Mutations of ribosomal protein S5 suppress a defect in late-30S ribosomal subunit biogenesis caused by lack of the RbfA biogenesis factor

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ABSTRACT
The in vivo assembly of ribosomal subunits requires assistance by maturation proteins that are not part of mature ribosomes. One such protein, RbfA, associates with the 30S ribosomal subunits. Loss of RbfA causes cold sensitivity and defects of the 30S subunit biogenesis and its overexpression partially suppresses the dominant cold sensitivity caused by a C23U mutation in the central pseudoknot of 16S rRNA, a structure essential for ribosome function. We have isolated suppressor mutations that restore partially the growth of an RbfA-lacking strain. Most of the strongest suppressor mutations alter one out of three distinct positions in the carboxy-terminal domain of ribosomal protein S5 (S5) in direct contact with helix 1 and helix 2 of the central pseudoknot. Their effect is to increase the translational capacity of the RbfA-lacking strain as evidenced by an increase in polysomes in the suppressed strains. Overexpression of RimP, a protein factor that along with RbfA regulates formation of the ribosome’s central pseudoknot, was lethal to the RbfA-lacking strain but not to a wild-type strain and this lethality was suppressed by the alterations in S5. The S5 mutants alter translational fidelity but these changes do not explain consistently their effect on the RbfA-lacking strain. Our genetic results support a role for the region of S5 modified in the suppressors in the formation of the central pseudoknot in 16S rRNA.

Keywords: ribosome assembly; 16S rRNA central pseudoknot; RbfA; RimP; translational accuracy

INTRODUCTION
Biogenesis of ribosomes, the cellular components responsible for protein synthesis, is the most energy intensive part of cellular metabolism consuming up to 80% of cellular energy in rapidly dividing bacteria, yeast, and human cells (Bremer and Patrick 1996; Warner 1999; Tschochner and Hurt 2003). Ribosomes also constitute by far the largest fraction of cellular RNA and protein in all cells in order to support production of thousands of ribosomes per minute in rapidly growing cells. Ribosomes are quite complex in structure, involving multiple RNAs and many integral ribosomal proteins as well as both RNAs and proteins undergoing many covalent modifications. The process of biogenesis, therefore, must be highly coordinated to achieve the required rate of production. Bacterial, but not eukaryotic ribosomal subunits can be reconstituted in vitro into active ribosomes from their components of rRNA and ribosomal proteins (r-proteins) but the process is slow and requires nonphysiological conditions (Nierhaus 1991) whereas biogenesis in vivo takes as little as 3 min (Champney 1977). This difference in efficiency can be attributed to the presence of accessory proteins that assist during cellular assembly of ribosomal subunits. Hundreds of factors facilitate biogenesis of eukaryotic ribosomes (Woolford and Baserga 2013), which along with their inability to be reconstituted in vitro argues for the complexity of their structure. In contrast, only around a dozen of biogenesis factors have been identified in bacteria but others are suspected to exist (Connolly and Culver 2009). However, even with so few bacterial factors, their functions are diverse including endonucleases that process the rRNA transcripts to mature rRNAs, enzymes that modify rRNA and r-proteins, DEAD box RNA helicases, GTPases, and many proteins with unknown functions (Connolly and Culver 2009). Known
proteins with a role in assembly of the 30S subunit in Escherichia coli include the GTPases Era (Nashimoto et al. 1985; Nashimoto 1993; Sayed et al. 1999; Inoue et al. 2003) and YjeQ/RsgA (Himeno et al. 2004; Campbell and Brown 2008), the rRNA and r-protein modifying enzymes KsgA (Connolly et al. 2008) and RimJ (Roy-Chaudhuri et al. 2010), the ribosome maturation factors RimM (Lövgren et al. 2004) and RimP (Nord et al. 2009), and the cold-shock protein RbfA (Dammel and Noller 1993). In addition, the DnaK chaperone system and the tRNA modifying enzyme YrdC (RimN) may participate in 30S maturation (Maki et al. 2002, 2003; Kaczanowska and Ryden-Aulin 2005), although their role is controversial (Alix and Nierhaus 2003; El Yacoubi et al. 2009). The complexity of the folding of the rRNAs (Stern et al. 1989) and the need to insert ribosomal proteins into this folded structure (Recht and Williamson 2004) present significant thermodynamic barriers to ribosome assembly. Although the precise role of individual factors is frequently unknown, the factors Era, RimM, and RimP have been shown to increase the rate of incorporation of specific r-proteins during in vitro reconstitution of the 30S ribosomal subunits (Bunner et al. 2010).

RbfA (ribosome binding factor A) was initially identified as a high-copy suppressor of the dominant cold-sensitive C23U mutation in 16S rRNA. The protein associates with free 30S ribosomal subunits but not with 70S ribosomes or polysomes (Dammel and Noller 1993, 1995). Thermus thermophilus RbfA binds to the 30S subunit near C23 in a position that overlaps with the binding sites for the A and P site tRNA (Datta et al. 2007) suggesting that the cold sensitivity of the C23U mutant might result from slowed recruitment of RbfA to this binding site. Consistent with that idea, a mutant lacking RbfA is also cold-sensitive and shows a constitutive cold-shock response (Jones and Inouye 1996). Mutations in both 16S rRNA and rbfA affect ribosome function and biogenesis in similar ways, with increased levels of free 50S and 30S subunits and decreased levels of 70S ribosomes and polysomes (Dammel and Noller 1993, 1995). An rbfA null mutant also showed slowed conversion of 17S rRNA to mature 16S rRNA (Bylund et al. 1998), which was exacerbated at lower temperatures (Xia et al. 2003). All of these phenotypes are shared by mutants lacking the 30S ribosomal subunit biogenesis factors Era (Nashimoto et al. 1985), KsgA (Connolly et al. 2008), RimM (Lövgren et al. 2004), RimP (Nord et al. 2009), and YjeQ/RsgA (Guo et al. 2011). Genetic interactions suggest that these factors act together to complete the final steps in 30S maturation (Shajani et al. 2011). For example, overexpressing RbfA improves the growth and translational efficiency at 37°C of a strain lacking the ribosome maturation factor RimM (Bylund et al. 1998) and overexpression of the GTPase Era suppresses partially the slow growth and cold-sensitive 30S subunit maturation deficiency of the mutant lacking RbfA (Inoue et al. 2003).

Ribosome assembly begins cotranscriptionally with local secondary structures folding in a 5′–3′ direction and many ribosomal proteins binding before the transcript is complete (for review, see Shajani et al. 2011). Assembly progresses through multiple parallel pathways, some of which may lead to kinetically trapped structures that cannot progress by further rRNA folding or ribosomal protein binding (Sykes and Williamson 2009). The role of the RimM and RbfA factors appears to be to bind nascent rRNAs and block the formation of trapped structures (Williamson 2003; Clatterbuck Soper et al. 2013).

Clatterbuck Soper et al. (2013) used in vivo footprinting to demonstrate that presumed chaperones RbfA and RimM are required for mature folding of the 3′ end of 16S rRNA including the “head” domain and helix 44, which forms important intersubunit bridges in the mature structure (Yusupov et al. 2001; Schuwirth et al. 2003; Selmer et al. 2006). Pre-30S subunits formed in the absence of these proteins lacked tertiary binding ribosomal proteins (S2, S3, and S21) and helices 1 and 2 (h1 and h2), which comprise the central pseudoknot, and helix 44 were significantly destabilized. These results were corroborated by Sashital et al. (2014) who used quantitative mass spectrometry and electron microscopy to demonstrate roles for several late biogenesis factors in formation of the central pseudoknot and anchoring the head domain in its mature position. These large scale assembly events are coordinated with the addition of individual ribosomal proteins, beginning with S5, which forms part of the interface with the head domain in its tethered position.

The last steps of rRNA processing occur during the final stage of 30S assembly. Mutants lacking the late-30S ribosome biogenesis factors RbfA (Inoue et al. 2003), Era (Nashimoto et al. 1985), RimM (Lövgren et al. 2004), KsgA (Connolly et al. 2008), RimP (Nord et al. 2009), and RsgA (Guo et al. 2011) accumulate an immature 17S rRNA carrying 5′ and 3′ extensions that are removed in generating mature 16S rRNA. Retaining as little as 10 nt of the 5′ leader is predicted to result in formation of an alternative structure lacking h1 of the central pseudoknot rRNA (Young and Steitz 1978; Dammel and Noller 1993), a structure located in the ribosomal A site near other structures associated with fidelity. In addition to accumulating 17S rRNA a mutant form of ribosomal protein S5 (G28D) and in rimM and ksgA mutants also cause reduced translational fidelity (Roy-Chaudhuri et al. 2010). Because overexpression of the biogenesis protein RimJ in the mutant expressing S5-G28D both restores translational fidelity and increases 16S maturation, Roy-Chaudhuri et al. (2010) have suggested that the rRNA extension is the cause of decreased fidelity. Clatterbuck Soper et al. (2013) showed that the 17S-containing pre-30S subunits can catalyze dipeptide formation in vitro but at a rate substantially lower than mature 30S subunits; whether the immature subunits participate in translation in vivo, however, has not been demonstrated. A recent structural study showed that immature 30S subunits isolated from an Arabidopsis mutant strain that lack of S5 also show increased mobility of the head domain and flexibility of h1 and the 5′ end of the rRNA.
suggesting an active role for S5 in both tethering of the head domain and formation of the central pseudoknot (Yang et al. 2014). This correlation, however, does not establish an active role for S5 in that process other than to comprise part of the stable structure formed under the control of late biogenesis factors like RbfA.

To further explore the relationship between RbfA and ribosome biogenesis and function we identified chromosomal mutations that suppress the cold sensitivity and slow growth of an rbfA::KmR null mutant. Several of these mutations target the rpsE gene, encoding S5. These suppressor mutations also increased the rate of translation and suppressed the lethality of overexpressing a second ribosome assembly protein, RimP, in the rbfA::KmR background. The rpsE suppressors increased the amount of polysomes in the rbfA::KmR background; this result is consistent with improved growth resulting from increased translational output. We found that the immature 17S-containing 30S subunits do not participate in translation since they were absent from the polysomes. The rpsE suppressors did not significantly reduce the proportion of 17S rRNA in the 30S or 70S monosomes but they did increase the amount of mature or near-mature 30S subunits engaged in translation in polysomes. Because of the suggested connection between late ribosomal biogenesis events and translational fidelity, we tested the effect of rbfA::KmR and the rpsE mutations on translational misreading. The rbfA::KmR mutation caused decreased fidelity as did four of the five rpsE suppressor mutations; the fifth suppressor actually increased fidelity. However, there was no consistent effect on accuracy of combining rbfA::KmR with the rpsE suppressors. This result suggests that the improved growth effect seen in the double mutants does not reflect a change in accuracy. Rather, the rpsE mutations appear to improve the ability of mature 30S subunits to engage in translation, probably by reducing the formation of improperly matured subunits.

RESULTS

Overexpression of RimP in the absence of RbfA causes synthetic lethality

In order to further characterize the function of RbfA we identified high-copy suppressors of the slow growth of a strain lacking RbfA. Plasmids from the NIG collection (Saka et al. 2005), from which the expression of the cloned genes could be induced with IPTG, were introduced into the rbfA::KmR mutant GOB162 and tested for the ability to increase growth rate. Consistent with the work of others (Bylund et al. 1998; Inoue et al. 2003), we found high-copy suppressor interactions between the rbfA::KmR mutant and only three other ribosome biogenesis protein genes. A plasmid carrying the era gene improved growth of the mutant (data not shown) as expected (Inoue et al. 2003). Overexpressing RimM did not alter growth of the rbfA::KmR mutant (Fig. 1) despite the fact that overexpressing RbfA suppresses the slow growth of a strain lacking RimM (Bylund et al. 1998). Surprisingly, overproducing the RimP ribosome maturation factor actually prevented growth of the rbfA::KmR mutant GOB162 at 30°C, 37°C, and 44°C (Fig. 1). All of these factors—Era, RbfA, RimM, and RimP—function late during the cotranscriptional association of r-proteins with the 16S rRNA (Bunner et al. 2010; Sashital et al. 2014) and RbfA, RimM, and RimP are all implicated in formation and stabilization of the central pseudoknot (Sashital et al. 2014). The synthetic lethality from overexpressing RimP in the absence of RbfA is particularly striking, perhaps suggesting that RimP can force bypass of an RbfA "checkpoint" (Connolly and Culver 2013) that coordinates other morphogenetic events with formation of the central pseudoknot.

Identifying rpsE mutations that suppress the slow growth phenotype of an rbfA rimP double mutant

To investigate the function of ribosome assembly proteins we identified second site mutations that suppressed the slow growth of mutants lacking the biogenesis proteins RbfA and RimP. The rbfA::KmR insertion mutant grows most
slowly at low temperature (2.5-fold at 26°C), while a strain carrying an in-frame deletion of rimP (ΔrimP135) grows most slowly at high temperature (2.5-fold at 44°C) (Dammel and Noller 1995; Nord et al. 2009). Attempts to isolate suppressors of the ΔrimP135 mutation were unsuccessful (data not shown) and there are no reports of chromosomal suppressors of the rbfA::KmR mutation. We chose to select suppressors of a rbfA::KmR ΔrimP135 double mutant because it grows more slowly than either single mutant and we reasoned that this more intense phenotype might make it easier to isolate suppressor mutations. To isolate faster-growing derivatives of the rbfA::KmR ΔrimP135 double mutant STN133, cultures were grown overnight in rich medium at 30°C, 37°C, and 44°C and samples were tested for colony size on rich medium plates incubated at the same temperature. Cells were subcultured repeatedly for 7 d to allow faster-growing clones to arise. In this way, 12, 11, and 9 independently isolated faster-growing derivatives were obtained at 30°C, 37°C, and 44°C respectively. Different classes of clones were identified based on colony size on rich medium plates (Table 1). In general, the difference in growth between the suppressor-containing clones and the rbfA::KmR ΔrimP135 parental strain was more pronounced for clones isolated at 30°C than for those isolated at the other two temperatures.

It seemed probable that some of the suppressor mutations (sar = suppressor to rbfA and rimP) would affect genes encoding small subunit ribosomal proteins, specifically, those dependent on RbfA for binding to the preribosome. Since two-thirds of ribosomal protein genes are found in a cluster at the 74 th minute of the E. coli chromosome (Berlyn 1998), we tested whether introducing this region from a suppressor-free strain could replace suppressor mutation in one of the faster-growing suppressor containing clones, STN174 (rbfA::KmR ΔrimP135 sar022). We grew the generalized transducing phage P1 on strain PW078, which contains a Tn10 insertion, zhc-2421::Tn10, conferring tetracycline resistance (TsC) downstream from the last gene of this r-protein gene cluster, rplQ. We used this stock of phage to transduce strain STN174 to TsC. rbfA is located at 71.4, too distant to be cotransduced with zhc-2421::Tn10. The majority of individual transductant colonies examined on rich medium plates at 37°C showed the slow growth characteristics of the original rbfA::KmR ΔrimP135 double mutant STN133 suggesting that the suppressor mutation in strain STN174 had been crossed out and therefore was located in this region of the chromosome. The linkage between zhc-2421::Tn10 and the suppressor mutation was determined to be 95% (data not shown). In order to identify the suppressor mutation, regions containing the genes for r-proteins S4, S11, S13, S5, S8, S14, and S19 were amplified by PCR and sequenced. Strain STN174 contained a T to C transition in position 380 of rpsE, which encodes S5, changing an alanine (GCC) to a valine (GTC) at codon 127; no other sequence changes were found in the other genes sequenced. To determine if the other suppressor mutations also occur in rpsE, the gene was sequenced from all strains listed in Table 1, except STN155, STN156, STN173, and STN185. Strain STN161 (rbfA::KmR ΔrimP135 sar012) contained the identical C to T transition in codon 127 as in STN174, while in STN187 (rbfA::KmR ΔrimP135 sar032), the same GCC alanine codon had been changed to ACC for threonine. STN176 (rbfA::KmR ΔrimP135 sar024) and STN186 (rbfA::KmR ΔrimP135 sar031) both contained a transition from G to A in position 259 changing codon 87 from Gly to Ser for serine. STN181 (rbfA::KmR ΔrimP135 sar027) the same codon was changed to GCT for glycine to GGT for glutamic acid. Thus, two mutations were found twice and for two codon positions there were different mutations resulting in different amino acid substitutions. Interestingly, the three different amino acid positions that were altered in the suppressor containing strain are clustered in the structure of S5 (Fig. 2). The three
growth, we investigated whether the suppressors were transferred to strains GOB162 (ΔrbfA::KmR but not ΔrimP135). The three amino acids altered in the S5 mutants described here (G87, G91, and A127) are shown in space-filling mode. The residues are located in a domain of S5 (in red) including a β sheet, composed of two β strands, and an adjoining strand. This domain is in close proximity to the central pseudoknot. The position of the spectinomycin-resistance mutant G28 is also shown in space-filling and is located near the region of h28 that is stacked on h2. The structure was modeled using VMD (Humphrey et al. 1996).

The rpsE mutations suppress the slow growth of the rbfA::KmR but not the ΔrimP135 mutant

Since both the rbfA and the rimP mutations confer slow growth, we investigated whether the rpsE mutations, isolated as suppressors of the slow growth of the double mutant, would improve the growth of either single mutant or have any negative effect on the growth of an rbfA+ rimP+ strain. The suppressors were transferred to strains GOB162 (rbfA::KmR), MW187 (ΔrimP135), and MW100 (wild type) by P1 transduction using the linked marker mutations. None of the five different rpsE mutations suppressed the slow growth of the ΔrimP135 mutant MW187 on solid or liquid rich medium at 44°C or had any detectable effect on the growth of the wild-type strain MW100 on solid rich medium at 21°C, 30°C, or 37°C (data not shown). However, all the rpsE mutations improved the growth on solid rich medium at 30°C, 37°C, and 44°C of the mutant lacking RbfA with the greatest effect at 30°C (data not shown). In liquid medium at 30°C, the rpsE mutations increased the growth rate of the rbfA::KmR mutant 1.3- to 1.6-fold (Fig. 3). Thus, the different rpsE mutations suppressed the growth deficit caused by the lack of RbfA and not RimP.

Lack of the cold-shock protein RbfA impairs the response to a shift from 37°C to 15°C causing slower growth than wild type after the cold shock (Jones and Inouye 1996). We tested the ability of the rpsE mutations to suppress the slow growth of the rbfA::KmR mutant after cold shock by comparing the growth of suppressor-free and suppressor-containing derivatives of the rbfA::KmR mutant after shifts from 37°C to 15°C in rich medium. The rbfA::KmR strain resumed growth soon after the shift, but at a rate several-fold lower than for the wild-type strain MW00 (data not shown). The rpsE mutations improved growth at 15°C after shock from 1.2- to 1.6-fold. The fact that the suppressors had the same effect on growth with or without cold-shock suggests that they may generally improve the growth rate rather than altering the response to cold shock per se.

The rpsE mutations increase the proportion of 30S subunits that take part in translation

A strain that lacks RbfA is deficient in the maturation of the 30S ribosomal subunits and shows an accumulation of free 50S and 30S ribosomal subunits and much less translating ribosomes than a wild-type strain probably as a result of this maturation deficiency (Dammel and Noller 1995; Bylund et al. 1998; Inoue et al. 2003; Xia et al. 2003). Polysome profiles obtained after centrifugation through sucrose gradients of total cell extracts of relevant strains grown at 30°C confirmed this observation. For the rbfA::KmR strain the size of the peaks corresponding to 70S ribosomes and polysomes were strongly reduced, whereas those for free 50S and 30S subunits were strongly increased relative to wild type (cf. Fig. 4A,B). The presence of the rpsE suppressors in the rbfA::KmR background resulted in increased amounts of
70S ribosomes and polysomes and reduced amounts of 30S and 50S subunits than in their absence (cf. Fig. 4A–F). To quantify the effect of the suppressors we calculated the ratio of the area of the 30S peak to the area of the peaks for 70S ribosomes and polysomes. The obtained ratio [30S/total 70S] was lower for the suppressor containing strains compared with the \textit{rbfA::Km\textsuperscript{R}} mutant by an average of 2.2-fold (Fig. 4H). The difference was greatest (2.8-fold) for the \textit{rbfA::Km\textsuperscript{R} rpsE-G91A} strain and least (1.8-fold) for the \textit{rbfA::Km\textsuperscript{R} rpsE-A127T} strain. The change in this ratio results from a reduction in the total amount of free subunits (30S reduced by an average of 28\% and 50S by 19\%) and an increase in associated subunits (70S increasing by 46\% and polysomes by 85\%). Thus, the presence of the \textit{rpsE} suppressors improved the fraction of 30S subunits competent to participate in translation but not to wild-type levels. The strongest suppressor (\textit{rpsE-G91A}), which caused the greatest growth rate, had the greatest improvement and the weakest (\textit{rpsE-A127T}) had the least; the three with intermediate growth phenotypes were also intermediate for this phenotype. The correlation between the two phenotypes suggests that improvement in growth results from improvement in ability of 30S subunits to participate in translation.

The \textit{rpsE} suppressor mutations do not reduce accumulation of 17S rRNA-containing immature 30S subunits

The RbfA factor is required for formation of the mature 5’ and 3’ ends of 16S rRNA. A strain lacking RbfA accumulates 17S rRNA, which is generated by cleavage by RNase III in a stem–loop formed by pairing of the region upstream of the mature 5’ end (the 5’ leader) and downstream from the mature 3’ (the 3’ trailer). This cleavage leaves a 115-nt region upstream and a 33-nt region downstream from the mature 16S rRNA; further maturation removing these regions converts the larger pre-rRNA that sediments as 17S, to a completely matured 16S rRNA (Gupta and Culver 2014). The strain lacking RbfA performs this further maturation inefficiently so the normally rare 17S form accumulates. The failure of \textit{rbfA::Km\textsuperscript{R}} to complete processing could slow or block the resulting 30S from participating in translation and the \textit{rpsE} suppressors could promote 70S formation by accelerating processing.

To determine if the gross phenotypic effect of the \textit{rpsE} suppressors reflects an increased rate of rRNA maturation, the relative amounts of 5’ ends corresponding to 17S and 16S rRNA were determined for suppressor-containing and suppressor-free \textit{rbfA::Km\textsuperscript{R}} mutant grown at 30°C using fluorescence-based primer extension on total RNA with FAM-labeled primers specific for 16S rRNA. The primer extension products were mixed with internal lane molecular weight markers before electrophoresis after which the fluorescence in relevant peaks was quantified. The relative amount of 17S rRNA [17S/(16S + 17S)] was 5.4-fold higher in the \textit{rbfA::Km\textsuperscript{R} mutant} STN409 than in the wild-type strain MW100 (Fig. 5) in agreement with previous findings (Bylund et al. 1998; Xia et al. 2003). The \textit{rpsE} mutations did reduce the relative amount of 17S rRNA but the effect was rather small, 1.1- to 1.2-fold (Fig. 5). This increase was not statistically

\textbf{FIGURE 4.} Polysome profiles of an \textit{rbfA::Km\textsuperscript{R}} mutant (A) and its derivatives expressing variants of r-protein S5 containing different amino acid substitutions (B–F), as well as of a wild-type strain (G). Different ribosomal particles are marked above corresponding peaks. 2x, 3x, and 4x indicate two, three, and four 70S ribosomes per mRNA, respectively. In H, the ratio is shown of the area of the peak for the 70S and 2x, 3x, and 4x polysomes to the total area of total ribosomal particles ("Associated subunits") and the ratio of the area of the peak for the 30S subunit to the areas of the peaks for the 70S, 2x, 3x, and 4x ("30S/total associated"). The error bars represent the standard error of the mean from three independent experiments as shown for strains STN397, STN399, STN401, and STN409, and from two independent experiments for strains STN403 and STN405.
significant in any case ($P > 0.05$ by Student’s $t$-test) and was insufficient to explain the much larger (average 1.5-fold) and statistically significant ($P < 0.005$) increase in amount of 70S ribosomes plus polysomes formed in the presence of the suppressors. A comparison of the two effects suggests that the $rpsE$ suppressors improve the ability of mature 30S subunits to engage in translation without affecting the probability of maturation of the 5′ end of the rRNA.

Immature 17S rRNA-containing 30S subunits do not participate in translation

The data on 5′ rRNA processing shows that under the conditions of growth used approximately half of the small subunit rRNA is mature 16S and half immature 17S and that this ratio is essentially unaffected by the presence of the suppressor mutant forms of S5. There are two ways that the $rpsE$ mutants might increase total translation given their failing to alter the efficiency of 16S rRNA maturation. The effect might be entirely independent of the maturation state of the rRNA, so the $rpsE$ mutant S5 proteins might allow 17S-containing 30S subunits to associate with 50S subunits and to participate in translation despite being incompletely processed. Translation by ribosomes comprised of incompletely processed 30S subunits has been invoked to explain the error-prone nature of mutants carrying the spectinomycin-resistant mutation of S5, G28D (Roy-Chaudhuri et al. 2010). But there is no evidence showing that 17S-containing 30S subunits participate in translation in vivo. The fact that the proportion of polysomes in the $rbfA::Km^R$ strain is reduced much more than 50% suggests that in the absence of RbfA even subunits with mature 16S rRNA may be defective in translation. Under this hypothesis, the increase in translation in the presence of the S5 mutant proteins might result from improvement in some step(s) in biogenesis independent of rRNA 5′ end maturation.

To determine if the 17S rRNA can participate in translation, we performed fluorescence-based primer extension on samples of rRNA isolated after separation by sucrose gradient centrifugation. The results fail to support this conclusion. The primer extension experiments revealed a third form of rRNA in addition to 16S mature and 17S immature in which the nearly mature 16S has short 1–8-nt 5′ extensions (labeled “NM” in Table 2). These extensions probably tend to be too short to interfere with formation of h1 (Young and Steitz 1978; Dammel and Noller 1993) so subunits with this rRNA might be expected to be largely functional. The clearest result from this analysis is that the 17S immature rRNA was entirely excluded from the polysomes (Table 2, column 9) despite the extremely large increase in all the mutant strains. While it remains possible that these subunits can translate in monosomes, the most likely conclusion is that they are not translationally competent. The very low dipeptide formation in vitro by ribosomes containing 17S rRNA would predict a very slow elongation rate, and a slowly elongating ribosome would block the progress of upstream wild-type ribosomes, which would tend to cause a shift toward higher polysomes. If these immature subunits are indeed incompetent for translation then the 70S ribosomes carrying 17S rRNA must be
inactivate couples or result from abortive attempts by such a ribosome to initiate translation, leading to an mRNA bound by a single such ribosome, which blocks further initiation events. We have not been able to distinguish between these two hypotheses.

The nearly mature (nm) 16S rRNA can participate in translation, as shown by its presence in polysomes. The fact that the nm16S is the predominant small subunit rRNA in nonpolysomal 70S ribosomes but in polysomes 16S predominates suggests that a proportion of these nm16S-containing 30S subunits is in inactive couples or result from abortive initiation. This could result from two populations of nm16S-containing 30S subunits with alternative 5′ structures with those with slightly longer 5′ extensions not being able to form h1 and being inactive.

The composition of polysomal small subunit rRNAs is similar in wild-type and in the rbfA::KmR mutant strain with and without the S5 suppressor protein. This underscores the concept that the difference among these strains is unrelated to 5′ end maturation. The quantity of polysomal ribosomes is greatest where RbfA is present. In the absence of RbfA the quantity is reduced a 7.2-fold but the presence of the S5 suppressor proteins the quantity increases an average of 85%, still very low compared with wild type, but apparently sufficient to substantially improve growth.

The rpsE mutations prevent the lethality of overexpressing RimP in the rbfA::KmR mutant

The current understanding of the role of ribosome biogenesis factors RbfA and RimM is that they superintend events late in ribosome biogenesis, possibly even slowing the process down to allow critical events to occur to complete biogenesis of translationally competent 30S subunits (Clatterbuck Soper et al. 2013). The need to slow down the last steps in biogenesis may explain the vulnerability of strains lacking RbfA to overexpression of other late-30S biogenesis factors. Our finding that overexpression of RimP is lethal in the absence of RbfA suggests that excess RimP might force pre-30S subunits through a step subsequent to RbfA action, producing defective 30S subunits. The rpsE suppressor mutations on the other hand, improve growth in the absence of RbfA suggesting that they might replace RbfA in slowing the progress through the RimP-sensitive step. Alternatively, the rpsE mutants might affect an RbfA-dependent step that is unrelated to sensitivity to RimP overexpression.

We tested the effect of the rpsE suppressors on the lethal effect of overexpressing the 30S maturation factor RimP in the rbfA::KmR mutant by introducing a rimP+ plasmid from the NIG collection (Saka et al. 2005) into the rbfA::KmR mutant containing the rpsE suppressor mutations. The expression of RimP was induced by addition of 0.03 mM IPTG, a concentration that in the rbfA::KmR background allowed some growth at 30°C but none at 37°C or 44°C (cf. Figs. 6, 1). Growth was improved at 30°C by all five rpsE suppressor mutations although the extent of improvement varied with the greatest effect being caused by the rpsE2342 (S5-G91A) mutant. Four of the rpsE mutants suppressed lethality at 37°C and 44°C, the exception being rpsE2344 (S5-A127T), which was also the weakest suppressor at 30°C. At 44°C only the strongest suppressor, rpsE2342 (S5-G91A) conferred strong growth (Fig. 6).

The similarity in the strength of improvement of growth of the strain lacking RbfA and of the suppression of the deleterious effect of overexpressing RimP in the absence of RbfA imply that the role of the S5 suppressor mutant proteins is to slow some event promoted by RimP. Given that the residues altered in S5 are close to the central pseudoknot and that RimP helps form that structure (Sashital et al. 2014), it is a reasonable conjecture that the S5 mutant proteins replace RbfA to an extent in modulating RimP activity in forming that structure. However, RimP also accelerates binding of the set of late binding ribosomal proteins (Bunner et al. 2010), including S5, which is required for binding of the other proteins. The S5 mutant proteins may, therefore bind especially slowly, thus retarding binding of the later binding proteins, which could preclude the formation of a kinetically trapped complex.

Effect of rpsE suppressors on translational accuracy

As we have explained above, various defects in late-30S assembly have been associated with decreased translational fidelity. It is logical, therefore, to suppose that one of the effects of the rpsE mutations is to alter translational accuracy. This is a reasonable hypothesis because mutations of rpsE were among the first to be shown to alter accuracy (Gorini

![FIGURE 6](image-url)
The \( rpsE \) mutations were identified as suppressors of mutations affecting ribosomal protein S12 that confer hyperaccuracy as well as dependence on the error-inducing antibiotic, streptomycin (Gorini 1974). The hyperaccurate mutations encode amino acids in the ribosomal A site, some of which participate directly in cognate tRNA recognition (Ogle et al. 2001). Ribosomal ambiguity (\( ram \)) mutations that alter S4 and S5 and suppress S12 streptomycin-dependence change amino acids at an interface between the two proteins. Ogle et al. (2002) proposed that the mutations destabilize the interface and lessen the energy barrier to formation of a high-affinity amino acid-tRNA-ribosome complex, however the validity of the model was questioned by the fact that the phenotype of the mutants corresponds poorly with their effect on the stability of the S4–S5 interface (Vallabhaneni and Farabaugh 2009). It may be significant that an rRNA nucleotide near the 5′ end of the 16S RNA intercalates within the S4–S5 interface, connecting the S4–S5 interface with the structure of the 5′ end and central pseudoknot, implicating these structures in translational accuracy as well. More recently, a \( ram \) mutation outside the S4–S5 interface was identified that changes Gly 28 of S5 to Asp (G28D), which is near the central pseudoknot (Roy-Chaudhuri et al. 2010). This mutation is also cold-sensitive and causes accumulation of the immature 17S rRNA.

We quantified translational accuracy using a system that exploits mutations of an active site codon from the gene encoding \( Photinus pyralis \) (firefly) luciferase (Fluc) (Kramer and Farabaugh 2007). Lys 529 (K529) coordinates the reactants, ATP and luciferin, in the active site of Fluc (Branchini et al. 2000) and mutations of that codon cause up to 5000-fold reductions in activity (Kramer and Farabaugh 2007). Several single nucleotide mutations of the K529 codon allow it to be misread frequently by tRNA\(_{\text{Lys}}\)CUU, which normally decodes both Lys codons, thereby inserting the wild-type amino acid while decoding the mutant codon. These misreading events result in the expression of fully functional, wild-type Fluc and the frequency of the events is equal to the ratio of activity from the mutant gene to the wild type. Most near cognate codon mutations (those differing from one of the two Lys codons by a single base change) result in activities averaging \( \sim 3 \times 10^{-4} \) times wild type; several codons result in greater activities, including the stop codons UAA and UAG, the Arg codons AGA and AGG, and the Asn codon AAU (Kramer and Farabaugh 2007). The greater activity when codons are present reflects more frequent near cognate decoding of these codons by tRNA\(_{\text{Lys}}\)CUU.

To measure accuracy, four error-prone codon mutant codon reporters were tested with the K529 codon changed either to UAG (stop), AGA (Arg), or AGG (Arg). Error bars are standard errors of the mean. Significance is indicated: (*) \( P \leq 0.05 \); (**) \( P \leq 0.005 \).

\( \text{FIGURE 7.} \) The activity of K529 mutants of firefly luciferase (Fluc) expressed in strains carrying various \( rpsE \) suppressor mutations in (A) the wild-type background or (B) in the presence of the \( \text{rflA::KmR} \) mutation. The Fluc activities produced for each of the mutants of Fluc K529 are expressed as a ratio to that of the wild-type enzyme. Activities are shown for the four known error-prone mutants replacing the wild-type Lys codon with AAU (Asn), UAG (stop), AGG (Arg), or AGA (Arg). Error bars are standard errors of the mean. Significance is indicated: (*) \( P \leq 0.05 \); (**) \( P \leq 0.005 \).
DISCUSSION

We have exploited the extremely slow growth phenotype of a double mutant lacking two ribosome biogenesis proteins, RbfA and RimP, to identify a large set of suppressor mutations that restore growth although not to wild-type rates. We had expected that some of these suppressors would affect ribosomal proteins since one of the roles of these two factors is to aid assembly of a set of late binding proteins. By screening for mutations in a ribosomal protein operon we identified a set of seven mutations comprising five unique sequence changes in ribosomal protein S5. The identity of the genes mutated in the other suppressor mutants is actively under investigation using whole-genome sequencing.

Finding suppressor mutants of S5 is significant since it is the first of the late assembly ribosomal proteins whose binding is promoted by RbfA and RimP (Bunner et al. 2010; Sashital et al. 2014). It is also significant that S5 binds to a portion of the central pseudoknot (Schuwirth et al. 2005), a structure whose assembly depends on RbfA and RimP (Sashital et al. 2014). The binding site for RimP on the 30S subunit is not known, but RbfA binds to three domains of the ribosome, the head (h28, h29, h30), body (h18 and ribosome protein S12) and 3′ minor (h44, h45) domains (Datta et al. 2007). By binding, RbfA deforms many of these structures and blocks both the A and P sites to exclude participation in translation. RbfA does not appear to be in contact with S5 or the central pseudoknot, implying that its effect on binding of S5 and stabilizing h1 of the pseudoknot is indirect. It is possible that RimP contacts both S5 and the pseudoknot but it is unclear why, then, the S5 mutations would suppress the defects associated with loss of RbfA, rather than RimP. As shown in Figure 2, S5 directly contacts both h1 and h2 of the central pseudoknot and also h28, which stacks on h2. The previously identified rpsE-G28D mutation alters an amino acid in close contact with h28, the nearest approach of S5 to the site of RbfA binding. The mutations identified here alter residues G87, G91, and A127, all of which are within a triple β-stranded domain that contacts the central pseudoknot and distant from RbfA. Gly-91, which is altered in the strongest suppressor mutant, is in the loop between β-strands 5 and 6 of S5 very close to the phosphodiester bond between nucleotides 19 and 20 of h2. The other two amino acids, G87 and A127, are in the other two β-strands of the domain. The substitution of the small Gly and Ala amino acids by bulkier amino acids could perturb packing of these strands with adjacent structures of the hydrophobic core of S5, altering the conformation of the interface between S5 and the central pseudoknot. Only one of the mutations introduces a more substantial structural change, altering Ala 127 to Val; the rather conservative nature of most of the changes suggests that less conservative changes might result in lethality or poor growth phenotypes.

Mutants lacking RbfA accumulate a small subunit rRNA, 17S, with extensions on both the 5′ and 3′ ends as do mutants lacking several other late-30S biogenesis factors, the S5–G28D
mutant and the destabilizing C23U mutant of h1. The presence of 17S in mature 30S subunits has been identified as a possible cause of the decreased fidelity of translation and decreased cellular fitness of the C23U mutant and by extension the others (Roy-Chaudhuri et al. 2010). One possibility for the mechanism by which the S5 suppressors improve growth in the absence of RbfA is by improving the maturation of 17S to 16S rRNA. If that were true, the suppressor mutants would also be expected to increase the fidelity of translation. Our results show that the S5 suppressors have no significant effect on 17S maturation; in the \( \text{rbfA}^\Delta \text{Km}^R \) strain either with or without the \( \text{rpsE} \) suppressor mutations ~50% of the 30S subunits had 17S rRNA with the rest consisting of 16S mature or without the 5′ terminal processing. An alternative model would suggest that the suppressors are bypass mutations, improving the fidelity of ribosomes with the 17S-containing 30S subunit. We showed that this is also not the case since the 17S-containing ribosomes do not participate in translation. Thus, the model in which 17S reduces fidelity is not viable.

If the S5 suppressors do not change the proportion of immature rRNA in the 30S subunits, then what could they do to improve growth and translational fidelity? The remaining model would suggest that the S5 suppressors improve some other step in the maturation of pre-30S subunits. We have observed that the suppressors increase the proportion of these subunits that participate in translation; the pool of ribosomes in polysomes improves in all suppressors and the effect is greatest in the strongest suppressors. The fact that apparently mature 30S subunits fail to participate as fully in translation as they do in a wild-type strain suggests that in the strain lacking RbfA a large proportion of apparently mature 30S subunits are in some way defective in translation. This defect could reflect the absence of one or more essential ribosomal proteins or the formation of inappropriate RNA secondary or tertiary structures in the absence of RbfA. This model is consistent with the role for RbfA in avoiding kinetically trapped complexes. In its absence during late-30S assembly some rRNA folding may occur prematurely, excluding one or more of the last ribosomal proteins to bind. Sashital et al. (2014) showed that in a \( \text{ΔrimP} \) strain, the 30S peak of a sucrose gradient largely consisted of subunits lacking one or more of the last four proteins, S2, S3, S12, and S21, but that using a pulse-chase approach they showed that these complexes are only delayed in assembly and not dead-end complexes. Experiments to test for deficits in late ribosomal proteins in the \( \text{rbfA}^\Delta \text{Km}^R \) strain with and without the suppressors are planned and should resolve this issue.

The third alternative is that the absence of RbfA results in misfolding of the rRNA that is not reflected in lack of binding of ribosomal proteins. It is possible that, in the absence of RimP or RbfA chaperones, the RNA may adopt a noncanonical structure that produces functional complexes with reduced fidelity. The S5 suppressors, through their interaction with the central pseudoknot, could limit the formation of those structures. The fact that the mutant S5 proteins reverse the lethal effect of overexpressing RimP in the absence of RbfA is consistent with this concept. RimP is known to stabilize the central pseudoknot (Sashital et al. 2014) and accelerate binding of S5 and S12 (Bunner et al. 2010). The lethality of overexpressing RimP may result from these processes proceeding too quickly, creating kinetically trapped complexes. The S5 suppressors presumably block the downstream effects that result subsequent to its binding, or slow the binding itself in mimicry to the presumed effect of RbfA.

We also show here that in addition to their effect on assembly the S5 suppressors alter translational accuracy. The evidence shows that both the \( \text{rbfA}^\Delta \text{Km}^R \) and four of the five \( \text{rpsE} \) suppressor mutants are error prone, with an effect of a similar magnitude as the canonical ram mutations (Gorini 1974; Kramer and Farabaugh 2007). Interestingly, the \( \text{rbfA}^\Delta \text{Km}^R \text{rpsE} \) double mutants do not show greater error frequencies, which suggests that both lack of RbfA and the mutant forms of S5 contribute to increasing error in the same mechanistic pathway. This similar error-prone effect is despite the fact that the \( \text{rbfA}^\Delta \text{Km}^R \text{rpsE} \) mutants have opposite gross phenotypes and that the suppressor with the strongest effect on growth of the \( \text{rbfA}^\Delta \text{Km}^R \) strain actually exhibits the opposite phenotype of hyperaccuracy. Thus, increased inaccuracy is not a necessary and direct cause of improving the growth of the strain lacking RbfA.

The observed increase in translational errors in a G28D 16S rRNA mutant has been proposed to result from 30S subunits carrying the 17S immature rRNA (Roy-Chaudhuri et al. 2010); the argument was that the extra RNA perturbs the function of the ribosomal A site within which it resides so as to increase the frequency of acceptance of incorrect aminoacyl-tRNAs. Since the 17S-containing ribosomes appear to be inactive in translation, incomplete 5′ processing must not be the cause of the error phenotype. The two effects are largely associated among mutants of several ribosome biogenesis proteins, the mutant expressing the S5-G28D and the mutant expressing the unstable C23U for of 16S rRNA. The diversity of primary defects in these biogenesis mutants—lacking GTPases, chaperones, and a protein methylase—suggest that the accuracy effect, like the 5′ processing must be distant from the site of action of the primary defect. This suggests that both 17S rRNA accumulation and inaccuracy result from incomplete or inaccurate assembly of the mature 30S subunit. Direct biochemical characterization of mature 30S subunits produced in these mutant strains could identify the mechanism underlying this effect.

**MATERIALS AND METHODS**

**Strains, phages, plasmids, oligonucleotides**

Relevant strains, phages, plasmids, and oligonucleotides used are listed in Table 3. Strain STN133 (Hfr P4X \( \text{rbfA}^\Delta \text{Km}^R \text{ΔrimP}135 \))
### TABLE 3. Bacterial strains, bacteriophages, plasmids, and oligonucleotides

| Name | Genotype or sequence | Source
|------|----------------------|--------|
| GOB162 | Hfr P4X rbfA::Km<sup>G</sup> | Bylund et al. (1998) |
| MW100 | Hfr P4X | Wilström et al. (1988) |
| STN042 | ΔrimP135 argG2424::miniTn10Cm ara Δgpt-lac<sup>S</sup> | |
| STN133 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar+ | |
| STN145 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar001 | |
| STN147 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar002 | |
| STN148 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar003 | |
| STN149 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar004 | |
| STN153 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar005 | |
| STN154 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar006 | |
| STN155 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar007 | |
| STN156 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar008 | |
| STN158 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar009 | |
| STN160 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar011 | |
| STN161 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar012 = rpsE2343 (GCC to GTC in codon 127; A127V) | |
| STN164 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar013 | |
| STN165 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar014 | |
| STN166 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar015 | |
| STN167 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar016 | |
| STN168 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar017 | |
| STN169 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar018 | |
| STN170 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar019 | |
| STN171 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar020 | |
| STN173 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar021 | |
| STN174 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar022 = rpsE2343 (GCC to GTC in codon 127; A127V) | |
| STN175 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar023 | |
| STN176 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar024 = rpsE2341 (GGT to AGT in codon 87; G87S) | |
| STN178 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar025 | |
| STN180 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar026 | |
| STN181 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar027 = rpsE2340 (GGT to GCT in codon 87; G87A) | |
| STN182 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar028 = rpsE2342 (GGT to GCT in codon 91; G91A) | |
| STN184 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar029 | |
| STN185 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar030 | |
| STN186 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar031 = rpsE2341 (GGT to AGT in codon 87; G87S) | |
| STN187 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar032 = rpsE2344 (GGC to ACC in codon 127; A127T) | |
| STN188 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 sar033 | |
| STN277 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 rpsE2343 (A127V) zbc-2421::Tn10 | |
| STN369 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 rpsE2341 (G87S) zbc-2421::Tn10 | |
| STN372 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 rpsE2344 (A127T) zbc-2421::Tn10 | |
| STN377 | Hfr P4X rpsE2343 (A127V) zbc-2421::Tn10 | |
| STN379 | Hfr P4X rpsE2340 (G87A) zbc-2421::Tn10 | |
| STN381 | Hfr P4X rpsE2342 (G91A) zbc-2421::Tn10 | |
| STN383 | Hfr P4X rpsE2341 (G87S) zbc-2421::Tn10 | |
| STN385 | Hfr P4X rpsE2344 (A127T) zbc-2421::Tn10 | |
| STN387 | Hfr P4X ΔrimP135 rpsE2343 (A127V) zbc-2421::Tn10 | |
| STN389 | Hfr P4X ΔrimP135 rpsE2340 (G87A) zbc-2421::Tn10 | |
| STN391 | Hfr P4X ΔrimP135 rpsE2342 (G91A) zbc-2421::Tn10 | |
| STN393 | Hfr P4X ΔrimP135 rpsE2341 (G87S) zbc-2421::Tn10 | |
| STN395 | Hfr P4X ΔrimP135 rpsE2344 (A127T) zbc-2421::Tn10 | |
| STN397 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 rpsE2343 (A127V) zbc-2421::Tn10 | |
| STN399 | Hfr P4X rbfA::Km<sup>G</sup> ΔrimP135 rpsE2340 (G87A) zbc-2421::Tn10 | |
| STN401 | Hfr P4X rbfA::Km<sup>G</sup> rpsE2341 (G91A) zbc-2421::Tn10 | |
| STN403 | Hfr P4X rbfA::Km<sup>G</sup> rpsE2341 (G87S) zbc-2421::Tn10 | |
| STN405 | Hfr P4X rbfA::Km<sup>G</sup> rpsE2344 (A127T) zbc-2421::Tn10 | |
| STN409 | Hfr P4X rbfA::Km<sup>G</sup> zbc-2421::Tn10 | |
| STN410 | Hfr P4X del(srfR-recA)306::Tn10 | |

Continued
was constructed by transfer of rbfA::Km\textsuperscript{4} from GOB162 (Hfr P4X rbfA::Km\textsuperscript{4}) into strain MW187 (Hfr P4X ΔrimP135) (Nord et al. 2009) by P1 transduction selecting for Km\textsuperscript{4} and screening for slow growth at 37°C. The slow-growing clones were confirmed by PCR analysis to contain rimP135ΔrimP135.

Strains having zhc2421::Tn10 linked to the rpsE mutations were constructed by P1 transduction using PW078 (Hfr P4X zhc2421::Tn10) (G Bylund, O Persson, M Lövgren, M Wikström, unpubl.) as donor and the rpsE mutants STN174, STN176, STN181, STN182, and STN187 as recipients, selecting for Tc\textsuperscript{8} and screening for fast growth. The obtained clones were used as donors in P1 transductions when zhc2421::Tn10 was transferred together with the rpsE mutations to STN133 (Hfr P4X rbfA::Km\textsuperscript{4} ΔrimP135) selecting for Tc\textsuperscript{8} and screening for faster growth than that of STN133 yielding strains STN360, STN363, STN366, STN369, and STN372. The rpsE mutations in these five strains were transferred to strains MW100 (Hfr P4X), MW187 (Hfr P4X ΔrimP135), and GOB162 (Hfr P4X rbfA::Km\textsuperscript{4}) by P1 transduction selecting for Tc\textsuperscript{8}. The obtained strains STN377, STN379, STN381, STN383, STN385, STN387, STN389, STN391, STN393, STN395, STN397, STN401, STN403, and STN405 (Table 3) were verified by DNA sequencing to contain the rpsE mutations.

The rbfA::Km\textsuperscript{4} ΔrimP135 mutations of the strains STN174, STN181, STN182, STN186, and STN187 containing rpsE mutations were replaced by the corresponding rbfA\textsuperscript{4} and rimP\textsuperscript{4} genes linked to argG2424::miniTn10Cm by P1 transduction using GOB375 (Hfr P4X argG2424::miniTn10Cm) (Bylund et al. 2001) as donor, selecting for Cm\textsuperscript{4} and screening for Km\textsuperscript{4}. The resulting strains STN419-423 and strain MW100 were made recA by P1 transduction using strain JC10289 (thr-1 leuB6 Δ(srlR-recA)306::Tn10) (Csonka and Clark 1979; Willis et al. 1981) selecting for Tc\textsuperscript{8}, yielding strains STN424-428 and STN410, respectively.

For the tests of translational missense error frequencies, the rbfA::Km\textsuperscript{4} and rpsE suppressor mutations were transferred by P1 transduction to the Xac genetic background (ara Δ[sec-proAB] gyrA (nal\textsuperscript{4}) rpoB(rif\textsuperscript{8}) argE[amber]) (Andersson et al. 1982; Dahlgren and Ryden-Aulin 2000).

**Growth conditions**

Rich medium used was LB (Bertani 1951). Cultures were grown at indicated temperatures and growth was monitored at 600 or 650 nm using a Beckman Coulter DU 730 spectrophotometer.

**Polysome profiles after sucrose gradient centrifugation**

Cell cultures were grown in LB at 30°C to A650 = 0.5. Preparation of polysome extracts and fractionation by sucrose gradient centrifugation were as described previously (Nord et al. 2009).

**Primer extension on rRNA**

Primer extension on 16S rRNA using a FAM-labeled primer and analysis of the obtained products were as described previously (Nord et al. 2009). Briefly, the amount of immature relative to mature 5'-ends of 16S rRNA was determined by running fluorescent-based primer extension on total RNA preparations with the primer FAM16S-R4 binding downstream from the 5'-end of mature 16S rRNA and analyzing the fluorescing extension products using a DNA sequencer. The fluorescence in the primer extension products corresponding to 16S and 17S rRNA was quantified using the GeneScan Analysis Software version 3.1 (Applied Biosystems).
**Translational misreading error frequency measurement**

Assays of the expression of dual-luciferase reporters cultures were performed as described (Kramer and Farabaugh 2007). Cultures of individual clones were grown to OD600 of from 0.5 to 0.8 in liquid LB medium with 100 µg/mL ampicillin at 37°C and then pelleted by centrifugation and resuspended in 200 µL 1 mg/mL lysozyme/10 mM Tris–HCl, pH 8.0/1 mM EDTA. Cells were incubated on ice for 10 min, frozen on dry ice and thawed on ice. Five microliter samples of this extract were assayed for *Photinus pyralis* (firefly) luciferase (Fluc) and *Renilla reniformis* (sea pansy) luciferase (Rluc) activities using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using the luminometer function of a Module II Microplate Multimode Reader (Promega). Three transformants for each construct were assayed, each in triplicate. The Fluc activity was used as an internal standard and standardized Fluc activity was calculated as the ratio of the Fluc to Rluc activity expressed in relative light units (RLU). The significance of differences in activity was determined by comparing values obtained in each *rpsE* suppressor mutant strain and the congenic *rpsE* strain using a two-tailed, homoscedastic Student’s *t*-test.

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