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Kinetics of paused ribosome recycling in *Escherichia coli*

Brian D. Janssen and Christopher S. Hayes*

Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, Santa Barbara, CA 93106-9610

Summary

The bacterial tmRNA•SmpB system recycles stalled translation complexes in a process termed ‘ribosome rescue’. tmRNA•SmpB specifically recognizes ribosomes that are paused at or near the 3' end of truncated mRNA, and therefore nucleolytic mRNA processing is required before paused ribosomes can be rescued from full-length transcripts. Here, we examine the recycling of ribosomes paused on both full-length and truncated mRNAs. Peptidyl-tRNAs corresponding to each paused translation complex were identified, and their turnover kinetics used to estimate the half-lives of paused ribosomes *in vivo*. Ribosomes were paused at stop codons on full-length mRNA using a nascent peptide motif that interferes with translation termination and elicits tmRNA•SmpB activity. Peptidyl-tRNA turnover from these termination-paused ribosomes was slightly more rapid in tmRNA⁺ cells ($T_{1/2} = 22 \pm 2.2$ s), compared to Δ tmRNA cells ($T_{1/2} = 32 \pm 1.6$ s). Overexpression of release factor-1 (RF-1) greatly accelerated peptidyl-tRNA turnover from termination-paused ribosomes in both tmRNA⁺ and Δ tmRNA cells, whereas other termination factors had little or no effect on recycling. In contrast to inefficient translation termination, ribosome recycling from truncated transcripts lacking in-frame stop codons was dramatically accelerated by tmRNA•SmpB. However, peptidyl-tRNA still turned over from nonstop-paused ribosomes at a significant rate ($t_{1/2} = 61 \pm 7.3$ s) in Δ tmRNA cells. Overexpression of RF-1, RF-3, and ribosome recycling factor (RRF) in Δ tmRNA cells failed to accelerate ribosome recycling from nonstop mRNA. These results indicate that tmRNA•SmpB activity is rate-limited by mRNA cleavage, and that RF-3 and RRF do not constitute a tmRNA-independent rescue pathway as previously suggested. Peptidyl-tRNA turnover from nonstop-paused ribosomes in Δ tmRNA cells suggests the existence of another uncharacterized ribosome rescue pathway.

Keywords

peptidyl-tRNA; release factor; ribosome pausing; tmRNA; translation termination

Introduction

Protein synthesis is terminated when the ribosome encounters a stop codon in the A site. In eubacteria, the three “universal” stop codons are recognized by two distinct protein release factors (RF). RF-1 decodes UAA and UAG, whereas RF-2 decodes UAA and UGA stop codons. Upon binding the A-site stop codon, RF-1 and RF-2 coordinate hydrolysis of the

*Correspondence should be addressed to: Christopher S. Hayes, Department of Molecular, Cellular and Developmental Biology, Life Sciences Technology Building, Room 3105, University of California, Santa Barbara, CA 93106-9610, Tel: 805-893-2028, Fax: 805-893-4724. chayas@lifesci.ucsb.edu.

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nascent protein chain from the P-site tRNA^{1; 2}, releasing the newly synthesized protein from the ribosome. Additional protein factors are required to disassemble and recycle the post-termination ribosome. Release factor-3 (RF-3) is a non-essential GTP-binding protein that facilitates dissociation of both RF-1 and RF-2 from the post-termination ribosome^{3; 4}, thereby enhancing translation termination by increasing the available pool of RF-1 and RF-2. After RF-1 or RF-2 are removed (or dissociate) from the post-termination ribosomal A site, ribosome recycling factor (RRF) and elongation factor G (EF-G) collaborate to separate the 70S ribosome into individual 50S and 30S subunits⁵. The free ribosomal subunits are then able to initiate protein synthesis on other messages.

It has long been recognized that nascent peptide sequences influence translation termination in *Escherichia coli*. Isaksson and colleagues extensively characterized the effect of C-terminal dipeptide sequences on translation termination, and found that nascent chains with Asp, Gly, and especially Pro residues significantly increased stop codon readthrough^{6; 7}. Subsequently, C-terminal Asp-Pro and Pro-Pro nascent peptide sequences were found to induce tmRNA•SmpB activity and A-site mRNA cleavage during translation termination in *E. coli*^{8; 9}. A-site cleavage activity truncates the message, producing a nonstop mRNA, which lacks an in-frame stop codon. Ribosomes stalled on nonstop mRNA are unable to undergo canonical translation termination, and are instead “rescued” by the tmRNA•SmpB system in eubacteria^{10; 11}. tmRNA (transfer-messenger RNA) is a stable RNA that functions both as a tRNA and an mRNA during ribosome rescue. tmRNA binds the A site of stalled ribosomes using its aminoacylated tRNA-like domain, and the nascent chain is rapidly transferred to tmRNA via peptidyl transferase activity. The truncated message is then released, and translation resumes using a small open reading frame within tmRNA^{10; 12}. This process results in the co-translational addition of the tmRNA-encoded SsrA peptide tag to the nascent protein, followed by translation termination and ribosome recycling. The SsrA peptide is a degradation tag recognized by several bacterial proteases^{13; 14; 15}. SmpB is a tmRNA-binding protein that is required for delivery of tmRNA to the ribosome, and translation of the SsrA peptide tag^{16; 17}. Thus, tmRNA•SmpB is a quality control system that recycles stalled ribosomes and targets incompletely synthesized proteins for degradation. Taken together, these observations suggest that specific nascent peptides inhibit RF-mediated translation termination, while still allowing suppressor tRNA and/or tmRNA•SmpB activity on paused ribosomes.

Heretofore, nascent peptide-induced ribosome pausing during translation termination has been inferred from A-site mRNA cleavage and SsrA-peptide tagging activities^{8; 9; 18; 19}. Although A-site mRNA cleavage and SsrA tagging are generally correlated with ribosome pausing, there are instances where strong translational arrest does not elicit either activity in *E. coli*^{20; 21}. Therefore, to more directly examine the kinetics of translational pauses, we used pulse-chase experiments to monitor peptidyl-tRNA turnover from paused ribosomes. During nascent peptide-mediated translational pausing at stop codons, peptidyl-tRNA turnover occurred at similar rates in Δ tmRNA and tmRNA⁺ cells. In contrast, ribosomes arrested at the ends of nonstop mRNA turned over much more rapidly in tmRNA⁺ cells than in Δ tmRNA cells. These results are consistent with data showing that tmRNA•SmpB-mediated ribosome rescue requires truncated mRNA²².

However, paused ribosomes were still recycled from nonstop mRNA in the absence of tmRNA. We demonstrate that RF-3 and RRF do *not* play a significant role in this tmRNA-independent ribosome recycling. These findings suggest the existence of another, uncharacterized ribosome-recycling pathway.

Results

Peptidyl prolyl-tRNA^{Pro} is a marker of termination-paused ribosomes

C-terminal Pro-Pro residues in the nascent peptide interfere with translation termination and induce a variety of alternative translation reactions in *Escherichia coli* 6⁸; 9. We reasoned that peptidyl prolyl-tRNA^{Pro} bound to the ribosomal P site should accumulate when ribosomes pause during translation termination. Peptidyl-tRNA has reduced mobility during gel electrophoresis, and can be readily distinguished from aminoacyl-tRNA by Northern blot. To facilitate peptidyl prolyl-tRNA analysis, we generated a mini-gene expression construct based on the previously characterized *ybeL-PP* gene from *E. coli* 8⁹. The *flag-(m)ybeL-PP* mini-gene encodes a 60-residue peptide composed of an N-terminal FLAG epitope fused to the C-terminal 49 residues of YbeL-PP (Fig. 1a). The C-terminal Pro residue of FLAG-(m)YbeL-PP was coded as CCC (Fig. 1a), which is decoded exclusively by tRNA₂^{Pro} in *E. coli* 23. The other five Pro residues in FLAG-(m)YbeL-PP were coded as CCG, which is decoded by either tRNA₁^{Pro} or tRNA₃^{Pro} 23. Therefore, upon overexpression of FLAG-(m)YbeL-PP, the accumulation of peptidyl prolyl-tRNA₂^{Pro} should be indicative of termination-paused ribosomes. Indeed, Northern blot analysis showed that the majority of tRNA₂^{Pro} was mobility shifted in cells expressing FLAG-(m)YbeL-PP, but not in uninduced cells (Fig. 1b). Peptidyl prolyl-tRNA₂^{Pro} did not accumulate in cells expressing the FLAG-(m)YbeL-EA (Figs. 1a & 1b), whose mRNA does not contain a CCC Pro codon, and should undergo efficient translation termination based on previous results with full-length YbeL-EA protein 8⁹. Moreover, peptidyl alanyl-tRNA_{1B}^{Ala} was not detected in cells expressing FLAG-(m)YbeL-EA, indicating that peptidyl-tRNA does not typically accumulate as a result of translation termination (Fig. 1b).

Although the observed peptidyl prolyl-tRNA₂^{Pro} is likely to be ribosome-associated, peptidyl-tRNA can “drop-off” of paused ribosomes under some circumstances. Drop-off products are rapidly hydrolyzed by peptidyl-tRNA hydrolase (Pth), whereas ribosome-bound peptidyl-tRNA is protected from Pth activity 24²⁵. However, because the prolyl-tRNA^{Pro} ester bond is apparently resistant to hydrolysis during translation termination, it is possible that Pth hydrolyzes this product slowly, allowing peptidyl prolyl-tRNA₂^{Pro} to accumulate off of the ribosome. To test this hypothesis, we used purified His₆-tagged Pth (Pth-His₆) to hydrolyze peptidyl prolyl-tRNA₂^{Pro} *in vitro*. At low Pth-His₆ concentrations (1 nM), peptidyl prolyl-tRNA₂^{Pro} was hydrolyzed more slowly than the control peptidyl glycyl-tRNA₃^{Gly} isolated from cells expressing the *secM'* mini-gene (Fig. 1c) 20²⁶. However, peptidyl prolyl-tRNA₂^{Pro} was rapidly and completely hydrolyzed at higher concentrations (50 nM) of Pth-His₆ (Fig. 1c). Because the intracellular concentration of Pth is ~ 1 μM in *E. coli* 27, we suspect that there is sufficient activity to efficiently hydrolyze any peptidyl prolyl-tRNA drop-off products *in vivo*. This conclusion was supported by experiments in which we overproduced wild-type Pth from an arabinose-inducible promoter, and observed no decrease in peptidyl prolyl-tRNA₂^{Pro} levels (Fig. 1b). Finally, we fractionated cell lysates using sucrose density ultracentrifugation, and found that peptidyl prolyl-tRNA₂^{Pro} partitioned with ribosomes in the pellet fraction, whereas most of the free tRNA₂^{Pro} remained in the supernatant fraction (Fig. 1d). We note that peptidyl prolyl-tRNA₂^{Pro} levels decayed significantly during the process cell lysis and ultracentrifugation (compare Figs. 1b & 1d). Taken together, these results strongly suggest that peptidyl prolyl-tRNA₂^{Pro} is bound the P site of paused ribosomes.

Peptidyl prolyl-tRNA₂^{Pro} accumulates in tmRNA⁺ cells

The C-terminal Pro-Pro nascent peptide induces SsrA-peptide tagging in *E. coli* 8. During the peptide tagging process, the nascent chain is transferred from tRNA^{Pro} to tmRNA, and the paused ribosome is recycled into individual 30S and 50S subunits. Therefore, tmRNA•SmpB-mediated ribosome rescue is predicted to diminish, or perhaps even prevent, the accumulation

of peptidyl prolyl-tRNA₂^{Pro}. However, the same levels of peptidyl prolyl-tRNA₂^{Pro} were observed in tmRNA⁺ and ΔtmRNA cells (Fig. 1b). It is possible that FLAG-mYbeL-PP overexpression overwhelmed the capacity of tmRNA•SmpB, allowing paused ribosomes to accumulate. To test this proposal, we overproduced tmRNA•SmpB from a multi-copy plasmid and examined peptidyl prolyl-tRNA₂^{Pro} levels. Though tmRNA•SmpB was overexpressed ~30-fold compared to wild-type cells, there was no significant decrease in peptidyl prolyl-tRNA₂^{Pro} by Northern analysis (data not shown).

To determine whether static peptidyl-tRNA levels reflect the kinetics of translational pausing, we examined peptidyl prolyl-tRNA₂^{Pro} turnover using pulse-chase analysis. Nascent chains were pulse-labeled with [³⁵S]-labeled methionine/cysteine, chased with excess unlabeled amino acids, and peptidyl-tRNA analyzed by gel electrophoresis and autoradiography. A ladder-like pattern of radiolabeled species was observed in samples taken from cells expressing FLAG-(m)YbeL-PP, whereas fewer products were observed in cells expressing FLAG-(m)YbeL-EA (Fig. 2a). In contrast, no high-molecular weight species were radiolabeled in uninduced cells (data not shown). To confirm that radiolabeled products were indeed peptidyl-tRNAs, we treated a sample with purified Pth-His₆ *in vitro*. Pth-His₆ converted most of the radiolabeled products into species of lower gel mobility (Fig. 2b). However, the smallest radiolabeled product was unaffected by Pth-His₆ treatment (Fig. 2b). These data indicate that the higher molecular weight species are peptidyl-tRNAs, whereas the smallest product is most likely radiolabeled methionyl-tRNA^{Met} and cysteinyl-tRNA^{Cys}, which are not Pth substrates 28; 29. The multiple peptidyl-tRNA species observed in cells expressing FLAG-(m)YbeL-PP is suggestive of ribosome queuing behind the termination-arrested ribosome. To identify which peptidyl-tRNA species corresponds to peptidyl prolyl-tRNA₂^{Pro}, we directly compared the migration position of blotted [³⁵S]-labeled peptidyl-tRNA with that of peptidyl prolyl-tRNA₂^{Pro} identified by Northern blot hybridization (Fig. 2b). The lower band of the prominent radiolabeled doublet co-migrated with peptidyl prolyl-tRNA₂^{Pro} (Fig. 2b); and therefore the decay of this radiolabeled species was quantified to determine the kinetics of paused ribosome recycling.

Pulse-chase experiments showed that peptidyl prolyl-tRNA₂^{Pro} turnover was similar in tmRNA⁺ and ΔtmRNA cells (Fig. 2c). Although peptidyl-tRNA turnover was adequately described by single exponential decay, we chose to fit double exponentials to the data because there are at least two peptidyl prolyl-tRNA₂^{Pro} decay processes in tmRNA⁺ cells: RF-mediated termination and tmRNA•SmpB-mediated ribosome rescue. For consistent analyses, and because the fits were generally better, double exponentials were also fitted to data from ΔtmRNA cells. The composite half-life (*T*_{1/2}) of peptidyl prolyl-tRNA₂^{Pro} decay was 22 ± 2.2 s in tmRNA⁺ cells, and 32 ± 1.6 s in ΔtmRNA cells (Table 1 and Fig. 2d). Overproduction of tmRNA•SmpB did not increase the rate of peptidyl prolyl-tRNA₂^{Pro} turnover (Table 1, Figs. 2c, and 2d), indicating that peptidyl-tRNA turnover is not limited by insufficient tmRNA•SmpB capacity. Moreover, overexpression of Pth had no effect on peptidyl prolyl-tRNA₂^{Pro} turnover in ΔtmRNA cells (Table 1).

Overexpression of RF-1 accelerates peptidyl prolyl-tRNA₂^{Pro} turnover during translation termination

Previous work has shown that RF-1 overexpression suppresses both A-site mRNA cleavage and SsrA-peptide tagging in the YbeL-PP system, presumably by increasing translation termination efficiency 8; 9. Because RF-1 decodes UAA and UAG, but not UGA stop codons, we predicted that peptidyl prolyl-tRNA₂^{Pro} levels would be reduced in a stop codon-specific manner upon RF-1 overexpression. However, RF-1 had no effect on peptidyl prolyl-tRNA₂^{Pro} accumulation during translation termination at all three stop codons (Fig. 3a). Despite this unexpected result, RF-1 overexpression clearly suppressed A-site cleavage at

UAG and UAA stop codons, but not at the UGA codon (Fig. 3a), consistent with codon-specific relief of pausing during translation termination. To resolve this apparent inconsistency, we analyzed peptidyl-tRNA turnover using pulse-chase experiments, which showed that RF-1 overexpression dramatically increased the rate of peptidyl-tRNA turnover in both Δ tmRNA and tmRNA⁺ cells (Fig. 3b, and data not shown). In fact, pulse-labeled peptidyl-tRNA was difficult to detect when RF-1 was overproduced (Fig. 3b). The disparity between static and kinetic views of peptidyl-tRNA may be a consequence of ribosome queuing. Soon after peptidyl-tRNA hydrolysis and/or paused ribosome recycling, a queued ribosome would rapidly advance to the stop codon and pause, thereby maintaining peptidyl prolyl-tRNA₂^{Pro} at high static levels. Because of the discrepancy between peptidyl-tRNA analyses in this instance, we implemented pulse-chase assays for the remainder of our studies.

The Pro-Pro nascent peptide inhibits RF-1 activity

In principle, the Pro-Pro nascent peptide could inhibit RF-mediated peptidyl-tRNA hydrolysis, or alternatively, decrease RF binding to the ribosomal A site. Based on two precedents, we hypothesized that this nascent peptide inhibits subsequent peptidyl-tRNA hydrolysis. The *E. coli* TnaC and the human cytomegalovirus UL4 uORF2 nascent peptides both contain C-terminal Pro residues that are critical for ribosome pausing during translation termination^{30; 31}. Both TnaC- and UL4-arrested ribosomes have RF bound stably in the A site, but fail to hydrolyze peptidyl-tRNA^{32; 33}. To determine whether RFs bind paused ribosomes in our system, we fractionated cell lysates using sucrose density ultracentrifugation, and analyzed the supernatant and pellet fractions by Western blot using antibodies specific for *E. coli* RF-1. We used *full-length* YbeL proteins in these experiments because the longer nascent chain helped stabilize paused ribosome complexes during ultracentrifugation (data not shown). In the absence of YbeL-PP expression, most RF-1 was found in the supernatant fraction (Fig. 4a). Induction of YbeL-PP expression in both tmRNA⁺ and tmRNA cells caused RF-1 to partition in the pellet fraction (Fig. 4a). In contrast, nearly all RF-1 was found in the supernatant fraction from cells expressing YbeL-EA (Fig. 4a). These results suggest that RF-1 is associated with termination-paused ribosomes. To determine whether RF-1 is bound in the ribosomal A site, we examined RF partitioning in cells expressing YbeL-PP from messages containing UAG, UAA, and UGA stop codons. RF-1 was found in the pellet fraction only when YbeL-PP was synthesized from messages containing UAG or UAA stop codons (Fig. 4b), indicating that RF-1 is likely bound to the A site in codon-specific manner. In contrast, RF-2 was found primarily in the supernatant fraction irrespective of stop codon (Fig. 4b).

Effect of RF-3 and RRF on ribosome pausing during translation termination

We have previously shown that RF-3 and RRF modulate SsrA-peptide tagging during inefficient translation termination⁸. Presumably, these factors facilitate efficient translation termination and canonical ribosome recycling; although several reports have shown that RF-3 and RRF collaborate to remove intact peptidyl-tRNAs from the ribosome^{21; 25; 34; 35}. We examined the effects of RF-3 and RRF on translational pausing during termination using pulse-chase analysis of peptidyl-tRNA turnover. RF-3 overexpression increased the rate of peptidyl prolyl-tRNA₂^{Pro} turnover in tmRNA⁺ cells, but had no significant effect on Δ tmRNA cells (Table 1 and Fig. 5a). This result perhaps suggests that RF-3 increases termination efficiency during translation of the SsrA peptide tag, which would allow tmRNA to recycle more rapidly. Overexpression of RRF did not dramatically change peptidyl prolyl-tRNA₂^{Pro} turnover in either tmRNA⁺ or Δ tmRNA cells, whereas the coexpression of RF-3 and RRF slightly increased turnover in Δ tmRNA cells (Table 1 and Fig. 5a). We also used density ultracentrifugation to examine RF-ribosome interaction in cells overexpressing RF-3 and/or RRF, and found no changes compared to control cells in which RF-3/RRF expression was not induced (Fig. 5b).

Peptidyl-tRNA turnover during translational pausing on nonstop mRNA

We next examined the kinetics of peptidyl-tRNA turnover from ribosomes paused on nonstop messages, which lack in-frame stop codons. We removed the stop codon from *flag-(m)ybeL-PP* and fused the open reading frame to the *rrnB* T1 transcription terminator (Fig. 6a). The *rrnB* T1 terminator effectively terminates T7 RNA polymerase transcription³⁶, and should produce an mRNA with no in-frame stop codons (Fig. 6a). Proteins synthesized from nonstop messages are efficiently tagged by the tmRNA•SmpB system^{12;37}, and therefore we confirmed SsrA-tagging of FLAG-(m)YbeL-rrnB in cells expressing tmRNA(His₆). tmRNA(His₆) encodes the SsrA(His₆) peptide tag, which is resistant to proteolysis and allows purification and identification of tagged proteins^{38;39}. We used mass spectrometry to identify SsrA(His₆)-peptide tagging sites after residues Gly74, Leu75, and Ser76 (Fig. 6b), consistent with translation of a nonstop message truncated at or near these codons. In Δ tmRNA cells, we identified peptidyl-tRNAs by Northern blot using probes specific for tRNA₁^{Ser}, tRNA₂^{Leu}, and tRNA₃^{Gly}, consistent with the expression of a *bona fide* nonstop message (Figs. 6a, 6c, and data not shown). Although each peptidyl-tRNA species was not abundant, its accumulation was dependent upon nonstop message expression and was not detected in tmRNA⁺ cells (Fig. 6c, and data not shown). Additionally, a negative control blot for tRNA_{1B}^{Ala} showed no evidence of peptidyl-tRNA (Fig. 6c). Pulse-chase analysis showed that nonstop-paused ribosomes turned over much more rapidly in tmRNA⁺ cells compared to tmRNA cells (Fig. 6d). Because the number of ribosome-recycling pathways is unknown for nonstop mRNA, we fitted single exponential decay equations to these data. Peptidyl-tRNA turned over with $t_{1/2} = 61 \pm 7.3$ s in Δ tmRNA cells, whereas peptidyl-tRNA could not be detected in tmRNA⁺ cells (Table 1 and Fig. 6d). These results are consistent with *in vitro* data showing that tmRNA•SmpB rescues ribosomes from truncated messages much more efficiently than from full-length mRNA²².

Translation termination factors do not influence ribosome pausing on nonstop mRNA

In contrast to translational pausing at stop codons, ribosomes arrested on nonstop mRNA are unable to turnover via canonical RF-mediated translation termination. Indeed, overexpression of RF-1 had no effect on peptidyl-tRNA turnover from nonstop-paused ribosomes (Table 1). RRF has recently been proposed to promote peptidyl-tRNA drop-off from nonstop-paused ribosomes arrested in both Δ tmRNA and tmRNA⁺ cells⁴⁰. However, we found that the rate of peptidyl-tRNA turnover from nonstop-paused ribosomes was unaffected in Δ tmRNA cells overexpressing either RF-3 or RRF; and the turnover rate actually decreased when RF-3 and RRF were co-expressed (Table 1). These results demonstrate that RRF and RF-3 do not accelerate ribosome recycling from nonstop mRNA. To further examine the role of RRF and RF-3 in paused ribosome recycling, we asked whether these factors influence tmRNA•SmpB activity on nonstop-arrested ribosomes. We reasoned that if RRF and RF-3 possess robust ribosome rescue activity, then they should effectively compete with tmRNA•SmpB for the ribosomal A site, and inhibit SsrA-peptide tagging. To test this hypothesis, we used a well-characterized nonstop mRNA encoding λ N-trpAt, which is the N-terminal domain of λ phage cI repressor fused to the *trp* operon attenuator-transcription terminator (Fig. 7a)^{13;37}. Nearly all λ N-trpAt chains are SsrA-tagged in tmRNA⁺ cells, and these products are stable in cells lacking the ClpP protease, which is responsible for the bulk of SsrA-tagged protein degradation in *E. coli*^{13;37}. In contrast, untagged λ N-trpAt accumulates to high levels in Δ tmRNA cells (Fig. 7a). SsrA-tagged and untagged λ N-trpAt proteins are readily resolved from one another by SDS-PAGE, allowing SsrA-tagging efficiency to be assessed by quantifying the ratio of tagged to untagged protein (Fig. 7a). We first used pulse-chase analysis to confirm that peptidyl-tRNA accumulated in Δ tmRNA cells expressing λ N-trpAt, but not in tmRNA⁺ cells (Fig. 7b). We then co-expressed λ N-trpAt with RF-1, RF-3, RRF, and RF-3/RRF in tmRNA⁺ cells deleted for the ClpP protease. Examination of whole cell lysates by SDS-PAGE and Coomassie staining showed that SsrA-peptide tagging was not inhibited by any of the

overexpressed translation factors (Fig. 7c). Analysis of purified λ N-trpAt by SDS-PAGE confirmed that SsrA-tagging efficiency was unaltered under each condition (Fig. 7c). We note that the overproduced translation factors were readily identified on Coomassie stained gels of cell lysates (Fig. 7c). In fact, RRF was expressed at approximately the same level as λ N-trpAt (Fig. 7c). These results indicate that RRF and RF-3 play no significant role in ribosome rescue from nonstop mRNA.

Effects of nonstop and nascent peptide-induced ribosome pausing on cell growth

Finally, we examined the effects of ribosome pausing on *E. coli* growth and viability. To avoid the general growth inhibition inherent to T7 RNA polymerase-based expression systems, we used IPTG-inducible P_{trc} promoter constructs based on plasmid pPW500¹³. Two new constructs were made, encoding λ N-PP and λ N-LA proteins, which are analogous to YbeL-PP and YbeL-EA described above. We confirmed that the C-terminal Pro-Pro nascent peptide of λ N-PP induced A-site cleavage of the stop codon and SsrA-peptide tagging, whereas no translational pausing or SsrA-tagging was apparent during λ N-LA expression (data not shown). Induction of both λ N-PP and λ N-LA in Δ tmRNA cells led to a loss of viability, although the effect was far more severe for λ N-PP (Figs. 8a & 8b). In contrast, tmRNA⁺ cells were unaffected by λ N-LA expression, whereas λ N-PP expression led to abnormal colony morphology but only a minor loss of tmRNA⁺ cell viability (Figs. 8a & 8b). We sought to rescue viability in Δ tmRNA cells by overproducing RF-1, RF-3, RRF, or a combination of both RF-3 and RRF. None of these translation factors had any effect on the viability of either Δ tmRNA or tmRNA⁺ cells expressing λ N-LA and λ N-PP (Figs. 8a & 8b). This result was unexpected given that RF-1 overexpression significantly increased peptidyl-tRNA turnover from termination-paused ribosomes (Fig. 3b). In fact, co-expression of λ N-PP and RF-1 actually *decreased* tmRNA⁺ cell viability, compared to cells expressing either of the proteins individually (Fig. 8b). This synthetic phenotype was not observed in tmRNA⁺ cells expressing both λ N-LA and RF-1 (Fig. 8a).

We also examined the viability of cells expressing nonstop mRNA using the λ N-trpAt expression construct (Fig. 7a). Expression of the λ N-trpAt nonstop mRNA decreased Δ tmRNA cell viability by approximately four orders of magnitude, but had no effect on tmRNA⁺ cells (Fig. 8c). As was found with λ N-LA and λ N-PP, the co-expression of RF-1, RF-3, and RRF was unable to restore viability to Δ tmRNA cells expressing λ N-trpAt (Fig. 8c). Finally, the overproduction of Pth, tRNA₂^{Pro}, and IF-3 had no effect on the viability of Δ tmRNA or tmRNA⁺ cells expressing any of the λ N protein variants (data not shown).

Discussion

These results represent the first comparative analysis of ribosome turnover kinetics during translational pausing on full-length and nonstop mRNAs. We used the previously characterized Pro-Pro nascent peptide to pause ribosomes during translation termination. Translational pausing at this nascent peptide motif resulted in the accumulation of peptidyl prolyl-tRNA₂^{Pro}, which was exploited as specific marker of termination-paused ribosomes. Pulse-chase analysis of peptidyl prolyl-tRNA₂^{Pro} turnover revealed the time scale of translational pausing, and uncovered evidence of ribosome queuing. Somewhat unexpectedly, ribosome turnover during inefficient translation termination was similar in Δ tmRNA and tmRNA⁺ cells. We propose that at least two processes account for these findings. Firstly, tmRNA•SmpB is not recruited to ribosomes paused on full-length mRNA, and therefore the message must be cleaved prior to ribosome rescue²². We have recently shown that this cleavage is largely mediated by 3'×5' exoribonucleases that degrade mRNA to the border of the paused ribosome (Garza-Sánchez *et al.*, in press). Therefore, tmRNA•SmpB-mediated peptidyl prolyl-tRNA₂^{Pro} turnover is probably rate-limited by exonucleolytic processing of mRNA. This

model is consistent with the very rapid peptidyl-tRNA turnover from nonstop mRNA-paused ribosomes in tmRNA⁺ cells. Because nonstop messages are “pre-cleaved”, ribosome rescue occurs immediately. Secondly, though its activity is reduced by the nascent peptide motif, RF-1 probably mediates the majority of ribosome turnover during inefficient termination in both tmRNA⁺ and ΔtmRNA cells. Approximately 30% of YbeL-PP nascent chains are SsrA-tagged in tmRNA⁺ cells⁸, and therefore the remaining 70% presumably undergo canonical RF-1 mediated translation termination. This reasoning implies that chains destined for SsrA-tagging in tmRNA⁺ cells are turned over by some other process in ΔtmRNA cells. This tmRNA-independent process may simply be canonical RF-mediated termination, or alternatively may be the activity of novel ribosome recycling system. We are unable to distinguish between these two possibilities because the protein products are presumably identical for each process. However, there is good evidence that tmRNA-independent ribosome recycling occurs during translation of nonstop messages in ΔtmRNA cells. Proteins are readily overproduced from nonstop mRNA in ΔtmRNA cells (see Fig. 7c), even though the translating ribosomes cannot undergo canonical termination. Moreover, peptidyl-tRNA turns over relatively rapidly from nonstop mRNA-arrested ribosomes. Taken together, these data suggest the activity of a tmRNA-independent ribosome recycling system. The existence of such an alternative ribosome rescue pathway was initially proposed when tmRNA activity was discovered, because tmRNA is not essential in *E. coli*^{12, 41}. This is not true of all bacteria as tmRNA is essential for the viability of *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and other species¹¹. Presumably, these bacteria lack a back-up system to recycle stalled ribosomes in the absence of tmRNA.

What is the tmRNA-independent ribosome recycling mechanism? A number of tmRNA-independent recycling pathways have been described over the past 15 years. Each pathway involves the activity of canonical translation factors, including RF-3, RRF, IF-1, IF-2, and IF-3^{21; 25; 34; 35}. In general, these translation factors remove small peptidyl-tRNAs (usually containing fewer than ten amino acid residues) from the ribosome in a phenomenon known as peptidyl-tRNA “drop-off”. It is unclear whether factor-mediated drop-off also occurs with larger peptidyl-tRNAs, such as the nascent polypeptides examined here. Moreover, direct peptidyl-tRNA drop-off is probably not feasible when the nascent chain contains a folded domain on the cytoplasmic side of the ribosomal exit tunnel. However, a recent report by Varshney and colleagues suggests that RRF mediates the drop-off of large peptidyl-tRNAs (>200 residues) from nonstop-arrested ribosomes in tmRNA⁺ cells⁴⁰. Our data are inconsistent with the conclusions of Singh *et al.*⁴⁰. Firstly, gratuitous overproduction of RRF did not inhibit SsrA-peptide tagging of λN protein synthesized from nonstop mRNA, indicating that RRF does not compete with tmRNA•SmpB for binding to nonstop-paused ribosomes. Secondly, overexpression of RF-3 and/or RRF did not accelerate peptidyl-tRNA turnover during ribosome pausing on nonstop mRNA. If ribosome recycling is an essential function as has been suggested⁴², then the ubiquitous RRF is an unlikely candidate for a recycling system that is apparently absent in several species¹¹. Based on this evidence, we propose that another uncharacterized tmRNA-independent recycling pathway(s) exists in *E. coli* and other bacteria. This pathway could be an intrinsic activity of the ribosome itself. Two recent reports provide evidence that *E. coli* ribosomes can recycle from truncated messages in the absence of exogenous factors^{43; 44}.

Singh *et al.* proposed RRF-mediated ribosome rescue, in part, based on evidence that RRF overexpression suppressed the toxicity of a nonstop message expressed in tmRNA⁺ cells⁴⁰. We are unsure why their nonstop message was lethal to tmRNA⁺ cells, because we observed no loss of cell viability upon expression of the well-characterized λN-trpAt nonstop mRNA in tmRNA⁺ cells. Perhaps the message described in Singh *et al.* is toxic for reasons unrelated to ribosome pausing. Because the ribosome arrest site was not experimentally confirmed in that paper, we cannot fully explain why our results differ. Regardless of mechanism, we note that the protective effect observed during RRF overexpression in Singh *et al.* was not dramatic

⁴⁰. Because plasmid-borne RRF is typically expressed at very high levels (see Fig. 7c) ⁸, perhaps RRF synthesis monopolized ribosome activity, leading to less translation of the toxic “nonstop” mRNA. In our viability assays, we observed no protective effect from RRF under any of the examined conditions. Instead, we find that tmRNA is the most important protective factor during ribosome pausing on both nonstop and full-length messages. Additionally, Δ tmRNA cells lost significant viability upon expressing a control message that causes no detectable ribosome pausing. This finding suggests that all heterologous protein overexpression is accompanied by subtle translational problems that tmRNA•SmpB typically resolves. Surprisingly, RF-1 overexpression failed to restore viability to cells expressing λ N-PP. In fact, tmRNA⁺ cells actually lost viability when co-expressing RF-1 and λ N-PP. This effect was not seen when either λ N-trpAt or λ N-LA were overexpressed, suggesting that the synthetic toxicity is specific to the nascent peptide-induced ribosome pause. This is perplexing because RF-1 was the only translation factor to dramatically increase peptidyl prolyl-tRNA₂^{Pro} turnover. We are unsure of the mechanism, but perhaps RF-1 prevents entry of the tmRNA-independent recycling factor into the ribosomal A site. For this model to be tenable, toxicity would have to result from a small subpopulation of ribosomes that require the proposed tmRNA-independent system for turnover. Clearly, cell viability losses are not necessarily related to the severity of translational pausing. These results illustrate that caution must be exercised when interpreting cell growth data in terms of molecular mechanism.

Our findings also show that the Pro-Pro nascent peptide does not inhibit RF-1 binding to the ribosomal A site. Indeed, essentially all of the cellular RF-1 can be isolated on ribosomes from cells overexpressing YbeL-PP. This is due to high-level YbeL-PP expression from the T7 RNA polymerase system, and suggests that the sequestration of RF-1 on paused ribosomes produces a second population of termination-paused ribosomes that have empty A sites. Overexpressed RF-1 presumably suppresses this secondary ribosome pausing, leading to more rapid peptidyl prolyl-tRNA₂^{Pro} turnover. RF-3 overexpression has a similar, yet less pronounced, effect because it promotes RF-dissociation from post-termination ribosomes, and thereby increases the free RF-1 concentration. Even in the absence of RF overproduction, RF-1 must be exchanging between ribosomal A sites *in vivo*, because YbeL-PP protein is expressed at very high levels despite the pause during termination ^{8; 9}. Ribosome complexes isolated by ultracentrifugation contained much less peptidyl prolyl-tRNA₂^{Pro} than expected given the high level of ribosome-associated RF-1. This result suggests that the isolated ribosomes were, in fact, mostly post-termination complexes containing RF-1 in the A site. Given that peptidyl prolyl-tRNA₂^{Pro} turned over with $T_{1/2} \sim 20 - 30$ s *in vivo*, we suspect that RF-1 continued hydrolysis of peptidyl prolyl-tRNA₂^{Pro} during the cell lysis and ultracentrifugation procedures. RF-1 probably remained bound to the ribosome due to a combination of low temperature, and physical separation from soluble RF-3 during ultracentrifugation. RF-3 accelerates RF-1 and RF-2 dissociation from the post-termination A site. Additionally, RF-1 has significantly higher affinity for the ribosomal A site than RF-2 ⁴⁵, which may explain why RF-1 readily co-purified with ribosomes whereas RF-2 did not. Nevertheless, RF-1 was not ribosome-associated during YbeL-EA overexpression, strongly suggesting that RF-1 binds to the A site during ribosome pausing, but is unable to efficiently hydrolyze the peptidyl-tRNA bond.

What is the basis of nascent peptide-induced ribosome pausing during translation termination? C-terminal Pro residues are important for regulated ribosome pausing in *E. coli* and human cells ^{31; 46}. Given the divergence between bacteria and mammals, we suspect that the inherently slow rate of peptidyl prolyl-tRNA^{Pro} hydrolysis has been exploited to regulate protein synthesis in these two systems. The C-terminal Pro-Pro sequence inhibits translation termination in several genetic contexts ⁸, suggesting that the intrinsic chemical properties of proline are responsible for translational pausing, rather than specific interactions between the nascent chain and ribosome. Given that Pro residues have the unique ability to adopt and stabilize *cis* peptide bonds in proteins, one possibility is that the C-terminal Pro residue facilitates

trans to *cis* isomerization of the final peptide bond, thereby inhibiting translation termination. Peptidyl-prolyl isomerization may also explain why the penultimate residue of the nascent chain also influences ribosome pausing^{6; 8}. Presumably, peptide bonds are formed by the ribosome in the *trans* conformation, which is the predominate stereoisomer in mature proteins. Isomerization to the *cis* conformation could conceivably alter the geometry of the peptidyl-tRNA ester bond and prevent nucleophilic attack by water during translation termination, or by aminoacyl-tRNAs during translation elongation. Indeed, recent data from the Ito and Rodnina groups have shown that peptidyl prolyl-tRNA reacts inefficiently with puromycin, which is a mimic of aminoacyl-tRNA^{47; 48}. However, we have been unable to detect ribosome pausing at internal Pro-Pro sequences, indicating that this nascent peptide sequence is not problematic during elongation. Perhaps the rapid delivery of ternary complexes does not allow enough time for peptidyl-prolyl isomerization during elongation. In contrast, translation termination is significantly slower than elongation⁴⁹, which may allow for *trans* to *cis* isomerization. Because translation termination is 10–100-fold more rapid than peptidyl-prolyl isomerization in solution, *trans-cis* isomerization would have to be accelerated on the ribosome for this model to be tenable. This is certainly plausible given that peptidyl-prolyl isomerization can be dramatically increased by desolvation, or by hydrogen bonding to the imide nitrogen of Pro residues^{50; 51}. Importantly, the peptidyl-prolyl isomerization is reversible, and would eventually allow RF-mediated termination to occur, consistent with our findings in this study. Although the translational termination pause characterized here has no known function, we propose that nascent peptidyl-prolyl isomerization can be used as a molecular switch to regulate translation termination.

Materials and Methods

Bacterial strains and plasmids

All bacterial strains were derivatives of *Escherichia coli* strain X90 and are listed in Table 2. The *prfB*(FLAG) allele encoding the FLAG epitope at the C-terminus of release factor-2 (RF-2) was introduced into the *prfB* locus by Red-mediated homologous recombination as described^{8; 52}. The resulting strain was confirmed by whole-cell PCR and anti-FLAG Western blot analyses. For routine isolation of RNA and protein, 10 mL cultures were grown at 37 °C with aeration in Luria broth (LB) supplemented with the appropriate antibiotics (150 µg/mL ampicillin, and 25 µg/mL tetracycline) to maintain plasmids. The expression of YbeL and λN proteins was induced with 1.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the expression of RF-1, RF-3, RRF, Pth, and IF-3 was induced with 0.2% to 0.4% L-arabinose.

Strains for viability plating assay were grown in LB medium (supplemented with 150 µg/mL ampicillin and 25 µg/mL tetracycline) to late log phase at 37 °C with aeration. Cell densities were adjusted to OD₆₀₀ = 1.0, and ten-fold serial dilutions were plated onto LB agar plates containing: i) 0.1% D-glucose; ii) 0.1% D-glucose + 0.05 mM IPTG; iii) 0.1% L-arabinose; or iv) 0.1% L-arabinose + 0.05 mM IPTG. In addition, all LB agar plates contained 150 µg/mL ampicillin and 5 µg/mL tetracycline. Plates were incubated for 15 – 17 h at 37 °C.

All plasmids used in this study are listed in Table 2. Plasmids pYbeL(PP), pYbeL(EA), pRF-1, ptRNA₂^{Pro}, and pPW500 have been described previously^{8; 9; 13; 20}. The (*m*)ybeL-PP and (*m*)ybeL-EA mini-genes were generated by PCR amplification of plasmids pYbeL(PP) and pYbeL(EA) using oligonucleotides, ybeL(49)-NdeI, (5' - GGA CCT CAA TCA TAT GGG GGT TTA TCA CAG) and pET-EcoRI, (5' - CGT CTT CAA GAA TTC TCA TGT TTG ACA GC), digested with NdeI and EcoRI (restriction endonuclease site underlined), and ligated to plasmid pFG11⁵³. The resulting plasmids express the C-terminal 49 residues of YbeL(PP) and YbeL(EA) fused to an N-terminal FLAG epitope. Full-length and mini-gene versions of ybeL-PP containing TAG and TGA stop codons were made by PCR using primer pET-PstI, (5' - CAA GGA ATG GTG CAT GCC TGC AGA TGG CGC CC); with ybeL(PP)uag-BamHI,

(5' - GCC GGA TCC TAC TAG GGC GGA AAC GGG), and **ybeL(PP)uga-BamHI**, (5' - GCC GGA TCC TAT CAG GGC GGA AAC GGG), respectively. The λ N-PP construct was generated by PCR of pPW500 using primers **lacI-HpaI** (5' - TAT CCC GCC GTT AAC TAG TAT CAA ACA GGA TTT TCG C), and **λ N-PP-SacI**, (5' - AAT GAG CTC AAT TAG GGC GGA TGA TGG TGA TGA TGG TGC). The λ N-LA PCR fragment was made using oligonucleotide, **λ N-LA-SacI**, (5' - ACC GAG CTC AAT TAT GCC AGA TGA TGG TGA TGA TGG), and **lacI-HpaI**. The resulting PCR fragments were digested with HpaI and SacI and ligated to plasmid pTrc99A (GE Healthcare).

The *E. coli pth* gene was PCR amplified with primers, **pth-NdeI**, (5' - GGA CAA AAA CAT ATG ACG ATT AAA TTG ATT GTC G), and **pth-KpnI**, (5' - GAC GGT ACC TTA TTG CGC TTT AAA GGC GTG C), digested with NdeI and KpnI and ligated to plasmid pCH410, which contains *araC* and the *araBAD* promoter⁹. The Pth-His₆ overexpression construct was generated by PCR of *pth* with primers, **pth-XhoI**, (5' - AAC CTC GAG TTG CGC TTT AAA GGC GTG CAA TCG G), and **pth-NdeI**, followed by ligation to plasmid pET21b (Novagen-EMD). The *prfC* gene was subcloned from plasmid pCH405::*prfC*8 into pCH450 using SacI and KpnI restriction sites, to generate pRF-3. The *E. coli frr* gene, encoding RRF, was PCR amplified using oligonucleotides, **frr-KpnI**, (5' - TCT GGT ACC ACT GAG ACA AGT TTT CAA GG), and **frr-PstI**, (5' - GAT CTG CAG GGA TCT ACT GAG CGG CG), followed by ligation to plasmids pCH450 and pRF-3 to generate plasmids pRRF and pRF-3/RRF, respectively. The *E. coli infC* gene, encoding IF-3, was PCR amplified using oligonucleotides, **infC-NdeI**, (5' - GAG GAA TAA CAT ATG AAA GGC GGA AAA CGA GTT CAA ACG); and **infC-XhoI**, (5' - CTT CTC GAG ATT ACT GTT TCT TCT TAG G), and ligated to pCH450 to generate plasmid pIF-3. The *E. coli smgB* and *ssrA* genes, encoding SmpB and tmRNA, were PCR amplified using oligonucleotides, **smgB-SacI**, (5' - CGC GAG CTC TTT AAA CAC GCG ACC AAA GGC G), and **ssrA-XhoI**, (5' - CAA CTC GAG GGC ACA AAA AAG CCC GCA GGG C), and ligated to pCH405 Δ to generate plasmid pSmpB-tmRNA. All plasmid constructs were confirmed by DNA sequencing.

Analysis of RNA and protein

Total RNA was isolated and Northern blot analyses were conducted as previously described^{20; 53}. The following oligonucleotides were 5' end-labeled with [γ -³²P]-ATP, and used as probes for Northern blot hybridizations: **pET-rbs probe**, (5' - GTA TAT CTC CTT CTT AAA GTT AAA C) for mRNA; **proL probe** (5' - CAC CCC ATG ACG GTG CG) for tRNA₂^{Pro}; **glyV probe**, (5' - CTT GGC AAG GTC GTG CT) for tRNA₃^{Gly}; **alaT probe**, (5' - TCC TGC GTG CAA AGC AG) for tRNA_{1B}^{Ala}; **serT probe**, (5' - AAC CCT TTC GGG TCG CCG GTT TTC) for tRNA₁^{Ser}; and **leuU probe**, (5' - CCC GTA AGC CCT ATT GGG CA) for tRNA₂^{Leu}.

Total cell protein was isolated by freeze-thaw lysis in buffered 8M urea as previously described³⁸. Protein samples were quantified by Bradford protein reagent and run on Tris-tricine-SDS 10% polyacrylamide gels. Western blotting was performed as described⁵³, and blots were imaged using a LI-COR[®] infrared imager and quantified using Odyssey software. Polyclonal antisera were raised against C-terminal His₆-tagged RF-1 from *E. coli*. Anti-FLAG monoclonal antibodies (Sigma-Aldrich) were used to detect FLAG-tagged RF-2 in double labeling Western blot experiments. All RF-2 experiments were verified using wild-type RF-2 and anti-RF-2 polyclonal antisera (a generous gift from Dr. Richard Buckingham).

Pulse-chase analysis of peptidyl-tRNA turnover

E. coli strains were grown at 37 °C with aeration in MOPS defined media⁵⁴ containing 0.4% glucose and supplemented with 100 μ g/mL L-arginine and 20 μ g/mL of all other amino acids except L-methionine and L-cysteine. Once cultures attained a cell density of OD₆₀₀ ~ 0.7,

mRNA synthesis was induced with 1.5 mM IPTG for 20 – 30 min, followed by pulse-labeling with 20 $\mu\text{Ci/mL}$ of [^{35}S]-radiolabeled L-methionine and L-cysteine (MP Biomedicals –1175 Ci/mmol) for 1 – 2 min. Radiolabel was chased with 0.2 mg/mL of unlabeled L-methionine and L-cysteine, and 1.0 mL samples were removed at the indicated time intervals and quenched with ice-cold trichloroacetic acid (1% final concentration). Cells were collected by centrifugation, washed once with 50 mM Tris-acetate (pH 7.0) – 1 mM EDTA, and lysed in 100 μL of 1% SDS – 50 mM Tris-acetate (pH 7.0) 1 mM EDTA for 20 min at ambient temperature. Cell lysates (45 μL) were mixed with 500 μL of 2% cetyltriethylammonium bromide (CTABr) and 500 μL of 0.5 M sodium acetate (pH 5.0), and placed on ice for 20 min. Samples were then incubated at 30 °C for 10 min and subsequently centrifuged at 13,000 rpm for 15 min in a microcentrifuge. The resulting pellets were washed with 1 mL of 100% ice-cold acetone, and dissolved in 8 M urea – 10 mM sodium acetate (pH 5.2) – 1 mM EDTA – 0.01% bromophenol blue for analysis on acid-urea polyacrylamide gels²⁰. Gels were dried onto filter paper, and the data visualized and quantified by phosphorimager using the Quantity One software package (BioRad). Exponential decay equations were fitted to the pulse-chase data using DeltaGraph (RedRock Software). Composite half-life values ($T_{1/2}$) from double-exponential decay fits were determined for termination-paused ribosomes, and simple half-lives ($t_{1/2}$) were determined from single-exponential fits to nonstop-paused turnover data.

Cell fractionation and ribosome isolation

Cells were grown in 100 mL of LB were grown to an OD_{600} of 0.7 – 1.0 and induced with 1.5 mM IPTG for 30 min. Cells over-producing RF-3 and/or RRF were grown in LB supplemented with 0.4% L-arabinose, and induced with IPTG as indicated above. Cultures were harvested over ice, centrifuged at 4 °C to collect cells, washed with S30 buffer [60 mM ammonium acetate – 10 mM Tris-acetate (pH 7.0) – 10 mM magnesium acetate], and frozen at –80 °C. Frozen cells were resuspended in 10 mL of S30 buffer and broken by French press passage at 20,000 psi. Lysates were clarified by centrifugation at 30,000 μg at 4 °C for 15 min, loaded onto 40% sucrose in S30 buffer, and centrifuged in either a SW50.1 or MLS50 rotor for 1 – 4 hr at 40,000 rpm. RNA and protein were isolated from high-speed supernatant and pellet fractions.

Purification and in vitro activity of Pth-His₆

His₆-tagged peptidyl-tRNA hydrolase (Pth-His₆) purification was performed according to Bonin *et al.* with minor modifications⁵⁵. Recombinant Pth-His₆ was overexpressed using a T7 RNA polymerase expression system in cells deleted for RNase I and SlyD⁵³. Cells were resuspended in Pth buffer [50 mM sodium phosphate (pH 8.0) – 300 mM sodium chloride – 5 mM β -mercaptoethanol – 10% glycerol], and broken by one passage through French press at 20,000 psi. Lysates were clarified by centrifugation at 30,000 μg for 15 min at 4 °C. Clarified lysates were incubated with nickel-nitrilotriacetic acid (Ni^{2+} -NTA) agarose resin for 4 h at 4 °C. Ni^{2+} -NTA resin was collected and batch washed twice with Pth buffer containing 20 mM imidazole. Washed resin was loaded into column and washed with Pth buffer containing 20 mM imidazole until the eluate contained no detectable protein. Pth-His₆ was eluted in Pth buffer containing 250 mM imidazole. Pooled fractions were exhaustively dialyzed against 2 μ Pth buffer, diluted with an equal volume of glycerol, and stored at –20 °C. Pth-His₆ was judged to be >95% pure by SDS-PAGE, and quantified by absorbance at 280 nm using a molar extinction coefficient of 13,980 $\text{M}^{-1} \text{cm}^{-1}$.

Total cellular RNA (500 $\mu\text{g/mL}$ final concentration) was treated with purified Pth-His₆ in Pth reaction buffer [20 mM Tris-acetate (pH 7.0) – 10 mM magnesium chloride – 0.2% BSA – 0.1 mM EDTA – 10 mM β -mercaptoethanol] at 37 °C. Reactions were quenched with an equal volume of gel loading buffer [8M urea – 10 mM sodium acetate – 1 mM EDTA – 0.01% bromophenol blue 0.01% xylene cyanol] and placed on ice. Samples were incubated at 95 °C for 3 min, then loaded onto acid-urea 6% polyacrylamide gels for blotting and Northern

hybridization²⁰. [³⁵S] pulse-labeled samples were treated similarly, but the acid-urea gel was dried onto filter paper and visualized by phosphorimaging.

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Figure 1. Peptidyl prolyl-tRNA₂^{Pro} accumulates during inefficient translation termination

(a) FLAG-(m)YbeL expression constructs. The *flag-(m)ybeL* message is shown schematically along with the Northern probe binding site. The 3' coding sequences of *flag-(m)ybeL-PP*, *-PP(uag)*, *-PP(uga)*, and *-EA* are shown. The boxed **P** and **A** indicate the positions of the ribosomal P and A sites during translation termination. **(b)** Northern blot analysis of peptidyl-tRNA. Total RNA was isolated from tmRNA⁺ and ΔtmRNA cells, resolved on acid-urea polyacrylamide gels, and analyzed by Northern blot hybridization using probes specific for tRNA₂^{Pro} and tRNA_{1B}^{Ala}. Expression of FLAG-(m)YbeL-PP (+ IPTG) resulted in the accumulation of peptidyl prolyl-tRNA₂^{Pro} in both tmRNA⁺ and ΔtmRNA cells, whereas no peptidyl-tRNA was detected in cells expressing FLAG-(m)YbeL-EA. Overexpression of Pth had no significant effect on peptidyl prolyl-tRNA₂^{Pro} levels. **(c)** Treatment of peptidyl-tRNA with Pth-His₆ *in vitro*. Total RNA from cells expressing FLAG-(m)YbeL-PP or FLAG-SecM' was treated with purified Pth-His₆ for the indicated time, and analyzed by Northern blot hybridization for tRNA₂^{Pro} or tRNA₃^{Gly}, respectively. **(d)** Fractionation of peptidyl-tRNA. Cells expressing FLAG-(m)YbeL-PP were broken by French press and fractionated by sucrose density ultracentrifugation. RNA was extracted from the lysate, pellet, and supernatant fractions, and analyzed by Northern blot for tRNA₂^{Pro} and tRNA_{1B}^{Ala}. Peptidyl prolyl-tRNA₂^{Pro} was only found in the high-speed pellet fraction.

Figure 2. Pulse-chase analysis of peptidyl-tRNA turnover

(a) Pulse labeling of nascent chains. Cells expressing FLAG-(m)YbeL-PP and FLAG-(m)YbeL-EA were pulse labeled with [³⁵S]-methionine/cysteine, then chased with unlabeled amino acids for the indicated times. Peptidyl-tRNA was precipitated from cell lysates, resolved on acid-urea polyacrylamide gels, and visualized by phosphorimaging. High molecular mass radiolabeled species were only detected in cells expressing FLAG-(m)YbeL-PP. (b) Peptidyl-tRNA identification. An [³⁵S]-labeled sample was treated with purified Pth-His₆ *in vitro*, confirming that higher molecular weight radiolabeled species were peptidyl-tRNAs. [³⁵S]-labeled and unlabeled samples from cells expressing FLAG-(m)YbeL-PP were resolved by acid-urea gel, and blotted to nylon membrane. The unlabeled sample lane was cut from the

membrane, hybridized to [^{32}P]-labeled tRNA $_2^{\text{Pro}}$ probe, and then re-aligned to the [^{35}S]-labeled sample blot for phosphorimaging. **(c)** Pulse-chase analysis of peptidyl-tRNA turnover. tmRNA $^+$ and Δ tmRNA cells expressing FLAG-(m)YbeL-PP were pulse labeled, and peptidyl-tRNA turnover examined as a function of time. The rates of peptidyl-tRNA turnover were similar in Δ tmRNA and tmRNA $^+$ cells, as well as cells overexpressing tmRNA•SmpB. **(d)** Kinetics of peptidyl prolyl-tRNA $_2^{\text{Pro}}$ turnover. Radiolabeled peptidyl prolyl-tRNA $_2^{\text{Pro}}$ was quantified by phosphorimager, and double exponential decay equations were fitted to calculate composite half-life ($T_{1/2}$) values. Averaged half-life values ($\pm$ standard error) were determined from at least three independent pulse-chase experiments.

(a) RF-1 and ribosome pausing **(b) Peptidyl-tRNA pulse-chase** **Figure 3. RF-1 overexpression and nascent peptide-induced ribosome pausing**

(a) Northern blot analysis of tRNA₂^{Pro} and mRNA during translational pausing. FLAG-(m) YbeL-PP was synthesized from constructs containing UAG, UAA, and UGA stop codons, with and without co-expression of RF-1. Peptidyl prolyl-tRNA₂^{Pro} levels were similar for all constructs, and were not affected by RF-1 overproduction. Northern blot of mRNA showed that RF-1 overexpression significantly reduced A-site cleavage at UAG (32% of control) and UAA (25% of control) stop codons, but not at the UGA (105% of control) codon. A-site cleavage products do not accumulate in tmRNA⁺ cells. **(b)** RF-1 overexpression and peptidyl-tRNA turnover in tmRNA cells. FLAG-(m)YbeL-PP nascent chains were pulse labeled with [³⁵S]-methionine/cysteine, then chased with unlabeled amino acid for the indicated times. RF-1

overexpression dramatically increased the rate of peptidyl prolyl-tRNA₂^{Pro} turnover in both Δ tmRNA and tmRNA⁺ cells (data not shown).

(a) Ribosome pausing and RF-1-ribosome interaction

(b) Specificity of RF-ribosome interaction

Figure 4. RF-1 interacts with the ribosome during translational pausing

(a) Anti-RF-1 Western blot. Cell lysates were fractionated by sucrose density ultracentrifugation, and the pellet (**P**) and supernatant (**S**) fractions analyzed by Western blot. RF-1 was found primarily in the supernatant fraction of uninduced cells (**no IPTG**), and cells expressing YbeL-EA. In contrast, RF-1 partitioned to the pellet fraction in tmRNA⁺ and ΔtmRNA cells expressing YbeL-PP. **(b)** Anti-RF-1/RF-2 Western blot. Lysates from tmRNA⁺ cells expressing YbeL-PP from messages with UAG, UAA, and UGA stop codons were fractionated by ultracentrifugation, and analyzed by Western blot. RF-1 partitioned in the pellet fraction upon expression of the UAG and UAA constructs, but was found in the supernatant with the UGA message. RF-2 was found in the supernatant fraction for all constructs.

Figure 5. Effect of RF-3 and/or RRF overexpression on nascent peptide-induced ribosome pausing
(a) Peptidyl-tRNA turnover in tmRNA⁺ cells overexpressing RF-3 and/or RRF. FLAG-(m) YbeL-PP nascent chains were pulse labeled with [³⁵S]-methionine/cysteine, then chased with unlabeled amino acids for the indicated time. The $T_{1/2}$ of peptidyl prolyl-tRNA₂^{Pro} turnover was 15 ± 0.3 s during RF-3 overexpression, 25 ± 3.2 s during RRF overexpression, and 20 ± 1.8 s during RF-3/RRF co-overexpression. **(b)** RF-3 and RRF overexpression and RF-ribosome interaction. Lysates from tmRNA⁺ cells co-expressing YbeL-PP (UAA) along with RF-3 and/or RRF were fractionated by density ultracentrifugation, and analyzed by Western blot. Expression of RF-3 and/or RRF (+ **L-Ara**) had no effect on the RF-1-ribosome interaction, compared to control cells without RF-3/RRF induction (+ **D-Glu**).

Figure 6. Characterization of the *flag-(m)ybeL-rrnB* nonstop message

(a) Schematic of the *flag-(m)ybeL-rrnB* nonstop mRNA. The nucleotide sequence of the *E. coli rrnB* T1 intrinsic transcription terminator is shown, along with the positions of codons encoding residues Gly74, Leu75, and Ser76. Ribosomes translate to the 3' end on nonstop messages, allowing efficient tmRNA•SmpB recruitment to the empty A site. (b) Identification of SsrA(His₆)-tagging sites by mass spectrometry. FLAG-(m)YbeL-rrnB was expressed in tmRNA(His₆) cells, and SsrA(His₆)-tagged proteins purified by Ni²⁺-NTA affinity chromatography. Mass spectrometry identified three species corresponding to tagging after residues Gly74 (calculated mass, 9,522.7 Da), Leu75 (calculated mass, 9,635.8 Da), and Ser76 (calculated mass, 9,722.9 Da). (c) Northern analysis of peptidyl-tRNA. Peptidyl seryl-tRNA₁^{Ser} was dependent upon FLAG-(m)YbeL-rrnB expression, and not observed in tmRNA⁺ cells. A negative control blot for tRNA_{1B}^{Ala} detected no peptidyl-tRNA. (d) Pulse-chase analysis of peptidyl-tRNA turnover during translational pausing on nonstop mRNA. Nascent chains were radiolabeled with [³⁵S]-methionine and cysteine, then chased with unlabeled amino acids. Radiolabeled peptidyl-tRNA decayed with $t_{1/2} = 61 \pm 7.3$ s in ΔtmRNA cells, but was not detected in tmRNA⁺ cells.

Figure 7. Translation termination and recycling factors do not induce peptidyl-tRNA “drop-off” during ribosome pausing on nonstop mRNA

(a) Schematic of the λ N-trpAt nonstop message and the fates of λ N-trpAt nascent chains during ribosome arrest. In tmRNA⁺ cells, nearly all λ N-trpAt chains are SsrA-tagged. In Δ tmRNA cells, untagged chains are released from the paused ribosome by an unknown mechanism. If overproduced translation factors facilitate peptidyl-tRNA “drop-off”, then untagged λ N-trpAt chains should accumulate in tmRNA⁺ cells. **(b)** Peptidyl-tRNA turnover during ribosome pausing on the λ N-trpAt nonstop mRNA. Nascent chains were pulse-labeled with [³⁵S]-methionine/cysteine in cells expressing λ N-trpAt, and chased with unlabeled amino acids for the indicated time. Peptidyl-tRNA was only detected in Δ tmRNA cells, consistent with ribosome pausing on a nonstop message. **(c)** SsrA-tagging of λ N-trpAt in cells overexpressing translation termination and recycling factors. Cell lysates and Ni²⁺-NTA purified λ N-trpAt were examined by Coomassie staining of SDS-polyacrylamide gels. Untagged λ N-trpAt accumulated in Δ tmRNA cells, whereas SsrA-tagged λ N-trpAt accumulated in tmRNA⁺ Δ ClpP cells. Overexpression of translation termination and recycling factors had no effect on SsrA-

tagging compared to control cells carrying vector plasmid (pCH450). The gel migration positions of RF-3, RF-1, RRF, and λ N-trpAt are indicated.

Figure 8. Translational pausing and cell viability

Cultures were serially diluted (10-fold), and plated on LB agar plates containing: glucose to suppress P_{BAD} expression; glucose + IPTG to induce λ N expression; arabinose to induce translation factor expression; or L-arabinose + IPTG to induce expression of λ N variants and P_{BAD} -dependent translation factors. **(a)** Cell viability during λ N-LA overexpression. Expression of λ N-LA had no effect on $tmRNA^+$ cells, but caused ~ 100-fold decrease $\Delta tmRNA$ cell viability. Expression of translation termination/recycling factors had no significant effect on cell viability under all conditions. **(b)** Cell viability during λ N-PP overexpression. Expression of λ N-PP resulted in ~ 10,000-fold decrease in $\Delta tmRNA$ cell viability, and led to altered colony morphology in $tmRNA^+$ cells. Overexpression of RF-3 and/or RRF had no effect on cell viability. However, co-expression of RF-1 with λ N-PP led to a significant loss of $tmRNA^+$ viability compared to cells expressing the proteins individually. **(c)** Cell viability during λ N-trpAt expression from nonstop mRNA. Expression of λ N-trpAt resulted in ~ 10,000-fold decrease in $\Delta tmRNA$ cell viability, but had no effect on $tmRNA^+$ cells. Expression of translation termination/recycling factors had no significant effect on cell viability under all conditions.

Table 1

Peptidyl-tRNA decay half-lives^a

Expressed translation factor	<i>Pro-Pro-stop</i> mRNA (<i>T</i> _{1/2} in sec)		nonstop mRNA (<i>t</i> _{1/2} in sec)	
	ΔtmRNA	tmRNA ⁺	tmRNA	ΔtmRNA
none	32 ± 1.6	22 ± 2.2	61 ± 7.3	
SmpB•tmRNA	ND ^b	24 ± 4.0	ND ^b	
RF-1	NF ^c	NF ^c	71 ± 10	
RF-3	33 ± 5.2	15 ± 0.3	60 ± 0.4	
RRF	28 ± 0.9	25 ± 3.2	65 ± 11	
RF3-RRF	26 ± 0.4	20 ± 1.8	84 ± 13	
Pth	30 ± 4.8	25 ± 2.9	54 ± 4.6	

^a*T*_{1/2} values were determined from fitted double-exponential, and *t*_{1/2} values from single-exponential decay equations as described in Methods. Reported values are the mean ± the standard error of measurement (SEM).

^bNot determined.

^cNot fitted. Peptidyl-tRNA decay was too rapid for exponential fitting.

Table 2

Bacterial strains and plasmids.

Strain or plasmid	Description ^a	Reference
Strains		
X90	F prime; <i>lac⁹ lac⁺ pro/ara A[lac-pro] nalA argE[am] rif^r thi-1</i>	
CH12	X90 (DE3)	(8)
CH22	X90 <i>ssrA::cat</i> , Cm ^r	(39)
CH94	X90 <i>clpP::cat</i> , Cm ^r	(13)
CH113	X90 (DE3) <i>ssrA::cat</i> , Cm ^r	(8)
CH2016	CH12 <i>Δrna ΔslyD::kan</i> , Kan ^r	(53)
CH4119	CH12 <i>prfB(FLAG)</i>	This study
CH4120	CH113 <i>prfB(FLAG)</i> , Cm ^r	This study
Plasmids		
pYbel-PP	pET11d::ybelL(E159P), T7 expression of Ybel-PP, Amp ^r	
pYbel-EA	pET11d::ybelL(P160A), T7 expression of Ybel-EA, Amp ^r	(8)
pYbel-PP(<i>uag</i>)	Ybel-PP expressed from mRNA with UAG stop codon, Amp ^r	This study
pYbel-PP(<i>uga</i>)	Ybel-PP expressed from mRNA with UGA stop codon, Amp ^r	This study
pFLAG-(m)Ybel-PP	Expresses C-terminal 49 residues of Ybel-PP fused to FLAG, Amp ^r	This study
pFLAG-(m)Ybel-PP(<i>uag</i>)	Expresses FLAG-(m)Ybel-PP from mRNA with UAG stop codon, Amp ^r	This study
pFLAG-(m)Ybel-PP(<i>uga</i>)	Expresses FLAG-(m)Ybel-PP from mRNA with UGA stop codon, Amp ^r	This study
pFLAG-(m)Ybel-PP(<i>rmB</i>)	Expresses FLAG-(m)Ybel-PP from a nonstop mRNA, Amp ^r	This study
pFLAG-(m)Ybel-EA	Expresses C-terminal 49 residues of Ybel-EA fused to FLAG, Amp ^r	This study
pET21b::pTh	pET plasmid expressing His ₆ -tagged <i>E. coli</i> Pth, Amp ^r	(20)
pSecM ^r	pFG21b::secM ^r , Amp ^r	(13)
pPW500	pTc99a derivative expressing the N-terminal domain of phage λ cI repressor (λN) from nonstop mRNA, Amp ^r	This study
pΔN-PP	pTc99a derivative expressing λN with C-terminal Pro-Pro, Amp ^r	This study
pΔN-LA	pTc99a derivative expressing λN with C-terminal Leu-Ala, Amp ^r	(20)
pCH405Δ	pACYC184 derivative containing multi-cloning site, Tet ^r	(9)
pCH450	pACYC184 derivative encoding <i>araC</i> and <i>araBAD</i> promoter, Tet ^r	This study & (9)
pPh	pCH410::pTh, arabinose-inducible Pth expression, Tet ^r	
pRF-1	pACYC184 derivative expressing RF-1 under <i>araBAD</i> promoter, Tet ^r	This study
pRF-3	pCH450::pTh, arabinose-inducible RF-3 expression, Tet ^r	This study
pRRF	pCH450::pTh, arabinose-inducible RF-3 expression, Tet ^r	This study
pRF-3/RRF	pCH450::pTh, arabinose-inducible RF-3 and RRF expression, Tet ^r	This study
pIF-3	pCH450::pTh, arabinose-inducible IF-3 expression, Tet ^r	This study
pSmpB•tmRNA	pCH405Δ::smpB-ssrA, constitutive expression of SmpB•tmRNA, Tet ^r	(20)
pIRNA _{Pro}	pCH405Δ::pTh constitutive expression of rRNA _{Pro} , Tet ^r	

^a Abbreviations used: Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant.