A physiological connection between tmRNA and peptidyl-tRNA hydrolase functions in *Escherichia coli*

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ABSTRACT

The bacterial *ssrA* gene codes for a dual function RNA, tmRNA, which possesses tRNA-like and mRNA-like regions. The tmRNA appends an oligopeptide tag to the polypeptide on the P-site tRNA by a *trans*-translation process that rescues ribosomes stalled on the mRNAs and targets the aberrant protein for degradation. In cells, processing of the stalled ribosomes is also pioneered by drop-off of peptidyl-tRNAs. The ester bond linking the peptide to tRNA is hydrolyzed by peptidyl-tRNA hydrolase (Pth), an essential enzyme, which releases the tRNA and the aberrant peptide. As the *trans*-translation mechanism utilizes the peptidyl-transferase activity of the stalled ribosomes to free the tRNA (as opposed to peptidyl-tRNA drop-off), the need for Pth to recycle such tRNAs is bypassed. Thus, we hypothesized that tmRNA may rescue a defect in Pth. Here, we show that overexpression of tmRNA rescues the temperature-sensitive phenotype of *Escherichia coli* (*pth*ts). Conversely, a null mutation in *ssrA* enhances the temperature-sensitive phenotype of the *pth*ts strain. Consistent with our hypothesis, overexpression of tmRNA results in decreased accumulation of peptidyl-tRNA in *E.coli*. Furthermore, overproduction of tmRNA in *E.coli* strains deficient in ribosome recycling factor and/or lacking the release factor 3 enhances the rescue of *pth*ts strains. We discuss the physiological relevance of these observations to highlight a major role of tmRNA in decreasing cellular peptidyl-tRNA load.

INTRODUCTION

For various physiological reasons, not all ribosomes that begin to translate an mRNA reach the termination codon. A significant population of the translating ribosomes stalls in between the decoding cycles (1,2). Stalled ribosomes pose a serious impediment to mRNA translation as they deplete pools of free tRNA and ribosomes, bringing protein synthesis to a halt. One of the cellular strategies to process stalled ribosomes involves peptidyl-tRNA drop-off (Figure 1, upper) (3–5). While the mechanisms responsible for the drop-off of peptidyl-tRNAs from the ribosomes are not very well understood (6), genetic and biochemical evidence (7–10) suggests that in *E.coli*, ribosome recycling factor (RRF) and EFG, as well as other factors such as RF3, IF1, IF2 and RelA contribute to this phenomenon. The peptidyl-tRNAs are recycled by peptidyl-tRNA hydrolase (Pth), which hydrolyzes the ester link between the tRNA and the peptide (11). As accumulation of peptidyl-tRNAs leads to cellular toxicity, Pth is an essential protein in bacteria (12,13).

Another cellular strategy that leads to the rescue of ribosomes stalled on fragmented mRNAs, involves *trans*-translation (14). Here the product of *ssrA* gene, tmRNA (also known as SsrA), is aminoacylated by alanyl-tRNA synthetase, and recruited to the ribosomal A site with the help of EFTu and an ancillary protein, SmpB. The peptidyl-transferase activity of the ribosome transfers the peptide from the P-site bound peptidyl-tRNA to the alanine on the –CCA end of the tmRNA. Consequently, the tRNA that was sequestered as peptidyl-tRNA in the stalled complex is released (15). The elongation process then resumes to translate a 30 nt long open reading frame (mRNA portion) of the tmRNA to encode the ANDENYALAA sequence tag (in *E.coli*). When the ribosomes reach the termination codon in the tmRNA, the C-terminally tagged peptide is released by the activity of release factors, and subjected to degradation by cellular proteases (16–18). Interestingly, as the tmRNA-mediated mechanism utilizes the peptidyl-transferase activity of the stalled ribosomes to liberate tRNA (as opposed to peptidyl-tRNA drop-off), the need for Pth to recycle such tRNAs is bypassed (Figure 1, lower). Since, a number of cellular processes result in accumulation of stalled ribosomes with fragmented mRNA (19–23), *trans*-translation may represent a major mechanism to recycle both the stalled ribosomes and the tRNAs. This raises an important question as to whether excess tmRNA in *E.coli* can alleviate the requirement for Pth activity.

Earlier studies have shown that RRF and RF3 are involved in peptidyl-tRNA drop-off from the ribosome. Thus, conditional lethal strains of *E.coli*, with a temperature-sensitive phenotype because of a mutation in *pth* could be rescued by downregulation of RRF expression (8). Conversely, overproduction of RRF in *E.coli* is toxic under conditions of limiting Pth activity (7). It was also observed that disruption of the *prfC* gene coding for RF3 resulted in suppression of the temperature-sensitive phenotype of a *pth*ts strain. *In vitro*

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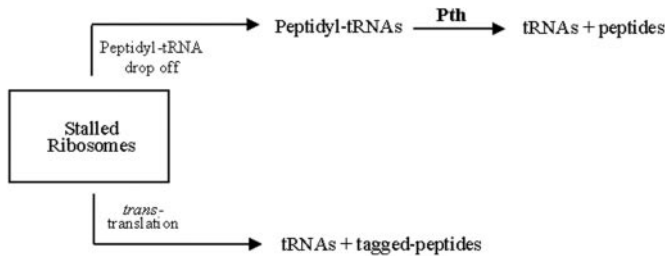
Figure 1. Pathways depicting release of the tRNAs from the peptidyl-tRNAs by processing of stalled ribosomes pioneered either by dissociation of peptidyl-tRNAs (upper) or by tmRNA-mediated *trans*-translation (lower). Stalled ribosomes for *trans*-translation may arise either by endonucleolytic cleavage of mRNA in stalled ribosomes or from the translation of the truncated mRNAs.

analyses suggested that disruption of *prfC* and downregulation of RRF result in decreased levels of peptidyl-tRNA drop-off (8). Also, the tRNA^{Lys} appears to be one of the first species of tRNAs that is depleted by its sequestration as peptidyl-tRNA in the *pth*^{ts} strains of *E.coli*. Overproduction of this tRNA increases the temperature threshold at which the strains continue to grow (4,24).

Here, we show that overproduction of tmRNA rescues the temperature-sensitive phenotype of a *pth*^{ts} strain and that a major cellular function of tmRNA is to lessen the peptidyl-tRNA load. Further, coupling of tmRNA overproduction with downregulation of RRF and depletion of RF3, results in a stronger suppression of *pth*^{ts}. However, the role of Pth in *E.coli* is central and it was not possible to dispense with *pth* function even upon integration of the known suppressors in a single strain.

MATERIALS AND METHODS

Plasmids, strains and growth conditions

Plasmids and strains used in this study are listed in Table 1. Luria–Bertani (LB) liquid or solid (with 1.5% agar) media (Difco, USA) were used for growth (25). The medium was supplemented with various antibiotics at the following final concentrations: tetracycline, 7.5 µg/ml; kanamycin, 25 µg/ml; chloramphenicol, 30 µg/ml; and ampicillin, 100 µg/ml as required.

Cloning

All constructs were made using standard recombinant DNA techniques (25) as follows:

E.coli ssrA gene. DNA primers 5′-GAAAAGCTTATTGGC-TATCAC-3′ and 5′-CACTAAGCTTGATTAACAGAG-3′ containing HindIII sites were used to amplify the *ssrA* gene (along with its promoter) from *E.coli* HB101 DNA using *Pfu* DNA polymerase by PCR consisting of 30 cycles of incubations at 95°C for 1 min, 40°C for 30 s and 72°C for 2 min, digested with HindIII and cloned between the same sites of pTrc99C.

E.coli smpB-ssrA. The linked *smpB* and *ssrA* genes (along with their promoters) were PCR amplified from *E.coli* HB101 DNA by *Pfu* DNA polymerase using 5′-AACGGATCCCGGC-GCTGGGT-3′, and 5′-CACTAAGCTTGATTAACAGAG-3′

primers containing BamHI and HindIII sites, respectively, by 30 cycles of incubations at 95°C for 1 min, 45°C for 30 s and 72°C for 2 min, digested with BamHI and HindIII, and cloned into similarly digested pTrc99C.

E.coli valU operon and generation of pTrc-valU-ssrA construct. The *valU* operon containing tRNA^{Lys} gene was cloned from *E.coli* HB101 DNA by PCR using *Pfu* DNA polymerase with 5′-GCGAATTCTTTAGAAAATGC-3′ and 5′-CAACTGGATCCTGCTGCAGGTTGG-3′ primers, involving 30 cycles of incubations at 95°C for 1 min, 50°C for 30 s and 72°C for 1 min 20 s. The PCR product was digested with EcoRI and BamHI, and cloned between the same sites of pTrc-*ssrA* to obtain pTrc-*valU-ssrA* construct. The pTrc-*valU* construct was generated by deleting *ssrA* gene from the pTrc-*valU-ssrA* by digestion with HindIII followed by religation with T4 DNA ligase. The veracity of the clone was established by its ability to complement *E.coli* AA7852 for its *pth*^{ts} phenotype.

E.coli and Bacillus subtilis pth genes. The *E.coli pth* gene along with its promoter was amplified from *E.coli* HB101 using 5′-CACGTCTTGAAGGCAAACGG-3′ and 5′-CGAA-CAACGTGACCGATCG-3′ primers, digested with EcoRI and EcoRV, and cloned into the same sites of pTrc99C. Similarly, the open reading frame of *B.subtilis pth* gene was PCR amplified from *B.subtilis* genomic DNA using 5′-CGCCATG-GTTGATTGCCGGTC-3′ and 5′-CATTGAAGCTTCGAG-ACTGCC-3′ primers. The PCR reaction involved 30 cycles of heating at 94°C for 1 min, 50°C for 45 s and 70°C for 1 min 45 s. The PCR product was digested with NcoI and HindIII, and cloned between the same sites of pTrc99C and pACDH.

Generation of *E.coli* strains

All strains described below were generated following the method of Datsenko and Wanner (30).

E.coli AA7852ssrA::Kn and E.coli CP78ssrA::Kn strains. pTrc-*smpB-ssrA* was digested with EcoRV and religated to delete a small fragment containing unwanted sequences of the plasmid and *smpB* origin to generate pTrc-*smpB-ssrA**. This was digested with PvuII to cleave within the *ssrA* gene for cloning of a HincII DNA fragment carrying Kn^R cassette from pUC4K. This construct (pTrc-*ssrA::Kn*) was digested with EcoRV–HincII to obtain a 2.1 kb DNA containing the disrupted *ssrA* gene for electroporation into *E.coli* DY330. The *ssrA* disruption in *E.coli* DY330 was confirmed by PCR using flanking primers. The *ssrA::Kn* locus from the DY330 strain was mobilized into *E.coli* AA7852 (*pth*^{ts}) and *E.coli* CP78 (*pth*$^+$, parent strain of AA7852) by P1-mediated transductions (31).

E.coli AA7852 ΔprfC::Kn, E.coli CP79 ΔprfC::Kn and E.coli CP79 ΔprfC strains. The Kn^R cassette from pKD4 was amplified with 5′-CGCGGCGCGATGCCGCTTACTCA-AGAAAGAATTGTGTAGGCTGGAGCTGCTTCG-3′ and 5′-CAGGGCGCAGCGCGTACCGCGCCCTGGAGGCAAG-AACATATGAATATCCTCCTTA-3′ primers using *Pfu* DNA polymerase and electroporated into *E.coli* TG1 harboring pKD46. Transformants were screened for replacement of *prfC* with Kn^R cassette by PCR with the flanking primers

Table 1. List of plasmids/strains

Plasmid/strain	Relevant details	Reference
pACDH	A cloning vector harboring ACYC ori of replication, which is compatible with ColE1 ori of replication in pTrc99C.	(26)
pTrc-*ssrA*	*E.coli ssrA* cloned into pTrc99C.	This work
pTrc-smpB-ssrA	*E.coli smpB* and *ssrA* genes cloned into pTrc99C.	This work
pTrc-valU-ssrA	*E.coli valU* operon containing tRNA^{Lys} gene cloned in pTrc-*ssrA*.	This work
pTrc-*valU*	Derived from pTrc-*valU-ssrA* by deletion of *ssrA* gene.	This work
pACD*Bsu*Pth	Open reading frame of *B.subtilis pth* gene cloned into pTrc99C.	This work
pTrc*Eco*Pth	*E.coli pth* gene cloned into pTrc99C.	This work
E.coli CP78	F$^-$, *arg$^-$*, *leu$^-$*, *thr$^-$*, *his$^-$*, *thiamine$^-$*, *relA$^+$*, T1^s	(27)
E.coli AA7852	Derivative of CP78, *pth-1*^{ts}	(13)
E.coli AA7852 *ssrA::Kn*	*E.coli* AA7852 containing disruption of *ssrA* gene with Kn^R marker.	This work
E.coli AA7852 Δ*prfC::Kn*	*E.coli* AA7852 wherein in *prfC* gene has been replaced by Kn^R marker.	This work
E.coli AA7852*frr1*	*E.coli* AA7852 wherein in Cm^R marker has been inserted at −32 position in the *frr* promoter and which results in downregulation of RRF expression.	This work
E.coli AA7852Δ*prfC::Kn, frr1*	*E.coli* AA7852 wherein in *prfC* gene has been replaced by Kn^R marker and Cm^R marker was inserted at −32 position in the *frr* promoter.	This work
E.coli CP79	F$^-$, leuB6(Am), fhuA2, glnV44(AS)?, gal-3, his-65, relA2, malT1(λ^R), xylA7, mtlA2, argH46, thi-1.	(28)
E.coli CP79Δ*prfC::Kn*	*E.coli* CP79 wherein in *prfC* gene has been replaced by Kn^R marker.	This work
E.coli CP79Δ*prfC*	*E.coli* CP79Δ*prfC::Kn* from where Kn^R marker has been excised.	This work
E.coli CP79Δ*prfC, frr1*	*E.coli* CP79Δ*prfC* wherein *frr1* allele was introduced by P1 transduction.	This work
E.coli CP79*frr1*	*E.coli* CP79 containing Cm^R marker at −32 position in the *frr* promoter.	This work
E.coli DY330	W3110 Δ*lacU*169 *gal*490 [λc1857Δ(*cro-bioA*)]	(29)
Ecoli TG1	*Sup*E *hsd*Δ5 *thi* Δ(*lac-proAB*) F$^-$ [*traD*36 *proAB$^+$ lacI^q lacZ*ΔM15]	(25)

(5′-GTCGAGCCAGGTAAACTC-3′ and 5′-GCGACGGCA-GAGGTCAAC-3′) followed by digestion with PstI. The Δ*prfC::Kn* was subsequently mobilized into *E.coli* AA7852 and *E.coli* CP79 by P1-mediated transductions to generate *E.coli* AA7852 Δ*prfC::Kn* and *E.coli* CP79 Δ*prfC::Kn* strains. The *E.coli* AA7852 Δ*prfC* and *E.coli* CP79 Δ*prfC* strains were obtained by excising the Kn^R cassette in the presence of pCP20 expressing the FLP recombinase (30,32).

E.coli AA7852 frr1 and E.coli CP79 frr1 strains. The strain (*frr1*) wherein RRF expression was down regulated was generated by insertion of a Cm^R cassette at the −32 position of *frr* promoter. The Cm^R cassette was PCR amplified from pKD3 using 5′-GGCAAATAAAATAGCCTAATAATCCA-GACGATTACCCGGTGTAGGCTGGAGCTGCTTC-3′ and 5′-GGAAGTGTGCTAAGTATAGCCCTGATTAAACATA-TTACATATGAATATCCTCCTTA-3′ primers and electroporated into *E.coli* DY330. Integration of the Cm^R cassette was verified by PCR using flanking primers (5′-CGCAG-CAACCGG-AGCGTAC-3′ and 5′-GTTTCGCACTCGCTT-CATCG-3′). *E.coli* AA7852 *frr1* and *E.coli* CP79 *frr1* strains were generated by P1-mediated transductions.

E.coli CP79 ΔprfC, frr1. This strain was generated by P1-mediated transduction of *frr1* locus (Cm^R) from AA7852*frr1* to CP79Δ*prfC*.

Generation of E.coli pth::kan. The Kn^R cassette was amplified from pUC4K using 5′-CTCCGCTGCGCGAAGAGGCTAA-ATTCTTTGGTTATACAAAGCCACGTTGTGTCTC-3′ and 5′-CAGATCCAGTTCGTCGTGGGCCACCAGAATTTCGT-CCGCGCTGAGGTCTGCCTCGTG-3′ primers and electroporated into *E.coli* DY330 harboring pACD*Bsu*Pth. Transformants (Kn^R) were checked for disruption of the *pth* gene by PCR using flanking primers (5′-GTACGGATCCAC-GATTAAATTGATTGTCGGCCTG-3′ and 5′-AGACAAGC-TTTTATTGCGCTTTAAAGGCGTGCAA-3′).

Analysis of SsrA expression

Total RNA was prepared from *E.coli* (33), electrophoresed on a 1.5% agarose gel and vacuum blotted to nytran membrane (25). The blot was probed with 5′ ^{32}P-labeled DNA oligomer (5′-GTTTCGCAAATCCCGTCGAAT-3′) complementary to SsrA. The same blot was then probed for a second time with 5′ ^{32}P-labeled DNA oligomer (5′-TACCATCGGCGC-TACGGCGTTTC-3′) complementary to 5S rRNA (25). Blots were subjected to BioImage analysis (FLA2000, Fuji).

Analysis of peptidyl-tRNAs

Luria–Bertani medium (5 ml) containing ampicillin was inoculated with 2% inoculum from overnight cultures and grown at 30°C to an OD of ∼0.6 (595 nm), and shifted to 37°C for 45 min. The cells were then chilled on wet ice. All the subsequent steps for the isolation of total tRNA under acidic conditions at 4°C, separation on acid urea gels and electroblotting onto nytran membrane were as described in (34). The blot was probed (7) with a 5′ ^{32}P-labeled DNA complementary to tRNA^{His}, and tRNA^{Tyr} (5′-GACAACTGGAATCACA-ATCC-3′ and 5′-TACAGTCTGCTCCCTTTGGCCGCTC-3′, respectively) and subjected to BioImage analysis (FLA2000, Fuji) using storage phosphor imager plates (BAS SR, Fuji).

Immunoblotting

The cell-free extracts from log-phase cultures were separated on 15% SDS–PAGE, transferred to PVDF membrane and probed with anti-RRF antibodies (7).

Analysis of essentiality of *pth*

Phage P1 lysate was raised on *E.coli* DY330 (*pth*::*Kn*) harboring pACD*Bsu*Pth and used to transduce *E.coli* CP79Δ*prfC*, CP79*frr1*, and CP79Δ*prfC, frr1* harboring pTrc99C, pTrc*Bsu*Pth, pTrc-*ssrA* or pTrc-*valU-ssrA*. The transductants

(Kn^R) were screened by PCR for *pth* locus with the primers (5′-CACGTCTTGAAGGCAAACGG-3′ and 5′-CGAACAA-CGTGACCGATCG-3′).

RESULTS

Overproduction of tmRNA (SsrA) rescues the temperature-sensitive phenotype of *E.coli* AA7852 (*pth*^{ts})

To test the hypothesis if overproduction of tmRNA could rescue the *pth*^{ts} phenotype of *E.coli* AA7852, we introduced into this strain, various plasmid constructs (Table 1) by genetic transformation and checked for the growth of the transformants at different temperatures. As shown in Figure 2A, true to its genotype, the strain AA7852 harboring the pTrc99C vector alone grew at the permissive and semi-nonpermissive temperatures of 30 and 37°C, respectively but ceased to grow as the temperature was raised to 40°C (sectors 2). As a control, when the strain harbored a plasmid-borne copy of *E.coli pth* gene (pTrc*Eco*Pth), it grew at 40°C (sectors 1). Interestingly, a plasmid-borne copy of *ssrA* gene (sectors 3) also rescued the growth of AA7852 at 40°C (compare sectors 2 and 3). Simultaneous presence of the extrachromosomal copy of *smpB* gene (coding for SmpB protein) along with *ssrA* (pTrc-*ssrA*-*smpB*) conferred no apparent advantage towards rescue by plasmid-borne *ssrA* (compare sectors 3 and 4), and was not analyzed any further. To examine the extent of tmRNA overproduction in the transformants, we analyzed the levels of tmRNA and 5S rRNA [Figure 2B, panels (i) and (ii)]. Based on the relative

levels of tmRNA and 5S rRNA [Figure 2B, panel (iii)], in the transformants harboring plasmid-borne copies of *ssrA* (pTrc-*ssrA* or pTrc-*ssrA*-*smpB*), production of tmRNA was about 4.5-fold higher than the chromosomal background suggesting that a moderate level of overproduction of tmRNA lessens the severity of the *pth*^{ts} phenotype in *E.coli*.

E.coli AA7852 (*pth*^{ts}) with *ssrA::Kn* allele are hypersensitive to temperature

In another approach to demonstrate that the chromosomally encoded tmRNA contributes to rescue of the *pth*^{ts} phenotype, we disrupted the *ssrA* gene in the *E.coli* AA7852 with *Kn* marker to generate *E.coli* AA7852*ssrA::Kn* strain. This procedure resulted in the complete absence of tmRNA in the AA7852*ssrA::Kn* strain [Figure 2B, panel (i)]. As seen in Figure 3A (sectors 3), the AA7852*ssrA::Kn* strain became hypersensitive to temperature and was not viable at 37°C, a temperature at which the parent strain AA7852 sustained good growth (Figure 2A, sector 2). Introduction of the plasmid-borne copy of *ssrA* into the AA7852*ssrA::Kn* strain, rescued its growth not only at 37°C but also at 40°C (Figure 3A, sectors 4) ruling out that lack of growth of AA7852*ssrA::Kn* strain at the semi-nonpermissive temperature was due to some non-specific effects emanating from the alterations at the chromosomal level. Further, as shown in Figure 3B, in liquid cultures also, while at 30°C, the AA7852*ssrA::Kn* strain harboring the vector alone, a plasmid-borne copy of *ssrA* or *pth* genes grew with similar rates; at 39°C, the AA7852*ssrA::Kn* strain harboring vector alone failed to show any significant growth.
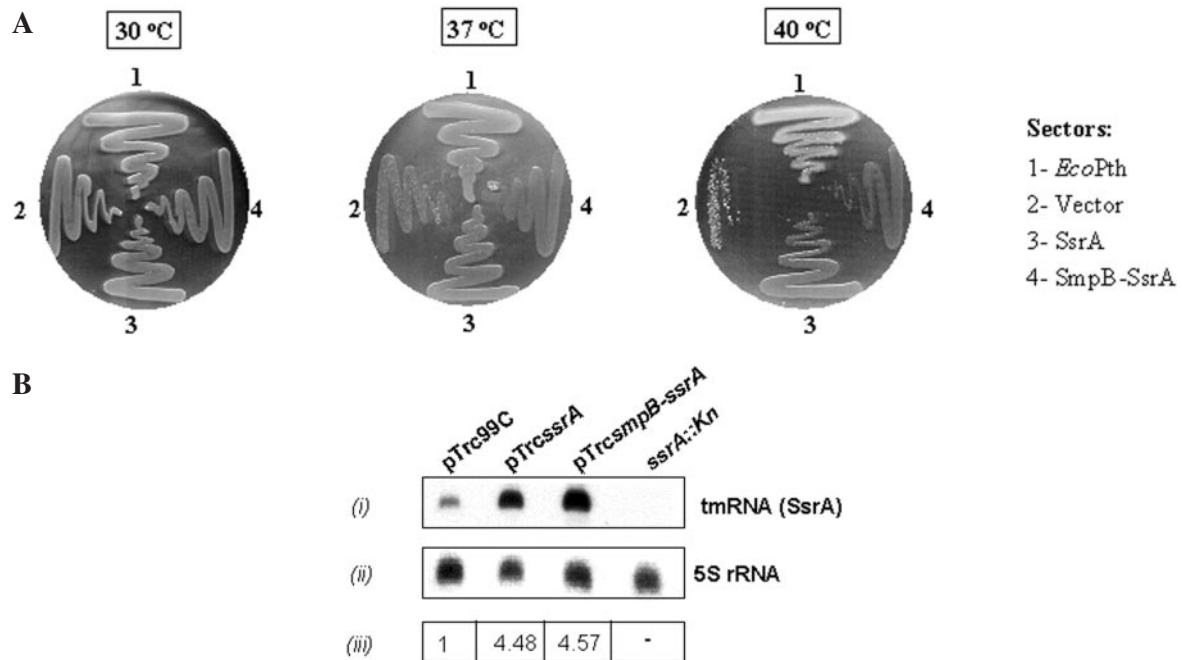
Figure 2. (**A**) Analysis of complementation of *E.coli* AA7852 (*pth*^{ts}) with *ssrA*. Various plasmids were introduced into *E.coli* AA7852 and the transformants were streaked on LB agar plates containing ampicillin, at various temperatures indicated above the plates. Sectors: 1, pTrc*Eco*Pth; 2, pTrc99C (vector); 3, pTrc-*ssrA*; and 4, pTrc-*ssrA*-*smpB*. (**B**) Expression of tmRNA (SsrA) in *E.coli* AA7852 harboring different plasmids indicated above the lanes (or a *ssrA::Kn* null strain) was detected by northern blotting using radiolabeled DNA probes for tmRNA [panel (i)] and 5 S rRNA [panel (ii)]. Relative levels of tmRNA (tmRNA/5S rRNA) shown in panel iii were obtained by quantification of signals by BioImage analyzer. Relative levels of tmRNA were normalized to transformants harboring pTrc99C vector (which was set as 1).
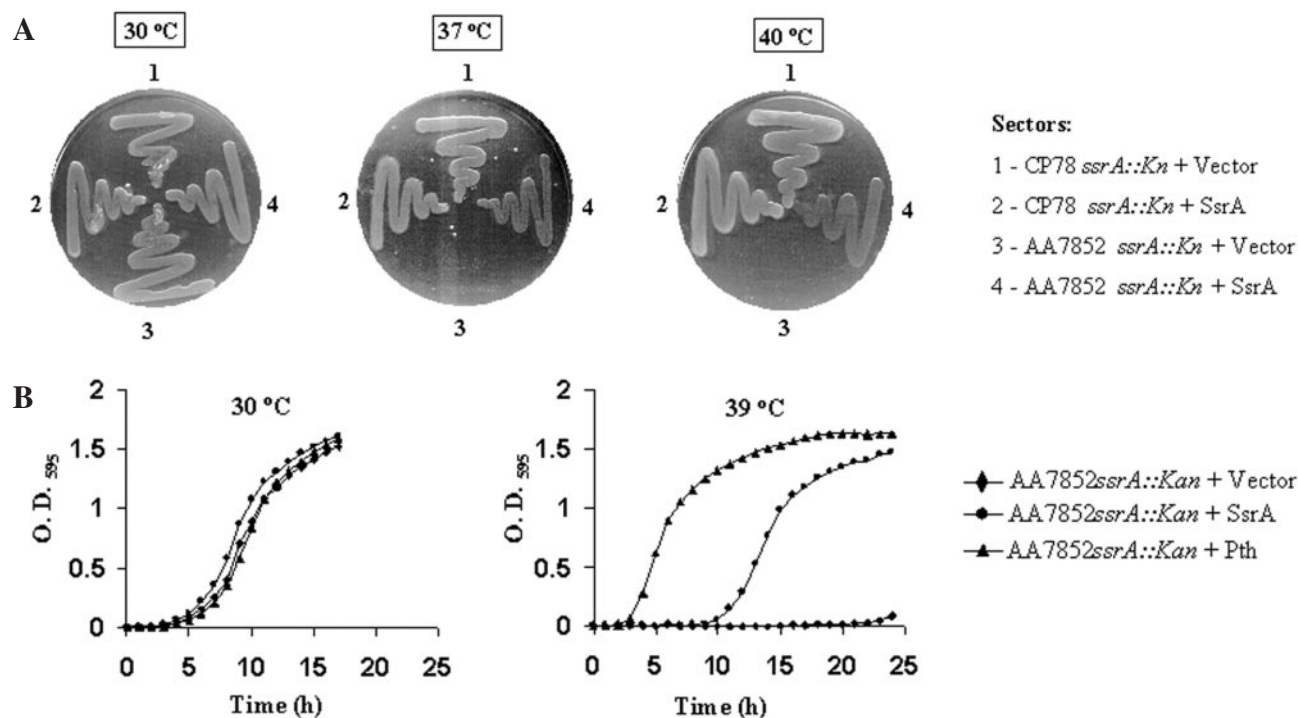
Figure 3. (**A**) Analysis of growth of *E.coli* CP78 (*pth+*, *ssrA::Kn*) harboring pTrc99C or pTrc-*ssrA* (sectors 1 and 2, respectively) and *E.coli* AA7852 (*pth*ts, *ssrA::Kn*) harboring pTrc99C or pTrc-*ssrA* (sectors 3 and 4, respectively) on LB plates containing ampicillin at various temperatures indicated above the plates. (**B**) Analysis of growth in LB medium. Overnight cultures (0.05%) were inoculated into fresh medium and grown under shaking conditions at 30 or 39°C. Progress in growth was monitored by taking absorbance of samples at 595 nm at different times.

However, the growth of the strain at 39°C was rescued by the presence of a plasmid-borne copy of *ssrA* or *pth*. Expectedly, the rescue by *pth* was better than that by *ssrA*. In a control experiment, when the *ssrA* locus was disrupted in *E.coli* CP78 (*pth+* isogenic strain of AA7852) in exactly the same manner, under the same conditions, no adverse effects of *ssrA* disruption were noticed (Figure 3A, compare sectors 1 and 2). Taken together, the observations in Figures 2 and 3 clearly suggest that *ssrA* is a suppressor of *pth*ts phenotype in *E.coli*.

Overexpression of tmRNA lowers the steady-state levels of peptidyl-tRNAs in *E.coli* AA7852

To understand the mechanism of tmRNA-mediated rescue of the *pth*ts phenotype, we analyzed the effect of its overexpression on the levels of peptidyl-tRNAs (peptidyl-tRNA^{His} and peptidyl-tRNA^{Tyr}) in the cells. To ensure preservation of the sensitive ester bond that links amino acid or the peptide to the tRNA, RNA was prepared under cold and acidic conditions, and analyzed using acid urea gels. In these gels, tRNAs attached to peptides of heterogeneous length migrate slower than the aminoacylated tRNA, often in the form of a smear (7). Further, since the absolute levels of total tRNA vary in the preparations, to compare changes in the steady-state levels of peptidyl-tRNAs, their abundance as percent fraction of total tRNA (for tRNA^{His} or tRNA^{Tyr}) was determined within each sample. As shown in Figure 4A, expectedly, at the permissive temperature of 30°C where the Pth is active, none of the transformants accumulate any significant levels of peptidyl-tRNA^{His} (lanes 1, 3 and 5). However, at the semi-nonpermissive temperature of 37°C, while the transformant harboring vector

alone accumulated ∼54% of the tRNA^{His} as peptidyl-tRNA^{His} (lane 4), the presence of *ssrA* construct in the strain decreased this to ∼37% (lane 6). Expressing a wild-type copy of *pth* in AA7852, reduced the extent of accumulation of peptidyl-tRNA^{His} at 37°C to ∼11% (lane 2). Similar results were obtained with tRNA^{Tyr} (Figure 4B). At 37°C, accumulation of tRNA^{Tyr} as peptidyl-tRNA^{Tyr} was 32% in transformants harboring vector alone (lane 4) which was decreased to ∼20% in transformants harboring the *ssrA* gene (lane 6). The level of peptidyl-tRNA^{Tyr} in the transformants with plasmid-borne copy of wild-type *pth* at 37°C, was ∼9% (lane 2). Thus, consistent with our hypothesis of *ssrA* serving as a suppressor of *pth*ts, overproduction of tmRNA at 37°C resulted in decreased accumulation of both the tRNA^{Tyr} and tRNA^{His} in the form of peptidyl-tRNAs.

ssrA-mediated suppression of *pth*ts in Δ*prfC*, and *frr* promoter down backgrounds

Heurgue-Hamard *et al*. (8) reported that disruption of *prfC* (RF3), or the promoter region of *frr* (RRF) resulted in suppression of *pth*ts phenotype in *E.coli*. In order to integrate these results and the tmRNA effect described above, and to further our understanding on the mechanisms that lead to suppression of the *pth*ts phenotype, we generated a Δ*prfC* derivative of AA7852 by replacement of the open reading frame of RF3 with *Kn*^{R} marker, and a promoter down *frr* by insertion of Cm^R marker at −32 position in the promoter region (*frr1*). Genetic manipulations in both strains were confirmed by PCR analysis (Materials and Methods). Further, an immunoblot analysis of the cell-free extracts obtained from the *frr1* derivative showed

A

B
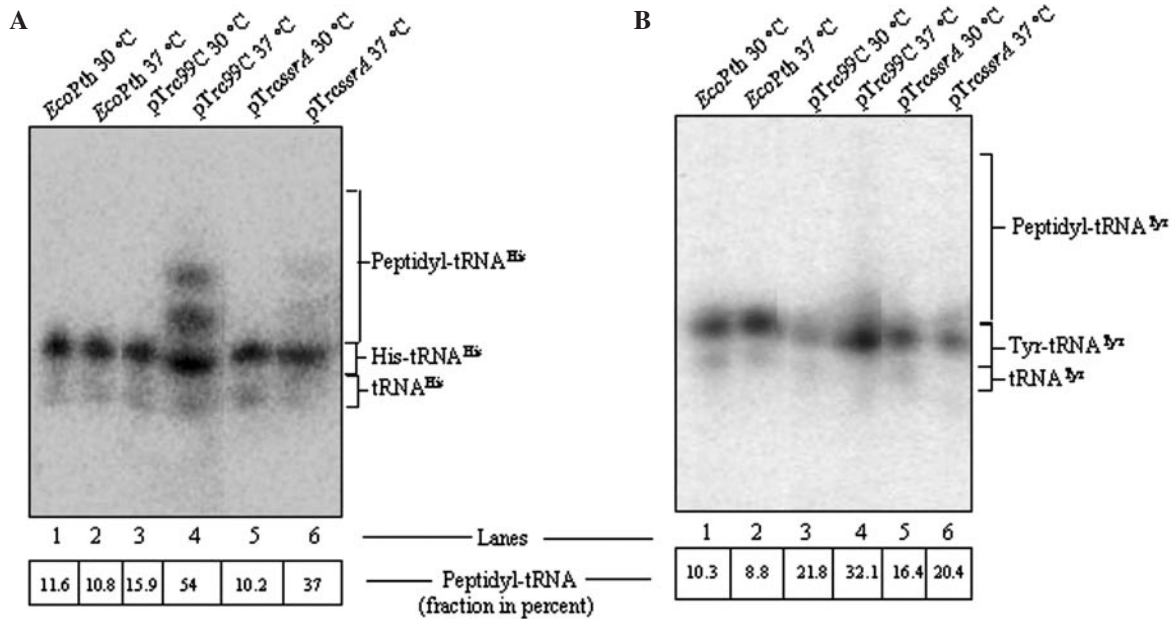
Figure 4. Analysis of the accumulation of peptidyl-tRNAs in *E.coli* AA7852 (*pth*^ts) in the presence of vector (pTrc99C) or pTrc-*ssrA*. Transformants were grown at permissive (30°C) or semi-nonpermissive (37°C) temperatures. Total tRNA was prepared under acidic conditions, fractionated on acid-urea gel, transferred to nytran membrane and hybridized with 5′ end ^{32}P-labeled anti-tRNA^{His} (**A**) or anti-tRNA^{Tyr} (**B**) probes. Signals were quantified by BioImage analyzer (Fuji) and % peptidyl-tRNA was calculated as [(peptidyl-tRNA/(peptidyl-tRNA + aminoacyl-tRNA + tRNA) × 100]. These are shown below the lanes.
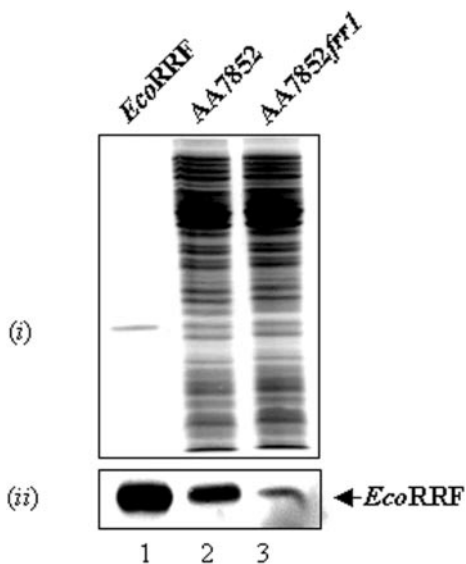

Figure 5. Immunoblot analysis of total cell-free extracts of *E.coli* AA7852 (lane 2) and its *frr1* derivative (lane 3) for RRF. Cell-free extracts (∼10 μg) were analyzed by SDS–PAGE and detected by Coomassie blue stain [panel (i)]. A similar gel was prepared for transfer to PVDF membrane and immunoblotted using anti-RRF antibodies [panel (ii)]. Lane 1 contains pure RRF as marker.

downregulation of expression of RRF [Figure 5 compare lanes 2 and 3, panel (ii)]. Yet another derivative of AA7852 that we generated possessed both the Δ*prfC* and *frr1* genetic alterations. As expected, when compared with the parent strain AA7852 (Figure 6, sector 2), all the three derivatives of AA7852 (Δ*prfC*, *frr1* and the double mutation of Δ*prfC* and *frr1*) rescued the phenotype of the *pth*^ts strain (sectors 4, 6 and 8, respectively, at 40 and 42°C). More importantly,

introduction of a plasmid-borne *ssrA* gene in all of these strains resulted in a higher efficiency of suppression of *pth*^ts in AA7852 (compare sectors 4, 6 and 8 with 3, 5 and 7, respectively at 42°C). At 42°C, it is seen that rescue by Δ*prfC* and *frr1* markers is somewhat better than that conferred by overproduction of tmRNA (compare sectors 1 with 4 and 6, respectively). Nevertheless, integration of Δ*prfC*, *frr1* with a plasmid-borne copy of *ssrA* (sector 7) resulted in a remarkable rescue of *E.coli* AA7852.

pth is essential in *E.coli*

With the identification of tmRNA as an additional suppressor of *pth*, there are now at least five factors (RRF, RF3, RelA, tRNA^{Lys} and tmRNA) whose influence on Pth is well characterized in *E.coli*. Of these, RF3, RRF and RelA are responsible for increased drop-off of peptidyl-tRNA, and tmRNA leads to a decrease in the level of peptidyl-tRNA. On the other hand, tRNA^{Lys} (in *valU* operon) functions at a step downstream of the peptidyl-tRNA release. These observations, together with the fact that both of the identifiable *pth* genes in *Saccharomyces cerevisiae* could be deleted, raise the question of whether the *pth* gene in *E.coli* can be dispensed with. To address this, we first integrated one or many of the conditions that lead to rescue of *pth*^ts, into *E.coli* CP79 (*relA*⁻). We also generated an *E.coli* strain wherein ∼0.1 kb segment of the chromosomal *pth* open reading frame was replaced with a kanamycin resistance marker (*pth::Kn*) in the presence of a helper plasmid harboring a *pth* gene of *B.subtilis* origin under the control of an *E.coli* promoter (Figure 7A). Phage P1 lysate was raised on this strain to attempt knockout of the *pth* gene in the *E.coli* CP79 derivatives by transduction. As shown in Table 2, a number of ∼1000 transductants on a *Kn* plate with a control strain harboring plasmid-borne *pth* gene of
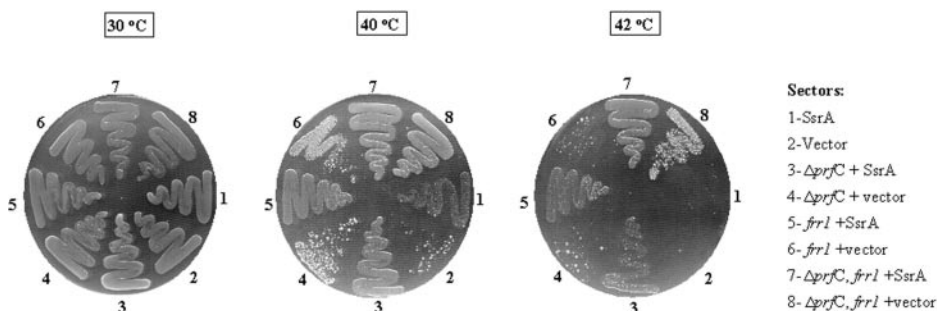
Figure 6. Analysis of growth of *E.coli* AA7852 (sectors 1 and 2), and its derivatives *E.coli* AA7852Δ*prfC::Kn* (sectors 3 and 4), *E.coli* AA7852*frr1* (sectors 5 and 6), and *E.coli* AA7852*frr1, ΔprfC::Kn* (sectors 7 and 8) harboring pTrc99C (sectors 2, 4, 6 and 8) or pTrc-*ssrA* (sectors 1, 3, 5 and 7) at various temperatures as indicated on LB agar plates containing ampicillin.
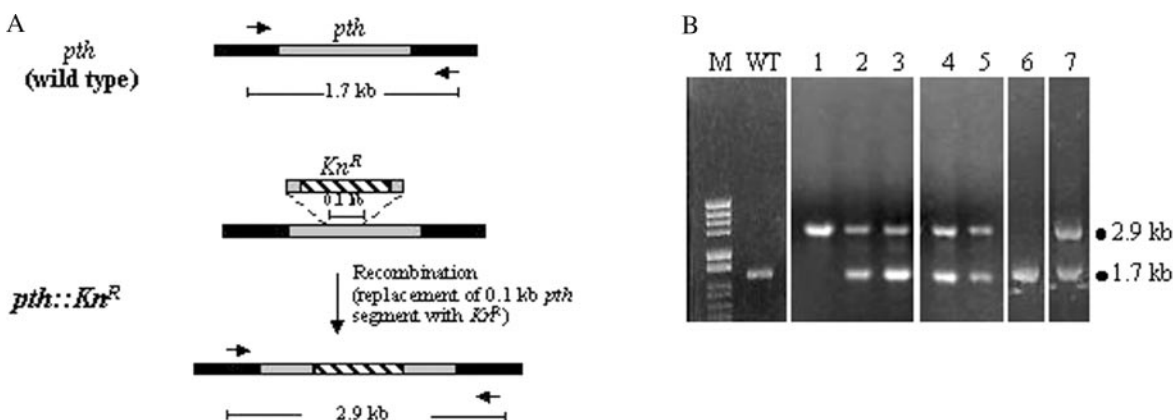
Figure 7. Analysis of *pth* gene disruption in *E.coli*. (**A**) Schematic representation of the *pth* locus and its disruption by *Kn* cassette in *E.coli*. Expected sizes of the PCR products from chromosomal DNAs of an intact and disrupted loci are as indicated. (**B**) PCR screening of representative transductants. Lanes: M, DNA size markers (λ DNA digested with HindII and HindIII); WT, wild-type locus; lane 1, positive control transductant (disrupted locus); lanes 2–7, transductants obtained on CP79Δ*prfC*, *frr1*, pTrc-*ssrA* (lanes 2 and 3), CP79 *frr1*, pTrc-*ssrA* (lanes 4 and 5) and CP79Δ*prfC*, *frr1*, pTrc-*valU-ssrA* (lanes 6 and 7) backgrounds.

Table 2. Analysis of *pth* gene disruption in *E.coli*

E.coli strains	Number of colonies upon transduction with phage P1 (*pth::Kn*)	PCR test for intact copy of *pth* in the chromosome
E.coli CP79, pTrc*Bsu*Pth	>1000	Negative
E.coli CP79 *frr1*, pTrc99C	0	—
E.coli CP79 *frr1*, pTrc-*ssrA*	9	Positive
E.coli CP79 Δ*prfC*, pTrc99C	0	—
E.coli CP79 Δ*prfC*, pTrc-*ssrA*	0	—
E.coli CP79 Δ*prfC, frr1*, pTrc99C	0	—
E.coli CP79 Δ*prfC, frr1*, pTrc-*ssrA*	3	Positive
E.coli CP79 Δ*prfC, frr1*, pTrc-*valU-ssrA*	24	Positive

B.subtilis origin suggests a high efficiency of tranduction. However, despite many attempts, the number of transductants on the test strains, which lacked a *pth* support plasmid but into which various suppressors of *pth* had been integrated, was very low (Table 2). Further, PCR screening (Figure 7A) of the entire test transductants obtained, revealed that they either possessed an intact copy, or both an intact and a disrupted copy of the *pth* genes in their chromosomes (a representative gel is shown in Figure 7B, and data are summarized in Table 2). On the other hand, the control transductants (obtained in the

presence of support plasmid) possessed only a disrupted copy of the *E.coli pth* gene (Figure 7B, lane 1). Use of several other strains of *E.coli* in such experiments yielded identical results (data not shown). Taken together, these observations suggest that *pth* plays a central role and is indispensable in *E.coli*.

DISCUSSION

In bacteria, the product of *ssrA* gene, SsrA is a well-conserved molecule of ~360 nt in length. In *E.coli*, SsrA was originally identified as 10Sa RNA upon further fractionation of the 10S RNA (35). Subsequently, SsrA was shown to function both as tRNA and mRNA, hence the new name tmRNA (14). The 3′ end of tmRNA terminates with a –CCA sequence to which alanine is ligated by alanyl-tRNA synthetase. The alanylated-tmRNA is recruited to the ribosomal A-sites of the stalled ribosomes for the process of *trans*-translation, which attaches an AANDENYALAA sequence (in *E.coli*) to the C-terminal of the incomplete peptides and directs the aberrant proteins for degradation by the cellular proteases (4,36–38). In this study, we have uncovered yet another facet of tmRNA function in decreasing the peptidyl-tRNA load in the cells.

Ribosomes may stall on either a full-length or a truncated mRNA. While the stalled complexes with truncated mRNAs (with no or very short 3′ extensions) are direct and preferred

substrates for tmRNA, even those with an intact mRNA may be directed to *trans*-translation following the action of codon-specific ribonuclease (19–22). The observation that in the wild-type *ssrA* background, overproduction of tmRNA raises the threshold of the temperature sensitivity of the *pth*^ts strain suggests that in the cells, there exists a population of stalled ribosomes, which is the substrate for *trans*-translation, but remains unprocessed by the available tmRNA. Consequently, this population of stalled ribosomes (which may possess either a truncated or intact mRNA) must be processed via alternate pathways resulting in dissociation of peptidyl-tRNAs from them, which in turn should be processed by Pth to recycle the tRNAs. Thus, we believe that rescue of the *pth*^ts strain AA7852, by overproduction of tmRNA is due to diversion of this population of the stalled ribosomes from dropping-off peptidyl-tRNAs to engaging them in *trans*-translation (Figure 1, lower). This will in turn, decrease the substrate load on Pth, and make the available Pth activity sufficient to process peptidyl-tRNAs that arise from other physiological processes. Further, although the exact mechanisms by which RRF and RF3 result in peptidyl-tRNA drop-off are not understood, it is clear that downregulation of RRF and/or absence of RF3 result in decreased peptidyl-tRNA load in the cell (7,8). Interestingly, as shown in Figure 6, overproduction of tmRNA in a strain harboring *frr1* and Δ*prfC* mutations in single or in combination, results in a synergistic effect on the rescue of the *pth*^ts phenotype. The synergism between the two mutations themselves and with *ssrA* suggests that the stalled ribosomes are channeled to utilization by all the three factors and, while the RRF and RF3 pathways rescue them by drop-off of peptidyl-tRNAs (Figure 1, upper), *ssrA* rescues them by *trans*-translation (Figure 1, lower).

Studies on mutants of *E.coli* and its phages have unraveled crucial aspects of the host's physiology (39). It was observed that *rap* (same as *pth*) mutation in *E.coli* resulted in defective growth of bacteriophage λ (40,41). Interestingly, yet another independent study, reported a similar phenotype for the *sip* (same as *ssrA*) mutation in *E.coli* (42,43). While no link has been established between these two separate studies, these observations support the importance of *ssrA* and the physiological relevance of the connection between *ssrA* and *pth* that we have established in this study. In fact, at least in *Neisseria gonorrhoeae*, the *ssrA* is an essential gene (44). It will be interesting to investigate the status of peptidyl-tRNA/Pth in this organism.

The genetic and biochemical analyses of the suppressor mutations (*bar* mutations) in phage λ that rescue the growth on *rap* (*pth*) mutants of *E.coli* have shown that the phage-encoded minigenes (wild-type *bar* regions, at least one of which is located in the *imm* region) are responsible for excess drop-off of the peptidyl-tRNAs and depleting the availability of tRNA for translation (45). Such peptidyl-tRNAs are recycled by Pth. Hence, the effect of Pth on phage growth is a direct one. Whether the effect of *ssrA* on phage growth is also a direct one (e.g. by *trans*-translation) is not clear. At least, the lack of tmRNA-mediated targeting of aberrant proteins to degradation is not responsible for the bacteriophage λ growth defect in *sip* (*ssrA*) mutants (38). On the other hand, based on recent reports that the ribosomes with truncated mRNAs with no or very short 3′ extensions are the real substrates for *trans*-translation by tmRNA (46,47) and that the minigene toxicity

is more a consequence of frequent re-initiation events (48), it appears that the *ssrA* effect on bacteriophage λ growth is an indirect one. The presence of *ssrA* most likely decreases peptidyl-tRNA load by engaging the stalled ribosomes into *trans*-translation (thereby withdrawing the chances of the factor-dependent or spontaneous processes that result in drop-off of peptidyl-tRNAs). This process, frees Pth to now hydrolyze the peptidyl-tRNAs arising out of the minigenes of the bacteriophage λ, or other host activities. However, this interpretation does not rule out a direct role for *ssrA* in the bacteriophage growth. Recently, it has been shown that at least within certain codon contexts, much of the peptidyl-tRNA population arising from minigene expression remained in the ribosomes (49). Such 'stand-by' stalled ribosomal complexes can be further processed to become substrates for *trans*-translation (19–22). Therefore, it will be interesting to study the mechanism by which *ssrA* contributes to the lambdoid phage growth.

Finally, biochemical studies show that *Saccharomyces cerevisiae* possesses at least two distinct types of Pth activities similar to those found in bacteria and archaea. Recently, it was shown that deletions in *S.cerevisiae* of the two identifiable *pth* genes (individually or in combination) were viable (50,51). A large number of factors in *E.coli* (RRF, RF3, IF1, IF2, EFG, tmRNA, RelA, tRNA^Lys, etc.) affect peptidyl-tRNA levels in cell. And, the fact that many of these have also been characterized as suppressors of Pth suggests that the role of Pth in *E.coli* (and possibly in other organisms) is central to the process of mRNA translation. On the other hand, the knowledge of a large number of suppressors that decrease the peptidyl-tRNA load in *E.coli* also makes it desirable to attempt to delete the only known *pth* gene in *E.coli*. Thus, we integrated many of the known suppressor markers in *E.coli* to attempt deletion of *pth* gene. However, our studies (Figure 7) clearly support the fact that the Pth activity is indeed an essential function. In fact, as has also been suggested by the authors of the *S.cerevisiae* study (51), there may well be several Pth-like activities in eukaryotes to recycle tRNAs. In rabbit reticulocyte lysates, one of the activities that recycles tRNAs from the peptidyl-tRNAs is a phosphodiesterase that cleaves between the C and A, the last two nucleotides in the tRNA (52). The tRNA is subsequently repaired by tRNA nucleotidyltransferase (53). Whether such Pth activity is present in yeast is not known. Certainly, the reaffirmation of the essentiality of Pth activity in *E.coli* is a good indicator of the presence of additional Pth activities in yeast.

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