Generation of pre-tRNAs from polycistronic operons is the essential function of RNase P in *Escherichia coli*

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**ABSTRACT**

Ribonuclease P (RNase P) is essential for the 5′-end maturation of tRNAs in all kingdoms of life. In *Escherichia coli*, temperature sensitive mutations in either its protein (*rnpA49*) and or RNA (*rnpB709*) subunits lead to inviability at nonpermissive temperatures. Using the *rnpA49* temperature sensitive allele, which encodes a partially defective RNase P at the permissive temperature, we show here for the first time that the processing of RNase P-dependent polycistronic tRNA operons to release pre-tRNAs is the essential function of the enzyme, since the majority of 5′-immature tRNAs can be aminoacylated unless their 5′-extensions ≥8 nt. Surprisingly, the failure of 5′-end maturation elicits increased polyadenylation of some pre-tRNAs by poly(A) polymerase I (PAP I), which exacerbates inviability. The absence of PAP I led to improved aminoacylation of 5′-immature tRNAs. Our data suggest a more dynamic role for PAP I in maintaining functional tRNA levels in the cell.

**INTRODUCTION**

Ribonuclease P (RNase P) is an essential riboendonuclease responsible for the 5′-end maturation of every tRNA and is found in all three domains of life (1–4). It is a ribozyme consisting of an RNA and one or more protein subunits (5,6), although protein-only variants of RNase P have recently been described (7–11). The recently identified obligatory parasitic archaeon *Nanoarchaeum equitans*, which does not have RNase P activity (4), is the single exception. In *Escherichia coli*, RNase P is comprised of two subunits, a small protein (C5 encoded by the *rnpA* gene) and a catalytic RNA (M1 encoded by the *rnpB* gene) (12) and is essential for cell viability. In addition to its well-characterized role in 5′-end maturation of tRNAs, RNase P is required for separation of pre-tRNAs from at least seven polycistronic tRNA transcripts in *E. coli* (13–15). RNase P has also been shown to process the precursors of 4.5S RNA and tmRNA as well as several mRNAs residing in polycistronic operons (16–18).

While a mature 3′-end with a CCA trinucleotide is required for aminoacylation of a tRNA (19), there is no data to suggest that 3′-maturation of a tRNA is dependent on 5′-end maturation by RNase P. The vast majority of *E. coli* pre-tRNAs (79/86) are matured at their 3′ termini using a combination of six 3′ → 5′ exonucleases (RNase T, RNase PH, RNase D, RNase BN/Z, RNase II and polynucleotide phosphorylase) (20–22), while the rest use an one-step RNase E endonucleolytic cleavage (23). When 3′-processing is compromised due to absence of RNase T and/or RNase PH, most tRNAs become substrates for polyadenylation by poly(A) polymerase I (PAP I, encoded by *pncB*) (24). However, the fate of the 5′-unprocessed pre-tRNAs that are observed in the absence of RNase P is unknown.

Due to the essential nature of RNase P, temperature sensitive *E. coli* mutants in either the protein or RNA subunit have been used extensively to determine its *in vivo* activity. The commonly used *rnpA49* allele was isolated from heavily mutagenized cells (25) and was shown to contain an A to G transition resulting in a change from an arginine to histidine residue in the C5 protein (26), leading to temperature sensitive RNase P activity. In this mutant, tRNAs with unprocessed 5′-ends as well as polycistronic tRNA transcripts accumulate rapidly upon shift to the nonpermissive temperature (13–15,25).

Of the 86 tRNAs found in *E. coli*, 36 are transcribed either as monocistronic or are the first tRNA in a polycistronic transcript that have 5′ leader sequences varying from 2 to 52 nucleotides (27). In addition, pre-tRNAs resulting from RNase E cleavages of polycistronic tRNA transcripts will retain their 5′ leaders in an RNase P mutant (28–30). However, a significant number of tRNAs will be present as unprocessed polycistronic transcripts, which are processed directly at their mature 5′ termini by RNase P (13–15). Thus, RNase P is required not only to process the 5′ leader sequences on pre-tRNAs, but also to process a significant number of polycistronic tRNA transcripts.

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The exact reason why RNase P is essential *E. coli* has not been determined. It has been suggested that the 5'-unprocessed tRNAs, which occur in the *rnpA49* mutant at the nonpermissive temperature, are not aminoacylated thereby failing to support the need for increased protein synthesis at the higher temperature (31). However, several reports have demonstrated that a pre-tRNA with unprocessed nucleotides at the 5'-end can be efficiently aminoacylated at its mature 3'-end. For example, yeast aspartic tRNA with a 5'-extension has been shown to be aminoacylated in vitro by aspartyl-tRNA synthetase with same *K_m*, but with 3-fold reduction in *K_cat* values compared to the 5'-mature species (32). Furthermore, it is now known that tRNA^His^ nearly universally contains an additional G residue at the −1 position at the 5'-end, which is essential for efficient aminoacylation by histidinyl-tRNA synthetase (33,34). In addition, complementation of a *leuU* deletion in *E. coli* with a tRNA^Leu^ containing one extra nucleotide at the 5'-end (G−) (13) suggested its functionality in vivo.

These data are consistent with the fact that successful aminoacylation is primarily dependent on the interaction between the anti-codon loop, the CCA terminal trinucleotide and the D-stem of the tRNA with its cognate aminoacyl tRNA synthetase (35). Furthermore, complementation of an *E. coli* RNase P mutant with an RNase P derived from *Arabidopsis thaliana*, where many aberrant 5' cleavages of tRNAs were observed (36), has indicated that 5'-maturation of tRNAs is not critical for aminoacylation and cell viability.

It has also been suggested that the essential function of RNase P is the processing an essential precursor RNA, such as 4.5S RNA encoded by *fss* (18). However, overexpression of 4.5S RNA from a plasmid that did not require RNase P processing at its 5'-terminus to restore cell viability (15). Furthermore, recent experiments have shown that unprocessed 5'-extended 4.5S RNA supports cell viability in an *E. coli* RNase P mutant (36). It is worth noting that overexpression of the M1 RNA has been shown to partially complement the *rnpA49* mutation (37), but the exact mechanism of this partial complementation has not been yet determined.

We have previously shown that at least seven primary polycistrionic tRNA transcripts are dependent on initial RNase P endonucleolytic cleavages to generate pre-tRNAs (13–15). Thus, it is expected that none of the tRNAs (at least 25) derived from these operons will be available for maturation at either end in the absence of RNase P, resulting in a significant reduction in the functional levels of these tRNAs. Accordingly, we have hypothesized that the essential function of RNase P is most likely related to the separation of pre-tRNAs from primary tRNA transcripts rather than 5'-end maturation.

In this report, we show that complementation of the *rnpA49* mutant by overexpression of M1 RNA at the permissive temperature is due to the restoration of wild type RNase P activity resulting in more efficient processing of RNase P-dependent polycistrionic tRNA operons and increased availability of tRNAs for aminoacylation. We also show that a significant number of 5'-unprocessed tRNAs are substrates for aminoacylation except those with 5'-extensions ≥8 nt, which supports the hypothesis that it is not the 5' maturation of pre-tRNAs, but the processing of RNase P-dependent polycistrionic tRNA operons that is the essential function of RNase P. In addition, the polyadenylation of 5'-unprocessed pre-tRNAs by PAP I exacerbates the slow growth of an *rnpA49* mutant at both the permissive and nonpermissive temperatures by destabilizing these tRNAs. Thus, deletion of the *pcnB* gene in the *rnpA49* genetic background leads to an improved growth rate that arises from both increased steady-state levels of pre-tRNAs as well as improved aminoacylation.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

All the strains used in this study were derived from MG1693 (*thyA715 rph-1*), which has a single base pair deletion in the *rph* gene, resulting in a frame-shift mutation and the loss of RNase PH activity (38). A wild-type control SK10153 (*thyA715*) containing a functional *rph* gene was generated by P1 transduction (24). SK2525 (*rnpA49 rbsD296::Tn10 rph-1*) has been previously described (30). The *rnpA49* allele encodes a temperature-sensitive RNase P enzyme and does not support cell viability at 44°C (25).

A P1 lysate grown on SK2525 (*rnpA49 rph-1*) was used to transduce SK10521 to obtain SK10521 (*rnpA49*). A P1 lysate grown on SK7988 (*ΔpcnB::kan rph-1*) (39) was used to transduce SK2525 and SK10521 to generate SK10297 (*rnpA49 ΔpcnB::kan rph-1*) and SK10522 (*rnpA49 ΔpcnB::kan*), respectively. A P1 lysate grown on SK10148 (*Δrnt::kan rph-1*) (30) was used to transduce SK2525 to generate SK10700 (*rnpA49 Δrnt::kan rph-1*).

The plasmids pAAK17 (*rnpB*/*Cm^R^) and pBKM83 (*hisR*/*Cm^S^) contain the p15A origin of DNA replication (15–20 copies/cell) and express the M1 RNA (*rnpB*) and tRNA^His^, respectively, under the control of the lacZ promoter. Both plasmids were constructed by cloning a PCR (polymerase chain reaction) fragment containing the lacZ promoter, either the *rnpB* or *hisR* coding sequences followed by a Rho-independent transcription terminator [derived from *leuU* gene (14)] into the BamHI/HindIII sites of pBKM11 (40). Both PCR fragments were generated by overlapping PCR. In order to avoid the RNase P requirement for 5' maturation of tRNA^His^, the *hisR* coding sequence was cloned so that it was transcribed starting at its mature 5'-end. SK10521 (*rnpA49*) was transformed with pAAK17 and pBKM83 to construct SK10521 and SK10779, respectively. No difference in the growth rate was observed in presence and absence of IPTG (data not shown) for either plasmid, suggesting leaky expression of both genes. pBKM14 (*Cm^R^) has been described previously (40). Plasmid pBKM14 (*Cm^R^) (with no *hisR*) (40) was transformed into SK10522 (*rnpA49*) to construct SK10778 and was used as the control.

**Bacterial growth and RNA isolation**

Bacterial strains were grown in Luria broth supplemented with thymine (50 μg/ml). When appropriate, tetracycline (20 μg/ml), kanamycin (25 μg/ml) or chloramphenicol (20 μg/ml) was added to the culture medium. Bacterial
growth was measured using a Klett–Summerson Colorimeter (No. 42 green filter). Unless mentioned otherwise, the cultures were initially grown at 30°C until they reached 50 Klett units above background and then were shifted to 44°C to inactivate RNase P. The cultures were maintained in exponential growth by periodic dilutions with pre-warmed Luria broth.

Steady-state RNA was isolated when the bacteria were either at 50 Klett units at 30°C (permissive temperature) or after 60 min at 44°C (nonpermissive temperature) following the shift. Total RNA for time-course analyses was isolated starting at 60 s (0 min) after the addition of rifampicin (500 μg/ml, dissolved in DMSO) and nalidixic acid (40 μg/ml, dissolved in water) to the bacterial culture. All RNA samples for northern analysis, poly(A) sizing assays and RT-PCR clonings were isolated using a modified RNAsnap® protocol and treated with DNase I as described previously (41). All RNA samples were initially quantified by measuring the OD260 using a Nanodrop (ND2000c) apparatus. To ensure equal loading during northern blotting, 500 ng of all RNA samples were separated in agarose mini gels and normalized to Vistra Green (Amersham Bioscience) stained 16S and 23S rRNA using a PhosphorImager (Storm 840, GE Healthcare) and quantified using ImageQuant TL (V7) software. All northern analyses were repeated at least three times. The quantification data (relative fold increase or decrease) mentioned in the text represent an average of three independent determinations.

**RESULTS**

The level of short poly(A) tails increased significantly in the *rnpA49* mutant

Previously, we have shown that 79/86 tRNAs in *E. coli*, which require 3′ → 5′ exonucleases for 3′-end maturation, become PAP I substrates in the absence of these exonucleases (24). In contrast, the three proline tRNAs, which are matured by an RNase E endonucleolytic cleavage at the CCA terminus, are not polyadenylated (23,24). Based on these findings, we sought to determine if failure to process the 5′-termini of pre-tRNAs might also make them susceptible to polyadenylation.

Initially, we performed a poly(A) sizing assay to compare the length and the distribution of poly(A) tails in *rnpA49* and 3′ → 5′ exonuclease mutants (Figure 1). The level of the poly(A) tails longer than 10 nts was almost identical in the wild type and *rnpA49* single mutant (lanes 3–4). Surprisingly, there was a 3 ± 1-fold increase in the amount of short poly(A) tails (≤10 nts) in the *rnpA49* mutant compared to the wild type control. In contrast, the loss of RNase PH and RNase T led to a 12 ± 2-fold increase in the amount of short poly(A) tails in the *Δrnt rplh-1* double mutant (lane 1), demonstrating that inhibition of 3′-end maturation was more critical than 5′-end maturation for polyadenylation to occur. However, the level of short poly(A) tails increased almost an additional 2-fold (23 ± 4-fold) in the *Δrnt rplh-1 rnpA49* triple mutant (lane 2), indicating a synergistic effect resulting from the failure of both 5′- and 3′-end maturation. The absence of all the poly(A) tails except a few of ≤6 nts in the *rnpA49 ΔpncB* double mutant (lane 5) was consistent with PAP I being responsible for the addition of the tails observed in lane 4.

5′-Unprocessed pre-tRNAs are susceptible to polyadenylation

We have shown previously that the poly(A) tails added to 3′-unprocessed tRNAs in the absence of RNase T and RNase PH are usually short (<10 nt) (24,45). Thus, the increase in short poly(A) tails in the *rnpA49* mutants (Figure 1) indicated that 5′-immature tRNAs were likely targets...
for polyadenylation upon inactivation of RNase P. Accordingly, we analyzed several tRNAs isolated from the rnpA49 mutant at the nonpermissive temperature by sequencing both their 3’- and 5’-ends as previously described (14,24,45).

tRNA<sub>leuX</sub> is processed from a monocistronic transcript encoded by <i>leuX</i>. We have previously shown that ∼19% (5/27 tRNAs) of the 5’-termini and ∼67% (18/27 tRNAs) of the 3’-termini of <i>leuX</i> tRNAs were immature in the <i>rph-1</i> strain, while ∼44% (8/18) of the 3’-immature termini contained short poly(A) tails (22) (Figure 2A). A similar analysis of the <i>leuX</i> tRNA isolated from the <i>rnpA49</i> <i>rph-1</i> double mutant showed that all the tRNAs (25/25 tRNAs) had immature 5’-leader sequences, while ∼92% (23/25 tRNAs) had immature 3’-termini (Supplementary Figure S1A). More importantly, ∼88% (22/25 tRNAs) of the immature tRNAs had 1–4 untemplated A residues (Figure 2A, Supplementary Figure S1A). The increase in the fraction of polyadenylated 5’-immature <i>leuX</i> tRNAs in the <i>rnpA49 rph-1</i> double mutant was consistent with previous results (22). However, no tRNAs with untemplated A residues beyond the three encoded A residues were observed in the <i>rnpA49 ΔpcnB rph-1</i> triple mutant (Supplementary Figure S1A), suggesting that all the tRNAs with untemplated A residues in the <i>rnpA49 rph-1</i> double mutant were generated post-transcriptionally by PAP I. The 5’-ends of all the sequenced tRNAs (23/23 tRNAs) isolated from the <i>rnpA49 ΔpcnB rph-1</i> triple mutant retained unprocessed 5’-ends. The unprocessed 5’-ends in both the double and triple mutants ranged between 7 and 13 nt (Supplementary Figure S1A) due to non-specific RNase E.

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**Figure 1.** Distribution of poly(A) tails in various strains. Total RNA (15 μg/lane) was isolated from various strains after one hour of growth at 44°C and separated on a 20% PAGE/8 M urea after digestion with RNase A and RNase T1 as described in the Materials and Methods. The genotype of each strain is listed at the top of each lane. Lane 6, 32P-labeled oligo d(A) size standards.

**Figure 2.** (A) Analysis of polyadenylated 3’-ends and (B) mature 3’-ends of tRNAs in various strains. The nucleotide sequences at the 3’- and 5’-ends of all tRNAs were identified using RT-PCR cloning of 5’-3’ self-ligated transcripts from two independent determinations as described in the Materials and Methods. Total RNA isolated from exponentially growing cultures at 44°C for 1 h after shifting from 30°C were used for self-ligation and RT-PCR. The percentages of polyadenylated 3’-ends were determined based on number of polyadenylated 3’-ends out of total number of immature 3’-ends for a particular tRNA. The percentages of mature 3’-ends were determined based on number of mature 3’-ends out of total number of sequenced 3’-ends (both mature and immature) for a particular tRNA. No polyadenylated 3’-ends was detected in the <i>rnpA49 ΔpcnB rph-1</i> mutant. The <i>rph-1</i> strain was used as control in this experiment. The identified sequences for all the tRNAs are shown in Supplementary Figure S1A–E.
cleavages within the 5′ leader region as has previously been reported (22).

Next, we analyzed the phεU and phεV tRNAs, which are transcribed as monocistronic transcripts with 5′-leader sequences of 3–4 nucleotides (46). Previously, we showed that ∼14% (4/28 tRNAs) of these species retained their 5′-leader sequences and ∼89% (25/28 tRNAs) of them had immature 3′-ends (retaining 1–3 nts trailer sequences downstream of CCA) in a rph-I strain (46). Approximately 36% (9/25 tRNAs) of the 3′-immature termini in the rph-I strain contained short poly(A) tails. In the rnpA49 rph-I double mutant, ∼96% (23/24 tRNAs) of the phεU and phεV tRNAs retained the 5′-immaute ends, while ∼67% (16/24 tRNAs) tRNAs retained the immature 3′-ends. However, the 3′-immature termini with short poly(A) tails increased to ∼81% (13/16 tRNAs) in the double mutant (Figure 2A) (46). In the rnpA49 ΔpcnB rph-I triple mutant, ∼92% (21/24 tRNAs) tRNAs retained their 5′-leader sequences and ∼42% (10/24 tRNAs) tRNAs had immature 3′ termini, but no immature 3′ ends had poly(A) tails (Supplementary Figure S1B).

Subsequently, we examined the hisR (Supplementary Figure S1C) and cysT (Supplementary Figure S1D) tRNAs, both of which are part of distinct polycistronic tRNA transcripts (30). RNase E endonucleolytic cleavages within the argX hisR leuT proM primary transcript release hisR pre-tRNAs with a 5′-leader of 8 nt and a 3′-trailer of 1–3 nt, while the pre-cysT is released with a 4 nt leader at the 5′-end and a 1–2 nt trailer at the 3′-end from the glyW cysT leuW primary transcript (13,14,28,30). About 25% (6/24 tRNAs) of the hisR pre-tRNAs retained the 8 nt 5′-leader sequence and ∼63% (15/24 tRNAs) had immature 3′-ends containing 1–3 encoded trailer nucleotides downstream of CCA in the rph-I strain. In contrast, all the 24 his tRNAs in the rnpA49 rph-I double mutant retained their 5′-leaders and 22/24 tRNAs (∼92%) had the immature 3′-termini. More importantly, the number of immature termini containing short poly(A) tails increased from 36% (4/11 tRNAs) in the rph-I strain to ∼59% (13/22 tRNAs) in the rnpA49 rph-I double mutant (Figure 2A). A similar analysis in the rnpA49 ΔpcnB rph-I mutant showed that ∼71% (20/28) 3′-ends retained the 1–3 nt downstream trailer sequence, but none had poly(A) tails. All 5′-ends except one were immature in the rnpA49 ΔpcnB rph-I triple mutant (Supplementary Figure S1C).

While all of the cloned cysT tRNAs were mature at their 5′-end in the rph-I strain, almost all of the cysT tRNAs retained their 5′ leader sequences in the rnpA49 rph-I double (98%, 49/50 tRNAs) and the rnpA49 ΔpcnB rph-I triple (35/35 tRNAs) mutants (Supplementary Figure S1D). Surprisingly, the number of immature 3′ termini was the highest in the rph-I strain (∼77%, 27/35 tRNAs) compared to the rnpA49 rph-I double (∼16%, 8/50 tRNAs) and rnpA49 ΔpcnB rph-I triple (∼6%, 2/35 tRNAs) mutants. However, the tRNAs with immature 3′-ends with short poly(A) tails was higher in the rnpA49 rph-I double mutant (∼50%, 4/8 tRNAs) compared to the rph-I strain (∼26%, 7/27 tRNAs) (Figure 2A, Supplementary Figure S1D). None of the immature 3′-ends in the rnpA49 ΔpcnB rph-I triple mutant were polyadenylated.

We also analyzed tRNAserV, encoded by serV, which was predicted to have the longest (52 nt) 5′-leader sequence of all the E. coli tRNAs (27). Surprisingly, sequencing analysis showed 5′-leader sequences ranging from 3–56 nt in all genetic backgrounds (Supplementary Figure S1E), suggesting it can have a 5′-leader sequence of up to 56 nts. The multiple cleavages upstream of the mature 5′ terminus in the rnpA49 mutants were most likely due to nonspecific endonucleolytic cleavages by RNase E as we have shown previously for the leuX tRNA (22). As expected, tRNAs in both the rnpA49 rph-I double mutant (17/27 tRNAs) and the rnpA49 ΔpcnB rph-I triple mutant (14/24 tRNAs) retained a higher percentage of the 5′-leader sequences ranging from ∼58–63% compared to ∼20% (4/20 tRNAs) in the rph-I strain. The processing of the 3′-ends was also similar in both rnpA49 mutants except ∼25% (3/12 tRNAs) of the immature tRNAs in the rnpA49 rph-I double mutant had short poly(A) tails (Figures 2A, Supplementary Figure S1E). Although no poly(A) tails were expected in the rnpA49 ΔpcnB rph-I triple mutant, the inability to detect any poly(A) tails in the rph-I strain (Supplementary Figure S1E) was unexpected. It is possible that either serV is not a preferred PAP I substrate or it undergoes faster 3′-end maturation thereby preventing polyadenylation.

Taken together, these data suggested that pre-tRNAs are more susceptible to polyadenylation by PAP I in the rnpA49 mutant (Figure 2A) in agreement with the poly(A) sizing analyses (Figure 1). Furthermore, the number of tRNAs with mature 3′-ends increased in the rnpA49 ΔpcnB rph-I triple mutant compared to rnpA49 rph-I double mutant for all the tRNAs studied (Figure 2B), although no improvement in their 5′ end maturation was observed. The fraction of leuX tRNAs with mature 3′-ends increased from 8% (2/25 tRNAs) in the rnpA49 rph-I mutant to 57% (13/23 tRNAs) in the rnpA49 ΔpcnB rph-I triple mutant (Supplementary Figure S1A). The number of phεU and phεV tRNA mature 3′-ends increased from 33% (8/24 tRNAs) in the rnpA49 rph-I mutant to 58% (14/24 tRNAs) in the rnpA49 ΔpcnB rph-I triple mutant (Figure 2B; [46]). The number of hisR tRNAs with mature 3′-ends increased from 8% (2/24 tRNAs) in the rnpA49 rph-I mutant to 29% (8/28 tRNAs) in the rnpA49 ΔpcnB rph-I triple mutant (Supplementary Figure S1C), while the number of cysT tRNAs with mature 3′-ends increased from 84% (42/50 tRNAs) in the rnpA49 rph-I mutant to 94% (33/35 tRNAs) in the rnpA49 ΔpcnB rph-I triple mutant (Supplementary Figure S1D).

**Suppression of the conditional lethality associated with the rnpA49 mutant**

Previous experiments have shown that the conditional lethality associated with the rnpA49 allele could be partially reversed at 42°C by overexpressing the M1 RNA (encoded by rnpB) (37,47,48), although the exact mechanism of this complementation was not established. Additionally, we observed that an rnpA49 ΔpcnB double mutant had a faster
growth rate compared to the rnpA49 single mutant at both permissive and nonpermissive temperatures (see below).

In a first step to determine if the improvement in growth in the presence of extra M1 RNA or the inactivation of PAP I was occurring by a similar mechanism, we cloned the rnpB gene into a 15–20 copy plasmid (pAAK17 (rnpB+/Cm\(^{5}\)) and transformed it into an rnpA49 mutant. Initially we compared the ability of RNase P mutants to grow on Luria agar plates at various temperatures (Supplementary Figure S2). As expected, all the strains grew up to a 10\(^{-5}\) dilution at 30°C. At 42°C, very weak growth of the rnpA49 strain was observed only when undiluted. In contrast, the rnpA49 ΔpcnB double mutant showed some growth up to a 10-fold dilution and rnpA49/pAAK17 strain showed growth up to a 100-fold dilution. At 44°C, the rnpA49 strain failed to grow, while both rnpA49 ΔpcnB and rnpA49/pAAK17 double mutants showed growth up to a 10-fold dilution.

Next, we compared the growth rate of the various rnpA49 mutants in Luria broth (Figure 3A). The rnpA49 single mutant grew slower at 30°C compared to the wild type control and ceased growth within 40–60 minutes after shifting to the nonpermissive temperature of 44°C. Overexpression of M1 RNA from a 15–20 copy plasmid (pAAK7/rnpB\(^{+}\)) in the rnpA49 strain restored the growth rate at 30°C to the wild type level. The rnpA49 ΔpcnB double mutant also grew faster than the rnpA49 single mutant, but slower than the rnpA49/pAAK17 strain at 30°C. Growth of both the rnpA49 ΔpcnB and the rnpA49/pAAK17 strains continued for about 60 minutes after shifting to 44°C before it significantly tapered off.

The growth phenotype of RNase P mutants shown in Figure 3A indicated that while the rnpA49 single mutant stopped growing completely at 44°C, both the rnpA49 ΔpcnB and rnpA49/pAAK17 strains kept growing slowly at the nonpermissive temperature. Thus, we determined if cessation in cell growth at the nonpermissive temperature resulted in a loss of cell viability. Viable cell counts in all strains increased until 150 min after shifting to the nonpermissive temperature (Figure 3B). The viable cell counts from the rnpA49 single mutant dropped significantly by 210 min and were ~20-fold less than the wild type control. In contrast, viable cell counts in both rnpA49 ΔpcnB and rnpA49/pAAK17 strains increased during this period. However, the total number of viable cells was significantly lower in all the rnpA49 mutants compared to the wild type control suggesting much slower cell division. Furthermore, significant differences in the growth rate and viable cell counts between rnpA49 ΔpcnB and rnpA49/pAAK17 strains (Figure 3A, B) suggested independent mechanisms of complementation in the two genetic backgrounds.

**Overexpression of the M1 RNA improved the processing of RNase P-dependent polycistronic (RNA transcripts at 30°C**

It has been shown previously that *in vitro* the RNase P holoenzyme containing the RnpA49 protein has a lower cleavage efficiency at the permissive temperature compared to the RNase P from a wild type control (31), suggesting that its reduced activity might be responsible for the mutant’s slower growth rate (Figure 3A). Consequently, the full complementation of the growth phenotype of rnpA49 single mutant after M1 RNA overexpression at the permissive temperature (Figure 3A) indicated that the RNase P activity had been restored to wild type levels. In order to directly test this hypothesis, we analyzed the processing of *valV valW*.
leuQ leuP leuV, and secG leuU operons in various rnpA49 mutants using Northern blot analysis (Figures 4, Supplementary Figures S3 and S4), since it has been shown that the initial processing of these three operons is completely dependent on RNase P (13,14).

As expected, Northern blot analysis of steady-state RNA showed no full-length valV valW transcripts (VW1 and VW2) at either 30°C or 44°C in the wild type control (Figure 4B). In contrast, a significant amount of unprocessed valV valW transcripts was observed in both the rnpA49 single and rnpA49 ΔpcnB double mutants at 30°C and increased dramatically at 44°C (Figure 4B). Consequently, the mature tRNA level (M, processed fraction, PF) in these strains was lower than in the wild type control at 30°C and dropped to below 15% at 44°C. In contrast, at 30°C the VW1 and VW2 species were barely detectable in the rnpA49/pAAK17 strain and the mature tRNA level was similar to the wild type control. However, at 44°C the levels of the VW1, VW2 and mature tRNA (M, PF) species were at the similar levels to the rnpA49 single mutant. Furthermore, a time-course analysis of the valV valW transcript (Figure 4C) showed that the VW1 and VW2 species could be detected up to 20 minutes after transcription was stopped by rifampicin addition in both the rnpA49 single and rnpA49 ΔpcnB double mutants at 30°C. However, no unprocessed transcripts were observed in the wild type control and the rnpA49/pAAK17 strain under similar conditions. At 44°C, the unprocessed transcripts (VW1 and VW2) were detected until up to 60 minutes after rifampicin addition in all strains except the wild type control (Figure 4C).

Northern analyses of leuQ leuP leuV and secG leuU operon transcripts showed similar results (Supplementary Figures S3 and S4). The processing of both operons was significantly inhibited in the rnpA49 and rnpA49 ΔpcnB strains at the permissive (30°C) temperature. In contrast, their processing in the rnpA49/pAAK17 strain was normal and similar to the wild type control. Although it was expected that the processing of all three operons would be dramatically impaired at 44°C in the rnpA49 single mutant, it was somewhat unexpected that processing did not improve in either the rnpA49 ΔpcnB or rnpA49/pAAK17 strains at 44°C (Supplementary Figures S3 and S4).

**The improvement in the growth rate of the rnpA49 ΔpcnB mutant was not related to changes in M1 RNA levels**

Since the catalytic activity of RNase P resides in the M1 RNA (49), partial complementation of the rnpA49 mutation in the presence of additional M1 RNA was not entirely unexpected. In fact, overexpression of rnpB led to ~5–6-fold increase in the steady-state M1 RNA level in the rnpA49/pAAK17 strain compared to the wild type control at both temperatures (Figure 5). However, no significant difference in the M1 RNA level was found between the rnpA49 single and rnpA49 ΔpcnB double mutants (Figure 5). The lower M1 RNA levels at the nonpermissive temperature in both strains compared to the wild type control was consistent with the reported shorter half-life of the rnpB transcript in the rnpA49 mutant (50).

**Chargeable tRNA levels were higher in the rnpA49 ΔpcnB mutant**

After ruling out that the changes in M1 RNA levels accounted for the improvement of the growth phenotype of rnpA49 ΔpcnB strain, we suspected that there was an improvement in the aminoacylation of tRNAs in the double mutant compared to the rnpA49 single mutant based on the significant improvement in the maturation of 3′-ends of tRNAs in the rnpA49 ΔpcnB double mutant without any change in the 5′-end maturation (Figures 2B, Supplementary Figure S1). Previous in vitro and in vivo studies have suggested that tRNAs with unprocessed nucleotides at the their 5′-ends, but a mature 3′-end could serve as substrates for aminoacylation (13,32,36). Accordingly, several tRNAs were analyzed in vivo for their aminoacylation patterns in wild type, rnpA49, rnpA49 ΔpcnB and rnpA49/pAAK17 strains at permissive (P, 30°C) and nonpermissive (NP, 44°C) temperatures.

Total RNAs isolated under acidic conditions were analyzed using acid urea gel electrophoresis. In this procedure the aminoacylated tRNA runs slower prior to deaminoacylation by Tris treatment. After Tris treatment, the aminoacylated tRNAs change mobility without any effect on the non-aminoacylated tRNAs, which may include polyadenylated as well as immature tRNAs (24). We determined the percentage of aminoacylated tRNA (PAT) in each strain as well as the relative amount of chargeable tRNA (RCT) compared to the wild type control. The PAT is calculated as the fraction of aminoacylated tRNA based on the total of the specific tRNA pool (charged and uncharged) available in that strain. The RCT is calculated as the net amount of chargeable tRNAs (those that changed in mobility after Tris treatment) in each strain compared to the wild type control.

We initially tested several tRNAs that are part of RNase P-dependent polycistronic operons. For example, tRNA^{Ser}_{32} encoded by the leuU gene requires RNase P for its separation from the secG leuU polycistronic transcript (14) (Supplementary Figure S3). The percentage of leuU tRNA aminoacylation was similar in all genetic backgrounds at the permissive temperature [Figure 6B, PAT(P)]. At the nonpermissive temperature, the percentage of aminoacylation in the wild type and rnpA49 ΔpcnB strain was almost identical and was only reduced slightly in the rnpA49 and rnpA49/pAAK17 strains [Figure 6B, PAT(NP)].

The amount of chargeable tRNAs remained almost identical in the wild type, rnpA49 ΔpcnB double mutant and rnpA49/pAAK17 strains at the permissive temperature [Figure 6A, lanes 5–8; 6B, RCT(P)]. However, it was already reduced in the rnpA49 single mutant compared to the wild type control at the permissive temperature [Figure 6A, lanes 1–4, 6B, RCT(P)]. At the nonpermissive temperature, the amount of chargeable tRNA [Figure 6B, RCT(NP)] was further decreased in both the rnpA49 single mutant (Figure 6A, lanes 11–12) and rnpA49/pAAK17 strains (Figure 6A, lanes 15–16). No significant reduction in the amount of chargeable tRNA in the rnpA49 ΔpcnB double mutant was observed (Figure 6A lane 13–14). Almost identical amounts of chargeable tRNA in the wild type control and rnpA49 ΔpcnB double mutant at both the permissive and non-
Figure 4. Analyses of valV valW transcripts processing in various strains by Northern blot analyses. (A) Graphical presentation (not drawn to scale) of valV valW operon in the genome. (B) Analysis of valV valW steady-state transcript level. Total RNA (10 μg/lane) isolated from exponentially growing cultures at 30 °C and after one hour of growth at 44 °C were used. (C) Comparison of valV valW transcript stability in various strains. Total RNA (10 μg/lane) isolated from exponentially growing culture (at 30 °C and after 1 h of growth at 44 °C) at various time points after addition of rifampicin and nalidixic acid were used. All RNA samples were separated on 6% PAGE with 8 M urea and transferred to nylon membrane. The blots were probed with a 32P-end labeled oligonucleotide probe (VALW, Supplementary Table S1). Genotypes are shown at the top of each blot. The processing intermediates (VW1 and VW2) and mature (M) tRNA are labelled to the right of the blot (13). ptRNAs marked with (*) are due to weak hybridization to Valine tRNAs independent of valV valW (13). PF (processed fraction) in blot B represents the fraction of the mature tRNA (M) relative to the total amount of the tRNA (processed and unprocessed combined) in the specific strain. The numbers were calculated based on the pixel counts of each of the band in the strain and represent the average of three independent determinations. Multiple blots in each panel were run separately and merged at the vertical and/or horizontal lines.
permisive temperature suggested that the 5'-unprocessed tRNAs in the double mutant were stabilized and aminoacylated in the absence of PAP I. Overexpression of rnpB (M1 RNA), which complemented the rnpA49 allele at the permissive temperature, failed to do so at the nonpermissive temperature (compare Figure 6, lanes 7–8 and 15–16).

It has been shown previously that the leuU pre-tRNAs that accumulate in the rnpA49 mutant contain 3'-mature termini and 5'-immature termini with 5–11 nt extensions due to non-specific RNome (14). Multiple higher molecular weight bands in the rnpA49 mutants at the nonpermissive temperature were consistent with the accumulation of such 5'-unprocessed pre-tRNAs (Figure 6A, lanes 11–16). Surprisingly, these bands also changed mobility and became smaller after alkaline treatment suggesting aminoacylation at the 3'-end. Similar results were also obtained for leuT, leuP, leuQ, leuV, valV, valW, metT, and metU tRNAs (data not shown), which are also transcribed as part of RNase P-dependent polycistronic transcripts (13, 43).

Next, we tested tRNAs that are independent of initial RNome processing, such as hisR, cysT, pheU and pheV. While hisR and cysT are unique tRNAs and are part of RNase E-dependent polycistronic tRNA operons (30), the pheU and pheV tRNAs are transcribed as monocistronic transcripts (46). RNase E cleaves 8 nt upstream and 1–2 nt downstream of hisR and 4 nt upstream and 1–2 nt downstream of cysT (14). The majority of the higher molecular weight hisR tRNAs prior to Tris treatment converted to mature tRNA after the Tris treatment, suggesting a negligible amount of unprocessed tRNA in the wild type control at both the permissive and nonpermissive temperature (Figure 6A, lanes 1–2 and 9–10). In contrast, a significant amount of the high molecular weight hisR tRNA in the rnpA49 ΔpcnB double mutant was not converted to the size of mature tRNA after alkaline treatment (lanes 5–6 and 13–14) at both temperatures, which was consistent with their retaining the 8 nt 5'-leader sequence in the absence of RNase P. Furthermore, unlike leuU 5'-unprocessed tRNAs, alkaline treatment had no effect on these hisR 5'-unprocessed tRNAs, indicating that these were not aminoacylated.

Surprisingly, there was a dramatic reduction in the total amount of the hisR tRNA in the rnpA49 single mutant, where bulk of the hisR tRNA retaining the 5'-leader sequence was missing at both temperatures (Figure 6A, lanes 3–4 and 11–12). Overexpression of rnpB restored hisR tRNA levels to almost wild type levels at the permissive temperature (Figure 6A, lanes 7–8), but not at the nonpermissive temperature (Figure 6A, lanes 15–16). The reduction in hisR tRNA species in the rnpA49 and rnpA49/pAAK17 strains at 44°C was most likely due to polyadenylation dependent turnover of these species, since the absence of PAP I stabilized these species significantly in the rnpA49 ΔpcnB double mutant (Figure 6A, lanes 5–6, 13–14).

The level of hisR tRNA aminoacylation was only minimally reduced in all the rnpA49 mutants at both the permissive and nonpermissive temperature [Figure 6C, PAT(P) and PAT(NP)]. The relative amount of chargeable tRNAs (RCT) was reduced significantly both at the permissive and nonpermissive temperature in the rnpA49 single mutant compared to the wild type strain [Figure 6C, RCT(P) and RCT(NP)]. Under identical conditions, the chargeable tRNA (RCT) level was significantly higher in the rnpA49 ΔpcnB double mutant compared to the rnpA49 single mutant. The percentage of aminoacylation in the rnpA49/pAAK17 mutant was higher than the rnpA49 single mutant and similar to the wild type and rnpA49 ΔpcnB double mutant at the permissive temperature [PAT(P)], but similar to the rnpA49 single mutant at the nonpermissive temperature [PAT(NP)]. Overexpression of rnpB, increased the amount of chargeable tRNAs available for aminoacylation in rnpA49/pAAK17 strain over ~4-fold at the permissive temperature [RCT(P)], but failed to do so at the nonpermissive temperature [RCT(NP)].

The results obtained for cysT, pheU and pheV tRNAs were similar to what was observed with hisR except that the 5' immature tRNAs seemed to be aminoacylated based on their reduction in size after alkaline treatment and their levels were not significantly reduced in the rnpA49 mutant (data not shown). Furthermore, all these pre-tRNAs were destabilized in the rnpA49 single mutant, but significantly stabilized in the rnpA49 ΔpcnB double mutant. In addition, M1 RNA overexpression in the rnpA49/pAAK17 strain led to improved aminoacylation levels of all tRNAs at the permissive temperature, but not at the nonpermissive temperature (data not shown).

5'-End maturation of tRNAs is not mandatory for aminoacylation.

The data presented above suggested that tRNAs with unprocessed 5'-ends were aminoacylated in the rnpA49 mutant at the nonpermissive temperature (Figure 6A). In order to obtain additional support for this conclusion, we analyzed the proline tRNAs encoded by proK, proL and proM. Both proK and proL are monocistronic transcripts with 5'-leaders of 5–7 nt (23). proM is the last gene in a polycistronic transcript that is separated by RNase E cleavages at 4–7 nt up-
stream of the mature 5'-end (23,28). These tRNAs are matured at their 3' termini by a one-step RNase E cleavage immediately downstream of the CCA determinant without the involvement of any 3' → 5' exonucleases (23). Accordingly, these tRNAs are not subject to polyadenylation, but are still dependent on RNase P for 5'-end maturation (23). Thus, most of the proline tRNAs were matured at the 3' end, but remained immature at the 5' end in an rnpA49 mutant at the nonpermissive temperature (23). The RNA isolated at the permissive temperature showed that there was only one high molecular weight aminoacylated tRNA band which returned to the size of the mature tRNA after Tris treatment in all genetic backgrounds (Figure 7A, lanes 1–8). In contrast, the RNA isolated under nonpermissive conditions showed that there were two high molecular weight aminoacylated tRNA bands in all the rnpA49 mutants (Figure 7A, lanes 11–16). After Tris treatment, both bands changed mobility with one still being larger than other. The smaller one was the fully matured tRNA identical to the wild type strain (lanes 9–10) and the larger one arose from the 5'-immature tRNA, which was observed only in the rnpA49 mutants (lanes 11–16).

Furthermore, the percentage aminoacylation of proline tRNAs was experimentally identical in each strain at both the permissive and nonpermissive temperature (Figure 7B, PAT). There was a minor reduction in the amount of chargeable proline tRNAs in the rnpA49 and rnpA49/pAAK17 strains, which was similar at both the permissive and nonpermissive temperature (Figure 7B, RCT). It has been shown that metV, metW, metY and metZ rep-
representing the metf1 and metf2 isotypes are also not subject to polyadenylation (24) and are believed to be matured at the 3′-end by one-step RNase E cleavage (23). An aminoacylation analysis of these tRNAs yielded essentially similar results to proK, proL, proM (data not shown).

**Pre-tRNAs with 5′-extension of < 8 nts and mature 3′ end are substrates for aminoacylation.**

Subsequently, we analyzed the aminoacylation patterns of 35 tRNA transcripts encoding 17 tRNAs in various RNase P mutants in this study (Table 1). Only two of these tRNAs (Met m and Val2) were part of RNase P-dependent operons where no significant amount of 5′-unprocessed pre-tRNAs was detected in the rnpA49 mutants. The rest of the tRNAs examined were processed mostly by initial endonucleolytic cleavages by enzymes other than by RNase P such that significant levels of 5′-unprocessed pre-tRNAs were detected in the rnpA49 mutants. Based on the mobility of aminoacylated tRNAs in acid-urea gels, it was apparent that the pre-tRNAs with unprocessed 5′-ends ≥8 nts (hisR, serV) were not effectively aminoacylated with relative amount of chargeable tRNAs (RCT) <15% (Table 1). In contrast, the 12 pre-tRNAs with unprocessed 5′-end of <8 nts had much higher RCT levels, suggesting that they were effectively aminoacylated.

**Inviability of rnpA49 mutant is not due to lack of efficient hisR aminoacylation**

Since tRNAHis is unique in *E. coli*, is very unstable in the rnpA49 mutant, and is not efficiently aminoacylated due to its longer unprocessed 5′-end (Figure 6A, C), it might be a possible candidate for causing the observed inviability. To answer this question, we overexpressed hisR from a plasmid (pBMK83) where hisR transcription was initiated at its mature 5′-end employing a lac promoter thereby avoiding the requirement of RNase P for 5′-end maturation. Northern analyses confirmed that there was ∼8 ± 1-fold increase in the mature tRNA level in rnpA49/pBMK83 strain compared to rnpA49/pBMK14 strain (Supplementary Figure S5). The relative chargeable tRNA (RCT) level of tRNAHis in rnpA49/pBMK83 was even higher than the wild type strain (Table 1). However, no growth complementation in rnpA49/pBMK83 was observed at 44°C (data not shown), ruling out the lack of the mature hisR as the cause of the inviability in the rnpA49 mutant.
Table 1. The leader lengths and the aminoacylation status of various tRNAs in the *rnpA49* mutant at nonpermissive temperatures

| tRNAs   | tRNA isotypes/operons | Leader length * | RCT** compared to the wild type strain (%) |
|---------|-----------------------|-----------------|------------------------------------------|
| Trp     | *trpT*                | 3               | 80 ± 3                                    |
| Phe     | *pheU, pheV*          | 3–4             | 94 ± 5                                    |
| Cys     | *cysT*                | 4               | 30 ± 5                                    |
| Ser1    | *serT*                | 5               | 90 ± 3                                    |
| Pro     | *proK, proL, proM*    | 5–7             | 90 ± 5                                    |
| Leu2    | *leuU*                | 5–11†           | 50 ± 5                                    |
| Asn     | *asnT, asnU, asnV, asnW* | 6–8         | 25†                                       |
| Leu1    | *leuT*                | <8              | 32 ± 5                                    |
| Leu3    | *leuW*                | <8              | 30†                                       |
| Leu5    | *leuX*                | 6–22†          | 90 ± 5                                    |
| Lys     | *lytO, lytT, lytV, lytW* | ND         | 20†                                       |
| Met2    | *metY*                | ND              | 67 ± 5                                    |
| His     | *hisR*                | 8               | 14 ± 3                                    |
| His9    | *hisR9*               | 0               | 110 ± 5                                   |
| Ser3    | *serV*                | 52              | 15–17†                                    |
| Met m   | *metT, metU*          | .b              | <10                                       |
| Val2    | *valV, valW*          | .b              | <10†                                      |
| Leu1    | *leuQ, leuP, leuV*    | .b              | ?                                         |
| Met f1  | *met2, metW, metT*    | .b              | ?                                         |

* Leader lengths are based on previous (14,23,27,46) and this study.
** Relative amount of chargeable tRNA (see text) in *rnpA49* mutant after one hour of growth at 44°C.
† Done once.
‡ 5′ leader lengths are due to non-specific RNase E cleavage in the *rnpA49* mutant (14,22).
§ RNR P dependent operons (13,14). No significant amounts of unprocessed pre-tRNAs were detected.
?: RCT could not be determined since these RNase P dependent tRNAs have RNase P independent tRNA isotypes.
ND: Not determined.
*†* tRNA isotype expressed from pBMK83 (his R+/CmR).

** DISCUSSION **

Since it was first shown in 1972 that RNase P was required for the 5′-end maturation of tRNAs in *E. coli* (51), it has been thought that the processing of tRNA precursors must be perfect at both the 5′ and 3′ termini to permit aminoacylation. It was therefore assumed that 5′-end maturation was the essential function of the enzyme. However, here we have shown that 66% of the *E. coli* pre-tRNAs we tested (12 out of 14 pre-tRNAs) are aminoacylated between 20 and 94% of their wild type levels in the absence of RNase P; if their unprocessed 5′-extensions are <8 nts in length (Table 1). Only pre-tRNAs with 5′-extensions ≥8 nts appeared to be poor substrates for aminoacylation (Table 1).

The fact that the aminoacylation levels for the majority of the tRNAs were not reduced significantly in the absence of RNase P, under conditions where the pre-tRNAs were not matured at their 5′-ends (Figures 6, 7, Table 1, data not shown), strongly suggests that the tRNA aminoacylation machinery in *E. coli* has considerable tolerance for the presence of extra sequences of up to 7 nts at the 5′-ends of tRNA substrates. *In vivo* aminoacylation of 5′-extended tRNAs is consistent with the *in vitro* aminoacylation of a yeast tRNA<sup>Amp</sup> with a 5′-extension (32) and the complementation of *E. coli* RNase P mutant with RNase P isozymes from *A. thaliana* (AtRNase P), which cleave several *E. coli* pre-tRNAs in aberrant locations (36). Furthermore, it has also been shown that aminoacylation of a tRNA by its cognate aminoacyl-tRNA synthetase depends only on a limited number of nucleotides, primarily at the amino acid acceptor stem and the anticodon nucleotides (52,53). Taken together, the data presented here appear to rule out the loss of pre-tRNA aminoacylation in the *rnpA49* mutants as the primary reason for the strains’ inviability at nonpermissive temperatures.

In contrast, our previous work has shown that at least seven polycistronic tRNA transcripts, encoding 25 tRNAs, in *E. coli* genome are absolutely dependent on initial RNase P processing to release pre-tRNAs that can be converted into aminoacylated species upon 3′-end maturation (13–15). When coupled with our recent finding using transcriptome analysis that three additional operons, encoding another 9 tRNAs, require initial RNase P processing (Moehanty and Kushner, manuscript in preparation), it appears that a significant drop in the steady-state levels of at least 34/86 tRNAs, including all seven valine species, will occur in the absence of RNase P. In fact, the data presented here shows a direct correlation between cessation of growth and a significant drop in the steady-state level of tRNAs, as low as ~10% of wild type levels, derived from the *valV, valW, secG*, *leuU* and *leuP, leuV* operons in the absence of RNase P (Figures 4, Supplementary Figures S3 and S4). Thus, the failure to generate pre-tRNAs from primary transcripts is the likely cause for the cessation of growth and loss of cell viability at nonpermissive temperatures.

This conclusion is supported by the results shown in Figure 4 and Supplementary Figures S3, S4 where the increased expression of the M1 RNA restored efficient processing of RNase P dependent polycistronic operons coupled with a restoration of a wild type growth rate for the *rnpA49* mutant at the permissive temperature (Figure 3A). Since the RNase P holoenzyme containing the mutant protein has been shown *in vitro* to have a lower cleavage efficiency compared to the wild type holoenzyme at the permissive temperature (31), the increased expression of the M1 RNA (Figure 5) clearly helped restore the full catalytic activity of RNase P at 30°C (Figure 4, Supplementary Figures S3, S4).

While the protein subunit plays critical role in stabilizing the catalytically active conformation of M1 RNA (54) and helps define the substrate specificity of the RNase P enzyme (55), the defective C5 protein has reduced solubility at the nonpermissive temperature via a M1 RNA mediated quality control mechanism (56). Thus, although the overexpression of the M1 RNA was sufficient to improve RNase P activity at the permissive temperature, it failed to improve the processing of the RNase P-dependent primary tRNA transcripts (Figure 4 and Supplementary Figures S2-S3) at the nonpermissive temperature, resulting in the loss of complementation (Figure 3).

The inactivation of RNase P not only results in the failure of pre-tRNA generation from various RNase P-dependent operons (13–15), but it also leads to the accumulation of 5′-immature tRNAs derived from polycistronic operons processed by RNase E (28,30). While such pre-tRNAs undergo normal 3′-end maturation by one or more of the 3′ → 5′ exonucleases (RNase T, RNase PH, RNase D, RNase BN and RNase II), in competition with poly(A) polymerase I (PAP
While aminoacylation of tRNAs requires a fully matured 3′-end terminated with the CCA trinucleotide, addition of a single nucleotide following the CCA determinant or additions to 5′-extensions (14, 22) made them substrates for aminoacylation provided they have mature 3′-extensions (14, 22), making them substrates for aminoacylation. The exact reason why tRNAs with 5′-extensions ≥8 nts are poor substrates for aminoacylation is not clear at this time. It is possible that the longer 5′-extensions change the three-dimensional structure of the tRNA in such a way that it is no longer recognized by its cognate tRNA synthetase.

Since at least 34/86 tRNAs from 11 polycistronic tRNA operons are affected in the absence of RNase P, it is not likely that complementation of any single tRNA isotype will be sufficient to restore growth. Taken together, the data presented here show that it is not the failure of pre-tRNA aminoacylation, but rather the dramatic reduction in pre-tRNA levels derived from primary transcripts dependent on RNase P for their initial processing that is most likely responsible for the viability of rnpA49 mutants. Thus, the processing of polycistronic tRNA operons by RNase P should be considered its essential function. Furthermore, our data show that PAP I-dependent polyadenylation, which was once considered to be most relevant to mRNA metabolism in E. coli, also plays a more prominent role in tRNA metabolism.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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