

Caulobacter crescentus RNase E condensation contributes to autoregulation and fitness

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ABSTRACT RNase E is the most common RNA decay nuclease in bacteria, setting the global mRNA decay rate and scaffolding formation of the RNA degradosome complex and BR-bodies. To properly set the global mRNA decay rate, RNase E from *Escherichia coli* and neighboring γ -proteobacteria were found to autoregulate RNase E levels via the decay of its mRNA's 5' untranslated region (UTR). While the 5' UTR is absent from other groups of bacteria in the Rfam database, we identified that the α -proteobacterium *Caulobacter crescentus* RNase E contains a similar 5' UTR structure that promotes RNase E autoregulation. In both bacteria, the C-terminal intrinsically disordered region (IDR) of RNase E is required for proper autoregulation to occur, and this IDR is also necessary and sufficient for RNase E to phase-separate, generating BR-bodies. Using in vitro purified RNase E, we find that the IDR's ability to promote phase separation correlates with enhanced 5' UTR cleavage, suggesting that phase separation of RNase E with the 5' UTR enhances autoregulation. Finally, using growth competition experiments, we find that a strain capable of autoregulation rapidly outcompetes a strain with a 5' UTR mutation that cannot autoregulate, suggesting autoregulation promotes optimal cellular fitness.

SIGNIFICANCE STATEMENT

- RNase E controls bacterial mRNA degradation and autoregulates its synthesis via its intrinsically disordered C-terminal domain; however, the role of the disordered region was unclear.
- The authors show that *Caulobacter* RNase E's C-terminal domain drives condensation with its 5' UTR accelerating RNA decay activity. Therefore, autoregulation is stimulated by RNase E condensation.
- The discovery that RNase E condensation with RNA appears to directly stimulate RNA cleavage activity suggests that mRNA decay enzyme phase separation with RNA substrates may be a more broadly utilized strategy for stimulating RNA decay activity and controlling cellular RNA lifetimes.

Monitoring Editor

Erin Goley
Johns Hopkins University

Received: Dec 19, 2023

Revised: May 29, 2024

Accepted: Jun 3, 2024



New Hypothesis

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E23-12-0493>) on June 12, 2024.

Author contributions: V.N., N.A., A.V., and K.D. conceived and designed the experiments; V.N., N.A., A.V., and K.D. performed the experiments; V.N., N.A., and A.V. analyzed the data; V.N. drafted the article; V.N. prepared the digital images.

Conflicts of interests: The authors declare no financial conflict of interest.

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Abbreviations used: ATP, adenosine triphosphate; AU, absorbance unit; BR bodies, bacterial ribonucleoprotein bodies; CTD, C terminal domain; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; IDR, intrinsically disordered region; IVT, invitro transcription; MgCl₂, magnesium chloride; mRNA, messenger ribonucleic acid; NaCl, sodium chloride; NTD, N terminal domain; PAGE, polyacrylamide gel electrophoresis;

pCp Cy5, Cytidine-5'-phosphate-3'-(6-aminohexyl)phosphate Cyanine 5; PNPase, polynucleotide phosphorylase; PYE, peptone yeast extract; qRT PCR, quantitative real transcription polymerase chain reaction; RNA, ribonucleic acid; RNAP, RNA polymerase; RNase E, ribonuclease E; rRNA, ribosomal ribonucleic acid; SD, standard deviation; SS, single strand; TAP seq, tobacco acid pyrophosphatase sequencing; tn seq, transposon sequencing; UTR, untranslated region.

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INTRODUCTION

In bacteria, mRNA decay is typically controlled by the protein RNase E (Kushner, 2002; Deutscher, 2006; Carposis *et al.*, 2009; Mackie, 2013; Chao *et al.*, 2017). RNase E is an endonuclease that performs the rate-limiting step of RNA cleavage, setting the global rate of mRNA decay (Ono and Kuwano, 1979; Al-Husini *et al.*, 2020). To do this, the levels of RNase E in *Escherichia coli* are carefully controlled through autoregulation activity whereby the protein binds to the 5' untranslated region (UTR) in its own mRNA and facilitates its cleavage (Jain and Belasco, 1995; Schuck *et al.*, 2009). Importantly, this mechanism of autoregulation allows the cell to adjust its mRNA decay demands to changes in mRNA abundance (Sousa *et al.*, 2001). While the 5' UTR is well conserved among γ -proteobacteria (Diwa *et al.*, 2000), it is currently not annotated outside this clade of bacteria in Rfam (Kalvari *et al.*, 2021). In *Caulobacter crescentus*, an α -proteobacterium, it was observed that RNase E can also autoregulate its expression (Al-Husini *et al.*, 2018); however, the 5' UTR secondary structure analysis and functional impact on autoregulation were not explicitly investigated. In *E. coli* and *C. crescentus*, it was found that the intrinsically disordered C-terminal domain (CTD) of RNase E is necessary for autoregulation (Jiang *et al.*, 2000; Al-Husini *et al.*, 2018). Interestingly, this C-terminal region of RNase E is also necessary and sufficient for the formation of BR-bodies, phase-separated biomolecular condensates that promote mRNA decay activity (Al-Husini *et al.*, 2020). However, the role of BR-bodies in the process of RNase E autoregulation has not been directly tested. Finally, while RNase E appears to be essential in *C. crescentus* (Al-Husini *et al.*, 2018), it has not been thoroughly tested as to how autoregulation of RNase E contributes to cellular fitness.

To examine the role of RNase E autoregulation in *C. crescentus*, we show that underexpression or overexpression of RNase E led to significant reductions in cell growth. We find that like the γ -proteobacteria, the α -proteobacteria likely also utilize a similar 5' UTR structure that is necessary for RNase E autoregulation. By

performing a growth competition experiment, a mutant whose 5' UTR was replaced by a synthetic 5' UTR, was rapidly outcompeted, suggesting that even mild overexpression of RNase E lowers fitness. Finally, using an *in vitro* purified system, we find that in the presence of *C. crescentus* RNase E condensates, 5' UTR cleavage is stimulated, suggesting phase separation of RNase E together with the 5' UTR promotes RNase E cleavage leading to autoregulation.

RESULTS

RNase E depletion or overexpression leads to loss of fitness

To determine the essentiality of the major mRNA endonuclease RNase E, we used a depletion strain (JS8) where the promoter of RNase E was replaced with a xylose-inducible promoter (Figure 1A). While a previous Tn-seq study found it was a high fitness cost gene in rich media, insertions were only found in the intrinsically disordered CTD and were absent in the catalytic N-terminal domain (NTD), suggesting its activity may be essential for growth (Christen *et al.*, 2011). In liquid culture, we found that the depletion strain had somewhat attenuated growth compared with a control strain in the presence of xylose, but the observed growth rate was slower in the absence of xylose (Figure 1A). The deceleration of growth likely arises from the slow depletion of the RNase E protein by cell division which takes ~4 h (Figure 1A). After 8 h of growth without xylose, the depletion strain shows an ~4 log reduction in the number of colonies formed compared with the control, suggesting the gene is indeed critical for colony formation (Figure 1B). Of note, the RNase E depletion strain colonies that grew in the absence of xylose were heterogeneous in size and the larger ones no longer required xylose to grow in liquid cultures (data not shown), suggesting they accumulated mutations that allow constitutive expression of RNase E in the absence of xylose. Additionally, our attempts to make a clean deletion of the RNase E gene failed in our hands (data not shown), suggesting the RNase E gene is likely essential, or at a minimum provides very high fitness cost in *C. crescentus*. Altogether, this suggests that depletion of RNase E leads to a strong reduction in cell growth.

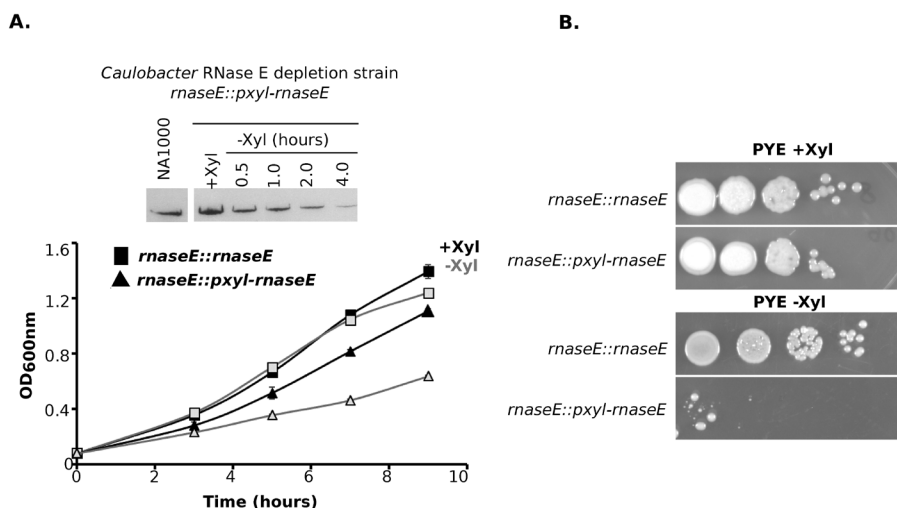
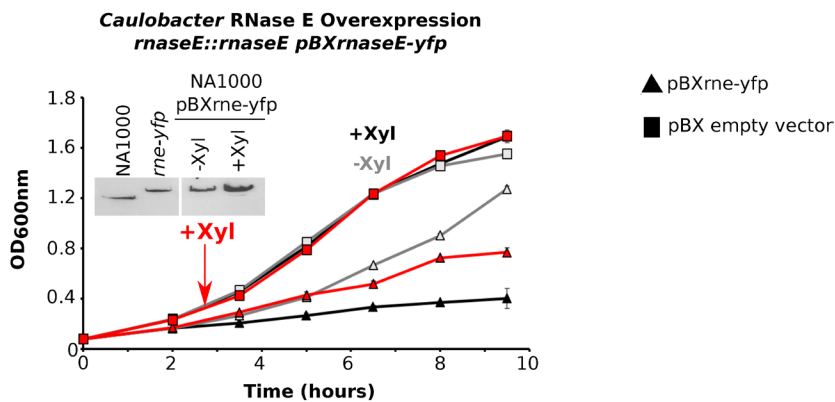


FIGURE 1: Depletion of *Caulobacter* RNase E leads to slow growth and loss in colony formation. (A) Growth curves of depletion strains of RNase E (triangles) and empty vector controls (squares) in the presence of xylose (black) or the absence of xylose (light gray). All cells were grown in PYE with kanamycin and each timepoint is the average OD₆₀₀ measured from three replicate cultures. Error bars represent SD. (B) Colony forming unit assay to determine cell viability in the depletion strains. Depletion strains were grown in PYE/Kan in the presence of xylose (black) or absence of xylose (light gray) for 8 h, then spotted on PYE/Kan/Xyl plates. Three replicates of the experiment were performed, and one was chosen as a representative image. EV = Empty vector; RNE = RNase E.

Next, we explored the functional consequences of artificial overexpression of RNase E. We generated a pBX multicopy plasmid containing the RNase E-YFP construct (JS89) (Figure 2A). Cells harboring an empty vector showed little difference in growth in the presence or absence of xylose (Figure 2A). In the strain harboring the plasmid with RNase E-YFP, we found that growth was significantly slower in the absence of xylose, likely due to leaky levels of expression that surpassed that of the wild-type cells (Figure 2A); however, growth was halted when the xylose inducer was added (Figure 2A). Additionally, we added the xylose inducer to cells as they approached an OD₆₀₀ of 0.3 in the middle of log-phase instead of at OD₆₀₀ of 0.05 and found that the growth rate was maintained similar to the uninduced cells for ~2 h, but then showed a significant reduction after 3 h (Figure 2A). The maximum induction of RNase E protein appeared to occur after 3 h of induction with xylose (Supplemental Figure S1), suggesting that the reduction in growth observed at 3 h is due to its peak in protein accumulation. Of note, we observed

A.



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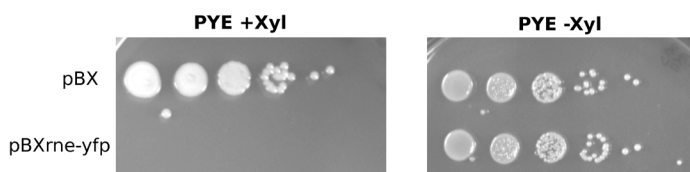


FIGURE 2: Overexpression of *Caulobacter* RNase E leads to growth arrest. (A) Growth curves of overexpression strains of RNase E (triangles), and empty vector controls (squares) grown in the presence of xylose (black) or the absence of xylose (light gray). All cells were grown in PYE with kanamycin and each time point is the average OD600 measured from three replicate cultures. Error bars represent SD. (B) Colony forming unit assay to determine cell viability in the overexpression strains harboring pBX vectors with the insert on the left. Three replicates of the experiment were performed, and one was chosen as a representative image.

that when blotted using anti-RNase E antibodies, the chromosomal copy of RNase E's expression was no longer detected after RNase E overexpression, suggesting that it was shut off in response to plasmid-based expression (Figure 2A). To examine whether the cells were losing viability after RNase E overexpression, we also performed dilution assays, plated the dilutions on solid media, and assayed for colony formation (Figure 2B). Here, induction of RNase E led to a complete loss in colony formation upon RNase E overexpression (Figure 2B). In conclusion, when RNase E is overexpressed strongly above wild-type levels, *C. crescentus* has a strong reduced growth rate and reduction in cell viability.

RNase E autoregulation ensures optimal cell fitness

RNase E is known to autoregulate its own levels in *E. coli* and other γ -proteobacteria (Mudd and Higgins, 1993; Jain and Belasco, 1995; Diwa *et al.*, 2000; Schuck *et al.*, 2009; Al-Husini *et al.*, 2018) and we previously observed autoregulation of *C. crescentus* RNase E when expressed from the *vanA* locus on the chromosome (Al-Husini *et al.*, 2018). Here, we noticed that when cells harbored an extra copy of RNase E-YFP on the pBX plasmid, chromosomal RNase E levels from the *rne* locus were no longer detected (Figure 2A). Further, when a second copy of RNase E-YFP with a plasmid-derived 5' UTR was expressed artificially from the vanillate promoter, we observed that the relative abundance of the wild-type RNase E protein was reduced by a corresponding amount (Figure 3A). To further examine the properties of RNase E autoregulation, we induced RNase E-YFP

from the *vanA* locus at different levels of vanillate and found that the more RNase E-YFP produced, the stronger the inhibition of native RNase E expression (Figure 3A). In *E. coli*, the 5' UTR of RNase E has an RNA structural element which is recognized by RNase E that is necessary for its autoregulation (Schuck *et al.*, 2009). To test whether the *C. crescentus* RNase E gene's 5' UTR is responsible for autoregulation, we generated a strain in which an active site mutant of RNase E that is unable to cleave RNA (Al-Husini *et al.*, 2020) was expressed from the chromosome (Figure 3A). This version failed to shut down expression of the native RNase E gene (Al-Husini *et al.*, 2018), suggesting that RNase E activity is required for autoregulation. Additionally, by using the strain in which the native RNase E's 5' UTR was replaced by a plasmid-derived ribosome binding site, we also observed a failure to autoregulate *rne* expression (Figure 3A). Altogether, this suggests that RNase E activity on its 5' UTR is necessary for autoregulation.

We investigated whether the *C. crescentus* RNase E had an annotated 5' UTR structure in Rfam (Kalvari *et al.*, 2021) but no 5' UTR annotations existed outside of γ -proteobacteria. To explore the 5' UTR's potential for structure formation, we extracted the 5' UTR sequence from our RNA sequencing (RNA-seq)-based annotation (Bharmal *et al.*, 2020) and performed secondary structure prediction using turbofold (Tan *et al.*, 2017). This yielded a putative secondary structure that was similar to the *E. coli* 5' UTR (Figure 3B), and that could be aligned to other α - and γ -proteobacterial

RNase E 5' UTRs (Supplemental Figure S2), suggesting that the *C. crescentus* RNase E 5' UTR and those in α -proteobacteria are likely a structural variant of the *E. coli* 5' UTR. Secondary structure comparison between the γ - and α -proteobacteria show that the RNase E 5' UTR likely exists with two different classes: class I occurs in the γ -proteobacteria with a larger single-stranded region I located between hairpins 1 and 2, while class II occurs in the α -proteobacteria with a shorter single-stranded region I and a larger single-stranded region 2 (Supplemental Figure S3A). To identify *C. crescentus* 5' RNA cleavage sites, we reanalyzed TAP-dependent global 5' end sequencing data which were used to identify 5' PPP-containing transcription start sites to identify enriched 5' P-cleavage sites (Zhou *et al.*, 2015) whereby we identified two RNA cleavage sites within the 5' UTR, located in the single-stranded region II (Figure 3B). The location of these cleavage sites differs from *E. coli*, where single-stranded region I is the location of an RNase E cleavage site (Figure 3B). This suggests that the variation in the single-stranded region may alter the preference where RNA cleavage by RNase E occurs between class I and II 5' UTRs.

To test whether autoregulation impacts the cellular fitness, we compared RNase E replacement strains expressing either RNase E-YFP with the natural 5' UTR or a plasmid-derived 5' UTR (Figure 4A). In RNase E replacement strains, the cells contain two copies of RNase E: the UTR variants were expressed from the *vanA* locus of the chromosome, while a xylose-inducible promoter integration plasmid was introduced at the native *rne* locus, thereby the 5' UTR

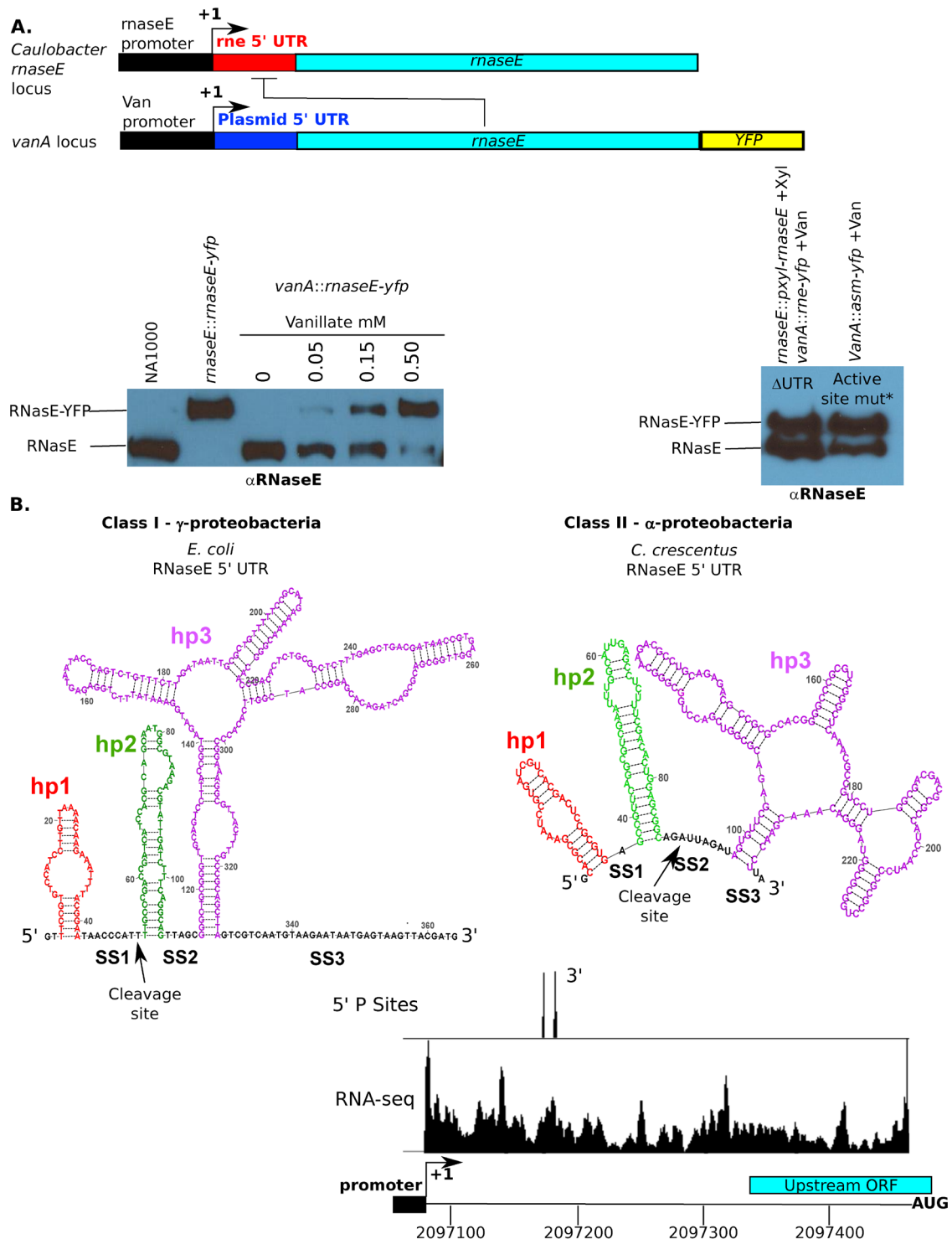
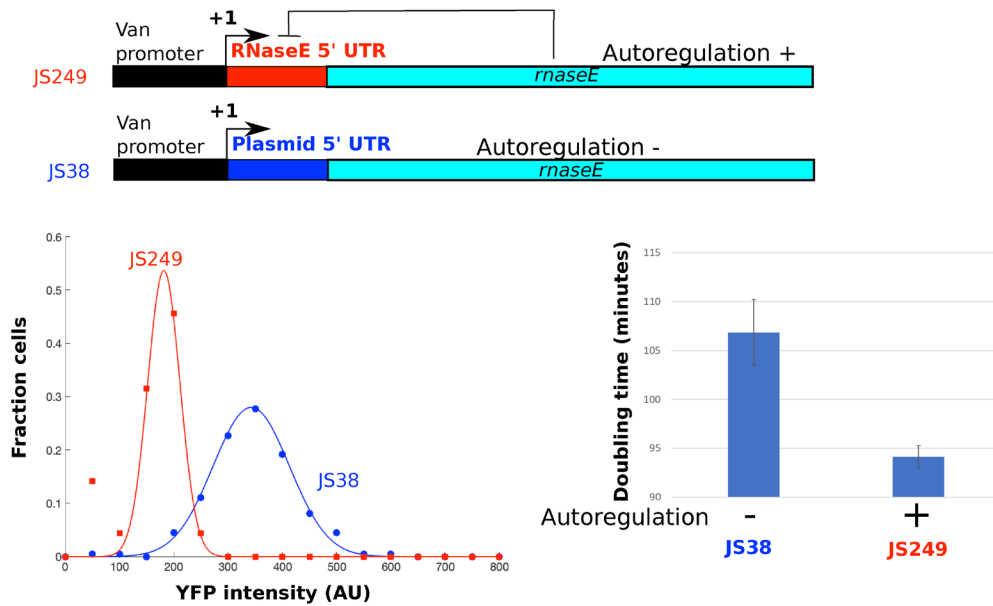


FIGURE 3: *Caulobacter* RNase E autoregulation requires activity on its 5' UTR. (A) Western blot of RNase E in the indicated strains. Vanillate-induced RNase E lacks the 5' UTR. RNase E-YFP and RNase E bands are indicated. Three replicates of the experiment were performed, and one was chosen as a representative image. (B) Predicted mRNA secondary structures generated by turbofold for *C. crescentus* (left) and for *E. coli* (right). RNA cleavage sites are mapped with arrows. RNA decay sites identification in the 5' UTR. 5' P-sites from (Zhou et al., 2015) and RNA-seq data indicating the mRNA 5' UTR from (Schrader et al., 2014).

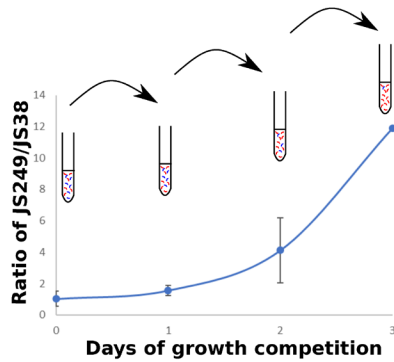
variants are the only expressed copies of RNase E when grown in peptone-yeast extract (PYE) (Al-Husini et al., 2018). The strain harboring the 5' UTR replaced with a plasmid 5' UTR (JS38) led to an ~2-fold higher RNase E-YFP levels than the strain harboring RNase

E's own 5' UTR (JS249) (Figure 4A). When grown in PYE-vanillate conditions, we found that JS38 (106 min doubling time) had a decrease in growth rate compared with JS249 (94 min doubling time) (Figure 4A), suggesting that RNase E autoregulation promotes faster

A.



B.



C.

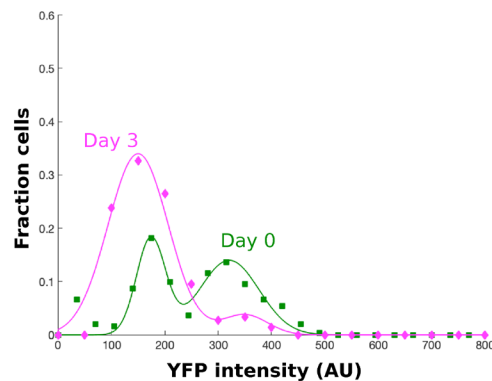


FIGURE 4: *Caulobacter* RNase E autoregulation contributes to cellular fitness. (A) Cartoon of strains JS249 containing the *rne* 5' UTR, and JS38 which has a 5' UTR from a plasmid expression system. YFP intensity distributions of strains JS249 and JS38, Gaussian fits were performed with kaleidagraph from >100 cells each. (Right) Doubling times of JS38 and JS249 as grown in PYE gent vanillate media. The average value is shown, with the error bars representing the SD of doubling times calculated from three replicate growth curves. A one-tailed *t* test with uneven variance yielded a *P* value of 0.025. (B) Growth competition of equally mixed cultures containing JS249 and JS38 after multiple days of growth. Error bars are from three biological replicates of the growth competition. The fraction of cells were calculated from the relative area under the curve from a dual Gaussian curve fit as shown in panel C based on a YFP quantitation >100 cells per replicate. (C) YFP distributions of the mixed cultures at the beginning of the growth competition or after 3 d of culture. Dual Gaussian fits were performed using kaleidagraph on >100 cells. The relative area under the JS249 peak compared with the area under the JS38 peak was calculated as the ratio of JS249/JS38.

growth. While the magnitude in growth rate difference was small, we sought to assay fitness between the strains more sensitively by performing a growth competition experiment. In this competition experiment, a 50:50 ratio of each strain was incubated together and grown for multiple generations and the fraction of JS38 and JS249 cells in the population were measured. To measure the fraction of each strain in the population, we imaged the cells during midlog-phase of growth and measured their YFP intensities, which yielded two peaks in fluorescence intensity that could be fit with a dual Gaussian curve fit, and the area under each peak representing JS249 maxima (low YFP centered around 200 AU) and JS38 (high YFP centered around 350 AU) were used to calculate the ratio of each strain in the mixed culture (Figure 4, B and C). Each day after incubating the mixed culture, we observed a growing fraction of JS249 cells and a corresponding reduction in JS38 cells (Figure 4B). After 3 days of growth competition, we observed ~12x more JS249 cells than JS38 cells, suggesting that autoregulation can promote a significant fitness advantage to *C. crescentus* cells (Figure 4, B and C).

RNase E's intrinsically disordered region promotes phase separation and 5' UTR cleavage

In *E. coli* and *C. crescentus*, the intrinsically disordered CTD [or intrinsically disordered region (IDR) henceforth] of RNase E was found to be necessary for autoregulation (Jiang *et al.*, 2000; Al-Husini *et al.*, 2018). While the IDR is also the scaffolding site for the RNA degradosome complex, we found an IDR mutant lacking the three degradosome proteins binding sites (Δ Aconitase, Δ RNase D, Δ PNPase) was capable of autoregulation (Al-Husini *et al.*, 2018), suggesting autoregulation does not require the formation of the RNA degradosome. We further dissected the N-terminal domain by dissecting it into its two subdomains, the N-terminal S1 domain (amino acid 1-176) and the E/G catalytic core (amino acid 177-450) (Al-Husini *et al.*, 2018). We also observed that deletion of the N-terminal S1 domain, which binds RNA, or deletion of the catalytic E/G domain also abolished autoregulation (Supplemental Figure S3C), suggesting a fully functional N-terminal domain is required for autoregulation. The IDR was found to be necessary and sufficient to form BR-bodies (Al-Husini *et al.*, 2018; Nandana *et al.*, 2023), which allows RNase E to phase-separate with RNA and stimulates RNA decay activity in vivo (Al-Husini *et al.*, 2018, 2020). To test whether the IDR promotes 5' UTR cleavage in vitro, we purified full-length RNase E (Supplemental Figure S4A) and RNase E lacking the IDR. To confirm purified RNase Es were active, we performed 9S ribosomal RNA processing assays with RNase E and RNase E Δ IDR since RNase E is required for processing 9S rRNA to precursor 5S ribosomal RNA (p5S rRNA) (Hardwick *et al.*, 2011). We observed that both RNase E and RNase E Δ IDR processes *C. crescentus* 9S rRNA to p5S rRNA (Supplemental Figure S3A), suggesting both proteins were functionally active. As *C. crescentus* 9S appears to only cleave on the 5' side of the 5S, we tested *C. crescentus* RNase E cleavage on the *E. coli* 9S RNA which is known to be cleaved on both 5' and 3' sides of the 5S, where we observed the same cleavage pattern [Supplemental Figure S4B, (Hardwick *et al.*, 2011)]. Next, we in vitro transcribed the *rne* 5' UTR and tested its cleavage by RNase E. Under conditions with low enzyme to substrate ratio, initial RNA cleavage fragment size was observed to be consistent with the in vivo 5'P cleavage site (Figure 3B; Supplemental Figure S3B), which appears to be followed by subsequent cleavage events upon prolonged incubation.

To test the role of the IDR and impacts of phase separation on RNase E autoregulation in vitro, we sought to incubate RNase E and RNase E Δ IDR under conditions in which they were incubated above or below the critical concentration of phase separation. In prior work,

C. crescentus RNase E's IDR was incubated between 2 and 48 μ M with poly-A RNA under a range of different monovalent salt concentrations, and it was observed to undergo phase separation at a critical concentration >2 μ M (Figure 5A) (Al-Husini *et al.*, 2018). Therefore, we incubated full-length RNase E reactions at two concentrations, one concentration above the critical threshold for RNase E phase separation (6 μ M RNase E), and one below the critical threshold for phase separation (1 μ M RNase E) (Figure 5A). We confirmed these results with full-length RNase E, where we observed that when incubated at 6 μ M with its 5' UTR RNA, it formed condensates under the microscope (Figure 5A); however, only a diffuse solution of RNase E Δ IDR at 6 μ M was observed in the presence of 5' UTR RNA, in line with the role of the C-terminal IDR driving phase separation in vivo (Al-Husini *et al.*, 2018). Additionally, incubating 1 μ M RNase E or RNase E Δ IDR with 5' UTR RNA did not lead to condensate formation (Figure 5A). We then performed single turnover in vitro 5' UTR RNA cleavage assays at both phase-separating concentrations (6 μ M) and non-phase-separating conditions (1 μ M) while keeping the enzyme/RNA ratio constant. We observed that at 1 μ M RNase E cleaved the RNA slightly faster than RNase E Δ IDR, as we observed increased amounts of RNA cleavage intermediates (Figure 5B); however, even after 30 min of incubation with RNase E, the amount of full-length 5' UTR RNA remained high, and only a small fraction of cleavage products was detected (Supplemental Figure S3B). When RNase E was incubated at 6 μ M, its 5' UTR was degraded rapidly, with only a small fraction of full-length RNA remaining after 15 min of incubation and the rest of the RNA being cleaved into decay fragments. In contrast, the RNase E Δ IDR mutant incubated at 6 μ M cleaved the RNA more slowly than full length, with the major population of RNA being uncleaved after 15 min of incubation, and a much lower fraction of RNA decay fragments accumulated than the full length. This was consistent with prior in vivo observations, showing the rates of 5' UTR decay were accelerated when expressing RNase E NTD fusions with the *S. meliloti* or *A. tumefaciens* IDRs which had stronger partitioning into foci in vivo (Figure 6; Al-Husini *et al.*, 2018). In addition, we observed that the deletion of the C-terminal IDR or the individual RNA degradosome binding sites (Δ DBS) led to a reduction in YFP-foci per cell and decelerated 5' UTR decay rates. Taken together, this suggests that RNase E's IDR accelerates 5' UTR cleavage under conditions that promote condensation.

DISCUSSION

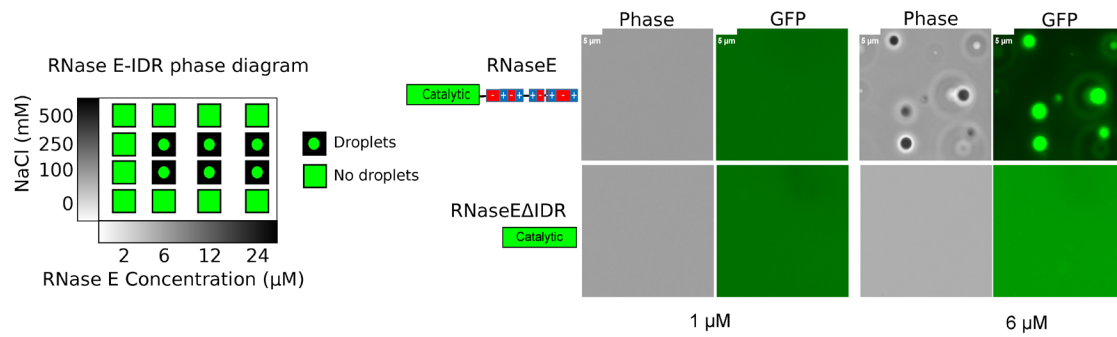
RNase E autoregulation tunes RNase E levels for optimal growth

RNase E is typically the rate-limiting enzyme controlling mRNA decay, so its protein levels must be carefully controlled to allow for the required mRNA decay activity for the cell. Negative autoregulation of RNase E appears to help fulfill this requirement by fine-tuning the amount of RNase E activity in the cell via cleavage of its own 5' UTR (Figures 3, 4, and 5). Levels of RNase E that are too low or too high fail to support cell growth (Figures 1 and 2), while mild overexpression of RNase E in cells lacking its native 5' UTR leads to a more subtle but significant defect in cell growth and fitness (Figure 4). This suggests that even minor alterations of RNase E levels can negatively alter cell physiology and fitness. Therefore, RNase E's 5' UTR appears to be more broadly conserved outside the γ -proteobacteria to precisely control RNase E levels that fine-tunes its cellular concentrations and sets the cellular mRNA decay rate.

RNase E condensates accelerate 5' UTR cleavage

Past studies found that autoregulation in vivo required the 5' UTR and the intrinsically disordered CTD of *E. coli* RNase E (Jiang *et al.*, 2000). This is the same IDR of RNase E that is necessary and sufficient

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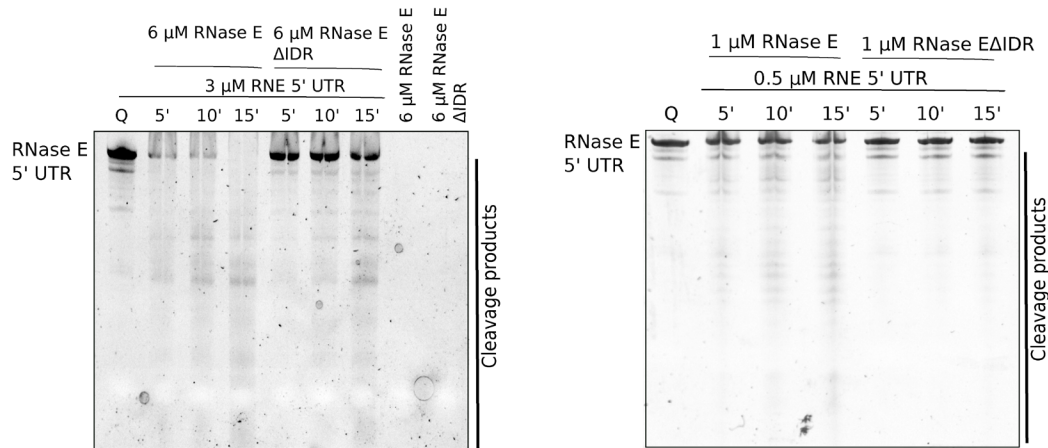


FIGURE 5: *Caulobacter* RNase E 5' UTR cleavage is stimulated by the IDR. (A) Purified solutions of full-length RNase E containing the IDR (left) and the ΔIDR variant (right) after incubated with RNA for 30 min. Scale bar is 5 μm. Three replicates of the experiment were performed, and one was chosen as a representative image. (B) RNase E 5' UTR cleavage assay. RNase E's 5' UTR was incubated with either full-length RNase E or the ΔIDR variant for the indicated time periods before running on a 7% denaturing PAGE and stained by SYBR gold. Each gel is a representative gel of three independent replicates.

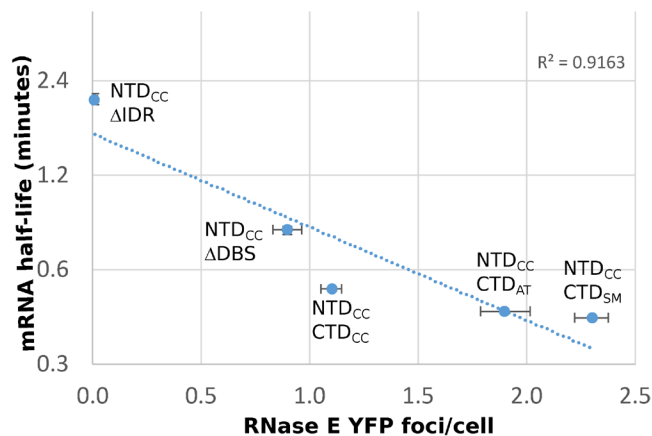


FIGURE 6: *Caulobacter* IDR mutants show a correlation between RNase E 5' UTR cleavage rate and foci formation of YFP-fusions. The RNase E 5' UTR half-life measured in vivo by rifampicin qRT-PCR time course is correlated with the YFP-foci per cell of various RNase E IDR mutants (data measured previously in Al-Husini et al. Mol Cell 2018 based on triplicate half-life measurements and YFP quantitation from >50 cells). The indicated species of the NTD-IDR fusions is indicated in lower case (CC = *C. crescentus*, AT = *A. tumefaciens*, and SM = *S. meliloti*). The ΔDBS mutant has deletions for the binding sites for

to phase-separate into BR-bodies, which are biomolecular condensates that promote faster RNA cleavage in vivo (Al-Husini et al., 2018, 2020). Interestingly, using in vitro reconstituted *C. crescentus* RNase E and *C. crescentus* RNase E ΔIDR incubated above and below the critical concentration for phase separation, we showed that the full-length version of RNase E can phase-separate with the 5' UTR in vitro yielding faster 5' UTR cleavage than RNase E ΔIDR, while below the critical concentration the proteins were more similar in their 5' UTR cleavage rates. This suggests that RNase E's phase separation with its 5' UTR promotes more rapid RNA cleavage, perhaps by increasing the local concentration of RNA and RNase E within the condensate and may also explain why RNase E IDR deletion mutants have been found to globally slow mRNA decay (Lopez et al., 1999; Al-Husini et al., 2018). We observed previously that RNase E condensates are observable within seconds after the addition of RNA, suggesting that phase separation of BR-bodies is also a rapid process (Nandana et al., 2023). Importantly, the RNA degradosome binding partner PNPase, which is a 3' to 5' exoribonuclease, was also shown to have increased exonucleolytic activity in the presence

RNase D, PNPase, and Aconitase in the IDR. The Y-axis is plotted on a log₂ scale, and the curve fit and R^2 was generated from an exponential equation in Microsoft excel.

of RNase E droplets (Collins *et al.*, 2023). This suggests that BR-bodies help coordinate the multistep RNA decay process by concentrating essential mRNA decay enzymes with their mRNA substrates, not only to accelerate the rate-limiting step of RNA decay by RNase E, but also to promote the subsequent 3' to 5' exonucleolytic steps that complete mRNA decay, thereby preventing the premature release of RNA decay intermediates. While *C. crescentus* provides an ideal model for the biochemical characterization of BR-bodies, the RNA degradosome machinery in multiple other bacteria and in mitochondria has been found to form foci in vivo (Strahl *et al.*, 2015; Zhou *et al.*, 2015; Hamouche *et al.*, 2020; Tejada-Arranz *et al.*, 2020; Griego *et al.*, 2022; Muthunayake *et al.*, 2020), and which contain large IDRs, suggesting BR-bodies are likely widespread condensates used for mRNA decay. Biomolecular condensation therefore provides a general organizational strategy for bacteria to organize their biochemical pathways in the absence of membrane-bound organelles. Indeed, many bacterial enzymes from various multistep biochemical pathways have been found to phase-separate and form biomolecular condensates (Heinkel *et al.*, 2019; Guilhas *et al.*, 2020; Harami *et al.*, 2020; Ladouceur *et al.*, 2020; Lasker *et al.*, 2020; McQuail *et al.*, 2020; Tejada-Arranz *et al.*, 2020; Azaldegui *et al.*, 2021; Nandana and Schrader, 2021; Goldberger *et al.*, 2022; Saurabh *et al.*, 2022; Tan *et al.*, 2022; Kryptou *et al.*, 2023; Nandana *et al.*, 2023; Ramm *et al.*, 2023) suggesting that this strategy of sub-cellular organization may be utilized to organize a variety of other biochemical pathways in bacteria.

MATERIALS AND METHODS

Cell growth

All strains used in this study were derived from the wild-type strain NA1000, and were grown at 28°C in PYE medium or M2 minimal medium supplemented with 0.2% D-glucose (M2G). When appropriate, the indicated concentration of Vanillate (5 µM), xylose (0.2%), gentamicin (0.5 µg/ml), kanamycin (5 µg/ml), chloramphenicol (2 µg/ml), oxytetracycline (2 µg/ml), spectinomycin (25 µg/ml), and/or streptomycin (5 µg/ml) were added. Strains were analyzed at mid-exponential phase of growth (OD 0.3–0.6). Optical density was measured at 600 nm in a cuvette using a NanoDrop 2000C spectrophotometer. For depletion, a strain containing a xylose-inducible copy of RNase E was first grown in media containing xylose overnight, then washed three times with 1 ml growth media, and resuspended in growth media without xylose and grown for 8 h overnight. log-phase cultures were then used for any downstream application. For overexpression, overexpression strains containing a xylose-inducible copy of RNase E variant were first grown in media without xylose overnight, then induced with xylose for 3.5 to 4 h. Autoregulation test strains containing a copy of *rne-yfp* integrated at the *vanA* locus were grown overnight in PYE, then induced with the indicated vanillate concentration for 6 hours. Replacements strains containing a xylose-inducible copy of RNase E and a Vanillate-inducible test construct were first grown in media containing xylose overnight. log-phase cells were washed three times with growth plain media and used to inoculate resuspended in growth media containing Vanillate, diluted, and grown overnight. log-phase cultures were then used for any downstream application.

Serial dilutions assay

For serial dilution assays, cells were grown overnight in PYE-kan media supplied with 0.2% xylose. log-phase cells were washed three times with PYE media and diluted in PYE without xylose to an OD 0.05. Serial dilutions were then performed and 5 µl of the selected dilutions were spotted on PYE-kan plates with and without xylose

and incubated at 28°C. For overexpression strains, the cells were grown in PYE-kan media without xylose overnight and log-phase cells were diluted in PYE-kan without xylose to an OD 0.05. Serial dilutions were then performed and 5 µl of the selected dilutions were spotted on PYE-kan plates with and without xylose and incubated at 28°C. For replacements strains, cells were first grown in PYE-kan-gent media supplied with 0.2% xylose overnight. log-phase cells were washed three times with plain PYE media and resuspended in plain PYE media to an OD 0.05. Serial dilutions were then performed and 5 µl of the selected dilutions were spotted on PYE-kan-Gent, PYE-kan-Gent-xylose, and PYE-kan-Gent-vanillate plates and incubated at 28°C.

Western blots

For determining the optimal RNE depletion time, JS8 cells were grown in PYE-kan media containing 0.2% xylose overnight, then washed three times with PYE media. The washed cells were used to inoculate 30 ml of PYE media without xylose. Samples were taken at 1, 2, 3, 4, and 8 h after xylose removal. The cells were pelleted and resuspended in 250 µl of 1x laemmli buffer for each 1.0 OD₆₀₀ unit. For determining the optimal RNE overexpression time, JS89 cells were grown in PYE media overnight, log-phase cells were used to inoculate 30 ml of PYE-kan media with 0.2% xylose. Samples were taken at 1, 2, 3, and 4 h after xylose addition. The cells were pelleted and resuspended in 250 µl of 1x laemmli buffer for each 1.0 OD₆₀₀ unit. The Western blotting was performed as in (Al-Husini *et al.*, 2018) using (1:1000) dilution of α-RNE antibodies.

5' UTR structure prediction

The 5' UTR of RNase E was extracted from (Bharmal *et al.*, 2020), and the sequence from the +1 site to the start codon was placed into turbofold (Tan *et al.*, 2017) for secondary structure prediction. As a control, the *E. coli* 5' UTR of RNase E from (Diwa *et al.*, 2000) was predicted in the same manner.

5' P site identification

For determining 5' P sites in the 5' UTR, we reanalyzed the TAP samples from Zhou *et al.* (2015) that occurred within the 5' UTR region.

Growth competition experiments

The strains used in the experiments included the strain with a mutated 5' UTR (JS38) and one with a WT 5' UTR (JS249). JS38 has the functional RNase E gene under the xylose-inducible promoter and an YFP-tagged RNase E with a mutated 5' UTR under the vanillate-inducible promoter. JS249 also has a functional RNase E gene under the xylose-inducible promoter, but it has an YFP-tagged RNase E with the wild-type 5' UTR region under the vanillate-inducible promoter.

A competition experiment was conducted to compare the growth efficiencies of the two strains. To start, both strains were grown in 5-ml overnight cultures at 28°C in PYE with xylose and antibiotics Kanamycin (Kan) and Gentamycin (Gent). The next day, cells were pelleted and washed to remove the xylose and then resuspended in 1 ml of PYE. An optical density (OD₆₀₀) was taken for each culture, and the cultures were diluted to 0.05 in 5-ml cultures of PYE/Kan/Gent and induced for 6 h with vanillate in 28°C shaker. After cells grew for 6 h, OD₆₀₀ was taken for each culture, and the culture with a greater OD₆₀₀ was diluted to match the culture with the lower initial OD₆₀₀. From here, a mixed culture of JS38 and JS249 was created. Cells were spotted on agarose pads

and imaged through fluorescence microscopy with an YFP filter cube and exposure time of 700 ms for both the cultures. These images represent the day 0 images. Serial dilutions of JS38, JS249, and JS38 versus JS249 were made and grown overnight with vanillate to ensure log phase imaging for the following day. Images were taken until day 3 for Trial 1 and day 2 for Trial 2. A master mix of PYE/Kan/Gent/Van was made to be used for cultures starting with the 6-h induction on day 0 and the controls were imaged every day.

Competition strain fluorescence measurements

The experiment resulted in six images of each strain (JS38, JS249, and JS38 vs. JS249) from each day. The fluorescence intensities were analyzed using the program ImageJ. The background intensity of each image, found using MicrobeJ, was subtracted from the fluorescent intensities. The resulting fluorescent intensities were used for the analysis.

Due to overlap between the median fluorescent intensities of the two strains, cells from the mixed strains could not be clearly separated by fluorescence intensities. Instead, using the program, Kaleidagraph, a Gaussian curve fit was created for both JS38 and JS249, and the functions of these graphs were summed to create a double Gaussian curve fit for the mixes each day. In each of the graphs, the values were normalized to account for differences in the number of cells imaged per day. The ratio of the areas under the curve was calculated, JS249:JS38 by taking the integral of both parts of the double Gaussian functions separately from zero to 1000. The expected result is a 1:1 ratio on day 0 and an increase in the ratio each following day.

Competition strain growth rate measurements

The experiment was conducted in a triplicate to ensure reproducibility. JS38 and JS249 were grown in 5-ml overnight cultures containing PYE/Kan/Gent and xylose at 28°C. The cells were washed to remove the xylose and induced overnight in serial dilutions with Van. The next day, an OD₆₀₀ was measured, and the cells in log phase were used to inoculate six 50-ml cultures, creating an OD₆₀₀ of around 0.05. OD₆₀₀ timepoints were taken for each of the six flasks every 90 min, and the data was used to analyze the doubling time of each strain. A master mix of PYE/Kan/Gent/Van was used starting with the overnight in Van and used for the cultures, as well as the media for blanking while taking OD₆₀₀.

RNase E purification

Full-length RNase E gene was amplified from *C. crescentus* genome using VN106F and VN107R primers and cloned into pET-GFP vector through ligation-independent cloning (LIC). The sequence verified plasmid was transformed into BL21(DE3) cells and the resultant colonies were inoculated into lysogeny broth (LB) media (50 ml) with 30 µg/ml Kanamycin and grown overnight at 37°C, 200 rpm. The saturated culture was reinoculated into 1.5-l LB media containing 30 µg/ml Kanamycin and grown at 37°C, 200 rpm until the OD reaches ~0.6. RNase E expression was induced with 0.5 mM IPTG at 37°C, 140 rpm for 3 h. The cells were harvested at 5000 rpm for 15 min and resuspended in 20 ml of Lysis buffer (20 mM Tris pH 7.4, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 10 µg/ml DNase I, two protease inhibitor cocktail tablets, and 1 mM PMSF). The cells were lysed in Sonicator at 4°C with 5 s on time and 10 s off time for 3 min. The lysate was centrifuged at 4°C, 14000 rpm for 45 min and the resultant supernatant was passed through pre-equilibrated Ni-NTA resin for binding of GFP-RNase E-His to the resin. After washing the protein bound resin with

5 column volumes each of low salt buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 10 mM imidazole) and high salt buffer (20 mM Tris pH 7.4, 1000 mM NaCl, 5% glycerol, 10 mM imidazole), the protein was eluted in elution buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 250 mM imidazole). The protein was concentrated using amicon concentrator with 30 kDa cutoff and the concentrated protein was passed through Superdex 200 increase 10/300 GL size exclusion column in size exclusion chromatography (SEC) buffer (20 mM Tris pH 7.4, 250 mM NaCl, 2% glycerol) for further purification. The resultant pure protein was concentrated to 15 mg/ml and stored in -80°C.

The GFP RNase E Δ IDR (RNase E Δ IDR) was the proteolyzed fragment recovered from size exclusion chromatography while performing GFP RNase E full-length (RNase E) purification.

In vitro transcription of *C. crescentus* RNase E 5' UTR and 9S rRNA

The plasmid containing RNase E 5' UTR (pVN053) was linearized using Nhe1 restriction enzyme, which served as template in in vitro transcription (IVT). The IVT reaction mixture contained 2.7 mg of linearized plasmid, 21 µg T7 RNA polymerase, 2.5 mM NTPs, 1X reaction buffer (50 mM Tris-Cl pH 7.4, 15 mM MgCl₂, 5 mM DTT, 2 mM spermidine), 0.01 U/µl yeast PPase in a total volume of 1000 µl and the reaction was carried out at 37°C for 4 h. The transcribed RNA loaded onto 7% urea gel was extracted using phenol-chloroform and ethanol precipitation methods. The precipitated RNA was resuspended in nuclease-free water for carrying out RNase E cleavage assays. The template for *C. crescentus* 9S rRNA IVT was created by PCR amplification (using VN108 F and VN109 R) consisting of T7 promoter and 9S rRNA sequence.

IVT of *E. coli* 9S rRNA

The plasmid containing 300 bp of the *E. coli* 9S rRNA (we included an additional 55 bases as a 3' extension compared with the 245 bp used in Hardwick *et al.* [Hardwick *et al.*, 2011]) under the T7 promoter was ordered from Twist biosciences. The plasmid was linearized by HindIII, followed by IVT by purified T7-RNAP and RNA purification was carried out as described for the *C. crescentus* RNAs.

pCp Cy5 labeling of RNase E 5' UTR

In vitro transcribed RNase E 5' UTR (4.5 µM) was incubated with 33 µM of pCp Cy5 (Jena Bioscience #NU-1706-CY5) in a total reaction volume of 100 µl consisting of 50 units of T4 RNA ligase 1 (NEB #M0204S), 1 mM ATP, 10% DMSO, 1X T4 RNA ligase reaction buffer (NEB #B0216S) for 16 h at 16°C. Following the reaction, T4 RNA ligase 1 was heat inactivated at 65°C for 15 min and the reaction mixture was subjected to Phenol-Chloroform extraction to remove the enzyme. The unincorporated pCp Cy5 was removed by passing the labeled RNA through Sephadex G-50 column (Cytiva # 28903408) and eluted in nuclease-free water.

In vitro RNase E condensate formation assay

6 µM of GFP RNase E or GFP RNase E Δ IDR were incubated with 25 ng/µl RNase E 5' UTR - Cy5 RNA in 20 mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT buffer in a total reaction volume of 10 µl for 30 min at room temperature. Entire 10 µl was spotted on a slide and covered with coverslip before imaging with Nikon Eclipse NI-E with CoolSNAP MYO-CCD camera and 100x Oil CFI Plan Fluor (Nikon) objective under phase contrast, GFP and Red channels with exposures of 30 ms, 50 ms, and 50 ms, respectively.

In vitro 9S rRNA processing assay

Indicated concentrations of RNase E or RNase E Δ IDR were incubated with indicated concentrations of *E. coli* or *C. crescentus* 9S rRNAs in 20 mM Tris pH 7.4, 150 mM NaCl, 2% glycerol, 1 mM DTT, 5 mM MgCl₂ buffer in a total reaction volume of 40 μ l at 28°C. At the end of each timepoint, 10 μ l of the reaction volume was mixed with 15 μ l of stop buffer (95% formamide, 50 mM ethylenediamine tetraacetic acid (EDTA), 0.1% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol). The samples were heated at 90°C for 3 min and resolved on 7% or 10% urea-acrylamide gel, stained with 1X SYBR Gold nucleic acid stain and scanned using GE Typhoon FLA 9000 gel scanner.

In vitro RNase E 5' UTR cleavage assay

1 μ M of GFP RNase E or GFP RNase E Δ IDR was incubated with 0.5 μ M of RNase E 5' UTR in 1X reaction buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2% glycerol, 1 mM DTT, 100 μ M MgCl₂) in a total reaction volume of 40 μ l. At the end of each timepoint, 10 μ l of this reaction mixture was added to 15 μ l of stop buffer (95% formamide, 50 mM EDTA, 0.1% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol). The samples were heated at 90°C for 3 min and loaded onto 7% urea-acrylamide gel. The gel was stained with 1X SYBR Gold nucleic acid stain (Thermo Fisher Scientific #S11494) for 20 min and scanned using Thermo Fisher iBright imaging system. RNA-Cy5 gel was imaged using 650 nm laser in Typhoon FLA 9000 gel scanner.

For the cleavage assay under condensate forming condition, 6 μ M RNase E or RNase E Δ IDR was incubated with 3 μ M RNase E 5' UTR and the reaction was carried out as mentioned above. At the end of each timepoint, the reaction is diluted 50 times so that the RNA loaded in each well is 100 ng.

PLASMID CONSTRUCTION

pVN053

RNase E 5'UTR along with the first 150 bases of coding sequence was amplified from *C. crescentus* genome using VN082F and VN083R primers. The amplified sequence was ligated into pMCS-2 vector under T7 promoter through Gibson assembly. Positive colonies were selected on LB-Kan plate followed by Sanger sequencing.

pVN059

Full-length RNase E gene was amplified from *C. crescentus* genome using VN106F and VN107R primers and cloned into pET-GFP vector through LIC.

Table of oligos

Oligo ID	Sequence
RNE5'UTR_F	CAAGATTGGATCCGCACGCGAAATCCGTGATCGTCACG
RNE5'UTR_R	GCATCTTCTTCGACATTAAGGTTCTGTTTGCCCTACCGCG
PVRNEYFPC-4_F	GGGACAAACGAACCTTAATGTCGAAGAAGATGCTGATCGACGCA
PVRNEYFPC-4_R	GGATTTGCGGTGCGGATCCAATCTTGATCGTAATCAAACGGACGT
VN082F	tcgcgagacgtccaattgcatatgTAATACGACTCACTATAGGGCACGCGAAATCC
VN083R	gtggatccccgggctgcagctagcAACGCGCGTCACCTTGGCGA
VN106F	TACTCCAATCCAATGCAatgtcgaagaagatgctgatcgacgca
VN107R	TTATCCAATTCCAATGTTATTAttaccggcgccaccagccccgacg
VN108 F	taatacgactcactatagggtcagtcacaaacccatgcaaacgcatg
VN109 R	tcagggtgtttgttgattgtgatggagg

pBXMCS-2 RNE YFP

RNase E-YFP from the pVRNEYFPC-4 plasmid was digested with NdeI and NheI and ligated into pBXMCS-2 cut with NdeI and XbaI. The plasmid was sequence verified (genewiz) before electroporation into *Caulobacter*.

Strain Construction

JS8: NA1000 RNE::pXRNEssrAC Kan^R

The strain was generated as described before (Al-Husini et al., 2018).

JS49: NA1000 vanA::FL-RNE-YFP Gent^R

The strain was generated as described before (Al-Husini et al., 2018).

JS61: NA1000 vanA::RNE(Δ E/G)-YFP Gent^R

The strain was generated as described before (Al-Husini et al., 2018).

JS62: NA1000 vanA::RNE(DRhIBBS)-YFP Gent^R

The strain was generated as described before (Al-Husini et al., 2018).

JS89

The strain was generated by transforming pBXMCS-2-RNE YFP plasmid into NA1000 cells and the positive colonies were selected on PYE-kan medium followed by YFP fluorescence in the cells.

JS249

To generate JS249, we inserted the 5' UTR into the pVRNEYFPC-4 plasmid via Gibson assembly. The 5' UTR was amplified from the NA1000 chromosome using RNE5'UTR_F and RNE5'UTR_R primers and the pVRNEYFPC-4plasmid containing RNaseE-YFP was amplified using PVRNEYFPC-4_F and PVRNEYFPC-4_R primers. We then performed Gibson assembly using both fragments, transformed into DH10B cells, and selected on LB-Kan plates. Colonies were then screened for the RNase E 5' UTR and sequence verified. The resulting pVRNE5'UTR-RNEYFPC-4 plasmid was then transformed into NA1000 cells by electroporation and selected on PYE-gent plates. GentR colonies were then subjected to phage transduction using JS8 lysates and selected on PYE-gent-kan-xylose plates. The resulting colonies were phenotyped for xylose- and vanillate-dependent growth.

JS 293: NA1000 vanA::RNE(ASMmut2)YFPC Gent^R

The strain was generated as described before (Al-Husini et al., 2018).

Table of Strains used

Strain name	Genotype
JS8	NA1000 RNE::pXRNEssrAC Kan ^R
JS49	NA1000 vanA::FL-RNE-YFP Gent ^R
JS61	NA1000 vanA::RNE(Δ E/G)-YFP Gent ^R
JS62	NA1000 vanA::RNE Δ S1-YFP Gent ^R
JS89	NA1000+pBXMCS2 with FLRNE-YFP Kan ^R
JS221	NA1000 vanA::RNase E Δ IDR-YFP integrated +RNE::pXRNEssrAC-2 Gent ^R Kan ^R
JS249	NA1000 vanA::RNE 5'UTR-YFP + RNE::pXRNEssrAC-2 Gent ^R Kan ^R
JS 293	NA1000 vanA::RNE(ASMmut2)YFPC Gent ^R

ACKNOWLEDGMENTS

The authors thank Wayne State University startup funds to J.M.S. Research reported in this publication was supported by NIGMS of the National Institutes of Health under award number R35GM124733. We thank Dr. Tamara Hendrickson for providing lab space and equipment to perform experiments while in revision.

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