JBC RESEARCH ARTICLE

Lack of polyamines leads to cotranslational degradation of the general stress factor RpoS in *Escherichia coli*

Received for publication, April 11, 2023, and in revised form, June 2, 2023 Published, Papers in Press, June 19, 2023,
https://doi.org/10.1016/j.jbc.2023.104943

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Reviewed by members of the JBC Editorial Board. Edited by Ursula Jakob

The specialized sigma factor RpoS mediates a general stress response in *Escherichia coli* and related bacteria, activating promoters that allow cells to survive stationary phase and many stresses. RpoS synthesis and stability are regulated at multiple levels. Translation of RpoS is positively regulated by multiple small RNAs in response to stress. Degradation of RpoS, dependent upon the adaptor protein RssB, is rapid during exponential growth and ceases upon starvation or other stresses, increasing accumulation of RpoS. *E. coli* carrying mutations that block the synthesis of polyamines were previously found to have low levels of RpoS, while levels increased rapidly when polyamines were added. We have used a series of reporters to examine the basis for the lack of RpoS in polyamine-deficient cells. The polyamine requirement was independent of small RNA–mediated positive regulation of RpoS translation. Mutations in *rssB* stabilize RpoS and significantly bypassed the polyamine deficit, suggesting that lack of polyamines might lead to rapid RpoS degradation. However, rates of degradation of mature RpoS were unaffected by polyamine availability. Codon optimization in *rpoS* partially relieved the polyamine dependence, suggesting a defect in RpoS translation in the absence of polyamines. Consistent with this, a hyperproofreading allele of ribosomal protein S12, encoded by *rpsL*, showed a decrease in RpoS levels, and this decrease was also suppressed by either codon optimization or blocking RpoS degradation. We suggest that *rpoS* codon usage leads it to be particularly sensitive to slowed translation, due to either lack of polyamines or hyperproofreading, leading to cotranslational degradation. We dedicate this study to Herb Tabor and his foundational work on polyamines, including the basis for this study.

Polyamines have been implicated in multiple processes in all organisms [reviewed in a thematic minireview series (1)]. These include pleotropic effects of polyamines on protein synthesis, growth, and survival of *Escherichia coli* cells in response to different stress conditions (2–7). These studies were done in a variety of strains and in different conditions, but many of them found that levels of the general stress sigma factor of *E. coli*, RpoS (σ^{38}), were among the proteins that appeared to be depleted in the absence of polyamines. Studies in the Tabor lab to understand the targets for polyamine function were carried out using HT873, a strain deleted for the genes for seven different enzymes in the multiple pathways that contribute to polyamine synthesis, referred to here as Δ7 (8) (Fig. S1*A*). Microarray analysis carried out with the Δ7 strain grown with or without exogenously added polyamines suggested that a major defect in the absence of polyamines was low levels of RpoS. When polyamines were added, RpoS rapidly increased, and RpoS-dependent genes such as those encoding glutamate dehydrogenase were expressed (8, 9).

RpoS is a sigma factor, the promoter recognition component of RNA polymerase. It mediates the general stress response in *E. coli*, leading to resistance to multiple stress treatments, including resistance to both low pH and high pH, resistance to oxidative stress, and to high osmolarity [reviewed in (10)]. RpoS levels also increase after various stresses, including upon starvation for carbon, magnesium, and potassium, in response to changing pH, oxidative stress, and high osmolarity. Particularly worth noting, induction by one of these stresses provides cross-resistance to others (10). In rich media, RpoS levels increase when cells enter stationary phase, and the protein has thus also been called the stationary phase sigma factor. The unexpected observation that the stress of polyamine starvation apparently decreases, rather than increasing, RpoS levels forms the basis for this study.

Some but not all of the signal transduction mechanisms for increasing RpoS in response to stress are well understood [reviewed in (10)]. Two major pathways for posttranscriptional regulation of RpoS have been studied. First, translation of RpoS is stimulated by three different sRNAs, each of which pairs with and restructures the long 5′ UTR of *rpoS* to allow ribosome access. Each sRNA is made in response to a different stress and therefore contributes to RpoS accumulation under different conditions. All three of these sRNAs require the RNA chaperone Hfq for *in vivo* stability and function. Second, RpoS is rapidly degraded in exponentially growing cells; degradation requires the ClpXP ATP-dependent protease and an adaptor protein, RssB, that binds RpoS and delivers it to ClpXP. RssB recognizes a region of RpoS around and including residue K173. The activity of RssB, a member of the response

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regulator family of proteins, can be modulated by phosphorylation, although the sources and *in vivo* importance of phosphorylation (by one or more histidine kinases and/or small molecules such as acetyl-phosphate) are not fully understood. Under starvation or stress conditions, RpoS becomes stable. Stress-induced proteins called antiadaptors bind the RssB adaptor, blocking degradation of RpoS. The three characterized antiadaptors are structurally unrelated, are induced under different conditions, and bind RssB differently (11, 12). Additional levels of regulation of translation initiation and translation within the open reading frame (ORF) have also been reported but are less well understood (13). The increase in RpoS in the Δ7 strain after polyamine supplementation was blocked by chloramphenicol treatment but not by rifampicin treatment (8), suggesting that polyamine addition does not act by increasing transcription of RpoS.

Here, we have compared expression of RpoS or an RpoS translational reporter in wildtype (WT) cells and cells devoid of polyamine, in situations in which different known mechanisms of RpoS regulation are perturbed. The results suggest that loss of polyamine or high levels of proofreading lead to cotranslational hyperdegradation of RpoS and that codon usage within *rpoS* make this gene particularly sensitive to perturbed translation.

Results

Fusions reflect effects on RpoS levels in the absence of polyamine

Both the role of sRNAs in translation of RpoS and the regulated proteolysis of RpoS can be measured using an RpoS-LacZ translational fusion in which the first 262 amino acids of RpoS are fused to LacZ. The fusion is expressed from a constitutive Cp17 promoter (14), initiating at the position of the upstream P_{rpoS} promoter and thus contains a 567 nt 5′ UTR (13), (Fig. 1*A*). This fusion, integrated into the bacterial chromosome at the *lacZ* site, was linked to a zeo^R cassette encoding resistance to zeocin (zeo^R) and transferred by P1 transduction into BW25113 and the polyamine-deficient derivative of BW25113 in which seven genes for polyamine synthesis are deleted, HT873X1, to create NM25000 and HT946 respectively. On Lactose MacConkey agar plates, the parental NM25000 strain was Lac^+ (red), while HT946 was Lac^- (white, Fig. 1*B*), consistent with low levels of the RpoS-

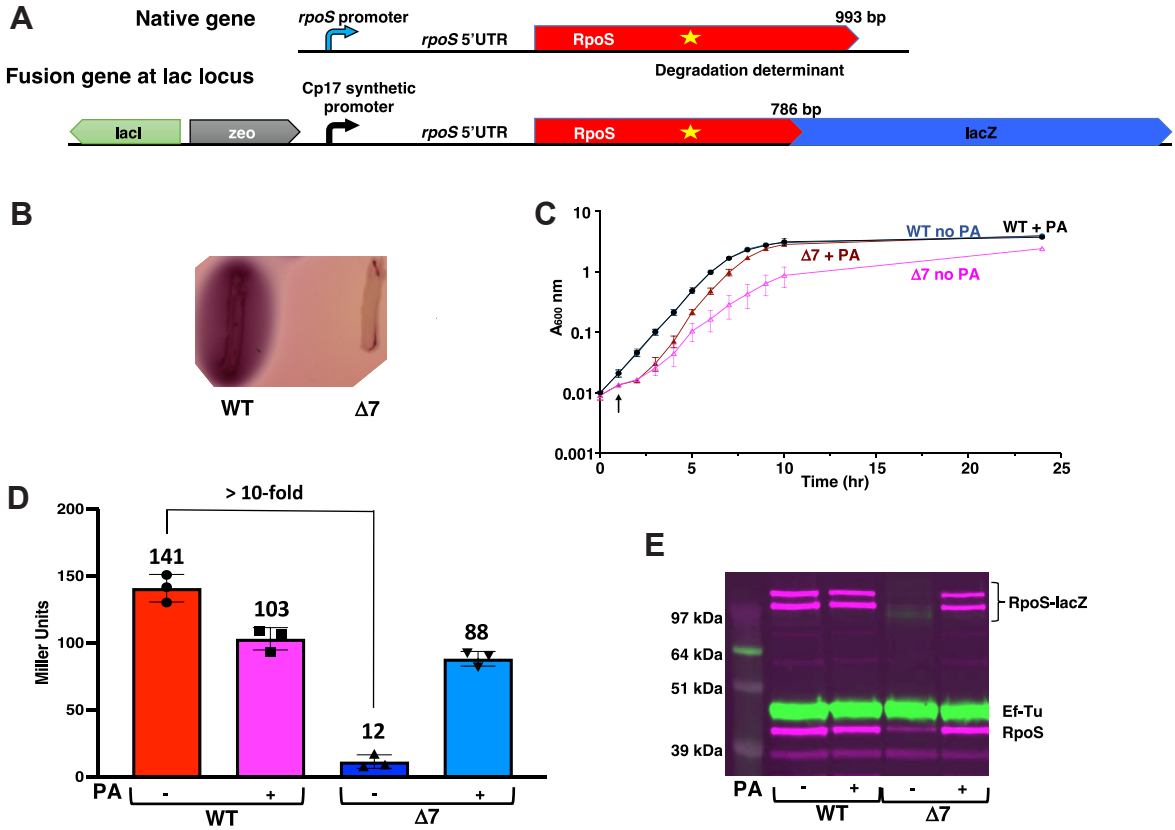

Figure 1. RpoS expression is low in the absence of polyamines. *A*, schematic of endogenous *rpoS* gene with native promoter found in the *middle* of the upstream *nlpD* gene, and fusion construct, inserted at the *lac* locus and expressed from the Cp17 synthetic promoter (14). Both copies carry the full 5′ UTR, as well as enough of the *rpoS* coding region to include the degradation determinant at the region of K173 (*yellow star*, recognized by adaptor RssB). *B*, lactose MacConkey plate of WT strain (NM25000) and Δ7 derivative (HT946), each carrying the RpoS-LacZ fusion, incubated overnight at 30 °C. *C*, growth of WT (NM25000) and Δ7 (HT946) strains in Vogel-Bonner (VB) polyamine-free medium at 37 °C with aeration. 100 μM each of spermidine and putrescine were added at the time indicated by the *arrow*, early in the growth. Experiment was done with two biological replicates. *D*, assay of RpoS-LacZ strains in C, grown in VB medium at 37 °C to A_{600} 0.3, with polyamines added for 20′ in cultures indicated as + PA. Beta-galactosidase was measured in Miller units (38). Assays are average of three independent experiments; error bars indicate standard deviation. *E*, Western blot of cultures in *D*, grown to A_{600} 0.3, and probed for RpoS; the samples with PA added were sampled after 20′ growth with polyamines. Both the fusion (RpoS-LacZ) and the native protein can be detected. A likely degradation product of the fusion was also detected in WT strains. Ef-Tu was used as a loading control.

LacZ fusion in the absence of polyamines. This result also shows that the effect of loss of polyamines is independent of the native promoter, not present in this fusion, confirming the previous finding that polyamines do not act at the level of *rpoS* transcription initiation (8).

Polyamine-deficient cells showed a clear growth defect when grown in polyamine-free minimal liquid media; this growth defect was overcome by the addition of polyamines (Fig. 1*C*). Measurement of the RpoS-LacZ fusion protein by beta-galactosidase activity showed a 12-fold decrease in the Δ7 strain. Polyamine addition for 20 min significantly increased the level of the fusion in the Δ7 strain but not in the WT strain (Fig. 1*D*). Both the fusion and the native RpoS, measured with anti-RpoS antibody from cells at A_{600} 0.3, were easily detectable in the WT strain but not in the Δ7 strain. After 20 min of polyamine treatment, both RpoS and RpoS-lacZ accumulated to a level visible in a Western blot, consistent with the increase in activity of the RpoS-LacZ fusion (Fig. 1, *D* and *E*). The significant amount of RpoS during exponential growth in the WT strain suggests that in this minimal medium, cells are somewhat stressed and thus accumulating RpoS. The general agreement in behavior of RpoS and RpoS-LacZ, the latter expressed from a constitutive synthetic promoter (Cp17), provides evidence that further analysis of RpoS-LacZ fusions can be used to dissect the polyamine dependence.

The genome of the Δ7 strain HT946 was sequenced and compared to the WT version, NM25000. This confirmed the expected deletions but revealed a 20 kb deletion as well as some rearrangements associated with the construction of the Δ7 derivative (Fig. S1*B*; discussed in legend to Fig. S1). Therefore, these strains cannot be considered strictly isogenic. Nonetheless, the full growth recovery of HT946 with long-term polyamine addition (Fig. 1*C*) and the significant recovery of RpoS with even short-term polyamine treatment (Fig. 1, *D* and *E*) suggest that HT946 does not contain changes that dramatically affect growth or RpoS expression, making a comparison with NM25000 appropriate. Finally, an examination of RpoS levels by Western blot in the precursors to HT946 carrying different numbers of polyamine pathway deletions demonstrated that deleting just *speA* and *speC*, unlikely to contain any genome rearrangements, was sufficient to significantly lower RpoS levels (Fig. S1, *C* and *D*).

Polyamine dependence of RpoS expression does not involve sRNAs and Hfq

The fusion used above measures both effects of sRNAs on translation initiation and degradation of RpoS. As a first step in analyzing the basis for low RpoS in the absence of polyamines, we examined the role of the 5′ UTR of *rpoS*, the site of action of sRNAs that activate RpoS translation. Three sRNAs, DsrA, ArcZ, and RprA, are known to positively regulate RpoS translation, by interacting with a region of an inhibitory hairpin in the 5′ UTR [reviewed in (15)]. A derivative of the RpoS-LacZ fusion was created in which the hairpin and most of the 5′ UTR were deleted, except for 24 nt upstream of the AUG (Fig. 2*A*). This fusion should be blind to the roles of the sRNAs and to the RNA chaperone Hfq that participates in sRNA function and, unlike deleting the sRNAs or Hfq, still expresses RpoS-LacZ at a reasonable level. The RpoS-LacZ leaderless fusion was expressed at a 10-fold lower level in the absence of polyamines and increased significantly when polyamines were added (Fig. 2, *B* and *C*), demonstrating that

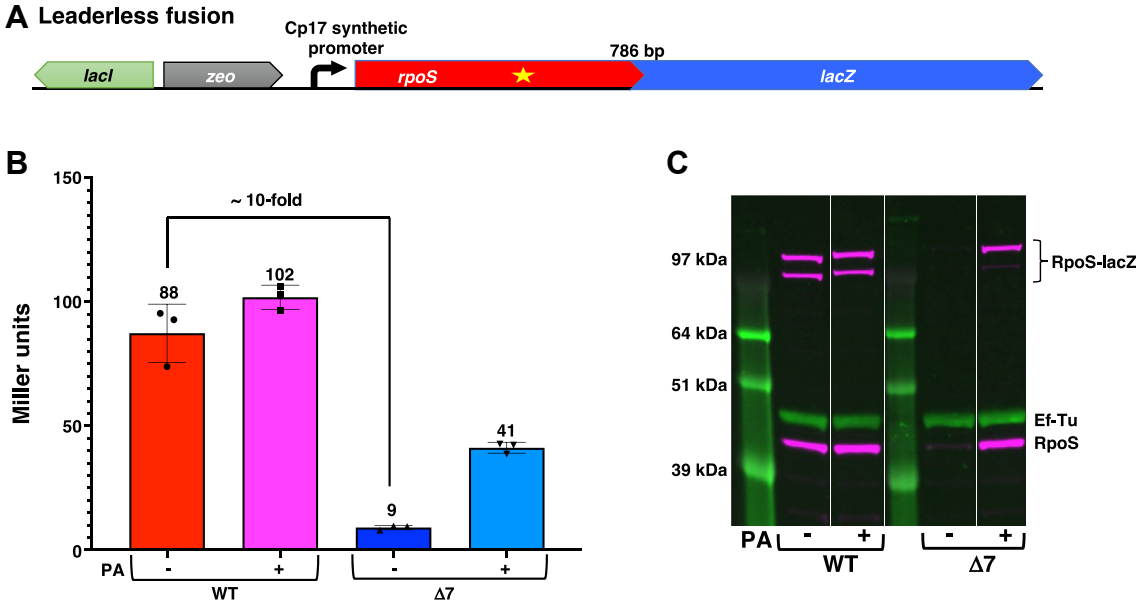

Figure 2. The *rpoS* 5′ UTR is not involved in the requirement for polyamine. *A*, the leaderless fusion contains 24 nt upstream of AUG; promoter and region downstream of AUG are the same as for fusion in Figure 1. *B*, assay of WT strain (NM25088) and Δ7 strain (NM25089), each carrying the leaderless fusion, grown as in Figure 1*D*. Assays are average of three independent experiments; error bars indicate standard deviation. *C*, Western blot of strains in Figure 2*B*, as in Figure 1*E*. *Vertical white spaces* indicate deletion from original gel of lanes with essentially identical results from two other biological replicates.

the upstream hairpin and sRNA-dependent translation initiation are not needed for the polyamine dependence or the effect of added polyamines.

The RpoS ORF and its coding region are required for polyamine dependence

Given that the 5′ UTR of RpoS was not needed for polyamine dependence, we asked whether translation initiation or the translation or stability of the RpoS ORF was the polyamine sensitive step. A fusion was created which retained the 5′ UTR but removed all but the first eight codons of the ORF (Fig. 3*A*); this fusion showed only a two-fold decrease in the Δ7 strain background and no significant improvement on addition of polyamines (Fig. 3*B*). As expected, the RpoS-LacZ fusion protein could not be detected in this strain by anti-RpoS antibody, but the native RpoS protein responded as expected to polyamines (Fig. 3*C*). Therefore, the RpoS ORF and not simply the signals in the region of the initiating AUG are required for polyamine dependence.

Degradation is the primary reason for loss of RpoS in absence of polyamines

One level of regulation specific to the RpoS ORF is proteolysis. RpoS is highly unstable when cells are grown under nonstress conditions; it becomes stable when cells are stressed or starved in various ways (reviewed in (15)). As described above, RpoS degradation requires that the RssB adaptor protein delivers it to the ClpXP ATP-dependent protease. RpoS can also be stabilized by interaction with core polymerase (16) and other proteins which help RpoS bind core, such as Crl, or otherwise change the availability of core can affect RpoS stability (17). RssB is a protein of the response regulator family and is thus subject to phosphorylation on a conserved aspartate in the N-terminal domain (D58); *in vitro*, phosphorylation significantly increases RpoS degradation (11, 16). However, phosphorylation is not the major regulator of RpoS turnover. Small protein antiadaptors, made under stress conditions, can interact with RssB, interfering with RpoS degradation by sequestering the adaptor protein [reviewed in (10, 18)].

Fusions of RpoS to LacZ that include the RpoS region that is the site of recognition by RssB (yellow star in schematics in Figs. 1 and 2) are degraded in the same manner as RpoS itself, dependent upon RssB and ClpXP. The role of degradation in the polyamine dependence was tested by deleting *rssB*, encoding the RssB adaptor, in the same strains assayed in Figure 1, blocking degradation. As expected, this increased the levels of the RpoS-LacZ fusion and the native RpoS protein (Fig. 4, *A* and *B*, compare lane one to lane 5). However, while the RpoS-LacZ fusion protein in the WT (polyamine synthesizing) strain increased about 2.5-fold, the increase in the Δ7 strain was significantly more (30-fold), leading to a loss of most of the polyamine dependence (from 40-fold to 3-fold) (Fig. 4*A*, compare lane 3 to lane 7). A similar significant increase was seen for the native RpoS protein (Fig. 4*B*).

While stabilization of RpoS with an *rssB* mutant partially suppressed the loss of RpoS, it was not sufficient to improve growth of the polyamine-deficient strain in the absence of polyamines (Fig. S2*B*). Deletion of *rpoS* also did not affect growth rate (Fig. S2*A*). Based on these results, the growth defect in the absence of polyamines is likely independent of levels of RpoS.

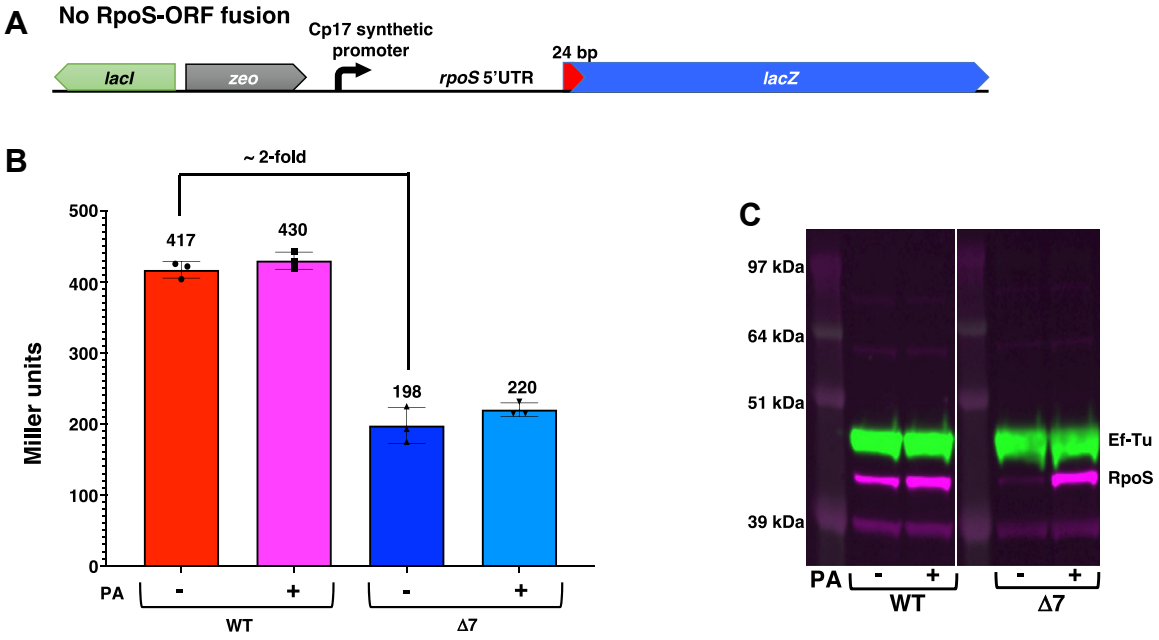

Figure 3. Polyamine dependence requires the *rpoS* ORF. *A*, schematic of fusion in which only the first eight codons of RpoS were fused to LacZ (ORF-less fusion), constructed as described in Table S1 and Experimental procedures. *B*, assay of WT strain (NM25092) and Δ7 strain (NM25083), each carrying the fusion shown in *panel A*, grown as in Figure 1*D*. Assays are average of three independent experiments; error bars indicate standard deviation. *C*, Western blot of strains in Fig 3*B*, as in Figure 1*E*. *Vertical white space* indicates deletion from original gel of lanes with essentially identical results from two other biological replicates.

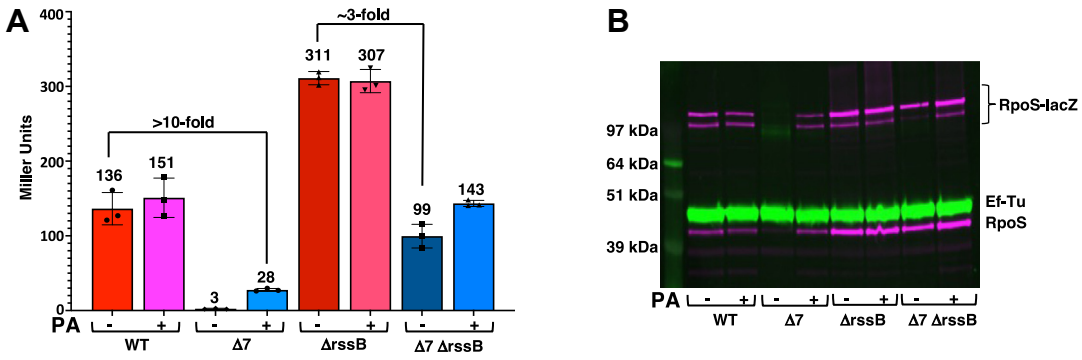

Figure 4. Polyamine dependence is suppressed in a strain unable to degrade RpoS. *A*, strains carry the fusion shown in Figure 1*A* and were grown as in Figure 1 and assayed for beta-galactosidase. Strains used: WT (NM25000); Δ7 (HT946); Δ*rssB*(NM25007); Δ7 Δ*rssB* (NM25008). Assays are average of three independent experiments; *error bars* indicate standard deviation. *B*, the same four strains were sampled for Western blotting with antibody to RpoS. Note that the first four samples in this figure (*rssB*$^+$ strains) are the same as in Figure 1*E*; the *rssB*$^+$ and Δ*rssB* were grown and analyzed together, as seen in this figure.

These results strongly suggest that rapid degradation of RpoS is the primary reason for loss of RpoS in the absence of polyamines, and RpoS accumulation after addition of polyamines reflects stabilization. In situations where RpoS or the RpoS-LacZ fusion is stable, loss of polyamines had only a modest effect, and addition of polyamines had essentially no effect.

To directly investigate the degradation of RpoS in the absence of polyamines, we carried out chase experiments in which cells were treated with chloramphenicol to stop new protein synthesis, and RpoS levels were followed over time by Western Blot (Fig. 5). Because the levels of RpoS were so low in the Δ7 strain, we modestly overexpressed RpoS from a pBAD plasmid to allow us to detect it so we could measure its degradation. Both RpoS and RpoS-LacZ were degraded in the WT strain. Unexpectedly, degradation of RpoS was somewhat slower, rather than faster, in the Δ7 strain; RpoS-LacZ could not be detected.

Translation as the basis for hyperdegradation of RpoS in the absence of polyamines

The experiments in Figure 5 measured the disappearance of the full-length RpoS and RpoS-LacZ protein bands during a chase and demonstrated that once translation was complete, the polyamine status did not affect the rate of protein degradation. One explanation for the lack of a polyamine effect in this experiment could be that polyamine only affects the stability of RpoS during translation. If, in the absence of polyamines, RpoS is being degraded cotranslationally, the amounts of full-length protein would be decreased, while the stability of full-length protein would not change, consistent with the observations in Figures 4 and 5. Because this rapid RpoS degradation is dependent upon RssB (Fig. 4), the translating polypeptide must be recognized by RssB and brought to the ClpXP protease only once the RssB binding epitope (star in Fig. 1*A*) is translated.

This model implies that something about translation of RpoS is different in the absence of polyamines, allowing degradation to happen much more readily than in the presence of polyamines. This could be a general defect in translation, but because RpoS is specifically depleted (8, 9), we tested whether the codon usage in *rpoS* contributed to the polyamine dependence. A codon-optimized RpoS-LacZ fusion, equivalent to the fusion in Figure 1*A*, was constructed, introduced into the same set of strains (WT and Δ7), and assayed (Fig. 6). As for the fusion blocked for degradation, the codon-optimized fusion lost much of the polyamine dependence (Fig. 6, *A* and *B*). This result suggested that translation within the *rpoS* ORF was slower or otherwise perturbed in the absence of

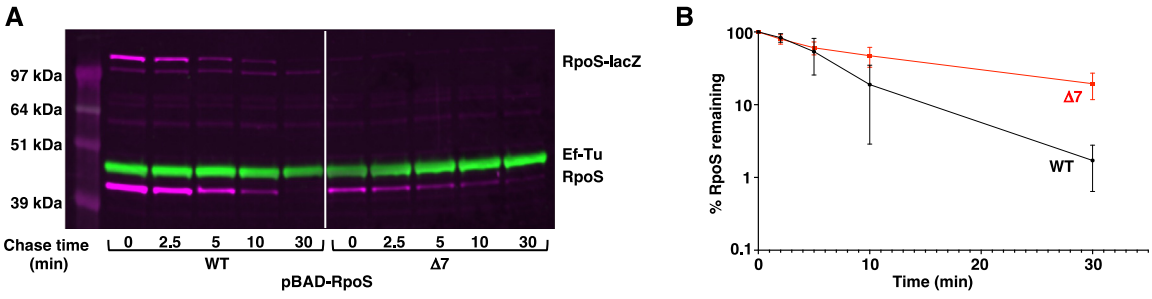

Figure 5. Loss of Polyamines does not increase degradation of mature RpoS. *A*, strains carrying a pBAD-RpoS plasmid (45), in which RpoS is expressed under control of the arabinose-inducible promoter, were grown to an A_{600} of 0.25 to 0.3, RpoS synthesis was induced by adding 0.02% arabinose for 15 min, and chloramphenicol added to stop further protein synthesis. Samples were taken at the indicated times and prepared for Western blotting (see Experimental procedures). Strains used: NM25096 (WT Δ*rpoS*); NM25084 (Δ7 Δ*rpoS*). *B*, RpoS remaining after antibiotic chase, derived from quantitation of three independent chase experiments, as in *panel A*.

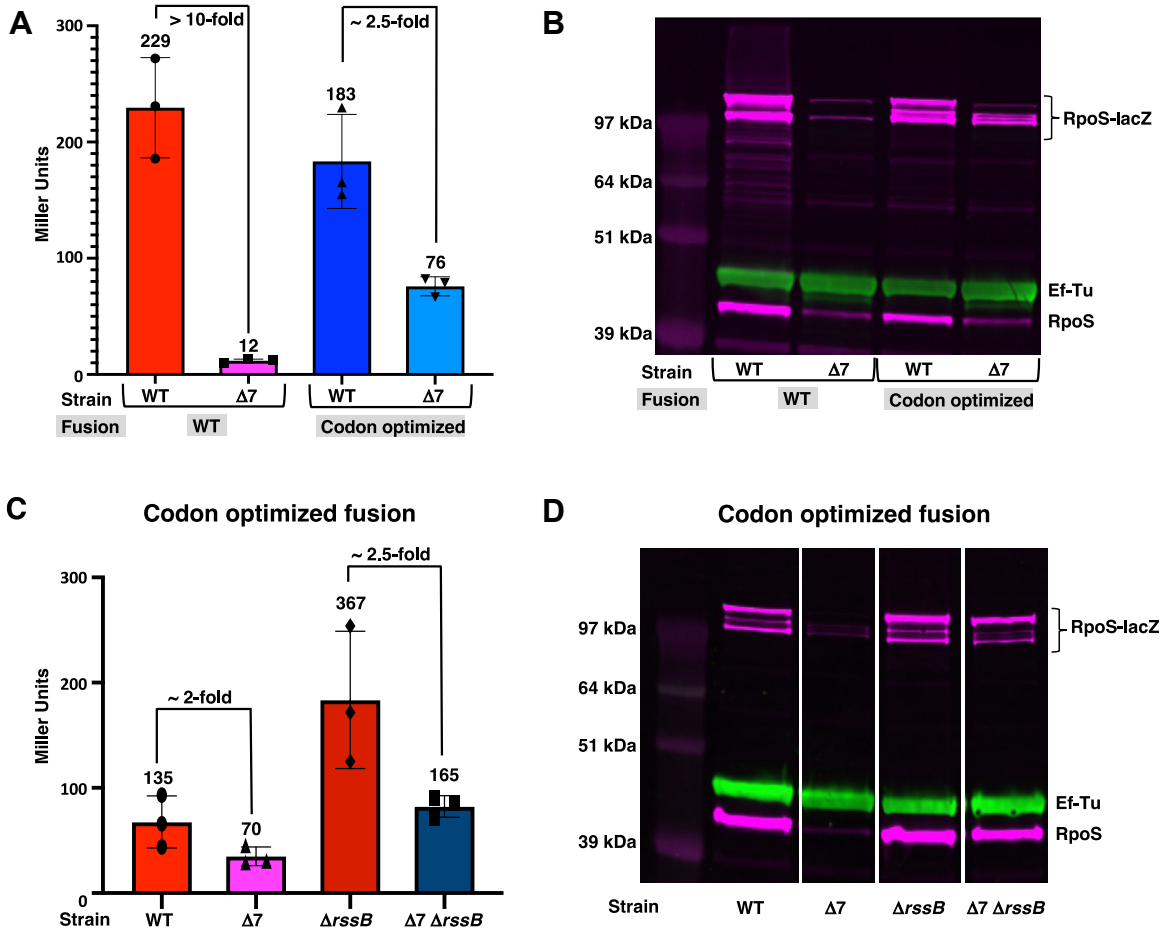

Figure 6. Codon optimization of RpoS decreases polyamine dependence. *A*, strains carrying either the native RpoS-Lac fusion (NM25000 or HT946) or an isogenic set in which the RpoS portion of the fusion was codon-optimized (WT: NM25105; Δ7: NM25106) were grown and assayed as in Figure 1. Assays are average of three independent experiments; *error bars* indicate standard deviation. *B*, samples of the strains grown in *A* were taken for Western blots. Note that the native RpoS is not codon-optimized and thus serves as a control for the codon-optimized RpoS-LacZ fusion protein. *C*, the codon-optimized strains tested in *A* (NM25105 and NM25106) were assayed in parallel to *rssB*::tet derivatives of these strains (WT: NM25111 and Δ7: NM25112). Assays are average of three independent experiments; *error bars* indicate standard deviation. *D*, samples of the strains grown in *C* were taken for Western blots. Note that the native RpoS is not codon-optimized and thus serves as a control for the codon-optimized RpoS-LacZ fusion protein. Lanes showing samples after addition of polyamines were omitted from the gel image shown here, with *vertical white spaces* shown for the missing lanes.

polyamines, affording RssB an opportunity to interact with the translating protein and deliver it to ClpXP. To confirm the role of RssB, the codon-optimized fusion was compared to the WT fusion in an *rssB* mutant (Fig. 6, *C* and *D*). In the absence of RssB, we expect an increase in the RpoS-LacZ fusion, as seen for a WT strain in Figure 4 (WT fusion increased from 136 units to 311 units, for a 2.3-fold increase), presumably reflecting the increase when the full-length protein is not being degraded. The codon-optimized fusion acted very similarly in the WT strain, increasing from 135 units to 367 units (2.7-fold) in the absence of *rssB* (Fig. 6*C*). However, unlike the polyamine-deficient strain in Figure 4, which increased dramatically in the absence of *rssB* (from 3 units to 99 units), the codon-optimized fusion, already high in the polyamine-deficient strain, increased 2.3-fold, from 70 to 165 units. Therefore, codon optimization blocked the co-translational degradation in the absence of polyamines but did not affect the degradation of the full-length protein.

We sought other ways to perturb *rpoS* translation to see if this would also lead to co-translational degradation, dependent on codon usage. Ribosomal mutants that either relax or increase proofreading have been described. Ling *et al.* have previously demonstrated that error-prone ribosomes, due to an *rpsD*I199N allele, had higher levels of RpoS, while a cell with hyperproofreading ribosomes, due to an *rpsL*K42N allele, had decreased RpoS (19). We tested the effect of two *rpsL*K42 alleles on expression of our RpoS-LacZ fusion protein (Fig. 7*A*). While the decrease in RpoS levels was relatively modest (2- to 3-fold, compared to the 10-fold decrease in the absence of polyamines), the effect was almost completely suppressed by an *rssB* mutant, stabilizing the fusion (Fig. 7*A*). Furthermore, the effect of the *rpsL* allele was significantly less in cells carrying the codon-optimized fusion (Fig. 7*B*). Both characteristics suggest that the *rpsLK42* mutations, like the lack of polyamines, leads to degradation of RpoS, and this is dependent on the nature of the codons within the ORF. We interpret this as most consistent with the *rpsL* hyperproofreading alleles also leading to an increase in cotranslational degradation of RpoS.

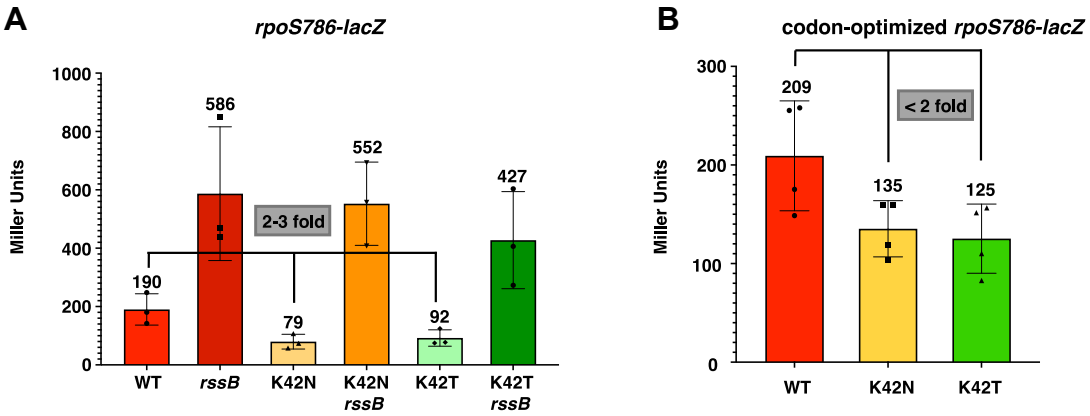

Figure 7. Hyperproofreading ribosomes also lead to cotranslational degradation of RpoS. *A*, derivatives of the *rpoS*786-*lacZ* fusion (see Fig. 1*A*) containing two different *rpsLK42* alleles, with and without a mutation in *rssB*, were grown and assayed as in Figure 1*B*. Strains used: WT: NM25000; *rssB*::tet: NM25007, *rpsIK42N*: NM25125; *rpsLK42N rssB*::tet: NM25127; *rpsIK42T*: NM25126; *rpsIK42T rssB*::tet: NM 25128. Assays are average of three independent experiments; *error bars* indicate standard deviation. *B*, derivatives of the codon-optimized *rpoS*786-*lacZ* strain NM25105 carrying the *rpsLK42N* (NM25129) or *rpsLK42T* (NM25130) alleles were grown and assayed as in Figure 1*B*.

The loss of RpoS in the absence of polyamines and in the *rpsL* mutant strains is at least partially specific—the RpoD vegetative sigma factor does not show a decrease in the absence of polyamines or in these mutants (Fig. S3). However, we did note that the RpoD antibody detected a lower molecular weight band, likely a truncated form of RpoD, that appeared in both the Δ7 strain and, at a lower level, in the K42N strain. The source of this band is not currently known but would suggest the possibility of a translation stop or strong pause at this position in the *rpoD* coding region.

Chimeras define regions within rpoS *important for polyamine dependence*

Based on these results, additional experiments were done to begin to define the relevant part(s) of *rpoS* that lead to *rpsL* sensitivity and polyamine dependence. In the degradable fusion used in most of the experiments in this paper, 786 nt of the 993 nt *rpoS* coding region, encoding 262 aa, were fused to *lacZ*. Codon optimization was only done for the *rpoS* portion, suggesting that translation of *lacZ* itself is not significantly affected by the *rpsL* mutation or the loss of polyamines. K173 in RpoS is needed for recognition by RssB and thus for degradation; shorter fusions are not subject to RssB-dependent degradation (20). An RpoS-LacZ fusion, containing 567 nt, encoding 189 aa of RpoS, was tested for its dependence on polyamines (Fig. S4). It was somewhat less sensitive to loss of polyamines (9-fold decrease compared to a 30-fold decrease in the longer RpoS786 fusion), suggesting that the region between aa 189 and aa 262 contributes somewhat to the likelihood of RpoS being degraded cotranslationally in the absence of polyamines. However, the shorter fusion is still quite sensitive to loss of polyamines, suggesting critical regions lie in the remaining portions of *rpoS*, most of which are N terminal to the K173 adapter recognition site.

We created and tested other chimeric versions of the RpoS786 fusion, changing portions of the codons to see if they were critical. We had previously found that the *rpoS* coding region is enriched for leu codons subject to MiaA-dependent tRNA decoding and that significantly less RpoS was made in a *miaA* mutant (21). In that case, changing leu codon usage was sufficient to bypass most of the MiaA requirement (22). To test whether these *miaA*-sensitive codons were playing a role in polyamine dependence, all leu and ser codons in the *rpoS* portion of the fusion were changed from *miaA*-sensitive codons (UXX) to *miaA*-insensitive codons. However, this chimera was still as polyamine dependent as the original RpoS-LacZ fusion (Fig. S5).

Two other chimeric RpoS-LacZ fusions were created. In one (WT/C-O, in Fig. 8), the region of RpoS before the RssB recognition region (from aa 9 to aa 173) was left with the native codons, and the sequence between aa 173 and aa 262 was codon optimized. In the second (C-O/WT in Fig. 8), the portion of the codon-optimized sequence from aa 9 to aa 173 (RssB recognition region) was used in place of the usual *rpoS* sequence, while the region beyond K173 was left with the native codons. In each of these chimeras, the first eight codons of RpoS were unchanged from the WT gene. The polyamine dependence of these new fusions was compared to the WT and a fusion in which all but the first eight amino acids were codon-optimized (C-O, Fig. 8). In both Figure 6, in which all of *rpoS* was codon optimized, and Figure 8, codon optimization reduced the expression in the WT host and significantly increased it in the Δ7 strain, reducing the polyamine dependence from 15-fold in the WT fusion to 1.9-fold in the C-O strain. Strikingly, while the WT/C-O optimized fusion still showed an 8.5-fold dependence on polyamine synthesis, the dependence of the C-O/WT chimera was reduced to 3.8-fold (Fig. 8). These results suggest that while sequences both before and after amino acid 173 contribute to polyamine dependence, the most significant contribution is from the codons in the first part of the gene. This suggests that translation and likely folding of the region of RpoS before aa173 normally protects the emerging polypeptide from RssB binding and degradation.

Discussion

Many stress treatments, including starvation for magnesium, potassium or carbon, lead to induction of the RpoS

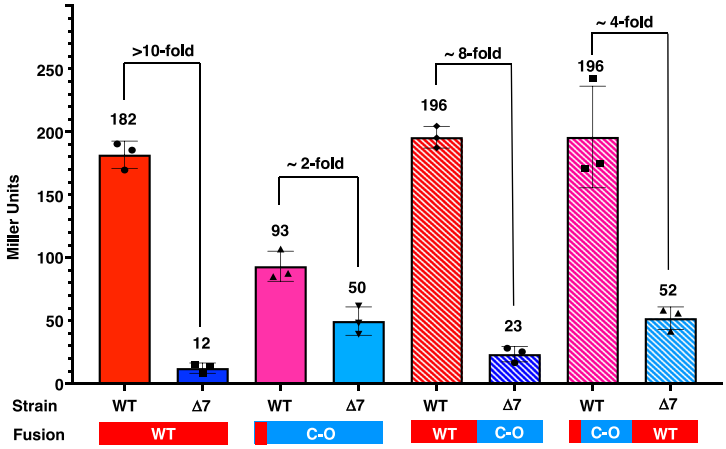

Figure 8. The first portion of *rpoS* is important for polyamine dependence. Chimeric versions of the RpoS-LacZ fusion were created carrying silent codon changes (codon optimization) in the regions indicated, in the context of the fusion shown in Figure 1*A*. The bars under the graph marked fusion show the portions of each chimera corresponding to the native WT *rpoS* sequence in *red*, with the codon-optimized (C-O) portion in *blue*. The first eight amino acids of native *rpoS* were retained in all codon-optimized constructs (short *red* bar at front of C-O construct and C-O/WT construct). Note that this was not the case for the codon-optimized fusion used in Figures 6 and 7. The transition point between *red* and *blue* in WT/C-O was at K173, as it was for the transition between *blue* and *red* in C-O/WT. Cells were grown and assayed as in Figure 1. Strains used, in order shown: "WT" codon set: WT: NM25000; Δ7: HT946; C-O set: WT: NM25168: Δ7: NM25172; WT/C-O set (aa 174-33- codon optimized) WT: NM25141; Δ7: NM25144; C-O/WT set (codons 9-173 optimized): WT,: NM24142; Δ7: NM25145. Assays are average of three independent experiments; *error bars* indicate standard deviation.

general stress response *via* increased synthesis and decreased degradation of RpoS (reviewed in (10)). Depletion of polyamines unexpectedly had the opposite effect, interfering with the accumulation of RpoS, even though cells are clearly growing slowly (Fig. S2*A*) (8). Here, we propose that cotranslational degradation of RpoS is responsible for the low RpoS levels. Degradation requires the RssB adaptor protein, the ATP-dependent ClpXP protease, and the RpoS region recognized by RssB; blocking the proteolysis pathway significantly suppressed the polyamine dependence of RpoS accumulation (Fig. 4). Because we do not see more rapid degradation of full-length RpoS in the polyamine deficient strain (Fig. 5), we propose that RpoS is not present because it is cotranslationally degraded (Fig. 9). Degradation is promoted by two interacting factors: (1) codon usage within the *rpoS* gene, particularly within the first portion of *rpoS*, leading to (2) hypersensitivity to defects or slowing in translation, as found in the absence of polyamines or in the *rpsL* hyper-proofreading mutants. These changes in translation in turn lead to changes in the folding or interactions of the emerging

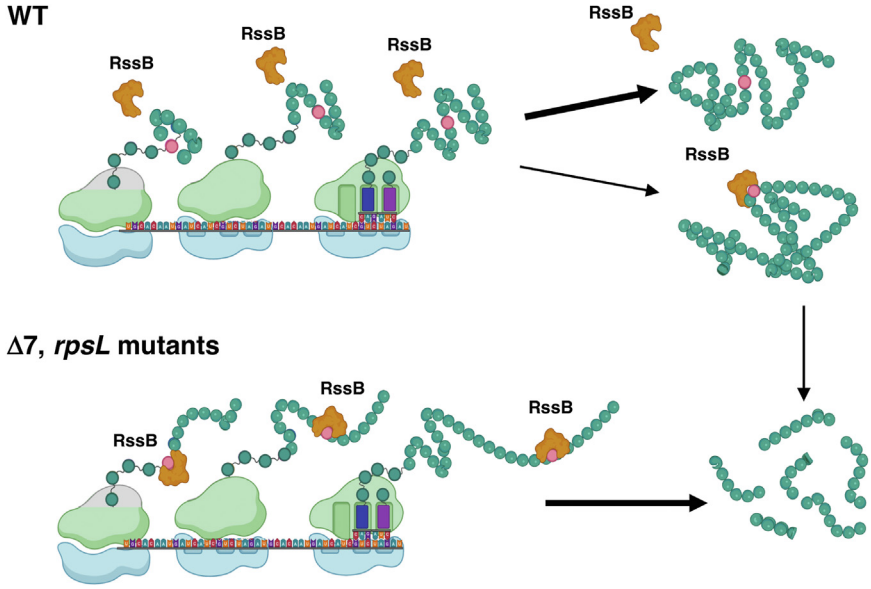

Figure 9. Model for cotranslational RpoS degradation when translation is perturbed. In WT cells, we suggest that folding of the emerging RpoS chain (*green balls*) leads to a conformation in which the epitope within the translating RpoS recognized by RssB (*pink ball* in polypeptide chain) is unavailable for RssB binding. As a result, the majority of RpoS is fully translated. Under nonstress conditions, RssB can bind to this fully translated version, leading to ClpXP-dependent degradation. In the polyamine-deficient strain, or in the *rpsL* mutants studied here, translation is perturbed, leading to a change in the folding of the protein and more accessibility of the RssB-binding epitope. As a result, significant amounts of RpoS are delivered to ClpXP and degraded before translation is completed.

RpoS polypeptide chain, leading to it being more accessible to binding by RssB and delivery to the ClpXP-dependent protease (Fig. 9).

Polyamines have been shown to have critical roles in translation in both prokaryotes and eukaryotes, with extensive evidence for effects on translational elongation [reviewed in (7, 23, 24)]. Studies in the Tabor lab demonstrated synthetic requirements for polyamines in *E. coli* in some *rpsL* alleles (25) and in cells mutant for tRNA modifying genes *mnmE/G* (26), as well as a requirement for polyamines for efficient amber suppression (27), all suggesting that polyamines play roles that are somewhat redundant with other factors that promote translation. The Tabor lab studied the effect of added polyamines on gene expression in polyamine-deficient *E. coli*; these experiments pointed to RpoS-dependent genes as most responsive to added polyamines (8). This study also demonstrated that cadaverine, putrescine, or spermidine were each sufficient to increase RpoS levels, with spermidine the most effective (8).

Here we find that translation of RpoS, dependent on the specific codon usage within RpoS, is changed in such a way in the absence of polyamines or in *rpsL* mutants that RpoS is rapidly degraded cotranslationally. The rapid effect of adding polyamines (Fig. 1) is consistent with this addition immediately feeding back to the translating ribosome, rather than, for instance, affecting the maturation of new ribosomes. While that may occur as well and may explain why addition of polyamines does not fully suppress the defect in the Δ7 strain, we suggest that is not the primary effect in our assays. A previous study had also implicated polyamines in affecting degradation of RpoS, based on the behavior of translational fusions, although they had not investigated the possibility of co-translational degradation (6).

In previous work, we and others have found that mutations that perturb translation, including mutations in *ssrA*, encoding tmRNA, with a role in quality control, or in genes involved in tRNA modification, including *miaA* and *trmL*, reduce translation of full-length RpoS. These results suggested that there may be pauses within *rpoS*, leading to a required release role for tmRNA and a need for tRNA modifications (encoded by *miaA*) for optimal translation of this gene (21, 22, 28, 29). The *miaA* effect has been found to be dependent, at least in part, on the leucine codon usage within *rpoS* (22). However, changing codons for leu and ser codons that require *miaA* to those that are independent of *miaA* did not relieve the dependence on polyamines of the RpoS-LacZ reporter (Fig. S4). Therefore, some other characteristic of this region must be responsible.

For RpoS to be degraded by RssB and ClpXP, as seen in the polyamine-deficient host, and for this to occur co-translationally, RssB must be able to access the region at and around K173 in the emerging polypeptide chain. However, this does not occur in the WT host or when polyamines are supplied. In our model, in WT cells polypeptide folding or interaction of the growing polypeptide chain with other proteins protects it from RssB binding. Absence of polyamines or hyperproofreading ribosomes change translation rate or pausing, interfering with this folding and making the growing chain susceptible to RssB binding and cotranslational degradation (Fig. 9). The region upstream of K173 must be important for this protective folding, since codon optimizing this is sufficient to significantly reduce polyamine dependence (Fig. 8, C-O/WT construct). It would make sense physiologically for the growing polypeptide chain to be protected—it would certainly be wasteful under most conditions for RpoS to be degraded even before it is fully synthesized. One possible binding partner could be core RNA polymerase. Core binding to RpoS protects it from RssB (16). Comparison to the well-studied vegetative RpoD sigma factor as well as recent structures of RpoS and core polymerase identify regions 2.1 and 2.2 of sigma as able to bind to the β′ subunit of RNA polymerase (30–32); this binding region is upstream of K173.

While our results suggest specific characteristics of *rpoS* make it particularly susceptible to the loss of polyamines, we assume translation of other genes may also show similar hypersensitivity to loss of polyamines. The decrease in RpoS was initially detected after demonstration of a decrease in expression of RpoS-dependent genes (the RpoS regulon) in the absence of polyamines (8). Changes in translation of genes that are not transcription factors may not have been detected in microarray experiments, although changes in translation rate can certainly affect mRNA stability and thus protein accumulation. In addition, the level of RpoS is only modestly decreased in the absence of polyamines if it cannot be degraded (3-fold in an *rssB* mutant *versus* 40-fold in *rssB*$^+$, Fig. 4). Thus, slower translation or pausing may have a significant but modest effect on amounts of many proteins; RpoS is particularly sensitive because it can be rapidly degraded while being translated. There is no evidence that the synthetic lethality seen by the Tabor lab with a polyamine-deficient strain in an *rpsL* mutant or tRNA-modifying mutant (25, 26) is due to changes in RpoS. Thus, increased RpoS levels in the *rssB* mutant or deleting *rpoS* neither improved nor decreased growth in the absence of polyamines (Fig. S2). Rather, the slow growth of polyamine-deficient cells may suggest that there is a general problem with translation. We note the appearance of an apparent fragment of RpoD in polyamine-deficient strains, although levels of the RpoD sigma factor were not significantly perturbed (Fig. S3).

Our results implicate a previously unsuspected level of regulation of RpoS, dependent upon codon usage within the ORF, but using the well-studied machinery that regulates degradation of the full-length protein. A major question still to be addressed is an understanding of when this is physiologically relevant in WT cells. The very low or total lack of polyamines studied here is not a situation that will normally be encountered. In fact, *E. coli* normally has relatively high levels of both putrescine and spermidine [reviewed in (7)]. Deletion of the genes for these synthesis pathways is also likely to affect the level of precursors as well as pathways involved in inactivation and breakdown of polyamines, all changing cell metabolism. It is thus difficult to know if the rapid restoration of RpoS upon polyamine addition reflects a direct effect of the polyamines upon translation. Nevertheless, the behavior of this

strain uncovered an unexpected aspect of RpoS regulation, the evolution of codon usage in *rpoS* that is sensitive to stresses in translation. Such stresses might include pressures (including antibiotics) slowing translation or selecting for *rpsL* alleles such as those studied, suggesting that under these conditions, low RpoS levels are advantageous.

The effect of codon optimization used here demonstrates that a gene with multiple silent mutations, without changes in the amino acid composition of the growing polypeptide, has a dramatically different fate in the absence of polyamines. This is not a unique finding, and the role of synonymous mutations in both prokaryotes and eukaryotes is a subject of growing interest (33, 34). A silent polymorphism in the human MDR1 gene encoding P-glycoprotein was found to change the timing of cotranslational folding, changing substrate specificity (35). In a recent study, mutagenesis within portions of 21 different yeast genes demonstrated fitness effects for many synonymous mutations (36). A variety of studies have demonstrated co-translational assembly of proteins and the importance of this for optimal function [reviewed in (37)]. Our results and those of others suggest the importance of looking at the coding sequence as well as the sequence of the resulting protein to understand all of the factors contributing to the level and activity of a translated protein.

Experimental procedures

Strain construction

All strains and their source are listed in Table S1. The RpoS-LacZ translational fusions were linked either to a Zeo^R or a Cm^R cassette upstream of the Cp17 constitutive promoter to allow selecting the fusions after P1 transduction. The fusions were introduced by P1 transduction into strains that were WT for polyamine synthesis genes or deleted for seven different genes (Δ7), (Fig. S1*A*), to create the parallel sets of strains used here.

All oligonucleotides and gBlocks (dsDNA fragments) were obtained from IDT and are listed in Table S2. Codon optimization to design the gblock "RpoS-codon optimized" was done using https://www.idtdna.com/pages/tools/codon-optimization-tool set for *E. coli*. Portions of the same codon-optimized sequence were used to design the chimeric RpoS gblocks. Most strains were constructed by P1 transduction as described by Miller (38) and some by recombineering (39). To construct the RpoS567-lacZ fusion, primers oNM1042 and oNM1043 were used to amplify the zeo-Cp17-UTR-rpoS567 fragment from BA754. This PCR product was then recombineered into NM1100. This MG1655 derivative carries the chromosomal mini-λ Red system (12). Cells were grown in LB at 32 °C to an A_{600} of ∼0.4 to 0.6. Cultures were transferred to a water bath at 42 °C to induce expression of the λ-Red system for 15 min, chilled immediately in an ice-water slurry for 10 min prior to washing four times in sterile ice-cold water to make electrocompetent cells. 100 ng of dsDNA were used in the electroporation, recovered at 37 °C for an hour, and plated on LB-zeo plates. After purification and confirmation by sequencing or PCR, the fusion was then transduced into appropriate strains. For the codon-optimized and chimeric fusions, we first constructed NM25101, a strain in which the *rpoS* portion of a *rpoS-lacZ* fusion was replaced with a counter-selectable kan-araC-pBAD-Kid cassette, expressing the Kid toxin under control of arabinose (40). Primers oNM1005 and oNM1007 were used to amplify the kan-araC-pBAD-Kid cassette from CRB-kid (40). This counter-selectable cassette was then recombineered into electrocompetent BA754 cells carrying the pSIM6 (Red-system, amp^R) plasmid (41). All steps were done as described above for NM1100. However, cells were incubated to recover at 32 °C on LB-1% glucose-kan plates to retain the pSIM6 plasmid and silence the pBAD promoter. A kan^R amp^R colony was selected and grown in LB-1% glucose-amp and made electrocompetent for recombineering. Electrocompetent cells were electroporated with 50 ng of a gBlock fragment from IDT, allowed to recover overnight on the bench in LB-1% glucose before plating on LB-1% arabinose. Fusions were sequenced and transduced into appropriate backgrounds selecting for zeocin resistance. To remove FRT-flanked markers, plasmid pCP20 carrying the FLP-recombinase was used as described in Datsenko and Wanner (42).

The *rpsL* K42 alleles, which render cells streptomycin resistant, were introduced into cells by P1 transduction from donor strains indicated in Table S1, plating for resistance to streptomycin (40 μg/ml). Note that K42, as it is referred to in the literature, is amino acid 43 of the *rpsL* ORF; amino acid numbers for this protein omit counting the initiating Met.

Growth conditions

Cells were grown in Vogel Bonner medium (43) containing 0.4% glucose (V-B medium) at 37 °C with aeration. Where mentioned, the media was supplemented with spermidine and putrescine to 100 μM final each. Some experiments were also performed in LB media as indicated. Antibiotics were used at final concentrations of 100 μg/ml ampicillin or carbenicillin, 40 μg/ml zeocin, 25 μg/ml tetracycline, and 25 μg/ml kanamycin.

Growing strains in the absence of polyamine

Strains were generally grown in minimal medium with addition of polyamines, and as indicated in Figure 1*C*, the Δ7 strains grow slowly under this condition. During the course of our experiments, we noticed that growth in glass flasks that were re-used after autoclaving led to relatively good growth of the mutant strains in minimal media without polyamines. We attribute this to the presence of traces of polyamines on the glass. While this improves growth, it does not seem to affect RpoS levels significantly, suggesting a different threshold for growth (even small amounts of polyamine suffice) and RpoS translation (higher levels are needed). In the experiments reported here, either acid-washed glass flasks or single use disposable plastic flasks were used for evaluating the polyamine dependence of RpoS synthesis.

Genome sequencing

A kanamycin-sensitive derivative of the Δ7 strain (HT873), HT873X1 and derivatives (HT946, HT947, HT948) were sequenced and compared to the WT parent (BW25113). DNA extraction for genome sequencing was performed using Promega's Wizard Genomic DNA purification kit according to manufacturer's instructions (Madison). Sequencing was performed using PacBio long-read machines, by the NCI Core Sequencing facility (Frederick). Sequence alignment was performed using Geneious Prime (Biomatters). Rearrangements and a deletion of 16 kb were found, likely the result of recombination between the FRT sites flanking the Kan marker used for the knock-outs during the removal of the final transposon insertion (see Fig. S1 legend) (42, 44). The rearranged genome is shown in Fig. S1. DNA sequencing data were deposited in the BioProject Database at NCBI with the following genome accession identifications: BW25113: CP122319; HT-946: CP122316; HT-947: CP122315; HT-948: CP122317; HT873X1: CP122318.

β-galactosidase assays

Three independent colonies were picked and inoculated into 7 ml of V-B minimal media and vortexed. Serial dilution of that stock was done by taking a 1 ml aliquot into 6 ml, vortexing, and transferring 1 ml to another tube with 6 ml. Four serially diluted tubes were made for each strain and grown overnight at 37 °C. In the morning, the tube with the A_{600} closest to 0.7 was selected and diluted into a flask of 12 ml V-B media to an A_{600} of 0.01. Flasks were incubated in a water bath at 37 °C with shaking and grown to an A_{600} of ∼0.3. A 1 ml aliquot was taken for TCA precipitation, and 200 μl were used for a Miller assay (38). Averaged values are presented.

Westerns blots

TCA-precipitated samples were resuspended in LDS sample buffer (Invitrogen) to give equal final concentrations, adjusting relative to absorbance of the culture. Ten microliter samples were loaded on a 10- or 12-well NuPage 12% gel (Invitrogen) and run for 1 h at 180 V. Upon completion, the gel was transferred to nitrocellulose membranes using the iBlot2 system (Invitrogen) according to manufacturer's instructions. Membranes were blocked for a minimum of 30 min with 1% casein (Bio-Rad) and incubated overnight at 4 °C with anti-RpoS rabbit polyclonal antibody at 1:5000 and mouse monoclonal anti-Ef-Tu at 1:10,000 (loading control). Blots were washed in PBST and incubated with StarBright Blue700 anti-rabbit and DyLight800 anti-mouse (Bio-Rad) for 2 to 3 h and exposed on a ChemiDoc MP imager (Bio-Rad).

Protein degradation assays

Cells carrying the pBAD-rpoS plasmid (29, 45) were grown in V-B media with ampicillin at 37 °C to an A_{600} of 0.25 to 0.3 prior to the addition of 0.02% arabinose for 15 min followed by chloramphenicol (200 μg/ml) to stop translation. Samples were taken at the indicated time points and processed for Western blots. ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, https://imagej.nih.gov/ij/, 1997–2018) was used to quantify the bands. The average of three independent chases is shown.

Data availability

All data are provided within the paper and supporting materials. The genomes sequenced as part of this project have been deposited in NCBI with the accession numbers indicated above.

Supporting information—This article contains supporting information, including the following citations: (8, 13, 44, 46–50).

Acknowledgments—This paper is dedicated to Herb Tabor, whose work on polyamine and its role in the bacterial stress response led to this paper; Herb was an active participant in planning and discussing much of this work. We wish to thank Irina Artsimovitch and Jiqiang (Lanny) Ling for *rpsL* alleles and advice, Deborah Hinton for advice, and members of the Gottesman laboratory for discussions and comments. We would also like to thank Gisela Storz, Anupama Khare, Taran Bauer, Song Tong, and Xing Luo for critical review of the manuscript.

Author contributions—N. M., M. C., and S. G. conceptualization; N. M. methodology; N. M. and C. K. investigation; N. M. formal analysis; S. G. supervision; S. G. writing-original draft.

Funding and additional information—This research was supported by the Intramural Research Program of the NIH, in part by the National Cancer Institute, Center for Cancer Research. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ORF, open reading frame; WT, wildtype.

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