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Interdependency and Redundancy Add Complexity and Resilience to Biogenesis of Bacterial Ribosomes

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Abstract

The pace and efficiency of ribosomal subunit production directly impact the fitness of bacteria. Biogenesis demands more than just the union of ribosomal components, including RNA and proteins, to form this functional ribonucleoprotein particle. Extra-ribosomal protein factors play a fundamental role in the efficiency and efficacy of ribosomal subunit biogenesis. A paucity of data on intermediate steps, multiple and overlapping pathways, and the puzzling number of functions that extra-ribosomal proteins appear to play in vivo make unraveling the formation of this macromolecular assemblage difficult. In this review, we outline with examples the multinodal landscape of factor-assisted mechanisms that influence ribosome synthesis in bacteria. We discuss in detail late-stage events that mediate correct ribosome formation and the transition to translation initiation and thereby ensure high-fidelity protein synthesis.

Contents

1. INTRODUCTION

In 1955, George Palade published the first article on the machines we now called ribosomes, entitled "A Small Particulate Component of the Cytoplasm" (65). Shortly after, these particles were identified as ribonucleoprotein (RNP) particles and designated as the site of protein synthesis (86). RNPs, and their two asymmetric subunits, were named based on sedimentation in a sucrose gradient. Each subunit of the ribosome is made up of ribosomal RNA (rRNA) and ribosomal proteins (r-proteins) and is synthesized by a process often termed biogenesis, biosynthesis, assembly, or maturation. An accurate ribosome maturation pathway is essential to ensure high-fidelity protein synthesis by establishing the correct architecture of active sites within the ribosome. Moreover, this process must be fast and robust, for allowing the fast doubling rates of certain bacteria (31).

In the 1970s, elaborate in vitro experiments had demonstrated that ribosomes can self-assemble from mature component parts (60, 88). (This review is focused on bacterial ribosome biogenesis; thus, broad statements are meant to only apply to these organisms unless otherwise noted.) The in vitro experiments paved the way to a hierarchical assembly model and were crucial, in combination with advances in structural biology in the 1990s–2000s, to understand detailed structural aspects of ribosomes (68). However, an important question has remained unanswered and has been the focus of the last few decades of research—Why are there dozens of extra-ribosomal assembly factors encoded in genomes if ribosomes are capable of self-assembly?

Prokaryotic ribosomes are made up of about 30% protein and 70% RNA (31). The small subunit (SSU or 30S subunit) contains one rRNA molecule (16S rRNA is 1,542 nucleotides) and 21 r-proteins. The large subunit (LSU or 50S subunit) contains two rRNA molecules (23S rRNA is 2,904 nucleotides and 5S rRNA is 120 nucleotides) and 34 r-proteins (75). The precursor rRNA is processed to remove sequences flanking each mature rRNA species. This is not accomplished in a single step and occurs cotranscriptionally and in coordination with other events of folding, r-protein binding, and RNA modification (75). In vivo pathways of rRNA processing have eluded studies due to several reasons—similar phenotypes of mutant strains, nonessentiality of factors, and the presence of thermodynamically stable intermediates in biogenesis-defective strains that are not representative of the true intermediates. For example, why does the rRNA retain precursor sequences well into the late stages of biogenesis (35)? Is there one dominant pathway or several equally preferred (or not) alternate pathways for ribosomal subunit maturation? How are

rRNA: ribosomal RNA

SSU or 30S subunit: the small subunit

r-proteins: ribosomal proteins

LSU or 50S subunit: the large subunit

Assembly, biosynthesis, biogenesis, maturation: The process of assembling ribosomal subunits from its components

mechanisms deployed to delay rRNA processing or r-protein binding? What triggers and what is involved in quality control of functional ribosome synthesis? These questions have begun to be addressed by slowing or blocking the maturation of ribosomes using nonstandard conditions or creation of mutant strains.

In bacteria, over 40 auxiliary proteins have been categorized as ribosome biogenesis factors (75). Despite extensive work on individual factors, there is a knowledge gap between the traditionally known hierarchical nature of assembly (60, 88) and the lesser-known kinetic bottlenecks that impact this cascade-like process (21, 23). In this review we look at the process of bacterial ribosome biogenesis with emphasis on auxiliary, nonribosomal factors that play a role in assembly in vivo. We discuss the interwoven pathways leading to a mature ribosome, challenges associated with studying them, the extent of interactions between factors, and new mechanisms that shed light on this complex, highly evolved process.

2. A RIBOSOME BIOGENESIS FACTOR GRID THAT IS REPRESENTATIVE OF THE COMPLEX INTERCONNECTIVITY OF THE BIOGENESIS SYSTEM

Despite the wealth of information gleaned from in vitro assembly studies, there are limitations to these insights. In vitro ribosomal subunit biogenesis does not truly reflect physiological conditions under which biogenesis happens for three reasons: (*a*) The large number of assembly factors available in vivo are not required in vitro; (*b*) in vitro experiments use mature components to initiate assembly, not precursor or unmodified components as happens in vivo; and (*c*) the optimum conditions (like temperature and ionic strength) for in vitro reconstitution are vastly different from in vivo conditions (60, 88). The *Escherichia coli* small subunit (SSU or 30S) can be fully assembled in under a minute in vivo (23, 50), and healthy bacterial cells accumulate ribosomal intermediates at the level of about 2% of the total RNA (50). Thus, the speed and plasticity of bacterial ribosome biogenesis make it difficult to isolate and analyze in vivo–formed intermediates. To date, the use of mutant phenotypes has been the most productive in vivo approach to understand ribosome biogenesis. Unfortunately, heterogeneity and overlapping patterns exist in the intermediates that accumulate in different biogenesis-defective mutant strains, adding to the difficulty of identifying substrates and assigning precise functions to biogenesis factors (85).

In the last 10 years, it has become clear that the bacterial ribosomal biogenesis pathway has many related functional nodes where factors act. These factors include r-proteins as well as those that transiently bind, act, and leave (nonribosomal proteins). The identified nonribosomal proteins include biogenesis factors, rRNA modification enzymes, GTPases, RNA helicases, and proteins with other assigned roles in vivo like translation factors (75). Here, we present a schematic to reveal known interactions and to allow for discussion of these interactions (**Figure 1**). This network is not all-inclusive but provides a broad perspective of the pathway.

The characterization of individual interactions has shed light on some striking features of assembly in *E. coli* ribosomes and enabled unraveling some of the intricacies of the in vivo process. **Table 1** provides a comprehensive list of the links shown in **Figure 1** along with some comments on interactions and the corresponding references. A list of all genes discussed along with their known functions is provided in the **Supplemental Appendix**. We focus here on themes that emerge from the network including, but not limited to, redundancy, suppressor mechanisms, and quality control mechanisms. We also discuss late-stage events that delineate the end of biogenesis and the beginning of translation initiation and, finally, the importance of multiple pathways that kinetically favor efficient mature ribosome formation.

Supplemental Material >

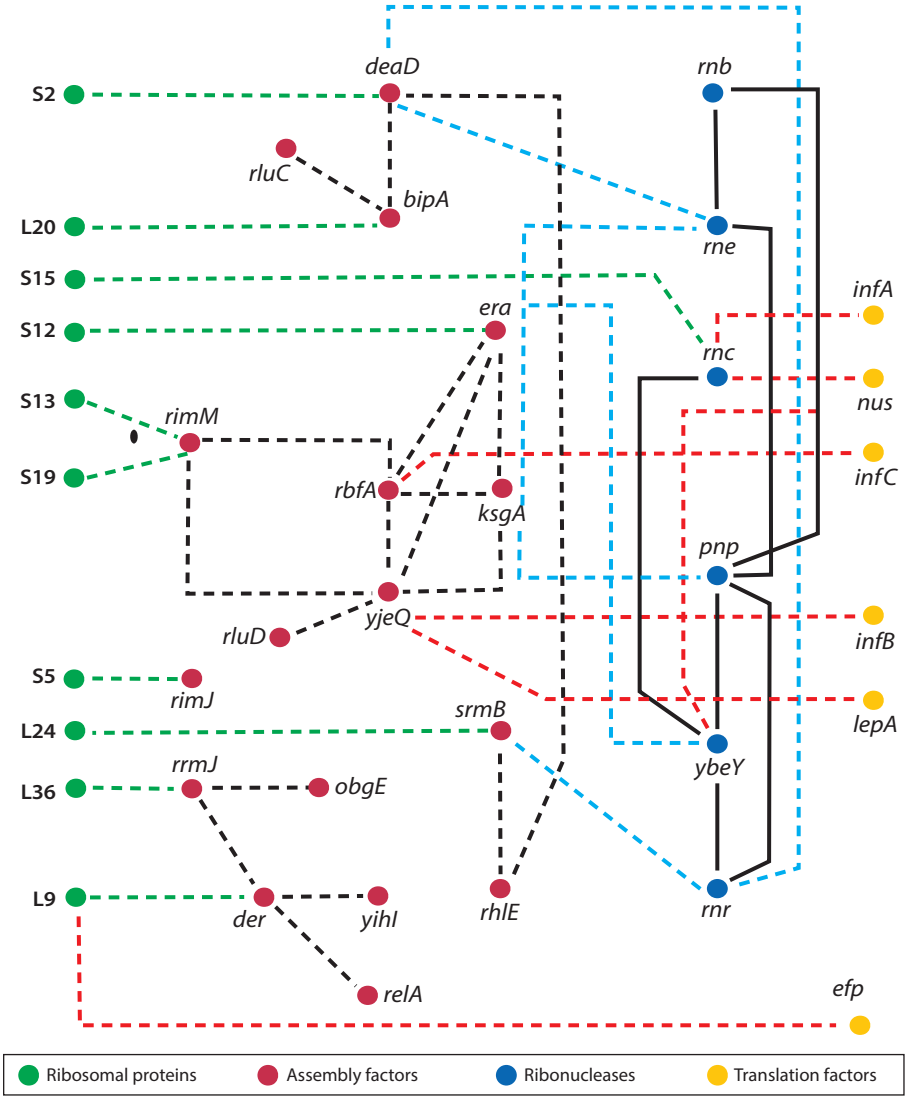

Figure 1

A ribosome biogenesis factor grid that is representative of the complex interconnectivity of the biogenesis system. The network is a representation of the complex web of interactions that are beginning to shed light on the intricacies bacterial assembly. Ribosomal proteins are denoted by green dots, all biogenesis factors (including helicases, rRNA modification enzymes, and GTPases) are denoted by red dots, ribonucleases by blue dots, and canonical translational factors by yellow dots. Green dashed lines represent interactions of ribosomal proteins with other factors. Black dashed lines indicate connections between biogenesis factors. Blue dashed lines indicate links where one end is a ribonuclease. Interactions where both ends are ribonucleases are denoted by solid black lines. Any interaction involving a translation factor is denoted by a red dashed line. The links in the network involve proteins previously associated with biogenesis but does not include direct protein-protein interactions or phenotypes with altered stoichiometry of proteins. The phenotypes indicated in this figure and **Table 1** are genetically identified, biochemically characterized, or both. Some interactions are better-studied than others. All gene names are provided except those of ribosomal proteins (*green dots*), for easy recognition. Alternate gene names and corresponding protein names are detailed in **Table 1** and in the **Supplemental Appendix**.

Supplemental Material >

Table 1 A noncomprehensive list of interacting partners involved in bacterial ribosome biogenesis[a]

Factors with a direct role in maturation	Gene (protein) linked to factor[b]	Interaction[c]	Reference(s)
***ybeY* (YbeY)** ■ Endoribonuclease ■ 16S rRNA processing ■ Stress regulation ■ Transcription antitermination	*nusA*, *nusB* (NusA, NusB)	YbeY plays a role in transcription antitermination. The growth defect of Δ*ybeY* is partially suppressed by overexpression of Nus factors	33
	pnp (PNPase)	Deletion of both *pnp* and *ybeY* exacerbates 16S rRNA processing defects	20
	rnc (RNase III)	Deletion of *rnc* exacerbates the growth defect and worsens 16S rRNA processing in Δ*ybeY*	20
	rnr (RNase R)	Deletion of *rnr* exacerbates the growth defect and worsens 16S rRNA processing in Δ*ybeY*. Deletion of *rnr* makes *yqfG* (*ybeY* homolog in *Bacillus subtilis*) deletion viable	*B. subtilis*, 5; *E. coli*, 20
	era (Era)	Overexpression of Era suppresses growth and processing defects in Δ*ybeY*	26
***rnc* (RNase III)** ■ Endoribonuclease ■ Initial rRNA processing ■ Cellular mRNA processing	*nus* (Nus)	Deletion of *rnc* suppresses cold sensitivity of *nus* mutants	9
	rpsO (S15)	Deletion of *rnc* partially suppresses cold sensitivity of Δ*rpsO*	55
	ybeY (YbeY)	Deletion of *rnc* exacerbates the phenotype associated with Δ*ybeY*	20
	infA (IF-1)	Deletion of *rnc* suppresses cold sensitivity of an IF-1 mutant	6
***pnp* (PNPase)** ■ 3′-to-5′ exoribonuclease ■ 16S rRNA processing ■ mRNA degradation	*rne* (RNase E)	RNase E influences the transcription of *pnp*	36
	rnb (RNase II)	Overexpression of RNase II suppresses growth in Δ*rph*Δ*pnp* and Δ*pnp* strains	3, 43
	rnr (RNase R)	Δ*pnp*Δ*rnr* is not viable. Overexpression of RNase R suppresses growth in a Δ*rph*Δ*pnp* strain	13, 43
	ybeY (YbeY)	Deletion of *pnp* and *ybeY* exacerbates 16S rRNA processing defects in *E. coli*	20
	rne (RNase E) *ksgA* (KsgA)	An RNase E mutant suppresses growth of a Δ*ksgA*Δ*pnp* strain	Unpublished data[d]
***rne* (RNase E)** ■ Endoribonuclease ■ All RNA processing ■ RNA degradation	*pnp* (PNPase)	RNase E influences the transcription of *pnp*	36
	rne (RNase E) *ksgA* (KsgA)	An RNase E mutant suppresses growth of a Δ*ksgA*Δ*pnp* strain	Unpublished data[d]
	rnb (RNase II) *pnp* (PNPase)	A triple mutant of *rne*/*pnp*/*rnb* is viable	64
	deaD/*csdA* (DeaD/CsdA)	Mutation in *deaD* suppresses an *rne* mutant	83
***rnr* (RNase R)** ■ 3′-to-5′ exoribonuclease ■ rRNA processing ■ mRNA and rRNA degradation	*pnp* (PNPase)	Δ*pnp*Δ*rnr* is not viable. Overexpression of RNase R suppresses growth in a Δ*rph*Δ*pnp* strain	13, 43
	deaD/*csdA* (DeaD/CsdA)	Deletion of *deaD* exacerbates growth and ribosome biogenesis defects in a Δ*rnr* strain	44
	srmB/*rhlA* (SrmB/RhlA)	Deletion of *srmB* exacerbates growth and processing defects in a Δ*rnr* strain	44
	ybeY (YbeY)	Deletion of *rnr* exacerbates growth and 16S rRNA processing in Δ*ybeY*. Deletion of *rnr* makes *yqfG* (*ybeY* homolog in *B. subtilis*) deletion viable	*B. subtilis*, 5; *E. coli*, 20

(*Continued*)

Table 1 (*Continued*)

Factors with a direct role in maturation	Gene (protein) linked to factor[b]	Interaction[c]	Reference(s)
***ksgA/rsmA* (KsgA)** ■ 16S rRNA methyltransferase ■ SSU maturation	*era* (Era)	Overexpression of KsgA suppresses the cold sensitivity of an *era* mutant	52
	pnp (PNPase) *rne* (RNase E)	An RNase E mutant suppresses growth of a Δ*ksgA*Δ*pnp* strain	Unpublished data[d]
	rbfA (RbfA)	Overexpression of RbfA exacerbates growth and biogenesis defect in a Δ*ksgA* strain	16
	yjeQ/rsgA (YjeQ/RsgA)	Deletion of *ksgA* exacerbates growth of a Δ*yjeQ* strain	11
***rrmJ/rlmE/FtsJ* (RrmJ)** ■ 23S rRNA methyltransferase ■ SSU maturation	*rpmJ* (L36)	Deletion of *rpmJ* (L36) exacerbates the growth defect of Δ*rrmJ*	2
	obgE/yhbZ/ctgA (ObgE)	Overexpression of ObgE suppresses growth and biogenesis defects in Δ*rrmJ*	84
	Der/engA (Der/EngA)	Overexpression of Der suppresses growth and biogenesis defect in Δ*rrmJ*	84
***rplI* (L9)** ■ Large subunit ribosomal protein	*Der/engA* (Der/EngA)	Mutated *der* causes dependency on L9	56
	efp (EF-P)	L9 improves growth in EF-P-deficient strains	57
***rpsE* (S5)** ■ SSU ribosomal protein	*rimJ* (RimJ)	Overexpression of RimJ suppresses the biogenesis defects of an S5 mutant	70, 71
Der/EngA ■ GTPase ■ LSU maturation	*yihI* (YihI)	Overexpression of YihI partially suppresses growth defect in a *der* mutant	56
	rplI (L9)	Mutated *der* causes dependency on L9	56
	relA (RelA)	Overexpression of RelA suppresses growth defect of a *der* mutant	38
	rrmJ (RrmJ)	Overexpression of Der suppresses growth and biogenesis defect in a Δ*rrmJ* strain	84
bipA/typA ■ GTPase ■ LSU maturation	*rluC* (RluC)	Deletion of *rluC* suppresses phenotypes in a Δ*bipA* strain	48
	deaD/csdA (DeaD/CsdA)	Deletion of *deaD* exacerbates the phenotype of a Δ*bipA* strain	15
	rplT (L20)	Overexpression of L20 (*rpsT*) restored growth and ribosome processing defects of a Δ*bipA* strain	14
***era* (Era)** ■ GTPase ■ SSU maturation	*rpsL* (S12)	Mutation in *era* suppresses an *rpsL* mutant	58
	ksgA (KsgA)	Overexpression of KsgA suppresses an *era* mutant	52
	rbfA (RbfA)	Overexpression of Era partially suppresses cold sensitivity of a Δ*rbf*A strain	39
	yjeQ/rsgA	Overexpression of Era partially suppresses growth of a Δ*yje*Q strain	11
	ybeY (YbeY)	Overexpression of Era suppresses growth of a Δ*ybeY* strain	26
yjeQ/rsgA ■ GTPase ■ SSU maturation	*lepA* (LepA/EF-4)	Deletion of *yjeQ* exacerbates the growth of a Δ*lepA* strain	4, 27
	ksgA/rsmA (KsgA/RsmA)	Deletion of *ksgA* exacerbates to growth of a Δ*yjeQ* strain	11
	era (Era)	Overexpression of Era suppresses growth and biogenesis defects in a Δ*yjeQ* strain	11
	rbfA (RbfA)	Mutations of *rbfA* suppress growth and biogenesis defects in a Δ*yjeQ* strain	30

(*Continued*)

Table 1 (*Continued*)

Factors with a direct role in maturation	Gene (protein) linked to factor[b]	Interaction[c]	Reference(s)
	infB (IF-2)	Overexpression of IF-2 suppresses growth and biogenesis defects in a Δ*yjeQ* strain	11
	rimM (RimM)	Deletion of *rimM* exacerbates growth of a Δ*yjeQ* strain	11
	rluD (RluD)	Deletion of *rluD* exacerbates growth of a Δ*yjeQ* strain	11
LepA/EF4 ■ GTPase ■ SSU maturation	*yjeQ/rsgA* (YjeQ/RsgA)	Deletion of *rsgA* exacerbates the growth of a Δ*yjeQ* strain	4, 27
***rimM* (*RimM*)** ■ GTPase ■ SSU maturation	*rpsM* (S13)	An S13 mutation suppresses growth and biogenesis defects in a Δ*rimM* strain	51
	rpsS (S19)	An S19 mutation suppresses growth and biogenesis defects in a Δ*rimM* strain	51
	yjeQ (YjeQ)	Deletion of *rimM* exacerbates growth of a Δ*yjeQ* strain	11
	rbfA (RbfA)	Overexpression of RbfA suppresses the growth and biogenesis defects of a Δ*rimM* strain	10, 51
***rbfA* (RbfA)** ■ SSU maturation	*era* (Era)	Overexpression of Era partially suppresses cold sensitivity of a Δ*rbfA* strain	39
	ksgA (KsgA)	Overexpression of KsgA exacerbates growth and biogenesis defect in a Δ*rbfA* strain	16
	yjeQ/rsgA (YjeQ/RsgA)	Mutations of *rbfA* suppress growth and biogenesis defects in a Δ*rsg*A strain	30
	rim (RimM)	Overexpression of RbfA suppresses the growth and biogenesis defects of a Δ*rimM* strain	10, 51
	infC (IF-3)	The expression of RbfA leads to toxicity in an IF-3 mutant strain	76
***deaD/csdA* (DeaD)** ■ RNA helicase ■ LSU maturation	*rpsB* (S2)	Overexpression of *deaD* restores growth in a *rpsB* mutant	54, 87
	rne (RNase E)	Mutation in *deaD* suppresses a *rne* mutant	83
	srmB, *rhlE*, *rhlB*, *dbpA* (SrmB, RhlE, RhlB, DbpA)	Deletion of all five helicases is viable	41
	Rnr (RNase R)	Deletion of *rnr* and *deaD* exacerbates growth and ribosome biogenesis defects	44
	bipA (BipA)	Deletion of *deaD* exacerbates phenotypes of a Δ*bipA* strain	15
***srmB/rhlA* (SrmB)** ■ RNA helicase ■ LSU maturation	*rnr* (RNase R)	Deletion of *srmB* exacerbates growth and processing defects in a Δ*rnr* strain	44
	rhlE (RhlE)	Overexpression of RhlE exacerbates the growth defect of a Δ*srmB* strain	42
	rplX (L24)	Overexpression of SrmB suppresses an L24 mutant	59
	srmB, *rhlE*, *rhlB*, *dbpA* (SrmB, RhlE, RhlB, DbpA)	Deletion of all five helicases is viable	41

Abbreviations: SSU, small subunit; LSU, large subunit.

[a]All genes and references are for *Escherichia coli* unless otherwise noted. Commonly used alternate gene names are also provided.

[b]The **Supplemental Appendix** provides a list of primary function for genes and proteins.

[c]Some of the interactions are repeated to provide a complete list of links for each gene listed.

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Supplemental Material >

Each interaction shown in **Figure 1** is one of four types (factor 1 and factor 2 being the two interacting partners in each node):

- Loss of factor 1 alleviates the phenotype associated with the loss of factor 2.
- Loss of factor 1 exacerbates the phenotype associated with the loss of factor 2.
- Overabundance of factor 1 alleviates the phenotype associated with the loss of factor 2.
- Overabundance of factor 1 exacerbates the phenotype associated with the loss of factor 2.

3. FUNCTIONAL REDUNDANCY AND SUPPRESSOR PHENOTYPES

In functional interactions that involve compensation of a biogenesis defect (the first and third types listed in the previous section), we note that most phenotypes are only partially suppressed [for example, Era/RbfA or RNase III/S15 (**Table 1**)]. It is possible that in such partially suppressed phenotypes, the loss of one factor produces intermediates that are more suitable for an alternate maturation pathway. Partial suppression can also be explained by the high functional redundancy of assembly factors. Most of these factors are nonessential for viability, and in many cases the deletion of individual genes has little effect on fitness (**Table 1**). Hence, on-path intermediates of ribosomal subunits that accumulate in a defective strain may be rescued partially or fully by suppressor mechanisms depending on whether the alternate pathway is kinetically favored. Evolutionarily speaking, partial suppression is not surprising (although not well understood) because less-preferred pathways could serve an important function during stress or starvation, when speed and efficiency may be compromised but ribosomes can be synthesized nonetheless. This hypothesis remains to be tested.

Factors with redundant functions are sometimes highly conserved and seem to make unique contributions to the pathway, yet specific roles have been hard to discern. For example, the enzymes that participate in rRNA maturation and processing are highly redundant (22), and despite the presence of specific cleavage sites on precursor 16S rRNA, the absence of some ribonucleases like RNase III and RNase G leads to no overt growth phenotypes in *E. coli*. Additionally, in these strains, 16S rRNA maturation can be completed effectively (46, 55). But a closer look at the network (**Figure 1**) reveals that ribonucleases make diverse connections and that their rRNA processing function is crucial to timely and accurate ribosome synthesis (40, 44, 55, 83) (**Table 1**). Interestingly, the interconnectivity between ribonucleases (**Figure 1**) suggests a highly evolved process and implicates them in correct rRNA processing with production of high-quality ribosomes. The connecting link between ribonucleases, YbeY, and RNase R led to the identification of a quality control system that is responsible for the removal of immature ribosomal subunits (20, 40). Further investigation will reveal whether the nodes connecting these enzymes to other factors in the network are suggestive of the same or a different regulatory mechanism. Another instance of difficulty assigning function can be seen in terms of rRNA modification. The *E. coli* ribosome contains 22 known rRNA base methylations (73, 74). The impact of these methylations in maturation is not clear, although there is strong evidence to suggest a quality control function, especially for 16S rRNA methylations (73). The N6,N6-dimethyl adenosines ($m^6{}_2$-A1518, $m^6{}_2$-A1519) in the SSU rRNA helix 45 stem-loop is the most conserved rRNA modification across kingdoms (18, 66). The methyltransferase responsible for this methylation, KsgA (alternative bacterial name RsmA and eukaryotic name Dim1p), is also universally conserved (67). However, KsgA function is not essential in *E. coli*, while Dim1p function appears to be essential (62). In the absence of *ksgA* or KsgA methylation activity, mature SSUs can be synthesized, supporting the idea that biogenesis can proceed in the absence of these conserved modifications and suggesting that other biogenesis factors are critical in a *ksgA* deletion. The unique contributions made by KsgA in bacteria, however, come to light upon examining the interactions with Era, RbfA, and YjeQ, all of which have

a putative biogenesis factor function (11, 17) (**Table 1**). Based on the individual functional interactions of KsgA, the factor has been assigned a quality control function in late-stage biogenesis (discussed further in the next section) (16, 17). Outside of the genetic interactions mentioned here, structural data have shown that the binding region of KsgA on SSUs is coincident with that of initiation factor 3 (IF-3), further supporting its role in the final steps of maturation and the importance of an interface between biogenesis and translation as a means of quality control (62). Thus, functional redundancy and suppressor mechanisms provide the flexibility, plasticity, and dexterity needed for in vivo ribosome biogenesis and for quality control.

4. QUALITY CONTROL MECHANISMS AND THEIR SIGNIFICANCE

A growing body of evidence has suggested that quality control mechanisms exist in later stages of ribosome biogenesis and perform a gatekeeping function to prevent premature ribosomes from initiating translation (2, 27, 30, 49, 69, 76). It is also well established that final subunit maturation events can continue after the formation of the 70S initiation complex (77) and that final steps of ribosome biogenesis are linked to successful translation initiation (9). There are two main ways that these quality control mechanisms work—by directly, sterically blocking the association of translation factors and by preventing key rRNA conformational changes necessary for function, thereby creating a functional checkpoint to ensure initiation only occurs when ribosomal subunits are prepared. The conformational changes required to pass the final checks are directly influenced by the binding and dissociation of assembly factors in vivo.

Despite considerable variability in the composition of ribosomal subunits between bacteria and eukaryotes, these organisms all share the common goal of ensuring that the functionally important ribosomal centers are correctly formed (9). In eukaryotes, a test-drive mechanism has been proposed where the pre-40S (SSU) subunit goes through a translation-like cycle to test association to the 60S (LSU) subunit as well as translation factors (81, 82). As a result of this test-drive, defective ribosomes can be targeted to degradation prior to authentic translation events. To our knowledge, such a trial run for bacterial subunits has not been reported, but studies by the Varshney group have identified a possible role of initiator tRNA in regulating 16S rRNA processing in the context of the 70S ribosome initiation complex (77). Thus, delaying the final maturation steps may itself serve a gatekeeping function. This close coupling of rRNA processing to the final stages of biogenesis is further demonstrated by the functional links involving late-stage biogenesis factors, ribonucleases, and initiation factors (e.g., YbeY/Era, YjeQ/IF-2, RbfA/IF-3) (**Figure 1**). Therefore, the main elements of quality control seem to be conserved, although factors involved, and the specific mechanisms, may differ.

The connected nodes in **Figure 1** (and **Table 1**) that link auxiliary factors and r-proteins to translation factors (L9/EF-P, YjeQ/IF-2, RbfA/IF-3) have helped unravel some events in the transition from immature subunits or immature ribosomes to translationally ready ribosomes. For example, IF-3 can displace the biogenesis factor RbfA from mature 30S ribosomal subunits (76). IF-3 can bind to both mature and immature SSUs but can only displace RbfA from mature 30S subunits (76). A late-acting GTPase and 30S subunit biogenesis factor, YjeQ/RsgA, is also able to release RbfA from mature 30S subunits (30). Additionally, discovery of a link between RbfA and KsgA provided a crucial clue in understanding a series of biogenesis events (16, 17). The methylation of 16S rRNA by the universally conserved protein KsgA is not essential in *E. coli*. Cells lacking KsgA do not exhibit severe phenotypes; however, the expression of RbfA in a *ksgA* deletion is toxic (16). Methylation by KsgA is not required for RbfA binding, suggesting that RbfA binding occurs prior to or regardless of KsgA function in the assembly cascade (30, 76). KsgA-dependent methylation is, however, required for GTPase-induced release of RbfA, indicating that YjeQ/RsgA and IF-3

may act downstream of KsgA (76). Thus, the characterization of multiple links between RbfA, IF-3, YjeQ/RsgA, and KsgA (**Figure 1**) in different studies culminated in identifying a mechanistic web that was undecipherable 10 years ago. As shown in **Figure 1**, YjeQ/RsgA is connected not only to RbfA but also to several other biogenesis factors, as well as translation factors IF-2 and LepA. A closer, more mechanistically focused examination of individual interactions will further clarify events associated with late-stage ribosomal subunit biogenesis in bacteria. In this example, multiple checkpoints exist—both RbfA release and RsgA's GTPase activation occur after KsgA methylation. Each checkpoint here can be compared to a handoff in a multidirectional relay race. In track relay races, each runner must hand off the baton to the next runner on the track (and generally in their lane). In this analogy, the late-stage intermediate would be the baton and the assembly factors, the runners. We know that in the absence of one of these factors (KsgA, RbfA, or RsgA), biogenesis does not come to a halt—so the baton is moved from one lane on the track to another that has a runner ready. How this nonstandard handoff occurs, in the absence of a runner (assembly factor), is unclear; however, it is now well recognized that factor binding, function, and then release are crucial at checkpoints (16, 17, 27, 28, 76, 85).

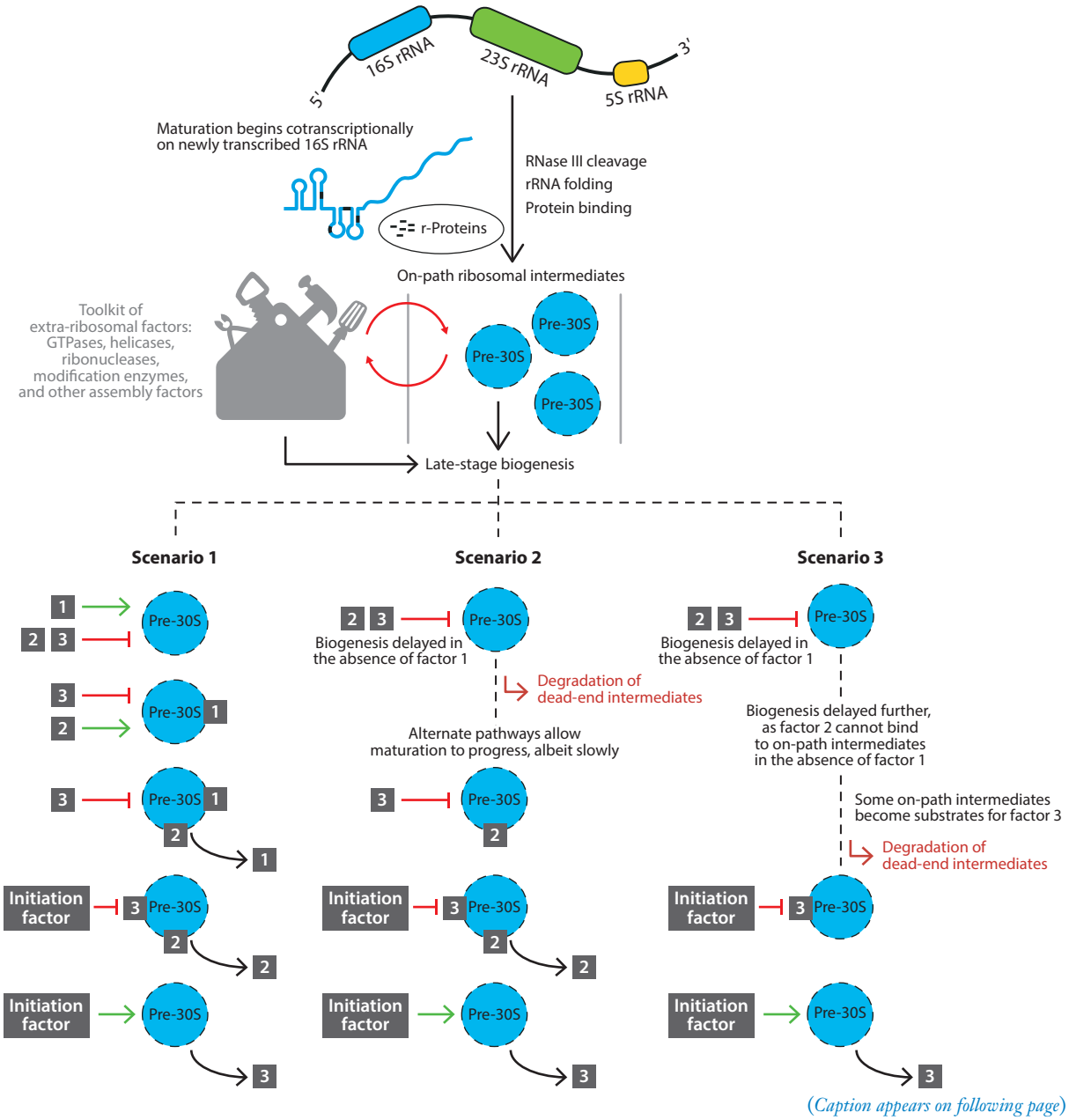
Some late-stage quality control mechanisms can regulate maturation even after subunit association via the 30S subunit (27, 77). Delay of 50S subunit biogenesis results in the accumulation of precursor 30S subunits, indicating that there is cooperativity between the two assembly cascades (14, 28, 35, 56, 72). Based on current evidence, the quality control checkpoints appear to be predominantly driven via the SSU pathway. This is further supported by comparing the effect of rRNA methylation profiles of 23S rRNA and 16S rRNA. 23S rRNA methylations appear to play a structural role (32), whereas many 16S rRNA methylations (like $m^6{}_2$A-1518, $m^6{}_2$-A1519, and m^5C967) have a role in quality control (73). Although there is evidence of a 50S subunit quality control mechanism (61), it is likely that there are fewer subunit conformational changes that serve as checkpoints in the 50S subunit compared to 30S subunits, but this remains to be established. Future studies, and the availability of additional data of late 50S assembly event intermediates, will be revealing.

To further illustrate quality control in bacterial ribosome biogenesis, we provide some examples in **Figure 2** related to the late-stage steps. Experimental and modeling studies reveal that the on-path ribosomal intermediates can take different routes based on thermodynamic stability (23, 34). Once pre-SSUs (**Figure 2**) have assembled, hypothetical biogenesis factors 1, 2, and 3 bind in a specific order, but it is not unnatural for alternative pathways to take shape in the absence of one or two factors. As illustrated, the binding of factor 2 requires that factor 1 first bind and then be released (scenario 1). In scenarios 2 and 3, factor 1 is absent. Although there are consequences to the absence of factor 1, there appears to be considerable latitude in how checkpoints are utilized during biogenesis. In bacteria, it does not seem that every checkpoint is traversed before ribosomes are sanctioned for translation. We conclude that the main function of quality control in bacterial ribosome biogenesis is not to have every checkpoint cleared but to allow for a broad survey of emerging ribosomal subunits so that certain structural requirements are met before initiating translation. In some intermediates, biogenesis is delayed to a point where rRNA degradation pathways can be triggered (**Figure 2**).

5. THE SIGNIFICANCE OF GTPase FUNCTION IN RIBOSOME BIOGENESIS

The role of GTPases in ribosome biosynthesis is well established across kingdoms (8, 45). Several GTPases in *E. coli* (YjeQ/RsgA, Der/EngA, Era, and YihA) are implicated in ribosome biogenesis, yet many are nonessential for cell viability (8). Does the nonessentiality of a GTPase mean that

other GTPases (or similar enzymes) can functionally substitute? This is not fully understood. The example of suppressors of strains lacking *rrmJ* reveals some of the issues with our understanding of these roles. RrmJ is a late-acting, 23S rRNA methyltransferase that is crucial to 50S subunit biogenesis (84). The overexpression of either of two GTPases, ObgE or Der, can suppress growth and biogenesis defects in an *rrmJ* null strain by unknown mechanisms (84). The suppression is associated with GTPase function and does not alter RrmJ's ability to methylate, indicating that the suppression mechanism directly impacts on-path intermediates. However, the overexpression



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

An illustration of possible pathways for bacterial ribosome biogenesis with a focus on late-stage events of 30S subunit biogenesis. Biogenesis begins as soon as rRNA is transcribed as a primary transcript (*top*) consisting of 16S rRNA (*blue*), 23S rRNA (*green*), and 5S rRNA (*yellow*). A toolkit of extra-ribosomal factors is responsible for ensuring proper rRNA processing, modification, and folding along with r-protein (*black dashes*) binding. For simplicity, only 30S subunit biogenesis is illustrated here. A series of pathways results in the formation of pre–30S ribosome intermediates (*blue circles*). Factors 1, 2, and 3 are representative of three hypothetical biogenesis factors that act on late intermediates and are assumed to bind in a specific order (1, 2, and then 3). Scenario 1 represents a genotypically wild-type pathway where all factors are available. In the absence of factor 1 (due to deletion or inactivation of function), biogenesis may be briefly delayed, but in at least some (if not all) cases the intermediates achieve full maturation. This is hypothesized to happen in several ways: Intermediates progress to stages that allow factor 2 binding (scenario 2) or factor 3 binding (scenario 3). These scenarios are indicative of the multiple avenues of quality control in 30S subunit assembly based on recently discovered mechanisms. They are not a complete representation of all events occurring in late-stage biogenesis.

of Era (a GTPase closely related to Der) is unable to rescue defects in an *rrmJ* null strain (84) (**Table 1**). Interestingly, the overexpression of Era can suppress defects in *rbfA*, *yjeQ*, and *ybeY* deletion strains (**Table 1**), clearly indicating that despite overlapping chemistry, specific GTPases may have unique ribosomal substrates in biogenesis.

The coupling of specific GTPase function to the ribosomal subunit assembly process has been recently characterized. In most cases, the GTPase activity is stimulated by binding to ribosomal subunit intermediates (YjeQ, Der, Era) (8). In some cases (e.g., YjeQ/RsgA), it has been shown that the activation of the enzyme sanctions the intermediate to the next step in the assembly line. The binding of RbfA alters helices 44 and 45 of 16S rRNA such that RbfA-bound pre-SSUs are not suitable for translation initiation (19, 76). For RbfA-bound intermediates to progress to the next stage, GTPase YjeQ/RsgA needs to bind in the helix 44 region to reverse the structural alterations to helix 44 made by RbfA binding. This reversal releases RbfA and promotes GTP hydrolysis in YjeQ/RsgA (30, 69, 85). Thus, GTPase activity is closely coupled to this checkpoint, ensuring that immature 30S subunits do not prematurely initiate translation when major structures have not been formed. The connecting links between GTPases and other members of the biogenesis pathway (**Figure 1**) will help to further reveal gatekeeping mechanisms.

The abovementioned GTPases are different from the translational GTPases (trGTPases) that have a direct role in translation (e.g., IF-2, EF-Tu, RF3, and EF-G) (9). More recently, trGTPases LepA and BipA have been implicated in SSU and LSU biogenesis, respectively (4, 27, 28). Unlike GTPases whose function are generally coupled to individual maturing subunits (e.g., YjeQ-SSU, Der-LSU, and Era-SSU), BipA and LepA bind to 50S and 30S subunits, respectively, but their GTPase activity is highly stimulated in the context of 70S ribosomes. The similarity in the protein composition and structure of intermediates in the absence of *lepA* or *yjeQ* strongly suggests overlapping function in late-stage biogenesis (27). Although the GTPases (LepA and YjeQ) seem to have a similar function, the timing of their action might be crucial. It has been hypothesized that LepA is employed to assist in quality control in the context of 70S whereas RsgA may act on SSUs before subunit association (27). These findings indicate that GTPases drive forward important events during biogenesis at different stages.

6. STUDY OF BIOGENESIS AND ANTIMICROBIALS

A large fraction of antibiotics bind to ribosomes and interfere with protein synthesis (89). Studies on antibiotics have been crucial to understanding fundamental mechanisms underlying decoding and peptide bond formation (63). Because of the complexity of interactions within the ribosome that are functionally crucial, the ribosome is a rich source of antimicrobial targets. Given the coupling between translation and ribosome biogenesis, it is not surprising that the inhibition of translation has an observable impact on rRNA processing and maturation (12). Whether

antibiotics directly influence biogenesis or their effects are merely a secondary effect of translational inhibition is debated (12, 24, 78). Several studies by the Champney lab have demonstrated that ribosome biogenesis can be directly influenced by antibiotics, making it a suitable target for antibiotic action (12, 24, 25). Interestingly, the ribosomal phenotypes of antibiotic-treated cells closely resemble that of biogenesis-defective mutants (24, 78). Moreover, sensitivity to antibiotics is influenced by changes to rRNA processing, modification, or disruptions to the maturation cascade (57, 76, 80, 91). For example, erythromycin results in a reduced population of free 50S subunits and inhibits biogenesis of LSUs (12, 89). Therefore, ribosome assembly has been more carefully considered as an antimicrobial target (53). Other antibiotics can directly bind to ribosomal intermediates (12). They bind in the context of a 70S ribosome, meaning that it is likely that rRNA nucleotides and r-proteins are themselves less important for antibiotic action than the secondary and tertiary structures formed in the intermediates (53). Bacterial RNases and biogenesis factors are currently being explored as potential targets of ribosome assembly (7, 25). A study by Yassin et al. (90) identified putative antibiotic-binding regions in 16S rRNA that were previously not known to be important. Therefore, shedding light on *trans*-acting biogenesis factors could be crucial to a deeper understanding of underlying mechanisms of biogenesis and antibiotic action and thus benefit the development of novel antimicrobials.

7. DISCUSSION

Broadly, ribosomal subunit maturation begins with the initial cleavage of the primary rRNA transcript (containing 16S rRNA, 23 rRNA, and 5S rRNA), perhaps by RNase III, which is followed by each rRNA species undergoing a sequence of folding, protein binding, modification, and cleavage events that results in functional 30S and 50S subunits and then 70S ribosomes (75). However, many folding and protein-binding events begin as soon as the rRNA is transcribed. Cotranscriptional 16S rRNA folding begins even before the pre–23S rRNA transcript is released by RNase III cleavage (9). These intricate rRNA processing pathways produce mature-length rRNAs, but the sequence of cleavage events and directionality of rRNA processing does not occur via one defined pathway (34, 79). Kitahara & Suzuki (47) have shown that the 5′-to-3′ directionality of transcription is not compulsory for ribosome biogenesis in *E. coli*. Moreover, the individual domains of the 30S subunit can be assembled in the absence of several r-proteins in vivo (1). While there is some degree of hierarchy to protein binding and the formation of intermediates, efficient biogenesis in vivo is achieved through a multifaceted landscape of events and pathways occurring parallelly. Assembly factors play an important role in this landscape to prevent misfolding and to avoid kinetic traps, even though they are only briefly part of a ribosomal intermediate during assembly. In this review, we highlight the cooperative and synergistic nature of *trans*-acting biogenesis factors and discuss the significance of quality control processes driven by these factors in biogenesis. We discuss genetic links that have identified new and previously uncharacterized functions by uncovering links between proteins where an obvious functional connection was lacking.

The absence of one or more biogenesis factors creates kinetic bottlenecks in the assembly cascade that to a large extent are overcome via alternate pathways or by the action of stand-in assembly factors (1, 10, 11, 14, 23, 28, 34, 85). Thus, ribosome synthesis is feasible in the absence of most biogenesis factors, and it appears that compensatory mechanisms play an important role in driving biogenesis forward. For example, a deletion strain lacking five genes encoding five RNA helicases (DeaD, SrmB, DpbA, RhlE, and RhlB), each of which has been implicated in biogenesis, is viable (41). RNA helicases have overlapping functions, and it is not fully clear what unique contribution each one makes. Therefore, the characterization of links between these enzymes and other factors (like those shown in **Figure 1**) will be crucial to dissecting redundant and overlapping functions.

7.1. Current Challenges and Future Directions

Many remaining challenges impede advancement in our understanding of the ribosomal subunit biogenesis process. First, precursor intermediates that accumulate in the absence of assembly factors are not wild-type substrates. They are thermodynamically stable, on-path intermediates; substrates with limited downstream options; dead-end intermediates; or a combination of these. Thus, isolation and visualization of biogenesis substrates formed in wild-type cells have been difficult. Second, despite high conservation of the composition of the ribosome and the fundamental process of translation, the biogenesis process is quite dissimilar between bacteria and eukaryotes. The biogenesis pathways in *E. coli* and *B. subtilis* exhibit distinctive rRNA processing and cleavage pathways (5, 22). Some bacteria (e.g., *M. tuberculosis*) have only one rRNA operon, compared to seven rRNA operons in *E. coli*. Although cotranscription of precursor rRNA species is a significant regulator of biogenesis, in *Thermus thermophilus*, the SSU and LSU rRNA transcription units are unlinked (29, 37). These differences within the bacterial kingdom suggest that environment and cellular conditions may have played an important role in the evolution of biogenesis processes and thus need to be taken into consideration while studying these processes, especially if there is a potential for antimicrobial development. Finally, ribosome biogenesis is closely coupled to other cellular processes in bacteria. Functional interactions between ribosomal and/or nonribosomal factors involved in maturation have helped to elucidate salient features like gatekeeping mechanisms of biogenesis. But how can we integrate data from individual studies to distinguish biogenesis function from a secondary effect? Identification of more links between factors will add to the network (**Figure 1**) and only reveal it to be even more interconnected. Genetic and biochemical studies combined with structural insights provide crucial pieces of information on phenotypes of biogenesis mutants. Techniques such as quantitative mass spectrometry high-throughput analysis of RNA folding and modification and structural data from cryo–electron microscopy all have been powerful to garner details about possible intermediates in these mutants. However, many questions remain unanswered. The path that a ribosomal intermediate takes during maturation may be influenced by clustering of functionally aligned factors. This idea is supported by the nodes in **Figure 1**. Studies by various groups on these clusters of interactions have shed light on new quality control mechanisms, beginning an exciting new chapter in the field of bacterial biogenesis.

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