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Authors: Tatsuya Akiyama, Kerry S. Williamson, Robert Schaefer, Shawna Pratt, Connie Chang, & Michael J. Franklin

This is a postprint of an article that originally appeared in Proceedings of the National Academy of Sciences of the United States of America on March 2017. https://doi.org/10.1073/pnas.1700695114.

Akiyama T, Williamson KS, Schaefer R, Pratt S, Chang CB, Franklin MJ, "Resuscitation of Pseudomonas aeruginosa from dormancy requires hibernation promoting factor (PA4463) for ribosome preservation," Proceedings of the National Academy of Sciences of the United States of America, 2017 March 21; 114(12):3204-3209. doi: 10.1073/pnas.1700695114.

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Resuscitation of *Pseudomonas aeruginosa* from dormancy requires hibernation promoting factor (PA4463) for ribosome preservation

Tatsuya Akiyama, Kerry S. Williamson, Robert Schaefer, Shawna Pratt Connie B. Chang, and Michael J. Franklin

Pseudomonas aeruginosa biofilm infections are difficult to treat with antibiotic therapy in part because the biofilms contain sub-populations of dormant antibiotic-tolerant cells. The dormant cells can repopulate the biofilms following alleviation of antibiotic treatments. While dormant, the bacteria must maintain cellular integrity, including ribosome abundance, to reinitiate the de novo protein synthesis required for resuscitation. Here, we demonstrate that the P. aeruginosa gene PA4463 [hibernation promoting factor (HPF)], but not the ribosome modulation factor (PA3049), is re-quired for ribosomal RNA preservation during prolonged nutrient starvation conditions. Single-cell–level studies using fluorescence in situ hybridization (FISH) and growth in microfluidic drops dem-onstrate that, in the absence of hpf, the rRNA abundances of starved cells decrease to levels that cause them to lose their ability to resuscitate from starvation, leaving intact nondividing cells. P. aeruginosa defective in the stringent response also had reduced ability to resuscitate from dormancy. However, FISH analysis of the starved stringent response mutant showed a bimodal re-sponse where the individual cells contained either abundant or low ribosome content, compared with the wild-type strain. The results indicate that ribosome maintenance is key for maintaining the ability of P. aeruginosa to resuscitate from starvation-induced dormancy and that HPF is the major factor associated with P. aeruginosa ribosome preservation.

dormancy | resuscitation | hibernation promoting factor | ribosome | rRNA

Biofilms are communities of microorganisms that are attached to surfaces through their secreted extracellular polymeric substance material (1, 2). Biofilms are found in most aqueous environments but become problematic when associated with in-fectious diseases (3). In particular, bacteria growing in biofilms on host tissue or artificial implant devices are difficult to eradi-cate with antibiotic treatments and often result in chronic in-fections (4). For example, Pseudomonas aeruginosa growing in biofilms on pulmonary tissue is associated with chronic infections of cystic fibrosis (CF) patients (5). Even though the population of P. aeruginosa associated with biofilm infections can be reduced with antibiotic treatments, it is rarely eliminated. Results of lon-gitudinal genomics studies of P. aeruginosa strains infecting CF pulmonary tissue show that strains within a patient are usually clonal over time (6, 7), suggesting that even though antibiotics reduce the bacterial loads of pulmonary biofilms, clones of the original infecting strains are able to re-emerge and establish new biofilm infections. One mechanism for enhanced tolerance of biofilm-associated bacteria to antibiotics is that biofilms contain heterogeneous populations of cells, including subpopulations of cells that are tolerant of the treatments (8, 9).

Bacterial heterogeneity in biofilms may arise by several mechanisms (10) including adaptation to local environmental conditions. Cells within regions of the biofilm with low nutrients or oxygen may enter a slow-growth or dormant state. Because antibiotics generally target active metabolic functions, dormant bacteria are tolerant of most antibiotic treatments. The dormant bacteria may then resuscitate and repopulate the biofilms fol-lowing alleviation of antibiotics. Supporting this mechanism for biofilm-associated antibiotic tolerance, in prior research, we differentially labeled P. aeruginosa cells with the green fluores-cent protein (GFP) and sorted them based on their metabolic activity (9). In those studies, the slow-growing P. aeruginosa cells in biofilms were tolerant to ciprofloxacin or tobramycin at con-centrations 10-fold greater than their minimum inhibitory con-centrations, whereas the active bacteria were killed by those antibiotics. We also used transcriptomics in combination with laser capture microdissection to identify mRNA transcripts that were abundant in the different biofilm subpopulations (9). As expected, most mRNA transcripts were in low abundance in the dormant subpopulation. However, the slow-growing antibiotic-tolerant subpopulation had a high abundance of mRNA tran-scripts for several genes, including PA4463 [a homolog to the Escherichia coli hibernation promoting factor (HPF)]. In E. coli, HPF along with ribosome modulation factor (RMF) inactivates ribosomes during stationary phase (11).

RMF and HPF have been well characterized in E. coli as ribosome-interacting proteins (12–15). RMF binds to the ribo-some near the mRNA exit tunnel on the 30S ribosomal subunit,

Significance

The dormant subpopulations of Pseudomonas aeruginosa biofilms are linked to chronic infections because dormant cells tolerate antibiotic treatment and then repopulate the infec-tions when conditions become favorable. Dormant cells must maintain cellular integrity, including preformed ribosomes, to resuscitate. The small-ribosome–binding proteins, ribosome modulation factor, and hibernation promoting factor (HPF) have evolved to maintain ribosomes in an inactive state. Using both population and single-cell–level studies, we show that HPF provides the primary mechanism used by P. aeruginosa to maintain ribosome integrity during dormancy, and that HPF is required for optimal P. aeruginosa resuscitation from dor-mancy. Preventing regrowth of the dormant subpopulation by targeting HPF may provide an effective means for eliminating the dormant subpopulations of P. aeruginosa infections.

and HPF binds at the channel of the 30S ribosomal subunit where tRNA and mRNA bind, thereby inhibiting translation (12, 16, 17). RMF and HPF also cause conformational changes to the ribosome, which results in dimerization of two ribosomes to form an inactive 100S form (18). Ueta et al. (11) developed a model for ribosome inactivation during stationary phase of *E. coli*. They concluded that RMF binds the ribosome, forming an inactive 90S dimer, and that HPF stabilizes the ribosome in an inactive 100S form. *E. coli* also encodes an HPF paralog, YfiA, that inactivates the 70S ribosome, but inhibits the formation of the 100S dimer (11).

Homologs to RMF and HPF are found in many bacterial taxa, but vary depending on the organism. *E. coli* and most other gamma Proteobacteria have genes for *rmf*, *hpf*, and *yfiA*, whereas bacteria other than the gamma Proteobacteria lack the gene for *rmf*. *Staphylococcus aureus* does not encode *rmf*, but has an *hpf* with an extended C-terminal tail, termed long HPF (19). Long HPF results in 100S ribosome formation in stationary-phase *S. aureus* cells, even in the absence of an RMF homolog (19). *P. aeruginosa* PAO1 contains genes for *rmf* (PA3049) and *hpf* (PA4463), but does not encode the *hpf* paralog, *yfiA* (20). The involvement of HPF and RMF on maintenance of cell viability also varies among bacterial species. In *E. coli*, RMF, but not the HPF homologs, is required to maintain cell viability during stationary phase (11, 14). *Vibrio cholerae*, which has an *rmf* and two *hpf* homologs, requires at least one copy of *hpf* to maintain cell viability during stationary phase (21). *Mycobacterium* spp. and *Listeria monocytogenes*, which encode long HPF, require HPF for cell viability maintenance during prolonged incubation and during hypoxic conditions (22, 23).

In our prior study (9), biofilms of *P. aeruginosa* PAO1 with deletions of *rmf* or *hpf* had increased uptake of the membrane-impermeable stain, propidium iodide, compared with wild-type cells, suggesting that the nutrient- or oxygen-starved *P. aeruginosa* cells may lose viability in the absence of these ribosome-interacting factors. Here, we investigated the roles of *hpf* and *rmf* in the maintenance of viability of *P. aeruginosa* undergoing nutrient-deprived conditions. Under starvation conditions, wild-type *P. aeruginosa* PAO1 maintained the ability to resuscitate for weeks with little loss of viability. Surprisingly, the Δ*rmf* mutant also had little loss of viability during extended starvation. However, the Δ*hpf* mutant was impaired in its ability to resuscitate from starvation. To investigate the molecular mechanism for impaired resuscitation of the mutant strain, we analyzed the rRNAs of the starved cells both at the population level and at the single-cell level. Unlike the wild-type cells and the Δ*rmf* mutant cells, the Δ*hpf* mutant strain lost most of its rRNA by day 4 of starvation. In addition, by using drop-based microfluidic approach, we show that most of the Δ*hpf* mutant cells were unable to divide and remained as single nonreplicating cells following extended starvation. Overall, the results demonstrate that, in *P. aeruginosa*, HPF is required to protect cells from ribosome loss during extended nutrient-deprived conditions and that ribosome protection by HPF is necessary for *P. aeruginosa* to resuscitate from dormancy.

Results

HPF, but Not RMF, Is Required for Maintenance of *P. aeruginosa* Viability Under Starvation Conditions. To characterize the physiological roles of HPF and RMF in *P. aeruginosa*, we tested the ability of *P. aeruginosa* PAO1 with Δ*hpf*, Δ*rmf*, and Δ*hpf*/Δ*rmf* mutations to resuscitate following prolonged nutrient deprivation. Wild-type and mutant cells were cultured to early stationary phase, washed in PBS, and then incubated in PBS with shaking at 37 °C. Aliquots of the cultures were sampled daily for their ability to form colonies on Tryptic Soy Agar (TSA) plates. The wild-type *P. aeruginosa* PAO1 was able to survive the starvation conditions with no apparent loss of viability ($P = 0.96$) (Fig. 1*A*). Based on results from *E. coli* (11, 14), RMF is predicted to play a role in ribosome inactivation. However, no observable phenotype with respect to recovery from starvation conditions ($P = 0.37$) was observed for *P. aeruginosa* Δ*rmf* (Fig. 1*A*). In addition, and in contrast to published results for RMF in *E. coli* (24–27), the *P. aeruginosa* Δ*rmf* mutant did not show an observable survival phenotype compared with the wild-type strain when exposed to osmotic shock, heat shock, acid stress, or sensitivity to gentamicin. In contrast, a deletion of PA4463 (Δ*hpf*) resulted in a decrease in cell recovery following starvation ($P < 0.0001$). The Δ*hpf* mutant strain initially produced 1.9×10^8 cfu × mL^{-1} after 30 min of starvation and reduced to 2.5×10^7 cfu × mL^{-1} by 5 d of starvation (Fig. 1*A*). When Δ*hpf* was complemented *in trans* (Δ*hpf* + *hpf*), survival under nutrient-limited conditions was restored to wild-type levels ($P = 0.25$), but not for the vector control strain (Δ*hpf* + VC) ($P < 0.0001$). The Δ*hpf*/Δ*rmf* double mutant also showed loss of recovery following starvation compared with the wild-type strain ($P = 0.0002$) (*SI Appendix*, Fig. S1). The Δ*hpf*/Δ*rmf* double mutant could be restored to wild-type levels of resuscitation with a plasmid containing *hpf* alone, but not with a plasmid containing *rmf* alone (*SI Appendix*, Fig. S1).

The stringent response plays a role in the ability of *P. aeruginiosa* to survive in stationary phase (28). In the stringent response, guanidine penta- and tetra-phosphate [(p)ppGpp], produced by the activities of RelA and SpoT when RelA interacts with stalled ribosomes (29), acts as a signaling molecule that induces expression of genes required for survival during stationary phase (30). We tested a Δ*relA*/Δ*spoT* mutant under the same starvation conditions as the Δ*rmf* and Δ*hpf* mutant strains. The Δ*relA*/Δ*spoT* mutant showed a similar response to starvation as the Δ*hpf* mutant, where cell recovery was impaired compared with the wild-type strain ($P = 0.03$) (Fig. 1*A*). The results indicate that HPF and the stringent response, but not RMF, is necessary for prolonged *P. aeruginosa* survival under nutrient-limited conditions.

HPF Is Required for Optimal Recovery of *P. aeruginosa* from Nutrient Starvation. During the starvation experiments, we noted that, in addition to the reduced number of cfu's for the Δ*hpf* mutant strain, the colonies that arose were heterogeneous in morphology (Fig. 1*B* and *SI Appendix*, Fig. S2). The starved wild-type strain produced large, uniform colonies. Although some colonies of the Δ*hpf* strain were similar to those of the wild type, many were small and required extended incubation time to become visible. The Δ*hpf* + *hpf* complemented strain had smaller colonies than the wild type, but the colonies were uniform (Fig. 1*B* and *SI Appendix*, Fig. S2). The Δ*relA*/Δ*spoT* strain had colony morphologies with heterogeneity in size, but did not require extended incubation time to become visible. When the small Δ*hpf* colonies were restreaked onto new TSA plates, the wild-type colony morphology was restored, indicating that heterogeneity in recovered colony size was not heritable. To determine if the reduced cfu counts following starvation of the Δ*relA*/Δ*spoT* mutant strain were due to the role of the stringent response in regulation of *hpf* expression, we introduced a plasmid copy of *hpf* under control of the isopropyl β-D-1-thiogalactopyranoside-inducible P_{trc} promoter into the Δ*relA*/Δ*spoT* strain. The Δ*relA*/Δ*spoT* + *hpf* strain had a similar survival phenotype as the Δ*relA*/Δ*spoT* strain (*SI Appendix*, Fig. S3), indicating that loss of cell viability in the Δ*relA*/Δ*spoT* mutant is not associated with loss of *hpf* expression.

HPF Is Required for Preservation of 23S rRNA During Nutrient Starvation. HPF and RMF are ribosome-associated proteins that in *E. coli* cause formation of inactive 100S ribosome dimers during stationary phase (11). To characterize the roles of RMF and HPF on ribosomes under starvation conditions of *P. aeruginosa*,

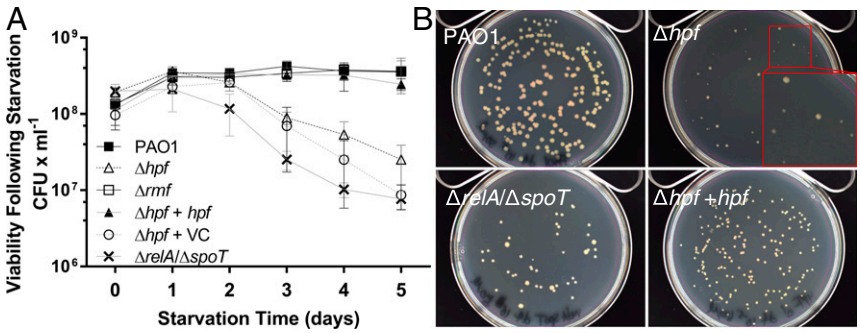

Fig. 1. Recovery of *P. aeruginosa* following extended incubation under nutrient-deprived conditions. (*A*) The cfu's on TSA agar following incubation in aerated PBS. (*B*) Colony morphology of *P. aeruginosa* on TSA, following 4 d of incubation in PBS for wild-type *P. aeruginosa* PAO1, PAO1 Δ*hpf*, PAO1 Δ*relA*/Δ*spoT*, and PAO1 Δ*hpf* + *hpf*. Colony morphologies of each strain and at each time point are shown in *SI Appendix*, Fig. S2.

we extracted total RNA from nutrient-deprived cultures and assayed the relative abundance of the 23S and 16S rRNAs using the Agilent Bioanalyzer. *P. aeruginosa* PAO1 cells cultured to stationary phase showed a 23S/16S rRNA ratio of ∼1.6 (Fig. 2 *A* and *B*). Remarkably, this ratio was maintained in *P. aeruginosa* PAO1 throughout the starvation period (Fig. 2*B* and *SI Appendix*, Fig. S4), indicating little loss of ribosome quality during prolonged nutrient deprivation. Similarly, the Δ*rmf* mutant maintained a 23S/16S rRNA ratio of ∼1.5 throughout starvation (Fig. 2*B* and *SI Appendix*, Fig. S4). In contrast, the Δ*hpf* strain had 23S/16S rRNA ratios of 1.4 before starvation, which rapidly decreased to 0.1 by day 4 of starvation (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S4). The differences between the Δ*hpf* mutant and the wild-type strain were significant throughout starvation, except on day 0 ($P < 0.000001$). When the Δ*hpf* mutant strain was complemented with *hpf*, the ratio of 23S and 16S rRNA was restored to levels that were not significantly different from the wild type ($P = 0.83$), whereas the vector control (Δ*hpf* + VC) did not restore the rRNA ratios ($P < 0.0001$) (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S4). We also determined the 23S/16S rRNA ratios of the Δ*hpf*/Δ*rmf* double mutant. As with the Δ*hpf* mutant, the Δ*hpf*/Δ*rmf* double mutant had loss of the 23S rRNA during nutrient starvation (*SI Appendix*, Fig. S5). The 23S/16S rRNA ratio for the double mutant was complemented by a plasmid copy of *hpf* without *rmf*, but not with *rmf* alone (*SI Appendix*, Fig. S5). The results indicate that HPF, but not RMF, is required for maintenance of the 23S rRNA under starvation conditions. Interestingly, although the Δ*relA*/Δ*spoT* mutant had reduced viability during starvation compared with wild type, the strain did not show selective loss of the 23S rRNA compared with wild type ($P = 0.30$) (Fig. 2*B* and *SI Appendix*, Fig. S4). The results indicate that the molecular mechanism for reduced viability in the starved Δ*relA*/Δ*spoT* mutant likely differs from that of the Δ*hpf* mutant.

During nutrient starvation of *E. coli*, ribosome degradation is initiated by site-specific endoribonuclease cleavages of the 16S and 23S rRNAs, leading to reduced 70S ribosome abundances and proteolysis of ribosomal proteins (31–33). To determine the fate of the rRNA during nutrient starvation of the *P. aeruginosa* Δ*hpf* strain, we performed time-course Bioanalyzer studies over the first day of starvation. The Δ*hpf* mutant showed reduction of the 23S peak and a concomitant increase in peak-associated small-molecular-weight RNAs (∼100 nt in length), characteristic of degraded rRNA fragments (*SI Appendix*, Fig. S6*A*). In contrast to the wild-type cells and the Δ*hpf* + *hpf* cells, the small-molecular-weight RNA comprised most of the cellular RNA of the Δ*hpf* mutant strain by day 4 of starvation (*SI Appendix*, Fig. S6*B*). The total RNA content of the Δ*hpf* culture also decreased, compared with the wild-type control, after 4 d of starvation ($P = 0.01$) (*SI Appendix*, Fig. S6*D*). We next analyzed the relative amounts of 16S and 23S rRNA of the Δ*hpf* mutant strain compared with wild-type strain following 0 d and 4 d of starvation by reverse transcription-quantitative PCR (RT-qPCR). The results showed a reduction of both rRNA subunits in the Δ*hpf* mutant cells on day 4 ($P = 0.03$), but with greater reduction of the 23S rRNA subunit than the 16S subunit (*SI Appendix*, Fig. S6*E*).

Heterogeneity of HPF-Based rRNA Protection Determined at the Single-Cell Level. Although cell viability was reduced in the Δ*hpf* mutant, viability was not completely eliminated during nutrient deprivation (Fig. 1). In addition, loss of the rRNA was characterized for the entire population of cells, which may have masked the amount of rRNA present in individual cells within the population. Therefore, we used FISH analysis to determine the 16S rRNA levels of individual nutrient-deprived cells. To analyze the FISH results quantitatively, we determined the average fluorescence intensity per pixel within the area of single cells using ImageJ (https://imagej.nih.gov/ij/index.html). The fluorescence intensity for each cell was background-subtracted and then normalized by the spike-in control cells, consisting of exponentially growing *P. aeruginosa* PAO1 (pMF230). The spike-in cells were differentiated from the starved test cells because they were larger, had higher abundances of rRNAs, and had GFP fluorescence

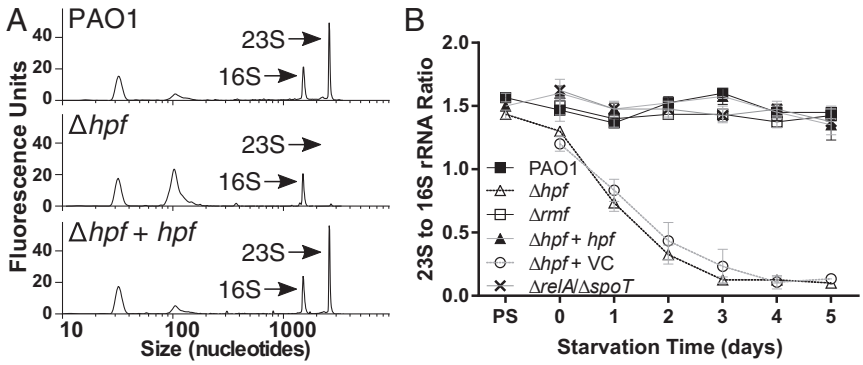

Fig. 2. *P. aeruginosa* 23S-to-16S rRNA ratios following extended incubation under nutrient-deprived conditions with shaking at 37 °C. (*A*) Bioanalyzer traces of 23S and 16S rRNA following 4 d of starvation for wild-type strain PAO1, the Δ*hpf* mutant, and the Δ*hpf* mutant complemented with *hpf* (Δ*hpf* + *hpf*). (*B*) *P. aeruginosa* 23S-to-16S rRNA ratios following extended starvation conditions. Representative bioanalyzer traces showing 23S and 16S rRNAs for all strains at each time point are shown in *SI Appendix*, Figs. S4 and S5.

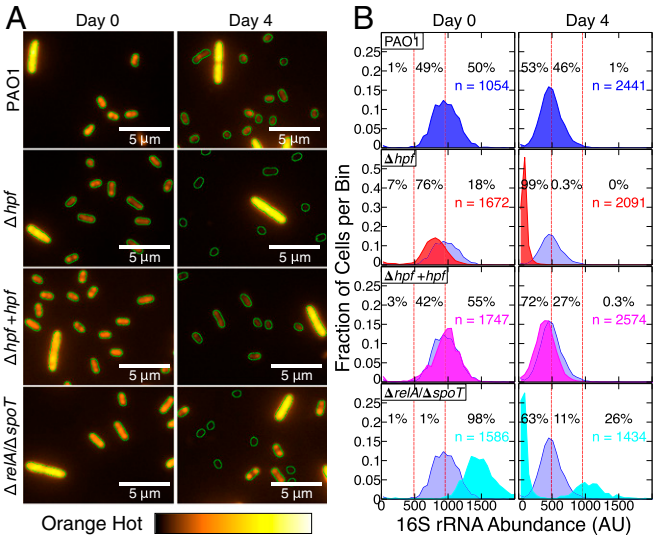

Fig. 3. (*A*) FISH analysis of 16S rRNAs for *P. aeruginsoa* PAO1 and mutant derivatives, starved for 4 d in PBS at 37 °C. The16S probe labeled with Cy3 is false-colored orange. Each field contains a spike-in control cell of exponential phase PAO1 (pMF230) (the large cell with high rRNA abundance). The edges of cells were determined by using bright-field microscopy and are outlined in green. (*B*) The mean fluorescence intensity for 16S-Cy3 probe was quantified, normalized to the spike-in controls, and binned based on the fluorescence intensity of individual cells. The dashed vertical lines represent the average FISH fluorescence intensity for the wild-type strain before starvation and after 4 d of starvation. Data for the wild-type strain are shown on each plot in blue. Data shown are from three independent biological replicates per strain at each time point with the total number of cells quantified indicated. Histograms showing the FISH fluorescence intensities for each day of starvation are shown in *SI Appendix*, Fig. S7. Box-whisker plots showing the average and range of these data are shown in *SI Appendix*, Fig. S8.

(Fig. 3*A*). The results from the wild-type cells showed a gradual reduction in the average FISH fluorescence intensity per cell over time ($P = 0.008$) (Fig. 3 and *SI Appendix*, Fig. S7). However, the FISH fluorescence of the wild-type cells was maintained at high levels throughout the starvation conditions, indicating that most cells maintained abundant ribosome content during starvation (Fig. 3 and *SI Appendix*, Fig. S7). The FISH-fluorescence intensities for the Δ*hpf* mutant strain were at similar levels to the wild-type cells before nutrient starvation ($P = 0.17$) (Fig. 3). However, the average FISH-fluorescence intensity of Δ*hpf* decreased following 1 d of starvation ($P < 0.001$) and continued to decrease compared with wild-type cells with continued starvation incubation (Fig. 3*B* and *SI Appendix*, Fig. S7 and Table S1). The complemented Δ*hpf* + *hpf* strain had restored FISH fluorescence, comparable to the wild type (Fig. 3 and *SI Appendix*, Fig. S7 and Table S2).

The FISH results for the Δ*relA*/Δ*spoT* mutant showed a strikingly different response to starvation than either the wild-type strain or the Δ*hpf* mutant strain. During the first 2 d of starvation, the Δ*relA*/Δ*spoT* cells had higher abundances of 16S rRNA than the wild-type cells (*SI Appendix*, Fig. S7). Continued incubation in nutrient-free medium resulted in Δ*relA*/Δ*spoT* mutant cells segregating into two populations with bimodal distribution. One subpopulation of Δ*relA*/Δ*spoT* cells continued to have higher 16S rRNA content than the wild-type cells, whereas the other subpopulation of cells had little detectable rRNA (Fig. 3 and *SI Appendix*, Fig. S7). By day 4 of starvation, most (~63%) Δ*relA*/Δ*spoT* cells had little signal for the 16S rRNA, whereas 26% had signals greater than the average of the wild-type cells.

The presence of outliers with high and low FISH fluorescence intensities were observed for all strains. The box and whisker plots (*SI Appendix*, Fig. S8) show a greater average decrease of 16S rRNA for the Δ*hpf* cells than for the wild-type cells. However, all strains had outliers where either high or low abundances of rRNAs were observed, including a small fraction of Δ*hpf* mutant cells that had fluorescence intensity as high as the average intensity of 4-d-starved wild-type cells. The observation of outliers at the single-cell level suggests that HPF may not be the sole mechanism for maintenance of ribosome integrity of *P. aeruginosa*.

Role of HPF in the Resuscitation of Individual Dormant Cells. The colony size and variable rRNA levels of the starved Δ*hpf* mutant cells suggested that there was heterogeneity in the resuscitating population. Therefore, we used a drop-based microfluidic approach (34) to quantify the heterogeneity in this population. These drop-based experiments allowed us to differentiate and quantify individual cells undergoing resuscitation from the nondividing cells within many drop-based bioreactors. We compared resuscitation of the wild-type cells and the Δ*hpf* mutant cells, both expressing GFP, so that they could be visualized by confocal scanning laser microscopy (CSLM) (Fig. 4 *A* and *B*). Cultures were starved using the conditions described above, and then individual cells were encapsulated in 15-µm-diameter, water-in-oil drops containing TSB medium as the dispersed phase in oil. The ratio of drops containing one cell to empty drops was set at ~1:5–1:10. The cells were then incubated in the TSB contained in each drop for 24–48 h to allow resuscitation of individual cells from the starvation-induced dormancy. Drops were then analyzed by CSLM and quantified by image analysis for cell growth (drops that were completely or partially filled with bacteria versus drops that contained only one cell) (Fig. 4). Similar to the cfu counts, there was an increase in total number of cells from day 0 to day 1 of starvation for both the wild-type ($P = 0.037$) and Δ*hpf* mutant cells ($P = 0.003$) (Fig. 1 and *SI Appendix*, Fig. S9), suggesting that the cells had divided in the PBS-starvation medium. To determine if cells were capable of division during the first day of starvation, we encapsulated and incubated cells in drops containing PBS. Under these conditions, most drops contained multiple cells after 24 h (*SI Appendix*, Fig. S10), indicating that the cells had undergone at least one round of cell division during the first day of incubation in PBS.

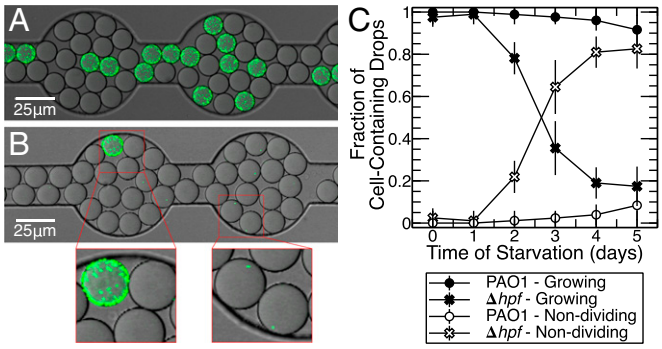

Fig. 4. Examples of cell resuscitation of individual *P. aeruginosa* (pMF230) cells within 15-µm-diameter oil drops with visualization by CSLM. Cells were incubated in PBS for 4 d, and encapsulated in drops containing TSB medium. (*A*) *P. aeruginosa* PAO1 following 4 d of starvation and then regrowth for 24 h in TSB. (*B*) *P. aeruginosa* PAO1 Δ*hpf* after 4 d of starvation and then regrowth for 24 h in TSB. (*Insets*) Cell regrowth and single nondividing cells in drops. (*C*) Percentage of cells that replicated inside of TSB-containing drops versus cells that remained as single intact nondividing cells following starvation in PBS. Cell counts of replicating and nonreplicating cells are shown in *SI Appendix*, Fig. S9. Regrowth of PAO1 and the Δ*hpf* mutant cells in drops over 24 h is shown in Movies S1 and S2.

Following the initial cell division in PBS from day 0 to day 1 of starvation, the total number of cells captured in drops remained essentially constant throughout the starvation period for both the Δ*hpf* mutant ($P = 0.41$) and wild-type cells ($P = 0.88$) (*SI Appendix*, Fig. S9), indicating little cell lysis during starvation. However, the percentage of cells that remained as single nonreplicating cells versus cells that grew within the drops differed over time for PAO1 ($P = 0.03$) and the Δ*hpf* mutant strains ($P = 0.01$) (Fig. 4*C* and *SI Appendix*, Fig. S9). Initially, ∼100% of the wild-type cells were capable of resuscitation inside of drops. The number of PAO1 cells capable of resuscitation decreased to 96% by 4 d of starvation (Fig. 4*C* and *SI Appendix*, Fig. S9). In contrast, the number of Δ*hpf* mutant cells capable of resuscitation inside of the drops decreased over time of starvation, with most cells (81%) remaining as single nondividing cells by day 4 of starvation (Fig. 4*C* and *SI Appendix*, Fig. S9). Additional incubation to 48 h inside drops did not result in a significant increase in the number of cells that could resuscitate ($P = 0.81$). The results indicate that the number of nondividing *P. aeruginosa* cells increases during starvation in the absence of a functional HPF.

The resuscitating Δ*hpf* mutant cells had heterogeneous colony morphology (Fig. 1), suggesting that some of the recovering Δ*hpf* cells either had an increased lag time or a reduced growth rate. To distinguish these two possibilities, we performed time-course imaging of single cells in microfluidic drops following 4 d of starvation. Most wild-type cells (98.7%) recovered from starvation and had a relatively uniform growth rate averaging 0.56 h^{-1} (Movie S1 and *SI Appendix*, Fig. S11), whereas most of the Δ*hpf* mutant cells (84.4%) did not recover following 4 d of starvation. The Δ*hpf* cells that recovered had a slightly lower average growth rate (0.44 h^{-1}) than the wild-type cells. However, the Δ*hpf* cells had highly variable lag times (Movie S2 and *SI Appendix*, Fig. S11). The results indicate that the small-colony variants of the Δ*hpf* cells as seen in Fig. 1 are likely caused by an increased lag time required for these cells to recover from starvation.

Discussion

Heterotrophic bacteria living in aqueous environments often experience conditions where nutrients are scarce, including the nutrient-depleted zones of biofilms (10). Rapidly growing bacteria contain sufficient ribosome concentrations for maximum protein biosynthesis during exponential phase (35, 36). When the cells switch to slow-growth conditions, the number of ribosomes per cell diminishes through degradation or by partitioning to daughter cells. However, slowly growing and dormant cells must have mechanisms to maintain sufficient quantities of premade macromolecules, including ribosomes, to allow de novo protein synthesis and cell regrowth when conditions become favorable (37). Mechanisms have evolved to avoid complete loss of these essential macromolecules when cells are dormant. Here, we demonstrate that the small-ribosome–interacting protein, HPF, is required for ribosome preservation during dormancy of *P. aeruginosa*. Surprisingly, although important for *E. coli* ribosome inactivation (13) and abundant in most *P. aeruginosa* biofilm transcriptomic experiments (38), RMF does not appear to play a significant role in ribosome preservation in *P. aeruginosa* under the conditions tested here. Mechanisms for starvation responses may be specific for a certain nutrient. In *P. aeruginosa*, *hpf* is located downstream of the nitrogen-stress–associated sigma factor, *rpoN*, and is expressed, in part, from the *rpoN* promoter. However, *hpf* also has its own promoter(s), allowing high expression under certain conditions.

In a previous study, we measured the ribosome abundance of cells at different vertical strata within *P. aeruginosa* biofilms by microdissecting the biofilms and then assaying 16S rRNA amounts from the different microzones using RT-qPCR (39). Because rRNA is rapidly degraded when not associated with ribosomes (40), quantification of rRNA provides an estimate of the ribosome copy number per cell. From that study, we showed that exponentially growing *P. aeruginosa* had ∼70,000 ribosomes per cell, whereas cells in the slowly growing subpopulation of biofilms had ∼20,000 ribosomes per cell (39). This relatively high concentration of ribosomes in the slow-growing cells was maintained even for cells with little transcriptional activity, suggesting that ribosomes are preserved in the cells in the interior of the biofilms. Identification of transcripts for the ribosomal accessory protein, *hpf*, as abundant in the interior of the biofilms indicated that the product of this gene may be important for ribosome preservation in the dormant subpopulation of cells (9). Here, we show that, in the absence of *hpf*, starved cells have reduced capacity to resuscitate following nutrient deprivation and have reduced rRNA levels. The population-level experiments of the Δ*hpf* strain showed greater loss of the 23S rRNA than of the 16S rRNA (Fig. 2), but with a significant loss of both rRNA species (*SI Appendix*, Fig. S6), compared with only a modest decrease of 16S and 23S rRNA levels for starved wild-type cells. The FISH single-cell results also showed loss of the 16S rRNA in the Δ*hpf* mutant, whereas the wild-type strain maintained relatively high levels of 16S rRNA during starvation (Fig. 3).

Ribosome degradation during nutrient starvation in *E. coli* is initiated by site-specific endoribonuclease cleavages of 16S and 23S rRNAs, leading to the loss of detectable free subunits as well as reduced 70S ribosome abundance (31, 33). Ribosomal proteins dissociated from rRNAs are susceptible to proteolysis and likely recycled as nutrients (32). We observed an additional peak in the Δ*hpf* mutant in the Bioanalyzer studies, which may indicate the product of 23S rRNA cleavage (Fig. 2 and *SI Appendix*, Fig. S6). Cleavage of 23S rRNA may initiate rRNA degradation to small fragments as observed here in the Bioanalyzer studies. The crystal structures of ribosomes with HPF and its paralog revealed that the HPF-binding site overlaps with the binding sites of translation initiation factors on the 30S subunit (12, 41). In vitro studies also indicate that the HPF paralog YfiA competes for 30S binding with IF-3 in the presence of polyamines (41). Binding of IF-3 to the 30S subunit prevents the association of the 50S subunit until IF-2 promotes subunit joining to form the 70S initiation complex (42). Because the interaction of ribosomal subunits with HPF induces subunit joining (12, 16, 41), HPF may protect unused ribosomes from endoribonucleases through 70S-HPF complex formation, whereas IF-3 binding to the 30S subunit in the absence of HPF would leave free 50S subunits available for endonuclease cleavage.

The heterogeneity observed in microfluidic studies of resuscitating cells indicates that HPF may not provide the sole mechanism for ribosome preservation. A percentage of the Δ*hpf* cells was capable of regrowth in microfluidic drops (Fig. 4). In addition, some Δ*hpf* cells maintained their ribosomal content even after extended starvation (*SI Appendix*, Fig. S8). Therefore, an alternative strategy to ensure ribosome preservation may exist in *P. aeruginosa*. The microfluidic studies of wild-type cells also showed that a percentage of cells (4% by day 4 of starvation) were unable to regrow following starvation. Whether these cells still had metabolic activity and were unable to grow (e.g., viable but nonculturable cells), or whether the cells were not viable, is not known yet. In either case, the results demonstrate that, even in a clonal population of cells, heterogeneity in ribosome content and cellular capacity for regrowth exists.

The stringent response mutant had a similar loss of recovery from starvation as the Δ*hpf* mutant. However, the mechanism for impaired recovery of the stringent response mutant differs from the Δ*hpf* mutant strain. Our preliminary data using a transcriptional reporter of *hpf* indicate that the stringent response has a small modulatory effect on *hpf* expression, but that *hpf* expression is not abolished in the Δ*relA*/Δ*spoT* mutant. A plasmid copy of *hpf* did not have any effect on the survival phenotype of the Δ*relA*/Δ*spoT* strain (*SI Appendix*, Fig. S3). Therefore, we conclude that expression of *hpf* is not solely regulated through the product of the stringent response alarmone, (p)ppGpp. Overall, the

results demonstrate that HPF provides the primary mechanism for ribosome preservation during nutrient starvation of *P. aeruginosa* and that ribosome preservation is needed for *P. aeruginosa* cells to resuscitate from dormancy.

Materials and Methods

Bacterial Strains and Growth Conditions. Studies were performed on *P. aeruginosa* strain PAO1 and its Δ*rmf*, Δ*hpf*, and Δ*hpf*Δ*rmf* mutant derivatives and on complemented strains as described in *SI Appendix, SI Materials and Methods*, and previously (9). The stringent response mutant, containing deletions of *relA* and *spoT* (28), was provided by Pradeep Singh, University of Washington, Seattle. Nutrient-starvation studies and the drop-plate method (43) used to quantify cfu's were performed as described in *SI Appendix, SI Materials and Methods*.

RNA Extraction and Determination of Relative rRNA Abundances. RNA was extracted as described previously (44) with modifications described in *SI Appendix, SI Materials and Methods*. RNA was analyzed using the Bioanalyzer 2100 (Agilent Technologies) as described in detail in *SI Appendix, SI Materials and Methods*. Total rRNA and 16S and 23S rRNAs were quantified using the NanoDrop1000 (Thermo Fisher Scientific) and by RT-qPCR as described in *SI Appendix, SI Materials and Methods*.

FISH for Quantification of rRNA. FISH, as described by Brileya et al. (45), and using a Cy3-labeled 16S rRNA probe described by Hogardt et al. (46), was used to quantify the relative amounts of 16S RNA from individual cells. A detailed description of the FISH method and analysis is provided in *SI Appendix, SI Materials and Methods*.

Drop Encapsulation and Monitoring Growth of Single Bacterial Cells. Single bacterial cells were encapsulated into microfluidic drops as described in detail in *SI Appendix, SI Materials and Methods* and in ref. 47. Drop-encapsulated cells were injected into a modified "Dropspots" immobilization device (48). Microscopic and statistical analyses of bacterial growth in drops are provided in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Phil Stewart, Joanna Borgogna, and Al Parker for their helpful discussions of this work; Geoffrey Zath for microfluidic drop growth rate analysis; Betsy Pitts, Kristen Brileya, and Amanda Richards for microscopy data acquisition; and Dr. Pradeep K. Singh for providing the Δ*relA*/Δ*spoT* mutant. This work was funded by National Institute of Allergy and Infectious Diseases Grant AI113330 (to M.J.F.), National Institute of General Medical Sciences Grant P20GM103474 (to S.P.), and Montana State University's Graduate School (T.A.).

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