*	Δ*vraR* carrying an integration vector pCL-*psmα-lux*	this study

Δ*vraR*::*vraX-lux*	Δ*vraR* carrying an integration vector pCL-*vraX-lux*	this study
Δ*vraR*/p-*vraR*	Δ*vraR* carrying plasmid p-*vraR*	this study
Δ*vraR*::*vraX-lacZ*	Δ*vraR* carrying an integration vector pCL-*vraX-lacZ*	this study
USA300-1	USA300 LAC carrying pMutin–HA-*pbp2'*	this study
USA300/p-*gfp-pbp2*	USA300 LAC carrying p-*gfp-pbp2*	this study
Δ*tarO*/p-*gfp-pbp2*	Δ*tarO* carrying p-*gfp-pbp2*	this study
JE2/pYJ335	JE2 carrying plasmid pYJ335	this study
sgtB	JE2 mutant with mariner-based transposon insertion at 562 bp after the start codon of *sgtB*.	laboratory stock
sgtB::*vraX-lux*	*sgtB* mutant carrying an integration vector pCL-*vraX-lux*	This study
sgtB/pYJ335	*sgtB* carrying plasmid pYJ335	this study
sgtB/p-*sgtB*	*sgtB* carrying plasmid p-*sgtB*	laboratory stock
USA300::*vraX-lux*/pYJ335	USA300::*vraX-lux* carrying plasmid pYJ335	this study
USA300::*vraX-lux*/p-*murJ*	USA300::*vraX-lux* carrying plasmid p-*murJ*	this study
Δ*mecA*::*vraX-lux*/pYJ335	Δ*mecA*::*vraX-lux* carrying plasmid pYJ335	this study
Δ*mecA*::*vraX-lux*/p-*mecA*	Δ*mecA*::*vraX-lux* carrying plasmid p-*mecA*	this study
RN4220::*vraX-lux*/pYJ335	RN4220::*vraX-lux* carrying plasmid pYJ335	this study
RN4220::*vraX-lux*/p-*murJ*	RN4220::*vraX-lux* carrying plasmid p-*murJ*	this study
RN4220::*vraX-lux*/p-*mecA*	RN4220::*vraX-lux* carrying plasmid p-*mecA*	this study
DH5α	for DNA cloning	laboratory stock
BL21 star (DE3)	F^-*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm met* (DE3)	laboratory stock

Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Erm^r, erythromycin resistance; Erm^s, erythromycin sensitive; Kan^r, kanamycin resistance

625 **Table S3. Primers used in this study.**

Primers	Sequence (5′to 3′)	Purpose
psmα-pro-F	CCGGAATTCCACTGCATAACCTCCTTATTTCTAA	for pCL-*psmα-lacZ*
psmα-pro-R	CGGGGTACCTAAGATTACCTCCTTTGCTTATGAGT	
vraX-pro-F	CCGGAATTCTGGATCACGGTGCATACAAC	for pCL-*vraX-lacZ*
vraX-pro-R	CGGGGTACCCCTATATTACCTCCTTTGCTACTCTAT	
luxABCDE-F	CCGGAATTCGAGCTCGAGCGCCACGTGATGAAGC AAGAGGAGGACTCTC	for pCL-*lux*
luxABCDE-R	CGGGGTACCGTCGACTTAACTATCAAACGCT	
psmα-pro-*lux*-R	CCGCTCGAGCTTTGCTTATGAGTTAACTTCATTG	for pCL-*psmα-lux*
vraX-pro-*lux*-R	CCGCTCGAGCCTTTGCTACTCTATGGTTATATTATA A	for pCL-*vraX-lux*
tarO-up-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGA ATGACAACTGAGAACTCTTC	for pKOR1::*tarO*
tarO-up-R	CCATACAGCTATGCTTTCATTCCTTATTCACCTTCAT CGATATTAATTG	
tarO-down-F	GGAATGAAAGCATAGCTGTATGG	
tarO-down-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAC ACTTAATGGCGCTATTTG	
tarO-F	ACAATTAATATCGATGAAGGTGAATAA	for p-*tarO*
tarO-R	AGGGGGCCCACAGCTATGCTTTCATTCCCTATT	
vraR-up-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTGG TTCAATGCTCATCTTAGTA	for pKOR1::*vraR*
vraR-up-R	TCTTAATTCGATATGAACTATTGAATTAACCACAAA CAATACTTTAATCGTCA	
vraR-down-F	TTAATTCAATAGTTCATATCGAATTAAGA	
vraR-down-R	GGGGACCACTTTGTACAAGAAAGCTGGGTATGTT GCTTTTCCTATTAACATTATTA	
vraR-F	CCTTTAAATAAGGAGGATTCGTATG	for p-*vraR*
vraR-R	AGGGGGCCCTTCGATATGAACTATTGAATTAAATT ATG	
His-*vraR*-F	CGCGGATCCATGACGATTAAAGTATTGTTTGTGG	for pET28a-His$_6$-*vraR*
His-*vraR*-R	CCGCTCGAGTTCGATATGAACTATTGAATTAAATTA TG	
mecA-up-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGA ACTTTATGTCCCGGACTCAT	for pKOR1::*mecA*
mecA-up-R	CTTTACCTGAGATTTTGGCATTGTCGACAACTACA ACTATTAAAATAAGTGG	
mecA-down-F	ACAATGCCAAAATCTCAGGTAAAG	
mecA-down-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTGC GATTAATGTGATAATACAGTTACG	

pYJ335-PCR-F	GATATCCCCTTGGCGCGCCAAAGGGGGCCCCCTG AATTCGGAGGCATATC	for pYJ335-1
pYJ335-PCR-R	GGGCCCCCTTTGGCGCGCCAAGGGGATATCAAGC TTATTTTAATTATACTCTATCA	
EMSA-*spa*-F	TATTACGCAAGTGTGCTGTATTCTA	for EMSA of *spa*
EMSA-*spa*-R	ATTAATACCCCCTGTATGTATTTGTAA	
EMSA-*vraX*-F	GAAAATTTCATACATATCGAACAATAC	for EMSA of *vraX*
EMSA-*vraX*-R	AACCTATATTACCTCCTTTGCTACTC	
EMSA-*vraRS*-F	GGTCCGATTTTAACGACAAAG	for EMSA of *vraRS*
EMSA-*vraRS*-R	TGAAATGACGCATTGATTGTG	
EMSA-*coa*-F	GTGTTGTCATGCTTTGTTACTCC	for EMSA of *coa*
EMSA-*coa*-R	GCGCCTAGCGAAATTATTTGC	
FAM-*spa*-R	FAM-ATTAATACCCCCTGTATGTATTTGTAA	for footprinting assay of *spa*
FAM-*vraX*-R	FAM-AACCTATATTACCTCCTTTGCTACTC	for footprinting assay of *vraX*
G152A-F	GCAATTAACTTAATTGATGCTCTCGATGGTTTGG	for p-*tarO*^{G152A}
G152A-R	CCAAACCATCGAGAGCATCAATTAAGTTAATTGC	
spa-RT-F	GAAAAAGAAAAACATTTATTCAATTCG	for qRT-PCR of *spa*
spa-RT-R	AGGCATATTTAAGACTTGATAAAAAGC	
psmα3-RT-F	GCCATTCACATGGAATTCGT	for qRT-PCR of *psmα3*
psmα3-RT-R	GATTAGTTGTTACCTAAAAATTTACCAAG	
psmβ1-RT-F	ATGGAAGGTTTATTTAACGCAAT	for qRT-PCR of *psmβ1*
psmβ1-RT-R	AATCCGAATAATTTACCTAATAAACC	
RNAIII-RT-F	GGAAGGAGTGATTTCAATGG	for qRT-PCR of RNAIII
RNAIII-RT-R	TTCACTGTGTCGATAATCCA	
vraX-RT-F	ATGATTATTTATCGACAGTATCACCAT	for qRT-PCR of *vraX*
vraX-RT-R	AGAGCAATTTGAATATTTCAGTATCAC	
pbp2-RT-F	GATTTAAACTTAGCGGAAGAAGC	for qRT-PCR of *pbp2*
pbp2-RT-R	TGTTTTTACGATCTTCAGCAGC	
saeR-RT-F	CACTTACTGATCGTGGATGATGA	for qRT-PCR of *saeR*
saeR-RT-R	ATCATTTGATAGTAAAGAAATTGCTTC	
saeS-RT-F	TAGAAGTCAAATCATTATTGGCGT	for qRT-PCR of *saeS*
saeS-RT-R	CAGCTTGTAATTATTGTCGTTAAGG	
16S-RT-F	GGCAAGCGTTATCCGGAATT	for qRT-PCR of 16S rRNA
16S-RT-R	GTTTCCAATGACCCTCCACG	
P*spac-pbp2*-F	CCCAAGCTTGATGAAAGTGAGGACCGCGT	for pMutin-HA-*pbp2'*
P*spac-pbp2*-R	GCGGGTACCATACTTAGCAGCAGCTTTAATACCTG	
gfp-F	GATGAAAGTGAGGACCGCGTATGTCAAAAGGAG AAGAATTATTTAC	for p-*gfp-pbp2*
gfp-R	TGAGAAGATCCTTTGTTTTCCGTCTTATATAATTCAT CCATTCCGTG	
pbp2-F	ACGGAAAACAAAGGATCTTCTCA	for pMutin-HA-*pbp2'*

pbp2-R	AGGGGGCCCAGTGGATTAGTTGAATATACCTGTTAATC	
sgtB-F	TTAAAAGAAGGAGCAAACGCAT	for p-*sgtB*
sgtB-R	ATGCAAGTATTTAACGATTTAATTGTG	
mecA-F	CTACAAATGTAGTCTTATATAAGGAGTATATTG	for p-*mecA*
mecA-R	TTACGGATTGCTTCACTGTTTT	
murJ-F	AATTAAAATAAGCTTATGAGATAGGGAGATTCGTA	for p-*murJ*
murJ-R	TATGCCTCCGAATTCTCATCGTAAAAACCTAACTC	

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