# Supporting Information

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### SI Experimental Procedures

**Bacterial Strains, Plasmid, and Antibiotics.** MIC of azithromycin for *S. aureus* NCTC 8325 was determined by E-test on the Mueller Hinton agar (BD Difco) plates following the manufacturer's instruction (Biomérieux). *S. aureus* strain USA300 (JE2) and its *hpf* knockout mutant NE838 were obtained from National Institutes of Health/National Institute of Allergy and Infectious Diseases-sponsored Network on Antimicrobial Resistance in *Staphylococcus aureus*. All *S. aureus* cells were grown in tryptic soy broth (TSB) at 37 °C unless otherwise noted. All antibiotics were purchased from Sigma-Aldrich. DNA and RNA oligonucleotides were obtained from IDT Integrated DNA Technologies.

**Anti-SaHPF Production.** SaHPF gene was PCR-amplified with primers 5′-GGCATATGATTAGATTTGAAATTCATGGAG-3′ and 5′-CGCTCGAGTTATTGTTCACTAGTTTGAATC-3′ using genomic DNA of RN4220 as template. The product was cloned into the NdeI and XhoI sites of pET28a (Novagen). The resulting $His_6$-SaHPF recombinant protein was overexpressed in *E. coli* BL21(DE3) and purified by Ni-NTA affinity column (MCLAB) under native conditions. Polyclonal anti-SaHPF was raised in rabbits (Josman) and used at a 1:4,000 dilution.

**Pulse-Chase Analysis.** The experiments were carried out as previously described (1). Briefly, *S. aureus* cells were grown in defined minimal medium 4 and adjusted to $OD_{600}$ ~0.3. After the incubation of antibiotics for 1 min, cells were pulse-labeled with 30 μCi/mL of Tran$^{35}$S-label (MP Biomedicals) for 3 min and chased with excess amounts of unlabeled casamino acids at various time points. Protein samples were TCA precipitated, resolved on AnykD TGX SDS/PAGE (BioRad), and autoradiographed.

**cDNA Libraries Construction.** Libraries were prepared as previously described, with some modifications (2–6). The procedures are detailed as follows.

***Cell growth and harvest.*** An overnight culture of *S. aureus* was diluted by adding 10 mL to 1 L of TSB. Negative and positive drug conditions were prepared simultaneously. Cells were shaker incubated at 37 °C and 225 rpm until cultures reached an $OD_{600}$ of 0.45–0.50. For the negative drug condition, cells were pretreated for 2 min with ~100 μg/mL chloramphenicol (Sigma-Aldrich). For the positive drug condition, 5 mg/mL AZ (Fluka Analytical) was added to the culture to a final concentration of 25 μg/mL, and cells were grown under standard growth conditions for 15 min. The culture was then pretreated for 2 min with ~100 μg/mL chloramphenicol. Immediately after chloramphenicol pretreatment the cultures were placed on ice, and ~600 mL of 100 μg/mL chloramphenicol ice cubes were added and mixed with vigorous shaking. The remaining, unmelted ice was removed by decanting the chilled cell cultures into centrifugation bottles. Cells were pelleted by centrifugation at 12,227 × *g* for 30 min at 4 °C. After decanting the supernatant, the cell pellets were resuspended in 10 mL of prechilled Resuspension Buffer [10 mM $MgCl_2$, 100 mM $NH_4Cl$, 20 mM Tris (pH 8.0), and 1 mM chloramphenicol] to wash the cells. The cellular suspension was spun at 4,000 × *g* for 10 min at 4 °C. Cellular pellets were then resuspended in 3.75 mL of prechilled Lysis Buffer [10 mM $MgCl_2$, 100 mM $NH_4Cl$, 20 mM Tris (pH 8.0), 0.1% IGEPAL CA-630 (Sigma Aldrich), 0.4% Triton X-100 (Sigma Aldrich), 100 U/mL RNase-free DNase I (Roche), 0.5 U/μL SUPERase•In (Ambion), and 1 mM chloramphenicol]. The cellular resuspensions were incubated on ice for 5 min before being dripped into a 50 mL conical vial containing liquid nitrogen. The resulting frozen cell pellets were transferred to prechilled 10 mL grinding jars and cryogenically milled by eight 3-min cycles at 15 Hz (Retsch MM400, 10 mL grinding jar, 15 mm grinding ball). Grinding jars were rechilled between each cycle in liquid nitrogen. Milled cells were transferred to 50 mL conical vials containing liquid nitrogen and placed in a −80 °C freezer to allow the liquid nitrogen to evaporate through the pierced cap of the vial.

***Lysate preparation.*** Cryogenically milled cells were thawed in a 30 °C water bath for 3–5 min and then immediately placed in an ice water bath for 10 min. Lysate was centrifuged at 20,000 × *g* for 10 min at 4 °C. Clarified lysate was recovered and transferred to prechilled microcentrifuge tubes and spun at 14,000 × *g* for 5 min at 4 °C to remove residual cellular debris. The concentration of the resulting lysate was determined by $A_{260}$ measurement using a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and a 1:100 dilution in 10 mM Tris (pH 8.0). Recovered lysate was divided into 50- and 200-μL aliquots, flash frozen in liquid nitrogen, and stored at −80 °C.

***Nuclease digestion and sucrose fractionation.*** Aliquots of clarified lysate, containing 90 (replicate 1) or 180 (replicate 2) $Abs_{260}$ ribosome units (1 $A_{260}$ = 12 μg/μL) (7) were supplemented with 5 mM $CaCl_2$ and sequentially digested (i.e., two sequential additions of 875 enzyme units) with a total of 1,750 enzyme units of S7 Micrococcal Nuclease (Roche) to remove the unprotected mRNA to generate the footprint fragments. Digestion reactions were incubated for 1 h at 25 °C and 1,400 rpm (Eppendorf Thermomixer) and quenched with the addition of EGTA to a final concentration of 6 mM.

Linear sucrose (Sigma) gradients [10–55% (wt/vol)] were prepared with Gradient Buffer [10 mM $MgCl_2$, 100 mM $NH_4Cl$, 20 mM Tris (pH 8.0), 0.2 mM chloramphenicol, and 2 mM DTT] in 14 × 89-mm Polyallomer Centrifugation tubes (Beckman Coulter) and equilibrated with a BioComp Gradient Master (BioComp Instruments) using the provided "SW41 LONG SUCR 5-50% wv" program. Undigested (reference standard) and digested samples were carefully loaded onto prepared gradients and spun on a Beckman Coulter L7-55 Ultracentrifuge for 2.5 h at 210,053 × *g* at 4 °C in a SW41 rotor. Sucrose gradients were fractionated using the Density Gradient Fractionation System (Brandel) with a set flow rate of 0.75 mL/min, sensitivity of 2 Å, a chart speed of 60 cm/hr, and an approximate 200-μL fraction size. Because the gradient was displaced from the bottom-up by the chase solution, Fluorinert FC-70 (Hampton Research), a continuous absorbance profile was obtained by monitoring the absorption at 254 nm. Fractions were manually collected, and an $A_{260}$ measurement was obtained for each fraction using a NanoDrop. Absorbance values were graphed in Microsoft Excel to determine the RPF1 and RPF2 associated fractions, which were consequently pooled.

***Library generation.*** RNA was purified from total lysate and the RPF1 and RPF2 fractions using the SDS/hot acid phenol method. Samples were first denatured by adding SDS (Amersco) to a final concentration of 1% (wt/vol). RNA was extracted with one volume of 65 °C preheated acid phenol (pH 4.5) (Amersco), one volume of acid phenol (pH 4.5), and 0.9 volume of chloroform/isoamyl alcohol (24:1) (Sigma Aldrich). Each mixture was vortexed and centrifuged for 15 min at 5,000 × *g* before recovering the aqueous phase of each sample. RNA was precipitated by isopropanol precipitation and resuspended in 20 μL of 10 mM Tris (pH 8.0). Concentration was determined by $A_{260}$ measurement using a NanoDrop. Because of elevated RNA concentrations, only half of each sample was used for the remaining experimental procedure.

The mRNA of the purified total lysate samples was enriched by removing the 16S and 23S rRNAs with the MICROB*Express* Kit (Ambion), as described in the manufacturer's provided protocol

without modification. Before enrichment, the samples were supplemented with 1 mM EDTA to prevent degradation of the RNA during the procedure. The resulting enriched mRNA samples were resuspended in 25 μL of 10 mM Tris (pH 8.0).

The enriched mRNA samples were randomly fragmented by adding an equal volume of 2× Alkaline Fragmentation Solution (1 mM EDTA, 5 mM $Na_2CO_3$, and 45 mM $NaHCO_3$, pH 9.3) and incubated for 20 min at 95 °C. Reactions were quenched by adding ice cold 3M sodium acetate, pH 5.2 (Amresco) to a final concentration of 300 mM. RNAs were precipitated by isopropanol precipitation and resuspended in 20 μL of 10 mM Tris (pH 8.0).

The fragmented mRNA, RPF1, and RPF2 samples were resolved on a denaturing polyacrylamide gel for size selection of the footprint fragments. RNA samples were prepared for electrophoresis by adding 2× Novex TBE-Urea Sample Buffer (Invitrogen). Oligonucleotide and ladder standards were prepared at 1 μM 28-nt marker oligo (5′-AUGUACACGGAGUCGACCCGCAACGCGA-3′) and 0.05 μg/μL 10-bp DNA Ladder (Invitrogen), respectively. These were prepared in Novex TBE-Urea Sample Buffer (2×) and 10 mM Tris (pH 8.0). With the exception of the 10-bp DNA Ladder, the resulting mixtures were denatured for 2 min at 80 °C, briefly centrifuged, and placed on ice. Samples were resolved on a 15% TBE-Urea gel (Invitrogen) in 1× TBE (Ambion) run for 65 min at 200 V. Gels were stained for 3 min in SYBR Gold Nucleic Acid Gel Stain (diluted from 10,000× in 1× TE; Invitrogen) and visualized by UV transillumination. Using the 10-bp DNA ladder and 28-nt marker as standards to identify the footprint fragment, a band between 25 and 40 nt was excised. RNA was recovered using the ZR small-RNA PAGE Recovery Kit (Zymo Research), as described in the manufacturer's provided protocol without modification. Ligated products were eluted from the final spin column with 15 μL of 10 mM Tris (pH 8.0).

***Dephosphorylation.*** RNA samples were denatured for 2 min at 80 °C and then placed on ice. The 3′ ends of the RNA were dephosphorylated by T4 Polynucleotide kinase (T4 PNK; NEB) in the presence of the following reaction components: 1× T4 PNK Reaction Buffer (without ATP), 20 U SUPERase•In, and 10 U T4 PNK. Reactions were incubated at 37 °C for 1 h. The enzyme was heat-inactivated for 10 min at 75 °C. RNA was precipitated by isopropanol precipitation and resuspended in 10 μL of 10 mM Tris (pH 8.0). Concentration was determined by $A_{260}$ measurement using a NanoDrop.

***Linker ligation.*** Dephosphorylated samples were diluted with 10 mM Tris (pH 8.0) and divided into 5-μL aliquots, containing RNA concentrations ranging between 2 and 10 pmol. One microliter of 1 μg/μL Linker-1 (5′-App/CTGTAGGCACCATCAAT/3 ddC-3′) was added to each of the RNA aliquots. Mixtures were denatured for 90 s at 80 °C and then cooled to room temperature for 15 min. Ligation of RNA to Linker-1 occurred with the following reaction components: 20% (wt/vol) sterile PEG MW 800, 1× T4 Ligase Reaction Buffer, 10% DMSO, 20 U SUPERase•In, and 10 U T4 Ligase 2, truncated (NEB). Reaction mixtures were incubated at 37 °C for 2.5 h. Aliquots of each sample were pooled. Ligation products were precipitated by isopropanol precipitation in the presence of 56 μg/mL of glycogen (Invitrogen), resuspended in 10 μL of 10 mM Tris (pH 8.0), and resolved on a 10% TBE-urea gel (Invitrogen) in 1× TBE run for 50 min at 200 V. Samples were prepared for electrophoresis by adding 2× Novex TBE-Urea Sample Buffer and denatured for 3 min at 70 °C. A three-oligonucleotide marker standard containing 1 μM 28-nt marker oligo, 1 μM 45-nt marker oligo (5′-TAACTTTAAGAAGGAGATATACCAATGTGCACCAGTATCGCAGTA-3′), and 1 μM 60-nt marker oligo (5′-GTAATAGCATAAAAAATTTATTTTTTCAGGAGGCGCAATGGTGTAGGCTGGAGCTGCTTC-3′) was prepared in Novex TBE-Urea Sample Buffer (2×) and 10 mM Tris (pH 8.0) and denatured for 3 min at 70 °C. The 10-bp DNA ladder was prepared as previously described. Gels were stained for 3 min in SYBR Gold Nucleic Acid Gel Stain and visualized by UV transillumination. Using the 10-bp DNA ladder and marker oligonucleotides as standards to identify the ligated product, a band between 25 and 65 nt was excised. Ligated RNA was recovered using the ZR small-RNA PAGE Recovery Kit, as described in the manufacturer's provided protocol without modification. Ligated products were eluted from the final spin column with 10 μL of 10 mM Tris (pH 8.0).

***Reverse transcription.*** The resulting gel products were mixed with 2 μL of 1.25 μM Link-1.2, the reverse transcription primer (5′-5phos/AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC/iSp18/CACTCA/iSp18/TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGCCTACAG-3′), resulting in a final volume of 12 μL. The RNA/Link-1.2 mixtures were denatured for 2 min at 80 °C and then quickly cooled on ice. Samples were incubated at room temperature for 10 min before being reverse transcribed by SuperScript III Reverse Transcriptase (Invitrogen) with the following reaction components: 1× First-strand buffer, 0.5 mM dNTPs (Invitrogen), 5 mM DTT, 20 U SUPERase•In, and 200 U SuperScript III Reverse Transcriptase (Invitrogen). Reaction mixtures (20 μL) were incubated for 30 min at 48 °C. RNA products were hydrolyzed by adding 1 mM NaOH to a final concentration of 0.1 mM and incubating for 15 min at 95 °C. The cDNA products were resolved from the unextended primer on a 10% TBE-Urea gel in 1× TBE run for 70 min at 200 V. Samples were prepared for electrophoresis by adding 2× Novex TBE-Urea Sample Buffer and denatured for 3 min at 70 °C. The 10-bp DNA ladder was prepared as previously described. Gels were stained for 3 min in SYBR Gold Nucleic Acid Gel Stain and visualized by UV transillumination. Using the 10-bp DNA ladder as a standard to identify the reverse transcription product, a band between 125 and 155 nt was excised. DNA was recovered using the ZR small-RNA PAGE Recovery Kit, as described in the manufacturer's provided protocol without modification. cDNA products were eluted from the final spin column with 15 μL of 10 mM Tris (pH 8.0).

***cDNA circularization.*** The resulting cDNA was circularized (8) with CircLigase (Epicentre) in the presence of the following reaction components: 1× CircLigase buffer, 0.05 mM ATP, 2.5 mM $MnCl_2$, and 100 U CircLigase. Reaction mixtures (20 μL) were incubated at 60 °C for 1 h. The enzyme was heat-inactivated for 10 min at 80 °C.

***PCR amplification and barcode addition.*** The resulting circDNA was PCR amplified with Phusion High-Fidelity DNA Polymerase (NEB). PCR mixtures contained the following reaction components: 5 μL of circDNA, 0.5 μM reverse library index primer/barcode (see below), 0.5 μM forward library primer (5′-AATGATACGGCGACCACCGAGATCTACAC-3′), 1× Phusion HF buffer, 0.2 mM dNTPs, and 2 U Phusion High-Fidelity DNA Polymerase. PCR amplification was performed as follows: 30 s at 98 °C (denaturation); 12, 14, 16, or 17 cycles of 30 s at 98 °C (denaturation), 10 s at 65 °C (annealing), and 5 s at 72 °C (extension). Amplified PCR products were resolved from unextended reverse transcription primer on an 8% TBE gel (Invitrogen) in 1× TBE for 40 min at 180 V. Samples were prepared for electrophoresis by adding 5× Novex Hi-Density TBE Sample Buffer (Invitrogen) to each reaction. The 10-bp DNA ladder was prepared as previously described but using Novex Hi-Density TBE Sample Buffer (5×). Gels were stained for 3 min in SYBR Gold Nucleic Acid Gel Stain and visualized by UV transillumination. Using the 10-bp DNA ladder as a standard to identify the PCR product of interest, a band between 160 and 170 nt was excised. Four hundred microliters of DNA Gel Extraction Buffer [300 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA] was added to the excised gel slices and frozen for 30 min on dry ice. The samples were placed on a Thermomixer at room temperature and agitated at 1,400 rpm overnight. After brief centrifugation, 400 μL of elutant was transferred into a clean, nonstick, RNase-free microcentrifuge tube and stored at −20 °C. The overnight gel extraction was repeated, and the elutants of each sample were pooled.

DNA was precipitated by isopropanol precipitation in the presence of 56 μg/mL of glycogen and resuspended in 15 μL of 10 mM Tris (pH 8.0). Concentration was determined by $A_{260}$ and $A_{280}$ measurements using a NanoDrop. The resulting DNA library was quantified and characterized by using the high-sensitivity DNA chip on the Agilent BioAnalyzer (Agilent Technologies), which was conducted at the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine.

***Indexed library PCR primers.*** Reverse index primers used to amplify the cDNA are listed below. Barcode sequences are underlined.

Index 1: 5′-CAA GCA GAA GAC GGC ATA CGA GAT CGT GAT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG-3′

Index 2: 5′-CAA GCA GAA GAC GGC ATA CGA GAT ACA TCG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG-3′

Index 3: 5′-CAA GCA GAA GAC GGC ATA CGA GAT GCC TAA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG-3′

Index 4: 5′-CAA GCA GAA GAC GGC ATA CGA GAT GAT CTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG-3′

Index 5: 5′-CAA GCA GAA GAC GGC ATA CGA GAT TCA AGT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG-3′

Index 6: 5′-CAA GCA GAA GAC GGC ATA CGA GAT CTG ATC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG-3′

***Sequencing.*** The resulting DNA libraries were sequenced using the Illumina HiSeq system and single-end reads. All sequencing was conducted at the University of Chicago Genomics Core. The same sequencing primer (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′) was used for all sample libraries.

***Global gene expression analysis.*** The Illumina libraries were preprocessed and aligned in Galaxy. Sequence libraries were preprocessed by clipping the adaptor sequence (5′-CTGTAGGCACCATCAAT-3′) and trimming the sequences by discarding the first base and all those extending beyond the 50th base. The sequencing reads were mapped to the rRNA reference genome with Bowtie2. Unmapped sequences were then aligned to the *S. aureus* NCTC 8325 genome (GenBank CP000253). Cufflinks was then used to assemble the transcripts and determine the relative expression levels in RPKM. Cufflinks was followed by Cuffdiff to calculate the differential expression at the gene and transcript levels of the negative and positive drug samples, per replicate. Geometric library normalization and pooled dispersion estimation methods were used with a 0.05 false discovery rate and a minimum alignment count of 10. Bias correction was performed during the analysis. Although RPKM is a widely accepted normalization for within-sample comparisons, RPM unit is useful for assessing overall expression levels between samples and has been used in all ChIP-seq, miRNA-seq, and ribosome profiling analyses. In this work, RPKM will be used for measuring differential gene expression, whereas RPM will be used for the rest of the ribosome density analyses.

The resulting Cuffdiff gene differential expression files were analyzed to determine the number of genes turned on, turned off, up-regulated, down-regulated, untranslated, and unchanged by drug treatment. Genes were sorted according to their RPKM values to identify those genes that are turned on, turned off, or untranslated. The value of the logarithm base twofold change was used to sort the genes into the remaining categories. The groups were further analyzed with arbitrary thresholds. Genes with only one RPKM value (~10 raw read counts) being expressed in either the negative or positive drug sample, and ≥1, were considered to be significant and were retained in their respective groups, which were either turned on or turned off; however, genes with only one RPKM value that was lower than 1, were reclassified as unchanged. Genes with an absolute $\log_2$-fold change value ≥1 were considered to be significant and were retained in their respective groups, either up-regulated or down-regulated; however, genes with absolute logarithm base twofold change values lower than 1 were reclassified as unchanged. Genes classified as untranscribed or untranslated had zero read counts in one of the negative and positive drug samples.

**In Vitro Translation of Previously Unidentified ORFs.** Newly identified ORFs were translated by the "Δribosome" PURExpress Kit (New England BioLabs) that was programmed with T7 promoter-containing DNA templates (10 ng/μL), 10 μCi Tran$^{35}$S-label (MP Biomedicals), and 15 pmol of *S. aureus* ribosomes in a total of 10 μL reaction. The reaction products were either subjected to acetone precipitation or fractionated by CTABr precipitations (9). Half of the reaction was treated with 1 μg of RNase A at 37 °C for 15 min before acetone precipitation. All samples were resolved on 12% NuPAGE minigels with Mes running buffer (Invitrogen). *S. aureus* S-30 extract was prepared from strain RN4220 as previously described (10). Ribosomes were collected by sedimenting through a 0.5 M sucrose/RNC buffer cushion in a Beckmann TLA100 rotor at 4 °C (435,000 × *g*, 10 min). The pellet was resuspended to the desired concentration in RNC buffer [20 mM Hepes (pH 7.5)/100 mM KOAc/14 mM $MgCl_2$].

**Toe-Printing Assay.** T7 promoter was incorporated into linear DNA templates by two-step crossover PCR. Five microliters of in vitro translation with and without addition of antibiotics were carried out with *E. coli* PURExpress Kit (New England BioLabs) according to the manufacturer's manual, followed by primer extension using a [γ[$^{32}$P]ATP]-labeled reverse primer that anneals 50–100 nt downstream of the anticipated stall site (11, 12). Antibiotics were used at a final concentration of 50 μM except for AZ (30 μM). Reversed-transcribed cDNAs were subjected to phenol/chloroform extractions and isopropanol precipitation. Pellets were dissolved in 6 μL formamide loading dyes and analyzed on a 6% PAGE/8 M Urea sequencing gel. Sequencing ladders were prepared with Thermo sequenase cycle sequencing kit (USB corp.) The control *dhfr* template in the PURExpress kit was used to determine the optimal inhibitory concentration of antibiotics in the vitro translation reactions.


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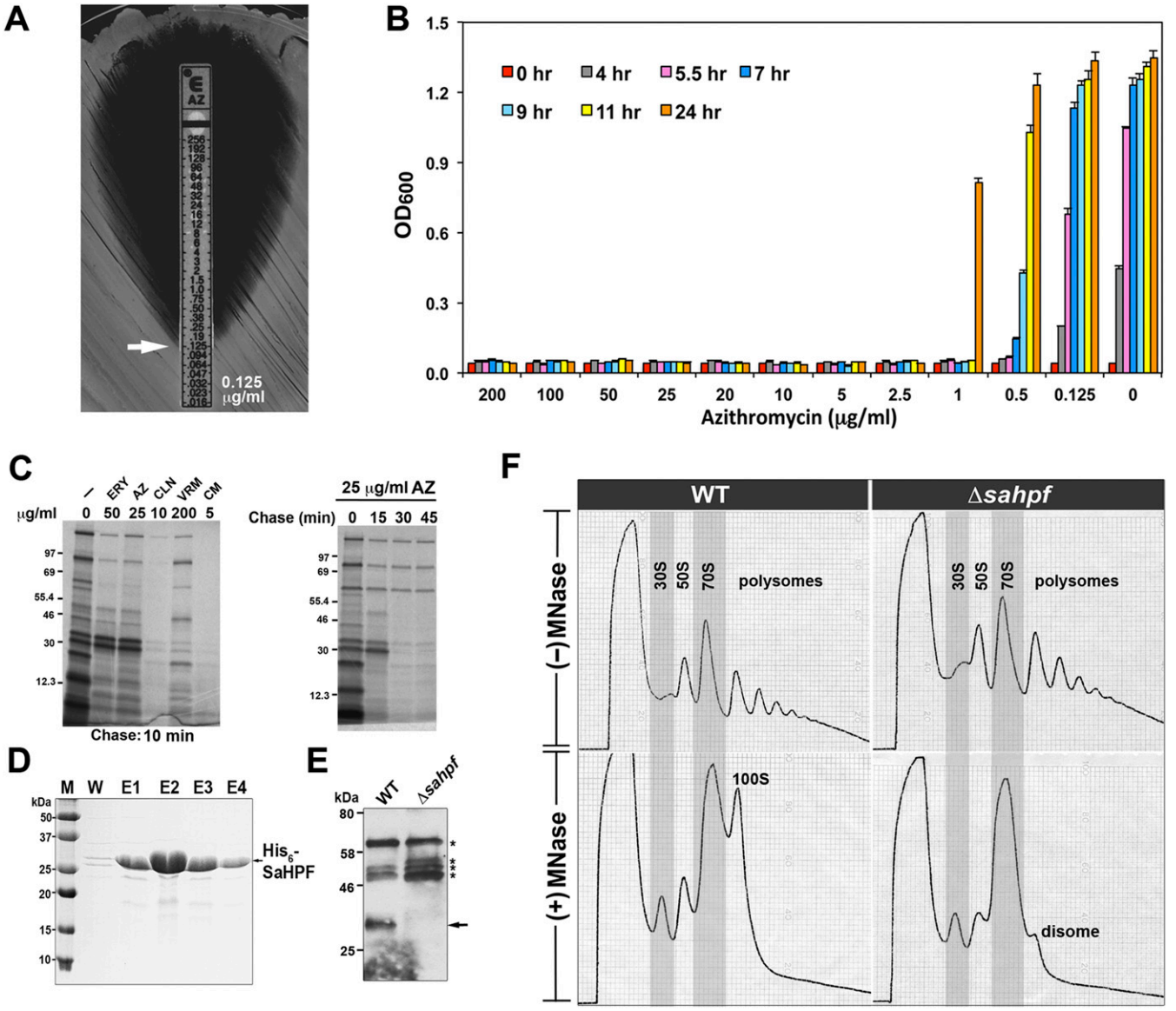


**Fig. S1.** Bactericidal activity of AZ and an unusual association of SaHPF with the translating ribosomes. (*A*) MIC of AZ for *S. aureus* was determined by placing an AZ containing E-strip on the agar plate. MIC is indicated by a clear zone around the strip (white arrowhead). (*B*) Time course of *S. aureus* growth in TSB supplemented with different concentrations of AZ. AZ concentrations above 1 μg/mL are completely bactericidal. (*C*) Pulse-chase analysis of *S. aureus* protein synthesis in the presence of various ribosome-targeting antibiotics (*Left*) and AZ exposure over a 45-min period (*Right*). Macrolides (ERY and AZ) and streptogramin (VRM) do not fully block protein synthesis, whereas clindamycin (CLN) and chloramphenicol (CM) bind to the PTC and effectively inhibit translation. (*D*) Overexpression and purification of $His_6$-tagged SaHPF. Purified proteins were analyzed on SDS/PAGE and used to raise SaHPF antibodies. M, protein marker; W, wash; E, elution fraction. (*E*) Western blot showing the specificity of anti-SaHPF (1:4,000 dilutions) in the whole-cell lysates of wild-type *S. aureus* and its *sahpf* knockout strain. The arrowhead marks SaHPF. The asterisks denote nonspecific cross-reactions. (*F*) The peak after the monosome peak in the MNase-digested samples primarily consists of the 70S dimer (100S ribosomes) and a trace amount of "disome." Cell lysates without AZ treatment from the wild-type *S. aureus* and its *sahpf* knockout strain were analyzed on a 10–55% sucrose density gradient. The "disome-like" peak diminished by more than 85% in the *sahpf* knockout.

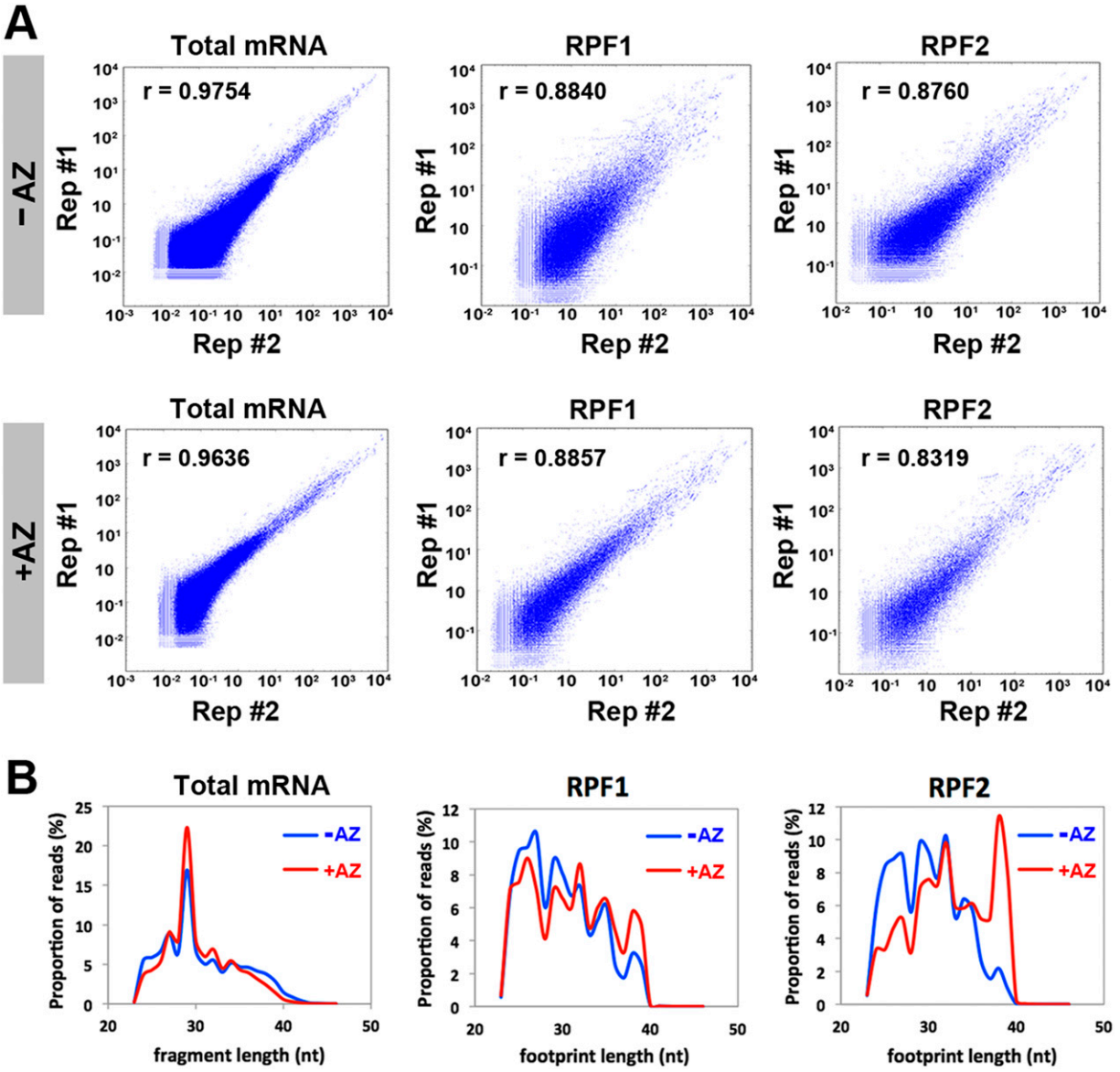


**Fig. S2.** Reproducibility of RNA sequencing and the size distribution of ribosome footprints. (*A*) Reproducibility between two replicates from two independent TSB cultures of *S. aureus*. Each dot corresponds to the number of sequencing RPM of a particular mRNA. (*B*) The length distribution of mRNA reads and RPFs. RPF1 and RPF2 libraries exhibit multimodal size distribution possibly due either to the ribosome pausing-mediated asymmetric extension of footprints at the 3′ end (1) or the strong 3′ A/T bias of MNase that generates longer footprints (2).

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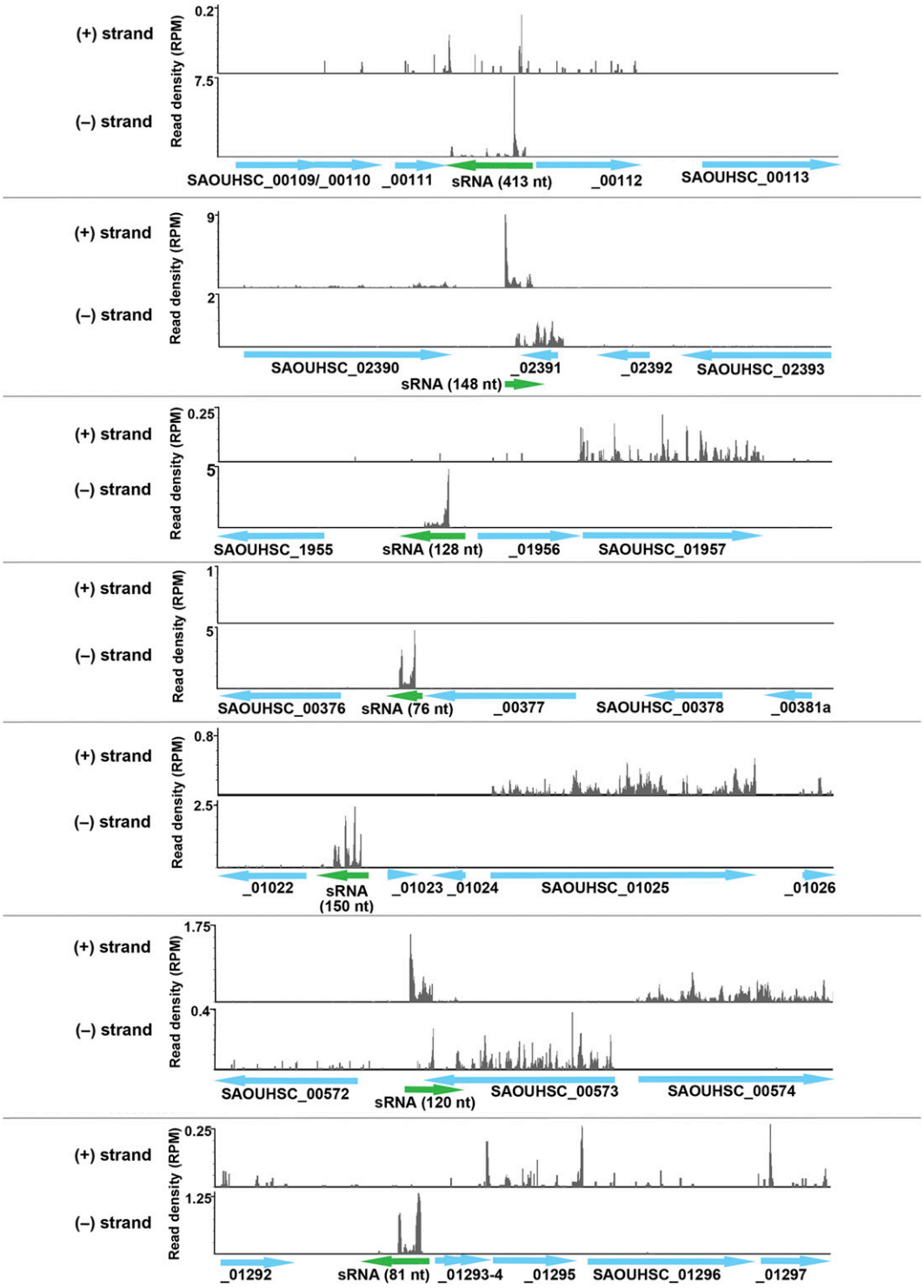


**Fig. S3.** Identification of noncoding small RNAs (sRNAs) from the mRNA-seq libraries. High read densities were detected in previously unannotated intergenic regions. The expression of these sRNAs was not affected by AZ treatment and was not detected in the ribosome footprint libraries. Read density profiles of the plus (*Upper*) and minus (*Lower*) strands are shown above the physical map. Genes are represented by thick arrows, which point in the direction of transcription. Green arrows mark the putative sRNAs along with their predicted lengths.

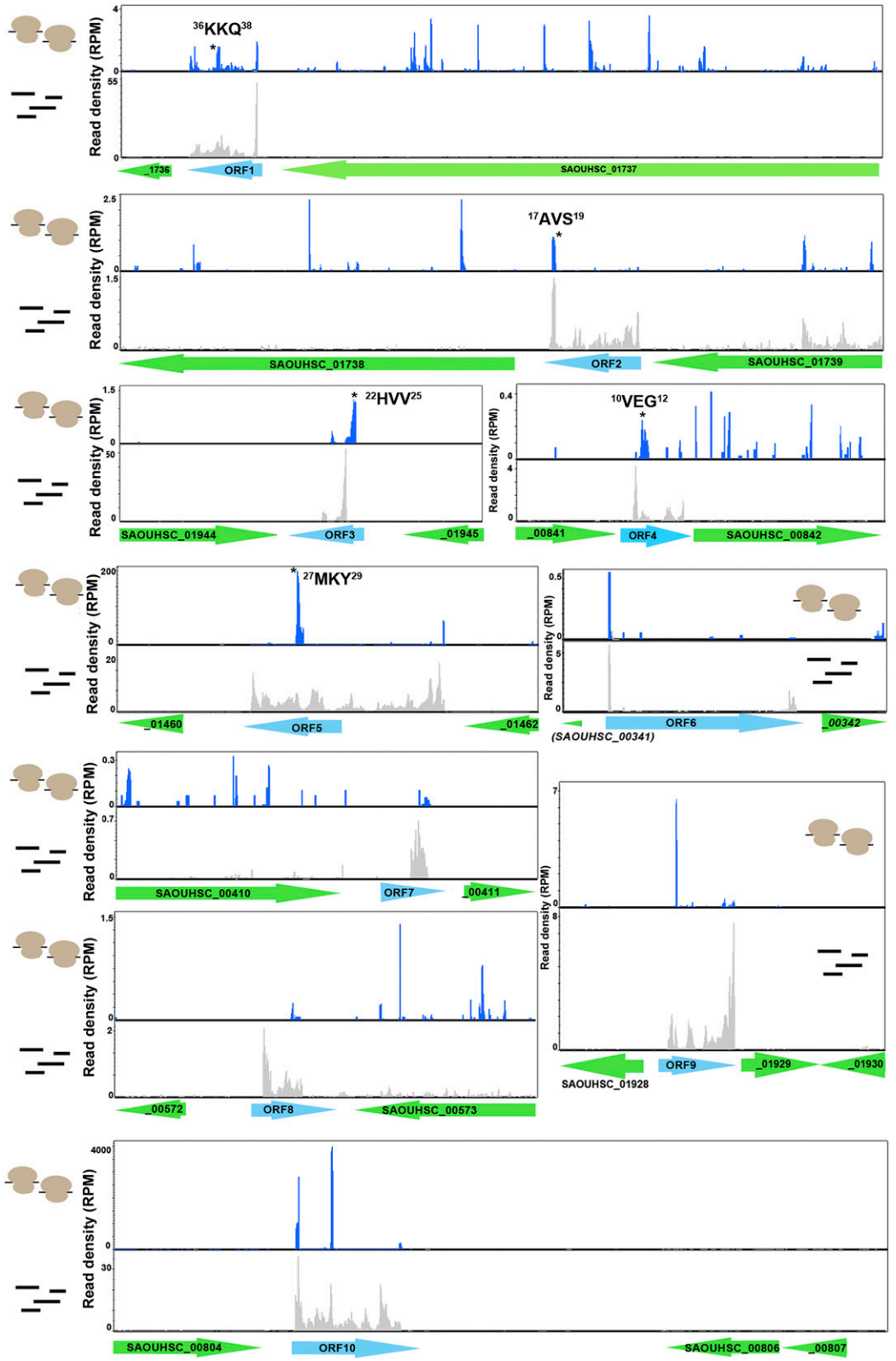


**Fig. S4.** Representative examples of newly identified small ORFs. The upper panels show the ribosome density as a function of position. The lower panels show the mRNA-seq density. Previously unidentified ORFs are marked in light blue arrows. The asterisks and tripeptide sequences denote the potential ribosome stalling sites in ORF1–ORF5. Only the density plots of samples without AZ treatment are shown because AZ inhibits the translation of these ORFs.

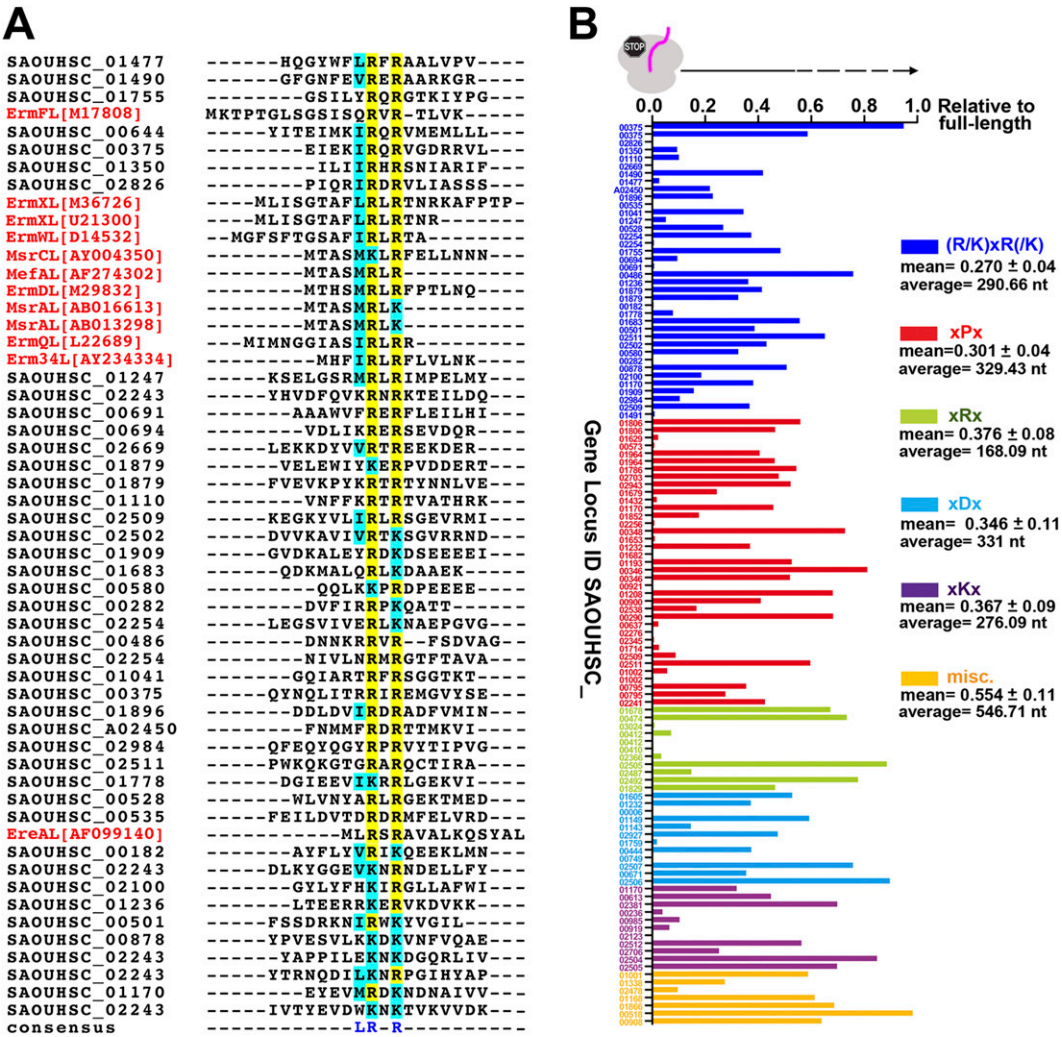


**Fig. S5.** Similarity of (R/K)x(R/K) containing proteins to the regulatory RLR leader peptides and ribosome processivity beyond the AZ-specific stalling sites. (*A*) Multiple sequence alignment of "RLR" regulatory peptides (in red) with the representative (R/K)x(R/K) containing *S. aureus* proteins. GenBank accession numbers of the leader peptides are shown in brackets. Conserved and similar residues are box shaded in yellow and blue, respectively. (*B*) Residual ribosome density (≥0.05 RPM) is detected beyond the strong ribosome stalling sites. On average, ribosome density extends 100–200 nt before a complete disappearance of ribosome occupancy.

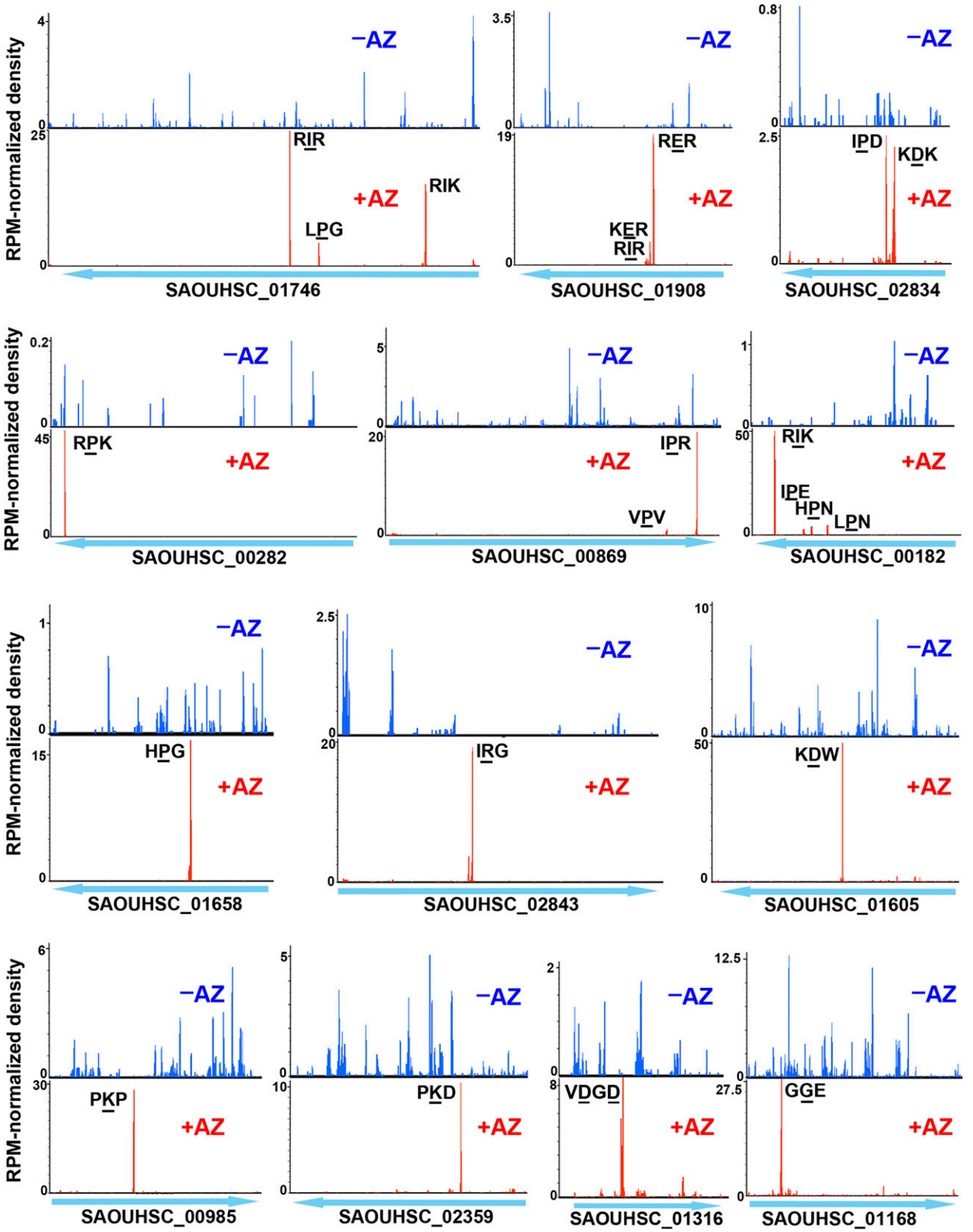


**Fig. S6.** Examples of ribosome density plot showing the AZ-induced ribosome stalling sites. ORFs from Table S2 representing each indicated motif class are randomly chosen. Tripeptide stalling sequences are shown on the (+) AZ panels. P-site codons are underlined. In many cases multiple stalling sites were identified in a single ORF.

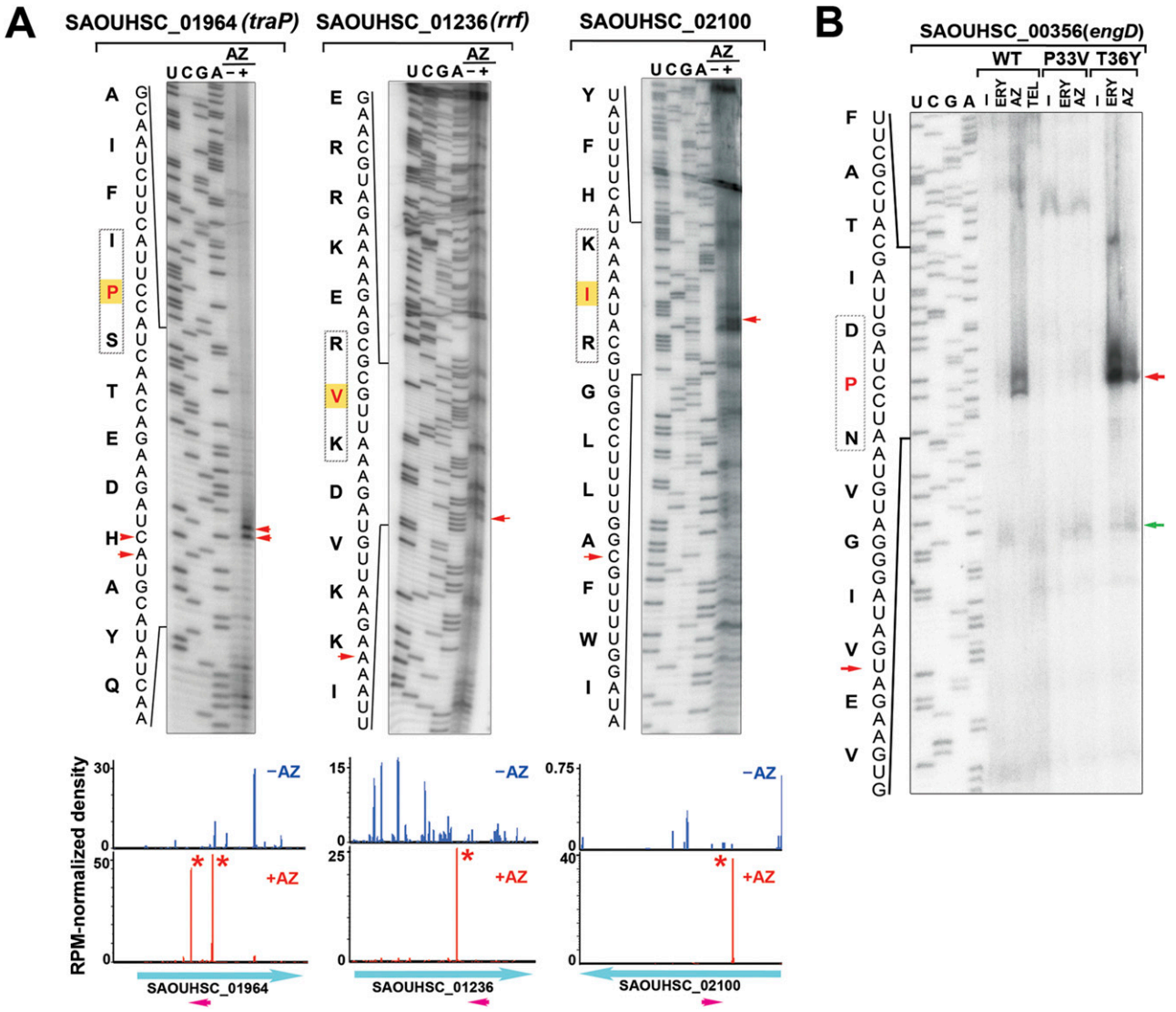


**Fig. S7.** Toe-printing analysis of drug-induced ribosome stalling. (*A*) Related to Fig. 3. Validation of additional xPx and (R/K)x(R/K) stalling sites. Labeling schemes are identical to those in Fig. 3. (*B*) Mutations in EngD that impair (P33V) and restore (T36Y) antibiotic-specific ribosome stalling. TEL, telithromycin.

## Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)