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Biochemical Analysis of SmpB and RNase R in RNA Quality Control Mechanisms

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Hina Zafar

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Dr. A. Wali Karzai Ph.D. – Thesis Advisor
Associate Professor, Department of Biochemistry and Cell Biology

Dr. Steven E. Glynn – Second Reader
Assistant Professor, Department of Biochemistry and Cell Biology

This thesis is accepted by the Graduate School

Charles Taber
Interim Dean of the Graduate School

Abstract of the Thesis

Biochemical Analysis of SmpB and RNase R in RNA Quality Control Mechanisms

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trans-translation is a quality control mechanism used by bacteria to rescue ribosomes stalled on aberrant mRNAs. The major contributors to this salvage mechanism are SmpB (Small Protein B) and tmRNA (transfer messenger RNA). The SmpB-tmRNA complex recognizes and binds stalled ribosomes. As a result, it helps release the stalled ribosomes while simultaneously marking the incomplete polypeptide for proteolysis by adding a degradation tag to its C-terminus. The SmpB protein has a C-terminal tail that is disordered in solution and is known to play an important role during *trans*-translation. I have incorporated several independent mutations into the corresponding region of the *smpB* gene that should enable *in vivo* UV crosslinking and identification of interacting partners of SmpB during various stages of the *trans*-translation process.

During *trans*-translation, the defective mRNA that causes ribosome stalling is targeted for degradation by the 3´-5´ exoribonuclease RNase R. RNase R has two distinct domains that are not present in other related exoribonucleases in the cell. An N-terminal domain

that contains a helix-turn-helix motif of unknown function, and a C-terminal domain that is important for *trans*-translation dependent degradation of the defective mRNA. RNase R is also involved in general RNA turnover in the cell. Recently, RNase R has been shown to associate with the RNA degradosome during stationary phase. I have investigated which of the two unique domains of RNase R play a role in its association with the degradosome. Based on the results, I have demonstrated that the N-terminal domain of RNase R is not required for this process.

This thesis is dedicated to Zafar Chaudry and Anika Zafar.

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List of Abbreviations

PAPI- Poly(A) Polymerase I

E. coli- Escherichia coli

Bpa- p-Benzoyl-L-phenylalanine

SmpB- Small Protein B

tmRNA- Transfer messenger RNA

MLD- mRNA-like domain of tmRNA

TLD- tRNA-like domain of tmRNA

tge- *Thermoanaerobacter tengcongensis*

rnr^{tge} – *rnr* gene from *Thermoanaerobacter tengcongensis*

RNR- RNase R

PNPase- Polynucleotide phosphorylase

mRNA- Messenger RNA

tRNA- Transfer RNA

λS- Lambda c-I-N stop reporter

λNS- Lambda c-I-N nonstop reporter

ORF- Open Reading Frame

CSD- Cold shock domain

HTH- Helix turn helix

PNPase- polynucleotide phosphorylase

Ppk- polyphosphate kinase

Acknowledgments

First, I would like to thank my thesis advisor Dr. A. Wali Karzai, who has helped me with every step in my research. I appreciate the time and effort he took to make sure of my progress. I am thankful for his guidance in developing critical thinking skills. I am particularly thankful for the assistance and guidance I have received from Krithika Venkataraman. She taught me most of the molecular biology lab skills that I have today. I am grateful for the friendships I have shared with Karzai lab members: Dr. Preeti Mehta, Neha Puri, Perry Woo, Devin Camenares, and Arthur Korman. Lastly, I am thankful for the support I have received from my family, specially my husband. Without his support it would not have been possible for me to go back to school.

Chapter 1: Introduction

1.1 Introduction

Bacteria have different quality control mechanisms that are important for their survival and pathogenesis especially under stress conditions. These mechanisms ensure the regulation of RNA and proteins as required for the cellular functions. Bacterial cells maintain the required pool of RNA under different physiological conditions by processing RNA precursors and degrading non-essential RNA. In most bacterial species, a large multi-enzyme complex called the RNA degradosome carries out this process of RNA metabolism and turnover to control post-transcriptional gene expression. The dual function of RNA degradosome in processing and degradation of RNA is analogous to the process of RNA turnover and quality control mechanisms in other forms of life.

All cell types from different organisms synthesize proteins by using the same basic mechanism known as *translation*. In general, translation is known to be a robust and accurate process. Nevertheless, there are instances when ribosomes become stalled on aberrant mRNAs (e.g., an mRNA lacking an in-frame stop codon). If these stalled ribosomes are not relieved they get sequestered on defective mRNA. Persistence of defective mRNA leads to futile cycles of translation initiation and elongation that result in formation of defective polypeptide thereby wasting energy and resources. Bacteria have evolved a unique quality control mechanism known as *trans*-translation to solve these problems.

Together, these two mechanisms contribute to maintaining cellular homeostasis.

1.2 *trans*-Translation

trans-translation is a rescue mechanism by which bacterial cells relieve stalled ribosomes. As a result of this action, the defective mRNA and the nascent polypeptide chain are targeted for degradation. The key players of *trans*-translation are SmpB (Small Protein B) and tmRNA (transfer-messenger RNA) (1). tmRNA is a chimeric molecule that has features of both tRNA and mRNA. The *E. coli ssrA* gene encodes for the 363 nucleotides long tmRNA. A representation of tmRNA structure is shown in Figure 1.1. The tRNA-like domain (TLD) of tmRNA consists of a D-loop, a T-arm, and an acceptor stem, but lacks the anti-codon stem. In place of the anti-codon stem, there is a connecting structure linking the tRNA-like domain with the mRNA-like domain (MLD). SmpB is a small protein (160 amino acids) cofactor that binds the tRNA-like domain of tmRNA and is required for the recruitment of tmRNA to stalled ribosomes (2). Together, SmpB and tmRNA play a crucial role in recognizing stalled ribosomes and going through subsequent stages of *trans*-translation.

The genes coding for SmpB and tmRNA, *smpB* and *ssrA* respectively, are present in all bacterial species sequenced to date (3). In some cases, it is shown to be important for bacterial survival and pathogenesis. Even though the SmpB-tmRNA system is not essential for growth in *E. coli* under ideal conditions, its absence causes the cells to become more sensitive to oxidative stress and sub-lethal concentration of translation-specific antibiotics (4). In several other bacterial species, such as *Y. pseudotuberculosis*, SmpB-tmRNA system is crucial for virulence. In this case, mutations in *smpB-ssrA* genes resulted in the pathogen losing the ability to cause lethal infection in mice, primarily because of defects in expression and

secretion of Yersinia outer proteins (Yops), which are essential for virulence (5, 6).

Ribosome stalling occurs in response to various causes such as during translation of an incomplete mRNA lacking a stop codon, secondary structure in the mRNA, shortage of required aminoacylated tRNA, etc. In case of internal stalling events resulting from the latter two, mRNA is cleaved in the ribosomal A-site leading to formation of an empty A-site. These stalled ribosomes with an empty A-site are recognized by a quaternary complex composed of elongation factor EF-Tu, GTP, alanyl-tmRNA, and SmpB (7, 8). Successful engagement of the stalled ribosome results in release of EF-Tu-GDP and accommodation of the tRNA-like domain of tmRNA into the A-site of the ribosome. The incomplete polypeptide is transferred onto the tRNA-like domain of tmRNA and the translation machinery is established with the ORF of tmRNA serving as the surrogate mRNA. The tmRNA ORF is decoded until the ribosome reaches the stop codon, which enables normal translation termination. Therefore, the *trans*-translation process adds an 11 amino acid degradation tag (known as tmRNA tag or SsrA tag) to the defective nascent polypeptide and rescues stalled ribosomes, making them available for future rounds of translation. As a corollary to this process, the tmRNA tag is recognized by C-terminal specific proteases such as ClpXP, resulting in degradation of the defective polypeptide. The defective mRNA is degraded specifically by RNase R (9) thus preventing further futile rounds of translation (Figure 1.2).

SmpB is present in 1:1 ratio with tmRNA on the ribosome and this ratio remains the same during the course of *trans*-translation (10), which suggests that SmpB and tmRNA stay as a complex during all stages of *trans*-translation. The C-terminal tail of SmpB has been shown to play a critical role in efficient tagging of SsrA-tagged proteins (11). My research involves studying interactions of SmpB C-terminal tail with the ribosome and/or other factors

during *trans*-translation. This study will shed light on the finer mechanistic details of the various stages of the *trans*-translation process.

1.3 RNA Degradosome

The RNA degradosome is a large multi-enzyme complex that plays a crucial role in RNA metabolism and post-transcriptional control of gene expression in most bacteria. It ensures proper RNA turnover as well as processing of structured RNA precursors during their maturation. Even though the composition of the degradosome varies among different species, its presence in most bacterial species is indicative of its crucial role as a regulatory hub for complex cellular processes.

RNA degradosome is composed of many different enzymes, scaffolding proteins, and chaperones. The core components of *E. coli* degradosome include RNase E, PNPase (polynucleotide phosphorylase), the RhlB helicase, and enolase (12-14). RNase E is an endoribonuclease with a predicted molecular mass of 118 kDa that provides the main scaffold of the degradosome. RNase E has two main domains: a globular N-terminal catalytic domain (NTD) that tethers the degradosome to bacterial inner membrane (15) and an unstructured C-terminal domain (CTD) that acts as a scaffold for assembly of other components of the degradosome through short recognition motifs as depicted in Figure 1.3 (16). The CTD also has two RNA-binding sites. The short recognition motifs, also called “microdomains”, in the C-terminal domain of RNase E are more highly conserved than the rest of the C-terminus. They are involved in protein-protein interactions between PNPase, RhIB helicase and enolase (16). PNPase is a 3´-5´ exoribonuclease with a molecular mass of 77 kDa that digests RNA fragments

produced by the initial RNase E cleavage with the help of RhIB (an ATP dependent helicase) that unwinds the RNA secondary structures (17). Other DEAD box RNA helicases such as SrmB, CsdA, and RhIE have also been found to be a part of the degradosome under stress conditions (18). Enolase is a glycolytic enzyme whose function in the degradosome is not known. However, it affects the degradation of specific mRNA transcripts such as those encoding for enzymes involved in glycolysis. The loss of RNase E-enolase interaction causes an increase in the half-life of such mRNA transcripts (19).

The structure of RNA degradosome is not completely understood so far, but its assembly seems to be under dynamic control. A model of RNA degradosome assembly is shown in Figure 1.4. The "microdomains" of RNase E CTD bind to multimeric core components. RNase E, itself exists as a tetramer by association of NTD from 4 monomeric units (20). The CTD of the homo-tetrameric RNase E binds to homotrimeric PNPase, protomeric enolase, and the RhIB helicase. The current model describes the RNA degradosome to be present in a filament like assembly tethered to the bacterial cytoplasmic membrane (18-21).

Different accessory proteins are also associated with the degradosome depending on the growth phase and environment of the cell. This provides additional ways of modulating the function of degradosome. Among these are different regulatory RNases and enzymes involved in mRNA decay such as poly(A) polymerase I (PAPI) (22). In addition, molecular chaperones such as GroEL, DnaK, Hfq, and polyphosphate kinase (Ppk) have also been shown to associate with the degradosome in substoichiometric amounts (23, 24). Recently, RNase R has also been shown to associate with a form of degradosome containing PAPI, especially during stationary phase (25).

My investigations are focused on dissecting the biochemical interactions of RNase R with the RNA degradosome. I am particularly interested in finding the domain(s) of RNase R that mediate its association with the degradosome, and its specific interacting partners in the complex during this process.

1.5 Figures

Figure 1.1

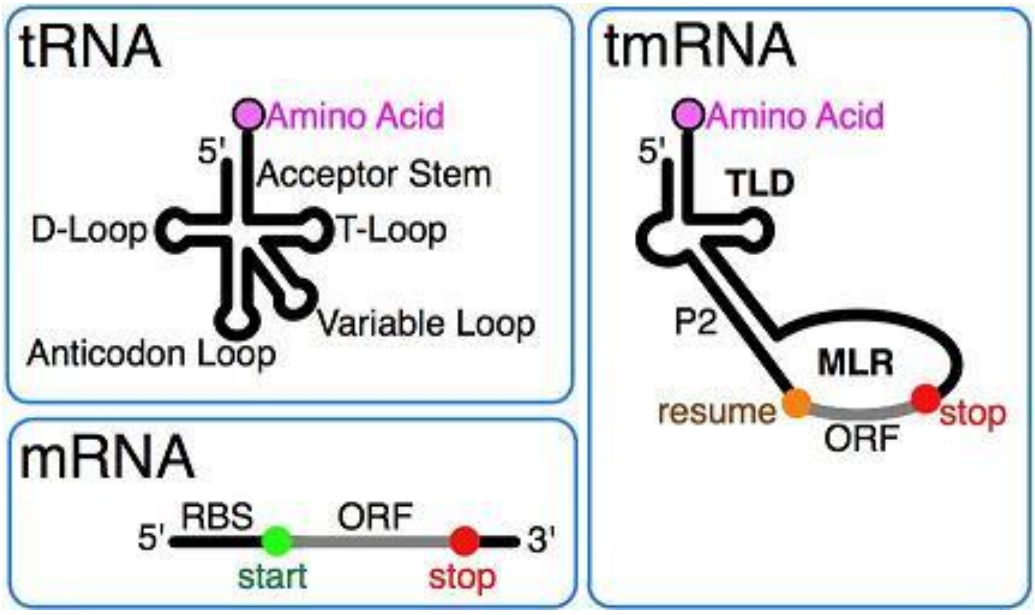


Figure 1.1: *Representation of a tmRNA combining the structural elements of both an mRNA and a tRNA.* The tRNA-like domain of tmRNA contains a D-loop, a T-arm, and an acceptor stem. As with all tRNA, the 3´ end of the acceptor stem of this domain terminates in CCA. However, the anticodon stem is absent, with a "connecting" structure linking the tRNA-like domain with the ORF of the mRNA-like domain. [Figure adapted from http://molbiol.grkraj.org/html]

Figure 1.2

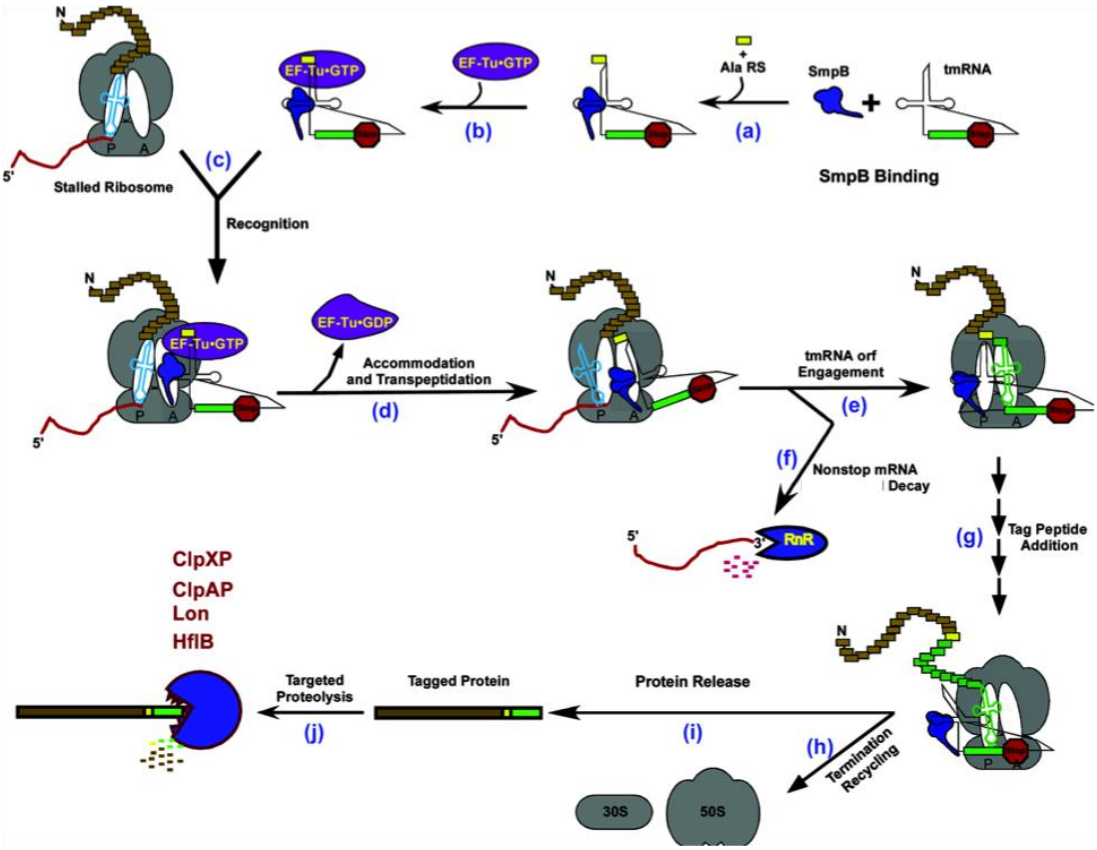


Figure 1.2: *Overview of the model of the translation quality control system.* **(a)** SmpB binding to the tRNA-like domain of tmRNA and aminoacylation by Alanyl tRNA synthatase. **(b)** Binding of EF-Tu-GTP and formation of a ribosome recognition complex then **(c)** recognizes and stably associates with stalled ribosomes leading to **(d)** hydrolysis of GTP on EF-Tu and accommodation of the SmpB-tmRNA complex in the A-site, which is followed by transpeptidation. **(e)** The mRNA-like domain is then decoded; **(f)** the defective message degraded by RNase R and **(g)** the degradation tag appended to the incomplete polypeptide. **(h)** The ribosome encounters the tmRNA stop codons releasing the stalled ribosomes, **(i)** the tagged protein is released and **(j)** specifically targeted for proteolysis by C-terminal specific proteases. [Figure Adopted from Dulebohn et al. 2007] (1)

Figure 1.3

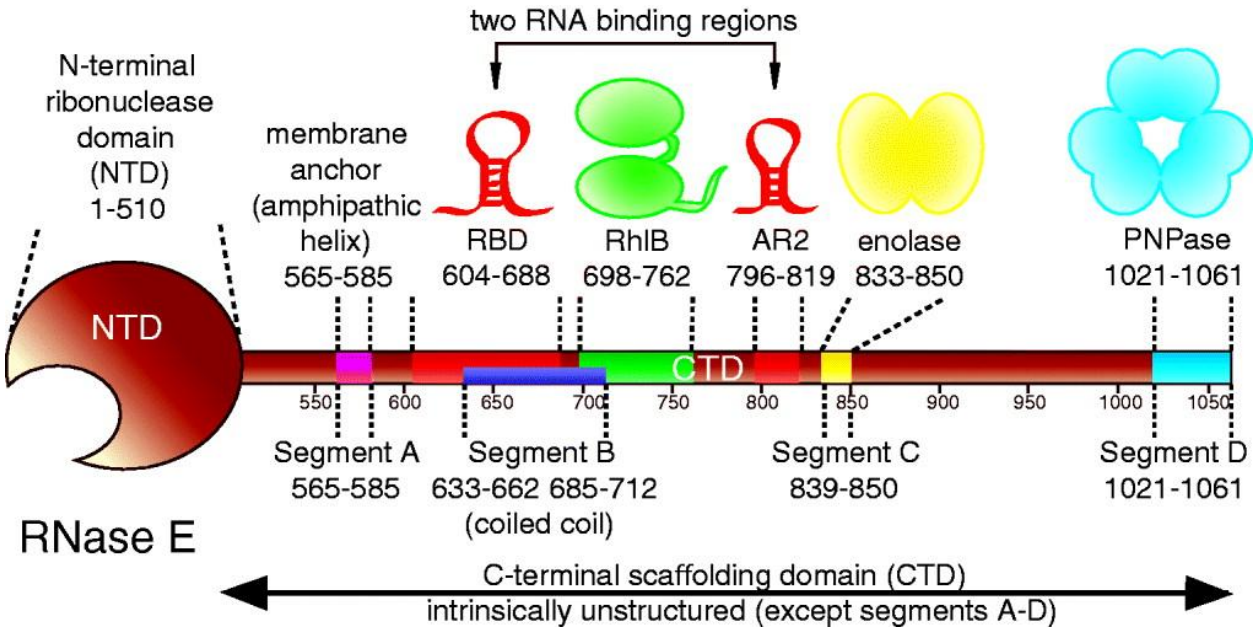


Figure 1.3: *Schematic representation of domain structure of RNase E and interaction sites with other core components of E. coli RNA degradosome.* RNase E provides the scaffold for assembly of the *E. coli* RNA degradosome. RNase E contains a globular catalytic NTD and a scaffolding CTD. Annotated are the RNA-binding sites (RBD, AR2) and the conserved microdomains mediating interactions with cytoplasmic membrane or other degradosome components. The indicated segments A through D are 'microdomains' mediating molecular recognition. [Figure adapted from M. W. Go´rna et al. 2012] (18)

Figure 1.4

A.

Component	Protomer size	Oligomeric state	Oligomeric species binding to RNase E
Ribonuclease E	1061 aa; 118 kDa	Tetramer	-
RNA helicase B	421 aa; 47 kDa	Monomer	RhlB monomer per RNase E
Enolase	432 aa; 46 kDa	Dimer	Enolase dimer per RNase E
Polynucleotide phosphorylase	711 aa; 77 kDa	Trimer	PNPase protomer per RNase E

B.

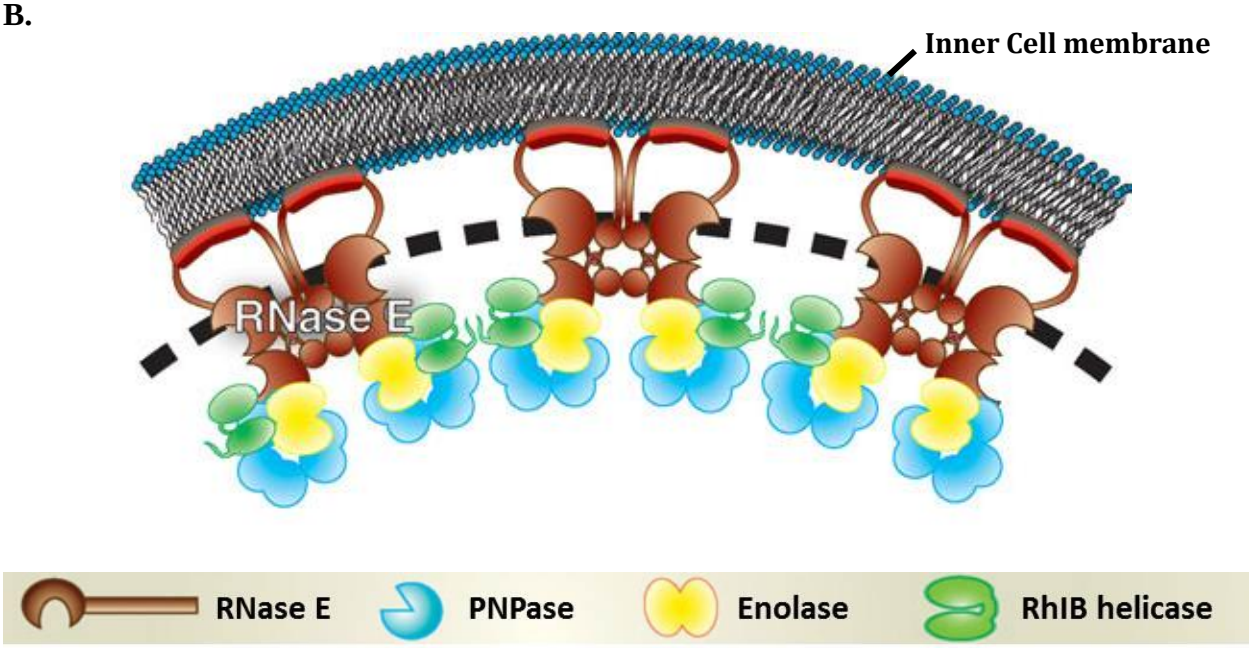


Figure 1.4: *RNA degradosome components and a proposed model of their assembly*. **(A)** Panel summarizing the canonical degradosome components and their physical properties. **(B)** A proposed model of the association of the RNA degradosome with the inner leaflet of the cytoplasmic membrane. The dashed lines indicate interactions between adjacent degradosomes in the filament-like assembly. Note that RNase E is a tetramer, but only two segment-A regions are shown for clarity. The molecular dimensions and stoichiometry of the assembly were arbitrarily chosen to simplify the figure. [Figure modified from M. W. Go´rna et al. 2012] (18)

Chapter 2: SmpB C-terminal tail interactions on the ribosome during *trans*-translation

2.1 Summary

The SmpB-tmRNA complex recognizes stalled ribosomes. Biochemical studies demonstrated that the C-terminal tail of SmpB is crucial for efficient recognition and engagement of stalled ribosomes (11). Recent structural data confirm these finding and show that the SmpB C-terminal tail occupies the ribosomal mRNA channel (26). To study the SmpB interacting partners on the ribosomes, I have made point mutants of a region of the *smpB* gene corresponding to the C-terminal tail of the protein, which will enable incorporation of the photoactivatable amino acid analogue p-Benzoyl-L-phenylalanine (Bpa). Using the pEVOL-Bpa orthogonal suppressor tRNA amino acid incorporation system, I have performed preliminary experiments to pinpoint interactions of the SmpB C-terminal tail with protein and RNA components of the rescued ribosome.

2.2 Introduction

SmpB is required for stable association of tmRNA with the ribosomes during *trans*-translation (27, 28). Figure 2.1 shows SmpB-tmRNA as a complex, along with EF-Tu-GTP, in the A-site of a stalled ribosome. Structural and biochemical analysis of SmpB-tmRNA complex have highlighted the importance of highly conserved residues within the core of the protein that interact directly with the tRNA-like domain (TLD) (27, 29, 30). The C-terminal domain of SmpB (residues 133-160) has been shown to be important for efficient tagging of tmRNA-tagged proteins (11). In addition, recent data from our lab point to the importance of SmpB C-terminal

tail in ORF establishment and accurate tagging. This implies that the residues required for this function lie in this region.

The C-terminal region of SmpB is separated form the core of the protein by a glycine residue at position 132. Gly132 is speculated to be working as a hinge. This residue is conserved throughout bacterial kingdom, and it tolerates only small, uncharged residues at this position (31). This suggests the importance of conformational flexibility in this region for SmpB function. Throughout the entire length of SmpB C-terminal tail (Figure 2.2), there are conserved amino acids that are critical in supporting tmRNA tagging activity (11). Changing these conserved residues abolishes tagging activity without affecting the binding of the SmpB-tmRNA complex to the stalled ribosomes. The tagging defect is severe in case of substituting residues Gly132, Lys139, Lys151, and Lys153. Moreover, changing both Ile154 and Met155 to negatively charged amino acids at the same time abolishes tagging completely. In addition, recent data from our lab highlight the importance of the SmpB C-terminal tail in defective mRNA degradation by RNase R (unpublished data). This highlights the importance of deciphering the molecular details of biochemical interactions of the C-terminal tail.

Although the C-terminal tail of SmpB is unstructured in solution, recent structural studies suggest that it adopts a conformation similar to that of an mRNA in the ribosomal mRNA channel (Figure 2.3). It is believed that the C-terminal tail makes crucial contacts with ribosomal proteins and/or RNA in this process. To study these interactions, I introduced UAG stop codons at selected positions in the C-terminal tail of SmpB that enable the incorporation of p-Benzoyl-L-phenylalanine at these sites during translation of SmpB. This was accomplished using the pEVOL–Bpa amino acid incorporation system. The pEVOL plasmid codes for the orthogonal aminoacyl tRNA synthatase-$tRNA_{CUA}$ pair that is necessary for the incorporation of Bpa (32,

33). p-Benzoyl-L-phenylalanine (Bpa) is an unnatural photoactivatable amino acid analog that can be efficiently incorporated by the translational machinery in response to the UAG codon. Upon near-UV irradiation (350-365nm wavelength), the benzophenone group of *p*Bpa preferentially reacts with carbon-hydrogen bonds (C—H) in proximal proteins and RNA (Figure 2.4) (34, 35).

The C-terminal tail conserved residues chosen for Bpa incorporation were Lys131, Lys133, Lys134, Lys151, and Arg153, referred to as K131B, K133B, K134B, K151B, and R153B, respectively. The residues K131, K133, and K134 flank the highly conserved Gly132 (Figure 2.4). Residues K151 and R153 are adjacent to Ile154 and Met155. The importance of these regions has been described earlier (11). Upon incorporation in the above-mentioned sites and subsequent UV irradiation, Bpa will crosslink to residues that are in close proximity. I have shown that this system can be successfully used to find potential SmpB crosslinks in my preliminary experiments.

2.3 Materials and Methods

2.3.1 Cloning of the smpB-ssrA genes in pPW600-λS and pPW600-λNS

BglII/AvrII restriction sites were introduced in pPW600 λ-cI-N stop (pPW600-λS) and pPW600 λ-cI-N nonstop (pPW600-λNS) reporter plasmids downstream of *λ-cI* ORF by using standard site-directed PCR mutagenesis protocol. The s*mpB-ssrA* gene cassette was cloned with its native promoter and a transcriptional terminator into pPW600-λS and pPW600-λNS using BglII/AvrII restriction sites in a way that the *smpB-ssrA* genes are transcribed in the opposite direction to that of *λ-cI* ORF (Fig 2.5). Primers used for cloning were:

5`ACTGCCCCTAGGCCGACTTCCGGTACAACCCGGCAAAAACTG3` and

5`ACTGCCAGATCTGGGGAGTCTCCCTGCGTTTAGGCCCAACGTAAAC3`

The presence of the insert was confirmed by sequencing. The confirmed clones were checked for expression after transforming them in W3110 Δ*smpBssrA* (DE3).

2.3.2 Making SmpB C-terminal tail variants

SmpB C-terminal tail variants were generated by introducing UAG stop codons, for Bpa incorporation, at the desired sites of the *smpB* gene. Table 2.1 lists the mutagenic primers used for site-directed PCR mutagenesis. All mutants were confirmed by sequencing the *smpB* gene in each plasmid. All mutants were checked for expression by co-transforming them with the pEVOL plasmid in W3110 Δ*smpBssrA* (DE3). To check for expression, 2 mL culture of each SmpB variant was grown in LB at 37°C with1 mM Bpa and 0.02% arabinose (w/v). Whole cell lysate samples were resolved by gel electrophoresis on a 15% Tris-Tricine gel. Western blots were performed using anti-SmpB antibody (rabbit, polyclonal antibody) and detected by the Odyssey Infrared Imaging System (LI-COR).

2.3.3 Small scale UV crosslinking

Five milliliter culture of each SmpB variant with either λS reporter or λNS reporter was grown to OD_{600} of 0.6 in 2XYT media (with 30 µg/mL chloramphenicol and 100 µg/mL ampicillin) at 37°C with 1 mM Bpa and 0.02% arabinose (w/v). The λ reporters were induced with 1 mM IPTG for one hour. Cells were harvested by centrifugation at 3500 rpm for 15 minutes at 4°C. Pellets were resuspended in 100 µL 1XPBS and were treated with long wavelength (365 nm) ultraviolet radiation for 30 minutes at 4°C. Crosslinked samples were diluted in SDS loading buffer. Samples were resolved by gel electrophoresis on a 15% Tris-Tricine gel. Western blots

were performed using anti-SmpB antibody (rabbit, polyclonal antibody) and detected by the Odyssey Infrared Imaging System (LI-COR).

2.4 Results and Discussion

Numerous experiments have been done to demonstrate the importance of the C-terminal tail of SmpB in *trans*-translation (11, 26). To look for the specific interacting partners during the rescue of stalled ribosomes, I incorporated the photoactivatable amino acid analogue Bpa at specific sites in the SmpB C-terminal tail. The experiment was performed in an *smpB-ssrA* deficient strain supplemented with plasmid borne tmRNA and SmpB variants co-expressed with pEVOL system. Expression of SmpB-tmRNA was from pPW600 plasmid bearing *smpB-ssrA* genes under native promoter. The same plasmid also has the λ reporter gene under IPTG inducible T7 promoter. The reporter gene was induced with 1 mM IPTG when the cells reached an OD_{600} ~ 0.5. The plasmid pEVOL has a tightly controlled arabinose inducible promoter. The mutants were expressed while inducing pEVOL with arabinose. The expression of SmpB variants was detected by Western blot analysis with SmpB-specific antibody.

The λNS reporter, pPW600 *λ-cI-N* nonstop, was expressed to promote ribosome stalling. This reporter contains the encoding sequence of N-terminal domain of the bacteriophage *λ-cI* gene, and lacks in-frame stop codon, followed by the strong *trp* operon transcriptional terminator. The construction of this reporter construct has been described previously (36). The related λS reporter construct containing an in-frame stop codon, pPW600 *λ-cI-N* stop, was used as a control, as it undergoes normal translation and recycling of the translational machinery. Figure 2.1 shows pPW600 plasmid map with *smpB-ssrA* ORF and *λ-cI-N* ORF in opposite orientations.

2.4.1 Mutants of SmpB C-terminal tail incorporate Bpa at different levels

Figure 2.6A shows the level of expression for each mutant. The two bands for each mutant in the anti-SmpB Western blot image correspond to the full-length product (higher molecular weight band) and the truncated product that did not incorporate Bpa (UAG is a stop codon in the absence of suppressor Bpa-tRNA). K131B shows the highest level of Bpa incorporation compared to all other mutants. Bpa incorporated and unincorporated (truncated) bands in case of SmpB mutants K151B and R153B are not distinguishable in the Western blot as the size difference is ~ 9 and 7 amino acids, respectively, in comparison to full length SmpB. Hence, the bands could not be resolved. An important point to be noted is that there is a considerable amount of SmpB that is truncated, i.e., it lacks Bpa incorporation. This suggests that Bpa incorporation is not 100% and it is inefficient. To address this issue, different concentrations of Bpa and arabinose, to induce pEVOL system, were tested to identify and optimize the condition for maximum Bpa incorporation in the SmpB variants. K134B was used for optimizing induction conditions because this mutant showed the highest levels of truncated product accumulation relative to the full-length product. However, changing Bpa or arabinose concentration had minimal effect on the amount of Bpa incorporation (Figure 2.6B, 2.6C).

2.4.2 SmpB C-terminal tail mutants show potential crosslinks after UV treatment

Cells expressing Bpa variants of the SmpB C-terminal tail were subjected to UV exposure for various length of time, (0 min, 10 min, 20 min, and 30 min) to find the optimal exposure time. Figure 2.7A shows a Western blot image of the crosslinking of an SmpB variant after different UV exposure times. The higher molecular weight bands increase in

intensity with an increase in UV exposure time, suggesting that they might be potential interacting partners that are cross-linked to the SmpB K131B variant. Figure 2.7B shows crosslinking of different SmpB variants in the presence of either λS or λNS reporter transcripts. Mutant K134B shows at least one crosslink in the presence of λS reporter while mutant K151B shows a potential crosslink in the presence of λNS reporter. Mutant R153B shows crosslink in the presence of both λS and λNS reporters. However, these data are preliminary, and further analysis has to be performed to confirm these results. Future experiments involve finding the identity of those crosslinked higher molecular bands. There is also the possibility that SmpB is interacting with the ribosomal RNA. High levels of RNA crosslinks (that cannot be detected on the Western blot) might explain the low levels of crosslinks seen in the Western blot analysis. Moreover, the results shown above have been done in the context of cell lysate. To further investigate crosslinks specific to ribosomal proteins and RNA, ribosome enrichment experiments need to be performed to isolate ribosomes stalled on the λNS reporter (37). Figure 2.8 depicts the principle behind the reporter based ribosome enrichment assay. Mass spectrometry and RNA sequencing will be used to confirm the identity of crosslinked proteins and RNA, respectively.

The crystal structure of the SmpB-tmRNA-ribosomes complex shows the C-terminal tail of SmpB occupying the mRNA channel beyond the 3´end of mRNA being translated (26). When SmpB-tmRNA complex is bound to the A-site of the ribosome, the mRNA channel adopts a tunnel-like conformation around SmpB C-terminal tail. Finding the interacting partners of SmpB C-terminal tail would provide insights into the molecular details of how C-terminal tail functions in the process of tmRNA ORF establishment. I predict that these interactions are dynamic in nature as SmpB C-terminal tail has to move out of the channel during ORF

establishment so that the mRNA-like domain of tmRNA can get accommodated in the mRNA channel, and the ribosome can resume translation of the tmRNA ORF. Other highly conserved residues in the C-terminal tail such as Asp137, Lys138, and Arg139 can also be mutated to incorporate Bpa for future crosslinking experiments.

2.5 Figures

Figure 2.1

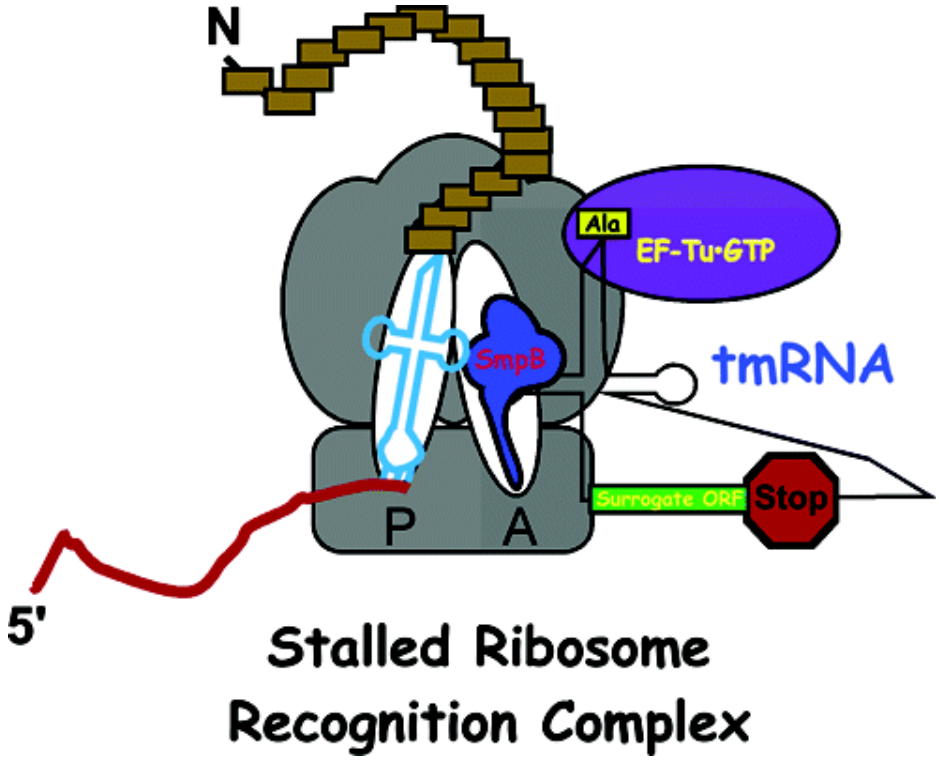


Figure 2.1: *Schematic representation of stalled ribosome recognition complex*. The quartnernary recognition complex consisting of tm-RNA, SmpB, EF-Tu, GTP bound to stalled ribosome. SmpB is occupying the empty A-site of the ribosome. [Figure adapted from Dulebohn et al. 2007] (1)

Figure 2.2

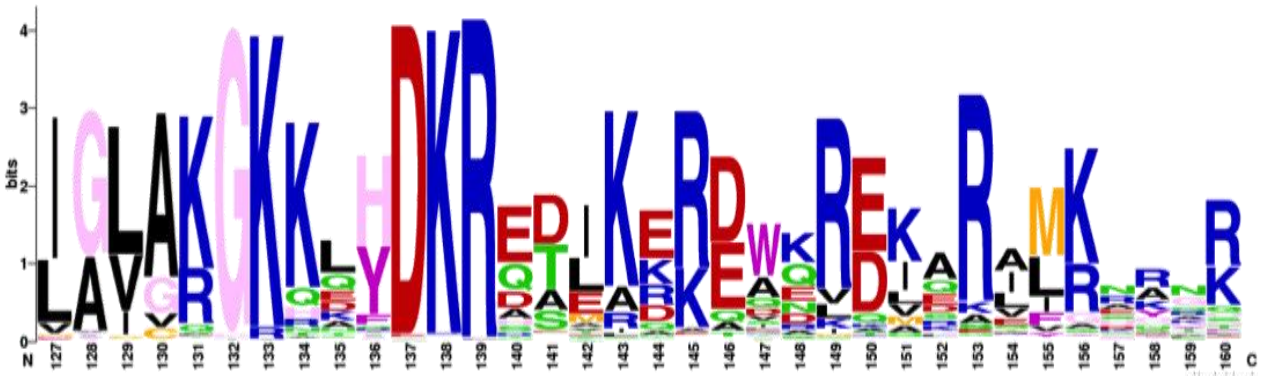


Figure 2.2: *Conserved residues in C-terminal region of SmpB.* Web Logo showing conserved residues in C-terminal tail of SmpB from 400 different species of bacteria. The residue position corresponds to the residue in *E. coli* SmpB. G132 acts as a hinge between the core of the protein and the C-terminal tail (133-160). **(**Figure courtesy of Devin Camenares)

Figure 2.3

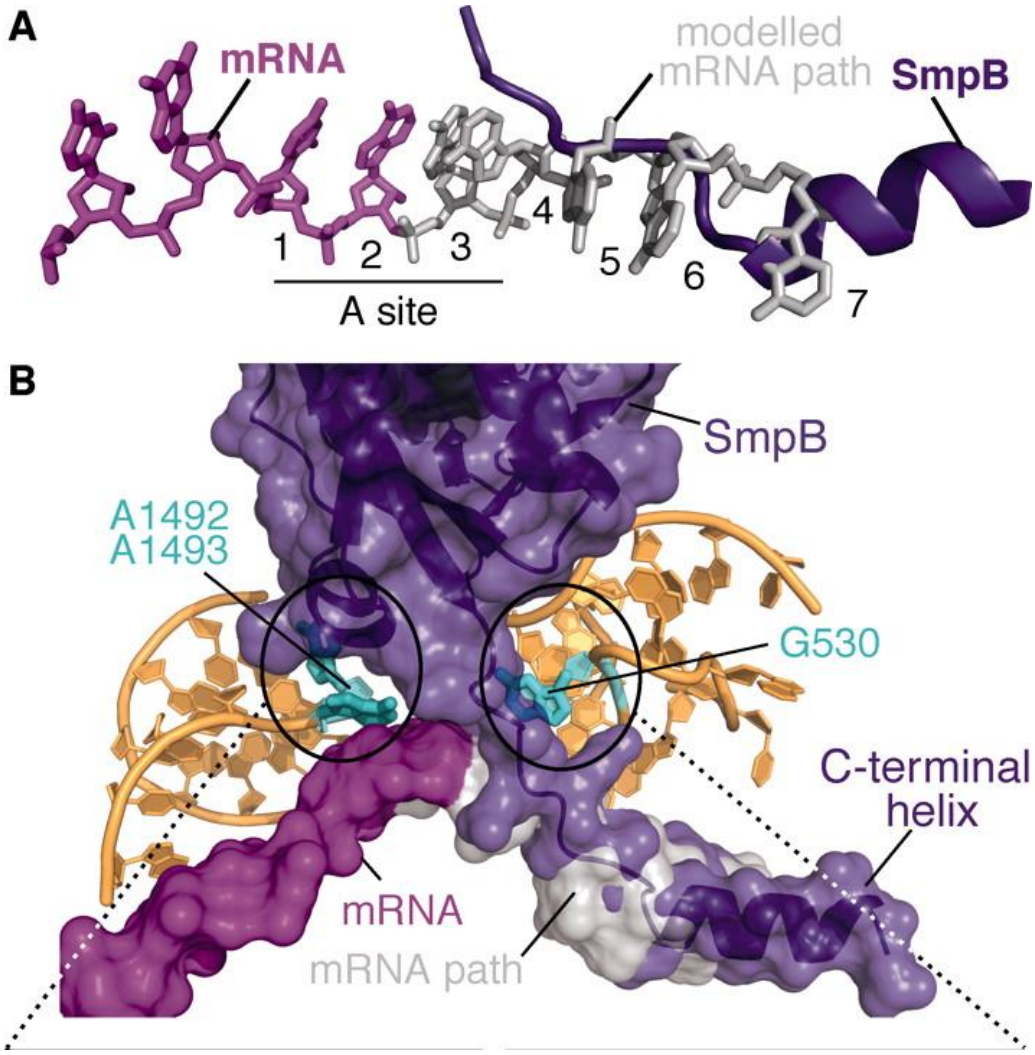
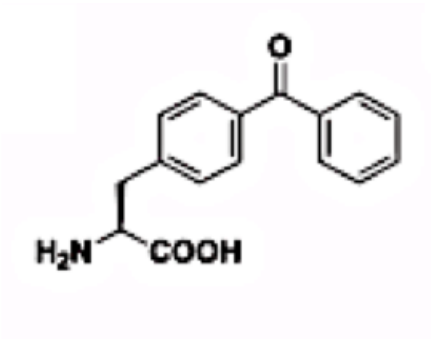


Figure 2.3: *A crystal structure showing the SmpB C-terminal tail occupying the mRNA channel on the ribosome.* (**A**) The C-terminal tail of SmpB would clash with mRNA downstream of the A-site codon. The mRNA used in this work is colored in magenta, and an extension based on the superposition of a longer mRNA is shown in gray. The mRNA nucleotides are numbered starting with the first nucleotide of the A-site codon. (**B**) SmpB interacts with both the shoulder domain and the 3′ major domain of 16*S* rRNA near the decoding center. [Figure adapted from Neubauer et al. 2012] (26)

Figure 2.4

A.



B.

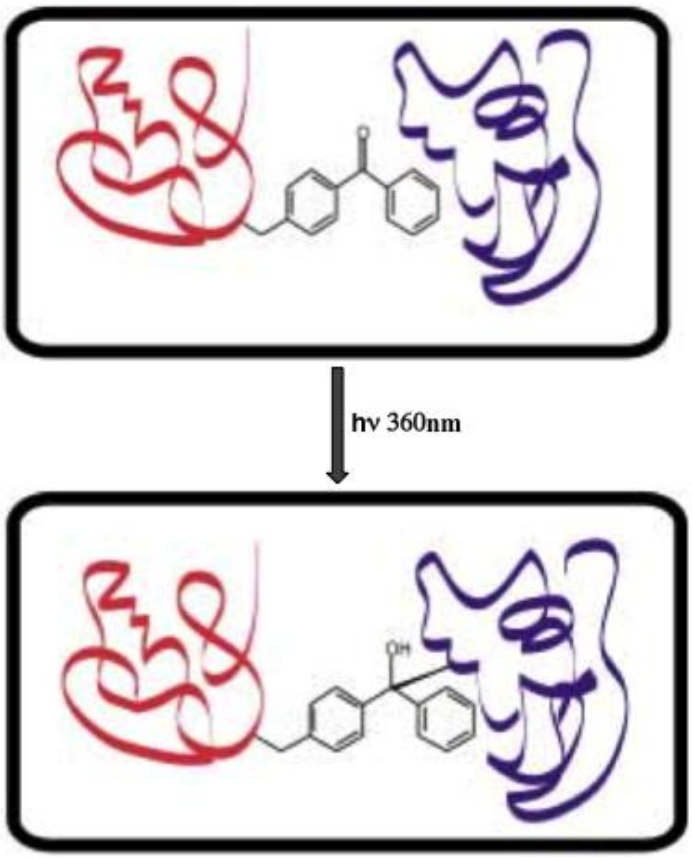
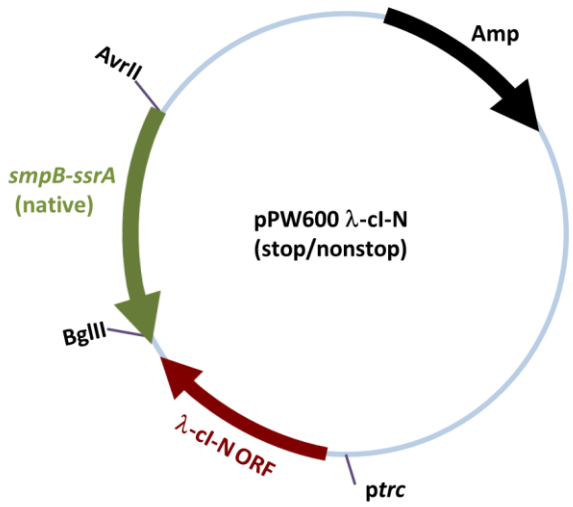


Figure 2.4: *Chemistry of in vivo photocrosslinking with Bpa.* **(A)** The chemical structure of *p*Bpa. C-H bonds within 3 Å of the carbonyl oxygen are targets for crosslinking. **(B)** The creation of covalent bonds between protein surfaces by in vivo photocrosslinking with *p*Bpa. A single amino acid in a protein is replaced with *p*Bpa in vivo. The cells are irradiated with near-UV light to crosslink proteins proximal to the surface of the *p*Bpa-containing protein. [Figures modified from Chin et al. 2002 and Farrell et al. 2005] (32, 34)

Figure 2.5

A.



B.

Figure 2.5: *Schematic representation of the plasmid and the λS or λNS reporters.* **(A)** pPW600 λ-cI-N reporter construct with or without a stop codon. *SmpB-SsrA* gene (with native promoter and a transcriptional terminator) was cloned between BglII/AvrII restriction sites such that its orientation is opposite to *λ-cI-N* ORF. **(B)** The *λ-cI-N* coding region is represented as a rectangle and the nucleotide sequence of the *trp*A terminator, located at the 3´ end of the transcript, is shown.

Figure 2.6

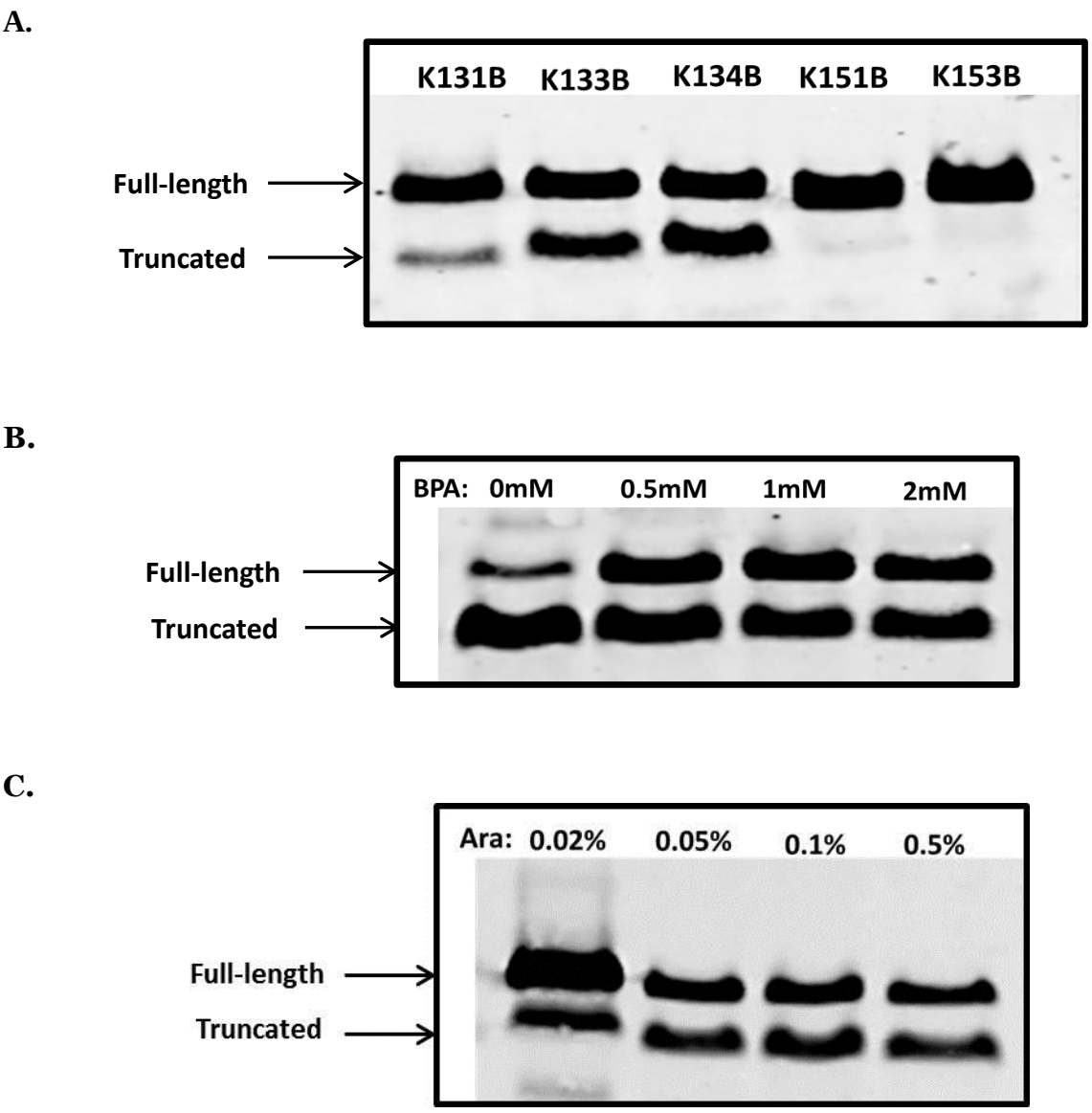
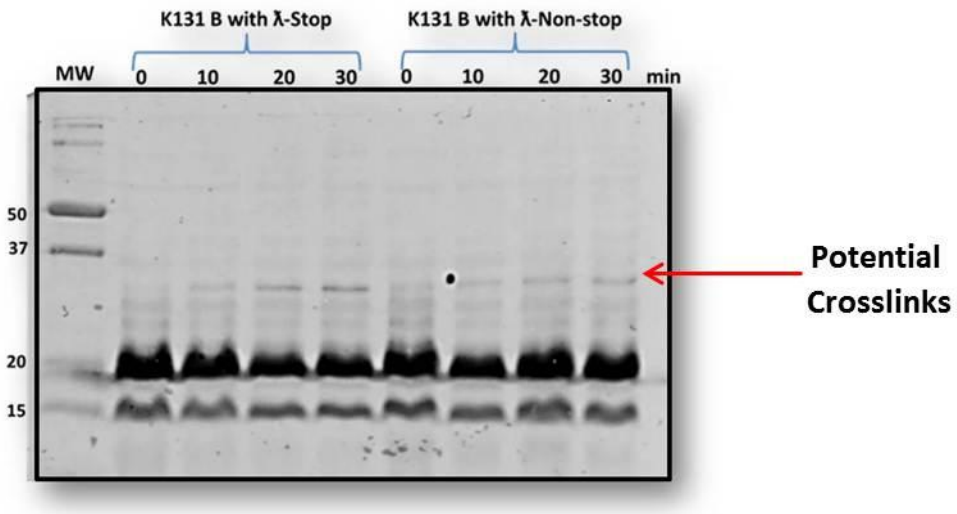


Figure 2.6: *Analysis of expression of SmpB C-terminal tail mutants under different induction conditions.* **(A)** Western blot using anti-SmpB antibodies showing the expression and Bpa incorporation in different SmpB C-terminal tail mutants. **(B)** Western blot using anti-SmpB antibodies showing the level of Bpa incorporation with different concentrations of Bpa in SmpB variant K134B. **(C)** Western blot using anti-SmpB antibodies showing the level of Bpa incorporation under different induction conditions for pEVOL in SmpB variant K134B.

Figure 2.7

A.



B.

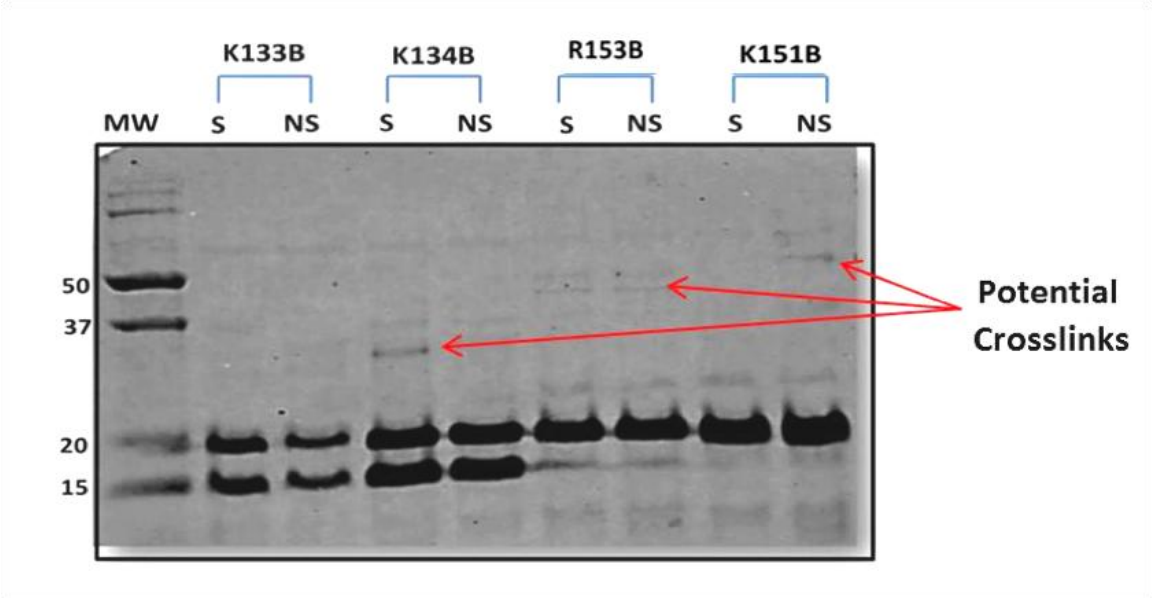


Figure 2.7: *Analysis of SmpB C-terminal tail mutants after UV crosslinking.* **(A)** Western blot with anti-SmpB antibodies showing the result of different amount of UV treatment. **(B)** Western blot with anti-SmpB antibodies to showing the result of small scale UV crosslinking on different C-terminal tail mutants of SmpB.

Figure 2.8

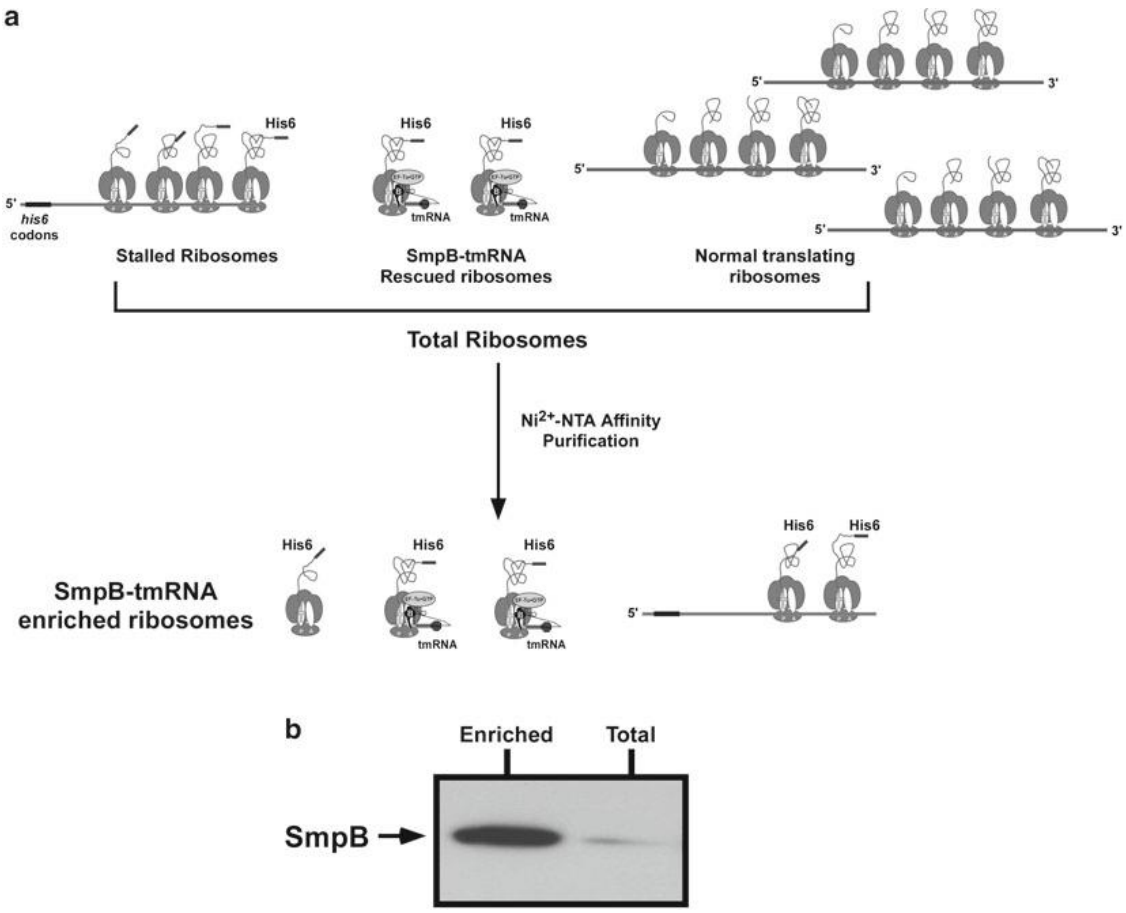


Figure 2.8 *Reporter based enrichment assay for ribosome-associated trans-translation factors.* **(a)** Schematic representation of a ribosome enrichment experiment. Total ribosomes can be obtained via any of the methods described in the main text. Isolated total ribosomes comprise a mixture of those translating normal cellular mRNA, or the reporter nonstop mRNAs encoding a His6 epitope tag. Stalled and rescued *trans*-translating ribosomes can be separated from the normal ribosome pool by using a suitable affinity column. The figure depicts enrichment of ribosomes translating a nonstop mRNA encoding an N-terminal His6-tagged reporter protein using a Ni^{2+}-NTA affinity column. Enriched ribosomes are subjected to Western blot analysis, using antibodies specific to the protein of interest. **(b)** Representative Western blot showing enrichment of SmpB on ribosomes translating λNS mRNA. Cushion purified total ribosomes were segregated into normal and stalled or *trans*-translating ribosomes using Ni^{2+}-NTA column chromatography. Total and eluted enriched ribosomes were normalized by A 260 and resolved by electrophoresis on a 10% SDS-acrylamide gel. The gel was used for electrophoretic transfer and Western blot analysis using anti-SmpB antibodies. SmpB was enriched on captured ribosomes translating reporter nonstop mRNA. [Figure adapted from Mehta et al. 2012] (37)

Table 2.1

Primers used for site-directed PCR mutagenesis to make mutations in C-terminal tail of SmpB at specific residues to incorporate unnatural amino acid (Bpa).

SmpB C-terminal tail variants	**Primers used for making the *smpB* mutant**
K131B	5´AAAGTGAAAATCGGCGTCGCCtagGGTAAGAAACAGCACGATAAAC3´ 5´GTTTATCGTGCTGTTTCTTACCctaGGCGACGCCGATTTTCAC3´
K133B	5´GAAAATCGGCGTCGCCAAAGGTtagAAACAGCACGATAAACGTTC3´ 5´GAACGTTTATCGTGCTGTTTctaACCTTTGGCGACGCCGATTTTC3´
K134B	5´AATCGGCGTCGCCAAAGGTAAGtagCAGCACGATAAACGTTC3´ 5´GAACGTTTATCGTGCTGctaCTTACCTTTGGCGACGCCG3´
K151B	5´CGCGAATGGCAGGTGGATtagGCACGTATCATGAAAAAC3´ 5´GTTTTTCATGATACGTGCctaATCCACCTGCCATTCGCG3´
K153B	5´GAATGGCAGGTGGATAAAGCAtagATCATGAAAAACGCCCACCG3´ 5´CGGTGGGCGTTTTTCATGATctaTGCTTTATCCACCTGCCATTC3´

“B” refers to Bpa.

Chapter 3: Determining the domains of RNase R responsible for association with the RNA degradosome during stationary phase

3.1 Summary

The *E. coli* RNA degradosome degrades defective and unwanted RNA by a combined effort of various enzymes associated with it. Poly (A) polymerase (PAPI), the enzyme responsible for polyadenylation of mRNA transcripts, is associated with the RNA degradosome under most conditions in addition to the core components of degradosome as described in Chapter 1. The polyadenylated RNA transcripts then become substrates for 3´-5´ exoribonucleases such as PNPase, RNase II, and under certain conditions RNase R. I have used different variants of RNase R to dissect the nature of biochemical interactions of RNase R in the PAPI-degradosome complex. My data shows that the unique N-terminal region of RNase R is not responsible for its association with the degradosome.

3.2 Introduction

3.2.1 PAPI and RNA degradosome

Components of the degradosome assemble on the RNase E scaffold via protein-protein interactions with microdomains present in the C-terminal domain of RNase E. One of the enzymes involved in these protein-protein interactions with RNase E is PAPI. Membrane association of degradosome is well established, and PAPI has also been shown to localize near the inner cell membrane in a growth phase dependent manner (38). Additionally, there is *in vitro* experimental evidence showing PAPI and RNase E interaction (22).

PAPI adds poly(A) tail to the 3´end of RNA. Unlike eukaryotes, where addition of the poly(A) tail stabilizes the mRNA and enhances translation efficiency, in prokaryotes Poly(A) tail serves as a signal for degradation of RNA transcripts by RNA degradosome and its associated RNases (39). After cleavage by the RNase E endoribonuclease, RNA transcripts become a substrate for polyadenylation by PAPI. These transcripts with a 3´ poly(A) tail are then degraded by 3´-5´ processive exoribonucleases such as PNPase and RNase II.

The structural organization of PAPI is similar to the eukaryotic PAPs with an N-terminal catalytic domain, a C-terminal RNA binding domain and sites for the interaction with other protein factors (22). In *E. coli*, PAPI is a 54 kDa protein encoded by the gene *pcnB*, which has a moderately strong promoter, but its expression is very low because of a non-canonical start codon (UUG) and a poor ribosome binding site (40). In addition, the coding sequence of *pcnB* contains at least four Shine-Dalgarno-like sequences that have been shown to cause translational pausing (41). These factors are responsible for limited protein synthesis of PAPI to the extent that there are only about 30-50 molecules of PAPI per cell in exponentially growing cells (42).

Recently, Carabetta et al. (25) provided *in vivo* evidence of protein-protein interactions of PAPI with the mRNA degradosome during both log and stationary phases. They also identified interacting partners of PAPI-GFP (PAPI–Green Fluorescent Protein fusion) in the degradosome by immunopurifications on magnetic beads followed by mass spectrometric analysis (Figure 3.1). Stationary-phase regulatory protein SprE was found to play a critical role in maintaining PAPI-degradosome association during stationary phase (25), which is consistent with the previously understood role of SprE in polyadenylation. In addition to the main components of the degradosome isolated from both log and stationary phase of wild type *E. coli*, RNase R was found to be part of PAPI-degradosome complex during stationary phase.

3.2.2 RNase R and RNA degradosome

RNase R belongs to the RNase II superfamily of 3´-5´ exoribonucleases (43). It shares similar domain architecture with RNase II. RNase R has a core catalytic domain, an S1 RNA binding domain and two cold shock domains (CSD) (Figure 3.2A). In addition to these domains, RNase R has an N-terminal helix-turn helix (HTH) domain of unknown function, and a C-terminal lysine rich (K-rich) domain that is important for its role in *trans*-translation. By itself, the core nuclease domain of RNase R can bind and degrade structured RNAs with lower efficiency. The CSD and S1 domains play a role in binding and positioning of the substrate (44-46). RNase R has been shown to degrade mRNAs with extensive secondary structures efficiently *in vivo* and *in vitro* (47). Because there has been no characterized role for the unique N-terminal domain of RNase R, we suspected that it might be responsible for its association with the degradosome. To address this question, I examined the ability of an $RNR^{\Delta N}$, an RNase R variant lacking the N-terminal domain, to associate with the RNA degradosome. From the results, I conclude that and $RNR^{\Delta N}$ is fully capable of associating with the degradosome.

3.3 Materials and Methods

3.3.1 Cloning of pcnB with a C-terminal his tag

pcnB was cloned with its native promoter by amplifying it from *E. coli* genomic DNA with a C-terminal 6XHis tag using primers

5´GCTTAGAGATCTCAGCGTCGAGCAAATCCTTCAG3´ and

5´AGGCTATCTAGATTAATGGTGATGGTGATGGTGTGCGGTACCCTCACGACGTGGT

GCGC3′.

The pET28b plasmid (Novagen) was used for cloning. The BglII and XbaI restriction sites were used to remove the T7 promoter. The presence of the insert in positive clone was confirmed by sequencing. The construct was checked for expression after transforming it in *ΔpcnBΔrnr* W3110 (DE3) cells. *ΔpcnBΔrnr* W3110 (DE3) strain was made by P1 transduction using individual gene knockouts strains from the Keio Collection (48).

3.3.2 Isolation of the RNA degradosome

The RNA degradosome was isolated from a 2 L culture of *ΔpcnBΔrnr* W3110 (DE3) cells that have the *pcnB* gene with its native promoter and a C-terminal his tag on a plasmid (pET28b) and the *rnr* gene on a separate plasmid (pACYC-duet) under the control of araBAD promoter. The cells were grown in LB (with 50 µg/mL kanamycin and 30 µg/mL chloramphenicol) at 37°C to OD_{600} of 0.6 for log phase and OD_{600} of 3-4 for stationary phase cultures and RNase R was induced with 0.01% arabinose (w/v). The cells were harvested by centrifugation at 3500 rpm for 45 minutes at 4˚C. The resulting cell pellets were stored at -80˚C. Frozen cell pellets were resuspended in lysis buffer (5 mL lysis buffer/gram of cells) containing: 20 mM HEPES (pH 7.4), 0.11 M potassium acetate, 2 mM $MgCl_2$, 0.1% Tween20, 1 µM $ZnCl_2$, 1 µM $CaCl_2$, 1% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 1:100 protease inhibitor cocktail, 1:200 PMSF, 10 mM Imidazole. Lysis was performed using a French press. Insoluble fraction was separated by centrifugation at 5000 rpm for 10 minutes. The soluble fraction was incubated with 200 µL Ni-NTA agarose resin (GE). Resin binding was allowed to go for an hour at 4˚C. Resin-sample slurries were subjected to column chromatography by gravity flow. The resin was washed 5-8 times with 10 mL of lysis buffer and PAPI-degradosome complex was eluted with 100 µL elution buffer (lysis buffer + 250 mM

imidazole).

3.3.3 RNase R detection

PAPI-degradosome elution samples were resolved by gel electrophoresis using 8% SDS-PAGE at 120 V. For Western blots, proteins were transferred to a PVDF membrane at 10 V for 90 minutes, blocked with 5% milk in TBST for an hour, incubated with anti-RNase R (rabbit, polyclonal) or anti-His (rabbit, polyclonal) antibody for an hour, washed, and incubated with goat anti-rabbit secondary antibody, respectively. Western blots were detected by Odyssey Infrared Imaging System (LI-COR).

3.4 Results and Discussion

To investigate whether N-terminal HTH domain of RNase R plays a role in its association with the degradosome, I have used wild type RNase R (RNR^{WT}) and an N-terminal truncation variant of RNase R ($RNR^{\Delta N}$) which has the 64 amino acids HTH domain deleted (Figure 2.3B).

The experiments were performed in a strain lacking the chromosomal copy of both *pcnB* and *rnr* that were supplemented with plasmids for expression of the PAPI and RNase R variants. Expression of PAPI (with a C-terminal 6XHis tag) was under the control of its native promoter. Expression of RNase R and its truncation variants was from a pACYC-based plasmid with a tightly controlled arabinose inducible promoter. The PAPI-degradosome complex was isolated from cells grown to stationary phase in medium containing arabinose for inducing RNase R variants. The complex associated with the His-tagged PAPI was isolated by Ni-NTA affinity chromatography. The presence of RNase R in degradosome complex was detected by Western blot analysis with polyclonal RNase R-specific antibody. The lysis

conditions for isolation of PAPI-degradosome complex have to be very stringent with considerable concentration of detergent (0.1% Tween20, 1% Triton X-100, 0.5% deoxycholate) due to the presence of membrane-associated components. The exact composition of lysis buffer is described in materials and methods section. Moreover, the cells have to be centrifuged at a low speed for a short time (5000 rpm for 10 min) to prevent disruption of the degradosome assembly.

PAPI level is highly regulated at both the transcription and translation stages of expression. There are about 30-50 molecules of PAPI per cell in exponentially growing cells (42). Because over expression of PAPI is toxic to the cells (49), it makes it very difficult to purify enough to study its interactions with other proteins, especially in the context of RNA degradosome. The presence of a multi-copy number plasmid (pET28b) with *pcnB* gene for PAPI expression from its native promoter causes the cells to have a slow-growth phenotype.

Isolated components of the PAPI-degradosome complex were resolved on 10% SDS-PAGE and stained with Coomassie brilliant blue dye as shown in Figure 3.3. There is no considerable difference in the profiles of isolated PAPI-degradosome components from cells expressing full length RNase R or N-terminal truncation of RNase R. Moreover, these profiles look very similar to those of PAPI-GFP-degradosome complex seen by Carabetta et al. 2010 (25). From the Western blot analysis I inferred that RNase R N-terminal truncation variant associates with the PAPI-degradosome (Figure 3.4). This suggests that one or more of the other domain(s) of RNase R must be playing a role in its association with the degradosome. I conclude that the N-terminal domain does not influence RNase R association with the degradosome. The role of this unique N-terminal domain, which is lacking in other RNase II family members, is yet to be understood.

Under physiological conditions, RNase R expression and activity is highly regulated. Under certain stress conditions such as entry into stationary phase, starvation, and cold shock, RNase R levels in cells can increase up to 10-fold (50, 51). This increase in RNase R levels could be a possible reason for its association with the degradosome specifically in the stationary phase.

The RNA degradosomes from other bacterial species have been shown to be composed of analogous enzymes and accessory proteins. For instance, in the degradosome of the psychrotrophic bacterium *Pseudomonas syringae Lz4W*, RNase R was found to co-purify with RNase E (52). However, the exact nature of protein-protein interactions of RNase R in the degradosome is a question that still remains unanswered. The ability of RNase R to degrade secondary structure RNA distinguishes it from other exoribonucleases of RNase II family and might be the reason for its degradosome-related function. The exact nature of RNase R interactions in the degradosome is a question that needs further investigation.

3.5 Figures

Figure 3.1

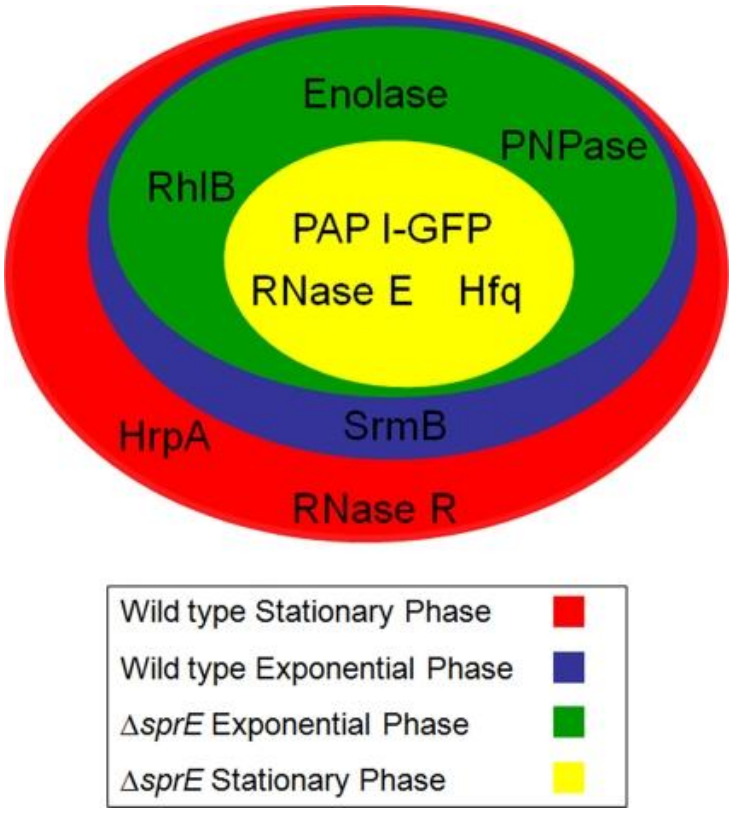


Figure 3.1: *Summary of PAPI-GFP interacting partners represented as a Venn diagram*. During stationary phase, in the absence of SprE, the only interacting partners of PAPI-GFP isolated were RNase E and Hfq. During exponential phase, the rest of the degradosome components were isolated, with the exception of SrmB. In a wild-type background, SrmB could be isolated in exponential phase, along with the other degradosome components. HrpA and RNase R were additionally isolated during stationary phase in the wild-type case. [Figure adapted from Carabetta et al. 2010] (25)

Figure 3.2

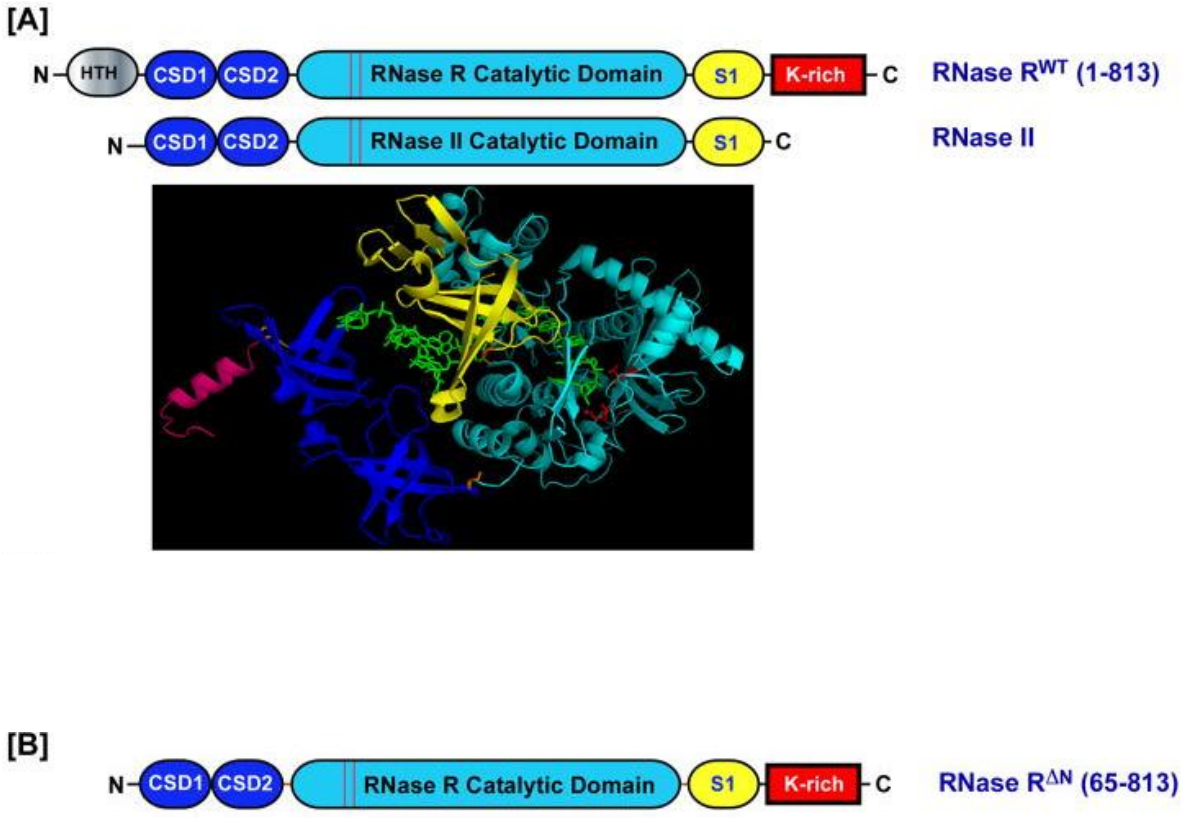


Figure 3.2: [A] *Schematic representation of the domain architecture of RNase R and RNase II*. RNase R and RNase II share extensive similarity in the N-terminal cold-shock, central nuclease and C-terminal S1 domains. RNase R has two additional domains, a N-terminal putative helix-turn-helix (HTH) domain and a C-terminal lysine-rich (K-rich) domain. **[B]** RNase R truncation variant, missing the N-terminal HTH domain [Figure modified from Ge et al., 2010] (53)

Figure 3.3

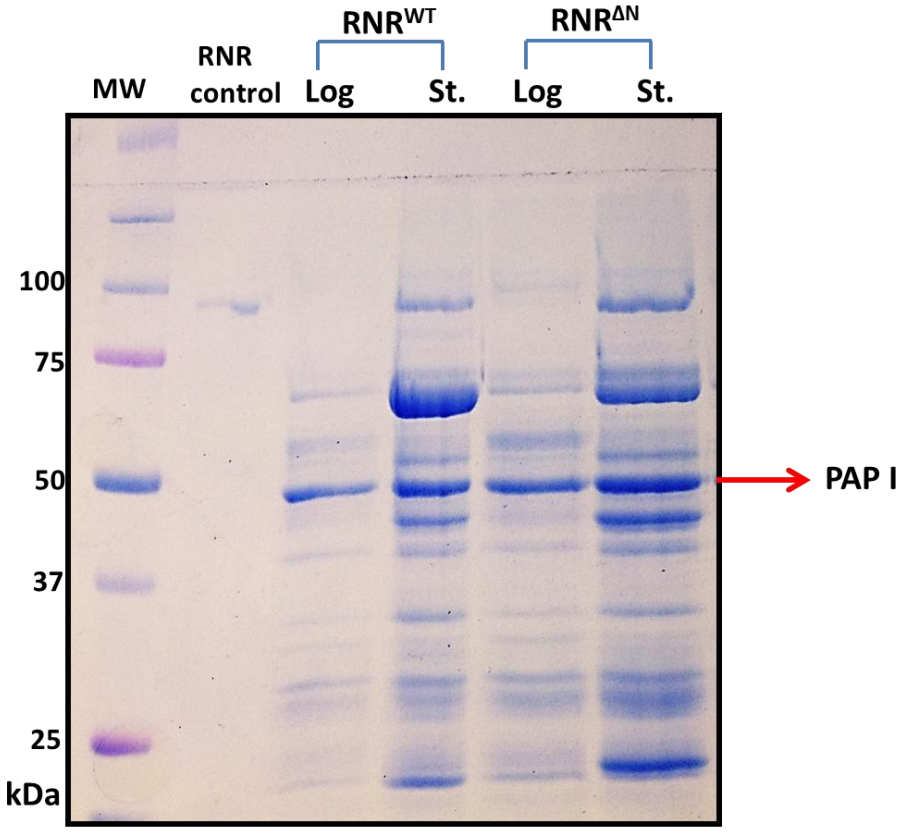


Figure 3.3: *Coomassie staining of isolated PAPI-Degradosome complex.* Coomassie stained SDS-PAGE (10%) showing components of the isolated PAPI degradosome complex from log and stationary phase cells expressing either full-length RNase R (RNR^{WT}) or an RNase R variant with an N-terminal truncation ($RNR^{\Delta N}$).

Figure 3.4

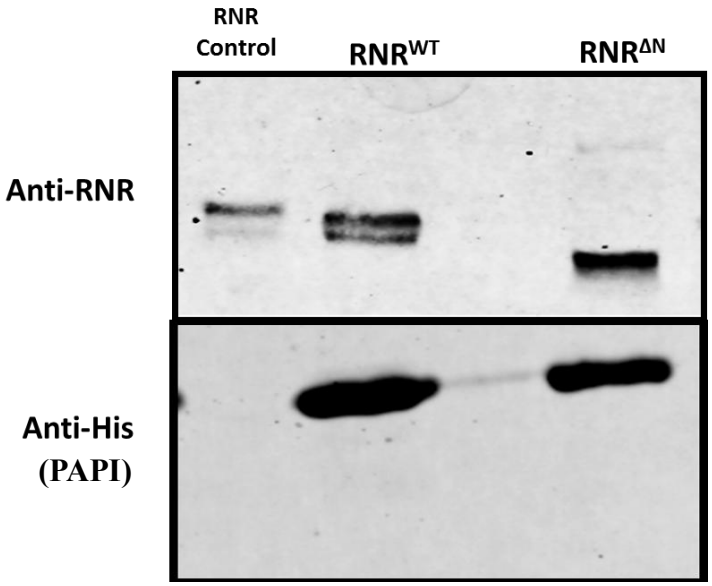


Figure 3.4: *Association of RNase R variants with the PAPI-degradosome complex during stationary phase.* Western blots show the association of RNR^{WT} and $RNR^{\Delta N}$ with the PAPI-degradosome complex during stationary phase. The presence of RNase was detected using an RNase R specific antibody and the presence of PAPI (with C-terminal 6XHis tag) was detected using anti-His antibody.

Chapter 4: Concluding Remarks and Future Directions

4.1 trans-Translation

This thesis has provided me with insights into the biochemical interactions of SmpB C-terminal tail in *trans*-translation. Specifically, I have identified amino acids (K131, K133, K134, K151, and R153) in the SmpB C-terminal tail conserved region to incorporate Bpa as they are close to the residues known to be important for tmRNA tagging activity. I optimized the induction conditions for incorporation of Bpa into those specific positions in SmpB tail. To determine if Bpa crosslinked to proteins present in close proximity to the SmpB C-terminal tail, I performed small-scale crosslinking experiments. I showed potential crosslinks in my preliminary data. Further analysis has to be done to confirm the identities of the crosslinked partners.

Upon binding stalled ribosomes, the C-terminal tail of SmpB adopts a helical conformation in the ribosomal mRNA channel. This conformation might be stabilizing the binding of SmpB to stalled ribosomes (26). It is thought to be making specific interactions with regions of the ribosome that would normally be occupied by mRNA. These initial contacts would be disrupted for the next step of engaging the tmRNA ORF, which would require SmpB to change its position such that the tmRNA ORF can get accommodated into the mRNA channel. These dynamic interactions would explain the importance of G132 as a hinge allowing the C-terminal tail flexibility to move and change its position throughout different stages of the *trans*-translation process.

To further investigate the residue-specific contacts using UV crosslinking, ribosome enrichment experiments need to be performed after UV treatment in the presence of SmpB C-terminal tail Bpa variants (Figure 2.6). Isolating SmpB crosslinks on the ribosomes would enrich

for the interactions specifically associated with *trans*-translation and/or ribosomes. Finding the crosslinks on enriched ribosomes would confirm the results and reveal the identity of interacting partners could be determined by mass spectrometry. Other highly conserved C-terminal tail residues such as Asp137, Lys138, and Arg139 can also be mutated to incorporate Bpa to study their specific interactions. Extensive analysis of crosslinked products would allow us to get a snap shot of SmpB interacting partners at various stages of *trans*-translation.

4.2 RNA degradosome

This thesis had also helped me understand the dynamic assembly of the RNA degradosome. I isolated the PAPI-degradosome complex containing the core components of the degradosome from both exponential and stationary phase cells. I have presented the results showing the presence of RNase R in stationary phase RNA degradosome. I determined that the distinct N-terminal region of RNase R is not involved in aiding its association with the degradosome. This suggests that one or more of the other domains of RNase R might be involved in its association with degradosome. This experiment needs to be repeated with variants of RNase R lacking other domains such as the C-terminal K-rich domain and the S1 domain. Once the domain responsible for its association with the degradosome is identified, we can use the pEVOL-Bpa system (described in chapter 2) to look for specific interacting partners of RNase R in RNA degradosome.

It would be interesting to find out if RNase R association with the degradosome is PAPI-dependent. There is *in vitro* evidence that RNase R prefers RNA substrates with a poly-A tail to substrates with a poly-U tail (53), which is consistent with the understanding that after PAPI polyadenylates the RNA it becomes a substrate of RNase R. Further work needs to be done to

test if PAPI and RNase R interact directly within the degradosome or if the interaction is indirect.

Despite a lot of recent work on the assembly of the RNA degradosome and the identity of its components under various growth conditions, the exact mechanism of the assembly of degradosome and its membrane localization still remains to be understood. It is still unknown whether the degradosome assembles on the C-terminal domain of RNase E first and then gets anchored to the membrane or RNase E localizes to the membrane and as a result all other components are recruited near the membrane. The reasons for how RNase R is recruited to the degradosome or why it binds to this complex only during stationary phase remain to be understood.

The two processes of *trans*-translation and RNA degradation by the degradosome might have more in common than meets the eye. For instance, a minimal RNase J-based degradosome was found to be associated with translating ribosomes in bacterial pathogen *H. pylori* (54). In addition, researchers have recently shown that the toxicity caused by increased PAPI levels is a direct result of polyadenylation of mature tRNAs (which are not a substrate of PAPI under normal conditions) leading to dramatic reduction of aminoacylated tRNAs, promoting ribosome stalling and cessation of protein synthesis (55, 56).

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