Ribosome Hibernation Facilitates Tolerance of Stationary-Phase Bacteria to Aminoglycosides

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Upon entry into stationary phase, bacteria dimerize 70S ribosomes into translationally inactive 100S particles by a process called ribosome hibernation. Previously, we reported that the hibernation-promoting factor (HPF) of *Listeria monocytogenes* is required for 100S particle formation and facilitates adaptation to a number of stresses. Here, we demonstrate that HPF is required for the high tolerance of stationary-phase cultures to aminoglycosides but not to beta-lactam or quinolone antibiotics. The sensitivity of a Δ*hpf* mutant to gentamicin was suppressed by the bacteriostatic antibiotics chloramphenicol and rifampin, which inhibit translation and transcription, respectively. Disruption of the proton motive force by the ionophore carbonyl cyanide *m*-chlorophenylhydrazone or mutation of genes involved in respiration also suppressed the sensitivity of the Δ*hpf* mutant. Accordingly, Δ*hpf* mutants had aberrantly high levels of ATP and reducing equivalents during prolonged stationary phase. Analysis of bacterial uptake of fluorescently labeled gentamicin demonstrated that the Δ*hpf* mutant harbored increased intracellular levels of the drug. Finally, deletion of the main ribosome hibernation factor of *Escherichia coli*, ribosome modulation factor (*rmf*), rendered these bacteria susceptible to gentamicin. Taken together, these data suggest that HPF-mediated ribosome hibernation results in repression of the metabolic activity that underlies aminoglycoside tolerance. HPF is conserved in nearly every bacterial pathogen, and the role of ribosome hibernation in antibiotic tolerance may have clinical implications.

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen that poses serious medical risks for pregnant women, immunocompromised individuals, and infants (1, 2). The significance of *L. monocytogenes* as a foodborne pathogen was recently highlighted when contaminated cantaloupes caused the deadliest U.S. foodborne disease outbreak since 1924 (3). This bacterium is hardy and able to survive in a variety of environments, including soil, groundwater, and processed meats and cheeses, and within mammalian cells (2, 4–6). *L. monocytogenes* can also form biofilms that can persist for years in food processing plants, despite repeated exposure to disinfectants (7, 8). Persistent *L. monocytogenes* contamination of food processing plants remains a significant source of foodborne illness (9–11). Understanding the molecular mechanisms that allow *L. monocytogenes* to survive and persist in harsh environments is crucial for developing strategies to ensure food safety.

Ribosome dimerization is a highly conserved yet relatively underappreciated arm of the classic ribosome cycle. During stress, bacteria are able to dimerize 70S ribosomes into inactive hibernating 100S ribosome dimers. These dimers lack mRNA and tRNA and have been shown to be translationally silent *in vitro* (12, 13). Upon introduction of fresh nutrients, 100S ribosomes are immediately dissociated into 70S ribosomes and available for translation (14–16). Thus, the rapid interconversion between 70S and 100S ribosomes is a potentially powerful mechanism for tuning translational capacity to environmental conditions.

Almost all bacteria possess genes that facilitate 100S ribosome formation; however, the distribution of these genes differs between Gram-positive and Gram-negative bacterial species. In *Escherichia coli* and other gammaproteobacteria, 100S ribosomes are formed through the sequential activities of ribosome modulation factor (RMF) and a short hibernation-promoting factor (HPF) to produce a 90S dimer that is then stabilized into the mature 100S form (17). In Gram-positive bacterial species, ribosome hibernation is mediated solely by a long HPF homolog with an N terminus common to all HPF homologs and a C-terminal extension thought to perform the activity of RMF (18, 19). The temporal profiles of 100S ribosomes formed by short and long HPFs are distinct. In *Escherichia coli*, the formation of 100S ribosomes is restricted to stationary phase, where they comprise the majority of the ribosome content (19, 20). In contrast, 100S ribosomes formed by long HPF homologs are detected at all phases of growth, with abundance peaking during the transition from logarithmic to stationary-phase growth, where they comprise 50% of the ribosome content (18, 21). The function of translationally silent 100S particles during logarithmic growth remains to be elucidated.

Ribosome hibernation is important for adaptation to various conditions, yet the mechanism by which 100S ribosomes mediate these adaptations is not clear. Ribosome hibernation is critical for survival during and recovery from a prolonged stationary phase (18, 20–23). Hibernation-associated factors are important for adaptation to a number of different stresses present during stationary phase, including deprivation of carbon, amino acids, and polyamines, as well as osmotic, heat, and acid stress (23–27). In addition, our lab recently described ribosome hibernation in *L. monocytogenes* and highlighted for the first time that 100S ribosomes contribute to bacterial pathogenesis (21). Because 100S ribosomes are induced during nutrient starvation, it has been pro-

TABLE 1 Strains used in this study

Strain	Description	Reference or source
L. monocytogenes		
10403S	WT reference strain	67
DP-L6181	Δ*hpf* mutant	21
DP-L6182	Δ*hpf* mutant carrying pPL2-HPF	This study
E. coli		
BW25113	WT reference strain	68
DP-E6184	Δ*rmf* mutant	This study
DP-E6185	Δ*hpf* mutant	This study

posed that ribosome hibernation serves to reduce the level of active translation to that commensurate with available nutrients and/or to store and protect ribosomes for use later, when favorable growth conditions return (13, 15, 23, 28). However, these roles have yet to be demonstrated experimentally, and the function that 100S ribosomes play in mediating adaptive processes remains unclear.

L. monocytogenes and many other bacteria naturally reside within complex microbial communities that include antibiotic-producing bacteria (29–33). Bacteria are inherently capable of evading killing by antibiotics (i.e., without acquiring genetic resistance) through various adaptive mechanisms that generally lead to reduced growth and cellular metabolism (34–36). Ribosome hibernation is implicated in bacterial states associated with high levels of tolerance to antibiotics, including stationary-phase, biofilm, and dormant persister cell states (13, 22, 37). We therefore considered the possibility that ribosome hibernation contributes to the natural ability of microbes to survive in the presence of antibiotics.

Here we demonstrate a role for ribosome hibernation in conferring tolerance to antibiotics. An *L. monocytogenes* Δ*hpf* mutant was sensitive specifically to the ribosome-acting class of aminoglycoside antibiotics but not other classes of bactericidal antibiotics. The susceptibilities of *L. monocytogenes* and *E. coli* ribosome hibernation mutants were compared, and the results suggested that the two species have a common mechanism by which 100S ribosomes protect the bacteria from the activity of aminoglycosides. Importantly, 100S ribosomes may be a new target against which therapies against chronic infections caused by other human pathogens that are recalcitrant to traditional antibiotic treatments may be developed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All *L. monocytogenes* strains used and generated in this study were in the strain 10403S background and are listed in Table 1. *L. monocytogenes* strains were cultured in filter-sterilized brain heart infusion medium (BHI) buffered to pH 7.3 (Difco, Detroit, MI). *E. coli* K-12 Keio knockout strains were obtained from the Yale Coli Genetic Stock Center, New Haven, CT. Each strain was transformed with the pCP20 helper plasmid that carries the gene for a flippase recombinase (38) to remove the kanamycin resistance cassette marking the gene deletions. The resulting clean deletion mutant strains are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani (LB) medium.

MIC determination. MICs were determined by a broth microdilution technique in BHI according to the guidelines of Wikler and the Clinical and Laboratory Standards Institute (CLSI) (39).

Long-term antibiotic susceptibility and suppression assay. Bacterial cells were cultured at 37°C for 16 h with shaking in 5 ml of the appropriate medium. Aliquots from these overnight cultures were removed and subjected to antibiotic treatment. Unless otherwise noted, the antibiotics were used at the following concentrations: 10 μg/ml gentamicin, 200 μg/ml carbenicillin, 30 μg/ml ciprofloxacin, 30 μg/ml norfloxacin, 160 μg/ml amikacin, 150 μg/ml kanamycin, and 20 μg/ml tobramycin. For suppression analyses, overnight aliquots were pretreated for 30 min with the appropriate chemical (20 μg/ml chloramphenicol, 20 μg/ml rifampin, or 2.5 μM carbonyl cyanide *m*-chlorophenylhydrazone [CCCP]) before gentamicin was added. At specified time points, aliquots of samples were removed, serially diluted, spot plated onto LB agar plates, and incubated overnight at 37°C to determine the numbers of CFU per milliliter.

ATP quantification. To determine the ATP levels of long-term stationary-phase bacteria, bacterial cells were cultured as described above in buffered BHI at 37°C with shaking for 72 h. The bacterial cells were diluted 100-fold in sterile phosphate-buffered saline (PBS) and combined 1:1 with the BacTiter-Glo reagent (Promega) in white-walled 96-well plates (PerkinElmer). Luminescence was measured on a SpectraMax L luminescence microplate reader (Molecular Devices). Relative ATP levels were calculated by normalizing the luminescence to the numbers of CFU and are reported as the fold change relative to that of the wild type (WT). The experiments were repeated two times, each of which had four biological replicates. The data presented are from a representative experiment. Significance was determined using an unpaired *t* test.

Tetrazolium salt (XTT) reduction assay. To determine the metabolic reducing power of long-term stationary-phase bacteria, the samples collected for ATP quantification (as described above) were subjected in parallel to an 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction assay. The XTT assay methodology was essentially the same as that described previously (40). Briefly, the bacterial cells were diluted 100-fold in sterile PBS and aliquoted into a 96-well microtiter plate (Sarstedt). A 1/10 volume of reagent (9:1 solution of XTT reagent [11.1 mM] and phenazine methosulfate [8 mM]) was added to the samples, and the resulting mixtures were incubated at 37°C in the dark for 1 h. The reduction of XTT to formazan was determined by measurement of the absorbance at 492 nm on an Infinite M1000 Pro microplate reader (Tecan). Values were normalized to the number of CFU and are reported as a multiple of the wild-type level. The experiments were repeated two times, each of which had four biological replicates. The data presented are from a representative experiment. Significance was determined using an unpaired *t* test.

Texas Red-gentamicin uptake assay. A Texas Red-gentamicin mixture was made as described previously (41), and Texas Red-gentamicin uptake was measured by flow cytometry as described previously (42). Briefly, the Texas Red fluorescent dye was conjugated to gentamicin sulfate and purified. The resulting Texas Red-gentamicin reagent was incubated with stationary-phase samples for 24 h in the same manner described below for the long-term gentamicin assay. Samples were then washed, resuspended in PBS, analyzed on a BD LSR Fortessa flow cytometer, and plated for determination of the numbers of CFU. Gentamicin uptake was calculated as the mean fluorescence intensity normalized to that for the untreated samples. Results are from two independent experiments, each of which had five biological replicates. Significance was determined using an unpaired *t* test.

Long-term gentamicin susceptibility of *E. coli* hibernation mutants. Bacterial cells were cultured at 37°C for 16 h with shaking in LB medium. Aliquots from these overnight cultures were removed and subjected to gentamicin (5 μg/ml) treatment. At specified time points, aliquots of samples were removed, serially diluted, spot plated onto LB agar plates, and incubated overnight at 37°C to determine the numbers of CFU per milliliter.

RESULTS

Stationary-phase Δ*hpf* mutants are susceptible to prolonged gentamicin exposure. Antibiotic-tolerant cells are characterized as phenotypic variants able to withstand antibiotic treatment

TABLE 2 MICs of different classes of antibiotics for *L. monocytogenes* 10403S WT and Δ*hpf* mutant strains

Antibiotic[a]	MIC (µg/ml)		
	WT strain 10403S	Δ*hpf* mutant	Δ*hpf* mutant carrying pPL2-HPF
Ampicillin	0.06–0.12	0.06–0.12	0.06–0.12
Carbenicillin	1–2	1–2	1–2
Vancomycin	1	1	1
Ciprofloxacin	1	1	1
Norfloxacin	8–16	8–16	8–16
Gentamicin	0.5–1	0.5–1	0.5–1
Kanamycin	4–8	4	4–8
Tobramycin	0.5–1	1	1
Amikacin	2–8	2–8	2–8
Chloramphenicol	4	4	4
Rifampin	0.03–0.06	0.03–0.06	0.03–0.06

[a] Boldface indicates a bactericidal antibiotic.

without acquiring genetic resistance (37). Mutants with mutations that affect the frequency of antibiotic-tolerant cells therefore do not display MICs different from those of their parental strains (43). To determine whether deletion of *hpf* affected the MIC of *L. monocytogenes*, several antibiotics from each of the bactericidal classes were tested. Deletion of *hpf* had no significant (greater than 2-fold) effect on the MIC of any of the antibiotics tested (Table 2).

MICs were determined using cultures at a low density (10^5 to 10^6 CFU/ml). *L. monocytogenes* 100S ribosomes, however, are most prominent during entry to stationary phase, where cells are dense and growth has begun to slow (21). We therefore tested the sensitivity of stationary-phase *L. monocytogenes* cultures to prolonged antibiotic treatment. Neither the WT nor the Δ*hpf* mutant in the untreated samples lost viability, even after an incubation of 3 days (Fig. 1). Both Δ*hpf* mutant and WT stationary-phase bacteria were completely refractory to treatment with the beta-lactam carbenicillin or the quinolones ciprofloxacin and norfloxacin at

levels greater than 10 times the MICs of these drugs (Fig. 1A). WT *L. monocytogenes* displayed a modest loss in the number of CFU when exposed to 10 times the MIC of gentamicin (10 µg/ml) for 72 h (Fig. 1B). Conversely, the Δ*hpf* mutant exhibited a dramatic 5-log₁₀-CFU/ml loss in viability. HPF mutant sensitivity was generalizable for all aminoglycosides tested, including amikacin, tobramycin, and kanamycin (Fig. 1B). Furthermore, killing by gentamicin occurred in a concentration-dependent manner. At the highest dose tested (20 µg/ml), the WT lost $2 \log_{10}$ CFU/ml after 72 h, whereas the Δ*hpf* mutant lost nearly $8 \log_{10}$ CFU/ml and its growth approached the limit of detection (Fig. 1C). Finally, complementation of the Δ*hpf* strain with pPL2-HPF fully rescued the gentamicin sensitivity phenotype (Fig. 1D). Taken together, these data indicate that stationary-phase Δ*hpf* mutant cultures are specifically sensitive to aminoglycosides.

Inhibition of translation and transcription suppresses Δ*hpf* mutant sensitivity. Translation is required for the bactericidal activity of aminoglycosides (44). Given the role of HPF in inactivating ribosomes during stationary phase, we hypothesized that inhibition of translation with a bacteriostatic translation inhibitor like chloramphenicol could suppress the sensitivity of the Δ*hpf* mutant to gentamicin. To test this idea, translation was inhibited by pretreating stationary-phase cultures with a subinhibitory concentration of chloramphenicol. Chloramphenicol treatment alone did not affect the number of CFU for either the WT or the Δ*hpf* mutant (data not shown). Chloramphenicol pretreatment significantly suppressed the sensitivity of the Δ*hpf* mutant to gentamicin (Fig. 2). To determine whether this suppression was specific to inhibition of translation, transcription was inhibited with subinhibitory levels of rifampin, a bacteriostatic inhibitor of RNA polymerase. Rifampin treatment alone did not affect the number of CFU for the WT or the Δ*hpf* mutant (data not shown). However, pretreatment with rifampin suppressed the sensitivity of the Δ*hpf* mutant to prolonged gentamicin exposure (Fig. 2). These
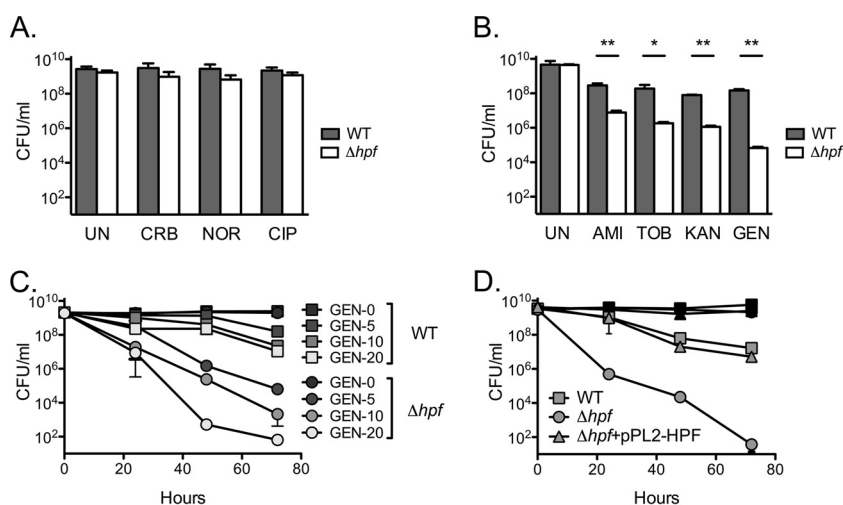

FIG 1 Stationary-phase Δ*hpf* mutant cultures are specifically susceptible to prolonged aminoglycoside exposure. (A) Stationary-phase Δ*hpf* mutant cultures are insensitive to prolonged beta-lactam or quinolone exposure. UN, untreated culture; CRB, carbenicillin (200 µg/ml); NOR, norfloxacin (150 µg/ml); CIP, ciprofloxacin (30 µg/ml). Data are from 72 h. (B) Stationary-phase Δ*hpf* mutant cultures are susceptible to prolonged aminoglycoside exposure. AMI, amikacin (160 µg/ml); TOB, tobramycin (10 µg/ml); KAN, kanamycin (150 µg/ml); GEN, gentamicin (10 µg/ml). Data are from 72 h. *, $P < 0.05$; **, $P < 0.01$. (C) Sensitivity to gentamicin is dose dependent. The gentamicin concentrations in the key are reported in micrograms per milliliter. (D) Sensitivity to gentamicin is fully complemented by the addition of pPL2-HPF. Means and SDs ($n \geq 3$) are presented.
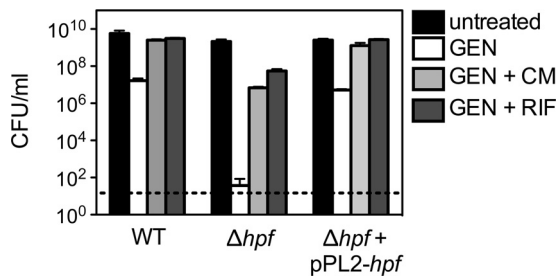
FIG 2 Aminoglycoside susceptibility is suppressed by chemically arresting translation or transcription. Translation and transcription were inhibited by pretreating stationary-phase cultures for 30 min with subinhibitory levels of chloramphenicol and rifampin, respectively. Data are from 72 h. GEN, gentamicin (10 μg/ml); CM, chloramphenicol (20 μg/ml); RIF, rifampin (20 μg/ml). Means and SDs ($n \geq 3$) are presented. Dashed line, limit of detection of 100 CFU/ml.

FIG 3 Disruption of cellular energy production suppresses the sensitivity of the Δhpf mutant to gentamicin. Chemical inhibition of the proton motive force with $0.5\times$ the MIC of CCCP (A) and disruption of genes involved in cellular energy production (B) suppress Δhpf mutant gentamicin sensitivity. Data are from 72-h cultures. Means and SDs ($n \geq 3$) are presented. Tn, transposon.

results confirm that active gene expression in the stationary-phase Δhpf mutant contributes to aminoglycoside susceptibility.

Disruption of the PMF suppresses Δhpf mutant sensitivity to gentamicin. The uptake and accumulation of aminoglycosides are energy-dependent processes and require a threshold proton motive force (PMF) even in nondividing cells (42, 44). We hypothesized that the Δhpf strain remains metabolically active during stationary phase in a manner that facilitates aminoglycoside uptake and, consequently, enhances bacterial killing. To test this hypothesis, stationary-phase cultures were pretreated with the proton ionophore CCCP in order to disrupt the PMF and inhibit aminoglycoside uptake. The MIC of CCCP was determined to be 5 μM. Treatment with $0.5\times$ the MIC of CCCP did not affect the number of CFU for either the WT or the Δhpf mutant (data not shown). Incubation with gentamicin for 72 h produced a modest reduction of 1.5 $\log_{10}$ CFU/ml for the WT, while the CFU counts for the Δhpf mutant were below the detection limit (10^2 CFU/ml) (Fig. 3A). Pretreatment with CCCP significantly rescued Δhpf mutant sensitivity. To further confirm that reduction of the PMF suppresses the susceptibility of the Δhpf mutant, genes involved in energy production were disrupted in the Δhpf mutant background. Transposon insertion into cytochrome quinol oxidase (*qoxA*), an ATP synthase subunit (*atpF*), or a member of the menaquinone biosynthetic pathway (*menC*) suppressed Δhpf mutant gentamicin sensitivity to levels near those for the WT (Fig. 3B). These data confirm that an active PMF is required for the sensitivity of the Δhpf mutant to gentamicin.

Long-term stationary-phase Δhpf mutant cultures exhibit high levels of energy production. In stationary phase, cells downregulate their levels of respiration and energy production to those commensurate with nutrient limitation and slow growth (45). Accordingly, overall ATP and NADH levels drop under nutrient starvation conditions (46–48). If ribosome hibernation facilitates the downregulation of energy production during stationary phase, then we predict that the Δhpf mutant will exhibit inappropriately high levels of ATP. To test this idea, overall ATP levels were quantified in a stationary-phase Δhpf mutant culture using the BacTiter-Glo microbial cell viability assay, which measures the amount of ATP present in a sample as a function of luminescence. Indeed, mutant cultures incubated for 72 h had 5-fold more ATP than the WT (Table 3). To measure the reducing potential of these strains, we employed an assay based on the reduction of the tetra-
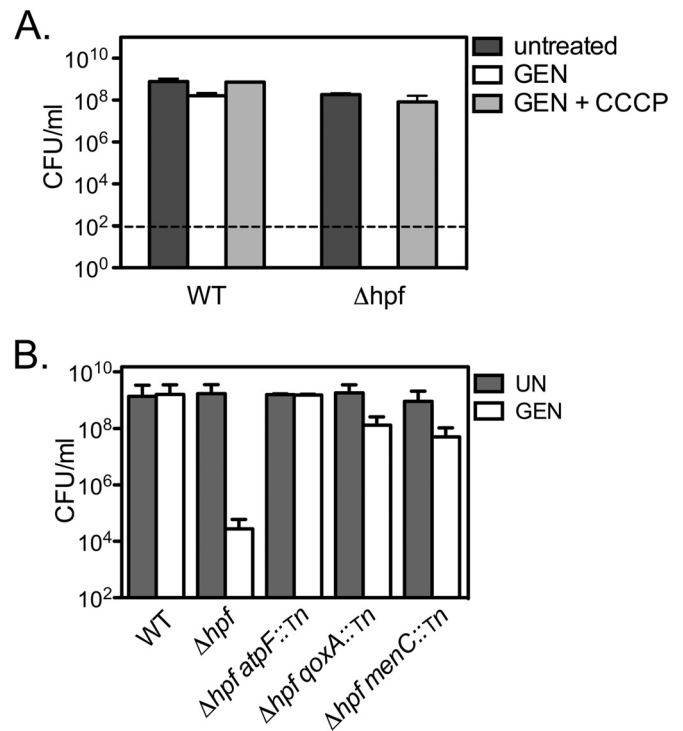
zolium dye XTT into a formazan, which is directly related to the intracellular NADH pool. Similar to the measurements of ATP, the reducing power of the aged Δhpf mutant cultures was 5-fold higher than that of the WT (Table 3). Taken together, these analyses indicate that the Δhpf mutant remained in an inappropriately energized state during prolonged stationary phase.

A Δhpf mutant accumulates high levels of gentamicin. Given that NADH drives the PMF required for aminoglycoside uptake, we hypothesized that enhanced uptake and enhanced accumulation of the drug underlie the mutant's sensitivity. Previous work has employed fluorescently labeled gentamicin to evaluate aminoglycoside uptake (42). Aminoglycoside uptake was quantified for the WT and the Δhpf mutant after an incubation of 24 h. The 24-h time point was chosen for analysis in order to avoid confounding of the results due to the dramatic loss in the number of CFU of the Δhpf mutant after 72 h of incubation. Texas Red-gentamicin killed the WT and Δhpf mutant at levels comparable to what we previously found for unlabeled gentamicin (Table 4). Quantification of the intracellular fluorescence for the Δhpf mutant showed that it was nearly 70% higher than that for the WT and correlated with a 1.5-$\log_{10}$-unit loss in the numbers of CFU per milliliter (Table 4). These data demonstrate that the high level of killing of the Δhpf mutant strain by gentamicin is related to increased intracellular concentrations of the drug.

An *E. coli* hibernation mutant exhibits sensitivity to gentamicin. In order to kill bacteria, aminoglycosides must bind ribosomes and interfere with translation. The crystal structure of 100S ribosomes formed by *E. coli* RMF and HPF shows that short HPF

TABLE 3 Relative intracellular ATP and NADH measurements for *L. monocytogenes* WT and Δ*hpf* mutant cultures at 72 h[a]

Strain or *P* value	ATP[b]	XTT reduction (NADH)[c]
WT	1.1 ± 0.1	1.3 ± 0.2
Δ*hpf* mutant	5.34 ± 1.7	5.04 ± 1.4
P value	0.022	0.011

[a] Each value is the mean ± standard deviation from four biological replicates.
[b] Relative luminescence units per CFU normalized to the value for the WT.
[c] Absorbance at 450 nm per CFU normalized to the value for the WT.

TABLE 4 Mean intracellular fluorescence and numbers of CFU of *L. monocytogenes* WT and Δ*hpf* mutant cultures after incubation with Texas Red-gentamicin for 24 h[a]

Strain or parameter	Relative fluorescence[b]	Log$_{10}$ CFU/ml
WT	45.8 ± 5.4	9.0 ± 0.1
Δ*hpf* mutant	76.1 ± 15.0	7.4 ± 0.2
P value	0.01	0.006
Fold change	1.7	0.8

[a] Values are means ± standard deviations from five biological replicates.
[b] Normalized to the value for untreated isolates.

binds ribosomes in a manner that occludes the aminoglycoside-binding site, while RMF binds near the peptide exit site (49). Given that short HPF and the N-terminal portion of long HPF are highly homologous, these data suggest the possibility that HPF homologs in general protect ribosomes from aminoglycosides by competing for binding. Because aminoglycosides bind to ribosomes very tightly (dissociation constant = 0.6 μM) (50), binding site competition could also explain why a Δ*hpf* mutant harbors more intracellular gentamicin than the wild-type does. To test this hypothesis, *E. coli* Δ*rmf* and Δ*hpf* deletion mutants were evaluated for sensitivity to gentamicin (Fig. 4). Wild-type *E. coli* was much more sensitive to gentamicin than *L. monocytogenes* and required that the gentamicin concentration be reduced by half in order not to sterilize the cultures. At 5 μg/ml gentamicin, stationary-phase WT *E. coli* exhibited a 2-log$_{10}$-unit loss in the numbers of CFU after 2 days. Contrary to the hypothesis of competitive binding, the Δ*rmf* mutant but not the Δ*hpf* mutant exhibited sensitivity to gentamicin. After 2 days, the numbers of CFU from the Δ*rmf* mutant were at levels below the limit of detection. Conversely, the Δ*hpf* mutant exhibited sensitivity similar to that of the WT. Given that RMF-bound ribosomes are translationally silent, these results suggest that ribosome hibernation protects bacteria by inactivating ribosomes rather than by blocking aminoglycoside binding.

DISCUSSION

The 100S ribosomes were first observed over 50 years ago (51), yet the precise function of ribosome hibernation remains unclear. This study demonstrated that ribosome hibernation is critical for the high level of tolerance to aminoglycosides exhibited by stationary-phase bacteria. Stationary-phase Δ*hpf* mutant bacteria remained inappropriately energized and, accordingly, exhibited enhanced gentamicin uptake. Bacterial killing was suppressed by genetically or chemically disrupting processes like energy production. Taken together, these data imply that during stationary phase the Δ*hpf* mutant fails to adequately downregulate cellular activities that contribute to the bactericidal activity of aminoglycosides.

On the basis of these data, we propose that ribosome hibernation is an important mechanism for energy conservation under conditions where nutrients are limiting. Exactly how ribosome hibernation contributes to aminoglycoside tolerance remains unclear. A simple model is that by inactivating a significant portion of ribosomes available for translation, ribosome hibernation reduces overall protein production, which has downstream effects on growth, metabolism, and energy production. The net result is a reduction in PMF, which blocks aminoglycoside import and subsequent killing.

The data presented here indicate that ribosome hibernation

influences the two requirements for the bactericidal activity of aminoglycosides: energy-dependent uptake and active translation. While aminoglycoside uptake has been extensively studied (44), the lethal mode of action for aminoglycosides remains a matter of debate. It has been proposed that aminoglycosides reduce translation fidelity and result in the production of toxic peptides that cause lethal membrane damage (52). It is possible that, in the absence of 100S ribosomes, translation fidelity is further reduced and potentiates the effect of these mistranslated proteins. Although the role of ribosome hibernation in translation fidelity has not been studied, there are many other examples of cellular mechanisms for ensuring translation fidelity, for instance, the stringent response mediated by (p)ppGpp, which is crucial for tuning the translational capacity so that it is commensurate with the amounts of nutrients available during periods of amino acid deprivation (53). The stringent response contributes to translation fidelity, survival, and antibiotic tolerance (53–56). Ribosome hibernation factors are regulated in response to (p)ppGpp (24, 57, 58), and the connection between 100S ribosomes, the stringent response, translation fidelity, and antibiotic tolerance remains to be explored.

The recently described crystal structure of heterologous 100S ribosomes comprising *Thermus thermophilus* 70S ribosomes and *E. coli* RMF and HPF demonstrated that the short HPF homolog binds the ribosome at a site directly overlapping the binding site of aminoglycosides (49). We therefore hypothesized that ribosomes bound by HPF would naturally be protected from aminoglycoside binding and subsequent death. Long HPF is thought to perform the activities of both short HPF and RMF and likely binds ribosomes in a similar manner. We therefore attempted to test our hypothesis by generating constructs that split the *L. monocytogenes* long HPF into the N-terminal portion, which corresponds to the short HPF, and the C-terminal portion, which is thought to serve the function of RMF. However, coexpression of these constructs failed to reconstitute the activity of HPF, and bacteria expressing these constructs did not form 100S ribosomes (data not shown). So, we tested ribosome hibernation mutants of *E. coli*, which provide a nice system for uncoupling the dimerization activity of RMF (which is required for 90S ribosome formation) and the binding activity of HPF. Contrary to our hypothesis of competitive binding, the *E. coli* Δ*rmf* mutant but not the Δ*hpf* mutant exhibited sensitivity to gentamicin. These data suggest that ribosome inactivation rather than blocking of ribosome binding protects bacteria from the bactericidal activity of aminoglycosides. Although short HPF does not contribute to aminoglycoside tolerance *per se*, it is possible that, by protecting ribosomes from irreversible aminoglycoside binding, HPF aids in recovery once the
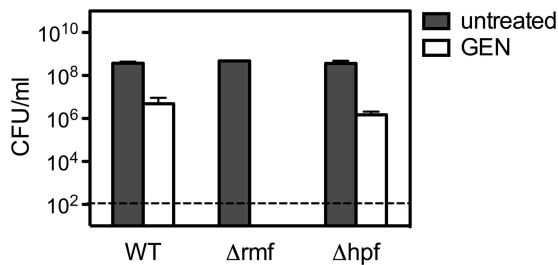
FIG 4 Stationary-phase *E. coli* Δ*rmf* hibernation mutant cultures are susceptible to prolonged gentamicin exposure. The numbers of CFU per milliliter after a 48-h incubation with gentamicin at 5 µg/ml are provided. Means and SDs ($n = 3$) are presented.

antibiotic stress is removed. Further studies on outgrowth after antibiotic treatment will be required to examine any additional roles that HPF may play in recovery from stress.

The bacterial growth rate is correlated with the translational capacity of bacteria (59) as well as ATP levels (60). Given that a Δ*hpf* mutant is unable to inactivate ribosomes into 100S particles (21), we hypothesized that the Δ*hpf* mutant may continue to divide even during stationary phase. However, we have been unable to demonstrate a significant effect of ribosome hibernation on growth. We previously reported that the doubling time of the Δ*hpf* mutant does not significantly differ from that of the WT (43 and 40 min, respectively) (21), and time-lapse microscopy studies failed to provide evidence that the Δ*hpf* mutant continues to divide during stationary phase (data not shown). Previous work has demonstrated that bacteria can remain metabolically active without dividing (42, 61, 62). Taken together, these data suggest that hibernating ribosomes do not influence cell division but probably do have effects on other cellular activities.

The reduction of ATP levels and the subsequent homeostasis of bacteria in no-growth states are important adaptive responses to nutrient limitation. In *E. coli*, ATP levels decrease 5-fold upon entry into stationary phase, which triggers an important homeostatic loop for regulating ribosome biogenesis (47). Furthermore, this drop in ATP levels is required for the stability and activation of RpoS and σ_B, which are the major regulators of adaptation to stationary phase in *E. coli* and *Bacillus subtilis*, respectively (63, 64). Finally, like *E. coli*, when *Mycobacterium tuberculosis* shifts to a nonreplicating state, ATP levels are reduced by 5-fold and maintained at this reduced level (46, 48). This ATP reduction is important for survival and correlated with dramatically enhanced antibiotic tolerance (46, 65). It will be interesting to determine whether ribosome hibernation contributes to maintaining the 5-fold drop in ATP levels and the notorious drug recalcitrance of nonreplicating *M. tuberculosis* cells.

This work supports the idea proposed by others that ribosome hibernation adjusts the cell's translational capacity according to the available nutrients (15, 18). Although *in vitro* studies have conclusively shown that 100S ribosomes are translationally silent (13, 17, 49), the effect of ribosome hibernation on protein production *in vivo* has not yet been determined and should be the focus of future work. While global translation is reduced during stationary phase, translation continues at low levels and is critical for survival and subsequent recovery (61, 62, 66). It is possible that, in addition to reducing the overall translation level, ribosome hibernation is required to facilitate the efficient translation of the

proteins required for stationary-phase survival. Future studies of the role of ribosome hibernation in translation regulation and adaptation are critical to obtain a complete understanding of this fundamental bacterial process.

In the broader sense, this work suggests that ribosome hibernation represents an Achilles heel for populations of slow-growing or latent bacteria. Pathogens like *M. tuberculosis* and *Pseudomonas aeruginosa* within biofilms that cause recurrent infections may employ ribosome hibernation to avoid being killed by antibiotics. The development of strategies that disrupt 100S ribosomes may vastly improve the efficacy of conventional antimicrobial agents.

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