



Decreased Expression of Stable RNA Can Alleviate the Lethality Associated with RNase E Deficiency in *Escherichia coli*

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ABSTRACT The endoribonuclease RNase E participates in mRNA degradation, rRNA processing, and tRNA maturation in *Escherichia coli*, but the precise reasons for its essentiality are unclear and much debated. The enzyme is most active on RNA substrates with a 5'-terminal monophosphate, which is sensed by a domain in the enzyme that includes residue R169; *E. coli* also possesses a 5'-pyrophosphohydrolase, RppH, that catalyzes conversion of 5'-terminal triphosphate to 5'-terminal monophosphate on RNAs. Although the C-terminal half (CTH), beyond residue approximately 500, of RNase E is dispensable for viability, deletion of the CTH is lethal when combined with an R169Q mutation or with deletion of *rppH*. In this work, we show that both these lethalitys can be rescued in derivatives in which four or five of the seven *rrn* operons in the genome have been deleted. We hypothesize that the reduced stable RNA levels under these conditions minimize the need of RNase E to process them, thereby allowing for its diversion for mRNA degradation. In support of this hypothesis, we have found that other conditions that are known to reduce stable RNA levels also suppress one or both lethalitys: (i) alterations in *relA* and *spoT*, which are expected to lead to increased basal ppGpp levels; (ii) stringent *rpoB* mutations, which mimic high intracellular ppGpp levels; and (iii) overexpression of DksA. Lethality suppression by these perturbations was RNase R dependent. Our work therefore suggests that its actions on the various substrates (mRNA, rRNA, and tRNA) jointly contribute to the essentiality of RNase E in *E. coli*.

IMPORTANCE The endoribonuclease RNase E is essential for viability in many Gram-negative bacteria, including *Escherichia coli*. Different explanations have been offered for its essentiality, including its roles in global mRNA degradation or in the processing of several tRNA and rRNA species. Our work suggests that, rather than its role in the processing of any one particular substrate, its distributed functions on all the different substrates (mRNA, rRNA, and tRNA) are responsible for the essentiality of RNase E in *E. coli*.

KEYWORDS RNA processing and decay, RNase E, stable RNA expression, ppGpp, stringent *rpoB* mutants

In all organisms, mRNA degradation has to be precisely regulated in order to regulate protein expression levels, to modulate protein expression according to the changing environment, and to recycle ribonucleotides for new RNA synthesis (1–3). mRNA degradation in *Escherichia coli* begins with progressive endonucleolytic cleavages by RNase E (with a 5'-to-3' polarity) followed by exonucleolytic digestion of the resulting fragments by the 3'-5' exoribonucleases polynucleotide phosphorylase (PNPase), RNase R, and RNase II. The 2- to 5-nucleotide (nt)-long oligonucleotides so generated are then converted to mononucleotides by an oligoribonuclease, Orn (4–6).

RNase E, which is essential for *E. coli* viability, is a crucial enzyme in mRNA decay, encoded by gene *rne* (7–9). It is a 1,061-amino-acid-long protein with catalytic activity

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residing in the N-terminal half of the protein (10); the C-terminal unstructured half of the polypeptide serves as a scaffold for binding of the proteins PNPase, RhlB helicase, and enolase, which together with RNase E, constitute the degradosome (11–15). The degradosome is proposed to assist in cleavage of structured RNAs (16, 17). RNase E also processes the precursors of stable RNAs (both rRNA and tRNA) and *ssrA* (encoding small stable RNA) to generate their mature and functional forms (18–21); it also degrades the stable RNAs in growing cells (22) and under starvation conditions (23). RNase E autoregulates its synthesis in response to changes in total cellular RNase E activity by cleaving its own mRNA in the 5' untranslated region (UTR) (24–26).

The reasons for the essentiality of RNase E in *E. coli* are unclear, and several alternative models have been proposed. While some studies suggest that it is the processing of tRNA but not that of 9S RNA or mRNA decay that renders RNase E essential (19, 27–30), others suggest that degradation of around 100 distinct mRNA species contributes to its essential function (31). Studies with *rne(Ts)* alleles have also indicated that mRNA degradation is one of the essential functions of RNase E in *Salmonella enterica* (32). Furthermore, association of proper FtsZ levels, maintained by the processing of the *ftsQAZ* transcript by RNase E, with viability is also debated (33, 34).

Structural studies with its N-terminal catalytic half (residues 1 to 529) have shown that RNase E exists as a dimer of dimers, with each protomer possessing a large domain (residues 1 to 400), a small domain (residues 415 to 510), and a CPXCXGXG motif (residues 400 to 415) that coordinates a Zn²⁺ ion between two monomers (35). Each large domain carries the catalytic site as well as a "5'-sensor" pocket (which includes the residues R169 and T170) that can accommodate 5'-monophosphorylated substrate RNA (35, 36). The presence of the 5'-sensor region explains the preferential cleavage of 5'-monophosphorylated substrates by RNase E (4), which may be because of an increase in substrate binding affinity and/or in activity of the enzyme (6, 37); an R169Q mutation in RNase E abolishes this activation (35, 36).

All RNA synthesis is initiated with a 5'-terminal triphosphate moiety, and *E. coli* possesses a Nudix hydrolase, RppH, that acts to remove the pyrophosphate group from 5'-triphosphorylated RNA to generate the 5'-monophosphorylated product which is required for the first cleavage by RNase E to occur efficiently (38, 39). 5'-monophosphorylated RNAs are also generated by endonucleolytic cleavage of RNA, for example, by RNase E itself, by its paralog RNase G, or by enzymes such as RNase III. With respect to RNase E, while only the first cleavage of transcript is expected to be defective in the absence of RppH, all the cleavage reactions will be defective in strains with RNase E-R169Q (40, 41), which would explain why precursors of rRNA accumulate in the latter mutant (40, 41).

Neither deletion of RppH nor the R169Q mutation in RNase E's 5'-sensor domain is lethal, and it is only a subset of mRNAs (~400) that become stabilized in a $\Delta rppH$ strain (39, 40). This suggests that the 5'-end-dependent pathway is not the sole pathway for RNA cleavage. Similarly, strains expressing RNase E variants with deletion of the C-terminal half (CTH) are viable but suffer defects in global mRNA decay (42), ribosome free mRNA degradation (43), and autoregulation (27, 44); these defects are likely contributed by one or more of several mechanisms, such as absence of degradosome assembly, reduced substrate binding affinity, loss of membrane interactions, and defective oligomerization. We (41) and others (40) have shown earlier that combining two mutations, namely, (i) deletion of *rppH* or the RNase E-R169Q mutation with (ii) deletion of the CTH (Δ CTH) of RNase E, imparts inviability to *E. coli*. We have interpreted these results to indicate the existence of two pathways for RNase E action (41). In this communication, we present evidence that the lethality associated with the R169Q- Δ CTH or Δ RppH- Δ CTH combination but not that associated with Δrne can be suppressed by various perturbations that are expected to reduce rRNA expression in the strains. We hypothesize that, by diverting RNase E from stable RNA processing to mRNA degradation, lowered stable RNA levels help in the optimal degradation of mRNA in strains with limiting RNase E activity. Our results therefore suggest that the distributed functions of RNase E contribute to its essentiality in *E. coli*.

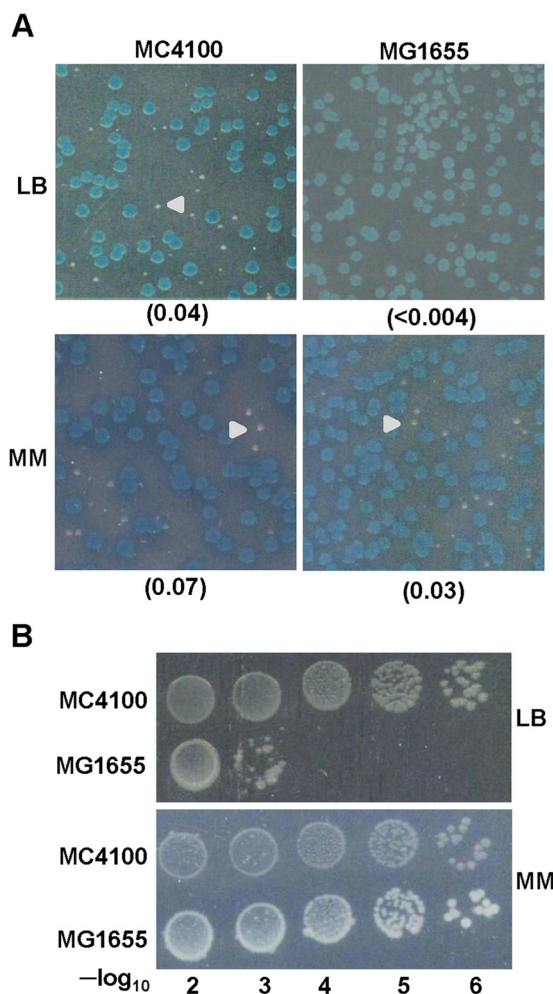


FIG 1 Growth characteristics of MC4100 and MG1655 derivatives of the *rne-169Q,ΔCTH* double mutant. (A) The GJ14067 (MC4100 Δrne) and GJ14033 [MG1655 *lacZ*(Am) Δrne] strains carrying the unstable plasmids pHYD1613 (*rne*⁺ *lacZ*⁺ Tp^r) and pHYD2373 (*rne-169Q,ΔCTH* Kan^r) were grown to stationary phase in LB with Tp and Kan, and suitable dilutions were plated on LB and MM (glucose-minimal A) with Kan and X-Gal (without Tp). Representative Lac⁻ colonies are shown by arrowheads. The ratio of Lac⁻ white colonies to total colonies is given in parentheses. (B) White colonies obtained on glucose-minimal A medium from GJ14067 and GJ14033 were grown in the same medium to stationary phase and were spotted at dilutions on LB and MM media.

RESULTS

Effect of basal ppGpp levels on RNase E-R169Q,ΔCTH and ΔRppH RNase E-ΔCTH double mutant lethality. We have reported earlier that RNase E-ΔCTH double mutants with either ΔRppH or RNase E-R169Q are inviable, presumably because of the combined loss of both the 5'-end-dependent and CTH-activated pathways of RNase E activity (41). In further studies, as described below, we have found that the inviability is apparently modulated by the basal ppGpp levels in the strains, as dictated by their genotypes at *relA* and *spoT* (the two genes involved in ppGpp synthesis [both *relA* and *spoT*] and degradation [*spoT* alone] [45, 46]).

For these studies, we employed the "shelter plasmid loss assay" as described in Materials and Methods, in which we scored for the ability of Δlac derivatives that had lost an unstable plasmid bearing *lacZ*⁺ and *rne*⁺ to remain viable and grow as white colonies (Lac⁻) on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)-supplemented plates. With this approach, we observed first that RNase E-R169Q, Δ CTH derivative of MG1655 were inviable on LB and viable on defined medium, while similar derivatives of MC4100 were viable on both media (Fig. 1A). The Lac⁻

colonies obtained on defined medium from MG1655 derivative failed to purify on LB medium (Fig. 1B), confirming that these derivatives exhibit rich-medium lethality.

Although the genotypes of strains MG1655 and MC4100 differ at several loci (47), we considered the possibility that it is the variations at *relA* and *spoT* between the two strains which are responsible for the observed phenotypic differences from the RNase E-R169Q, Δ CTH derivatives. Whereas MG1655 has the genotype *relA*⁺ *spoT*⁺, MC4100 has the genotype *relA1 spoT1*, with *relA1* being associated with reduced ppGpp synthase activity and reduced ppGpp levels and *spoT1* with reduced ppGpp hydrolase activity and increased ppGpp levels. Overall, basal ppGpp levels are higher in MC4100 than in MG1655 (48), and that growth rate is inversely correlated with intrinsic ppGpp levels (49, 50).

Accordingly, we constructed a set of isogenic strains with different *relA* (*relA*⁺, *relA1*, or Δ *relA*) and *spoT* (*spoT*⁺, *spoT1*, or Δ *spoT*) alleles and tested them for viability with the RNase E-R169Q, Δ CTH and Δ RppH RNase E- Δ CTH combinations. These constructions were made in the genomic background of strain MC4100. The Δ *relA* Δ *spoT* combination could not be reliably tested because of rapid accumulation of suppressors, many of which are presumably RNA polymerase mutants (51). The basal ppGpp levels in different derivatives have been inferred from their genotypes based on the earlier studies (49, 50, 52), and no direct ppGpp measurements were made in this work. Growth rates of the strains on LB medium (see Fig. S1 in the supplemental material) were also correlated with the inferred intrinsic ppGpp levels, as reported earlier (53).

The results, presented in Table 1, indicate that RNase E-R169Q, Δ CTH derivatives are inviable at low basal ppGpp levels (*relA1 spoT*⁺ and Δ *relA spoT*⁺) on all media, viable only on defined medium at intermediate levels (*relA*⁺ *spoT*⁺), and viable on both LB and defined media at high ppGpp levels (*relA*⁺ *spoT1* and *relA1 spoT1*) (Table 1). On the other hand, the Δ RppH RNase E- Δ CTH derivatives were inviable with all combinations of *relA* and *spoT* alleles tested (Table 1). With respect to control strains, the Δ *rne* mutant was inviable with all the *relA-spoT* combinations (see Table S2, rows 11 to 15, in the supplemental material), while the RNase E-R169Q (Table S2, rows 1 to 5) and RNase E- Δ CTH (Table S2, rows 6 to 10) single mutants were viable under all conditions.

The RNase E C-terminal truncation employed in the experiments described above extends from residue 494, and hence the truncated form is also missing part of the enzyme's small domain, which could conceivably have affected its oligomerization. Accordingly, we also examined the effects of basal ppGpp status on the viability of an RNase E-R169Q, Δ 529 mutant, that is, where the R169Q mutation is borne on a protein with Δ CTH extending from residue 530; Garrey and Mackie have earlier reported that this is inviable in MG1655 (40).

In a comparison across isogenic strains, the *rne*-R169Q, Δ 529 double mutant was inviable on LB at intermediate levels of basal ppGpp (*relA*⁺ *spoT*⁺) (Table 1). It was also inviable at low basal ppGpp levels (*relA1 spoT*⁺ and Δ *relA spoT*⁺) on both LB and minimal media but was viable on both media at high basal ppGpp levels (*relA*⁺ *spoT1* and *relA1 spoT1*) (Table 1). The results therefore indicate that deletions of the CTH beyond residue 494 or 530 are equivalent in terms of their effects on compromising RNase E function.

Since ppGpp is known to regulate the expression of many genes (54), we have tested whether the reason for suppression at elevated ppGpp levels is an increase in RNase E expression and thereby its activity. For this, we measured the expression of β -galactosidase from a single-copy *me-lac* fusion as described earlier (41), where β -galactosidase expression is inversely correlated with cellular RNase E levels (24). The results indicate that RNase E activity is unaffected by the basal ppGpp levels (Fig. 2A), which was also confirmed by immunoblot analysis of RNase E protein (Fig. 2A). Thus, increased RNase E expression at high basal ppGpp levels is not the cause of suppression.

Suppression of Δ RppH RNase E- Δ CTH and RNase E-R169Q, Δ CTH lethality by mutations in *rho* and *nusG* is also conditional on basal ppGpp levels. In our earlier study (41), we had shown that missense mutations in *rho* and *nusG*, which adversely affect the process of Rho-dependent transcription termination, suppress the lethality

TABLE 1 Effects of different combinations of *relA* and *spoT* alleles on the *rne-169Q,ΔCTH* and $\Delta rppH$ *rne-ΔCTH* double mutant lethality and their suppression by *rho* and *nusG* mutations^a

Genotype ^b	No. of Lac ⁻ colonies/total (ratio) for the given mutations in the indicated strain and medium											
	<i>rne-R169Q,ΔCTH</i>					$\Delta rppH$ <i>rne-ΔCTH</i>						
	WT		rho mutant,		nusG mutant,		WT, LB		rho mutant,		nusG mutant,	
	LB	MM	LB	LB	LB	LB	LB	LB	LB	LB	LB	MM
<i>relA</i> ⁺ <i>spoT</i> ⁺	8/198 (0.04)	6/232 (0.03)	12/116 (0.103)	13/165 (0.07)	0/261 (<0.003)	16/164 (0.1)	13/125 (0.10)	48/288 (0.17)	56/176 (0.31)			
<i>relA</i> ⁺ <i>spoT</i> ^{1c}	6/226 (0.02)	6/130 (0.04)	16/164 (0.09)	16/148 (0.11)	0/240 (<0.004)	14/197 (0.07)	10/179 (0.05)	16/176 (0.09)	28/180 (0.15)			
<i>relA</i> ⁺ <i>spoT</i> ⁺	0/199 (<0.005)	6/192 (0.03)	32/236 (0.13)	0/176 (<0.005)	0/203 (<0.004)	16/148 (0.11)	0/124 (<0.008)	0/186 (<0.005)	14/838 (0.02)			
<i>relA</i> ⁺ <i>spoT</i> ⁺	0/136 (<0.007)	0/109 (<0.009)	0/199 (<0.005)	0/208 (<0.005)	0/153 (<0.006)	0/128 (<0.005)	0/112 (<0.009)	0/284 (<0.003)	0/480 (<0.002)			
$\Delta relA$ <i>spoT</i> ⁺	0/108 (<0.009)	0/96 (<0.01)	0/206 (<0.004)	0/213 (<0.004)	0/106 (<0.009)	0/176 (<0.005)	0/168 (<0.005)	0/160 (<0.006)	0/520 (<0.001)			

^aThe shelter plasmid loss assay was done as described in the legend to Fig. 1. Strains with the indicated genotypes (listed in Table S1 in the supplemental material) carry plasmids pHYD1613 and pHYD2373 (*rne-R169Q,ΔCTH* strains), plasmid pHYD1613 ($\Delta rppH$ *rne-ΔCTH* strains), or plasmids pHYD1613 and pRne-SG21 (*rne-R169Q,ΔCTH* strains) (a kind gift from George A. Mackie).

^bGenotypes are arranged in decreasing order of the strains' ppGpp levels as deduced from the earlier reports (49, 50, 52, 53).

^cThe strain with this genotype gave plasmid-free white colonies in 4 out of 7 experiments, and we speculate that the presence of borderline ppGpp levels in this background is the cause of the fluctuating growth phenotype.

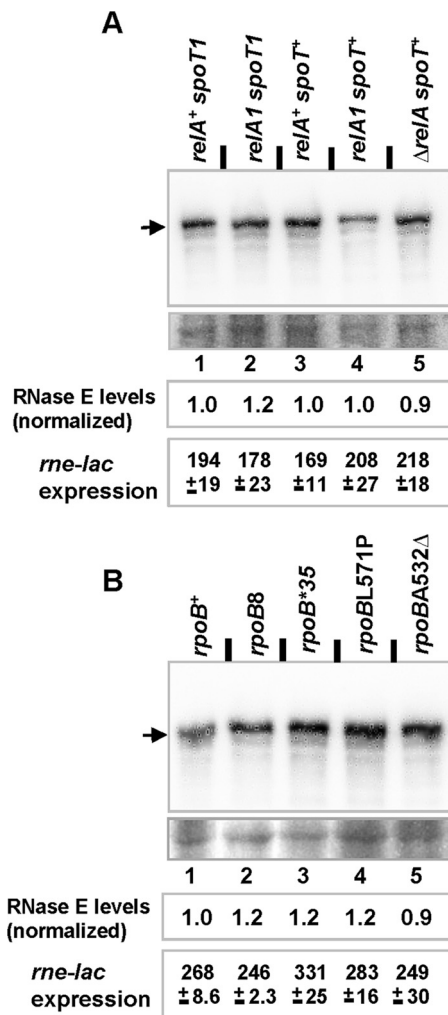


FIG 2 Effects of basal ppGpp levels and *rpoB* genotype on RNase E levels and *rne-lac* expression. RNase E levels were determined by immunoblotting following growth to mid-log phase of cultures with either various combinations of *relA* and *spoT* alleles (A) or different *rpoB* alleles (B). The upper image in each panel shows the results from probing with anti-RNase E antibody, and the band for RNase E is marked by an arrow; the lower image shows a representative area from the same blot after staining with amido black to serve as a loading and transfer control. For each of the strains, RNase E levels were quantitated (average from two independent experiments) as the ratio of densitometric intensities of the band for RNase E to that of a suitable band (common to all lanes) in the loading control; further, the values have also been normalized to the ratio calculated for lane 1 in each of the two panels. *rne-lac* expression values (expressed in Miller units) for strains of the indicated genotypes were determined in LB-grown cultures. The strains used in these experiments are listed in Table S1 in the supplemental material.

associated with both Δ RppH RNase E- Δ CTH and RNase E-R169Q, Δ CTH mutations. These findings were interpreted in the context of a model in which formation of excessive RNA-DNA hybrids (R-loops) in the *rho* and *nusG* mutants (55, 56) provides an alternative means of mRNA degradation in the RNase E-deficient strains.

In the present study, we have now tested the suppression ability of *rho* and *nusG* mutations in the different isogenic strain backgrounds with various basal ppGpp levels (Table 1). Our results indicate that for the Δ RppH RNase E- Δ CTH combination, which is inviable at all ppGpp levels, *rho* and *nusG* suppress lethality at high basal levels of ppGpp (*relA⁺ spoT1* and *relA1 spoT1*) but not at the low ppGpp levels (*relA⁺ spoT⁺* and Δ *relA spoT⁺) (Table 1). At the intermediate level of ppGpp (*relA⁺ spoT⁺*), only the *rho* mutation suppressed the inviability. For the RNase E-R169Q, Δ CTH combination, inviability in the *relA⁺ spoT⁺* background alone (but not in the *relA1 spoT⁺* or Δ *relA spoT⁺* background) was suppressed by the *rho* mutation (Table 1).*

TABLE 2 Suppression of *rne*-169Q, Δ CTH and Δ *rppH rne*- Δ CTH double mutant lethality by stringent RNA polymerase mutations and effect of deleting *rrn* in RNA polymerase mutants^a

Genotype	No. of Lac ⁻ colonies/total (ratio) for the indicated mutation				
	<i>rpoB</i> ⁺	<i>rpoB8</i>	<i>rpoB</i> *35	<i>rpoBL571P</i>	<i>rpoBA532Δ</i>
<i>rne</i> -R169Q, Δ CTH (<i>relA</i> ⁺ <i>spoT</i> ⁺)	0/241 (<0.001)	0/206 (<0.004)	8/184 (0.04)	28/224 (0.13)	36/216 (0.16)
<i>rne</i> -R169Q, Δ CTH (<i>relA1 spoT</i> ⁺)	0/208 (<0.004)	0/152 (<0.006)	6/162 (0.03)	14/270 (0.05)	16/402 (0.04)
Δ <i>rppH rne</i> - Δ CTH (<i>relA1 spoT1</i>)	0/291 (<0.003)	0/240 (<0.004)	6/222 (0.03)	24/224 (0.107)	12/280 (0.04)
Δ <i>rppH rne</i> - Δ CTH (<i>relA1 spoT</i> ⁺)	0/167 (<0.003)	0/128 (<0.007)	32/188 (0.17)	24/144 (0.17)	40/184 (0.21)
Δ <i>rne</i> (<i>relA</i> ⁺ <i>spoT1</i>)	0/140 (<0.007)	ND ^b	0/220 (<0.004)	0/282 (<0.003)	0/312 (<0.003)
Δ <i>rne</i> (<i>relA1 spoT1</i>)	0/224 (<0.004)	ND	0/264 (<0.003)	0/253 (<0.003)	0/160 (<0.006)
Δ <i>rrn rne</i> -R169Q, Δ CTH (<i>relA</i> ⁺ <i>spoT</i> ⁺)	0/148 (<0.006)	ND	36/428 (0.08)	18/157 (0.11)	12/124 (0.09)
Δ <i>rrn \Delta</i> <i>rppH rne</i> - Δ CTH (<i>relA1 spoT1</i>)	ND	ND	8/177 (0.04)	16/180 (0.09)	20/126 (0.15)
Δ <i>rrn \Delta</i> <i>rppH rne</i> - Δ CTH (<i>relA1 spoT</i> ⁺)	ND	ND	16/148 (0.11)	16/244 (0.15)	19/125 (0.07)

^aThe shelter plasmid loss assay for strains carrying either only pHYD1613 (rows 3, 4, 5, 6, 8, and 9) or pHYD1613 and pHYD2373 (rows 1, 2, and 7) was done as described in the legend to Fig. 1. The strains used are listed in Table S1 in the supplemental material.

^bND, not determined, as the *rpoB8* mutation was not suppressing the double mutant lethality.

We also tested whether the transcription polarity relief phenotypes of *rho* and *nusG* mutants were affected by the basal ppGpp status of the strains. For this purpose, we determined the expression of *lacZ* downstream of the Rho-dependent terminator λ t_{R1} as previously described (57). The results indicate that the extent to which transcription termination is rendered defective by *rho* and *nusG* mutations is the same at high and low ppGpp levels (see Table S3 in the supplemental material). The missense *rho* and *nusG* single mutants were viable but sick at low ppGpp levels (see Table S2 [rows 16 to 25] and Fig. S2 in the supplemental material).

“Stringent” RNA polymerase (*rpoB*) mutations also suppress RNase E-R169Q, Δ CTH and Δ RppH RNase E- Δ CTH double mutant lethality. As we found that basal ppGpp levels modulate the growth of the strains with limiting RNase E activity, we also tested whether “stringent” *rpoB* mutations, which mimic the effect of high ppGpp levels (51, 58–60), could restore the viability of RNase E-R169Q, Δ CTH and Δ RppH RNase E- Δ CTH mutants. For this, we chose the stringent *rpoB**35, *rpoBL571P*, and *rpoBA532 Δ* RNA polymerase mutants and the nonstringent *rpoB8* mutant (61) as a control. The stringent *rpoB* mutations alone suppressed lethality of both RNase E-R169Q, Δ CTH (at both intermediate [*relA*⁺ *spoT*⁺] and low [*relA1 spoT*⁺] ppGpp levels) and Δ RppH RNase E- Δ CTH combinations (at high [*relA1 spoT1*] and low [*relA1 spoT*⁺] basal ppGpp levels) (Table 2). None of them suppressed Δ *rne* lethality (Table 2).

We also tested the stringent *rpoB* mutation *rpoBA532 Δ* for its ability to suppress *rne*-R169Q, Δ 529 Δ double mutant lethality, and the data indicated that viability was restored in these derivatives at levels of ppGpp that were intermediate (ratio of Lac⁻ colonies to total = 0.26) or low (ratio of Lac⁻ colonies to total = 0.27 and 0.13 in *relA1 spoT*⁺ and Δ *relA spoT*⁺ backgrounds, respectively).

That increased RNase E expression is not the cause of suppression by stringent *rpoB* mutations was evident from both immunoblotting experiments and *rne-lac* expression assays; these measurements were performed in strains carrying either full-length RNase E (Fig. 2B) or the protein truncated for its CTH beyond residue 494 (see Fig. S4 in the supplemental material). We then studied whether the conditions conferring viability under situations of limiting RNase E activity, namely, presumptive basal high ppGpp levels or presence of stringent *rpoB* mutations, reduce *rrn* operon transcription, as measured with an *rrnB* P1-*lacZ* fusion in the appropriate strains (Table 3). This assay was performed in the MG1655 background in which the suppression phenotype is observed. As reported earlier (58, 60), β -galactosidase expression from the *rrnB* P1-*lacZ* fusion was reduced significantly (3-fold) in the stringent *rpoB* mutants compared to the *rpoB*⁺ strain and control *rpoB8* mutant. Furthermore, there was a modest reduction in transcription from the *rrnB* promoter (1.2-fold) at high basal ppGpp levels (*relA*⁺ *spoT1*).

Taken together, these results suggest that reduction in stable RNA transcription is associated with the rescue of inviability under conditions of limiting RNase E activity and that when there is a small reduction in *rrn* transcription (as with high basal ppGpp

TABLE 3 Effects of various *rpoB* alleles and high basal ppGpp levels on expression of the *rrnB* P1 promoter determined using *rrnB* P1-*lacZ* gene fusion

Mutation ^a	<i>rrnB</i> P1- <i>lacZ</i> sp act ^b
<i>rpoB</i> ⁺	1
<i>rpoB8</i>	1.30
<i>rpoBA532Δ</i>	0.33
<i>rpoB</i> *35	0.34
<i>rpoBL571P</i>	0.34
<i>relA</i> ⁺ <i>spoT1</i> <i>rpoB</i> ⁺	0.8

^aExcept for the last row, the genotype of the strains is *relA*⁺ *spoT*⁺. From top to bottom, the strains used were RLG1350 (a kind gift from Richard L. Gourse), GJ14359, GJ14360, GJ14363, GJ14362, and GJ14365.

^bThe specific activities of β -galactosidase of the strains with the indicated genotypes were determined after growth in LB medium, and the values were normalized to that of the wild-type strain. Values are averages from three independent experiments.

levels), lethality of RNase E-R169Q, Δ CTH but not that of Δ RppH RNase E- Δ CTH is suppressed, while on the other hand when the reduction is more significant, both the lethalitys are suppressed.

Inviability due to limiting RNase E generated by other means is also rescued by stringent *rpoB* mutations. A strain in which RNase E is expressed from the P_{lac} promoter is viable only in medium supplemented with $\geq 3 \mu\text{M}$ isopropyl- β -D-thiogalactopyranoside (IPTG) (62). We found in this study that stringent *rpoB* mutations enable such a strain to grow without IPTG (Fig. 3A). Suppression by the *rpoBA532Δ* mutation was most robust, followed by that by the *rpoBL571P* and *rpoB**35 mutations.

RNase E levels were determined by immunoblotting in colonies of P_{lac} -*rne* strains carrying the stringent *rpoB* mutations that were grown on plates with 0 μM IPTG, and these levels were shown to be lower than the levels in colonies of the control *rpoB*⁺

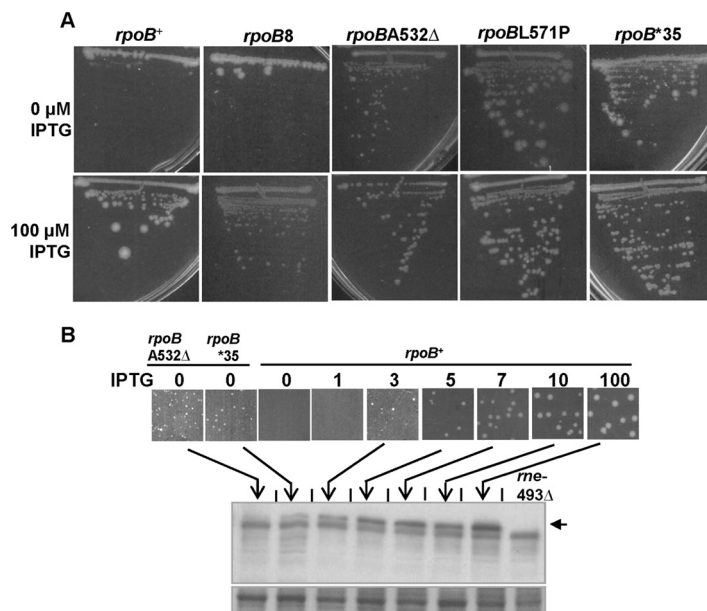


FIG 3 Stringent *rpoB* mutations rescue the inviability of a P_{lac} -*rne* strain grown without inducer (IPTG). (A) Wild-type and *rpoB* mutant derivatives of P_{lac} -*rne* strains were streaked on a pair of LB plates with the indicated micromolar IPTG concentrations and incubated at 37°C until the growth on plate without IPTG was clear (for 2 days). The strains used, from left to right, were GJ6974, GJ17402, GJ17403, GJ17404, and GJ17405. (B) Cultures of strains GJ17403, GJ17405, and GJ6974 were grown in LB with IPTG (100 μM), washed, and plated at suitable dilutions on LB plates with the indicated IPTG concentrations, and RNase E levels in the cells of colonies harvested from the plates after 48 h were determined by Western blotting. In the upper panel, the RNase E band is marked by the arrow; the lower panel shows a representative section of the same blot stained with amido black, which serves as a loading and transfer control. The band seen immediately below that of RNase E in this experiment was inferred to be a nonspecific band, since it is also observed in the lane for RNase E- Δ CTH (see also Fig. S4 in the supplemental material).

TABLE 4 Role of DksA and RNase R in the viability of the *rne*-R169Q, Δ CTH mutant at high basal ppGpp levels (*relA*⁺ *spoT1*)^a

Mutation	No. of Lac ⁻ colonies/total (ratio) for the indicated <i>rne</i> -R169Q, Δ CTH (<i>relA</i> ⁺ <i>spoT1</i>) strain		
	WT	<i>rho</i> mutant	<i>nusG</i> mutant
WT	12/218 (0.05)	52/272 (0.19)	30/166 (0.18)
Δ <i>dksA</i>	0/144 (<0.006)	6/136 (0.04) ^b	11/159 (0.07) ^b
Δ <i>pnp</i>	14/262 (0.05)	ND ^c	ND
Δ <i>rnb</i>	6/154 (0.04)	ND	ND
Δ <i>rnr</i>	0/544 (<0.002)	6/312 (0.02)	9/320 (0.03)

^aThe shelter plasmid loss assay was done as described in the legend to Fig. 1. The following strains carrying plasmids pHYD1613 and pHYD2373 were employed: row 1, GJ15232, GJ15234, and GJ15235; row 2, GJ16159, GJ16157, and GJ16158; row 3, GJ15217; row 4, GJ15216; row 5, GJ15259, GJ15260, and GJ15261.

^bThe Lac⁻ colonies are considered inviable, as they did not grow or revive upon restreaking.

^cND, not determined, as the corresponding wild-type strains were viable.

strain grown on plates with 3 μ M IPTG (Fig. 3B). These results indicate that suppression by the stringent *rpoB* mutations is not because of an IPTG-independent increase in RNase E expression in the P_{*lac*}-*rne* strain (Fig. 3B).

Therefore, it appears that stringent *rpoB* mutations can suppress growth defects of limiting RNase E conditions that have arisen by any of the several different ways.

DksA dependence for viability of RNase E-R169Q, Δ CTH. The protein DksA is required to potentiate the regulatory effects of ppGpp on transcription from *rnn* operons (reviewed in references 46 and 63). Therefore, we tested the requirement of DksA for viability of RNase E-R169Q, Δ CTH at high basal ppGpp levels.

Deletion of *dksA* from wild-type (WT), *rho*, and *nusG* derivatives of the RNase E-R169Q, Δ CTH mutant at high basal ppGpp levels (*relA*⁺ *spoT1*) resulted in inviability (Table 4), analogous to that observed for *dksA*⁺ at low ppGpp levels. On the other hand, viability conferred by the stringent *rpoB* mutation *rpoBA532* Δ was retained even in the Δ *dksA* derivative (ratio of Lac⁻ colonies to total = 0.11); Vinella et al. (64) have also reported that the suppression by *rpoBA532* Δ of other low-[ppGpp] phenotypes is retained even in the absence of DksA.

We also have checked whether overexpression of DksA can suppress the lethality associated with limiting RNase E at otherwise nonpermissive ppGpp levels, since Potrykus et al. (65) have demonstrated that such overexpression leads to inhibition of transcription initiation from the *rnnBP1* promoter in a ppGpp-independent manner. Our results suggest that overexpression of DksA does suppress RNase E-R169Q, Δ CTH lethality at both intermediate and low ppGpp levels but that it does not suppress Δ RppH RNase E- Δ CTH lethality (Table 5).

TABLE 5 Effect of overexpression of DksA on *rne*-R169Q, Δ CTH and Δ *rppH rne*- Δ CTH lethality^a

Genotype	No. of Lac ⁻ colonies/total (ratio) for the indicated mutations	
	<i>rne</i> -R169Q, Δ CTH	Δ <i>rppH rne</i> - Δ CTH
<i>relA</i> ⁺ <i>spoT</i> ⁺ /pHR53	0/186 (<0.005)	0/106 (<0.009)
<i>relA</i> ⁺ <i>spoT</i> ⁺ /pJK537 ^b	17/138 (0.12)	2/265 (0.007) ^c
<i>relA1 spoT</i> ⁺ /pHR53	0/203 (<0.005)	0/180 (<0.005)
<i>relA1 spoT</i> ⁺ /pJK537 ^b	32/204 (0.14)	14/320 (0.044) ^c

^aThe shelter plasmid loss assay for strains carrying pHYD1613 and pHYD2373 (*rne*-R169Q, Δ CTH) or only pHYD1613 (Δ *rppH rne*- Δ CTH) was done as described in the legend to Fig. 1. The strains carrying either pHR53 or pJK537 employed were as follows: rows 1 and 2, GJ15233 and GJ16022 for *rne*-R169Q, Δ CTH and Δ *rppH rne*- Δ CTH, respectively; rows 3 and 4, GJ14175 and GJ14157 for *rne*-R169Q, Δ CTH and Δ *rppH rne*- Δ CTH, respectively.

^bOverexpression of the *dksA* gene from this plasmid is brought about by the non-feedback-regulated promoter present upstream from the native *dksA* promoter (88).

^cThe Lac⁻ colonies are considered inviable, as they did not grow or revive upon restreaking.

TABLE 6 Reducing the number of *rrn* operons suppresses both *rne*-R169Q, Δ CTH and Δ *rppH rne*- Δ CTH lethalties^a

No. of <i>rrn</i> operons deleted (deleted operon[s])	No. of Lac ⁻ colonies/total (ratio) for the indicated mutations	
	<i>rne</i> -169Q, Δ CTH	Δ <i>rppH rne</i> - Δ CTH
1 (Δ <i>rrnG</i>)	0/62 (<0.02)	0/160 (<0.006)
4 (Δ <i>rrnGADE</i>)	20/140 (0.14)	52/252 (0.21)
5 (Δ <i>rrnGADEH</i>)	16/80 (0.20)	21/141 (0.12)

^aThe shelter plasmid loss assay for strains carrying pHYD1613 and pHYD2373 (*rne*-169Q, Δ CTH) or only pHYD1613 (Δ *rppH rne*- Δ CTH) was done as described in the legend to Fig. 1. The strains used for the one-, four-, and five-operon deletions, respectively, were GJ16183, GJ16028, and GJ16030 (*rne*-169Q, Δ CTH) and GJ16184, GJ16124, and GJ16125 (Δ *rppH rne*- Δ CTH).

Deletion of four or five *rrn* operons suppresses lethality associated with RNase E deficiency. Based on the above observations, we considered the possibility that it is the reduction in the expression and levels of stable RNA that is associated with the rescue of lethality in strains with limiting RNase E activity. To test this hypothesis, we examined their viability in derivatives in which the *rrn* copy number (seven in the wild type) has been reduced (66).

We found that both combinations, Δ RppH RNase E- Δ CTH and RNase E-R169Q, Δ CTH, remain inviable when only one *rrn* operon is deleted (Δ *rrnG*) but are rendered viable upon deletion of four (Δ *rrnGADE*) or of five (Δ *rrnGADEH*) *rrn* operons (Table 6). Inviability of the *rne*-R169Q, Δ 529 Δ double mutant also was suppressed upon deletion of four or five *rrn* operons (ratio of Lac⁻ colonies to total = 0.05 and 0.18, respectively). These results strongly suggest that it is the lowered stable RNA levels brought about by various perturbations that can impart viability to strains with very low RNase E activity.

Rescue of lethality in strains with limiting RNase E is not because of a reduction in growth rate. All conditions described above that restored viability to strains with limiting RNase E, namely, high basal ppGpp levels, stringent *rpoB* mutations, and decreased *rrn* copy number, are also associated with a decrease in the growth rate of the strains. We therefore tested whether a similar rescue of lethality could be enacted by any other perturbation which decreases the growth rate of the strains.

Deletion of neither *crp* nor *hfq*, both of which are associated with a reduction in growth rate (67, 68), rescued the lethality associated with limiting RNase E activity of the RNase E-R169Q, Δ CTH mutant (ratio of Lac⁻ colonies to total = <0.003 and <0.002, respectively). Whereas the *hfq* mutant does exhibit a higher growth rate than the stringent *rpoB* mutants or the *rrn* operon-deleted strains, the *crp* mutant is as compromised for growth as is the *rpoB**35 stringent mutant or the Δ 4-*rrn* strain (see Table S4 in the supplemental material). These results indicate that a mere reduction in growth rate does not rescue the lethality associated with limiting RNase E.

Global repression of transcription does not rescue lethality caused by RNase E deficiency. We then tested whether sublethal concentrations of rifampin would restore the viability to derivatives with RNase E-R169Q, Δ CTH (at intermediate and low basal ppGpp levels) or Δ RppH RNase E- Δ CTH (at high basal ppGpp levels). The lethalties could not be rescued at any of the rifampin concentration tested (see Table S5 in the supplemental material), suggesting that a global reduction in transcription is not equivalent to the suppressors described above, such as elevated basal ppGpp levels, stringent *rpoB* mutations, or decrease in *rrn* copy number. Sublethal rifampin also did not rescue the inviability of the P_{*lac*}-*rne* strain on plates with 0 μ M IPTG (data not shown).

Role of RNase R in viability of strains with limiting RNase E activity. As mentioned above, three exoribonucleases, PNPase, RNase II, and RNase R, are involved in mRNA turnover (13, 69–71) following endonucleolytic cleavage by RNase E, and we tested their role, if any, in the suppression of lethalties associated with limiting RNase E at high basal ppGpp levels or with stringent *rpoB* mutations.

Depletion of RNase R (but not of PNPase or RNase II) resulted in inviability of RNase E-R169Q, Δ CTH at high basal (*relA*⁺ *spoT1*) ppGpp levels (Table 4, compare Δ *rrn* with

WT). However, deletion of *rnr* in the presence of *rho* or *nusG* mutations (for RNase E-R169Q, Δ CTH at high ppGpp levels) (Table 4) and stringent *rpoB* mutations (for both the mutants) (Table 2) did not lead to loss of viability.

We also found that depletion of RNase R from the P_{lac} -*rne* derivative with high basal ppGpp levels (*relA1 spoT1*) resulted in a 100-fold reduction in CFU at 2 μ M IPTG (see Fig. S3 in the supplemental material), confirming the need for RNase R to support growth under conditions of low RNase E activity and high ppGpp. The possible role of RNase R during RNase E-limited growth is discussed below.

DISCUSSION

RNase E is essential for viability and plays important roles in both mRNA degradation and stable RNA processing. Combined perturbations of both its 5'-end-dependent pathway (occurring in Δ RppH or RNase E-R169Q strains) and CTH-activated pathway (in RNase E Δ CTH) lead to deficiency in RNase E activity that results in growth inhibition (40, 41). In this work we report that high basal ppGpp levels, *rpoB* mutations that encode stringent RNA polymerases, or reduced *rnr* copy numbers alleviate the growth defects associated with limiting RNase E activity. We propose that reduced *rnr* transcription under these conditions results in diversion of the enzyme activity to achieve efficient mRNA turnover as a consequence of the lowered requirement of RNase E for processing of stable RNA, since the cellular levels of the latter are now reduced.

Evidence for suppression of inviability associated with limiting RNase E activity by the reduction of stable RNA levels. Three observations support the hypothesis that reduced stable RNA levels result in the suppression of inviability associated with RNase E-R169Q, Δ CTH and RNase E- Δ CTH Δ RppH double mutants that have limiting RNase E activity. First, all *rpoB* mutations (*rpoBA532* Δ , *rpoB**35, and *rpoBL571P*) that suppressed both the lethality also conferred reduced transcription from the *rnrB* P1 promoter (Table 3). Other groups have also shown earlier that stringent *rpoB* mutations reduce transcription from the *rnrB* P1 promoter *in vivo* and *in vitro* (58, 60).

We also obtained a modest reduction (1.2-fold) in transcription from the *rnrB* P1 promoter at high basal ppGpp levels (where RNase E-R169Q, Δ CTH is viable) compared to that at intermediate ppGpp levels (*relA*⁺ *spoT*⁺) where the strain is inviable. Ryals et al. (72) had reported a small increase in *rnr* transcription in *relA* mutants during exponential growth relative to that in *relA*⁺ strains, with this difference becoming more pronounced during the stringent response. Likewise, Barker et al. (73) had also shown that *rnr* transcription in Δ *relA* Δ *spoT* mutants is only marginally elevated compared to that in the wild-type strain.

The second line of evidence in support of our hypothesis is that DksA overexpression suppressed RNase E-R169Q, Δ CTH lethality at both intermediate and low ppGpp levels (Table 5). Overproduction of DksA reduces transcription initiation from the *rnrB* P1 promoter in either the presence or absence of ppGpp (65). Conversely, deletion of *dksA* is associated with an increase in transcription from the *rnrB* P1 promoter during steady-state growth (65, 74). Consistent with these reports, we find that the RNase E-R169Q, Δ CTH double mutant, like its *rho* and *nusG* derivatives, is inviable upon DksA depletion even at high basal ppGpp levels (Table 4).

The third and most important finding to support the notion that reduction of stable RNA levels is the cause of suppression is that deletion of four or five *rnr* operons suppressed both RNase E-R169Q, Δ CTH and RNase E- Δ CTH Δ RppH lethality (Table 6). The decrease in *rnr* operon copy number is correlated with decreases both in the RNA/protein ratio and in growth rates in nutrient-rich media (75, 76). At the same time, we have also shown that it is not a reduction in growth rate *per se* which is responsible for suppression of inviability in strains with limiting RNase E.

Why do perturbations expected to lower stable RNA levels rescue strains with limiting RNase E activity? Stable RNA constitutes the majority of total RNA in the cell (77), and the requirement of RNase E for processing of stable RNA is high compared to that needed for degradation of mRNAs; it is estimated that in each generation RNase E makes around 400,000 and 90,000 cleavages, respectively, for processing of rRNA/

tRNA and for degradation of mRNA (5). We therefore speculate that in the mutants with limiting RNase E activity, a reduction in stable RNA levels leads to diversion of the enzyme activity to degrade mRNA and thus to restore viability.

The reduction in *rnr* transcript levels is greater with stringent *rpoB* mutations than it is with high basal ppGpp levels (Table 3). It is perhaps for this reason that RNase R is important at high basal ppGpp levels for the suppression of RNase E-R169Q, Δ CTH lethality (Table 4) but is dispensable in the presence of the stringent *rpoB* mutations (Table 2). Based on both our earlier studies (41) and this work, it also appears that RNase E- Δ CTH Δ RppH is more defective in RNA metabolism than is RNase E-R169Q, Δ CTH, so that the former's viability is not restored at high ppGpp or high DksA levels.

Distributed functions of RNase E as the reason for its essentiality. Given that RNase E participates in processing/degradation of numerous substrates, the reason for its essentiality has remained unclear and several alternative models have been proposed in this regard, as mentioned above (19, 27, 29–31). On the basis of our present studies, we hypothesize that there is an optimal threshold level of the enzyme that is required for each of the substrate families (mRNA, rRNA, and tRNA) and that inviability can arise if there is a perturbation in fulfilling any one or more of these requirements. We believe that this model can also satisfactorily account for the discrepant observations and conclusions from other groups (19, 27–32) to explain RNase E's essentiality.

Role of RNase R in strains with limiting RNase E. Taking these findings together with our earlier results, it would appear that the viability of strains with limiting RNase E activity can be restored if sufficient mRNA turnover can be achieved in the cells. Thus, mutations in *rho* and *nusG*, which are postulated to provide R-loop-mediated RNA degradation (41), and a decrease in stable RNA content, which allows diversion of RNase E for mRNA turnover (this study), confer viability on strains with limiting RNase E activity.

In this context, our finding that suppression of RNase E-R169Q, Δ CTH is abolished in *rnr* mutants can perhaps be explained as follows. RNase R is reported to degrade full-length mRNA in various situations, such as in stationary phase (78) and under RNase E-deficient conditions (79). The levels of RNase R are also elevated in different *rne* mutants (80). Accordingly, we speculate that RNase R also contributes to mRNA degradation, especially in cells with limiting RNase E activity.

MATERIALS AND METHODS

Growth media, bacterial strains, and plasmids. LB medium and glucose-minimal A (MM) medium were routinely used as rich and defined media (81) with appropriate concentrations of antibiotics, namely, ampicillin (Amp) (40 μ g/ml for plasmid and 20 μ g/ml for chromosomal marker), kanamycin (Kan) (25 μ g/ml), chloramphenicol (Cm) (30 μ g/ml), trimethoprim (Tp) (60 μ g/ml), and tetracycline (Tet) (15 μ g/ml), wherever necessary. X-Gal was used at 50 μ g/ml, and the isopropyl- β -D-thiogalactoside (IPTG) concentration varied from 2 μ M to 100 μ M according to the requirement of the experiment. Strains were routinely grown at 37°C.

The *E. coli* K-12 strains used in this work are listed in Table S1 in the supplemental material. The C-terminal half truncated derivative of RNase E that is used in our work is 493 amino acids long and is designated RNase E- Δ CTH. The RNase E-R169Q, Δ CTH double mutant carries RNase E- Δ CTH with an R169Q mutation, and the Δ RppH RNase E- Δ CTH double mutant is devoid of RppH and carries RNase E- Δ CTH.

Several plasmids used in this study are derivatives of pMU575, which is an unstable single-copy-number plasmid with an IncW replicon and Tpr^r antibiotic marker: pHYD1613 (*rne*⁺) (41), pHYD2411 (*Salmonella enterica* serovar Typhimurium *rho*⁺ gene) (55), and pHYD2385 (*Salmonella* Typhimurium *nusG*⁺) (J. Krishna Leela, unpublished data). Other plasmids (with salient features in parentheses) employed include pHYD2373 (pWSK129 derivative carrying *rne*-R169Q, Δ CTH [encoding RNase E-R169Q, Δ CTH]), the pSC101 replicon, Kan^r, and Cm^r (41), pRne-SG21 (pWSK129 derivative carrying *rne*-R169Q,529 Δ [encoding RNase E-R169Q,529 Δ]), the pSC101 replicon, and Kan^r (40), pCP20 (pSC101-based Ts replicon encoding Flp recombinase, Cm^r, and Amp^r) (82), pJK537 (1.8-kb fragment carrying *dksA* cloned in pBR322, pMB1, and Amp^r) (83), and pHR53 (pJK537 derivative carrying truncated *dksA* gene, pMB1, and Amp^r) (84).

Screening methodology for synthetic lethal phenotypes. A “shelter plasmid loss assay” using an unstable single-copy-number plasmid (85) based on vector pMU575, which is Tpr^r and *lacZ*⁺, was routinely employed as described previously to check whether particular mutant combinations in strains were lethal (41). For example, a Δ *lac* but otherwise wild-type strain carrying plasmid pHYD1613 (a pMU575 derivative carrying *rne*⁺) yields ~10% plasmid-free white colonies on X-Gal-containing medium, whereas a Δ *rne* strain fails to yield viable white colonies. Serial dilutions of stationary-phase cultures

carrying the pMU575 derivative grown in LB-Tp were plated on LB with X-Gal (without Tp) to obtain ~200 colonies, and the ratio of white to total colonies was determined.

Immunoblot analysis of *in vivo* RNase E levels. Procedures for lysate preparation, electrophoresis through sodium dodecyl sulfate-polyacrylamide gels, electroblotting to a polyvinylidene difluoride (PVDF) membrane, and staining with amido black (to determine the quantity of protein loading in different lanes) were as described earlier (41). Blocking of PVDF membrane was done with 5% fat-free milk, and the membrane was probed with primary anti-RNase E antibody (rabbit, polyclonal; a gift from A. J. Carpousis), washed, and probed with secondary antibody (anti-rabbit IgG raised in goat) conjugated to horseradish peroxidase as described previously (86). The membrane was then washed and probed for antibody-reactive bands with the aid of a chemiluminescence detection system according to the manufacturers' protocols (Amersham ECL Prime and Sigma Chemical Co., St. Louis, MO).

Other techniques. Procedures for P1 transduction (81), *in vitro* DNA manipulations and transformation (86), and β -galactosidase assays (81) were as described previously. Quantitation of the extent of polarity relief in *rho* and *nusG* mutants by using P_{lac} -*lacZ* and $P_{lac-t_{R1}}$ -*lacZ* (where t_{R1} is a Rho-dependent terminator from lambdaoid phage H19B) fusion construct pairs was performed as reported previously (57). Derivatives with an autoregulated *rne-lac* fusion were used to determine the status of cellular RNase E activity as described previously (41). An *rnmB* P1-*lacZ* construct was used for measurements of transcriptional expression and regulation of the *rnm* operons (58). The *dksA*, *mr*, *pnp*, *rnb*, *crp*, *hfq*, and *rppH* gene deletions were sourced from the Keio collection (87).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00724-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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