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# Supporting Information for

## SpbR Controls Lipoteichoic Acid Length by Directly Inhibiting Signal Peptidase SpsB in *Staphylococcus aureus*

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## Supporting Information Text

### Materials

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich. 6x non-reducing Laemmli SDS-Sample Buffer and 6x reducing Laemmli SDS-Sample Buffer were purchased from Boston BioProducts. Tryptic Soy Broth (TSB) powder, lysogeny broth (LB) powder, and Bacto Agar were purchased from Becton Dickinson. Terrific Broth (TB) powder was purchased from VWR. Kanamycin, carbenicillin, and isopropyl β-d-1-thiogalactopyranoside (IPTG) were purchased from Gold Biotechnology. Tris-Glycine (TGX) SDS-PAGE gels, TGX running buffer, immunoblot PVDF, and 0.2 mm pore nitrocellulose were purchased from Bio-Rad. Anhydrotetracycline (aTc) was purchased from Cayman Chemical. Imperial Protein Stain, Superblock T20 (TBS), Pierce ECL Western Blotting Substrate, Immobilon-FL PVDF membrane, dithiobis(succinimidyl propionate) (DSP) were purchased from ThermoFisher. α-mouse-LTA was purchased from HycultBiotech. α-rabbit-HRP and α-mouse-HRP secondary antibody were purchased from Cell Signaling Technology. α-mouse-AlexaFluor488 and α-rabbit-AlexaFluor647 were purchased from Abcam. Amersham ECL Prime Western Blotting Detection Reagent was purchased from GE. n-Dodecyl-β-D-maltoside (DDM) was purchased from Avanti Polar Lipids. 1xFLAG peptide were purchased from GenScript. DNA polymerases for PCR (Q5 and Phusion), restriction enzymes, T4 ligase, proteinase K and buffer were purchased from New England Biolabs. Stellar competent cells and In-Fusion HD Cloning Mix were purchased from Takara Bio. Oligos used for cloning were purchased from Integrated DNA Technologies. Buffers for DNA plasmid and PCR purification were purchased from Qiagen. Nunc maxisorp 96-well plates were used for ELISAs. BD OptEIA solutions were purchased from BD Biosciences Pharmingen. Recombinant Lysostaphin and M1-α-Flag resin were prepared by the Walker Lab. The α-eLtaS antibody which is specific for the extracellular domain of LtaS was provided by the laboratory of Angelika Gründling (Imperial College London).

### Plasmid construction

#### pLOW expression plasmids

To construct pLOW plasmids containing IPTG-inducible constructs, the gene sequence with the *rpoB* ribosome-binding site (−25) was amplified by PCR. The fragment was inserted into pLOW between restriction sites SalI and BamHI. For pMS7, the *spbR* gene was amplified from RN4220 gDNA with the *rpoB* RBS. For pMS12, the *spbR* gene was amplified from RN4220 gDNA with the *rpoB* RBS and N-terminal FLAG sequence. For pMS16, the *GFPmut2* was amplified from plasmid pTM186 with the *rpoB* RBS and N-terminal FLAG sequence. For pMS18, pMS24, pMS26, pMS28, pMS29, pMS30, pMS32, and pMS33, various mutations were introduced by site-directed mutagenesis (SDM) in the conserved CAAX protease residues of *spbR* and using pMS12 as template for the PCR reactions. See *SI Appendix*, Table S7 for the primer information.

#### pMS50

To construct the plasmid pMS50 for SpbR overexpression and purification, the gene sequence for *spbR* was amplified from RN4220 gDNA by PCR using primers oMS154 and oMS155. The plasmid backbone was amplified from pAM205 by PCR using primers ATP146 and 147. The two PCR products were ligated by in-Fusion. The resulting plasmid pMS50 expresses $His_6$-SUMO-FLAG-SpbR (N2-A200).

#### pMS46

To construct plasmid pMS46 for SpsB overexpression and purification, the gene sequence for *spsB* was amplified from RN4220 gDNA by PCR using oMS73 and oMS139. The fragment was inserted into pET28b(+) between restriction sites HindIII and NdeI. $His_6$-SpsB can be expressed from the resulting plasmid pMS46.

#### pMS52

To construct plasmid pMS52 for SpbR and SpsB co-expression and co-purification, the gene sequence for $His_6$-SUMO-FLAG-*spbR* (N2-A200) was amplified by PCR using oMS162 and oMS163. The plasmid backbone was amplified from pETDuet by PCR using primers oMS160 and oMS161. The two PCR products were ligated by in-Fusion. The gene sequence for *spsB* was amplified from RN4220 gDNA by PCR using oMS72 and oMS73. The fragment was inserted into pETDuet containing *spbR* between restriction sites EcoRI and HindIII. $His_6$-SUMO-FLAG-SpbR (N2-A200) and $His_6$-SpsB and be expressed from the resulting plasmid pMS52.

**pYS042**
To construct plasmid pYS042, cysteine mutations were introduced at the AlphaFold2-predicted interface between SpbR and SpsB using SDM. The gene sequences containing the desired cysteine mutations were amplified from pMS52 by PCR using primers oYS200 and oYS203, and oYS201 and oYS202, and PCR products were purified by DNA agarose gel. Two gene fragments were ligated by in-Fusion. pYS042 expresses $His_6$-SUMO-FLAG-SpbR$^{K104C}$ and $His_6$-SpsB$^{F177C}$.
**pYS043**
To construct plasmid pYS043, cysteine mutations were introduced at the AlphaFold2-predicted interface between SpbR and SpsB using SDM. The gene sequences containing the desired cysteine mutations were amplified from pMS52 by PCR using primers oYS204 and oYS207, and oYS205 and oYS206, and PCR products were purified by DNA agarose gel. Two gene fragments were ligated by in-Fusion. pYS043 expresses $His_6$-SUMO-FLAG-SpbR$^{W103C}$ and $His_6$-SpsB$^{F180C}$.
**pYS044**
To construct plasmid pYS044, cysteine mutations were introduced at the AlphaFold2-predicted interface between SpbR and SpsB using SDM. The gene sequences containing the desired cysteine mutations were amplified from pMS52 by PCR using primers oYS208 and oYS211, and oYS209 and oYS210, and PCR products were purified by DNA agarose gel. Two gene fragments were ligated by in-Fusion. pYS044 expresses $His_6$-SUMO-FLAG-SpbR$^{V190C}$ and $His_6$-SpsB$^{I18C}$.
**pYS045**
To construct plasmid pYS045, cysteine mutations were introduced at the AlphaFold2-predicted interface between SpbR and SpsB using SDM. The gene sequences containing the desired cysteine mutations were amplified from pMS52 by PCR using primers oYS212 and oYS215, and oYS213 and oYS214, and PCR products were purified by DNA agarose gel. Two gene fragments were ligated by in-Fusion. pYS045 expresses $His_6$-SUMO-FLAG-SpbR$^{I197C}$ and His6-SpsB$^{I16C}$.
**pYS046**
To construct plasmid pYS046, cysteine mutation was introduced on SpbR using SDM. The gene sequence containing the desired cysteine mutation was amplified from pMS52 by PCR using primers oYS200 and oYS201, and PCR products were purified by DNA agarose gel. The template plasmid was digested by Dpn1, and the PCR products were ligated by directly transforming into Stella cells. pYS046 expresses $His_6$-SUMO-FLAG-SpbR$^{K104C}$ and $His_6$-SpsB.
**pYS047**
To construct plasmid pYS047, cysteine mutation was introduced on SpbR using SDM. The gene sequence containing the desired cysteine mutation was amplified from pMS52 by PCR using primers oYS205 and oYS206, and PCR products were purified by DNA agarose gel. The template plasmid was digested by Dpn1, and the PCR products were ligated by directly transforming into Stella cells. pYS047 expresses $His_6$-SUMO-FLAG-SpbR$^{W103C}$ and $His_6$-SpsB.
**pYS049**
To construct plasmid pYS049, cysteine mutation was introduced on SpbR using SDM. The gene sequence containing the desired cysteine mutation was amplified from pMS52 by PCR using primers oYS213 and oYS214, and PCR products were purified by DNA agarose gel. The template plasmid was digested by Dpn1, and the PCR products were ligated by directly transforming into Stella cells. pYS049 expresses $His_6$-SUMO-FLAG-SpbR$^{I197C}$ and $His_6$-SpsB.
**pYS062**
To construct plasmid pYS062, mismatched cysteine mutations were introduced on SpbR and SpsB using SDM. The gene sequences containing the desired cysteine mutations were amplified from pMS52 by PCR using primers oYS201 and oYS202, and oYS213 and oYS214, and PCR products were purified by DNA agarose gel. Two gene fragments were ligated by in-Fusion. pYS062 expresses $His_6$-SUMO-FLAG-SpbR$^{K104C}$ and $His_6$-SpsB$^{I16C}$.
**pYS058**
To construct plasmid pYS058 for IsaA expression and purification, the gene sequence for *isaA* was amplified from RN4220 gDNA by PCR using oYS229 and oYS230. The plasmid backbone was amplified from pET22b by PCR using primers oYS191 and oYS231. The two PCR products were ligated by in-Fusion. The resulting pYS058 expresses $His_6$-IsaA-FLAG.

**Strain constructions**

Marked deletions and plasmids were first introduced into RN4220 by electroporation, then transferred into the HG003 strain background via Φ85 phage transduction. For protein expression and purification in *E. coli*, plasmids carrying the target genes were introduced into Stellar competent cells and subsequently into C43 strains. Colony PCR was used to verify the genotypes of the strains.

To construct *S. aureus* strains that overexpress SpbR variants in the *ΔspbR* background, the desired pLOW plasmid was first introduced into RN4220 and then phage transduction was used to introduce it into the HG003 Δ*spbR* mutant strain SW0201. Using this approach, strains MS49, MS85, MS87, MS111, MS120, MS141, MS143, MS144, MS145, MS147, and MS148 were generated.

To construct a strain expressing LtaS-FLAG from its native genomic site, a one-step gene deletion method using PCR product transformation, originally developed for *E. coli*, was modified for application in *S. aureus* (1). Electrocompetent cells were made of the *S. aureus* RN4220-derived strain Sa07 which contains plasmid pTW07 (2). This plasmid encodes ATc-inducible homologs of the λ-Red β and Exo protein, consisting of a RecT family single-stranded DNA-annealing protein from *Clostridium* sp. FS41 (NCBI Protein: WP_152649059) and a prophage exonuclease from the PD-(D/E)XK superfamily from the same organism (NCBI Protein: WP_045093217). These two genes were cloned into a plasmid backbone derived from pRAB11 (3). Homology arms were designed to flank the *ltaS* stop codon in the genome, allowing for the insertion of a FLAG sequence just upstream of the stop codon. PCR products containing 50 bp homology arms flanking the FLAG tag, the *ltaS* stop codon, and a KanR resistance marker were amplified from the plasmid pJP60 using primers oMS284, oMS285, oMS302, and oMS303. A total of 1 µg of the PCR product was electroporated into competent Sa07 cells, followed by recovery in TSB and plating on TSA plates containing Kan/Neo. The plates were incubated at 30 °C. Confirmed mutants were subsequently transduced by phage into HG003 WT, SW0201, and MS49 strains to generate stain MS277, MS278, and MS279, respectively.

To construct a strain expressing IsaA-FLAG or SsaA-FLAG from its native site in the genome, the one-step gene replacement method involving PCR product transformation described earlier was used with modifications to the design of the PCR product. For IsaA-FLAG, PCR products containing 50 bp homology arms flanking FLAG, the *isaA* stop codon, and a KanR resistance marker were amplified from plasmid pJP60 using oTS487 and oTS488. This PCR product was then amplified to extend the homology arms using primers oTS508 and oTS509. The PCR product was electroporated into Sa07 cells as described above and phage transduced into HG003 WT, SW0201, and MS49 to generate strains TAS399, TAS400, and TAS401. For SsaA-FLAG, PCR products containing 50 bp homology arms flanking *FLAG* the *ssaA* stop codon, and a KanR resistance marker were amplified from plasmid pJP60 using oTS252 and oTS253. The PCR product was electroporated into Sa07 competent cells as described earlier and phage transduced into the HG003 WT, SW0201, and MS49 to generate strains TAS216, TAS376, and TAS377.

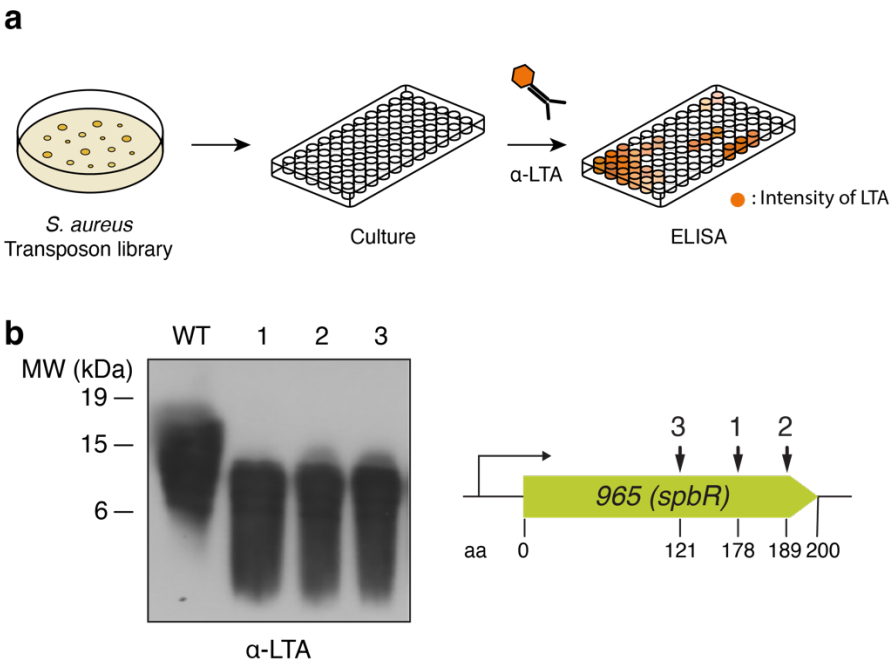



**Figure S1. SpbR was identified in a whole-cell ELISA screen as factor modulating LTA levels in *S. aureus*.**

a. Schematic showing the experimental procedure for the detection of LTA levels in different *S. aureus* transposon mutants using a whole-cell ELISA method.

b. Immunoblot showing the LTA length of three mutant strains identified in the ELISA screen based on a decrease in signal (Left). The transposon insertion was mapped to *SAOUHSC_00965* (*spbR*) in all three mutants (Right).

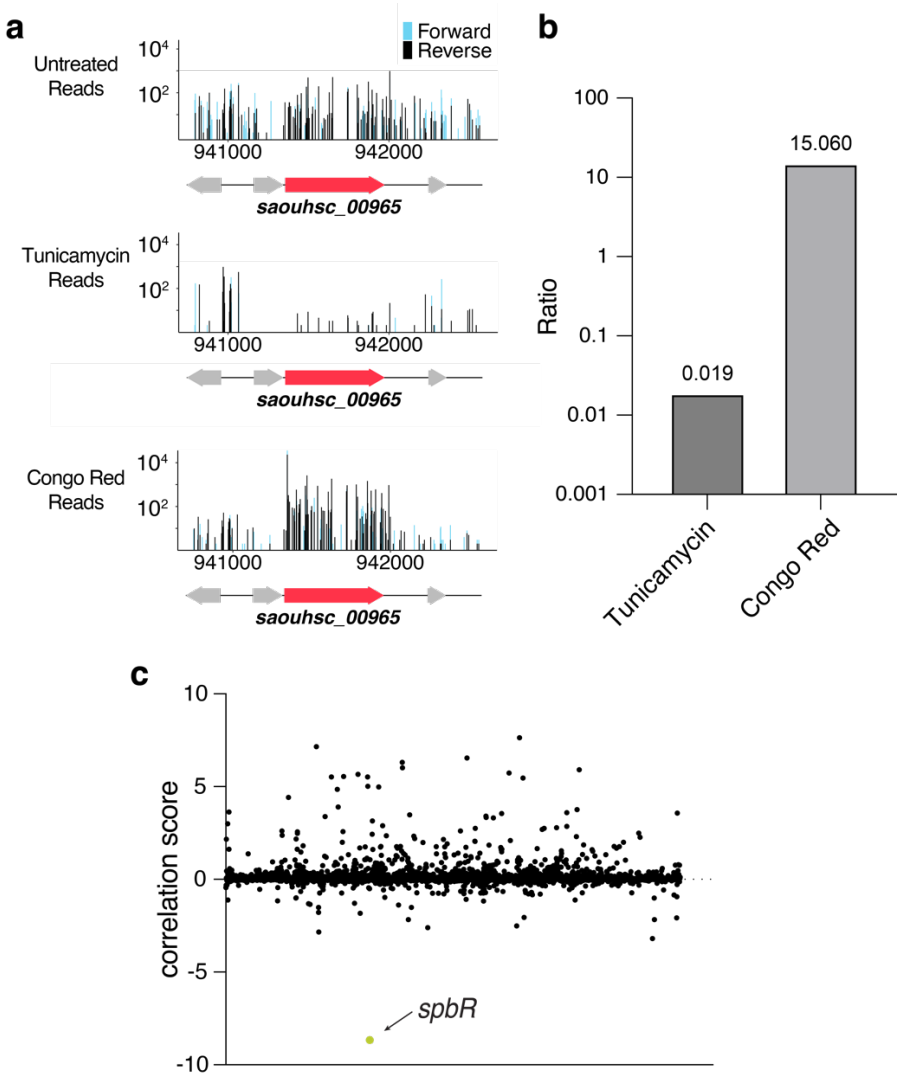



**Figure S2. *spbR* showed an anti-correlated pattern upon tunicamycin and Congo Red treatment.**

a. A plot showing the number of transposon insertions in *spbR* in both the forward and reverse directions. The reads in untreated cells serve as the control and were matched to the reads of tunicamycin or Congo red treated cultures. For simplicity, only matched untreated control of the tunicamycin sample is shown.

b. Following tunicamycin treatment, reads mapping to *SAOUHSC_00965 (spbR)* were significantly depleted compared to the untreated control (ratio = 0.019, q-value = $4.84 \times 10^{-16}$). After Congo red treatment, reads were significantly enriched compared to the untreated control (ratio = 15.060, q- value = $7.84 \times 10^{-9}$).

c. Plot showing correlation scores for each gene. Enrichment and depletion ratios from Figure 1b were log-transformed then multiplied to yield the correlation score (Y-axis). Genes are plotted numerically by locus tag (X-axis). More negative values indicate stronger anti-correlation.

| | % D-Ala | # GroP |
|---|---|---|
| WT | 74.74 ± 3.17 | 32.53 ± 2.76 |
| *spbR::Tn* | 79.31± 0.78 | 19.80 ± 1.88 |

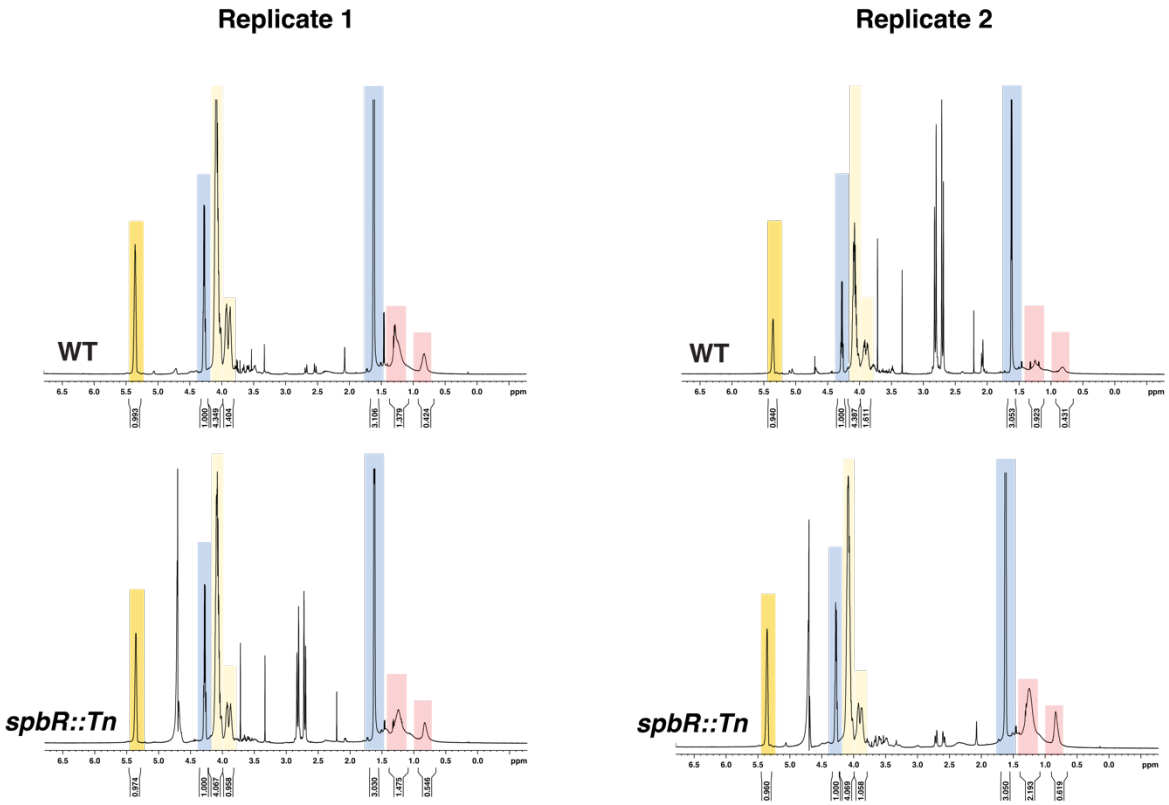



**Figure S3. Analysis of $^{1}$H-NMR spectra shows that *spbR::Tn* mutants produce shorter LTAs than WT, while their D-alanylation levels remain similar.**
The table provides mean ± S.D. for the % D-alanylation and the number of GroP units in LTA for WT and *spbR::Tn*, based on the analysis of three independent spectra obtained from separate LTA extractions. Shown are two independent $^{1}$H-NMR spectra of LTA extracted from wildtype *S. aureus* (top) and *spbR::Tn* (bottom), in addition to the spectra set presented in Fig. 1f. Selected NMR signals are color coded to correspond to the protons shown in Fig. 1e, and the integration values are shown below the chemical shift axis.

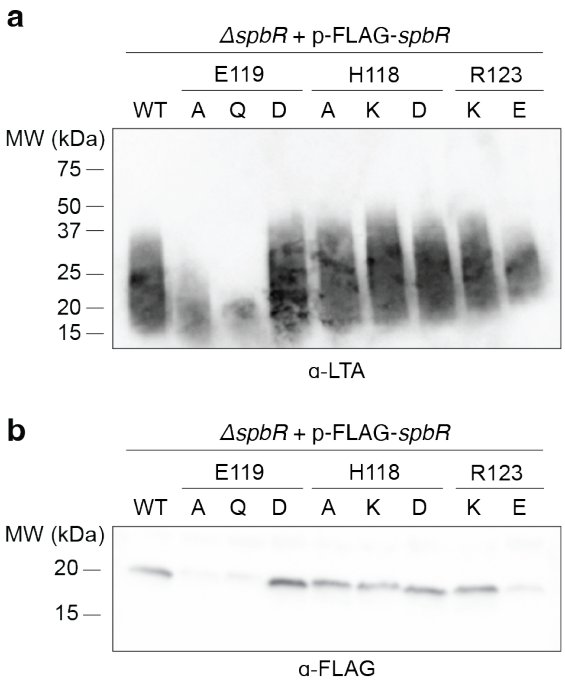



**Figure S4. Conserved CAAX protease residues in SpbR are not required for LTA length modulation.**

a. Immunoblot showing the LTA signal for strains expressing SpbR variants in the *ΔspbR* background strain. The SpbR variants are mutated for residues in the conserved CAAX protease motif shown in Fig. 2a.

b. Immunoblot showing the expression level of the SpbR variants in the strains used in (a).

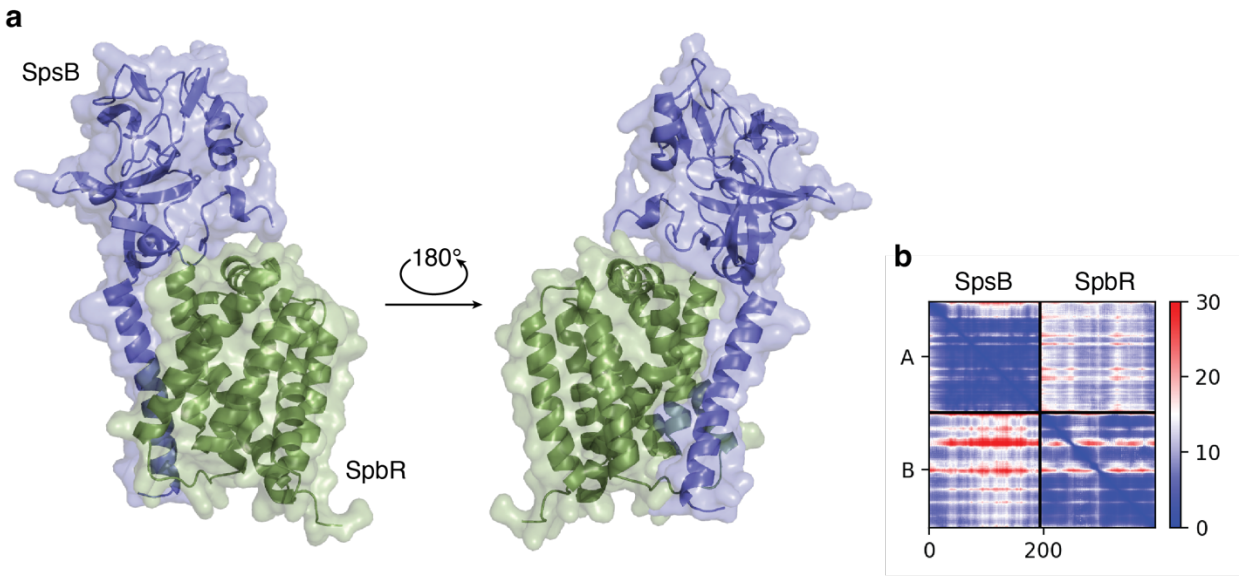



**Figure S5. AlphaFold2 predicted structure of the *S. aureus* SpsB-SpbR complex.**

a. The top-ranked AlphaFold2 model of the *S. aureus* SpsB-SpbR complex, with proteins colored as in Fig. 2d. The predicted local Distance Difference Test (plDDT) is a metric of model confidence, with plDDT values > 90 representing very high confidence, 70–90 representing moderate confidence. The pLDDT of the predicted structure is 83.24, representing moderate confidence. The predicted template modelling (pTM) score is an integrated measure of how well AlphaFold has predicted the overall structure of the complex. The pTM score above 0.5 means the overall predicted fold for the complex might be similar to the true structure. The pTM score below 0.5 means the predicted structure is likely wrong. The pTMscore of the predicted structure is 0.72, implying that the complex structure might be similar to the true structure.

b. The relative positions of the two proteins are reliable based on the Predicted Aligned Error (PAE). In the PAE graphs, the value at each (x, y) coordinate represents the expected position error at residue x if the predicted and true structures were aligned on residue y. Low values (blue) indicate a low expected position error. The x-axis numbers correspond to the residue numbers of the concatenated protein complex sequence in the order of SpsB and SpbR, while the y-axis would start at '0' at the top.

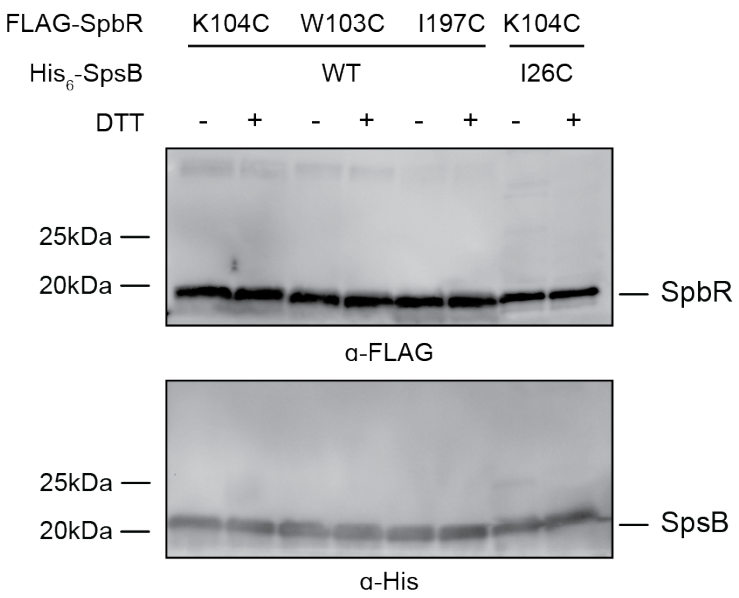



**Figure S6. SpbR with a cysteine mutation is unable to form a disulfide bond with SpsB unless SpsB also has a matching cysteine mutation.**
Immunoblot analysis of FLAG-SpbR with cysteine mutations and $His_6$-SpsB without matching cysteine mutations. $SpbR^{W104C}$, $SpbR^{W103C}$, and $SpbR^{I197C}$, which were confirmed to form disulfide bonds with SpsB through their matching cysteine mutations in Fig. 2f, were co-purified with wild-type SpsB. In the fourth lane, $SpbR^{W104C}$ was co-purified with $SpsB^{I26C}$, a mismatching mutant. The indicated SpbR and SpsB variants were co-expressed in *E. coli* and purified on anti-FLAG column following a 1 hour treatment with copper(II) phenanthroline. Samples were prepared for SDS-PAGE with and without DTT.

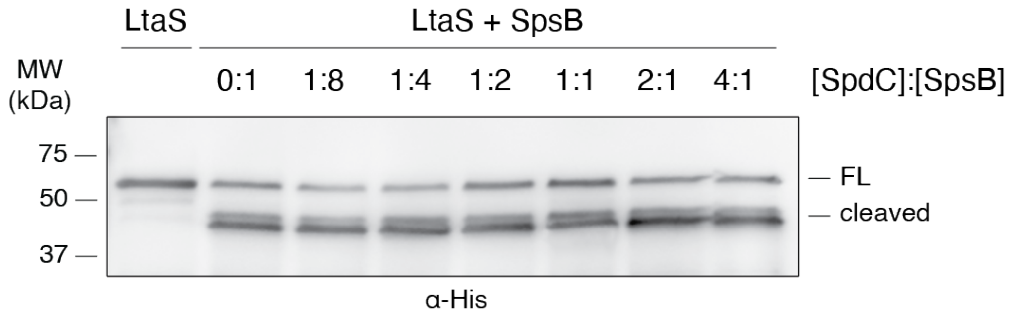



**Figure S7. SpdC, another CAAX protease homolog in *S. aureus*, does not affect LtaS cleavage.**
*In vitro* cleavage of $His_6$-LtaS by SpsB (1:4 ratio of LtaS:SpsB) in the presence of increasing amounts of SpdC. Reactions were incubated for 1 hour at 30 ˚C and analyzed on an α-His immunoblot.

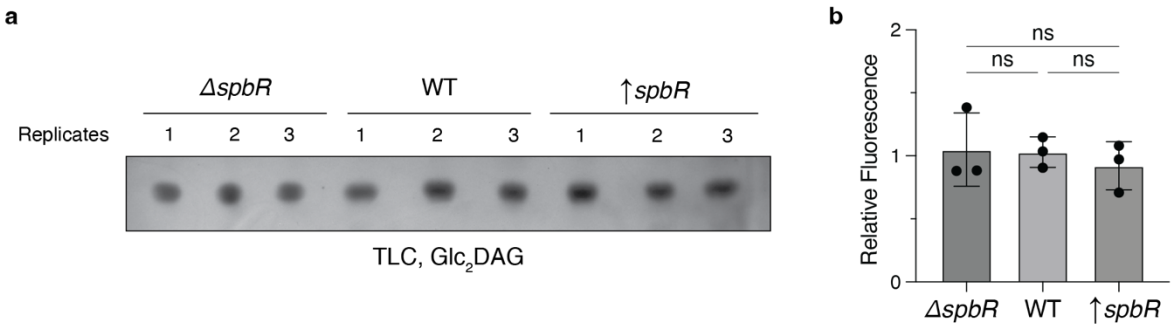



**Figure S8. SpbR does not impact the level of $Glc_2DAG$ in *S. aureus*.**
TLC analysis and quantification of the amount of $Glc_2DAG$ in total lipid extracts derived from the indicated *S. aureus* strains (n = 3, mean ± S.D. ns: not statistically significant, by 1-way ANOVA).

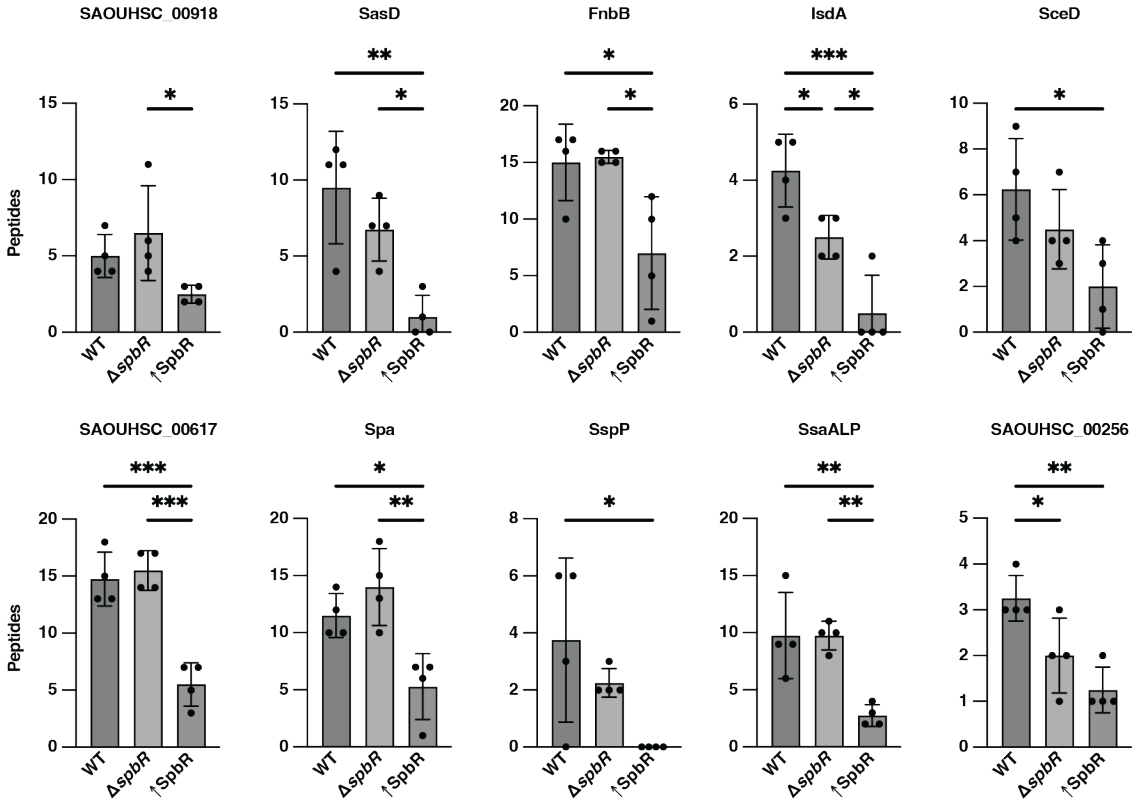



**Figure S9. Overexpression of SpbR decreases the secretion levels of proteins containing signal peptides.**

Peptide counts for ten proteins with signal peptides were plotted based on mass spectrometry data. Individual values are plotted mean ± 1 standard deviation from the mean Significance was determined using a one-way ANOVA followed by a Tukey's post-hoc analysis. ✱ = $p < 0.05$; ✱✱ = $p < 0.01$, ✱✱✱ = $p < 0.005$. SsaALP stands for SsaA-like protein (i.e. SAOUHSC_00671). In addition to their N-terminal signal peptides, Spa, FnbB, SceD, and IsdA contain LPxTG sequences near their C-termini for attachment to the cell wall. These were likely shed into the supernatant.

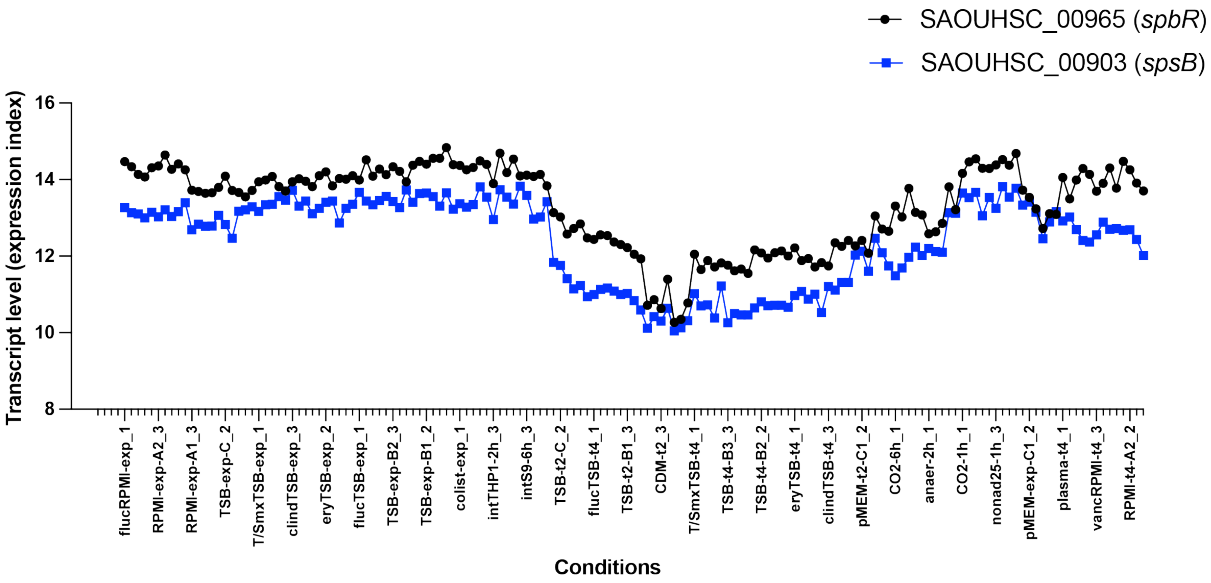



**Figure S10. Expression of *spbR* and *spsB* are highly correlated under various conditions**
Expression profiles of spbR and spsB under various conditions (4). The x-axis represents the expression condition, and the y-axis represents the transcript level of the gene of interest.

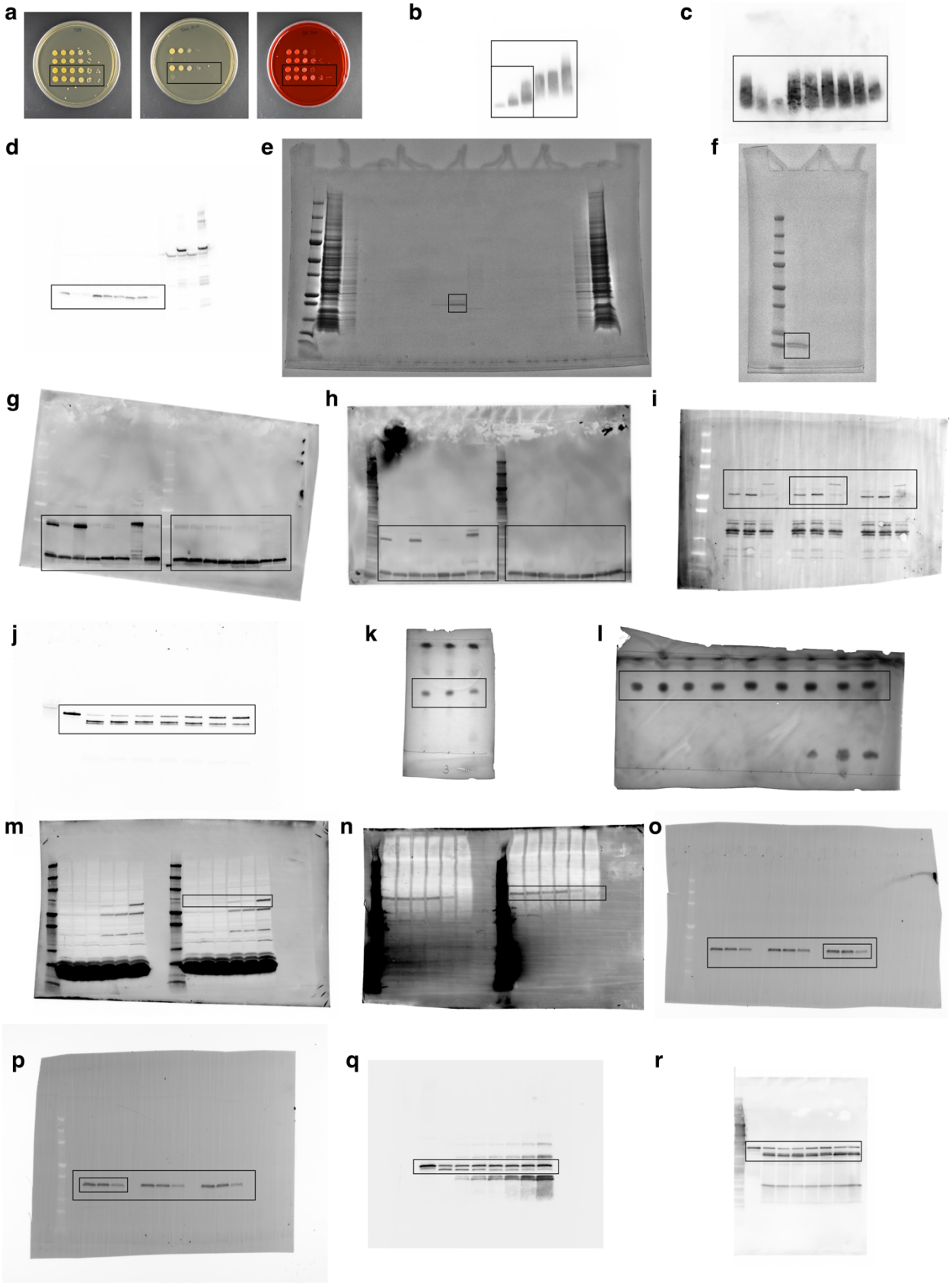

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**Figure S11. Full images used in this paper.**

a. Full image from Fig. 1c.
b. Full images from Fig. 1d (small box) and Fig. 3e (large box).
c. Full image from Fig. S4a.
d. Full image from Fig. S4b.
e. Full image from Fig. 2c.
f. Full image from Fig. 2d.
g. Top images of Fig. 2f and Fig. S6.
h. Bottom images of Fig. 2f and Fig. S6.
i. Full image from Fig. 3b (large box indicating triplicates).
j. Full image from Fig. 3c.
k. Full image from Fig. 3d.
l. Full image from Fig. S8.
m. Middle image of Fig. 3e.
n. Bottom image of Fig. 3e.
o. Full image from Fig. 4a (large box indicating triplicates).
p. Full image from Fig. 4b (large box indicating triplicates).
q. Full image from Fig. 4c.
r. Full image from Fig. S7.

**Notes**: Black boxes indicate the cropped regions shown in the respective figures or the areas used for statistical analysis of triplicated result.

**Table S1.** Peptides identified in the lower band in co-immunoprecipitation experiment with FLAG-SpbR as bait. DSP crosslinker was used in this experiment. The lower band in Fig. 2c was excised and peptides were identified by mass spectrometry. The highlighted genes are the ones examined in this study.

| Unique | Total | Reference | Gene | MW (kDa) | Coverage |
|---|---|---|---|---|---|
| 2 | 19 | Q2FZN5_Q2FZN5_STAA8 | *SAOUHSC_00965* | 23.29 | 7.50% |
| 10 | 12 | Q2FZT7_Q2FZT7_STAA8 | *spsB* | 17.59 | 51% |
| 6 | 6 | Q2FY84_Q2FY84_STAA8 | *SAOUHSC_01580* | 23.81 | 31.40% |
| 5 | 5 | Q2FW66_ASP23_STAA8 | *asp23* | 19.18 | 21.30% |
| 4 | 4 | Q2G247_Y1855_STAA8 | *SAOUHSC_01855* | 17.99 | 32.50% |
| 4 | 4 | Q2FYL0_Q2FYL0_STAA8 | *SAOUHSC_01430* | 17.95 | 35.50% |
| 4 | 4 | Q2FWE7_ATPD_STAA8 | *atpH* | 20.49 | 21.80% |
| 4 | 4 | Q2FW64_Q2FW64_STAA8 | *SAOUHSC_02443* | 20.79 | 14.60% |
| 3 | 3 | Q2FXL6_Y1819_STAA8 | *SAOUHSC_01819* | 18.46 | 24.10% |
| 3 | 3 | P48940_RS7_STAA8 | *rpsG* | 17.78 | 28.80% |
| 3 | 3 | Q2FXS8_RL21_STAA8 | *rplU* | 11.33 | 37.30% |
| 3 | 3 | Q2FW23_RS5_STAA8 | *rpsE* | 17.73 | 30.10% |
| 3 | 3 | Q2FVL3_Q2FVL3_STAA8 | *SAOUHSC_02698* | 26.43 | 14.20% |
| 1 | 3 | Q2G1K8_Q2G1K8_STAA8 | *SAOUHSC_00114* | 24.84 | 5.40% |
| 2 | 2 | Q2FYK0_Q2FYK0_STAA8 | *SAOUHSC_01440* | 26.01 | 11.10% |
| 2 | 2 | Q2G2R9_Y2131_STAA8 | *SAOUHSC_02131* | 22.94 | 9% |
| 2 | 2 | Q2FXH8_Q2FXH8_STAA8 | *SAOUHSC_01869* | 15.72 | 15% |
| 2 | 2 | Q2FXQ1_RL20_STAA8 | *rplT* | 13.68 | 16.10% |
| 2 | 2 | Q2FW11_RL22_STAA8 | *rplV* | 12.83 | 17.90% |
| 2 | 2 | Q2FYV8_Q2FYV8_STAA8 | *SAOUHSC_01316* | 20.32 | 10.20% |
| 2 | 2 | Q2FZ29_HSLV_STAA8 | *hslV* | 19.56 | 11% |
| 2 | 2 | Q2FW07_RL4_STAA8 | *rplD* | 22.45 | 10.10% |
| 2 | 2 | Q2FVD0_Y2783_STAA8 | *SAOUHSC_02783* | 30.78 | 6.10% |
| 2 | 2 | Q2FW33_RL17_STAA8 | *rplQ* | 13.74 | 19.70% |
| 1 | 2 | Q2FW38_RL13_STAA8 | *rplM* | 16.32 | 8.30% |
| 1 | 1 | Q2FVC5_Y2788_STAA8 | *SAOUHSC_02788* | 30.31 | 5% |
| 1 | 1 | Q2FW31_RS11_STAA8 | *rpsK* | 13.87 | 10.10% |
| 1 | 1 | Q2FW14_RL29_STAA8 | *rpmC* | 8.09 | 18.80% |
| 1 | 1 | Q2FW18_RL5_STAA8 | *rplE* | 20.25 | 8.40% |
| 1 | 1 | Q2FXT7_Q2FXT7_STAA8 | *SAOUHSC_01747* | 9.67 | 12.80% |
| 1 | 1 | Q2FXP9_IF3_STAA8 | *infC* | 20.2 | 6.90% |
| 1 | 1 | Q2FYS6_PLSY_STAA8 | *plsY* | 22.25 | 6.90% |
| 1 | 1 | Q2FYZ0_Q2FYZ0_STAA8 | *SAOUHSC_01282* | 18.11 | 8.20% |
| 1 | 1 | P0A0F8_RL15_STAA8 | *rplO* | 15.59 | 8.20% |
| 1 | 1 | Q2FXZ9_Y1676_STAA8 | *SAOUHSC_01676* | 35.16 | 4% |
| 1 | 1 | Q2FVC4_Y2789_STAA8 | *SAOUHSC_02789* | 30.29 | 4.30% |
| 1 | 1 | Q2G2G7_Y1054_STAA8 | *SAOUHSC_01054* | 24 | 4.90% |
| 1 | 1 | Q2G1Q1_Y052_STAA8 | *SAOUHSC_00052* | 29.64 | 3.50% |

**Table S1** (Continued).

| Unique | Total | Reference | Gene | MW (kDa) | Coverage |
|---|---|---|---|---|---|
| 1 | 1 | P0A0G2_RL30_STAA8 | *rpmD* | 6.55 | 23.70% |
| 1 | 1 | Q2FW39_RS9_STAA8 | *rpsI* | 14.82 | 7.60% |
| 1 | 1 | Q2FW08_RL23_STAA8 | *rplW* | 10.6 | 11% |
| 1 | 1 | Q2G0N0_EFTU_STAA8 | *tuf* | 43.08 | 3% |
| 1 | 1 | Q2G2A4_Q2G2A4_STAA8 | *SAOUHSC_01042* | 46.33 | 2.80% |
| 1 | 1 | Q2G1P9_Y054_STAA8 | *SAOUHSC_00054* | 29.56 | 3.50% |
| 1 | 1 | Q2G257_Q2G257_STAA8 | *SAOUHSC_01477* | 24.57 | 4.90% |
| 1 | 1 | Q2FWA5_Q2FWA5_STAA8 | *SAOUHSC_02393* | 44.69 | 3.70% |
| 1 | 1 | Q2G1I7_Q2G1I7_STAA8 | *SAOUHSC_00135* | 19.24 | 6.50% |
| 1 | 1 | P0A0B7_AHPC_STAA8 | *ahpC* | 20.96 | 6.30% |
| 1 | 1 | Q2FYT7_Q2FYT7_STAA8 | *SAOUHSC_01338* | 11.02 | 10.50% |
| 1 | 1 | Q2FZH7_Q2FZH7_STAA8 | *SAOUHSC_01027* | 20.08 | 6.10% |

**Table S2.** Peptides identified in the upper band in a co-immunoprecipitation experiment with FLAG-SpbR as bait. DSP crosslinker was used in this experiment. The upper band in Fig. 2c was excised and peptides were identified by mass spectrometry. The highlighted genes are the ones examined in this study.

| Unique | Total | Reference | Gene | MW (kDa) | Coverage |
|---|---|---|---|---|---|
| 9 | 10 | Q2FW18_RL5_STAA8 | *rplE* | 20.25 | 46.90% |
| 9 | 9 | Q2FX90_Q2FX90_STAA8 | *SAOUHSC_01987* | 22.33 | 35.20% |
| 5 | 5 | Q2FW66_ASP23_STAA8 | *asp23* | 19.18 | 21.90% |
| 5 | 5 | Q2FW21_RL6_STAA8 | *rplF* | 19.77 | 28.10% |
| 5 | 5 | Q2FXJ7_Q2FXJ7_STAA8 | *SAOUHSC_01837* | 23.06 | 23.90% |
| 4 | 4 | Q2FWE7_ATPD_STAA8 | *atpH* | 20.49 | 21.80% |
| 4 | 4 | P0A0B7_AHPC_STAA8 | *ahpC* | 20.96 | 28.60% |
| 4 | 4 | Q2G0Z9_Q2G0Z9_STAA8 | *SAOUHSC_00362* | 23.65 | 21.60% |
| 4 | 4 | Q2FYM8_Q2FYM8_STAA8 | *SAOUHSC_01407* | 24.69 | 15.80% |
| 3 | 3 | Q2FZT7_Q2FZT7_STAA8 | *spsB* | 17.59 | 21.90% |
| 3 | 3 | Q2G247_Y1855_STAA8 | *SAOUHSC_01855* | 17.99 | 23.30% |
| 3 | 3 | Q2FW64_Q2FW64_STAA8 | *SAOUHSC_02443* | 20.79 | 14% |
| 3 | 3 | Q2G105_Q2G105_STAA8 | *SAOUHSC_00356* | 21.29 | 15.30% |
| 3 | 3 | Q2FY84_Q2FY84_STAA8 | *SAOUHSC_01580* | 23.81 | 15.70% |
| 3 | 3 | Q2FXZ8_Q2FXZ8_STAA8 | *SAOUHSC_01677* | 24.99 | 11.50% |
| 3 | 3 | Q2FVL3_Q2FVL3_STAA8 | *SAOUHSC_02698* | 26.43 | 14.20% |
| 3 | 3 | Q2FVG1_Q2FVG1_STAA8 | *SAOUHSC_02753* | 27.88 | 11.20% |
| 2 | 2 | Q2FXS8_RL21_STAA8 | *rplU* | 11.33 | 26.50% |
| 2 | 2 | Q2FW11_RL22_STAA8 | *rplV* | 12.83 | 17.90% |
| 2 | 2 | Q2FXQ1_RL20_STAA8 | *rplT* | 13.68 | 16.10% |
| 2 | 2 | Q2FW33_RL17_STAA8 | *rplQ* | 13.74 | 15.60% |
| 2 | 2 | Q2FXL6_Y1819_STAA8 | *SAOUHSC_01819* | 18.46 | 13.30% |
| 2 | 2 | Q2FZ77_PYRR_STAA8 | *pyrR* | 19.84 | 12% |
| 2 | 2 | Q2FXP9_IF3_STAA8 | *infC* | 20.2 | 13.70% |
| 2 | 2 | Q2G1F1_Q2G1F1_STAA8 | *SAOUHSC_00174* | 21.92 | 8.90% |
| 2 | 2 | Q9RQQ0_ICAR_STAA8 | *icaR* | 21.97 | 12.90% |
| 1 | 2 | Q2FVI3_Q2FVI3_STAA8 | *SAOUHSC_02727* | 22.27 | 5.80% |
| 2 | 2 | Q2FW07_RL4_STAA8 | *rplD* | 22.45 | 10.10% |
| 2 | 2 | Q2G2R9_Y2131_STAA8 | *SAOUHSC_02131* | 22.94 | 9% |
| 2 | 2 | Q2FZN5_Q2FZN5_STAA8 | *SAOUHSC_00965* | 23.29 | 7.50% |
| 2 | 2 | Q2G1K8_Q2G1K8_STAA8 | *SAOUHSC_00114* | 24.84 | 10.80% |
| 1 | 2 | Q2G2F4_ATP6_STAA8 | *atpB* | 27.61 | 4.10% |
| 2 | 2 | Q2FVC5_Y2788_STAA8 | *SAOUHSC_02788* | 30.31 | 7.30% |
| 2 | 2 | Q2FVD0_Y2783_STAA8 | *SAOUHSC_02783* | 30.78 | 5.30% |
| 2 | 2 | Q2FW36_Q2FW36_STAA8 | *ecfT* | 30.81 | 8.60% |
| 2 | 2 | Q2FXZ9_Y1676_STAA8 | *SAOUHSC_01676* | 35.16 | 7% |
| 1 | 1 | P0A0G2_RL30_STAA8 | *rpmD* | 6.55 | 23.70% |
| 1 | 1 | Q2FW14_RL29_STAA8 | *rpmC* | 8.09 | 18.80% |

**Table S2** (Continued).

| Unique | Total | Reference | Gene | MW (kDa) | Coverage |
|---|---|---|---|---|---|
| 1 | 1 | Q2FXT7_Q2FXT7_STAA8 | *SAOUHSC_01747* | 9.67 | 12.80% |
| 1 | 1 | Q2FW08_RL23_STAA8 | *rplW* | 10.6 | 11% |
| 1 | 1 | Q2FW17_RL24_STAA8 | *rplX* | 11.53 | 9.50% |
| 1 | 1 | Q2FW31_RS11_STAA8 | *rpsK* | 13.87 | 10.10% |
| 1 | 1 | Q2FW39_RS9_STAA8 | *rpsI* | 14.82 | 9.80% |
| 1 | 1 | P0A0F8_RL15_STAA8 | *rplO* | 15.59 | 8.20% |
| 1 | 1 | Q2FXH8_Q2FXH8_STAA8 | *SAOUHSC_01869* | 15.72 | 9.30% |
| 1 | 1 | Q2G0N9_RL10_STAA8 | *rplJ* | 17.7 | 6.60% |
| 1 | 1 | Q2FW23_RS5_STAA8 | *rpsE* | 17.73 | 14.50% |
| 1 | 1 | P48940_RS7_STAA8 | *rpsG* | 17.78 | 7.70% |
| 1 | 1 | Q2G252_RLMH_STAA8 | *rlmH* | 18.29 | 5.70% |
| 1 | 1 | Q2FZH7_Q2FZH7_STAA8 | *SAOUHSC_01027* | 20.08 | 6.10% |
| 1 | 1 | Q2G0P2_NUSG_STAA8 | *nusG* | 20.65 | 5.50% |
| 1 | 1 | Q2FWD0_RPOE_STAA8 | *rpoE* | 20.87 | 7.40% |
| 1 | 1 | Q2FYG4_Q2FYG4_STAA8 | *SAOUHSC_01488* | 22.17 | 6.30% |
| 1 | 1 | Q2G2M7_Q2G2M7_STAA8 | *SAOUHSC_00510* | 23.74 | 4.70% |
| 1 | 1 | Q2G2G7_Y1054_STAA8 | *SAOUHSC_01054* | 24 | 4.90% |
| 1 | 1 | Q2FW12_RS3_STAA8 | *rpsC* | 24.09 | 3.70% |
| 1 | 1 | Q2G0H4_Q2G0H4_STAA8 | *SAOUHSC_00592* | 25.56 | 4.70% |
| 1 | 1 | Q2FWX0_Q2FWX0_STAA8 | *SAOUHSC_02151* | 28.12 | 2.40% |
| 1 | 1 | Q2FZ25_RS2_STAA8 | *rpsB* | 29.08 | 4.70% |
| 1 | 1 | Q2G1P9_Y054_STAA8 | *SAOUHSC_00054* | 29.56 | 3.50% |
| 1 | 1 | Q2G2E2_Q2G2E2_STAA8 | *SAOUHSC_00651* | 29.9 | 2.90% |
| 1 | 1 | Q2FVC4_Y2789_STAA8 | *SAOUHSC_02789* | 30.29 | 4.30% |
| 1 | 1 | Q2G2A3_Q2G2A3_STAA8 | *SAOUHSC_01043* | 49.45 | 2.40% |
| 1 | 1 | Q2FWF0_ATPB_STAA8 | *atpD* | 51.37 | 2.30% |
| 1 | 1 | Q2G093_LTAS_STAA8 | *ltaS* | 74.35 | 2.20% |
| 1 | 1 | Q2G0P5_CLPC_STAA8 | *clpC* | 90.98 | 1.60% |
| 1 | 1 | Q2FZS8_Q2FZS8_STAA8 | *SAOUHSC_00912* | 98.27 | 1.20% |

**Table S3.** Strains used in this study.

| Strain | Description | Source or Reference |
|---|---|---|
| Stellar (HST08) | *E. coli* host strain for plasmid cloning | Takara bio |
| C43(DE3) | *E. coli* BL21(DE3) derivative for protein expression | (5) |
| RN4220 | *S. aureus* laboratory strain for phage transduction | (6) |
| HG003 | *S. aureus* NCTC8325 derivative laboratory strain | (7) |
| Sa07 | *S. aureus* RN4220 pTW07 for recombineering | Tim Wannier |
| SW0201 | *S. aureus* HG003 Δ*spbR::FRT* | (8) |
| MS49 | S. aureus HG003 Δ*spbR::FRT* pMS7 | This study |
| MS85 | S. aureus HG003 Δ*spbR::FRT* pMS12 | This study |
| MS87 | S. aureus HG003 Δ*spbR::FRT* pMS16 | This study |
| MS111 | *S. aureus* HG003 Δ*spbR::FRT* pMS18 | This study |
| MS120 | *S. aureus* HG003 Δ*spbR::FRT* pMS24 | This study |
| MS141 | *S. aureus* HG003 Δ*spbR::FRT* pMS26 | This study |
| MS143 | *S. aureus* HG003 Δ*spbR::FRT* pMS28 | This study |
| MS144 | *S. aureus* HG003 Δ*spbR::FRT* pMS29 | This study |
| MS145 | *S. aureus* HG003 Δ*spbR::FRT* pMS30 | This study |
| MS147 | *S. aureus* HG003 Δ*spbR::FRT* pMS32 | This study |
| MS148 | *S. aureus* HG003 Δ*spbR::FRT* pMS33 | This study |
| SW0044 | *S. aureus* HG003 Δ*ugtP*::KanR | (9) |
| MS102 | *S. aureus* HG003 Δ*spbR::FRT* Δ*ugtP*::KanR | This study |
| MS182 | *S. aureus* HG003 Δ*spbR::FRT* Δ*ugtP*::KanR pMS7 | This study |
| MS277 | *S. aureus* HG003 *ltaS-flag::KanR* | This study |
| MS278 | *S. aureus* HG003 Δ*spbR::FRT ltaS-flag::KanR* | This study |
| MS279 | *S. aureus* HG003 Δ*spbR::FRT ltaS-flag::KanR* pMS7 | This study |
| TAS399 | *S. aureus* HG003 *isaA-flag::KanR* | This study |
| TAS400 | *S. aureus* HG003 *ΔspbR::FRT isaA-flag::KanR* | This study |
| TAS401 | *S. aureus* HG003 *ΔspbR::FRT isaA-flag::KanR* pMS7 | This study |
| TAS216 | S. aureus HG003 *ssaA-flag::KanR* | This study |
| TAS376 | S. aureus HG003 *ΔspbR::FRT ssaA-flag::KanR* | This study |
| TAS377 | S. aureus HG003 *ssaA-flag::KanR* pMS7 | This study |
| ARH255 | E. coli C43 pET28b *ltaS*-$His_6$ | (10) |
| KS05 | E. coli C43 pETDuet $His_6$-SUMO-FLAG-*spdC* | (11) |
| ANG314 | *S. aureus* RN4220 *Δspa* | (12) |
| ANG403 | ANG314 with transposon insertion in *SAOUHSC_00965* | This study |

**Table S3** (Continued).

| Strain | Description | Source or Reference |
|---|---|---|
| ANG415 | ANG314 with transposon insertion in *SAOUHSC_00965* | This study |
| ANG416 | ANG314 with transposon insertion in *SAOUHSC_00965* | This study |
| ANG448 | ANG448 with transposon insertion in *SAOUHSC_02858* (constrol strain) | This study |

**Table S4.** Plasmids used in this study.

| Plasmid | Description | Source or Reference |
|---|---|---|
| pTP63 | integrative vector for ATc-inducible expression in *S. aureus; CamR* | (13) |
| pLOW | low-copy vector for IPTG-inducible expression in *S. aureus; ErmR* | (14) |
| pET22b | high-copy vector for IPTG-inducible expression in *E. coli; CarbR* | Novagen |
| pMS7 | pLOW vector expressing the *rpoB* RBS and *spbR* | This study |
| pMS12 | pLOW vector expressing the *rpoB* RBS and *flag-spbR* | This study |
| pMS16 | pLOW vector expressing the *rpoB* RBS and *flag-gfp* | This study |
| pMS18 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(E119A)* | This study |
| pMS24 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(E119Q)* | This study |
| pMS26 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(E119D)* | This study |
| pMS28 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(H118A)* | This study |
| pMS29 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(H118K)* | This study |
| pMS30 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(H118D)* | This study |
| pMS32 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(R123K)* | This study |
| pMS33 | pLOW vector expressing the *rpoB* RBS and *flag-spbR(R123E)* | This study |
| pTW07 | vector encoding ATc-inducible homologs of λ-Red β and *exo*: a RecT family single-stranded DNA-annealing protein from *Clostridium* sp. FS41 (NCBI Protein: WP_152649059) and the PD-(D/E)XK superfamily prophage exonuclease from the same organism (NCBI Protein: WP_045093217) | (2) |
| pJP60 | Vector containing KanR flanked by FRT sites | Julia Page |
| pTM186 | Vector containing *GFPmut2* sequence | (15) |
| pAM205 | vector containing $His_6$-SUMO-FLAG sequence; CarbR | (16) |
| pAM174 | vector expressing Ulp1 protease; CamR | (16) |
| pET28b(+) | high-copy vector for IPTG-inducible expression in *E. coli*; KanR | Novagen |
| pETDuet | high-copy vector for IPTG-inducible expression in *E. coli*; CarbR | Novagen |
| pMS50 | pAM205 vector expressing $His_6$-SUMO-FLAG-SpbR | This study |
| pMS46 | pET28b(+) vector expressing $His_6$-SpsB | This study |
| pMS52 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR and $His_6$-SpsB | This study |
| pYS042 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR K104C and $His_6$-SpsB F177C | This study |
| pYS043 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR W103C and $His_6$-SpsB F180C | This study |
| pYS044 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR V190C and $His_6$-SpsB I18C | This study |
| pYS045 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR I197C and $His_6$-SpsB I16C | This study |

**Table S4** (Continued).

| Plasmid | Description | Source or Reference |
|---|---|---|
| pYS046 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR K104C and $His_6$-SpsB | This study |
| pYS047 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR W103C and $His_6$-SpsB | This study |
| pYS049 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR I197C and $His_6$-SpsB | This study |
| pYS062 | pETDuet vector expressing $His_6$-SUMO-FLAG-SpbR K104C and $His_6$-SpsB I16C | This study |
| pYS058 | pET22b vector expressing $His_6$-IsaA-FLAG | This study |

**Table S5.** Primers used in this study.

| Primer | Description | Sequence (5' to 3') |
|---|---|---|
| oMS21 | Primer used to amplify *spbR* and add a 3' BamHI cut site for insertion into pLOW vectors | GCGGCGGGATCCGCTGAGCGAACACATAC GTCTAAGAAAAGCTTATTTAAG |
| oMS22 | Primer used to amplify *spbR* with the *rpoB* RBS and a 5' SalI cut site for insertion into pLOW vectors | GCGGCGGGTACCGTCGACCATAATTTTTGA GGGGTGAATCTGTATGAATAAAATCTCGAA GGCTTTAACTTGG |
| oMS51 | Primer used to amplify *spbR* with an N- terminal *flag* sequence | ATGGACTACAAAGACGATGACGACAAGAAT AAAATCTCGAAGGCTTTAACTTGG |
| oMS52 | Primer used to amplify *GFPmut2* with an N-terminal *flag* sequence | ATGGACTACAAAGACGATGACGACAAGAGT AAAGGAGAAGAACTTTTCACTGG |
| oMS53 | Primer used to amplify *GFPmut2* and add a 3' BamHI cut site for insertion into pLOW vectors | ATCATGGATCCGCTCAGCATCATCTATTTGT ATAGTTCATCCATGCCATG |
| oMS56 | Primer used to amplify *flag-spbR* or *flag- GFPmut2* with the *rpoB* RBS and a 5' SalI cut site for insertion into pLOW vectors | ATGATGTCGACCATAATTTTTGAGGGGTGA ATCTGTATGGACTACAAAGACGATGACGAC |
| oMS64 | SDM primer used with oMS65 to make pMS18 | TGTCACGCATTATATATGAGAACTGTTTTAC AAA |
| oMS65 | SDM primer used with oMS64 to make pMS18 | ATATAATGCGTGACATGGAATGACAAAAAG |
| oMS80 | SDM primer used with oMS81 to make pMS24 | ATGTCACCAATTATATATGAGAACTGTTTTA CAAA |
| oMS81 | SDM primer used with oMS80 to make pMS24 | TATAATTGGTGACATGGAATGACAAAAAGTA AAG |
| oMS87 | SDM primer used with oMS88 to make pMS26 | TGTCACGATTTATATATGAGAACTGTTTTAC |
| oMS88 | SDM primer used with oMS87 to make pMS26 | ATATAAATCGTGACATGGAATGACAAAAAG |
| oMS91 | SDM primer used with oMS92 to make pMS28 | TCCATGTGCAGAGTTATATATGAGAACTGTT TT |
| oMS92 | SDM primer used with oMS91 to make pMS28 | AACTCTGCACATGGAATGACAAAAAGTAAA G |
| oMS93 | SDM primer used with oMS94 to make pMS29 | TCCATGTAAAGAGTTATATATGAGAACTGTT TT |
| oMS94 | SDM primer used with oMS93 to make pMS29 | AACTCTTTACATGGAATGACAAAAAGTAAAG |
| oMS95 | SDM primer used with oMS96 to make pMS30 | TCCATGTGATGAGTTATATATGAGAACTGTT TT |
| oMS96 | SDM primer used with oMS95 to make pMS30 | AACTCATCACATGGAATGACAAAAAGTAAAG |
| oMS99 | SDM primer used with oMS100 to make pMS32 | ATATATGAAAACTGTTTTACAAAAAGAATTAA CACA |
| oMS100 | SDM primer used with oMS99 to make pMS32 | ACAGTTTTCATATATAACTCGTGACATG |
| oMS101 | SDM primer used with oMS102 to make pMS33 | ATATATGGAAACTGTTTTACAAAAAGAATTA ACACATTTT |

**Table S5** (Continued).

| Primer | Description | Sequence (5' to 3') |
|---|---|---|
| oMS102 | SDM primer used with oMS101 to make pMS33 | ACAGTTTCCATATATAACTCGTGACATGG |
| oMS284 | Primer used with oMS285 to amplify *flag*-stop-KanR from pJP60 flanked by 36 bp *ltaS* stop codon homology arms | AAATATGAAACAGGACCTAAAGCAAACTCTA AAAAAGACTACAAAGACGATGACGACAAGT AAGAGGTGTGGATGAAGTTCC |
| oMS285 | Primer used with oMS284 to amplify *flag*-stop-KanR from pJP60 flanked by 36 bp *ltaS* stop codon homology arms | ATTGATATTTTATCAATCCGAGTTCGTGTTT AGATAGCTTATCAAAGAAGTTCCTATACTTT C |
| oMS302 | Primer used with oMS303 to extend *ltaS-flag*-KanR homology arms to 50 bp | ATCCTTCGAAGTATAAATATGAAACAGGACC TAAAGC |
| oMS303 | Primer used with oMS302 to extend *ltaS-flag*-KanR homology arms to 50 bp | TAATAAAAACCCGGATTGATATTTTATCAAT CCGAGTTC |
| oMS9 | Primer used with oMS10 for *spbR* colony PCR | GCATGCCTCGAATCTTCGTATTGC |
| oMS10 | Primer used with oMS9 for *spbR* colony PCR | TAATGACCTTGATTTACCGGAAAAAACTG |
| oMS58 | Primer used with oMS59 for *ltaS* colony PCR | CTATAATTATGACAATGTTAACTTTTAAACG CAC |
| oMS59 | Primer used with oMS58 for *ltaS* colony PCR | GAACATAATAAAAACCCGGATTGATATTTTA TC |
| oMS18 | Primer used with oMS19 for pLOW colony PCR and sequencing | GACTTTATCTACAAGGTGTGGC |
| oMS19 | Primer used with oMS18 for pLOW colony PCR and sequencing | TTCAGGCTGCGCAACTGTTG |
| oMS154 | Primer used with oMS155 to make pMS50 | CCTGGGGGGTCATCCAATAAAATCTCGAAGG CTTTAACTTGG |
| oMS155 | Primer used with oMS154 to make pMS50 | TGCAGTCACCCGGGCTTAAGCATTAAAAATTA ATAACAATATAATAGCTACAA |
| ATP146 | Primer used with ATP147 to make pMS50 | GCCCGGGTGACTGCAGGA |
| ATP147 | Primer used with ATP146 to make pMS50 | GGATGACCCCCCAGGGCC |
| oMS73 | Primer used with oMS139 to make pMS46 | CCGCAAGCTTTTAATTTTTAGTATTTTCAGGAT TGAAATTATGTTTAAATT |
| oMS139 | Primer used with oMS73 to make pMS46 | CATATGAAAAAAGAAATATTGGAATGGATTATT TCAATT |
| oMS160 | Primer used with oMS161 to make pMS52 | GGATCCGAATTCGAGCTC |
| oMS161 | Primer used with oMS160 to make pMS52 | GGTATATCTCCTTCTTAAAGTTAAAC |
| oMS162 | Primer used with oMS163 to make pMS52 | AGAAGGAGATATACCATGCGTGG |
| oMS163 | Primer used with oMS162 to make pMS52 | CTCGAATTCGGATCCTTAAGCATTAAAAATTAA TAACAATATAATAGCTACAA |
| oMS72 | Primer used with oMS73 to make pMS52 | ATCCGAATTCGAAAAAAGAAATATTGGAATGG ATTATTTCAATTG |

**Table S5** (Continued).

| Primer | Description | Sequence (5' to 3') |
|---|---|---|
| oMS73 | Primer used with oMS72 to make pMS52 | CCGCAAGCTTTTAATTTTTAGTATTTTCAGGATTGAAATTATGTTTAAATT |
| oYS200 | Primer used with oYS203 to make pYS042 | TTTGCCAACACCAATTGACACCTGTTCTTGCTAAT |
| oYS201 | Primer used with oYS202 to make pYS042 | CAATTGGTGTTGGCAAATGTTAGTAACTTTACTTTTTGTCA |
| oYS202 | Primer used with oYS201 to make pYS042 | ATTCACTACATGGCCAGAACCTAAATGAAACTTTAC |
| oYS203 | Primer used with oYS200 to make pYS042 | CTGGCCATGTAGTGAATTTAAACATAATTTCAATCCTGAAAATAC |
| oYS204 | Primer used with oYS207 to make pYS043 | GCCACTTACAATTGACACCTGTTCTTGCTAATTCTTTAAT |
| oYS205 | Primer used with oYS206 to make pYS043 | TGTCAATTGTAAGTGGCAAATGTTAGTAACTTTACTTTTTG |
| oYS206 | Primer used with oYS205 to make pYS043 | AATTATGTTTACATTCACTAAATGGCCAGAACCTAAATG |
| oYS207 | Primer used with oYS204 to make pYS043 | TGAATGTAAACATAATTTCAATCCTGAAAATACTAAAAATTAAGG |
| oYS208 | Primer used with oYS211 to make pYS044 | ATATAATAGCACAAATTTGTGCAACGGATGTTGTTGCTA |
| oYS209 | Primer used with oYS210 to make pYS044 | AATTTGTGCTATTATATTGTTATTAATTTTTAATGCTTAAGGATC |
| oYS210 | Primer used with oYS209 to make pYS044 | TAAATAAACAGACAAAAGCGACTGCAATTGAAATAATC |
| oYS211 | Primer used with oYS208 to make pYS044 | TTTTGTCTGTTTATTTATAGTAGGTAAATTTATTGTTACGCCATA |
| oYS212 | Primer used with oYS215 to make pYS045 | CATTAAAACATAATAACAATATAATAGCTACAATTTGTGCAACG |
| oYS213 | Primer used with oYS214 to make pYS045 | GTTATTATGTTTTAATGCTTAAGGATCCGAATTCGAG |
| oYS214 | Primer used with oYS213 to make pYS045 | GCGTAACACAAAATTTACCTACTATAAATAAAATGACAAAAGCGA |
| oYS215 | Primer used with oYS212 to make pYS045 | TAAATTTTGTGTTACGCCATATACAATTAAAGGTGAATC |
| oYS191 | Primer used with oYS231 to make pYS058 | AAGACGATGACGACAAGTGAGATCCGGCTGCTAAC |
| oYS229 | Primer used with oYS230 to make pYS058 | CACCACCACCACCACCATAAAAAGACAATTATGGCATCATCATTA |
| oYS230 | Primer used with oYS229 to make pYS058 | TTGTCGTCATCGTCTTTGTAGTCGAATCCCCAAGCACCTAAACCT |
| oYS231 | Primer used with oYS191 to make pYS058 | GTGGTGGTGGTGGTGCATATGTATATCTCCTTCTTAAAGTTAAAC |

**Table S5** (Continued).

| Primer | Description | Sequence (5' to 3') |
|---|---|---|
| oMS284 | Primer used with oMS285 to amplify *flag*-stop-KanR from pJP60 flanked by 36 bp *ltaS* stop codon homology arms | AAATATGAAACAGGACCTAAAGCAAACTCTA AAAAAGACTACAAAGACGATGACGACAAGT AAGAGGTGTGGATGAAGTTCC |
| oMS285 | Primer used with oMS284 to amplify *flag*-stop-KanR from pJP60 flanked by 36 bp *ltaS* stop codon homology arms | ATTGATATTTTATCAATCCGAGTTCGTGTTT AGATAGCTTATCAAAGAAGTTCCTATACTTT C |
| oMS302 | Primer used with oMS303 to extend *ltaS-flag*-KanR homology arms to 50 bp | ATCCTTCGAAGTATAAATATGAAACAGGACC TAAAGC |
| oMS303 | Primer used with oMS302 to extend *ltaS-flag*-KanR homology arms to 50 bp | TAATAAAAACCCGGATTGATATTTTATCAAT CCGAGTTC |
| oTS487 | Primer used with oTS488 to amplify flag-stop-KanR from pJP60 flanked by short homology arms to the *isaA* locus | TAAACATTACTTTTATTTATTATGAAGGAACATA AAAACAACTCGTAGCTTATCAAAG |
| oTS488 | Primer used with oTS487 to amplify flag-stop-KanR from pJP60 flanked by short homology arms to the *isaA* locus | AAAGCACAAGGTTTAGGTGCTTGGGGATTCGA CTACAAAGACGATGACGACAAGTAACAATGAC CTAAGAGGTGTGG |
| oTS508 | Primer used with oTS509 to extend isaA-flag-KanR homology arms to 50bp | GTTGACCAACAAATCAACGCAGCTGTTAAAGC ATACAAAGCACAAGGTTTAGG |
| oTS509 | Primer used with oTS508 to extend isaA-flag-KanR homology arms to 50bp | AATTTCTATGGGAAGAGCTATATATTTAATGTC TAAACATTACTTTTATTTATTATGAAG |
| oTS252 | Primer used with oTS253 to amplify flag-stop-KanR from pJP60 flanked by homology arms to the *ssaA* locus | CCTTGTTGGCGTAAACATAGCCATCACTAACA TAAAAACAACTCGTAGCTTATCAAAG |
| oTS253 | Primer used with oTS252 to amplify flag-stop-KanR from pJP60 flanked by homology arms to the *ssaA* locus | AGCCAAGCTGCTGGTTATAACTTCATTCACGA CTACAAAGACGATGACGACAAGTAACAATGAC CTAAGAGGTGTGG |

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