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Ribosome Hibernation

Thomas Prossliner, Kristoffer Skovbo Winther, Michael Askvad Sørensen, and Kenn Gerdes

Department of Biology, University of Copenhagen, DK-2200 Copenhagen, Denmark; email: kgerdes@bio.ku.dk

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Abstract

Protein synthesis consumes a large fraction of available resources in the cell. When bacteria encounter unfavorable conditions and cease to grow, specialized mechanisms are in place to ensure the overall reduction of costly protein synthesis while maintaining a basal level of translation. A number of ribosome-associated factors are involved in this regulation; some confer an inactive, hibernating state of the ribosome in the form of 70S monomers (RaiA; this and the following are based on *Escherichia coli* nomenclature) or 100S dimers (RMF and HPF homologs), and others inhibit translation at different stages in the translation cycle (RsfS, YqjD and paralogs, SRA, and EttA). Stationary phase cells therefore exhibit a complex array of different ribosome subpopulations that adjusts the translational capacity of the cell to the encountered conditions and ensures efficient reactivation of translation when conditions improve. Here, we review the current state of research regarding stationary phase-specific translation factors, in particular ribosome hibernation factors and other forms of translational regulation in response to stress conditions.

INTRODUCTION

Stationary phase: the growth phase of a bacterial culture during which the net number of viable cells has ceased to increase

Ribosome-associated factors: proteins that are transiently and/or substoichiometrically bound to ribosomes and are therefore not considered canonical ribosomal proteins

Ribosome hibernation: the active formation of idling ribosome complexes in stationary phase cells

General stress response: reprogramming of global transcription by alternative sigma factor 38, increasing tolerance to a variety of stresses

Protein synthesis is a fundamental cellular process in all organisms and has been extensively studied over many years. While astounding progress has been made in understanding the structure and function of the cellular protein synthesis apparatus, the ribosome, many aspects still need to be elucidated. In particular, the ribosomal response of microorganisms to adverse conditions that lead to growth arrest and stationary phase remains only partly understood. Under such conditions, bacteria need to reduce energy consumption by decreasing global protein synthesis to a fraction of its full potential while maintaining the ability to rapidly resume growth. This is thought to be mediated, at least in part, by a set of stationary phase-specific ribosome-associated factors that convert actively translating 70S ribosomes to inactive 70S monomers or 100S dimers. This mechanism, referred to as ribosome hibernation, is almost ubiquitous in bacteria (29, 106, 125), and some evidence suggests the existence of similar mechanisms in eukaryotes (54, 88). In addition to ribosome hibernation, other mechanisms of translational modulation have been discovered recently that may contribute to the reprogramming of global translation during stress conditions (14, 42, 47, 122). The aim of this review is to provide an in-depth analysis of the current state of research regarding ribosome modulation in stationary phase with emphasis on ribosome hibernation in gammaproteobacteria such as *Escherichia coli* as well as its counterparts in other bacteria. We address the factors involved and the structural basis and physiological significance of this intriguing phenomenon, and we discuss related mechanisms, open questions, and possible future directions of research.

Stationary Phase and the Stringent Response

The term stationary phase was coined over 70 years ago by one of the pioneers of microbial growth physiology, Jacques Monod (67). Monod described the fundamental laws underlying bacterial growth in a controlled laboratory environment and proposed the division of the life cycle of a bacterial culture into distinct stages, which have remained nearly unchanged until today: (*a*) lag phase; (*b*) exponential or log phase; (*c*) transition to stationary phase; (*d*) stationary phase; (*e*) death phase; and (*f*) prolonged stationary phase (67, 68). These stages apply neatly to the highly artificial and optimized culture environment in the laboratory. In natural habitats, bacteria encounter vastly different conditions: Nutrient limitation and harsh, highly variable conditions are the norm, and, thus, the majority of naturally occurring microorganisms are thought to be in a dormant or slow-growing state comparable to the stationary phase (53). Estimates assume that up to 60% of the global biomass can be attributed to such resting microorganisms (38), underscoring the importance of a deeper understanding of this phenomenon.

Although stationary phase is a highly heterogeneous state, some common morphological and metabolic changes can be observed upon entry to this growth phase in most cases. *E. coli* cells decrease in size and change from their characteristic rod-like shape to an almost spherical shape. The cytoplasm condenses, the volume of the periplasm increases, and the compositions of cell wall and membrane are altered. Additionally, DNA replication and cell division cease, and DNA reorganizations take place in the form of nucleoid condensation and changes in the superhelical structure of plasmids (12, 35, 53, 68). These changes are governed by a substantial rearrangement of gene expression by general as well as specific stress response regulatory pathways. In *E. coli*, one of the major factors involved in transcriptional regulation under such conditions is the alternative sigma factor 38, responsible for inducing the so-called general stress response (56, 68). In addition, several stress-specific sigma factors regulate transcription in response to nitrogen and iron limitation, heat shock, or envelope stress (95).

Other stress-induced regulators, collectively referred to as second messengers, are the molecules cyclic AMP (cAMP), cyclic di-GMP, and, in particular, (p)ppGpp. The latter, which is the collective term for the stringent response regulators guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp), exerts its regulatory function on multiple levels, affecting transcription, translation, and even replication (41, 59). In *E. coli* and other gammaproteobacteria, pppGpp and ppGpp are synthesized by addition of a phosphate group to GTP (or GDP, respectively) by RelA and SpoT in an ATP-dependent manner. RelA exhibits only synthetase activity and is activated by elevated levels of uncharged transfer RNAs (tRNAs) encountered under starvation, whereas SpoT can both synthesize and hydrolyze (p)ppGpp in response to specific signals. (p)ppGpp regulates transcription via association with RNA polymerase and thereby inhibits the synthesis of stable RNAs [tRNAs and ribosomal RNAs (rRNAs)], ribosomal proteins and other components of the translation machinery. Transcription of genes that encode factors involved in adaptation to nutrient limitation on the other hand is stimulated (41, 76).

Stringent response: (p)ppGpp-mediated reprogramming of cell physiology in response to stress, such as amino acid, carbon, and fatty acid starvation and increased oxidation during infection

100S complex: ribosome dimer consisting of two 70S ribosomes, resulting in a particle with a sedimentation coefficient of 100S

Sucrose gradient sedimentation: method for the separation of macromolecules according to their sedimentation coefficient, which is dependent on size and shape of the particle

The Bacterial Ribosome and the Canonical Translation Cycle

Ribosomes (70S) are large complexes that consist of two heterogeneous ribonucleoprotein subunits. In bacteria, the large 50S subunit consists of two rRNAs, the 23S rRNA and the 5S rRNA, as well as around 30 ribosomal proteins. The small 30S subunit contains a single rRNA of 16S and more than 20 ribosomal proteins. Three tRNA binding sites span the two subunits: the A-, P-, and E-sites. Decoding of the genetic information stored in the messenger RNA (mRNA) takes place by interaction between mRNA codons and cognate tRNAs in the A-site of the small subunit. The 50S subunit contains the catalytic center of the ribosome, the peptidyl transferase center, where the peptide bond is formed between the nascent polypeptide chain bound to the P-site tRNA and an amino acid presented by tRNA in the A-site. After translocation, the resulting uncharged tRNA temporarily resides in the E-site before dissociating from the complex (94).

The full translation cycle is a complex chain of reactions involving a multitude of auxiliary factors and is often divided into four stages: initiation, elongation, termination, and recycling. In the initiation step, a 70S initiation complex is formed consisting of the assembled subunits, mRNA, and an initiator formyl-methionine (fMet)-tRNA^{fMet} in the P-site. The elongation stage is essentially the continuous incorporation of amino acids into a growing polypeptide chain using the mRNA as a template. When the translational complex encounters a stop codon on the mRNA, termination of translation is induced, resulting in the release of the completed polypeptide and a 70S ribosome carrying an mRNA and a deacylated tRNA in the P-site. Finally, this posttermination complex is returned to its initial state in the recycling step to make components of the ribosomal machinery available for initiation of another round of translation (81, 86). While the regular translation cycle has been extensively studied and is therefore relatively well-understood, it has become clear that diversions from the canonical mechanism are the rule rather than the exception and provide an important additional level of translation regulation in response to adverse conditions.

RIBOSOME HIBERNATION AND THE FACTORS INVOLVED

100S Ribosome Dimers as Physiologically Relevant Macromolecules

In bacteria, one of the most striking alterations of the translational apparatus taking place during stress is the dimerization of 70S ribosomes to 100S complexes. These particles were initially observed almost 60 years ago in sucrose gradient sedimentation profiles of *E. coli* cell lysates

Hibernation factors: small, ribosome-associated protein factors that confer a hibernating state upon binding to ribosomes

(63, 100) and electron micrographs of high-magnesium ribosome preparations (40). Remarkably, it was already then speculated that ". . .100-S ribosomes may be regarded as inert ribonucleoprotein which can rapidly be converted into protein-synthesising machinery" (63, p. 564). However, in part due to the focus of research on exponentially growing cells, it has become clear only more recently that 100S dimers indeed represent a physiologically relevant state of the ribosome (72, 115, 118).

The 100S complexes consist of two 70S ribosomes arranged in a 50S–30S–30S–50S conformation and they can constitute up to 60% of the total ribosome pool in the cell (11a, 72, 112, 115, 118, 123). 100S ribosomes are translationally inactive in vitro and devoid of mRNA (11a, 49, 72, 113). The appearance of 100S dimers in *E. coli* follows a growth phase-dependent pattern: Although 100S dimers are absent during exponential growth, they appear upon transition to stationary phase and remain present for a period that varies by growth medium and strain (90, 108, 112, 114, 115). Strikingly, 100S formation is rapidly reversed upon stress relief: Within one minute of transferring cells to fresh growth medium, the entire subpopulation of inactive dimerized ribosomes dissociates, and the cell can rapidly resume growth (3, 112). Based on the properties of these complexes, the phenomenon of idling ribosome dimers (and, later, also inactive 70S ribosome monomers) in stationary phase has been dubbed ribosome hibernation (125).

Small Ribosome-Associated Translation Factors

A variety of small, ribosome-associated factors have been identified that are not considered bona fide ribosomal proteins because they are associated with the ribosome either transiently or at a substoichiometric ratio (or both). These factors enable the translation apparatus to overcome a variety of stresses ranging from mRNA damage, antibiotic stress, ribosome stalling, temperature shock, and oxidative stress to nutrient deprivation and other stationary phase conditions (93). Several of these factors are specifically involved in modulating ribosome activity during growth arrest and are discussed here (**Table 1**).

The Hibernation Factors of *Escherichia coli*: Ribosome Modulation Factor, Hibernation Promoting Factor, and Ribosome-Associated Inhibitor A

Ribosome hibernation has been shown to be almost ubiquitously present within the bacterial domain as well as in plastids of plants (29, 88, 106). However, the factors and mechanisms involved vary. In *E. coli*, three factors have been associated with the occurrence of hibernating 100S and 70S ribosomes and are therefore collectively referred to as hibernation factors (**Table 1**): ribosome modulation factor (RMF), hibernation promoting factor (HPF), and ribosome-associated inhibitor A (RaiA). RMF is a small, basic protein of 55 amino acids and has been identified as the essential component for 100S formation (112, 115). The protein is exclusively associated with 100S dimers and is both sufficient and essential for the dimerization mechanism; deletion of *rmf* results in abolishment of 100S formation (114, 118), and RMF can dimerize 70S ribosomes to a 90S dimer complex in vitro (104, 108). The 90S dimer intermediate is in turn converted to its mature form by the second factor, HPF, resulting in a stable 100S particle (60, 113) (**Figure 1*a***). The supporting role of HPF is reflected by the fact that it is not sufficient to dimerize ribosomes on its own in vitro (104). However, despite this merely auxiliary function in vitro, an *E. coli hpf* mutant is deficient in forming ribosome dimers in vivo (108). This is thought to be due to the presence of the third hibernation factor, RaiA, which has been shown to stabilize vacant 70S ribosomes in an inactive state (1, 109) (**Figure 1*b***). In the absence of HPF, RaiA most likely shifts the balance between 70S monomers and 100S dimers toward the former. In agreement with this proposal, a double mutant

Table 1 Selected factors of *Escherichia coli* that interact with the ribosome under nutrient limitation and stationary phase conditions

Factor	Alternative name(s)	Length (aa)	Putative function	Select reference(s)
Ribosome modulation factor (RMF)	Res, RimF	55	Dimerization of 70S ribosomes to 90S dimers	112, 113, 115
Hibernation promoting factor (HPF)	YhbH	95	Maturation of 90S–RMF ribosome dimers to translationally silent 100S complexes	60, 104
Ribosome-associated inhibitor A (RaiA)	YfiA, pY, Urf1	113	Stabilization of 70S ribosomes in an inactive state	2, 60
Ribosome silencing factor S (RsfS)	YbeB, RsfA	105	Inhibition of ribosome subunit association	42
Stationary phase-induced ribosome-associated protein (SRA)	RpsV, Protein D	45	Binds to 70S ribosomes in stationary phase; function unknown	47
Energy-dependent translational throttle A (EttA)	YjjK	555	Translation inhibition in response to low ATP levels	14, 21
YqjD	NA	101	Localization of 70S and 100S ribosomes to the inner cell membrane	122
ElaB	YfbD	101	Analogous to YqjD	39
YgaM	NA	109	Analogous to YqjD	122

Abbreviations: aa, amino acid; NA, not applicable.

lacking both *hpf* and *raiA* exhibits 90S dimer formation, and HPF and RaiA share a binding site on the ribosome (see the section titled Structural Basis for Ribosome Modulation by Hibernation Factors). These findings suggest mutually exclusive binding of the two factors (108). Importantly, both forms of hibernating ribosomes, RMF–HPF–100S dimers and RaiA–70S monomers, are likely to coexist in the cell (60, 108).

The Single Hibernation Factor in Other Organisms: Long Hibernation Promoting Factor

The formation of 100S dimers by the concerted action of RMF and HPF and the stabilization of 70S ribosomes by RaiA are confined to gammaproteobacteria (106, 125). Most other bacteria do not possess RMF and RaiA but instead have an HPF homolog that is necessary and sufficient to promote the formation of translationally silent 100S ribosomes (4, 52, 78, 106). Compared with the HPF of *E. coli*, long HPFs (lHPFs) contain a C-terminal extension and mediate dimerization by direct interaction between the two lHPF molecules in the complex (**Figure 1*c***): Two 70S ribosomes are tethered together by an lHPF homodimer spanning the contact areas between the 30S subunits.

Organisms that produce lHPF also exhibit dimerization of ribosomes in response to growth arrest. Interestingly, lHPF–100S dimers have also been detected in exponentially growing cells, albeit at lower levels than in stationary phase, suggesting that their role is not restricted to slow growth and stationary phase (4, 52, 78, 106, 107). Furthermore, lHPF–100S complexes appear to be more stable than RMF–HPF–100S particles (106). In addition to the distinct nature of RMF–HPF–100S and lHPF–100S assemblies, there are also noticeable differences within each

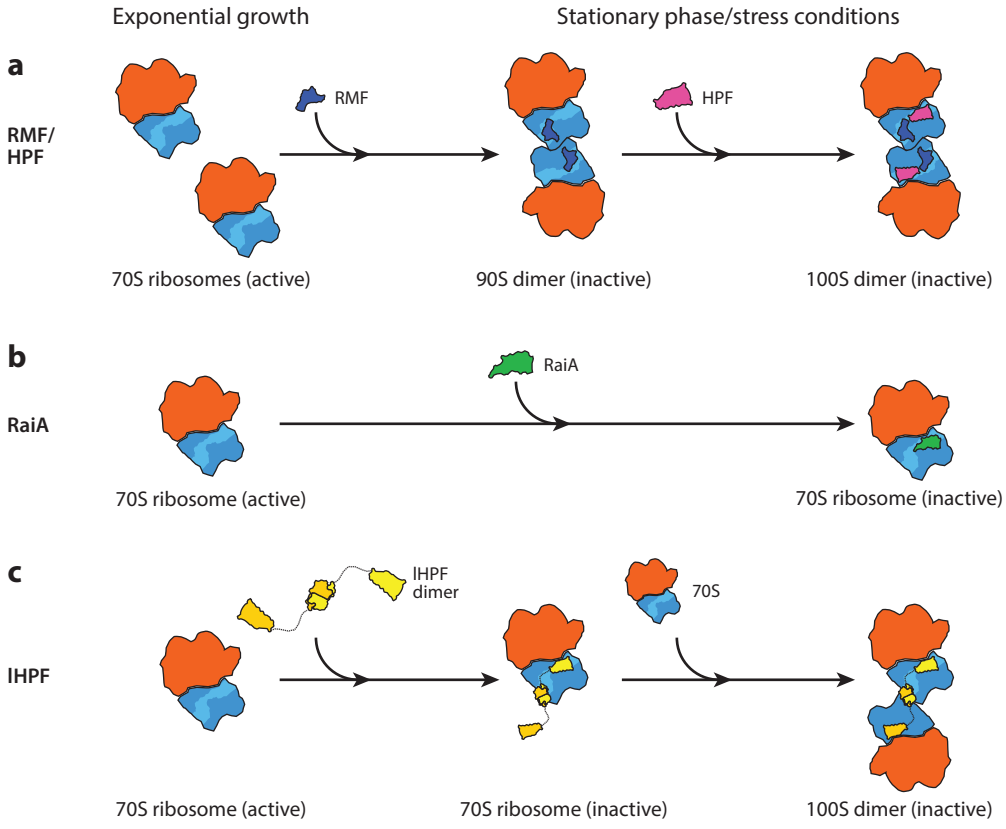


Figure 1

Models of the mechanism of action of hibernation factors in *Escherichia coli* and other bacteria. (*a*) Formation of the 100S ribosome particle mediated by ribosome modulation factor (RMF) and hibernation promoting factor (HPF) in *E. coli* and other gammaproteobacteria. Upon growth arrest, both factors are synthesized. RMF triggers the transient dimerization of two 70S ribosomes to a translationally inactive 90S dimer, which in turn is converted to its mature 100S form by binding of HPF. (*b*) Transition to stationary phase and other stress conditions leads to an increase in ribosome-associated inhibitor A (RaiA) levels. RaiA binds to and stabilizes the 70S ribosome in an inactive state. (*c*) In most other organisms, long HPF (lHPF) is responsible for the dimerization of ribosomes. Free lHPF is present as a dimer and binds in this conformation to a 70S ribosome, thereby inhibiting translation. A second 70S particle joins the complex and the complete 100S dimer is formed. Note that 100S dimers formed by lHPF are also present at some level during exponential growth; also, the lHPF-bound 70S ribosome is not merely an intermediate state but is thought to exist as a separate form of the ribosome in the cell.

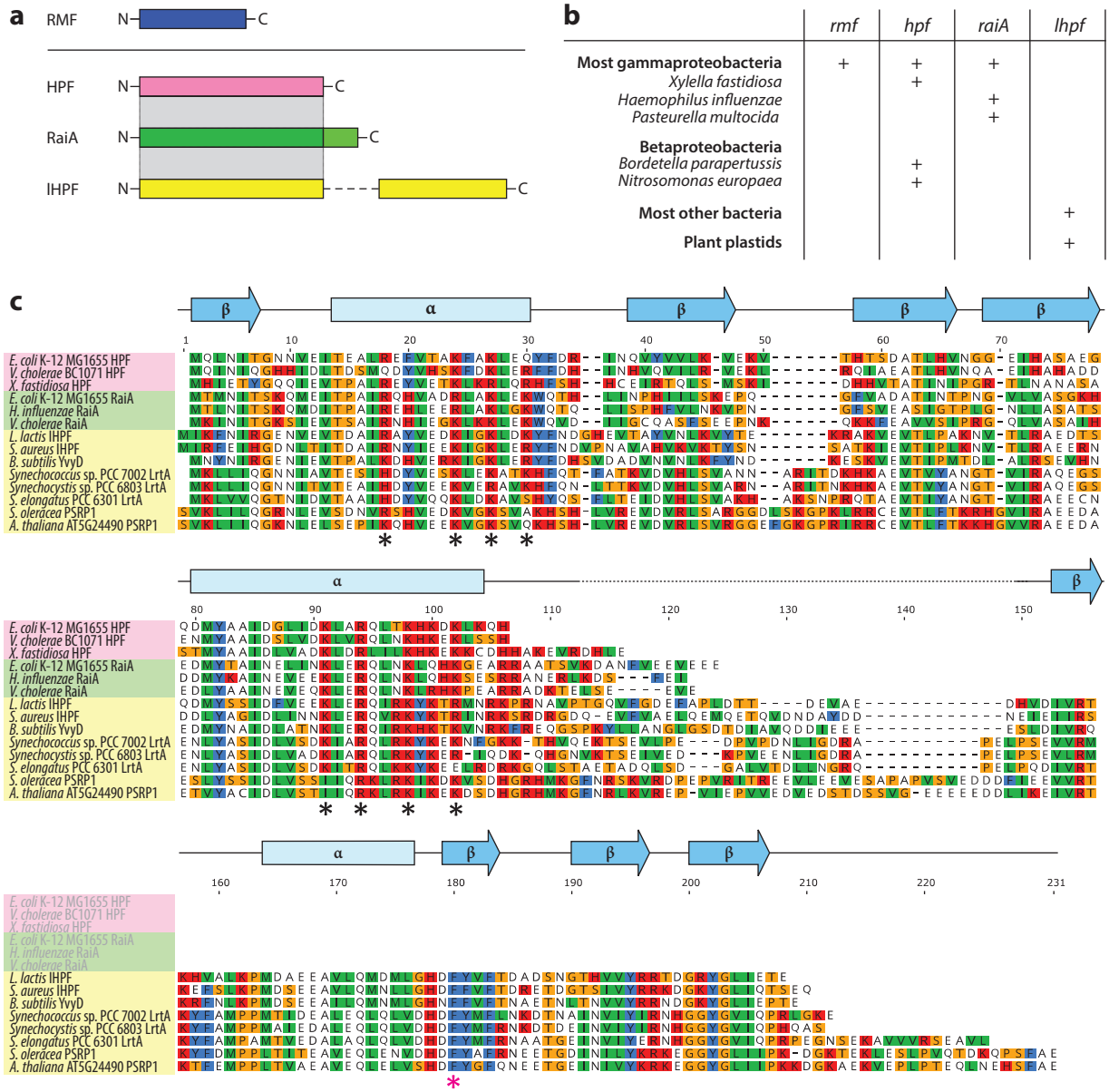
of these two groups of 100S dimers. Significant variations have been reported in the stability and growth phase-dependent patterns of formation of these assemblies across organisms (106, 107). For example, high salt treatment, which releases loosely associated proteins, tRNAs, and mRNA from ribosome preparations, does not lead to dissociation of lHPF–100S dimers isolated from *Lactobacillus paracasei*, whereas *Staphylococcus aureus* lHPF–100S complexes readily dissociate. Also, the time course of RMF–HPF–100S formation in gammaproteobacteria is highly dependent on the species (106, 107).

In contrast to RMF and short HPF, lHPF is present not only in the 100S fraction of the ribosome pool but also in the 70S fraction (9, 52, 106, 107) (**Figure 1*c***). These lHPF-bound 70S

complexes may constitute a second population of hibernating ribosomes in organisms carrying an *lhpf* homolog, reminiscent of the RaiA-stabilized 70S monosomes in *E. coli*.

Phylogeny of Hibernation Factors

While RMF is confined to the gammaproteobacteria, HPF homologs can be found in almost all bacteria and even plant plastids. HPF homologs have been divided into three distinct groups, based on protein sequence and length: HPF, RaiA, and lHPF (**Figure 2*a***). Conserved sequences in *E. coli* HPF are also found in the N-terminal domain (NTD) of RaiA and lHPFs. RaiA is similar



(Caption appears on following page)

Figure 2 (*Figure appears on preceding page*)

Hibernation factors across bacterial domains. (*a*) The phylogenetic relationship between hibernation factors. Ribosome modulation factor (RMF) is unrelated to hibernation promoting factor (HPF), the homologs of which can be divided into short HPF, ribosome-associated inhibitor A (RaiA), and long HPF (lHPF). The homologous regions (*gray*) are limited to the N-terminal domains (NTDs) of the factors. RaiA contains a 13-residue extension not present in short HPF. lHPF homologs contain an additional C-terminal domain (CTD) that is absent in both short HPF and RaiA. A linker region of variable length connects the lHPF NTD and CTD. (*b*) Presence of hibernation factor genes in bacteria and plant plastids. The presence of *rmf*, *hpf*, and *raiA* is confined to gammaproteobacteria. In several cases, only *hpf* (e.g., *Xylella fastidiosa*) or only *raiA* (e.g., *Haemophilus influenzae*, *Pasteurella multocida*) is present. Some betaproteobacteria also contain only *hpf* (e.g., *Bordetella parapertussis*, *Nitrosomonas europaea*). Most other bacteria and plant plastids carry *lhpf* members. *lhpf* in plastids encodes an lHPF variant that is not capable of dimerization of ribosomes and is therefore thought to be a functional homolog of RaiA. (*c*) Multiple sequence alignment of selected HPF homologs across the domains of life. ClustalW (36, 57) in Geneious software version 11.0.4 (50) was used to align HPFs (*magenta*) of *Escherichia coli* str. K-12 substr. MG1655 (NP_417670.1), *Vibrio cholerae* (WP_001166643.1), and *X. fastidiosa* (WP_004083778.1); RaiAs (*green*) of *E. coli* (NP_417088.1), *H. influenzae* (WP_005689430.1), and *V. cholerae* (WP_000700179.1); and lHPFs (*yellow*) of *Lactococcus lactis* (WP_011834629.1), *Staphylococcus aureus* (WP_000617735.1), *Bacillus subtilis* (WP_003228031.1), *Synechococcus* sp. PCC 7002 (ACA98273.1), *Synechocystis* sp. PCC 6803 (BA000022.2), *Synechococcus elongatus* PCC 6301 (BAD79941.1), *Spinacia oleracea* (XP_021858263.1), and *Arabidopsis thaliana* (NP_568447.1). N-terminal chloroplast import sequences of the nucleus-encoded *S. oleracea* and *A. thaliana* plastid specific ribosomal protein 1 (PSRP1) have been omitted for the sake of clarity. Schematic depiction of secondary structure is based on the determined solution structures of *E. coli* HPF (NTD) and *S. aureus* lHPF (CTD) (51, 85). Black asterisks mark conserved charged residues forming basic patches in the two α helices of the NTD that are involved in ribosome binding. The magenta asterisk marks a conserved phenylalanine residue at position 160 (*S. aureus* numbering) that is essential for the dimerization of the lHPF CTD.

to HPF in both sequence and structure with the exception of a short, C-terminal extension of 18 residues. Interestingly, some gammaproteobacteria encode only *raiA* but not *hpf* nor *rmf*, while others have only *hpf*, suggesting that HPF has a role other than facilitating dimerization by RMF in these organisms (125) (**Figure 2*b***). Indeed, the *hpf* gene is located in the *rpoN* [nitrogen limitation sigma factor 54 (sigma 54)] operon and has been shown to negatively modulate transcription from sigma 54–dependent promoters, an effect that may represent an alternative function (48, 65).

lHPFs are approximately twice the size of short HPF and consist of the HPF homologous NTD and an extended C-terminal domain (CTD) that is exclusive to lHPF proteins. Both domains are connected by a highly variable linker region of 16 to 62 residues (typically 24 to 34) (29). Particularly important for the function of HPF homologs are a series of highly conserved hydrophobic residues in the two α helices of the NTD that form basic patches that interact with the 16S rRNA of the small subunit (see the section titled Structural Basis for Ribosome Modulation by Hibernation Factors). The CTD of lHPF contains a conserved phenylalanine residue at position 160, which has been shown to be essential for the formation of the lHPF homodimer (11) (**Figure 2*c***).

Plastid specific ribosomal protein 1 (PSRP1) is the long form of HPF in plant plastids, which, surprisingly, does not mediate the dimerization of chloroplast ribosomes and fails to mediate dimerization in vitro with purified *E. coli* ribosomes. In contrast, PSRP1 stabilizes 70S ribosomes, and therefore it may be considered a functional homolog of *E. coli* RaiA (15, 88, 89). Interestingly, the lHPF homolog found in mycobacteria, mycobacterial specific protein Y (MPY), seemingly also does not induce ribosome dimerization (58a).

Regulation of Hibernation Factor Expression

The expression of hibernation factors is under tight control by various regulatory mechanisms. In rapidly growing *E. coli* cells, *rmf* mRNA is undetectable but appears upon transition into stationary phase concomitantly with the appearance of 100S ribosomes (3, 91, 118). Transcription of *rmf* is inversely correlated with the growth rate and is induced by a variety of stresses, including

amino acid starvation, heat and cold shock, ethanol and ethidium bromide treatment, changes in pH, osmotic stress, and envelope stress (26, 33, 46, 66, 79, 118). In addition, biofilm formation in *Pseudomonas aeruginosa* triggers a high level of *rmf* transcription (116). The high level of *rmf* expression in stationary phase cultures of *E. coli* is not maintained but instead is downregulated during prolonged stationary phase (8).

In *E. coli*, induction of *rmf* transcription has been shown to be mainly mediated by the global regulators (p)ppGpp and (to some extent) cAMP (46). Interestingly, *rmf* expression is independent of sigma 38 (46, 91, 118), and in vitro experiments suggest that housekeeping sigma 70 recognizes the *rmf* promoter only inefficiently (118). A putative binding motif for sigma 54 has been identified in the promoter region of *rmf* (16). Therefore, differential sigma factor recognition may contribute to transcriptional regulation in addition to the second messengers (p)ppGpp and cAMP. Interestingly, *rmf* transcript stability is highly dependent on the growth phase: Its half-life is unusually long in early stationary phase, at approximately 24 min, and it increases even further to approximately 120 min with continued incubation in the same conditions (3). Transfer of starved cells to fresh medium results in a more than 20-fold reduction of transcript levels within 10 min. This rapid degradation is dependent on active transcription, indicating that de novo synthesized RNA could be involved in destabilization of the transcript (3).

In agreement with the transcriptional regulation of *rmf* expression, RMF protein is detected only during stationary phase (112, 115). Concomitantly with the disappearance of 100S dimers, RMF becomes undetectable within one min of transferring stationary phase cells to fresh medium. Interestingly, disappearance of RMF precedes the depletion of *rmf* mRNA, suggesting the existence of an active mechanism of RMF degradation (3). Active degradation of RMF is further supported by the observation that RMF was not detected in slow growing cells although *rmf* mRNA was present (46). The latter observation could also be explained by the elevated levels of polyamines in stationary phase cells (102). Polyamines have been shown to strongly stimulate RMF synthesis in a concentration-dependent manner by changing the secondary structure in the 5′ terminus of the *rmf* mRNA (99, 102).

While the regulation of *rmf* expression is relatively well-studied, less is known about *hpf* and *raiA*. Like *rmf*, neither *hpf* nor *raiA* are under the control of sigma 38 (46, 91, 118). According to microarray data, both genes are induced by (p)ppGpp (25, 103). In addition, quorum sensing inducer AI-2 moderately induces *hpf* transcription (22). In agreement with its auxiliary role in the RMF-mediated formation of 100S dimers, HPF protein is coexpressed with RMF: Although HPF is detected only scarcely in exponentially growing cells, it is abundant in stationary phase and disappears following transfer of starved cells to fresh medium in under 30 min (60).

Transcription of *raiA* is induced by cAMP–CRP (92) and the Cpx envelope stress response in *E. coli* (79) and in *P. aeruginosa* biofilms (116); however, in contrast to *rmf* and *hpf*, *raiA* promoter activity is also high during exponential growth in *E. coli* (92). Accordingly, RaiA protein can be detected in exponentially growing cells, albeit levels do increase upon transition to stationary phase and during cold shock-induced growth arrest (1, 2, 60). The *raiA* homolog in *Vibrio cholerae*, *vrp*, is downregulated by a small RNA named VrrA in an Hfq-dependent manner. Depletion of VrrA and thus increase of Vrp results in decreased levels of the *V. cholerae* HPF homolog, VC2530. A *vc2530* mutant shows increased levels of Vrp, consistent with a mechanism that maintains the balance between the two factors (82).

Given the different habitats and wide variety of organisms possessing only *lhpf* variants, it is not surprising that the environmental cues and genetic mechanisms to induce and regulate hibernation vary significantly. However, certain common patterns have been identified: In contrast to the hibernation factors of *E. coli*, *lhpf* is generally expressed throughout exponential growth at a basal

level that increases under different stress conditions (106, 107). In *S. aureus*, *lhpf* is induced mainly by the general stress response sigma-B (10), which is also responsible for *lhpf* induction in *Bacillus subtilis* and *Listeria monocytogenes* in response to heat, salt, and ethanol stress and glucose limitation (4, 24, 52). Additionally, amino acid limitation leads to induction of *lhpf* by sigma-H via the stringent response (4, 24, 97). The *lhpf* gene is also induced in biofilm-forming cells of *Bacillus cereus* as compared with planktonic cells (71), and lHPF is the main protein synthesized under glucose starvation in *Lactococcus lactis* (17).

In cyanobacteria, the *hpf* homolog was first described in *Synechococcus* sp. PCC 7002 as light repressed transcript (*lrtA*) (98). As the name implies, *lrtA* mRNA is not present under illuminated conditions but appears upon transferring cells to the dark (83, 98). In *Synechococcus elongatus*, a mechanism analogous to the stringent response results in (p)ppGpp accumulation and thereby induction of *lrtA* upon light depletion (43). Interestingly, *lrtA* mRNA of both *Synechococcus* and *Synechocystis* sp. PCC 6803 is highly stable in the dark, whereas light exposure results in destabilization dependent on an unknown factor and the 5′-untranslated region (UTR) of the mRNA (31, 83). Given the observed regulation of *vrp* in *V. cholerae* by a small RNA that is dependent on the 5′-UTR of the *vrp* transcript (82), it is tempting to speculate that *lrtA* expression may be regulated in a similar manner.

STRUCTURAL BASIS FOR RIBOSOME MODULATION BY HIBERNATION FACTORS

Two Types of Structures of 100S Dimers

Ribosome dimers formed by either RMF and HPF or lHPF differ not only in complex stability and temporal pattern of formation but also in overall structure (**Figure 3**). *E. coli* 100S complexes display a top-to-top orientation, whereas dimers formed by lHPF in other organisms are in a side-to-side conformation (11, 11a, 29, 49, 51, 62, 72) (**Figure 3*a*,*b*,*d***). As a result, the 30S–30S interface in the RMF–HPF–100S complex is markedly larger than in the lHPF–100S (**Figure 3*c***). The direct contact between the two lHPF molecules in the latter ensures stable association between the 70S components of the dimer, consistent with higher stability of the complex compared with the *E. coli* dimer (106).

Dimer Formation in *Escherichia coli* and Other Gammaproteobacteria

All three factors involved in ribosome hibernation of *E. coli* are relatively small monomeric proteins. RMF consists of two α helices connected by a 13-amino acid linker region (75) (**Figure 4*a***). HPF and RaiA share similar structural features: Both adopt β-α-β-β-β-α folds in which the two parallel α helices are in close proximity to a β sheet formed by the four β strands (11a, 75, 80, 85, 121) (**Figure 4*a***). RaiA contains a flexible extension of 18 residues at the C terminus likely to be responsible for the functional differences of the two factors (75, 80). First insight into the structural basis of ribosome hibernation was gained by a high-resolution structure of the *Thermus thermophilus* ribosome in complex with each of the hibernation factors of *E. coli*, which predicted RMF binding on the small subunit (75). However, RMF could also be detected in the 50S fraction after in vitro dissociation of 100S complexes (115), and the results of crosslinking and chemical footprinting studies suggested that RMF covers the peptidyl transferase center and the entrance of the peptide exit tunnel on the large subunit (123, 126). In addition, it should be considered that *T. thermophilus* does not possess *rmf* but instead contains an *lhpf* homolog (106). A recent study

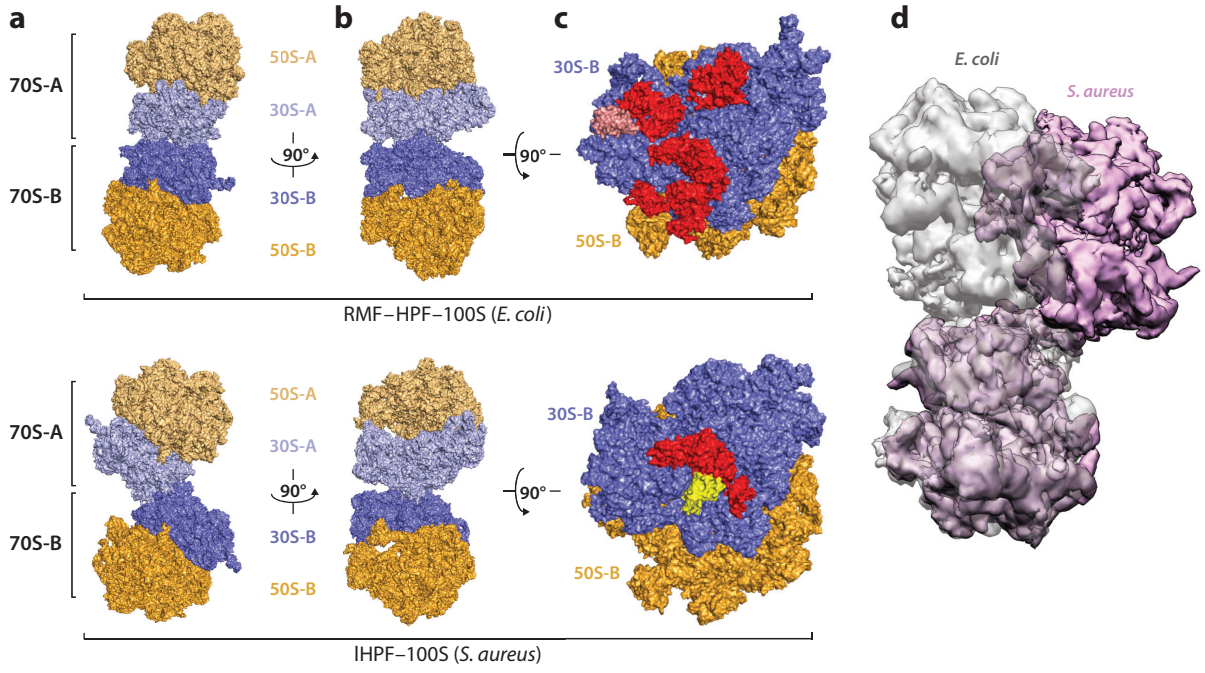
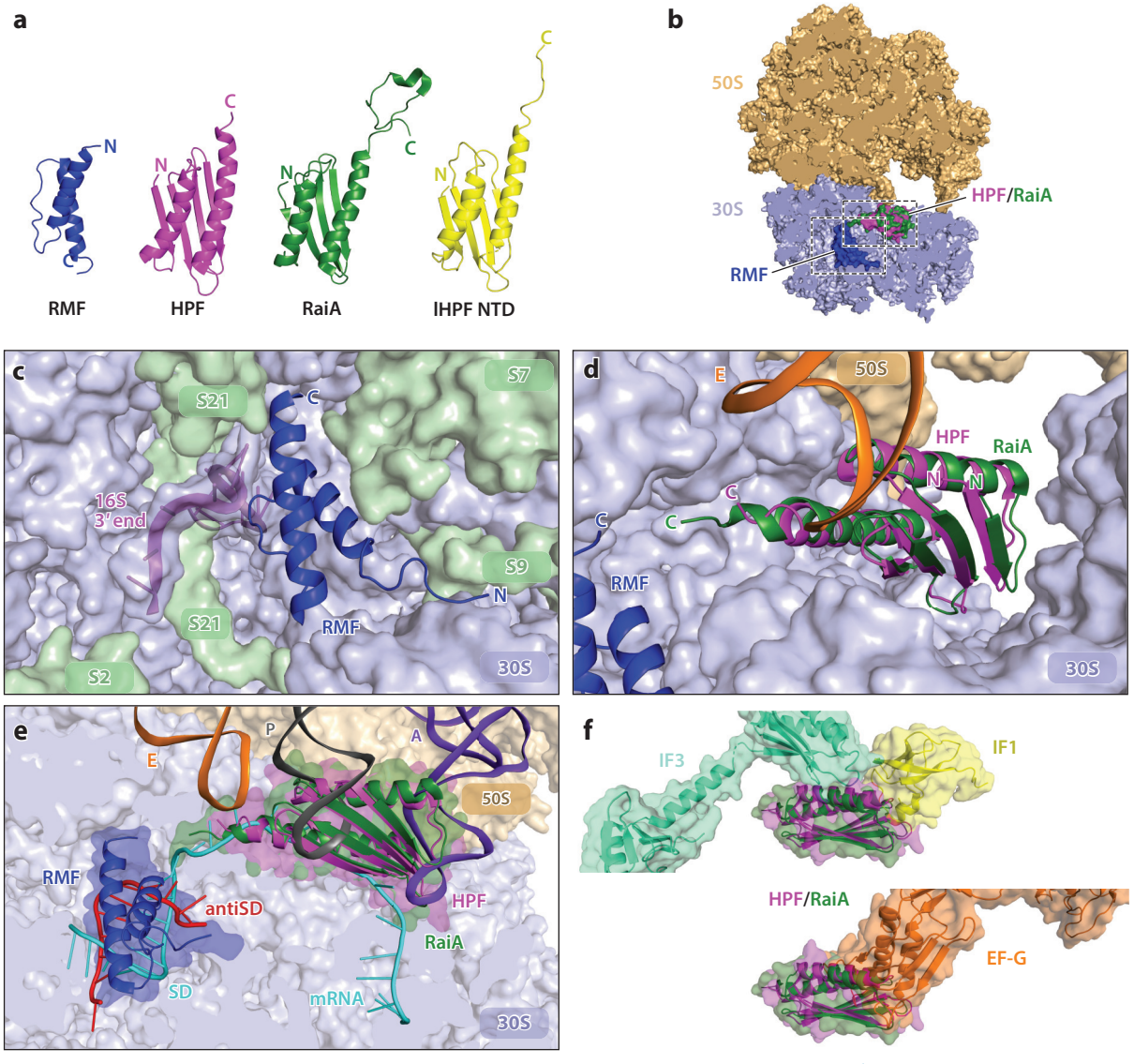


Figure 3

Different conformations of 100S dimers. (*a*) Comparison of the top-to-top conformation found in ribosome modulation factor (RMF)–hibernation promoting factor (HPF)–100S complexes (*top*) and the side-to-side conformation exhibited by long HFP (lHPF)–100S complexes (*bottom*). The model for the RMF–HPF–100S dimer was obtained from a recently published cryo-electron microscopy (cryo-EM) structure of the *Escherichia coli* 100S particle in complex with RMF, HPF, and an E-site transfer RNA (tRNA) (PDB ID: 6H58) (11a). The deposited cryo-EM structure of the *Staphylococcus aureus* 100S dimer (PDB ID: 5NG8) (62) was used as an example of an lHPF–100S complex. Structures were visualized with Pymol version 2.0.6 (87). (*b*) Rotated (90° counterclockwise) view of the complexes shown in panel *a*. (*c*) Top view of the complexes shown in panels *a* and *b*. 70S-A is not shown for visualization of the dimer interface on the 70S-B. The primary interaction sites (*red*) on the 30S involve ribosomal proteins S1, S2, S3, and S4 in *E. coli* and S2 and helix h26 of the 16S rRNA in *S. aureus*. The RMF–HPF–100S complex contains a second putative interaction area consisting of S10 (*salmon*) and an exposed domain of S1 (*not depicted*). The C-terminal domain of lHPF (*yellow*) in the lHPF–100S complex is located at the 30S interface. (*d*) Direct overlay of the cryo-EM density maps of the *E. coli* 100S dimer (EMDB ID: 0139) (11a) and the *S. aureus* 100S dimer (EMDB ID: 3637) (62). Maps were aligned and image was generated using UCSF Chimera version 1.12 (73).

presented high-resolution cryo-electron microscopy (cryo-EM) structures of *E. coli* RMF–HPF–70S and RMF–HPF–100S particles purified from stationary phase cells (11a). The complex appears to be devoid of mRNA, but, in contrast to previously obtained cryo-EM maps of the 100S complex (49, 72), it contains a deacylated tRNA in the E-site. The structure confirms binding of RMF on the 30S subunit, albeit in a different location than previously predicted (11a). RMF was shown to bind between the head and the platform domains of the small subunit, in a pocket created by ribosomal proteins S2, S7, S9, and S21 and 16S rRNA helices h28, h37, and h40 (**Figure 4*b*,*c***). Ribosomal protein S1 covers this cavity in an unusually compacted conformation, which presumably prevents it from exerting its functions during translation initiation. The binding site of RMF overlaps with the interaction region between the mRNA Shine-Dalgarno (SD) sequence and the 3′-terminal antiSD sequence of the 16S rRNA. RMF interacts with three nucleotides (C1535, C1536, and U1537) of the antiSD sequence, thus sterically interfering with the formation of the antiSD/SD helix that occurs during the initiation stage of translation (**Figure 4*e***).

Consistent with their structural similarity, HPF and RaiA share a binding site on the ribosome at the interaction site of tRNAs and mRNA in the channel between the head and body domains of the small subunit (**Figure 4*b*,*d***). HPF directly interacts with the anticodon stem loop of the deacylated tRNA present in the E-site. The binding site occupies both A-sites and P-sites, and overlaps with the known binding sites of initiation factor 1 (IF1), IF3, and elongation factor G (EF-G) (11a, 75, 85, 109) (**Figure 4*e*,*f***). The extended C terminus of RaiA could be only partially modeled but is thought to protrude further into the mRNA channel, reaching into the putative binding site of RMF (**Figure 4*d*,*e***). Thus, it mutually excludes the binding of both factors and explains the opposing effects of HPF and RaiA on the formation of the dimer (11a, 75).



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Dimerization by ribosome modulation factor (RMF) and hibernation promoting factor (HPF) and 70S stabilization by ribosome-associated inhibitor A (RaiA). (*a*) Structures of hibernation factors in *Escherichia coli*. RMF consists of two helices connected by a linker region (PDB ID: 4V8G) (75). HPF (PDB ID: 4V8H), RaiA, and the N-terminal domain (NTD) of long HPF (lHPF) (PDB 5NG8) (62) adopt a β-α-β-β-β-α fold, and RaiA contains an extended, partially flexible C-terminal domain (CTD) (PDB ID: 1N3G) (75, 80). (*b*) Binding of *E. coli* hibernation factors RMF, HPF, and RaiA to the ribosome (*cross section*). Superimposition of the *Thermus thermophilus* ribosome in complex with *E. coli* RaiA (PDB ID: 4V8I) (75) on the *E. coli* RMF–HPF–70S ribosome (PDB ID: 6H4N) (11a). (*c*) Close-up of the RMF (*blue*) binding site depicted in panel *b*. The small subunit ribosomal proteins S2, S7, S9, and S21 in proximity to RMF are depicted in light green, and the 3′-terminal domain of the 16S ribosomal RNA (rRNA) is drawn in purple. For better visibility, S1 has been omitted. (*d*) Close-up of the overlapping binding sites of HPF (*magenta*) and RaiA (*green*). HPF interacts with the deacylated transfer RNA (tRNA) residing in the E-site (*orange*). The flexible 17 C-terminal residues of RaiA are not depicted but are thought to protrude into the binding site of RMF. For the sake of clarity, S7, S9, and parts of the 16S rRNA have been omitted. (*e*) Superimposition of messenger RNA (mRNA) (PDB ID: 4V4Z) (127) and tRNAs in the A- and P-sites (PDB ID: 4V5C) (110) on the binding sites of hibernation factors. The RMF binding site overlaps with the location of the antiShine-Dalgarno (antiSD)/SD helix formed by the 3′ end of 16S rRNA and the mRNA. Both HPF and RaiA bind to the site of interaction between mRNA and tRNAs in the A- and P-sites. (*f*) Superimposed translation factors on the binding sites of HPF and RaiA. Initiation factor 1 (IF1) and IF3 (PDB ID: 5LMT; *top*) (44) and the CTD of elongation factor G (EF-G) (PDB ID: 4V5F; *bottom*) (32) overlap with the binding sites of HPF and RaiA.

Overall, dimerization and concomitant inactivation of 70S ribosomes are thought to be facilitated by stabilization of S1 and S2 upon binding of RMF and HPF: RMF and S1 in its compact conformation prevent translation by interaction with the antiSD sequence of the 16S. S2 extends into the mRNA exit channel of the second ribosome in the dimer, thereby interfering with the binding of mRNA to the ribosome (11a).

Dimerization by Long Hibernation Promoting Factor

The NTD of lHPF homologs is very similar to *E. coli* HPF and RaiA and adopts the described β-α-β-β-β-α fold (**Figure 4*a***), whereas the CTD exhibits a β-α-β-β-β topology (51). A number of recently published high-resolution cryo-EM structures have provided detailed insight into the structural basis for 70S dimerization in organisms encoding lHPF, including *B. subtilis*, *L. lactis* and two variants of *S. aureus* (11, 29, 51, 62) (see **Figure 5** for an example). According to these structures, the NTD of lHPF binds to the same region as *E. coli* HPF (**Figure 5*a*,*b***), which is consistent with the high degree of similarity in their structure and sequence: The binding site overlaps the site of codon–anticodon interaction between mRNA and tRNAs in the A- and P-sites (11, 29, 51, 62). There, the NTD resides in a pocket created by several 16S rRNA helices and ribosomal proteins and contacts the former at various residues (29, 62) (**Figure 5*b***): The NTD β sheet interacts with the 16S rRNA at the head domain via stacking interactions, and conserved positively charged residues in the basic patches of the two α helices contact the body domain of the 30S subunit.

The structure of the linker region between the NTD and CTD of lHPF could not be determined due to its highly flexible nature. However, a short fragment distal to the NTD was shown to reach into the mRNA channel and toward the assigned binding site of the C terminus (11, 29, 51, 62) (**Figure 5*b***). At the 30S–30S interface, the CTD of lHPF bound to the top 70S in the complex forms a homodimer with the CTD bound to the bottom 70S ribosome (11, 29, 51, 62) (**Figure 5*c–e***). Two hydrophobic patches stabilize the interaction, resulting in two parallel β sheets consisting of the first β strand of CTD-A and the last three β strands of CTD-B, and vice versa (29, 51) (**Figure 5*d***). A conserved phenylalanine residue in the second α helix of CTD-A (phe160 in *B. subtilis* and *S. aureus*) forms stacking interactions with phe160 of CTD-B that are essential for the dimerization (**Figure 5*d***). The formation of the CTD homodimer is independent of the NTD of lHPF: Both the full-length lHPF and the CTD-only fragment form dimers in solution and are capable of triggering the dimerization of ribosomes (11, 51).

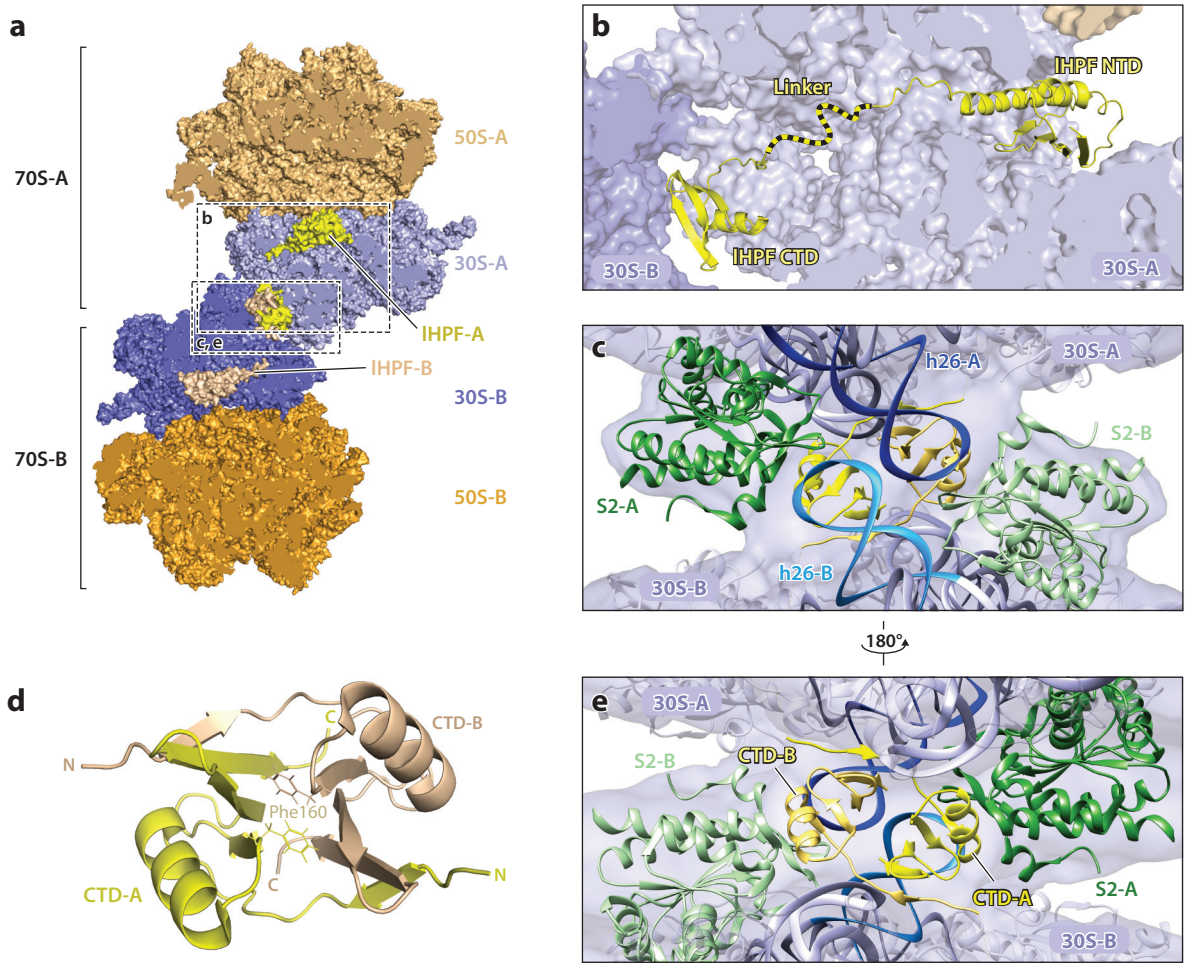


Figure 5

Ribosome dimerization by long hibernation promoting factor (lHPF). (*a*) Display of lHPF binding in the 100S dimer (*cross section*), using the example of the *Staphylococcus aureus* 100S cryo-electron microscopy structure (PDB ID: 4NG8) (62). (*b*) Close-up view of the binding sites of the N-terminal domain (NTD) and C-terminal domain (CTD) of lHPF in the top 70S ribosome of the complex (70S-A). The NTD binds in the messenger RNA (mRNA) binding channel at the interaction site of mRNA and transfer RNAs (compare also with **Figure 4*d*,*e***). The CTD binds at the cytosolic surface of the 30S subunit (the second CTD in the dimer is not depicted for clarity). A flexible linker region (*dashed line*) connects the CTD and NTD. (*c*,*e*) Close-up view of the 30S–30S interface rotated 180°. The 16S ribosomal RNA helices h26 of the top (30S-A) and bottom (30S-B) 30S subunits interact with each other. The 100S complex is tethered together by a homodimer that forms between the CTDs of the two lHPF molecules in the complex. CTD-A interacts with S2 of the same 30S subunit (i.e., CTD-A with S2-A, CTD-B with S2-B). (*d*) Solution structure of the lHPF CTD homodimer (PDB ID: 5NKO) (51). The CTD of lHPF adopts a β-α-β-β-β fold. In the dimer, the first β strand of CTD-A forms a β sheet with the last three β strands of CTD-B, and vice versa. The interaction is stabilized by stacking interactions of a conserved phenylalanine residue (phe160) in the fourth β strand of the CTD that is essential for dimerization. Panels *c–e* adapted from Reference 60 with permission.

The interactions taking place at the 30S interface vary slightly between the published lHPF–100S structures and in some cases even within the same organism, as several distinct conformations of 100S dimers have been observed in *L. lactis* and *S. aureus*. In these conformations designated closed, intermediate, and open in *L. lactis* and tight and loose in *S. aureus*, the respective positions of the two 70S monomers differ: The open and loose conformation exhibits an increased degree of rotation between 70S monomers, resulting in a wider space between the two 30S subunits as compared with the closed or tight state (29, 51). Regardless, common key elements can be identified when comparing the structures (**Figure 5*c,e***). Ribosomal proteins S2 and S18 and helix h16 of the 16S rRNA appear to have central roles in the CTD–30S and 30S–30S interactions. In both *B. subtilis* and *L. lactis*, CTD-A interacts with S2 of 70S-A, and both CTD-A and S2 contact the N-terminal part of 70S-B S18 (11, 29, 62). In the closed conformation in *L. lactis* and in the tight conformation in *S. aureus*, helices h26 of both 30S subunits appear to face each other (29, 51). In the open 100S conformation of *L. lactis*, this interaction is lost; instead, S2 of 70S-A contacts h26 of 70S-B (29). A second structure of the 100S complex of *S. aureus* indicates that overall conformational changes take place in the 30S subunit upon lHPF binding: The head domain rotates with respect to its body domain, resulting in reduced contact surface area between the domains. However, these changes are not sufficient to induce dimerization because lHPF NTD is not capable of forming 100S complexes on its own. Instead, the changes are thought to stabilize the otherwise highly flexible S2 and thereby facilitate the interactions that result in dimerization (62). Notably, lHPF of *S. aureus* can dimerize ribosomes independently of their individual rotational state: In both tight and loose dimers, ribosomes were present in unrotated and rotated states (51).

PSRP1, the lHPF homolog found in plant plastids, represents a special case in the different forms of hibernating ribosome factors. Although PSRP1 is an lHPF homolog based on its sequence, it does not induce dimerization but instead stabilizes ribosomes in their 70S state (15). Therefore, PSRP1 can be considered a functional homolog of *E. coli* RaiA. This discrepancy may be explained by the distinct nature of the chloroplast ribosome (chloro-ribosome); in comparison to the bacterial ribosome, the chloro-ribosome is larger, while the ratio of rRNA to ribosomal protein content is lower (119, 120). Chloro-ribosomes also contain a tightly bound S1 protein with an N-terminal extension at the cytosolic face of the 30S subunit that may interfere with dimerization (15). In vitro assays have shown that *Spinacia oleracea* PSRP1 does not induce the dimerization of *E. coli* ribosomes, however, in contrast to bacterial lHPF variants (88, 106). This suggests that the inability of PSRP1 to form dimers lies in the protein itself rather than in the chloro-ribosome. The NTD of PSRP1 binds to the subunit interface, analogous to HPF, RaiA, and the NTD of other lHPFs. Only weak density was observed for the CTD of PSRP1, indicating that it is flexible (13, 37, 88, 89). In addition, the CTD alone does not bind to the 70S ribosome, whereas PSRP1 lacking a large fragment of the CTD does bind. This, again, appears to contrast with what can be observed for CTDs of lHPF homologs in bacteria (19, 88). A recent study has presented similar observations for the lHPF homolog in *Mycobacterium smegmatis*, MPY, which binds to the ribosome analogous to PSRP1, and does not induce dimerization (58a).

Reactivation Mechanisms

Very little is known about the dissociation mechanisms underlying the reactivation of ribosomes constituting 100S particles. Two possibilities seem obvious: first, passive dissociation of 100S dimers by spontaneous dissociation (and subsequent degradation) of the hibernation factors and thus destabilization of the complex, and, second, active dissociation induced by an unknown mechanism.

The surprisingly rapid disappearance of 100S dimers in *E. coli* upon refeeding may be attributed in part to the apparent equally rapid degradation of the main dimerization factor, RMF (3, 112). However, it seems improbable that proteases can access RMF for degradation while still associated with the ribosome, and passive dissociation therefore seems unlikely to be responsible for the rapid splitting of 100S complexes.

Active dissociation may be occurring in several ways: first, allosteric modulation of the complex by an unknown factor; second, competitive binding of other translation factors resulting in displacement of bound hibernation factors; and third, modifications of the ribosome that lead to reduced binding affinity of the hibernation factors. A recent publication has provided the first insight into active dissociation mechanisms of lHPF–100S ribosomes: The widely conserved GTPase HflX was shown to trigger the dissociation of 100S particles in a GTP-dependent manner in vitro (10). Deletion of *hflX* had only a moderate effect on the fraction of dimerized ribosomes, however, suggesting that HflX is not solely responsible for dissociation. Interestingly, artificially increased levels of lHPF in exponential phase do not necessarily lead to increased 100S formation (78, 97). In conditions that allow rapid growth, translation factors IF1, IF3, and EF-G, which have overlapping binding sites with the NTD of HPF homologs, may prevent excessive binding of lHPF to the ribosome. Consistent with this hypothesis, IF3 promotes HPF dissociation in *E. coli* in vitro assays (124). Interestingly, binding of EF-G to the ribosome is GTP-dependent in the elongation and recycling steps (86). Together with the observed dependence of HflX splitting activity on GTP hydrolysis, this may imply a role for GTP homeostasis [and possibly (p)ppGpp homeostasis] in the formation of 100S dimers. First evidence for ribosome modifications affecting the binding affinity of hibernation factors was found in *M. smegmatis:* MPY exclusively binds to a specialized form of the 70S ribosome, which contains paralogs of ribosomal proteins exchanged in response to zinc starvation (58a). Moreover, ribosomes isolated from stationary phase *E. coli* cells have been shown to be more prone to RMF-mediated dimerization in vitro than exponential phase ribosomes, indicating that a change in the ribosome itself may facilitate dimerization (113). 100S formation in *E. coli* has been shown to be dependent on the presence of the nonessential ribosomal protein L31 (105). L31, which is only loosely associated with the ribosome, is also one of several ribosomal proteins that can be exchanged in 70S ribosomes in vitro and in vivo, demonstrating the flexible nature of ribosome composition (77). Finally, the cytosolic level of polyamines may directly influence the equilibrium between dissociation and association of hibernating ribosomes. These polyvalent cations are important modulators of the translation machinery (45) and are present at increased levels during the transition to stationary phase (102). Importantly, RaiA binding to the ribosome in vitro in the presence of translation factors has been shown to be dependent on the presence of polyamines (109).

Physiological Role of Ribosome Hibernation

The exact role of hibernating ribosomes in cell physiology is not entirely clear. *E. coli* mutants lacking one or several of the hibernation factors do not show any growth impairment during exponential growth, which is in agreement with the observed stationary phase-specific expression patterns (23, 84, 108, 118). Surprisingly, although the formation of 100S dimers in other organisms is not restricted to stationary phase, most studies show that deletion of *lhpf* in these organisms does not affect growth rate either (9, 28, 31, 78).

Ribosome hibernation is frequently cited as an important mechanism for maintenance of viability in stationary phase, although some published results are contradictory. Whereas several reports have shown strongly decreased survival of mutants lacking one or several factors when compared with wild-type cells (7, 46, 90, 118), others have shown that deletion of *rmf*—even in

combination with deletion of other hibernation factors *hpf*, *raiA*, and *sra*—has no effect on survival in stationary phase, although fitness in direct competition with wild-type cells is decreased (18). Deletion of both *hpf* and *raiA* is seemingly also without phenotype, and single deletions even lead to slightly increased viability after several days of incubation (108). These discrepancies may be due to the employment of different *E. coli* strains and the diverse conditions (i.e., growth medium composition) under which viability has been assessed in these studies. The level of 100S formation has been shown to differ even within different strains of the same species and could therefore have variable effects on viability (112). Also, the types of stresses that cells are exposed to in stationary phase are very diverse and dependent on the growth medium, and ribosome hibernation may be needed to survive these conditions. In more defined stress conditions, hibernation factors—in particular RMF—are essential for survival; *rmf* mutants are highly sensitive to heat, acid, and osmotic stress (27, 33, 69). In addition, deletion of *rmf* in *P. aeruginosa* leads to compromised membrane integrity in biofilm-forming cells (116). However, the *rmf* mutant does not show decreased viability in stationary phase or increased susceptibility to osmotic shock, heat shock, acid stress, or gentamicin treatment. Instead, HPF appears to be the factor required for long-term survival of *P. aeruginosa* (5). Similarly, lHPF deficiency in *S. aureus* can lead to decreased survival in stationary phase (9) and under heat shock (62).

Ribosome hibernation has also repeatedly been implicated in the ability of cells to resume growth upon stress relief. In *P. aeruginosa*, deletion of *hpf* leads to delayed resuscitation of nutrient-starved cells (5). A similar role for efficient regrowth has been shown for lHPF in several organisms including *L. lactis*, *B. subtilis*, and *Synechocystis* sp. PCC 6803 (4, 11, 31, 78).

Especially interesting in the context of pathogenic bacteria are the reported observations that implicate ribosome hibernation in persistence and virulence. Deletion of the *lhpf* homolog in *M. smegmatis* leads to decreased tolerance to kanamycin and streptomycin (58a). Similarly, deletion of *lhpf* in *L. monocytogenes* and *rmf* in *E. coli* renders the cells more susceptible to the antibiotic gentamicin, and the *rmf* mutant exhibits decreased tolerance to netilmicin (64, 101, 102). These antibiotics belong to the aminoglycoside family, which targets the translation machinery (117). Tolerance to β-lactam antibiotics and fluoroquinolones is, in contrast, observed to be maintained in the *lhpf* mutant (64). Consistent with a possible protective effect of lHPF against aminoglycosides, the binding site of the lHPF NTD is in close proximity to the described binding site of gentamycin, thus likely interfering with its mechanism of action (51). In addition, ribosome-bound lHPF appears to occlude the reported binding sites of several other aminoglycoside antibiotics as well as those of tetracyclin, the peptide antibiotic edein, and pactamycin (51). Importantly, *lhpf* deletion in *L. monocytogenes* leads to decreased colonization of a mouse infection model (52), and the HPF homolog in the facultative intracellular pathogen *Francisella tularensis* was identified as one of several putative virulence factors (20). In addition, HPF of the plant pathogen *Erwinia amylora* is indispensable for virulence (6). However, this may be due to the proposed alternative function of HPF in modulating transcription from sigma 54–dependent promoters (48, 65).

The reasons for these observed phenotypes are only partly understood. Some reports suggest that hibernation is involved in maintaining the stability of ribosomes: *rmf* and *lhpf* mutants show decreased rRNA content in response to stationary phase, zinc depletion, heat stress, and acid treatment (10, 27, 30, 58a, 69, 90). The 100S dimer may protect ribosomes from degradation by preventing dissociation into free subunits, which have been shown to be the primary substrate for ribonucleoprotein degradation (74, 96, 128). Alternatively, absence of hibernation factors could lead to gratuitous protein synthesis in restrictive conditions and could therefore decrease fitness. However, an *S. aureus* mutant lacking lHPF exhibits only a modest increase of translation upon transition to stationary phase, whereas an *L. lactis* mutant is not affected at all (9, 17). This suggests that the physiological role of ribosome hibernation is of a protective rather than an inhibitory nature.

How Other Ribosome-Associated Factors Silence Translation

Apart from hibernation factors RMF, HPF, and RaiA, several additional factors have been proposed to have complementary functions or serve as alternative forms of translation modulation in stationary phase: ribosome silencing factor S (RsfS), stationary-phase-induced ribosome-associated protein (SRA), the transmembrane domain protein YqjD and its paralogs, and the energy-dependent translational throttle A (EttA) (**Table 1**).

Ribosome silencing factor S. RsfS is highly conserved within bacteria and eukaryotes (42). RsfS binds to the 50S subunit via ribosomal protein L14 and adopts an α-β-β-α-β-β-α fold, in which the β strands form a β sheet that is flanked by the two first α helices on one side and the last α helix on the other (42, 58). While RsfS is a dimer in solution, cryo-EM structures of *Mycobacterium tuberculosis* RsfS bound to the ribosome have shown that binding occurs in a monomeric form (58). Mutants lacking *rsfS* exhibit decreased fitness in direct competition with wild-type cells, in particular during nutrient deprivation (i.e., downshift to poor medium). Similar to the other ribosome-associated factors, RsfS does not appear to be necessary for rapid growth. Upon downshift from rich to poor medium, however, a striking phenotype could be observed: The *rsfS* mutant cells initially grew as rapidly as wild-type cells but then abruptly arrested growth in mid-exponential phase. Growth was resumed only after more than 12 h of continued incubation (42). Intriguingly, lack of RsfS leads to increased protein synthesis upon transition to stationary phase, indicating that the protein has a role in reducing translation upon encountering unfavorable conditions. This was further corroborated by in vitro results showing strong translation inhibition by RsfS via prevention of subunit association, in both bacterial translation systems and mitochondrial translation systems (42, 58). Importantly, RsfS does not act on already assembled 70S ribosomes and 100S complexes (10, 42, 58). RsfS may therefore be a crucial inhibitor of translation initiation under nutrient-limited conditions, acting through prevention of subunit association (**Figure 6*a***).

Stationary-phase-induced ribosome-associated protein (SRA). Very little is known about enterobacterial SRA. It is a small, basic protein with a molecular weight of approximately 5 kDa, consisting of 45 amino acids, and it has been identified as one of four proteins that are tightly associated with the 30S subunit (111). The expression level of SRA in laboratory and wild-type *E. coli* strains increases upon transition to stationary phase from 0.1 to 0.4 molecules per ribosome and remains fairly constant for several days under this condition (47, 122). Transcription of *sra* in stationary phase is partially dependent on sigma 38 and positively regulated by (p)ppGpp, cAMP, and the DNA-binding transcriptional regulators H-NS and FIS (47, 55). No apparent phenotype has yet been observed for a mutant lacking *sra*, and SRA does not seem to have an influence on distribution of ribosomes in their different states (18, 47). Recently, *sra* has been identified as one of several stress-related genes that are downregulated in fast-growing *E. coli* isolates from cystic fibrosis patients (61). Based on this observation and on the tight association of the protein with the ribosome in stationary phase, it has been speculated that SRA may have a regulatory role similar to that of hibernation factors, making it a promising subject for future research.

YqjD/ElaB/YgaM. YqjD is a stationary phase-specific inner membrane protein that binds to both 70S ribosomes and 100S ribosomes and has been proposed to mediate the localization of hibernating ribosomes to the cell membrane (122) (**Figure 6*b***). The two paralogs of YqjD, ElaB and YgaM, are also associated with ribosomes and are upregulated in stationary phase and during slow growth in a sigma 38–dependent manner (39, 122). Polyamines induce *yqjD* expression (102).

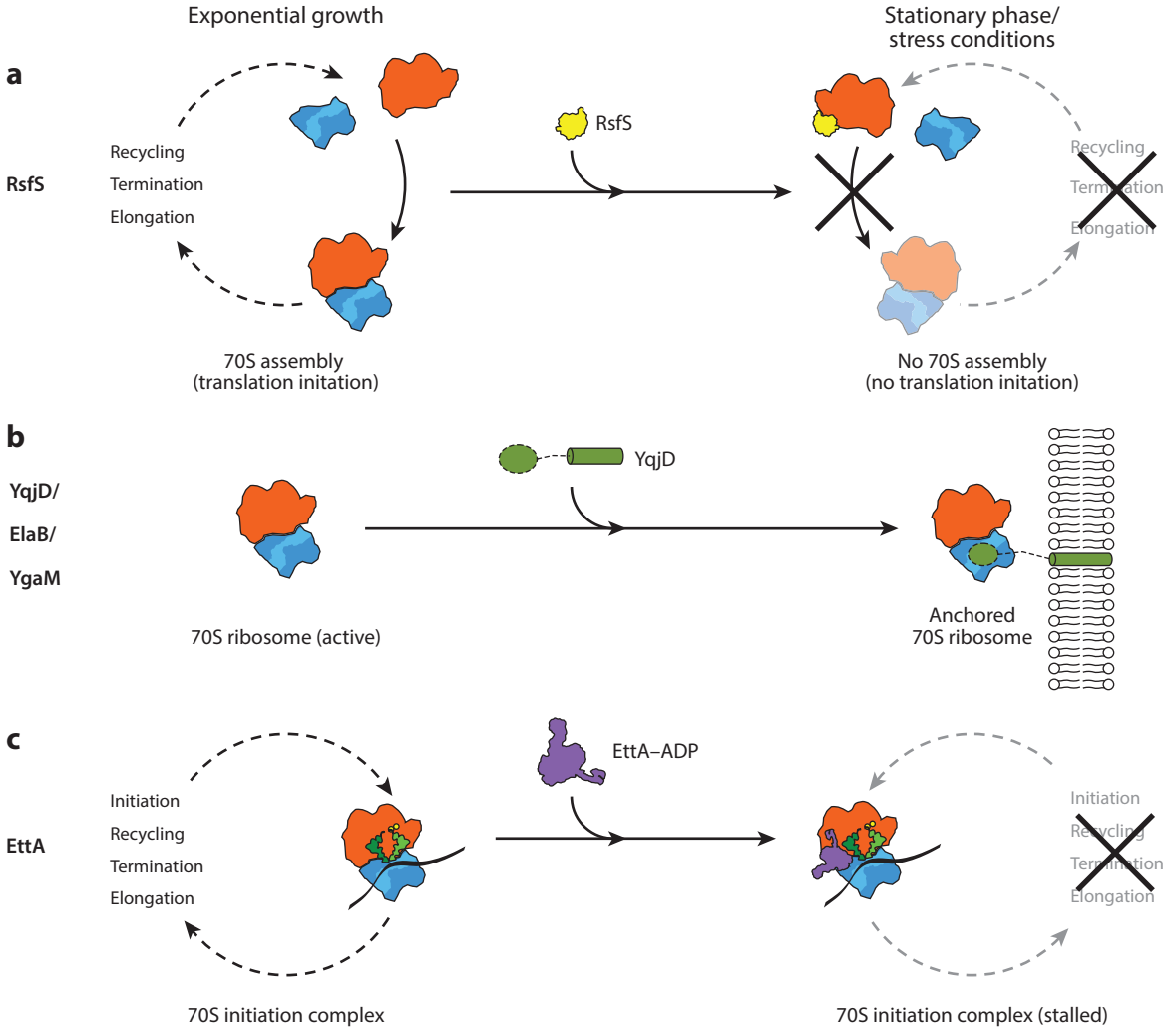


Figure 6

Simplified models of ribosome modulation by factors other than hibernation promoting factor (HPF), ribosome modulation factor (RMF), and ribosome-associated inhibitor A (RaiA). (*a*) Inhibition of subunit association and, thereby, translation initiation by ribosome silencing factor S (RsfS). RsfS binds to the L14 protein of the large subunit and sterically interferes with the formation of the 70S initiation complex. (*b*) The transmembrane domain protein YqjD and its paralogs ElaB and YgaM bind to the 70S ribosome via their C-terminal domain, while their N terminus contains the transmembrane domain, likely resulting in tethering of 70S (and 100S) ribosomes to the cell membrane in stationary phase. (*c*) The ATPase energy-dependent translational throttle A (EttA) binds to an initiating 70S complex at the E-site. When EttA is ADP-bound, it remains on the ribosome, thereby stalling the complex in a hibernating state.

The NTD of YqjD binds to the 30S subunit, whereas the CTD contains a transmembrane motif that is responsible for integration into the inner membrane (122). Cells lacking *yqjD* do not have an altered growth rate or altered proteome, morphology or ribosome composition. However, the artificial increase of YqjD levels results in abrupt growth arrest that is dependent upon the ribosome-binding capability of the protein (122). In contrast, ElaB does not inhibit cell growth when overexpressed. However, an *elaB* mutant is highly sensitive to both heat stress and oxidative

stress. A mutant strain lacking *yqjD* exhibits decreased persister formation when treated with the aminoglycoside netilmicin (102). Conversely, ElaB appears to have a negative effect on the formation of persisters: The *elaB* mutant showed significantly increased numbers of cells tolerant to ciprofloxacin and ampicillin (39). It has not been investigated if YgaM-deficient cells have similar phenotypes.

Energy-dependent translational throttle A (EttA). One of the most interesting factors that confer transient inactivation of ribosomes is EttA, an ATP binding protein of the ABC-F family. Although all eukaryotic and most bacterial organisms encode several members of this protein family, information about protein function is scarce, and only relatively recently has light been shed on a possible regulatory role of EttA in translation (14, 21). EttA is presumably present as a monomer in vivo, both freely and in association with the 70S ribosome. An EttA variant that constitutively binds to ATP due to substitutions in its ATPase domain inhibits growth upon overexpression by rapidly silencing protein synthesis. The precise point of interference with translation has been identified as right after the formation of the first peptide bond and before the first translocation step induced by EF-G. This suggests that ribosomes can enter an idle, hibernating state not only before initiation or after completion of a full translation cycle (as is the case with RMF, HPF, or RaiA) but also as assembled initiation complexes. Furthermore, inhibition of translation by wild-type EttA has been shown to be dependent on the ATP/ADP ratio, with inhibition at high ADP levels and relief at high ATP levels. These findings led to a model in which EttA modulates the translation rate in response to the energy level of the cell. Consistently, EttA levels increase in stationary phase, and an *ettA* mutant exhibits reduced fitness in competition with wild-type cells when grown in LB medium (14).

A cryo-EM study of ATP-bound EttA showed that the protein binds at the E-site of the ribosome in the pretranslocation state, where it is thought to promote peptide bond formation. Subsequently, EttA dissociates from the ribosome upon ATP hydrolysis. It was proposed that under nutrient-limited conditions in which ADP levels are elevated, EttA instead remains bound to the ribosome and thereby maintains the ribosome in a stalled, hibernating state until ATP levels increase again (21) (**Figure 6*c***).

SUMMARY AND CONCLUSIONS

Over the past 25 years, the phenomenon of ribosome hibernation has gained more and more attention with respect to understanding translation in bacterial cells under stress. Considerable progress has been made in understanding the How and Why of translation modulation by small protein factors, allowing us to summarize the current state of research in an extended model (**Figure 7**). In gammaproteobacteria such as *E. coli*, unfavorable environmental conditions lead to a reduced translation rate and slow growth. Hibernation factors RMF and HPF are synthesized and promote dimerization of 70S ribosomes to translationally inactive 100S dimers. In addition, RaiA stabilizes 70S ribosomes in an inactive state. Thus, stationary phase cells seemingly contain at least three different subpopulations of ribosomes: inactive 100S dimers, inactive 70S monosomes, and actively translating ribosomes (34). In many other bacteria, diversification of the ribosome pool takes place during exponential growth: Continuous expression of lHPF maintains a considerable fraction of ribosomes in an inactive dimerized state. This fraction increases when stress conditions are encountered. It has been proposed that lHPF-bound 70S ribosomes also exist, which would constitute a third form of idle ribosomes in these organisms (29, 51). Finally, other stress-induced translation factors may give rise to additional subpopulations of inactive ribosomes (EttA, SRA), modulate existing hibernating complexes (YqjD and paralogs), or act independently as translational

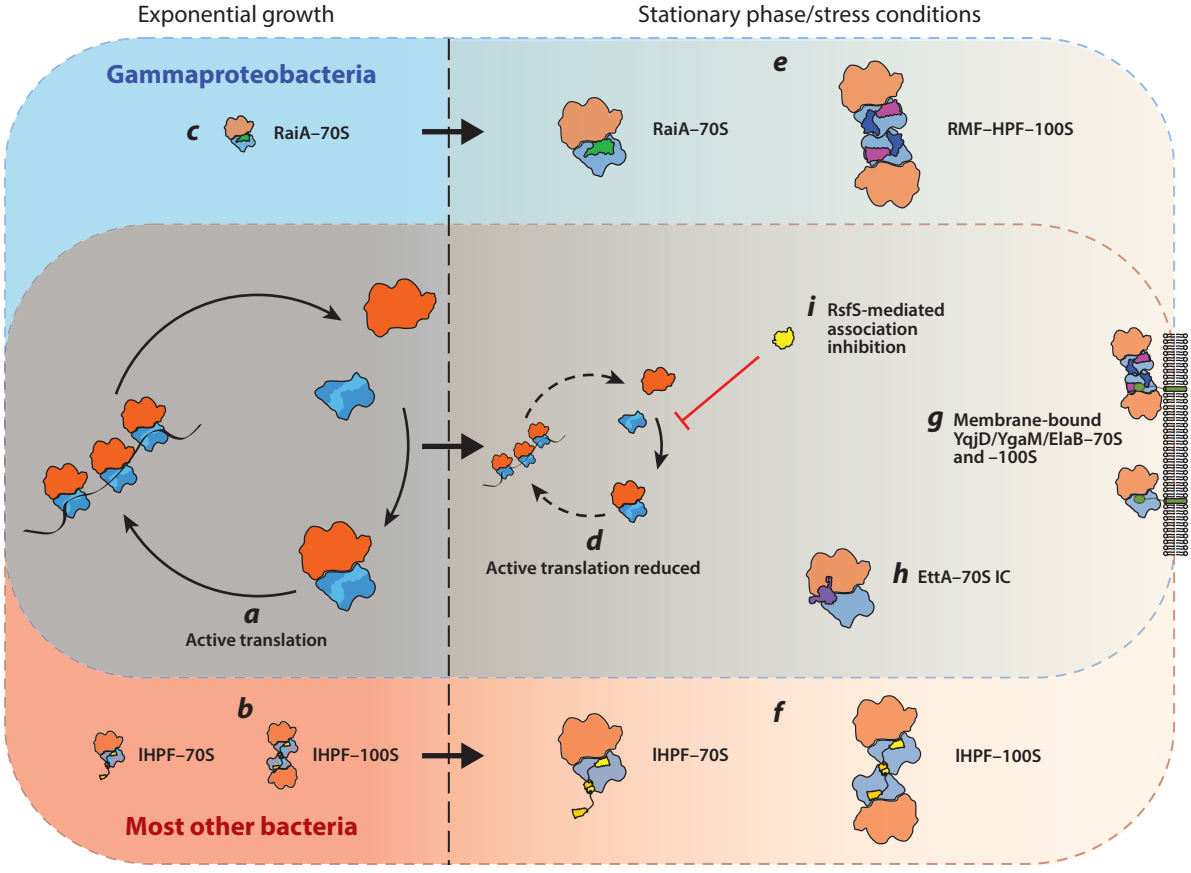


Figure 7

Current model of ribosome hibernation in gammaproteobacteria and other bacteria. (*a*) In exponential phase, the majority of ribosomes are engaged in active translation. (*b*) However, small pools of hibernating 70S monomers and 100S dimers [and (*c*) only 70S monomers in gammaproteobacteria] are present. (*d*) Upon transition to stationary phase, levels of hibernation factors and other ribosome modulators increase and active translation is reduced. (*e*) In gammaproteobacteria, hibernating 70S ribosomes are increasingly formed by ribosome-associated inhibitor A (RaiA), and hibernating 100S complexes are formed by the concerted action of ribosome modulation factor (RMF) and hibernation promoting factor (HPF). (*f*) In other bacteria, long HPF (lHPF) forms inactive 70S monomers and/or 100S dimers. (*g*) The transmembrane domain protein YqjD and its paralogs localize 100S dimers and 70S monomers to the cell membrane, (*h*) energy-dependent translational throttle A (EttA)–ADP stalls 70S initiation complexes (ICs), and (*i*) ribosome silencing factor S (RsfS) exerts its inhibitory function on the association of ribosomal subunits to complete 70S ribosomes.

regulators (RsfS). Thus, an important task in the coming years will be to untangle the complex network of translational regulation during stationary phase and other stressful conditions.

FUTURE ISSUES

1. Regulation of hibernation factor expression is only partly understood. Thus, future work will focus on obtaining a more complete picture of the regulatory pathways and degradation mechanisms that determine the levels of hibernation factors as a function of the phases of cell growth.

2. We should gain additional insight into the physiological function or functions of the different subpopulations of inactive and translating ribosomes during stationary phase and other stressful conditions.
3. How—and at which point in the translation cycle—does the dimerization of ribosomes by RMF (or long hibernation promoting factor) occur?
4. How are 100S dimeric ribosome complexes reactivated?
5. Finally, our knowledge about a number of stationary phase-specific ribosome-associated factors including ribosome silencing factor S, stationary-phase-induced ribosome-associated protein, energy-dependent translational throttle A, and the YqjD paralogs remains limited. Further investigation of these factors will unravel more about their biological functions within the complex translation machinery in stationary phase and under stress conditions.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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