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Two pathways for RNase E action in *Escherichia coli in vivo* and bypass of its essentiality in mutants defective for Rho-dependent transcription termination

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Summary

The endonuclease RNase E of *Escherichia coli* is essential for viability, but deletion of its C-terminal half (CTH) is not lethal. RNase E preferentially acts on 5′-monophosphorylated RNA whose generation from primary transcripts is catalysed by RppH, but ΔRppH strains are viable. Here we show that the RNase E-ΔCTH ΔRppH combination is lethal, and that the lethality is suppressed by *rho* or *nusG* mutations impairing Rho-dependent transcription termination. Lethality was correlated with defects in bulk mRNA decay and tRNA processing, which were reversed by the *rho* suppressor. Lethality suppression was dependent on RNase H1 or the helicase UvsW of phage T4, both of which act to remove RNA–DNA hybrids (R-loops). The *rho* and *nusG* mutations also rescued inviability of a double alteration R169Q (that abolishes 5′-sensing) with ΔCTH in RNase E, as also that of conditional RNase E deficiency. We suggest that the ΔCTH alteration leads to loss of a second 5′-end-independent pathway of RNase E action. We further propose that an increased abundance of R-loops in the *rho* and *nusG* mutants, although ordinarily inimical to growth, contributes to rescue the lethality associated with loss of the two RNase E cleavage pathways by providing an alternative means of RNA degradation.

Introduction

The endoribonuclease RNase E is conserved among the proteobacteria and is essential for viability in *Escherichia coli*. Encoded by the *rne* gene, it is a homotetramer of a 1061-amino acid-long polypeptide that is comprised of an N-terminal half with the catalytic domain and a C-terminal half (CTH) that serves as a scaffold for assembly of a protein complex called the degradosome (see Deutscher, 2006; Carpousis, 2007; Condon, 2007; Carpousis *et al*., 2009; Arraiano *et al*., 2010; Kaberdin *et al*., 2011 for reviews). In addition to RNase E, the degradosome includes as its major stoichiometric components an RNA helicase (RhlB), a phosphorolytic 3′-5′ exoribonuclease (polynucleotide phosphorylase or PNPase) and a glycolytic enzyme (enolase). The assembled degradosome by itself is not essential for viability as deletion of the CTH of RNase E (ΔCTH) does not confer lethality in *E. coli*. Other proteins have also been reported to bind to the CTH of RNase E in sub-stoichiometric amounts and to assemble in alternative degradosome complexes; these proteins include RhlE, CsdA, SrmB, ribosomal protein L4, Hfq, DnaK, GroEL, polyphosphate kinase, poly-A polymerase, MinD, RraA and RraB (reviewed in Carpousis, 2007; Arraiano *et al*., 2010; Kaberdin *et al*., 2011).

RNase E acts on a large variety of RNA molecules including mRNA, small non-coding RNA, and precursors of tRNA and rRNA (reviewed in Deutscher, 2006; Condon, 2007; Carpousis *et al*., 2009; Arraiano *et al*., 2010). Deficiency of RNase E *in vivo* is associated with prolongation of half-lives of more than 1500 mRNAs (Bernstein *et al*., 2004), as well as defective processing and maturation of several tRNA and rRNA species (Lopez *et al*., 1999; Li and Deutscher, 2002; Ow and Kushner, 2002; Perwez *et al*., 2008). As the 5′-untranslated region of *rne* mRNA is itself a substrate for cleavage by RNase E, the activity of the enzyme is subject to autoregulation in *E. coli* (Jain and Belasco, 1995; Jiang *et al*., 2000; Sousa *et al*., 2001; Schuck *et al*., 2009). Another well-studied substrate of RNase E is RNA-I, which is involved in antisense replication control of plasmids of the ColE1 family (Tomcsanyi and Apirion, 1985; Lin-Chao and Cohen, 1991; McDowall *et al*., 1994).

In light of the multitude of its substrates, various alternative hypotheses have been proposed to explain why RNase E is essential for viability, such as its role in turnover of bulk or particular species of mRNA, generation of mature tRNAs and rRNAs, or as yet unidentified functions (Ow *et al*., 2000; Lee *et al*., 2002; Li and Deutscher, 2002;

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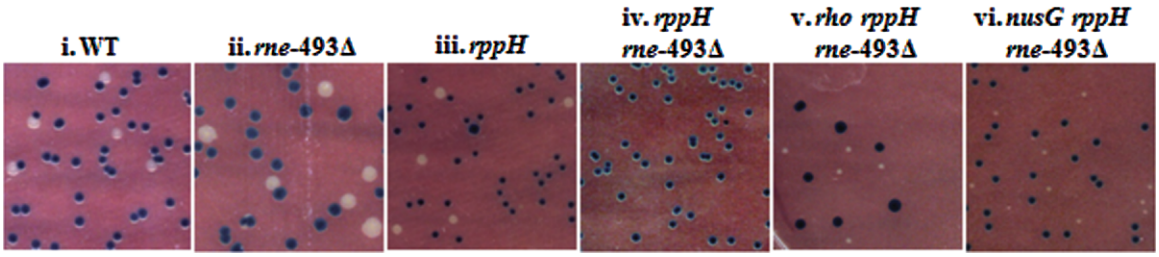

Fig. 1. Inviability of Δ*rppH rne*-493Δ strains and rescue by *rho*-A243E or *nusG*-G146D mutations. Strains with the indicated genotypes (all Δ*lac*) and carrying the unstable plasmid pHYD1613 (*rne*$^{+}$ *lacZ*$^{+}$ Tp^{R}) were grown to stationary phase in LB with Tp and plated at a suitable dilution on LB with Xgal (without Tp). Strains for the different panels were pHYD1613-bearing derivatives of: i, GJ3161; ii, GJ5108; iii, GJ6964; iv, GJ9592; v, GJ6967; and vi, GJ6968.

Ow and Kushner, 2002; Deana and Belasco, 2004; Takada *et al*., 2005; Tamura *et al*., 2006; Perwez *et al*., 2008; Chung *et al*., 2010). *E. coli* also possesses a second endonuclease RNase G which is paralogous to the N-terminal half of RNase E (Li *et al*., 1999; Wachi *et al*., 1999); RNase G is dispensable for viability, but certain variants of the protein can substitute for the essentiality of RNase E in the cell (Lee *et al*., 2002; Deana and Belasco, 2004; Chung *et al*., 2010).

Although RNase E is an endonuclease, its activity is strongly affected by the nature of the 5′-end of the substrate, with 5′-monophosphorylated RNA being the species most efficiently cleaved by the enzyme (Mackie, 1998; Jiang *et al*., 2000). Because all primary transcripts contain a 5′ triphosphate, the latter's prior conversion to 5′-monophosphate increases the efficiency with which RNase E catalyses its first cleavage of many substrates *in vivo*, and an enzyme RppH has been identified that possesses such RNA 5′-pyrophosphohydrolase activity (Celesnik *et al*., 2007; Deana *et al*., 2008). The crystal structure of the N-terminal half of RNase E has also revealed the existence of a pocket to accommodate the 5′-monophosphate moiety that includes a critical Arg residue at position 169 of the protein (Callaghan *et al*., 2005; Koslover *et al*., 2008). Consistent with this finding, an R169Q variant of RNase E fails to be activated by 5′-monophosphate in the RNA substrate (Garrey *et al*., 2009).

Unlike a null *rne* mutant that is inviable, both the null *rppH* and the *rne*-R169Q mutants are viable (Deana *et al*., 2008; Garrey *et al*., 2009), suggesting that RNase E can be sufficiently catalytically active *in vivo* even in absence of the activating effect of 5′-monophosphate. At the same time, transcriptome data indicate that the null *rppH* mutant does exhibit prolongation of half-lives of a significant number of mRNA species in *E. coli* (Deana *et al*., 2008). RNase G is also activated by 5′-monophosphate in much the same way as RNase E (Tock *et al*., 2000).

In this study, we show that although neither of the individual alterations RNase E-ΔCTH or ΔRppH is lethal, a combination of the two confers inviability. Furthermore, this synthetic lethality is suppressed or rescued by several mutations in the genes *rho* or *nusG* that are associated with defective factor-dependent (also called Rho-dependent) transcription termination (for reviews, see Adhya and Gottesman, 1978; Richardson, 2002; Roberts *et al*., 2008; Sen *et al*., 2008; Peters *et al*., 2011). The suppression required the presence in the strains of either RNase H1 (which hydrolyses RNA in R-loops, that is, RNA–DNA hybrids) or UvsW (an R-loop helicase of phage T4; see Carles-Kinch *et al*., 1997; Dudas and Kreuzer, 2001; Nelson and Benkovic, 2007; Kreuzer and Brister, 2010). The *rho* and *nusG* mutations were also able to suppress the recently described inviability of the combination of ΔCTH and R169Q mutations in RNase E (Garrey and Mackie, 2011), as well as the inviability of conditionally expressing *rne* strains at the restrictive conditions. Our results serve to confirm the existence of a 5′-end-independent pathway of RNase E action that requires its CTH, and furthermore suggest that an R-loop-dependent pathway of RNA degradation in *rho* and *nusG* mutants can serve as a bypass pathway in RNase E-deficient cells.

Results

Synthetic lethality of RNase E-ΔCTH with ΔRppH

Strains bearing either RNase E-ΔCTH or ΔRppH are viable, but we found that the combination conferred a lethal phenotype. The initial demonstration employed an RNase E variant truncated after amino acid 493 (RNase E-493Δ), that was obtained in this study as described in *Experimental procedures*. An unstable plasmid pHYD1613 bearing *rne*$^{+}$ (and is also *lacZ*$^{+}$, hence colonies of a Δ*lac* strain bearing it are blue on Xgal medium while plasmid-free colonies are white) is spontaneously lost at about 10% frequency from wild-type, RNase E-493Δ or ΔRppH strains (see Fig. 1, panels i to iii; data quantified in Table 1, strains GJ3161, GJ5108 and GJ6964, respectively). On the other hand, with the ΔRppH RNase E-493Δ double mutant [Table 1 (GJ9592), and Fig. 1, panel iv], no plasmid-free colonies were obtained indicating that the combination was

Table 1. Proportion of cells in different strains exhibiting spontaneous loss of *rne*$^+$ *lac*$^+$ Tp^R plasmid pHYD1613[a].

Strain	Relevant genotype[b]	Lac$^-$ colonies/total (Ratio)
GJ3161	Wild type	19/159 (0.11)
GJ5108	*rne*-493Δ	35/145 (0.24)
GJ6964	Δ*rppH*	11/176 (0.06)
GJ9592	*rne*-493Δ Δ*rppH*	0/371 (< 0.003)
GJ9595[c]	*rne-131* Δ*rppH*	0/551 (< 0.002)
GJ9596	*rne-Δ23* Δ*rppH*	76/1000 (0.076)
GJ13701	Δ*rppH* Δ*pnp*	43/473 (0.09)
GJ13702	Δ*rppH* Δ*rhlb*	79/451 (0.17)
GJ13703	Δ*rppH* Δ*pnp* Δ*rhlB*	60/530 (0.11)
GJ6967	*rne*-493Δ Δ*rppH rho*	11/53 (0.21)
GJ6968	*rne*-493Δ Δ*rppH nusG*	32/152 (0.21)
GJ13713	*rne*-493Δ Δ*rppH* Δ*rng rho*	36/246 (0.15)
GJ13714	*rne*-493Δ Δ*rppH* Δ*rng nusG*	41/181 (0.23)
GJ12087/pSG-Rne-R169Q	*rne*-R169Q	38/434 (0.08)
GJ12087/pHYD2373	*rne*-R169Q,493Δ	0/371 (< 0.003)
GJ12175/pSG-Rne-R169Q	*rne*-R169Q Δ*rhlB* Δ*pnp*	35/413 (0.08)
GJ12089/pHYD2373	*rho rne*-R169Q,493Δ	30/174 (0.17)
GJ12088/pHYD2373	*nusG rne*-R169Q,493Δ	8/131 (0.06)
GJ9592/pHYD1810	Δ*rppH rne*-493Δ/*rppH*$^+$	37/502 (0.07)
GJ12089	Δ*rne rho*	0/939 (< 0 .001)
GJ12166	Δ*rne* Δ*rppH rho*	0/522 (< 0.001)

a. Derivatives of the indicated strains carrying plasmid pHYD1613 were grown and plated on Xgal medium without Tp supplementation as described in the legend to Fig. 1, and numbers of total and of Lac$^-$ (white) colonies for each were determined.
b. Designations *rho* and *nusG* refer to *rho*-A243E and *nusG*-G146D mutations respectively.
c. GJ9595 yielded Lac$^-$ microcolonies (at a ratio of 0.09), which, however, failed to grow upon re-streaking.

synthetically lethal. The synthetic lethality was complemented by a plasmid bearing *rppH*$^+$ (Table 1, GJ9592/pHYD1810).

Synthetic lethality was also observed of ΔRppH with another RNase E-ΔCTH variant (truncated after residue 584) encoded by the *rne-131* allele (Lopez *et al*., 1999), and in this case small white colonies that had lost plasmid pHYD1613 were obtained, which could not be subcultured (Table 1, GJ9595). On the other hand, a smaller truncation Δ585–693 internal to RNase E-CTH encoded by *rne-Δ23* (Leroy *et al*., 2002) was not lethal with ΔRppH (Table 1, GJ9596).

Deficiency of other degradosome components is not lethal with ΔRppH

The CTH of RNase E serves as scaffold for assembly of the degradosome, and we considered the possibility that it is the failure of degradosome formation or function that renders RNase E-ΔCTH lethal with ΔRppH. However, ΔRppH strains were viable even after two major components of the degradosome, PNPase and RhlB, had been deleted either individually (Table 1, strains GJ13701 and GJ13702 respectively) or simultaneously (Table 1, GJ13703). (The third component, enolase, could not be tested because it is an essential glycolytic enzyme in *E. coli*.) These results indicate that RNase E-ΔCTH lethality with ΔRppH is likely not because of a defect in degradosome assembly or function.

Suppression of RNase E-ΔCTH ΔRppH lethality by rho *and* nusG *mutations*

The proteins Rho and NusG are critically important for Rho-dependent transcription termination, and are essential for viability (reviewed in Adhya and Gottesman, 1978; Richardson, 2002; Roberts *et al*., 2008; Sen *et al*., 2008; Peters *et al*., 2011); however, NusG is dispensable in a 'reduced genome' *E. coli* strain MDS42 (Cardinale *et al*., 2008). The missense mutations *rho*-A243E and *nusG*-G146D are both associated with a greatly reduced efficiency of transcription termination (Harinarayanan and Gowrishankar, 2003; Chalissery *et al*., 2011; Saxena and Gowrishankar, 2011a,b).

We were able to show by a number of approaches that these two mutations could suppress the inviability associated with RNase E-493Δ in combination with ΔRppH. First, in the assay involving *rne*$^+$ plasmid pHYD1613, viable plasmid-free segregants were obtained with the *rho* or *nusG* derivatives of the ΔRppH RNase E-493Δ strain [Table 1 (GJ6967, GJ6968); Fig. 1, panels v and vi]. In a second approach, the Δ*rppH*::Kan allele was transduced into a pair each of the *rho* and *nusG* mutant strains (carrying the cognate *rho*$^+$ or *nusG*$^+$ gene on an IPTG-dependent Amp^R replicon) and bearing either *rne*$^+$ or *rne*-493Δ; in all four instances, Kan^R transductants were obtained on IPTG-supplemented plates but upon subsequent testing, those from the *rne*-493Δ derivatives failed to grow on medium with IPTG and Amp (that is, where the

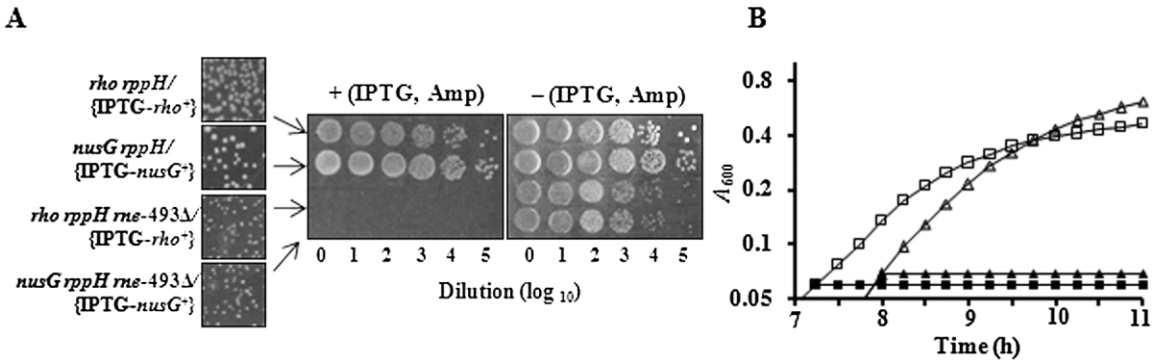

Fig. 2. Inviability of Δ*rppH rne*-493Δ strains and rescue by *rho*-A243E or *nusG*-G146D mutations.
A. The column of four panels at left shows colonies of the indicated genotypes that were obtained following P1 transduction of Δ*rppH*::Kan into derivatives of strains GJ3110 (*rho*), GJ3107 (*nusG*), GJ5073 (*rho rne*-493Δ), and GJ5123 (*nusG rne*-493Δ) carrying the Amp^R IPTG-dependent plasmids with cognate *rho*$^+$ (pHYD1201) or *nusG*$^+$ (pHYD751) genes, as appropriate. Transductants were selected on Amp- and IPTG-supplemented LB plates and then subcultured by spotting at dilutions on a pair of LB plates without or with Amp and IPTG supplementation, as shown on the pair of panels at right.
B. Cells of the strains GJ9593 (■, □) and GJ9594 (▲, △) that are, respectively, *rho* and *nusG* derivatives of a Δ*rppH rne*-493Δ strain and carrying the cognate P_{ara}-*rho*$^+$ or P_{ara}-*nusG*$^+$ constructs were grown to stationary phase in LB with 0.2% glucose, washed, and inoculated at 10^5 cells ml^{-1} into fresh LB supplemented with either 0.2% glucose (open symbols) or 0.2% Ara (closed symbols), for growth rate measurements. In the curves shown, A_{600} values for the Ara-grown cultures remained below 0.05 and have been arbitrarily adjusted to 0.06.

rho$^+$ or *nusG*$^+$ plasmid would be retained) and were viable only on medium without IPTG (Fig. 2A).

Finally, in ΔRppH RNase E-493Δ strains with either the *rho* or *nusG* mutation and the cognate P_{ara}-*rho*$^+$ or P_{ara}-*nusG*$^+$ construct integrated in single copy on the chromosome, growth was observed only in medium not supplemented with Ara (that is, when *rho*$^+$ and *nusG*$^+$ are not expected to be expressed) (Fig. 2B). In LB medium, doubling times for the *rho* and *nusG* suppressed derivatives of the ΔRppH RNase-493Δ strain were each around 48 min, while those for the wild-type strain and the viable single and double mutant combinations ranged from around 20 to 35 min (Table 2). However, the suppressed strains failed to grow in defined media (data not shown). Interestingly, the *rho rppH* double mutant exhibited a growth rate in LB medium that was faster than that for either of the single mutants *rho* or *rppH* (Table 2), the reasons for which are at present not known.

Table 2. Growth rates of strains with Δ*rppH, rne*-493Δ, *rho*-A243E and *nusG*-G146D mutations[a].

Mutation	Wild type	*rho*-A243E	*nusG*-G146D
Nil	19	29	24
rne-493Δ	21	35	36
Δ*rppH*	28	20	30
Δ*rppH rne*-493Δ	>300[b]	48	47

a. Values given are doubling times (in min) in LB, determined as averages from three replicate cultures. Strains employed were (in order, wild type, *rho*-A243E, *nusG*-G146D): Nil, GJ3161, GJ3110, GJ3107; *rne*-493Δ, GJ5108, GJ5073, GJ5123; Δ*rppH*, GJ6964, GJ6965, GJ6966; and Δ*rppH rne*-493Δ (*rho, nusG* only), GJ6967, GJ6968.
b. Estimated from the conditional lethal experiments following Ara-supplementation to cultures of GJ9593 and GJ9594, as described in Fig. 2B.

Several observations pointed to the generality of this suppression by *rho* and *nusG* mutations of ΔRppH RNase E-493Δ lethality. Thus, the *rho*-A243E and *nusG*-G146D mutations also suppressed the lethality of ΔRppH with RNase E-584Δ that is encoded by *rne-131* (data not shown). Furthermore, various other missense *rho* mutations (G51V, G53V, Y274D, P279L, P279S, G324D, N340S or I382N) that interfere with Rho-dependent termination (Chalissery *et al.*, 2007) were able to suppress the ΔRppH RNase E-493Δ lethality to the same extent as did the *rho*-A243E allele (Fig. 3A).

We also tested for suppression by a null mutation in *nusG* in the MDS42 background (Cardinale *et al.*, 2008), for which a pair of isogenic *nusG*$^+$ and *nusG*::Kan derivatives of MDS42 *rppH rne*-493Δ carrying *rne*$^+$ and *nusG*$^+$ on Ts and IPTG-dependent replicon plasmids respectively were plated on either ITPG-supplemented medium at 30°C (positive controls) or without IPTG at 39°C (where neither plasmid will replicate). When colonies from the latter plates were subcultured, only those from the *nusG*::Kan derivative exhibited viability (Fig. 3B), indicating that the null *nusG* allele suppresses ΔRppH RNase E-493Δ lethality.

Several RNase E substrates exhibit defective processing in vivo *in the synthetic lethal mutant that is reversed upon suppression by* rho

The substrates for RNase E include mRNA and various precursors of tRNA and rRNA, and we determined the efficiency of RNase E action *in vivo* in various strains

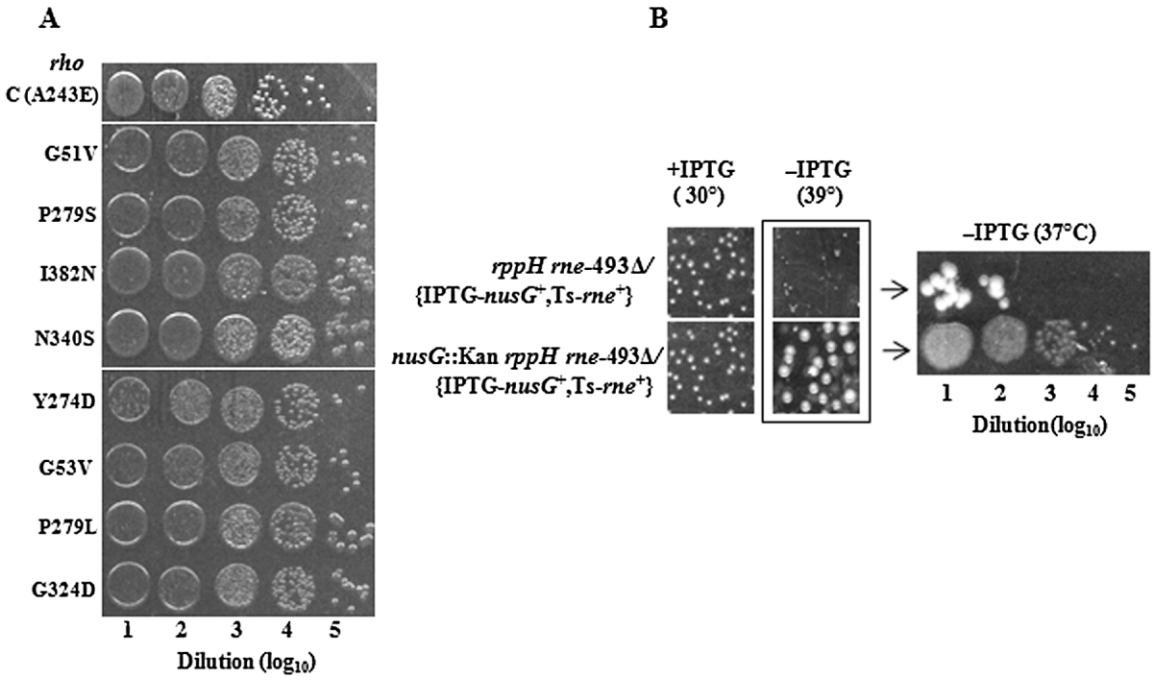

Fig. 3. Suppression of ΔRppH-RNase E-493Δ inviability by other *rho* mutations and by Δ*nusG*.
A. Derivatives of strain GJ13708 (Δ*rho*::Kan *rne*-493Δ Δ*rppH*) carrying the Sp^R plasmids with different *rho* mutations as indicated (Chalissery *et al*., 2007) were subcultured by spotting at dilutions on LB supplemented with Sp, as shown. No transformants were obtained with the *rho*$^+$ plasmid control. The top row [C (A243E)] depicts the control strain GJ6973 (*rho*-A243E *rne*-493Δ Δ*rppH*) transformed with the Sp^R vector plasmid pCL1920.
B. Isogenic *nusG*$^+$ (GJ9523) and *nusG*::Kan (GJ13710) derivatives of MDS42 Δ*rppH rne*-493Δ carrying plasmids pHYD751 (*nusG*$^+$ on Amp^R IPTG-dependent replicon) and pHYD2903 (*rne*$^+$ on Cm^R Ts replicon) were incubated after plating at a suitable dilution on LB medium with IPTG at 30°C (for 24 h) or without IPTG at 39°C (for 48 h), as marked. Colonies from the latter were then subcultured by spotting at dilutions on LB without IPTG followed by incubation at 37°C for 24 h.

including the ΔRppH RNase E-493Δ synthetic lethal mutant and its *rho*-suppressed derivative. For this purpose, a conditional lethal approach was employed as described in *Experimental procedures*, in which expression of P_{ara}-*rho*$^+$ was induced to confer lethality (restrictive condition) in a strain (GJ9593) carrying the synthetic lethal combination along with a chromosomal *rho*-A243E mutation; a culture of the same strain grown without Ara represented one in which the lethality had been suppressed by the *rho* mutation (permissive condition).

Bulk mRNA chemical half-lives were determined, as described in *Experimental procedures*, in cultures of the strain GJ9593 grown under the restrictive and permissive conditions (that is, with *rho*$^+$ and *rho*-A243E, respectively); the values obtained were compared with those for the wild-type as well as the single mutant strains (Table 3). The data indicate that (i) the single mutant RNase E-493Δ exhibits moderate increase in bulk mRNA half-life, consistent with the results from other groups (Lopez *et al*., 1999; Bernstein *et al*., 2004), whereas ΔRppH is not significantly

Table 3. Bulk mRNA decay rates in strains with *rppH, rne* and *rho* mutations.

Strain (genotype)	Half-life (min)[a]
GJ3161 (wild type)	2.5 ± 0.6
GJ5108 (*rne*-493Δ)	3.8 ± 0.4
GJ6964 (Δ*rppH*)	2.7 ± 0.4
GJ9593 (Δ*rppH rne*-493Δ *rho*-A243E [λP_{ara}-*rho*$^+$])[b]	8.7 ± 1.2 (with Ara)
	5.6 ± 0.8 (with glucose)
GJ12103/pSG-Rne-R169Q (*rne*-R169Q)	2.6 ± 0.1
GJ12103/pHYD2373	6.4 (with Ara)
(*rne*-R169Q,493Δ *rho*-A243E [λP_{ara}-*rho*$^+$])[b]	3.8 (with glucose)

a. The bulk mRNA chemical half-life (mean ± standard error) was calculated for each strain from at least three independent experiments, as described in *Experimental procedures*. Values reported for GJ12103/pHYD2373 were obtained from a single experiment.
b. Values are given for cultures grown with Ara and with glucose (that is, with and without induction of P_{ara}-*rho*$^+$ respectively).

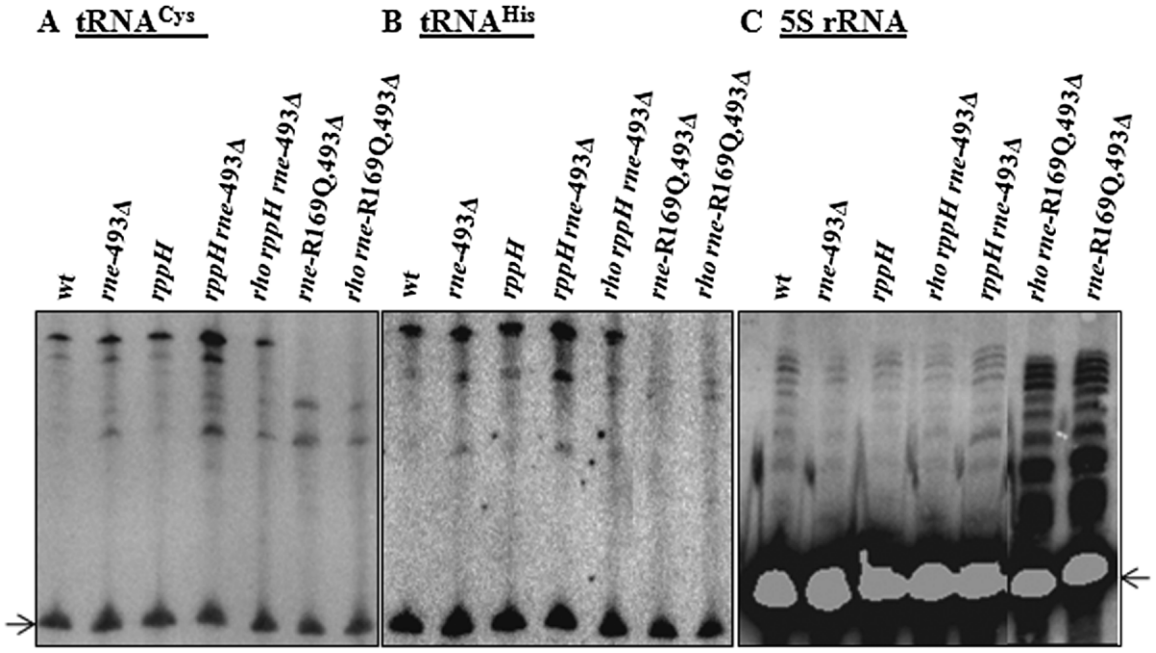

Fig. 4. Processing/maturation of stable RNA species in ΔRppH RNase E-493Δ and RNase E-R169Q,493Δ strains and their *rho* derivatives. See *Experimental procedures* for details of methods employed. Strains were: WT, GJ3161; *rne*-493Δ, GJ5108; *rppH*, GJ6964; *rppH rne*-493Δ and *rppH rne*-493Δ *rho*, GJ9593 cultured with and without 0.2% Ara, respectively; and *rne*-R169Q,493Δ and *rne*-R169Q,493Δ *rho*, GJ12103/pHYD2373 cultured with and without 0.2% Ara respectively. The mature RNA species in the panels are indicated by arrows.
A. Processing of $tRNA^{Cys}$.
B. Processing of $tRNA^{His}$.
C. Maturation of 5S rRNA.

different from the wild-type strain; (ii) the synthetic lethal combination of RNase E-493Δ with ΔRppH is more severely compromised for mRNA degradation (that is, in strain GJ9593 grown with Ara); and (iii) the *rho* suppressor mutation partially reverses the mRNA degradation defect in the synthetic lethal double mutant (that is, in strain GJ9593 grown without Ara).

We also examined the processing of tRNA and rRNA precursors in the cultures above. In the processing of both $tRNA^{Cys}$ (Fig. 4A) and $tRNA^{His}$ (Fig. 4B), the *rppH* mutant (lane 3) was not significantly different from the wild-type strain (lane 1); the *rne*-493Δ single mutant (lane 2) was moderately defective as has been reported earlier (Ow and Kushner, 2002), with evidence for accumulation of precursor species in the cells; and the synthetic lethal combination *rppH rne*-493Δ (that is, strain GJ9593 grown with Ara) was even more severely affected (lane 4). The tRNA processing defects in the double mutant were partially corrected by the *rho* mutation (that is, by culturing GJ9593 without Ara, lane 5). On the other hand, maturation of 5S rRNA from its 9S precursor (Fig. 4C) was unaffected in the two single mutants (lanes 2–3) and even in the ΔRppH RNase E-493Δ double mutant grown under the permissive (without rho^+ expression, lane 4) or the restrictive (with rho^+ expression, lane 5) conditions.

Suppression by rho *or* nusG *is neither RNase G-dependent nor correlated with alterations in RNase E activity or levels*

The results obtained so far had shown that the synthetic lethality of RNase E-ΔCTH with ΔRppH is correlated with reduced activity of RNase E on several of its substrates *in vivo* and that this reduction is apparently reversed when Rho-dependent transcription termination is rendered defective in the strain. Because defective Rho-dependent termination and the associated relief of transcriptional polarity leads to increased expression of a large number of genes (Cardinale *et al*., 2008; Peters *et al*., 2009), we considered the possibility that the suppression above is a consequence of increased synthesis and activity of RNase E (or of its paralogue RNase G) in the strains.

RNase E expression is autoregulated at the level of mRNA stability, such that β-galactosidase expression from a single-copy *rne-lac* fusion is inversely related to the activity of RNase E in the strain (Sousa *et al*., 2001). We observed that *rne-lac* expression was elevated twofold in the RNase E-ΔCTH strain compared with that in the strain with full-length RNase E (Table 4), which is consistent with the earlier data (Leroy *et al*., 2002). The *rppH* single mutant exhibited a twofold decrease in *rne-lac* expres-

Table 4. Expression of *rne-lac* in *rho, nusG, rppH* and *rne*-493Δ strains[a].

Mutation	Wild type	*rho*-A243E	*nusG*-G146D
Nil	243	278	268
rne-493Δ	490	554	510
Δ*rppH*	127	271	264
Δ*rppH rne*-493Δ	N.D.[b]	517	424

a. Values given are specific activities of β-galactosidase, expressed in the units described by Miller, (1992), in LB-grown cultures of *rne-lac* strains. Strains employed were (in order, wild type, *rho*-A243E, *nusG*-G146D): Nil, 6842, GJ9576, GJ9579; *rne*-493Δ, GJ11578, GJ9582, GJ9585; Δ*rppH*, GJ11574, GJ9586, GJ9587; and Δ*rppH rne*-493Δ (*rho, nusG* only), GJ9588, GJ9589.
b. N.D., not determined (as the indicated combination of mutations is lethal).

sion, the reasons for which are at present unclear. However, the *rho* and *nusG* mutations had no significant effect on *rne-lac* expression in the *rne*$^+$ strain, nor too in the *rne*-493Δ derivatives where the expression levels (with or without Δ*rppH*) were similar to that in the *rne*-493Δ single mutant (Table 4). Thus, the *rho* and *nusG* mutations do not alter the *in vivo* activity of RNase E or RNase E-493Δ.

Western blot experiments also demonstrated that the immunoreactive RNase E polypeptide levels were not elevated in strains with mutations that affect either RppH or Rho-dependent termination, both singly and in combination (Fig. 5); this was true for both the *rne*$^+$ and *rne*-493Δ derivatives (Fig. 5A and B, respectively), although the band intensities in the latter were weaker because of poor reactivity of the polyclonal anti-RNase E antibody against RNase E-ΔCTH (Leroy *et al*., 2002; Garrey and Mackie, 2011). Finally, suppression by *rho* or *nusG* of ΔRppH RNase E-493Δ lethality was observed even in a Δ*rng* derivative (lacking RNase G), indicating that the latter enzyme is not required for this effect (Table 1, GJ13713 and GJ13714).

Requirement for R-loop (RNA–DNA hybrid) removing enzymes in suppression

Based on several lines of genetic evidence including (i) over-replication of R-loop-dependent ColE1-family plasmids in *rho* and *nusG* strains and (ii) the synthetic lethalities of these mutations with deficiencies of R-loop removing enzymes such as RNase H or RecG, it has been proposed earlier that strains with inefficient Rho-dependent transcription termination suffer an increased abundance of RNA–DNA hybrids or R-loops (Harinarayanan and Gowrishankar, 2003; Gowrishankar and Harinarayanan, 2004). As the results above had indicated that neither the expression nor activity of RNase E is altered in *rho* and *nusG* strains, we considered the alternative hypothesis that suppression by these mutations of the lethality associated with ΔRppH RNase E-ΔCTH is because of a bypass pathway for RNA degradation offered by the increased occurrence of R-loops in these strains.

We found that suppression by the *rho* or *nusG* mutations of lethality of RNase E-493Δ with ΔRppH was diminished in derivatives that were also deficient for RNase H1, which specifically degrades RNA in RNA–DNA hybrids. For example, in an experiment in which RNase H1 was expressed solely from a chromosomal P_{ara}-*rnhA* construct, the growth rates of *rho* and *nusG* derivatives of a ΔRppH RNase E-493Δ strain were reduced upon RNase H1 depletion (that is, in cultures without Ara) compared with the growth rates in RNase H1-proficient cultures (with Ara supplementation) (Fig. 6A); this effect was more pronounced in the *rho*-suppressed strain. In further genetic experiments, we noted that the quadruple mutant strains (that is, with RNase E-493Δ; ΔRppH; Rho-A243E or NusG-G146D; and ΔRNase H1) were viable in the presence of plasmids expressing either RNase H1 itself (complementation), or the R-loop helicase UvsW from phage T4, but not with the vector plasmids or one expressing RecG (Fig. 6B). Neither RNase H1 nor UvsW expression restored viability in the ΔRppH RNase E-493Δ strain (that is, in the absence of *rho* or *nusG* mutations) (data not shown). These data indicate that one of the R-loop removing enzymes, RNase H1 or UvsW, is required for efficient suppression of the synthetic lethality of ΔRppH RNase E-ΔCTH by *rho* or *nusG* mutations.

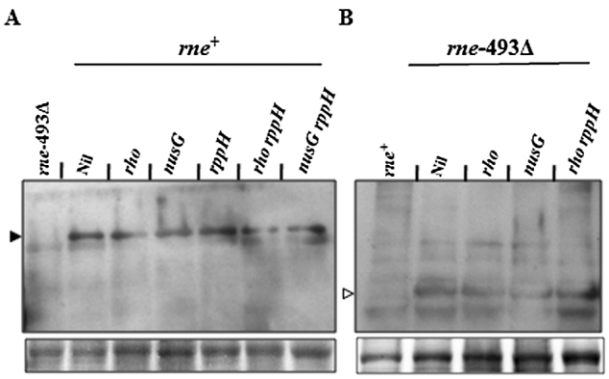

Fig. 5. Immunoblot analysis for intracellular levels of RNase E in *rne*$^+$ (A) and *rne*-493Δ (B) strains. The upper image in each panel depicts the results from immunoblotting with anti-RNase E antibody (full-length and CTH-truncated RNase E bands marked by the filled and open arrowheads in panels A and B, respectively), while the lower image is a representative section of the same blot stained with amido black to serve as loading and transfer control. The lanes at extreme left of the two panels represent negative controls (*rne*-493Δ in panel A, and *rne*$^+$ in panel B). The following strains with mutations as indicated were employed. *rne*$^+$: Nil, GJ3161; *rho*, GJ3110; *nusG*, GJ3107; *rppH*, GJ6964; *rho rppH*, GJ6965; and *nusG rppH*, GJ6966; and *rne*-493Δ: Nil, GJ5108; *rho*, GJ5073; *nusG*, GJ5123; and *rho rppH*, GJ6967.

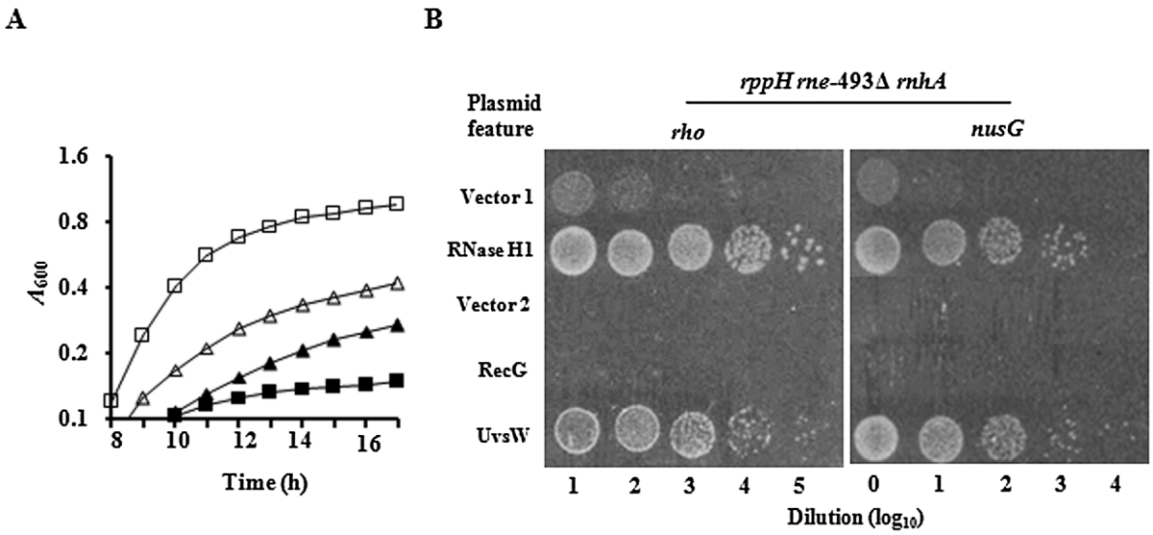

Fig. 6. Facilitation by RNase H1 and UvsW of suppression by *rho* and *nusG* mutations of ΔRppH RNase E-493Δ inviability.
A. Cells of the strains GJ13716 (■, □) and GJ13717 (▲, △) that are, respectively, *rho* and *nusG* derivatives of a *rnhA*::Cm Δ*rppH rne*-493Δ strain carrying P_{ara}-*rnhA*$^+$ were grown to stationary phase in LB with 0.2% Ara, washed, and inoculated at 10^4 cells ml^{-1} into fresh LB supplemented with 0.2% Ara (open symbols) or 0.2% glucose (closed symbols), for growth rate measurements.
B. Strains GJ9527 and GJ9551 (that are, respectively, *rho* and *nusG* derivatives of a *rnhA*::Cm *rne*-493Δ strain) carrying each of the Amp^R plasmids indicated at left were transduced with P1 (Δ*rppH*::Kan), and the Kan^R transductants were then subcultured by spotting at dilutions as shown on LB supplemented with 0.2% Ara, Kan and Amp. Plasmids used were: Vector 1, pBR322; RNase H1, pSK760; Vector 2, pBAD18; RecG, pHYD583; and UvsW, pHYD2368.

Suppression by rho *or* nusG *mutations of inviability associated with RNase E-R169Q,ΔCTH*

In *E. coli*, activation of the 5′-end-dependent pathway of RNase E function requires both the action of RppH to generate 5′-monophosphate ends on the primary transcripts as well as presence of the 5′-sensor domain in RNase E. An R169Q substitution in the 5′-sensor domain of RNase E abolishes the enzyme's activation by 5′-monophosphate, raising the possibility that it may phenocopy some of the features of an RppH deficiency. Providing support to this notion have been the findings of Garrey *et al.*, (2009) that as with ΔRppH, the RNase E-R169Q single mutant is viable, and more recently of Garrey and Mackie (2011) that the double mutant RNase E-R169Q,ΔCTH is inviable (which may be considered as analogous to the inviability of ΔRppH RNase E-ΔCTH identified in our studies above). We therefore asked whether the lethality of RNase E-R169Q,ΔCTH would also be suppressed by *rho* and *nusG* mutations.

For this purpose, we constructed an *rne* mutant allele encoding RNase E-R169Q,493Δ. This was achieved by engineering a –493Δ alteration in a plasmid (pSG-Rne-R169Q) bearing the *rne*-R169Q allele; the resultant plasmid was designated pHYD2373. In a strain with this double RNase E alteration sheltered by *rne*$^+$ on the unstable plasmid pHYD1613, no plasmid-free segregants (white colonies) were obtained, whereas such segregants were obtained in the control strain bearing RNase E-R169Q alone [Fig. 7, compare panels i and ii; Table 1 (GJ12087 with plasmids pSG-Rne-R169Q or pHYD2373)]. These results indicate that the combination of RNase E alterations R169Q with 493Δ is synthetically lethal, and hence is similar to the combination of ΔRppH and RNase E-493Δ described above and of RNase E-R169Q,584Δ described by Garrey and Mackie (2011). Again like ΔRppH, the RNase E-R169Q mutation by itself was not synthetically lethal with mutations affecting other degradosome components such as RhlB and PNPase (Table 1, GJ12175 with pSG-Rne-R169Q). Finally, as with ΔRppH RNase E-493Δ, the inviability of the RNase E-R169Q,493Δ combination was also suppressed by the *rho*-A243E or *nusG*-

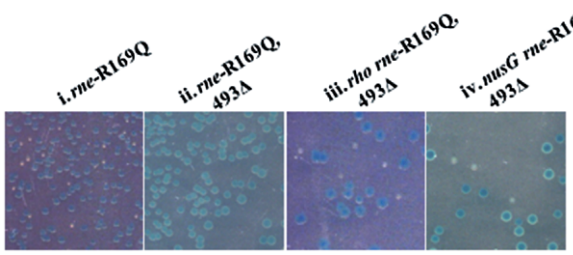

Fig. 7. Inviability of *rne*-R169Q,493Δ strains and rescue by *rho*-A243E or *nusG*-G146D mutations. See legend to Fig. 1 for description of the experimental approach. The strains for the different panels were pHYD1613-bearing derivatives of:
i, GJ12087/pSG-Rne-R169Q; ii, GJ12087/pHYD2373;
iii, GJ12089/pHYD2373; and iv, GJ12088/pHYD2373.

G146D mutations for growth on LB medium [Fig. 7, panels iii and iv; Table 1 (pHYD2373 derivatives of GJ12089 and GJ12088, respectively)], but the suppressed strains did not grow on defined media.

We also measured bulk mRNA decay rates in an RNase E-R169Q single mutant and in the RNase E-R169Q,493Δ strain with *rho*-A243E mutation and P_{ara}-*rho*$^+$ (GJ12103/pHYD2373) grown with glucose (that is, under suppressing conditions with *rho*$^+$ repressed) or Ara (with *rho*$^+$ expressed). The results given in Table 3 indicate that the bulk mRNA half-life in the RNase E-R169Q mutant (2.6 min) is similar to that in the wild-type strain (2.5 min), but that the value is substantially increased in the double mutant RNase E-R169Q,493Δ (6.4 min), which is then partially corrected by the *rho* mutation (3.8 min).

In assays to determine the efficiency of stable RNA processing in cultures of GJ12103/pHYD2373 grown without or with Ara supplementation (that is, with or without *rho* suppression of the RNase E-R169Q,493Δ lethality, respectively), we observed that the maturation of $tRNA^{Cys}$ and $tRNA^{His}$ was largely unaffected (although the precursor abundance patterns were different from those in the wild type, for reasons which are at present unclear), whereas 9S RNA processing was defective, in both the cultures (Fig. 4, last two lanes from left in each of the panels A–C). These findings for the RNaseE-R169Q,493Δ strain were quite the opposite of those described above for the ΔRppH RNase E-493Δ mutant (in which the processing of tRNAs was defective while that of 9S RNA was normal), and are discussed below. Mackie and coworkers have also previously shown that 9S RNA processing is defective while tRNA maturation is normal in the RNase E-R169Q single mutant strain (Garrey *et al*., 2009; Garrey and Mackie, 2011). Importantly, our data demonstrating that the *rho* suppressor does not correct the 9S RNA processing defect provide good evidence that lethality is not simply a consequence of inefficiency of maturation of 5S rRNA.

Our results therefore established that the lethality of strains with loss of the 5′-end-dependent pathway of RNase E action (by either R169Q substitution or ΔRppH) combined with deletion of the CTH of the enzyme can be rescued by rendering Rho-dependent transcription termination defective in these strains.

Inviability of RNase E-deficient strains is also suppressed by rho *or* nusG *mutations*

Given the essentiality of RNase E in *E. coli*, a strain in which *rne* is expressed from the P_{lac} promoter is IPTG-dependent for growth (Sousa *et al*., 2001; Jain *et al*., 2002; Leroy *et al*., 2002). A P_{lac}-*rne* strain (in which autoregulation by RNase E has been abolished) exhibits progressively lower RNase E levels as the IPTG concentration is decreased below 10 μM, and fails to grow at IPTG < 3 μM (Jain *et al*., 2002). Likewise, a P_{ara}-*rne* strain is viable only on Ara-supplemented media (Lee *et al*., 2002).

We found that introduction of the *rho*-A243E or *nusG*-G146D mutation permitted good growth of the P_{lac}-*rne* strain on IPTG-unsupplemented medium provided that the strain was also ΔRppH, with the *rho* allele being somewhat more effective in this suppression (Fig. 8A). Similarly, the *rho* mutation permitted healthy Ara-independent growth of the P_{ara}-*rne* strain, but again only if the strain was also ΔRppH (Fig. 8B).

Immunoblot experiments with anti-RNase E antibody were then undertaken with a pair of isogenic Δ*rppH* P_{lac}-*rne* strains without or with the *rho* mutation, which had been plated on media supplemented with different IPTG concentrations; the results are shown in Fig. 8C. Consistent with the observations of Jain *et al*. (2002), colonies of the *rho*$^+$ strain were obtained at concentrations of 3 μM or higher but were not observed at 1 μM IPTG, while the *rho* mutant yielded colonies even at 0 μM IPTG. The RNase E level in cells of the *rho* strain harvested from plates without IPTG was lower than that in cells of the *rho*$^+$ *rne-lac* control grown at 3 μM IPTG, even though colony growth was more robust in the former than the latter (Fig. 8C). These results therefore indicated that suppression was not achieved merely by gratuitous expression of RNase E in the *rho* mutant.

Thus, these data established that the *rho*, and to a less extent *nusG*, mutations are able to suppress the inviability associated with RNase E deficiency. At the same time, these mutations could not restore growth in either *rppH*$^+$ or Δ*rppH* strains that were Δ*rne*, that is, with an absolute deficiency of RNase E (Table 1, strains GJ12089 and GJ12166 respectively).

Discussion

Garrey and Mackie (2011) have recently reported that a strain in which the 5′-end-dependent pathway of RNase E action has been abolished by an R169Q alteration in RNase E is rendered inviable upon deletion of the CTH of RNase E. In this study, we have shown (i) that RNase E-ΔCTH is synthetically lethal also with ΔRppH (which is yet another means to inactivate the 5′-end-dependent pathway of RNase E); and (ii) that the inviabilities above of RNase E-ΔCTH in combination with either R169Q mutation or ΔRppH, as also the inviability associated with RNase E deficiency, is rescued by mutations affecting Rho-dependent transcription termination in *E. coli*. Each of these findings is further discussed below.

Two pathways of RNase E action

For RNase E to exhibit its property of preferential cleavage of 5′-monophosphorylated RNA in *E. coli*, it is

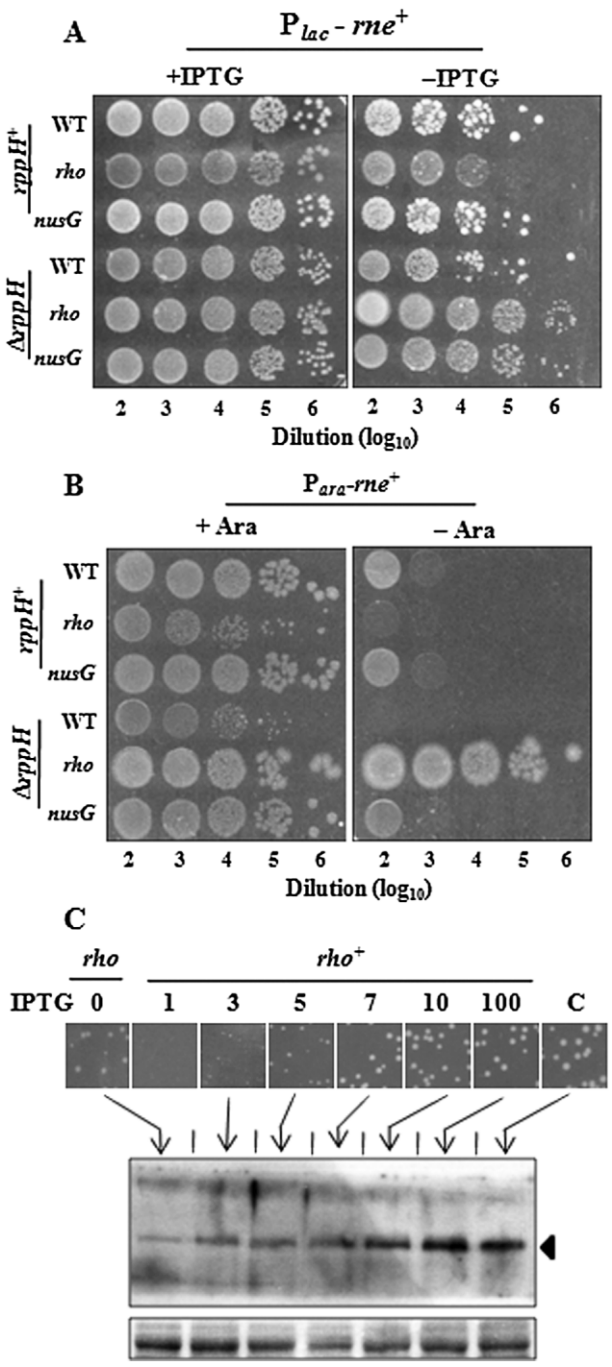

Fig. 8. Inducer-independent growth of P_{lac}-*rne* and P_{ara}-*rne* strains with *rho*-A243E or *nusG*-G146D mutations.
A. Wild-type (WT), *rho* and *nusG* derivatives, which were either *rppH*$^+$ or Δ*rppH*, of the P_{lac}-*rne* strains were grown to stationary phase in LB medium supplemented with IPTG and then spotted at dilutions on a pair of LB plates with or without IPTG, as shown. Strains used were (in order, WT, *rho, nusG* backgrounds): *rppH*$^+$, GJ6974, GJ6975, GJ6976; and Δ*rppH*, GJ6978, GJ6979, GJ6980.
B. As for panel A, except that P_{ara}-*rne* strains were used instead of P_{lac}-*rne* strains and 0.2% Ara instead of IPTG as inducer. Strains used were pBAD-RNE-carrying derivatives of (in order, WT, *rho, nusG*): *rppH*$^+$, GJ9466, GJ9467, GJ9468; and Δ*rppH*, GJ9469, GJ9470, GJ9471.
C. Immunoblot analysis for intracellular levels of RNase E in P_{ara}-*rne* strains. Cultures of strains GJ6978 and GJ6979 that are, respectively, *rho*$^+$ and *rho* derivatives of a P_{lac}-*rne* Δ*rppH* strain, were grown in LB supplemented with IPTG, washed, and plated at a suitable dilution on LB plates with the indicated IPTG concentrations (μM). Cells were harvested after 24 h from the plates shown, and intracellular levels of RNase E were determined by immunoblotting as described in *Experimental procedures*. The upper and lower panels of the blot show, respectively, the RNase E band recognized by anti-RNase E antibody (marked by arrowhead) and a representative section of the amido black-stained blot as a loading and transfer control. The last plate and lane at right, marked C, represents the control *rne*$^+$ Δ*rppH* strain GJ6964 grown in LB without IPTG.

required that the enzyme is proficient for 5′-sensing and that the 5′-triphosphorylated precursors are acted upon by RppH in the cells. Strains deficient for RppH or those that express RNase E-R169Q are viable (Deana *et al.*, 2008; Garrey *et al.*, 2009), indicating that the 5′-end-dependence for RNase E action is not absolute and that the enzyme is able to act through a second redundant pathway as well.

Based on such considerations, as also on the data from *in vitro* studies that RNase E does cleave RNA substrates without 5′-monophosphate, several groups in the last 15 years have postulated the existence of a second 'internal entry' or 'direct entry' pathway of RNase E action that may be masked by translating ribosomes (Marchand *et al.*, 2001; Baker and Mackie, 2003; Deana and Belasco, 2005; Koslover *et al.*, 2008; Kime *et al.*, 2010; Garrey and Mackie, 2011; reviewed in Dreyfus, 2009). The results reported here provide evidence in support of the conclusion that this second pathway requires the presence *in vivo* of the CTH of RNase E but not the other components of the degradosome such as PNPase and RhlB, which we accordingly refer to as the CTH-activated pathway. Furthermore, our data indicate that whereas strains with either pathway functional are viable, those with both pathways abrogated are inviable.

By *in vitro* studies, Kime *et al.* (2010) have shown that RNase E-ΔCTH can also act on some substrates through the 5′-end-independent pathway; however, they had employed a high ratio of enzyme to substrate in their assays, which may suggest (based on our genetic data) that the efficiency of this pathway is substantially higher in presence of the full-length RNase E. In other studies, Dreyfus and coworkers had shown that RNase E cleaves ribosome-free transcripts *in vivo*, and that such cleavage does not occur with RNase E-ΔCTH (Marchand *et al.*, 2001; Leroy *et al.*, 2002; Dreyfus, 2009). However, it was not established in their work whether such cleavage was 5′-end-dependent or -independent.

RppH deficiency and RNase E-R169Q mutation: similarities and differences

As discussed above, both RppH deficiency and the R169Q alteration in RNase E impair the 5′-end-dependent pathway of RNase E action *in vivo*, and the inherent similarity between these perturbations is illustrated by the two major findings of this study, namely that each of them is lethal in combination with RNase E-ΔCTH and that the lethality is suppressed by mutations affecting Rho-dependent transcription termination. At the same time, several differences also exist between them, as outlined below.

Conceptually, RppH deficiency would render inefficient only the first cleavage of a transcript by RNase E but not the subsequent cleavages (because RNase E action itself will generate substrates with 5′-monophosphate ends); on the other hand, RNase E-R169Q will be defective for all (that is, the first as well as later) cleavage reactions through the 5′-end-dependent pathway. As a corollary, an RNA molecule whose 5′-monophosphate has been generated by means other than through the action of RppH will be cleaved by RNase E as efficiently in the Δ*rppH* strain as in the wild type, whereas it would remain a poor substrate for RNase E-R169Q. This may explain our observation that 5S rRNA maturation is unaffected in the Δ*rppH* strain (Fig. 4C), because its 9S precursor is generated with 5′-monophosphate through the action of RNase III (Apirion and Gegenheimer, 1981); on the other hand, in strains with RNase E-R169Q, maturation of 5S rRNA is defective (Garrey *et al*., 2009; Garrey and Mackie, 2011; and this study, Fig. 4C). Additionally, Garrey and Mackie (2011) have reported that RNase E-R169Q expression *in vivo* is abnormally elevated because of defective autoregulation, while our studies and those of Deana *et al*. (2008) indicate that RNase E expression is not increased in the ΔRppH strains; this comparison would therefore suggest that whereas the first cleavage of the *rne* transcript by RNase E likely occurs by a 5′-end-independent mechanism (Schuck *et al*., 2009), the second and subsequent cleavages (whose efficiency will be impaired in the R169Q but not ΔRppH mutant) also contribute to its normal autoregulation (Garrey and Mackie, 2011).

Conversely, it may be expected that the degradation of transcripts that are redundantly cleaved by RNase E and RNase G (as also those by RNase G alone) will be inefficient in RppH-deficient strains (as both these RNases are activated by 5′-monophosphate) but not in a strain with RNase E-R169Q. In this context, we have found that the combined presence of RNase E-R169Q and ΔRNase G mutations confers pronounced sickness (data not shown), providing support to the notion of a redundant action of these enzymes on some of their substrates in *E. coli*. Whether this would also explain our observations (Fig. 4A and B) that maturation of $tRNA^{Cys}$ and $tRNA^{His}$ is defective in an RNase E-493Δ derivative with ΔRppH but not with the R169Q mutation is not clear, as previous reports have demonstrated no role for RNase G in processing of tRNA precursors in *E. coli* (Ow *et al*., 2003; Deana and Belasco, 2004; Chung *et al*., 2010). Other possible explanations for an altered phenotype that is observed only in the ΔRppH but not RNase E-R169Q mutant could relate either to perturbations in the former of RNA 3′-polyadenylation (which is also 5′-monophosphate-dependent; Feng and Cohen, 2000) or to the existence of additional biochemical activities and functions of RppH, as have been demonstrated for both the *E. coli* enzyme and its orthologue in *Bdellovibrio bacteriovorus* (Bessman *et al*., 2001; Steyert *et al*., 2008; Messing *et al*., 2009).

Suppression of the inviability of RNase E deficiency by mutations affecting Rho-dependent transcription termination

Transcription of protein-coding genes in *E. coli* is closely coupled to translation (Proshkin *et al*., 2010). Rho-dependent termination is the process by which translation-uncoupled transcripts both within and outside the coding regions are terminated, which requires the participation of, among others, the Rho and NusG proteins (Cardinale *et al*., 2008; Peters *et al*., 2009; Burmann *et al*., 2010). However, the detailed mechanism of Rho-dependent transcription termination remains unclear (Roberts *et al*., 2008; Sen *et al*., 2008; Peters *et al*., 2011).

We have shown in this study that a variety of mutations in *rho* or *nusG*, each associated with defective Rho-dependent termination, are able to suppress the inviability arising out of deficient RNase E activity in four different contexts: combination of ΔRppH RNase E-ΔCTH; RNase E-R169Q ΔCTH; P_{lac}-*rne* strain in medium without IPTG; and P_{ara}-*rne* strain in medium without arabinose. The latter two contexts also require that the strain be deficient for RppH and at least for the P_{lac}-*rne rho* derivative, the immunoblot data indicate that growth in medium lacking IPTG occurs despite the fact that RNase E levels are very low in these cells. A *nusA* mutation (-R258C), which has recently been shown to confer defective Rho-dependent termination (Saxena and Gowrishankar, 2011b), was also able to suppress the inviability of ΔRppH RNase E-ΔCTH (data not shown).

The suppression of ΔRppH RNase E-ΔCTH inviability by the *rho*-A243E and *nusG*-G146D mutations was characterized in detail in this study. Our results indicate that the suppression is RNase G-independent and is not also associated with alterations in either RNase E activity as determined by assay of autogenously regulated *rne-lac* expression or levels of immunoreactive RNase E

polypeptide. Furthermore, in the *rho* derivative where such experiments were undertaken, the suppression was correlated with reversal of the defects in bulk mRNA decay rate and in tRNA maturation that are exhibited by the ΔRppH RNase E-ΔCTH double mutant. These results suggest that defective Rho-dependent termination does not simply lead to increased *rne* expression and instead that it is able to provide a pathway for bypassing the essentiality of RNase E in *E. coli*. Other putative interactions between Rho and RNase E functions have been described earlier in both *E. coli* (Sozhamannan and Stitt, 1997; Sozhamannan *et al*., 1999) and other bacteria (Jäger *et al*., 2001), but their relevance to the results of the present study is unclear.

Our results also indicate that suppression of the ΔRppH RNase E-ΔCTH inviability by *rho* or *nusG* mutations is facilitated by the presence of either the R-loop hydrolase RNase H1 or helicase UvsW. Earlier studies have provided evidence for an increased occurrence of RNA–DNA hybrids or R-loops in *rho* and *nusG* mutants defective in Rho-dependent termination (Harinarayanan and Gowrishankar, 2003; Gowrishankar and Harinarayanan, 2004). Therefore, one model for the rescue of inviability associated with RNase E deficiency by mutations that compromise Rho-dependent termination is that RNA–DNA hybrids contribute to a bypass pathway of RNA degradation in these cells. There is evidence that RNase H and UvsW perform common functions in the biology of phage T4 as well (Woodworth and Kreuzer, 1996). Further, RNase E-independent and RNase H1-mediated global RNA degradation following R-loop formation has been described earlier in *topA* mutants of *E. coli* (Baaklini *et al*., 2008).

Even if one assumes that the model of increased R-loop occurrence in the *rho* and *nusG* mutants providing an alternative pathway for RNA degradation in RNase E-deficient cells is correct, several questions remain to be addressed. The first concerns the mechanism by which the abundance of R-loops is increased in mutants defective in Rho-dependent termination. It has been suggested earlier that *rho* and *nusG* mutants suffer increased R-loops from their failure to terminate nascent untranslated transcripts, which then go on to anneal with the template DNA strand in the negatively supercoiled region upstream of the moving RNA polymerase (Harinarayanan and Gowrishankar, 2003; Gowrishankar and Harinarayanan, 2004). Based on the recent finding that speed of ribosome movement directly controls the rate of transcription elongation in *E. coli* (Proshkin *et al*., 2010), one possibility that may be considered is that release of the ribosome at the termination codon after translation of a gene leads to RNA polymerase backtracking and arrest on the DNA template downstream (Dutta *et al*., 2011), and that a failure of Rho and NusG to act at this stage (Burmann *et al*., 2010) results in generation of R-loops. The absence of RNase E function and consequent stabilization of the ribosome-free transcript segment (Leroy *et al*., 2002) may also contribute to the propensity of R-loop formation under these conditions.

With the R-loop model, another issue that awaits explanation is our finding that tRNA maturation defects in the ΔRppH RNase E-ΔCTH strain are reversed by the *rho* mutation in an RNase H-dependent manner. The formation of RNA–DNA hybrids and consequent degradation of the RNA component in *rho* and *nusG* mutants, either directly by RNase H1 or indirectly by one or more exonucleases RNase II, RNase R and PNPase after unwinding by UvsW, would ordinarily be expected to account only for restoration of mRNA half-lives in the RNase E-deficient cells.

One model to explain our results is to assume that the 3′-ends of precursor tRNA transcripts also form RNA–DNA hybrids in the *rho* mutants, with RNase H1 then acting to initiate their processing and maturation. Alternatively, it is possible that the RNase H-mediated degradation of mRNA in R-loops has a sparing effect on scarce RNase E that is now made available to catalyse the maturation reactions of tRNA and rRNA in the cells. This may explain why the suppression by *rho* of inviability of P_{lac}-*rne* and P_{ara}-*rne* strains in inducer-unsupplemented medium also requires presence of the Δ*rppH* mutation, as it is likely that the rRNA and tRNA precursor substrates are more efficient in utilizing the CTH-dependent RNase E pathway than are the mRNA substrates. A complementary model of substrate competition for scarce enzyme availability has been proposed by Garrey and Mackie (2011) to explain defective processing of 9S RNA by RNase G in the RNase E-R169Q ΔCTH mutant.

It may also be noted that the *rho* and *nusG* mutations were unable to suppress the inviability of a Δ*rne* strain (Table 1). Thus, when Rho-dependent termination is defective, the need for RNase E in *E. coli* is very greatly reduced but not eliminated.

In this context, our results may also help to unify the alternative models proposed earlier as to why RNase E is essential for viability in *E. coli* (Ow *et al*., 2000; Lee *et al*., 2002; Li and Deutscher, 2002; Ow and Kushner, 2002; Deana and Belasco, 2004; Perwez *et al*., 2008; Chung *et al*., 2010). Given our interpretation that the various substrates compete with one another for RNase E (and perhaps RNase G) within the cells, we suggest that the essentiality of RNase E is dictated not just by a single category of reactions catalysed by it (and RNase G), but instead by the need for a certain minimum level of activity that is sufficient for all its reactions.

Conclusions

From the results above, we conclude that the essential endonuclease RNase E can act on its various substrates

in vivo by two redundant pathways that are 5′-end-dependent and CTH-activated respectively, and that simultaneous loss of both pathways (the former by RppH deficiency or R169Q substitution in RNase E; the latter by ΔCTH alteration of RNase E) is lethal. Furthermore, the inviability associated with RNase E deficiency is suppressed by a variety of mutations that render Rho-dependent transcription termination defective. This suppression appears to be mediated by the increased occurrence of RNA–DNA hybrids in the mutants, but the detailed mechanisms in this process remain to be determined. Thus, although R-loop formation is ordinarily growth-inhibitory in *E. coli* (Harinarayanan and Gowrishankar, 2003; Gowrishankar and Harinarayanan, 2004; Drolet, 2006; Boubakri *et al.*, 2010), in this situation it would act to suppress the inviability associated with RNase E deficiency.

Experimental procedures

Growth media, bacterial strains, phages and plasmids

The routine defined and rich growth media were, respectively, glucose-minimal A and Luria–Bertani (LB) medium, as described (Miller, 1992). Where necessary, kanamycin (Kan), ampicillin (Amp), spectinomycin (Sp) or trimethoprim (Tp) were used at 50 μg ml^{-1}, and tetracycline (Tet) or chloramphenicol (Cm) at 15 μg ml^{-1}. L-arabinose (Ara), isopropyl β-D-thiogalactoside (IPTG) and XgaI were used at 0.2%, 0.5 mM and 25 μg ml^{-1} respectively. Unless otherwise indicated, the growth temperature was 37°C.

The *E. coli* K-12 strains used in the study are described in Table 5. Phages T4gt7 (Wilson *et al.*, 1979), λ1098 for Tn*10*dTet transpositions (Miller, 1992), and λInCh for plasmid-chromosome shuttling (Boyd *et al.*, 2000), have been described earlier. The following plasmids have been described or cited in an earlier publication (Harinarayanan and Gowrishankar, 2003) from this laboratory (salient features of each in parentheses): pBR322 (pMB9 replicon, Amp^R Tet^R); pACYC184 (p15A replicon,Tet^R Cm^R); pBAD18 (pMB9 replicon, Amp^R, for Ara-induced expression of target genes); pHYD583 (pBAD18 derivative with *recG*$^+$ cloned downstream of P_{ara}, Amp^R); pMAK705 (pSC101-derived Ts replicon, Cm^R); pSK760 (pMB9 replicon carrying *rnhA*$^+$, Amp^R); pHYD751 and pHYD1201 (pMB9-derived IPTG-dependent replicons with, respectively, *nusG*$^+$ and *rho*$^+$ genes, Amp^R). Other plasmids previously described include pMU575 (IncW single-copy-number replicon with promoter-less *lacZ* gene, Tp^R) (Andrews *et al.*, 1991); pBluescriptII-KS (pMB9 replicon, Amp^R) (Stratagene, USA); pWSK30 (pSC101 replicon, Amp^R) (Wang and Kushner, 1991); pRS632 and pRS695 [pMB9 replicons, Amp^R, carrying P_{ara}-*rho* and P_{ara}-*nusG* (S60A), respectively] (Kalarickal *et al.*, 2010; Chalissery *et al.*, 2011); pSG-Rne-R169Q (pSC101 replicon with gene encoding RNase E-R169Q, Kan^R) (Garrey *et al.*, 2009); pBAD-RNE (pSC101 replicon with P_{ara}-*rne*$^+$, Kan^R) (Lee *et al.*, 2002); pRNE101 (p15A replicon with *rne*$^+$ gene, Kan^R) (Jain and Belasco, 1995); pKD3 (*ori*-R6Kγ replicon, with Cm^R cassette flanked by FRT sites, Cm^R Amp^R) (Datsenko and Wanner, 2000); and pCP20 (pSC101-based Ts replicon encoding Flp recombinase, Cm^R Amp^R) (Datsenko and Wanner, 2000). Plasmids with different mutations in the *rho* gene, as well as the *rho*$^+$ and vector (pCL1920) controls (pSC101 replicons, Sp^R), were those described in Chalissery *et al.* (2007). Plasmids that were constructed in this study are described in Table 6.

Isolation and characterization of RNase E-493Δ mutant

As mentioned above, it is known that pACYC184 transformants of the *rho*-A243E strain are inviable because of plasmid over-replication in them (Harinarayanan and Gowrishankar, 2003). Following pACYC184 transformation into a *rho*-A243E mutant, a spontaneous suppressor was identified as a survivor clone and the responsible mutation in the latter was then mapped with the aid of a transposon-tagging approach as follows.

Random transpositions of Tn*10*dTet were generated in the suppressor-bearing strain after infection with λ1098, as described (Miller, 1992). A P1 lysate was prepared on the pool of Tet^R colonies and used to transduce the *rho*-A243E mutant GJ3110 to Tet^R, followed by transformation with pACYC184. One surviving clone, designated GJ5073, was shown to carry a Tn*10*dTet insertion 76% linked to the original suppressor mutation, and the position of Tn*10*dTet was identified by an inverse-PCR approach (Higashitani *et al.*, 1994) to be in the *yceF* gene at 24.7 min on the *E. coli* chromosome (Rudd, 1998).

The *rne* gene is located in this region, and is known to regulate the replication of plasmids of the ColE1 family including pACYC184 (Lin-Chao and Cohen, 1991; Xu *et al.*, 1993; McDowall *et al.*, 1994). Therefore, we undertook PCR amplification and sequencing of the *rne* gene from the mutant, and the results showed that the sequence for codon 494 had been converted from GAA (glutamate-encoding) to TAA (chain-terminating). The RNase E variant encoded in the mutant has accordingly been designated RNase E-493Δ, and its truncated size was confirmed by immunoblotting experiments (data not shown). The mutant also exhibited several phenotypes expected of RNase E-ΔCTH, including reduced copy number of ColE1 family plasmids (Kido *et al.*, 1996) and increased expression of *lacZ* fused to a phage T7 polymerase-driven promoter (Lopez *et al.*, 1999) (data not shown).

Construction of Δ(flgK-rne)::Cm mutant

In order to construct an *rne* deletion allele that could not revert to *rne*$^+$ by recombination with plasmids such as pHYD1613 or pRNE101 that carry *rne*$^+$ on the 5.8 kb PstI fragment, the procedure of Datsenko and Wanner (2000) was used to engineer a deletion beginning from codon 1 of *rne* and extending beyond the downstream PstI site to the middle of the *flgK* gene. The deleted region was replaced with the Cm^R cassette from pKD3, and the Cm^R deletants were selected in a strain carrying an *rne*$^+$ plasmid. The primer pairs used were 5′-GACGGCAGGCAATATTGAGATCCCGGAG AAATTACTGAATACCGGGTCGCCATATGAATATCCTCCTT AG-3′ and 5′-TTAGTCGTCAATGTAAGAATAATGAGTAAGT

Table 5. List of *E. coli* K-12 strains.

Strain[a]	Genotype[b]
BW27783	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR*514 Δ(*araBAD*)*567* Δ(*rhaBAD*)*568* Δ(*araFGH*) Ø(Δ*araEp* P_{cp8}-*araE*)
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*
MDS42	derivative of wild-type with 42 defined deletions
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ3161	MC4100 *galEp3*
GJ3107	MC4100 *galEp3 nusG*-G146D
GJ3110	MC4100 *galEp3 rho*-A243E
GJ5073	GJ3110 *yceF*::Tn*10*dTet *rne*-493Δ
GJ5108	GJ3161 *yceF*::Tn*10*dTet *rne*-493Δ
GJ5123	GJ3107 *yceF*::Tn*10*dTet *rne*-493Δ
GJ6842	GJ3161 [λP_{rne}-*lac*]
GJ6964	GJ3161 Δ*rppH*::Kan
GJ6965	GJ3110 Δ*rppH*::Kan
GJ6966	GJ3107 Δ*rppH*::Kan
GJ6967	GJ3110 *yceF*::Tn*10*dTet Δ*rppH*::Kan *rne*-493Δ
GJ6968	GJ3107 *yceF*::Tn*10*dTet Δ*rppH*::Kan *rne*-493Δ
GJ6973	GJ3110 *yceF*::Tn*10*dTet Δ*rppH rne*-493Δ
GJ6974	GJ3161 (P_{lac}-*rne*,Amp) / F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ6975	GJ3110 (P_{lac}-*rne*,Amp)/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ6976	GJ3107 (P_{lac}-*rne*,Amp)/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ6978	GJ3161 Δ*rppH*::Kan (P_{lac}-*rne*,Amp)/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ6979	GJ3110 Δ*rppH*::Kan (P_{lac}-*rne*,Amp)/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ6980	GJ3107Δ*rppH*::Kan (P_{lac}-*rne*,Amp)/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]
GJ9466[c]	BW27783 Δ*rne*::Cm
GJ9467[c]	BW27783 *rho*-A243E Δ*rne*::Cm
GJ9468[c]	BW27783 *nusG*-G146D Δ*rne*::Cm
GJ9469[c]	BW27783 Δ*rppH* Δ*rne*::Cm
GJ9470[c]	BW27783 *rho*-A243E Δ*rppH* Δ*rne*::Cm
GJ9471[c]	BW27783 *nusG*-G146D Δ*rppH* Δ*rne*::Cm
GJ9523[d]	MDS42 Δ*rppH rne*-493Δ
GJ9527	GJ3110 *araD*$^+$ *rnhA339*::Cm *yceF*::Tn*10*dTet *rne*-493Δ
GJ9551	GJ3107 *araD*$^+$ *rnhA339*::Cm *yceF*::Tn*10*dTet *rne*-493Δ
GJ9576	GJ3161 *rho*-A243E [λP_{rne}-*lac*]
GJ9579	GJ3161 *nusG*-G146D [λP_{rne}-*lac*]
GJ9582	GJ3161 *rho*-A243E *rne*-493Δ *yceF*::Tn*10*dTet [λP_{rne}-*lac*]
GJ9585	GJ3161 *nusG*-G146D *rne*-493Δ *yceF*::Tn*10*dTet [λP_{rne}-*lac*]
GJ9586	GJ3161 *rho*-A243E Δ*rppH*::Kan [λP_{rne}-*lac*]
GJ9587	GJ3161 *nusG*-G146D Δ*rppH*::Kan [λP_{rne}-*lac*]
GJ9588	GJ3161 *rho*-A243E *rne*-493Δ Δ*rppH*::Kan *yceF*::Tn*10*dTet [λP_{rne}-*lac*]
GJ9589	GJ3161 *nusG*-G146D *rne*-493Δ Δ*rppH*::Kan *yceF*::Tn*10*dTet [λP_{rne}-*lac*]
GJ9592[d]	GJ3161 *rne*-493Δ Δ*rppH*::Kan *yceF*::Tn*10*dTet
GJ9593	GJ3110 *araD*$^+$ *rne*-493Δ Δ*rppH*::Kan *yceF*::Tn*10*dTet *attλ*::(P_{ara}-*rho*,Amp)
GJ9594	GJ3107 *araD*$^+$ *rne*-493Δ Δ*rppH*::Kan *yceF*::Tn*10*dTet *attλ*::(P_{ara}-*nusG*,Amp)
GJ9595[d]	GJ3161 Δ*rppH*::Kan *rne-131*
GJ9596	GJ3161 Δ*rppH*::Kan *rne-Δ23*
GJ11574	GJ3161 Δ*rppH*::Kan [λP_{rne}-*lac*]
GJ11578	GJ3161 *rne*-493Δ *yceF*::Tn*10*dTet [λP_{rne}-*lac*]
GJ12087[d]	GJ3161 *araD*$^+$ Δ(*flgK-rne*)
GJ12088[d]	GJ3107 *araD*$^+$ Δ(*flgK-rne*)
GJ12089[d]	GJ3110 *araD*$^+$ Δ(*flgK-rne*)
GJ12103[d]	GJ3110 *araD*$^+$ Δ(*flgK-rne*) *attλ*::(P_{ara}-*rho*,Amp)
GJ12166[d]	GJ3110 *araD*$^+$ Δ*rppH* Δ(*flgK-rne*)
GJ12175[d]	GJ3161 *araD*$^+$ Δ(*flgK-rne*) Δ*rhlB* Δ*pnp*
GJ13701	GJ3161 Δ*pnp* Δ*rppH*::Kan
GJ13702	GJ3161 Δ*rhlB* Δ*rppH*::Kan
GJ13703	GJ3161 Δ*rhlB* Δ*pnp* Δ*rppH*::Kan
GJ13708[e]	GJ3161 Δ*rho*::Kan *yceF*::Tn*10*dTet Δ*rppH rne*-493Δ
GJ13710[f]	MDS42 *rne*-493Δ Δ*rppH nusG*::Kan
GJ13713	GJ3110 *rne*-493Δ Δ*rppH* Δ*rng*::Kan *yceF*::Tn*10*dTet
GJ13714	GJ3107 *rne*-493Δ Δ*rppH* Δ*rng*::Kan *yceF*::Tn*10*dTet
GJ13716	GJ3110 *araD*$^+$ *rnhA339*::Cm Δ*rppH*::Kan *rne*-493Δ *yceF*::Tn*10*dTet *attλ*::(P_{ara}-*rnhA*,Amp)
GJ13717	GJ3107 *araD*$^+$ *rnhA339*::Cm Δ*rppH*::Kan *rne*-493Δ *yceF*::Tn*10*dTet *attλ*::(P_{ara}-*rnhA*,Amp)

a. Strains described earlier include BW27783 (Khlebnikov *et al.*, 2001); MC4100, GJ3107, GJ3110 and GJ3161 (Harinarayanan and Gowrishankar, 2003); MDS42 (Posfai *et al.*, 2006); and XL1-Blue (Sambrook and Russell, 2001). All other strains were constructed in this study.
b. Genotype designations are as described (Berlyn, 1998; Rudd, 1998). All strains are F$^-$ unless otherwise indicated. References or sources for the episomes and mutations that were introduced by transduction into the strains are as follows: *rho*-A243E, *nusG*-G146D, *nusG*::Kan and *rnhA339*::Cm (Harinarayanan and Gowrishankar, 2003); Δ*rne*::Cm (Lee *et al.*, 2002); [P_{lac}-*rne*, Amp] (Jain *et al.*, 2002); [λP_{rne}-*lac*] also called (λ*ez1*) (Jain and Belasco, 1995); *rne-131* and *rne-Δ23* (Leroy *et al.*, 2002); and F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*] (Sambrook and Russell, 2001). The Δ*rppH*, Δ*rng*, Δ*pnp*, and Δ*rhlB* mutations were introduced as Kan^R deletion-insertion mutations from the Keio knockout collection (Baba *et al.*, 2006); where necessary, the Kan^R marker at any of these loci, as also the antibiotic marker from the Δ(*flgK-rne*)::Cm insertion-deletion constructed in this study, was then excised by site-specific recombination with the aid of plasmid pCP20, as described (Datsenko and Wanner, 2000). The latter mutations are shown without the Kan^R or Cm^R designations in the table.
c. Each of the indicated strains was maintained as a derivative with plasmid pBAD-RNE.
d. Each of the indicated strains was maintained as a derivative with plasmid pHYD1613.
e. Viable derivatives of GJ13708 carried the mutant *rho* Sp^R plasmids of Chalissery *et al.* (2007), shown in Fig. 3A.
f. GJ13710 was maintained as a derivative with plasmids pHYD751 and pHYD2903.

Table 6. Plasmids constructed in this study.

Plasmid	Description (replicon, antibiotic markers)
pHYD1613, pHYD2903	5.8 kb PstI fragment carrying *rne*$^+$ gene cloned from plasmid pRNE101 into the PstI sites of pMU575 (IncW; Tp) and pMAK705 (pSC101 Ts; Cm), respectively
pHYD1810[a]	Derivative of pWSK30 with 7.9 kb BclI-KpnI fragment from *E. coli* chromosome (genomic co-ordinates 2963866 to 2971793; Rudd, 1998) carrying the *rppH*$^+$ gene cloned into the BamH1-KpnI sites of the vector (pSC101; Amp^R)
pHYD2361	Derivative of pBAD18 with 0.55 kb EcoRI-HindIII fragment encompassing ribosome-binding site and coding region of *rnhA* gene (obtained by PCR amplification with primers 5′-GTTTTACAGTTGAATTCAATTACAGG-3′ and 5′-ATTCGCCAGAAGCTTGGAGCCA-3′ from pSK760, followed by digestion with EcoRI and HindIII) downstream of P_{ara} promoter (pMB9; Amp^R)
pHYD2368[b]	Derivative of pBAD18 with 1.7 kb fragment encompassing ribosome-binding site and coding region of *uvsW* gene (obtained by PCR amplification with primers 5′-AAAGAGATTCTCGAGGCAAATTTAG-3′ and 5′-ACCTTTAGTCTCGAGTTTTGGCCCTT-3′ from T4gt7 phage) downstream of P_{ara} promoter (pMB9; Amp^R); the fragment was introduced into pBAD18 after an intermediate cloning step into pBluescriptII-KS
pHYD2373	Derivative of pSG-Rne-R169Q in which sequence corresponding to codon 494 of *rne* has been converted from GAA to TAA and that from codons 495 to 1061 has been deleted and substituted by a Cm^R cassette from pKD3 by the procedure of Datsenko and Wanner (2000), with the aid of the primer pairs 5′-CGTGCGTAAAGGGGAATAAACCCCAACCTTAAGCTA CATGCTGCCGAAGGTGTAGGCTGGAGCTGCTTC-3′ and 5′-TAATTATTACTCAACAGGTTGCGGACGCGCAGGAGCG GCAGAGGCATGATCATATGAATATCCTCCTTAG-3′ (pSC101; Kan^R)

a. Plasmid pHYD1810 was constructed by Abhijit Sardesai (unpublished).
b. *uvsW* is comprised of two cistrons encoding polypeptides UvsW and UvsW.1 that are 502 and 76 amino acids long, respectively (Kerr *et al.*, 2007; Nelson and Benkovic, 2007). For convenience, the two together are referred to as UvsW in this study. UvsW encoded by pHYD2368 has an I431S substitution in the larger polypeptide; it retains the ability to suppress lethality associated with combined deficiency of the two R-loop removing enzymes of *E. coli* RNase H1 and RecG (Carles-Kinch *et al.*, 1997), but unlike the native protein is not toxic when overexpressed.

TACGATGAAAAGAATGGTGTAGGCTGGAGCTGCTTC-3′. The deletion allele has been designated Δ(*flgK-rne*)::Cm.

Construction of derivatives with chromosomal P_{ara}-rho, P_{ara}-nusG *and* P_{ara}-rnhA *fusions*

The plasmid-chromosome shuttling procedure described by Boyd *et al.* (2000) was used to transfer the P_{ara}-*rho*, P_{ara}-*nusG* and P_{ara}-*rnhA* constructs from plasmids pRS632, pRS695 and pHYD2361, respectively, with the aid of phage λInCh into the *attλ* site of the chromosome. The *nusG* gene downstream of P_{ara} encodes an S60A substitution that does not affect the function of the protein (Chalissery *et al.*, 2011). All three constructs (that are each designated in Table 4 with the appropriate suffix after '*attλ*::P_{ara}-') were able to complement the corresponding chromosomal mutations in an Ara-inducible manner (data not shown).

Determination of synthetic lethality phenotypes

Two approaches were used to examine phenotypes of synthetic lethality and their suppression. In the first, which was based on the strategy earlier described by Bernhardt and de Boer (2004) and Rudolph *et al.* (2010), lethality or synthetic lethality associated with mutations in *rne* was assessed by the inability of these mutant strains bearing the *rne*$^+$ plasmid pHYD1613 (which exists in single copy number and exhibits inefficient partitioning to daughter cells during cell division) to generate plasmid-free segregants that are viable upon subculturing. The mutants were also Δ*lac* and because pHYD1613 carries the *lacZ* gene, plasmid-bearing and plasmid-free colonies could be distinguished as blue and white, respectively, on Xgal-supplemented medium. Typically, the proportion of white colonies obtained from a culture of the wild-type strain grown with Tp (that is, with selection for the plasmid) and then plated on medium without Tp was between 5% and 15%.

In the second approach, a strain in which the gene of interest was conditionally expressed, either by placing it downstream of the inducible P_{lac} or P_{ara} promoter or by cloning it in plasmid pAM34 that is IPTG-dependent for replication, was plated or spotted at suitable dilutions on a pair of plates corresponding to the permissive and restrictive conditions, respectively, for such expression.

Measurement of chemical half-lives of bulk mRNA

Chemical half-lives of bulk mRNA in different strains were measured by a modification of the method of Donovan and Kushner (1986). Briefly, cultures grown to mid-exponential phase in LB medium were pulse-labelled with ^{3}H-uracil for 2 min before addition of an excess of cold uracil along with rifampicin and nalidixic acid. Aliquots taken at different times thereafter were processed to determine the radioactivity present following precipitation with 20% trichloroacetic acid.

For pulse-labelling of RNA in the synthetic lethal mutants, conditional lethality was imposed as follows. Strains GJ9593 and GJ12103/pHYD2373 carry, respectively, the synthetic lethal combinations ΔRppH RNase E-493Δ and RNase E-R169Q,493Δ as also the *rho*-A243E mutation and the P_{ara}-*rho*$^+$ construct on the chromosome. Both strains are viable in glucose-supplemented LB medium (when P_{ara} is repressed and the chromosomal *rho* mutation suppresses the synthetic lethality) and are killed upon Ara induction. Each strain was grown to an A_{600} of 0.1 in LB-glucose, after which the cells were washed and resuspended in fresh LB supplemented in one case with 0.2% glucose and in another with 0.2% Ara. Pulse-labelling was then performed after growth to an A_{600} of around 0.4, as described above.

Because the measured radioactivity represents incorporation of label into both stable RNA and unstable mRNA

(Donovan and Kushner, 1986; Lopez *et al*., 1999), the chemical half-life of the latter was calculated from a plot of radioactivity versus time by applying the equation

$$y = S + U^{*}\exp[-(\ln 2)\,x/T]$$

where '*y*' is the radioactivity measured at time '*x*'; 'U' and 'S' are the fractional components of radioactivity incorporated in unstable mRNA at time 0 and in stable RNA respectively; and 'T' is the chemical half-life of bulk mRNA. A few representative RNA decay curves are presented in Fig. S1. The software OriginPro8 v8.0724 was used to fit the curve and solve for the values of 'S', 'U' and 'T' in each labelling experiment.

Determination of in vivo *efficiencies of tRNA maturation and 9S RNA processing*

The in vivo efficiencies of maturation of $tRNA^{His}$ and $tRNA^{Cys}$, as well as of 9S RNA processing, were assessed by the protocols described by Mohanty *et al*. (2008), using cultures that had been grown similarly as for the mRNA half-life measurements. For each strain, 5 μg of total RNA was subjected to electrophoresis on denaturing 8% polyacrylamide gels with 7 M urea, and the Northern blots prepared therefrom were hybridized to 5′-end-labelled oligonucleotide probes for the three stable RNA species as described (Mohanty *et al*., 2008). The probes employed corresponded to nucleotide regions 42–63, 38–59 and 50–69 of mature $tRNA^{His}$, $tRNA^{Cys}$ and 5S rRNA respectively (Ow *et al*., 2000; Li and Deutscher, 2002).

Immunoblot analysis of in vivo *RNase E levels*

The protocol for immunoblotting was essentially the same as that described earlier (Singh *et al*., 2009). Cells obtained from mid-log phase cultures were lysed by heating to 95°C for 5 min in the presence of 2% sodium dodecyl sulphate and 10 mM EDTA. The cell lysates were subjected to electrophoresis through 8% polyacrylamide gels with sodium dodecyl sulphate, followed by electroblotting to a polyvinylidene difluoride membrane, blocking (with 8% fat-free milk powder and 0.2% bovine serum albumin), staining with amido black, and reactions with primary anti-RNase E antibody (rabbit, polyclonal, a gift from A.J. Carpousis), and secondary antibody (anti-rabbit IgG raised in goat, peroxidase-conjugated), as described (Sambrook and Russell, 2001). Immunoreactive bands on the blot were visualized with the aid of a chemiluminescence detection system as per the manufacturer's protocol (Sigma Chemical, USA).

Other methods

Procedures for P1 transduction and conjugation (Miller, 1992), *in vitro* DNA manipulations and transformation (Sambrook and Russell, 2001), and β-galactosidase assays (Miller, 1992) were as described. Different *rho, nusG* and *rne* alleles were introduced into strains by transductional linkage with the *ilv, argE*, and *pyrC* loci respectively. The episome F′ [*proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15 Tn*10*] was introduced from XL1-Blue into different strains by conjugation. Growth rates of cultures were determined with the aid of an automated growth analyser (Varioskan, Fisher Scientific, USA).

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