Supporting Information

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SI Experimental Procedures

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich and Fisher, and the restriction enzymes from New England Biolabs.

Bacteria. *Staphylococcus aureus* strain Newman is a human clinical isolate (1) that stably maintains an *agr*$^{+}$ phenotype (i.e., quorum-controlled toxin secretion and the ability to cause animal disease) (2). *S. aureus* was propagated in tryptic soy broth (TSB; Difco) with shaking at 250 rpm or on tryptic soy agar (TSA; Difco) supplemented with 10 μg/mL chloramphenicol, where appropriate. *S. aureus* strains used in this study are listed in Table S1.

Phage Transduction and Determination of Transposon Insertion Sites. The *S. aureus bursa aurealis* insertion mutant strains were lysed with phage Φ85, and transposon insertions were transduced into wild-type Newman. The transduced *bursa aurealis* insertion sites were mapped by PCR and DNA sequencing. All experiments were performed as previously described (3).

Construction of the *S. aureus* Δ*stp1* Strain. To construct an in-frame, unmarked *stp1*-null mutant (Δ*stp1*), gene replacement vector pKOR1 (4) was used. PCRs were performed to amplify sequences upstream (1,910 bp) and downstream (1,633 bp) of the intended deletion. The upstream fragment was amplified from *S. aureus* strain Newman genomic DNA using primers FD1219upF and FD1219upR (with SacII site) (Table S2), and the downstream fragment was amplified with primers FD1219downF (with SacII site) (Table S2) and FD1219downR. PCR products were digested with SacII, mixed together and ligated by T4 DNA ligase (New England Biolabs). The ligation product was used for recombination with pKOR1 and recombination products were introduced to DH5a. The resulting plasmid pKOR1::Δ*stp1* was transferred by electroporation to *S. aureus* RN4220, and subsequently to Newman. The allelic replacement was preformed as described previously (4) and the deletion of *stp1* was further confirmed by PCR and DNA sequencing.

Preparation of Cell-Free Extracts. For Gram-postive bacteria, cell-free extracts were prepared as previously described (5) with some modifications. *S. aureus* or *B. subtilis* cells (OD_{600} = 0.9) from 50-mL cultures were harvested by centrifugation, washed once with buffer A [20 mM Tris-HCl, 50 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 5% (vol/vol) glycerol, pH 7.8], and frozen at −70 °C overnight. The pellet was suspended in 10 mL of buffer A containing lysostaphin or lysozyme at a concentration of 0.1 mg/mL. The bacterial suspension was incubated on ice for 3.5 h, and then frozen at −70 °C overnight. After thawing on ice, 6 mL of buffer A containing 1.3 M KCl was added and incubation on ice was continued for 30 min. The bacterial lysate was further disrupted by mechanical disruption (Fast Prep FP120 instrument; Qbiogene) and then centrifuged at 14,000 × *g* for 15 min to remove debris, and dialyzed against water for 3 h. The supernatant was filtrated through 0.45-μm filter, divided into small aliquots, and stored at −70 °C until use. For Gram-negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa*, cell-free extracts were prepared in a similar way except that cells were lysed by sonication.

Protein Phosphorylation and Dephosphorylation. Phosphorylation was performed by adding 10 μCi of [γ-^{32}P] ATP to cell-free extracts (0.03 μg) in the phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM $MnCl_2$) in the presence or absence of protein samples, as indicated. In a typical reaction, 2 μg of protein was used as the substrate and the final reaction volume was 20 μL. The reaction was incubated at 37 °C for 30 min and then quenched by the addition of SDS/PAGE loading buffer. For the dephosphorylation reaction, Stp1 was added subsequently to the in vitro phosphorylation reaction for an additional 10 min, as indicated. The reaction was stopped by the addition of SDS/PAGE loading buffer. The products were analyzed on a 13% (wt/vol) SDS/PAGE followed by autoradiography. To ensure the quality of the loaded protein, all PAGEs were subject to Coomassie blue staining after autoradiography.

To separate the phosphorylated protein, we incubated the purified His-tagged proteins (His_6-SarA) with the crude cell extract prepared from Δ*stp1* in the presence of 1 mM ATP disodium salt trihydrate. After incubation at room temperature for 30 min, we repurified the staphylococcal accessary regulator A (SarA) protein by HisTrap HP column. The fractions with pure protein were poured together, concentrated, and run on a de-salting column with buffer W [10 mM Tris (pH 7.4), 300 mM NaCl, 5 mM $MgCl_2$]. The phosphorylated and unphosphorylated forms of protein were further separated with the phosphoprotein purification kit (Qiagen) following the manufacturer's instructions.

In all cases, the purified proteins were washed twice with buffer W by using protein desalting spin columns and then used for subsequent assays.

LC-MS/MS Identification of Phosphorylation Sites on MgrA and SarA. The digests were analyzed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) coupled with a nanoLC Ultra (Eksigent). Each sample (5 μL) was loaded onto a phosphotrap column (TiO_2-C_{18} PepMap100, 200 μm × 20 mm; Dionex) at a flow rate of 4 μL/min for 5 min. Nonphosphorylated peptides elution and separation was carried out on a C_{18} column (Acclaim PepMap C_{18}, 15 cm × 75 μm × 3 μm, 100 Å; Dionex) at a flow rate of 0.26 μL/min. Peptides were separated using a 40-min linear gradient ranging from 2 to 50% (vol/vol) B (mobile phase A, 0.1% formic acid; mobile phase B, 0.1% formic acid in ACN). After elution of the nonphosphorylated peptides, an injection of 5 μL of elution buffer was performed to analyze the phosphopetides using the same analytical column and gradient described above. The mass spectrometer was operated in positive ionization mode. The MS survey scan was performed in the FT cell from a mass range of 300–2,000 *m/z*. The resolution was set to 30,000 at 400 *m/z* and the automatic gain control was set to 500,000 ions. Collision-induced dissociation fragmentation is used for MS/MS and the 20 most intense signals in the survey scan are fragmented. Detection was done in the ion trap with an isolation window of 1.5 *m/z* and a target value of 10,000 ions. Fragmentation was performed with normalized collision energies of 35% and activation time of 30 ms. Dynamic exclusion was performed with a repeat count of 1 and exclusion duration of 12 s, and a minimum MS signal for triggering MS/MS is set to 1,000 counts.

Data were processed using Mascot Distiller v2.2 and searched using Mascot Daemon 2.3.2 (Matrix Science). Searches were performed against the local database including the protein sequences for MarR family global transcriptional regulator A (MgrA) and SarA, using the following settings: trypsin as cleavage enzyme; two missed cleavages; methionine oxidation, serine, threonine, cysteine and tyrosine phosphorylation as variable modifications. The mass error tolerance for precursor ions was set to 10 ppm and 0.8 Da for fragment ions.

Antibiotic Susceptibility Assays. Minimum inhibitory concentration (MIC) was determined by the microdilution method in 96-well plates. Antibiotics were serially diluted twofold in 100 μL of TSB. Overnight cultures were diluted with 1× PBS to cell density of 10^7 CFU/mL To each well of the 96-well plate, aliquots of 5 μL were added for a final inoculum of $\sim 5 \times 10^4$ CFU per well. After incubation at 37 °C for 24 h, the MICs were determined visually. For the vancomycin resistance plate assay, TSA agar plates were made with designated amounts of vancomycin. Overnight cultures were diluted and 10-fold serial dilutions were made. Aliquots (10 μL) of the diluted cultures for each strain were spotted onto the solid media and grown at 37 °C for 24 h.

Semiquantitative Plate Assays for Hemolytic Activity. Hemolytic activity was analyzed on 5% (vol/vol) sheep blood agar plates. Zones of clearance surrounding the bacterial colonies indicate hemolysis and were determined 24 h after inoculation at 37 °C and then 24 h at 4 °C.

Western Blot Analysis of α-Hemolysin in Supernatant. *S. aureus* strains (Δ*stp1-sarA*/pYJ335, Δ*stp1-sarA*/pYJ335::*sarA,* Δ*stp1-sarA*/pYJ335::*sarA*C9S and Δ*stp1-sarA*/pYJ335::*sarA*C9E) were grown at 37 °C overnight in TSB containing 10 μg/mL chloramphenicol and 10 μg/mL erythromycin, diluted 100-fold in fresh 5 mL TSB (containing 10 μg/mL chloramphenicol and 10 μg/mL erythromycin) in a tube, and incubated at 37 °C with shaking at 250 rpm for 24 h (OD_{600} = 10). Supernatants were harvested by centrifugation and stored at −80 °C until use. Ten microliters of supernatant of each sample mixed with 10 μL of 2× SDS loading buffer was subject to 10% (wt/vol) SDS/PAGE. PageRuler Prestained Protein Ladder #SM0672 (Fermentas) was used as a molecular weight reference. Thereafter, proteins were transferred to a PVDF membrane at 350 mA for 60 min. The membrane was washed three times with TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) followed by the blocking step [20 mL of 5% (wt/vol) skim milk in TBS buffer] at room temperature for 2 h. The membrane was briefly rinsed by 20 mL of TBST buffer, incubated with the antibody solution (Sigma) [1/10,000 dilution of antibody in blocking buffer containing 5% (wt/vol) skim milk] at 4 °C overnight, and then washed three times at room temperature for 15 min in TBST buffer. Membranes were incubated with the secondary antibody (ECL Rabbit IgG, HRP-linked Wwole Ab; GE Healthcare) for 2 h at room temperature and washed three times for 15 min in TBST. The chemiluminescent detection reaction was performed and the membrane was exposed to X-ray film (Kodak) according to the manufacturer's recommendation.

Mouse Infection Models of Abscess Formation. All *S. aureus* strains were grown at 37 °C overnight in TSB, diluted 100-fold in fresh media, and incubated at 37 °C for 2.5–3.0 h until the cultures reached OD_{600} 1.0. Bacteria were collected by centrifugation, washed, and suspended in PBS buffer. Viable staphylococci were enumerated by colony formation on TSA plates to quantify the infectious dose. One-hundred microliters of bacterial suspension (1×10^7 CFU) was administered intravenously via retro orbital injection into each of 10 6-wk-old BALB/c mice. Five days after injection, animals were killed by CO_2 asphyxiation, and organs removed. The organs were homogenized in 1 mL of PBS, and aliquots of the homogenates were plated on TSA. A Student *t* test was performed for statistical analysis using Microsoft Excel. All mouse experiments were performed in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Western Blot Analysis of Phosphorylated SarA in Cell Extract. *S. aureus* strains (Δ*stp1-sarA*/pYJ335::*sarA* and Δ*stp1-sarA*/pYJ335::*sarA*C9S) were grown at 37 °C overnight in TSB containing 10 μg/mL chloramphenicol, diluted 100-fold in fresh 20 mL TSB (containing 10 μg/mL chloramphenicol) in a 50-mL conical tube (BD Biosciences), and incubated at 37 °C with shaking at 250 rpm for 2 h (OD_{600} = 0.6). Anhydrotetracycline (1 μg/mL) was added to induce the tetracycline-inducible *xyl*/*tetO* promoter in pYJ335 and then the bacteria culture was incubated for additional 6 h. Bacteria were harvested by centrifugation and stored at −80 °C until use. Cell extract in lysis buffer (10 mM Tris, pH = 8.0, 1 mM $MnCl_2$, 0.5 mM ATP, and phosphatase inhibitor mixture) was prepared with FastPrep-24 (Qbiogene) following the manufacturer's instructions. His-tag proteins were enriched by Ni-NTA beads from 1 mL of cell lysates, washed twice with 50 μL of lysis buffer supplemented with 50 mM imidazole, and eluted with the lysis buffer containing 250 mM imidazole. To detect Cys-phosphorylation of SarA, we took advantage of the promiscuity of antiphospho-Thr antibody (Abcam). Ten microliters of protein sample mixed with 10 μL of 2× SDS loading buffer was subject to 13% (wt/vol) SDS/PAGE. Trail Mix Western Markers (Novagen) was used as a molecular weight reference. Thereafter, proteins were transferred to a nitrocellulose membrane at 25 V for 80 min. The membrane was washed three times with TBST buffer followed by the blocking step [20 mL of 5% (wt/vol) BSA in TBST buffer] at room temperature for 1 h. The membrane was briefly rinsed by 20 mL of TBST buffer, incubated with the antibody solution (Abcam) [1/50,000 dilution of antibody in TBST buffer containing 3% (wt/vol) BSA] at 4 °C overnight, and then washed four times at room temperature for 30 min in TBST buffer. Membranes were incubated with the secondary antibody (ECL Rabbit IgG, HRP-linked whole Ab from GE Healthcare) at room temperature for 1 h and washed with TBST four times for 30 min. The chemiluminescent detection reaction was performed and the membrane was exposed to X-ray film (ISC BioExpress) according to the manufacturer's recommendation. To ensure the quality of the loaded protein sample, InVision His-tag In-gel Stain (Invitrogen) was used to stain the SDS/PAGE.

EMSA. For EMSA, the purified proteins were washed twice with buffer W (10 mM Tris, pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$) using protein desalting spin columns. The electrophoretic mobility shift experiments were performed as described previously (6). Briefly, DNA probes were PCR-amplified from *S. aureus* genomic DNA using primers listed in Table S2. The *sarV* promoter region was used for the MgrA binding assay. The *hla* promoter region was used for the SarA and SarZ binding assays. DNA fragments were labeled using [γ-^{32}P] ATP (Perkin-Elmer) and a T4 polynucleotide kinase (New England Biolabs). Unincorporated ATP was removed using illustra MicroSpin G-50 Columns (GE Healthcare). EMSAs were performed by adding 10 nM of ^{32}P-labeled duplex DNA to 20 μL of reaction buffer [10 mM Hepes at pH 8.0, 1 mM EDTA, 50 mM KCl, 0.05% Triton X-100, 10% (wt/vol) glycerol, 10 μg/mL salmon sperm DNA] in the presence of designated amounts of purified protein. Solutions were incubated at room temperature for 30 min before loading. Gels were run in 0.5 × TBE at 85 V at room temperature. The gels were dried and subject to autoradiography using the storage phosphor screen (Fuji) and the Molecular Imager PharosFX Plus System (Bio-Rad).

Construction, Expression and Purification of Stp1, Stk1, MgrA, SarZ, SarA, CymR, MgrAC12S, MgrAC12E, SarAC9S, SarAC9E, SarZC13S, SarZC13E, and CymRC25S. Full-length of *stp1* was cloned into the target vector pMCSG19 by ligation independent cloning (LIC) method (7). The resulting plasmid was transformed into BL21 star (DE3) competent cells containing pRK1037 (Science Reagents) by heat shock. Positive colonies were selected with 150 μg/mL Ampicillin and 30 μg/mL Kanamycin. One-liter of cells was grown at 37 °C from a 1:100 dilution of an overnight culture. The cells were induced with 1 mM of isfopropyl-β-D-thiogalactopyranoside at OD_{600} 0.6–0.8. After overnight growth at 16 °C with shaking, cells were collected by centrifugation and suspended in 40 mL of Ni-

NTA buffer A [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 50 mM imidazole, 10 mM β-ME, and 5% (wt/vol) glycerol] with protease inhibitor mixture. After loaded to a Ni-NTA column, the protein was eluted with a 0–100% gradient of buffer B [50 mM Tris-HCl pH 8.0, 250 mM NaCl, 500 mM imidazole, 10 mM β-ME, and 5% (wt/vol) glycerol]. The eluted protein was mixed with 6× SDS/PAGE buffer, boiled for 5 min and analyzed by 12% (wt/vol) SDS/PAGE. The fractions with pure protein were poured together, concentrated, and run on a desalting column with desalting buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, and 0.5 mM DTT). The protein was treated with tobacco etch virus protease at 4 °C for 48 h. The protein solution was passed through a HisTrap HP column again to remove the His-tag. The purified protein was > 90% pure as estimated by a 12% (wt/vol) SDS/PAGE gel. CymR was cloned using the same LIC strategy.

Stk1 and *sarA* were cloned into pET28a with a thrombin-cleavable N-terminal His_6-tag and yielded pET28a::His_6-*stk1* and pET28a::His_6-*sarA*, respectively. Primers used for amplification of DNA fragments from *S. aureus* strain Newman genomic DNA are listed in Table S1. Briefly, 1220-NdeI/1220-XhoI for *stk1* gene and SarA_NdeI_For/ SarA_XhoI_Rev for *sarA* gene. All of the constructs were sequenced to rule out unwanted mutations. The protein was expressed in *E. coli* strain BL21 star (DE3) and purifications were performed as described previously (8, 9).

MgrA was cloned into pET30a and yielded pET30a::*mgrA*. The protein (His-tag–free) was expressed in BL21 star (DE3) and purified by Heparin column (GE Healthcare).

Stratagene's QuikChange Site-Directed Mutagenesis Kit was used to construct all mutant proteins. Primers for mutagenesis are listed in Table S2. The protein expression and purification procedures are the same as described above. All proteins were subject to buffer exchange with the buffer containing 0.5 M NaCl, 10 mM Tris (pH = 7.4) and 10% (wt/vol) glycerol to remove imidazole before subsequent assays.

Crystallization and Structure Determination of SarZC13E. Crystallization of SarZC13E was done following procedures described previously (10). Purified SarZC13E in buffer A (10 mM Tris, pH 7.4, 200 mM NaCl) crystallized at room temperature using the hanging-drop vapor-diffusion method against a reservoir solution of 0.1 M Bis-Tris (pH 5.3) and 25% (wt/vol) polyethylene glycol 3,350. SarZC13E crystals appeared within 1 wk and were frozen in liquid nitrogen following cryoprotection with the reservoir solution containing 20% (wt/vol) glycerol. The data were collected to 2.0 Å at the General Medicine and Cancer Institutes Collaborative Access Team Beamline 23-ID-B at the Advanced Photon Source, Argonne National Laboratory, and processed using HKL2000. The model was built and improved as described previously (10), using the program Coot (11) and CNS (12).

Molecular Dynamics Simulations. To obtain starting structures for molecular dynamics (MD) simulations, coordinate of SarZ wild-type was taken from the crystal structure (PDB ID: 3HSE) (10). The coordinates of residues missing because of lack of interpretable electron density were generated by the loop-search method implanted in the Homology module of Insight II (Accelrys), including referring the other crystal structures of SarZ. Subsequently, the Cys13 residues in the two chains were mutated into the glutamate residues and modified by introduction of phosphate groups to the S atom, respectively. The three types of SarZ structures (wild-type, C13E, Cys-phosphorylated) were minimized to preclude the steric clash to make them reasonable starting structures.

MD simulations were carried out with the GROMACS 4 (13) package using the isothermal–isobaric (NPT) ensembles and periodic boundary condition. The Amber Parm99 force field was applied to the three structures of amino acids, and the parameters of phosphorylated-cysteine were obtained by using scripts provided by ANTECHAMBER/GAFF-ffamber port. In each simulation, the protein was solvated in a cubic box with TIP3P water molecules, keeping the boundary of the box at least 15 Å away from any protein atoms. The resulting system was then submitted to energy minimization. Counterions were subsequently added for charge neutralization. Energy minimization was then repeated on the whole system. After convergence had been reached, the solvent, counterions, and protein were sequentially coupled to a temperature bath as 310 K with a coupling time of 0.1 ps by the Berendsen thermostat. The pressure was maintained at 1 bar by the Berendsen barostat. Electrostatic interactions were calculated using the particle mesh Ewald algorithm. The bond lengths, including the hydrogen atoms, were restrained using the LINCS method, allowing an integration step of 2 fs. The coordinates of the whole system were saved every 100 ps.

Construction of Plasmids for Constitutive Expression or Overexpression of *stp1*, *stk1*, and *sarA*. The previously constructed shuttle plasmid pYJ335 (14) was used in this study. The transcription of gene is driven by *xyl*/*tetO* promoter in pYJ335 with a low basal activity (constitutive expression) or the inducible activity in the presence of inducer aTc (for overexpression). To construct pYJ335::*stp1*, an 810-bp DNA fragment containing *stp1* was amplified from Newman's genomic DNA with primers SAV1219F and SAV1219R. To construct pYJ335::*stk1*, a 2,062 bp DNA fragment containing *stk1* was amplified with primers *stk*BF and *stk*BR. To construct pYJ335::*sarA*, a DNA fragment containing His_6-*sarA* was amplified from pET28a::His_6-*sarA* with T7 promoter primer and T7 terminator primer. Primer pairs of tetRFor399/SAV1219R, tetRFor399/*stkB*R, and tetRFor399/T7 terminator primer were used to select the plasmid clones in which the genes are located downstream of the tetracycline-inducible *xyl*/*tetO* promoter as previously described (8, 9), respectively, where appropriate. The selected plasmid clones were sequenced to confirm that no additional mutations were introduced by PCR reactions. The correct plasmids were transformed into RN4220 and then into the Newman derivatives by electroporation. The pYJ335 vector alone was transformed into RN4220 and then into the Newman derivatives as the control, where appropriate.

RNA Isolation and Northern Blotting. To isolate RNA for Northern blot analysis, all *S. aureus* strains were grown at 37 °C overnight in TSB, diluted 100-fold in fresh 10 mL TSB in a 50-mL conical tube (BD Biosciences), and incubated at 37 °C with shaking at 250 rpm for 2.5 h (OD_{600} = 0.8). The bacteria were harvested and disrupted by mechanical disruption (Fast Prep FP120 instrument; Qbiogene). The RNeasy Mini Kit (Qiagen) was used for subsequent RNA purification. The concentration and purity of RNA were determined by absorbency at 260 and 280 nm. Northern blotting was performed following the reported procedures. Primers (*stk*BF/*stk*BR for *stk1*) used for amplification of DNA fragments in Northern blotting are listed in Table S2.

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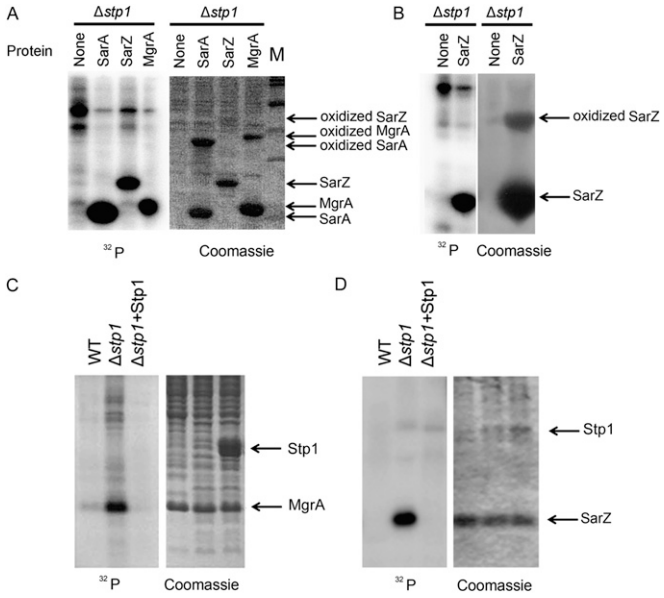

Fig. S1. (*A* and *B*) Phosphorylation occurs exclusively to the reduced proteins. (*A*) In vitro phosphorylation assays (*Left*, lane 1) cell extract alone as a negative control, indicated as "None"; (lane 2) SarA + Δ*stp1* cell extract; (lane 3) SarZ +Δ*stp1* cell extract; (lane 4) MgrA +Δ*stp1* cell extract. The gel stained with Coomassie blue (*Right*): oxidized SarA and oxidized MgrA are indicated by arrows. M, protein size marker. In the lane of SarZ, no significant SarZ oxidition was observed. (*B*) In vitro phosphorylation assay with an increased amount of SarZ. (*C* and *D*) Stp1 dephosphorylates Cys-phosphorylated MgrA (*C*) and SarZ (*D*). The phosphorylated product was treated with Stp1 (3 μM) before gel analysis. WT, cell extract from the wild-type Newman; Δ*stp1*, cell extract from the Δ*stp1* deletion mutant; Stp1, the recombinant protein Stp1 expressed from *E. coli*.

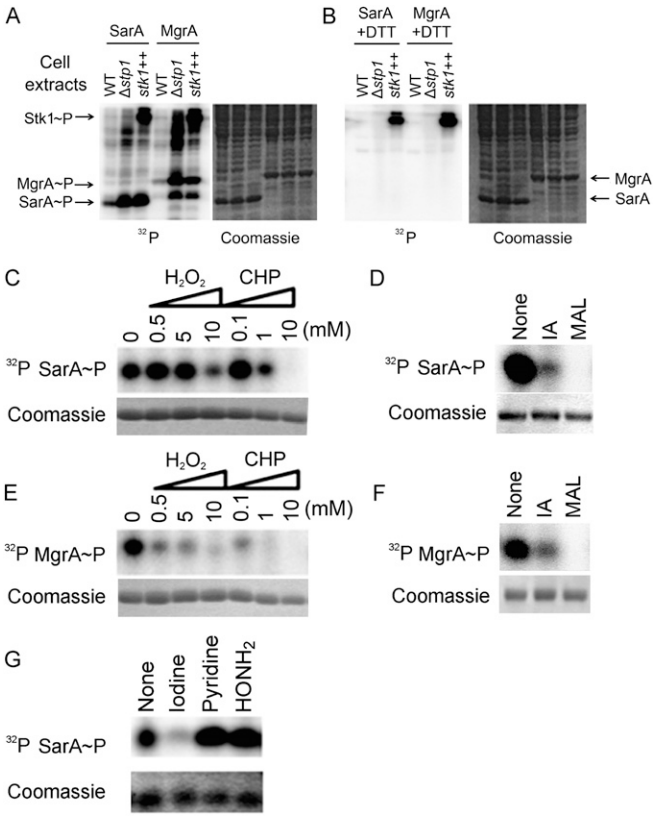

Fig. S2. Unique properties of Cys-phosphorylation. (*A* and *B*) The in vitro Cys-phosphorylation of SarA and MgrA is significantly enhanced by either deletion of *stp1* or overexpression of *stk1*, but inhibited by excess of DTT (10 mM). WT, cell extract from the wild-type Newman; Δ*stp1*, cell extract from a deletion mutant of *stp1*; *stk1*$^{++}$, cell extract from the strain (Newman/pYJ335::*stk1*) overexpressing *stk1* induced by 1 μg/mL aTc. (*C* and *E*) Cys-phosphorylation is blocked by oxidants. SarA or MgrA (10 μM) was treated with various amounts of H_2O_2, cumene hydrogen peroxide (CHP) at room temperature for 20 min followed by in vitro phosphorylation assays. (*D* and *F*) Cys-phosphorylation is blocked by alkylators. To test effects of alkylators on Cys-phosphoryaltion, the SarA or MgrA protein (10 μM) was treated with 2 mM of iodoacetamide (IA) or 2 mM of maleimide (MAL) at 37 °C for 30 min. Excess of alkylators were removed by desalting before in vitro phosphorylation assays. The proteins not treated with alkylators were used as positive controls, which were indicated as "None." (*G*) Cys-phosphorylation is susceptible to iodine but stable toward pyridine and hydroxylamine ($HONH_2$). The phosphorylated MgrA was treated with iodine (1 mM), pyridine (50 mM), or hydroxylamine (50 mM) at 37 °C for 10 min and then subject to SDS/PAGE analyses.

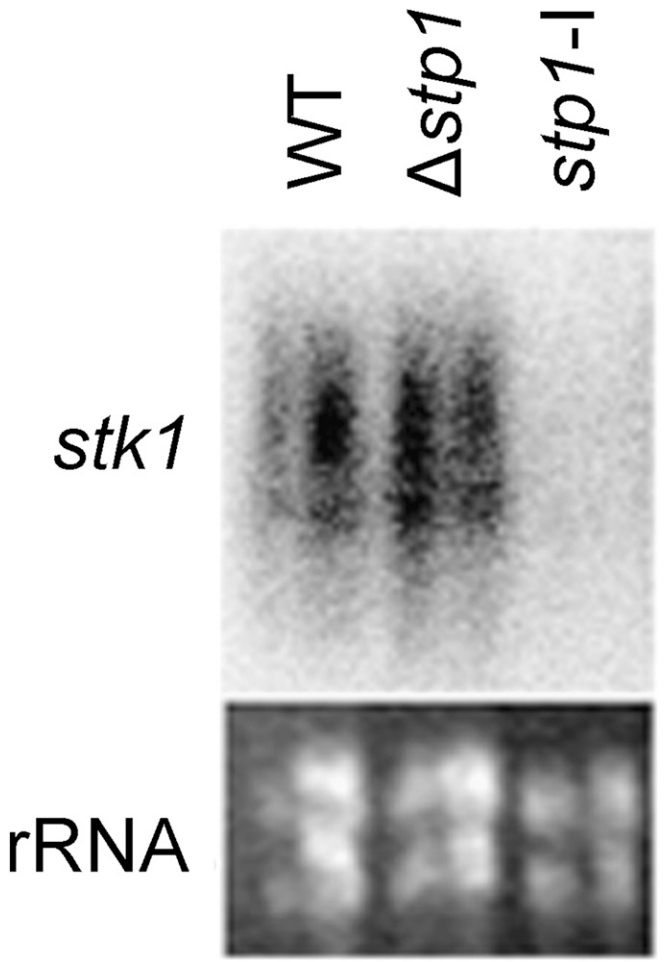

Fig. S3. Northern blot showing transposon insertion of the *stp1* gene abolishes the expression of *stk1*. Bacteria were harvested after incubating at 37 °C with shaking at 250 rpm for 3 h (OD_{600} = 0.8) and total RNA was isolated. Five micrograms of the total cellular RNA were used in each experiment with the ethidium bromide stained gel picture of the loaded RNA sample shown below each lane. *stp1*-I, *bursa aurealis* transposon insertion mutant of *stp1*.

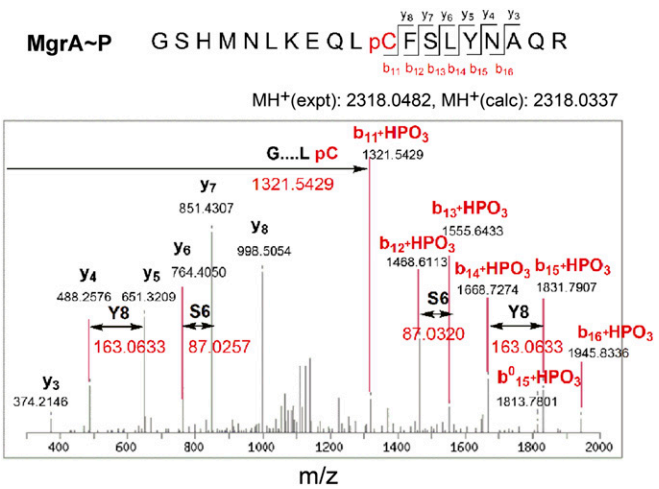

Fig. S4. LC-MS/MS of a miscleaved phospho-peptide from MgrA-P. Observed *m/z* 2318.0482–2238.0640 (the corresponding apo-peptide theoretical *m/z*) = 79.9842. The b_{11} fragment indicates the presence of phospho-Cys.

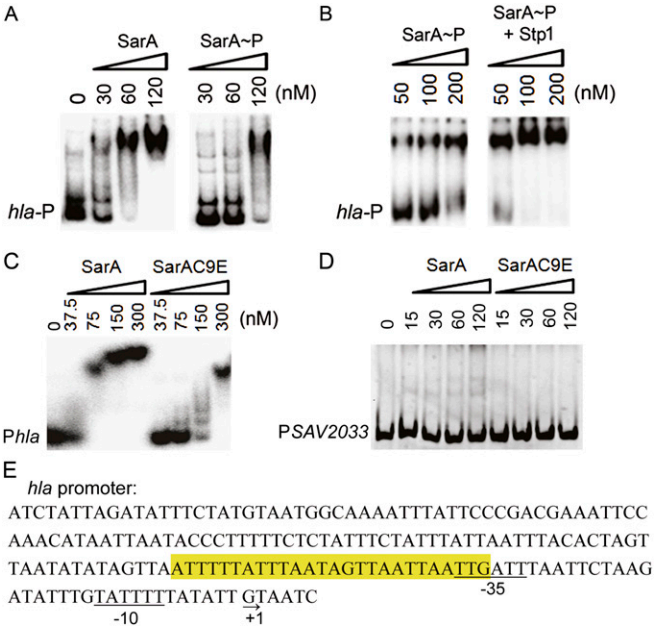

Fig. S5. Cys-phosphorylation and phosphomimetic mutation of Cys to Glu weaken the DNA-binding ability of SarA to *hla* promoter region. (*A* and *B*) EMSAs show that Cys-phosphorylation attenuates the binding of SarA to the *hla* (α-hemolysin) promoter DNA [K_d (SarA) = ~32 nM, and K_d (SarA~P) = ~120 nM] (*A*), which can be restored by treating phospho-SarA with phosphatase Stp1 (*B*). (*C* and *D*) SarA specifically binds to the *hla* promoter region (*C*) but not the promoter region of *SAV2033* (*D*), a gene not regulated by SarA, which serves as a negative control. The dissociation constants of SarA to *hla*: K_d (SarA) = ~32 nM, K_d (SarAC9E) = ~134 nM. All experiments were repeated at least twice with similar results obtained. (*E*) The promoter region of *hla*. The conserved SarA-binding site is colored in yellow. K_d was determined by a densitometry analysis.

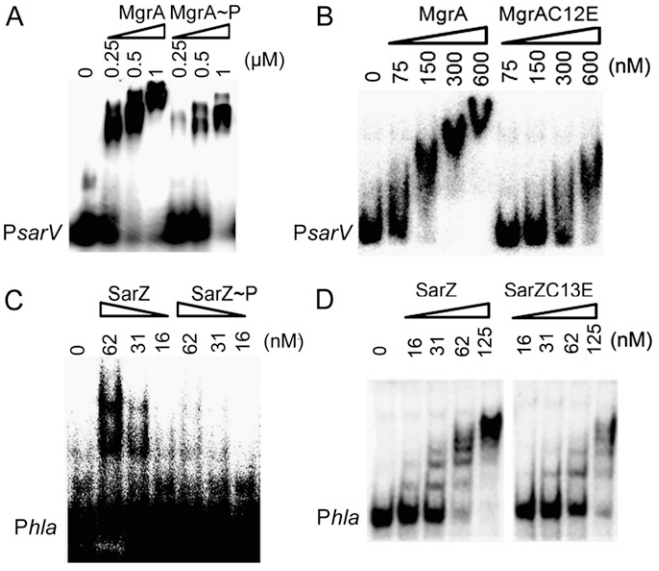

Fig. S6. Both phoshorylation and phosphomimetic mutation of Cys to Glu attenuates the binding abilities of MgrA (*A* and *B*) and SarZ (*C* and *D*) to their cognate promoter regions. The dissociation constants: K_d (MgrA) = ~102 nM, K_d (MgrA~P) = ~500 nM, K_d (MgrAC12E) = ~480 nM, K_d (SarZ) = ~35 nM, K_d (SarZ~P) = ~120 nM, and K_d (SarZC13E) = ~110 nM. All experiments were repeated at least twice with similar results obtained. K_d was determined by a densitometry analysis.

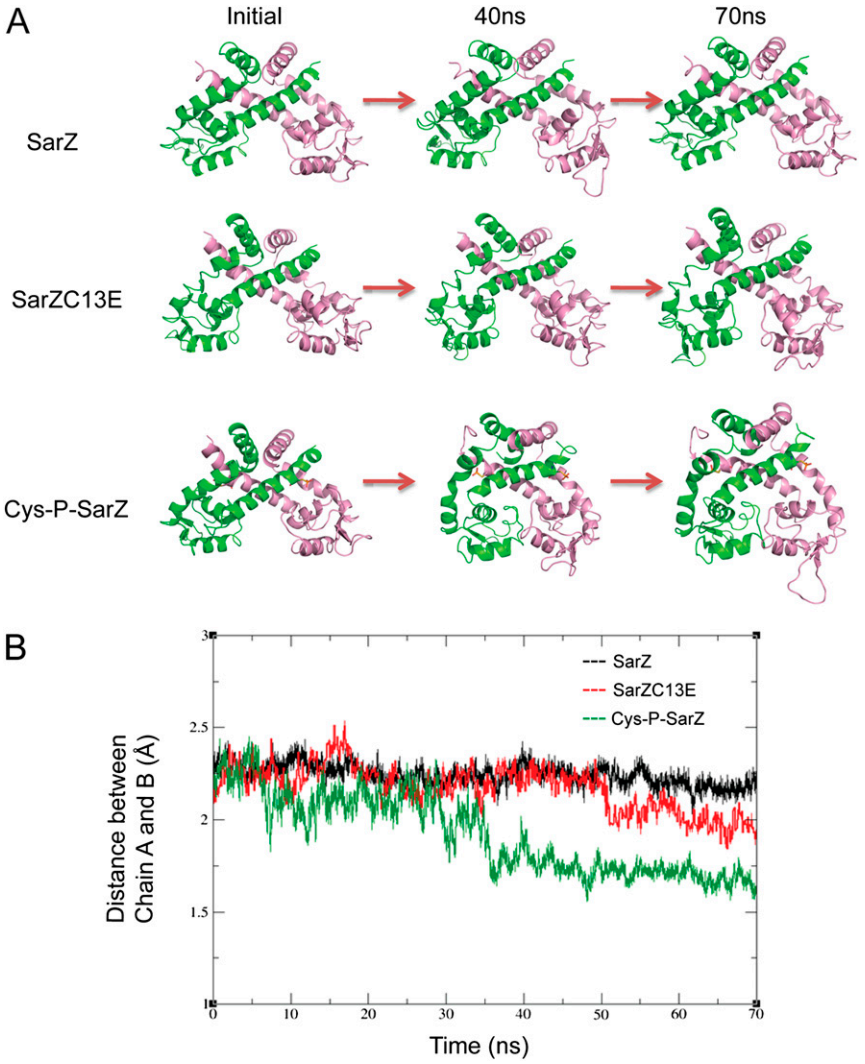

Fig. S7. Structural comparison of wild-type SarZ, SarZC13E, and Cys-P-SarZ during MD simulations. (*A*) Snapshot structures of wild-type SarZ, SarZC13E, and Cys-P-SarZ at t = 0 ns, t = 40 ns, and t = 70 ns. (*B*) Time evolutions of centroid distance between chain A and B for three types of SarZ from their initial conformation (t = 0) were recorded.

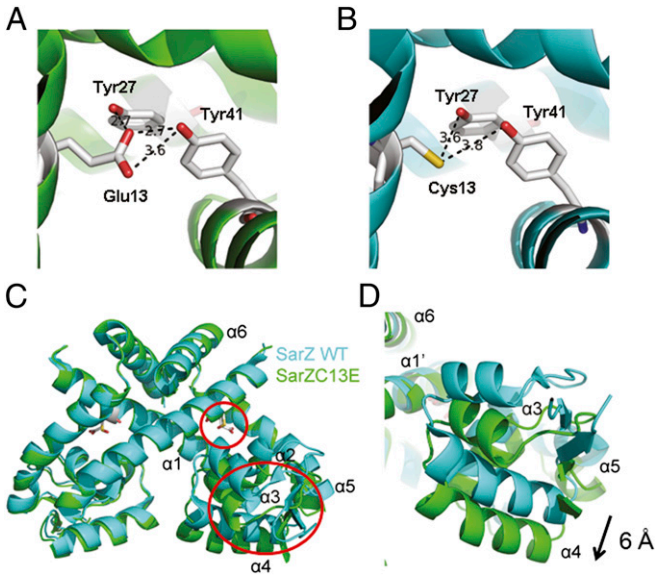

Fig. S8. Crystal structure of a phosphomimetic mutant (Cys to Glu) of SarZ. (*A*) The Cys-13 pocket in SarZ. Atoms are colored gray (carbon), red (oxygen), and yellow (sulfur). Hydrogen bonds are shown as black dashed lines: Cys-13 to Tyr-27, 3.6 Å; Cys-13 to Tyr-41, 3.8 Å. (*B*) The Glu-13 pocket in SarZC13E. Atoms are colored gray (carbon) and red (oxygen). Hydrogen bonds are shown as black dashed lines: Glu-13 to Tyr27, 2.7 Å; Glu-13(Oα) to Tyr-41, 2.7 Å; and Glu-13(Oβ) to Tyr-41, 3.6 Å. (*C*) Superimposition of the left monomer of the wild-type SarZ (cyan) and SarZC13E (green). The changes of the other monomers are highlighted. (*D*) Close view of the conformational change in the HTH domain between the two structures.

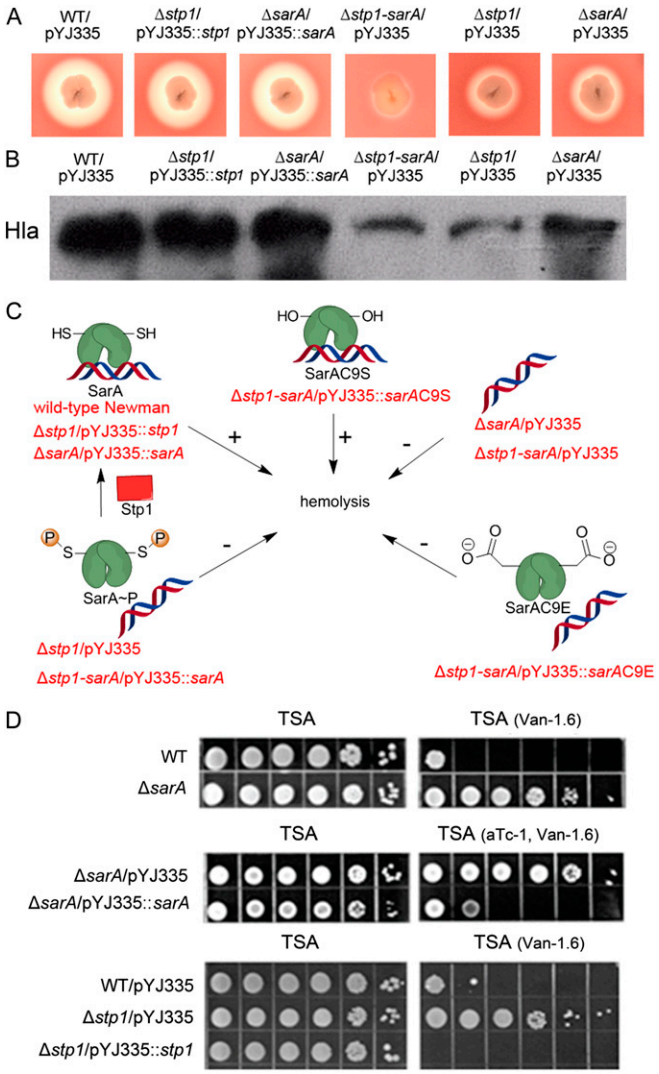

Fig. S9. SarA and Stp1 affect hemolysis and bacterial susceptibility to vancomycin. (*A*) Stp1 and SarA positively regulate hemolysis. The tested strains were spotted and then inoculated on a 5% (vol/vol) sheep blood agar plate at 37 °C for 24 h, and then 4 °C for 24 h. Clearance of zones indicates hemolysis. (*B*) Western blot analysis of the production of α-hemolysin. (*C*) The model depicting the effect of Cys-phosphorylation of SarA on hemolysis. Both SarA and Stp1 are positive regulators of hemolysis. Cys-phosphorylation of SarA is accumulated in both Δ*stp1* and Δ*stp1-sarA*/pYJ335::*sarA,* thus leading to decreased hemolysis. SarAC9S is not subject to Cys-phosphorylation and therefore Δ*stp1-sarA*/pYJ335::*sarA*C9S showed high hemolytic activities. (*D*) Stp1 and SarA negatively regulate bacterial resistance to vancomycin. Aliquots (10 μL) of the diluted overnight cultures for each strain were spotted onto the TSA plates without (*Left*) or with 1.6 μg/mL vancomycin (*Right*).

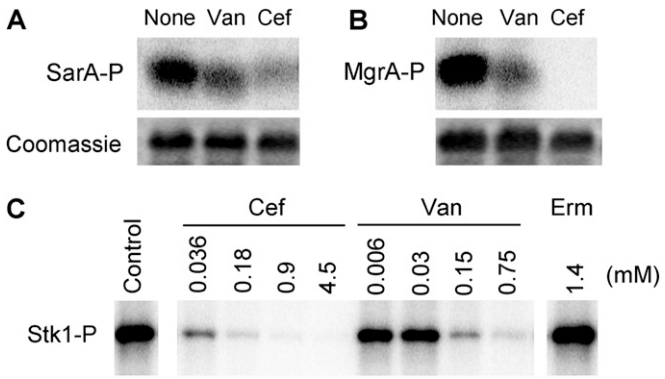

Fig. S10. Cell wall-targeting antibiotics impact Cys-phosphorylation. (*A* and *B*) Cys-phosphorylation of SarA and MgrA mediated by Δ*stp1* cell extract in the presence of vancomycin (0.75 mM) and ceftriaxone (0.9 mM). Cys-phophorylation was repressed by these cell wall-targeting antibiotics. (*C*) The autokinase activity of Stk1 in the presence of various amounts of antibiotics. Autophosphorylation of Stk1 was inhibited by cell wall antibiotics including vancomycin and ceftriaxone. The activity of Stk1 was not affected by a high concentration of erythromycin (1.4 mM), an antibiotic targeting protein synthesis.

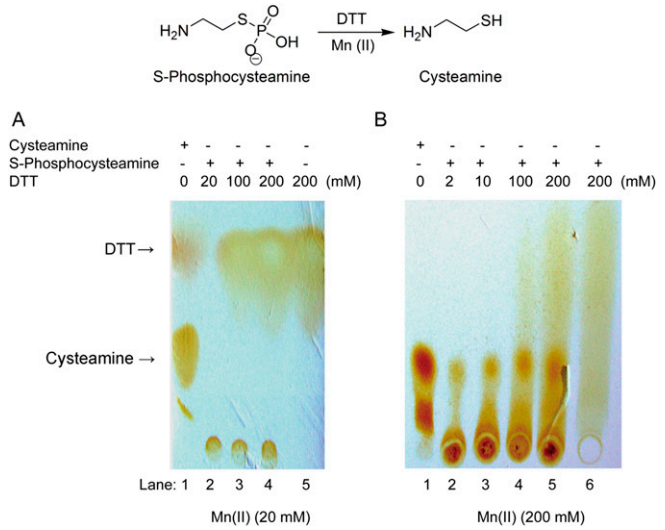

Fig. S11. DTT facilitates dephosphorylation of S-phosphocysteamine. S-Phosphocysteamine was used as a model compound to investigate the stability of phospho-Cys toward DTT. S-Phosphocysteamine was incubated with various amounts of DTT and $MnCl_2$ [20 mM (*A*) or 200 mM (*B*)] in the buffer containing 10 mM Tris, pH 7.4, and 10 mM NaCl at 37 °C for 3 h. The resulting mixtures were analyzed by thin-layer chromatography with silica gel plates (ethanol:propane-2-ol:1 N HCl 10:10:1), and visualized by iodine tank. Pure cysteamine (lane 1) was added into the reaction buffer as control. Cysteamine and DTT were indicated by arrows.

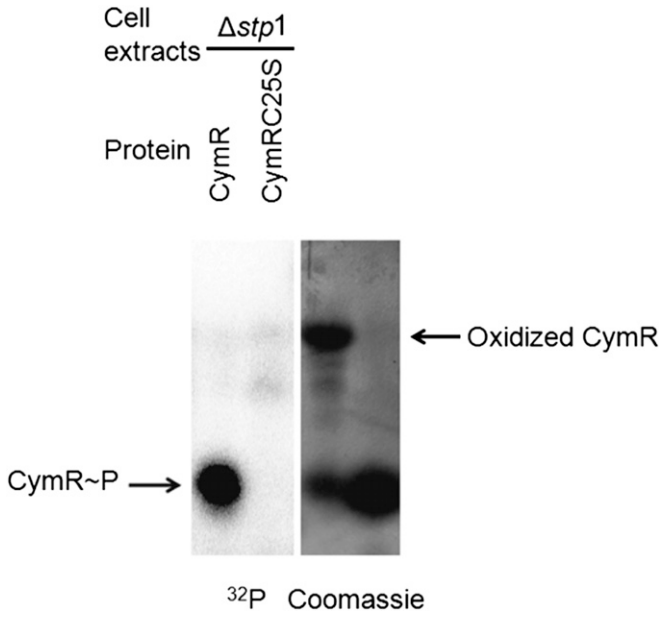

Fig. S12. Cys-Phosphorylation of CymR. The CymR protein contains a sole Cys-25 residue. The covalently linked dimer CymR was caused by oxidation. Phosphorylation only occurs to the reduced wild-type CymR.

Table S1. Bacterial strains and plasmids used in this study

Plasmids or strains	Relevant characteristics	Source
Plasmids		
pET28a	T7 *lac* promoter-operator, N-terminal His tag, kan^r	Novagen
pET30a	T7 *lac* promoter-operator, kan^r	Novagen
pMCSG19	T7 promoter, Ligation independent cloning (LIC) site, Ap^r	(1)
pKOR1	Gene replacement vector for *S. aureus* genes, Amp^r, Cm^r	(2)
pYJ335	Tet-inducible promoter; Ap^r in *E. coli*; Cam^r in *S. aureus*	(3)
pMCSG19::His-*stp1*	pMCSG19 derivative carrying *stp1*	Present study
pET28a::His-*sarA*	pET28a derivative carrying *sarA*	Present study
pET28a::His-*sarZ*	pET28a derivative carrying *sarZ*	(4)
pET30a::*mgrA*	pET30a derivative carrying *mgrA*	Present study
pYJ335::*stp1*	pYJ335 derivative carrying *stp1* in the downstream of the *xyl/tet* promoter region	Present study
pYJ335::*stk1*	pYJ335 derivative carrying *stk1* in the downstream of the *xyl/tet* promoter region	Present study
pYJ335::*mgrA*	pYJ335 derivative carrying *His_6-mgrA* in the downstream of the *xyl/tet* promoter region. Previously termed pYJ335-His-*mgrA*.	(5)
pYJ335::*sarA*	pYJ335 derivative carrying *His_6-sarA* in the downstream of the *xyl/tet* promoter region.	Present study
pKOR1::Δ*stp1*	pKOR1 derivative, for deletion of *stp1* gene.	Present study
Strains		
S. aureus		
RN4220	Restriction deficient.	(6)
Newman	Wild-type, human clinical isolate.	(7)
Δ*stp1*	In-frame, unmarked *stp1*-null mutant.	Present study
Stp1-I	*S. aureus* Newman with *bursa aurealis* insertion in *SAV1219*, transduced from strain ΦNΞ 14673.	Present study
Δ*stp1-sarA*	a double mutant with in-frame, unmarked *stp1* deletion and *bursa aurealis* transposon insertion in *sarA*	Present study
Δ*sarA*	*S. aureus* Newman with *bursa aurealis* insertion in *SAV0616*, transduced from strain ΦNΞ 10017.	Present study
WT/pYJ335	*S. aureus* Newman carrying plasmid pYJ335.	Present study
Δ*stp1*/pYJ335	Δ*stp1* mutant strain carrying plasmid pYJ335.	Present study
Stp1-I/pYJ335	*Stp1*-I mutant strain carrying plasmid pYJ335.	Present study
Δ*sarA*/pYJ335	Δ*sarA* mutant strain carrying plasmid pYJ335.	Present study
Δ*stp1-sarA*/pYJ335	*Stp1 and sarA* double mutant strain carrying plasmid pYJ335	Present study
WT/pYJ335::*stk1*	*S. aureus* Newman carrying plasmid pYJ335::*stk1*	Present study
Δ*stp1*-C	Δ*stp1* mutant strain carrying plasmid pYJ335::*stp1* (Δ*stp1*/pYJ335::*stp1*)	Present study
Δ*sarA*-C	Δ*sarA* mutant strain carrying plasmid pYJ335::*sarA* (Δ*sarA*/pYJ335::*sarA*)	Present study
Δ*stp1-sarA*/pYJ335::*sarA*	Δ*stp1-sarA* double mutant strain carrying plasmid pYJ335::*sarA*	Present study
Δ*stp1-sarA*/pYJ335::*sarA*C9S	Δ*stp1-sarA* double mutant strain carrying plasmid pYJ335::*sarA*C9S	Present study
Δ*stp1-sarA*/pYJ335::*sarA*C9E	Δ*stp1-sarA* double mutant strain carrying plasmid pYJ335::*sarA*C9E	Present study
E. coli		
DH5a	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* (Φ*80dlac*Δ(*lacZ*)*M15*)	Laboratory stock
BL21	F^- *ompT hsd*S_B (r_B^- m_B^-) *gal dcm met* (DE3)	Laboratory stock

Table S2. Primers used in this study

Primers	Sequence (5′ to 3′)
SarA_NdeI_For	aatct gacat atggc aatta caaaa atcaa tgatt gc
SarA_XhoI_Rev	aatct gactc gagtt atagt tcaat ttcgt tgttt gc
MgrA_NdeI_For	gtgtacacatatgaatttaaaagaacagctatgctttag
MgrA_XhoI_For	gtaggcactcgagttattcatcaaatgcatgaatgac
CymR_LIC_For	tacttccaatccaatgccatgaaaatttctactaaagggagatatgg
CymR_LIC_Rev	ttatccacttccaatgttaaatataaaacatgtatccgtc
CymRC25SF	gctaaaaaagaggggcaaggatctatatcattaaagtcaattgc
CymRC25SR	gcaattgactttaatgatatagatccttgccccctttttttagc
T7 promoter primer	aaataatacgactcactatagg
T7 terminator primer	atgctagttattgctcagcgg
*tetR*For399	caatacaatgtaggctgctctacacc
*sarA*C9S_F	caaaa atcaa tgata gcttt gagtt gttat caatg gtcac ttatg
*sarA*C9S_R	cataa gtgac cattg ataac aactc aaagc tatca ttgat ttttg
*sarA*C9E_F	gcaattacaaaaatcaatgatgaatttgagttgttatcaatggtc
*sarA*C9E_R	gaccattgataacaactcaaattcatcattgatttttgtaattgc
*sarZ*C13E_F	ta tcttagcaaa cagttggaat ttttatttta tgtttcttc
*sarZ*C13E_R	gaagaaacataaaataaaaattccaactgtttgctaagata
*mgrA*C12S_F	gaacagctaagctttagtttgtacaatgctcaaagac
*mgrA*C12S_R	gtctttgagcattgtacaaactaaagcttagctgttc
*mgrA*C12F_F	gaa cag cta ttt ttt agt ttgtacaatgctcaaagac
*mgrA*C12F_R	gtctttgagcattgtacaaactaaaaaatagctgttc
*mgrA*C12E_F	cataatttaaaagaacagctagaatttagtttgtacaatgctcaaag
*mgrA*C12E_R	ctttgagcattgtacaaactaaattctagctgttcttttaaattatg
*sarZ*C13E_F	ta tcttagcaaa cagttggaat ttttatttta tgtttcttc
*sarZ*C13E_R	gaagaaacataaaataaaaattccaactgtttgctaagata
*SAV1219*_1_For	tacttccaatccaatgccatgctagaggcacaatttttactgatactg
*SAV1219*_247_Rev	ttatccacttccaatgttatcatactttatcaccttcaatagccgcg
SAV1220-NdeI	ttggacacatatgataggtaaaataataaatgaacga
SAV1220-XhoI	cacactcgagttatacatcatcatagctgacttcttt
*stkB*F	tactcgcggctattgaaggt
*stkB*R	ttccaaatagaaacctcggtaca
SAV1219F	gatattgacgcagcttgtgg
*SAV1219*R	tcatactttatcaccttcaatagcc
*FD1219*upF	ggggaccactttgtacaagaaagctgggtctgacgcgagcatcattaga
*FD1219*upR	aggtttcccgcggatctctatgttgtccagtatcagt
*FD1219*downF	aggtttcccgcggtatttattattaaattcagatgga
*FD1219*downR	ggggacaagtttgtacaaaaaagcaggctatgtatccttttggcgcttg
P*SAV2033*	cctttgcttcaactgtgtg
P*SAV2033*	cttctttagtaaagaatac
P*hla*F	gtgtacaaacgaaaaagtatcgtatgtatttttaatatag
P*hla*R	tccggtacctacgagtttcattaacgtcaca
P*sarV*F	gaatatcatttctatcccactc
P*sarV*R	caaacttagcattttatctattac

Table S3. Data collection and refinement statistics for the SarZC13E structure

Data collection and crystal parameters	SarZC13E
Space group	$P2_12_12_1$
Cell dimensions	
a, b, c (Å)	45.44, 55.79, 119.26
α, β, γ (°)	90, 90, 90
Wavelength (Å)	1.07818
Resolution (Å)	20.00–2.05 (2.12–2.05)
R_{merge} (%)	8.6 (65.4)
I/σI	19.5 (2.0)
Completeness (%)	98.8 (92.5)
Redundancy	6.0 (4.5)
Refinement	
Resolution (Å)	20.00–2.05 (2.12–2.05)
R_{work}/R_{free} (%)	27.0/31.8 (29.4/31.4)
Rmsd bond lengths (Å)	0.014
Rmsd bond angle (°)	1.48
Mean B factor ($Å^2$)	40.1

Highest-resolution shell is shown in parenthesis.