Ribosome hibernation factor promotes Staphylococcal survival and differentially represses translation

Arnab Basu and Mee-Ngan F. Yap*

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA

Received November 25, 2015; Revised March 03, 2016; Accepted March 07, 2016

ABSTRACT

In opportunistic Gram-positive Staphylococcus aureus, a small protein called hibernation-promoting factor (HPFSa) is sufficient to dimerize 2.5-MDa 70S ribosomes into a translationally inactive 100S complex. Although the 100S dimer is observed in only the stationary phase in Gram-negative gammaproteobacteria, it is ubiquitous throughout all growth phases in S. aureus. The biological significance of the 100S ribosome is poorly understood. Here, we reveal an important role of HPFSa in preserving ribosome integrity and poising cells for translational restart, a process that has significant clinical implications for relapsed staphylococcal infections. We found that the hpf null strain is severely impaired in long-term viability concomitant with a dramatic loss of intact ribosomes. Genome-wide ribosome profiling shows that eliminating HPFSa drastically increased ribosome occupancy at the 5′ end of specific mRNAs under nutrient-limited conditions, suggesting that HPFSa may suppress translation initiation. The protective function of HPFSa on ribosomes resides at the N-terminal conserved basic residues and the extended C-terminal segment, which are critical for dimerization and ribosome binding, respectively. These data provide significant insight into the functional consequences of 100S ribosome loss for protein synthesis and stress adaptation.

INTRODUCTION

Protein synthesis is the most energy-consuming cellular process (1–3). Thus, suppression of translation during stress conditions is a universal adaptation mechanism used by all living organisms to mitigate wasteful energy consumption. In bacteria, the majority of non-translating ribosomes accumulate as 70S monomers and dimers of 70S (100S complex). Gammaproteobacteria possess three 70S ribosome-silencing factors that bind to the 30S subunit near the interface of the 30S and 50S subunits (4–5). In Escherichia coli, RMF Ec and HPF Ec (formerly YhbH) concertedly induce the formation of 100S ribosomes, whereas YfiA Ec (also known as pY or RaiA) (6) stabilizes the inactive 70S ribosome analogously to PSRP1 in plant plastids (7). Crystal structures of the T. thermophilus ribosome in complex with RMF Ec, HPF Ec and YfiA Ec have suggested that translational inactivation is due to the steric interference of these factors with the binding of tRNA, mRNA and initiation factors to the ribosomal decoding center on the 30S subunit (4). Electron microscopy studies have shown in vitro and in situ that the two 70S monomers in the 100S dimer are joined together via the 30S subunits (8–10). Of note, E. coli hibernating ribosomes are exclusively found during the stationary growth phase when nutrients are limited (11–13). Upon starvation relief, they are split into subunits and presumably are recycled for new rounds of translation by a poorly defined mechanism (11,14–16). Although an E. coli rmfEc mutant is reduced in long-term viability and stress tolerance during stationary phase growth (13,17,18), the deletion of hpfEc or yfiAEc does not appear to elicit strong cellular defects (19).

In stark contrast to E. coli, the clinically important Gram-positive bacterium Staphylococcus aureus does not contain RMF or YhA homologs; instead, it carries only a long form of HPF (HPFsa) that is twice the size of HPF Ec (Figure 1) (20–23). In fact, the long C-terminal extension, which shares some degrees of homology with RMF Ec, is a hallmark of the HPF family in plant plastids and all Gram-positive bacteria (10,22,24,25). Curiously, HPFsa-mediated 100S ribosomes are present throughout all growth phases even when nutrients are abundant (23,26). The physiological relevance of these translationally incompetent ribosomes during exponential growth remains unknown. Nevertheless, the deletion of hpf in Listeria monocytogenes leads to attenuated virulence in a mouse model of infection (24) and sensitivity to prolonged antibiotic exposure (27).

*To whom correspondence should be addressed. Tel: +1 314 977 9241; Fax: +1 314 977 9206; Email: myap1@slu.edu

© The Author(s) 2016. Published by Oxford University Press on behalf of Nucleic Acids Research. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
The \textit{Lactococcus lactis} \textit{hpf} mutant fails to resuscitate from nutrient-depleted growth (10).

In this study, we sought to define the role of \textit{HPF}_{Sa} in a methicillin-resistant \textit{S. aureus} (MRSA) strain by examining the biochemical features of \textit{HPF}_{Sa} and the effect of the \textit{hpf}_{Sa} null mutation on \textit{in vivo} global translation. We found that the \textit{hpf}_{Sa} mutant has an extremely low survival rate in long-term cultures that coincides with the rapid degradation of ribosomes. We identified \textit{HPF}_{Sa} regions that are critical for ribosome dimerization and binding. Using \textit{in vivo} ribosome profiling and \textit{in vitro} cell-free translation, we provided experimental evidence that \textit{HPF}_{Sa} attenuates translation of a subset of genes.

**MATERIALS AND METHODS**

**Bacterial strains and reagents**

Strains JE2 and NE838 are derivatives of the community-associated methicillin-resistant (CA-MRSA) \textit{S. aureus} isolate USA300 (28). The \textit{hpf}_{Sa} mutant strain (NE838) carries a \textit{bursa aurealis} transposon insertion at 816475 that disrupts the synthesis of \textit{hpf}_{Sa}. Complementing plasmid pL150hpf was constructed by PCR to amplify the promoter and coding region of \textit{hpf}_{Sa} with primers P651 (5'-CGG GAT CCA TAC AAC TGG ATT AAC ATC TCG TGC AGG GTG-3') and P627 (5'-TGA AGC TTT AAA CTT AAT TTA TTG TTC ACT AGT TTG AAT CAA GCC-3') and the genomic DNA of JE2 as a template. The PCR fragment was then cloned into the BamHII and HindIII sites of pLI50 (29). The overexpression of recombinant His\textsubscript{6}-tagged HPF on pET28a (Novagen) in \textit{E. coli} BL21(DE3) has been previously described (26). To prepare ribosomal protein S7 and L22 antibodies, the \textit{rpsG} region was PCR amplified using primers P524 (5'-GCC ATA TGC CTC GTAAAG GAT CAG TAC CTA-3') and P525 (5'-CGG TCG AGT TAC CAA CGG TAG TGA GCA AAT GCT-3'). The \textit{rplV} region was PCR amplified using P522 (5'-GCC ATA TGC CTC GTAAAG GAT CAG TAC CTA-3') and P525 (5'-CGG TCG AGT TAC CAA CGG TAG TGA GCA AAT GCT-3') and the genomic DNA of JE2 as template. The \textit{rpsG} fragment was cloned into the NdeI and XhoI sites, whereas the \textit{rplV} fragment was cloned into the Nhel and XhoI sites of pET28a and overexpressed in \textit{E. coli} BL21(DE3). The N-terminally His\textsubscript{6}-tagged proteins were purified under native conditions as previously described (26). Polyclonal anti-S7 and anti-L22 were raised in rabbits (Josman, LLC). Polyclonal anti-\textit{HPF}_{Sa} was prepared as previously described (26). \textit{S. aureus} strains were routinely grown in tryptic soy broth (TSB, BD Difco), brain heart infusion (BHI, BD Difco), or chemically defined minimal medium (30) supplemented with chloramphenicol (10 \mu g/ml) when necessary. \textit{E. coli} bearing pET28a derivatives was grown in LB supplemented with 50 \mu g/ml kanamycin. All strains were cultured at 37°C unless otherwise noted.

**Stress assays**

\textit{S. aureus} strains were grown in tryptic soy broth (TSB) or minimal medium (MM) at 37°C until the mid-log or stationary phase, followed by exposure to various concentrations of stressors. Cell viability was measured at 1, 2 and 4 hr after stress exposure by enumerating colony-forming units (CFU). Cells treated with antimicrobials were washed twice with 1 ml of sterile 1x phosphate buffered saline (PBS) to eliminate carryover; the cells subsequently underwent serial dilution and were spotted on TSB agar plates. The final concentrations of stressors were as follows: rifampicin (5–50 \mu g/ml), mitomycin (0.5–2 \mu g/ml), kanamycin (5–50 \mu g/ml), streptomycin (5–50 \mu g/ml), tetracycline (50–200 \mu g/ml), 5% ethanol and hydrogen peroxide (5–20 mM). Cell survival was also evaluated under conditions of low pH (pH 4.3), osmosis (2.5 M NaCl), heat shock (52°C) and cold shock (10°C).

**Purification of recombinant protein \textit{HPF}_{Sa}**

Recombinant \textit{HPF}_{Sa} variants were purified with nickel affinity chromatography (MCLAB) under native conditions according to the manufacturer’s manual with the following modifications: sodium phosphate buffer was replaced by 25 mM HEPES (pH 7.5) and NaCl was substituted with 500 mM KCl. Eluted proteins were stabilized in 25% (v/v) glycerol. His\textsubscript{6}-tag was removed by the thrombin-CleanCleave Kit (Sigma) in 1x cleavage buffer (20 mM HEPES (pH 7.5)/14 mM MgOAc) and incubated at room temperature overnight. The cleaved products were collected by centrifugation at 500 × g for 2 min.

**Preparation of salt-washed ribosomes**

The \textit{hpf}_{Sa} mutant was grown in 1 l of BHI at 37°C until \textit{OD}_{660} = 0.6. The cells were poured over an equal volume of sterile ice cubes and harvested by centrifugation at 20,000 × g for 2 min.
11 540 ×g (Beckman JLA 8.1 rotor) for 20 min at 4°C. The cell pellets were washed once with buffer A (20 mM HEPES (pH 7.5)/14 mM MgOAc/100 mM KOAc/6 mM 2-marcaploethanol/0.5 M PMSF) and then resuspended in buffer B (20 mM HEPES (pH 7.5)/14 mM MgOAc/100 mM KOAc/1 mM DTT/0.5 mM PMSF) to a concentration of 0.5 g wet cells/ml. The cell pellet was flash-frozen in liquid nitrogen and pulverized on a cryomiller (Retch MM400) using eight 3 min cycles at 15 Hz in a 10-ml grinding jar with a 15-mm grinding ball. Grinding jars were re-chilled between each cycle in liquid nitrogen. Milled cells containing liquid nitrogen were placed in a −80°C freezer to allow for evaporation. The resulting milled cells were thawed in a 30°C water bath for 5–8 min and then immediately placed in an ice bath for 10 min. The lysate was centrifuged at 20 000 ×g for 10 min at 4°C. Clarified lysate was recovered and spun at 20,817 ×g at 4°C for 5 min to remove residual debris. To remove factors associated with ribosomes, equal volumes of lysate and 2× high-salt buffer (28 mM MgOAc/1 M KOAc/40 mM HEPES (pH 7.5)/1 mM EDTA) were mixed and incubated at 4°C on a rotator for 1 h. The mixture was layered on 1 M sucrose composed of 1× high-salt buffer and spun in a Beckman TLA-100 rotor at 4°C and 435 000 ×g for 30 min. The ribosome pellets were resuspended in 1× volume of buffer B. The ribosome concentration was determined with a NanoDrop spectrophotometer (Thermo Scientific) and was calculated using 1 Abs260 = 23 pmol/ml 70S ribosome (31).

**Crude lysate preparation and sucrose density gradient fractionation**

TSB cultures at 37°C were harvested at 2, 28 and 96 h. Crude lysate was prepared by cryomilling as described above. Five Abs260 units of lysate were layered on 5–25% sucrose gradients made in buffer C. Ultra-centrifugation, fractionation and immunoblotting were as described above.

**Ribosome profiling**

Exponentially grown *S. aureus* were harvested at OD_{600} = 0.4–0.5 after 2 min pre-treatment with 100 μg/ml of chloramphenicol. Ribosome footprints were prepared exactly as described (26), except that Ribo-Zero rRNA removal kit (Epicentre) was used in place of the MICROBExpress (Ambion). Raw FastQ sequencing data were processed using a locally installed Galaxy platform. The rRNA-less reads were aligned to the USA300_FPR3757 reference genome (GenBank CP000255.1) using Bowtie v.0.12.0. The alignment .map files were used as inputs for the modified Python scripts (32). The normalized ribosome densities, measured as reads per million reads (RPM), were visualized in MochiView (33). The gene expression levels (reads per kilobase per million mapped reads, RPKM) were calculated using Python scripts (32). Translation efficiency (TE) was calculated as the relative number of ribosome footprints to mRNA-seq reads in log2 ratios (34). Sequencing data were deposited in the NCBI GEO database with accession number GSE74197.

**Hybrid in vitro translation**

The hybrid in vitro translation system consists of the salt-washed *S. aureus* HPFSa ribosome and purified translation factors from PURExpressRibosome Kit (New England BioLabs). Ribosomes were pre-incubated with or without HPFSa in 1x binding buffer at 37°C for 30 min. HPFSa targets were translated by programming T7 promoter-containing DNA templates (final 10 ng/μl), 10 μCi Tran35S-label (MP Biomedicals) and 0.8 μM of ribosome/HPFSa, or ribosome alone premix. After 1 hr incubation at 37°C, protein samples were precipitated by 4× volumes of acetone, resolved on 4–20% TGX SDS-PAGE (BioRad) and autoradiographed.

**RESULTS**

HPFSa is required for long-term viability of *S. aureus*

The phenotypic manifestations of hpfsa, remain unexplored, and studies from other bacterial counterparts point to hibernating ribosomes playing a role in the stress response. We subjected wild-type and hpfsa mutant strains to a variety of agents that are commonly used to assess virulence. All of the tested stressors, including hydrogen peroxide, high salt, low pH, transcription inhibition (rifampicin), DNA damage induction (mitomycin) and ribosome-targeting antibiotics (kanamycin and tetracycline), sensitized the wild-type and mutant cells equally, regardless of the dosage, bacterial growth phase or medium type. However, we found that the viability of the hpfsa mutant declined rapidly in long-term minimal medium (MM) culture and eventually lost >95% of cell viability at day eight, yielding approximately 400-fold fewer CFU than the wild-type strain (Figure 2A). A similar downturn was observed in rich tryptic soy broth (TSB)
Figure 2. HPFSa mediates long-term cell survival. (A) Cell viability in minimal medium (MM) over an 8-day period. (B) Cell viability in tryptic soy broth (TSB) over a 6-day period. Viability was assessed by enumerating colony-forming units (CFU) from wild-type, hpf null and complemented strain carrying pLI50hpf. Error bars indicate the standard deviation of three independent experiments. An arrow denotes the detection limit.

medium, although cell death was less pronounced (approximately 10-fold) than in MM (Figure 2B). In both cases, complementation of the hpfSa mutant with a plasmid-borne copy of hpfSa under control of its native promoter fully rescued the survival defect, confirming that hpfSa is essential for S. aureus long-term viability.

Loss of hpfSa sharply reduces the intact ribosome pool during the stationary phase

To investigate the link between 100S ribosome formation and the reduced survival rate in the hpfSa mutant, we monitored changes in ribosome species during stationary culturing by sucrose density gradient fractionation. Crude ribosomes were prepared from cells grown at different stages and extracted by cryomilling, a method known to faithfully preserve ribosome complexes and translational states (26,35,36). Consistent with previous studies (23,26), we observed an accumulation of 100S ribosomes in rich medium during early logarithmic growth (OD600 ∼ 0.3–0.4) (Figure 3 (top left), S1A). Western blots showed that the majority of HPFSa was associated with the 100S complex and 70S ribosome (Figure 3 (top left)). In the complemented strain in which plasmid-borne HPFSa was expressed approximately 10-fold more than wild type, the 100S ribosome peak was substantially elevated and a fraction of HPFSa also bound to the 30S subunits (Figure 3 (top right)). Curiously, the increased production of 100S ribosomes did not significantly slow bacterial growth over 24 h in the TSB culture (Supplementary Figure S1A). During the stationary phase, a substantial amount of the ribosome pool diminished in the wild-type and complemented strains (Figure 3 (middle and bottom)), presumably due to massive ribosome degradation to provide cells with energy and nutrients. The disappearance of the 100S complex and the basal 70S ribosome level in these strains suggested that 100S particles may have been redistributed to form translationally competent ribosomes. The exact sequence of 100S to 70S inter-conversion is unclear. In contrast, most of the ribosomes in the hpfSa mutant were degraded, which coincided with the onset of cell death in prolonged culture (Figure 2B). Free 30S and 50S subunits are more prone to ribonuclease attack than the 70S ribosome because of their exposed rRNA interface (37). The accumulation of 70S dimers in the wild-type cells may thus serve as a nuclease-resistant ribosome repository.

HPFSa alone is sufficient to induce the formation of 100S ribosomes in vitro

To confirm that HPFSa is the sole contributor to the formation of 100S ribosomes in vivo, we recapitulated 70S ribosome dimerization in vitro using purified HPFSa and S. aureus ribosomes isolated from the hpfSa null strain. The ribosomes were subjected to a high-salt wash to remove any associated factors. Hexahistidine-tagged HPFSa was overexpressed in E. coli and purified by nickel affinity chromatography. The affinity tag was removed by thrombin cleavage because 6His-HPFSa was prone to aggregation, possibly due to misfolding, and was nonfunctional in 100S complex formation (Figure 4A). Purified HPFSa was incubated with high-salt washed ribosomes at different molar ratios, and the ribosome profiles were obtained by sucrose density gradient fractionation. At a 1:1 ribosome to HPFSa ratio, HPFSa induced the dimerization of 70S ribosomes to a level comparable to that observed in vivo (Figure 3 (top left), 4B (middle)). Increasing the amount of HPFSa did not enhance dimerization, although immunoblotting showed that the protein effectively bound to the 70S ribosome. In fact, we found that a modest increase of the ribosome to HPFSa ratio to 1:2 completely abrogated dimerization but did not affect ribosome association (Figure 4B, bottom). The observation that a higher concentration of HPFSa is counterproductive could be attributable to HPFSa self-oligomerization or and non-specific binding of HPFSa to the ribosome when HPFSa is in large molar excess. Nevertheless, our data demonstrate that optimal in vitro formation of the 100S ribosome occurs when HPFSa and the ribosome are present at a 1:1 molar ratio, which is in agreement with previous in vivo estimation of the E. coli 100S ribosome (23).
Ribosome dimerizing activity of HPF_{Sa} is highly sensitive to perturbation mutagenesis

Our \textit{in vitro} dimerization results showed that HPF_{Sa} binding to the ribosome is not sufficient to promote 100S formation. The result prompted us to investigate the domains that are critical for dimerization. HPF_{Ec} adopts a \beta\alpha\beta\beta\alpha\alpha fold, with two basic patches each on the two \alpha-helices (Supplementary Figure S2) (4). These positively charged residues are conserved in all HPF and YfiA homologs (Figure 1) and have been implicated in direct interactions with the 16S rRNA bases at the decoding channel (4,5). \textit{In vitro} dimerization was repeated using purified HPF_{Sa} mutant proteins harboring the desired mutations in the basic patches (Figure 4A). Although the double mutation K27A/R30A on the first \alpha-helix did not completely impair dimerization, the double mutation K84A/R87A on the second \alpha-helix did but did not abolish ribosome binding, as a large fraction of mutant HPF_{Sa} was still associated with the 70S ribosomes (Figure 5A and B). To abrogate HPF_{Sa} binding to the ribosome, concurrent mutations (K27A/R30A/K84A/R87A) on both helices were required, as judged by the sedimentation of mutant HPF_{Sa} in the light sucrose fraction (Figure 5B, bottom). Notably, several mutations outside the \alpha-helices did not perturb 70S dimerization (Supplementary Figure S3). HPF_{Sa} contains a long C-terminal tail that is absent in HPF_{Ec} (Figure 1). Deletion of the last 42-amino acid segment reduced ribosome binding, whereas a larger truncation (up to 90 amino acids) exacerbated the binding defect further (Supplementary Figure S4). The data suggest that the basic residues on the second \alpha-helix (K84/R87) are critical for dimerization, whereas both \alpha-helices and the C-terminal tail are important for ribosome binding and/or the stabilization of binding.
Loss of *hpfSa* causes unusual ribosome occupancy around start codons under nutrient-limited conditions

The paradoxical observation that 100S ribosomes are prevalent in *S. aureus* growing logarithmically in rich culture medium with a growth rate that is unaffected by HPF*Sa* loss (Supplementary Figure S1A, 3 (top)) is inconsistent with the notion that HPF acts as a global ribosome inhibitor (4,20,22). It is conceivable that ribosomes are in excess in rich medium, and the subpopulation of 100S ribosomes is unable to titrate the bulk of ribosomes from normal translation, resulting in a lack of growth defect. It may be that the 100S ribosome is simply a quiescent product to conserve ribosome and has no direct role in regulating translation. Alternatively, the formation of 100S ribosomes may downregulate the translation of only a fraction of mRNAs that are not essential for exponential growth when nutrients are plentiful. This prediction is supported by the observation that when nutrients were limited, both wild-type and complemented strains were delayed in entering the stationary phase compared with the *hpfSa* mutant (Supplementary Figure S1B). It seems plausible that HPF*Sa* directly participates in translational repression and leads to a slower growth rate in minimal medium. To test this model, we performed a deep sequencing-based ribosome profiling analysis to compare the translatomes of exponentially growing wild-type and *hpfSa* mutant strains in both minimal and rich media. Ribosome profiling captures a global snapshot of ribosome positioning and density on template mRNAs with single-nucleotide resolution (34,36). A high number of ribosome-protected footprints (RPFs) mapping to the transcriptome (mRNA-seq) at a unique position or being distributed across the transcript is indicative of ribosome stalling or active translation, respectively.

Our initial attempt to construct RPF libraries from stationary phase cells was unsuccessful. We opted for mid-log cells, as the 100S ribosomes are already prevalent at this stage. Exponentially grown *S. aureus* were collected at OD600 0.4–0.5. Unlike other cell lysate preparations described in this study, the cells subject to ribosome profiling were pretreated with 100 μg/ml chloramphenicol for 2 min to stabilize the polysomes (translating ribosomes) before harvest (Supplementary Figure S5). Consistent with previous observations (26), the addition of chloramphenicol appears to trap HPF*Sa* in all ribosomal fractions (Supplementary Figure S5). Cryogenic cell disruption, construction of cDNA libraries and data analyses have been previously described in detail (26,32,36). A total of 16 RPFs and total mRNA-seq libraries were generated from the wild-type and *hpfSa* mutant strains, representing two independent biological replicates from rich and minimal media (Supplementary Table S1).

To obtain an overview of global differences in ribosome density at the beginning and end of open reading frames (ORFs), metagene analysis was performed to analyze the distribution of ribosomes along an average transcript of the most abundantly expressed genes (≥50 reads/transcript). Elevated ribosome density around the start and stop codons corresponds to slower kinetics of initiation and termination than those of the elongation rate (36). Based on the prediction that HPF*Sa* inhibits translation, we expected that...
Figure 5. Mutations that affect HPFSa-mediated 100S dimer formation and binding to the ribosome. (A) Sucrose density gradient (5-25%) profiles of salt-washed ribosomes reacted with an equimolar ratio of purified HPFSa and its mutant variants. (B) Western blots showing the association of HPFSa in each ribosomal fraction. The 30S ribosomal subunit protein S7 serves as a control. A K27A/R30A double mutant has reduced 100S dimer formation. A K84A/R87A double mutant impairs dimerization but not binding. The quadruple mutant abolishes binding.

loss of HPFSa would lead either to clustering of ribosomes at the very N-terminus of ORFs, reflecting derepression of translation initiation, or to disappearance of occupancy at unique positions within ORFs, suggesting a relief of elongation arrest, or to an increase in ribosome occupancy along the transcripts, indicating elevated translation efficiency. As predicted, the loss of HPFSa disproportionately increased ribosome occupancy around start codons transcriptome-wide in cells grown in minimal medium relative to the wild-type (Figure 6A, Supplementary Figure S6). Unexpectedly, despite a feature that suggests higher translation initiation, downstream translation in the hpfsa mutant did not increase to a level that positively correlates with the elevated ribosome density at the initiation site (Figure 6A, top). The unusual accumulation of ribosomes at the 5′-end could be an artifact derived from the chloramphenicol treatment as a recent study showed that the addition of translation inhibitors could distort ribosome coverage profiles in eukaryotes (38). This is highly unlikely in our case because the same elevated ribosome density was not observed in rich medium samples (Figure 6A, bottom) and MM-grown WT samples (Figure 6A, top). In rich TSB medium, a modest increase of ribosome density along the entire transcript was observed in the hpfsa mutant, suggesting moderate inhibition of translation by HPFSa (Supplementary Figure S6).

hpfsa null mutation increases translational efficiency of a subset of mRNAs

To evaluate how efficiently an individual transcript was translated, we calculated the TE in the wild-type and mutant strains by dividing ribosome footprints by mRNA-seq density (Figure 6B). Plotting the TEs from the hpfsa mutant against WT provides information about the changes in ribosome occupancy between strains. An improvement
Figure 6. Effects of \( \text{hpf}_{Sa} \) null mutation on global translome in minimal and rich culture conditions. (A) Ribosome density as a function of position. Metagene analysis of read densities in minimal medium (MM, top) and tryptic soy broth (TSB, bottom) samples. The normalized reads per million mapped reads (RPM) correspond to the average ribosome density across the most abundantly translated ORFs (\( >50 \) reads), which were aligned relative to the start and stop codons. Ribosome density was drastically elevated around start codons in the \( \text{hpf}_{Sa} \) mutant grown in MM, whereas such an increase was not observed in TSB culture. (B) Comparison between translation efficiency (TE) in the wild-type and \( \text{hpf}_{Sa} \) mutant in MM (left) and TSB (right). Each dot corresponds to a single gene. Genes with TE improvement upon \( \text{hpf}_{Sa} \) loss are shown in red. TE was moderately increased in the \( \text{hpf}_{Sa} \) mutant and more pronounced in MM cultures. TE was calculated as the log\(_2\) ratios of the RPFs to the mRNA reads that were measured in RPKM.
of hpfSa, TE will be indicated by a log2 TE value of hpfSa (x-axis) that is larger than the log2 TE value of the wild-type (y-axis), whereas high R² correlations suggest that TE is less affected by the loss of hpfSa. In general, TE in most genes remained unchanged between the wild-type and mutant. TE improvement in the hpfSa mutant was more pronounced in MM than in TSB culture (R² = 0. 67 (MM) and 0.81 (TSB)). Consistent with the metagene analysis, we found that genes displaying high ribosome density at the 5'-end were not always correlated with the overall improvement of TE. As a result, fewer than 10% of the actively transcribed mRNAs exhibited a significant TE increase (2-fold) in the absence of HPFSa (Supplementary Tables S2 and S3). Thus, the loss of hpfSa appears to have a modest impact on overall protein output during exponential growth.

**HPF**<sub>Sa</sub>**-mediated translational inhibition in vitro**

To determine the effects of HPF<sub>Sa</sub> on the protein yield from transcripts that were identified from ribosome profiling, we used a cell-free hybrid translation system that combines purified *E. coli* translation factors and ΔhpfSa ribosomes. Because the sequence context at the 5'-end might influence the translation efficiency of individual genes, we preserved the native Shine-Dalgarno (SD) sequence and start codon in T7 promoter-driven DNA templates. Representative genes whose TEs were elevated by >2-fold were randomly selected based on known function (Figure 7A and B). Translation reactions were programmed with <sup>35</sup>S-methionine and various molar ratios of ribosomes to HPF<sub>Sa</sub>. The protein production varied because each template carried a different translation initiation signal sequence. The intensities of the anticipated protein products were quantitated by normalization to an ~17 kDa non-specific band, whose levels remained constant in a minus template control and throughout HPFS<sub>a</sub> titration (Figure 7C). The non-specific band did not appear to be aminoacyl-tRNAs as the labile ester bond would be hydrolyzed on the alkaline SDS-PAGE. In brief, the inhibition of translation by HPFS<sub>a</sub> could be recapitulated in vitro, but the effects were less pronounced than the in vivo ribosome profiling effects, presumably due to subtle incompatibility of the purified E. coli components with the *S. aureus* ribosome. The inhibition was specific to HPF<sub>Sa</sub> targets because the same inhibition was not seen with the non-target *prmA* or the heterologous *E. coli dhfr*.

**DISCUSSION**

The bacterial 100S ribosome was first observed more than five decades ago (40,41). Its function has remained obscured despite its pervasiveness at different bacterial growth phases. Likewise, little is known about the biochemical properties of the factors (e.g. HPF, RMF) that promote 100S formation. Here, we integrate biochemistry and ribosome profiling to characterize the biological and biochemical features of HPF<sub>Sa</sub> in the opportunistic pathogen *S. aureus*. HPF<sub>Sa</sub> is a peculiar homolog in that it is unusually long, is constitutively expressed, and is functionally equivalent to RMF<sub>Ec</sub> and HPF<sub>Ec</sub> combined. Whereas 100S formation in *E. coli* is stationary phase-specific, it occurs in *S. aureus* as early as the post-lag phase. We show that the loss of hpfSa causes massive ribosome breakdown upon entering the stationary phase that correlates with the onset of cell death. It seems likely that 100S ribosomes provide a ’readiness plan’ in advance of future growth constraints when they can be redistributed for translation reinitiation (Figure 8). Beyond their role as a ’reservoir’, the formation of 100S ribosomes in logistically growing cells reduces the translation efficiency of a fraction of genes. Furthermore, structure-guided mutagenesis suggests important roles of the C-terminal segment of HPF<sub>Sa</sub> in ribosome binding and of the conserved basic residues in dimerization.

Although the 100S ribosomes are prevalent in bacteria, translationally silent ribosomes in eukaryotes exist as 80S monomers held by the clamping protein Stm1 (43). The mammalian equivalent of 100S, a dimer of 80S monomers (110 complex), occurs infrequently except under nutrient starvation conditions (42).

The rapid degradation of ribosomes in *hpf* null mutants has not been reported. The degradation of *S. aureus* ribosomes likely involves RNase III and RNase J1/J2 (44), as implicated by the accumulation of 100S dimers in *Streptomyces rnc* and *rnpj* mutants (45). Curiously, decay of the GFP-tagged *E. coli* ribosome is not aggravated in *hpfEc* and *hpfSa* mutants. Under our experimental conditions, the *S. aureus* USA300 strain enters the stationary phase much earlier than do other Gram-positive bacteria (10,22,24). The differences are likely due to the choice of medium, culture conditions and strains. The strain-specific abundance of 100S ribosomes has been observed in *E. coli* (47). In our hands, *S. aureus* RN4200 and N315 produce 30% more 100S ribosomes than the strain (USA300) we used in this study under the same conditions.

We propose a model for the role of HPF<sub>Sa</sub> (Figure 8). In minimal medium, the translation of a fraction of genes is suppressed because a subpopulation of ribosomes is sequestered away from active translation, leading to a slower growth rate. The reason underlying the specific targeting of mRNA has yet to be determined. It is possible that some mRNAs are more sensitive to altered cellular concentrations of active ribosomes; e.g. the translation of genes with highly structured mRNA or weaker SD sequence may require higher concentrations of ribosome. A loss of hpfSa liberates the ribosome for translation initiation but only partially increases the overall translational output, presumably due to low cellular concentrations of charged-tRNAs and other translation factors. Both wild-type and hpfSa null strains undergo ribosome degradation, but the former retains a fraction of the functional ribosomes that are derived from the 100S pool (Figure 8A). The process by which 100S dimers are disassembled and converted to active 70S ribosome is unclear, although the participation of the initiation factors IF1 and IF3 and ribosome recycling factor RRF have been implicated in *E. coli* (4,11,14). In rich medium, excess unused ribosomes are preserved as silent 100S dimers. The formation of the 100S complex does not significantly affect wild-type cell growth but is important for viability during the stationary phase, when the ribosome pool is low. The absence of HPF<sub>Sa</sub> increases ribosome occupancy but only modestly upregulates translation efficiency (Figure 8B). The reason is unclear, although it is possible that elevated ribosome occupancy causes ribosomal traffic
Figure 7. In vitro translation with S. aureus ribosomes recapitulates HPF$_{Sa}$-mediated translational repression. (A) Ribosome density profiles showing the strong increase in ribosome occupancy around start codons of selected genes in the hpf$_{Sa}$ mutant grown in minimal medium (MM). (B) Ribosome density plot of genes derived from tryptic soy broth (TSB) samples that exhibit an overall increase in ribosome occupancy along the gene body. (C) In vitro translation validating the repression of translation in the genes shown in panels A and B. Translation products were resolved on an alkaline Tris-glycine-based 4–20% TGX SDS-PAGE. An asterisk depicts the non-specific band used as an internal reference for intensity quantitation. A cursor indicates a second non-specific band that was fortuitously intensified in a specific translation reaction. The predicted gene product of SAUSA3000432 is a 50 kDa sodium transporter; it runs aberrantly faster on SDS-PAGE, which is not uncommon for membrane proteins.

Jams, which in turn may perturb polysome organization (48) and limit translation.

Although an increase in ribosome density at the 5’ regions of coding sequences suggests elevated translation initiation, it is possible that secondary effects in the absence of hpf$_{Sa}$, such as proteotoxic stress, may lead to ribosome stalling at 5’ proximal regions (49). Why is only a subset of genes derepressed in the hpf$_{Sa}$ null strain, particularly at the initiation phase? These genes do not belong to a specific functional category, although several important virulence factors are among the list (Supplementary Tables S2 and S3). No correlation with gene length or the mRNA’s abundance was observed. Sequence context, mRNA secondary structure and codon usage bias may shape the expression levels of individual genes (50). Moreover, the first 50 codons have been shown to dictate translation efficiency (51). Our attempts to identify unique sequence signatures upstream and downstream of the start codons were unsuccessful, although we noted that the spacer between the SD sequence and start codon in HPF$_{Sa}$ targets is highly enriched in uracil in contrast to the non-targets’ enrichment in adenine. Furthermore, mRNA secondary structures that occlude the SD sequence and initiation site appear to be overrepresented in
Figure 8. A model depicting the role of HPFSa. (A) In minimal medium, HPFSa-mediated formation of 100S dimers causes a reduction of translation capacity, and the cells grow slower than the hpfSa mutant. Upon entering the stationary phase, 100S dimers in the wild-type are converted to recyclable ribosome subunits to support a viable state, whereas all post-termination ribosomes in the hpfSa mutant are degraded, leading to cell death. (B) In rich medium, the sequestration of excess ribosomes in the form of 100S complexes does not affect cell growth, but the translation efficiency of a small set of genes is reduced. hpfSa mutation increases the active ribosome pool and increases the ribosome occupancy of specific genes. Hyperactive translation in the hpfSa mutant leads to rapid degradation of ribosomes during the stationary phase. A black circle denotes HPFSa and a star denotes an unknown factor that splits the 100S dimer.

the HPFSa-affected genes. The significance of these features remains to be determined.

The formation and stability of 100S ribosomes are sensitive to ionic strength and magnesium concentration (21). The binding of RMFEc and HPFEc induces a structural rearrangement of the 30S subunit head domain that is necessary for dimerization (4). Whereas dimerization is impaired by minor perturbation of the conserved basic patches of HPFSa, ribosome binding is abrogated when a large deletion or concurrent mutations on the two predicted α-helices are introduced. These data imply that HPFSa interacts with the ribosome at multiple sites and that a single-site defect does not abolish binding. Likewise, dimerization does not occur prior to the stabilization of ribosome binding. Previous work (10) has shown that the L. lactis HPF11 C-terminal tail (residues 126–185) is functionally equivalent to that of RMFEc. Truncation of this region does not impair ribosome binding, which is completely opposite to HPFSa. These differences suggest some idiosyncrasy of long-form HPF homologs in interacting with their cognate ribosomes.

Our data imply that HPF/100S ribosomes serve two purposes in S. aureus. The first is to suppress the translation of specific mRNA through titrating active ribosomes concomitantly with reducing energy consumption. The second is to avoid rapid ribosome turnover by dimerizing 70S monomers so that the dimers can be dissociated and reassembled into active ribosomes during stationary phase. Bacterial persistence has been linked to the suppression of macromolecule biosynthesis and translation (52–54) and S. aureus is particularly adept at establishing long-term survival in the host, which leads to recalcitrant and relapsing infections (55). Given that the hpfSa null strain is non-viable in long-term minimal medium culture, strategies that disrupt the 100S ribosome may vastly synergize the efficacy of conventional antibiotics and may serve as a new antistaphylococci drug target to treat recalcitrant infections.

ACCESSION NUMBER
NCBI GEO GSE74197.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank David Gohara and Hye-Sook Kim for providing analysis tools. S. aureus USA300 derivatives were acquired from the NIH/NIAID-funded Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) for distribution by BEI Resources.

FUNDING
National Institutes of Health [R00GM094212 to M.N.Y.]; Edward Mallinckrodt Jr. Foundation; Saint Louis University faculty start-up fund. M.N.Y. is a Pew Scholar in
the Biomedical Sciences, supported by the Pew Charitable Trusts. Funding for open access charge: The Pew Charitable Trusts [00002920].

Conflict of interest statement. None declared.

REFERENCES


